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Expression and Characterization of *Campylobacter jejuni* Benzoylglycine Amidohydrolase (Hippuricase) Gene in *Escherichia coli*

ERIC KURT HANI AND VOON LOONG CHAN*

Department of Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

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The basis for the difference between *Campylobacter jejuni* and *Campylobacter coli* is the presence and expression of the *N*-benzoylglycine amidohydrolase (hippuricase) gene only in *C. jejuni*. A pBR322 recombinant clone (pHIP-O) of *C. jejuni* TGH9011 capable of converting hippuric acid into benzoic acid and glycine, the hallmark of hippuricase activity, was characterized and sequenced. The hippuricase gene (*hipO*) was identified by use of deletion subclones and insertional inactivation. The transcription start point of the hippuricase gene was determined by primer extension analysis. A hippuricase-specific gene fragment was used to determine the presence of the gene in *Campylobacter* species. Maxicell analysis of recombinant plasmid pHIP-O by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated the production of a 42-kDa protein corresponding to the HipO gene product, in excellent agreement with the predicted molecular mass of the protein.

In the genus *Campylobacter*, one species, *Campylobacter jejuni*, has become recognized as a leading cause of human enteritis (30). It is currently accepted that the number of campylobacter infections approaches, and may exceed, those caused by *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae* (4). The *N*-benzoylglycine amidohydrolase (hippuricase) test is of paramount importance in the differentiation of *C. jejuni* (hippuricase positive) from the other campylobacters; in contemporary practice, the hippuricase (EC 3.5.1.32) assay is the only reliable biochemical assay that can discriminate *C. jejuni* from *Campylobacter coli* (25). Hippuricase acts to cleave *N*-benzoylglycine (hippuric acid) into the constituent products glycine and benzoic acid. Glycine formation is detected by using a ninhydrin-based reagent system (12, 23, 31), whereas detection of benzoic acid is achieved by precipitation with a ferric chloride reagent (9) or, more reliably, by gas-liquid chromatography (3, 33). The occurrence of hippuricase-negative variants of *C. jejuni* and the inability of the assays to detect low-level producers of hippuricase with whole-cell extracts (32) have been problematic in the separation of these species. Also, the phenotypic expression of enzymes in *Campylobacter* spp. is dependent on inoculum size (24), another factor not strictly controlled in these assays.

Therefore, there exists the possibility that *C. coli* could produce hippuricase at a level insufficient to be detected in the biochemical assay. This question could be resolved only by cloning the gene from *C. jejuni* and determining its presence in *C. coli* by Southern analysis. The cloned gene would permit the development of a genetic probe potentially specific for *C. jejuni* and the use of a genetic approach to investigate the physiological role of the *hip* gene and hippuricase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *C. jejuni* TGH9011 was obtained from J. L. Penner, University of Toronto, Toronto, Canada, and is the serotype reference strain for O:3 (ATCC 43431). *C. jejuni* ATCC 33560^T and *C. coli* ATCC 33559^T were purchased from the American Type Culture Collection. *C. jejuni* hippuricase-negative strains D594, D603, D941, D1713, and D1916 (32) were obtained from C. M. Patton, Centers for Disease Control, Atlanta, Ga. *C. jejuni* and *C. coli* strains were grown routinely on Columbia agar (Difco) supplemented with 5% horse blood (Woodlyn Labs, Guelph, Ontario, Canada) or in Mueller-Hinton agar or broth (Merck) in a 5% CO_2 incubator at 37°C. *Escherichia coli* JM101 [Δ(*lac-pro*) *thi rspL supE endA sbcB hsdR* F′ *traD36 proAB lacI*^{q} *ZΔM15*] (21) and *E. coli* DR1984, a nonmucoid derivative of CSR603 (*recA1 uvrA6 phr-1*) (27), were laboratory stocks. *E. coli* was grown in Luria-Bertani broth or agar aerobically at 37°C and supplemented with 100 μg of ampicillin per ml where appropriate. The plasmids used in this study are listed in Table 1. The *C. jejuni* pBR322 genomic library was prepared from strain TGH9011 (ATCC 43431) as described previously (8).

Materials. Ampicillin and hippuric acid were purchased from Sigma Chemical Co., St. Louis, Mo. Ninhydrin was purchased from Calbiochem, San Diego, Calif. Ferric chloride was purchased from Fisher Scientific Corp., Fair Lawn, N.J. The radioactive compounds purchased consisted of [α-^{32}P]dATP, [γ-^{32}P]ATP, and [^{35}S]methionine, all from ICN Biomedicals Inc., Irvine, Calif., and α-^{35}S-dATP (1,350 Ci/mmol), from Dupont-NEN, Mississauga, Ontario, Canada. Restriction enzymes were purchased from Boehringer (Mannheim, Germany), Gibco-BRL (Mississauga, Ontario, Canada), and Pharmacia (Uppsala, Sweden). Moloney murine leukemia virus reverse transcriptase and avian myeloblastosis virus reverse transcriptase were purchased from Pharmacia. Sequencing was done with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio). Nick translation was performed with the nick translation kit from Bethesda Research Laboratories (Mississauga, Ontario, Canada).

Cloning of the *hipO* gene. *hipO* was isolated by analysis of hippuricase activity from whole-cell extracts of *E. coli* containing individual recombinants of a *C. jejuni* pBR322 genomic library. A total of 1,500 recombinant plasmids were grown in collections of 10 in liquid media containing 100 μg of ampicillin per ml. One milliliter of overnight culture was harvested at 12,000 × *g* for 2 min at room temperature. The cells were washed in 1% sodium hippurate (pH 7.2) and resuspended in 0.1 ml of 1% sodium hippurate. The cells were incubated at 37°C for 1 h and pelleted. The supernatant was transferred to a 96-well microtiter plate and assayed for the presence of glycine by the addition of 50 μl of ninhydrin reagent (3.5% in 1:1 acetone-butanol) according to the method of Hwang and Ederer (14). A single collection of 10 recombinants with hippuricase activity was isolated in this manner. The 10 clones composing this group were analyzed individually, resulting in the isolation of a single recombinant capable of hydrolyzing hippuric acid, designated pHIP-O (see Fig. 1). This clone also gave a positive reaction for benzoic acid formation in the ferric chloride assay (data not shown).

Nucleotide sequencing. The DNA sequences of both strands of *hipO* were determined from overlapping clones by the dideoxy chain termination method (29) using nested deletions obtained with exonuclease III and S1 nuclease (13).

Nucleotide sequence accession number. The nucleotide sequence data for *hipO* are accessible in the EMBL Nucleotide Sequence Database under accession no. Z36940.

Southern analysis of genomic DNA. Genomic DNAs from *C. jejuni* and *C. coli* were digested to completion with restriction enzymes. The fragments were separated by electrophoresis through a 0.7% agarose gel at 10 V/cm and transferred to a GeneScreen nylon membrane by using a Vacublot apparatus (Pharmacia). The filter was probed with a 0.8-kb *Hin*dIII fragment of plasmid pHIP-H0.8

* Corresponding author. Phone: (416) 978-6077. Fax: (416) 978-4761. Electronic mail address: vlchan@gpu.utcc.utoronto.ca.

TABLE 1. Plasmids

Plasmid	Relevant characteristics	Source or reference
pUC19	Amp^r cloning vector	35
pBR322	Amp^r Tet^r cloning vector	5
pBlueScript	Amp^r cloning vector	Stratagene
pGEM-7f$^-$	Amp^r cloning vector	Stratagene
pHIP-O	Hippuricase-positive pBR322 recombinant	This work
pHIP-C	pBlueScript cut with *Cla*I containing *Cla*I(pBR322)-*Cla*I(2192) fragment of pHIP-O; *hip*$^+$	This work
pHIP-CR	pBlueScript cut with *Cla*I containing insert of pHIP-C in reverse orientation; *hip*$^+$	This work
pHIP-CΔX	pHIP-C deleted to *Xba*I(1160); *hip*$^+$	This work
pHIP-CRΔRV	pHIP-CR deleted to *Eco*RV(−635); *hip*$^+$	This work
pHIP-C/S2.0	pGEM7f$^-$ cut with *Cla*I and *Sph*I containing *Cla*I(pBR322)-*Sph*I(294) fragment of pHIP-O; *hip*	This work
pHIP-C/S1.8	pGEM7f$^-$ cut with *Cla*I and *Sph*I containing *Sph*I(294)-*Cla*I(2192) fragment of pHIP-O; *hip*	This work
pHIP-H0.8	pBlueScript cut with *Hin*dIII containing *Hin*dIII(pUC19)-*Hin*dIII(2433) fragment of pHK1; *hip*	This work
pHK1	Kan^r::*hipO* insertion into *Sph*I(1634) of pHIP-C	11

which was labeled by nick translation and contains only *hipO*-encoding sequences (see Fig. 1). The hybridization conditions were 50% formamide, 5% dextran sulfate, 1 M NaCl, and 0.1% sodium dodecyl sulfate (SDS) at 42°C. The filter was washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature twice and exposed to Kodak XAR-5 film at −70°C for 4 h (see Fig. 4).

Primer extension mapping of the transcription start site of the *hipO* mRNA. RNA was isolated from late-exponential-phase cultures of *E. coli* containing pHIP-O and from 24-h cultures of *C. jejuni* TGH9011 by the hot-phenol method (1). Oligonucleotides hpr1 (5′-GATGACGAATTTTTTCAAATTCGCC-3′) and hpr2 (5′-CATAATTCATCAAAACCAAGCTCAGG-3′) are complementary to the coding strand of *hipO* and are located from nucleotides 34 to 58 and 73 to 98 downstream from the Met codon of *hipO*, respectively. The oligonucleotides were end labeled with polynucleotide kinase and mixed with 25 μg of total RNA. The mixture was hybridized and extended by using avian myeloblastosis virus reverse transcriptase as described previously (7) or by using Moloney murine leukemia virus reverse transcriptase and adjusting the incubation temperature to 37°C. For analysis, 2 μl of the newly synthesized DNA (previously suspended in 4 μl of formamide loading buffer) was loaded onto a 6% polyacrylamide sequencing gel alongside dideoxy sequencing ladders of the upstream flanking region of the *hipO* gene generated with the same oligonucleotide (see Fig. 5).

Preparation of crude extracts. *C. jejuni* ATCC 43431, *C. coli* ATCC 33559^T, *E. coli* JM101, and *E. coli* JM101 containing recombinant plasmid pHIP-O were grown overnight in their respective media. Cells were harvested at 12,000 × *g* for 30 min, washed twice in 20 ml of 1× phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.3]), and resuspended in 2 ml of 1× PBS. The cell suspension was passed through a French press twice at a pressure of 16,000 lb/in^2. The extract was cleared of cell envelope by centrifugation at 20,000 × *g* for 40 min and stored frozen at −20°C.

Hippuricase. The incubation mixture for the hippuricase assay contained 1 ml of 10 mM sodium hippurate, 0.5 ml of 0.1 M Tris buffer (pH 8.0), and 0.5 ml of appropriately diluted enzyme solution. Incubation at 37°C for 0, 10, and 20 min was followed by termination of 0.2-ml aliquots by boiling for 2 min. One-half

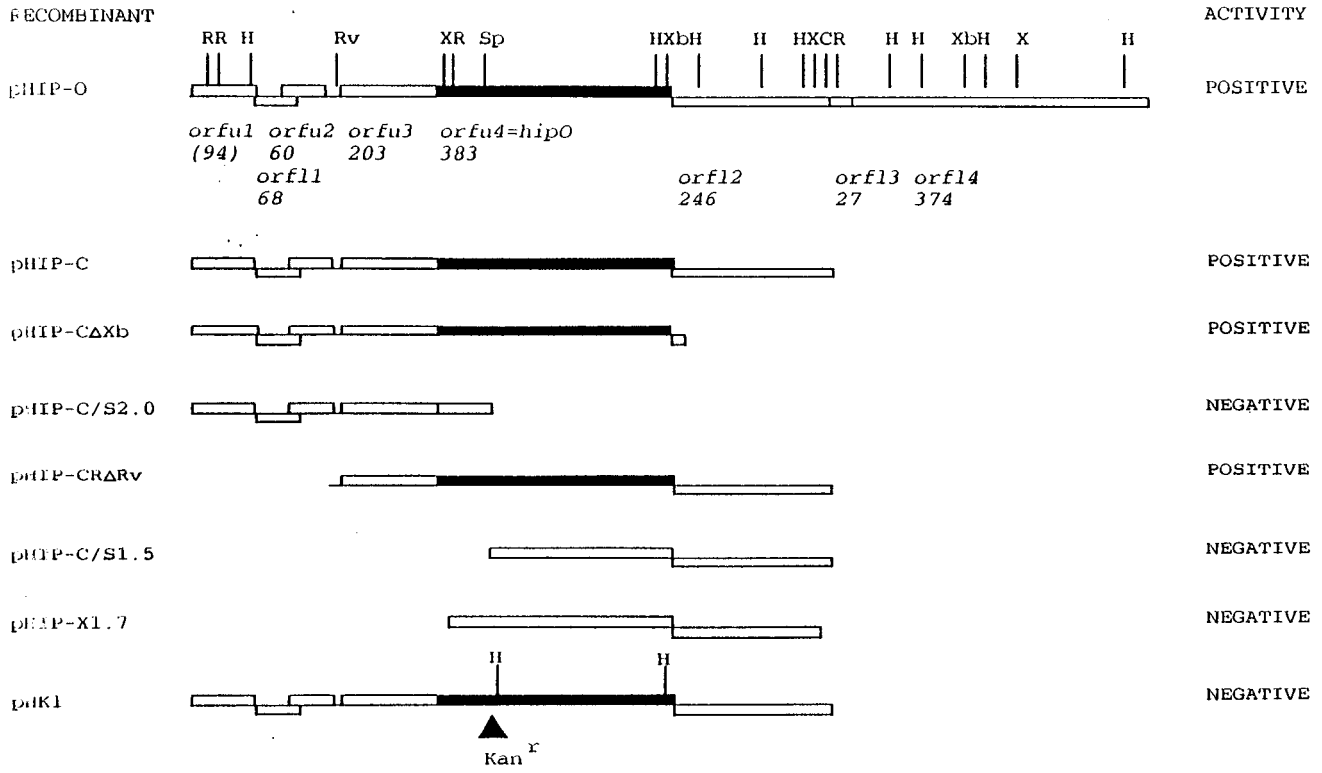

FIG. 1. Restriction enzyme map of recombinant plasmid pHIP-O. Potential open reading frames (open boxes) and the hippuricase gene (filled box) are indicated. The sizes of the open reading frames are shown (in parentheses if incomplete). Hippuricase activity is shown qualitatively for pHIP-O and relevant deletion subclones. Abbreviations: C, *Cla*I; H, *Hin*dIII; R, *Rsa*I; Rv, *Eco*RV; Sp, *Sph*I; X, *Xmn*I; Xb, *Xba*I.

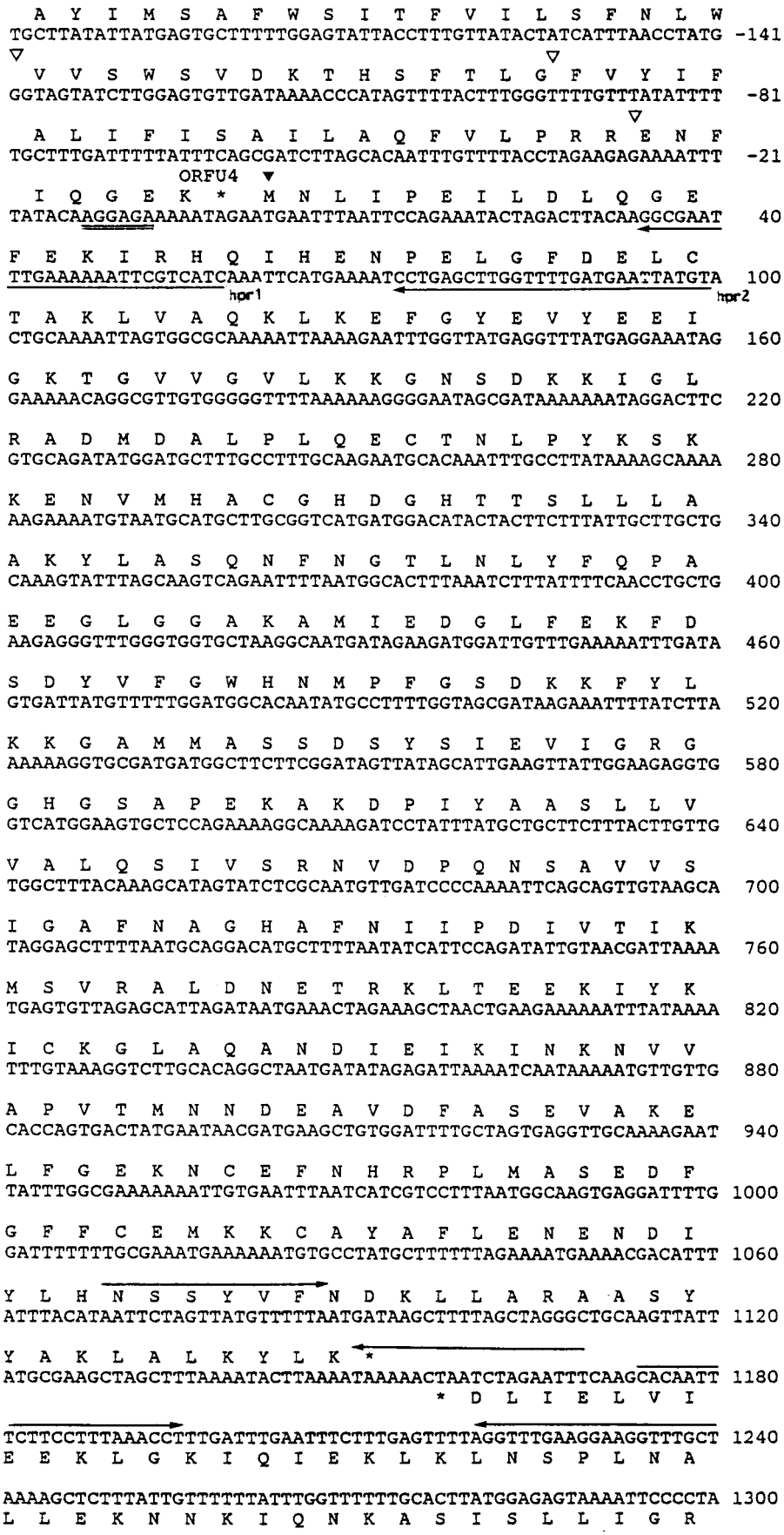
A Y I M S A F W S I T F V I L S F N L W
TGCTTATATTATGAGTGCTTTTTGGAGTATTACCTTTGTTATACTATCATTTAACCTATG -141
V V S W S V D K T H S F T L G F V Y I F
GGTAGTATCTTGGAGTGTTGATAAAACCCATAGTTTTACTTTGGGTTTTGTTTATATTTT -81
A L I F I S A I L A Q F V L P R R E N F
TGCTTTGATTTTTATTTCAGCGATCTTAGCACAATTTGTTTTACCTAGAAGAGAAAATTT -21
ORFU4
I Q G E K * M N L I P E I L D L Q G E
TATACAAGGAGAAAAATAGAATGAATTTAATTCCAGAAATACTAGACTTACAAGGCGAAT 40
F E K I R H Q I H E N P E L G F D E L C
TTGAAAAAATTCGTCATCAAATTCATGAAAATCCTGAGCTTGGTTTTGATGAATTATGTA 100
hpr1
hpr2
T A K L V A Q K L K E F G Y E V Y E E I
CTGCAAAATTAGTGGCGCAAAAATTAAAAGAATTTGGTTATGAGGTTTATGAGGAAATAG 160
G K T G V V G V L K K G N S D K K I G L
GAAAAACAGGCGTTGTGGGGGTTTTAAAAAAGGGGAATAGCGATAAAAAAATAGGACTTC 220
R A D M D A L P L Q E C T N L P Y K S K
GTGCAGATATGGATGCTTTGCCTTTGCAAGAATGCACAAATTTGCCTTATAAAAGCAAAA 280
K E N V M H A C G H D G H T T S L L L A
AAGAAAATGTAATGCATGCTTGCGGTCATGATGGACATACTACTTCTTTATTGCTTGCTG 340
A K Y L A S Q N F N G T L N L Y F Q P A
CAAAGTATTTAGCAAGTCAGAATTTTAATGGCACTTTAAATCTTTATTTTCAACCTGCTG 400
E E G L G G A K A M I E D G L F E K F D
AAGAGGGTTTGGGTGGTGCTAAGGCAATGATAGAAGATGGATTGTTTGAAAAATTTGATA 460
S D Y V F G W H N M P F G S D K K F Y L
GTGATTATGTTTTTGGATGGCACAATATGCCTTTTGGTAGCGATAAGAAATTTTATCTTA 520
K K G A M M A S S D S Y S I E V I G R G
AAAAAGGTGCGATGATGGCTTCTTCGGATAGTTATAGCATTGAAGTTATTGGAAGAGGTG 580
G H G S A P E K A K D P I Y A A S L L V
GTCATGGAAGTGCTCCAGAAAAGGCAAAAGATCCTATTTATGCTGCTTCTTTACTTGTTG 640
V A L Q S I V S R N V D P Q N S A V V S
TGGCTTTACAAAGCATAGTATCTCGCAATGTTGATCCCCAAAATTCAGCAGTTGTAAGCA 700
I G A F N A G H A F N I I P D I V T I K
TAGGAGCTTTTAATGCAGGACATGCTTTTAATATCATTCCAGATATTGTAACGATTAAAA 760
M S V R A L D N E T R K L T E E K I Y K
TGAGTGTTAGAGCATTAGATAATGAAACTAGAAAGCTAACTGAAGAAAAAATTTATAAAA 820
I C K G L A Q A N D I E I K I N K N V V
TTTGTAAAGGTCTTGCACAGGCTAATGATATAGAGATTAAAATCAATAAAAATGTTGTTG 880
A P V T M N N D E A V D F A S E V A K E
CACCAGTGACTATGAATAACGATGAAGCTGTGGATTTTGCTAGTGAGGTTGCAAAAGAAT 940
L F G E K N C E F N H R P L M A S E D F
TATTTGGCGAAAAAAATTGTGAATTTAATCATCGTCCTTTAATGGCAAGTGAGGATTTTG 1000
G F F C E M K K C A Y A F L E N E N D I
GATTTTTTTGCGAAATGAAAAAATGTGCCTATGCTTTTTTAGAAAATGAAAACGACATTT 1060
Y L H N S S Y V F N D K L L A R A A S Y
ATTTACATAATTCTAGTTATGTTTTTAATGATAAGCTTTTAGCTAGGGCTGCAAGTTATT 1120
Y A K L A L K Y L K *
ATGCGAAGCTAGCTTTAAAATACTTAAAATAAAAACTAATCTAGAATTTCAAGCACAATT 1180
* D L I E L V I
TCTTCCTTTAAACCTTTGATTTGAATTTCTTTGAGTTTTAGGTTTGAAGGAAGGTTTGCT 1240
E E K L G K I Q I E K L K L N S P L N A
AAAAGCTCTTTATTGTTTTTTATTTGGTTTTTTGCACTTATGGAGAGTAAAATTCCCCTA 1300
L L E K N N K I Q N K A S I S L L I G R

FIG. 2. Nucleotide sequence of *C. jejuni hipO* (ORFU4) and flanking sequences (ORFU3 and ORFL2), with the corresponding translated sequences shown in one-letter amino acid code. The *hipO* ATG codon (inverted solid triangle), the potential Shine-Delgarno sequence (double underlined), termination codons (asterisks), the transcription start points of *hipO* (inverted open triangles), oligonucleotides hpr1 and hpr2 (arrows), and potential stem-loop structures (overlined inverted arrows) are indicated.

volume of ninhydrin reagent (3.5% in 1:1 acetone-butanol) was added, and the mixture was boiled for 20 min. The solution was diluted with 60% ethanol, and the absorption intensity relative to a glycine standard was measured at 570 nm. The results of triplicate experiments are expressed as micromoles of glycine formed per minute per milligram of protein.

Maxicell analysis. Plasmid-encoded proteins were labeled in UV-irradiated *E. coli* DR1984 cells as described previously (27, 28). Cells were UV irradiated with a germicidal lamp (15 W) at a height of 50 cm. Survival was between 10^{-6} and 10^{-7} CFU/ml following 12 to 15 h of incubation with 200 μg of D-cycloserine per ml. Irradiated cells were washed twice with Hershey salts (27, 28) and then labeled with [^{35}S]methionine (40 μCi/ml) for 1 h in Hershey medium. Cells were lysed by being boiled for 3 min in 50 μl of 2× Laemmli SDS sample buffer, and labeled proteins were separated by 0.1% SDS–13% polyacrylamide gel electrophoresis (PAGE) as described previously (19). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, dried on 3MM cellulose paper, and then exposed to Kodak XAR-5 film at −70°C (see Fig. 6).

RESULTS

Cloning and characterization of the hippuricase gene. Gene *hipO* was isolated from a library of *C. jejuni* TGH9011 DNA fragments by screening for hippuricase activity as described in Materials and Methods. Strain TGH9011 was chosen because in the hippuricase assay it is strongly positive for glycine formation. The hippuricase-positive pBR322 recombinant was designated pHIP-O (Fig. 1). The specific activity for hippuricase from the recombinant pHIP-O was 2.05 μmol/min/mg of protein, whereas no activity was detectable in extracts from *E. coli* JM101 without the recombinant plasmid. The specific activity for hippuricase from *C. jejuni* TGH9011 was 0.038 μmol/min/mg. There was no detectable hippuricase activity observed in the *C. coli* type strain extracts or in the hippuricase-negative *C. jejuni* isolates (32).

Nucleotide sequence of *hipO*. The nucleotide sequence of the insert in recombinant pHIP-O was determined on both strands to completion. The sequence of the hippuricase gene and flanking regions is shown in Fig. 2. Lines of evidence supporting the conclusion that ORFU4 (Fig. 1) constitutes the hippuricase gene are as follows: (i) deletion subclones retaining DNA sequences between *Eco*RV (nucleotide −635) (pHIP-CRΔRV) and *Xba*I (nucleotide 1160) (pHIP-CΔX) also retain hippuricase activity (Fig. 1, numbering with respect to Fig. 2); (ii) hippuricase activity is abolished in clone pHK1, which has an insertion of a kanamycin resistance determinant into the *Sph*I (nucleotide 294) site within the coding sequence for ORFU4 (Fig. 1); (iii) insertion of a kanamycin resistance determinant into blunt-end restriction enzyme sites within ORFU4 also abolishes hippuricase activity (data not shown); and (iv) there is significant homology (2) between ORFU4 (27% over 70 amino acid residues) and a protein possessing an analagous catalytic activity: *Pseudomonas* sp. *N*-carbamoyl amino acid amidohydrolase protein (34) cleaves L-amino acids from *N*-carbamoyl L-amino acids, and hippuricase removes glycine from *N*-benzoylglycine. The homology between hippuricase and the *Bacillus subtilis* amino acid amidohydrolase protein (26) is 27.7% over 370 amino acid residues (Fig. 3).

The hippuricase gene consists of a single open reading frame of 1,149 bp that encodes a polypeptide of 383 amino acids with an estimated M_r of 42,596 and a pI of 6.00. Upstream from the

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   MNLIPEILDLQGEFEKIRHQIHENPELGFDELCTAKLVAQKLKEFGYEVYEEIGKTG  57
MTKEEIKRLVDEVKTDVIAWRRHLHAHPELSFQEEKTAQFVYETLQSFGHLELSRPTKTS  60
     .   .  .       *. .*  *** * *  **  * . *  **        **

VVGVLKKGNSDKKIGLRADMDALPLQECTNLPYKSKKENVMHACGHDGHTTSLLLAAKYL 117
VMARLIGQQPGRVVAIRADMDALPEQEENTFEFASKNPGVMHACGHDGHTAMLLGTAKIF 120
*.  *      . . .******** **     . **   ***********. ** .**

ASQNFNGTLNLY  FQPAEEGL GGAKAMIEDGLFEKFDSDYVFGWHNMPFGSDKKFYLK 174
SQLRDDIRGEIRFLFQHAEELFPGGAEEMVQAGVMDGV  DVVIGTHLWSPLERGKIGIV 178
.    .    .   ** ***   ***  *.. *.      * * * *        *  .

KGAMMASSDSYSIEVIGRGGHGSAPEKAKDPIYAASLLVVALQSIVSRNVDPQNSAVVSI 234
YGPMMAAPDRFFIRIIGKGGHGAMPGQTIDAIAIGAQVVTNLQHIVSRYVDPLEPLVLSV 238
 * ***. * . * .**.****. *  . * *   . .*  ** **** ***    *.*.

GAFNAGHAFNIIPDIVTIKMSVRALDNETRKLTEEKIYKICKGLAQANDIEIKINKNVVA 294
TQFVAGTAHNVLPGEVEIQGTVRTFDETLRRTVPQWMERIVKGITEAHGASYEFRFDYGY 298
  * ** * *..*  * *  .**. *   *.   . . .* **...*         .

PVTMNNDEAVDFASEVAKELFGEKNCEFNHRPLMASEDFGFFCEMKKCAYAFLENENDIY 354
RPVINYDEGDPRHGGNGVRAVRRRGSGPLETEHGRRRFLRLFAKSARQLFLRRRGQCRKR 358
   .* **               .                 *       .     .

LHNSSYVFNDKLLARAASYYAKLALKYLK                                383
HRLPAPPPALYD                                                 370
 .  .
```

FIG. 3. Amino acid sequence homology of *C. jejuni* ORFU4 with the *Bacillus stearothermophilus N*-acyl-L-amino acid amidohydrolase protein (gp|X74289) (26). Identical amino acids and similar amino acids are indicated by asterisks and periods, respectively. Gaps have been introduced to optimize the alignment.

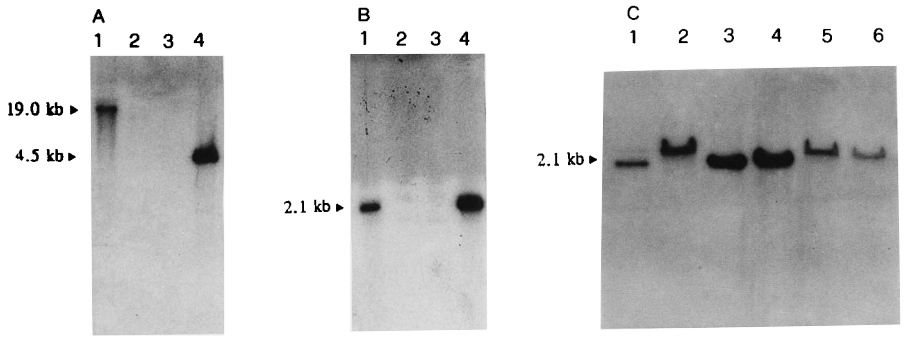

FIG. 4. (A and B) Southern blot analysis of chromosomal DNA of *C. jejuni* ATCC 33560^T and TGH9011 and *C. coli* ATCC 33559^T digested with *Cla*I (A) and *Hin*dIII (B). The fragments were separated by gel electrophoresis, transferred to GeneScreen, and probed with a radiolabeled 0.8-kb *Hin*dIII *hipO*-specific fragment. Lanes: 1, ATCC 33560^T; 2, ATCC 33559^T; 3, molecular weight marker; 4, TGH9011. The *C. jejuni* DNA fragments that hybridized with the probe are indicated (arrowheads). (C) Southern blot analysis of chromosomal DNA of *C. jejuni* ATCC 33560^T and hippuricase-negative variants of *C. jejuni*. The DNAs were digested with *Hin*dIII and probed with the same *hipO*-specific fragment as for panel A. Lanes: 1, ATCC 33560^T; 2, strain D594; 3, strain D603; 4, strain D941; 5, strain D1713; 6, strain D1916.

proposed start codon, sequence AGGAGA is representative of a typical Shine-Dalgarno sequence (Fig. 2). The codon usage of hippuricase is similar to that of other genes from *C. jejuni* TGH9011 which have been sequenced (references 6, 10, 15, 20 and unpublished results).

The organization of the open reading frames within the insert of pHIP-O is compact. Two of the theoretical open reading frames overlap by 16 nucleotides (ORFU2 and ORFL1) on complementary strands. Overlapping genes have already been described for *C. jejuni* (7, 10). The intergenic regions between the coding sequences are small, which reflects the size of the genome of *C. jejuni* (17).

Genomic hybridizations. Southern hybridizations were performed to determine the presence of the relevant gene in *Campylobacter* strains. The hippuricase gene has already been localized (16) on the physical map of *C. jejuni* TGH9011 (18) and was shown to be present in single copy (data not shown). The type strain of *C. jejuni* contains a 19-kb *Cla*I genomic DNA band and a 2.1-kb *Hin*dIII genomic DNA band which hybridize to the hippuricase probe (Fig. 4A and B, lanes 1). *C. coli* type strain genomic DNA does not hybridize with the hippuricase probe (Fig. 4A and B, lanes 2). *C. jejuni* TGH9011 genomic DNA produces 4.5-kb *Cla*I and 2.1-kb *Hin*dIII bands which hybridize with the hippuricase probe (Fig. 4A and B, lanes 4). The 2.1-kb *Hin*dIII fragment of *C. jejuni* TGH9011 was of the size expected from the nucleotide sequence. The size of the *Cla*I hybridizing band was consistent with the known sequence obtained from recombinant pHIP-O.

Genomic DNAs from *C. jejuni* ATCC 33560^T and hippuricase-negative *C. jejuni* isolates were digested with *Hin*dIII and examined by Southern blot analysis (Fig. 4C). All isolates gave a single hybridizing signal with the 0.8-kb *Hin*dIII hippuricase-specific fragment. As expected, the *C. jejuni* type strain identified a 2.1-kb *Hin*dIII hybridizing band (Fig. 4C, lane 1). The hippuricase-negative variants all gave a single hybridization signal with the hippuricase probe. The hybridizing bands for three of the mutants (D594, D1713, and D1916 [Fig. 4C, lanes 2, 5, and 6, respectively]) are larger than that of the *C. jejuni* wild-type strain, but the size of the hippuricase-encoding sequence is unchanged in these strains (data not shown). The other two *hip* mutant strains (D603 and D941 [Fig. 4C, lanes 3 and 4, respectively]) presumably suffered point mutations, as the size of the hybridizing band is unchanged.

Primer extension analysis. The 5′ end of the *hipO* mRNA was determined via primer extension using 25-mer oligonucleotide hpr1 and 26-mer oligonucleotide hpr2, both complementary to *hipO* mRNA. The sizes of the extended products were determined on a sequencing gel, with dideoxy sequencing reaction mixtures using the same primer as size markers. As shown in Fig. 5, multiple extended products corresponding to the transcription start points for *hipO* were produced from hpr2. When hpr1 was used with independent samples of RNA, the identical bands were produced (data not shown). In addition, the products are produced in both *C. jejuni* and *E. coli*,

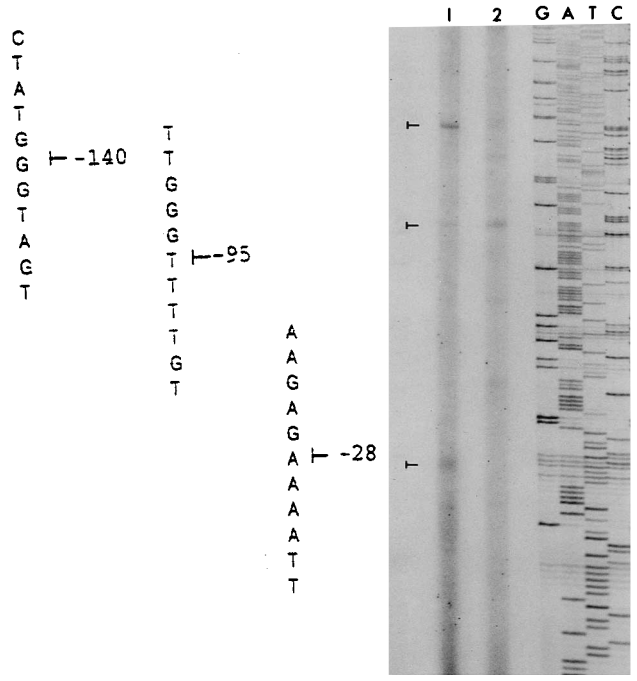

FIG. 5. Mapping of the 5′ end of the *hipO* transcript by primer extension. The reverse transcriptase products of *hipO* mRNAs from *C. jejuni* TGH9011 (lane 1) and *E. coli* JM101 containing plasmid pHIP-O (lane 2) are indicated (horizontal bars). The results of dideoxy chain termination sequence reactions in the region encompassing the promoter are also shown (lanes G, A, T, and C). The sequence of the [γ-^{32}P]ATP-labeled primer hpr2 (5′-CATAATTCATC AAAACCAAGCTCAGG-3′) is complementary to nucleotides 73 to 98 of *hipO*. Numbers indicate nucleotide positions.

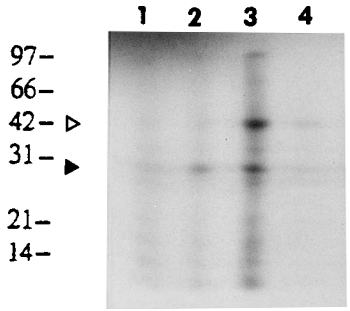

FIG. 6. Maxicell analysis for the elucidation of plasmid-encoded protein corresponding to *hipO* gene sequences, as analyzed by SDS-PAGE. The protein band produced in the recombinant pBR322 construct (open arrowhead) and the position of the ampicillin resistance product (solid arrowhead) are indicated. Lanes: 1 to 3, *E. coli* DR1984 with no plasmid, with plasmid pBR322, and with pHIP-O, respectively; 4, protein standard molecular mass markers (sizes [in kilodaltons] are indicated on the left).

albeit at different intensities, possibly reflecting the strength of the heterologous promoters.

Maxicell analysis. Plasmid proteins were detected by maxicell analysis as shown in Fig. 6. Plasmid pHIP-O produces protein bands in SDS-PAGE at 42 and 30 kDa. The 30-kDa protein is also produced by the pBR322 control plasmid and represents the ampicillin resistance determinant. The 42-kDa protein is in excellent agreement with the molecular mass of 42.6 kDa predicted for the hippuricase protein.

DISCUSSION

This study demonstrates the presence of the hippuricase gene in the human enteropathogen *C. jejuni* and the absence of the gene from the most closely related species, *C. coli*, and thus resolves the basis for the difference between these two species in the biochemical test. The phenotypic (and now genotypic) characteristic is key in discriminating the two species. Twelve strains of *C. jejuni* were positive with the hippuricase gene probe, and 17 *C. coli* isolates were negative with the probe (data not shown). In addition, we have shown that the gene is also absent from other campylobacteria, including *Campylobacter lari*, *Campylobacter upsaliensis*, and *Campylobacter sputorum*, and *Helicobacter pylori* (data not shown). By virtue of the lack of this genetic element in campylobacters other than *C. jejuni*, we are now in the position to develop a potentially species-specific diagnostic DNA probe for human gastrointestinal infections. The DNA probe offers a more sensitive and reliable means of distinguishing *C. jejuni* than the hippuricase assay yet can still be correlated with the biochemical test. We have determined that hippuricase-negative variants of *C. jejuni* which were confirmed by DNA-DNA hybridization can be detected with our hippuricase probe. This raises speculation on the classification of *C. coli* isolates which may be hippuricase-negative *C. jejuni*. The nature of the defect in hippuricase activity in these organisms has not yet been established.

The size of *C. jejuni* hippuricase closely matches that of *Pseudomonas putida* hippuricase (42 kDa) (22), as determined from maxicell experiments (Fig. 5) and purification of the hippuricase protein (data not shown). The physiological role of hippuricase and its possible relevance to the pathogenicity of *C. jejuni* remain to be elucidated.

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