*J. Microbiol. Biotechnol.* (2001), **11**(1), 35–39

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY



# Enzyme-Linked, Biotin-Streptavidin Bacterial-Adhesion Assay for *Helicobacter pylori* Lectin-Like Interactions with Cultured Cells

**GUZMAN-MURILLO, MARIA-ANTONIA AND FELIPE ASCENCIO***

*Center for Biological Research, Department of Marine Pathology, La Paz, BCS 23000, Mexico*

Received: June 14, 2000
Accepted: September 20, 2000

**Abstract** A simple method for studying the lectin-like interactions between *Helicobacter pylori* and cultured human epithelial cell lines was developed using an enzyme-linked, biotin-streptavidin bacterial-adhesion assay. The present study suggests that this method is suitable for evaluating the participation of lectin interactions in the adhesion of *H. pylori* to cultured HeLa S3 and Kato III cells, both fixed and glycosidase-treated cells, as well as assessing glycoconjugated binding inhibition studies. The time-course and dose-dependent kinetics of the biotin-labeled *H. pylori* adhesion to the formaldehyde-fixed Hela S3 and Kato III cell lines exhibited saturation. In addition, the binding of the biotin-labeled *H. pylori* to the formaldehyde-fixed cultured cells was partially blocked by pre-incubation with glycoconjugates and polyclonal antibodies against a heparan sulfate binding protein from *H. pylori*.

**Key words:** *Helicobacter pylori*, adhesion, lectins, HeLa S3, Kato III, heparan sulfate, proteoglycans

*H. pylori* is a causative agent of peptic ulcer diseases, chronic gastritis, and gastric cancer in humans [7]. About 95% of patients with duodenal ulcers and perhaps 80% of patients with gastric ulcers are infected with this bacterium and its eradication greatly diminishes the recurrence of these ulcers [3]. Of those infected, the subsequent development of disease is influenced by the virulence of the infecting *H. pylori* strain, the genetic susceptibility of the host, and environmental co-factors [2]. Although the vacuolating toxin of *H. pylori* is know to be a major pathogenetic factor, adhesion factors are also important for host colonization, especially during the early stages of the infection process.

Many putative adhesins of *H. pylori* have been previously identified. These include haemaglutinins [12, 16], adhesin binding to GM3 ganglioside, sulfatides, and glycosaminglycans [1, 19, 22], and extracellular matrix-binding proteins [15, 26]. The role, if any, of these adhesins and receptors in the colonization of the gastric mucosa has not been fully determined. Due to the lack of a convenient animal model, there is a heavy dependence on *in vitro* methods to study the adherence of *H. pylori*. These methods include the use of an enzyme-linked immunosorbent assay (ELISA) [18], microscopy [17, 18], and flow cytometry [14]. In spite of the strict tissue tropism exhibited *in vivo*, *H. pylori* has been shown to bind to a large range of cell types *in vitro*. These cells include human gastric epithelial cells [4, 9], gastric carcinoma cells [5, 14], and non-gastric epithelial cells, such as Hep-2 cells, Int-407 cells [21], and HeLa cells [6].

Accordingly, since most of the interactions of bacterial pathogens with host cell receptors are mediated by lectin-like interactions, the aim of this study was to set up an *in vitro* model to study the lectin-like interactions between *H. pylori* and gastric and non-gastric epithelial cells. The results suggest that the proposed enzyme-linked, biotin-streptavidin bacterial binding assay significantly reduces the need to work with live cells or bacteria.

## Materials and Methods

### Bacteria and Growth Conditions

The *H. pylori* strain 25 was obtained from Professor T. Wadström, Department of Medical Microbiology, University of Lund, Sweden. The strain was cultured on a GAB-CAMP (BBL, Becton, Dickinson Comp., U.S.A.) agar medium supplemented with 8.5% lysed human blood (80°C, 20 min), 10% inactivated horse serum (56°C, 30 min), 0.05% cysteine hydrochloride, 0.35% IsoVitaleX, and the following antibiotics: vancomycin (6 μg/ml), nalidixic acid (20 μg/ml), and ketoconazole (3 μg/ml), and incubated at 37°C for 2–3 days under microaerophilic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) [23].

*Corresponding author
Phone: 52-112-5-3633; Fax: 52-112-5-4710;
E-mail: ascencio@cibnor.mx

For the cell-adhesion studies, *H. pylori* grown on GAB-CAMP agar plates, as described above, was harvested and washed twice in PBS. The bacterial cell suspensions were adjusted to a density of $10^9$ cfu/ml and then labeled with n-biotin, as described elsewhere [8]. The stock cultures of *H. pylori* were stored at 80°C in trypticase soy broth containing 15% glycerol.

### HeLa S3 and Kato III Cell Lines

HeLa S3 cells, derived from a human epithelioid carcinoma, and Kato III, a gastric adenocarcinoma cell line, were obtained from the American Type Culture Collection (Mannasas, VA, U.S.A.). The HeLa S3 and Kato III cells were grown in an RPMI-1640 medium (Sigma Chemical Co., St. Louis, U.S.A.) containing 40 µg gentamicine per ml, 20 mmol/l HEPES buffer, and 12 mmol/l sodium bicarbonate supplemented with either 15% (vol/vol) fetal calf serum for the Kato III cells, or 10% (vol/vol) for the HeLa S3 cells, in 75 $cm^2$ polystyrene tissue culture flasks at 37°C (95% humidity and 5% $CO_2$). For the adherence assays, the cells attached to the culture flask were scraped with a rubber policeman (Costar, Cambridge, MA, U.S.A.) and centrifuged at 200 ×g for 5 min. The cells were then suspended in a fresh cell medium to a concentration of $4\times10^7$ cells/ml and then plated onto 96-well tissue culture plates and incubated at 37°C until the formation of a semiconfluent cell monolayer.

### Enzyme-Linked, Biotin-Streptavidin Bacterial-Adhesion Assay

The semiconfluent cell layers (0.5 to $1.0\times10^5$ cells/well) grown on 96-well tissue culture plates were washed 3 times in an RPMI-1640 medium without antibiotics and incubated for 1 h at 25°C with a suspension of biotin-labeled *H. pylori* cells in an RPMI-1640 medium without antibiotics ($10^9$ CFU $ml^{-1}$). After incubation, the plates were washed 3 times with PBS containing 0.05% Tween-20 to remove any non-adhering bacteria. One-hundred µl of horseradish peroxidase (POD)-conjugated streptavidin (diluted 1:2,000 in PBS) was added to each well and the plates were incubated for 90 min at 25°C. After washing the plates 3 times with PBS-Tween 20, 100 µl of o-phenylenediamine were added to each well, and the plates were incubated for an additional 20 min at 22°C in the dark. The reaction was stopped by the addition of 100 µl of 2 M $H_2SO_4$ and the color development was measured at 495 nm. The results of the adhesion of the biotin-labeled *H. pylori* to the HeLa S3 and Kato III cell lines were expressed as an optical density.

Other adhesion studies were performed as follows. (A) semiconfluent monolayers were fixed with either (i) methanol for 5 min at 4°C, (ii) 2.5% glutaraldehyde in a 0.1 $mol^{-1}$ phosphate buffer (pH 7.2) for 15 min at 37°C, or (iii) 0.5% formaldehyde in a 0.1 $mol^{-1}$ phosphate buffer (pH 7.2) for 30 min at 37°C, washed five times with PBS-Tween 20, and then 200 µl of 3% bovine serum albumin were added to each well. After 1 h of incubation at 37°C, the plates were washed three times with PBS-Tween 20 and used for the enzyme-linked, biotin-streptavidin bacterial-adhesion assay, as described above. (B) Glycosidase-treated cell monolayers. Formaldehyde-fixed cell monolayers were treated with several glycosidases. *Bacteroides fragilis* keratanase (Endo-β-galactosidase) (6 mU/ml) digestion was performed at 37°C for 24 h in a 0.05 M sodium acetate buffer (pH 5.8) containing bovine serum albumin (0.2 mg/ml). *Escherichia coli* β-galactosidase (1 U/ml) digestion was performed in a 0.1 M sodium citrate buffer (pH 4.3) and the reaction was run at 37°C for 16 h. *Bacillus stearothermophilus* α-glucosidase (0.5 mg/ml) digestion was performed in a 0.2 M sodium acetate buffer (pH 5) at 37°C for 16 h. The cells was treated with *Clostridium perfringens* type V neuraminidase (5 U/ml) at 37°C for 16 h in a 50 mM sodium acetate buffer (pH 5.5) containing 4 mM $CaCl_2$ and 0.1 mg of bovine serum albumin. Digestions with *Flavobacterium heparinum* heparinase I (0.1 U/ml), *Proteus vulgaris* chondroitinase ABC (0.1 U/ml), and bovine testes hyaluronidase Type VI-S (1 U/ml) were performed at 37°C for 24 h in a 0.05 M sodium acetate buffer (pH 5.8) containing bovine serum albumin (0.2 mg/ml). After each treatment, the monolayers were washed twice three times with PBS and blocked with 3% bovine serum albumin for 1 h at 37°C. Following the washing with PBS-Tween 20, the plates were then used for the enzyme-linked, biotin-streptavidin bacterial-adhesion assay, as described above.

For bacterial adherence inhibition studies, 100 µl of the biotinated bacterial suspension were pre-incubated with 100 µl of either *Fucus vesiculosus* fucoidan, Type IV lambda carrageenan, bovine milk lactoferrin, porcine intestinal mucosa heparin, Type I fetal calf serum asialofetuin, or dextran sulfate at a final concentration of 1 mg per ml. Similarly, HeLa S3 cells were pre-incubated with 100 µl of rabbit antiserum against a 71.5-kDa heparan sulfate binding protein isolated from *H. pylori* (Ruiz-Bustos *et al.*, in press), diluted 1:10 in PBS. After a 1 h pre-incubation, the bacterial adherence assay proceeded as described above. The results are expressed as a percentage of the inhibition of the bacterial adhesion relative to the control (without an inhibitor).

## RESULTS

### Fixation of Semiconfluent Cell Monolayer

Because of the nature of the adhesion assays in this study, the first objective was to standardize an adhesion assay that would allow for the preparation of a semiconfluent cell monolayer on 96-well tissue culture plates, such that

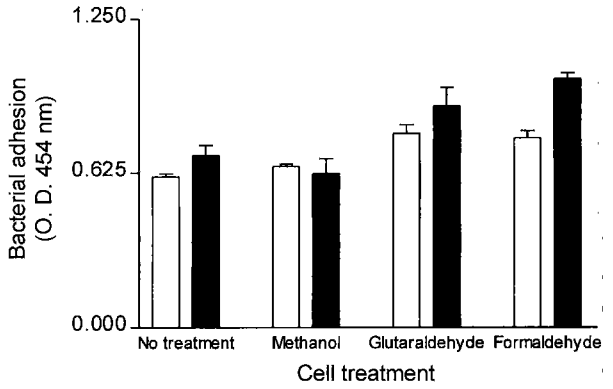


**Fig. 1.** Adhesion of *H. pylori* to live and fixed HeLa S3 (empty columns) and Kato III (full columns ) cell lines.

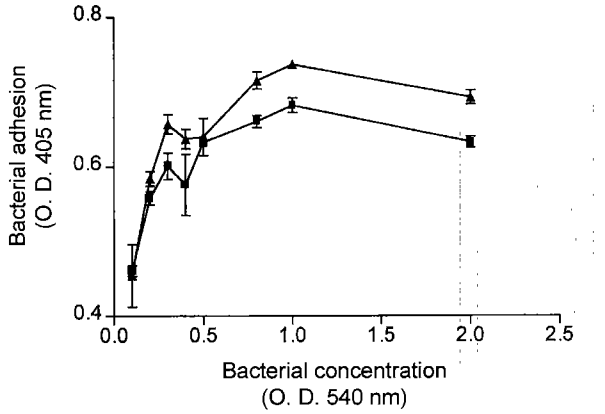


**Fig. 3.** Dose-course kinetics of *H. pylori* adhesion to formaldehyde fixed HeLa S3 (■) and Kato III (▲) cell lines.

the cells remained bound to the plastic under different conditions of pH, ionic strength, and buffer composition.

Accordingly, the semiconfluent HeLa S3 and Kato III cell monolayers were treated with methanol, glutaraldehyde, and formaldehyde, as described in the Materials and Methods. Once the semiconfleunet cell monolayers were fixed and blocked with 3% BSA, the biotinated *H. pylori* cell suspension was added and the bacterial binding assay continued, as described in the Materials and Methods. The treatment with methanol resulted in a decrease in the adhesion of the *H. pylori* to the HeLa S3 and Kato III cells (Fig. 1), whereas the glutaraldehyde and formaldehyde fixation procedures induced a small increment in the adhesion of the *H. pylori* to both the HeLa S3 and Kato III cells (Fig. 1).

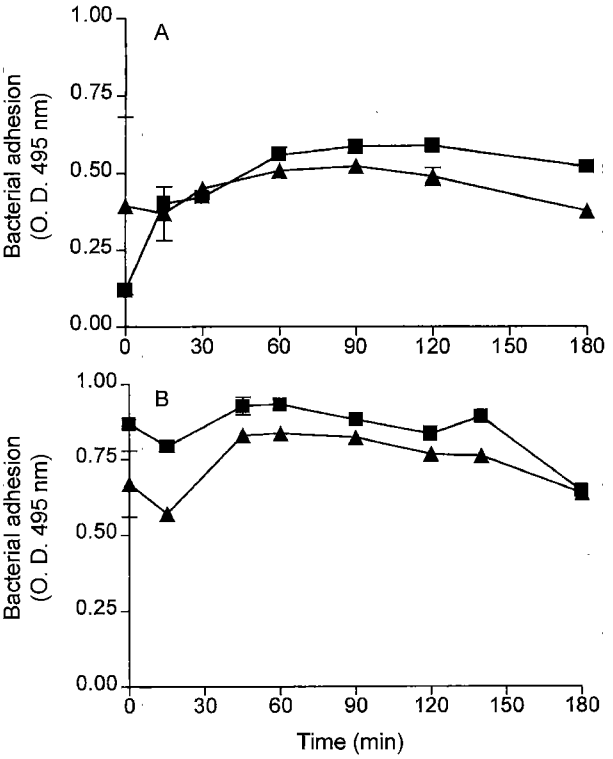


**Fig. 2.** Time-course kinetics of *H. pylori* adhesion to live (Panel A) and formaldehyde-fixed (Panel B) HeLa S3 (■) and Kato III (▲) cell lines.

### Time-Course Kinetics of *H. pylori* 25 Adhesion to HeLa S3 and Kato III Cell Lines

The saturation of the biotin-labeled *H. pylori* adherence to the HeLa S3 and Kato III cells occured withing 1 h of incubation, with both live cultured cells (Fig. 2A) and formaldehyde-fixed cultured cells (Fig. 2B). However, the data suggested that *H. pylori* strain 25 showed a tendency to adhere more to the formaldehyde-fixed cultured cell preparations.

### Dose-Course Adherence of *H. pylori* 25 to Formaldehyde-Fixed HeLa S3 and Kato III Cell Lines

Figure 3 shows that *H. pylori* strain 25 tended to adhere more to the HeLa S3 cells than to the Kato III cells; however, there was a dose-dependant effect in the bacterial adhesion kinetics of both cell lines.

### Effect of Glycolytic Treatment of Cultured Cells on *H. pylori* Attachment

Assuming that lectin-like interactions are important in the adhesion of *H. pylori* to human gastric epithelial cells, it was also tested whether the formaldehyde-fixed HeLa S3 and Kato III cells could withstand the glycosidase removal of the carbohydrate moieties exposed on the cultured cells and still remain attached to the plastic 96-well plate and allow *H. pylori* adhesion. Figure 4 shows that both glycosidic treatments affected the carbohydrates exposed on the cultured cells thereby inhibiting the *H. pylori* cell-adhesion. This was particularly true for the chondroitinase-treated cells, where a significant inhibition of the adhesion of the biotin-labeled *H. pylori* to the HeLa S3 cells was

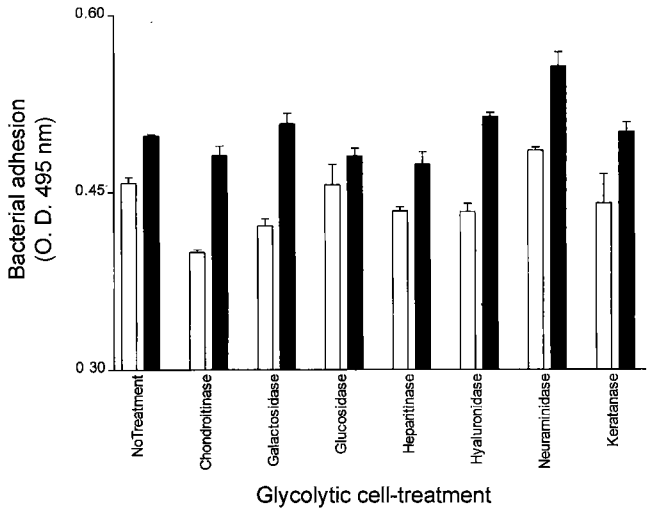


**Fig. 4.** Effect of glycolytic treatments of HeLa S3 (empy columns) and Kato III (full columns) cell lines on *H. pylori* attachment.

observed, an well as the heparitinase treatment of the Kato III cells, which also reduced the binding of the biotin-labeled *H. pylori* (Fig. 4).

To demonstrate the value of the proposed assay for studying the surface-exposed lectin-like activity in *H. pylori*, blockade studies with glycoconjugates and polyclonal antibodies against a heparan sulfate-binding protein (HSBP) from *H. pylori* were performed. The pre-incubation of *H. pylori* with both the glycoconjugates and the anti-HSBP reduced the adhesion of the bacteria to the cultured cells (Table 1).

## DISCUSSION

The ability of *H. pylori* to adhere and colonize gastric cells is believed to play a central role in the infection of the human stomach [25]. The cellular events associated with the *H. pylori* colonization of gastric epithelial cells are complex and include the adhesion of the pathogen to gastric epithelial cells by means of surface exposed adhesive components, invasion of the gastric epithelium via an integrin-mediated pathway [25], reorganization of the host cell actin, tyrosin phosphorylation of a 145-kDa protein, and cytokine release [20].

**Table 1.** Inhibition of *H. pylori* adhesion to HeLa S3 cells.

| Inhibitor | % Inhibition of bacterial adherence |
|---|---|
| No Inhibitor | 0 |
| Asialofetuin | 0 |
| λ-Carragenin | 0 |
| Dextran sulfate | 43 |
| Fucoidan | 0 |
| Heparin | 44 |
| Lactoferrin | 0 |
| Anti-HSBP | 51 |

However, the adhesion factors seem to be most important, because the successful colonization of the host and the subsequent expresion of pathogenicity stems from the ability of the pathogen to adhere to its target host gastric mucosa receptors. This is one of the reasons why alternative treatments for the prevention and control of *H. pylori* infections have focused on blocking the bacterial adhesion.

Lectin-like interactions are reported to play a key role in the adhesion of *H. pylori* to gastric epithelial cells [4, 24]. Therefore, this study was conducted to standardize an alternative approach to flow cytometric, microscopic, ELISA, and radiolabeled methods to study lectin-like interactions.

The present work showed that the adherence of biotin-labeled *H. pylori* to formaldehyde-fixed culture cells is an alternative methodology for studying the lectin-like interactions involved in the bacterial adherence process. Time-course studies demonstrated the binding saturation of biotin-labeled *H. pylori* within 1 h. Similarly, the dose-course kinetics of biotin-labeled *H. pylori* adhesion to formaldehyde-fixed Hela S3 and Kato III cell lines also showed saturation. In addition, the binding of biotin-labeled *H. pylori* to formaldehyde-fixed cultured cells was partially blocked by pre-incubation with glycoconjugates and polyclonal antibodies against a Heparan sulfate binding protein from *H. pylori*.

Su *et al.* [24] demonstrated that the adherence of *cag*-positive *H. pylori* to formalin-fixed normal and metaplastic cells of human gastric tissue is dependent on the expression of $Le^b$ oligosaccharide in the tissue and on the ability of an FITC-labeled microbe to express one or more adhesions factors. In addition, they found that the attachment of *H. pylori* to cultured cells is independent of the $Le^b$ epitope, and that the adherence of a *cag*-positive *H. pylori* strain, rather than *cag*-negative *H. pylori* strains, was significantly more prominent to viable AGS cells than formalin-fixed cells, thereby suggesting that the adherence of *cag*-positive *H. pylori* strains to cultured AGS cells requires viable target cells and bacteria with an intact protein synthesis.

It has not yet been determined whether the *H. pylori* strain used in the present work includes the *cag* gene. However, since the adherence to the formaldehyde-fixed Hela S3 (which does not express the $Le^b$ antigen) and Kato III cells was not significantly different from the adherence to live cells, it would appear that *H. pylori* strain 25 is *cag*-negative. Most importantly, however, neither the biotinization of *H. pylori* nor the formaldehyde fixation of the cultured cells interfered with the studies of the lectin-like interactions between the *H. pylori* and the cultured cells. Accordingly, this provided the opportunity to prepare both bacteria and cultured cells in a single batch for a set of studies without the need to prepare further fresh cells or

bacteria cells for the lectin studies, since after the fixation of the cultured cells with formaldehyde, these cells could be stored for several days at 4°C without any apparent deterioration of the cell monolayer (data not shown). Similarly, the biotin-labeled *H. pylori* suspension could be stored at 4°C for a least 3 days and still used for the lectin studies (data not shown).

## Acknowledgments

This work was supported by grants from the Center for Biological Research (ABM-11 and ABM-27). MAGM was the recipient of a doctoral scholarship from the Mexican Council of Science and Technology. The authors also wish to thank Dr. T. Zenteno-Savin for her comments and editing of the English-language text.

## REFERENCES

1. Ascencio, F., L.-Å. Fransson, and T. Wadström. 1993. Affinity of the gastric pathogen *Helicobacter pylori* for the N-sulphated glycosaminoglycan heparan sulphate. *J. Med. Microbiol.* **38:** 240–244.
2. Atherton, J. C. 1998. *H. pylori* virulence factors. *Brit. Med. Bull.* **54:** 105–120.
3. Calam, J. 1998. Clinical science of *Helicobacter pylori* infection: Ulcer and NSAIDs. *Brit. Med. Bull.* **54:** 55–62.
4. Clyne, M. and B. Drumm. 1993. Adherence of *Helicobacter pylori* to primary human gastrointestinal cells. *Infect. Immun.* **61:** 4051–4057.
5. Dunn, B. E., M. Altman, and G. P. Campbell. 1991. Adherence of *Helicobacter pylori* to gastric carcinoma cells: Analysis by flow cytometry. *Rev. Infect. Dis.* **13 (Suppl. 8):** 657–664.
6. Fauchere, J. L. and M. J. Blaser. 1990. Adherence of *Helicobacter pylori* cells and their surface components to HeLa cell membranes. *Microb. Pathog.* **9:** 2896–2906.
7. Forman, D. 1998. *Helicobacter pylori* infection and cancer. *Brit. Med. Bull.* **54:** 71–78.
8. Harlow E. and D. Lane. 1988. *Antibodies. A Laboratory Manual*, pp. 319–359. Cold Spring Harbor Laboratory Press, New York, U.S.A.
9. Hemalatha, S. G., B. Drumm, and P. Sherman. 1991. Adherence of *Helicobacter pylori* to human gastric epithelial cells *in vitro*. *J. Med. Microbiol.* **35:** 197–202.
10. Huang, J., C. J. Smyth, N. P. Kennedy, J. P. Arbuthnott, and P. W. N. Keeling. 1988. Haemagglutination activity of *Campylobacter pylori*. *FEMS Microbiol. Letters* **56:** 109–112.
11. Huang, J., P. W. N. Keeling, and C. J. Smyth. 1992. Identification of erythrocyte binding antigens in *Helicobacter pylori*. *J. Gen. Microbiol.* **138:** 1503–1513.
12. Lelwala-Guruge, J., Å, Ljungh, and T. Wadström. 1992. Haemagglutination patterns of *Helicobacter pylori*: Frequency of sialic acid-specific and non-sialic acid-specific haemagglutinins. *Acta Pathol. Microbiol. Immun. Scan.* **100:** 908–913.
13. Lingwood, C. A., M. Huesca, and A. Kukiss. 1992. The glycerolypid receptor for *Helicobacter pylori* (and exoenzyme S) is phosphatidylethanolamine. *Infect. Immun.* **60:** 2470–2474.
14. Logan, R. P. H., A. Robins, G. A. Turner, A. Cockayne, S. P. Borriello, and C. J. Hawkey. 1998. A novel flow cytometric assay for quantitating adherence of *Helicobacter pylori* to gastric epithelial cells. *J. Immun. Meth.* **213:** 19–30.
15. Moran, A. P., P. Kuusela, and T. U. Kosunen. 1993. Interaction of *Helicobacter pylori* with extracellular matrix proteins. *J. Appl. Bacteriol.* **75:** 184–189.
16. Nakazawa, T., M. Ishibashi, H. Konishi, T. Takemoto, M. Sigeeda, and T. Kochiyama. 1989. Hemagglutination activity of *Campylobacter pylori*. *Infect. Immun.* **57:** 989–991.
17. Ogata, M., K. Araki, and T. Ogata. 1998. An electron microscopic study of *Helicobacter pylori* in the surface mucus gel layer. *Histology and Histopathology* **13:** 347–358.
18. Osaki, T., H. Yamaguchi, H. Taguchi, M. Fukuda, H. Kawakami, H. Hirano, S. Watanabe, A. Takagi, and S. Kamiya. 1998. Establishment and characterization of a monoclonal antibody to inhibit adhesion of *Helicobacter pylori* to gastric epithelial cells. *J. Med. Microbiol.* **47:** 505–512.
19. Saitoh, T., H. Natomi, W. L. Zhao, K. Sugano, M. Iwamori, and Y. Nagai. 1991. Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. *FEBS Lett.* **282:** 385–387.
20. Segal, E. D., C. Lange, C. Covacci, L. S. Tompkins, and S. Falkow. 1997. Induction of host signal transduction pathways by *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* **94:** 7595–7599.
21. Simon, P. M., P. L. Goode, A. Mobasseri, and D. Zopf. 1997. Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect. Immun.* **65:** 750–757.
22. Slomiany, B. L., J. Piotrowski, A. Samanta, K. VanHorn, V. L. N. Murty, and A. Slomiany. 1989. *Campylobacter pylori* colonization factor shows specificity for lactosylceramine sulfate and GM3 ganglioside. *Biochem. Int.* **19:** 929–936.
23. Soltesz, V., C. Schalen, and P.-A. Mardh. 1988. New selective medium for *Campylobacter pylori*. *In* B. Kaijser and E. Falsen (eds.), *Proceeding of the Fourth International Workshop on Campylobacter infection*, pp. 433–436. Gothenbörg, Sweden.
24. Su, B., M. Hellström, C. Rubio, J. Çelik, M. Granström, and S. Normark. 1998. Type I *Helicobacter pylori* shows Lewis$^b$-independent adherence to gastric cells requiring *de novo* protein synthesis in both host and bacteria. *J. Infect. Dis.* **178:** 1379–1390.
25. Su, B., S. Johansson, M. Fällman, M. Patarroyo, M. Granström, and S. Normark. 1999. Signal transduction-mediated adherence and entry of *Helicobacter pylori* into cultured cells. *Gastroenterology* **117:** 595–604.
26. Trust, T. J., P. Doing, L. Emödy, Z. Kienle, T. Wadström, and P. O'Toole. 1991. High-affinity binding of the basement membrane proteins collagen type IV and laminin to the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* **59:** 4398–4404.