APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1996, p. 1116–1119 Vol. 62, No. 3
0099-2240/96/$04.00+0

Cold Stress Proteins Induced in *Listeria monocytogenes* in Response to Temperature Downshock and Growth at Low Temperatures

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Received 1 September 1995/Accepted 3 January 1996

Listeria monocytogenes **is a food-borne pathogen with the ability to grow at refrigerator temperatures. Twelve cold shock proteins (Csps) with apparent M_rs of 48,600, 41,000, 21,800, 21,100, 19,700, 19,200, 18,800, 18,800, 17,200, 15,500, 14,500, and 14,400 were induced by cold shocking *L. monocytogenes* 10403S from 37 to 5°C, as revealed by labeling with L-[^{35}S]methionine followed by two-dimensional gel electrophoresis. Strain SLCC53 showed a similar response. Cold acclimation proteins were observed in cultures of strain 10403S growing at 5°C, and four of these proteins, with apparent M_rs 48,000, 21,100, 19,700, and 18,800, were also Csps. Two cold-sensitive transposon-induced mutants were labeled less efficiently than the parent strain, but the Csp response of the mutant examined was very similar to that of the parent strain.**

Listeria monocytogenes is a gram-positive bacterium that is ubiquitous in the environment. The organism is the causative agent of listeriosis, a disease primarily affecting pregnant women and their neonates as well as patients who are immunocompromised (17). In the 1980s, several outbreaks of listeriosis were shown conclusively to be caused by the consumption of contaminated food (6). The range of temperatures over which *L. monocytogenes* can grow, 2 to 45°C, is unusual for a pathogenic bacterium (23). One of the salient features of *L. monocytogenes* as a food-borne pathogen is its ability to grow at low temperatures, thus allowing refrigeration at 4°C to act as an effective enrichment for the organism. One of the goals of the U.S. Department of Agriculture's hazard analysis critical control point policy regarding food-borne pathogens is to evaluate hazards imposed by a particular pathogen and then act to control it by implementing specific strategies (5). Effectiveness of the employed strategies is evaluated at various points during the processing of the food in its journey from field to market. Since refrigeration is a commonly used method of extending food life, it is important to understand how *L. monocytogenes* is able to grow at low temperatures. This knowledge may suggest strategies to control its growth. We have undertaken a study to examine the proteins produced by *L. monocytogenes* in response to cold shock and growth at low temperatures.

The role that proteins synthesized in increased amounts at low temperatures play in alleviating low-temperature stresses is only now beginning to be elucidated. Proteins produced in response to a temperature downshock have been given the name cold shock proteins (Csps), while proteins exhibiting increased synthesis during balanced growth at low temperatures have been called cold acclimation proteins (Caps). Jones et al. (12) have identified 13 proteins induced in *Escherichia coli* when exposed to a temperature shock from 37 to 10°C. Several of the *E. coli* Csps have been identified, and they are involved in diverse cellular processes (11, 12). NusA is involved in termination and antitermination of transcription, initiation factor 2 mediates the binding of charged $tRNA^{fMet}$ to the 30S ribosome subunit, and polynucleotide phosphorylase may be involved in the degradation of mRNA. Further identified Csps include RecA, which is involved in recombination and the SOS response, H-NS, a DNA-binding protein, and gyrase A, the α subunit of DNA gyrase.

The major *E. coli* Csp, on the basis of level of induction upon cold shock, has been designated CspA (8, 12). CspA has sequence homology with a family of Csps in *E. coli*, although some members of the family are not cold inducible (11). On the basis of sequence similarities with eukaryotic Y-box proteins it is proposed that Csps may be involved in activating transcription or unwinding or masking RNA molecules (11, 13). Homologs of CspA have been identified in *Bacillus subtilis* (25), *Streptomyces* species (2), and antarctic psychrotrophic bacteria (21).

In comparison with knowledge of the cold stress response in *E. coli*, knowledge of this response in other bacteria is at a much more rudimentary stage. However, Csp and Cap responses have been identified in a limited number of diverse psychrophilic, psychrotrophic, and mesophilic bacteria, including a *Vibrio* sp. (1), *Aquaspirillum articum* (22), *Bacillus psychrophilus* (24), *Pseudomonas fragi* (9), *Lactococcus lactis* (18), *B. subtilis* (15), and *Vibrio vulnificus* (16). Various numbers of proteins were designated as Csps and Caps, and some of the Caps were also Csps.

This report represents our initial characterization of the production of Csps and Caps by *L. monocytogenes* as revealed by two-dimensional gel electrophoresis. Information obtained from mutants unable to grow at low temperatures is also presented.

Strains used and mutant isolation. *L. monocytogenes* 10403S and DP-L910 (10403S carrying the Tn*917* plasmid pLTV3) were supplied by D. A. Portnoy (4), and strain SLCC53 was provided by T. Chakraborty (14). *L. monocytogenes* transposon mutants were generated by the procedure of Camilli et al. (4). Mutagenized cells were plated onto brain heart infusion agar plates containing erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}) and allowed to grow overnight at 37°C, after which they were sealed and incubated for 4 weeks at 4°C. Colonies

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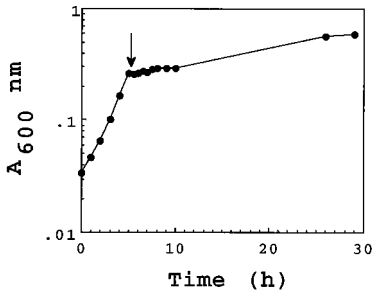

FIG. 1. Growth of *L. monocytogenes* 10403S following a temperature downshift from 37 to 10°C. The strain was grown in defined medium at 37°C and downshifted to 10°C at the point indicated by the arrow. The experiment was performed twice with strain 10403S and two other strains, with similar results.

which did not increase in size or obtain the morphology of the wild-type colonies were labeled as cold-sensitive mutants.

Csp and Cap labeling. For labeling Csps and Caps, we used a defined medium based on that of Pine et al. (20), with the following modifications: choline was not included in the medium, the methionine concentration was reduced to 30 μM for radiolabeling, and glucose (0.5%, wt/vol) was used as the carbon and energy source. In order to label Csps and Caps, 25 ml of defined medium was inoculated with an overnight culture of *L. monocytogenes*. The culture was incubated at 37°C in a rotary shaker (150 rpm) until the culture reached mid-exponential growth (A_{600} = 0.4). Cell culture (0.5 or 1 ml) was transferred to a 10-ml tube containing 150 μCi of [^{35}S]-TranS-label ([^{35}S]methionine and [^{35}S]cysteine; 1,208 Ci mmol^{-1}; ICN, Costa Mesa, Calif.) per ml, preequilibrated to the appropriate temperature. For cold shocks from 37 to 5°C, cells were labeled for 0 to 30 min, 30 to 60 min, and 60 to 120 min at 5°C. Control cells were labeled for 5 min at 37°C. Cap labeling was done in essentially the same fashion as Csp labeling except that the cells were grown in defined medium at 5°C to an A_{600} of 0.4 and were then labeled for 5 h at 5°C. Following the labeling period, the cells were harvested, the supernatant was decanted, and the cell pellet was treated with acetone and lysed as described by Fliss et al. (7). Incorporation of [^{35}S]-TranSlabel into trichloroacetic acid-precipitable protein was quantitated by the method of Berg et al. (3).

Two-dimensional gel electrophoresis. Protein samples were subjected to two-dimensional gel electrophoresis as described by Hochstrasser et al. (10), using a Bio-Rad Mini-Protean II apparatus; 200,000 cpm of activity in an equivalent volume of buffer was loaded onto each isoelectric focusing gel. Isoelectric focusing gels were cast with 5.5 g of urea, 3 ml of 30% acrylamide-bisacrylamide stock, 500 μl of detergent solution {0.15

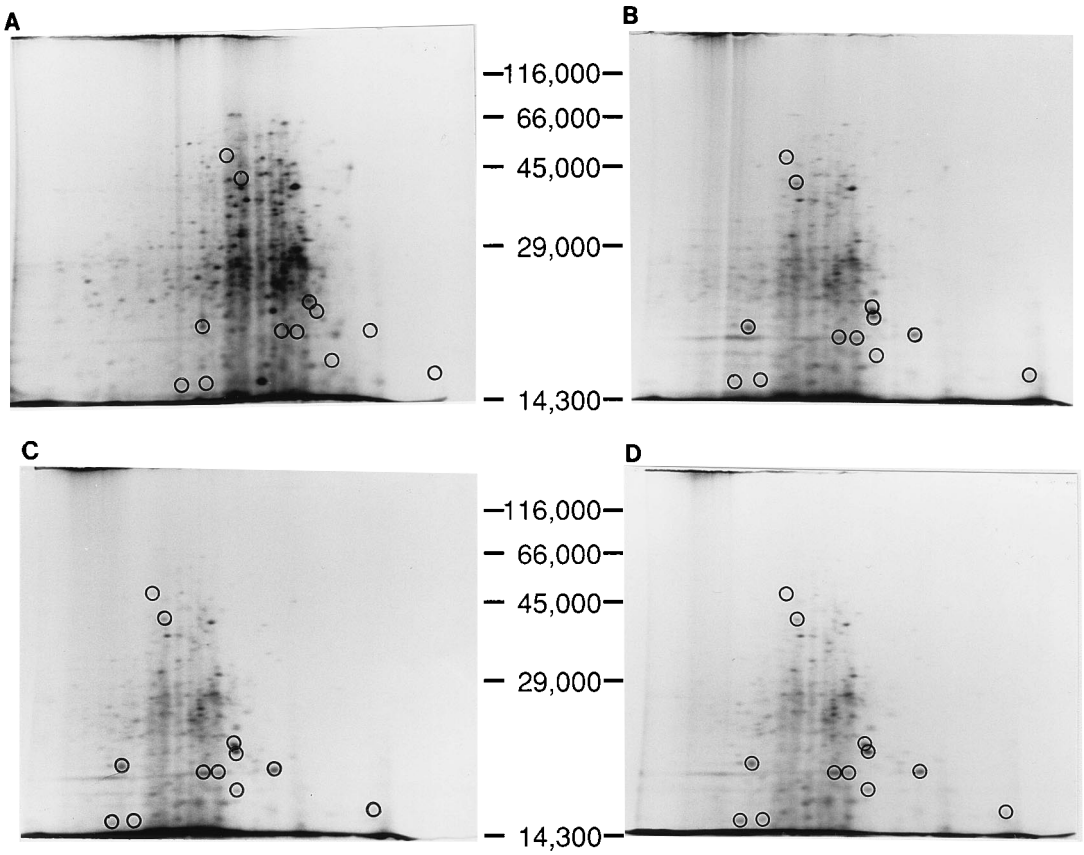

FIG. 2. Induction of Csps in *L. monocytogenes* 10403S following a temperature downshift from 37 to 5°C. Cells were labeled with ^{35}S-TranSlabel (ICN) at a final concentration of 150 μCi ml^{-1}. Each sample was quantitated for trichloroacetic acid-precipitable radioactivity, and equivalent amounts (200,000 cpm) of trichloroacetic acid-insoluble radioactivity were loaded onto the gels. Circles indicate the positions of Csps. Csps were identified on the basis of increased intensity of the protein spot on the gel compared with the non-cold-shocked control. Most of the Csps appeared to be synthesized at a relatively constant rate during the shock phase, and the spots do not increase in intensity as a result of loading equivalent amounts of radioactivity. The few spots that showed increased intensity later in the shock phase appeared to be exhibiting increased synthesis with time. The positions of molecular weight standards are indicated. (A) 37°C, 5-min preshift control; (B) 5°C cold shock, 0 to 30 min; (C) 5°C cold shock, 30 to 60 min; (D) 5°C cold shock, 60 to 120 min.

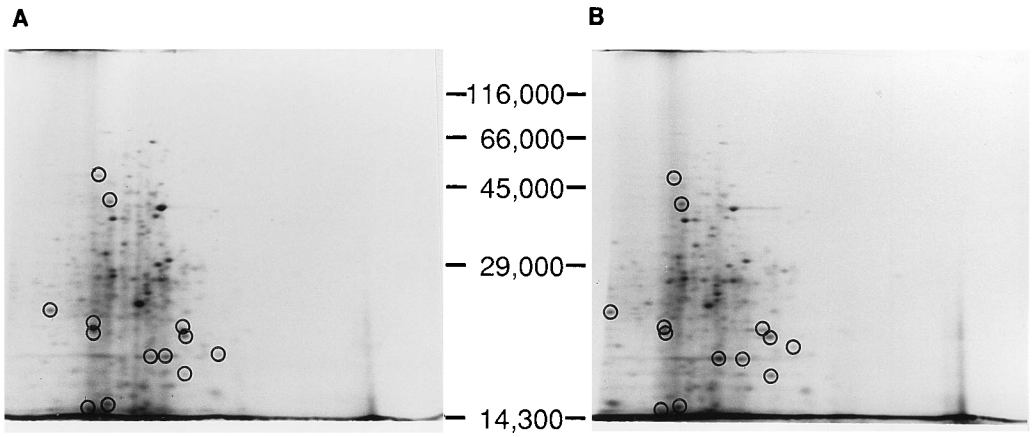

FIG. 3. Induction of Csps in *L. monocytogenes* SLCC53 following a temperature downshift from 37 to 5°C. Cells were labeled with ^{35}S-TranSlabel (ICN) at a final concentration of 150 μCi ml^{-1}. Each sample was quantitated for trichloroacetic acid-precipitable radioactivity, and equivalent amounts (200,000 cpm) of trichloroacetic acid-insoluble radioactivity were loaded onto the gels. Circles indicate the positions of Csps (identified as in Fig. 2). The positions of molecular weight standards are indicated. (A) 5°C cold shock, 0 to 30 min; (B) 5°C cold shock, 60 to 120 min.

g of 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 50 μl of Nonidet P-40, 450 μl of H_2O}, 100 μl of 5/8 ampholyte (Fisher Scientific), 400 μl of 3/10 ampholyte (Fisher Scientific), 20 μl of 10% (wt/vol) ammonium persulfate, and 10 μl of *N,N,N′,N′*-tetramethylethylenediamine. Following isoelectric focusing, gels were extruded from the tube cells and frozen at −80°C. Tube cells were briefly equilibrated in 30 μl of transfer buffer (40 ml of 0.5 M Tris-HCl [pH 8.8], 80 ml of 10% sodium dodecyl sulfate [SDS], 8 ml of 0.05% bromophenol blue, 150 ml of H_2O) and then loaded onto the second-dimension gel. Second-dimension gels were 12% continuous separating gels without SDS. ^{14}C-labeled protein standards (Sigma Chemical Co.) were run in the reference lane when appropriate. Gels were fixed, rinsed, and vacuum dried. Dried gels were allowed to expose a sheet of Kodak BioMax film for 65 h.

Csps. When cultures were shocked from 37 to 10°C, they entered a lag phase of about 2 h before growth resumed (Fig. 1). The lag phase was extended if the culture was shocked from 37 to 5°C, and the growth rate achieved upon resumption of growth was much slower. *L. monocytogenes* 10403S was cold shocked, and the proteins were labeled with [^{35}S]methionine. Proteins labeled preshock for 5 min at 37°C (Fig. 2A) provided a control by which changes in protein synthesis upon temperature downshock were evaluated. Cultures were shocked from 37 to 5°C and were labeled from 0 to 30, 30 to 60, and 60 to 120 min postshock. At least 12 proteins with apparent M_rs of 48,600, 41,000, 21,800, 21,100, 19,700, 19,200, 18,800, 18,800, 17,200, 15,500, 14,500, and 14,400 showed increased synthesis in cold-shocked cultures (Fig. 2B to D). During the shock period, synthesis of the majority of the cellular proteins was less than at 37°C. Only minor differences in labeling of the 12 Csps were observed in cells labeled at different times during the shock period. The Csp response of strain SLCC53 was also studied. The broad picture was similar to that of strain 10403S. However, strain SLCC53 lacked the 19,700- and 15,500-M_r Csps seen in strain 10403S and had additional proteins with apparent M_rs of 23,700, 22,000, and 21,400. In strain SLCC53, 13 proteins with apparent M_rs of 48,600, 41,000, 23,000, 22,000, 21,800, 21,400, 21,100, 19,200, 18,800, 18,800, 17,200, 14,500, and 14,400 showed increased synthesis in cold-shocked cells (Fig. 3).

Caps. Cells growing in defined medium at 5°C were labeled for 5 h with [^{35}S]methionine to identify Caps (Fig. 4). Four proteins with apparent M_rs of 48,000, 21,100, 19,700, and 18,800 that were identified as Csps were also Caps, exhibiting increased synthesis during balanced growth at 5°C compared with 37°C.

Screening of approximately 5,000 transposon mutants resulted in identification of 28 *L. monocytogenes* mutants with impaired ability to grow at 4°C. Two mutants (cld1 and cld2) impaired in the ability to grow at 4°C were cold shocked from 37 to 5°C and labeled for various lengths of time postshock. The mutants exhibited reduced labeling efficiency in that about twice as many mutant cells as wild-type cells were required to achieved 200,000 cpm of radiolabeled protein. The Csp response of cld2 was very similar to that of the wild-type strain 10403S in that no major Csp appeared to be missing in the mutant.

In this study, 12 proteins have been identified as Csps in *L. monocytogenes* 10403S. This number of Csps is similar to those

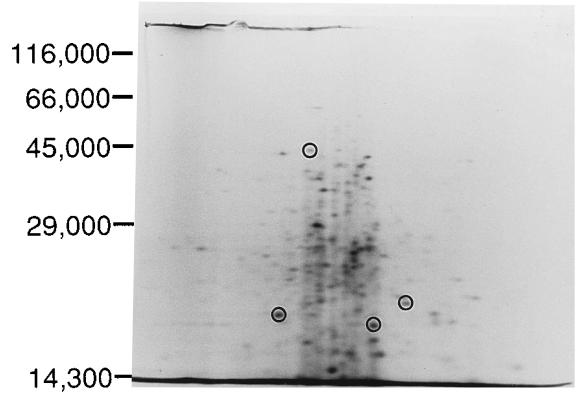

FIG. 4. Two-dimensional gel autoradiogram of *L. monocytogenes* 10403S proteins labeled during exponential growth at 5°C. Cells were labeled for 5 h with ^{35}S-TranSlabel (ICN) at a final concentration of 150 μCi ml^{-1}. Each sample was quantitated for trichloroacetic acid-precipitable radioactivity, and equivalent amounts (200,000 cpm) of trichloroacetic acid-insoluble radioactivity were loaded onto the gels. Circles indicate the positions of Caps (proteins exhibiting increased synthesis compared with non-cold-shocked controls) which were also identified as Csps. The positions of molecular weight standards are indicated.

identified in other bacteria, e.g., 13 in *E. coli* (12), 9 in *B. psychrophilus* (24), and 12 in *L. lactis* (18), although 52 Csps have been found in *B. subtilis* (15) and 40 have been found in *V. vulnificus* (16). The criteria for designation of a protein as a Csp and the means used to compare the intensities of spots corresponding to proteins in gels vary among investigators and the protein separating systems used. Little progress has been made in identification and characterization of Csps in bacteria other than *E. coli*. We view our designation of 12 Csps in *L. monocytogenes* 10403S as a conservative estimate of the total number of Csps in this organism. During the preparation of this paper, a report of the Csps of *L. monocytogenes* by Phan-Thanh and Gormon (19) appeared. Using a different strain of *L. monocytogenes*, they reported 32 proteins to be induced threefold or more upon cold shock. Proteins showing a fivefold or greater level of induction had M_rs of 94,700, 89,500, 87,000, 70,000, 68,500, 68,200, 55,300, 40,600, 37,000, 36,800, 34,800, and 17,600. While some of these proteins appear to correspond with proteins designated by us as Csps, many are different. This discrepancy may be due to the study of different strains and use of different experimental conditions.

In the present study, no obvious difference in the Csp profiles of a cold-sensitive mutant and the parent strain was observed. In contrast, a cold-sensitive mutant of the psychrotrophic bacterium *B. psychrophilus* showed no induction of two Csps produced by the parent organism. Zheng and Kathariou (25) have described transposon-induced cold-sensitive mutants of *L. monocytogenes*. Two independently isolated Tn*916* and Tn*916*ΔE mutants had a single insertion in the same 1.8-kb *Eco*RI fragment. The mutants were unaltered in a variety of phenotypic characteristics, including invasion in a cell culture model. The cloning of three genes essential for cold growth was reported (26).

We are currently using protein microsequencing to further characterize and identify prominent *L. monocytogenes* Csps.

This work was supported by award 93-37201-9560 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture and by grant 1 R15 AI35778-01 from the National Institutes of Health to B.J.W.

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