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Acid stress response in *Helicobacter pylori*

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Abstract

To determine the existence of an acid stress response in *Helicobacter pylori* the global changes in the proteins synthesized by the bacterium when subjected to an acid stress were studied. *H. pylori* ATCC43504 previously adapted to pH 7 did not show an acid stress response as detected by the two-dimensional electrophoretic pattern of ^{35}S-labeled proteins when incubated at pH 3. This was probably due to the neutralization of the external medium by the action of urease. However, *H. pylori* DW504UreI-negative, a mutant strain unable to transport urea into the cell, showed a large number of proteins changed, as is typical in an acid stress response. Some of these proteins were identified by N-terminal sequencing.

Keywords: *Helicobacter pylori*; Acid stress response; Acid tolerance response

1. Introduction

Over 50% of all humans worldwide are infected with *Helicobacter pylori*. The infection is acquired during childhood and persists lifelong if not eradicated, and is also associated with chronic gastritis and an increased risk of peptic ulcer and gastric or duodenal neoplasm [1]. *H. pylori* colonizes the gastric mucosa of humans [2], where the pH is thought to be 4.5–5.0, but may occasionally drop to 2 [3,4]. During primary colonization, the microorganism can stand pH values of 1–2 in the gastric lumen before reaching the protective mucosa. *H. pylori* exhibits a strict tropism towards gastric mucus-secreting cells in vivo and in vitro [5,6]. The acidic pH in the mucosa is probably one characteristic that makes this niche inaccessible to bacteria other than *H. pylori*. The bacterium must be well adapted to grow under acidic conditions and must employ specialized acid-protective mechanisms. The ability to survive under these low pH conditions enables *H. pylori* to colonize the stomach.

One striking characteristic of *H. pylori* is the constitutive expression of a neutral pH optimum urease at high levels [7]. The enzyme has a K_m for urea of 0.8 mM [8]. Urease elevates the pH of the bacterial microenvironment by the conversion of urea into ammonia and carbon dioxide. 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, ranging in the stomach from 1 to 14 mM [9,10]. It is thought that urease plays a role in protecting the microorganism from the harmful effects of acid, since the ammonia generated by the bacterium protects it by buffering the gastric acid. However, urease also plays a role in *H. pylori* metabolism [11,12].

Various approaches have been used to investigate the resistance of enteric bacteria to pH stress [13]. One approach is to examine the bacterial viability following an acid challenge. When *Salmonella typhimurium* cells are grown in vitro at pHs between 6.0 and 5.5, a process referred to as acid tolerance response is triggered, protecting the cells against much stronger acid conditions [14]. Hall et al. [15] proposed that the term acid tolerance should be reserved for the inducible adaptation to acid challenge. Based on their finding that *H. pylori* cells briefly adapted at pH 6 or 5 survived exposure to pH 3 much better than did unadapted (exposed to pH 7) cells, Karita and Blaser [16] proposed that *H. pylori* has an acid toler-

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Table 1
Acid tolerance response

Time at pH 3 (min)	*H. pylori* strain			
	ATCC43504 adapted to		DW504 adapted to	
	pH 7	pH 6	pH 7	pH 6
0	(100)	(100)	(100)	(100)
15	6×10^{-4}	5	5.14	52
30	6×10^{-6}	0.3	3.4	3
45	2.7×10^{-6}	2.5×10^{-2}	0.1	1.6
60	3.3×10^{-6}	5×10^{-3}	2×10^{-2}	2.9×10^{-2}
90	1.3×10^{-8}	5×10^{-3}	4×10^{-3}	4×10^{-2}

The pH suspension was adjusted by adding HCl without supplement of urea. Results are expressed as the percent of time 0 value of approx. 3.5×10^{8} bacteria ml^{-1} (mean value of at least three experiments).

ance response. It is known that *H. pylori* varies its gene expression when grown under acidic conditions [17–21], but nothing is known about the specific acid response proteins in this pathogen. To obtain additional information regarding an acid survival mechanism in *H. pylori*, we investigated the global changes in gene expression of the bacterium in response to acid stress. Results employing a DW504UreI-negative mutant strain (unable to incorporate urea) indicate that in addition to an acid tolerance response system, a urease-independent acid stress response system exists in *H. pylori*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *H. pylori* strains used for most experiments were ATCC43504, obtained from the American Type Culture Collection, and the isogenic *ureI*-negative mutant, DW504UreI-negative [22], obtained from Dr. G. Sachs. Stock cultures were maintained at −80°C in trypticase soy broth supplemented with 20% glycerol. *H. pylori* strains were cultured in a microaerobic atmosphere on Dent trypticase soy agar plates containing 5% defibrinated sheep blood, as indicated by ATCC, and the *ureI*-negative mutant was grown on Dent kanamycin (20 μg ml^{-1}) medium. After 2 days of growth, bacteria were harvested by scraping plates and suspended in RPMI 1640 medium (Gibco BRL). Quantitation of bacteria in suspension was done by optical density measurements at 560 nm or by viable counts on Petri dishes. Appropriate dilutions of the bacterial suspensions were spread on Dent trypticase soy agar plates, and after incubation at 37°C for 3–5 days, colonies were counted.

2.2. In vivo protein synthesis

Cell suspensions of about 3×10^{8} bacteria ml^{-1} (OD_{560} nm about 0.4) were used. After 2 days of growth on Dent trypticase soy agar plates, bacteria were harvested by scraping plates and suspended in RPMI 1640 methionine-free medium adjusted to pH 6 or 7, and supplemented with 150 μCi ml^{-1} of [^{35}S]methionine, of EXPRE^{35}S^{35}S Protein Labeling Mix (>1000 Ci $mmol^{-1}$) from NEN Life Science Products, before or during the acid stress, in the presence or absence of 10 mM urea. The extent of incorporation of [^{35}S]methionine was estimated by determining the trichloroacetic acid-insoluble material after boiling and cooling at 4°C.

2.3. Two-dimensional gel electrophoretic analysis of proteins

Cells to be used for two-dimensional polyacrylamide gel electrophoresis analysis were grown under the same conditions outlined above. Cells were labeled with 150 μCi ml^{-1} of [^{35}S]methionine for 90 min at pH 7.0 or pH 3.0 in the presence of 10 mM urea. Each sample of radioactively-labeled cells was pelleted and suspended in sonication buffer, sonicated at 20 W by 30 s 3 times at intervals of 1 min, in the 60 Sonic Dismembrater from Fisher Scientific. Those samples were run in a pH 3–10 isoelectric

Table 2
Protein synthesis in *H. pylori* 43504

Conditions for the second incubation	[^{35}S]Methionine incorporation (% control)	
	pH of 1 h preincubation	
	7.0	6.0
No incubation	(100)	(100)
pH 7.0	255 ± 5.68	–
pH 6.0	–	196 ± 6.31
pH 3.0	33 ± 1.25	61 ± 1.89
pH 3.0+10 mM urea	167 ± 4.27	286 ± 9.34
pH 3.0+10 mM urea+[^{35}S]meth	82 ± 3.98	193 ± 8.21
pH 3.0+[^{35}S]meth	23 ± 3.56	53 ± 5.76

For the first five lines, a suspension of 3×10^{8} cells in 1 ml of RPMI 1640 medium adjusted to pH 6.0 or 7.0 and in the presence of 150 μCi of [^{35}S]methionine was used. Cells were exposed to these pH values by a preincubation of 60 min. After this period, a second 60-min incubation was followed as indicated, except the first was incubated only the first 60 min. Samples in the last two tubes were first adapted to pH like the other ones, except that no [^{35}S]methionine was present during this adaptation period. The second 60-min incubation was done as indicated. Results are expressed as the mean value of three experiments. 100% values were 195 and 169 pmol of [^{35}S]methionine incorporated/3×10^{8} cells at pH 7 and pH 6, respectively.

focusing non-equilibrium pH gel electrophoresis system followed by SDS 11.5% PAGE as described [23].

3. Results and discussion

3.1. Acid tolerance response is elicited by exposure of cells to mild acid pH

To find out if under our experimental conditions the *H. pylori* 43504 showed the acid tolerance response previously reported [16], agar-grown microorganisms were harvested as described above. One-ml aliquots of approximately 3×10^8 bacteria ml^{-1} were first adapted to pH 6 or 7 for 1 h in RPMI 1640 medium. After this, they were transferred to pH 3 without supplement of urea for different times and the viability of the cells was determined (Table 1). The data indicate that the number of bacteria recovered after the pH 3 treatment was much higher in adapted cells (incubated at pH 6) compared to that of unadapted cells (incubated at pH 7). However, the DW504UreI-negative mutant showed higher acid tolerance when compared with the adapted or non-adapted parent ATCC43504 cells. On the other hand, when mutant cells were preincubated at pH 6, the percentage of bacteria recovered after the pH 3 treatment was even larger than in cells preadapted at pH 7, suggesting that these cells also have an acid tolerance response (Table 1). The results of a higher acid tolerance were confirmed by monitoring the capacity of the bacteria for protein synthesis by determining the incorporation of [^{35}S]methionine into whole cells. Adapted cells (pH 6) that subsequently underwent acid shock (pH 3) in the presence or absence of 10 mM urea incorporated larger amounts of [^{35}S]methionine than unadapted cells (pH 7) (Table 2). The pre-acid shocked (pH 6) cells being more resistant to acid pH could adapt for long-term survival at low pH by having an acid stress response. If this response exists in *H. pylori*, the bacterium should induce acid shock proteins amongst the proteins synthesized during the low pH stress (see below).

3.2. Survival of H. pylori DW504UreI-negative mutant in acidic conditions

When *H. pylori* ATCC43504 was incubated at pH 3 in the presence of 10 mM urea, the pH of the medium increased rapidly, reaching a value of 7 by 45 min (as seen in Fig. 1). In contrast, the DW504UreI mutant strain did not modify external medium pH. However, DW504UreI-negative strain was more resistant to acid pH than wild-type (Table 1) in spite of the lack of urea incorporation. Although *H. pylori* has the capability to survive under extremely acidic conditions and this survival is mediated by the constitutive urease, urease-negative mutants survived a 60-min exposure at pH 3.5 [24], suggesting the existence of additional mechanisms to cope with acid

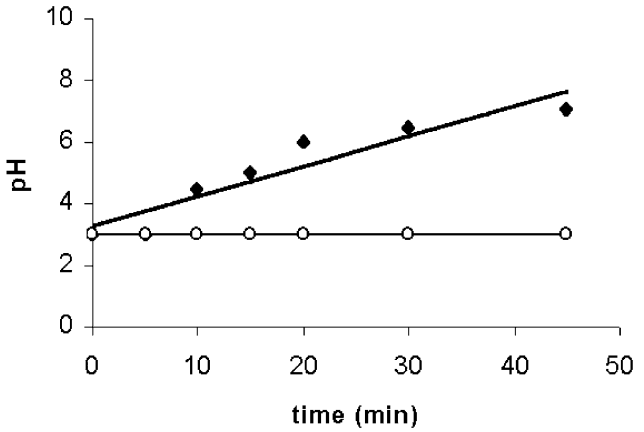

Fig. 1. Effect of the *ureI*-negative mutation on the ability of *H. pylori* to alkalinize the external medium. *H. pylori* ATCC43504 (♦) and DW504UreI-negative (○) were suspended to 3×10^8 cells ml^{-1} in medium containing 10 mM urea and were incubated at 37°C. The medium was supplemented with 15 mg ml^{-1} methionine to avoid methionine starvation. The pH of the medium was monitored at the times indicated.

stress. Although such pathways have not yet been elucidated, one possible mechanism of this sort is the arginine decarboxylase (designated HP0422 in the entire genome sequence) [25] in *H. pylori*, which converts arginine to agmatine, consuming a proton in the process. This could allow the removal of excess protons, such as occurs in the acid resistance system 3 from *Escherichia coli* [26], which requires arginine and an inducible arginine decarboxylase. Another possible alternative way of acid adaptation has been suggested by McGowan et al. [19] on the basis of subtractive RNA hybridization. They proposed that structural changes in the LPS might be an important acid-induced urease-independent adaptation at pH 4.

3.3. Characterization of the acid stress response

To characterize putative acid shock proteins, global changes in protein synthesis was studied by proteomic analysis employing 2-D isoelectric focusing non-equilibrium pH gel electrophoresis [23]. The ^{35}S-labeled protein pattern of *H. pylori* ATCC43504 incubated at pH 7 (Fig. 2A) differs only in a few protein spots that changed their levels of synthesis when these cells were labeled during incubation at pH 3 in presence of urea (Fig. 2B). This contrasts with the acid stress response of *S. typhimurium*, in which around 48 proteins change their expression levels [15,27]. A possible explanation for this lack of a multi-protein response under our experimental conditions is the rapid increase in the external pH due to the urease activity of *H. pylori* (Fig. 1), relieving the acid stress situation. The DW504UreI-negative mutant is not able to increase the external pH and under these conditions, the mutant cells show a multi-protein acid stress response (Fig. 2C,D). When comparing the labeling pattern of the DW504UreI-negative mutant incubated at pH 7 (C) with

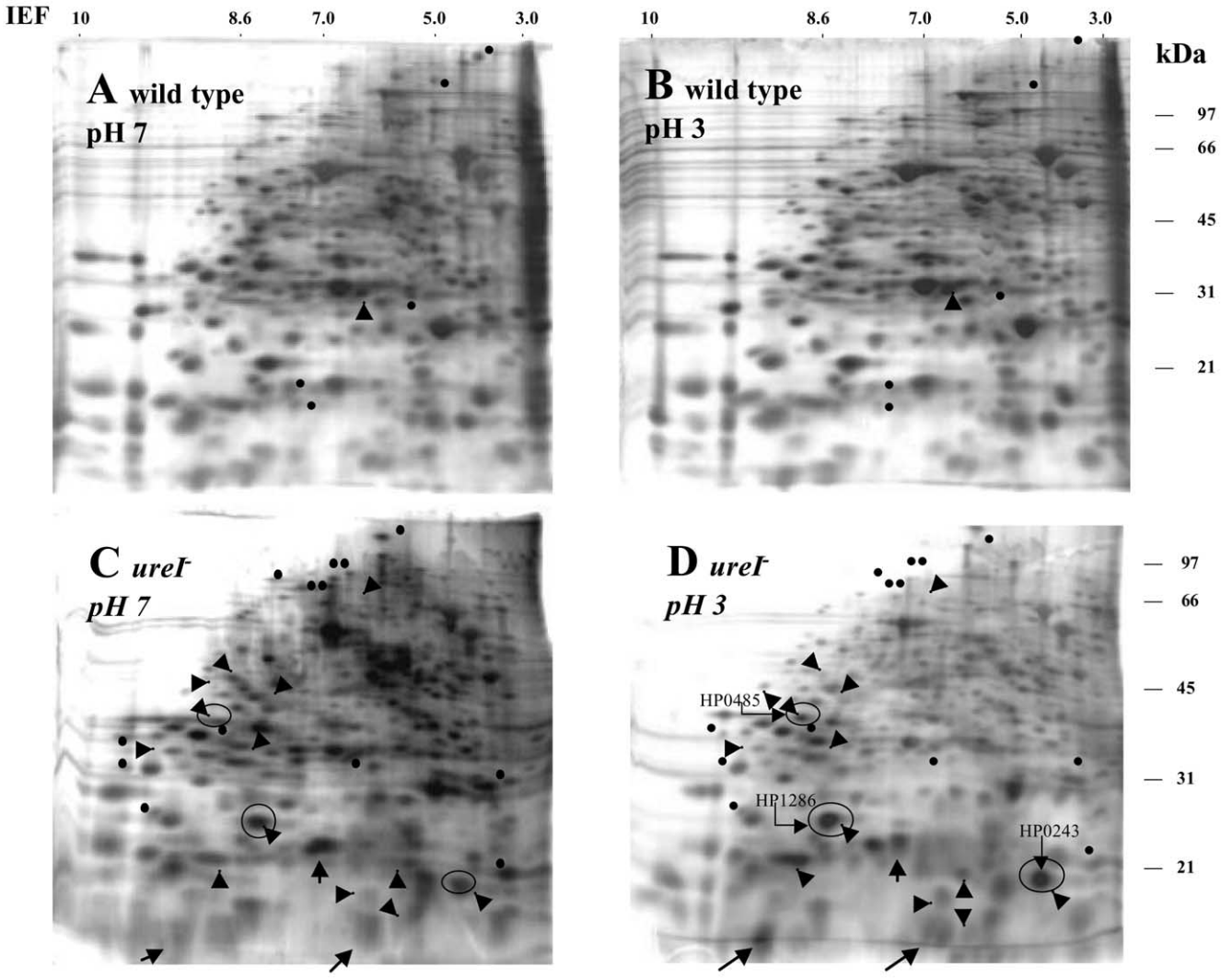

Fig. 2. Changes in protein content of *H. pylori* subjected to acid stress. *H pylori* ATCC43504 (A, B) and DW504UreI (C, D) were suspended to 3×10^8 cells ml^{-1} in RPMI 1640 medium supplemented with [^{35}S]methionine at pH 7 (A, C) or pH 3 plus 10 mM urea (B, D) for 90 min. The total cell proteins were separated by 2-D isoelectric focusing non-equilibrium pH gel electrophoresis followed by autoradiography. Arrowheads indicate proteins pre-existing at pH 7 increasing their synthesis at pH 3. Arrows point to proteins present only at pH 3; and dots signal those proteins whose synthesis is lower at pH 3. The indicated changes were observed in at least three different experiments. Circles indicate proteins that were subjected to microsequencing of N-termini.

that of the mutant labeled at pH 3 in the presence of urea (D), several protein changes were clearly apparent, reminiscent of a typical acid stress response. These results also show that *H. pylori* can adapt to acid conditions in a urease-independent fashion, since the urease activity is still present in the mutant. The number of proteins changing their levels of expression due to the acidic pH is around 30 in our experimental conditions which is lower than those reported for *S. typhimurium* [15,27]. To identify some of the proteins whose synthesis was clearly increased by exposure to pH 3, those spots present in the 2-D gel of Fig. 2 (indicated by circles) were located by their migrating coordinates in Coomassie blue-stained 2-D gels and were removed from the gels for N-terminal microsequencing. Table 3 shows the ORFs corresponding to the proteins identified: HP1286, HP0485 and HP0243, according to the genome sequence of strain 26695 [27]. There were some differences in the theoretical p*I* values listed in Table 3 with those that can be estimated from the 2-D gels. This is due to the fact that the non-equilibrium pH gel electrophoretic system used does not allow a correct determination of p*I* values [23]. None of these proteins has a demonstrated function. HP0243 could exhibit proadhesive activity in host cells and may perform analogous functions in iron detoxification and storage as that of animal ferritins (by similarity). On the other hand, HP0485, a catalase-like protein, could have a similar function to catalase, an enzyme that has been implicated in general stress responses of some bacteria, including acid pH [28]. In this regard, several studies have shown that acid adaptations confer resistance to a wide range of stress conditions including heat, salt, and H_2O_2 [29,30]. However, adaptation

Table 3
N-terminal amino acid sequences of proteins induced by acid in strain DW504ureI-negative

Peptide sequence	*H. pylori* 26695		
	ORF[a]	p*I*[b]	MW[b]
KPYTIDKANSSV	HP1286	9.8	20 614
MKTFEILK	HP0485	10.0	35 782
HEVSAEEI	HP0243	5.8	16 933

[a]Peptide sequences were compared against the TIGR gene sequence database of *H. pylori* 26695. The spots analyzed were taken from different Coomassie blue-stained 2-D gels, loaded and run in an SDS–10% polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride, and subjected to microsequencing (Laboratoire de Microséquençage des Protéines, Institut Pasteur, Paris, France).
[b]The theoretical p*I* and MW values were obtained from the TIGR gene sequence database.

to other stresses does not typically induce significant acid tolerance. This implies that exposure to acid may be perceived by bacteria as a more general stress. Further experiments will be required for a complete characterization of this complex response.

The results reported here indicate the presence of an acid stress response in *H. pylori*. It will be of great interest to identify these proteins and to establish their role during this acid pH adaptation and survival in the stomach, especially considering that these factors at the protein level are essential virulence factors.

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