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RESEARCH ARTICLE

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Changes in protein synthesis and acid tolerance in *Clostridium* perfringens type A in response to acid shock

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Summary The induction of acid-shock proteins and the degree of acid resistance conferred on *Clostridium perfringens* by acid shock, and the kinetics of this resistance were determined. A sublethal acid shock at pH 4.5 for 20 min increased the acid tolerance of cells at least fifteenfold. The acquired tolerance was maintained for 3 h after acid treatment. The response of the microorganism to acid shock was also examined by analysis of pulse-labeled proteins. Five acid-shock proteins (molecular weights 120, 84, 58, 45 and 17 kDa) were identified by polyacrylamide gel electrophoresis.

Introduction

Bacterial survival in stressful environments is an intriguing biological problem, with applications toward understanding pathogenic and environmentally important microorganisms. A number of stressful conditions such as starvation [14, 20, 23], heat shock [1, 12], cold shock [8, 23], and extreme pH [3, 22] have been documented for a number of microbial pathogens. In each case global physiological changes were shown to occur within the cell when they were exposed to the stressful condition.

An important stress condition that must be faced by many pathogenic microorganisms is low pH. When ingested, food- and waterborne microbial pathogens are exposed to an acid pH environment in the stomach and the small intestine [9]. Adaptation to and survival in a low pH environment may be an important prerequisite for the production of disease by many gastrointestinal pathogens. The response to low pH environments has been studied in *Salmonella typhimurium* [2–5], *Escherichia coli* [21], *Streptococcus mutans* [10], *Helicobacter pylori* [19], and *Aeromonas hydrophila* [15], among other species.

Among a wide variety of potentially foodborne gastrointestinal microbial pathogens, *Clostridium perfringens* is the most prolific toxin-producing member of the clostridial group. Toxins are responsible for a wide variety of human and veterinary diseases, many of which can be lethal. *C. perfrigens* causes two different food-related human diseases, the most common of which is food poisoning. The incidence of this disease is among the highest of all diseases caused by the consumption of contaminated food [16].

Foodborne illness is produced 8 to 24 h after the ingestion of food contaminated with large numbers of vegetative bacteria (>10^5 colony forming units [CFU]/g). Many of the ingested cells may die when exposed to stomach acidity, but if the food vehicle is sufficiently contaminated, some vegetative cells survive passage through the stomach and enter the small intestine, where they multiply, sporulate, and produce an enterotoxin that causes diarrhea [17].

Recently we determined that heat shock induced physiological changes in *C. perfringens* such as the production of heat shock proteins, the acquisition of heat-tolerance by vegetative and sporulating cells, and a decrease and delay in enterotoxin production [11, 12]. In this study, we determine the effect of acid shock on protein synthesis and acid tolerance of *C. perfringens*.

Materials and methods

Culture conditions The enterotoxin-positive strain FD-1041 of *C. perfringens* was maintained as a stock spore culture in cooked meat medium (Difco Laboratories, Detroit, MI, USA), at –20°C. Active cultures were obtained by transferring two drops of the stock culture into test tubes containing 10 ml of fluid thioglycolate medium (FTG; Difco), heat-activated at 75°C for 15 min, and incubated overnight (16 to 18 h) at 37°C [7]. Experiments were done at least in triplicate.

Acid tolerance assay When the cultures reached an A_{600} of 0.3 to 0.4, they were acid shocked at pH 2 to pH 5 (in 0.5 increments) by adding 2 M HCl (Sigma-Aldrich Química, México, D.F. México) for 20 min. During treatment, viability of the cells decreased at pH values below 4.5 (not shown). Thus, for the acid tolerance assay, the cells were shocked at pH 4.5 for 20 min and then challenged at pH 3.5. Cell viability at this pH was determined by plate counts in nutrient agar. For this purpose, aliquots were obtained at 0, 10, 20 and 30 min [6]. The plates were incubated at 37°C for 24 to 36 h in a mixture of N_2 and CO_2 (95:5). The duration of the acquired tolerance was determined as follows: after the cultures were acid shocked at pH 4.5, they were returned to pH 7.0 by adding 2 M NaOH, and after 2, 3 and 3.5 h they were challenged at pH 3.5 as mentioned above. The D value, defined as the time required to inactivate 90% of the population [13], was determined from the acid death curves. The *t* test was used to determine differences between the slopes of the curves [24].

Radiolabeling of proteins Vegetative cells were grown in test tubes (13 × 100 mm) containing 4 ml of brain and heart infusion broth (BHI, Difco) at pH 7.0 and incubated at 43°C. When the cell cultures reached an A_{600} of 0.30 to 0.35 (mid-exponential phase), they were acid-shocked at the indicated pH. After 5 min of acid shock, 100 µCi of a mixture of ^{35}S-labeled methionine and cysteine (Trans-^{35}S label, ICN Pharmaceuticals, Costa Mesa, CA, USA) was added to each sample, and incubation continued at the same pH for 15 min. Then unlabeled amino acids (40 µg methionine plus 10 µg cysteine per ml [Sigma-Aldrich Química, México, D.F. México], final concentration) were added to the tubes to quench uptake, and the samples were cooled rapidly on ice. Cells were pelleted by centrifugation for 10 min at 10,000× *g* at 4°C. Culture supernatants were kept at –20°C. The pellet was washed twice with 30 mM Tris-HCl buffer (pH 7.6).

The cells were solubilized as described by Heredia et al. [12]. The pellet was resuspended in 30 mM Tris-HCl buffer (pH 7.6) containing 500 µg of egg white lysozyme and 50 µg DNase per milliliter (Sigma-Aldrich Química). The mixture was incubated at 37°C for 30 min and then frozen at –20°C for 12 to 14 h to disrupt the cells. To determine the amount of radioactively labeled methionine and cysteine incorporated into protein, 5 µl of the sample was placed in the center of a Whatman GF/A filter (Whatman, Maidstone, England). The sample was allowed to dry, and then the filter was placed in 10% trichloroacetic acid for 5 min to precipitate the protein. Then the filter was washed 10 times with saline, dehydrated with absolute ethanol and dried. Radioactivity was measured using a scintillation counter (Model Delta 300, TM Analytic, Elk Grove Village, IL, USA).

Gel electrophoresis Radioactive samples were mixed with 4× sample buffer (pH 6.8; 3% Tris, 20% β-mercaptoethanol, 10% sodium dodecylsulfate [SDS], 0.02% bromophenol blue, and 40% glycerol) and heated at 95°C for 3 min, then centrifuged to remove debris. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli [18] with a 4% (w/v) stacking gel and 10% (w/v) separating gel. Protein samples containing 100,000 cpm were applied to each lane. Myosin (molecular weight [MW] 205 kDa), β-galactosidase (MW 116 kDa), phosphorylase b (MW 97.4 kDa), bovine albumin (MW 66 kDa), ovalbumin (MW 45 kDa) and carbonic anhydrase (MW 29 kDa) were obtained from Sigma-Aldrich Química and used as molecular weight standards. Gels were stained with Coomassie brillant blue R-250 and then dried at 60°C under a vacuum before exposure to Kodak X-OMAT AR film for 3 days at –70°C.

Results and Discussion

The results indicated that acid shock increased the subsequent acid tolerance of cells (p<0.05). For example, after acid shock (0 h), the D value (at pH 3.5) of strain FD-1041 increased from 5 to 75 min (Fig. 1, Table 1). The acquired acid tolerance was

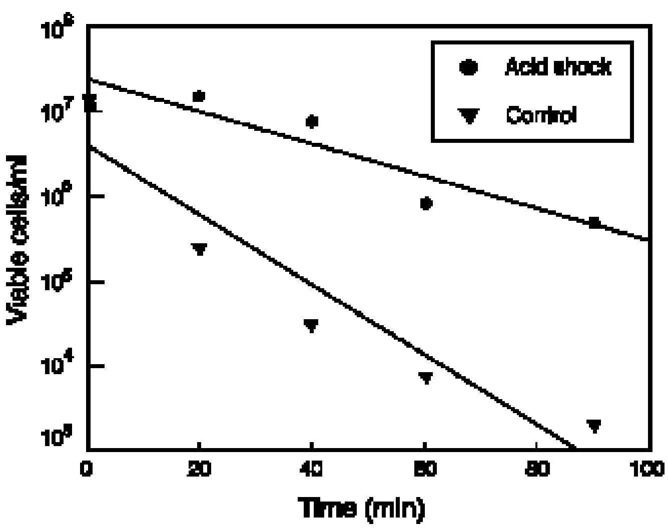

Fig. 1 Acidic death curves at pH 3.5 of cells of *Clostridium perfringens* FD-1041. Acid shock was applied when the culture population reached 0.3–0.4 A_{600} and then (0 h) acid tolerance at pH 3.5 was determined

maintained for 3 h after acid-shock treatment. At 3.5 h no significant difference was observed in comparison to the control.

Table 1 D values of acid-shocked cells of *Clostridium perfringens* FD-1041. Cells were acid-shocked at pH 4.5 for 20 min, and then challenged at pH 3.5 (0 h) or returned to pH 7.0 for 2, 3 and 3.5 h before challenge

Time after acid shock (h)	$D_{3.5}$ (min)	
	Control	Acid shocked
0	4.8±2.7	75±15.4
2	5.0±1.8	35±1.0
3	11±0.9	42±12.5
3.5	9.4±0.1	12±0.1

Analysis of cellular protein synthesis on the basis of SDS–PAGE and autoradiography results clearly demonstrated the induction of a set of acid shock proteins (Fig. 2). Strong induction of five proteins (120, 84, 58, 45 and 17 kDa) was observed.

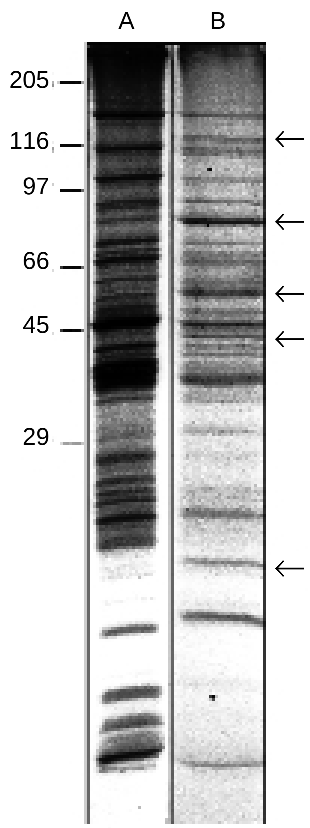

Fig. 2 Protein profiles of *Clostridium perfringens* after acid shock (pH 4.5 / 20 min). Cells were radiolabeled for 15 min by the addition of [^{35}S]methionine and [^{35}S]cysteine 5 min after pH downshift. (A) Control, (B) treatment. The migration positions and sizes (in kDa) of protein standards are indicated on the left; arrows at the right indicate acid shock proteins

This study shows that *C. perfringens* can be adapted to survive in severely acid environments. This adaptation requires prior exposure to a relatively mild pH (4.5) before being challenged at a lower pH (3.5). Also, acid adaptation in *C. perfringens* occurs concomitant to the synthesis of at least 5 proteins. It has been suggested that these new proteins play a significant role in protecting the cells at low pH. Adaptation to mild changes in pH may act as a signal for the cell indicating potentially lethal pH changes in the external environment, and allowing the cell to produce new protective proteins required for survival at more acidic levels. This represents a global cellular response at both, the physiological and the genetical levels similar to those described in other bacterial species [4, 5]

Further studies of the genetic mechanism(s) whereby this bacterial pathogen can adapt and survive in harshly acidic environments may provide insight into its ability to cause disease in humans.

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