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Characterization of *Helicobacter pylori dapE* and Construction of a Conditionally Lethal *dapE* Mutant

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***Helicobacter pylori* colonizes the human gastric mucosa and causes gastritis, ulceration, or gastric cancer. A previously uncharacterized region of the *H. pylori* genome was identified and sequenced. This region includes a putative operon containing three open reading frames termed *gidA* (1,866 bp), *dapE* (1,167 bp), and *orf2* (753 bp); the *gidA* and *dapE* products are highly homologous to other bacterial proteins. In *E. coli*, *dapE* encodes *N*-succinyl-L-diaminopimelic acid desuccinylase, which catalyzes the hydrolysis of *N*-succinyl-L-diaminopimelic acid to L-diaminopimelic acid (L-DAP) and succinate. When wild-type *H. pylori* strains were transformed to select for *dapE* mutagenesis, mutants were present when plates were supplemented with DAP but not with lysine; *orf2* mutants were selected without DAP supplementation. Consistent with the finding that GidA is essential in *Escherichia coli*, we were unable to obtain a *gidA* mutant in *H. pylori* despite evidence that insertional mutagenesis had occurred. The positions of *gidA*, *dapE*, and *orf2* suggest that they form an operon, which was supported by slot blot RNA hybridization and reverse transcriptase PCR studies. The data imply that the *H. pylori dapE* mutant may be useful as a conditionally lethal vaccine.**

Helicobacter pylori is a gram-negative enteric bacterium that colonizes the human gastric mucosa and is implicated in peptic ulcer disease (6, 11, 15) and malignant neoplasms of the stomach (5, 25, 29, 37). The chromosomal gene, *cagA*, which is commonly present in *H. pylori* strains, is strongly associated with the development of these diseases (1, 5, 11, 12, 39). In an effort to locate adjacent genes that also might be involved in *H. pylori* virulence, we used *cagA* to probe a plasmid library of *Eco*RI-digested *H. pylori* chromosomal DNA (40). One of the clones identified (pAK2) ultimately showed no association with *cagA*, but it contained an uncharacterized region of the *H. pylori* genome, which we now have further analyzed.

The deduced amino acid sequence of the deduced product of one open reading frame (ORF) in this clone was found to be highly homologous to *N*-succinyl-L-diaminopimelic acid desuccinylase (SDAP-deacylase) (23). In *Escherichia coli*, SDAP-deacylase, encoded by *dapE* (7, 45), an enzyme of the diaminopimelic acid-lysine pathway, catalyzes the hydrolysis of *N*-succinyl-L-diaminopimelic acid to L-diaminopimelic acid (L-DAP) and succinate. L-DAP is a precursor of *meso*-DAP, which is a structural component of the cell wall peptidoglycan of most bacteria (17). Both forms of DAP are precursors of lysine in many bacterial species (30, 35).

We now report the molecular cloning of the ORF encoding the *H. pylori* SDAP-deacylase (which we now call *dapE*) and its flanking genes, and we show that loss of *dapE* function is lethal for cells grown without DAP supplementation.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *H. pylori* 60190 was used for the molecular cloning studies, and 21 well-characterized clinical *H. pylori* strains from the Vanderbilt *Helicobacter*/*Campylobacter* culture collection were used to determine the conservation of the cloned genes. Stock cultures were maintained at −70°C in brucella broth (BBL Microbiology Systems, Cockysville, Md.) supplemented with 15% glycerol. *E. coli* DH5α, XL-1blue, and Dam$^-$ strains were used for transformation, and pBluescript (Stratagene, La Jolla, Calif.) was used as a cloning vector. *E. coli* strains were routinely cultured in Luria-Bertani medium with shaking at 37°C, and the clinical *H. pylori* isolates were cultured on Trypticase soy agar plates containing 5% sheep blood in a microaerobic atmosphere, as described previously (13). For transformation of *H. pylori* (14), strains were grown at 37°C in a microaerobic atmosphere on brucella agar plates containing 5% fetal calf serum (FCS) and 30 μg of kanamycin per ml and supplements of 0 to 2 mM DAP (a racemic mixture of all three DAP isomers; Sigma Chemical Co., St. Louis, Mo.) or 1 mM lysine (Sigma).

Genetic techniques and nucleotide sequence analysis. Chromosomal DNA was prepared as described previously (40). All other standard molecular genetic techniques including Southern and colony hybridizations were performed as described previously (33, 39). For molecular cloning, positive plaques were purified from a bank of approximately 5-kb random chromosomal fragments of *H. pylori* 60190 by using lambdaZapII, and recombinant DNA was prepared as described previously (40). Restriction enzyme cleavage maps were generated, and a 5-kb fragment was subcloned into pBluescript to create pAK2 (Fig. 1). Another 5-kb fragment carrying a portion of the *H. pylori* genome overlapping only the *orf2* region of pAK2 was subcloned into pBluescript to create pAK1 (Fig. 1). The nucleotide sequence was determined unambiguously on both strands, using double-stranded DNA templates and an automated DNA sequencer (model ABI377; Perkin-Elmer, Foster City, Calif.) with a ThermoSequenase dye primer reaction kit (Amersham, Arlington Heights, Ill.). Oligonucleotide primers were synthesized at the Vanderbilt Cancer Center DNA core facility with a model ABI392 DNA synthesizer-sequencer (Perkin-Elmer). Nucleotide sequences were compiled and analyzed by using programs in the Genetics Computer Group package (16). Amplifications were conducted in a Perkin-Elmer Thermal Cycler. PCR conditions used in this study were 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, with a terminal extension at 72°C for 10 min; the primers used in this study are listed in Table 1.

Construction of recombinant plasmids with insertion of *km* cassettes into targeted genes. A *Campylobacter coli* kanamycin resistance (*km*) gene (22) was ligated into the unique *Bcl*I site of pAK2 within the *gidA* ORF to create pAK2:*gidA*:*km* (pME36) (Fig. 1). An *E. coli km* cassette from pUC4K (38) was inserted into the unique *Nde*I site within the *dapE* ORF to create pAK2:*dapE*:*km* (pMAK36) (Fig. 1). *orf2* contained no unique sites for *km* insertion, but three *Hin*dIII sites were present within 107 bp. Therefore, to create *orf2*:*km*, we PCR amplified the *orf2* ORF from pAK1 and subcloned the amplified fragment into pT7Blue (Novagen, Madison, Wis.) to create pAK7. The 430-bp insert was subcloned into pCR-Script Cam SK (+) (Stratagene), a pBluescript derivative encoding chloramphenicol resistance, to create pAK8. After *Hin*dIII digestion of pAK8, the *km* cassette from pUC4K was inserted into *orf2* to create pAK8:*orf2*:*km* (pAKQ) (Fig. 1).

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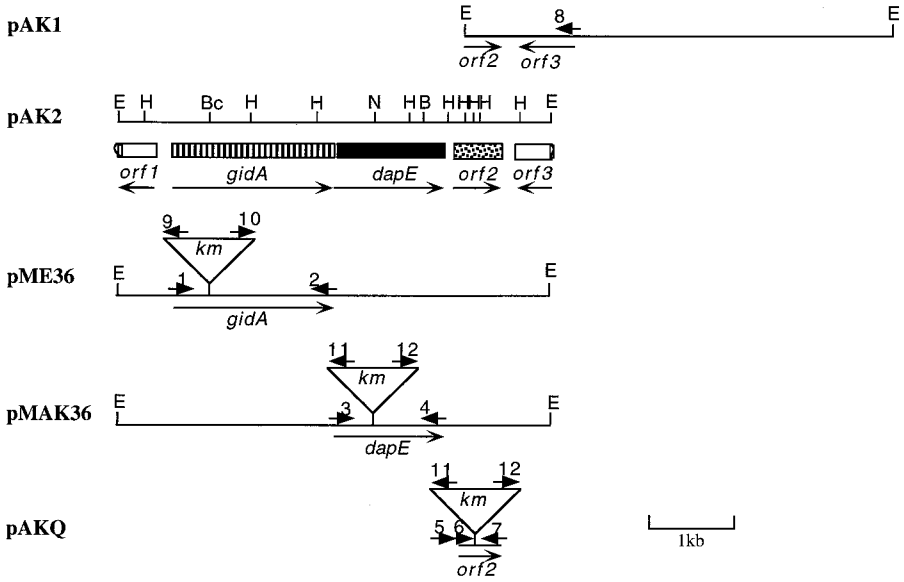

FIG. 1. Physical maps of recombinant pBluescript plasmids containing *gidA*, *dapE*, and *orf2*. pAK1 contains the 3′ region of *orf2* plus approximately 4 kb downstream. pAK2 contains a 5-kb *Eco*RI fragment that includes *gidA*, *dapE*, and *orf2*. Boxes and arrows beneath the plasmids represent the location of the genes and the presumed direction of translation, and *km* represents a cassette encoding kanamycin resistance. Arrowheads with numbers represent sites of oligonucleotide primers used in PCR. Restriction endonuclease cleavage sites: Ba (*Bam*HI), Bc (*Bcl*I), N (*Nde*I), E (*Eco*RI), and H (*Hin*dIII).

Construction of *H. pylori dapE* and *orf2* mutants. The constructs pAK2:*gidA*:*km* (pME36), pAK2:*dapE*:*km* (pMAK36), and pAK8:*orf2*:*km* (pAKQ), all of which are unable to replicate in *H. pylori*, were introduced into *H. pylori* 60190 by natural transformation; pCTB8:*km* containing *vacA*:*km* was used as a positive control (14). The transformants were selected on brucella broth agar plates containing 5% FCS and 30 μg of kanamycin per ml. In certain experiments, plates were supplemented with 1 mM DAP to determine the conditions necessary for *dapE* mutant viability. To determine the minimum concentration needed for growth of the *dapE* mutant, strains were grown on medium supplemented with 0 to 2.0 mM DAP or 1.0 mM lysine. To provide genetic evidence in the transformed strain of *dapE* disruption by the *km* insertion, DNA isolated from both the *H. pylori* mutant strain 60190 pAK2:*dapE*:*km* and wild-type strain 60190 was digested with *Bam*HI and hybridized to *dapE* and *km* probes. The authenticity of the mutant strain also was verified by PCR, using primers based on *dapE* and *km* (Fig. 1 and Table 1). The authenticity of the *orf2* mutants also was verified by Southern hybridization and PCR using parallel methods.

Evidence of homologous recombination between pAK2:*gidA*:*km* and *H. pylori* strain 60190 chromosomal DNA. We were not able to obtain any viable *gidA* mutants in this study, even with selection on medium supplemented with DAP or lysine. To provide genetic evidence that double-crossover events had occurred during the preselective growth phase, allowing for the insertion of *km* in the *H. pylori* chromosome within *gidA*, PCR was performed with a forward primer specific for *km* (primer 10 [Fig. 1 and Table 1]) and a reverse primer (primer 8 [Fig. 1 and Table 1]) specific for a region of the *H. pylori* chromosome present in pAK1 that is beyond the fragment cloned in pAK2. We examined DNA (0.1 μg) isolated from wild-type strain 60190 after overnight incubation with pAK2:*gidA*:*km* (0.1 μg). As negative controls, we used DNA from wild-type strain 60190 and a mixture of DNA from wild-type strain 60190 and pAK2:*gidA*:*km*. As a positive control, the forward *km* primer (primer 10 [Fig. 1 and Table 1]) and a confirmed *vacA* reverse primer (primer 17 [Table 1]) (14) were tested on DNA isolated from wild-type strain 60190 after overnight incubation with pCTB8:*vacA*:*km*.

RNA isolation, RT-PCR, and slot blot analysis. To determine whether *gidA*, *dapE*, and *orf2* are cotranscribed, wild-type and mutant *H. pylori* strains were cultured for 24 h on brucella agar plates containing 5% FCS supplemented with 1 mM DAP, cells were harvested, and RNA was recovered for reverse transcriptase PCR (RT-PCR) by two rounds of hot-phenol extraction, as described previously (40). cDNA was synthesized from 1 μg of DNase-treated total RNA by priming with 1 μg of random hexamer (Pharmacia, Inc., Piscataway, N.J.), 1 mM each deoxynucleoside triphosphate, 20 U of RNase inhibitor, and avian myelo-

TABLE 1. PCR primers used in this study

Designation	Gene	Position[a]	Strand	Length (bp)	Sequence (5′-3′)	Reference
1	*gidA*	569–586	+	18	CAGGAAAAAGAGTGGTAA	This work
2	*gidA*	2428–2445	−	18	TTAAGAGTTTTTTCGCAA	This work
3	*dapE*	2445–2462	+	18	AAGGATATTTAATGAACG	This work
4	*dapE*	3613–3633	−	21	GTTTATTTATTTTATGCCTCA	This work
5	*orf2*	3801–3819	+	19	TAATTTAGGCATAGAGAGC	This work
6	*orf2*	4024–4044	+	20	TATAACGGACAAGGCGTATCT	This work
7	*orf2*	4429–4450	−	24	GTTCTATTTTCAATTCCTTGAGAG	This work
8	*orf3*	5086–5103	−	18	GCGTGAATGAATACGATA	This work
9	*km*	689–712	−	24	CTCCCACCAGCTTATATACCTTAG	22
10	*km*	1336–1356	+	21	CTGGGGATCAAGCCTGATTGG	22
11	*km*	572–591	−	20	GACCGTTCCGTGGCAAAGCA	38
12	*km*	1601–1622	+	22	CTTGTGCAATGTAACATCAGAG	38
13	*gidA*	2300–2317	+	18	GCATTCCAGGCTTAAGCT	This work
14	*dapE*	2631–2650	−	20	TGCATGTTCTTTTTCTGCAT	This work
15	*dapE*	3506–3523	+	18	GAGTTTGGCGTTATTAAT	This work
16	*orf2*	3850–3866	−	17	GCTTTTTCAAAATGCGT	This work
17	*vacA*	4116–4134	−	16	AAGCTTGATCACTCC	14

[a] Position in sequence shown in Fig. 2 except for *km* and *vacA* primers. Positions refer to those in the cited publications.

blastosis virus reverse transcriptase (Promega, Madison, Wis.) in a final volume of 20 μl at 42°C for 15 min. PCRs were performed as described above. For slot blot analysis, DNase-treated RNA samples (12 μg) were transferred to nylon membranes. Hybridization used probes specific for *gidA* (1.9-kb PCR-amplified *gidA*-specific fragment), *dapE* (1.1-kb PCR-amplified *dapE*-specific fragment), *orf2* (0.7-kb PCR-amplified *orf2*-specific fragment), or, as a positive control, *cagA* (0.5-kb PCR-amplified *cagA*-specific fragment). The amount of radiolabel (50,000 cpm) was standardized for each probe, and experiments were performed as previously described (31).

Nucleotide sequence accession number. The complete sequence shown in Fig. 2 has been deposited in GenBank under accession no. AF008565.

RESULTS

Isolation of *H. pylori dapE*. A 5-kb *Eco*RI genomic fragment from *H. pylori* 60190 was cloned into pBluescript to create pAK2 (Fig. 1), and the nucleotide sequence of this fragment was determined (Fig. 2). Analysis of translation of the 5,050-bp nucleotide sequence in all possible reading frames revealed five complete or partial ORFs. The three complete ORFs, consisting of 1,866, 1,167, and 753 nucleotides, were oriented in the same direction and opposite the partial ORFs present at the ends of the fragment (Fig. 1 and 2). The first complete ORF begins with GTG as the initiation codon and encodes a 621-amino-acid polypeptide, yielding a predicted product with a molecular mass of 69,665 Da. The second ORF begins with an ATG codon 10 bp after the termination of the first ORF and encodes a 388-amino-acid polypeptide, yielding a predicted 42,822-Da product. The third ORF begins with ATG 80 bp after the termination of the second ORF and encodes a 250-amino-acid polypeptide with a predicted molecular mass of 27,585 Da. Potential ribosome binding sites begin 6 or 7 bp upstream of each ORF. Upstream of the translational start of the first ORF is the sequence TATTTT, which resembles the consensus σ^{70} −10 sequence (32) and is 19 bp downstream of the sequence TTGGCA that shares five of six bases with the corresponding −35 consensus sequence (32). Nucleotides 4456 to 4654 following the third ORF exhibit the sequence of a putative three-hairpin stem-loop structure ($\Delta G = -40.2$) that could permit a strong mRNA transcriptional terminator. The single putative promoter and transcription terminator and the close location and orientation of the ORFs suggest that they may represent an operon.

Analysis of the deduced products of the ORFs. We compared the translated amino acid sequences for genes in pAK2 with databases, using the FASTA, FASTDB, and BLAST network services of the National Center for Biotechnology Information. The deduced product from the first complete ORF showed significant homology throughout the translated amino acid sequence (48.3% identity and 66.5% similarity) with the glucose-inhibited division protein, encoded by *gidA* in *E. coli* (9, 36, 43), *Haemophilus influenzae* (18). (47.1% identity and 67.5% similarity) (Fig. 2A), *Pseudomonas putida* (27) (47.9% identity and 68.9% similarity), and *Bacillus subtilis* (26, 27) (46.1% identity and 64.4% similarity). The putative product of the second ORF showed significant homology with SDAP-deacylase (encoded by *dapE*) of *E. coli* (37.9% identity and 61.0% similarity) (Fig. 2B) (7, 45) and *H. influenzae* (39.1% identity and 58.8% similarity) (Fig. 2B) (18). There was no substantial overall homology between the products of the other complete or the two incomplete ORFs and other known sequences. These genes are tentatively identified as *orf1*, *orf2*, and *orf3*, as shown in Fig. 1.

Conservation of *gidA*, *dapE*, and *orf2* among *H. pylori* strains. To determine whether other *H. pylori* strains possess sequences homologous to *gidA*, *dapE*, or *orf2*, we studied 21 strains (10 *cagA*$^+$ and 11 *cagA* strains) by colony hybridization, using PCR-amplified *gidA*-, *dapE*-, and *orf2*-specific fragments. A positive signal was obtained from each of these strains (data not shown), indicating that these genes are conserved in *H. pylori* and are not related to *cagA* status. To determine whether the position and orientation of *gidA* and *dapE* relative to one another were conserved, PCR was performed using primers specific for *gidA* (primer 13 [Table 1]) and for *dapE* (primer 14 [Table 1]). A 0.35-kb band was amplified from the DNA from each of the 21 strains, the same as in 60190, indicating the conservation of position and orientation of these two genes.

Characterization of a *dapE* mutant. To create a *dapE* mutant, we transformed *H. pylori* 60190 with pMAK36 (*dapE*:*km*) and plated transformants on kanamycin-containing medium including 1 mM DAP. Southern and PCR analysis of the kanamycin-resistant transformants indicated that the *km* cassette was stably incorporated into the single chromosomal *dapE* gene, creating a *dapE* mutant (data not shown). However, in repeated experiments, transformation of *H. pylori* with pMAK36 (*dapE*:*km*) on plates lacking DAP did not yield any transformants (Table 2). Similarly, the *dapE* mutants obtained on DAP-containing plates were unable to grow when replated on Trypticase soy agar or brucella agar with 5% FCS without the addition of DAP. Transformation of *H. pylori* with pCTB8: *vacA*:*km* yielded a similar number of transformants whether or not DAP was present in the selective media. The minimum DAP concentration required for survival of the *dapE* mutant was found to be 0.2 mM (Table 3). The *dapE* mutant was unable to grow on media supplemented with lysine only (Table 2), emphasizing the specific DAP requirement of *H. pylori* for growth and/or survival. To determine whether suppression of the *dapE* null mutation is possible, we plated approximately 10^7 *dapE* mutant cells on brucella medium without DAP; the lack of any growth indicates that the *dapE* mutant cannot be overcome via another pathway.

Characterization of an *H. pylori* mutant lacking *orf2*. The *orf2* ORF begins only 80 bp downstream from *dapE* and lacks its own consensus promoter, suggesting that these genes could be cotranscribed and their products could be functionally related. To test this hypothesis, we disrupted *orf2* by allelic replacement and confirmed the insertion into *H. pylori* mutant 60190 *orf2*:*km* by Southern hybridization and PCR (data not shown). However, the *orf2* mutant was found to grow well with or without exogenous DAP in the growth medium (Table 2), demonstrating that *orf2* is not required in the metabolic pathway leading to DAP formation.

Evidence that mutation of *gidA* is lethal in *H. pylori*. The *dapE* ORF is separated by only 10 bp from *gidA*, suggesting cotranscription and a functional relationship between these two genes. To determine whether the *gidA* product in *H. pylori* is associated with *dapE* synthesis, we sought to insertionally inactivate *gidA*. However, efforts to inactivate *gidA* by transforming *H. pylori* 60190 with pAK2:*gidA*:*km* were unsuccessful. No transformants were observed even on media supplemented with DAP (or lysine), while parallel transformations that led to the inactivation of *dapE* or *vacA* yielded more than 100 transformants for each. Since these data suggested that interruption of *gidA* was lethal for *H. pylori*, PCR was performed to determine whether insertion of the *km* cassette within *gidA* had occurred to transiently create 60190 *gidA*:*km*, but this organism was not viable. As a positive control, wild-type strain 60190 was incubated in parallel with pCTB8:*vacA*:*km* to create an insertion in *vacA*. When the forward *km* primer (primer 10 [Fig. 1 and Table 1]) and reverse *vacA* primer (primer 17 [Table 1]) were used, a 3.1-kb band was amplified, as expected (Fig. 3). Using a forward *km* primer (primer 10) and a reverse primer (primer 8 [Fig. 1 and Table 1]) that is not present in pAK2 (Fig. 1), a 4.2-kb band was amplified in DNA isolated from

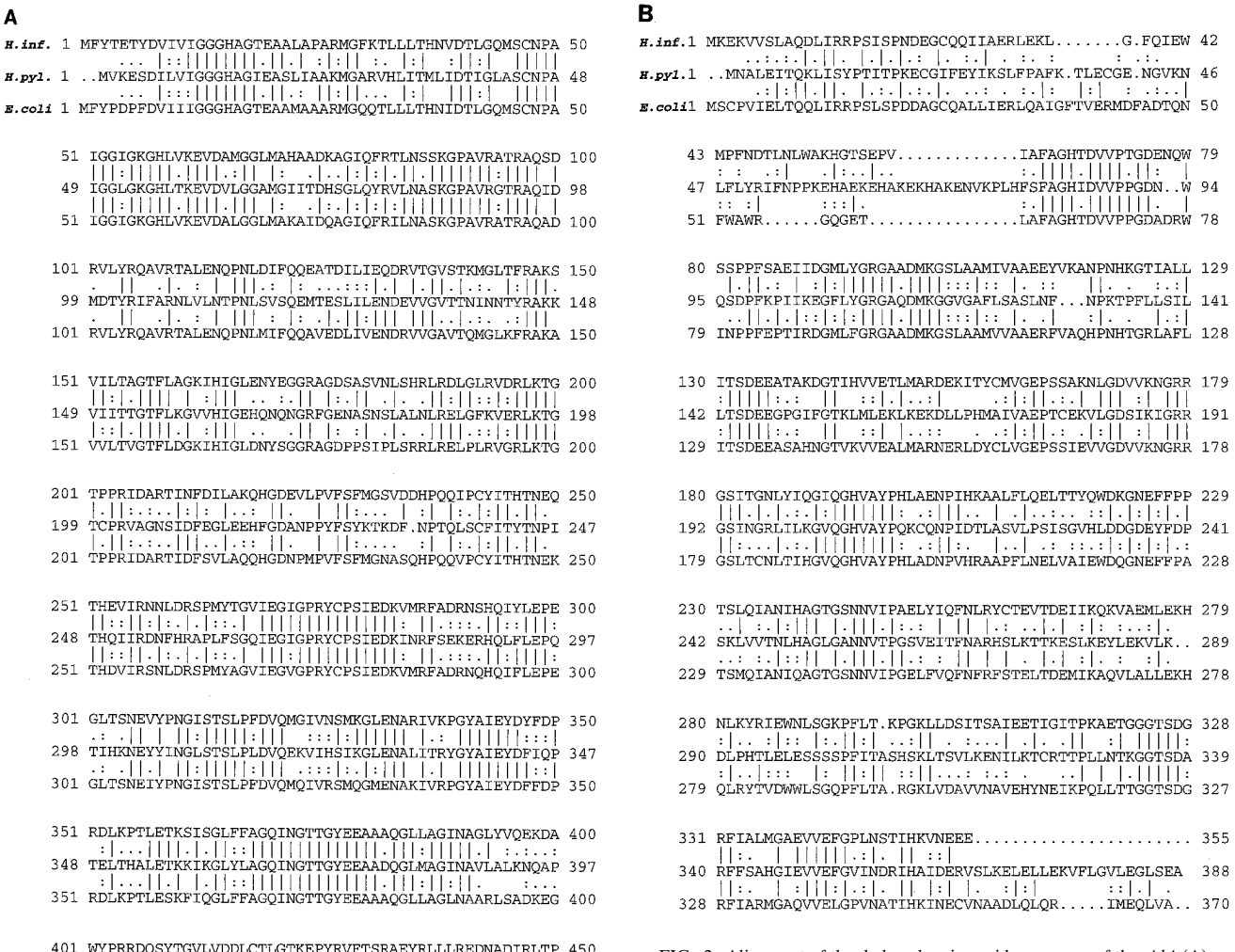

FIG. 2. Alignment of the deduced amino acid sequences of the *gidA* (A) and *dapE* (B) products in *E. coli* and *H. influenzae* (*H.inf.*) and the respective *H. pylori* (*H.pyl.*) homologs. To optimize the alignments, gaps were introduced when necessary. Vertical lines between residues indicate identity, whereas two dots represent a conservative substitution.

wild-type strain 60190 that had been incubated overnight with pAK2:*gidA*:*km* (Fig. 3). No band was present in DNA from wild-type strain 60190 alone or if 60190 DNA was mixed with pAK2:*gidA*:*km* in the absence of *H. pylori* cells (Fig. 3). These results indicate that homologous recombination had occurred between the chromosomal DNA of the wild-type strain 60190 and pAK2:*gidA*:*km*, leading to *km* insertion within *gidA*, and provided further evidence that this transformation event was lethal to *H. pylori.*

We also attempted to generate *gidA* knockout mutants by using pAK2:*gidA*:*km* in four other *cagA*$^+$ (J99, J116, J178, and 3401) and four *cagA* (J154, J262, 86-313, and Tx30a) strains. No transformants were observed, even with plating to medium containing 1 mM DAP or lysine, while parallel transformations with pAK2:*dapE*:*km*, leading to *dapE* knockout, led to more than 50 transformants in each case. Thus, *gidA* appears essentially in *H. pylori.*

RT-PCR and slot blot analysis. To ascertain whether *gidA*, *dapE*, and *orf2* are cotranscribed, RNA was extracted for analysis from wild-type *H. pylori* 60190 and its *dapE* and *orf2* mutants. We used RT-PCR of cDNA template with a pair of primers bridging the *gidA* and *dapE* ORFs (primers 13 and 14 [Table 1 and Fig. 4]). A 0.35-kb product was detected for each strain, as expected. In RT-PCR using primers bridging the *dapE-orf2* ORFs (primers 15 and 16), no product was detected

TABLE 2. Growth of wild-type and mutant *H. pylori* strains on brucella agar in the presence or absence of DAP

Strain	Growth in indicated medium[a]		
	No supplement	Supplemented with:	
		1 mM DAP	1 mM lysine
60190	−	−	−
60190 *vacA*:*km*	+	+	+
60190 *dapE*:*km*	−	+	−
60190 *orf2*:*km*	+	+	+

[a] Brucella broth with 1.5% agar supplemented with 5% FCS, 30 μg of kanamycin per ml, and DAP or lysine as indicated.

in the *dapE* mutant, as expected, but both the wild-type strain and the *orf2* mutant showed a product of the expected size (0.4 kb) (Fig. 4). Negative-control PCR using RNA as the template showed no products. To provide further evidence that *orf2* is cotranscribed with *dapE*, we used slot blot RNA analysis. As expected, the positive control *cagA* probe hybridized with equal intensity to the wild-type strain and its *dapE* and *orf2* mutants (Fig. 5). The *gidA* probe hybridized to RNA with similar intensities for the wild-type strain and its *dapE* and *orf2* mutants. The *dapE* probe hybridized equally well to wild-type and *orf2* mutant RNAs and less well to RNA of the *dapE* mutant. The *orf2* probe hybridized well to RNA from the wild-type strain but only weakly to RNA from the *dapE* and *orf2* mutants (Fig. 5). The results of both sets of experiments indicate that *orf2* can be cotranscribed with *dapE.*

DISCUSSION

In this study, we determined the nucleotide sequence of a previously uncharacterized 5-kb region of the *H. pylori* chromosome containing *dapE* and surrounding genes. The pathway for DAP biosynthesis in *H. pylori* has not been determined, nor are the other relevant genes known. We found that disruption of *dapE* in *H. pylori* results in cell death unless exogenous DAP is provided, presumably due to lysis based on inability to synthesize the cell wall peptidoglycan (17). This observation, and the homology of *H. pylori dapE* to genes in the DAP-succinylase pathways of other bacteria (7, 45), suggests that *H. pylori* may use the succinylase pathway to synthesize DAP. Methods

TABLE 3. Minimum concentration of DAP required for growth of the *dapE H. pylori* mutant

Strain	Inhibition at DAP concn[a] of:						
	2 mM	1.5 mM	1 mM	0.5 mM	0.2 mM	0.1 mM	0 mM
60190	−	−	−	−	−	−	−
60190 *dapE*:*km* mutant strains							
1	+	+	+	+	+	−	−
2	+	+	+	+	+	+	−
3	+	+	+	+	+	+	−
60190 *vacA*:*km* mutant strains							
1	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+

[a] DAP concentration in brucella broth with 1.5% agar supplemented with 5% FCS and 30 μg of kanamycin per ml.

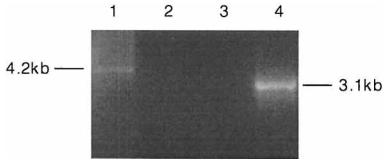

FIG. 3. Agarose gel electrophoresis of PCR-amplified products from DNA (0.1 μg) isolated from wild-type strain 60190 after overnight incubation and transformation with or without specified plasmid (0.1 μg of DNA). All PCRs used a forward primer specific for *km* (primer 10 [Fig. 1 and Table 1]). The reverse primer was either specific for a region of the *H. pylori* chromosome not included in the fragment cloned in pAK2 (primer 8 [Fig. 1 and Table 1]) or specific for *vacA* (primer 17 [Table 1]) as a control. Lane 1, template is DNA from strain 60190 after overnight incubation with pAK2:*gidA*:*km*, plus primers 10 and 8; lane 2, template is DNA isolated from strain 60190, plus primers 10 and 8; lane 3, template is a mixture of DNA from strain 60190 and pAK2:*gidA*:*km*, plus primers 10 and 8; lane 4, template is DNA from strain 60190 after overnight incubation with pCTB8:*vacA*:*km*, plus primers 10 and 17.

that other bacteria use for DAP biosynthesis include the succinylase, dehydrogenase, and acetylase pathways (10). *E. coli* is thought to synthesize DAP solely via the succinylase pathway (30), whereas the dehydrogenase and acetylase pathways are found in *Bacillus* (2, 44) and *Corynebacterium* (34) species. Among *Bacillus* species, some use just one pathway for DAP biosynthesis whereas others utilize both (2, 44). The maintenance of more than one pathway for DAP synthesis may indicate the importance of DAP for bacterial survival. The results in this study suggest that the ability of *H. pylori* to synthesize DAP is based only on the succinylase pathway, or that its synthesis via the dehydrogenase and/or acetylase pathways is too low to allow for survival.

The *gidA*, *dapE*, and *orf2* ORFs are closely spaced and oriented oppositely to the flanking genes (Fig. 1), suggesting that they form an operon. A sequence bearing strong homology to the σ^{70} promoter is present 5′ to *gidA*, but no promoter-like elements were observed upstream of *dapE* and *orf2*. The presence of a strong putative transcriptional terminator downstream of *orf2* also is consistent with the notion that these three genes form an operon, and RT-PCR and slot blot data indicate that *dapE* and *orf2* may be cotranscribed. The presence of another putative transcriptional terminator, an 80-nucleotide palindromic sequence ($\Delta G = -2.9$), beginning at nucleotide 3623 to 3702 in the region between *dapE* and *orf2*, may, under

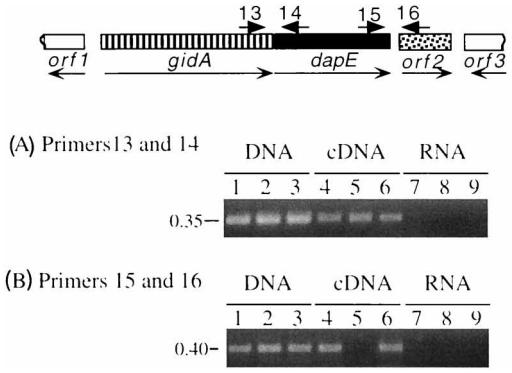

FIG. 4. Agarose gel electrophoresis of specific RT-PCR-amplified products from *H. pylori* wild-type and mutant strains, using primers within *gidA*, *dapE*, or *orf2*. Lanes 1, 4, and 7, wild-type strain 60190; lanes 2, 5, and 8, *dapE* mutant strain (60190E$^-$); lanes 3, 6, and 9, *orf2* mutant strain (60190-2$^-$). (A) PCR using primers 13 and 14 and DNA (lanes 1 to 3), cDNA (lanes 4 to 6), or RNA (lanes 7 to 9) as templates; (B) PCR using primers 15 and 16 and DNA (lanes 1 to 3), cDNA (lanes 4 to 6), or RNA (lanes 7 to 9) as templates.

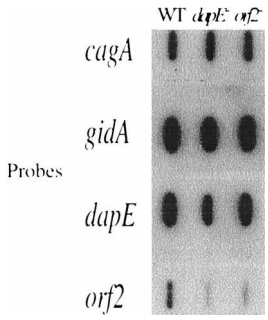

FIG. 5. Slot blot of mRNA transcripts of *gidA*, *dapE*, and *orf2*. RNA (12 μg) from wild-type (WT) *H. pylori* 60190 or from its *dapE* or *orf2* mutant was transferred to a nylon membrane and hybridized with equal amounts (50,000 cpm) of radiolabeled *cagA*, *gidA*, *dapE*, or *orf2* probe.

certain conditions, allow for transcription of *gidA* and *dapE* without *orf2*.

The *dapE* ORF is separated by only 10 bp from *gidA*. In *E. coli*, *gidA* lies near the origin of replication (*oriC*) (28); inactivation of *gidA* by transposon insertion reduces the *E. coli* growth rate by 20% and causes filamentation of cells in media containing glucose (41, 42). The arrangement of *gidA* and *dapE* in the same operon in *H. pylori* suggests that the products of these two genes could be functionally related. However, the inability of DAP or lysine supplementation to permit *gidA* mutants to survive suggests that its critical activity does not involve the DAP/lysine pathway. Complementation of *gidA* mutations in *E. coli* with the cloned *H. pylori gidA*, or vice versa, may help in addressing functional issues.

The presence of *orf2* 80 bp downstream from *dapE*, with no unique promoter sequence, suggests that these two genes may be cotranscribed and that their protein products may be functionally related. RT-PCR and slot blot results support this hypothesis, since *orf2* RNA was not transcribed in the *dapE* mutant. That the *orf2* mutant strain grew normally without exogenous DAP indicates that the *orf2* product is not required for DAP biosynthesis.

Because mammalian cells do not synthesize DAP, DAP biosynthetic enzymes are essential for *H. pylori* survival in the human host (23). The observations made in this study suggest that the enzymes involved in DAP biosynthesis represent targets for the development of novel agents against *H. pylori* (3, 4, 20, 21). DAP biosynthetic genes also may be used to stabilize shuttle plasmids for use in *H. pylori* in the absence of antibiotic markers (24). *H. pylori* strains carrying mutations in DAP biosynthesis genes could be transformed with plasmids carrying the respective complementing gene. Such plasmids may lead to the ability to stably maintain recombinant DNA in humans for the expression of *H. pylori* or heterologous antigens, and they may provide tools in the investigation of *H. pylori* pathogenesis as well as for the development of new anti-*H. pylori* agents.

Our results also suggest that coadministration of *H. pylori dapE* mutant strains with a DAP supplement may serve as an immunization strategy, extending that described for *Salmonella* species (19). After sufficient time for evoking immune responses directed at this *H. pylori* strain and the antigens that it is carrying, cessation of DAP supplementation would lead to its death. Ideally, optimal timing of supplementation could result in the establishment of long-term immunity to either or both *H. pylori* antigens or heterologous antigens delivered by the conditionally lethal form of this superb mucosal colonizer. Construction of other conditionally lethal mutants could extend the range of well-tolerated and widely available supplements.

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