Identification and Characterization of a *Streptococcus pyogenes* Operon Involved in Binding of Hemoproteins and Acquisition of Iron

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The hemolytic *Streptococcus pyogenes* can use a variety of heme compounds as an iron source. In this study, we investigate hemoprotein utilization by *S. pyogenes*. We demonstrate that surface proteins contribute to the binding of hemoproteins to *S. pyogenes*. We identify an ABC transporter from the iron complex family named *sia* for streptococcal iron acquisition, which consists of a lipoprotein (*siaA*), membrane permease (*siaB*), and ATPase (*siaC*). The *sia* transporter is part of a highly conserved, iron regulated, 10-gene operon. SiaA, which was localized to the cell membrane, could specifically bind hemoglobin. The operon's first gene encodes a novel bacterial protein that bound hemoglobin, myoglobin, heme-albumin, and hemoglobin-haptoglobin (but not apo-haptoglobin) and therefore was named Shr, for streptococcal hemoprotein receptor. PhoZ fusion and Western blot analysis showed that Shr has a leader peptide and is found in both membrane-bound and soluble forms. An M1 SF370 strain with a polar mutation in *shr* was more resistant to streptonigrin and hydrogen peroxide, suggesting decreased iron uptake. The addition of hemoglobin to the culture medium increased cell resistance to hydrogen peroxide in SF370 but not in the mutant, implying the *sia* operon may be involved in hemoglobin-dependent resistance to oxidative stress. The *shr* mutant demonstrated reduced hemoglobin binding, though cell growth in iron-depleted medium supplemented with hemoglobin, whole blood, or ferric citrate was not affected, suggesting additional systems are involved in hemoglobin utilization. SiaA and Shr are the first hemoprotein receptors identified in *S. pyogenes*; their possible role in iron capture is discussed.

Iron deprivation hinders the attempts of pathogenic bacteria to colonize the human body, and bacteria that can use heme and hemoglobin can take advantage of a major iron-reservoir in their host environment. Consequently, mechanisms for iron acquisition from hemoproteins are very common among bacterial pathogens (19, 70) and often are found to be important for full virulence (1, 4, 24, 40, 61, 68).

Hemoprotein utilization is best understood in gram-negative bacteria, since most use TonB-dependent outer membrane receptors that directly bind to hemoproteins, extract, and transport the heme or the iron through the receptor cavity (19, 70). A few bacteria produce hemophores that remove the heme and convey it to surface receptors (8, 30, 31, 50). Surface or secreted proteases that can release heme from hemoglobin are produced by *Vibrio vulnificus*, pathogenic *Escherichia coli*, and *Porphyromonas gingivalis* (32, 38, 41). Heme or iron crosses the cytoplasmic membrane by binding protein-dependent transport systems (PBT), which consist of a periplasmic substrate-binding protein, membrane-spanning protein(s), and hydrophilic ATPase(s) that energize the transport process (19, 70). The proteins of heme-PBT systems share homology and sequence signatures with transporters of siderophores and vitamin B_{12}; the most conserved components are the membrane permeases and the ATPases (28, 52).

In gram-positive bacteria, proteins, and genes involved in heme or hemoglobin utilization have been described only in *Corynebacterium* species (11, 53) and *Streptococcus pneumoniae* (4, 64, 65). The first bacterial heme oxygenase (HmuO), the enzyme that carries out heme oxidative cleavage, was discovered and characterized in *Corynebacterium* (53). Mutants in *hmuO* cannot utilize heme or hemoglobin to satisfy their iron needs. Complementation of *Corynebacterium ulcerans hmuO* mutants with the *C. diphtheriae* genomic library led to the discovery of a heme ABC transporter (HmuTUV) (11, 54). Disruption of *hmuT*, the binding protein, in *C. diphtheriae* had no effect on heme utilization, suggesting this bacterium may have additional heme uptake pathways. Direct evidence for the role of HmuTUV proteins came from the related bacterium *C. ulcerans*, in which a mutation in *hmuT* is significantly impaired in heme utilization (54).

S. pneumoniae can utilize heme and hemoglobin as an iron source (64). A surface heme-binding protein of about 43 kDa was described, but its physiological role has not been established (65). Recently, two pneumococcal operons *piu* and *pia* (formerly *pit1* and *pit2*), encoding homologous ABC transporters, were identified (4, 5). Growth and iron transport assays indicated that the *piu* and *pia* transporters are required for the transport of inorganic iron, as well as for iron capture from hemoglobin. Significant defects in hemoglobin utilization were observed only when both loci where inactivated. Mutants in *piu* and/or *pia* transporters were also attenuated in a mouse pneumonia model, with the most dramatic effect observed in the double mutant strain (4).

The gram-positive pathogen *S. pyogenes* is capable of producing a diverse array of skin and mucus membrane infections, as well as aggressive deep tissue diseases and streptococcal

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TABLE 1. *S. pyogenes* strains used in this study

Strain (M serotype)	Description[a] (mo/day/yr)	Source or reference[b]
JRS4 (M6)		57
NZ131 (M49)		58
SF370 (M1)		16
ZE11 (M1)	SF370 *shr::pCB2*	This study
GA02581 (M1)	Clinical isolate (5/5/94)	GaEIP
GA03198 (M11)	Clinical isolate (9/24/94)	GaEIP
GA04425 (M1)	Clinical isolate (2/6/95)	GaEIP
GA05982 (M1)	Clinical isolate (4/20/96)	GaEIP
GA06439 (M114)	Clinical isolate (10/14/96)	GaEIP
GA08067 (M12)	Clinical isolate (4/11/97)	GaEIP
GA07346 (M22)	Clinical isolate (8/20/97)	GaEIP
GA10156 (M75)	Clinical isolate (3/10/98)	GaEIP
GA10878 (M18)	Clinical isolate (11/16/98)	GaEIP

[a] Clinical strains were isolated from sterile sites of patients with *S. pyogenes* infections.

[b] GaEIP, Georgia Emerging Infections Program.

toxic shock syndrome. Untreated streptococcal infections can lead to the serious complications of rheumatic fever and acute glomerulonephritis (10). The role of iron in the physiology and virulence of *S. pyogenes* is poorly understood. We have previously established that iron is necessary for growth of the *S. pyogenes* class I strain JRS4 (M6 serotype), and iron limitation results in multiple changes in protein production and secretion, including the secretion of the streptococcal plasmin receptor, Plr (13). Reduced iron availability also resulted in decreased transcription of the *emm6.1* gene encoding the antiphagocytic M6 protein (36). Recent analysis revealed that in *S. pyogenes* cells grown at 40°C, iron restriction had a widespread effect on gene expression, including the induction of genes encoding a putative iron transporter, several hemolysins, a putative superoxide dismutase, a bacterioferritin homologue, and iron-dependent repressors (60). Unlike the other hemolysins, *slo* expression was downregulated under these conditions (60). Together, these results suggest that iron plays a significant role in the physiology of *S. pyogenes* and is used as an important regulatory cue by this bacterium.

Among the abundant iron complexes in the human body, hemolytic *S. pyogenes* could use the hemoglobin-haptoglobin complex, hemoglobin, myoglobin, heme-albumin, and catalase but not transferrin or lactoferrin (14, 18). The means by which *S. pyogenes* obtains iron from hemoproteins have not been defined. In the present study, we show that *S. pyogenes* can directly bind heme and hemoproteins to its surface. We have identified an ABC transporter (named *siaABC* for streptococcal iron acquisition) with homology to transporters that mediate the uptake of iron complexes and investigate its role in iron acquisition.

MATERIALS AND METHODS

Strains, media, and growth conditions. *S. pyogenes* strains used in the present study are listed in Table 1. *S. pyogenes* was grown in Todd-Hewitt broth (TH; Difco Laboratories), TH with 0.2% yeast extract (THY), or TH medium with 10 mM Tris, adjusted to pH 6.9 prior to autoclaving (ZTH) (14). ZTH was depleted of iron by the addition of the chelating agent nitrilotriacetic acid (NTA; Sigma-Aldrich). Medium containing 10 mM NTA was supplemented with 0.55 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, and $ZnCl_2$. *S. pyogenes* was grown statically at 37°C in screw-cap polypropylene tubes or Klett flasks. Defibrinated sheep blood was purchased from Colorado Serum Company (lot 2170) and horse serum from

either Sigma (lot 61k8407) or Atlas Biologicals (lot H20919A). All glassware was washed by soaking for 30 min in chromic-sulfuric acid (Sulfuric Acid and Chromerge; Fisher Scientific) and rinsing with double-distilled water. The *E. coli* strains JM83, DH5α, and Top10 (Invitrogen) were used for cloning and gene expression. *E. coli* cells were grown in Luria broth (LB). When necessary, the antibiotic spectinomycin was used at 100 μg/ml, ampicillin was used at 100 μg/ml, and chloramphenicol was used at 15 μg/ml for *E. coli* and at 5 μg/ml for *S. pyogenes*. Human hemoglobin (Sigma) was prepared as a 10-mg/ml stock solution in phosphate-buffered saline (PBS) and filter sterilized. Heme (0.4 mg/ml; Sigma) was mixed with 4 mg of bovine serum albumin (BSA; Sigma)/ml in PBS and filter sterilized.

Whole-cell binding assay. Cells grown overnight on TH (*S. pyogenes*) or LB (*E. coli*) were harvested, washed, and resuspended in 20 mM phosphate buffer (pH 7.4) containing 0.14 M NaCl (PBS). Cell suspensions were each adjusted to an optical density at 600 nm (OD_{600}) of 1, diluted in PBS, and applied to a nitrocellulose membrane (100 μl/dot, by using BioDot apparatus; Bio-Rad). Biotinylated hemoglobin-binding assays were done as previously described (62). Briefly, cell blots were blocked (with PBS, 0.0025% Tween 20, and 5% skim milk), rinsed, and incubated with 50 nM biotinylated hemoglobin (prepared according to manufacturer's protocol). Binding to hemoglobin was visualized with streptavidin conjugated to alkaline phosphatase (Roche). Heme-binding assays were performed as described by Mazoy and Lemos (35). In short, membranes were incubated for 5 h at room temperature with 10 μM heme (in double-distilled H_2O) and then rinsed; the color was developed in dimethoxybenzidine buffer (7.11 mM *o*-dianisidine, 50 mM sodium citrate [pH 4.4], 0.067% hydrogen peroxide).

Binding assays with proteinase K. Cells from overnight cultures were harvested, washed, resuspended in 1 ml of buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 10 mM NaCl) containing 0.04 μg of proteinase K (Sigma), and incubated for 1 h at 37°C with periodic mixing. The protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Roche) was subsequently added (to a final concentration of 8.2 mM) to stop the reaction, and the cells were harvested and washed three times with 1.5 ml of saline containing 4.2 mM AEBSF. Cell suspensions were then adjusted to an OD_{600} of 1, applied to membranes, and tested for hemoglobin as described above.

DNA manipulations. Cloning, plasmid construction, chromosomal DNA extraction, restriction analysis, DNA transformation, and Southern hybridization were performed by published methods (15, 43, 51). The primers used in the present study are listed in Table 2.

TABLE 2. Primers used in this study

Primer	Location	Sequence
204Y-S	*shr*	5′-TGAAGACGTTACTATTACCG-3′
204Y-A	*shr*	5′-TCTACCTGATAACCACTCCA-3′
204X-S	*spy1796*	5′-AGCATTCCTTTGATATTGGT-3′
204X-A	*spy1796*	5′-GGCTTTTGACTGTCTTTTAC-3′
204A-Fwd	*siaA*	5′-GCTATGATGCTGTTAAGCGTGTGG-3′
204A-Rev	*siaA*	5′-TCTGGAATGGCATGAGCTGTTC-3′
204B-Fwd	*siaB*	5′-GTTATTGCCTTATCCCTTGGTGG-3′
204B-Rev	*siaB*	5′-TACTTCCAACCAGCAAGCGG-3′
204C-Fwd	*siaC*	5′-ACTATTATGGGGGCAAATGG-3′
204C-Rev	*siaC*	5′-GATCAAGGAGGTCTCTTGGTTG-3′
204-ORF1-A	*spy1791*	5′-CCCCAAACTGCAGAATTCCCAGATACATAACTGCA
204-ORF5-S	*spy1787*	5′-ATTGCCAAGCAGGCAGAGACAGTGT-3′
204-ORF5-A	*spy1787*	5′-TCCAAGCCCGTTTGATCCTG-3′
ORFX-3-Fwd	*shr*	5′-GCCAGCATGTTAAAAACTGACTTAGCATCTG-3′
CP-ShuA-S	*siaA*	5′-CCCGGATCCCGGTCTAAAAAAGAC-3′
CP-ShuA-A	*siaA*	5′-CCCCTGCAGGTAAATCTGATGCTG-3′
ORFX-DEL-S	*shr*	5′-CCCGAATTCAAAAGATATCAATGGTAGCT-3′
ORFX-DEL-A	*shr*	5′-CCCGAATTCTGATTGAACTTCACATCTAA-3′
Y-His-Fwd	*shr*	5′-CCCCGAATTCAAATCACAAGAGCCTTTAGTT-3′
Y-His-Rev	*shr*	5′-CCCCGAATTCAAATCACAAGAGCCTTTAGTT-3′
ShuAF	*siaA*	5′-CCCCAACTCGAGGTGAATCAGCACCCTAAAAC-3′
ShuAR	*siaA*	5′-CCCCTTGAATTCTTACGGATGATCTCCCACG-3′
Spec-F	*aad9*	5′-GTGAGGAGGATATATTTGAATACATACGAA-3′
Spec-R	*aad9*	5′-GTCCATTCAATATTCTCTCCAAGATAACTAC-3′
SRAR	*recA*	5′-CTGATGCTACTGCCATAGCAG-3′
SRAL	*recA*	5′-GCGTTCAGGAGGTCTAGCTC-3′
X-Pho-Fwd	*shr*	5′-GGGGGAAGCTTATTGTGATGAATATGCAG-3′
X-Pho-Rev	*shr*	5′-GGGGGAAGCTTACTGATTGACTAGCGTAAGGG-3′

PCR analysis of the *sia* locus in *S. pyogenes* isolates. PCR analysis was done with *S. pyogenes* chromosomal DNA extracted from different strains as a template. The fragments *shr-spy1796*, *spy1796-siaC*, and *siaC-spy1787* were amplified from each strain by using the primer pairs 204Y-S and 204X-A, 204X-S and 204C-Rev, and 204C-Fwd and 204-ORF5-A, respectively.

Construction of plasmids and strains. (i) Construction of SiaA expression vector (pSiaA-His). *siaA* was amplified from the chromosome of SF370 without its leader peptide by using the primers ShuAF and ShuAR and cloned into *Xho*I and *Eco*RI sites in the plasmid pBAD/His (Invitrogen). The resulting plasmid, pSiaA-His, expresses the rSiaA (SiaA N-terminal fusion to His-Xpress epitope) from the arabinose-regulated promoter, P$_{BAD}$ (23). An enterokinase site exists downstream of the His-Xpress epitope.

(ii) Construction of SiaA under the P23 promoter (pCPSiaA). A fragment that carries the *siaA* gene was amplified with CP-ShuA-S and CP-ShuA-A primers and was cloned into the *Bam*HI and *Pst*I sites of pOri23 (46). The resulting plasmid, pCPSiaA, expresses the native SiaA protein from the lactococcal P23 promoter.

(iii) Construction of Shr expression vector (pCB1). *shr* was amplified from SF370 chromosome without its putative signal sequence by using the Y-His-Fwd and Y-His-Rev primers. The PCR product was cloned into the *Eco*RI site of pBAD/His (Invitrogen), resulting in pCB1, where the expression of an amino-terminal fusion of the His-Xpress epitope to Shr (rShr) is placed under control of the P$_{BAD}$ promoter. An enterokinase site exists downstream of the His-Xpress epitope.

(iv) Construction of the Shr-PhoZ fusion (pCW1). A 550-bp fragment of 5′ *shr* was amplified with X-Pho-Fwd and X-Pho-Rev primers and cloned into the *Hin*dIII site of pDC123 (7), ligating a fragment containing the ribosome-binding site and the first 62 amino acids of Shr to *phoZ* coding sequence. This construct, pCW1, carries the in-frame fusion of the leader sequence of *shr* with *phoZ*, replacing PhoZ's native leader with that of Shr. Restriction enzyme analysis determined the insert orientation in the resulting clones. Plasmid pCW1R carries the *shr* fragment in the reverse orientation.

(v) Construction of ZE11 mutant strain. A 1,068-bp internal fragment of *shr* was amplified from SF370 by using the ORFX-DEL-S and ORFX-DEL-A primers and cloned into the *Eco*RI site of the vector pUCSpec, which carries the spectinomycin resistance gene, *aad9* (26). The resulting construct, pCB2, was introduced into SF370 cells by electroporation (Bio-Rad Gene Pulser). Spectinomycin-resistant clones, resulting from the integration of pCB2 into the chromosome via homologous recombination, were selected by plating on TH agar plates containing spectinomycin. The structure of the chromosomal *shr::pCB2* mutation was verified by PCR and Southern blot analysis (Fig. 6). The 204Y-S and Y-His-Rev primer set was used to amplify the *shr* fragment outside of the insertion from the wild type and from the mutant strain (ZE11), and the 204Y-S and Spec-R primer set was used to verify the formation of the chromosome-pCB2 junction in the mutant strain. The Southern blot analysis was done with both an *aad9* specific probe, amplified with the Spec-F and Spec-R primers, and an *shr* probe, amplified with the ORFX-DEL-S and ORFX-DEL-A primers.

RNA methods. (i) RNA preparation. *S. pyogenes* cells were harvested at the mid-log phase (40 Klett units) after the addition of 0.06% sodium azide. RNA was extracted by using the Fast RNA Kit-Blue and FastPrep FP120 (Bio 101, Inc.). RNA was treated with DNase I and quantified by measuring the absorbance at 260 nm, and its integrity was verified on agarose gel. PCR analysis done with 2 μg of RNA as a template was used to confirm the absence of DNA contamination.

(ii) RT-PCR analysis. cDNA was generated with Superscript II reverse transcriptase (RT; Life Technologies) according to the manufacturer's recommendations by using 2 μg of total RNA as a template. The reactions were terminated by heat inactivation of the enzyme. The 204-ORF5-A and 204-ORF1-A primers were used to produce two cDNA molecules, and 1/10 of each RT reaction (2 μl) served as a template in the subsequent PCRs, which was done with the following primer pairs: 204Y-S and 204Y-A, 204X-S and 204X-A, 204A-Fwd and 204A-Rev, 204B-Fwd and 204B-Rev, 204C-Fwd and 204C-Rev, and 204C-Fwd and 204-ORF1-A.

(iii) RNA slot blot. RNA was transferred to a Zeta probe membrane (Bio-Rad) as previously described (67) by using a BioDot SF apparatus (Bio-Rad) and was cross-linked by baking for 2 h at 80°C. The membrane reacted with probes generated by PCR amplifications and was labeled with [α-^{32}P]dATP by random priming (Roche). Probes were made by using the following primer pairs: 204Y-S and 204Y-A for *shr*, 204X-S and 204X-A for *spy1796*, 204A-Fwd and 204A-Rev for *siaA*, 204B-Fwd and 204B-Rev for *siaB*, 204C-Fwd and 204C-Rev for *siaC*, 204-ORF5-S and 204-ORF5-A for *spy1787*, and SRAR and SRAL for *recA*. PCR products were gel purified by using the Concert extraction system (Life Technologies).

Purification of rSiaA. rSiaA was purified from Top10/pSiaA-His cells by using the HisTrap Kit (Amersham), according to the manufacturer's instructions. Expression was induced with 0.02% arabinose for 4 h. Cells were lysed by cycles of sonication, freezing, and thawing in the presence of a protease inhibitor cocktail (Sigma). After elution, rSiaA was concentrated by a Centricon spin column (10,000-molecular-weight cutoff) and quantified by Bradford assay (Bio-Rad). When digested with enterokinase, 20 μg of rSiaA was incubated with 1 U of EnterokinaseMax (Invitrogen) in 50 ml of EkMax buffer for 16 h at 37°C.

Purification of rShr. rShr was purified from DH5α/pCB1 by using the Probond purification system (Invitrogen) according to the manufacturer's protocol. Protein expression was induced with 0.002% arabinose for 4 h. Cells were lysed in lysis buffer (6 M guanidine-HCl, 20 mM sodium phosphate, 500 mM sodium chloride; final pH 7.8) by sonication. rShr protein was eluted from the column by using the lysis buffer at pH 4.0. After elution, the lysis buffer was subsequently replaced with 10 mM Tris (pH 7.4) by using Centricon-10 concentrators (10,000-molecular-weight cutoff, Amicon). The protein concentration was measured by using the Bradford assay (Bio-Rad). When digested with enterokinase, 1.2 μg of rShr were incubated with 0.01 U of EnterokinaseMax (Invitrogen) in 50 μl of EkMax buffer for 16 h at room temperature.

PhoZ enzyme activity in cell-free culture supernatant. Samples from SF370 cultures grown in TH to a density of 40 Klett units were obtained, and the cells were removed by centrifugation. Then, 200 μl of cell-free supernatant were incubated for 1.5 h with 800 μl of alkaline phosphatase substrate, 10% *p*-nitrophenyl phosphate in 1.0 M Tris-HCl (pH 8.03; Sigma). The absorbance was measured at 405 nm.

SDS-PAGE, Western blotting, and in vitro hemoprotein-binding assays. Proteins were prepared in Laemmli sample buffer, separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), and stained with Coomassie brilliant blue (29). For immunological analysis, proteins were transferred to a nitrocellulose membrane and reacted with the indicated antibody (6). For hemoprotein-binding assays, 3 μg of SiaA and 0.3 μg of Shr were resuspended in a sample buffer (65 mM Tris-HCl [pH 6.8], 10% glycerol, 0.03% bromophenol blue), fractionated at 4°C by SDS-PAGE without preboiling (17), and transferred to a nitrocellulose membrane. Membranes were blocked for 2 h, rinsed, and reacted with 50 nM biotinylated hemoproteins for 1 h. Binding was visualized with streptavidin-conjugated alkaline phosphatase (Roche).

Antiserum preparation. The purified protein (rSiaA or rShr) was emulsified with Freund complete adjuvant (Sigma) and injected into New Zealand female rabbits. The rabbits were immunized with two booster injections (in Freund incomplete adjuvant) at 3 to 4 weeks after each injection. Antiserum was collected 1 week after each booster. This protocol was approved by the Georgia State University Institutional Animal Care and Use Committee.

Protein preparation from *S. pyogenes* cells and culture supernatant. Protein preparation was done based on the method of Pancholi and Fischetti (42). Cells from overnight cultures were harvested, washed with saline, and resuspended in either PED (20 mM phosphate buffer, 5 mM EDTA, 10 mM dithiothreitol) for the preparation of total cellular proteins or in PERD (PED with 40% raffinose) for cellular fractionation. For total protein extract, cells were treated with the muralytic enzyme lysin for 1 h at 37°C to dissolve the cell wall and then boiled in sample buffer for 10 min. For cell fractionation, 20 mM MgCl$_2$ (final concentration) was added to lysin-treated cells, and protoplasts were separated from the cell surface fraction by centrifugation, resuspended in PE (20 mM phosphate buffer, 5 mM EDTA), and lysed by successive rounds of flash freezing, thawing, and vortexing. Membrane proteins were separated from the soluble intracellular proteins by centrifugation and were dissolved by boiling in sample buffer. Proteins were prepared from culture supernatant by microfiltration by using a Centricon-10 (10-molecular-weight cutoff; Amicon) at 4°C. Proteins extracted from whole cells, different cellular fractions, or supernatant were standardized with respect to the cell number in the corresponding culture.

Streptonigrin sensitivity. TH medium containing 1.6 μM streptonigrin was inoculated with cells from a glycerol stock (1:1,000 dilution) and incubated at 37°C. Cell growth was determined after overnight incubation.

β-Glucuronidase activity. Plasmid pNZ276 is a shuttle vector that carries the *gusA* gene fused to the *lacA* promoter of *L. lactis*; this promoter is significantly expressed in *S. pyogenes* in the absence of lactose (15a). Enzyme activity associated with ZE11 cells harboring plasmid pNZ276 and in the corresponding supernatant was determined as previously described (15a). Briefly, cells from 6-ml portions of overnight cultures grown in THY were harvested, resuspended in 0.4 ml of lysis buffer, and lysed with a FastPrep cell disrupter (model EP120; Bio 101, Inc.). Then, 5 ml of cell-free supernatant was concentrated ca. 100-fold by using Centricon and Microcon (Amicon) YM10 centrifugal filter devices. Next, 0.1 ml of cell lysate and all of the concentrated culture supernatant were assayed for β-glucuronidase activity by placement in 0.9 ml of reaction buffer and adding 0.2
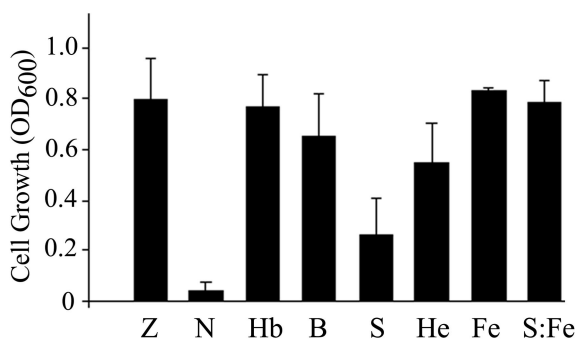
FIG. 1. Use of host iron sources by *S. pyogenes* strain SF370. Cells were used to inoculate (1:1,000 dilution from glycerol stocks) fresh ZTH medium (Z), ZTH medium containing 10 mM NTA (N), ZTH-NTA medium supplemented with 22 μM hemoglobin (Hb), 0.125% whole sheep blood (B), 0.125% horse serum (S), 6 μM heme-BSA (He), 10 mM ferric citrate (Fe), or 0.125% serum and 10 mM ferric citrate (S:Fe). Cell growth is expressed as the culture OD_{600} after overnight incubation (17 h). The results are from at least four separate experiments; the error bars indicate the standard deviation.

ml of 4 mg of *p*-nitrophenyl-β-D-glucuronidase/ml and incubation at 28°C until a yellow color developed in the samples from the whole cells. The reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3, and the OD_{420} was determined. The enzyme activity was corrected for the size of the processed sample (1.5 ml of whole cells and 5 ml for supernatant), and the OD_{420} obtained with ZE11 was used to determine the background values. β-Glucuronidase activity (in units) is expressed as 1,000 times the increase in the OD_{420} per min per the OD_{600} of the original culture.

Hydrogen peroxide susceptibility. Cells from overnight cultures were used to inoculate THY medium to an OD_{600} of 0.01 to 0.02 and then incubated at 37°C. Hydrogen peroxide was added (5 mM final concentration) to the culture at the mid-log phase of growth (OD_{600} 0.3 to 0.45). Culture samples, taken immediately before and 30 min after the addition of hydrogen peroxide, were plated on THY plates to determine viable counts. For hemoglobin protection assays, cells were grown in THYB with 15 μM human hemoglobin. Cell growth is expressed as the fraction (percentage) of CFU/ml obtained after 30 min of incubation with hydrogen peroxide versus that obtained before treatment.

Computer analysis of nucleic and amino acid sequence. Preliminary *S. pyogenes* sequence data was obtained from the streptococcal genome database (http://dna1.chem.ou.edu/strep.html) and from the NCBI microbial genome website (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html) when it became available later. The streptococcal numbers used in the present study represent the gene numbers in the SF370 database. The sequence data was analyzed and manipulated by using MacVector (International Biotechnologies) and Vector NTI (InforMax). BLAST searches of available nucleotide and protein databases and of incomplete microbial genomes were performed by using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). Cell blots were quantified by using the program Image Gauge version 3.0 (Fuji Film).

RESULTS

Heme compounds support *S. pyogenes* M1 SF370 growth in iron-depleted medium. We previously reported that the chelating agent NTA inhibited the growth of *S. pyogenes* M6 strain JRS4 on complete medium (ZTH) and that growth inhibition was specifically reversed by the addition of iron and heme compounds such as hemoglobin (13, 14). In the present study, we used a similar method to characterize the growth of *S. pyogenes* strain SF370 (serotype M1) on iron-depleted medium; this strain was chosen since its genome sequence has been determined (16). Growth of SF370 in ZTH containing 10 mM NTA and a mixture of bivalent cations was inhibited (Fig. 1). The concentration that allows optimal bacterial growth

under these conditions was found to be 22 μM hemoglobin, 6 μM heme-albumin, and 0.125% whole blood. Hemoglobin iron is the most abundant source of iron provided by whole blood (11 g/100 ml, for adult female sheep blood) (2). Addition of 0.125% whole blood provides ca. 2 μM hemoglobin, and it does not add significantly to the concentration of other metals such as Mg, Ca, Mn, and Zn, which are already present in concentrations of 0.5 mM in the ZTH-NTA medium. Therefore, streptococcal growth in the medium supplemented with blood is likely to result from the presence of hemoglobin and heme. Cell growth in 0.125% serum was only partial. It is likely that the inability of serum to support full streptococcal growth is the result of iron limitation rather than serum sensitivity since 10 mM ferric citrate restores full growth in the presence of NTA and serum.

***S. pyogenes* produces surface proteins that directly bind hemoglobin and other hemoproteins.** Since surface receptors are the most prevalent mechanism used by bacteria to capture iron from hemoproteins, we first sought to determine whether *S. pyogenes* could directly bind hemoglobin and free heme. Cells from SF370, JRS4, and nine clinical isolates (that represent seven different M serotypes; Table 1) were filtered onto a nitrocellulose membrane and tested for binding to biotinylated hemoglobin by using a streptavidin alkaline phosphatase reporter system (62). Each of the examined streptococcal strains bound hemoglobin in a cell concentration-dependent manner (Fig. 2A and data not shown). In the control, only very weak binding directly to streptavidin was observed (JRS4-S, in Fig. 2A), indicating that binding is specific to hemoglobin. In contrast to the *S. pyogenes* strains, the *E. coli* strain DH5α exhibited significantly lower binding to hemoglobin. Using the same method, we have also determined that *Streptococcus* binds heme-albumin and myoglobin (data not shown). Conversely, *S. pyogenes* did not bind transferrin and bound lactoferrin poorly, both of which cannot support its needs for iron (14). To test for heme binding, we used a method that is based on the intrinsic peroxidase activity of heme and was used several times to detect heme binding with whole cells or purified proteins (35). Cell blots were incubated with free heme (10 μm) and subsequently stained with the chromogenic substrate dimethoxybenzidine (DMB). All of the tested streptococcal strains bound heme, although to a different extent (Fig. 2B).

To determine the nature of the streptococcal receptors for hemoproteins, we tested the binding sensitivity to protease treatment. Treatment of intact cells with proteinase K reduced hemoglobin binding by 60% $\pm$ 9% ($n = 6$, $P < 0.01$, Fig. 2C), and a similar reduction in heme-albumin binding was observed as well (data not shown). A small but statistically insignificant reduction in binding to heme was observed following the treatment (19 $\pm$ 3, $n = 6$, $P < 0.2$; Fig. 2D). These observations suggest that *S. pyogenes* produces surface proteins that bind hemoglobin and heme-albumin, whereas other constituents of the cell surface are responsible for most of the heme binding under the tested conditions.

Streptococcal iron acquisition (*sia*) transporter. We initiated reverse genetics to identify streptococcal transporters that may be involved in iron acquisition from hemoproteins. A search of the streptococcal genome database and subsequent sequence analysis identified a three-gene transporter (*spy1795*, *spy1794*, and *spy1793*) with significant homology to a bacterial
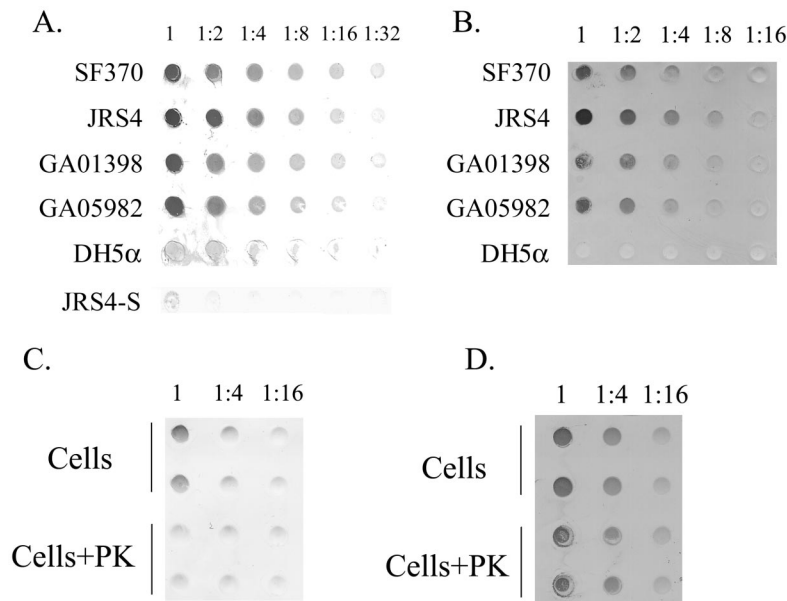
FIG. 2. Hemoglobin and heme binding to *S. pyogenes* surface. (A) Binding to human hemoglobin by *S. pyogenes* isolates. Cell suspensions at an OD_{600} of 1 were immobilized onto a nitrocellulose membrane from a serial dilution. Binding to biotinylated hemoglobin was detected with streptavidin-conjugated alkaline phosphatase. Binding to *S. pyogenes* strains SF370, JRS4, GA01398, and GA05982 and to *E. coli* DH5α is shown. JRS4-S represents JRS4 cells treated only with streptavidin. (B) Heme binding to *S. pyogenes* surface. The results here are the same as in panel A except that the membranes were stained with the chromogenic substrate DMB. (C) Surface proteins mediate hemoglobin binding to *S. pyogenes*. SF370 cells were incubated for an hour in buffer (cells) or were treated with proteinase K (cells+PK) prior to membrane immobilization in duplicates. Hemoglobin binding was assayed as in panel A. (D) Nonprotein constituent(s) mediates most of the heme binding to *S. pyogenes*. The results here are the same as in panel C except that the membrane was stained with DMB.

PBT system involved in iron complex uptake (Table 3). The transporter designated *siaABC* (for streptococcal iron acquisition) is distinct from the transporter for metal cations recently identified in *S. pyogenes* (27). The predicted protein sequence of SiaA (*spy1795*) shows homology to periplasmic binding proteins. The sequence of the first 20 amino acids has the characteristics of a lipoprotein signal with the LVAC sequence that conforms to the consensus sequence recognized by signal pep-

TABLE 3. Characteristics of the *sia* locus

sia protein (mass [kDa], pI)[a]	Protein type[b]	Homologue (organism)	DNA GenBank accession no.	% Similarity/ % identity	Function
Shr (144, 8.5)					Hemoprotein receptor
Spy1796 (32, 8.4)	Unknown	NA[e]			
SiaA (33, 5.8)	Binding protein	SirA (*Staphylococcus aureus*)	AF079518	37/18	Siderophore[c]
		HmuT (*Corynebacterium diphtheriae*)	AF109162	43/22	Heme
		HutB (*Vibrio cholerae*)	AF016580.1	35/17	Heme
SiaB (36, 9.8)	Membrane	FhuG (*Bacillus subtilis*)	AJ223978	56/35	Ferrichrome[c]
	Permease	HemU (*Yersinia enterocolitica*)	X77867	54/31	Heme
		HutC (*Vibrio cholerae*)	AF016580.1	54/30	Heme
		SirB (*Staphylococcus aureus*)	AF079518	53/28	Siderophore[c]
		HutD (*Vibrio cholerae*)	AF016580.1	46/24	Heme
SiaC (32, 6.4)	ATP binding	HmuV (*Yersinia pestis*)	U60647	53/32	Heme
		HemV (*Yersinia enterocolitica*)	X77867	49/28	Heme
Spy1791 (66, 9)	Permease and ATP binding	CydD (*H. influenzae*)	U32749	48/28	Cytochrome[c,d]
		HlyB (*Escherichia coli*)	AB011549	46/24	Hemolysin[e]
Spy1790 (63, 8)	Permease and ATP binding	CydC (*Haemophilus influenzae*)	U32795	46/26	Cytochrome[c,d]
		LktB (*Mannheimia haemolytica*)	M24197	44/24	Leukotoxin
		APXIB (*Actinobacillus pleuropneumoniae*)	X68595	45/24	RTX toxin
Spy1789 (22, 9.6)	Membrane				
Spy1788 (26, 10.3)	Membrane	*CbiQ2* (*Thermoanaerobacter tengcongensis*)	AE013114		Cobalt[c]
Spy1787 (54, 6)	ATP binding	MTH454 (*Methanobacterium thermautotrophicus*)	AE000829		Coenzyme M reductase[c] Cobalt[c]

[a] Calculated molecular masses and pI values are given in parentheses.
[b] Based on sequence homology.
[c] Putative.
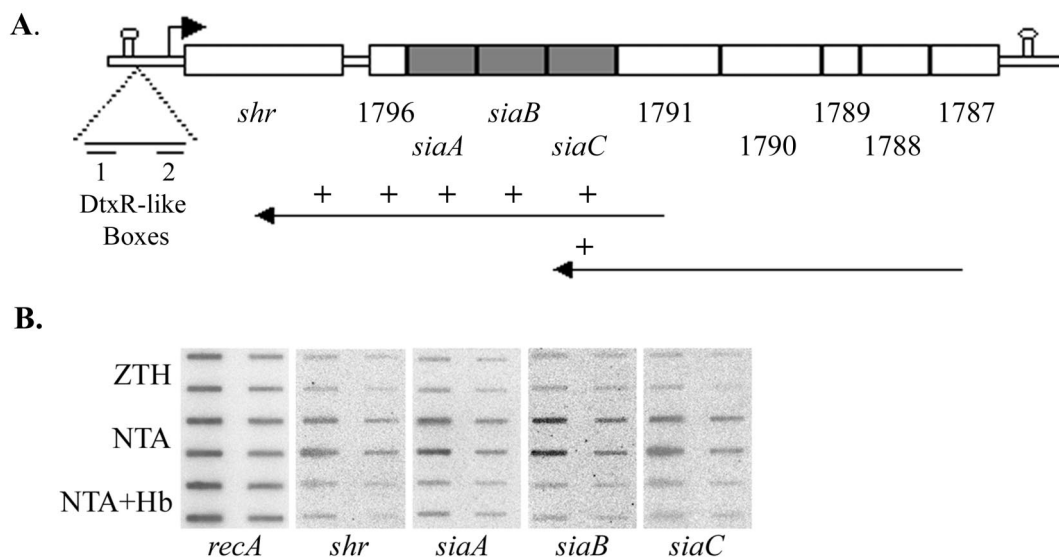[d] Required for cytochrome synthesis.
[e] NA, not applicable.

FIG. 3. *S. pyogenes* iron acquisition (*sia*) locus. (A) The *sia* operon consists of 10 genes flanked by predicted transcription terminators (represented by stem-loops); the numbers below indicate the SF370 gene number, and the small arrow shows the location of the putative *shr* promoter. The long arrows represent the cDNAs produced for the RT-PCR analysis and the "+" symbols represent *sia* sequences found on the cDNAs. (B) RNA slot blots with RNA extracted from mid-log-phase SF370 grown in ZTH, ZTH and NTA (NTA), or ZTH with NTA and hemoglobin (NTA+Hb). We applied 2 and 0.4 μg of RNA from each sample to the membrane in duplicates.

tidase II, LX$_1$X$_2$C (where X$_1$ is typically A, S, V, Q, or T, and X$_2$ is typically G or A) (63). In addition, a region within the N terminus of SiaA contains the signature sequence of binding proteins from the iron-complex family (82-PDIELIASLKPT WILSPNS-101) (66). The deduced amino acid sequences of SiaB (*spy1794*) and SiaC (*spy1793*) have high homologies to permeases and ATPases (respectively) of heme- or sidero-phore-PBT systems. *siaB* encodes a very hydrophobic protein with a sequence in its C terminus (222-KTCNLLILDDQVIRH LGID ATALRLGISLVAVL LASVATS-261) that conforms to the conserved signature of membrane permeases of the siderophore and heme family (52). *siaC* codes to a hydrophilic ATP-binding protein, with Walker A (40-GANGSGKST-49) and Walker B (167-LDEPT-171) motifs and the typical ABC signature (144-LSGG-147).

The *siaABC* transporter is part of a conserved 10-gene cluster. Sequence analysis suggests that *siaABC* genes are present as genes three through five in a 10-gene operon (Fig. 3A). The first gene, *spy1798*, later named *shr*, is separated from the second by 200 bp, whereas the other genes are very close or overlap. Potential promoter signals are found about 40 bp upstream of *shr*. A conserved ribosomal binding site is present in the immediate vicinity of all of the genes. Putative transcriptional termination signals are located about 400 bp upstream of *shr* (with ΔG of −11 kcal/mol) and 200 bp downstream of the last gene, *spy1787* (ΔG of −20 kcal/mol) (Fig. 3A).

S. pyogenes strains are known for their allelic variations and diversity in gene content. Primers generated on the basis of the sequence of *shr*, *spy1796*, *siaC*, and *spy1787* were used in a series of PCRs to determine the presence and structure of the *sia* locus in SF370, JRS4, NZ131, and nine clinical isolates (see Materials and Methods and Table 2). The presence and organization of the *sia* locus was confirmed in all tested strains. Sequence analysis of the operon in the three streptococcal

strains whose genome is available (SF370, MGAS8232, and MGAS315) revealed that the operon sequence is highly conserved. The amino acid residues of Shr, Spy1796, and SiaA are 99.3% identical in the three strains; 99.1% identity was found for SiaB, 98.6% identity was found for SiaC, 98.6% identity was found for Spy1791, 99.1% identity was found for Spy1790, 99.0% identity was found for Spy1789, 99.1% identity was found for Spy1788, and 96.9% identity was found for Spy1787. Spy1796 and SiaA overlap in SF370 and JRS4 (unpublished data) but appear to be in frame in the published sequences of MGAS8232 and MGAS315. It has yet to be determined whether this observation results from a sequencing error or represents a difference between the strains.

The *sia* locus is expressed as an iron-regulated operon. The expression from the *sia* locus was investigated by RT-PCR analysis (Fig. 3A). RNA was extracted from cultures grown on iron-depleted medium, and two cDNA molecules were produced by using primers specific to the complementary strands of *spy1791* and *spy1787*. Subsequent PCR analysis with primers for the coding regions of *shr*, *spy1796*, *siaA*, *siaB*, *siaC*, and *spy1791* was used to determine the transcripts encoded by each of the cDNA products (see Materials and Methods and Table 2). This analysis revealed that cDNA produced with *spy1791* primer codes to all genes upstream of *spy1791* and that the cDNA produced with the *spy1787* primer encompasses five genes and is extended to *siaC*. PCR performed directly on the mRNA without previous reverse transcription reaction did not result in any products, indicating that the RNA was not contaminated with chromosomal DNA. These observations suggest that the genes encoded by the *sia* locus are transcriptionally linked.

Negative regulation via an iron-responsive repressor is a common characteristic of iron uptake systems. We investigated the expression from the *sia* locus in response to iron by RNA
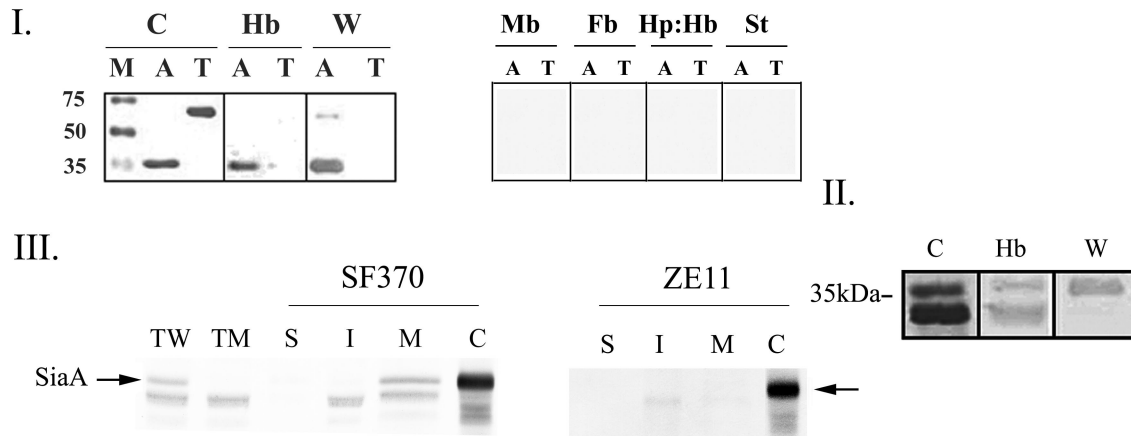
FIG. 4. (I) Purified recombinant SiaA (rSiaA) binds hemoglobin in vitro. Purified rSiaA (His-Xpress-SiaA, A) and human transferrin (T) were fractionated by SDS-PAGE under seminative conditions. A Coomassie blue-stained gel (C), Western blot analysis with anti-express antibodies (W, 1:5,000 dilution), and binding blots to biotinylated hemoglobin (Hb), myoglobin (Mb), fibrinogen (Fb), haptoglobin-hemoglobin (Hp:Hb), or streptavidin (St)-conjugated alkaline phosphatase are presented. M, molecular weight marker. (II) rSiaA binding to hemoglobin is independent of the His-Xpress epitope. The results here are the same as in panel A, except the rSiaA was digested with enterokinase prior to analysis. Both the digested and the undigested rSiaA can be seen in the Coomassie blue stain and the hemoglobin blot. (III) SiaA is associated with the *S. pyogenes* envelope. Total protein extracts prepared from the wild-type SF370 (TW) and the mutant ZE11 (TM) were analyzed by Western blot analysis with anti-rSiaA serum (1:5,000 dilution). Proteins from various cell fractions of SF370 and ZE11 strains are included: S, surface proteins; I, intracellular proteins; and M, membrane proteins. Total protein extracts from whole *S. pyogenes* SF370/pCPSiaA cells expressing the native SiaA from P23 promoter is included as a positive control (C). The arrows indicate SiaA.

slot blot analysis. The amounts of *shr* and each of the *siaABC* transcripts produced in cells grown in complete medium (ZTH) were compared to those produced in cells grown on iron-restricted medium (6 mM NTA) and to those grown on iron-restricted medium supplemented with hemoglobin (6 mM NTA + 8 μM Hb; Fig. 3B). All membranes were probed with a *recA*-specific probe to ensure that equal amounts of mRNA from each sample were loaded. Steady-state levels of transcripts from the *sia* locus were three to four times higher when cells were grown in iron-depleted medium than when cells were grown in complete medium. The addition of hemoglobin to the medium resulted in reduction in the NTA-induced expression from the operon, suggesting that the negative regulation of the operon is specific to iron.

SiaA is a hemoglobin-binding protein that is associated with the cell membrane. SiaA-N-terminal fusion to the His tag Xpress epitope (Invitrogen) was cloned under the control of P$_{BAD}$, the arabinose promoter (23). The recombinant protein (rSiaA) was expressed and purified from *E. coli* extracts. A protein band in the predicted size of 35 kDa (SiaA with the addition of the epitope) that reacted with the anti-Xpress antibodies was observed, confirming the production and purification of rSiaA (Fig. 4I). In the absence of an outer membrane, substrate-binding proteins may function as surface receptors; the ability of *C. diphtheria* HmuT to bind heme and hemoglobin supports this notion (11). We sought to determine whether rSiaA, the binding protein of the *siaABC* transporter, could directly bind hemoglobin. A solid-phase binding assay with purified rSiaA fixed to a membrane demonstrated that it could bind hemoglobin (Fig. 4I). Transferrin was included as a control, as was biotinylated fibrinogen. Human transferrin did not bind hemoglobin, demonstrating that hemoglobin binding is specific to rSiaA, and rSiaA did not bind biotinylated fibrinogen or streptavidin alone (Fig. 4I). This finding indicates that

rSiaA specifically binds hemoglobin and does not recognize the biotin group nor does it bind proteins nonspecifically. In addition to hemoglobin, other heme compounds can support growth of *S. pyogenes* in iron-depleted medium. Using the same binding assay, we determined that SiaA could bind neither myoglobin nor hemoglobin-haptoglobin complexes, indicating that the utilization of these heme compounds requires other proteins (Fig. 4I).

Histidine residues are common heme axial ligands in hemoproteins. Histidine residues conserved among heme/hemoprotein receptors were demonstrated to function in heme transport in the *Yersinia enterocolitica* HemR receptor (3); thus, we were concerned that the His-Xpress epitope could have contributed to hemoglobin binding. The His-Xpress epitope was removed by using enterokinase digest as indicated by the production of a predominant protein band smaller than rSiaA that did not react with the anti-Xpress antibodies (Fig. 4II). A less-abundant protein band representing intact rSiaA was also observed, indicating that the digest was not complete. Subsequent hemoglobin-binding assays showed that both rSiaA and rSiaA without the epitope bound hemoglobin, demonstrating that the binding ability of rSiaA is inherent to the native SiaA protein (Fig. 4II).

Sequence analysis suggests that the mature SiaA carries a lipid modification, which is likely to anchor it to the cell membrane. The in vivo production of SiaA and its cellular location were determined by using cell fractionation and Western blot analysis with anti-rSiaA antiserum. Proteins were prepared from different fractions of *S. pyogenes* cells, by using lysin, a bacteriophage muralytic enzyme (42). *S. pyogenes* cells expressing the native SiaA protein in *trans* from the P23 promoter (46) were used as a positive control. The rSiaA antiserum reacted with a protein band in the expected size found in both SF370 cells (TW, Fig. 4III) and in the positive control, SF370/
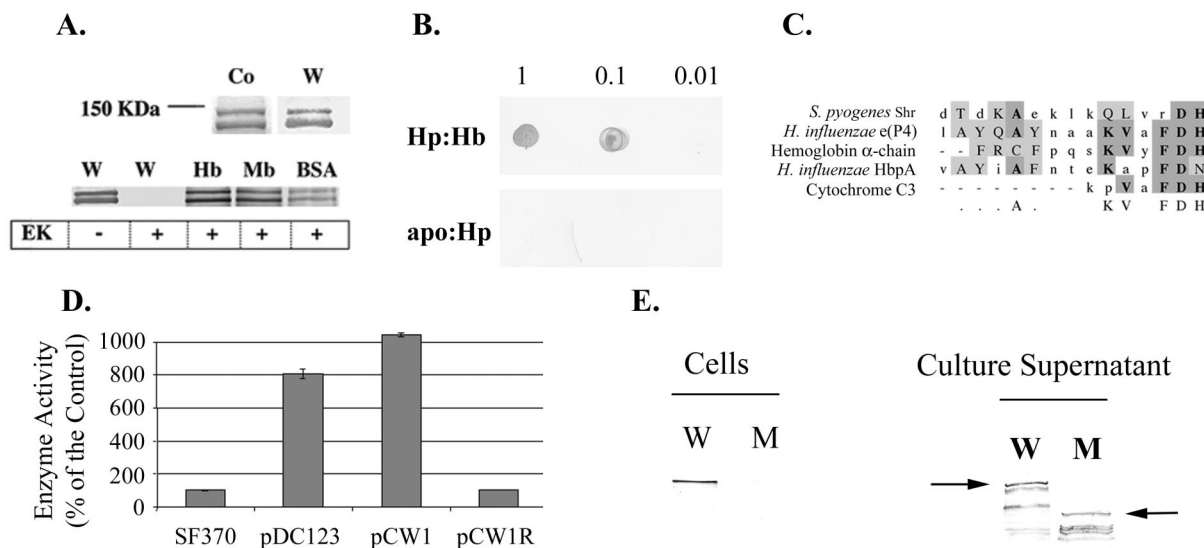
FIG. 5. (A) Purified rShr (His-Xpress-Shr) was fractionated by SDS-PAGE under seminative conditions. Coomassie blue-stained gel (Co), Western blot analysis with anti-Xpress antibodies (W, 1:5,000 dilution) and binding blots to biotinylated hemoglobin (Hb), myoglobin (Mb), and heme-BSA (BSA) are shown. The "+" sign indicates digestion of rShr with enterokinase prior to analysis. (B) rShr was spotted onto a membrane (1, 0.1, and 0.01 μg/spot) and reacted with biotinylated haptoglobin-hemoglobin (Hp:Hb) or apo-haptoglobin (apo:Hp). (C) Shr heme-binding motif. (D) PhoZ enzyme activity in cell-free culture supernatant of *S. pyogenes* harboring Shr-PhoZ fusion. Enzyme activity is expressed as a percentage of the background activity measured in SF370. Activity in cultures of cells harboring PhoZ expressed with its native leader peptide (pDC123, positive control), Shr-PhoZ fusion (pCW1), and Shr-PhoZ fusion in a reverse orientation (pCW1R, negative control) are shown. The mean and standard deviation of at least two independent experiments, each conducted in duplicates, are shown. (E) Western blot analysis of proteins prepared from SF370 (W) and ZE11 (M) strains with anti-rShr serum (1:5,000 dilution). Proteins prepared from cells and cell-free culture supernatant are included. The arrows point to the full-length Shr in wild-type and mutant strains.

pCPSiaA (C, Fig. 4III). A lower-molecular-weight band that cross-reacts with anti-SiaA was also found in the streptococcal protein preparations. In agreement with the predicted lipid modification, SiaA protein was found only in the membrane fraction of SF370 (M, Fig. 4III). As expected from the RNA analysis, iron restriction induced the production of SiaA (data not shown).

Shr is a hemoprotein binding receptor. The first gene in the cluster, *shr* (for streptococcal hemoprotein receptor [*spy1798*]), encodes a large hydrophilic protein that has no significant homologues in other bacteria but shares partial homology with eukaryotic receptors such as Toll and G-protein-dependent receptors (gi 15675635 [GenBank]). Additional sequence analysis identified a domain with leucine-rich repeats and a heme-binding motif described by Reidl and Mekalanos (47) that is shared by both prokaryotic and eukaryotic heme-binding proteins (Fig. 5C).

The *shr* gene was cloned into pBAD-His vector (Invitrogen), and the recombinant protein (rShr) was expressed and purified from *E. coli* extracts. SDS-PAGE and Western blot analysis with the anti-Xpress antibodies confirmed the production of Shr (Fig. 5A). Partial degradation during protein purification was apparent, producing a smaller band. This cleavage took place at the C terminus of rShr, since the resulting bands reacted with the anti-Xpress antibodies. The His tag Xpress epitope was removed by enterokinase (as indicated by Western analysis), and the binding of Shr to biotinylated hemoproteins was investigated. Shr directly bound hemoglobin, myoglobin, heme albumin, and the hemoglobin-haptoglobin complex but not apo-haptoglobin or fibrinogen (Fig. 5A and B and data not

shown). Together, these observations suggest that Shr specifically recognizes heme-containing proteins.

Amino acid sequence analysis suggested that Shr contains a leader peptide sequence. To determine whether Shr has a functional leader peptide, we constructed a translational fusion between the 5′ *shr* region and the alkaline phosphatase gene (*phoZ*) of *Enterococcus faecalis* (7) and tested for the secretion of the fusion protein. The construct carrying the fusion protein (plasmid pCW1) was introduced into *S. pyogenes* SF370 strain, and the alkaline phosphatase activity was measured by using a colorimetric detection assay. The PhoZ activity in the culture supernatant of cells harboring pCW1 was 10 times higher than the activity observed in cells that do not carry PhoZ or in cells that carry the fusion in the reverse orientation (pCW1R), indicating that Shr has a functional leader peptide (Fig. 5D).

Western blot analysis demonstrated the in vivo production of Shr (Fig. 5E). A protein band of the expected size (~140 kDa) that reacted with the anti-Shr antibodies was found both in the total cell extract and free in the culture supernatant. In addition to the full-length Shr, partial degradation was apparent in the supernatant, producing a banding pattern that is similar to that obtained when Shr was purified from *E. coli* extract, implying that the protein is cleaved by common proteases. To estimate the cell lysis occurring in the assayed cultures, we used the enzyme GusA carried by plasmid pNZ276 (15b). Since GusA does not have a leader peptide, it may be used as a cytoplasmic marker, and β-glucuronidase activity in the culture supernatant may be proportional to cell lysis. Enzyme activity associated with whole cells was compared to that found in the corresponding cell-free supernatant of *S. pyogenes*
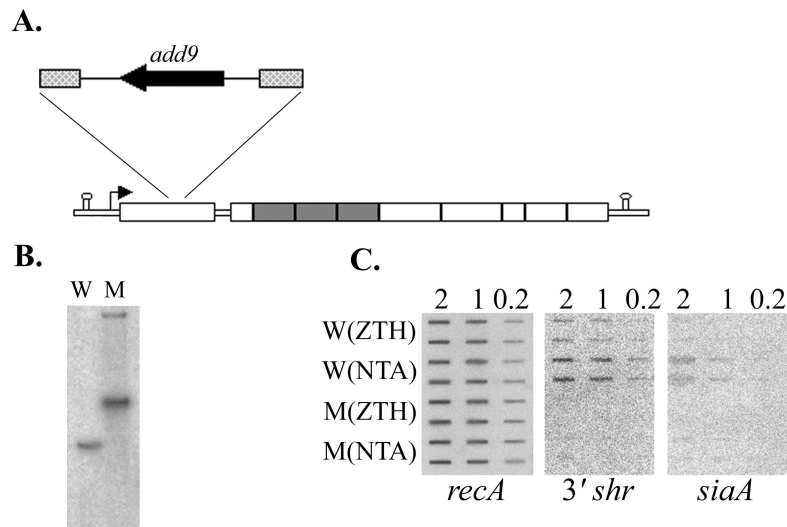
FIG. 6. Construction and analysis of an insertion duplication mutant in the *sia* operon. (A) Schematic presentation of the *shr::pCB2* mutation. The insertion site and the structure of plasmid pCB2 are indicated. The dashed boxes indicate the internal *shr* fragment cloned into pCB2. (B) Southern blot analysis. Chromosomal DNA of the parent strain SF370 (W) and the mutant strain ZE11 (M) was digested with *Bgl*II and hybridized with a [α-^{32}P]dATP-labeled probe specific to the *shr* gene. (C) RNA slot blot analysis of *sia* genes in both the wild-type (W) and the mutant (M) strains. The same as in Fig. 3C, RNA was extracted from mid-log-phase SF370 grown in complete medium (ZTH) or iron-restricted medium (NTA).

cultures. In cells that harbor pNZ276, β-glucuronidase activity associated with whole cells was 24 ± 13 U, which is about 11 times the background level (2.25 ± 0.35 U). In the culture supernatant, β-glucuronidase activity was 0.45 ± 0.49 U, which is comparable to the background level (0.65 ± 0.07 U). Based on difference in the activity found between whole cells and culture supernatant, we estimate that about 2% of the cells in the culture may have lysed. Two percent may represent an underestimate of the actual cell lysis, since it is possible some of the GusA activity may have been lost due to the presence of proteases. However, as the amounts of soluble Shr appears comparable to the amount of cell associated Shr (Fig. 5E), it seems reasonable that the presence of Shr in the supernatant results from secretion rather then cell lysis. Treatment of whole cells with proteinase K prior to protein extraction and Western blot analysis resulted in the disappearance of the Shr band from the total protein extract (data not shown). The susceptibility of Shr in intact cells to protease digest indicates that it is associated with the cell surface. The hydrophobic region found in the C terminus of Shr may help anchor the protein to the cell membrane.

Inactivation of the *sia* operon results in decreased iron uptake and hemoglobin binding to the surface. To further characterize the role of the *sia* locus, an insertion duplication mutation was constructed in the *shr* gene (strain ZE11, Fig. 6A). The structure of the chromosomal mutation was verified by PCR and Southern blot analysis. A chromosomal fragment that reacted with the *shr* specific probe was produced in the wild-type strain (W, Fig. 6B). In agreement with the integration of the pCB2 plasmid into the *shr* gene, the corresponding fragment in the mutant strain was larger and reacted with the *aad9*-specific probe (M, Fig. 6B and data not shown). Western blot analysis with Shr antiserum revealed the production of a truncated Shr that is found predominantly in the supernatant (Fig. 5E).

RT-PCR analysis indicated that genes in the *sia* gene cluster are transcriptionally linked (Fig. 3A). We investigated the effect of the *shr::pCB2* mutation on the expression from the *sia* operon by RNA slot blot analysis. The amounts of 3′ *shr* and *siaA* mRNAs produced in the wild-type cells grown in iron-rich and iron-depleted media were compared to those produced in the mutant cells. All membranes were probed with a *recA*-specific probe as a control for the mRNA amounts. A dramatic decrease in the production of the 3′ portion of the *shr* transcript was observed in ZE11, indicating that pCB2 integration into the *shr* gene does not allow transcription into the 3′ end of *shr*. Accordingly, almost no *siaA* mRNA was observed in the mutant (Fig. 6C) and expression of *siaB* and *spy1791* was significantly reduced as well (data not shown). These observations indicate that, under the tested conditions, the *shr* promoter provides most of the transcription of the *sia* operon. This conclusion was further supported by Western blot analysis with anti-SiaA antibodies, which demonstrated that SiaA protein was not detected in the mutant strain, although the lower-molecular-weight cross-reacting band is observed (TM and ZE11 panel, Fig. 4III).

The growth of ZE11 was compared to that of the parent strain in complete medium, iron-restricted medium, and iron-restricted medium supplemented with hemoglobin. The mutant growth rate was similar to that of the wild type under all conditions (Fig. 7A). In addition, no difference in the ability of the mutant to use whole blood, heme-albumin, or ferric citrate was observed (data not shown), indicating that iron acquisition from hemoproteins in *S. pyogenes* is complex and involves multiple pathways.

Since both Shr and SiaA proteins bind hemoglobin in vitro, we tested the ability of the mutant strain to bind hemoglobin. In agreement with the loss of SiaA and the production of a truncated Shr, ZE11 cells exhibited 40% ± 8% ($n = 6$, $P <$ 0.01) reduction in hemoglobin binding (Fig. 7B). Therefore,
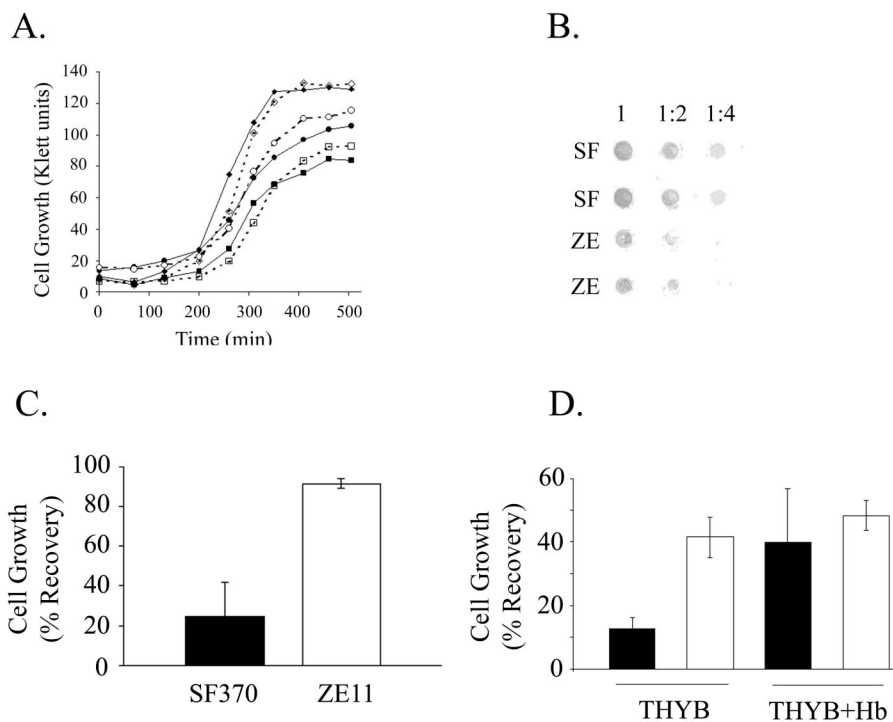
FIG. 7. Analysis of strain ZE11. (A) Growth of the wild-type strain SF370 (solid shapes) and the mutant strain ZE11 (open shapes). Cells were used to inoculate (1:100 dilution) fresh ZTH medium (diamonds), NTA containing medium (squares), and NTA-medium supplemented with 8 μM hemoglobin (circles). (B) Binding of biotinylated hemoglobin to cells of SF370 (SF) and ZE11 (ZE). The hemoglobin-binding assay was done as described in Fig. 2A. (C) Sensitivity to streptonigrin of the wild-type and the mutant strains. Cells were used to inoculate (1:1,000 dilution) fresh ZTH medium and medium containing 1.6 μM streptonigrin. The culture optical density (OD_{600}) after overnight incubation (17 h) was determined. The data are presented as the percent growth of each strain in ZTH. The results are from seven independent experiments; the error bars indicate the standard deviation ($P < 0.01$, t test). (D) Sensitivity of SF370 and ZE11 to hydrogen peroxide. Overnight cultures were used to inoculate THYB or THYB containing 15 μM hemoglobin. Cells in mid-log phase were challenged with 5 mM hydrogen peroxide for 30 min. Viable counts were determined by plating samples taken before and after the challenge, and cell recovery was expressed as the percentage. The data represent results obtained from at least three experiments and plating was done in triplicate. Error bars represent the standard error of the mean ($P < 0.01$ for SF370 and ZE11 in THYB and $P < 0.02$ for SF370 in THYB and SF370 in THYB+Hb according to the Student t test).

the sia operon contributes to the in vivo binding of hemoglobin to the surface of S. pyogenes, but additional proteins are likely to be involved.

The antibiotic streptonigrin interacts with the intracellular iron pool, causing the formation of reactive oxygen species. The toxicity of streptonigrin is proportional to the intracellular iron pool (71), and mutants in iron uptake systems are more resistant to the drug (4, 53). We compared the sensitivity of the shr mutant to that of the wild-type strain. Complete medium (TH) or medium containing increasing concentrations of streptonigrin were inoculated with S. pyogenes, and the culture growth after overnight incubation was determined. ZE11 cells were able to grow in higher concentrations of streptonigrin than the wild-type SF370, indicating that the mutant is more resistant to streptonigrin. Figure 7C shows that with 1.6 μM streptonigrin the growth of the wild type was dramatically inhibited (the culture reached 20% ± 14% of the growth on TH), whereas growth of the mutant was not significantly affected (88% ± 8%). These results suggest that the intracellular iron concentration in the sia mutant is lower than that of the wild type.

The susceptibility ZE11 to hydrogen peroxide stress was compared to that of the wild-type strain. Cells grown in THY to their mid-logarithmic phase were treated with 5 mM hydro-

gen peroxide for 30 min, and the bacterial recovery was determined. ZE11 recovery was higher than that of the wild-type strain (42 and 13%, respectively). This observation is also consistent with a decrease in intracellular iron content in the ZE11 mutant, since iron enhances oxidative stress. It was previously reported that the addition of hemoglobin to the culture medium resulted in a small growth stimulation of strain HSC5 in the presence of methyl violgen, a phenomenon attributed to the intrinsic peroxidase activity of hemoglobin (27a). We investigated the effect of the presence of hemoglobin on the cell susceptibility to hydrogen peroxide. Hemoglobin in the culture medium increased the recovery of SF370 from 13 to 40%, whereas no such stimulation was observed in the ZE11 mutant. This result is in agreement with the observed reduction in hemoglobin binding by ZE11 and suggests that the sia locus may be involved in hemoglobin-dependent resistance to hydrogen peroxide.

DISCUSSION

The growth of most organisms requires iron, which serves as a catalyst for electron transfer and is an essential component of many important enzymes. The use of heme and hemoproteins as a source of iron is ubiquitous in bacteria; however, the

bacterial mechanisms involved are not fully understood. *S. pyogenes* is an obligate parasite of the human body that requires iron for growth and can make use of hemoglobin, myoglobin, heme-albumin, and the hemoglobin-haptoglobin complex (13, 14, 18). *S. pyogenes* cannot use transferrin and lactoferrin, the ferric carriers in the serum and secretions (14). Therefore, it is likely that heme compounds are an important source of iron for *Streptococcus* during infection. The means by which *S. pyogenes* obtains iron from hemoproteins or transports any form of iron complex have not been characterized. The present study describes a molecular genetic investigation of iron acquisition from hemoproteins in *S. pyogenes*.

The study of *S. pyogenes* strain SF370 (M1 serotype) shows that it, like JRS4 (M6 serotype), can use heme compounds to support its needs for iron (Fig. 1). We have also demonstrated for the first time that whole blood can support *S. pyogenes* growth in iron-depleted medium; this observation is consistent with the hemolytic nature of this pathogen and suggests that hemolysis is used by *S. pyogenes* to obtain iron. Iron restriction was reported to induce hemolytic activity in *S. pyogenes* (22), and recent analysis of SF370 revealed that it induced the expression of SLS and other genes encoding putative hemolysins (60). The optimal heme concentration required when provided by whole blood or by heme-albumin is similar (6 to 8 μM), whereas it is about 10-fold higher when provided in the form of hemoglobin solution (Fig. 1). It is possible that in solution hemoglobin is oxidized to different forms (oxyhemoglobin and methemoglobin [19]) affecting its utilization by *S. pyogenes*, whereas in the blood hemoglobin is kept in the reduced form because of the presence of methemoglobin reductase.

Cell-binding assays revealed that *S. pyogenes* binds heme-containing proteins such as hemoglobin and heme-albumin to its surface (Fig. 2A and data not shown). Proteins mediate hemoprotein binding as was shown by the protease sensitivity assays (Fig. 2C). Streptococcal binding of hemoproteins is probably the first step in iron capture by the bacteria. The increase in bacterial recovery after hydrogen peroxide challenge observed in the presence of hemoglobin (Fig. 7D) suggests that binding to hemoglobin and possibly other hemoproteins may serve a protective role as well, since *S. pyogenes* produces hydrogen peroxide during aerobic growth (21) and may encounter it during infection.

S. pyogenes can also bind free heme (Fig. 2B). Hyaluronic acid may contribute to heme binding to *S. pyogenes* surface due to the electrostatic interactions between the polyanionic polysaccharide and the cationic heme (20); this macromolecule can also bind human hemopexin, a heme-binding glycoprotein found in the serum (25). Bacterial protein receptors for hemoproteins can often bind free heme as well (19, 70), which may also be the case for *S. pyogenes*. This assumption is supported by our observation that heme can compete, although not efficiently, with hemoglobin binding to the surface (data not shown). However, resistance to protease treatment suggests that heme binding to a nonprotein constituent is the dominant mechanism.

Using a genomic approach, we identified the *siaABC* transporter that shares significant homology with transporters of heme and siderophores. The *siaABC* transporter is located in the middle of a 10-gene cluster, which was confirmed by RT-PCR and ZE11 mutant analysis to be expressed as an operon

(Fig. 3A and 6C). The loss of SiaA expression in strain ZE11 (TM and ZE11 panel in Fig. 4III) and the dramatic decrease in the transcription of the *sia* genes (Fig. 6C) suggests that most of the transcription in the *sia* locus initiates from the promoter of the first gene, *shr*. This region contains a possible -35 box (GTGATA) that corresponds to that of the closely related species *L. lactis* (-TG-T-) (69), a 16-bp spacer, and a putative -10 box (TATAAA).

RNA analysis shows that, as in many iron acquisition systems, iron restriction induces expression from the *sia* operon, although low constitutive expression is observed when the cells are growing in complete medium. The ability of hemoglobin to repress the *sia* expression back to the background level indicates that the regulation is specific for iron. Putative sequence elements that may participate in the iron regulation of the operon are found in the upstream region of *shr*. These sequences have imperfect inverted repeats sharing 12 and 13 of 19 bp with the consensus sequence of the DtxR-repressor binding box (55). One of the binding boxes overlaps the -10 sequence of the inferred *shr* promoter mentioned above (Fig. 3A). Homologues to both Fur and DtxR (*spy0187* and *spy0450*, respectively) can be identified in the genome of *S. pyogenes*, but the presence of DtxR-like binding sequences implies that a DtxR homologue participates in the *sia* regulation. A second iron complex transporter found in SF370 genome (*spy0383*, *spy0384*, *spy0385*, and *spy0386*) is induced by iron restriction in cells growing at 37°C (unpublished data) or 40°C (60). Interestingly, regulation of this transporter is probably mediated by a Fur homologue, as evidenced by the presence of a potential Fur-box in the *spy0383* promoter region. Iron regulation is only poorly understood in *S. pyogenes*; additional work is required to reveal the physiological role of the different repressors and the regulatory network in which they are involved.

We demonstrated that SiaA, the transporter binding protein, is associated with the cell envelope and can produce a stable complex with hemoglobin (Fig. 4I). In addition, the *sia* operon codes for Shr, a surface-associated protein that binds in vitro to a variety of heme-compounds (Fig. 5A and B). Cells of strain ZE11 bind significantly less hemoglobin (Fig. 7B), indicating that the *sia* operon contributes to hemoglobin binding in vivo. The increased resistance to streptonigrin of the ZE11 mutant strain (Fig. 7C) suggests that this strain takes up less iron. Streptonigrin toxicity is proportional to the internal iron concentration (71); consequently, mutants in iron uptake pathways are typically more resistant to this antibiotic (4, 53). Together, the decrease in binding and the increased resistance to streptonigrin argue that the *sia* operon is involved in iron capture from hemoproteins. ZE11 was also more resistant to hydrogen peroxide when grown on THYB (Fig. 7D). This observation supports reduced iron uptake in this strain, since intracellular iron can augment oxidative stress. The addition of hemoglobin to the medium did not enhance ZE11 resistance to hydrogen peroxide as it did for SF370; this observation suggests that the *sia* operon may mediate hemoglobin-dependent resistance to hydrogen peroxide, possibly by binding hemoglobin (which has intrinsic peroxidase activity) to the cell surface, or by some other mechanism that may utilize the heme molecule.

It is likely, however, that *S. pyogenes* SF370 contains hemoprotein utilization machinery in addition to the *sia* locus, since

ZE11 was able to use heme compounds similarly to the wild-type strain (Fig. 7A) and since binding to hemoglobin or hemoglobin-haptoglobin was not eliminated (Fig. 7B). Redundancy in hemoprotein utilization systems was observed in other pathogenic bacteria, including *C. diphtheriae* (54), *S. pneumoniae* (4), and strains of *Neisseria* (33, 61) and *Haemophilus* (48). The second iron complex transporter found in the genome of SF370 (*spy0383*, *spy0384*, *spy0385*, and *spy0386*) is a likely candidate for an additional heme uptake system.

To the best of our knowledge, HmuT and SiaA are the only substrate-binding proteins in gram-positive bacteria that were demonstrated to bind hemoglobin. The ability of SiaA to directly bind hemoglobin corresponds to the model proposed by Drazek et al., which suggests that in gram-positive bacteria, binding proteins function as surface receptors for heme and hemoglobin (11). Schneider and Hantke previously proposed a similar model for siderophore uptake systems (56). However, unlike the situation in siderophore uptake, binding proteins involved in iron-heme capture from hemoproteins do not interact directly with their ligand; rather, they must extract it from a high-affinity protein carrier. Therefore, the function of such binding proteins in gram-positive bacteria is conceptually similar to that of outer membrane heme-hemoprotein receptors. Invariant histidine residues, conserved glutamic acid residues, and a highly conserved receptor domain containing two amino acid motifs (FRAP and NPNL) were identified in outer membrane receptors engaged in heme acquisition (3, 59). SiaA does not share sequence homology with hemoglobin-heme outer membrane receptors, nor does it carry the sequence motifs mentioned above; additional work is required to determine how SiaA recognizes and binds hemoglobin.

The identification of Shr is intriguing, since this protein that binds hemoglobin, hemoglobin-haptoglobin, myoglobin, and heme-albumin does not share homology with any of the known heme or hemoprotein receptors. Shr did not bind apo-haptoglobin, fibrinogen, or streptavidin, indicating that Shr specifically recognizes hemoproteins. Although Shr lacks overall homology to hemoprotein receptors, a heme-binding motif is found in the carboxy terminus of Shr (Fig. 5C). This motif is shared by several heme-binding proteins, including *H. influenzae* eP4 and HbpA, which are required for heme acquisition, and the heme-containing proteins hemoglobin (alpha-chain) and cytochrome C3 (47). Shr may be able to recognize both the heme moiety and the protein portion of hemoproteins; this recognition may be facilitated by Shr's leucine-rich repeats. The ability to discriminate between apo-haptoglobin and hemoglobin loaded haptoglobin was also demonstrated in the *P. gingivalis* hemoprotein receptor HmuR (39). This property may allow the receptor to unload the apo-haptoglobin after the capture of heme.

The linkage of *shr* to the *sia* operon that is conserved among streptococcal isolates argues that Shr is also functionally related to the SiaABC transporter. One might speculate that Shr and SiaABC may compose a multicomponent iron uptake system, in which Shr functions to obtain heme or iron from hemoproteins and to deliver it to the SiaABC transporter. Only a few bacterial multicomponent systems involved in heme capture are known. These include HpuAB, the bipartite hemoglobin-haptoglobin receptor (33), two types of hemophore-dependent systems (70), the *Porphyromonas* Kgp and HRgpA

proteases that interact with the outer membrane receptor HmuR (39), and the Hbp hemoglobin protease of *E. coli* (EB10), which was proposed to function as a shuttle protein of a heme uptake system (41). It is interesting that, like the hemophore HxuA and the Kgp hemoglobinase, Shr is found both on the cell surface and in the culture supernatant (9, 45). Analysis of the Shr-PhoZ fusion demonstrated that Shr has a functional leader peptide; however, the mechanism that anchors Shr to the surface remains unclear. Shr lacks the LPXTG motif commonly used to anchor proteins to the cell wall in gram-positive bacteria (37). Shr may be threaded through the membrane via its hydrophobic C terminus. Although we favor the hypothesis that *shr* is involved in iron acquisition, it is also possible that it may have other functions. The ability to bind hemoproteins and perhaps to acquire heme raises the possibility that it uses heme for redox sensing.

In addition to *shr* and *siaABC*, the operon codes for six other genes whose characteristics are described in Table 3. Two ATP-binding proteins (*spy1791* and *spy1790*) with homology to hemolysin secretion permeases such as *hlyB* (34) and to the cytochrome biosynthesis genes *cydCD* (44) are found downstream of the *siaABC* genes. *S. pyogenes* is a facultative anaerobe, and early studies determined that it does not contain cytochrome-like NADH oxidase enzymes (49). The two related species *E. faecalis* and *Lactococcus lactis* code to a *cydABCD* gene cluster and are capable of aerobic respiration when grown in the presence of heme (12, 72). However, we could not identify any *cydAB* homologues in the SF370 chromosome. In conclusion, the *sia* locus is a conserved iron regulated operon involved in iron acquisition and hemoprotein binding and includes genes that may be involved in the acquisition and/or use of heme.

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