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COLD TOLERANCE AND CHANGES IN RATES OF SYNTHESIS OF INDIVIDUAL PROTEINS OF *CLOSTRIDIUM PERFRINGENS* AS A RESULT OF COLD PRE-TREATMENT

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Clostridium perfringens is a widely distributed foodborne pathogen. Its ability to survive cold encounters could contribute to its persistence in foods and the potential to cause disease. In this work five cold-shock proteins (101, 82, 70, 45 and 10 kDa) were induced by cold-shocking *C. perfringens* FD-1041 from 43 °C to 28 °C, as revealed by labeling with L-[^{35}S]methionine and cysteine followed by gel electrophoresis. Cold shock also increased the cold tolerance of the cells at least fifteen fold. The acquired tolerance was maintained for 2 h after the cold treatment. This ability of *C. perfringens* could improve the survival in foods and present a significant hazard.

Keywords: *Clostridium perfringens*, cold shock, cold tolerance

Clostridium perfringens, a Gram-positive spore-forming anaerobe, causes various clinical problems, one of which, food-poisoning is an important cause of foodborne disease in the world (BEAN et al., 1996; HEREDIA & LABBE, 2001). Food preservation techniques can produce a variety of stresses (shocks) that interfere with bacterial homeostasis in order to prevent growth or to kill the bacteria. However, as a result of the stress response, bacteria can survive and grow after the stress condition has passed (JONES & INOUYE, 1994). Recently, we determined that this bacterium responds against heat and acid shock producing several physiological changes such as production of acid and heat shock proteins, inducing acid and heat-tolerance in vegetative and sporulating cells and decreasing and delaying enterotoxin production (HEREDIA et al., 1997; HEREDIA et al., 1998; VILLARREAL et al., 2000).

Responses to different stresses have been and continue to be studied in a large diversity of organisms from prokaryotes to humans (WELCH, 1993). The cold-shock response in bacteria describes a specific pattern of gene expression in response to a downshift in temperature; this pattern includes the induction of several proteins called cold-shock proteins and the continued synthesis of transcriptional and translational proteins despite the lag period that is induced (JONES & INOUYE, 1994). Some of these proteins have been involved in diverse cellular processes (JONES et al., 1987). Cold-

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inducible tolerance occurs in several prokaryotic organisms. This is a phenomenon by which cell survival at lethal cold conditions is significantly enhanced by a short pretreatment at sublethal temperatures (THAMMAVONGS et al., 1996).

It has been shown that *C. perfringens* is sensitive to cold temperatures (TRACI & DUNCAN, 1974). However, no information is available about the cold tolerance of this foodborne pathogen. In this work we evaluated protein synthesis and the development, duration and degree of cold resistance conferred on *C. perfringens* by a cold shock.

1. Materials and methods

1.1. Culture conditions

The enterotoxin positive strain FD-1041 of *C. perfringens* was used. It was maintained as a stock spore culture in sterile cooked meat medium (Difco Laboratories, Detroit, Mich) at –20 °C. Active cultures were obtained by transferring two drops of the stock culture into test tubes containing 10 ml of fluid thioglycollate medium (FTG; Difco), heat-activated at 75 °C for 15 min and incubated overnight (16 to 18 h) at 37 °C (GARCIA-ALVARADO et al., 1992a; 1992b). Experiments were done at least in triplicate.

1.2. Radiolabeling of proteins

Vegetative cells were grown in test tubes containing 4 ml of FTG and incubated at 43 °C. When the cultures reached an A_{600} of 0.30 to 0.35 (1.0×10^7 CFU ml^{-1}, mid-log phase), cells were pre-treated at 15–28 °C for different length of time. During pre-treatment, cells died at temperatures below 28 °C (not shown). Thus, the cells were pre-treated at 28 °C for 60 min. After 5 min of cold shock, 100 μCi of a mixture of ^{35}S-labeled methionine and cysteine (Trans-^{35}S label, ICN Pharmaceuticals, Inc., Costa Mesa, CA) were added to each sample, and the incubation was resumed at the same temperature for 55 more 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 the incorporation, and the samples were cooled rapidly on ice. Cells were pelleted by 10 min centrifugation 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).

Cells were solubilized as described by HEREDIA and co-workers (1998). The pellet was resuspended in 30 mM Tris-HCl buffer (pH 7.6) containing 500 μg of egg white lysozyme and 50 μg of DNAse per ml (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. For determination of the amount of radioactively labeled methionine and cysteine incorporated into protein, 5 μl of the sample were placed in the center of a Whatman GF/A paper filter (Whatman International, 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 solution,

dehydrated with absolute ethanol and dried. Radioactivity was measured using a scintillation counter (Model Delta 300, TM Analytic, Elk Grove Village, Ill).

1.3. Gel electrophoresis

Radioactive samples were mixed with 4 X sample buffer (pH 6.8; 3% Tris, 20% b-mercaptoethanol, 10% sodium dodecyl sulfate [SDS], 0.02% bromophenol blue and 40% glycerol) and heated at 95 °C for 3 min, and then centrifugated to remove debris. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of LAEMMLII (1970) with a 4% (w/v) stacking gel and a 10% (w/v) separating gel. Protein samples containing 100,000 cpm were applied to each line. 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, Sigma-Aldrich Quimica, México, D.F., México) were used as molecular weight standards. Gels were stained with Coomassie brillant blue R-250 and then dried at 60 °C under vacuum before exposure to Kodak X-OMAT AR film for 3 days at –70 °C.

1.4. Cold tolerance assay

When the cultures reached an A_{600} of 0.3 to 0.4, they were cold shocked at 28 °C for 60 min and then challenged at 10 °C. Cellular viability at the last temperature was determined from aliquots at 0, 20, 40 and 60 min by plate counts in a nutrient agar (GARCIA-ALVARADO et al., 1992a). 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 cold shocked at 28 °C, they were reheated to 43 °C, and after 1, 2 and 3 h they were challenged at 10 °C as mentioned above. D values, defined as the time required to inactivate 90% of the population (HEREDIA et al., 1997) were determined from the cold death curves. The *t* test was used to determine differences between the slopes from the curves (ZAR, 1996).

2. Results and discussion

2.1. Protein synthesis in cold-shocked cells

Analysis of protein synthesis by cells using SDS-PAGE and autoradiography clearly demonstrated the induction of a set of cold-shock proteins (Fig. 1). A strong induction of five proteins (101, 82, 70, 45 and 10 kDa) was observed.

2.1. Cold tolerance assay

Results indicated that a cold shock increased the subsequent cold tolerance of *C. perfringens* cells ($P<0.05$). For example, after cold shock (0 h), the $D_{10\,°C}$ of strain FD-1041 increased from 30 to 455 min (Table 1). The acquired cold tolerance was maintained for 2 h after the cold-shock treatment. At 2.5 h no significant difference was observed compared to the control.

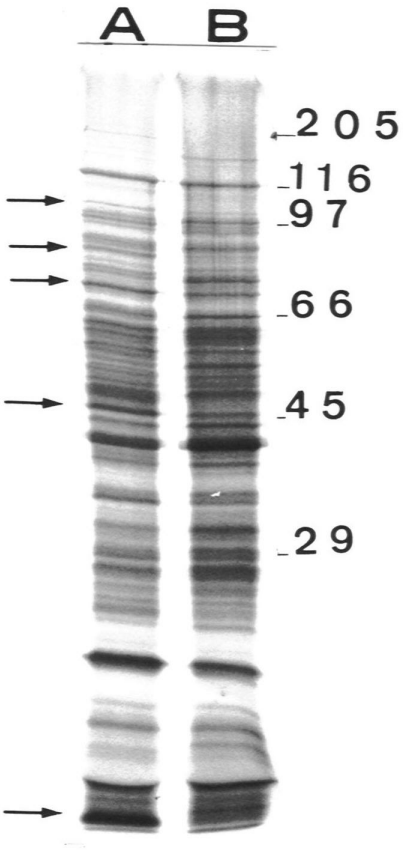

Fig. 1. Protein profiles characterizing cold shock proteins induction in *C. perfringens* after cold shock (28 °C/60 min). Cells were radiolabeled for 55 min by the addition of [^{35}S] methionine and cysteine 5 min after temperature downshift. (A) Treatment, (B) Control. The migration positions and sizes (in kDa) of protein standards are indicated on the right; arrows on the left indicate cold shock proteins

In the present study we have shown that *C. perfringens* can be adapted to survive cold environments. This adaptation requires prior exposure to a relatively mild cold temperature (28 °C) before challenging at a much lower temperature (10 °C). Also, cold adaptation in *C. perfringens* was concomitant with the increased synthesis of at least five proteins. The number of cold shock proteins found in *C. perfringens* is relatively lower compared to those identified in other bacteria, for example, 13 in *E. coli* (JONES et al., 1987), 9 in *B. psychrophilus* (WHYTE & INNISS, 1992), 12 in *L. lactis* and *L. monocytogenes* (BAYLES et al., 1996; PANOFF et al., 1994), 52 in *B. subtilis* (LOTTERING & STREIPS, 1995) and 40 in *V. vulnificus* (MCGOVERN & OLIVER, 1995).

Table 1. D values of cold-shocked *C. perfringens* FD-1041. Cells were cold shocked at 28 °C for 60 min, and then challenged at 10 °C (0 h) or returned to 43 °C for 2 and 2.5 h before challenging

Time after cold shock (h)	D_{10} (min)	
	Control	Cold-shocked
0	30 ± 15	455 ± 127
2	16 ± 2	130 ± 16
2.5	23 ± 2	20 ± 1.5

However, the criteria for designation of a protein as a cold shock protein and the means used to compare the intensities of spots corresponding to proteins in gels vary among investigators and so does the protein separating system used. Independently of the number of these proteins, in all the cases, including *C. perfringens*, the new proteins may have a significant role in protecting the cells at low temperatures.

Cold shock enhanced the cold tolerance of *C. perfringens* at least 15 fold. It is known that increased phenotypic tolerance to cold temperatures is accompanied by the synthesis of proteins involved in different functions such as lipid denaturation or DNA conformation modification (JONES & INOUYE, 1994; THAMAVONGS et al., 1996). Cold tolerance obtained by low temperature adaptation is in accordance with modifications in the cell's physical properties; a thermal downshift leads to the synthesis of cold shock proteins and also to an increase of fatty acid desaturation, resulting in control of the membrane fluidity which is essential in order to maintain the physiological function of biological membranes (MURATA & WADA, 1995). H-NS and the alpha subunit of the DNA gyrase are cold shock proteins involved in the control of several procedures by acting on DNA conformation (JONES et al., 1992; LA TREANA et al., 1991). Further studies on the genetic mechanism(s) whereby this bacterial pathogen can adapt and survive in cold environments may provide insight into its ability to cause disease in humans.

3. Conclusions

Five cold-shock proteins (101, 82, 70, 45 and 10 kDa) were induced by cold shocking *C. perfringens* FD-1041 from 43 °C to 28 °C. Cold shock also increased the cold tolerance of the cells at least fifteen fold. The acquired tolerance was maintained for 2 h after the cold treatment.

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