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Identification of a Two-Component Signal Transduction System from *Corynebacterium diphtheriae* That Activates Gene Expression in Response to the Presence of Heme and Hemoglobin

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***Corynebacterium diphtheriae*, the causative agent of diphtheria, utilizes various host compounds to acquire iron. The *C. diphtheriae hmuO* gene encodes a heme oxygenase that is involved in the utilization of heme and hemoglobin as iron sources. Transcription of the *hmuO* gene in *C. diphtheriae* is controlled under a dual regulatory mechanism in which the diphtheria toxin repressor protein (DtxR) and iron repress expression while either heme or hemoglobin is needed to activate transcription. In this study, two clones isolated from a *C. diphtheriae* chromosomal library were shown to activate transcription from the *hmuO* promoter in *Escherichia coli*. Sequence analysis revealed that these activator clones each carried distinct genes whose products had significant homology to response regulators of two-component signal transduction systems. Located upstream from each of these response regulator homologs are partial open reading frames that are predicted to encode the C-terminal portions of sensor kinases. The full-length sensor kinase gene for each of these systems was cloned from the *C. diphtheriae* chromosome, and constructs each carrying one complete sensor kinase gene and its cognate response regulator were constructed. One of these constructs, pTSB20, which carried the response regulator (*chrA*) and its cognate sensor kinase (*chrS*), was shown to strongly activate transcription from the *hmuO* promoter in a heme-dependent manner in *E. coli*. A mutation in *chrA* (*chrAD50N*), which changed a conserved aspartic acid residue at position 50, the presumed site of phosphorylation by ChrS, to an asparagine, abolished heme-dependent activation. These findings suggest that the sensor kinase ChrS is involved in the detection of heme and the transduction of this signal, via a phosphotransfer mechanism, to the response regulator ChrA, which then activates transcription of the *hmuO* promoter. This is the first report of a bacterial two-component signal transduction system that controls gene expression through a heme-responsive mechanism.**

Corynebacterium diphtheriae is a gram-positive, nonsporulating bacterium that is the causative agent of diphtheria. The primary virulence determinant in *C. diphtheriae* is the diphtheria toxin (DT), a 58,000-Da secreted protein which has been extensively studied (31). The *tox* gene, which encodes DT, is regulated at the transcriptional level by the diphtheria toxin repressor protein (DtxR) and iron (2, 38). DtxR, which is functionally similar to the *Escherichia coli* ferric uptake repressor protein (Fur) (13), is a global iron-dependent repressor that regulates the expression of at least eight genes in *C. diphtheriae* (2, 23, 36, 38, 40, 41, 45). The importance of iron in the regulation of bacterial virulence determinants has been well established, and the ability to acquire sufficient iron during infection has been shown to be important for a number of bacterial pathogens to be fully virulent (10, 24, 49).

Systems involved in the acquisition of iron by bacteria include high-affinity siderophore transport systems (3) and siderophore-independent mechanisms in which bacterial pathogens utilize iron from various host sources, such as transferrin, lactoferrin, heme, or hemoglobin (22, 26). The molecular mechanism involved in the transport of heme and its subsequent utilization as an iron source has been examined in several gram-negative pathogens (15, 18, 22, 28, 30, 43, 44, 46, 51). These systems include a heme-specific outer membrane receptor, which is required for the uptake of heme into the periplasm, and an ATP-binding cassette transporter complex that is involved in the transport of heme through the cytoplasmic membrane. It was proposed that these bacteria contain a heme oxygenase-like enzyme that functions in the removal of the heme-bound iron (22, 44). However, proteins with a heme-degrading activity have not been identified in any of these gram-negative species, and the mechanism involved in the extraction of iron from heme remains to be determined.

In *C. diphtheriae*, the ability to utilize iron from transferrin was shown to be siderophore dependent, while the use of iron from heme and hemoglobin was independent of the siderophore uptake system (36). Mutants of *C. diphtheriae* and *Corynebacterium ulcerans* that were unable to utilize heme and hemoglobin as iron sources have been isolated and characterized (36). Clones carrying the *C. diphtheriae hmuO* gene were shown to complement several of the *Corynebacterium* heme utilization mutants. The product of the *hmuO* gene has significant amino acid homology to eukaryotic heme oxygenases. Heme oxygenases, which had not been previously identified in bacteria but are well known in eukaryotic systems, are involved in the oxidative degradation of heme through the cleavage of the heme porphyrin ring and the subsequent production of CO, iron, and biliverdin (25). The HmuO protein from *C. diphtheriae* was purified and shown to have an enzymatic activity that is similar to that observed for eukaryotic heme oxygenases (50). It is proposed that the role of HmuO in the utilization of heme as an iron source in *C. diphtheriae* is in the

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degradation of heme and the subsequent release of the heme-bound iron. It is believed that *Corynebacterium* mutants deficient in HmuO activity are unable to extract the iron from heme and, therefore, defective in their ability to use heme as an iron source. Bacterial heme oxygenases have recently been identified in species of *Cyanobacterium* (5).

In gram-negative bacteria, most of the systems involved in the transport and utilization of heme-bound iron are repressed in high-iron environments; this repression is mediated through the Fur protein (22, 30, 51). In pathogenic species of *Haemophilus*, the expression of the transferrin and hemoglobin receptors is repressed by heme (8, 19, 29). The mechanism involved in this regulation has not been determined. Expression studies with the *C. diphtheriae hmuO* gene revealed that transcription from the *hmuO* promoter was under a dual regulatory mechanism, which involved repression by DtxR and iron and activation by heme (37). DNase I footprinting experiments showed that purified DtxR, in the presence of a divalent metal, bound to an approximately 30-bp region that overlapped the *hmuO* promoter. Expression of the *hmuO* promoter from a promoter-probe plasmid in *C. diphtheriae* revealed that only low levels of transcription were observed unless a heme source, either heme or hemoglobin, was added to the growth medium. Northern blot analysis and primer extension studies provided additional evidence that transcription of the *hmuO* gene was activated by heme (37). Genes that are activated by heme or other heme-containing compounds have not been previously reported for bacteria; however, several genes in eukaryotic systems, including those encoding certain heme oxygenases, are regulated at the transcriptional level by heme (52).

In this study, the mechanism involved in heme activation of the *hmuO* promoter was investigated. Two independent clones from a *C. diphtheriae* library were shown to activate expression of an *hmuO* promoter-*lacZ* fusion construct in *E. coli*. The recombinant plasmids were shown to encode the genes designated *chrA* and *cstA*, whose predicted products are homologous to response regulators of two-component signal transduction systems. Immediately upstream from *chrA* and *cstA* are open reading frames that are predicted to encode the cognate sensor kinase genes, which have been designated *chrS* and *cstS*, respectively. A construct carrying the entire coding region for *chrS* and its cognate response regulator *chrA* was shown to activate expression of the *hmuO* promoter in *E. coli* in the presence of heme. This is the first report of a two-component signal transduction system in which transcriptional activation is mediated through heme.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used throughout this study in the analysis of the *hmuO* promoter-*lacZ* reporter fusion constructs (P*hmuO-lac*) and for routine plasmid isolation. *C. diphtheriae* C7(−) (17) was originally obtained from the strain collection of Randall K. Holmes. Luria-Bertani (LB) medium (27) was used for culturing of *E. coli*, while heart infusion broth (Difco, Detroit, Mich.) containing 0.2% Tween 80 (HIBTW) was used for growth of *C. diphtheriae* C7(−). Permanent stocks of bacterial strains were maintained in 20% glycerol at −70°C. When needed, antibiotics were added to LB medium for *E. coli* as follows: 10 μg of tetracycline/ml, 34 μg of chloramphenicol/ml, and 100 μg of ampicillin/ml. Chloramphenicol at 2 μg/ml was added to HIBTW for growth of *C. diphtheriae* strains which harbor plasmids. LB medium and HIBTW were made low iron by the addition of ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) which was deferrated by the method of Rogers (33). EDDA was added to HIBTW-containing media at 50 μg/ml and to LB media at 2.5 μg/ml. Hemin (bovine) was added to *C. diphtheriae* cultures at 25 μg/ml and to *E. coli* cultures at 100 μg/ml, and hemoglobin (human) was added to cultures of both types at 10 μM. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Bethesda Research Laboratories) was used at 0.5 mM. Antibiotics, EDDA, Tween 80, hemin, and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Plasmid construction and DNA manipulation. The promoter probe vector pCM502 (Cm^r) (37), which contains a promoterless *lacZ* gene, was used for the construction of the six P*hmuO-lac* fusion plasmids (see Fig. 1). The DNA inserts present in the six P*hmuO-lac* fusion plasmids were generated by using PCR and seven different oligonucleotide primers. Six of the primers, designated PO-1, PO-2, PO-3, PO-4, PO-5, and PO-6, are 32 nucleotides (nt) in length: the 17-nt sequences at their 3′ ends are complementary to unique sequences upstream of the *hmuO* promoter (sense strand), and the 15-nt sequences at the 5′ ends contain a *Sal*I site. The primer on the antisense strand, designated PO-*Bam*, was used in PCRs with each of the six primers described above to generate the inserts in the various P*hmuO-lac* fusions. The primer PO-*Bam* contains sequences at its 3′ region that are complementary to a sequence 240 bp downstream from the *hmuO* promoter, and it contains a unique *Bam*HI site in a 5′ tail region. The six PCR products each have a unique *Sal*I site in the region upstream of the *hmuO* promoter and a unique *Bam*HI site in the downstream sequence. The six fragments were digested with *Bam*HI and *Sal*I and then ligated into the corresponding sites in pCM502 to generate the P*hmuO-lac* fusions for which maps are shown in Fig. 1. All of the fragments terminated at different locations upstream of the *hmuO* promoter but shared a common downstream terminus. The DNA sequences of the inserts in the six promoter fusion plasmids were determined, and it was confirmed that no sequence changes had occurred during construction of these plasmids.

Plasmids pWKS30 and pWSK29, which carry ampicillin resistance determinants, are low-copy-number plasmids that contain the pSC101 origin of replication (47) and were used in the construction of the plasmids pTSB20, pTSB50, and pTSB20-50. The plasmid pTSB20 was constructed in a two-step process, as follows: the 1.5-kb *Bam*HI-*Hin*dIII fragment was excised from pTSB2.B (Fig. 2) and ligated into the *Bam*HI-*Hin*dIII site of pWKS30 to generate plasmid pW2-1.5. In the second step, the 1.5-kb *Hin*dIII fragment in pKSH51 (identified from the colony hybridization; see below) was ligated into the *Hin*dIII site of pW2-1.5 to create pTSB20. The plasmid pTSB50 was also generated in a two-step process as follows: the 2.4-kb *Pst*I-*Eco*RI insert in pTSB5.R (Fig. 3) was ligated into the *Pst*I-*Eco*RI site of pWSK29 to produce plasmid pW5-2.4. In the second step, a 1.2-kb *Pst*I fragment in pR5-38 (obtained from the colony hybridization; see below) was ligated to the *Pst*I site in pW5-2.4 to create pTSB50. Plasmid pTSB20-50 was constructed by ligating the 3.5-kb insert of pTSB20 (present on a *Pvu*II fragment) into a *Hin*dIII site of pTSB50. The *Hin*dIII site, which is present in vector sequences, was made blunt prior to ligation. All of the genes present on pTSB20, pTSB50, and pTSB20-50 are oriented such that they are predicted to be under transcriptional control of the *lac* promoter on the vector.

The plasmid pTSB2-5 was constructed by ligating the 2-kb *Bam*HI fragment from pTSB2.B into the *Bam*HI site of pTSB5.R. The 2-kb *Bam*HI fragments in pTSB2-5 and in pTSB2.B are in the same orientation relative to the *trc* promoter present on plasmid p*Trc*99A. The pSHU9 plasmid (Tc^r, pAT153 replicon) was a gift from Shelley M. Payne, University of Texas at Austin, and it contains a 9-kb insert that carries genes for the heme transport system from *Shigella dysenteriae* (28). To optimize expression of the heme transport genes on pSHU9, EDDA was added to the growth medium at 2.5 μg/ml. The plasmid pBluescript KS (Stratagene, La Jolla, Calif.) was used for routine cloning experiments and for preparing DNA for sequencing. Plasmids were transformed into *C. diphtheriae* by electroporation (14) and into *E. coli* as previously described (12).

Construction of the *C. diphtheriae* chromosomal library. The expression vector p*Trc*99A (Amp^r) (Pharmacia, Milwaukee, Wis.), which was used in the construction of the *C. diphtheriae* library, contains the pBR322 origin of replication and the $lacI^q$ gene. The plasmid library was constructed as follows. *C. diphtheriae* C7(−) chromosomal DNA was isolated as previously described (42) and then partially digested with *Sau*3AI. DNA fragments of 3 to 7 kb were excised from a 1% agarose gel and purified by using a Gene Clean Spin kit from Bio 101 (Vista, Calif.). The DNA fragments were ligated into the *Bam*HI site of the p*Trc*99A vector that had been treated with shrimp alkaline phosphatase (Amersham, Cleveland, Ohio).

Identification and cloning of fragments from the C7(−) chromosome that carry the sensor kinase genes. Since plasmids pTSB2 and pTSB5 carry only the 3′ portions of the coding regions for the *cstS* and *chrS* genes, additional restriction mapping analysis of the *C. diphtheriae* chromosome was done to identify restriction fragments that might carry the 5′ regions of these two genes. ^{32}P-labeled DNA fragments obtained from either plasmid pTSB2 or pTSB5 were used as probes to hybridize to a chromosomal digest of *C. diphtheriae* C7(−) DNA (21, 34). Hybridization studies using the pTSB2 probe identified a 1.5-kb *Hin*dIII fragment that was predicted to contain the 5′ region of the sensor kinase gene *chrS*. Similarly, hybridization analysis with a pTSB5 probe indicated that a 1.8-kb *Eco*RV fragment present in the *C. diphtheriae* chromosome contains the 5′ portion of the *cstS* gene. Both the 1.5-kb *Hin*dIII fragment and the 1.8-kb *Eco*RV fragment contain approximately 500-bp sequences that are present on the cloned sequences of pTSB2 and pTSB5, respectively. The 1.5-kb *Hin*dIII fragment and the 1.8-kb *Eco*RV fragment were cloned into the pBluescript KS vector as follows. *C. diphtheriae* chromosomal DNAs were digested separately with either *Hin*dIII or *Eco*RV, and DNA fragments in the size range of 1 to 3 kb were excised from a 1% agarose gel, purified, and ligated into the appropriate restriction sites in the KS vector. The recombinants were transformed into DH5α, and then clones that carried the insert of interest were identified by

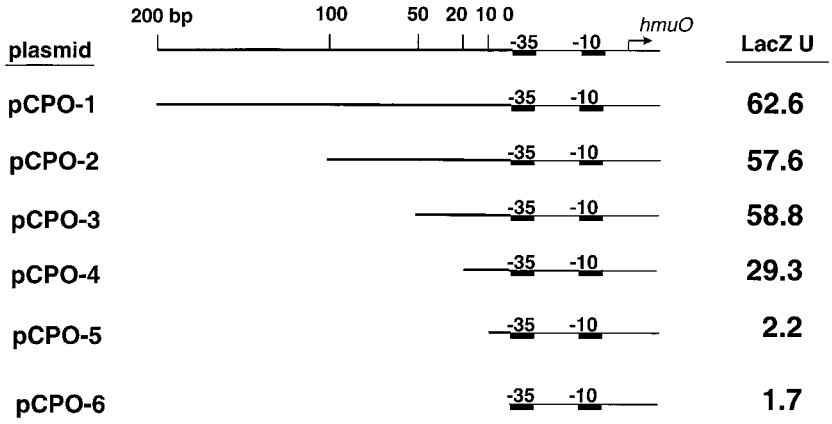

FIG. 1. Linear maps of the *hmuO* promoter region present on the six promoter-*lac* fusions. The *hmuO* promoter is indicated by the −10 and −35 elements. The top line indicates the distance from the −35 sequence in base pairs. The arrow indicates the start site of transcription of the *hmuO* gene (transcription begins at an A residue 40 bp downstream from the 5′ end of the −35 sequence [37]). LacZ activity (LacZ U) for each fusion was determined in *C. diphtheriae* C7(−) grown in low-iron medium in the presence of 25 μg of hemin/ml. LacZ units were determined as previously described (39). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean.

colony hybridization (34). Plasmid pKSH51 carried the 1.5-kb *Hin*dIII insert and plasmid pR5-38 carried the 1.8-kb *Eco*RV insert.

DNA sequence analysis. The DNA sequences for both strands of a 2,021-bp region that includes the complete sequence of the *chrS* and *chrA* genes were determined. Double-stranded DNA templates were sequenced by the chain termination method of Sanger et al. (35) by using a DNA sequencing kit from Amersham. The complete DNA sequences for both strands of a 2,404-bp region containing the *cstS* and *cstA* genes were also determined. Sequences were compiled and analyzed by using the Genetics Computer Group (GCG) program (Madison, Wis.). Amino acid homologies were identified by using a BLAST search of the SwissProt protein database. Amino acid alignments were done by the GAP program (GCG), and putative transmembrane regions were identified by the TMpred program (16).

LacZ assays. Cultures (18 h) of *E. coli* and *C. diphtheriae* were used to inoculate fresh medium at a 1:100 dilution which was then grown for 16 to 18 h at 37°C with shaking. Supplements were added to the medium as indicated. LacZ activities were determined for *E. coli* by the method of Miller (27) and for *C. diphtheriae* as previously described (39).

Mutant construction. A point mutation was introduced into the *chrA* gene by using inverse PCR and utilizing the useful properties of the class II restriction enzyme *Bsa*I (New England BioLabs, Beverly, Mass.). The mutation results in replacement of the Asp residue at position 50 (D50) in the wild-type gene product (ChrA) with an Asn (N50) in the mutant gene product (ChrAD50N). The mutagenesis procedure utilized two 33-bp oligonucleotide primers, MUTN50T, 5′-CGCGGTCTCACC**A**ACATCCAAATGCCAGGCACC-3′ (sense strand), and MUTN50B, 5′-CGCGGTCTCGT**T**GGTGACAACAACGTCGATGCC-3′ (antisense strand), which each contain a unique *Bsa*I recognition site (underlined) and a single base change from the wild-type *chrA* sequence (boldface type; G to **A** for MUTN50T and C to **T** for MUTN50B). The 24 nt sequences at the 3′ ends of the two primers are complementary to sequences on opposite strands of the *chrA* gene, while the 9 nt at the 5′ end contain noncomplementary sequences and include a *Bsa*I site. The primers are designed to anneal to circular template DNA containing the cloned *chrA* gene in a tail-to-tail inverted manner, such that there exists a 6-bp complementary overlap between the primers in the region that is immediately 3′ to the *Bsa*I site. The template DNA used for the PCR was the plasmid pKBH1.2, which contains the 1.2-kb *Bgl*II-*Hin*dIII fragment from pTSB2 (*chrA*$^+$) ligated into the *Bam*HI-*Hin*dIII sites of pBluescript KS. Inverse PCR was done by using Vent polymerase (New England BioLabs), and the reaction mixture contained 10 ng of template DNA, 0.5 μg of each primer, and 300 μM deoxynucleoside triphosphates in 1× Vent polymerase buffer (New England BioLabs). The reaction was run under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 6 min for 28 cycles and a final cycle of 72°C for 10 min. The 4-kb linear PCR product was digested with *Bsa*I, which generated two large fragments, each of approximately 2 kb (*Bsa*I cuts within the ampicillin resistance gene on the vector and also in the 5′ tail regions of the primers). The two fragments were ligated, and Amp^r transformants were isolated. Since *Bsa*I has a cut site that is adjacent to, but does not overlap, its recognition sequence, digestion of the PCR product with *Bsa*I followed by ligation will result in the removal of the *Bsa*I recognition sequence from the 5′ tail regions of the primers without affecting any of the sequences 3′ of the recognition site and will generate complementary overhangs. Therefore, *Bsa*I digestion of the PCR product followed by ligation of the two fragments is predicted to reconstitute the sequence of the original plasmid, pKBH1.2, with the incorporation of a single nucleotide substitution. Plasmid DNA was obtained from one of the transformants, and the DNA sequence of the 1.2-kb insert was determined, confirming the presence of the point mutation within the *chrA* gene and further showing that no other sequence changes had occurred. The resulting plasmid was designated pKBH1.2-D50N.

The plasmids pWBH20 and pWBH20-D50N were constructed by using a two-step process that was similar to that used for the construction of pTSB20. In the first step, the inserts in plasmids pKBH1.2 and pKBH1.2-D50N were excised with *Pvu*II and ligated into the *Eco*RV site of the low-copy-number vector pWKS30 to produce pWBH1.5 and pWBH1.5-D50N, respectively. In the second step, 1.5-kb *Hin*dIII fragments from pKSH51 were ligated into the *Hin*dIII sites of pWBH1.5 and pWBH1.5-D50N to generate plasmids pWBH20 and pWBH20-D50N, respectively. The P*trc99A* expression vector was used to construct plasmids pPBH2 and pPBH2-D50N, which contain the 1.2-kb inserts from pKBH1.2 and pKBH1.2-D50N, respectively.

Nucleotide sequence accession numbers. The sequences of the 2,021-bp region containing *chrS* and *chrA* and the 2,404-bp region containing *cstS* and *cstA* were assigned GenBank accession no. AF161327 and AF161328, respectively.

RESULTS

Sequences required for heme activation of the *hmuO* promoter. To identify sequences needed for the heme-dependent activation of the *hmuO* promoter, six *hmuO* promoter-*lacZ* transcriptional fusion constructs (P*hmuO-lac*) that contained various amounts of *C. diphtheriae* DNA sequences upstream of the *hmuO* promoter were created. Plasmid pCPO-1 contained 200 bp of upstream sequences, while plasmids pCPO-2 through pCPO-6 contained decreasing amounts of the native *C. diphtheriae* sequences upstream of the *hmuO* promoter (Fig. 1). The six P*hmuO-lac* fusion plasmids were examined for transcriptional activity in *C. diphtheriae* C7(−) that was grown in low-iron medium in the presence of heme. Expression of the P*hmuO-lac* fusion on plasmids pCPO-1, pCPO-2, and pCPO-3 resulted in similar levels of LacZ activity (Fig. 1). However, the P*hmuO-lac* fusion on plasmid pCPO-4, which contained only 20 bp of upstream sequence, exhibited a twofold decrease in expression relative to the fully induced levels seen with pCPO-1 (Fig. 1). The plasmids pCPO-5 and pCPO-6 exhibited little if any heme-dependent activation. C7(−) carrying pCPO-1 gave 2.3 U of LacZ activity in low-iron medium in the absence of heme, and similar LacZ levels in the same medium were observed for DH5α carrying pCPO-2 through pCPO-6 (data not shown). Hemoglobin also activated the expression of the *hmuO* promoter in a manner similar to that seen in the presence of heme (data not shown). These findings indicate that sequences upstream of the *hmuO* promoter are required for heme induction and further suggest that the upstream

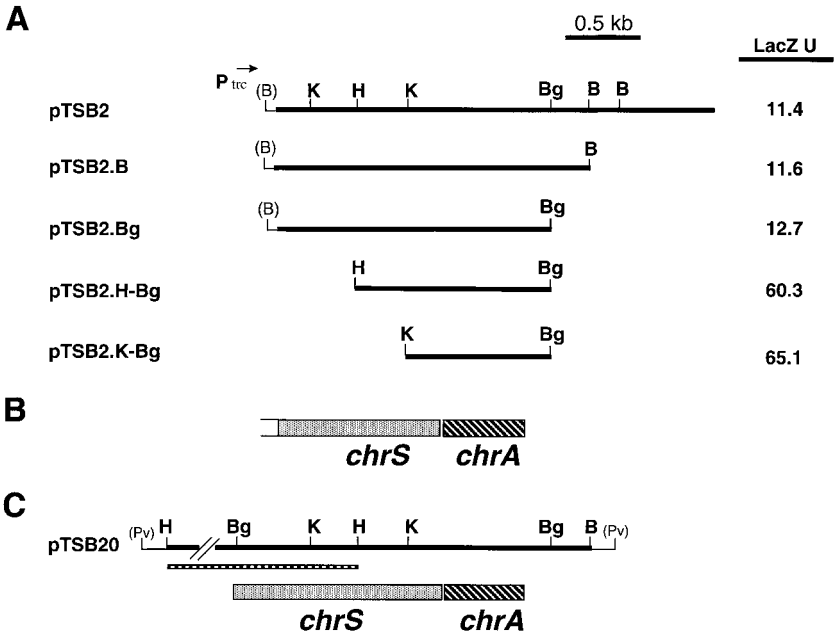

FIG. 2. (A) Restriction maps of plasmid pTSB2 and various subclones. The direction of transcription from the plasmid-encoded *trc* promoter is indicated by the arrow. LacZ assays were done with *E. coli* DH5α/pCPO-1 that carried the various activator clones. Bacteria were grown in LB medium in the presence of 0.5 mM IPTG, and LacZ units were determined by the method of Miller (27). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean. (B) Genetic map of the *chrS* and *chrA* genes present on plasmid pTSB2. The genetic map is aligned with the restriction maps shown in panel A. (C) Restriction and genetic maps of plasmid pTSB20. The thin boxed region below the restriction map indicates the location of the 1.5-kb *Hin*dIII fragment that is present in plasmid pKSH51 and contains the 5′ portion of the *chrS* gene. Only a portion of the *Hin*dIII fragment is shown. The restriction and genetic maps are aligned with each other and with the maps shown in panels A and B. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; Pv, *Pvu*II. Sites shown in parentheses indicate restriction sites present in vector sequences.

region may contain a binding site for a factor involved in the heme-responsive activation.

Identification of genes involved in the activation of the *hmuO* promoter. In an earlier study, it was shown that the *hmuO* promoter is poorly expressed in *E. coli* DH5α (37). Consistent with this earlier finding, the P*hmuO-lac* fusion on pCPO-1 is also expressed at low levels in DH5α (Table 1), and colonies of DH5α carrying pCPO-1 plated onto LB agar medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were white, which is indicative of low-level LacZ expression (data not shown). To identify the gene(s) that activates the expression of the *hmuO* promoter, a *C. diphtheriae* chromosomal library, which was constructed by using the expression plasmid p*Trc*99A, was transformed into DH5α carrying the plasmid pCPO-1. Transformants were plated onto LB agar medium containing X-Gal and IPTG (IPTG was used to induce the *trc* promoter on p*Trc*99A). Since DH5α carrying pCPO-1 produces white colonies on LB medium containing X-Gal and IPTG, blue colonies should represent transformants that contain recombinant clones capable of activating the expression of the *hmuO* promoter present on pCPO-1. Four blue colonies were identified after screening of approximately 10,000 library transformants. The four unique clones were designated pTSB1, pTSB2, pTSB3, and pTSB5 and had inserts of different sizes that ranged from 2.8 kb for pTSB2 to 5.6 kb for pTSB1. Restriction mapping analysis of these clones indicated that the entire 2.8-kb insert of the plasmid pTSB2 was contained within the larger inserts of the plasmids pTSB1 and pTSB3 (Fig. 2A and data not shown). Additionally, the inserts in pTSB1, pTSB2, and pTSB3 all shared the same left end terminus relative to the map of pTSB2 shown in Fig. 2A. Restriction analysis of the 3.7-kb insert in the plasmid pTSB5 indicated that it did not share sequences with the other three plasmids (Fig. 3A). DH5α carrying pCPO-1 and carrying each of the four putative activator clones produced blue colonies on X-Gal-containing medium only in the presence of IPTG (data not shown). This indicated that expression from the IPTG-inducible *trc* promoter, present on p*Trc*99A, was essential for each of these clones to activate the *hmuO* promoter. The dependence on the *trc* promoter indicated that either the putative activator gene(s) on the four clones lacked their native *C. diphtheriae* promoter or the native promoter was inadequately active in DH5α.

Effect of the activator clones on expression of the *hmuO* promoter. Expression of the *hmuO* promoter on pCPO-1, pCPO-4, and pCPO-5, in the presence of the various activator clones, was quantitated in liquid culture medium by measuring β-galactosidase levels (27). Only low levels of promoter activity were seen with the four clones in the absence of IPTG (Table 1 and data not shown). Greater than 10-fold induction of LacZ activity was seen when DH5α carried both pCPO-1 and either pTSB2 or pTSB5 and was grown in the presence of IPTG. Similar LacZ levels were seen with the clones pTSB1 (13.2 U) and pTSB3 (9.6 U) in the presence of IPTG. The plasmids pTSB1 and pTSB3 were not characterized further since these results suggested that all of the sequences needed for activation of the *hmuO* promoter reside in pTSB2. The P*hmuO-lac* fusion on pCPO-4 was less responsive than that on pCPO-1 to IPTG induction in the presence of plasmids pTSB2 and pTSB5; the LacZ levels were two- to threefold lower than those observed with pCPO-1 (Table 1). The plasmids pTSB2 and pTSB5 did not induce the expression of the P*hmuO-lac* fusion on pCPO-5. These results showed that there exists a similar trend for the heme activation obtained with pCPO-1,

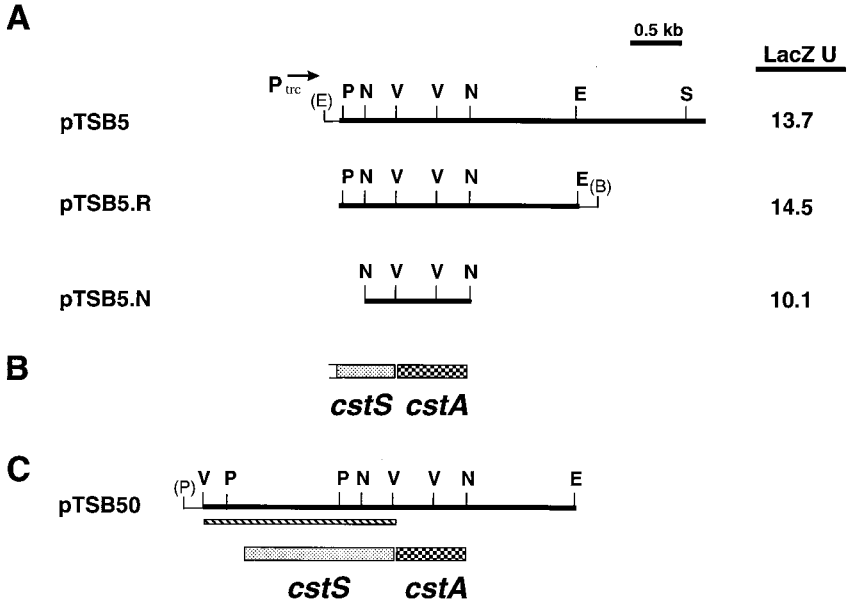

FIG. 3. (A) Restriction maps of plasmid pTSB5 and various subclones. Direction of transcription from the plasmid encoded *trc* promoter is indicated by arrow. LacZ assays were done with *E. coli* DH5α/pCPO-1 that carried the various activator clones. Bacteria were grown in LB medium in the presence of 0.5 mM IPTG, and LacZ units (U) were determined by the method of Miller (27). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean. (B) Genetic map of the *cstS* and *cstA* genes present on plasmid pTSB5. The genetic map is aligned with the restriction maps shown in panel A. (C) Restriction and genetic maps of plasmid pTSB50. The thin boxed region below the restriction map indicates the location of the 1.8-kb *Eco*RV fragment that is present in plasmid pR5-38 and contains the *cstS* gene. The restriction and genetic maps are aligned with each other and to the maps shown in panels A and B. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; N, *Nru*I; P, *Pst*I; S, *Sal*I; V, *Eco*RV. Sites shown in parentheses indicate restriction sites present in vector sequences.

pCPO-4, and pCPO-5 in *C. diphtheriae* (Fig. 1) and for the induction caused by pTSB2 and pTSB5 in *E. coli*.

Subclones of the plasmids pTSB2 and pTSB5 were constructed in p*Trc*99A to identify the smallest region that would maintain induction of the *hmuO* promoter on pCPO-1. The smallest such subclone from pTSB2 identified was pTSB2.K-Bg, which contained a 900-bp *Kpn*I-*Bgl*II fragment (Fig. 2A). Subclones of pTSB2 which contained deletions at the left end had a five- to sixfold increase in LacZ expression of the P*hmuO-lac* fusion on pCPO-1 (Fig. 2A, plasmids pTSB2.H-Bg and pTSB2.K-Bg). The reason for this increase in activation is not clear, although it may be due either to the loss of a repressor function or to the more proximal location of the putative activator gene to the *trc* promoter present on the vector. The smallest subclone of pTSB5 maintaining induction was an approximately 1-kb *Nru*I fragment on pTSB5.N (Fig. 3A). No significant differences in the activation of the *hmuO* promoter were observed for any of the pTSB5 subclones.

TABLE 1. Effect of activator clones on expression of P*hmuo-lac* fusions in DH5α

Plasmid(s)	IPTG[a]	LacZ U[b]
pCPO-1 (P*hmuO-lac*)	+	0.8
pCPO-1, pTSB2	–[c]	0.9
pCPO-1, pTSB2	+	11.4
pCPO-1, pTSB2, pSHU9 (+He)[d]	+	9.3
pCPO-4, pTSB2	+	4.3
pCPO-5, pTSB2	+	0.7
pCPO-1, pTSB5	–	0.9
pCPO-1, pTSB5	+	13.7
pCPO-1, pTSB5, pSHU9 (+He)[d]	+	10.4
pCPO-4, pTSB5	+	5.6
pCPO-5, pTSB5	+	0.6
pCPO-1, pTSB2-5	–	55.7
pCPO-1, pTSB2-5	+	352.1
pCPO-1, pTSB2-5, pSHU9 (+He)[d]	+	323.7

[a] IPTG was added as indicated (+) at 0.5 mM.
[b] LacZ units (U) were determined by the method of Miller (27). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean.
[c] pCPO-1, pCPO-4, and pCPO-5 showed similar LacZ activities in the absence of IPTG.
[d] pSHU9 enables DH5α to transport hemin; hemin (+He) was added at 100 μg/ml.

To assess the effect that both activator genes together would have on the expression of the *hmuO* promoter in *E. coli*, DNA fragments from pTSB2 and pTSB5 were placed in tandem onto the p*Trc99A* vector to generate plasmid pTSB2-5. The presence of pTSB2-5 in DH5α carrying pCPO-1 resulted in a high level of induction (352.1 U) of the *hmuO* promoter in the presence of IPTG (Table 1). This level of expression was 25-fold higher than the LacZ activity seen with either of the activators alone: 11.4 U for pTSB2 and 13.7 U for pTSB5. This finding suggested that the presence of both activators on the same plasmid caused a synergistic effect on the activation of the *hmuO* promoter. Relatively high levels of LacZ activity (55.7 U) were also detected with pTSB2-5 even in the absence of IPTG induction (Table 1), which may have been caused by low-level expression from the "leaky" *trc* promoter.

Almost all laboratory strains of *E. coli*, including DH5α, are unable to transport heme through their outer membranes unless a heme transport system, such as those present in certain bacterial pathogens, is provided in *trans*. The activation of the *hmuO* promoter in DH5α carrying pCPO-1 and either pTSB2,

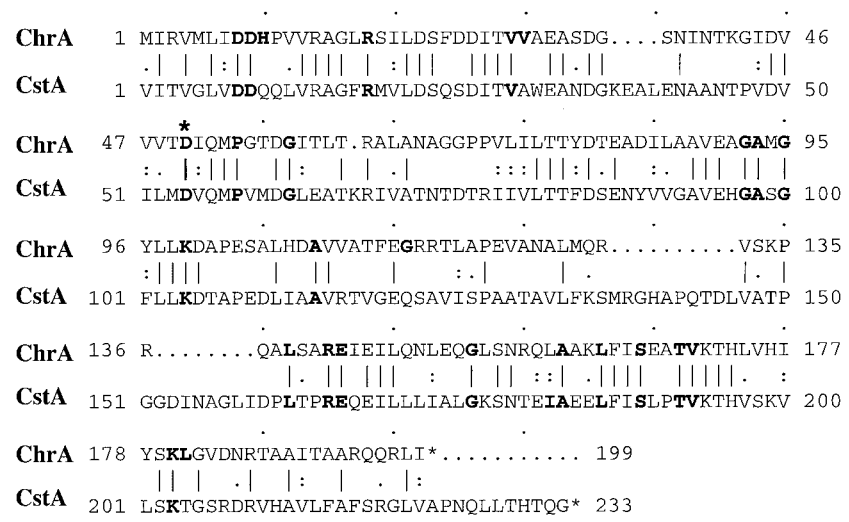

FIG. 4. Amino acid sequence alignment of ChrA and CstA. Amino acid residues that are highly conserved among other response regulators within the NarP and NarL family (1) are shown in boldface. The conserved Asp (D) residues marked with an asterisk (D50 in ChrA and D54 in CstA) are known to be the sites of phosphorylation in other response regulators.

pTSB5, or pTSB2-5 was not affected by the addition of heme to the medium even in the presence of the plasmid pSHU9 (28), which encodes the *S. dysenteriae* heme transport system and enables DH5α to transport heme (Table 1).

Sequence analysis of the activator genes on pTSB2 and pTSB5. To identify the genes present on pTSB2 and pTSB5 that are required for activation of the *hmuO* promoter, the DNA sequences of the 1.7-kb *Bam*HI-*Bgl*II insert on pTSB2.Bg and the 1.2-kb left end region of plasmid pTSB5.R (Fig. 2A and Fig. 3A, respectively) were determined. DNA sequence analysis indicated that a single open reading frame was present in the *Kpn*I-*Bgl*II fragment from pTSB2.Bg (the smallest subcloned region that maintains activation). This open reading frame was designated *chrA* (*Corynebacterium* heme-responsive activator) (Fig. 2B) and is predicted to encode a product of 199 amino acids that has significant homology to response regulators of two-component signal transduction systems. Immediately upstream from *chrA* is a partial open reading frame for a gene that is designated *chrS* (*Corynebacterium* heme-responsive sensor), whose product has homology to the sensor kinase component of two-component signal transduction systems (Fig. 2B). The *chrS* open reading frame is predicted to encode a product containing 340 amino acids; however, the 5′ portion of the *chrS* coding region is not present on pTSB2 or the other two related clones, pTSB1 and pTSB3. The *chrS* termination codon TGA overlaps the ATG start codon for *chrA*, suggesting that the genes are organized as an operon similar to other genes encoding two-component systems. Since *chrA* is the only complete gene present on the *Kpn*I-*Bgl*II fragment on plasmid pTSB2.K-Bg, this suggests that only the response regulator is needed for activation of the *hmuO* promoter in *E. coli* and this effect is observed only when the IPTG-inducible *trc* promoter is active.

Sequence analysis of the 1.2-kb region of pTSB5.R revealed that a single open reading frame was present on the 1-kb *Nru*I fragment, and this open reading frame, designated *cstA* for *Corynebacterium* signal transduction activator, also has significant homology to response regulators of two-component systems (Fig. 3B). Upstream from *cstA* is a partial open reading frame for a gene designated *cstS*, which is predicted to encode the C-terminal 137 amino acids of a sensor kinase. The predicted amino acid sequences of CstA and ChrA show the highest homology to proteins in the NarL and NarP family of response regulators (both ChrA and CstA show homologies to proteins in this family that range from 30 to 40% amino acid identity over the entire length of the protein). Proteins in this family of response regulators are known to bind DNA and function as transcriptional activators (32). ChrA and CstA are greater than 40% identical to each other at the amino acid level and share numerous residues that are conserved among all response regulators within the NarL and NarP family (Fig. 4) (1). The conserved amino acids include an aspartate residue (Asp50 for ChrA and Asp54 for CstA), which is the site of phosphorylation in other response regulators (32).

Cloning and sequence analysis of the sensor kinase homologs encoded by *chrS* and *cstS*. Although the *chrA* and *cstA* genes were able to activate the expression of the *hmuO* promoter in *E. coli*, the heme-dependent activation of *hmuO* observed in *C. diphtheriae* could not be reconstituted in *E. coli* with either of these genes. To determine if the sensor kinase genes, *chrS* and *cstS*, are needed for heme-dependent activation in *E. coli*, the 5′ portions of these genes along with upstream regions were cloned from the chromosome of *C. diphtheriae*. A 1.5-kb *Hin*dIII fragment, which carries the 5′ region of the *chrS* gene, was used to construct the plasmid pTSB20, which contains the complete coding region for *chrS* and *chrA* (Fig. 2C). Similarly, a 1.8-kb *Eco*RV fragment that was cloned from the chromosome of *C. diphtheriae* was used to construct the plasmid pTSB50, which contains the complete coding region for *cstS* and *cstA* (Fig. 3C). The plasmids pTSB20 and pTSB50 contain the pSC101 origin of replication and replicate at a low copy number in *E. coli*. Low-copy-number plasmids were used in the analysis of the *chrS* and *cstS* genes since the predicted products of these genes are presumed to be membrane associated and could be deleterious at high levels.

Sequence analysis of the *chrS* and *cstS* genes indicated that they are predicted to encode proteins of 417 and 408 amino acids, respectively. Both proteins showed the highest homology (approximately 30% identity) in their C-terminal halves to the UhpB and DegS sensor kinases (data not shown). The putative sensor domain of CstS, located at the N-terminal region, had no significant homology with any proteins in the GenBank

TABLE 2. Effects of sensor and/or activator clones on the expression of P*hmuO-lac* fusions in the presence and absence of heme in DH5α

Plasmids	Heme[a]	LacZ U[b]
pTSB20, pCPO-1 (P*hmuO-lac*)	−	5.3
pTSB20, pCPO-1	+	6.7
pTSB20, pCPO-1, pSHU9 (He^+)[c]	+	102.5
pTSB20, pCPO-1, pSHU9 (He^+)	−	4.6
pTSB20, pCPO-4, pSHU9 (He^+)	+	52.3
pTSB20, pCPO-4, pSHU9 (He^+)	−	7.3
pTSB20, pCPO-5, pSHU9 (He^+)	+	3.4
pTSB20, pCPO-5, pSHU9 (He^+)	−	0.8
pTSB50, pCPO-1, pSHU9 (He^+)	+	1.7
pTSB50, pCPO-1, pSHU9 (He^+)	−	1.3

[a] Hemin was added to cultures (+) at 100 μg/ml.
[b] LacZ units (U) were determined by the method of Miller (27). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean.
[c] Plasmid pSHU9 (He^+) enables DH5α to transport heme.

database. The N-terminal sensor domain of ChrS had 28% identity with the N-terminal region of the SenR protein from *Streptomyces reticuli* (GenBank accession no. Y14336). The ChrS and SenR proteins are 36% identical at the amino acid level over their entire sequences. A specific function for SenR has not been reported, although it is proposed to function as the sensor component in a two-component system. Analysis of the amino acid sequences for both ChrS and CstS, using the TMpred program, predicts both proteins to have multiple transmembrane helices in their N-terminal 200 amino acids (data not shown).

Effect of *chr* and *cst* operons on the expression of the *hmuO* promoter. Plasmid pTSB20 (*chrS*$^+$, *chrA*$^+$) and plasmid pTSB50 (*cstS*$^+$, *cstA*$^+$) were transformed into DH5α carrying pCPO-1 to determine what effect these genes had on the expression of the P*hmuO-lac* fusions in the presence and absence of heme. Plasmid pTSB20 in DH5α carrying pCPO-1 showed relatively low LacZ activity regardless of the presence heme (Table 2). However, when pSHU9, which carries a heme transport system, was moved into this strain, greater than 20-fold induction was seen in the presence of heme (the LacZ activity was 4.6 U in the absence of heme and 102.5 U in the presence of heme [Table 2]). This high level of heme induction was dependent on the presence of a functional heme transport system supplied by the pSHU9 plasmid. Heme activation in *E. coli* also required the presence of both the *chrS* and *chrA* genes, since clones (constructed on the same low-copy-number plasmids) containing only the *chrA* gene did not exhibit heme induction (data not shown). Furthermore, the presence of pTSB20 also conferred heme induction on the P*hmuO-lac* fusion on pCPO-4, but only very low LacZ activity was seen with pCPO-5 (Table 2). The relative levels of heme-induced expression from the P*hmuO-lac* fusions on pCPO-1, pCPO-4, and pCPO-5 by pTSB20 (*chrA*$^+$, *chrS*$^+$) in *E. coli* are similar to those observed for the same P*hmuO-lac* fusions in *C. diphtheriae* (Fig. 1). The plasmid pTSB50 transformed into DH5α carrying pCPO-1 and pSHU9 showed only a low level of LacZ activity that was not affected by the presence of heme (Table 2). Plasmid pTSB20-50, which contained the inserts of both pTSB20 and pTSB50 on the same low-copy-number plasmid, showed a level of heme-induced LacZ activity in DH5α carrying pCPO-1 and pSHU9 (96 U) that was similar to that observed for pTSB20.

Effect of the *chrAD50N* mutant allele on expression of the *hmuO* promoter. Site-directed mutagenesis was used to introduce a point mutation into the *chrA* gene which resulted in the replacement of the aspartate residue at position 50 with an asparagine. Based on sequence homologies with other response regulators, the Asp50 residue in ChrA is the presumed site of phosphorylation by its cognate sensor kinase ChrS. The plasmid pWBH20-D50N, which carries both the mutant *chrA* allele (*chrAD50N*) and the wild-type *chrS* gene on the low-copy-number vector pWKS30, was examined to determine the effect of the *chrAD50N* mutation on the heme-dependent activation of the *hmuO* promoter. DH5α carrying both pCPO-1 and pWBH20-D50N showed only low LacZ activity and very weak heme-dependent activation of the P*hmuO-lac* fusion present on pCPO-1 (Table 3). Plasmid pWBH20, which carries the wild-type copies of the *chrA* and *chrS* genes on pWKS30, showed levels of heme-dependent activation of the P*hmuO-lac* fusion that were greater than 18-fold higher than those observed for the *chrAD50N* mutant allele (the LacZ activities were 66.4 and 3.6 U, respectively [Table 3]). These results indicated that replacement of the Asp residue at position 50 with an Asn in the *chrAD50N* gene product virtually abolished all heme-responsive activation when *chrAD50N* was expressed from a low-copy-number plasmid.

However, when the *chrAD50N* allele was present on the higher-copy-number vector p*Trc99A* (pPBH2-D50N) and expressed from the strong IPTG-inducible *trc* promoter, the *chrAD50N* gene product exhibited a capacity to activate the *hmuO* promoter that was only twofold lower than the activation observed for the wild-type *chrA* gene on plasmid pPBH2 (the activities were 32.4 and 61.8 U, respectively [Table 3]). Activation of the P*hmuO-lac* fusion by both the wild-type *chrA* gene and the *chrAD50N* allele required the presence of IPTG, and this activation occurred in the absence of the *chrS* gene, since *chrS* is not present on the plasmid pPBH2-D50N or pPBH2. This finding indicates that the *chrAD50N* gene product has the capability to activate transcription, although at reduced levels relative to those induced by the wild-type gene product, and that the Asp50 residue is not essential for transcriptional activation if the gene is expressed at high levels.

TABLE 3. Effect of the *chrAD50N* mutation on the expression of a P*hmuO-lac* fusion in DH5α

Plasmids	Heme[a]	IPTG	LacZ U[b]
pWBH20-D50N, pCPO-1, pSHU9	−	−	1.1
pWBH20-D50N, pCPO-1, pSHU9	+	−	3.6
pWBH20, pCPO-1, pSHU9[c]	−	−	8.1
pWBH20, pCPO-1, pSHU9	+	−	66.4
pPBH2-D50N, pCPO-1	−	−	1.4
pPBH2-D50N, pCPO-1	−	+	32.4
pPBH2, pCPO-1	−	−	1.5
pPBH2, pCPO-1	−	+	61.8

[a] Hemin was added to cultures (+) at 100 μg/ml.
[b] LacZ units (U) were determined by the method of Miller (27). Values are means of three independent experiments, and standard deviations did not vary by greater than 15% from the mean.
[c] Plasmid pSHU9 enables DH5α to transport heme.

DISCUSSION

Bacteria utilize a variety of mechanisms to adapt to and interact with the environment, including the well-characterized two-component signal transduction systems (for a review see reference 32). Two-component regulatory systems typically consist of a sensor protein that monitors the environment and a cognate response regulator which is involved in controlling gene expression. In this study, the mechanism involved in the heme-responsive activation of the *hmuO* promoter has been reconstituted in *E. coli*. The genes, *chrS* and *chrA*, that encode homologs of two-component signal transduction systems were shown to activate the expression of a P*hmuO-lac* fusion construct in *E. coli*. The activation required heme or hemoglobin in the growth medium and a functional heme transport system that was provided in *trans*. Although this system has been reconstituted in an *E. coli* background, it very closely mimics the heme-dependent activation observed in *C. diphtheriae*, which strongly suggests that *chrS* and *chrA* are the relevant activators in *C. diphtheriae*.

The *hmuO* gene is the only bacterial gene whose expression is known to be activated by heme, and this is the first report in which heme has been identified as the environmental stimulus for a two-component regulatory system. Relatively little is known about heme-regulated gene expression in other bacterial systems. Numerous bacterial pathogens can transport and utilize heme as an iron source, and most of the genes that encode products mediating these heme transport functions are regulated by iron (22, 30, 51). However, the expression of certain proteins involved in the transport of heme and iron by pathogenic species of *Haemophilus* (8, 19, 29) and *Porphyromonas* (9, 20) has been shown to be heme repressible. The mechanisms involved in the regulation of genes encoding heme-repressed proteins in these organisms have not been described.

Sequences upstream from the *hmuO* promoter were shown to be important for heme-dependent transcription both in *C. diphtheriae* and in *E. coli*. The P*hmuO-lac* fusion on plasmid pCPO-3, which contained only 50 bp of *C. diphtheriae*-specific sequences upstream of the −35 sequence (which is 90 bp upstream from the start of transcription [37]), was fully induced by heme. The P*hmuO-lac* fusions on pCPO-4 and pCPO-5, which had fewer upstream sequences than pCPO-3, showed significantly reduced heme induction. These findings indicate that sequences within the 90-bp region upstream from the start site of transcription from the *hmuO* promoter, and most likely upstream of the −35 element, are required for heme induction and may contain the binding site for the response regulator encoded by the *chrA* gene. In support of this possibility, the related response regulators, NarP, NarL, and UhpA, bind to sequences within 80 bp of the transcriptional start sites of some of the genes that they regulate (6, 7).

The activator clones pTSB2 and pTSB5, which contain the *chrA* and *cstA* genes, respectively, were isolated from a plasmid library due to their ability to activate the expression of the P*hmuO-lac* fusion on pCPO-1 in *E. coli*. The cloned *chrA* and *cstA* genes in *E. coli* are able to activate the transcription of the *hmuO* promoter in the absence of their cognate sensor kinase genes, *chrS* and *cstS*, respectively. Several factors may contribute to the capacity of the *chrA* and *cstA* genes to activate transcription in the absence of their cognate sensor kinases. These include (i) low-level phosphorylation of ChrA and CstA by endogenous nonspecific kinases in *E. coli*, (ii) cross talk with other two-component systems, and (iii) overexpression of the products of the *chrA* and *cstA* genes due to the presence of these genes on high-copy-number plasmids. The expression of these genes is further enhanced since they are under the transcriptional control of the IPTG-induced *trc* promoter. Other investigators have made observations similar to those described here showing that multicopy plasmids carrying genes encoding response regulators are able to activate transcription in the absence of their cognate sensor kinases (11, 48).

The deletion constructs pTSB2.H-Bg and pTSB2.K-Bg, which carry the *chrA* gene and a portion of the 3′ region of *chrS*, exhibited an enhanced activity relative to the activity of the parent clone, pTSB2 (Fig. 2A). This enhanced activity may be due to the more proximal location of the *chrA* gene to the *trc* promoter or to the deletion of sequences containing the *chrS* gene. The truncated *chrS* gene present on either pTSB2.H-Bg or pTSB2.K-Bg lacks the codon for the conserved histidine (H215), which is proposed to be the site of phosphorylation and is predicted to be essential for the activities of sensor kinases. The truncated *chrS* gene on the parent clone pTSB2 contains this conserved histidine (H215)-encoding codon, and it is possible that any putative peptide produced from this truncated *chrS* gene (either initiating from a weak internal start codon or present as part of a translational fusion with upstream vector sequences) could potentially have activity. Since sensor kinases are known to have both positive and negative effects on the activities of their cognate response regulators (32), it is possible that a truncated ChrS product produced from pTSB2 may exert a repressor effect on the ChrA activator. However, when the conserved histidine codon is removed from the *chrS* sequence, as in the two-deletion constructs, the repressor effect is alleviated and enhanced expression is observed.

The *chrAD50N* mutant allele, when expressed at high levels, was also able to activate transcription of the *hmuO* promoter in the absence of its cognate sensor kinase. Similarly, other investigators have shown that a mutation in the gene encoding the *E. coli* UhpA response regulator, in which the Asp54 residue (the site of phosphorylation by its cognate sensor kinase) is replaced with an Asn, results in the loss of all activity when the gene is present in low copy number but that transcriptional activity is maintained when the gene is expressed from a multicopy plasmid (48). The presumed DNA binding domain for ChrA, based on sequence homologies with other response regulators in the NarP and NarL family, is predicted to be located in the C-terminal portion of the protein and would not be directly affected by the D50N mutation. The results obtained with the ChrAD50N and UhpAD54N response regulators indicate that phosphorylation at the conserved Asp residue is not essential for activating transcription when these proteins are strongly expressed.

The findings in this study indicate that the two genes *chrA* and *cstA* are able to activate expression at the *hmuO* promoter. Additionally, both genes showed similar levels of activation when expressed in the various promoter deletion constructs (Table 1), which suggests that the products of *chrA* and *cstA* may have DNA binding sites that are very near each other. Plasmid pTSB2-5, which carries both the *chrA* and *cstA* genes under control of the *trc* promoter, activates transcription of the P*hmuO-lac* fusion on pCPO-1 to levels greater than 25-fold higher than those produced by either pTSB2 or pTSB5, each of which carries only one of the activators (Table 1). Evaluation of the results for the tandem construct is difficult since protein levels are not known. While it is clear that additional studies are required to fully understand the mechanism of this enhanced activity of the *chrA* and *cstA* genes on pTSB2-5, one possible mechanism by which the expression may be increased could involve the formation of mixed dimers or multimers of the ChrA and CstA proteins. Since the DNA binding sites of ChrA and CstA may be close to each other, the presence of

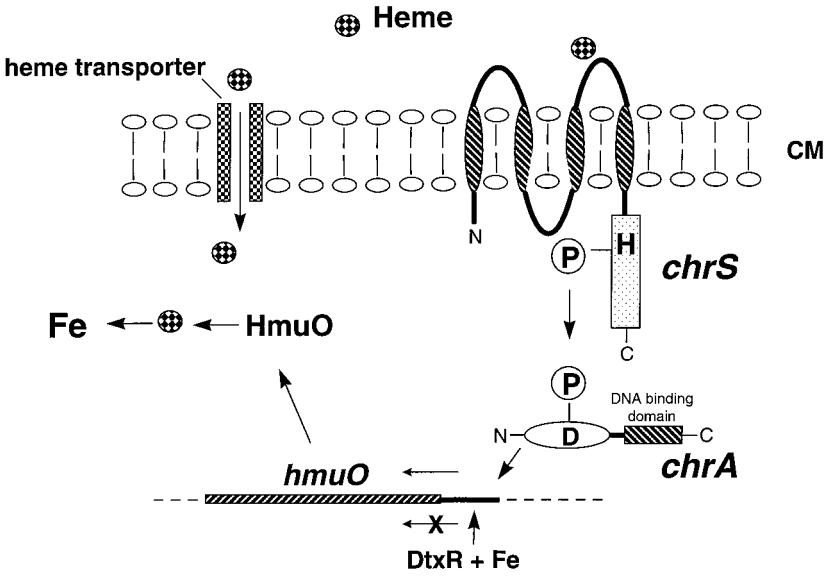

FIG. 5. Proposed mechanism of heme-responsive activation at the *hmuO* promoter in *C. diphtheriae*. The sensor kinase ChrS is proposed to detect extracellular heme at its N-terminal sensor domain, which is predicted to contain at least two transmembrane helices (indicated by striped ovals) and two extracellular loop regions. The detection of heme by ChrS is proposed to result in autophosphorylation at a conserved histidine (H, H215) that is located in the cytosolic kinase domain of ChrS (boxed region). The phosphoryl group (indicated by a circled P) is then transferred to a conserved Asp residue (D, D50) on ChrA. Phosphorylation is proposed to activate the DNA binding ability of ChrA and allows ChrA to bind upstream of the *hmuO* promoter and activate transcription. Transcription of the *hmuO* promoter can also be repressed by DtxR during growth in the presence of iron. The *hmuO* gene, therefore, is optimally expressed in low-iron environments in the presence of heme. Additionally, a *C. diphtheriae* heme-specific transporter has been proposed to be involved in the transport of heme into the cytosol (7a), where the HmuO protein is proposed to degrade the cytosolic heme and liberate the heme-bound iron.

mixed multimers may result in either greater stability of a protein-DNA complex or an alteration of the conformation of the proteins so that there exists a more optimal interaction with RNA polymerase, which results in the enhanced transcription.

The presence of both response regulators, *chrA* and *cstA*, and their cognate sensors, *chrS* and *cstS*, together on plasmid pTSB20-50 did not result in a synergistic effect similar to that seen for pTSB2-5, which contained only the response regulator genes. Since the presence of *cstA* and *cstS* on the same plasmid, pTSB50, failed to activate the expression of the *hmuO* promoter, it is unclear what role, if any, the *cstA* and *cstS* genes have in the regulation of the *hmuO* promoter in *C. diphtheriae*. Since *cstA* did not activate the *hmuO* promoter in the presence of the *cstS* gene but did in the absence of *cstS*, it is possible that the product of *cstS* may repress the activity of *cstA*. It is possible that an environmental factor other than heme may be required for the *cstA-cstS* system to activate transcription at the *hmuO* promoter. Additional studies are needed to define the role of the *cstA* and *cstS* genes in *C. diphtheriae*.

It is clear from the results of this study that heme-dependent activation of the *hmuO* promoter in *E. coli* requires the presence of both the *chrA* and *chrS* genes and a functional heme transport system, which serves to transport heme through the outer membrane. The evidence strongly suggests that the putative sensor kinase encoded by *chrS* is involved in the detection of heme, which is presumed to be the environmental signal. Alternatively, it is possible that the actual environmental stimulus is not heme but is a factor that is produced in response to the presence of heme in the medium. While additional studies are required to determine the mechanism by which ChrS detects heme (or other signals), it is likely that sequences in the N-terminal portion or sensor domain of ChrS are involved in the detection of the environmental stimulus. Amino acid sequence analysis using the TMpred program predicts that there are at least four transmembrane helices in the N-terminal 180 amino acids of ChrS. Extracytoplasmic loop regions between transmembrane helices have been proposed to be involved in the detection of environmental stimuli by other sensor kinases (4, 32), and it is plausible that regions between the putative membrane-spanning regions in ChrS may have a role in the detection of heme. In the reconstituted system in *E. coli*, it is proposed that the ChrS protein resides in the cytoplasmic membrane and that the extracytoplasmic loop regions are involved in the detection of heme that has been transported into the periplasm by means of the heme transport system encoded by the genes present on the plasmid pSHU9. In the gram-positive organism *C. diphtheriae*, ChrS is also predicted to reside in the cytoplasmic membrane; however, the extracytoplasmic loop regions would be involved in detecting extracellular heme. If the activity of ChrS is like that of other related sensor kinases, it is presumed that detection of an environmental stimulus by ChrS should result in autophosphorylation at the conserved histidine residue, H215. The phosphoryl group on H215 could then be transferred to the conserved Asp residue (D50) on ChrA, which would allow ChrA to activate transcription at the *hmuO* promoter. The findings from this study indicate that the D50 residue in ChrA is needed for heme-responsive activation of the *hmuO* promoter, since the ChrAD50N protein had little if any capacity to activate the *hmuO* promoter in the presence of heme (Table 3). This observation supports the sequence homology data which predicts that the D50 residue of ChrA functions as the site of phosphorylation by ChrS and, therefore, should have a direct role in a heme-dependent signal transduction mechanism. A model depicting how this phosphotransfer signaling mechanism may function to control *hmuO* transcription in *C. diphtheriae* is presented in Fig. 5.

While the construction of defined mutations in the *chrS* and/or *chrA* genes in the chromosome of *C. diphtheriae* should provide additional evidence for the function of these genes in *C. diphtheriae*, the capability to perform allelic replacement or transposon mutagenesis in *C. diphtheriae* is not yet available due to the lack of genetic tools. The findings in this study expand our knowledge as to the variety of environmental factors that can function as stimuli for two-component systems. The regulatory systems reported in this study are the first two-component signal transduction systems described for the genus *Corynebacterium*, and future research will focus on identifying additional genes controlled by these regulatory systems and the characterization of functional domains present in these regulatory proteins.

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