The impairment of superoxide dismutase coordinates the derepression of the PerR regulon in the response of *Staphylococcus aureus* to HOCl stress

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The response of *Staphylococcus aureus* to hypochlorous acid (HOCl) exposure was investigated. HOCl challenges were performed on cultures interrupted in the exponential phase. Pretreatment with HOCl conferred resistance to hydrogen peroxide in a PerR-dependent manner. Derepression of the PerR regulon was observed at low HOCl concentration (survival > 50%), using several fusions of different stress promoters to lacZ reporter genes. At least four members of the PerR regulon (*katA, mrgA, bcp* and *trxA*) encoding proteins with antioxidant properties were strongly induced following exposure to various HOCl concentrations. A striking result was the link between the derepression of the PerR regulon and the decreased superoxide dismutase (SOD) activity following exposure to increased HOCl concentrations. The *sodA* mutant was more resistant than the wild-type and also had a higher level of 3-phosphoglycerate dehydrogenase (a measure of PerR regulon activity) without exposure to HOCl. Together, these results imply that derepression of PerR by HOCl is dependent on the level of SOD and protects exponentially arrested cells against HOCl stress.

INTRODUCTION

Hypochlorous acid (HOCl) is a potent, low-cost disinfectant active against a wide variety of micro-organisms even at micromolar concentrations, due to the fact that micro-organisms do not possess specific enzymic mechanisms for its detoxification. The mechanism by which HOCl exerts its lethal effects has been documented in Gram-negative bacteria (Dukan & Touati, 1996; Dukan et al., 1996, 1999). Briefly, in *Escherichia coli*, it has been shown that oxygen plays an aggressive role during recovery from HOCl stress, which may be due to a HOCl-dependent loss of antioxidant defences such as glutathione reductase, catalase and superoxide dismutase (SOD) (Dukan et al., 1999). Interestingly, the redox regulon SoxRS, known to be activated by superoxide (Demple, 1991), was induced by sublethal HOCl concentration (Dukan et al., 1996), while the OxyR regulon, known to be activated by hydrogen peroxide (H2O2) was not induced. Moreover, pretreatment of bacteria with sublethal HOCl concentration conferred resistance to H2O2, but not to higher HOCl concentration, in an OxyR-independent manner (Dukan & Touati, 1996). Together these results suggest that part of the toxicity HOCl to *E. coli* is mediated by reactive oxygen species. *Staphylococcus aureus* is an important Gram-positive human pathogen causing a wide spectrum of diseases, from wound infections to severe infections such as septicemia, osteomyelitis and endocarditis (Easmon & Adlam, 1983). Eradication of the organism is extremely difficult, particularly in hospitals, due to its multiple drug resistances and its ability to survive in extreme conditions (Clements & Foster, 1999; Kloos & Bannerman, 1994; Sean et al., 1998). Upon starvation or entry into stationary phase, protective functions against heat shock and H2O2 are induced under the control of the sigma B regulon (Chan et al., 1998; Kullik & Giachino, 1997; Wu et al., 1996). In exponentially growing cultures, *S. aureus* also displays an adaptive response to low levels of H2O2 (Horsburgh et al., 2001a, b). Genetic evidence has revealed that the major regulatory circuit involved is the PerR regulon, which is a member of the ferric uptake repressor (Fur) family of metal-dependent DNA-binding proteins (Horsburgh et al., 2001a, b). This regulon includes catalase *(KatA)*, alkyl hydroperoxide reductase *(AhpCF)*, bacterioferritin comigratory protein *(Bcp)*, thioredoxine reductase *(TrxB)* and PerR itself (Horsburgh et al., 2001a, b). Studies with *lacZ* reporter fusions have also demonstrated that some of these genes (*ahpC, bcp, ft* and *katA, mrg* and *trxB*) are strongly derepressed by 500 μM H2O2, while others (fur, perR) show no induction. Furthermore, PerR, by the control
of the genes encoding the iron-storage proteins ferritin (Ftn) and the ferritin-like Dps homologue MrgA, coordinate the intracellular availability of free iron with the level of antioxidant proteins present in the cell (Horsburgh et al., 2001a, b). SOD also forms part of the bacteria’s armoury against reactive oxygen species by catalysing dismutation of the superoxide (O$_2^-$) (Clements et al., 1999). Two cytoplasmic SODs have been identified in S. aureus, a manganese SOD (MnSOD) and an iron SOD (FeSOD), encoded by SodA and SodB, respectively. MnSOD regulation is oxygen and growth-phase dependent. SodA has a role in starvation (Watson et al., 1999) and acid tolerance but not in pathogenicity (Clements et al., 1999). To date, there is no evidence of the regulatory mechanism of SodA in S. aureus, since SOD activity was not affected by either perR or sigB inactivation (Clements et al., 1999).

In this study, we demonstrate that the PerR regulon also controls HOCl stress resistance in S. aureus, probably by its derepression through the impairment of SOD. The transcriptional responses monitored by gene expression in stressed cells using lacZ reporter fusions were compared with unstressed control cells. The identity of genes induced provides new insights into the mechanism of HOCl toxicity and the cellular protection against this compound in Gram-positive bacteria.

**METHODS**

**Bacterial strains and culture conditions.** The S. aureus strains used in this study were obtained from Professor S. J. Foster (University of Sheffield, England) and are listed in Table 1. Cells were grown in brain heart infusion (BHI; Pasteur Institute production) at 30°C in a Heidolph UNIMAX 1010 incubator at 200 r.p.m. When appropriate, the medium was supplemented with erythromycin (5 µg ml$^{-1}$), tetracycline (5 µg ml$^{-1}$) or kanamycin (50 µg ml$^{-1}$).

**Chlorine assays.** Cells in the exponential (OD$_{600}$ 0-8) or stationary (OD$_{600}$ 10) growth phase were spun down at 4000 r.p.m. for 15 min at 4°C, washed in PBS and resuspended at a cell density of about 0.5 x 10$^8$ c.f.u. ml$^{-1}$. Samples of 5 ml were distributed to Falcon tubes (50 ml). To ensure that no organic material reacted with HOCI, Erlenmeyer flasks were previously heated at 500°C for 4 h.

Fresh HOCl (Prolabo Chemical Company) was added to cells at various concentrations from 0 to 8 mg l$^{-1}$ (<60 µl). The concentration of HOCl was determined iodometrically (Czapski et al., 1992). The cell suspension was incubated at 30°C in the dark with gentle shaking (100 r.p.m.), 100 µl was removed at intervals of 0 and 15 min and HOCl was quenched by the addition of 100 µl sterile sodium thiosulfate (5 x 10$^{-3}$ M). Culturable bacteria were assayed by plating on BHI plates after serial dilutions in cold PBS buffer. Colonies were counted after 48 h incubation at 37°C.

**HOCI adaptation experiments.** When exponential-phase cultures reached an OD$_{600}$ of 0-8, cells were washed twice and resuspended in PBS buffer to which 0 or 1 mg HOCl l$^{-1}$ was added. After 60 min incubation at 30°C with gentle shaking, cells were challenged with H$_2$O$_2$ at 200 mM; 0.1 ml samples were taken at regular intervals, and reactions were stopped by adding 0-1 ml catalase (200 U ml$^{-1}$).

**β-Galactosidase activity assays.** Following 15 min exposure to different HOCl concentrations, 2 ml of fourfold-concentrated BHI solution was added to 6 ml of cells and the mixture was incubated at 30°C under gentle shaking. At regular intervals, 0-1 ml samples were harvested and β-galactosidase assays were performed as described by Miller (1972).

**SOD measurements.** For preparation of crude extracts, cells were washed twice in PBS buffer pH 7-1 and resuspended in lysis buffer (10 mM Tris/HCl pH 8, 1 mM EDTA, 20 µg lysozyme ml$^{-1}$). After repeated freeze–thawing until cell lysis was observed by microscopic examination, cell wall debris was discarded by centrifugation (10 min, 14 000 g, 4°C), and the crude lysates stored at −20°C until analysis. The total protein concentration was determined by the Bradford assay (Bio-Rad) with bovine serum albumin as a standard (Bradford, 1976). SOD activities were revealed by staining polyacrylamide gels as previously described by Beauchamp & Fridovich (1977) and quantified using the Image Quant software. Total SOD activity was determined by adding crude cell lysate to nitroblue tetrazolium (NBT), methionine, riboflavin, sodium azide and potassium phosphate pH 7-8 according to the method of Beauchamp & Fridovich (1977). One unit of SOD activity was defined as the amount of enzyme causing a 50% inhibition in the rate of NBT oxidation.

**3-Phosphoglycerate dehydrogenase activity.** The level of 3-phosphoglycerate dehydrogenase (3-PGDH; EC.1.1.1.95) activity was measured in 40 mM Tris/HCl (pH 8-8), 1.0 mM EDTA, 1.0 mM NAD$^+$, 10-0 mM 3-phosphoglycerate by following the increase in absorbance at 340 nm (Sugimoto & Pizer, 1968). One unit of enzyme activity was defined as the formation of 1 nmol NADH min$^{-1}$ at 37°C (Zhao & Winkler, 1996).

**RESULTS**

**Definition of assay conditions: chlorine concentrations and culturability**

The experimental approach outlined above required assay conditions under which the damage exerted by HOCl would be sublethal. We looked for conditions which would expose the cells to HOCl concentrations high enough to induce cellular defence circuits but not enough to cause massive cell mortality. With colony-forming ability as the viability parameter, Fig. 1 presents the effect of different HOCl concentrations using the finalized procedure (5 x 10$^7$ cells ml$^{-1}$, 15 min exposure, 50 mM phosphate buffer, pH 7-1, and 30°C). Concentrations up to 5 mg l$^{-1}$ caused an insignificant

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**Table 1. S. aureus strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>8325-4</td>
<td>Wild-type strain cured for prophages</td>
</tr>
<tr>
<td>SPW1</td>
<td>sodA::Tn917-LTV1 Ery$^+$</td>
</tr>
<tr>
<td>ST16</td>
<td>katA::Tn917-LTV1 Ery$^+$</td>
</tr>
<tr>
<td>MJH001</td>
<td>perR::kan</td>
</tr>
<tr>
<td>MJH002</td>
<td>ahpC::pAZ106 ahpC$^+$ Ery$^+$</td>
</tr>
<tr>
<td>MJH003</td>
<td>bcp::pAZ106 bcp$^+$ Ery$^+$</td>
</tr>
<tr>
<td>MJH006</td>
<td>katA::pAZ106 katA$^+$ Ery$^+$</td>
</tr>
<tr>
<td>MJH007</td>
<td>mrgA::pAZ106 mrgA$^+$ Ery$^+$</td>
</tr>
<tr>
<td>MJH009</td>
<td>trxB::pAZ106 trxB$^+$ Ery$^+$</td>
</tr>
<tr>
<td>MJH107</td>
<td>perR::kan mrgA::pAZ106 mrgA$^+$</td>
</tr>
</tbody>
</table>
drop in viability, while higher levels had a pronounced lethal effect. Hence, in the experiments the cells were exposed to HOCl at concentrations of 5 mg l\(^{-1}\) or less.

**Effect of HOCl pretreatment on resistance to H\(_2\)O\(_2\): role of the PerR regulon**

Since HOCl pretreatment induces an OxyR-independent resistance to H\(_2\)O\(_2\) in *E. coli* (Dukan & Touati, 1996), we wondered whether non-lethal doses of HOCl would also induce H\(_2\)O\(_2\) resistance in *S. aureus*. As shown in Fig. 2(a), HOCl-pretreated cells showed increased resistance to an H\(_2\)O\(_2\) challenge. To analyse whether increased resistance to H\(_2\)O\(_2\) by HOCl pretreatment was mediated by derepression of the PerR regulon involved in the defence against H\(_2\)O\(_2\) (Horsburgh *et al.*, 2001a, b), the experiment was repeated in the perR mutant. However, as also shown for *E. coli* (Dukan & Touati, 1996), protection against HOCl stress could not be observed in these conditions (data not shown). As depicted in Fig. 2(b), HOCl pretreatment had no effect on the survival of the perR-defective mutant after H\(_2\)O\(_2\) exposure. However, the survival curves of the perR mutant and HOCl-pretreated wild-type were similar. Taken together, these results suggest that derepression of the PerR regulon by HOCl protects against H\(_2\)O\(_2\) or that derepression of the PerR regulon in a perR mutant will not allow us to detect more resistance after HOCl pretreatment.

**Induction of PerR-regulated genes with HOCl**

In order to investigate derepression of the PerR regulon by HOCl, we analysed whether some genes under the control of the PerR regulon were also induced by HOCl treatment. We first analysed a *mrgA–lacZ* fusion. Following HOCl exposure in phosphate buffer as outlined in Methods, no increase in \(\beta\)-galactosidase above the uninduced levels was observed (data not shown). After the addition of BHI, however, a very clear induction took place, as depicted in Fig. 3. Fig. 3(a) shows the kinetics of induction, while Fig. 3(b) shows the response ratios over the uninduced control. The response was dose dependent, with maximal induction occurring at 3 mg l\(^{-1}\). Activity reached a maximum after 60 min and then declined. The response was *perR* dependent since no induction was observed in the *perR* mutant MJH107 (Fig. 3b). Next we compared the time-course expression of *katA*, *trxB*, *bcp–pdh*, *mrgA* and *ahpC* encoding, respectively, the catalase, the thioredoxin reductase, the bacterioferritin comigratory protein, 3-PGDH, the ferritin-like Dps, and the alkyl hydroperoxide reductase, using 3 mg HOCl l\(^{-1}\). As shown in Fig. 3(c), except for *ahpC*, which did not respond, other gene fusions were induced at a maximum between 40 and 60 min and then declined. These results suggest that the PerR regulon is activated after exposure to HOCl.
Effect of defences against reactive oxygen species on HOCl resistance

The ability of HOCl to generate hydroxyl radicals in vitro (Candeias et al., 1994), to decrease defence against reactive oxygen species in vivo (Dukan et al., 1999) and to trigger the PerR regulon, involved in H2O2 resistance, led us to test whether defences against reactive oxygen species participated in HOCl resistance. Thus, we analysed the sensitivity of sodA (superoxide dismutase) and katA (catalase) mutants compared to the wild-type strain to 6 mg HOCl ml−1. As depicted in Fig. 4, after 15 min HOCl exposure, the katA mutant became more sensitive than the wild-type strain, while – very interestingly – the sodA mutant remained more resistant than the wild-type strain. These results suggested that (i) the lack of SodA trigger genes rendered the strain more resistant to HOCl challenge, and (ii) catalase was involved directly or indirectly in resistance to HOCl, which is consistent with the fact that the PerR regulon is induced by HOCl.

HOCl-dependent PerR induction is mediated by SOD inactivation

The fact that E. coli SOD activities were sensitive to HOCl exposure (Dukan et al., 1999) led us to test whether this was also the case in S. aureus. Indeed, as depicted in Fig. 5(a), cytoplasmic SOD activities showed a HOCl dose-dependent decrease, indicating a clear impairment of this enzyme. Interestingly, the sodA mutant of S. aureus was more resistant than the wild-type to HOCl, indicating that low-level SOD activities contribute to HOCl resistance. This result led us to test whether HOCl-dependent PerR induction is mediated by SodA inactivation. We measured, in strain MJH003 (bcp, pdh-lacZ) total SOD activity after 15 min exposure to different concentrations of HOCl and maximal PerR induction in terms of β-galactosidase units of the bcp gene. As demonstrated in Fig. 5(b), cytoplasmic SOD showed an HOCl-dependent increased sensitivity, indicating inactivation via the oxidant. However, the transcriptional level of the bcp gene increased with the decreased level of sod activity. Under our experimental conditions, at 3 mg HOCl l−1, the perR repression was completely relieved and bcp...
induction was found to be as great as the level of transcription resulting from perR inactivation. In order to confirm that PerR HOCl-dependent induction is mediated by SOD level, we tested the effect of sodA mutation on 3-PGDH (pdh) induction, which is under the control of the PerR regulon (Horsburgh et al., 2001a, b). Fig. 6 shows that the basal level was higher in a sodA mutant and that the maximal response was shifted from a concentration of 3 mg l⁻¹ in the wild-type to 1 mg l⁻¹ in the sodA mutant. These results confirm that PerR induction by HOCl is dependent on the level of SOD.

**DISCUSSION**

The ability of microbial pathogens to develop a complex mechanism and adapt to environmental stress conditions such as HOCl contributes to their survival in the natural environment. HOCl adaptation probably plays an important role in the dissemination of bacteria and their increased frequency in nosocomial infections. In this study we analysed transcriptional responses involved in the defence of *S. aureus* 8325-4 against HOCl stress. Using this approach, we found that in the exponential phase PerR is an important regulator of genes which is induced by low levels of HOCl via SOD inactivation. Moreover, PerR activation protected exponentially arrested cells against HOCl stress.

Indeed, our work provides evidence for PerR activation in exponential-phase cells exposed to HOCl stress. Four individual promoters, bcp, katA, mrgA and trxB, controlled by PerR, were induced by HOCl. Most transcriptional effects were maximal at about 60 min after the addition of HOCl and there was a rapid return to lower expression levels within 20–30 min. Transient induction of PerR has been previously documented only by Helmann *et al.* (2003).

Consistent with the hypothesis that HOCl toxicity is due to oxidative stress (Dukan *et al.*, 1999; Mokgatla *et al.*, 2002), we have found that several enzymes with antioxidant properties are induced by HOCl treatment and particularly enzymes involved in H₂O₂ degradation (catalase, TrxB) and DNA protection from oxidative damage (MrgA, Bcp) (Grant *et al.*, 1998; Jeong *et al.*, 2000; Wolf *et al.*, 1999). Interestingly, the *S. aureus* 3-PGDH gene (pdh), located in the same operon as bcp (Horsburgh *et al.*, 2001a), forms part of the bacterium’s armoury against HOCl stress. Thus pdh may collaborate in the reduction and detoxification of ROS generated in the cytoplasm through regeneration of the NADH pool, which drops in the presence of HOCl (Leyer & Johnson, 1997).

We observed that HOCl pretreatment conferred resistance to H₂O₂ mainly via PerR activation. This HOCl-induced protection against H₂O₂ suggested that cells could adapt to...
HOCl. However, while HOCl pretreatment provides protection against H2O2, we were unable, under the same experimental conditions, to obtain clear protection against a challenge with higher concentrations of HOCl. This failure may be related to the phenomenon of HOCl consumption by bacteria and buffer and the fact that the HOCl concentration used for pretreatment was negligible compared with the challenge concentration.

Several lines of evidence indicate that PerR activation after HOCl exposure was mediated by an increase in superoxide anion via SOD inactivation. We observed a correlation between the decreased level of SOD activity following HOCl stress and the extent of derepression of the PerR regulon, as judged by the bcp gene. We found that activation of the PerR regulon, shown by 3-PGDH activity, involves superoxide radicals, since it was affected by sodA mutation. Moreover, we observed that the sodA mutant was more resistant than the wild-type and also had an increased level of PerR regulon activity. The ability of PerR to be activated by H2O2 and superoxide anions (Horsburgh et al., 2001a; this study) was not unexpected, since a recent paper described that PerR was induced in response to both stresses in Bacillus subtilis (Mostertz et al., 2004). PerR does not appear to control functions that might be involved in maintaining intracellular superoxide radicals at low levels. The mechanism by which S. aureus protects itself against HOCl is presumably mediated by decreasing the levels of species that could react with HOCl to generate toxic reactive oxygen radicals. This decrease may be a critical factor in causing the eventual tolerance of S. aureus upon exposure to sublethal doses of HOCl.

The results presented in this work shed some new light on the poorly understood effects of HOCl on the bacterial response; much more work is needed in order to understand the global bacterial response. In future work, proteome analysis based on two-dimensional gel electrophoresis should be used to examine the global response of S. aureus to HOCl.

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REFERENCES


