

# The ABC Transporter HrtAB Confers Resistance to Hemin Toxicity and Is Regulated in a Hemin-Dependent Manner by the ChrAS Two-Component System in *Corynebacterium diphtheriae*<sup>∇</sup>

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*Corynebacterium diphtheriae*, the causative agent of the severe respiratory disease diphtheria, utilizes hemin and hemoglobin as iron sources for growth in iron-depleted environments. Because of the toxicity of high levels of hemin and iron, these compounds are often tightly regulated in bacterial systems. In this report, we identify and characterize the *C. diphtheriae* *hrtAB* genes, which encode a putative ABC type transporter involved in conferring resistance to the toxic effects of hemin. Deletion of the *hrtAB* genes in *C. diphtheriae* produced increased sensitivity to hemin, which was complemented by a plasmid harboring the cloned *hrtAB* locus. The HrtAB system was not involved in the uptake and use of hemin as an iron source. The *hrtAB* genes are located on the *C. diphtheriae* genome upstream from the *chrSA* operon, which encodes a previously characterized two-component signal transduction system that regulates gene expression in a heme-dependent manner. The *hrtB* promoter is activated by the ChrAS system in the presence of hemin or hemoglobin, and mutations in the *chrSA* genes abolish heme-activated expression from the *hrtB* promoter. It was also observed that transcription from the *hrtB* promoter is reduced in a *dtxR* deletion mutant, suggesting that DtxR is required for optimal expression of *hrtAB*. Previous studies proposed that the ChrS sensor kinase may be responsive to an environmental signal, such as hemin. We show that specific point mutations in the ChrS N-terminal transmembrane domain result in a reduced ability to activate the *hrtB* promoter in the presence of a heme source, suggesting that this putative sensor region is essential for the detection of a signal produced in response to hemin exposure. This study shows that the HrtAB system is required for protection from hemin toxicity and that expression of the *hrtAB* genes is regulated by the ChrAS two-component system. This study demonstrates a direct correlation between the detection of heme or a heme-associated signal by the N-terminal sensor domain of ChrS and the transcriptional activation of the *hrtAB* genes.

*Corynebacterium diphtheriae* is a Gram-positive bacterium and a well-known human pathogen that causes the severe upper respiratory tract disease diphtheria. *C. diphtheriae* colonizes the upper respiratory tract and is rarely found outside this region during infection in humans. Much of the severe morbidity and mortality associated with diphtheria is due to the production and secretion of diphtheria toxin, which disseminates throughout the host (12). Numerous studies with *C. diphtheriae* have focused on the genetic regulation of diphtheria toxin, and it is known that transcription of the structural gene *tox* is repressed by iron and the diphtheria toxin repressor protein, DtxR, a global iron-dependent regulator (6, 10, 18, 37, 39). While it is well established that iron levels can serve as a signal to regulate expression of bacterial virulence factors, the ability to acquire iron as a nutrient source by pathogenic bacteria is also important for virulence (7, 9). Numerous bacterial pathogens, including *C. diphtheriae*, utilize various host iron sources for growth, including transferrin, lactoferrin, and heme sources such as hemoglobin (7, 26, 35). Bacteria have developed a variety of mechanisms for the uptake and utilization of iron, including siderophore uptake systems (9, 19, 50) and

binding protein-dependent transporters that extract iron from various host sources (7, 50). Hemin is thought to be one of the primary host iron sources used by bacterial pathogens, and hemin iron transport and utilization systems in numerous bacterial species have been described (21, 45). Uptake and utilization of hemin as an iron source in *C. diphtheriae* involve the iron- and DtxR-regulated *hmu* hemin transport locus, which contains the *hmuTUV* genes, which encode an ABC hemin transporter, and the *htaA*, *htaB*, and *htaC* genes (1, 13, 18). HtaA and HtaB were recently shown to be surface-anchored hemin binding proteins, and mutations in *htaA* and in the *hmuTUV* genes result in reduced ability to use hemoglobin as an iron source, suggesting that factors encoded by these genes are important in hemin transport (1).

The use of heme as an iron source in *C. diphtheriae* also involves the *hmuO* gene, which encodes a heme oxygenase that degrades intracellular heme, which results in the release of the heme-associated iron (31, 49). Transcription of the *hmuO* gene in *C. diphtheriae* is repressed in high-iron conditions by DtxR and is activated by heme or hemoglobin (32). Heme-dependent activation of *hmuO* is primarily mediated by the ChrA-ChrS (ChrAS) two component signal transduction system, which is composed of the ChrS sensor kinase and the response regulator ChrA (4, 33). The HrrS-HrrA (HrrAS) two-component system is also involved in the heme-dependent transcriptional activation of the *hmuO* promoter and contributes ap-

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proximately 20% of the activity observed at this promoter (5). Moreover, HrrAS, as well as the ChrAS regulatory system, is involved in the heme-dependent repression of the *hemA* gene, the promoter-proximal gene in a heme biosynthetic operon. Mutations in either *chrA* or *chrS* result in reduced ability to activate expression of *hmuO* and in an increased sensitivity to hemin (4). The hemin sensitivity observed in the *chrSA* mutants was not due to the reduced expression of *hmuO*, since an *hmuO* mutant shows no increased sensitivity to hemin (4). It was proposed that the heightened hemin sensitivity observed in the *chrSA* mutants was likely due to reduced expression of an as yet identified gene(s) that is regulated by ChrAS (4).

Recent studies with *Staphylococcus aureus* and *Bacillus anthracis* identified an ABC type transporter, designated HrtAB, which is proposed to protect these bacteria from the toxic effects of high levels of hemin (15, 40, 41, 42). Mutations in the *S. aureus hrtA* and *hrtB* genes result in an increased sensitivity to hemin (40). The HrtAB system, which is composed of an ATPase (HrtA) and a membrane permease (HrtB), is hypothesized to either directly export excess heme from the cytosol or remove toxic metabolites that accumulate in response to hemin exposure (40, 42). The mechanism by which the HrtAB system reduces heme stress has not been determined (40). The *S. aureus hrtAB* genes are found adjacent to and transcribed divergently from the recently described *hssR-hssS* genes, which encode a two-component signal transduction system that controls the expression of the *hrtAB* genes in a heme-dependent manner (40, 41, 42).

In this study, we have identified a putative ABC type transporter in *C. diphtheriae* that is involved in protection from the toxic effects of hemin. We have designated this ABC transporter HrtAB to be consistent with previously described orthologous systems (15, 40, 41). A *C. diphtheriae hrtAB* deletion mutant exhibited an increased sensitivity to hemin; expression of the *hrtAB* genes is increased in the presence of heme or hemoglobin, and this regulation is mediated by the ChrAS two-component signal transduction system. Optimal expression from the *hrtB* promoter also requires DtxR. Mutations in the sensor region of ChrS were shown to affect heme-dependent activation of *hrtAB* transcription, demonstrating a direct association between detection of a heme-associated signal by ChrS and transcription of the *hrtAB* genes.

#### MATERIALS AND METHODS

**Bacterial strains and media.** *Escherichia coli* and *C. diphtheriae* strains used in this study are listed in Table 1. Luria-Bertani (LB) medium was used for culturing *E. coli*, and heart infusion broth (Difco, Detroit, MI) containing 0.2% Tween 80 (HIBTW) was used for routine growth of *C. diphtheriae* strains. Bacterial stocks were maintained in 20% glycerol at  $-80^{\circ}\text{C}$ . Antibiotics were added to LB medium at 34  $\mu\text{g}/\text{ml}$  for chloramphenicol, 50  $\mu\text{g}/\text{ml}$  for kanamycin, and 100  $\mu\text{g}/\text{ml}$  for spectinomycin; for *C. diphtheriae* cultures in HIBTW medium antibiotic concentrations were 2  $\mu\text{g}/\text{ml}$  for chloramphenicol, 100  $\mu\text{g}/\text{ml}$  for spectinomycin, and 50  $\mu\text{g}/\text{ml}$  for kanamycin. HIBTW was made low iron by the addition of ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) at 12  $\mu\text{g}/\text{ml}$  (unless indicated otherwise). Modified PGT (mPGT) is a semidefined low-iron medium that has been previously described (46). Antibiotics, EDDA, Tween 80, hemin (bovine), and hemoglobin (human) were obtained from Sigma Chemical Co. (St. Louis, MO).

**Mutant construction.** A previously described allelic replacement technique was used to construct in-frame deletions of *dtxR* and the *hrtAB* locus in *C. diphtheriae* strain C7 (47). Mutant construction utilized PCR to clone DNA fragments located upstream and downstream of the region targeted for deletion. The deleted region in the *hrtAB* mutant is predicted to encode a peptide of

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid              | Relevant characteristics or use                                | Reference or source |
|--------------------------------|----------------------------------------------------------------|---------------------|
| <i>Corynebacterium</i> strains |                                                                |                     |
| C7 (–)                         | <i>C. diphtheriae</i> wild type                                | 3                   |
| 1737                           | <i>C. diphtheriae</i> wild type, Gravis biotype                | 29                  |
| C7chrSΔ                        | <i>chrS</i> deletion mutant of C7 (–)                          | 4                   |
| C7ChrSΔΔ                       | <i>chrSA</i> deletion mutant of C7 (–)                         | 5                   |
| C7hrrSΔΔ                       | <i>hrrAS</i> deletion mutant of C7 (–)                         | 5                   |
| C7hrtABΔ                       | <i>hrtAB</i> deletion mutant of C7 (–)                         | This study          |
| C7dtxRΔ                        | <i>dtxR</i> deletion mutant of C7 (–)                          | This study          |
| CU44                           | <i>C. ulcerans hmuU</i> mutant                                 | 31                  |
| <i>E. coli</i> strains         |                                                                |                     |
| DH5α                           | Cloning strain                                                 | Invitrogen          |
| S17-1                          | RP4 mobilization functions                                     | 47                  |
| TOP10                          | Cloning of PCR fragments                                       | Invitrogen          |
| XL1-Gold                       | QuikChange cloning                                             | Stratagene          |
| Plasmids                       |                                                                |                     |
| pCR-Blunt-II                   | Cloning of PCR fragments, Kan <sup>r</sup>                     | Invitrogen          |
| pCM502                         | Promoter probe vector, Cm <sup>r</sup>                         | 32                  |
| pSPZ                           | Promoter probe vector, Spc <sup>r</sup>                        | 24                  |
| phrtB-lacZ                     | <i>hrtB</i> promoter-lacZ fusion on pSPZ                       | This study          |
| pCMtox                         | <i>tox</i> promoter-lacZ fusion on pCM502                      | 32                  |
| pKN2.6                         | <i>C. diphtheriae</i> shuttle vector, Kn <sup>r</sup>          | 37                  |
| pKN2.6Z                        | <i>C. diphtheriae</i> shuttle vector, Kn <sup>r</sup>          | 13                  |
| pCM2.6                         | <i>C. diphtheriae</i> shuttle vector, Cm <sup>r</sup>          | 37                  |
| pECK18mob2                     | <i>Corynebacterium</i> cloning vector, Kn <sup>r</sup>         | 30                  |
| pECK-chrSA                     | <i>chrSA</i> genes cloned into pECK18mob2                      | 5                   |
| pTrc99A                        | <i>E. coli</i> cloning and expression vector, Amp <sup>r</sup> | Pharmacia           |
| pKN-chrS                       | <i>chrS</i> cloned into pKN2.6                                 | This study          |
| pKN-hrtAB                      | <i>hrtAB</i> cloned into pKN2.6Z                               | This study          |
| pMnt-hrtAB                     | Plasmid pKN-hrtAB with <i>mntA</i> promoter                    | This study          |
| p2.6-dtxR                      | <i>dtxR</i> gene cloned into pKN2.6Z                           | This study          |

approximately 21 amino acids that contains residues derived from the N terminus of HrtB and the C terminus of HrtA. The *dtxR* mutant is predicted to produce a truncated peptide of 42 amino acids. Primers used for the construction of the mutants are listed in Table 2. PCR was used to confirm the mutation in all of the deletion mutants (not shown).

**Growth of *C. diphtheriae* cultures in the presence of hemin.** The effect of hemin on the growth of various *C. diphtheriae* strains was determined by inoculating overnight cultures, at an optical density at 600 nm (OD<sub>600</sub>) of 0.1, into fresh HIBTW medium that contained various hemin concentrations, as shown in Fig. 4. Cultures were grown aerobically at 37°C, and growth was assessed by measuring the optical density of cultures after 48 h.

**Hemoglobin-iron utilization assays.** The hemoglobin-iron utilization assay has been described previously (20). Briefly, *Corynebacterium* strains were grown overnight (20 to 22 h at 37°C) in HIBTW and then inoculated to an OD<sub>600</sub> of 0.2 into fresh HIBTW that contained 12  $\mu\text{g}/\text{ml}$  of the iron chelator EDDA. Strains were grown for several hours at 37°C until log phase, at which time bacteria were recovered by centrifugation, resuspended in mPGT medium, and then inoculated at an OD<sub>600</sub> of 0.03 into fresh mPGT medium that contained various supplements. After 20 to 22 h of growth at 37°C, the OD<sub>600</sub> of the cultures was determined.

**Plasmid construction.** Plasmids used in this study are listed in Table 1. PCR-derived DNA fragments were initially cloned into the pCR-Blunt II-TOPO vector (Invitrogen), and genomic DNA derived from *C. diphtheriae* strain C7 or 1737 was used as a template for PCRs. Promoter probe vectors pCM502 (31) and pSPZ (24) were used for the construction of lacZ promoter fusions; these plasmids contain a promoterless lacZ gene and replicate at low copy number in *C. diphtheriae*. To construct plasmid phrtB-lacZ, which contains the *hrtAB* promoter region fused to a promoterless lacZ gene, a 334-bp fragment encompass-

TABLE 2. Primers used in this study

| Primer                                             | Amplified region           | Sequence <sup>a</sup> (5'-3')     |
|----------------------------------------------------|----------------------------|-----------------------------------|
| <b>Deletion mutants</b>                            |                            |                                   |
| 23/24KO-Ds-F                                       | Downstream of <i>hrtAB</i> | GCATTCTAGATCATCACATTCAGCAATGC     |
| 23/24KO-Ds-R                                       |                            | TAGCGTCGACATGGCCGACGGAAAAGCC      |
| 23/24KO-U5-F                                       | Upstream of <i>hrtAB</i>   | GACTGTTCGACACGAATGTCGCGGATTCC     |
| 23/24KO-U5-F                                       |                            | GACTGTTCGACACGAATGTCGCGGATTCC     |
| dtxR-UP1                                           | Upstream of <i>dtxR</i>    | GGCAGTACTTCAATGCTGAAGATGAGG       |
| dtxR-UP2                                           |                            | GGCGTCGACGATCCTAGCGCGAAGAGG       |
| dtxR-DN1                                           | Downstream of <i>dtxR</i>  | GGCGTCGACGCCACTACTATTCGTATC       |
| dtxR-DN2                                           |                            | GGCACTAGTAGTACTGACCGAATATCCTTCACC |
| <b><i>hrtB</i> promoter fusion</b>                 |                            |                                   |
| DIP2324-PO-R                                       | <i>hrtB</i>                | GGATCCGGCCGGTCAGCATAACG           |
| DIP2324-PO-F                                       | Promoter                   | GTCGACGGGTTCTACGCTGACC -3'        |
| <b><i>hrtAB</i> cloning</b>                        |                            |                                   |
| DIP2323-R                                          |                            | CTGAGGATCCGACTAGCACACCAAATTCC     |
| DIP2324-PO-F(RV)                                   |                            | GGCGATATCGGGTTCCTACGCTGACC        |
| 2324P                                              |                            | GGCGATATCCCTTACTTTACAAACAAT       |
| mntP-Rev                                           |                            | GGCGATATCTCTGTTTGACAAACTTG        |
| <b><i>chrS</i> cloning</b>                         |                            |                                   |
| chrS-kin-R                                         |                            | GTCGACGGATCATGAAGTCTCCCG          |
| chrS-wt-F                                          |                            | GGATCCGCAGGAGATCTTTTATGC          |
| <b><i>ChrS</i> C-terminal expression construct</b> |                            |                                   |
| chrS-kin-ab-F2                                     |                            | CTACCTCTGAACATATGGCAGGCCGG        |
| chrS-kin-R                                         |                            | GTCGACGGATCATGAAGTCTCCCG          |
| <b><i>dtxR</i> cloning</b>                         |                            |                                   |
| DTRB                                               |                            | AAAGACATAGGATCCTATTAAAAGCAATC     |
| DTRS                                               |                            | ATCTAATTTGTCGACTTATGATTTAGAG      |

<sup>a</sup> Restriction sites are underlined.

ing the entire *hrtB-cmrA* intergenic region plus flanking sequence was amplified with primers DIP2324-PO-F and DIP2324-PO-R and cloned into pSPZ with a BamHI-SalI digest.

Plasmid pKN2.6 is an *E. coli-Corynebacterium* shuttle vector with a kanamycin resistance cassette that replicates at low copy number in *Corynebacterium* and has been previously described (37). Plasmid pKN2.6Z is a derivative of pKN2.6 that has the multiple cloning sequence from pBluescript KS and has been previously described (13). Plasmid pKN2.6(-EcoRI) is a derivative of pKN2.6 in which the EcoRI site has been blunt ended using standard techniques. To create plasmid pKN-*chrS*, a DNA fragment was amplified from either C7 and 1737 using primers *chrS*-wt-F and *chrS*-kin-R and ligated into the BamHI-SalI sites in pKN2.6(-EcoRI). Plasmid vector pECK18mob2 (30) was used to construct plasmids utilized in the *chrS* complementation studies. Plasmid pECK-*chrS* has a copy of the *chrS* gene from C7 or 1737 which was derived from plasmid pKN-*chrS* constructs.

To construct a plasmid with *hrtA* and *hrtB* for complementation, primers DIP2324-PO-F(RV) and DIP2323-R were used to amplify *hrtAB* and the upstream region containing the putative promoter region. This fragment was moved into plasmid pKN2.6Z with a BamHI-EcoRV digest to make pKN-*hrtAB*. To construct pMnt-*hrtAB*, the *mntA* promoter region from plasmid pPO1 (34) was amplified by PCR using primers mntP-Rev and PO1 to generate an approximately 260-bp fragment, which was subsequently ligated upstream of the *hrtB* gene on plasmid pKN-*hrtAB*. The DNA insert in plasmid pKN-*hrtAB*2 contains the complete coding region and translational signals for *hrtAB* but lacks the ChrAS-regulated promoter. The insert in plasmid pKN-*hrtAB*2 was derived from a PCR product using primers DIP2323R and 2324P and C7 chromosomal DNA as the template. The insert was ligated into pKN2.6Z at the BamHI-EcoRV sites.

Plasmid p2.6-dtxR, which carries the *C. diphtheriae* C7 *dtxR* gene, was constructed by using PCR and primers DTRB and DTRS to amplify a 901-bp fragment that contains the *dtxR* gene and upstream elements needed for expression of the gene product. The 901-bp DNA fragment was first cloned into pCR-Blunt II-TOPO and then moved into pKN2.6Z at the SalI-BamHI sites.

To construct PhoA fusions to the sensor region of ChrS, the *phoA* gene was obtained from plasmid pSS1324 (43) with a SalI-HindIII digest. The DNA

fragment containing *phoA* was ligated into pUC18. Fragments containing various segments of the *chrS* gene were amplified using various primer sets. Fragments were first cloned into pCR-Blunt II-TOPO and then moved into pUC18 as XbaI-SalI fragments. The *chrS-phoA* fusions were then cloned into the pTrc99A vector (Pharmacia) with an XbaI-HindIII digest. To construct LacZ fusions to the sensor region of ChrS, a DNA fragment harboring the *lacZ* gene was first amplified by PCR from plasmid pSS1462 (43) and subsequently ligated into the pTrc99A vector at the HindIII-SalI sites (pTrc99A-*lacZ*). Fragments containing various segments of the *chrS* gene were amplified using various primer sets and subsequently ligated into pTrc99A-*lacZ* at the XbaI-SalI sites.

The plasmid used to express the ChrS C-terminal kinase domain was constructed as follows. PCR was used to amplify a 670-bp product using primers *chrS*-kin-ab-F2 and *chrS*-kin-R and C7 chromosomal DNA as the template. This DNA fragment, which encodes the ChrS C-terminal kinase domain, was moved into the expression vector pET28a to allow production of a His-tagged fusion protein. The His-ChrS-C terminus fusion protein was expressed at high levels in *E. coli* by following standard techniques and purified by nickel affinity chromatography as previously described (1). Polyclonal antiserum against the ChrS-His fusion was obtained from guinea pigs by following standard procedures (Cocalico Inc.). Immunoblot analysis of *C. diphtheriae* whole-cell lysates was performed as described previously (1). The DNA inserts in all plasmid constructs used in this study were confirmed by DNA sequence analysis.

**LacZ assays.** *C. diphtheriae* strains containing promoter-*lacZ* fusion constructs were grown overnight in HIBTW medium and then inoculated at an OD<sub>600</sub> of 0.2 into fresh HIBTW medium in the presence of various supplements. Cultures were grown for several hours until they reached log phase, and LacZ activity was determined as previously described (38).

**PhoA and LacZ fusion analysis.** The various ChrS-PhoA and -LacZ fusion proteins were constructed to map the membrane topography of the ChrS N-terminal domain. DH5 $\alpha$  cells carrying the pTrc-ChrS-PhoA or -LacZ fusion construct were inoculated from frozen stocks into 1-ml (LB medium with ampicillin) overnight cultures. Overnight cultures were then inoculated into LB medium with ampicillin at an OD<sub>600</sub> of 0.05. Cultures were grown for 2 to 2.5 h at 37°C (for PhoA fusions only, isopropyl- $\beta$ -D-thiogalactopyranoside [IPTG] was

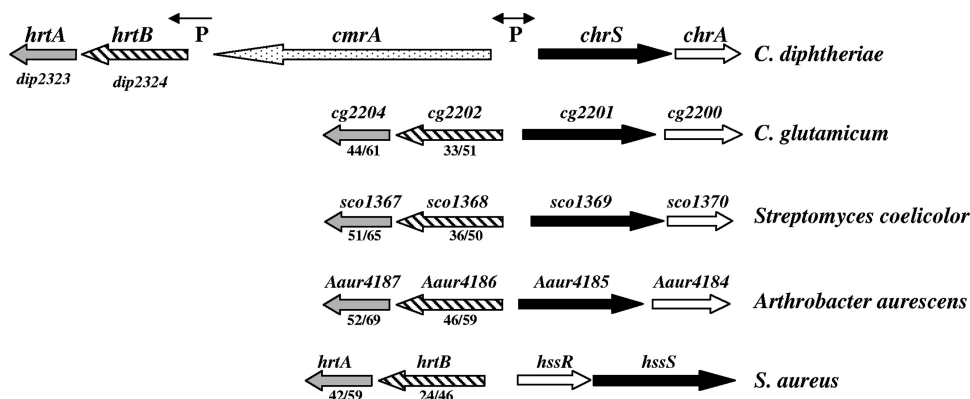


FIG. 1. Genetic map of the *hrtAB-chrSA* loci in *C. diphtheriae* and in related organisms. A genetic map of the *S. aureus hrtAB-hssRS* region is also shown for comparison (40). The percentages of amino acid similarity/identity to the *C. diphtheriae* HrtA and HrtB proteins are indicated below the gene designations. Genes with homology to *hrtA* are indicated by gray arrows, and homologs of *hrtB* are shown with hatched arrows. P indicates the locations of known promoters in *C. diphtheriae* and the directions of transcription. Genes encoding proteins with homology to sensor kinases are indicated in black, and response regulator homologs are shown with open arrows.

added at a final concentration of 0.1 mM and the cultures were incubated at 37°C for an additional 30 min. Alkaline phosphatase and LacZ activities were determined as previously described (8, 23).

**Site-directed mutagenesis.** Site-directed mutants of C7 ChrS were made using the QuikChange or QuikChange Lightning kit (Stratagene) according to the manufacturer's instructions. Briefly, 125 ng of each primer containing the targeted base change (designed using the QuikChange primer design program at <http://www.stratagene.com/sdmdesigner/default.aspx>) and 50 ng of plasmid template pKN-chrS were used in the QuikChange reaction mixture (50- $\mu$ l total volume). Methylated template DNA was removed from the reaction mixture by digestion with DpnI restriction endonuclease. Mutagenized DNA was recovered by transformation into XL1-Blue (QuikChange) or XL1-Gold (QuikChange Lightning) competent cells (2  $\mu$ l of DpnI-treated reaction mixture in 45  $\mu$ l of cells). The presence of the base changes was confirmed by sequencing. The mutated *chrS* genes on pKN-chrS were moved into pECK18mob2 for the complementation studies with C7chrS $\Delta$ , which harbors phrtB-lacZ.

**Computer analysis.** Amino acid sequence similarity searches were done using the BLAST program (2) at the National Center for Biotechnology Information and also using the BLAST server provided at the online site for the Sanger Institute [http://www.sanger.ac.uk/Projects/C\\_diphtheriae](http://www.sanger.ac.uk/Projects/C_diphtheriae). The annotated genome sequence for *C. diphtheriae* strain NCTC13129 (11) is accessible in the EMBL/GenBank database at accession number BX248353. The online transmembrane programs TMpred ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) and DAS (<http://www.sbc.su.se/~miklos/DAS/>) were used in the analysis of the ChrS N-terminal region, and sequence alignments were done with ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html/>).

## RESULTS

**Identification of a *C. diphtheriae* ABC transporter that confers protection from hemin toxicity.** Upstream of the *chrSA* operon is the *cmrA* gene, which is predicted to encode a large zinc-regulated surface-anchored protein that is divergently transcribed from *chrSA* (Fig. 1) (4). Downstream of *cmrA* are two genes, *dip2324* and *dip2323*, which are predicted to encode the permease and ATPase components, respectively, of an ABC transporter (Fig. 1). The predicted gene products of *dip2323* and *dip2324* have sequence similarity to the previously described *Staphylococcus aureus* gene products HrtA (DIP2323, 42% identity/59% similarity) and HrtB (DIP2324, 24% identity/46% similarity), respectively (15, 48). In *S. aureus*, the *hrtAB* genes are located adjacent to and divergently transcribed from the *hssS-hssR* genes, which encode a two-component signal transduction system that is required for the heme-dependent expression of *hrtAB* (Fig. 1) (42). A similar

genetic locus is present in *Bacillus anthracis* (41). Because of the sequence similarities between DIP2324 and DIP2323 and the orthologous proteins in *S. aureus*, we have renamed these *C. diphtheriae* genes *hrtB* and *hrtA*, respectively.

Genomic analysis of various Gram-positive species, some of which are closely related to *C. diphtheriae*, revealed the presence of putative gene products that share sequence similarity to HrtAB (Fig. 1). All of these *hrtAB*-related genes were genetically linked to a two-component signal transduction system (Fig. 1). The *S. aureus* HssS sensor kinase showed no significant sequence similarity with ChrS in either the C-terminal kinase domain or in the N-terminal sensor region, which anchors these proteins to the membrane and is proposed to be critical for recognition of an environmental signal. The amino acid sequence of HssR showed approximately 40% similarity and 23% identity with that of ChrA. Although the genetic organizations of the *hrt-chr* loci among the species that are closely related to *C. diphtheriae* are similar, only *C. diphtheriae* contains the *cmrA* gene, which resides between the coding sequences for the two-component system and the ABC transporter (Fig. 1). No gene products with significant amino acid similarity to CmrA are predicted to be encoded in any of these organisms, and a function for CmrA is not known (M. P. Schmitt, unpublished observation).

**Heme-dependent regulation of *hrtAB* expression is mediated by ChrAS.** Previous studies in our laboratory demonstrated that the ChrAS two-component signal transduction system was critical for the control of heme-dependent transcription at promoters upstream of the *hmuO* and *hemA* genes (4, 5). To determine if ChrAS also regulates transcription of *hrtAB* in *C. diphtheriae*, an *hrtB* promoter-lacZ fusion construct, phrtB-lacZ, was generated and promoter activity in various *C. diphtheriae* strains was assessed. These studies revealed that *hrtB* promoter activity is increased almost 50-fold in the presence of hemoglobin and that only minimal levels of activity are observed in the absence of a heme source (Fig. 2A). Hemin gave results similar to those for hemoglobin in the activation of the *hrtB* promoter (data not shown); however, because of the toxicity associated with hemin, hemoglobin was used for all experiments shown. Hemoglobin-dependent transcription at the

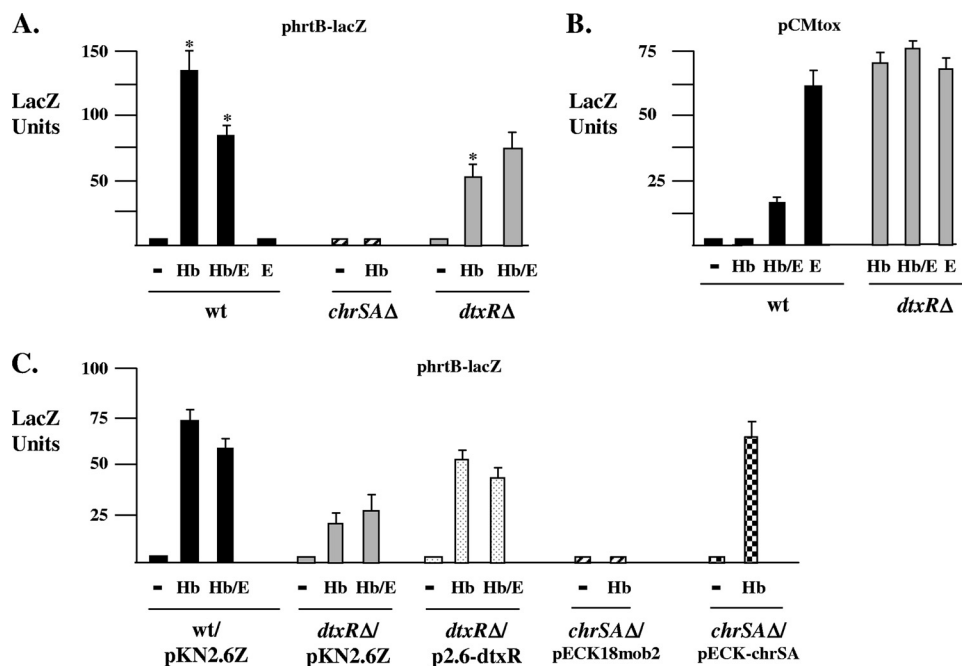


FIG. 2. (A) The *hrtB* promoter is regulated by ChrSA in the presence of hemoglobin. Plasmid phrtB-lacZ was used to measure *hrtB* promoter activity in C7 (wild type [wt]), C7chrSAΔ, and C7dtxRΔ. Strains were grown in HIBTW medium containing no supplement (–), hemoglobin (Hb; 200 μg/ml), EDDA (E; 12 μg/ml), or EDDA and hemoglobin (Hb/E). The results are the means of at least three independent experiments (±standard deviations). Asterisks indicate that results with wt grown in Hb are significantly different from results with wt grown in Hb/E ( $P < 0.05$ ) and with C7dtxRΔ grown in Hb ( $P < 0.02$ ). (B) Expression from the iron- and DtxR-regulated *tox* promoter was assessed using plasmid pCMtox. Conditions were the same as those for panel A. The results are the means of at least three independent experiments (±standard deviations). (C) Expression of the *hrtB* promoter in the *C. diphtheriae* mutants C7dtxRΔ and C7chrSAΔ in the presence of the cloned *dtxR* and *chrSA* genes. All strains carried plasmid phrtB-lacZ and a second plasmid, which was either the vector control or a plasmid containing the *dtxR* gene or the *chrSA* genes. Conditions were the same as those describe for panel A. wt/pKN2.6Z indicates the C7 wild type harboring vector pKN2.6Z (C7 wt with vector pECK18mob2 gave similar results [data not shown]). The results are the means of at least three independent experiments (±standard deviations).

*hrtB* promoter is abolished in strains carrying deletions in the *chrSA* genes, which indicates that the heme-responsive activation of *hrtAB* requires the ChrAS regulatory system (Fig. 2A). Heme-responsive expression at the *hrtB* promoter is restored in the *chrSA* deletion mutant in the presence of the cloned *chrSA* genes (Fig. 2C). The *C. diphtheriae* HrrAS two-component system, which is an additional heme-dependent activator of *hmuO* expression (5), is not involved in the regulation of the *hrtB* promoter (not shown).

Expression of the *hrtB* promoter is reduced approximately 40% in medium containing the iron chelator EDDA and hemoglobin (Fig. 2A), which suggests that reduced iron levels affect *hrtB* promoter activity. No activity is seen with medium supplemented only with EDDA, since a heme source is required for expression from the *hrtB* promoter. Since it was previously shown that the ChrAS-regulated *hmuO* promoter was affected by iron and *dtxR*, we examined the expression of the *hrtB* promoter in a *dtxR* deletion mutant. Expression of the *hrtB* promoter in the presence of hemoglobin was reduced 2- to 3-fold in the *dtxR* deletion mutant compared to expression under these conditions in the wild-type strain (Fig. 2A), suggesting that DtxR is required for wild-type levels of transcription at the *hrtB* promoter. There was no significant difference in expression at the *hrtB* promoter between the wild type and the *dtxR* mutant in the presence of EDDA and hemoglobin. Expression from the *hrtB* promoter in a *dtxR* mutant was in-

creased to nearly wild-type levels in the presence of the cloned *dtxR* gene, which suggests that the *dtxR* mutation is the reason for reduced *hrtB* expression (Fig. 2C). The inability of the cloned *dtxR* gene to fully restore *hrtB* promoter activity to wild-type levels may be due to low expression of the cloned *dtxR* gene on plasmid pKN2.6Z. In Fig. 2C, it was noted that expression of the *hrtB* promoter in the wild-type strain and in the *dtxR* mutant was approximately one-half the levels observed in these same strains in Fig. 2A. The reason for the difference in expression is likely the presence of two plasmids residing in the strains used in the studies in Fig. 2C. The presence of two plasmids in these strains may lower the copy number of phrtB-lacZ, which would be expected to result in reduced levels of LacZ activity. Appropriate antibiotics were present at all times during the expression studies to ensure maintenance of all plasmids.

Expression at the *hrtB* promoter cannot be assessed under the low-iron conditions that our laboratory has used previously to measure iron-regulated gene expression in *C. diphtheriae* (32). This is because heme functions as not only a heme source for *hrt* expression but also an iron source for *C. diphtheriae*, which results in increased intracellular iron levels. In previous studies, HIBTW medium supplemented only with EDDA was used to generate an optimal low-iron condition for assessing gene expression in *C. diphtheriae* (32). In order to assess the biologically relevant iron levels under the conditions used in

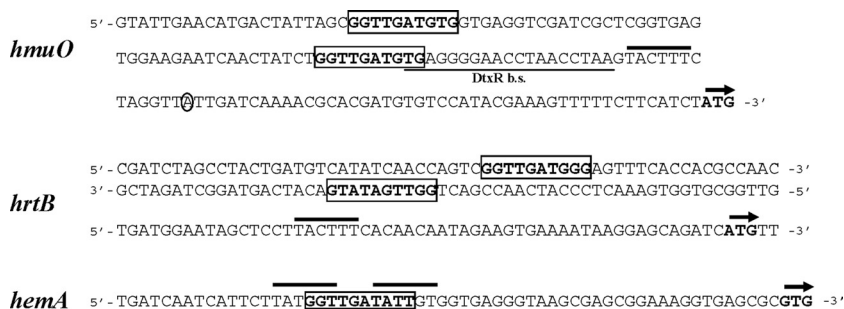


FIG. 3. DNA sequence of the region upstream of the *hmuO*, *hrtB*, and *hemA* genes. Boxed sequences indicates the 10-bp conserved region present in all three upstream sequences. The underlined region in the *hmuO* sequence indicates the location of the DtxR binding site (DtxR b.s.), and the circled A residue denotes the start site of transcription. The solid bar above the sequence indicates the location of putative  $-10$  promoter elements, and arrows identify start codons.

Fig. 2A, expression from the iron-responsive and DtxR-regulated *tox* promoter on plasmid pCMtox was examined (32). As expected, expression from the *tox* promoter in a *dtxR* deletion mutant was derepressed under all conditions tested (Fig. 2B). In the wild-type strain, expression of *tox* was fully repressed in high-iron medium (HIBTW medium that contained hemoglobin or no supplement); however, in medium supplemented with hemoglobin and EDDA (Hb/E medium), *tox* expression was approximately 25% of the activity seen under the optimal low-iron conditions (EDDA alone [E medium]) (Fig. 2B, compare wild-type E with Hb/E). Although these results indicate that optimal low-iron conditions are not achieved in the Hb/E medium, a reduction in biologically relevant iron levels is obtained in Hb/E medium, as demonstrated by an increase in expression at the *tox* promoter compared to the level of expression observed in high-iron conditions (Fig. 2B, compare wild-type Hb with Hb/E). This finding supports the observation that *hrtB* expression is affected by iron levels and further suggests that optimal expression of *hrtAB* requires DtxR, hemoglobin, and an iron-rich medium.

**Sequence analysis of the promoter regions for ChrA-regulated genes.** Sequence analysis of the region upstream of the ChrA-regulated genes, *hrtB*, *hmuO*, and *hemA*, revealed the presence of a conserved 10-bp region with the consensus sequence 5'-GGTTGATGTG-3' (Fig. 3). Upstream of the *hmuO* promoter are two invariant copies of this 10-bp sequence separated by 40 bases, while upstream of *hrtB* are two copies that match 9 of 10 bases of the consensus sequence and are positioned on opposite strands to create a potential stem-loop structure. A single copy of this conserved sequence, which matches 8 of 10 bases of the consensus, is also present upstream of *hemA* (Fig. 3). This conserved sequence in the *hemA* promoter region overlaps putative  $-10$  promoter elements, which is consistent with a regulatory protein, such as ChrA, functioning as a repressor at this promoter. While it has not been determined whether this conserved 10-bp sequence functions as a binding site for ChrA or another regulatory factor, the presence of this sequence upstream of the three known ChrA-regulated genes strongly suggests that this sequence has a role in transcriptional regulation.

**Deletion of the *hrtAB* genes results in increased hemin sensitivity.** To determine the function of the HrtAB system, a nonpolar deletion of the *hrtAB* genes was constructed in *C. diphtheriae* strain C7, and the mutant is designated C7hrtAB $\Delta$ .

C7hrtAB $\Delta$  exhibited growth similar to that of the wild-type strain in HIBTW medium, indicating that the deletion strain is not defective for growth in nutrient-rich broth (Fig. 4A). However, growth of C7hrtAB $\Delta$  was restricted in the presence of elevated levels of hemin. Hemin concentrations above 2.5  $\mu$ M strongly limited the growth of the mutant, whereas growth of the wild-type strain was not affected by hemin levels as high as 10  $\mu$ M (Fig. 4A). The hemin sensitivity of C7hrtAB $\Delta$  was complemented by a plasmid carrying the cloned *hrtAB* genes on plasmid pKNhrtAB, confirming that the hemin-sensitive phenotype observed in this mutant is specific to *hrtAB* (Fig. 4A). It was further noted that the presence of the *hrtAB* cloned genes in C7hrtAB $\Delta$  and in the wild-type strain resulted in a resistance to hemin toxicity that was greater than that observed in the wild-type strain carrying only the pKN2.6Z vector, sug-

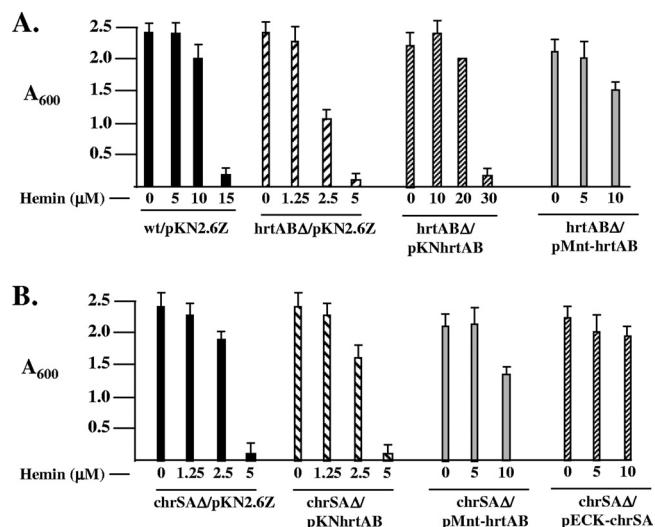


FIG. 4. (A) Deletion of the *C. diphtheriae* *hrtAB* genes results in increased sensitivity to hemin. Strains were grown in HIBTW medium containing various amounts of hemin as indicated. Growth of bacterial cultures was measured after 48 h (optical density at 600 nm [ $A_{600}$ ]). (B) The heme sensitivity of C7chrS $\Delta$  is complemented by the cloned *hrtAB* genes on plasmid pMnt-hrtAB. Growth conditions are the same as for panel A. A *chrS* deletion mutant showed a hemin-sensitive phenotype similar to that of C7chrS $\Delta$  (5) (data not shown). Results are the averages of three independent experiments  $\pm$  standard deviations.

gesting that multiple copies of the *hrtAB* genes (or increased expression or both) result in enhanced tolerance to hemin (Fig. 4A; data not shown for the wild type harboring the cloned *hrtAB* genes). C7hrtAB $\Delta$  exhibited a sensitivity to hemin similar to that of the *C. diphtheriae chrSA* mutant C7chrSA $\Delta$ , which was previously shown to be sensitive to elevated levels of hemin (5) (Fig. 4B). Because *hrtAB* expression is positively regulated by ChrAS in a heme-dependent manner, we sought to determine if the hemin sensitivity seen in *chrA* and *chrS* mutants was caused by reduced expression of *hrtAB*. The pKN-hrtAB plasmid, which carries the *hrtAB* genes, did not restore hemin tolerance in C7chrSA $\Delta$  (Fig. 4B); however, the *hrtAB* genes on this clone are under the control of the native *hrtB* promoter, which is poorly expressed in *chrSA* mutants (Fig. 2A). Plasmid pMnt-hrtAB contains the *hrtAB* genes under transcriptional control of the *mntA* promoter, a manganese-repressible promoter that is independent of ChrAS regulation (34). When plasmid pMnt-hrtAB is moved into C7hrtAB $\Delta$  and C7chrSA $\Delta$ , both of these strains are able to grow at hemin levels as high as 10  $\mu$ M, which is comparable to the growth observed in the wild-type strain at this hemin concentration (Fig. 4A and B). This finding strongly suggests that the hemin sensitivity observed in the *chrSA* mutant is due to reduced expression of the ChrAS-regulated *hrtAB* genes. The hemin-sensitive phenotype was not complemented in strains C7hrtAB $\Delta$  and C7chrSA $\Delta$ , harboring pMnt-hrtAB, in the presence of 20  $\mu$ M MnCl<sub>2</sub> (data not shown). The absence of complementation in the presence of MnCl<sub>2</sub> suggests that the Mn<sup>2+</sup>-repressible *mntA* promoter drives transcription of the *hrtAB* genes on plasmid pMnt-hrtAB. Hemin tolerance is fully restored in C7chrSA $\Delta$  when the cloned *chrSA* genes are provided *in trans* on plasmid pECK-*chrSA* (5) (Fig. 4B).

**HrtAB does not use hemoglobin as an iron source.** The *C. diphtheriae* C7 *hrtAB* deletion mutant exhibited no defect in the ability to use hemoglobin as an iron source (data not shown), which suggests that the HrtAB system does not function as a heme uptake and heme-iron utilization system like the previously described HmuTUV ABC transporter in *C. diphtheriae* and *Corynebacterium ulcerans* (13, 36). Furthermore, the cloned *hrtAB* genes on plasmid pKN-hrtAB were unable to complement the heme utilization defect in *C. ulcerans* strain CU44, which contains a defective permease component (HmuU) of the heme transport system (data not shown) (36).

**Analysis of the ChrS N-terminal region and effect of ChrS mutations on *hrtB* promoter activity.** (i) **Topology mapping of the ChrS N-terminal domain.** The N-terminal region of ChrS is proposed to respond to an environmental signal, which is presumed to be hemin or factors produced in response to hemin (33). It is further postulated that, upon interaction with external stimuli, ChrS autophosphorylates at the conserved histidine residue H215. The phosphate moiety on ChrS is transferred to an aspartate residue on the cognate response regulator ChrA, which then affects transcription of regulated promoters such as the *hrtB* promoter. A more complete description of the membrane architecture at the N terminus of ChrS is critical for understanding the mechanism of signal transduction and the interaction of ChrS with an activating signal. Analysis of the structure of the ChrS protein reveals that it is composed of a conserved C-terminal kinase domain

that is linked to an N-terminal membrane region that exhibits minimal sequence conservation with other proteins (4, 5, 33). The precise number and location of membrane-spanning regions in ChrS have not been reported, and transmembrane prediction programs (TMPred and DAS) indicate that there are either 5 or 6 regions in the N-terminal domain that may traverse the membrane (5). We used PhoA and LacZ translational fusions to facilitate topology mapping of the ChrS N-terminal region. These constructs contained various N-terminal segments of the ChrS protein fused in frame to either PhoA or LacZ. The fusion points were located at specific ChrS sequences that are predicted to reside between membrane-spanning regions, which would result in the C-terminal PhoA or LacZ portion of the fusion protein being inserted either into the cytosol or outside the cytoplasmic membrane. The rationale for membrane mapping with PhoA and LacZ fusions is based on the observation that higher levels of PhoA activity are detected when PhoA is inserted outside the cytoplasmic membrane, while increased LacZ activity is observed for fusions in which LacZ is cytosolic (43). The results of the mapping studies shown in Fig. 5 and Table 3 indicate that six transmembrane regions (TM 1 to 6) are located in the N terminus of ChrS. The even number of transmembrane segments observed with ChrS is consistent with that reported for many related sensor kinases; this predicts that the N terminus (sensor domain) and C terminus (kinase domain) are both located in the cytosol (27).

(ii) **Mutations in the ChrS N-terminal domain affect *hrtB* promoter activity.** Promoters upstream of *hmuO* and *hemaA* were previously shown to be regulated by ChrAS in a heme-dependent manner; however, heme regulation at both of these promoters also required the activity of the HrrAS system (5). In this report, we have shown that deletion of the *chrS* gene abolishes heme-dependent activation at the *hrtB* promoter, which demonstrates that only the ChrAS system is required for heme regulation of *hrtAB*. Mutations in *chrS* that affect signal transduction are predicted to directly impact heme-dependent expression from the *hrtB* promoter. To identify specific sequences important for the function of the N-terminal sensor region of ChrS, point mutations within this region were constructed. To assist in the identification of residues to target for mutagenesis, the ChrS sensor region was aligned with the N-terminal domains from proteins similar in sequence and genetic organization to ChrS (Fig. 1 and 5). It is not known if any of these orthologous proteins have a function similar to that of ChrS. Several highly conserved residues were identified from this alignment, including H21 and Y61; histidine and tyrosine residues are known to be associated with the coordination of hemin in other proteins (28). These two residues, as well as several other conserved or charged amino acids located primarily in putative loop regions, were targeted for site-directed mutagenesis (Fig. 5). Clones carrying the mutated *chrS* genes were moved into the C7 *chrS* deletion mutant, and the effect of these mutations on ChrS function was assessed by measuring heme-dependent activation at the *hrtB* promoter on phrtB-lacZ. Immunoblotting, which used a polyclonal antiserum raised against the C-terminal kinase domain of ChrS, showed that all of the mutated *chrS* genes expressed the ChrS protein at levels similar to that at which the wild-type gene was expressed (data not shown). Several of the site-directed mutations in the sensor domain strongly reduced heme-dependent

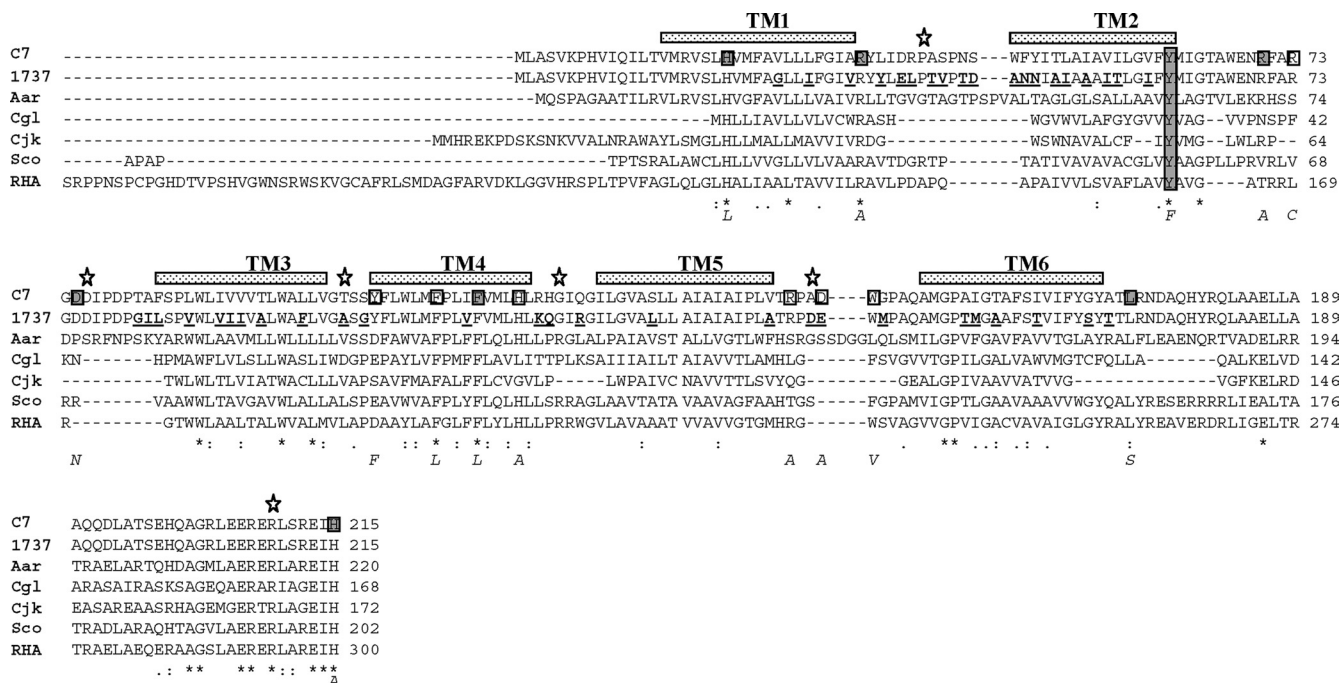


FIG. 5. Amino acid sequence of the N-terminal region of ChrS from *C. diphtheriae* aligned with orthologous proteins from related species. Aar, *Arthrobacter aurescens* TC1 Aaur4185; Cgl, *Corynebacterium glutamicum* 13032 cg2201; Cjk, *Corynebacterium jeikeium* jk1162; Sco, *Streptomyces coelicolor* A3, SCO1369; RHA, *Rhodococcus* species RHA1, RoO3970 (portions of the N terminus of the RHA ortholog are not shown). Stippled bars above the aligned sequences indicate predicted locations of the six transmembrane (TM) regions in *C. diphtheriae* C7, and stars denotes fusion points for PhoA and LacZ translational fusion constructs. Boxed amino acids in the C7 sequence indicate residues that were subjected to site-directed mutagenesis, and the resulting amino acid changes are indicated below the alignment. Substituted residues that resulted in a phenotype different from the wild type are shaded, and the conserved tyrosine, Y61, is shaded in all lanes. Amino acid differences between the C7 and 1737 *C. diphtheriae* strains are underlined and in boldface in the 1737 sequence.

*hrtB* promoter activity, with mutations in Y61 and R70 producing the strongest defect in ChrS function (Table 4). All of the site-directed mutants that were constructed in this study are indicated in Fig. 5, but only data for those mutants that exhibited *hrtB* promoter activity that was different from the wild type are presented in Table 4. It should be noted that 48 of the approximately 170 amino acids in the N-terminal transmembrane region of ChrS are different between the C7 and 1737 strains (changes in 1737 are indicated in boldface and

underlined in Fig. 5). It is assumed that none of the changes in 1737 significantly affect heme-dependent signaling in ChrS, since the cloned 1737 *chrS* gene is able to restore heme-dependent activation at the *hrtB* promoter in the C7 *chrS* mutant (Table 4). These findings suggest that amino acid substitutions that reduced heme-responsive promoter activity disrupt the recognition of a signaling molecule or affect transmission of the signal. No highly conserved residues were present in the

TABLE 3. PhoA and LacZ activity observed with ChrS translational fusions

| Fusion site <sup>a</sup> | Activity <sup>b</sup> (avg ± SD) of: |               | Location <sup>c</sup> |
|--------------------------|--------------------------------------|---------------|-----------------------|
|                          | PhoA                                 | LacZ          |                       |
| Vector                   | 7.7 (1.2)                            | 1.5 (0.43)    | NA <sup>d</sup>       |
| P40                      | 387.7 (46.6)                         | 145.0 (19.5)  | O                     |
| D76                      | 64.7 (15.6)                          | 673.3 (143.8) | I                     |
| T101                     | 2,121.0 (377)                        | 110.7 (36.5)  | O                     |
| G122                     | 11.4 (0.9)                           | 330.7 (53.5)  | I                     |
| A146                     | 1,275.4 (133.5)                      | 61.1 (6.6)    | O                     |
| R209                     | 23.6 (4.0)                           | 479.7 (182.2) | I                     |

<sup>a</sup> Site of fusion junction in ChrS-PhoA and -LacZ fusions (vector or amino acid).  
<sup>b</sup> PhoA and LacZ activities were determined as described previously (8, 23). Results are the averages of at least three independent experiments.  
<sup>c</sup> Predicted cellular location of fusion junction. O, outside membrane; I, intracellular.  
<sup>d</sup> NA, not applicable.

TABLE 4. Amino acid substitutions in the ChrS sensor region affect *hrtB* promoter activity

| <i>chrS</i> version or amino acid substitution <sup>a</sup> | LacZ activity |              |
|-------------------------------------------------------------|---------------|--------------|
|                                                             | +Hb           | -Hb          |
| Vector pECK18mob2                                           | 1.8 (0.6)     | 2.1 (0.3)    |
| Wild type (C7)                                              | 37.3 (8.4)    | 1.9 (0.1)    |
| Wild type (1737)                                            | 32.5 (4.4)    | 2.9 (0.5)    |
| H21L                                                        | 162.6 (34.7)  | 175.4 (28.4) |
| R34A                                                        | 6.9 (2.2)     | 3.0 (1.2)    |
| Y61F                                                        | 2.9 (0.7)     | 2.2 (0.4)    |
| R70A                                                        | 4.2 (0.8)     | 2.5 (0.3)    |
| D75N                                                        | 10.5 (3.1)    | 2.3 (0.6)    |
| F114L                                                       | 9.0 (1.8)     | 2.9 (0.7)    |
| L173S                                                       | 131.6 (14.0)  | 2.4 (0.4)    |
| H215A                                                       | 2.0 (0.2)     | 2.2 (0.3)    |

<sup>a</sup> *chrS* wild-type and mutant alleles in pECK18mob2 were tested for LacZ activity in C7chrSΔ/phrtB-lacZ in HIBTW medium in the presence and absence of hemoglobin (Hb).



loop regions after TM 3, 4, or 5, and none of the constructed mutations (or the 8 changes between C7 and 1737 in this region) had any effect on *hrtB* promoter activity (Fig. 5). A mutation in the highly conserved H21 residue (H21L) resulted in high-level, hemoglobin-independent activity, suggesting that this mutation causes a conformational change rather than having a specific effect on signal interaction. Additionally, a mutation in the partially conserved L173 residue, which is located after TM 6 (L173S), maintained heme-dependent regulation but resulted in a significant increase in promoter activity in the presence of hemoglobin. The H215 residue is proposed to be the site of phosphorylation and is assumed to be essential for the kinase activity (33), and as expected, the H215A mutation abolished activity (Table 4).

## DISCUSSION

High levels of heme or iron are toxic to many bacteria, including *C. diphtheriae*, and the expression of genetic systems involved in the uptake and utilization of iron or iron-containing compounds such as heme is often tightly regulated in bacteria (7, 9). Iron and heme transport systems in *C. diphtheriae* are regulated by the DtxR repressor in an iron-dependent manner (18, 19). While high iron levels repress the expression of numerous genes in *C. diphtheriae*, the presence of extracellular heme or heme sources like hemoglobin results in both activation and repression of gene expression, which are mediated by the ChrAS and HrrAS two-component systems (4, 5, 18). In this study, we have identified in *C. diphtheriae* an ABC transport system, HrtAB, that is required for resistance to the toxic effects of high levels of heme and whose genes are regulated by the ChrAS two-component system in a heme-dependent manner. The recent report that identified the *hrtAB* genes in *S. aureus* showed that mutations in these genes resulted in heme sensitivity, that expression of these genes is heme activated, and that heme activation is mediated by the HssRS system (40, 48). The *hrtAB* genes in *C. diphtheriae* appear to have a function similar to those reported in *S. aureus* and *B. anthracis*, which is the detoxification of high levels of heme. Pfam analysis (14) of the *C. diphtheriae* HrtB permease indicates that it shares similarity with a family of proteins involved in the export of compounds through the cytoplasmic membrane, suggesting that the heme-sensitive phenotype observed in an *hrtAB* mutant is associated with a defect in the ability to export excess heme or compounds produced in response to heme exposure. Recent studies with *S. aureus* using mass spectrometry-based tracking did not confirm a function for HrtAB in heme secretion, suggesting that heme metabolites or other toxic compounds may be the substrate for the HrtAB system in that species (40).

It was recently reported that proteins orthologous to HrtAB were present in various bacterial species within the phylum *Firmicutes*, including important human pathogens within the genera *Bacillus*, *Staphylococcus*, and *Listeria* (40, 42). To determine the prevalence of the HrtAB genes in other bacterial phyla, we performed a BLAST search using the *C. diphtheriae* HrtB sequence to identify orthologous proteins in the 1,200 completed bacterial genomes available in the NCBI database ([http://www.ncbi.nlm.nih.gov/sutils/genom\\_table.cgi](http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi)). The results identified two predominant phyla that contained organ-

isms encoding orthologs of HrtB: the above-mentioned *Firmicutes* and also the *Actinobacteria*, which include genera related to *Corynebacterium*, *Streptomyces*, *Arthrobacter*, and *Mycobacterium*; however, no HrtB orthologs were identified in any *Mycobacterium* species. No organisms outside these two groups of Gram-positive bacteria and no Gram-negative bacteria encoded proteins with significant sequence similarity to HrtB. In addition to the HrtAB system, other factors are known to protect bacteria from heme toxicity, including the unusual HbpS protein in *Streptomyces reticuli*, which has been proposed to degrade heme at the cell surface (25). Various systems in Gram-negative bacteria have also been identified, including ShuS and HemS in *Shigella dysenteriae* and *Yersinia enterocolitica*, respectively (44, 51). The mechanisms of protection conferred by these proteins have not been determined, although it is proposed that these intracellular proteins bind heme and function as either heme storage or shuttle proteins.

We previously reported that *C. diphtheriae* strains with mutations in the *chrSA* genes are more sensitive to heme than the wild-type strain (4), and it was further noted that this enhanced sensitivity was not associated with decreased expression of HmuO, an enzyme involved in heme degradation and previously shown to be activated by the ChrAS two-component system (4, 33). It was presumed that the increase in heme sensitivity in a ChrAS mutant was due to reduced expression of an as yet identified gene(s) whose transcription is elevated by the ChrAS regulatory proteins. In this study, we provide strong evidence that the increased heme sensitivity observed in *chrSA* mutants is due to reduced expression of the ChrAS-regulated *hrtAB* genes. The heme sensitivity in the *chrSA* mutant C7chrSΔ was alleviated in the presence of plasmid pMnt-hrtAB, which contains the cloned *hrtAB* genes under transcriptional control of the *mntA* promoter, a promoter that is independent of ChrAS regulation (34).

The heme-activated *hrtB* promoter is the third promoter identified that is regulated by the ChrAS system and the only promoter in the ChrA regulon that is not also affected by the response regulator HrrA. Sequence analysis of the three promoter regions regulated by ChrA, which include *hmuO*, *hrtB*, and *hemA*, revealed the presence of the 10-bp sequence 5'-GTTGATGTG-3', which may serve as a possible binding site for ChrA. The *hrtB* promoter contained two copies of this sequence (9/10 match with consensus), which form a potential stem-loop structure located upstream of the putative -10 element. No sequences matching a strong -35 element were identified at an appropriate distance from the putative -10 region within the *hrtB* promoter, which is consistent with the requirement for an activator involved in the expression of these genes. Two copies of 5'-GGTTGATGTG-3' separated by 40 bp are present upstream of the -10 element for the *hmuO* promoter, and a single copy that matches 8 of 10 residues for this sequence overlaps putative -10 sequences for the *hemA* promoter (Fig. 3). While additional studies are needed to confirm that these regions are important recognition sequences for the ChrA response regulator, the different relative locations and arrangements of these sequences suggest differences in the ChrA binding configurations at these various promoters. It is possible that the interaction of DtxR and HrrA influences ChrA binding at the *hmuO* promoter in a manner that is not observed with ChrA binding at the *hrtB* promoter,

where HrrA and DtxR do not appear to have any direct role in regulation.

In *C. diphtheriae*, it has been well established that DtxR functions in an iron-dependent manner to repress expression of *tox* and numerous other genes involved in iron uptake and metabolism (6, 18, 37). While DtxR functions as an iron-dependent repressor at the ChrAS-regulated *hmuO* promoter, this regulatory protein appears to function as an activator at the *hrtB* promoter. It was observed that expression of the *hrtB* promoter is reduced in a *dtxR* mutant, and it was further noted that the presence of the iron chelator EDDA resulted in a 40% decrease in expression of *hrtB* in the wild-type strain. Since hemoglobin or heme is required to activate expression of *hrtB*, the presence of the chelator resulted in only a modest reduction in biologically relevant iron levels, as measured by expression of the *tox* gene, so the difference between expression under high iron levels and that under very low levels is not possible to determine. Nevertheless, these findings suggest that DtxR functions as an activator at *hrtB* and that optimal expression occurs under iron-replete conditions. Analysis of the region upstream of the *hrtB* gene failed to identify sequences with significant similarity to the consensus DtxR binding site, which suggests that DtxR may affect expression of the *hrtB* promoter through indirect mechanisms. DtxR has not been shown to function as an activator of gene expression in *C. diphtheriae*. However, orthologs of DtxR in *Mycobacterium tuberculosis* (16) and in *Corynebacterium glutamicum* (10) are reported to function as both repressors and activators. IdeR, the DtxR ortholog in *M. tuberculosis*, activates transcription by directly binding to regions upstream of regulated promoters (16).

A comparison of *C. diphtheriae* ChrAS with *S. aureus* and *B. anthracis* HssRS suggests that these signal transduction systems have similar roles in the regulation of gene expression through a heme-responsive mechanism. However, several notable differences exist between these systems. The HssRS systems have been shown to regulate only the *hrtAB* promoter, while ChrAS controls the expression of at least three promoters, all of which are associated with various aspects of heme metabolism. Although the response regulators ChrA and HrrR exhibit some sequence homology, the similarity is primarily in regions that are commonly conserved in numerous other response regulators within this group of related regulatory proteins (27). Surprisingly, the cognate sensor kinases for these systems, ChrS and HssS, share no significant sequence similarities (data not shown) (40). The N-terminal sensor domains of these proteins, which are thought to be critical in the detection of the environmental signal and in anchoring the proteins to the membrane, are remarkably different not only in sequence but also in their predicted structures. We show in this study that ChrS contains 6 transmembrane helices in its N-terminal domain, joined by short loop regions, whereas the sequence of HssS is predicted to contain only two transmembrane domains and one large extracellular loop region. A recent analysis of point mutations in conserved residues in the *B. anthracis* HssS N-terminal region identified mutants that had reduced activity, suggesting that this region is important in signal transduction and in responding to heme exposure; however, in an expanded analysis of all HssS orthologs, no conserved residues that are known to coordinate with the axial iron atom of heme were

identified (40). We have also identified conserved residues in the ChrS N-terminal region that are important for activity; the most notable finding from this analysis is that a mutation in a highly conserved tyrosine (Y61) results in virtually complete loss of ChrS function. Tyrosine residues are known to be important in the coordination of heme in numerous proteins (28). While it is possible that the Y61 residue may be critical in recognition of heme or a heme metabolite, additional studies will be needed to confirm the function of this specific residue in signal transduction.

A recent report described the expression and purification of the ChrS protein in *E. coli* and showed that autophosphorylation of ChrS was enhanced in the presence of heme (17). Purified ChrS that was incorporated into proteoliposomes gave an absorbance peak of 405 nm in the presence of heme, suggesting that ChrS directly binds heme, although the specific nature of the binding and location of the binding site were not determined. It was also noted that the proteoliposomes in the absence of ChrS also bound heme and gave significant background absorbance in these studies. Numerous attempts in our laboratory to overexpress and purify ChrS have not been successful, even when using techniques very similar to those previously reported (17). Even moderate expression of ChrS appears to be detrimental to *E. coli* strains used in expression studies, presumably due to the presence of the multiple membrane-spanning regions. Small amounts of ChrS that were expressed in our studies could not be purified as glutathione *S*-transferase (GST) fusions, presumably due to unusual structural conformations. These fusion proteins also failed to bind to heme agarose, a technique we previously employed to demonstrate heme binding to the HmuT protein (13; M. P. Schmitt, unpublished results).

In this study, we show that transcriptional activation of the *hrtB* promoter is mediated by the ChrAS signal transduction system in response to heme. As depicted schematically in Fig. 6, it is postulated that heme or heme-associated factors interact with ChrS, resulting in autophosphorylation at H215, which is followed by the transphosphorylation of ChrA. Phosphorylated ChrA is presumed to undergo a conformational change allowing it to interact with sequences upstream of the *hrtB* promoter, resulting in activation of transcription. The products of *hrtAB* are predicted to function as an efflux pump to remove excess levels of heme or factors produced in response to heme exposure. If heme directly interacts with ChrS, as proposed in a recent study (17), then amino acid residues that we have identified that are critical for activity may be associated with heme binding. Interestingly, 3 of the 5 residues that reduce heme-responsive ChrS function, including the highly conserved Y61, are clustered in a loop region that is predicted to be intracellular, suggesting that the sensing of the signal may occur in the cytosol. Membrane-anchored histidine kinases that detect cytosolic signals are unusual but have been previously described (22). Confirmation that ChrS detects a signal in the cytosol at its N terminus will require additional studies, since other residues that are important for function, such as the conserved R34, are predicted to be outside the membrane. A sensor that is responsive to intracellular heme levels would be consistent with the proposed function of the various ChrAS-regulated systems, which either interact directly with intracellular heme (HmuO and HrtAB) or are involved in the main-

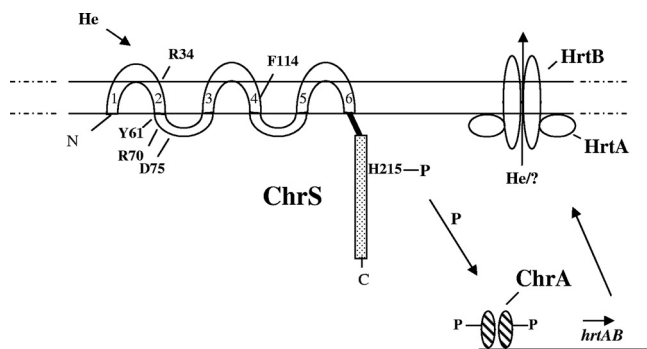


FIG. 6. Schematic representation of ChrSA regulation of *hrtAB* and proposed function of HrtAB. In the presence of a heme source, hemin or heme-associated factors are proposed to interact with the membrane-anchored N terminus of ChrS, which results in autophosphorylation at H215 and the subsequent transphosphorylation of ChrA. Phosphorylated ChrA is proposed to bind upstream of the *hrtB* promoter to activate transcription. The products of *hrtAB* are predicted to be components of an ABC transporter that may function as an efflux pump to export hemin or factors produced in response to hemin exposure. The locations of amino acids that were identified from mutagenesis studies and are critical for ChrS function are indicated in the ChrS sensor domain. The six transmembrane regions in the N-terminal of ChrS are numbered 1 to 6.

tenance of heme homeostasis (HemA and associated heme biosynthetic enzymes).

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