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Analysis of F_1F_0-ATPase from *Helicobacter pylori*

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The adaptive mechanisms that permit *Helicobacter* species to survive within the gastric mucosa are not well understood. The proton-translocating F_1F_0-ATPase is an important enzyme for regulating intracellular pH or synthesizing ATP in many other enteric bacteria; therefore, we used degenerate primers derived from conserved bacterial F_1F_0-ATPase sequences to PCR amplify and clone the gene (*atpD*) encoding the *H. pylori* F_1F_0-ATPase β subunit. The deduced amino acid sequences of the F_1F_0-ATPase β subunits from *H. pylori* and *Wolinella succinogenes* were 85% identical (91% similar). To characterize a potential functional role of F_1F_0-ATPase in *H. pylori*, *H. pylori* or *Escherichia coli* cells were incubated for 60 min in buffered solutions at pH 7, 6, 5, or 4, with or without 100 μM *N,N′*-dicyclohexylcarbodiimide (DCCD), a specific inhibitor of F_1F_0-ATPase. At pH 5 and 4, there was no significant decrease in survival of *H. pylori* in the presence of DCCD compared to its absence, whereas incubation with DCCD at pH 7 and 6 significantly decreased *H. pylori* survival. *E. coli* survival was unaffected by DCCD at any pH value tested. We next disrupted the cloned β-subunit sequence in *E. coli* by insertion of a kanamycin resistance cassette and sought to construct an isogenic F_1F_0-ATPase *H. pylori* mutant by natural transformation and allelic exchange. In multiple transformations of *H. pylori* cells grown at pH 6 or 7, no kanamycin-resistant F_1F_0 mutants were isolated, despite consistently successful mutagenesis of other *H. pylori* genes by using a similar approach and PCR experiments providing evidence for integration of the kanamycin resistance cassette into *atpD*. The sensitivity of *H. pylori* to DCCD at pH 7 and 6, and failure to recover F_1F_0 *H. pylori* mutants under similar conditions, suggests that the function of this enzyme is required for survival of *H. pylori* at these pHs.

H+-translocating adenosine 5′-triphosphate synthases (F_1F_0-ATPases) are multisubunit enzymes found in the cytoplasmic membranes of a variety of bacteria as well as mitochondria and chloroplasts (15, 16, 41). In bacteria, these enzymes can utilize the electrochemical gradient generated by respiration to synthesize ATP, or they may catalyze the reverse reaction, generating an electrochemical gradient by the hydrolysis of ATP (15, 16, 41). F_1F_0-ATPases from different sources have essentially the same structure. The catalytic portion of the enzyme (F_1) consists of five subunits, α, β, γ, δ, and ε, and can be detached from membranes as a soluble ATPase. The integral membrane sector (F_0) contains three different subunits, *a*, *b*, and *c*, and functions as a proton channel that translocates H+ across the membrane. Of the eight subunits, β subunits have the most conserved primary structures, with 40% amino acid identity in the bacterial sequences studied (15). The conserved nucleotide-binding motif, GXXXXGKT, is consistently found in the β subunit of F_1F_0-ATPases and is essential for catalytic activity (14, 15).

Helicobacter pylori is now recognized as the causative agent of chronic superficial gastritis and is a major factor contributing to the pathogenesis of peptic ulcer disease and gastric malignancies (5, 33). *H. pylori* colonizes the mucus layer overlying the gastric epithelium, where it is exposed to a wide pH gradient ranging from approximately 2 to 7 (4, 36). As no other bacteria are known to persist in the human stomach, *Helicobacter* species appear unique in their ability to adapt to this inhospitable ecological niche. Survival of *H. pylori* may be attributed to the development of several specialized characteristics (26). Spiral shape and multiple flagellae permit penetration and movement within the viscous mucus layer, allowing the organism to escape prolonged exposure to extremely low pH and clearance by peristalsis. Microaerobic metabolism enables *H. pylori* to live within the mucus layer, where oxygen levels are low but not absent. *H. pylori* produces a potent urease, which hydrolyzes urea to NH_3 and H_2O and which is required for the organism to colonize the stomach (8, 9). Survival of *H. pylori* below pH 4 in vitro is dependent on urease activity, whereas urease-independent mechanisms appear to be involved in survival above pH 4 (11, 29, 31, 34, 38). *H. pylori* may grow in vitro over a pH range of 4.5 to 8 (19, 21). Thus, although *H. pylori* must possess various mechanisms that allow adaptation to acid stress, these pathways have not yet been elucidated.

Since *H. pylori* survival above pH 4 in vitro is similar to that of other enteric organisms such as *Salmonella typhimurium* and *Escherichia coli* (31), *H. pylori* may possess analogous acid survival mechanisms (30). For *S. typhimurium*, a brief exposure to moderate acid stress (pH 6) induces synthesis of novel proteins, which improve survival upon subsequent challenge at a pH of ≤4.5 (12, 13). This phenomenon, known as the acid tolerance response, requires a proton-translocating F_1F_0-ATPase (12, 13). Low pH also induces biosynthesis of F_1F_0-ATPase in *Enterococcus faecalis* (24, 42). Oral streptococci exhibit high but varying levels of acid tolerance, which appears to depend on the relative F_1F_0-ATPase activity in transporting protons from cells (2). Thus, for several organisms that inhabit the gastrointestinal tract, the F_1F_0-ATPase is important in tolerance to low pH.

Total ATPase activity of *H. pylori* cells has been assayed in vitro and found to be most susceptible to inhibitors such as sodium azide, fluoroaluminate, and *N,N′*-dicyclohexylcarbodiimide (DCCD), which typically are most active against F_1F_0-

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ATPases; inhibitors of P-type and vacuolar ATPases had relatively little effect on *H. pylori* ATPase activity (1). To determine whether *H. pylori* possesses an F_1F_0-ATPase, we used degenerate PCR to amplify the F_1F_0-ATPase β-subunit gene from *H. pylori* chromosomal DNA and sought to compare its nucleotide sequence with those of other known bacterial F_1F_0-ATPase β subunits. Next, to investigate a potential functional role of F_1F_0-ATPase in *H. pylori*, we sought to determine whether inhibition of this enzyme's activity altered bacterial survival after exposure to a range of pH values. Finally, as another approach for characterizing the role of F_1F_0-ATPase in *H. pylori* pH homeostasis, we attempted to construct an isogenic mutant deficient in this enzyme activity by insertional mutagenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* 60190 (ATCC 49503), 84-183 (ATCC 53726), and 86-313 are well-characterized clinical isolates that were used in this study (7, 35, 47). *H. pylori* 60190-4, a spontaneous urease-negative mutant that does not produce either subunit of the urease protein, was used for comparative experiments (34). A clinical isolate of *E. coli* (strain 94-11) was used as a control in the survival studies. *H. pylori* was cultured on Trypticase soy agar plates containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at 37°C in a 5% CO_2 atmosphere. *E. coli* 94-11 was cultured on blood agar plates at 37°C in ambient air.

Genetic techniques. Restriction enzymes and Klenow fragment of *E. coli* DNA polymerase I were purchased from either New England Biolabs (Beverly, Mass.) or Promega (Madison, Wis.) and used according to the manufacturer's directions. *H. pylori* chromosomal DNA was prepared as described previously (28). Nucleotide sequences of plasmid DNA were determined unambiguously on both strands, using the primer walking technique and an ABI automated sequencer, by the Vanderbilt University Cancer Center Core Facility. Analysis of DNA and protein sequences was accomplished via the use of the BLASTX program in conjunction with the National Center for Biotechnology Information.

PCR amplification and cloning of the gene encoding the *H. pylori* F_1F_0-ATPase β subunit. Degenerate oligonucleotide primers were designed on the basis of conserved sequences of bacterial F_1F_0-ATPase β subunits. One primer (5′-GC NGGNGTNGGNAARAC-3′) was based on the amino acid sequence AGVG KT, and the second primer (5′-TCRTCNGCNGGNACRTA-3′) was based on the reverse complement of the amino acid sequence YVPADD (15). A 0.5-kb PCR fragment was amplified from chromosomal DNA from *H. pylori* 60190, using the following thermocycler parameters: 1 min at 94° (denaturation), 1 min at 50° (primer annealing), and 2 min at 72° (extension). The degenerate PCR product was excised from a 1% agarose gel, purified by using a Qiaex gel extraction kit (Qiagen, Chatsworth, Calif.), and subcloned into pT7Blue (Novagen, Madison, Wis.). To clone the entire gene encoding the β subunit, the 0.5-kb fragment was labeled with [α-^{32}P]dATP (650 Ci/mmol) by random priming (Amersham, Little Chalfont, England) and used as a probe to screen a cosmid library derived from partially *Sau*3A-digested genomic DNA from *H. pylori* NCTC11638, which was generously made available by Douglas Berg (3). Following identification of a reactive cosmid (cosmid 40), the cosmid DNA was digested with restriction endonucleases, and Southern hybridization with the ^{32}P-labeled 0.5-kb probe was performed as previously described (40).

Acid survival studies. Log-phase or stationary-phase bacteria were harvested from culture plates and suspended in 150 mM sodium chloride (pH 7) to yield a final suspension of approximately 10^8 CFU/ml. To evaluate the effect of DCCD (Sigma Chemical Co., St. Louis, Mo.) on survival of *H. pylori* and *E. coli* 94-11 under a variety of pH conditions, the cell suspensions were diluted 1:10 in McIlvain's buffer (titrations of 200 mM Na_2HPO_4 plus 100 mM citric acid; pH 4, 5, or 6), or 100 mM sodium phosphate buffer (pH 7), with or without DCCD (10 or 100 μM), and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. A stock solution of DCCD was freshly prepared in methanol prior to each experiment. To examine the effect of urease activity on *H. pylori* 60190 survival in the presence of DCCD, parallel experiments were done with the addition of 10 mM urea. After incubation, serial dilutions were made in 100 mM sodium phosphate buffer (pH 7), inoculated onto blood agar plates, and incubated for 72 to 96 h at 37°C in 5% CO_2 (*H. pylori* strain) or 24 h at 37°C (*E. coli* strain), and colonies were counted to determine the number of viable bacteria. The statistical significance of differences in survival was determined by using the paired *t* test.

Mutagenesis of the cloned *H. pylori* F_1F_0-ATPase β-subunit gene. Once the entire F_1F_0-ATPase β-subunit gene sequence of *H. pylori* NCTC11638 was determined, oligonucleotide primers derived from the sequences flanking the β-subunit open reading frame (ORF) were chosen (5′-GGTGGTAGATGTGG AGTT-3′) and 5′-AGCACATTGCGATCAATT-3′ [Fig. 1]), and the entire 1.4-kb subunit gene sequence was PCR amplified from *H. pylori* 60190, 84-183, and 86-313. The PCR products were subcloned into pT7Blue, yielding plasmids pCM101, pCM102, and pCM103, respectively, each of which was linearized at a unique *Nhe*I site in the midregion of the β-subunit ORF and end filled with Klenow enzyme. A 1.4-kb *Sma*I fragment derived from pILL600 was used as the source of a *Campylobacter coli* kanamycin resistance (*km*) gene (25). The *km* cassette was then cloned into the *Nhe*I site of each plasmid to yield pCM101:*km*, pCM102:*km*, and pCM103:*km*. Proper construction of each plasmid was verified by restriction endonuclease analysis.

Natural transformation of *H. pylori* strains. Natural transformation of *H. pylori* cells was done essentially as described previously (48). Log-phase *H. pylori* cells (24 h of growth) were harvested from four blood agar plates and concentrated into 100 μl of brucella broth (BB) containing 10% fetal bovine serum (FBS). Following placement of 25-μl aliquots of *H. pylori* for 4 h on nonselective BB plates supplemented with 10% FBS (plate pH of 7 or 6), 10 μl of purified plasmid in sterile H_2O was added to the cells, and the mixture was incubated overnight at 37°C in 5% CO_2. After 18 h, cells were harvested and replated on kanamycin (30 μg/ml)-containing BB plates with 10% FBS at pH 7 or 6. Kanamycin-resistant (Km^r) colonies were selected after 4 to 5 days of growth at 37°C in 5% CO_2. Plasmid pCTB8:*km*, a 1.4-kb *vac*A fragment containing the same *km* cassette (6), was used as a positive control for transformations.

Transformation of *H. pylori* in broth culture was carried out by using an adaptation of a previously described protocol (20). A 980-μl aliquot of BB containing 10% FBS and 50 mM potassium phosphate buffer with no additions (pH 7) or titrated with HCl (pH 5) was added to a 24-well microtiter plate. Log-phase *H. pylori* cells were harvested from four blood agar plates and concentrated into 150 μl of BB containing 10% FBS, and 20 μl of the cell suspensions (approximately 10^7 CFU/ml) was added to each well at the desired pH. After the cells were allowed to grow for 8 h at 37°C in 5% CO_2, 10 μl of purified plasmid was placed in the bottom of each well, and the microtiter plates were incubated overnight. After 18 h, 20-μl aliquots were removed to determine viable counts, and the remaining cells were harvested and plated on kanamycin (30 μg/ml)-containing BB plates with 10% FBS at pH 7, 6, or 5. Km^r colonies were selected as described above.

Electroporation of *H. pylori* strains. Electroporation of *H. pylori* was performed essentially as described previously (39). Log-phase *H. pylori* cells were harvested from five blood agar plates (24 h of growth) and washed twice in cold H_2O. Cells were resuspended in cold buffer (15% glycerol, 10% sucrose), washed twice, and suspended in a final volume of 300 μl. Aliquots (50 μl) of bacteria were added to prechilled 0.1-cm electroporation cuvettes, followed by the addition of 5 μl of plasmid (1 μg/μl, initial concentration). Conditions of electroporation using a Bio-Rad Gene Pulser were 1,800 V, 25-μF capacitor, and 200 ohms (in parallel with the sample channel). Immediately after electroporation, 800 μl of ice-cold BB was added to each sample, and aliquots were placed on blood agar plates and incubated overnight at 37°C in 5% CO_2. The following morning, cells were harvested and transferred to BB plates containing kanamycin (30 μg/ml), and Km^r colonies were selected after 4 to 5 days growth.

PCR amplification of *H. pylori* chromosomal DNA after integration of pCM101:*km*. Natural transformations of *H. pylori* 60190 were performed on blood agar plates as described above. After overnight incubation of the cells with plasmid pCM101:*km*, chromosomal *H. pylori* DNA was isolated. PCR amplification of this DNA was performed by using forward and reverse primers derived from *km* cassette DNA sequences paired with primers derived from *H. pylori* genomic sequences located upstream and downstream of the cloned β subunit and thus not present in pCM101:*km* (see Fig. 4). Chromosomal DNA isolated from log-phase *H. pylori* cells was used as a negative control.

Nucleotide sequence accession number. The sequence shown in Fig. 1 has been deposited in GenBank (accession no. AF 004014).

RESULTS

PCR amplification and cloning of the *H. pylori* F_1F_0-ATPase β-subunit gene. To isolate a fragment of the *H. pylori* F_1F_0-ATPase, we performed PCR using degenerate primers designed on the basis of an alignment of conserved protein sequences of known bacterial F_1F_0-ATPase β subunits (14, 15). Degenerate PCR of *H. pylori* 60190 chromosomal DNA performed with these primers (described in Materials and Methods) resulted in the amplification of a product of the expected size (480 bp), which was subcloned into pT7Blue. Computer database analysis indicated that the sequence of the subcloned PCR product was highly homologous with bacterial F_1F_0-ATPase β-subunit genes. To isolate the entire β-subunit gene, we screened a cosmid library of *H. pylori* NCTC11638 chromosomal DNA, using the 480-bp PCR-amplified fragment as a probe, and identified one reactive cosmid (cosmid 40). Southern hybridization of *Hin*dIII-digested cosmid DNA with the 480-bp probe yielded a 4.0-kb reactive band, which was subcloned into pBluescript to yield pCM100.

Features of *H. pylori* F_1F_0-ATPase β-subunit gene sequences. Nucleotide sequence analysis of pCM100 revealed

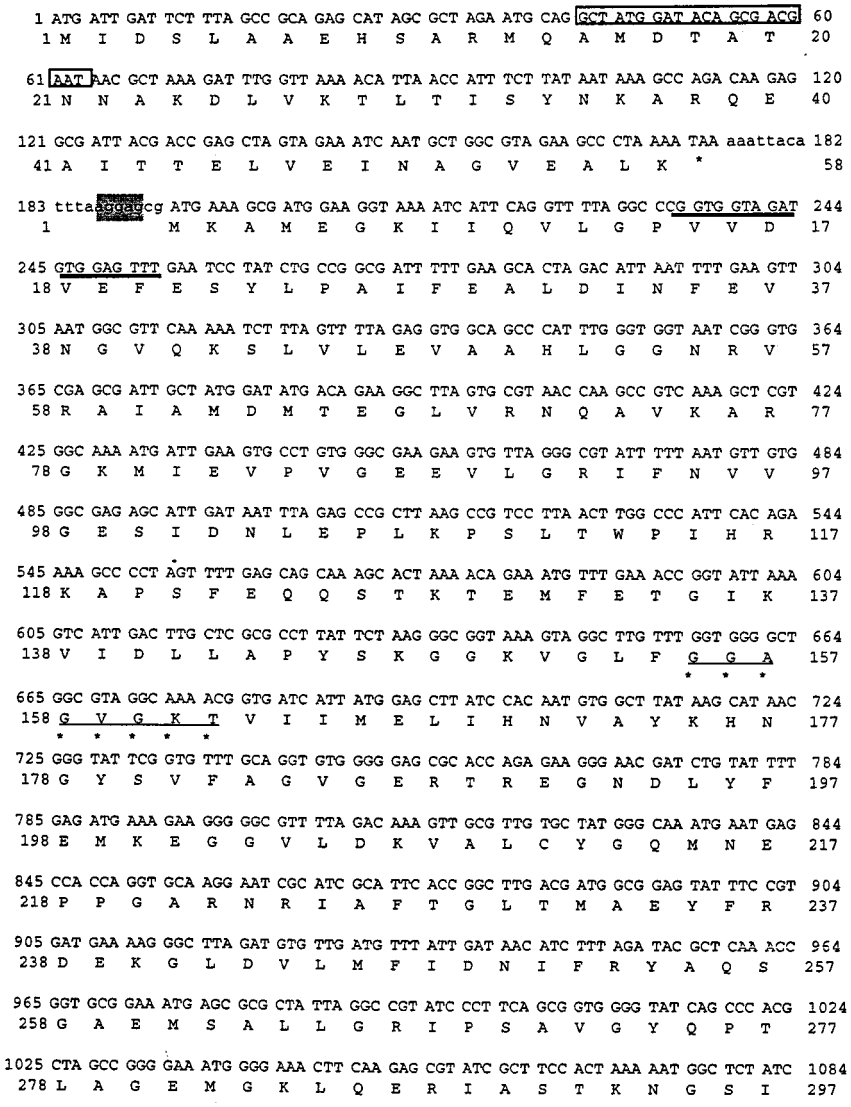

FIG. 1. Sequence of the *H. pylori* F_1F_0-ATPase β-subunit gene (*atpD*) and flanking regions from pCM100. The conserved β-subunit amino acid sequences on which the degenerate PCR primers were based are lightly underlined. The putative nucleotide-binding site (residues 155 to 162) is indicated by asterisks. The nucleotide sequences of the primers used to PCR amplify the β-subunit gene (yielding plasmids pCM101, pCM102, and pCM103) are heavily underlined. The *Nhe*I site into which the *km* cassette was inserted is located between codons 277 and 278. The 5′-flanking DNA sequence of *H. pylori atpD* contains a truncated ORF for which the deduced amino acid sequence is homologous to *atpG* (nucleotides 1 to 171), and the 3′-flanking sequence contains a truncated ORF corresponding to the first 62 amino acid residues of *atpC*. Upstream and downstream primers (γ for and ε rev [Fig. 4]) used in the PCR amplification of chromosomal *H. pylori* DNA after integration of pCM101:*km* are indicated by boxes. A putative ribosome-binding site for *atpD* is shaded.

the presence of an ORF of 1,407 nucleotides which encoded a putative 469-amino-acid protein with a predicted molecular mass of 51.4 kDa (Fig. 1). The deduced protein sequence was highly homologous with sequences of the β subunits of multiple bacterial F-type ATPases. The highest degree of homology was found with the F_1F_0-ATPase β subunit of *Wolinella succinogenes*, which was 85% identical and 91% similar to that of *H. pylori*, whereas the *E. coli* F_1F_0-ATPase β subunit was 67% identical and 79% similar. Thus, we assigned this *H. pylori* gene the name *atpD*, based on the subunit and structural gene nomenclature widely used for F_1F_0-ATPases (16). The putative ATP-binding motif in the *H. pylori* F_1F_0 β subunit (GGAGVG KT [residues 155 to 162]) was 100% identical to the corresponding sequences in *W. succinogenes* (residues 152 to 159) and *E. coli* (residues 149 to 156). The 5′-flanking DNA sequence of *H. pylori atpD* contained an ORF which was homologous to the gene (*atpG*) encoding bacterial F_1F_0-ATPase γ subunits, and the 3′-flanking sequence contained an ORF corresponding to the gene (*atpC*) encoding F_1F_0-ATPase ε-subunit gene sequences. Thus, the genes encoding the *H. pylori* F_1F_0-ATPase appear to be organized in an operon that may be similar to that described for *E. coli* and other enteric bacteria, in which there are nine genes encoding eight enzyme subunits (15, 42).

Effect of DCCD on bacterial survival. *H. pylori* is able to survive over a pH range of 4 to 7 in vitro, regardless of whether urea is present (31). To determine whether proton-translocating F_1F_0-ATPase activity is required for survival of *H. pylori* at these pH values, stationary-phase cells (from 48-h cultures) of *H. pylori* 60190 were incubated in buffered solutions at pH 4, 5, 6, or 7 in the presence or absence of various concentrations of DCCD, a specific inhibitor of F_1F_0-ATPase activity. Station-

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1085 ACT TCG GTT CAA GCG GTG TAT GTG CCA GCA GAC GAC TTG ACT GAC CCA GCC CCT GCT TCG  1144
 298 T   S   V   Q   A   V   Y   V   P   A   D   D   L   T   D   P   A   P   A   S    317

1145 GTG TTT GCG CAT TTA GAT GCG ACT ACG GTG TTG AAT AGA AAG ATC GCT GAA AAA GGG ATT  1204
 318 V   F   A   H   L   D   A   T   T   V   L   N   R   K   I   A   E   K   G   I    337

1205 TAT CCG GCG GTG GAT CCT TTG GAT TCC ACT TCA AGG ATT TTA AGC CCT CAA ATG ATT GGC  1264
 338 Y   P   A   V   D   P   L   D   S   T   S   R   I   L   S   P   Q   M   I   G    357

1265 GAG AAG CAC TAT GAA ATC GCC ACC GGT ATC CAG CAA GTT TTG CAA AAA TAC AAG GAT TTG  1324
 358 E   K   H   Y   E   I   A   T   G   I   Q   Q   V   L   Q   K   Y   K   D   L    377

1325 CAA GAT ATT ATT GCG ATT TTG GGA TTG GAC GAA TTG AGC GAA GAG GAT AAA AAA ACG GTT  1384
 378 Q   D   I   I   A   I   L   G   L   D   E   L   S   E   E   D   K   K   T   V    397

1385 GAA AGG GCC AGA AAA ATT GAG AAG TTT TTA TCC CAG CCG TTT TTT GTG GCT GAA GTG TTT  1444
 398 E   R   A   R   K   I   E   K   F   L   S   Q   P   F   F   V   A   E   V   F    417

1445 ACA GGA AGT CCC GGT AAG TAT GTG ACT CTC CAA GAG ACT TTA GAG GGC TTT GGA GGG ATT  1504
 418 T   G   S   P   G   K   Y   V   T   L   Q   E   T   L   E   G   F   G   G   I    437

1505 TTA GAG GGC AAA TAC GAT CAC ATT CCT GAA AAC GCG TTT TAC ATG GTG GGC AGC ATT CAA  1564
 438 L   E   G   K   Y   D   H   I   P   E   N   A   F   Y   M   V   G   S   I   Q    457

1565 GAG GTT TTA GAA AAA GCT AAA AAC ATG AAA AAT TCC TAA gggttttgtgatggctttgttgaaaatt 1631
 458 E   V   L   E   K   A   K   N   M   K   N   S   *                               470

1632 agtgtggtagttcctgaggggggaagtttatacaggagaggttaaaagcgttgtgttgccaggattgaaggggaatttggg 1711

1712 gtgctttatgggcatagcaac ATG ATT ACT TTG CTT CAG GCG GGA ATG GTT GAG ATT GAA ACC   1774
   1                     M   I   T   L   L   Q   A   G   M   V   E   I   E   T     14

1775 GAA AAC CAA AAA GAG CAC ATT GCG ATC AAT TGG GGC TAT GCA GAA GTT ACT AAT GAA CGG  1834
  15 E   N   Q   K   E   H   I   A   I   N   W   G   Y   A   E   V   T   N   E   R    34

1835 GTG GAT ATT TTA GCC GAT GGG GCG GTC TTT ATT AAC AAA GAA TCA GAC GAC AGA GAT GAT  1894
  35 V   D   I   L   A   D   G   A   V   F   I   N   K   E   S   D   D   R   D   D    54

1895 GCT ATC TCT AGG GCT AAA AAG CTT                                                 1918
  55 A   I   S   R   A   K   K   L                                                    62
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FIG. 1—*Continued.*

ary-phase cells (from a 24-h culture) of *E. coli* 94-11 were tested in parallel as a control. In the absence of DCCD, both *H. pylori* and *E. coli* survived well at each of the pH values tested (Fig. 2), which is in accordance with previous studies (31). The addition of 10 μM DCCD had an insignificant effect on survival of *H. pylori* at a pH of ≤6, whereas incubation at pH 7 with 10 μM DCCD markedly decreased *H. pylori* survival (3.7 ± 0.5 $\log_{10}$ CFU/ml decrease, $P = 0.01$) (Fig. 2A). Higher

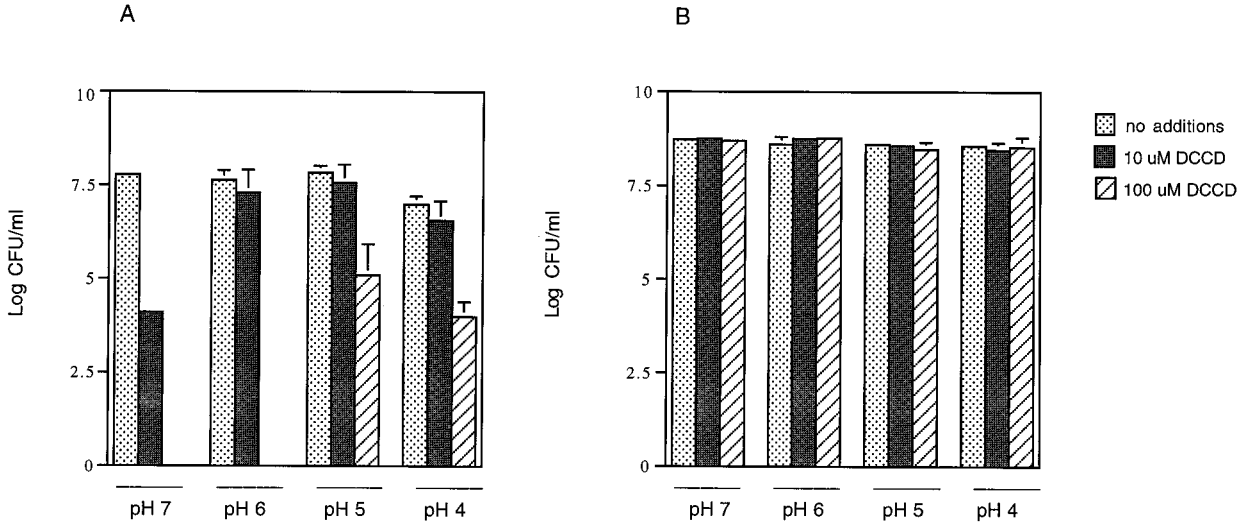

FIG. 2. Effects of DCCD on survival of stationary-phase *H. pylori* 60190 (A) and *E. coli* 94-11 (B) at various pH values. *H. pylori* 60190 and *E. coli* 94-11 (initial inocula of approximately 10^8 CFU/ml) were incubated for 1 h in buffers ranging from pH 4 to 7 in the absence or presence of 10 or 100 μM DCCD. The survival of *H. pylori* at pH 7 was significantly decreased in the presence of 10 μM DCCD, and no organisms were recovered at pH 6 or 7 after exposure to 100 μM DCCD (note absence of hatched bars at pH 6 and 7). In contrast, *E. coli* survival was unaffected by DCCD at any pH tested. The results represent the means ± standard errors of the means of three experiments. The standard error of the mean was small (≤0.1) for several pH values in experiments with both *H. pylori* and *E. coli*, and thus the error bars are not visible above the bar graph.

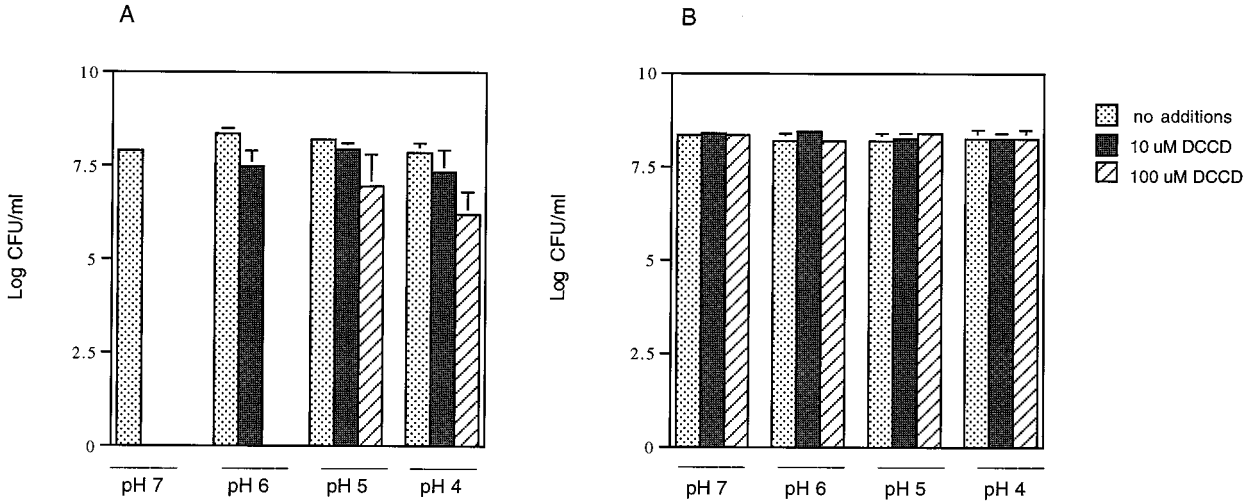

FIG. 3. Effect of DCCD on survival of log-phase *H. pylori* 60190 (A) and *E. coli* 94-11 (B). The conditions were the same as indicated in the legend for Fig. 2. No viable *H. pylori* were recovered at pH 6 or 7 after exposure to 100 μM DCCD (note absence of hatched bars). Log-phase *H. pylori* cells were significantly more susceptible to 10 μM DCCD at neutral pH than were stationary-phase cells (see Fig. 2 for comparison). In the absence of DCCD, however, no difference in survival between log- and stationary-phase cells was noted at any pH tested. Survival of log-phase *E. coli* was unaffected by DCCD.

concentrations of DCCD (100 μM) completely inhibited *H. pylori* survival at a pH of ≥6 (7.7 $\log_{10}$ decrease), but this concentration caused only a 3-$\log_{10}$ decrease in survival at pH 5 and 4. In contrast, survival of *E. coli* was unaffected by the addition of either 10 or 100 μM DCCD at any pH value tested (Fig. 2B). Total ATPase activity has been previously measured for *H. pylori* cells *in vitro* and was observed to decrease by 60% in the presence of 100 μM DCCD (1). Therefore, based on the known inhibitory effects of DCCD on ATPase from many different sources (37), it seems likely that inhibition of this enzyme is being achieved with the concentrations of DCCD used in the current experiments. These results suggest that F_1F_0-ATPase activity is required for in vitro survival of *H. pylori* at a pH of ≥6 but may be less essential at lower pH values.

To address the possibility that the absence of *H. pylori* inhibition by DCCD at low pH may reflect inactivation of the compound under acidic conditions, cells were suspended in 0.9% NaCl and preincubated in the presence of the desired concentration of DCCD (10 or 100 μM) for 15 min before exposure to acidic conditions. Results in these experiments were similar to those obtained with the previous methodology (data not shown). Irreversible inactivation of *E. coli* F_1F_0-ATPase by DCCD in vitro occurs within minutes (37); thus, these results suggest that the effects of DCCD were not inactivated at low pH and that *H. pylori* was maximally sensitive to DCCD at neutral pH.

Effect of growth phase on bacterial survival. For many other enteric bacteria, stationary-phase cells are more resistant than log-phase cells to various environmental stresses and to acid stress in particular (27, 43). Recent data suggest that pH-induced responses of *H. pylori* also may be dependent on the growth phase of the organism (22). To determine whether the effect of DCCD on *H. pylori* survival at the pH values tested was affected by growth phase, we next sought to compare the effects of DCCD on *H. pylori* cells harvested after 18 to 24 h of culture (log phase) (Fig. 3) to the effects on cells from a 48-h culture (stationary phase) (Fig. 2). The addition of 10 μM DCCD to log-phase *H. pylori* cells at pH 7 resulted in complete loss of bacterial viability, suggesting that *H. pylori* from log-phase cultures are more sensitive to the effects of F_1F_0-ATPase inhibition at neutral pH than are stationary-phase cells (Fig. 3A). This same effect also was noted for cells incubated at pH 8 with 10 μM DCCD (data not shown). In contrast, survival of log-phase cells in the presence of 10 μM DCCD at a pH of ≤6 was similar to that of stationary-phase cells. Increasing the concentration of DCCD to 100 μM caused complete loss of viability of log-phase *H. pylori* cells at pH 7 and 6, but survival below pH 6 was not significantly affected. In the absence of DCCD, no difference in acid susceptibility was observed between the log- and stationary-phase *H. pylori* cells. When log-phase *E. coli* cells (from a 4-h culture) were incubated with and without 10 or 100 μM DCCD, no difference in survival was observed compared to *E. coli* from stationary-phase culture (Fig. 3B). Thus, although growth phase had no effect on *H. pylori* survival in the absence of DCCD at the pH values tested, log-phase organisms were significantly more sensitive to the effects of DCCD at pH 7 than were stationary-phase cells.

Effect of urease activity on *H. pylori* survival in the presence of DCCD. To determine if urease activity affects the inhibition of *H. pylori* by DCCD, survival experiments also were carried out in the presence of 10 mM urea. Survival of *H. pylori* at pH 7 in the presence of 10 μM DCCD was increased slightly (approximately 1 $\log_{10}$) when urea was added to the buffer compared to its absence, but the result was not statistically significant (data not shown). This mildly protective effect of urea was not observed when the DCCD concentration was increased to 100 μM, as no viable bacteria were recovered at this concentration. The isogenic urease-negative mutant (strain 60190-4 [34]) demonstrated survival similar to that of the wild-type *H. pylori* 60190 in the presence of DCCD at all pH values tested. These experiments suggest that the inhibitory effect of DCCD on *H. pylori* survival is unrelated to urease activity.

Failure to isolate *H. pylori* F_1F_0-ATPase mutants. To further characterize the role of F_1F_0-ATPase in *H. pylori* pH homeostasis, we attempted to construct an *H. pylori* mutant that lacks F_1F_0-ATPase activity. We first inserted a *km* gene into the unique *Nhe*I site of PCR-amplified F_1F_0 β-subunit genes from several *H. pylori* strains, which would result in premature termination of translation of *atpD*. The resulting plasmids were used as donor DNAs for the homologous transformation of *H.*

TABLE 1. Number of colonies of Km^r *H. pylori* resulting from natural transformation with plasmids containing either *H. pylori atpD* or *vacA* disrupted by a *km* cassette

H. pylori strain used for transformation	Mean no. of transformants ($n = 3$)/μg of plasmid DNA							
	pCM101:*km*[a]		pCM102:*km*[b]		pCM103:*km*[c]		pCTB8:*km*[d]	
	pH 7	pH 6	pH 7	pH 6	pH 7	pH 6	pH 7	pH 6
60190	0	0					214	128
84-183			0	0			132	120
86-313					0	0	121	50

[a] *atpD* from *H. pylori* 60190, containing a *km* cassette.
[b] *atpD* from *H. pylori* 84-183, containing a *km* cassette.
[c] *atpD* from *H. pylori* 86-313, containing a *km* cassette.
[d] *vacA* from *H. pylori* 60190, containing a *km* cassette.

pylori 60190, 84-183, and 86-313. Multiple transformations were performed, but no viable F_1F_0 mutants were isolated, despite successful mutagenesis in all three strains by using a *vacA* fragment of similar size that contains the same *km* cassette (Table 1). Since inhibition of F-type ATPase activity with 10 μM DCCD completely inhibited survival of 24-h *H. pylori* cells at pH 7, we hypothesized that mutation of the F_1F_0-ATPase gene at neutral pH might be lethal to the organism. Therefore, additional transformations of *H. pylori* were performed on BB agar plates prepared at pH 6, which were known to support normal growth of the wild-type strains (data not shown). No viable F_1F_0-ATPase transformants could be isolated under these pH conditions, whereas mutagenesis with the control *vacA* plasmid (pCTB8:*km*) yielded approximately the same number of mutants as observed at pH 7 (Table 1). Successful transformation of all three *H. pylori* strains with pCTB8:*km* was achieved with a minimum amount of 1 μg of plasmid DNA/transformation of approximately 10^9 CFU/ml *H. pylori* cells. However, even higher concentrations of F_1F_0-ATPase plasmid DNA (≥10 μg/transformation) failed to yield viable mutants.

Transformations of *H. pylori* 60190 grown in broth culture (BB with 10% FBS) were also attempted at pH 7 and 5. Mutagenesis with the *vacA* plasmid (pCTB8:*km*) at pH 7 yielded an average of 212 Km^r mutants per transformation, whereas no viable bacteria were recovered following transformations at pH 7 with plasmid pCM101:*km* (data not shown). No viable mutants were recovered from transformations performed at pH 5 with either plasmid, suggesting that this pH condition may be less permissive for natural transformation of *H. pylori.*

Since electroporation may be more efficient than natural transformation, we next attempted to generate an F_1F_0-ATPase mutant by using electroporation at neutral pH. This approach yielded approximately 1,200 *H. pylori* mutants per experiment, using the control plasmid pCTB8:*km*, but again no viable mutants were recovered from electroporations with pCM101:*km*. Thus, although electroporation was fivefold more efficient than natural broth transformation in producing control mutants, mutagenesis of the β-subunit gene was still unsuccessful.

To determine whether uptake and integration of the cloned β-subunit gene containing the *km* cassette had occurred during the transformation experiments, chromosomal DNA was isolated from *H. pylori* cells after overnight incubation with pCM101:*km* and used as the template DNA for PCR amplifications (Fig. 4). We performed PCR using primers unique to pCM101:*km* and unique to the chromosomal DNA; by this approach, a product could be amplified only if there had been integration of the *km* cassette into the chromosome of strain 60190. Primers complementary to the upstream (γ-subunit) and downstream (ε-subunit) F_1F_0-ATPase gene sequences (relative to the β-subunit gene) were paired with forward and reverse *km* cassette primers, respectively, to yield 1.0- and 1.5-kb bands of the expected sizes for the transformed 60190 but not 60190 alone (Fig. 4B). These data indicate that integration of pCM101:*km* does occur within the *H. pylori* chromosome, but that the resultant mutants are nonviable.

DISCUSSION

Although *H. pylori* has the ability to persist in the gastric mucosa, the mechanisms permitting the organism to respond to pH stress are not well understood. To begin to determine

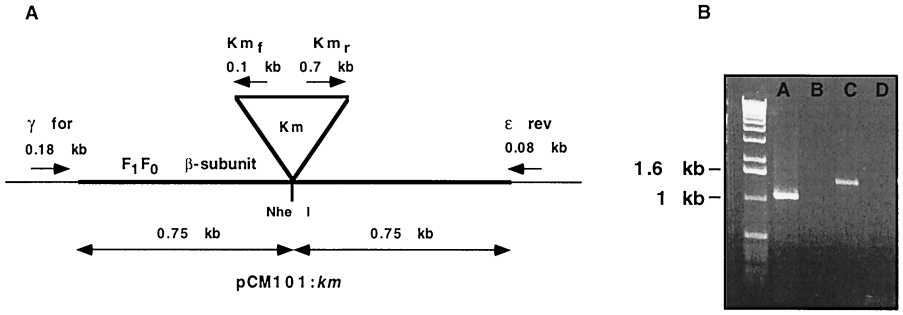

FIG. 4. (A) Construction of pCM101:*km* and location of the PCR primers used to confirm integration of the mutated F_1F_0 β-subunit gene into *H. pylori* chromosomal DNA by natural transformation; (B) PCR of *H. pylori* 60190 chromosomal DNA extracted after integration of pCM101:*km* (lanes A and C). Chromosomal *H. pylori* 60190 DNA was used as a negative control (lanes B and D). Primer pairs used were γ for-Km_f (lanes A and B) and ε rev-Km_r (lanes C and D), yielding bands of 1.0 and 1.5 kb, respectively. These data confirm integration of the cloned *H. pylori* β-subunit containing the *km* cassette into the chromosome of 60190.

mechanisms of pH homeostasis in *H. pylori*, we first sought to learn whether *H. pylori* possesses an F_1F_0-ATPase, an enzyme complex that has been demonstrated to be important for pH regulation or ATP production in several bacterial species. As expected, we found that *H. pylori* possesses homologs of known F_1F_0-ATPase subunits; that the translated protein sequence of the β subunit has 85% identity with that of *W. succinogenes* is consistent with the known taxonomy of *H. pylori*, which is closely related to *W. succinogenes* on the basis of 16s rRNA sequencing (18). The *E. coli* F_1F_0-ATPase enzyme complex is composed of eight subunits that assemble in various stoichiometries and that are encoded by the *atp* operon. The clustering of *atpG*, *atpD*, and *atpC* in *H. pylori* resembles the organization of the *atp* operon in *E. coli* (*a*, *c*, *b*, δ, α, γ, β, and ε) (15, 16, 41). Thus, genes encoding the F_1F_0-ATPase in *H. pylori* may be encoded by an operon similar to the *atp* operon described for *E. coli* and other bacteria.

Despite the similarities with the *E. coli* enzyme, several observations in this study suggest that the F_1F_0-type ATPase may serve a modified function in *H. pylori*. First, that inhibition of *H. pylori* F_1F_0-type ATPase by DCCD substantially diminished survival at pH values of ≥6, whereas survival of *E. coli* under similar conditions was unaffected, suggests that the *H. pylori* F_1F_0-ATPase may play an important role in the physiology of the organism under conditions near pH neutrality. This hypothesis also is supported by the failure to obtain viable cells in which the *H. pylori* F_1F_0-type ATPase β subunit was mutated. In contrast, mutants of the β subunit (and other subunits) have been successfully constructed in *E. coli* (14, 15, 41).

To optimize the conditions for transformation and mutagenesis of *atpD* of *H. pylori*, homologous donor DNA was used for several *H. pylori* strains that previously have been shown to be consistently competent in our laboratory for the uptake of other insertionally mutagenized *H. pylori* genes. Since inhibition of F_1F_0-type ATPase by 10 μM DCCD was lethal to log-phase *H. pylori* at neutral pH, we also conducted transformations at pH 5 and 6, conditions which might be better able to support growth of the mutant *H. pylori*. No mutants were isolated at pH 6 for any strain, despite successful mutagenesis with a comparable-size insert (*vacA*) containing the same *km* cassette. This result is consistent with the complete loss of viability of *H. pylori* at pH values of ≥6 in the presence of DCCD (100 μM) and suggests that the F_1F_0-ATPase is important for survival at pH values of ≥6. Broth transformations performed at pH 5 failed to yield any mutants, even with the control plasmid, suggesting that this pH condition is less permissive for transformation of *H. pylori*. Since the transformation strategy used in this study is similar to that which has been used successfully in our laboratory for mutagenesis of other *H. pylori* genes, including *cagA*, *vacA*, and *recA* (45, 46), our results suggest that mutagenesis involving the F_1F_0-ATPase is lethal under the conditions tested. An alternative hypothesis is that the failure to obtain mutants is due to the lack of DNA uptake sequences in the subcloned β subunit. However, mutagenesis by electroporation should not require the presence of uptake sequences, and this technique failed to generate F_1F_0-ATPase *H. pylori* mutants, despite a fivefold increase in transformation efficiency for the control plasmid. Additionally, PCR amplification of chromosomal *H. pylori* DNA isolated after incubation with pCM101:*km* indicated that uptake and chromosomal integration of the cloned *H. pylori* β subunit had indeed occurred; that no viable mutants were recovered after integration of the mutagenized β subunit strongly suggests that this is a lethal mutation in *H. pylori*. Using the same natural transformation strategy, Ge and Taylor have recently identified a lethal mutation in the *H. pylori ftsH* (filamentation temperature-sensitive) gene, which encodes a protein belonging to a family of putative ATPases associated with diverse cellular activities (17). Thus, *H. pylori* is likely to possess many genes that are essential for survival and growth.

H. pylori is able to grow *in vitro* between pH 4.5 and 8 (19, 21) and to survive between pH 4 and 8 in the absence of urea, but survival at pH less than 4 requires the presence of urease activity (11, 29, 31, 34, 38). In assays using the pH-sensitive dye probe BCECF, *H. pylori* has been determined to have an intracellular pH of 8.4 in the presence of an external pH of 7, but the actual intracellular pH has not been measured at more acidic external pH values. These data suggest that *H. pylori* may be a neutrophilic organism which is acid tolerant due to its urease production (32). In other neutrophilic enteric bacteria, the F_1F_0-ATPase contributes to the maintenance of intracellular pH. For example, F_1F_0-ATPase activity is required for full expression of the acid tolerance response in *S. typhimurium*, and it functions to maintain intracellular pH in a nonlethal range (12, 13). Similarly, in *Enterococcus faecalis*, the F_1F_0-ATPase is important in acid tolerance, and its biosynthesis and activity are increased in response to acid pH (24, 42). *E. faecalis* is a facultative anaerobe that hydrolyzes ATP to generate a proton motive force (PMF) across its cytoplasmic membrane, thereby regulating intracellular pH (42, 44). Thus, F_1F_0-ATPase may operate in either direction (in different bacteria) to serve the same function of pH regulation. The DCCD and mutagenesis studies presented here indicate that the F_1F_0-ATPase does not play a critical role in acid survival of *H. pylori* but suggest that activity of this enzyme is required for survival of the organism when the external pH is near neutral.

Meyer-Rosberg and colleagues have investigated the effect of external pH on the PMF of *H. pylori* (32). In these experiments, *H. pylori* was able to maintain a constant PMF over the same range of pH values which permit bacterial survival (pH 4 to 8) by increasing its transmembrane potential difference. In many aerobic bacteria such as *E. coli*, the PMF provides a flux of protons across the plasma membrane which is coupled to the synthesis of ATP catalyzed by F_1F_0-ATPase. Thus, one hypothesis is that inhibition of *H. pylori* F_1F_0-ATPase by DCCD at pH 7 or 6 may dissipate the PMF, leading to cellular death, possibly due to ATP depletion; in contrast, a proton gradient across the membrane could possibly compensate for the inhibition of ATPase at more acidic pH. However, in the studies by Meyer-Rosberg et al., *E. coli* also was found to have a PMF similar to that of *H. pylori* at pH 7, but the present study shows that inhibition of *E. coli* F_1F_0-ATPase by DCCD did not cause loss of cellular viability at this (or any) pH tested. Thus, if inhibition of F_1F_0-ATPase in *H. pylori* leads to loss of the PMF and death of the organism, it appears to be a response different from that seen in *E. coli*. Therefore, in addition to potentially altering the organism's capacity to maintain pH homeostasis or generate a PMF, we speculate that F_1F_0-ATPase inhibition by DCCD may lead to ATP depletion and associated inhibition of other metabolic pathways that are important for *H. pylori* survival.

In conclusion, the sequence data in this study suggest that *H. pylori* possesses an F_1F_0-ATPase operon similar to those found in other bacteria. However, in contrast with *E. coli* and other enteric bacteria, the F_1F_0 enzyme appears to play an essential role in survival of *H. pylori* at pH values near neutrality. Identification of potential lethal targets such as these in *H. pylori* may lead to the development of unique treatment strategies.

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