Characterization of Lipoprotein IRP1 from *Corynebacterium diphtheriae*, Which Is Regulated by the Diphtheria Toxin Repressor (DtxR) and Iron

MICHAEL P. SCHMITT,[†] BRENDA G. TALLEY,[‡] AND RANDALL K. HOLMES*

Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences,
Bethesda, Maryland 20814

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The *Corynebacterium diphtheriae* *irp1* gene is negatively regulated by DtxR and iron. The nucleotide sequence of *irp1* revealed that it has homology with genes involved in iron acquisition. Expression of the *irp1* gene showed that it encodes a lipoprotein (IRP1) with a predicted size of 38 kDa. Northern blot experiments indicated that transcription from the *irp1* promoter is repressed in high-iron medium and suggested that *irp1* is part of an iron-regulated operon.

Corynebacterium diphtheriae is the causative agent of diphtheria. The primary virulence determinant of this bacterium is diphtheria toxin, a 59-kDa secreted protein that possesses ADP-ribosyltransferase activity (10). The structural gene for diphtheria toxin, *tox*, is negatively regulated at the level of transcription by the diphtheria toxin repressor protein (DtxR) and iron (1, 18). DtxR is a 25.3-kDa iron-dependent regulatory protein that negatively controls the expression of at least seven genes in *C. diphtheriae* (6, 17, 21, 22, 26). DtxR is functionally similar to the ferric uptake repressor protein (Fur) in *Escherichia coli* (4).

The corynebacterial siderophore is regulated by DtxR, but the chromosomal genes required for synthesis and transport of the siderophore have not been identified (18, 25). *C. diphtheriae*, in addition to utilizing its own siderophore to acquire iron, can also utilize aerobactin for iron uptake (12). Other iron sources that are used by *C. diphtheriae* include heme, hemoglobin, and transferrin (17). Chemically derived mutants of *C. diphtheriae* that are defective in iron transport or siderophore biosynthesis have been described (2, 12). The specific genes mutated in the *C. diphtheriae* iron-uptake mutants have not been determined.

In a previous study, we identified two promoter/operator regions upstream of the *irp1* and *irp2* genes that are regulated by DtxR and iron in *E. coli* (21). In vitro footprinting experiments indicated that DtxR binds in a metal-dependent manner to sequences that overlap these promoters. A partial sequence of the 5' region of the *irp1* gene was shown to be homologous with a family of periplasmic binding proteins from gram-negative bacteria that are associated with high-affinity iron acquisition systems. IRP1 also has homology with the ferrichrome receptor FhuD from *Bacillus subtilis* (23). FhuD is a lipoprotein and is proposed to be anchored to the cell surface by an N-terminal lipid moiety embedded in the cytoplasmic mem-

brane. Like FhuD, IRP1 has a leader sequence that is similar to signal sequences found in lipoproteins (21). Because of the homology of IRP1 with FhuD and with periplasmic binding proteins from gram-negative bacteria, it was proposed that IRP1 functions as a cell surface receptor for a ferric-siderophore complex in *C. diphtheriae*. Here we present the complete sequence of *irp1*, describe its regulation, and characterize its product.

Cloning and sequence analysis of the *irp1* gene. To obtain the complete sequence of the open reading frame for *irp1*, a *C. diphtheriae* C7(–) (5) chromosomal library was constructed in the vector LambdaGem-12 (Promega, Madison, Wis.). *C. diphtheriae* strains were routinely grown in heart infusion broth containing 0.2% Tween 80 (HIBTW). *C. diphtheriae* chromosomal DNA was purified as described previously (16, 18). A *Sau*3AI partial digest of the C7(–) chromosomal DNA was size fractionated by ultracentrifugation through a sucrose gradient (14), and fractions of 15 to 22 kb were isolated and ligated to the LambdaGem-12 vector following a protocol described by the manufacturer. Lambda plaques were transferred to nitrocellulose, and clones carrying sequences homologous to the *irp1* gene were identified by in situ hybridization with a 460-bp *Alu*I fragment containing *irp1* sequences (21). DNA probes were labeled with [³²P]dCTP by using a random primer kit (Stratagene Inc., La Jolla, Calif.). A lambda clone carrying an insert that hybridized with the DNA probe specific for *irp1* was designated λIRP1. A portion of the restriction map of λIRP1 was generated, and the approximate location of the *irp1* coding region and promoter are shown in Fig. 1A. The complete insert for the λIRP1 clone was approximately 17 kb.

To obtain the complete DNA sequence of the *irp1* coding region, DNA fragments carrying the *irp1* gene were subcloned into plasmid vectors. Since DNA fragments carrying the complete coding region for the *irp1* gene were found to be unstable in high-copy-number plasmids, the *irp1* gene was subcloned into the low-copy-number plasmid pWKS30 (27). *E. coli* K-12 DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used throughout this study for all cloning purposes and for routine isolation and maintenance of plasmid DNA. DNA was sequenced by the chain termination method of Sanger et al. (15) with a DNA sequencing kit purchased from United States Biochemicals Inc., Cleveland, Ohio. The DNA sequence of *irp1* and the deduced amino acid sequence of its product are shown in Fig. 1B. It was previously demonstrated that the

* Corresponding author. Present address: Department of Microbiology, University of Colorado Health Sciences Center, Campus Box B-175, 4200 East Ninth Ave., Denver, CO 80262. Phone: (303) 315-7903. E-mail: Randall.Holmes@uchsc.edu.

[†] Present address: Laboratory of Bacterial Toxins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

[‡] Present address: Radiation Biology Branch, National Cancer Institute, Bethesda, MD 20892.

FIG. 1. (A) Restriction map of clones carrying the *irp1* gene. λ IRP1 is a LambdaGem-12 clone that carries the *irp1* gene. The hatched box represents the *irp1* coding region, P indicates the location of the *irp1* promoter, and the arrowhead shows the direction of transcription. Only a portion of the 17-kb insert is shown. Plasmids pWES33 and pWR382 are subclones of λ IRP1 which contain 3.3- and 8-kb fragments, respectively, that are present in the low-copy-number vector pWKS30. Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Sal*I. (B) Nucleotide sequence of *irp1* and the deduced amino acid sequence of the gene product. Upstream of the coding region of *irp1* are -10 and -35 sequences. The DtxR binding site is indicated by inverted arrows. The putative ribosome binding site (underlined) is located 7 bp upstream of the ATG start codon. The transcriptional start site is a T residue (arrowhead and asterisk) located seven bases downstream from the -10 sequence. The amino acid sequence LAACGN (underlined) is the presumed signal peptidase II recognition sequence. Primers used for PCR to generate DNA probes used for Northern blot analysis are indicated (P1A and P1B).

promoter region of the *irp1* gene contains a DtxR binding site and its expression is iron regulated in *E. coli* (21). The complete coding region of *irp1* is predicted to encode a protein of 38.1 kDa. In an earlier study from this laboratory, the deduced amino acid sequence of the N-terminal 85 amino acids of the IRP1 protein was shown to be homologous to those of proteins involved in high-affinity iron acquisition systems, including the FhuD lipoprotein from *B. subtilis* (23). It was also revealed that the IRP1 protein contains a putative 23-amino-acid leader that has homology to recognition sequences for signal peptidase II,

an enzyme which processes secreted lipoproteins in bacteria. A recent homology search of the GenBank and SwissProt databases with the complete IRP1 amino acid sequence revealed that two additional proteins, FeuA from *B. subtilis* (11) and a protein of unknown function from *Mycobacterium tuberculosis* H37Rv (GenBank accession no. Z86089), were also homologous with IRP1. FeuA and the *M. tuberculosis* protein are presumed to be lipoproteins, and like FhuD and IRP1 they are homologous to iron-regulated periplasmic binding proteins in gram-negative bacteria. While functions for FeuA, IRP1, and the *M. tuberculosis* protein have not been determined, the homology between these proteins and the *B. subtilis* ferri-chrome receptor FhuD suggests that these proteins may also function as receptors for siderophores or related compounds in their respective hosts. Furthermore, the homologies found among these iron-regulated proteins indicate that the utilization of lipoproteins as receptors for ferric siderophores may be a common mechanism in the transport of iron by gram-positive bacteria.

Primer extension analysis of the *irp1* gene. Total cell RNA isolated from *C. diphtheriae* C7(–) was used to map the transcriptional start site for the *irp1* gene. *C. diphtheriae* strains were grown overnight in HIBTW medium and then diluted 1:50 into fresh HIBTW medium which contained either 100 μ g of EDDA/ml (low-iron medium) or no added EDDA (high-iron medium). The bacteria were grown for an additional 6 to 8 h at 37°C with aeration, and then total cell RNA was isolated and purified as described previously (7, 19). Primer extension experiments were done as described by Sambrook et al. (14). A 30-bp single-stranded oligonucleotide, PEX1 (5'-CGATGGA CCCGGTGGCGAGTGCAGTGTCA-3'), which is located approximately 60 bp downstream from the putative -10 sequence was utilized for the primer extension analysis. PEX1 is specific for the *irp1* gene and is complementary to the predicted mRNA. Primers were labeled with ^{32} P at their 5' termini with T4 DNA kinase (14). Analysis of *irp1* revealed a single primer extension product that was seen only in RNA preparations that were isolated from strains grown in low-iron medium (data not shown). Transcription is predicted to initiate at a T residue located 7 bp downstream from the predicted -10 sequence of the *irp1* promoter (Fig. 1B).

Northern blot analysis. RNA preparations that were used for the primer extension studies were also analyzed in Northern blot experiments by using a procedure that was described previously (14, 20). RNA (25 μ g) was transferred to nitrocellulose and then probed with a ^{32} P-labeled PCR-generated 613-bp DNA fragment that is internal to the *irp1* coding region (Fig. 1B). The *irp1* probe hybridized to mRNA products of 3.4, 2.2, and 1.1 kb (Fig. 2). These products were detected only in RNA obtained from low-iron cultures. The minimal RNA predicted to contain the entire *irp1* open reading frame is approximately 1.1 kb, and the presence of larger mRNA products suggests that the *irp1* gene is part of an iron-regulated operon.

The 1.1-kb band identified in Northern blots had the strongest intensity, while the 3.4-kb mRNA was the weakest. It is unclear why three distinct bands with different intensities were detected by the *irp1* probe in the RNA blots. Pause sites for RNA polymerase or weak transcriptional terminators may be present in intergenic regions within the operon. However, no well-defined rho-independent transcriptional terminator was observed within the 100-bp region downstream of the *irp1* gene (unpublished observation). The different concentrations of the three distinct RNA products may provide a means for differentially regulating the transcription of the genes within this operon.

FIG. 2. Northern blot analysis with *irp1* mRNA. -Fe, RNA from low-iron cultures; +Fe, RNA from high-iron cultures. Experimental procedures are described in the text.

Expression of the *irp1* gene. DNA fragments carrying the *irp1* gene were subcloned from the lambda vector λ IRP1 into the low-copy-number expression vector pWKS30 to generate plasmids pWES33 (3.3-kb *SalI* fragment) and pWR382 (8-kb *EcoRI* fragment) (Fig. 1A). Transcription of the *irp1* gene on these plasmids is under the control of the T7 promoter. Proteins were analyzed with the T7 expression system as described previously (24). Expression of the *irp1* gene from plasmids pWES33 and pWR382 revealed a unique doublet band that migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a size of approximately 42 kDa (Fig. 3, lanes P). When excess unlabeled methionine was added to the labeling reaction, only a single band which corresponded to the faster-migrating product seen in the doublet in lane P was detected (Fig. 3, lanes C). This suggests that the slower-migrating band seen after the 5-min pulse was processed to a lower-molecular-

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S -labeled proteins synthesized with the T7 expression system. The *irp1* gene on plasmid pWES33 or pWR382 was expressed from the T7 promoter. The location of the IRP1 lipoprotein is indicated. P, proteins detected after a 5-min labeling with [^{35}S]methionine (pulse); C, proteins detected after excess unlabeled methionine was added after the 5-min labeling period (chase); G, proteins detected when globomycin was added 1 h prior to labeling. Numbers on the left are molecular masses, in kilodaltons.

weight protein. When globomycin (a specific inhibitor of signal peptidase II) was added 1 h prior to labeling of the proteins, only the slower-migrating band was observed (Fig. 3, lanes G). This finding provides strong evidence that IRP1 is a lipoprotein. Two additional proteins, which migrate with predicted sizes of 65 and 29 kDa, were expressed only from plasmid pWR382 (Fig. 3).

Complementation of iron acquisition mutants. Clones carrying the *irp1* gene were tested for the capability to complement the defect in various iron uptake or siderophore synthesis mutants of *C. diphtheriae*. The 8-kb *EcoRI* fragment from λ IRP1 was moved into the *E. coli*-*C. diphtheriae* shuttle vector pCM2.6 (18), and the resulting clone was then transformed by electroporation into the *C. diphtheriae* C7(β) mutants HC1, HC3, HC4, HC5, and HC6 and into PW8. The shuttle vector without any insert was also transformed into these strains and tested as a control for siderophore expression. Low-iron modified PGT medium (25) was used for the detection of siderophores produced by *C. diphtheriae* strains. *C. diphtheriae* strains HC1, HC3, HC4, and HC5 are defective in iron uptake and overproduce siderophore in medium containing excess iron (2), whereas HC6 (12) and PW8 (13) fail to produce siderophore. The plasmid carrying the *irp1* gene had no effect on siderophore production or on the regulation of the siderophore in the strains examined (data not shown).

Explanations as to why these genes may have failed to complement include the following. (i) The relevant mutation(s) in these strains resides in a gene other than (or in addition to) *irp1*. (ii) The cloned *irp1* gene was not sufficiently expressed in the mutant strains. (iii) Defective proteins produced in the mutant strains interfered with the function of the proteins expressed from the cloned wild-type genes. While it seems likely that the phenotypes of the mutant strains are due to mutations in genes associated with the corynebacterial siderophore system, it is possible that genes not directly involved with siderophore transport or biosynthesis are responsible for the iron uptake defects. Although a defined mutation in the *irp1* gene will ultimately be needed for determining the role of IRP1 in iron transport, a mechanism for allelic replacement in *C. diphtheriae* has not yet been developed.

The ability to acquire iron during an infection is an important virulence determinant for many pathogenic bacteria (8). Little is known concerning the molecular mechanisms by which gram-positive bacterial pathogens acquire iron in the human host. Siderophore high-affinity iron uptake systems are known to have important roles in the pathogenesis of diseases caused by a number of gram-negative bacteria (3, 9, 28). However, the importance of siderophores in the pathogenesis of *C. diphtheriae* or in any gram-positive organism is not known. The findings in this study revealed that the IRP1 protein from *C. diphtheriae* is a lipoprotein that likely functions as a cell surface ferric-siderophore receptor. Additional studies of the iron acquisition systems in *C. diphtheriae* and in other medically relevant gram-positive bacteria are needed to determine the role of iron uptake in the virulence of this important group of organisms.

Nucleotide sequence accession number. The nucleotide sequence of the *irp1* gene was assigned GenBank accession no. U02617.

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