Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH

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Abstract
Background—Helicobacter pylori produces large amounts of urease presumably to be prepared for the rare event of a sudden acid exposure. The hypothesis that *H pylori* is acid sensitive and protein production is inhibited by low pH was examined.
Methods—H pylori or its soluble enzymes were incubated buffered or unbuffered at a pH ranging from 2–7 in the presence of 5 mM urea for 30 minutes. After exposure, urease and catalase activities of whole cells, supernatants, and soluble enzyme preparations were measured at pH 6·8. Newly synthesised enzyme was quantified by immunoprecipitation of [^{35}S]-methionine labelled protein.
*Results—*Exposure to buffer below pH 4 resulted in loss of intracellular urease activity. In soluble enzyme preparations and supernatant, no urease activity was measurable after incubation at pH<5. In contrast, catalase in whole cells, supernatant, and soluble enzyme preparations remained active after exposure to pH≥3. Exposure below pH 5 inhibited synthesis of total protein including nascent urease and catalase. At pH 6 or 7, urease represented 10% of total protein, catalase 1·5%. Exposure of *H pylori* to unbuffered HCl (pH>2) resulted in an immediate neutralisation; urease and catalase activities and synthesis were unchanged.
*Conclusion—*Low surrounding pH reduces activity of urease and synthesis of nascent urease, catalase, and presumably of most other proteins. This suggests that *H pylori* is not acidophilic although it tolerates short-term exposure to low pH.
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Helicobacter pylori causes chronic active gastritis, and is closely associated with the development of peptic ulcer disease.[1] [2] The bacterium exclusively colonises gastric mucosa including areas with and without acid secretion. In vivo and in vitro, *H pylori* produces large quantities of urease,[3–6] which converts urea into ammonium permitting neutralisation of gastric acid.[7] Native urease of *H pylori* has a molecular mass of approximately 550 kilodalton (kDa) and is a nickel containing protein consisting of six copies each of two subunits (UreA–30 kDa and UreB–62 kDa) in a one:one molar ratio.[8] [9] The low Michaelis constant (K_m) of *H pylori* urease permits this enzyme to be catalytically efficient even at low urea concentrations.[8] [9] Urease is clearly central to the pathogenesis of *H pylori* infection. This activity is necessary for the survival of *H pylori* in acidic environment in vitro.[10] Although urease negative *H pylori* mutants were unable to colonise the gastric mucosa in an animal model,[11] this was pH independent. Thus, the role of urease in vivo is probably not only its acid neutralising capacity.

Nevertheless, protection against acidity seems sufficiently important, that *H pylori* produces urease in large quantities. This is even more surprising because *H pylori* is usually found underneath the mucous layer where pH approaches neutrality.[12] Thus, exposure to low acidity seems to be a very rare event occurring only if the mucous layer is damaged mechanically or by chemical agents such as aspirin[13] or during first infection. However, the route of infection is unknown and it might happen during food intake, when gastric pH is already raised.[14] Thus, although neutralisation of acid is probably rarely necessary, large amounts of urease is synthesised even in a situation where no acid is present.

Based on these findings we hypothesised that *H pylori* is very sensitive to acid exposure and the large stock of urease is necessary to protect *H pylori* against a sudden drop in pH where no time is available to up regulate production of the enzyme.

In contrast, in *Streptococcus salivarius* a urease positive bacterium found in dental plaque, regulation of urease levels by pH has been demonstrated.[15] Other species regulate urease synthesis in response to urea[16–18] or available nitrogen concentration.[19] In *H pylori* and *Escherichia coli* containing cloned *H pylori* urease genes, neither nitrogen limitation nor addition of urea seen to stimulate synthesis of the urease structural subunits.[8] [20] [21]

The aim of this study was to evaluate the effect of exposure of *H pylori* cells to pH values, which vary within the physiological gastric range from 2 to 7, on the synthesis and activity of urease. The direct effect of different pH values on the activity of crude enzyme was tested by exposing fresh preparations of soluble *H pylori* enzymes to the same pH values as intact *H pylori* cells. Experiments were conducted in buffered and unbuffered solutions both containing urea. Exposure to buffered solutions allowed us to evaluate the effect of exposure to a constant pH even in the presence

of urea hydrolysis. In experiments using the unbuffered solutions, bacterial ammonia was expected to increase the pH gradually. These latter experiments allowed us to study the effect of a transient exposure to low pH as it may occur in the stomach.

For comparison with urease, catalase was used in this study to control for non-specific effects of low surrounding pH on enzyme synthesis and activity. By analogy to other bacteria, catalase is assumed to protect *H pylori* against the damaging effect of hydrogen peroxide[22] by converting H_2O_2 to H_2O+O_2. The native catalase of *H pylori* has a molecular mass of 165–200 kDa; subunits have a molecular mass of 50–52 kDa.[22] No evidence exists that catalase production is regulated by the surrounding pH in *H pylori* or other bacterial species.

Methods

Bacterial strains

H pylori strain UMAB41 was isolated from gastric biopsy specimens from a patient undergoing endoscopy at the University of Maryland, School of Medicine, Baltimore, Maryland. The strain has been characterised as reported earlier.[8 23]

Culture conditions and preparation of bacterial suspensions

Cultures were grown on 10% sheep blood agar supplemented with vancomycin (10 μg/ml), polymyxin (2·5 IU/ml), and trimethoprim lactate (5 μg/ml).[24] Cultures were incubated at 37°C in a microaerobic environment generated by an activated Campypak (Becton-Dickinson, Baltimore, MD). *H pylori* were cultured for 72 hours before use in experiments.

Exposure of H pylori cells to various pH steps and measurement of urease and catalase activity in cells and supernatant

Bacteria were suspended to an OD_{600} of 2·5 in 2 ml HCl adjusted to an initial pH of 2, 3, 4 or 5, or in 0·1 M sodium phosphate at pH 6 or 7 or in 0·1 M citrate-phosphate buffer at a pH of 2, 3, 4, 5, 6 or 7. All solutions contained 5 mM urea (Sigma) and had an osmolarity of 300 mmol. The pH was controlled and corrected if necessary before each experiment. *H pylori* cells were incubated in the various solutions for 30 minutes under microaerobic conditions at 37°C. After incubation, samples were taken for determination of viable counts (CFU/ml) by direct plating of 10-fold dilutions. Cells and supernatant were separated by centrifugation (5000 rpm, 20 min, 4°C). The pH of the supernatant was determined using a pH meter (Corning, model 320). Cells were washed twice and lysed in a French pressure cell at 20 000 psi (SLM Aminco, Urbana, IL). Proteins in the supernatant were concentrated by filtration using a centrifugal concentrator (Centrisart I, Sartorius, Goettingen, Germany) with a cut off size of 20 kDa.

Concentrated proteins were solubilised in 20 mM sodium phosphate buffer, pH 6·8. Enzyme activities were determined for whole cells, soluble enzyme preparations, and supernatants as described below immediately after each experiment.

Exposure of H pylori whole cells to various pH values and measurement of urease and catalase synthesis by immunoprecipitation

In a second series of experiments that involved radiolabelling of proteins, *H pylori* cells were deprived of L-methionine by preparing suspensions of the bacteria (OD_{600}=2·5) in a L-methionine free medium (Difco) and incubating the suspensions for 90 minutes at 37°C under microaerobic conditions. After incubation of the cells in the various pH solutions, incubations were continued after addition of 63 μCi of [^{35}S]-methionine (>600 Ci/mmol; Dupont, New England Nuclear, Boston, MA). After incubation for 30 minutes, labelling was stopped by addition of unlabelled L-methionine to a final concentration of 8 mg/ ml. Cells and supernatant were separated; cells were washed twice and resuspended in 200 μl of 50 mM TRIS-HCl buffer containing 1% SDS, pH 8, and lysed by boiling for 10 minutes. Soluble protein was used for immunoprecipitation as described below.

Exposure of H pylori cytosolic enzymes to solutions of various pHs and measurement of urease and catalase acitivity

H pylori cells were harvested from plates and lysed by passage through a French pressure cell at 20 000 psi. After centrifugation (12 000 rpm, 5 min, 4°C), 50 μl of soluble enzyme (approximately 150 μg protein) were added to 2 ml of each HCl, phosphate, or citrate buffer adjusted to various pH values; solutions were incubated for 30 minutes as described in the first experiment. After incubation, cellular proteins were separated from the buffer solutions as described above. Enzyme activities were determined as described below immediately after each experiment.

Urease activity

Urease activity was measured using the phenol red assay of Hamilton-Miller and Gargan[25] as calibrated for quantitative determination as described previously.[26] Rates of urea hydrolysis are expressed as mmol NH_3/min/mg protein. For each experiment, protein concentration was determined by the BCA method according to the instructions of the manufacturer (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Catalase activity

Catalase activity was determined by observing the disappearance of H_2O_2 as measured by its absorbance at 240 nm.[27] The 2 ml reaction mixture contained 11 mM H_2O_2 in 10 mM potassium phosphate, pH 6·8. The reaction

was started by adding 5 μl of soluble enzyme preparation or concentrated supernatants. Absorption was monitored for one minute at 23°C. Based on the initial rate, catalase activity was expressed as the disappearance of H_2O_2 in mmol/min/mg protein. The calculation was based on a standard curve determined from the absorption of 0–11 mM H_2O_2 under the same conditions. The results were normalised for the protein concentration of the enzyme preparations.

Antiserum

Polyclonal antisera were raised against the small (UreA) and large (UreB) subunits of the *H pylori* urease in rabbits using purified urease subunits as previously described.[28] For immunoprecipitation of catalase, polyclonal goat antiserum against *H pylori* catalase was used. The specificity of the antisera was confirmed by western blot. Pre-immune sera were used as controls.

Immunoprecipitation

H pylori bacterial cells, labelled with [^{35}S]-methionine as described above, were used for immunoprecipitation. Cells were washed twice and lysed by boiling for 10 minutes.

Immunoprecipitation was performed according to a standard protocol.[29] Briefly, 10 μl of cell lysate or supernatant were counted to determine total counts per μl in each sample. For immunoprecipitation the different amounts of total cpms were standardised by using 100 000 cpms in each set up. [^{35}S]-labelled soluble proteins were incubated for one hour at 4°C in 400 ml of 50 mM TRIS-HCl (pH 8·0), 2% (v/v) Triton X 100, 0·15 M NaCl, 0·1 M EDTA containing urease antisera (1:50 v/v of anti-UreB (recognises the large subunit of urease)), 1:25 v/v of anti-UreA (recognises the small subunit of urease), anti-catalase serum (1:50 v/v), or pre-immunise control sera. After one hour, 50 μl protein A (10% w/v) (Gibco BRL, Gaithersburg, MD) was added and incubation was continued at 4°C for 30 minutes. For immunoprecipitation of catalase, protein G (Gibco BRL, Gaithersburg, MD) was used because previous tests showed a higher yield for precipitation of the catalase antiserum than protein A. After incubation, precipitated antibody-antigen complex and supernatant were separated by centrifugation (5000 rpm, 5 min, 23°C), washed twice with the Triton X-100 containing buffer, resuspended in 100 μl of 50 mM TRIS-HCl, pH 8·0 containing 1% SDS, boiled for 10 minutes, and counted by liquid scintillation. Counts obtained from incubation with pre-immunised control sera were subtracted from the counts obtained for specific anti-urease or anti-catalase sera. Values for immunoprecipitated urease and catalase were then expressed as percentage of total counts. Thus, the results represent the percentage of protein as urease or catalase from total protein produced by the *H pylori* cells during the period of incubation in the solutions of various pH.

Statistical analysis

Results for enzyme activities and results of immunoprecipitation studies were compared by the non-parametric Kruskal-Wallis test.[30] If this test showed statistical significance (p<0·05), individual comparisons were performed using the test procedure described by Nemenyi.[30]

Results

Duration of acid exposure to H pylori

Conditions were established whereby *H pylori* was either briefly exposed to acidic conditions or where acidic conditions were held constant despite active urea hydrolysis. When *H pylori* was incubated in unbuffered HCl of pH 2 containing 5 mM urea, pH increased during five minutes to values around 4 (Fig 1). Incubation in HCl of pH 3, 4 or 5 containing 5 mM urea increased the pH to values above 7 within the first minute. The increase in pH was assumed to be due to neutralisation of unbuffered HCl by ammonia produced by hydrolysis of urea. The pH of citrate or phosphate buffers, however, remained unchanged during 30 minutes of incubation with *H pylori*. Although all buffers contained 5 mM urea, neutralisation by ammonia did not change the pH.

Viability of H pylori after incubation at various pHs

Survival of *H pylori* after incubation for 30 minutes in citrate buffer, phosphate buffer, or unbuffered HCl is shown in Figure 2. With a starting inoculum of approximately 2×10^9 cfu/ml, only exposure to pH 2 citrate buffer reduced viability significantly (p<0·05). A tendency for reduced viability was also found after exposure to pH 3 citrate buffer or pH 2 unbuffered HCl compared with viability after exposure to pH 7. However, these differences were not statistically significant.
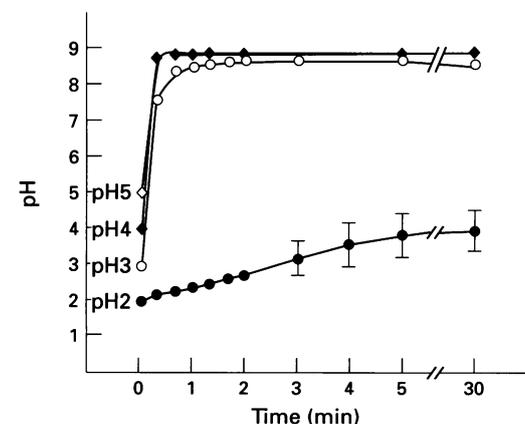

Figure 1: pH of unbuffered HCl solutions of pH 2, 3, 4, and 5 during incubation of H pylori cells. H pylori was suspended in HCl solutions of various pH containing 5 mM urea. pH was measured over time; the initial pH is given at the start of each curve. Each point represents the mean (SD) of four experiments, unless SD is smaller than the point.
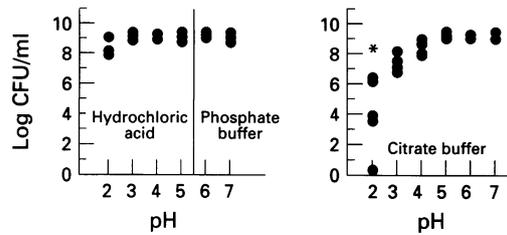
Figure 2: Survival of H pylori *in citrate buffer, unbuffered HCl, or phosphate buffer containing 5 mM urea at various pHs. Suspensions of* H pylori *UMAB41 (10⁹ cfu/ml) were incubated for 30 minutes in unbuffered HCl at pH 2–5 or phosphate buffer at pH 6 and 7 (left graph) or citrate buffer at pH 2–7 (right graph); all solutions contained 5 mM urea. Viability was determined by plating 10-fold dilutions of the suspensions. The x axis represents the initial pH of the solution; the y axis represents the viability after incubation for 30 minutes at 37°C given as $\log_{10}$ cfu. Each point represents one experiment. Survival was significantly reduced when* H pylori *was exposed to pH 2 in citrate buffer compared with pH 7 (*, p<0·01 tested by Kruskal-Wallis test, followed by Nemenyi test). There was also a tendency for reduced survival at pH 3 in citrate buffer and at pH 2 in HCl but these differences were not statistically significant.*

Urease and catalase activity

Activities of urease and catalase of whole *H pylori* cells incubated for 30 minutes in buffered or unbuffered solutions of various pHs are shown in Figure 3. Urease activity was expressed as mmol NH_3/min/mg protein. Catalase activity was measured as decomposition of H_2O_2 in mmol/min/mg protein. All enzyme measurements were determined at pH 6·8 and 23°C. Urease activity was reduced in the cells after incubation of *H pylori* cells in citrate buffer at pH 2 or 3 or after incubation in HCl at pH 2. For all other conditions, urease activity was similar to those of samples incubated in neutral citrate or phosphate buffers.

Catalase activity was reduced when *H pylori* cells were incubated in citrate buffer at pH 2. In contrast with the results obtained for urease
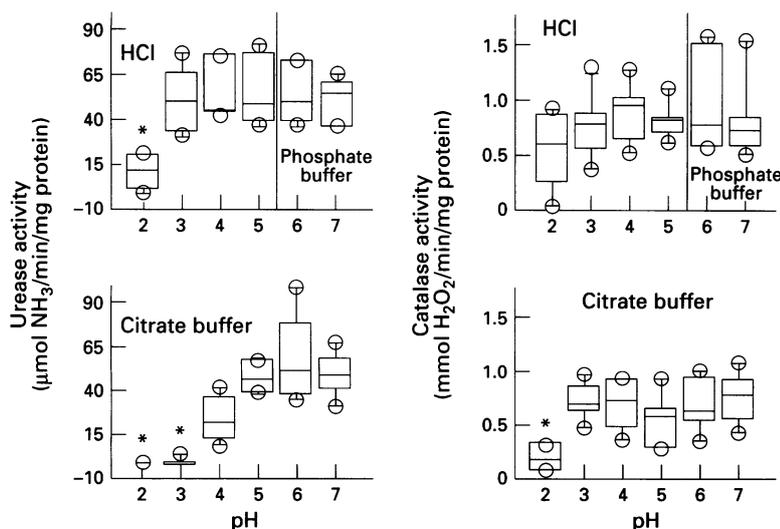
activity, catalase activity was not affected by incubation at pH 3 or after incubation in HCl at pH 2.

To evaluate whether release of enzyme from whole cells was responsible for the reduction in enzyme activity at certain pH levels, enzyme activity was also measured in the supernatant. Similar to the results in the intracellular enzyme activity, reduced urease activity was found after incubation in citrate buffer at pH 2 and 3. Catalase activity was also reduced in the supernatant after incubation in citrate buffer at pH 2. Under none of the conditions was an increase of enzyme activity found as compared with the results at pH 7.

Synthesis of nascent urease and catalase

Newly synthesised enzyme was measured by immunoprecipitation of [³⁵S]-methionine labelled protein. Labelling was performed during incubation in solutions of various pH. As shown in Figure 4, incubation in unbuffered HCl or phosphate buffer of pH 6 or 7 did not affect urease or catalase production. Urease and catalase represented approximately 10% and 1% respectively, of newly synthesised protein in unbuffered HCl (pH 2-5) and phosphate or citrate buffer (pH 6–7). In contrast, incubation in citrate buffer, pH 2–5 significantly inhibited nascent synthesis of urease and catalase.

To control for any loss of newly synthesised enzyme by release into the supernatant, immunoprecipitation of urease and catalase was also performed using the supernatant. Although comparatively more urease and catalase was found in the supernatant at low pH in citrate buffer, the total amount of precipitated counts at pH 2, 3, 4 and 5 in citrate buffer was lower than 10% compared with the counts at pH 6 or 7. Thus, the reduced amount of precipitated urease or catalase from the cells at low pH was not due to a loss of proteins into the supernatant.

Urease and catalase stability

The Table shows the activity of the crude cytosolic enzyme after incubation in solutions of various pH values. Enzyme activities were determined after raising the pH in all groups to 6·8. Urease activity was reduced after exposure of the enzyme to citrate buffer at a pH below 5. In contrast, catalase activity was only affected by exposure to pH 2 in HCl. Thus, exposed urease is not resistant to the effects of low pH unless it is cell associated or cytoplasmic, while catalase is relatively more resistant.

Figure 3: Urease and catalase activities in H pylori *whole cells after incubation in HCl, phosphate buffer, or citrate buffer at different pHs.* H pylori *was suspended in HCl at pH 2, 3, 4, 5 or phosphate buffer at pH 6, 7 (upper panels) or in citrate buffer at pH 2–7 (lower panels) and incubated for 30 minutes. All solutions contained 5 mM urea. Cells were lysed and activity of urease (left panels) and catalase (right panels) activities were measured at pH 6·8 at 23°C. Results of six experiments are displayed as box whisker plots. The median is represented by the horizontal line; the top and bottom edge of the box represent the 25 and 75 percentile; the whisker represents the 10 and 90 percentile and the circles extending away from the boxes represent minimal and maximal values.*
*Significant difference from value obtained at pH 7.

Discussion

The natural environment of *H pylori* is the human gastric mucosa. The bacterium is usually found beneath the mucus layer in an area where the pH approaches neutrality.[12] Nevertheless, it could be assumed that *H pylori* might be exposed suddenly for short periods to lower pH values either during disruption of
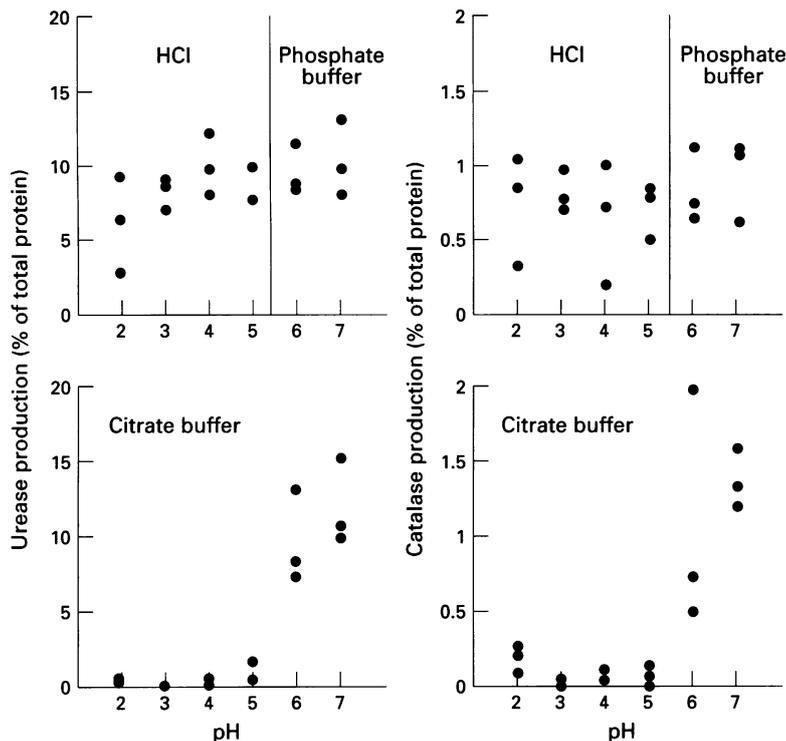
Figure 4: Nascent enzyme synthesis during incubation of H pylori *at various pHs. Suspensions of* H pylori *in HCl at pH 2, 3, 4, 5 or phosphate buffer at pH 6, 7 (upper panels) or in citrate buffer at pH 2–7 (lower panels) were labelled with [^{35}S]-methionine. All solutions contained 5 mM urea. Urease and catalase synthesis was determined by immunoprecipitation using polyclonal antiserum directed against the small (UreA) and large (UreB) structural subunit of* H pylori *urease and against the* H pylori *catalase holoenzyme. Each data point represents one of three independent experiments.*

the mucous layer or during transmission from person to person. *H pylori* must survive low surrounding pH, at least transiently, by its ability to hydrolyse urea into ammonia, which in turn neutralises HCl and increases the pH adjacent to the bacterium. This finding was reported earlier[10] and confirmed in this study. In contrast, urease negative *H pylori* does not survive exposure to low pH[31] and is unable to colonise the stomach in an animal model.[11]

As urease activity seems crucial for the survival and transmission of the bacterium and exposure to very low pH may happen within

Effect of exposure to various pH values on soluble urease and catalase activities

Buffer	pH	Enzyme activity* (% of activity at pH 7)	
		Urease	Catalase
HCl	2	0 sig	9 sig
HCl	3	65	95
HCl	4	77	93
HCl	5	87	82
Phosphate	6	75	84
Phosphate	7	100	100
Citrate	2	0 sig	55
Citrate	3	0 sig	42
Citrate	4	0 sig	103
Citrate	5	18	102
Citrate	6	81	108
Citrate	7	100	100

*H pylori soluble enzyme preparations were incubated in HCl, phosphate buffer, or citrate buffer containing 5 mM urea. After 30 minutes, the pH was adjusted to pH 6·8 and enzyme activities were measured. Median results from four experiments are expressed as a percentage of enzyme activity obtained at pH 7·0. (sig, significantly different from the pH 7 value, p<0·05).

seconds, we hypothesised that urease is constitutively produced. As the bacterium produces large quantities of urease even during periods where there is no exposure to low pH, we hypothesised that survival and its vital functions depend on the availability of urease and its protective function. Thus, we hypothesised that *H pylori*'s protein synthesis and urease activity are very sensitive to low pH. To test this hypothesis, *H pylori* cells were exposed to pH values ranging from 2 to 7, the same pH range that is likely to occur in the human stomach.[14] We used buffered and unbuffered solutions to obtain conditions of stable pH as well as gradually increasing pH, respectively. Exposure to unbuffered hydrochloric acid mimicked the probable physiological events in the stomach where the pH may be quickly changed by neutralisation with ammonia.

The effect of pH on the enzymes was examined at three levels: (a) the effect on the enzyme activity when crude cytosolic enzyme preparations were exposed directly to low pH; (b) the effect on intracellular enzyme activity when intact *H pylori* cells were exposed to such conditions; and (c) the effect on the synthesis of nascent enzyme during exposure of intact *H pylori* cells to various pH levels.

The results of this study supports the initial hypothesis. As also shown by others,[31] prolonged exposure to low pH reduces viability. Furthermore, exposure of *H pylori* to low pH decreases protein synthesis, including urease and catalase. Exposure to low pH also abolishes activity of free and cellular urease. The detrimental effect of low pH depends on the duration of exposure and acid concentration. However, the inhibition of protein production seems to be reversible because viability was not affected by exposure to pH 3, 4, or 5. Free catalase seemed to be more stable than urease when exposed directly to low pH; only exposure to pH 2 reduced catalase activity significantly.

Intracellular enzyme activity is defined by the sum of the rates of new synthesis, degradation, and release. Low pH inhibited production of both urease and catalase. Thus, intracellular enzyme activity measured after acid exposure was due to preformed enzyme. After exposure to citrate buffer at pH 2, bacterial viability and activities of both enzymes were considerably reduced. Under this condition, many bacterial cells were lysed and the released enzyme was inactivated by the low pH as shown in the experiments where free enzymes were exposed to low pH. The most interesting events occurred after exposure to pH 3. Here, viability was not significantly decreased, and catalase activity was only mildly decreased. As only intracellular urease activity was reduced, it could be assumed that the intracellular pH was reduced to a level where the intracellular enzyme was inactivated. As shown in the experiments with free enzyme, this presumably occurs below a pH of 5. After exposure of the *H pylori* cells to citrate buffer of pH 4, intracellular urease remained active whereas free enzyme was destroyed showing

that the cells were able to cope with that extracellular pH and maintain the intracellular pH at a physiologically functional level. Although we did not measure intracellular pH, the comparison of intracellular urease activity and activity of free urease at various pH levels allows us to speculate indirectly on the intracellular pH of *H pylori* after exposure to low pH. *H pylori* seems to maintain an intracellular pH above 5 if exposed to a pH of 4. This also explains why *H pylori* is able to grow at low pH values.[32] It further seems that *H pylori* restores its normal intracellular pH after a short-term exposure to pH below 4 permitting normal protein production and survival. The precise mechanisms of maintenance of intracellular pH in *H pylori* are unknown. However, a *H pylori* P type ATPase has recently been characterised,[33] which is probably a cation transporter and might be responsible for the regulation cytoplasmic pH.

Although *H pylori* can survive transient periods of low surrounding pH, both urease production and urease stability are sensitive to low pH. In contrast with other urease positive bacteria such as *Streptococcus salivarius*,[15] urease production is not stimulated during exposure to low pH. The amount of enzyme activity in the cell is reduced when *H pylori* is exposed to low pH depending on duration of exposure and level of acidity. Brief exposure to low unbuffered acidity above pH 2 neither completely inhibited urease activity nor stimulated it. These findings are in some contrast with the idea that the major role of *H pylori* urease is its protective function against acidity. However, the extreme range of pH in its natural environment and the acid sensitivity of *H pylori* explains why such high amounts of urease are prepared even without preceding acid exposure. *H pylori* seems not to be acidophilic and needs the stock of urease for its protection against a sudden drop in pH. As soon as the short-term episode of low pH is overcome, the bacterium is able to restore its normal intracellular pH and to replace the destroyed proteins.

Our findings support the concept that *H pylori* is not acidophilic and does not seem to thrive in an acidic environment. A key enzyme of the organism, urease, is not acid stable and its synthesis is not stimulated by low pH. In addition, the metabolism of the bacterium, as measured by incorporation of radiolabelled methionine into protein, functions optimally at near neutral pH and is dramatically reduced at low pH. Indeed, the preferred environment would seem to be under the mucous layer of the gastric mucosa where the pH may approach neutrality. There is no evidence that the ulcerogenic activity of acid is amplified by stimulation of the bacterial virulence factor urease.

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