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Insertional Inactivation of *Streptococcus pyogenes sod* Suggests that *prtF* Is Regulated in Response to a Superoxide Signal

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In establishing an infection, *Streptococcus pyogenes* has the capacity to bind to the host extracellular matrix protein fibronectin via its protein F adhesin. Previous studies have suggested that the expression of protein F is stimulated during aerobic growth or upon addition of superoxide-generating agents to the culture under O_2-limited conditions. To further explore the role of superoxide, we have examined the transcription of the gene which encodes protein F (*prtF*), as well as the expression of superoxide dismutase (SOD) under conditions which promote or repress protein F expression. These studies show that *prtF* transcription is regulated in response to superoxide concentration and that SOD is regulated in different environments in a manner which directly parallels the expression of protein F. A mutant deficient in SOD activity was constructed by insertional mutation into the gene which encodes SOD (*sod*). The resulting mutant was sensitive to superoxide and aerobic conditions, showed hypersensitive induction of *prtF* in response to superoxide, and expressed *prtF* under normally unfavorable O_2-limited conditions. These findings suggest that a streptococcal signal transduction system which senses superoxide may coordinately control expression of *prtF* and *sod*.

Many pathogenic bacteria have evolved sophisticated mechanisms of signal transduction that recognize and respond to environmental cues during the course of an infection. These mechanisms ensure the appropriate expression of gene products required for adaptation to environmental conditions within specific host compartments (11, 21). Regulation of virulence-associated genes likely plays an important role in the generation of the different diseases caused by *Streptococcus pyogenes* (group A streptococcus). The bacterium is the causative agent of numerous types of infections of the skin (erysipelas, impetigo, and cellulitis) and pharynx (pharyngitis). If left untreated, these infections may sometimes lead to the more serious postinfection sequelae of acute glomerulonephritis or rheumatic fever. Reports of fatal cases of the necrotizing fasciitis of the soft tissues (37) and the rising incidence of a toxic shock-like syndrome caused by severely invasive streptococcal strains (37) emphasize the importance of understanding the pathogenesis of streptococcal infections; however, very little is known concerning the regulation of genes which may contribute to these diseases.

S. pyogenes must continually monitor its surroundings throughout an infection and regulate the expression of virulence-associated factors in response to the changing environmental signals within the host. For example, in the cutaneous tissues, *S. pyogenes* transiently colonizes the surface of human skin and typically does not cause disease unless implanted into deeper tissues through traumatic injury (42). Therefore, to cause a productive infection, the streptococcus must be able to distinguish between the surface of the skin and deeper tissues and to adjust accordingly. Recent evidence suggests that recognition of the environmental concentrations of CO_2 and O_2 may regulate the expression of important *S. pyogenes* virulence-associated genes, including those of the M protein family, protein F, and C5a peptidase (4, 6, 23, 26, 34, 38).

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The *S. pyogenes trans*-acting regulatory protein Mga (formerly Mry) responds to elevated levels of CO_2 and increases expression of *emm*, the gene which encodes the antiphagocytic M protein, as well as genes for other M-like proteins and the wall-associated C5a peptidase (3, 4, 23, 26, 34). In contrast to the genes under the control of Mga, atmospheric concentrations of O_2 are used by *S. pyogenes* as a signal to regulate the expression of the gene for protein F, a 120-kDa surface protein that binds fibronectin and promotes attachment to the host extracellular matrix (16, 17). In vitro expression of the gene encoding protein F, *prtF*, is regulated at the transcriptional level and is maximal under ambient levels of O_2 or repressed in environments of reduced O_2 (38). To date, only one gene has been characterized as a regulator of *prtF* transcription. The gene, designated *rofA*, was identified in a strain which constitutively expresses high levels of protein F even in an environment that is unfavorable for *prtF* expression. Within this unfavorable environment the concentration of O_2 is apparently reduced below that of the ambient atmosphere, but the environment is not strictly anaerobic and is referred to as O_2 limited (15). Interestingly, disruption of *rofA* in this host background abolishes O_2-limited expression of protein F but has no influence on O_2 regulation of *prtF*, since the *rofA* mutant expresses normal levels of protein F when cultured under aerobic conditions (15). These data suggest that *rofA* may not be an essential component of the O_2 regulation pathway.

Although the genes responsible for regulation of *prtF* in response to O_2 have not been identified, some insight into the signal transduction pathway which responds to O_2 has been gained through the analysis of the abilities of various substances to stimulate expression in normally unfavorable O_2-limited environments. Both superoxide stress, induced by the addition of paraquat to O_2-limited cultures, and an altered redox environment, generated by the addition of $Fe(CN)_6^{3-}$, result in increased fibronectin-binding activity in comparison with that in O_2-limited cultures alone (38). These data suggest that a signal transduction system which senses superoxide and/or redox potential might regulate *prtF* expression. However, conclusions concerning a redox signal have been compli-

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype (comment)[a]	Reference or source
E. coli		
DH5α	*recA1 endA1 hsdR17*	BRL[b]
HB101	*recA13 proA2*	1a
S. pyogenes		
JRS4	*prtF rofA emm6.1* (constitutive Fn binding)	32a
HSC5	*prtF emm5* (regulated Fn binding)	17
HSC13[c]	HSC5ΩpPTF34 (contains *prtF100*)	38
HSC14[c]	HSC5ΩpCMG5	This work
HSC15[c]	HSC5ΩpCIV23′	This work

[a] Fn, fibronectin.
[b] BRL, GIBCO Bethesda Research Laboratories, Inc., Gaithersburg, Md.
[c] Strain derived by transformation of the indicated host with the designated integrational plasmid.

cated by the recent observation that $Fe(CN)_6^{3-}$ has been shown to stimulate a previously unrecognized protein F-independent pathway of fibronectin binding (19). In this study, to further examine the regulation of *prtF* in response to superoxide and redox signals, we have examined the transcription of *prtF* and the expression of superoxide dismutase (SOD) in response to both paraquat and $Fe(CN)_6^{3-}$, and we have analyzed the expression of *prtF* in a *sod* null mutant. While alterations in redox conditions do not appear to be involved in *prtF* regulation, our data support a role for superoxide as a signal used by *S. pyogenes* to regulate the expression of both *prtF* and *sod*.

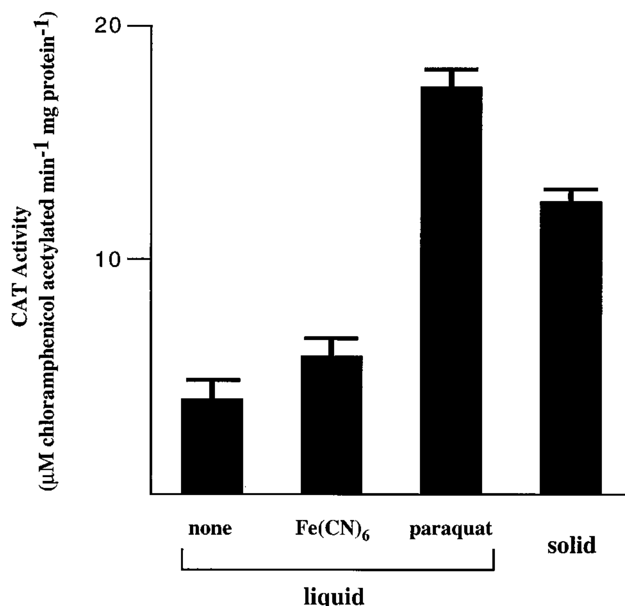

FIG. 1. Stimulation of *prtF* transcription by oxidative stress. *S. pyogenes* HSC13 contains a promoterless CAT allele from *Bacillus pumilis* (*cat-86*) fused to the *prtF* promoter and integrated into the HSC5 chromosome (38). The bars show CAT specific activities of HSC13 cultured in liquid THY medium with the indicated medium supplements and on solid THY medium in an ambient atmosphere. The data represent the means and standard errors of the means obtained from at least two independently prepared extracts, each of which was analyzed in duplicate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *Escherichia coli* DH5α was used for molecular cloning experiments, and *E. coli* HB101 was used in fibronectin-binding assays. *E. coli* was cultured in Luria-Bertani broth (32), and *S. pyogenes* was cultured in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY medium) as previously described (5). Solid medium was produced by adding Bacto agar (Difco) to THY medium at a final concentration of 1.4%. *S. pyogenes* strains grown in liquid medium were incubated overnight at 37°C without agitation in sealed culture bottles. Streptococci grown on solid medium were incubated in ambient air (20% O_2, 0.03% CO_2) or in an anaerobic environment (<0.03% O_2, 10% CO_2) generated by a commercial gas generator (GasPak, catalog no. 70304; BBL) in a sealed jar. For some experiments the liquid medium was altered by the addition of paraquat (methyl viologen) (Sigma) to a final concentration of 0.2 or 10 mM or of $Fe(CN)_6^{3-}$ to a final concentration of 1 mM. When appropriate, antibiotics were added to the medium at the following final concentrations: ampicillin, 50 µg/ml for *E. coli*; kanamycin, 25 µg/ml for *E. coli* and 500 µg/ml for *S. pyogenes*; and streptomycin, 1,000 µg/ml for *S. pyogenes*.

DNA techniques. Plasmid DNA was purified by standard techniques and transformed into *E. coli* by the method of Kushner (18). Restriction endonucleases, ligases, and polymerases were used according to the recommendations of the manufacturers. Chromosomal DNA was purified from *S. pyogenes* after growth in THY medium supplemented with 20 mM glycine (5). A modified T7 DNA polymerase and the dideoxy-chain termination method were used for all DNA sequence analyses (30), and all reported sequence data represent analysis of both strands of DNA. In cloning experiments, incompatible restriction fragment ends were ligated following treatment with T4 DNA polymerase to provide blunt fragment ends.

Determination of CAT specific activities. The determination of chloramphenicol acetyltransferase (CAT) specific activities was performed as described previously with [^{14}C]chloramphenicol (Amersham) and the chromatographic assay of Shaw (33). The data presented represent the means and standard errors of the means derived from two independently prepared extracts.

Determination of SOD activity. Streptococcal cells were cultured overnight in either solid or liquid THY medium in the presence or absence of paraquat. The preparation of cell extracts was performed as described previously (15), and the total protein concentration in each cell extract was determined by the bicinchoninic acid method (35) with bovine serum albumin as a standard. Whole-cell extracts were separated by electrophoresis through duplicate 12.5% native polyacrylamide gels, one of which was stained with Coomassie blue stain to determine the protein profile of each sample. The other gel was subjected to the assay of Beauchamp and Fridovich (1), which utilizes photochemical generation of superoxide by riboflavin to visualize the quantity of SOD present. Superoxide catalyzes the formation of blue formazan from nitroblue tetrazolium; therefore, in the presence of nitroblue tetrazolium, SOD appears as a clear band due to inhibition of blue formazan accumulation in the region immediately surrounding the enzyme.

Construction of integrational plasmids. A 511-bp internal region of the Mn-SOD gene (SODint) was amplified by PCR from *S. pyogenes* by using the primers SOD-For (5′-TTACCAGATC TTCCATATGC TTATGACGCA CTTGAACC-3′) and SOD-Rev (5′-GAGATAATAA GCGTGTTCCC AGACATCAAG TGCTAAAATT GG-3′) (see Fig. 5). SODint was inserted into pCRII (Invitrogen) to form pSOD1. A 518-bp *Xba*I-*Sst*I fragment of pSOD1 containing SODint was ligated with *Xba*I-*Sst*I-digested DNA of pUC19 (39) to produce pSOD19. To eliminate the β-lactamase gene and to provide a suitable selectable marker for *S. pyogenes*, a 2.2-kb *Sma*I fragment containing ΩKm-2 (25) was inserted between the *Sca*I and *Ssp*I sites of pSOD19, resulting in the chimeric plasmid pCMG5 (see Fig. 5).

To determine the nucleotide sequence of the region of *sod* 3′ to SODint, purified *S. pyogenes* chromosomal DNA was subjected to *Hin*dIII digestion, ligation, and inverse PCR according to the method of Eaton et al. (13) with the primers SOD-R4 (5′-GCAGCATTCG CATTGGCAAC ATAAGTGGCA TG GTGTTT-3′) and SOD-F2 (5′-CCAATTTTAG CACTTGATGT CTGG-3′). The product was cloned into pCRII, generating pIP6, and this plasmid was used as a template to determine both the nucleotide sequence 3′ of SODint and the chromosomal region immediately downstream of the *sod* coding region. This sequence was used to design a primer, SOD-R6 (5′-CTAAAAAATGATGTCC CCAC ACTG-3′), which was then used in conjunction with SOD-For to amplify a fragment which includes SODint, the region of *sod* 3′ to SODint, and the adjacent noncoding region (see Fig. 5). The resulting product was inserted into pCRII to form pSOD3′. The pSOD3′ 675-bp *Eco*RI fragment containing the region derived by PCR was inserted into the *Eco*RI site of pCIV2 (23) to form pCIV23′. This plasmid includes SODint plus the entire 3′ end of the *sod* coding region and 83 bp downstream, but it lacks the 5′ end and promoter of *sod* (see Fig. 5).

Construction of mutants. *S. pyogenes* was transformed by electroporation as described previously (5). Southern blot analysis (36) with ^{32}P-labeled probes of appropriate sequences was used to confirm the structures of all *S. pyogenes* strains with modified chromosomes resulting from transformation by integrational plasmids.

Analysis of fibronectin-binding activity. *S. pyogenes* strains were cultured as described above, harvested by centrifugation (6,500 × *g*, 10 min, 14°C), and washed once in phosphate-buffered saline (PBS). The streptococci were then resuspended in PBS containing 1% (vol/vol) Tween 20 to an optical density at 600 nm of 0.1. The fibronectin-binding activity of the bacteria was then deter-

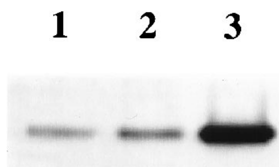

FIG. 2. *S. pyogenes* SOD activity increases in aerobic culture. SOD specific activity was analyzed for HSC5 cultured in liquid THY medium with no addition (lane 1) or with the addition of 10 mM paraquat (lane 2) and on solid THY medium (lane 3). Cell extracts were prepared from all cultures, and SOD activities were determined by separating samples containing 25 μg of protein from cell extracts on a nondenaturing 10% polyacrylamide gel and staining for SOD activity. A negative image of the actual gel is shown.

mined by using ^{125}I-fibronectin as described previously (16). In selected experiments, a whole extract of *E. coli* DH5α cells was included in the binding assay as described previously (16). While this treatment does not inhibit the binding of fibronectin to protein F (16), it inhibits a second O_2-induced fibronectin-binding activity that is unrelated to protein F (19).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database under accession number U43776.

RESULTS

Effect of paraquat and $Fe(CN)_6^{3-}$ on *prtF* transcription. It has been previously shown that because of differential diffusion of O_2, protein F is expressed when *S. pyogenes* is cultured on solid media in the ambient atmosphere but is not expressed when *S. pyogenes* is cultured in liquid media, in which oxygen is apparently present at a reduced concentration (38). Furthermore, the addition of either $Fe(CN)_6^{3-}$ or paraquat stimulated fibronectin binding in the normally unfavorable liquid culture (38). To determine if the same medium supplements also stimulate transcription of *prtF*, transcription in different environments was analyzed by determination of CAT specific activities by using the strain HSC13, which contains a promoterless CAT allele from *Bacillus pumilis* (*cat-86*) fused to the *prtF* promoter and integrated into the HSC5 chromosome (38). HSC13 grown in liquid medium with the addition of $Fe(CN)_6^{3-}$ shows little difference in CAT specific activity compared with growth in liquid medium alone (Fig. 1). In contrast, the addition of 10 mM paraquat causes a substantial increase in *prtF*::*cat-86* expression, comparable to that obtained following growth on solid medium (Fig. 1).

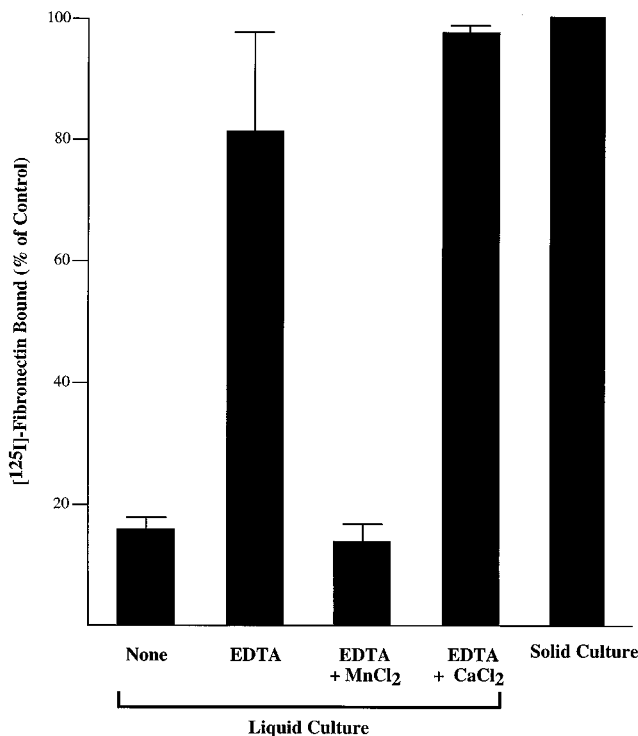

FIG. 3. Mn^{2+} sequestration induces superoxide stress and increases fibronectin-binding activity. The ability of HSC5 to bind ^{125}I-fibronectin was determined following growth in liquid THY medium with the indicated medium supplements at a final concentration of 1 mM. Fibronectin-binding activities relative to the activity of HSC5 grown on solid medium are shown. The data presented represent the means and standard errors of the means derived from at least two independent determinations.

FIG. 4. Comparison of amino acid sequences of *S. pyogenes* SOD and SODs from other bacteria. The amino acid sequences of Mn-SODs from *E. coli* (*E. c*) (GenBank accession number M94879), *Lactococcus lactis* (*L. l*) (GenBank accession number U17388), and *S. mutans* (*S. m*) (GenBank accession numbers S39782 and D01037) are compared with that of *S. pyogenes* (*S. p*) (GenBank accession number U43776). Amino acid residues identical to those of the *E. coli* Mn-SOD are indicated by dashes, and gaps are represented by dots. Characteristic residues of Mn-SODs are in boldface, and the shaded bars indicate conserved residues essential for binding the Mn^{2+} cofactor (24).

HSC5 SOD activity is greatest under aerobic conditions. To further explore the relationship between superoxide stress and expression of *prtF*, we next examined the expression of another gene likely to be regulated by superoxide, using conditions which are favorable or unfavorable for expression of *prtF*. For several organisms SOD, which catalyzes the dismutation of the superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), has been shown both to be regulated in response to superoxide levels and to play an important role in protection against oxidative stress (reviewed in reference 14). While gram-negative bacteria typically possess a manganese-containing SOD (Mn-SOD) and an iron-containing SOD (Fe-SOD), studies with *Streptococcus mutans* have demonstrated that streptococci possess only an Mn-type SOD (22). To determine and compare the levels of SOD expression under various atmospheric conditions and the levels of *prtF* expression, native polyacrylamide gels were run to visualize SOD

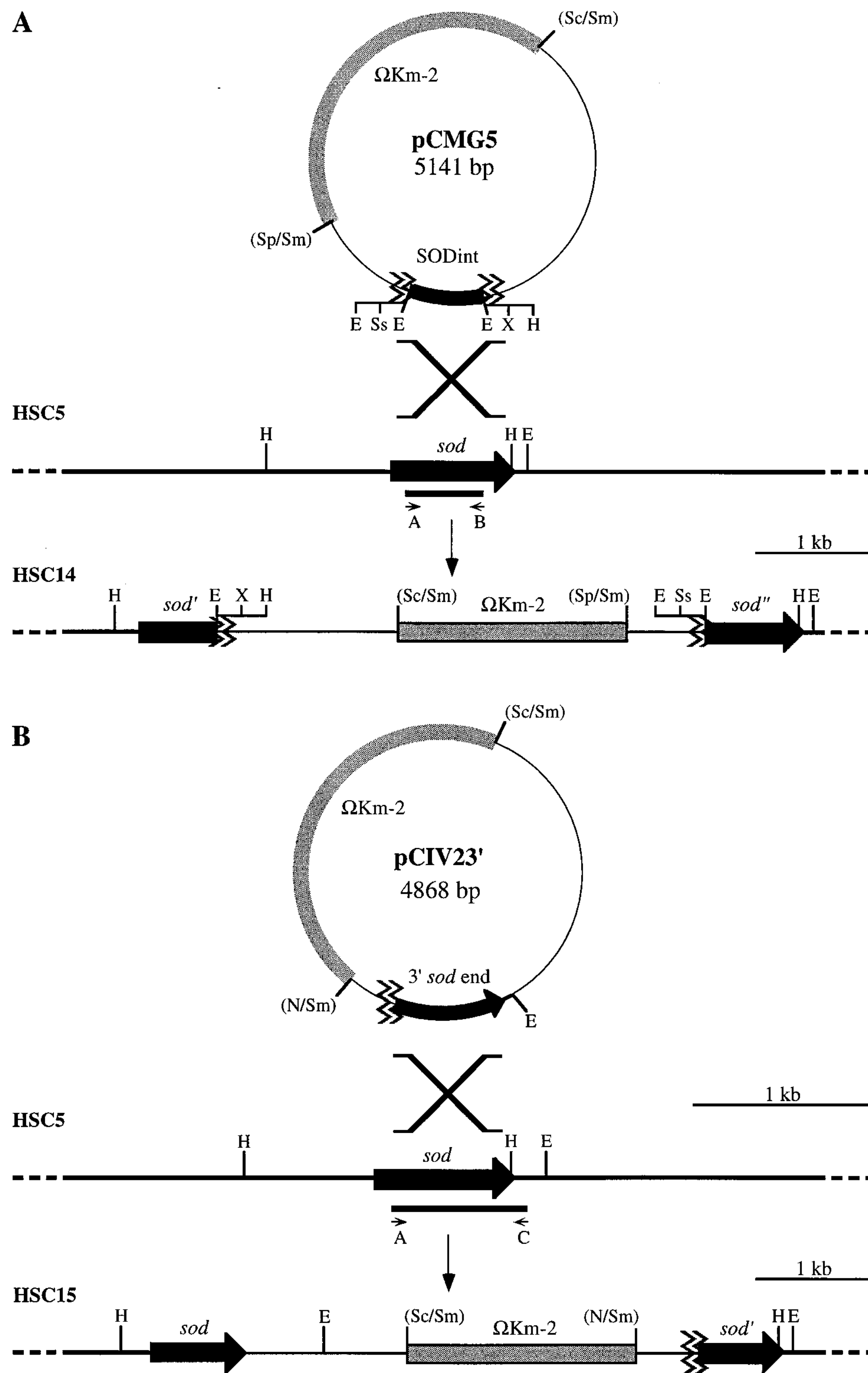

FIG. 5. Construction of the mutant strains. (A) The SODint region amplified from *S. pyogenes* by using the SOD-For (A) and SOD-Rev (B) primers is depicted beneath the HSC5 chromosomal restriction map. Integrational plasmid pCMG5 contains a kanamycin resistance determinant (shaded bar) and the SODint region of HSC5 (black bar). Homologous recombination between the plasmid and the HSC5 chromosome (indicated by the large X between the plasmid and chromosomal restriction maps) generated HSC14, which has a chromosomal structure that contains two truncated and inactive versions of *sod* (indicated by the double zigzag line). (B) The 3′ *sod* product amplified from *S. pyogenes* by using the SOD-For (A) and SOD-R6 (C) primers is depicted beneath the HSC5 chromosomal restriction map. Integrational plasmid pCIV23′ also contains a kanamycin resistance determinant (shaded bar) which includes strong transcriptional and translational terminators in addition to the 3′ region of *sod* (black bar). Homologous recombination between pCIV23′ and the HSC5 chromosome (indicated by the large X) generates HSC15, which contains one functional *sod*, a downstream polar element (ΩKm-2), and one truncated *sod* (indicated by the double zigzag line). Southern blot analysis (36) with ^{32}P-labeled probes of appropriate sequences was used to confirm the structures of all *S. pyogenes* strains with modified chromosomes resulting from transformation by integrational plasmids. Sc, *Sca*I; Sm, *Sma*I; Sp, *Ssp*I; E, *Eco*RI; Ss, *Sst*I; X, *Xba*I; H, *Hin*dIII; N, *Nde*I.

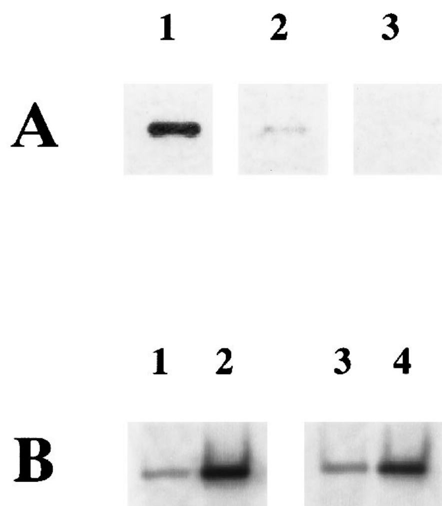

FIG. 6. SOD activity in mutant strains. (A) SOD specific activities of HSC5 cultured on solid THY medium (lane 1) and in liquid THY medium (lane 2) and of HSC14 cultured in liquid THY medium (lane 3). (B) SOD specific activities of HSC5 and HSC15 cultured in liquid THY medium (lanes 1 and 3, respectively) and on solid THY medium (lanes 2 and 4, respectively). Cell extracts (25 μg of protein) prepared from all cultures were subjected to electrophoresis through a nondenaturing 12.5% polyacrylamide gel and then stained for SOD specific activity as described in Materials and Methods. Negative images of the actual gels are shown.

specific activity by the assay of Beauchamp and Fridovich (1). As expected, *S. pyogenes* HSC5 was shown to contain a single SOD (Fig. 2). Furthermore, SOD specific activity was directly correlated to the level of oxidative stress to which the cells were exposed. SOD specific activity was minimal in cultures in liquid medium alone (Fig. 2, lane 1), intermediate in cultures in liquid medium plus paraquat (Fig. 2, lane 2), and greatest when *S. pyogenes* was grown on solid medium (Fig. 2, lane 3). In *S. pyogenes* JRS4, which constitutively expresses *prtF* (15, 38), the SOD specific activity in liquid culture was identical to that of HSC5 cultured under all conditions tested (data not shown), providing additional evidence that the O_2-sensing system is not affected by the mutation responsible for constitutive expression in JRS4.

Sequestration of Mn^{2+} increases fibronectin-binding activity. Since superoxide is a prominent by-product of numerous essential redox reactions, including those catalyzed by D-lactate dehydrogenase, glutathione reductase, and ribonucleotide reductase (reviewed in reference 14), we hypothesized that any alteration of the activity of SOD itself would stimulate expression of protein F in normally unfavorable environments as a result of the accumulation of aberrantly high intracellular levels of superoxide. To test this hypothesis, protein F expression was analyzed following growth in liquid medium to which EDTA had been added to reduce the concentration of divalent cations, including Mn^{2+}, which is an essential cofactor for the dismutation reaction catalyzed by SOD (27, 31). The concentration of EDTA used did not hinder *S. pyogenes* growth in liquid medium alone but inhibited growth in liquid medium containing paraquat. Significantly, cells from EDTA-treated cultures expressed protein F fibronectin-binding activity strongly, at levels nearly equivalent to those in cells from aerobic solid cultures (Fig. 3). The addition of Mn^{2+}, but not Ca^{2+}, to EDTA-treated cultures resulted in an inhibition of the expression of binding activity, which was equivalent to that observed in liquid culture alone (Fig. 3).

TABLE 2. Mutant growth characterization

Culture conditions[a]	Paraquat concn (mM)	Growth[b]		
		HSC5	HSC14	HSC15
Liquid medium	0	+++	+	+++
	0.2	+++	+	+++
	10	+++	−	+++
Solid medium				
Aerobic	0	+++	−	+++
Anaerobic	0	+++	+	+++
	0.2	+++	−	+++

[a] Bacterial cultures were grown in liquid THY medium or on solid THY medium under the ambient atmosphere (aerobic) or in an anaerobic chamber (anaerobic).

[b] The optical density at 600 nm of bacteria grown under different liquid culture conditions was recorded after 15 h of incubation at 37°C: +++, >0.725, +, 0.100 to 0.001; −, <0.001. Solid medium was inoculated with 100-μl aliquots of an 18-h liquid culture and exposed to the indicated culture conditions for 15 h, and the number of CFU was then recorded: +++, >10,000; +, 1 to 20; −, 0.

Construction of a *sod* null mutant. To more conclusively investigate the effect of superoxide stress on *prtF* expression, we constructed a *sod* null mutant. Primers from regions of the *S. mutans sod* gene that are highly conserved among other prokaryotic SOD genes were used to amplify a 500-bp internal region of *sod* (SODint) from *S. pyogenes* HSC5 (see Materials and Methods). Nucleotide sequence analysis of this product as well as of the 3′ end and downstream region of *sod* (see below) revealed a nucleotide sequence 76% identical to that of the Mn-SOD gene of *S. mutans*, with an amino acid identity of 75% between the two proteins (22). Invariant amino acid residues characteristic of all Mn-SODs (24) were present in the *S. pyogenes* SODint sequence, which confirms that the *S. pyogenes* SOD is also of the Mn type (Fig. 4). SODint was inserted into pUC19, and ΩKm-2 was used to replace the β-lactamase gene of the vector as a selectable marker usable in both *E. coli* and *S. pyogenes*. The resulting plasmid, pCMG5, was then used to transform HSC5 to kanamycin resistance. Since pCMG5 is unable to replicate in *S. pyogenes*, kanamycin-resistant transformants can be obtained only through integration of the plasmid into the HSC5 chromosome via a single homologous crossover between SODint and the chromosomal *sod*, which results in the insertional inactivation of *sod* (Fig. 5). One such transformant was chosen for further analysis, and the new strain was designated HSC14.

To verify that the effects seen in HSC14 were due to an inactive SOD and not to a polar effect on a downstream gene, a strain was constructed to contain a polar insertion immediately 3′ of *sod*. This was accomplished by using the chimeric plasmid pCIV23′ to transform HSC5 to kanamycin resistance via a single homologous crossover. One resulting transformant was chosen for further study and denoted HSC15. The chromosomal structure HSC15 is similar to that of the *sod* null mutant, except that HSC15 contains a functional copy of *sod* upstream of the polar insertion (Fig. 5).

Mutant characterization. The inactivation of SOD in HSC14 was verified by SOD activity gel analysis (Fig. 6). The SOD specific activity of the parent strain under aerobic conditions (Fig. 6A, lane 1) and O_2-limited conditions (Fig. 6A, lane 2) was apparent, whereas that of the *sod* null mutant under O_2-limited conditions was not detectable (Fig. 6A, lane 3). HSC14 is unable to grow on solid media; therefore, the SOD specific activity of this strain grown within this environment could not be tested. The SOD specific activity of HSC15 mimics the activity observed in the parent strain, HSC5, under

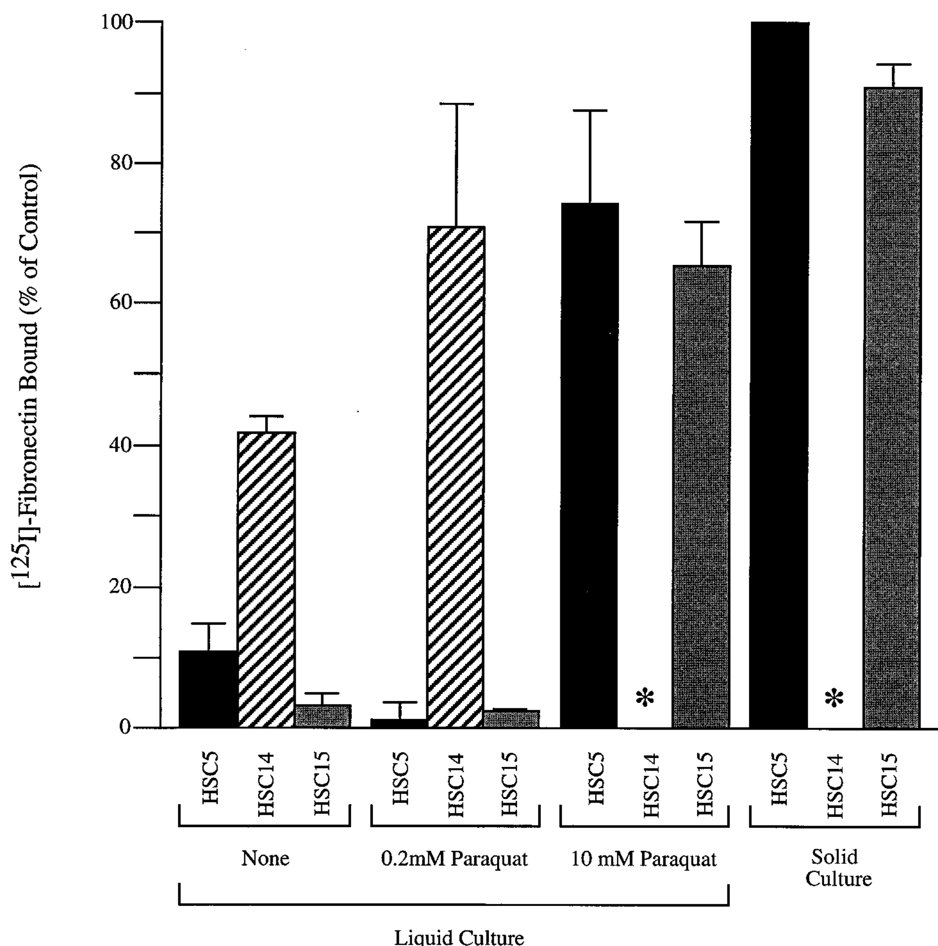

FIG. 7. The *sod* null mutant constitutively expresses protein F. The ability of HSC5, HSC14, or HSC15 to bind ^{125}I-fibronectin was determined following growth in liquid THY medium with the indicated medium supplements or on solid THY medium in the ambient atmosphere. An asterisk indicates that HSC14 is hypersensitive to O_2 and therefore unable to grow on solid medium or in liquid medium with the addition of paraquat in concentrations greater than 0.2 mM. Fibronectin-binding activities relative to the activity of HSC5 grown on solid THY medium are shown. The data represent the means and standard errors of the means derived from at least two independent determinations.

all conditions tested (Fig. 6B). The protein profile of HSC14 seemed to equal that of the wild-type parent strain and HSC15 as determined by Coomassie blue staining. Protein F, however, cannot be detected on Coomassie blue-stained gels.

The *sod* null mutant HSC14 is hypersensitive to oxidative stress and grows at a much lower rate than its parent strain, HSC5, in liquid culture (Table 2), a phenotype similar to that of the *S. mutans sod* mutant (22). However, unlike the *S. mutans* mutant, which required 2 days of growth on solid medium under aerobic conditions to form colonies the same size as those of its parent strain (22), HSC14 did not grow at all when incubated aerobically. The polar insertion strain HSC15 grows under aerobic conditions and is phenotypically indistinguishable from its parent strain, HSC5 (Table 2).

Increased protein F expression in the *sod* null mutant. The fibronectin-binding profile of polar insertion strain HSC15, which contains an intact *sod* gene, closely resembled that of wild-type HSC5, since high levels of activity were observed following culture on solid medium or in liquid medium in the presence of paraquat (Fig. 7). As expected, minimal activity of both HSC5 and HSC15 was observed in cells grown in unsupplemented liquid medium. In contrast, the *sod* mutant HSC14 expressed substantial binding activity under all conditions tested, including the unsupplemented liquid culture conditions which were unable to promote binding in the other strains (Fig. 7). HSC14 *prtF* expression was additionally stimulated by 0.2 mM paraquat, the maximal concentration in which the *sod* null mutant could grow. However, this concentration did not stimulate binding in either HSC5 or HSC15, which required much higher concentrations of paraquat (10 mM) to have the same effect (Fig. 7). Since HSC14 was unable to grow on solid medium under aerobic conditions or in the presence of 10 mM paraquat, fibronectin binding could not be tested under these conditions. These data indicate that in the absence of a functional *sod* gene, *prtF* is expressed constitutively.

DISCUSSION

As a consequence of its inability to synthesize heme, *S. pyogenes* lacks catalase and various cytochromes that are important for survival in the presence of O_2. Thus, it is currently not well understood how *S. pyogenes* is able to survive and grow aerobically. However, its capacity to tolerate aerobic atmospheres strongly suggests that this ability is essential for survival during infection of its human host. In addition, the results

of this study have demonstrated that O_2 in the form of superoxide stress is a signal used by *S. pyogenes* to regulate its ability to bind fibronectin via protein F. Thus, not only is *S. pyogenes* able to grow aerobically, but it also utilizes O_2 in a signaling pathway that plays a crucial role in regulating the expression of a key determinant of virulence.

Inactivation of SOD is considered to be a powerful method to induce superoxide stress for analysis of superoxide-regulated genes (reviewed in reference 14). Analysis of the *S. pyogenes sod* null mutant HSC14 suggests that in contrast to an *S. mutans sod* null mutant, which grows aerobically on solid media (albeit more slowly than its parent strain) (22), the *S. pyogenes* mutant cannot grow aerobically on solid media and grows poorly even under anaerobic conditions on solid media. The colony morphology of HSC14 grown under anaerobic conditions is also irregular and small, whereas the *S. mutans sod* mutant develops colonies the same size as those of its parent strain grown anaerobically (22). In addition, considerable care had to be employed when working with HSC14 because of the strong selective pressure for reversion. These observations might indicate that *S. pyogenes* is more susceptible to oxidative stress than other species in the same genus. On the other hand, these observations may reflect differences in culture conditions, as we observed that THY media from different manufacturers varied considerably in their abilities to support growth of the *sod* mutant. The most consistent growth of the mutant was obtained in THY medium from Difco. In some cases, growth of the mutant in liquid medium from other manufacturers could be supported by the addition of horse serum to a final concentration of 5%. Some component found in permissive (Difco) medium or in horse serum may stimulate the expression of gene products which can provide protection against oxidative stress. An example of such a gene product is the hyaluronic acid capsule, which has previously been shown to provide protection against oxidative stress (10).

Other than capsule and SOD, no gene products that protect against oxidative stress in *S. pyogenes* have been described. Another gram-positive facultative anaerobe, *Enterococcus faecalis*, contains a superoxide dismutase (*sod* gene) but no capsule. In addition, *E. faecalis* contains an NADH oxidase (*nox* gene) and an NADH peroxidase (*npr* gene) which may provide protection against oxidative stress. These enzymes catalyze the two-electron reduction of hydrogen peroxide to H_2O (NADH peroxidase) or the four-electron reduction of O_2 to H_2O (NADH oxidase) (9). While previous studies have detected the activities of these two enzymes in *S. pyogenes* (2, 44), we failed to detect genes homologous to *nox* and *npr* from the *S. pyogenes* chromosome by PCR with primers based on the *nox* and *npr* sequences (28, 29), suggesting that *S. pyogenes* does not contain genes highly homologous to either of the *E. faecalis* genes.

Pathogens frequently utilize global stress sensory signal transduction pathways for the regulation of virulence genes (11, 14, 21). While global O_2 stress regulation has not been well characterized for gram-positive bacteria, for *Bacillus subtilis* two different groups of stress proteins have been shown to be induced in response to environmental conditions (40). The first group is referred to as the general stress proteins (Gsps), which are induced in response to multiple stresses such as heat shock, salt stress, glucose and oxygen limitation, or oxidative stress. The regulation of these Gsps appears to be dependent on the alternative sigma factor SigB (40, 41). The second group consists of proteins which are induced by a specific stress and includes the hydrogen peroxide-sensing regulon of *B. subtilis* (reviewed in reference 12). This hydrogen peroxide-sensing regulon has recently been shown to be under the control of a repressor that specifically senses both hydrogen peroxide and the availability of Mn^{2+} (8). Interestingly, as with protein F, low levels of Mn^{2+} promote expression of the genes in the hydrogen peroxide regulon (7, 8). However, an operator sequence homologous to the Per box of genes in the *B. subtilis* peroxide regulon (8) is not found upstream of *prtF*.

A previous fibronectin-binding analysis demonstrated that in *S. pyogenes*, protein F expression was induced only in response to O_2 stress and specifically in response to superoxide stress or alterations in the redox environment (38). However, the lack of *prtF* transcription in response to $Fe(CN)_6^{3-}$ observed in this study, in addition to the results of a recent investigation which shows that $Fe(CN)_6^{3-}$ induces a previously unrecognized *prtF*-independent fibronectin-binding activity (19), suggests that protein F expression is induced as a component of a specific stress response to superoxide.

For *S. pyogenes*, since SOD activity is regulated in a manner which directly parallels expression of *prtF*, the possibility exists that both SOD and *prtF* may be under the control of a common regulator which responds to superoxide. Such a regulator exists in *E. coli* (43) and is part of a two-regulatory-gene system known as the *soxRS* regulon. SoxR senses either increased levels of superoxide or a decreased $NADPH/NADP^+$ ratio and in turn activates the expression of *soxS*, a transcriptional regulator (14, 20, 43). However, a similar regulon has not yet been described for any gram-positive bacterium. The identification of regulatory loci which control expression of *prtF* and SOD in response to superoxide stress will be required to test this hypothesis.

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