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Cloning and Genetic Analysis of the *Vibrio vulnificus fur* Gene and Construction of a *fur* Mutant by In Vivo Marker Exchange

CHRISTINE M. LITWIN[1]* AND STEPHEN B. CALDERWOOD[1,2]

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

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***Vibrio vulnificus* infections have been associated with iron overload and preexisting liver disease. Iron may play a major role in the pathogenesis of *V. vulnificus* infections. Many virulence genes, as well as genes involved in the transport of iron by bacteria, are regulated by iron, with increased expression under low-iron conditions. In *Escherichia coli* and *Vibrio cholerae*, transcriptional regulation by iron depends on the *fur* gene. We utilized Southern hybridization under low- and high-stringency conditions with both *E. coli* and *V. cholerae fur* gene probes to demonstrate that there are *fur*-homologous sequences in the DNAs of *V. vulnificus*, *Vibrio fischeri*, and *Aeromonas* sp. but not in the DNAs of the other bacterial species tested. We developed a restriction map and cloned the *fur*-homologous sequence from *V. vulnificus*. The hybridizing clone of *V. vulnificus* chromosomal DNA complemented a *V. cholerae fur* mutant. DNA sequence analysis confirmed the presence of a 149-amino-acid open reading frame that was 77% homologous to *E. coli* Fur and 93% homologous to *V. cholerae* Fur. Primer extension localized a single promoter for the *V. vulnificus fur* gene. Northern (RNA) blot analysis and β-galactosidase assays of an operon fusion to *lacZ* suggested that there was not significant regulation of transcription of *V. vulnificus fur* by iron or the *E. coli* Fur protein. We used marker exchange to construct a *V. vulnificus fur* deletion mutant and confirmed its phenotype by observing overexpression of iron-regulated outer membrane proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The *fur* deletion mutant of *V. vulnificus* will be helpful in future studies of the role of iron in *V. vulnificus* pathogenesis.**

Vibrio vulnificus is a halophilic, marine pathogen that has been associated with septicemia and serious wound infections in immunocompromised individuals and patients who have hemochromatosis, cirrhosis, or alcoholism (3, 41, 42). Many of the *V. vulnificus* infections occur in patients with iron overload. Septicemia is often acquired by eating shellfish, and the mortality rates of patients with septicemia often exceed 50% (26).

A number of factors have been proposed as possible virulence determinants for *V. vulnificus*, including an extracellular cytolysin (20, 21, 30), an elastolytic protease (27, 28), siderophores (54), a phospholipase (61), the presence of a polysaccharide capsule (29, 31, 56, 64, 65), resistance to the bactericidal effects of sera (25, 64, 65), resistance to phagocytosis (25, 29, 65), and the ability to acquire iron from transferrin (44, 55, 56). Wright et al. directly correlated virulence of *V. vulnificus* with the availability of iron (63).

The importance of iron for microorganisms has long been recognized and the ability to scavenge iron is often important in pathogenesis (33). Free iron is extremely limited in the tissues and fluids of mammalian hosts (6). A shift from a high-iron to a low-iron environment may function as an important regulatory signal to bacteria that they have entered a mammalian host. Several virulence-associated determinants in pathogenic bacteria are regulated by the iron status of the organisms, with increased gene expression occurring under conditions of low iron availability. Such iron-regulated virulence determinants include Shiga-like toxin I of enterohemorrhagic *Escherichia coli*, diphtheria toxin of *Corynebacterium diphtheriae*, and certain iron-regulated outer membrane proteins of *Vibrio anguillarum* and *Vibrio cholerae* (1, 5, 7, 19). Iron restriction in the bioluminescent (light organ) symbiosis of *Vibrio fischeri* with monocentrid fish may also serve as a signal for increased expression of the *lux* genes required for luminescence (24). In *E. coli* and *V. cholerae*, coordinate regulation of genes involved in iron uptake, as well as iron-regulated virulence determinants, depends on the regulatory gene *fur*, whose protein product represses gene transcription in the presence of sufficient iron (7, 23, 53). A consensus DNA binding site for the Fur protein, consisting of a 19-bp dyad symmetric sequence, has been suggested previously (7–10).

The gene encoding the cytolysin of *V. vulnificus* has been cloned and found to contain possible binding sites for a Fur protein in its promoter (62). Since iron plays a major role in the pathogenesis of *V. vulnificus* infections, the present study was conducted to determine whether *V. vulnificus* contains a gene homologous to the *fur* gene and to construct a mutation in this gene by in vivo marker exchange.

(These studies were presented in part at the 92nd Annual Meeting of the American Society for Microbiology, New Orleans, La., 27 to 30 May 1992.)

MATERIALS AND METHODS

Bacterial strains and plasmids. Characteristics of the bacterial strains and plasmids used in this study are described in Table 1. Identification of strain 80363 as a *V. vulnificus* strain, which was performed in the Clinical Microbiology Laboratory at Massachusetts General Hospital, was kindly confirmed by Glenn Morris, who used DNA hybridization

* Corresponding author.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
V. cholerae strains		
0395	Classical strain, Sm^r	34
C6706	El Tor isolate from Peru	CDC[a]
C6709	El Tor isolate from Peru	CDC
MBG40	0395 *irgA*::Tn*phoA* Sm^r Km^r	19
CML13	0395 *irgA*::Tn*phoA fur*::pCML13 Sm^r Km^r Ap^r	This study
V. vulnificus strains		
80363	Clinical isolate	This study
80363Sm	80363 Sm^r	This study
CML17	80363 Δ(*fur*)	This study
E. coli strains		
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* (ϕ80d *lacZ M15*)λ^-	22
SY327λ*pir*	Δ(*lac pro*) *nalA recA56 araD argE*(Am) λ*pir*R6K	40
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*R6K Km^r	40
SM796	F^- *araD139* Δ(*araABC-leu*)*7697 galE galK* Δ(*lac*)*X74 rpsL thi phoA*Δ*Pvu*II *phoR* Sm^r	8
SBC796	SM796 *fur*::Tn*5* Sm^r Km^r	8
A. hydrophila 33191		This study
H. influenzae 1022		This study
Pseudomonas aeruginosa 996604		This study
N. gonorrheae FA19		P. F. Sparling
Porphyromonas gingivalis HG66		C. Genco
V. fischeri MJ-1		Paul Dunlap
Plasmids		
pUC19	Cloning vector, Ap^r	Laboratory stock
pLAFR3	Cloning vector, Tc^r	59
pCML10	1.0-kbp *Hin*dIII *V. cholerae fur* clone in pLAFR3, Tc^r	32
pCML14	1.6-kbp *Bam*HI *V. vulnificus fur* clone in pLAFR3, Tc^r	This study
pCML16	1.1-kbp *Bam*HI-*Hin*dIII *V. vulnificus fur* clone in pLAFR3, Tc^r	This study
pGP704	pJM703.1 with 1.5-kbp *Sph*I-*Pvu*II deletion and 75-bp insertion of M13tg131 polylinker, Ap^r	18
pCML12	Internal fragment of *V. cholerae fur* gene generated by PCR and cloned into pUC19, Ap^r	This study
pCML13	pGP704 with 450-bp *Xba*I-*Sal*I insertion of pCML12, Ap^r	This study
pSBC60	Internal fragment of *E. coli fur* gene generated by PCR and cloned into pUC19, Ap^r	32
pMLB1109	Promoterless *lacZ* gene with four transcriptional terminators and polylinker preceding *lacZ* gene, Ap^r	M. L. Berman
pCML22	pMLB1109 with 239-bp *Eco*RI insertion from pCML16, forward with respect to *fur* promoter, Ap^r	This study
pCML23	pMLB1109 with 239-bp *Eco*RI insertion of pCML16, reverse with respect to *fur* promoter, Ap^r	This study
M13del	M13mp19 with *Bam*HI-*Hin*dIII insertion of *V. vulnificus fur*, containing an internal 377-bp *Sca*I-*Eco*RV deletion	This study
pCVD442	Positive selection suicide vector, pGP704 with *sacB* gene inserted in multiple cloning site, Ap^r	12
pCML21	pCVD442 with a 990-bp *Sac*I-*Pvu*II fragment of M13del inserted between *Sac*I and *Sma*I sites, Ap^r	This study

[a] CDC, Centers for Disease Control, Atlanta, Ga.

with a probe specific to the *V. vulnificus* cytolysin-hemolysin (43). The following strains are clinical isolates obtained from the Clinical Microbiology Laboratory at Massachusetts General Hospital: *Aeromonas hydrophila* 33191, *Haemophilus influenzae* 1022, and *Pseudomonas aeruginosa* 996604. *Neisseria gonorrheae* FA19 was a gift from P. F. Sparling. *Porphyromonas gingivalis* HG66 was a gift from C. Genco. *V. fischeri* MJ-1 was a gift from Paul Dunlap. *V. vulnificus* 80363 was made resistant to streptomycin by selection on LB plates containing 100 μg of streptomycin per ml, to yield strain 80363Sm.

Media. Tris-buffered medium with or without addition of 10 μM $FeSO_4$ and Luria-Bertani (LB) medium with or without addition of the iron chelator 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.2 mM were used to assess the effect of iron concentration on gene expression, as described previously (19). For *E. coli* strains, Tris-buffered medium was supplemented with thiamine (10 μg/ml) and the L amino acids arginine (40 μg/ml) and leucine (40 μg/ml). *V. fischeri* was grown in modified LB medium supplemented with 2% NaCl and 0.3% glycerol in 50 mM Tris HCl adjusted to pH 7.5; tap water was used instead of distilled water to prepare this medium (13). The following antibiotic concentrations were used as needed: ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; kanamycin, 45 μg/ml; and tetracycline, 15 μg/ml for *E. coli* and 5 μg/ml for *V. cholerae* strains.

DNA and RNA manipulations. Rapid isolation of plasmid

DNA was done as described by Birnboim and Doly (2). Standard recombinant DNA techniques were performed as described by Sambrook et al. (51). DNA restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were used according to the manufacturers' specifications. Except as noted below, plasmids were transformed into *E. coli* strains. Electroporation was used in the transformations of the pLAFR3 minilibrary into strain DH5α and of pLAFR3, pCML10, pCML14, and pCML16 into *V. cholerae* CML13. Electroporation was performed in a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's protocol under the following conditions: 2,500 V at a 25-μF capacitance, producing time constants of 4.5 to 4.9 ms.

Bacterial genomic DNA was isolated by a sodium dodecyl sulfate-proteinase K method (51). Plasmid restriction enzyme fragments and restriction enzyme-digested genomic DNA fragments of appropriate sizes were isolated from 1.0% agarose gels by electroelution and were purified further by phenol and chloroform extractions. Radiolabeled probes were generated by using a random priming labeling kit (Prime Time C kit; International Biotechnologies, Inc., New Haven, Conn.). The oligonucleotides used as probes were end labelled with T4 polynucleotide kinase. Radioactively labelled deoxynucleotides were obtained from Du Pont, NEN Research Products, Boston, Mass.

The *E. coli fur* gene probe consisted of an internal fragment of *E. coli fur* generated by the polymerase chain reaction (PCR) and cloned in plasmid pSBC60 as described previously (32). The *V. cholerae fur* gene probe consisted of an internal fragment of the *V. cholerae fur* gene generated by the PCR by using a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Emeryville, Calif.). Oligonucleotide primers were constructed so that the amplified *fur* gene fragment would be internal to the start and stop codons of the *V. cholerae fur* gene and would have the restriction enzyme *Xba*I and *Sal*I sites at the ends to facilitate subsequent subcloning. Plasmid pCML10 was used as a template. The agarose gel-purified *Xba*I-*Sal*I PCR fragment was subcloned into *Xba*I-*Sal*I-digested pUC19 to yield plasmid pCML12. The DNA sequence of the amplified fragment in pCML12 was confirmed following the PCR.

Hybridizations. Restriction enzyme-digested genomic and plasmid DNA fragments were resolved through 1.0% agarose gels, and DNA was transferred to GeneScreen Plus membranes (Du Pont, NEN Research Products) by the method of Southern (57). High-stringency hybridizations were performed at 42°C in a buffer containing 1 M NaCl, 1% sodium dodecyl sulfate, and 50% formamide; the buffer used for low-stringency hybridizations contained 25% formamide instead of 50% formamide. After 6 to 24 h of hybridization, the membranes were washed according to the manufacturer's recommendations and visualized by autoradiography.

Colony blot hybridizations were performed by using Gene Screen Plus colony-plaque membranes as described previously (32).

Genetic constructions. (i) Construction of a *fur* mutation in strain MBG40. *V. cholerae* CML13, which contains an insertion mutation in *fur*, was constructed from strain MBG40 in the following manner. Plasmid pCML13 was transferred from strain SY327λ*pir* into strain SM10λ*pir* by transformation. Strain SM10λ*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 (40). Strain SM10λ*pir* containing pCML13 was conjugated with strain MBG40, with double selection for ampicillin resistance (encoded by pCML13) and streptomycin resistance (encoded by strain MBG40). Because strain MBG40 does not contain the *pir* gene, pCML13 is unable to replicate in this strain, so that doubly resistant colonies arise by homologous recombination between the internal fragment of *fur* on pCML13 and the corresponding chromosomal gene on the recipient, causing insertional inactivation of *fur*. Proper chromosomal integration within *fur* was confirmed by Southern hybridization (data not shown).

(ii) Construction of *lacZ* operon fusions. Plasmid pCML22, which contains an operon fusion between the promoter of the *V. vulnificus fur* gene and a promoterless *lacZ* gene, was constructed by inserting the upstream 239-bp *Eco*RI fragment of *V. vulnificus fur* into the polylinker upstream of the *lacZ* gene in pMLB1109. Confirmation of the correct orientation of the *fur* promoter in relation to *lacZ* was obtained by restriction enzyme analysis and double-stranded DNA sequencing. Plasmid pCML23 consists of the upstream 239-bp *Eco*RI fragment inserted into the polylinker of pMLB1109, with the *fur* promoter in reverse orientation with respect to *lacZ*.

(iii) Construction of a *fur* deletion mutant of *V. vulnificus* by in vivo marker exchange. A *fur* deletion was constructed in *V. vulnificus* by in vivo marker exchange as described previously (4). Plasmid pCVD442 is a suicide vector containing the *sacB* gene, which allows positive selection with sucrose for the loss of plasmid sequences after homologous recombination into the chromosome (12). The *Bam*HI-*Hin*dIII fragment of pCML16, containing *V. vulnificus fur* and surrounding DNA, was subcloned in M13mp19; a 377-bp *Sca*I-*Eco*RV fragment internal to *fur* was deleted by digestion and religation, and the deletion was confirmed by DNA sequencing to yield M13del. The 990-bp *Sac*I-*Pvu*II fragment of M13del was ligated into *Sac*I-*Sma*I-digested pCVD442, yielding pCML21. In vivo marker exchange was used to replace the chromosomal copy of *fur* in *V. vulnificus* with the internally deleted copy in pCML21 without any remaining integrated plasmid sequences, as described previously (4, 12), to generate strain CML17.

Alkaline phosphatase assays. Alkaline phosphatase activities in strains grown overnight in LB medium with or without added 2,2-dipyridyl were determined by measuring hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells, as described previously (35). Activity was normalized to the optical density at 600 nm of the bacterial cells.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (36), except that the cells to be assayed were grown overnight in Tris-buffered medium without or with iron (10 μM). Additional supplements added to Tris-buffered medium included thiamine (10 μg/ml), proline (40 μg/ml), leucine (40 μg/ml), arginine (40 μg/ml), and ampicillin (100 μg/ml). Activity was normalized to the optical density at 600 nm of the bacterial cells. Parental strains SM796 and SBC796 had less than 1 U of β-galactosidase activity in the absence of plasmids carrying *lacZ*.

DNA sequencing. The DNA sequence was determined by the dideoxy chain termination method of Sanger et al. (52), using both plasmid and single-stranded templates and a Sequenase kit obtained from United States Biochemical Corp., Cleveland, Ohio.

The synthetic oligonucleotides used as primers for the PCR, DNA sequencing, and primer extension were a generous gift from Brian Seed (Massachusetts General Hospital).

DNA and protein analyses and data base searches. The DNA sequence was entered and analyzed on the GenBank

On-line Service by using IntelliGenetics Suite software (IntelliGenetics, Inc., Mountain View, Calif.). Data base searches and protein alignments were performed by searching the Swiss-Protein data bank (version 18; University of Geneva) with the FASTA algorithm for protein homology (46).

RNA analysis. RNAs from cultures grown under high-iron conditions (LB medium) and low-iron conditions (LB medium containing 2,2-dipyridyl) were prepared as described previously (18). A Northern (RNA) blot analysis was performed by using standard molecular biological techniques (51); equivalent amounts of RNA, as calculated from the optical density at 260 nm, were loaded into all of the lanes. The internal *Sca*I-*Eco*RV fragment of the *V. vulnificus fur* gene was used as the probe. Primer extension and primer extension sequencing were performed as described by Miller et al. (38), except that the oligonucleotide primers were hybridized to RNA in a mixture containing 0.4 M NaCl and 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 6.4) but no formamide at 60°C for 2 h. RNasin and avian myeloblastosis virus reverse transcriptase were obtained from Bethesda Research Laboratories Life Technologies, Gaithersburg, Md.

Preparation and analysis of outer membrane proteins. Enriched outer membrane proteins were prepared by using previously described procedures (23) from cells grown to late logarithmic phase in LB medium with and without added 2,2-dipyridyl. The outer membrane proteins were separated on sodium dodecyl sulfate–10% polyacrylamide gels and were stained with Coomassie blue, as described previously (19).

Nucleotide sequence accession number. The GenBank accession number for the sequence presented in this paper is L06428.

RESULTS

Detection of *fur*-homologous sequences by Southern blotting in various bacterial species. Because iron plays an important role in the regulation of gene expression in pathogenic bacteria, as well as in the regulation of the bioluminescent symbiosis of *V. fischeri*, we examined a variety of bacterial species in addition to *E. coli* and *V. cholerae* for sequences homologous to *fur*. We performed Southern blots of digests of chromosomal DNAs from a number of bacterial species, probing with internal fragments of both the *V. cholerae* and *E. coli fur* genes under conditions of low and high stringency (Fig. 1). Under conditions of low stringency, both probes hybridized to single bands of DNAs from *V. vulnificus* 80363, *V. fischeri* MJ-1, *A. hydrophila* 33191, and other *V. cholerae* strains, but under conditions of high stringency, only the *V. cholerae* probe hybridized with DNAs from *V. vulnificus* 80363 and *V. fischeri* MJ-1. No hybridization was detected with either probe, even under conditions of low stringency, with DNA from *Porphyromonas gingivalis* HG66, *N. gonorrheae* FA19, *Pseudomonas aeruginosa* 996604, or *H. influenzae* 1022. Since the *V. cholerae fur* probe hybridized with DNAs of other *Vibrio* species under high-stringency conditions, it was used to construct a restriction map of the *V. vulnificus fur* gene and to screen a minilibrary of *V. vulnificus* DNA for the cloned *fur* gene by using high-stringency colony hybridization.

Restriction map of *V. vulnificus* chromosomal DNA around the *fur* gene. Genomic DNA from *V. vulnificus* 80363 was hybridized with a probe internal to the *V. cholerae fur* gene (derived from plasmid pCML12). Under conditions of high stringency, single discrete bands of hybridization were seen with each of a variety of restriction enzymes (data not shown). By using the sizes of the hybridizing fragments obtained in single and double digests, a restriction map of the DNA around the hybridizing region was developed. A strongly hybridizing 1.6-kbp *Bam*HI fragment was identified at the center of this map (Fig. 2).

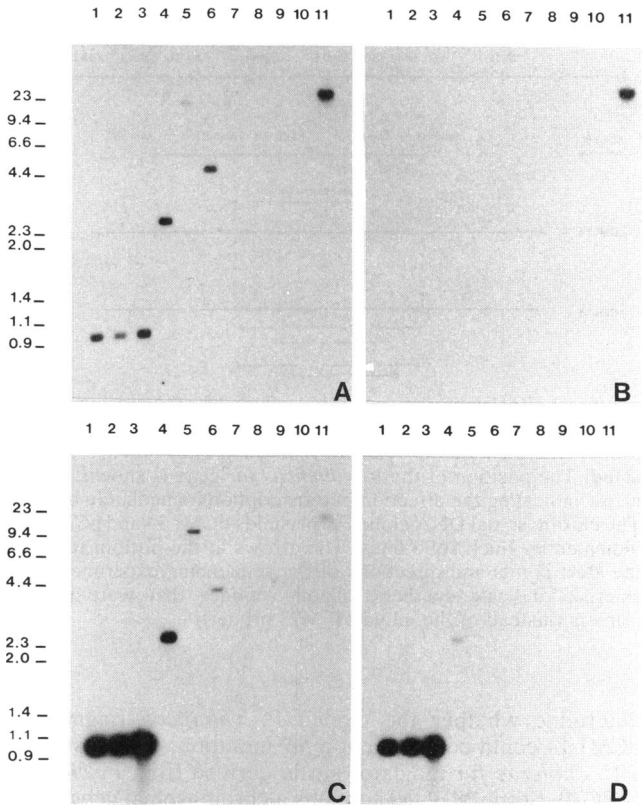

FIG. 1. Southern blot analysis of genomic DNA digested with various restriction endonucleases. (A and B) Southern blots probed with an internal *E. coli fur* gene probe under conditions of low (A) and high stringency (B). (C and D) Southern blots probed with an internal *V. cholerae fur* gene probe under conditions of low (C) and high stringency (D). Lanes 1, *V. cholerae* 0395 digested with *Hin*dIII; lanes 2, *V. cholerae* El Tor strain C6706 digested with *Hin*dIII; lanes 3, *V. cholerae* El Tor strain C6709 digested with *Hin*dIII; lanes 4, *V. vulnificus* 80363 digested with *Hin*dIII; lanes 5, *V. fischeri* MJ-1 digested with *Hin*dIII; lanes 6, *A. hydrophila* 33191 digested with *Eco*RI; lanes 7, *Pseudomonas aeruginosa* 996604 digested with *Eco*RI; lanes 8, *Porphyromonas gingivalis* HG66 digested with *Bam*HI; lanes 9, *N. gonorrhoeae* FA19 digested with *Cla*I; lanes 10, *H. influenzae* 1022 digested with *Eco*RI; lanes 11, *E. coli* DH5α digested with *Hin*dIII. The positions of molecular size standards (in kilobase pairs) are indicated on the left.

Cloning of the *V. vulnificus fur* gene. The 1.6-kbp *Bam*HI fragment was cloned by screening a minilibrary of *Bam*HI fragments of *V. vulnificus* chromosomal DNA, gel-purified to encompass 1.0 to 2.0 kbp and ligated into the *Bam*HI site of vector pLAFR3. Transformants in strain DH5α were screened by colony hybridization, using the internal *V. cholerae* probe under conditions of high stringency. A hybridizing clone (pCML14) contained the desired 1.6-kbp insert and was verified by Southern hybridization (data not shown).

Construction of a *V. cholerae fur* indicator strain. To

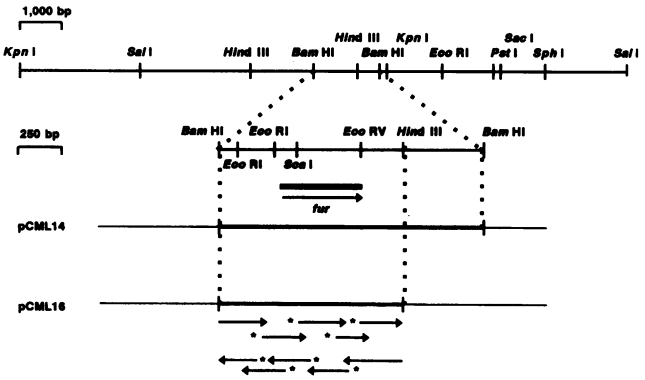

FIG. 2. Restriction map of *V. vulnificus* chromosomal DNA around the region that hybridizes with the internal *V. cholerae fur* probe. The locations of relevant restriction enzyme sites are indicated. The position of the *V. vulnificus fur* gene is shown, with an arrow indicating the direction of transcription immediately below it. The chromosomal DNA cloned in plasmids pCML14 and pCML16 is indicated by thick solid lines. The arrows at the bottom represent the start points and directions of the sequencing experiments; the asterisks indicate synthetic oligonucleotides that were used as primers (instead of the universal M13 primer).

determine whether the cloned *V. vulnificus* fragment in pCML14 could complement a *fur* mutation, we constructed a *V. cholerae fur* indicator strain derived from *V. cholerae* MBG40. Strain MBG40 contains a chromosomal gene fusion between *irgA* and Tn*phoA* and possesses strongly iron-regulated alkaline phosphatase activity. *V. cholerae fur* indicator strain CML13 was constructed by integrating suicide plasmid pCML13 into the chromosomal *fur* gene of strain MBG40, with disruption of the gene. Integration of the suicide plasmid into *fur* was confirmed by Southern hybridization (data not shown) and alkaline phosphatase activity in LB medium with or without 2,2-dipyridyl (Table 2). Strain CML13 showed substantial loss of the normal iron regulation of alkaline phosphatase activity present in strain MBG40; this regulation was restored to normal levels by introduction of a *V. cholerae fur* clone in *trans* on pCML10.

Complementation of a *V. cholerae fur* mutant with the *V. vulnificus fur* gene. When pCML14 was introduced into strain CML13, regulation of alkaline phosphatase activity by iron was restored to a level identical to the level of regulation seen when the *V. cholerae fur* gene was introduced into strain CML13. A subclone of pCML14, pCML16 (Fig. 2), also restored normal levels of iron regulation of alkaline phosphatase activity.

TABLE 2. Alkaline phosphatase activities of *V. cholerae* in high- and low-iron media

Strain	Alkaline phosphatase activity (U/OD_{600} unit)[a]		Induction ratio
	Low-iron medium	High-iron medium	
MBG40	950 (810–1,200)	4 (2–6)	240
CML13	1,600 (1,400–2,200)	500 (230–780)	3
CML13(pLAFR3)	900 (770–1,100)	340 (200–420)	3
CML13(pCML10)	290 (230–330)	2 (1–3)	150
CML13(pCML14)	300 (270–360)	2 (1–2)	150
CML13(pCML16)	520 (250–770)	2 (1–3)	260

[a] The values are the averages (ranges) from three separate experiments. OD_{600}, optical density at 600 nm.

DNA sequence of *V. vulnificus fur* gene. We determined the DNA sequence of the *V. vulnificus fur* gene from both strands of the complementing *Bam*HI-*Hin*dIII fragment in pCML16 (Fig. 3). A 447-bp open reading frame begins downstream of an *Eco*RI restriction site. A putative Shine-Dalgarno sequence is located just upstream from the initiating methionine. A perfect inverted repeat, suggestive of a bidirectional transcriptional terminator (60), is indicated beyond the termination codon. An additional open reading frame was also identified upstream from and in the opposite orientation to the *fur* gene, starting at base 206 and continuing beyond the *Bam*HI site (data not shown).

Homology of *V. vulnificus* Fur to *V. cholerae* Fur and *E. coli* Fur. The deduced amino acid sequence encoded by the 447-bp open reading frame in Fig. 3 was analyzed for homologous proteins in the Swiss-Protein data base and was found to be highly homologous to both the *E. coli* Fur sequence and *V. cholerae* Fur sequence (Fig. 4). Optimal alignment of the deduced 149-amino-acid sequence of *V. vulnificus* Fur with the 150 amino acids in *V. cholerae* Fur and the 148 amino acids in *E. coli* Fur revealed that 93% of the *V. cholerae* residues and 77% of the *E. coli* residues were identical to the *V. vulnificus* residues. Considerable conservation was observed in the amino two-thirds of all three proteins. The divergent open reading frame upstream of *V. vulnificus fur* showed no significant homology to proteins in the Swiss-Protein data base.

Primer extension analysis to localize the start site of *V. vulnificus fur* transcription. Primer extension analysis of RNA from *V. vulnificus* 80363 grown under low-iron conditions was done by using a synthetic oligonucleotide complementary to the DNA sequence just downstream of the initiating codon (Fig. 3, bases 416 through 436). A single, strong primer extension product corresponding to base 356 of the sequence was identified (Fig. 5 and Fig. 3, asterisk). A −10 box upstream of the transcriptional start site was identified, but a sequence homologous to the *E. coli* −35 box was not identified.

Northern blot analysis of the *fur* transcript in *V. vulnificus*. Northern blot analysis was performed with RNA prepared from *V. vulnificus* grown in low- and high-iron media. The blot was probed with the *Sca*I-*Eco*RV fragment contained in the *V. vulnificus fur* gene. One transcript of approximately 550 bases was seen under both low- and high-iron conditions (Fig. 6), consistent with the size predicted by the DNA sequence information. Under high-iron conditions (Fig. 6, lane 1) slightly less RNA transcript was seen, suggesting possible autoregulation of *fur* transcription by iron. However, we were not able to identify a dyad sequence homologous to an *E. coli* Fur box in the vicinity of the *V. vulnificus* promoter.

Analysis of transcriptional regulation by iron of *V. vulnificus fur*, utilizing a *lacZ* operon fusion. In order to quantitate possible regulation of *fur* transcription by iron, we constructed an operon fusion between the *fur* promoter and *lacZ* (in plasmid pCML22), as well as a control operon fusion with the *fur* promoter fragment in the opposite orientation (and presumably containing the promoter of the upstream open reading frame; plasmid pCML23). As shown in Table 3, we did not detect significant iron regulation of the *fur* promoter in either a *fur*$^+$ or *fur* background, suggesting that there was no autoregulation of transcription from the *V. vulnificus fur* promoter.

Construction of a mutant of *V. vulnificus* with an internal

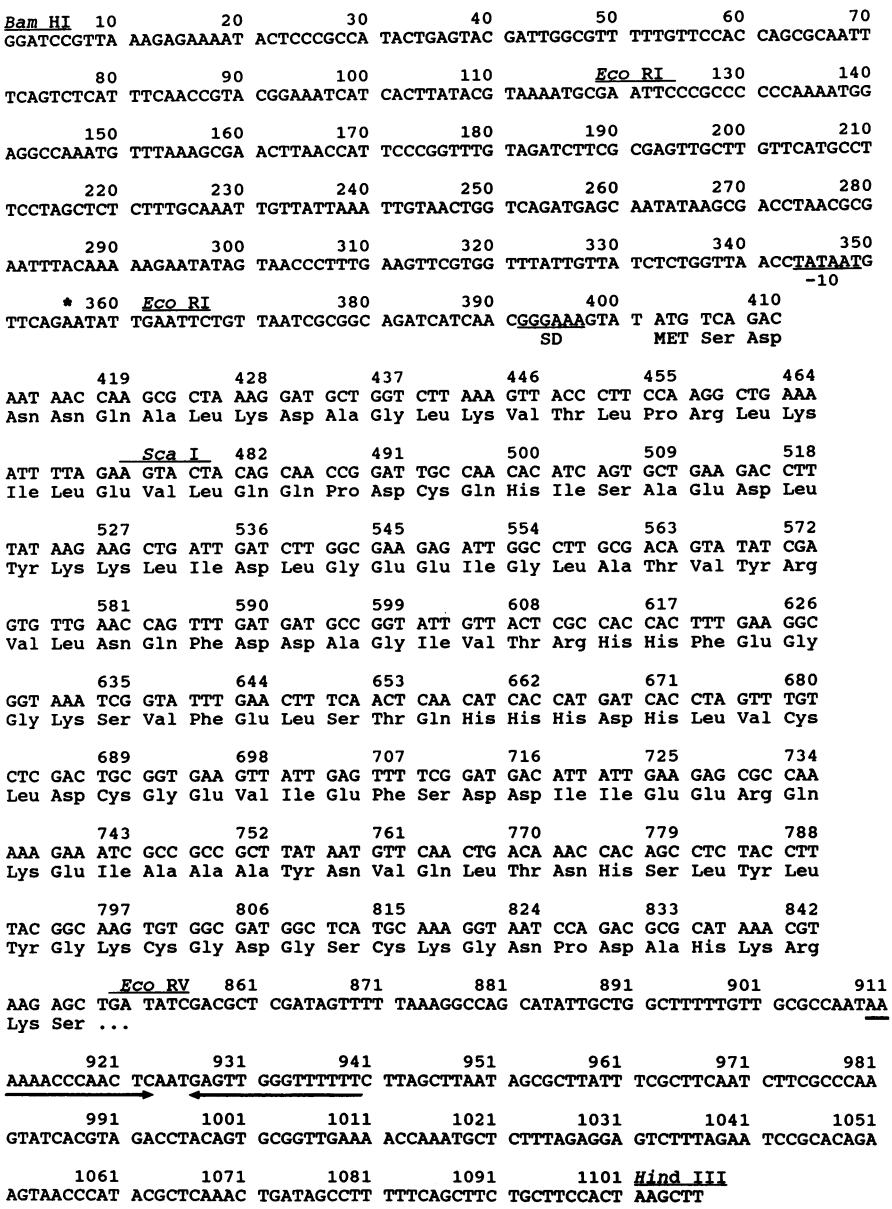

FIG. 3. Nucleotide sequence of *V. vulnificus fur* and its promoter region starting at the upstream *Bam*HI site. The locations of certain restriction enzyme sites are indicated. The deduced amino acid sequence of *V. vulnificus* Fur is shown below the *fur* sequence. The approximate start site of transcription is indicated by an asterisk, and the positions of the −10 box of the promoter (−10) and the Shine-Dalgarno sequence (SD) are also indicated. The termination codon of Fur is indicated by three dots; this codon is followed by a probable transcription terminator (indicated by convergent arrows near the end of the sequence).

deletion of *fur* (strain CML17). To introduce an internal deletion of *fur* into the chromosome of *V. vulnificus* by marker exchange, we constructed plasmid pCML21, a suicide vector containing the *fur* gene with a 377-bp internal deletion. Plasmid pCML21 was transferred by conjugation into *V. vulnificus* 80363Sm, with selection on medium containing ampicillin and streptomycin for the merodiploid state in which pCML21 had integrated into the chromosomal *fur* gene by homologous recombination. The resulting merodiploid strain was grown without selection to late logarithmic phase, spread on plates containing 10% sucrose, and incubated overnight at 30°C (as described above). A total of 200 sucrose-resistant colonies were screened for sensitivity to ampicillin, suggesting that vector sequences were lost. Of the 14 colonies that were ampicillin sensitive, 1 was negative for the deleted *fur* sequence as determined by colony hybridization (data not shown), and this organism was designated strain CML17.

(i) Verification of strain CML17 by Southern blotting. We verified the genetic construction of strain CML17 by Southern hybridization of *Bam*HI-*Hin*dIII-digested chromosomal DNA, probing with the cloned *Bam*HI-*Hin*dIII *fur* fragment, and comparing the results with results for wild-type *V. vulnificus* DNA and the deleted *fur* fragment in M13del (Fig. 7).

(ii) Verification of strain CML17 by analysis of iron-regu-

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                      10        20        30        40        50
       E. coli MTDNNTALKKAGSKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEI
               :.::: :::.:::::::::::::::::.:: .:.:::::::.:::.::::
 V. vulnificus MSDNNQALKDAGLKVTLPRLKILEVLQQPDCQHISAEDLYKKLIDLGEEI
               :::::::::::::::::::::::::::::.::::::.:::::::::.:::
   V. cholerae MSDNNQALKDAGLKVTLPRLKILEVLQQPECQHISAEELYKKLIDLSEEI

                      60        70        80        90       100
       E. coli GLATVYRVLNQFDDAGIVTRHNFEGGKSVFELTQQHHHDHLICLDCGKVI
               :::::::::::::::::::::.::::::::::. :::::::.:::::.::
 V. vulnificus GLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVI
               ::::::::::::::::::::::::::::::::::::::::::::::::::
   V. cholerae GLATVYRVLNQFDDAGIVTRHHFEGGKSVFELSTQHHHDHLVCLDCGEVI

                     110       120       130       140       150
       E. coli EFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCA-EGDCREDEHAHEGK
               ::::: ::.::.:::: ...:::::::::::.:. .:.:... .::. :
 V. vulnificus EFSDDIIEERQKEIAAAYNVQLTNHSLYLYGKCG-DGSCKGNPDAHKRKS
               :::::.::.::::::: ::::::::::::::::: :::::.::.:::.:
   V. cholerae EFSDDVIEQRQKEIAAKYNVQLTNHSLYLYGKCGSDGSCKDNPNAHKPKK
```

FIG. 4. Homology among *E. coli*, *V. vulnificus*, and *V. cholerae* Fur proteins. Amino acids are indicated by single-letter designations. Identical amino acids are indicated by two dots, and conservative substitutions are indicated by single dots. When *V. vulnificus* Fur was aligned with *E. coli* Fur, the two sequences were identical in 77% of their residues. When *V. vulnificus* Fur was aligned with *V. cholerae* Fur, the two sequences were identical in 93% of their residues. A space (indicated by a dash) was introduced into both the *E. coli* Fur protein sequence and the *V. vulnificus* Fur protein sequence by the computer to optimize alignments.

lated outer membrane proteins. We additionally confirmed the *fur* phenotype of mutant CML17 by comparing the outer membrane proteins of wild-type *V. vulnificus* and strain CML17 after growth in low- and high-iron media (Fig. 8). In wild-type *V. vulnificus*, at least two new proteins having apparent molecular masses of 72 to 78 kDa appeared after growth under low-iron conditions (Fig. 8, lane 2). In contrast, mutant CML17 expressed both of these proteins under high- and low-iron conditions (Fig. 8, lanes 3 and 4).

DISCUSSION

The ability of bacterial pathogens to acquire iron is often essential to their virulence. Many bacteria use the low concentration of iron present in a host as an important signal to enhance the expression of a wide variety of bacterial toxins and other virulence determinants (33). Iron restriction may also play a role in the bioluminescent (light organ) symbiosis of *V. fischeri* with monocentrid fish by providing the fish with a way of restricting the growth of *V. fischeri* while still maintaining a high level of bacterial light production (45, 49). Several bacteria have a Fur-like system for gene regulation in response to iron. Fur homologs have been identified in various gram-negative bacteria, including *Salmonella typhimurium*, *Serratia marcescens*, *V. cholerae*, *Yersinia pestis*, and *Pseudomonas aeruginosa* (16, 32, 47, 48, 58). We report here the cloning and sequencing of a *fur* gene from *V. vulnificus*, an organism for which iron also plays a major role in pathogenesis. The *dtxR* gene, which is responsible for regulation of diphtheria toxin expression by

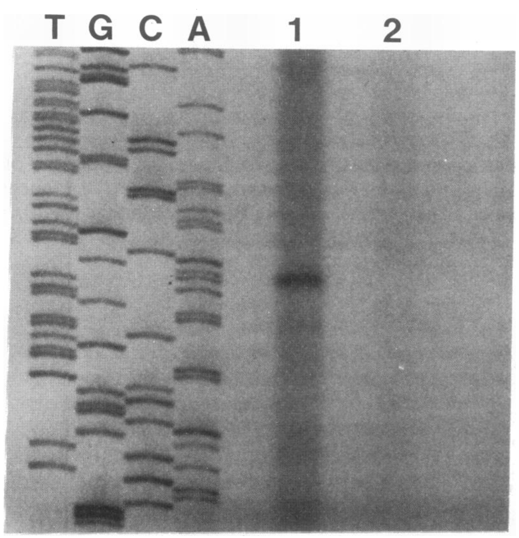

FIG. 5. Primer extension analysis of RNA from *V. vulnificus fur*. Lanes T, G, C, and A correspond to lanes of the DNA sequencing ladder. Lane 1 contained the primer extension reaction mixture, and lane 2 contained the control without added primer. A strong primer extension product, corresponding to base 356 of the sequence shown in Fig. 3, was identified.

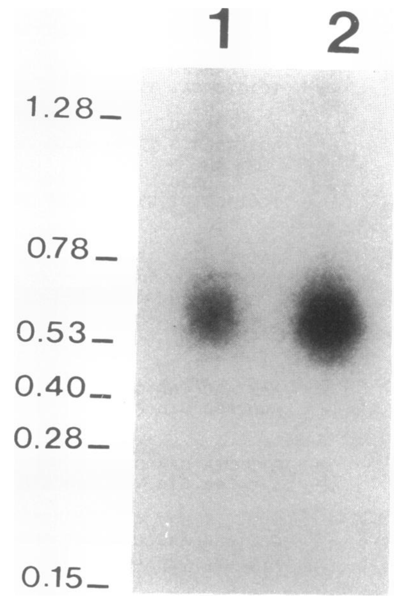

FIG. 6. Northern blot analysis of RNA prepared from *V. vulnificus* after growth in high-iron medium (lane 1) and low-iron medium (lane 2) and probed with a *Sca*I-*Eco*RV fragment internal to *fur*. The positions of RNA standards (in kilobases) are shown on the left.

TABLE 3. Results of β-galactosidase assays performed with various strains of *E. coli* by using Tris-buffered media with and without iron

Strain	Plasmid	Concn of iron added (μM)	β-Galactosidase activity (U/OD_{600} unit)[a]
SM796 (*fur*$^+$)	pMLB1109	None	6 (1–15)
		10	9 (1–23)
	pCML22	None	4,700 (2,300–7,200)
		10	3,300 (2,100–5,500)
	pCML23	None	2,200 (1,700–2,800)
		10	1,600 (1,400–1,700)
SBC796 (*fur*)	pMLB1109	None	11 (1–27)
		10	11 (1–21)
	pCML22	None	3,100 (1,300–4,300)
		10	3,300 (3,000–3,400)
	pCML23	None	1,600 (1,000–2,000)
		10	1,000 (810–1,200)

[a] The values are the averages (ranges) from three separate experiments. OD_{600}, optical density at 600 nm.

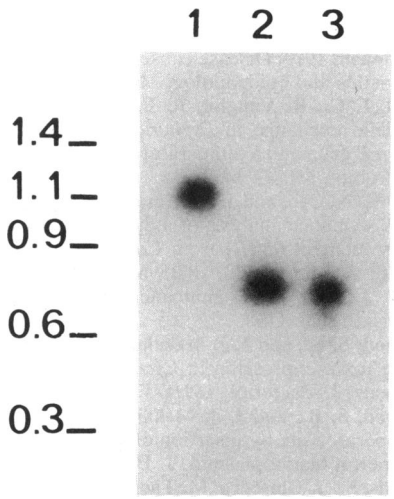

FIG. 7. Southern blot confirmation of the construction of a *fur* deletion mutant in *V. vulnificus*, using suicide vector pCML21. The preparation was probed with the *Bam*HI-*Hin*dIII DNA fragment containing the *fur* gene. Lanes 1, wild-type *V. vulnificus* digested with *Bam*HI and *Hin*dIII; lane 2, *V. vulnificus* CML17 digested with *Bam*HI and *Hin*dIII; lane 3, M13del digested with *Bam*HI and *Hin*dIII. The positions of molecular size standards (in kilobase pairs) are indicated on the left.

iron in *C. diphtheriae*, has been cloned and found to be homologous to the *E. coli fur* gene at both the amino acid and nucleotide sequence levels (5). The conservation of a Fur-like system for iron regulation in both gram-negative and gram-positive bacteria suggests that such a system is of central importance in many bacterial species.

In *Vibrio* species, gene regulation by iron may be complex; some iron-regulated genes require other factors that work in concert with Fur. In *V. cholerae*, *irgA* is the most thoroughly studied iron-regulated gene. IrgA is an iron-regulated outer membrane protein which has been found to be a virulence determinant in an infant mouse model (19). Transcription of *irgA* requires the positive transcriptional activator *irgB*, which is upstream from and in the inverse orientation to *irgA*; transcription of *irgA* and transcription of *irgB* are both negatively regulated by iron, and the overlapping promoters of these genes contain a dyad repeat homologous to an *E. coli* Fur box (18). The *V. cholerae fur* gene has been cloned previously (32). In the present study, we constructed a mutation in the *fur* gene of *V. cholerae* by insertional inactivation. This mutation substantially reduced the regulation by iron of an *irgA*::Tn*phoA* fusion. However, *irgA*::Tn*phoA* expression was not completely constitutive in the *fur* mutant, suggesting that negative regulation of *irgA* expression by iron in *V. cholerae* may depend on factors other than the Fur protein. Further studies will be needed to determine the effect of Fur and other regulators on the expression of *irgA* and *irgB* in *V. cholerae*.

Iron regulation in *V. anguillarum* has also been studied in relation to virulence and appears to be complex. A major virulence factor in this organism is the presence of a specific plasmid which encodes an iron acquisition system mediated by the siderophore anguibactin (1). Proteins AngR and Taf regulate the production of anguibactin by activating transcription of biosynthetic genes under iron-limiting conditions; these two factors together activate anguibactin biosynthesis in a synergistic manner (50). Transcriptional analysis of *angR* revealed that this gene is also negatively regulated by iron (17). This suggests that a Fur-like protein may be responsible for regulation of *angR* transcription by iron in *V. anguillarum*.

The role of iron limitation in the luminescence of *V. fischeri* is also very complex. In this organism, restriction of iron leads to a slower growth rate and earlier autoinduction of luminescence (24). However, no direct connection between *lux* gene transcription and iron has been established (14). Rather, iron appears to control luminescence in *V. fischeri* by an indirect mechanism involving cyclic AMP and cyclic AMP receptor protein (15). Dunlap found no difference between the induction of luminescence by an *E. coli fur* mutant and the induction of luminescence by its parent strain containing the cloned *lux* genes, suggesting that Fur is not involved in the control of luminescence by iron (14). The identification in the present study of a sequence in *V. fischeri* that is highly homologous to the *fur* gene of *V. cholerae* suggests that a *fur* gene is also present in *V. fischeri*. Cloning of the *fur* gene of *V. fischeri* and construction of *fur* mutants of this organism should be useful in studies to analyze further the effect of iron and/or Fur on *lux* gene transcription.

A number of virulence factors have been suggested to be important in the pathogenesis of *V. vulnificus*. However, regulation of virulence determinants in *V. vulnificus* by iron has not been studied in detail. Since the promoter of the cytolysin gene of *V. vulnificus* may contain possible binding sites for Fur (62), the Fur protein may play a role in the regulation of cytolysin expression. In addition, our results

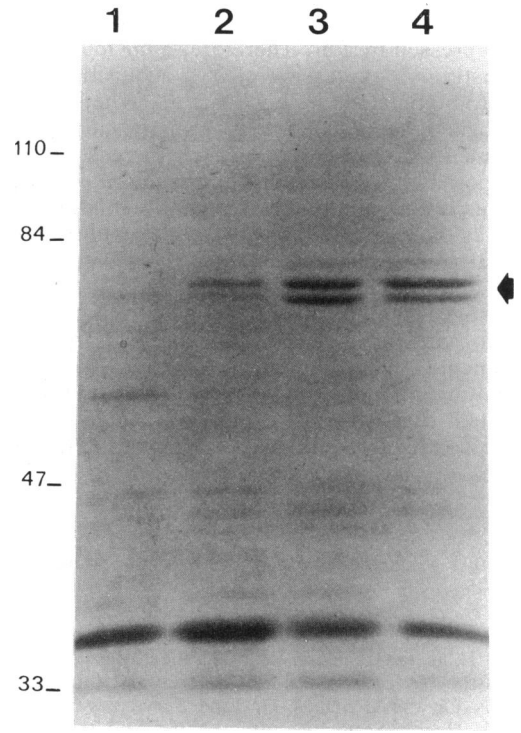

FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins. Lane 1, wild-type *V. vulnificus* grown in high-iron medium; lane 2, wild-type *V. vulnificus* grown in low-iron medium; lane 3, strain CML17 grown in high-iron medium; lane 4, strain CML17 grown in low-iron medium. The numbers on the left indicate the positions of protein standards (in kilodaltons). The arrow indicates the position of iron-regulated proteins.

suggest that at least two iron-regulated outer membrane proteins in *V. vulnificus* are regulated by Fur. Further analysis of iron-regulated genes in *V. vulnificus* may reveal factors in addition to Fur that are important in the regulation of individual genes.

Previous work with *E. coli* has suggested that transcription of the *fur* gene in this species is autoregulated by the Fur protein, as well as by the catabolite activator protein (11). In a previous study, we were not able to identify an apparent Fur box in either of the two promoters of the *V. cholerae fur* gene, and Northern blot analysis did not suggest that iron had a strong effect on *fur* transcription (32). In the present study, we were also not able to identify a Fur box in the single promoter of *V. vulnificus fur*, and Northern blot analysis did not suggest that iron had a strong effect on *fur* expression. We constructed an operon fusion to the *V. vulnificus fur* promoter to quantitate the effect of iron and Fur on *fur* gene expression, but again, no significant effect was seen. However, the results of these experiments do not totally exclude the possibility that *fur* autoregulation occurs. It is possible that the *V. vulnificus fur* promoter on multicopy plasmid pCML22 might have titrated out the *E. coli* Fur protein, dissipating any autoregulation. Also, it is possible that the heterologous *E. coli* Fur may not have functioned on the *V. vulnificus fur* promoter. Further experiments will be needed to examine the question of *fur* autoregulation in *V. vulnificus*.

The construction of a *fur* mutant of *V. vulnificus* will be helpful in analyzing the role of iron metabolism and acquisition in the virulence of this pathogen. It is not clear what effect a mutation on a global regulatory gene like *fur* might have on virulence. The iron-regulated genes studied previously are repressed by Fur. Therefore, a *fur* mutant should exhibit constitutive expression of iron acquisition systems and other iron-regulated virulence factors. Whether the loss of physiologic gene regulation by iron in such a mutant impairs virulence is unknown. However, there is precedence for such a possibility. In *Salmonella typhimurium*, the *phoP* genetic locus is part of a two-component regulatory system that activates the expression of genes essential for virulence and survival within macrophages (37). However, a mutant strain with a *phoP* constitutive phenotype is attenuated for virulence, suggesting either that PhoP also represses genes important for virulence or that a normal regulatory response to environmental signals is required for effective pathogenesis (39). Future studies involving the analysis of virulence in the *fur* mutant of *V. vulnificus* will help clarify the role of physiologic iron regulation in the pathogenesis of this organism.

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