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Iron-Responsive Genetic Regulation in *Campylobacter jejuni*: Cloning and Characterization of a *fur* Homolog

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The Fur protein of *Escherichia coli* represses transcription from Fur-responsive genes in an iron-dependent manner. We have demonstrated a Fur-like iron-responsive genetic regulatory activity operating in *Campylobacter jejuni* by using a chloramphenicol acetyl transferase reporter gene separated from its promoter by a synthetic Fur-responsive operator. A *fur*-like gene has been cloned from *C. jejuni* by partial functional complementation of an *E. coli fur* mutation. Sequence analysis has shown that, at the amino acid level, the *C. jejuni* Fur protein is 35% identical with its *E. coli* counterpart.

Pathogenic and commensal bacteria possess high-affinity iron-scavenging systems allowing them to obtain sufficient quantities of this essential trace metal in the iron-limiting environment of the vertebrate host (9, 17, 23). Genes responsible for iron scavenging are coordinately and negatively regulated in response to the iron status of the bacterial cell (2). In addition, genes encoding virulence determinants not directly involved in iron scavenging may also be regulated by iron (4, 5, 11, 20, 32). In *Escherichia coli* more than 30 genes are repressed by the Fur protein (ferric uptake regulator; product of the *fur* gene) (2), which utilizes ferrous iron as a corepressor and binds to specific operators (12, 24). Highly conserved homologs of *fur* have been cloned from *Vibrio cholerae* (27), *Vibrio vulnificus* (28), *Vibrio anguillarum* (40), *Yersinia pestis* (36), *Pseudomonas aeruginosa* (34), and *Neisseria gonorrhoeae* (3), and iron-regulated genes in *Neisseria meningitidis* (39) and *Bacillus subtilis* (15) are preceded by promoters containing sequences with similarity to Fur-responsive operators in *E. coli*. A less highly conserved *fur* homolog, *dtxR*, has been found in the gram-positive organism *Corynebacterium diphtheriae* (7). The DtxR protein binds to AT-rich operators which have similarity to Fur-binding sequences (37).

Campylobacter jejuni is a leading cause of bacterial diarrhea worldwide, causing a spectrum of disease from watery diarrhea to acute colitis (6, 10). Extraintestinal infections including meningitis (22), cholecystitis (18), septicemia (29), and urinary tract infection (19) also occur occasionally. The mechanisms by which *C. jejuni* causes disease are poorly understood. The only genes to have been convincingly demonstrated to contribute to virulence are the flagellin genes (42). Expression of flagellin genes is subject to phase (13) and antigenic (26) variation and is environmentally regulated (1), but little is known about the genetic regulation of other potential virulence-determinant genes.

In common with other bacteria, *C. jejuni* synthesizes new envelope-associated proteins in response to iron stress (21). One such protein is probably a component of a high-affinity uptake pathway for hemin and hemoglobin (33); thus an iron-responsive regulatory circuit similar to the Fur system probably regulates a subset of virulence-associated genes in *C. jejuni*. We report the demonstration of a Fur-like activity in *C. jejuni* and the cloning and characterization of the *C. jejuni fur* homolog.

E. coli strains were grown at 37°C with agitation in Luria broth, on Luria agar, or on MacConkey agar supplemented with 60 μM $FeSO_4$. Where appropriate, ampicillin (100 μg/ml), kanamycin (50 μg/ml or 300 μg/ml for selection of Tn*phoA* in high copy number) or rifampin (50 μg/ml) was included. Luria broth was supplemented with 200 μM 2,2′-dipyridyl (Sigma) to achieve iron-restricted conditions; 60 μM $FeSO_4$ was added to achieve high-iron conditions. *C. jejuni* strains were grown in a variable-atmosphere incubator at 42°C in an atmosphere of 6% O_2, 5% CO_2, 6% H_2, and 83% N_2. Liquid cultures were grown with agitation in Mueller-Hinton broth (Unipath). Solid medium was Mueller-Hinton agar. Kanamycin (50 μg/ml) was included where appropriate. $FeSO_4$ (60 μM) was added to broth cultures to achieve high-iron conditions; 25 μM desferrioxamine mesylate (Desferal; CIBA GEIGY) was included to achieve iron-restricted conditions.

Chromosomal DNA was prepared as described by Wassenaar et al. (42). Plasmid and M13 replicative form and single-stranded DNA were prepared by standard techniques (35). Restriction enzymes were used according to the manufacturer's instructions. Electrotransformation of *E. coli* was performed by standard methodology (35), and electrotransformation of strain 81116 (31) was performed as described by Wassenaar et al. (42).

Demonstration of a Fur-like activity in *C. jejuni*. We have been unable to detect a protein in *C. jejuni* extracts which cross-reacts in Western blots (immunoblots) with an antiserum raised against the purified *E. coli* Fur protein (data not shown). To search for a protein with Fur-like activity, we constructed an intracellular probe consisting of the coding sequence of a chloramphenicol acetyltransferase (*cat*) gene of *Campylobacter* origin, just upstream of which we inserted a synthetic Fur-responsive operator sequence based on the *E. coli* Fur-binding consensus sequence 5′-GATAATGATAATCATTATC-3′ (11). The final construct, pKG6, was a derivative of pUOA14 (a *Campylobacter-E. coli* shuttle vector) (41), into which a synthetic double-stranded DNA was inserted downstream of the promoter site and 20 bp upstream of the ATG start codon of *cat*. The synthetic DNA was prepared by annealing two complementary oligonucleotides, 5′-GATCGATAATGATAATCATTATCG-3′ and 5′-GATCCGATAATGATTATCATTATC-3′. A control construct, pKG61, lacked the synthetic Fur-binding operator but was otherwise identical to pKG6. pKG6 and pKG61 were introduced into *C. jejuni* 81116 by electroporation.

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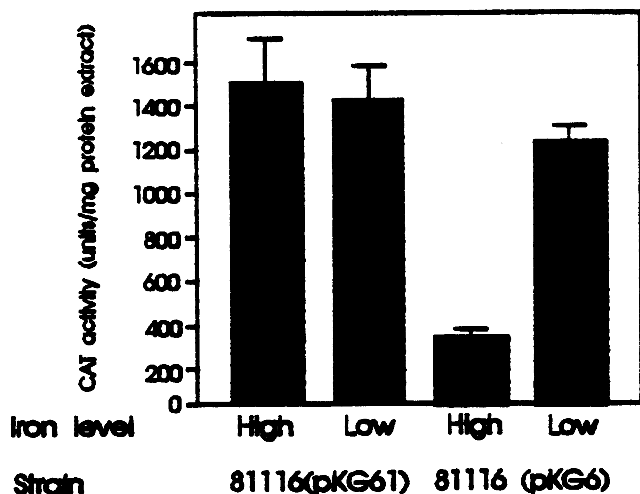

FIG. 1. CAT activity in extracts of 81116(pKG6) and 81116 (pKG61) grown in high-iron or low-iron medium (Mueller-Hinton broth supplemented with either 60 μM $FeSO_4$ or 25 mM desferrioxamine mesylate, respectively). Each column represents an average of three determinations; error bar represents one standard deviation.

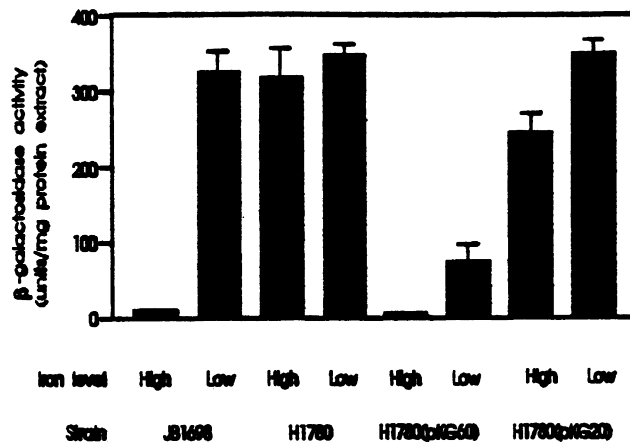

FIG. 2. β-Galactosidase activity in *E. coli* cells of strain JB1698 (*fiu*::λ*plac*Mu53), H1780 (like JB1698 but *fur*), H1780(pKG60), or H1780(pKG20) grown in high-iron or iron-restricted medium (Luria broth supplemented with either 60 μM $FeSO_4$ or 200 μM dipyridyl, respectively). Each column represents an average of three determinations; error bar represents one standard deviation.

Cells of *C. jejuni* 81116 containing either plasmid pKG6 or plasmid pKG61 from 5-ml exponential-phase cultures, grown in either high-iron or iron-restricted media, were harvested by centrifugation, resuspended in 200 μl of 250 mM Tris-HCl (pH 8.0), and disrupted by sonication. Insoluble debris was removed by ultracentrifugation at 100,000 × *g*. The protein content of each extract was determined by the method of Bradford (8), and CAT activity was determined by using a Promega CAT enzyme assay system according to the manufacturer's instructions. Significant CAT activity was detected in extracts of cells containing plasmid pKG6 or plasmid pKG61 (Fig. 1). While the level of CAT activity in extracts prepared from pKG61 transformants was not significantly affected by the iron status of the cells, extracts prepared from pKG6 transformant cells which had been grown in low-iron conditions consistently contained approximately fourfold-higher levels of CAT activity than did those prepared from cells grown in high-iron medium (Fig. 1).

Cloning of a *C. jejuni fur* homolog. In order to clone a putative *fur* homolog from *C. jejuni*, total genomic DNA from *C. jejuni* 81116 was digested with *Sau*3AI, size-selected by agarose gel electrophoresis for fragments between 1 and 10 kb, and ligated into the *Bam*HI site of pUC19 (43). Ligation products were used to electrotransform cells of *E. coli* H1780 (25), and transformed cells were plated onto MacConkey agar plates supplemented with $FeSO_4$ and ampicillin. *E. coli* H1780 contains a gene fusion between the Fur-repressible gene *fiu* and *lacZ* (25). In addition the strain is a *fur* mutant and thus, even in high-iron conditions, there is no repression of the gene fusion, resulting in high levels of β-galactosidase expression. However, providing a functional *fur* gene in *trans* results in iron-dependent repression of the gene fusion and consequent reduction in β-galactosidase expression. High expression of β-galactosidase causes colonies to appear bright red on MacConkey agar, whereas reduced expression results in a pale colony color. One such pale colony was detected among the background of red transformant colonies. Plasmid DNA prepared from this clone was reintroduced into strain H1780, and transformants were grown on MacConkey agar to confirm the phenotype. The plasmid contained an insert of approximately 5 kb and was designated pKG20.

Partial complementation of the *E. coli fur* mutation by the cloned *C. jejuni fur* homolog. To quantify the activity of the cloned *C. jejuni fur* homolog in *E. coli*, β-galactosidase activity of JB1698 (*fur*$^+$ parent of H1780) (25), H1780, and H1780(pKG20) cells grown in high- and low-iron conditions were determined by the method of Miller (30) (Fig. 2). As an additional control, the *E. coli fur* gene was subcloned from plasmid pMH15 (25) on a 2.3-kb *Xho*II fragment into the *Bam*HI site of pUC19, and the resulting plasmid, pKG60, was introduced into H1780. In strain JB1698, expression of the *fiu-lacZ* fusion was reduced 96% under high-iron conditions compared with low-iron conditions. No significant reduction was observed in the isogenic *fur* mutant strain H1780. When strain H1780(pKG60) was grown in iron-restricted conditions, the β-galactosidase level was only 20% of that seen in strain JB1698. Nonetheless, under high-iron conditions, a substantial reduction in β-galactosidase activity (89%) was observed. Strain H1780(pKG20), carrying the putative *C. jejuni fur*-like gene, had a high level of β-galactosidase activity under low-iron conditions; increased iron caused a 37% decrease in β-galactosidase activity.

Proteins encoded by plasmid pKG20. Plasmid pKG20 was used to program a Promega in vitro transcription/translation kit. Synthesis of proteins of approximately 16.5, 17.5, and 21 kDa was detected (Fig. 3). The *E. coli* Fur protein is 17 kDa.

Sequence analysis of the *C. jejuni fur* gene. Transposon mutagenesis using Tn*phoA* was used to facilitate sequencing of the *fur* homolog on plasmid pKG20. To create Tn*phoA* insertion mutants of pKG20, the plasmid was first transferred to strain CC118 (38), a kanamycin-sensitive strain. Strain SM10λ*pir* (38) harboring suicide vector pRT733 was used as the Tn*phoA* donor and was mated with CC118(pKG20) as described by Taylor et al. (38). Kanamycin- and rifampin-resistant clones resulting from the mating were pooled, and plasmid DNA was prepared from them for transformation of strain H1780. Transformed cells were plated onto $FeSO_4$-supplemented MacConkey agar containing ampicillin. Two plasmids, pKG31 and pKG32, isolated from red colonies, were used as templates for chain termination sequencing by using

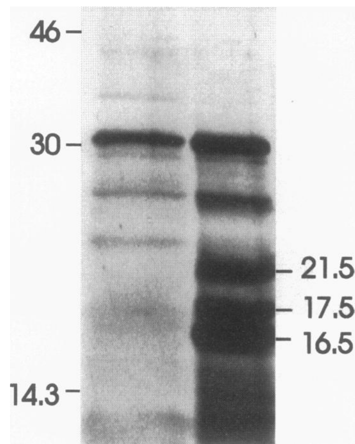

FIG. 3. Autoradiograph showing the products produced in an in vitro transcription/translation assay programmed with pUC19 (left) or pKG20 (right). Sizes (in thousands) of the radiolabeled molecular weight markers are indicated to the left, and deduced sizes of the major translation products unique to pKG20 are indicated to the right.

the primers 5′-CTGAGCAGCCCGGTT-3′ and 5′-GTTAG GAGGTCACATG-3′, which are specific for the left and right ends, respectively, of Tn*phoA*. The nucleotide sequence determined in this way identified a *Cla*I site upstream and an *Apa*I site downstream of the *fur* gene, and these sites were used to subclone an 805-bp fragment from pKG20 into the *Hin*cII site of pUC19 after the ends were converted to blunt ends by using DNA polymerase I. The M13 forward and reverse primers (U.S. Biochemical) were then used as sequencing primers to confirm sequence deduced from the Tn*phoA*-mutagenized genes. The nucleotide sequence of the *C. jejuni fur* gene is shown in Fig. 4. The predicted mass of the putative *C. jejuni* Fur protein, on the basis of the deduced amino acid sequence, is 17,990 Da. The 17.5-kDa product observed in the in vitro transcription/translation assay (Fig. 3) is probably the product of this gene. When comparing the nucleotide sequence of the *C. jejuni fur* gene with sequences deposited in the EMBL/GenBank databases, we noticed that the sequence encoding the C-terminal 57 amino acids had been reported previously as the region upstream of the lysyl-tRNA synthetase gene (14). The coding sequences of the two genes are in frame and separated by a single stop codon.

Two sequences, each containing 11 of the 19 bases making up the *E. coli* Fur-binding consensus sequence (12), were identified upstream of the structural gene (Fig. 4). This raises the possibility that the *C. jejuni fur* gene, like its counterpart in *E. coli*, may be autoregulated.

The Fur homologs of other gram-negative bacteria are highly conserved (Fig. 5). By contrast, the Fur-like protein of *C. jejuni* is relatively highly diverged; only the Fur homolog DtxR of the gram-positive organism *Corynebacterium diphtheriae* is less like the other gram-negative Fur proteins. All other known Fur proteins are at least 50% identical to each other, whereas the *C. jejuni* Fur protein is less than 40% identical to its closest match (the Fur protein of *P. aeruginosa*). The motif CXYCG, which is present within metal-binding centers of many proteins, has been suggested as a metal-binding motif within the Fur protein (3, 16). This motif was found within the *C. jejuni* Fur-like protein.

The degree of dissimilarity of the *C. jejuni* Fur protein with respect to its *E. coli* counterpart is reflected both antigenically,

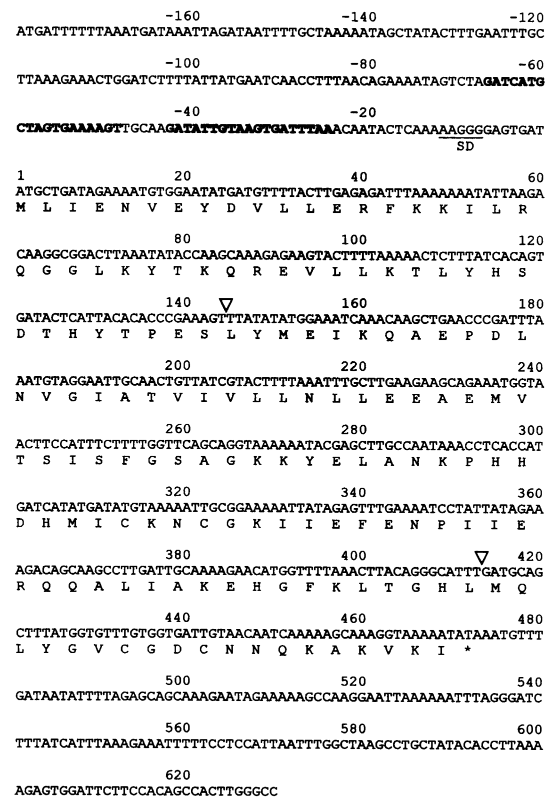

FIG. 4. Nucleotide and deduced amino acid sequence of the *C. jejuni fur* gene. A potential Shine-Dalgarno (SD) sequence is underlined. Sequences with similarity to the *E. coli* Fur-binding consensus sequence and overlapping the promoter region are shown in boldface. Open arrows at positions 144 and 413 indicate the points of insertion of Tn*phoA* within plasmids pKG32 and pKG31, respectively (see text).

by its lack of reactivity in Western blots, and functionally, by the low level of repression of the *E. coli fiu* promoter compared with that afforded by the *E. coli* Fur protein. If *C. jejuni* Fur-like protein is truly the major iron-dependent regulator in this organism, its recognition sequence, as well as the repressor itself, may have significantly diverged between the two species. To determine whether a *C. jejuni* Fur-binding consensus sequence which is divergent from that of *E. coli* exists, it will be necessary to sequence more *Campylobacter* Fur-responsive operators.

We have shown that *C. jejuni* is able to respond to the availability of iron in its environment and that it codes for a protein homologous to the Fur proteins of *E. coli* and other gram-negative bacteria. Since Fur and Fur-like repressors are known to regulate some virulence-determinant genes in other bacteria, the *C. jejuni* Fur-like repressor protein may also regulate a subset of genes with a role in pathogenesis. As a corollary, genes repressed by Fur in *C. jejuni* would be good candidate virulence determinants. We are therefore attempting to clone genes from *C. jejuni* with Fur-repressible promoters which could be tested individually for a role in virulence after mutagenesis and in vivo marker exchange.

Nucleotide sequence accession number. The sequence of the *C. jejuni fur* gene has been deposited with the EMBL Data Library and has been assigned the accession number X78965.

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C. jejuni          MLIENVEYDV LLERFKKILR QGGLKYTKQR EVLLKTLYHS DT-HYTPESL
N. gonorrhoeae              M EKFSNIAQ.K DS...V.GP. LKI.DLFEKH AEE.LSA.DV
P. aeruginosa                  MVENSE.. KA...V.LP. VKI.QM.DSA EQR.MSA.DV
Y. pestis                     MTDNN.A.K NA...V.LP. LKI.EV.QNP ACH.VSA.D.
E. coli                       MTDNNTA.K KA...V.LP. LKI.EV.QEP .NH.VSA.D.
V. vulnificus                 MSDNNQA.K DA...V.LP. LKI.EV.QQP .CQHISA.D.
V. cholerae                   MSDNNQA.K DA...V.LP. LKI.EV.QQP ECQ.ISA.E.

C. jejuni          YMEIKQAEPD LNVGIATVIV LLNLLEEAEM VTSISFGSAG KKYELANKPH
N. gonorrhoeae     .RILLEEGVE --I.V..IYR V.TQF.Q.GI LQRHH.ETGK AV...DKGD.
P. aeruginosa      .KALME.GE. --V.L...YR V.TQF.A.GL .VRHN.DGGH AVF...DSG.
Y. pestis          .KILIDIGEE --I.L...YR CSEQFDD.GI ..RHN.EGGK SVF..TQQH.
E. coli            .KRLIDMSEE --I.L...YR V..QFDD.GI ..RHN.EGGK SVF..TQQH.
V. vulnificus      .KKLIDLGEE --I.L...YR V..QFDD.GI ..RHH.EGGK SVF..STQH.
V. cholerae        .KKLIDLSEE --I.L...YR V..QFDD.GI ..RHH.EGGK SVF..STQH.

C. jejuni          HDHMICKNCG KIIEFENPII ERQQALIAKE HGFKLTGHLM QLYGVCGDCN
N. gonorrhoeae     ...IV.VK.. EVT..HN.E. .AL.DK..E. N.YRIVD.AL YM....-SDC
P. aeruginosa      ...MV.VDT. EV...MDAE. .KR.KE.VR. R..E.VD.NL V..VRKKK
Y. pestis          ...L..LD.. .V...S.ES. .SL.RE...Q ..I...N.SL Y...H.E-TG
E. coli            ...L..LD.. .V...SDDS. .AR.RE..AK ..IR..N.SL Y...H.A-EG
V. vulnificus      ...LV.LD.. EV...SDDI. .ER.KE..AK YNVQ..N.SL Y...K..SDG
V. cholerae        ...LV.LD.. EV...SDDV. .QR.KE..AK YNVQ..N.SL Y...K..SDG

C. jejuni          NQKAKVKI
N. gonorrhoeae     QA.G.R
P. aeruginosa
Y. pestis          .CREDESAHS KR
E. coli            DCREDEHAHE GK
V. vulnificus      SC.GNPDAHK RKS
V. cholerae        SCKDNPNAHK PKK
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FIG. 5. Alignment of the deduced amino acid sequence of the *C. jejuni* Fur protein with Fur proteins of *N. gonorrhoeae*, *P. aeruginosa*, *Y. pestis*, *E. coli*, *V. vulnificus*, and *V. cholerae*. The sequences were aligned by using the multiple sequence alignment program PILEUP from the Genetics Computer Group sequence analysis software package (University of Wisconsin, Madison) and were inverted to display the most highly diverged *C. jejuni* Fur protein at the top. Dots indicate identity with the *C. jejuni* Fur protein; dashes indicate gaps introduced into the sequences by the PILEUP software package.

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