Analysis of a Heme-Dependent Signal Transduction System in *Corynebacterium diphtheriae*: Deletion of the chrAS Genes Results in Heme Sensitivity and Diminished Heme-Dependent Activation of the hmuO Promoter

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The *Corynebacterium diphtheriae* hmuO gene encodes a heme oxygenase that is involved in the utilization of heme as an iron source. Transcription of *hmuO* is activated by heme or hemoglobin and repressed by iron and DtxR. Previous studies with *Escherichia coli* showed that heme-dependent transcriptional activation of an *hmuO* promoter-lacZ fusion was dependent on the cloned *C. diphtheriae* chrA and chrS genes (chrAS), which encode the response regulator and sensor kinase, respectively, of a two-component signal transduction system.

In this study, nonpolar deletions in the chrAS genes were constructed on the chromosome of *C. diphtheriae*. Mutations in chrAS resulted in marked reduction in heme-dependent transcription of *hmuO*, which indicates that the ChrA/S system is a key regulator at the *hmuO* promoter. However, low but significant levels of heme-specific transcriptional activity were observed at the *hmuO* promoter in the chrAS mutants, suggesting that an additional heme-dependent activator is involved in *hmuO* expression. The chrAS mutants were also sensitive to heme, which was observed only in stationary-phase cultures and correlated with reduced cell viability. The heme sensitivity of the mutants was not due to reduced expression of *hmuO*, and these results suggest that additional factors controlled by the ChrA/S system may be involved in protection against heme toxicity. Transcriptional analysis of the chrAS operon revealed that it was not autoregulated or affected by iron or heme levels.

The ability to acquire iron is essential for many bacterial pathogens to cause disease (14, 17). *Corynebacterium diphtheriae*, the causative agent of the severe respiratory disease diphtheria, can use a variety of compounds as iron sources, and recent reports have identified several iron uptake transporters in this organism (4, 5, 10, 11, 20, 26, 27), including systems involved in the utilization of iron from heme and hemoglobin (5, 10, 23). The *hmuO* gene in *Corynebacterium* species encodes a heme oxygenase that is involved in the degradation of heme and the subsequent release of the heme-bound iron (23, 35). Heme oxygenases have been studied in eukaryotic systems for over 30 years (13, 34) but were only recently identified as important factors for iron acquisition. Indeed, the high-affinity heme uptake systems for *C. diphtheriae* interact with the ChrA/S system to modulate the expression of at least 20 promoters distributed throughout the genome (3, 7, 10, 11, 20, 28). A DtxR binding site at the *hmuO* promoter was shown to overlap the −10 and −35 sequences, and DNase I protection experiments confirmed that DtxR binds to this region (24). Additional studies showed that transcription of *hmuO* supports growth in iron-limited conditions, where DtxR and iron repress expression, and a heme source, such as heme or hemoglobin, is required for activation (24). A 50-bp region downstream of the *hmuO* promoter −35 sequence was required for heme-dependent activation, which suggests that this sequence harbors an activator binding site. Analysis of a plasmid library carrying *C. diphtheriae* genomic DNA identified two independent genes, designated cstA and chrA, that activated expression of an *hmuO* promoter-lacZ reporter construct in *Escherichia coli* DH5α (25). The predicted products of *chrA* and *cstA* shared sequence similarity with the ChrA/S system, suggesting that these activators were involved in the modulation of iron-dependent expression of the *hmuO* operon.

The diphtheria toxin repressor, DtxR, is a global iron-dependent repressor in *C. diphtheriae*, where it is known to regulate the expression of at least 20 promoters distributed throughout the genome (3, 7, 10, 11, 20, 28). A DtxR binding site at the *hmuO* promoter was shown to overlap the −10 and −35 sequences, and DNase I protection experiments confirmed that DtxR binds to this region (24). Additional studies showed that transcription of *hmuO* supports growth in iron-limited conditions, where DtxR and iron repress expression, and a heme source, such as heme or hemoglobin, is required for activation (24). A 50-bp region downstream of the *hmuO* promoter −35 sequence was required for heme-dependent activation, which suggests that this sequence harbors an activator binding site. Analysis of a plasmid library carrying *C. diphtheriae* genomic DNA identified two independent genes, designated cstA and chrA, that activated expression of an *hmuO* promoter-lacZ reporter construct in *Escherichia coli* DH5α (25). The predicted products of *chrA* and *cstA* shared sequence similarity with the ChrA/S system, suggesting that these activators were involved in the modulation of iron-dependent expression of the *hmuO* operon.

Analysis of the heme sensitivity of the mutants suggested that additional factors controlled by the ChrA/S system may be involved in protection against heme toxicity. Transcriptional analysis of the chrAS operon revealed that it was not autoregulated or affected by iron or heme levels.

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its outer membrane. The cstA gene, in the presence of its cognate sensor gene, cstS, failed to significantly activate expression of hmuO in E. coli, and the function of the cstAS system has not been determined.

It was proposed from these studies that the detection of a heme source on the cell surface by the C. diphtheriae ChrS sensor kinase initiates a signaling cascade, which is similar to the mechanism by which other sensor kinase-response regulator pairs transduce signals in response to environmental stimuli (19). This model of regulation proposes that ChrS is phosphorylated in response to a heme source; the phosphate is then transferred to the response regulator ChrA, which activates transcription at the hmuO promoter. While the previous findings suggest that the ChrA/S system has a role in heme-dependent activation of the hmuO promoter, direct experimental evidence for this function in C. diphtheriae has been lacking.

In this report, we have used allelic replacement to construct nonpolar deletions in the chrA and chrS genes in the chromosome of C. diphtheriae strain C7(-). These mutations resulted in reduced heme-activated transcription of hmuO and a heightened sensitivity to heme. These observations indicate that the ChrA/S regulator is the primary activator of the hmuO promoter but also suggest that an additional heme-dependent activator is involved in hmuO expression. Moreover, the ChrA/S system appears to control the expression of factors involved in protecting the bacteria from the toxic effects of heme.

**Materials and Methods**

**Bacterial strains and media.** Bacterial strains are listed in Table 1. Luria-Bertani (LB) medium was used for culturing of E. coli, and heart infusion broth (Difco, Detroit, MI) containing 0.2% Tween 80 (HIBTW) was used for growth of C. diphtheriae strains. Antibiotics were added to LB medium at 34 μg/ml for chloramphenicol and 50 μg/ml for kanamycin and to HIBTW at 2 μg/ml for chloramphenicol. Ethylenediamine di(o-hydroxyphenylacetic acid) (EDDA) and N,N,N',N'-tetraakis (2-pyridylmethyl) ethylenediamine (TPEN) were added to HIBTW medium to chelate iron and zinc, respectively. Antibiotics, EDDA, TPEN, Tween 80, hemin (bovine), and hemoglobin (human) were from Sigma Chemical Co. (St. Louis, MO).

**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 C7(-) genomic DNA was used as a template for all PCRs. The promoter probe vector pCM502 (24), which was used for the construction of the lacZ promoter fusions, contains a promoterless lacZ gene and replicates at low copy number in C. diphtheriae. The promoter-lacZ fusions on plasmids pchrS-PO and pchrA-PO contained the identical 287-bp DNA insert and included the complete chrS-cmrA intergenic region and flanking sequences. The DNA fragment in these two constructs was cloned in opposite orientations relative to the lacZ gene.

Plasmids pchrS and pchrA, which contained the complete coding region for the chrS and chrA genes, respectively, were constructed in vector pCM2.6. PCR was used to produce the 1.8-kb DNA fragment that contained the chrS gene, while the chrA gene was present on a 1.2-kb SalI fragment derived from plasmid pT502 (25). Primers used for plasmid constructions are listed in Table 2.

**Mutarient construction.** Deletions in the C7(-) chrS, chrA, cstA, and hmuO genes were constructed using an allelic-replacement technique that has been previously described (10). Mutant construction utilized PCR to clone DNA fragments located upstream and downstream of the region targeted for deletion. The chrS mutant chrSΔ2 has a 606-bp deletion within the S' region; the deleted DNA encodes a portion of ChrS predicted to be involved in heme detection. The chrSΔ2 deletion is predicted to generate an in-frame fusion between 8 residues at the N terminus and 215 amino acids from the C-terminal domain. The chrA mutation of chrAΔ deletes 93% of the coding region and is predicted to generate a 13-amino-acid product that contains 9 amino acids at the N terminus fused in frame to 4 residues from the C terminus (Fig. 1). The chrA mutation has a deletion similar to that in chrAΔ. The hmuO deletion was constructed using PCR to amplify a 2.4-kb fragment, followed by the removal of an internal 600-bp Stul fragment, which deleted 96% of the coding region for hmuO. This construct was then used for allelic replacement as described previously (10). 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 strains in the presence of heme.** The ability of C. diphtheriae strains to grow on heme agar plates was determined by streaking or spread plating overnight cultures of C. diphtheriae C7(-) wild type or the deletion mutants chrSΔ, chrAΔ, and C7hmuO-Δ onto HIBTW agar plates that contained either 2.5 μM heme, 10 μM heme, or no added heme. The plates were incubated at 37°C for 24 h and examined for growth.

To determine the affects of heme on broth cultures, C. diphtheriae strains were grown overnight at 37°C in 4 ml of HIBTW and then inoculated at an optical density at 600 nm (OD600) of 0.1 into fresh HIBTW that contained either 2.5 μM heme, 10 μM heme, or no added heme. Cultures were grown at 37°C with shaking, and OD600 measurements of the bacterial culture were made. At various time points, bacterial viability was also evaluated by plating dilutions of each culture onto HIBTW agar plates.

The effect of hemoglobin on C. diphtheriae growth was similarly examined using hemoglobin concentrations of 140 μg/ml, 460 μg/ml, and 700 μg/ml. To assess the effect of adding heme after inoculation, C. diphtheriae strains were grown overnight at 37°C in 4 ml of HIBTW and then inoculated at an optical density at 600 nm (OD600) of 0.1 into fresh HIBTW that contained either 2.5 μM heme, 10 μM heme, or no added heme. At various times after inoculation (1 h, 2 h, and 3 h), aliquots of the nontsupplemented cultures were provided with 10 μM heme. Cultures were grown at 37°C with shaking, and OD600 measurements were made to assess bacterial growth.

**LacZ assays.** Overnight cultures of C. diphtheriae strains grown in HIBTW were inoculated at an OD600 of 0.1 into fresh HIBTW medium that contained various supplements. After 5 to 6 h of growth at 37°C with shaking, LacZ activity was determined as previously described (29).

**Computer analysis.** Nucleic acid and amino acid sequences were analyzed using DNA analysis software provided by the Genetics Computer Group Wisconsin Package, version 10.3. Amino acid sequence similarity searches were done using the BLAST program (1) 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. The annotated genome sequence for C. diphtheriae strain NCTC13129 (4) is accessible in the EMBL/GenBank database at accession number BX248353.
RESULTS

Genetic organization of the chrAS region. Analysis of the genetic organization of the chrS and chrA genes suggests they constitute an operon that is flanked by two genes, cmrA and dip2328, which are predicted to encode membrane proteins that show no significant amino acid similarity to known proteins (Fig. 1). The predicted amino acid sequence encoded by cmrA indicates that the protein is secreted and contains a possible sortase recognition motif at its C terminus, suggesting that the protein is secreted and contains a possible sortase recognition motif at its C terminus, suggesting the product of cmrA may be anchored to the cell wall (18). The dip2328 and cmrA genes are transcribed in the opposite direction from the chrAS operon, and a 129-bp intergenic region separates chrS and cmrA while a 2-bp gap is located between chrA and dip2328 (Fig. 1). Nonpolar deletions were constructed in the chromosomal chrA and chrS genes in C. diphtheriae C7(-) using a previously described allelic-replacement procedure (10). In strain chrAΔ, 93% of the coding region of chrA was removed, while chrSΔ2 carries a deletion in the 5′ portion of chrS, a region that is proposed to function in the detection of heme (Fig. 1).

The chrA and chrS mutants are heme sensitive. Initial attempts to examine the effect of the chrA and chrS mutations on expression of the hmuO promoter revealed that chrAΔ and chrSΔ2 failed to grow in HIBTW agar medium that contained 10 μM heme but exhibited wild-type levels of growth on medium with 2.5 μM heme or no supplement (Table 3). The ability of the mutants to grow on 10 μM heme was complemented by the relevant cloned chrA or chrS gene, which indicates that the specific chrA or chrS mutation was responsible for the heme sensitivity phenotype and confirmed that the deletion in chrS did not have a polar effect on chrA. Since the ChrAS system is an activator of hmuO expression, it was possible that reduced levels of HmuO, a heme oxygenase involved in heme degradation, may be the cause for the heme sensitivity. However, the presence of the cloned hmuO gene on plasmid pCD293 in chrAΔ did not diminish the heme sensitivity in the mutant (Table 3). (Transcription of hmuO on pCD293 is directed from constitutively expressed vector promoters and is independent of ChrAS regulation [28]). Moreover, strain C7hmuOΔ, a mutant of C7 that is deleted for hmuO, was not heme sensitive, indicating that heme sensitivity in the chrAS mutants is not due to poor expression of hmuO but likely involves another gene(s) that is regulated by the ChrAS system.

To better define the nature of the heme sensitivity, growth measurements were done in liquid medium for the wild type and deletion mutants in the presence and absence of heme. Since the chrS deletion mutant showed similar results to the

FIG. 1. Genetic map of the chrAS region. The regions deleted in the C. diphtheriae C7 chrSΔ2 and chrAΔ mutants are indicated below the map. P indicates putative promoter, and arrows indicate direction of transcription. Sizes of predicted gene products are indicated in kilodaltons.
TABLE 3. Growth on heme medium

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Growth(^a) at a heme concn (µM) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>C7 wt(^c)</td>
<td>+</td>
</tr>
<tr>
<td>chrAΔ</td>
<td>+</td>
</tr>
<tr>
<td>chrAΔ (pchA)(^e)</td>
<td>+</td>
</tr>
<tr>
<td>chrAΔ (pchS)</td>
<td>+</td>
</tr>
<tr>
<td>chrAΔ (pCD293 [hmuO(^-)])</td>
<td>+</td>
</tr>
<tr>
<td>C7 hmuOΔ</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) +, growth on HIBTW agar medium containing heme; -, no growth. All strains grew in the absence of heme.

\(^b\) chrA is transcribed from vector promoters on pchrA (28).

\(^c\) wt, wild type.

Strain (plasmid)  Growth\(^a\) at a heme concn (µM) of:

- C7 wt
- chrAΔ
- chrAΔ (pchA)
- chrAΔ (pchS)
- chrAΔ (pCD293 [hmuO\(^-\)])
- C7 hmuOΔ

\(^a\) +, growth on HIBTW agar medium containing heme; -, no growth. All strains grew in the absence of heme.

\(^b\) chrA is transcribed from vector promoters on pchrA (28).

\(^c\) wt, wild type.

**chrA** mutant, only data for chrAΔ are presented. The chrAΔ mutant grew like the wild type in HIBTW medium containing 2.5 µM heme (data not shown) or in the absence of heme but failed to grow at 10 µM heme (Fig. 2). In 5 µM heme, the chrAΔ mutant exhibited a significant lag phase, although by 24 h chrAΔ reached a cell density similar to wild type. The C7 wild-type strain showed some reduced growth at the highest heme level during log-phase growth. The presence of hemoglobin at concentrations as high as 700 µg/ml (approximately 40 µM heme content) caused only a slight reduction in the growth of the mutants in HIBTW medium (data not shown). While the reason for this difference in toxicity between these two heme sources is not known, it is possible that different transport mechanisms may account for the toxicity differences.

To determine the effect of heme on cell survival, dilutions of the cultures were plated onto HIBTW agar medium to quantitate viable bacteria. The results shown in Fig. 3 indicate that growth in 10 µM heme caused a marked decrease in cell viability in the chrAΔ mutant when compared to the parental C7 strain. After 6 hours of growth in 10 µM heme, the number of viable cells for chrAΔ was 5 logs less than that of the wild-type strain and was at least 4 logs lower than the number of viable cells present in the initial inoculum. Growth in 5 µM heme also resulted in decreased viability for chrAΔ, but the reduction was not as significant as that seen at 10 µM heme (data not shown). These findings indicate that the presence of 10 µM heme results in cell death in the chrAS mutants.

Further analysis of the toxic effects of heme on the chrAS mutants revealed that the sensitivity to heme was significantly alleviated when heme is added to log-phase cultures. As shown in the growth curves in Fig. 4, the toxic effects of heme on chrAΔ were greatly reduced when 10 µM heme was added to the cultures 2 hours (or longer) after inoculation of the HIBTW medium. A similar effect was seen with chrAΔ (data not shown). This observation suggests that one or more factors present in log-phase cultures protect the chrAS mutants from heme toxicity.

**Analysis of hmuO promoter activity.** Expression from the hmuO promoter in the deletion mutants, chrAΔ and chrAS2, and in the wild-type strain was measured using the hmuO-lacZ reporter construct pCP0-1 (25). Only data for chrAΔ are presented here since similar results were observed with chrAS2. Bacteria were grown in HIBTW medium that contained supplements, and LacZ activity was determined after 6 hours of growth from log-phase cultures. Because of the toxic effects of heme on stationary-phase cultures, heme (10 µM) and hemo-

**chrA** deletion mutant is sensitive to heme. Wild-type C7(-) (solid symbols with solid lines) and the chrA deletion mutant chrAΔ (open symbols with dashed lines) were grown in HIBTW medium with no added heme (diamond), 5 µM heme (triangle), or 10 µM heme (circle). Data shown are the mean of three experiments ± standard deviations.

**Heme sensitivity of chrAΔ is reduced in log-phase cultures.** Growth curves of wild-type C7(-) (solid symbols with solid lines) and chrAΔ (open symbols with dashed lines) are shown. Strains were grown in HIBTW, and heme was added to both C7(-) and chrAΔ at a final concentration of 10 µM at various times (T) after inoculation of the medium: T = 0 h (square), T = 1 h (triangle), T = 2 h (circle), and T = 3 h (×). Results shown are from a representative experiment. Wild-type C7 without heme (solid diamond) is shown as a control.
globin (140 μg/ml) were added 2 hours after inoculation of the cultures. As shown in Table 4, transcription of the hmuO promoter was repressed in high-iron conditions (+Fe) in both chrAΔ and the wild-type strain, and only a slight increase in activity was observed in low-iron medium (−Fe). When wild-type cells were grown in low-iron conditions with heme or hemoglobin, a greater-than-70-fold increase in activity was observed. This observation is consistent with previous studies that showed that DtxR and iron repress the hmuO promoter in high-iron conditions, and increased levels of hmuO promoter activity are observed in the presence of a heme source, regardless of the iron content of the medium (Table 4) (24). In the chrAΔ mutant, a 10-fold increase in promoter activity was observed in low-iron conditions in the presence of hemoglobin compared to low iron without a heme source and a 3-fold increase was seen with heme (Table 4). These findings indicate that the ChrA/S system is a critical heme-dependent regulator of hmuO transcription, since promoter activity is markedly reduced in the chrAΔ mutants. However, the significant levels of hemoglobin-dependent activation (and to a lesser extent heme activation) observed in the chrAΔ mutants, suggest that an additional activator(s) functions in these mutants. Furthermore, LacZ assays performed on stationary-phase cultures that were grown in the presence of hemoglobin showed that activity in wild-type and chrAΔ strains decreased two- to fourfold relative to levels observed in log phase (data not shown), which suggests that expression of the hmuO promoter is also affected by growth phase. When promoter activity was assessed in high-iron conditions, in the presence of heme or hemoglobin, a four- to fivefold decrease in promoter activity was observed in the wild-type strain compared to levels seen in low-iron medium (in the presence of a heme source) (Table 4). No hmuO promoter activity was detected in the chrA mutant under high-iron conditions even in the presence of a heme source (Table 4).

In an earlier study, we identified a second response regulator, designated cstA, which was capable of activating expression of the hmuO-lacZ fusion in E. coli (25). To determine if the cstA gene has a role in the expression of the hmuO promoter in C. diphtheriae, we constructed a deletion in cstA and also generated a cstA chrA double mutant. LacZ studies revealed that the cstA single mutant had no effect on expression of the hmuO promoter when tested under the same conditions used with chrAΔ (data not shown). Expression from the hmuO promoter in the cstA chrA double mutant was similar to that observed with the chrA single mutant (Table 4 and data not shown), which indicates that CstA is not involved in the low-level hemoglobin/heme-dependent activation that is observed in the chrAΔ mutants.

Transcriptional analysis of the chrS-cmrA intergenic region. The start codons for chrS and cmrA are separated by 129 base pairs, and transcriptional elements that control expression of these genes are predicted to be located in this region. Previous studies have noted that certain two-component signal transduction systems are autoregulated and also control the expression of adjacent genes (12, 15). To analyze transcription of the chrS-cmrA region, we cloned the entire chrS-cmrA intergenic region upstream of the lacZ gene on the promoter probe vector pCM502. The DNA fragment was cloned in both possible orientations relative to lacZ, and the resulting constructs, designated pchrS-PO and pcmrA-PO, were analyzed for promoter activity in the wild-type and chrAΔ mutant strains. In HIBTW medium, only low constitutive levels of transcription were observed from pchrS-PO in the wild-type and chrAΔ strains, regardless of supplementation (Table 5) or growth phase (data not shown). These findings indicate that the chrA/S genes are not autoregulated, nor is their expression appreciably affected by iron or hemoglobin.

Analysis of pcmrA-PO in wild-type C7 or in the chrAΔ strain revealed no detectable expression in HIBTW medium in high- or low-iron conditions or in the presence of a heme source (Table 5). A BLAST search of the C. diphtheriae genome using the predicted CmrA amino acid sequence identified four open reading frames that were flanked by conserved sequences, which were sufficient to activate expression in E. coli (data not shown). Two of these genes were circularly permuted with the lacZ gene (pchrS-PO and pcmrA-PO), and the resulting constructs, designated pchrS-PO and pcmrA-PO, were analyzed for promoter activity in the wild-type and chrAΔ mutant strains (Table 5). Both constructs showed high levels of transcription in the presence of heme or hemoglobin, indicating that the chrA/S genes are autoregulated.

### Table 4. hmuO promoter activity in C7 wild type and chrAΔ

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>+Fe</th>
<th>−Fe</th>
<th>Hb</th>
<th>Heme</th>
<th>+Fe</th>
<th>−Fe</th>
<th>Hb</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7wt/pCP0-1 (hmuO-lacZ)</td>
<td>&lt;0.5</td>
<td>2.9 ± 0.4</td>
<td>209.6 ± 31.2 (72.2)</td>
<td>373.5 ± 45.8 (128.7)</td>
<td>40.3 ± 6.5 (&gt;80)</td>
<td>107.8 ± 14.3 (&gt;215)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chrAΔ/pCP0-1</td>
<td>&lt;0.5</td>
<td>2.7 ± 0.3</td>
<td>26.4 ± 3.2 (9.8)</td>
<td>9.1 ± 1.2 (3.4)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* LacZ activity was determined as described previously (29). Values represent the means of three experiments ± standard deviations. Numbers in parentheses are increases in activity over medium that does not contain a heme source.

### Table 5. chrS and cmrA promoter activity in C7 wild type and chrAΔ

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>+Fe</th>
<th>−Fe</th>
<th>−Fe + Hb</th>
<th>+TPEN*</th>
<th>+TPEN Zn*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7wt (pCMtox)</td>
<td>&lt;0.5</td>
<td>105 ± 22</td>
<td>ND</td>
<td>&lt;0.5</td>
<td>ND</td>
</tr>
<tr>
<td>C7wt (pcmrS-PO)</td>
<td>6 ± 1</td>
<td>8 ± 2</td>
<td>5 ± 1</td>
<td>9 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>chrAΔ (pcmrS-PO)</td>
<td>5 ± 1</td>
<td>7 ± 1</td>
<td>5 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C7wt (pcmcmrA-PO)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>135 ± 26</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>chrAΔ (pcmcmrA-PO)</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>128 ± 33</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

* LacZ activity was determined as described previously (29). Values represent the means of three or more experiments ± standard deviations.

a +Fe, HIBTW medium.

b −Fe, HIBTW medium with 12.5 μg/ml EDDA.

c Hb, hemoglobin at 140 μg/ml.

d Heme at 10 μM.

e Heme, HIBTW medium with 12.5 μg/ml EDDA and 140 μg/ml hemoglobin.

f +TPEN, HIBTW medium with 15 μM TPEN.

g +TPEN Zn, HIBTW medium with 15 μM TPEN and 10 μM ZnSO4.

h ND, not determined.

i C7wt, C7 wild type.
reading frames, of unknown function, which encode predicted products that showed low but significant similarity with CmrA. Three of these orfs, dip439, dip442, and dip443, were located in a putative metal transport operon that we had previously found to be repressed by zinc (M. P. Schmitt and C. A. Kunkle, unpublished observation). Because of the similarity between CmrA and the products of these zinc-regulated orfs, we examined the effect of the zinc chelator TPEN on expression from the cmrA promoter. The presence of TPEN at 15 μM resulted in transcription from the cmrA promoter in HIBTW medium, and the addition of zinc to medium containing TPEN fully repressed expression of the cmrA promoter. The presence of TPEN did not affect the expression from the chrS promoter, nor did it have any effect on the iron-regulated tox promoter on pCMtox in high-iron conditions (Table 5). No significant difference in expression of the cmrA promoter was observed between the wild-type strain and chrAΔ. These findings indicate that transcription of the cmrA promoter is regulated by zinc but is not affected by iron, heme, or mutations in chrA.

**DISCUSSION**

Two-component signal transduction systems are ubiquitous in bacteria and enable microorganisms to rapidly adapt to changes in their environment through activation of gene expression. In *C. diphtheriae*, heme and hemoglobin are environmental stimuli that activate gene expression through the ChrA/S signal transduction system. In this report we extend our earlier study (25) and provide direct evidence for a role of the chrAS genes in the heme-dependent regulation of hmuO. Analysis of chromosomal deletions in the *C. diphtheriae* chrA and chrS genes demonstrated that the ChrA/S system is a critical heme-dependent regulator of hmuO expression. However, the significant levels of heme/hemoglobin-activated expression from the hmuO promoter in the chrAS mutants suggest that an additional regulator is involved in the heme-specific transcription of hmuO. Transcription from the hmuO promoter in the chrAS mutants was detected only under low-iron conditions, and optimal activation was observed when hemoglobin served as the heme source. In contrast, heme-dependent expression of hmuO in the wild-type strain was observed under high- and low-iron conditions, and optimal activation was observed with heme, rather than hemoglobin. These findings suggest that a putative second activator is either not expressed or is not functional in high-iron conditions and is more responsive to hemoglobin than heme.

While heme-regulated gene expression has been well documented in eukaryotes (13, 34), heme activation in bacteria is unusual. Heme-responsive activators have been identified in four bacterial species, including *C. diphtheriae*, *Serratia marcescens*, and two different *Bordetella* species. The heme transport genes in *Bordetella pertussis* and *Bordetella avium* are regulated by heme compounds through a signaling cascade that requires an extracytoplasmic function (ECF) sigma factor (9, 32). The has operon in *Serratia marcescens*, which is involved in heme uptake and encodes a small secreted heme-binding protein, known as a hemophore, is also regulated by an ECF sigma factor (22). These heme uptake systems are also repressed by iron. The hiaA-hmuTUV heme transport locus in *C. diphtheriae* is also iron regulated, but heme had no effect on the expression of this system (10). Similarly, the expression of bacterial heme oxygenases in *Neisseria* and *Pseudomonas* species is regulated only by iron (16, 36). While two-component signal transduction systems that utilize sensor kinases and response regulators are abundant in bacteria, the ChrA/S regulators are the only examples of this type of system that are responsive to heme and hemoglobin. We have identified orthologs of the ChrS gene in the recently completed genomes of various species related to *C. diphtheriae*, including *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Streptomyces coelicolor*, and *Streptomyces reticuli*. Whether these putative sensor kinases are responsive to heme compounds remains to be determined.

Promoter activity in the chrS-cmrA intergenic region revealed that transcription of chrS is not appreciably affected by iron, heme, or mutations in chrAS, but rather expression appears to occur at a low constitutive level under the conditions examined. In contrast, transcription of cmrA was repressed by zinc but was not influenced by iron, heme, or mutations in chrA. Zinc-dependent repression in other bacteria is regulated by the Fur-like repressor Zur (8), and although a gene which encodes a predicted product with sequence similarity to Fur-like proteins was identified in the *C. diphtheriae* genome, a function for this gene has not been determined (Kunkle and Schmitt, unpublished observation).

Cytoxicity associated with high levels of heme and hemoglobin is due to the production of reactive oxygen species (6), and although the reason for the heme sensitivity in the chrAS mutants is not known, the findings suggest these mutants are deficient in a chrAS-regulated gene product involved in protecting the cells from heme toxicity. Mutations that result in heightened sensitivity to heme have been observed in other bacterial pathogens, including virulent strains of *Neisseria*, where heme sensitivity has been associated with mutations in genes encoding the heme oxygenase (36). Additionally, mutations in the *Yersinia enterocolitica hemS* gene, which was proposed to encode a heme-degrading enzyme, have been associated with heme sensitivity (30). The product of the *ahuS* gene in *Shigella dysenteriae* is able to bind heme and DNA, and it is proposed that AhuS may protect the DNA from heme-mediated oxidative damage (33). All of these genes are present in heme transport operons and are involved in the utilization of heme as an iron source. Orthologs for *ahuS* and *hemS* were not identified in BLAST searches of the *C. diphtheriae* genome (M. P. Schmitt, unpublished observation).

The hmuO gene is the only gene known to be regulated by chrAS, and we have shown that hmuO has no role in heme sensitivity in *C. diphtheriae*. It is possible that a putative factor involved in protecting the cells from heme toxicity may function in the oxidative stress response or in the degradation, binding, or storage of heme. The heme sensitivity of the chrAS mutants resulted in loss of cell viability and occurred primarily when heme was added to stationary-phase cultures. While the reason for the diminished sensitivity to heme seen in log-phase cultures in the chrAΔ strain has not been determined, it could be the differential expression of this putative protective factor, such that expression of the factor in log phase does not require the chrAS system. Alternatively, an increase in the number of heme binding proteins may occur during log-phase aerobic growth, and these proteins may provide a repository for heme, thus reducing potentially toxic intracellular heme levels.
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REFERENCES


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