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CorA Affects Tolerance of *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium to the Lactoperoxidase Enzyme System but Not to Other Forms of Oxidative Stress

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The enzyme lactoperoxidase is part of the innate immune system in vertebrates and owes its antimicrobial activity to the formation of oxidative reaction products from various substrates. In a previous study, we have reported that, with thiocyanate as a substrate, the lactoperoxidase system elicits a distinct stress response in *Escherichia coli* MG1655. This response is different from but partly overlapping with the stress responses to hydrogen peroxide and to superoxide. In the current work, we constructed knockouts in 10 lactoperoxidase system-inducible genes to investigate their role in the tolerance of *E. coli* MG1655 to this antimicrobial system. Five mutations resulted in a slightly increased sensitivity, but one mutation (*corA*) caused hypersensitivity to the lactoperoxidase system. This hypersensitive phenotype was specific to the lactoperoxidase system, since neither the sensitivity to hydrogen peroxide nor to the superoxide generator plumbagin was affected in the *corA* mutant. *Salmonella enterica* serovar Typhimurium *corA* had a similar phenotype. Although *corA* encodes an Mg^{2+} transporter and at least three other inducible open reading frames belonged to the Mg^{2+} regulon, repression of the Mg stimulon by Mg^{2+} did not change the lactoperoxidase sensitivity of either the wild-type or *corA* mutant. Prior exposure to 0.3 mM Ni^{2+}, which is also transported by CorA, strongly sensitized MG1655 but not the *corA* mutant to the lactoperoxidase system. Furthermore, this Ni^{2+}-dependent sensitization was suppressed by the CorA-specific inhibitor Co(III) hexaammine. These results indicate that CorA affects the lactoperoxidase sensitivity of *E. coli* by modulating the cytoplasmic concentrations of transition metals that enhance the toxicity of the lactoperoxidase system.

The potential of natural antimicrobial systems to be used as biopreservatives in foods, cosmetics, pharmaceuticals, and other industrial products has attracted much attention in recent years. Although they are generally not very powerful when used alone, their antimicrobial efficacy can sometimes be strongly enhanced when they are combined with other treatments, an approach called hurdle technology (28). For example, we have demonstrated earlier that sublethal treatment with high hydrostatic pressure strongly sensitizes a wide range of vegetative food spoilage and pathogenic bacteria to the lactoperoxidase-thiocyanate enzyme system (11, 41). Lactoperoxidase (LP) is a heme-containing enzyme that catalyzes the oxidation by hydrogen peroxide of a wide range of substrates. LP is part of the innate immune system in vertebrates and is found in secretions such as milk, saliva, tears, and airway mucus (16, 26, 44). In view of its occurrence and function in the human body, together with its natural presence in numerous foods, there is little or no toxicological concern for the use of LP in foods, provided that its uptake remains within the range that occurs from normal food exposure (6).

The antimicrobial activity of the LP system is nonspecific and stems from the oxidative power of the enzymatic reaction products formed. The major physiological substrate of LP is thiocyanate (SCN^-), which is oxidized to hypothiocyanite ($OSCN^-$). LP can also oxidize bromide and iodide anions to the corresponding hypohalogenous acids but, in contrast to myeloperoxidase and chloroperoxidase, cannot oxidize chloride ions (1, 10, 25). The LP system is believed to induce oxidation of cellular sulfhydryl (—SH) groups into disulfides (—S—S—). When the homeostatic capacity of the cell is exceeded, this may result in structural and functional damage, reflected by loss of pH gradient, K^+ leakage, inhibition of respiration, and inhibition of protein and nucleic acid synthesis (2, 8, 24, 27, 30). As opposed to oxidants like H_2O_2 and superoxide (O_2^-), $OSCN^-$ causes no DNA damage and is considered nontoxic for the host cells producing it (43). In fact, the LP system is thought to protect host cells by consuming the potentially harmful H_2O_2 produced during oxidative burst (19).

Bacteria, like other living organisms, have developed defense systems against oxidative stress. In *Escherichia coli* and related bacteria, OxyRS and SoxRS are the key regulators of the transcriptional oxidative stress response to (hydrogen) peroxide and superoxide, respectively (37). We recently characterized the stress response in *E. coli* after challenge with the LP/SCN^- system. Thirteen open reading frames (ORFs) were identified that were induced by the enzyme system but not by H_2O_2 or the superoxide generator plumbagin. In addition, some genes that are inducible by H_2O_2 (*recA*) or by O_2^- (*sodA*) were also induced by the LP/SCN^- system. We concluded that the LP/SCN^- system elicits a specific and unique stress response different from but partly overlapping with other oxidative stress responses (29). Similarly, Hansen et al. (13)

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
MG1655	Wild-type strain; *rph-1*, F^-, λ^-	12
QC2411	MG1655 Δ*recA306 srl*::Tn*10*; Tet^r	7
QC909	MG1655 *sodA*::*cat* and *sodB*::*kan*	3
BW26742	Δ*pstS625*::*kan*	*E. coli* Genetic Stock Center
FB20382	MG1655 *rstA*::*kan*	F. R. Blattner
FB21757	MG1655 *intB*::*kan*	F. R. Blattner
FB21746	MG1655 *mgtA*::*kan*	F. R. Blattner
FB21469	MG1655 *corA*::*kan*	F. R. Blattner
LMM-JS03-14	MG1655 *ydjN*::*kan*	This study
LMM-JS03-66	MG1655 *ybjG*::*kan*	This study
LMM-JS03-64	MG1655 *yhdA*::*kan*	This study
LMM-JS03-15	MG1655 *cysJ*::*cat*	This study
LMM-JS03-60	MG1655 *fdx*::*kan*	This study
S. enterica serovar Typhimurium		
MM2089	Wild-type strain	M. E. Maguire
MM2242	MM2089 *corA*52::Tn10d16d17; Tet^r	M. E. Maguire
Plasmids		
pKD3	Template plasmid containing FRT-*cat*-FRT for the one step inactivation protocol; Ap^r	5
pKD4	Template plasmid containing FRT-*kan*-FRT for the one-step inactivation protocol; Ap^r	5
pKD46	Red recombinase expressing plasmid; Ap^r	5
pJS121	pFPV25 containing *E. coli corA*; Ap^r	This study
pASV_AOD12	*gfp-mgtA* transcriptional fusion	29
pASV_CIC2	*gfp-corA* transcriptional fusion	29
pASV_ACB1	*gfp-rstA* transcriptional fusion	29
pASV_ATC9	*gfp-ybjG* transcriptional fusion	29
pMAS29	Contains serovar Typhimurium *corA*; Ap^r	M. E. Maguire

characterized the stress response in *E. coli* after challenge with the *Curvularia* haloperoxidase, using bromide as a substrate, and succeeded in establishing a link between one of the induced genes and bacterial tolerance to the haloperoxidase system. In the current work, we investigated the potential role of 10 of the LP/SCN^--inducible ORFs in tolerance of *E. coli* against this enzyme system.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. All strains and plasmids used in this work are listed in Table 1. Luria-Bertani (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl, 10 g/liter agar for solid medium) was used as the standard growth medium. Antibiotics (Sigma-Aldrich, Bornem, Belgium) were added as appropriate at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 30 μg/ml. When indicated, medium was supplemented with $MgSO_4$ from a filter-sterilized 1 M stock solution. Tryptone soy agar (30.12 g/liter tryptone soy broth, 10 g/liter agar) was used as the growth medium in sensitivity assays.

Enzymes and chemicals. Stock solutions of LP (Sigma-Aldrich) (10 mg/ml) and glucose oxidase (Sigma-Aldrich) (100 U/ml) were prepared in a 50% glycerol solution in phosphate-buffered saline (2.87 mM KH_2PO_4, 7.12 mM K_2HPO_4, 0.151 M NaCl, pH 6.0). Stock solutions of plumbagin (5-hydroxy-2-methyl-1,4-naphtoquinone) (Sigma-Aldrich) (200 mM) and of Co(III) hexaammine (Sigma-Aldrich) (200 mM) were prepared in dimethyl sulfoxide and in ultrapure deionized water, respectively. All these stock solutions were stored at −18°C. Every week, fresh stock solutions of potassium thiocyanate (KSCN; 25 mM), hydrogen peroxide (H_2O_2; 25 mM), and $NiCl_2$ (1 M) were prepared in ultrapure deionized water and stored at 4°C.

Construction of knockout mutants. Knockout mutants in our laboratory strain MG1655 background were constructed either by P1 transduction of mutant alleles from *E. coli* strains obtained from elsewhere (Table 1) or by using the standard one-step inactivation protocol of Datsenko and Wanner (5). In the latter procedure, a chloramphenicol (*cat*) or kanamycin (*kan*) resistance gene was amplified using platinum *pfx* polymerase (Invitrogen, Merelbeke, Belgium) from template plasmids pKD3 or pKD4, respectively, using the mutagenesis primers (Isogen Biosciences, Maarsen, The Netherlands) specified in Table 2. These primers contained at their 5′ ends 45-bp regions homologous to the gene of interest, which allows homologous recombination between the PCR fragment containing the resistance gene and the genomic region of interest. Amplified fragments were transferred to MG1655 by electroporation, and recombination was promoted by inducing the λ *red* genes provided on plasmid pKD46 with 1 mM arabinose. Transformants were isolated and grown at 42°C to cure plasmid pKD46. To avoid any secondary mutations, all mutant alleles were transferred again to MG1655 by P1 transduction. Purified mutants were verified by PCR (*Taq* polymerase; Fermentas, St. Leon-Rot, Germany) using combinations of control primers (Table 2) (Eurogentec, Seraing, Belgium) upstream and downstream of the genomic region of interest and resistance gene-specific primers C1 and C2 for *cat* and K1 and K2 for *kan* (5).

Cloning of *corA* for complementation experiments. The *corA* gene of MG1655 was amplified by PCR using Platinum *pfx* polymerase with cloning primers PcorAclUP and PcorAclDWN (Eurogentec) containing XbaI and HindIII restriction sites, respectively (Table 2). Plasmid pFPV25 (40) was prepared by removing the 700-bp XbaI-HindIII fragment containing *gfp*, followed by dephosphorylation with calf intestinal alkaline phosphatase (Epicenter, Landgraaf, The Netherlands) and purification from agarose gel. Subsequently, the amplified wild-type *corA* fragment was cloned as an XbaI-HindIII fragment in pFPV25, resulting in plasmid pJS121. This construct was verified by PCR using a vector-specific primer PgfpFWD and primer PcorAclDWN.

Sensitivity assays for LP system, H_2O_2, or plumbagin. Overnight cultures were diluted to 10^6 CFU/ml in 10 mM HEPES-KOH, pH 7.0, containing 10 μg/ml LP, 0.5 U/ml of glucose oxidase, 0.4% glucose, and 0.75 mM KSCN. Control samples contained cells together with glucose oxidase, glucose, and KSCN, and blank samples contained cells in HEPES-KOH buffer only. After incubation at 30°C for different times, survivors were counted by a rapid agar spot method: 10-fold dilutions of the bacterial suspensions in 10 mM HEPES-KOH buffer were spotted (5 μl/spot) on tryptone soy agar trays, which were then incubated at 37°C for 22 h. The reduction in CFU/ml could be determined with about 0.5-log unit accuracy by assessing the formation of colonies in the spots corresponding to the

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)
Mutagenesis primers[a]	
PcysJmutUP	GACACAGGTCCCACCTTCCGCGTTGCTTCCGTTGAACCCGGAGCAAgtgtaggctggagctgcttc
PcysJmutDWN	CTCACTTAAAAATTCATCCGCCGCTTCGGTGTCCATGCCACCAAATTCGcatatgaatatcctcctta
PydjNmutUP	ATGAACTTTCCATTAATTGCGAACATCGTGGTGTTCGTTGTACTGCgtgtaggctggagctgcttc
PydjNmutDWN	GGTCAGCGTGCCAGCTGTCATCGAGCCACTAACGTTTAACGCCGcatatgaatatcctcctta
PfdxmutUP	ACGAGGTTTAATATGCCAAAGATTGTTATTTTGCCTCATCAGGATgtgtaggctggagctgcttc
PfdxmutDWN	AATTTCGCGGCTATCGGTCCACTTAAGTCCCATACTAACTCTGTcatatgaatatcctcctta
PyhdAmutUP	TTTCGGCCTTTGTTACGCTGCTCACCGGGTTAACAATTTTTGTGAgtgtaggctggagctgcttc
PyhdAmutDWN	ATCTTTGTGAATATTTTTTCACGTTAGTATCAAGTGGCTGTGAGGcatatgaatatcctcctta
PybjGmutUP	TTTGAATCTCTCTCTATTCTCTCTTATTAACGCGACGCCAGACTCgtgtaggctggagctgcttc
PybjGmutDWN	GCGGATCGGTAATGCAAAACAGACGCGATACCACGATTGCAGACGcatatgaatatcctcctta
Control primers	
PcysJctrUP	GACGATAAAACCGCCGTAGA
PcysJctrDWN	GGTTCGAGGTGCAGAGTACG
PydjNctrUP	GTTCGTTGGTCATCGCAAAT
PydjNctrDWN	GAATGGGAAGTGGTGTGCTT
PfdxctrUP	GGTCTGACCGATAGCGAAAT
PfdxctrDWN	TATGTGACTGGGGTGAACGA
PyhdActrUP	CGCTAATGGAAAAGCAAAGC
PyhdActrDWN	ACAAGGCGCAAAAATCATCT
PybjGctrUP	GCGGTCATCGAAGTAGGAAC
PybjGctrDWN	TAACAAACGGCGGTAAAAGG
C1[b]	TTATACGCAAGGCGACAAGG
C2[b]	GATCTTCCGTCACAGGTAGG
K1[b]	CAGTCATAGCCGAATAGCCT
K2[b]	CGGTGCCCTGAATGAACTGC
PgfpFWD	AAAAATAGGCGTATCACGAGG
Cloning primers	
PcorAclUP	TCGA**TCTAGA**CACCTTCTGCTGACCGTTTT[c]
PcorAclDWN	CAGT**AAGCTT**GGGGGCAACACCAGAATATAA[d]

[a] Sequence in uppercase, 45-bp homologous fragment; sequence in lowercase, template plasmid specific primer.
[b] Datsenko and Wanner (5).
[c] **TCTAGA**, XbaI recognition site.
[d] **AAGCTT**, HindIII recognition site.

subsequent dilutions for each treatment and for the control and blank samples. All experiments were repeated at least two times with three replicates per treatment. Results obtained with replicates did not differ by more than 0.5 log units. To determine inactivation by H_2O_2 or the O_2^--generating agent plumbagin, the same method was used, but the enzyme systems were replaced by 0.5 mM H_2O_2 or 0.5 mM plumbagin and 0.4% glucose, respectively.

Promoter activity assay for genes belonging to the Mg^{2+} regulon. Strains carrying transcriptional *gfp* fusions to *mgtA*, *corA*, *rstA*, and *ybjG* (Table 1) were grown overnight in LB with or without the extra addition of 10 mM $MgSO_4$. After 20 h of growth, cells were harvested by centrifugation (6,000 rpm for 5 min) and resuspended in 10 mM HEPES-KOH (pH 7.0), and 200 μl from each sample was transferred to a 96-well microplate. Gfp production was then quantified by measuring fluorescence at 520 nm with a Fluoroskan Ascent FL microplate fluorescence reader (Thermo Labsystems, Brussels, Belgium) using an excitation wavelength of 485 nm. The results were expressed as relative fluorescence units per unit of optical density at 600 nm.

RESULTS

Construction and LP sensitivity of knockout mutants in LP-inducible genes. In an earlier study we identified promoters specifically induced upon exposure to the antimicrobial LP/SCN^- system (29). The first objective of the present work was to evaluate the possible contribution of the corresponding genes in the protection of *E. coli* against the LP system. Strains with knockouts in some of the corresponding genes were obtained from F.R. Blattner (*rstA*, *intB*, *mgtA*, and *corA*) and B. L. Wanner (*pstS*). These mutant alleles were transferred to our own laboratory *E. coli* MG1655 strain using P1 transduction. *ydjN*, *ybjG*, *yhdA*, *cysJ*, and *fdx* knockout mutants were constructed using the standard gene inactivation protocol from Datsenko and Wanner (5). All these mutants were then tested for sensitivity to the LP system (Fig. 1A). Only the *corA* mutant was severely affected by the LP treatment, showing complete inactivation (>3.7 log CFU/ml) after 120 min of exposure. In contrast, the other mutants and the wild-type strain were still at their initial level of CFU/ml after 120 min, except for a small reduction of 0.5 log CFU/ml for the *rstA* mutant. During further exposure all other mutants and the wild-type strain became gradually inactivated by the LP/SCN^- system but not all to the same extent. After 210 min, only small differences in inactivation existed among the strains, but after 300 min, when the inactivation of MG1655 was 2.0 log CFU/ml, the *rstA*, *ydjN*, *yhdA*, *cysJ*, and *fdx* mutants were also completely inactivated. In contrast, the *pstS*, *intB*, *ybjG*, and *mgtA* mutants showed the same reduction as MG1655.

The increased sensitivity of the *corA* mutant was not caused by a trivial growth retardation since the growth curve of the mutant was indistinguishable from that of the wild-type strain under the same growth conditions as used for the sensitivity experiments (results not shown). When the same experiment was conducted with omission of the LP enzyme (Fig. 1B), much lower levels of inactivation were observed. This indicates

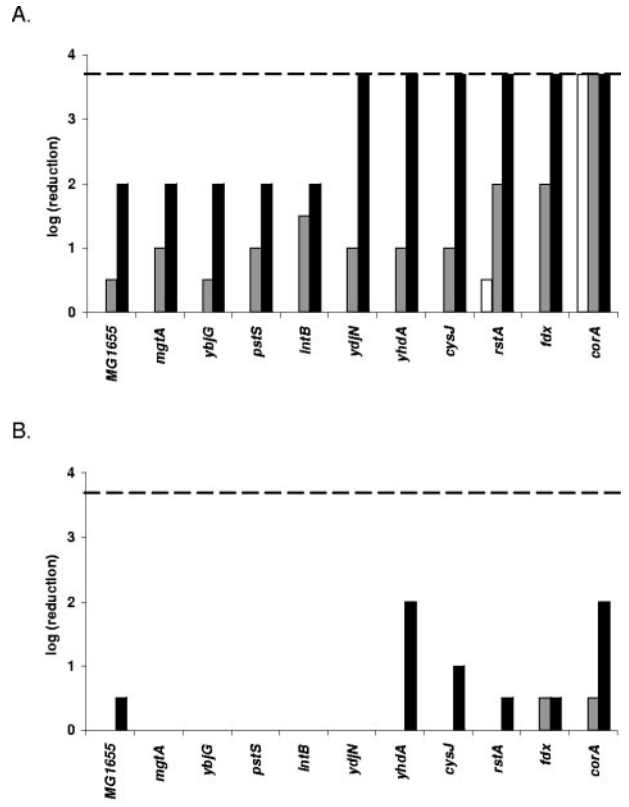

FIG. 1. Susceptibility of knockout mutants to the complete LP/SCN^- system (A) and to the control treatment (B). Samples were diluted and spotted after 120 min (white bars), 210 min (gray bars), and 300 min (black bars). The detection limit (dashed line) was 200 CFU/ml. All strains are gradually killed by the antibacterial LP/SCN^- system, but *corA* shows a specific hypersensitivity toward the LP/SCN^- system and only slight sensitivity to H_2O_2 produced by the glucose/glucose oxidase system in the control treatment.

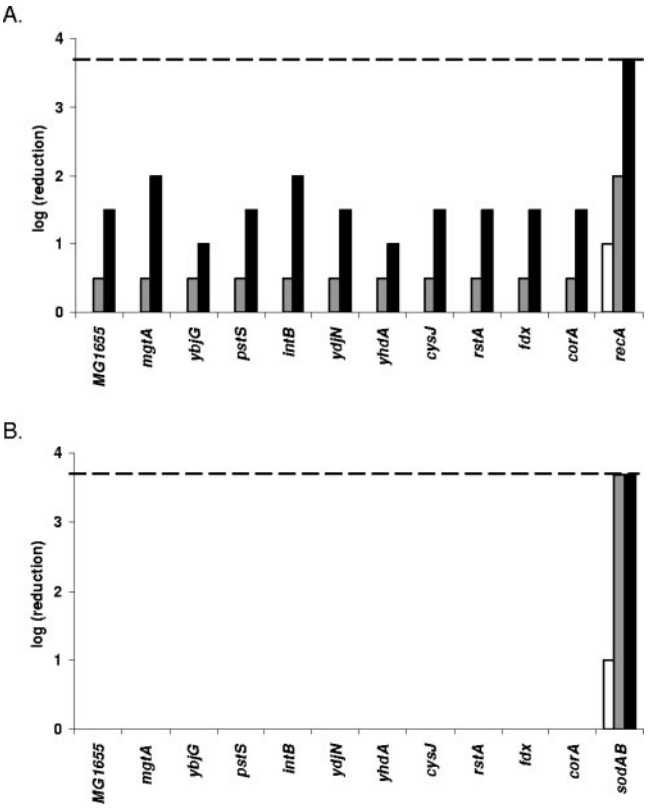

FIG. 2. Susceptibility of knockout mutants to H_2O_2 (A) and to plumbagin (B). After treatment with H_2O_2, samples were diluted and spotted after 120 min (white bars), 210 min (gray bars), and 300 min (black bars). The positive control strain *recA* was severely inactivated, but none of the other tested mutants was hypersensitive to H_2O_2. For the plumbagin-treated samples, samples were taken after 210 min (white bars), 300 min (gray bars), and 420 min (black bars). Only the positive control strain *sodAB* was killed by the plumbagin treatment. The detection limit (dashed line) for both treatments was 200 CFU/ml.

that bacterial inactivation in the presence of the complete LP/SCN^- system was caused by the antibacterial effect of this enzyme system and not, or only in small part, by the H_2O_2 generated by the glucose/glucose oxidase system. The true contribution of H_2O_2 to bacterial inactivation by the LP/SCN^- system is likely to be even smaller than what is suggested by Fig. 1B, since the presence of the LP enzyme will reduce the effective concentration of H_2O_2. Nevertheless, the results suggest a somewhat increased H_2O_2 sensitivity of the *corA* and *yhdA* mutants.

Sensitivity to H_2O_2 or plumbagin of knockout mutants in LP-inducible genes. To further examine the specificity of the bactericidal effect of the LP/SCN^- system, we studied the sensitivity of the 10 knockout strains toward a direct challenge with H_2O_2 or to the O_2^--generating agent plumbagin. A *recA* and a *sodAB* mutant were included in the experiment as an H_2O_2-sensitive and O_2^--sensitive strain, respectively. The *recA* strain clearly suffered from an attenuated resistance towards H_2O_2, but all other mutants showed a level of sensitivity to H_2O_2 similar to that of the wild-type strain (Fig. 2A). Thus, the somewhat increased sensitivity to the glucose/glucose oxidase system noticed above for the *corA* and *yhdA* knockout strains could not be reproduced in a direct challenge with 0.5 mM H_2O_2.

As expected, the O_2^--sensitive *sodAB* strain was rapidly inactivated by plumbagin treatment. However, none of the other strains showed any inactivation under the conditions of the treatment (Fig. 2B). Although this result precludes the observation of possible small differences in sensitivity toward O_2^-, it can be concluded that none of the mutants was hypersensitive to O_2^-.

These results and the results of the LP sensitivity assay described above indicate that among all the mutants tested, only the *corA* mutant is hypersensitive toward the LP system. This hypersensitivity is specific to the LP/SCN^- system, since it is not linked to hypersensitivity to H_2O_2 or O_2^-. The specificity of the antibacterial effect of the LP system is further illustrated by the finding that *recA* and *sodAB* mutants, which are hypersensitive to H_2O_2 and O_2^-, respectively, did not exhibit altered sensitivity to the LP system (results not shown). The other mutants in which an LP-inducible gene was knocked out did not show altered sensitivity or showed only moderately increased sensitivity to the LP system and no altered sensitivity to other oxidants.

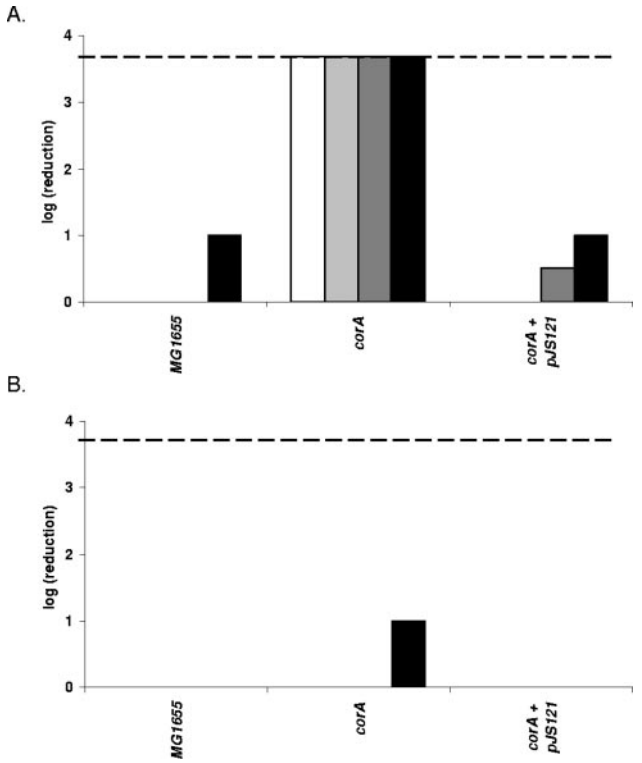

FIG. 3. Sensitivity of *E. coli* MG1655, *corA*, and *corA* containing plasmid pJS121 to the LP/SCN$^-$ system (A) and the control treatment (B). When a wild-type copy of the *E. coli corA* gene is provided on a plasmid, the hypersensitivity of *corA* to the LP/SCN$^-$ system is completely counteracted. Samples were taken 120 min (white bars), 165 min (light gray bars), 210 min (dark gray bars), and 255 min (black bars) after treatment. The detection limit (dashed line) was 200 CFU/ml.

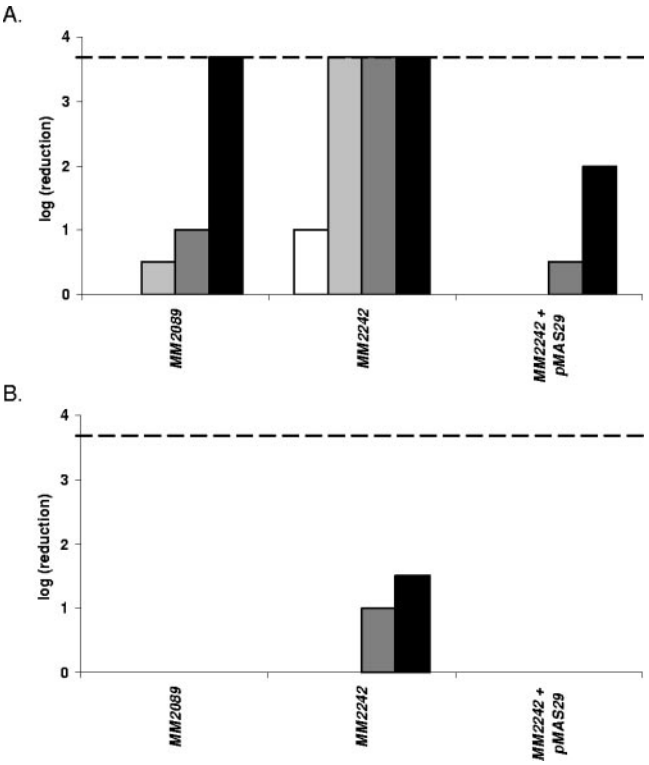

FIG. 4. Sensitivity of *S. enterica* serovar Typhimurium MM2089, MM2242 (*corA*), and MM2242 containing plasmid pMAS29 to the LP/SCN$^-$ system (A) and the control treatment (B). The *S. enterica* serovar Typhimurium *corA* gene provided on plasmid pMAS29 renders *corA* more resistant to the LP system than the corresponding wild-type strain. Samples were taken 120 min (white bar), 165 min (light gray bars), 210 min (dark gray bars), and 255 min (black bars) after treatment. The detection limit (dashed line) was 200 CFU/ml.

Sensitivity of the *Salmonella enterica* serovar Typhimurium *corA* mutant to the LP/SCN$^-$ system and complementation of *E. coli* and *S. enterica* serovar Typhimurium *corA* mutants with a cloned *corA* gene. Contrary to what we found in *E. coli*, a *corA* knockout mutant of *S. enterica* serovar Typhimurium was earlier reported to be sensitive to H_2O_2 (K. M. Papp, J. Lin, D. G. Kehres, L. M. Kucharski, M. E. Maguire, Abstr. 103th Gen. Meet. Am. Soc. Microbiol., abstr. I-068, 2003). It cannot be excluded that this discrepancy is due to different assay methods. Therefore, to sort out whether CorA indeed affects H_2O_2 sensitivity differently in both bacteria and to determine its contribution to tolerance against the LP/SCN$^-$ system in *S. enterica* serovar Typhimurium, we compared a wild-type strain, a *corA* knockout, and a *corA* knockout strain carrying a cloned intact *corA* gene of both species for resistance to the LP/SCN$^-$ system and to the control treatment containing only the H_2O_2-generating glucose oxidase/glucose system and KSCN. The results for *E. coli* (Fig. 3) and *S. enterica* serovar Typhimurium (Fig. 4) were very similar. In both bacteria, knockout of *corA* caused hypersensitivity to the LP/SCN$^-$ system but no hypersensitivity or only very slight hypersensitivity to H_2O_2. Complementation with an intact *corA* gene restored the resistance to the wild-type level or even a slightly higher level in both organisms.

Involvement of the Mg stimulon in sensitivity of *E. coli* to the LP/SCN$^-$ system. CorA forms a homotetrameric inner membrane transporter of Mg^{2+} and, to a lesser extent, some other bivalent cations (15, 42). Interestingly, our screen for LP-inducible promoters also yielded three other genes (*mgtA*, *ybjG*, and *rstA*) belonging to the Mg stimulon of *E. coli* (18, 29). This suggested a possible link between Mg^{2+} concentration and resistance to the LP/SCN$^-$ system. In contrast to the constitutively expressed *corA*, these genes are inducible by Mg^{2+} shortage. Unexpectedly, we found that the clones containing the *mgtA*, *ybjG*, and *rstA* promoters fused to the *gfp* reporter gene became induced during overnight growth (20 h) in normal LB but not in LB supplemented with 10 mM Mg^{2+} (Fig. 5). Therefore, to investigate the role of the Mg stimulon in resistance toward the LP system, the wild-type strain and the *corA*, *mgtA*, *ybjG*, and *rstA* mutants were subjected to treatment with the LP system after growth in LB containing 10 mM Mg^{2+}. As was the case for cultures grown in unsupplemented LB, only the *corA* mutant was hypersensitive to the LP system and no differences in sensitivity were observed compared to results obtained earlier (Fig. 6). Also, the addition of 10 mM Mg^{2+} during treatment with the LP system did not significantly change these results (data not shown).

Effect of pharmaceutical inhibition of CorA on sensitivity of *E. coli* to the LP/SCN$^-$ system. Co(III) hexaammine is a com-

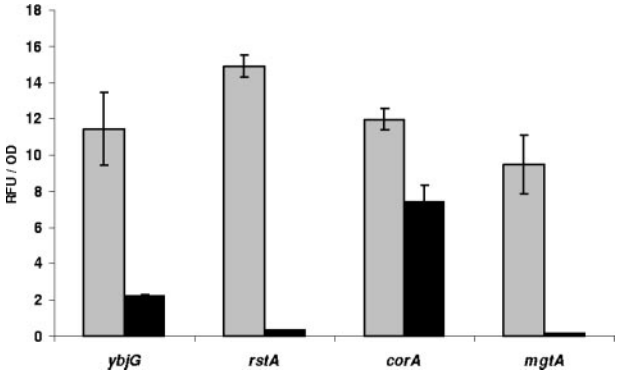

FIG. 5. Promoter activity measured using *ybjG*, *rstA*, *corA*, and *mgtA gfp*-fusions. After 20 h of growth in normal LB (gray bars) or LB supplemented with 10 mM $MgSO_4$ (black bars), cells were harvested to measure fluorescence (for Gfp production) and optical density (for bacterial growth) as described in Materials and Methods. Results shown are means ± standard deviations of three replicates.

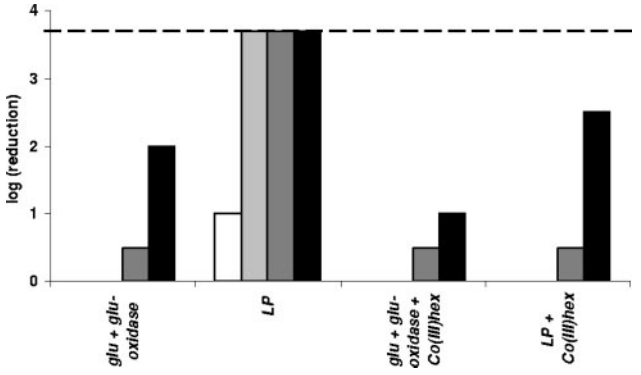

FIG. 7. Influence of 0.3 mM Co(III) hexaammine on sensitivity of *E. coli* MG1655 to the LP system in the presence of 0.3 mM Ni^{2+}. Prior exposure to 0.3 mM Ni^{2+} severely increased sensitivity of *E. coli* to the LP system and to the control treatment with the glucose/glucose oxidase system. Inhibition of CorA by addition of 0.3 mM Co(III) hexaammine before addition of Ni^{2+} almost completely suppressed this sensitization. Samples were taken 60 min (white bars), 120 min (light gray bars), 180 min (dark gray bars), and 240 min (black bars) after LP treatment. The detection limit (dashed line) was 200 CFU/ml.

pound reported to specifically inhibit CorA-mediated cation transport (17). Initial experiments in which we added 0.03 to 3 mM of this compound during growth of the bacterial cultures or during exposure to the LP system showed no effect. This concentration of Co(III) hexaammine was sufficient to block CorA since it could completely inhibit the growth of an *mgtA* knockout strain (data not shown). However, with a modified experimental procedure, a distinct influence of Co(III) hexaammine on LP sensitivity could be demonstrated. In this modified procedure, 0.3 mM Ni^{2+} was added to the cell suspensions in HEPES-KOH buffer 1 h before the bacteria were exposed to the LP treatment. This concentration of Ni^{2+} did not cause any inactivation of the strains in itself (data not shown). The addition of Co(III) hexaammine (0.3 mM), when applicable, was done 1 h before the Ni^{2+} addition. Comparison of the results shown in Fig. 7 with those of the former experiments in the absence of Ni^{2+} (Fig. 1 and 3) reveals that the addition of Ni^{2+} severely increased the lethality of the LP system. After 120 min, MG1655 was completely inactivated by the LP treatment, whereas without Ni^{2+} after 300 min inactivation was only 2 log CFU/ml (Fig. 1). Inactivation due to H_2O_2 produced by the glucose/glucose oxidase system was also slightly increased in the presence of Ni^{2+}. However, when 0.3 mM Co(III) hexaammine was added before the Ni^{2+} treatment, the enhancing effect of Ni^{2+} on the LP lethality was completely suppressed. The enhancing effect of Ni^{2+} on the lethality of the glucose/glucose oxidase treatment was reduced to a lesser extent.

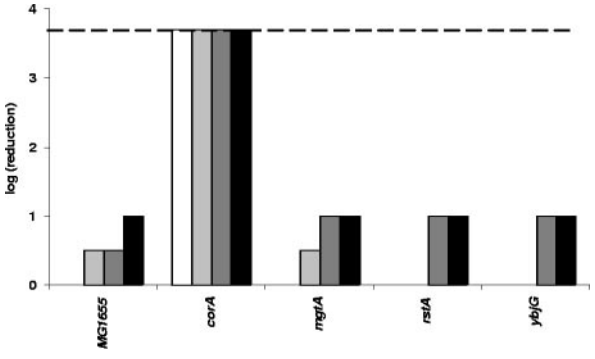

FIG. 6. Sensitivity of MG1655, *corA*, *mgtA*, *rstA*, and *ybjG* to the LP/SCN^- system after growth in LB supplemented with 10 mM Mg^{2+}. As seen before, only *corA* is hypersensitive to the antibacterial LP/SCN^- system. Samples were taken 120 min (white bars), 165 min (light gray bars), 210 min (dark gray bars), and 255 min (black bars) after treatment. The detection limit (dashed line) was 200 CFU/ml.

These results suggest that Ni^{2+} uptake sensitizes the cells for the LP system. Consequently, one would predict a *corA* mutant to be less sensitive to this Ni^{2+} enhancing effect because it has a reduced Ni^{2+} uptake capacity. To confirm this, we reduced the dosage of KSCN substrate of the LP enzyme system from 0.75 mM to 0.15 mM for practical reasons, because otherwise the *corA* mutant is inactivated too rapidly (Fig. 1 and 3). Under these conditions, MG1655 was inactivated by 2 log CFU/ml after 240 min in the presence of Ni^{2+}, while inactivation of the *corA* mutant was only 1 log CFU/ml after 300 min (Fig. 8). Also, for treatment with the glucose/glucose oxidase system only, the *corA* mutant was more resistant to the LP system in the presence of Ni^{2+} than MG1655 in this experiment.

DISCUSSION

In this work we constructed 10 *E. coli* MG1655 knockout mutants in genes or ORFs identified earlier to be specifically inducible by the LP/SCN^- system (29) and tested their susceptibility to the antibacterial activity of this enzyme system. Several mutants showed slightly to moderately increased sensitivity (*ydjN*, *yhdA*, *cysJ*, *rstA*, and *fdx*), but only one mutant (*corA*) exhibited hypersensitivity to the LP/SCN^- system. The genes that were most strongly induced (*cysJ* and *ydjN*) had only a small contribution to the resistance against the LP/SCN^- system. Similar conclusions were reached by Hansen et al. (13), who identified 25 upregulated ORFs (including *cpxARP*) upon exposure of *E. coli* to the *Curvularia* haloperoxidase system with bromide as an electron donor. Only one of the con-

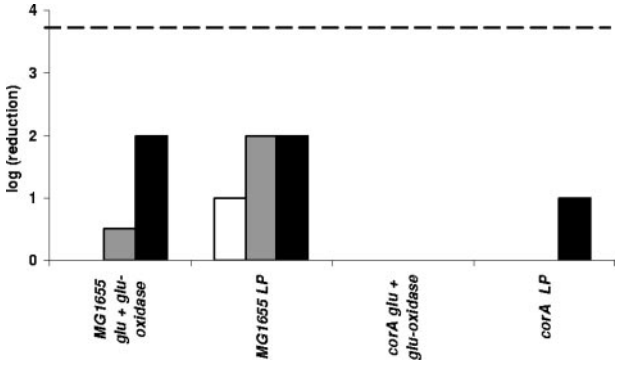

FIG. 8. Sensitivity of *E. coli* MG1655 and *corA* to the LP system (10 μg/ml LP, 0.5 u glucose oxidase, 0.4% glucose, and 0.15 mM KSCN) in the presence of 0.3 mM Ni^{2+}. Prior exposure to 0.3 mM Ni^{2+} increased the sensitivity of *E. coli* MG1655 to the LP system and to the control treatment with the glucose oxidase system. The *corA* mutant was much less sensitized to the LP system by Ni^{2+}. Samples were taken 180 min (white bars), 240 min (gray bars), and 300 min (black bars) after treatment. The detection limit (dashed line) was 200 CFU/ml.

structed corresponding knockout mutants (a *cpxARP* triple mutant) was found to be sensitive to the enzyme system. Apparently, the majority of the induced genes are not required for specific defense against these enzyme systems. This can be either because they are simply not involved or because the cellular defense systems against these stresses are redundant (13, 39). Interestingly, *corA* mutants of both *E. coli* and *S. enterica* serovar Typhimurium were specifically sensitive to the LP/SCN^- system, and not, or to a much lower degree, to H_2O_2 and the superoxide-generating agent plumbagin (Fig. 1 and 2). In an earlier study, S. *enterica* serovar Typhimurium was reported to be peroxide sensitive (Papp et. al, Abstr. 103th Gen. Meeting ASM, 2003). However, to our knowledge experimental details of this study were not published, and it is therefore difficult to compare our results with those of the earlier study. It is quite possible that the apparent discrepancy stems from the use of different experimental procedures.

The specific sensitivity of *corA* mutants to an LP challenge, together with the specificity of the LP-induced stress response that we reported earlier (29), strongly suggests that $OSCN^-$, the primary antimicrobial reaction product of the LP enzyme system, targets other cellular constituents than H_2O_2 and O_2^- target. As a consequence, bacteria may have developed specific defense systems to cope with this form of oxidative stress.

Bacterial magnesium transporters were first identified in *E. coli* (20, 21, 23, 31, 32) and have also been well characterized in *S. enterica* serovar Typhimurium (15, 22, 35, 42). CorA is a 37-kDa integral membrane protein that forms the primary constitutive Mg^{2+} uptake system in many *Bacteria* and some *Archaea* (15) and lacks homology to any other known transporter. It has an affinity for Mg^{2+} of 15 to 20 μM, and Mg^{2+} uptake is driven by the cellular membrane potential (9, 15, 42). However, there is no straightforward clue for the role of CorA in bacterial sensitivity to the LP system, since there are no indications for a direct involvement of Mg^{2+} in oxidative stress resistance. On the other hand, CorA can also mediate the influx of other bivalent cations, including some transition metal ions that have been linked to the generation of or protection against oxidative stress. The affinity of CorA for Co^{2+} and Ni^{2+} is 20 to 40 μM and 200 to 400 μM, respectively, which is in the toxic concentration range of these cations for bacteria like *S. enterica* serovar Typhimurium. Nevertheless, in view of the very small cellular requirements for these cations, their leakage through CorA might be physiologically relevant (15). Mn^{2+}, Zn^{2+}, and Ca^{2+} are probably not transported by CorA and do not interfere with Mg^{2+} transport via *corA* (33, 36), but for Fe^{2+} the situation is less clear. Hantke (14) reported reduced uptake of Fe^{2+} in *corA* mutants of *E. coli* and *S. enterica* serovar Typhimurium. Concomitantly, an increased resistance of these mutants was found against Fe^{2+}-mediated oxidative stress caused by the Fenton reaction. Similarly, Chamnongpol and Groisman (4) revealed that *phoP* mutation rendered *S. enterica* serovar Typhimurium extremely sensitive to Fe^{2+}. This phenotype was rescued in a *corA phoP* double mutant. In both studies, the increased resistance of a *corA* mutant was attributed to reduced Fe^{2+} uptake. However, the increased Fe^{2+} resistance of *S. enterica* serovar Typhimurium *corA* mutants could not be reproduced more recently, and direct measurements with radioisotopes indicated that Fe^{2+} is not transported by CorA and does not inhibit CorA-mediated Ni^{2+} transport. In addition, cellular Fe^{2+} uptake was not affected by Co(III) hexaammine, a selective inhibitor of CorA (22). Therefore, a link between CorA, the Fe^{2+} status of the cell, and LP resistance remains speculative.

Knockout of the three other Mg^{2+}-related ORFs that are induced by the LP system had different effects: there was no change in LP sensitivity for *ybjG* and a slight and moderate increase in sensitivity for *mgtA* and *rstA*, respectively (Fig. 1A). These ORFs belong to the Mg stimulon and are strongly derepressed under Mg^{2+} limitation, together with a whole set of other genes under the control of the PhoP/Q two-component regulatory system (18), among which several are also induced after challenge with the LP system (29). Therefore, we investigated whether repression or derepression of these regulons by Mg^{2+} would affect LP sensitivity. Although the Mg stimulon was reported to remain repressed during growth in standard LB broth (22, 34, 38), expression of *mgtA*, *rstA*, and *ybjG* was clearly derepressed when cells were analyzed after 20 h of growth. Addition of 10 mM Mg^{2+} was required to maintain the repressed state (Fig. 5) during the complete growth time of the cultures. It is possible that this derepression in LB starts only in an advanced growth phase, when Mg^{2+} becomes depleted. However, since the purpose of our experiment was only to compare LP resistance of cells with a repressed and a derepressed Mg stimulon, we did not further investigate the causes of this derepression in LB medium. Addition of Mg^{2+} to the growth medium to repress the Mg stimulon, or even during LP challenge, did not significantly affect the LP sensitivity of wild type or *corA*, *mgtA*, *rstA*, and *ybjG* mutants of *E. coli*.

As opposed to Mg^{2+}, Ni^{2+} severely sensitized *E. coli* MG1655 to the LP system. This enhancing effect was clearly mediated by CorA, since it was suppressed by the CorA inhibitor Co(III) hexaammine (Fig. 7) and in a *corA* mutant (Fig. 8). An enhancing effect of several transition metal cations, particularly Fe^{2+}, on the toxicity of H_2O_2 is well known and is ascribed to the Fenton reaction. In contrast, the enhancement of LP system toxicity by transition metals has not been described to our knowledge, and its mechanism remains unclear

to date. Nevertheless, our results indicate that CorA affects the LP sensitivity of *E. coli* by its effect on the cytoplasmic concentrations of transition metals that enhance the toxicity of the LP system. Taking into account that CorA can mediate both uptake and efflux of bivalent cations, this allows us to propose the following model. When cells are loaded with a transition metal prior to exposure to the LP system, strain MG1655 will be more sensitive than a *corA* mutant because of a higher accumulation of the metal. This is the case in the experiment shown in Fig. 8. Conversely, when LB-grown cells are resuspended in HEPES buffer without added metal ions, some release of transition metal ions from the cytoplasm through CorA may take place in MG1655, resulting in an enhanced tolerance of this strain compared to a *corA* mutant. Of course, for this efflux to occur, at least some of the transition metal cations that can be transported by CorA must occur in a free form in the cytoplasm. Because these metals can bind tightly to proteins and nucleic acids, their free cellular concentrations are normally very low. An alternative explanation is that the loss of CorA results in an increased production or activity of one or more of the other divalent cation uptake systems that exist in *E. coli* and, thus, in an increased cellular concentration of one or more divalent cations. Further experimentation is necessary to distinguish between these possibilities.

It was already noted previously that although loss of CorA does not produce any significant Mg^{2+}-dependent growth phenotype, it causes a surprising variety of other phenotypes, including altered expression of genes encoded by *Salmonella* pathogenicity island I; increased sensitivity to heat shock and peroxide; defective invasion, survival, and proliferation within macrophages and epithelial cells; and diminished virulence (unpublished results from M. E. Maguire laboratory, mentioned in references 15 and 22). Although the causes of these effects have not been elucidated precisely, it has been observed that loss of CorA affects transcription of several genes. For example, a number of genes from the PhoP/Q regulon are derepressed while others remain PhoP/Q- and Mg^{2+}-dependent (15, 22). On the other hand, other genes including *mgtA* could no longer be fully induced by low Mg^{2+} concentrations in a *corA* background (34; our preliminary results). These observations suggest a pleiotropic effect of a mutation in *corA* that could explain the wide range of associated phenotypes. Our work reveals LP sensitivity as an additional phenotype of *corA* mutants in *E. coli* and *S. enterica* serovar Typhimurium. This phenotype may well be the basis for the observed virulence-related defects in the latter organism, since LP and the closely related myeloperoxidase are important components of the vertebrate innate immune system.

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