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Mutation of the Cytotoxin-Associated *cagA* Gene Does Not Affect the Vacuolating Cytotoxin Activity of *Helicobacter pylori*

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***Helicobacter pylori* now is recognized as an etiological agent in chronic superficial gastritis and peptic ulcer disease. Although only about 60% of *H. pylori* isolates produce an immunodominant 128-kDa antigen (CagA; cytotoxin-associated gene product), virtually all *H. pylori*-infected patients with duodenal ulceration develop a serologic response to the 128-kDa protein, which suggests an association of this gene with ulceration. The cloned *cagA* gene from *H. pylori* 84-183 was disrupted by insertion of a kanamycin resistance gene, and this inactivated *cagA* construct was introduced into *H. pylori* 84-183 by electrotransformation. Southern hybridization of kanamycin-resistant *H. pylori* transformants demonstrated that the wild-type *cagA* gene had been disrupted by insertion of the kanamycin cassette, and immunoblot analysis showed that the mutant strains no longer produced the 128-kDa CagA protein. Similar results were obtained when the *cagA* mutation was introduced by natural transformation into *H. pylori* 60190, a high-level toxin-producing strain. The *cagA*-negative *H. pylori* strains showed cytotoxin, urease, and phospholipase C activities, C3 binding, and adherence similar to those of the isogenic wild-type strains. These findings demonstrate that the *cagA* gene product does not affect the vacuolating cytotoxin activity of *H. pylori*.**

Helicobacter pylori is the major causative agent of chronic superficial gastritis (24) and plays an important role in the pathogenesis of peptic ulcer disease (2, 29) and gastric cancer (26, 27). The pathogenic mechanisms whereby *H. pylori* causes human disease are not yet well understood. Several pathogenic determinants have been proposed for *H. pylori*, including motility (15, 21), adhesins (16), urease activity (12, 13, 14, 18, 19, 22, 33), cytotoxin production (6, 25), and the presence of a 128-kDa antigen (5, 7, 11, 34). The 128-kDa protein (CagA) is highly immunogenic, and its presence is strongly associated with cytotoxin production (5, 7, 10, 34). However, the *cagA* and vacuolating toxin (*vacA*) genes are localized about 300 kb apart on the *H. pylori* chromosome (4). Among *H. pylori*-infected patients, both serum and local antibody recognition of CagA is significantly more common among those with peptic ulcer disease than among those with chronic gastritis alone (7, 11). In addition, mucosal neutrophil infiltration and epithelial surface degeneration were significantly greater in patients with a local gastric immune response to CagA than in those without such a response (11). In previous studies, we cloned and characterized the gene encoding CagA (34). In this study, we sought to construct an isogenic mutant strain in which production of CagA is eliminated and to characterize the role of CagA in expression of cytotoxin activity or other putative virulence attributes.

A 3.6-kb fragment that contained the first 2,577 nucleotides of the 3,543-bp *cagA* open reading frame was previously cloned into pBluescript to create pMC3 (34). In the present study, we inserted a *Campylobacter coli* kanamycin resistance gene (23) into the unique *NdeI* site of pMC3 to create pMC3:*km* (Fig. 1). Based upon Western blotting (immunoblotting) with human antiserum, pMC3:*km* produced a 40-kDa CagA fragment (Fig. 2, lane c). In addition, we cloned the 3.6-kb fragment of pMC3 into the suicide vector pILL570 (18) to create pIC1; this construct was subjected to random insertional mutagenesis using mini-Tn*3*-Km (18). One of these derivatives, designated pIC1::19 (Fig. 1), produced no detectable CagA fragment (Fig. 2, lane e).

To inactivate the *cagA* gene of *H. pylori*, the construct pMC3:*km*, which is unable to replicate in *H. pylori*, was introduced directly into *H. pylori* 84-183 (ATCC 53726) by electroporation. In brief, *H. pylori* cells grown on blood agar plates for 24 to 48 h were harvested, washed three times in 15% glycerol–5% sucrose solution, and suspended in 200 μl of the same solution. Plasmid DNA isolated (1) from pMC3:*km* was incubated on ice with the *H. pylori* cells for 5 min. The cells and DNA were transferred to a 0.2-cm cuvette in a gene-pulsar apparatus (Bio-Rad), and high-voltage pulses (25 F, 2.5 kV, and 2,000 Ω) were delivered as described previously (18). Electroporation of 10^{10} CFU of 84-183 with 500 ng of pMC3:*km* DNA yielded 3×10^3 kanamycin-resistant transformants. Forty of the kanamycin-resistant *H. pylori* 84-183 transformants were replica plated, and separate colony blot hybridizations were done with probes specific for the pBluescript vector and for the kanamycin resistance gene. None of the kanamycin-resistant transformants hybridized to the pBluescript probe, which indicated that they did not contain vector sequences; in contrast, all the transformants hybridized to the Km probe. These studies suggested that the kanamycin resistance gene had been rescued from the nonreplicating plasmid by incorporation into the bacterial chromosome at a frequency of approximately 10^{-7}, as expected. However, in three separate experiments when *H. pylori* 60190 was electrotransformed using the identical DNA, no kanamycin-resistant transformants were obtained.

To provide genetic evidence that the *cagA* gene had been disrupted in the transformed strains, Southern hybridizations were performed. DNA isolated (31) from wild-type strain 84-183 and *H. pylori* mutants 84-183:M21 and 84-183:M1 (both

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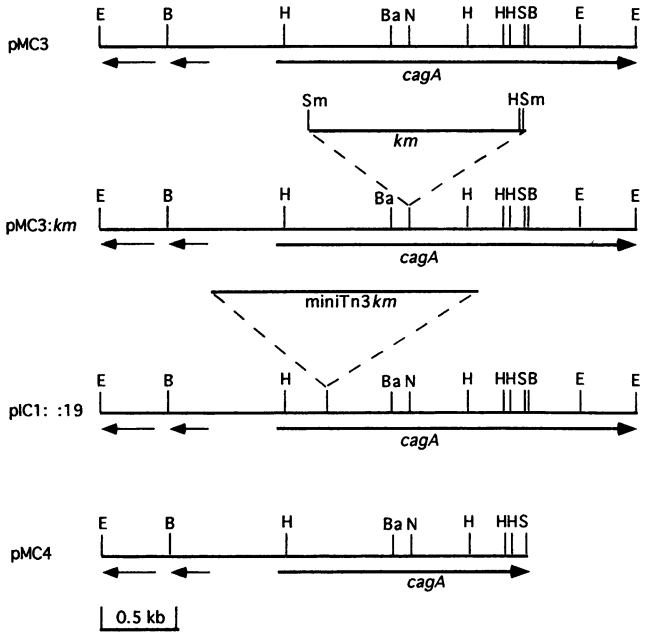

FIG. 1. Restriction maps of *cagA* constructs used in construction of *H. pylori* mutants. The Km cassette from pILL600 was ligated into the *Nde*I site of pMC3 to create pMC3:*km*. The arrows represent open reading frames, including the truncated *cagA* open reading frame. pIC1::19 represents pILL570 containing the pMC3 insert into which mini-Tn*3*-Km has been inserted at the location shown. The insert from plasmid pMC4 was used as a probe in hybridization experiments. Restriction sites are as follows: E, *Eco*RI; B, *Bgl*II; H, *Hin*dIII; Ba, *Bam*HI; N, *Nde*I; S, *Sac*I; Sm, *Sma*I.

of which contained the Km sequence by colony hybridization) was digested with *Bam*HI and *Sac*I or with *Hin*dIII, and the resulting fragments were electrophoresed on 0.7% agarose gels and transferred to nylon membranes (30). Hybridizations were performed using a ^{32}P-radiolabeled probe (17) in a solution of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 100 μg of salmon sperm DNA per ml at 68°C, and the blots were washed for 30 min at 60°C in 0.5× SSC–0.1% SDS. A *cagA* probe (pMC4, Fig. 1) hybridized to approximately 20- and 1.0-kb *Bam*HI-*Sac*I fragments in the wild-type strain, but in each mutant strain, the 1.0-kb fragment was lost and a new 2.3-kb hybridizing fragment was observed without disruption of the other band (Fig. 3). Similarly, a 1.2-kb *Hin*dIII fragment was lost and two new bands (2.1 and 0.4 kb) were observed in the kanamycin-resistant mutant, as expected, since the Km cassette contains an internal *Hin*dIII site. The Km probe hybridized with the 2.3-kb *Bam*HI-*Sac*I and 2.1-kb *Hin*dIII fragments in mutant strains M21 and M1, which indicates that insertions had occurred within *cagA*. Thus, we now had direct evidence that the *cagA* gene of strain 84-183 had been mutagenized by insertion of the Km cassette.

Since electroporation of strain 60190 (ATCC 49503) with a mutant *cagA* construct (pMC3:*km*) was not successful, we sought to create a *cagA* mutation in this high-level toxin-producing strain, using natural transformation. *H. pylori* 60190 was grown for 24 h in brucella broth supplemented with 5% fetal bovine serum, and then DNA isolated from the *cagA H. pylori* mutant strain 84-183:M21 was added to the culture. The cells were harvested after 30 min, plated on blood agar plates, and incubated in 5% CO_2 overnight at 37°C. These cells were replated on blood agar plates containing kanamycin (40 μg/ml), and transformants were selected after 2 to 3 days of incubation. When we incubated 1 μg of chromosomal DNA

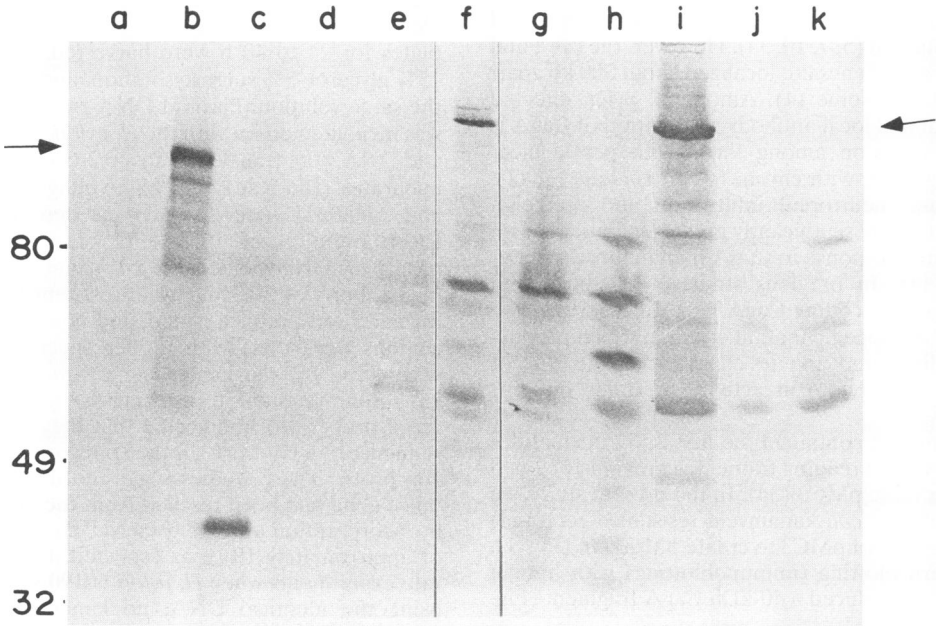

FIG. 2. Immunoblot of *E. coli* XL1-Blue (lanes a to e) and *H. pylori* (lanes f to k) strains with serum from an *H. pylori*-infected person. *E. coli* contains pBluescript (lane a), pMC3 (lane b), pMC3:*km* producing a 40-kDa CagA fragment (lane c), pILL570 (lane d), and pIC1::19 producing no detectable CagA fragment (lane e). *H. pylori* strains are 84-183 (lane f), 84-183:M21 (lane g), 84-183:M22 (lane h), 60190 (lane i), 60190:M21 (lane j), and 60190:M22 (lane k). The right arrow points to the *cagA* product in *H. pylori*, and the left arrow points to the truncated (96-kDa) *cagA* product in pMC3.

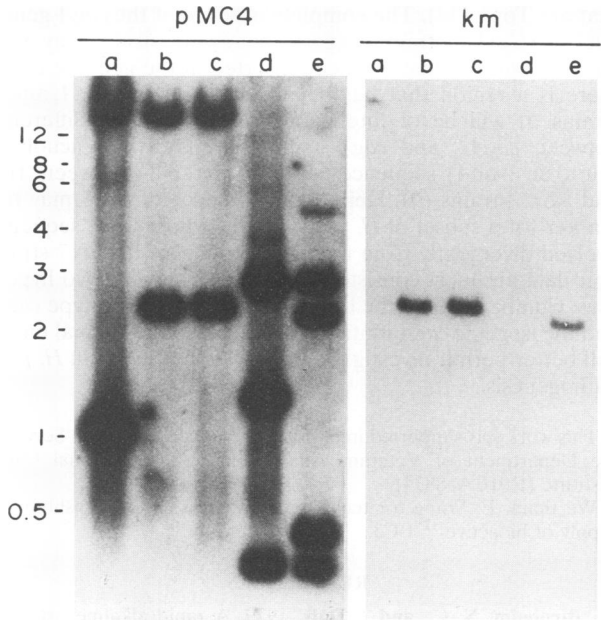

FIG. 3. Southern hybridization of wild-type and mutant *H. pylori* strains. DNA from wild-type strain 84-183 and kanamycin-resistant transformants 84-183:M21 and 84-183:M1 was digested with *Bam*HI and *Sac*I (lanes a to c) or with *Hin*dIII (lanes d and e). Filters were hybridized with ^{32}P-labeled pMC4 or the 1.3-kb Km cassette under conditions of high stringency. Lanes a and d, 84-183; lanes b and e, 84-183:M21; lane c, 84-183:M1.

isolated from the 84-183 *cagA* mutant (84-183:M21) with 10^7 cells of strain 60190, approximately 200 kanamycin-resistant transformants were obtained. Southern hybridization of chromosomal DNA from one of the transformants (60190:M21) showed the expected Km insertion resulting in allelic replacement (results not shown).

Since the mutants derived from pMC3:*km* could potentially produce a truncated *cagA* product of 40 kDa, we next sought to create mutants that produced a smaller gene product. Thus, mutants of 84-183 and 60190 were constructed using pIC1::19, which encodes a truncated *cagA* product of 10 kDa (Fig. 1). As described above, it was necessary to use natural transformation to create a mutant of 60190. The resulting mutants were named 84-183:M22 and 60190:M22.

To determine whether the kanamycin-resistant *H. pylori* mutants were able to express the *cagA* product, whole cells from the wild-type strain 84-183, mutants 84-183:M21 and 84-183:M22 (Fig. 2, lanes f, j, and h, respectively), the wild-type strain 60190, and the mutants 60190:M21 and 60190:M22 (lanes i, j, and k, respectively) were immunoblotted (28), using serum from an *H. pylori*-infected person that strongly recognized the CagA antigen (7). Wild-type cells expressed a 128-kDa protein that was strongly recognized by the human serum, whereas none of the mutants produced the 128-kDa CagA antigen (Fig. 2). These experiments indicated that both mutations of the *cagA* locus ablated expression of CagA.

Since several studies indicate that the expression of CagA is associated with expression of cytotoxin activity, we sought to compare the cytotoxin activities of the wild-type and isogenic CagA$^-$ mutant strains. Quantitation of HeLa cell vacuolation was accomplished using concentrated supernatants, standardized by protein concentration (32). Supernatants were incubated for 24 h with HeLa cells, and vacuolation was quantified by a neutral red uptake assay as previously described (8). Culture supernatants from the wild-type and isogenic mutant strains induced essentially identical vacuolation of HeLa cells, whereas the supernatant from the naturally occurring Tox$^-$ strain Tx30a had no effect (Fig. 4). As expected, the two wild-type strains, 60190 and 84-183, differed in the level of expression of cytotoxin activity (9). Thus, expression of cytotoxin activity in both *H. pylori* 60190 and 84-183 was not dependent on the presence of the CagA protein.

To assess whether mutation of the *cagA* gene affected urease production, the wild-type and mutant strains were grown on blood agar plates and urease activity was determined as described previously (13). These studies showed no significant difference in the urease activity of wild-type and mutant strains (Table 1). Similar experiments were done to detect phospholipase C activity (35) of strains 84-183, 84-183:M21, 60190, and 60190:M21. Again, there were no significant differences between the wild-type and the derived strains (data not shown).

Earlier studies have suggested that the 128-kDa protein may be a marker of bacteria which are better adapted to colonize the duodenum (11). To test whether CagA facilitates adher-

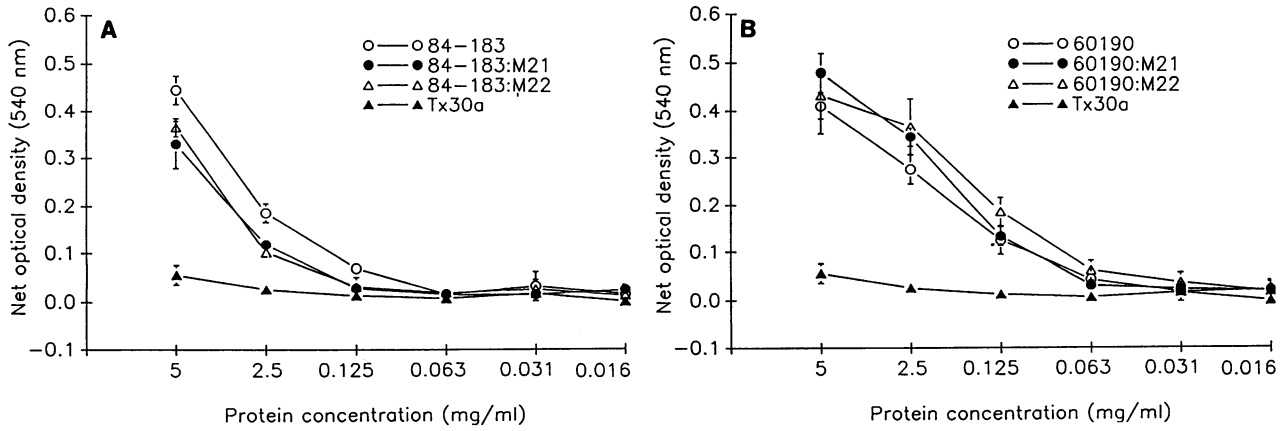

FIG. 4. Vacuolating cytotoxin activity of wild-type and isogenic *cagA* mutant *H. pylori* strains. HeLa cells were incubated for 18 h with concentrated culture supernatant from wild-type *H. pylori* 84-183 or isogenic mutants (A) or wild-type *H. pylori* 60190 or isogenic mutants (B). In each experiment, culture supernatant from Tox$^-$ *H. pylori* Tx30a was tested as a control. Cell vacuolation was quantitated spectrophotometrically using a neutral red uptake assay (8) and is expressed as net optical density.

TABLE 1. Effect of *cagA* mutation on ^{125}I-C3 binding and urease activity

Strain	Characteristic	C3 binding (mean ± SEM)[a]	Urease activity (mean ± SEM)[b]
C. fetus			
23D	Wild type	182 ± 17	0.000
23B	Spontaneous mutant	1,329 ± 236	0.000
H. pylori			
60190	*cagA*$^+$ wild-type	293 ± 90	0.012 ± 0.006
60190:M21	*cagA* mutant derivative of 60190	169 ± 119	0.013 ± 0.005
84-183	*cagA*$^+$ wild type	1,637 ± 351	0.011 ± 0.004
84-183:M21	*cagA* mutant derivative of 84-183	1,303 ± 85	0.013 ± 0.007

[a] ^{125}I-C3 counts bound to bacterial cell pellet after incubation in the presence of pooled normal human serum for 15 min. Net binding reflects subtraction of nonspecific binding in the presence of heat-inactivated (56° for 30 min) normal human serum. Results shown are means of three assays on different days.

[b] Results shown as units per minute are means of three assays.

ence of bacteria to epithelial cells, we performed adherence assays using wild-type and *cagA* mutant strains. The adherence assay used in these experiments was essentially the method of Grant et al. (20). The number of bacteria adherent to AGS cells was quantified by plating serial dilutions of the bacteria on blood agar plates and counting the resulting colonies. There was no significant difference in the adherence of the wild-type and mutant strains (data not shown).

Finally, since infection with a CagA-expressing strain appears to be associated with higher levels of neutrophil infiltration into tissues (11), we examined the effect of *cagA* mutation on interactions of *H. pylori* with complement component 3 (C3), a critical factor in opsonophagocytosis. C3 binding assays were done as previously described (3) using ^{125}I-C3 in the presence of normal human serum (NHS) or heat-inactivated normal human serum. *Campylobacter fetus* 23B and 23D were used as positive and negative controls, respectively (3). The two wild-type strains differed markedly in their binding of C3 (Table 1), but the mutants behaved similarly to the parental strains.

In conclusion, our studies now show that ablation of CagA expression does not have any apparent effect on vacuolating cytotoxin activity. In contrast, insertional mutation of the vacuolating cytotoxin gene (*vacA*) results in absence of cytotoxin production (9). There are several possible explanations for the dichotomy between the association of *cagA* with cytotoxin expression and our current findings. (i) The mutations did not completely delete CagA activity, and even the N-terminal region of CagA is sufficient to enhance cytotoxin expression. Although unlikely, this remains a possibility. (ii) The Km insertion was lost, and the strains reverted to the wild-type phenotype. However, in the absence of duplicate copies of the gene, there is no mechanism for loss of the Km cassette. In addition, on repeated subcultures, the mutant strains remain negative for *cagA* and are resistant to kanamycin even in the absence of continued selection. This is expected since neither the Km cassette nor mini-Tn*3*-Km can be mobilized in *H. pylori*. (iii) More than one copy of *cagA* was present, and only one was mutated. However, the Southern analyses demonstrate that only a single copy was present, and Western blotting shows that there is no production of the 128-kDa protein. (iv) *cagA* may only be a marker for other genetic elements that affect cytotoxin expression. Such a hypothesis is consistent with the observation that there are *cagA*$^+$ strains that are Tox$^-$ (34). The complete absence of the *cagA* gene in 40% of *H. pylori* strains suggests that *cagA*$^+$ strains may either have a sequence that has been inserted in the genome or that there is a region that has been deleted in the *cagA* mutant strains. It will be of interest to better map the differences between *cagA*$^+$ and *cagA* mutant strains. (v) Vacuolating cytotoxin (*vacA*) sequences diverge markedly between Tox$^+$ and Tox$^-$ strains (9). Hence, the presence of *cagA* may be a marker for a subset of *H. pylori* strains whose *vacA* sequences evolved divergently from the *vacA* sequences of Tox$^-$ strains. Our data are most consistent with one of the last two hypotheses. Comparisons of the biological activity of wild-type *cagA*$^+$ and the isogenic *cagA* mutant strains in relevant animal models will better permit investigation of the role of *cagA* in *H. pylori* pathogenesis.

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REFERENCES

1. **Birnboim, N. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Blaser, M. J.** 1992. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology **102**:720–727.
3. **Blaser, M. J., P. A. Smith, J. E. Repine, and K. A. Joiner.** 1988. Pathogenesis of *Campylobacter fetus* infections. Failure to bind C3b explains serum and phagocytosis resistance. J. Clin. Invest. **81**:1434–1444.
4. **Bukanov, N. O., and D. E. Berg.** 1994. Ordered cosmid library and high resolution physical genetic map of *Helicobacter pylori* strain NCTC 11638. Mol. Microbiol. **11**:509–523.
5. **Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, and R. Rappuoli.** 1993. Molecular characterization of the 128 kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA **90**:5791–5795.
6. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. **267**:10570–10575.
7. **Cover, T. L., C. P. Dooley, and M. J. Blaser.** 1990. Characterization of human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. Infect. Immun. **58**:603–610.
8. **Cover, T. L., W. Puryear, G. I. Pérez-Pérez, and M. J. Blaser.** 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59**:1264–1270.
9. **Cover, T. L., M. K. R. Tummuru, P. Cao, S. A. Thompson, and M. J. Blaser.** Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. J. Biol. Chem., in press.
10. **Crabtree, J. E., N. Figura, J. D. Taylor, M. Bugnoli, D. Armellini, and D. S. Tompkins.** 1992. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. J. Clin. Pathol. **45**:733–734.
11. **Crabtree, J. E., J. D. Taylor, J. I. Wyatt, R. V. Heatley, T. M. Shallcross, D. S. Tompkins, and B. J. Rathbone.** 1991. Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration and gastric pathology. Lancet **338**:332–335.
12. **Cussac, V., R. L. Ferrero, and A. Labigne.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J. Bacteriol. **174**:2466–2473.
13. **Dunn, B. E., G. P. Campbell, G. I. Pérez-Pérez, and M. J. Blaser.** 1990. Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265**:9464–9469.
14. **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.

15. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. **57:**1119–1125.
16. **Evans, D. G., D. J. Evans, 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.
17. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
18. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174:**4212–4217.
19. **Ferrero, R. L., and A. Lee.** 1991. The importance of urease in acid protection for the gastric colonizing bacteria *Helicobacter pylori* and *Helicobacter felis* sp. nov. Microb. Ecol. Health Dis. **4:**121–134.
20. **Grant, C. C. R., M. E. Konkel, W. Cieplak, Jr., and L. S. Tompkins.** 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. **61:**1764–1771.
21. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. **153:**658–663.
22. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173:**1920–1931.
23. **Labigne-Roussel, A., P. Courcoux, and L. Tompkins.** 1988. Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. J. Bacteriol. **170:**1704–1708.
24. **Lee, A., J. Fox, and S. Hazell.** 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. **61:**1601–1610.
25. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.
26. **Nomura, A., G. N. Stemmerman, P. H. Chyou, I. Kato, G. I. Pérez-Pérez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma in a population of Japanese-Americans in Hawaii. N. Engl. J. Med. **325:**1132–1136.
27. **Parsonnet, J., G. D. Friedman, and D. P. Vandersteen, et al.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127–1131.
28. **Pérez-Pérez, G. I., and M. J. Blaser.** 1987. Conservation and diversity of *Campylobacter pyloridis* major antigens. Infect. Immun. **55:**1256–1263.
29. **Peterson, W.** 1991. *Helicobacter pylori* and peptic ulcer disease. N. Engl. J. Med. **324:**1043–1048.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning; a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology, p. 98–105. Springer Verlag, New York.
32. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzand, E. K. Fujimoto, N. M. Goekf, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using Bicinchoninic acid. Anal. Biochem. **150:**76–85.
33. **Smoot, D. T., H. L. T. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect. Immun. **58:**1992–1994.
34. **Tummuru, M. K. R., T. L. Cover, and M. J. Blaser.** 1993. Cloning and expression of a high molecular mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect. Immun. **61:**1799–1809.
35. **Weitkamp, J.-H., G. I. Pérez-Pérez, G. Bode, P. Malfertheiner, and M. J. Blaser.** 1993. Identification and characterization of *Helicobacter pylori* phospholipase C activity. Zentralbl. Bakteriol. **280:** 11–27.