Induction of Oxidative Stress by High Hydrostatic Pressure in Escherichia coli

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Using leaderless alkaline phosphatase as a probe, it was demonstrated that pressure treatment induces endogenous intracellular oxidative stress in Escherichia coli MG1655. In stationary-phase cells, this oxidative stress increased with the applied pressure at least up to 400 MPa, which is well beyond the pressure at which the cells started to become inactivated (200 MPa). In exponential-phase cells, in contrast, oxidative stress increased with pressure treatment up to 150 MPa and then decreased again, together with the cell counts. Anaerobic incubation after pressure treatment significantly supported the recovery of MG1655, while mutants with increased intrinsic sensitivity toward oxidative stress (katE, katF, oxyR, sodAB, and soxS) were found to be more pressure sensitive than wild-type MG1655. Furthermore, mild pressure treatment strongly sensitized E. coli toward t-butylhydroperoxide and the superoxide generator plumbagin. Finally, previously described pressure-resistant mutants of E. coli MG1655 displayed enhanced resistance toward plumbagin. In one of these mutants, the induction of endogenous oxidative stress upon high hydrostatic pressure treatment was also investigated and found to be much lower than in MG1655. These results suggest that, at least under some conditions, the inactivation of E. coli by high hydrostatic pressure treatment is the consequence of a suicide mechanism involving the induction of an endogenous oxidative burst.

High hydrostatic pressure (HHP) treatment is an emerging food preservation process that is commercially used for the nonthermal pasteurization of an increasing number of food products (21, 28). The technique is based on the use of ultrahigh pressures in the range of 200 to 800 MPa to kill foodborne pathogens and spoilage organisms and to inactivate deteriorative enzymes. Pressurized foods generally retain a superior quality than thermally pasteurized foods because, for a comparable effect, HHP treatment can be performed at considerably lower temperatures, thus reducing thermal damage. Research on the effect of pressure on the microbial safety and stability of foods has vastly increased over the last years, and a finding that emerged from several independent studies is that the microbialicidal efficacy of HHP treatment can be strongly increased by the application of specific additional hurdles such as mild heat and various (bio)preservatives (5, 20, 32). This has stimulated researchers to pursue a fundamental insight in the perception of HHP stress by microorganisms to be able to explain why certain combinations of hurdles work better than others.

Although the precise mechanisms of bacterial killing by HHP remain unknown, progress has been made in identifying the major cellular HHP targets, based on in vitro thermodynamic studies of biomolecules under pressure. These studies have indicated that hydrophobic and electrostatic interactions in and between proteins are especially pressure sensitive, making the tertiary and quaternary protein structure highly prone to pressure denaturation, although there are large differences among proteins (22, 25). Besides proteins, HHP also affects biological membranes by increasing the packing density of lipid molecules and inducing phase separations due to differences in compressibility between lipid species and between lipids and membrane proteins (10, 31). Both protein denaturation and membrane disruption have been amply demonstrated in bacterial cells treated with HHP and shown to result in inactivation of key enzymes and processes (18, 39, 43) and functional and structural disruption of cellular structures and membranes (19, 35, 37, 46). Currently, the accumulation of protein and membrane damage is believed to be at the basis of bacterial inactivation by HHP, and both chaperones and membrane fluidity have recently been linked with increased pressure resistance (2, 13). However, whether these types of damage are the direct and only cause of cell death remains uncertain.

An alternative model that has been proposed, based on thermal inactivation studies with Salmonella, states that sublethal damage may result in a state of metabolic imbalance that in its turn causes a lethal burst of reactive oxygen species (ROS). This is called the suicide hypothesis because not the environmental stress itself but specific enzymatic cellular processes are the ultimate cause of cell death (3, 4). Because HHP treatment, like heat treatment but unlike other nonthermal treatments, including high electric field pulses, UV light, high-intensity white light pulses, and high-pressure homogenization, causes high levels of sublethal injury in bacterial cells (48) and because it has been proposed that the fate of sublethally injured cells depends on scavengers of reactive oxygen species in resuscitation media, indicating an oxidative burden to be associated with these cells (9), we speculated that bacterial inactivation by HHP might involve a similar suicide mechanism. In the present work, we present several lines of evidence supporting this hypothesis.

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Materials and Methods

Strains and growth conditions. All experiments were conducted in an Escherichia coli strain MG1655 background (8, 23). Pressure-resistant mutants LMM1010, LMM1020, and LMM1030 of this strain originate from earlier work in this laboratory (24). E. coli katE (katE::Tn10) (30), katF (katF::Tn10) (30), oxyR (ΔoxyR::Km) (45), sodAB (sodA::Cm sodB::Km) (12), and sodS (sodS::Tn10) (47)-deficient backgrounds were kindly donated by Danièle Touati (Institut Jacques Monod, Paris, France) and were crossed into MG1655 by P1 transduction. Stationary-phase cultures were obtained by growth in Luria-Bertani (LB) broth (42) for 21 h at 37°C under well-aerated conditions by placing them on an orbital shaker (200 rpm). For late-exponential-phase cultures, stationary cultures were diluted 1/100 in fresh LB and incubated further until the optical density at 600 nm reached 0.6. Ampicillin (100 μg/ml) was added to stationary cultures when the cell density reached 0.6. Cultures were grown to stationary or exponential phase in the presence of IPTG and subsequently pressurized. Directly after pressurization, cells were collected by centrifugation, resuspended in 100 mM iodoacetamide, which alkylates free sulfhydryl groups and prevents any further disulfide bridge formation (38), and kept on ice for 20 min. Afterwards, the cells were resuspended in Tris-HCl buffer (1.0 M, pH 8.0) and the optical density at 600 nm was measured using an optical density microplate reader (Multiskan RC; Thermo Electron Corporation, Vantaa, Finland).

RESULTS

Evidence for pressure-induced oxidative stress in the cytoplasm. Wild-type AP is initially synthesized with a leader peptide that targets the polypeptide to the periplasm, where active enzyme dimers are formed by disulfide bond formation. The Δ2-22 AP lacks the leader peptide and therefore remains in the reducing environment of the cytoplasm, where disulfide bond formation and consequent activation of the enzyme only occur when the cell encounters oxidative stress, allowing it to be used as a probe to measure intracellular oxidative stress (15, 38). Stationary-phase cultures of E. coli MG1655 expressing the leaderless AP clearly showed a pressure-dependent activation of the enzyme, indicative of an HHP-induced oxidative stress in the cytoplasm (Fig. 1). The AP activation increased with the pressure applied, even beyond 200 MPa, which is the point where the number of culturable survivors started to decrease. Treatment at 400 MPa for 15 min resulted in a 12-fold increase in AP activity compared to nonpressurized cells.

The experiment was repeated with exponential-phase cells of MG1655, since in this growth phase, cells are much more pressure sensitive than in stationary phase. Again, AP activation initially increased with pressure, up to 11-fold at 150 MPa. However, AP activation then decreased again at higher pressures (Fig. 2). As can be seen in the same figure, 150 MPa coincides with the onset of inactivation for the exponential phase cells.

HHP-induced oxidative stress contributes to pressure inactivation of E. coli. Since HHP treatment was shown to induce cytoplasmic oxidative stress, we further investigated whether this stress actually contributed to the inactivation of E. coli by HHP. If this is the case, reducing the exposure of the pressurized cells to oxygen should improve their recovery. Therefore, we compared the number of colonies formed when HHP-treated cells were either plated on TSA and incubated aerobically or plated on TSA containing cysteine as an oxygen scavenger and incubated anaerobically. Figure 3 shows that anaerobic incubation significantly improved cellular survival by approximately 100-fold at 400 MPa (15 min, 20°C), indicating that the presence of oxygen is in part responsible for HHP-mediated inactivation of E. coli.

Furthermore, the extremely pressure-resistant mutant of MG1655, LMM1010 (24), showed significantly less oxidative stress (Fig. 1) (5-fold increase in AP activity at 400 MPa compared to a 12-fold increase for MG1655), providing further evidence for the role of pressure-induced oxidative stress in E. coli inactivation.
indication that reduced levels of HHP-induced oxidative stress may correlate with cellular pressure resistance.

Conversely, mutants of MG1655, impaired in peroxide (katE, katF, oxyR) and superoxide (sodAB, soxS) stress management, were found to be more prone to HHP inactivation (Fig. 4). Without exception, all of these mutants were hypersensitive toward HHP, showing a 10-fold-higher inactivation than the wild type for oxyR to a more-than-10³-fold-higher inactivation for katF upon treatment at 350 MPa (15 min, 20°C). We also attempted to rescue these mutants by anaerobic incubation in a similar way as described above for wild-type strain MG1655. For most of the mutants, a higher number of colonies was obtained under anaerobic incubation conditions, but the increase was generally lower than for MG1655 and not always statistically significant (data not shown). Possibly, our procedure of anaerobic incubation is insufficient to achieve higher rescue levels in these mutants because they are much more sensitive to low levels of oxidative stress.

**Synergy between HHP and oxidants with respect to cellular inactivation.** While it is clear from Fig. 3 that an anaerobic/reducing environment supports the recovery of MG1655, we proceeded to investigate whether HHP treatment could increase the sensitivity of E. coli for subsequent oxidative stress. Untreated and pressurized (250 MPa, 15 min, 20°C) stationary-phase cells of MG1655 were treated with sublethal doses of t-BHP (0.11 M) or the superoxide generator plumbagin (1 mM). The pressure treatment alone caused a viability reduction of ca. 1 log₁₀ unit. Treatment of nonpressurized cells with the oxidants caused only very little inactivation (<2-fold). However, the cells that survived pressure treatment were strongly sensitized toward t-butylhydroperoxide and plumbagin, showing, respectively, 3 × 10⁻³-fold- and >3 × 10⁻⁴-fold-higher inactivation (Fig. 5).
tional cause of cell death by HHP, at least under some conditions.

A first indication that HHP treatment induces oxidative stress was obtained from experiments with leaderless alkaline phosphatase, which has been used previously as a sensitive and direct cellular reporter of oxidative stress in aging cells of *E. coli* (15). Using this probe, we were able to demonstrate cytoplasmatic oxidative stress as a result of HHP treatment in stationary- and exponential-phase cells of *E. coli* MG1655 (Fig. 1 and 2). Recently, a mechanism has been proposed by Aldsworth et al. (4) stating that, upon disturbing cellular homeostasis, inimical processes can result in uncoupling growth and metabolic rate. As a consequence of this imbalance of anabolism and catabolism, a burst of ROS production occurs that is held responsible for cell death. Interestingly, in exponential-phase cells challenged with higher pressures, a fall in oxidative burst was observed, indicating that the ROS-generating metabolic routes themselves probably become affected by these pressures. In stationary-phase cells, however, for reasons that are not yet clear, ROS production continues to increase with pressure treatments up to at least 400 MPa.

Aldsworth et al. (4) also suggested that this intrinsic suicide mechanism could in part explain the higher resistance to inimical processes of stationary-phase cells compared to exponential-phase cells by assuming that this oxidative burst is more prominent, and thus destructive, in actively respiring cells (3). Our results are in good agreement with this hypothesis because we observe that at up to 150 MPa the relative increase in oxidation for exponential-phase cells (Fig. 2) is greater than that of stationary-phase cells (Fig. 1). Alternatively, stationary-phase cell suspensions, due to their slightly higher (<3-fold) cell density than late-exponential-phase cell suspensions, might scavenge the available oxygen in the pressurization buffer more rapidly, leading to reduced oxygen toxicity during pressurization and increased survival. However, this possibility could be excluded, since exponential phase cells tagged with a chloramphenicol resistance cassette in lacZ exhibited the same level of inactivation when they were pressurized in the presence and in the absence of a threefold excess of untagged stationary-phase cells and plated on TSA supplemented with chloramphenicol (data not shown).

While the previous experiments clearly demonstrated the generation of endogenous oxidative stress upon HHP treatment, several subsequent observations provided convincing evidence for its contribution to bacterial inactivation by HHP treatment. First, when pressurized cells were incubated anaerobically, their survival increased significantly (about 100-fold at 400 MPa, 15 min, 20°C) (Fig. 3) compared to when they were aerobically incubated. Since a reduction of oxygen availability will reduce the endogenous formation of reactive oxygen species in cells after HHP treatment, this result suggests that a significant subpopulation of cells dies after HHP treatment due to endogenous oxidative stress. A similar situation has been reported for several other bacteria after exposure to heat or freezing stress (4, 44), and the existence of a subpopulation of HHP-treated bacteria that is still viable but unable to form colonies under standard plating conditions has recently also been reported for *Lactobacillus rhamnosus* (6). This finding also has several ramifications for the application of HHP in the food industry, where aerobic plate counts may lead to an over-

![Diagram](https://example.com/diagram.png)

**FIG. 6.** Survival of stationary-phase cultures of MG1655 (■) and its pressure-resistant mutants LMM1010 (▲), LMM1020 (●), and LMM1030 (○) upon exposure to 0.8 mM plumbagin (A) or 0.22 M tert-butylhydroperoxide (B), expressed as \( \frac{N}{N_0} \times 100\% \), with \( N_0 \) and \( N \), respectively, being the plate count before and after treatment. Open symbols represent counts below the detection limit. Representative results from two independent experiments are shown.

**Increased tolerance of pressure-resistant mutants toward superoxide.** The data presented so far suggest an oxidative pathway to be involved in pressure-mediated killing at least for relatively mild pressures (<400 MPa). Conversely, we investigated whether the previously isolated pressure-resistant mutants of MG1655 (24) are more resistant toward exogenous oxidative stress. All three pressure-resistant mutants indeed displayed increased tolerance towards superoxide generated by plumbagin (Fig. 6A) but not to tert-butylhydroperoxide (Fig. 6B).

**DISCUSSION**

In the last 10 years, elaborate evidence has accumulated describing membrane leakage and protein denaturation as two key processes in microbial inactivation by HHP (7). In this study with *E. coli*, however, we report the generation of endogenous oxidative stress and the sensitization to oxidative stress subsequent to HHP treatment. In addition, we present indications that HHP resistance is related to oxidative-stress resistance in *E. coli*. These findings suggest that, aside from proteome or membrane damage, the subsequent oxidative burst that is a consequence of this damage might be an addi-

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estimation of bacterial inactivation by HHP and thus to unstable or even unsafe foods. Specific plating media and procedures should therefore be used that support recovery of highly oxygen-sensitive injured survivors. Furthermore, the effect of packaging techniques, such as vacuum packaging, and of natural oxygen scavengers in foods on bacterial inactivation by HHP treatment deserves further investigation.

Second, five different mutants affected in oxidative-stress management (katE, katF, oxyR, sodA4B, and ssoX) were significantly more sensitive to HHP inactivation (Fig. 4). Of these, katF (pcoS) was found to be the most sensitive toward HHP treatment, a finding that correlated well with earlier observations of Robey et al. (40), who discovered a firm correlation between an attenuated RpoS function and HHP sensitivity in natural isolates of *E. coli* O157:H7. Although the involvement of RpoS in oxidative-stress defense (17) might well form the basis for this phenomenon, it should be noted that RpoS also plays an important role in the defense against osmotic and acid stress and therefore confers resistance toward a number of other inimical processes used in the food industry (14).

A third observation is that wild-type cells were strongly sensitized to sublethal doses of t-butylhydroperoxide and plumbagin by HHP treatment (Fig. 5), indicating that HHP treatment not only induces an oxidative burst in *E. coli* but at the same time makes the cells hypersensitive to subsequent oxidative stress. The exact cause of this is not known, but it may be that the pressure-induced boost of ROS exceeds the capacity of the cellular machinery dealing with oxidative stress or that this machinery is very pressure sensitive and thus inactivated by HHP treatment. This sensitization toward oxidative stress may explain the synergistic effect of HHP and the oxidative stress-generating lactoperoxidase enzyme system on bacterial inactivation (20). In the context of hurdle-based minimal processing of foods, discovering and understanding such synergies between hurdles becomes increasingly important (29, 34, 41).

Finally, it was shown that the pressure-resistant strain LMM1010 (24) suffers less oxidative stress upon HHP treatment than its pressure-sensitive parent MG1655 (Fig. 1). Indeed, after treatment at 400 MPa, the AP activity increased only 5-fold in LMM1010 compared to 11-fold in MG1655. This finding also fits well into the suicide hypothesis for HHP inactivation of *E. coli* by suggesting that strain LMM1010 owes its pressure resistance to a reduced ROS production and/or an enhanced ROS scavenging mechanism. Moreover, three pressure-resistant mutants of MG1655, although they were isolated independently and solely on the basis of pressure resistance (24), proved to be more resistant to lethal concentrations of plumbagin than their parental strain (Fig. 6A). Again, this can be explained either by an increased protection against or a decreased production of superoxide in the presence of plumbagin in these mutants, but in either case, this suggests a link with pressure resistance in light of the suicide hypothesis. It should be noted that the same mutants did not exhibit increased t-butylhydroperoxide resistance (Fig. 6B), although another study recently revealed increased hydrogen peroxide resistance in a pressure-resistant mutant of *Listeria monocytogenes* (26).

In recent years, evidence that oxidation plays a terminal role in all moribund cells has increased (1, 36). Dukan and Nyström (15) demonstrated that bacterial stasis due to senescence results in increased oxidation of cytoplasmic proteins. In addition, these authors proved that developmental induction of heat shock proteins resulted from oxidation of target proteins. Furthermore, it is speculated that certain chaperones such as DnaK are acting as a molecular shield, protecting specific proteins against oxidation at their own expense (16). Interestingly, we most recently demonstrated HHP-induced expression of heat shock proteins in *E. coli* (2). Whether heat shock protein induction is a consequence of oxidative protein damage in HHP treated cells, as was suggested for senescent cells, is difficult to assess, since due to its protein-denaturing effect, HHP treatment may also induce a heat shock response in a more direct way. However, expression of heat shock proteins and oxidative-stress resistance seem not to be totally unrelated in the context of HHP treatment because, in the same study, the pressure-resistant mutants shown here to have a higher resistance to plumbagin and to support a lower level of ROS production upon HHP treatment were found to express elevated basal levels of heat shock proteins. Moreover, in *L. monocytogenes*, pressure resistance was also found to be related to enhanced expression of class III heat shock genes due to a mutation in the negative regulator CtsR (27) and was also accompanied by increased peroxide resistance (26).

One possible model of bacterial HHP inactivation emerging from our results that incorporates and further refines the suicide hypothesis formulated earlier by Aldsworth et al. (3) consists of the following steps: (i) pressure-sensitive cytoplasmic or membrane enzymes are inactivated by HHP; (ii) cellular metabolism becomes imbalanced and an excess of reducing power and derailing electron transfer reactions result in generation of ROS; (iii) cytoplasmic proteins denatured by pressure and/or oxidized by ROS induce a heat shock response; (iv) accumulation of oxidative damage due to ROS causes unculturability and cell death. In this model, and given their elevated basal expression of chaperones, the high pressure resistance of pressure-resistant mutants can be explained by an increased protection of cellular proteins against pressure-induced denaturation, thus reducing ROS generation and preventing cellular suicide, and/or by an increased chaperone-mediated resilience against pressure-induced oxidative stress. Nevertheless, further evidence is necessary to corroborate this model.

Together, these data provide convincing indications of endogenous oxidative stress as a possible cause of death for *E. coli* upon mild HHP treatment. Besides inducing an oxidative burst, HHP treatment also sensitizes *E. coli* to reactive oxygen, while pressure resistance seems to coincide with increased tolerance to reactive oxygen. These findings provide an additional case for the emerging insight that bacterial death as a result of starvation or other mild stresses may be triggered by oxidation and that bacteria burn out rather than fade away. While this study emphasizes proteome oxidation during or directly after HHP treatment, future research has to determine the metabolic origin of this HHP-induced oxidative stress and specify the cellular targets or compartments impeded by this oxidation.

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REFERENCES


