JOURNAL OF BACTERIOLOGY, Apr. 2000, p. 2350–2353 Vol. 182, No. 8
0021-9193/00/$04.00+0

Identification of an Operon Required for Ferrichrome Iron Utilization in *Vibrio cholerae*

MARC B. ROGERS,[1]† JESSICA A. SEXTON,[1]‡ G. JOEL DECASTRO,[1]§ AND STEPHEN B. CALDERWOOD[1,2]*

Infectious Disease Division, Massachusetts General Hospital, Boston, Massachusetts 02114,[1] and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 15 November 1999/Accepted 24 January 2000

Mutagenesis of *Vibrio cholerae* with Tn*phoA*, followed by screening for fusions that were activated under low-iron conditions, led to the identification of seven independent fusion strains, each of which was deficient in the ability to utilize ferrichrome as a sole iron source for growth in a plate bioassay and had an insertion in genes encoding products homologous to *Escherichia coli* FhuA or FhuD. Expression of the gene fusions was independent of IrgB but regulated by Fur. We report here a map of the operon and the predicted amino acid sequence of FhuA, based on the nucleotide sequence. Unlike those of the *E. coli fhu* operon, the *V. cholerae* ferrichrome utilization genes are located adjacent and opposite in orientation to a gene encoding an ATP-binding cassette transporter homolog, but this gene, if disrupted, does not affect the utilization of ferrichrome in vitro.

Vibrio cholerae requires iron (0.5 to 1 μM) in a bioavailable form for growth and survival within the environment and within an animal host. Iron not only is necessary for bacterial multiplication within the host but also serves as an important environmental signal that regulates expression of other virulence determinants unrelated to iron acquisition (for reviews, see references 8, 13, 14, and 17). Many iron transport systems characterized to date involve iron-repressible outer membrane proteins (IROMPs) which bind a specific iron-containing compound and transport either free iron or the iron-bound ligand into the cell. Expression of many of these receptor proteins is mediated at the transcriptional level by the iron-binding repressor protein called Fur (ferric uptake regulator), which requires ferrous iron as a cofactor and acts as a repressor when environmental iron levels are high. Homologs of the *Escherichia coli fur* gene have been identified for at least 32 other bacterial species, including *V. cholerae* and *Vibrio vulnificus* (9, 11).

When *V. cholerae* is grown in vitro under iron-restricted conditions, the catechol siderophore vibriobactin is produced, as are six or more IROMPs. Only three of these IROMPs, ViuA, HutA, and IrgA, have been characterized. ViuA is the 74-kDa ferric vibriobactin receptor that allows internalization of iron from vibriobactin by an undefined mechanism (1, 20). HutA, HutB, and TonB1 have been characterized, and are all required for utilization of heme by *V. cholerae* (16). IrgA is a 77-kDa IROMP of unknown function that shares significant homology to TonB-dependent outer membrane proteins of gram-negative bacteria (4). Strains with a mutation in *irgA* show no defect in transport or utilization of iron from vibriobactin, heme, hemoglobin, ferrichrome, or ferric citrate, yet they show a 100-fold virulence defect in an infant mouse model of cholera (5). In vivo, a mutation in *irgA* leads to a more severe growth defect than a mutation in either *hutA* or *viuA* (21). The *irgA* gene is regulated by a positive transcriptional activator of the LysR family called IrgB (3). TonB1 is required not only for utilization of heme by *V. cholerae* but also for utilization of ferrichrome iron (16). The receptor for ferrichrome, however, has remained unidentified. We now present evidence that *V. cholerae* contains an operon consisting of genes homologous to those in the *E. coli* ferrichrome utilization system and that the products of these genes are required for the utilization of ferrichrome as a sole iron source in vitro.

The strains used in this study are listed in Table 1. Tn*phoA* fusions to iron-regulated genes of classical *V. cholerae* strain O395 were constructed and screened as described previously (5). Mutant strains containing Tn*phoA* fusions activated under low-iron conditions were individually screened for the ability to grow with ferrichrome as a sole iron source, using a growth stimulation assay (20). Ferrichrome is a siderophore produced by the rust fungus *Ustilago sphaerogena* and was purchased from Sigma Chemical Co. (no. F8014; St. Louis, Mo.). Ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) was deferrated as described previously (20) and incorporated into Luria-Bertani (LB) agar in such a way that there was no growth of the indicator strain in the absence of a usable exogenous iron source placed on the surface of the agar. This usually required 30 μg of deferrated EDDA/ml, with the indicator strain seeded at 10^5 CFU/ml. Indicator plates were spotted with 10 μl of various iron sources, and after 24 h, zones of growth were measured. Unlike the parent strain, O395, seven Tn*phoA* fusion strains were found to produce no zone of growth specifically around either 1 or 10 mM ferrichrome but were able to utilize vibriobactin (produced by the O395 strain), 1 mM hemin, 0.233 mM hemoglobin, or 36 mM ferric sulfate normally. Results for three representative fusion strains and controls are listed in Table 2. MBG14, deficient in the vibriobactin receptor, was unable to utilize vibriobactin but could grow in the presence of all other iron sources tested, including ferrichrome, as reported previously (20). CA40130, a vibri-

* Corresponding author. Mailing address: Infectious Disease Division, Jackson 504, Massachusetts General Hospital, 55 Fruit St., Boston, MA 02114. Phone: (617) 726-3811. Fax: (617) 726-7416. E-mail: scalderwood@partners.org.

† Present address: EcoSoil Systems, Inc., San Diego, CA 92127.

‡ Present address: Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110.

§ Present address: College of Physicians and Surgeons, Columbia University, New York, NY 10032.

TABLE 1. Bacterial strains used in this study

V. cholerae strain	Relevant characteristics[a]	Reference
O395	Classical; Sm^r	15
MBG14	O395 *viuA*::Tn*phoA* Sm^r Kn^r	5
MBG40	O395 *irgA*::Tn*phoA* Sm^r Kn^r	5
CML19	O395 *fur*::Kn^r Sm^r Kn^r	10
CA40130	Classical CA401 vibriobactin synthesis mutant	19
BE	O395 *fhuA*::Tn*phoA* Sm^r Kn^r	This study
BG	O395 *fhuA*::Tn*phoA* Sm^r Kn^r	This study
JAS73	O395 *fhuA*::Tn*phoA* Sm^r Kn^r	This study
JAS73 *fur*	JAS73 *fur*::pCML13 Sm^r Kn^r Ap^r	This study
JAS73 *irgB*	JAS73 *irgB*::pMBG111 Sm^r Kn^r Ap^r	This study
PAC6[b] (MBG18)	O395 *fhuD*::Tn*phoA* Sm^r Kn^r	5
PAC6 *fur*	PAC6 *fur*::pCML13 Sm^r Kn^r Ap^r	This study
PAC6 *irgB*	PAC6 *irgB*::pMBG111 Sm^r Kn^r Ap^r	This study
PAC10 (MBG39)	O395 *fhuD*::Tn*phoA* Sm^r Kn^r	5
PAC12 (BD)	O395 *fhuA*::Tn*phoA* Sm^r Kn^r	5
PAC13 (MBG27)	O395 *fhuA*::Tn*phoA* Sm^r Kn^r	5

[a] Abbreviations: Ap, ampicillin; Gn, gentamicin; Kn, kanamycin; Sm, streptomycin.

[b] PAC strains were derived from the previously isolated iron-regulated Tn*phoA* insertion strains shown (5), by curing of the Gn^r delivery plasmid pPH1JI.

TABLE 2. Utilization of various iron sources by wild-type and mutant *V. cholerae*[a]

Indicator strain	Growth on:					
	O395 supernatant (vibriobactin)	Hemin	Hemoglobin	Ferrichrome		$FeSO_4$
				1 mM	10 mM	
O395	+	+	+	+	+	+
CA40130	+	+	+	+	+	+
MBG14	−	+	+	+	+	+
MBG40	+	+	+	+	+	+
PAC6	+	+	+	−	−	+
PAC12	+	+	+	−	−	+
JAS73	+	+	+	−	−	+

[a] Zones of growth (+) were all >5 mm in diameter. Indicator strains were seeded into low-iron LB agar containing 30 μg of deferrated EDDA/ml at a density of approximately 10^5 CFU/ml. Iron sources (10 μl), including culture supernatant of O395 containing vibriobactin, 1 mM hemin, 0.233 mM hemoglobin, 1 and 10 mM ferrichrome, and 36 mM ferric sulfate, were spotted onto the surface of the agar.

obactin biosynthetic mutant, was able to grow on all five iron sources tested, as was MBG40, a strain with a mutation in *irgA*.

Chromosomal DNA was isolated from the seven fusion strains and was digested with *Xba*I and *Eco*RV, which do not cut within Tn*phoA*, and Southern analysis was performed as previously described (5), using a probe internal to Tn*phoA*, to demonstrate that each strain contained only a single insertion (data not shown). Inverse PCR was used to obtain DNA flanking the Tn*phoA* insertions as follows. Three to four micrograms of chromosomal DNA from each mutant strain was digested with various enzymes, including *Xho*I, *Pst*I, *Sac*II, *Sfu*I, and *Taq*I, followed by phenol-chloroform extraction and ethanol precipitation. Aliquots (25 to 50 ng) of this digested DNA were self ligated in 50-μl reaction mixtures with 1 U of T4 DNA ligase (Boehringer Mannheim), followed by heat inactivation at 70°C for 10 min. Aliquots of the ligation reaction product were then used in PCRs with 200 to 250 pmol of each primer (divergently oriented and both binding to either the 5′ or 3′ end of Tn*phoA*). Flanking DNA both upstream (*Sfu*I derived) and downstream (*Taq*I derived) of the PAC12 insertion revealed homology to a 626-bp fragment (gvc.dg01f) in the nonannotated genomic DNA sequence collection of *V. cholerae* El Tor N16961 at The Institute for Genomic Research (TIGR). This collection of 5,523 sequences was downloaded from the TIGR website into a local server in the Department of Molecular Biology, Massachusetts General Hospital, in August 1997 and used for additional analyses described below. BLASTX analysis of this fragment in turn displayed 23% identity and 43% similarity to the *E. coli* FhuA protein, amino acids 245 to 415 (of 747 total). Similarly, inverse-PCR products from fusion strain PAC13 also indicated that Tn*phoA* had been inserted into the region encompassed by gvc.dg01f (Fig. 1).

We then used a λZAP II phagemid library containing 5- to 10-kbp fragments from *V. cholerae* O395, derived by partial *Sau*3A1 digestion (courtesy of Shelley Trucksis), to isolate larger genomic fragments with homology to inverse-PCR fragments from the remaining five fusions, which showed no similarity to any of the 5,523 TIGR fragments available at that

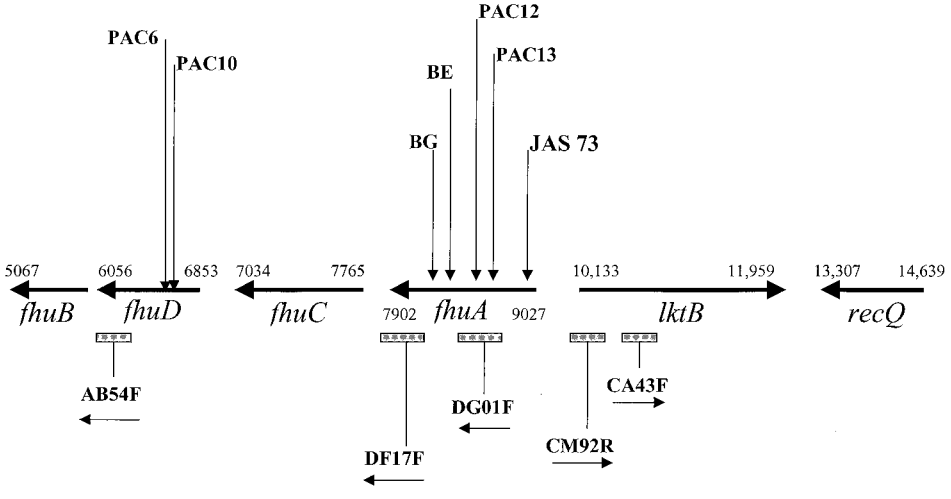

FIG. 1. Map (not to scale) of the *V. cholerae* O395 gene cluster with homology to *fhuACDB* of *E. coli*. Shown are bp 5067 to 14639 (of 48,695) from *V. cholerae* N16961 TIGR contig asm818 (see URL in the text). Locations of the seven Tn*phoA* fusions are indicated with arrows, and original TIGR fragments are indicated with boxes. Open reading frame designations other than *fhuA* are presumptive, based on TIGR contig asm818 regions of similarity to the *E. coli fhu* genes (see the text).

FIG. 2. Amino acid sequence alignment of *E. coli* FhuA (eco), *S. enterica* serovar Paratyphi B FhuA (spara), *P. agglomerans* FhuA (paa) (6), *Erwinia chrysanthemi* ferrichrysobactin receptor (fct) (18), *B. japonicum* FegA (feg) (7), and *V. cholerae* O395 FhuA (Vc), using the Pileup program in the Genetics Computer Group package with the default settings. Amino acids representing the adjacent loop and the gating loop are shaded, as are the TonB box sequences, as in reference 6. Only the first 525 to 572 residues of each protein are shown.

time. Restriction mapping of overlapping phage clones was combined with PCR analysis using gvc.dg01f as a reference point. Ordering of the Tn*phoA* fusions was accomplished using PCR analysis with a primer to the 3′ end of Tn*phoA* and an opposing primer to the 3′ end of TIGR fragment gvc.ab54f, resulting in the map shown in Fig. 1. This provided the first suggestion of an operon structure, with the JAS73 fusion as the most proximal fusion relative to the orientation of the *fhuA* ortholog. In addition, the fact that all seven Tn*phoA* fusions were inserted into either *fhuA* or *fhuD* homologs is consistent with the requirement for the N terminus of the fusion protein to provide a signal sequence for the export of PhoA to the periplasm or outer membrane to yield an active fusion protein in our screen. It is thus not surprising that no fusions were isolated from *fhuC* or *fhuB*, which are localized to the cytoplasmic membrane.

Appropriate regions of phage clones encompassing the *fhuA* ortholog were amplified by PCR and sequenced. The DNA sequence of this gene from strain O395 was very similar to that of *V. cholerae* El Tor strain N16961, now available in the TIGR database, bp 229508 to 231607 of contig 1752 (chromosome 1). There were two areas of substantial difference in otherwise virtually identical predicted proteins, however: amino acids (aa) 193 to 200 (ITRIKTVP) in O395 were DYANQDGS in the N16961 sequence in the TIGR database, and aa 214 to 223 (WAVERYAKPA) in O395 were GQLNG*TQTS in the TIGR database (the asterisk denotes a stop codon). Each of these areas of substantial difference resulted from frameshift mutations in the sequences in N16961 compared to sequences in O395. It is uncertain if these frameshifts (and the resulting stop codon at amino acid 219 of FhuA in N16961) are correct or might represent sequencing errors yet to be corrected in the TIGR database. The *fhuA* open reading frame from strain O395 was 2,100 bp, encoding a protein of 700 aa. The predicted FhuA protein sequence shared homologies with a variety of siderophore receptors from many bacteria but was most similar to *E. coli* FhuA (AE000124) (33% identity; 53% similarity), *Bradyrhizobium japonicum* FegA (U61401) (34% identity; 53% similarity), the ferrichrome receptors for *Pantoea* (*Enterobacter*) *agglomerans* (Y14026) and *Salmonella enterica* serovar Paratyphi (Y14067) (33% identity; 52% similarity), and a hydroxamate-type ferrisiderophore receptor for *Pseudomonas aeruginosa* (AF051691) (36% identity; 54% similarity). The peptide sequence is shown aligned to known FhuA homologs in Fig. 2. Certain features were conserved, but the largest differences were seen in a region known to form the "gating loop" (6); the significance of these differences for FhuA function in *V. cholerae* is uncertain. It also appeared that the *V. cholerae* FhuA protein may contain an "adjacent loop" that may be an alternate binding site that allows the transport of ferrichrome and albomycin (6).

We examined whether expression of the *fhu* operon was regulated by Fur in an iron-dependent fashion (as in other organisms) and analyzed whether the *V. cholerae* transcriptional activator IrgB played a role in expression of this operon. Fusion strains JAS73 (which has the most proximal insertion in *fhuA*) and PAC6 (which has the most distal insertion in *fhuD*) were chosen for this analysis. For each strain, either *irgB* or *fur* was disrupted by allelic exchange with pMBG111 or pCML13, respectively, as described previously (3, 9), and these mutations were confirmed by Southern hybridization (data not shown). Alkaline phosphatase assays were performed using strains grown overnight in LB medium with or without 2,2-dipyridyl (200 μM) to limit iron availability. Results shown in Table 3 clearly indicate that both fusions were induced 12- and 16-fold under low-iron conditions but were more constitutively expressed if *fur* was disrupted. Disruption of *irgB* had no effect on the expression of the fusions. In addition, Northern blot analysis of *fhuA* expression was performed using total RNA isolated from O395 grown in LB medium (high iron) and LB

TABLE 3. Alkaline phosphatase activities of representative fusion strains and their corresponding regulatory mutant strains under high- and low-iron conditions

Strain	Activity (U/OD_{600}) in[a]:		Avg fold induction[b]
	High-iron medium	Low-iron medium	
JAS73	21 (2.5)	223 (18)	11
JAS73::*irgB*	25 (3.7)	252 (84)	10
JAS73::*fur*	123 (16)	320 (42)	2.6
PAC6	3 (0.8)	40 (5.0)	13
PAC6::*irgB*	3 (0)	52 (34)	17
PAC6::*fur*	15 (2.1)	36 (1.3)	2.4

[a] Values are the means of at least five separate experiments, with standard errors of the means in parentheses. OD_{600}, optical density at 600 nm. High-iron medium is LB broth and low-iron medium is LB with 200 μM 2,2-dipyridyl.
[b] Between low- and high-iron media.

medium with 200 μM 2,2-dipyridyl (low iron), probed with a 593-bp PCR fragment internal to TIGR fragment gvc.dg01f (bp 23 to 615). As a control, total RNA was isolated from a *fur* mutant derivative, CML19, under high- and low-iron conditions and probed in parallel. A hybridization signal was detected in wild-type cells under low-iron conditions only but under both low- and high-iron conditions in CML19 (data not shown).

More recent searching of the nonannotated *V. cholerae* N16961 genomic sequence at the TIGR website (http://www.tigr.org/cgi-bin/BlastSearch/blastcgi?organism=v_cholerae) revealed a much larger, 48,695-bp contig, asm818, that contained the entire ferrichrome uptake operon and neighboring genes. This region (bp 5067 to 14639) is shown in Fig. 1 (not to scale). The *V. cholerae* genome can now be searched as two contigs: 1752, which is 2,962,721 bp, and 1741, which is 1,072,915 bp (see URL above); the *fhu* operon is in contig 1752. BLASTX searches (GenBank release 112.0) using portions of contig asm818, bp 5067 to 14639, as a query allowed us to establish the positions and orientations of the remainder of the *fhu* operon. Interestingly, bp 10133 to 11959 shared 50% identity with and 65% similarity to *H. influenzae* LktB aa 5 to 612 (of 614 aa). An internal 1,167-bp portion of *lktB* was amplified by PCR, cleaved with *Hin*cII (this encompasses the region encoding aa 125 to 511), blunt end ligated into the *Eco*RV site of suicide vector pGP704 (3), and integrated into the O395 chromosome by a single crossover as previously described (9); integration was confirmed by Southern analysis (data not shown). Insertion into this gene did not abrogate the ability to use ferrichrome in a growth stimulation assay (data not shown).

In summary, multiple Tn*phoA* fusions to iron-regulated genes were isolated. Inverse PCR was used to determine that seven fusions had been inserted into genes with similarity to *E. coli fhuA* and *fhuD*. Larger genomic fragments isolated from a phagemid library showed that these genes were clustered into an operon structure. The seven fusions were mapped and ordered with respect to the most distal known TIGR sequence at the time (gvc.ab54f) by PCR. All Tn*phoA* insertions prevented the utilization of ferrichrome (but not other substances) as an iron source in a plate growth stimulation assay. The ability to bind and transport vibriobactin was not required for ferrichrome utilization. The most proximal fusion in *fhuA* and the most distal fusion in *fhuD* were shown to be regulated by Fur (but not IrgB) at the level of transcription. The gene for an interesting ABC transporter protein homologous to LktB was identified immediately upstream of and opposite in orientation to *fhuA* in *V. cholerae*. Disruption of this gene, however, did not affect ferrichrome utilization in a plate bioassay. The sequence of the 700-aa *V. cholerae* FhuA protein adds to the large amount of information on this multifunctional outer membrane protein, a paradigm for ligand-specific gated channel proteins (2, 6, 12).

Nucleotide sequence accession number. The sequence of O395 *fhuA* determined here has been submitted to GenBank (accession no. AF203702).

This work was supported by NIAID grant R01AI34968 (S.B.C.) and NIDDK grant F32 DK09651 (M.B.R.).

We thank Shelley Trucksis for the packaged λZAP II O395 library. Preliminary sequence data were obtained from the TIGR website at http://www.tigr.org. Sequencing of *V. cholerae* N16961 by TIGR was accomplished with support from NIAID.

REFERENCES

1. **Butterton, J. R., J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of *viuA*, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3729–3738.
2. **Ferguson, A. D., E. Hofmann, J. W. Coulton, K. Diederichs, and W. Welte.** 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science **282:**2215–2220.
3. **Goldberg, M. B., S. A. Boyko, and S. B. Calderwood.** 1991. Positive transcriptional regulation of an iron-regulated virulence gene in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **88:**1125–1129.
4. **Goldberg, M. B., S. A. Boyko, J. R. Butterton, J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Characterization of a *Vibrio cholerae* virulence factor homologous to the family of TonB-dependent proteins. Mol. Microbiol. **6:**2407–2418.
5. **Goldberg, M. B., V. J. DiRita, and S. B. Calderwood.** 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect. Immun. **58:**55–60.
6. **Killmann, H., C. Herrmann, H. Wolff, and V. Braun.** 1998. Identification of a new site for ferrichrome transport by comparison of the FhuA proteins of *Escherichia coli*, *Salmonella paratyphi* B, *Salmonella typhimurium*, and *Pantoea agglomerans*. J. Bacteriol. **180:**3845–3852.
7. **LeVier, K., and M. L. Guerinot.** 1996. The *Bradyrhizobium japonicum fegA* gene encodes an iron-regulated outer membrane protein with similarity to hydroxamate-type siderophore receptors. J. Bacteriol. **178:**7265–7275.
8. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
9. **Litwin, C. M., and S. B. Calderwood.** 1993. Cloning and genetic analysis of the *Vibrio vulnificus fur* gene and construction of a *fur* mutant by in vivo marker exchange. J. Bacteriol. **175:**706–715.
10. **Litwin, C. M., and S. B. Calderwood.** 1994. Analysis of the complexity of gene regulation by Fur in *Vibrio cholerae*. J. Bacteriol. **176:**240–248.
11. **Litwin, C. M., S. A. Boyko, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897–1903.
12. **Locher, K. P., B. Rees, R. Koebnik, A. Mitschler, L. Moulinier, J. P. Rosenbusch, and D. Moras.** 1998. Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell **95:**771–778.
13. **Martinez, J. L., A. Delgado-Iribarren, and F. Baguero.** 1990. Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol. Rev. **75:**45–56.
14. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
15. **Mekalanos, J. J., D. J. Swartz, and G. D. N. Pearson.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature **306:**551–557.
16. **Occhino, D. A., E. E. Wyckoff, D. P. Henderson, T. J. Wrona, and S. M. Payne.** 1998. *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB*, *exbB*, *exbD* genes. Mol. Microbiol. **29:**1493–1507.
17. **Payne, S. M.** 1988. Iron and virulence in the family Enterobacteriaceae. Crit. Rev. Microbiol. **16:**81–111.
18. **Sauvage, C., T. Franza, and D. Expert.** 1996. Analysis of the *Erwinia chrysanthemi* ferrichrysobactin receptor gene: resemblance to the *Escherichia coli fepA-fes* bidirectional promoter region and homology with hydroxamate receptors. J. Bacteriol. **178:**1227–1231.
19. **Sigel, S. P., J. A. Stoebner, and S. M. Payne.** 1985. Iron-vibriobactin transport system is not required for virulence of *Vibrio cholerae*. Infect. Immun. **47:**360–362.
20. **Stoebner, J. A., J. R. Butterton, S. B. Calderwood, and S. M. Payne.** 1992. Identification of the vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3270–3274.
21. **Tashima, K. T., P. A. Carroll, M. B. Rogers, and S. B. Calderwood.** 1996. Relative importance of three iron-regulated outer membrane proteins for in vivo growth of *Vibrio cholerae*. Infect. Immun. **64:**1756–1761.