Inactivation of the **Lactococcus lactis** high-affinity phosphate transporter confers oxygen and thiol resistance and alters metal homeostasis

Bénédicte Cesselin, Djae Ali,† Jean-Jacques Gratadoux,‡ Philippe Gaudu, Patrick Duwat,§ Alexandra Gruss and Meriem El Karoui

INRA, UR 888 Unité des Bactéries Lactiques et Pathogènes Opportunistes-UBLO, F-78350 Jouy en Josas, France

Numerous strategies allowing bacteria to detect and respond to oxidative conditions depend on the cell redox state. Here we examined the ability of **Lactococcus lactis** to survive aerobically in the presence of the reducing agent dithiothreitol (DTT), which would be expected to modify the cell redox state and disable the oxidative stress response. DTT inhibited ***L. lactis*** growth at 37 °C in aerobic conditions, but not in anaerobiosis. Mutants selected as DTT resistant all mapped to the **pstFEDCBA** locus, encoding a high-affinity phosphate transporter. Transcription of **pstFEDCBA** and a downstream putative regulator of stress response, **phoU**, was deregulated in a **pstA** strain, but amounts of major oxidative stress proteins were unchanged. As metals participate in oxygen radical formation, we compared metal sensitivity of wild-type and **pstA** strains. The **pstA** mutant showed approximately 100-fold increased resistance to copper and zinc. Furthermore, copper or zinc addition exacerbated the sensitivity of a wild-type ***L. lactis*** strain to DTT. Inactivation of **pstA** conferred a more general resistance to oxidative stress, alleviating the oxygen- and thermo-sensitivity of a **clpP** mutant. This study establishes a role for the **pst** locus in metal homeostasis, suggesting that **pst** inactivation lowers intracellular reactivity of copper and zinc, which would limit bacterial sensitivity to oxygen.

**INTRODUCTION**

Oxygen and derivative radical oxygen species (ROS) such as superoxide anion radicals, hydrogen peroxide (H$_2$O$_2$), or hydroxyl radicals are toxic for cells. In the reducing environment of the cytosol, they act as oxidative agents and their accumulation may lead to growth arrest in the absence of cell protection. Detoxification enzymes (e.g. catalase, superoxide dismutase; Imlay, 2008), which remove oxygen or ROS, constitute a major means of escape from oxidative stress conditions. Oxygen toxicity is intimately associated with metals. Reduced metals, in particular iron and copper, can convert H$_2$O$_2$ to highly toxic hydroxyl radicals (Fenton reaction). However, other metals can increase oxidative stress independently of the Fenton reaction. For example, zinc is not redox-active by itself but interacts with redox-active cysteines in bacterial proteins (such as Hsp333; Graf & Jakob, 2002). It has been shown that zinc deficiency or overload can lead to oxidative stress, whereas intermediate cellular concentrations of zinc have an antioxidant effect (Maret, 2006). Oxidative stress is also exacerbated by high temperature. Increased temperature triggers an oxidative burst of superoxide, which causes a profound loss of *Escherichia coli* viability (Benov & Fridovich, 1995). Thus, a connection between response to oxidative stress and heat shock is expected. As such, defects in Clp proteins, chaperones or RecA dampen resistance to oxidative stress in bacteria (Dwat et al., 1995; Frees et al., 2003; Robertson et al., 2002).

Bacteria are programmed to coordinate numerous strategies to respond to damage induced by the presence of oxygen. Interestingly, stress sensors rely on reactivity between oxygen or metal and the sensor itself. Oxidation of cysteines in disulfide-bond-containing proteins (RSH→RSSR), redox balance of cofactors (e.g. iron–sulfur clusters), or metal binding, allows regulators to be active...
and thereby govern transcriptional expression of genes under their control. Note that one stress protein may belong to several regulons, and thus respond to several stress conditions. This is exemplified by SodA, which is induced by metal, heat, and oxidative or acid stress (Budin-Verneuil et al., 2005).

*Lactococcus lactis* is a Gram-positive mesophilic bacterium of industrial interest, related to its uses in cheese production (O’Connor, 2007), and recently in novel bioprotein delivery strategies (see Steidler & Rottiers, 2006). Several *L. lactis* defence proteins have been characterized, and its genome contains stress protein homologues, including RecA (Duwat et al., 1995), Mn-SodA (Sanders et al., 1995), TrmA (Turner et al., 2007), the thioredoxin reductase–thioredoxin system (Vido et al., 2005) and a glutathione reductase (Li et al., 2003), although *L. lactis* reportedly does not synthesize glutathione. *L. lactis*, which is generally known for its fermenting capacities, can also undergo aerobic respiration when haem is provided (Duwat et al., 2001; Gaudu et al., 2002, 2003; Vido et al., 2004); in this condition, respiration chain activity eliminates oxygen and decreases the occurrence of ROS (Rezaiki et al., 2004). Analyses of the *L. lactis* genome revealed that conserved CXXC motifs are present in numerous *L. lactis* ORFs encoding repair or stress response proteins, suggesting that these factors might be modulated by the oxidative state of the cytoplasm.

In this study, we examined the ability of *L. lactis* to survive aerobically in the presence of dithiothreitol (DTT), a membrane-diffusible thiol reducing agent. DTT would be expected to modify the cell redox state and disable the oxidative stress response. Mutants conferring resistance to DTT were found to map exclusively to genes in the *pstFEDCBA* locus (referred to as the *pst* locus) involved in phosphate uptake. Our results indicate that *pstA* inactivation impacts on copper and zinc toxicity. We explain these effects by the modified homeostasis of metals which may limit bacterial sensitivity to oxygen.

### METHODS

**Bacterial strains, plasmids, and general growth conditions.**

Bacterial strains, plasmids are listed in Supplementary Table S1, available with the online version of this paper. *L. lactis* strain MG1363 (wild-type, WT), or strain MG1363 carrying low-copy plasmid pIL252 conferring EryR (referred to as WT-Ery) was used as control. Details of strain constructions are described below. *L. lactis* strains were grown at 30 °C under static growth conditions in M17 liquid medium (Difco) supplemented with 1% glucose (GM), or on solid GM that contained 1.5% agar. M17 modified medium (GMm), containing 1% glucose, but lacking β-glycerophosphate and beef extract (and therefore essentially phosphate-free), and buffered to pH 7 with 200 mM MOPS was used as indicated. Plates were incubated aerobically or under anaerobic conditions in jars (containing the ‘GENbox anaer’ generator for culture of anaerobic bacteria, bioMérieux). Erythromycin (Ery) was used at 2.5 mg ml⁻¹, and 1 μg Ery ml⁻¹. Cultures were then shifted to 37 °C with aeration (230 r.p.m.) in the presence or absence of 30 mM DTT, for 1 h and 30 min. Cells were then collected for protein extraction (Guillot et al., 2003) and proteins were subjected to denaturing 1D gel electrophoresis following standard protocols.

**H₂O₂ sensitivity tests.** The WT-Ery and *pstA*::Ery strains were grown for 24 h in GMm supplemented with 20 mM K₂HPO₄ and 1 μg Ery ml⁻¹. For oxidative challenge, cultures were diluted 10-fold and incubated with 10 mM H₂O₂ for 1 h. H₂O₂ was then removed by addition of bovine catalase (10 U ml⁻¹, Sigma), and c.f.u. were determined on GMm plates containing 20 mM K₂HPO₄. Reported results correspond to the mean of three assays.

**Construction of the *pstA*::Ery mutant and the *pstA*::Ery clpP double mutant.** Single-stranded DNA oligonucleotide primers 5'-TTG ACC GCA AGG ACA CG-3' and 5'-CAT TAC GAA TGT GCT GG-3' were used to PCR-amplify a 305 bp fragment from the MG1363 chromosome. DNA fragments were treated with T4 and Klenow polymerases and cloned into Smal-linearized pRV300 (Leloup et al., 1997), giving pFstAin (Supplementary Table S1). The chromosomal *pstA* gene was then inactivated by single-crossover recombination. To do this, *L. lactis* MG1363 electrocomptent cells were transformed with pFstAin, selecting on GM plates containing 20 mM K₂HPO₄, monitoring growth for 24 h using a plate reader (EL808, BioTek Instruments).

**Protein expression**

**1D gel electrophoresis.** Protein expression levels were assessed by growing the WT and *pstA*::ISS1 strains to OD₆₀₀ 0.05, then shifted to 37 °C for 1 h under static growth conditions, and finally plated aerobically on GM containing different DTT concentrations at 37 °C or 35 °C. Selection concentrations were determined by finding the lowest DTT concentrations that led to near-total mortality of WT-Ery in the presence of Ery (1 μg ml⁻¹), as seen by the absence of c.f.u. after 48 h: 30 mM DTT at 35 °C or 25 mM DTT at 37 °C.

**2D gel electrophoresis.** Protein expression levels were assessed by growing the WT-Ery and *pstA*::ISS1 strains to OD₆₀₀ 0.4 under static conditions in GM. Cultures were then shifted at 37 °C with aeration (230 r.p.m.) in the presence or absence of 30 mM DTT, for 1 h and 30 min. Cells were then collected for protein extraction (Guillot et al., 2003) and proteins were subjected to denaturing 1D gel electrophoresis following standard protocols.
Ery. The same strategy was used to construct a clpP pstA double mutant, starting from the markerless clpP strain (Frees & Ingmer, 1999). Strain constructions were verified by Southern hybridization using pRV300 containing the pstA fragment as probe.

**Metal sensitivity assays.** The WT-Ery and pstA::Ery strains were grown overnight at 30 °C in GM, and dilutions were spotted (5 μl of 10⁻¹ to 10⁻³ dilutions) on GM plates containing or not different metals, as indicated in the text. Plates were incubated for 24 h at 30 °C and photographed.

To test the effects of metal addition on DTT toxicity, dilutions (5 μl spots) of overnight WT-Ery and pstA::Ery cultures (grown at 30 °C in GM) were deposited on solid GM containing or not 0.1 mM Cu, 1 mM Zn and 10 mM DTT. All plates were incubated at 37 °C for 48 h and then photographed.

**RESULTS AND DISCUSSION**

*L. lactis* is sensitive to DTT under aerobic, but not anaerobic growth conditions at elevated temperature

To explore the role of thiol stress in *L. lactis* survival, we examined MG1363 (WT) survival in the presence of different concentrations of the thiol reducing agent DTT added to solid medium in aerobic conditions. We expected that DTT addition during aerobic growth might prevent cells from responding to oxidative stress; DTT might also cause cell damage by reducing oxygen to form ROS. We found that *L. lactis* plating efficiency was unaffected by concentrations of up to 50 mM DTT at 30 °C (data not shown). However, at 37 °C the number of c.f.u. was reduced at least 10³-fold in the presence 25 mM DTT (the optimum growth temperature of *L. lactis* is around 30 °C). This result suggested that the combination of DTT and high temperature is toxic for aerobically grown lactococci.

The contribution of oxygen to mortality due to DTT was evaluated by comparing plating efficiencies of WT cells grown in the presence of DTT, in aerobic versus anaerobic conditions (Table 1). In contrast to the >10³-fold drop in viability in aerobic conditions, full viability was observed after incubation in anaerobic conditions at 37 °C. Oxygen is thus an important factor in DTT-mediated toxicity at high temperature.

A proteomic approach was used to examine expression levels of known oxidative stress response proteins of aerobic 37 °C cultures of *L. lactis* grown without or with DTT. In the presence of DTT at 37 °C, levels of SodA, thioredoxin reductase and the peroxide-detoxifying enzyme alkylhydroperoxide reductase (encoded by sodA, trxB1, and ahpC, respectively) were clearly decreased compared to those observed in cells grown in the same conditions without DTT (Fig. 1). In the case of sodA, lower expression in cultures grown with DTT was also confirmed at the transcriptional level by Northern blotting (data not shown). These results are in keeping with our initial hypothesis, that DTT impairs the cellular response to oxidative stress. They are also supported by a study in *E. coli* indicating that another thiol agent, homocysteine, has a damping effect on oxidative stress response (Fraser et al., 2006). We did not observe appreciable differences in the amounts of heat-shock proteins DnaK and GroEL, despite the elevated growth temperature; this may reflect the reported transient nature of the heat-shock response (Arnau et al., 1996). These results suggest that DTT disables the normal oxidative stress response of *L. lactis*.

**Table 1.** DTT resistance of the pstA::Ery mutant in aerobic conditions at high temperature

WT-Ery and pstA::Ery strains were grown under static liquid growth conditions in GMm containing 20 mM K2HPO4 to mid-exponential phase (OD₆₀₀ 0.2–0.3). Dilutions were plated on solid GMm medium containing 20 mM K2HPO₄, with or without 30 mM DTT. C.f.u. were determined after 48 h incubation. Results are the means of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DTT Survival (c.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic 30 °C</td>
</tr>
<tr>
<td>WT-Ery</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>4.5 × 10⁸</td>
</tr>
<tr>
<td>pstA::Ery</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>5.0 × 10⁸</td>
</tr>
</tbody>
</table>

**Fig. 1.** Oxidative stress response protein levels are significantly decreased in the presence of DTT. Protein extracts of WT *L. lactis* grown with and without DTT were subjected to 2D gel proteome analysis. Levels of known oxidative stress response proteins SodA, TrxB1 and AhpC are decreased in cells grown in the presence of DTT. The FbaA protein was used as reference to compare total protein amounts on the gels. Two independent experiments were performed; a representative gel is shown.
DTT-resistant insertional mutants all map in the pst locus

We selected for transposition insertion mutants that could grow aerobically in the presence of DTT. Two independent pG + host9::ISS1 insertional mutageneses of MG1363, performed in the presence of DTT at 35 °C or 37 °C, resulted in the selection of 20 mutant colonies. DTT-resistant phenotypes were reconfirmed on purified mutants, and insertion sites were identified by chromosomal junction cloning and DNA sequencing. All 20 insertions were independent, and all interrupted the pst locus, comprising the pstFEDCBA genes. The precise insertion points of 10 mutants were determined by sequencing the chromosomal junctions: they all mapped in pstA, pstB, pstC, pstD and pstE (Fig. 2). This locus shows strong similarity to the E. coli pst locus, which encodes a high-affinity phosphate carrier (Wanner, 1996). The pst locus is followed by phoU, encoding a homologue of the E. coli PhoU protein. In E. coli PhoU is a negative regulator of the pho regulon (Wanner, 1996; Steed & Wanner, 1993) and was shown to be a global negative regulator that shuts down several hundred genes involved in bacterial metabolism (Li & Zhang, 2007).

Stabilized excision mutants of two of the initial pG + host9::ISS1 DTT-resistant isolates corresponding to insertions in pstA and pstE were constructed, and the DTT-resistant phenotype at 37 °C was confirmed on both. To avoid possible secondary mutations during selection, an independent pstA strain containing an erythromycin resistance marker was constructed by single-crossover mutation (pstA::Ery). This strain showed DTT resistance (Table 1), indicating that the observed phenotype was not due to a secondary event during selection.

Physiological characterization of the pstA::Ery mutant

To confirm that the pst locus was involved in phosphate transport in L. lactis, growth of the WT strain containing an erythromycin resistance marker (WT-Ery) and the pstA::Ery strain was compared in phosphate-limiting medium (GMm, see Methods) and phosphate-rich medium (GMm supplemented with 20 mM K2HPO4) (Fig. 3). Under phosphate-limiting conditions, the pstA::Ery mutant showed a lower growth rate than the WT-Ery strain. In contrast, pstA::Ery grew nearly as well as WT-Ery in phosphate-rich medium, probably due to activity of the low-affinity phosphate transport system predicted from the genome sequence. These results are in keeping with a role for the pst locus in phosphate uptake at low-phosphate concentrations in L. lactis.

Rallu et al. (2000) reported that acid stress resistance of a pstE mutant (also called pstS) of L. lactis was due to decreased intracellular phosphate concentration. We therefore examined the influence of phosphate depletion on DTT resistance. We compared WT-Ery and pstA::Ery cultures for survival in aerobic conditions in the presence of DTT on GMm plates containing or not 20 mM phosphate (Table 2). The pstA mutant remained highly DTT resistant in the presence of excess phosphate. It is thus likely that a low cytoplasmic phosphate concentration is not the cause of DTT resistance of the pstA mutant.

Mutants in the pst locus were reported as being resistant to H2O2 (Rallu et al., 2000). We checked whether a pstA mutant was resistant to oxidative stress independently of the presence of DTT. The ability of WT-Ery versus pstA::Ery cells to survive an H2O2 shock at 30 °C was examined in GMm liquid medium containing 20 mM phosphate. The pstA::Ery mutant showed fivefold greater survival than the control strain upon 1 h exposure to 10 mM H2O2 in stationary phase. Thus, pstA inactivation results in greater resistance to H2O2-provoked oxidative stress.

http://mic.sgmjournals.org
Table 2. DTT resistance of pstA::Ery is independent of phosphate concentration

WT and pstA::Ery strains were grown under static liquid growth conditions in GMm with or without 20 mM K₂HPO₄, to mid-exponential phase (OD₆₀₀ 0.2–0.3). Dilutions were plated on solid GMm medium with or without 20 mM K₂HPO₄, and 30 mM DTT. C.f.u. were determined after 48 h incubation. Results are the means of three independent experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DTT</th>
<th>Survival (c.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aerobic (37 °C)</td>
</tr>
<tr>
<td>WT-Ery</td>
<td>−</td>
<td>1.8 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.8 × 10⁴</td>
</tr>
<tr>
<td>pstA::Ery</td>
<td>−</td>
<td>4.0 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.4 × 10⁸</td>
</tr>
</tbody>
</table>

Stress response protein expression is not induced by pstA inactivation

We compared protein expression in response to DTT treatment of WT and pstA strains grown aerobically at 37 °C, by 1- and 2D gel electrophoresis. Proteins were identified by MALDI-TOF mass spectrometry. We observed a strong expression of PstF, a putative lipoprotein component of the high-affinity phosphate transport system, which was also confirmed by transcriptional analysis (see supplementary material). A similar finding was recently reported in a proteomic study of lactococcal pstS mutant (Budin-Verneuil et al., 2007). However, other than this difference, protein expression in the pstA::Ery mutant and WT-Ery strains was similar, in both normal and DTT-stress conditions (data not shown). In particular, in the presence of DTT, the low levels of oxidative stress response proteins SodA, TrxB1 and AhpC remained low in both WT and pstA::Ery strains. We conclude that the pstA mutation has little, if any, impact on the induction of several characterized oxidative stress response genes.

The pstA::Ery mutant is resistant to copper and zinc

Studies in Saccharomyces cerevisiae and E. coli suggested that a phosphate transporter could be implicated in transport of metals such as manganese, zinc and cobalt (Jensen et al., 2003; van Veen et al., 1994). Metals catalyse ROS production (Schutzendubel & Polle, 2002; Teitzel et al., 2006) and disulfide bond formation (Hiniker et al., 2005; Maret, 2006). It was thus possible that pst inactivation confers oxygen resistance in the presence of DTT by affecting metal availability. We compared the metal sensitivities of L. lactis WT-Ery and pstA::Ery strains. The pstA::Ery mutant showed approximately 100-fold greater survival in the presence of copper and zinc compared to the control strain (Fig. 4). No differences were observed in the presence of nickel, cobalt, iron or manganese (data not shown). These results suggest that pstA has an impact on copper and zinc homeostasis.

DTT sensitivity of the WT strain is exacerbated by copper or zinc

We hypothesized that greater copper and zinc tolerance of the pstA strain could be implicated in DTT resistance. The effect of copper or zinc addition on the sensitivity of the WT and pstA::Ery strains to DTT at 37 °C was tested on solid medium. In these tests, we lowered the amount of DTT to 10 mM (from 25 mM above). The presence of 0.1 mM copper or 1 mM zinc alone had no effect on survival of either the WT or the pstA::Ery strain. However, the combination of each metal and DTT had a marked effect on the WT, compared to the pstA::Ery strain (Fig. 5). These results indicate that DTT toxicity is exacerbated in the presence of copper and zinc in the WT strain. We propose that a pstA mutation would result in altered homeostasis of free metal loads in the cell. In the case of copper, decreased intracellular free metal levels would result in lower ROS formation, thereby improving survival in the presence of DTT. In the case of zinc, which is not directly redox-active by itself, we assume that the pst mutation results in a modified balance between redox-active cysteines and zinc–cysteine complexes in proteins (Maret, 2006), which would limit DTT toxicity. A role of pst in metal homeostasis would not require induction of oxidative stress response proteins, in keeping with our observations.

Fig. 4. The pstA mutation confers resistance to zinc and copper. Tenfold dilutions of WT-Ery and pstA::Ery overnight cultures were spotted on GM plates containing or not 0.5 mM copper chloride or 7 mM zinc chloride. Plates were incubated 24 h at 30 °C and photographed. ud, undiluted.
The *pstA* mutation relieves thermosensitivity of a *clpP* mutation

If *pstA* inactivation affects free metal availability and lessens DTT sensitivity, we predicted that it might have similar effects in different situations where thiol homeostasis is modified. This unexpectedly occurs in a *clpP* mutant. In *Bacillus subtilis*, a *clpP* mutant was shown to accumulate thioredoxin reductase (TrxB), thioredoxin (TrxA) and thiol peroxidase (Tpx) at high temperature (Kock et al., 2004). We reasoned that *clpP* inactivation would thus modulate disulfide bond formation in a way analogous to that seen when DTT is added to a WT culture. As in *B. subtilis*, *clpP* is thermosensitive in *L. lactis* (Frees & Ingmer, 1999). First, we observed that loss of *clpP* viability at 38 °C is alleviated in anaerobic conditions (Fig. 6A) suggesting that the observed thermosensitivity is due at least in part to impaired oxidative stress response. [In a previous study this phenotype was not reported but tests were performed in conditions where even the WT strain did not grow (Frees et al., 2001)]. Then we asked whether *pstA* inactivation might rescue a *clpP* mutant. We constructed a *clpP* *pstA::Ery* double mutant, and examined growth of WT, *clpP*, and *clpP* *pstA::Ery* strains at 30 °C and 38 °C (Fig. 6B). The *pstA* mutation fully restored growth of a *clpP* mutant at 38 °C in the presence of oxygen. These results show that *pstA* inactivation alleviates both *clpP* thermosensitivity and WT strain DTT sensitivity.

Conclusions

The physiological and genetic responses of *L. lactis* to DTT stress were examined. Our main findings are that DTT toxicity in lactococci is affected by (i) oxygen, (ii) the availability of metals copper and zinc, and (iii) activity of the *pst* locus. We suggest that the high-affinity phosphate uptake system encoded by *pst* genes is, in addition to its role in phosphate transport, involved in copper and zinc homeostasis. Mutants in the *pst* locus were recently isolated in a screening for tellurite resistance (Turner et al., 2007), suggesting that *pst* might also affect tellurite homeostasis and possibly that of other metals. Interestingly, results suggestive of metal transport via phosphate uptake systems were previously reported in other species (Alvarez & Jerez, 2004; Beard et al., 2000; van Veen et al., 1994, 1993), and could explain the resistance to metal toxicity. In our mutagenesis, we did not obtain mutants affected in known or putative metal transporters (Gostick et al., 1999; Scott et al., 2000; Turner et al., 2007), nor did we obtain mutants in regulators of such transporters, e.g. ZitR and the FNR-like proteins of *L. lactis* (Gostick et al., 1999; Llull & Poquet, 2004; Scott et al., 2000). This might suggest that the transporters are redundant or that *pst* mutation has a pleiotropic role affecting intracellular availability of several metals, which would not be obtained by inactivation of a single transporter.

This study establishes a role for the *pst* locus in copper and zinc homeostasis in *L. lactis*. Experiments measuring total intracellular metal loads did not reveal significant differences between WT and *pstA* strains, even when the growth medium was supplemented with copper or zinc (data not shown). This would suggest that *pst* does not control total zinc and/or copper pools. To explain this, we speculate that *pst* may be involved in controlling the pool of free (reductive) metals; the existence of free and bound iron was shown in *E. coli* (Keyer & Imlay 1996).


Edited by: D. J. Jamieson