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Purification of *Vibrio cholerae* Fur and Estimation of Its Intracellular Abundance by Antibody Sandwich Enzyme-Linked Immunosorbent Assay

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The *Vibrio cholerae fur* gene was previously cloned and sequenced. A putative Fur box was identified in the divergent promoters of *irgA*, a virulence factor of *V. cholerae*, and *irgB*, a transcriptional activator of *irgA*. In this work, *V. cholerae* Fur was overexpressed in *Escherichia coli* and purified to approximately 95% homogeneity. The purified protein bound a DNA fragment containing the *irgA-irgB* promoter in a gel shift assay. The purified protein was used to raise monoclonal and polyclonal antibodies to *V. cholerae* Fur, and a Fur sandwich enzyme-linked immunosorbent assay was developed to estimate the intracellular abundance of Fur under a variety of growth conditions. The number of Fur molecules per cell during exponential growth was approximately 2,500, which is higher than most measurements for other bacterial repressors but comparable to the intracellular concentration of the leucine-responsive regulatory protein. The number of Fur molecules per cell increased in the late logarithmic and stationary phases. Growth of *V. cholerae* in low-iron medium did not alter the intracellular abundance of Fur significantly. Growth under microaerophilic conditions resulted in a significant, approximately twofold decrease in the intracellular levels of Fur. The measurements of intracellular Fur abundance indicate that a large amount of this repressor is produced constitutively and that the concentration of Fur in the cell varies by less than a factor of 2 under the conditions studied. We hypothesize that the high constitutive expression of Fur is necessary for its role as an iron-responsive regulator.

Iron is an essential nutrient for bacterial growth. In the mammalian host, iron is tightly bound by high-affinity iron-binding proteins synthesized by the host. This leads to limited iron availability, which bacteria overcome by producing their own high-affinity iron chelators, known as siderophores, or by directly utilizing the mammalian iron-binding proteins as sources of exogenous iron (24).

In 1977, the repression of siderophore synthesis in the presence of sufficient iron was noted in *Escherichia coli* (21). Subsequently, a mutation which resulted in iron-independent expression of siderophores was discovered in *Salmonella typhimurium* (7). The gene encoding the repressor responsible for iron-dependent regulation was eventually isolated from *E. coli* and named *fur*, for ferric uptake regulator (28), and a 19-bp, dyad symmetric consensus DNA sequence for binding of *E. coli* Fur, called the Fur box, was identified (2, 5). Homologs of the *E. coli fur* gene have been identified in many other gram-negative bacteria, including *Yersinia pestis* (30), *Vibrio cholerae* (17), *Vibrio vulnificus* (18), *Pseudomonas aeruginosa* (25), *Neisseria meningitidis* (33), and *Legionella pneumophila* (14).

Fur regulates not only genes involved in iron uptake pathways but also those encoding virulence factors. A change in iron concentration therefore may be a signal to the bacterium that it has entered a mammalian host. In fact, Fur (or DtxR, a protein of similar function in *Corynebacterium diphtheriae*) has been shown to regulate several important virulence determinants of bacterial pathogens, including Shiga toxin 1 (formerly Shiga-like toxin I) of enterohemorrhagic *E. coli* (2), Shiga toxin of *Shigella dysenteriae* type 1 (6, 31), diphtheria toxin of *C. diphtheriae* (29), and exotoxin A of *P. aeruginosa* (25).

In *V. cholerae*, a large number of genes are regulated by Fur and/or iron, including those encoding at least 22 proteins which are repressed by Fur in the presence of iron. In addition, a *fur* mutant of *V. cholerae* is unable to use several carbon sources for growth, suggesting the importance of Fur as a global regulatory element in this pathogen (19). Sequences homologous to the Fur box of *E. coli* have been identified in several iron-regulated genes in *V. cholerae*, and the *V. cholerae fur* gene is able to complement a *fur* mutation in *E. coli* (17), suggesting that *V. cholerae* recognizes a DNA binding site similar to that in *E. coli.*

IrgA, a 77-kDa outer membrane protein involved in virulence, and its transcriptional activator, IrgB, are two proteins which are negatively regulated by iron in *V. cholerae. irgA* and *irgB* are divergently transcribed from overlapping promoters. A putative Fur box has been identified in these overlapping promoters, and iron regulation of these two genes is lost in *fur* mutants of *V. cholerae* (9–12), suggesting that iron regulation is mediated by Fur.

Physical evidence of the interaction between *V. cholerae* Fur and promoter sequences in *V. cholerae* has not previously been presented, and no direct measurement of the intracellular abundance of Fur has been reported, although regulation of *fur* expression has been studied in a number of bacterial species (4, 18). In this paper, we report the purification of *V. cholerae* Fur to 95% homogeneity, the direct confirmation of Fur binding to a DNA fragment containing the overlapping promoters of *irgA* and *irgB*, the production of high-affinity polyclonal and monoclonal antibodies to *V. cholerae* Fur, the development of a sensitive and specific sandwich enzyme-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
V. cholerae strains		
O395	Classical strain; Sm^r	22
CML19	O395 *fur*::Km^r; Sm^r Km^r	19
JRB3	O395 Δ*irgB*; Sm^r	Joan R. Butterton
E. coli strains		
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* (ϕ*d lacZ*Δ*M15*)	13
BL21	F^- *ompT* r_B^- m_B^-	32
BL21(DE3)	DE3 lysogen of BL21 encoding *lacI*, the *lacUV5* promoter, and the gene for T7 RNA polymerase	32
CC118	Δ(*ara-leu*)*7679* Δ(*lac*)*X74 araD139 phoA*Δ20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	20
Plasmids		
pET11a	Expression vector; Ap^r	Novagen
pLysS	T7 lysozyme gene in *Bam*HI site of pACYC184; Cm^r	23
pPIW1	pET11a with *fur* gene inserted between the *Nde*I and *Bam*HI sites; Ap^r	This study
pCML7	1.5-kbp *Pst*I *V. cholerae fur* clone in pUC19; Ap^r	17
pMBG47	*Bam*HI fragment of *V. cholerae* chromosomal DNA containing *irgB* and the 5′ end of *irgA* cloned in pUC19; Ap^r	10

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance.

linked immunosorbent assay (ELISA) for determining Fur concentrations, and the use of this ELISA to estimate the number of Fur molecules in the *V. cholerae* cell under a number of different growth conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Bacterial strains were stored at −80°C in Luria-Bertani (LB) medium containing 15% glycerol until use. LB medium was used for routine growth of all bacterial strains. Syncase medium without added iron was used for growth under iron-restricted conditions (9). Streptomycin (100 μg/ml), chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), or 10 mM cyclic AMP was added to the media as indicated. A microaerophilic environment was created by growing bacteria in 150 ml of LB medium in a 250-ml Erlenmeyer flask incubated with gentle agitation.

Genetic methods. Rapid isolation of plasmid DNA was done as described by Birnboim and Doly (1). Digestion of DNA with restriction enzymes, DNA ligations, and separation of DNA fragments by agarose gel electrophoresis were performed according to standard molecular biological techniques (27). DNA was recovered after gel electrophoresis by using GenElute agarose spin columns (Supelco, Bellefonte, Pa.). Plasmids were electroporated into *E. coli* and *V. cholerae* with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (11). PCR (16) and sequencing methods (17) were as previously described. For PCR, template DNA was prepared by boiling a colony of the appropriate bacterial strain. *E. coli* DH5α(pCML7) and CC118(pMBG47) provided templates for amplification of *V. cholerae fur* and the *irgA-irgB* intergenic region, respectively. Oligonucleotide primers for amplification of *fur* were designed to span the region from the initial ATG to the *Hin*dIII site downstream of the gene, incorporating *Nde*I and *Bam*HI sites at the 5′ and 3′ ends, respectively, to facilitate insertion into pET11a. For amplification of the overlapping promoters of *irgA* and *irgB*, primers spanned the region from bp 912 to 1100 of the previously published sequence (10), incorporating *Pst*I and *Xba*I sites at the 5′ and 3′ ends, respectively, to facilitate radioactive labelling for gel shift assays.

Overexpression and purification of *V. cholerae* Fur. The *V. cholerae fur* gene was cloned into the pET11a vector (Novagen, Madison, Wis.) to construct plasmid pPIW1, and the sequence of the insert was confirmed including the junctions of the insert. Plasmid pPIW1 was then electroporated into the expression vector, BL21(DE3)(pLysS). BL21(DE3) containing pLysS and pPIW1 was grown overnight in LB medium with added chloramphenicol and ampicillin. Five milliliters of this culture was inoculated into 500 ml of LB medium with chloramphenicol and ampicillin. The cells were grown in a 2-liter baffled flask with vigorous aeration and induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the culture reached an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 (approximately 2 h). The cells were allowed to grow for another 4 h and then pelleted by centrifugation at 6,000 rpm for 15 min in a Sorvall RC5B centrifuge at 4°C.

The pellet of cells was frozen at −80°C until needed and then thawed and resuspended in approximately 1/10 the original volume in 20 mM Tris, pH 8.0. Fragmentation of chromosomal DNA was accomplished by sonication at a setting of 60 (Biosonik; Bronwell Scientific, Rochester, N.Y.) in three 5-s bursts separated by 1-min cooling intervals on ice. The extracts were spun at 14,000 rpm in an Eppendorf 5415C centrifuge for 30 min. The supernatant was recovered and spun for 1 h at 36,000 rpm in a Beckman L8-55 ultracentrifuge. The supernatant was passed through a 0.2-μm-pore-size filter to remove any debris prior to loading onto an anion-exchange column (Hi-trap Q; Pharmacia Biotech, Piscataway, N.J.). Chromatography was carried out using a Consep LC 100 liquid chromatography system (PerSeptive Biosystems, Framingham, Mass.). Proteins were eluted with a salt gradient of 0 to 500 mM NaCl in 20 mM Tris, pH 8.0. The partially purified Fur protein eluted between 250 and 310 mM NaCl. The protein was then loaded onto a nickel affinity column (Hi-trap chelating; Pharmacia) and eluted with a histidine gradient in 20 mM Tris, pH 8.0. Dialysis against 20 mM Tris was necessary to remove histidine and excess metal after purification. The purified protein was concentrated to 300 μg/ml with a Centriplus 10 concentrator (Amicon, Inc., Beverly, Mass.). Progression of purification was monitored at each step by Western immunoblot and Coomassie brilliant blue staining of the crude protein after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by the Bradford assay (Pierce, Rockford, Ill.) as well as amino acid analysis (performed by a core facility at the Massachusetts General Hospital). To confirm purification of an active Fur protein, we tested purified Fur for DNA binding utilizing a gel shift assay with a fragment containing the divergent *irgA* and *irgB* promoters (and putative Fur box) essentially as previously described (3).

Induction of polyclonal antibodies to *V. cholerae* Fur. The purified Fur protein was run on an SDS–15% PAGE gel. The protein band was visualized with a copper stain (Pharmacia), excised, dried, ground to a fine powder, and resuspended in phosphate-buffered saline (PBS) to obtain a thin slurry. Protein prepared in this fashion was mixed in a 1:1 ratio with Freund's adjuvant (complete for the first injection only; Sigma Chemical Co., St. Louis, Mo.) immediately prior to injection. Two mature New Zealand White male rabbits weighing between 2.5 and 3.0 kg were injected subcutaneously with a total of 1 mg of Fur protein divided in four aliquots. Preimmune serum was obtained from the central ear artery prior to the first injection, and the rabbits were boosted by subcutaneous injection three times at 2- to 4-week intervals prior to euthanasia and final harvesting of serum.

To estimate the titer of anti-Fur antibodies in the rabbit sera, a microtiter plate was exposed to 1 μg of purified Fur per ml overnight at 4°C. After blocking, the Fur-coated plates were incubated sequentially with serial dilutions of the rabbit sera in PBS and then anti-rabbit immunoglobulin G (IgG)–alkaline phosphatase conjugate. One milligram of *p*-nitrophenyl phosphate (Amresco, Solon, Ohio) per ml in 1 M Tris, pH 8.0, was added to each well. The OD_{405} was read after approximately 15 min. For this procedure, a positive reading was taken as an OD_{405} greater than 0.1 (with controls of approximately 0.07). By this procedure, the polyclonal sera gave a positive result to a dilution of 1:100,000.

Induction of monoclonal antibodies to *V. cholerae* Fur. Primary immunization was accomplished by intraperitoneal injection of purified Fur protein (100 μg per mouse in 500 μl of 50% complete Freund's adjuvant) into female BALB/c mice. The secondary immunizations were performed at 4, 6, 8, and 10 weeks after primary immunization (50 μg per mouse in 500 μl of Freund's incomplete adjuvant). Splenocytes were fused with the myeloma cell line P3/NSI/1-Ag4-1 4 days after the final immunization (intravenous injection of 50 μg of Fur protein per mouse in 100 μl of PBS). Hybridomas that produced antibodies with anti-Fur binding activity were selected by ELISA using goat anti-mouse polyvalent immunoglobulins (IgG, IgA, and IgM) conjugated with horseradish peroxidase (Sigma). One of the anti-Fur-secreting hybridomas, 10H4 (IgM isotype), was

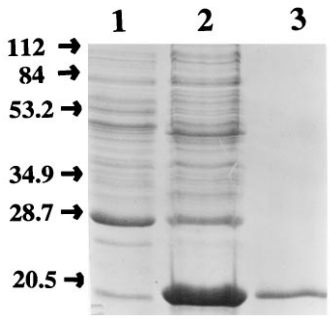

FIG. 1. SDS-PAGE of various cell extracts and purified *V. cholerae* Fur stained with Coomassie blue. Lanes: 1, BL21(DE3)(pLysS), 12 μg of protein loaded; 2, BL21(DE3)(pLysS, pPIW1) harvested 4 h after induction with IPTG, 12 μg of protein loaded; 3, purified Fur, 1.5 μg of protein loaded. Sizes are given on the left in kilodaltons.

further selected by two cycles of limiting dilution and cloning. Ascitic fluid was produced by injecting 3×10^6 10H4 hybridoma cells intraperitoneally into BALB/c mice that had been previously stimulated with 2,6,10,14-tetra-methylpentadecane (Pristane).

Immunoblotting of Fur. Cell extracts or purified protein were run on an SDS–15% PAGE gel and then transferred to a NitroBind transfer membrane (Micron Separations Inc., Westboro, Mass.) with a semidry blotting apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Immunoreactive proteins were visualized after sequential incubation with anti-Fur polyclonal rabbit antiserum (dilution, 1:10,000) or mouse ascitic fluid containing an anti-Fur monoclonal antibody (dilution, 1:2,000), goat anti-rabbit IgG-conjugated alkaline phosphatase, or goat anti-mouse IgM-conjugated alkaline phosphatase as appropriate, followed by staining for phosphatase activity. Polyclonal Fur antiserum was preadsorbed prior to use in immunoblots with a sonicated CML19 culture. Preadsorption of polyclonal antisera resulted in significantly less background.

Preparation of cell extracts for ELISA. Cultures were grown to the indicated OD_{600} in LB medium or syncase with appropriate antibiotics. An aliquot of the culture was set aside for measurement of CFU by plating serial dilutions. Ten milliliters of culture was pelleted by centrifugation at 4°C, freeze-thawed, and then resuspended in 250 μl to 1 ml of 20 mM Tris, pH 8.0, depending on the OD_{600} of the culture. Cell extracts were sonicated to complete lysis with three 10-s bursts with several minutes of cooling on ice between each burst. Control experiments demonstrated that additional sonication for up to a total of 2 min did not significantly increase the amount of protein released from cells. The cell lysate was spun for 30 min in an Eppendorf 5415C centrifuge at 14,000 rpm, and the supernatant was harvested and frozen at −80°C until use.

Development of a sandwich ELISA for quantitation of *V. cholerae* Fur. Anti-Fur monoclonal antibody in mouse ascitic fluid was diluted 1:1,000 in 60 mM carbonate buffer, pH 9.6. The diluted antibody was aliquoted into 96-well plates (Immunosorb MaxiSorb; Nunc, Roskilde, Denmark) and incubated overnight at 4°C. The following day, additional protein binding was blocked by a 1-h incubation with 2% bovine serum albumin in PBS–0.05% Tween 20. Dilutions of either purified Fur or cell extracts in PBS-Tween 20 were added to each of the wells. The plates were covered and incubated at 42°C overnight. The ELISA was particularly sensitive to the temperature at which Fur was incubated with the monoclonal antibody. The signal intensity was maximum after incubation at 42°C and dropped to background levels at room temperature. The polyclonal anti-Fur rabbit antiserum was subsequently added at a dilution of 1:500, and the plates were incubated at room temperature for 2 h. The alkaline phosphatase conjugate of anti-rabbit IgG developed in goats (Sigma) was added at a dilution of 1:7,500, and the plate was incubated at room temperature for an additional 2 h. *p*-Nitrophenyl phosphate (2 mg/ml) in 1 M Tris, pH 8.0, was added, and the reaction was allowed to proceed for approximately 45 min. Plates were then read with a Vmax ELISA plate reader (Molecular Devices, Sunnyvale, Calif.) at a wavelength of 405 nm. Volumes added in each step were 100 μl, and plates were washed with four well volumes of PBS-Tween 20 between each step.

One row of wells in each ELISA plate contained concentrations of purified Fur varying from 0.5 to 100 ng/ml. A least-squares routine was used to derive a linear equation relating OD_{405} readings to Fur concentration. The OD_{405} values measured for various dilutions of cell extracts were fit to $y = \log(x)$. The theoretical OD_{405} of a dilution in the initial portion of the curve was then calculated and used to determine the amount of Fur present in the undiluted extract.

RESULTS AND DISCUSSION

The inducible T7*lac* promoter of pET11a provided tight control and good induction of *V. cholerae* Fur expression. Following induction with IPTG, Fur was the predominant protein band in cell extracts analyzed by SDS-PAGE (Fig. 1, lane 2). Purification of Fur from 1 liter of cells yielded 10 to 15 mg of approximately 95% pure Fur, which ran as a single band of approximately 17 kDa by SDS-PAGE (Fig. 1, lane 3). The composition of the purified protein as determined by amino acid analysis matched the composition of the *V. cholerae* Fur protein predicted from the DNA sequence quite closely, with the number of most amino acid residues falling within 1 of the predicted number. As expected, the measured number of histidine residues was artificially high due to coelution with the Tris buffer. The purified Fur protein reacted strongly with polyclonal anti-*E. coli* Fur antiserum (a gift from Michael Vasil) (data not shown) and altered the mobility of a PCR-amplified *irgB-irgA* intergenic DNA fragment in the presence of Mn^{2+} but not in its absence (data not shown).

The rabbit polyclonal antisera used in the ELISA reacted specifically with the cloned, overexpressed *V. cholerae* Fur and the purified protein by Western blot (Fig. 2A). We also verified that the polyclonal antisera recognized native *V. cholerae* Fur by comparing immunoblots of extracts from wild-type *V. cholerae* O395 and its isogenic *fur* mutant derivative, CML19 (Fig. 2B). Similar results were obtained for monoclonal antibody 10H4 (data not shown). In both the Western blots utilizing monoclonal antibodies and those utilizing polyclonal antibodies, a second immunoreactive band was seen at approximately 34 kDa in the extracts of the *E. coli* expression strain and, to a

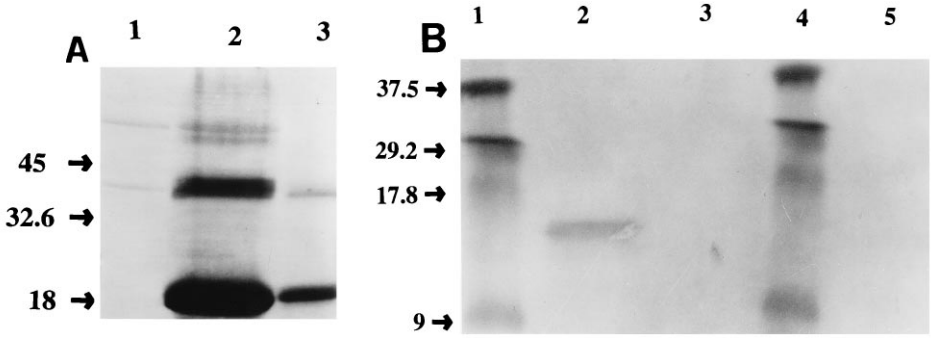

FIG. 2. Immunoblots of Fur in various cell extracts and purified protein. (A) Western blot using anti-Fur rabbit polyclonal antisera in a dilution of 1:10,000 as the primary antibody. Lanes: 1, BL21(DE3)(pLysS), 12 μg of protein loaded; 2, BL21(DE3)(pLysS, pPIW1) harvested 4 h after induction with IPTG, 12 μg of protein loaded; 3, purified Fur, 1.5 μg of protein loaded. (B) Western blot of cytoplasmic extracts of *V. cholerae* strains using anti-Fur polyclonal antisera preadsorbed with CML19, an isogenic *fur* mutant of *V. cholerae* O395, used in a dilution of 1:20,000. Lanes: 1, molecular size markers (marked in kilodaltons on the left); 2, O395, 50 μg total protein loaded; 3, empty; 4, molecular size markers; 5, CML19 (a *fur* mutant derivative of O395), 50 μg of total protein loaded.

TABLE 2. Measurements of the intracellular abundance of Fur in *V. cholerae* O395 cultures at various stages of growth

OD_{600}	Fur (ng/ml)/OD_{600} in culture (mean ± SD)	No. of Fur molecules/bacterial cell (mean ± SD)
0.671	188 ± 39	2,618 ± 551
1.140	308 ± 71	5,909 ± 1,364
2.050	529 ± 84	7,414 ± 1,084

much lesser extent, in the purified protein. We believe that this band represents an aggregated form of Fur, which is removed to some extent by purification. Although this Fur aggregate may be a disulfide-linked dimer, efforts to reduce disulfide bonds by incubation with 100 mM dithiothreitol at 60°C for several hours did not eliminate this band.

The Fur protein concentration for use in standardizing our ELISA was determined by the Bradford assay. The protein concentration was also measured by amino acid analysis and found to be within 5% of the Bradford assay result. The linear range of the optimized ELISA is approximately 10 to 200 ng/ml. A typical standard curve gave an equation of [Fur (ng/ml)] = $-17 + 105 \times OD_{405}$ ($R^2 = 0.981$).

We used this optimized ELISA to measure the intracellular abundance of Fur in *V. cholerae* O395 at different stages of growth. When the concentration of Fur in cell extracts was corrected for the OD_{600} of the culture (as an estimate of the total cell mass), a threefold increase in the intracellular abundance of Fur was observed as the culture progressed from mid-log to stationary phase (Table 2). The number of viable cells at each of these time points (expressed as CFU per milliliter) was linearly related to the OD_{600}, suggesting that the mass per cell did not change under these conditions. This allowed us to estimate the number of Fur monomers per bacterial cell. During the mid-logarithmic phase of growth, the number of Fur monomers per cell was measured to be approximately 2,500. Similar types of measurements have been made for other bacterial repressor proteins, including the nitrogen assimilation regulator I (5 to 70 molecules/cell [26]), the *trp* repressor (50 to 300 molecules/cell [15]), and the leucine-responsive regulatory protein (Lrp; 6,000 molecules/cell [34]). The number of Fur molecules per cell is higher than those of several other repressor proteins but comparable to that of Lrp. Two-dimensional gel electrophoresis has demonstrated that the Lrp (8) and Fur (19) regulons are similarly large and complex. The bacterial cell therefore may require large numbers of these regulatory molecules for proper functioning.

The number of Fur monomers per cell increased as cells reached the late logarithmic and stationary phases, with a maximum in stationary phase of approximately 7,500 Fur monomers per cell (Table 2). This is in agreement with the studies of De Lorenzo et al. with *E. coli* using a *fur*::*lacZ* fusion (4). In *E. coli*, it has been suggested that catabolite activator protein also plays a role in Fur regulation, and a catabolite activator protein binding site has been identified in the promoter of *E. coli fur* (4). The addition of cyclic AMP to the growth medium, however, did not significantly alter the level of *V. cholerae* Fur in cells (not shown).

TABLE 3. Measurement of the abundance of Fur at mid-log phase for various strains and growth conditions

Strain	Growth condition	Fur (ng/ml)/OD_{600} in culture (mean ± SD)
O395	LB medium	188 ± 39
CML19	LB medium	<1
O395	Syncase	265 ± 80
O395	Microaerophilic	87 ± 2

We also measured the abundance of Fur under different growth conditions and in different strains, some of which are shown in Table 3. To standardize these comparisons, all measurements were made in extracts of cells harvested at mid-log phase. As expected, the amount of Fur in an extract of CML19, a *fur* mutant of O395, was unmeasurable by our ELISA and significantly smaller than in strain O395 ($P = 0.002$). Growth of *V. cholerae* O395 in syncase, a low-iron medium, showed a small increase in the abundance of Fur which did not reach statistical significance ($P = 0.2$). An experiment in *V. vulnificus* which used a *fur*::*lacZ* fusion on a plasmid to assess regulation of *fur* expression by iron also found no significant effect (18). In contrast, previous results in *E. coli* have suggested an increase in *fur* expression when cells are grown in low-iron medium (4).

Growth of *V. cholerae* O395 in microaerophilic conditions decreased the abundance of Fur by approximately a factor of 2 ($P = 0.06$), indicating a possible interaction between expression of *fur* and regulatory proteins responsive to anaerobic conditions. Additional studies are necessary to determine the nature of this effect. There was no significant difference between Fur intracellular abundance in *V. cholerae* O395 and that in JRB3 (an *irgB* mutant of O395) (data not shown).

These studies show that the basal expression of Fur in the cell is quite high and is altered by less than an order of magnitude under the conditions described above. At least two possible explanations for the high basal expression of Fur may be considered. First of all, Fur may serve in more than just a regulatory capacity in the cell. For instance, it may provide an iron storage mechanism for the cell. This seems unlikely, since Fur expression is not increased in the presence of high iron and low oxygen, both conditions under which an Fe(II) repository would probably be most needed. A second explanation would be that Fur requires high intracellular concentrations to function effectively as a regulator because of (i) the large number of genes that it regulates, (ii) the affinity of Fur for iron in vivo, (iii) the concentration of Fur necessary for dimerization to occur, and/or (iv) the affinity of the Fur-Fe complex for some or all of its promoters in vivo. If the affinity of the Fur-Fe complex differed for different promoters, this might provide a mechanism by which a single regulatory protein could produce a graded response to intracellular iron concentration. The small variation in Fur concentration under the conditions studied suggests that the degree of repression of other genes by Fur is governed primarily by changes in iron concentration rather than by changes in the concentration of Fur itself. Experiments to test these hypotheses are under way.

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