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Differentiation between Cold Shock Proteins and Cold Acclimation Proteins in a Mesophilic Gram-Positive Bacterium, *Enterococcus faecalis* JH2-2

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Transfer of *Enterococcus faecalis* to a cold temperature (8°C for 4 to 30 h) led to increased expression of 11 cold shock proteins (CSPs). Furthermore, this mesophilic prokaryote synthesized 10 cold acclimation proteins, five of them distinct from CSPs, during continuous growth (4 days) at the same temperature (8°C).

Stress responses have been extensively studied (35) and seem to be implicated in important phenomena such as cellular survival, species perpetuation, and evolution of genera (33). In this context, the cold stress response of mesophilic bacteria is a widely studied subject (16, 26) which can be subdivided into two parts, depending on whether the low temperature is above or below 0°C. (i) At subzero temperatures, the stress response is passive and characterized by membrane damage and DNA denaturation leading to cell death (9). (ii) At low positive temperatures, the stress response is active; the main feature of this phenomenon is the synthesis of a specific set of proteins (19) regulated at transcriptional (17, 20) and translational (5, 12, 18, 25) levels. This synthesis supports a dramatic metabolic differentiation.

Furthermore, exposure to low positive temperatures may act as an "adapter" to freezing temperatures, leading, as a possible consequence of the synthesis of specific proteins, to decreased lethality, a phenomenon called cryotolerance (27, 34, 37).

Study of low-temperature stress responses in bacteria is tackled in two ways, depending on the type of transfer of organisms to cold temperature. It is a shock when the transfer of bacteria from the optimal growth temperature to a growth-permissive cold temperature leads to an immediate and transient response of the microorganisms. It is an acclimation when the transfer is durable and leads to a delayed and continuous response. CSP (cold shock protein) (19, 21) synthesis is induced in response to a shock or a shift to a low positive temperature, whereas CAPs (cold acclimation proteins) (2, 30, 36) are proteins specifically synthesized during continuous growth at cold temperatures (acclimation). Nevertheless, Graumann and Marahiel (13) have proposed other definitions of CSP and CAP and introduced a new term, cold-induced protein (CIP). Cold-induced proteins are proteins previously called CSPs, and CSPs are limited to the small, acidic proteins.

Interestingly, while CAPs are clearly distinct from CSPs in psychrotrophic and psychrophilic microorganisms (2, 7, 30, 36), the synthesis of CAPs in mesophilic bacteria in response to continuous growth at low temperatures has not been studied.

Enterococcus faecalis is interesting for its ability to resist numerous stresses (4, 8, 10). It is an allochthonous, gram-positive bacterium (31) which lives in the intestinal tracts of many animals and is commonly present in cold biological environments (3, 29, 32).

This report aims to show, with the example of an enteric microorganism, that it is possible to distinguish CAPs from CSPs in a mesophilic bacterium.

Strain and culture conditions. Studies were performed with *E. faecalis* JH2-2, derived from parental strain JH2 (15). Cultures were carried out in brain heart infusion medium at 8 or 37°C, depending on the experiments.

Protein sample preparations. (i) Unlabelled proteins. Cultures were grown at 8 or 37°C from 2×10^7 to 2×10^8 cells · ml^{-1} (an optical density at 600 nm of 1 corresponds to 4×10^8 cells · ml^{-1}). Exponential-phase cells (10 ml) were then harvested by centrifugation (3,040 × *g*, 10 min), and proteins were extracted as described by Flahaut et al. (10). The composition of the resuspending protein buffer was modified (4.4 mM Tris base, 5.6 mM Tris-HCl, 120 mM dithiothreitol, 0.006% sodium dodecyl sulfate (SDS), 7.92 mM urea, 3.2% Nonidet P-40, 1.76% [vol/vol] ampholytes [pH 4 to 7]).

(ii) Radiolabelled proteins. Cultures were grown at 37°C to an optical density at 600 nm of 0.25 or 0.5, depending on the experiments. A 1-ml sample was then transferred to a microtube (37 or 8°C) and mixed with [^{35}S]methionine-[^{35}S]cysteine (100 or 200 μCi, respectively) (New England Nuclear Corp.). After 35 min of labelling at 37°C or 4 to 30 h at 8°C, proteins were extracted as described above.

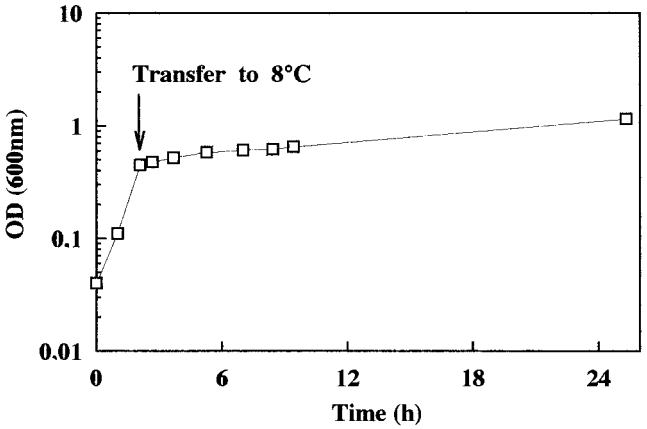

FIG. 1. Growth of *E. faecalis* JH2-2 in brain heart infusion medium. Cells were incubated at 37°C before a transfer to 8°C.

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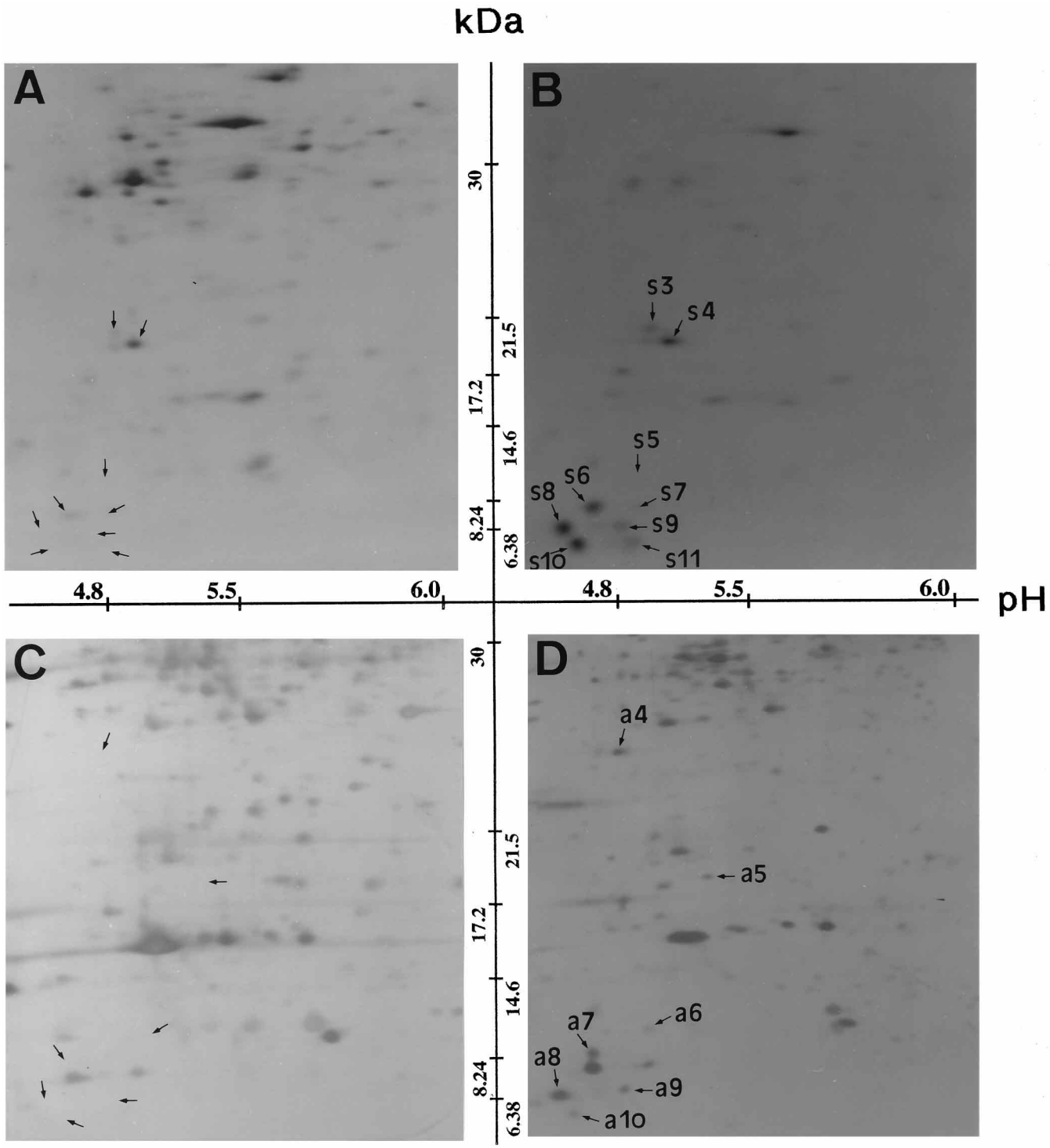

FIG. 2. 2-D gel electrophoresis (16% polyacrylamide) of proteins of *E. faecalis* JH2-2 following a temperature downshift from 37°C (A and C) to 8°C (B, 8 h; D, 4 days). (A and B) Proteins labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine. (C and D) Proteins visualized by silver staining. a, CAP; s, CSP.

Two-dimensional (2-D) gel electrophoresis. Protein electrophoresis was carried out by the method developed by O'Farrell (24) and modified as described by Flahaut et al. (10). The first-dimension gel separation was carried out with Immobiline Dry Strips (pH 4 to 7) by following the manufacturer's (Pharmacia, Uppsala, Sweden) advice. (Previous work has shown that proteins are acidic in *E. faecalis* [11].) The second dimension was realized on SDS–10 and 16% polyacrylamide gels by using the Millipore Investigator 2-D electrophoresis system.

Unlabelled proteins were visualized by silver staining of 2-D gels by the method of Morrissey (23) modified as described by

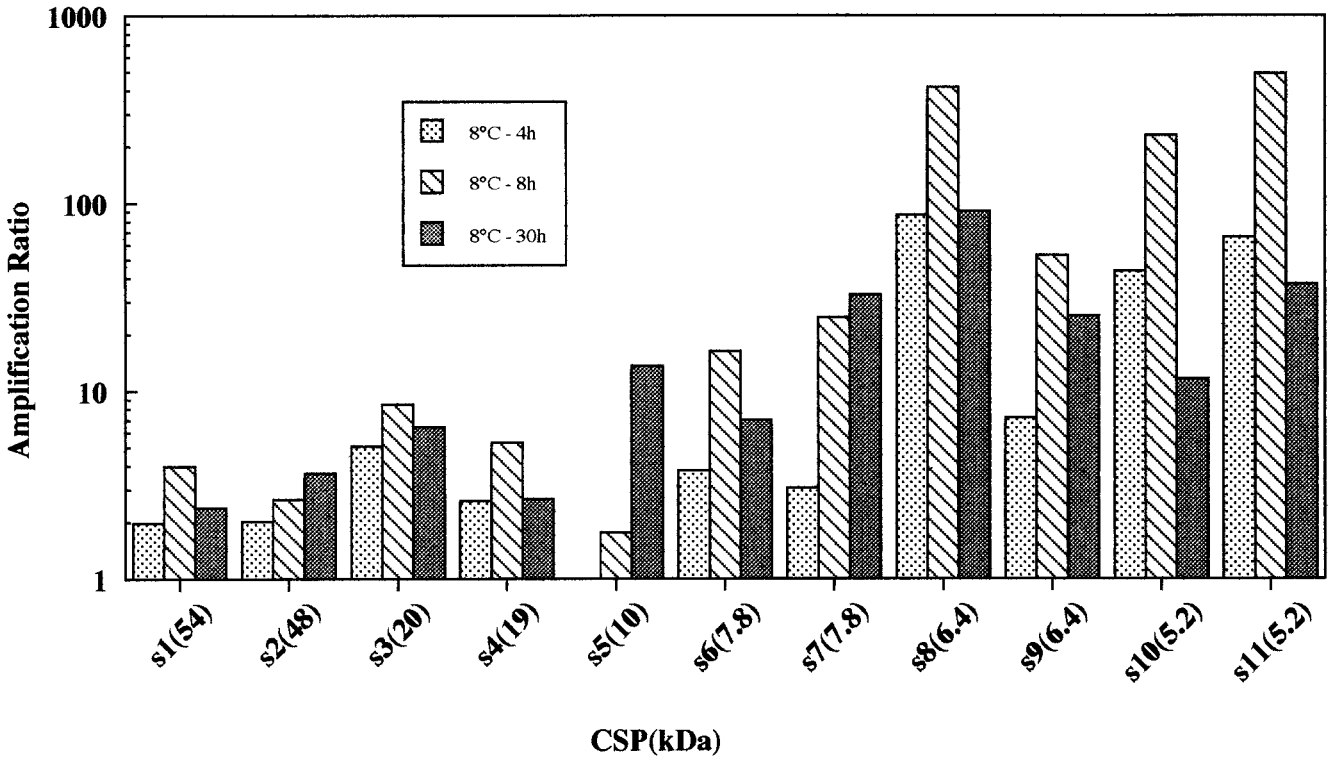

FIG. 3. Kinetics of CSP synthesis in *E. faecalis* JH2-2 at 8°C (for 4 to 30 h). The CSPs are those observed by 2-D gel electrophoresis as shown in Fig. 2, except for s1 and s2.

Giard et al. (11). Radioactive protein gels were dried and exposed as described by Flahaut et al. (10).

Spot quantifications were realized by computing scanning densitometry with the 2-D Analyzer Computer Program (Bio Image Systems Corp.). The densities of individual proteins were determined by surface integration. Proteins with increased relative synthesis (compared with the whole-spot density) at a cold temperature compared to the optimum growth temperature were referred to as CSPs or CAPs, depending on the experiments.

CSP induction and kinetics. Compared with *Escherichia coli* (19), the transfer of *E. faecalis* cells to a low temperature does not lead to any growth lag phase: when the culture is shifted to 8°C, cells start growing with a generation time of 29 h (34) (Fig. 1).

When *E. faecalis* JH2-2 cell cultures are transferred from 37 to 8°C (for 4 to 30 h), some protein syntheses are repressed whereas others are amplified, according to radiolabelling experiments (Fig. 2A and B). This phenomenon, which has been described in numerous species, shows some characteristics in this bacterium. (i) Eleven CSPs are amplified (more than threefold) after a culture is transferred to a cold temperature. Only two of them (s1 and s2) have a molecular mass of more than 30 kDa (Fig. 3). s1 and s2 were visualized on an SDS–10% (wt/vol) polyacrylamide gel (data not shown). (ii) Optimally increased expression of eight of the CSPs occurred after 8 h of exposure to 8°C, and that of the other three occurred after 30 h (Fig. 3). (iii) Proteins with low molecular masses (less than 15 kDa) had levels of relative increased synthesis clearly higher (more than 10-fold) than proteins with medium or high molecular masses (more than 15 kDa) (Fig. 3). (iv) The pK of these CSPs is acidic, with values below 5.5 (Fig. 2).

CAP synthesis and comparison with CSPs. Visualization of silver-stained proteins on 2-D electrophoretic gels after 4 days of slow exponential growth at 8°C ($\mu = 0.003\ h^{-1}$) shows that 10 CAPs are synthesized in *E. faecalis* JH2-2 (Fig. 2C and D and Table 1). In parallel with the characteristics of the CSPs described above, it was noted that seven of the CAPs have molecular masses below 30 kDa with increased expression higher than that of the other three, whereas only the three CAPs with molecular masses of more than 30 kDa are amplified at 8°C compared to 37°C, with an amplification ratio lower than 3 (a1, a2, and a3 were visualized on an SDS–10% [wt/vol] polyacrylamide gel; data not shown). Six of the seven proteins with molecular masses of less than 30 kDa are amplified from 10- to 50-fold.

Comparison of the CSPs and CAPs showed that among the 10 CAPs synthesized, 5 correspond to CSPs (Table 1). Since the work of Jones et al. in 1987 (19), CSP and/or CAP synthesis

TABLE 1. CAPs of *E. faecalis*

Electrophoresis (% polyacrylamide)	CAP spot reference[a]	CSP spot correspondence[a]	Molecular mass (kDa)	Relative intensity (%) at:		Amplification ratio
				37°C	8°C	
10	1		66	0.057	0.132	2.32
10	2	1	54	0.076	0.196	2.58
10	3		32	0.070	0.187	2.67
16	4		25	<0.005	0.171	>34.2
16	5		18	<0.005	0.054	>10.8
16	6	5	10.0	0.032	0.097	3.03
16	7		8.8	<0.005	0.202	>40.4
16	8	8	6.4	0.049	0.491	10.0
16	9	9	6.4	<0.005	0.186	>37.2
16	10	10	5.2	<0.005	0.239	>47.8

[a] The numbers correspond to spots in Fig. 2.

in prokaryotes (mesophilic bacteria or psychrobacteria) has been examined in a variety of organisms by silver staining or radiolabelling. It is usual to differentiate between CSPs and CAPs in psychrotrophic bacteria (temperatures: minimum, <5°C; optimum, >15°C; maximum, >20°C) (2, 7, 36) and psychrophilic bacteria (temperatures: minimum, <0°C; optimum, <15°C; maximum, <20°C) (30), even if some investigators have not made this distinction among cold stress proteins (1, 6, 14, 22, 28). If CSP synthesis is induced in response to a shift to a low positive temperature, CAPs are proteins specifically synthesized during continuous growth at cold temperatures and have never been studied in a mesophilic bacterium (temperatures: minimum, >5°C; optimum, <45°C; maximum, <50°C). Transfer of *E. faecalis* from the optimal growth temperature to a growth-permissive cold temperature leads relatively quickly to synthesis of CSPs by the organism. In addition, CAPs are synthesized during constant growth of this mesophilic prokaryote at low positive temperatures, as shown in this report.

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