INFECTION AND IMMUNITY, July 1994, p. 2687–2694
0019-9567/94/$04.00+0

Vol. 62, No. 7

# Genetic, Enzymatic, and Pathogenic Studies of the Iron Superoxide Dismutase of *Campylobacter jejuni*

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Received 4 February 1994/Returned for modification 16 March 1994/Accepted 11 April 1994

***Campylobacter jejuni* is a microaerobic bacterium that produces an acute, self-limiting, watery or bloody diarrhea in humans. Little is known about how *C. jejuni* causes disease or even what specific capabilities it requires for survival in vivo. The enzyme, superoxide dismutase (SOD), which catalyzes the breakdown of superoxide radicals to hydrogen peroxide and dioxygen is one of the bacterial cell's major defense mechanisms against oxidative damage. A PCR-based search for *sod* genes in *C. jejuni* 81-176 revealed that this bacterium contained at least one *sod* gene. We cloned and sequenced a *sod* gene from 81-176 and determined that its predicted protein product was most similar to that of FeSODs (*sodB* genes). Transcriptional analysis indicated that this gene is monocistronic and may be transcribed from a $\sigma^{70}$-like promoter. Nondenaturing polyacrylamide gels stained to reveal SOD activities, accompanied by inhibition studies, demonstrated that *C. jejuni* produces five electrophoretically distinct bands of SOD activity, all of which appeared to be FeSODs. Analysis of an 81-176 *sodB* strain revealed that all of these FeSOD activities may be products of the one *sodB* gene that we cloned. The expression and enzymatic activity of the respective *sodB* and FeSOD produced by both *C. jejuni* and *Helicobacter pylori* were examined in *Escherichia coli*. Both genes were expressed in *E. coli*, and the proteins produced were enzymatically active. Finally, the ability of the 81-176 *sodB* strain to survive INT407 cell invasion was found to be significantly decreased (12-fold) compared with that of the parent, suggesting a potential role for SodB in *C. jejuni* intracellular survival.**

*Campylobacter jejuni*, a major causative agent of human diarrheal disease, is one of only a few bacterial species with a known requirement for microaerobic growth conditions. The enteric disease caused by *C. jejuni* occurs commonly in both developed and underdeveloped countries (7), causing, for example, an estimated 2 million cases of enteritis each year in the United States (49). *C. jejuni* usually generates an acute, self-limiting infection in healthy humans, with symptoms consisting of watery or bloody diarrhea accompanied by fever, cramping, and nausea (8). The mechanisms by which *C. jejuni* produces disease are not well understood, but it is thought that colonization and invasion of intestinal epithelial cells are important capabilities (30, 57). A variety of toxins, including cytolethal distending toxin, and a hemolysin have also been mentioned as possible pathogenic factors (3, 27, 45).

*Helicobacter pylori*, a close relative to *C. jejuni* (19), is also a microaerobic human pathogen. It has been recently recognized as a cause of chronic type B gastritis in humans and may contribute to the development of gastric ulcers and gastric cancer (34). Some aspects of the ability of *H. pylori* to survive and colonize the human stomach are known, but many questions about its basic metabolism as well as specific pathogenic mechanisms still remain to be answered (34).

Superoxide dismutases (SODs) catalyze the breakdown of superoxide molecules to hydrogen peroxide and dioxygen and thus constitute one of the cell's major defense mechanisms against oxidative stress (22). These enzymes are widely distributed in nature, having been found in most, if not all, aerobic organisms and some anaerobic organisms. Three general types of SODs have been described: those containing iron cofactors (FeSOD or SodB), those with manganese cofactors (MnSOD or SodA), and those with both copper and zinc cofactors (CuZnSOD or SodC). They have been shown to specifically protect several cell components, including cytoplasmic enzymes, DNA, and membrane factors, from damage caused by oxygen radicals (26, 53). CuZnSOD appears to be found primarily in eukaryotes and a few prokaryotes, while MnSOD and FeSOD are found primarily in prokaryotes (22). MnSOD and FeSOD have very similar amino acid sequences (42), and the monomers can combine to form active homodimeric or heterodimeric (hybrid) enzymes (14). Studies of *sod* genes from several different organisms have been initiated, yet data characterizing the possible role of bacterial SODs in vivo are limited. The available data suggest that SODs may play a role in pathogenesis in some in vivo environments or in some bacteria, but perhaps not in others. For example, the SodB of *Shigella flexneri* and a secreted, surface-associated SOD of *Nocardia asteroides* have been reported to play an important role in protection against killing by human polymorphonuclear leukocytes (4, 5, 17). *sodB* mutants of *S. flexneri* were also shown to be less pathogenic than wild-type bacteria when tested with the rabbit ligated ileal loop model (17). However, another report suggests that *sodB* is not an important virulence factor for a serum-resistant *Escherichia coli* K-12 derivative (41).

There are brief accounts of SOD activity from *C. jejuni*, but the enzymes were not characterized (25, 28), and two recent reports have characterized a *sodB* gene from *H. pylori* (43, 51). In an effort to learn more about the lifestyle and survival of these microaerobic organisms, both in vivo and in vitro, we have initiated a study of the SOD(s) that they possess. In this paper, we report the cloning, sequencing, initial characterization, and mutagenesis of the *sodB* gene from *C. jejuni*. We also report on the expression and activity in *E. coli* of the *C. jejuni*

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and *H. pylori sodB* genes and SodB proteins, respectively. Finally, we report that the FeSOD of *C. jejuni* may be required for intracellular survival in INT407 cells.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and plasmids.** *C. jejuni* 81-176 has been described before (32). *H. pylori* 60190 (11) was obtained from the American Type Culture Collection. *E. coli* DH5αMCR (Gibco/BRL, Gaithersburg, Md.) and SURE (Stratagene, La Jolla, Calif.) were used as recipients in transformations according to the manufacturer's instructions. The *E. coli* minicell-producing strain, P678-54, is described by Adler et al. (1). *C. jejuni* was grown on brucella agar, as described previously (45). *H. pylori* was grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 10% defibrinated sheep blood (Colorado Serum Co., Denver, Colo.) and 6 μg of vancomycin, 20 μg of nalidixic acid, and 2 μg of amphotericin B per ml. *C. jejuni* and *H. pylori* were grown on plates at 42 and 37°C, respectively, in a microaerobic atmosphere of 5% $O_2$–10% $CO_2$–85% $N_2$. *E. coli* strains were grown aerobically on L agar or broth (35) at 37°C. When necessary, ampicillin was added to media to a final concentration of 50 μg/ml.

Plasmid pBluescript IISK$^-$ was obtained from Stratagene. Plasmids pCSD035, pCSD1.2, and pHSD1.3 were isolated during the course of this work. pCSD035 consists of a 2.9-kb *Bcl*I fragment of *C. jejuni* chromosomal DNA inserted into pBluescript. pCSD1.2 consists of a 1.2-kb *Bsa*BI-*Bsm*I fragment derived from pCSD035, containing an intact *sodB* gene and putative promoter region, inserted into pBluescript. pHSD1.3 is a derivative of pHSD141 (43), a plasmid consisting of a 7-kb *Nsi*I-*Bam*HI fragment of *H. pylori* chromosomal DNA inserted into pBluescript. pHSD1.3 has a 1.3-kb *Bsa*BI-*Bsm*I fragment which contains the intact *H. pylori sodB* gene and its putative promoter region cloned into pBluescript. Both pCSD1.2 and pHSD1.3 have their respective *sodB* genes oriented so that transcription of these genes is in the opposite direction of transcription from the *lac* promoter present on pBluescript.

**DNA techniques.** Whole-cell DNA was purified as described by Silhavy et al. (48). Plasmid DNA was purified with an alkaline extraction procedure (35). DNA fragments were purified from agarose gels with QIAEX (Qiagen, Studio City, Calif.). Southern blot and DNA-DNA hybridizations were performed as described in Maniatis et al. (35). Colony blots and their use in hybridizations were as described by Wahl and Berger (56). When necessary, DNA was radiolabeled by nick translation, using [α-$^{32}$P]dATP (NEN Research Products, Boston, Mass.) and a nick translation kit (Promega, Madison, Wis.).

**PCR.** Degenerate oligonucleotide primers corresponding to amino acids 124 to 130 (FGSGWAW) and 167 to 174 (DVWE HAYY) of the *E. coli* MnSOD (53) were synthesized by the Macromolecular Structure Analysis Facility at the University of Kentucky. Two oligonucleotide pools, 5′-TT(CT)GGNAG (CT)GGNTGGGCNTGG-3′ (SOD1A) and 5′-TT(CT)GGN TCNGGNTGGGCNTGG-3′ (SOD1B), were based on the amino acid sequence FGSGWAW, and one pool, 5′-TA(AG) TANGC(AG)TG(CT)TCCCANAC(AG)TC-3′ (SOD2), was based on the amino acid sequence DVWEHAYY. These primers correspond to regions that are highly conserved among both Mn- and FeSODs from seven different bacterial species (38). A PCR with these primers and template DNA containing a *sodA* or a *sodB* gene was expected to produce a product of about 150 bp. Whole-cell DNA from *C. jejuni* 81-176 was used as template DNA for the reactions. The PCR reactions contained 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 1× *Taq* DNA polymerase buffer (Promega), a 0.4 μM concentration of each primer, 0.5 μg of template DNA, and 5 U of *Taq* DNA polymerase (Promega). Reaction parameters were 30 cycles, as follows: 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min. PCR products were ligated into pBluescript, recombinant plasmids were transformed into *E. coli* DH5αMCR, and sequence analysis was performed on 14 plasmids that appeared to contain inserts of about 150 bp.

**Nucleotide sequencing.** The DNA sequence of both strands of the relevant insert region of pCSD035 was obtained. The dideoxy chain termination procedure was followed for sequencing reactions (47) with the use of Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) and α-$^{35}$S-dATP (NEN Research Products). DNA and predicted protein sequences were analyzed by PCgene (IntelliGenetics, Inc., Mountain View, Calif.). Amino acid alignments were performed with the CLUSTAL multiple alignment method of Higgins and Sharp (24).

**RNA isolation and analysis.** *C. jejuni* was grown for 18 h, and RNA was isolated by following the procedure described by Reddy (46). Aurintricarboxylic acid (Sigma Chemical Co., St. Louis, Mo.) was left out of the stop solution when RNA to be used in primer extensions was isolated. Northern (RNA) blot analysis was performed according to the procedure of Brown (9). Briefly, RNA was separated by electrophoresis at 20 V for 19 h on a gel (1.5% agarose, 6.2% formaldehyde). A 0.24- to 9.5-kb RNA ladder obtained from Gibco/BRL was used as a size marker. The blot was probed with radiolabeled, cloned PCR product released from pCSD19. Primer extension was carried out as described by Kingston (29). The primer 5′-CCTGCAAATTCAGTATCT-3′ (bases 427 to 410 of Fig. 1), 100 ng, was end labeled with [γ-$^{32}$P]ATP (NEN Research Products) and polynucleotide kinase (Boehringer GmbH, Mannhein, Germany) as described by Maniatis et al. (35). Labeled primers were added to 20 μg of aurintricarboxylic acid-free RNA, and extension was completed with a Superscript II RNase H$^-$ reverse transcriptase (Gibco/BRL). The extended primer was loaded onto a sequencing gel next to sequencing reactions performed with the same primer.

**Construction of a *C. jejuni sodB* mutant.** A *sodB* derivative of *C. jejuni* 81-176 was constructed according to the basic procedure developed by Labigne-Roussel et al. (33). The sequence of the *C. jejuni sodB* gene revealed a single *Bgl*II restriction site (unique on pCSD035) centered 152 bp downstream (positions 431 to 436, Fig. 1) of the start codon. A 1.4-kb *Eco*RI-*Hin*dIII fragment from pILL550 (33), which carries a kanamycin resistance gene that is expressed in both *E. coli* and *C. jejuni*, was inserted into the unique *Bgl*II site to create pCSD035km. The *oriT* region from pILL550, present on a 1-kb *Sal*I-*Hin*dIII fragment, was then inserted into a unique *Sca*I restriction site within the ampicillin resistance gene of pCSD035km, to generate pCSD035kmt. Plasmid pCSD035kmt was subsequently transformed into *E. coli* HB101, which contained the mobilizing plasmid pRK212.1 (16). The resulting strain, HB101 (pRK212.1, pCSD035kmt) was used as a donor in a conjugation with *C. jejuni* 81-176. The conjugation was carried out according to the protocol of Labigne-Roussel et al. (33) with the slight modifications described by Miller et al. (37). Kanamycin-resistant transconjugants presumably represented derivatives of 81-176 that contained the kanamycin resistance gene within the *sodB* gene. Putative *sodB* mutants were examined in a series of hybridization experiments designed to identify the desired mutants in which a double-crossover event had occurred (see Results).

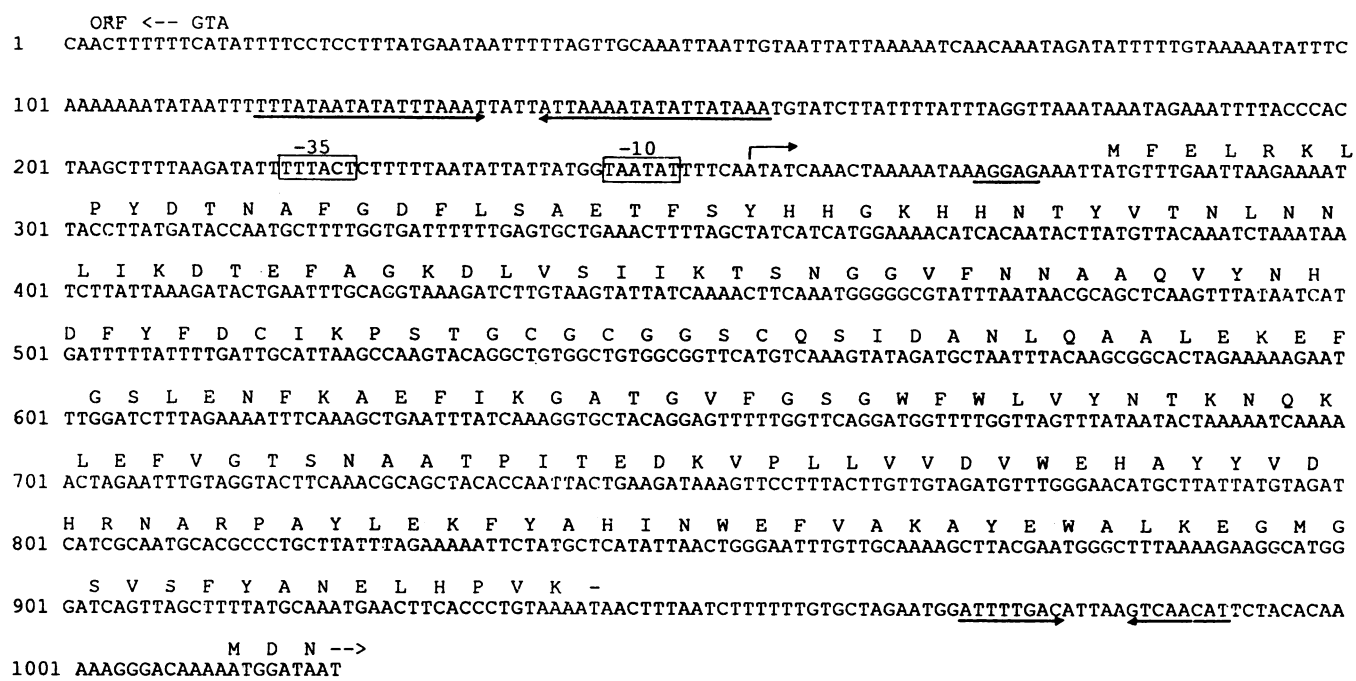


FIG. 1. Nucleotide sequence of the *C. jejuni sodB* gene. The predicted amino acid sequence is shown above the nucleotide sequence. The starts of potential upstream and downstream ORFs are indicated with dashed arrows in the direction of translation. A potential ribosome binding site for *sodB* is underlined. The regions underlined with inverted arrows have the potential to form stem-and-loop structures. The most abundant mRNA start site determined by primer extension (see Fig. 4) is indicated by an arrow pointing in the direction of transcription. Putative promoter regions have been boxed.

**SOD gels.** Cell lysates were prepared according to the method of Hoffman et al. (25). Nondenaturing, high-pH, polyacrylamide gels were made by following the procedure of Hames (21) and were subjected to electrophoresis for 20 h at 5 mA. Gels were stained for SOD activity according to the method of Beauchamp and Fridovich (6). For the SOD gel inhibition studies, KCN or $H_2O_2$ was added to the riboflavin staining solution at 1.9 or 3.7 mM, respectively (52).

**Invasion assays.** The human embryonic intestinal cell line INT407 was obtained from the American Type Culture Collection. INT407 cells were grown in Eagle's minimal essential medium (Mediatech-Fisher, Pittsburgh, Pa.) supplemented with 10% fetal bovine serum and incubated in 6% $CO_2$ at 37°C. Invasion assays were performed essentially as described by Oelschlaeger et al. (40). For these experiments, bacteria stored at −70°C were passaged twice on brucella agar and a 20-h culture on brucella agar was harvested with B broth, washed twice with Eagle's minimal essential medium, and resuspended in Eagle's minimal essential medium to a concentration of $7 \times 10^6$ cells per ml. Confluent monolayers of INT407 cells were inoculated with approximately $7 \times 10^6$ bacteria to achieve a multiplicity of infection of 100. Inoculated monolayers were incubated for 2 h, to allow invasion, and were subsequently incubated for 2 h in the presence of 100 μg of gentamicin per ml to kill extracellular bacteria before lysis of the INT407 cells with 0.01% Triton X-100. Control experiments showed that no viable bacteria remained after the 2-h gentamicin treatment (data not shown) and that 0.01% Triton X-100 lysed all INT407 cells but had no effect on bacterial viability (data not shown). Eagle's minimal essential medium was used for all washes during the assays. Recovered bacteria were enumerated by triplicate plate counts. The results of duplicate assays repeated at least four times were averaged, expressed as the percentage of inoculum recovered after the gentamicin treatment, and statistically analyzed by Student's *t* test.

**Minicell experiments.** Minicell experiments were performed by the method of Meagher et al. (36). Minicells were labeled with 50 μCi of $^{35}$S-methionine and cysteine (American Radiolabeled Chemicals, Inc., St. Louis, Mo.) per ml of minicell suspension. Labeled protein of approximately 200,000 trichloroacetic acid-precipitable cpm per sample was applied to a sodium dodecyl sulfate-polyacrylamide (12%) gel (35). Following electrophoresis, gels were dried and autoradiographed to visualize labeled proteins.

**Nucleotide sequence accession number.** The nucleotide sequence of the *C. jejuni sodB* gene has been entered in GenBank under accession number U08132.

## RESULTS

**Cloning the *C. jejuni sodB* gene.** PCR with the primers SOD1B and SOD2 and *C. jejuni* whole-cell DNA as template yielded a product of approximately 150 bp. This PCR product was cloned, and sequence analysis of the inserts from 14 transformants revealed that all 14 contained identical inserts that were most similar to previously reported *sodB* sequences. No PCR product similar to a *sodA* gene was found (see Discussion). One recombinant plasmid, pCSD19, was used to probe Southern blots of *C. jejuni* whole-cell, restriction enzyme-digested DNA. A 2.9-kb *BclI* fragment of *C. jejuni* DNA hybridized to the probe; therefore, *BclI* fragments of about 2.9 kb were purified and cloned into pBluescript. Colony blots of 800 transformants identified the positive transformant, pCSD035, used in further studies.

**Nucleotide sequence analysis.** The nucleotide sequence of the relevant region of pCSD035 revealed that a complete *sodB*

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E. coli MnSOD      SYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALESLPEFAN    50
E. coli FeSOD      SFELPALPYAKDALAPHISAETIEYHYGKHHQTYVTNLNNLIKG-TAFEG    49
C. jejuni FeSOD    MFELRKLPYDTNAFGDFLSAETFSYHHGKHHNTYVTNLNNLIKD-TEFAG    49
H. pylori FeSOD    MFTLRELPFAKDSMGDFLSPVAFDFHHGKHHQTYVNNLNNLIKG-TDFEK    49
                    ..*. **.. .... .. ... *..***.***.* *. ... ..*.

E. coli MnSOD      LPVEELITKLDQLPADKKTVLRNNAGGHANHSLFWKGLKKGTT-------    93
E. coli FeSOD      KSLEEIIRS-------SEGGVFNNAAQVWNHTFYWNCLAPNAGGEPTG--    90
C. jejuni FeSOD    KDLVSIIKT-------SNGGVFNNAAQVYNHDFYFDCIKPSTGCGCGGSC    92
H. pylori FeSOD    SSLFDILTK-------SSGGVFNNAAQIYNHDFYWDCLSPKAT-------    85
                      . ... .       ... . ***.   **..... .   ..

E. coli MnSOD      --LQGDLKAAIERDFGSVDNFKAEFEKAAAAASRFGSGWAWLV--LKGDK   139
E. coli FeSOD      -----KVAEAIAASFGSFADFKAQFTDAAIKN--FGSGWTWLV-KNSDGK   132
C. jejuni FeSOD    QSIDANLQAALEKEFGSLENFKAEFIKGATGV--FGSGWFWLVYNTKNQK   140
H. pylori FeSOD    -ALSDELKGALEKDFGSLEKFKEDFIKSATTL--FGSGWNWAAYNLDTQK   132
                         .. .*.. .*** ..**..* ..*     ***** * .   .. *

E. coli MnSOD      LAVVSTANQDSPLMGEAISGASGFPILGLDVWEHAYYLKFQNRRPDYIKE   189
E. coli FeSOD      LAIVSTSNAGTPLTTDAT------PLLTVDVWEHAYYIDYRNARPGYLEH   176
C. jejuni FeSOD    LEFVGTSNAATPITEDKV------PLLVVDVWEHAYYVDHRNARPAYLEK   184
H. pylori FeSOD    IEIIQTSNAQTPVTDKKV------PLLVVDVWEHAYYIDHKNARPVYLEK   176
                   .. . *.*. .*. .. .      *.* .********.. .* ** *...

E. coli MnSOD      FWNVVNWDEAAARFAAAKK------------------   208
E. coli FeSOD      FWALVNWEFVAK----------------------NLAA   192
C. jejuni FeSOD    FYAHINWEFVAKAYEWALKEGMGSVSFYANEL-HPVK   220
H. pylori FeSOD    FYGHINWHFVSQCYEWAKKEGLGSVDYYINELVHKKA   213
                   *.. .**. ..
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FIG. 2. Amino acid alignment of *E. coli* MnSOD and FeSOD, *C. jejuni* FeSOD, and *H. pylori* FeSOD. Identical residues (*) and conserved residues (·) are indicated under the alignments. Gaps produced by the alignment are represented with dashes. Amino acid alignments were performed with the aid of the CLUSTAL program of Higgins and Sharp (24).

gene had been cloned from *C. jejuni* (Fig. 1). The gene consisted of a 662-bp open reading frame (ORF) that was predicted to encode a protein with 220 amino acids, an $M_r$ of 24,800, and a pI of 5.79. A search of the BLAST data base (BLAST; Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine) revealed that the predicted protein had a very high degree of similarity to other bacterial SODs, with the greatest similarity being to FeSODs (Fig. 2). The *C. jejuni sodB* nucleotide sequence was 64 and 59% identical to that of the *H. pylori* (43) and *E. coli sodB* (10) nucleotide sequences, respectively. The upstream region of the *C. jejuni sodB* gene had a potential ORF on the opposite strand 268 bp upstream from the *sodB* start codon. The A of the upstream ORF start codon corresponded to the T at position 13 of Fig. 1. This potential ORF could encode a 60-amino-acid protein with a molecular weight of 6,500 (data not shown). The noncoding region between the potential upstream ORF and *sodB* had a very high A+T content (86%), which is about 15% higher than the average A+T content of the *C. jejuni* genome (50). Within this A+T-rich region is a relatively long inverted repeat (Fig. 1), which consists entirely of A's and T's. The significance of this inverted repeat, if any, is unknown. A putative ribosome binding site for *sodB* was centered 7 bp upstream from its start codon. The region downstream from *C. jejuni sodB* had a potential ORF on the same strand as *sodB*. This ORF began 68 bp downstream from the 3′ end of *sodB* (Fig. 1). It could encode a 177-amino-acid protein with an $M_r$ of 20,251 (data not shown). The two ORFs adjacent to *C. jejuni sodB* were not homologous to proteins in the BLAST data base or to the potential ORFs adjacent to the *H. pylori sodB* gene (44).

The amino acid alignments of the *C. jejuni*, *H. pylori*, and *E. coli* FeSODs and the *E. coli* MnSOD are presented in Fig. 2. The *C. jejuni* FeSOD had gaps after residues 58 and 158 (Fig. 2) which are typical of FeSODs. It was 64, 58, and 36% identical to the *H. pylori* FeSOD, *E. coli* FeSOD, and *E. coli* MnSOD, respectively. Finally, the FeSOD from *C. jejuni* had a 23-amino-acid tail at its carboxyl terminus, which is very similar to the 24-amino-acid tail at the carboxyl terminus of the *H. pylori* FeSOD (Fig. 2 [43]).

***sodB* transcript analysis.** Northern blot analysis of *C. jejuni* RNA showed that an mRNA of approximately 700 nucleotides was transcribed from the *sodB* gene (Fig. 3). This result indicated that, as in *E. coli* (15) and *H. pylori* (43), the *C. jejuni sodB* gene exists as a monocistronic operon. Primer extension analysis repeatedly produced four extended products that

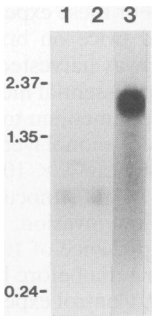


FIG. 3. Northern blot analysis of *C. jejuni* RNA. RNA size standards, in kilobases, are indicated on the left. Lanes 1 and 2 were hybridized to a *C. jejuni sodB* probe. Lanes 1 and 3 contain 10 μg, and lane 2 contains 20 μg, of *C. jejuni* RNA. Lane 3 is a control duplicate blot in which the probe was a PCR product (2) generated from the *flaA* gene of *C. jejuni* 81-176 and which recognizes the very abundant *flaA* transcript.

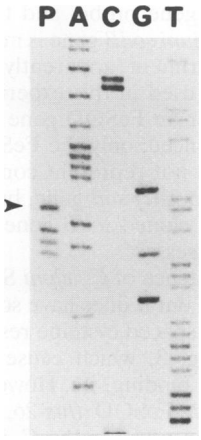


FIG. 4. Primer extension analysis of the 5′ end of the *C. jejuni sodB* transcript. Sequencing reaction lanes are labeled according to nucleotide (A, C, G, or T), and the extended primer reaction lane is labelled P. The arrowhead points to the most abundant extension product.

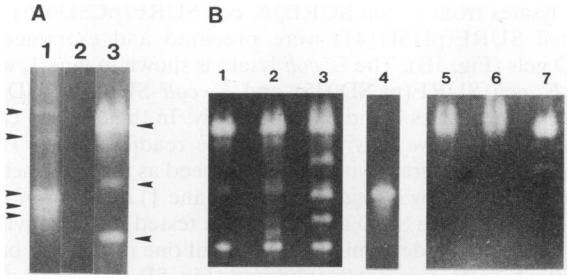


FIG. 5. Nondenaturing polyacrylamide gel stained for SOD activity. (A) Lanes: 1, cell lysates from *C. jejuni* 81-176 (1.3 mg of total protein); 2, *C. jejuni sodB* (1.5 mg); 3, *E. coli* SURE (480 μg). The SOD activity bands produced by each species are identified by arrowheads. (B) Expression and activity of the FeSODs in *E. coli*. Lanes 1 to 4 and 5 to 7 were stained for SOD activity in the absence or presence of $H_2O_2$, respectively. Lanes: 1 and 5, *E. coli* SURE (480 μg of total protein per lane); 2 and 6, *E. coli* SURE(pCSD035) (630 μg per lane); 3 and 7, *E. coli* SURE(pHSD141) (580 μg per lane); 4, *H. pylori* 60109 (871 μg). All lysates were loaded onto the negative electrode end (top) of a 12% nondenaturing polyacrylamide gel. Unequal amounts of total protein were loaded in order to optimize visualization of the SOD activities and to help ensure that any lighter bands present in the *sodB* lysate would be visible.

ended at nucleotide positions 254, 256, 257, and 258 of Fig. 1 (Fig. 4); the most abundant product was that which ended at position 254 (Fig. 1). A putative promoter was found upstream of the transcriptional start site and is indicated in Fig. 1. This potential promoter matched 4 of 6 bases with *E. coli* $\sigma^{70}$-like promoter −35 (TTGACA) and −10 (TATAAT) consensus sequences. The potential −35 and −10 regions were separated by 19 bases, and the start site for the most abundant transcript was 6 bases after the −10 region (Fig. 1).

**Isolation of an isogenic *C. jejuni sodB* mutant.** One hundred ninety-nine kanamycin-resistant transconjugants, presumably the result of homologous recombination between DNA sequences flanking the kanamycin gene on pCSD035kmt and the chromosome, were screened by colony blot hybridizations in which pBluescript was the probe. Thirty-six percent of the transconjugants did not hybridize with pBluescript, indicating that a double-crossover event had probably occurred. Whole-cell DNA from *C. jejuni* and four transconjugants (three potential double crossovers and one single crossover) was digested with *Bcl*I and used to make Southern blots. The blots were probed sequentially with the 1.4-kb kanamycin resistance fragment, the *sodB* fragment from pCSD19, and pBluescript (data not shown). Hybridization with the *sodB* fragment confirmed that the *sodB* gene is on a 2.9-kb *Bcl*I fragment in *C. jejuni*. In mutants suspected of undergoing a double-crossover event, a 4.3-kb *Bcl*I fragment hybridized to this probe, and in the mutant suspected of undergoing a single-crossover event, an 11.4-kb *Bcl*I fragment hybridized to the *sodB* probe. The 8.5-kb increase in size seen in the single-crossover mutant is approximately the size of pCSD035kmt (8.3 kb), which is not cut by *Bcl*I. The fragment containing the kanamycin resistance gene hybridized to a 4.3- and an 11.4-kb fragment in the mutants containing double and single crossovers, respectively, but did not hybridize to the parental *C. jejuni* strain. Finally, pBluescript did not hybridize to DNA from either *C. jejuni* or the double-crossover mutants but did hybridize to an 11.4-kb fragment in the single-crossover mutant. These results indicated that the three suspected double-crossover mutants carried the 1.4-kb kanamycin resistance gene fragment within the *sodB* gene and that they did not possess an intact copy of *sodB*.

**SOD activity assays.** Cell lysates from *C. jejuni* 81-176, *C. jejuni* 81-176 *sodB*, and *E. coli* were subjected to electrophoresis on a nondenaturing polyacrylamide gel and then stained for SOD activity (Fig. 5A). The inhibitors, KCN and $H_2O_2$, were added to the staining solution in some experiments to differentiate the types of SOD activity. CuZnSODs and FeSODs are inhibited by KCN and $H_2O_2$, respectively, while MnSODs are resistant to both of these inhibitors (12, 18). Previous reports have shown that *E. coli* produces three electrophoretically distinct SOD activity bands (55), and our results are in agreement with this (Fig. 5A, lane 3). The slowest and fastest migrating bands are an MnSOD and an FeSOD, respectively, while the middle band is a hybrid Fe/MnSOD (42). Figure 5B, lane 5, shows the effect of $H_2O_2$ on the *E. coli* SOD activities (cf. Fig. 5B, lane 1). Our *C. jejuni* lysate appeared to contain at least five electrophoretically distinct bands of SOD activity (Fig. 5A, lane 1). Three of these bands appeared as an easily visible triplet at the positive terminal end of the gel. Two other bands of SOD activity from *C. jejuni* migrated more slowly and are marked with the top two arrowheads on Fig. 5A, lane 1. They were very much weaker in intensity than the triplet bands but were consistently visible. The *C. jejuni sodB* cell lysate did not appear to contain any SOD activities (Fig. 5A, lane 2). All five SOD activities produced by *C. jejuni* appeared to be inhibited by $H_2O_2$, thus producing a result that looked exactly like that of the *sodB* mutant in lane 2 of Fig. 5A (data not shown directly, but see discussion of Fig. 5B below).

**Expression and enzymatic activity of the *C. jejuni* and *H. pylori sodB* genes in *E. coli*.** The plasmids containing only the intact *sodB* genes from *C. jejuni* and *H. pylori*, pCSD1.2 and pHSD1.3, respectively, were transformed into the *E. coli* minicell-producing strain, P678-54, to determine if the *sodB* genes were expressed in *E. coli*. The minicell results showed that one major protein with an apparent $M_r$ of 28,500 was unique to pCSD1.2, and similarly, a single major protein with an apparent $M_r$ of 27,300 was unique to the *H. pylori* clone, pHSD1.3 (data not shown). The orientation of the *sodB* genes in these two subclones is opposite to the direction of transcription from the *lac* promoter in pBluescript. These results clearly indicated that the *C. jejuni* and *H. pylori sodB* genes were readily expressed in *E. coli*, possibly from their own promoters.

We were interested in knowing whether or not the *C. jejuni* and *H. pylori* SodB proteins produced in *E. coli* were active.

Cell lysates from *E. coli* SURE, *E. coli* SURE(pCSD035), and *E. coli* SURE(pHSD141) were prepared and examined on SOD gels (Fig. 5B). The *E. coli* lysate is shown in lane 1, while the *E. coli* SURE(pCSD035) and *E. coli* SURE(pHSD141) lysates are in lanes 2 and 3, respectively. In the last two cases, at least three new activity bands were readily visible. These bands did not migrate with the same speed as the SOD activity bands produced by *C. jejuni* (Fig. 5A, lane 1) or *H. pylori* (Fig. 5B, lane 4). These SOD activities were tested for sensitivity to $H_2O_2$, and it was determined that all but one of the new bands of activity were completely inhibited (Fig. 5B, cf. lanes 2 and 3 with lanes 6 and 7). This indicated that these SOD activities were either FeSODs or hybrid Fe/MnSODs. All of the bands of activity seen in Fig. 5B were resistant to KCN (data not shown). Although these new bands did not comigrate with the FeSODs produced by *C. jejuni* or *H. pylori*, these results nevertheless indicated that the *C. jejuni* and *H. pylori* SODs produced in *E. coli* were active.

**Invasion of INT407 cells by strain 81-176 *sodB*.** The ability of *C. jejuni* 81-176 to invade INT407 cells has been demonstrated (31, 40), and invasive ability is hypothesized to be an important component of *C. jejuni* pathogenesis (20, 30, 57). The ability of the *sodB* mutant to survive invasion of INT407 cells was tested and compared with that of the parent strain, *C. jejuni* 81-176. In this invasion assay, the number of bacteria that invade and survive intracellularly after a 2-h gentamicin treatment are quantified. The results of these assays showed that the means of the percentage of initial inoculum recovered for *C. jejuni* 81-176 and *C. jejuni* 81-176 *sodB* were 0.00537 (standard error = 0.00113) and 0.00046 (standard error = 0.00009), respectively. These recovery rates showed that the *sodB* mutant had an approximately 12-fold decrease in ability to survive INT407 cell invasion compared with *C. jejuni* 81-176. This decrease represents a significant difference ($P = 0.0225$) between the parent and mutant strains with respect to invasion survival capability and suggests a role for SOD in intracellular survival by *C. jejuni*. In control experiments, the effect of the assay conditions (6% $CO_2$ and 37°C) on the survival of the parent and mutant strains in the presence and absence of INT407 cells was tested. These controls showed that neither the atmosphere nor the presence or absence of INT407 cells caused a difference between the survival of parent and mutant during the incubation periods used in the invasion assays (data not shown).

## DISCUSSION

We have cloned and sequenced a gene from *C. jejuni* that has a predicted amino acid sequence that is most similar to that of other reported FeSODs. This gene has been designated *sodB* in accordance with the names of other FeSOD genes. Hoffman et al. (25) and Kikuchi and Suzuki (28) reported that *C. jejuni* lysates contained either three or two electrophoretically distinct SOD activities, but they did not characterize these activities further. We report here that *C. jejuni* 81-176 produces at least five electrophoretically distinct bands of SOD activity, all of which appeared to be inhibited by $H_2O_2$ and resistant to KCN. Two other pieces of evidence suggest that all of the SOD activities seen on the SOD gels may be FeSOD activities. First, extracts from *C. jejuni* 81-176 *sodB* failed to show any bands of activity on these gels, suggesting that all of the activities seen in *C. jejuni* 81-176 extracts are derived from the one *sodB* gene that was inactivated. The possibility that our mutant might be directly affected in more than one gene seems remote, since the hybridizations used to characterize the mutant showed that only one DNA fragment hybridized to the kanamycin resistance gene probe, and the Northern analysis indicated that the *C. jejuni sodB* gene is monocistronic. Second, the initial PCR experiment apparently produced only one product. The primers used in this experiment could theoretically have found either an FeSOD gene or an MnSOD gene, yet of 14 clones sequenced, only the FeSOD gene was found. These experiments do not represent conclusive proof that *C. jejuni* does not have another *sod* gene, but they are consistent with the idea that the cloned *sodB* gene is the only *sod* gene that this organism possesses.

The amino acid sequence of *C. jejuni* SodB is very similar to that of other FeSODs, but it does have some unusual features. There are four closely spaced cysteine residues at positions 79, 86, 88, and 92 of Fig. 3, which cause this region to look somewhat like a metal binding site. However, the iron binding residues of the *E. coli* FeSOD (*his-26*, *his-73*, *asp-156*, and *his-160* [10]) are all conserved in the *C. jejuni* FeSOD (*his-26*, *his-73*, *asp-164*, and *his-168*). In addition, the *C. jejuni* and *H. pylori* FeSODs had C-terminal tails of 23 and 24 amino acids, respectively, thus making them two of the largest FeSODs reported. Whether these extended carboxyl termini serve a particular function is not known.

Since there is a general lack of information about how well *C. jejuni* and *H. pylori* genes are expressed in *E. coli*, we tested both our *C. jejuni* and *H. pylori* clones for the ability to be expressed in *E. coli* minicells. The results indicated that the *sodB* genes from both species were expressed in *E. coli*, possibly from their own promoters, since expression was independent of the orientation of the *sodB* genes in pBluescript. The putative *C. jejuni* promoter identified here, as well as the promoter for the *H. pylori sodB* gene (43), is similar to *E. coli* $\sigma^{70}$ promoters (23). Since SodB appears to be the only SOD produced by *C. jejuni*, it seems plausible to expect that the *sodB* gene is expressed constitutively; hence $\sigma^{70}$-like promoters would not be surprising. Whether additional levels of regulation might exist, so that increases or decreases in expression of the *C. jejuni sodB* gene occur in certain environments, is not known. In *E. coli*, the regulation of *sodB* expression is incompletely understood; it appears to be made constitutively, yet a *fur* mutant synthesizes much less SodB than the wild-type parental strain (39). We have examined the putative promoter region of the *C. jejuni sodB* gene for sequences that resemble the Fur-binding consensus sequence (13). The sequence 5′-GATATTTTTACTCTTTTTA-3′ that starts at nucleotide position 212 (Fig. 1) matches 13 of 19 nucleotides found in the consensus sequence for Fur-binding sites. In addition, this site is centered on the putative −35 region identified in our primer extension experiments, a plausible location for the Fur-binding site. Further experimentation will be needed to determine whether iron regulation of the *C. jejuni sodB* gene occurs and if the −35 region is important for that regulation.

Both *C. jejuni* and *H. pylori* exhibited multiple FeSOD activity bands on the SOD gels (Fig. 5). Since the activities detected in the *C. jejuni* extracts are apparently all produced by the *sodB* gene described here, the multiple bands cannot be the products of multiple *sodB* genes. Although a *sodB* mutant of *H. pylori* has not yet been reported, it seems probable that it also does not possess multiple *sodB* genes. The closely migrating triplet bands in both species may represent some slight modifications to the proteins that occur posttranslationally. Alternatively, these bands might be an artifact of the nondenaturing, nonreducing conditions that are required for the isolation and visualization of active SODs. The *C. jejuni* extracts also contained two slower-migrating FeSOD activities that were not present in extracts of the mutant strain and therefore are presumably products of the *sodB* gene. These bands were not

as abundant as the triplet bands and perhaps represent some sort of association of the FeSODs with other cell components. Slower-migrating bands similar to these were not observed on the *H. pylori* SOD activity gels (Fig. 5B, lane 4) (42).

The SOD activity gels of extracts from *E. coli* carrying the *C. jejuni* or *H. pylori* clones are intriguing. The change in migration of the *C. jejuni* and *H. pylori* FeSOD activities suggests either that they are largely participating in the various possible hybrids with the *E. coli* SODs or that some modification of the SODs that occurs in *C. jejuni* and *H. pylori* is not occurring to the same extent in *E. coli*.

Finally, our invasion experiments clearly and consistently demonstrated that *C. jejuni* requires SodB either for entry into the INT407 cells or for survival intracellularly. In further experiments not reported here, we extended the incubation period following gentamicin treatment to 6 h. By this time, we were rarely able to recover live *sodB* cells. (The numbers of recoverable parental bacteria sometimes declined up to twofold during this time period but were never eliminated.) The percentage of *C. jejuni* 81-176 cells recovered in our assays is similar to that in some reports (e.g., reference 20) but lower than that in others (40). Since we reproducibly obtained this percentage in repeated experiments, and since the recovery of the *sodB* cells was always at least a factor of 10 less than that of the parent, *C. jejuni* 81-176, it seems reasonable to conclude that our results represent a valid and significant difference between the abilities of these two strains to invade or survive within these epithelial cells. Presumably, the FeSOD is protecting *C. jejuni* from some oxygen-mediated intracellular killing and may also protect during invasion of the epithelial cells. In order for this type of protection to occur, it would be beneficial for the FeSOD of *C. jejuni* to be present on its surface. In this regard, it is of interest to note the results of Spiegelhalder et al. (51), who have recently shown that the FeSOD of *H. pylori* is at least partially localized on the bacterium's surface, including on the flagellar sheath. Whether the FeSOD of *C. jejuni* can also be found on the surface of the cell will be determined in future experiments.

## ACKNOWLEDGMENTS

We thank Sue Straley and Greg Plano for their interest and advice. We also thank Mike Russ and Karen Fortenberry of the Macromolecular Structure Analysis Facility for synthesizing oligonucleotides.

This work was supported in part by National Institutes of Health grant AI-27908.

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