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# Growth and Morphological Transformations of *Helicobacter pylori* in Broth Media

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***Helicobacter pylori*, a cause of peptic ulcer disease and certain types of gastric cancers, has usually been cultured on diverse agar-based media, resulting in a requirement for 2 to 4 days of growth at 37°C. We have developed a novel broth medium consisting of a base medium supplemented with 2% newborn calf serum, $Mg^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, and 1 mg of lysed human erythrocytes per ml. This medium supports rapid growth of *H. pylori*, with a doubling time of about 50 min. Optimal growth was obtained in a pH range higher than that supporting most other gram-negative bacteria (at pH 8.5). *H. pylori* cultured in this supplemented broth retains the spiral morphology seen in both histological sections and cultures from agar-based media and also retains a high urease activity. After 18 h in this broth, *H. pylori* transforms to a coccal form with a complete loss of urease activity. Previously these cocci have been reported to be senescent, since they could not be subcultured on agar medium. Our experiments suggest that some of the cocci can revert back to the spiral morphology with full recovery of urease activity when subcultured in fresh microaerobic broth medium.**

Increasing evidence has linked the presence of *Helicobacter pylori*, a gram-negative curved-rod- or spiral-shaped bacterium, with the development of gastritis and peptic ulcer disease (4, 10, 20). *H. pylori* resides primarily in the gastric mucosa without invading the gastric epithelium, causing persistent low-grade gastric inflammation (2). Diagnosis of active *H. pylori* infection usually requires visualization of the bacteria in sections of gastric biopsy material, culture of *H. pylori* from gastric biopsies, and/or testing of such biopsies for urease activity (CLO test) (12, 13, 16, 18). Noninvasive methods include the use of a labeled urea breath test (14, 22) and an enzyme-linked immunosorbent assay for measuring levels of anti-*H. pylori* immunoglobulin G antibodies in patient serum (3, 18, 20, 21).

The culture results obtained from gastric biopsies are highly dependent upon the accuracy of the biopsy sampling and the bacterial load in the tissue sample (18). The conditions for optimal growth are of major importance, since this organism does not tolerate prolonged exposure to air (6) and biopsy growth requires 3 to 6 days in special cultivation medium (1). *H. pylori* assumes a coccal form, which may not be recognized in histological sections, under certain conditions (13). Older cultures transform to the coccal form, which was previously thought to be senescent, with no urease activity and an associated decrease in ability to be subcultured (12).

The aim of this study was to develop an appropriate liquid culture medium that supports faster growth of *H. pylori* as well as permitting the growth of large quantities of bacteria for protein purification or RNA-DNA isolation. We present data on the composition of such a liquid medium. We also report our observations that the coccal form of *H. pylori* is not senescent but rather is a dormant form of the bacterium which can revert to spiral morphology when conditions are appropriate. This morphological reversal is accompanied by full recovery of urease activity and the ability to be subcultured.

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## MATERIALS AND METHODS

Freeze-dried cultures of *H. pylori* were purchased from the American Type Culture Collection (ATCC 43504) and propagated after rehydration on ATCC medium 1115. The bacteria were either frozen at −70°C by the ATCC protocol or maintained on either Skirrows medium (BBL Microbiology Systems, Cockeysville, Md.) or CHOC II medium (BBL). Plates were incubated at 37°C in a microaerobic environment supplied by the Campy Pak Plus system (BBL) or by the Bio-Bag environmental chamber (BBL).

The basic liquid medium contained 25 mg of Bacto Tryptone (Difco, Detroit, Mich.) per ml, 7.5 mg of Bacto Yeast extract (Difco) per ml, 0.4 μM $CuSO_4$ (Sigma, St. Louis, Mo.), 0.35 μM $ZnSO_4$ (Fisher, Springfield, N.J.), 0.36 μM $FeSO_4$ (Mallinckrodt, St. Louis, Mo.), 0.24 μM $MnSO_4$ (Sigma), and 20 mM Tris base (Sigma) (pH 8.5); the mixture was autoclaved for 20 min, and 5 μl of sterile filtered 1 M $MgCl_2$ (Sigma) was added. The final liquid medium contained the basic liquid medium plus 1 mg of erythrocyte lysate per ml and 2% newborn calf serum. The system used to cultivate the organisms consisted of a 250-ml Erlenmeyer flask holding 50 ml of medium; the flask was sealed with a rubber stopper, and the medium was equilibrated with a Campy-Pak cartridge.

Erythrocyte lysate, prepared from citrated human blood, was sedimented (2,500 × *g* for 10 min), washed twice in saline, lysed with a 2× volume of distilled water, and centrifuged (40,000 × *g* for 15 min), and the supernatant was filtered through a 0.22 μm-pore-size sterile filter. The protein concentration was determined by refractometer and adjusted to 80 mg/ml, and the lysate was stored at −20°C.

Newborn calf serum was obtained from Gibco BRL (Grand Island, N.Y.) and sterile filtered prior to use.

Urease activity was detected in saline-washed bacteria by either the CLO test (Delta West Pty. Ltd.) or spectrophotometrically, with 100 mM urea (Ultrapure; Sigma), 0.9% NaCl, and 0.2 mM phenol red (Becton Dickinson; Franklin Lakes, N.J.) at 560 nm and jack bean urease type VI (100,000 to 150,000 U/g; Sigma) as a standard.

Growth was measured by reading the optical density of saline-washed bacterial cultures at 600 nm ($OD_{600}$).

Microscopic control of the cultures was performed by using Gram stain (Difco) on a Zeiss inverted microscope.

Transformation of the *H. pylori* coccal form to the spiral form was performed with 72-h-old cultures devoid of any detectable urease activity. The bacteria were washed twice in phosphate-buffered saline (PBS), resuspended in the base medium, and assayed in a glass-bottomed microwell tray (MatTek Corp., Ashland, Mass.) covered with a glass coverslip to reduce oxygen diffusion. The experimental chambers were then placed on a microscope stage maintained at 37°C. *H. pylori* cells were monitored with a Leitz diavert microscope (Leitz, Wetzlar, Germany) equipped with Nomarski differential interference (DIC) optics. Im-

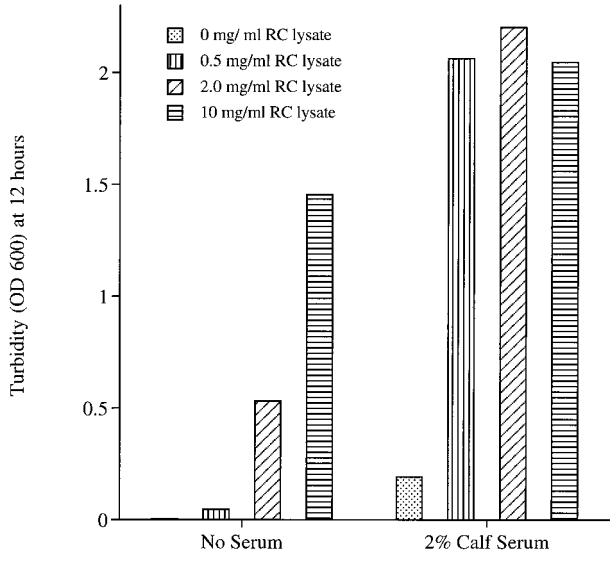


FIG. 1. Basic broth medium, prepared as described in Materials and Methods, was supplemented with human erythrocyte (RC) lysate and/or 2% newborn calf serum. Growth was measured after 12 h of incubation at 37°C with shaking. A representative experiment ($n = 4$) is shown.

ages were obtained with a video camera (CCD-72; Dage-MTI Inc., Michigan City, Ind.) at intervals of 2 min, for a total of 50 min. These images were then processed by using Adobe Photoshop (Mountainview, Calif.) and printed by a Phaser IISDX printer (Tektronix Inc., Beaverton, Oreg.).

## RESULTS

*H. pylori* cells obtained from the ATCC and stored at −70°C were cultured microaerobically at 37°C on commercial Skirrows or CHOC II medium. Visible colonies appeared as approximately 1-mm translucent colonies at day 2 to 3 on Skirrows medium and 1 day later on CHOC II. However, colonies from frozen (−70°C) cells were generally observed at day 5. Gram stains revealed mostly spiral or curved-rod morphology at day 2 to 3, with the coccal morphology dominant at day 5. A documented urease activity test showed decreased activity at day 5 compared to day 3.

The broth medium first tested was a mixture of Bacto Tryptone, Bacto Yeast extract, and 20 mM Tris (pH 7.5) containing 5 mM $MgCl_2$ and maintained microaerobically at 37°C. The inoculum for this broth was derived from bacteria cultured on Skirrows medium for 3 days, when urease activity was at maximum. This medium yielded minimal bacterial growth, with high urease activity for at least five days. In the absence of $Mg^{2+}$, no growth was observed (data not shown). The effect of erythrocyte lysate added to the basic medium was an increase in both culture yield and urease activity. Figure 1, giving results of a representative experiment (of the four that were performed), shows that the addition of erythrocyte lysate and 2% newborn calf serum substantially increased the turbidity. Also, the urease activity was affected, as shown in Fig. 2 (results of a representative experiment of the three that were performed). Two percent calf serum in the presence of erythrocyte lysate gave the highest yield of *H. pylori*. When *H. pylori* was cultured in the presence of concentrations of erythrocyte lysate above 1 mg/ml, a precipitate formed, which interfered with the yield of *H. pylori* when harvested. This precipitate did not occur in controls without bacteria. The base medium supplemented with trace elements ($Cu^{2+}$, $Zn^{2+}$, $Fe^{2+}$, and $Mn^{2+}$) supported growth of the organism at a level 30% of that obtained in medium supplemented with 0.5 mg of erythrocyte lysate per ml but five times the level obtained with 2% calf serum alone (data not shown). The fastest growth was observed when serum, trace elements, and erythrocyte lysate were all used.

The optimum pH of *H. pylori* was tested in a medium containing 2% serum, trace elements, and 1 mg of erythrocyte lysate per ml in a pH range of 6 to 10. The flasks were left to equilibrate in the microaerobic environment for 15 h with shaking at 37°C before the inoculum of *H. pylori* was added. During equilibration, the pH changed due to the increased $CO_2$ from the cartridge supplying the microaerobic environment. Figure 3 shows results of a representative experiment (of the six that were performed), demonstrating the turbidity obtained at 12 and 24 h after inoculation. Urease activity and growth rate are related; thus, cultures at pH 6 and 7 show very slow growth and minimal urease activity. At pH 8 there was a 35-fold increase in urease activity (data not shown) compared to pH 7 at the 8-h time point. Even at the optimal pH, the urease activity was reduced during the lag phase. At 24 h the urease activity was absent in cultures of pH 8 to 9.5 and all cells had changed to the coccal morphology. Slower but sustained growth was observed at lower pHs, and urease activity was still present at pH 7.0 after 24 h (data not shown) but disappeared after 2 to 3 days as full growth was accomplished. Our measurements of the commercial media often used to culture *H. pylori*, such as Skirrows and CHOC II, gave pHs of 6.9 and 7.0, respectively, without adjustment for the $CO_2$ effect on pH, indicating suboptimal growth conditions.

The optimal medium for best yield and urease activity was thus determined to be a Bacto Tryptone (25 mg/ml)–Bacto Yeast extract (7.5 mg/ml)-based medium with trace minerals and 20 mM Tris (pH 8.5) supplemented with 5 mM $MgCl_2$, 2% newborn calf serum, and 1 mg of erythrocyte lysate per ml. This medium provides a maximal growth rate, with a doubling time of 50 min, and a maximal $OD_{600}$ of 2 to 3 (data not shown). The cells obtained during the first 12 h produced a white pellet of mostly spiral morphology and were positive for urease activity, as seen by the CLO test (within seconds).

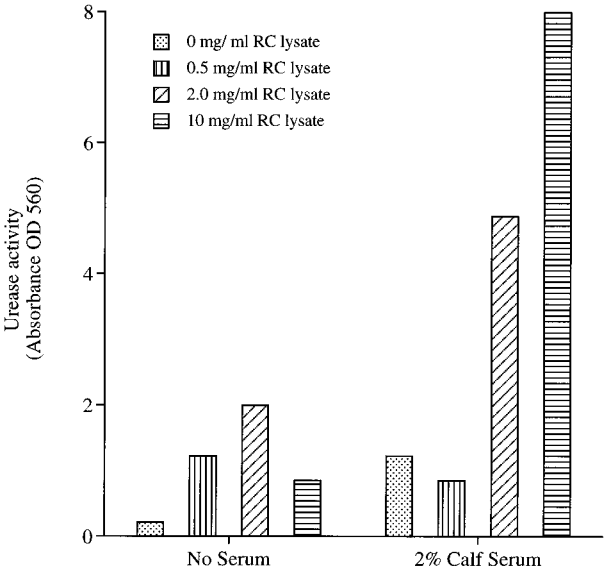


FIG. 2. Basic broth medium, prepared as described in Materials and Methods, was supplemented with human erythrocyte lysate (RC) and/or 2% newborn calf serum. Urease activity was measured after 12 h of incubation at 37°C with shaking. A representative experiment ($n = 3$) is shown.

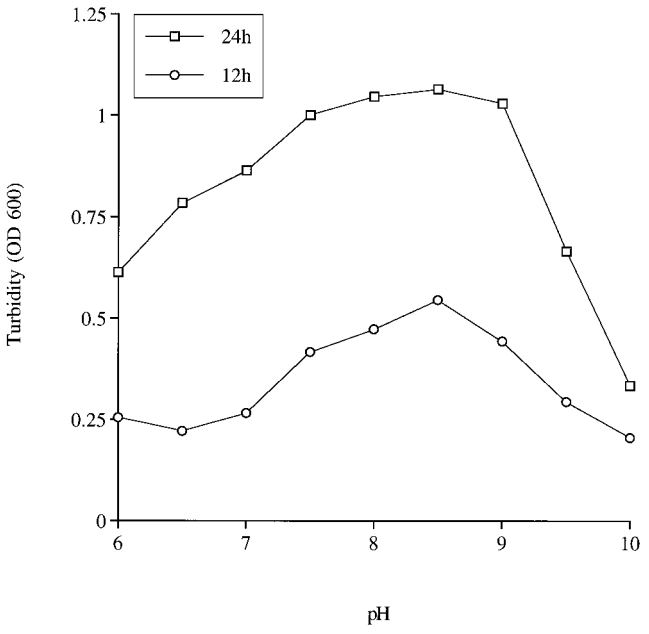


FIG. 3. Growth of *H. pylori* as a function of medium pH. The growth of the bacteria is displayed at the pH of the medium before equilibration with the microaerobic cartridge. The maximal growth rate is at a pH of 8.5. A representative experiment ($n = 6$) is shown.

However, at 24 to 36 h the pellet became completely black and devoid of urease activity, as tested by the CLO test. The morphology of *H. pylori* cells in the latter pellets was confirmed microscopically to be of the coccal form, as previously reported for the 5- to 6-day-old agar-based cultures. Subculturing of the coccal form was not successful on either Skirrows or CHOC II medium. Subculturing of the coccal form from 5-day-old cultures was successful in the liquid medium. At the zero time point urease activity was $(0.16 \pm 3.6) \times 10^{-3}$ (mean ± standard deviation of five separate experiments), and full recovery of urease activity, $(13.73 \pm 5.33) \times 10^{-3}$, was obtained within 6 h of transfer to fresh liquid medium. The reversion from coccal to spiral morphology was monitored microscopically (Fig. 4). Transformation from the coccal to the spiral morphology was first observed at 15 to 20 min at 37°C after transfer to fresh medium when the cells were suspended between two glass coverslips to decrease the diffusion of oxygen. Figure 4A shows a 12-min incubation of the coccal form in fresh medium. Bacteria are seen mostly as individual spherical cells. After a 40-min incubation (Fig. 4B), clustering of cells as well as individual spirals were seen. Both panels of Fig. 4 show the same sample and field. However, note that full recovery of urease activity takes place at between 4 and 6 hours. The exact percentage of cocci reverting to spiral form is hard to quantify, but it is clear than many do change within this time span.

## DISCUSSION

*H. pylori* is typically grown on various agar media for 3 to 4 days at 37°C. Since this bacterium is sensitive to oxygen (7) and cannot be subcultured even after limited exposure to air, it is grown microaerobically. Despite the fact that several selective and nonselective agar-based media have been described (5, 7, 8, 9, 19, 23), there are no descriptions of broth media that permit the growth of large quantities of bacteria. We have designed a broth-based medium that supports growth of a Skirrows-derived inoculum.

We initially employed a basic broth medium which allowed minimal growth of a Skirrows-derived inoculum. The cultures were maintained at 37°C for 5 days at pH 7.5. This medium yielded few cells; however, it retained high urease activity. Addition of newborn calf serum to the base medium improved the yield but not the urease activity. Serum alone did not affect the doubling time within the first 24 hours. Supplementation with erythrocyte lysate alone significantly improved both the yield and urease activity. Thus, our results indicate that addition of erythrocyte lysate is a requirement for optimal *H. pylori*

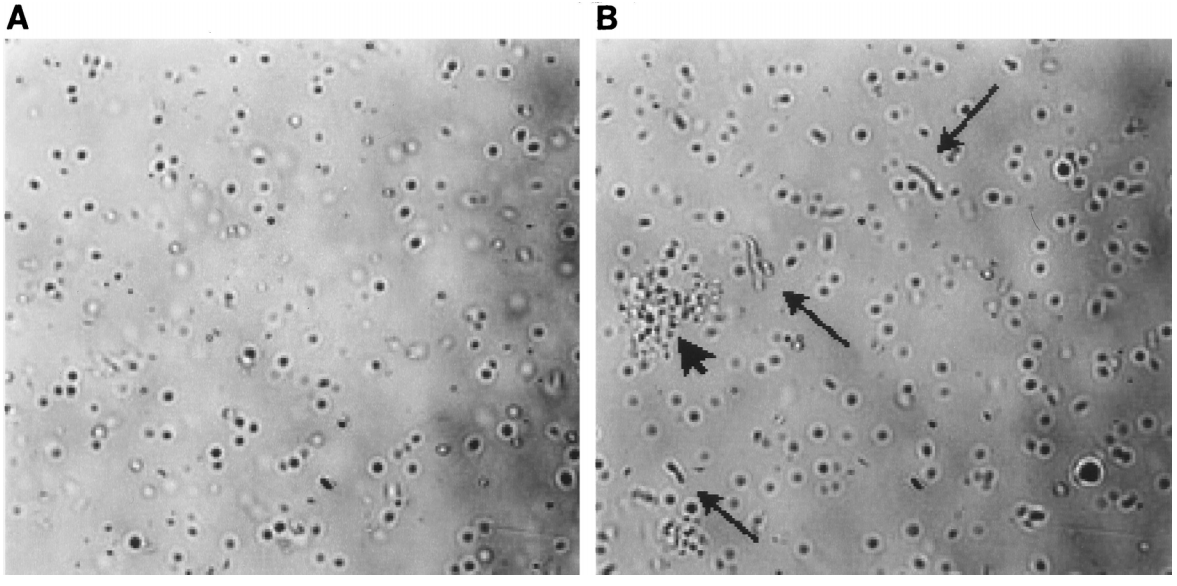


FIG. 4. A 4-day-old culture of *H. pylori*, completely transformed to cocci, was washed in PBS, resuspended in fresh medium, and assayed as described in Materials and Methods. (A) *H. pylori* at 10 min after being washed, showing no cells of spiral or rod-shaped morphology. (B) Same field as that in panel A at 40 min. Note the spiral morphology of the cells (arrows) and the clusters of spiral *H. pylori* cells (arrowhead).

growth, but the addition of newborn calf serum to the erythrocyte lysate provides the highest yield.

*H. pylori* cultured in the presence of erythrocyte lysate forms a sticky brown precipitate. This precipitate is not seen when the bacteria are grown either in the absence of the erythrocyte lysate or in controls without bacteria. The optimal concentration of the erythrocyte lysate depends on whether serum is present or absent. While 2 mg of erythrocyte lysate per ml is optimal in the absence of serum, in the presence of serum a maximal yield is obtained with 10 mg of erythrocyte lysate per ml. Due to the presence of the precipitate, a substitute for the erythrocyte lysate was examined. Since erythrocyte lysate is rich in iron, we studied the effect of a mixture of trace elements ($Cu^{2+}$, $Zn^{2+}$, $Fe^{2+}$, and $Mn^{2+}$) on bacterial growth. We had already found that $Mg^{2+}$ was a requirement, since the base medium supplemented with serum and erythrocyte lysate did not support growth if $Mg^{2+}$ was absent. Addition of trace elements permitted the reduction of erythrocyte lysate to a level at which there was no precipitate but did not compromise the quality of the medium. Our fully supplemented medium permits relatively high quantities of bacteria to be obtained at a much higher growth rate than that previously reported (12). This medium permits an $OD_{600}$ of $>1$ while retaining a high urease activity if harvested within 12 h of inoculation, whereas solid media require 2 to 3 days of growth with a small yield per plate. Harvesting *H. pylori* from broth medium requires higher centrifugal force (5,000 to 10,000 $\times$ *g*) than do other gram-negative bacteria, like *Escherichia coli* (1,500 $\times$ *g*). The cell pellet formed is very soft, white, and easily resuspendable. The coccal form also requires a high centrifugal force and is as easily resuspended, but the sedimented pellet exhibits a black color (unpublished observation). Thus, it is possible that differences in specific gravity between *H. pylori* and other bacteria could be exploited for *H. pylori* enrichment. It should be noted that all experiments were performed with *H. pylori* obtained from ATCC and, thus, we can only assume that this broth medium will also support growth of *H. pylori* from infected samples of human gastric mucosa.

We examined the effect of pH on the growth of *H. pylori* in our supplemented medium. We found that during the 15 to 18 h of equilibration to obtain the microaerobic environment, the pH changed, most likely due to the release of $CO_2$. After these changes were adjusted for, the optimal pH for the starting broth was established to be 9; after equilibration and at the time of adding the inoculum, the optimal pH was found to range between 8.3 and 8.5. It is of interest that during growth, the bacterium will adjust the pH up or down to maintain an optimal pH of 8.5. It has been reported by others (17) that urease activity is lower at pH 8.2 than at a neutral or acidic pH. This finding may suggest that bacteria at this higher pH convert to cocci at a higher rate than at a lower pH, which would be consistent with our findings. We found that complete conversion with subsequent total loss of urease activity takes place within 24 h in the microaerobic medium at pH 8.3. If oxygen is present or the bacteria are starved, the conversion would be expected to be even faster. We and others have found that bacteria exposed to air for a couple of hours cannot be subcultured on agar plates, probably due to cocci conversion. In our broth medium a significant proportion of cocci reverted to spirals, with full recovery of urease activity. Miederer and Grübel (17) found that regardless of the initial pH (5.0 to 8.2), the final pH was always selfadjusted to 8.0 to 8.4. We saw a similar effect of pH selfadjustment, approximately 0.5 pH units, in our medium during growth (the absence of urea in this medium may explain the less dramatic results). The greatest yield and maximal urease activity were obtained at pH 8.5, with a doubling time of 50 min.

Cultures at pH 8.5 mature faster and, at 24 h, contain only the nondividing coccal form, with the associated loss of urease activity. It is possible that a factor is produced by *H. pylori* at this higher pH that enhances the transformation to the coccal form since conversion takes place within a couple of hours. Previously published studies have reported that this coccal morphology cannot be subcultured in vitro, and it has been speculated that this dormant form plays a role in the transmission of *H. pylori* and in relapses after antibiotic therapy (15). Others have speculated that the coccal form is either a contaminant or dead bacteria (15). We found that in our liquid medium, conversion from coccal to spiral morphology does occur if the coccal form is washed in PBS and subcultured in fresh liquid medium, pH 8.5. The rate of conversion from the coccal morphology to spiral or curved-rod shape excludes the possibility that the coccal form is a contaminant. The combination of morphological conversion, recovery of urease activity, and growth to high turbidity document that the coccal form represents a viable form of *H. pylori*. The reason for the clustering seen under the microscope is unknown, but it is possible that diffusing oxygen may have reached unacceptable levels, in which case these aggregates may assist the bacteria in creating a microenvironment conducive to growth.

In conclusion, we have described a liquid medium that supports the growth of *H. pylori*. This medium permits good bacterial yield in less than 24 hours with sustained urease activity. In addition, we report that the coccal form of *H. pylori* will proliferate in this medium, with conversion to spiral form, recovery of urease activity, and the ability to be subcultured on solid media.

## REFERENCES


1. **Andersen, L. P., and F. Espersen.** 1992. Immunoglobulin G antibodies to *Helicobacter pylori* in patients with dyspeptic symptoms investigated by the Western immunoblot technique. J. Clin. Microbiol. **30:**1743–1751.
2. **Blaser, M. J.** 1992. Helicobacter pylori: its role in disease. Clin. Infect. Dis. **15:**386–393.
3. **Bolton, F. J., D. N. Hutchinson, P. M. Hinchliffe, and A. V. Holt.** 1989. Distribution in various clinical groups of antibodies to C. pylori detected by enzyme-linked immunoabsorbent assay, complement fixation and microagglutination test. Serodiagn. Immunother. Infect. Dis. **3:**41–50.
4. **Chodos, J. E., B. Dworkin, and F. Smith, et al.** 1988. Campylobacter pylori and gastroduodenal disease: a prospective endoscopic study and comparison of diagnostic tests. Am. J. Gastroenterol. **83:**126–130.
5. **Dent, J. C., and C. A. M. McNulty.** 1988. Evaluation of a new selective media for Campylobacter pylori. Eur. J. Clin. Microbiol. Infect. Dis. **7:**555–568.
6. **Goodwin, C. S.** 1989. Campylobacter pylori: detection and culture, p. 60–62. *In* B. J. Rathbone and R. V. Heatley (ed.), Campylobacter pylori and gastroduodenal disease. Blackwell Scientific Publications Ltd., Oxford, England.
7. **Goodwin, C. S., and B. W. Worsley.** 1993. Microbiology of Helicobacter pylori. Gastroenterol. Clin. North Am. **22:**5–19.
8. **Goodwin, C. S., E. D. Blincow, J. R. Warren, T. E. Waters, C. R. Sanderson, and L. Easton.** 1985. Evaluation of cultural techniques for isolation of Campylobacter pyloridis from endoscopic biopsies of the gastric mucosa. J. Clin. Pathol. **38:**1127–1131.
9. **Goodwin, C. S., and B. W. Worsley.** 1993. The Helicobacter genus: the history of *H. pylori* and taxonomy of current species, p. 1–13. *In* C. S. Goodwin and B. W. Worsley (ed.), Helicobacter pylori: biology and clinical practice. CRC Press, Inc., Boca Raton, Fla.
10. **Goodwin, C. S., J. A. Armstrong, and B. J. Marshall.** 1986. Campylobacter pyloridis, gastritis and peptic ulceration. J. Clin. Pathol. **39:**353–365.
11. **Hazeil, S. L., and A. Lee.** 1986. Campylobacter pyloridis, urease, hydrogen ion back diffusion and gastric ulcers. Lancet **ii:**15–17.
12. **Jerris, R. C.** 1995. Helicobacter, p. 492–498. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, sixth ed. ASM Press, Washington, D.C.
13. **Jones, D. M., and A. Curry.** 1990. The genesis of coccal forms of Helicobacter pylori, p. 29–37. *In* P. Malfertheiner and H. Ditschuneit (ed.), Helicobacter pylori, gastritis and peptic ulcer. Springer-Verlag KG, Berlin, Germany.
14. **Klein, P. D., and D. Y. Graham.** 1989. Detection of Campylobacter pylori by the $^{13}C$-breath test, p. 94–105. *In* B. J. Rathbone and R. V. Heatley (ed.),

Campylobacter pylori and gastroduodenal disease. Blackwell Scientific Publications Ltd., Oxford, England.
15. **Kusters, J. G., M. M. Gerrits, and C. M. J. E. Vandenbrouke-Grauls.** 1996. The morphologic conversion of *H. pylori* from bacillary to coccoid forms is not an active process, abstr. B20, p. 25. *In* Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.
16. **McNulty, C. A. M.** 1989. Detection of Campylobacter pylori by the biopsy urease test, p. 69–73. *In* B. J. Rathbone and R. V. Heatley (ed.), Campylobacter pylori and gastroduodenal disease. Blackwell Scientific Publications Ltd., Oxford, England.
17. **Miederer, S. E., and P. Grübel.** 1996. Profound increase of Helicobacter pylori urease activity in gastric antral mucosa at low pH. Dig. Dis. Sci. **41:**944–949.
18. **Mobley, H. L. T., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol. **26:**831–836.
19. **Penner, J. L.** 1991. *Campylobacter*, *Helicobacter*, and related spiral bacteria, p. 402–409. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
20. **Perez-Perez, G. I., B. M. Dworkin, J. E. Chodos, and M. J. Blaser.** 1988. Campylobacter pylori antibodies in humans. Ann. Intern. Med. **109:**11–18.
21. **von Wulffen, H., J. Heesemann, G. H. Bützow, T. Löning, and R. Laufs.** 1986. Detection of *Campylobacter pyloridis* in patients with antrum gastritis and peptic ulcers by culture, complement fixation test, and immunoblot. J. Clin. Microbiol. **24:**716–720.
22. **Weil, J., and G. D. Bel.** 1989. Detection of Campylobacter pylori by the $^{14}$C-breath test, p. 83–93. *In* B. J. Rathbone and R. V. Heatley (ed.), Campylobacter pylori and gastroduodenal disease. Blackwell Scientific Publications Ltd., Oxford, England.
23. **Westblom, T. U., E. Madan, and B. R. Midkiff.** 1991. Egg yolk emulsion agar, a new medium for the cultivation of *Helicobacter pylori*. J. Clin. Microbiol. **29:**819–821.