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Cloning of a *Corynebacterium diphtheriae* Iron-Repressible Gene That Shares Sequence Homology with the AhpC Subunit of Alkyl Hydroperoxide Reductase of *Salmonella typhimurium*

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To understand how *Corynebacterium diphtheriae* responds to iron limitation, we compared the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of both wild-type cells and iron uptake mutants grown in either high- or low-iron medium. The removal of iron by ethylene diamine di-(*o*-hydroxyphenyl acetic acid) from the growth medium of wild-type cells resulted in induction of at least 14 polypeptides. DirA, a major iron-repressible polypeptide, was purified from wild-type cells by preparative SDS-PAGE, and the *dirA* structural gene was isolated from a genomic library of nontoxigenic *C. diphtheriae*. The nucleotide sequence of *dirA* was determined, and the deduced amino acid sequence of DirA revealed strong homologies with the AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase and polypeptides of other microorganisms associated with oxidation reduction activity. Like AhpC, cloned DirA reduced the susceptibility of an *Escherichia coli ahp* mutant to cumene hydroperoxide, suggesting that DirA has alkyl hydroperoxide reductase activity.

Iron plays an essential role in the growth of almost all organisms as a cofactor for enzymes or as an integral part of cytochromes in oxidation reduction and energy generating systems. However, its profound insolubility and tendency to associate with iron-binding proteins under physiological conditions reduce the availability of free iron for the growth of bacteria in infected animals. In response to iron limitation, bacterial pathogens have evolved various survival strategies, such as induction of a high-affinity iron uptake system to scavenge iron or an increase in toxin production to damage host tissue.

For decades, it has been known that the production of diphtheria toxin (DT) by *Corynebacterium diphtheriae* is maximal when cells are infected with toxigenic phages and grown under iron-limited conditions (11, 15, 17, 30). The molecular mechanism regulating DT synthesis was not clear until recently (2, 22, 23, 27). The genetic determinant for DT, *tox*, is located on the phage genome, and its expression is controlled by a host repressor protein, DtxR, in an iron-dependent fashion. After its association with iron, DtxR binds to a 9-bp inverted repeat located in the −10 region of *tox* and turns off transcription. Iron-dependent regulation of the phage *tox* gene by bacterial DtxR led us to study the cellular response of *C. diphtheriae* to iron limitation.

Induction of a high-affinity iron uptake system is a major low-iron response. Our knowledge of iron metabolism is mainly derived from studies of gram-negative bacteria, especially *Escherichia coli* (for recent reviews, see references 1, 5, and 9). Like *E. coli*, *C. diphtheriae* produces high-affinity iron-chelating siderophores which stimulate the growth of bacteria under iron-limited conditions (18, 19). Though how *C. diphtheriae* acquires iron is still unknown, a working model for coordinate synthesis of DT and siderophore has been proposed and indirectly supported by analysis of a *C. diphtheriae* mutant, C7(β)hm723, which can produce both DT and siderophore under iron-rich conditions (26). Further studies have demonstrated that this mutant has a point mutation at the *dtxR* allele (3, 21). The introduction of plasmid-encoded wild-type DtxR into C7(β)hm723 restores regulation of both DT synthesis and siderophore synthesis by iron (22). The role of DtxR in the *C. diphtheriae* iron acquisition system was supported by recent identification of two DtxR-regulated open reading frames (ORFs) and by sequence similarity at the amino acid level between one of these ORFs and iron acquisition genes, FecB, FcpB, and FhuD of *E. coli* and FhuD of *Bacillus subtilis* (24).

In this communication, we studied the low-iron response of *C. diphtheriae* by examining the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of wild-type cells and a set of iron uptake mutants grown under either high- or low-iron conditions. In addition, we cloned and determined the nucleotide sequence for a diphtherial iron-repressible gene, *dirA*, from wild-type cells. A search of the GenBank database revealed that DirA had strong homologies with a family of polypeptides, including the AhpC subunit of *Salmonella typhimurium* alkyl hydroperoxide reductase. Like AhpC, cloned DirA reduced the susceptibility of an *E. coli ahp* mutant to cumene hydroperoxide.

MATERIALS AND METHODS

Strains, media, and growth conditions. Wild-type *C. diphtheriae* C7, lysogen C7(β), and iron uptake mutants HC1, HC3, HC4, HC5, and C7(β)hm723 were from our stock collections, originally obtained from Randall Holmes and stored in 10% glycerol at −70°C (18, 26). *C. diphtheriae* cells used in this study were cultured at 37°C in PGT-maltose medium (11) supplemented with either 400 μmol of ethylene diamine di-(*o*-hydroxyphenyl acetic acid) (EDDA; low-iron medium) (Sigma, St. Louis, Mo.) or 36 μmol of added Fe^{3+} per ml (high-iron medium). *E. coli* DH5α was used as the host for plasmid cloning, and strain Y1090 was used in construction and screening of the *C. diphtheriae* genomic

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pST348	pGEM7f(−) containing a 4.8-kb *Eco*RI fragment from λgt11 *dirA*	This study
pST419	Derivative of pST438; pGEM7f(−) carrying the coding sequence of DirA on a 2.2-kb *Cla*I-*Bam*HI fragment	This study
pST435	Derivative of pST419; pGEM7f(−) carrying the coding sequence of DirA on a 1.2-kb *Bal*I-*Bam*HI fragment	This study
pST446	pACYC184 carrying the coding sequence of DirA on a 2.2-kb *Cla*I-*Bam*HI fragment	This study
pAQ27	pUC18 carrying the entire *ahp* operon on a 4.1-kb *Bgl*II-*Kpn*I fragment	28
pGS01	Derivative of pAQ27; the *ahpC* gene was eliminated and expression of AhpF protein was due to transcription from other promoters of pUC18	G. Storz
pST449	Derivative of pAQ27; the *Sma*I-*Eco*RV fragment of pAQ27 was removed to abolish the activity of AhpF and expression of *ahpC* was driven from its own promoter	This study

library. Strain TA4315, with the entire *ahp* locus deleted, was kindly provided by Gisela Storz and used to study the function of cloned DirA (25). All *E. coli* cells were cultured in Luria-Bertani broth at either 30, 37, or 42°C, as indicated. The following supplements were added to growth medium as needed: ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 10 mM). Plasticware was used in the cultivation of low-iron cells. The plasmids used in this study are listed in Table 1. Plasmids pAQ27 and pGS01 were provided by G. Storz.

Preparation of crude cell lysates of *C. diphtheriae*. The removal of iron from medium by EDDA restricted the growth of *C. diphtheriae*. To increase the yield of proteins for analysis, cell lysates of *C. diphtheriae* were prepared as follows. An aliquot (0.2 ml) of an overnight *C. diphtheriae* culture was transferred into 30 ml of fresh PGT-maltose medium and grown with shaking at 37°C to early log phase (approximately 40 Klett units). Then the culture was split into two parts (20 and 10 ml) and harvested by centrifugation at 10,000 × *g* for 10 min. The former was resuspended in 20 ml of low-iron medium, and the latter was suspended in 10 ml of high-iron medium. High-iron cell cultures were incubated for another 3 h, and low-iron cultures were incubated for 6 h before being harvested. Cells were washed, suspended in 1/10 volume of 50 mM Tris-chloride buffer (pH 8.0) containing 1 mM EDTA and 50 μM dithiothretol, and disrupted with a Mini-Bead Beater (BioSpec, Bartlesville, Okla.) for 3 min. After cell debris and unbroken cells had been cleared by centrifugation at 8,000 × *g* for 10 min, supernatants were collected and used as crude cell lysates of *C. diphtheriae.*

Isolation of DirA and antibody preparation. The cell lysate of *C. diphtheriae* grown in low-iron medium was electrophoretically separated on a preparative gel containing SDS and a 7 to 15% gradient of polyacrylamide. The Coomassie blue-stained band with an apparent molecular mass of 22 kDa was excised and further purified by SDS-PAGE on an 18% polyacrylamide gel. The purified protein, named DirA, was excised, electroeluted from the gel, and concentrated on a Centricon apparatus with a YM-10 membrane (Amicon, Beverly, Mass.). The partial amino acid sequence of DirA was determined with an automatic sequencer. Polyclonal antibodies for DirA were prepared in rabbits.

Construction and screening of the genomic library of *C. diphtheriae*. Our interest was focused on the iron-repressible polypeptides of *C. diphtheriae*. The major concerns in cloning genes for these polypeptides in *E. coli* were uncertainties as to whether these genes would be transcribed from their own promoters and if they were expressed, whether these polypeptides would be lethal to the *E. coli* host. To circumvent these uncertainties, we constructed a genomic library of nontoxigenic *C. diphtheriae* C7 by using phage expression vector λgt11 (12). Briefly, chromosomal DNAs were sheared to between 3 and 8 kb by repeated passages through a 30-gauge needle. The endogenous *Eco*RI sites on genomic DNA fragments were modified by *Eco*RI methylase. The sheared ends of DNA fragments were made flush with T4 DNA polymerase and ligated with *Eco*RI linker. After digestion with *Eco*RI, processed DNAs were ligated into *Eco*RI-digested, dephosphorylated λgt11 DNA arms and packaged into phage particles with an in vitro packaging kit (Promega). All packaged phage particles were used to infect *E. coli* Y1090 cells and were plated on solid Luria-Bertani media. The plaques on all of these plates were pooled and used as the genomic library of *C. diphtheriae*. The cloning efficiency of this genomic library was approximately 88%, as estimated by the ratio of white plaques to total plaques on plates containing IPTG and X-Gal.

Screening of the *C. diphtheriae* genomic library with polyclonal anti-DirA antibodies was performed as described previously (12, 32). Positive clones were identified by an alkaline phosphatase-based color developing system, with naphthol biphosphate and fast blue as substrates. The lysogen of the positive recombinant phage clone was constructed in *E. coli* Y1090 by standard techniques. The lysogenization of phages was confirmed by reisolating phages by thermal induction at 42°C.

Genetic and biochemical techniques. All recombinant DNA procedures were carried out by standard methods. Restriction fragments were purified from the excised agarose gel with a GeneClean II kit (Bio 101, La Jolla, Calif.). The nucleotide sequence of *dirA* was determined by the chain termination method, with synthetic oligonucleotides as primers (20). The protein concentrations of samples were quantitated by Coomassie blue binding assays, with bovine serum albumin as the standard (4). Protein profiles were analyzed by SDS-PAGE (7 to 15% gradient of polyacrylamide) with a Laemmli buffer system (14). Immunoblot analysis was performed and visualized with an alkaline phosphatase-based color developing system. The susceptibilities of cells to cumene hydroperoxide were measured by disc inhibition assay, as described previously, except that 2 μl of 5% cumene hydroperoxide was used (25).

Nucleotide sequence accession number. The GenBank database accession number for *dirA* is U18620.

RESULTS

Identification of iron-repressible polypeptides in *C. diphtheriae*. To study the cellular response of *C. diphtheriae* to iron limitation, we compared the SDS-PAGE protein profiles of both wild-type cells and iron uptake mutants grown in either high- or low-iron medium. The removal of iron by EDDA from the growth medium of wild-type C7 cells increased the levels of at least 14 polypeptides. The accumulation of these polypeptides was reduced when ferric iron was added to EDDA-containing medium (Fig. 1). The lysogenization of *C. diphtheriae* with toxigenic phage β had no significant effect on the differences in the protein profiles of high- and low-iron cells. The SDS-PAGE protein profiles of iron uptake mutants grown in either high- or low-iron medium were different from those of wild-type cells. The results are summarized in Table 2. Mutant cells were unable to produce these 14 iron-repressible polypeptides at the same levels as wild-type cells did. The most dramatic differences were observed for the patterns of five polypeptides. Four of them had apparent molecular masses that ranged from 22 to 18 kDa, and the molecular mass of the fifth was 64 kDa. The levels of all five of these polypeptides in mutants HC3 and HC5, those of the 22-, 19-, and 17-kDa polypeptides in mutants HC1 and C7(β)hm723, and that of the 64-kDa polypeptide in mutant HC4 were significantly lower than they were in wild-type cells.

Characteristics of DirA. To investigate the function of these iron-repressible polypeptides, we chose to focus on the 22-kDa species (DirA). It was a major iron-repressible polypeptide of wild-type cells but was not produced at an elevated level by iron uptake mutants grown under low-iron conditions. We purified DirA by two consecutive preparative SDS-PAGE runs, determined the partial amino acid sequence, and prepared polyclonal antibodies for this polypeptide. The purified polypeptide exhibited a single Coomassie blue staining band on gels and reacted with prepared antibodies. The sequence of the first 30 amino acids of purified DirA was found to be SerIleLeuThrValGlyGluLysPheProGluPheAsnLeuThrAla LeuLysGlyGlyAspLeuHisAspValAsnAlaSerGlnPro.

Cloning and expression of *dirA* in *E. coli*. Anti-DirA antisera did not cross-react with proteins in *E. coli* Y1090 and were used directly in screening the genomic library of *C. diphtheriae* without further purification. After we had initially screened

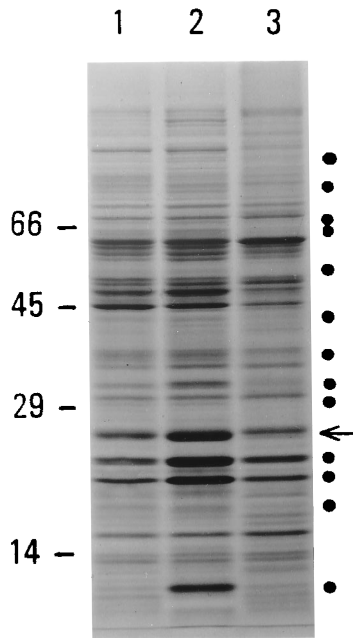

FIG. 1. Effects of iron on the protein profile of wild-type *C. diphtheriae* C7 cells. Crude lysates of cells cultured in three media were prepared and analyzed by SDS-PAGE. An equal amount of protein (30 μg) was loaded in each lane. Lane 1, medium containing both EDDA and ferric iron; lane 2, medium containing EDDA only; lane 3, medium containing ferric iron only. Dots indicate the positions of 13 of 14 iron-repressible polypeptides (excluding DirA), and an arrow indicates the position of DirA. Molecular mass markers (in kilodaltons) are given on the left.

approximately 60,000 recombinant λgt11 phages, we identified five positive clones. The crude lysate for a lysogen of these clones was analyzed by immunoblot. A polypeptide with an apparent molecular mass of 22 kDa was shown to cross-react with anti-DirA polyclonal antibodies. The addition of IPTG to the growth medium of the lysogen had no effect on production of cloned DirA. These results indicated that expression of cloned *dirA* in *E. coli* was driven from its own promoter and that cloned DirA was apparently identical in molecular size to DirA produced by *C. diphtheriae.*

Sequence analysis of *dirA*. The 4.8-kb *Eco*RI DNA insert in the positive recombinant phage was isolated and cloned into plasmid vector pGEM7f(−), and the coding region for *dirA* was determined by sequential deletion of DNA fragments from both ends of the insert in conjunction with immunoblot anal-

TABLE 2. Synthesis of iron-repressible polypeptides in iron uptake mutants of *C. diphtheriae*[a]

Strain	Synthesis of polypeptide species (molecular mass [kDa])[b]													
	99	90	74	64	53	44	38	32	28	22	20	19	18	15
HC1	S	S	S	S	S	S	S	S	S	R	S	R	R	S
HC3	S	S	S	R	R	S	S	S	R	R	R	R	R	S
HC4	S	S	S	R	S	S	S	S	S	S	S	S	S	S
HC5	S	S	S	R	S	S	S	S	S	R	R	R	R	S
C7(β)hm723	S	S	S	S	S	S	S	S	S	R	S	R	R	S

[a] The protein profiles of wild-type *C. diphtheriae* and iron uptake mutants grown in either high- or low-iron medium were analyzed by SDS-PAGE on a 7 to 15% gradient polyacrylamide gel. The effects of iron on the levels of iron-repressible polypeptides in iron uptake mutants were examined.

[b] S, regulated and produced at the same level as in wild-type cells; R, produced at a reduced level in iron uptake mutants, compared with that in wild-type cells.

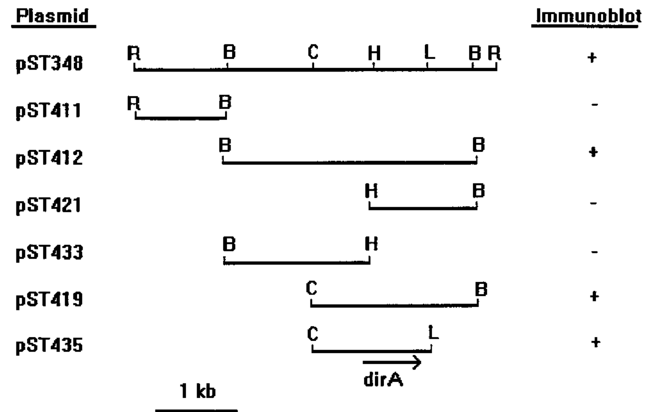

FIG. 2. Subcloning process of DirA. The relevant restriction enzyme sites are indicated. B, *Bam*HI; C, *Cla*I; H, *Hin*cII; L, *Bal*I; R, *Eco*RI. These sites, except for *Cla*I, are unique sites. Lysates of *E. coli* cells harboring these plasmids were analyzed by immunoblotting DirA-specific antibodies.

yses of lysates of these constructed clones. The steps in subcloning *dirA* are shown in Fig. 2.

The nucleotide sequence of the cloned fragment in plasmid pST435 was determined by the dideoxy chain termination method and analyzed by using the PC/gene sequence analysis program. The nucleotide and deduced amino acid sequences of DirA are shown in Fig. 3. This reading frame was selected on the basis of the partial amino acid sequence of purified DirA, determined as described above. DirA had 198 amino acid residues and a molecular mass of 22,280 Da. The first amino acid residue of the deduced sequence of DirA was valine, while that of the purified DirA polypeptide was serine. There was no significant ORF within 600 bp (upstream) of *dirA*. The −10 and −35 regions of *dirA* were selected by using a computer program.

Sequence homology analysis. The amino acid sequence of DirA was analyzed for homology to previously reported pro-

FIG. 3. Complete nucleotide sequence of *dirA* and its deduced amino acid sequence. SD, Shine-Dalgarno sequence. ★★★, end of amino acid sequence.

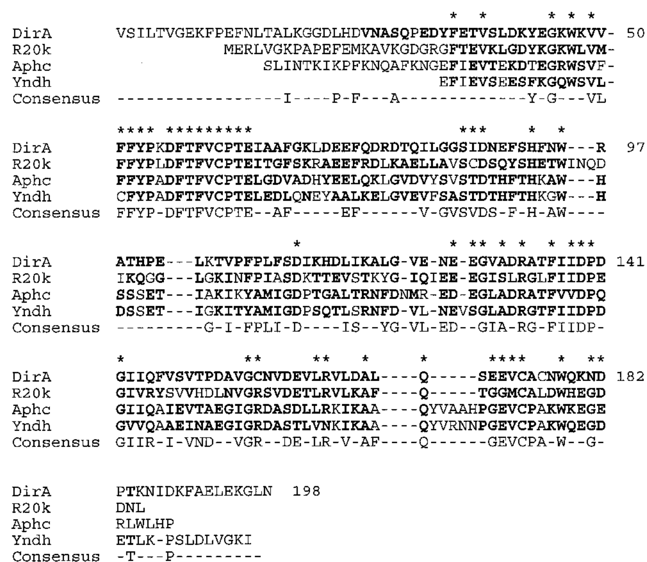

FIG. 4. Alignment of the amino acid sequences of DirA and its bacterial homologs involved in oxidation reduction reactions, as well as the consensus sequence of AhpC/TSA family proteins (7). Similar amino acid residues are in boldface. Conserved (minimum, 3 of 4) residues among bacterial homologs are denoted by asterisks. R20k, 20-kDa protein in the rubredoxin operon of *Clostridium pasteurianum*; Aphc, alkyl hydrogen peroxide reductase AhpC protein of *S. typhimurium*; Yndh, hypothetical polypeptide in the *Bacillus* NAD dehydrogenase operon. Dashes represent gaps.

tein sequences by using the Genetics Computer Group FASTA program. This analysis showed that DirA shares identity with at least 25 proteins that are widely distributed among organisms of all kingdoms and have various biological functions. Some of these homologs are associated with oxidation reduction activity. DirA had identities of 44.1% with ORF C of the *Clostridium pasteurianum* rubredoxin operon, 37.1% with yeast thiol-specific antioxidant, 35.1% with the AhpC subunit of *S. typhimurium* alkyl hydroperoxide reductase, and 32.7% with ORF A of the *Bacillus* sp. strain YN-1 NAD dehydrogenase operon. An alignment of the amino acid sequences of DirA and its bacterial homologs, as well as the consensus sequence of all known homologs, is shown in Fig. 4.

Function of cloned DirA in *E. coli*. The alkyl hydroperoxide reductase of *S. typhimurium* and *E. coli* consists of two subunits, AhpC and AhpF, and renders cells resistant to cumene hydroperoxide (13). Since DirA had amino acid sequence homology with the AhpC subunit of *S. typhimurium* and *E. coli*, we investigated whether these two proteins had similar activities. Plasmids pST435 (encoded DirA), pAQ27 (encoded both AhpC and AhpF), pST449 (encoded AhpC), and pGS01 (encoded AhpF) were separately transformed into *E. coli* TA4315, which has the entire *ahp* locus deleted. The susceptibilities of these transformants to cumene hydroperoxide were tested by disc inhibition assays. As shown in Table 3, TA4315 cells were sensitive to cumene hydroperoxide; this sensitivity was reduced when cells were transformed with pST449 and further reduced when cells were transformed with pAQ27. The introduction of plasmid pGS01 into TA4315 had no effect on its susceptibility to cumene hydroperoxide. The resistance of TA4315 cells harboring plasmid pST435 to cumene hydroperoxide was similar to that of TA4315 cells harboring pST449. The similarity in biological activity between AhpC and DirA was further examined by complementation in a two-plasmid system. Plasmid pST446 was transformed into TA4315 cells which already carried pGS01. The resistance of the resultant transformant to cumene hydroperoxide was similar to that of the transformant harboring plasmid pST446 only. These results indicated that like AhpC, DirA had partial alkyl hydroperoxide reductase activity, but cloned DirA did not interact with the AhpF subunit of *E. coli.*

TABLE 3. Functional analysis of cloned DirA in *E. coli* TA4315

Plasmid	Plasmid-encoded polypeptide(s)	Zone of inhibition (diameter [mm])[a]
None		24.9
pAQ27	AhpF, AhpC	10.5
pGS01	AhpF	24.9
pST449	AhpC	14.2
pST435	DirA	14.1
pST446	DirA	15.3
pGS01, pST446	AhpF, DirA	17.3

[a] An aliquot (0.2 ml) of an overnight culture was mixed with 3 ml of molten 0.8% top agar and poured onto Luria-Bertani plates. After the top agar solidified, filter discs (diameter, 6 mm) were placed on top of the agar and 2 μl of 5% cumene hydroperoxide was spotted onto the filter. The zone of inhibition of growth was measured after overnight incubation at 37°C. Data are averages from three experiments, with three measurements for each experiment.

DISCUSSION

Iron limitation triggers a series of adaptive responses in microorganisms. The scope of low-iron response is not well understood. Here we have reported that wild-type nontoxigenic *C. diphtheriae* cells increased production of at least 14 polypeptides when iron was removed from growth medium. Iron uptake mutants produced only some of these iron-repressible polypeptides at the levels observed for wild-type cells. They had reduced levels of five polypeptides, with apparent molecular masses of 64, 22, 20, 19, and 18 kDa. Since these iron uptake mutants have not been clearly characterized, it is not known whether reduced production of these five polypeptides is the cause or result of iron uptake deficiency. To investigate the function of these polypeptides, we cloned the genetic determinant for DirA from the λgt11 genomic library of *C. diphtheriae. dirA* encoded a polypeptide of 198 amino acid residues and did not have a leader sequence at its amino terminus. Sequence and hydropathic analyses showed that 42.9% of these amino acid residues were hydrophobic, with the majority of them located in two clusters at positions 45 to 70 and 130 to 177. However, it was not a membrane protein; it was detected in the cytoplasm of *C. diphtheriae* by immunoblot analysis with DirA-specific antibodies (data not shown). Though production of DirA by *C. diphtheriae* was affected by the concentration of iron in growth medium, DNA sequence alignment did not reveal any consensus sequence for DtxR binding sites in the region upstream of *dirA*. The molecular mechanism regulating DirA synthesis remains to be investigated.

Amino acid sequence homology analysis had shown that DirA belonged to an antioxidant family which includes the AhpC subunit of *S. typhimurium* alkyl hydroperoxide reductase, yeast thiol-specific antioxidant (TSA), and at least 25 proteins (6, 7). The most striking homologous regions were three stretches of amino acid residues, positions 49 to 65, 127 to 140, and 171 to 174 of DirA. They were located within the hydrophobic amino acid clusters described above. Members of the AhpC/TSA family have either one or two conserved cysteine residues. DirA had four cysteine residues; two of them (positions 61 and 174) were located in highly conserved re-

gions. As is the case for other two-cysteine family members, the sequence surrounding the first cysteine is FVCP. Cysteine residues are important for oxidation reduction activities. Biochemical or genetic alteration of conserved cysteine residues results in inactivation of both *S. typhimurium* alkyl hydroperoxide reductase and yeast thiol-specific antioxidant (8, 13). This strong homology between DirA and AhpC/TSA proteins suggests that DirA may be involved in an oxidation reduction reaction.

Among bacterial AhpC homologs, only the biochemical activity of *S. typhimurium* alkyl hydroperoxide reductase has been characterized. This enzyme consists of two subunits and confers alkyl hydroperoxide resistance by reducing this compound to alcohol (13). The AhpC subunit is responsible for reduction of the substrate, while the AhpF subunit is responsible for reduction of oxidized AhpC, with an electron transfer from NAD(P)H. Our results have shown that plasmid-encoded DirA can reduce the sensitivity of an *E. coli ahp* mutant to cumene hydroperoxide to a level similar to that of a cell carrying plasmid-encoded AhpC only. Plasmid-encoded *E. coli* AhpF further reduces the sensitivity of cells carrying cloned AhpC, but not that of cells carrying cloned DirA. Like AhpC, DirA has partial alkyl hydroperoxide reductase activity but cannot accept electrons from *E. coli* AhpF. Alkyl hydroperoxide reductase is a member of a group of detoxification enzymes that can remove and repair damage in nucleic acids, proteins, and lipids caused by reactive oxygen intermediates, such as oxygen and hydroxide radicals and peroxides (for a review, see reference 10). Iron participates in the synthesis of these reactive oxygen intermediates. Coupling the synthesis of alkyl hydroperoxide reductase with a siderophore-mediated iron acquisition system may be beneficial for the growth of *C. diphtheriae* under iron-limited conditions. The control of superoxide dismutase by the iron-dependent regulator Fur in *E. coli* also illustrates this coordinated survival strategy (16).

Analysis of the nucleotide sequence of *dirA* and flanking regions suggests that *dirA* is part of a polycistronic operon. No significant ORF was detected within 600 bp upstream of the coding region of *dirA*. Nevertheless, 5 bases after the termination codon of *dirA*, an ORF coding for a polypeptide of 18,814 Da, which in turn was followed by another significant ORF, was detected (data not shown). With the exception of genes whose flanking sequences have not been determined, all bacterial DirA homologs are parts of polycistronic operons which also share sequence homology at another gene. For example, DirA is related to the AhpC subunit of *S. typhimurium*. The downstream AhpF subunit has amino acid sequence similarity with a *Bacillus* NAD dehydrogenase and ORF A of the *Clostridium pasteurianum* rubredoxin operon (29, 31). All AhpF homologs have a nucleoside (flavin adenine dinucleotide or NADH) binding motif. It is not known whether the *dirA* operon has an AhpF homolog which is responsible for the reduction of oxidized DirA in oxidation reduction reactions. Currently, we are analyzing sequences for downstream genes of the *dirA* operon. Analysis of these genes may help us to delineate the physiological function of the *dirA* operon and the cellular response of *C. diphtheriae* to iron limitation.

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