Microbiology (2006), **152**, 855–861 DOI 10.1099/mic.0.28385-0

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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Received 28 July 2005
Revised 25 November 2005
Accepted 6 December 2005

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 (H_2O_2) was not induced. Moreover, pretreatment of bacteria with sublethal HOCl concentration conferred resistance to H_2O_2, 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 septicaemia, 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 H_2O_2 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 H_2O_2 (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*, *ftn*, *katA*, *mrgA* and *trxB*) are strongly derepressed by 500 μM H_2O_2, while others (*fur*, *perR*) show no induction. Furthermore, PerR, by the control

Abbreviations: 3-PGDH, 3-phosphoglycerate dehydrogenase; SOD, superoxide dismutase.

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 ($O_2^{\bullet -}$) (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 survival (Watson *et al.*, 1998) 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 $0{\cdot}5 \times 10^8$ c.f.u. ml^{-1}. Samples of 5 ml were distributed to Falcon tubes (50 ml). To ensure that no organic material reacted with HOCl, 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 (Czpaski *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 (5×10^{-4} 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.

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

Strain	Genotype
8325-4	Wild-type strain cured for prophages
SPW1	*sodA*::Tn*917*-LTV1 Ery^r
ST16	*katA*::Tn*917*-LTV1 Ery^r
MJH001	*perR*::*kan*
MJH002	*ahpC*::pAZ106 *ahpC*$^+$ Ery^r
MJH003	*bcp*::pAZ106 *bcp*$^+$ Ery^r
MJH006	*katA*::pAZ106 *katA*$^+$ Ery^r
MJH007	*mrgA*::pAZ106 *mrgA*$^+$ Ery^r
MJH009	*trxB*::pAZ106 *trxB*$^+$ Ery^r
MJH107	*perR*::*kan mrgA*::pAZ106 *mrgA*$^+$

HOCl 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_2O_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 (1971) 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 (1971). 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 dithiothreitol, 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 (5×10^7 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

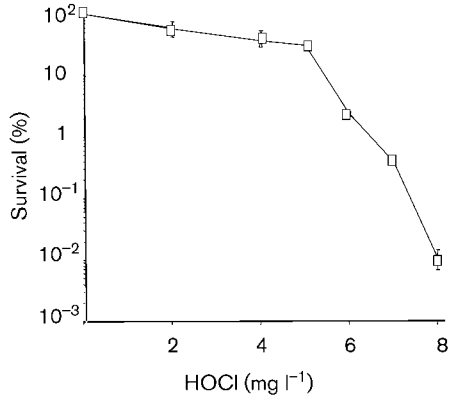

Fig. 1. Effect of HOCl on viability. Survival of wild-type *S. aureus* 8325-4 stopped in exponential growth phase was challenged with increased HOCl concentrations as described in Methods. Samples were collected 15 min post-challenge and culturable counts were performed. The results are expresssed as percentage survival (means ± SD of three independent experiments).

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_2O_2: role of the PerR regulon

Since HOCl pretreatment induces an OxyR-independent resistance to H_2O_2 in *E. coli* (Dukan & Touati, 1996), we wondered whether non-lethal doses of HOCl would also induce H_2O_2 resistance in *S. aureus*. As shown in Fig. 2(a), HOCl-pretreated cells showed increased resistance to an H_2O_2 challenge. To analyse whether increased resistance to H_2O_2 by HOCl pretreatment was mediated by derepression of the PerR regulon involved in the defence against H_2O_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_2O_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_2O_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 β-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.

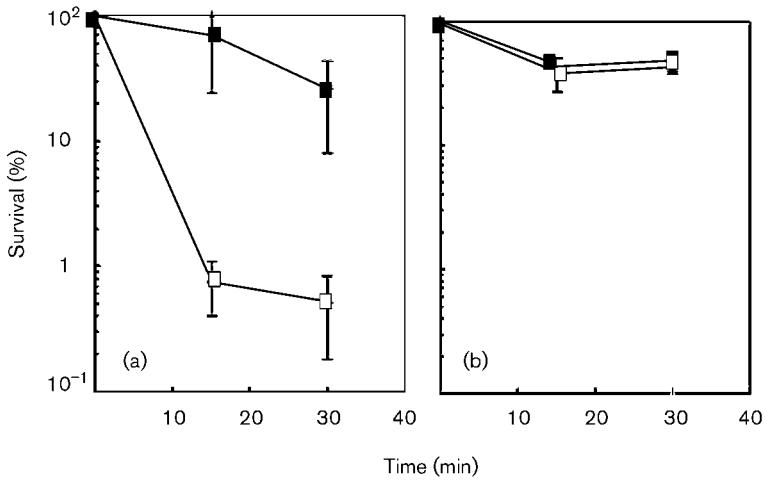

Fig. 2. Adaptative response of *S. aureus* to HOCl and H_2O_2 stresses. Cultures of the wild-type (a) and *perR*::*kan* mutant (b) stopped in exponential phase were pretreated or not by 1 mg HOCl l^{-1} and challenged with 200 mM H_2O_2. ■, Pretreated cells; □, no pretreatment. Values are means ± SD of three independent experiments.

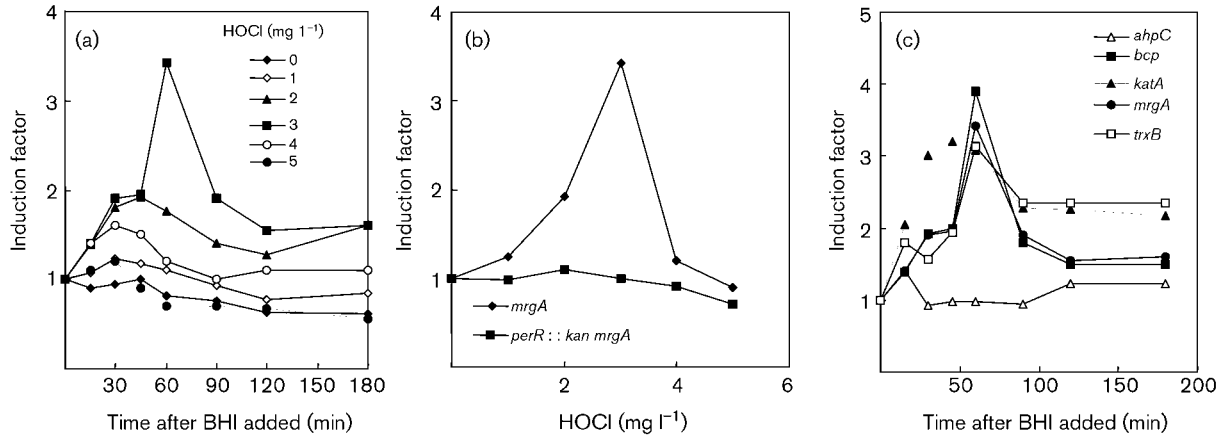

Fig. 3. PerR induction. Cells (10^8 ml^{-1}) of strain MJH007 (*mrgA–lacZ*) were treated for 15 min with HOCl at the indicated concentrations. BHI was then added, samples were removed at the times indicated and analysed for β-galactosidase activity. (a) Kinetics of *mrgA* transcription from promoter-*lacZ* fusion (fold induction relative to the time-zero control sample); (b) maximal induction factor of the *mrgA–lacZ* in the wild-type or in the *perR* mutant in the same background. (c) Comparison of the time-courses of expression for selected members of the PerR regulon. Induction factor (relative to the time zero control sample) is plotted for cells exposed to 3 mg HOCl l^{-1}.

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 H_2O_2 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*

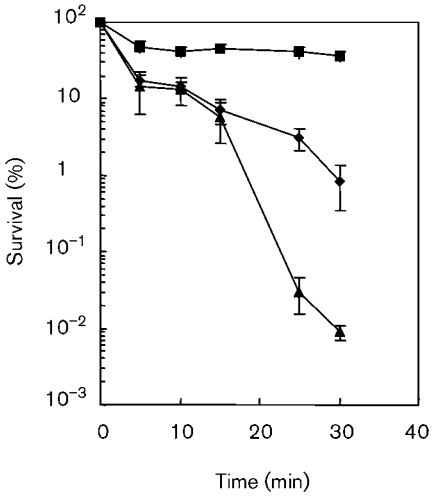

Fig. 4. Effect of defences against reactive oxygen species on HOCl resistance. Exponential-phase cells of SPW1 (*sodA*), ST16 (*katA*) and 8325-4 (wild-type) were challenged for 15 min with 6 mg HOCl l^{-1} as described in Methods. Percentage survivals are the means ± SD of three independent experiments. ■, PC400 (*sodA* : : Tn*917*-LTV1 Ery^r); ▲, ST16 (*katA* : : Tn*917*-LTV1 Ery^r); ◆, 8325-4 (wild-type).

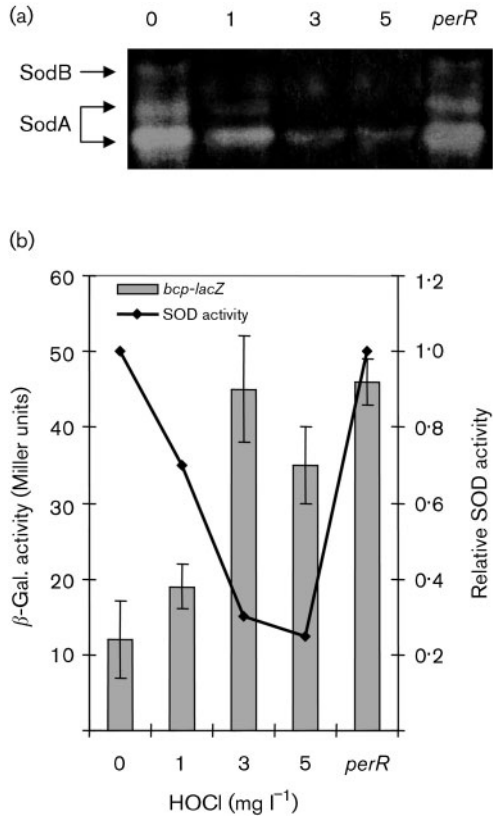

Fig. 5. Effect of HOCl exposure on SOD activity and *perR* induction in *S. aureus*. (a) Cells of strain MJH003 (*bcp*::pAZ106 *bcp*$^+$ Ery^r) containing the *bcp–lacZ* fusion were exposed for 15 min to the indicated concentrations (mg l^{-1}) of HOCl, then samples were removed to visualize SOD activity on non-denaturing 12 % (w/v) polyacrylamide gel (60 μg protein in all lanes) and monitor total SOD activity. (b) BHI was then added and maximal *bcp* transcription from promoter–*lacZ* fusion in terms of β-galactosidase activity was monitored.

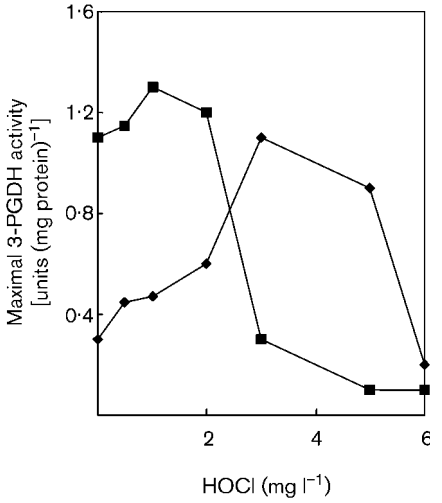

Fig. 6. Lack of SodA affects 3-PGDH induction after HOCl exposure. Cells of strains 8325-4 (wild-type, ◆) and SPW1 (*sodA* mutant, ■) containing 3-PGDH activity were treated with different concentrations of HOCl and 3-PGDH activity was measured as described in Methods.

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 H_2O_2 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 H_2O_2 mainly via PerR activation. This HOCl-induced protection against H_2O_2 suggested that cells could adapt to

HOCl. However, while HOCl pretreatment provides protection against H_2O_2, 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 H_2O_2 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.

ACKNOWLEDGEMENTS

We thank Professor S. J. Foster for the generous gifts of strains.

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