Aerotolerance and Peroxide Resistance in Peroxidase and PerR Mutants of Streptococcus pyogenes

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Survival in aerobic conditions is critical to the pathogenicity of many bacteria. To investigate the means of aerotolerance and resistance to oxidative stress in the catalase-negative organism Streptococcus pyogenes, we used a genomics-based approach to identify and inactivate homologues of two peroxidase genes, encoding alkyl hydroperoxidase (ahpC) and glutathione peroxidase (gpoA). Single and double mutants survived as well as the wild type under aerobic conditions. However, they were more susceptible than the wild type to growth suppression by paraquat and cumene hydroperoxide. In addition, we show that S. pyogenes demonstrates an inducible peroxide resistance response when treated with sublethal doses of peroxide. This resistance response was intact in ahpC and gpoA mutants but not in mutants lacking PerR, a repressor of several genes including ahpC and catalase (katA) in Bacillus subtilis. Because our data indicate that these peroxidase genes are not essential for aerotolerance or induced resistance to peroxide stress in S. pyogenes, genes for a novel mechanism of managing peroxide stress may be regulated by PerR in streptococci.

Adaptation to an aerobic environment provides organisms with a wider selection of ecological niches for survival. The major cost associated with this evolutionary gain is constant exposure to the deleterious effects of oxygen, particularly the partially reduced forms of oxygen that include peroxide, superoxide, and hydroxyl radicals (23). In response to this toxic exposure, aerobic organisms have evolved powerful strategies to defend themselves against the damaging effects of oxygen.

The group of organisms collectively known as the lactic acid bacteria provides a useful example of adaptation to the aerobic environment. Unlike organisms that have evolved a system of oxidative phosphorylation, these bacteria have an exclusively fermentative metabolism, and they lack many of the well-characterized enzymes thought to be essential for aerobic survival (13). Nevertheless, most lactic acid bacteria are aerotolerant to some degree and thus are considered facultative anaerobes. Surprisingly, growth is unaffected or even enhanced by exposure to oxygen for some species (38).

Recent investigations have suggested that interaction with oxygen plays an important role in the pathogenesis of infections caused by the lactic acid bacterium Streptococcus pyogenes. This bacterium is an important human pathogen that is responsible for various diseases, including soft tissue infections that range from minor and self-limiting (pharyngitis, impetigo) to severely destructive and life-threatening (necrotizing fasciitis). Toxigenic infections (toxic shock syndrome) and several autoimmune diseases (rheumatic fever, acute postinfectious glomerulonephritis) are also associated with infection by S. pyogenes. Adhesion to host tissues is likely to be an early event in pathogenesis for all of these diseases. Expression of a major adhesin, the fibronectin-binding protein known as protein F or Sfb, is environmentally regulated, being preferentially expressed in aerobic environments (51). Regulation occurs at the level of transcription and is apparently linked to a pathway that senses oxidative stress (22). These observations suggest that S. pyogenes encounters and reacts to an aerobic environment during infection.

Consistent with an at least partially aerobic lifestyle, most isolates of S. pyogenes are highly aerotolerant, and growth yields can actually be increased under aerobic conditions when grown on certain substrates (21). To thrive in an aerobic environment, it is likely that S. pyogenes must possess mechanisms for defense against reactive oxygen species. A critical issue concerns defense against hydrogen peroxide (H₂O₂), a highly reactive molecule that can readily diffuse across cellular membranes and oxidatively damage a number of vital cellular components, including membrane lipids, enzymes, and DNA (39, 44). Not only may S. pyogenes encounter peroxide during the infection of tissue but, like many other lactic acid bacteria, S. pyogenes also has the capacity to produce large amounts of peroxide endogenously when it is growing under aerobic conditions (21). A large body of evidence collected from analysis of multiple bacterial species has implicated catalase, a heme-containing peroxidase, as the major factor responsible for defense against peroxide (19). The importance of catalase is further emphasized by the fact that most facultative and aerobic bacteria contain multiple catalases (14). However, as is characteristic of lactic acid bacteria, S. pyogenes does not synthesize heme and lacks catalase (13). The specific mechanisms by which S. pyogenes adapts to peroxide stress are unknown.

In this study, we have examined the adaptive response of S. pyogenes to peroxide stress and the potential contribution of alternative peroxidases to this response. Using a genomics-based approach, we found that S. pyogenes contains homologues of two peroxidases that have been characterized in other species. Construction and analysis of several mutants revealed that these peroxidases contribute to defense against high-level oxidative stress. In contrast, the inactivation of these genes neither impairs growth under aerobic conditions nor hinders the ability of S. pyogenes to mount an adaptive response against peroxide. Interestingly, this adaptive response was constitutively induced in mutants lacking PerR, a negative regulator of several genes, including ahpC and catalase (katA) in Bacillus subtilis. However, because these latter two genes do not appear to be essential to the S. pyogenes adaptive response,
PerR may control expression of genes responsible for a potentially novel strategy for adaptation to aerobic growth environments in S. pyogenes.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The host for molecular cloning experiments was Escherichia coli DH5α. For peroxide resistance experiments involving E. coli, strain CH34 was used. For probing of αβ genes by PCR, the following bacterial strains were used: Staphylococcus aureus Newman (16), Listeria monocytogenes 103935 (45), and B. subtilis Marburg 166 (34). Luria-Bertani broth (LB) or 2×YT or 1×T broth (137 mM NaCl, 2% yeast extract [wt/vol], and 1.4% agar) were used to culture E. coli. Strains of S. pyogenes were grown in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (THY medium) at 37°C to prepare solid medium for S. pyogenes. Bacto Agar (Difco) was added to THY medium to a final concentration of 1.4%. Various atmospheres for cultures of S. pyogenes were created using conditions established in prior investigations (22). Briefly, for aerobic cultures, streptococci were grown on solid medium in ambient air (20% O2, 0.03% CO2) or in liquid medium in a 125-ml Erlenmeyer flask filled to 8% of its total volume on an orbital shaker rotating at 225 rpm. For near-anaerobic cultures, streptococci were grown statically in 10-ml volumes of liquid medium in a sealed 15-ml culture tube with a commercial additive to produce anaerobic conditions (Oxyrase). When appropriate, the 10-ml volume of liquid media in a sealed jar with orbital shaker rotating at 225 rpm. For near-anaerobic cultures, streptococci were incubated on solid media in a sealed jar with a commercial additive to produce anaerobic conditions (Oxyrase). When appropriate, the 10-ml volume of liquid media in a sealed 15-ml culture tube with a commercial additive to produce anaerobic conditions (Oxyrase). When appropriate, the 10-ml volume of liquid media in a sealed 15-ml culture tube with a commercial additive to produce anaerobic conditions (Oxyrase).

Construction of reporter plasmids. A 1.1-kb genomic fragment upstream and including the first 10 codons of abpC were amplified by PCR using the primers 5′AhpXhoI (GGTT GAAA TCCTC CGAGG ATGTC ATCAA AGC) and 3′PerSacI (GGGATCC CACGG TGACG TAAGT CCTAA AGC) and the reporter plasmids pABG5 (CTTG TGCCA TATGG CTACCA TCTCC GC) and pApB6 (GAGC ACAGG TCGA TTTT TCGA TCTCC GC) (see Fig. 1). This region included all of the intervening sequence between abpC and the next upstream and divergently transcribed open reading frame that showed homology to a cold shock protein E gene. Similarly, the 335-bp genomic fragment upstream and including the first 35 codons of the oligoendopeptidase F gene that immediately precedes gpoA in an apparent operon were amplified using the primers 5′AhpPro (CCTAA ATAAT CAGGA TCCGT ATTAT TTTGA) and 3′GpxPro2 (GGGATCC CACGG TGACG TAAGT CCTAA AGC) and the reporter plasmids pGpoPho, pMrgPho, and pAhpPho (see Fig. 2). This region includes the intervening region between the oligoendopeptidase gene and the upstream and divergently transcribed phosphoethanolamine carboxylase gene. The 411-bp genomic fragment upstream of and including the first 36 coding sequences of mrga was amplified by PCR using the primers 5′MrgSacI (GGCT CGAAG ATCAG TTAGT CCTAA AGTAG) and 3′MrgPro (GCGC AGAGA ATCTC GCAGC GTTAT TTTTA) and the reporter plasmids pMrgPho, pApB6 (GAGC ACAGG TCGA TTTT TCGA TCTCC GC), and pApB5 (see Fig. 2). This region encompasses the entire region between MrgA and a divergent upstream open reading frame with homology to a type 4 prepilin-like gene. Each of these constructs contained a BamHI restriction site (underlined). In addition, the 3′ primers contained a stop codon in the relevant reading frame to prevent translational fusions with the reporter (boldface). These PCR products were inserted into the EcoRI and BamHI restriction sites of pABG5 (24) to place the fragment upstream of the pABG5 vector (24).

Expression of abpC and gpoA. The abpC, gpoA, and mrga reporter plasmids constructed in the previous section were introduced into various S. pyogenes hosts and used to assess expression in the presence or absence of inducing concentrations of H2O2 (50 μM), ethanol (5%), cumene hydroperoxide (0.025%), or methyl viologen (10 mM). Activities of the various reporters were also compared between the wild type and perR mutants. The plasmids pABG5, in which a rofA promoter controls photoZ, and pABG6, a derivative of pABG5 in which the rofA promoter has been inactivated, were used in parallel experiments as positive and negative controls, respectively (24). Expression was monitored by determination of alkaline phosphatase activity in cell-free culture supernatants as described previously (24).

Analysis of methyl viologen sensitivity. Sensitivity to the oxidative stress-promoting compound methyl viologen (paraquat) was assayed as follows. Methyl viologen (Sigma) was added to liquid THY medium up to a final concentration of 10 mM. This medium was then inoculated with 5 μl of an overnight culture grown in near-anaerobic conditions. The culture density was determined by measuring optical density at 600 nm (OD600). Cultures were incubated at 37°C for 18 h at 37°C. The same analysis was conducted in the presence of catalase (1 mg/ml; Sigma) or in the presence of hemoglobin (1 mg/ml; Sigma).
The activity of superoxide dismutase in *S. pyogenes* was assayed as previously described (22).

**Sensitivity to peroxides.** The strain of interest was cultured under near-anerobic conditions to mid-log phase and a 50-μl aliquot was spread evenly over the surface of a THY agar plate. A 10-μl aliquot of various concentrations of hydrogen peroxide (Sigma) diluted in water or cumene hydroperoxide (Sigma) at a concentration of 20% (vol/vol [in ethanol]) was then added to a sterile 10-mm-diameter filter disk placed at the center of each plate. After incubation at 37°C for 16 h under aerobic conditions, the area of the zone of inhibition was calculated for each mutant and compared to that of the respective wild-type strain.

**Characterization of resistance to hydrogen peroxide challenge.** The ability of various strains to survive a lethal challenge by H2O2, with or without prior H2O2 exposure, was determined as follows: a 10-μl aliquot of an anaerobic overnight liquid culture was used to inoculate 10 ml of medium which was then incubated under near-anerobic conditions at 37°C. When the absorbance (OD600) of this culture reached 0.040, H2O2 was added to a sublethal concentration (50 mM), and the incubation continued until the OD600 reached 0.070. At this time, H2O2 was added to a final concentration of 4 mM. After an additional 3 h of incubation, the number of CFU was determined under anaerobic conditions. The ability of other agents to induce resistance to H2O2 was assessed by substitution of the initial inducing dose of peroxide with either ethanol (5% [vol/vol]), nalidixic acid (250 μM; Sigma), or mitomycin C (200 nM; Sigma). The inducing dose used for each agent represents the highest concentration of that agent which did not inhibit bacterial growth during an overnight incubation under near-anerobic conditions. For comparison, the sensitivity of *E. coli* CH734 to H2O2 was determined using the same assay except that Luria-Bertani medium was substituted for THYB. The data reported represent the mean and standard error of the mean derived from at least two independent experiments, each of which was conducted in triplicate.

In selected experiments, the viability of cultures was assessed by using a vital stain (Live/Dead Cell Viability Kit; Molecular Probes) and microscopic observation or by determining numbers of CFU following brief sonication (Branson model W-185, 22°C, microprobe at setting of 6, five bursts of 5 s each) to disrupt the streptococcal chains. However, since the differences between viable cells counted before or after challenge were similar to that derived by the direct determination of CFU, neither procedure was routinely performed.

**RESULTS**

**Identification of peroxidases in the *S. pyogenes* genome.** Since *S. pyogenes* lacks catalase, we hypothesized that it contains an alternative peroxidase. The available *S. pyogenes* genome information was evaluated for the presence of potential homologues to other known bacterial peroxidases (see Materials and Methods). Only three sequences in the *S. pyogenes* genome database showed significant homology to the peroxidase genes used in the searches (*P* < 0.05). The first sequence appeared homologous to NADH peroxidase (GenBank accession no. 1942624) of *B. subtilis*; however, genetic and biochemical studies have identified this gene as NADH oxidase (21). Of the other two open reading frames identified, the first showed homology to the peroxidase subunit of alkyl hydroperoxide reductase (*ahpC*) (17) (see Fig. 1). Subsequently, the reading frame downstream of this *ahpC* homologue was found to be similar to *ahpF*. In *B. subtilis*, these two genes encode a two-subunit protein complex and are known to form an operon (3). The other open reading frame identified showed homology to the glutathione peroxidase (*gpoA*) of *Lactococcus lactis* (see also Fig. 2). In addition to a high overall level of homology, each of these two putative peroxidases contained highly conserved residues located in the active sites of these enzymes known to be essential for their respective peroxidase activities (Fig. 1 and 2).

**Construction of mutants.** In order to analyze the contribution of these putative peroxidases to the aerobic growth of *S. pyogenes*, we constructed mutants in which these genes were inactivated. Since it was anticipated that these mutants may have growth defects and since the transcriptional organization of these loci has not been characterized, in-frame deletions were constructed to create stable nonpolar mutations. For alkyl hydroperoxide reductase, a deletion was introduced into *ahpC* which removed 45 amino acids from the central region of the polypeptide that was anchored amino terminally in a highly conserved domain immediately adjacent to the putative active site residue C46 (Fig. 1). For *gpoA*, a region encompassing 57 amino acid residues was removed, including a cysteine (C36) and the highly conserved residues surrounding it that are cons- tants of the active site (Fig. 2) (17). These mutations were introduced into two unrelated strains of *S. pyogenes* (JRS4 and HSC5) to construct mutants defective in one or both of these peroxidases (Table 1).

**Characterization of stress phenotypes.** Alkyl hydroperoxide reductase catalyzes the pyridine nucleotide-dependent reduction of organic hydroperoxides and H2O2 (43). Recent evidence also implicates these proteins in enzymatic defense...
against reactive nitrogen species (9). Mutants lacking functional AhpC or AhpF in some other organisms are hypersensitive to organic hydroperoxides such as cumene hydroperoxide (3). The *S. pyogenes* *ahpC* mutants were examined for a similar phenotype in a disk diffusion assay which revealed an enhanced sensitivity to cumene hydroperoxide, with zones of growth inhibition approximately 130% larger than those of the wild type when tested over a broad range of concentrations (5 to 20% cumene hydroperoxide). Glutathione peroxidases have been best characterized from mammalian cells and are also thought to provide protection from both H$_2$O$_2$ and organic hydroperoxides (18, 49). Their function in prokaryotic cells has been less well studied; however, glutathione peroxidase-defective mutants of *Neisseria meningitidis* are hypersensitive to oxidative stress induced by methyl viologen (40). Similarly, *gpoA* mutants of *S. pyogenes* demonstrated an enhanced sensitivity to methyl viologen which was apparent as the inability to grow in the presence of a concentration of methyl viologen that did not affect the growth of the wild-type strain (see below). Strains with mutations in *ahpC* also demonstrated this sensitivity (see below).

**Growth phenotypes of peroxidase mutants.** The gram-positive bacterium *B. subtilis* contains both a catalase and an alkyl hydroperoxide reductase (5). Mutations in the genes encoding either peroxidase have no effect on the ability of the organism to grow under aerobic conditions (5). However, a mutant that is simultaneously defective for both grows very poorly aerobically (5). Since *S. pyogenes* is naturally catalase deficient, it was expected that an *ahpC* mutant would be equivalent to the *B. subtilis* peroxidase double mutant and would demonstrate poor aerobic growth characteristics. However, the *S. pyogenes* *ahpC* mutants did not demonstrate any observable growth restrictions when examined under several types of aerobic culture, including culture on solid medium in ambient air and culture in liquid medium in a shaking flask (data not shown). In addition, the *ahpC* mutants did not demonstrate any increased sensitivity to H$_2$O$_2$ when analyzed both by a disk diffusion assay and by determination of MICs (data not shown). Identical results were obtained for *gpoA*-deficient mutants.

**Peroxidase mutants are sensitive to methyl viologen.** Although the various peroxidase mutants were proficient for growth under aerobic conditions, they did demonstrate sensitivity to certain forms of extreme oxidative stress. Specifically, when cultured in the presence of a concentration of methyl viologen that has minimal impact on the growth of the wild-type strains (10 mM) neither the *ahpC*- nor the *gpoA*-deficient mutants showed any growth deficiencies. The *ahpC* mutants of *S. pyogenes* were compared with wild type for their susceptibility to methyl viologen over a broad range of concentrations. The wild-type strain was able to grow at concentrations up to 100 mM, whereas the *ahpC*-defective mutants were no longer able to grow at concentrations greater than 50 mM.

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

**FIG. 2.** Glutathione peroxidase gene in *S. pyogenes*. The position and direction of *gpoA* is shown relative to its upstream neighbor. The percent amino acid identity and similarity for the closest match found in a TBlastN search are shown. The region inserted into the reporter plasmid for expression studies is underlined. Below is a partial alignment of four glutathione peroxidase genes, showing the conserved cysteine (C36), asparagine (N99), and C-terminal region (including N125). The region of the in-frame deletion is indicated by arrows. In the process of making the deletion, a proline and a glycine (underlined) were replaced by arginines.

**TABLE 1.** *S. pyogenes* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Characteristic</th>
<th>Source or reference</th>
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<tr>
<td>HSC5</td>
<td>Wild type</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>HAHP</td>
<td><em>ahpC</em>&lt;sub&gt;D&lt;/sub&gt;133–292</td>
<td><em>ahpC</em> inactivated in HSC5</td>
<td>This work</td>
</tr>
<tr>
<td>HGLT</td>
<td><em>gpoA</em>&lt;sub&gt;D&lt;/sub&gt;42–212</td>
<td><em>gpoA</em> inactivated in HSC5</td>
<td>This work</td>
</tr>
<tr>
<td>HAG&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>ahpC</em>&lt;sub&gt;D&lt;/sub&gt;133–292; <em>gpoA</em>&lt;sub&gt;D&lt;/sub&gt;42–212</td>
<td><em>ahpC</em> and <em>gpoA</em> inactivated in HAG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>JRS4</td>
<td>Wild type</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td>JAHP</td>
<td><em>ahpC</em>&lt;sub&gt;D&lt;/sub&gt;133–292</td>
<td><em>ahpC</em> inactivated in JRS4</td>
<td>This work</td>
</tr>
<tr>
<td>JAG</td>
<td><em>ahpC</em>&lt;sub&gt;D&lt;/sub&gt;133–292; <em>gpoA</em>&lt;sub&gt;D&lt;/sub&gt;42–212</td>
<td><em>ahpC</em> and <em>gpoA</em> inactivated in JAG</td>
<td>This work</td>
</tr>
<tr>
<td>HAΔPer</td>
<td><em>perR</em>&lt;sub&gt;D&lt;/sub&gt;198</td>
<td><em>perR</em> inactivated in HAΔPer</td>
<td>This work</td>
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<sup>a</sup> Strained derived by integration of the given plasmid into the indicated strain.
mutants demonstrated any detectable growth (Fig. 3). The ahpC gpoA double mutant was even more sensitive to methyl viologen than the single mutants, with no growth at a concentration of 1 mM (data not shown). Methyl viologen acts to increase intracellular levels of superoxide (27). Since mutants expressed superoxide dismutase at levels equivalent to those for the wild-type cells (data not shown), it is unlikely that superoxide is the source of lethal stress to the mutants. Rather, since H₂O₂ is a product of the dismutation of superoxide, high levels of superoxide could also result in increased concentrations of H₂O₂ and, in turn, other reactive species generated from the reaction of H₂O₂ with a variety of substrates. This hypothesis is supported by the observation that the addition of catalase could rescue the growth of the peroxidase mutants in the presence of methyl viologen, but the same effect was not seen with the addition of hemoglobin (Fig. 3). A small stimulation of growth above background values in the presence of methyl viologen was observed when hemoglobin was added (Fig. 3), which may be due to the weak peroxidase activity of hemoglobin (20). These data indicate that AhpC and GpoA contribute to defense under conditions of extreme and continuous oxidative stress and that both peroxidase gene products are required for optimal resistance.

AhpC and GpoA are not required for an induced resistance to peroxide. The observation that neither AhpC nor GpoA were required for normal growth under aerobic conditions suggested that S. pyogenes may have alternative strategies for resistance to peroxide. To examine this question, the kinetics of interaction with peroxide were examined in greater detail. When a culture of a wild-type strain was challenged at mid-log phase (approximately 10⁶ CFU) with 4 mM H₂O₂ and the number of viable CFU was examined after 2 h, it was observed that the number of viable CFU decreased ca. 0.4 log. When examined at 3 h, the number of viable CFU had decreased by >3.0 log (data not shown). These values were actually less than those obtained for a culture of E. coli CH734 under these same conditions, for which the number of CFU decreased by about 2.0 log after 2 h and >4 log after 3 h (data not shown). These data suggest that, under these conditions, S. pyogenes is not hypersensitive to peroxide compared to E. coli, a catalase-containing bacterium. Furthermore, when ahpC and gpoA mutants were examined, instead of demonstrating an enhanced sensitivity, they were in fact more resistant to peroxide challenge, with viability decreasing by only about 1.5 log 3 h after challenge (data not shown). A similar phenomenon has been reported for alkyl hydroperoxide reductase-deficient mutants of B. subtilis, where it appears that inactivation of the ahpC results in low-level oxidative stress sufficient to induce an adaptive resistance response to peroxide (3).

These data suggest that S. pyogenes may also possess an adaptive resistance response to peroxide. To test this, growing cultures were treated with a sublethal dose of H₂O₂ (50 μM) 1 h prior to challenge with a lethal dose (4 mM). As before, viability was examined after an additional 3 h of incubation. When treated in this fashion, wild-type strains were >100-fold more resistant to lethal peroxide challenge (Fig. 4; wild type, H₂O₂ induction). Similar to the wild type, both ahpC and gpoA mutants were protected by pre-exposure to a sublethal concentration of H₂O₂ (Fig. 4; AHP⁻, H₂O₂ induced; GPO⁻, H₂O₂ induced). Bacteria containing mutations in both ahpC and gpoA were approximately 10 times more sensitive to peroxide challenge than wild-type bacteria (Fig. 4; AHP⁻ GPO⁻, no induction). Nonetheless, the double mutants demonstrated a striking resistance response; when pretreated with sublethal peroxide, they were up to 1,000 times more resistant to lethal peroxide challenge than without pretreatment (Fig. 4; AHP⁻ GPO⁻, H₂O₂ induced). Taken together, these data indicate that S. pyogenes can mount a vigorous adaptive response to peroxide stress and that this response does not require the contributions of AhpC or GpoA.

As an additional test for involvement of AhpC and GpoA in inducible peroxide resistance, we studied expression of the peroxidase genes. Reporter plasmids were constructed by inserting the genomic regions upstream of ahpC and gpoA before a promoterless alkaline phosphatase reporter. Expression of the two reporter alleles in S. pyogenes was comparable with that of the well-characterized rofA promoter in this vector, indicating that functional promoters were identified by this analysis.
However, expression of the \( \text{ahpC} \) and \( \text{gpoA} \) reporters did not increase in wild-type cells in the presence of inducing levels of \( \text{H}_2\text{O}_2 \) or ethanol (data not shown). In addition, alkaline phosphatase activity from both reporters was not higher in wild-type cells that were incubated in the presence of 10 mM methyl viologen or 0.025% cumene hydroperoxide (data not shown). These findings lend further support to the observation that AhpC and GpoA are not part of the adaptive response to peroxide stress.

**Peroxide resistance can be induced by ethanol.** Insight into the pathway which controls an adaptive response can often be gained from an examination of whether the response is induced by a specific stress or whether other stress-promoting agents can induce the protective response against the agent of interest (19). The SOS response is a highly regulated response involved in resistance to agents which damage DNA, including \( \text{H}_2\text{O}_2 \) (56). However, treatment with two different agents known to induce the SOS response (mitomycin C and nalidixic acid) (56) resulted in a level of killing by \( \text{H}_2\text{O}_2 \) that did not differ from untreated cultures (Fig. 4; compare wild type un-induced to treatment with nalidixic acid or mitomycin C). Similar results were obtained in parallel experiments with the \( \text{ahpC} \) and \( \text{gpoA} \) mutants (Fig. 4). In contrast, treatment with a sublethal concentration of ethanol (5% [vol/vol]) was highly effective at inducing a protective response to subsequent challenge with a lethal dose of peroxide in wild-type bacteria and in both peroxide mutants (Fig. 4). In fact, under these conditions, ethanol was a more effective inducing agent than \( \text{H}_2\text{O}_2 \) itself (Fig. 4, wild type).

**\( \text{S. pyogenes} \) may lack \( \sigma^B \).** In gram-positive bacteria, a large number of stress-induced genes are regulated by a pathway known as the general stress response (53). Since induction by ethanol is a signature of the general stress response (52), the possibility existed that the observed adaptive response to \( \text{H}_2\text{O}_2 \) was a manifestation of this pathway. A key element in the regulation of the general stress response is the alternative sigma factor \( \sigma^B \) and its interaction with the anti-sigma factor RbsW (25, 53). This sigma factor is highly conserved among gram-positive organisms, including \( \text{S. aureus} \), \( \text{L. monocytogenes} \), and \( \text{B. subtilis} \) (36, 53, 54). The genes encoding \( \sigma^B \) in each of these three species are between 59 and 66% identical at the amino acid level. However, when the available \( \text{S. pyogenes} \) genome information was examined, there were no sequences with significant \( P < 0.33 \) similarity to the \( \text{S. aureus} \) \( \sigma^B \) gene other than the housekeeping sigma factor \( \sigma^A \). Similarly, no clear homologues to other elements of the \( \sigma^B \) pathway were apparent, including RsbU and RsbW. As an alternative approach, primers for PCR amplification were designed based on an examination of highly conserved elements of the \( \sigma^A \) gene sequence. While a PCR product of the expected size was amplified using these primers from several different gram-positive organisms, no product was amplified when the chromosome of \( \text{S. pyogenes} \) strains that display the adaptive response was used as a template (data not shown). These data indicate that while the adaptive response of \( \text{S. pyogenes} \) to peroxide shares many phenotypic characteristics of the general stress response, the \( \sigma^B \) pathway is either highly divergent or may be missing altogether.

**Regulation of the inducible peroxide resistance response involves PerR.** In addition to the \( \sigma^B \) general stress pathway, \( \text{B. subtilis} \) also harbors a specific response to hydrogen peroxide stress. The peroxide-sensitive PerR regulon in that species is known to take part in the transcriptional regulation of peroxidasises such as catalase and alkyl hydroperoxidase and of protective proteins such as MrgA, a DNA-binding protein (4, 10). Mutational analyses have indicated that PerR is a negative regulator of katA, ahpC, and mrgA expression in \( \text{B. subtilis} \). Notably, mutants lacking PerR were found to be hyper-resistant to hydrogen peroxide in a disk diffusion assay. Examination of the genome information and other published work suggests that \( \text{S. pyogenes} \) contains homologues for \( \text{perR} \) and \( \text{mrgA} \) in addition to \( \text{ahpC} \) (4). To analyze the role of PerR, we constructed an in-frame deletion that removes the N-terminal one-third of \( \text{perR} \), including a region that encodes most of the putative DNA-binding region of the protein (Fig. 5). The resulting mutant \( \text{HΔPer} \) proliferated as well as the wild type in aerobic growth conditions; however, no significant derepression of the \( \text{ahpC} \), \( \text{gpoA} \), and \( \text{mrgA} \) reporter con-
structs were observed in HΔPer compared to the wild type (data not shown). These findings indicate that, in contrast to what has been shown in B. subtilis, PerR does not appear to repress transcription of ahpC, gpoA, and mrgA in S. pyogenes. However, the PerR mutant was derepressed for the inducible peroxide resistance response and survived lethal hydrogen peroxide challenge approximately 100 times better than the wild-type cells, even without preinduction with a sublethal dose of hydrogen peroxide or ethanol (Fig. 6). Interestingly, while the high level of resistance in the PerR mutant was not affected by pretreatment with peroxide, an additional level of resistance to lethal peroxide challenge could be induced by ethanol (Fig. 6). This observation is consistent with the observation that ethanol consistently induced higher levels of resistance to peroxide than did peroxide itself (see above) and suggests that the response induced by ethanol includes an additional regulatory component independent of PerR.

DISCUSSION

We employed functional genomics to investigate the mechanisms of defense against toxic oxygen species in the lactic acid bacterium S. pyogenes. Using this approach, we have shown...
that while alkyl hydroperoxide reductase (AhpC) and glutathione peroxidase (GpoA) contribute to resistance against methyl viologen and cumene hydroperoxide, neither enzyme is required for growth under aerobic conditions or for the ability of S. pyogenes to mount an induced response to peroxide stress. We have also found that the induced response shares many characteristics with the $\sigma^d$ general stress response pathway but that S. pyogenes may lack $\sigma^d$. On the other hand, we have discovered that PerR is a negative regulator of the inducible characteristics with the katA or the oxidoreductase encoded by $\text{ahpC}$ comes from comparison of these data to similar studies conducted on other organisms. For example, S. mutans mutants deficient in either the catalase encoded by katA or the oxidoreductase encoded by $\text{ahpC}$ grow normally under aerobic conditions. However, a katA $\text{ahpC}$ double mutant exhibits a number of striking phenotypes, including slow growth in liquid media and accelerated lysis on certain solid media. Significantly, while the Bacillus double mutant can grow as well as the wild type under microaerobic conditions on rich medium, it grows very poorly under these conditions on minimal medium and fails to grow at all under normal aerobic conditions. Since S. pyogenes is naturally catalase deficient, the observation that the $\text{ahpC}$ mutant is not defective for growth suggests that S. pyogenes has alternative strategies for management of peroxide stress.

Further support for the involvement of a novel mechanism comes from comparison of these data to similar studies conducted in the gram-positive bacterium B. subtilis. For example, B. subtilis mutants deficient in the catalase encoded by katA or the oxidoreductase encoded by $\text{ahpC}$ grow normally under aerobic conditions. However, a katA $\text{ahpC}$ double mutant exhibits a number of striking phenotypes, including slow growth in liquid media and accelerated lysis on certain solid media. Significantly, while the Bacillus double mutant can grow as well as the wild type under microaerobic conditions, B. subtilis $\text{ahpC}$ mutants are only slightly more sensitive to cumene hydroperoxide than is the wild type, again suggesting a possible alternate mechanism for peroxide resistance in streptococci.

While resistance to peroxide has not been well studied in most lactic acid species, mechanisms of aerotolerance appear to be diverse among this family, and novel mechanisms of defense against peroxide are not unprecedented. For example, one species of lactobacilli (Lactobacillus sake) produces a catalase that utilizes heme scavenged from the organism’s environment. A second lactobacillus species, Lactobacillus plantarum, harbors a pseudocatalase that uses two manganese atoms in place of heme at its active site. In Enterococcus faecalis, a cystinyl redox center-containing NADH peroxidase has been well characterized, and several oral streptococcal species, including Streptococcus gordonii, contain a gene with homology to the periplasmic thiol peroxidase of E. coli. As noted above, examination of the available genome information failed to reveal any clear homologues of these other classes of peroxidases in S. pyogenes. However, given this diversity in mechanisms of achieving aerotolerance among lactic acid bacteria, it is not unreasonable to expect that S. pyogenes may have evolved other unique and as-yet-uncharacterized pathways for dealing with oxidative stress.

Identification of such alternative strategies will be facilitated by our studies of the PerR homologue in S. pyogenes. Even though the peroxidases known to be repressed by PerR in B. subtilis, namely, katA and $\text{ahpC}$, are not involved in the inducible response to hydrogen peroxide in S. pyogenes, mutants lacking the PerR homologue are constitutively resistant to hydrogen peroxide challenge. This finding suggests that PerR represses alternate genes important for the inducible response. MrgA has been shown to be essential for the $\sigma^d$-dependent response to oxidative stress in that species. However, our in vivo expression studies suggest that PerR is not an important regulator for MrgA in S. pyogenes. PerR itself is a transcription repressor with homology to the iron-sensitive Fur protein of E. coli and binds to a well-conserved operator sequence in its target promoters (“Per box”) in B. subtilis. This sequence can be found in the promoter regions of genes repressed by PerR in that species, including katA, $\text{ahpC}$, and mrgA. However, a search for Per boxes in $\text{ahpC}$ and mrgA of S. pyogenes did not reveal any such sequences, which is likely a significant observation given that the putative DNA binding regions of PerR in S. pyogenes and B. subtilis are identical at 12 of 13 positions. Thus, the targets of PerR-dependent gene repression have not yet been determined. Identification of such targets in S. pyogenes will provide further insight about potentially novel effectors of protection against hydrogen peroxide.

The ability of ethanol to induce resistance to peroxide provides some insight into the regulation of this response. Stress responses in prokaryotes are known to involve redundant and overlapping functions. For example, in E. coli, hydrogen peroxide exposure leads to the expression of a number of proteins, some of which are also induced as part of the heat shock response. However, the peroxide response regulon also includes several proteins not regulated by the heat shock regulon. For example, in E. coli, hydrogen peroxide exposure leads to the expression of a number of proteins, some of which are also induced as part of the heat shock response. However, the peroxide response regulon also includes several proteins not regulated by the heat shock regulon. In gram-positive bacteria, induction by ethanol is commonly used as a probe for regulation by the $\sigma^d$-dependent general stress response. This regulon is widely distributed among pathogenic gram-positive bacteria, including Staphylococcus and Listeria spp. However, our examinations using several different criteria, including examination of the database for the $\sigma^d$ gene, $\sigma^d$ promoters, and genes which regulate $\sigma^d$ and PCR analyses, suggest that S. pyogenes may lack a clear homologue of $\sigma^d$. In B. subtilis, a few stress response genes that are induced by multiple stresses are regulated independently of $\sigma^d$. These include the heat shock genes regulated by the CIRCE element and the genes for the various Clp-family proteases that are regulated by the recently described transcription repressor CtsR. However, there is no evidence that the genes regulated by these responses specifically play major roles in resistance to peroxide. The $\text{ahpC}$ and $\text{gpoA}$ mutants are sensitive to high-level oxidative stress in the form of challenge with methyl viologen but are not more sensitive to hydrogen peroxide in a disk diffusion or MIC assay. This apparent contradiction may be due to the fact that methyl viologen catalyzes the continuous production of superoxide and hydrogen peroxide in the culture medium, whereas exogenously added hydrogen peroxide is reduced over time. In addition, our findings suggest that different mechanisms of resistance may be involved in protection from different levels of stress. The notion of a response tailored to the degree of stress is consistent with studies suggesting that different levels of peroxide generate different types of cellular damage in E. coli. Peroxide killing is bimodal with respect to peroxide concentration in E. coli, where it appears that low-level hydrogen peroxide exposure leads to DNA damage, whereas high levels of hydrogen peroxide may directly oxidize some other cellular target. Damage to different constituents would likely require different types of repair.
and/or resistance mechanisms. The existence of multiple pathways in S. pyogenes is suggested by the observation that ethanol can induce an additional degree of resistance in PerR mutants.

The regulation and manifestation of resistance to oxidative stress in S. pyogenes appear to involve several novel features. Since streptococcal lesions in tissue are highly inflammatory and since the production of toxic oxygen species is an important component of the inflammatory response, the inducible response may serve as a virulence determinant that specifically allows the bacterium to survive oxidative stresses encountered in the host environment. Further work is being undertaken to characterize the components of this system and to understand its importance in bacterial survival and virulence.

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