Elucidation of the Antibacterial Mechanism of the *Curvularia* Haloperoxidase System by DNA Microarray Profiling

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A vast of array of antimicrobial compounds are used in the fight against microorganisms. For instance, disinfection of surfaces in the clinical sector or in pharmaceutical and food processing settings involves the use of compounds such as chlorite, hydrogen peroxide, iodophors, or quaternary ammonium compounds. Many of these compounds have adverse side effects, such as leaving active residues, causing corrosion and degradation of surfaces and equipment, or being unsafe for the personnel exposed thereto. Hence, there has been a drive for development of milder, more environmentally friendly antimicrobial agents (14, 20). One such agent is the *Curvularia* haloperoxidase system, which has a lethal effect against bacteria, yeasts, and filamentous fungi and is active against both planktonic microorganisms and surface-associated microorganisms (16). In the presence of hydrogen peroxide, *Curvularia* haloperoxidase facilitates the oxidation of halides, such as chloride, bromide, and iodide, to antimicrobial compounds. The level of hydrogen peroxide used in the enzyme system is much lower than if hydrogen peroxide is used as the sole compound, thus reducing the corrosive action of the system. It has been hypothesized that the antimicrobial effect of the *Curvularia* haloperoxidase system is due to the production of highly oxidative intermediates; however, this is not known factually. Further assessment of areas of application of the *Curvularia* system would be greatly facilitated by an understanding of its mechanism of action. Also, understanding the mechanism would allow low evaluation of potential side effects such as development of resistance to the system.

Elucidating the mechanisms of action of antimicrobial compounds can be approached in different ways, including creation of mutants that are resistant or hypersensitive to the effect, determining cellular damage (e.g., respiration effects and leakage of intracellular materials), or using stress-gene reporter fusions (2, 10, 12, 17, 26, 32, 38). Comparison of global gene transcripts as performed by using DNA microarrays offers a unique way of analyzing the effects of antimicrobial compounds on microorganisms. The expression profiles may reveal the mode of action of the compound and may also provide information on potential resistance mechanisms. Examples of areas investigated by use of microarray analysis are oxidative stress and effect of antibacterial compounds intended for both disinfection and pharmaceutical use (27, 40, 43).

Bacteria are able to respond to stress conditions in order to circumvent stress factors such as oxidative stress, heat shock, cold shock, etc. Oxidative stress is caused by exposure to reactive oxygen intermediates and has been shown to cause damage to proteins, nucleic acids, and lipids (13). The regulatory protein SoxRS controls the defense against superoxide, whereas OxyR regulates the hydrogen peroxide response (13, 36, 43). Another defense mechanism is the synthesis of heat shock proteins that stabilize and protect intracellular proteins not only from heat but also from other stresses such as oxidative stress. *Escherichia coli* strains overexpressing the molecular chaperone DnaK or the small heat shock proteins IbpA and IbpB exhibit increased tolerance to hydrogen peroxide or superoxide, respectively (12, 19).

In the present study, we exposed the *E. coli* K-12 strain MG1655 to the *Curvularia* haloperoxidase system. The com-
pletion of the full genome sequence of this strain (3) has allowed the development of commercial DNA microarray chips, and the vast biological knowledge of this organism facilitates the interpretation of expression profiles from the microarray analysis. Other *E. coli* strains such as verotoxigenic *E. coli* O157:H7 are important human pathogens, and outbreaks have been caused by contaminated drinking water (15) and cross-contamination of foods probably due to improper disinfection of food process equipment (5, 22). *E. coli* is therefore an important organism when the need arises to evaluate a potential antimicrobial agent.

Since the *Curvularia* haloperoxidase system has an almost instantaneous bactericidal effect, we analyzed the changes in gene expression during a short-term sublethal exposure of *E. coli* strain MG1655 to this system. This analysis allowed the identification of both single genes and gene clusters affected by the system, and we subsequently generated knockout mutants to confirm their role in the response of *E. coli* to the enzyme system. Our data allow us to suggest a mechanism by which the enzyme system may exert its killing effect. This study presents a novel method for investigating new antimicrobial compounds with the aim of elucidating the mechanism of antibacterial action.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The *E. coli* K-12 reference strain MG1655 was used in this work (1). Cells were routinely grown at 37°C on solid or in liquid Luria-Bertani medium supplemented with the appropriate antibiotics. Cells for stress induction experiments were grown at 37°C in morpholinepropanesulfonic acid (MOPS) minimal medium supplemented with 0.4% glucose (25). MOPS minimal medium was supplemented with 50 µg of kanamycin per ml when used as growth medium for knockout mutants.

**Monitoring growth and enumeration of bacteria.** Growth of bacterial strains was followed by measurements of optical density at 450 nm (OD450). Flasks were kept under agitation (~100 rpm) during sampling to ensure constant growth conditions. Plate counting was used to determine cell numbers after stress induction. Serial 10-fold dilutions were prepared in sterile physiological saline (0.85% NaCl) with 0.1% peptone (21677; BD). The first 10-fold dilution was prepared in sterile physiological saline with 0.1% peptone and 0.5% bovine serum albumin (A-7906; Sigma) to inhibit any residual antimicrobial effect of the *Curvularia* haloperoxidase enzyme (16). Appropriate dilutions were surface plated on Tryptone soy agar (CM131; Oxoid), and all plates were incubated at 37°C overnight at 45°C.

**Growth of bacterial strains** was described previously by Hansen et al. (16). A 24-h *E. coli* cell culture was diluted to an OD450 of 0.05 in a total volume of 10 ml and grown to an OD450 of 0.4 under agitation (150 rpm) at 37°C. Separate solutions of *Curvularia* haloperoxidase, hydrogen peroxide (1.0720; Merck), and KBr (P-9881; Sigma) were made 30.3 times stronger than the final concentration used in the experiment. Stock solutions of *Curvularia* haloperoxidase and KBr were prepared in MOPS minimal medium, and the hydrogen peroxide solution was mixed in Milli-Q water. A volume of 900 µl of cell suspension, 33 µl of *Curvularia* haloperoxidase, 33 µl of hydrogen peroxide, and 100 µl of KBr were mixed in a sterile Eppendorf tube. The final concentrations varied between 0.6 and 1.0 mg of enzyme liter⁻¹, 0.7 and 1.0 mM hydrogen peroxide, and 3 and 5 mM bromide. Suspensions of bacteria were incubated with 10 ml and grown to an OD450 of 0.4 under agitation (150 rpm) at 37°C. The mixture was cooled to 4°C and treated with NaOH (65°C for 30 min) to degrade the RNA strands, followed by neutralization with HCl. cDNA was further purified by using a QIAquick PCR purification kit (Qiagen). Fragmentation was performed with DNase I (0.6 U/µg of cDNA) in One-Phor-All buffer (Amscherm Pharmacia Biotech). DNase I was inactivated by heating at 98°C for 10 min, and the 3'-term of fragmented cDNA products was labeled with biotin by using the Enzo BioArray terminal labeling kit with biotin dUTP (Affymetrix).

**DNA microarray analysis.** *E. coli* GeneChip microarrays were purchased from Affymetrix (Santa Clara, Calif.). An antisense oligonucleotide array essentially the same as that described by Selinger et al. (34) (with the exception that probe sequences were the same as the coding region sequence) was employed. Biotin-labeled fragmented cDNA was hybridized to *E. coli* GeneChip microarrays overnight at 45°C in morpholineethanesulfonic acid (MES) buffer containing herring sperm DNA (100 µg/ml) and bovine serum albumin (500 µg/ml). Probe array washing and staining procedures were carried out as described by using GeneChip analysis suite software (version 4.0). Hybridized cDNA was fluorescently labeled in a three-step affinity binding procedure that involved binding of streptavidin to biotin-labeled binding of biotin-labeled antibody to streptavidin, and finally, binding of phycoerythrin-conjugated streptavidin to biotin-labeled antibodies. Probe arrays were scanned twice at 570 nm at a 3-µm resolution with an Affymetrix scanner, and a quantitative analysis of hybridization patterns and intensities was performed by using the GeneChip analysis suite software's expression analysis window.

**Data analysis.** Gene expression data was analyzed by using Microarray Suite 5.0 software (Affymetrix). The software calculates change calls, change in P values, and signal-log ratios. Change calls indicate an increase or decrease between a baseline array and an experimental array, and change in P value indicates the statistical significance for the change calls. The signal-log ratio is the relative change between the baseline and the experimental array expressed as the log ratio. In these studies, the signal-log ratios were calculated as the difference between expression levels after stress induction and expression values before stress induction. All experiments were performed in duplicate or triplicate unless indicated otherwise. The fluorescence of each array was normalized by scaling total chip fluorescence intensities to a common value of 5,000. Changes in expression levels that had a change call of decrease or increase together with a P value of <0.001 and a signal-log ratio of <2 or >2 were considered significant.

Expression ratios of all genes for each experiment along with all the results are available at http://www.dtu.min.dk/micro. **Construction of knockout mutants.** Mutant strains were constructed by using the λ Red recombinase gene replacement system (9). Briefly, the kanamycin gene from plasmid pKD4 was amplified by primers containing 50-nucleotide homology extensions for recombination (P1, 5'–AACACCGGCCGTTATGTAAGCATTGCCAGCAGCCGTCAGCGGTTGCTGGGCGATGGTGTGCTCGAAGCAGACGCCGCACCGGATATATACGACTCAAGAGA–3'); and P2, 5'–TCAATTACCCAGGACGACCCAGCAGCCGATAATACGACTCAAGAGA–3').
RESULTS

Killing of *E. coli* MG1655 with *Curvularia* haloperoxidase system. In order to assess the killing kinetics of the *Curvularia* haloperoxidase system, cells of *E. coli* MG1655 were exposed to a range of different enzyme concentrations (Fig. 1). The initial cell number was 8.4 ± 0.1 log CFU ml\(^{-1}\), corresponding to an OD\(_{450}\) of 0.4. Low concentrations of the enzyme system (0.6 mg of enzyme liter\(^{-1}\), 0.7 mM hydrogen peroxide, 3 mM bromide) had no effect on cell numbers following a 20-min exposure time. However, increasing the concentration of all three components (1 mg of enzyme liter\(^{-1}\), 1 mM hydrogen peroxide, 5 mM bromide) resulted in a log reduction of 3.2 ± 0.5.

Stress induction of *E. coli* MG1655 with *Curvularia* haloperoxidase system. *E. coli* strain MG1655 was subsequently exposed to sublethal levels of the *Curvularia* haloperoxidase system (0.4 mg of *Curvularia* haloperoxidase liter\(^{-1}\), 0.8 mM hydrogen peroxide, and 2.0 mM KBr). The *Curvularia* haloperoxidase system caused a temporary arrest of growth, after which the normal growth rate was resumed (Fig. 2A) (Table 1). The doubling times of *E. coli* MG1655 before and after stress induction were 63.3 and 69.3 min, respectively. During the 17 min of growth arrest, the doubling time increased to 630.1 min. Each of the individual components of the *Curvularia* haloperoxidase system was also tested for growth interference. Hydrogen peroxide affected growth, although not to the same extent as did the *Curvularia* haloperoxidase system (Fig. 2B) (Table 1). Exposure to hydrogen peroxide caused an increase in the doubling time (from 64.2 to 161.2 min, over an arrest time of 19 min) before returning to 71.5 min. Induction with either *Curvularia* haloperoxidase or bromide alone did not significantly affect the growth rate (Fig. 2C and D) (Table 1).

DNA microarray analysis of *E. coli* MG1655 stress induced with *Curvularia* haloperoxidase system. The *Curvularia* haloperoxidase enzyme system consists of three individual components, and gene induction or repression can be a consequence of individual components of the system or of the combined effect when *Curvularia* haloperoxidase is mixed with bromide and hydrogen peroxide. DNA microarray analysis revealed that several genes were affected either by the complete enzyme system or by the individual components, especially hydrogen peroxide. In order to evaluate genes affected by the *Curvularia* haloperoxidase system, it was therefore essential to differentiate between genes which were also altered in expression after induction with each of the individual components administered alone.

Identification of genes altered in expression in response to stress induction with hydrogen peroxide. Upon exposure to hydrogen peroxide, the regulatory protein OxyR induces the expression of several genes belonging to the OxyR regulon. Therefore, we specifically addressed genes belonging to the OxyR regulon after exposure to 0.8 mM hydrogen peroxide (Table 2). The expression of 73% of the genes in the OxyR regulon was induced with a log factor of >2 in the present study. Similarly, Zheng et al. (43) found a four-fold or greater induction of 73% of the genes in the OxyR regulon after stress induction with 1 mM hydrogen peroxide. Most of the previously described OxyR-regulated genes that were not induced in the present study (e.g., *fur*, *gor*, *dsbG*, *flu*, and *fluF*) were also not induced in the study by Zheng et al. (43). The strong correlation between the two data sets serves as an additional internal control to indicate the reliability of our microarray data.

Identification of genes altered in expression in response to stress induction with *Curvularia* haloperoxidase or bromide. Several genes were affected by stress induction with pure *Curvularia* haloperoxidase, but only *yieC*, which is a putative membrane protein, was down-regulated in both experiments. No genes were up-regulated in both experiments. In the response to bromide, 50 genes were either up-regulated or down-regulated. The down-regulated genes were primarily ribosomal genes. Other genes had a low baseline expression level, and a relatively small increase in expression level resulted in a log ratio of >2.

Identification of genes altered in expression in response to stress induction with the *Curvularia* haloperoxidase system. When identifying genes that were up-regulated by the *Curvularia* haloperoxidase system, we specifically addressed genes that were affected by the complete enzyme system and not by any of the components individually. This resulted in only a limited number of genes being involved in the response to the *Curvularia* haloperoxidase system (Table 3).

Based on known or proposed functions, a number of the genes induced by the *Curvularia* haloperoxidase system were of immediate specific interest. The *ibpA* and *ibpB* genes encode...
small heat shock proteins in \textit{E. coli}. In one of the three experiments, \textit{ibpA} was induced with a log \textsubscript{e} ratio of only 1.5, but the other two experiments showed log \textsubscript{e} inductions of 3.4 and 4.0, respectively. Ibpa and Ibpb are part of the cellular response to denatured proteins (35, 39). In \textit{E. coli}, the Cpx pathway also senses and responds to misfolded proteins (6, 8, 28). The overlapping open reading frames designated b3913 and b3914 have recently been shown to comprise the \textit{cpxP} gene (7). For simplification purposes, we have therefore referred to these two open reading frames as the \textit{cpxP} gene throughout the remainder of this paper. The \textit{cpxP} gene lies immediately adjacent to the \textit{cpxAR} sensor response regulatory genes and encodes a periplasmic protein involved in combating extracytoplasmic protein-mediated toxicity. The transcription of \textit{cpxP} was increased by a log \textsubscript{e} ratio of between 2.0 and 3.8 after the stress induction compared to the expression level before stress induction. Expression levels of \textit{cpxAR} were not affected by the stress induction. Additionally, we identified a cluster of six

![Diagram](http://aem.asm.org.org/)

**FIG. 2.** Stress induction of MG1655 with \textit{Curvularia} haloperoxidase system (A), hydrogen peroxide (B), \textit{Curvularia} haloperoxidase (C), and bromide (D). Arrows indicate times of stress induction. Data are means, and error bars indicate standard deviations of duplicate determinations.
adjacently linked genes (b0301 to b0306) to be induced at significantly high levels. The function of these genes is unknown.

A number of other single genes were also up-regulated after exposure to the *Curvularia* haloperoxidase system. Some of these genes encode known or putative membrane- or periplasm-located proteins that may be directly involved in the stress-induced response. For example, nlpA encodes an inner membrane lipoprotein that may reduce cell permeability (29, 42), while the tauA gene encodes a periplasmic protein whose expression is regulated by sulfate starvation (37). Other significantly enhanced genes included the gloA gene (which encodes a protein associated with enhanced tolerance to methylglyoxal) (23), nemA (which encodes an N-ethylmaleimide [NEM] reductase) (24), and yqhD and ytfG (both of which encode putative oxidoreductases).

The growth curve of *E. coli* after stress induction with the *Curvularia* haloperoxidase system indicated that the effect was only transient, as normal exponential growth resumed hereafter. In line with this observation, we assumed that the induced genes observed from our DNA microarray profiling would also return to levels observed in the uninduced state. Indeed, 1.5 h after stress induction, the expression level of all the up-regulated genes (except for b1970) returned to levels observed before stress induction (Table 3).

No clear tendency could be observed in the down-regulation of genes in the response to the *Curvularia* haloperoxidase system.

**Mutants lacking cpx or ykg gene clusters.** To evaluate whether the up-regulated cpx and ykg gene clusters were involved in a specific defense mechanism against the *Curvularia* haloperoxidase system or were part of an un specific response, knockout mutants MS669, MS710, and MS719 were constructed in which ykgB-D, cpxP, and cpxARP, respectively, were inactivated. MS669 and MS710 were stress induced with the *Curvularia* haloperoxidase system and compared to the parent MG1655 strain (Fig. 3A and B) (Table 1). As observed for MG1655, the growth rate of both MS669 and MS710 decreased for a period of approximately 15 to 16 min, after which it returned to normal. The similar reaction pattern of MS669, MS710, and MG1655 to stress induction with the *Curvularia* haloperoxidase system suggests that the ykgB-D gene cluster and the cpxP gene are dispensable with regard to the specific

### TABLE 1. Doubling times of *E. coli* MG1655 and mutant strains MS669, MS710, and MS719 before, during, and after stress induction with the *Curvularia* haloperoxidase system or individual components thereof

<table>
<thead>
<tr>
<th>Strain</th>
<th>Stress-inducing component</th>
<th>Concen of</th>
<th>Doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Curvularia haloperoxidase (mg/liter)</td>
<td>Bromide (mM)</td>
</tr>
<tr>
<td>MG1655</td>
<td><em>Curvularia</em> haloperoxidase system</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td><em>Curvularia</em> haloperoxidase</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Bromide</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>MS669</td>
<td><em>Curvularia</em> haloperoxidase system</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>MS710</td>
<td><em>Curvularia</em> haloperoxidase system</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>MS719</td>
<td><em>Curvularia</em> haloperoxidase system</td>
<td>0.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$ Growth arrested for approximately 17 min before resuming.
$^b$ Growth arrested for approximately 19 min before resuming.
$^c$ Growth arrested for approximately 16 min before resuming.
$^d$ Growth arrested for approximately 15 min before resuming.
$^e$ Growth arrested for approximately 110 min before resuming.
$^f$ Growth arrested for approximately 26 min before resuming.

$^a$ ND, not determined.

### TABLE 2. Induction of genes in the OxyR regulon of *Escherichia coli* MG1655 after exposure to H$_2$O$_2$

<table>
<thead>
<tr>
<th>Gene</th>
<th>b no.</th>
<th>Log induction ratio in the present study$^a$</th>
<th>Log induction ratio observed by Zheng et al.$^b$</th>
<th>Induction ratio observed by Zheng et al.</th>
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</thead>
<tbody>
<tr>
<td>dps</td>
<td>b0812</td>
<td>3.3</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>katG</td>
<td>b3942</td>
<td>5.2</td>
<td>44</td>
<td>44</td>
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<tr>
<td>grxA</td>
<td>b0849</td>
<td>4.7</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>ahpF</td>
<td>b0606</td>
<td>3.3</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>trxC</td>
<td>b2582</td>
<td>4.8</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>ahpC</td>
<td>b0605</td>
<td>2.3</td>
<td>20</td>
<td>20</td>
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<tr>
<td>fur</td>
<td>b0683</td>
<td>1.0</td>
<td>2.9</td>
<td>2.9</td>
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<tr>
<td>gor</td>
<td>b3500</td>
<td>1.4</td>
<td>2.1</td>
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<td>dsbG</td>
<td>b0604</td>
<td>−0.2</td>
<td>0.7</td>
<td>0.7</td>
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<td>flu</td>
<td>b2000</td>
<td>−3.1</td>
<td>1.0</td>
<td>1.0</td>
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<td>fhuF</td>
<td>b4367</td>
<td>1.3</td>
<td>0.4</td>
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<td>hemH</td>
<td>b0475</td>
<td>2.0</td>
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<td>ydcC</td>
<td>b1684</td>
<td>4.4</td>
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<td>ynhE</td>
<td>b1683</td>
<td>4.0</td>
<td>16</td>
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<td>ynhD</td>
<td>b1682</td>
<td>3.8</td>
<td>12</td>
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<tr>
<td>ynhC</td>
<td>b1681</td>
<td>4.0</td>
<td>8.3</td>
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<tr>
<td>b1680</td>
<td>b1680</td>
<td>4.3</td>
<td>3.5</td>
<td>3.5</td>
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<tr>
<td>ynhA</td>
<td>b1679</td>
<td>4.2</td>
<td>8.2</td>
<td>8.2</td>
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<td>b0006</td>
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<td>yuiA</td>
<td>b0389</td>
<td>2.9</td>
<td>56</td>
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<td>yhiM</td>
<td>b0848</td>
<td>−1.3</td>
<td>15</td>
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</tr>
<tr>
<td>yfhA</td>
<td>b0881</td>
<td>2.9</td>
<td>11</td>
<td>11</td>
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</table>

$^a$ Growth induction with 0.8 mM hydrogen peroxide. Significant log$_e$ ratios are indicated in boldface type.

$^b$ Growth induction with 1 mM hydrogen peroxide (43).
TABLE 3. Log_e ratios of E. coli MG1655 genes significantly up-regulated only by treatment with the Curvularia haloperoxidase system in sublethal concentrations and not by the single components of the enzyme system

<table>
<thead>
<tr>
<th>Gene cluster induced</th>
<th>b no.</th>
<th>Description</th>
<th>Log_e ratio for Curvularia haloperoxidase system in:</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
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<tbody>
<tr>
<td>yylN</td>
<td>b0395</td>
<td>Putative alpha helix chain</td>
<td>2.3 2.4 2.6 1.1 1.1 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0</td>
<td>2.1 2.2 2.3 1.4 1.4 1.4 1.4 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0</td>
<td>2.0 2.2 2.3 1.2 1.2 1.2 1.2 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0</td>
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</tr>
<tr>
<td>tauA^c</td>
<td>b0365</td>
<td>TauA transport system periplasmic protein</td>
<td>2.3 2.4 2.6 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4</td>
<td>2.1 2.2 2.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3</td>
<td>2.0 2.2 2.3 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2</td>
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<tr>
<td>ycfR</td>
<td>b1112</td>
<td>ORF, hypothetical protein</td>
<td>5.0 5.0 5.0 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6</td>
<td>4.4 4.1 4.1 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4 0.4</td>
<td>4.3 4.1 4.1 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3</td>
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<tr>
<td>b1649</td>
<td>b1560</td>
<td>ORF, hypothetical protein</td>
<td>3.1 3.1 3.1 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9 0.9</td>
<td>2.1 2.6 2.6 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3</td>
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<td>b1651</td>
<td>Lactoylglutathione lyase</td>
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<td>b1846</td>
<td>ORF, hypothetical protein</td>
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<td>b1987</td>
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Gene clusters induced with the Curvularia haloperoxidase system: 1. Expt 1, Expt 2, and Expt 3 are three independent experiments.

Gene descriptions are from the Affymetrix expression analysis sequence information database. ORF, open reading frame.

- Gene listed although one of the three log_e ratios was not >2.

The log_e ratio of transcript level 9 to 10 min after stress induction with the Curvularia haloperoxidase system versus transcript level before stress induction. Expt 1, Expt 2, and Expt 3 are three independent experiments.

In contrast, growth of MS719 (ΔcpxARP) (Fig. 3C) (Table 1) was significantly affected after stress induction with the Curvularia haloperoxidase system, and the growth did not resume until approximately 2 h after stress induction (albeit very slowly). Plate counting of MS719 revealed that cell numbers before and 19 min after stress induction were the same, indicating that growth was arrested after stress induction rather than that the cells were killed because of the treatment. E. coli strains containing mutations in the cpxAR gene locus are known to be more sensitive to several compounds, including hydrogen peroxide (P. De Wulf, unpublished data). To confirm that the altered stress response of MS719 was caused by the complete enzyme system and not by hydrogen peroxide alone, MS719 was stress induced only with hydrogen peroxide with the same concentration as that used in the enzyme system (Fig. 3D) (Table 1). Growth of MS719 was arrested for 26 min, after which the doubling time returned to the level observed before stress induction. The increased sensitivity to hydrogen peroxide does not resemble the severe growth arrest observed after treatment of MS719 with the Curvularia haloperoxidase system, which seems to be a response unique to this system.

**DISCUSSION**

That the Curvularia haloperoxidase system has a lethal effect against bacteria, yeasts, and filamentous fungi and is active against both planktonic and surface-associated microorganisms has been previously demonstrated (16). When evaluating a novel antimicrobial compound, it is crucial to understand its mechanism of action. This information allows for assessment of potential side effects such as the development of resistance and is also required in the approval process of the antimicrobial compound. The Curvularia haloperoxidase system is potentially useful as a disinfectant in many areas, such as the...
disinfection of contact lenses, equipment used in ocul- 
science, or medical devices, including pacemakers and urinary 
tract catheters.

Bacteria possess a wide range of defense mechanisms 
against antibacterial compounds and are therefore able to neu-
tralize certain harmful components, such as hydrogen per-
oxide, to repair damaged cell components (e.g., denatured pro-
teins) or to mutate and develop specific resistance 
mechanisms. By studying induced defense mechanisms, it may 
be possible to elucidate how an antimicrobial compound af-
facts the bacterial cell and thereby determine its mechanism of 
action. Most previous studies (10, 17, 18) of mechanisms of 
antimicrobial compounds have focused on changes in micro-
bial physiology or morphology, e.g., leakage of cell compo-
ients, changes in respiration, changes in intracellular pH, al-
terations in membrane structure, etc. DNA microarrays have 
previously been used to evaluate the stress response in E. coli 
when exposed to hydrogen peroxide (4, 43). In the present 
study, we demonstrate how DNA microarrays can be used to 
investigate gene expression in response to stress induction with 
sublethal doses of the Curvularia haloperoxidase system. Sub-
sequently, mutants were created in which some of the induced 
genes were deleted so as to identify whether the reaction 
mechanisms were specific for the enzyme system.

To analyze the expression of genes in E. coli that are altered 
upon exposure to the Curvularia haloperoxidase system, we 
used an Affymetrix DNA chip based on the E. coli reference 
strain MG1655. The chip used here was a custom-designed 
antisense oligonucleotide array that requires the conversion of 
mRNA to cDNA as part of the process for the generation of 
the labeled target sequence. We observed an induction of the 
ibpA, ibpB, and cpxP genes (among others) upon exposure to 
the Curvularia haloperoxidase system. Significantly, these three 
genes encode proteins that are known to play a role in the 
bacterial response to denatured proteins. ibpA and ibpB en-
code small heat shock proteins, and in vitro studies have shown 
that the IbpB protein can bind to heat-denatured proteins and 
hold them in a nonaggregating state and furthermore deliver 
them to the DnaK/DnaJ/GrpE chaperone system for subse-
quent refolding (35, 39). This function has been con-
firmed by in vivo studies in E. coli, which showed that overproduction 
of IbpA and IbpB stabilizes aggregates of heat-denatured pro-
teins (21). IbpA- and IbpB-overproducing strains of 
E. coli have also been shown to acquire higher levels of resistance to 
heat and superoxide stress (19). Furthermore, the expression 
of the ibpA and ibpB genes has also been observed to increase 
during stationary-phase growth (33). Increased expression of 
these two genes is therefore consistent with the pause in ex-
ponential growth induced by the Curvularia haloperoxidase 
system. It should be noted that of all of the genes induced by 
the Curvularia haloperoxidase system, only ibpA and ibpB 
were also induced during stationary-phase growth. Taken together, 
these findings indicate that the genes identified by our microar-
ray studies are specifically activated by the Curvularia haloper-
oxidase system.

The cpxP gene lies immediately adjacent to cpxRA (7). The 
CpxRA system senses and responds to envelope protein dis-
tress in E. coli by regulating the synthesis of several enzymes 
involved in the folding and degradation of periplasmic proteins 
(6, 8, 28). The exact mechanism of action of CpxP is unknown. 
CpxP is a repressor of the Cpx system, but during stress con-
ditions CpxP is strongly induced (11). It has been proposed that 
CpxP functions as a negative regulator during nonstress 
conditions but is inactivated during stress, perhaps by binding 
to misfolded proteins, allowing for activation of the pathway

FIG. 3. Stress induction of MS669 (MG1655 ΔykgBICD) (△) and 
MG1655 (○) with Curvularia haloperoxidase system (A). MS710 
(MG1655 ΔpxP) (▲) and MG1655 (○) with Curvularia haloperoxidase 
system (B). MS719 (MG1655 ΔcpxARP) (□) and MG1655 (○) with 
Curvularia haloperoxidase system (C), and MS719 (□) with 
hydrogen peroxide (D). Arrows indicate times of stress induction. Data are 
means, and error bars indicate standard deviations of duplicate deter-
minations.
(11, 30). In addition, CpxP may have a chaperone function under stress conditions (11). The Curvularia haloperoxidase system induced the transcription of cpxP, but it apparently did not affect the expression of the cpxAR genes, which was not induced by the stress induction.

The up-regulation of ibpA, ibpB, and cpxP indicates that the "Curvularia" haloperoxidase system damages proteins. This observation is in agreement with the fact that the addition of protein to the enzyme reaction mixture interferes with the antimicrobial effect by increasing the survival of microorganisms exposed to the "Curvularia" haloperoxidase system (16). The "Curvularia" haloperoxidase system probably damages proteins through oxidation. Other peroxidase systems such as the lactoperoxidase system are well described in previous studies (31, 41). In the presence of hydrogen peroxide, lactoperoxidase oxidizes thiocyanate (SCN⁻) to thioyanogen ([SCN]₂⁻), which hydrolyzes rapidly to hypothiocyanic acid (HOSCN) or hypothiocyanate (OSC²⁻). (SCN⁻), and HOSCN are able to oxidize protein sulfhydryl groups to sulfenyl thiocyanate derivatives. We believe that "Curvularia" haloperoxidase (like lactoperoxidase) oxidizes halides (for example, bromide to hypobromite), thereby causing oxidative stress in microorganisms, although this supposition has not been experimentally verified.

In addition to protein oxidation, the gene expression profile of E. coli exposed to the "Curvularia" haloperoxidase system may indicate that the enzyme system also caused lipid peroxidation. nemA (encoding an NEM reductase) was induced, and it has previously been postulated that lipid peroxidation is involved in the induction of NEM reductase (24). Miura et al. (24) found that NEM reductase activity in E. coli was induced by linoleic acid but not by oleic acid, which is less susceptible to lipid peroxidation than is linoleic acid. Furthermore, menadione, which generates superoxides, also induced NEM reductase activity. Phadture et al. (27) exposed E. coli to 4,5-dihydroxy-2-cyclopenten-1-one and observed an induction of nemA in conjunction with other genes known to respond to oxidative stress.

The results from our DNA microarray experiments prompted us to construct deletion mutants within the ykg gene cluster and the cpxP gene. These mutants did not have an altered sensitivity to the enzyme system compared to the wild type. An explanation of these results may be that they are part of a general stress response that is not specific to the "Curvularia" haloperoxidase system or are rather part of a broad defense specifically against the enzyme system. Alternatively, these proteins may possess a redundant phenotype that can be compensated for by as yet undefined proteins from similar or parallel functional pathways. Consequently, a mutant with a more extensive deletion of the Cpx pathway was constructed. In contrast to the cpxP mutant, the cpxARP mutant was notably more sensitive to the "Curvularia" haloperoxidase system than was the wild type, indicating a direct role for this pathway in coping with the stress induced by the enzyme system. These results demonstrate that the DNA microarray technology cannot be used as the sole technique when investigating mechanisms of action of new antimicrobial compounds. However, the array technique provides a very powerful and unique tool for insight into the system. By combining DNA microarray analysis and subsequent creation of knockout mutants, we were able to pinpoint one of the specific responses of E. coli—namely, the Cpx pathway, which is important for managing the stress from the "Curvularia" haloperoxidase system. The study shows that this novel method is suitable for investigating novel antimicrobial compounds with an aim to elucidate their mechanisms of action.

ACKNOWLEDGMENTS

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REFERENCES


