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Transcriptomic Analysis of Enterohaemorrhagic *Escherichia coli* O157:H7 in Response to Plant Extracts

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) are a group of food and contact-borne pathogens responsible for haemorrhagic colitis. The bacteria can be transmitted by contaminated meat, but importantly, also by plants. The bacteria can use plants as an alternative host, where they associate with both the leaves and the roots. Colonisation in the rhizosphere of plants is thought to be the main habitat for colonisation.

Four different plant species, commonly associated with EHEC outbreaks, were infected with EHEC O157:H7 isolates Sakai and TUV 93-0 over ten days to assess the colonisation potential of the bacteria in both the phyllosphere and rhizosphere of plants. The rhizosphere was found to sustain a higher population level of bacteria over time in comparison to the phyllosphere, yet both strains were unable to utilize root exudates for growth.

Global gene expression changes of EHEC O157:H7 strain Sakai were measured in response to plant extracts such as leaf lysates, root exudates and leaf cell wall polysaccharides from spinach cultivar Amazon and lettuce cultivar Salinas. Microarrays analysis showed a significant change in expression of 17 % of genes on exposure to leaf lysates of spinach. A more specific response was seen to spinach leaf cell wall polysaccharides with only a 1.5 % change. In contrast, when exposed to lettuce leaf cell wall polysaccharides a higher change of 4.8 % was seen. Genes that were differentially expressed belonged to multiple functional groups, including metabolism, indicating the utilization of plant-specific polysaccharides.

Several areas of further investigation have been determined from this project, including the importance of culturing bacterial strains at a relevant temperature, the proposed lack of the type III secretion system in plant colonization by EHEC O157:H7 and the utilization of plant components for growth and persistence in the plant environment.

Declaration of original authorship

Declaration:

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed …… Date ……

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List of Abbreviations

A/E	Attaching and effacing lesions
AAF	Aggregative adhesion fimbria
ABC	ATP-binding cassette
ANOVA	Analysis of variance
ARDB	Antibiotic resistance database
B & H	Benjamini & Hochberg
Bp	Base pair
BPEX	Bacteria-plant extraction
cDNA	Complementary DNA
CDS	Coding DNA sequence
CDTA	Cyclohexane Diamine Tetraacetic Acid
cfu	Colony forming unit
Cm	Chloramphenicol
C_T	Cycle threshold
cv	Cultivar
DAEC	Diffusely-adherent *E. coli*
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DWA	Distilled water agar
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EAHEC	Enteroaggregative-haemorrhagic *E. coli*
*Ec*O104	*E. coli* serotype O104:H4
*Ec*O157	*E. coli* serotype O157:H7
ECP	*E. coli* common pilus
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*

ET	Ethylene
ETEC	Enterotoxigenic *E. coli*
ETI	Effector-triggered immunity
F	Forward primer
FC	Fold change
g	gram
Gb3	Globotriaosylceramide
gDNA	Genomic DNA
GO	Gene ontology
GC-MS	Gas chromatography – mass spectrometry
GX	GeneSpring GX
HPLC	High performance liquid chromatography
HUS	Haemolytic uremic syndrome
Hz	Hertz
IAA	Indole-3-acetate
JA	Jasmonic acid
K12/MG1655	K-12
Kan	Kanamycin
Kb	Kilobase pair
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
LL	Leaf lysates
MAC	MacConkey
MS	Murashige & Skoog
MCT	Multiple comparison test
MTC	Multiple testing correction
N/A	Not applicable
NA	Nalidixic acid
ncRNA	Non-coding RNA
nle	non-LEE encoded

NMR	Nuclear magnetic resonance
OD	Optical density
P/S	Polysaccharides
PAGDB	Pathogen-associated gene database
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PR	Pathogen resistance
qPCR	Quantitative/real-time PCR
^{R}	Resistant
R	Reverse primer
RE	Root exudates
RIN	RNA integrity number
Rpm	Revolutions per minute
ROS	Reactive oxygen species
RT	Reverse transcriptase
^{S}	Sensitive
SA	Salicylic acid
SAR	Systemic acquired resistance
SDW	Sterile distilled water
SEM	Standard error of the mean
SMAC	Sorbitol MacConkey
STEC	Shiga-toxin producing *E. coli*
Stx	Shiga-toxin
T2SS	Type II secretion system
T3SS	Type III secretion system
Tc	Tetracycline
TE	Tris-EDTA
TFA	Trifluoracetic acid
Tir	Translocated intimin receptor

Tm	Melting temperature
TUV	EHEC strain TUV 93-0
UV	Ultraviolet
VBNC	Viable but non-culturable
VFDB	Virulence Factor Database
VTEC	Verotoxin-producing *E. coli*
w/v	Weight/volume

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1 Introduction

1.1 Food safety

Foodborne illness cases are recorded at high numbers each year, with bacterial species such as *Salmonella* and *Escherichia coli* (*E. coli*) pathogenic strains accounting for approximately 42,000 and 265,000 cases respectively each year in the USA alone (Centers for Disease Control and Prevention, 2012a; Centers for Disease Control and Prevention, 2012b), although the number of total cases is predicted to be higher as not all illnesses are reported. The cause of these outbreaks can be linked to a variety of different food products, with the most common being undercooked, contaminated meat products, specifically chicken for *Salmonella* (De Buck *et al.*, 2004) and beef for *E. coli* pathogenic species (Elder *et al.*, 2000). However, it has now been recognised within the past decade that a large proportion of these outbreaks are attributed to a variety of fruits and vegetables; in fact, 33 % of salmonellosis cases recorded from 2004–2008 in the USA and 21 % of *Escherichia coli* serotype O157:H7 (*Ec*O157) from 1982–2002 were linked to fresh produce (Rangel *et al.*, 2005). This was regarded as unusual when this was first reported, despite the fact that many closely-related bacteria within the *Enterobacteriaceae* are plant-associated microbes, such as *Pectobacterium atrosepticum* (Toth *et al.*, 2006). *Klebsiella* is another genus of bacteria that is closely related to *Salmonella* and *E. coli*, which contains strains that are pathogenic to human but can also be found on plants (Rosenblueth *et al.*, 2004).

The occurrence of human pathogens on plant material was assumed to be a result of accidental post-harvest cross-contamination; however in recent years many studies have shown that these human pathogenic bacterial strains can actively colonise plant tissue both pre- and post-harvest and persist within the plant and surrounding environment for long periods of time (Islam *et al.*, 2004).

Contaminated fresh produce related cases may represent such a high number of cases of all foodborne pathogens due to the fact they are consumed raw, such as in a salad, whereas contaminated meat products only tend to result in illness if the food is consumed when

undercooked. As well as this, a variety of different food products have been implicated in outbreaks of these illnesses, meaning that these human pathogens can not only survive in the environment but can also persist within a wide range of environmental conditions.

1.2 Outbreaks relating to the consumption of fresh produce

Many large outbreaks have been recorded from these human pathogens that have occurred as a result of consuming contaminated fresh produce within the past twenty years. One outbreak which highlighted the importance of human pathogens on plant tissue occurred in Osaka, Japan in 1996, in which 7,900 school children were infected and 12 died (Michino *et al.*, 1999). The source of the outbreak was predicted to be white radish sprouts, a fact which was surprising to many since previous outbreaks had only been reported from consuming contaminated, undercooked meat products. The bacterial isolate of *Ec*O157 that was isolated was designated as "Sakai" in reference to the area of the outbreak. Since then, many outbreaks of *Salmonella* and pathogenic *E. coli* have been linked to contaminated fresh produce. A summary of plant-associated outbreaks for these strains can be seen in Table 1-1. It is evident from the list of outbreaks that there are a variety of different sources of *Ec*O157 and *Salmonella* infection and that it is difficult to isolate a single vector. The examples given demonstrate a small selection of the outbreaks that have occurred over the past twenty years that have been linked to fresh produce. Many outbreaks are small, with little long term consequences; however, some outbreaks can affect a large number of people, with significant health and economic consequences.

During the outbreak of another *E. coli* serotype, O104:H4 (*Ec*O104:H4), which resulted in 3,842 reported cases (including 53 deaths) in Europe (Struelens *et al.*, 2011) different produce and manufacturing companies were blamed for the contamination, resulting in large economic consequences for the businesses and affected countries. *Ec*O104:H4, like *Ec*O157, is a Shiga-toxin producing strain of *E. coli*; however it is an enterohaemorrhagic-enteroaggregative type and found to cause a significantly higher number of haemolytic uremic syndrome (HUS; characterised by acute kidney failure) cases compared to *Ec*O157 outbreaks. More surprisingly,

2/3 of the HUS cases were in adult women (Struelens *et al.*, 2011); this differs from *Ec*O157, which usually leads to HUS in children, although it can happen in adults.

There have been a number of recalls of fresh produce in the USA and Europe over the past few years due to *Ec*O157 outbreaks. Therefore, there are not only serious health consequences as a result of human pathogen contamination in plant food products but also substantial economic consequences for the companies and countries implicated as the source of these outbreaks (Roberts, 2000).

Table 1-1. Examples of outbreaks of *Salmonella* and *E. coli* infections that have been linked to plants. All outbreak data was acquired from ProMed-Mail.

			Number of affected individuals				
Bacteria	***Year***	***Location***	***Total cases***	***Hospitalised***	***HUS***	***Death***	***Source of Infection***
*Ec*O157	2014	Canada	24	5	0	0	Bean sprouts
*Ec*O157	2014	USA	15	0	0	0	Cabbage
Salmonella	2013	USA	84	17	-	0	Cucumber
*Ec*O169	2012	Korea	1642	Unknown	Unknown	Unknown	Radish kimchi
*Ec*O157	2012	USA	28	10	2	0	Spinach
*Ec*O157	2012	Japan	110	Unknown	Unknown	5	Pickled cabbage
*Ec*O157	2012	USA	25	6	0	0	Sprouts
Salmonella	2012	USA	261	94	-	3	Cantaloupe
*Ec*O157	2011	USA	60	30	2	0	Romaine lettuce
*Ec*O157	2011	USA	31	Unknown	Unknown	Unknown	Salad
*Ec*O104	2011	Europe	3842	Unknown	855	53	Fenugreek sprouts
*Ec*O157	2011	USA	51	Unknown	Unknown	Unknown	Salad
*Ec*O157	2011	UK	250	74	4	1	Raw leeks and potatoes
*Ec*O157	2011	USA	15	7	3	1	Strawberries
Salmonella	2007	UK	55	3	-	0	Basil
*Ec*O157	2006	USA	204	Unknown	Unknown	3	Spinach
Salmonella	2006	USA	183	22	-	0	Tomato
*Ec*O157	1996	Japan	7900	Unknown	Unknown	12	White radish sprouts

1.3 *Escherichia coli* O157:H7: pathogenicity

Pathogenic *E. coli* can be classified as one of six pathotypes: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely-adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Kaper *et al.*, 2004).

EHEC are responsible for the cause of acute diarrhoea and haemorrhagic colitis. Infections usually last a few days and patients fully recover; however, in some cases infection with EHEC results in HUS, a disease in which the kidneys lose function due to haemorrhaging. One of the most commonly seen EHEC strains is *Ec*O157. This serotype of *E. coli* was first seen in an outbreak of contaminated hamburgers in 1982, and later identified and classified in 1983 as *E. coli* O157:H7 (Wells *et al.*, 1983). The "O" refers to the cell wall or somatic antigen, whereas the "H" denotes the flagellar antigen. *Ec*O157 is unusually different from other pathogenic *E. coli* serotypes for several reasons: most isolates are unable to ferment sorbitol within 24 hours, whereas 93% of all known *E. coli* are able to, and so this is one characteristic tested for when identifying serotypes (Wells *et al.*, 1983); it is negative for invasiveness (again, usual in identification of a sample by use of the Sereny test); it adheres through the *E. coli* common pilus (ECP) (Rendón *et al.*, 2007); and it does not produce heat stable/labile toxins.

*Ec*O157 species are of significant interest as a low infectious dose of ~20-700 bacteria is all that is required for human infection (Tuttle *et al.*, 1999; Willshaw *et al.*, 1994): in comparison, strains of EPEC have been reported as requiring as a high a dose as 10^8 (Donnenberg *et al.*, 1993). Furthermore, despite *Ec*O157 strains only being detected in <1% of cattle in two different studies, yet non-O157 Shiga-toxin producing *E. coli* (STEC) strains isolated from 10-45 % of the cattle (Johnson *et al.*, 1996; Montenegro *et al.*, 1990), only 25 % of HUS cases were linked to non-O157 strains in the USA in 1996 (Johnson *et al.*, 1996), suggesting that *Ec*O157 strains may be more virulent than non-O157 strains.

*Ec*O157 contains several virulence factors, some of which are found on a plasmid, pO157, but the majority of which are located on genomic islands within the chromosome, acquired by

bacteriophage. For example, the genes required for the characteristic attaching and effacing lesions (A/E lesions) necessary to bind to the host epithelial tissue are encoded by a chromosomal pathogenicity island referred to as the locus of enterocyte effacement (LEE) (Garmendia *et al.*, 2005). The LEE is present in both EHEC and EPEC, although there are slight differences in the LEE between the two serotypes (Perna *et al.*, 1998). If the LEE from EHEC is transformed into *E. coli* K-12, there is no development of the A/E lesions; however, if the LEE from EPEC is used, A/E lesions are able to be formed by *E. coli* K-12 (Elliott *et al.*, 1999), suggesting that the virulence factors encoded in the EHEC LEE require another factor encoded elsewhere for its pathogenicity.

The LEE is made up of several distinct regions, one of which encodes for secreted proteins such as EspA, EspB and EspD; another for the type three secretion system (T3SS) apparatus; and another contains the *eae* gene, coding for intimin. All of these virulence factors play a role in the infection process in humans. Many other genes are encoded but at present their function in the infection process is still unclear (Law, 2000).

Contact with the host cell induces the expression of genes involved in actin pedestal formation, a characteristic feature of A/E lesions (Garmendia *et al.*, 2005). The EspA secreted protein provides a link in the form of a filamentous type three secretion structure that links the bacterial cell with the host cell through which secreted proteins are transferred (Crepin *et al.*, 2005), such as EspB and EspD. The secretion of EspB and EspD through the EspA filament results in the formation of a translocon between the two cells (Daniell *et al.*, 2001) allowing the transfer of other effector proteins into the host cell. One of the first bacterial effectors that is secreted into the host cell is the translocated intimin receptor (Tir). Tir inserts into the membrane of the host cell, where it becomes phosphorylated, to act as a receptor for intimin, expressed by the bacterial cell (Hartland *et al.*, 1999). Binding to this receptor results in the intimate attachment of the bacterial cell to the epithelial cell and secretion of subsequent effectors initiate changes in the host cytoskeleton, inducing the formation of the characteristic actin pedestals seen in the infection.

*Ec*O157 also possesses two Shiga-like toxins (Stx), known as *Stx1* and *Stx2*, which bind to the globotriaosylceramide (Gb3) receptor in the endothelium cells of the gastrointestinal tract. Collectively these are also termed verotoxins in the literature; therefore EHEC can also be referred to as verotoxin-producing *E. coli* (VTEC). The Stx toxins block protein synthesis by irreversibly damaging ribosomal RNA synthesis, halting the cellular processes within the host cell and thus inducing cell death (Endo *et al.*, 1988). Continued cell death within an area as a result of these toxins weakens the epithelial layer and results in haemorrhaging, hence the characteristic bloody diarrhoea seen in *Ec*O157 infections.

Gb3 receptors are also present in the glomerulus of the kidney: therefore if the bacteria get into the circulatory system of the body and reach the kidneys this is another site for the *Stx* toxins to bind to, resulting in haemorrhaging in the kidneys. This receptor is not present in cattle, hence why these toxins do not affect them and the bacteria are not pathogenic (Pruimboom-Brees *et al.*, 2000).

Stx1 has been shown to have a higher binding affinity for Gb3 compared to *Stx2*, although *Stx2* is 1000 times more cytotoxic than *Stx1* (Schuller, 2011). Both of these toxins are similar to the Shiga toxins produced by *Shigella dysentariae* serotype 1, although *Stx1* is much more highly conserved with a larger sequence similarity to the Shiga toxins. Studies have found that by making antibodies directed against the *Stx* of *Shigella* and subjecting *Ec*O157 cells to them has resulted in a neutralisation of *Stx1*, but not in *Stx2* (Smith *et al.*, 2006). Even though it is the *Stx1* which has a higher affinity for Gb3 and this can be neutralised with antibodies, another study found that the *Stx2* is essential for the pathogenicity of *Ec*O157, as it is found in every outbreak that has been tested (Bhunia, 2008). Interestingly, as HUS was found to affect children more than adults in *Ec*O157 outbreaks it was thought that children contained a larger concentration of the Gb3 receptor in their kidneys, and this concentration decreased with age. However, since then recent work testing this hypothesis has demonstrated that the number of Gb3 receptors in the kidney within an individual does not decrease with age (Ergonul *et al.*, 2003); therefore it as yet unclear why *Ec*O157 infection is more likely to result in the development of HUS in children

rather than adults. Furthermore, different variants of *stx2* have been isolated and differences in potency between them has been observed, with the variants *stx2a*, *stx2c* and *stx2d* more frequently associated with the development of HUS (Fuller *et al.*, 2011). The presence of these variants, along with other identified virulence factors, results in hypervirulent strains. An *Ec*O157 strain isolated from an outbreak in 2009 in which 22 % of people infected developed HUS was found to be a hypervirulent strain as it contained two variants of the *stx2* gene and was absent for the *stx1* gene (Underwood *et al.*, 2013). Thus, the evolving nature of the *stx* toxins is also an important area of note regarding these human pathogens.

The addition of antibiotics to an *Ec*O157-infected individual has found to cause significantly more damage, as the bactericidal effects caused lead to a large release of the toxin, which in turn causes severe haemorrhaging (Dahan *et al.*, 2004). Thus, there is still no treatment option available for those infected – only if the development of HUS occurs can treatment be offered to the damaged kidneys, but again not to remove the bacteria, only to counteract the damage.

1.4 Transmission of *Ec*O157 within the environment

There are a number of routes of transmission of human pathogens from their animal host to humans, both directly and via a plant host intermediate (Figure 1-1). The most well-known method of transmission of *Ec*O157 from animal to human is via the ingestion of contaminated, undercooked meat. Zoonotic pathogens can also be transmitted by direct contact and can pose a threat to farm workers or in petting zoos. These bacteria reside mainly within cattle (and other ruminants) commensally, with no pathogenic effect due to a lack of the Gb3 receptor within these species. *Ec*O157 can be spread between cattle, especially via the presence of "super-shedders" – cattle which deposit large numbers of *Ec*O157 (> 10^4 colony forming units (cfu)/gram (g)) into the environment via faeces (Chase-Topping *et al.*, 2008). Cattle are one of the main reservoirs of *Ec*O157 and can not only pose a risk by the consumption of beef products but also by their ability to deposit *Ec*O157 into the environment.

Once *Ec*O157 is transmitted into the soil via faeces from cattle, the bacteria can persist within this environment for long periods of time, with studies reporting viable and recoverable bacteria six months after inoculation (Islam *et al.*, 2005). Another study found that a strain of *Salmonella* could be isolated from the soil five years after it was originally detected as the source of an outbreak (Uesugi *et al.*, 2007).

From the soil *Ec*O157 can be transmitted to produce plants if deposited into soil near growing fields or they can be spread via contaminated irrigation water (Solomon *et al.*, 2002). *Ec*O157 and *Salmonella* can persist within water sources. For example, *Salmonella enterica* serovar Newport was involved in an outbreak in 2002, affecting 510 people and again in 2005 affecting 72 individuals. The source of the outbreaks was traced back to contaminated irrigation ponds at a set of fields and the same strain from 2002 was found to be the source of the 2005 outbreak (Greene *et al.*, 2008).

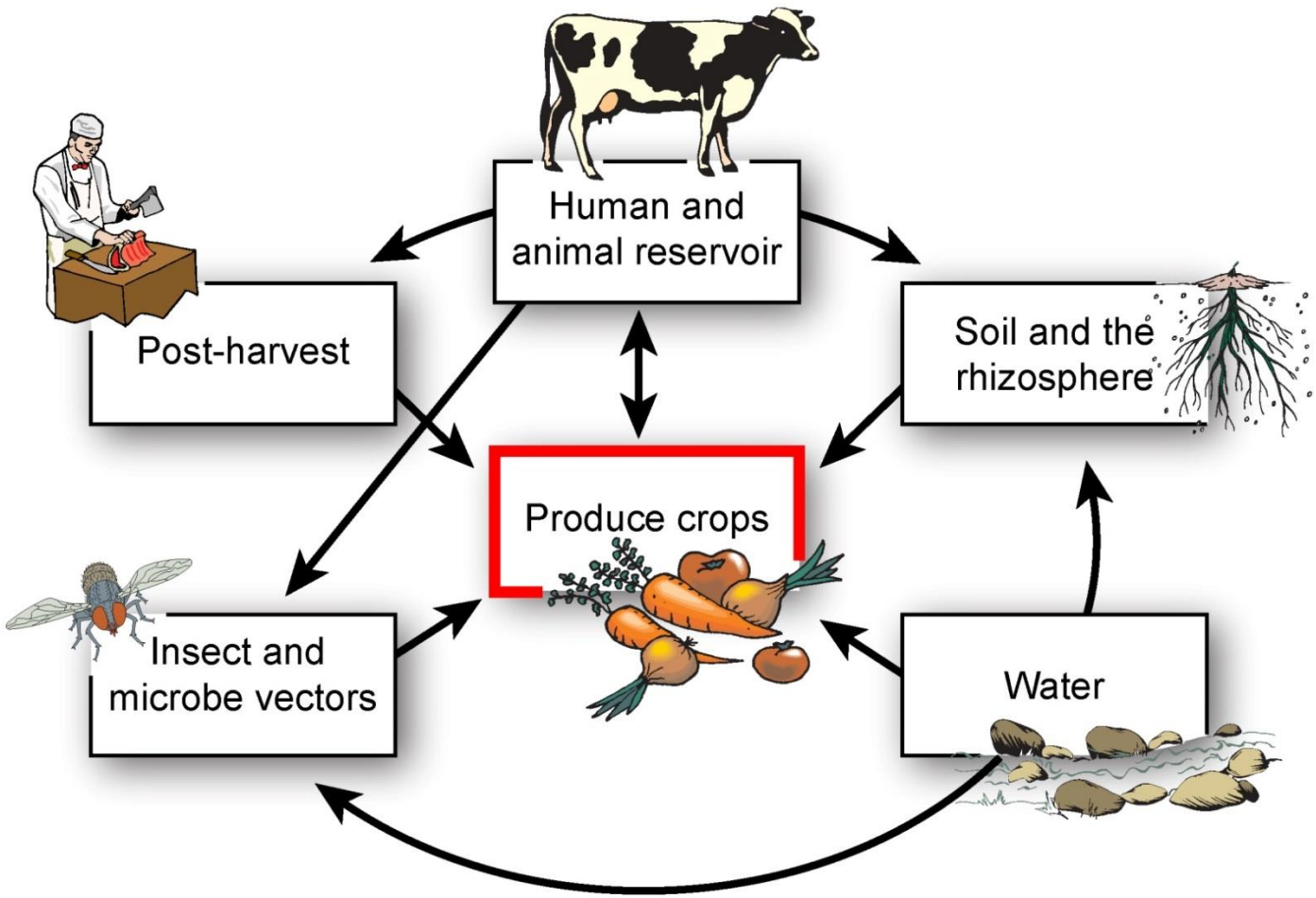


Figure 1-1. Modes of transmission of human pathogens from their mammalian hosts to humans via the environment.

Figure designed and published by Nicola Holden (Holden, 2010).

Furthermore, other modes of transmission that have been reported as possible include vectors such as birds and insects. Starlings in the USA were able to transmit *Ec*O157 between crops (Kauffman & LeJeune, 2011), and fruit flies were shown to contaminate apples with *Ec*O157 (Janisiewicz *et al.*, 1999a). Seed contamination is another area of concern: seeds contaminated with *Salmonella* and *Ec*O157 contained viable bacteria after two years of storage and subsequent germination into seedlings led to the isolation of these pathogens from the plant (Van der Linden *et al.*, 2013). Finally, wild animals have been implicated in transmitting *Ec*O157 to plants: one outbreak of *Ec*O157 from strawberries was attributed to wild deer faeces near the plants (Laidler *et al.*, 2013).

Therefore there are many routes of transmission that can occur for human pathogens from a mammalian host to a plant host. However, contamination of plants does not only occur pre-harvest: studies have found that many foods can be exposed to the bacteria during the process and packaging procedures, e.g. from cross-contamination of infected material, unsanitary machinery and human error (Buchholz *et al.*, 2012). As these food products are able to become contaminated in a variety of ways both pre- and post-harvest, prevention is not an easy task. As a result, many studies have focused on the removal of the bacteria once they are on the plant tissue.

1.5 Prevention of Human Pathogens on Plants

Current washing and sanitizing procedures used in the food processing industry include the use of hypochlorite (sodium or calcium) solutions to reduce the number of human pathogenic bacteria present on the plant tissue (Sapers, 2001). One challenge of the sterilising procedure is that the method employed needs to be effective enough to remove the human pathogen but without being too damaging to the food product itself by altering the taste or texture. Hypochlorite treatments do not affect the vegetable products at relatively low concentrations, however studies have reported low efficiencies in the reduction of *Ec*O157 and *Salmonella* (Keskinen & Annous, 2011; Niemira, 2007) and an inability to prevent regrowth of the bacteria after treatment using the hypochlorite treatment (Lang *et al.*, 2000), leading others to investigate new methods for the treating fresh produce.

Other sanitising treatments have been proposed, such as a follow-up step to the washing procedure by a 2 % L-lactic acid treatment at 55 °C (Neal *et al.*, 2012). This treatment resulted in a 2.7 log cfu/g reduction of *Ec*O157 on spinach leaves. Another study proposed the use of ultraviolet (UV) radiation combined with H_2O_2 treatment: this method resulted in a reduction of *Salmonella* and *Ec*O157 on the surface of and within lettuce leaf tissue with no obvious discolouration of the leaf (Hadjok *et al.*, 2008). An alternative treatment method that avoids the need for wash solutions is an oscillation method. A 3.73 log cfu reduction was seen on spinach leaves after a 20 hertz (Hz) treatment for ten seconds (Kim *et al.*, 2011); however, as was the case for the L-lactic acid and the UV/H_2O_2 treatments, complete removal of the bacteria was not observed using this method.

As well as sanitising agents, biocontrol using phytopathogens has also been proposed as a method for reducing the association of human pathogens with plants. A 10 fold reduction was reported for *Ec*O157 on wounded apples that were co-inoculated with *Pseudomonas syringae* (Janisiewicz *et al.*, 1999b). Both *Ec*O157 and *Salmonella* were reduced at least 100-fold when tested on lettuce and *Arabidopsis* when *Enterobacter asburiae* was added (Cooley *et al.*, 2003; Cooley *et al.*, 2006). In both cases, the mechanism of prevention is unknown: the reduction in

colonisation of *Ec*O157 may be due to the presence of anti-microbial compounds produced by these added bacteria, by induction of the plant defence system or, perhaps most likely, by out-competing the human pathogen for important macronutrients required from the plant. In contrast, many studies have reported that some epiphytic bacteria can actually increase the proliferation of human pathogens, as detailed further in section 1.6.

The use of bacteriophages as a method of prevention of human pathogens on plants has also been suggested. This method has also reported some mixed results, as a complete reduction of the bacterial population has not been achieved. *Salmonella* inoculated on alfalfa sprouts were seen to have only a ten-fold reduction in their population when bacteriophage were added (Kocharunchitt *et al.*, 2009). A 2.5 log reduction in the cfu counts were reported when three different *Ec*O157 bacteriophages were used together on *Ec*O157 colonising lettuce and cantaloupe (Sharma *et al.*, 2009b). As no complete reduction in the bacterial population in seen it is not clear if the use of bacteriophage is a viable method. It is proposed that the bacteriophage are unable to completely remove the bacterial population due to either the bacteria having protection within a biofilm (Chekabab *et al.*, 2013) or an equilibrium is reached in the infection process by the phage, meaning that as more host cells are destroyed there are less available for the phage to infect.

As mentioned previously, the fact that these human pathogens can internalise within plant tissue presents another issue in terms of developing a method to remove these bacteria. It has been reported that once the bacteria internalise into the plant tissue, many chemical sanitizers prove ineffective at removing the bacteria (Takeuchi & Frank, 2000). Therefore, we need to understand how the bacteria interact with the plant environment and how they are able to internalise into plant tissue so as to develop an effective method of prevention and removal of human pathogens from plants.

1.6 Interaction of Human Pathogens with the Environment and Plants

In order to survive and persist for long periods of time in the environment and on plants, *E. coli* must be able to metabolise many of the compounds present within the plant and soil. *E. coli* species are facultative anaerobes, which are classed as mesophilic in relation to temperature and neutrophiles in relation to pH (Neidhardt, 1996). There are specific metabolic pathways differences however within the *E. coli* strains, including within the *Ec*O157. The metabolic profiles of 18 different bovine and clinical *Ec*O157 strains were found to differ in terms of their oxidative activity of 192 carbon sources, with some of the strains also able to survive for longer periods of time in the soil (Franz *et al.*, 2011). Another study looking at *E. coli* strains, including *Ec*O157, found that only 36 % of carbohydrate metabolism pathways were found in the "core" genome, i.e. were well conserved between *E. coli* strain; these metabolic pathways outside the core genome, i.e. present in only certain species, included pathways in amino acid metabolism and alternate carbon source metabolism (Monk *et al.*, 2013). Little work has been carried out on the metabolic changes that occur in *Ec*O157 species when they are in the environment, including in the presence of plants. However, due to the large metabolic flexibility of *E. coli* as a species and the wide variety of nutrients present in rhizosphere, including sugars, amino acids (Moe, 2013) and organic acids (Oburger *et al.*, 2009), it could be speculated that there would be suitable nutrients for survival; however, other conditions such as pH, temperature and humidity will all play an important role in *Ec*O157 survival in the environment.

As *Ec*O157 can be introduced to the plant via the soil, water or directly onto the leaves this bacterial species will need to have adapted to survive in a variety of conditions. Previous studies have shown this human pathogen can survive in the soil environment for 25-92 days, depending on the type of soil used (Gagliardi & Karns, 2002). Further to this, phenotypic microarray examination of an environmental strain of *E. coli* in comparison to the reference laboratory strain, *E. coli* K-12, found that the environmental strain could utilise 161 substrates at 15 °C whereas K-12 could only use 67 (Brennan *et al.*, 2013). The majority of these substrates were sources of sulphur, phosphorus and nitrogen, although many carbon sources were also seen to be utilised by the environmental strain and not the laboratory-adapted strain. Interestingly,

another study found the application of ammonium nitrate enhanced the growth of *Ec*O157 on lettuce leaves, whereas the addition of glucose had no effect (Brandl & Amundson, 2008), suggesting that nitrogen utilisation may play an important role in the survival of this human pathogen on plants. The fact that human pathogens can survive and colonise the rhizosphere and roots of plants is not surprising due to the commonalities between roots and mammalian guts (Ramírez-Puebla *et al.*, 2012): both contain large, diverse microbiota and microhabitats that are influenced by factors such as pH, nutrients, water and oxygen gradients.

Survival of human pathogens within the rhizosphere and on plant tissue can be enhanced by certain plant-associated microbes, especially those that contain enzymes to degrade plant tissue. For example, a three year survey of produce in supermarkets found that out of 400 samples tested from 40 different fruits and vegetables, approximately 60 % that were affected by bacterial soft rot also harboured *Salmonella* (Wells & Butterfield, 1997). Populations of *Ec*O157 grew ten times higher on intact lettuce leaves when co-inoculated with *Erwinia chrysanthemi* (*Dickeya dadantii*), a plant pathogen (Brandl, 2008). Finally, higher cfu counts of *Salmonella* were seen when colonising tomato fruits that were infected with *Pectobacterium carotovorum* compared to the uninfected fruits (Noel *et al.*, 2010b).

It is thought that this increase in numbers of *Salmonella* and *Ec*O157 when co-colonising with other microbes is due to the accessibility of nutrients as a result of the plant cell wall degrading enzymes secreted by the plant pathogens. They could form associations with other microbes in the plant environment in the form of a biofilm: however, although the presence of *Dickeya dadantii* increased the population of *Ec*O157, no difference was seen for *Dickeya* populations when inoculated with or without *Ec*O157 (Brandl, 2008).

As well as being able to colonise the roots, leaves and fruits of many plants, studies have shown that *Ec*O157 can also internalise into the plant tissue, residing within the mesophyll, stomata, cortex and root hairs (Deering *et al.*, 2012). Internalisation of bacteria has been seen to occur via natural plant openings such as stomata (Gomes *et al.*, 2009; Kroupitski *et al.*, 2009a) or via plant wounds (Burnett *et al.*, 2000), including by damage induced by plant pathogens such as nematodes (Beuchat *et al.*, 2003). Root hairs have been implicated as an area of considerable

susceptibility to bacterial internalisation, with both *Ec*O157 (Wright *et al.*, 2013) and *Salmonella* (Schikora *et al.*, 2008) detected within these appendages after inoculation into the rhizosphere.

There have been many conflicting reports of internalisation of human pathogens into plant tissue, with some suggesting it is an active process and others claiming it is passive. Studies have shown that these human pathogens are chemotactically attracted towards these openings in the plant, such as plant lesions (Brandl, 2008) and stomata (Kroupitski *et al.*, 2009a). It is predicted that photosynthesis may play an integral role in the chemotaxis process as the bacteria did not migrate towards stomata that were artificially induced to remain open in the dark (Kroupitski *et al.*, 2009a). Stomatal colonisation and internalisation is reduced in *Ec*O157 that have an impaired T3SS (Shaw *et al.*, 2008), suggesting that this is an integral component required for survival within the plant. However, many others have proposed that internalisation is not mediated by the bacteria and is a result of transpiration processes by the plant. One study examined the uptake and subsequent internalisation of *Ec*O157 cells and fluorescent microspheres by growing lettuce plants and found that both were located within plant tissue after five days in similar numbers (Solomon & Matthews, 2005), suggesting that the internalisation process is mediated by the plant and not by the bacteria. Furthermore, studies have implicated a low efficiency of internalisation, with 99.7 % of lettuce samples grown in *Ec*O157-inoculated soil having no internalised bacteria after 60 days (Zhang *et al.*, 2009). Another study looking at field-grown spinach found that 16 % of the samples contained internalised *Ec*O157 seven days post-inoculation – however, no internalised *Ec*O157 could be detected after 14 or 21 days post-inoculation, suggesting the bacteria could not persist within the plant (Erickson *et al.*, 2010).

In order to persist on or within the plant, these human pathogens would need to evade or circumvent the plant defence response. Both *Ec*O157 and *Salmonella* contain pathogen-associated molecular patterns (PAMPs) that can be recognised by the plant defence response, such as flagellin. The flagellin of *Salmonella* has been shown to be recognised by FLS2 in *Arabidopsis*, leading to activation of the plant immune response (Garcia *et al.*, 2013). Stomatal closure, one of the first lines of defence against plant pathogens, was induced by both *Ec*O157

and *Salmonella*; however, *Ec*O157 appeared to induce a stronger defence response, with the stomata remaining closed yet stomata responding to *Salmonella* infection were open after four hours (Roy *et al.*, 2013). *Salmonella* induced the plant defence genes *PFD1.2* and *PR1* after two hours of exposure indicating jasmonic acid (JA) and ethylene (ET) resistance pathways were upregulated in response to this human pathogen (Schikora *et al.*, 2008). Finally, *Salmonella* has also been shown to induce effector-triggered immunity (ETI) in *Nicotiana benthamiana*, as the T3SS effector protein sseF was recognised by the plant, eliciting HR-like symptoms (Üstün *et al.*, 2012). Finally, a pathogenic role for *Salmonella* has been suggested for cowpea, as inoculation of seeds and sprouts resulted in a reduced germination rate and defective sprout production (Singh *et al.*, 2007). The plant defence response to *Ec*O157 has not been studied in as great detail as *Salmonella*, with no studies suggesting a plant pathogenic role for *Ec*O157. Therefore, perhaps this bacterial species relies on the presence of other plant-associated microbes that can suppress the plant immune response.

Overall, there have been a number of studies looking at how *Ec*O157 can survive on different plant species over time. However, little is known about how this human pathogen has adapted to interact with and colonise the plant environment and what genes are necessary for its survival. A few transcriptomic studies have been carried out on this pathogen in response to parts of the plant, which gives a starting point for understanding this interaction (as detailed further in Chapter 5). However, most of the work has focused on conditions that are optimal for the bacteria to survive, rather than focused on optimal plant conditions.

A further point of note is that viable but non-culturable (VBNC) *Ec*O157 in plants may present a significant risk in fresh produce outbreaks, as colony-screening methods cannot detect the bacteria when they are in this dormant state. A large proportion of a population of *Ec*O157 have been shown to be VBNC shortly after inoculation onto plants (Moyne *et al.*, 2013), suggesting that this initial exposure induces very large changes in the gene expression of the bacteria. Studying this initial exposure to the plant environment may identify genes of biological significance for this environment.

Therefore, my project aims to give an understanding of how this human pathogen adapts to different plant environments by examining the gene expression changes occurring when *Ec*O157 is incubated with different plant extracts (after a one hour exposure, to understand the adaptive response before the bacteria underwent cell division) using a transcriptomic approach. This will allow us to understand how *Ec*O157 responds to different components of the plant, how it utilises them to its advantage and identify any gene clusters which may be important for the colonisation of plants.

Beginning to understand the interaction between *Ec*O157 and plants will hopefully lead to new developments in the future to prevent the association of this bacterial strain with fresh produce. As no fully effective treatment is available for removing the bacteria from fresh produce and no treatment method is available for the infection in humans, it is essential that we understand this process to combat this interaction in the future. The work also adds information regarding the lifestyle of microbes outwith their primary host, which is still of much question.

1.7 Aims of this project

Two strains of *Ec*O157 were chosen for this project: a bovine isolate, EDL933, which was first isolated from contaminated hamburgers in 1983 (Wells *et al.*, 1983) and since then has been the focus of much study for both cattle and human infection; and a plant-associated outbreak isolate, Sakai, which was isolated from an individual who had consumed contaminated white radish sprouts (Makino *et al.*, 1999). Both strains are well characterised in terms of their ability to infect humans and colonise cattle; however, little work has been looked at these strains in the environment. As there is a wealth of information on these strains in the mammalian host, I decided these strains would be ideal for my project, as it would allow comparisons between strains of *Ec*O157 and between the adaptation to different hosts (mammalian versus plant). Other plant-associated outbreak strains of *Ec*O157 are available, such as the spinach outbreak strain TW14359 (CDC, 2006), but less is known about their metabolic and virulence capabilities at this point in time. However, it could be a consideration for the future to use the methods in

this project with strain TW14359 and compare it to *Ec*O157 Sakai, to test for differences between plant-associated *Ec*O157 species.

As *Ec*O157 strains have been isolated from a number of different plant sources, it is thought that these bacteria must be well adapted to survive in a wide range of environmental conditions. However, it is unknown if *Ec*O157 Sakai and EDL933 strains can persist and colonise plant tissue a) to the same extent as each other and b) to a similar level for different plant species. Furthermore, it is unknown how *Ec*O157 strains adapt to and survive within the different environmental niches present when colonising plants, especially in terms of their metabolism and ability to utilise plant substrates. As a starting point for examining this question, I decided to study the response of *Ec*O157 Sakai, an *Ec*O157 strain which had previously been linked to plants, to plant extracts of spinach and lettuce, two well-known carriers of *Ec*O157 strains. These plant extracts would help represent different areas of the plant that the bacteria may be exposed to (detailed further in Chapter 5).

Therefore, the aims of this project were:

A. To assess the ability of *Ec*O157 to colonise living plant tissue and utilise plant extracts from four plant species (Chapter 3).

B. To identify genetic differences between a bovine-derived isolate and a plant-associated isolate of *Ec*O157 (Chapter 4).

C. To identify groups of genes that were differentially expressed in *Ec*O157 when incubated at an environmentally-relevant temperature (18 °C) compared to the human body temperature (37 °C) (Chapter 5).

D. To identify groups of genes that were differentially expressed in response to plant extracts of spinach and lettuce, including leaf lysates, root exudates and leaf cell wall polysaccharides (Chapter 5 and Chapter 7).

E. To identify metabolic pathways that were significantly upregulated in *Ec*O157 in response to plant extracts (Chapter 6).

The overall aim of this project is to identify genes that are differentially expressed in *Ec*O157 Sakai when in the plant environment, whether in the presence of living plant tissue or with plant extracts. It is planned that any genes identified as significantly expressed in these conditions could be studied further in future work, via the use of knockout studies for example, to verify the necessity of these genes for colonisation of these human pathogens on plants. Therefore, genes identified could eventually be a target for prevention studies in the future. Individual aims for each area of work are detailed in the relevant chapters.

2 Materials and Methods

2.1 Bacterial strains/plasmids and culture conditions

Strains and plasmids used in this study are listed in Table 2-1. Strains were grown overnight in Luria-Bertani (LB) media at 37 °C, 200 revolutions per minute (rpm) then subcultured in a 1:100 dilution into MOPS media (10 x MOPS solution (stock of 1L contains: 400 ml of 1 M potassium morpholinopropane sulfonate (MOPS), pH 7.4; 40 ml of 1 M N-Tris(hydroxymethyl)-methyl glycine (tricine), pH 7.4; 10 ml of 0.01 M $FeSO_4$; 50 ml of 1.9 M NH_4Cl; 10 ml of 0.276 M K_2SO_4; 10 ml of 0.5 mM $CaCl_2$; 10 ml of 0.528 M $MgCl_2$; 100 ml of 5 M NaCl; 10 ml of micronutrients (3 µM $(NH_4)_6MO_7O_{24}.H_2O$; 0.4 mM H_3BO_3; 0.03 mM $CoCl_2$; 0.01 mM $CuSO_4$; 0.08 mM $MnCl_2$; 0.01 mM $ZnSO_4$); 360 ml sterile distilled water); 0.2 % glycerol; 132 mM K_2HPO_4; 0.02 M thiamine HCl; 50x essential amino acids and 100x non-essential amino acids (Sigma Aldrich, St. Louis, USA)) (Neidhardt *et al.*, 1974) at 18 °C, 200 rpm until stationary phase.

For all RNA extractions, bacterial strains were grown in M9 media (20 ml M9 salts (x 5) (5 x M9 salts: 64 g of $Na_2HPO_4.7H_2O$; 15 g of KH_2PO_4; 2.5 g of NaCl; 5 g of NH_4Cl; dissolved in 1L sterile distilled water) 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; 0.2 % glycerol; pH 7.0) (Miller, 1972), at 37 °C, 200 rpm then subcultured in a 1:100 dilution into fresh M9 media containing 0.2 % glycerol and grown at 18 °C, 200 rpm unless otherwise stated.

Table 2-1. Bacterial strains and plasmids used in this study.

Designation	Details	Reference
*Ec*O157:H7 Sakai	Kan^R, insertional inactivation of verotoxin genes	(Dahan *et al.*, 2004)
*Ec*O157:H7 TUV 93-0	NA^R. EDL933 derivative, deleted for verotoxin genes	(Donohue-Rolfe *et al.*, 2000)
E. coli DH5α	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Invitrogen, Life Technologies, USA
TUVΔ*lacZY*	TUV 93-0 deleted for *lacZY;* NA^R, Tet^R	This study
SakaiΔ*araBAD*	Sakai deleted for *araBAD* locus	This study
SakaiΔ*ECs1335*	Sakai deleted for *ECs1335*	This study
SakaiΔ*emrD*	Sakai deleted for *emrD*	This study
SakaiΔ*dctA*	Sakai deleted for *dctA*	This study
pUC19	High-copy number cloning vector, Amp^R	Invitrogen, Life Technologies, USA
pTOF24	Temperature-sensitive cloning vector, Cm^R	(Merlin *et al.*, 2002)
pTOF1	Contains tetracycline cassette at *Not*I sites, Tet^R	Ashleigh Holmes

2.2 Primer design

A list of primers used in this study can be found in Table 2-2. All primers were designed using Primer3Plus (Untergasser *et al.*, 2012), checked for their specificity using NCBI Primer-BLAST (Ye *et al.*, 2012) and any hairpins, dimers and cross-dimers were predicted using Netprimer (PremierBiosoft).

All primers for qPCR were designed in the same manner as above, but with the following parameters: 100-200 base pair (bp) product size; paired towards 3' end; GC content of 50-60 %; optimum melting temperature (Tm) of 60 °C.

2.3 PCR Reaction Setup and Cycling Conditions

Different polymerases and reagents were used for the different PCR tests carried out: GoTaq PCR conditions were used for diagnostic tests; Phusion for all cloning steps due to its proof-reading ability; and Expand for fragments amplified which were longer than 3 kb. Cycling conditions for all three polymerases are detailed below.

2.3.1 GoTaq PCR conditions

GoTaq buffers and polymerase (Promega, Madison, USA) were used to set up a PCR in the following protocol: 5 µl 5x GoTaq buffer; 3 µl 2.5 mM dNTPs; 0.5 µl of each 50 µM forward and reverse primer; 0.2 µl GoTaq polymerase; 0.5–1 µl template; molecular biology grade water to 25 µl.

Cycling conditions were as follows: initial denaturation at 95 °C for two minutes; 30 cycles of denaturation at 95 °C for one minute, annealing at 50–65 °C (depending on primer pair used) and extension at 72 °C at one minute/kb; and a final extension step of 72 °C for five minutes.

PCR products were migrated on a 0.7–2 % agarose gel, depending on the expected product size, by running at 80 V, 300 mA, 50 W for 30 minutes and visualised under ultraviolet (UV) light.

2.3.2 Phusion PCR conditions

Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) buffers and enzyme were used as required for the generation of constructs, mutants and sequencing. The reaction was prepared as follows: 10 µl 5x Phusion HF buffer; 4 µl 2.5 mM dNTPS; 1 µl of each 50 µM forward and reverse primer; 0.5 – 1 µl template; 0.5 µl Phusion polymerase (1 unit/50 µl); molecular biology water to 50 µl.

Cycling conditions were as follows: initial denaturation of 98 °C for 30 seconds; 30 cycles of 98 °C for ten seconds, 50–70 °C (dependent on primers used) for 30 seconds, 72 °C at 30 seconds/kb; and a final extension of 72 °C for ten minutes.

2.3.3 Expand PCR conditions

Expand High Fidelity PCR System, (Roche Applied Science, West Sussex, UK) buffers and enzymes were used following the manufacturer's recommended setup. Briefly, two master mixes were prepared: the first contained 1 µl 10 mM dNTPs, 0.5-1 µl forward and reverse primers, 0.5-1 µl of template and molecular biology grade water to 25 µl; the second mix contained 19.25 µl of molecular biology grade water, 5 µl of Expand Hi-Fi 15 mM $MgCl_2$ 10x buffer and 0.75 µl of Expand polymerase (1 unit/1 µl). The two solutions were mixed and aliquoted into sterile PCR tubes and incubated in a thermo cycler with the following program: initial denaturation of 94 °C for two minutes; ten cycles of denaturation at 94 °C for 15 seconds, annealing at 50-65 °C (depending on primers used) for 30 seconds, elongation at 68 °C if product was bigger than 3 kb, or 72 °C if it was smaller, at a rate of 45 seconds/kb; 20 cycles of denaturation at 94 °C for 15 seconds, annealing at 50-65 °C for 30 seconds, and an elongation step at 72 °C at a rate of 45 seconds/kb, but with a five second extension time each cycle; followed by a final elongation step of 72 °C for seven minutes.

Table 2-2. Primers used in this study, subcategorised into diagnostic, cloning, and qPCR primers.

Target	Primer Name	Sequence	Tm (° C)	Product Size (bp)	Application	Reference
Diagnostic Primers						
gadA	gadA_for gadA_rev	ACCTGCGTTGCGTAAATA GGGCGGGAGAAGTTGATG	56	670	*E.coli* diagnostic primers	(Kim *et al.*, 2006)
stx2	Stx2_F Stx2_R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	55	255	VTEC diagnostic primers	(Aranda *et al.*, 2004)
Cloning Primers						
lacZY 500bp N flank	NoLacZY_*Pst*I NiLacZY_*Not*I	AAAAACTGCAGGATACTACCCGCGCCAATAA CGCTCTTGCGGCCGCTTGGAACGGCGGAATGATCGCAATGAAC	65	502	*LacZY* knockout	This study
lacZY 500bp C flank	CoLacZY_*Sal*I CiLacZY_*Not*I	AAAAAGTCGACCAACAAACCATGCAAATGCT CCGTTCCAAGCGGCCGCAAGAGCGTATCCGCTCACAATTCCACA	65	504	*LacZY* knockout	This study
lacZY	LacZY-F.ext LacZY-R.ext	GCCTGCTTTTATTCTTTCTGTCA AGTGAGCGCAACGCAATTA	59	4608	*LacZY* knockout confirmation	This study
araBAD 500bp N flank	ECs0066No_for ECs0066Ni_rev	AAAAACTGCAGCGGTCGGTCGATAAAAAA CGCTTCTTGCGGCCGCTTGGAACGGAAAGCTCGCACAGAATCA	65	500	*araBAD* knockout	This study
araBAD 500bp C flank	ECs0066Ci-for ECs0066Co_rev	CCGTTCCAAGCGGCCGCAAGAGCGGCCAACTCACCGACTACT AAAAAGTCGACCATTTGATTGGCTGTGGTT	65	500	*araBAD* knockout	This study
araBAD	AraBADSeqF AraBADSeqR	GCCTGGTTTCATTTGATTGG TTAGCGGATCCTACCTGACG	58	4227	*araBAD* knockout confirmation	This study
araBAD	AraBAD-F.ext AraBAD-R.ext	CACGCTGAAAATCCATCAAA GCGCTTCAGCCATACTTTTC	58	4537	*araBAD* knockout confirmation	This study
ECs1335 500bp N flank	ECs1335No_for ECs1335Ni_rev	AAAAACTGCAGCAACGAACCCGCATAAAC CGCTCTTGCGGCCGCTTGGAACGGCCGGCGCAACAGAATAAA	65	500	*ECs1335* knockout	This study
ECs1335 500bp C flank	ECs1335Ci_for ECs1335Co_rev	CCGTTCCAAGCGGCCGCAAGAGCGCGTTTCCAGAAGGCTCAT AAAAAGTCGACGTGTACAAATCTCTGGCGG	65	500	*ECs1335* knockout	This study
ECs1335	ECs1335-F.ext ECs1335-R.ext	CTGTGTATTCCCGGCAGACT GCGGTTTCGCAAATCTGTAT	58	1614	*ECs1335* knockout confirmation	This study
emrD 500bp N flank	ECs4614No_for ECs4614Ni_rev	AAAAACTGCAGTGTGTTATGTCAACGAGC CGCTCTTGCGGCCGCTTGGAACGGCACGAGTAATACCAACATCA	65	500	*emrD* knockout	This study
emrD 500bp C flank	ECs4614Ci_for ECs4614Co_rev	CCGTTCCAAGCGGCCGCAAGAGCGATTGTTGATCGTGCTGTG AAAAAGTCGACACCTGCCGATTTTTATCAC	65	500	*emrD* knockout	This study
emrD	EmrD-F.ext EmrD-R.ext	GCAACATTTATTGCCGCTTT GGGTGATGCGTGATCTGATA	59	1420	*emrD* knockout confirmation	This study
dctA 500bp N flank	ECs4408No_for ECs4408Ni_rev	TGGATGATTTCGGCATGG CGCTCTTGCGGCCGCTTGGAACGGAGAGGTTTTCATAGGGTGT	65	500	*dctA* knockout	This study
dctA 500bp C flank	ECs4408Ci_for ECs4408Co_rev	CCGTTCCAAGCGGCCGCAAGAGCGGAACTGGACCACAAAAAAC AAAAAGTCGACGAATTTCAACACGATCGCT	65	500	*dctA* knockout	This study

dctA	DctA-F.ext DctA-R.ext	GAGCGAACCGGTATTAACCA TTCCTTTTTGCTCGTCCACT	58	1956	*dctA* knockout confirmation	This study
Vector insert	M13F M13R	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	55	Dependent on insert	Sequencing primers for vectors	Promega, USA
pTOF24 vector	pTOF.F pTOF.R	GTCGTTAAATAGCCGCTTATG GCGGTCACGCTGCGCGTAAC	55	Dependent on insert	Confirm insert in pTOF24 vector	Ashleigh Holmes
TetR cassette	TetR.F	GCCTTCAACCCAGTCAGCTC	55	Dependent on primers	Confirm tetracycline resistance cassette	Ashleigh Holmes
qPCR reference gene primers						
mfd	1492-F 1492-R	CCGTGAGTATGACAGCCTGG TCATCACCCGTTCCAGTTCG	60	194	Housekeeping gene for qPCR	This study
rpoN	4081-F 4081-R	AACCAGCGTAAACAGCTCGT TTGCATCTGAGGTGTGGGTG	60	203	Housekeeping gene for qPCR	This study
gadA	GadA-F GadA-R	CCGCGTGTGAAATCGATCAGTGC GGCAAAAGTACCAATTTGACCACCC	60	150	Housekeeping gene for qPCR	This study
gyrB	gyrB.RT.F gyrB.RT.R	CATCAGAGAGGTCGGCTTCC CATGGAGCGTCGTTATCCGA	60	80	Housekeeping gene for qPCR	Ashleigh Holmes
invA	InvA-F InvA-R	ACCAGCAAAGGCGAGCAGCC GCCGGCTCTTCGGCACAAGT	60	150	Housekeeping gene for qPCR	This study
ipxL	lpxL-F.1 lpxL-R.1	TCCCTTTATTTGCCGTTGAG TCGACCACTTTGTTCATCCA	60	202	Housekeeping gene for qPCR	This study
parE	parE-F.1 parE-R.1	GATGCGGACTCTGATGGTTT TGCTCAAGTACGCCCTCTTT	60	182	Housekeeping gene for qPCR	This study
rnc	rnc-F.1 rnc-R.1	AGTGCCAGCAGCAAACATAAC TCATATCGCCTTCATCCACA	60	115	Housekeeping gene for qPCR	This study
arcB	arcB-F.1 arcB-R.1 arcB-F.2 arcB-R.2	TCATCAGTAACGCCGTCAAA GCCGATACCAGAATCTTCCA TATTGGTCTGGCCGTTTCTC ACGCATCATCGACCTCTTCT	60 60	107 137	Housekeeping gene for qPCR	This study
qPCR primers						
aaeX	4115-F 4115-R	ATCGTGGTGTTTGGGCTGTC GCAATAGAGCGCGGTGTTGA	60	150	qPCR for gene expression levels	This study
araA	araA-F.1 araA-R.1 araA-F.2 araA-R.2	CGGTCACTGGCAGGATAAAC GACGGAGAAACCGAACTTGA CTGAAACAGCTTCCTGGTCTG CACCAGGTCATTGCCTTTCT	60 60	175 186	qPCR for gene expression levels	This study
araB	0067-F 0067-R 0067-F2	GCGCGTTTGACTGCCATATG GACCGATAAATCCAGGCACCA AGCGCCGAACAACACTATCT	60 60	180 209	qPCR for gene expression levels	This study

	0067-R2 araB-F.1.2. araB-R.1 araB-F.1.2. araB-R.2 araB-F.3 araB-R.3	GTTTCGCTTCCGTATTCAGC GAGCGCCGAACAACACTATC AGATGCTGGCTGCCAATTAC GAGCGCCGAACAACACTATC GGCTGCCAATTACAAACCAC CCGAATAACCAGTTCCGTCA GGTACTGTCAACGCCAATCC	60 60 60	133 126 129		
araD	araD-F.1 araD-R.1 araD-F.2 araD-R.2 araD-F.3 araD-R.3	GATACGCCAACTCACCGACT GTTGCTGGAATCGACTGACC GCAAACGCTGCTGGATAAAC GCCTGGTTTCATTTGATTGG CACGCCGACTATTTCTACGG CCGTGAGAATGGACCAGAAC	60 60 60	116 103 164	qPCR for gene expression levels	This study
araJ	0446-F 0446-R	TGATTGGTAGCCGTTCTGGT GAGTGAGTAGCGGCTGGAAA	60	236	qPCR for gene expression levels	This study
atpA	4676-F 4676-R	TCCTGGAAACCAACCTGTTC TGTTTCAGCAGTTCGGTCAC	60	200	qPCR for gene expression levels	This study
caiF	0037-F 0037-R	CGGTGGGTGATAGCAAGAGAG TTCCCGACTGTTATTGCGCA	60	222	qPCR for gene expression levels	This study
carA	0035-F 0035-R	ACTTCTGCGGAAGATGTGCT TTCGAGGAATTTCTGGATGG	60	114	qPCR for gene expression levels	This study
csgA	1420-F 1420-R	AACGGTGCAGATGTTGGTCA GCGGTCGCGTTGTTACCAA	60	215	qPCR for gene expression levels	This study
csgE	1416-F 1416-R	CACGCCGTTGAGGTAGAAGT CGTCCTGATTGACCGTTATTG	60	181	qPCR for gene expression levels	This study
cspA	4441-F 4441-R	GGTTCAACGCTGACAAAGGC TACAGGCTGGTTACGTTGCC	60	181	qPCR for gene expression levels	This study
cysJ	3619-F 3619-R	AGCGTTACGTCAAAGAGGGC AGTGCCTGCTCAACGTCTTT	60	190	qPCR for gene expression levels	This study
dctA	dctA-F.1.2 dctA-R.1.3 dctA-F.1.2 dctA-R.2 dctA-F.3.4 dctA-R.1.3 dctA-F.3.4 dctA-R.4 dctA-F.5 dctA-R.5	AGGTCATCTTCGGCATCATC GAACCCAATACCAGCACCAC AGGTCATCTTCGGCATCATC GCGATTGAACCCAATACCAG TCGAAAGTTTCTCGCAGGTC GAACCCAATACCAGCACCAC TCGAAAGTTTCTCGCAGGTC AGCGATTGAACCCAATACCA ATCCCGACAGGCTACTCGTT TAACAGCGTGATTTGGTGGA	60 60 60 60 60	172 178 187 194 120	qPCR for gene expression levels	This study
ECs0664	0664-F	CCGTGTTGATGCTGGTAATG	60	185	qPCR for gene	This study

	0664-R	TTGACCAGTGGTGCTCTTTG			expression levels	
ECs0845	0845-F 0845-R	AAGTGGGCAGTGATACAGGC CCTGCTCCGGAATAATGCCA	60	184	qPCR for gene expression levels	This study
ECs3027	3027-F 3027-R 3027.RT.F 3027.RT.R	TTTGCGCTGTATGAGTCGGT TTTCCAGCCGTACAACCACTC AGTGACACGCGCAGTAATTG ACCGCATCCATCATGGTAAT	60 60	177 182	qPCR for gene expression levels	This study
ECs3167	3167-F 3167-R	ATGGGGTTGTGCTTTCTTTG ATCAGGGTGGCGATAAACAG	60	126	qPCR for gene expression levels	This study
ECs3238	3238-F 3238-R	TCATTGACAGGACTCGTGGGT CGTAACGGAATCTCGGATCCTC	60	238	qPCR for gene expression levels	This study
ECs3674	3674-F 3674-R	TGATCGAGAAAGAGGGCAAT GTTCTGGGTTTGTGCCAGAT	60	227	qPCR for gene expression levels	This study
ECs3926	3926-F 3926-R	TTCCAGTTGCTGCATCTCAC ATTCCCACGGATACAGCTTG	60	202	qPCR for gene expression levels	This study
ECs4474	4474-F 4474-R	GCGTTGGGAGTGCTGATCTT TCGCCCAGATCCACAGAAAC	60	181	qPCR for gene expression levels	This study
ECs5162	5162-F 5162-R	CCTGGTTTGAGGGTGAAAAA TCCAGCTACCGATGAGTGTG	60	216	qPCR for gene expression levels	This study
ECs5290	5290-F 5290-R	AGGCTGGTGGGTGAATAATGG GCTCCCGGTGTAAATTGCGA	60	201	qPCR for gene expression levels	This study
emrD	4614-F 4614-R emrD-F.1.2 emrD-R.1 emrD-F.1.2 emrD-R.2 emrD-F.3 emrD-R.3 emrD-F.4 emrD-R.4	GTGACCTTCAGTATGGCCCG GACCGTCATACTGCTCAGCC TTCTGCCGCTAAACAATTCC CGGCCACGAGTAATACCAAC TTCTGCCGCTAAACAATTCC ACGGCCACGAGTAATACCAA GCAACATTTATTGCCGCTTT TGCGGGAGAGGAATCTATCA GGATTGTTGATCGTGCTGTG ATGGAGCTGATGACGATGCT	60 60 60 60 60 	213 188 189 107 103	qPCR for gene expression levels	This study
fadE	0248-F 0248-R fadE.RT.F fadE.RT.R	GCGTTGGTGGATGTGATTGC AACTTTACCGGCTTCGTCGC ATATCGGTCACGTCGGTAGC CTGCCATCGAGACATCAGAA	60 60	230 162	qPCR for gene expression levels	This study
fis	4133-F 4133-R	CGTACTGACCGTTTCTACCGTT GTACCACGGTTGATGCCCAT	60	234	qPCR for gene expression levels	This study
flhD	2602-F 2602-R	TGCATACCTCCGAGTTGCTG GCCAGCTTAACCATTTGCGG	60	172	qPCR for gene expression levels	This study
gadB	2098-F	GCTGGGTTATCTGGCGTGAT	60	151	qPCR for gene	This study

	2098-R	CCGAGGCGCAGGAATTCATA			expression levels	
gntT	4257-F 4257-R	AGAAGAGATGCCGAGCTTTG CCAGCGTGTCGTTAATCTGA	60	230	qPCR for gene expression levels	This study
ler	4588-F 4588-R	CGAGAGCAGGAAGTTCAAAGTGT TCTGCCCTTCTTCATTGCGG	60	160	qPCR for gene expression levels	This study
lsrB	2123-F 2123-R	TGGGCGTTGATGTGACCTAC ATCAGAGTCCCAGGTCAGCA	60	188	qPCR for gene expression levels	This study
malE	5017-F 5017-R	TTCACGAGCACTTCACCAAC CCTTTATCGCCGTTAATCCA	60	159	qPCR for gene expression levels	This study
mglA	3041-F 3041-R	ATTAGGGGCTTCCCATGAGT TGACAATTAACCCTGCCACA	60	207	qPCR for gene expression levels	This study
murB	4899-F 4899-R	AAGGGATGCAAATGGGTGGG AAGCGGACTTCAGGCTCAAG	60	160	qPCR for gene expression levels	This study
osmY	5334-F 5334-R	ACAACGCGCAGACTACCAAT GCCTGGCTTTCAACGAAACC	60	202	qPCR for gene expression levels	This study
phoH	1266-F 1266-R	AGCCAACCCTCTGATGTTCG GCGAGGTGACCGATTCTTCTT	60	187	qPCR for gene expression levels	This study
purF	3196-F 3196-R	TGTATACTTTGCTCGCCCGG CACACGAGGTTTCCGGGATC	60	152	qPCR for gene expression levels	This study
sodC	2355-F 2355-R	GGGTTGGGCAGTCAATTGGT TGGCCCTTCATGTTTACCGG	60	215	qPCR for gene expression levels	This study
spoT	4525-F 4525-R	ATGGGATAAAGAGACGGCGC CGCATGATATTCGCCAGATGC	60	210	qPCR for gene expression levels	This study
uhpC	4604-F 4604-R	CCGGGATGATGATTGCTGGT CGCCCGAACCACATAGACCA	60	248	qPCR for gene expression levels	This study
xylA	4448-F 4448-R	CGATGCTGCGACAGTCTATG AGCGCATTCTCTTCCACACT	60	225	qPCR for gene expression levels	This study
yhcN	4111-F 4111-R	CACGAAACAAAGGCCCAGC CGAAGACGCCACACCACTTA	60	189	qPCR for gene expression levels	This study

2.4 Plant growth conditions

Plants used were spinach cultivar (cv) Amazon (*Spinacia oleracea*) (Suttons Seeds, Paignton, UK), lettuce cv Salinas (*Lactuca sativa*) (Tozer Seeds, Surrey, UK), prickly lettuce (*Lactuca serriola*) (Tozer Seeds, Surrey, UK), and pea (*Pisum sativum*) (Dr Rob Jackson, Reading). Plants were grown in vermiculite (William Sinclair Holdings, Lincoln, UK) containing Osmocote Start six-week short-term base fertiliser or in compost (containing peat, sand, limestone, perlite, calcote, Sincrostart and Multicote 4) depending on the assay, at 75 % humidity, light intensity of 150 µmol m^2 s^{-1} (16 hour photoperiod: day temperature of 26 °C, night temperature of 22 °C).

For hydroponic assays, ~30 seeds were sterilised by first soaking in sterile distilled water (SDW) for two hours, followed by incubation with 2 % calcium hypochlorite ($(Ca(OCl)_2$); Sigma Aldrich, St. Louis, USA) for 15 minutes. Seeds were washed in SDW six times then left to soak for a further two hours. Seeds were germinated on distilled water agar (DWA) (0.5 %) plates for 16 hours in the dark at 22 °C, before being placed in a growth chamber with the conditions of 22 °C temperature, 16/8 hr light/dark cycle, 75 % humidity.

Once germinated, seedlings were transferred into hydroponic pots (Greiner, Frickenhausen, Germany) with 10 g of 2.5 mm perlite (William Sinclair Holdings, Lincoln, UK) and sterile 0.5 x Murashige and Skoog (Sigma Aldrich, USA) media without sucrose (referred to as 0.5 x MS) and left to grow for three weeks.

2.5 Colonisation Assay of the Leaves and Phyllosphere

Plants were grown in compost as described above. The plants were then moved to a growth chamber (22 °C, 75 % humidity, 16/8 hour light/dark cycle) to acclimatise three days prior to the assay. Bacterial strains were grown as described in section 2.1. For each assay the bacterial culture was washed with sterile 1 x phosphate buffered saline (PBS) (1 L of 10 x PBS contains 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4.12H_2O$, 2 g KCl; 1 x PBS has a pH of 7.4) once to remove the MOPS media and resuspended at an optical density at 600 nm (OD_{600}) of 1. A soft marker pen with indelible ink was used to mark 1 mm spots onto the leaves on both the adaxial and abaxial sides (separate leaves were used to test the different sides). Only two leaves (one marked

adaxially and one abaxially) were used per plant, with a total of three technical replicates taken at each time point. Sterile pipette tips were used to apply 2 µl of the bacterial culture onto the spots and the leaves were left to dry for one hour. 2 µl of sterile PBS was pipetted onto the spots for uninoculated, control plants.

At each time point, infected and negative control leaves were excised and placed into sterile microcentrifuge tubes. The fresh weight of the leaves was measured and 1 ml of PBS was added to each. The leaves were then macerated to a slurry using a sterile micropestle. Ten-fold dilutions (10^0 to 10^{-3}) were made and 10 µl of each dilution was spot plated onto Sorbitol-MacConkey (SMAC) plates containing antibiotics (25 µg/ml kanamycin for *Ec*O157 strain Sakai; 25 µg/ml nalidixic acid for *Ec*O157 strain TUV 93-0) in triplicate. The agar plates were left to grow for 16 hours at 37 °C and colonies were counted for each sample. The average (from the triplicate samples) was determined and each sample was then normalised by dividing by the fresh weight of the leaf. The data was transformed ($\log_{10}$) for statistical analysis and graphical presentation, and analysed using by ANOVA with the Tukey MCT in GraphPad Prism version 5.0 for Windows (GraphPad software, San Diego, California, USA; www.graphpad.com).

2.6 Colonisation Assay of the Roots & Rhizosphere

2.6.1 Root Colonisation of Plants Grown in Hydroponics

Seeds were surface sterilised and grown in hydroponic tubes in perlite and 0.5 x MS as described in section 2.4. The bacterial culture was grown as described in section 2.1. Prior to inoculation, the bacterial culture was washed using sterile 0.5 x MS and resuspended at an OD_{600} of 0.02 (~ 1.6×10^7 colony forming units (cfu) ml^{-1}). The 0.5 x MS was removed from the hydroponic tubes via pipetting and replaced with 10 ml of the 0.5 x MS + bacteria suspension. The plants were left for one hour. Negative control samples contained only 0.5 x MS i.e. no bacterial suspension. A positive control of fresh 0.5 x MS with bacteria (OD_{600} 0.02) was also set up to observe any carryover effect from the hydroponic solution.

At each time point, the plants were removed from the tubes, the roots were excised and each sample placed into sterile microcentrifuge tubes. They were processed in the same way as the phyllosphere colonisation assay.

2.6.2 Root Colonisation of Plants Grown in Compost

The plants were moved to a growth chamber (18 °C, 75 % humidity, 16/8 hour light/dark cycle) to acclimatise three days prior to the experiment. The bacterial overnight culture was prepared as in section 2.1, washed with PBS and resuspended at an OD_{600} of 0.02 in 1 L of SDW. Six individual potted plants (which had not been watered for 24 hours) were immersed in the 1 L SDW and bacteria mixture, which covered the bottom few centimetres of the pot. The plants were left for one hour and then placed back into normal trays. For a negative control, uninoculated potted plants were placed onto a tray containing sterile distilled water for one hour.

At each time point, the roots were excised from each sample using a sterile scalpel. As much compost was removed as possible without breaking the roots by gently placing the detached roots into 20 ml PBS and mixing on the vortex machine, on a low speed for ten seconds. The roots were then macerated using a mortar and pestle in 2 ml PBS with sand and the slurry placed into sterile microcentrifuge tubes and prepared as for the phyllosphere colonisation assay.

2.7 Preparation of Leaf Lysates

Plants were grown in compost as stated in section 2.4. After three to four weeks growth, the leaves were removed and snap-frozen in liquid nitrogen. Upon evaporation of the liquid nitrogen, the leaves were ground using a sterile mortar and pestle until they formed a fine powder. 10 g of the leaf powder was resuspended in 40 ml SDW and centrifuged for 15 minutes at 5,000 x *g*. The supernatant was heated at 50 °C for 30 minutes and clarified by centrifugation at 5,000 x *g* for 20 minutes and the final supernatant passed through a 0.22 μm sterile filter. The lysates were stored at -20 °C.

2.8 Preparation of Root Exudates

Seeds were surface sterilised as described above and grown on approximately 10g of rockwool (Progrow Ltd, Devon, UK) standing in 10 ml of 0.5 x MS media. After two to three weeks growth, the exudates were removed from 24 plants by three aqueous extractions of the rockwool with 50 ml SDW each time. The 0.5 x MS solution from the plant hydroponics was mixed to the crude exudate preparation and the total mixture clarified by passing through a 0.22 μm sterile filter and stored at -20 °C until further use.

2.9 Preparation of Root Lysates

Seeds were germinated in seed trays containing vermiculite with Osmocote Start for one week. The seedlings were then transplanted into individual pots containing vermiculite and Osmocote Start and left to grow for a further three weeks. The phyllosphere was removed using a sterile scalpel and the roots washed briefly in SDW to remove as much vermiculite as possible. The roots were macerated to a fine powder in liquid nitrogen using a mortar and pestle (as described above). 10 g of the root powder was resuspended in 40 ml SDW and the root debris pelleted by centrifugation for fifteen minutes at 5,000 x *g*. At this point the supernatant was collected and the protocol was carried out as for the preparation of leaf lysates.

2.10 Preparation of Plant Cell Wall Polysaccharides

Plants were prepared as for the root lysates protocol. Whole leaves and roots were excised using a sterile scalpel. Roots were washed first in SDW to remove as much of the vermiculite as possible. As a negative control, vermiculite only was also prepared using this method to subtract any residual carry-over from the vermiculite.

The roots and leaves were treated separately to prepare batches of root cell wall polysaccharides and leaf cell wall polysaccharides. Roots/leaves were frozen in liquid nitrogen and ground to a fine powder using a prechilled mortar and pestle (described above). The 'alcohol insoluble residue' protocol was used to process the plant powder (Popper, 2011). Briefly, 70 % ethanol was added to the plant powder in a 5:1 ratio, mixed on a vortex machine briefly, then on a shaking rotator for ten minutes (80 rpm). The samples were pelleted by

centrifugation at 5,000 x *g* for ten minutes and the supernatant discarded. The addition of ethanol and centrifugation steps were repeated five times or at least until the supernatant was clear. 100 % acetone was added to the powder and mixed on a shaking rotator for ten minutes. The acetone wash step was repeated twice. Following this, the supernatant was discarded and the polysaccharide powder was left to air dry for sixteen hours in a fume cabinet.

2.11 Growth Curves in Plant Extracts

Bacterial strains were adjusted to an OD_{600} of 0.02 in 0.5 x MS and incubated in an Erlenmeyer flask containing M9 media supplemented with 40 % normalised (for protein content) plant extracts (leaf lysates/root exudates/root lysates) at 18 °C, 200 rpm. Extracts were normalised by performing a Bradford assay using the Micro BCA™ Protein Assay kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions to measure protein levels and dilute extracts to similar concentrations if necessary. Extracts were diluted to the same range as that of the extract with the lowest protein concentration so to compare between plant species.

The flasks were incubated at 18 °C, 200 rpm for two days with plant lysates and four days with plant exudates. 1 ml of culture was taken at each time point and measured in a spectrophotometer at OD_{600}. Samples were set up in triplicate and triplicate readings were taken for each.

2.12 Chemotaxis & Motility Assays

2.12.1 Chemotaxis assay

Motility plates were prepared by mixing together 2 x DWA with 2 x minimal MOPS media (i.e. lacking the essential and non-essential amino acids) and 25 µg/ml kanamycin. Plates were left to dry and the centre of the plate was marked on the base using a marker pen.

Filter paper discs (10 mm) (Sigma Aldrich, St. Louis, USA) were soaked in the root exudates. A single disc was placed on top of the semi-solid agar in the centre of the plate, corresponding to the mark.

Three single colonies of *Ec*O157 Sakai (grown on LB with kanamycin at 25 μg/ml) were selected and inoculated into the plate, at equidistance from each other and from the filter paper disc in the centre. The plates were incubated at either 37 °C or 18 °C for seven days. Motility was calculated by measuring the distance from the centre of the bacterial colony to the filter disc after seven days incubation. Samples were set up in triplicate.

2.12.2 Motility assay

Motility plates were prepared as above except instead of using the 10 mm filter paper discs 1 ml of root exudates was added to the media of each plate. A single colony of *Ec*O157 Sakai was inoculated into the centre of the agar and motility was calculated over seven days by measuring the distance (largest radial point) from the colony as described above.

2.13 Plant Environment Assays – Experimental Setup

2.13.1 Temperature condition

*Ec*O157 Sakai was grown in M9 minimal media containing 0.2 % glycerol (see section 2.1) at either 37 °C or 18 °C until early stationary phase (OD_{600} of ~1). Each 10 ml culture was washed in 0.5 x MS once and adjusted to OD_{600} of 0.5 and sub-inoculated into 10 ml fresh M9 media with 0.2 % glycerol, which had been adjusted to 37 °C or 18 °C. The cultures were incubated for one hour at 37 °C or at 18 °C, with aeration (200 rpm). After one hour, the cultures were harvested and RNA isolated using the RNA Protect and purification protocol (section 2.15.1).

2.13.2 Treatment conditions

*Ec*O157 Sakai was grown in M9 minimal media containing 0.2 % glycerol at 18 °C, as described in section 2.1, until early stationary phase. The culture was washed once in 0.5 x MS and subcultured to an OD of 0.5 into 10 ml fresh M9 media containing either spinach leaf lysates or spinach root exudates (40 % of media). Cultures were incubated at 18 °C with aeration (200 rpm) for one hour, harvested and then subjected to the RNA Protect and purification protocol (see section 2.15.1). *Ec*O157 Sakai grown as described in section 2.13.1 (M9 minimal media with 0.2 % glycerol) without any plant extracts was used as the *in vitro* control.

2.13.3 Polysaccharide conditions

The experimental procedure was as per that for the plant extract array, except *Ec*O157 Sakai culture was subcultured into an Erlenmeyer flask containing 10 ml fresh M9 minimal media (no glycerol) with one of three supplements: 1 % weight/volume (w/v) vermiculite polysaccharides, to be used as the control; 1 % (w/v) spinach leaf cell wall polysaccharides; or 1 % (w/v) lettuce (*L. sativa*) leaf cell wall polysaccharides. Flasks were incubated at 18 °C, 200 rpm for one hour. Total RNA was then extracted using the protocol described in section 2.15.1.

2.14 In planta Assays – Experimental Setup

2.14.1 Leaf infiltrations

Three week old plants (grown as described in section 2.4) were moved to a temperature-controlled chamber (set at 22 °C, 70 % humidity) three days prior to the infection for the plants to acclimatise.

E. coli strain Sakai was grown in MOPS media as described in section 2.1. Prior to plant inoculation, the bacterial culture was pelleted by centrifugation to remove the MOPS media and resuspended in 1 mM of $MgCl_2$ to a cell density of OD_{600} 0.2 (equivalent to ~2×10^8 cfu ml^{-1}).

A sterile needle was used to gently scrape a small section on the underside of the leaf. A 1 ml sterile syringe was placed against the underside of the leaf and the bacterial culture infiltrated into the leaf via the small wound. This procedure was repeated for several areas of the leaf tissue until 1 ml total was infiltrated into the leaf. Plants were left for one hour after which time whole leaves were excised, wrapped in aluminium foil and immediately stored in liquid nitrogen. RNA was extracted as described in section 2.15.2.

2.14.2 Root infections

Plants were grown in hydroponic pots in 0.5 x MS as described in section 2.4. Whole plants were removed from the hydroponic solution and perlite with sterile forceps. *Ec*O157 Sakai was grown as described in section 2.1, washed in PBS to remove the growth media and resuspended in PBS to a cell density of OD_{600} 0.2. Five plants were placed together in 20 ml bacterial

suspension in 50 ml falcons and incubated in static conditions at 18 °C. Following incubation, the plants were removed from the falcons, the roots aseptically detached and immediately placed into RNA Stop solution (95 % ethanol: 5 % phenol, pH 4.0) for five minutes. Roots were then stored in liquid nitrogen until further processing. RNA was extracted as described in section 2.15.3.

2.15 RNA extractions

2.15.1 RNA Extractions from Bacterial Cultures

To prevent degradation of bacterial transcripts, the RNA Protect Bacterial Reagent (QIAGEN, Limburg, Netherlands) was used according to the manufacturer's instructions. Samples were placed in individual tubes preloaded with an equal volume of reagent, mixed using a vortex machine immediately for ten seconds and left for ten minutes at room temperature. The bacterial culture was pelleted by centrifugation at 5000 x *g* for ten minutes. The supernatant was discarded and the pellet either processed immediately or stored for later use at -20 °C. Total RNA was then extracted from the samples using the RNeasy Plant Mini kit clean-up protocol (QIAGEN, Limburg, Netherlands), with the addition of the optional DNaseI digestion stage for fifteen minutes (QIAGEN, Limburg, Netherlands). The concentration of total RNA was estimated using a NanoDrop (Wilmington, USA) spectrophotometer and visualised for quality using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) following electrophoresis on a 1.2 % RNase-free agarose gel loaded with RNase-free 2 x RNA loading dye (New England Biolabs, Ipswich, USA).

2.15.2 RNA Extractions from Infiltrated Leaves

Samples were stored in liquid nitrogen in foil packets until all were collected. Each sample was removed from liquid nitrogen and crushed in the foil packet. 0.5 g of plant tissue was weighed and ground to a fine powder using a pre-chilled mortar and pestle. The powder was then placed into a sterile 15 ml falcon and the remainder of the sample was stored at -80 °C for future use. 2 ml of lysis buffer (RLT buffer, QIAGEN RNeasy Plant Mini Kit; QIAGEN, Limburg, Netherlands)

containing 10 µl ml^{-1} β-mercaptoethanol (Sigma Aldrich, St. Louis, USA) was added to the plant tissue and mixed vigorously on a vortex machine for three minutes. The mixture was divided into four and each aliquot was passed through a QIAGEN shredder column (RNeasy Plant Mini Kit). Material was passed through the columns by centrifugation for two minutes at 10,000 x *g*. The eluent from two columns was collected and mixed with an equal volume of DNase-, RNase-free ethanol. This mixture was then passed through an RNeasy spin column (RNeasy Plant Mini Kit) and the rest of the protocol followed according to the manufacturer's instructions. RNA was visualised as for the in vitro extractions (section 2.15.1).

2.15.3 RNA Extractions from Colonised Roots

Several methods for RNA extraction were tested to acquire bacterial mRNA from infected plant roots. Originally the bacterial-plant-extraction (BPEX) buffer method was used, adapted from that previously reported (Schenk *et al.*, 2008). More commonly known as the "hot phenol" method, this protocol involves extraction through the use of BPEX buffer (0.35 M glycine; 0.7 M NaCl; 2 % (w/v) polyethylene glycol 20,000; 40 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 50 mM NaOH; 4 % (w/v) sodium dodecyl sulphate; 100 mM β-mercaptoethanol supplemented just before use) added to plant tissue which has been ground to a fine powder in liquid nitrogen. This mixture was mixed in a vortex machine and incubated at 95 °C for 90 seconds. 750 µl phenol/chloroform mix (5:1; pH 4.0) was added and the mixture was mixed by inversion at room temperature for five minutes. Following this, extraction was carried out in several steps: the two phases separated by centrifugation at 16,000 x *g*, 20 °C for seven minutes. The upper layer was transferred to a fresh tube containing 675 µl phenol/chloroform mix (5:1; pH 4.0), left for five minutes at room temperature and separated as for the previous step. The upper phase was transferred to a third tube, this time containing 575 µl phenol/chloroform/isoamyl alcohol mix (25:24:1; pH 4.0). This was left for five minutes and separated again as above. The upper phase was transferred to a fourth tube, preloaded with 550 µl chloroform/isoamyl alcohol mix (24:1). 55 µl of CTAB/NaCl solution (10 % (w/v) hexadecyltrimethylammonium bromide; 0.7 M NaCl) which had been prewarmed to 55 °C was added to the tube, left for five minutes, and then separated once more. The upper layer was

transferred to a microcentrifuge tube preloaded with 145 µl 8 M LiCl solution. The tube was inverted six times to mix and stored at -20 °C for 30 minutes to precipitate the RNA. Following this, the RNA was harvested by centrifugation at 16,000 x *g*, 4 °C for 20 minutes.

Following this, the pellet was resuspended in 100µl molecular biology grade water. 350µl lysis buffer (RLT; RNeasy Plant Mini Kit, QIAGEN) and 250µl 100% molecular biology grade ethanol was added to the sample and mixed by pipetting. The RNA was purified using an RNeasy spin column (RNeasy Plant Mini kit, QIAGEN, Limburg, Netherlands) and the RNA clean-up protocol as per manufacturer's instructions. Once the purified RNA had been eluted, it was stored at -80 °C until further use.

When testing the BPEX method (developed by Schenk et al, 2008) high levels of plant-derived RNA were found in the samples, with very low yields of bacterial RNA, as assessed on a Agilent Bioanalyser 2100 chip. Insufficient RNA in these samples meant that they were unsuitable for qPCR to study *Ec*O157 Sakai gene expression changes *in planta*. As a result, a modified protocol was developed in this study in conjunction with another lab member, which added a bead beating step to shear the epidermal layers of the root and minimise plant RNA carryover while enhancing bacterial RNA recovery. The full protocol is published (Holmes *et al.*, 2014) and an outline of the protocol can be found in Figure 2-1 .

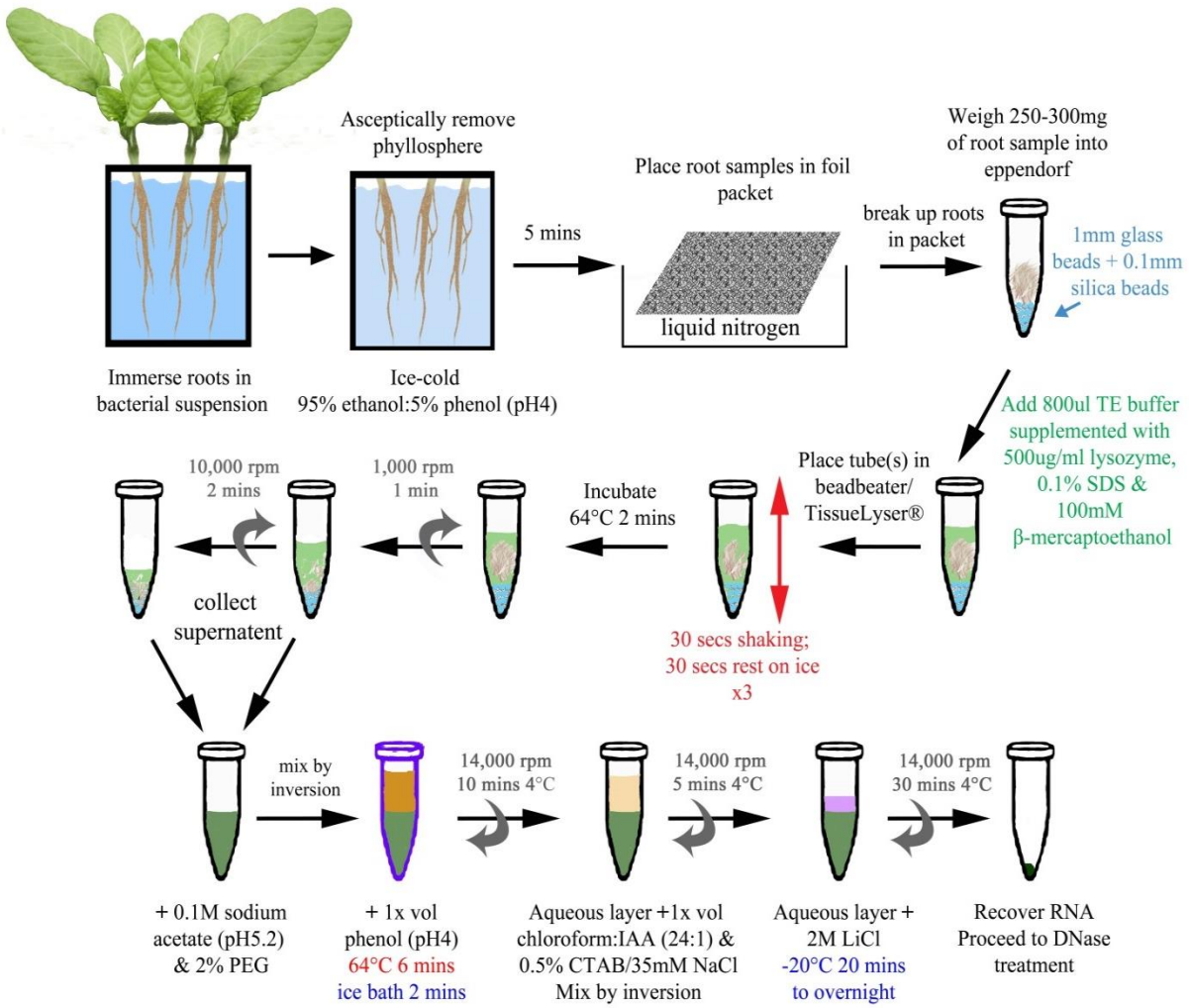


Figure 2-1. Protocol for the optimised method of bacterial RNA extraction from infected plant roots as published in Holmes et al, 2014.

Coloured text has been used to identify different stages of the process: blue for the addition of 1 mm & 0.1 mm beads/storage at low temperature; green for lysis of the tissue sample; red for heating/vortexing.

Briefly, samples were removed from the liquid nitrogen and crushed in the aluminium foil packet. 0.25 g of the crushed root tissue was weighed out into a microcentrifuge tube (DNase, RNase free; Ambion, Life Technologies, Carlsbad, USA) containing 0.25 g 1 mm glass and 0.1 mm silica beads (both ThermoScientific Ltd, Waltham, USA). 800 µl of Tris-EDTA (TE; 10 mM Tris-HCl; 1 mM EDTA at pH 8.0; 500 µg ml^{-1} lysozyme) was added to root tissue to start the lysis step. 0.1 % SDS and 100 mM β-mercaptoethanol was added to each sample and the tubes shaken via a TissueLyser (QIAGEN, Limburg, Netherlands) for thirty seconds followed by a thirty second chill on ice, repeated for three cycles. Samples were cooled on ice for two minutes, and then heated to 64 °C for two minutes. The beads were collected from the samples by centrifugation at 100 x *g* for one minute and the supernatant transferred to a fresh RNase-free microcentrifuge tube. A second centrifugation step was performed at a faster speed of 8,600 x *g* and the two supernatants pooled. 100 mM NaOAc (pH 5.2) and 2 % (w/v) PEG 20,000 was added to the supernatant, mixed by inversion three times and then mixed with an equal volume of phenol (pH 4.0). The sample was heated to 64 °C for six minutes, with the tube being inverted at least every 30 seconds. The sample was cooled on ice for two minutes and then extracted by centrifugation at 16,000 x *g* for ten minutes in a pre-chilled centrifuge (4 °C). Following centrifugation, the sample split into two layers: the upper aqueous layer was transferred to an RNase-free microcentrifuge tube pre-loaded with an equal volume of chloroform/IAA mix (24:1). 0.5 % CTAB/NaCl (prewarmed to 55 °C) was added to the tube and mixed by inversion. A further chilled centrifugation step at 16,000 x *g* (4 °C) was carried out for five minutes to separate the layers once more. The upper aqueous layer was transferred to a fresh RNase-free microcentrifuge tube which was preloaded with 2 M LiCl. The sample was mixed by inversion and stored at -20 °C overnight to precipitate the nucleic acids.

The precipitate was collected by centrifugation at 4 °C for 30 minutes at 16,000 x *g*, resuspended in 100 µl molecular biology grade water and the clean-up protocol followed using the RNeasy Plant Mini Kit (QIAGEN, Limburg, Netherlands). 5 µl of RNA was visualised as for the 'in vitro' RNA preparations.

2.16 Microarray set-up and conditions

All complementary DNA (cDNA) labelling and microarray hybridisation steps were carried out by Mrs Jenny Morris, The James Hutton Institute, described below. Initial data extraction and quality control analysis was carried out by Dr Pete Hedley, The James Hutton Institute, also described below.

2.16.1 Labelling of Prokaryote RNA

5 µg of total RNA (verified to have an RNA integrity number (RIN) of at least 8.5 via running on an Agilent Bioanalyser 2100) was combined with 100 ng/µl of the ten 11mer oligos (Fislage *et al.*, 1997) on ice. The mixture was incubated for ten seconds at 70 °C then returned to ice.

A master mix of 5x first-strand buffer, 0.1 M Dithiothreitol (DTT), 25 x aa-dNTP labelling mix and Superscript RT (all Superscript, Life Technologies, USA) was added to each of the samples and the mixture incubated at 42 °C for two hours. 1 M NaOH and 0.5 M EDTA (pH 8.0) were added to hydrolyse the RNA with an incubation step of fifteen seconds at 65 °C. Finally, 1 M HCl was added to neutralise the reaction.

450 µl of phosphate buffer (0.95 M K_2HPO_4; 0.5 M KH_2PO_4; pH 8.6) was added to the cDNA, mixed and applied to a MiniElute column (MiniElute PCR Purification Kit, QIAGEN, Limburg, Netherlands). The mixture was centrifuged at 13,000 x *g* for five seconds and the supernatant discarded. 750 µl phosphate wash buffer (0.25 M phosphate buffer; 7.7 ml molecular grade SDW; 42.2 ml 100 % molecular grade ethanol) was added to the column, and centrifuged as before. Following discarding of the supernatant, the column was centrifuged once more at 15,000 x *g* for ten seconds.

The column was transferred to an amber 1.5 ml collection tube (Sigma Aldrich, St.Louis, USA) and 10 µl of phosphate elution buffer (0.2 M phosphate buffer; 49.8 µl molecular grade SDW) was added to the centre of the membrane. The column was centrifuged at 13,000 x *g* for one minute. The elution step was repeated once more with fresh phosphate elution buffer to give 20 µl total eluted cDNA.

The following steps were all prepared in a low light room and incubations were stored in the dark. 2 M sodium carbonate buffer was added to the purified cDNA, followed by 1 µl of the Cy-dye (resuspended in dimethyl sulfoxide (DMSO); Cy3/Cy5 (GE Healthcare, UK)). The mixture was incubated in the dark for one hour at room temperature.

The labelled cDNA was purified by adding 12 M hydroxylamine hydrochloride and incubating in the dark for thirty minutes at room temperature. Each reaction was made up to 100 µl with molecular grade SDW. 500 µl of phosphate buffer was added and the total mixture was applied to a MiniElute column. At this point, the clean-up protocol was carried out according to the manufacturer's instructions.

Successful Cy3/Cy5 incorporation was assessed by running samples on a NanoDrop spectrophotometer using the microarray application to measure absorbance of each dye.

2.16.2 Preparation of Prokaryotic Hybridisation Samples for Arrays

600 ng labelled cDNA was added to a 1.5 ml amber tube, with the addition of molecular grade SDW to bring the total volume up to 20 µl. 5 µl of 10 x blocking agent (Agilent, Santa Clara, USA) was added to each tube, followed by 25 µl of 2 x GE hybridisation buffer HI-RPM (GE healthcare, UK). The sample was mixed carefully to avoid introducing bubbles and then centrifuged at 13,000 x *g* for one minute at room temperature.

The sample was immediately loaded onto the array (Agilent #G4813A-020097) by pipetting 40 µl of each mixture onto the gasket slide and placing the Agilent array slide on top. The chamber was assembled and placed into the hybridisation oven to incubate for seventeen hours at 65 °C, 10 rpm.

2.16.3 Data Extraction and Normalisation

Agilent *E. coli* v.2 microarrays (8 x 15K slides) were used for both the plant extract and the polysaccharide experiments. These arrays have 15,208 *E. coli* probes, covering four genomes in total, and were manufactured using Agilent 60mer SurePrint technology. The content of the array is devised from the TIGR comprehensive microbial resources database and uses the

following information for each *E. coli* genome: K12-MG1655 (herein referred to as K12) version 1 from December, 2000; O157:H7 VT2-Sakai version 3 from April 2001; CFT073 version 11 from August 2003; and EDL933 version 2 April 2001.

Data was extracted and imported into GeneSpring GX (herein referred to as GX; Agilent, USA) by Pete Hedley. A quality control report was generated to check the histogram for each sample. Principal component analysis was performed to see how the replicate samples cluster together and to help identify any outliers.

For both microarray experiments, the data was subjected to the following analysis: firstly, the data was filtered on flags to be present or marginal (i.e. the value for a gene must appear in two out of the three replicate samples). Secondly, statistical analysis of the datasets was carried out using GX by performing a Volcano plot on each condition with a two-fold minimum cut off for the fold change and multiple testing correction (MTC) of Benjamini and Hochberg (B&H).

The transcriptomic raw datasets have been uploaded to ArrayExpress under the accession E-MTAB-3249. For ease of understanding, edited datasets showing the fold change for each of the transcriptomic datasets can be found in the appendix (Appendix files S-3 to S-8).

2.16.3.1 Plant Extract Array Normalisation

The plant extract array was performed with four replicate samples over two slides, in which the samples were randomised to minimise error due to differences in the slides. A single colour array approach was used for the plant extract array.

For the temperature condition, data was normalised by dividing by the control dataset, which for this condition was the average of the "37 °C" condition. Volcano plot analysis was carried out as described above, but with a *p* value of 0.005.

The treatment conditions (leaf lysates and root exudates) were normalised by dividing by the "18 °C" temperature condition and volcano plot analysis was performed with a *p* value of 0.005.

2.16.3.2 Polysaccharide Array Normalisation

The polysaccharide array was set-up using a two-colour array approach, meaning a dye-swap stage was added to the normalisation process. Four replicate samples were used for each condition (spinach leaf cell wall polysaccharide exposure and lettuce leaf cell wall polysaccharide exposure) and eight control samples (vermiculite polysaccharide exposure).

For the spinach polysaccharide dataset, a volcano plot was performed as described above, but with a *p* value of 0.05. For the lettuce polysaccharide dataset, after initial statistical analysis the number of genes that were deemed to be statistically significantly different from the control was more than could be feasibly analysed; as a result, it was decided to increase the stringency for this dataset by performing a volcano plot with a *p* value of 0.01.

2.16.4 Microarray Analysis

Datasets were analysed using gene ontology (GO) mapping using GX software (Agilent Technologies, Santa Clara, USA). Lists were compiled for significant gene expression changes (as calculated by volcano plot analysis in GX) and exported into Microsoft Excel. Genes were sorted on fold change to identify those with the highest changes in expression between conditions.

Datasets were uploaded to EcoCyc (Keseler *et al.*, 2011; Keseler *et al.*, 2013) and metabolic maps generated for each dataset linking to *E. coli* K12 and *Ec*O157 Sakai cellular maps by uploading the transcriptomics dataset onto the cellular overview for each organism.

Comparisons between datasets were carried out by overlapping datasets using GX, and Venn diagram comparisons made using Venny software (Oliveros, 2007).

2.17 Preparation of RNA for qPCR analysis

2.17.1 Removing genomic DNA contamination

Once RNA samples had been assessed for quantity on a NanoDrop spectrophotometer and quality on an RNA denaturing agarose gel, a GoTaq PCR (see PCR reaction setup and cycling conditions) was performed on all samples to test for the presence of genomic DNA (gDNA). The

majority of samples tested had contaminating gDNA, especially in the *in vitro* samples. This required a second round of DNA removal, using the routine DNase treatment in the TURBO DNA-free protocol (Ambion, Life Technologies, Carlsbad, USA). gDNA removal was verified by performing a GoTaq PCR on the samples: a negative sample after 30 cycles of PCR amplification led to the assumption that the RNA was free of gDNA contamination (alongside positive, gDNA and negative no-template controls).

2.17.2 Converting RNA to cDNA

1μg total RNA was converted into cDNA samples by using the Superscript II reagents and protocol for using random primers as described by the manufacturer (Life Technologies, Carlsbad, USA), but included the 11mer primers, described above (Fislage *et al.*, 1997).

Successful cDNA synthesis was confirmed by the presence of a band after performing a GoTaq PCR.

2.18 qPCR Analysis

2.18.1 qPCR Setup, Cycling Conditions and Analysis

All qPCR reactions were set up using iTaq™ Universal SYBR© Green Supermix (herein referred to as iTaq) (Bio-Rad, Hercules, USA) as follows : 10 μl 2 x iTaq SYBR; 1.6 μl 300 nM forward primer; 1.6 μl 300 nM reverse primer; 1.6 μl cDNA at 1/10 dilution; DNase-, RNase-free molecular biology grade water to 20 μl, in 96-well plates. Reactions were carried out using a StepOnePlus™ machine (Applied Biosystems, Life Technologies, Carlsbad, USA) with the following cycling conditions: initial denaturation at 95 °C for ten minutes; 40 cycles of denaturation at 95 °C for fifteen seconds, primer annealing and product extension at 60 °C for one minute. Melt curve analysis was performed immediately afterwards with an initial denaturation at 95 °C for fifteen seconds, then a step-and-hold analysis of annealing at 60 °C for one minute with a 0.3 °C increase, followed by a final incubation at 95 °C for fifteen seconds. Data was analysed by averaging three technical and three biological replicates and applying the

formula $2^{-\Delta\Delta CT}$, with the data being normalised to the calibrator (control) sample and to a selected reference gene to obtain the fold-change in product levels.

2.18.2 Identifying a suitable reference gene

A suitable reference gene was identified using the geNorm 6-gene kit with SYBR (Primer Design Ltd, Southampton, UK). Reactions were set up as described above (section 2.18.1), however the data was analysed using the geNorm protocol. Cycle threshold (C_T) values were collected for each of the samples and all primer sets tested on a single plate. Three technical replicates and two biological replicates of each sample were assessed. Data was first transformed for the input file for the VBA by subtracting the highest C_T from all C_T values to calculate ΔC_T. The formula $2^{(-\Delta CT)}$ was all applied to all values. The data was then imported into the geNorm Excel VBA and analysed to generate graph "M". A suitable reference gene is identified as one that has an "M" value of lower than 1.5 (Primer Design, Southampton, UK).

Three GeNorm checks were carried out and the results of each are presented in Figure 2-2. The M value for *gyrB* was <1.5 for all three conditions tested thus satisfying the conditions that this gene could be used for qPCR normalisation for all conditions.

2.18.3 Optimisation of Primer Concentration

The optimum concentration for each qPCR primer set was determined by setting up a $\Delta\Delta C_T$ qPCR plate as described (see qPCR Setup and Cycling Conditions) with four different concentrations of primers: 300nM F-300nM R; 300nM F-900nM R; 900nM F-300nM R; 900nM F-900nM R.

The optimum concentration was taken as the lowest concentration of primers (to minimise the chance of primer dimers) that resulted in a product in the lowest number of cycles (C_T value). Optimum concentrations for each primer are presented in Table 2-3I.

2.18.4 Optimisation of Primer Efficiency

Each qPCR primer set was tested for its efficiency in the generation of product by performing a standard curve qPCR plate as described (see qPCR Setup and Cycling Conditions). Five 2-fold dilutions of cDNA were made in molecular biology grade water, starting at a concentration of 20 ng/µl cDNA. The cDNA was prepared from the calibrator sample of *Ec*O157 Sakai in M9 minimal media at 18°C, 200 rpm for one hour (as described for the treatment control condition in section 2.13.2). A standard curve was obtained by plotting the cDNA concentration against the C_T value for each primer set and the slope (*m*) of the line used to determine the efficiency of the primers using the formula $(10^{(-1/slope)})-1$.

All qPCR primers detailed in Table 2-II were tested, however only primers with an efficiency of 95-100 % were used for further qPCR. Primers which passed this efficiency test can be seen in Table 2-3.

Primers were verified in the same manner using the *in planta* cDNA samples to check for primer inhibition in these samples. No inhibition of the primers was observed in the *in planta* samples.

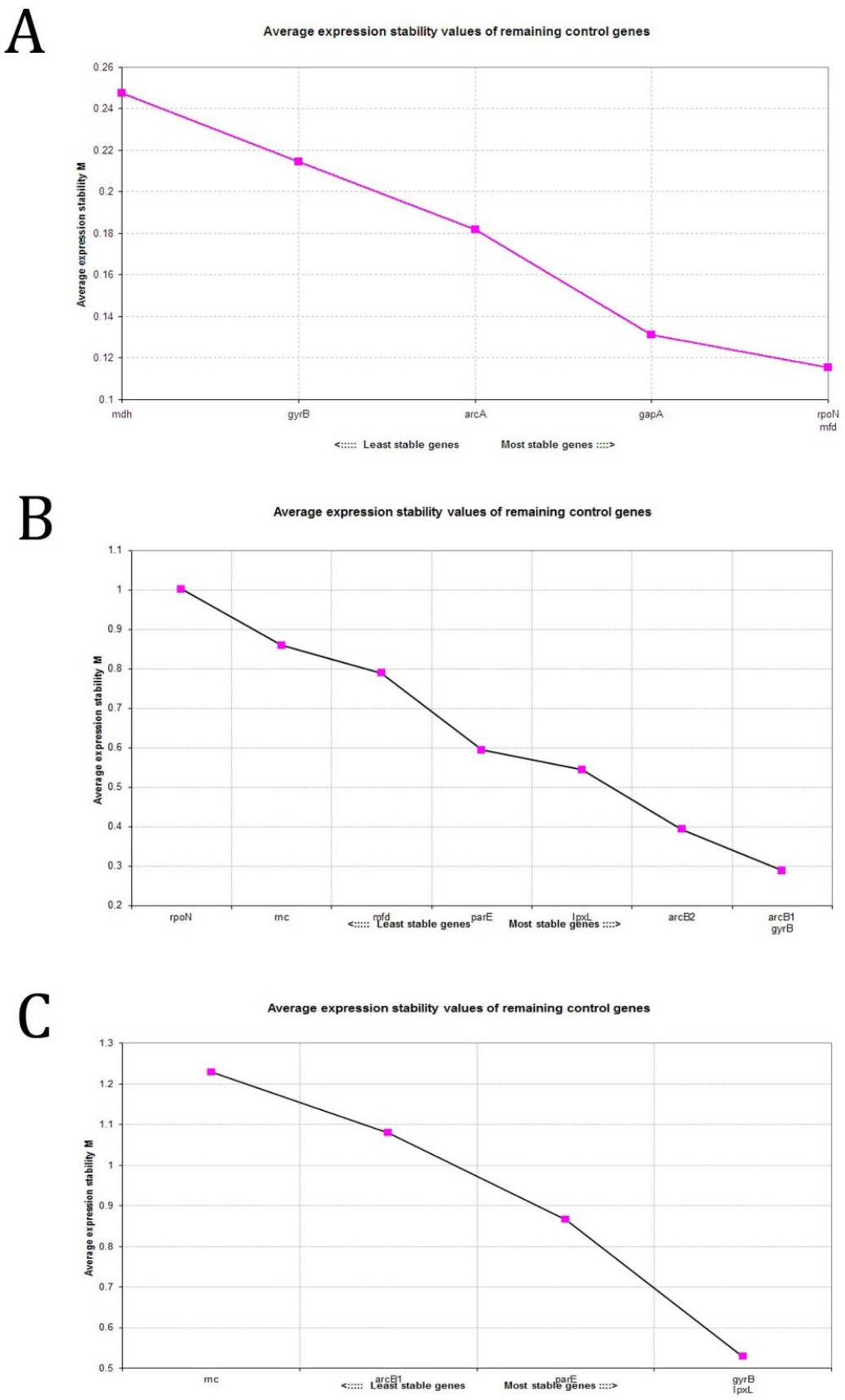


Figure 2-2. GeNorm analysis of three experimental conditions.

GeNorm analysis was carried on **A)** the plant extract array samples (containing the samples for the temperature and treatment conditions, including the controls); **B)** polysaccharide array samples (the two leaf cell wall polysaccharide conditions and the control samples; and **C)** the *in planta* conditions (infiltrated leaves and a culture only control). M>1.5 for *gyrB* in all three conditions.

Table 2-3. qPCR primers that passed all efficiency tests.

Primers were tested for their optimum concentration and efficiency. Those with an efficiency of 95-100% were then compared to the reference gene, *gyrB* by subtracting the Ct values of the reference gene to that of the target gene for each of the five dilution points and plotted. The M value of the line generated must be lower than 0.1 to be used for qPCR.

Gene Target	qPCR Primers	Optimum Concentration	Efficiency (%)	M value
AraA	araA-F1/-R1	300nM F::300nM R	97.6	0.0411
AraD	araD-F2/-R2	300nM F::300nM R	95.1	0.0172
AraJ	0446-F/-R	300nM F::300nM R	101	0.0293
CsgE	1416-F/-R	300nM F::300nM R	98.7	0.0168
DctA	dctA-F5/-R5	300nM F::300nM R	100.7	0.0324
ECs0664	0664-F/-R	300nM F::300nM R	99.5	0.0035
ECs0845	0845-F/-R	300nM F::300nM R	102	0.059
ECs3238	3238-F/-R	300nM F::300nM R	103	0.0741
ECs3926	3926-F/-R	300nM F::300nM R	95.5	0.0011
ECs4474	4474-F/-R	300nM F::300nM R	95.2	0.0921
EmrD	EmrD-F4/R4	300nM F:: 900nM R	99.5	0.0351
GyrB	gyrB.RT-F/R	300nM F::300nM R	99.6	N/A
Fis	4133-F/-R	300nM F::300nM R	102	0.0709
FlhD	2602-F/-R	300nM F::300nM R	98.2	0.034
Ler	4588-F/-R	900nM F:: 300nM R	99.9	0.0076
MalE	5017-F/-R	300nM F::300nM R	103	0.0476
PhoH	1266-F/-R	300nM F::300nM R	102	0.0468
SpoT	4525-F/-R	300nM F::300nM R	102	0.0431

2.19 Gene knockout mutagenesis

Selected genes were knocked out using the allelic exchange method (Merlin *et al.*, 2002). Briefly, ~500 bp regions either 5' or 3' to the gene(s) of interest were amplified by PCR with the appropriate N_i/N_o and C_i/C_o primers for each selected target, using Phusion polymerase and reaction conditions (see Phusion PCR conditions). PCR products were purified using Wizard SV Purification kit (Promega, Madison, USA). The primers contained restriction sites of *Not*I for the N_i and C_i primers, *Pst*I for the N_o and *Sal*I for the C_o primers. The N_i and C_i primers also contained regions of complementary sequence to fuse the 500 bp products together to make a 1 kb product, with the *Not*I site in the middle. This 1 kb product was then ligated into pTOF24 cloning vector, pre-cut with *Pst*I and *Sal*I (Promega, Madison, USA) and treated with alkaline phosphatase (T-SAP; Promega, Madison, USA) according to manufacturer's instructions. Ligations were set up in a 5:3 vector to insert ratio using T4 DNA ligase protocol (Promega, Madison, USA).

The pTOF24::1kb linker construct was transformed into chemically competent DH5α (Invitrogen, Life Technologies, Carlsbad, USA). Cells were recovered at 27 °C (with 25 μg/ml chloramphenicol as the plasmid contains a chloramphenicol resistance cassette) to enable replication of the temperature sensitive origin of replication on the plasmid, a feature later exploited to induce recombination.

The original protocol (Merlin *et al.*, 2002) describes the use of the kanamycin antibiotic resistance cassette as a feature to insertionally inactivate the gene of interest. However, since *Ec*O157 Sakai already contains a kanamycin resistance cassette inserted in place of the *stx2* gene an alternative was required. Instead, a tetracycline resistance cassette obtained from pTOF1 was used to insert into the gene of interest.

The tetracycline resistance cassette was removed from the pTOF1 plasmid using *Not*I sites that flanked the gene. The pTOF24::1kb linker was cut with *Not*I (Promega, Madison, USA) and the vector was treated with T-SAP. The vector and the antibiotic resistance cassette were ligated using T4 DNA ligase and transformed into chemically competent DH5α.

The pTOF24::1kb linker::Tet cassette plasmid construct was purified using Qiaprep Spin Miniprep kit (QIAGEN, Limburg, Netherlands). This was electroporated into *Ec*O157 Sakai (*stx*-Kan^R) and grown overnight in LB-Tc at 27 °C. The culture was plated onto LB plates containing 12.5 μg/ml tetracycline and 25 μg/ml kanamycin and incubated at 27 °C and 42 °C for sixteen hours. A viability drop was confirmed at 42 °C, as the plasmid is unable to autonomously replicate at this temperature, thus indicating that the plasmid had integrated completely into the chromosome via one of the homologous regions, and retained the tetracycline resistance gene.

To resolve the plasmid and complete the recombination process, 10 colonies of each integrant were selected and inoculated into LB supplemented with tetracycline at 12.5 μg/ml and 25 μg/ml kanamycin at 37 °C, repeated for four times. The resulting culture was serially diluted (10-fold) and inoculated onto LB plates containing no NaCl (to create a hypotonic environment) and 6 % sucrose, as well as tetracycline and kanamycin. The addition of the sucrose results in the formation of levans by the *sacB* gene (encoded on the pTOF24 plasmid), build-up of which in

a hypotonic environment is toxic to the cell. This creates a positive selection for the surviving cells to lose the *sacB* gene but maintain the tetracycline cassette, thus inducing the second recombination step. Successful recombination of the second homologous region results in the tetracycline cassette integrated in place of the gene of interest, which is integrated into the plasmid and lost through dilution during active cell division.

Successful gene knockout is confirmed by PCR and sequencing of the region of interest.

2.20 Comparative Genomic Analysis

2.20.1 DotPlot Analysis using Gepard

Genomes of *Ec*O157 Sakai and *Ec*O157 EDL933 were downloaded from NCBI (NCBI Resource Coordinators, 2013) in FASTA format and analysed for their similarity by dotplot analysis using Gepard free software version 1.30 (Krumsiek *et al.*, 2007).

2.20.2 Identification of Genomic Islands using Island Viewer

Genes that were associated with genomic islands were identified using Island Viewer software (Langille & Brinkman, 2009), combining the predicted results from three programs: SIGI-HMM (Waack *et al.*, 2006), IslandPath-DIMOB and IslandPick (Langille *et al.*, 2008). Genes from the virulence factor database (VFDB) (Chen *et al.*, 2012), the antibiotic resistance gene database (ARDB) (Liu & Pop, 2009) and the pathogen-associated gene database (PAGDB) (Ho Sui *et al.*, 2009) were overlayed onto the results by Island Viewer.

2.20.3 Genetic & Metabolic Comparison using EcoCyc

A full genomic comparison was carried out by using the comparative genomic analysis feature on EcoCyc to compare *E. coli* K-12, *Ec*O157 Sakai and *Ec*O157 EDL933. This generates a list of genes found in all organisms followed by a list of unique genes to each organism. As well as this, metabolic pathway classes and individual pathways that are present in one, two or three of the organisms are detailed.

2.21 HPLC Analysis of Plant Extracts

A total of twenty plant extract samples were analysed via high performance liquid chromatography (HPLC) to identify their monosaccharide content. Samples included leaf lysates, root exudates and root lysates for all four plant species, one rockwool control, one vermiculite polysaccharide control and leaf and root polysaccharide samples from three plant species (spinach and two types of lettuce: *L. sativa* and *L. serriola*).

2.21.1 Preparation of samples for HPLC

All plant extract samples (leaf lysates, root lysates, root exudates) were prepared by an ethanol extraction. Briefly, 10 ml extract samples were freeze dried for 16 hours in a freeze drier and resuspended in 80 % ethanol. The mixture was centrifuged at 5,000 x *g* for 30 minutes and 2 ml of the supernatant transferred to a sterile microcentrifuge tube. The ethanol was removed by drying in a speed vac for five hours. The extract was freeze dried once more for 16 hours, with a final suspension step in 2 ml molecular biology grade water.

TFA hydrolysis was carried out on the polysaccharide samples. 10 mg of polysaccharide sample was weighed into sterile microcentrifuge tubes. 2M trifluoracetic acid (TFA) was added to each sample and boiled at 100 °C for one hour. The TFA was removed by evaporation for sixteen hours and the sample freeze dried for five hours in a freeze drier. Extracts were resuspended in 1 ml molecular biology grade water.

2.21.2 HPLC Setup and Analysis

All samples were run at a 1/10 dilution on a Dionex chromatography machine using the Chromeleon software with a PA100 column (all Dionex, Thermo Fischer, Carlsbad, USA). Three buffers were used to create the gradient: buffer A contained ultra-pure water (Merck Millipore, Darmstadt, Germany); buffer B contained 200nM NaOH; and buffer C 1M NaOAc. The PA100 column was used to detect glucose, fructose, sucrose, arabinose and rhamnose. The polysaccharide samples were also run on a PA20 column (Dionex, Thermo Fischer, Carlsbad, USA) to check for fucose, galactose, xylose, mannose, glucuronic acid and galacturonic acid.

Standards were made up for all monosaccharides described in a 5-point gradient of 5 μg/ml to 25 μg/ml.

Data were extracted based on the area of the peak and exported into Excel. Each value was calculated for content based on the dilution factor and the amount of extract prepared for HPLC and the total monosaccharide content for each sample displayed as μg/ml for the lysate and exudate samples, and as μg/mg dry weight for the polysaccharide samples.

3 Bacterial Colonisation on Different Plant Species and Utilisation of Plant Extracts

3.1 Introduction

The prevalence of human pathogens such as *Salmonella* and *E. coli* O157:H7 (*Ec*O157) in the environment and on a variety of different plant species, including many types of fresh produce, has become more apparent in the last two decades as more outbreaks occurring from the ingestion of contaminated produce have been reported since the first in 1991 (Heaton & Jones, 2008; Lynch *et al.*, 2009; Rangel *et al.*, 2005). For *Ec*O157 it was shown that 21 % of the outbreaks recorded over a twenty year period from 1982 were fresh produce-related (Rangel *et al.*, 2005). The increase in fresh-produce related outbreaks can be attributed to a variety of factors, such as an increased consumption of fruit and vegetables due to the 5-a-day, 7-a-day and 9-a-day campaigns implemented in the UK and the USA. As well as this, improvements to the surveillance of outbreaks across the world means more cases are being identified, although it is thought that there are still many cases that go unrecorded each year (Werber *et al.*, 2012).

Of the outbreaks recorded for both *Salmonella* and *Ec*O157 since 1991 a wide range of plant hosts have been implicated, ranging from leafy greens such as lettuce and spinach (Ackers *et al.*, 1998; CDC, 2006) to fruit/vegetables (Hedberg *et al.*, 1999) to nuts (Miller *et al.*, 2012). Thus these human pathogens are not only able to survive outside the mammalian host in the soil for long periods of time (Zhang *et al.*, 2009) but they can also colonise different plant hosts, making prevention a very difficult task. *Ec*O157 has adapted to a wide range of environments, including within different tissues of the plant host.

3.1.1 Colonisation of Human Pathogens on Plants

Previous studies looking at the colonisation potential of human pathogens on plants have shown that these bacterial species have strong associations with specific areas of the leaf tissue, especially natural openings such as stomata (Brandl & Amundson, 2008; Kroupitski *et al.*, 2009b), trichomes (Barak *et al.*, 2011; Burnett *et al.*, 2000) and epidermal cell wall junctions,

similar to other plant-associated bacteria (Beattie & Lindow, 1999). Colonisation of leaves by *Ec*O157 has mostly been studied on post-harvest, pre-packaged food material, which has shown high colony forming unit (cfu) counts after a short amount of time (Abdul-Raouf *et al.*, 1993; Brandl, 2008; Chang & Fang, 2007); however, this is not surprising as other studies have shown *Ec*O157 has a higher colonisation and internalisation potential if the plant is wounded (Brandl, 2008; Hora *et al.*, 2005), presumably due to the exudation of nutrients from the cut material. While postharvest contamination of fresh produce is still an important area to study to identify prevention strategies, any decontamination procedures that only acted on exposed surfaces would most likely be ineffective on populations that have colonised and internalised into the plant tissue when the plant was developing.

As a result, few studies have been carried out on the survival of human pathogens on living plants. However, it is an area of great importance as *Salmonella* and *Ec*O157 do not require plant wounding to survive in the plant environment. One study found that *Salmonella* was able to survive on lettuce for 63 days when the bacteria was added to the compost used for growing the plant (Islam *et al.*, 2004). Other studies have also shown that *Ec*O157 and *Salmonella* are able to internalise into plant tissues (Gu *et al.*, 2011; Solomon *et al.*, 2002), usually in the extracellular spaces at plant cell junctions, via entry through the stomata (Saldaña *et al.*, 2011), although there is still some debate that this is a passive process due to water uptake (Johannessen *et al.*, 2005; Zhang *et al.*, 2009). As well as this, internalisation of human pathogens into epidermal cells has been observed in the roots of plants (Wright *et al.*, 2013).

Little work has been carried out on colonisation of the roots of plants by human pathogens, despite this being proposed site of initial colonisation and eventual transmission to the phyllosphere, whether through cross-contamination during handling of the food for processing or via the movement of the bacteria from the roots to the leaves up the stem by flagella-mediated motility (Cooley *et al.*, 2003). As this movement from root to leaf has only been demonstrated in *Arabidopsis* so far it is still not clear if this is the main route of transmission to the leaves for these human pathogens on other plant species. Other studies have implicated

irrigation water as the main cause of leaf colonisation by these bacteria and many other plant-associated bacteria also (Oliveira *et al.*, 2012; Solomon *et al.*, 2002).

Other plant-associated bacteria are also known to play a key role in the colonisation ability of human pathogens, as studies have shown that co-infection of a human pathogen with a plant-associated bacteria/fungi on plants can increase the prevalence of *Salmonella* and *E. coli*. Increased cfu counts by co-inoculation have been demonstrated in a number of studies, such as: *Salmonella* with *Erwinia carotovora* (*Pectobacterium caratovorum*) (Wells & Butterfield, 1997); *Salmonella* with fungal pathogens *Alternaria* or *Cladosporium* in tomatoes (Wade & Beuchat, 2003); *Salmonella* with *Pseudomonas syringae* on tomato (Meng *et al.*, 2013); *Ec*O157 with *Glomerella cingulata* (Riordan *et al.*, 2000); and *Ec*O157 with *Wausteria paucula* on lettuce leaf, where a 6-fold increase was observed compared to *Ec*O157 on lettuce alone (Cooley *et al.*, 2006).

3.1.2 Aims of this Area of Study

A. To assess the ability of two strains of *Ec*O157 to colonise plant tissue.

B. To assess the ability of *Ec*O157 Sakai to utilise plant extracts.

C. To identify any chemotactic response from *Ec*O157 towards plant root exudates.

Outbreaks of *Ec*O157 have occurred from a variety of plant hosts, indicating that this serotype is adapted to a variety of environments and conditions. Therefore, I hypothesise that EcO157 strain Sakai will be able to persist on four different plant species for ten days as similar strains have been previously linked to several different plant products. To test this, colonisation assays were developed to understand firstly how two different *Ec*O157 strains survived in different plant environments. Bacteria were drop-inoculated onto the leaves of four different plant species to mimic an irrigation water method of contamination and the number of recoverable bacteria enumerated over ten days by direct plating. The ability of these strains to colonise the roots was also tested in two different systems: firstly on hydroponics so as to limit competing bacterial/fungal species and control the nutrients available to both plant and *Ec*O157; and

secondly in compost-grown plants via a soil-soak procedure to observe if the two strains were able to colonise roots in the presence of potentially competing microflora.

Studies using other plant-associated bacteria help develop the understanding of how these human pathogens have adapted to the environment, including to different tissue types and to different extractions of plant material. As previous studies had shown that many plant-associated bacteria exhibit a positive chemotaxis response towards plant extracts (leaf lysates to mimic plant wounds (Kyle *et al.*, 2010)) and root exudates (Klerks *et al.*, 2007; Yao & Allen, 2006), it was hypothesised that *Ec*O157 would have a similar chemotactic response. The transmission pathway for the colonisation of plants by *Ec*O157 is thought to occur via the soil as a result of contamination by cattle or other farm animal faeces (Chekabab *et al.*, 2013), from where the bacteria can traverse to the plant roots (although studies have not been able to show this for *Ec*O157 in spinach (Sharma *et al.*, 2009a)). This means that root exudates will be the first point of contact between *Ec*O157 and the plant. Thus, root exudates may induce a positive chemotactic response towards the plant roots.

To study the interaction of *Ec*O157 with plant extracts, two representative strains were incubated with different extracts over three days firstly to test if the human pathogen could grow in the extracts, as previous reports (Jayaraman *et al.*, 2014; Kyle *et al.*, 2010) have shown this for other plant species. Further to this, positive tropism towards root exudates had previously been demonstrated for *Salmonella* towards lettuce root exudates (Klerks *et al.*, 2007). Therefore, motility and chemotaxis of *Ec*O157 to plant extracts was also tested. To assess chemotaxis by *Ec*O157 towards the root exudates of the four plant species used, motility assays were set up in two formats: one using filter discs soaked in root exudates to assess the chemotactic response towards the substrate; and a second using motility agar plates containing root exudates in the media to see if the addition of the exudates alone affected motility of the *Ec*O157 strain.

Colonisation assays were set up for two strains of *Ec*O157 on the leaves and also for the rhizosphere and roots, over ten days on four different plant species: spinach (*S. oleracea*), domesticated lettuce (*L. sativa*), prickly lettuce (*L. serriola*) and pea (*P. sativum*). Two strains of

*Ec*O157 were tested: *Ec*O157 strain Sakai and strain TUV 93-0. *Ec*O157 Sakai is a clinical isolate from an outbreak from Sakai City, Japan in 1996 in which 10,000 school children were affected (Michino *et al.*, 1999), the cause of which was contaminated white radish sprouts. In contrast, *Ec*O157 strain TUV 93-0 (a *stx*-inactivated variant of EDL933) is a bovine isolate originating from the hamburger outbreak in the USA (Donohue-Rolfe *et al.*, 2000). They were selected because they represent either a plant-derived or animal-derived outbreak strain, which may have implications on their colonisation ability in the different plant environments. Since *Ec*O157 Sakai was isolated from two different fresh produce outbreaks it is expected that it is better adapted to the plant environment than *Ec*O157 TUV 93-0 and therefore will colonise both leaves and roots at a higher level.

3.2 Results

3.2.1.1 Colonisation Assays of Ec*O157 on Four Plant SpeciesSurvival of* Ec*O157 Sakai on the Leaves of Four Plant Species*

Colonisation was assessed at four time points: 0, 1, 2 and 10 days. At each time point the whole leaf was aseptically excised and processed as described in section 2.5. Bacterial counts obtained for each time point represent total counts, i.e. both for surface-adhering bacteria and any internalised cells. The survival of each strain was tested on the adaxial (top) and the abaxial (bottom) surfaces of the leaf, as a previous study had shown that *Salmonella* colonises the underside of the leaf in higher numbers compared to the top (Kroupitski *et al.*, 2011). Data from all time points were analysed for differences between the adaxial and abaxial counts for each of the plant species tested by one-way ANOVA, followed by the Tukey multiple comparison test (MCT) (GraphPad Prism, version 5.01).

Colonisation of the phyllosphere and leaves for *Ec*O157 Sakai showed higher numbers of bacteria were recovered following abaxial rather than adaxial inoculations for all four plant species that after ten days, although statistical tests showed that this difference was not significant. The number of *Ec*O157 recovered from leaves of all the plant species tested followed a trend of decreasing counts from the initial inoculum level over the ten day period. The final count on the abaxial side of the leaf for *L. sativa* lettuce and pea was approximately 3 $\log_{10}$ cfu/ml/g (Figure 3-1) and 2 $\log_{10}$ cfu/ml/g on spinach. *L. serriola* supported the lowest numbers of *Ec*O157 Sakai at the ten day timepoint on both the adaxial and abaxial sides, significantly lower than that for *L. sativa* (*L. sativa* abaxial compared to *L. serriola* adaxial, $p < 0.05$) and pea (adaxial, $p < 0.01$; abaxial, $p < 0.05$). Furthermore, bacteria were only recovered from the *L. serriola* leaves at the ten day time point in one and two samples out of nine for the adaxial and abaxial surfaces, respectively. In all cases, it was possible to isolate *Ec*O157 Sakai from each of the plant species tested, showing it is able to survive in this environment for the duration of this test, albeit there is a large amount of plant to plant variation that appears to be affecting the ability of *Ec*O157 Sakai to persist on the leaves.

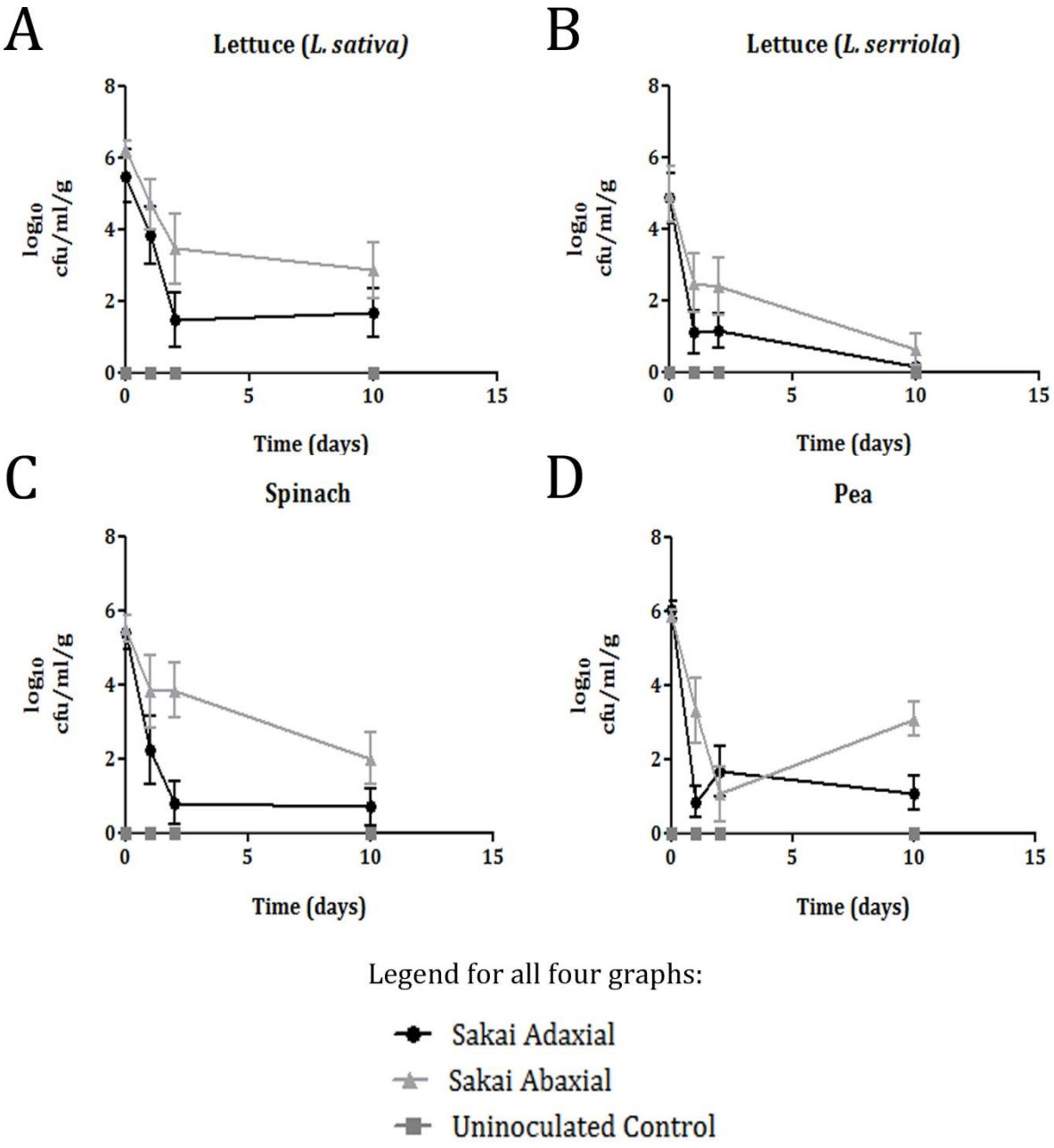


Figure 3-1. Colonisation assays of *Ec*O157 Sakai on the leaves over ten days.

*Ec*O157 Sakai was drop-inoculated onto the surface of the adaxial and abaxial sides of the leaves of **A)** lettuce (*L. sativa*), **B)** lettuce (*L. serriola*), **C)** spinach and **D)** pea. Three samples were collected from three biological replicate experiments to give a total of nine samples. Uninoculated control samples were tested at each timepoint and no *Ec*O157 counts were recorded. It should be noted that culture methods used here have a limit of detection (>100) which varies between media used; therefore "zero" counts may still contain colonies. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0). Data points represent the mean of nine samples; error bars represent standard error of the mean (SEM).

3.2.1.2 Survival of Ec*O157 TUV 93-0 on the Leaves of Four Plant Species*

The numbers of *Ec*O157 TUV 93-0 recovered from the leaves of the plant tested decreased at each time point as for *Ec*O157 Sakai, but the numbers fell below the limit of detection after ten days for both species of lettuce tested, although they could still be recovered at low levels on the spinach and pea leaves (Figure 3-2).

Statistical tests showed no significant differences between the counts obtained from adaxial- and abaxial-inoculated leaves over ten days, both within plant species and between plant species. Analysis of the final ten day time point counts alone also did not yield any significant results, which was as expected as most samples were below the limit of detection by this time point. As for *Ec*O157 Sakai, despite higher abaxial counts from each timepoint, no significant difference was seen between the adaxial and abaxial counts from all four of the plant species.

As it was possible to detect *Ec*O157 Sakai and not *Ec*O157 TUV 93-0 after ten days from leaves of both lettuce species, and much lower numbers of *Ec*O157 TUV 93-0 were recovered in comparison to *Ec*O157 Sakai (Figure 3-1) (Figure 3-2) a statistical comparison was made between the two strains in terms of their colonisation ability. Despite higher average numbers of *Ec*O157 Sakai recovered compared to *Ec*O157 TUV 93-0, the only significant difference between the counts of *Ec*O157 Sakai and *Ec*O157 TUV 93-0 was for *L. sativa* lettuce, not for spinach, pea or *L. serriola* lettuce. *Ec*O157 Sakai was recovered at significantly higher numbers from *L. sativa* lettuce compared to *Ec*O157 TUV 93-0 ($p < 0.01$), on both the adaxial and abaxial sides after ten days.

Following this examination of the colonisation ability of these two strains on the phyllosphere and leaves, the same strains were also tested on the rhizosphere and roots of the same plant species to see if they could also colonise this environment.

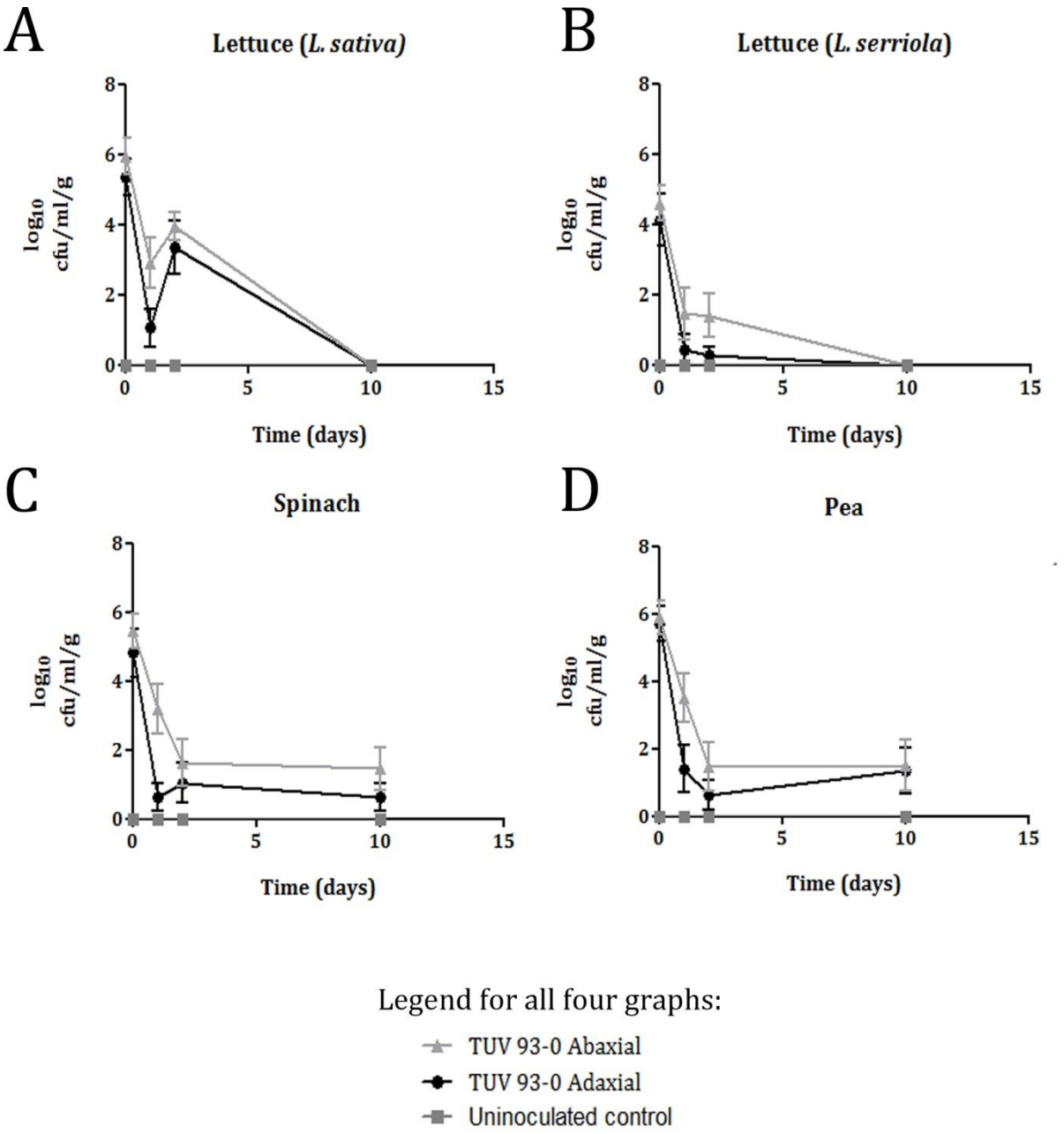


Figure 3-2. Colonisation assays of *Ec*O157 TUV 93-0 on the phyllosphere and leaves over ten days.

*Ec*O157 TUV 93-0 was inoculated onto the adaxial and abaxial sides of the leaves of **A)** lettuce (*L. sativa*), **B)** lettuce (*L. serriola*), **C)** spinach and **D)** pea. Three samples were collected from three biological replicate experiments to give a total of nine samples. Uninoculated control samples were tested at each timepoint and no *Ec*O157 counts were recorded. It should be noted that culture methods used here have a limit of detection (>100) which varies between media used; therefore "zero" counts may still contain colonies. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0). Data points represent the mean of nine samples; error bars represent standard error of the mean (SEM).

3.2.1.3 Survival of Ec*O157 Sakai on Roots Grown in Hydroponics for Three Plant Species*

*Ec*O157 Sakai was inoculated onto intact, living plant roots for a root colonisation assay. Three plant species (spinach, domesticated lettuce and prickly lettuce) were grown under hydroponics conditions to assess bacterial colonisation with only a limited number of competitors. Surface sterilised pea seeds did not yield sufficient numbers of plants free of extensive fungal association: therefore, this species was not assessed in the same manner. As a non-plant control, *Ec*O157 Sakai was also inoculated into sterile 0.5 x MS, which was used as the hydroponic growth media, and the numbers counted after one, two and ten days. Uninfected plant samples were processed to check for any contaminating *Ec*O157.

Bacterial counts were determined over 10 days for spinach and lettuce roots (Figure 3-3). *Ec*O157 Sakai was able to colonise the roots and could be recovered in similar number to the initial inoculum after 10 days for all three plant species. Bacterial counts from the inoculation in 0.5 x MS only (with no sucrose) decreased over the ten day period, but could still be recovered at ten days at very high levels. Interestingly, the high plant to plant variation that was observed from the leaf samples did not occur for the roots grown in hydroponics. *Ec*O157 Sakai was not detected in the uninfected plants.

Differences between bacterial counts from the roots and in the 0.5 x MS solution were analysed by one-way ANOVA, followed by the Tukey MCT (GraphPad Prism, version 5.01). Statistical analysis showed that after 10 days there was a significant difference between the EcO157 Sakai recovered from the roots compared to the 0.5 x MS control, for all three plant species (all $p < 0.001$), with significantly higher numbers of *Ec*O157 Sakai on *L. serriola* lettuce in comparison to the domesticated lettuce, *L. sativa*, after ten days ($p < 0.01$).

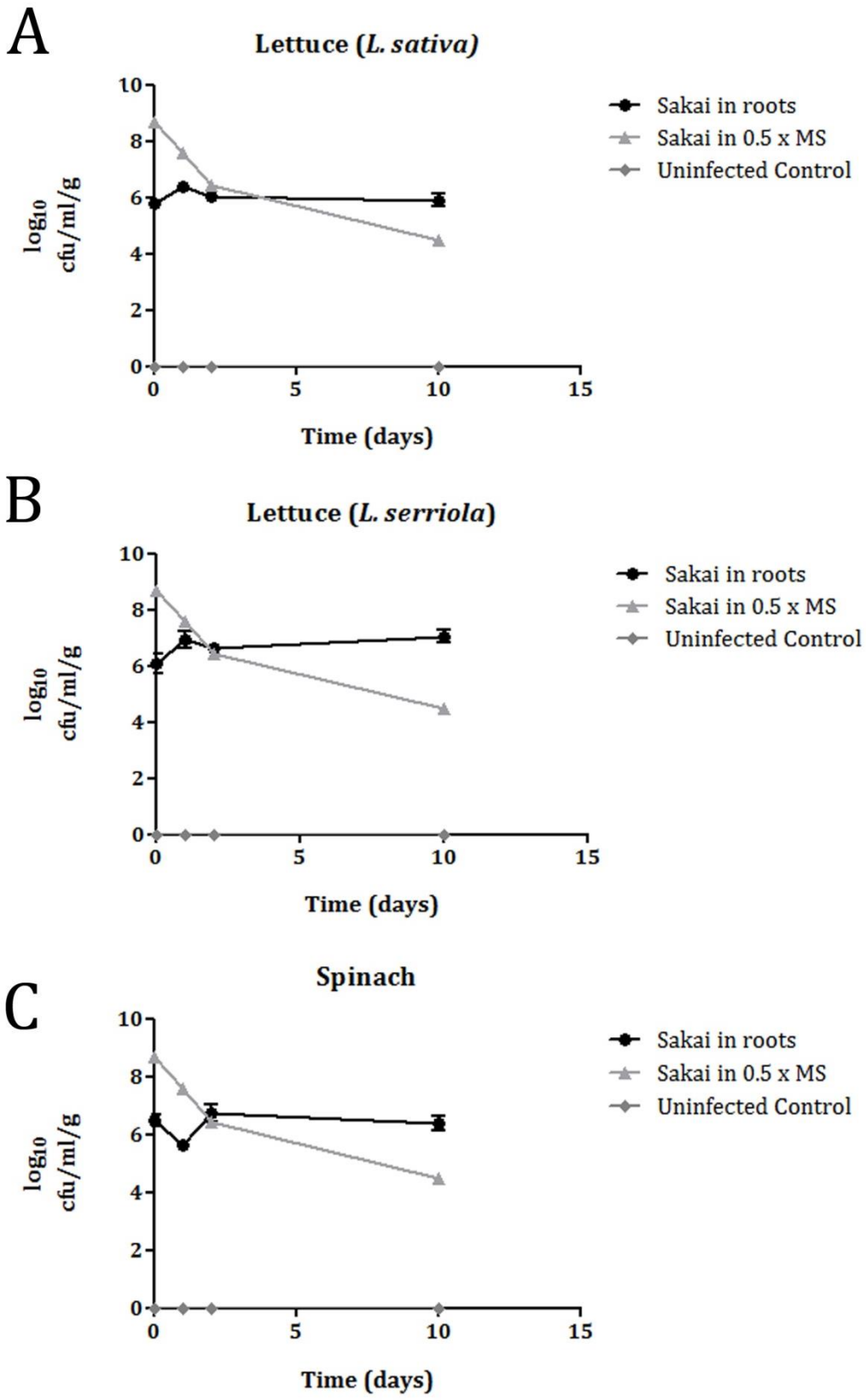


Figure 3-3. Colonisation assays of *Ec*O157 Sakai on the rhizosphere and roots from hydroponic-grown plants over ten days.

*Ec*O157 Sakai was inoculated onto the roots of **A)** lettuce (*L. sativa*), **B)** lettuce (*L. serriola*) and **C)** spinach. Three samples were collected from three biological replicate experiments to give a total of nine samples. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0). Data points represent the mean of nine samples; error bars represent standard error of the mean (SEM).

3.2.1.4 Survival of Ec*O157 TUV 93-0 on the Roots of Four Plant Species*

The hydroponic root colonisation assay was set up to assess the colonisation ability of the bovine strain, *Ec*O157 TUV 93-0. *Ec*O157 TUV 93-0 was recovered from the roots of all three plant species tested after ten days, with counts being similar to that of the initial inoculum (Figure 3-4), as was the case for *Ec*O157 Sakai. *Ec*O157 TUV 93-0 was recovered in similar numbers (~ 6 log cfu/ml/g) after one, two and ten days. As for *Ec*O157 Sakai in 0.5 x MS, the population of *Ec*O157 TUV 93-0 in 0.5 x MS decreased over time. A significant difference in the counts was observed for *Ec*O157 TUV 93-0 recovered from the roots of the two lettuce species, with higher counts observed in the roots of *L. sativa* ($p < 0.001$) and *L. serriola* ($p < 0.01$) compared to the 0.5 x MS control. No significant difference in the counts was observed for *Ec*O157 TUV 93-0 in 0.5 x MS compared to those isolated from spinach roots after ten days. Finally, the number of bacteria recovered after ten days from the roots of *L. sativa* was significantly higher than those recovered from spinach ($p < 0.05$).

As no significant difference was seen for *Ec*O157 TUV 93-0 on spinach hydroponic roots compared to the media control and the colonisation assays on the leaves with this bacterial species had yielded low counts on the plant species tested, it was decided to focus most of the work for this project on *Ec*O157 Sakai since it is a clinical isolate associated with plants and appears to be well adapted to the phyllosphere, leaves, rhizosphere and roots of spinach and lettuce. *Ec*O157 TUV 93-0 would only be used for comparative purpose in future experiments.

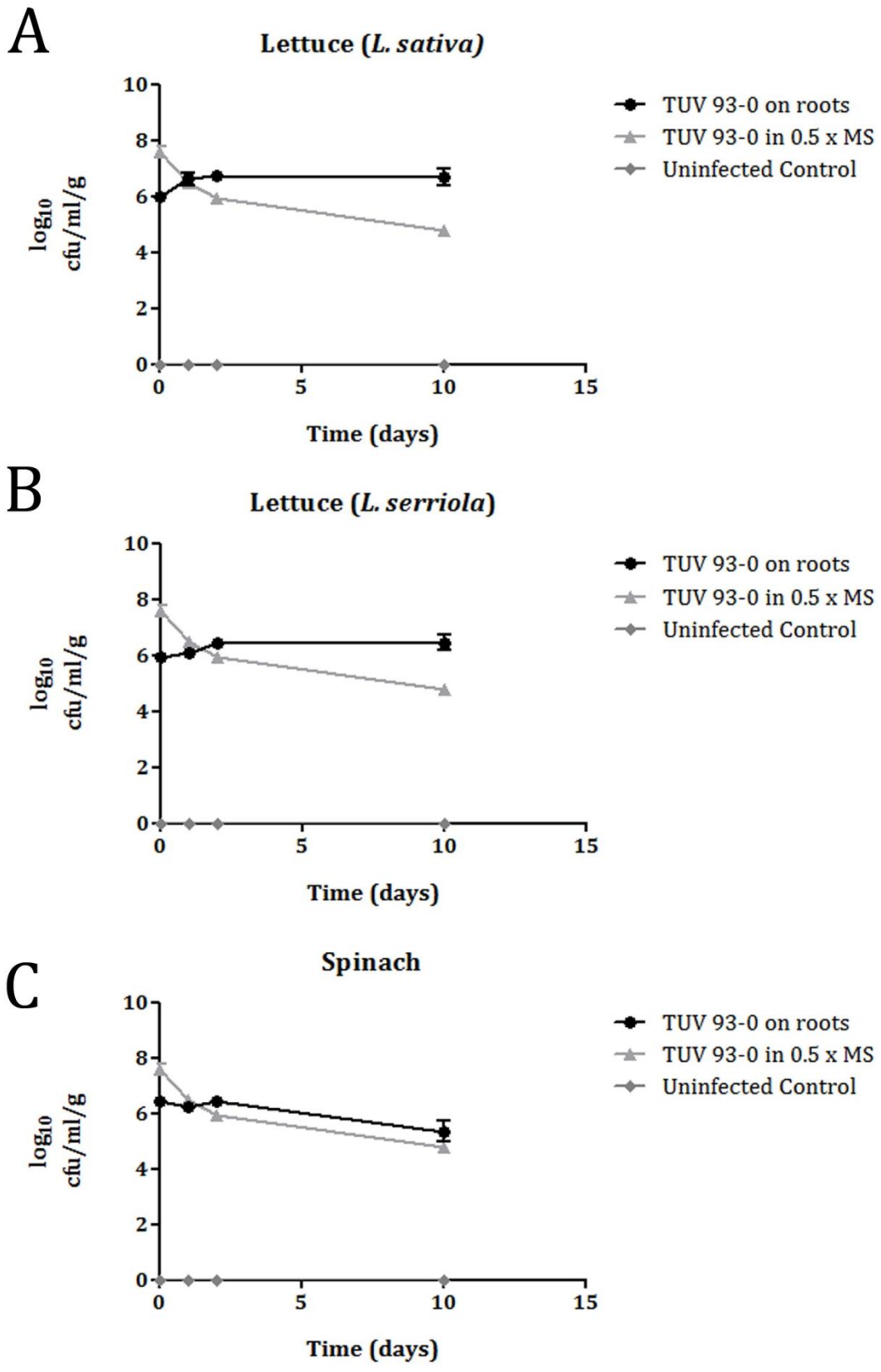


Figure 3-4. Colonisation assays of *Ec*O157 TUV 93-0 on the rhizosphere and roots from hydroponic-grown plants over ten days.

*Ec*O157 TUV 93-0 was inoculated onto the roots of **A)** lettuce (*L. sativa*), **B)** lettuce (*L. serriola*) and **C)** spinach. Three samples were collected from three biological replicate experiments to give a total of nine samples. The line represents the mean of the samples for each timepoint. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0). Data points represent the mean of nine samples; error bars represent standard error of the mean (SEM).

3.2.1.5 Survival of Ec*O157 Sakai on Roots Grown in Compost for Four Plant Species*

As high numbers of *Ec*O157 Sakai were recovered from the roots of spinach and lettuce when grown in hydroponic conditions, I decided to test the association of this bacterial species with the roots of compost-grown plants. This would allow us to compare colonisation of *Ec*O157 Sakai from the hydroponically grown plants with an environment that contained a more competitive microflora. Therefore the assays were repeated using plants grown in individual pots containing compost, as described in section 2.6.2. As seeds did not need to be sterilised prior to growing as they did in the hydroponic assay, root colonisation assays were also carried out for pea.

Roots were separated and removed from the pots for processing at each time point and gently washed three times in 1 x PBS solution to remove as much of the soil as possible, as well as any loosely-associated bacteria. Thus any counts should represent *Ec*O157 Sakai that are strongly associated or internalised into the root tissue. *Ec*O157 Sakai was recovered by plating on MacConkey agar supplemented with kanamycin. Uninfected plants were processed and plated to test for any kanamycin-resistant contaminating species present in the plants.

*Ec*O157 Sakai was able to colonise the roots of spinach, lettuce and pea when the plants were grown in compost (Figure 3-5). In all cases, a similar trend of relatively stable numbers were observed, although the number of cfu recovered at each time point was lower than what was observed from the hydroponic roots.

The lowest numbers of *Ec*O157 Sakai were isolated from the roots of *L. sativa* lettuce and pea, with approximately 2-3 $\log_{10}$ cfu/ml/g plant tissue after two and ten days. The numbers of *Ec*O157 Sakai from the roots of *L. serriola* lettuce and spinach was significantly higher than for the roots of pea (both $p < 0.05$). No significant difference was seen between the two lettuce species.

In all cases *Ec*O157 Sakai was isolated from the roots from compost-grown plants, albeit at a much lower level than when the plants were grown in hydroponics. Regardless, the same stable

population trend was observed for all four of the plant species, although lettuce and spinach roots had significantly higher numbers than that of pea.

It is notable that for three of the species tested, the numbers of bacteria increased from the day two to the day 10 timepoint, indicative of growth.

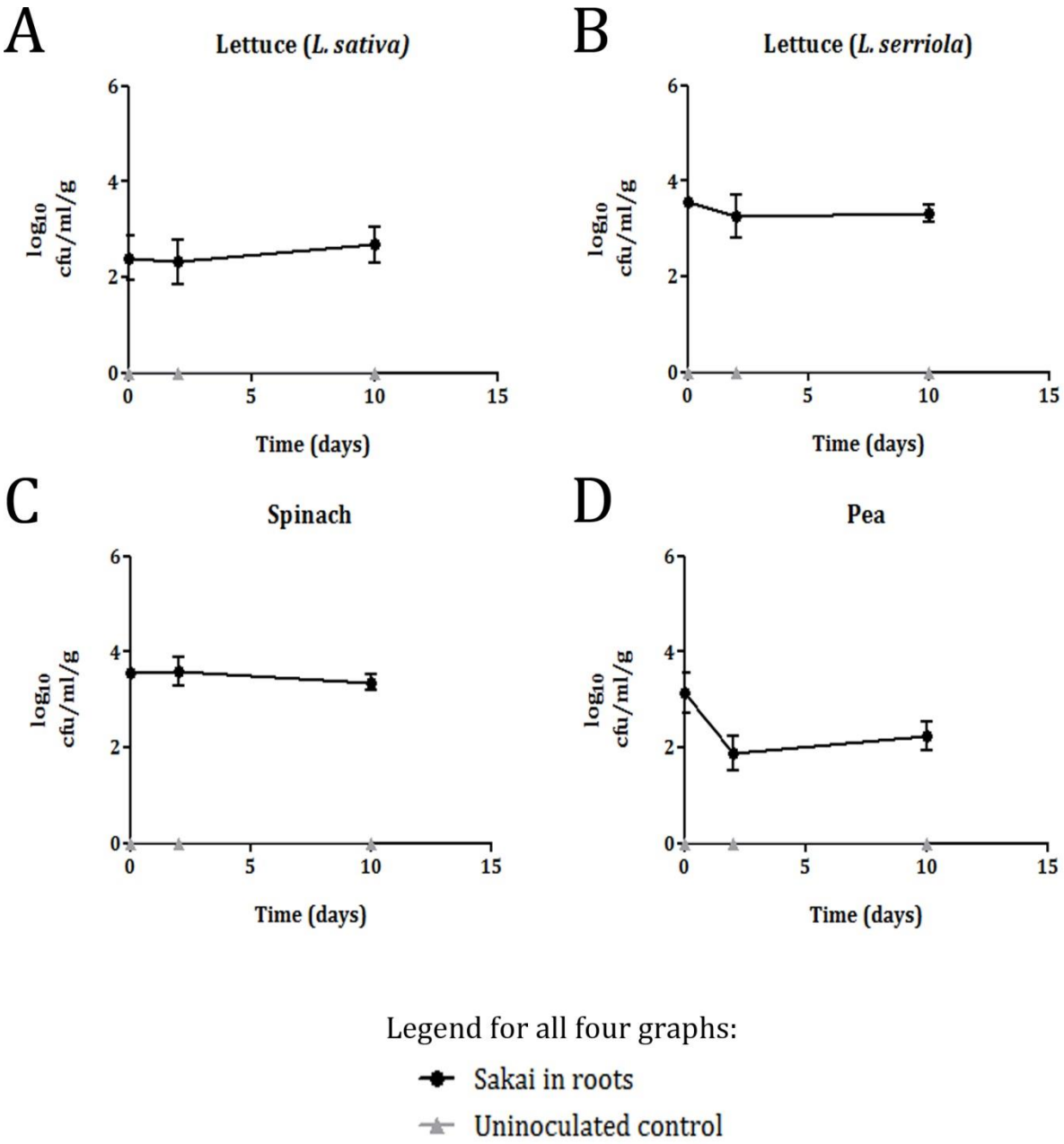


Figure 3-5. Colonisation assays of *Ec*O157 Sakai on the rhizosphere and roots from compost-grown plants over ten days.

*Ec*O157 Sakai was inoculated the soil of pots containing **A)** lettuce (*L. sativa*), **B)** lettuce (*L. serriola*), **C)** spinach and **D)** pea. Three samples were collected from three biological replicate experiments to give a total of nine samples. The line represents the mean of the samples for each timepoint. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0). Data points represent the mean of nine samples; error bars represent standard error of the mean (SEM).

3.2.2 Growth & Motility of Ec*O157 Sakai in Plant Extracts*

As *Ec*O157 Sakai had been shown to survive in the plant environment for ten days for all of the plant conditions tested, I next decided to determine whether *Ec*O157 Sakai could use plant metabolites and grow in plant extracts. Three different plant extracts were made from all four plant species: leaf lysates, root exudates and root lysates (as described in sections 2.7, 2.8 and 2.9, respectively) to represent the three distinct niches of leaves (internal & external tissues); roots (internal & external tissues) and rhizosphere. Further to this, motility tests were carried out with *Ec*O157 Sakai in response to root exudates only, as similar extracts have previously been shown to induce a positive chemotaxis response from many plant-associated bacteria (Somers *et al.*, 2004; Yao & Allen, 2006).

3.2.2.1 Growth of Ec*O157 Sakai in Minimal Media with Plant Extracts*

Growth of *Ec*O157 Sakai was assessed in M9 minimal media supplemented with plant extracts over three days and measured using a spectrophotometer (OD_{600nm}). All extracts had been normalised on the basis of their protein content, in a Bradford assay (see section 2.11), to allow growth of the bacterial strain to be compared between each of the different plant extracts. Data was analysed using one-way ANOVA with the Tukey MCT.

*Ec*O157 Sakai was able to grow in both leaf and root lysates, with the highest cell density recorded for *L. sativa* leaf lysates. Growth was almost as high in spinach, with no significant difference compared to *L. sativa* lettuce (Figure 3-6). Interestingly, significantly higher growth of *Ec*O157 Sakai occurred in the *L. sativa* extracts compared to *L. serriola* ($p < 0.01$). Leaf lysates of both species of lettuce and spinach were seen to induce significantly higher rates of growth of *Ec*O157 Sakai compared to the pea leaf lysate condition (all $p < 0.001$). *Ec*O157 Sakai was unable to use the pea leaf lysate-supplemented media for growth to the same extent as for the other plant species, and only reached an OD_{600nm} of ~0.2. In comparison, when *Ec*O157 Sakai was incubated in M9 minimal media with 0.2 % glycerol it reached a cell density of ~ 1.5 after 48 hours when grown at 18 °C (Figure 3-7).

In root lysates however the opposite was seen: *Ec*O157 Sakai incubated with pea root lysates reached the highest cell density of OD_{600nm} ~0.6, whereas with the other root lysate extracts growth was only observed to an OD_{600nm} of ~0.2 (Figure 3-6). The growth of *Ec*O157 Sakai in pea root lysates was significantly higher than in spinach, *L. sativa* lettuce and *L. serriola* lettuce root extracts (all $p < 0.001$).

In summary, *Ec*O157 Sakai can utilise plant lysates for growth, both of the leaf and the root, but a difference can be seen between the different plant species. Furthermore, a difference is seen between the ability to utilise leaf and root lysates for growth within each plant species.

As the root colonisation assays had shown that *Ec*O157 Sakai could persist in the roots of both hydroponic and compost grown plants to high levels, it was hypothesised that the bacteria would be able to utilise plant root exudates as it was able to use root lysates. However, as shown in Figure 3-7 (**A**), *Ec*O157 Sakai was unable to grow in media containing root exudates from any of the four plants. To determine whether there was insufficient available carbon, or whether root exudates were inhibitory to growth of the bacteria a second experiment was carried out where 0.2 % glycerol was added to the root exudate-supplemented media (**B**; Figure 3-7). No difference was seen in the growth rates between *Ec*O157 incubated with 0.2 % glycerol and with 0.2 % glycerol + root exudates. This suggests that the inability to grow in root exudates is not due to an inhibitory effect but due to a lack of nutritional carbon source.

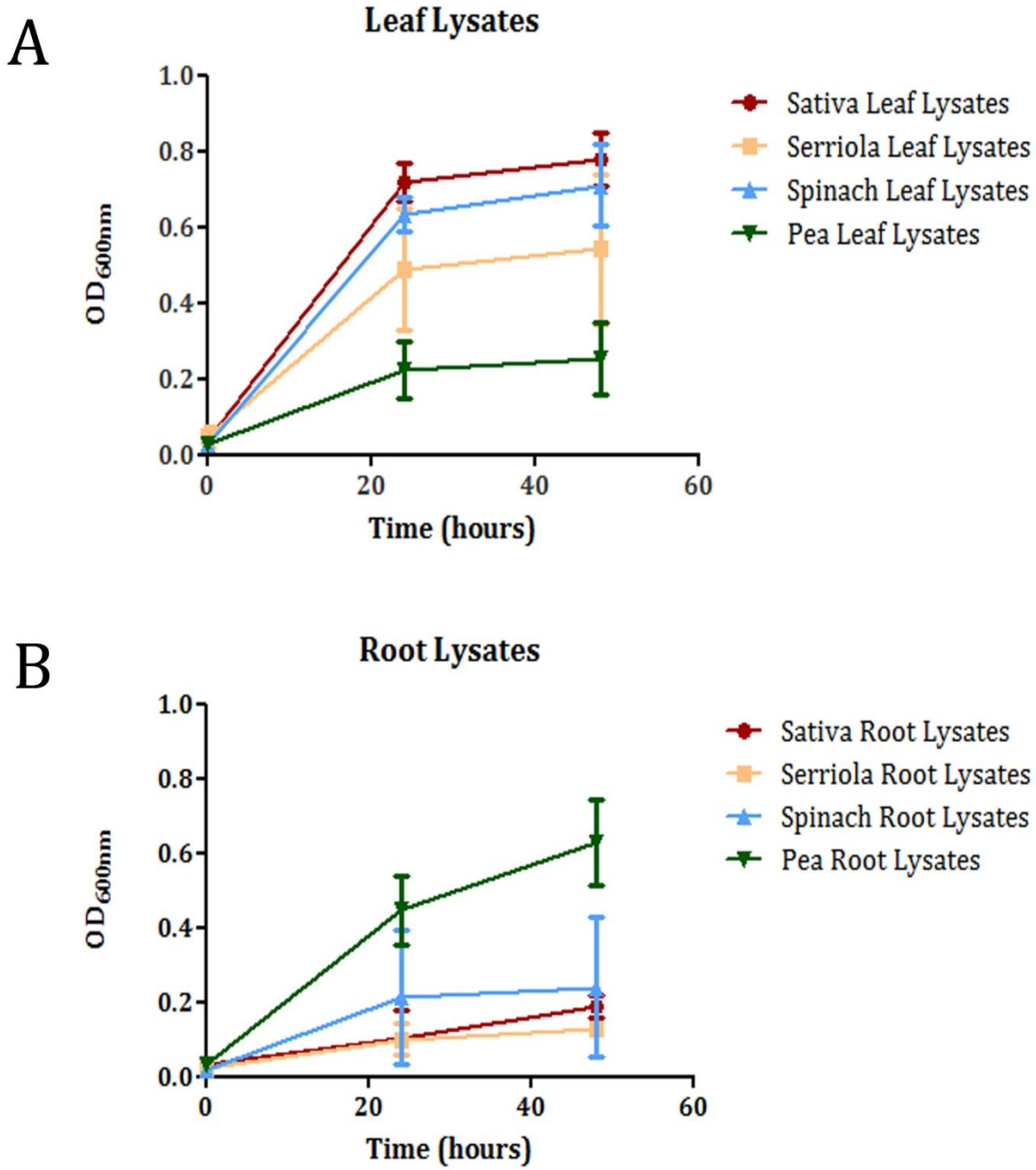


Figure 3-6 Growth of *Ec*O157 Sakai in M9 media supplemented with plant lysate extracts.

Media was supplemented with **A)** leaf lysates and **B)** root lysates from spinach, lettuce (*L. sativa* and *L. serriola*) and pea. Growth was measured at OD_{600nm}. Data points represent average of three biological repeats each with three technical replicates; error bars represent SEM. Data was analysed by one-way ANOVA, followed by the Tukey MCT if a significant difference was seen (GraphPad Prism, version 5.0).

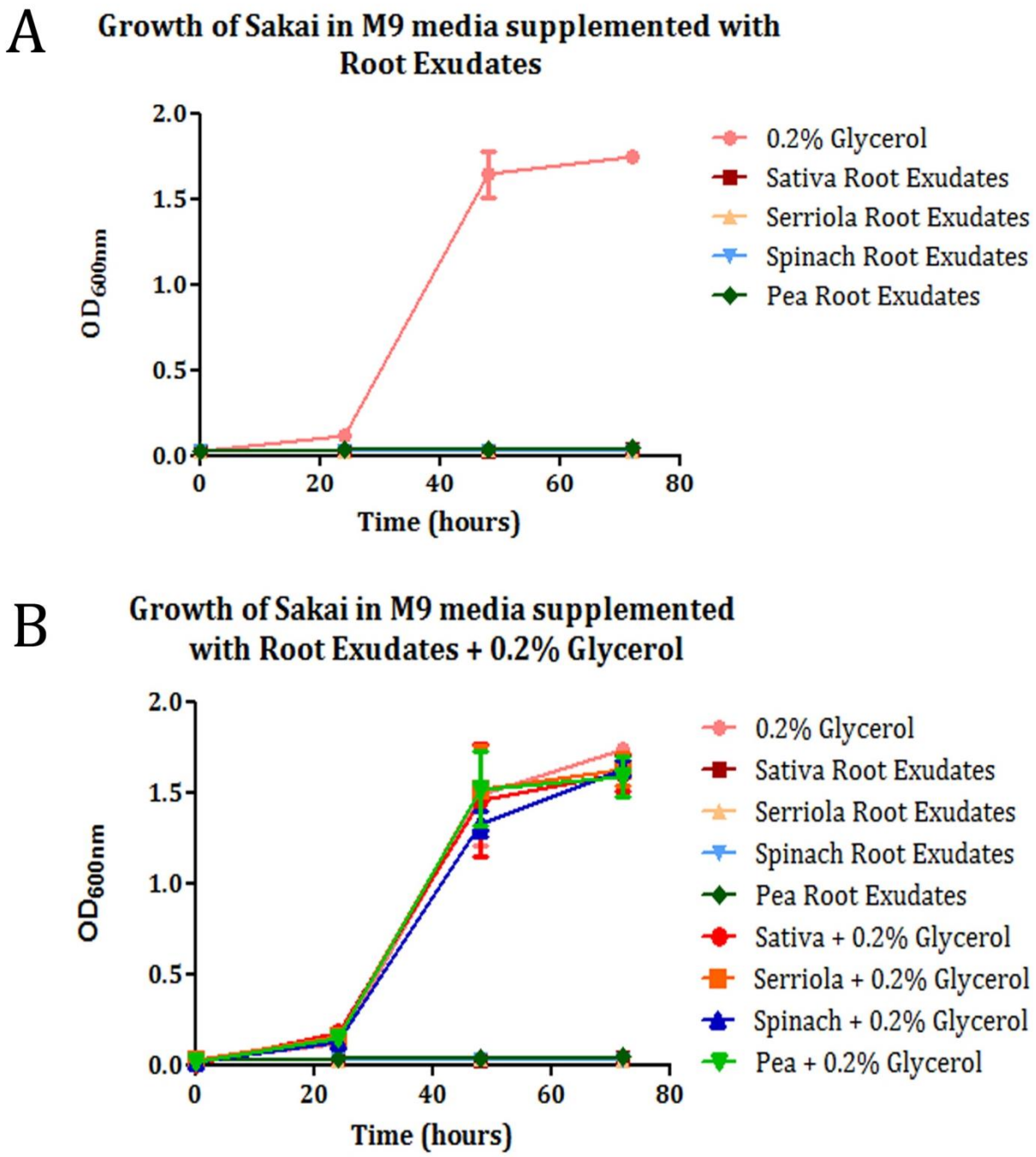


Figure 3-7. Growth of *Ec*O157 Sakai in M9 media supplemented with plant extracts.

Media was supplemented with **A)** root exudates from spinach, lettuce (*L. sativa* and *L. serriola*) and pea. *Ec*O157 Sakai was grown in media containing root exudates ± 0.2 % glycerol **(B)** to test if lack of growth was due to inhibition or an unavailable carbon source. Growth was measured at OD_{600nm}. Data points represent average of three biological repeats each with three technical replicates; error bars represent SEM.

3.2.2.2 Motility of Ec*O157 Sakai in Response to Plant Extracts*

To test if the root exudates from spinach, lettuce or pea had an effect on the motility of *Ec*O157 Sakai, two assays were set up: a chemotaxis assay to assess the ability of the bacteria to migrate from a specified distance on an agar plate towards a filter disc soaked in substrate in the centre of the plate; and a motility assay to measure the motility of the bacteria in agar that had been supplemented with the different plant extracts. In this way the induction of any positive chemotaxis response or an impact on the motility of *Ec*O157 Sakai from root exudates was examined.

Both assays were also carried out at two different temperatures to determine whether any temperature-sensitive effects on motility occurred when incubated with root exudates. All colonisation and growth assays had been carried out at lower temperatures (18 - 22 °C) which were relevant for plant growth. The chemotaxis and the motility assays were tested at both 18 °C and at 37 °C to determine for any differences to motility and the interaction with root exudates as a result of the temperature.

3.2.2.2.1 Chemotaxis Assay Using Filter Paper Discs Soaked in Root Exudates

There was no obvious sign of chemotaxis towards filter paper soaked with root exudates seen after seven days, i.e. no directional growth was seen, only uniform spread from the initial point of inoculation at 18 °C or at 37 °C.

However, differences were seen in the size of the area of motility in *Ec*O157 Sakai when incubated on motility agar containing filter discs soaked in root exudates (data not shown). As a result, a second assay was performed to test if the motility of *Ec*O157 Sakai was affected when root exudates were present within the agar.

3.2.2.2.2 Motility Assay Using Media containing Root Exudates

As no chemotaxis was observed towards the filter discs, a second assay was set up to determine whether the same effects on motility were seen in media containing the root exudates. This

assay was carried out at both 18 °C and 37 °C to observe any temperature-dependent effects. Set-up conditions for the motility assay are described in section 2.12.2.

Motility areas were observed for the water control samples at both 18 °C (36 mm^2) and 37 °C (187 mm^2) (Figure 3-8). Despite larger areas of motility recorded when *Ec*O157 Sakai was inoculated into media containing root exudates, no significant difference was seen between the SDW control and the root exudate samples at 18 °C and at 37 °C. The increase in motility of *Ec*O157 Sakai when incubated with filter discs soaked in root exudates could not be repeated by the addition of root exudates to the motility media.

The motility of *Ec*O157 TUV 93-0 was also assessed to determine whether there was any difference compared to *Ec*O157 Sakai in the presence of root exudates. No significant difference in motility was observed when this strain was exposed to root exudates at 18 °C (**A,** Figure 3-9). When the bacteria was inoculated onto motility plates and stored at 37 °C however an increase was seen in the area of motility measured on plates containing *L. serriola* root exudates ($p < 0.05$; **B**, Figure 3-9). No significant difference was seen for *Ec*O157 TUV 93-0 on plates containing *L. sativa*, spinach or pea root exudates at 37 °C, although none of the large variation in motility was observed for *Ec*O157 TUV 93-0 in response to pea root extracts.

In summary, motility appeared to increase for several of the samples where *Ec*O157 Sakai was inoculated in the presence of root exudates, however no chemotaxis towards root exudates was observed, nor was any significant increase in the spread of the colony compared to a water control.

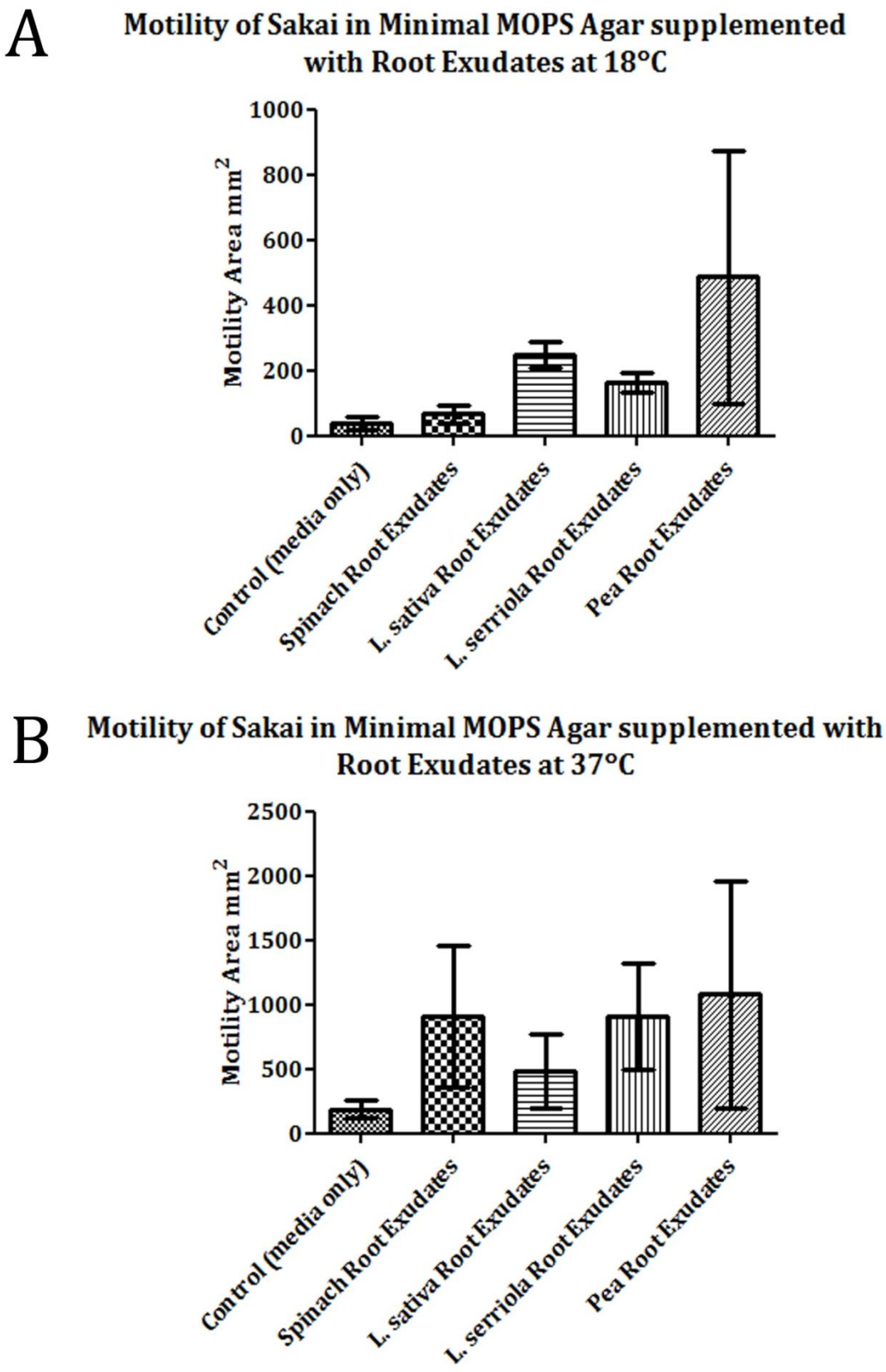


Figure 3-8. Motility assay results for *Ec*O157 Sakai in motility agar supplemented with root exudates over seven days.

Motility assays were carried out at **A)** 18 °C and **B)** 37 °C to check for differences due to temperature. Bars represent an average of three replicates, with error bars showing SEM. Significance was calculated using one-way ANOVA with a Dunnet MCT if significance was seen; * = $p<0.05$. n = 9.

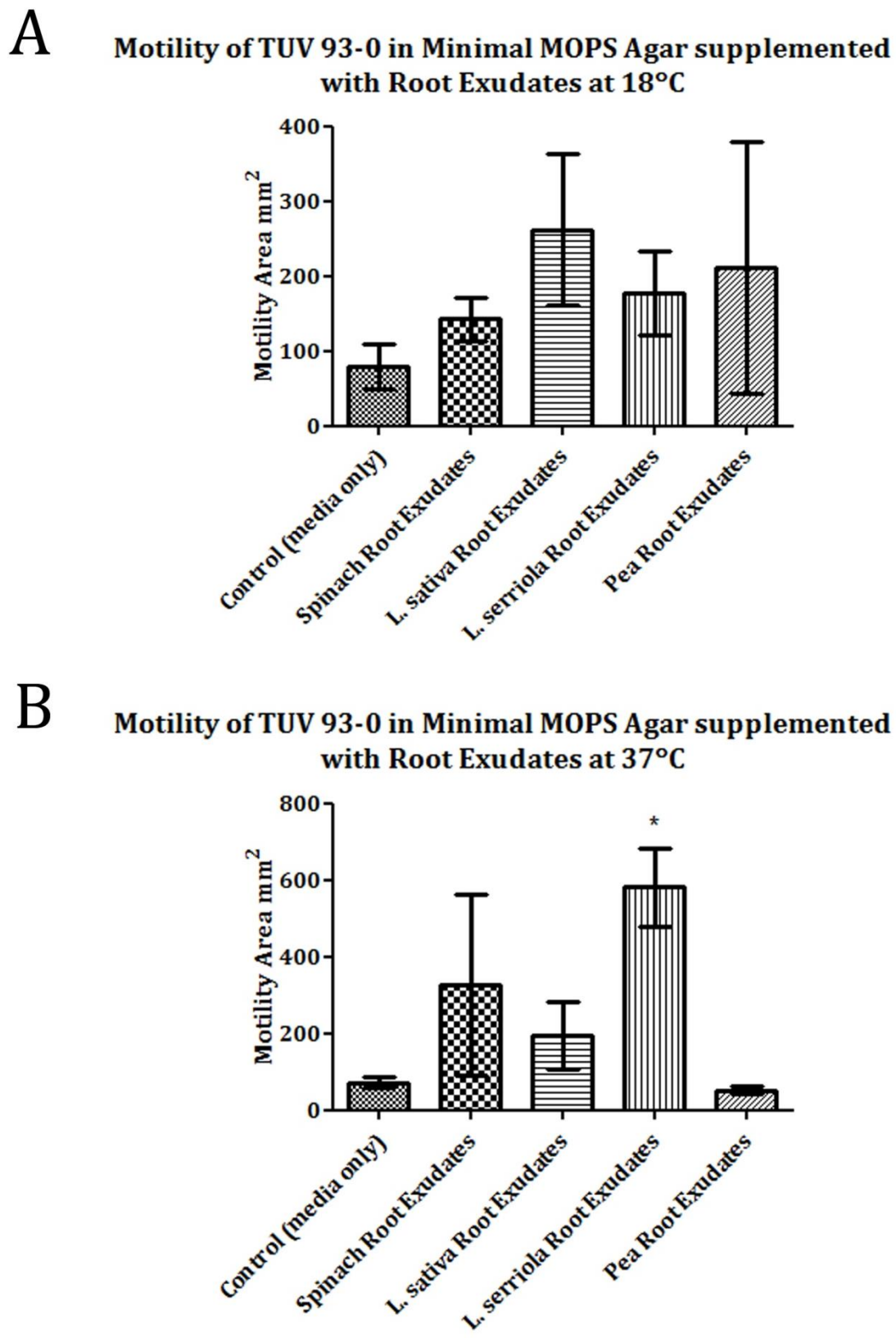


Figure 3-9. Motility assay results for *Ec*O157 TUV 93-0 in motility agar supplemented with root exudates over seven days.

Motility assays were carried out at **A)** 18 °C and **B)** 37 °C to check for differences due to temperature. Bars represent an average of three replicates, with error bars showing SEM. Significance was calculated using one-way ANOVA with a Dunnet MCT if significance was seen; * = $p<0.05$. n = 9.

3.3 Discussion

3.3.1 EHEC can colonise leaves & roots of different plant species

The prevalence of human pathogens on different plant food crops has caused a number of outbreaks over the past few years. Previous studies have shown that human pathogens have positive tropism towards many natural openings in plants such as stomata, and also for plant wounds (Kyle *et al.*, 2010), similar to many plant-associated bacteria (Yu *et al.*, 2013). Further to this, these bacteria can survive in the roots as well as the leaves (Hou *et al.*, 2012) and can even internalise into plant tissue of both leaves and roots as seen in a number of studies (Erickson *et al.*, 2010; Gomes *et al.*, 2009; Wright *et al.*, 2013; Zheng *et al.*, 2013). Internalisation into the plant tissue further decreases the chance of removing these bacteria from the plant prior to processing for consumption. As most prevention strategies developed involve a surface sterilisation treatment of the leaf (Hadjok *et al.*, 2008; Keskinen & Annous, 2011; Neal *et al.*, 2012; Seo *et al.*, 2012), any internalised bacteria will not be exposed to the treatment and will remain within the plant. As a result, it is extremely important to further understand how these bacteria interact with plants, both in living plants and with different plant extracts, in order to develop applicable prevention strategies.

*Ec*O157 strains have been implicated in many of these fresh produce-related outbreaks. Therefore, I decided to test the ability of two *Ec*O157 strains to colonise four different plant species to determine any differences in the colonisation ability between the strains and secondly any differences in the survival of each strain across the four plant species. The survival of each strain was tested on the adaxial (top) and the abaxial (bottom) surfaces of the leaf, as a previous study had shown that *Salmonella* colonises the underside of the leaf in higher numbers compared to the top (Kroupitski *et al.*, 2011), perhaps due to an increased number of stomata and trichomes on the underside of the leaf (Yadav *et al.*, 2005).

Colonisation assays of two strains of *Ec*O157 on leaves demonstrated that not only does an irrigation water-style method of contamination appear to very applicable for the transmission and establishment of human pathogens on plants, but also that the colonisation potential of

*Ec*O157 within the phyllosphere and leaf environment is strain specific in certain plant species. For *Ec*O157 Sakai, a decline in numbers from that of the initial inoculum was observed in all of the plant species tested; however, *Ec*O157 TUV 93-0 counts are seen to continue to decline until they are unable to be detected after ten days in both species of lettuce. This inability to detect this bovine strain of *E. coli* after ten days on lettuce suggests that it may lack metabolic enzymes or adherence factors for example that may be necessary for survival within the phyllosphere and leaf environment, or that the numbers of bacteria present after ten days are below the limit of detection using plate counts. Furthermore, although a drop off from the initial inoculum was seen for *Ec*O157 Sakai in all cases, this is similar to work carried out by Glyn Barrett (University of Reading), where a similar pattern of colonisation was seen when testing *Ec*O157 Sakai on pea leaves (Barrett, 2013). This 'carrying capacity' or threshold of bacteria the leaves of ~ 2 – 3 log cfu/g could be down to a number of factors affecting bacterial survival in the phyllosphere, such as a lack of nutrient availability, UV exposure, low water levels and competing epiphytic species present on the leaves. Presumably far lower levels of inoculum would result in an increase in bacteria counts until this threshold was reached.

As *Ec*O157 Sakai had significantly higher counts than *Ec*O157 TUV 93-0 after ten days in lettuce this supports the hypothesis that *Ec*O157 Sakai is better adapted to the plant environment and may encode specific genetic elements or regulation that allow it to colonise these leaves in higher numbers. However, as no difference was seen in the ability of these two strains to colonise spinach and pea, this suggests that there is also some common genetic elements to both that are responsible for survival on plants (discussed further in Chapter 4).

*Ec*O157 Sakai and TUV 93-0 were both recovered from spinach leaves after ten days. As spinach has been implicated in *Ec*O157 outbreaks (Wendel *et al.*, 2009) and has been shown to harbour *Ec*O157 strains (Choi *et al.*, 2011; Lopez-Velasco *et al.*, 2012; Patel *et al.*, 2010), perhaps this environment is more susceptible to colonisation by human pathogens than other plant species, or provides a preferential habitat. As a result, spinach may provide an ideal plant species for studying the interaction of *Ec*O157 with plants to show how they are able to survive within the plant environment.

In contrast, very low/no counts of both strains were recovered from the wild, prickly lettuce *L. serriola* after ten days. *Ec*O157 Sakai was detected on *L. sativa*, the domesticated lettuce, after ten days, but the bovine strain was not. This not only shows the impact of using certain lettuce species, but also could implicate *L. serriola* as an ideal target for studying inhibition of human pathogens on plants, by testing for antimicrobial compounds for example. This inability to survive after ten days on *L. serriola* lettuce could be due to differences in the plant leaf surface, differences in secondary metabolites produced, since this species was shown to be enriched in polyphenols in comparison to edible lettuce (Chadwick, 2013) or dependent on which epiphytic species are present. This difference between species correlates with other work, which has found differences even at the cultivar level for bacterial potential colonisation and interaction with plants (Macarisin *et al.*, 2013; Mitra *et al.*, 2009; Quilliam *et al.*, 2012).

In contrast to the phyllosphere, both *Ec*O157 species were able to colonise the rhizosphere, and this occurred on both hydroponic-grown and compost-grown plants for *Ec*O157 Sakai, including for *L. serriola*, suggesting that the rhizosphere is an easier habitat for colonisation for this bacterial species. However, the numbers of recovered bacteria after ten days were similar from that of the initial inoculum. As a high inoculum was used, it is not clear from these results if the bacteria recovered are new cells or if the bacteria are unable to grow and instead persist over time. Either way, as *Ec*O157 was recovered from both compost- and hydroponic-grown plant roots this adds weight to the possibility that the rhizosphere is a more beneficial environment for the survival and proliferation of *E. coli* O157 strains than the phyllosphere – such that even bovine strains which are not adapted to the plant environment are able to survive in high numbers. For both species, bacterial counts from the inoculation in the plant growth medium 0.5 x MS only (containing no sucrose) decreased over the ten day period, but could still be recovered at ten days at very high levels, an example of how these bacteria can persist in low nutrient environments.

Although bacterial counts of *Ec*O157 Sakai recovered from root grown in hydroponics were higher than counts recovered from the compost-grown plants for all plant species tested, it is thought that this could be due to the presence of other competing microorganisms present in the

compost. Therefore growth/persistence of *Ec*O157 Sakai in compost-grown plants is slower than that in a hydroponic system, but it can still occur. This demonstrates the importance of mimicking the appropriate plant growth conditions as closely as possible.

The persistence of human pathogens within the rhizosphere has been studied previously (Gagliardi & Karns, 2002; Quilliam *et al.*, 2012), as it is proposed that root-to-shoot colonisation by these human pathogens could occur as another mode of transmission to the leaves (Cooley *et al.*, 2003). Therefore it is essential to understand both interactions with the leaf tissue and with the root tissue and surrounding rhizosphere with *Ec*O157 further as no one plant tissue has been implicated as a target for colonisation.

As a difference was seen between the two strains used in this study in terms of their colonisation ability on the leaves, it was concluded that *Ec*O157 Sakai would be suitable to study its interactions with plants in more detail, to understand how a human pathogen could survive in the plant environment, by using a bacterial strain that appears to be well adapted to this environment.

To investigate different areas of this interaction, plant extracts were prepared from both the leaf and root environment. Although the work was carried out under *in vitro* conditions rather than *in planta*, analysis of the response of *Ec*O157 Sakai to the plant extracts should give an indication of firstly how the bacteria responds and secondly what genes are important for this response. These can then be applied to *in planta* conditions in the future. Leaf lysates were used as a mimic for plant wounds in a similar method to Kyle et al, 2010, as plant wounds have been shown to be an area of high colonisation for *Ec*O157 strains (Brandl, 2008). However, there are limitations to using leaf lysates as a representation of plant wounds. Firstly, the bacteria will be exposed to different concentrations of the sugars, organic acids and amino acids present within the leaf lysates compared to whole plant tissue, partly due to the preparation process and the half-life of many metabolic enzymes. Furthermore, preparing a solution of leaf lysates will dilute out many of the anti-microbials that were present on and within the leaf tissue. One could speculate that phenolic compounds may have been focused around the area of wounding on the plant, potentially at toxic concentrations to *Ec*O157 – however, this effect would be lost in the leaf

lysates. Finally, any chemotactic and motile response would not be induced when in the presence of the leaf lysates solution, as the nutrients would be accessible to the bacteria at all times. However, despite these caveats the leaf lysates do help to provide an initial understanding of if *Ec*O157 can use substrates present within the leaf tissue and gene expression work in this area (Chapter 5) can help identify genes important for utilisation of plant substrates.

3.3.2 Ec*O157 Sakai can utilise some plant extracts for growth*

Root exudates have been shown to play important roles in influencing the microbiome of the rhizosphere, through their nutritional content, antimicrobial properties and pH differences (Bulgarelli *et al.*, 2012). The content of root exudates is hard to determine due to their ever changing nature in response to environmental cues and age of the plant (Badri & Vivanco, 2009; Carvalhais *et al.*, 2011). Furthermore, differences are seen in the root exudate content between plant species (Sauer *et al.*, 2006). Nevertheless, studies have suggested that in general plants will pass 20 – 50 % of the carbon in the leaves to the roots (Kuzyakov & Domanski, 2000), and 2 – 70 % of this carbon will be translocated from the roots into the soil via rhizodeposition (Jones *et al.*, 2004; Kuzyakov & Domanski, 2000; Lynch & Whipps, 1990). Root exudates have been shown in the past to be rich in organic acids such as citrate, malate, oxalate and succinate (Jones *et al.*, 2009), all of which can be used by *E. coli* (Neidhardt, 1996). To date, no study has investigated the composition of spinach root exudates; however, there has been work looking at lettuce root exudates from lettuce grown in different soils, where the authors found that there was an 11-fold difference in overall amino acid concentration between the different soils (Neumann *et al.*, 2014). The authors analysed the lettuce root exudates by GC-MS and found that the most prevalent amino acids were glycine, serine and valine over the three soil types; glucose, fructose, maltose, trehalose, sucrose and glycerol for sugars in the root exudates; and succinate and benzoic acid were the most common organic acids found (Neumann *et al.*, 2014). *E. coli* is able to use all these metabolites for its own survival and growth (Neidhardt, 1996) – therefore, it would be presumed that the root exudates would provide a suitable environment for this bacteria to survive.

However, my results showed that *Ec*O157 Sakai is able to utilise leaf and root lysates for growth, but is unable to grow in the presence of root exudates alone. It is expected that this bacteria would be able to use the lysates for growth as the process for preparing the lysates of both leaves and roots released all the potential metabolites and cell contents and diluted out any anti-microbial compounds that may have been present in high concentrations on the surface of the leaf or in the apoplast. Studying this further may give some clues of how this human pathogen is able to metabolise plant components, especially in the case of processed foods that have been cut. Previous studies have shown that VTEC and *Salmonella* display enhanced tropism towards open wounds and cuts on the leaf surface (Brandl, 2008); thus, studying leaf lysates further may help understand which genes are being upregulated and are important for this interaction.

The inability of *Ec*O157 Sakai to use root exudates for growth is in marked contrast with reports for plant-associated bacteria such as *Pseudomonas*, which have been shown to exhibit tropism towards root exudates and utilise them for growth (Mark *et al.*, 2005). There could be three potential explanations for this relating to the low concentrations and/or inaccessible forms of carbon-based metabolites. The first explanation is that as the root exudates were prepared from surface-sterilised seeds and grown in sterile hydroponic conditions there will be little plant-associated bacteria/fungi present in the rhizosphere, making it a very different environment from that in compost. Perhaps this lack of plant-associated species (whether this is pathogenic or commensal species) is the reason *Ec*O157 Sakai is unable to grow in root exudates, as the other competing species may break down plant material into accessible forms for uptake and degradation by *Ec*O157 Sakai. In fact, other studies have shown that the presence of certain microorganisms in the plant environment can greatly aid the colonisation of VTEC and *Salmonella* in both the leaves (Lopez-Velasco *et al.*, 2012; Meng *et al.*, 2013) and the roots (Cooley *et al.*, 2006). A second explanation for this lack of response to root exudates may be that Sakai is unable to use root exudates to survive in the rhizosphere, with or without the presence of other species; however, this would force the bacteria to associate with the roots of the plant in order to survive and may explain why such high colonisation levels are seen for the rhizosphere colonisation assay results. If *Ec*O157 Sakai was able to grow in root exudates, there would be

little need for the bacteria to associate with the plant itself and they could continue to proliferate in the soil. Therefore, this strong association with the roots could be due to the roots of the plant providing a much more nutrient rich environment than just the exudates alone (further supported by the HPLC results in section 4.2.2.). Finally, it is possible that other microbes were present within the root exudates and they have depleted all the available nutrients prior to *E. coli* inoculation. Sterile media and containers were used throughout the process; however, it has been shown in the past that validation methods are necessary to determine if the root exudates are free of microbes (Kuijken *et al.*, 2015). These validation methods were not carried out in this project; therefore microbial contamination remains a possibility.

3.3.3 Root exudates do not influence the motility of Ec*O157*

As other plant-associated bacteria and fungi display positive tropism towards root exudates (Yao & Allen, 2006), it was suggested that *Ec*O157 may do the same. However, despite *Ec*O157 Sakai having a strong association with the roots, no obvious chemotaxis phenotype was observed upon exposure to any of the root exudates tested. Greater motility was observed for selected samples; however, this could not be repeated at a different temperature or between assays (motility and chemotaxis assays), indicative of variation between the samples. As a result, a clear answer could not be determined and further studies would need to be carried out in the future, perhaps testing alongside plant-associated bacteria for a comparison. Therefore, it is proposed that root exudates do not affect the motility of *Ec*O157 Sakai, as no clear trend could be established.

3.3.4 Summary

Overall, it would appear that the plant-associated strain of *Ec*O157 (Sakai) can persist within the leaves of lettuce, spinach and pea over ten days and can sustain a higher population than the bovine-strain of *Ec*O157, TUV 93-0. Both strains are able to survive within the rhizosphere at higher levels than when in the phyllosphere and this population is stable over ten days, with evidence for population growth.

*Ec*O157 Sakai has been shown to utilise leaf and root lysates for growth, but is unable to use root exudates. Addition of 0.2 % glycerol to the root exudate media allowed this bacterial strain to grow similar to the control, suggesting that the root exudates were not having an inhibitory effect on the growth of the bacteria and merely lacked sufficient carbon for growth. It was proposed that root exudates may induce positive tropism from the bacteria towards the roots; however, root exudates did not appear to influence the motility of *Ec*O157 Sakai.

To help explain the findings from these colonisation, growth and motility experiments, further study is necessary: firstly, to identify differences between the two bacterial strains, *Ec*O157 Sakai and TUV 93-0, e.g. to determine what genetic differences between the two could cause *Ec*O157 Sakai to be better adapted to the plant environment than *Ec*O157 TUV 93-0; and secondly, differences between the plants used (spinach, lettuce (*L. sativa* and *L. serriola*) and pea) and in the composition of their extracts, which may affect the response and the colonisation ability of the bacteria.

4 Comparisons between Bacterial Strains and Plant Extracts

4.1 Introduction

4.1.1 Differences Between Human Pathogen Species On Plants

A number of pathogenic strains of *Escherichia coli* have been linked to the consumption of infected food in the past twenty years, with the most prevalent serotype of verotoxigenic *E. coli* being O157:H7. Outbreaks have been linked to a variety of food products, including ground beef, where the serotype *Ec*O157:H7 was first isolated from in 1982 (Wells *et al.*, 1983). However, outbreaks have also been linked to many different types of fresh produce, including leafy greens such as lettuce (Ackers *et al.*, 1998), spinach (CDC, 2006), fruits such as strawberries (Laidler *et al.*, 2013), and vegetables such as leek and potato (ProMED-mail, 2011). *Ec*O157:H7 is a serotype of the VTEC pathogroup, which is isolated with the highest incidence from patients with HUS worldwide (Tarr *et al.*, 2005). A strain of *E. coli* with the serotype O104:H4 was seen in a large outbreak in Germany in 2011, which resulted in 4,075 cases, 908 of which developed HUS and 50 resulted in death (CDC, 2013). The serotype was rarely detected in outbreaks before, and is essentially a newly emerged pathotype, EAHEC, evolved from enteroaggregative *E. coli* (EAEC), carrying the aggregative adhesion fimbria (AAF) encoded on the pAA plasmid, as well as the bacteriophages encoding the Shiga-toxin genes, which are present in *E. coli* O157:H7 and *Shigella* species (Brzuszkiewicz *et al.*, 2011). Transfer of genetic elements between bacteria has been described in the past as an important cause of pathogenicity to both humans and plants (Jackson *et al.*, 2011); for example in *Ec*O157:H7, the genomic island OI-57, which encodes for virulence genes such as *adfO* and *chf*, was identified as the part of the 'virulome' of this bacterial serotype (Imamovic *et al.*, 2010).

Mobile genetic elements such as plasmids and genomic islands are normally acquired because they confer some sort of an advantage, such as in fitness or transmission in the mammalian host: therefore, it is logical to predict that there are acquired genes that would give organisms an advantage in other areas such as proliferation in the environment. In section 3.2.1, two strains of *Ec*O157 were assessed for their ability to colonise the leaves and roots of plants: a bovine

isolate TUV 93-0 (a *stx*-inactivated variant of *Ec*O157 EDL 933 from Dahan et al, 2004) and a clinical isolate from a plant-associated outbreak, Sakai (Hayashi *et al.*, 2001). The bovine strain numbers dropped below the limit of detection after ten days on lettuce leaves, whereas the plant-associated strain could be recovered from the leaves of all plant species tested after ten days. This suggests that *Ec*O157 Sakai possesses some genetic component that gives it an advantage in the plant environment, specifically the leaves. As this strain has been implicated in an outbreak with white radish sprouts (Michino *et al.*, 1999), it has been exposed to the plant environment previously. Soil and water environments are known to be areas of high incidence of transfer of plasmids and other mobile genetic elements (Baquero *et al.*, 2008; van Elsas & Bailey, 2002), therefore it is possible that repeated exposure to the plant environment may increase the chances of acquiring genetic elements from the native environmental microflora that may provide an advantage in adaptation to plants.

Phylogenetic comparisons of *Ec*O157 strains EDL933 and Sakai reveals that the strains have only a 3 % difference in nucleotide sequence observed between the two (Manning *et al.*, 2008). Both strains contain the large 92 kb pO157 plasmid, which encodes 83 genes, including some of the virulence genes necessary for their pathogenic lifestyle in humans (Burland *et al.*, 1998). This includes genes encoding for haemolysin and the *toxB* gene, which has been shown to promote the adherence to human epithelial cells through stimulation of the T3SS (Tatsuno *et al.*, 2001). However, *Ec*O157 Sakai also possesses a small 3.3 kb plasmid (pOSAK1) (Makino *et al.*, 1998). Only three genes are present on this extra plasmid in *Ec*O157 Sakai: a plasmid mobilisation gene known as *mobA* and two hypothetical proteins, simply known as pOSAK1_02 and pOSAK1_03. To date no studies have implicated a role for these hypothetical proteins in environmental colonisation and BLAST searches for these hypothetical proteins reveal they are similar to hypothetical proteins encoded in plasmids present in *Salmonella* species. Therefore perhaps other genetic differences must be present between the *Ec*O157 strains to account for the difference in environmental colonisation.

Alongside this, there have been contradictory studies published looking at the environmental colonisation potential of *E. coli* K-12, the laboratory strain of *E. coli*. One study showed this

strain of *E. coli* was able to colonise plant tissue better than *Ec*O157 EDL933 (Fink *et al.*, 2012), whereas another showed the internalisation of *Ec*O157 Sakai into root tissue in high numbers in contrast to very low levels for *E. coli* K-12 (Wright *et al.*, 2013). As *E. coli* K-12 is not a pathogenic strain little work has been carried out to look at its colonisation in the environment. However, if *E. coli* K-12 can colonise plant tissue such as leaves then perhaps the "core" genome, i.e. the metabolism genes common to *E. coli* K-12 and other strains that are necessary for growth and survival, plays an important role in the adaptation to the plant environment. As a result, it is important to study the response of these core genes, as well as any *Ec*O157-specific genes that may confer additional advantages.

4.1.2 Differences Between Plants Used In This Study

Although there are differences in the experimental setups and bacterial strains used between the two studies reporting conflicting work (Fink *et al.*, 2012; Wright *et al.*, 2013), another important factor is the plant species used for the experiment. Previous studies have demonstrated the importance that different cultivars of plants can have on the survival of both *Ec*O157 and *Salmonella* strains in the plant environment (Barak *et al.*, 2011; Klerks *et al.*, 2007; Macarisin *et al.*, 2013; Quilliam *et al.*, 2012). *Ec*O157 and *Salmonella* have been found in outbreaks with many different types of plants, indicating that these bacterial strains are well adapted to a wide variety of environments.

Four plant species were used in this study: spinach (*Spinacia oleracea* var. Amazon) and lettuce (*Lactuca sativa* cv Salinas), since multiple *Ec*O157 outbreaks have been associated with leafy salad vegetables; a wild species of lettuce (*L. serriola*) to investigate plant species differences; and pea, which although it has not been implicated in any human pathogen outbreaks to date, *E. coli* performs well on it (G. A. Barrett and R. W. Jackson, pers. comm.). There are many morphological differences between the plants in terms of the surface structure of the leaf, as seen in Figure 4-1. Differences can also be seen between the domesticated (*L. sativa*) and the wild (*L. serriola*) lettuce, especially in terms of the stems, where a prickly morphology can be seen for the wild lettuce. As well as phenotypic differences, there are also many differences in

the biochemical content between the species. Analysis of the leaves of the two lettuce species, by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR), found the greatest difference lay in the polyphenols (Chadwick, 2013), which could offer some explanation for the colonisation results seen in section 3.2.1. It is proposed that the *L. sativa* species contains less phenolics due to the selective breeding process as these have a bitter flavour and thus less phenolics in lettuce would make the product more appealing for general consumption (Drewnowski & Gomez-Carneros, 2000). It is possible that lower concentrations of phenolic compounds, such as flavonoids, tannins and phenolic acid which have antimicrobial properties against *E. coli* (Daglia, 2012), could provide an explanation for increased leaf colonisation in *L. sativa* compared to *L. serriola*.

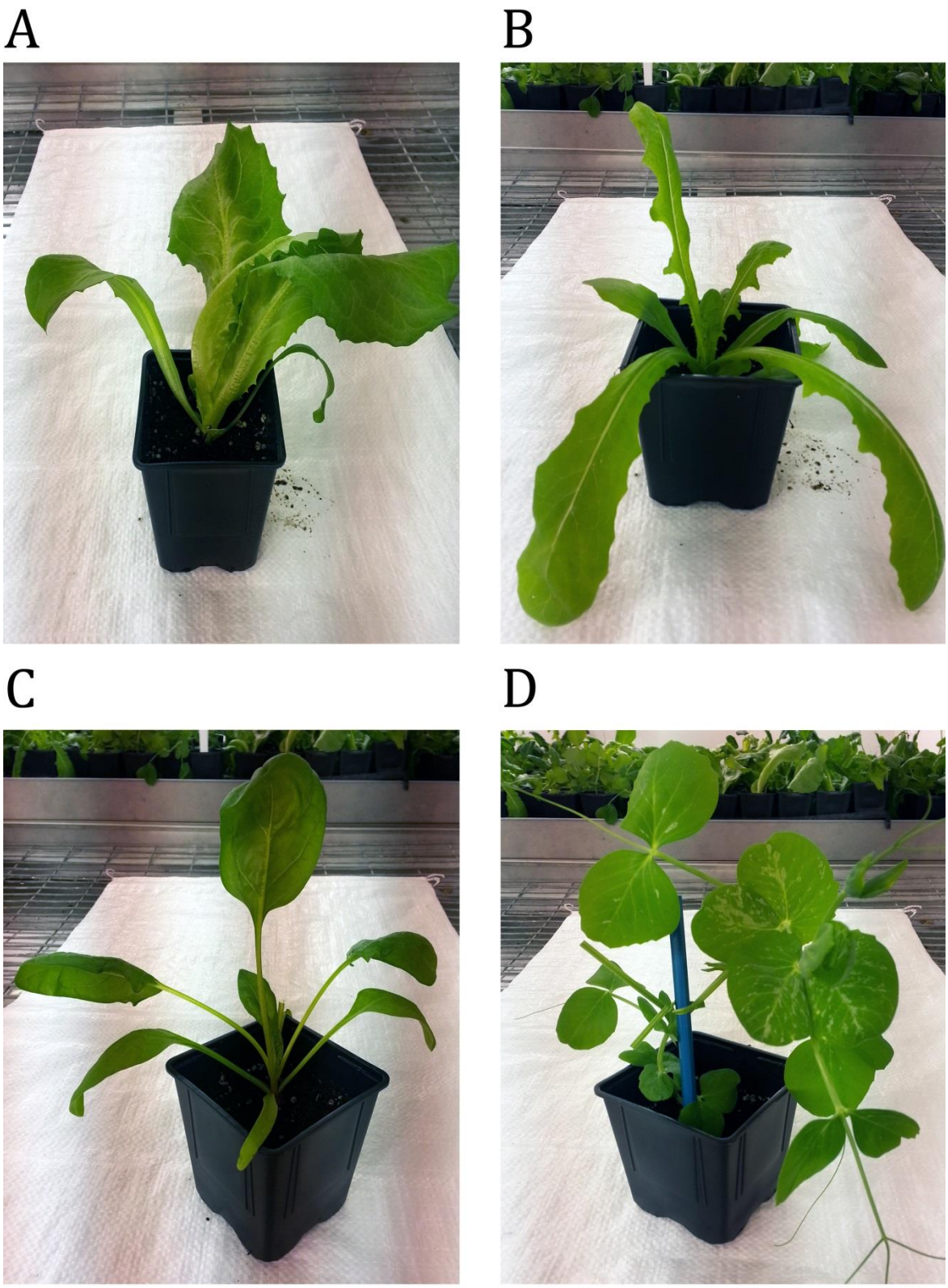


Figure 4-1. Plants used in this study.

All plants were grown for 2 and a half weeks at 75 % humidity, 22 °C, 16/8 hour light/dark cycle. Plant species are as follows: **A**) Lettuce (*Lactuca sativa* cv Salinas), otherwise known as domesticated lettuce; **B**) Lettuce (*Lactuca serriola),* otherwise known as wild, prickly lettuce; **C**) Spinach (*Spinacia oleracea* cv Amazon); and **D**) Pea (*Pisum sativum*).

4.1.3 Aims of this Area of Study

A. To identify genetic differences between *E. coli* isolates Sakai and EDL933 that might highlight the genetic basis of superior plant colonisation by Sakai.

B. To identify metabolic pathways that differ between *Ec*O157 Sakai and EDL933 that might confer an advantage in the plant environment.

C. To determine the biochemical content of the plant extracts to look for differences between the plant species, which may affect growth and colonisation of the bacteria.

Since differences were seen in the colonisation ability and growth of *Ec*O157 Sakai on the different plants used in this study, HPLC analysis was carried out to determine the monosaccharide content. Differences in the lysate extracts may explain differences in the growth profiles of *Ec*O157 Sakai, whereas the total content of sugars in the root exudate extracts may explain the lack of bacterial growth (section 3.2.2.1).

In addition, transcriptome analysis had shown large differences in the response of *Ec*O157 Sakai to cell wall polysaccharide from spinach and lettuce tissue (section 5.2.2). Therefore, HPLC analysis was carried out to assess and quantify a number of monosaccharides in these samples. The overall aim of the biochemical analysis was to determine whether it was possible to correlate the plant extract monosaccharide composition with the *Ec*O157 Sakai gene expression response to the extracts (detailed in Chapter 5).

4.2 Results

4.2.1 Comparative Genomic Analysis of Ec*O157 EDL933 and Sakai*

As *Ec*O157 Sakai and TUV 93-0 (EDL933) had different levels of colonisation after ten days on the leaves of certain plant species, it was proposed that there would be genetic differences between the two strains that may confer an advantage or disadvantage in one strain over the other. Therefore, to compare the two genomes of *Ec*O157 EDL933 and Sakai, the sequences were downloaded from the National Centre for Biotechnology Information (NCBI) (NCBI Resource Coordinators, 2013) and analysed using the Gepard dotplot comparison program (Krumsiek *et al.*, 2007). As expected, a high degree of similarity occurred between the two sequences (Figure 4-2). In this analysis one genome is plotted on the X-axis and the other on the Y-axis and sections where the two genomes overlap are coloured black generating a black diagonal line. Disruption to the line is caused by the presence of insertion elements or genetic deletions, a feature which has been analysed in many of the *Ec*O157 and related *E. coli* strains (Kotewicz *et al.*, 2007). The high degree of similarity confirms other phylogenetic analysis (Zhou et al, 2010).

*Ec*O157 isolates have acquired many of their virulence genes via the uptake of genetic elements, for example via the insertion of the 933 phage, or the exchange of virulence plasmids (Ohnishi *et al.*, 2000). These horizontally acquired genetic elements, such as genomic islands, may contain some of the genes necessary for environmental survival and colonisation. Comparative analysis of genes predicted to be present on a genomic island was tested using Island Viewer (Langille & Brinkman, 2009), which combines the statistical probabilities of three different prediction methods (IslandPick (Langille *et al.*, 2008), SIGI-HMM (Waack *et al.*, 2006) and IslandPath-DIMOB (Hsiao *et al.*, 2003)) to determine the output.

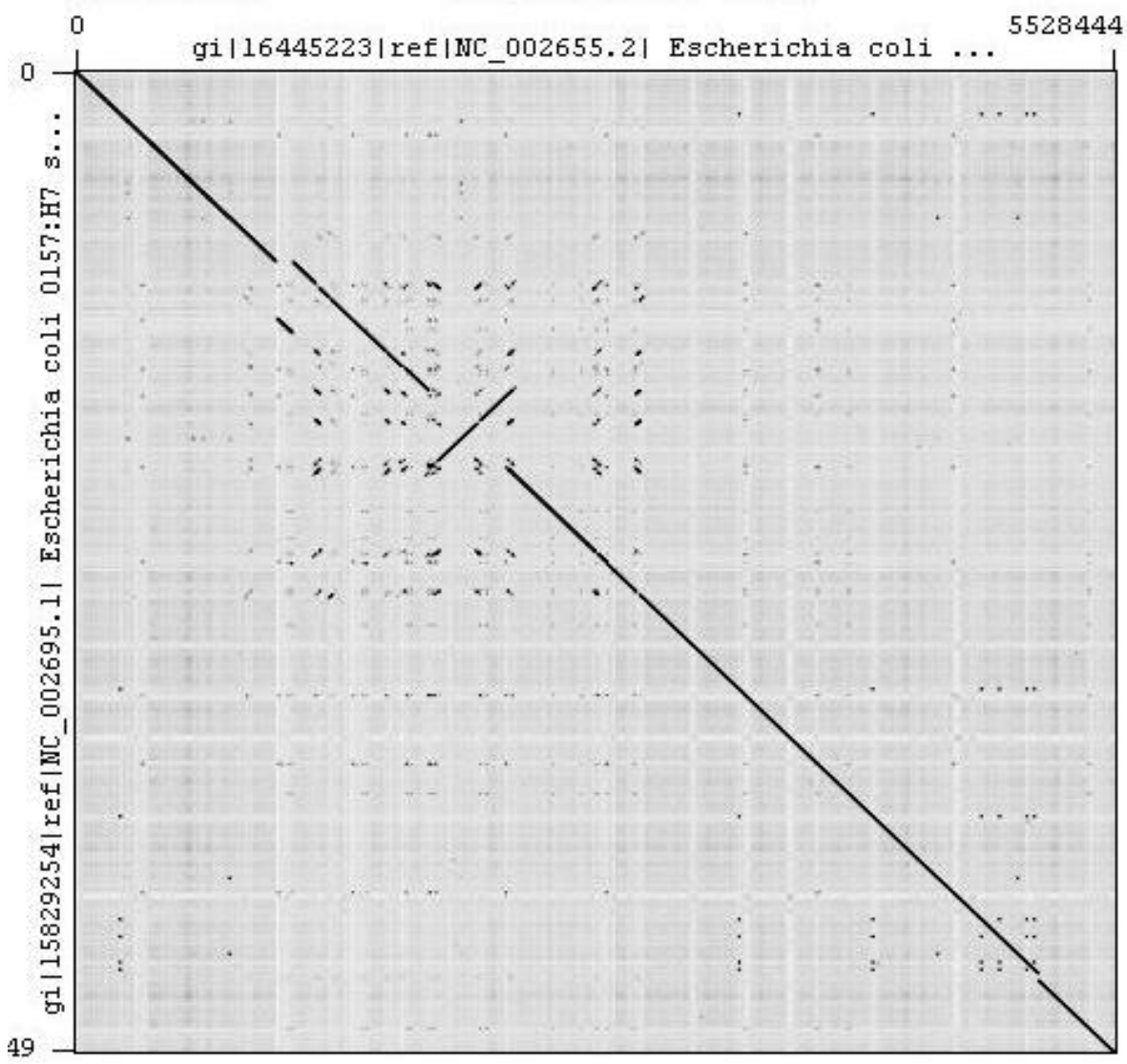


Figure 4-2. Dotplot analysis of *Ec*O157 EDL933 versus Sakai.

*Ec*O157 EDL933 is represented on the X-axis and *Ec*O157 Sakai on the Y-axis, with a black dot representing areas of homology between the two. Dotplot analysis and final output was calculated using Gepard (version 1.3) (Krumsiek *et al.*, 2007).

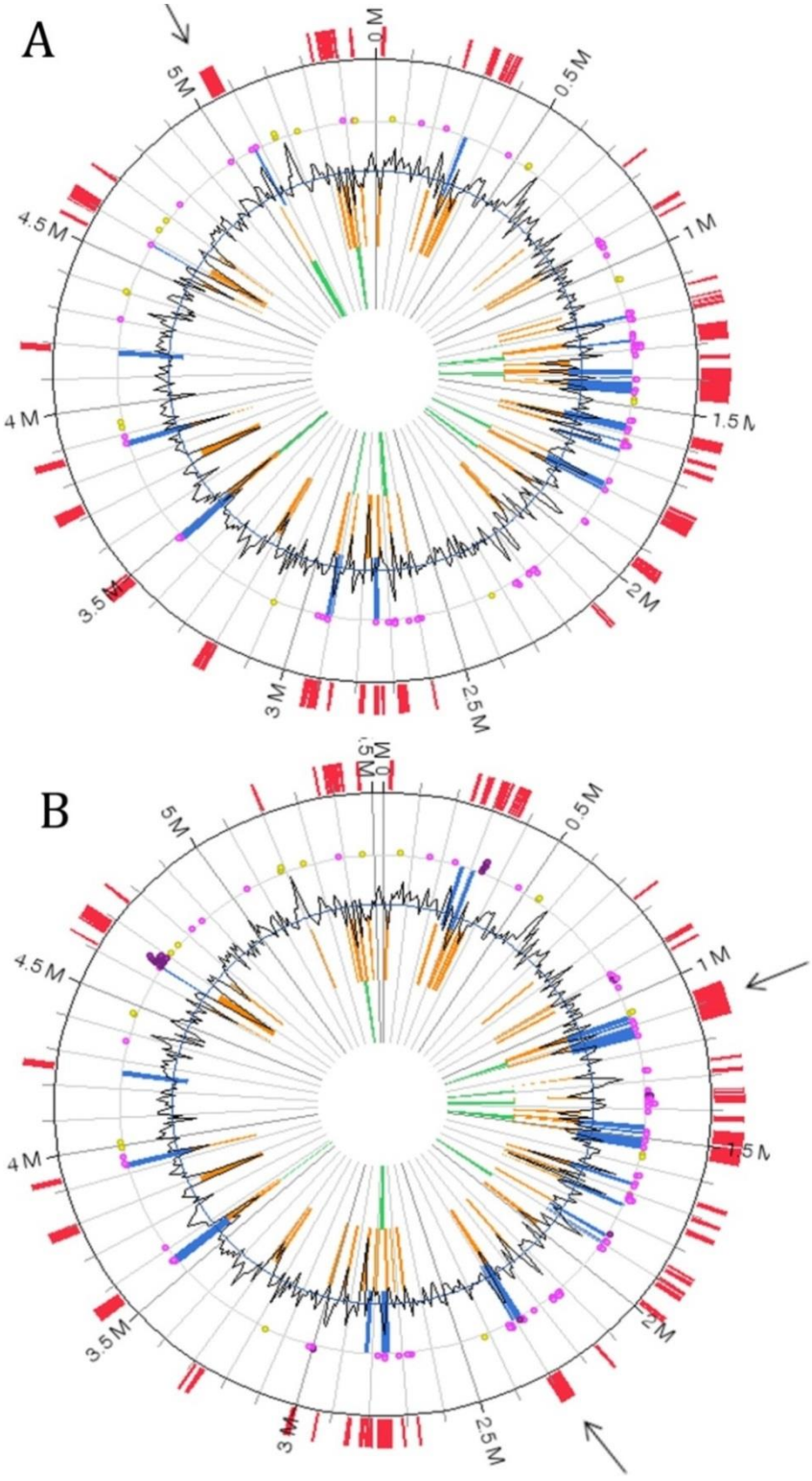


Figure 4-3. Comparison of predicted genomic islands present in *Ec*O157 Sakai (A) and EDL933 (B).

The genome of *Ec*O157 Sakai (A) and EDL933 (B) were mapped onto IslandViewer to predict acquired genomic islands present within the genome. Predicted genomic islands are displayed on the outside of the circle in red and represent the integrated results from three separate prediction programs (Langille & Brinkman, 2009). Results from each of the individual programs can be seen in green (IslandPick (Langille *et al.*, 2008)), orange (SIGI-HMM (Waack *et al.*, 2006)) and blue (IslandPath-DIMOB (Hsiao *et al.*, 2003)). The genomic island map is overlayed with known virulence factors described in the virulence factor database (VFDB (Chen *et al.*, 2012)) shown as small purple circles, and antimicrobial resistance genes described in the antibiotic resistance gene database (ARDB (Liu & Pop, 2009)), small yellow circles. Known pathogen-associated genes are depicted as small pink circles (Ho Sui *et al.*, 2009). Black arrows represent differences in genomic islands between the two strains of *Ec*O157. Maps were created using Island Viewer (Langille & Brinkman, 2009). Full lists of genes that are present on genomic islands as predicted by the Island Viewer program can be found in the appendix (File S-1).

Genomic island analysis can also be overlayed with hits from the virulence factor database (VFDB (Chen *et al.*, 2012), the antibiotic resistance database (ARDB (Liu & Pop, 2009)) and the pathogen-associated gene database (PAGDB (Ho Sui *et al.*, 2009)) to look for areas with known pathogenicity factors.

The genomic comparison shows that there are subtle differences in the genes located on genomic islands (Figure 4-3). *Ec*O157 EDL933 has two regions of island-located genes not present in *Ec*O157 Sakai (**B**, Figure 4-3, highlighted by the black arrows). Similarly, *Ec*O157 Sakai contains a region of predicted genes located on a genomic island not present in *Ec*O157 EDL933 (**A**; Figure 4-3). Many of the pathogen-associated genes are seen to occur in regions of predicted genomic islands.

The fact that human pathogen species have not been shown to be pathogenic in plants raises the hypothesis that the pathogen-associated genes are only required for symptomatic human and animal infection and those genomic islands that do not appear to contain pathogen-associated genes may contain genes that are important for environmental colonisation.

Table 4-1. Comparative genomic analysis of *E. coli* K-12, *Ec*O157 EDL933 and *Ec*O157 Sakai.

Data was predicted using EcoCyc comparative genomics browser (Keseler *et al.*, 2013). Genes are classified as having unknown function if there is no experimental evidence for their function. Genes unique to *Ec*O157 Sakai, as predicted by EcoCyc, can be found in the appendix (File S-2).

	***E. coli* K-12**	***Ec*O157 EDL933**	***Ec*O157 Sakai**
Chromosomes	1	1	1
Plasmids	0	1	2
Genes	4501	5476	5442
Genes of Known/Predicted Function	3150	1424	1890
Genes of Unknown Function	1351	4052	3542
Pathways	409	361	479
Metabolic Reactions	1564	1366	1692
Transport Reactions	400	161	212
Transcription Units	3538	3531	3404

To identify which genes and metabolic pathways were found in both strains of *Ec*O157 and which were unique to *Ec*O157 Sakai, a genomic comparison was carried out using the EcoCyc comparative genomics feature (Keseler *et al.*, 2013), where a comparison was made for each of the two *Ec*O157 strains and the laboratory strain, *E. coli* K-12. Both strains of *Ec*O157 contained approximately 900 extra genes compared to *E. coli* K-12; however a large number of these are of unknown function (Table 4-1).

416 of these genes were unique to *Ec*O157 Sakai (Appendix File S-2), the majority of which were hypothetical genes. Of these, a total of 192 genes overlapped with genes predicted to be on genomic islands by Island Viewer, potential targets that may have been acquired from the native environmental microflora through horizontal gene transfer. However, these genes may be important for human infection, as differences in adherence and infection of bovine epithelial cells has been found between different isolates of *Ec*O157 (Abu-Ali *et al.*, 2010).

Most of the *Ec*O157 Sakai-specific genes were of unknown function. Therefore, a different approach was used to identify differences between the strains of *E. coli*, with the comparative genomic browser in EcoCyc to identify metabolic pathways present in each organism. A summary of all the pathway classes present in *E. coli* K-12, *Ec*O157 EDL933 and Sakai can be seen in Table 4-2.

As expected, the majority of pathway classes were conserved across all three strains. However, several pathway classes were isolate- or *Ec*O157-unique, including hormone biosynthesis, secondary metabolites biosynthesis, chlorinated compounds degradation and protein degradation (Table 4-2). Analysis of these pathway classes that are unique to *Ec*O157 Sakai, i.e. not present in *E. coli* K-12 or *Ec*O157 EDL933 revealed eleven metabolic pathways that are found in other plant- or environmental-associated bacteria (as predicted by EcoCyc), or are involved in the utilisation of environmental components (highlighted in Table 4-2; detailed in Table 4-3).

Table 4-2. Comparative genomic analysis of the different metabolic pathway classes present in *E. coli* K-12, *Ec*O157 EDL933 and *Ec*O157 Sakai.

Comparative analysis was performed using EcoCyc comparative genomics browser. Pathways highlighted with an asterisk contains reactions involving plant/environmental components, detailed further in Table 4-III.

Pathway Class	***E. coli* K-12**	***Ec*O157 EDL933**	***Ec*O157 Sakai**
Biosynthesis	205	203	254
Amines and Polyamines Biosynthesis	8	9	9
Amino Acids Biosynthesis	28	28	33
Aminoacyl-tRNA Charging	1	1	1
Aromatic Compounds Biosynthesis	4	4	4
Carbohydrates Biosynthesis	15	18	19
Cell Structures Biosynthesis*	9	8	10
Cofactors, Prosthetic Groups, Electron Carriers Biosynthesis	47	45	59
Fatty Acids and Lipids Biosynthesis	19	19	22
Hormones Biosynthesis*	0	0	2
Metabolic Regulators Biosynthesis	1	1	1
Nucleosides and Nucleotides Biosynthesis	28	27	31
Other Biosynthesis	5	6	6
Secondary Metabolites Biosynthesis*	2	0	2
Degradation/Utilisation/Assimilation*	139	116	172
Alcohols Degradation	5	6	7
Aldehyde Degradation	5	3	2
Amines and Polyamines Degradation	10	7	12
Amino Acids Degradation	18	19	22
Aromatic Compounds Degradation	4	4	7
C1 Compounds Utilisation and Assimilation	1	3	5
Carbohydrates Degradation*	23	20	29
Carboxylates Degradation	20	14	17
Chlorinated Compounds Degradation*	0	0	1
Degradation/Utilisation/Assimilation – Other*	0	1	2

Fatty Acid and Lipids Degradation	4	6	6
Inorganic Nutrients Metabolism	8	7	19
Nucleosides and Nucleotides Degradation	13	8	11
Polymeric Compounds Degradation	2	2	5
Protein Degradation*	0	0	2
Secondary Metabolites Degradation*	16	9	13
Steroids Degradation	0	0	1
Generation of Precursor Metabolites and Energy	30	29	51
Signal Transduction Pathways	30	0	0
Total	335	283	388

Table 4-3. Metabolic pathways present in *Ec*O157 Sakai with links to environmental/plant-associated compounds.

No homologous pathways were predicted to be present in *E. coli* K-12 or *Ec*O157 EDL933 using EcoCyc.

Pathway Class	***Pathway Name***	***Associated Environmental Compound***	***Genes Involved***
Cell Structures Biosynthesis	Polymyxin resistance	Polymyxins	*ECs3141-3143, ECs3145*
Chlorinated Degradation	Chlorosalicylate degradation	Chlorosalicylate	*ECs1877, ECs3027*
Secondary Metabolites Degradation	Betanidin degradation	Betacyanins (2 betanidin)	*ECs3342*
Hormones Biosynthesis	Indole-3-acetate biosynthesis III	Indole-3-acetate (IAA/auxin)	*ECs0664, ECs3674, ECs3926, ECs5162*
	Indole-3-acetate biosynthesis IV	Indole-3-acetate (IAA/auxin)	*ECs0664, ECs3674, ECs3926, ECs5162*
Protein Degradation	Wound-induced proteolysis I	Unknown	*ECs1015, ECs3265, ECs5237*
	Seed germination protein turnover	Unknown	*ECs1015, ECs3265, ECs5237*
Degradation/Utilisation/Assimilation – Other	Methylsalicylate degradation	Methylsalicylate	*ECs3027*
	Salicylate degradation I	Salicylate	*ECs3027*
Carbohydrate Degradation	Pectin degradation III	Pectin	*ECs0800, ECs3974*
	Acetoin degradation	Acetoin	*ECs3024, ECs4466, ECs2490, ECs2082, ECs1741, ECs0411*
Secondary Metabolites Biosynthesis	Linalool Biosynthesis	Linalool	*ECs0475*

Three *Ec*O157 Sakai-unique pathways were identified that involved the degradation of salicylate, chlorosalicylate and methylsalicylate; esterified forms of salicylic acid, an important plant defence hormone (Vlot *et al.*, 2009). Degradation of salicylate by the *ECs3027* salicylate hydroxylase enzyme present in *Ec*O157 Sakai is predicted to result in the formation of catechol (based on the metabolic pathway prediction software from MetaCyc (Caspi *et al.*, 2010)). This product is then converted in a catechol degradation pathway into 3-oxoadipate (β-ketoadipate). Interestingly, the chlorosalicylate degradation pathway is also predicted to result in the product 3-oxoadipate by MetaCyc (Caspi *et al.*, 2010). These salicylate degradation pathways are present in other plant- and soil-associated bacteria and fungi, including many *Pseudomonas* species (Sazonova *et al.*, 2008).

Two other pathways identified in Table 4-3 produce the hormone indole-3-acetate (IAA), which is also referred to as auxin. This pathway is also present in many plant-associated bacteria and fungi, shown to produce functional IAA hormone and have an effect on plant growth and the secretion of carbon and nitrogen sources into the rhizosphere, which the microbes can subsequently use for growth (Ali *et al.*, 2009; Kazan & Manners, 2009; Spaepen & Vanderleyden, 2011). The presence of this pathway in *Ec*O157 Sakai may provide an example of how this strain survives in the plant environment. A more detailed analysis of the plant hormone degradation and biosynthesis pathways is found in section 7.2.2.

The degradation of some plant-specific components can also be seen in Table 4-3, such as betacyanins, the indole-derived red and violet pigments that are found in many plant species such as beets (Sakuta, 2014). Furthermore, enzymes have recently been identified in EcoCyc that are predicted to be involved in pectin degradation. A closely related plant pathogen *Pectobacterium atrosepticum* contains pectic lyases, which have been shown to be secreted in response to plant extracts (Mattinen *et al.*, 2007). Therefore, this pathway could be an example of acquired genes from plant pathogens in the environment that are allowing this human pathogen to persist in plants.

In the past, the ability of *E. coli* to degrade aromatic acids was suggested as evidence of this bacterial strain's ability to survive outside the mammalian host (Burlingame & Chapman, 1983),

a fact which has now become apparent by the contaminated fresh produce outbreaks. This coupled with the identification of pathways involved in plant hormone synthesis and degradation as well as potentially plant cell wall degrading enzymes suggests that *Ec*O157 can not only survive in the plant environment, but also manipulate the environment for its own survival.

4.2.2 Comparative Analysis of Plant Extracts of Four Plant Species

The different plant species used to study bacterial-plant interactions dramatically affect the colonisation potential between the strains of *E. coli*. Outbreaks have been seen with a number of plant species, including from spinach (CDC, 2006), lettuce (Ackers *et al.*, 1998), and sprouts (CDC, 2013). Differences between the sugar content of plant species could provide an indication as to why one plant species may be more susceptible to colonisation of human pathogens than another. Growth curve analysis had shown the bacterial populations were able to reach higher numbers when in spinach and lettuce leaf lysates compared to pea, whereas the inverse was seen for root lysates (section 3.2.2.1). Incubation with root exudates of any of the plant species yielded no growth of the bacteria; however the addition of 0.2 % glycerol with root exudates allowed the bacteria to grow as normal, suggesting that growth arrest was not an inhibitory effect from the root exudates but merely insufficient carbon source to sustain bacterial growth.

Therefore, to assess the carbon content in the root exudates and to determine any differences in the monosaccharide content of the lysates, HPLC analysis was performed for selected monosaccharides that should be abundant in the plant environment and which *E. coli* can use for growth. Samples were run on a P20 column (Dionex) with standards to detect for five monosaccharides: arabinose, rhamnose, glucose, fructose and sucrose.

Similar concentrations were detected for the spinach and pea leaf lysate extracts (**A**; Figure 4-4) for the five monosaccharides tested, suggesting that the increase in growth in spinach may be due to another factor not tested, for example the presence and quantity of amino acids in each extract.

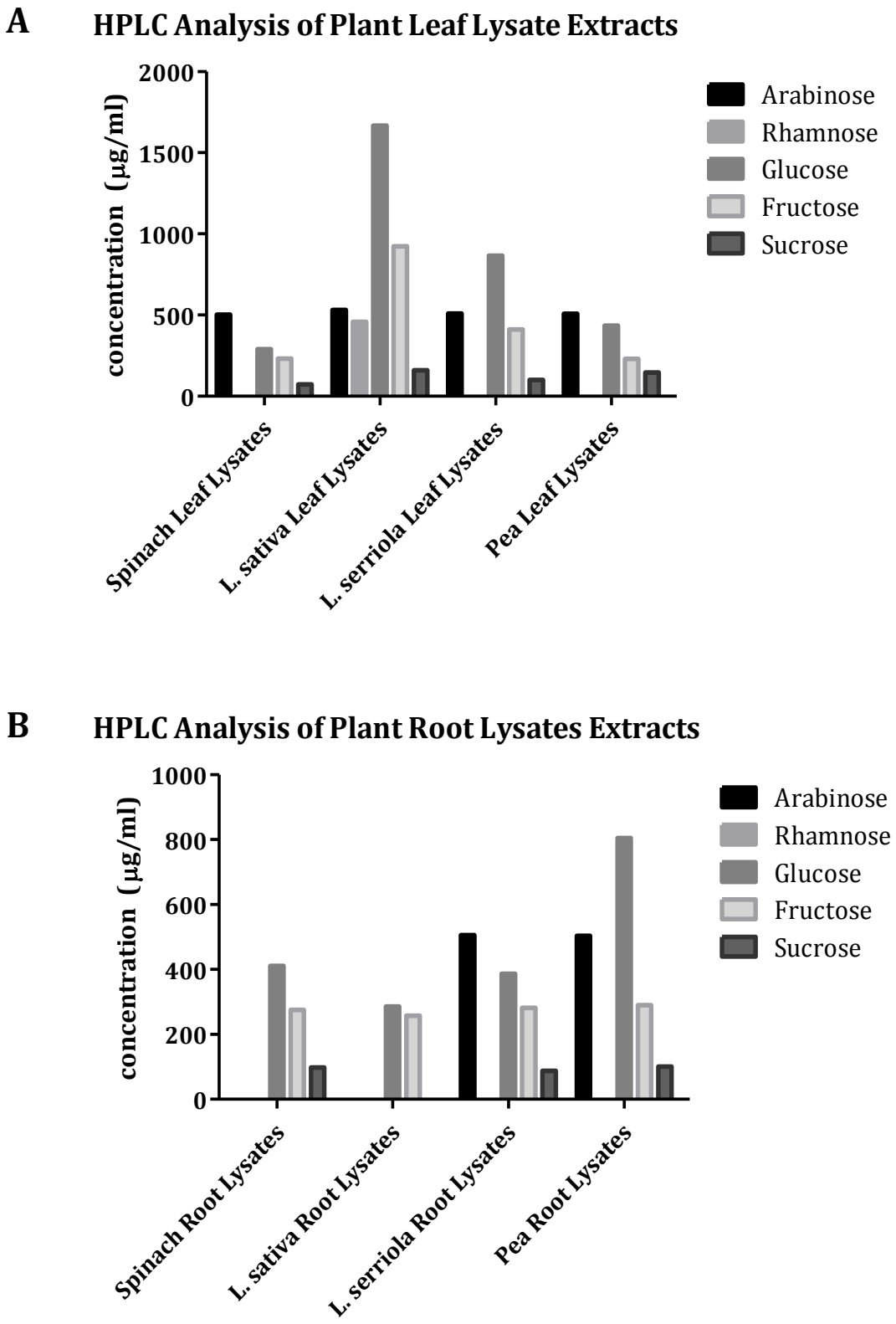


Figure 4-4. HPLC analysis of leaf and root lysate extracts.

Samples were run on a Dionex P20 column to test for arabinose, rhamnose, glucose, fructose and sucrose in the **A**) leaf lysates and **B**) root lysates of spinach, *L. sativa* lettuce, *L. serriola* lettuce and pea. Each column represents one sample.

L. sativa lettuce appeared to have higher levels of glucose compared to the other species tested and was the only species for which rhamnose could be detected in the leaf lysates. In contrast, highest levels of glucose were seen in the pea root lysates compared to the other root lysate extracts (**B;** Figure 4-4). No rhamnose was detected from any of the root lysate extracts. The lowest concentrations were seen in *L. sativa* root lysates, with only ~225µg/ml glucose and fructose being detected out of the five monosaccharides tested for.

In the root exudates all five of the monosaccharides were detected. However, the concentrations were very low and in a similar range to that of the rockwool media only control.

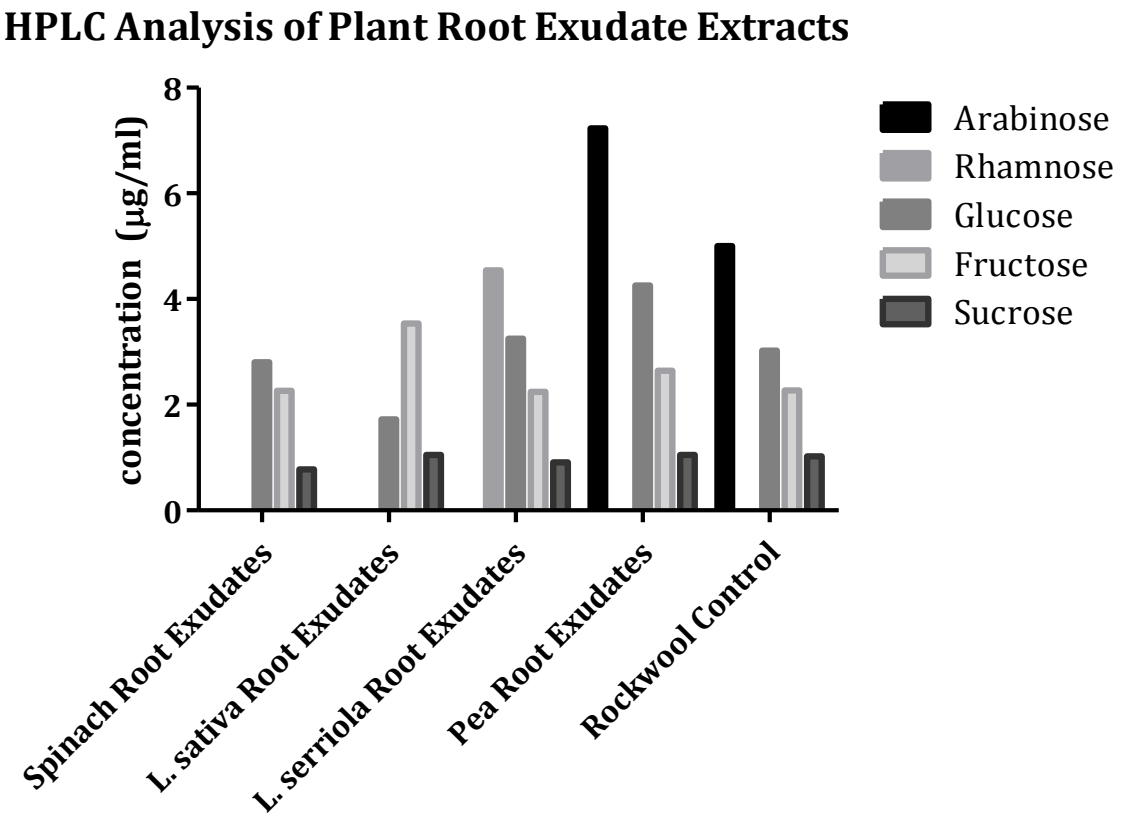


Figure 4-5. HPLC analysis of root exudate extracts.

Samples were run on a p20 column to test for arabinose, rhamnose, glucose, fructose and sucrose in the root exudates of spinach, *L. sativa* lettuce, *L. serriola* lettuce and pea. A rockwool media sample was used as a control. Each column represents one sample.

4.2.3 Comparative Analysis of Plant Cell Wall Polysaccharide Extracts of Four Plant Species

Cell wall polysaccharide extracts of the leaves and roots of the spinach and lettuce plants were made to study the response of the bacteria to specific components of the plant cell (described in detail in section 5.2.2), as *Ec*O157 has been shown to target residues within the plant cell wall for its adherence and subsequent colonisation (Rossez *et al.*, 2014). The plants were grown in vermiculite prior to the extracts being made, thus as a control vermiculite polysaccharides were also prepared to check for any carryover. Samples were run on two HPLC columns: P100 to check for mannose, xylose, fucose, galactose, glucuronic acid and galacturonic acid; and P20 to check for fructose, glucose and sucrose, as well as arabinose and rhamnose, which co-elute on the P100 column.

The leaf cell wall polysaccharide extracts contained high concentrations of fructose, glucose and fucose, in comparison to minimal readings from the vermiculite control (**A**; Figure 4-6). Interestingly, the highest concentrations of fructose, glucose and fucose monosaccharides were in the wild, prickly lettuce (*L. serriola*), with much lower concentrations in the *L. sativa*.

Much lower concentrations of the other monosaccharides were detected, with no mannose or galacturonic acid present in any of the leaf cell wall polysaccharide extracts samples, including the control. The highest monosaccharide detected after fructose, glucose and fucose was arabinose, which was found to be highest in spinach leaves compared to both lettuce extracts, and trace amounts detected in the vermiculite control (**B**; Figure 4-6).

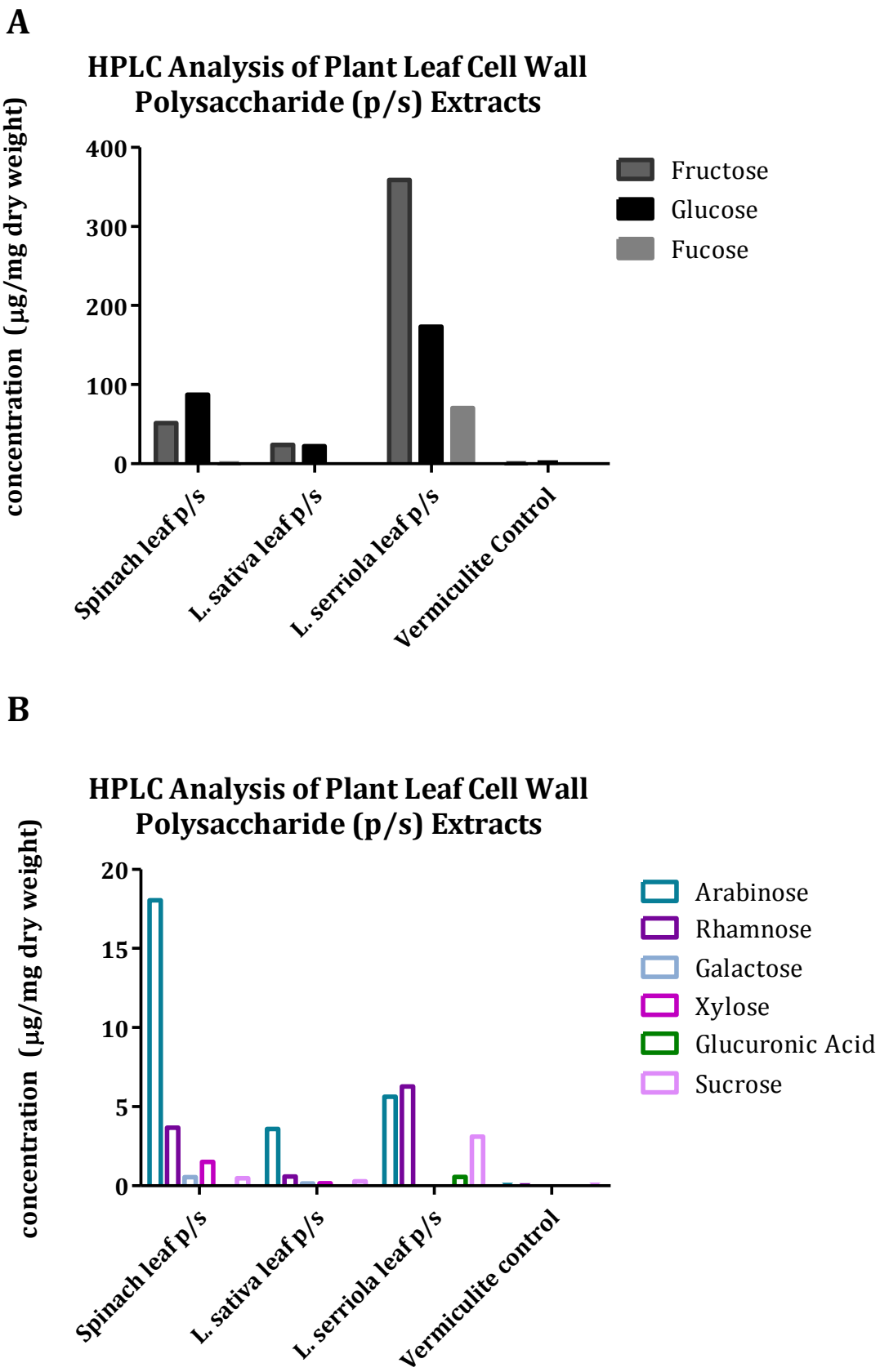


Figure 4-6. HPLC analysis of leaf cell wall polysaccharide extracts.

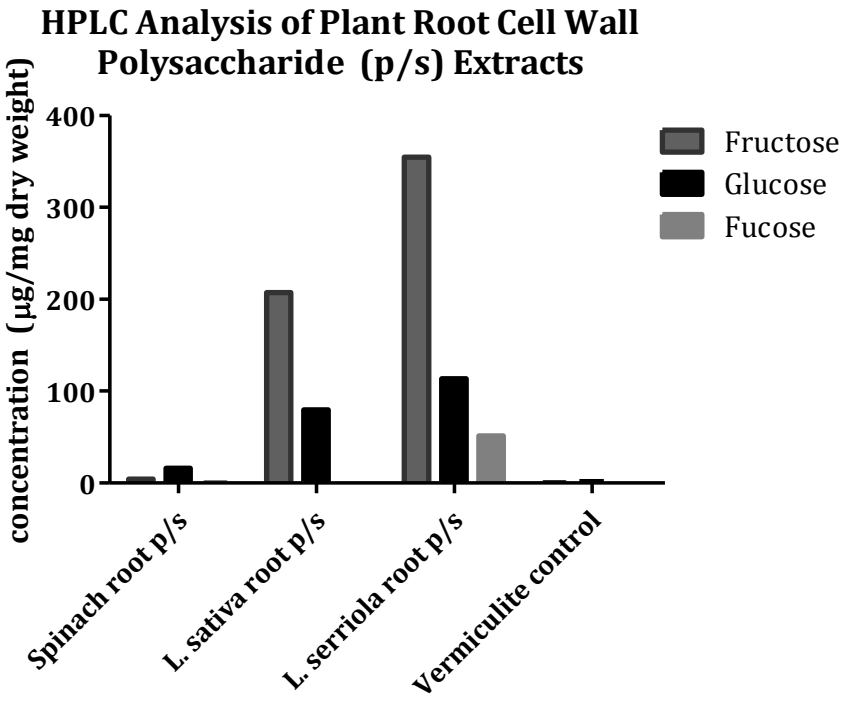
Samples were tested for glucose, fructose and fucose (**A**) and for arabinose, rhamnose, galactose, xylose, glucuronic acid and sucrose (**B**) on a P20 and P100 column, respectively. A vermiculite polysaccharide sample was used as a control. Each column represents one sample.

Rhamnose was also detected in the extracts, with the highest seen in the *L. serriola*. The remaining monosaccharides occurred in very low concentrations in all three of the samples, with only spinach containing xylose and *L. serriola* containing sucrose.

A similar profile was seen for the root cell wall polysaccharide extracts, with the highest concentrations of fructose, glucose and fucose in *L. serriola* (**A;** Figure 4-7). The spinach root polysaccharide extracts contained much lower concentrations of these three monosaccharides compared to lettuce and also lower than in the spinach leaf extracts.

A small difference was seen for the monosaccharides tested for on the p100 column for the spinach root polysaccharide extracts compared to the control, although the concentrations were quite low. As for the leaf polysaccharide extracts, arabinose was the next highest monosaccharide to be detected for all three of the plant species tested (**B**; Figure 4-7), although, the highest concentration occurred in *L. serriola* root extracts. Similar concentrations of rhamnose, galactose, xylose and sucrose were seen for spinach and *L. sativa* root polysaccharides, with only rhamnose and sucrose being detected in the *L. serriola* extracts.

A



B

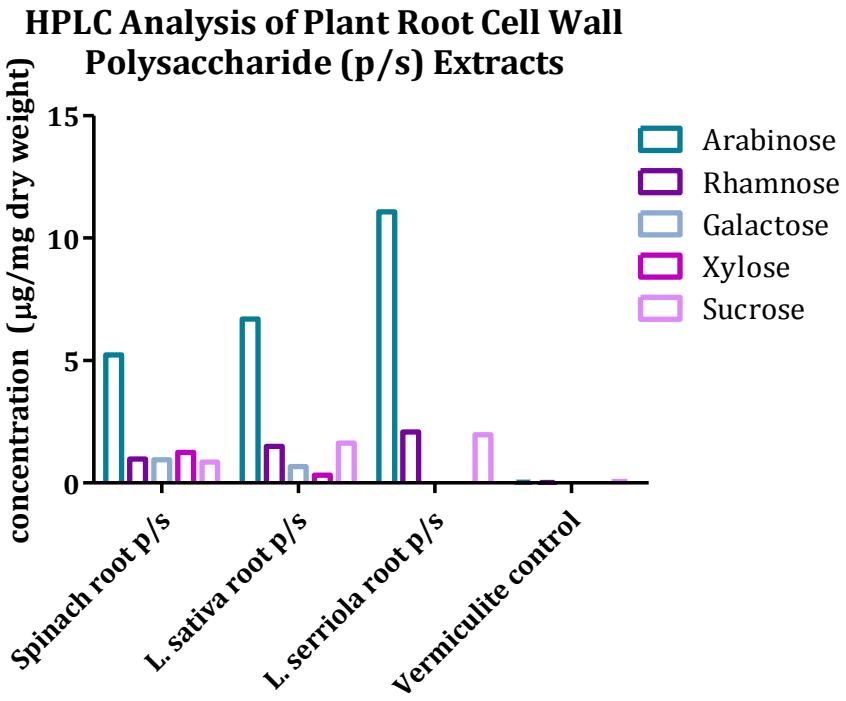


Figure 4-7. HPLC analysis of root cell wall polysaccharide extracts.

Samples were tested for glucose, fructose and fucose (**A**) and for arabinose, rhamnose, galactose, xylose, and sucrose (**B**) by running root cell wall polysaccharide samples of spinach and lettuce on a p20 and p100 column. A vermiculite polysaccharide sample was used as a control. Each column represents one sample.

4.3 Discussion

Comparative genomic analysis showed that *Ec*O157 strains EDL933 and Sakai are closely related, as described previously (Zhou *et al.*, 2010). However, some genetic differences were evident: 416 genes were present in the plant-isolated strain *Ec*O157 Sakai that were absent from the bovine strain *Ec*O157 EDL933, based on the parameters set by EcoCyc (Keseler *et al.*, 2013). Just under half of those genes were found to be associated with genomic islands, potentially horizontally transferred from the environment such as soil and water, previously demonstrated to be an area of high exchange of genetic material due to the large diversity of organisms present (Baquero *et al.*, 2008; van Elsas & Bailey, 2002).

*Ec*O157 Sakai has several metabolic pathways that are common to other plant-associated bacteria which *Ec*O157 EDL933 does not, possibly providing evidence for this human pathogen being environmentally adapted. For example, the salicylate degradation pathways identified in *Ec*O157 Sakai are present in other plant- and soil-associated bacteria and fungi, including many *Pseudomonas* species (Sazonova *et al.*, 2008). However, *Pseudomonas* species contain a further 3-oxoadipate degradation pathway that converts this compound into succinyl-CoA and acetyl Co-A for use in the TCA cycle (Kaschabek *et al.*, 2002). No pathway utilising 3-oxoadipate has been identified in *Ec*O157 Sakai or in *E. coli* K-12, so perhaps this compound is exported from the cell via one of the acid efflux pumps and utilised by other plant species such as *Pseudomonas*.

One other pathway that was identified as present in *Ec*O157 Sakai yet not in *Ec*O157 EDL933 was the polymyxin resistance pathway. Interestingly, this pathway was seen to be induced when *Ec*O157 was cultured in M9 minimal medium at 18 °C compared to 37 °C. Polymyxins are antibiotics produced by Gram positive bacteria such as *Paenibacillus polymyxa*. These bacteria can be found in soil and plant root environments as plant-growth-promoting bacteria which have antagonistic effects on plant pathogens such as *Phytophthora* (Timmusk *et al.*, 2009). Polymyxins are lethal to Gram negative bacteria by disrupting the permeability of the outer membrane, as the polymyxins have specificity for the lipid A phospholipids. The production of 4-amino-4-deoxy-L-arabinose (L-Ara4N) by the polymyxin resistance pathway increases the

charge of the lipid A molecules, reducing this specificity with polymyxin (Tran *et al.*, 2005). This pathway is present in *Yersinia* also, and it has been shown to be increased at lower temperatures in this bacterial species (Reines *et al.*, 2012). Therefore, not only does this pathway appear to be upregulated in response to an environmentally relevant temperature, but it is also an example of how *Ec*O157 Sakai may survive in the rhizosphere.

All these similarities to plant-associated bacteria do however beg the question: if *Ec*O157 Sakai has become more adapted to survival in the environment, has this strain lost some of its pathogenicity in humans as a result? It is clearly still pathogenic in humans as it been linked to large outbreaks (Michino *et al.*, 1999); however a comparison between Sakai and another plant strain did find differences in the ability of the strains to adhere to human intestinal epithelial cells (Abu-Ali *et al.*, 2010). Thus, it would be interesting to test if the pathogenicity of *Ec*O157 Sakai can be altered by passaging this bacterial strain through plants.

As previous colonisation assays had shown that despite a difference being seen in the number of bacteria colonising the leaves of plants between the two strains, no difference was seen when these same strains were inoculated onto the roots and rhizosphere of four plant species. Thus, while there may be Sakai-specific genes that give it an advantage in the phyllosphere, perhaps different genes that are common to both strains are advantageous for root colonisation and survival.

Based on this, it was decided that a transcriptomic approach would be a beneficial way to study the interaction of *Ec*O157 Sakai with different plant extracts to identify if Sakai-specific/*Ec*O157-specific genes are induced due to the plant environment. A commercial microarray was used to study this interaction, details of which are found in section 2.16.3. As a result, four genomes were represented on the microarray, meaning that the core genes (i.e. those present in all of the *E. coli* on the microarray) and any genes specific to *Ec*O157/Sakai could be identified.

As detailed in chapter 5, gene expression analysis of *Ec*O157 Sakai was studied in response to different plant extracts. HPLC analysis provides an analysis of the levels of selected monosaccharides present in the extracts, which can then be correlated with gene expression

changes. From the results, it was determined that the leaf and root lysates contained high concentrations of arabinose, glucose and fructose, all of which can be used by *E. coli* for growth. This gives further indication as to why plant lesions and wounding promotes the persistence and growth of this bacterial species (Brandl, 2008), as there are sugars present within the leaf that are accessible to the bacteria.

The growth curve profiles seen in section 3.2.2.1 can be explained by the HPLC data obtained from this study, where leaf and root lysates were seen to have high concentrations of glucose and fructose. The root exudate samples contained similar concentrations to that of the rockwool media control with only trace amounts of the five monosaccharides found, which would explain why no growth was observed in section 3.2.2.1. However, other peaks were detected on the HPLC chromatogram, suggesting the presence of other compounds that could induce a response from the bacteria. It should be noted that there is the possibility that the exudates were depleted of the essential carbon sources by contaminating seed-borne or other rhizo-bacteria that may have been present on the plants (Kuijken *et al.*, 2015).

The plant cell wall polysaccharide extracts contained a variety of monosaccharides, with spinach leaves containing the highest concentration of arabinose compared to the other species tested. Analysis of arabinose and the connection to bacterial colonisation is detailed further in section 6.2.6. Interestingly, differences were seen in the monosaccharide composition in the polysaccharide samples between the two lettuce species, with *L. serriola* having the highest concentration of fructose, glucose and fucose in the leaf polysaccharide extracts. *L. serriola* also contained higher concentrations of fructose and fucose in the root extract polysaccharides compared to *L. sativa*. No difference was seen between the two lettuce species for monosaccharide concentrations in the leaf lysates, presumably why no difference was seen in the growth profiles of *Ec*O157 Sakai incubated with the lettuce leaf lysate extracts. As the preparation of the cell wall polysaccharide extracts results in an insoluble powder, growth assays were unable to be performed using a spectrophotometry or plating approach to determine the utilisation of these extracts. However, sequential extraction with CDTA and NaOH results in soluble fractions of the pectic and hemicellulosic components of the extracts.

Therefore, as differences were seen in the monosaccharide composition between the lettuce species it would be interesting to examine the growth of *Ec*O157 Sakai in response to these soluble fractions and determine any differences in the utilisation of the extracts.

For the root cell wall polysaccharide extracts, low levels of monosaccharides were detected in the vermiculite control samples compared to the plant samples, except for the spinach samples. This could suggest that there is a lot of vermiculite carryover in the spinach root polysaccharide extracts compared to the lettuce root polysaccharide extracts. No further analysis using the root cell wall polysaccharides was carried out due to time constraints of the project, but any future work may need to take this information into account.

Previous work of *Ec*O157 in response to plant tissue has not reported concentrations of monosaccharides present with the extracts, therefore it is not possible to identify if differences between gene expression are down to varying concentrations between the extracts used in this study compared to previously published reports. Furthermore, due to external constraints, only one HPLC run could be performed in this study, so further analysis will be needed in the future to confirm these results. However, the same HPLC analysis was carried out on leaf and root lysate and root exudate extracts from spinach and a different lettuce cultivar by another lab member, which gave a similar pattern of which monosaccharides were present to that of this study, although at higher concentrations than reported here (Bernhard Merget, unpublished data).

As well as this, further analysis by GC-MS would provide information on the chemical linkages between the glycans, which may inform on the ability of the bacterial utilisation of these sugars. A GC-MS approach would also be useful to identify the metabolic changes in plant extracts prior to and after inoculation with *Ec*O157, in order to confirm some of the metabolic pathways upregulated in Chapter 6 showing the utilisation of plant components. Finally, NMR analysis would be beneficial for identifying some of the key differences in secondary metabolites between the plant species, especially for compounds such as polyphenols that could affect the survival of the bacteria. Therefore there are still many approaches as well as the HPLC method

used in this study that could further compare the plant species and show the interaction of *Ec*O157 with the different plant extracts.

In summary, it appeared that bacterial growth was dependent on glucose and fructose levels more than the other monosaccharides tested, as these were found in high concentrations in all extracts tested apart from the root exudates, therefore correlating with the growth profiles from Chapter 3. The ability to utilise the extracts for growth and the presence of certain monosaccharides such as arabinose were found to have significant effects on the gene expression of *Ec*O157 Sakai, detailed in both Chapter 5 and Chapter 6.

5 Global Transcriptomic Response to the Plant Environment and a Plant-relevant Temperature

5.1 Introduction

Differences were found in the colonisation potential and ability of *Ec*O157 to utilise plant extracts for growth between plant species (see section 3.2.1 and 3.2.2). To determine the regulatory basis to these differences and examine differential gene expression in more detail a transcriptomic approach was taken to identify which genes and metabolic pathways were induced in certain conditions, with an aim of identifying why *Ec*O157 Sakai fared better in some plant species over others. Gene expression was linked to the monosaccharide content of the plant extracts that was previously determined by HPLC (see sections 4.2.2 and 4.2.3). Finally, no temperature shift was included for the plant adaptation stage to remove a temperature-dependent effect. To determine which genes were differentially regulated at the environmentally relevant temperature (18 °C), I studied the expression differences between *Ec*O157 Sakai cultured at 18 °C compared to 37 °C, the optimal temperature for this bacterium.

5.1.1 Importance of Culturing Human Pathogenic Bacteria at Environmentally Relevant Temperatures for in planta *Interactions*

Previous studies have analysed the transcriptome changes of human pathogens to different environmental conditions, including plant extracts such as leaf lysates: a full list of these studies can be seen in Table 5-1. As well as this, there have been recent publications showing the interaction of different strains of *E. coli* with the leaves and roots of living lettuce plants (Fink *et al.*, 2012; Hou *et al.*, 2012; Hou *et al.*, 2013). These datasets are very useful for a reference to look for key genes that may be involved in the interaction: however, one caveat of the work is that the bacteria-plant interaction was studied at 37 °C, which is the optimum temperature for *E. coli*, but not an environmentally relevant temperature for the plant. Therefore, interactions studied at these temperatures may give an advantage to the bacteria and also skew any data returned due to the presence of temperature-sensitive genes. As well as this, the majority of studies looking at the association of human pathogens on plants not only carry out the

interaction at 37 °C but also culture the bacterial strain at 37 °C (Bergholz *et al.*, 2009; Fink *et al.*, 2012; Hou *et al.*, 2012; Hou *et al.*, 2013; Landstorfer *et al.*, 2014; Visvalingam *et al.*, 2013). There are many studies which do carry out the bacterial-plant interaction at an environmentally relevant temperature of 18-25 °C, yet they culture the bacteria at 30-37 °C prior to this (Jayaraman *et al.*, 2014; Kyle *et al.*, 2010; Thilmony *et al.*, 2006). This would result in a sudden temperature change for the bacteria, which would affect the gene expression results obtained and thus incur many false positives in the data which would be attributed to a reaction to plants instead of temperature.

Recent work has also found that it is important to study plant-bacteria interactions at the appropriate temperature as temperature has been shown to have an effect on the expression of a wide range of plant defence genes, including *PR1* and those involved in salicylic acid production, in both inoculated and control plants (Rivera-Domínguez *et al.*, 2012; Wang *et al.*, 2009; Zhu *et al.*, 2010). This is another factor that will influence the ability of *E. coli* O157 strains to colonise the plant environment and so by studying this at 37 °C when plant defence genes are downregulated slightly, this gives the bacteria yet another advantage in the interaction.

Taking this information into account, the gene expression of *Ec*O157 Sakai was tested by microarray at two temperatures to study the effect of culturing the bacteria at 18 °C compared to 37 °C. For all experiments carried out, bacterial strains were cultured at 18 °C prior to inoculating onto plants/with plant extracts and the interaction was studied at this same temperature. In this way any temperature-related effects should be minimised, and by carrying out the interaction at an environmentally relevant temperature it should mean that the results obtained will be more applicable to environmental conditions.

5.1.2 Transcriptomic Analysis of Interactions of Human Pathogens with Plants and their Extracts

The initial aim of this project was to study the interaction between *Ec*O157 Sakai and living plant roots to understand how the bacteria colonise roots, which they have been shown to do in very

high numbers both in hydroponics and in compost (section 3.2.1.4 and 3.2.1.6). However, any RNA extracted using already published techniques (Bowtell, 2003; Schenk *et al.*, 2008) was found to be dominated by plant nucleic acid and what little bacterial RNA was recovered was partially degraded. As a result an optimised method for extracting bacterial RNA from plant roots was developed and subsequently published (Holmes *et al.*, 2014), as detailed in section 2.15.3. However, this technique only yielded sufficient bacterial *m*RNA for single gene expression (e.g. by qRT-PCR), so as an alternative, differential gene expression of *Ec*O157 Sakai was examined following exposure to different plant extracts. The extracts provided a suitable proxy to examine the bacterial genetic response, without any complications of plant defence response, and in a homogeneous matrix. The growth and motility of *Ec*O157 Sakai in response to plant extracts had already been tested (section 3.2.2). Identification of genes differentially expressed in plant extracts such as root exudates would highlight important genes and metabolic pathways required for adaptation to plant hosts.

Spinach extracts were chosen as the plant substrate for *Ec*O157 Sakai interactions, as not only have *Ec*O157 been implicated in a previous outbreaks with spinach (CDC, 2006) but also because *Ec*O157 Sakai was able to colonise both the leaves and roots of spinach in high numbers (section 3.2.1). In addition, *Ec*O157 Sakai was able to utilise spinach leaf and root lysates for growth (section 3.2.2.1). The bacteria were unable to grow in the root exudates of spinach or any of the three other plant species tested. HPLC analysis of the spinach extracts allowed correlation with monosaccharide content and differential gene expression.

Table 5-1. Comparison of transcriptomic studies looking at human pathogens in response to the plant environment.

Bacterial Strain	***Plant Species***	***Condition***	***Exposure Time***	***Culture Temp***	***Exposure Temp***	***Reference***
*Ec*O157 Sakai	N/A	Model apple juice	10 minutes	37 °C	37 °C	(Bergholz *et al.*, 2009)
E. coli MG1655	Lettuce	Detached leaves	3 days	37 °C	25 °C	(Fink *et al.*, 2012)
*Ec*O157 EDL933	Lettuce	Detached leaves	3 days	37 °C	25 °C	(Fink *et al.*, 2012)
S. enterica sv. Typhimurium	Coriander	Shredded leaves	18 hours	28 °C	28 °C	(Goudeau *et al.*, 2013)
S. enterica sv. Typhimurium	Lettuce	Shredded leaves	18 hours	28 °C	28 °C	(Goudeau *et al.*, 2013)
E. coli MG1655	Lettuce	Living roots	3 days	37 °C	25 °C	(Hou *et al.*, 2012)
*Ec*O157 EDL933	Lettuce	Living roots	3 days	37 °C	25 °C	(Hou *et al.*, 2013)
*Ec*O157 EDL933	Lettuce	Leaf lysates	15/30 minutes	37 °C	28 °C	(Kyle *et al.*, 2010)
*Ec*O157 EDL933	Spinach	Leaf lysates	5 hours	37 °C	37 °C	(Landstorfer *et al.*, 2014)
*Ec*O157 EDL933	Radish sprout	Leaf lysates	5 hours	37 °C	37 °C	(Landstorfer *et al.*, 2014)

One further plant substrate was examined to determine the response of the bacteria to cell wall polysaccharide preparation, in order to highlight any genes that were specific for the initial 'point of contact' in foliar tissue. To assess any differences between the plant species, lettuce cell wall polysaccharides were compared to that of spinach. Differences have already been reported in bacterial colonisation between cultivars of plant species (Macarisin *et al.*, 2013; Quilliam *et al.*, 2012), so it was hypothesised that different gene expression patterns would be seen in response to spinach and lettuce polysaccharides. Furthermore, differences were found in the concentrations of the monosaccharide content between the two extracts in section 4.2.3, indicating that the response may be different.

Therefore, five different conditions were selected for transcriptomic analysis: lower temperature; spinach leaf lysates; spinach root exudates; spinach leaf cell wall polysaccharides and lettuce cell wall polysaccharides.

5.1.3 Aims for this Area of Study

The aims for this area of study were:

A. To identify temperature-responsive genes that are present within *Ec*O157 Sakai.

B. To identify differences from culturing *Ec*O157 Sakai in minimal medium at an environmentally relevant temperature compared to the optimal growth temperature.

C. To identify genes that are induced when incubated with plant extracts, both within and between datasets.

D. To compare results from this study to previously published work to narrow down which genes are important for interaction with the plant environment.

5.2 Results

To address the aims of this area of study, several experiments were designed. Firstly, *Ec*O157 Sakai was cultured in M9 media + 0.2 % glycerol at 18 °C and at 37 °C; following one hour incubation, RNA was extracted and processed for microarray analysis (detailed in Chapter 2). Four replicate samples were prepared of each condition and analysed using a single-colour microarray approach. The "37 °C" condition was set as the control to identify genes that were up/down regulated at the lower, environmentally relevant temperature of 18 °C.

To assess gene expression changes in *Ec*O157 Sakai when incubated with different plant extracts, two separate experiments were carried out. As before, *Ec*O157 Sakai was set up in M9 minimal media for a one hour exposure, however this time the media was supplemented with spinach whole leaf lysates or spinach root exudates. The control condition for these samples was *Ec*O157 Sakai in M9 media + 0.2 % glycerol at 18 °C. All plant extracts experiments were carried out at 18 °C; as a result no temperature shift occurred between the control and the plant extract conditions and any differences seen are due to growth and response. Four replicate samples were prepared for each of the leaf lysate and root exudate conditions. These samples were run on the same microarray as for the temperature conditions; therefore the same single colour approach was used.

Finally, the response of *Ec*O157 Sakai to further specialised plant extracts was examined. Leaf cell wall polysaccharides were prepared from spinach and lettuce (*L. sativa*) plants. These extracts retained the shape of the leaf and when supplemented to M9 media allowed an extract for the bacteria to utilise and to adhere to if necessary. Therefore this condition would help assess how *Ec*O157 Sakai responds to polysaccharides of the leaf cell wall, both in terms of growth and in adherence. *Ec*O157 Sakai was incubated in M9 media containing the polysaccharide extracts (1 % w/v) for one hour at 18 °C. As a control, vermiculite (the growth media used to prepare the plants) polysaccharides were prepared and added to M9 media containing *Ec*O157 Sakai for one hour at 18 °C. A two-colour microarray analysis was set up for this experiment: as a result, four replicate samples for the spinach and lettuce polysaccharide

conditions and eight replicate vermiculite control samples were prepared. All of the conditions in this experiment were carried out at 18 °C; thus, any differences in gene expression are not a result of a temperature shift and not a result of the plant growth media (vermiculite) and represent a response to the plant polysaccharides.

In summary, the conditions are a one hour exposure of *Ec*O157 Sakai to:

1) M9 minimal medium + 0.2 % glycerol at 18 °C compared to 37 °C. The 37 °C condition was set as the control.
2) M9 minimal medium + spinach leaf lysates at 18 °C compared to M9 minimal medium + 0.2 % glycerol at 18 °C. The 0.2 % glycerol condition was set as the control.
3) M9 minimal medium + spinach root lysates at 18 °C compared to M9 minimal medium + 0.2 % glycerol at 18 °C. The 0.2 % glycerol condition was set as the control.
4) M9 minimal medium + spinach leaf cell wall polysaccharides at 18 °C compared to M9 minimal medium + vermiculite polysaccharides at 18 °C. The vermiculite condition was set as the control.
5) M9 minimal medium + *L. sativa* lettuce leaf cell wall polysaccharides at 18 °C compared to M9 minimal medium + 0.2 % glycerol at 18 °C. The vermiculite condition was set as the control.

For all conditions tested, the Agilent v2 *E. coli* microarray was used. This microarray contains information of four genomes of *E. coli: E. coli* K-12; *Ec*O157 Sakai; *Ec*O157 EDL933 and *E. coli* CFT 073. As a result, multiple hits could be observed for a single gene and some cross-hybridisation could occur. The transcriptomic raw datasets have been uploaded to ArrayExpress under the accession E-MTAB-3249. Edited datasets showing the fold change for each of the transcriptomic datasets can be found in the appendix (Files S-3 to S-8).

5.2.1 Plant Extract Array of Temperature/Treatment Conditions

Gene expression differences were measured following a temperature shift and in response to plant extracts, using a single colour microarray. The conditions were as follows: one hour exposure of *Ec*O157 Sakai in M9 minimal media supplemented with 0.2 % glycerol incubated at 18 °C or at 37 °C; with spinach leaf lysates at 18 °C; and with spinach root exudates at 18 °C (section 2.13 for experimental details). This experimental setup was referred to as the "plant extract microarray" and contained conditions for temperature (18 °C versus 37 °C in minimal media) and treatment (minimal medium with 0.2 % glycerol versus minimal medium containing spinach plant extracts).

A large number of genes passed the quality control tests for all of the conditions tested (Figure 5-1). To assess temperature-dependent differences, the gene expression results obtained for *Ec*O157 Sakai at 18 °C were compared against expression at 37 °C (control): in this experiment genes that have "upregulated" gene expression are those induced in lower temperature conditions (18 °C), and those with "downregulated" gene expression would be repressed at 18 °C. Genes were filtered using a volcano plot analysis with a two-fold cut-off and using the MTC of Benjamini & Hochberg ($p > 0.005$). The decision to filter the genes based on a two-fold threshold should include significantly different expression profiles that are also likely to be biologically significant. Those genes with a lower fold-change than the threshold may still be biologically significant at the single cell level, but are likely to be masked when an "averaging" approach is used, such as whole transcriptome analysis.

After data normalisation and statistical analysis (section 2.16), 1333 genes were found to have differential expression at 18 °C compared to at 37 °C in minimal medium, representing 8.76 % of the total probes present: 575 genes had increased expression at 18 °C, whereas 758 genes were downregulated at the lower temperature.

To determine whether there was any effect of leaf lysates affecting gene expression, the gene expression data obtained from the leaf lysates samples were compared against the control sample of *Ec*O157 Sakai in M9 medium + 0.2 % glycerol. A total of 1736 genes (11.41 % of the

total probes) were found to have significantly different expression to the control samples upon exposure to spinach leaf lysates, with 824 of these being upregulated and 912 downregulated.

To determine whether there was any effect of root exudates affecting gene expression of *Ec*O157 Sakai, the gene expression data obtained from the spinach root exudates were compared against the control sample of *Ec*O157 Sakai in M9 medium + 0.2 % glycerol at 18 °C: 2257 genes had differential expression compared to the 0.2 % glycerol control. Of these 2257, 1113 genes were upregulated, whereas 1144 were downregulated. This large change in expression represented a change of 14.84 % of the total probes present on the microarray.

An inverse pattern of gene expression emerged for the leaf lysate compared to the root exudate samples, i.e. genes that are upregulated (red in the heatmap) in leaf lysates are downregulated (green) in root exudates and vice-versa (Figure 5-1). Different plant extracts induce quite different gene expression responses, suggesting that adaptation of *Ec*O157 to one condition may be quite distinct to another.

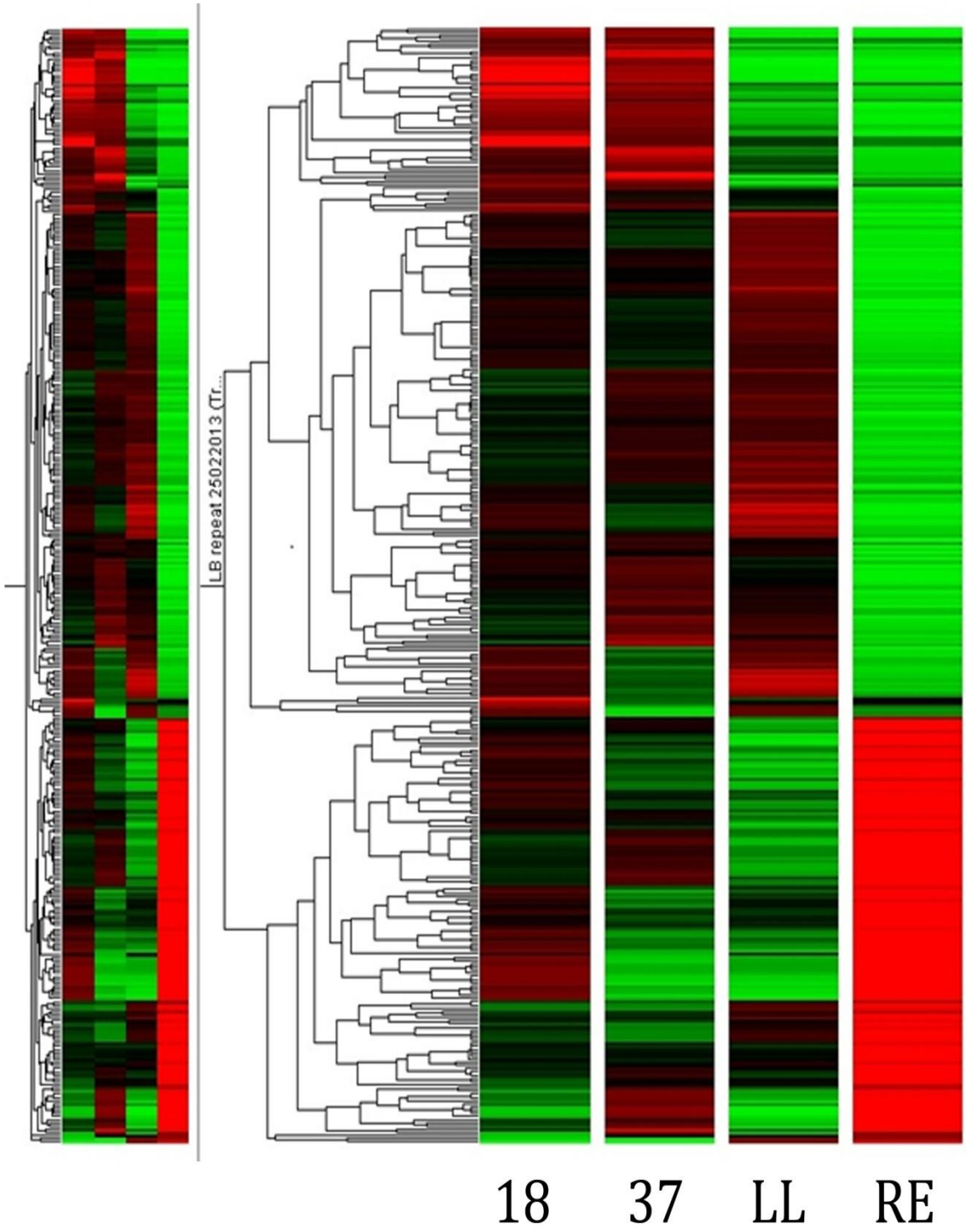


Figure 5-1. Heatmap showing gene expression changes to spinach plant extracts and temperature.

Columns represent the four conditions tested of Sakai in the following media: **18** = M9 media + 0.2% glycerol at 18°C; **37** = M9 media + 0.2% glycerol at 37°C; **LL** = M9 media + spinach leaf lysates at 18°C; **RE** = M9 media + spinach root exudates at 18°C. Red colouring is used to highlight genes with significantly upregulated genes, whereas green was used for significantly downregulated genes. Black was used to show genes with no change to the control. Significance was determined by volcano plot analysis with a threshold cut-off of 2-fold change, multiple testing correction Benjamini & Hochberg, $p = 0.005$. Heat map was produced using GeneSpring software, version 7.0.

5.2.1.1 Temperature condition – 18 °C Minimal Media vs 37 °C Minimal Media

To identify which categories of genes were being upregulated in each of the conditions tested, the data were first analysed using the gene ontology (GO) browser feature of GeneSpring (GX) version 7.1 software package. GO classification designates a primary category and any subsequent sub-categories that the gene in question has been shown experimentally to be involved in. However, this GO mapping has not been carried out for many prokaryotic organisms and in this case this feature was only available for genes with an accession number from *E. coli* K-12. As there were sometimes up to quadruple hits for a single gene due to the four genomes present on the array, the total number of genes returned was not accurate. Nevertheless, using GO is very useful for identifying which categories of genes are being upregulated as a general overview and can help to spot patterns in the dataset that would be unnoticeable by looking at the total gene lists alone.

Figure 5-2 shows the GO maps for the temperature dataset: many of the genes with differential expression related to metabolism, as is the case across all the conditions tested in this study, with the largest number of genes with differential activity related to cellular and primary metabolism. These numbers will include genes that are repressed as well as upregulated, so to understand which components of metabolism are being affected by the temperature, metabolic pathway analysis was necessary. Details of metabolic pathways that are induced under the temperature, treatment and polysaccharide conditions are described in Chapter 6.

In addition to the metabolism class, the largest subset of genes, was related to “response to abiotic stress”, which was expected since temperature is an abiotic factor. The second largest subset in this category was “response to stress” and sub-classified further with osmotic stress being the most prominent factor.

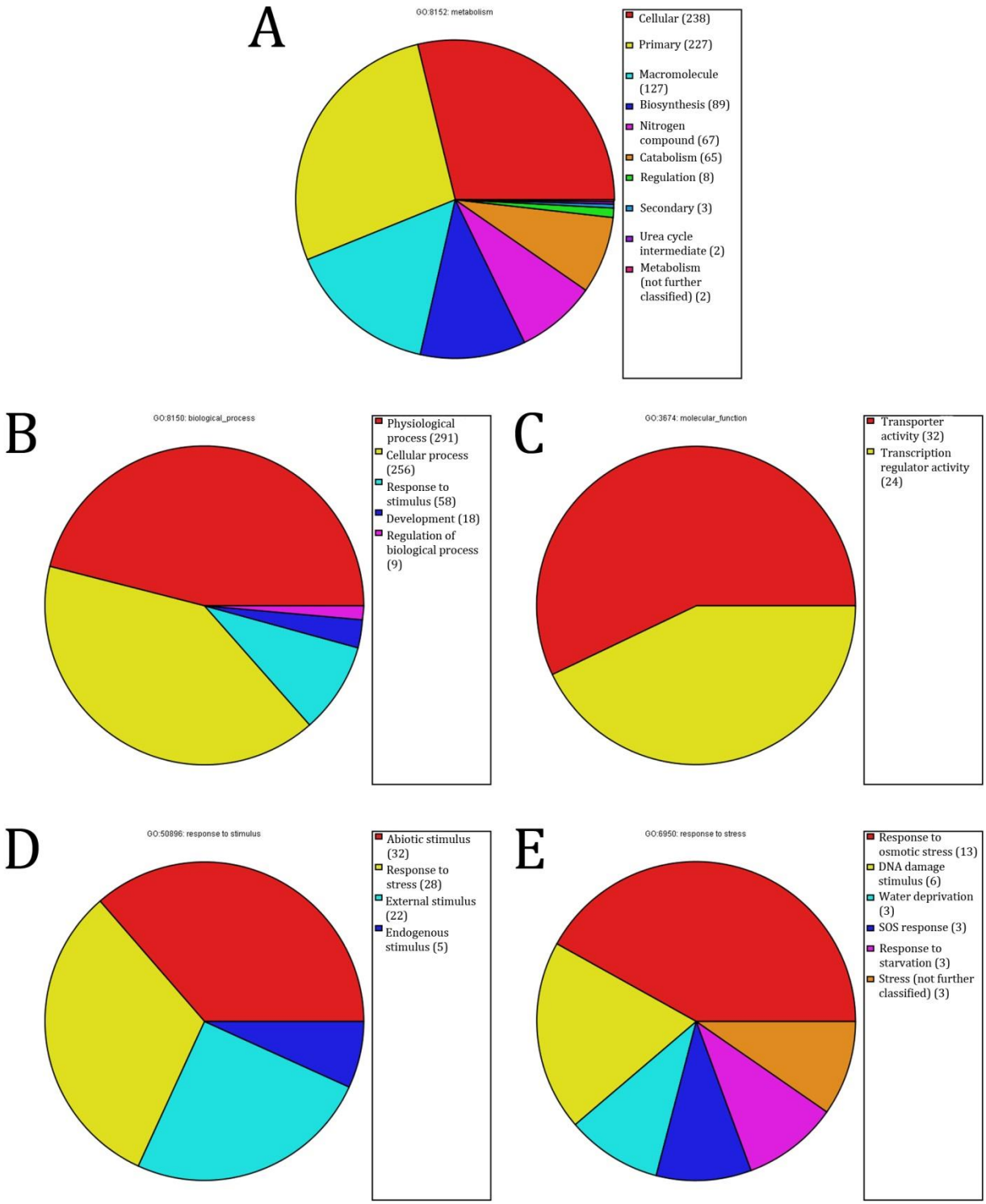


Figure 5-2. Gene ontology maps for genes with significant expression changes at 18°C compared to 37°C in M9 minimal medium.

Gene ontology maps were highlighted for **A)** metabolism (828 genes), **B)** biological process (632 genes), **C)** molecular function (56 genes), **D)** response to stimulus (87 genes) and **E)** response to stress (31 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

Genes related to membrane stress and fluidity had significantly upregulated expression at the lower temperatures (Table 5-2).

Many of the GO categories and the individual genes found from the temperature dataset were as predicted. However, there were also some interesting genes that were induced by the lower temperature, such as the *stx1a* gene, but not the *stx2* genes. It is unclear at present why these toxin genes, which are essential for the pathogenicity of *Ec*O157 in humans. are induced at low temperature. However, it should be noted that this strain contains a deletion of the *stx1* coding DNA sequence (CDS) and an insertional inactivation of *stx2*, which may affect promoter activity (Donohue-Rolfe *et al.*, 2000).

Another set of genes which play an essential role for the pathogenic lifestyle of *Ec*O157 in humans are the type III secretion system (T3SS) genes. A selected set of genes from the T3SS are detailed in Table 5-2, including regulators of the T3SS and their secreted effector proteins. All genes involved in the T3SS were found to be downregulated at 18 °C compared to 37 °C. Furthermore, the T3SS was not significantly upregulated in any of the conditions tested in this study (discussed further in section 7.2.1).

Table 5-2. Selected genes with significantly different expression in M9 media at 18 °C compared to 37 °C.

Fold change was calculated using the normalised 37°C dataset as the control; i.e. genes with upregulated gene expression represent genes that are induced at lower temperatures. Metabolic pathways that each gene is involved in are detailed, with not applicable (N/A) denoted if no metabolic pathways have been identified for a gene. Genes were determined to be conserved (i.e. present in *E. coli* K-12) or only present in selected *E. coli* groups (i.e. O157) using the multiple operon browser in EcoCyc (Keseler *et al.*, 2013). The full list of differentially expressed genes can be found in the appendix (File S-3).

Gene Category	Primary Accession	Gene Name	Fold Change	Description	Metabolic Pathways Involved In	Conserved
Type III Secretion System	ECs4550	*espF*	-10.34	EspF protein	N/A	O157
	ECs4565	*sepQ*	-7.17	type III secretion system protein	N/A	O157
	ECs4573	*escJ*	-11.84	type III secretion system protein	N/A	O157
	ECs4575	*escC*	-10.28	type III secretion system protein	N/A	O157
	ECs4582	*escS*	-20.16	type III secretion system protein	N/A	O157
	ECs4583	*ECs4583*	-15.03	type III secretion system protein	N/A	O157
	ECs4588	*ler*	-130.37	Ler protein; regulator of T3SS	N/A	O157
Toxins	ECs1205	*stx2a*	-5.23	Shiga toxin 2 subunit A	N/A	O157
	ECs1206	*stx2b*	-8.15	Shiga toxin 2 subunit B	N/A	O157
	ECs2974	*stx1a*	5.69	Shiga toxin I subunit A	N/A	O157
Motility, Chemotaxis & Adherence	B0942	*ycbU*	2.36	predicted fimbrial-like adhesion protein	N/A	K-12, O157
	b1073	*flgB*	-59.39	flagellar component of cell-proximal portion of basal-body rod	N/A	K-12, O157
	b1074	*flgC*	-40.47	flagellar component of cell-proximal portion of basal-body rod	N/A	K-12, O157
	b1075	*flgD*	-31.37	flagellar hook assembly protein	N/A	K-12, O157
	b1076	*flgE*	-26.47	flagellar hook protein	N/A	K-12, O157
	b1077	*flgF*	-11.57	flagellar component of cell-proximal portion of basal-body rod	N/A	K-12, O157
	b1078	*flgG*	-10.58	flagellar component of cell-distal portion of basal-body rod	N/A	K-12, O157
	b1079	*flgH*	-17.32	flagellar protein of basal-body outer-membrane L ring	N/A	K-12, O157
	b1080	*flgI*	-8.01	predicted flagellar basal body protein	N/A	K-12, O157
	b1880	*flhB*	-5.70	predicted flagellar export pore protein	N/A	K-12, O157
	b1937	*fliE*	-26.37	flagellar basal-body component	N/A	K-12, O157
	b1938	*fliF*	-9.86	flagellar basal-body MS-ring and collar protein	N/A	K-12, O157
	b1939	*fliG*	-7.81	flagellar motor switching and energizing component	N/A	K-12, O157
	b1944	*fliL*	-16.28	flagellar biosynthesis protein	N/A	K-12, O157
	b1945	*fliM*	-14.85	flagellar motor switching and energizing component	N/A	K-12, O157

	b1946	*fliN*	-10.69	flagellar motor switching and energizing component	N/A	K-12, O157
	ECs2686	*fliO*	-8.00	flagellar biosynthesis protein	N/A	K-12, O157
	b1948	*fliP*	-8.47	flagellar biosynthesis protein	N/A	K-12, O157
	b1950	*fliR*	-9.12	flagellar export pore protein	N/A	K-12, O157
	Z1676	*csgA*	-3.78	cryptic curlin major subunit	N/A	K-12, O157
	b1041	*csgB*	-2.94	curlin nucleator protein, minor subunit in curli complex	N/A	K-12, O157
	b2289	*lrhA*	-8.24	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes; regulator of type 1 fimbriae	N/A	K-12, O157
Efflux Systems/ Transporters	b0343	*lacY*	34.40	lactose/galactose transporter	N/A	K-12, O157
	b0574	*cusB*	56.31	copper/silver efflux system, membrane fusion protein	N/A	K-12, O157
	ECs0610	*cusC*	26.39	copper/silver efflux system outer membrane protein CusC	N/A	K-12, O157
	b2046	*wzxC*	12.32	colanic acid exporter	N/A	K-12, O157
	b3513	*mdtE*	14.52	multidrug resistance efflux transporter	N/A	K-12, O157
	b3514	*mdtF*	17.92	multidrug transporter, RpoS-dependent	N/A	K-12, O157
	b1599	*mdtI*	7.48	multidrug efflux system transporter	N/A	K-12, O157
	b4148	*sugE*	33.48	multidrug efflux system protein	N/A	K-12, O157
Hypothetical Proteins	ECs1653	*ECs1653*	14.01	hypothetical protein	unknown	O157
	ECs1654	*ECs1654*	27.78	hypothetical protein	unknown	O157
	ECs1655	*ECs1655*	23.09	hypothetical protein	unknown	K-12, O157
	ECs2623	*ECs2623*	-251.86	hypothetical protein	unknown	O157
	ECs2624	*ECs2624*	-256.68	hypothetical protein	unknown	O157
	ECs2625	*ECs2625*	-190.70	hypothetical protein	unknown	O157
	ECs2713	*ECs2713*	7.25	hypothetical protein	unknown	Sakai only
	ECs4474	*ECs4474*	-9.92	hypothetical protein	unknown	K-12, O157
Other Interesting Genes	ECs2085	*bdm*	57.74	biofilm-dependent modulation protein	N/A	K-12, O157
	ECs2098	*gadB*	120.61	glutamate decarboxylase isozyme	glutamate degradation IX; glutamate dependent acid resistance	K-12, O157
	b0344	*lacZ*	55.17	β-D-galactosidase	Lactose degradation II/ lactose degradation III	K-12, O157
	b1739	*osmE*	19.39	DNA-binding transcriptional activator	N/A	K-12, O157
	b4376	*osmY*	110.45	periplasmic protein	N/A	K-12, O157
	b4045	*yjbJ*	57.05	predicted stress response protein	N/A	K-12, O157

Finally, many hypothetical proteins were differentially expressed and small selections of these are shown in Table 5-2. A few of the hypothetical proteins that are present in *Ec*O157 but are not found in *E. coli* K-12 were seen to be downregulated at lower temperatures. These genes could be important for human infection or animal colonisation, since they were subject to >100-fold down regulation at the lower temperature. However, some hypothetical genes were upregulated at 18 °C, one of which was specific to *Ec*O157 Sakai. BLAST analysis identified this hypothetical protein in other associated *Ec*O157 strains, including the spinach-associated outbreak strain *Ec*O157 TW14359 (CDC, 2006). A 100% match was observed for two regions of the gene: a 24 bp alignment at the 5' end to a predicted cytochrome and the remaining 304 bp at the 3' end to another hypothetical protein.

Therefore, large gene expression differences were seen as a result of culturing at the two different temperatures: 37 °C, optimal for the bacteria; and 18 °C, optimal for the plant.

5.2.1.2 Treatment conditions – 18 °C Minimal Media vs 18 °C Spinach Leaf Lysates

As many outbreaks of *Ec*O157 have been linked to leafy produce, I decided to test the gene expression changes that were induced in the plant-adapted strain *Ec*O157 Sakai when exposed to whole leaf lysate extracts. As spinach has been implicated in previous *Ec*O157 outbreaks and colonisation data found spinach leaves were susceptible to colonisation by two *Ec*O157 strains, I decided to use this plant species for this experiment.

Once the baseline level of gene expression at 18 °C was determined, it was possible to examine expression in response to plant extract, independently of any temperature effects. Response to the spinach leaf lysates was mainly in metabolism (Figure 5-3). The primary and cellular metabolism GO groupings contained the largest number of genes with differential expression after a one-hour exposure to spinach leaf lysates. Within both of these categories, one of the largest groups related to nucleobase, nucleoside, nucleotide and nucleic acid metabolism. The second largest grouping of genes (117 genes) related to carbohydrate metabolism. The cellular metabolism GO as seen in Figure 5-3 (**C**) details the different areas of metabolism affected upon exposure to leaf lysates.

GO analysis of the leaf lysates for four additional categories was carried out: biological process; molecular function; response to stimulus; and classifying the response to stimulus further, response to stress (Figure 5-4).

The GO category of "biological process" displayed a similar pattern as metabolism, with the largest number of differentially expressed genes in the physiological and cellular processes class. The "molecular function" category contained the next largest number of genes, involved in regulation of transcription and transportation. In the "response to stimulus" category, the two most represented groupings were abiotic stimulus and stress.

As large gene expression changes were seen in *Ec*O157 Sakai when exposed to spinach leaf lysates, we decided to study this response further by analysing these samples by RNA-Seq.

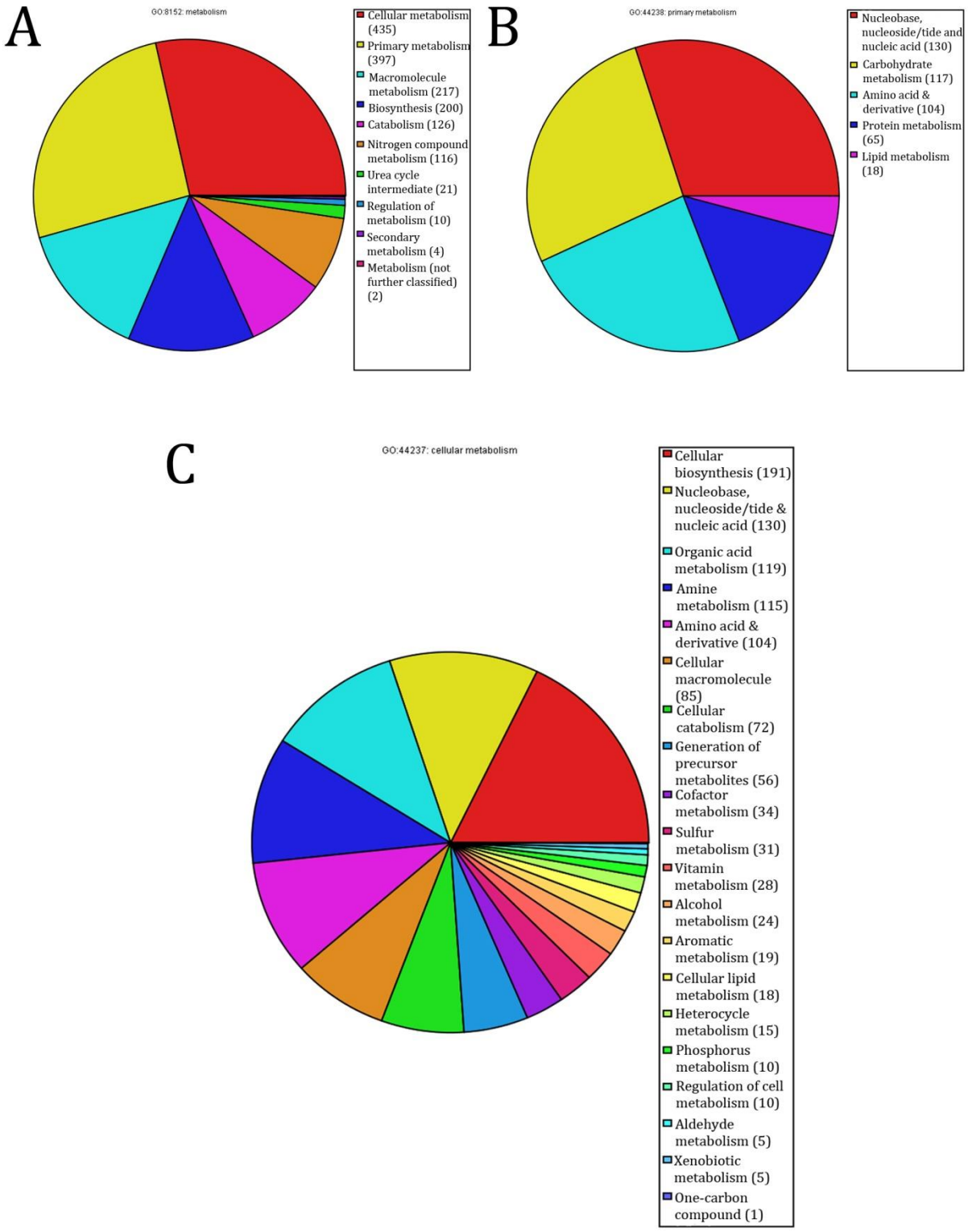


Figure 5-3. Gene ontology maps for genes with significant expression changes in response to spinach leaf lysates compared to 0.2% glycerol in M9 minimal media.

Gene ontology maps were highlighted for **A)** overall metabolism (1528 genes), and then further classified into **B)** primary metabolism (434 genes) and **C)** cellular metabolism (1072 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

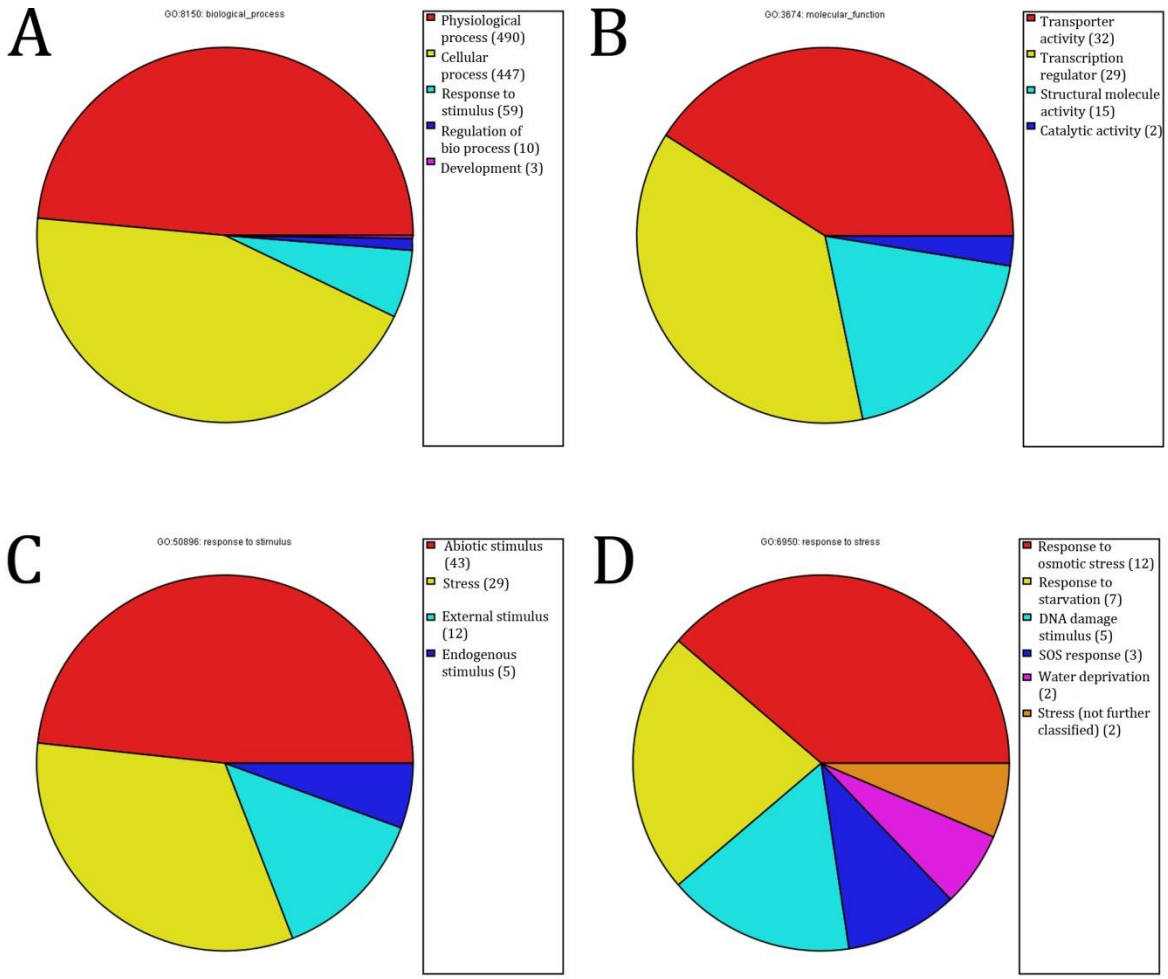


Figure 5-4. Gene ontology maps for genes with significant expression changes in response to spinach leaf lysates compared to 0.2% glycerol in M9 minimal media.

Gene ontology maps were highlighted for **A)** biological process (1009 genes), **B)** molecular function (78 genes), **C)** response to stimulus (89 genes) and **D)** response to stress (31 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

5.2.1.3 Transcriptome Analysis via Microarray versus RNA-Seq

Whole transcriptomic data using a microarray is limited by the number of genes represented on the array. Furthermore, analysis can be complicated by the presence of other hybridisation targets, e.g. for other species or isolates (as is the case for the *E. coli* v2 array). Additionally, microarrays rely on knowledge of the genome and its genetic elements (e.g. genes, different RNA species, alternative coding regions). Alternative approaches can therefore be used to overcome these issues, including RNA-seq. This method not only gives entire representation of expressed genes, but provides additional information that can be used for additional analysis including the transcriptional start sites and the expression of any non-coding RNA (ncRNA). Therefore, RNA-

Seq analysis was carried out on the same RNA samples used for the microarray analysis of *Ec*O157 Sakai to spinach leaf lysates.

As an initial step, analysis was carried out to determine how well the microarray and RNA-Seq datasets correlated, both in terms of expression profiles and sensitivity. Comparison of the datasets and RNA-Seq analysis was carried out by Ewa Grabowiecka (as detailed in section 2.17) to validate the datasets and thus justify using the data from RNA-seq.

Correlation of the $\log_2$ fold change obtained from the microarray versus the RNASeq can be seen in Figure 5-5 (Grabowiecka, 2014). A direct correlation can be seen between the two techniques, validating the microarray dataset and justifying the use of the RNASeq dataset. Fold change values from the microarray dataset (as determined from this study) and from the RNASeq (as determined from Ewa Grabowiecka's study) for selected genes are detailed in Table 5-3. Differences between values could be attributed to the different techniques (hybridisation of the microarray versus direct sequencing of the cDNA), but could result from the analysis methods, as the RNASeq dataset was analysed in a different approach (detailed in section 2.17) to determine the fold change values.

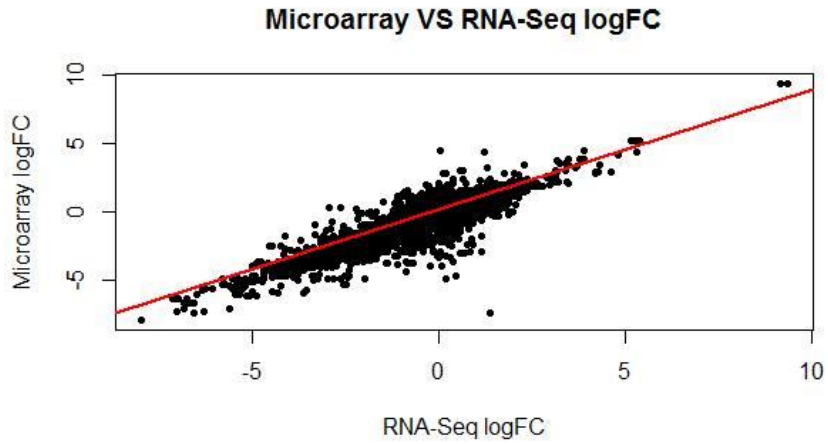


Figure 5-5. Scatterplot correlation between results obtained from microarray versus RNASeq.

Log_2 fold change (FC) of genes obtained from microarray (Y-axis) was compared against $\log_2$ fold change from RNASeq (X-axis), both of which were analysed using the four replicate samples of *Ec*O157 Sakai exposed to spinach leaf lysates for one hour used in this study. A direct correlation could be seen between the two methods. Data was obtained, interpreted and the final graph produced by Ewa Grabowiecka as part of an honours project using the samples produced in this study (Grabowiecka, 2014).

Table 5-3. Selected *E. coli* O157 Sakai genes identified by microarray and RNASeq with differential expression in M9 medium containing spinach leaf lysates compared to 0.2% glycerol.

Fold change was calculated using the normalised 18°C, M9 + 0.2% glycerol dataset as the control; i.e. genes with upregulated gene expression represent genes that are induced in response to spinach leaf lysates. Fold change is shown for results from the microarray (MA) and RNASeq analysis. Metabolic pathways that each gene is involved in are detailed, with not applicable (N/A) denoted if no metabolic pathways have been identified for a gene. Genes were determined to be conserved (i.e. present in *E. coli* K-12) or only present in selected *E. coli* groups (i.e. O157) using the multiple operon browser in EcoCyc (Keseler *et al.*, 2013). Gene expression denoted as "NP" represents genes that were not present on the microarray. The full list of differentially expressed genes can be found in the appendix (File S-4 for the microarray dataset; File S-5 for the RNA-Seq dataset).

Category	Primary Accession	Gene Name	Fold Change MA	Fold Change RNASeq	Description	Metabolic Pathways Involved In	Conserved
Toxins	ECs0819	*ECs0819*	2.70	1.35	endolysin	N/A	K-12, O157
	L7048	*hlyA*	3.51	2.08	enterohemolysin	N/A	O157
Motility, Chemotaxis & Adherence	b1891	*flhC*	-9.17	-8.52	regulator of flagellar biosynthesis acting on class 2 operons; transcription initiation factor	N/A	K-12, O157
	b1892	*flhD*	-7.43	-9.63	regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor	N/A	K-12, O157
	b2289	*lrhA*	3.20	2.10	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes	N/A	K-12, O157
	b3142	*yraH*	2.44	2.22	Predicted fimbrial-like adhesin protein	N/A	K-12, O157
	ECs2914	*ECs2914*	-6.93	1.24	putative type-1 fimbrial protein	N/A	K-12, O157
Efflux Systems/ Transporters	b0396	*araJ*	2.22	1.37	involved in either transport or processing of arabinose polymers	N/A	K-12, O157
	b0448	*mdlA*	3.48	2.18	fused predicted multidrug transporter subunits of ABC superfamily: ATP-binding components	N/A	K-12, O157
	b0449	*mdlB*	2.48	2.24	fused predicted multidrug transporter subunits of ABC superfamily: ATP-binding components	N/A	K-12, O157
	b1117	*lolD*	3.22	1.57	outer membrane-specific lipoprotein transporter subunit	N/A	K-12, O157
	b1516	*lsrB*	-16.25	-14.52	AI2 transporter	N/A	K-12, O157
	b1528	*ydeA*	6.86	3.55	predicted arabinose transporter	N/A	K-12, O157
	b3514	*mdtF*	-4.91	1.23	multidrug transporter, RpoS-dependent	N/A	K-12, O157
	b3241	*aaeA*	21.22	10.59	p-hydroxybenzoic acid efflux system component	N/A	K-12, O157
	b3240	*aaeB*	19.20	9.57	p-hydroxybenzoic acid efflux system component	N/A	K-12, O157
	b3242	*aaeX*	23.58	7.37	membrane protein of efflux system	N/A	K-12, O157

	b2182	*bcr*	3.11	2.01	bicyclomycin/multidrug efflux system	N/A	K-12, O157
	b0842	*cmr*	4.73	4.42	multidrug efflux system protein	N/A	K-12, O157
	b2685	*emrA*	9.07	7.45	multidrug efflux system	N/A	K-12, O157
	b2686	*emrB*	10.20	4.61	multidrug efflux system protein	N/A	K-12, O157
	b3673	*emrD*	33.69	18.13	multidrug efflux system protein	N/A	K-12, O157
	b3513	*mdtE*	-3.08	-5.10	multidrug resistance efflux transporter	N/A	K-12, O157
	b1065	*mdtH*	2.81	2.56	predicted drug efflux system	N/A	K-12, O157
	b2684	*mprA*	7.76	6.28	DNA-binding transcriptional repressor of microcin B17 synthesis and multidrug efflux	N/A	K-12, O157
	b2170	*setB*	4.25	3.08	lactose/glucose efflux system	N/A	K-12, O157
Stress	b3510	*hdeA*	-11.58	-19.92	stress response protein acid-resistance protein	N/A	K-12, O157
	b1482	*osmC*	-10.97	-13.26	osmotically inducible, stress-inducible membrane protein	N/A	K-12, O157
	b1743	*spy*	-6.36	-7.16	envelope stress induced periplasmic protein	N/A	K-12, O157
	b3495	*uspA*	-3.90	-5.19	universal stress global response regulator	N/A	K-12, O157
	b1895	*uspC*	-3.15	-4.33	universal stress protein	N/A	K-12, O157
	b1376	*uspF*	-18.09	-3.45	stress-induced protein, ATP-binding protein	N/A	K-12, O157
	b4045	*yjbJ*	-11.09	-9.26	predicted stress response protein	N/A	K-12, O157
Hypothetical Proteins	ECs0304	*ECs0304*	3.49	2.74	hypothetical protein	unknown	O157
	ECs0317	*ECs0317*	11.85	5.83	hypothetical protein	unknown	K-12, O157
	ECs1376	*ECs1376*	6.18	4.96	hypothetical protein	unknown	O157
	ECs1521	*ECs1521*	NP	3.61	hypothetical protein	unknown	Sakai only
	ECs1653	*ECs1653*	-15.29	-22.23	hypothetical protein	unknown	O157
	ECs1654	*ECs1654*	-32.08	-34.07	hypothetical protein	unknown	O157
	ECs1655	*ECs1655*	-18.13	-22.28	hypothetical protein	unknown	K-12, O157
	ECs2304	*ECs2304*	12.97	11.18	hypothetical protein	unknown	K-12, O157
	ECs2713	*ECs2713*	-2.89	-1.47	hypothetical protein	unknown	Sakai only
	ECs3138	*ECs3138*	5.16	3.45	hypothetical protein	unknown	K-12, O157
	ECs3535	*ECs3535*	5.13	3.21	hypothetical protein	unknown	O157
	ECs3864	*ECs3864*	NP	2.16	hypothetical protein	unknown	Sakai only
	ECs3865	*ECs.3865*	NP	2.03	hypothetical protein	unknown	Sakai only
	ECs4330	*ECs4330*	4.98	3.91	hypothetical membrane protein	unknown	O157
	ECs4474	*ECs4474*	-3.10	-3.20	hypothetical protein	unknown	K-12, O157

	ECs4964	*ECs4964*	NP	2.52	hypothetical protein	unknown	Sakai only
	b1722	*ydiY*	47.86	19.96	conserved protein	unknown	K-12, O157
Other Interesting Genes	b0343	*lacY*	-30.06	-32.17	lactose/galactose transporter	N/A	K12, O157
	b0344	*lacZ*	-77.98	-47.58	β-D-galactosidase	Lactose degradation	K-12, O157
	b0880	*cspD*	-7.55	-5.46	cold shock protein homolog	N/A	K-12, O157
	b0990	*cspG*	10.47	13.49	DNA-binding transcriptional regulator	N/A	K-12, O157
	b2313	*cvpA*	-8.98	-7.34	membrane protein required for colicin V production	N/A	K-12, O157
	b2764	*cysJ*	-136.85	-145.60	sulfite reductase, alpha subunit, flavoprotein	methionine biosynthesis; sulphate assimilation and cysteine biosynthesis; sulphate reduction I/IV	K-12, O157
	b3261	*fis*	3.20	4.70	global DNA-binding transcriptional dual regulator	N/A	K-12, O157
	b3556	*cspA*	18.17	21.58	major cold shock protein	N/A	K-12, O157
	b3650	*spoT*	2.32	1.67	bifunctional (p)ppGpp synthetase II/ guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase	ppGpp biosynthesis	K-12, O157
	b4376	*osmY*	-19.05	-24.73	periplasmic protein	N/A	K-12, O157
	ECs1837	*ECs1837*	NP	-11.42	Trp operon leader peptide		
	ECs2098	*ECs2098*	-19.16	-17.48	glutamate decarboxylase isozyme	Glutamate degradation IX (via 4-aminobutyrate); glutamate dependent acid resistance	K-12, O157
	ECs2819	*ECs2819*	NP	-2.13			
	ECs3461	*ECs3461*	NP	-2.03	Leader peptide of chorismate mutase-P-prephenate dehydratase	unknown	
	ECs4929	*purH*	-22.75	-18.89	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	Purine nucleotides *de novo* biosynthesis I/II; histidine, purine and pyrimidine biosynthesis; inosine-5'-phosphate biosynthesis (I & II)	K-12, O157

Regulators of flagella and motility (*flhDC, lrhA*) were significantly downregulated in response to spinach leaf lysates, with no structural flagellar genes seen to be significantly expressed. Upregulation of sugar transporters, including ATP-binding cassette (ABC) types of transporters, and efflux systems was observed.

Universal stress protein production was also repressed upon exposure to leaf lysates, similar to many of the stress-related genes.

For all the conditions tested, a large proportion of hypothetical proteins were induced/repressed, some of which have been selected to show the range of expression, including some hypothetical proteins that are specific to *Ec*O157 Sakai (when compared against other *Ec*O157, K-12 and related *E. coli* strains).

As there is no experimental evidence available to determine their function, reciprocal best BLAST hits are the only bioinformatics tool available to help identify a possible function for these proteins. Further identification and characterisation of the gene expression profiles of selected hypothetical proteins is detailed in section 7.2.5.

Therefore, the spinach leaf lysates induce large gene expression changes in *Ec*O157 Sakai over a broad range of categories when compared to the 0.2 % glycerol control. RNA-Seq analysis of these same samples demonstrated a strong correlation between the data obtained from microarray analysis (Grabowiecka, 2014), therefore validating the use of the microarray in this experiment. The data from the RNA-Seq analysis will be helpful in future projects for further study of this transcriptome response, especially by studying ncRNA, a feature which is unavailable using microarrays.

5.2.1.4 Treatment conditions – 18 °C Minimal Medium vs 18 °C Root Exudates

*Ec*O157 Sakai can survive and proliferate to high numbers in the roots and rhizosphere of spinach plants. Therefore, the bacterial response to root exudates was examined. It is possible that exposure to root exudates primes the bacteria for root colonisation despite an inability to grow on purified exudates.

GO analysis of the root exudates dataset revealed categories of genes with differential patterns of expression similar to that of the spinach leaf lysates dataset, with large changes in metabolism seen (Figure 5-6). The largest changes were seen in primary and cellular metabolism with 517 and 559 genes significantly different in expression compared to the control, respectively. Within primary metabolism, nucleic acid and carbohydrate metabolism contained the largest number of genes with differential expression. As GO only highlights significantly expressed genes, it is uncertain whether these genes with the GO designation "carbohydrate metabolism" are up and down-regulated without looking at the gene datasets. Many of the genes in this category were downregulated due to the inability of *Ec*O157 Sakai to use root exudates as a carbon source. Individual gene expression can be seen in Table 5-4, and metabolic pathways that are induced are discussed in section 6.2.3.

Within the category of "biological process" 81 K-12-annotated genes were classed as "response to stimulus", of which 54 were sub-classed as "response to abiotic stimulus". Forty-one K-12 genes were related to osmotic stress, DNA damage, the bacterial SOS response and starvation.

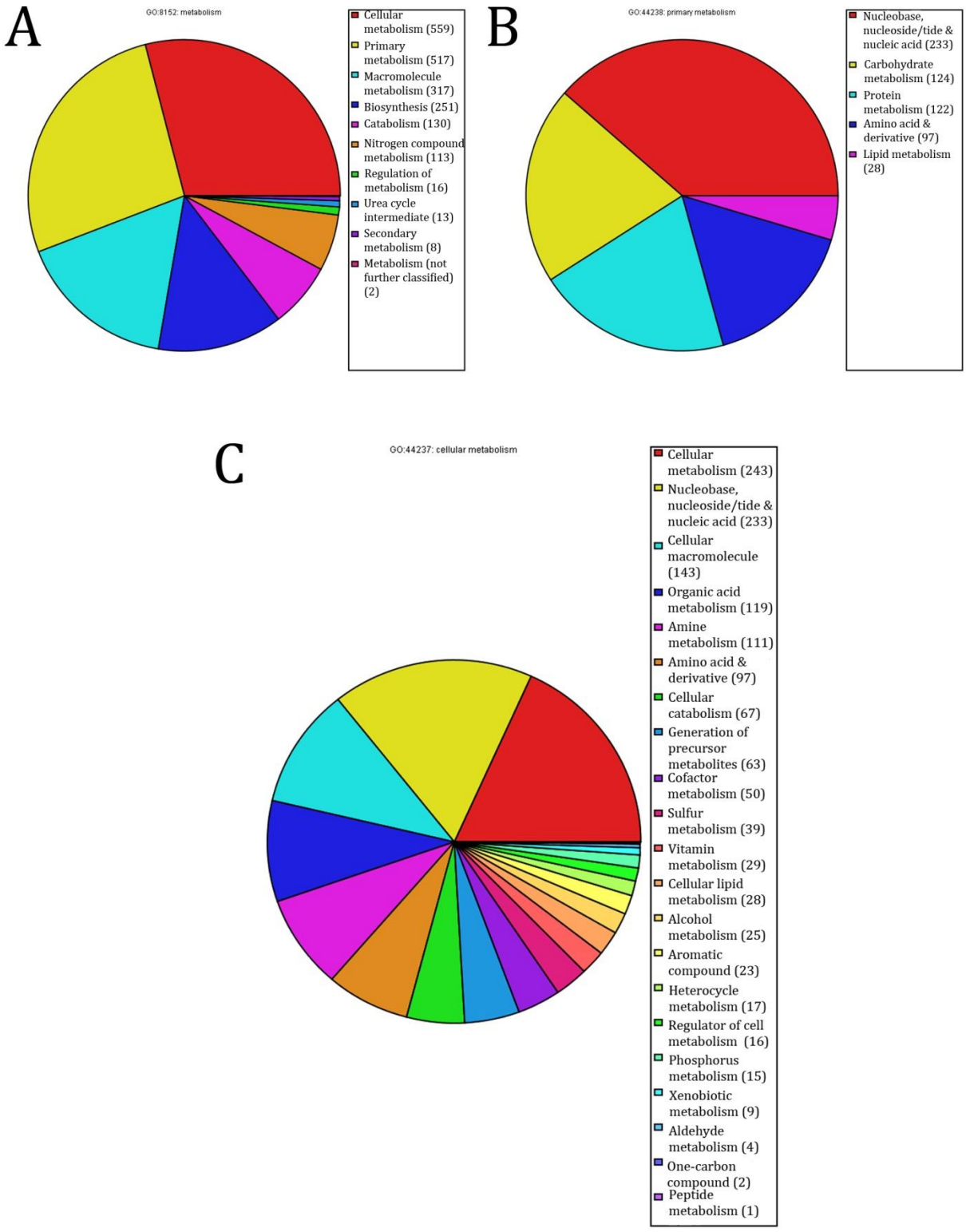


Figure 5-6. Gene ontology maps for genes with significant expression changes in response to spinach root exudates compared to 0.2% glycerol in M9 minimal media.

Gene ontology maps were highlighted for **A)** overall metabolism (1926 genes), and then further classified into **B)** primary metabolism (604) and **C)** cellular metabolism (1334 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

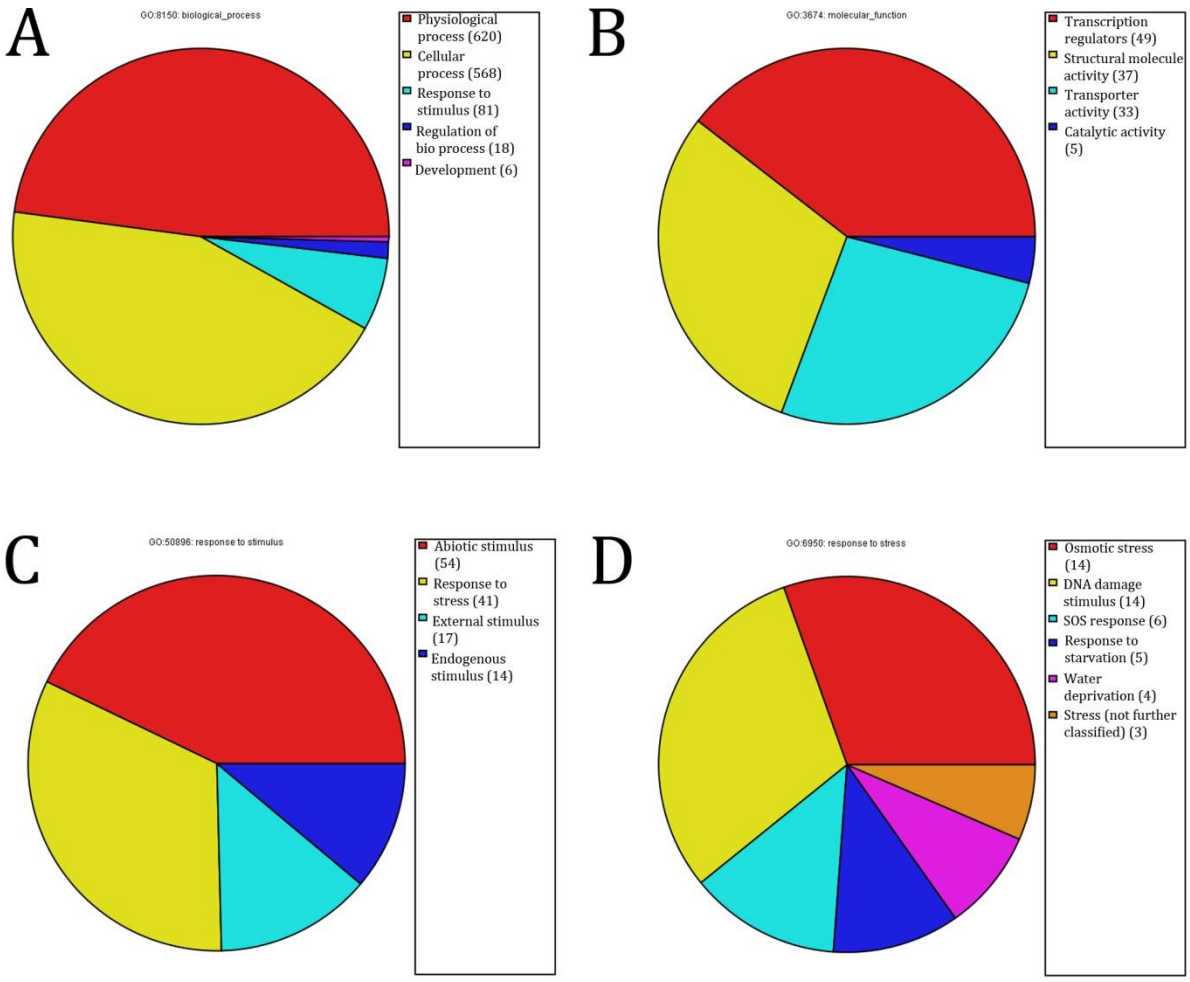


Figure 5-7. Gene ontology maps for genes with significant expression changes in response to spinach root exudates compared to 0.2% glycerol in M9 minimal media.

Gene ontology maps were highlighted for **A)** biological process (1293 genes), **B)** molecular function (124 genes), **C)** response to stimulus (126 genes) and **D)** response to stress (46 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

Table 5-4. Selected *E. coli* O157 Sakai genes with significantly different expression in M9 media containing spinach root exudates compared to 0.2% glycerol.

Fold change was calculated using the normalised 18°C, M9 + 0.2% glycerol dataset as the control; i.e. genes with upregulated gene expression represent genes that are induced in response to spinach root exudates. Metabolic pathways that each gene is involved in are detailed, with not applicable (N/A) denoted if no metabolic pathways have been identified for a gene. Genes were determined to be conserved (i.e. present in *E. coli* K-12) or only present in selected *E. coli* groups (i.e. O157) using the multiple operon browser in EcoCyc (Keseler *et al.*, 2013). The full list of differentially expressed genes can be found in the appendix (File S-6).

Category	Primary Accession	Gene Name	Fold Change	Description	Metabolic Pathways Involved In	Conserved
Type II Secretion System	L7032	*etpC*	2.17	type II secretion protein	N/A	K-12, O157 (not EDL933)
	L7033	*etpD*	2.55	type II secretion protein	N/A	K-12, O157 (not EDL933)
Toxins	ECs1206	*stx2b*	6.62	Shiga toxin 2 subunit B	N/A	O157
	L7050	*hlyA*	2.47	hemolysin transport protein	N/A	O157
	b4225	*chpB*	2.98	toxin of the ChpB-ChpS toxin-antitoxin system	N/A	K-12, O157
	b4224	*chpS*	5.26	antitoxin of the ChpB-ChpS toxin-antitoxin system	N/A	K-12, O157
	Z0056	*Z0056*	2.31	putative antitoxin of gyrase inhibiting toxin-antitoxin system	N/A	O157
	Z0057	*Z0057*	2.81	putative toxin of gyrase inhibiting toxin-antitoxin system	N/A	O157
Motility, Chemotaxis & Adherence	b1072	*flgA*	-2.48	assembly protein for flagellar basal-body periplasmic P ring	N/A	K-12, O157
	b2321	*flk*	-4.21	predicted flagella assembly protein	N/A	K-12, O157
	ECs2680	*fliI*	3.54	flagellum-specific ATP synthase	N/A	K-12, O157
	b1891	*flhC*	-11.93	DNA-binding transcriptional dual regulator with FlhD	N/A	K-12, O157
	b1892	*flhD*	-27.84	DNA-binding transcriptional dual regulator with FlhC	N/A	K-12, O157
	b1421	*trg*	5.52	methyl-accepting chemotaxis protein III, ribose and galactose sensor receptor	N/A	K-12, O157
	ECs1420	*csgA*	4.05	cryptic curlin major subunit	N/A	K-12, O157
	b1041	*csgB*	3.55	curlin nucleator protein, minor subunit in curli complex	N/A	K-12, O157
	ECs0142	*ECs0142*	2.31	putative fimbrial protein	N/A	K-12, O157

	ECs0744	*ECs0744*	2.16	putative fimbrial-like protein	N/A	K-12, O157
	ECs2914	*ECs2914*	-9.28	putative type-1 fimbrial protein	N/A	K-12, O157
	ECs4020	*ECs4020*	-2.80	putative fimbrial-like protein	N/A	K-12, O157
	ECs4023	*ECs4023*	2.53	truncated putative fimbrial protein	N/A	O157
Efflux Systems/ Transporters	b0575	*cusA*	-2.33	copper/silver efflux system, membrane component	N/A	K-12, O157
	b0572	*cusC*	-2.27	copper/silver efflux system, outer membrane component	N/A	K-12, O157
	b0752	*zitB*	6.83	zinc efflux system	N/A	K-12, O157
	b0462	*acrB*	-2.33	multidrug efflux system protein	N/A	K-12, O157
	b3266	*acrF*	-2.27	multidrug efflux system protein	N/A	K-12, O157
	Z1870	*emrE*	2.12	multidrug efflux protein	N/A	
	b2074	*mdtA*	2.56	multidrug efflux system, subunit A	N/A	K-12, O157
	b1901	*araF*	10.45	L-arabinose transporter subunit	N/A	K-12, O157
	Z3708	*eutH*	2.58	ethanolamine utilization; homolog of Salmonella putative transport protein	N/A	
	b0343	*lacY*	-21.56	lactose/galactose transporter	N/A	K-12, O157
	b1515	*lsrD*	63.17	AI2 transporter	N/A	K-12, O157
	b1528	*ydeA*	-4.34	predicted arabinose transporter	N/A	K-12, O157
	b4228	*ytfR*	48.51	predicted sugar transporter subunit: ATP-binding component of ABC superfamily	N/A	K-12, O157
	b4230	*ytfT*	52.15	predicted sugar transporter subunit: membrane component of ABC superfamily	N/A	K-12, O157
Stress	b0607	*uspG*	4.46	universal stress protein UP12	N/A	K-12, O157
	b1333	*uspE*	16.29	stress-induced protein	N/A	K-12, O157
	b1376	*uspF*	8.60	stress-induced protein, ATP-binding protein	N/A	K-12, O157
	b1743	*spy*	14.13	envelope stress induced periplasmic protein	N/A	K-12, O157
	b1895	*uspC*	3.58	universal stress protein	N/A	K-12, O157
	b3494	*uspB*	14.53	predicted universal stress (ethanol tolerance) protein B	N/A	K-12, O157
	b3923	*uspD*	13.87	stress-induced protein	N/A	K-12, O157
	b4045	*yjbJ*	5.92	predicted stress response protein	N/A	K-12, O157
Hypothetical Proteins	ECs0884	*ECs0884*	37.03	hypothetical protein	unknown	K-12, O157
	ECs1191	*ECs1191*	7.46	hypothetical protein	unknown	Sakai only

	ECs2304	*ECs2304*	20.21	hypothetical protein	unknown	K-12, O157
	ECs3238	*ECs3238*	11.80	hypothetical protein	unknown	O157
	ECs3750	*ECs3750*	34.86	hypothetical protein	unknown	K-12, O157
	ECs4474	*ECs4474*	267.91	hypothetical protein	unknown	K-12, O157
	ECs5165	*ECs5165*	60.15	hypothetical protein	unknown	K-12, O157
	L7002	*L7002*	2.03	hypothetical protein	unknown	O157
	L7060	*L7060*	2.63	hypothetical protein	unknown	O157
Other Interesting Genes	b0061	*araD*	3.58	L-ribulose-5-phosphate 4-epimerase	L-arabinose degradation I; L-lyxose degradation	K-12, O157
	b0062	*araA*	8.93	L-arabinose isomerase	L-arabinose degradation I	K-12, O157
	b0063	*araB*	19.98	L-ribulokinase	L-arabinose degradation I	K-12, O157
	b0064	*araC*	9.14	DNA-binding transcriptional dual regulator	N/A	K-12, O157
	b0221	*fadE*	183.35	acyl coenzyme A dehydrogenase	Fatty acid β-oxidation I	K-12, O157
	b0344	*lacZ*	-80.39	β-D-galactosidase	Lactose degradation III	K-12, O157
	b0875	*aqpZ*	6.30	aquaporin	N/A	K-12, O157
	b1020	*phoH*	97.77	conserved protein with nucleoside triphosphate hydrolase domain	N/A	K-12, O157
	b1818	*manY*	4.82	mannose-specific enzyme IIC component of PTS	N/A	K-12, O157
	b2764	*cysJ*	-257.08	sulfite reductase, alpha subunit, flavoprotein	sulphate assimilation and cysteine biosynthesis; sulphate reduction I (assimilatory)	K-12, O157
	b3261	*fis*	-27.78	global DNA-binding transcriptional dual regulator	N/A	K-12, O157
	b3564	*xylB*	5.37	xylulokinase	Xylose degradation I	K-12, O157
	b3565	*xylA*	5.06	D-xylose isomerase	Xylose degradation I	K-12, O157
	b3599	*mtlA*	29.53	fused mannitol-specific PTS enzymes: IIA components/IIB components/IIC components	N/A	K-12, O157
	ECs3027	*ECs3027*	2.42	salicylate hydroxylase	salicylate degradation I; chlorosalicylate degradation; methylsalicylate degradation	O157

From the gene expression data, genes involved in the type II secretion system (T2SS) were seen to be induced 2-fold in *Ec*O157 Sakai when exposed to spinach root exudates.

As well as this, many genes involved in stress were upregulated, as was expected based on the previous growth experiments, including the universal stress proteins. Finally, many motility genes were downregulated, with no clear chemotaxis response seen from the gene expression data. However, genes encoding for curli were seen to be induced in root exudates.

5.2.2 Plant Cell Wall Polysaccharide Array

Analysis of the gene expression changes in response to spinach leaf lysates and root exudates provided an overall view of how the bacteria were interacting with the different components of the plant extracts and reinforced the colonisation, growth and motility data obtained (see Chapter 3).

However, as these extracts were made by macerating leaves or collecting exudates after a three-week period of plant growth, it was unclear what was in each of the extracts. Initial HPLC analysis was performed on the extracts to quantify selected monosaccharide content; however, secondary metabolite analysis would help to narrow down what components of the extracts are causing some of the gene expression changes seen.

The plant cell wall will be one of the first components of the plant environment that the bacteria will encounter when colonising plant species. Plant cell wall polysaccharides are known to affect the gene expression and behaviour of bacteria, such as for *Bacillus subtilis*, in which plant cell wall polysaccharides were shown to induce biofilm production in this bacterial species (Beauregard *et al.*, 2013). To elucidate whether the interaction of *Ec*O157 Sakai with the plant cell wall influences gene expression, leaf cell wall polysaccharides extractions were made (section 2.10). It should be noted however that this preparation excludes the apoplastic fluid and the intracellular components.

*Ec*O157 Sakai was incubated with leaf cell wall polysaccharides (1 % w/v in M9 media) for a one hour exposure in the same manner as for the leaf lysate and root exudate experiments (section

2.13.3). A comparison of the *Ec*O157 Sakai response was made for extracts from spinach and lettuce to determine any plant species-specific effects. The overall *Ec*O157 Sakai transcriptional response was much greater to lettuce polysaccharides compared with spinach (Figure 5-8). The total change in gene expression compared to the vermiculite polysaccharide control for lettuce was 4.8 %, whereas for spinach it was only 1.5 %.

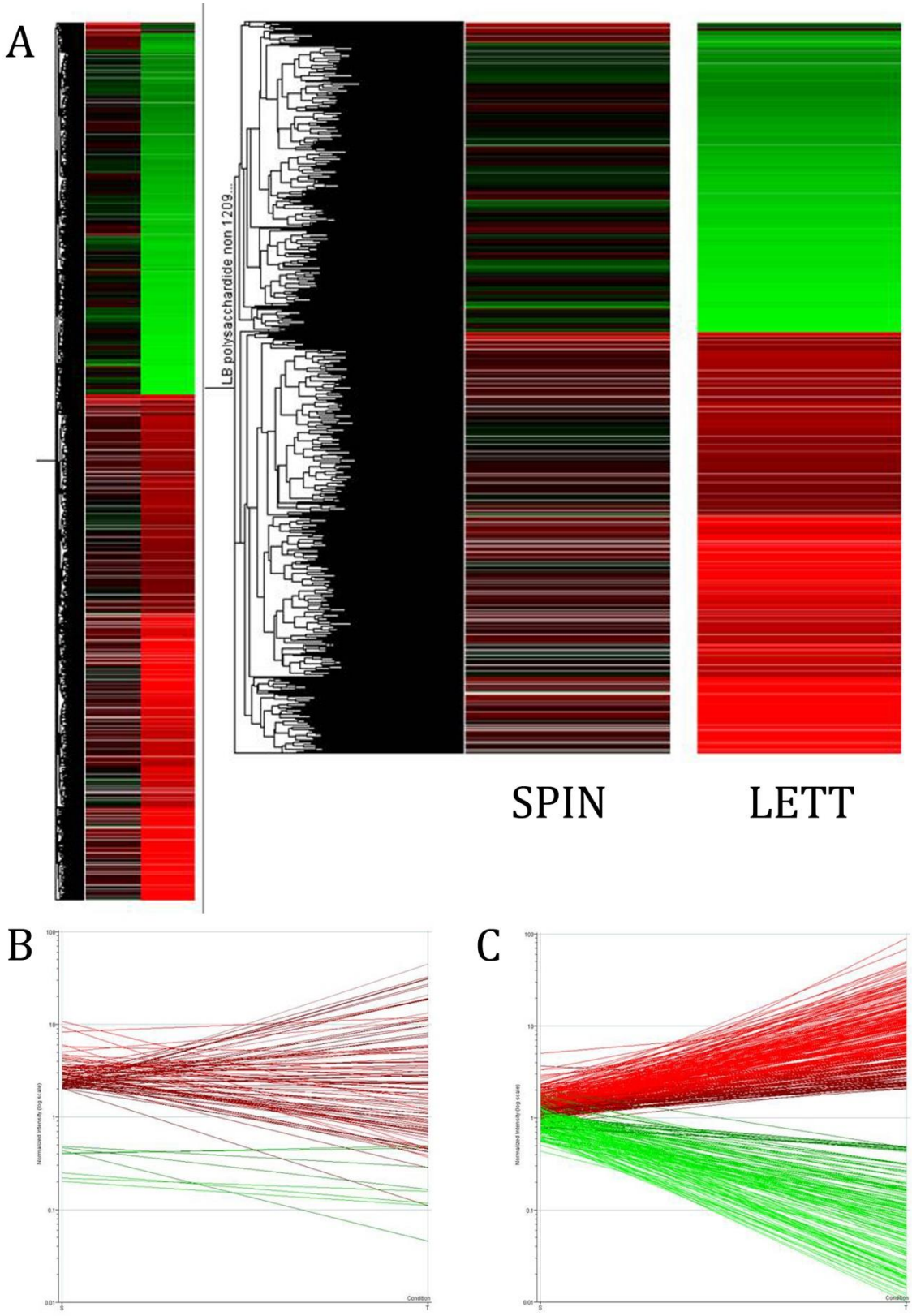


Figure 5-8. Gene expression changes for Sakai in response to leaf cell wall polysaccharides.

A) Heatmap of overall gene expression changes of Sakai in response to spinach (SPIN) and lettuce (LETT) leaf cell wall polysaccharides. As a two-colour array approach was used for this experiment there is no separate bar to show gene expression changes of the control (vermiculite polysaccharides). Significant gene expression changes for the spinach condition can be seen in **B)** and for the lettuce condition in **C)**. Red represents significantly upregulated genes, green for downregulated and black for no significant difference to the control.

5.2.2.1 Spinach Leaf Cell Wall Polysaccharide Condition

As only 1.5 % of the total genes were seen to have differential expression to the vermiculite polysaccharide control this resulted in a dataset of only 111 genes. GO analysis of the dataset was limited to those genes with a K-12 primary accession number. Most of the changes seen were in metabolism, with primary and cellular metabolism the largest groups of genes, although 18 % of the total genes with significantly different expression to the control were seen to be involved in "biosynthesis" (Figure 5-9). The largest group of genes within primary metabolism were designated "carbohydrate metabolism", most of which were induced, including those involved with the processing of the plant-specific polysaccharide arabinose (Table 5-5) (as discussed further in chapter 6.2.6).

No genes related to stress or "response to stimulus" were significantly expressed in this dataset, which may explain why only 1.5 % difference in gene expression changes from the control condition was seen in this condition.

Many of the genes with differential expression were related to metabolism, transport and processing of various sugars such as arabinose, galactose, gluconate and maltose (Table 5-5). Details of the metabolic pathways upregulated in this dataset are discussed further in section 6.2.4. As well as this, some hypothetical genes were seen to be upregulated, although none of these were specific to *Ec*O157 Sakai, i.e. they were present in other O157 or K-12.

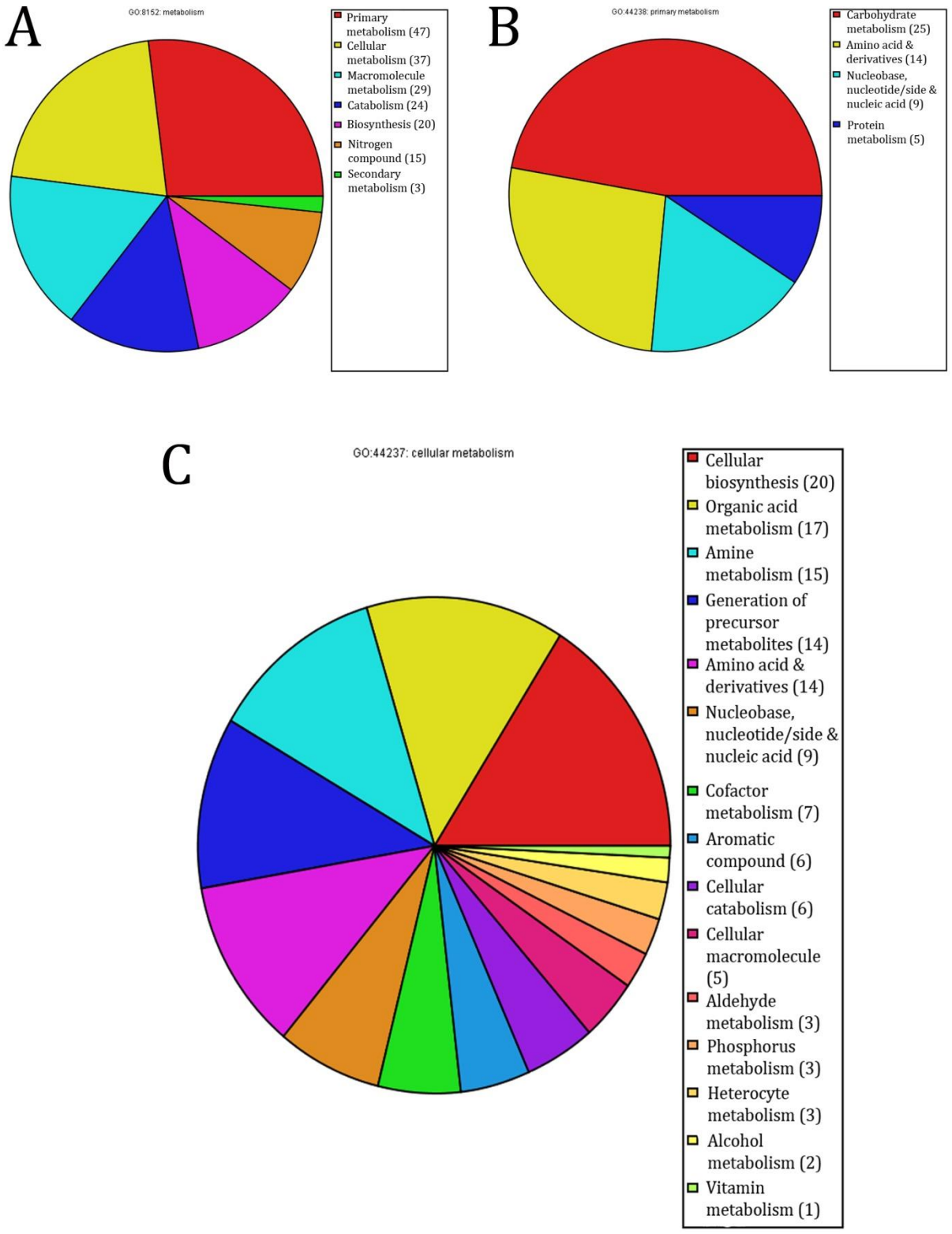


Figure 5-9. Gene ontology maps for genes with significant expression changes in response to spinach leaf cell wall polysaccharides compared to vermiculite polysaccharides in M9 minimal media.

Gene ontology maps were highlighted for **A)** overall metabolism (175 genes), and then further classified into **B)** primary metabolism (53 genes) and **C)** cellular metabolism (125 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

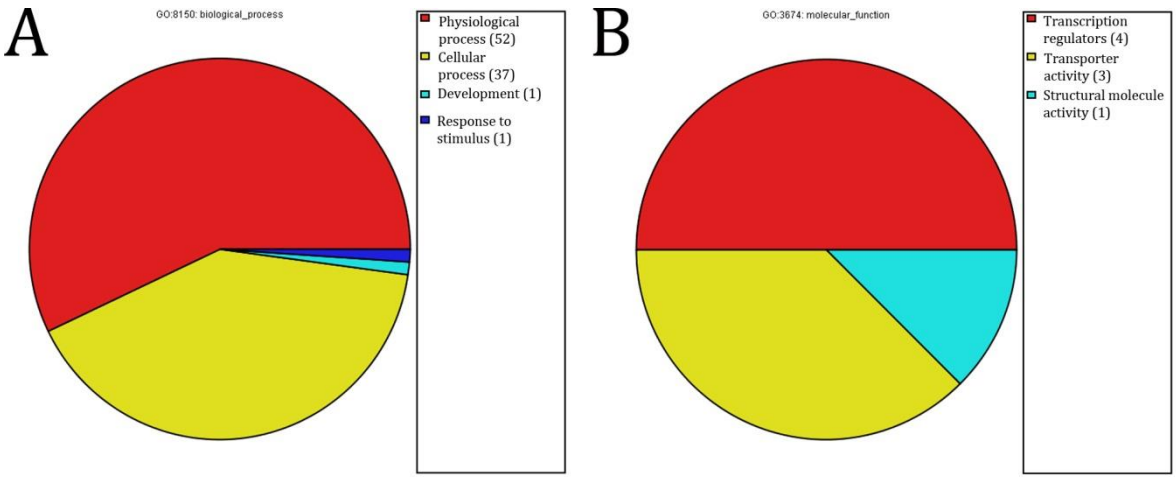


Figure 5-10. Gene ontology maps for genes with significant expression changes in response to spinach root exudates compared to 0.2% glycerol in M9 minimal media.

Gene ontology maps were highlighted for **A)** biological process (91 genes) and **B)** molecular function (8 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

Table 5-5. Selected genes with significantly different expression in M9 medium containing spinach leaf cell wall polysaccharides compared to vermiculite polysaccharides.

Fold change was calculated using the normalised 18°C, M9 + vermiculite polysaccharides dataset as the control; i.e. genes with upregulated gene expression represent genes that are induced in response to spinach leaf cell wall polysaccharides. Metabolic pathways that each gene is involved in are detailed, with not applicable (N/A) denoted if no metabolic pathways have been identified for a gene. Genes were determined to be conserved (i.e. present in *E. coli* K-12) or only present in selected *E. coli* groups (i.e. O157) using the multiple operon browser in EcoCyc (Keseler *et al.*, 2013). The full list of differentially expressed genes can be found in the appendix (File S-7).

Category	Primary Accession	Gene Name	Fold Change	Description	Metabolic Pathways Involved In	Conserved
Motility, Chemotaxis & Adherence	b1892	*flhD*	2.21	regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor	N/A	K-12, O157
Transporters	b0451	*amtB*	-2.51	ammonium transporter	N/A	K-12, O157
	b1006	*rutG*	-2.14	predicted transporter	N/A	K-12, O157
	b1039	*csgE*	2.26	predicted transport protein	N/A	K-12, O157
	b1900	*araG*	2.16	fused L-arabinose transporter subunits of ABC superfamily: ATP-binding components	N/A	K-12, O157
	b1901	*araF*	6.01	L-arabinose transporter subunit	N/A	K-12, O157
	b2148	*mglC*	3.25	methyl-galactoside transporter subunit	N/A	K-12, O157
	b2149	*mglA*	5.07	fused methyl-galactoside transporter subunits of ABC superfamily: ATP-binding components	N/A	K-12, O157
	b2150	*mglB*	3.43	methyl-galactoside transporter subunit	N/A	K-12, O157
	b3415	*gntT*	4.66	gluconate transporter, high-affinity GNT I system	N/A	K-12, O157
	b3460	*livJ*	2.30	leucine/isoleucine/valine transporter subunit	N/A	K-12, O157
	b3528	*dctA*	8.24	C4-dicarboxylic acid, orotate and citrate transporter	N/A	K-12, O157
	b4033	*malF*	2.32	maltose transporter subunit	N/A	K-12, O157
	b4034	*malE*	2.70	maltose transporter subunit	N/A	K-12, O157
	b4035	*malK*	3.20	fused maltose transport subunit, ATP-binding component of ABC superfamily/regulatory protein	N/A	K-12, O157
	b4227	*ytfQ*	2.03	predicted sugar transporter subunit: periplasmic-binding component of ABC superfamily	N/A	K-12, O157
	ECs0413	*fbpC*	4.33	ferric transporter ATP-binding subunit	N/A	K-12, O157
	Z2019	*oppA*	2.04	oligopeptide transport; periplasmic binding protein	N/A	K-12, O157
Hypothetical Proteins	ECs1254	*ECs1254*	-4.92	hypothetical protein	unknown	K-12, O157
	ECs2489	*ECs2489*	2.50	hypothetical protein	unknown	K-12, O157
	L7059	*L7059*	2.21	hypothetical protein	unknown	O157

	L7060	*L7060*	2.48	hypothetical protein	unknown	O157
	Z2820	*yeaD*	2.15	hypothetical protein	unknown	K-12, O157
Metabolism & Other Interesting Genes	b0062	*araA*	10.68	L-arabinose isomerase	L-arabinose degradation I	K-12, O157
	b0063	*araB*	9.37	L-ribulokinase	L-arabinose degradation I	K-12, O157
	b0077	*ilvI*	2.25	acetolactate synthase III, large subunit	leucine, valine and isoleucine biosynthesis	K-12, O157
	b0344	*lacZ*	2.37	□-D-galactosidase	Lactose degradation III	K-12, O157
	b0432	*cyoA*	2.68	cytochrome o ubiquinol oxidase subunit II	Succinate/proline/NADH to cytochrome *bo* oxidase electron transfer	K-12, O157
	b0729	*sucD*	3.33	succinyl-CoA synthetase, NAD(P)-binding, alpha subunit	glycolysis, pyruvate dehydrogenase, TCA and glyoxylate bypass	K-12, O157
	b1008	*rutE*	-2.43	predicted oxidoreductase	Uracil degradation III	K-12, O157
	b2021	*hisC*	3.32	histidinol-phosphate aminotransferase	N/A	K-12, O157
	b2151	*galS*	3.65	DNA-binding transcriptional repressor	N/A	K-12, O157
	b2607	*trmD*	2.13	tRNA (guanine-1-)-methyltransferase	N/A	K-12, O157
	b2704	*srlB*	2.20	glucitol/sorbitol-specific enzyme IIA component of PTS	N/A	K-12, O157
	b2890	*lysS*	2.02	lysine tRNA synthetase, constitutive	tRNA charging	K-12, O157
	b3236	*mdh*	2.57	malate dehydrogenase, NAD(P)-binding	glycolysis, pyruvate dehydrogenase, TCA and glyoxylate bypass	K-12, O157
	b3603	*lldP*	3.01	L-lactate permease	N/A	K-12, O157
	b3734	*atpA*	2.54	F1 sector of membrane-bound ATP synthase, alpha subunit	histidine, purine and pyrimidine biosynthesis; purine/adenosine nucleotides *de novo* biosynthesis II	K-12, O157
	b4014	*aceB*	2.52	malate synthase A	glycolysis, pyruvate dehydrogenase, TCA and glyoxylate bypass	K-12, O157
	b4018	*iclR*	2.34	DNA-binding transcriptional repressor	N/A	K-12, O157
	b4037	*malM*	3.65	maltose regulon periplasmic protein	N/A	K-12, O157
	b4142	*groS*	2.04	Cpn10 chaperonin GroES, small subunit of GroESL	N/A	K-12, O157
	b4153	*frdB*	2.18	fumarate reductase (anaerobic), Fe-S subunit	NADH to fumarate electron	K-12, O157

					transfer; mixed acid fermentation; respiration (anaerobic)	
	b4240	*treB*	3.22	fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component	N/A	K-12, O157
	ECs2014	*acpD*	2.06	azoreductase	N/A	K-12, O157
	Z4240	*gcvP*	2.00	glycine dehydrogenase	Glycine cleavage	K-12, O157
	Z4805	*gntK*	2.08	gluconate kinase 1	D-gluconate degradation	K-12, O157

5.2.2.2 Lettuce Leaf Cell Wall Polysaccharide Condition

In contrast to the dataset of gene expression changes to spinach leaf cell wall polysaccharides, the results obtained after a one hour exposure to lettuce (*L. sativa*) leaf cell wall polysaccharides showed a much higher return (4.8 %) in the number of significantly different expressed genes compared to the vermiculite polysaccharide control.

Many of the genes with differential expression were involved in metabolism (Figure 5-11), similar to the other plant extract conditions tested. The biggest changes within the metabolism GO category were involved in primary and cellular metabolism, macromolecule metabolism and biosynthesis.

Unlike the spinach polysaccharide dataset, within the GO classification "biological process", 15 genes were differentially expressed that were involved in "response to stimulus" (Figure 5-12). Within this category, 11 genes were involved in the "response to stress", including osmotic stress, DNA damage, SOS response and in response to starvation.

Selected genes are displayed in Table 5-6 that are differentially expressed in *Ec*O157 Sakai in response to lettuce polysaccharides. Selected universal stress genes were found to be downregulated overall in the dataset. Of the categories displayed, efflux systems and transporters were seen to have the highest induction/repression compared to the other categories, which is similar to the spinach polysaccharide dataset.

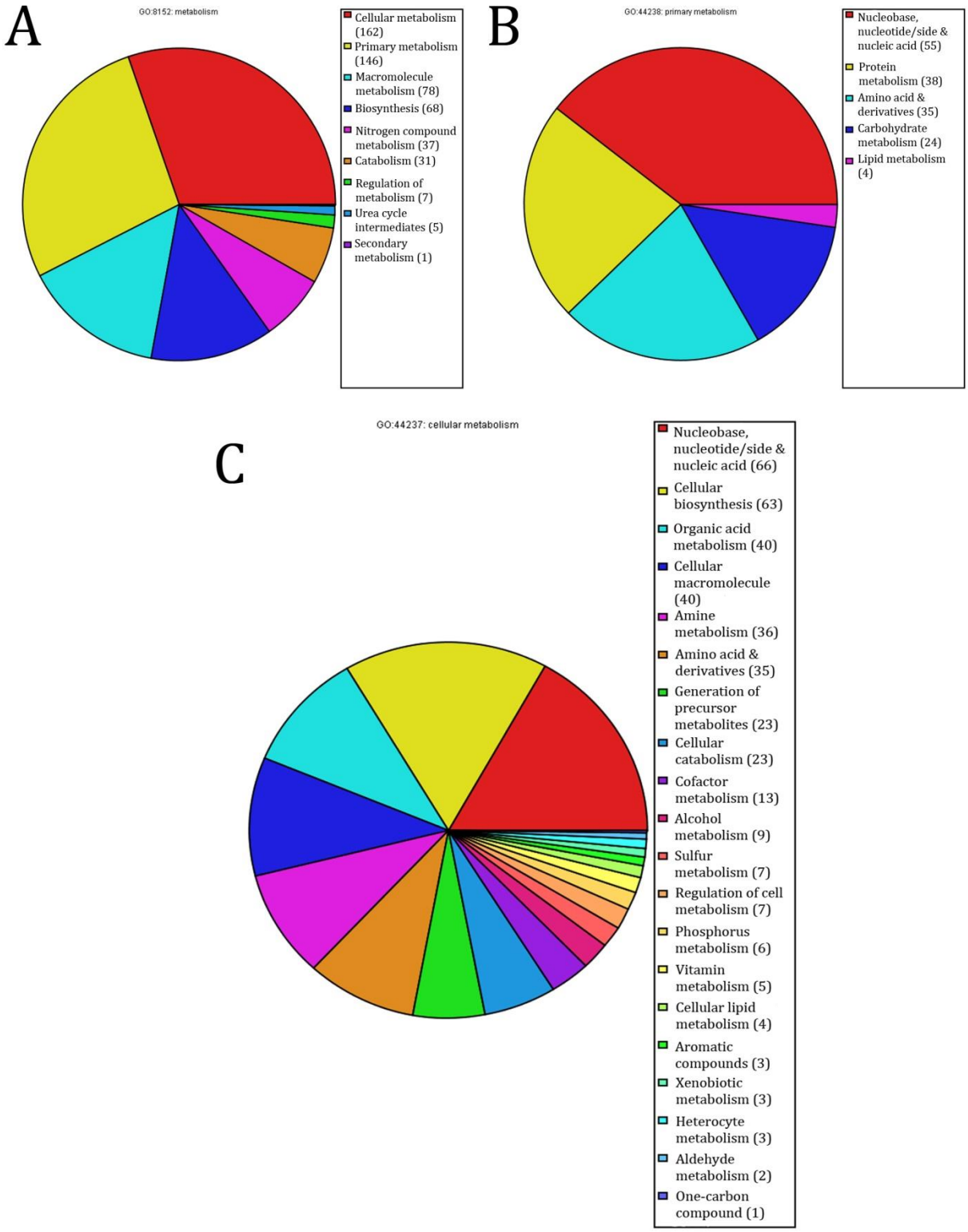


Figure 5-11. Gene ontology maps for genes with significant expression changes in response to lettuce leaf cell wall polysaccharides compared to vermiculite polysaccharides in M9 minimal media.

Gene ontology maps were highlighted for **A)** overall metabolism (535 genes), and then further classified into **B)** primary metabolism (156 genes) and **C)** cellular metabolism (389 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

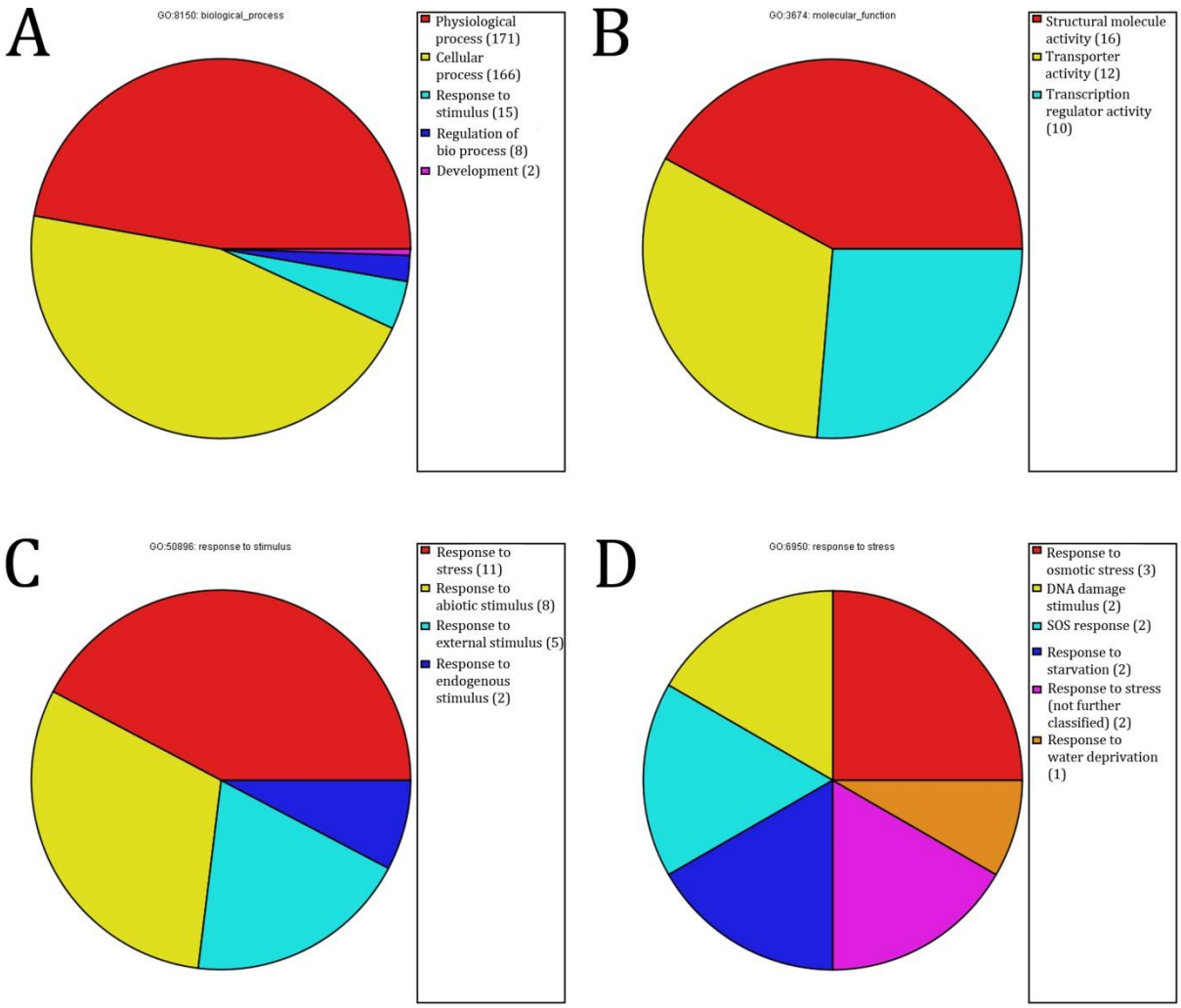


Figure 5-12. Gene ontology maps for genes with significant expression changes in response to lettuce leaf cell wall polysaccharides compared to vermiculite polysaccharides in M9 minimal media.

Gene ontology maps were highlighted for **A)** biological process (362 genes), **B)** molecular function (38 genes), **C)** response to stimulus (26 genes) and **D)** response to stress (12 genes). Genes identified via the gene ontology browser in GX are only those associated with *E. coli* K-12. The number of genes in each category is given in parenthesis.

Many hypothetical genes were differentially expressed, including those that were conserved in *E. coli* K-12 and that were specific to *Ec*O157 species. Finally, as was observed for the spinach dataset, *flhD*, a master regulator of flagella and chemotaxis, was found to be significantly upregulated, although at a much higher induction than in the spinach polysaccharide condition, suggesting flagella induction may be triggered by the presence of plant cell wall material, either for moving towards nutrients or for playing a role in adherence.

Full gene lists of those with significant changes in each of the conditions tested can be found in appendix (Files S-3 to S-8).

Table 5-6. Selected genes with significantly different expression in M9 medium containing lettuce leaf cell wall polysaccharides compared to vermiculite polysaccharides.

Fold change was calculated using the normalised 18°C, M9 + vermiculite polysaccharides dataset as the control; i.e. genes with upregulated gene expression represent genes that are induced in response to lettuce leaf cell wall polysaccharides. Metabolic pathways that each gene is involved in are detailed, with not applicable (N/A) denoted if no metabolic pathways have been identified for a gene. Genes were determined to be conserved (i.e. present in *E. coli* K-12) or only present in selected *E. coli* groups (i.e. O157) using the multiple operon browser in EcoCyc (Keseler *et al.*, 2013). The full list of differentially expressed genes can be found in the appendix (File S-8).

Category	Primary Accession	Gene Name	Fold Change	Description	Metabolic Pathways Involved In	Conserved
Type II Secretion System	L7033	*etpD*	-2.05	type II secretion protein	N/A	K-12, O157 (not EDL933)
Toxins	b2782	*chpA*	2.82	toxin of the ChpA-ChpR toxin-antitoxin system, endoribonuclease	N/A	K-12, O157
	ECs1206	*ECs1206*	-6.16	Shiga toxin 2 subunit B	N/A	O157
Motility, Chemotaxis & Adherence	b1892	*flhD*	44.36	regulator of flagellar biosynthesis, acting on class 2 operons; transcriptional initiation factor	N/A	K-12, O157
Efflux Systems/ Transporters	b3241	*aaeA*	2.55	p-hydroxybenzoic acid efflux system component	N/A	K-12, O157
	b0197	*metQ*	12.40	DL-methionine transporter subunit	N/A	K-12, O157
	b0860	*artJ*	13.77	arginine transporter subunit	N/A	K-12, O157
	b1126	*potA*	10.95	polyamine transporter subunit	N/A	K-12, O157
	b1515	*lsrD*	-63.35	AI2 transporter	N/A	K-12, O157
	b1516	*lsrB*	-29.90	AI2 transporter	N/A	K-12, O157
	b2149	*mglA*	10.87	fused methyl-galactoside transporter subunits of ABC superfamily: ATP-binding components	N/A	K-12, O157
	b2423	*cysW*	14.83	sulfate/thiosulfate transporter subunit	N/A	K-12, O157
	b2796	*sdaC*	7.07	predicted serine transporter	N/A	K-12, O157
	b2943	*galP*	9.95	D-galactose transporter	N/A	K-12, O157
	ECs3296	*ECs3296*	23.64	thiosulfate transporter subunit	N/A	K-12, O157
	Z3608	*fadL*	-8.58	long-chain fatty acid outer membrane transporter	N/A	K-12, O157
Stress	b1376	*uspF*	-26.31	stress-induced protein, ATP-binding protein	N/A	K-12, O157
	b1743	*spy*	-7.89	envelope stress induced periplasmic protein	N/A	K-12, O157
	b3494	*uspB*	-29.32	predicted universal stress (ethanol tolerance) protein B	N/A	K-12, O157
	b3923	*uspD*	-12.16	stress-induced protein	N/A	K-12, O157
Hypothetical Proteins	ECs0988	*ECs0988*	12.06	hypothetical protein	unknown	K-12, O157
	ECs1344	*ECs1344*	2.65	hypothetical protein	unknown	O157

	ECs1503	*ECs1503*	-2.26	hypothetical protein	unknown	Sakai only
	ECs1567	*ECs1567*	-2.04	hypothetical protein	unknown	Sakai only
	ECs1654	*ECs1654*	-71.95	hypothetical protein	unknown	O157
	ECs2473	*ECs2473*	11.65	hypothetical lipoprotein	unknown	O157
	ECs2547	*ECs2547*	-13.06	hypothetical protein	unknown	K-12, O157
	ECs3134	*ECs3134*	-2.06	hypothetical protein	unknown	O157 (not EDL933)
	ECs3521	*ECs3521*	-8.91	hypothetical protein	unknown	K-12, O157
	ECs4324	*ECs4324*	5.20	hypothetical lipoprotein	unknown	O157
	ECs4474	*ECs4474*	-92.51	hypothetical protein	unknown	K-12, O157
	ECs4519	*ECs4519*	6.28	hypothetical protein	unknown	K-12, O157
	ECs4847	*ECs4847*	-10.03	hypothetical protein	unknown	K-12, O157
	ECs4970	*ECs4970*	4.87	hypothetical protein	unknown	Sakai only
	ECs4976	*ECs4976*	7.13	hypothetical protein	unknown	Sakai only
	ECs5109	*ECs5109*	-44.91	hypothetical protein	unknown	K-12, O157
Other Interesting Genes	b0032	*carA*	89.37	carbamoyl phosphate synthetase small subunit, glutamine amidotransferase	Glutamine degradation I; arginine and polyamine biosynthesis; histidine, purine and pyrimidine biosynthesis	K-12, O157
	b1020	*phoH*	-38.02	PhoB-dependent, ATP-binding pho regulon component	N/A	K-12, O157
	b1512	*lsrR*	-21.03	lsr operon transcriptional repressor	N/A	K-12, O157
	b1621	*malX*	8.00	fused maltose and glucose-specific PTS enzymes: IIB component - IIC component	N/A	K-12, O157
	b2890	*lysS*	20.68	lysine tRNA synthetase, constitutive	tRNA charging	K-12, O157
	b3517	*gadA*	-23.01	glutamate decarboxylase A, PLP-dependent	Glutamate dependent acid resistance	K-12, O157
	b3958	*argC*	48.62	N-acetyl-gamma-glutamylphosphate reductase, NAD(P)-binding	arginine and polyamine biosynthesis; ornithine biosynthesis	K-12, O157
	b4376	*osmY*	-36.64	periplasmic protein	N/A	K-12, O157
	ECs3197	*ECs3197*	28.02	colicin V production protein	N/A	K-12, O157
	ECs3311	*ECs3311*	-10.92	ethanolamine ammonia-lyase small subunit	Ethanolamine utilization	K-12, O157

5.2.3 Comparison between array datasets

Overlapping the datasets obtained from the four plant-associated conditions (i.e. *Ec*O157 Sakai in a one hour exposure with spinach leaf lysates, spinach root exudates, and spinach and lettuce leaf cell wall polysaccharides) showed that only six genes were found to be differentially expressed within all four of the plant extract conditions tested (**A**; Figure 5-13). A larger overlap of 158 genes was seen between the spinach root exudates and the two polysaccharide datasets. Thirty-five genes were commonly found in the spinach leaf polysaccharide and the spinach leaf lysates dataset.

Removing the lettuce polysaccharide dataset from the analysis (**B**; Figure 5-13) showed that 13 genes were common to all three of the spinach conditions.

Gene lists for those that are differentially expressed in all four of the conditions and in the spinach extract conditions only can be found in Appendix (File S-9). Different categories of genes were analysed for their expression changes in the temperature condition and the four plant-associated categories, and are detailed in their respective sections in chapter 6 and 7.

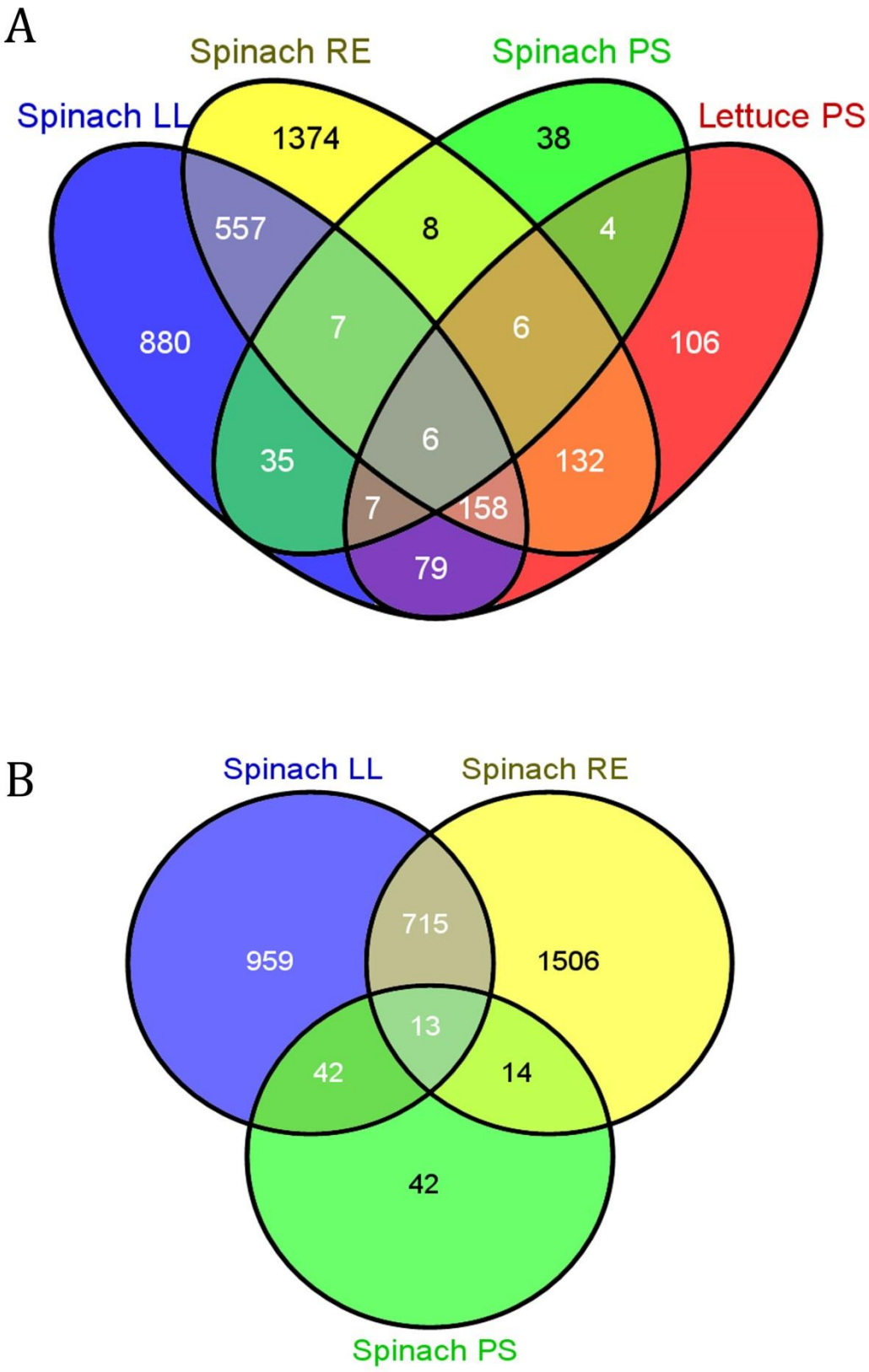


Figure 5-13. Genes with differential expression across the microarray datasets.

A) Comparisons were made of all four of the plant-associated microarray datasets tested: lettuce leaf cell wall polysaccharides (lettuce P/S); spinach leaf cell wall polysaccharides (spinach P/S); spinach leaf lysates (spinach LL); and spinach root exudates (spinach RE). **B)** Comparisons were made between the spinach datasets only to identify any plant-specific genes that may be induced. Venn diagrams were produced using Venny (Oliveros, 2007).

5.2.4 Microarray validation by qPCR

Gene expression from the microarray data was confirmed by qPCR with a selection of optimised primers. Validation was performed using two sets of samples: the first using the "array samples", i.e. the same RNA that was used for the microarray to confirm correct hybridisation and identify any technical errors from the microarray; and the second was carried out using "repeat samples", in which RNA was extracted from new bacterial cultures set up in the one hour exposure to a fresh batch of plant extract/media, so as to confirm the results could be replicated between batches.

qPCR was carried out using primers that had been verified to have efficiency between 95-100 %, similar to that for the 'reference' gene, *gyrB*. This reference gene was validated and selected since it was found to be stably expressed in all five of the conditions (verified by the GeNorm kit (Primer Design, Southampton, UK; section 2.18.4)).

Validation of the plant extract array (containing the conditions for temperature, spinach leaf lysates and spinach root exudates) by qPCR was carried out using a range of primers as detailed in section 2.19.4. A high level of correlation was seen for the temperature condition, between the microarray and qPCR datasets, for both the array samples (r^2 = 0.9994) and the repeat samples (r^2 = 0.9998) (**A; B;** Figure 5-14). A strong, but lower level of correlation was found for the leaf lysates dataset when using the repeat samples (r^2 = 0.89) although the array samples were in a similar range to that of the temperature validation results (r^2 = 0.9851) (**C; D;** Figure 5-14). This difference was not seen for the temperature condition and may be a result of batch variation between the spinach leaf lysates.

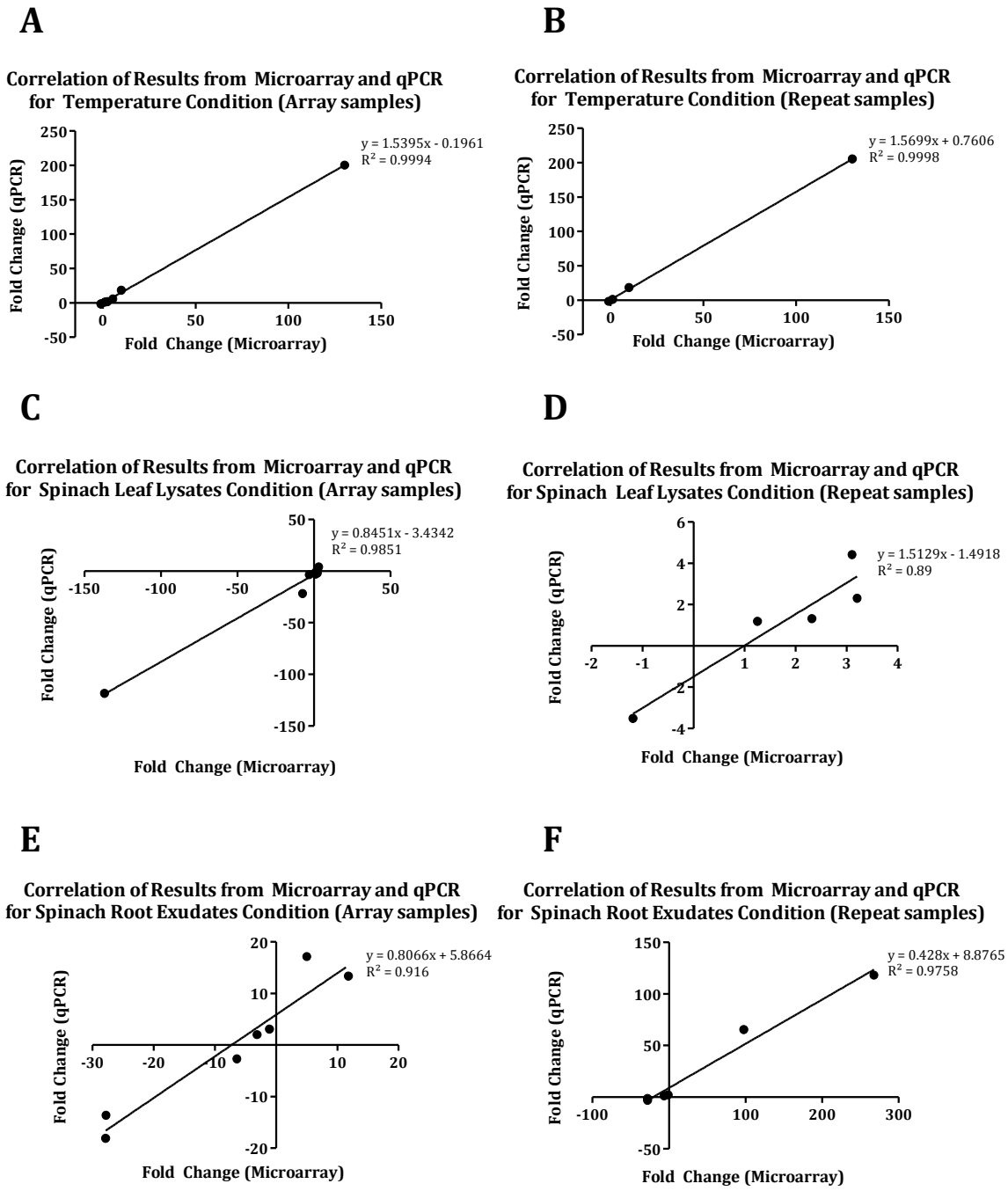


Figure 5-14. Validation scatterplots of gene expression results obtained from microarray versus qPCR for the temperature, leaf lysates and root exudates conditions.

Microarray results were validated by qPCR for sets of genes using the samples used for the microarray (array samples) and using fresh RNA extractions of Sakai in the different media after a one hour exposure (repeat samples). Microarray results were validated for the temperature dataset (**A**; **B**), the spinach leaf lysates dataset (**C**; **D**) and the spinach root exudates dataset (**E**; **F**). Result were analysed by linear regression using GraphPad Prism software (version 5.0).

Linear regression analysis also showed a strong correlation for the spinach root exudates condition between the microarray results and qPCR results from both samples (array samples r^2 = 0916; repeat samples r^2 = 0.9758) (**E; F;** Figure 5-14).

The same validation was carried out for the polysaccharide array for spinach and lettuce leaf cell wall polysaccharides. A high correlation was seen for the spinach polysaccharide for both the array samples (r^2 = 0.9733) and the repeat samples (r^2 = 0.9477) (**A; B;** Figure 5-15). Similarly strong correlation occurred for the lettuce polysaccharide samples, with linear regression analysis giving an r^2 value of 0.9211 for the array samples and 0.9801 for the repeat samples. Individual gene values for the lettuce polysaccharide dataset had lower fold change results in the repeat samples compared to the array samples for the majority of genes tested, although the expression was still significantly different to that of the control. This change could be attributed to batch variation between the plant extracts once more and is a factor to consider for future experimental work in this area.

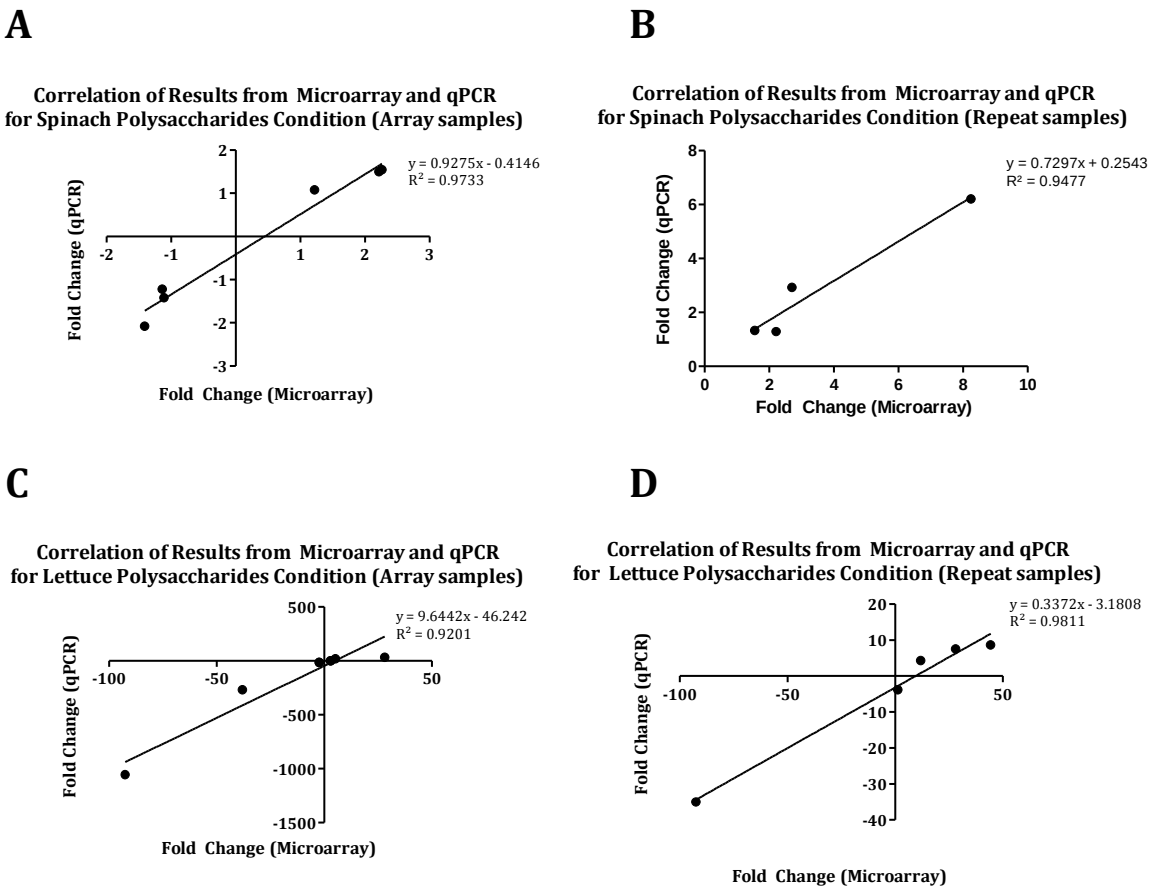


Figure 5-15. Validation scatterplots of gene expression results obtained from microarray versus qPCR for polysaccharide treatments.

Microarray results were validated by qPCR using the samples used for the microarray (array samples) and using fresh RNA extractions of Sakai in the different media after a one hour exposure (repeat samples). Microarray results were validated for the spinach leaf cell wall polysaccharides dataset (**A**; **B**) and the lettuce leaf cell wall polysaccharides dataset (**C**; **D**). Result were analysed by linear regression using GraphPad Prism software (version 5.0).

5.3 Discussion

The transcriptome response of *Ec*O157 Sakai was determined to a number of plant extracts *in lieu* of expression changes *in planta*. Analysis of the effect of different extracts of the plant on *Ec*O157 Sakai genes may give an indication as to which genes and metabolic pathways are essential for surviving in the plant environment. There are some previous reports of transcriptome changes for different strains of *E. coli*, including K-12 and O157:H7 strain EDL933, in the plant environment or in response to certain extracts (Bergholz *et al.*, 2009; Fink *et al.*, 2012; Hou *et al.*, 2012; Hou *et al.*, 2013; Kyle *et al.*, 2010), making comparative analysis between datasets and between strains possible.

From these large datasets generated from the five different conditions, a number of duplicate gene entries were seen. As a commercial Agilent microarray was used for this experiment, probes representing four *E. coli* genomes (*E. coli* K-12; *Ec*O157 Sakai; *Ec*O157 EDL933; *E. coli* CFT 073) were present. This led to a number of replicate hits for the same gene due to the core genome having differential expression. Thus, one caveat of using the commercial microarray is the total number of unique genes with differential expression is lower than that of the total numbers returned.

5.3.1 The importance of culturing bacteria at an appropriate temperature

Differences between the published studies and this one include bacterial strains, plant species and cultivars and plant exposure time. One of the most important and most common factors found in studies looking at human pathogens on plants however is the use of temperature: published studies have cultured the bacterial strains at the optimum growth temperature of 37 °C prior to plant infection, before studying the bacterial-plant interaction at a lower, more environmentally relevant temperature thus inducing a cold-shock response in the bacteria (Fink *et al.*, 2012; Hou *et al.*, 2013; Kyle *et al.*, 2010). Others (Bergholz *et al.*, 2009; Landstorfer *et al.*, 2014) study the interaction at 37 °C, which may be optimal for the bacteria but is not environmentally relevant and would affect the physiology of the plant, including the plant

defence response (Wang *et al.*, 2009). *E. coli* is an adaptable organism that can grow at a wide range of temperatures, making it possible to culture the bacteria at an environmentally/plant-growth relevant temperature such as 18 °C. Culturing at this lower temperature removes any temperature-dependent effects caused by the shift in temperature for the bacterial-plant interaction. This will help to eliminate any misleading gene expression changes that may be induced due to the high temperature or the temperature shift, depending on the experimental setup.

There were a number of significant gene expression changes seen between the two temperature conditions, with one of the most significant changes seen in genes involved in the T3SS. All T3SS genes, whether they were locus of enterocyte and effacement (LEE)-encoded or non-LEE-encoded (*nle*), were found to be downregulated at 18 °C compared to the higher temperature. The T3SS is a virulence factor required for animal infection and colonisation that is known to be induced under a very specific set of conditions (Mills *et al.*, 2013; Savage *et al.*, 2007). In contrast to a previously published study in which the authors have cultured the bacteria at 37 °C prior to exposure to leaf lysates (Kyle *et al.*, 2010), the T3SS was not significantly expressed in the plant extract conditions, perhaps suggesting this upregulation seen in other studies is a carryover effect from culturing at 37 °C – this is discussed further in sections 7.2.1 and 7.3.1.

Genes involved in osmotic shock and membrane stress were found to have expression differences at the lower temperature. The osmotic stress response at the lower temperature ties in with data previously published (Casadei *et al.*, 2002; Mansilla *et al.*, 2004) where it has been demonstrated that a lower temperature affects the composition and fluidity of the bacterial membrane. One gene, *osmY*, was significantly and substantially upregulated at 18 °C. This gene has been implicated as important for *Pseudomonas fluorescens* SBW25, where it was found that increased expression of this gene occurred at 14 °C compared to 28 °C, suggesting this is a temperature-sensitive gene (Knight *et al.*, 2010). As well as this, the authors also demonstrated that a deletion mutant of *osmY* had a significant fitness cost to the bacterium during colonisation of the plant (Knight *et al.*, 2010).

A number of hypothetical proteins also showed differential gene expression at 18 °C compared to 37 °C, with some being induced at the lower temperature. Further study of these hypothetical genes that are upregulated at the lower temperatures may be of use since they could play a role in the adaptation to the environment.

This emphasises the need for not only culturing the bacteria prior to exposure at this lower temperature to the plant environment but for studying the interaction between bacteria and plant at the most environmentally relevant temperature. A shift in temperature would have dramatic effects on the membrane fluidity of the bacterial cell, as shown by the gene expression results in Table 5-2. This sudden change in temperature and thus effects on the membrane will further induce any stress responses that the bacteria may be having already in response to the plant environment. This could in turn alter the response of the bacteria to the condition that is being tested, e.g. leaf lysates, as for Kyle et al, 2010.

5.3.2 Plant extracts of spinach induce large changes in the gene expression of Ec*O157 Sakai*

Inoculation of *Ec*O157 Sakai in spinach leaf lysates induced significant gene expression changes, similar to that seen by Kyle et al, 2010, with 19.85 % gene expression changes in *Ec*O157 EDL933 when incubated with lettuce leaf lysates after a 30 minute exposure. Their study found that many of the flagella genes were upregulated in response to leaf lysates after a one hour exposure; however, microarray analysis of *Ec*O157 Sakai genes in response to spinach leaf lysates in this study found that the flagella genes were downregulated. This difference in expression could be down to differences in the experimental setup as lettuce leaf lysates were used instead of spinach or due to differences in the leaf lysates preparation and content. However it could also be a carryover effect of temperature. The temperature dataset showed that flagella genes were downregulated at the lower temperature: thus, perhaps this upregulation seen in the study by Kyle et al is a carryover effect as a result of culturing the bacteria at 37 °C immediately prior to interaction with the leaf lysates. In fact, a transcriptomic study looking at *Pseudomonas syringae,* cultured at 25 °C prior to infection, on plant bean

Phaseolus vulgaris found that genes involved in flagella synthesis and motility were found to be downregulated in the apoplast, suggesting that these genes are not required within the leaf tissue (Yu *et al.*, 2013). However, they did find that these genes were upregulated on the epiphytic layer of the leaf, which relates to work highlighting the importance of flagella for *E. coli* adherence on the surface of the plant tissue (Rossez *et al.*, 2013). Further study of the expression of flagellar genes in these plant extract conditions and also in infiltrated leaves is discussed in section 7.2.3

Genes in many GO categories were seen to be differentially regulated such as the category of nucleobase, nucleoside, nucleotide and nucleic acid metabolism in response to spinach leaf lysates. This GO category also had differential expression after a 15 minute and 30 minute exposure to romaine lettuce leaf lysates in another study (Kyle et al. 2010), suggesting that this is a common response to this plant environment.

Another category of GO that contained differentially expressed genes was "carbohydrate degradation". As the HPLC analysis of leaf lysates had previously shown large amounts of the monosaccharides present (section 4.2.2), it was anticipated that genes involved in the metabolism and processing of these carbohydrates would be induced in this environment. Large changes in the metabolism of *Ec*O157 Sakai in response to spinach leaf lysates can signify utilisation of plant substrates, as well as a stress response.

As well as the monosaccharide sugars present within the extracts many anti-microbial compounds exuded from the leaves and upon the leaf surface are presumed to be present in the leaf lysates due to the induction of several genes in *Ec*O157 related to antimicrobial stress, such as the *emr* and *acr* efflux systems. This induction of these genes is similar to the expression of antimicrobial stress genes in *Ec*O157 in lettuce leaf lysates (Kyle *et al.*, 2010). These plant-derived compounds could induce stress on the bacterial membrane and hence cell survival, drastically affecting the expression of many genes. It is not known what is being exported from the bacterial cell without further study; however, the *emr* system could be involved in the efflux of phenolics, as demonstrated for *Erwinia chrysanthemi* (*Dicketa dadantii*), which had induced expression of *emrA*, a homolog of *emrA* in *E. coli*, when in the presence of the phenolic acids

salicylic acid and benzoic acid (Ravirala *et al.*, 2007). Therefore, the composition of leaf lysates need to be studied further to test for the presence of phenolics, as upregulation of *emr* genes suggests that some may be present in the spinach leaf lysates tested. However, as *Ec*O157 Sakai was able to utilise the leaf lysates of spinach for growth (section 3.2.2.1), it would be expected that the bacteria would not be under significant stress so as to deter their viability and reproduction.

Many phenolic compounds may also be exuded from the plant roots, such as *trans*-cinnamic acid (*t*-cinnamic acid) as demonstrated by other studies (Lanoue *et al.*, 2010). As with the leaf lysate extracts, many efflux systems were induced when *Ec*O157 Sakai was incubated with spinach root exudates, such as *emr* and *mdt;* however the *acr* genes were downregulated in root exudates. Expression of these efflux pumps suggests that some of the compounds in the leaf lysates may also be present in the root exudates.

In addition, some of the genes found specific to *Ec*O157 Sakai that are associated with plant compounds (section 4.2.1, Table 4-III) were found to be upregulated in root exudates, including *ECs3027*, which is a salicylate hydroxylase. This not only suggests the presence of salicylic acid (SA) hormone and its derivatives but upregulation of this gene may be an example of a human pathogen utilising a plant hormone to its advantage. The presence of SA and its derivatives such as chlorosalicylate and methylsalicylate is to be expected in root exudates, as these hormones are produced in the roots to not only prime the plants for invading organisms, but they also play important roles in plant growth and development (Rivas-San Vicente & Plasencia, 2011).

Since salicylic acid is likely to be detrimental to the survival of *Ec*O157 Sakai in the environment by activating the systemic acquired resistance (SAR) plant defence response, upregulation of this enzyme may help to combat this response. Alternatively, the enzyme may simply be involved in SA degradation for metabolism. Further analysis of the response of *Ec*O157 Sakai to these plant hormones is detailed in section 7.2.2.

Recently published work has demonstrated the response of *E. coli* K-12 and *Ec*O157 EDL933 in response to the lettuce roots and surrounding rhizosphere after a three day incubation period

(Hou *et al.*, 2012; Hou *et al.*, 2013). These data found a 7.6 % change in *E. coli* K-12 gene expression change when exposed to lettuce roots after three days (Hou *et al.*, 2012), whereas *Ec*O157 EDL933 showed a much smaller response of 1.4 % (Hou *et al.*, 2013). It was also demonstrated that *E. coli* K-12 had a higher total colonisation potential on the leaves and roots compared to *Ec*O157 EDL933 (Fink *et al.*, 2012; Hou *et al.*, 2013); however, the internalisation potential of *E. coli* K-12 has been reported as much lower than that of *Ec*O157 (Wright *et al.*, 2013). To date, no study has demonstrated the gene expression changes that are induced for *Ec*O157 Sakai when in the plant environment, including the roots. Thus, although looking at root exudates *in vitro* will have its limitations as it is very different to environmental conditions, this dataset of genes that are induced/repressed after a one-hour exposure to root exudates should help to identify genes that are necessary for this plant-associated *E. coli* O157 isolate, some of which may not be present in *Ec*O157 EDL933 and so may help explain why there was little gene expression changes in the other studies (Hou *et al.*, 2013).

Despite the differences between the studies, there were some overlaps between the datasets, where many genes involved in stress and also selected hypotheticals were upregulated (see section 7.2.5 for further description of these genes). However, a much larger response was seen in the bacterium incubated in root exudates in this study, with a 14.84 % change seen overall. Samples were only incubated for one hour to examine the initial adaptive response, whereas other studies have looked at a longer term exposure time of three days on the roots.

In the root exudates experiment, many genes related to stress were upregulated, which was as expected since they were unable to grow in minimal medium that contained root exudates as their only carbon source (section 3.2.2.1). As shown in section 4.2.2, the root exudates contained very low levels of arabinose, rhamnose, fructose, glucose and sucrose, all within similar ranges to the rockwool media control, presumably explaining why the bacteria were unable to grow. Despite an inability to use root exudates for metabolism, high colonisation levels on the roots of spinach were seen on compost and hydroponically-grown plants (sections 3.2.1.4; 3.2.1.6). Therefore, it is possible that exposure to root exudates primes the bacteria for

root colonisation despite an inability to grow on purified exudates, as previously demonstrated for endophytic rhizosphere strains (Shidore *et al.*, 2012).

Despite the bacteria being unable to use spinach root exudates for growth, many sugar transporters and some metabolic pathways were seen to be upregulated after a one hour exposure, such as for arabinose and xylose transport and processing, both of which are plant-specific carbohydrates. A further look of the interaction of *Ec*O157 Sakai with arabinose and the role of this in plant colonisation is detailed in section 6.2.6.

Finally, many motility genes were downregulated, with no clear chemotaxis response seen from the gene expression data, which would explain why chemotaxis was not observed in the assays carried out in section 3.2.2.2. Genes encoding for curli were seen to be induced in root exudates however, suggesting curli are important for interactions with the roots. The importance of curli has already been demonstrated for the leaves (Fink *et al.*, 2012; Macarisin *et al.*, 2012) so perhaps it plays a similar role in association with the roots.

This inability to grow in the root exudates may explain the inverse pattern of gene expression seen in the heatmap for expression changes in root exudates versus leaf lysates. As *Ec*O157 Sakai is able to grow in one condition but not the other this will cause differential expression of genes involved in many aspects of cell physiology and growth, and thus explains why the majority of genes in the leaf lysates condition are found to be downregulated in the root exudate condition.

5.3.3 Differences are seen in the response of Ec*O157 Sakai to leaf cell wall polysaccharide extracts of spinach and lettuce*

As the leaf lysates provided a large overview of the response to leaf tissue, it was decided to narrow the interaction further by studying the reaction of *Ec*O157 Sakai to leaf cell wall polysaccharides after a one hour exposure, because the cell wall is most likely the first 'point of contact' in the interaction. Both spinach and lettuce (*L. sativa*) were tested to make a comparison between extracts (polysaccharides versus lysates) and between plant species.

An important note is that the polysaccharide preparation resulted in an insoluble fraction, which meant it was not possible to obtain an accurate growth curve via optical density or plating counts. Further solubilisation of the polysaccharide extracts into the hemicellulose and pectin components could be performed to analyse the growth of *Ec*O157 Sakai into these different fractions in a future study. However, for this purpose the complete cell wall material was of interest. As a result, it is only by looking at the gene expression data that the growth potential of the bacteria can be determined. As a one hour time point was selected, this was less than one generation time in the minimal medium; thus gene expression changes indicated the initial response of the bacteria and were not masked by gene expression changes of actively dividing cells.

A small change of 1.5 % of the total gene expression was seen in the spinach polysaccharide samples compared to the vermiculite polysaccharide control. As *Ec*O157 Sakai has been implicated in an outbreak with spinach (CDC, 2006) it was hypothesised that this bacterial strain may be well adapted to this plant. From looking at the GO categories and the gene expression changes for this dataset no response to stress was seen, suggesting that the presence of spinach cell wall polysaccharides does not have a detrimental effect on the bacteria. Only eight genes were downregulated, with the majority of upregulated genes involved in the utilisation and transport of different sugars, such as arabinose, which was present in high levels in the spinach leaf cell wall polysaccharides using HPLC analysis (section 4.2.3). As well as this, genes involved in the regulation of flagella and curli were upregulated in spinach leaf cell wall polysaccharides, although no genes in flagellar assembly were changed in expression. As flagella and curli have been suggested to play an important role in adherence to leaf tissue (Macarisin *et al.*, 2012; Rossez *et al.*, 2013; Saldaña *et al.*, 2011), it was thought that these leaf cell wall polysaccharides may induce upregulation of certain adherence factors. No adherence factors were seen to be upregulated in the spinach dataset, suggesting that these extracts do not invoke an adherence phenotype in *Ec*O157 Sakai, unless one of the hypothetical proteins seen to be induced is found to play a role in adherence. Alternatively, this approach of examining the total transcriptome

effectively 'averages' gene expression in a population and may mask differential expression that occurs on a single cell basis.

A larger change in the total gene expression was seen for *Ec*O157 Sakai incubated with lettuce leaf cell wall polysaccharides, accounting for 4.5 % of the total. This is similar to other published work looking at the exposure of *Fusarium graminearum* to plant polysaccharides, in which 5% of the total gene expression changed (Carapito *et al.*, 2013). The lettuce leaf cell wall polysaccharides had lower concentrations of most of the monosaccharides tested for than the spinach polysaccharide extracts (section 4.2.3), so perhaps this difference in concentration would account for some of the differences between these datasets. Most importantly, only a selected few monosaccharides were tested for by HPLC analysis, so there is still doubt about much of the content within these two extracts as there could be many other monosaccharides to be accounted for. Further analysis by GC-MS could also help to define the nature of the bonds found in certain compounds, which would indicate if they are suitable for use by the bacteria. As all polysaccharide extractions and experimental setups were carried out concurrently it is hypothesised that differences between the datasets are due to differences within the extracts. Further to this, stress-related genes were seen to be downregulated in the lettuce dataset, suggesting that the difference in datasets is not due to a stress response from the lettuce extracts.

5.3.4 Validation of and comparisons between microarray datasets

Before any gene expression analysis could be studied further the results from the microarray for all five conditions were validated by qPCR. For all of the conditions tested, qPCR results were found to be of a similar range to the microarray for both array and repeat samples. Any differences in the level of expression seen were thought to be down to differences in the sensitivity of the techniques and batch variation between the plant extracts. As a result, the data from the microarray was verified and could be analysed in further detail.

By analysing the overlapping genes that are differentially expressed between datasets it was predicted that genes important for the plant environment would be identified. However,

comparative analysis of all 4 plant-associated datasets yielded few genes, within which the genes were found to be differentially expressed between conditions. It was observed that some categories of genes were found to be upregulated in one condition but down in another: for example flagella gene expression, which was induced in response to spinach and lettuce polysaccharides, but repressed in spinach leaf lysates and root exudates. Thus each condition may need to be considered separately due to the different environments and stresses that each of them provides.

Due to the contrasting natures of the different extracts, it was clear that it was more beneficial to study the datasets separately and check for overlaps within different categories of genes. As well as this, any genes that were identified as potential factors that were important for the plant environment, whether through the use of plant polysaccharides or survival in plant root exudates, would need to be validated *in planta* to confirm their role, avoiding the need for relying solely on transcriptomic analysis, in which any post-translational modifications are not seen.

Finally, analysis of the metabolic pathways that are induced within and between datasets, as well as between other published studies give an indication of not only how the bacteria are responding to different plant extracts, but also what plant components are being utilised for the bacterial survival.

6 Metabolic & Regulatory Changes in *Ec*O157 Sakai in Response to Plant Extracts

6.1 Introduction

6.1.1 The Importance of Studying Metabolism Changes in Plant-Bacteria Interactions

Transcriptomic changes occurring in human pathogens when exposed to the plant environment help to identify genetic elements that are important for adaptation to plants. Many transcriptomic studies have been carried out on human pathogens, such as *Salmonella enterica* subsp. *enterica* and *Escherichia coli*, and plant-associated bacteria within the plant environment, to both living plants and plant extracts. The importance of studying the metabolic networks between plants and associated phytopathogens to combat plant disease has already been well documented and is a focus of much research (Duan *et al.*, 2013; Karpinets *et al.*, 2014; van de Mortel *et al.*, 2012).

However, when looking at human pathogens on plants much of the focus has looked at the adherence of these bacteria to plant cells and the persistence of these microbes within plant tissue (Berger *et al.*, 2009; Gagliardi & Karns, 2002; Patel & Sharma, 2010; Shaw *et al.*, 2008; Shaw *et al.*, 2011). Only a few studies have analysed the metabolic changes of the bacteria within the plant environment; for example, one study found *Salmonella* had differential expression within many areas of its cellular metabolism and the expression of the gene *fadH* (fatty acid oxidation complex α subunit) was influenced by many of the different metabolites present in tomato fruits (Noel *et al.*, 2010a). It is important to understand the metabolic changes occurring within the bacteria to different plant components as it highlights what the bacteria may be utilising to persist within the plant environment.

Unlike some plant pathogens, there is no experimental evidence of *E. coli* and *Salmonella* containing any plant cell wall degrading enzymes and therefore it is predicted that these bacteria would need to utilise any simple sugars that are available within the plant apoplast and

the rhizosphere. However, as mentioned previously, co-colonisation with plant-associated bacteria can increase the prevalence of human pathogens within plants, whether through the formation of a biofilm or by degradation of plant material via a combination of their metabolic networks (Teplitski *et al.*, 2011). Work described here made use of filter-sterilised plant extracts in minimal medium, to identify whether any metabolic pathways induced are a direct response of the *Ec*O157 to plant extracts and not influenced by other competing microbial species. Therefore analysis of the metabolic pathways that are induced under these conditions will help to understand the bacterial response and show the differential expression that occurs without the need for degradation of plant components by other microbes.

6.1.2 Aims of this Area of Study

A. To identify metabolic pathways that are upregulated in response to each of the plant extracts used in this study.

B. To identify regulatory changes that are induced in *Ec*O157 Sakai in response to each of the plant extracts.

C. To identify pathways/genes that may involve the utilisation of plant compounds for the growth and survival of *Ec*O157 Sakai.

The overall aim of this area of study was to identify the metabolic and regulatory changes that occur in *Ec*O157 Sakai in response to each of the plant extract conditions tested: spinach leaf lysates; spinach root exudates; spinach leaf cell wall polysaccharides; and lettuce leaf cell wall polysaccharides. Furthermore, a second aim was to identify any metabolic pathways induced in these conditions that involved the utilisation of plant metabolites, which may be important for the proliferation and survival of *Ec*O157 Sakai in that environment.

6.2 Results

6.2.1 Metabolic Changes in Ec*O157 Sakai in Response to Plant Extracts*

6.2.1.1 Changes in the core metabolism pathways

The transcriptomic datasets discussed in Chapter 5 were imported into the EcoCyc cellular overview browser to determine the metabolic pathways that were differentially expressed when in the presence of plant extracts. The results of each dataset were mapped onto a representation of the *E. coli* cell to show changes across the different areas of the cell. Full lists of the metabolic pathways can be found in the appendix when mapped onto *E. coli* K-12 and *Ec*O157 Sakai: Folder S-9 for the temperature dataset; Folder S-10 for spinach leaf lysates; Folder S-11 for spinach root exudates; Folder S-12 for spinach polysaccharides; and Folder S-13 for lettuce polysaccharides.

Gene expression analysis of the response of *Ec*O157 Sakai to spinach leaf lysates in M9 minimal medium after a one hour exposure revealed a large change in the transcriptome of this bacterial strain (section 5.2.1.2). Significant gene expression changes were seen to occur across the entire cell (Figure 6-1), with many of the core metabolism processes affected, such as amino acid biosynthesis, which was mostly downregulated. As the comparator for the microarray analysis *Ec*O157 Sakai was cultured in M9 medium supplemented with 0.2% glycerol. *Ec*O157 Sakai was previously shown to grow in spinach leaf lysates extract (section 3.2.2.1); it is likely that downregulation of these important core metabolism processes is merely a result of differences in growth rate: faster under M9 + 0.2 % glycerol conditions. As well as the core metabolism, it is apparent from the cellular overview that many of the genes with differential expression were found to encode for proteins involved at the bacterial membranes or periplasmic space (Figure 6-1). Many of these proteins are involved in transport systems, including both importer and efflux systems of a variety of compounds, which is evident from the gene expression data in section 5.2.1.2.

Similarly, large changes in the overall gene expression occurred when *Ec*O157 Sakai was exposed to spinach root exudates (section 5.2.1.3). As the bacteria were unable to utilise root exudates as the sole carbon source for growth (section 3.2.2.1), due to the lack of accessible carbon (section 4.2.2), the transcriptomic data was mapped onto the metabolic pathway analysis to determine if any genes related to metabolism were induced, despite the lack of growth.

The majority of genes displayed on the cellular overview are downregulated (depicted as blue on the diagram) (Figure 6-2). This was as expected due to the lack of growth; however some pathways were highly induced in the root exudates, including certain enzymes (as seen by the purple, pink and red boxes on the diagram) involved in the metabolism categories fatty acid degradation, detoxification, amino acid degradation, aldehyde degradation and carbohydrate degradation. Both up and downregulation was found for the transporters (visualised on the edge of the diagram to represent the bacterial membranes and the periplasmic space), with some being highly induced in the presence of root exudates.

As large changes were seen across the cell in response to the whole leaf extracts, I decided to focus on the response to certain components of the plant by studying the interaction of *Ec*O157 and leaf cell wall polysaccharides. The preparation of the plant cell wall polysaccharides excluded any plant intracellular components and apoplastic fluid, resulting in an interaction with a specific component of the plant cell wall. This extraction represented the tissue type that is likely to be the first encountered by *E. coli* in the leaves. The response to spinach and lettuce leaf cell wall polysaccharides was studied to determine any differences between the two species.

The bacterial response to spinach leaf cell wall polysaccharides resulted in a small number of differentially regulated genes, when compared to a vermiculite (the plant medium used for growth in this study) polysaccharide control, the majority of which were induced. Categories of metabolic pathways that were induced included those involved in amino acid biosynthesis and degradation, fermentation, the TCA cycle and carbohydrate degradation (Figure 6-3). Many transporters were also differentially regulated: for example two of the arabinose transporters, *araF* and *araG*, were upregulated, which ties in to the utilisation of arabinose, discussed further in section 6.2.2.1.

In comparison to the response to spinach polysaccharide, a much larger proportion of genes were differentially expressed in *Ec*O157 Sakai when incubated with lettuce leaf cell wall polysaccharides. Cellular overview analysis revealed several metabolic pathways that were upregulated in the lettuce polysaccharide dataset were involved in nucleosides and nucleotides biosynthesis, amino acid degradation and fatty acids and lipids biosynthesis (Figure 6-4). These metabolic categories contained genes with the highest fold change difference from the control, as well as a few selected transporters. Cellular processes across the entire bacterial cell were differentially regulated, suggesting that the lettuce polysaccharides induced a stronger change in gene expression in *Ec*O157 Sakai compared to the spinach polysaccharides.

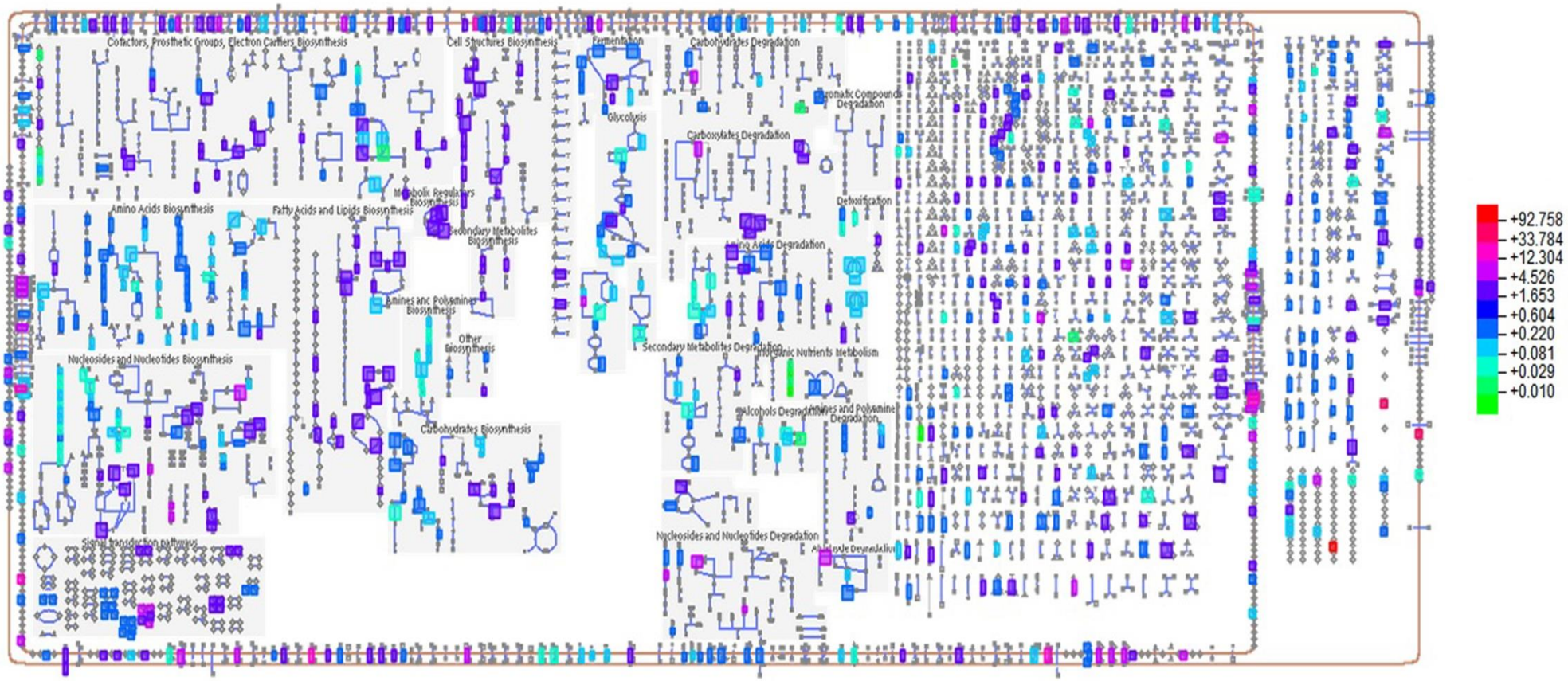


Figure 6-1. Cellular overview of metabolic pathways significantly differentially expressed in minimal medium supplemented with spinach leaf lysates compared to 0.2 % glycerol.

Transcriptomic data for the leaf lysates dataset was mapped onto the cellular metabolic map profile for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013). Each enzyme that was differentially expressed is represented by a coloured box to show the level of expression. Data was normalised to 1, therefore values >1 are upregulated expression and <1 are downregulated expression.

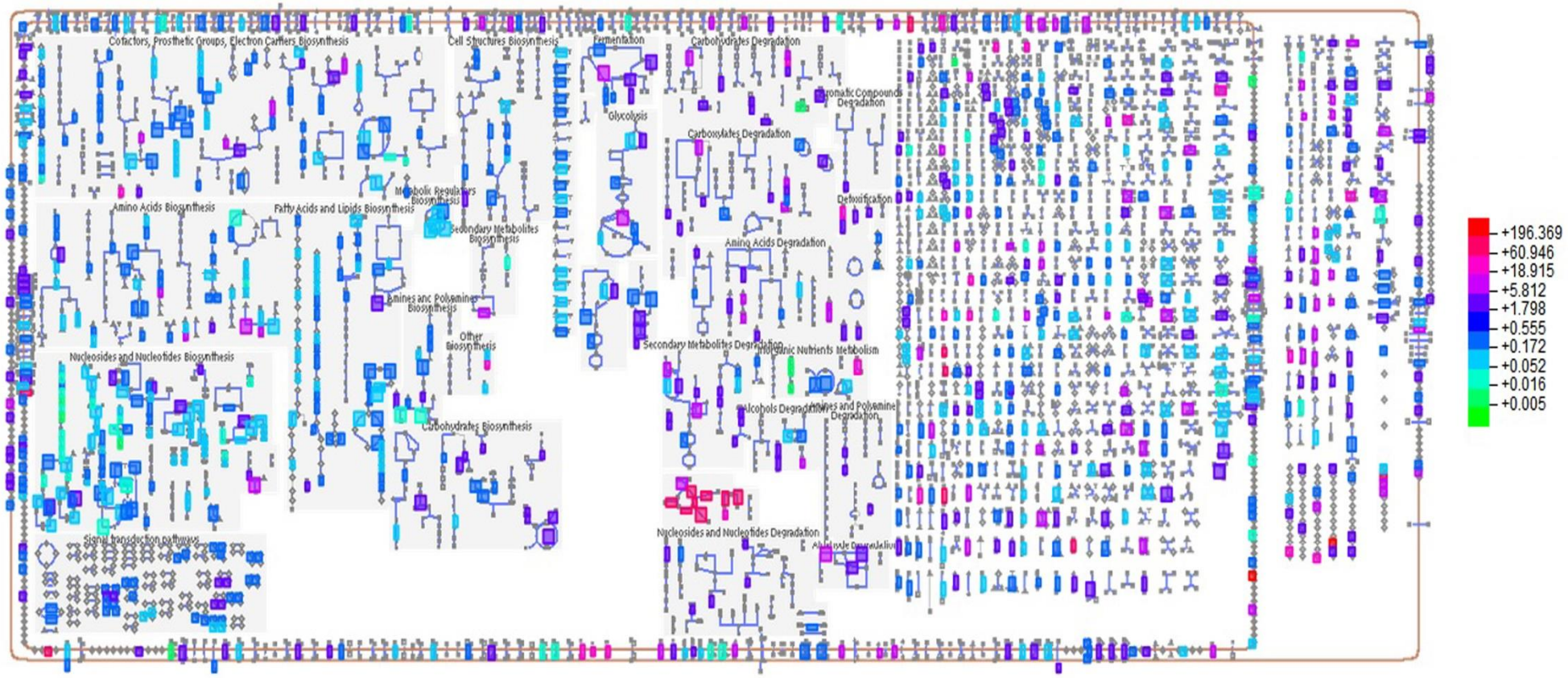


Figure 6-2. Cellular overview of metabolic pathways significantly differentially expressed in minimal medium supplemented with spinach root exudates compared to 0.2 % glycerol.

Transcriptomic data for the root exudates dataset was mapped onto the cellular metabolic map profile for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013). Each enzyme that was differentially expressed is represented by a coloured box to show the level of expression. Data was normalised to 1, therefore values >1 are upregulated expression and <1 are downregulated expression.

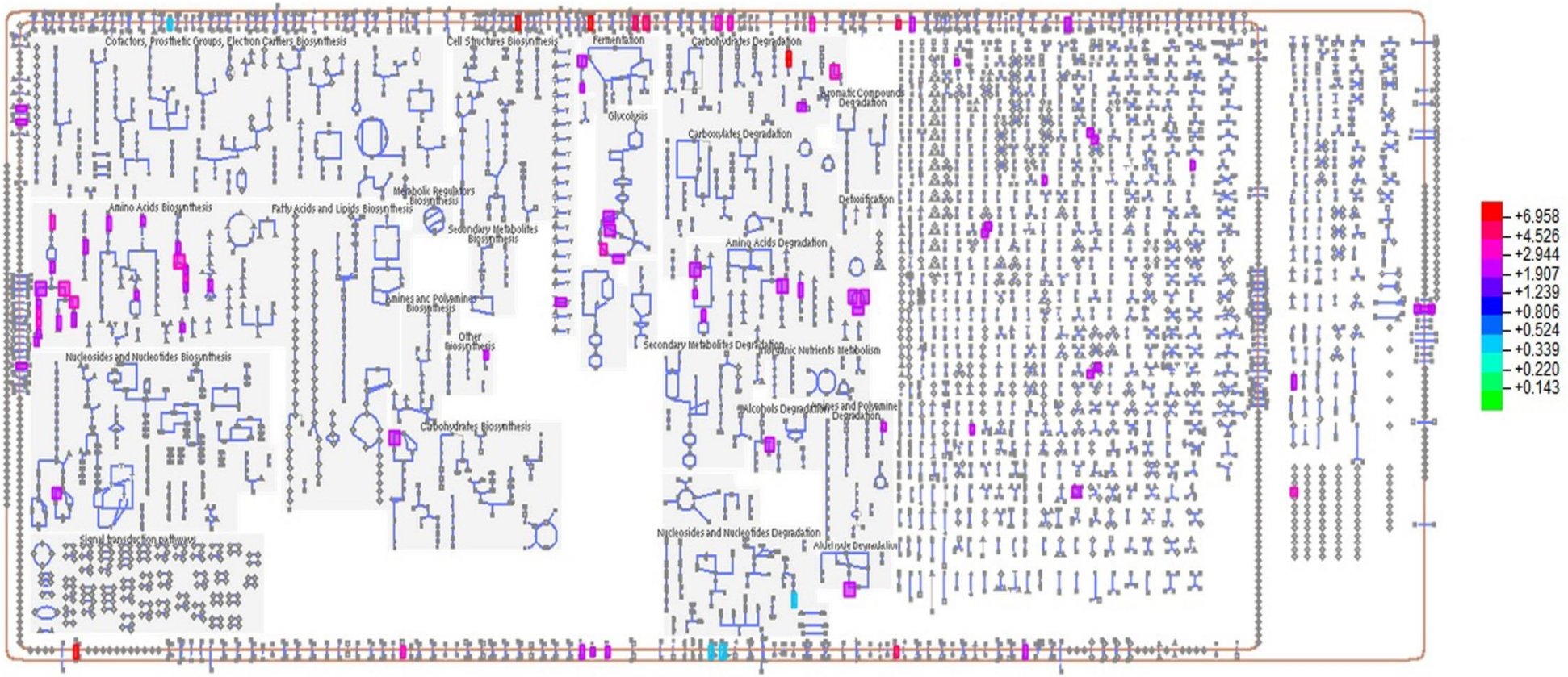


Figure 6-3. . Cellular overview of metabolic pathways significantly differentially expressed in minimal medium supplemented with spinach leaf cell wall polysaccharides compared to a vermiculite polysaccharide control.

Transcriptomic data for the spinach polysaccharides dataset was mapped onto the cellular metabolic map profile for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013). Each enzyme that was differentially expressed is represented by a coloured box to show the level of expression. . Data was normalised to 1, therefore values >1 are upregulated expression and <1 are downregulated expression.

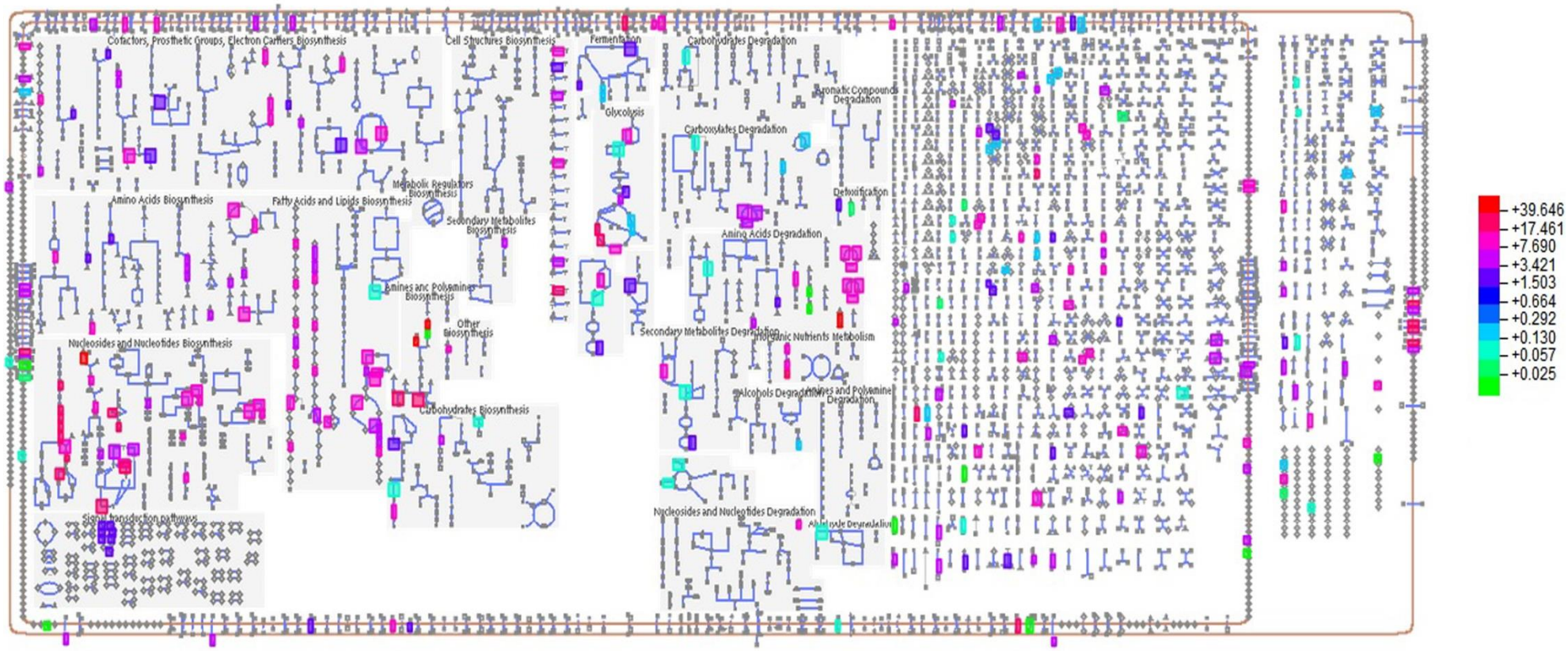


Figure 6-4. Cellular overview of metabolic pathways significantly differentially expressed in minimal medium supplemented with lettuce leaf cell wall polysaccharides compared to a vermiculite polysaccharide control.

Transcriptomic data for the lettuce polysaccharides dataset was mapped onto the cellular metabolic map profile for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013). Each enzyme that was differentially expressed is represented by a coloured box to show the level of expression. . Data was normalised to 1, therefore values >1 are upregulated expression and <1 are downregulated expression.

As well as the metabolic pathway analysis, an overview of the regulation processes that were differentially expressed helps to give an understanding of the overall response of the bacteria to the different plant extracts. As expected based on the metabolic pathway analysis, large changes were seen in the regulome of *Ec*O157 Sakai after a one hour exposure to the different plant extracts, with the largest changes seen in response to spinach leaf lysates (Figure 6-5) and spinach root exudates (Figure 6-6). The hierarchy of the regulators is depicted by the different ellipses, with the most central ellipse representing regulators at the highest level of hierarchy, i.e. with many regulatees.

For the leaf lysates dataset some of the regulators in the highest level of hierarchy were differentially expressed (Figure 6-5). This included the transcription factors *flhC* and *flhD*, which together form the principal regulator of flagellum biosynthesis and motility. These transcription factors were both downregulated after one hour, hence explaining the down regulation of many of the motility genes (section 5.2.1.2). *crp* is another regulator in the top level of the hierarchy that was differentially expressed. This regulator is known to affect the expression of 180 genes that code for a variety of processes, including those involved in catabolism of secondary carbon sources, biofilm formation, virulence, nitrogen assimilation, iron uptake, multidrug resistance and the stringent response (Zheng *et al.*, 2004). Another master regulator that was upregulated in spinach leaf lysates compared to a 0.2% glycerol control was *fis*, which affects the transcription of approximately 21% of the genome (Cho *et al.*, 2008), including those involved in translation, virulence, stress and biofilm formation. Furthermore, Fis is normally a marker for exponential growth since its expression is growth phase-specific (Bradley *et al.*, 2007). Many of the regulators with the highest difference in expression compared to the control were seen on the outside of the ellipses, representing genes that have no direct regulatees. Many of these genes will be affected by *fis, flhDC,* and *crp*, although some will be triggered by other specialised factors.

Mixed expression was also seen in the regulatory pathways for root exudates (Figure 6-6), much more so than for leaf lysates dataset. Presumably these strong differences between the datasets for the regulation of the bacteria are due to the growth differences in the two extracts. There

were some similarities however, with downregulation of *flhC* and *flhD*, the master regulators for flagellar biosynthesis and motility, in both spinach root exudates and leaf lysates. Another set of regulators that affects the expression of a large number of genes that were induced in root exudates was *ihfA* and *ihfB* (integration host factor). These genes are responsible for maintaining the structure of DNA, by playing a role in DNA supercoiling and duplex destabilisation (Dhavan *et al.*, 2002).

In contrast to the leaf lysates and root exudates datasets, regulatory analysis revealed that only one gene in the highest level of regulation was induced in the presence of spinach leaf cell wall polysaccharides: *flhD*. *FlhD* regulates the expression of 80 genes, most of which are involved in flagellar biosynthesis and motility; however, it does this by forming a complex with *flhC*. As can be seen from the regulatory analysis (Figure 6-7), *flhC* is not induced in this condition. As well as this, of the 80 genes that are regulated by *flhDC* only a small number are seen to have differential expression (as verified using EcoCyc's regulatory analysis online viewer), which could be induced via another regulator/mechanism. Therefore, it is possible that this apparent expression of *flhD* is perhaps a result of cross-hybridisation on the microarray.

The second level of regulation (the middle ellipse on the diagram) contains three genes with differential expression: *galS*, *iclR* and *lldR*. *GalS* expression is induced by β-D-galactose, the polymer of which, galactan, is found in the hemicellulose component of plant cell walls. This gene regulates the expression of ten genes that are involved in galactose and maltose degradation and transport. The transcriptional repressor IclR (isocitrate lyase regulator) is involved in regulating the expression of the glyoxylate bypass genes (Sunnarborg *et al.*, 1990). Finally, the lactate regulator *lldR* has two roles: it is induced upon biofilm formation, and a mutant in *lldR* has been shown to have impaired biofilm formation (Beloin *et al.*, 2004); and it is responsible for regulating genes involved in the degradation and utilisation of L-lactate (Aguilera *et al.*, 2008).

Thus, as can be seen from both the metabolic pathway analysis and the regulatory analysis there were small changes in the transcriptome that occurred in *Ec*O157 Sakai after a one hour exposure to these spinach polysaccharide extracts. The metabolic pathway with the highest

induction was L-arabinose degradation I. The utilisation of arabinose in the plant extract conditions is discussed in further detail in section 6.2.6.

Finally, only two genes were differentially regulated in *Ec*O157 Sakai in the presence lettuce leaf cell wall polysaccharides that are at the highest level of regulation: *flhD* and *lrp* (Figure 6-8). As mentioned previously, *flhD* creates a complex with *flhC* to affect the transcription of a large number of genes. *FlhC* fold change did not pass the stringency tests significance level in this dataset; however, many of the targets of *FlhDC* were differentially expressed. *Lrp* (Leucine-responsive regulatory protein) regulates the transcription of 103 genes (Tani *et al.*, 2002), which code for processes involved in nutrient transport, pili synthesis, one-carbon metabolism and amino acid biosynthesis (Brinkman *et al.*, 2003).

Many of the regulators that were differentially expressed at the lower level of regulation were related to ribosomal protein synthesis and processing, such as *deaD*. Overall however, most of the gene expression changes were seen in those which have no direct regulatees, represented as the outer layer of the circle in Figure 6-8.

Therefore, for all four conditions large changes were seen in the metabolic pathways and in the regulatory network of *Ec*O157 Sakai. Individual metabolic pathways that were of interest were examined further for each condition.

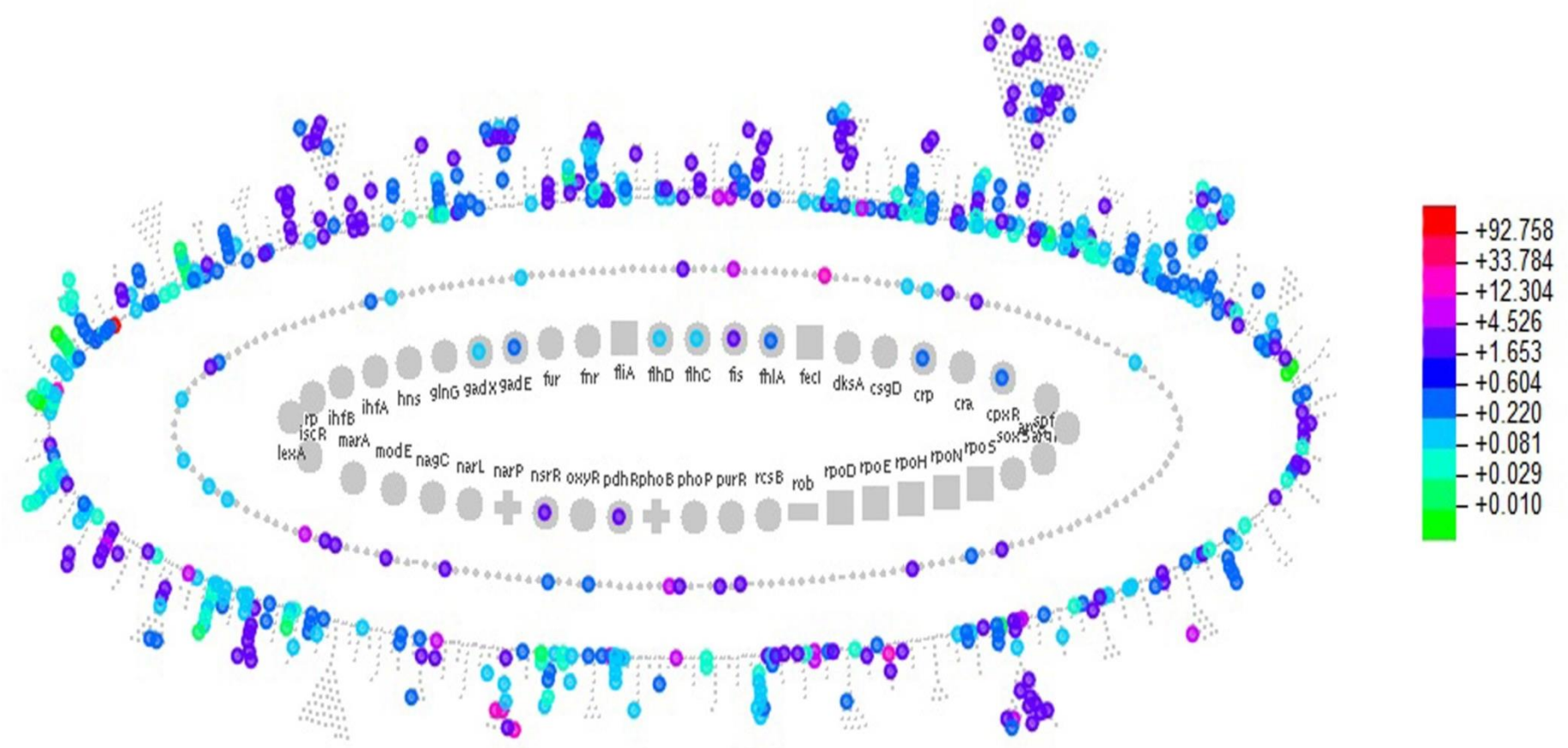


Figure 6-5. Regulatory map overview showing significantly differentially expressed regulators to spinach leaf lysates compared to 0.2 % glycerol.

Transcriptomic data for the spinach leaf lysates dataset was mapped onto the regulatory overview for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013), which represents known regulators (depicted in grey). Each coloured circle represents a regulator that is differentially expressed in response to spinach root exudates. The inner ellipse depicts master regulators and sigma factors; the outer ring represents genes that are regulatees only; the middle ring represents all remaining regulatory genes. Shapes in the inner circle depict the type of regulator: a plus sign for a regulator that has activators only; a minus sign for a regulator that has inhibitors only; a circle for a regulator that has activators and inhibitors; and a square for a gene that encodes a sigma factor. Data was normalised to 1; therefore values >1 are genes that are upregulated and <1 are genes that are downregulated.

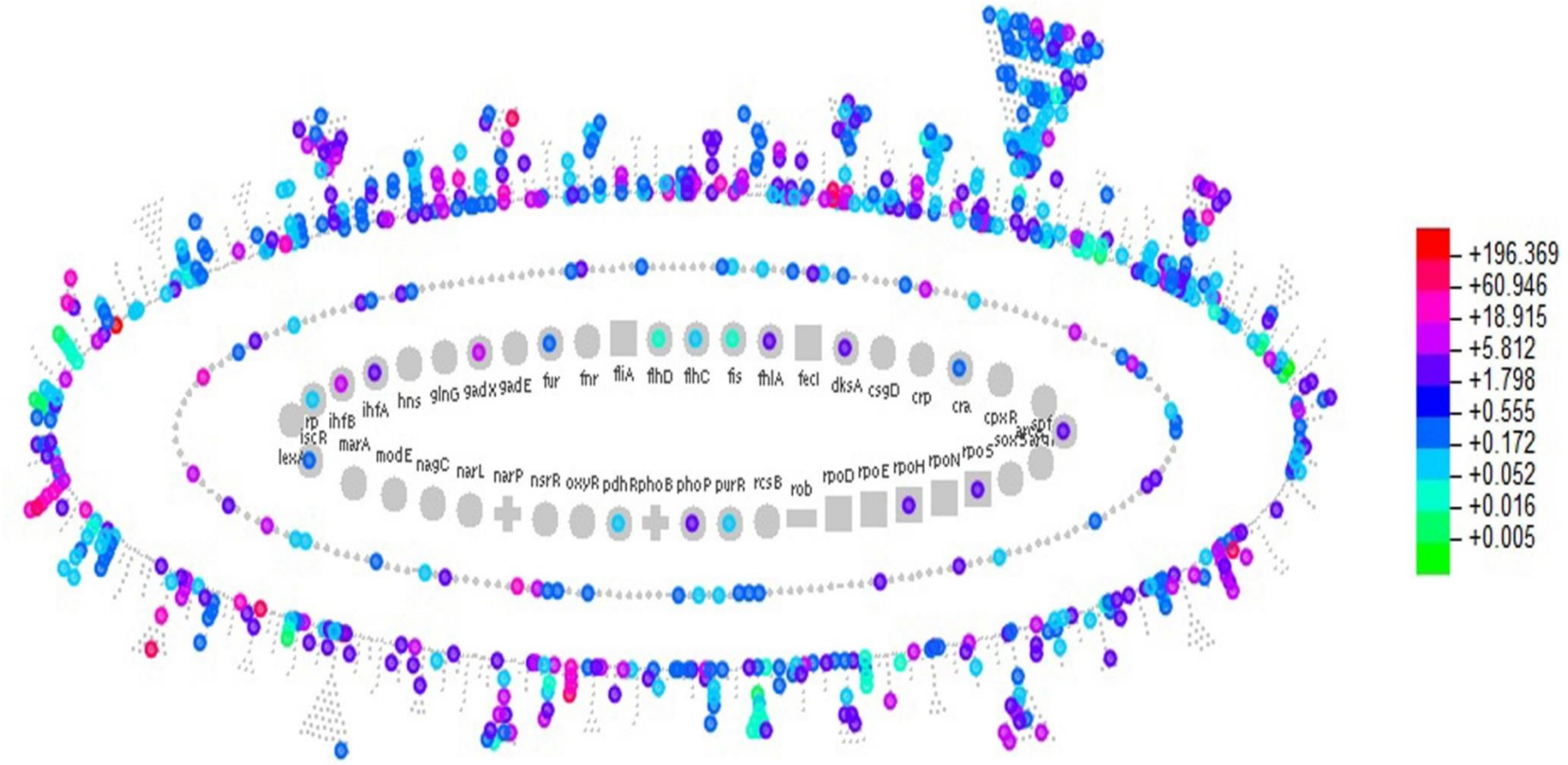


Figure 6-6. Regulatory map overview showing significantly differentially expressed regulators to spinach root exudates compared to 0.2 % glycerol.

Transcriptomic data for the spinach root exudate dataset was mapped onto the regulatory overview for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013), which represents known regulators (depicted in grey). Each coloured circle represents a regulator that is differentially expressed in response to spinach root exudates. The inner ellipse depicts master regulators and sigma factors; the outer ring represents genes that are regulatees only; the middle ring represents all remaining regulatory genes. Shapes in the inner circle depict the type of regulator: a plus sign for a regulator that has activators only; a minus sign for a regulator that has inhibitors only; a circle for a regulator that has activators and inhibitors; and a square for a gene that encodes a sigma factor. Data was normalised to 1; therefore values >1 are genes that are upregulated and <1 are genes that are downregulated.

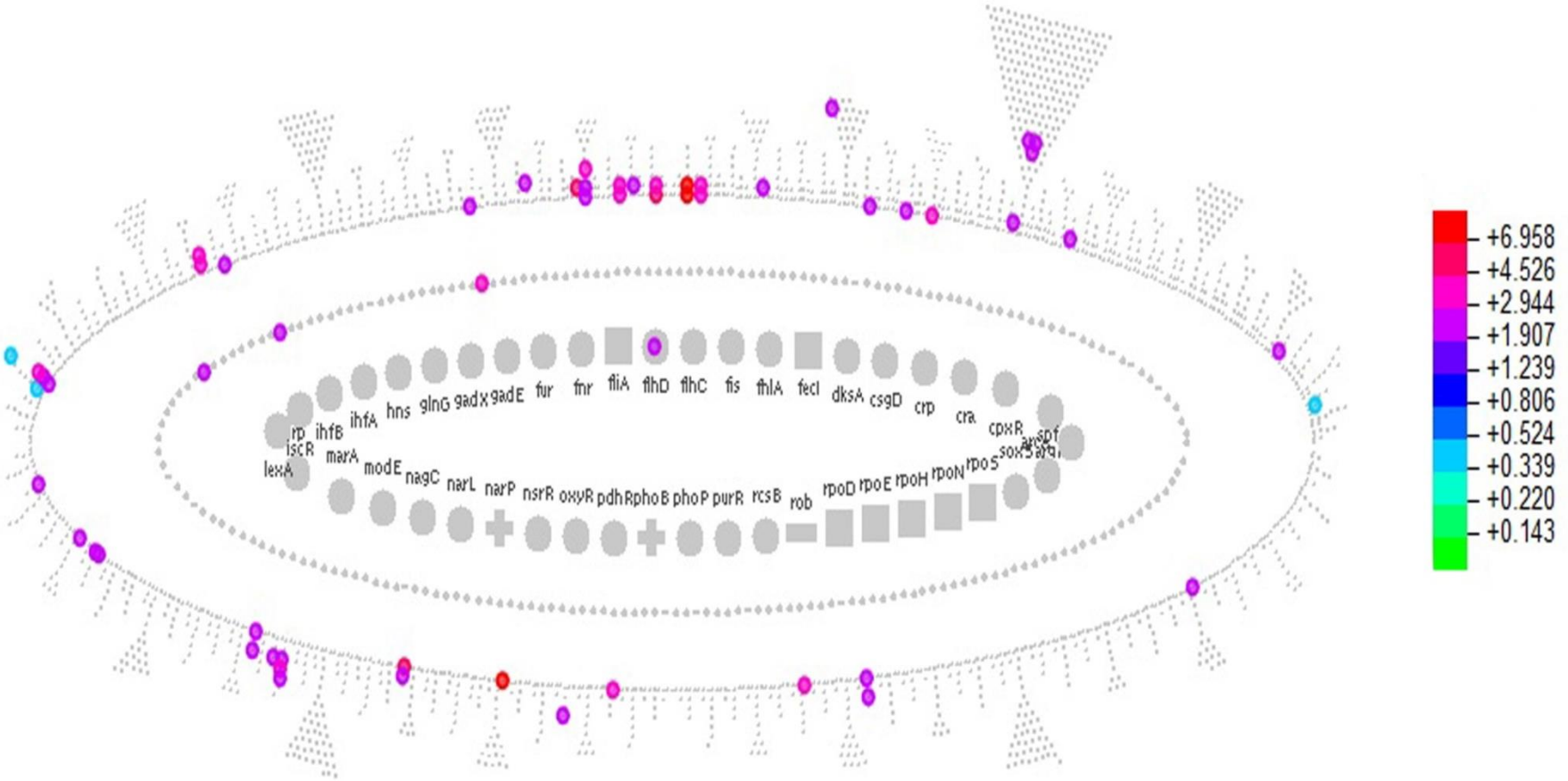


Figure 6-7. Regulatory map overview showing significantly differentially expressed regulators to spinach leaf cell wall polysaccharides compared to a vermiculite control.

Transcriptomic data for the spinach root exudate dataset was mapped onto the regulatory overview for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013), which represents known regulators (depicted in grey). Each coloured circle represents a regulator that is differentially expressed in response to spinach root exudates. The inner ellipse depicts master regulators and sigma factors; the outer ring represents genes that are regulatees only; the middle ring represents all remaining regulatory genes. Shapes in the inner circle depict the type of regulator: a plus sign for a regulator that has activators only; a minus sign for a regulator that has inhibitors only; a circle for a regulator that has activators and inhibitors; and a square for a gene that encodes a sigma factor. Data was normalised to 1; therefore values >1 are genes that are upregulated and <1 are genes that are downregulated.

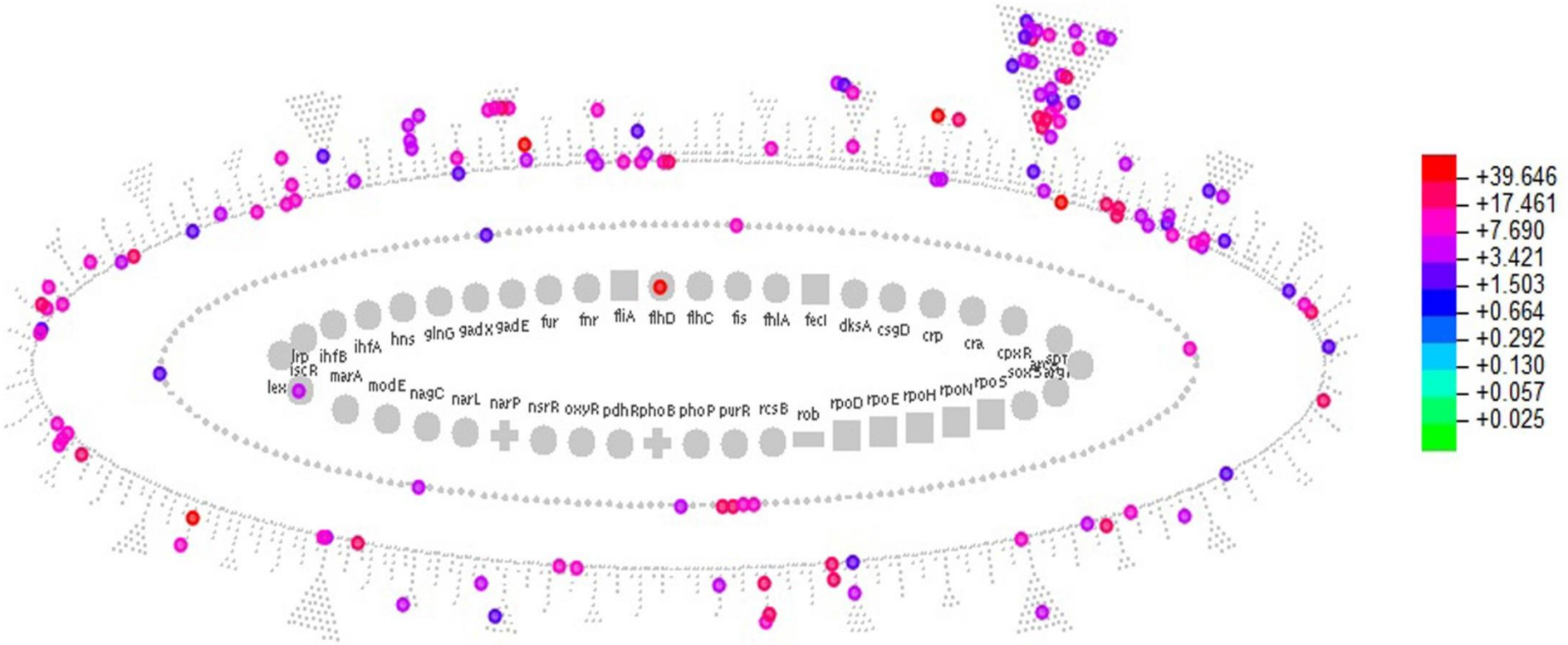


Figure 6-8. Regulatory map overview showing significantly differentially expressed regulators to lettuce leaf cell wall polysaccharides compared to a vermiculite control.

Transcriptomic data for the spinach root exudate dataset was mapped onto the regulatory overview for *E. coli* K-12 using EcoCyc (Keseler *et al.*, 2013), which represents known regulators (depicted in grey). Each coloured circle represents a regulator that is differentially expressed in response to spinach root exudates. The inner ellipse depicts master regulators and sigma factors; the outer ring represents genes that are regulatees only; the middle ring represents all remaining regulatory genes. Shapes in the inner circle depict the type of regulator: a plus sign for a regulator that has activators only; a minus sign for a regulator that has inhibitors only; a circle for a regulator that has activators and inhibitors; and a square for a gene that encodes a sigma factor. Data was normalised to 1; therefore values >1 are genes that are upregulated and <1 are genes that are downregulated.

6.2.1.2 Expression of genes in the TCA cycle

The TCA cycle was used as an indicator of growth in the plant extracts, when compared to a 0.2 % glycerol control for the spinach leaf lysates and root exudates conditions and when compared to a vermiculite polysaccharide control for the spinach and lettuce leaf cell wall polysaccharide conditions (Figure 6-9).

The expression of genes involved in the TCA cycle were found to be downregulated from 3- to 12-fold in response to spinach leaf lysates and 4.5- to 16-fold to spinach root exudates (Figure 6-9). In contrast, the same genes were found to be induced 2.25- to 3.34-fold in the presence of leaf cell wall polysaccharides of spinach and 16- to 30-fold in lettuce polysaccharides, although the *acnA* gene, which is involved in the conversion of citrate into D-*threo*-isocitrate, was downregulated 7-fold in response to the lettuce extract. Studies have shown that these enzymes are individually expressed at different conditions (Cunningham *et al.*, 1997), hence why mixed expression may be seen in the lettuce polysaccharide dataset for this metabolic pathway.

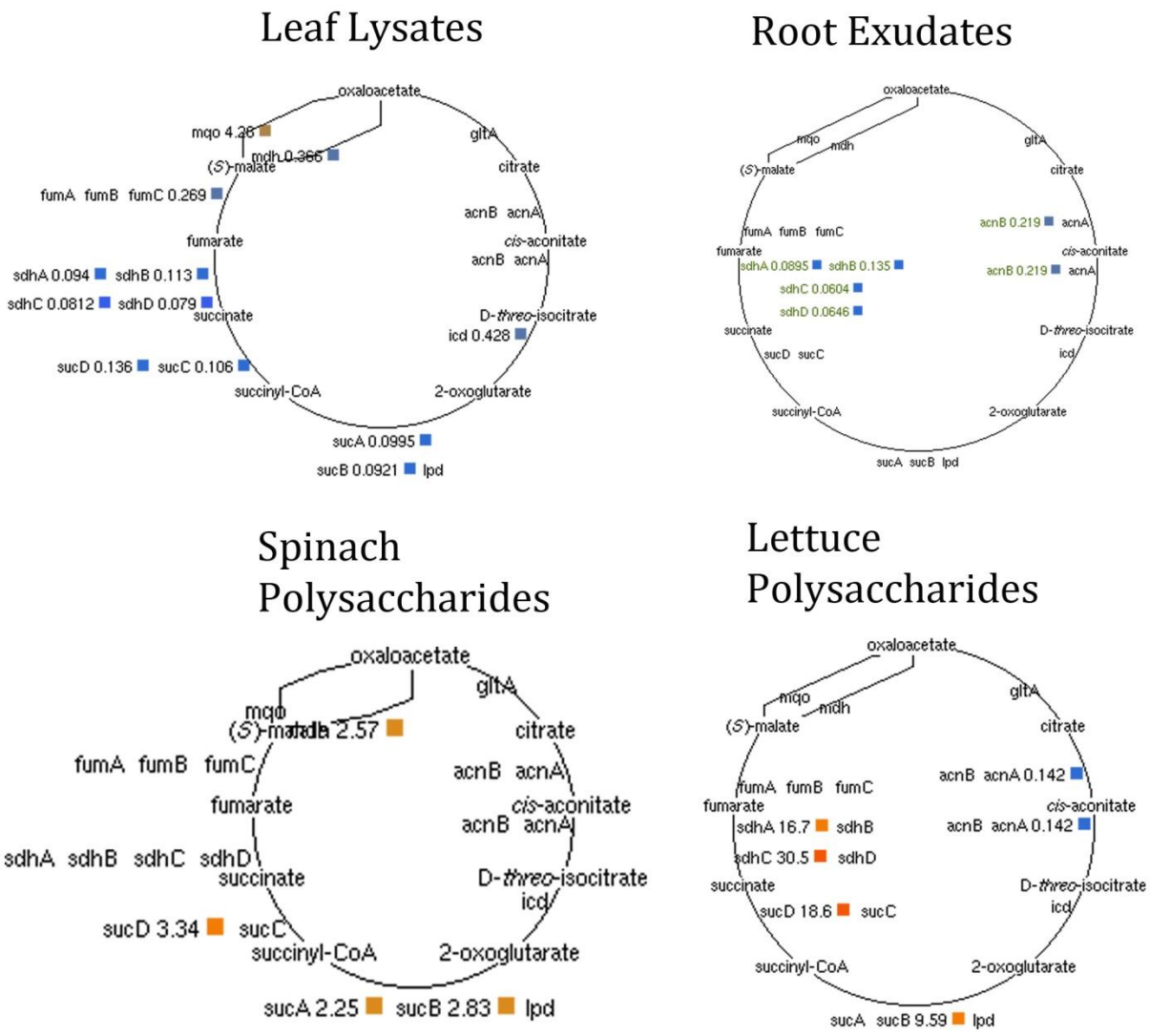


Figure 6-9. Expression of genes involved in the TCA cycle when *Ec*O157 was incubated with plant extracts.

Gene expression datasets were mapped onto the metabolic pathways of *E. coli* using the EcoCyc cellular overview browser. A red/orange to blue/green scale was used to depict up and down regulated gene expression, respectively. Images were generated using EcoCyc (Keseler *et al.*, 2011).

6.2.1.3 Induction of fatty acid β-oxidation genes

The fatty acid β-oxidation pathway was induced in *Ec*O157 Sakai when incubated with spinach root exudates for one hour (Figure 6-10). Genes were upregulated from 12-fold (*fadJ*) up to 183-fold (*fadE*) in this pathway, indicating the breakdown of short- and long-chain fatty acids. This pathway removes two carbons each turn, eventually resulting in acetyl-CoA when starting with an even-number of carbons and propionyl-CoA with an odd-number of carbons. These compounds can then be utilised in other metabolic pathways to generate energy for the cell. This pathway was only upregulated overall in spinach root exudates: the gene *fadD* was upregulated 4-fold, however *fadA* was downregulated 3-fold in spinach leaf lysates. No genes in this pathway were differentially expressed in response to spinach leaf cell wall polysaccharides and the genes *fadA*, *fadD* and *fadI* were all downregulated 8 to 14-fold in the lettuce polysaccharides dataset.

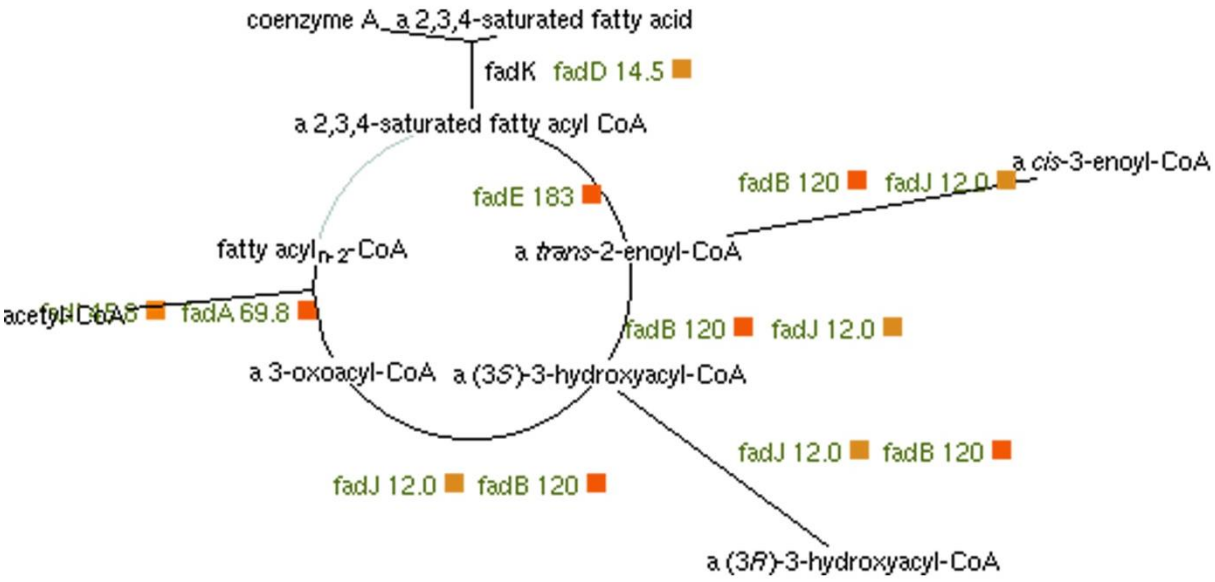


Figure 6-10. Expression of genes in the fatty acid β-oxidation I pathway in *Ec*O157 Sakai exposed to spinach root exudates.

Gene expression datasets were mapped onto the metabolic pathways of *E. coli* using the EcoCyc cellular overview browser. A red/orange to blue/green scale was used to depict up and down regulated gene expression, respectively. Images were generated using EcoCyc.

6.2.1.4 Uptake of Compounds for Metabolic Processes

As was seen for the cellular overviews for *Ec*O157 Sakai in the four different plant extracts, many transporters were seen to be differentially expressed. This not only included efflux pumps for survival but also transporters involved in the uptake of substrates within the plant extract media.

One gene, *malE*, encodes the protein-binding domain for the maltose ABC transporter, making it an integral component for the uptake of malto-oligosaccharides (Boos & Shuman, 1998). *malE* was upregulated when *Ec*O157 Sakai was incubated with spinach leaf cell wall polysaccharides, but not in the other conditions. This expression was verified by qPCR analysis on the microarray RNA samples and on fresh RNA extractions of *Ec*O157 Sakai incubated with spinach leaf cell wall polysaccharides (referred to as repeat samples). *malE* was shown to be upregulated compared to the vermiculite polysaccharide control when in the presence of spinach leaf cell wall polysaccharides when qPCR was carried out on the array and the repeat samples (**A**; Figure 6-11). As this gene was not differentially expressed in response to lettuce leaf cell wall polysaccharides, this provides an example of the differences between the plant species in terms of their extracts and the subsequent effect on the gene expression of *Ec*O157 Sakai.

Another transporter that was induced in the presence of the polysaccharide extracts was *dctA*, a c4-dicarboxylate transporter. DctA transports fumarate, succinate, malate and orotate into the cell across the inner membrane. The expression of this gene was seen to be differentially expressed in *Ec*O157 Sakai incubated with spinach and lettuce leaf cell wall polysaccharides, but was not expressed in response to spinach leaf lysates or root exudates. Validation of the expression of *dctA* seen on the microarray was carried out by qPCR using the microarray RNA samples and repeat RNA samples. *dctA* was seen to be upregulated compared to the vermiculite polysaccharide control in the qPCR analysis of both the array samples and the repeat samples for both polysaccharide extracts (**B**; Figure 6-11).

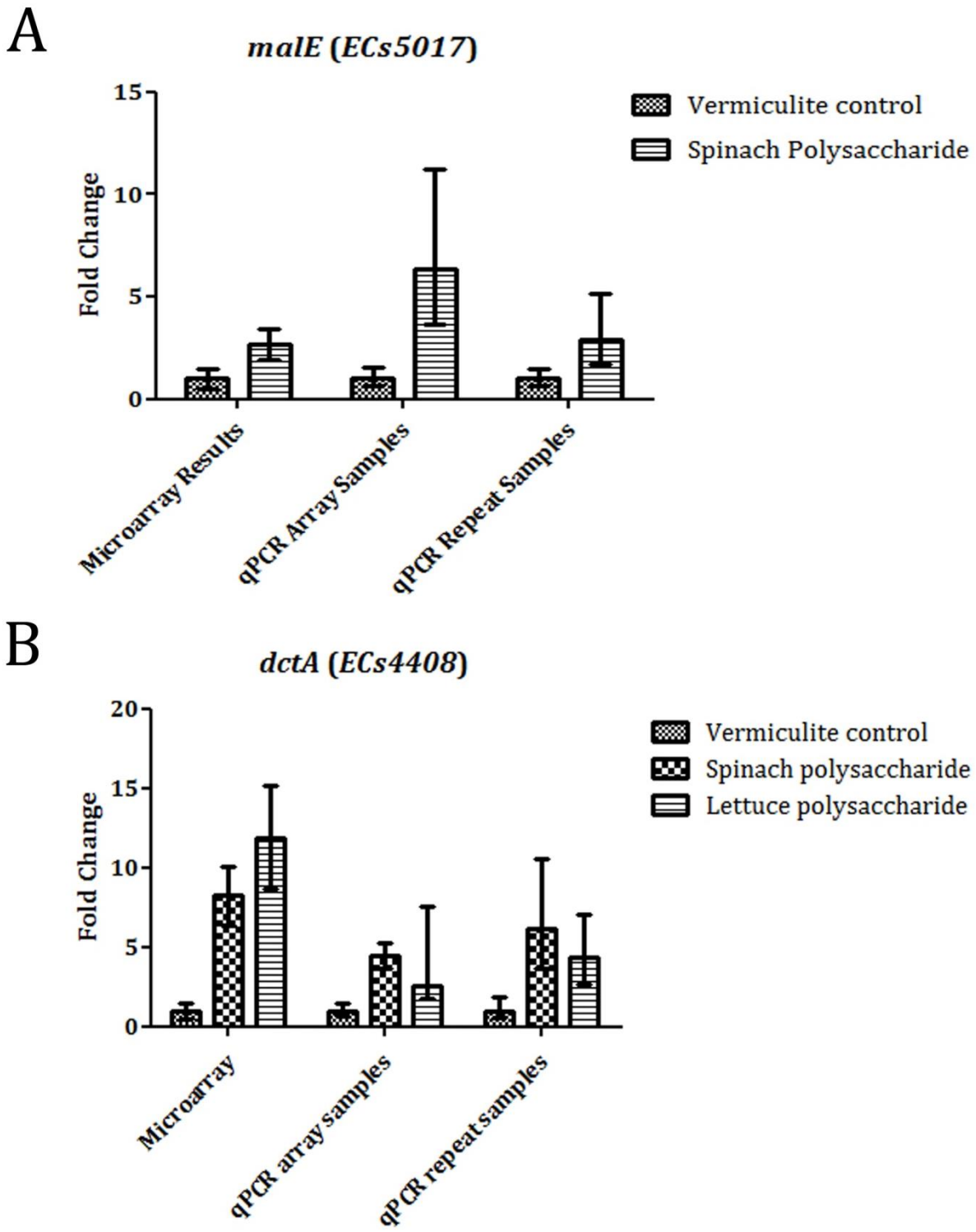


Figure 6-11. qPCR validation of *malE* and *dctA* expression in the presence of polysaccharide extracts.

qPCR was performed using the ΔΔCT method with *gyrB* as a validated reference gene for normalisation. All data were normalised to the control sample (vermiculite polysaccharides) to give a value of 1. Bars represent an average of three biological replicates, each with three technical replicates. Error bars represent SEM.

6.2.1.5 Lactose metabolism changes in EcO157 Sakai

Lactose degradation genes were differentially expressed in response to four out of five of the conditions tested by microarray, as detailed in Table 6-1. *LacA*, *lacZ* and *lacY* were upregulated at 18 °C compared to 37 °C and in response to lettuce leaf cell wall polysaccharides, whereas these genes were all downregulated when incubated with spinach leaf lysates and root exudates.

As differential expression was seen, I decided to generate a mutant in the *lacZY* genes in TUV 93-0 for two reasons: firstly to determine if it affected the colonisation of *Ec*O157 in the leaves and roots of spinach and lettuce plants since differential expression was observed; and secondly to use in a future project in a competition assay. The competition assay would involve inoculating *Ec*O157 Sakai and TUV 93-0 strains together into the plant environment to determine if one strain would outcompete the other. As colony counts were used to determine the number of bacteria within the plant, a method of differentiating between the two strains was necessary. As a result, a *lacZY* mutant was generated in TUV 93-0 (named TUVΔ*lacZY*) to make use of blue/white colony screening to differentiate between the two strains.

Table 6-1. Expression of lactose degradation genes in response to lower temperature, spinach leaf lysates, spinach root exudates and leaf cell wall polysaccharides of spinach and lettuce.

Cells were shaded red for upregulated genes and green for downregulated expression. NS = not significant.

	Fold Change				
Gene Name	***Lower Temperature***	***Leaf Lysates***	***Root Exudates***	***Spinach Polysaccharides***	***Lettuce Polysaccharides***
lacA	10.92	-5.51	-6.02	NS	NS
lacY	34.40	-30.07	-21.74	NS	8.95
lacZ	55.17	-58.17	-83.33	NS	9.14

TUVΔ*lacZY* was tested for its colonisation ability compared to TUV 93-0 wild type (WT) on the leaves and root of spinach and lettuce (*L. sativa* and *L. serriola*) over ten days. For all time points sampled (0, 1, 2 and 10 days) no significant difference was determined between the number of TUV 93-0 WT and the *lacZY* mutant on the abaxial or adaxial sides of the leaves of spinach or lettuce (Figure 6-12). Similarly, when these strains were analysed for their colonisation ability on the roots of spinach and lettuce no significant difference was seen between the mutant and the wild type (Figure 6-13). Therefore, as no significant difference was seen, not only are these genes not essential for survival and proliferation in the plant environment but also results in the availability of a mutant to use for a competition assay in a future project.

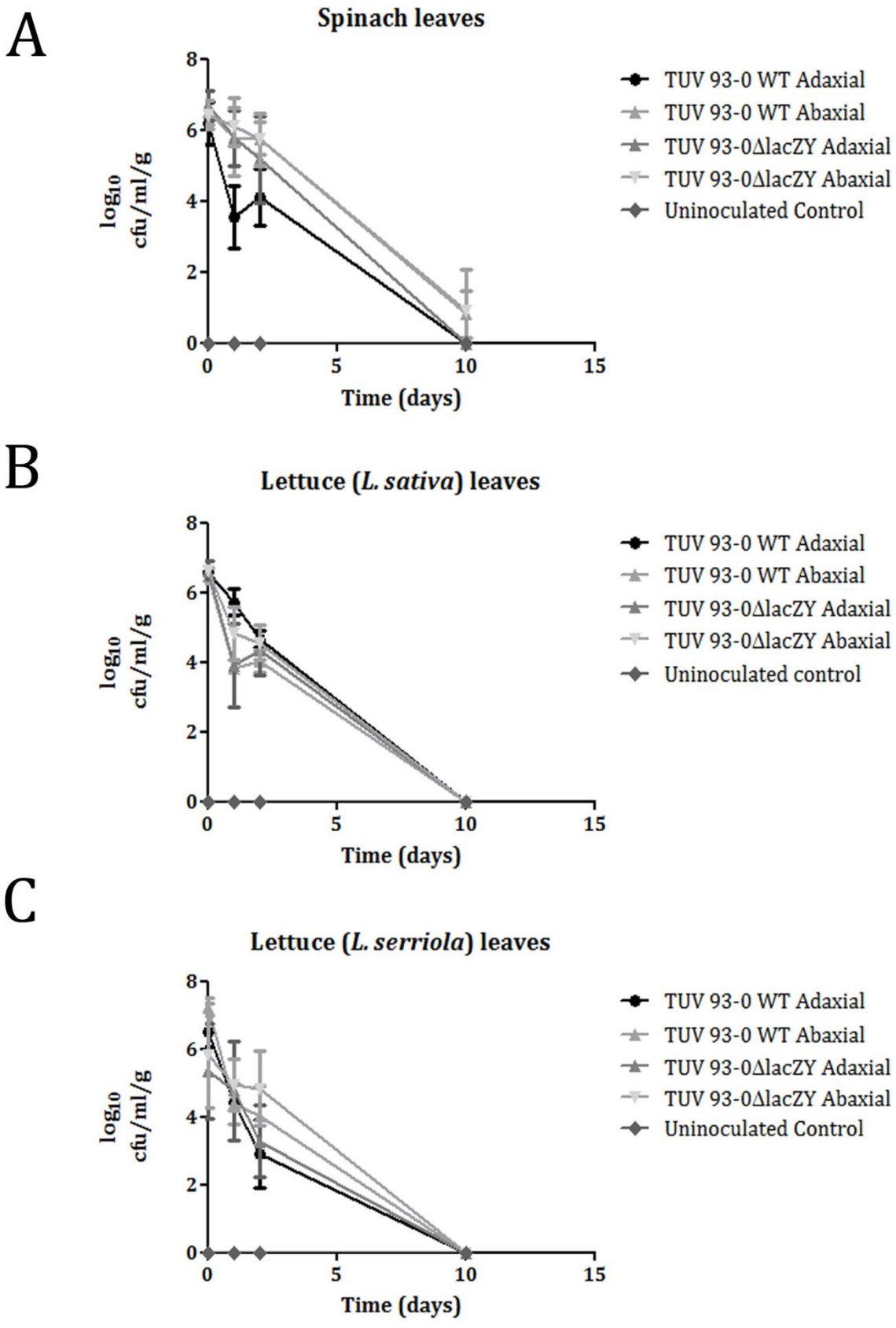


Figure 6-12. Colonisation of TUV 93-0 WT compared to TUVΔ*lacZY* on the leaves of spinach and lettuce.

Log cfu counts/g plant tissue were recovered for 0, 1, 2 and 10 day time points for the two bacterial strains on the adaxial and abaxial sides of the leaves of **(A)** spinach, **(B)** *L. sativa* lettuce and **(C)** *L. serriola* lettuce. Error bars represent SEM; *n* = 9.

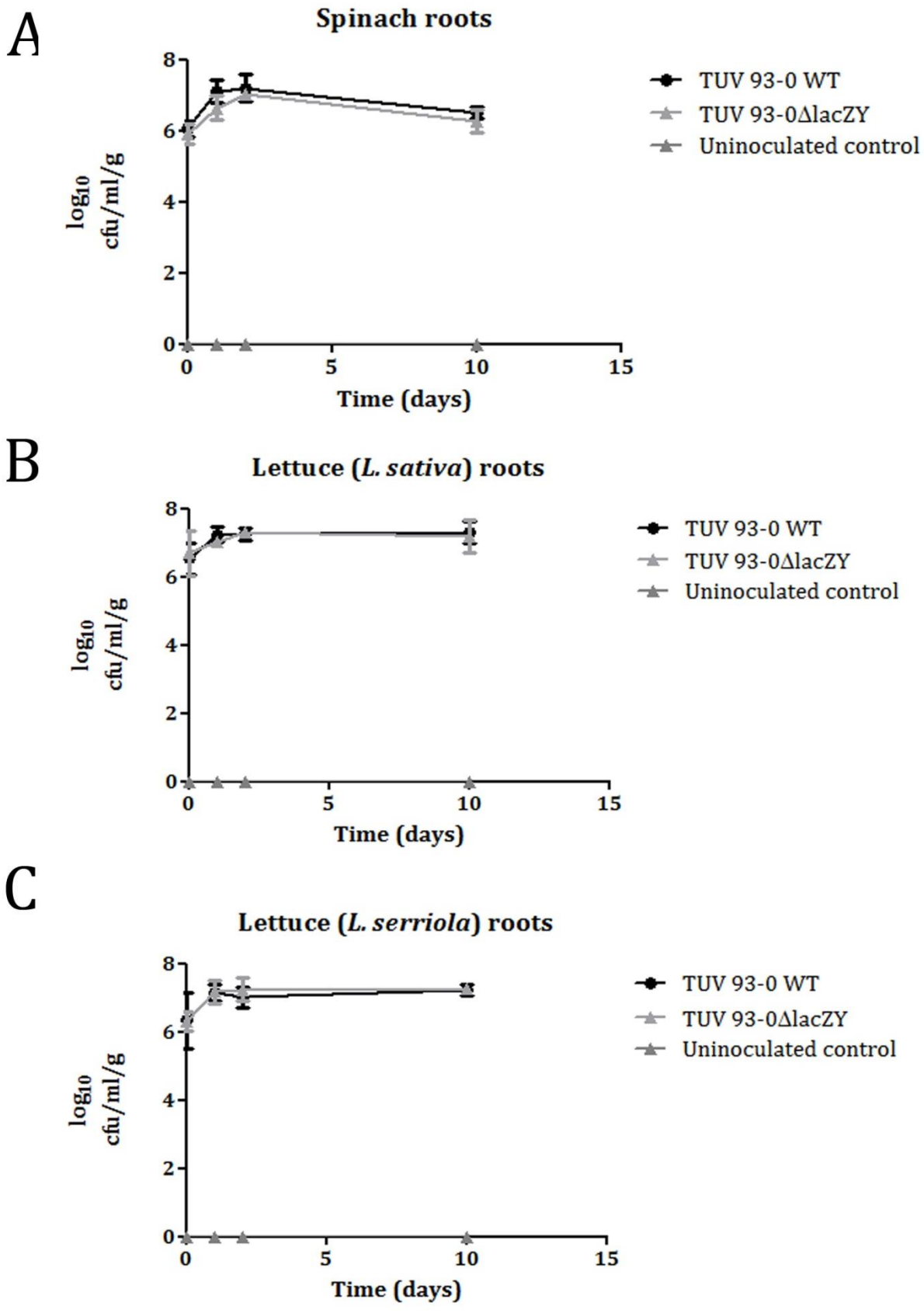


Figure 6-13. Colonisation of TUV 93-0 WT compared to TUVΔ*lacZY* on the roots of spinach and lettuce.

Log cfu counts/g plant tissue were recovered for 0, 1, 2 and 10 day time points for the two bacterial strains on the roots of hydroponically-grown **(A)** spinach, **(B)** *L. sativa* lettuce and **(C)** *L. serriola* lettuce. Error bars represent SEM; *n* = 9.

6.2.2 Utilisation of Plant-derived Substrates

Of the total metabolic pathways that were seen to have differential expression in response to the four different plant extracts, a select few were found to involve the utilisation of compounds that could have been derived from the plants, such as curcumin, xylose and arabinose.

6.2.2.1 Utilisation of curcumin, fructose and xylose

Curcumin and related curcuminoids are the main yellow pigment present in turmeric that can be degraded by *E. coli* (Hassaninasab *et al.*, 2011). The pathway for curcumin degradation was seen to be induced after one hour in response to spinach root exudates, with the gene *curA* upregulated 2.25 fold (Figure 6-14), but was not induced in any of the other plant extract conditions tested.

The degradation of xylose was induced in spinach root exudates, with a 5-fold increase (Figure 6-14). Xylose, a component of the hemicellulose fraction of the cell wall, is degraded into D-xylulose 5-phosphate, which then enters the central metabolism pathways of the cell, providing the bacterial cell with energy. Upregulation of this pathway not only suggests the presence of xylose within the root exudates (which would be expected since xyloglucans are a fundamental component of the cell wall), but also is another example of the bacteria trying to metabolise a plant component for its own survival.

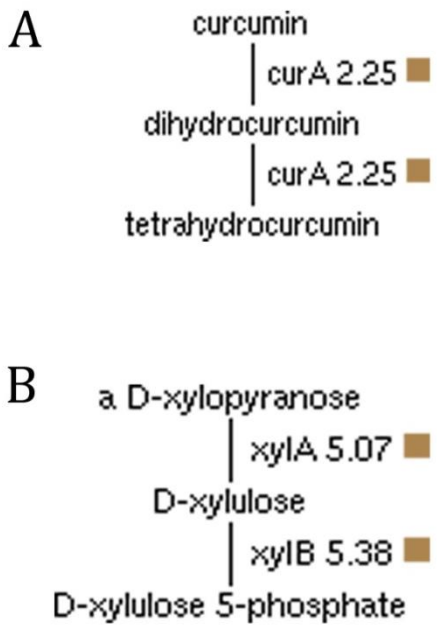


Figure 6-14. Metabolic pathways of curcumin and xylose degradation.

The spinach root exudate gene expression dataset was overlayed onto the metabolic pathways to show expression of each of the enzymes in this condition using EcoCyc. Red (upregulated) to green (downregulated) colour scale was used.

6.2.2.2 Arabinose Utilisation by EcO157 Sakai in Plant Extracts

From the cellular overview data it is clear that *Ec*O157 Sakai undergoes large gene expression changes in response to the different plant extracts. Sets of genes may be essential or advantageous in one extract over the other, due to the different environment each presents. The expression of genes related to the transport, processing and utilisation of arabinose as a carbon source were upregulated after a one-hour exposure to spinach root exudates and spinach leaf cell wall polysaccharides (Table 6-2). In contrast, only *araJ* was differentially expressed in the spinach leaf lysates dataset. Furthermore, despite upregulation of some of these arabinose genes on exposure to the spinach polysaccharides, downregulation of *araB*, *araC* and *araF* was seen in the lettuce leaf cell wall polysaccharides (the remainder were not significantly different from the vermiculite control). None of the genes were induced at the lower temperature (18 °C) indicating the response was solely to the plant extract.

Table 6-2. Expression of genes related to the processing, transport and utilisation of arabinose.

Boxes have been shaded red for upregulated gene expression and green for downregulated gene expression of the selected arabinose metabolism, utilisation and transport genes. NS = not significantly different from the control..

	Fold Change (Results from Microarray)				
Gene Name	***Temperature***	***Spinach Leaf Lysates***	***Spinach Root Exudates***	***Spinach Polysaccharides***	***Lettuce Polysaccharides***
araA	-3.21	NS	8.94	10.68	NS
araB	-4.94	NS	19.98	9.37	-2.18
araC	-5.76	NS	9.14	NS	-4.97
araD	NS	NS	3.58	NS	NS
araE	NS	NS	NS	NS	NS
araF	NS	NS	10.45	6.01	-2.70
araG	NS	NS	NS	2.16	NS
araJ	NS	2.22	-3.10	NS	NS

L-arabinose is the common form of this monosaccharide found in nature and metabolism of this plant monosaccharide is via the L-arabinose degradation pathway, widely conserved in prokaryotes, including *E. coli*. This pathway was upregulated in *Ec*O157 Sakai after a one hour exposure to spinach root exudates (**A**; Figure 6-15) and to spinach leaf cell wall polysaccharides (**B**; Figure 6-15). *AraD* was not deemed significantly different based on the stringency tests used (see section 2.16.3 for analysis methods) for the spinach polysaccharide dataset.

All microarray datasets were validated by qPCR using two different sets of samples: those used for the microarray (referred to as "array samples") and from fresh RNA extractions using the same experimental setup (referred to as "repeat samples"). All microarrays were validated using this method for each of the five conditions tested (section 5.2.4). Expression of arabinose-processing genes was validated for the polysaccharide datasets via qPCR by testing the expression of *araA*, the first enzyme in the L-arabinose degradation pathway (**C**; Figure 6-15). The vermiculite polysaccharide sample was used as the control and the expression of this gene was normalised to one. For the spinach polysaccharide dataset, *araA* expression was upregulated compared to the vermiculite control as determined by microarray and by qPCR of array and repeat samples. Expression measured from the repeat samples was lower than from the array sample: 2.78 fold compared 5.93 fold. Despite the lower fold change for *araA* seen in the repeat samples, *araA* was found to be upregulated in all three of the tests.

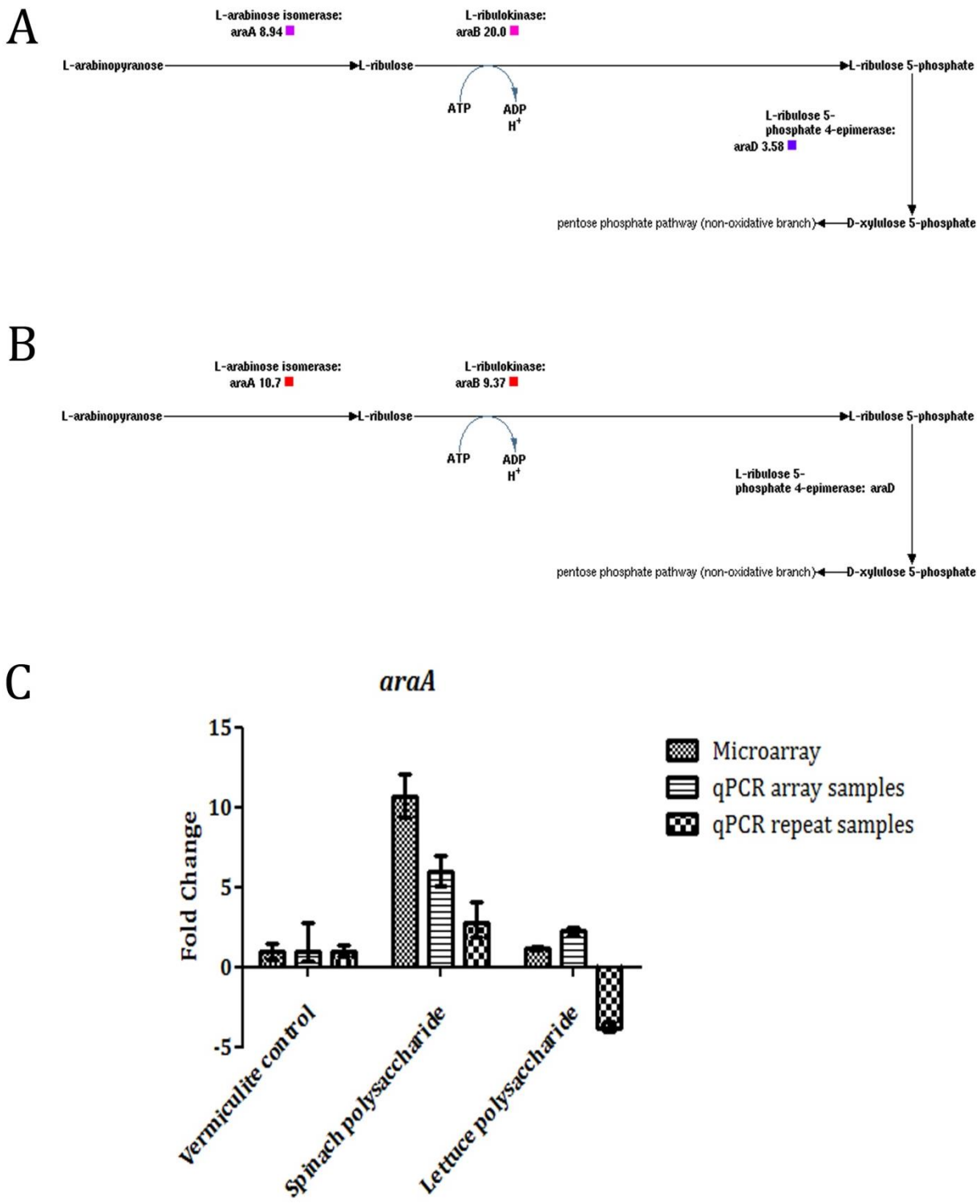


Figure 6-15. Expression of arabinose utilisation genes in plant extracts.

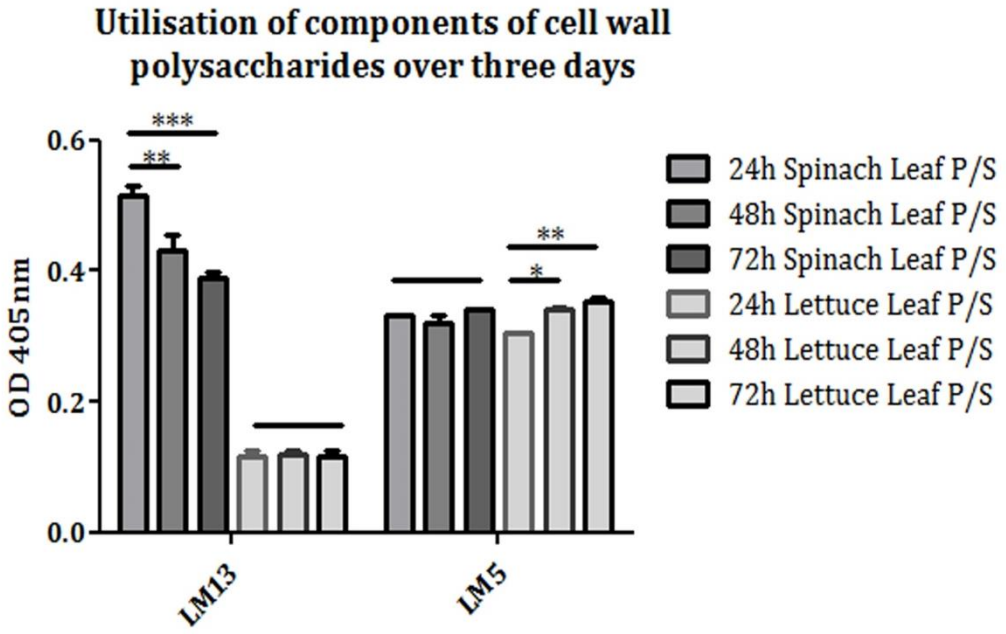
A) L-arabinose degradation metabolic pathway overlayed with transcriptomic data from the spinach root exudates dataset (EcoCyc); **B)** L-arabinose degradation metabolic pathway overlayed with transcriptomic data from the spinach leaf cell wall polysaccharides dataset (EcoCyc); **C)** qPCR validation of *araA* using array and repeat samples for the spinach and lettuce leaf cell wall polysaccharide conditions. Bars represent an average of three biological replicates, each with three technical replicates. Error bars represent SEM.

In contrast, expression of *araA* was not induced in lettuce leaf cell wall polysaccharides and was not significantly different from the vermiculite control. In all cases, *araA* was not induced in the presence of lettuce leaf cell wall polysaccharides, unlike the spinach polysaccharide extract.

To confirm if expression of the L-arabinose degradation pathway resulted in the utilisation of arabinose an ELISA was performed by Yannick Rossez using the batch of spinach and *L. sativa* lettuce leaf cell wall polysaccharide samples that were used for the microarray experiments. Cultures of *Ec*O157 Sakai were grown in M9 medium with 1 % (w/v) spinach/lettuce polysaccharides and incubated at 18 °C. Samples were taken at 24, 48 and 72 hrs and tested by ELISA for the presence of selected sugars using specific monoclonal antibodies (Plant Probes, University of Leeds). (1, 5)-α-arabinan was detected with LM13 and (1, 4)-β-galactosyl was detected with LM5 (Megazyme International Ireland, Wicklow, Ireland). Detection of LM13 decreased after 48 hrs (p = 0.0391) and 72 hrs (p = 0.0016) in the spinach polysaccharide samples (**A**; Figure 6-16), whereas no changes in LM13 binding occurred on lettuce polysaccharides, indicating *Ec*O157 Sakai-mediated degradation of arabinans in spinach.

Interestingly, another difference between the plant species was observed from the ELISA for LM5 antibody binding, which recognises 1,4-galactan. For the spinach polysaccharides, no difference was seen in the absorbance for LM5 binding across the three timepoints (Figure 6-16). However detection increased in the lettuce leaf cell wall polysaccharide samples after 48 hours (p = 0.0367) and 72 hours (p = 0.0014), perhaps indicating the release of galactans over time.

A



B

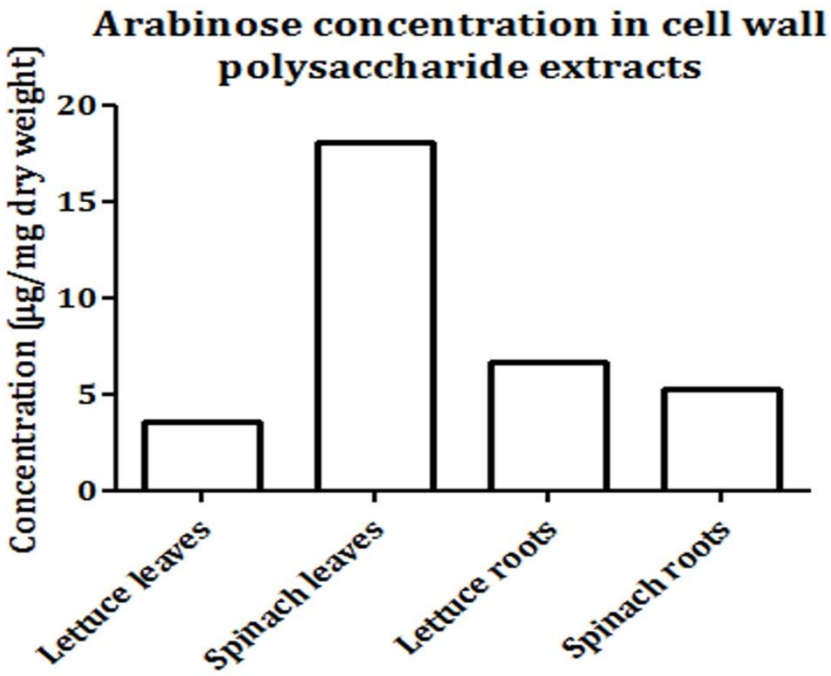


Figure 6-16. Utilisation of arabinose by *Ec*O157 Sakai from spinach leaf cell wall polysaccharide extracts.

A) Utilisation of leaf cell wall polysaccharide extracts over three days as determined by ELISA analysis, checking the level of binding of antibodies LM13 (which binds to (1-5)-α-arabinans) and LM5 (recognises (1-4)-β-D-galactosyl residues. Uninfected polysaccharide control samples were set up for three days and no change in antibody binding was seen (data not shown). ELISA experimental set-up and analysis was carried out by Yannick Rossez using the spinach and leaf cell wall polysaccharides samples from this study. **B)** HPLC analysis of arabinose content (µg/mg dry weight polysaccharide) in the leaves and roots of spinach and *L. sativa* lettuce cell wall polysaccharides. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$.

HPLC analysis was carried out as detailed in section 4.2.3 to measure the monosaccharide content of the different polysaccharide samples. Arabinose content (measured as μg/mg dry weight of plant extract) was analysed in this method to compare to the results generated from the transcriptomic analysis. Spinach leaf cell wall polysaccharide extracts were found to have the highest arabinose content (18 μg/mg dry weight), although the spinach root cell wall polysaccharide samples were much lower (5 μg/mg dry weight). *L. sativa* lettuce polysaccharide extracts showed no difference in arabinose levels between the leaves and the roots, and contained a similar concentration of arabinose to that of the spinach root polysaccharides.

As *Ec*O157 Sakai was shown to utilise arabinose and this plant polysaccharide was seen to vary in terms of its concentration between plant species, I decided to study which forms of arabinose this human pathogen was responding to. One-hour exposures were set up of *Ec*O157 Sakai in M9 minimal medium containing 0.2 % of different commercially available forms of arabinose. After this time, the RNA of *Ec*O157 Sakai was extracted, converted to cDNA and qPCR analysis performed to study the response of three arabinose-processing genes to the different conditions: *araA* and *araD*, from the L-arabinose degradation pathway and *araJ*, which has been proposed to transport arabinose polymers, although no direct evidence of this reaction has been found as of yet (Reeder & Schleif, 1991).

The expression of these three genes was tested in response to 0.2 % arabinose monosaccharide; 1,5-α-linked backbone to which 1,3- α-linked (and possibly some 1,2- α-linked) L-arabinofuranosyl residues are attached (herein referred to as branched); 1,5- α-linked backbone which has been treated with α-L-arabinofuranosidase to remove all branch units (debranched); 1,5- α-L-Arabinobiose (arabinobiose); 1,5-α-L-Arabinoheptaose (arabinoheptaose) (Megazyme International Ireland, Wicklow, Ireland). M9 medium only was used as a control.

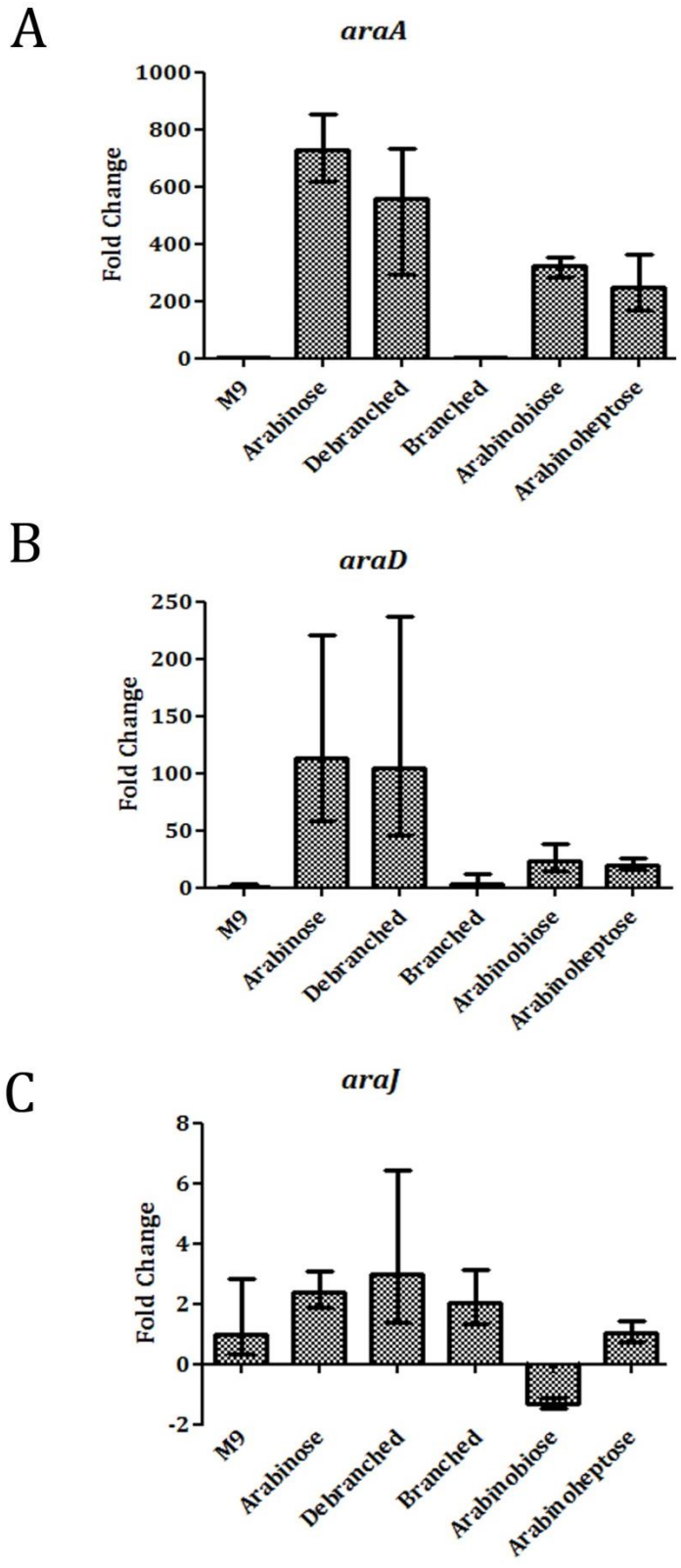


Figure 6-17. Expression of arabinose-associated genes in response to different forms of arabinose.

Genes related to arabinose utilisation/transport (**A** - *araA;* **B** – *araD;* **C** – *araJ*) were tested by qPCR to see their expression changes to different forms of arabinose. Samples were set up in M9 minimal medium only (M9), and with the addition of 0.2%: arabinose monosaccharide; (1-5)-α-linked backbone treated with α-L-arabinofuranosidase to remove branch units (debranched); (1-5)-α-linked backbone to which (1-3)-α-linked and possible some (1,2)-α-linked L-arabinofuranosyl residues are attached (branched); (1-5)-α-L-arabinobiose (arabinobiose); and (1,5)-α-L-arabinoheptose (arabinoheptose). *GyrB* was used as a reference gene for qPCR analysis. Bars represent an average of three biological replicates, each with three technical replicates. Error bars represent SEM.

AraA expression was significantly upregulated compared to a M9 minimal medium only control when *Ec*O157 Sakai was exposed to arabinose, debranched arabinan, arabinobiose and arabinoheptaose after one hour (**A**; Figure 6-17). No significant change in gene expression was seen for *araA* in response to the branched condition after a one hour exposure. Both the arabinobiose and arabinoheptaose conditions induced a lower level of expression of *araA* compared to the arabinose monosaccharide.

A similar pattern of expression was seen for the third enzyme in the L-arabinose degradation pathway, *araD*, with arabinose and the debranched condition inducing the highest levels of expression (**B;** Figure 6-17). No significant change was seen for the branched condition, as for *araA*. The level of expression in terms of fold change was smaller for *araD* compared to *araA*, although both were still significantly different compared to the control.

Finally, the expression of the gene *araJ* was tested in response to the same conditions. Gene expression of *araJ* in response to all of the conditions tested was found to be not significantly different to that of the M9 medium control (**C**; Figure 6-17).

As a final gene expression test to validate the expression results seen from these plant extracts, qPCR was carried out on *in planta* samples. *Ec*O157 Sakai was infiltrated into the leaves of spinach and *L. sativa* lettuce for one hour prior to bacterial RNA extraction and cDNA synthesis. The expression of *araA* was tested by qPCR as this gene had shown strong expression profiles to spinach polysaccharide extracts and to the different forms of arabinose tested. Cultures of *Ec*O157 Sakai in the plant infiltration buffer for one hour were used as a control.

After one hour of infiltration inside the leaves of spinach and lettuce, *araA* expression was not significantly different to that of the culture control. Large variation was seen between the three samples tested, with one of the three showing downregulation of *araA* expression *in planta*. No upregulation of this gene was seen in either plant condition, contrasting the results previously found looking at plant extracts.

Figure 6-18. Expression of *araA* in infiltrated spinach and lettuce leaves compared to a culture control after one hour.

*Ec*O157 Sakai was infiltrated into the leaves of spinach and lettuce and left for one hour before RNA extraction. Fold change was calculated by qPCR, using *gyrB* as a reference gene. The culture control was incubated in the $MgCl_2$ infiltration medium for one hour as a comparison. Bars represent an average of three biological replicates, each with three technical replicates. Error bars represent SEM.

6.3 Discussion

Metabolic analysis of pathways that are differentially expressed within certain conditions can provide valuable insights into the adaptation of microbes to many environments, whether via a phenotypic or a transcriptomic microarray approach. Transcriptomic analysis of metabolic pathways provides an overview of the processes happening at a specific time point and is subject to translational regulation. Nevertheless, overlapping the gene expression changes onto the metabolic pathways present using EcoCyc software provides a wealth of information of the overall status of the bacteria, especially in terms of stress and ability to utilise the plant extracts tested. Several studies have analysed the metabolic changes and interface between the bacteria and plant, to try and understand this relationship more effectively (Brader *et al.*, 2014; Karpinets *et al.*, 2014; Rico *et al.*, 2011), with some looking closely at bacterial metabolism of plant extracts (Rico & Preston, 2008). Little focus has been given to the metabolism and utilisation of plant extracts for the survival of human-pathogenic bacteria, with most transcriptional work focused on the adherence of the bacteria and the expression of the T3SS during colonisation (Fink *et al.*, 2012; Kyle *et al.*, 2010; Thilmony *et al.*, 2006). Understanding the adherence of the bacteria is very crucial, but so too is the adaptation and colonisation of *Ec*O157 to the different plant niches, in order to develop any preventative measures or interventions. Further to this, recent work has highlighted the importance of metabolic enzymes in regulating certain virulence factors in *Ec*O157:H7 (Beckham *et al.*, 2014), including the expression of many adherence and infiltration factors, some of which may play a key role in plant colonisation.

6.3.1 Expression of Genes Involved in the TCA Cycle

Changes in the expression of several metabolic pathways were seen when *Ec*O157 Sakai was exposed to four different plant extracts for one hour. Many of the core metabolism genes involved in energy production and growth, such as glycolysis and the TCA cycle, were differentially expressed in *Ec*O157 response to spinach leaf lysates, spinach root exudates, spinach polysaccharides and lettuce polysaccharides.

The growth of *Ec*O157 Sakai in leaf lysates was tested previously (section 3.2.2.1) and it was shown that the bacteria were able to utilise leaf lysates of spinach for growth. However, the gene expression data used in this chapter was compared to *Ec*O157 Sakai utilising 0.2 % glycerol, which was shown to result in a higher growth rate compared to leaf lysates. Thus, downregulation of the TCA cycle when in comparison to a 0.2 % glycerol control would correlate with the growth profiles of this bacterial species in these conditions.

This suggests that *Ec*O157 Sakai can utilise these leaf lysates extracts for its growth and survival to a similar extent as for the *in vitro* condition of minimal M9 medium supplemented with 0.2 % glycerol.

Genes in the TCA cycle were downregulated in response to root exudates, which correlates with the lack of growth seen in *Ec*O157 Sakai incubated with root exudates alone. However, the TCA cycle was induced in response to both spinach and lettuce leaf cell wall polysaccharides. Due to the method of preparation of the cell wall polysaccharides, growth assays were unable to be performed using optical density or colony counts. The upregulation of these genes does suggest that the bacteria are actively metabolising the polysaccharides compared to the vermiculite only control and so we can speculate that the polysaccharides may result in the eventual growth of the bacterial population.

6.3.2 Fatty acid β-oxidation genes are induced by spinach root exudates

Induction of the fatty acid β-oxidation was only seen in the spinach root exudates dataset. As well as this, it was also one of the most highly induced pathways in this dataset. Expression of this metabolic pathway is induced by long-chain fatty acids, although the pathway is used to break down long and short-chain fatty acids (Clark, 1981). Studies have shown that long-chain fatty acids are exuded from plant species such as cucumber and corn (Windstam & Nelson, 2008). No evidence of fatty acids exuded from plant roots has been described for spinach and this was not tested for in the course of this project. However, as this pathway has been induced it could be speculated that spinach, like other plant species, exudes many fatty acids from the roots. Therefore the root exudates induce the expression of the fatty acid β-oxidation pathway

to break down these compounds into acetyl- and propionyl-CoA, which can be used in further metabolic pathways to generate energy for the bacterial cell. Therefore this is another example of the bacteria trying to utilise the root exudates for their survival.

6.3.3 Transporters were induced in response to polysaccharide extracts

The induction of *malE* and *dctA* occurred in response to spinach leaf cell polysaccharide extracts and for lettuce polysaccharide extracts for *dctA* only. The induction of *malE* in one polysaccharide extract but not another is interesting as it demonstrates differences in the polysaccharide content between these two species, therefore affecting the gene expression of *Ec*O157 Sakai. The presence of certain oligosaccharides in one plant species yet not another may impact upon the survival of *Ec*O157 Sakai, as is suggested for arabinose also in section 6.3.6.

In contrast, *dctA* was induced in response to both spinach and lettuce polysaccharide extracts, therefore the expression of this gene does not seem plant-specific. However, homologues of this gene have been identified for other plant-associated bacteria in the plant environment, such as *Pseudomonas syringae*, where *in vivo* expression technology (IVET) analysis of this bacterial species in the plant environment identified a predicted dicarboxylate transport protein (Rainey, 1999). Furthermore, a study looking at *Pseudomonas chlororaphis* found that a mutant in *dctA* had reduced colonisation on tobacco roots compared to the wild type (Nam *et al.*, 2006).

During the course of this project, a mutant in *dctA* in *Ec*O157 Sakai was developed but due to time constraints was not tested for its colonisation ability in the plant environment. Thus, as plant-associated bacterial strains have demonstrated a reduction in colonisation when impaired for *dctA* and this gene was induced in the presence of plant polysaccharide extracts, it would be interesting in the future to test this mutant in spinach and lettuce leaves and roots to help determine the importance of this gene.

6.3.4 Expression of lacZY *is not necessary for survival in the plant environment*

Lactose transport and degradation genes were upregulated in response to growth at 18 °C compared to 37 °C and also when incubated with lettuce leaf cell wall polysaccharides.

However, when the colonisation ability of an *Ec*O157 TUV 93-0 strain lacking the *lacZY* was assessed, no difference was seen compared to the wild type. Therefore, I suggest that expression of these genes is due to a carryover effect from the other metabolism processes, perhaps due to degradation of plant material. Cell wall components have been shown to induce expression of β-galactosidases in other fungal plant-associated species, such as *Aspergillus niger* (Manzanares *et al.*, 1998), therefore perhaps this upregulation demonstrates another similarity between these human pathogens and environmental species.

6.3.5 Metabolic pathways involving the degradation of curcumin and xylose were induced in Ec*O157 Sakai*

Exposure to spinach root exudates for one hour induced several different metabolic pathways compared to the leaf lysates. The extracts present very contrasting environments: the leaf extract provides many nutrients to support growth and potentially anti-microbials, whereas the root exudates contained little accessible sugars (section 4.2.2). However, genes required for the degradation of curcumin and xyloses were induced in root exudates.

Curcumin degradation was upregulated in response to the root exudates after one hour. The concentration of curcumin was not determined in the exudates, as it was not measured during the HPLC analysis. Further analysis of the root exudates would help to identify this response. Upregulation of the curcumin degradation pathway is interesting as the addition of curcumin has been shown to reduce the pathogenicity of *Pseudomonas aeruginosa* PAO1 on *Arabidopsis thaliana*, by inhibiting virulence factors present in PAO1, such as those required for biofilm formation (Rudrappa & Bais, 2008).

Xylose degradation was also upregulated in the spinach root exudates dataset. Xylose utilisation involves degradation of the hemicellulose fraction of plant cell walls, a trait which is present in plant-associated bacteria such as *Xanthomonas* (Déjean *et al.*, 2013; Santos *et al.*, 2014). Interestingly, these genes were not induced in response to the plant cell wall polysaccharide extracts.

Therefore, although *Ec*O157 Sakai was unable to grow in root exudates, it is possible that it was still able to degrade several plant-related components as demonstrated by the induction of these pathways. This suggests that although the root exudates did not contain enough accessible carbon to support growth, the exudates do contain a variety of plant-derived compounds which the bacteria appear to be trying to degrade for their own use.

6.3.6 Arabinose Utilisation in the Plant Environment

L-arabinose degradation was highly induced in response to spinach leaf cell polysaccharides and to spinach root exudates; in fact it was the most upregulated metabolic pathway in the spinach leaf cell wall polysaccharides dataset, suggesting that the bacteria are degrading components of the plant cell wall to survive. In contrast, this pathway was downregulated/not differentially expressed in *Ec*O157 Sakai incubated with spinach leaf lysates or lettuce leaf cell wall polysaccharides. This suggests that induction of these metabolic pathways is specific to certain plant species, presumably due to differences in the content of the polysaccharide extracts.

The lack of differential expression of the L-arabinose degradation pathway in leaf lysates may be due to the presence of other freely accessible sugars, such as glucose, fructose and sucrose. Due to carbon catabolite repression mechanisms in bacteria the presence of these sugars will induce metabolic pathways involved in their utilisation and repress others involved in degradation of other sugars, such as arabinose (Brückner & Titgemeyer, 2002).

Interestingly, the only gene that was upregulated in the spinach leaf lysates dataset that is linked to arabinose was *araJ*, which is proposed to be an arabinose transport protein. However, when expression of this gene was measured on response to different forms of arabinan, there was no change in expression compared to the media-only control. Little is known about *araJ*, but it is proposed to play a role in arabinose utilisation as it is under the control of an arabinose-inducible regulator, *araC* (Reeder & Schleif, 1991). As no change in expression was seen for this gene in this study, one could speculate that this gene plays a different role perhaps in the transport of another metabolite. The fact that this gene was the only "arabinose"-related gene to be upregulated in response to spinach leaf lysates further strengthens this idea. In contrast,

araA and *araD*, which encode enzymes necessary for the L-arabinose degradation pathway, were highly induced on exposure to all of the forms of arabinose tested, except for the branched glycan. This allows us to speculate on the importance of other plant-associated species in the co-colonisation with human pathogens on plants, as plant species such as *Pectobacterium* that contain plant cell wall degrading enzymes may degrade the branched forms of arabinose.

The absence of induction of arabinose enzymes in response to the lettuce polysaccharides may be due to availability. HPLC analysis showed higher concentrations of arabinose in the spinach leaf cell wall polysaccharides compared to the lettuce polysaccharide extracts. Changes in polymeric forms of arabinose were observed following three days exposure of *Ec*O157 Sakai spinach polysaccharide extracts, measured by a decrease in antibodies specific for (1,5)-α-arabinan. In contrast, no change was observed in the lettuce polysaccharide samples (Yannick Rossez). As well as this, there appeared to be accumulation of galactosyl residues in lettuce. Galactan, the polymer of galactose, is an important component of hemicellulose and galactose degradation genes were induced in the spinach polysaccharide dataset but not in the lettuce dataset. Together the data suggest that polymers in hemicellulose may be utilised by *Ec*O157 Sakai after three days, although there is also the possibility that the hemicellulose is bacteria-derived.

Confirmation of this arabinose utilisation was tested *in planta* by testing expression of the gene *araA*, the first enzyme in the L-arabinose degradation pathway in *Ec*O157 Sakai that had been infiltrated into the leaves of lettuce and spinach for one hour. However, as for the leaf lysates dataset, these genes were not differentially expressed *in planta* (compared to an infiltration buffer only control). Perhaps as the bacteria were infiltrated into the leaf the expression of the arabinose utilisation genes was not important for two reasons: firstly due to the presence of other sugars, similar to the situation for the leaf lysates extracts; and secondly, as the bacteria were infiltrated into the leaves, adherence on the surface of the leaf was bypassed. As a result, in the future this experiment should be repeated by testing the expression of bacteria that are adhering to the surface of the leaf to see if L-arabinose degradation is involved during the adherence and colonisation process. One caveat that should be noted is that an optimised

method would need to be developed further, as only "loosely"-adherent bacteria on leaf surfaces have been recovered and subsequent gene expression tested previously, with low bacterial RNA recovery for "strongly"-adherent bacteria (van der Linden, 2014).

The utilisation of arabinose in the plant extracts is an interesting response from the bacteria, as it not only shows the use of plant components for the growth of the bacteria but arabinose has also been implicated as playing a role in the adherence of the bacteria to spinach. Recent work has shown that an adherence factor known as *E. coli* common pilus (ECP) present in *Ec*O157 Sakai binds to arabinosyl residues present in pectin (Rossez *et al.*, 2014). Furthermore, a greater number of bacteria were found to attach to spinach tissue compared to lettuce, supporting differences in terms of the colonisation potential of these plants by human pathogenic bacteria. The link between the utilisation of arabinose via the *araBAD* enzymes and adherence to arabinosyl residues within the plant cell via ECP is at present unclear, therefore further work will be required. Deletion mutants for *araBAD* were made during this project in *Ec*O157 Sakai and in *E. coli* K-12; therefore, there is potential for a future project to check if the inability to metabolise arabinose is important for plant adherence/colonisation.

Studies have shown that arabinose will only be used by *E. coli* when glucose is not available, under a carbon catabolite repression mechanism (Miyada *et al.*, 1984), hence why no induction of these genes was seen in the spinach leaf lysates, which were rich in glucose (Chapter 4). Very little free glucose was detected in the spinach root exudates, therefore induction of the arabinose genes occurred. Furthermore, Desai and colleagues found that arabinose was also preferentially used by *E. coli* over xylose (Desai & Rao, 2010). Therefore, *E. coli* will use any free glucose that is present within the plant tissue first, before moving on to arabinose and finally xylose. Due to these catabolite repression mechanisms in *E. coli*, we are able to infer details of the nutrient status of the different extracts: the leaf lysates are plentiful in sugars such as glucose, which is why we do not see induction of arabinose or xylose genes in this condition. In contrast, in the spinach leaf cell wall polysaccharides condition, *Ec*O157 Sakai switches on genes for L-arabinose degradation after the one hour incubation period, suggesting no glucose is available at this time. These arabinose genes are not induced in lettuce, either do the presence

of glucose or the absence of arabinose – this is unclear at this time. Finally, the spinach root exudates are the least nutrient-accessible condition, with *E. coli* cells switching on arabinose gene expression and a sub-set of the population switching on xylose metabolism genes. Therefore the bacteria are scavenging as much as possible in this environment in order to survive. As mentioned previously, this may highlight the importance of other plant-associated microbes that could degrade many plant substrates into more accessible forms of the sugars that the bacteria could use.Overall, for each of the conditions tested many metabolic pathways were seen to be induced in response to different components of the plant. Analysis of these metabolic pathways will help give insight into how this human pathogenic bacterial strain has adapted and can survive in the plant environment, for example via the use of plant polysaccharides such as arabinan. Furthermore, differences were seen in the metabolic profiles between extracts, such as between spinach leaf lysates and spinach leaf cell wall polysaccharides. This could be due to repression by compounds present in one extract yet not another; therefore, it is important to consider different responses to each part of the plant to help gain an overall understanding of the interaction.

As well as metabolism, many other bacterial processes were affected, as shown by the regulation overviews for each condition. Details of additional processes that may confer an advantage to survival in the environment are detailed in Chapter 7.

7 Other Notable Responses

7.1 Introduction

Incubation of *Ec*O157 Sakai with plant extracts for one hour resulted in very large transcriptomic changes, with most of the changes seen in the different areas of metabolism. In addition to temperate-dependent and plant-extract dependent changes in metabolism gene expression, several other notable genes of interest were differentially expressed in the five conditions. This included genes involved in the type three secretion system (T3SS), manipulation of the plant defence response, adherence factors and many hypothetical proteins. The response of each of these categories to the different plant extracts will be covered in detail in the relevant sections.

7.1.1 The role of the T3SS in planta

The T3SS is an important adherence and colonisation factor for many plant-associated and mammalian-associated bacteria, and is especially well-described for pathogenic bacteria (Büttner & He, 2009; Coburn *et al.*, 2007). The role of the T3SS is to inject bacterial effectors directly into the host cell, bypassing the bacterial and host cell membranes. These bacterial effectors manipulate several processes of the host cell, such as effectors ExoS and ExoT in *Pseudomonas aeruginosa*, disrupting the actin cytoskeleton of human epithelial cells (Engel & Balachandran, 2009). As the T3SS is utilised by bacteria in the colonisation of a wide variety of hosts it has been suggested that this system is not only important for the pathogenic lifestyle, but also symbiotic relationships and opportunistic infections (Preston, 2007).

For enteric pathogens such as *Salmonella* and *Ec*O157, the T3SS has been determined as an important virulence factor for the colonisation of intestinal epithelial cells and subsequent infection in humans. Induction of the characteristic attaching and effacing (A/E) lesions in mammalian tissue by *Ec*O157 is mediated by T3SS effectors, which are encoded on a genomic island known as the locus of enterocyte effacement (LEE); however there are some T3SS

effectors that are located outside of this genomic island, termed non-LEE encoded (*nle*), similar to the T3SS-1 of *Salmonella* (Tobe *et al.*, 2006).

While the importance of the T3SS has been demonstrated for interaction with human intestinal epithelial cells for enteric pathogens, the role of the T3SS in these bacteria for plant colonisation is still unclear. Therefore, the expression of several T3SS genes encoding for regulators, structural proteins and effectors was investigated when in the presence of plant extracts to test if this system is induced in the presence of plant material.

7.1.2 Evading the Plant Defence Response

A dual role for the T3SS was postulated in adherence and in suppressing the plant immune response, as seen by the development of HR in T3SS deficient strains of *S. enterica* (Schikora *et al.*, 2012b). While the mechanism of action of *Ec*O157 T3SS effectors *in planta* is still unknown and even whether they are secreted, there are other factors that have been implicated in both the activation and suppression of the plant defence response.

The plant immune response is complex, consisting of a basal defence response recognising pathogen-associated molecular patterns (PAMPs), including flagellin and lipopolysaccharides, and specific ETI, induced by factors such as effectors (Jones & Dangl, 2006). Few studies have looked at the response of the plant to colonisation by human pathogens such as *Salmonella* and *Ec*O157, with much of the focus centred on the bacterial adaptation. One study demonstrated that an inoculum as low as 2 cfu/plant of *Ec*O157 or *Salmonella enterica*, inoculated into the rhizosphere without mechanical disruption of the plant tissue, induced a defence response which was still observed after ten days incubation (Jayaraman *et al.*, 2014). Genes upregulated in response to *Ec*O157 colonisation included three pathogen resistance (PR) genes and two peroxidases. Differential gene expression was also seen in defence genes such as cell wall modification components, protein kinases and plant hormones (Jayaraman *et al.*, 2014). Both bacterial species were seen to induce similar responses from the plant, suggesting perception of conserved PAMPs.

The plant innate immunity has been reported to be the main point of defence against these bacterial species (Melotto *et al.*, 2014). Flg22, a conserved motif in the N-terminal of flagellin, in *Salmonella enterica* has been reported to induce the plant immune response in *Arabidopsis* as it is a PAMP (Garcia *et al.*, 2013). *Salmonella* flg22 has also been reported to induce the production of reactive oxygen species (ROS) in tomato leaves (Meng *et al.*, 2013).

As well as this, *PR1* gene expression in *Arabidopsis* was measured in response to either *Ec*O157 or *Salmonella* colonisation. Both species were seen to induce expression of this gene, albeit at different levels, with *Salmonella*-infected plants having lower expression of *PR1* (Roy *et al.*, 2013). Many studies have reported suppression of the plant immune system by *Salmonella* in order for it to survive and colonise the plant environment, although to date there is no evidence of how this occurs. Many reports suggest the use of T3SS effectors to suppress the immune response for *Salmonella* (Schikora *et al.*, 2012a); however, perhaps there are other mechanisms present in the bacteria to manipulate the plant immune response.

The use of these alternative mechanisms is the proposed case for *Ec*O157, as this study identified several metabolic pathways present in *Ec*O157 Sakai that appeared to play a role in surviving in the plant environment (Chapter 4, section 4.2.1), including in the synthesis and degradation of plant hormones. Some pathways have been identified as present in *Ec*O157 Sakai yet absent in *Ec*O157 EDL933, whereas others are found in both; those pathways present in both species in *Ec*O157 may be indicative of an important pathway for human pathogen survival in plants. It is hypothesised that expression of these pathways may play an important role in evading the plant immune response when colonising the plant environment.

7.1.3 Adherence factors present in EcO157

*Ec*O157 strains possess a variety of adherence factors to allow them to colonise host tissue, whether this is mammalian or plant. Virulence factors such as the Shiga-like toxins (*stx1* and *stx2*) present in *Ec*O157 that facilitate the adherence, colonisation and subsequent infection of human epithelial cells have not been implicated as playing a role *in planta*. However, other

factors involved in adherence and motility of *Ec*O157 have been implicated in the colonisation of plant tissue, such as *E. coli* common pilus (ECP) fimbria, curli and flagella.

ECP has been shown to mediate the adherence of *Ec*O157 strains to not only human epithelial cells (Rendón *et al.*, 2007) but also in plant tissue. One study found that deletion of *ecpA-D* genes resulted in a reduced level of adherence in *Ec*O157 Sakai to both spinach leaves and roots (Rossez *et al.*, 2014). As well as this, ECP has been shown to play a role in biofilm formation as *ecpR* inhibits flagellar expression by repressing *flhDC* (Lehti *et al.*, 2012).

As well as ECP, another fimbria known as curli, the main structural subunit of which is encoded for by *csgA*, has been shown in several studies to play an important role for plant colonisation. Mutants in *csgA* in *Ec*O157 were unable to adhere to the leaves of spinach to the same level as the wild type, although some adherence did still occur (Macarisin *et al.*, 2012; Saldaña *et al.*, 2011).

Finally, flagella are also required for the adherence to plant tissue by *Ec*O157 (Rossez *et al.*, 2013; Saldaña *et al.*, 2011) and by *Salmonella* (Berger *et al.*, 2009). Interestingly, deletion of *fliC*, encoding for flagellin, was only seen to affect the adherence of *Salmonella enterica* sv. Senftenberg, a clinical isolate from a basil outbreak, to plant tissue and not *S. enterica* sv Typhimurium (Berger *et al.*, 2009).

As individual deletions of the three adherence factors have shown a reduction but not a complete loss in adherence it is assumed that bacterial adherence to plant tissue is mediated by a concert of these factors. Previous studies have demonstrated plant extracts such as root exudates may prime bacteria for colonisation of plant tissue (Mark *et al.*, 2005), therefore it is hypothesised that the plant extracts used in this study would induce the expression of these factors after a one-hour exposure. However, it is worth noting that there are potentially other adherence factors present in *Ec*O157 that have not been studied yet and therefore many hypothetical proteins may play a role in this process.

7.1.4 Expression of Hypothetical Proteins

One final category of interest of genes that may be differentially expressed in response to the plant environment is those encoding for hypothetical proteins, or proteins of unknown function. As the name suggests, no experimental evidence is present for these genes to determine their role, therefore analysis is limited. However, the nucleotide sequence can be compared to other closely-related species to try and determine a possible function. Those of the most interest however could be any genes that are specific to *Ec*O157 Sakai, as they could potentially encode factors that enable this bacterial strain to persist in the plant environment at a higher level than other strains such as *Ec*O157 TUV 93-0. Therefore, identification of hypothetical proteins induced in response to the plant environment may provide examples of important genes required for plant colonisation by this bacterial species.

7.1.5 Aims of this Area of Study

A. To assess the expression changes in the T3SS (LEE and *nle*) of *Ec*O157 Sakai in response to temperature and plant extracts.

B. To examine the expression changes of genes involved in plant hormone degradation/synthesis when in the presence of plant material.

C. To assess the expression changes of genes encoding for and involved in the regulation of ECP, curli and flagella adherence factors when the bacteria are exposed to plant extracts.

D. To identify any genes encoding for hypothetical proteins that are induced in response to plant extracts, including any that are present in *Ec*O157 Sakai and not in *Ec*O157 TUV 93-0.

7.2 Results

7.2.1 Expression of the T3SS

As other transcriptomic studies of enteric pathogens in the plant environment had found an increase in expression of the T3SS *in planta*, it was hypothesised that these genes would be induced in response to the different plant extracts tested. Firstly, transcriptomic analysis of the response of *Ec*O157 Sakai in response to an environmentally relevant temperature was assessed to identify any temperature-induced genes that may affect the results of the plant extract transcriptomic datasets.

T3SS-related genes were selected on the basis of genome annotations and from published reports. Genes related to the T3SS system of *Ec*O157 Sakai were downregulated after a one hour incubation in minimal media with 0.2 % glycerol at 18 °C compared to 37 °C (**A**, Figure 7-1). Six of the T3SS-associated genes were selected to show the overall expression profile: three from the LEE, *escN* (the main structural subunit of the T3SS), *espA* (an effector) and *ler* (one of the master regulators of the T3SS); and three that were non-LEE-encoded, *nleH1*, *nleA* and *nleH2* (all effectors). All six genes were downregulated at 18 °C compared to 37 °C, with the largest difference seen for *ler,* which was downregulated 130-fold. The remainder of the T3SS genes showed a similar expression profile to the six represented, with all downregulated at the lower temperature.

To identify any upregulation of the T3SS in response to plant extracts, expression in minimal media at 18 °C was used as a base-line to remove any temperature-specific effects. No significant change in gene expression for any T3SS genes was seen in *Ec*O157 Sakai incubated for one hour with spinach leaf lysates, spinach root exudates, and spinach or lettuce leaf cell wall polysaccharides. Interestingly, this coincided with results produced by another lab member who found no significant change in expression of the T3SS for *Ec*O157 Sakai adhering to the surface of spinach leaves (van der Linden, 2014).

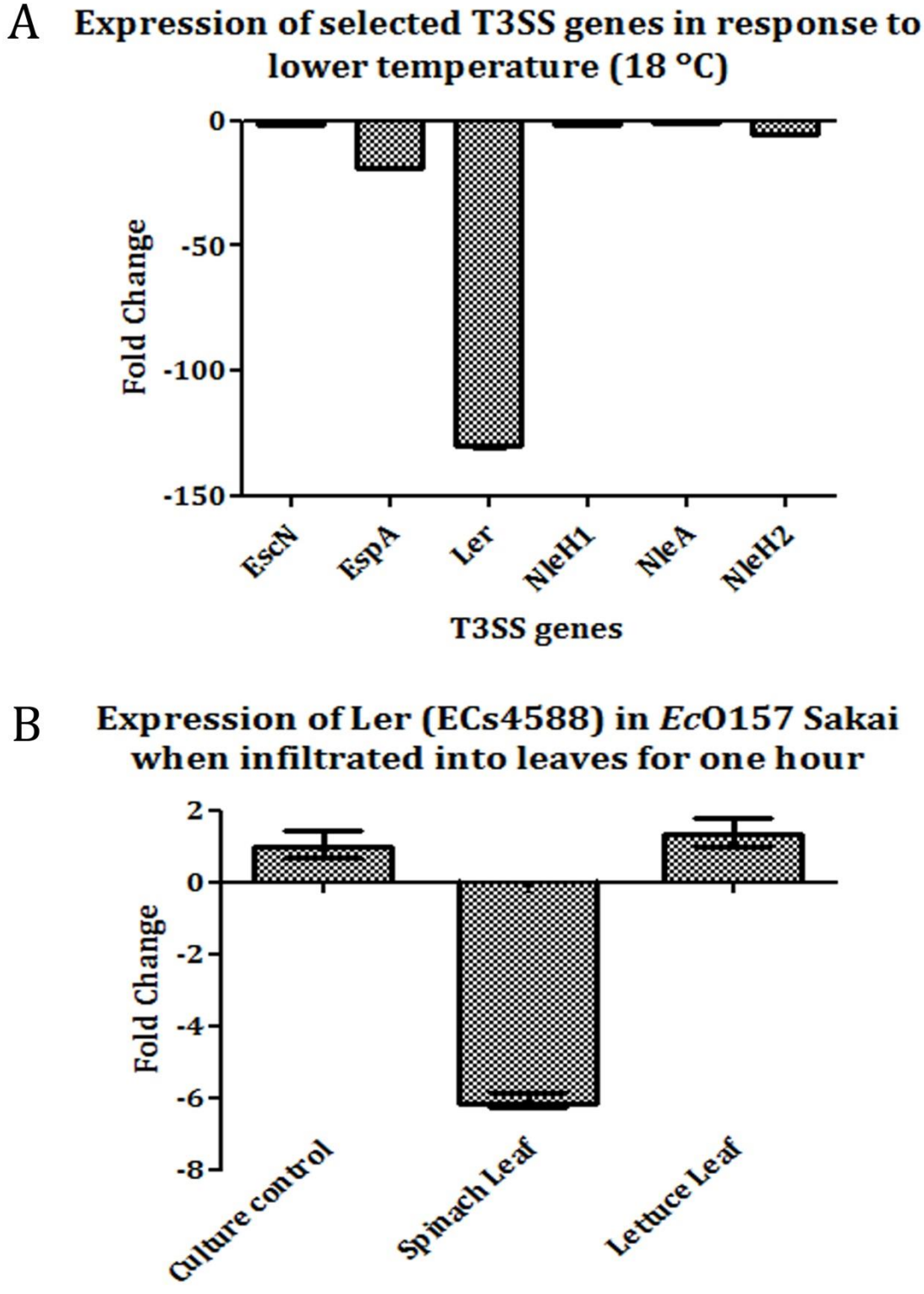


Figure 7-1. Expression of T3SS genes in *Ec*O157 Sakai in response to temperature and the plant environment.

A) The expression of six selected T3SS genes was assessed when *Ec*O157 Sakai was incubated at 18 °C compared to 37 °C for one hour in minimal media. *N* = 4. **B)** The expression of *ler* (*ECs4588*) was examined using qPCR after one hour incubation in infiltrated spinach and lettuce leaves compared to an incubation buffer culture control. *N* = 3. Error bars represent the standard error of the mean.

As this was in contrast to work that had been previously published demonstrating the importance of the T3SS for plant colonisation (Shaw *et al.*, 2008), I decided to carry out gene expression analysis of *ler* when infiltrated into spinach and lettuce leaves, as perhaps the extracts were not a suitable inducer for this system. Infiltration of the bacteria into the leaves allowed us to study the expression of a more homogeneous population, whereas inoculation of the surface may result in sub-populations adhering to the surface and others internalising, giving mixed expression profiles. *Ec*O157 Sakai was suspended in a $MgCl_2$ infiltration buffer and infiltrated into the apoplastic space of the leaves of spinach and lettuce (*L. sativa*), incubated for one hour at 20 °C under glasshouse conditions and the bacterial RNA extracted for qPCR analysis.

Quantification of the *ler* gene by qPCR revealed no significant change when *Ec*O157 Sakai was infiltrated into lettuce, compared to a $MgCl_2$ infiltration buffer only culture control (**B**, Figure 7-1), whereas infiltration into spinach leaf apoplast resulted in 6-fold downregulation of expression, compared to the control.

To confirm these transcriptomic results and test the hypothesis that the T3SS does not play a role in plant colonisation an *escN* knockout in *Ec*O157 Sakai was made (Ashleigh Holmes) for on-going experimentation.

7.2.2 Plant Defence Evasion/Manipulation

Induction with different plant extracts showed induction of certain metabolic pathways in *Ec*O157 Sakai involved in the utilisation and degradation of plant compounds, such as components of the cell wall (Chapter 6). This led us to investigate the response of *Ec*O157 Sakai to other plant compounds, such as those related to the plant defence response.

Three metabolic pathways were found in *Ec*O157 Sakai that involved the degradation of the plant hormones salicylate (salicylic acid; SA), methylsalicylate and chlorosalicylate (Figure 7-2). The degradation of all three of these plant hormones is mediated by a single enzyme, *ECs3027*. The expression of this enzyme was only upregulated in response to spinach root exudates,

where it was upregulated 2.37 fold. No differential expression of this enzyme was observed in response to temperature, spinach leaf lysates or spinach/lettuce leaf cell wall polysaccharides.

Two metabolic pathways were identified in *Ec*O157 Sakai that are not predicted to be present in a different *Ec*O157 isolate, EDL933. These were also involved in the potential manipulation of plant hormones, but via the synthetic pathways rather than the degradation. Both pathways result in the synthesis of indole-3-acetate (indole-3-acetic acid; IAA), either from L-tryptophan (**A**) or indole-3-acetonitrile (**B**) (Figure 7-3). Four enzymes are involved in the synthesis of IAA from the common intermediate indole-3-acetamide: *ECs0664*, *ECs3674*, *ECs3926* and *ECs5162*. Probes for these enzymes were not present on the commercial Agilent *E. coli* v2 microarray used in this project; therefore the expression of these enzymes in response to plant extracts and temperature could not be determined in this manner. However, as RNA-Seq analysis was carried out on the samples derived from inoculated spinach leaf lysates, the expression of these enzymes in response to these plant extracts was calculated. Three of the four enzymes were significantly differentially expressed in response to spinach leaf lysates: *ECs0664* and *ECs3926* were downregulated 2.16 fold and 2.62 fold respectively, whereas *ECs3674* was upregulated 2.06 fold.

Primers were designed to *ECs0664* and *ECs3674* to determine the expression of these genes by qPCR when *Ec*O157 Sakai was infiltrated into the leaves of lettuce and spinach. However, only the primers for *ECs0664* passed the qPCR quality control tests for efficiency. As no evidence has been reported on the regulation of these enzymes I decided to test the expression of *ECs0664 in planta*, despite it showing downregulation in response to spinach leaf lysates. *Ec*O157 Sakai was infiltrated into spinach or lettuce leaves in a $MgCl_2$ buffer and left for one hour. *Ec*O157 Sakai incubated in $MgCl_2$ buffer for one hour was used as a control. The expression of *ECs0664* in *Ec*O157 Sakai in lettuce or spinach leaves was not significantly different to that of the culture control. As a result, it is still unclear what induces these metabolic pathways in *Ec*O157 Sakai and if they play an important role in the survival of this bacteria in the plant environment.

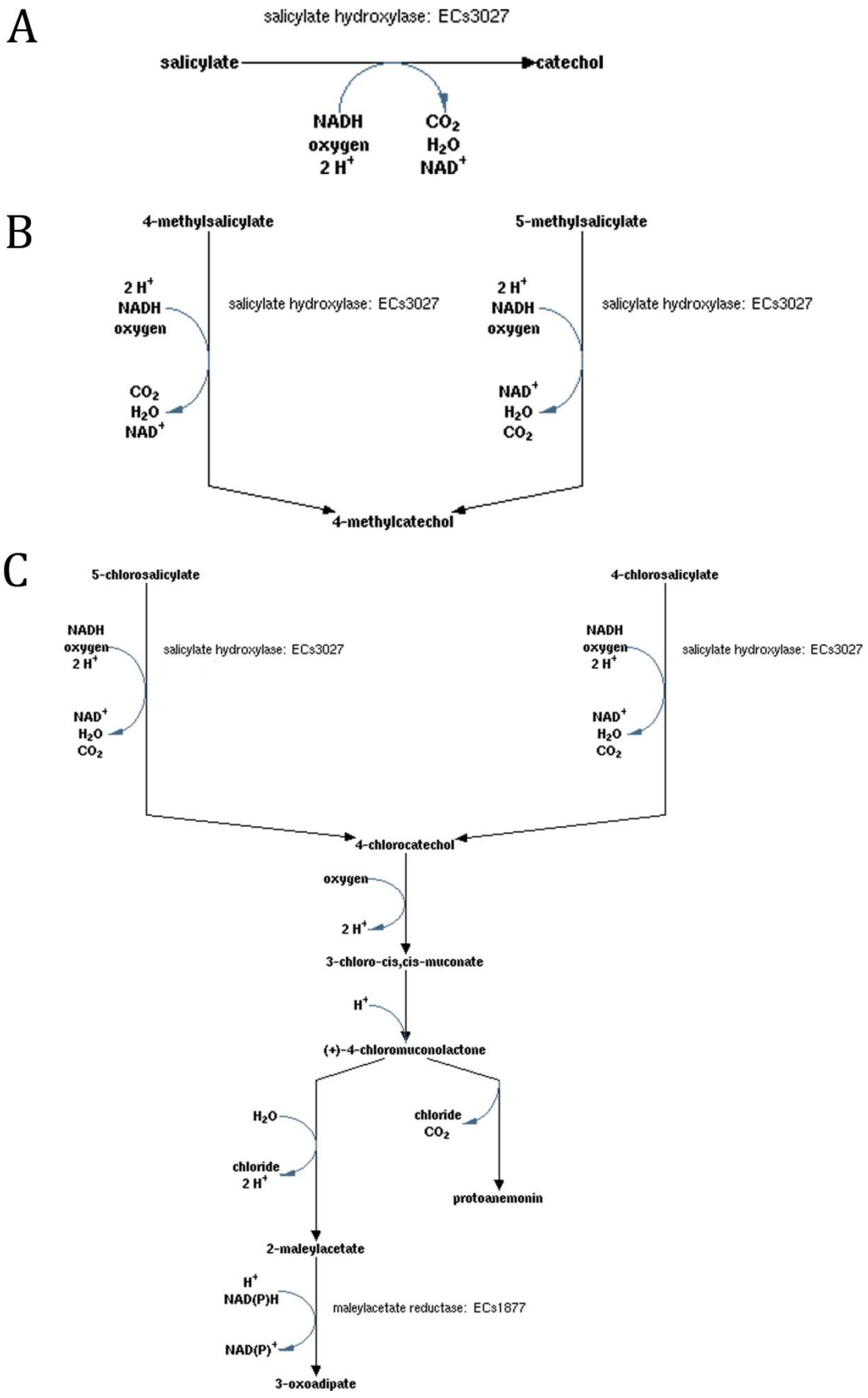


Figure 7-2. Metabolic pathways involved in the degradation of salicylate.

The enzyme *ECs3027* is involved in the pathways salicylate degradation (**A**), methylsalicylate degradation (**B**) and chlorosalicylate degradation (**C**). Pathways were generated using EcoCyc (Keseler *et al.*, 2013).

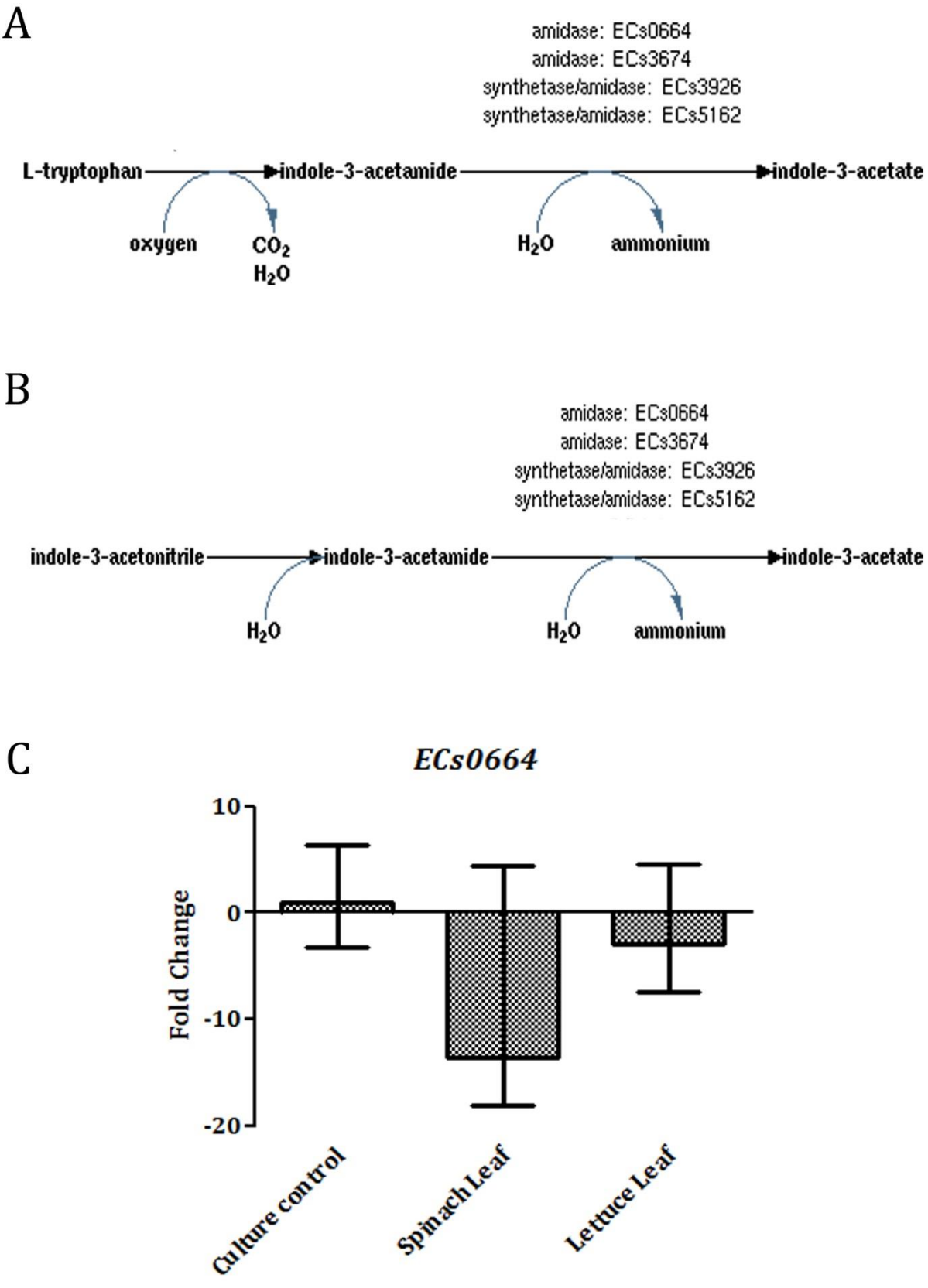


Figure 7-3. Metabolic pathways producing IAA and their expression *in planta*.

Two metabolic pathways present in *Ec*O157 Sakai, known as indole-3-acetate biosynthesis III (**A**) and IV (**B**) that result in the production of IAA (auxin) hormone. Gene expression of *ECs*0664 enzyme present in both these pathways was measured by qPCR after *Ec*O157 Sakai was infiltrated into spinach and lettuce leaves for one hour (**C**). *N* = 3. Error bars represent the standard error of the mean.

7.2.3 Expression of Selected Adherence factors

Fimbrial genes play an important role in the adherence of *Ec*O157 to different surfaces, including to host cells (Low *et al.*, 2006). An overview of the expression of fimbrial genes was determined using GeneSpring (GX), looking at those differentially expressed in the plant extract conditions (Figure 7-4). Four fimbrial genes were differentially expressed in response to spinach leaf lysates, and twelve in response to spinach root exudates. No difference in fimbrial gene expression was seen in the spinach or lettuce polysaccharide datasets. Many of the genes may not be annotated as fimbrial genes as the commercial microarray uses information from an *Ec*O157 Sakai dataset from 2001 (Agilent). Furthermore, fimbrial genes such as curli do not appear in the "fimbria" search as they have been renamed. The selection of adherence factors was therefore mostly based on published reports and eventually led to the selection of three adherence systems to be studied in further detail: ECP, curli and flagella. All three have been shown in previous studies to facilitate adherence of *Ec*O157 to plant tissue, therefore it was hypothesised that these genes may be induced when *Ec*O157 Sakai was exposed to plant extracts.

As a consequence of the Agilent (*E. coli* v2) microarray design, some genes present in the *Ec*O157 Sakai genome are not represented on the microarray and it was found that this was the case for all of genes in the *ecp* operon, expect for *ecpD*, which was shown to be induced in response to spinach root exudates (Figure 7-4). However, the level of expression was low and examination of the RNA-Seq dataset for spinach leaf lysates showed that none of the genes involved in ECP synthesis, function and regulation were differentially expressed > 2-fold compared to the 0.2 % glycerol control.

Expression of the main curli subunit, *csgA*, was downregulated in response to the lower temperature, as well as spinach leaf lysates and lettuce leaf cell wall polysaccharides (Table 7-1). In contrast, the expression of *csgA* and *csgB* was induced over 3.5 fold when the bacteria were inoculated into media containing spinach root exudates. *csgE* was the only curli gene that was differentially expressed in response to polysaccharides: upregulated in spinach polysaccharides

and downregulated in lettuce polysaccharides. The only curli gene that was induced in the lettuce polysaccharide was the regulator *crl*, whereas the other genes were downregulated in this condition.

Finally, the expression of genes involved in the assembly, structure, function and regulation of flagella was examined for each of the conditions tested in this study (Table 7-1). The class II master regulator *flhD* was induced in spinach polysaccharides and both regulators *flgE* and *fliN* were induced in lettuce polysaccharides. In contrast, genes were not differentially expressed or were downregulated for spinach leaf lysates, with spinach root exudates, or at 18 °C. The polysaccharide extracts appeared to be the only condition in which induction of flagellar genes occurred, although *fliC* was not seen to be significantly expressed in either of the polysaccharide conditions.

To confirm this expression profile seen on the microarray, quantitative analysis of *flhD* by qPCR was carried out with *Ec*O157 Sakai inoculated with spinach leaf lysates, spinach root exudates and lettuce polysaccharides. The same pattern of expression was seen for the qPCR analysis as for the microarray, with down regulation of *flhD* occurring in spinach lysates and root exudates but upregulation of this gene when in the lettuce polysaccharides (**A**; Figure 7-5). Therefore the results of the microarray were verified by the qPCR analysis, showing that the polysaccharide condition induces flagellar gene expression whereas the leaf lysates and root exudates repress these genes.

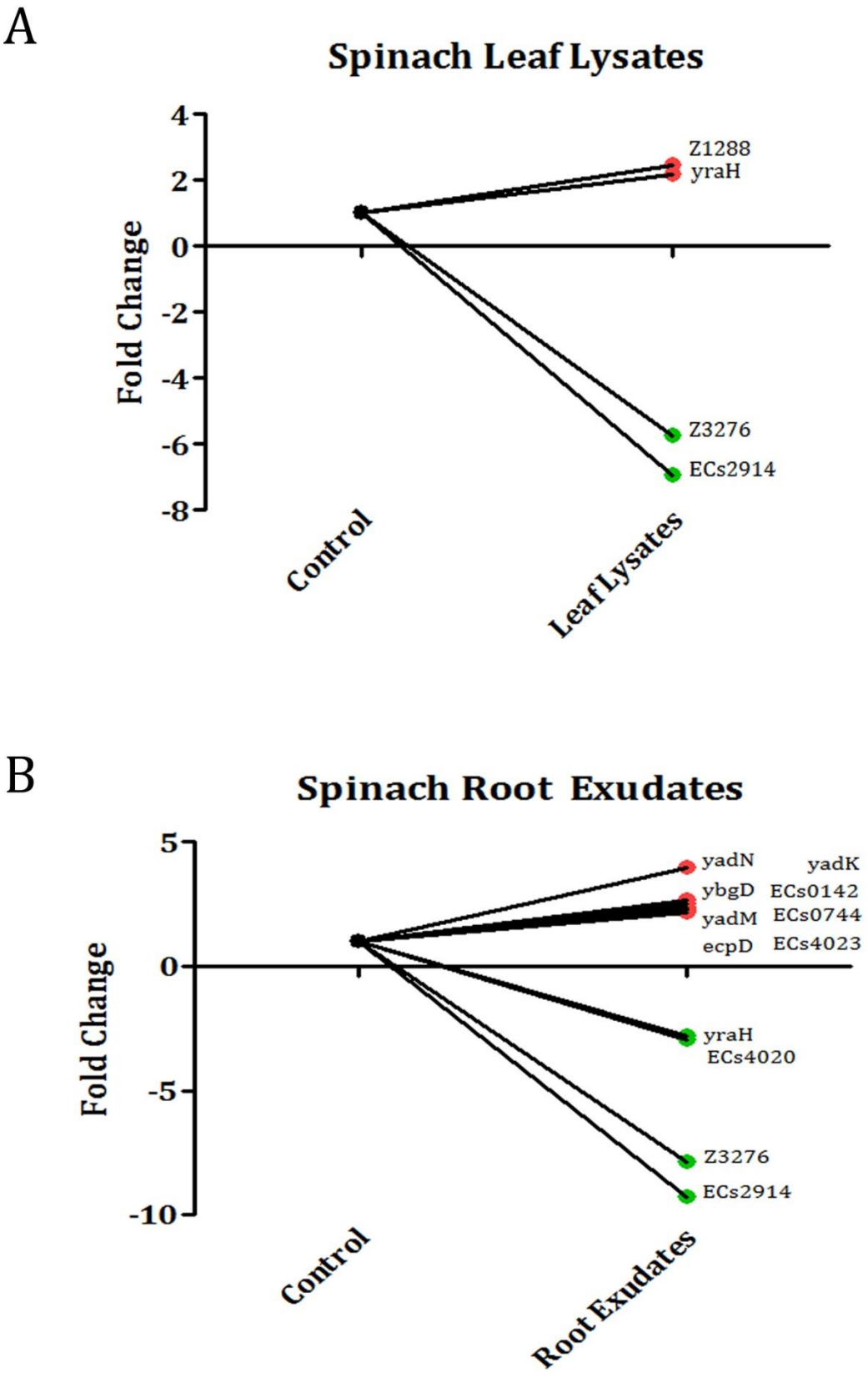


Figure 7-4. Fimbrial genes that were differentially expressed in *Ec*O157 Sakai in spinach leaf lysates and root exudates.

Genes that were significantly expressed and annotated as "fimbrial" genes were identified in GeneSpring (version 7.0) for the **A)** spinach leaf lysates and **B)** the spinach root exudates datasets. The control dataset was normalised to 1 and values represent the fold change in expression of each selected gene. Each gene is coloured red for upregulated and green for downregulated expression.

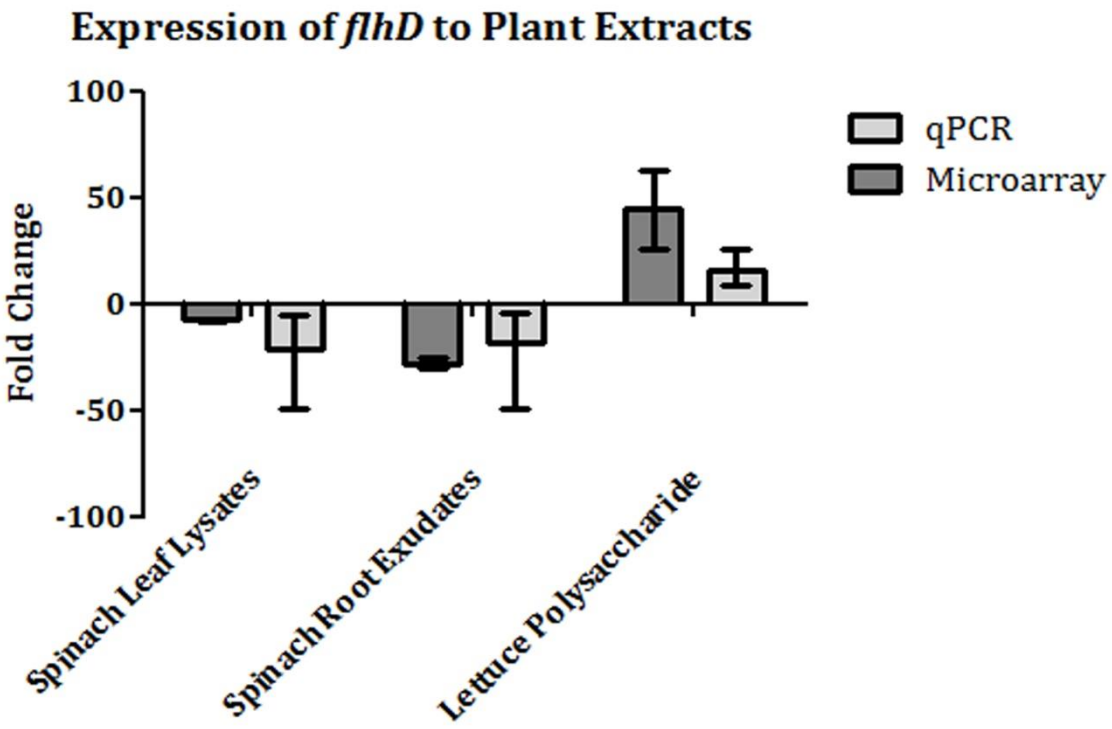
Table 7-1. Expression of selected flagellar and curli genes in the five transcriptomic datasets of *Ec*O157 Sakai.

NS denotes genes that were not expressed significantly different from the control.

		Fold Change (Results from Microarray)				
Adherence Factor	***Gene Name***	***Temperature***	***Spinach Leaf Lysates***	***Spinach Root Exudates***	***Spinach Polysaccharides***	***Lettuce Polysaccharides***
Curli	*csgA*	-3.78	-2.24	3.97	NS	-2.34
	csgB	-2.94	NS	3.55	NS	-2.74
	csgC	NS	NS	NS	NS	NS
	csgD	NS	NS	NS	NS	-2.26
	csgE	NS	NS	NS	2.26	-2.37
	csgF	NS	NS	NS	NS	NS
	csgG	NS	NS	NS	NS	NS
	crl	NS	NS	NS	NS	7.38
Flagella	*flgA*	NS	NS	-2.48	NS	NS
	flgB	-59.39	NS	NS	NS	NS
	flgC	-40.47	NS	NS	NS	NS
	flgD	-31.37	NS	NS	NS	NS
	flgE	-.26.47	NS	NS	NS	3.36
	flgF	-11.57	NS	NS	NS	NS
	flgG	-10.58	NS	NS	NS	NS
	flgH	-17.32	NS	NS	NS	NS
	flgI	-8.01	NS	NS	NS	NS
	flhC	NS	-9.17	-11.93	NS	15.98
	flhD	NS	-7.43	-27.84	2.21	44.36
	fliC	NS	NS	NS	NS	NS
	fliE	-26.37	NS	NS	NS	NS
	fliF	-9.86	NS	NS	NS	NS
	fliG	-7.81	NS	NS	NS	NS
	fliL	-16.28	NS	NS	NS	NS
	fliM	-14.85	NS	NS	NS	NS
	fliN	-10.69	NS	NS	NS	4.83

To test the expression of this gene *in planta*, *Ec*O157 Sakai was infiltrated into the leaves of spinach and lettuce in $MgCl_2$ infiltration buffer and incubated for one hour. *Ec*O157 Sakai in $MgCl_2$ buffer only was used as a control. The bacterial RNA was extracted from the leaves and analysed by qPCR. In contrast to the observation for the polysaccharide extract microarray data, expression of *flhD* was down regulated when *Ec*O157 was infiltrated into spinach and lettuce leaves, with similar levels of expression seen for both samples (**B**; Figure 7-5).

A



B

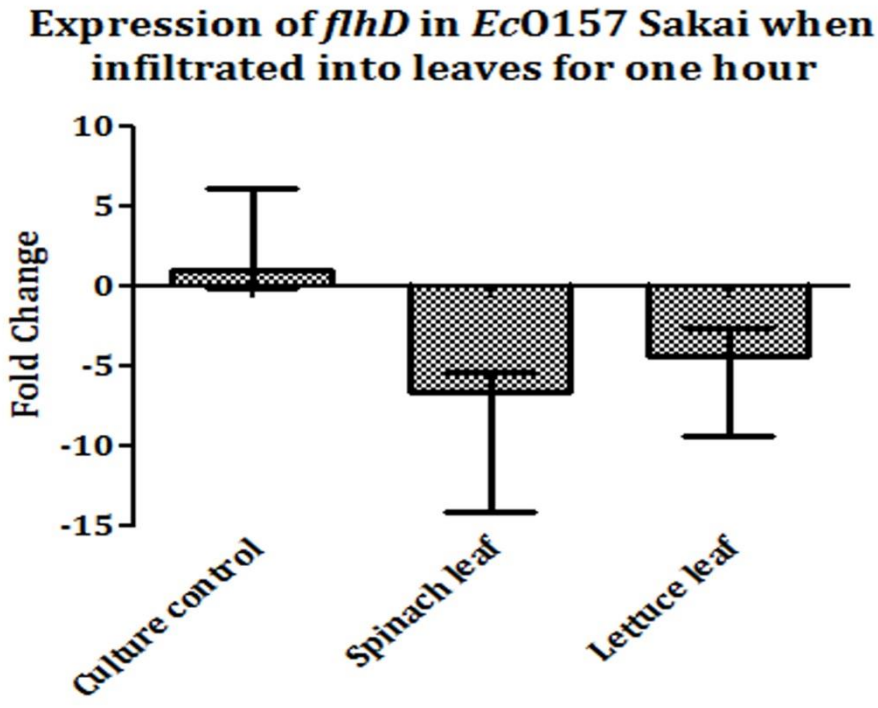


Figure 7-5. Expression of *flhD* in *Ec*O157 Sakai inoculated into plant extract media and infiltrated into leaves.

A) qPCR analysis of *flhD* in *Ec*O157 Sakai samples that were exposed to spinach leaf lysates, spinach root exudates or lettuce leaf cell wall polysaccharides for one hour. **B)** qPCR analysis of *flhD* in *Ec*O157 Sakai samples that were inoculated into $MgCl_2$ buffer and infiltrated into spinach or lettuce leaves for a one hour incubation. *Ec*O157 Sakai in $MgCl_2$ buffer only was used as a culture control.

7.2.4 Hypothetical-genes expression

One category of genes that were seen to be differentially expressed in all five of the conditions tested by microarray in Chapter 5 were those classed as hypothetical. A total of 245 hypothetical genes (18.37 % of the total expression change) were found to be differentially expressed in *Ec*O157 Sakai when incubated at 18 °C compared to 37 °C, indicating temperature sensitivity. A high proportion of the hypothetical genes were differentially expressed in response to plant extracts, with 216 (13.35 %) genes expressed in response to spinach leaf lysates and 277 (13.28 %) to spinach root exudates. A slightly lower percentage of hypothetical genes were differentially expressed in response to spinach leaf cell wall polysaccharides (7 genes; 6.6 %) and lettuce leaf cell wall polysaccharides (36 genes; 8.53 %).

There was no overlap for differential expression for any of the hypothetical genes between all five of the conditions. However, gene *ECs2489*, which is conserved within *E. coli* and *Shigella,* was differentially expressed in response to all of the plant extracts (Table 7-2). Other genes that were significantly upregulated in response to plant extracts included *ECs2473*, which was only upregulated in response to lettuce leaf cell wall polysaccharides, and *ECs2304* which was induced in response to spinach leaf lysates and root exudates. BLAST analysis was carried out to identify any sequence homologues and showed that many of the genes that were differentially expressed in response to plant extracts were similar to phage proteins present in other *Escherichia* species or in different phage (Table 7-2). Genes *ECs1375, ECs2713, ECs4970, ECs4976* and *ECs5247* are specific to *Ec*O157 Sakai i.e. not present in *E. coli* K-12 or in *Ec*O157 EDL933, located within genomic islands (see Appendix File S-1).

Table 7-2. Expression of selected genes of *Ec*O157 Sakai encoding for hypothetical proteins in five transcriptomic datasets.

Homologous genes were checked for in *E. coli* K-12 and *Ec*O157 EDL933 using EcoCyc comparative browser. The nucleotide sequence for each gene was compared to the NCBI database using the BLAST feature, searching for highly similar sequences outside of *E. coli*. Results shown display the top hit from the results of the BLAST search and the % of the coding nt sequence query that was identical to the target. The expression of each gene was measured by microarray analysis. Upregulated genes are depicted by red shading and downregulated by green shading (> 2-fold).

					Fold Change (Results from Microarray)				
Gene Name	***In K-12***	***In EDL933***	***Output from BLAST comparisons***	***% Hit***	***18 °C***	***Spinach Leaf Lysates***	***Spinach Root Exudates***	***Spinach P/S***	***Lettuce P/S***
ECs0317	✔	✔	Membrane protein - *Escherichia albertii* KF1	97	NS	11.85	4.81	NS	NS
ECs0844	*x*	✔	Phage tail fibre protein - *Escherichia albertii* KF1	94	-2.82	NS	NS	NS	NS
ECs0845	*x*	✔	Unknown - phages HUN/2013, TL-2011C, Min27	98	NS	NS	5.00	NS	NS
ECs0988	*x*	✔	Conserved hypothetical protein YeaO - *Shigella flexneri*	99	NS	2.49	-18.16	NS	12.06
ECs1254	*x*	✔	Putative enzyme - *Shigella dysenteriae*	90	NS	NS	NS	-4.92	-8.98
ECs1335	*x*	✔	Unknown - *Shigella flexneri* plasmid pSFxv_1	90	NS	3.42	NS	NS	4.53
ECs1375	*x*	*x*	No highly similar BLAST hits outside *Escherichia coli*	-	NS	2.87	NS	NS	NS
ECs1653	*x*	✔	Hypothetical protein - *Citrobacter freundii* CFNIH1	95	14.01	-15.29	9.20	NS	-13.23
ECs1654	*x*	✔	Hypothetical protein - *Citrobacter freundii* CFNIH1	94	27.78	-32.08	8.79	NS	-71.95
ECs1655	✔	✔	Hypothetical protein - *Citrobacter freundii* CFNIH1	94	23.09	-18.13	8.85	NS	-8.21
ECs2304	✔	✔	Conserved hypothetical protein - *Shigella dysenteriae* Sd197	99	NS	12.97	20.21	NS	-3.27
ECs2473	*x*	✔	Hypothetical protein - *Escherichia albertii* KF1	81	NS	NS	NS	NS	11.65
ECs2489	✔	✔	Conserved hypothetical protein YeaD - *Shigella dysenteriae* Sd197	99	NS	-3.60	-2.96	2.50	3.30
ECs2713	*x*	*x*	Putative cytochrome - *Shigella boydii*	97	7.25	-2.89	NS	NS	-4.18
ECs2940	*x*	*x*	Phage tail fibre protein - *Escherichia albertii* KF1	99	NS	NS	4.72	NS	NS
ECs3238	*x*	✔	No highly similar BLAST hits outside *Escherichia coli*	-	-5.31	NS	11.80	NS	-2.29
ECs3521	✔	✔	FAD dependent oxidoreductase CsiD – *Shigella boydii*	97	17.66	-12.88	NS	NS	-8.91
ECs3750	✔	✔	Conserved hypothetical protein – *Shigella boydii* Sb227	99	NS	NS	34.86	NS	-12.47
ECs4115	✔	✔	AaeX protein – *Shigella flexneri* 2002017	99	NS	24.15	2.86	NS	NS
ECs4474	✔	✔	Conserved protein YibI – *Shigella flexneri* Shi06HN006	99	-9.92	-3.10	267.91	NS	-92.50
ECs4491	✔	✔	M23 peptidase domain protein – *Shigella boydii* CDC 308394	98	NS	NS	-19.12	NS	10.08
ECs4970	*x*	*x*	Galactidol-1-phosphatol dehydrogenase – *Citrobacter rodentium*	94	NS	NS	NS	NS	4.87
ECs4976	*x*	*x*	Galactidol-1-phosphatol dehydrogenase – *Citrobacter rodentium*	92	-10.59	NS	NS	NS	7.13
ECs5165	✔	✔	Biofilm stress and motility protein A – *Shigella flexneri* Shi06HN006	99	NS	-6.01	60.15	NS	-7.03
ECs5247	*x*	*x*	Putative magnesium transporter – *Pantoea ananatis* AJ13355	97	4.51	NS	NS	NS	NS

Thus several genes with unknown function are differentially expressed in response to each of the five conditions tested within this study, some of which are specific to *Ec*O157 species and some which are present on genomic islands. BLAST analysis of these genes found similar hits to many *Shigella* species and some environmental species, such as *ECs5247* which was similar to a gene of *Pantoea ananatis*. These genes could be important for the utilisation of plant extracts and the colonisation of plants by *Ec*O157; however, further work will need to determine their function.

7.3 Discussion

Whole transcriptomic analysis enables differential gene expression of every functional class to be examined. As well as large changes in metabolism, gene expression changes in functional groups relating to the T3SS, adherence, plant defence compounds and those genes with an as yet undefined function were observed when *Ec*O157 Sakai was incubated with different plant extracts. Each of these different groups will be discussed separately because they are functionally quite distinct from each other.

7.3.1 The T3SS is not induced upon exposure to the plant environment

The T3SS has been shown to play an important role in symbiotic, commensal and pathogenic bacterial strains to both mammalian and plant tissue (Preston, 2007). For human pathogenic bacteria within the plant environment, mixed reports have emerged. Some studies have reported the necessity of the T3SS for *in planta* survival, as mutants of *Salmonella typhimurium* in both of the T3SS were seen to induce the hypersensitive response (HR) when in *Arabidopsis*, whereas the wild type did not (Schikora *et al.*, 2012b). *Ec*O157 T3SS mutants were also seen to have a lower colonisation and internalisation potential in spinach leaves, whereas an *E. coli* K-12 carrying a plasmid encoding for the LEE resulted in 3.2–fold increase in bacterial counts *in planta* than the wild type (Saldaña *et al.*, 2011). The use of T3SS knockout mutants showed a decrease in viable bacteria *in planta* (Schikora *et al.*, 2011; Seo & Matthews, 2012).

One study demonstrated the T3SS was important for binding to the guard cells of rocket leaves via the EspA filament, encoded within the LEE (Shaw *et al.*, 2008). This difference was only seen when the bacterial strain TUV 93-0 was grown at 37 °C in LEE-inducing media and adherence did not occur in bacteria grown in LB at 37 °C or at 20 °C, leading to the conclusion that the T3SS was essential for stomatal adherence at 37 °C. This result together with the results found in this study showing repression of the T3SS at lower temperature suggests that the T3SS is not essential for stomatal colonisation at 18 – 20 °C. Analysis of selected effectors produced by *Salmonella* and *Ec*O157 were found to have high homology to those present in plant-associated bacteria such as *Pseudomonas syringae* (Tobe *et al.*, 2006). These results suggest the T3SS may

play an important role *in planta* – however, there is no reported evidence for the injection of T3SS effectors into plant cells. Furthermore, one study has suggested it is not possible for the T3SS of *Ec*O157 and *Salmonella* to penetrate the plant cell wall because of the fundamental difference in overall structure of the complex: in animal pathogenic bacteria the T3SS apparatus is relatively short and rigid, whereas in phytopathogens, the apparatus is longer and more flexible, presumably to enable penetration into the plant cell wall (He *et al.*, 2004). Therefore, not only is the necessity of the T3SS of animal pathogens unknown for plant cell interactions, but also its expression and function.

Published transcriptomic studies of *Ec*O157 in response to plant extracts/plant tissue reveal contradictory results regarding the T3SS. Short-term interactions appeared to induce expression, e.g. selected T3SS genes were upregulated in *Ec*O157 incubated with lettuce leaf lysates after a 15 minute and 30 minute exposure (Kyle *et al.*, 2010) and after a 10 minute incubation with apple juice media for both LEE- and non-LEE-encoded genes (Bergholz *et al.*, 2009). However, longer-term interactions had no effect, e.g. inoculation of *Ec*O157 to shredded lettuce leaves for one and three days (Fink *et al.*, 2012), or living plant roots for three days (Hou *et al.*, 2013) did not result in differential expression of the T3SS. Finally, another study found that expression of T3SS genes was downregulated in *Ec*O157 incubated with spinach leaf lysates after five hours of growth and when adhering to radish sprouts after a five day incubation (Landstorfer *et al.*, 2014). Differences between studies appear to be due to the incubation time, supporting the idea that the T3SS plays a role in the initial adherence of the bacteria (Shaw *et al.*, 2008). However, another important factor is the temperature in which the bacteria are cultivated. All studies listed above used bacteria cultured at 37 °C immediately prior to inoculating onto the plant/into plant extracts. As shown in this study, expression of the T3SS is very sensitive to temperature, with genes being downregulated at the lower temperature compared to 37 °C. Interestingly, these studies also used a short inoculation period for the *Ec*O157 strains with the plant environment, from 10 to 30 minutes (Bergholz *et al.*, 2009; Kyle *et al.*, 2010). Other studies that have cultured the bacteria at 37 °C that do not report differential expression of the T3SS had longer exposure times, such as one to three days (Fink *et al.*, 2012;

Hou *et al.*, 2013). Therefore, it is possible that any functional role of the T3SS during the short incubation periods is due to a 'carry-over' effect of culturing the bacteria at 37 °C, and may be as a result of adherence. This effect would be lost as the bacteria adapt to the new environment if they are incubated for long periods of time at plant-relevant temperatures.

The data presented in this study demonstrate the importance of temperature in the regulation of the T3SS. Interestingly, a similar result was found for *Bradyrhizobium*, with higher T3SS expression at 30 °C compared to 15 °C (Wei *et al.*, 2010). As 37 °C is the human body temperature and the T3SS is an essential factor for virulence of *Ec*O157 in this host it is understandable that this temperature would induce expression of this system.

Not only was the expression of the T3SS downregulated at the environmentally relevant temperature, but its expression was not induced after a one hour exposure to any of the plant extracts tested or when infiltrated into spinach or lettuce leaves. This was supported by work investigating leaf surface-adhering *Ec*O157 Sakai where no significant change in the expression of the T3SS was observed (van der Linden, 2014). Future work will investigate the response of an *Ec*O157 SakaiΔ*escN* strain in its ability to adhere and colonise plant tissue at 18 °C, in the absence of any temperature effects.

7.3.2 Ec*O157 has the potential to degrade/synthesise plant hormones*

Metabolic pathways were identified as present in *Ec*O157 Sakai that involved the degradation of plant defence hormones such as SA, or the biosynthesis of plant-growth promoting hormones such as IAA. Expression of the salicylate degradation pathways were only observed in *Ec*O157 Sakai incubated with root exudates, indicating that the enzymes are not responsive to leaf lysates (or leaf polysaccharides). However, SA levels were not determined in this work and it may be that the levels were insufficient to induce enzyme expression. Other pathways involved in the degradation of plant compounds were also upregulated in response to root exudates, as described in Chapter 6, such as arabinose and curcumin degradation (section 6.2.3). SA is a core signalling hormone, fundamental to the PAMP response in plants. The degradation of salicylic acid is found in many plant-associated bacteria such as *Pseudomonas* in order for them to

survive within the plant environment by disrupting these signalling molecules (Sazonova *et al.*, 2008). As these plant hormones are unlikely to be present within the mammalian gut I speculate that these pathways have been acquired, for example via horizontal gene transfer, within the soil environment by plant-associated microbes and utilised to persist within this environment. Further work in the future would need to be carried out to determine the concentration of these hormones that induces upregulation of these pathways and confirm that degradation does occur. Nevertheless, this could be a potential gene that enhances the survival of *Ec*O157 within the rhizosphere.

Pathways leading to the synthesis of the hormone IAA were also present in *Ec*O157 Sakai; however, no expression was seen in lettuce or spinach leaves. Four enzymes are predicted to catalyse the reaction resulting in IAA production but due to technical limitations only one was tested *in planta*. Two of these enzymes, one of which was *ECs0664*, were downregulated in spinach leaf lysates; therefore perhaps this result is not surprising, as *Ec*O157 infiltrated into the leaves would encounter many aspects of the leaf lysates extract. As no functional work on the enzymes has been reported, I hypothesise that the four enzymes may be induced in different conditions or play slightly different roles in the reaction, or the timing may not be correct for a response.

Another reason for this lack of differential expression *in planta* could be down to the method of inoculation. IAA has been reported to inhibit stomatal closing by regulating ethylene production (Tanaka *et al.*, 2006); therefore it is hypothesised that IAA synthesis by the bacteria may prevent stomatal closing and provide an entry point for these microbes, similar to other virulence factors affecting stomatal regulation produced by plant-associated bacteria (Zeng *et al.*, 2010). However in the experiment carried out in this study, the bacteria were infiltrated into leaves of spinach and lettuce to determine their expression thus bypassing this stomatal entry step, perhaps meaning that there is no need for IAA production. Therefore future work would need to determine the expression of IAA synthesis on *Ec*O157 Sakai adhering to the surface of the leaf as well as testing the different enzymes in the pathways.

7.3.3 The expression of adherence factors is induced by certain plant extracts

ECP, curli and flagella have all been shown to play an important role in the adherence of *Ec*O157 strains to plant tissue. Expression of ECP genes was not significantly changed in response to spinach leaf lysates. The spinach leaf lysates condition did not induce the expression of any of the three adherence factors tested, which is perhaps not surprising: not only do the spinach leaf lysates mimic an area of plant wounding, with accessible sugar and little environmental stress but also do not represent the surface of the leaf. Therefore there is perhaps no need for the induction of these adherence factors in this condition and in infiltrated leaves since the adherence step has been bypassed. Therefore it would be interesting to test the expression of ECP to the other conditions, especially root exudates, which were shown to induce curli expression, and leaf cell wall polysaccharides, which were shown to induce flagellar gene expression. However, one point of note is that all the transcriptomic approaches used in this study (microarray analysis, qPCR and RNA-Seq) quantify gene expression changes as an average of the total population and not at the single cell level. The regulator for ECP, *ecpR*, contains three promoters (Lehti *et al.*, 2013), therefore the expression of this system may be variable depending on which of the promoters is active; for example, *ecpR* had variable expression *in planta* (Rossez *et al.*, 2014). Since whole transcriptomic analysis presents the averaged expression of a population, any subtle difference would be masked.

ECP has been shown to mediate adherence to both root and leaves of spinach (Rossez *et al.*, 2014), therefore root exudates could prime the expression of ECP genes to facilitate adherence. Furthermore, it would be hypothesised that ECP genes would be induced in response to leaf cell wall polysaccharides, as previous work has demonstrated that the adhesin, EcpD, targets $\alpha 1 \rightarrow 5$ polyarabinan in plant cell walls (Rossez *et al.*, 2014). Interestingly, this links to a preference in location of colonisation by the bacteria: stomatal colonisation is reported as a preferential site of occupation over other areas of the leaf (Kroupitski *et al.*, 2011; Saldaña *et al.*, 2011) and other studies have previously shown that the guard cells of the stomata contain high levels of arabinose (Jones *et al.*, 2003). Our lab also showed ECP-dependent attachment to guard cells (Rossez *et al.*, 2014). Therefore this suggests the link between stomatal colonisation on spinach

is via ECP adherence to the arabinose present (Rossez *et al.*, 2014). As arabinose was detected in high levels in the spinach polysaccharide samples used in this project it would be expected that ECP expression would be induced in this condition. Expression analysis of *ecp*A and *ecpD* genes in the spinach polysaccharides would help to confirm this hypothesis.

Curli have been shown to play an important role in binding to plant tissue, in both the leaves and the roots. However, in this study the main structural subunit *csgA* was only upregulated in response to root exudates. If curli is necessary for adhering to roots and root exudates induce the expression of curli genes, this would add to the hypothesis that root exudates 'prime' the bacteria for plant colonisation. In fact, studies have shown that tomato root exudates have induced the expression of amyloid fibres in *Bacillus subtilis* (Hufnagel *et al.*, 2013) and so the results from this study correlate with those looking at other microbes in the rhizosphere.

The expression of flagellar genes was repressed in spinach leaf lysates and spinach root exudates yet induced in response to spinach and lettuce leaf cell wall polysaccharides. Furthermore, when *Ec*O157 Sakai was infiltrated into the leaves of spinach and lettuce for one hour *flhD* gene expression was repressed. This supports the result seen with spinach leaf lysates, which will contain a high proportion of apoplastic fluid.

As flagella are a known adherence factor to plants, as they have been demonstrated to interact with plant membranes in the initial adherence (Rossez *et al.*, 2013), the expression of flagellar genes to the polysaccharide extracts suggests that components of the plant cell wall induce this expression and hence this interaction. However, many studies have shown that flagella are downregulated by human pathogenic bacteria within the plant environment as flagellin is a known PAMP (Garcia *et al.*, 2013). The expression of the flagellar genes in this study follows this hypothesis: flagellar genes need to be induced in response to the plant cell wall polysaccharides in order to adhere to cell surfaces, but upon penetration of plant tissue, expression is downregulated as they do not require this adherence factor in these conditions and thus it is detrimental to the bacterial survival; therefore, it is downregulated to evade the plant immune response.

Finally, other adherence factors may be present in *Ec*O157 Sakai that are induced in response to the plant environment. For example, one study found that fimbrial gene expression was affected by ethanolamine and choline in the human gut (Gonyar & Kendall, 2014); however, these compounds are also synthesised by plants so presumably fimbrial gene expression may be induced by plant extracts. There are also many putative fimbrial genes present in *Ec*O157 Sakai that could be involved in the adherence to plants, although only a few were seen to be upregulated in response to plant extracts in this study. Further study of known adherence factors to human epithelial cells when in the plant environment would be interesting, as well as analysis of genes with unknown function.

7.3.4 Hypothetical genes

A large proportion of the genes that were induced in each of the conditions tested by microarray were genes which were annotated as hypothetical proteins. This is similar to previous work looking at genes important for the adherence of *S. enterica* to alfalfa sprouts, where of the 20 transposon mutants with reduced adherence identified, 13 were uncharacterised (Barak *et al.*, 2005). Many of the hypothetical genes differentially expressed in response to plant extracts are also encoded by *Ec*O157 EDL933 but not by *E. coli* K-12, with some present on horizontally acquired genomic islands. Furthermore, some of the genes are unique to *Ec*O157 Sakai, and may potentially confer an advantage for *Ec*O157 Sakai in the plant environment compared to *Ec*O157 EDL933. In the future, knockout mutants of selected hypotheticals could be tested for their ability to utilise plant extracts and colonise to plants to a) begin to elucidate their overall function and b) understand if these hypothetical proteins play a role in the persistence of *Ec*O157 on plants, and c) to work out if they are real proteins that are expressed and not just hypothetical. Potentially, these genes could be the most interesting as a role has yet to be characterised for them. As the majority of work on VTEC species has been studied in animal hosts, these hypothetical genes may only be expressed in environmental conditions and thus could be necessary for the colonisation of plants as secondary hosts or alternative habitats.

8 Final Discussion

It is now well established that human pathogens such as *Ec*O157 and *Salmonella* are able to survive outside the mammalian host and interact with and subsequently colonise plant tissue. However, it is still not clear how these human pathogens adapt to survive within the environment and on plants although recent transcriptomic studies have provided much information, allowing some speculation to be made on the mode of colonisation.

The overall aim of this project was to understand the gene expression changes that occurred in the human pathogenic strain *Ec*O157 Sakai when the bacteria were introduced to the plant environment. Initially it was planned to study this interaction with living plant roots; however a suitable method of extracting bacterial RNA from plant roots was not available at this time. During this project, an optimised method of bacterial RNA extraction *in planta* was developed and subsequently published, meaning that this pursuit can be explored further in the future to clarify some of the results obtained in this project from the *in vitro* studies.

Instead of looking at the interaction with living plants, I decided to study how *Ec*O157 Sakai responded to different plant extracts to identify genes that were important for the survival and proliferation of this human pathogen within the plant environment using a transcriptomic approach. Large changes in the gene expression of *Ec*O157 Sakai were seen when the bacteria were incubated at a lower temperature and with plant extracts, including leaf lysates, root exudates and cell wall polysaccharides. Categories of genes were seen to be induced in one condition, yet repressed in another, indicating the need to study the different niche areas of the plant that these bacteria are able to occupy, as different genes were induced in each.

Many metabolic pathways were induced in *Ec*O157 Sakai, highlighting that these bacteria were able to utilise components of the plant extracts for their own survival, including plant-specific components such as arabinose and xylose. As well as this, other pathways were identified utilising other plant components; these pathways were specific to *Ec*O157 species and not present in *E. coli* K-12, such as pectin degradation, or were present in *Ec*O157 Sakai but not in

the bovine isolate *Ec*O157 TUV 93-0, such as the synthesis of hormones such as IAA. I also found that *Ec*O157 Sakai could be recovered from the leaves and roots of three plant species, including spinach, after ten days, whereas the bovine strain was below the level of detection in the leaves but was recoverable from the roots at this time point.

As most of the work in this study focused on the interaction of *Ec*O157 Sakai with spinach we can begin to understand how this human pathogen colonises this particular plant species. The information obtained from this study, coupled with previously published findings looking at *Ec*O157 strains in the environment, helps us to speculate on a model of colonisation for these bacteria in the roots and leaves of spinach.

8.1 Survival and Persistence of *Ec*O157 Sakai in the Leaves of Spinach

Based on the information obtained from this project, a model of colonisation of the leaves of spinach can be determined (Figure 8-1). Transcriptomic analysis of *Ec*O157 Sakai in response to spinach leaf lysates and spinach leaf cell wall polysaccharides was investigated in this study, identifying genes that were important for each of these interactions. Furthermore, the identification of Sakai-specific and *Ec*O157-specific genes highlights areas of interest which may play a key role in the colonisation of plants. Therefore, the colonisation of spinach leaves is speculated to be as follows:

1) *Ec*O157 Sakai is inoculated onto the surface of spinach leaves due to droplets of contaminated irrigation water. Irrigation water has been implicated as an important vector of transmission of this pathogen in the environment (Heaton & Jones, 2008; Kljujev *et al.*, 2012; Oliveira *et al.*, 2012). Other vectors such as insects have also been implicated in leaf contamination (Wasala *et al.*, 2013) and there is also the potential for many post-harvest methods of contamination during the processing of the food product. Furthermore, some studies have hypothesised that leaves can become contaminated by *Ec*O157 by movement of the bacteria from the roots/soil to the leaves via the stem (Cooley *et al.*, 2003), although this has not been demonstrated for *Ec*O157 species on

spinach. An irrigation water method of contamination is presumed for this model of colonisation.

2) *Ec*O157 Sakai needs to adapt to survive on the surface of the leaf. Flagellar genes are upregulated in response to plant cell wall polysaccharides (section 5.2.2) and flagella are used to adhere to plant cells (Rossez *et al.*, 2013). Spinach has been shown to contain higher levels of arabinose in the leaf polysaccharide extracts compared to lettuce (section 4.2.3, 6.3.6; (Rossez *et al.*, 2014)). *Ec*O157 Sakai upregulates genes involved in arabinose degradation when in the presence of spinach leaf polysaccharides (5.2.2.1) and utilises arabinose for its initial survival, along with any other available sugars.
3) The leaf surface is presumed to be a harsh environment for *Ec*O157 survival due to the low nutrient availability, low humidity levels and UV exposure. *Ec*O157 is able to utilise components of the plant cell for its survival such as arabinose and xylose (6.2.2.1). However, more accessible sugars are present within the leaf itself. Therefore to persist within this environment, *Ec*O157 Sakai is chemotactically attracted towards openings in the leaf, such as plant wounds (Brandl, 2008) or stomata (Kroupitski *et al.*, 2009b). *Ec*O157 can then internalise into the plant tissue via these plant openings (Deering *et al.*, 2012) .
4) Once *Ec*O157 Sakai has internalised into the plant tissue via the stomata, it can colonise the openings between plant cells and utilise the apoplastic fluid or leaf cell components (leaf lysates) for its survival. High levels of glucose and fructose are present within these extracts, which are accessible to and used by *Ec*O157 for its survival (section 4.2.2). As these sugars are present, genes encoding for arabinose and xylose degradation are downregulated (section 5.2.1.2) due to catabolite repression. Flagellar genes are downregulated once the bacteria have internalised (section 5.2.1.2, 7.2.3) as flagella are a known PAMP that can trigger the plant defence response (Seo & Matthews, 2012).

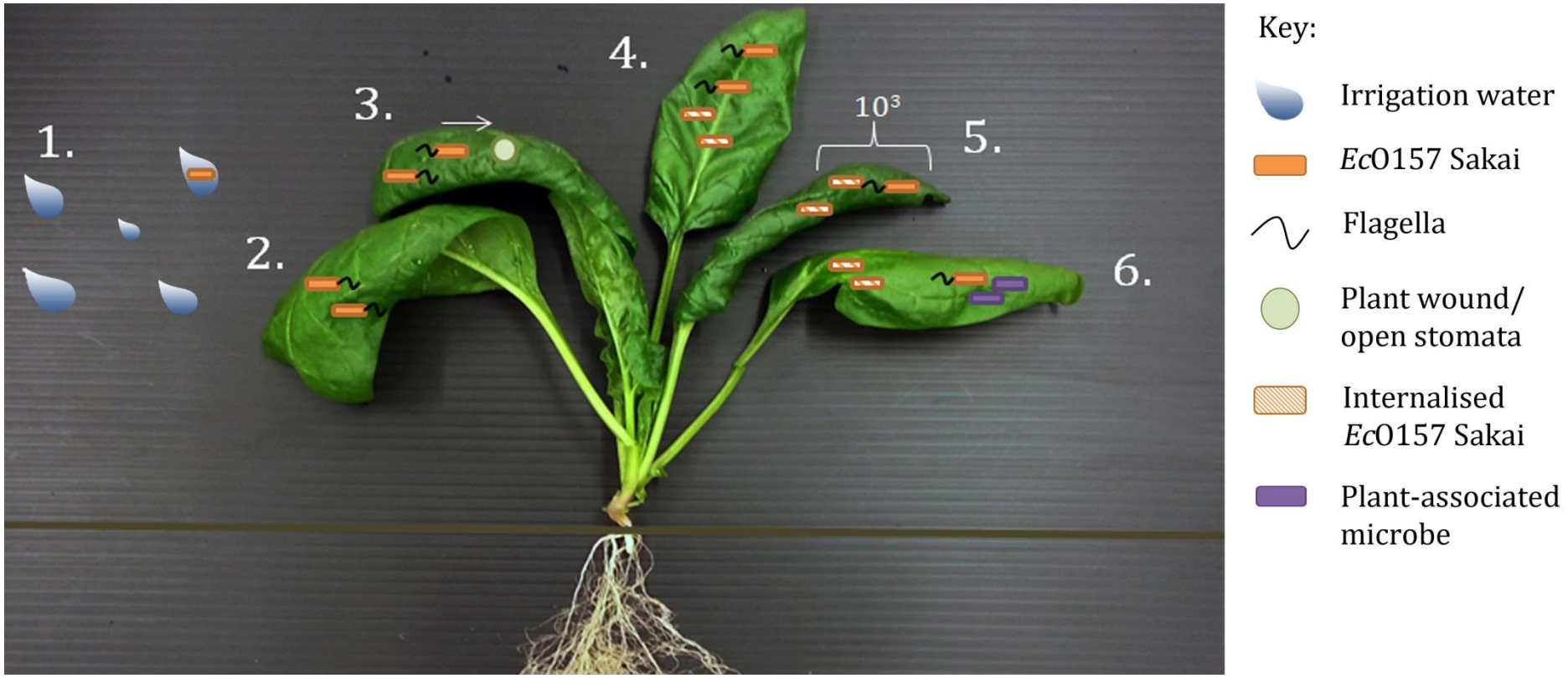


Figure 8-1. Model of spinach leaf colonisation by *Ec*O157 Sakai via irrigation water.

1) *Ec*O157 Sakai is transferred to the surface of the leaf by contaminated irrigation water. **2)** Flagellar genes are induced and facilitate adherence to the plant cells by interacting with their plasma membrane. **3)** *Ec*O157 Sakai migrates towards open stomata/plant wounds and uses these to eventually internalise into the leaf tissue. **4)** *Ec*O157 Sakai uses sugars present within the leaf tissue for growth and downregulates its flagella expression to evade the plant immune response. **5)** The overall population of *Ec*O157 Sakai stabilises to 10^3 per leaf. **6)** *Ec*O157 Sakai can persist as an internalised population or by forming associations with other plant-associated microbes such as in a biofilm.

5) The total number of bacteria present within the leaf stabilises at approximately 10^3 after ten days. Colonisation assays of *Ec*O157 Sakai on the leaves of different plant species that were inoculated by mimicking an irrigation water method of contamination found that the total bacterial population after 10-14 days was approximately 10^3 (section 3.2.1.1; (Barrett, 2013)). A further test to quantify internalised population versus the total population recovered would be interesting. If the majority of the population recovered were found to be internalised it would be assumed that this is imperative to the survival of human pathogens within spinach leaves.
6) *Ec*O157 Sakai persists within the phyllosphere, either by survival on the leaf surface within biofilms with other plant-associated microbes or by internalising into the spaces between plant cells. No visible effect on the plant is observed, therefore it is unknown if a plant is contaminated with this human pathogen. The leaves can then be harvested, processed and packaged. Any sterilisation treatments used will not remove the internalised bacteria. The contaminated leaves are then passed to the consumer and ingested, which can result in infection within this individual.

8.2 Survival and Persistence of *Ec*O157 Sakai within the Roots of Spinach

As the phyllosphere and the rhizosphere present very different challenges to the survival of *Ec*O157 Sakai, a separate model for root colonisation of spinach is proposed (Figure 8-2). Transcriptomic analysis of *Ec*O157 Sakai in response to spinach root exudates provided an insight into how these bacteria may adapt their gene expression to adhere to and colonise the roots of spinach.

1) *Ec*O157 Sakai are present within the soil as a result of defecation from nearby cattle or spread via contaminated water. Root exudates are excreted from the roots of spinach and these exudates are the first point of contact between *Ec*O157 Sakai and the spinach plant.
2) *Ec*O157 Sakai are unable to use these root exudates for growth due to a lack of a carbon source (section 3.2.2.1); as a result, these bacteria must directly associate with the roots

in order to survive. The root exudates induce gene expression changes in the bacteria, including many stress genes due to the lack of a nutritional source. Curli genes are induced upon exposure to root exudates for the bacteria to adhere to the root cells. The root exudates also contain signalling molecules including plant hormones such as salicylic acid responsible for priming the plant defence response. *Ec*O157 possesses a salicylate hydroxylase that can degrade variations of this plant hormone (section 4.1.1); this gene is induced in this environment (section 5.2.1.4) and the salicylic acid is taken up by the bacteria and degraded in order to suppress the plant defence response.

3) *Ec*O157 Sakai adheres to the root cells via curli fibres and utilises sugars present in the plant cell wall such as arabinose and xylose for growth (section 5.2.1.4). Populations of *Ec*O157 Sakai are established on the root surface as part of a biofilm in association with other plant-associated microbial species, which contain plant cell wall degrading enzymes that release many of the sugars for use by the microbial species.
4) A proportion of the bacteria may internalise into the plant tissue via natural openings, such as emerging roots (Wright *et al.*, 2013), if they are unable to survive on the root surface in a biofilm. Once internalised they have access to other sugars and can use these for their persistence in this environment.
5) *Ec*O157 Sakai is able to associate with and subsequently colonise the roots and populations remain stable after ten days (sections 3.2.1.3, 3.2.1.5). A high population of *Ec*O157 Sakai in the roots may result in cross-contamination during the post-harvest stages of processing of the plant material, causing *Ec*O157 to adhere to and colonise the spinach leaves, which may result in infection if consumed.
6) A small proportion of the bacterial population may migrate from the roots, up the stem of the spinach plant and eventually to the leaves via tropism towards different metabolites. This could then result in subsequent colonisation of the leaves.

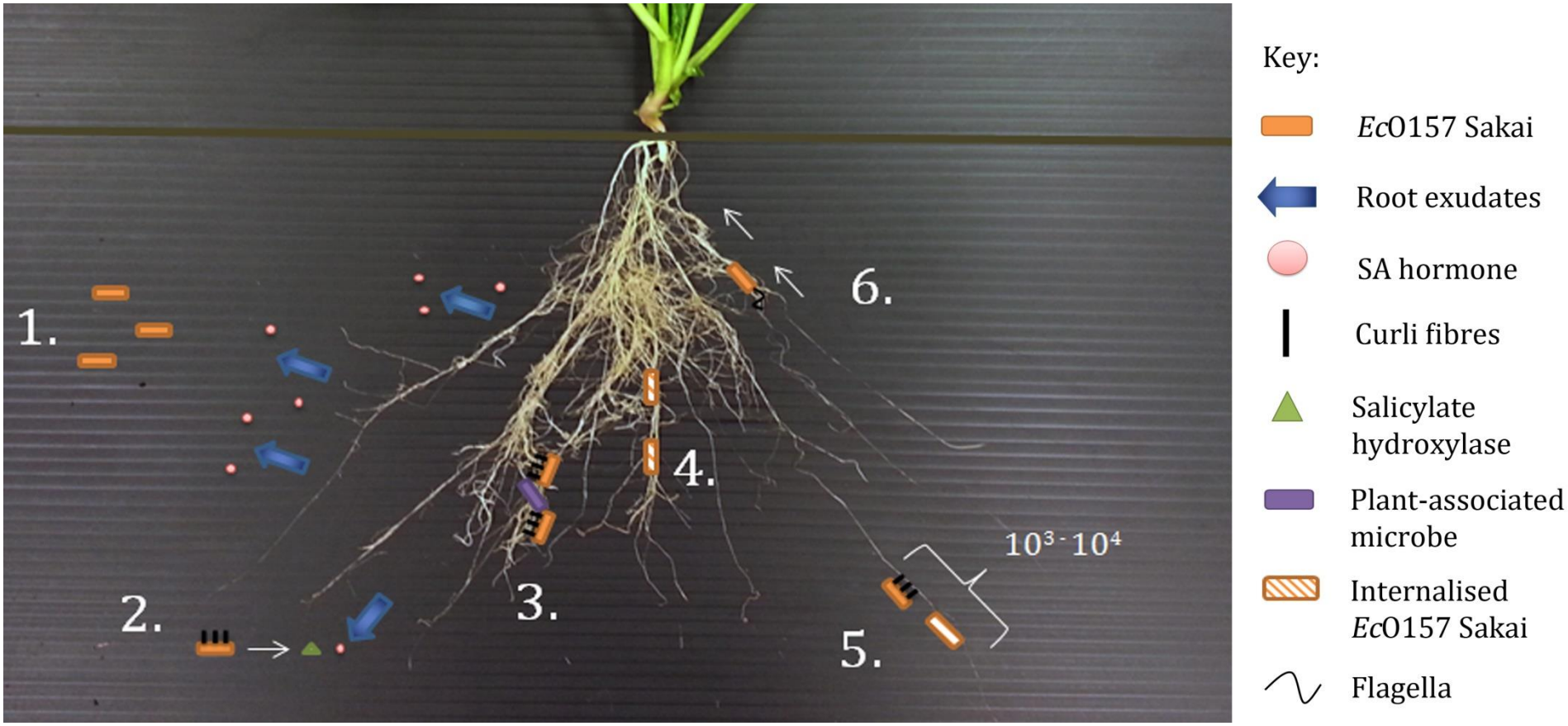


Figure 8-2. Model of colonisation of spinach roots by *Ec*O157 Sakai.

1) *Ec*O157 Sakai is present within the soil, via faeces or contaminated water. **2)** Root exudates induce upregulation of curli genes and defence genes such as a salicylate hydroxylase to degrade plant hormones. Salicylic acid is taken up by the bacteria and degraded by the bacterial salicylate hydroxylase (note – for viewing ease this interaction has been demonstrated outside the bacterial cell). **3)** *Ec*O157 Sakai adheres to the roots via its curli fibres and can eventually survive within a biofilm with other plant-associated microbes present on the roots. **4)** A proportion of the bacterial population may internalise into and persist within the root tissue. **5)** The bacteria colonise the roots to approximately 10^3-10^4 per gram of root tissue, including internalised and surface-adhering bacteria. **6)** Some bacteria may not internalise or establish populations within the roots and instead migrate up the stem of the plant to the leaves via chemotaxis using their flagella.

8.3 Conclusion and Future Work

The data generated from this study, coupled with that of previously published data, has allowed the development of a speculative model of colonisation and has identified several different categories of genes that could be investigated in the future to further understand this interaction. Many of the categories of genes that were differentially expressed in response to plant extracts were involved in motility (flagellar genes) and nutrient assimilation and utilisation. This is similar to published work looking at an *rpoN* mutant in *Pseudomonas fluorescens*, which was shown to be aflagellate and unable to utilise many carbon and nitrogen sources for growth (Jones *et al.*, 2007). *Pseudomonas fluorescens* SBW25Δ*rpoN* was significantly impaired in its ability to colonise the roots of sugar beets, suggesting that the genes regulated by *rpoN* play an important role in the colonisation of these plants. *rpoN* regulates the activity of 125 genes in *E. coli*, including the master regulators *flhD* and *flhC* (Dong *et al.*, 2011), which have been shown in this study to be differentially expressed in response to plant extracts. Therefore, many similarities can be found between human pathogens and plant-associated strains, suggesting that the results from this study may not only help understand how the human pathogen *Ec*O157 has adapted to the plant environment, but may also help identify important genes and metabolic pathways required by other plant-associated bacteria for the colonisation of plants.

As mentioned previously, five different conditions were tested over the course of this study, providing information of how the bacteria adapt to each of these niche environments. However, a limitation of the work is that the transcriptomic analysis was carried out on *in vitro* samples of *Ec*O157 Sakai, by analysing the response of the bacteria to a minimal media supplemented with plant extracts. There are drawbacks to this method as the transcriptome of the bacteria will be affected by other environmental factors present in the soil and by other microbes when in the field; therefore *in vitro* analysis may only present a fraction of the interaction. Nevertheless, the datasets generated offer many areas to target for further investigation, which can be tested in *in vivo* studies at a future date.

Due to the nature of this project there are several routes of future research that can be pursued. Gene knockout mutants have been generated during the course of the project for *araBAD* (the genes encoding for the three enzymes in the L-arabinose degradation pathway) in *E. coli* K-12 and in *Ec*O157 Sakai. Thus, these mutants can be tested for their ability to colonise and adhere to the leaves and roots of plants compared to the wild type. I would hypothesise that the *araBAD* mutant would be reduced in its colonisation ability in spinach as this pathway was induced in response to spinach extracts; therefore, it would be interesting to also test for any plant differences, such as if the mutant is impaired in lettuce colonisation also.

Three single gene knockout mutants were also generated in this project based on the results of the transcriptomic datasets: *ECs1335*, *emrD* and *dctA*. *ECs1335* is a hypothetical protein, which is predicted to be encoded on an acquired genomic island in *Ec*O157 Sakai. Adjacent genes are also hypothetical – as this region of genes have not been identified as having a role in human infection, perhaps they are important for environmental survival. In contrast, *emrD* is well characterised and is an efflux pump in *E. coli*. *emrD* is interesting as it has been previously shown to play a role in the efflux of arabinose (Koita & Rao, 2012) and *emr* genes have also been upregulated in *Erwinia amylovora* when inoculated into pear tissue (Zhao *et al.*, 2005). Finally, *dctA* encodes a dicarboxylate transporter. This gene was upregulated in response to both spinach and lettuce leaf cell wall polysaccharides. It has also been shown that a *dctA* mutant in *Pseudomonas* is impaired in its ability to colonise roots (Nam *et al.*, 2006).

Further transcriptomic analysis would also be very beneficial: as a new method for extracting bacterial RNA from plant roots was developed during this project the original aim of this project could be tested in the future. This would highlight any gene expression changes induced by the root exudates that are also seen when the bacteria are associated with the roots. Finally, it would also be beneficial to study the interaction of *Ec*O157 with the leaves of spinach, by extracting the RNA from bacteria adhering to the surface of the leaf. This would allow us to understand how this human pathogen survives upon the leaf surface, rather than within the leaf itself, which is a very different environment.

In conclusion, I have shown that *Ec*O157 Sakai is able to colonise a variety of plant niche environments and adapts its gene expression differentially to each. As it is able to persist in the roots and the leaves and has been shown to internalise into the tissue of both, this human pathogen presents a serious issue due to contaminated plant consumption. Identification of how *Ec*O157 Sakai adapts to each environment provides information which may help in the future for the development of prevention strategies. This study has shown the induction of many different cellular processes, including many aspects of metabolism, adherence factors and stress responses. I have also identified genes that are present in *Ec*O157 species which are hypothesised to play a role in the plant environment, such as pectin degradation, salicylate degradation and IAA production. Therefore there are several gene targets highlighted in this study that could be investigated further in the future to develop the models of spinach colonisation further and apply them to other plant species.

Abdul-Raouf, U. M., Beuchat, L. R. & Ammar, M. S. (1993). Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Applied and Environmental Microbiology* **59**, 1999-2006.

Abu-Ali, G. S., Ouellette, L. M., Henderson, S. T., Whittam, T. S. & Manning, S. D. (2010). Differences in adherence and virulence gene expression between two outbreak strains of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* **156**, 408-419.

Ackers, M.-L., Mahon, B. E., Leahy, E., Goode, B., Damrow, T., Hayes, P. S., Bibb, W. F., Rice, D. H., Barrett, T. J., Hutwagner, L., Griffin, P. M. & Slutsker, L. (1998). An Outbreak of *Escherichia coli* O157:H7 Infections Associated with Leaf Lettuce Consumption. *Journal of Infectious Diseases* **177**, 1588-1593.

Aguilera, L., Campos, E., Gimenez, R., Badia, J., Aguilar, J. & Baldoma, L. (2008). Dual role of LldR in regulation of the lldPRD operon, involved in L-lactate metabolism in *Escherichia coli*. *Journal of Bacteriology* **190**, 2997-3005.

Ali, B., Sabri, A. N., Ljung, K. & Hasnain, S. (2009). Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum*. *Letters in Applied Microbiology* **48**, 542-547.

Aranda, K. R. S., Fagundes-Neto, U. & Scaletsky, I. C. A. (2004). Evaluation of Multiplex PCRs for Diagnosis of Infection with Diarrheagenic *Escherichia coli* and *Shigella* spp. *Journal of clinical microbiology* **42**, 5849-5853.

Badri, D. V. & Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant Cell Environ* **32**, 666-681.

Baquero, F., Martínez, J.-L. & Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current opinion in biotechnology* **19**, 260-265.

Barak, J. D., Gorski, L., Naraghi-Arani, P. & Charkowski, A. O. (2005). *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. *Applied and Environmental Microbiology* **71**, 5685-5691.

Barak, J. D., Kramer, L. C. & Hao, L. Y. (2011). Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. *Applied and Environmental Microbiology* **77**, 498-504.

Barrett, G. (2013). Disentangling the molecular mechanisms for persistence of *Escherichia coli* O157:H7 in the plant environment, Doctor of Philosophy. University of Reading.

Beattie, G. A. & Lindow, S. E. (1999). Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology* **89**, 353-359.

Beauregard, P. B., Chai, Y., Vlamakis, H., Losick, R. & Kolter, R. (2013). *Bacillus subtilis* biofilm induction by plant polysaccharides. *Proceedings of the National Academy of Sciences* **110**, E1621-E1630.

Beckham, K. S., Connolly, J. P., Ritchie, J. M., Wang, D., Gawthorne, J. A., Tahoun, A., Gally, D. L., Burgess, K., Burchmore, R. J., Smith, B. O., Beatson, S. A., Byron, O., Wolfe, A. J., Douce, G. R. & Roe, A. J. (2014). The metabolic enzyme AdhE controls the virulence of *Escherichia coli* O157:H7. *Molecular Microbiology* **93**, 199-211.

Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J. A., Molin, S., Prensier, G., Arbeille, B. & Ghigo, J. M. (2004). Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular Microbiology* **51**, 659-674.

Berger, C. N., Shaw, R. K., Brown, D. J., Mather, H., Clare, S., Dougan, G., Pallen, M. J. & Frankel, G. (2009). Interaction of *Salmonella enterica* with basil and other salad leaves. *International Society for Microbial Ecology Journal* **3**, 261-265.

Bergholz, T. M., Vanaja, S. K. & Whittam, T. S. (2009). Gene expression induced in *Escherichia coli* O157:H7 upon exposure to model apple juice. *Applied and Environmental Microbiology* **75**, 3542-3553.

Beuchat, L. R., Scouten, A. J., Allen, R. I. & Hussey, R. S. (2003). Potential of a plant-parasitic nematode to facilitate internal contamination of tomato plants by *Salmonella*. *Journal of Food Protection* **66**, 1459-1461.

Bhunia, A. K. (2008). *Foodborne Microbial Pathogens: Mechanisms & Pathogenesis*, 1st edition edn: Springer.

Boos, W. & Shuman, H. (1998). Maltose/Maltodextrin System of *Escherichia coli*: Transport, Metabolism, and Regulation. *Microbiology and Molecular Biology Reviews* **62**, 204-229.

Bowtell, D. (2003). *DNA Microarrays: A Molecular Cloning Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Brader, G., Compant, S., Mitter, B., Trognitz, F. & Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Current opinion in biotechnology* **27**, 30-37.

Bradley, M. D., Beach, M. B., de Koning, A. P., Pratt, T. S. & Osuna, R. (2007). Effects of Fis on *Escherichia coli* gene expression during different growth stages. *Microbiology* **153**, 2922-2940.

Brandl, M. T. (2008). Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on postharvest lettuce. *Applied and Environmental Microbiology* **74**, 5285-5289.

Brandl, M. T. & Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella enterica*. *Applied and Environmental Microbiology* **74**, 2298-2306.

Brennan, F. P., Grant, J., Botting, C. H., O'Flaherty, V., Richards, K. G. & Abram, F. (2013). Insights into the low-temperature adaptation and nutritional flexibility of a soil-persistent *Escherichia coli*. *FEMS Microbiology Ecology* **84**, 75-85.

Brinkman, A. B., Ettema, T. J., de Vos, W. M. & van der Oost, J. (2003). The Lrp family of transcriptional regulators. *Molecular Microbiology* **48**, 287-294.

Brückner, R. & Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS microbiology letters* **209**, 141-148.

Brzuszkiewicz, E., Thurmer, A., Schuldes, J., Leimbach, A., Liesegang, H., Meyer, F. D., Boelter, J., Petersen, H., Gottschalk, G. & Daniel, R. (2011). Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC). *Archives of Microbiology* **193**, 883-891.

Buchholz, A. L., Davidson, G. R., Marks, B. P., Todd, E. C. & Ryser, E. T. (2012). Quantitative transfer of *Escherichia coli* O157:H7 to equipment during small-scale production of fresh-cut leafy greens. *Journal of Food Protection* **75**, 1184-1197.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F. O., Amann, R., Eickhorst, T. & Schulze-Lefert, P. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**, 91-95.

Burland, V., Shao, Y., Perna, N. T., Plunkett, G., Sofia, H. J. & Blattner, F. R. (1998). The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Research* **26**, 4196-4204.

Burlingame, R. & Chapman, P. J. (1983). Catabolism of phenylpropionic acid and its 3-hydroxy derivative by *Escherichia coli*. *Journal of Bacteriology* **155**, 113-121.

Burnett, S. L., Chen, J. & Beuchat, L. R. (2000). Attachment of *Escherichia coli* O157:H7 to the Surfaces and Internal Structures of Apples as Detected by Confocal Scanning Laser Microscopy. *Applied and Environmental Microbiology* **66**, 4679-4687.

Büttner, D. & He, S. Y. (2009). Type III Protein Secretion in Plant Pathogenic Bacteria. *Plant Physiology* **150**, 1656-1664.

Carapito, R., Vorwerk, S., Jeltsch, J.-M. & Phalip, V. (2013). Genome-wide transcriptional responses of *Fusarium graminearum* to plant cell wall substrates. *FEMS microbiology letters* **340**, 129-134.

Carvalhais, L. C., Dennis, P. G., Fedoseyenko, D., Hajirezaei, M.-R., Borriss, R. & von Wirén, N. (2011). Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *Journal of Plant Nutrition and Soil Science* **174**, 3-11.

Casadei, M. A., Mañas, P., Niven, G., Needs, E. & Mackey, B. M. (2002). Role of Membrane Fluidity in Pressure Resistance of *Escherichia coli* NCTC 8164. *Applied and Environmental Microbiology* **68**, 5965-5972.

Caspi, R., Altman, T., Dale, J. M., Dreher, K., Fulcher, C. A., Gilham, F., Kaipa, P., Karthikeyan, A. S., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L. A., Paley, S., Popescu, L., Pujar, A., Shearer, A. G., Zhang, P. & Karp, P. D. (2010). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research* **38**, D473-D479.

CDC (2006). Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach -- United States, September 2006. *MMWR Morbidity and Mortality Weekly Report* **55**, 1045-1046.

CDC (2013). Outbreak of *Escherichia coli* O104:H4 infections associated with sprout consumption - Europe and North America, May-July 2011. *MMWR Morbidity and Mortality Weekly Report* **62**, 1029-1031.

Centers for Disease Control and Prevention (2012a). *Salmonella*, http://www.cdc.gov/salmonella/general/. 03/12/14.

Centers for Disease Control and Prevention (2012b). *E. coli* (*Escherichia coli*), http://www.cdc.gov/ecoli/index.html. 03/12/14.

Chadwick, M. (2013). Development of molecular markers linked to quantitative and qualitative assessment of bitterness in lettuce, PhD. The University of Reading.

Chang, J. M. & Fang, T. J. (2007). Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars Typhimurium in iceberg lettuce and the antimicrobial effect of rice vinegar against *E. coli* O157:H7. *Food Microbiology* **24**, 745-751.

Chase-Topping, M., Gally, D., Low, C., Matthews, L. & Woolhouse, M. (2008). Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nature Reviews Microbiology* **6**, 904-912.

Chekabab, S. M., Paquin-Veillette, J., Dozois, C. M. & Harel, J. (2013). The ecological habitat and transmission of *Escherichia coli* O157:H7. *FEMS microbiology letters* **341**, 1-12.

Chen, L., Xiong, Z., Sun, L., Yang, J. & Jin, Q. (2012). VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Research* **40**, 641-645.

Cho, B. K., Knight, E. M., Barrett, C. L. & Palsson, B. O. (2008). Genome-wide analysis of Fis binding in *Escherichia coli* indicates a causative role for A-/AT-tracts. *Genome Research* **18**, 900-910.

Choi, S., Bang, J., Kim, H., Beuchat, L. R. & Ryu, J. H. (2011). Survival and colonization of *Escherichia coli* O157:H7 on spinach leaves as affected by inoculum level and carrier, temperature and relative humidity. *Journal of Applied Microbiology* **111**, 1465-1472.

Clark, D. (1981). Regulation of fatty acid degradation in *Escherichia coli:* analysis by operon fusion. *Journal of Bacteriology* **148**, 521-526.

Coburn, B., Sekirov, I. & Brett Finlay, B. (2007). Type III Secretion Systems and Disease. *Clinical Microbiology Reviews* **20**, 535-549.

Cooley, M. B., Miller, W. G. & Mandrell, R. E. (2003). Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and Enterohemorrhagic *Escherichia coli* O157:H7 and Competition by *Enterobacter asburiae*. *Applied and Environmental Microbiology* **69**, 4915-4926.

Cooley, M. B., Chao, D. & Mandrell, R. E. (2006). *Escherichia coli* O157:H7 Survival and Growth on Lettuce Is Altered by the Presence of Epiphytic Bacteria. *Journal of Food Protection* **69**, 2329-2335.

Crepin, V. F., Shaw, R., Abe, C. M., Knutton, S. & Frankel, G. (2005). Polarity of Enteropathogenic *Escherichia coli* EspA Filament Assembly and Protein Secretion. *Journal of Bacteriology* **187**, 2881-2889.

Cunningham, L., Gruer, M. J. & Guest, J. R. (1997). Transcriptional regulation of the aconitase genes (acnA and acnB) of *Escherichia coli*. *Microbiology* **143** 3795-3805.

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current opinion in biotechnology* **23**, 174-181.

Dahan, S., Knutton, S., Shaw, R. K., Crepin, V. F., Dougan, G. & Frankel, G. (2004). Transcriptome of Enterohemorrhagic *Escherichia coli* O157 Adhering to Eukaryotic Plasma Membranes. *Infection and Immunity* **72**, 5452-5459.

Daniell, S. J., Takahashi, N., Wilson, R., Friedberg, D., Rosenshine, I., Booy, F. P., Shaw, R. K., Knutton, S., Frankel, G. & Aizawa, S. (2001). The filamentous type III secretion translocon of enteropathogenic *Escherichia coli*. *Cell Microbiol* **3**, 865-871.

De Buck, J., Van Immerseel, F., Haesebrouck, F. & Ducatelle, R. (2004). Colonization of the chicken reproductive tract and egg contamination by *Salmonella*. *Journal of Applied Microbiology* **97**, 233-245.

Deering, A. J., Mauer, L. J. & Pruitt, R. E. (2012). Internalization of *E. coli* O157:H7 and *Salmonella* spp. in plants: A review. *Food Research International* **45**, 567-575.

Déjean, G., Blanvillain-Baufumé, S., Boulanger, A., Darrasse, A., de Bernonville, T. D., Girard, A.-L., Carrére, S., Jamet, S., Zischek, C., Lautier, M., Solé, M., Büttner, D., Jacques, M.-A., Lauber, E. & Arlat, M. (2013). The xylan utilization system of the plant pathogen *Xanthomonas campestris* pv campestris controls epiphytic life and reveals common features with oligotrophic bacteria and animal gut symbionts. *New Phytologist* **198**, 899-915.

Desai, T. A. & Rao, C. V. (2010). Regulation of arabinose and xylose metabolism in Escherichia coli. *Appl Environ Microbiol* **76**, 1524-1532.

Dhavan, G. M., Crothers, D. M., Chance, M. R. & Brenowitz, M. (2002). Concerted binding and bending of DNA by *Escherichia coli* integration host factor. *Journal of Molecular Biology* **315**, 1027-1037.

Dong, T., Yu, R. & Schellhorn, H. (2011). Antagonistic regulation of motility and transcriptome expression by RpoN and RpoS in *Escherichia coli*. *Molecular Microbiology* **79**, 375-386.

Donnenberg, M. S., Tacket, C. O., James, S. P., Losonsky, G., Nataro, J. P., Wasserman, S. S., Kaper, J. B. & Levine, M. M. (1993). Role of the eaeA gene in experimental enteropathogenic *Escherichia coli* infection. *Journal of Clinical Investigation* **92**, 1412-1417.

Donohue-Rolfe, A., Kondova, I., Oswald, S., Hutto, D. & Tzipori, S. (2000). *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. *Journal of Infectious Diseases* **181**, 1825-1829.

Drewnowski, A. & Gomez-Carneros, C. (2000). Bitter taste, phytonutrients, and the consumer: a review. *The American journal of clinical nutrition* **72**, 1424-1435.

Duan, G., Christian, N., Schwachtje, J., Walther, D. & Ebenhöh, O. (2013). The Metabolic Interplay between Plants and Phytopathogens. *Metabolites* **3**, 1-23.

Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, G. A., Koohmaraie, M. & Laegreid, W. W. (2000). Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences* **97**, 2999-3003.

Elliott, S. J., Yu, J. & Kaper, J. B. (1999). The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. *Infection and Immunity* **67**, 4260-4263.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. & Igarashi, K. (1988). Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. *European Journal of Biochemistry* **171**, 45-50.

Engel, J. & Balachandran, P. (2009). Role of *Pseudomonas aeruginosa* type III effectors in disease. *Current Opinion in Microbiology* **12**, 61-66.

Ergonul, Z., Clayton, F., Fogo, A. & Kohan, D. (2003). Shigatoxin-1 binding and receptor expression in human kidneys do not change with age. *Pediatric Nephrology* **18**, 246-253.

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., Payton, A. S., Liao, J., Ma, L. & Doyle, M. P. (2010). Infrequent internalization of *Escherichia coli* O157:H7 into field-grown leafy greens. *Journal of Food Protection* **73**, 500-506.

Fink, R. C., Black, E. P., Hou, Z., Sugawara, M., Sadowsky, M. J. & Diez-Gonzalez, F. (2012). Transcriptional Responses of *Escherichia coli* K-12 and O157:H7 Associated with Lettuce Leaves. *Applied and Environmental Microbiology* **78**, 1752-1764.

Fislage, R., Berceanu, M., Humboldt, Y., Wendt, M. & Oberender, H. (1997). Primer design for a prokaryotic differential display RT-PCR. *Nucleic Acids Research* **25**, 1830-1835.

Franz, E., van Hoek, A. H., Bouw, E. & Aarts, H. J. (2011). Variability of *Escherichia coli* O157 strain survival in manure-amended soil in relation to strain origin, virulence profile, and carbon nutrition profile. *Appl Environ Microbiol* **77**, 8088-8096.

Fuller, C. A., Pellino, C. A., Flagler, M. J., Strasser, J. E. & Weiss, A. A. (2011). Shiga Toxin Subtypes Display Dramatic Differences in Potency. *Infection and Immunity* **79**, 1329-1337.

Gagliardi, J. V. & Karns, J. S. (2002). Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. *Environmental Microbiology* **4**, 89-96.

Garcia, A. V., Charrier, A., Schikora, A., Bigeard, J., Pateyron, S., de Tauzia-Moreau, M. L., Evrard, A., Mithöfer, A., Martin-Magniette, M. L., Virlogeux-Payant, I. & Hirt, H. (2013). *Salmonella enterica* flagellin is recognized via FLS2 and activates PAMP-triggered immunity in *Arabidopsis thaliana*. *Molecular Plant* **7**, 657-674.

Garmendia, J., Frankel, G. & Crepin, V. F. (2005). Enteropathogenic and Enterohemorrhagic *Escherichia coli* Infections: Translocation, Translocation, Translocation. *Infection and Immunity* **73**, 2573-2585.

Gomes, C., Da Silva, P., Moreira, R. G., Castell-Perez, E., Ellis, E. A. & Pendleton, M. (2009). Understanding *E. coli* internalization in lettuce leaves for optimization of irradiation treatment. *International journal of food microbiology* **135**, 238-247.

Gonyar, L. A. & Kendall, M. M. (2014). Ethanolamine and choline promote expression of putative and characterized fimbriae in enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **82**, 193-201.

Goudeau, D. M., Parker, C. T., Zhou, Y., Sela, S., Kroupitski, Y. & Brandl, M. T. (2013). The *Salmonella* transcriptome in lettuce and cilantro soft rot reveals a niche overlap with the animal host intestine. *Applied and Environmental Microbiology* **79**, 250-262.

Grabowiecka, E. (2014). Microbiologist guide to transcriptomics – comparison of computational approaches to adaptation of pathogenic *E. coli* to spinach leaves, BSc Honours Microbiology. BSc Honours Dissertation. University of Dundee.

Greene, S. K., Daly, E. R., Talbot, E. A., Demma, L. J., Holzbauer, S., Patel, N. J., Hill, T. A., Walderhaug, M. O., Hoekstra, R. M., Lynch, M. F. & Painter, J. A. (2008). Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiology and infection* **136**, 157-165.

Gu, G., Hu, J., Cevallos-Cevallos, J. M., Richardson, S. M., Bartz, J. A. & van Bruggen, A. H. C. (2011). Internal Colonization of *Salmonella enterica* Serovar Typhimurium in Tomato Plants. *PLoS ONE* **6**, e27340.

Hadjok, C., Mittal, G. S. & Warriner, K. (2008). Inactivation of human pathogens and spoilage bacteria on the surface and internalized within fresh produce by using a combination of ultraviolet light and hydrogen peroxide. *Journal of Applied Microbiology* **104**, 1014-1024.

Hartland, E. L., Batchelor, M., Delahay, R. M., Hale, C., Matthews, S., Dougan, G., Knutton, S., Connerton, I. & Frankel, G. (1999). Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Molecular Microbiology* **32**, 151-158.

Hassaninasab, A., Hashimoto, Y., Tomita-Yokotani, K. & Kobayashi, M. (2011). Discovery of the curcumin metabolic pathway involving a unique enzyme in an intestinal microorganism. *Proceedings of the National Academy of Sciences* **108**, 6615-6620.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.-G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M. & Shinagawa, H. (2001). Complete Genome Sequence of Enterohemorrhagic *Escherichia coli* O157:H7 and Genomic Comparison with a Laboratory Strain K-12. *DNA Research* **8**, 11-22.

He, S. Y., Nomura, K. & Whittam, T. S. (2004). Type III protein secretion mechanism in mammalian and plant pathogens. *Biochim et Biophysica Acta* **1694**, 181-206.

Heaton, J. C. & Jones, K. (2008). Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *Journal of Applied Microbiology* **104**, 613-626.

Hedberg, C. W., Angulo, F. J., White, K. E., Langkop, C. W., Schell, W. L., Stobierski, M. G., Schuchat, A., Besser, J. M., Dietrich, S., Helsel, L., Griffin, P. M., McFarland, J. W. & Osterholm, M. T. (1999). Outbreaks of salmonellosis associated with eating uncooked tomatoes: implications for public health. *Epidemiology and infection* **122**, 385-393.

Ho Sui, S. J., Fedynak, A., Hsiao, W. W. L., Langille, M. G. I. & Brinkman, F. S. L. (2009). The Association of Virulence Factors with Genomic Islands. *PLoS ONE* **4**, e8094.

Holden, N. J. (2010). Plants as reservoirs for human enteric pathogens. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **5**.

Holmes, A., Birse, L., Jackson, R. W. & Holden, N. (2014). An optimised method for the extraction of bacterial mRNA from plant roots infected with *Escherichia coli* O157:H7. *Frontiers in microbiology* **5**.

Hora, R., Warriner, K., Shelp, B. J. & Griffiths, M. W. (2005). Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *Journal of Food Protection* **68**, 2506-2509.

Hou, Z., Fink, R. C., Black, E. P., Sugawara, M., Zhang, Z., Diez-Gonzalez, F. & Sadowsky, M. J. (2012). Gene expression profiling of *Escherichia coli* in response to interactions with the lettuce rhizosphere. *Journal of Applied Microbiology* **113**, 1076-1086.

Hou, Z., Fink, R. C., Sugawara, M., Diez-Gonzalez, F. & Sadowsky, M. J. (2013). Transcriptional and functional responses of *Escherichia coli* O157:H7 growing in the lettuce rhizoplane. *Food Microbiology* **35**, 136-142.

Hsiao, W., Wan, I., Jones, S. J. & Brinkman, F. S. L. (2003). IslandPath: aiding detection of genomic islands in prokaryotes. *Bioinformatics* **19**, 418-420.

Hufnagel, D. A., Tükel, Ç. & Chapman, M. R. (2013). Disease to Dirt: The Biology of Microbial Amyloids. *PLoS Pathogens* **9**, e1003740.

Imamovic, L., Tozzoli, R., Michelacci, V., Minelli, F., Marziano, M. L., Caprioli, A. & Morabito, S. (2010). OI-57, a genomic island of *Escherichia coli* O157, is present in other seropathotypes of Shiga toxin-producing *E. coli* associated with severe human disease. *Infection and Immunity* **78**, 4697-4704.

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P. & Jiang, X. (2004). Persistence of *Salmonella enterica* serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *Foodborne pathogens and disease* **1**, 27-35.

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P. & Jiang, X. (2005). Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiology* **22**, 63-70.

Jackson, R. W., Vlnatzer, B., Arnold, D. L., Dorus, S. & Murillo, J. (2011). The influence of the accessory genome on bacterial pathogen evolution. *Mobile Genetic Elements* **1**, 55-65.

Janisiewicz, W. J., Conway, W. S., Brown, M. W., Sapers, G. M., Fratamico, P. & Buchanan, R. L. (1999a). Fate of *Escherichia coli* O157:H7 on fresh-cut apple tissue and its potential for transmission by fruit flies. *Applied and Environmental Microbiology* **65**, 1-5.

Janisiewicz, W. J., Conway, W. S. & Leverentz, B. (1999b). Biological control of postharvest decays of apple can prevent growth of *Escherichia coli* O157:H7 in apple wounds. *Journal of Food Protection* **62**, 1372-1375.

Jayaraman, D., Valdés-López, O., Kaspar, C. W. & Ané, J.-M. (2014). Response of *Medicago truncatula* Seedlings to Colonization by *Salmonella enterica* and *Escherichia coli* O157:H7. *PLoS ONE* **9**, e87970.

Johannessen, G. S., Bengtsson, G. B., Heier, B. T., Bredholt, S., Wasteson, Y. & Rorvik, L. M. (2005). Potential uptake of *Escherichia coli* O157:H7 from organic manure into crisphead lettuce. *Applied and Environmental Microbiology* **71**, 2221-2225.

Johnson, R. P., Clarke, R. C., Wilson, J. B., Read, S. C., Rahn, K., Renwick, S. A., Sandhu, K. A., Alves, D., Karmali, M. A., Lior, H., McEwen, S. A., Spika, J. S. & Gyles, C. L. (1996). Growing Concerns and Recent Outbreaks Involving Non-O157:H7 Serotypes of Verotoxigenic *Escherichia coli*. *Journal of Food Protection* **59**, 1112-1122.

Jones, D. L., Hodge, A. & Kuzyakov, Y. (2004). Plant and mycorrhizal regulation of rhizodeposition. *New Phytologist* **163**, 459-480.

Jones, D. L., Nguyen, C. & Finlay, R. D. (2009). Carbon flow in the rhizosphere: carbon trading at the soil–root interface. *Plant and Soil* **321**, 5-33.

Jones, J., Studholme, D. J., Knight, C. G. & Preston, G. M. (2007). Integrated bioinformatic and phenotypic analysis of RpoN-dependent traits in the plant growth-promoting bacterium *Pseudomonas fluorescens* SBW25. *Environmental Microbiology* **9**, 3046-3064.

Jones, J. D. G. & Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323-329.

Jones, L., Milne, J. L., Ashford, D. & McQueen-Mason, S. J. (2003). Cell wall arabinan is essential for guard cell function. *Proceedings of the National Academy of Sciences* **100**, 11783-11788.

Kaper, J. B., Nataro, J. P. & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology* **2**, 123-140.

Karpinets, T. V., Park, B. H., Syed, M. H., Klotz, M. G. & Uberbacher, E. C. (2014). Metabolic Environments and Genomic Features Associated with Pathogenic and Mutualistic Interactions Between Bacteria and Plants. *Molecular Plant-Microbe Interactions* **27**, 664-677.

Kaschabek, S. R., Kuhn, B., Muller, D., Schmidt, E. & Reineke, W. (2002). Degradation of aromatics and chloroaromatics by *Pseudomonas* sp. strain B13: purification and characterization of 3-oxoadipate:succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. *Journal of Bacteriology* **184**, 207-215.

Kauffman, M. D. & LeJeune, J. (2011). European Starlings (*Sturnus vulgaris*) challenged with *Escherichia coli* O157 can carry and transmit the human pathogen to cattle. *Letters in Applied Microbiology* **53**, 596-601.

Kazan, K. & Manners, J. M. (2009). Linking development to defense: auxin in plant–pathogen interactions. *Trends in Plant Science* **14**, 373-382.

Keseler, I. M., Collado-Vides, J., Santos-Zavaleta, A., Peralta-Gil, M., Gama-Castro, S., Muñiz-Rascado, L., Bonavides-Martinez, C., Paley, S., Krummenacker, M., Altman, T., Kaipa, P., Spaulding, A., Pacheco, J., Latendresse, M., Fulcher, C., Sarker, M., Shearer, A. G., Mackie, A., Paulsen, I., Gunsalus, R. P. & Karp, P. D. (2011). EcoCyc: a comprehensive database of *Escherichia coli* biology. *Nucleic Acids Research* **39**, D583-D590.

Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martínez, C., Fulcher, C., Huerta, A. M., Kothari, A., Krummenacker, M., Latendresse, M., Muñiz-Rascado, L., Ong, Q., Paley, S., Schröder, I., Shearer, A. G., Subhraveti, P., Travers, M., Weerasinghe, D., Weiss, V., Collado-Vides, J., Gunsalus, R. P., Paulsen, I. & Karp, P. D. (2013). EcoCyc: fusing model organism databases with systems biology. *Nucleic Acids Research* **41**, D605-D612.

Keskinen, L. A. & Annous, B. A. (2011). Efficacy of adding detergents to sanitizer solutions for inactivation of *Escherichia coli* O157:H7 on Romaine lettuce. *International journal of food microbiology* **147**, 157-161.

Kim, J., Demeke, T., Clear, R. M. & Patrick, S. K. (2006). Simultaneous detection by PCR of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium* in artificially inoculated wheat grain. *International journal of food microbiology* **111**, 21-25.

Kim, S. Y., Sagong, H. G., Ryu, S., Mah, J. H. & Kang, D. H. (2011). Development of oscillation method for reducing foodborne pathogens on lettuce and spinach. *International journal of food microbiology* **145**, 273-278.

Klerks, M. M., Franz, E., van Gent-Pelzer, M., Zijlstra, C. & van Bruggen, A. H. (2007). Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *ISME J* **1**, 620-631.

Kljujev, I., Raicevic, V., Andrews, S., Jackson, R., Lalevic, B. & Dorati, F. (2012). Transmission of *E. coli* from contaminated irrigation water and soil to plant tissue. *Journal of Hygienic Engineering and Design* **1**, 83-87.

Knight, C. G., Zhang, X. X., Gunn, A., Brenner, T., Jackson, R. W., Giddens, S. R., Prabhakar, S., Zitzmann, N. & Rainey, P. B. (2010). Testing temperature-induced proteomic changes in the plant-associated bacterium *Pseudomonas fluorescens* SBW25. *Environmental microbiology reports* **2**, 396-402.

Kocharunchitt, C., Ross, T. & McNeil, D. L. (2009). Use of bacteriophages as biocontrol agents to control *Salmonella* associated with seed sprouts. *International journal of food microbiology* **128**, 453-459.

Koita, K. & Rao, C. V. (2012). Identification and analysis of the putative pentose sugar efflux transporters in *Escherichia coli*. *PLoS ONE* **7**, e43700.

Kotewicz, M. L., Jackson, S. A., LeClerc, J. E. & Cebula, T. A. (2007). Optical maps distinguish individual strains of *Escherichia coli* O157:H7. *Microbiology* **153**, 1720-1733.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D. & Sela, S. (2009a). Internalization of *Salmonella enterica* in Leaves Is Induced by Light and Involves Chemotaxis and Penetration through Open Stomata. *Applied and Environmental Microbiology* **75**, 6076-6086.

Kroupitski, Y., Pinto, R., Brandl, M. T., Belausov, E. & Sela, S. (2009b). Interactions of *Salmonella enterica* with lettuce leaves. *Journal of Applied Microbiology* **106**, 1876-1885.

Kroupitski, Y., Pinto, R., Belausov, E. & Sela, S. (2011). Distribution of *Salmonella typhimurium* in romaine lettuce leaves. *Food Microbiology* **28**, 990-997.

Krumsiek, J., Arnold, R. & Rattei, T. (2007). Gepard: a rapid and sensitive tool for creating dotplots on genome scale. *Bioinformatics* **23**, 1026-1028.

Kuijken, R. P., Snel, J. H., Heddes, M., Bouwmeester, H. & Marcelis, L. M. (2015). The importance of a sterile rhizosphere when phenotyping for root exudation. *Plant and Soil* **387**, 131-142.

Kuzyakov, Y. & Domanski, G. (2000). Carbon input by plants into the soil. Review. *Journal of Plant Nutrition and Soil Science* **163**, 421-431.

Kyle, J. L., Parker, C. T., Goudeau, D. & Brandl, M. T. (2010). Transcriptome Analysis of *Escherichia coli* O157:H7 Exposed to Lysates of Lettuce Leaves. *Applied and Environmental Microbiology* **76**, 1375-1387.

Laidler, M. R., Tourdjman, M., Buser, G. L., Hostetler, T., Repp, K. K., Leman, R., Samadpour, M. & Keene, W. E. (2013). *Escherichia coli* O157:H7 infections associated with consumption of locally grown strawberries contaminated by deer. *Clinical Infectious Diseases* **57**, 1129-1134.

Landstorfer, R., Simon, S., Schober, S., Keim, D., Scherer, S. & Neuhaus, K. (2014). Comparison of strand-specific transcriptomes of enterohemorrhagic *Escherichia coli* O157:H7 EDL933 (EHEC) under eleven different environmental conditions including radish sprouts and cattle feces. *BMC Genomics* **15**, 353.

Lang, M. M., Ingham, B. H. & Ingham, S. C. (2000). Efficacy of novel organic acid and hypochlorite treatments for eliminating *Escherichia coli* O157:H7 from alfalfa seeds prior to sprouting. *International journal of food microbiology* **58**, 73-82.

Langille, M., Hsiao, W. & Brinkman, F. (2008). Evaluation of genomic island predictors using a comparative genomics approach. *BMC Bioinformatics* **9**, 329.

Langille, M. G. & Brinkman, F. S. (2009). IslandViewer: an integrated interface for computational identification and visualization of genomic islands. *Bioinformatics* **25**, 664-665.

Lanoue, A., Burlat, V., Schurr, U. & Rose, U. S. (2010). Induced root-secreted phenolic compounds as a belowground plant defense. *Plant Signaling and Behavior* **5**, 1037-1038.

Law, D. (2000). Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *Journal of Applied Microbiology* **88**, 729-745.

Lehti, T. A., Bauchart, P., Dobrindt, U., Korhonen, T. K. & Westerlund-Wikstrom, B. (2012). The fimbriae activator MatA switches off motility in *Escherichia coli* by repression of the flagellar master operon *flhDC*. *Microbiology* **158**, 1444-1455.

Lehti, T. A., Bauchart, P., Kukkonen, M., Dobrindt, U., Korhonen, T. K. & Westerlund-Wikstrom, B. (2013). Phylogenetic group-associated differences in regulation of the common colonization factor Mat fimbria in *Escherichia coli*. *Molecular Microbiology* **87**, 1200-1222.

Liu, B. & Pop, M. (2009). ARDB - Antibiotic Resistance Genes Database. *Nucleic Acids Research* **37**, D443-D447.

Lopez-Velasco, G., Tydings, H. A., Boyer, R. R., Falkinham, J. O., 3rd & Ponder, M. A. (2012). Characterization of interactions between *Escherichia coli* O157:H7 with epiphytic bacteria in vitro and on spinach leaf surfaces. *International journal of food microbiology* **153**, 351-357.

Low, A. S., Holden, N., Rosser, T., Roe, A. J., Constantinidou, C., Hobman, J. L., Smith, D. G., Low, J. C. & Gally, D. L. (2006). Analysis of fimbrial gene clusters and their expression in enterohaemorrhagic *Escherichia coli* O157:H7. *Environmental Microbiology* **8**, 1033-1047.

Lynch, J. M. & Whipps, J. M. (1990). Substrate flow in the rhizosphere. *Plant and Soil* **129**, 1-10.

Lynch, M. F., Tauxe, R. V. & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. *Epidemiology & Infection* **137**, 307-315.

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A. & Sharma, V. K. (2012). Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. *Foodborne pathogens and disease* **9**, 160-167.

Macarisin, D., Patel, J., Bauchan, G., Giron, J. A. & Ravishankar, S. (2013). Effect of Spinach Cultivar and Bacterial Adherence Factors on Survival of *Escherichia coli* O157:H7 on Spinach Leaves. *Journal of Food Protection* **76**, 1829-1837.

Makino, K., Ishii, K., Yasunaga, T., Hattori, M., Yokoyama, K., Yutsudo, C. H., Kubota, Y., Yamaichi, Y., Iida, T., Yamamoto, K., Honda, T., Han, C. G., Ohtsubo, E., Kasamatsu, M., Hayashi, T., Kuhara, S. & Shinagawa, H. (1998). Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. *DNA Research* **5**, 1-9.

Makino, K., Yokoyama, K., Kubota, Y., Yutsudo, C. H., Kimura, S., Kurokawa, K., Ishii, K., Hattori, M., Tatsuno, I., Abe, H., Iida, T., Yamamoto, K., Onishi, M., Hayashi, T., Yasunaga, T., Honda, T., Sasakawa, C. & Shinagawa, H. (1999). Complete nucleotide sequence of the prophage VT2-Sakai carrying the verotoxin 2 genes of the enterohemorrhagic Escherichia coli O157:H7 derived from the Sakai outbreak. *Genes Genet Syst* **74**, 227-239.

Manning, S. D., Motiwala, A. S., Springman, A. C., Qi, W., Lacher, D. W., Ouellette, L. M., Mladonicky, J. M., Somsel, P., Rudrik, J. T., Dietrich, S. E., Zhang, W., Swaminathan, B., Alland, D. & Whittam, T. S. (2008). Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. *Proceedings of the National Academy of Sciences* **105**, 4868-4873.

Mansilla, M. C., Cybulski, L. E., Albanesi, D. & de Mendoza, D. (2004). Control of Membrane Lipid Fluidity by Molecular Thermosensors. *Journal of Bacteriology* **186**, 6681-6688.

Manzanares, P., de Graaff, L. H. & Visser, J. (1998). Characterization of galactosidases from *Aspergillus niger*: purification of a novel alpha-galactosidase activity. *Enzyme and Microbial Technology* **22**, 383-390.

Mark, G. L., Dow, J. M., Kiely, P. D., Higgins, H., Haynes, J., Baysse, C., Abbas, A., Foley, T., Franks, A., Morrissey, J. & O'Gara, F. (2005). Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. *Proceedings of the National Academy of Sciences* **102**, 17454-17459.

Mattinen, L., Nissinen, R., Riipi, T., Kalkkinen, N. & Pirhonen, M. (2007). Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. *PROTEOMICS* **7**, 3527-3537.

Melotto, M., Panchal, S. & Roy, D. (2014). Plant innate immunity against human bacterial pathogens. *Frontiers in microbiology* **5**.

Meng, F., Altier, C. & Martin, G. B. (2013). *Salmonella* colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. *Environmental Microbiology* **15**, 2418-2430.

Merlin, C., McAteer, S. & Masters, M. (2002). Tools for Characterization of *Escherichia coli* Genes of Unknown Function. *Journal of Bacteriology* **184**, 4573-4581.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A. & Yanagawa, H. (1999). Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *American journal of epidemiology* **150**, 787-796.

Miller, B. D., Rigdon, C. E., Ball, J., Rounds, J. M., Klos, R. F., Brennan, B. M., Arends, K. D., Kennelly, P., Hedberg, C. & Smith, K. E. (2012). Use of Traceback Methods To Confirm the Source of a Multistate *Escherichia coli* O157:H7 Outbreak Due to In-Shell Hazelnuts. *Journal of Food Protection* **75**, 320-327.

Miller, J. H. (1972). *Experiments in Molecular Genetics*: Cold Spring Harbor Laboratory.

Mills, E., Baruch, K., Aviv, G., Nitzan, M. & Rosenshine, I. (2013). Dynamics of the Type III Secretion System Activity of Enteropathogenic *Escherichia coli*. *MBio* **4**.

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S. & Fletcher, J. (2009). Effect of Route of Introduction and Host Cultivar on the Colonization, Internalization, and Movement of the Human Pathogen *Escherichia coli* O157:H7 in Spinach. *Journal of Food Protection* **72**, 1521-1530.

Miyada, C. G., Stoltzfus, L. & Wilcox, G. (1984). Regulation of the araC gene of Escherichia coli: catabolite repression, autoregulation, and effect on araBAD expression. *Proc Natl Acad Sci U S A* **81**, 4120-4124.

Moe, L. A. (2013). Amino acids in the rhizosphere: from plants to microbes. *Am J Bot* **100**, 1692-1705.

Monk, J. M., Charusanti, P., Aziz, R. K., Lerman, J. A., Premyodhin, N., Orth, J. D., Feist, A. M. & Palsson, B. Ø. (2013). Genome-scale metabolic reconstructions of multiple Escherichia coli strains highlight strain-specific adaptations to nutritional environments. *Proceedings of the National Academy of Sciences* **110**, 20338-20343.

Montenegro, M. A., Bülte, M., Trumpf, T., Aleksić, S., Reuter, G., Bulling, E. & Helmuth, R. (1990). Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. *Journal of clinical microbiology* **28**, 1417-1421.

Moyne, A.-L., Harris, L. J. & Marco, M. L. (2013). Assessments of Total and Viable *Escherichia coli* O157:H7 on Field and Laboratory Grown Lettuce. *PLoS ONE* **8**, e70643.

Nam, H. S., Anderson, A. J., Yang, K. Y., Cho, B. H. & Kim, Y. C. (2006). The dctA gene of *Pseudomonas chlororaphis* O6 is under RpoN control and is required for effective root colonization and induction of systemic resistance. *FEMS microbiology letters* **256**, 98-104.

NCBI Resource Coordinators (2013). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research* **41**, D8-D20.

Neal, J. A., Marquez-Gonzalez, M., Cabrera-Diaz, E., Lucia, L. M., O'Bryan, C. A., Crandall, P. G., Ricke, S. C. & Castillo, A. (2012). Comparison of multiple chemical sanitizers for reducing *Salmonella* and *Escherichia coli* O157:H7 on spinach (*Spinacia oleracea*) leaves. *Food Research International* **45**, 1123-1128.

Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture Medium for *Enterobacteria*. *Journal of Bacteriology* **119**, 736-747.

Neidhardt, F. C. (1996). *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd Edition edn: ASM Press.

Neumann, G., Bott, S., Ohler, M. A., Mock, H. P., Lippmann, R., Grosch, R. & Smalla, K. (2014). Root exudation and root development of lettuce (*Lactuca sativa* L. cv. Tizian) as affected by different soils. *Frontiers in microbiology* **5**, 2.

Niemira, B. A. (2007). Relative efficacy of sodium hypochlorite wash versus irradiation to inactivate *Escherichia coli* O157:H7 internalized in leaves of Romaine lettuce and baby spinach. *Journal of Food Protection* **70**, 2526-2532.

Noel, J. T., Arrach, N., Alagely, A., McClelland, M. & Teplitski, M. (2010a). Specific Responses of *Salmonella enterica* to Tomato Varieties and Fruit Ripeness Identified by *In Vivo* Expression Technology. *PLoS ONE* **5**, e12406.

Noel, J. T., Joy, J., Smith, J. N., Fatica, M., Schneider, K. R., Ahmer, B. M. & Teplitski, M. (2010b). *Salmonella* SdiA recognizes N-acyl homoserine lactone signals from *Pectobacterium carotovorum* in vitro, but not in a bacterial soft rot. *Molecular Plant Microbe Interactions* **23**, 273-282.

Oburger, E., Kirk, G. J. D., Wenzel, W. W., Puschenreiter, M. & Jones, D. L. (2009). Interactive effects of organic acids in the rhizosphere. *Soil Biology and Biochemistry* **41**, 449-457.

Ohnishi, M., Murata, T., Nakayama, K., Kuhara, S., Hattori, M., Kurokawa, K., Yasunaga, T., Yokoyama, K., Makino, K., Shinagawa, H. & Hayashi, T. (2000). Comparative Analysis of the Whole Set of rRNA Operons Between an Enterohemorrhagic *Escherichia coli* O157:H7 Sakai Strain and an *Escherichia coli* K-12 Strain MG1655. *Systematic and Applied Microbiology* **23**, 315-324.

Oliveira, M., Vinas, I., Usall, J., Anguera, M. & Abadias, M. (2012). Presence and survival of *Escherichia coli* O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. *International journal of food microbiology* **156**, 133-140.

Oliveros, J. (2007).VENNY. An interactive tool for comparing lists with Venn diagrams.

Patel, J., Millner, P., Nou, X. & Sharma, M. (2010). Persistence of enterohaemorrhagic and nonpathogenic *E. coli* on spinach leaves and in rhizosphere soil. *Journal of Applied Microbiology* **108**, 1789-1796.

Patel, J. & Sharma, M. (2010). Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. *International journal of food microbiology* **139**, 41-47.

Perna, N. T., Mayhew, G. F., Posfai, G., Elliott, S., Donnenberg, M. S., Kaper, J. B. & Blattner, F. R. (1998). Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* **66**, 3810-3817.

Popper, Z. A. (2011). *The Plant Cell Wall - Methods & Protocols*: Humana Press.

PremierBiosoft NetPrimer, http://www.premierbiosoft.com/netprimer/. 23/06/2014.

Preston, G. M. (2007). Metropolitan Microbes: Type III Secretion in Multihost Symbionts. *Cell Host & Microbe* **2**, 291-294.

ProMED-mail (2011). *E. coli* O157 - United Kingdom, Leeks and Potatoes, http://www.promedmail.org/?archiveid=O1578

Pruimboom-Brees, I. M., Morgan, T. W., Ackermann, M. R., Nystrom, E. D., Samuel, J. E., Cornick, N. A. & Moon, H. W. (2000). Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proceedings of the National Academy of Sciences* **97**, 10325-10329.

Quilliam, R. S., Williams, A. P. & Jones, D. L. (2012). Lettuce Cultivar Mediates Both Phyllosphere and Rhizosphere Activity of *Escherichia coli* O157:H7. *PLoS ONE* **7**, e33842.

Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environmental Microbiology* **1**, 243-257.

Ramírez-Puebla, S. T., Servín, L. E., Jiménez-Marín, B., Bolaños, L. M., Rosenblueth, M., Martínez, J., Rogel, M. A., Ormeño-Orrillo, E. & Martínez-Romero, E. (2012). Gut and root microbiota commonalities. *Applied and Environmental Microbiology*.

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. & Swerdlow, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerging infectious diseases* **11**, 603-609.

Ravirala, R. S., Barabote, R. D., Wheeler, D. M., Reverchon, S., Tatum, O., Malouf, J., Liu, H., Pritchard, L., Hedley, P. E., Birch, P. R., Toth, I. K., Payton, P. & San Francisco, M. J. (2007). Efflux pump gene expression in *Erwinia chrysanthemi* is induced by exposure to phenolic acids. *Molecular Plant Microbe Interactions* **20**, 313-320.

Reeder, T. & Schleif, R. (1991). Mapping, sequence, and apparent lack of function of *araJ*, a gene of the *Escherichia coli* arabinose regulon. *Journal of Bacteriology* **173**, 7765-7771.

Reines, M., Llobet, E., Llompart, C. M., Moranta, D., Perez-Gutierrez, C. & Bengoechea, J. A. (2012). Molecular basis of *Yersinia enterocolitica* temperature-dependent resistance to antimicrobial peptides. *Journal of Bacteriology* **194**, 3173-3188.

Rendón, M. a. A., Saldaña, Z., Erdem, A. L., Monteiro-Neto, V., Vázquez, A., Kaper, J. B., Puente, J. L. & Girón, J. A. (2007). Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proceedings of the National Academy of Sciences* **104**, 10637-10642.

Rico, A. & Preston, G. M. (2008). *Pseudomonas syringae* pv. tomato DC3000 Uses Constitutive and Apoplast-Induced Nutrient Assimilation Pathways to Catabolize Nutrients That Are Abundant in the Tomato Apoplast. *Molecular Plant-Microbe Interactions* **21**, 269-282.

Rico, A., McCraw, S. L. & Preston, G. M. (2011). The metabolic interface between *Pseudomonas syringae* and plant cells. *Current Opinion in Microbiology* **14**, 31-38.

Riordan, D. C., Sapers, G. M. & Annous, B. A. (2000). The survival of *Escherichia coli* O157:H7 in the presence of *Penicillium expansum* and *Glomerella cingulata* in wounds on apple surfaces. *Journal of Food Protection* **63**, 1637-1642.

Rivas-San Vicente, M. & Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany* **62**, 3321-3338.

Rivera-Domínguez, M., Astorga-Cienfuegos, K. R., Tiznado-Hernández, M. E. & González-Aguilar, G. A. (2012). Induction of the expression of defence genes in *Carica papaya* fruit by methyl jasmonate and low temperature treatments. *Electronic Journal of Biotechnology* **15**.

Roberts, J. A. (2000). Economic aspects of food-borne outbreaks and their control. *British medical bulletin* **56**, 133-141.

Rosenblueth, M., Martinez, L., Silva, J. & Martinez-Romero, E. (2004). *Klebsiella variicola*, a novel species with clinical and plant-associated isolates. *Systematic and Applied Microbiology* **27**, 27-35.

Rossez, Y., Holmes, A., Wolfson, E. B., Gally, D. L., Mahajan, A., Pedersen, H. L., Willats, W. G., Toth, I. K. & Holden, N. J. (2013). Flagella interact with ionic plant lipids to mediate adherence of pathogenic *Escherichia coli* to fresh produce plants. *Environmental Microbiology* **16**, 2181-2195.

Rossez, Y., Holmes, A., Lodberg-Pederson, H., Birse, L., Marshall, J., Willats, W. G. T., Toth, I. K. & Holden, N. J. (2014). *Escherichia coli* common pilus (ECP) targets arabinosyl residues in plant cell walls to mediate adhesion to fresh produce plants. *Journal of Biological Chemistry* **289**, 34349-34365.

Roy, D., Panchal, S., Rosa, B. A. & Melotto, M. (2013). *Escherichia coli* O157:H7 Induces Stronger Plant Immunity than *Salmonella enterica* Typhimurium SL1344. *Phytopathology* **103**, 326-332.

Rudrappa, T. & Bais, H. P. (2008). Curcumin, a Known Phenolic from *Curcuma longa*, Attenuates the Virulence of *Pseudomonas aeruginosa* PAO1 in Whole Plant and Animal Pathogenicity Models. *Journal of Agricultural and Food Chemistry* **56**, 1955-1962.

Sakuta, M. (2014). Diversity in plant red pigments: anthocyanins and betacyanins. *Plant Biotechnology Reports* **8**, 37-48.

Saldaña, Z., Sánchez, E., Xicohtencatl-Cortes, J., Puente, J. L. & Girón, J. A. (2011). Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:H7. *Frontiers in microbiology* **2**, 119.

Santos, C. R., Hoffmam, Z. B., Martins, V. P., Zanphorlin, L. M., Assis, L. H., Honorato, R. V., Oliveira, P. S., Ruller, R. & Murakami, M. T. (2014). Molecular mechanisms associated with xylan degradation by *Xanthomonas* plant pathogens. *Journal of Biological Chemistry* **289**, 32189-32200.

Sapers, G. M. (2001). Efficacy of Washing and Sanitizing Methods for Disinfection of Fresh Fruit and Vegetable Products. *Food Technology & Biotechnology* **39**, 305-311.

Sauer, D., Kuzyakov, Y. & Stahr, K. (2006). Spatial distribution of root exudates of five plant species as assessed by 14C labeling. *Journal of Plant Nutrition and Soil Science* **169**, 360-362.

Savage, P. J., Campellone, K. G. & Leong, J. M. (2007). Interaction of enterohemorrhagic *Escherichia coli* (EHEC) with mammalian cells: cell adhesion, type III secretion, and actin pedestal formation. *Current protocols in microbiology* **Chapter 5**, Unit 5A 1.

Sazonova, O. I., Izmalkova, T. Y., Kosheleva, I. A. & Boronin, A. M. (2008). Salicylate degradation by *Pseudomonas putida* strains not involving the "Classical" nah2 operon. *Microbiology* **77**, 710-716.

Schenk, A., Weingart, H. & Ullrich, M. S. (2008). Extraction of high-quality bacterial RNA from infected leaf tissue for bacterial in planta gene expression analysis by multiplexed fluorescent Northern hybridization. *Molecular plant pathology* **9**, 227-235.

Schikora, A., Carreri, A., Charpentier, E. & Hirt, H. (2008). The Dark Side of the Salad: *Salmonella typhimurium* Overcomes the Innate Immune Response of *Arabidopsis thaliana* and Shows an Endopathogenic Lifestyle. *PLoS ONE* **3**, e2279.

Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A. V., Nilau, T., Charrier, A., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P. & Hirt, H. (2011). Conservation of *Salmonella* Infection Mechanisms in Plants and Animals. *PLoS ONE* **6**, e24112.

Schikora, A., Garcia, A. V. & Hirt, H. (2012a). Plants as alternative hosts for *Salmonella*. *Trends in Plant Science* **17**, 245-249.

Schikora, M., Neupane, B., Madhogaria, S., Koch, W., Cremers, D., Hirt, H., Kogel, K.-H. & Schikora, A. (2012b). An image classification approach to analyze the suppression of plant immunity by the human pathogen *Salmonella Typhimurium*. *BMC Bioinformatics* **13**, 171.

Schuller, S. (2011). Shiga toxin interaction with human intestinal epithelium. *Toxins (Basel)* **3**, 626-639.

Seo, H. S., Bang, J., Kim, H., Beuchat, L. R., Cho, S. Y. & Ryu, J. H. (2012). Development of an antimicrobial sachet containing encapsulated allyl isothiocyanate to inactivate *Escherichia coli* O157:H7 on spinach leaves. *International journal of food microbiology* **159**, 136-143.

Seo, S. & Matthews, K. R. (2012). Influence of the Plant Defense Response to *Escherichia coli* O157:H7 Cell Surface Structures on Survival of That Enteric Pathogen on Plant Surfaces. *Applied and Environmental Microbiology* **78**, 5882-5889.

Sharma, M., Ingram, D. T., Patel, J. R., Millner, P. D., Wang, X., Hull, A. E. & Donnenberg, M. S. (2009a). A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. *Journal of Food Protection* **72**, 1513-1520.

Sharma, M., Patel, J. R., Conway, W. S., Ferguson, S. & Sulakvelidze, A. (2009b). Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. *Journal of Food Protection* **72**, 1481-1485.

Shaw, R. K., Berger, C. N., Feys, B., Knutton, S., Pallen, M. J. & Frankel, G. (2008). Enterohemorrhagic *Escherichia coli* exploits EspA filaments for attachment to salad leaves. *Applied and Environmental Microbiology* **74**, 2908-2914.

Shaw, R. K., Berger, C. N., Pallen, M. J., Sjoling, A. & Frankel, G. (2011). Flagella mediate attachment of enterotoxigenic *Escherichia coli* to fresh salad leaves. *Environmental microbiology reports* **3**, 112-117.

Shidore, T., Dinse, T., Ohrlein, J., Becker, A. & Reinhold-Hurek, B. (2012). Transcriptomic analysis of responses to exudates reveal genes required for rhizosphere competence of the endophyte *Azoarcus* sp. strain BH72. *Environmental Microbiology* **14**, 2775-2787.

Singh, B. R., Chandra, M., Agarwal, R. & Babu, N. (2007). Interactions between *Salmonella enterica* supspecies *enterica* serovar Typhimurium and Cowpea (*Vigna unguiculata* variety Sinensis) Seeds, Plants and Persistance in Hay. *Journal of Food Safety* **27**, 169-187.

Smith, M. J., Carvalho, H. M., Melton-Celsa, A. R. & O'Brien, A. D. (2006). The 13C4 monoclonal antibody that neutralizes Shiga toxin Type 1 (Stx1) recognizes three regions on the Stx1 B subunit and prevents Stx1 from binding to its eukaryotic receptor globotriaosylceramide. *Infection and Immunity* **74**, 6992-6998.

Solomon, E. B., Yaron, S. & Matthews, K. R. (2002). Transmission of *Escherichia coli* O157:H7 from Contaminated Manure and Irrigation Water to Lettuce Plant Tissue and Its Subsequent Internalization. *Applied and Environmental Microbiology* **68**, 397-400.

Solomon, E. B. & Matthews, K. R. (2005). Use of fluorescent microspheres as a tool to investigate bacterial interactions with growing plants. *Journal of Food Protection* **68**, 870-873.

Somers, E., Vanderleyden, J. & Srinivasan, M. (2004). Rhizosphere bacterial signalling: a love parade beneath our feet. *Critical reviews in microbiology* **30**, 205-240.

Spaepen, S. & Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harbor perspectives in biology* **3**.

Struelens, M. J., Palm, D. & Takkinen, J. (2011). Enteroaggregative, Shiga toxin-producing *Escherichia coli* O104:H4 outbreak: new microbiological findings boost coordinated investigations by European public health laboratories. *Eurosurveillance* **16**.

Sunnarborg, A., Klumpp, D., Chung, T. & LaPorte, D. C. (1990). Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. *Journal of Bacteriology* **172**, 2642-2649.

Takeuchi, K. & Frank, J. F. (2000). Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *Journal of Food Protection* **63**, 434-440.

Tanaka, Y., Sano, T., Tamaoki, M., Nakajima, N., Kondo, N. & Hasezawa, S. (2006). Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in *Arabidopsis*. *Journal of Experimental Botany* **57**, 2259-2266.

Tani, T. H., Khodursky, A., Blumenthal, R. M., Brown, P. O. & Matthews, R. G. (2002). Adaptation to famine: a family of stationary-phase genes revealed by microarray analysis. *Proceedings of the National Academy of Sciences* **99**, 13471-13476.

Tarr, P. I., Gordon, C. A. & Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **365**, 1073-1086.

Tatsuno, I., Horie, M., Abe, H., Miki, T., Makino, K., Shinagawa, H., Taguchi, H., Kamiya, S., Hayashi, T. & Sasakawa, C. (2001). *toxB* gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. *Infection and Immunity* **69**, 6660-6669.

Teplitski, M., Warriner, K., Bartz, J. & Schneider, K. R. (2011). Untangling metabolic and communication networks: interactions of enterics with phytobacteria and their implications in produce safety. *Trends in Microbiology* **19**, 121-127.

Thilmony, R., Underwood, W. & He, S. Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. tomato DC3000 and the human pathogen *Escherichia coli* O157:H7. *The Plant Journal* **46**, 34-53.

Timmusk, S., Van West, P., Gow, N. A. R. & Paul Huffstutler, R. (2009). *Paenibacillus polymyxa* antagonizes oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*. *Journal of Applied Microbiology* **106**, 1473-1481.

Tobe, T., Beatson, S. A., Taniguchi, H., Abe, H., Bailey, C. M., Fivian, A., Younis, R., Matthews, S., Marches, O., Frankel, G., Hayashi, T. & Pallen, M. J. (2006). An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdoid phages in their dissemination. *Proceedings of the National Academy of Sciences* **103**, 14941-14946.

Toth, I. K., Pritchard, L. & Birch, P. R. (2006). Comparative genomics reveals what makes an enterobacterial plant pathogen. *Annual Review of Phytopathology* **44**, 305-336.

Tran, A. X., Lester, M. E., Stead, C. M., Raetz, C. R. H., Maskell, D. J., McGrath, S. C., Cotter, R. J. & Trent, M. S. (2005). Resistance to the Antimicrobial Peptide Polymyxin Requires Myristoylation of *Escherichia coli* and *Salmonella typhimurium* Lipid A. *Journal of Biological Chemistry* **280**, 28186-28194.

Tuttle, J., Gomez, T., Doyle, M. P., Wells, J. G., Zhao, T., Tauxe, R. V. & Griffin, P. M. (1999). Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiology & Infection* **122**, 185-192.

Uesugi, A. R., Danyluk, M. D., Mandrell, R. E. & Harris, L. J. (2007). Isolation of *Salmonella Enteritidis* phage type 30 from a single almond orchard over a 5-year period. *Journal of Food Protection* **70**, 1784-1789.

Underwood, A. P., Dallman, T., Thomson, N. R., Williams, M., Harker, K., Perry, N., Adak, B., Willshaw, G., Cheasty, T., Green, J., Dougan, G., Parkhill, J. & Wain, J. (2013). Public Health Value of Next-Generation DNA Sequencing of Enterohemorrhagic *Escherichia coli* Isolates from an Outbreak. *Journal of clinical microbiology* **51**, 232-237.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. & Rozen, S. G. (2012). Primer3 - new capabilities and interfaces. *Nucleic Acids Research* **40**, e115.

Üstün, Ş., Müller, P., Palmisano, R., Hensel, M. & Börnke, F. (2012). SseF, a type III effector protein from the mammalian pathogen *Salmonella enterica*, requires resistance-gene-mediated signalling to activate cell death in the model plant *Nicotiana benthamiana*. *New Phytologist* **194**, 1046-1060.

van de Mortel, J. E., de Vos, R. C., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., van Loon, J. J., Dicke, M. & Raaijmakers, J. M. (2012). Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiology* **160**, 2173-2188.

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M. & Heyndrickx, M. (2013). Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead

lettuce seeds, and their subsequent survival and growth on the seedlings. *International journal of food microbiology* **161**, 214-219.

van der Linden, I. (2014). Survival and virulence of *E. coli* O157:H7 and *Salmonella* during greenhouse butterhead lettuce production, PhD. Ghent University.

van Elsas, J. D. & Bailey, M. J. (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiology Ecology* **42**, 187-197.

Visvalingam, J., Hernandez-Doria, J. D. & Holley, R. A. (2013). Examination of the Genome-Wide Transcriptional Response of *Escherichia coli* O157:H7 to Cinnamaldehyde Exposure. *Applied and Environmental Microbiology* **79**, 942-950.

Vlot, A. C., Dempsey, D. A. & Klessig, D. F. (2009). Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annual Review of Phytopathology* **47**, 177-206.

Waack, S., Keller, O., Asper, R., Brodag, T., Damm, C., Fricke, W., Surovcik, K., Meinicke, P. & Merkl, R. (2006). Score-based prediction of genomic islands in prokaryotic genomes using hidden Markov models. *BMC Bioinformatics* **7**, 142.

Wade, W. N. & Beuchat, L. R. (2003). Metabiosis of proteolytic moulds and *Salmonella* in raw, ripe tomatoes. *Journal of Applied Microbiology* **95**, 437-450.

Wang, Y., Bao, Z., Zhu, Y. & Hua, J. (2009). Analysis of Temperature Modulation of Plant Defense Against Biotrophic Microbes. *Molecular Plant-Microbe Interactions* **22**, 498-506.

Wasala, L., Talley, J. L., DeSilva, U., Fletcher, J. & Wayadande, A. (2013). Transfer of *Escherichia coli* O157:H7 to Spinach by House Flies, *Musca domestica* (Diptera: Muscidae). *Phytopathology* **103**, 373-380.

Wei, M., Takeshima, K., Yokoyama, T., Minamisawa, K., Mitsui, H., Itakura, M., Kaneko, T., Tabata, S., Saeki, K., Omori, H., Tajima, S., Uchiumi, T., Abe, M., Ishii, S. & Ohwada, T. (2010). Temperature-dependent expression of type III secretion system genes and its regulation in *Bradyrhizobium japonicum*. *Molecular Plant-Microbe Interactions* **23**, 628-637.

Wells, J. G., Davis, B. R., Wachsmuth, I. K., Riley, L. W., Remis, R. S., Sokolow, R. & Morris, G. K. (1983). Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *Journal of clinical microbiology* **18**, 512-520.

Wells, J. M. & Butterfield, J. E. (1997). *Salmonella* Contamination Associated with Bacterial Soft Rot of Fresh Fruits and Vegetables in the Marketplace. *Plant Disease* **81**, 867-872.

Wendel, A. M., Johnson, D. H., Sharapov, U., Grant, J., Archer, J. R., Monson, T., Koschmann, C. & Davis, J. P. (2009). Multistate Outbreak of *Escherichia coli* O157:H7 Infection Associated with Consumption of Packaged Spinach, August–September 2006: The Wisconsin Investigation. *Clinical Infectious Diseases* **48**, 1079-1086.

Werber, D., Krause, G., Frank, C., Fruth, A., Flieger, A., Mielke, M., Schaade, L. & Stark, K. (2012). Outbreaks of virulent diarrheagenic *Escherichia coli* - are we in control? *BMC medicine* **10**, 11.

Willshaw, G. A., Thirlwell, J., Jones, A. P., Parry, S., Salmon, R. L. & Hickey, M. (1994). Vero cytotoxin-producing *Escherichia coli* O157 in beefburgers linked to an outbreak of diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome in Britain. *Letters in Applied Microbiology* **19**, 304-307.

Windstam, S. & Nelson, E. B. (2008). Temporal Release of Fatty Acids and Sugars in the Spermosphere: Impacts on *Enterobacter cloacae*-Induced Biological Control. *Applied and Environmental Microbiology* **74**, 4292-4299.

Wright, K. M., Chapman, S., McGeachy, K., Humphris, S., Campbell, E., Toth, I. K. & Holden, N. J. (2013). The Endophytic Lifestyle of *Escherichia coli* O157:H7: Quantification and Internal Localization in Roots. *Phytopathology* **103**, 333-340.

Yadav, R. K., Karamanoli, K. & Vokou, D. (2005). Bacterial colonization of the phyllosphere of mediterranean perennial species as influenced by leaf structural and chemical features. *Microbial ecology* **50**, 185-196.

Yao, J. & Allen, C. (2006). Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum*. *Journal of Bacteriology* **188**, 3697-3708.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134.

Yu, X., Lund, S. P., Scott, R. A., Greenwald, J. W., Records, A. H., Nettleton, D., Lindow, S. E., Gross, D. C. & Beattie, G. A. (2013). Transcriptional responses of *Pseudomonas syringae* to growth in epiphytic versus apoplastic leaf sites. *Proceedings of the National Academy of Sciences* **110**, E425-E434.

Zeng, W., Melotto, M. & He, S. Y. (2010). Plant stomata: a checkpoint of host immunity and pathogen virulence. *Current opinion in biotechnology* **21**, 599-603.

Zhang, G., Ma, L., Beuchat, L. R., Erickson, M. C., Phelan, V. H. & Doyle, M. P. (2009). Lack of internalization of *Escherichia coli* O157:H7 in lettuce (*Lactuca sativa* L.) after leaf surface and soil inoculation. *Journal of Food Protection* **72**, 2028-2037.

Zhao, Y., Blumer, S. E. & Sundin, G. W. (2005). Identification of *Erwinia amylovora* Genes Induced during Infection of Immature Pear Tissue. *Journal of Bacteriology* **187**, 8088-8103.

Zheng, D., Constantinidou, C., Hobman, J. L. & Minchin, S. D. (2004). Identification of the CRP regulon using *in vitro* and *in vivo* transcriptional profiling. *Nucleic Acids Research* **32**, 5874-5893.

Zheng, J., Allard, S., Reynolds, S., Millner, P., Arce, G., Blodgett, R. J. & Brown, E. W. (2013). Colonization and internalization of *Salmonella enterica* in tomato plants. *Applied and Environmental Microbiology* **79**, 2494-2502.

Zhou, Z., Li, X., Liu, B., Beutin, L., Xu, J., Ren, Y., Feng, L., Lan, R., Reeves, P. R. & Wang, L. (2010). Derivation of *Escherichia coli* O157:H7 from Its O55:H7 Precursor. *PLoS ONE* **5**, e8700.

Zhu, Y., Qian, W. & Hua, J. (2010). Temperature Modulates Plant Defense Responses through NB-LRR Proteins. *PLoS Pathogens* **6**, e1000844.