UREA -INDEPENDENT ACID TOLERANCE RESPONSE (ATR) IN *HELICOBACTER PYLORI*

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INTRODUCTION

Helicobacter pylori is a Gram negative spirillum found in close association with the gastric mucosa and has been identified to play a potential role in causing gastritis and gastrointestinal ulcerative diseases in humans. The primary source of infection by this pathogen in humans has been attributed to contaminated ground water. This organism has even been detected in deep well water. A number of reports have proposed that adaptation and survival of this pathogen in a gastric acid environment is due to their ability to utilize urea to form ammonia through urease activity, creating a microenvironment of relatively higher pH. The mechanism by which this bacterium can survive the fairly low pH conditions that are often encountered in ground water, which is normally relatively urea-free, has not yet been determined, and this is the subject of this study. It has been reported that adaptation to an acidic pH environment in many of the microbial pathogens such as *Salmonella typhimurium*, *Escherichia coli,* and *Aeromonas hydrophila* requires exposure to relatively mild pH (pH 6.0-4.5) which results in alteration of the cellular protein profile. Some of these proteins have been characterized as "stress" proteins and others as "acid responsive" proteins. Therefore, these microorganisms manifest physiological adaptation (possibly with underlying genetic mechanisms) to acidic pH environment. In addition to these microbial pathogens, naturally occurring microorganisms that colonize in the human gastric environment and are urease positive, such as *Lactobacillus*, have survival mechanisms that have not been attributed to their ability to catalyze urea to ammonia and CO_2 to increase the surrounding pH to an alkaline level. *H. pylori* produces massive quantities of urease at the cell surface and urease activity remains highly active in an acidic pH environment. Whether *H. pylori* has adapted to the acidic pH environment using underlying physiological and genetic mechanisms like other acidophilic microorganisms such as *Lactobacillus*, or has adapted via activity of its urease enzyme alone, is a question that needs to be addressed in order to better understand how this organism is able to survive in the natural environment.

MATERIALS AND METHODS

Microbial Strains

Helicobacter pylori strain ATCC 49503 was used for all acid experiments. Cells were plated onto 10% SBA (BRL or Difco TSA blood agar base & defibrinated sheep blood from Colorado Serum) and incubated plated side up at 37° C in a CO_2 incubator with 8% CO_2 & 100% humidity for 3-4 days.

Acid Tolerance

Preparation of Initial Cell Suspension. 3 to 4 plates (lawn inoculum) of cells were removed from 3 to 4 day cultures of *H. pylori* with an inoculating loop and transferred to a centrifuge tube containing 3 ml Dulbecco's formula PBS (D-PBS = 0.137 M NaCl, 0.0026 M KCl, 0.008 M Na_2HPO_4, 0.0014 M KH_2PO_4, pH 7.3). Dulbecco's PBS contains no urea. The cells were suspended well by repeated pipetting and the cell suspension was transferred to a centrifuge bottle containing 250 ml of D-PBS (pH 7.3). The cell suspension was vortexed for 1 min to remove any remaining cell clumps and an aliquot was used to perform an OD_{450} reading in a Perkin-Elmer Lambda II spectrophotometer. The optical density of the cell suspension was then adjusted to approximately 0.8 by adding more cells or PBS as needed. 300 μl of cells from this suspension were transferred to one well of a microtiter plate and dilutions of -1,-2,-3, & -4 were made from this well into D-PBS. 10 μl of cell suspension from the direct well and each dilution well were spot plated in triplicate onto 10% SBA, and colonies were counted after 4 days of incubation in order to obtain an initial viable plate count.

Exposure to Acidic pH, Dilution, and Plating. The acid tolerance test was performed within 30 min from the time of the initial suspension of the cells in D-PBS. 20 ml aliquots of the initial cell suspension were distributed to 11 centrifuge tubes, and the pH of each tube was immediately adjusted such that a total of 12 suspensions, pH 7.3 (original suspension), 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, and 1.0 were prepared. All pH decreases were made by the addition of 0.6 N, 0.4 N, or 0.2 N HCl. Minor pH increases were made as required by the addition of 0.4 N or 0.2 N NaOH. The approximate amount of acid required for each

pH change was predetermined in D-PBS without cells in order to minimize the time required for the pH changes. At 15 min intervals over a total period of 2 hrs, 300 μl aliquots of cells from each tube were transferred to a microtiter plate and dilutions of 10^{-1},10^{-2},10^{-3}, & 10^{-4} were made into D-PBS (300 μl total volume per well). 10 μl of cell suspension from the direct well and from each dilution well were spot plated in triplicate onto 10% SBA and colonies were counted after 4 days of incubation in order to obtain viable plate counts. The cell suspensions were kept in the CO_2 incubator between spot platings and pH changes.

Acid Induction

The same procedure as used for preparation of the initial cell suspension for the acid tolerance test was used for preparation of the cells for the acid induction experiment, only the volume of cell suspension that was prepared was adjusted as required. A spot plating in order to obtain an initial viable cell count was performed. Immediately afterward, 20 ml aliquots of the initial cell suspension were distributed to 4 centrifuge tubes, and the pH of each tube was immediately adjusted such that a total of 4 suspensions, tube 1 = pH 7.3 (original suspension), tube 2 = pH 5.5 (induction pH control), tube 3 = pH 5.5 (induction pH), and tube 4 = pH 4.5 (direct challenge pH) were prepared. All pH decreases were made by the addition of 0.6 N, 0.4 N, or 0.2 N HCl. Minor pH increases were made as required by the addition of 0.4 N or 0.2 N NaOH. The approximate amount of acid required for each pH change was predetermined in D-PBS without cells in order to minimize the time required for the pH changes. After 30 min 300 μl aliquots of cells from each tube were transferred to a microtiter plate and dilutions of 10^{-1},10^{-2},10^{-3}, & 10^{-4} were made into D-PBS (300 μl total volume per well). 5 μl of cell suspension from the direct well and from each dilution well were spot plated in triplicate onto 10% SBA. Immediately after plating, the pH of tube 3 was lowered to a *challenge* pH of 4.5 . Afterwards, at 15 minute intervals, dilutions and spot platings were made from each tube up until 105 min after the initial change of pH. Colonies were counted after 4 days of incubation in order to obtain viable plate counts. The cell suspensions were kept in the CO_2 incubator between spot platings and pH changes. The exact same procedure as described above was then repeated, using an induction pH of 5.5 and a challenge pH of 4.5.

RESULTS AND DISCUSSION

Acid Tolerance and Induction

To determine the susceptibility of *H. pylori* to acidic pH in the absence of urea, aliquots of a culture suspended in a phosphate buffered saline solution were exposed to solution pH ranging from pH 1.5 to pH 7.3, in approximately 0.5 pH increments. Samples from each aliquot were spot plated and after incubation, viable plate counts were performed. The viable plate counts (Figure 1) indicated that after 30 min of exposure there was approximately a 1-log decrease in viability at pH 4.5, a 2-log decrease in viability at pH 4.0, and a 2.5-log decrease at pH 3.5. Above pH 4.5 there was very little decrease in viability even after 1 hour. At pH 3.0 and below no viable cells were detectable in the culture aliquots after only 15 minutes. Based upon these results, an induction pH of 5.0 was chosen to test whether incubation of the cells at this induction pH would induce an acid tolerance response (ATR) in the cells, such that they would adapt to the induction pH of 5.0 and hopefully better survive when challenged with a pH of 4.0. Cells at pH 7.3 and cells directly challenged to pH 4.0 without adaptation at pH 5.0 were kept as controls. Viable plate counts (Figure 2) indicated that after 90 min of exposure the unadapted cultures showed no survival, while the adapted cultures survived at a level approximately 1-log lower than the pH 7.3 control. The cells in the unadapted culture directly challenged to pH 4.0 began losing viability after about 15 min. When the procedure was repeated using an induction pH of 5.5 and a challenge pH of 4.5, no decrease in viability was indicated after exposure to the challenge pH after 105 min after exposure to pH 4.5 (Figure 3). The results of this experiment suggest that *H. pylori* possesses a urea-independent acid tolerance mechanism which contributes to survival of the organism in low pH environments.

CONCLUSION

Helicobacter pylori infections are at an epidemic level in many developing nations, and the primary source of infection by this pathogen in humans has been attributed to contaminated ground water. A number of reports have suggested that adaptation and survival of this pathogen in a gastric acid environment is due to their potent urease activity; however, the mechanism by which this bacterium can survive low pH conditions in ground water is unknown, since ground water normally contains too little urea to be of any use to the organism. It has been reported that adaptation to an acidic pH environment in many other microorganisms can be attributed to physiological adaptation, possibly with underlying genetic mechanisms. The results of this experiment suggest that *H. pylori* possesses a urea-independent acid tolerance response mechanism which may contribute to survival of the organism in low pH environments that are urea-free, which offers a possible explanation for the persistence of this organism in ground water. Although this mechanism does appear to possess some type of acid tolerance response to deal with low pH conditions in the absence of urea, the response appears to be weak when compared with other organisms that are known to have a urea-independent acid tolerance response. Whether this ATR system is unique or is physiologically similar to

ATR systems found in other organisms remains to be investigated.

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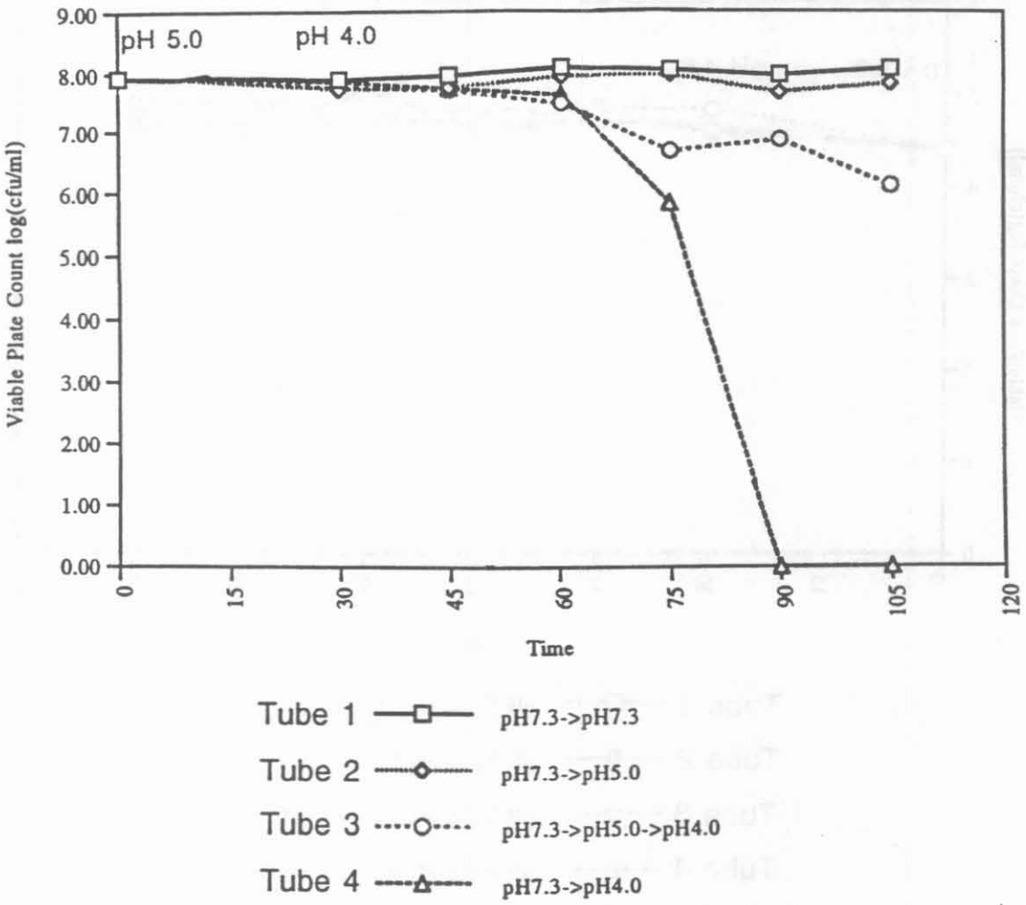

Figure 1: *H. pylori* acid induction at pH 5.0 in Dulbecco's phosphate buffered saline followed by acid challenge at pH 4.0. Four tubes of cell culture were prepared at pH 7.3. At time zero the pH in tubes 2 and 3 was lowered to pH 5.0. After allowing 30 min of acid adaptation in tube 3, the pH in tubes 3 and 4 was lowered to pH 4.0. Aliquots from each tube were spot plated onto 10% SBA at timed intervals, and viable plate counts were obtained after 4 days of incubation.

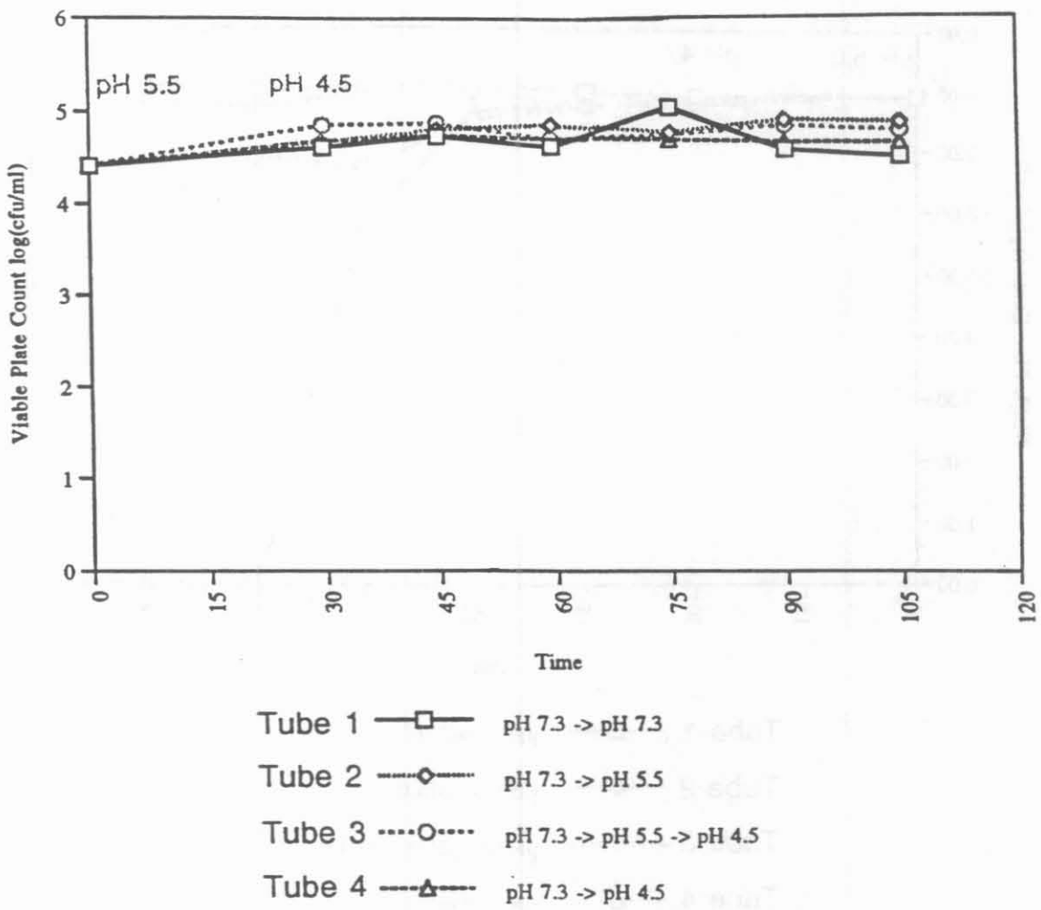

Figure 2: *H. pylori* acid induction at pH 5.5 in Dulbecco's phosphate buffered saline followed by acid challenge at pH 4.5. Four tubes of cell culture were prepared at pH 7.3. At time zero the pH in tubes 2 and 3 was lowered to pH 5.5. After allowing 30 min of acid adaptation in tube 3, the pH in tubes 3 and 4 was lowered to pH 4.5. Aliquots from each tube were spot plated onto 10% SBA at timed intervals, and viable plate counts were obtained after 4 days of incubation.