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Microbiological Aspects of *Helicobacter pylori (Campylobacter pylori)*

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The human gastric pathogen *Campylobacter pylori* has recently been reclassified as *Helicobacter pylori*, and a related spiral bacterium found in the stomach of ferrets has been designated *Helicobacter mustelae*. The general microbiological features of *Helicobacter pylori* are delineated here, with details of phenotypic differences between *Helicobacter pylori* and *Helicobacter mustelae*; comparisons are made with *Wolinella succinogenes* and *Campylobacter jejuni*. The *Helicobacter* organisms possess an external glycocalyx which can be visualised by electron microscopy, and which may be involved in bacterial adherence. The finding of soluble and cell-associated haemagglutinins of *Helicobacter pylori* is reported. Detection of *Helicobacter pylori* in clinical specimens, susceptibility of the organism to antibacterial agents, and other aspects of practical and clinical significance are briefly reviewed.

Campylobacter pyloridis was the name given originally to a gram-negative, urease positive, curved or slightly spiral bacterium first isolated in Western Australia in 1982 from the stomach of patients with gastritis lesions and peptic ulceration (1). The specific epithet was later amended to *Campylobacter pylori* (2); however, characterisation of the organism's properties, including ribonucleic acid sequencing studies (3), indicated it could not properly be included within the genus *Campylobacter*. A possible relationship to the genus *Wolinella* was considered, but phenotypic differences militated against that, and most recently the organism was renamed *Helicobacter pylori*, representing the type species of a proposed new genus *Helicobacter* (4). Another distinctive gastric organism isolated from ferrets (5) was compared with *Helicobacter pylori*; the ferret organism has now been designated *Helicobacter mustelae* (4). Curved and spiral bacteria, apparently very similar, if not identical, to *Helicobacter pylori* have been recovered from the stomach in non-human primates including *Macaca nemestrina* (6), *Macaca mulatta* (7), and also from a baboon and a pig (8). All the above-mentioned organisms differ in important respects from the various and more tightly spiral ("corkscrew-like") bacteria commonly present in relation to the stomach epithelium and associated glands in cats, dogs, rodents, monkeys and (occasionally) man (9, 10, 11).

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Morphology

Findings on electron microscopy are shown in Figures 1, 2 and 3. Electron microscopy of thin sections and of negatively stained specimens (Figures 1A and 2) shows *Helicobacter pylori* to be a unipolar, multi-flagellate, rodlike organism with bluntly rounded ends, measuring 0.5–1.0 μm in width and 2.5–4.0 μm in length (12, 13). When observed colonising the human gastric mucosa, where it is usually most prolific in the antrum, it is a curved, sinuous or gently spiral bacterium (Figure 2). Occasional organisms may contain bacteriophages, and exhibit incipient or overt bacteriolysis. When cultured on solid media or in broth media, true spiral forms may be few or absent.

The outer cell wall appears smooth, closely following the underlying cytoplasmic membrane, in contrast to the looser, wrinkled cell walls of the true campylobacters (Figures 1C). Towards the flagellar pole, in relation to the inner surface of the cytoplasmic membrane, is a conical shell structure (12); similar polar submembranous complexes are described in various flagellate spiral bacteria (14) but their functional significance is still undetermined. Studies of the external surface of *Helicobacter pylori* utilising tannic acid or ruthenium red for stabilisation of polysaccharide components (15, 16) reveal that in vivo, and also in pure cultures, the organism has, external to the cell wall membrane, a distinctive glycocalyx, which is inapparent in standard thin section preparations (Figures 3A and B). The glycocalyx, up to 40 nm

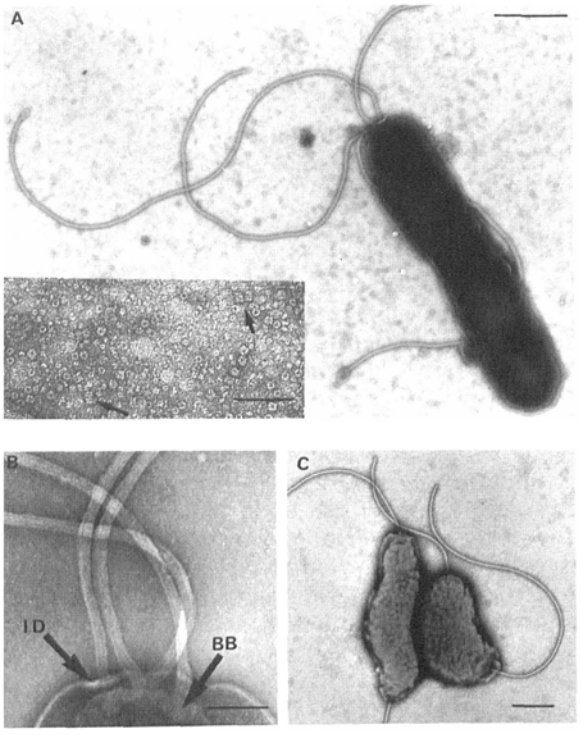

Figure 1: A: *Helicobacter pylori* as seen in vitro by negative staining with 3 % ammonium molybdate (pH 6.5). The rod-like organism exhibits four unipolar flagella. Inset: Higher magnification reveals abundant annular subunits (ca. 13 nm diameter) adherent to the bacterial cell wall, and stacks of four or five subunits in the side view (arrows). Bar = 500 nm (inset 100 nm). B: Close-up view of the flagellar pole of *Helicobacter pylori* showing the sheathed character of the flagella. An insertion disc (ID) and a basal body (BB) are indicated in relation to the cell wall and cytoplasmic membrane respectively. Bar = 100 nm. C: Negative stain general view of cultured *Campylobacter jejuni* organisms showing a wrinkled cell wall pattern and bipolar unsheathed flagella, which readily distinguish this true *Campylobacter* species from *Helicobacter pylori*. Bar = 500 nm.

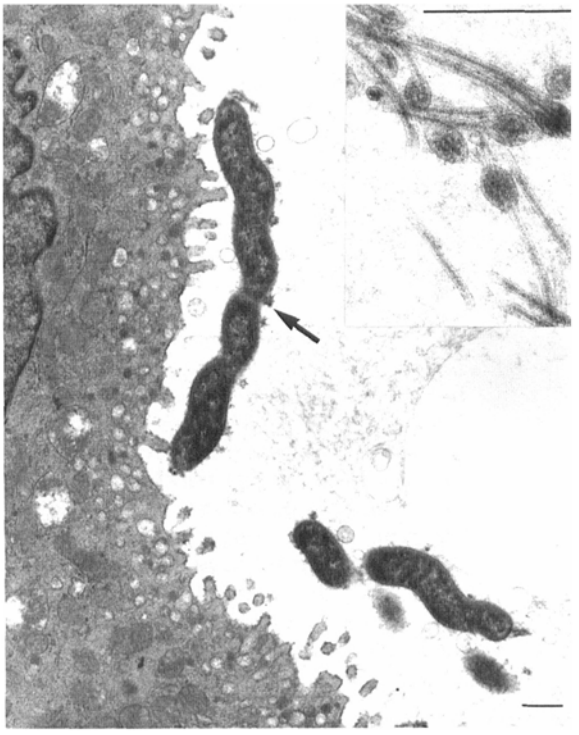

Figure 2: Thin section features of a human gastric biopsy specimen with *Helicobacter pylori* colonization. Bacterial cells have a gently spiral form, and the epithelium exhibits depletion of mucus granules and microvilli. Bacteria undergoing fission (arrow) may account for occasional reports of individual very long organisms. Inset: Close-up showing detailed sectional view of the sheathed flagella with terminal bulbs. Bars = 500 nm.

in thickness, is thicker in vivo than in vitro; its rather diffuse outer region is removed by vigorous washing to reveal rows of radiating peg-like projections with a periodicity of about 14 nm (Figures 3B). On negative staining (Figure 1A – inset) the bacterial cell wall is seen to be festooned with regular, ring-like proteinaceous subunits (13) 12 to 15 nm in diameter. Cylindrical stacks of four or five such subunits are to be seen (16) corresponding in their location to radial pegs in the glycocalyx; this suggests a surface structural layer of glycoprotein composition, analogous to the regularly arranged "S-layers" described in several other gram-negative bacterial species (17, 18). In gastric specimens the surface glycocalyces of the organisms and the epithelial microvilli are often linked by thread-like bridges or more extensive points of confluence. At sites of extensive contact between the bacteria and epithelial membranes (so-called adherence pedestals) the opposing glycocalyces appear fused (Figure 3C). Bacterial adherence has been proposed as a likely factor in *Helicobacter pylori* pathogenesis (19, 20, 21), and strategies aimed at its impairment could perhaps lead to improved measures for lasting eradication of *Helicobacter pylori* from the gastric mucosa.

Four to six sheathed flagella are normally attached at one pole, each 2.5 µm long and about 30 nm in thickness, with a membranous terminal bulb that is an extension of the flagellar sheath (Figures 1A, 1B and 2 inset). The flagellar core fibre reaches to the tip of the terminal bulb and proximally ends in a basal body associated with the cytoplasmic membrane (Figure 1B). Circular "insertion discs", 90 nm in diameter and with a central hole through which the core fibre passes, are located at the level of the cell wall. The discs have been most clearly seen in negatively stained sarcosinate extract preparations (13). *Helicobacter pylori* in culture

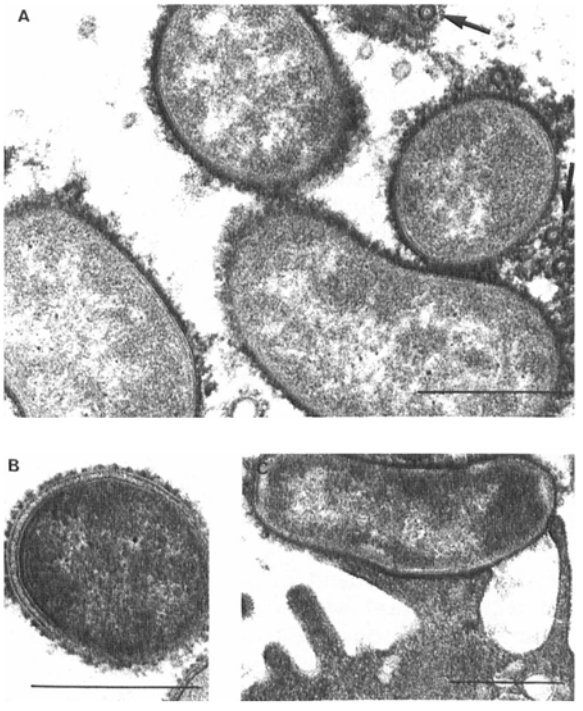

Figure 3: A: *Helicobacter pylori* in a gastric biopsy specimen as seen after tannic acid visualization of the glycocalyx. Note that the coating extends onto the flagella (arrows) Its outermost part is diffuse and flocculent. B: Periodic peg-like projections present in deeper levels of the glycocalyx attached to the outer aspect of the cell wall membrane. C: Pedestal-like site of intimate adherence between the bacterial glycocalyx and antral epithelium, as commonly found in biopsy specimens. Bars = 500 nm.

often exhibits aberrant or deficient flagellar expression, and flagella are sometimes entirely absent.

The ferret organism, *Helicobacter mustelae*, is morphologically somewhat different (5). It takes the form of slightly curved rods, somewhat shorter than *Helicobacter pylori*. There are multiple sheathed flagella with terminal bulbs, but they are variable, often being bipolar and also lateral in location. A "regularly ordered" external surface is revealed in negatively stained preparations (5), and there is a well-developed glycocalyx (4) suggestive of a glycoprotein S-layer, as in *Helicobacter pylori*.

Helicobacter pylori in culture (Figure 1A) occurs usually in the form of straight or slightly curved rods (22, 23). Prolonged culture gives rise to emergence of coccoid forms, possibly reflecting a transformation in response to unfavorable conditions or nutrition. Such forms occur in faeces and may be responsible for an oro-faecal mode of transmission between human hosts, but they are difficult to culture (24).

Genome and Plasmids

The DNA composition of 32 strains of *Helicobacter pylori* was found to yield an average of 35.2 mol % G + C, with a range of 34.1–37.5 mol % (25); in *Helicobacter mustelae* the DNA composition was found to be 35–41 mol % G + C (4).

It is not known whether fresh strains of *Helicobacter pylori* contain plasmids which are rapidly lost after subculture. Of 84 strains of *Helicobacter pylori* from Australia and the UK, 40 contained plasmids (26), and of 41 isolates from Western Australia, 24 contained plasmids (27), To visualise the plasmids, the method of Kado and Liu (28) was modified by the addition of chloroform and phenol (27). Most plasmids were small – 1.8 to 22 – kilobase pair (kbp), with an occasional large plasmid of 40 kbp. Many isolates contained two or more plasmids. Antibiotic resistant strains and urease-negative strains showed no difference in plasmid content. It was not possible to ascribe a phenotype to a particular plasmid (27). In a study of *Helicobacter pylori* strains from South Africa, 20 of 42 had plasmids; most isolates had more than one plasmid (29). Biopsy specimens taken from the body and antrum of the stomach of one patient yielded isolates with distinctly different plasmid profiles, suggesting the presence of two subtypes of *Helicobacter pylori* in the same patient (29). Similar evidence of a mixed infection has been obtained by endonuclease analysis and outer membrane analysis (26).

Outer Membrane Proteins and Lipopolysaccharides

In 1985 Megraud et al. (30) reported that the major protein bands were of 12,000, 17,000, 21,500, 43,500, 58,000, 64,000 and 74,000 molecular weight, but others reported that the major bands were 26,000, 29,000, 56,000 and 62,000 (31). Our acid glycine preparation showed dominant bands at 57 kDA, 62 kDA and 64 kDA with other strong bands at 24.5 kDA, 28 kDA, 33 kDA and 84 kDA (32). Newell (33) reported constant outer membrane proteins at 31 kDA, 54 kDAand 61 kDA, and several variable proteins. Proteinase K-treated lysates showed different lipopolysaccharide (LPS) profiles, ranging from rough to smooth with multiple repeating side chains (31), which suggests that *Helicobacter pylori* has core LPS group antigens and strain-specific LPS side chain antigens (31).

Cellular Fatty Acid Composition, Menaquinones and Lipid A

The major cellular fatty acids of *Helicobacter pylori* are tetradecanoic acid (14:0) and 19-carbon cyclopropane fatty acid (19:0 cyc), with a very small amount of hexadecanoic acid (16:0) (12). In addition, *Helicobacter pylori* is unique in possessing 3-hydroxyoctadecanoic acid (3-OH-18:0) (34). The 19-carbon fatty acid has been shown to be cis-11,12-methylene octadecanoic acid (35). *Helicobacter mustelae* does not possess 3-OH-18:0, and has much less 14:0 and much more 16:0 than *Helicobacter pylori* (36). The fatty acids of *Helicobacter pylori, Campylobacter jejuni, Helicobacter mustelae* and the *Helicobacter pylori*-like organisms (HPLOs) from the baboon and macaque monkeys have been compared (36). The second type of organism isolated from *Macaca nemestrina* (nemestrina type B) has a unique profile without 19:0 cyc but with 3-OH-14:0 and 15:0; the latter two acids are not present in other HPLOs. For this organism the name *Helicobacter bronsdoniae* has been proposed (Bronsdon, Goodwin, Sly, Chilvers, Schoenknecht, in press).

Respiratory quinones are also important chemotaxonomic markers (37). *Helicobacter pylori* lacks the methylated menaquinone-6 (38) which is found in all other campylobacters in a quantity of 20–50 % (39). We have reported that *Helicobacter mustelae* possesses only a small amount (5 %) of methylated menaquinone-6 (4). This and other features of *Helicobacter mustelae* underline the conclusion that *Helicobacter mustelae* is a unique species (4).

With regard to lipid A, in contrast to *Campylobacter jejuni* and other enterobacteria *Helicobacter pylori* lipid A contains 3-OH-16:0 and 3-OH-18:0. The antigenic similarity between lipid A of *Helicobacter pylori* and *Campylobacter jejuni* is less than that between *Campylobacter jejuni* and *Salmonella typhimurium* based on the amounts needed for 50 % inhibition of anti-lipid A activity (40).

Hydrophobicity

Strains of *Escherichia coli* have been reported to have retention values between 34 % and 86 % (41), as have strains of *Campylobacter jejuni*. We have found values of greater than 90 % in nearly all strains of *Helicobacter pylori*, with increased hydrophobicity at low pH in the presence of urea, and after mild protein extraction by pre-treatment with glycine hydrochloride at pH 2.5 (42). Bacteria with hydrophobic surfaces are known to have an increased affinity for mucosal membranes (41). The experiments mentioned above suggest the presence of a proteinaceous outer layer of *Helicobacter pylori* which confers negative charge and hydrophobicity. In acid conditions in the stomach this proteinaceous layer would be partially removed and would expose hidden hydrophobic regions on the bacterial surface, thus enhancing mucus penetration and adherence.

Haemagglutinins

A cell-bound haemagglutinin (HA) with a fibrillar structure has been purified from clinical isolates of *Helicobacter pylori* by Evans et al. (43). Ten isolates showed HA activity with all the human and animal erythrocytes tested. HA activity was destroyed by pronase and papain, but was resistant to pepsin and trypsin. HA inhibition was observed with the sialoprotein fetuin and similar proteins, but not with asialofetuin. The HA bound preferentially to the NeuAc(2-3)Gal isomer of NeuAc-lactose. It has been proposed that this antigen should be designated as a putative colonization factor antigen. Immunogold-labelling of the HA revealed a thick layer around *Helicobacter pylori* which contained the HA. However, other workers have reported that some strains of *Helicobacter pylori* do not agglutinate all species of erythrocytes (44, 45). We recently detected the presence of soluble haemagglutinins in supernatants of cultures harvested into phosphate buffered saline for one hour (unpublished observations). After removal of these supernatants, the remaining bacteria exhibited cell-associated haemagglutinins. This observation is similar to the situation with *Escherichia coli*, where soluble and cell-associated haemagglutinins have been found. However, we found that some strains of *Helicobacter pylori* showed the presence of soluble haemagglutinins only for a short time after isolation. After multiple subculture in the laboratory, the ability to produce soluble haemagglutinins was lost. Other strains retained this feature at a high titre, even after multiple subculture over many years.

Growth Requirements

Helicobacter pylori can be subcultured without blood or serum, but with 0.2 % charcoal or 1 % cornstarch (46); primary isolation has been achieved with brain-heart infusion agar (BHIA) and 20 % horse serum (47). We have found

luxuriant growth with egg yolk or casein in BHIA. In liquid media a thin layer of fluid yields growth after three days (48), but shaking yields growth after 24 hours, even in deep liquid media (49). Heavy growth for primary isolation can be obtained after 48 hours on BHIA plus 7 % horse blood lysed with saponin, plus 1 % IsoVitaleX (BBL, USA), but such heavy growth is not achieved if the blood is lysed by freezing and thawing.

pH and Temperature Range

In a Brucella medium modified by the addition of agar and tetrazolium chloride, a red precipitate indicating growth was found at pH 2.9 to 9.2 for each of seven isolates; no growth was found at pH 2.6, nor at pH 9.4 (50). In Christensen's urea broth medium, urease activity was observed through a pH range of 3.1 to 7.1 but at the lowest and highest pH *Helicobacter pylori* could not be subcultured, suggesting that the colour reactions were due to preformed urease in the medium (50). In brain-heart infusion broth plus 10 % horse serum and 0.25 % yeast extract, *Helicobacter pylori* grew only between pH 6.6 and 8.4, and between 33 °C and 40.5 °C, but not at 25 °C or at 42 °C (38). Some strains grow at 42 °C (51), which may be due to the use of different media. *Helicobacter pylori*, but not *Campylobacter jejuni*, can remain alive at pH 1.5 or 2 in the presence of 5 mM urea (52, 53).

Motility in Viscous Environments

In solutions of methyl cellulose with a range of 0.1.–1.5 % in 1 % peptone water, *Escherichia coli* was found to be immobilised at 20 centipoise (cp) whereas *Helicobacter pylori* moved faster at 10 cp than at 1 cp and was still motile at 200 cp (54). In the more viscous medium *Helicobacter pylori* appeared more spiral in form.

Bile Sensitivity

On blood agar *Helicobacter pylori* failed to grow in the presence of 5 % bile, with reduced growth on 0.5–1 % bile, but full growth occurred on 0.1 % bile (52), suggesting that concentrations of bile found in the duodenum may be inhibitory for *Helicobacter pylori*. However, we have found that exposure of *Helicobacter pylori* to 5 % bile in a liquid medium for 30 minutes killed only 25 % of strains (unpublished observation). Also, in vivo *Helicobacter pylori* may be immersed in gastric mucus, and eruption of bile into the duodenum is intermittent. Thus, *Helicobacter pylori* could survive passage through the duodenum. One report has appeared of *Helicobacter pylori*-like organisms seen histologically in the mucosa of the rectum (55).

Protease and Lipase

Incubation of a filtrate of *Helicobacter pylori* with gastric mucus led to a 35 % loss of mucus viscosity after 48 hours (56), indicating the possession of a protease that may account for the mucus depletion of colonized gastric mucosal cells. A dialysed filtrate of a *Helicobacter pylori* culture released labelled fatty acids indicating lipolytic activity, as well as phospholipase A activity (57).

Toxins

Broth culture filtrates of 50 % of isolates of *Helicobacter pylori* were found to produce non-lethal cytopathic effects in seven of nine mammalian tissue culture cell lines (58). In these broth culture filtrates urease activity was not detected. However, we have found that plate and broth culture supernatants do contain urease activity, and in the presence of low concentrations of urea intracellular vacuolization is produced by all strains of *Helicobacter pylori* (data to be published).

Detection in Clinical Specimens

Helicobacter pylori can occur in a patchy distribution in the antrum of the stomach (47). Endoscopy of the stomach and duodenum allows multiple biopsies to be taken from different areas. To ensure that the presence of *Helicobacter pylori* is not missed, at least two specimens should be taken from the antrum, in addition to the body of the stomach. Chemical agents used during endoscopy can be antibacterial to *Helicobacter pylori*. Benzocaine is inhibitory for *Helicobacter pylori* (59), but lidocaine is not. Simethicone is also inhibitory to *Helicobacter pylori*, and if a cimetidine tablet has been ingested before endoscopy this could provide a sufficiently high concentration to at

least partially inhibit *Helicobacter pylori*. Biopsy forceps can be contaminated with glutaraldehyde (60).

With regard to different liquid transport media, *Helicobacter pylori* rapidly loses viability at room temperature in 0.9 % NaCl (61). To retain the mucus layer on the specimen, 0.5 ml of 20 % glucose solution was used in one study (47). Other transport media include thioglycolate broth, nutrient broth, Brucella broth (62) or Stuart's transport medium (63). A transport medium consisting of Brucella broth with 10 % horse serum and 1 % IsoVitaleX, plus a solid phase of brain-heart infusion agar with 10 % bovine blood and 1 % IsoVitaleX on a 20 ° slant was found to have a slightly higher recovery rate than Brucella broth with horse serum (64). A biopsy specimen may be kept in transport medium for five hours at 4 °C without any loss of viability of *Helicobacter pylori* (47). If the specimen is put into − 70 °C in a medium of broth or 1 % peptone water and 25 % glycerol, *Helicobacter pylori* may remain viable for months. Gastric brush biopsy specimens yield a low rate of detection of *Helicobacter pylori*.

Microscopy and Monoclonal Antibodies

In the laboratory a corner of the biopsy specimen should be cut off to prepare a smear on a slide for Gram stain to detect spiral bacteria. Alternatives to Gram stain that have been used successfully include acridine orange and Giemsa stains.

Grinding specimens in a ground glass grinder in 0.3 ml 20 % glucose prior to inoculation of culture media produces a much heavier growth of *Helicobacter pylori* than mincing the specimen with sterile scalpel blades and subsequent inoculation (47). Maceration of a biopsy in two to three drops of transport solution with the tip of an unsealed glass pipette may be more useful than employing a ground glass grinder for preparation of specimens of the gastric mucosa. After grinding the specimen to prepare it for inoculation of culture media, a Gram stain will not reveal spiral bacteria even when they are present (47). Phase contrast microscopy can be more sensitive for the detection of *Helicobacter pylori* than smear (65). The specimen is minced in two drops of saline and a portion pressed firmly between two glass slides. As shown under 400 times magnification, the morphology of *Helicobacter pylori* may be retained for at least five hours in preparations kept in a moist chamber at room temperature. Using monoclonal antibodies prepared against *Helicobacter pylori* for direct immunofluorescence assays a high rate of detection of *Helicobacter pylori* was found, although it is not clear whether a higher rate is obtained than with culture (66). The main advantage of monoclonal antibodies is that only one hour is needed for the technique.

Culture Media

Although some authors recommend freshly poured media, plates kept at room temperature in a closed plastic box for 6 to 19 days were found to give equally good culture growth as freshly poured plates (47). Whatever medium is used, the incorporation of tetrazolium chloride (40 mg/l) will yield unique golden colonies which may be more easy to detect if there are many commensals (67). However, experience culturing *Helicobacter pylori* usually enables the worker to detect colonies without any difficulty. Brain-heart infusion agar or Brucella agar without any supplementation will not support the growth of *Helicobacter pylori*, but such media supplemented with activated 0.2 % charcoal or 1 % cornstarch will allow the growth of *Helicobacter pylori* (46). Culture on blood agar, chocolate agar or lysed blood agar yields similar rates of isolation, although we have found that 7 % horse blood lysed with saponin plus 1 % IsoVitaleX yields a heavier growth of *Helicobacter pylori* more rapidly than other media (unpublished observation). Selective media with antibiotics are usually essential to detect *Helicobacter pylori*. In one series of 29 cultures yielding *Helicobacter pylori*, using media without antibiotics five positive cultures were missed (47). The maximum concentration of vancomycin and amphotericin B which should be incorporated in media is 6 mg/l and 10 mg/l respectively. In lysed blood agar 5 mg/l cefsulodin (68) is very useful, and 20 mg/l trimethoprim may also be useful; trimethoprim may be synergistic with other antibiotics in unlysed blood agar. Nalidixic acid should not be used as 14 % of isolates can be inhibited (68), and polymyxin can inhibit 5 % (68); thus Skirrow's medium should not be used. Sodium metabisulphite, which is contained in the growth supplement FBP (ferrous sulphate, bisulphite, pyruvate; Oxoid, UK), is inhibitory to *Helicobacter pylori* (48). Horse blood or sheep blood, 5–10 %, have been used to enrich media.

For primary isolation the cefsulodin medium has been used alone, but most workers also use a non-selective medium in addition; incubation should be in a microaerobic atmosphere with 5–7 % O_2. Although *Helicobacter pylori* will grow after subculture in the laboratory in a 10 % CO_2 incubator in air, some primary isolates will not

grow unless the oxygen concentration is reduced (47). We prefer to evacuate an anaerobic jar to 220 mm Hg pressure and fill it with an anaerobic gas mixture (10 % CO_2, 10 % H_2 and 80 % N_2) giving an atmosphere of 5 % O_2, 7 % CO_2, 8 % H_2 and 80 % N_2, which yields growth as good as, and occasionally better than, a standard campylobacter gas mixture (Campy-Pak Systems; BBL Microbiology Systems, USA).

Helicobacter pylori does not grow aerobically and isolates left for longer than 45 minutes on a laboratory bench may die. The optimum temperature for isolation is 35–37 °C. For primary isolation plates may need to be examined after two, three and up to five days. Colonies of *Helicobacter pylori* are 1–2 mm in diameter, translucent and not markedly haemolytic. Gram stain of the colony may show rods or curved forms, but subculture of these organisms into shaken liquid media can yield spiral forms such as are seen in Gram smears of biopsy specimens. Replacement of the gas mixture at 24 hours appears to enhance the growth of *Helicobacter pylori* (69).

The highest culture rate of *Helicobacter pylori* in the laboratory is only achieved by skillful workers after months of experience. In our laboratory we saw spiral bacteria in Gram stains in 7 % of specimens in 1984 when we did not culture the organism (47). However, by 1987 we always cultured *Helicobacter pylori* when we saw it on Gram stain, and in up to 10 % of specimens we failed to see spiral bacteria but cultured *Helicobacter pylori*. Subculture of single colonies of *Helicobacter pylori* is difficult, but can be achieved if one colony is subcultured in an area of only 1 cm diameter. The plate is incubated for three days and the growth obtained is then spread over a wider area. If a single colony is originally spread over a wide area on a plate, growth may not follow. Similarly, successful subculture in liquid media usually requires a larger inoculum than for other bacteria. Brain-heart infusion broth plus 0.25 % yeast extract with or without 10 % horse serum can be shaken in a jar containing a microaerobic atmosphere, and a heavy growth is obtained after 18 hours of incubation; 0.1 ml of this broth can be used as an inoculum for other liquid media (23). *Helicobacter pylori* can be grown in batch culture and continuous culture in liquid media in volumes of 1 l in the presence of serum and a gas mixture of 5 % O_2, 10 % CO_2 and 85 % N_2, with stirring. With hyclone bovine calf serum obtained from 14-week-old animals, cultures can reach a count of 1.9×10^9 at 20 hours after inoculation (Klaasen, Tuinder and Zandvliet, personal communication).

Presumptive and Confirmatory Identification

Gram-stain of colonies may show curved bacteria which are typical of *Helicobacter pylori*; biochemical tests – catalase positive, oxidase positive, and particularly urease positive results – strongly indicate that the colonies are *Helicobacter pylori*. Further biochemical features are negative reactions for hippurate hydrolysis and nitrate reduction, with susceptibility to cephalothin. Resistance to nalidixic acid has been described as a feature of *Helicobacter pylori*, but a few strains are sensitive to low concentrations; most strains grow on 40 mg/l, but all are inhibited at 48 mg/l (69). In a few patients, *Helicobacter pylori* may be mixed with campylobacters, including *Campylobacter sputorum* from the mouth or *Campylobacter jejuni* from the small intestine. The biochemical differences between *Helicobacter pylori*, *Helicobacter mustelae*, *Campylobacter jejuni* and *Wolinella succinogenes* are shown in Table 1.

Enzyme Production

Helicobacter pylori is oxidase-positive and produces a large amount of catalase; this can be detected with a 5 % solution of hydrogen peroxide in 10 % Tween-80, in which only one loopful of *Helicobacter pylori* causes vigorous frothing. However, after repeated subculture in the laboratory, catalase activity can be reduced or lost. *Helicobacter pylori* also possesses large amounts of extracelluar superoxide dismutase (70). These enzymes could confer resistance to the oxidative killing mechanisms of the host macrophages and polymorphonuclear neutrophils.

The extremely powerful urease of *Helicobacter pylori* is a highly characteristic feature of the organism (71), but urease-negative strains can be obtained after multiple subculture in the laboratory both in human isolates (24) and in *Helicobacter mustelae* (4). There may be two urease enzymes of *Helicobacter pylori* with different maximal pH values of 5.0 and 7.5 (72); the major urease enzyme has a high molecular weight of 600,000 (73). Tissue culture cells show severe cytopathic effects of *Helicobacter pylori* when a high concentration of urea is added to culture media, the effect being due to ammonia produced by the urease (74). However, we have recently found that when lower concentrations of urea are used, with addition of the urea on four occasions at two-hour intervals to simulate the in vivo situation, every strain of *Helico-*

Table 1: Biochemical differences between *Campylobacter jejuni, Helicobacter pylori, Helicobacter mustelae* and *Wolinella succinogenes.*

	C. jejuni NCTC 11351	*H. pylori* NCTC 11637	*H. mustelae* NCTC 12032	*W. succinogenes* NCTC 11488
Oxidase production	+	+	+	+
Catalase production	+	+	+	–
Urease production	–	+	+	–
Hippurate hydrolysis	+	–	–	–
Nitrate reduction (microaerophilic)	+	–	+	+
H_2S production in triple sugar iron agar	–	–	–	W
Gammaglutamyltranspeptidase production	–	+	+	–
Alkaline phosphatase production	W	+	+	–
Growth in CO_2 incubator	+	+	–	–
Growth anaerobically at 37 °C	+	–	+	+
Growth on 0.5 % glycine	+	+	+	–
1 % glycine	+	–	+	–
1 % bile	+	–	–	+
Growth on brain-heart infusion agar + 1 % galactose	–	–	–	+
Growth on charcoal/casein/ 0.1 % deoxycholate	+	–	–	+

W = weak.

bacter pylori produces intracellular vacuolisation of Vero cells. In Christensen's medium a colour change occurs very rapidly, often in less than two minutes. Weak urease activity is exhibited by *Campylobacter nitrofigilis*, urease-positive thermophilic campylobacters, and some strains of *Campylobacter laridis.*

All *Helicobacter pylori* strains appear to produce alkaline phosphatase, acid phosphatase, phosphoamidase and glutamyl transpeptidase (22). Four different biotypes have been described (75); biotype I possesses esterases but the other biotypes do not, biotype II possesses phosphohydrolase but biotypes III and IV do not. Biotype IV does not possess leucine arylamidase. Other arylamidases are produced only by some strains. Biotype I may be more common in gastritis specimens, and biotype II more common in duodenal ulcer specimens. *Helicobacter pylori* is sensitive to the vibriostatic compound O_{129} and does not hydrolyse hippurate. *Helicobacter pylori* does not produce H_2S in FBP medium, is tolerant to tetrazolium chloride at 0.4 mg/ml and 1 mg/ml, and tolerant to 0.1 % sodium selenite. *Helicobacter pylori* does not ferment glucose or other carbohydrates.

Antibiotic Susceptibility

Helicobacter pylori has been found in vitro and in vivo to be sensitive to a wide range of antibiotics (23, 48, 76, 77). Indeed, this organism appears sensitive to almost all antibiotic agents except vancomycin, cefsulodin, trimethoprim and sulphonamides. Classical patterns of transformation of the spiral and bacillary organisms into bizarre rounded spheroplast-like forms are induced by beta-lactam antibiotics (Figure 4A). Colloidal bismuth subcitrate is also active against *Helicobacter pylori* both in vivo and in vitro (21, 23), and is considered an essential component of multiple drug therapy to achieve a high rate of elimination of these organisms from the stomach (20). Single drug therapy is remarkably ineffective, and even double or triple drug therapy cannot be guaranteed to always achieve eradication of *Helicobacter pylori* from the colonized human stomach. It is therefore necessary to carefully evaluate whether the treatment has been entirely successful four weeks after completion of antibacterial therapy.

Susceptibility to Low pH

Unusual tolerance of an acidic environment does not seem to be an inherent property of

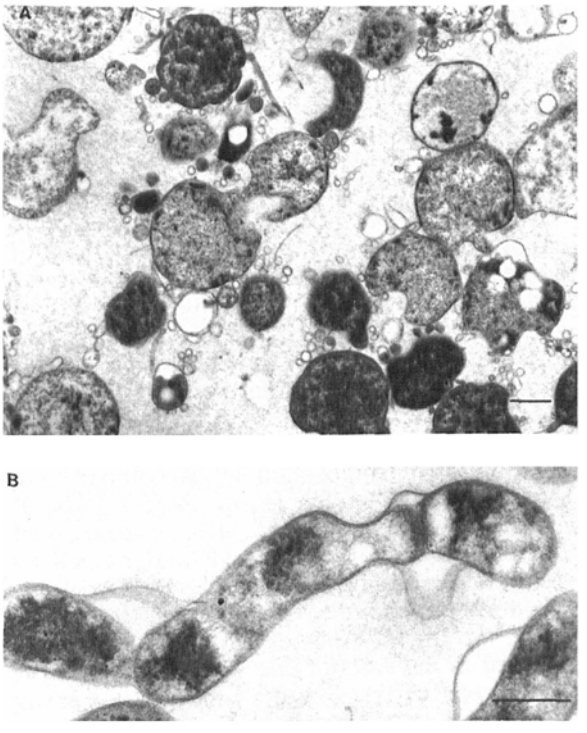

Figure 4: A: Degenerating spheroplast-like forms of *Helicobacter pylori*, resulting from exposure in liquid culture to amoxicillin (0.5 mg/l) for 24 hours. B: Cell wall separation and cytoplasmic coagulation in *Helicobacter pylori*, resulting from exposure in vitro to an environment of pH 3.5 for 15 minutes. Bars = 500 nm.

Helicobacter pylori cells, notwithstanding their distinctive adaptation to a gastric habitat beneath the mucus layer. In tests with broth cultured strains under a range of decreasing pH conditions (78), minor cell wall swelling was seen at pH5, and exposure for 10 minutes or more to conditions of pH4 and less caused gross ballooning of the cell walls, with flagellar fragmentation and cytoplasmic coagulation (Figure 4B). This pattern of morphological change closely resembled that seen in endoscopic gastric biopsy specimens obtained from patients shortly after commencement of oral colloidal bismuth subcitrate therapy (21), in which the bacteria were no longer confined to the deep aspect of the mucus layer.

Preservation

We have preserved *Helicobacter pylori* and related animal bacteria by drying suspensions directly from the liquid state (L-drying); freeze-drying harms *Helicobacter pylori*. L-drying may be done more simply, safely and economically than freeze-drying. The most successful drying solution was L-glutamic acid 20 % w/v per 100 ml de-ionised water. Cultures have been kept for seven years at – 20 °C with successful reconstitution. Five per cent glutamic acid solution and 20 % glucose solution were slightly less successful than 20 % glutamate, and storage at + 4 °C was less successful than that at – 20 °C.

Using a storage medium of 1 ml of 1 % peptone water containing 25 % glycerol, we have preserved *Helicobacter pylori* for more than 13 months at – 70 °C. Another method is storage of *Helicobacter pylori* at – 70 °C in 1 ml defibrinated horse blood which retains viable organisms for at least six months (79). Other workers have successfully used tryptone soy broth containing 15 % glycerol and beads in cryopreservative fluid (80).

Typing Schemes

Attempts at typing *Helicobacter pylori* have been relatively unsuccessful. Biotyping with preformed enzymes was shown not to be feasible as all isolates gave the same reactions (81). Because plasmids are found in only 50 % of isolates (26, 27, 28), they could not be used for a typing scheme.

Coagglutination

Preparation of antisera against individual strains of *Helicobacter pylori* and subsequent agglutination of other strains by means of protein A has been reported by Danielsson et al. (82) to generate a practical typing scheme of at least six types (I–VI). For coagglutination whole bacterial cells, untreated or treated with formalin, or heated at 100 °C in a waterbath for 30 minutes were used as antigens. Protein A-containing *Staphylococcus aureus* was coated with rabbit antibodies raised against *Helicobacter pylori* or with IgG from unimmunized rabbits. The coagglutination tests were performed as slide agglutination tests. All the *Helicobacter pylori* strains tested gave clear cut moderate or strong reactions with the homologous antiserum, and equally strong reactions with untreated or heated cells, indicating that heat stable antigens are involved. Some of the reactions appeared only after the organisms were boiled. Appropriate absorption of antisera with homologous organisms showed that heat labelled antigens were involved as well. Some strains showed broader cross-

reactions than others. Isolates with two different *Helicobacter pylori* coagglutination types were obtained from the same specimen in one patient.

Immunoblotting

Antibody was raised in rabbits against the protein extract of a single isolate of *Helicobacter pylori* by Burnie et al. (83). One hundred and fifty isolates of *Helicobacter pylori* were each run as a protein profile and then immunoblotting performed with the rabbit antiserum. Nine typing groups were defined on the basis of 11 protein bands, however 66 % of isolates were either in Group 1 or Group 2. Immunoblotting can be useful in localised epidemiological studies, but is not entirely suitable as an international method of classification because the rabbit antiserum may be unique.

Serotyping

Helicobacter pylori has both protein and core LPS group antigens, and strain-specific protein and LPS side chain antigens (29). Immunoblotting of *Helicobacter pylori* LPS with immune serum demonstrated that most *Helicobacter pylori* strains share core antigens, but that the side chain antigens are unique (29). The heterogeneity observed in LPS profiles of *Helicobacter pylori* may be used to develop a serotyping system based on heat stable antigens.

Outer Membrane Protein Analysis

Helicobacter pylori outer membrane profiles have more variability between strains than do whole cell or acid-extractable preparations (29). Sarcosyl insoluble preparations can yield reproducible protein bands. We have typed organisms on the basis of four bands at 31 kDA, 70 kDA, 90 kDA and 110 kDA because these show the greatest variation between strains (unpublished observation). Eighteen typing groups were detected, and we found a wide scatter of strains among these groups. Such a method has potential for use internationally since there could be a standardised method of preparing sarcosyl-insoluble preparations.

Restriction Endonuclease Analysis

The chromosomal DNA from 24 isolates of *Helicobacter pylori* was studied by *Hind*III endonuclease analysis by Langenberg et al. (84). Majewski and Goodwin (26) developed a rapid extraction method to analyse the chromosomal DNA of *Helicobacter pylori*; they tested nine endonucleases but only three gave satisfactory digestion, *Hind*III, *Eco*RI and *Sac*I. The latter two produced fewer, high molecular weight bands whereas *Hind*III produced many, low molecular weight bands which allowed clearer comparisons between isolates. A ten-fold increase in the lysozyme concentration was required for successful extraction of *Helicobacter pylori* chromosomal DNA compared to that of *Campylobacter jejuni*. Both groups of workers reported that the isolate of *Helicobacter pylori* from each patient had its own unique endonuclease profile. This suggests considerable genomic variation of *Helicobacter pylori* at the subspecies level (26). Isolates obtained at intervals of one year could have the same profile (81), but on occasions after an interval of three or six months the second isolate from a patient can have a different profile (26). However, it has been reported that when *Helicobacter pylori* was eradicated from a patient, after an interval of one year another isolate was obtained which had the same profile as the original one (84); thus this technique can be of epidemiological use.

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