The relationship between membrane damage, release of protein and loss of viability in Escherichia coli exposed to high hydrostatic pressure

Article

Accepted Version

Ms. Ref. No.: FOOD-D-09-00627 Rev 1

The relationship between membrane damage, release of protein and loss of viability in *Escherichia coli* exposed to high hydrostatic pressure

Bernadette Klotz[1], Pilar Mañas[2] and Bernard M. Mackey*

Department of Food and Nutrition Sciences, The University of Reading, PO Box 226, Whiteknights, Reading RG6 6AP, UK

*Corresponding author
Tel: +44(0)1183788727
b.m.mackey@reading.ac.uk

[1] Present address:
Universidad de La Sabana, Ingeniería de Producción Agroindustrial, Campus Universitario Puente del Común, Chía, Colombia

[2] Present address:
Departamento de Producción Animal y Ciencia de los Alimentos
Facultad de Veterinaria
Miguel Servet 177
50013, Zaragoza
Spain

Abstract

The aim of this work was to examine a possible association between resistance of two *Escherichia coli* strains to high hydrostatic pressure and the susceptibility of their cell membranes to pressure-induced damage. Cells were exposed to pressures between 100 and 700 MPa at room temperature (~20°C) in phosphate-buffered-saline. In the more pressure-sensitive strain *E. coli* 8164, loss of viability occurred at pressures between 100 MPa and 300 MPa and coincided with irreversible loss of membrane integrity as indicated by uptake of propidium iodide (PI) and leakage of protein of molecular mass between 9 and 78 kDa from the cells. Protein release increased to a maximum at 400 MPa then decreased, possibly due to intracellular aggregation at the higher pressures. In the pressure-resistant strain *E. coli* J1, PI was taken up during pressure treatment but not after decompression indicating that cells were able to reseal their membranes. Loss of viability in strain J1 coincided with the transient loss of membrane integrity between approximately 200 MPa and 600 MPa. In *E. coli* J1 leakage of protein occurred before loss of viability and the released protein was of low molecular mass, between 8 and 11 kDa and may have been of periplasmic origin. In these two strains differences in pressure resistance appeared to be related to differences in the ability of their membranes to withstand disruption by pressure. However it appears that transient loss of membrane integrity during pressure can lead to cell death irrespective of whether cells can reseal their membranes afterwards.

Key words: *E. coli*, hydrostatic pressure, membrane damage, protein leakage

Abbreviated title: Release of protein from pressure-treated *Escherichia coli*

1. Introduction

A range of non-thermal methods for preserving food have been investigated to satisfy growing consumer demands for minimally-processed high-quality foods that contain little or no chemical preservatives but are safe to eat (Mañas and Pagán 2005). High hydrostatic pressure (HHP) is generally regarded as one of the more promising of these emerging technologies and many new products have appeared on the market including fruit juices, smoothies, guacamole, seafood, snacks and prepared meals (Rastogi et al., 2007). HHP can inactivate vegetative microorganisms but is largely ineffective against spores, at least when applied at ambient temperatures (San Martín et al., 2002). In this sense it is essentially a pasteurization process and it is therefore essential that pressure treatments used in food preservation can inactivate the most resistant vegetative foodborne pathogens. To this end, considerable effort has been spent to determine the intrinsic pressure resistance of different microorganisms and to understand the physiological, environmental and processing factors that modify that resistance (Smelt, 1998; Hoover, et al., 1989; Rastogi et al., 2007). Resistance to high pressure varies between species of microorganism but does not always correlate with resistance to other preservation treatments such as heat (Metrick et al., 1989; Alpas, 2000). Strains within a given species can also differ widely in pressure resistance. This is particularly true of *E. coli,* some strains of which are inactivated by pressures as low as 200 MPa whereas others can survive exposure to 600 MPa in neutral media (Benito et al., 1999; Robey et al., 2001). This is of considerable practical importance because some strains of *E. coli* O157 are among the most pressure resistant vegetative cells examined to date (Patterson et al., 1995; Benito et al., 1999).

Many cellular components are affected by pressure including cell membranes and membrane proteins, enzymes, ribosomes and the nucleoid (Hoover et al., 1989;

74 Mackey and Mañas, 2008). Details of the mechanisms of inactivation have been
75 investigated in several bacterial species but the particular events leading to loss of
76 viability are not known for certain. In *E. coli* three processes seem to be especially
77 important. These are protein denaturation, oxidative stress and disruption of the
78 cytoplasmic membrane (Mackey and Mañas, 2008). Several lines of evidence point to
79 the importance of protein denaturation in microbial cell death. At the thermodynamic
80 level pressure-temperature diagrams of cellular inactivation rates of *E. coli* and other
81 microorganisms show a strong resemblance to the elliptic pressure-temperature phase
82 diagrams for protein denaturation (Sonoike, 1992). Supporting evidence comes from
83 biochemical studies showing that heat shock proteins are synthesized in cells during
84 exposure to sublethal pressures and in cells recovering from pressure treatment (Welch
85 et al., 1993; Aertsen et al., 2004). The heat shock proteins synthesized include
86 chaperones (DnaK, GrpE, GroES, and GroEL) and proteases that are involved in
87 degradation of denatured proteins (ClpB, ClpP and Lon). There is also strong
88 circumstantial evidence that protection against protein denaturation may enhance
89 cellular pressure resistance since exposure to mild heat shock increases resistance to
90 pressure whilst pressure-resistant mutants of *E. coli* selected by successive cycles of
91 pressure treatment and outgrowth had increased levels of heat-shock proteins (Aertsen
92 et al., 2004).
93 Oxidative stress appears to play an important role in cell death under some
94 circumstances. The lethality effect of pressure was increased by mutations in *oxyR* and
95 *soxS,* coding for oxidative stress regulatory elements, and in *katE* and *sodAB* coding for
96 HPII hydroperoxidase and superoxide dismutase respectively (Aertsen et al., 2005).
97 Conversely recovery of pressure-treated cells under anaerobic conditions enhanced
98 survival. It has been suggested that pressure treatment results in the release of iron

from Fe-S clusters leading to the generation of hydroxyl free radical via the Fenton reaction (Malone et al., 2006).

Finally, there is strong evidence that membrane damage can lead to cell death. Exponentially growing cells of *E. coli* are much more pressure sensitive than stationary phase ones and in these cells loss of viability coincides with irreversible disruption of cytoplasmic membrane integrity as measured by uptake of the non-permeant fluorescent dye propidium iodide (PI) and loss of osmotic responsiveness (Pagán and Mackey, 2000, Mañas and Mackey 2004). Pressure resistance is influenced by membrane fluidity and fatty acid composition such that cells with more fluid membranes are more pressure resistant (Casadei et al., 2002). In stationary-phase cells the picture is more complicated. Some weak strains undergo irreversible disruption of the cytoplasmic membrane similar to that in exponential phase cells but more robust strains are able to re-seal their membranes after decompression (Pagán and Mackey, 2000). The role of membrane damage in stationary phase cells of the more pressure resistant strains of *E. coli* is thus far from clear.

Further work is needed to unravel the contribution of the three types of mechanism outlined above to cell death which may depend on the properties of individual strains, their physiological state at the time of exposure to pressure and the conditions during pressure treatment and recovery. The aim of this work was to investigate the role of membrane damage in cell death of stationary-phase cells, specifically to examine the relationship between loss of membrane integrity and loss of viability in a two strains of *E. coli* with wide differences in pressure resistance. Two different indicators of membrane damage were used: uptake of PI and loss of protein from the cell. A preliminary characterization of released protein was also carried out using 1-D gel electrophoresis.

2. Materials and methods

2.1. Bacterial strain and growth conditions.

Escherichia coli NCTC 8164, *E. coli* J1 and *E. coli* NCTC 8003 were stored at -70°C in bead vials (Protect Technical Service Consultants Limited, Lancashire, United Kingdom). *Escherichia coli* NCTC 8164 was used in previous studies of the mechanisms of thermal inactivation (Mackey et al., 1991), the role of membrane fluidity in pressure resistance (Casadei et al., 2002) and kinetics of inactivation by pressure (Klotz et al., 2007). *Escherichia coli* J1 is a commensal strain with high pressure resistance used previously to study morphological changes caused by exposure to high pressures (Mañas and Mackey, 2004). *Escherichia coli* NCTC 8003 was previously used in studies of membrane damage in pressure-treated cells (Pagán and Mackey, 2000). To activate the strains one frozen bead was transferred to 9 ml Tryptone Soya Broth (TSB; Oxoid CM129, Basingstoke, United Kingdom) and incubated in shaken culture (140 rpm; Aquatron, Infors UK, Reigate, Surrey, United Kingdom) at 37^{o}C for approximately 6 h. The culture was then diluted 1:1000 into 100 ml fresh TSB and incubated in shaken flasks (250 mL) at 37^{o}C for approximately 18 h. The resulting stationary-phase culture contained approximately 3 x 10^{9} cells/mL

2.2. Pressure treatment.

Samples of stationary-phase cells were centrifuged at 2800 x g for 15 min at 5^{o}C (Biofuge 28 RS15; Heraeus Sepatech, Osterode, Germany), resuspended in an equal amount of phosphate-buffered-saline (PBS; Oxoid BR0014, Basingstoke, United Kingdom) and dispensed in volumes of 2 mL in plastic sachets, heat sealed, and placed

on ice before treatment. Samples were treated in a 300 ml pressure-vessel (Foodlab Plunger Press model S-FL-850-9W; Stansted Fluid Power, Stansted, Essex, United Kingdom). The pressure- transmitting fluid was ethanol: castor oil (80:20). The come-up rate was approximately 330 MPa / min and the deviation at targeted pressure was ± 10 MPa. After treatment, the pressure was released quickly in two steps. In the first step the pressure decreases to 30 MPa in about 15 seconds. The total decompression takes about 35 seconds. The transient increase in temperature of the pressurization fluid due to adiabatic heat during the treatment is measured with a thermocouple located near the vessel closures attached to the inside of the vessel lid. The average temperature rise was 4.3 (± 0.4) ^{o}C/100 MPa. Experiments were carried out at room temperature.

2.2. Viable counts

Sample bags were opened with sterile scissors and cell suspensions were diluted ten-fold in Maximum Recovery Diluent (MRD; Oxoid CM733, Basingstoke, United Kingdom). Appropriate dilutions were plated on TSA plus 0.1% sodium pyruvate as recovery medium and colonies were counted after incubation at 37^{o}C for 24 and 48 h. Two to four counts at relevant dilutions were performed for each sample. The mean was calculated and expressed as CFU/mL (colony-forming unit per mL sample). The lower limit of accurate measurements was 25 CFU/mL**.**

2.3. Preparation of the supernatant from suspensions of pressure-treated cells.

Cultures were centrifuged for at 5°C for 15 min at 2800 x g, resuspended in PBS, dispensed in sterile stomacher bags (Seward Limited, Worthing, West Sussex, United Kingdom), heat-sealed without head space, and placed on ice. Pouches were pressure-

177 treated in the range of 125 to 700 MPa. After decompression, pouches were removed
178 from the unit and wiped clean of any residual pressurising fluid. The bags were opened
179 with a sterile scissors and the content was centrifuged (2800 x g, 15 min, 5^{o}C). The
180 supernatant was collected, filtered (Minisart High Flow syringe filters, 0.2 µm; Sartorius
181 Mechatronics UK Limited, Epsom, Surrey, United Kingdom) and stored at -70^{o}C for
182 protein electrophoretic analysis.
183
184 *2.4. Osmotic shock treatment*
185
186 The osmotic shock treatment was performed according to Vázquez-Laslop et al.
187 (2001). Samples of stationary-phase *E. coli* NCTC 8164 cultures were centrifuged (2800
188 x g, 15 min, 5^{o}C) and resuspended to an OD_{680} of 10 in ice-cold TSE buffer (10 mM Tris-
189 HCl, pH 7.5, 20% sucrose, 2.5 mM Na-EDTA). After 10 min incubation on ice, cells were
190 centrifuged for 10 min at 5000 x g at 4^{o}C. The supernatant was decanted and the
191 pelleted cells were resuspended in an equal amount of ice-cold water. After 10 min
192 incubation on ice cell suspensions were centrifuged again and the supernatant with the
193 released proteins was collected, filtered (Sartorius Minisart High Flow syringe filters, 0.2
194 µm,), and saved for electrophoretic analysis at -70^{o}C.
195
196 *2.5. Determination of the protein content of supernatants*
197
198 The protein determination was performed using the Bradford Reagent according
199 to the Micro 2 mL assay protocol described in the technical bulletin (B 6916, Sigma-
200 Aldrich Company Ltd., Gillingham, Dorset, United Kingdom.). One ml of Bradford reagent
201 was added to 1 mL sample containing 1-10 µg protein and the samples were incubated
202 at room temperature for 5 min. The absorbance was measured at 595 nm in a

spectrophotometer (model CE 2020, Cecil Instruments Ltd., Cambridge, United Kingdom). Bovine serum albumin (BSA; Sigma-Aldrich P-0834) was used as the protein standard at concentrations between 1-10 µg/mL.

2.6. Electrophoretic analysis of proteins

Characterisation of the proteins present in the supernatant was performed with pre-cast gels (Novex, 1.0 mm x 10 well, Invitrogen Ltd., Paisley, United Kingdom.) according to the manufacturer's electrophoresis guide. Tricine gels (Novex) were used for low molecular weight peptides and proteins. The protein gels were stained using a silver staining kit (Cat. no.161-0449; Bio-Rad Laboratories, Hemel Hempstead, United Kingdom). The amount of extract from strain J1 loaded onto the gels was twice that from NCTC 8164 to allow protein bands to be visualized at similar densities. The silver stained gels were digitally photographed with the computer-based automated gel imaging system Gene Snap from Syngene V. 3.00.15 (Syngene, Cambridge, UK). The gray-scale files were quantified with Gene Tools from Syngene with the subtraction of the background. According to the gel resolution and characteristics the software was operated automatically or manually. Results were expressed in Microsoft Excel charts.

2.7. Assessment of cell membrane damage

The fluorescent dye propidium iodide (PI; Sigma-Aldrich, 287075) was used to evaluate cell membrane damage in stationary-phase cultures of *E. coli* NCTC 8164 and J1. A stock solution of 1 mg PI in 1 mL water (ISO grade 2) was prepared. Samples of cell suspensions in PBS with an OD_{680} of 0.2 (spectrophotometer model CE 2020, Cecil Instruments) were mixed with PI solution to a final concentration of 2.9 µM before or

after pressure treatment for 10 min at 100, 125, 150, 200, 300, 400, 500, 600, or 700 MPa. For evaluation of PI uptake after pressure treatment, cells were incubated with PI for 10 min, then centrifuged (10 000 x g) at 4^{o}C and washed twice in PBS. When PI was present during pressure treatment the cells were centrifuged and washed immediately after decompression. Fluorescence was measured at an excitation wavelength of 495 nm and an emission wavelength of 615 nm in a fluorimeter (Model LS-5B, PerkinElmer, Massachusetts, USA). The data were normalized by subtracting fluorescence values obtained from untreated cells and against OD_{680}. The normalized data were plotted as percentages of PI uptake during and after pressure treatment at different pressures.

3. Results

3.1. Pressure resistance of stationary-phase cells of E. coli NCTC 8164 and E. coli J1

The two strains showed large intrinsic differences in pressure resistance. The onset of extensive cell inactivation occurred at a pressure that was about 200 MPa higher in *E. col*i J1 than in *E. coli* NCTC 8164 (Fig 1). To reduce viable numbers of *E. coli* NCTC 8164 by 90% required a pressure treatment of only 300 MPa for 10 min, compared with 500 MPa needed to achieve the same effect in strain J1.

3.2. Loss of membrane integrity

The uptake of the PI by pressure-treated cells is shown in Fig 2. The dye was added to the cell suspensions either before pressure treatment or after decompression. Uptake of dye added before pressure treatment was taken to indicate loss of cytoplasmic

membrane permeability under pressure whilst uptake of dye added after decompression was taken to indicate permanent loss of membrane integrity. Uptake of PI began between 100 and 125 MPa in *E. coli* NCTC 8164 and between 200 and 300 MPa in *E. coli* J1. In *E. coli* NCTC 8164 there was little difference in the amount of PI taken up during or after pressure treatment indicating a permanent loss of membrane integrity. In *E. coli* J1 PI was also taken up during pressure treatment but very little after indicating that the permeability barrier to PI was restored after decompression.

3.3. Uptake of propidium iodide by single cells

Propidium iodide staining of single cells of *E. coli* J1 is shown in Fig 3. Cells were pressure treated at 400 MPa for 10 min and PI was added either before pressure treatment (Fig 3A) or after decompression (Fig 3B). Under these conditions some individual cells in the population take up PI during pressure treatment, but few cells do so after decompression.

3.4. Release of protein from pressure-treated cells

Loss of protein into the extracellular fluid began at 100-125 MPa in *E. coli* 8164 and 125-150 MPa in *E. coli* J1 but the total amount of protein released was greater in *E. coli* NCTC 8164 than in *E. coli* J1 (Fig 4). In *E. coli* NCTC 8164 the amount of protein released increased to a maximum at 300-400 MPa then decreased whereas in *E. coli* J1 the amount of protein released increased to a maximum at 200 MPa then remained constant.

3.5. Relationship between loss of membrane integrity, loss of protein and cell death in E. coli strains.

The relationship between loss of membrane integrity, loss of cellular protein and loss of viability in *E. coli* strains J1 and NCTC 8164 is shown in Figs 5A and 5B respectively. In *E. coli* J1 loss of viability coincided with uptake of PI during pressure treatment but not with uptake of PI after pressure treatment, which occurred at higher pressures; or with release of protein, which occurred at lower pressures. In *E. coli* NCTC 8164 loss of viability, uptake of PI and release of protein all occurred over more or less the same pressure range although uptake of PI after pressure took place at somewhat higher pressures than the other measured events. The only event that was correlated with loss of viability in both strains was therefore the uptake of PI during pressure treatment.

The relationship between uptake of PI during pressure treatment and loss of viability in *E. coli* J1, *E. coli* 8164 and an additional strain, *E. coli* NCTC 8003, is shown in Fig 6. The correlation between PI uptake under pressure and loss of viability was reasonable for the combined data (coefficient of determination = 0.94), consistent with there being an association between loss of membrane integrity during pressure treatment and cell death in all three of the tested strains of *E. coli*.

3.6. Characterisation of proteins released from E. coli strains during pressure treatment

The electrophoretic profiles of the proteins released from *E. coli* J1 and *E. coli* NCTC 8164 and are shown in Figs. 7A and 7B. Twelve protein bands were identified from *E. coli* NCTC 8164 and sixteen from *E. coli* J1 (Table 1). The approximate

molecular masses ranged from 6 to 64 kDa in *E. coli* J1 and from 9 to 78 kDa in *E. coli* 8164. Ten of the proteins released were of similar molecular mass in both strains.

Protein release started between about 100-150 MPa in both strains with three proteins from *E. coli* 8164 being released and one from *E. coli* J1. Visual inspection of the gel showed that the protein from *E. coli* J1 was a 9 kDa protein that comprised most of the released material from this strain. Further groups of proteins were released from each strain at successively higher pressures though the pattern was different in each strain (Table 1).

The amounts of each protein released at different pressures were estimated by measuring the optical density of the bands. This is only semi-quantitative but does give an indication of the relative amounts of particular proteins released at different pressures. With many proteins, the amount released increased with pressure, as for example those in strain 8164 with an apparent molecular mass of 15-16, 19-20, 20-21,21-22 and 22-23 kDa. In other cases the amounts released increased initially but then decreased at higher pressures. This was the case for the higher molecular mass proteins in strain 8164, for example the bands at 52-53, 56-65, and 66-78 kDa. In general more different proteins were released from *E. coli* NCTC 8164 than from *E. coli* J1 and much of the protein released from *E. coli* J1 was of low molecular mass (Fig 7A and 7B).

3.7. A comparison of proteins released by pressure and osmotic shock treatment

Figure 8 shows a comparison of the proteins released by pressure and osmotic shock from *E. coli* NCTC 8164. Proteins of molecular mass 64-65, 49, 41, 28, 24 and 9 kDa were present in the supernatant of both pressure-treated and osmotically-shocked

cells but additional bands at 37, 21 and 15 kDa were present only in the supernatant from pressure-treated cells.

4. Discussion

The large difference in pressure-resistance between stationary phase cells of *E. coli* NCTC 8164 and *E. coli* J1 appears to be due to a difference in the resilience of their cytoplasmic membranes towards high pressure stress. The membrane of *E. coli* NCTC 8164 became disrupted at a lower pressures than that of *E. coli* J1 and was unable to reseal after release of pressure whereas the cell membrane of *E. coli* J1 appeared to undergo less severe disruption and could reseal afterwards. Previous work by Pagán and Mackey (2000) showed that stationary phase cells of *E. coli* O157 strain C9490 and *E. coli* NCTC 8003 underwent transient membrane permeabilization during pressure treatment. Cells of strain C9490 retained their ability to plasmolyse and remained alive whilst in cells of strain NCTC 8003, 50% of the cells lost their osmotic responsiveness and 99% of the cells died. Mañas and Mackey (2004) showed that at high pressures above 400 MPa a proportion of stationary phase cells of *E. coli* J1 died without loss of osmotic responsiveness. Moussa et al. (2007) found that pressure treatment: at subzero temperatures induced mainly reversible permeabilization in *E. coli* while both reversible and irreversible permeabilization occurred at room temperature. A qualitative relationship was noted between membrane permeabilization and cell death. In the present work a direct relationship was observed between transient loss of membrane integrity and cell death in three different strains of *E. coli.*

From the above findings we can discern a spectrum in the resilience of stationary-phase *E. coli* membranes towards pressure. At one extreme we have strains such as *E. coli* NCTC 8164 which have fragile cell membranes that undergo permanent

disruption under pressure. These strains bear some resemblance to exponential phase cells which are also unable to reseal after decompression (Benito et al., 1999; Pagán and Mackey, 2000). In another group of strains cells undergo transient permeabilization but nevertheless die; whilst at the other extreme we have unusually resistant strains such as *E. coli* O157 C9490 which are able to recover from transient permeabilization (Pagán and Mackey, 2000). The basis of this spectrum of behaviour among the different strains is unknown. Although physical integrity of the cell membrane can apparently be regained under some circumstances, there may be other irreversible changes that can lead to cell death. Possibilities are: subtle changes in permeability control preventing restoration of homeostasis; disruption of electron transport components leading to oxidative stress; denaturation of critical membrane or cytoplasmic proteins; loss of critical intracellular components or an irreversible change in the intracellular environment that prevents recovery. With regard to the last point it is interesting that near-complete recovery of *E. coli* after electroporation is possible if cells are quickly transferred from electroporation buffer to recovery medium, but if cells remain in the electroporation medium viability is rapidly lost (Dower et al., 1988). The composition of the suspending medium may thus be critical in survival of transiently permeabilized cells. Though not investigated here, transient changes in the outer membrane of *E. coli* have also been reported (Hauben et al., 1996; Chilton et al., 2001; Ganzle and Vogel, 2001). Outer membrane damage is not believed to be lethal but does allow entry of antimicrobial substances such as lysozyme or nisin that can enhance lethality of pressure treatments (Garcia-Graells, 1999).

In Gram-positive bacteria the relationship between membrane damage and death of pressure-treated cells is unclear. Pressure-treated populations of *Listeria monocytogenes*, *Lactobacillus rhamnosus,* and *Staphylococcus aureus* in which more than 99% of cells were dead, still contained appreciable proportions of cells with intact

382 membranes as indicated by lack of staining with propidium iodide (Arroyo et al., 1999;
383 Ritz et al., 2001; Ananta and Knorr 2009). Ulmer et al. (2000) concluded from their
384 studies of the kinetics of pressure inactivation and PI uptake in *L. plantarum* that
385 irreversible membrane damage occurred after cell death. By contrast Smelt et al. (1994)
386 reported a very good correlation between PI uptake and cell death in *L. plantarum*. The
387 possible effects of growth phase and transient membrane permeabilization on survival
388 after pressure treatment appear not to have been investigated in Gram-positive bacteria
389 and it may be significant that Smelt et al. (1994) used exponential phase cells whereas
390 the other authors used cells in stationary phase. Studies using pulsed electric fields at
391 pH 7.0 found that Gram-positive bacteria were able to recover after transient
392 permeabilization whereas Gram-negative ones were not (Garcia et al., 2006). These
393 studies also suggest that an ability to reseal cell membranes is necessary but not
394 sufficient for cell survival.

395 Both *E. coli* strains released cellular proteins into the suspending medium as a
396 result of pressure treatment but the amount of protein released from *E. coli* J1 was
397 considerably less than from *E. coli* NCTC 8164 and the proteins were fewer and smaller
398 in size. This supports the conclusion that damage to the cytoplasmic membrane in *E.*
399 *coli* J1 was less extensive than in *E. coli* NCTC 8164. Loss of protein was coincident
400 with loss of cytoplasmic membrane integrity in *E. coli* NCTC 8164 but not in *E. coli* J1. A
401 large proportion of the total protein leaking from strain J1 consisted of a protein of 9 kDa
402 that appeared in the supernatant before any uptake of PI or loss of viability. This small
403 protein may therefore have come from the periplasm or outer membrane and its loss
404 does not appear to be lethal to the cell.

405 Release of protein from *E. coli* under pressure was previously reported by Mañas
406 and Mackey (2004) but the proteins were not characterized and there appears to be no
407 information on this in the literature. The preliminary analysis of released proteins by 1-D

gel electrophoresis revealed sixteen protein bands from *E. coli* NCTC 8164 and thirteen from *E. coli* J1. Several proteins released from *E. coli* NCTC 8164 had the same molecular mass as those released by osmotic shock and may therefore have come from the periplasm. These include the protein of approximately 9 kDa; however, given the disruption of the cytoplasmic membrane in *E. coli* NCTC 8164 it is likely that some of the released proteins were of cytoplasmic origin. The real number of different proteins released under pressure is likely to be greater than that detectable on 1-D gels and further studies using 2-D gel electrophoresis are desirable to identify the proteins and their origin.

All of the proteins released from pressure-treated cells had a molecular mass below 80 kDa. This cut-off point is consistent with the suggestion of Vázquez-Laslop et al. (2001) that the peptidoglycan of the cell wall acts as a molecular sieve for proteins leaking from bacterial cells. The results obtained by Vázquez-Laslop et al., (2001) in a study of osmotically-shocked cells indicated that the peptidoglycan mesh was comparable in porosity to a 100 kDa cut-off cellulose membrane. The amount of protein released from *E. coli* NCTC 8164 increased with pressure intensity up to 300-400 MPa then decreased. We believe this may be due to the formation of intracellular aggregates at the higher pressures that are unable to pass through the peptidoglycan. In *E. coli* J1 the amount of protein released increased with pressure then remained more or less constant. This would be consistent with the released proteins originating from a region outside the peptidoglycan. In this case the release of any aggregated protein would not be impeded by the sieving effect of the peptidoglycan so no reduction in released protein would be expected at high pressures.

Although the membrane disruption by high pressure is acknowledged as a critical event in microbial inactivation by pressure the role of membrane damage in death of stationary phase cells has been unclear. This work shows that stationary phase

membranes of different *E. coli* strains differ quite widely in their ability to resist disruption by pressure treatment and in their ability to recover integrity after decompression. This has a major influence on the ability of cells to survive high hydrostatic pressure. It is now clear that the pressure at which membrane disruption begins is more important for cell survival than the ability to reseal membranes after decompression. Even temporary loss of membrane integrity can lead to cell death. Since the degree of membrane permeabilization, protein loss and resealing varies between different strains of *E. coli*, differences in the efficacy of combined processes which rely in the entrance of an antimicrobial substances during pressurization might be expected. It would be interesting for example to examine whether such combined treatments could overcome the pressure resistance of strains that have more resilient cell membranes.

Acknowledgements

We are grateful to the European Commission for support to Bernadette Klotz under the ALFA Network II and for providing Pilar Mañas with a Marie Curie postdoctoral grant in the Quality of Life program. We also thank the Universidad de La Sabana, Bogota, Colombia.

References

Aertsen, A., Vanoirbeek, K., De Spiegeleer, P., Sermon, J., Hauben, K., Farewell, A., Nystrom, T., Michiels, C.W., 2004. Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli.* Applied and Environmental Microbiology 70, 2660-2666.

Aertsen, A., De Spiegeleer, P., Vanoirbeek, K., Lavilla, M., Michiels, C.W., 2005. Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli.* Applied and Environmental Microbiology 71, 2226-2231.

Alpas, H., Kalchayanand, N., Bozoglu, F., Ray, B., 2000. Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. International Journal of Food Microbiology 60, 33-42.

Ananta, E., Knorr,D., 2009. Comparison of inactivation pathways of thermal or high pressure inactivated *Lactobacillus rhamnosus* ATCC 53103 by flow cytometry analysis. Food Microbiology 26, 542-546.

Arroyo, G., Sanz, P.D., Préstamo, G., 1999. Response to high-pressure low-temperature treatment in vegetables: determination of survival rates of microbial populations using flow cytometry and detection of peroxidise activity using confocal microscopy. Journal of Applied Microbiology 86, 544-556.

Benito, A., Ventoura, G., Casadei, M., Robinson, T. Mackey, B.M., 1999. Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat and other stresses. Applied and Environmental Microbiology 65, 564-1569.

Casadei, M.A., Mañas, P., Niven, G., Needs, E., Mackey, B.M., 2002. The role of membrane fluidity in pressure resistance of *Escherichia coli* 8164. Applied and Environmental Microbiology 68, 5965-5972.

Chilton, P., Isaacs, N.S., Mañas, P., Mackey, B.M., 2001. Metabolic requirements for the repair of pressure-induced damage to the outer and cytoplasmic membranes of *Escherichia coli*. International Journal of Food Microbiology 71, 101-104.

Dower, W.J., Miller, J. F., Ragsdale,C.W., 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Research 16, 6127-6145.

Ganzle, M.G., Vogel, R.F., 2001. On-line fluorescence determination of pressure mediated outer membrane damage in *Escherichia coli.* Systematic and Applied Microbiology 24, 477-85.

Garcia-Graells, C., Masschalck, B., Michiels, C.W., 1999. Inactivation of *Escherichia coli* in milk by high-hydrostatic pressure treatment in combination with antimicrobial peptides. Journal of Food Protection 62,1248-1254.

García, D., Gómez, N., Mañas, P., Raso, J., Pagán, R., 2006. Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. International Journal of Food Microbiology 113, 219–227.

Hauben, K.J.A., Wuytack, E.Y., Soontjens, C.C.F., Michiels, C.W., 1996. High pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. Journal of Food Protection 59, 350-355.

Hoover, D.G., Metrick, C., Papineau, A.M. Farkas, D.F., Knorr, D., 1989. Biological effects of high hydrostatic pressure on food microorganisms Food Technology. 43, 99-107.

Klotz, B., Pyle, D.L., Mackey, B.M., 2007. A new mathematical modeling approach for predicting microbial inactivation by high hydrostatic pressure. Applied and Environmental Microbiology 73, 2468-78.

501 Mackey, B.M., Miles, C.A., Parsons, S.E., Seymour, D.A., 1991. Thermal denaturation
502 of whole cells and cell components of *Escherichia coli* examined by differential
503 scanning calorimetry. Journal of General Microbiology 137, 2361-2374.
504 Mackey, B.M., Mañas, P., 2008. Inactivation of *Escherichia coli* by high pressure. In:
505 Michiels,C., Aertsen, A., Bartlett, D., and Yayanos, Y. (Eds), High Pressure
506 Microbiology. ASM Press, Washington DC, pp 53-85.
507 Malone, A.S., Chung Y-K., Yousef, A.E., 2006. Genes of *Escherichia coli* O157:H7 that
508 are involved in high-pressure resistance. Applied and Environmental
509 Microbiology 72, 2661-2671.
510 Mañas, P., Pagán, R., 2005. Microbial inactivation by new technologies of food
511 preservation. Journal of Applied Microbiology 98,1387-1399.
512 Mañas, P., Mackey, B.M., 2004. Morphological and physiological changes induced by
513 high hydrostatic pressure in exponential- and stationary-phase cells of
514 *Escherichia coli*: relationship with cell death. Applied and Environmental
515 Microbiology 70, 1545-1554
516 Metrick, C., Hoover, D.G., Farkas, D.F.,1989. Effects of hydrostatic pressure on heat-
517 resistance and heat-sensitive strains of *Salmonella*. Journal of Food Science 54,
518 1547-1549.
519 Moussa, M., Perrier-Cornet, J-M. and Gervais, P., 2007. Damage in *Escherichia coli*
520 cells treated with a combination of high hydrostatic pressure and subzero
521 temperature. Applied and Environmental Microbiology. 73, 6508–6518.
522 Págan, R., Mackey, B.M., 2000. The relationship between membrane damage and cell
523 death in pressure-treated *Escherichia coli* cells: differences between exponential
524 and stationary phase cells and variation among strains. Applied and
525 Environmental Microbiology 66, 2829-2834.

526 Patterson, M.F., Quinn, M., Simpson, R., Gilmour, A., 1995. Sensitivity of vegetative
527 pathogens to high hydrostatic pressure treatment in phosphate-buffered saline
528 and foods. Journal of Food Protection 58, 524-529.
529 Rastogi, N.K., Raghavarao, K.S.M.S., Balasubramaniam, V.M., Niranjan, K., Knorr, D.,
530 2007.Opportunities and challenges in high pressure processing of foods. Critical
531 Reviews in Food Science and Nutrition 47, 69-112.
532 Ritz, M., Tholozan, J.L., Federighi, M., Pilet, M.F., 2001. Morphological and physiological
533 characterization of *Listeria monocytogenes* subjected to high hydrostatic
534 pressure. Applied and Environmental Microbiology 67, 2240-2247.
535 Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S. F., Mackey, B.M., 2001.
536 Variation in resistance to high hydrostatic pressure and *rpoS* heterogeneity in
537 natural isolates of *Escherichia coli* 0157:H7. Applied and Environmental
538 Microbiology 67, 4901-4907.
539 San Martín, M.F., Barbosa-Cánovas, G.V., Swanson, B.G., 2002. Food processing by
540 high hydrostatic pressure. Critical Reviews in Food Science and Nutrition 42,
541 627-645.
542 Smelt, J.P.P.M., Rijke, A.G.F., Hayhurst, A.,1994. Possible mechanism of high pressure
543 inactivation of microorganisms. High Pressure Research 12, 199-203.
544 Smelt, J.P.P.M., 1998. Recent advances in the microbiology of high pressure
545 processing. Trends in Food Science and Technology 9, 152-158.
546 Sonoike, K., Setoyama, T., Kuma, Y., Kobayashi, S., 1992. Effect of pressure and
547 temperature on the death rates of *Lactobacillus casei* and *Escherichia coli*. In:
548 Balny,C., Hayashi,R., Heremans,K., Masson, P. (Eds.), High Pressure and
549 Biotechnology. Colloque INSERM/John Libbey Eurotext Ltd., 224, pp. 297-301.

Ulmer, H.M., Gänzle, M.G., Vogel, R.F., 2000. Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1.460. Applied and Environmental Microbiology 66, 3966-3973.

Vázquez-Laslop, N., Lee, H., Hu, R., Neyfakh, A.A., 2001. Molecular sieve mechanism of selective release of cytoplasmic proteins by osmotically shocked *Escherichia coli*. Journal of Bacteriology 183, 2399–2404

Welch, T.J., Farewell, A.F., Neidhardt, F.C., Bartlett, D.H., 1993. Stress response of *Escherichia coli* to elevated hydrostatic pressure. Journal of Bacteriology 175, 7170-7177.

Legends to Figures.

Fig 1. Loss of viability of *E. coli* J1 (■) and *E. coli* NCTC 8164 (●) after treatment for 10 min at different pressures. Plotted values are means from three replicate trials ± standard deviation.

Fig 2. Uptake of propidium iodide during (closed symbols) and after (open symbols) treatment of *E. coli* J1 (■,□) and *E. coli* NCTC 8164 (●,O) for 10 min at different pressures. Plotted values are means from three replicate trials ± standard deviation.

Fig 3. Microscopy of *E. coli* J1 cells stained with propidium iodide present during (A) and after (B) pressure treatment at 400 MPa for 10 min. Bar marker 1 μm.

Fig 4. Release of protein from cells of *E. coli* J1 (■) and *E. coli* NCTC 8164 (●) after treatment for 10 min at different pressures. Plotted values are means from three replicate trials ± standard deviation.

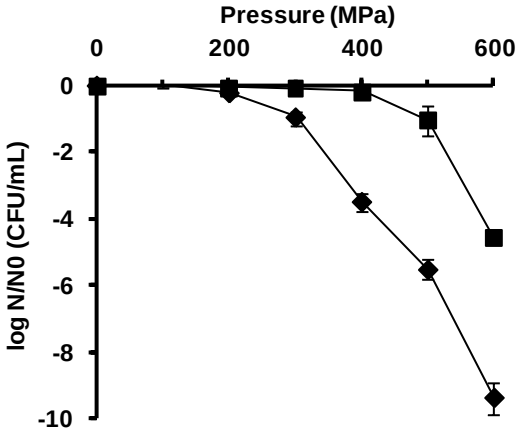
Fig 5. Relationship between loss of viability (O), uptake of propidium iodide during pressure treatment (■), uptake of propidium iodide after pressure treatment (□) and release of protein (▲) in *E. coli* NCTC 8164 (A) and *E. coli* J1 (B).

Fig 6. Relationship between propidium iodide uptake during pressure treatment and loss of viability in *E. coli* strains J1, NCTC 8164 and NCTC 8003.

Fig 7. Gel electrophoresis of proteins released following 10 min treatment at different pressures from *E. coli* J1 (A) and *E. coli* NCTC 8164 (B). Indicated pressures are in MPa. Molecular mass markers are shown in the right hand lane.

Fig 8. Comparison of proteins released from *E. coli* NCTC 8164 after pressure treatment at 400 MPa for 10 min (solid bars) or osmotic shock induced by transfer from TSE buffer containing 20% sucrose to distilled water (open bars).

585 **Fig 1.**

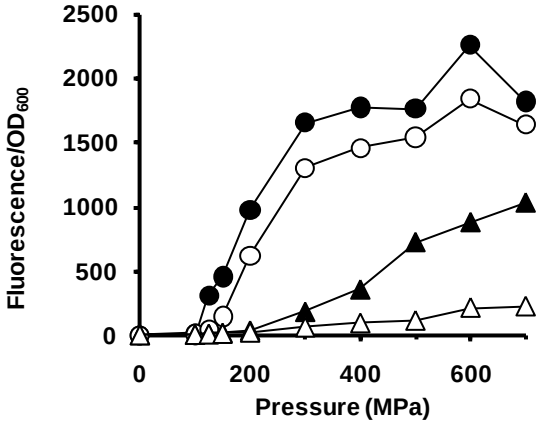


586

587

588

589 **Fig. 2**

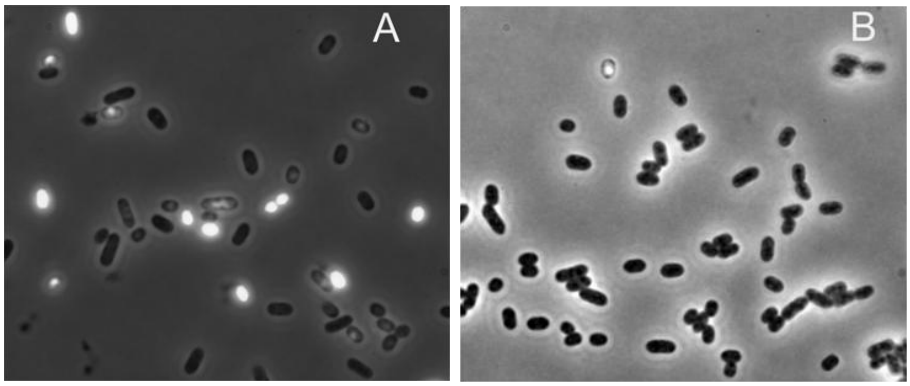


590

591

592

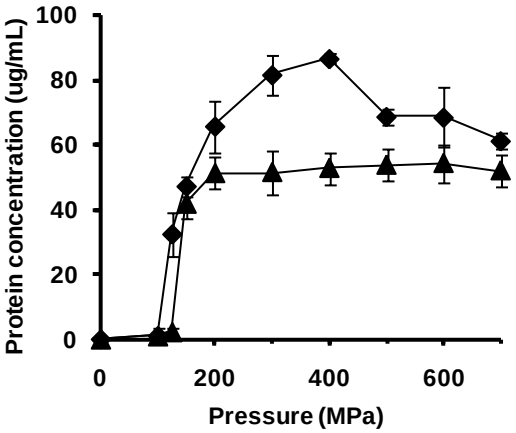
593 **Fig 3.**



594

595

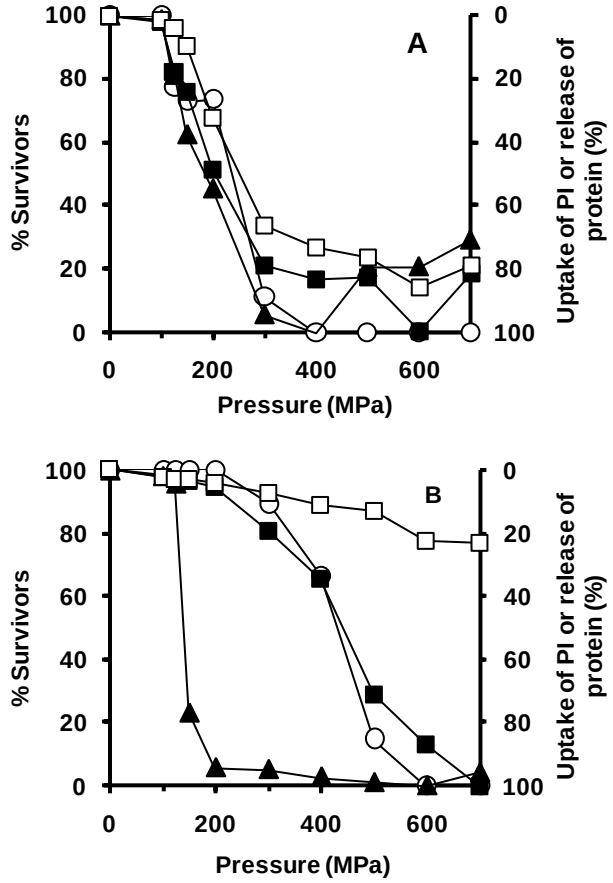
596 **Fig 4.**



597

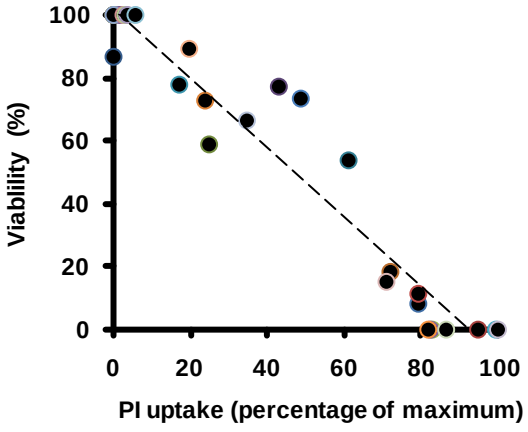
598

599 **Fig 5.**



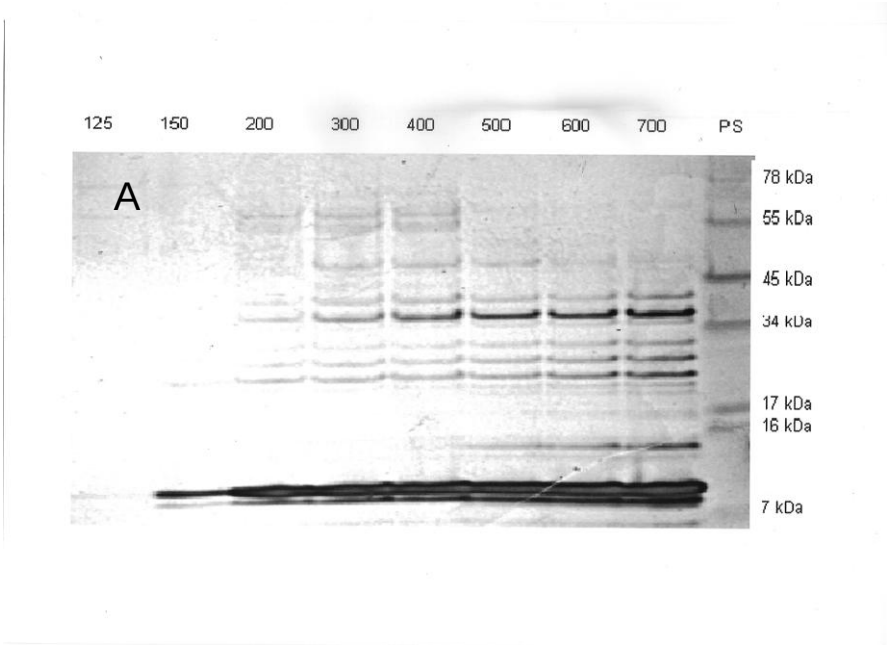
600 **Fig 6.**

601

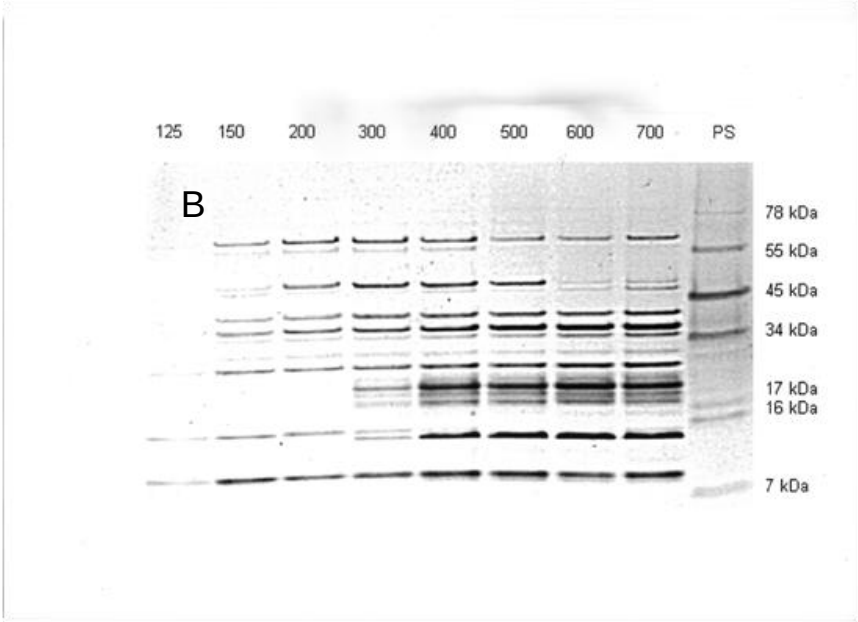


602

603 **Fig 7A**



604 **Fig 7B.**



605

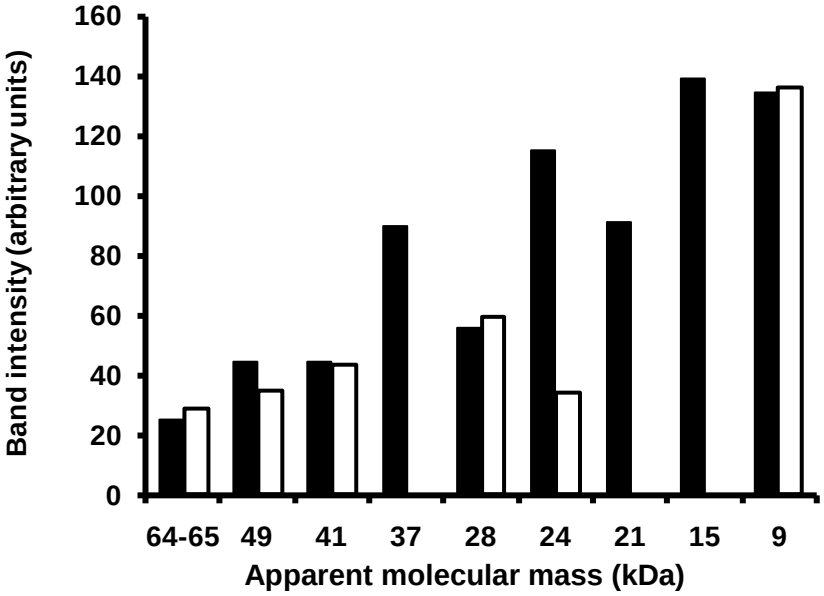
606

607

608

609

610 **Fig 8.**



611