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A Flagellar Sheath Protein of *Helicobacter pylori* Is Identical to HpaA, a Putative *N*-Acetylneuraminyllactose-Binding Hemagglutinin, but Is Not an Adhesin for AGS Cells

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The gene encoding a 29-kDa flagellar sheath protein was cloned and found to be similar to *hpaA*, reported to encode an *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin (D. G. Evans, T. K. Karjalainen, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee, J. Bacteriol. 175:674–683, 1993). The transcriptional start was mapped by primer extension from *Helicobacter pylori* mRNA, indicating an active consensus promoter at a location different from that suggested by Evans et al. Immunogold labelling of the flagellar sheath with a monoclonal antibody to HpaA was demonstrated in four strains, contrary to previous reports of a surface (D. G. Evans, T. K. Karjalainen, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee, J. Bacteriol. 175:674–683, 1993) or a cytoplasmic (P. W. O'Toole, L. Janzon, P. Doig, J. Huang, M. Kostrzynska, and T. J. Trust, J. Bacteriol. 177:6049–6057, 1995) locale. Agglutination of erythrocytes and adherence to AGS cells by a Δ*hpaA* mutant were no different from those of the parent strain, confirming a recent finding of O'Toole et al.

Helicobacter pylori is widely accepted to cause chronic type B gastritis (21) and most peptic ulcers (4, 18) and is associated with the development of gastric cancer (1, 20). Motility is apparently essential for the colonization of the gnotobiotic pig model (7–9), as is urease production (5, 6). Relatively little is known about the flagellar sheath, a bilayered membrane (12) which, morphologically, is a continuation of the cell wall's outer membrane. Its function is not understood, but suggested roles have included the protection of the filament from depolymerization by gastric acid (14) and the promotion of adhesion to the gastric mucosa (17).

Recently, a basic 29-kDa protein has been identified which is localized specifically to the flagellar sheath, as shown by immunogold electron microscopy (17). We now describe the cloning and analysis of the gene which encodes this protein, which we have found shows identity with *hpaA*, the gene encoding a putative receptor-binding subunit of the *N*-acetylneuraminyllactose-binding hemagglutinin first described by Evans et al. (10, 11). Recently, O'Toole et al. (19) have shown that HpaA is a 29-kDa lipoprotein and have highlighted significant sequence discrepancies in the *hpaA* locus between strain 8826, used by Evans et al., and strain CCUG 17874, equivalent to NCTC 11637. In addition, O'Toole et al. demonstrated that the protein was not an adhesin with a surface locale, as first reported, and suggested it was a cytoplasmic protein.

In this study, we corroborate the report by O'Toole et al. (19), detailing major differences between the nucleotide sequence of *hpaA* and that first reported by Evans et al. (11), and extend the sequence to include the promoter and transcriptional terminator regions. Furthermore, we provide immunogold-labelling evidence to confirm that the in situ location of the protein is the flagellar sheath, add to the data which suggests that the protein is lipid modified, and show that the protein is not an adhesin for erythrocytes and AGS cells.

Cloning and expression of the gene encoding a flagellar sheath protein. A representative genomic library was constructed in the lambda expression vector Zap Express (Stratagene). Plaque replicas were screened by incubation overnight, with an undiluted monoclonal antibody (culture hybridoma supernatant derived from whole *H. pylori* Roberts cells, a clinical isolate obtained in Manchester, United Kingdom) containing 0.1% Tween 20. The antibody, designated GF6, has been described previously as recognizing a 29-kDa flagellar sheath protein (17). *Escherichia coli* harboring plasmids (pBK-CMV derivatives) excised from several antibody-reactive lambda clones by using the ExAssist/XLOLR system (Stratagene) expressed a protein with a molecular mass identical to the 29-kDa flagellar sheath protein. The smallest plasmid, pACJ193, was sequenced.

A region was found to be 94% identical to a fragment sequenced by Evans et al. (11) which contained the gene *hpaA*. An open reading frame (ORF) of 783 bp was identified, which encompassed two regions termed ORF 2 and ORF 3 by Evans et al., confirming the data of O'Toole et al. (19). In addition, a typical Shine-Dalgarno ribosome binding site was identified 11 bp upstream of *hpaA*, and plausible σ^{70} consensus promoter elements CTGATA and TAAAAT, separated by 17 bp, were identified at appropriate positions 111 and 88 bp upstream of the start codon. A potential rho-independent transcriptional terminator, TAAAAAGGGTTTTTTAGCGTTCTTTT, was located 43 bp downstream of the stop codon, and a second ORF 269 bp downstream of *hpaA* and preceded by a plausible σ^{70} consensus promoter was identified, indicating that *hpaA* is a single gene operon.

Mapping of the transcriptional start. The transcriptional start point of *hpaA* was mapped by the extension of *H. pylori* mRNA. Briefly, 4.5 pmol of ^{32}P-labelled oligonucleotide D8153 (Table 1) was hybridized to 20 μg of RNA (or DNase-treated RNA) in 30 μl of hybridization buffer [40 mM piper-

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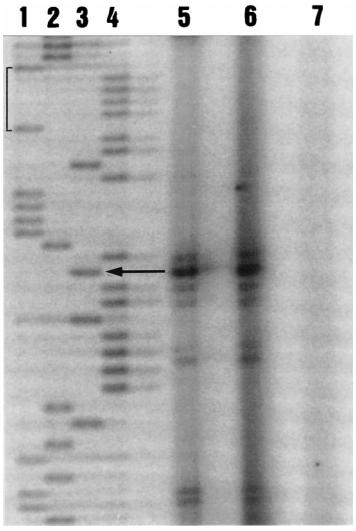

FIG. 1. Mapping of the transcriptional start of *hpaA*. Lanes 1 to 4, sequencing reaction of pACJ193 (in the order TGCA); lanes 5 to 7, primer extension of *H. pylori* mRNA, *H. pylori* mRNA treated with DNase, and tRNA control, respectively. The position of the −10 promoter element is indicated by a bracket, and the transcriptional start is indicated by the arrow.

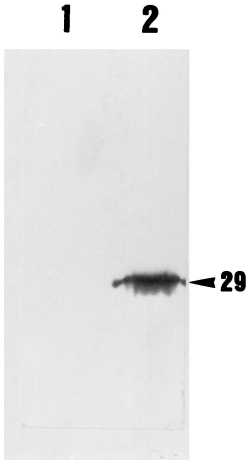

FIG. 2. Partitioning of HpaA into detergent. Western blot (immunostained with GF6) of *H. pylori* cells incubated with 10% (vol/vol) Triton-X114 at 4°C and centrifuged to produce an aqueous supernatant (lane 1) and a detergent pellet (lane 2). The molecular mass (in kilodaltons) of the reactive band is indicated.

azine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM EDTA, 400 mM NaCl, 80% formamide] by heating at 85°C for 5 min and then cooling to 45°C overnight. The primer was extended with 400 U of Superscript RNase H^- reverse transcriptase (Life Technologies) in buffer supplemented with deoxynucleoside triphosphates (2.2 mM), dithiothreitol (22 mM), and 40 U of RNasin (RNase inhibitor; Promega) for 1 h at 42°C. The cDNA was precipitated, suspended in loading buffer, heated to 85°C for 3 min, and placed on ice prior to electrophoresis on a 6% sequencing gel alongside the products of a double-stranded sequencing reaction of pACJ193 with the primer D8153. As a control for primer specificity, a sample containing tRNA from baker's yeast was included. A prominent band which ran level with a cytosine residue 72 nucleotides upstream of the start codon and 11 nucleotides downstream of the proposed −10 element (Fig. 1) indicated the transcriptional start and confirmed the presence of a σ^{70} consensus promoter at a location different from that suggested by Evans et al. (11). There were no bands in the tRNA control (lane 7), indicating that the oligonucleotide used to prime the extension reaction did not hybridize nonspecifically to unrelated RNA species.

HpaA is a lipoprotein. We confirm the findings of O'Toole et al. (19), which suggest that HpaA is a lipoprotein: (i) a predicted hydrophobic domain at the amino terminus consistent with the existence of a signal sequence; (ii) the amino acid motif LVGC, which is common among lipoproteins as the cleavage and acylation site of a signal sequence; and (iii) globomycin inhibition of processing of the recombinant protein by signal peptidase II. Furthermore, by using a method based upon that of Brusca and Radolf (3), we partitioned the mature nonrecombinant protein into the detergent phase of a Triton X-114 two-phase system (Fig. 2). Samples were solubilized and analyzed by immunoblot with GF6. Since the hydropathy plot of HpaA (data not shown) suggests that the mature protein is predominately hydrophilic, partitioning into the detergent phase is compatible with the lipid modification of the mature protein.

Localization of the 29-kDa protein. The location of HpaA in *H. pylori* has proved controversial. First, HpaA was described as a surface antigen by Evans et al. on the basis of immunogold-labelling studies with a rabbit polyclonal antiserum to the purified hemagglutinin (10). Specific labelling of the cell surface, but not of the flagella, which appeared to lack sheaths, was observed. Subsequent immunogold-labelling studies with a polyclonal antibody prepared against a synthetic dodecapeptide containing the hexapeptide motif KRTIQK, similar to a motif conserved among bacterial sialic-acid hemagglutinins, appeared to reinforce these findings (11). However, other surface-associated antigens or adhesins of *H. pylori* may contain the same amino acid motif and thus bind to the antibody. Contradicting our partitioning studies demonstrating the hydrophobic nature and probable membrane location of HpaA, O'Toole et al. (19) reported the detection of HpaA predominantly in the cytoplasmic fraction of Sarkosyl-solubilized cells, with only trace amounts in the inner membrane and none in the outer membrane. The means of detection was immunoblotting with an antibody raised against the same dodecapeptide as that used by Evans et al. (11). Furthermore, a cytoplasmic locale for a bacterial lipoprotein has not previously been reported (2).

The monoclonal antibody GF6, elicited with cellular antigens including intact HpaA, provides a more valid means of detection and was used by Luke and Penn (17) to demonstrate specific immunogold labelling of the flagellar sheath in the Roberts strain (the strain against which the antibody was derived). To confirm this finding, further immunogold electron microscopy was performed on strain NCTC 11637 and on two other clinical isolates, A654/91 and A681/91 (a gift from J. Bickley, Colindale, United Kingdom) in addition to the Roberts strain. Carbon-stabilized Formvar-coated Ni grids bearing bacterial cells were blocked by floating on 3% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h, labelled with GF6 diluted 1/10 in PBS containing 0.1% (wt/vol) BSA and 0.1% (vol/vol) Tween 20 (PAT) for 16 h at 4°C, and washed five times for 5 min each with 3% BSA. The grids were subsequently incubated with goat anti-mouse immunoglobulins conjugated to 10-nm colloidal gold (British BioCell International, Cardiff, United Kingdom) diluted 1/10 in PAT for 5 h, washed five times for 5 min each with 3% BSA and 5 min with Milli-Q water, and briefly negatively stained with 1.7% sodium phosphotungstate. Samples were examined

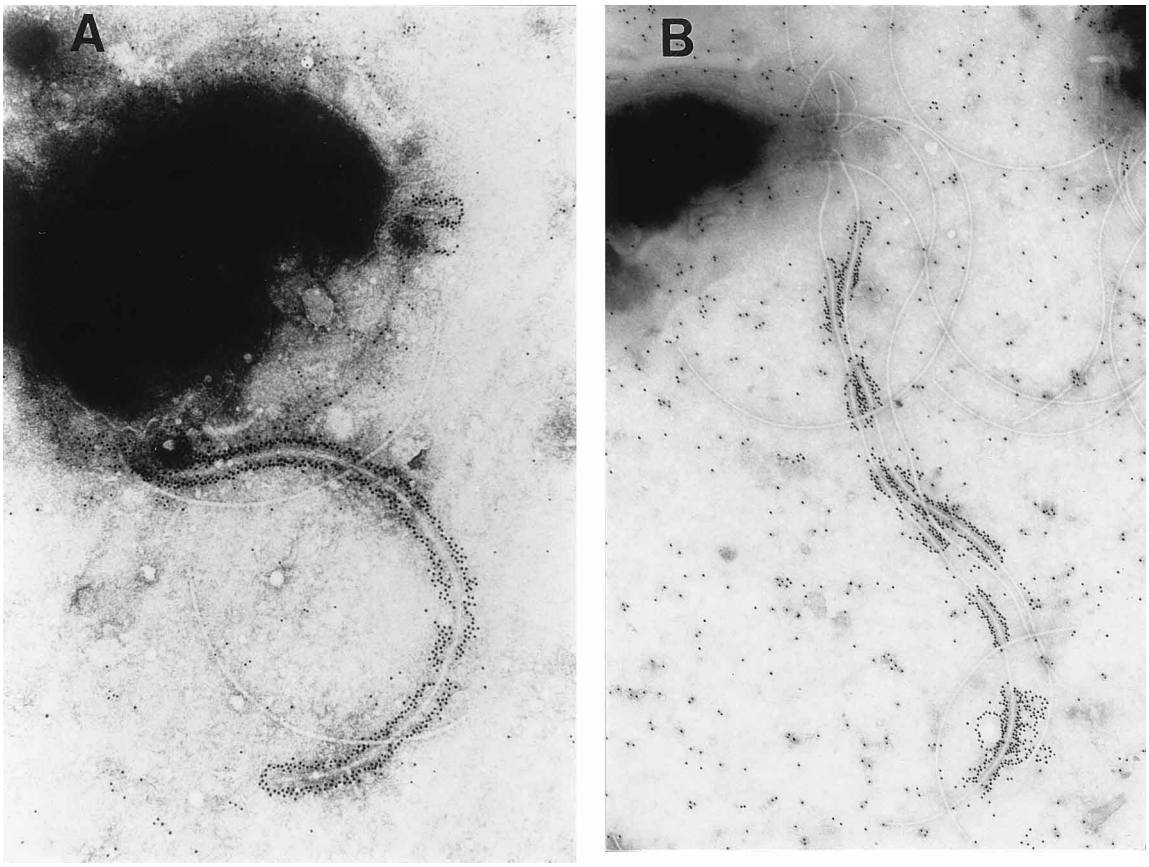

FIG. 3. Transmission electron micrographs of *H. pylori* cells incubated with GF6 and labelled with 10-nm colloidal gold particles. (A) Strain NCTC 11637; (B) strain Roberts. Note specific labelling of the flagellar sheaths. Magnification, ×15,000.

by using a JEOL 1200 EX (Hertfordshire, United Kingdom) transmission electron microscope. In all strains, GF6 specifically bound only to the flagellar sheath and not to naked filaments or to the remainder of the cell surface at any comparable level (Fig. 3). Flagellar sheaths lacking label were also observed, which suggests that the protein may be highly labile and readily removed during sample preparation. It is possible that HpaA is also present on the surface of the bacterium but, contrary to its position in the sheath, oriented such that the GF6 epitope is not exposed.

Characterization of Δ*hpaA* mutants. The complete gene *hpaA* was precisely deleted, and a unique *Bgl*II site was engineered into a plasmid construct which contained *hpaA* together with 280 bp of upstream and 124 bp of downstream flanking sequences and also an ampicillin resistance gene by inverse PCR mutagenesis (22) with primers A1 and C1 (Table 1). A cassette containing a *Campylobacter coli aphA-3* kanamycin resistance gene, originally from plasmid pILL512 (15), was ligated into the *Bgl*II site of the amplified product. This plasmid was sequenced to confirm that the appropriate manipulations had been made. Clonal populations of naturally competent *H. pylori*, strains 11637 and Roberts, were transformed with 1 to 2 μg of the plasmid by the method of Haas et al. (13). Recombinants were selected on the basis of Km^r and Ap^s to ensure that a double crossover event had occurred.

One transformant from each strain was demonstrated to be a Δ*hpaA* mutant by immunoblotting with GF6 (data not shown). Allelic replacement of the wild-type *hpaA* gene was confirmed by whole-cell PCR with primers P1 and P3 (Table 1). The size of the PCR products generated by the mutants (1.8 versus 1.2 kbp for the wild types) was consistent with the replacement of *hpaA* (0.783 kbp) with the kanamycin resis-

TABLE 1. Summary of oligonucleotides

Primer	Nucleotide position[a]	Strand	Sequence[b]
D8153	573–597	−	GCTCTCATCGTTCTATCCTTGATTG
A1	567–589	−	gcgagatctCGTTCTATCCTTGATTGTAATAT
C1	1373–1395	+	gcgagatctAAACAAATAACGCATAAGAAAAG
P1	305–325	+	gcgtCTagAGCTAAAACGGCCGTAG
P3	1457–1478	−	GTAAGTAACCAGATCATCAGCC

[a] Numbers refer to accession no. X92502 in the EMBL database.
[b] Lowercase letters indicate nucleotides which are not homologous to the sequence. Underlined nucleotides indicate restriction endonuclease sites.

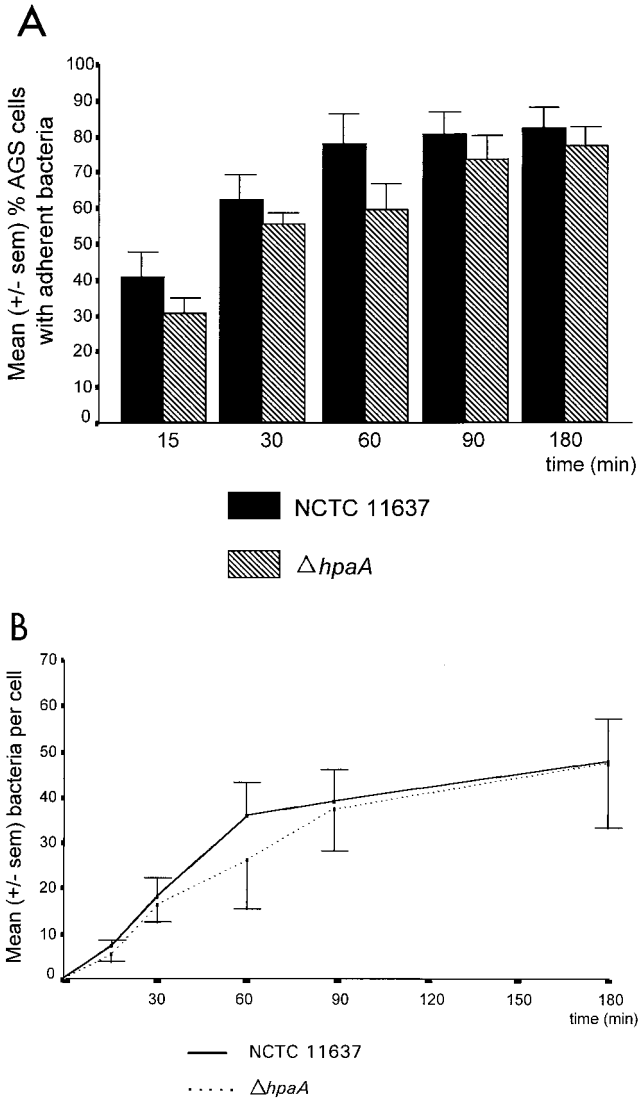

FIG. 4. (A) Proportion of AGS cells with adherent wild-type and Δ*hpaA* mutant bacteria. (B) Number of wild-type and Δ*hpaA* mutant bacteria per AGS cell. sem, standard error of the mean.

tance cassette (1.4 kbp) (data not shown). Furthermore, a probe for *aphA-3* hybridized with Southern blots of *Ssp*I-restricted DNA from the mutants but did not hybridize with wild-type DNA (data not shown). The size of the hybridized fragment was consistent with the occurrence of the desired recombinant event.

Further immunogold-labelling experiments revealed that the flagellar sheaths of the Δ*hpaA* mutants were not recognized by GF6. The absence of HpaA does not appear to be deleterious for flagellar sheath synthesis, as negatively stained mutants observed by transmission microscopy possessed numerous sheathed flagella and appeared to be morphologically identical to the parent strains.

Adherence of Δ*hpaA* mutants to gastric epithelial cells. Adherence of strain NCTC 11637 and the corresponding Δ*hpaA* mutant to gastric epithelial cells and to human erythrocytes was assessed by fluorescence-activated cell sorting (FACS) and a quantitative hemagglutination assay, respectively, as previously described (16). For the hemagglutination assay, washed standardized suspensions of bacteria grown in broth culture or harvested from chocolate blood agar plates were interacted with washed human erythrocytes (Group O) under saturating conditions. Hemagglutination was quantitated by an automated spectrophotometric method. For hemagglutination inhibition studies, bacteria were preincubated with *N*-acetylneuraminyllactose (3,500 mg/ml). Both Δ*hpaA* mutants agglutinated human erythrocytes. Quantitatively, the extent of agglutination was not significantly different from that seen for each respective wild-type strain (data not shown). In addition, the extent of inhibition of hemagglutination by *N*-acetylneuraminyllactose was similar for both mutant and wild-type strains.

The FACS assay was performed only with strain NCTC 11637 and the corresponding mutant. Tissue culture flasks (75 ml) were seeded with 2×10^5 AGS cells/flask. After overnight incubation, the culture medium was discarded and replaced with 2 ml of RPMI medium buffered with 25 mM HEPES. *H. pylori* from 24-h broth cultures was labelled fluorescently by incubation with 0.9 mM carboxy-fluorescein-diacetate succinimidyl ester (CFDA-SE) at 37°C for 20 min. After they were washed (5 min in PBS [Dulbecco A; Oxoid]) to remove excess CFDA-SE, bacteria were resuspended in PBS, and a standardized number were coincubated with AGS cells (ratio, 10:1) at 37°C for 3 h. Nonadherent bacteria were removed by two further washes with PBS before the AGS cells with adherent bacteria were detached from the flasks with 2 mM EDTA and fixed with 3% formaldehyde prior to FACS. The percentage of AGS cells with adherent bacteria and the number of bacteria adhering to the AGS cells were quantitated with reference to the fluorescence of bacteria alone.

Both the mutant and wild-type strains were able to adhere to AGS cells. After 15 min of coincubation, approximately one-third of the AGS cell population had adherent bacteria, while after 3 h of coincubation, bacteria were present on nearly all AGS cells (Fig. 4A). The adherence phenotype was also assessed by the calculation of the number of bacteria adhering to each AGS cell. After 15 min of coincubation, the mean (± standard error of the mean) numbers of bacteria per cell were 6.9 (±1.0) and 5.7 (±1.9) for NCTC 11637 and the Δ*hpaA* mutant, respectively (not significant; Fig. 4B). The number of bacteria per cell increased rapidly for both strains, such that after 3 h of coincubation, the mean (± standard error of the mean) numbers of bacteria per cell were 44.4 (±8.1) and 43.7 (±11.3) for NCTC 11637 and the Δ*hpaA* mutant, respectively (not significant). At no time point was the difference between the mutant and the wild-type strain significant regarding the proportion of AGS cells with adherent bacteria or the numbers of adherent bacteria per cell.

Similar observations have been reported by O'Toole et al. (19) in a study involving the characterization of Δ*hpaA* mutants of strains CCUG 17874, CCUG 915, and A5, which indicated that these mutants were no different from the respective wild types in terms of hemagglutination activity and ability to adhere to eukaryotic cell lines.

Nucleotide sequence accession number. The nucleotide sequence reported here has been entered in the EMBL database under accession no. X92502.

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REFERENCES

1. **Blaser, M. J., and J. Parsonnet.** 1994. Parasitism by the 'slow' bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. J. Clin. Invest. **94:**4–8.
2. **Braun, V., and H. C. Wu.** 1994. Lipoproteins, structure, function, biosynthesis and model for protein export, p. 319–341. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science B. V., Amsterdam, The Netherlands.
3. **Brusca, J. S., and J. D. Radolf.** 1994. Isolation of integral membrane-proteins by phase partitioning with Triton-X114. Methods Enzymol. **228:**182–193.
4. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135–145.
5. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59:**2470–2475.
6. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect. Immun. **62:**3604–3607.
7. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. **57:**1119–1125.
8. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. **37:**123–127.
9. **Eaton, K. A., S. Suerbaum, C. Josenhans, and S. Krakowka.** 1996. Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. Infect. Immun. **64:**2445–2448.
10. **Evans, D. G., D. J. Evans, Jr., J. J. Moulds, and D. Y. Graham.** 1988. *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. Infect. Immun. **56:**2896–2906.
11. **Evans, D. G., T. K. Karjalainen, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee.** 1993. Cloning, nucleotide sequence, and expression of a gene encoding an adhesin subunit protein of *Helicobacter pylori*. J. Bacteriol. **175:**674–683.
12. **Geis, G., S. Suerbaum, B. Forsthoff, H. Leying, and W. Opferkuch.** 1993. Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. J. Med. Microbiol. **38:**371–377.
13. **Haas, R., T. F. Meyer, and J. P. M. van Putten.** 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Mol. Microbiol. **8:**753–760.
14. **Jones, D. M., and A. Curry.** 1992. The ultrastructure of *Helicobacter pylori*, p. 29–41. *In* B. J. Rathbone and R. V. Heatley (ed.), *Helicobacter pylori* and gastroduodenal disease, 2nd ed. Blackwell Scientific Publications, Oxford, United Kingdom.
15. **Labigne-Roussel, A., J. Harel, and L. Tompkins.** 1987. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. **169:**5320–5323.
16. **Logan, R. P. H., A. Cockayne, A. Robbins, S. P. Borriello, and C. J. Hawkey.** 1995. Development of a quantitative assay for adherence of *H. pylori*. Gut **47**(Suppl. 1)**:**A64.
17. **Luke, C. J., and C. W. Penn.** 1995. Identification of a 29 kDa flagellar sheath protein in *Helicobacter pylori* using a murine monoclonal antibody. Microbiology **141:**597–604.
18. **Nomura, A., G. N. Stemmermann, P. H. Chyou, G. I. Perez-Perez, and M. J. Blaser.** 1994. *Helicobacter pylori* infection and the risk for duodenal and gastric ulceration. Ann. Intern. Med. **120:**977–981.
19. **O'Toole, P. W., L. Janzon, P. Doig, J. Huang, M. Kostrzynska, and T. J. Trust.** 1995. The putative neuraminyllactose-binding hemagglutinin HpaA of *Helicobacter pylori* CCUG 17874 is a lipoprotein. J. Bacteriol. **177:**6049–6057.
20. **Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127–1131.
21. **Warren, J. R., and B. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **ii:**1273–1275.
22. **Wren, B. W., J. Henderson, and J. M. Ketley.** 1994. A PCR-based strategy for the rapid construction of defined bacterial mutants. BioTechniques **16:**994–996.