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Effect of Different Temperature Downshifts on Protein Synthesis by *Aeromonas hydrophila*

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Abstract. The psychrotrophic bacterium *Aeromonas hydrophila* 7966 was subjected to cold shocks from 30°C to 20°C, 15°C, 10°C, or 5°C, or were incubated at low temperature to determine its adaptative response. The cell protein patterns analyzed by two-dimensional electrophoresis revealed that only a few proteins were underexpressed, whereas numerous new proteins appeared with the decrease of temperature, and some others were overexpressed. Among them, a few constituted cold shock proteins because they were transiently induced, whereas others belong to the acclimatation family proteins. Two cold shock proteins of 11 kDa were synthesized at low level because they were visualized only after radiolabeling or silver staining. Moreover, under our experimental conditions, no major cold shock protein of a molecular mass similar to that of *E. coli* (7.4 kDa) could be identified.

Bacteria belonging to the genus *Aeromonas* are currently classified in the family *Vibrionaceae.* Most are oxidase positive, contrary to *Enterobacteriaceae,* but evidence of a common evolutionary origin for *Vibrionaceae* and *Enterobacteriaceae* has been achieved from genetic analysis. *Aeromonas hydrophila* is ubiquitous and has been frequently selected from various food products [13]; it is a psychrotrophic spoilage organism capable of competitive growth at 5°C [10–12]. Interest in this microorganism has been increasing steadily ever since it was recognized as a food-borne opportunistic pathogen, and some authors showed that many virulence factors such as exotoxins are expressed at 5°C [9–14]. Many food products are commonly stored at refrigeration temperatures. It was, therefore, interesting to examine the cold shock response of this bacterium, leading to the synthesis of proteins that are essential for its survival. The effect of cold temperature on bacteria has been extensively investigated in the mesophilic species *Escherichia coli* [2–8] and in some psychrotrophic and psychrophilic bacteria [14]. Low-temperature response depends on the type of transfer of organisms to cold temperature. Acclimatation is evoked when the transfer is durable and leads to a delayed and continuous response. Specific proteins expressed under these conditions were named cold acclimatation proteins (Caps). Shock is mentioned when the transfer of bacteria is a rapid shift and leads to an intermediate and transient response of the microorganism. It involves the transient induction of a subset of cold shock proteins (Csps) [6]. Little is known about the cold shock response of *A. hydrophila.* Nevertheless, Francis and Stewart [4] detected *csp* genes in a variety of bacteria including *A. hydrophila,* using a PCR method with primers derived from the major cold shock protein CspA of *E. coli.* The aim of this study was to resolve the extent of the cold shock response of *A. hydrophila* by a two-dimensional gel analysis system.

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Materials and Methods

Bacterial strain and growth conditions. *A. hydrophila* ATCC 7966 from the American Type Culture Collection (Rockville, MD, USA) was cultivated in synthetic medium/per liter: Na_2HPO_4, 6.5 g; $NaH_2PO_4{\cdot}2H_2O$, 3.6 g; $(NH_4)_2SO_4$, 1 g; $C_6H_5Na_3O_7{\cdot}2H_2O_2$,0.3 g; KCl, 0.4 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; $FeSO_4$, 1.7×10^{-4} g; $ZnSO_4{\cdot}7H_2O$, 2×10^{-4} g; $CuSO_4$, 5×10^{-7} g; $MnSO_4$, 5×10^{-5} g; and 0.4 mL of trace element solution containing, per liter, H_3BO_4, 0.4 g; $CaCl_2$, 0.5 g; $NiCl_2$, 2 g; Na_2MoO_4, 3 g. After autoclaving, the synthetic medium was supplemented with separately sterilized glucose and yeast extract at final concentrations of 0.2% and 0.0001%, respectively.

Cold growth profile, thermal shock, and acclimatation treatments. Precultures were grown overnight at 30°C with shaking in synthetic medium and were used to inoculate experimental 1-L culture medium. Cultures were inoculated to 0.1 optical density at 600 nm (OD_{600}) and

were incubated for cold growth at 20°C to 5°C until they reached the mid-exponential growth phase (OD_{600} ~0.4). For thermal shocks and acclimatation treatments, samples of 100 mL were removed at the mid-exponential phase and were incubated in a water bath at 20°C, 10°C, or 5°C for 2 or several hours. A control before shock was done with 100 mL of culture. Cells were harvested by centrifugation and were washed with 50 mM Tris-HCl buffer, pH 8.0 (buffer A). The cell pellets were frozen before protein extraction. Experiments were repeated three times.

Radioactive labeling of proteins. A culture was carried out at 30°C in 10 mL of synthetic medium with shaking. When the OD_{600} was close to 0.4, 1.5-mL samples were transferred into Eppendorf tubes and were centrifuged. Cells were resuspended in 500 μL of the same medium cooled to the desired shock temperature. *De novo* protein synthesis was examined by adding 10 μCi (3.7 MBq) of L[^{35}S]methionine (specific activity >37 TBq/mmol) to each sample. The cells were radiolabeled for (i) 30 min at 30°C for the control; (ii) the last 30 min for the cold shocks. The radiolabeling was stopped by centrifugation at 12,000 *g* for 5 min at 4°C. Cell pellets were washed with 500 μL of the buffer A, and proteins were immediately extracted as described below except that buffer volumes were adjusted. Radioactivity amounts were determined with 1 μL of the SDS-solubilized protein extract dropped on a glass-fiber filter, rinsed with 3 mL of cold 25% trichloracetic acid (TCA) and then with 10 mL of cold 8% TCA. Filters were dipped into a liquid scintillation cocktail and analyzed with a Beckman LS-2800 liquid scintillation spectrometer.

Sample preparation. The cell pellets were washed with 5 mL of buffer A and then treated with 2.5 mL of cold acetone (−20°C) for 10 min on ice. Cells were harvested by centrifugation and, after drying, were resuspended in 5 mL of buffer A. The bacteria were then disrupted by sonication and cooled on ice for 2 × 3 min with continuous pulses. Cell-free extracts were obtained by centrifugation. After 3% TCA precipitation for 2 h at −20°C, proteins were recovered by centrifugation. Protein pellets were rinsed with cold acetone and dissolved in 100 μL of a buffer containing (per 100 mL): urea, 57 g; CHAPS, 0.06 g; K_2CO_3, 0.069 g; dithiothreitol, 0.5 g; 3–10 ampholytes, 1 mL; and sodium dodecylsulfate (SDS), 1.25 g.

Total cellular proteins were measured on 1 μL of acetone-precipitated extract with the Bio-Rad DC protein assay kit, with crystalline bovine albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE) of proteins. Two-dimensional PAGE was performed as described by O'Farrel [11]. Succinctly, equal amounts of samples equivalent in protein content (100 or 400 μg) or TCA-precipitable radioactivity (10^6 cpm) were loaded onto the electrofocusing (1 mm or 1.5-mm diameter according to amount of protein) gels. The gel mixture contained 10 g urea, 2.5 mL of acrylamide/piperazine diacrylamide (PDA) solution (100 mL of 40% acrylamide, 1 g of PDA complemented with distilled water to 134 mL), 0.9 mL of 3–10 ampholytes, 0.1 mL of 5–7 ampholytes, 2 mL of 10% Triton X-100, 40 μL of ammonium persulfate, and 10 μL of TEMED. Gels were run for 17 h at 1200 V and for 0.5 h at 1500 V. Focalization gels were equilibrated for 1 h in 2.3% SDS, 0.5 M Tris-HCl, pH 6.8, buffer containing bromophenol blue. These gels were loaded on 12.5% SDS polyacrylamide gels and run at 350 V until bromophenol blue reached the bottom of the gel. Proteins separated in the gels were stained either with Coomassie blue G-250, blue colloidal method (Sigma-Aldrich), or silver. The pH gradient was monitored by using two-dimensional electrophoresis standards. Molecular weights were estimated by the comigration in the second dimension of mass markers.

Results

Cold shock protein synthesis. For demonstration of significant cold response, *A. hydrophila* was grown either at 20°C, 15°C, 10°C, or 5°C, and the content of proteins was analyzed. The more the temperature was lowered the higher number of new proteins (from at least 22 proteins at 20°C to 30 at 5°C). Among them, new proteins P_{33a} (33 kDa) and P_{62} (62 kDa) were common at all the cold temperatures of growth (data not shown).

To show evidence of cold shock proteins, cells were grown at 30°C and then shifted to either 20°C or 5°C for 2 h. Compared with the control (Fig. 1A), at least 26 cold shock-related polypeptides were induced at 20°C (squares in Fig. 1B), whereas at least 47 were induced at 5°C (squares in Fig. 1C). They presented apparent molecular masses ranging from 25 to 65 kDa, and most of the polypeptides induced at 5°C were acidic. On the other hand, most of the ten proteins common to 20°C and 5°C displayed a pH_i near neutrality (P_{33a}, P_{34b}, P_{38}, P_{39}, P_{40}, P_{55a}, P_{55b}). As the proteins P_{34a} and P_{62} were also synthetized during growth at low temperature, they may therefore constitute Cap proteins. The other eight proteins could constitute a part of the cold shock proteins.

Some proteins (arrows in Fig. 1B and 1C) showed an increased level of synthesis. Nine were overexpressed at 20°C and 5°C, whereas expression of 17 additional proteins was increased at 5°C. To determine whether progressive decrease in temperature would result in the progressive overexpression of some proteins, cells growing at 30°C were shifted to additional temperatures (15°C or 10°C) for 2 h. Two polypeptides (P_{33b} and P_{36}) of 33 and 36 kDa respectively were specifically enhanced with the decrease in temperature (Fig. 2). The polypeptide P_{33b} still present after 21 h of cultivation (Fig. 2; 15°C was given as an example) was also found during growth at low temperature and probably belongs to the family of acclimatation proteins. On the contrary, the polypeptide P_{36} completely disappeared when time was extended up to 21 h, suggesting it was a Csp protein. This result was confirmed by its absence during cold growth.

Radiolabeling of newly synthesized proteins. Different electrophoresis conditions (higher percentage of acrylamide, gradient SDS polyacrylamide gel, urea-polyacrylamide gel, etc.) did not show evidence of an acidic protein of low molecular mass similar to the major cold shock protein CspA of *E. coli.* This could be owing to a low amount of protein with regard to the cellular protein pool. With this aim, we measured only newly synthesized proteins by labeling experiments. Strain 7966 was grown at 30°C and was shifted to either 15°C, 10°C, or

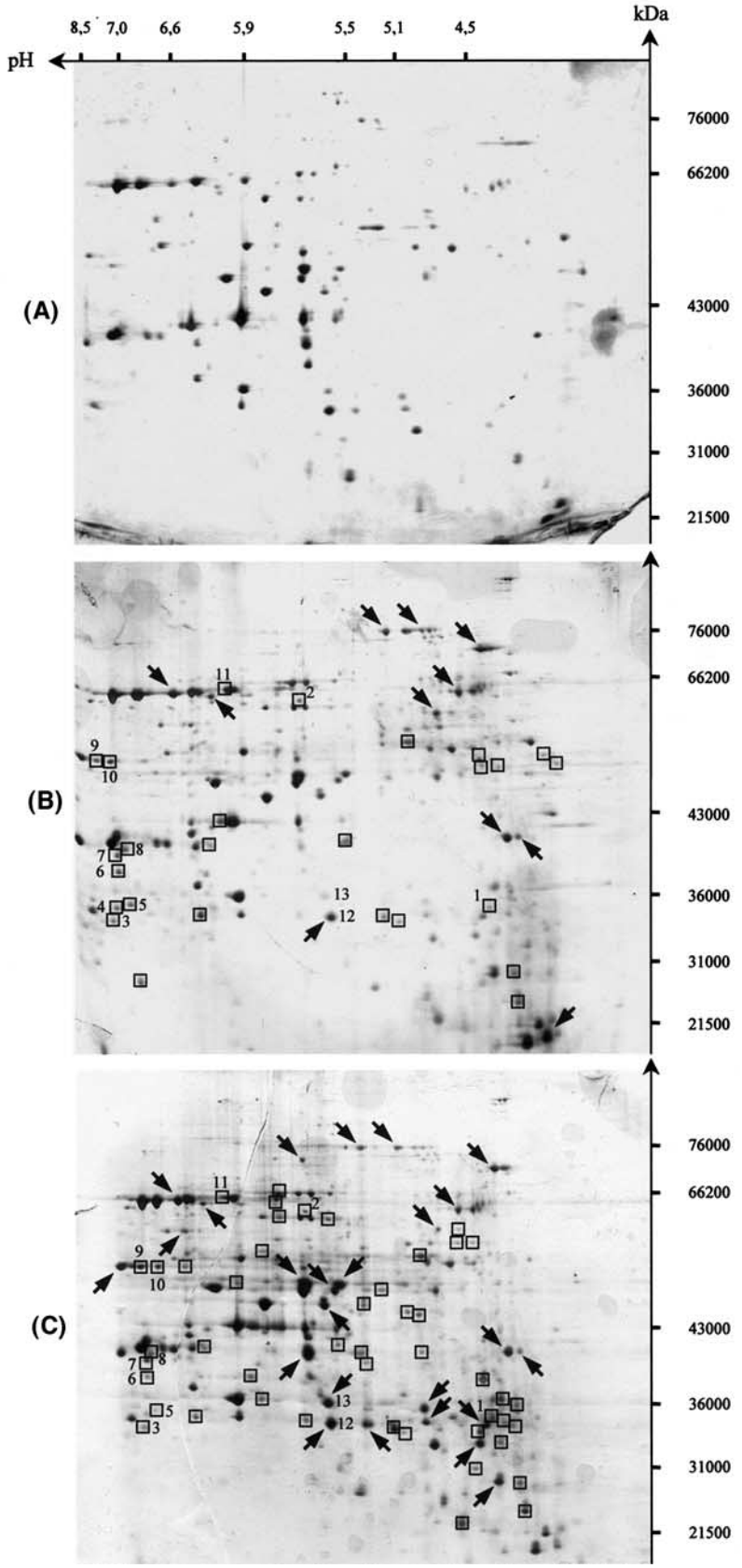

Fig. 1. Two-dimensional analysis of total cellular proteins of *A. hydrophila* 7966 (A) grown at 30°C-control; (B) after 2 h downshift from 30°C to 20°C; (C) after 2 h downshift from 30°C to 5°C. Overexpressed proteins are pointed out, newly synthesized protein are squared. Numbered proteins are common to many shifts (1: P_{34a}, 2:P_{62}, 3:P_{33a}, 4:$P_{33.5}$, 5:P_{34b}, 6:P_{38}, 7:P_{39}, 8:P_{40}, 9:P_{55a}, 10:P_{55b}, 11:P_{64}, 12: P_{33b}, 13:P_{36}).

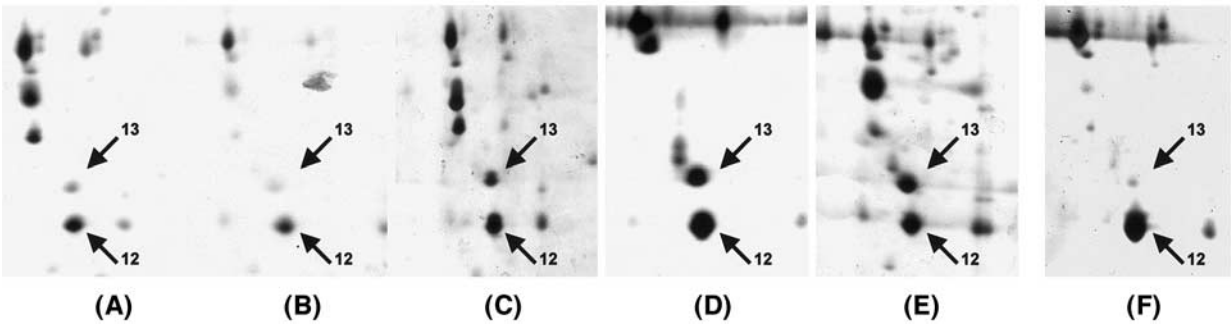

Fig. 2. Two-dimensional overexpression of proteins P_{33b} and P_{36} at different temperatures of shift. (A) Control culture; (B) 2 h shift from 30°C to 20°C; (C) 2 h shift from 30°C to 15°C; (D) 2 h shift from 30°C to 10°C; (E) 2 h shift from 30°C to 5°C; (F) 21 h shift from 30°C to 15°C. Numbers 12 and 13 correspond to P_{33b} and P_{36}, respectively.

5°C for 1 h. The analysis of autoradiograms did not permit superimposing them with the gels obtained from non-labeled experiments because the profiles were different. Nevertheless, the comparison of the autoradiograms with that of the control culture demonstrated that the synthesis remained important throughout the three temperature shifts (data not shown). As previously observed, more proteins were induced at low temperatures. Among these proteins, one (R_{11b}; 11 kDa) was significantly induced (Fig 3B, C, D), and another one of similar molecular mass (R_{11a}; 11 kDa) already present at a very low level at 30°C (Fig. 3A) also exhibited a strong incorporation of radioactivity with the decrease of temperature. ^{35}S incorporation in these two polypeptides was much more important when the shock was prolonged up to 2 h, (Fig. 3E). R_{11a} and R_{11b} disappeared when the cells were allowed to stand at 5°C for 3 weeks (data not shown).

R_{11a} and R_{11b} peptides were probably not observed in the non-radioactive experiments in accordance with the low amount of protein loaded. Then, new shifts were performed when the protein content was increased to 400 μg and electrophoresis was performed in thicker gels (1.5 mm). Gels were stained either with Coomassie blue, brilliant colloidal blue, or silver. The expected protein profile was obtained only with silver stain, and two spots corresponding to 11 kDa were seen.

Discussion

Under low temperatures, *A. hydrophila* 7966 overexpressed some proteins and synthesized new ones. As described by Berger et al. [1], they can be classified into two categories: i) Csps proteins transiently induced, such as P_{36}, R_{11a} and R_{11b}, ii) Caps proteins (Caps), such as P_{34a}, P_{62}, P_{33b}, corresponding to a durable cold transfer.

Contrary to *E. coli,* in which protein synthesis decreases with transfer from 37°C to 4°C [10], the levels of very few proteins were altered when *A. hydrophila* was transferred from 30°C to 5°C. Most housekeeping proteins were similarly expressed at 30°C and 5°C. This

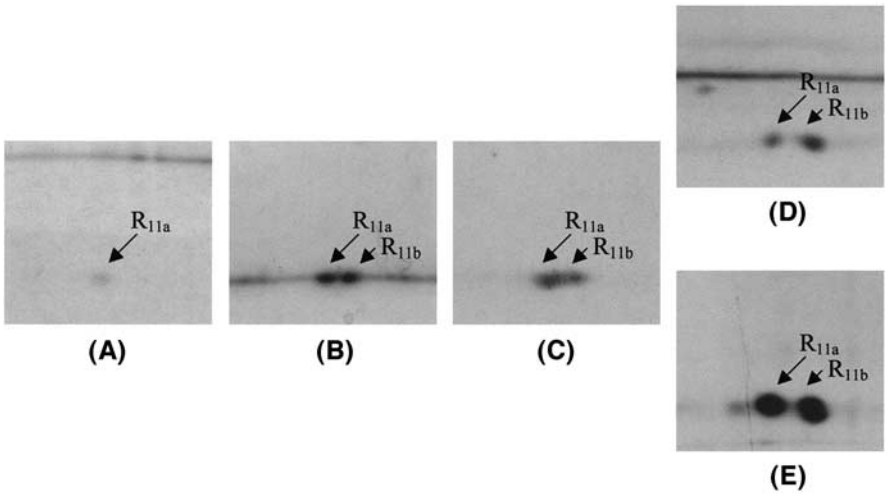

Fig. 3. Two-dimensional gel electrophoresis of cellular proteins of *A. hydrophila* 7966 labeled with ^{35}S methionine. Cells were grown to 30°C (A) or shifted from 30°C to 15°C for 1 h (B); to 10°C for 1 h (C); to 5°C for 1 h (D); and to 5°C for 2 h (E); and labeled for the last 30 min of incubation.

result was in accordance with those of Berger et al. [14] from the psychrotrophic bacterium *Arthrobacter globiformis.* More Csps are overexpressed in psychrotrophic bacteria than in mesophilic ones.

Under our experimental conditions, no major cold shock protein of molecular mass similar to that of *E. coli* (7.4 kDa) [5] was evidenced. This was previously observed in *Vibrio vulnificus* by Carroll et al. [3], who suggested that the lack of this Csp is related to the ability of this organism to enter into a viable but not culturable state (VBNC) when the temperature of water falls below 15°C in winter and autumn. *A. hydrophila* still grows at 5°C but can also be induced into a VBNC state by incubation at low temperature [14]. We postulate that the nature of proteins induced at low temperatures differs from those previously reported as cold-shock proteins in *E. coli.*

ACKNOWLEDGMENTS

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