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Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*

(transcriptional activation/LysR family/Fur/IrgA/IrgB)

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ABSTRACT **We have previously described a virulence gene in *Vibrio cholerae* (*irgA*) that is more than 850-fold regulated in response to iron. Negative regulation of *irgA* by iron occurred at the transcriptional level, and there was a dyad symmetric nucleotide sequence in the vicinity of the *irgA* promoter homologous to the Fur binding site in *Escherichia coli*. When *irgA* was cloned into *E. coli*, we showed that transcription of *irgA* required 900 base pairs of DNA upstream of the *irgA* promoter that contained an open reading frame in inverse orientation to *irgA*. In the present study, we show that this upstream region of DNA encodes a gene in inverse orientation to *irgA* (named *irgB*) that is also negatively regulated by iron. Insertional inactivation of *irgB* on the *V. cholerae* chromosome leads to loss of expression of a chromosomal *irgA'-'phoA* fusion (in which the primes indicate truncated genes), which is restored to normal by provision of *irgB* on a plasmid in trans. DNA sequencing of *irgB* shows that the protein product (IrgB) is homologous to the LysR family of positive transcriptional activators, and secondary structure analysis of IrgB predicts a helix-turn-helix DNA binding motif. The promoters of *irgB* and *irgA* are divergent but overlap each other and the previously defined Fur-binding site. We propose a model for iron regulation of *irgA* expression in *V. cholerae*. In the presence of sufficient iron, transcription of both *irgA* and *irgB* is negatively regulated by a Fur-like protein. In low iron conditions, negative regulation of transcription is removed, and production of IrgB leads to positive transcriptional activation of *irgA*. It seems likely that the high induction ratio of *irgA* expression under low- and high-iron conditions (850-fold) relates to the fact that its cognate positive transcriptional activator (*irgB*) is itself negatively regulated by iron.**

Vibrio cholerae infection in humans may cause a severe dehydrating diarrhea. Illness occurs following ingestion of the organism in contaminated fresh or salt water. The bacteria must pass through the acid barrier of the stomach to reach the small intestine, where they penetrate the mucus gel and adhere to the brush border of intestinal epithelial cells by specific adhesins, including toxin coregulated pilus (TcpA) and other accessory colonization factors. At the intestinal brush border, *V. cholerae* produces a number of virulence factors, including cholera toxin, neuraminidase, hemolysin, and other extracellular secreted proteins. The genetic regulation of cholera toxin and TcpA in response to environmental stimuli has been described (1, 2). The genetic regulation of other virulence determinants in response to environmental signals is less well understood.

Many bacterial virulence determinants are regulated by the environmental concentration of free iron, with increased expression occurring under low-iron conditions. Examples of iron-regulated virulence determinants include diphtheria toxin of *Corynebacterium diphtheriae* (3), Shiga toxin of *Shigella dysenteriae* 1 (4), Shiga-like toxin I of enterohemorrhagic *Escherichia coli* (5), exotoxin A of *Pseudomonas aeruginosa* (6, 7), and several outer membrane proteins of *Vibrio anguillarum* (8) and *Yersinia* species (9).

We have described an iron-regulated virulence determinant in *V. cholerae* (10). Strain MBG40 of *V. cholerae*, which contains a Tn*phoA* insertion mutation in the iron-regulated gene *irgA*, has reduced virulence in a newborn mouse model and has lost the 77-kDa major iron-regulated outer membrane protein (10). The deduced amino acid sequence of the amino terminus of IrgA is homologous to FepA, the ferrienterochelin outer membrane receptor of *E. coli*, suggesting that IrgA could be the iron-vibriobactin outer membrane receptor of *V. cholerae* (11). Expression of the *irgA'-'phoA* gene fusion (in which the primes indicate truncated genes), as measured by alkaline phosphatase activity, increases 850-fold in low- versus high-iron media (10). Negative regulation of *irgA* by iron occurs at the transcriptional level, and its promoter region, defined by primer extension, contains a dyad symmetric sequence homologous to dyad elements in *E. coli* that bind the Fur protein, an iron-responsive repressor of transcription (11).

When the *irgA'-'phoA* gene fusion was cloned from *V. cholerae* onto a plasmid in *E. coli*, expression of alkaline phosphatase activity and transcription of the gene fusion required an additional 900 base pairs (bp) of DNA upstream of the *irgA* promoter (11); this region of DNA, which we call *irgB*,§ contains an open reading frame in inverse orientation to *irgA* (Fig. 1). We wished to test the model in which this open reading frame encodes a positive activator for *irgA* transcription.

MATERIALS AND METHODS

Bacterial Strains. Bacterial strains and plasmids used in this study are shown in Table 1. *V. cholerae* strain MBG260 and plasmids pSBC45 and pMBG111 were constructed as described below.

Media. Luria–Bertoni (LB) medium with or without the addition of the iron chelator 2,2-dipyridyl (final concentration, 0.2 mM) was used to evaluate the effect of iron concentration on gene expression as described (10).

Construction of plasmids. Strain MBG40 contains a chromosomal gene fusion between *irgA* and *phoA*, constructed by Tn*phoA* mutagenesis (10). Plasmid pMBG59 contains a subclone of the intact *irgA*::Tn*phoA* gene fusion from MBG40 into plasmid pBR322. Plasmid pMBG59 also contains all of the open reading frame upstream of *irgA* (subsequently named *irgB*) (see *Results*) (Fig. 1).

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55988).

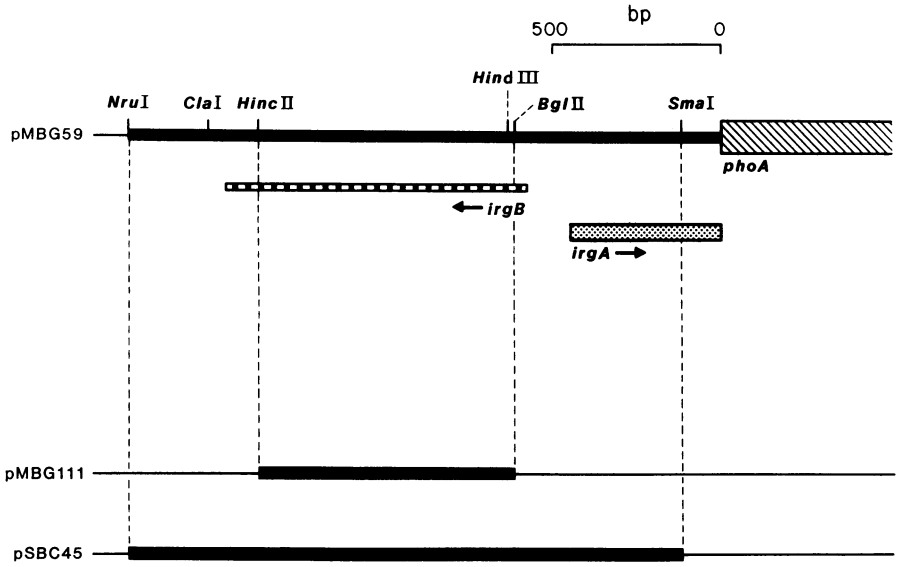

FIG. 1. Partial restriction map of the chromosomal DNA upstream of the Tn*phoA* fusion junction in *V. cholerae* strain MBG40, subcloned as described (11) to make plasmid pMBG59. Locations of relevant restriction enzyme sites are indicated, as well as fragments used to construct plasmids pMBG111 and pSBC45. The solid bar represents MBG40 chromosomal DNA. The diagonally cross-hatched bar represents Tn*phoA* DNA. The stippled bar indicates the position and direction of transcription of *irgA* upstream of the fusion with *phoA*. The striped bar indicates the position and direction of transcription of *irgB*.

Plasmids pSBC45 and pSBC46 are derivatives of pACYC184, a plasmid encoding chloramphenicol resistance. Plasmids pSBC45 and pSBC46 were constructed by isolating the *Nru* I–*Sma* I fragment of plasmid pMBG59 by electroelution from a gel and ligation into *Nru* I-digested pACYC184; the two plasmids differ only in the orientation of the inserted fragment, which contains the intact gene *irgB* (Fig. 1; see *Results*).

Plasmid pMBG111 was derived from pGP704, a broad-host-range plasmid containing the ampicillin-resistance gene from pBR322, the mobilization domain of plasmid RP4 (14), the origin of replication from plasmid R6K (15), and a polylinker from phage M13 tg131 (Amersham). Plasmid pGP704 was a gift of Gregory D. N. Pearson and is itself derived from plasmid pJM703.1 (13). Plasmid pGP704 and its derivatives are able to replicate only in strains containing the *pir* gene, which encodes the π protein necessary for the function of the R6K origin (15). To construct pMBG111, a 676-bp *Hin*cII–*Bgl* II fragment of pMBG59 internal to *irgB* (Fig. 1) was ligated into the *Eco*RV and *Bgl* II sites of the pGP704 polylinker.

Genetic Methods. *V. cholerae* strain MBG260, which contains an insertion mutation in *irgB*, was constructed from strain MBG40 in the following manner. Plasmid pMBG111 was transferred from strain SY327 λ *pir* into SM10 λ *pir* by transformation. SM10 λ *pir* contains a chromosomally-integrated RP4-2 (Tc::Mu), which encodes trans-acting factors necessary to mobilize pGP704 derivatives into a broad range of recipients without RP4 itself being transferred (13). SM10 λ *pir* containing pMBG111 was conjugated with MBG40, with double selection for ampicillin resistance (encoded by pMBG111) and streptomycin resistance (encoded by MBG40). Because MBG40 does not contain the *pir* gene, pMBG111 is unable to replicate in this strain, so that doubly resistant colonies arise by homologous recombination between the internal fragment of *irgB* on pMBG111 and the corresponding chromosomal gene on the recipient, causing insertional inactivation of *irgB*. To confirm that chromosomal integration occurred within *irgB* on the recipient, we performed Southern hybridization of chromosomal DNA digests with *Eco*RV, an enzyme that does not cut within either *irgB* or pMBG111. The blot was probed with the *Hin*cII–*Bgl* II fragment of *irgB*, radioactively labeled by random primer extension with a commercial kit (Prime Time, International Biotechnologies).

Plasmids were transformed into *E. coli* strains by standard techniques (16). Plasmids pACYC184 and pSBC45 were introduced into *V. cholerae* strains by electroporation by using the protocol of the manufacturer (Gene Pulser, Bio-Rad), with the exception of substitution of 2 mM $CaCl_2$ as the buffer for resuspending cells during preparation, rather than water or Hepes buffer. Plasmid content of the electroporants was confirmed by restriction enzyme digestion of plasmid

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Ref. or source
V. cholerae strains		
0395	Sm^r	27
MBG40	*irgA*::Tn*phoA*, Sm^r Km^r	10
MBG260	*irgA*::Tn*phoA irgB*::pMBG111,	This study
E. coli strains		
CC118	Δ(*ara leu*)7697 Δ(*lac*)*X74 araD139 phoA*Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	12
SY327 λ *pir*	Δ(*lac pro*) *nalA recA56 araD argE*(Am) λ *pir*R6K	13
SM10 λ *pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ *pir*R6K, Km^r	13
Plasmids		
pMBG59	pBR322 with 4.6-kb *irgB irgA*::Tn*phoA* insert from MBG40	11
pJM703.1	*oriR6K mobRP4*, Ap^r	13
pGP704	pJM703.1 with 1.5-kb *Sph* I–*Pvu* II deletion and 75-bp insert of M13 tg131 polylinker	G. D. N. Pearson
pMBG111	pGP704 with 676-bp *Hin*cII–*Bgl* II insert of pMBG59	This study
pSBC45	pACYC184 with 1.6-kb *Nru* I–*Sma* I insert of pMBG59	This study

Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance.

minipreps, analyzed by agarose gel electrophoresis as described (11).

Assays. The enzymatic activity of alkaline phosphatase encoded on Tn*phoA* permitted the comparison of fusion gene expression when strains were grown in low- versus high-iron media. Strains were grown overnight in LB medium with or without added 2,2-dipyridyl. Alkaline phosphatase activity was determined as described (10).

DNA and RNA Analysis. Analysis of DNA and RNA, including DNA and RNA preparation, restriction mapping, DNA sequencing, RNA (Northern) blot analysis, and primer extension were performed as described (11). For Northern blot analysis, an equivalent quantity of RNA, as calculated from OD_{260}, was loaded into each lane.

Synthetic oligonucleotides used as probes for Northern blot analysis and as primers for DNA sequencing and primer extension were the gift of Brian Seed (Massachusetts General Hospital).

Protein Analysis and Protein Data Base Searches. Protein analysis and protein data base searches were performed by using IBI-Pustell sequence analysis software (International Biotechnologies). The hydropathicity index profile of IrgB was calculated by the formula of Kyte–Doolittle (17). The protein secondary structure prediction of IrgB was calculated by the algorithm of Chou–Fasman (18). Data base searches and protein alignments were performed by searching the Protein Identification Resource, National Biomedical Research Foundation data base (release 19) using the FASTP algorithm for protein homology (19).

RESULTS

Northern Blot Analysis of the Open Reading Frame Upstream of *irgA*. To determine whether an RNA transcript was associated with the upstream open reading frame, and if present, to determine the size of the transcript and whether or not transcription was regulated by iron, we performed Northern blot analysis of RNA prepared from strains 0395 and MBG40 following growth in low- and high-iron media (Fig. 2). The blot was probed with an oligonucleotide complementary to the DNA sequence near the 5′ terminus of the open reading frame. A single band of approximately 1.1 kilobases (kb) in size was seen in RNA prepared from 0395 and MBG40 grown under low-iron conditions (Fig. 2, lanes 2 and 4), showing that a transcript was associated with the open reading frame. No bands were seen in either strain grown under high-iron conditions (Fig. 2, lanes 1 and 3), showing that transcription of the open reading frame was negatively regulated by iron. The size of the transcript, in relation to the size of the open reading frame (894 bp, see below), suggests that the transcript is monocistronic. The iron-regulated gene encoded by this open reading frame was designated *irgB*.

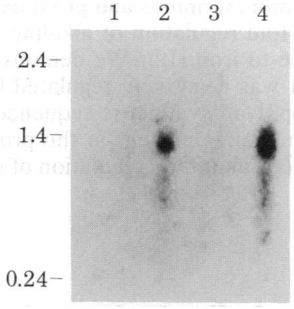

FIG. 2. Northern blot analysis of RNA from 0395 and MBG40 probed with an oligonucleotide complementary to the 5′ terminus of *irgB*. Lanes: 1, 0395 grown in high-iron medium; 2, 0395 grown in low-iron medium; 3, MBG40 grown in high-iron medium; 4, MBG40 grown in low iron medium. The positions of single-stranded RNA molecular weight markers (in kilobases) are indicated on the left.

Trans-Complementation of *irgB* and *irgA*. We have previously demonstrated, by deletion subcloning of pMBG59 in an *E. coli* background, that deletion of any portion of *irgB* eliminates transcription of *irgA* (11). We wished to examine whether *irgB* could restore expression of *irgA* in trans.

To examine trans-complementation of *irgB* and *irgA* in the *V. cholerae* background, we constructed the *irgB* mutant strain MBG260 from MBG40. Highly expressed, iron-regulated alkaline phosphatase activity seen in strain MBG40 was almost completely eliminated with the introduction of the *irgB* mutation to make strain MBG260 (Table 2). Iron-regulated, alkaline phosphatase activity was completely restored by the introduction of *irgB* in trans on plasmid pSBC45 (Table 2) or pSBC46 (data not shown), while the introduction of the vector pACYC184 had no effect (data also not shown). Strain 0395, which does not contain an *irgA′-′phoA* fusion, had negligible alkaline phosphatase activity, with or without the introduced plasmids (data not shown). The higher alkaline phosphatase activities seen in MBG260(pSBC45) and MBG40(pSBC45), as compared with MBG40 (Table 2), may result from the high copy number of *irgB* carried on pSBC45. These data suggest that *irgB* is a trans-acting factor that positively regulates *irgA*.

DNA Sequence of *irgB* and Deduced Protein Sequence of IrgB. Fig. 3 shows the DNA sequence of the chromosomal insert of pMBG59 (reading 5′ to 3′ from right to left in Fig. 1), starting approximately 60 bp beyond the transcription start site of *irgA* (11) and extending up to the *Cla* I site of pMBG59, including the 894-bp open reading frame of *irgB*. A Shine–Dalgarno sequence is indicated just upstream of the initiating methionine (20). A perfect inverted repeat, suggestive of a transcription terminator, is indicated just beyond the termination codon of the *irgB* open reading frame. The deduced protein sequence for IrgB is shown below the nucleotide sequence.

Primer-Extension Analysis of the Start Site of *irgB* Transcription. Primer-extension analysis of RNA prepared from MBG40 and 0395 following growth in low-iron medium was done with a synthetic oligonucleotide complementary to the DNA sequence located between 3 bases upstream and 17 bases downstream of the methionine start codon (data not shown). The same transcription start site was identified in both MBG40 and 0395 and is indicated by an asterisk in Fig. 3. A promoter homologous to the *E. coli* consensus sequence (21) was located upstream of the transcription start site (Fig. 3). The 19-bp interrupted dyad symmetric sequence that is homologous to the Fur binding consensus sequence of *E. coli* (5, 22) and is located immediately downstream of the *irgA* transcription start site (11) also overlaps the *irgB* transcription start site and −10 box (Fig. 3). Fig. 4 shows the overlapping but divergent *irgA* and *irgB* promoters and the location of the Fur-like box in relation to each.

Table 2. Complementation of *irgB* and *irgA′-′phoA* in *V. cholerae*

Strain	Alkaline phosphatase activity, units	
	Low iron	High iron
MBG40	683	3
MBG260	4	1
MBG40(pSBC45)	1420	11
MBG260(pSBC45)	1573	8

Alkaline phosphatase assays were in LB medium with and without added 2,2-dipyridyl which chelates iron. Units of alkaline phosphatase were normalized by using the absorbance of the cell culture at 600 nm.

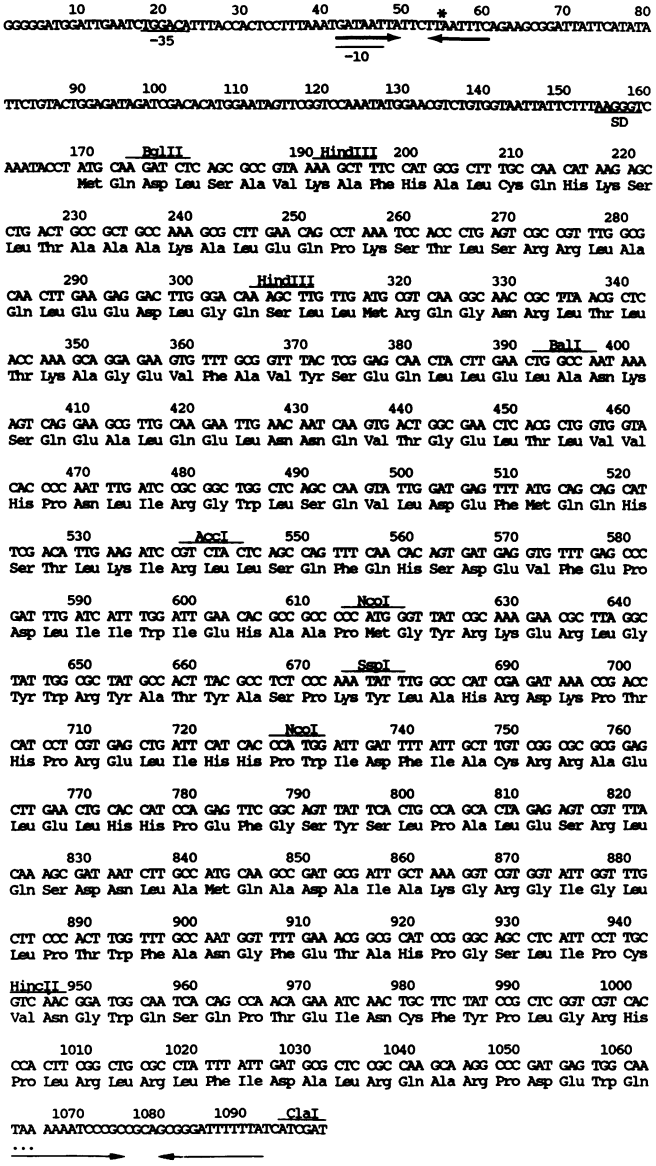

FIG. 3. Nucleotide sequence of the chromosomal DNA in pMBG59 (reading 5′ to 3′ from right to left in Fig. 1), starting downstream of the start site of *irgA* transcription and extending up to the *Cla* I restriction site in pMBG59. The locations of restriction enzyme sites are noted. The deduced amino acid sequence of IrgB is shown in three-letter code. The approximate start site of transcription (*), the −10 and −35 boxes of the promoter, and the putative Shine–Dalgarno sequence (SD) are indicated. A 19-bp interrupted dyad symmetric element homologous to the Fur box of *E. coli* is indicated by heavy horizontal arrows below the sequence in the vicinity of the promoter. The termination codon of IrgB is indicated (. . .), followed by a probable transcription terminator (light horizontal arrows at end of sequence).

IrgB Protein Analysis. The predicted amino acid sequence of IrgB is shown below the nucleotide sequence in Fig. 3.

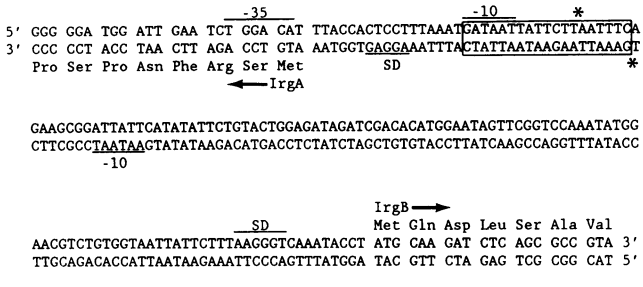

FIG. 4. Detail of the overlapping, divergent promoters of *irgA* and *irgB*. The promoter of *irgB* (−35, −10), start site of transcription (*), Shine–Dalgarno sequence (SD), and open reading frame are indicated on the upper strand, while the corresponding features of *irgA* are noted on the bottom strand. Note that *irgA* does not have a consensus −35 box. The dyad symmetric element homologous to the *E. coli* Fur-binding site is enclosed within a box.

Hydropathicity profile. The hydropathicity profile of IrgB showed no stretches of hydrophobic residues that would be suggestive of either a signal sequence or a transmembrane domain (data not shown).

Homology of IrgB to the LysR family of positive transcriptional activators. Comparison of IrgB to the Protein Identification Resource, National Biomedical Research Foundation (release 19) data base demonstrated significant homology between IrgB and the LysR family of positive transcriptional activator proteins in bacteria (23). The best match in this family was to the *E. coli* positive activator protein IlvY; the optimized score between IrgB and IlvY was 217. The regions of highest homology between IrgB and the LysR family were near the amino terminus and were at roughly the same positions in each protein. The homology near the amino terminus of IrgB with several members of the LysR family is shown in Fig. 5. All of the proteins shown are of approximately the same size (IrgB is 298 amino acids long; IlvY, LysR, AmpR, NodD, and CysB are 297, 300, 291, 300, and 301 amino acids long, respectively).

Prediction of the secondary structure of IrgB. The secondary structure of IrgB was predicted by using the Chou–Fasman algorithm (18). A helix-turn-helix motif was seen in the same region of IrgB as in the other members of the LysR family (Fig. 5) (23).

DISCUSSION

The expression of many bacterial virulence determinants is regulated by the concentration of iron in the environment, with increased expression occurring under low-iron conditions. We have previously described such an iron-regulated virulence determinant in *V. cholerae* named *irgA* (10). *V. cholerae* strain MBG40, which contains a Tn*phoA* gene fusion with the amino terminus and promoter of *irgA*, shows several-hundred-fold regulation of alkaline phosphatase activity in response to iron (10). We demonstrated that transcription of *irgA* was negatively regulated by iron and that there was a 19-bp dyad symmetric sequence homologous to *E. coli* Fur-binding sites present in the promoter region of *irgA* (11). Questions about the regulation of *irgA* expression,

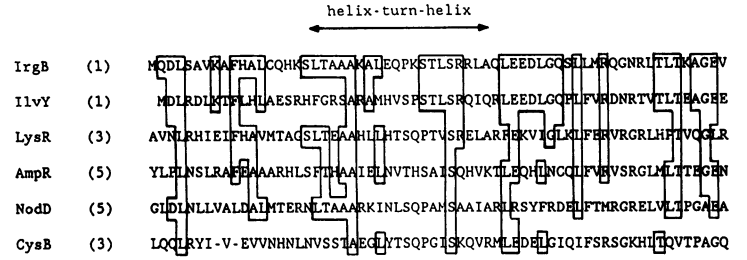

FIG. 5. Homology between the amino terminus of IrgB and several members of the LysR family of positive transcriptional activators. Amino acids are identified in single letter code and identical residues are enclosed in boxes. The conserved helix-turn-helix domain of these proteins is indicated above the sequences.

however, remained. (*i*) Why was the induction ratio of *irgA* expression in relation to iron so large compared with iron-regulated genes in other bacteria? (*ii*) Why was there no consensus −35 box in the *irgA* promoter, a feature seen in genes requiring a positive activator? (*iii*) Why was 900 bp of DNA upstream of the *irgA* promoter required for transcription of the *irgA'-'phoA* fusion when cloned on a plasmid into *E. coli*?

The data in the present study suggest that the open reading frame upstream of *irgA*, now named *irgB*, is a positive activator of *irgA* transcription that is itself negatively regulated by iron at the transcriptional level (Fig. 2). We have previously shown that *E. coli* CC118 (pMBG59) expresses iron-regulated alkaline phosphatase activity, while deletion subclones into any portion of *irgB*, which still maintain an intact *irgA'-'phoA* fusion, lose alkaline phosphatase activity (11). We examined the effect of *irgB* on *irgA* expression in single copy in the *V. cholerae* background by introducing an *irgB* mutation into the chromosome of strain MBG40. The resulting mutant, MBG260, showed marked reduction of *irgA'-'phoA* fusion activity that was fully restored by complementation with *irgB* in trans (Table 2). In addition, trans-complementation with *irgB* in *V. cholerae* also restored full iron regulation to expression of the *irgA'-'phoA* fusion.

The deduced amino acid sequence of IrgB is homologous to the LysR family of bacterial transcriptional activator proteins (Fig. 5). Homologous proteins have been identified as members of this family from several species of Enterobacteriacae, including *E. coli*, *Salmonella typhimurium*, and *Enterobacter cloacae* (23). All proteins in this family have a helix-turn-helix motif near the amino terminus, and several of the proteins are transcribed divergently from an operon or gene that is regulated by the protein (23). IrgB is similar in both of these respects.

The genes *irgA* and *irgB* are divergently transcribed from overlapping promoters that contain a single 19-bp dyad symmetric element homologous to *E. coli* Fur-binding sites (Fig. 4), suggesting that a *V. cholerae* Fur-like protein might simultaneously repress the transcription of both *irgA* and *irgB* in the presence of iron. We have shown previously that the amino-terminal portion of IrgA is homologous to FepA, the *E. coli* ferrienterochelin receptor (11). *irgA* is similar to *fepA* in that its transcription is negatively regulated by iron and there is a Fur-like box in its promoter region. In addition, each of these genes has another iron-regulated gene that is divergently transcribed from a promoter that overlaps the same Fur box (24). *irgA* differs from *fepA*, however, in that the gene divergently transcribed from it (*irgB*) is itself a trans-acting positive regulator of *irgA* transcription, whereas the gene that is divergently transcribed from *fepA* (*fes*) does not appear to be involved in *fepA* regulation (24).

A trans-acting factor, *angR*, has recently been described in the plasmid-mediated iron-regulated anguibactin iron-uptake system of *V. anguillarum* (25). *angR* is a positive regulator of this system and is itself negatively regulated by iron at the transcriptional level (26). *angR* is not divergently transcribed from any of the genes it is known to regulate (26). It is not yet known whether or not AngR has any homology to IrgB or other members of the LysR family.

We have developed the following model for regulation of *irgA* expression in *V. cholerae*. In the presence of sufficient iron, transcription of both *irgA* and *irgB* is negatively regulated by a Fur-like protein. In low-iron conditions, negative regulation of transcription is removed, and production of IrgB leads to positive transcriptional activation of *irgA*. We do not yet know whether transcription of *irgB* might also be positively autoregulated. It seems likely that the explanation of the very high induction ratio of *irgA* expression under low- and high-iron conditions relates to the fact that its cognate positive transcriptional activator (*irgB*) is itself negatively regulated by iron. We are currently investigating whether other iron-regulated proteins in *V. cholerae* are also positively activated by IrgB. In addition, we are examining other bacterial species to look for further examples of dual positive and negative iron regulatory systems.

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