

Reactive Oxygen Species Are Partially Involved in the Bacteriocidal Action of Hypochlorous Acid

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Hypochlorous acid (HOCl) is probably the most widely used disinfectant worldwide and has an important role in inflammatory reaction and in human resistance to infection. However, the nature and mechanisms of its bacteriocidal activity are still poorly understood. Bacteria challenged aerobically with HOCl concentrations ranging from 9.5 to 76 μM exhibit higher ability to form colonies anaerobically than aerobically. Conversely, aerobic plating greatly increased lethality after an anaerobic HOCl challenge, although anaerobic survival did not depend on whether HOCl exposure was aerobic or anaerobic. Even a short transient exposure to air after anaerobic HOCl challenge reduced anaerobic survival, indicative of immediate deleterious effects of oxygen. Exposure to HOCl can cause lethal DNA damage as judged by the fact that *recA* sensitivity to HOCl was oxygen dependent. Anti-oxidant defenses such as reduced glutathione and glucose-6-phosphate dehydrogenase were depleted or inactivated at 10 μM HOCl, while other activities, such as superoxide dismutase, dropped only above 57 μM HOCl. Cumulative deficiencies in superoxide dismutase and glucose-6-phosphate dehydrogenase rendered strains hypersensitive to HOCl. This indicates that part of HOCl toxicity on *Escherichia coli* is mediated by reactive oxygen species during recovery.

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Uncombined chlorine, in the form of un-ionized hypochlorous acid (HOCl),³ is an extremely potent bacteriocidal agent, even at micromolar concentrations. During inflammatory reactions, HOCl is generated within phagosomes of activated neutrophils by the myeloperoxidase-catalyzed peroxidation of chloride ion (1, 2). Surprisingly, however, despite the fact that HOCl is probably the most widely used disinfectant worldwide, and despite its important role in inflammatory reactions and in human resistance to infection, the nature and mechanisms of its bacteriocidal activity are still poorly understood.

Aerobic organisms must deal with reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals that are generated from sequential univalent reductions of oxygen during the normal course of aerobic metabolism. To prevent the harmful effects of ROS, cells are equipped with several defenses including antioxidants, such as glutathione; enzymes, including catalases, peroxidases, and superoxide dismutases (SOD); as well as general defense systems activated in response to oxidative stress (3, 4). Several reports have suggested the involvement of *Escherichia coli* anti-oxidation defenses in the protection against HOCl stress. It has been shown that genes that are part of *E. coli*'s defenses against hydrogen peroxide are also involved in HOCl resistance, indicating a possible overlap in the defense circuits (5). Consistent with this observation, it has been reported that the addition of catalase enhances the colony-forming ability of HOCl-stressed cells (6). The inactivation of catalase and superoxide dismutase activities by HOCl

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³ Abbreviations used: HOCl, hypochlorous acid; G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase; GSH, reduced glutathione; ROS, reactive oxygen species; GSSG, oxidized glutathione; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NBT, nitroblue tetrazolium.

have been described *in vitro* (7, 8) and also *in vivo* for *E. coli* catalase (6). Recently, Chesney *et al.* (9) have shown that reduced glutathione (GSH) plays an important role in the resistance of *E. coli* cells to HOCl. Moreover, HOCl, can mobilize intracellular transition metals such as iron *in vivo* (10), via some oxidative process, which can lead to production of the highly reactive hydroxyl free radical via the Fenton reaction. Finally, the *soxRS* regulon, the multigene defense system against superoxide ($O_2^{\cdot-}$) (11–13) or nitric oxide (14), was shown to be induced after HOCl exposure (15).

In this study, we investigate the possible involvement of ROS in the lethal effect of HOCl on *E. coli*. We show that oxygen plays an aggressive role during recovery from HOCl stress, which may be due to a HOCl-dependent loss of antioxidant activity. Our results indicate that this oxygen-dependent toxicity was due, at least in part, to lethal DNA damage.

MATERIALS AND METHODS

Bacterial strains. All strains are *E. coli* K-12 derivatives. Mutations in various genes were introduced by P1 transduction in the same parental strain, MG1655 (16), using P1 vir and selecting for an antibiotic resistance associated with the mutation: resistance to chloramphenicol for Φ (*sodA'-'lacZ*)49 (17), resistance to kanamycin for Φ (*sodB-kan*) Δ 2 (17), and resistance to tetracycline for *soxS3::Tn10* (18). The Δ (*edd-zwf*)₂₂ (19) strain was generated by cotransduction of the *zwf* deletion with *zeb-1::Tn10*; selection was for tetracycline resistance and cotransduction of the *zwf* deletion was screened by glucose-6-phosphate dehydrogenase enzymatic measurement. The Δ *recA306* (20) strain was generated by cotransduction of the Δ *recA* allele with *srl::Tn10*; selection was for tetracycline resistance and cotransduction of the *recA* allele was screened by UV sensitivity. Standard procedures were used for genetic manipulations (21).

Reagents and hypochlorous acid assay. All chemicals used were of analytical grade. *N,N*-diethyl-*p*-phenylenediamine, NaClO, sodium thiosulfate, peroxidase, oxidized glutathione (GSSG), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), riboflavin, and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co. NADP and NADPH were purchased from Boehringer-Mannheim GmbH. Fresh solutions were prepared daily in distilled water and used immediately. NaClO solution in distilled water was stable for several hours. The concentration of hypochlorous acid was determined both iodometrically and colorimetrically (22); we use the term hypochlorous acid (HOCl) throughout the paper to include both undissociated acid and hypochlorite ion.

Growth and hypochlorous acid challenge conditions. Growth conditions and challenge were as previously described with small changes (5). Briefly cells were grown in LB (21) at 25°C in a rotary shaking waterbath at 200 rpm. Erlenmeyers flasks for HOCl treatment were washed with sulfochromic acid. At an optical density of 0.3 at 600 nm (10^8 bacteria/ml) the cells were sedimented by centrifugation at 3000g for 10 min at 4°C, washed twice with cold 0.05 M phosphate buffer (pH 7), and resuspended in the same volume of phosphate buffer. The number of colony-forming units (CFU) of the culture was not reduced by this washing procedure. Samples were distributed in 25-ml Erlenmeyer flasks (2.5 ml each) and fresh hypochlorous acid was added at various concentrations from 0 to 76 μ M (≤ 100 μ l). After 20 min incubation at 25°C in the dark with gentle shaking, hypochlorous acid was quenched by the addition of

sterile sodium thiosulfate to 5×10^{-4} M. Culturable bacteria were assayed by plating on LB plates containing 0.4% glucose after serial dilutions in phosphate buffer. Colonies were counted after 48 h incubation at 37°C in the air or in the Forma Scientific anaerobic chamber (Model 1024). Anaerobic cultures and media were supplemented with 0.4% glucose. All media and materials were equilibrated in the anaerobic chamber for at least 1 day before use.

Antioxidant measurements. Crude extracts were prepared by ultrasonic treatment and used for enzymatic assays. Bicinchoninic acid reagent (Pierce Chemical Co.) was used to measure protein concentration (23), with bovine serum albumine as standard. SOD activities were revealed by staining polyacrylamide gels as previously described by Beauchamp and Fridovich (24) and quantified using the Image Quant software. Catalase activities were revealed on polyacrylamide gels as previously described by Gregory and Fridovich and quantified as above (25). G6PD was measured in 50 mM Tris buffer (pH 7.6), 10 mM MgCl₂, 0.6 mM glucose 6-phosphate, and 0.2 mM NADP by following the increase in absorbance at 340 nm (26). Glutathione reductase was measured in 0.1 M phosphate buffer (pH 7), 2.5 mM GSSG, and 0.2 mM NADPH by following the decrease in absorbance at 340 nm (27).

Bacterial GSH concentration was determined colorimetrically on the basis of the absorbance of the reaction product of GSH and DTNB as described (28) (molar absorptivity coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ at 412 nm).

RESULTS

Lethal Effects of HOCl Are Enhanced by Oxygen

To investigate whether oxidative stress contributed to the toxicity of HOCl in *E. coli*, we tested whether plating under anaerobic conditions bacteria grown and challenged aerobically could increase the surviving fraction. Anaerobic plating increased survival, the effect becoming quite pronounced above the 19 μ M HOCl concentration (a 15-fold increase in survival at 38 μ M) (Fig. 1A). To determine whether oxygen was necessary during treatment to observe oxygen-dependent killing, cells were challenged in anaerobiosis. As for challenge in aerobiosis the plating efficiency was lower in aerobiosis than anaerobiosis, while oxygen-independent lethal effects (recorded by anaerobic survival) were similar regardless of the treatment condition (Fig. 1B). Since we showed previously that no residual HOCl is present in the cells after treatment (5), these results suggested that oxygen-dependent damage occurred during recovery from HOCl treatment.

To rule out a possible difference in plating efficiency due to a differential sensitivity to HOCl of factors involved in anaerobic and aerobic general metabolism, we exposed cells anaerobically challenged by HOCl to air for short periods before being plated in the absence of oxygen. An exposure to air as short as 10 min significantly reduced survival during subsequent plating in anaerobiosis (Table I). Thus, this indicated that oxygen-dependent damage is rapidly and irreversibly produced upon oxygen exposure during cell recovery. It rules out the possibility that increase in HOCl toxicity is due to the loss of some specific function necessary for general aerobic metabolism.

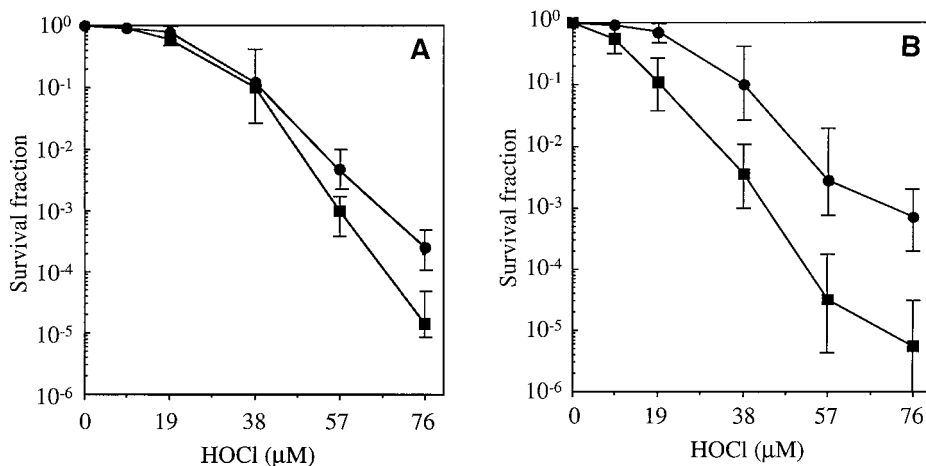


FIG. 1. Effect of anaerobiosis on survival after HOCl challenge. Cultures of wild-type bacteria were grown and challenged with various HOCl concentrations in aerobiosis (A) or anaerobiosis (B) and plated anaerobically (●) and aerobically (■).

Role of Recombinational DNA Repair in Protection from Oxygen-Dependent HOCl Lethal Effects

We previously reported that *recA* and *recB* mutants, deficient in DNA recombinational repair were slightly more sensitive than the wild type to HOCl treatment, indicative of DNA attack (5). To test whether the DNA damage was mediated by oxidative species generated by HOCl treatment, the sensitivity of a *recA* mutant to HOCl was assessed in anaerobiosis (Fig. 2). The increased sensitivity of the *recA* strain to HOCl observed in air was suppressed in anaerobiosis. This indicates that DNA strand breaks which could be repaired by recombination can be generated after HOCl treatment via reactive oxygen species.

Oxidative Stress after HOCl Treatment

Impairment of antioxidative defence after HOCl treatment. A possible explanation of the exacerbation of sensitivity to HOCl in the presence of oxygen could

TABLE I

Effect of a Transient Aerobic Exposure on Anaerobic Survival after Growth and HOCl Challenge in Anaerobiosis

Duration of aerobic exposure	Survival fraction (%)
0 min	50
10 min	6
60 min	5
48 h ^a	0.1

Note. Wild-type anaerobically grown bacteria were arrested in exponential phase (10^8 CFU/ml) and challenged under anaerobic conditions with 38 μ M HOCl. After plating, plates were transferred for various times to an aerobic atmosphere at 37°C and then returned to the anaerobic chamber and incubated for 48 h.

^aPlates were not returned to anaerobic chamber and counted directly after 48 h in aerobiosis.

be an impairment of enzyme activities or compounds involved in protection against ROS. We therefore assayed SOD, catalase, glutathione reductase and G6PD activities, and the GSH concentration immediately after an aerobic HOCl challenge (Fig. 3). One group, including G6PD, GSH, and the catalase activity of hydroperoxidase I (catalase–hydrogen peroxidase), showed high sensitivity to low concentrations of HOCl (Fig. 3A). A second group, including SOD, glutathione reductase, and hydroperoxidase II (catalase), was sensitive only at higher HOCl concentrations (57 μ M) (Fig. 3B).

Effect of mutations in protective systems against oxidative stress on survival after HOCl treatment. The above results indicate that antioxidative defence are impaired at various degrees after HOCl treatment. To further investigate whether those defects result in HOCl toxicity, the effect of HOCl on strains mutated in protective systems was assayed. We previously reported that deficiencies in protections against hydrogen peroxide did not sensitize to HOCl treatment. We also reported that the protective global defence against superoxide, implying genes of the *soxRS* regulon, was triggered by HOCl treatment. The SoxRS regulon is governed by SoxR and SoxS. SoxR is a sensor protein activated upon a signal of oxidative stress. It activates, in turn, the transcription of SoxS regulatory protein that itself activates transcription of numerous genes of functions involved in protection against superoxide. These include MnSOD, repair functions, and metabolic functions that will permit the bacteria to survive during the stress. We thus questioned whether mutants deficient in defence against superoxide, SOD-deficient mutants, and mutants unable to induce *soxRS* regulon were more sensitive in aerobiosis to HOCl. The *sodA sodB* mutant, deficient in cytoplasmic SODs, was not

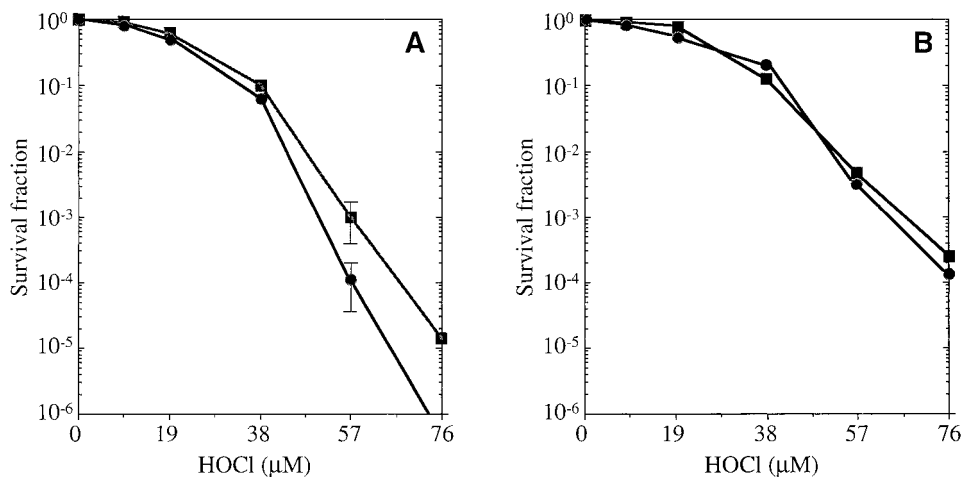


FIG. 2. Oxygen-dependent sensitivity of *recA* mutants to HOCl treatment. Cells were challenged in aerobiosis with HOCl and plated in aerobiosis (A) and anaerobiosis (B). ■, wild type; ●, *recA*.

significantly more sensitive than the wild type to HOCl exposure (not shown). The *soxS3* mutant was slightly more sensitive at concentrations of HOCl greater than 57 μ M (Fig. 4). The triple *sodA sodB soxS3* mutant

showed a significant increase in sensitivity, even at low concentrations, suggesting that protections against oxidative stress are involved in resistance to HOCl. Among *soxRS*-dependent functions, G6PD, which con-

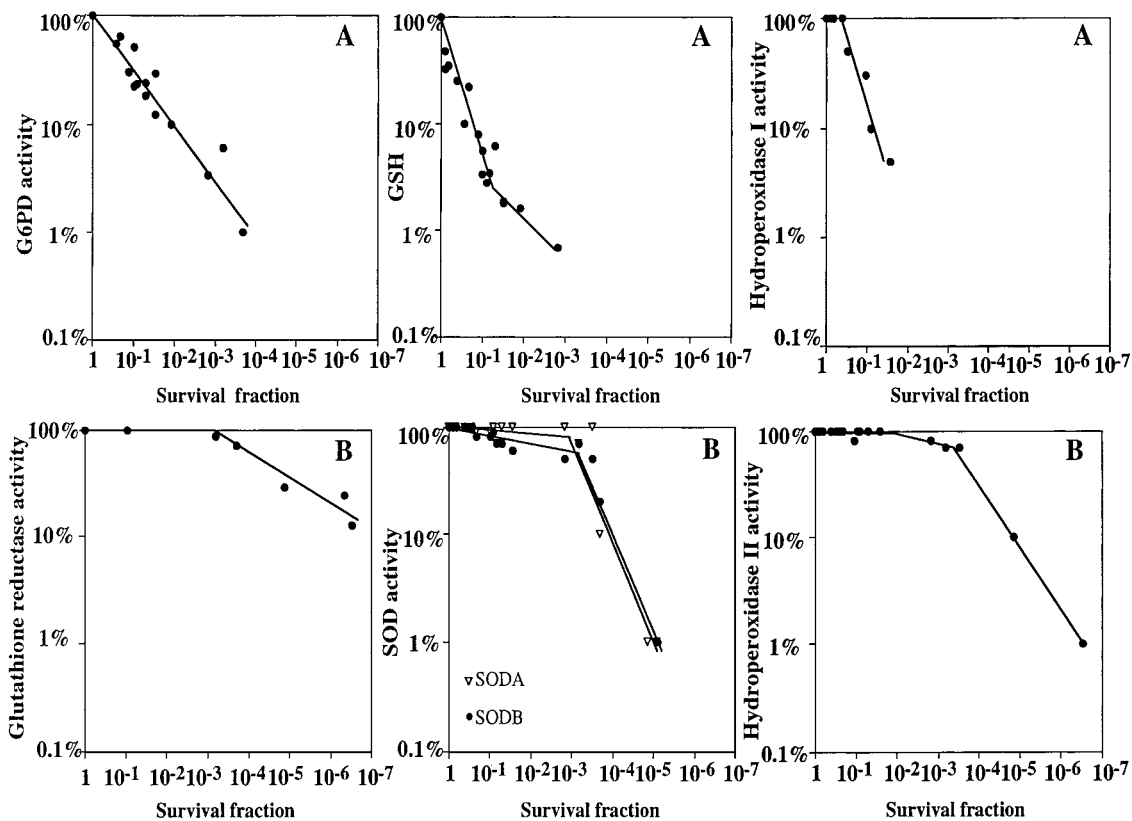


FIG. 3. Measurement of antioxidants after HOCl challenge. Wild-type cells were treated with various HOCl concentrations as described in aerobiosis. Cells were chilled and aliquots were removed for count of survival and GSH content determination. Crude extracts were prepared on the remaining fraction for measurements of enzymatic activities. Each point represents the mean of duplicate determinations; the variation between the two measurements was less than 20%.

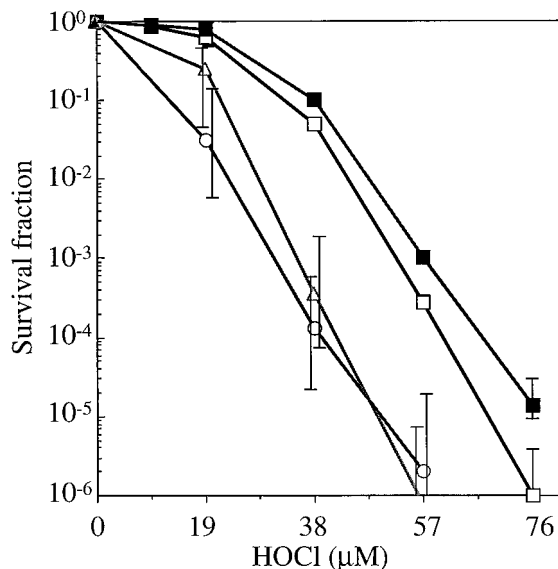


FIG. 4. Effect of mutations in protective systems against superoxide on HOCl lethality. Cultures stopped in exponential growth phase were challenged in aerobiosis with various concentrations of HOCl as described. Survival fractions are the mean values of four independent experiments. Error bars are indicated (average \pm SD). Wild-type, ■; *soxS3::Tn10*, □; $\Delta(edd-zwf)_{22} \Phi(sodA'-lacZ)_{49} \Phi(sodB-kan)\Delta 2$, △; *soxS3::Tn10* $\Phi(sodA'-lacZ)_{49} \Phi(sodB-kan)\Delta 2$, ○.

tributes in regenerating the NADPH pool, could be important for HOCl resistance. In fact, dehydrogenase activities have been reported to be sensitive to HOCl *in vivo* (29). We thus tested a *zwf* mutant, deficient in G6PD. While the single mutant showed no significant increase in sensitivity (not shown), the *zwf sodA sodB* mutant was as sensitive as *sodA sodB soxS3*, supporting the idea that the noninducibility of G6PD in the *sodA sodB soxS3* mutant associated to the increase level of superoxide is largely responsible for its HOCl sensitivity.

DISCUSSION

In this study we have shown that a part of the lethal damage produced by hypochlorous acid is mediated by ROS during cell recovery, even though hypochlorous acid is no longer present.

Impairment of several antioxidant defenses may be the cause of the oxygen-dependent killing following HOCl challenge. The effect of oxygen increases with HOCl concentration, becoming significant from about 40 μ M. At low HOCl concentrations (leading to more than 10^{-1} survival), HOCl-mediated oxidative killing of wild-type cells is minor, although there is a drastic drop in G6PD and hydroperoxidase I catalase activities and the GSH content. This suggests that other components of defence systems, such as SOD, are sufficient to protect cells. As the HOCl concentration increases, en-

zymes such as SOD and hydroperoxydase II are inactivated. Thus, the reduction in available GSH along with the drop of catalase and SOD activities and the loss of general defence against oxidative stress should cause an accumulation of superoxide and hydrogen peroxide. This, together with the increase in free iron level observed after a HOCl challenge (10), should favor the Fenton reaction, leading to the formation of highly deleterious hydroxyl radicals.

This view is supported by our mutant analysis, although it should be kept in mind that mutations lead to permanent inactivation, while HOCl-inactivated proteins can be resynthesized in surviving cells since HOCl was rapidly eliminated. The mutant deficient in SOD (*sodA sodB*), despite its high steady-state level of superoxide, is not more sensitive to HOCl than wild type, but is drastically sensitized, even at low HOCl concentrations, when the *soxRS* protective response is mutated (in the *sodA sodB soxS3* mutant). Notably, although it has been shown that the *soxRS* regulon is induced at low HOCl concentrations (15), the *soxS3* mutation alone did not increase HOCl sensitivity, indicating that the basal level of proteins under SoxRS regulation is sufficient to protect against HOCl-mediated oxidative damage, at least at low HOCl concentration. Among *soxRS*-dependent protective functions, G6PD appears to play a major role, as shown by the hypersensitivity of the *zwf sodA sodB* mutant. The synergy between the *zwf* and the *sodA sodB* mutations is interesting and may be coupled to NADPH regeneration. NAD(P)H might contribute indirectly to maintaining superoxide and hydrogen peroxide at low levels. Indeed, GSH and surface-exposed methionine residues can scavenge ROS via catalytic cycles driven by an NAD(P)H oxidation reactions (4, 30). G6PD, which is in a large part responsible for the regeneration of NADPH pool, might thereby contribute to the maintenance of a low ROS level. Thus, in addition to the SOD deficiency resulting in a high increase of superoxide steady state, the pool of reduced GSH which drops in the presence of HOCl and the methionine residues which might be oxidized (31), should be poorly reconstituted in *zwf* mutants, resulting in negligible scavenging of ROS.

One consequence of ROS and free iron level increases is that at least part of the oxygen-dependent toxicity of HOCl challenge could be due to DNA damage via the formation of highly deleterious hydroxyl radicals. A prior study supported the generation upon HOCl exposure of hydroxyl radicals via a Fenton reaction, based in part on the increased sensitivity of mutants in DNA recombinational repair (5). This increased sensitivity is not observed in anaerobiosis, where *recA* behaves as wild type, supporting the idea of HOCl induced lethal DNA damage. However, it should be noted that while *recA* mutants are more sensitive to HOCl than wild-

type cells, it is not clear whether HOCl-inflicted oxidative DNA damage is involved in the killing of wild-type cells.

Involvement of ROS in the deleterious effects produced by various stresses may be a general phenomenon. Oxidative stress is implicated in bacterial killing during lethal heat shock. *E. coli* superoxide dismutase-deficient mutants are hypersensitive to heat killing (32), and catalase is partially inactivated by heat shock (33). Conversely, catalase enhances the ability of bacteria to form colonies following heat shock and other stresses such as freezing, desiccation, and dehydration (33–37). In eukaryotes oxidative stress plays a major role in the lethal effect of heat, as recently observed in yeast, in which the deleterious effects of heat stress is drastically reduced by anaerobiosis or by overexpression of antioxidants (38). Free radical mechanisms also appear to be implicated in the toxicity of ethanol (39, 40). Thus, other stresses, like HOCl, may leave cells in a vulnerable physiological state in which atmospheric oxygen, during the recovery period, increases the toxic effect of the primary stressor, which could maybe explain the viable but nonculturable phenomenon (41).

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REFERENCES

1. Foote, C. S., Goynes, T. E., and Lehrer, R. I. (1983) *Nature* **301**, 715–716.
2. Klebanoff, S. J. (1968) *J. Bacteriol.* **95**, 2131–2138.
3. Fridovich, I. (1978) *Science* **201**, 875–880.
4. Halliwell, B. (1974) *New Phytol.* **73**, 1075–1086.
5. Dukan, S., and Touati, D. (1996) *J. Bacteriol.* **178**, 6145–6150.
6. Calabrese, J. P., and Bissonnette, G. (1990) *Appl. Environ. Microbiol.* **56**, 3558–3564.
7. Aruoma, O. I., and Halliwell, B. (1987) *Biochem. J.* **248**, 973–976.
8. Mashino, T., and Fridovich, I. (1988) *Biochim. Biophys. Acta* **956**, 63–69.
9. Chesney, J. A., Eaton, J. W., and Mahoney, J. R. JR. (1996) *J. Bacteriol.* **178**, 2131–2135.
10. Rosen, H., and Klebanoff, S. J. (1982) *J. Biol. Chem.* **257**, 13731–13735.
11. Greenberg, J. T., and Demple, B. (1989) *J. Bacteriol.* **171**, 3933–3939.
12. Hidalgo, E., Ding, H., and Demple, B. (1997) *Trends Biochem. Sci.* **22**, 207–210.
13. Tsaneva, I. R., and Weiss, B. (1990) *J. Bacteriol.* **172**, 4197–4205.
14. Nunoshiba, T., DeRoja-Walker, T., Wishnok, S. J., and Tannenbaum, S. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9993–9997.
15. Dukan, S., Dadon, S., Smulki, D. R., and Belkin, S. (1996) *Appl. Environ. Microbiol.* **62**, 4003–4008.
16. Bachmann, B. J. (1987) in *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology (Neidhardt, F. C., Ed.), pp. 1190–1219, Am. Soc. Microbiol., Washington, DC.
17. Carlioz, A., and Touati, D. (1986) *EMBO J.* **5**, 623–630.
18. Wu, J., and Weiss, B. (1991) *J. Bacteriol.* **173**, 2864–2871.
19. Barcak, G. J., and Wolf, R. E., Jr. (1988) *J. Bacteriol.* **170**, 365–371.
20. Dutreix, M., Moreau, P. L., Bailone, A., Galibert, F., Battista, J. R., Walker, C. G., and Devoret, R. (1989) *J. Bacteriol.* **171**, 2415–2423.
21. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. AFNOR (Eds.) (1994) pp. 391–402, AFNOR NF T 90-037.
23. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
24. Beauchamp, B., and Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287.
25. Gregory, E. M., and Fridovich, I. (1974) *Anal. Biochem.* **58**, 57–62.
26. Fraenkel, D. G., and Levisohn, S. R. (1967) *J. Bacteriol.* **93**, 1571–1578.
27. Lopez-Barea, J., and Lee, C. Y. (1979) *Eur. J. Biochem.* **98**, 487–499.
28. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
29. Venkobachar, C., Iyengar, L., and Prabhakara Rao, A. V. S. (1977) *Water Res.* **11**, 727–729.
30. Levine, R. L., Mosoni, L., Berlett, B., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 15036–15040.
31. Reddy, V. Y., Desrochers, P. E., Pizzo, S. V., Sahakian, S. L., Levine, R. L., and Weiss, S. J. (1994) *J. Biol. Chem.* **269**, 4683–4691.
32. Benov, L., and Fridovich, I. (1995) *J. Bacteriol.* **177**, 3344–3346.
33. Mackey, B. M., and Seymour, D. A. (1987) *J. Gen. Microbiol.* **133**, 1601–1610.
34. Gram, L., Pederson, P., and Sogaard, H. (1984) *Int. J. Food Microbiol.* **1**, 155–162.
35. Marthi, B., Shaffer, B. T., Lighthart, B., and Ganio, L. (1991) *Appl. Environ. Microbiol.* **57**, 2775–2776.
36. Martin, S. E., Flowers, R. S., and Ordal, Z. J. (1976) *Appl. Environ. Microbiol.* **32**, 731–734.
37. McDonald, L. C., Hackney, C. R., and Ray, B. (1983) *Appl. Environ. Microbiol.* **45**, 360–365.
38. Davidson, J. F., Whyte, B., Bissinger, P. H., and Schiestl, R. H. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5116–5121.
39. Kurose, I., Higuchi, H., Kato, S., Miura, S., and Ishii, H. (1996) *Alcohol Clin. Exp. Res.* **20**, 77A–85A.
40. Nordmann, R., Ribiere, C., and Rouach, H. (1992) *Free Radic. Biol. Med.* **12**, 219–240.
41. Bloomfield, S. F., Stewart, S. A. B., Dodd, C. E. R., Booth, I. R., and Power, E. G. M. (1998) *Microbiology* **144**, 1–3.