Prolonged Survival and Cytoplasmic pH Homeostasis of Helicobacter pylori at pH 1

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In the presence of urea, Helicobacter pylori survived for at least 3 h at pH 1. Under these conditions, the cells maintained their cytoplasmic pH at 5.8. De novo protein synthesis during acid shock was not essential for survival of H. pylori at pH 1.

The human pathogen Helicobacter pylori grows in vitro between pH 5.5 and 8.5 (10). During primary colonization, it has to overcome pH values of 1 to 2 in the gastric lumen before reaching the protective mucosa, with a pH close to neutral. It is likely that only a few cells suffice for infection and that such a low concentration of bacteria in the stomach cannot change bulk values of pH and urea concentration, which amounts to 1.7 to 3.4 mM for humans (9). H. pylori produces huge amounts of urease (11). Originally, it was thought that the enzyme is extracellular and that in the stomach protection occurs by the creation of a cloud of ammonia around the cells (3). However, urease activity is cytoplasmic, and UreI regulates its activity by mediating acid-triggered urea uptake (16). The formed NH₃ is believed to increase the buffering capacity of the periplasm (7, 17).

From previous experiments, evidence is lacking that H. pylori survives at pH 1 to 2. In situations in which cells have been suspended at high concentrations in nonbuffered, urea-containing solutions of low pH, extracellular pH returned to neutral within minutes due to NH₃ formation, relieving the acid stress situation (1, 2, 7). In buffered solutions of low pH, cell counts decreased dramatically even in the presence of urea (1, 6, 7). Under these conditions, urease activity was abolished (17). With an improved cultivation procedure, we show that the acid tolerance of H. pylori has been widely underestimated. In the presence of urea, the pathogen survived for several hours at pH 1, and cytoplasmic pH (pHₘ) remained almost neutral.

H. pylori wild-type strain DSM 4867 (NCTC 11637) was cultivated in acid-precipitated medium (APM) at 37°C by shaking at 140 rpm under microaerobic conditions in a 2.5-liter anaerobic jar using Anaerocult C (Merck). APM was prepared by supplementing brucella broth (Difco) with 5% fetal calf serum (Fisher Scientific) and titration with HCl to pH 3. Precipitated protein was removed, and the supernatant was neutralized with NaOH to pH 7. Cells grown for 24 to 48 h were subcultured at least three times in fresh prewarmed and 10% CO₂-equilibrated medium (pH 6.5, calculated optical density at 578 nm [OD₅₇₈] of inoculation of ~0.001), and finally grown to an OD₅₇₈ of ~0.2. All experiments were performed at 37°C under continuous gassing (5% O₂, 10% CO₂, 85% N₂). Acid shock was exerted by diluting the cells 50- to 100-fold in APM medium of pH 1. Under these condition, the cells did not increase external pH or exhaust urea (added at 3 mM) within 3 h. For the determination of pHₘ or protein synthesis, cell suspensions of about 7 × 10⁷ CFU/ml (OD₅₇₈ ~ 0.2) were used. Urea was added at a concentration of 50 or 40 mM followed immediately by titration with HCl to pH 1 or 2, respectively. Subsequently, medium pH was kept constant by occasional titration with HCl. For the acid shock experiment in citric acid, cells were first collected by centrifugation, resuspended to an OD₅₇₈ of about 2 in 150 mM NaCl, and diluted 10-fold with 100 mM citric acid (pH 2). Urease activity was monitored by the decrease of urea concentration in the medium, determined as described elsewhere (12). CFU counts were determined by diluting samples in prewarmed brucella broth containing 5% fetal calf serum, followed by plating on prewarmed agar medium. Colonies were counted after 3 to 5 days. Protein synthesis was determined by measuring the incorporation of [³⁵S]methionine into acid-precipitable material. For this purpose, either methionine-free assay medium (Difco) supplemented with 150 mM NaCl (pH 6.5) or citric acid (pH 2) containing 40 mM urea was used. pHₘ was estimated from the distribution of [¹⁴C]salicylate across the cell membrane (5, 15), using the silicone oil filtration method (8).

Using 50- to 100-fold-diluted cell suspensions (equivalent to a calculated OD₅₇₈ of 0.004 to 0.002), which mimics the situation in the stomach most closely, about 45% H. pylori cells survived acid shock from pH 6.5 to pH 1 for 3 h in the presence 3 mM NaCl (Fig. 1). However, such a low cell concentration was not suitable for measuring urease activity, pHₘ, or protein synthesis of H. pylori. Thus, those experiments were performed at about 7 × 10⁷ CFU/ml (OD₅₇₈ ~ 0.2) and started with 40 to 50 mM urea to guarantee urea supply for at least 1 h after acid shock to pH 2 or 1. Under these conditions, H. pylori survived for 1 h without any significant reduction of CFU (Fig. 2A). In the absence of urea no viable cells, were detected after 30 min of exposure to pH 1 or 2 (data not shown). Urease activity amounted to 0.63 ± 0.07 and 0.38 ± 0.07 μmol of urea · min⁻¹ · 10⁻⁶ CFU at 1 and pH 2, respectively.

At pH 6.5, prior to acid shock the pHₘ of H. pylori was 6.9 (Fig. 2B), confirming previous results (5). After acid shock to pH 2 or 1, pHₘ remained almost neutral (5.8) in the presence of urea (Fig. 2B). In citric acid of pH 2, pHₘ was 5.5 and the
cells also survived for at least 1 h (Fig. 2). Addition of 1 mM of the uncoupler 2, 4-dinitrophenol lowered the pHin to 4.1. Similar results have been obtained with acidophilic organisms at low pH (8). In the absence of urea pHin was also close to 4.

Addition of chloramphenicol (50 μg/ml) 15 min prior to acid shock did not reduce the viability of H. pylori at pH 1 or 2 (Fig. 2A). At pH 6.5, protein synthesis was inhibited by more than 95% by the inhibitor. Compared to pH 6.5, incorporation of [35S]methionine at pH 2 without inhibitor was only 0.5% and even less when chloramphenicol was present. These results suggest that protein synthesis during acid shock was not essential for the survival of H. pylori at low pH.

In conclusion, first, we demonstrated a much higher level of acid tolerance of H. pylori than reported previously. Most likely, the use of rapidly growing cultures (with a doubling time of about 2.7 h) and as little stress and manipulation of the cells during the acid shock procedure as possible were essential for our success. Second, urease activity appears to trigger pHin homeostasis. We favor the mechanism proposed in reference 18 for other bacteria in which the NH3 formed in the cytoplasm buffers away protons leaking in through the cytoplasmic membrane and NH4+ leaves the cells electrogenically. Finally, our data also indicate that H. pylori can effectively transit the gastric acid barrier without any previous acid adaptation or the formation of coccolid forms as has been postulated by others (4, 7, 14).

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REFERENCES


