Helicobacter pylori comb. nov. Exhibits Facultative Acidophilia and Obligate Microaerophilism

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Modified brucella broth medium was used to study the growth of Helicobacter pylori at varied pHs and partial pressures of oxygen and to determine the effect of urea on culture pH. Our findings suggested that the pHs of the media remained stable with or without urea and that H. pylori showed facultative acidophilia and obligate microaerophilism.

Helicobacter pylori comb. nov. is associated with human gastroduodenal diseases such as gastroduodenitis (4, 12), peptic ulcer (9), and gastric carcinoma (17). Even though H. pylori colonizes human stomach tissue with pHs as low as 1.5 to 1.8, this bacterium is reported to be extremely sensitive to gastric acidity (5, 13). Thus, it is generally assumed that H. pylori is not acidophilic or acid tolerant. It is postulated that the organism survives the acidic environment of the human stomach mainly because (i) the organism occupies a protected niche in the stomach, below the mucus layer (7), which has a near-neutral pH (2); and (ii) H. pylori has a very potent urease which hydrolyzes urea, producing ammonia, which could neutralize acidity in the stomach (6). In order to understand the colonization and survival strategies of H. pylori in the human stomach, it is critical to determine whether H. pylori can grow at acidic pHs and to directly determine whether urease activity of H. pylori can significantly alter its environmental pH.

Partial pressure of oxygen (pO2) is another environmental parameter that is important for the growth of H. pylori (6, 15). However, very little work has been done to study the range of pO2s that supports the growth of this bacterium. One of the main reasons for the paucity of information about the growth of H. pylori is the lack of a liquid culture technique that is adequate for the cultivation of this fastidious bacterium. In this study, the first task was to develop a liquid culture technique to adequately grow H. pylori and then to investigate the effects of pH, pO2, and urea on the growth of the organism.

Commercial brucella broth (Difco) was modified by adding 15 mM (each) KH2PO4 and K2HPO4, 10 μM NH4Cl, and 0.5 μg of nitrapyrin [2-chloro-6-(trichloromethyl) pyridine] (Sigma Chemical Co.) ml−1. Nitrapyrin was filter sterilized (0.2 μm pore size) and added at 40 to 50°C after being autoclaved (11). The medium modification was based on our previous finding that nitrapyrin together with NH4+ induces rapid growth of many bacteria (11) and on the results of our preliminary studies to develop a liquid medium for H. pylori.

Serum vials (24 ml), fitted with rubber stoppers, were partially evacuated (20 ml) with a 22-gauge hypodermic needle fitted on a 20-ml syringe to reduce the time required to flush the vials. The vials were flushed for ca. 2 min with N2 through hypodermic needles before being autoclaved for 15 min. Ten milliliters of medium was added to the sterile vials, and the pressure was neutralized. After 2 drops (50 to 60 μl) of 5% (wt/vol) cysteine were added per vial, the vial contents were allowed to equilibrate for ca. 1 h with periodic gentle agitation. Seven milliliters of the headspace gas was replaced with 2.4 ml of CO2 and 4.6 ml of N2, thus adjusting the gas composition in the vials to approximately 5 to 7 kPa of O2, 10 kPa of CO2, and 83 to 85 kPa of N2. The vial contents were allowed to equilibrate for at least 4 h (preferably overnight) before H. pylori (ATCC 43504/NCTC 11637) inoculum was added. The vials were incubated at 37°C on a platform shaker at 130 to 150 rpm.

For pH experiments, the pH of the modified medium was adjusted to 4.0, 4.5, 5.0, 6.0, 7.0, or 8.0 with sterile dilute HCl. Inoculum was added to an initial optical density at 660 nm (OD660) of approximately 0.085 by using a 48-h-old liquid culture of H. pylori (the weight of cells in 1.0 ml of culture at an OD660 of 1.0 is equivalent to a dry weight of 0.15 μg). The culture vials (two replicates per treatment) were incubated as specified above, and observations of OD660 and culture pH were recorded every 6 h between 12 and 24 h of incubation and at 12-h intervals up to 72 h of incubation by using a spectrophotometer (Beckman DU-65) and a pH meter (Beckman Φ 44), respectively. For these measurements, 0.75-ml samples were removed each time and replaced with the same volume of sterile headspace gas mixture.

To study the effect of urea on culture pH, the growth medium without phosphate buffer was amended with 5 mM urea and the pH was adjusted to 5.0, 6.0, or 7.0. H. pylori was added to an initial OD660 of approximately 0.12 as described above. Control vials contained no urea. All treatments were performed in duplicate. Observations of OD660 and culture pH were made at 24-h intervals up to 72 h of incubation.

For experiments with pO2, brucella broth (pH 7.0) was constituted by using its components (Difco), except sodium bisulfite, and modified as described above but without cysteine. After 10 ml of the warm medium (40 to 45°C) was added to 24-ml vials prefilled with nitrogen, further flushing was done by bubbling nitrogen through the medium for about 2 to 3 min before the medium was autoclaved for 15 min. Rubber stoppers were temporarily held with autoclavable tape during autoclaving. The pO2 levels were adjusted to 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, and 20.0 (standard deviation of ±

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having the ability to grow well at pH 4.5. Any pH change in the growing cultures consistently tended towards a relatively stable pH of 5.0 to 5.5. The trend may be toward the cytoplasmic pH of 5.2, indicating the presence of a pH gradient across the plasma membrane. The cytoplasmic pH determined by flow dialysis with [14C]salicylic acid remained constant regardless of external pH (10a).

The type strain of *H. pylori* used in this study produced active urease (1, 16). The popular hypothesis that urease activity nullifies the acidity of the growth environment and causes back diffusion of protons (10) has no reliable experimental evidence. In this study, when *H. pylori* was grown with 5 mM urea in the culture media at pHs of 5.0, 6.0, and 7.0, there was no significant change in the medium pHs compared with those of controls, even into the mid-stationary phase (72 h) of growth (Fig. 2). Five millimolar urea was chosen so as to exceed the human blood urea level of 1.7 to 3.4 mM (14). The data here suggest that even at very high inoculum densities (e.g., an OD of 1.0 at 72 h) and in the presence of 5 mM urea, *H. pylori* did not significantly alter the pH of its growth medium by urease activity. It is possible that the buffering action of ammonia produced by urease activity takes place in the periplasmic space or on the wall surface (3) instead of in the outer macroenvironment, thereby maintaining an electrochemical gradient across the plasma membrane to overcome external acidity.

It is known that *H. pylori* does not grow under totally anaerobic conditions (2). *H. pylori* grew well in a PO2 range (standard deviations appear in parentheses) of 2.0 to 10.0 (± 1.0) kPa, with a sudden and progressive drop in growth rate above 10.0 (± 1.0) kPa and no growth at 18.0 or 20.0 (± 1.0) kPa (Fig. 3). With its optimal PO2 requirements in the range of 2.0 to 10.0 (± 1.0) kPa and its inability to grow anaerobically or at or near ambient PO2, *H. pylori* should be categorized as an obligate microaerophile.

Our findings, apart from providing useful information on the growth and survival of *H. pylori*, can be of significance to physiologists, pharmacologists, and clinicians. Of particular significance is the demonstrated acidophilic nature of *H. pylori*. The importance of this information to future therapy is that new drugs can be aimed at specific targets of *H. pylori* for its eradication, such as the electron transport chain components and/or the permeases, which are highly selective in acidophiles. Further work is needed to identify such selective targets.

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FIG. 2. Effect of 5 mM urea on the pH of an *H. pylori* culture during 72 h of growth. The initial pHs were 5.0 (A), 6.0 (B), and 7.0 (C).
REFERENCES


10a. Kangatharalingam, N. Unpublished data.


