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Identification of an Iron-Regulated Virulence Determinant in *Vibrio cholerae*, Using Tn*phoA* Mutagenesis

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Several virulence determinants of bacteria are regulated by the concentration of iron in the medium, with increased expression occurring under low-iron conditions. Iron-regulated virulence factors have not been previously described in *Vibrio cholerae*. We used the transposon vector Tn5 IS50$_L$::*phoA* (Tn*phoA*) to obtain insertion mutations in iron-regulated genes of *V. cholerae* 0395. One strain, carrying an insertion mutation in iron-regulated gene *irgA*, had reduced virulence in an animal model and had lost the 77-kilodalton major iron-regulated outer membrane protein. The 50% lethal dose of this mutant strain (MBG40) in suckling mice was 3 × 10^5 bacteria compared with 4 × 10^3 bacteria for 0395 wild type. In an in vivo competition assay, the wild-type strain out competed the mutant almost 10-fold, suggesting a possible colonization defect in MBG40. The in vitro competitive index and in vitro growth curves in low- and high-iron media showed that the mutant strain had no discernable growth defect. These data suggest that the 77-kilodalton iron-regulated outer membrane protein may play a role in the pathogenesis of *V. cholerae* infection. It is not yet clear whether the reduction of virulence in MBG40, as assessed by 50% lethal dose analysis, is explained by a colonization defect or whether it relates to another defect associated with loss of the IrgA protein.

Vibrio cholerae is a gram-negative bacterium that causes a severe secretory diarrheal illness in humans. The organism is found in fecally contaminated fresh and coastal waters. After oral inoculation, bacteria must pass through the gastric acid barrier of the stomach to colonize the small intestine. There, the organism penetrates the mucus gel, adheres to the brush border of intestinal epithelial cells via specific adhesins (Tcp pili and other accessory colonization factors) (25, 33), and produces a number of extracellular secreted proteins, including cholera toxin, neuraminidase, and hemolysin. Full virulence of *V. cholerae* depends on the coordinate regulation of these and perhaps additional, as yet undescribed, pathogenic factors (3).

There is little free iron available in the mammalian host, in contrast to the comparably high levels of free iron present in the environment. The shift from a high- to low-iron environment, therefore, may serve as a signal to the bacterium that it has entered the human host. Several virulence determinants of bacteria are regulated by the concentration of iron in the medium, with increased expression occurring under low-iron conditions. These virulence factors include Shiga toxin of *Shigella dysenteriae* 1 (10), diphtheria toxin of *Corynebacterium diphtheriae* (24), Shiga-like toxin I of enterohemorrhagic *Escherichia coli* (6), certain outer membrane proteins of *Vibrio anguillarum* (1) and *Yersinia* species (8), and exotoxin A of *Pseudomonas aeruginosa* (4, 5).

Iron-regulated virulence factors have not been previously described in *V. cholerae*. Synthesis of several outer membrane proteins (16, 27, 28) and of hemolysin (31) have been shown to be regulated by iron in this organism, but no role in virulence has yet been shown for them. In this report, we describe the use of the transposon vector Tn5 IS50$_L$::*phoA* (Tn*phoA*) to select a series of transposon insertions into iron-regulated genes of *V. cholerae* that are expressed at the cell surface. Tn*phoA* insertion in one of these mutant strains (MBG40) is associated with loss of the major 77-kilodalton (kDa) iron-regulated outer membrane protein. Strain MBG40 has no apparent growth defect in low- or high-iron conditions in vitro but has significantly decreased virulence in a mouse model of cholera infection.

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MATERIALS AND METHODS

Bacterial strains. *V. cholerae* 0395 Sm^r was a gift of John J. Mekalanos, and *E. coli* CC118 has been described previously (6). Mutant strain MBG40 was derived from 0395 by the methods described below. Strains were maintained at −70°C in LB medium (19) containing 15% glycerol.

Media. Two types of media were used to assess the effect of iron concentration on gene expression: (i) LB medium with or without the addition of the iron chelator 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 0.2 mM and (ii) Tris-buffered medium (T medium) (30) prepared with highly purified water (Barnstead Nanopure water purification system [Sybron, Boston, Mass.]) and supplemented with 4 g of sucrose per liter, with or without the addition of 36 μM $FeSO_4$. The concentrations of iron in growth medium were verified by using 1,10-phenanthroline (Aldrich Chemical Co., Inc., Milwaukee, Wis.) (18). By this assay, T medium contained less than 0.5 μM iron and LB medium contained 10 μM iron.

LB agar was used for high-iron plates. Chelex-LB agar supplemented with 0.3% glucose was used for low-iron plates. Chelex-LB agar was prepared by stirring the iron-chelating resin Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) in fivefold concentrated LB medium overnight at 4°C, filtering through Whatman filter paper (no. 1) to remove the resin, and then adding highly purified water and Noble agar (Difco Laboratories, Detroit, Mich.) before sterilization. Streptomycin (100 μg/ml), kanamycin (45 μg/ml), gentamicin (30 μg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (XP) (Amresco; 40 μg/ml) were added where appropriate. XP is a chromogenic substrate for alkaline phosphatase.

Genetic methods. The transposon vector Tn*phoA* was used

to obtain random insertions into the chromosome of *V. cholerae* 0395 by methods previously described (32, 33). Fusion-containing colonies were screened for the $PhoA^+$ phenotype under low-iron growth conditions by the presence or absence of blue color on Chelex-LB agar containing streptomycin, kanamycin, gentamicin, glucose, and XP. They were subsequently screened for iron regulation of alkaline phosphatase activity by streaking on Chelex-LB agar with the same supplements and by adding a filter paper disk spotted with 10 μl of 10 mM $FeSO_4$ in the streak. Strains that showed a distinct zone of white color surrounding the disk, with blue color peripherally, contained fusions in genes whose expression was negatively regulated by iron.

Confirmation of single Tn*phoA* insertions into the chromosome of 0395 was examined by Southern hybridization of digests of chromosomal DNA by using the restriction enzymes *Xba*I and *Eco*RV, which do not cut within the Tn*phoA* insert, and probing with an internal fragment of Tn*phoA* that had been radioactively labeled by random primer extension with a commercially available kit (Prime Time [International Biotechnologies, Inc.]).

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 dipyridyl and in T medium with or without added $FeSO_4$. Alkaline phosphatase activity was calculated as described previously (21) from measurement of hydrolysis of *p*-nitrophenyl phosphate (Amresco) by permeabilized cells. Activity was expressed in units per A_{600} of bacterial cells, with 1 U of activity defined as by O'Callaghan et al. (23).

The amount of cholera toxin produced was assayed by using the GM-1-dependent enzyme-linked immunosorbent assay as previously described by Holmgren (15) with slight modifications. Assays were performed on serial dilutions of supernatant fluids from cells grown to saturation in low- and high-iron media at 30°C, using 96-well GM-1 ganglioside-coated microdilution plates.

Preparation and analysis of outer membrane proteins. Enriched outer membrane proteins were prepared by procedures previously described (14) from cells grown to late logarithmic phase in LB medium with and without added dipyridyl. Outer membrane proteins were separated on sodium dodecyl sulfate–10% polyacrylamide gels and stained with Coomassie blue.

Assessment of virulence by competition and LD_{50} assays. 0395 wild type and mutant strain MBG40 both demonstrated equal plating efficiencies on LB agar and Chelex-LB agar (data not shown). Competition assays between strains were performed essentially as described by Freter et al. (12) and modified by Taylor et al. (33). In vitro competition was determined by growth at 37°C for 24 h in LB medium with dipyridyl, from a starting density of 4×10^5 CFU/ml. In vivo competition was determined by intraintestinal growth in 6- to 7-day-old suckling CD-1 mice (Charles River Breeding Labs, Inc., Wilmington, Mass.) inoculated orally with 4×10^5 CFU. The mice were sacrificed 24 h later. The input ratio was approximately 1.0 in both types of competition experiments; competitive indices were corrected for the input ratio. Viable cell counts and the ratio of the two strains were determined by plating dilutions onto Chelex-LB agar containing streptomycin, glucose, and XP and scoring for the $PhoA^+$ blue colony phenotype of the mutant Tn*phoA* fusion strain and the $PhoA^-$ white colony phenotype of the wild type. The in vivo competitive index was the average of values from five individual mouse experiments.

The 50% lethal dose (LD_{50}) assays were performed by oral inoculation of 3- to 5-day-old suckling CD-1 mice with various doses of viable bacteria grown in LB medium at 30°C, pelleted, washed twice in LB medium, and suspended in 0.15 M $NaHCO_3$ (pH 8.15). Four or more mice were used per dose of bacteria. Survival was determined at 36 h, and results were analyzed as described previously (26).

RESULTS

Tn*phoA* is a derivative of the transposon Tn*5* that contains a portion of *phoA*, the *E. coli* gene for alkaline phosphatase (20). After random insertion of Tn*phoA* into chromosomal DNA, those insertions that yield in-frame fusions between a target gene and *phoA* encode hybrid proteins that have a carboxy-terminal fragment of PhoA fused to an amino-terminal portion of the target protein product. These hybrid proteins display alkaline phosphatase activity only if the target gene encodes a protein expressed at the cell surface (a secreted, transmembrane, or outer membrane protein) and so provides the requisite signals for transport of the carboxy-terminal PhoA fragment into the periplasmic space (20). Because most bacterial virulence determinants are expressed at the cell surface, this technique selects for Tn*phoA* insertions into such genes (11, 17, 25, 32, 33).

Isolation and characterization of Tn*phoA* fusions in iron-regulated genes of *V. cholerae*. (i) Construction and isolation of Tn*phoA* insertion mutant strains. After random insertion of Tn*phoA* into the chromosome of *V. cholerae* 0395, colonies were screened for the $PhoA^+$ phenotype by blue color on a low-iron agar plate containing XP. Of 300 individual blue colonies examined, we obtained 25 Tn*phoA* insertion mutant strains whose blue colony phenotype was repressed around an iron-containing disk.

(ii) Alkaline phosphatase assays. Iron regulation of these Tn*phoA* gene fusions was confirmed by measuring alkaline phosphatase activity after growth in LB medium with or without added dipyridyl (Table 1). All strains showed a significant increase in alkaline phosphatase activity in low-iron conditions compared with high-iron conditions, with induction ratios ranging from 6-fold (MBG38) to more than 850-fold (MBG40). The differing alkaline phosphatase activities and induction ratios of the 25 mutant strains suggest that we had isolated Tn*phoA* insertions into a number of different genes on the *V. cholerae* chromosome. To confirm further the iron regulation of the Tn*phoA* gene fusion in MBG40, alkaline phosphatase activity of both this strain and wild-type 0395 was also determined after growth in T medium with and without added iron: alkaline phosphatase activity of MBG40 increased from 1 U after growth in iron-supplemented T medium to 126 U after growth in T medium without added iron. As expected, wild-type 0395 had low levels of alkaline phosphatase activity in both low- and high-iron growth conditions.

(iii) Outer membrane proteins. Since most bacterial virulence determinants are expressed at the cell surface, we analyzed outer membrane proteins for wild-type 0395 and each of the 25 mutant strains after growth in low- and high-iron media. In wild-type 0395, at least four new proteins of apparent molecular mass from 75 to 81 kDa appeared after growth in low-iron conditions compared with high-iron conditions (Fig. 1). The most prominent of these iron-regulated proteins, with an apparent molecular mass of 77 kDa, was lost in two of the 25 mutant strains: MBG15 (data not shown)

TABLE 1. Alkaline phosphatase assays in LB medium with and without added dipyridyl

Strain	Alkaline phosphatase activity (U/A_{600})		Induction ratio
	Without dipyridyl	With dipyridyl	
0395	1	1	1
MBG13	2	773	387
MBG14	1	50	50
MBG15	1	689	689
MBG17	3	1,147	382
MBG18	3	39	13
MBG19	1	113	113
MBG20	1	268	268
MBG21	1	259	259
MBG22	1	338	338
MBG23	1	386	386
MBG24	1	370	370
MBG25	1	353	353
MBG26	1	300	300
MBG27	6	344	57
MBG28	1	620	620
MBG29	1	318	318
MBG30	1	437	437
MBG31	3	72	24
MBG33	1	136	136
MBG34	1	86	86
MBG35	1	25	25
MBG37	2	197	99
MBG38	100	575	6
MBG39	4	39	10
MBG40	1	857	857

and MBG40 (Fig. 1). Several other mutant strains had lost other individual iron-regulated outer membrane protein bands (data not shown).

(iv) Mapping of Tn*phoA* insertions to single chromosomal fragments. Because *Xba*I and *Eco*RV do not cut within the Tn*phoA* vector, Southern hybridization of *Xba*I- or *Eco*RV-digested chromosomal DNA, probed with an internal fragment of Tn*phoA*, was performed for each of the 25 mutant strains. This analysis verified that only single Tn*phoA* chromosomal insertions are present in each of the mutant strains (data not shown). Moreover, single Tn*phoA* insertions were present in at least eight distinct *Xba*I chromosomal fragments, ranging in size from 9.0 to 30.0 kilobases of DNA (including the inserted Tn*phoA* [7.6 kilobases]); there was no hybridization signal for chromosomal DNA of parent 0395 (data not shown). Figure 2 shows a Southern blot of *Eco*RV-digested chromosomal DNA from 10 of the mutant strains, including MBG40.

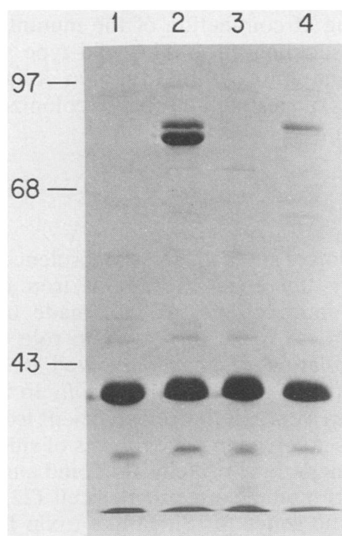

FIG. 1. Outer membrane proteins for *V. cholerae* 0395 wild type and MBG40 grown in both low- and high-iron media. Lanes: 1, 0395 wild type grown in high-iron medium; 2, 0395 wild type grown in low-iron medium; 3, MBG40 grown in high-iron medium; 4, MBG40 grown in low-iron medium. The numbers to the left of the gel indicate the apparent molecular masses (in kilodaltons) of the bands.

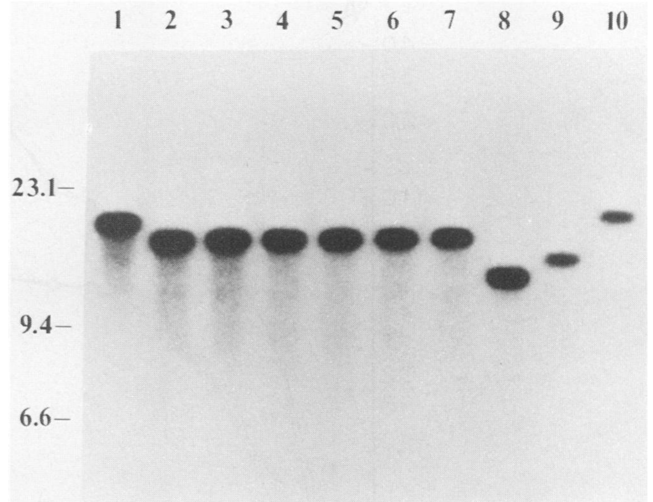

FIG. 2. Southern blot analysis of chromosomal DNA from 10 mutant strains, digested with *Eco*RV and probed with a ^{32}P-labeled internal fragment of Tn*phoA*. Lanes: 1, MBG18; 2, MBG19; 3, MBG20; 4, MBG21; 5, MBG24; 6, MBG25; 7, MBG26; 8, MBG34; 9, MBG37; 10, MBG40. *Eco*RV does not cut within Tn*phoA*. The numbers to the left of the gel indicate the sizes (in kilobases) of the bands.

We selected strain MBG40 for further study for the following two reasons. (i) The expression of the gene fusion in this strain, as measured by alkaline phosphatase activity, was highly regulated by iron, with an induction ratio of more than 850 after growth in low-iron conditions compared with high-iron medium (Table 1). (ii) The Tn*phoA* insertion in strain MBG40 was associated with loss of the major 77-kDa iron-regulated outer membrane protein of *V. cholerae* 0395 (Fig. 1). The iron-regulated gene in strain MBG40 that contains the Tn*phoA* insertion was designated *irgA*.

In vitro characterization of iron-regulated (*irgA*) fusion strain MBG40. (i) In vitro growth curves. To determine whether MBG40 was defective for growth in vitro, growth curves of 0395 wild type and MBG40 in LB medium with and without added dipyridyl and in T medium with and without added iron were determined. The two strains grew similarly in each in vitro growth condition (Fig. 3). The enhancement of growth for both strains after the addition of iron to T medium demonstrates that growth in unsupplemented T medium was indeed limited by iron.

(ii) In vitro competition assays. To determine whether MBG40 was able to compete effectively in vitro with 0395 wild type, in vitro competition of the two strains was performed in LB medium with added dipyridyl. Low-iron media was chosen because previous data has suggested that the intraintestinal environment has limited iron availability (27). The in vitro competitive index of 0.98 (Table 2) con-

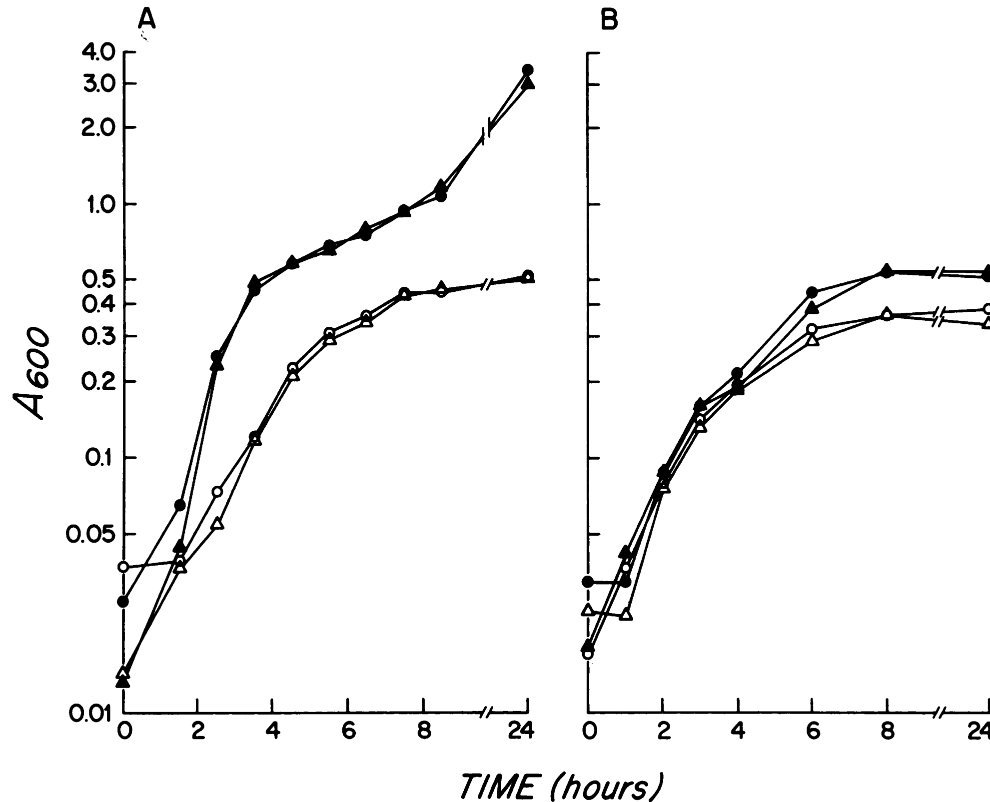

FIG. 3. In vitro growth curves of 0395 wild type and MBG40 in low- and high-iron media. (A) Growth in LB medium with and without added dipyridyl. (B) Growth in T medium with and without added iron. Symbols: ●, 0395 wild type in high-iron medium; ○, 0395 wild type in low-iron medium; ▲, MBG40 in high-iron medium, △, MBG40 in low-iron medium.

firmed the results of the in vitro growth curves, suggesting the absence of an in vitro growth defect in the mutant strain.

(iii) Cholera toxin assays. To control for any differences in cholera toxin production that might affect the virulence of parental and mutant strains under different growth conditions, cholera toxin activity was assayed from the supernatant fluids of 0395 and MBG40 grown to saturation in T medium with and without added iron and in LB medium with and without added dipyridyl. Cholera toxin activity of the two strains was similar under the various growth conditions (data not shown).

In vivo characterization of iron-regulated (*irgA*) fusion strain MBG40. (i) LD_{50} assays. To determine whether *irgA* has a role in virulence of 0395, LD_{50} assays of 0395 wild type and MBG40 were performed. The LD_{50} of mutant strain MBG40 was 3×10^5 bacteria, compared with 4×10^3 bacteria for parental strain 0395 (Table 2). This increase in LD_{50} of 2 orders of magnitude clearly suggests that *irgA* is important for virulence in this animal model. Previous data have shown that 0395 strains carrying either a randomly selected chromosomal Tn*phoA* insertion or Tn*phoA* insertion in the structural gene for the outer membrane protein OmpV maintain full virulence for suckling mice (33), demonstrating that chromosomal Tn*phoA* insertion by itself has no effect on virulence.

TABLE 2. Virulence assays of wild-type and mutant strains

Strain	Competing strain	Competitive index		LD_{50} (no. of bacteria)
		In vitro	In vivo	
0395				4×10^3
MBG40	0395	0.98	0.11	3×10^5

(ii) In vivo competition assays. Mutant strain MBG40 was tested for a colonization defect by an in vivo competition assay consisting of coinfection of the mutant with parental strain 0395 in suckling mice. The wild-type strain outcompeted the mutant almost 10-fold (in vivo competitive index, 0.11 [Table 2]), suggesting a probable colonization defect in the mutant strain.

DISCUSSION

The production of several bacterial virulence determinants is regulated by the concentration of iron in the growth medium, with more of the product made under low-iron conditions (1, 4, 5, 6, 8, 10, 24). The role of iron in the coordinate regulation of genes involved in virulence has been most thoroughly studied in *E. coli*. In this organism, several genes are repressed when sufficient iron is available, including genes involved in biosynthesis of siderophores and production of membrane proteins that bind and transport the iron-siderophore complexes into the cell (22). In addition, expression of the genes for Shiga-like toxin I in *E. coli* are similarly repressed by iron (6). The coordinate regulation of these diverse genes by iron depends on a single regulatory gene, *fur* (2, 6, 7, 9, 13, 14), whose protein product acts to repress transcription of these genes when sufficient iron is present. We wished to understand whether a similar system of iron-regulated virulence determinants existed in *V. chol-*

erae and whether the mechanism of iron regulation in this organism was similar to that of *E. coli*.

Sigel and Payne (28) have described five iron-regulated outer membrane proteins in *V. cholerae*, having apparent molecular masses of 62, 73, 75, 76, and 77 kDa. Sigel et al. (29) have found that *V. cholerae* mutants with defects in the synthesis of the siderophore vibriobactin or its transport into the cell retain full virulence, as measured by in vivo fluid accumulation in the mouse intestine, suggesting that these genes involved in iron metabolism are not required for virulence. In addition, Sciortino and Finkelstein (27) have described seven iron-regulated outer membrane proteins in several *V. cholerae* strains, having apparent molecular masses of 16, 18, 25, 46, 48, 58, 66, and 68 kDa. Jonson et al. (16) have described eight envelope proteins in *V. cholerae* 01 that are induced during in vivo cultivation in rabbits and are immunogenic, but which do not appear to be regulated by iron. The specific role of any of these outer membrane or envelope proteins in the virulence of *V. cholerae* has not been previously examined.

We have described a Tn*phoA* insertion in *V. cholerae* 0395 into a gene (*irgA*) that is regulated by iron and that affects the virulence of this organism. Mutant strain MBG40 has an LD_{50} in suckling mice that is 2 orders of magnitude higher than that for parental strain 0395. Production of cholera toxin by MBG40 is similar to that of the wild type, suggesting that the virulence defect of the mutant is distinct from cholera toxin.

Phenotypically, strain MBG40 shows loss of a single 77-kDa iron-regulated outer membrane protein. Because our Tn*phoA* mutagenesis procedure selects for insertions in genes encoding surface proteins and a single outer membrane protein appears to be lost in strain MBG40, we favor the hypothesis that *irgA* is the structural gene for this outer membrane protein. We cannot exclude the possibility that *irgA* is a regulatory gene or that Tn*phoA* inserted in *irgA* acts by a polar effect on the expression of a downstream gene. If *irgA* were a regulatory gene, we might have expected to see a more global regulatory effect, with a change in the expression of more than one iron-regulated outer membrane protein, as occurs with the *fur* regulatory locus in *E. coli* (13). The virulence determinant lacking in strain MBG40 may be the 77-kDa iron-regulated outer membrane protein.

Competition of the mutant and wild-type strains in vivo produces a competitive index of 0.11, suggesting that the mutant may be defective in its ability to colonize the mouse intestine and that the 77-kDa major iron-regulated outer membrane protein may play some role in the colonization process. It is not yet clear whether the reduction of virulence in the mutant strain as assessed by LD_{50} is explained by this colonization defect or whether it relates to another defect associated with loss of the IrgA protein.

The molecular mechanism of iron regulation of this virulence determinant and of other iron-regulated genes in *V. cholerae* is unknown. These factors may be coordinately regulated in a fashion similar to the coordinate regulation of many iron-regulated genes by the *fur* locus in *E. coli*. Further experiments to define the mechanism of iron regulation in *V. cholerae* are under way.

We have described a simple technique for the identification of iron-regulated genes in *V. cholerae*. Tn*phoA* is randomly inserted in the chromosome, giving rise to fusions between target genes and the *E. coli* gene for alkaline phosphatase. Hybrid proteins encoded by such gene fusions display alkaline phosphatase activity only if the target gene product is a protein expressed at the cell surface (20). Screening of mutant strains on low-iron agar for alkaline phosphatase activity that is repressed around an iron-containing disk allows rapid isolation of Tn*phoA* insertions in iron-regulated genes expressed at the cell surface. This technique can also be applied to the identification of iron-regulated genes in other bacterial species.

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