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Hypochlorous Acid Activates the Heat Shock and *soxRS* Systems of *Escherichia coli*

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A series of plasmids, containing fusions of different stress promoters to *lux* reporter genes, was used in an attempt to monitor the defense circuits activated upon exposure of *Escherichia coli* to sublethal doses of free chlorine. A significant level of activation was exhibited by promoters of three heat shock genes (*grpE*, *dnaK*, and *lon*), in an *rpoH*-dependent manner. The promoter of *micF*, a gene under the control of the *soxRS* regulon, was also strongly induced, but not in a *soxR* mutant. This induction was not affected by *sodA* and *sodB* mutations, implying that it did not involve oxygen radical activity. Free-chlorine activation of both heat shock and *soxRS* regulons required an exposure of less then 1 s in duration. The *oxyR* or the SOS regulons were apparently not induced by free chlorine (as judged by lack of activation of *katG* and *recA*, respectively), and neither was the universal stress (*uspA*) protein.

Uncombined chlorine, in the form of un-ionized hypochlorous acid (HOCl), is an extremely potent bactericidal agent even at concentrations lower than 0.1 mg/liter (29). As a direct consequence, chlorination is the most widely used method for disinfection of water and wastewater. Nevertheless, the mechanism by which HOCl exerts its lethal effects on microorganisms has never been fully elucidated experimentally. Specific and general damages caused by exposure to free chlorine have been documented in many reports (5, 9, 10, 14, 15, 22–24, 27, 39, 40, 48, 49), but in most cases it is difficult to differentiate between primary and secondary effects. Some of the more reactive hypochlorous acid targets seem to be membrane and protein associated (19, 42). They appear to be mostly protein amino and sulfhydryl groups (42), nonheme Fe-S clusters, cytochromes, and conjugated polyenes (20).

Recently presented data (16) indicate that the bactericidal effect of free chlorine involves, at least in part, hydroxyl radicals generated by a Fenton-type reaction. It has also been shown that genes which are a part of *Escherichia coli*'s defenses against hydrogen peroxide are involved in free-chlorine resistance, indicating a possible overlap in the defense circuits. A different set of experiments suggested a specific, *oxyR*-independent adaptive response to HOCl (16).

In contrast to bacterial responses to free chlorine, the ability of bacteria to resist and to adapt to the presence of hydrogen peroxide has been extensively studied. In *E. coli*, a major regulatory circuit involved is the *oxyR* regulon (13, 41). One of the genes positively regulated by the OxyR protein is *katG*, coding for the hydroperoxidase I (HPI) catalase. Another catalase (HPII, encoded by *katE*) is regulated by *rpoS* (or *katF* [26]); the latter circuit apparently functions in *katG* regulation as well (21). It has also been shown that hydrogen peroxide activates many genes that are part of the heat shock (11, 33, 44) and SOS (47) responses.

In order to begin to decipher the mode of action of free chlorine, we have attempted to identify the genes induced following a low-dosage HOCl exposure. Several genes, belonging to different regulatory systems known to be involved in the defenses against oxidative stress, were selected for this purpose. To monitor their induction in real time, we have used a set of plasmids in which the promoters of the studied genes were fused to the bioluminescence operon derived from the marine bacterium *Vibrio fischeri* (30). The parental plasmid used, pUCD615 (37), contained *luxCDABE* genes downstream from a multiple cloning site, into which promoters of the selected genes were inserted. Members of this set of plasmids have been previously described (6–8, 43–46), and the advantages of their uses as general and specific reporters for bacterial stress have been documented elsewhere.

The *E. coli* genes thus tested were the heat shock genes *dnaK*, *grpE*, and *lon*, *micF* (controlled by the redox regulon *soxRS*), *katG* (under the control of *oxyR*), *recA* (SOS), and the universal stress protein (encoded by *uspA*). The results indicated that all three heat shock genes as well as *soxRS*, but not *oxyR*, *recA*, or *uspA*, were induced.

MATERIALS AND METHODS

Plasmids and bacterial strains. All strains were *E. coli* K-12 derivatives and are listed in Table 1, as are the different plasmids used. Construction of the plasmids was reported elsewhere (see references in Table 1) except for pMic FLux1 (5a) and pRecALux3 (46a), descriptions of which are in preparation. In all plasmids used, the presence of the correct promoters was verified by (i) complete sequencing of the promoter region and (ii) induction by specific inducing agents (6–8, 43–46). Standard procedures were used for genetic manipulations (38).

Growth and hypochlorous acid challenge conditions. Cells were grown in LB medium (32) containing 50 or 100 μg of kanamycin monosulfate or ampicillin per ml, respectively, at 26°C in a rotary shaker (200 rpm). Following overnight growth, cells were diluted to 10^7/ml in the same medium lacking antibiotics and allowed to grow for a few generations under the same conditions. At an early exponential phase (25 Klett units, as measured with a Klett-Summerson colorimeter), the cells were spun down at 10,000 rpm (Eppendorf 5415C centrifuge) for 2 min, washed twice with 0.05 M phosphate buffer (pH 7.0), and resuspended in the same buffer at a cell density of 10^8/ml. Samples (1 ml) were distributed into glass test tubes, and a freshly prepared hypochlorous acid stock solution (<30 μl) was added to the desired final concentration. After 20 min of incubation at 26°C in the dark with gentle shaking, free chlorine was quenched by the addition of sterile sodium thiosulfate to 0.5 mM. Culturable bacteria were assayed by plating on LB plates after serial dilutions in phosphate buffer. Free chlorine (hypochlorous acid and hypochlorite ion) and total chlorine concentrations were determined (3) by the *N*,*N*-diethyl-*p*-phenylenediamine colorimetric method and by

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TABLE 1. *E. coli* plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pUCD615	Amp^r, Kan^r, MCS[a] upstream of *luxCDABE*	37
pKatGLux2	Same as pUCD615, but *katG′*::*luxCDABE*	6
pMicFLux1	Same as pUCD615, but *micF′*::*luxCDABE*	5a
pRecALux3	Same as pUCD615, but *recA′*::*luxCDABE*	46a
pUspALux2	Same as pUCD615, but *uspA′*::*luxCDABE*	46
pGrpELux5	Same as pUCD615, but *grpE′*::*luxCDABE*	44
pRY002	Same as pUCD615, but *dnaK′*::*luxCDABE*	44
pLonLux2	Same as pUCD615, but *lon′*::*luxCDABE*	45
Parental strains		
RFM443	*galK2 lac74 rpsL200*	31
GC4668	F$^-$ Δ*lac4169 rpsL*	18
JHC1092	Same as GC4668, but Δ(*soxR-zjc-2205*) *zjc-2204*::Tn*10* Km	18
MC4100	F$^-$ *araD139* Δ(*laclPOZA*)*U169 rpsL thi*	25
CAG9333 (R40)	Same as 4100, but Δ*rpoH*::*Kan groE* constitutive	25
MG1655 (QC1301)	F$^-$	4
QC2472	ϕ(*sodA′-′lacZ*)*49* (*sodB-kan*)Δ*2*	16
Other strains		
TV1061	pGrpELux.5/RFM443	44
DPD1590	pGrpELux.5/MC4100	This study
DPD1581	pGrpELux.5/CAG9333	This study
DPD1592	pRY002/MC4100	This study
DPD1583	pRY002/CAG9333	This study
DPD2519	pMicFLux1/RFM443	5a
DPD1571	pMicFLux1/GC4668	This study
DPD1565	pMicFLux1/JHC1092	This study
DPD1587	pMicFLux1/MC4100	This study
DPD1578	pMicFLux1/CAG9333	This study
S9	pMicFLux1/MG1655	This study
S8	pMicFLux1/QC2472	This study
DE135	pUspALux2/RFM443	46
DPD2794	pRecALux3/RFM443	46a
DPD1006	plonLux2/RFM443	45

[a] MCS, multiple cloning site.

an iodometric method, respectively. All glassware used for HOCl treatment was washed with sulfochromic acid.

Measurement of bioluminescence. Bioluminescence at 26°C was monitored with a microtiter plate luminometer (Lucy1; Anthos Labtec Instruments, Salzburg, Austria), following the addition of 75 μl of cells (untreated or treated as described above) to 25 μl of a fourfold-concentrated LB solution containing different additions, where required. All experiments were conducted in duplicate in opaque white microtiter plates (Dynatech, Denkendorf, Germany). Results are presented either as the average numbers of relative light units measured by the instrument or as the response ratios (luminescence of the treated cells/that of the untreated controls). To avoid possible side effects of either known or cryptic mutations, the experiments were done in several different genetic backgrounds.

Reagents. All chemicals used were of analytical grade. *N,N*-Diethyl-*p*-phenylenediamine and methyl viologen were purchased from Sigma Chemical Co.; sodium hypochlorite (NaClO) was purchased from Aldrich Chemical Company, Milwaukee, Wis.

RESULTS

Definition of assay conditions: chlorine concentrations and viability. The experimental approach outlined above required assay conditions under which the damage exerted by free chlorine would be sublethal. Conditions which would expose the cells to HOCl concentrations sufficiently high to induce cellular defense circuits but not high enough to cause massive cell mortality were therefore sought. With colony-forming ability as the viability parameter, Fig. 1 presents the effect of different HOCl concentrations under the conditions of the finalized procedure (10^8 cells per ml, 20-min exposure, 50 mM phosphate buffer [pH 7.0], and 26°C). Concentrations as high as 1 mg/liter caused a relatively insignificant drop in viability, while higher levels had a pronounced lethal effect. Hence, in the experiments the results of which are described below the cells were exposed to free chlorine at a concentration of 1 mg/liter or less. Curves similar to the one displayed in Fig. 1 were obtained for all *E. coli* strains employed in this study. Interest-

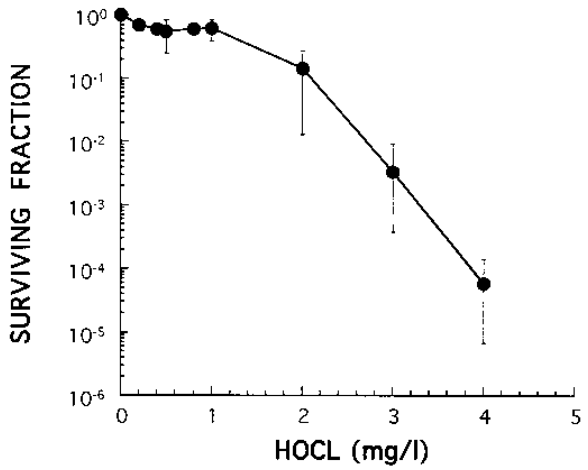

FIG. 1. Effect of free chlorine on viability. *E. coli* (strain MG1655) cells (10^8/ml, in 0.05 M phosphate buffer) were exposed for 20 min to different HOCl concentrations as described under Materials and Methods. CFU on LB were counted after 24 h. Standard errors (bars) represent data from at least five independent experiments.

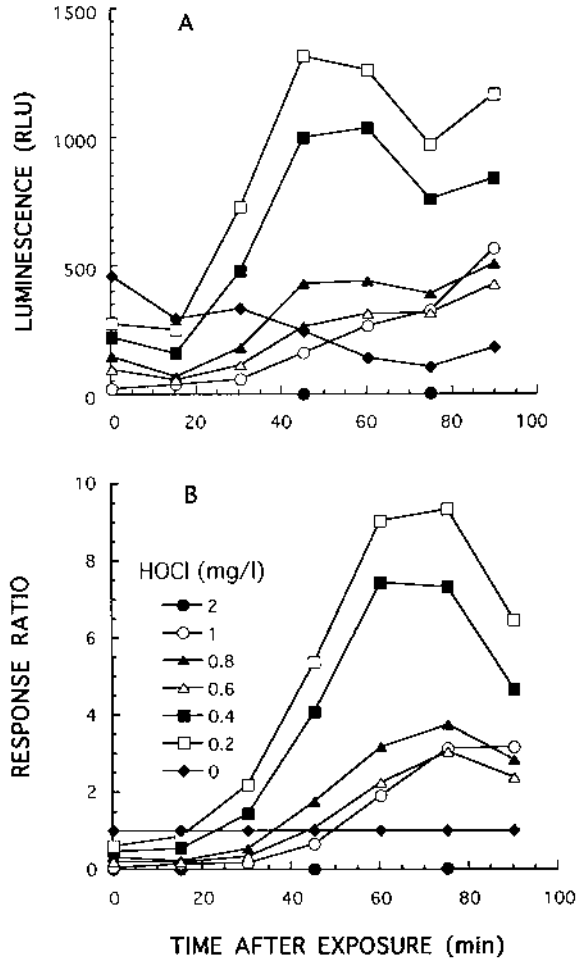

FIG. 2. Heat shock induction. Strain TV1061 (*grpE′::lux*) cells (10^8/ml) were treated (20 min) with HOCl at the designated concentrations (symbols defined in panel B). LB was then added, and luminescence was monitored for 90 min. (A) Kinetics of light emission (in relative light units [RLU]); (B) ratio to value for untreated control.

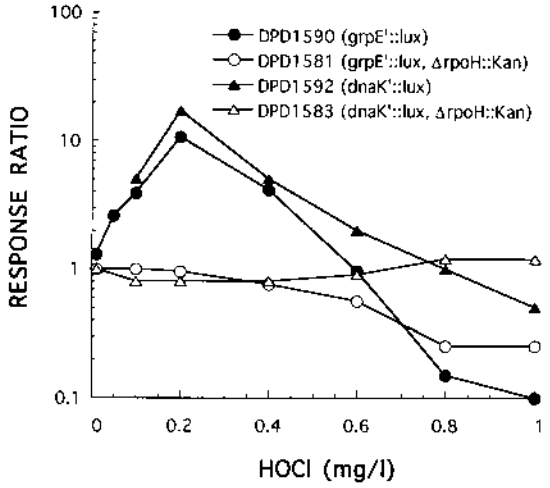

FIG. 3. HOCl induction of *grpE* and *dnaK* is *rpoH* dependent. The conditions for this experiment were the same as those described in the legend to Fig. 2, with a pair of isogenic hosts, either an *rpoH* mutant (open symbols) or the wild-type control (MC4100) (solid symbols). Maximal response ratios obtained for each HOCl concentration are presented.

ingly, higher chlorine concentrations, while drastically affecting viability as well as light emission, caused in some of the strains a delayed peak in luminescence which appeared a few hours after exposure (not shown). The causes for this late response will be discussed elsewhere (15a).

Heat shock induction. *E. coli* TV1061 contains a plasmid in which the promoter of the heat shock gene *grpE* is fused to the structural part of the *V. fischeri lux* operon (44). Following chlorine exposure in phosphate buffer as outlined in Materials and Methods, no increase in luminescence above the uninduced levels was observed (data not shown). After the addition of LB, however, a very clear induction took place, as depicted in Fig. 2. Figure 2A presents actual kinetics of light development, while Fig. 2B presents the response ratios over the uninduced control. The response was dose dependent, with maximal induction occurring at 0.2 mg/liter. Activity reached a maximum after 40 to 60 min and then declined.

The same induction pattern was displayed following the exposure to HOCl of *E. coli* cells in which *lux* genes were fused to two other heat shock promoters, *lon* and *dnaK*. Figure 3 presents the results for *grpE* and *dnaK* in a pair of isogenic hosts, one of them defective in *rpoH*, the gene coding for the heat shock regulator σ^{32}. The data are presented in this case as the maximal response ratios for each strain as a function of HOCl concentration. In addition to displaying the identity in the response pattern of the two promoters, the data in Fig. 3 clearly demonstrate the dependency of the reaction to free chlorine on *rpoH*. The responses of the *lon′::lux* fusion were similar (not shown).

***soxR* activation after HOCl challenge.** As demonstrated in Figure 4, another gene clearly induced by HOCl exposure is *micF*. The kinetics of induction were different from those of the heat shock genes, and the rate of light production was maximal about 120 min after the addition of the hypochlorous acid (Fig. 4A). The induction was maximal at 1 mg of hypochlorous acid per liter, a concentration which resulted in an approximately 20-fold induction. As is clear from Fig. 4B, the response was *soxR* dependent: no induction was observed in a *soxR* mutant. The same *soxR* mutation did not affect the activation of other *lux* fusions, such as *katG′::lux* induced with H_2O_2 (8).

The standard experimental procedure used throughout the study described here for exposing the cells to HOCl involved a 20-min exposure. Figure 5 demonstrates that the HOCl exposure needed to subsequently induce *micF* was actually shorter then 1 s. Similar data for another *lux*-fused promoter tested in this manner, that of the heat shock protease *lon*, were also obtained. In fact, it can be observed that luminescence values displayed after exposures longer then 1 s were lower, suggesting that such treatments could be suboptimal for gene induction.

The HOCl effect is not superoxide mediated. In contrast to genes controlled by the *rpoH* and the *soxRS* regulons, genes controlled by other regulatory circuits were not affected by HOCl treatment. These were the universal stress protein *uspA* (36, 46), *recA* (47), and *katG* (13, 41). Data for the first two are not shown. The lack of response of the third, depicted in Fig. 6, is particularly significant, since it is a catalase gene under the positive control of *oxyR*. *E. coli* cells harboring plasmids containing the *katG′::lux* fusion were shown to be sensitively induced by either hydrogen peroxide (6) (Fig. 6) or the redox cycling agent methyl viologen (6). The latter response was explained by superoxide dismutase-mediated generation of

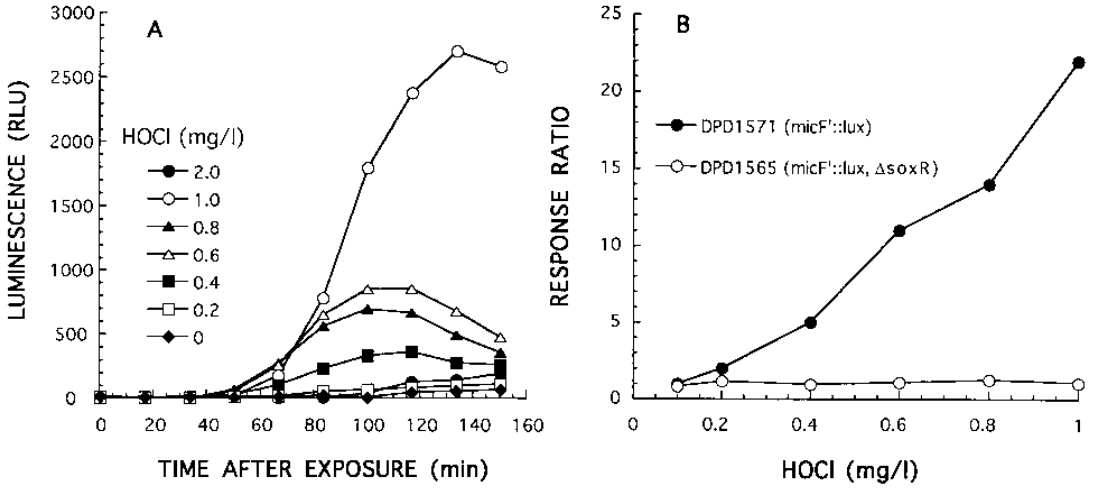

FIG. 4. *soxRS* induction. (A) Kinetics of light development (in relative light units [RLU]) in strain S9 (*micF'*::*lux*) after HOCl treatment as described in the legend to Fig. 2; (B) maximal response ratios of the *micF'*::*lux* fusion in the wild type (●) or the *soxR* mutant (○) in the same background.

peroxides from the superoxide created by the presence of methyl viologen.

The lack of *oxyR* induction by hypochlorous acid (Fig. 6), therefore, indicates that the inductive effects of this compound are not due to the presence of hydrogen peroxide, suggesting that superoxide radicals were not involved. To confirm that there is no increase of superoxide oxygen after hypochlorous acid stress, we tested the effects of superoxide dismutase mutations on *micF'*::*lux* induction. From Fig. 7A it is clear that the double mutation in the genes for two forms of this enzyme, *sodA* and *sodB*, had no effect on HOCl induction of *micF*. Figure 7B, showing the results of a control, demonstrates the very dramatic effect these mutations had on the induction of the same promoter by methyl viologen: maximal responses were shifted to a concentration 1,000-fold lower. These results confirm that *micF* induction by HOCl is not superoxide mediated and point to the possibility of either a direct activation of *soxR* by HOCl or its derivative or an indirect activation mediated by an affected cellular component.

DISCUSSION

Bacteria have developed several complex mechanisms, with a considerable degree of overlap, to allow them to cope with potential oxidative hazards (13). Two *E. coli* global regulatory circuits which appear to be dedicated to the fight against deleterious oxygen species are *oxyR* and *soxRS*, which are responsible for the H_2O_2 and superoxide responses, respectively. Other global regulators involved are *rpoH* (34), *rpoS* (26), *soxQ* (18), *fur*, *arcA*, *fnr* (12), and possibly other circuits.

Most reports on bacterial antioxidative metabolism concentrate on two major oxidants: superoxides and peroxides. The defenses against hypochlorous acid have been investigated relatively little, in spite of hypochlorous acid's very strong oxidative capacity, its widely utilized bactericidal potential, and its

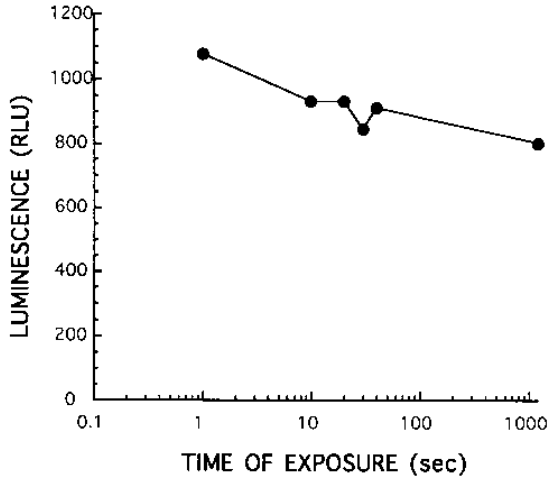

FIG. 5. Activation of *micF* requires less then 1 s. Cells of *E. coli* S9 containing *micF'*::*lux* were HOCl-treated for different periods (1 to 1,000 s), after which thiosulfate and LB were added and luminescence (in relative light units [RLU]) was monitored for 180 min. The highest luminescence values obtained during this period are presented.

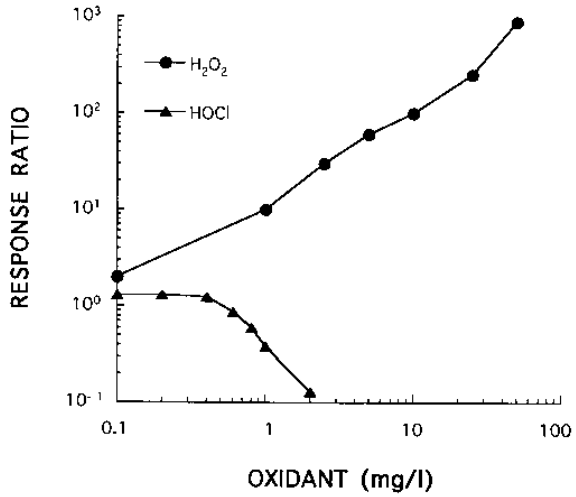

FIG. 6. HOCl does not induce *oxyR*. Strain DPD2511 (*katG'*::*lux*) was treated with H_2O_2 or HOCl. The data represent the maximal response ratios obtained in the course of the experiment (90 min).

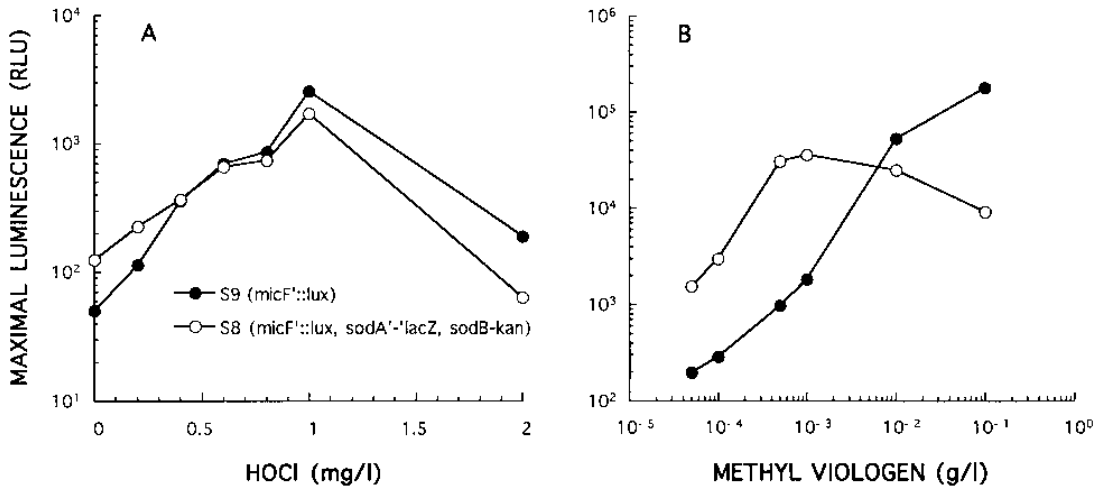

FIG. 7. Lack of superoxide dismutase does not affect *micF* induction by HOCl. Cells of strains S9 (wild type [MG1655]) and S8 (*sodA sodB* mutant) containing *micF'::lux* were induced by treatment with either HOCl (A) or methyl viologen (B). RLU, relative light units.

apparent existence in neutrophils and macrophages (17). A recent study (16) has indicated similarities between and overlaps in the defense systems against hypochlorous acid and hydrogen peroxide and suggested that both compounds generate common active oxidative species. It has also been shown that the induction of *oxyR* provided protection from HOCl exposure but that HOCl pretreatment conferred H_2O_2 resistance in an *oxyR*-independent manner.

In this study we have confirmed that *oxyR* is not involved in the adaptive response of *E. coli* to free chlorine (16) by demonstrating that exposure to sublethal doses of HOCl did not induce *oxyR*, at least as judged by monitoring the *oxyR*-controlled *katG* (Fig. 6). Two other genes which appeared not to be induced were *uspA* and *recA*. This was monitored by using plasmids carrying either the *katG*, *uspA*, or *recA* promoter fused to *V. fischeri lux* genes, which enabled the use of light emission as a convenient real-time indicator of gene induction.

Using similar plasmids in which *lux* served as a reporter for several other promoters, we have shown for the first time the induction by free chlorine of two other regulatory circuits, as summarized below.

(i) Induction by the *rpoH* (σ^{32})-controlled heat shock system. Three individual promoters, *grpE*, *dnaK*, and *lon*, were induced by HOCl, with a maximal response at an HOCl concentration of 0.2 mg/liter. The response was not observed in *rpoH* mutants (Fig. 2 and 3). It has been shown (42) that hypochlorous acid reacted rapidly with proteins to generate abnormal proteins and polypeptides. It is therefore not surprising that the heat shock circuit, whose primary function is to conserve protein conformation, was induced in response to HOCl. Nevertheless, the heat shock circuit responds in a very sensitive manner to highly varied environmental injuries, including oxidative ones (34, 44), the effects of which do not necessarily involve modifications in protein structure. The heat shock response to HOCl could therefore be a part of a more global pattern.

(ii) Induction by the *soxRS* regulon. This was evidenced by the *soxR*-dependent induction of *micF*, with maximal activity at 1 mg of HOCl per ml (Fig. 4 and 5). Activation by the *soxRS* regulon appeared not to involve superoxide radicals, since it was not affected by double *sod* mutations (Fig. 7). It is therefore possible that the activation of *soxRS* occurs by direct interaction with either hypochlorous acid or a free-chlorine derivative or occurs indirectly via a chlorine-induced change in another cellular component. The *soxRS* regulon was previously shown to be activated by one-electron reactions of either superoxide (13) or nitric oxide (35). A direct activation by hypochlorous acid, a two-electron redox agent, is therefore questionable. A more likely hypothesis is that exposure to free chlorine limits the availability of compounds, such as NADPH (28) or reduced flavodoxins or ferrodoxins, that are necessary to maintain SoxR in its reduced form. At least in vitro, plant and bacterial ferrodoxins were shown to be very sensitive to free chlorine (2).

The deleterious effects of hypochlorous acid were previously shown to occur very rapidly. It has been demonstrated, for instance, that less then 0.1 s was required for a bactericidal effect (1). In the study reported here, with sublethal HOCl levels, the activation of the defense circuits studied occurred in the same time frame; neutralization of the free chlorine after a 1-s exposure was already too late to prevent full induction.

The results presented in this work shed some new light on the little-understood effects of free chlorine on bacterial metabolism; much more work is needed in order to understand its actual interactions with *rpoH*, *soxRS*, or any other circuit it could activate. We believe that as demonstrated here, plasmids containing *lux* fusions to promoters of interest are very powerful tools for such studies.

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