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The Cold Shock Response of the Psychrotrophic Bacterium *Pseudomonas fragi* Involves Four Low-Molecular-Mass Nucleic Acid-Binding Proteins

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The psychrotrophic bacterium *Pseudomonas fragi* was subjected to cold shocks from 30 or 20 to 5°C. The downshifts were followed by a lag phase before growth resumed at a characteristic 5°C growth rate. The analysis of protein patterns by two-dimentional gel electrophoresis revealed overexpression of 25 or 17 proteins and underexpression of 12 proteins following the 30- or 20-to-5°C shift, respectively. The two downshifts shared similar variations of synthesis of 20 proteins. The kinetic analysis distinguished the induced proteins into cold shock proteins (Csps), which were rapidly but transiently overexpressed, and cold acclimation proteins (Caps), which were more or less rapidly induced but still overexpressed several hours after the downshifts. Among the cold-induced proteins, four low-molecular-mass proteins, two of them previously characterized as Caps (CapA and CapB), and heat acclimation proteins (Haps) as well as heat shock proteins (Hsps) for the two others (TapA and TapB) displayed higher levels of induction. Partial amino acid sequences, obtained by microsequencing, were used to design primers to amplify by PCR the four genes and then determine their nucleotide sequences. A *Bam*HI-*Eco*RI restriction fragment of 1.9 kb, containing the complete coding sequence for *capB*, was cloned and sequenced. The four peptides belong to the family of small nucleic acid-binding proteins as CspA, the major *Escherichia coli* Csp. They are likely to play a major role in the adaptative response of *P. fragi* to environmental temperature changes.

Microbial responses to physical or chemical stresses are characterized by qualitative and quantitative adjustments in protein synthesis. Such phenomena, such as the heat shock response, have been extensively studied, particularly in *Escherichia coli*, the best-characterized bacterium at the physiological and molecular levels. Its heat shock response is now well known: proteins involved have been characterized, and the way they are regulated has been elucidated (52). The effects of cold shock on growth and protein synthesis have been also investigated in *E. coli*. After a downshift from 37 to 10°C, bacterial growth stops for 4 h while the relative rate of synthesis of 14 proteins increases greatly (18). These proteins, termed cold shock proteins (Csps), are completely different from previously characterized heat shock proteins (Hsps). Ten of these Csps have been identified. They are involved in various cellular processes such as transcription (NusA and CspA), translation (initiation factor 2), degradation of mRNA (polynucleotide phosphorylase), condensation of the chromosome (H-NS), and DNA superhelicity (GyrA). More recently, four new Csps have been identified. One of them, CsdA, is a ribosome-associated protein which unwinds the double-stranded RNA formed at low temperatures (21). The two others are Hsc66 (HscA) and HscB, which have high homology with the Hsp chaperones DnaK and DnaJ, respectively, and are involved in the regulation of the cold shock response (27). The last one is RbfA, another ribosome-associated factor, which enhances the translational capacity of the ribosome and so allows growth resumption (17). Among the *E. coli* Csps, the main one is CspA or CS7.4, a DNA- and RNA-binding protein induced more than 200-fold after a 37-to-10°C downshift (16). It belongs to the family of Y-box factors, whose members include such eukaryotic DNA- and RNA-binding proteins as YB-1 and FRGY2 (24). CspA belongs to a multigenic family of seven members whose genes, named *cspA* to *cspG*, have been sequenced (26, 33, 51). Among them, *cspB* and *cspG* are also cold shock inducible (26, 33), whereas the regulation of the four other genes remains to be elucidated. CspA plays a central role in the cold shock response of *E. coli*, as it activates the transcription of other cold shock genes (*hns* and *gyrA*) (20, 25). It can also selectively stimulate the translation of its own mRNA and possibly of other cold shock mRNAs (5). *cspA* analogs have been found in numerous bacteria (2, 3, 12, 30, 40, 45, 50), but the existence of a multigenic family of *cspA*-like genes has been shown only in *Bacillus cereus* (30) and *B. subtilis* (12).

Pseudomonas fragi is a psychrotrophic bacterium able to grow over a wide range of temperatures (from 2 to 35°C). This is the main surface-spoiling microorganism of refrigerated meat (7). During chilling, the temperature at the surface of the carcass decreases rapidly (35). The faster the bacterium overcomes this stress, the faster it is able to spoil meat. The growth response of *P. fragi* to a sudden downshift from 30 or 20°C to a low (5°C) temperature has been investigated. Evolution of *E. coli* H-NS and RpoS immunorelated proteins and general protein patterns (i.e., the relative synthesis of newly synthesized polypeptides and kinetics of variations) has been monitored for several hours after 30-to-5°C and 20-to-5°C cold shocks. Four classes of proteins have been distinguished according to their variation in type and kinetics. Among the variations, four peptides, previously described as CspA immunorelated (13), were the main cold-induced proteins. They have been identified by partial microsequencing, and their genes have been

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amplified by PCR. A restriction fragment of 1.9 kb, containing one of the four genes, has been cloned and sequenced. The roles and modes of action of the four peptides, homologs to CspA, are discussed in connection with the previous results observed upon heat shocks (32) and at different steady-state temperatures (13).

MATERIALS AND METHODS

Bacterial strain and medium. *P. fragi* K1 was isolated in our laboratory from minced beef. Cells were grown in M9 liquid synthetic medium (pH 7.5) containing (per liter) 6 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 3 g of glucose, 0.5 g of yeast nitrogen base, and 0.25 g of $MgSO_4$.

Cold shock experiments. Cold shock experiments were carried out in flasks where aeration and agitation were performed by injecting sterile air through sintered glass. Cells in mid-log phase (optical density of 0.5 at 600 nm) were transferred from a water bath at the preshift temperature (30 or 20°C) to a water bath maintained at 5°C. Five minutes after the transfer, the temperature in the culture medium reached 10.5 or 8.5°C, and the final shock temperature was reached in 12 or 10.5 min from the 30 or 20°C cultures, respectively. This delay time was considered the initial time for the cold shock labelings.

Radiolabeling of newly synthesized proteins. Labeling was accomplished by adding 100 μCi (3.7 MBq) of L-[^{35}S]methionine (specific activity, >37 TBq $mmol^{-1}$; ICN) to 2-ml aliquots of cells transferred from the culture flasks to tubes precooled to 5°C. Incorporation of the radioactive amino acid in newly synthesized proteins was performed during one generation time at the preshift temperature, i.e., 50 min at 30°C or 120 min at 20°C. Several sequential labelings were carried out, and their duration, established by preliminary experiments in order to cover all variations in protein synthesis, varied from 30 min to 3 h.

Sample preparation. Incorporation of L-[^{35}S]methionine was stopped by centrifuging 2-ml aliquots at 15,500 × *g* for 5 min. Pellets were resuspended in 200 μl of extraction buffer (pH 6.8) composed of 10 mM Tris HCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 25 U of RNase A per ml, and 50 U of DNase I per ml. Cells were disrupted by ultrasonic treatment, and cell walls were removed by centrifugation at 15,500 × *g* for 5 min. The incorporated radioactivity was determined by precipitating 5 μl of the cell extracts onto glass fiber filters (Whatman GF/C) with cold 25% (vol/vol) trichloroacetic acid. Radioactivity was measured with a liquid scintillation counter (Packard) by using a scintillation cocktail (BCS; Amersham).

2-DE. Radiolabeled proteins were separated by two-dimensional electrophoresis (2-DE) according to the method of O'Farrell (36), with the modifications described previously (13). Equal quantities of radioactivity (10^6 cpm) for each labeling experiment were first subjected to isoelectric focusing in 150- by 1.5-mm gels containing 5% carrier ampholytes (LKB) (2 parts pH 5 to 7, 3 parts pH 3 to 10). The second dimension was a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli (23). The slab gels were composed of a 12.5% polyacrylamide resolving gel overlaid with a 5% polyacrylamide stacking gel. After migration, gels were fixed with 10% (vol/vol) acetic acid for 1 h, and autoradiograms were obtained by exposing dried gels to Amersham X-ray Hyperfilm-MP. Proteins, whose relative rate of synthesis varied at any given time, were designated with an alphanumeric code according to their locations in the pH gradient and their estimated molecular masses as previously described (13).

Western blot analysis. Immunochemical analyses were performed with *P. fragi* K1 cell extracts separated by SDS-PAGE. The proteins were transferred from the gels to BA-S 85 nitrocellulose membranes (Schleicher & Schuell) in a semi-dry electrophoretic transfer apparatus (X-Blot; Cera-Labo). Transfer was done for 1 h at 0.8 mA/cm^2 in 48 mM Tris HCl (pH 8.3)–39 mM glycine–0.1% (wt/vol) SDS–and 20% (vol/vol) isopropanol. Membranes were pretreated overnight at 4°C with 1% (wt/vol) bovine serum albumin in Tris-buffered saline (50 mM Tris HCl [pH 7.4], 150 mM NaCl). Blots were washed three times with 0.05% (vol/vol) Tween 20 in Tris-buffered saline, incubated for 2 h at room temperature with a polyclonal antibody, and then treated with goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase. Antigen-antibody associations were revealed with 4-chloro-1-naphthol as the substrate.

Antisera against *E. coli* Csp H-NS and *E. coli* sigma factor RpoS were tested on *P. fragi* cell extracts recovered before and at various times after a 30-to-5°C cold shock. A positive control for the H-NS antiserum was simultaneously carried out with total protein extracts of *E. coli* MC4100 (*hns*$^+$) recovered 4 h after a cold shock from 37 to 10°C.

N-terminal and internal amino acid sequencing. After 2-DE of total protein extracts from cells subjected to a 30-to-5°C 2-h cold shock, proteins were electrotransferred onto a ProBlott membrane (Applied Biosystems) with 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid [pH 11])–10% (vol/vol) methanol as the transfer buffer. Proteins were visualized by staining with 0.1% (wt/vol) Coomassie blue R-250 in 50% (vol/vol) methanol and destaining with several changes of 50% (vol/vol) methanol–10% (vol/vol) acetic acid. Each blot was extensively rinsed with distilled water and air dried, and the spots, corresponding to the four peptides, were cut off and stored at −20°C. The N-terminus sequence of each protein was tentatively determined by placing individual spots in the cartridge of a pulsed-liquid gas-phase sequencer (Applied Biosystems) with on-line analysis of phenylthiohydantoin derivatives. For peptides with blocked N termini, an in situ enzymatic digestion was carried out with endoprotease Lys-C. The resulting fragments were separated on a Nucleosil C_{18} column (150 by 46 mm) by a linear gradient from 0.1% (vol/vol) trifluoroacetic acid to 60% of an 80% (vol/vol) acetonitrile–0.096% (vol/vol) trifluoroacetic acid solution. The gradient spread out during 40 min at a flow rate of 1 ml/mn. The absorbance of the eluate was monitored at 214 nm. Some of the peptides were submitted to microsequence analysis.

DNA extraction. *P. fragi* cells were recovered in late exponential phase from a culture carried out at 30°C. Total genomic DNA was extracted according to the procedure of Brenner et al. (6).

PCR amplification. Chromosomal DNA amplifications of the *P. fragi* K1 genes encoding for the four highly cold induced peptides were performed with two combinations of primers. The primer sequences were designed from the partial amino acid sequences of the four *P. fragi* peptides, the known nucleotide sequences, and codon usage of the *E. coli cspA* family genes. The upstream primers were either the 25-mer PFW25 5′-GGTTCAACGAYGARAAAGGYTTCG G-3′ or the 20-mer PFMS20 5′-ATGTCYCARCGYCARTCYGG-3′; the downstream primer was the 23-mer PFG23 5′-TCWGCTTGCATGCCTTTTTGRC C-3′ (where R = A or G, W = A or T, and Y = C or T). The amplification reactions (100 μl) were performed with *Taq* DNA polymerase (Appligene) in a PTC-100 thermocycler (MJ Research Incorporation). The amplification reactions consist of 25 cycles of denaturation (1 min at 95°C), primer annealing (1 min at 50 or 55°C), and primer extension (1 min at 72°C). A step of elongation (5 min at 72°C) was carried out after the last cycle to allow the polymerase to add a deoxyadenosine at the 3′ end of PCR products in order to clone them in the 3′-protruding dT ends of the pCRII cloning vector (InVitrogen). Then 10-μl aliquots of amplification mixtures were separated on a 2.5% agarose gel run in TAE buffer (40 mM Tris HCl, 40 mM acetic acid, 1 mM EDTA [pH 8.0]) at 10 V cm^{-1}. The bands were visualized under a 260-nm UV source after a 30-min incubation of gels in an ethidium bromide solution (0.5 μg ml^{-1}).

Southern blot hybridization. Genomic DNA was digested with different restriction enzymes, and DNA fragments were separated on a 0.8% agarose gel at 2 V cm^{-1}. They were transferred by capillarity onto a nylon membrane (Sigma) with a 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then fixed by a 30-s exposure to UV. The membrane was prehybridized in 5× SSC with 0.1% (wt/vol) *N*-lauroylsarcosine sodium salt–0.02% (wt/vol) SDS–1% (wt/vol) blocking reagent (Boehringer Mannheim) for 1 h at 55°C. The *capB* PCR-amplified partial gene was 5′-end labeled with [γ-^{32}P]ATP (specific activity, 167 TBq mol^{-1}; ICN) with T4 polynucleotide kinase as instructed by the supplier (Promega). Hybridization was carried out with *capB* for 12 h at 55°C. The Southern blot was washed at the hybridization temperature in 2× SSC–0.1% (wt/vol) SDS for twice for 15 min each time and then for 10 min each in 1× SSC–0.1% (wt/vol) SDS and 0.1× SSC–0.1% (wt/vol) SDS. Autoradiography was obtained by exposing the membrane to Kodak X-Omat film.

DNA cloning and sequencing. DNA fragments and amplified DNAs were extracted from agarose gels with the Prep-A-Gene kit (Bio-Rad) as recommended by the manufacturer. DNA fragments were ligated into the pUC18 vector linearized previously with suitable endonucleases. PCR products were ligated into the pCRII cloning vector (InVitrogen) as recommended by the supplier. The plasmids were used to transform competent cells of *E. coli* JM109 by standard procedures (42). Colonies harboring recombinant plasmids were selected by blue-white screening, and plasmids were purified by minipreparations (plasmid kit from Qiagen, Hilden, Germany) through alkaline lysis of the host strains. To identify plasmids containing the DNA fragment of interest, recombinant pUC18 DNAs were transferred to a nylon membrane and screened by dot blotting using the hybridization and wash conditions described above.

DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (43), with a Sequenase kit (version 2.0; United States Biochemicals) and either universal −40 or reverse primers, or by primer walking.

Nucleotide sequence accession number. The nucleotide sequences reported in this work have been assigned the following GenBank database accession numbers: U62985 for *capA*, U62986 for *capB*, U62987 for *tapA*, and U62988 for *tapB*.

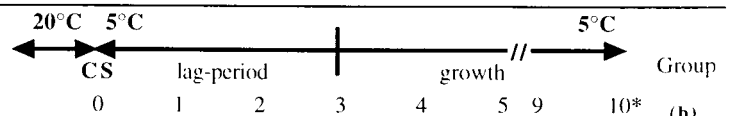
RESULTS

Effect of temperature downshifts on *P. fragi* growth. When mid-exponential-phase cultures of *P. fragi* K1 were submitted to cold shocks from 20 or 30 to 5°C, growth ceased immediately for 3 or 5 h, respectively. After the lag period, growth resumed with the generation time usually observed in steady-state conditions at 5°C (i.e., about 7 h).

Effects of temperature downshifts on protein patterns. Sequential labelings were carried out to monitor the evolution of gene expression after the cold shocks. Tables 1 and 2 compile the variations observed on 2-DE protein patterns after the downshifts in temperature from 20°C (Fig. 1) and 30°C (Fig. 2)

TABLE 1. Proteins whose relative rates of synthesis varied following cold shock from 20 to 5°C.

Protein number (a)	Estimated coordinates	pI	Group (b)
1	A17.8	4.93	IIb
2	B31.0	5.15	IIa
3	B47.0	5.17	IIa
4	C7.0	5.93	IIb
5	C8.0	5.86	IIb
6	C13.0	5.46	Ib
7	C15.9	5.92	IVb
8	C16.0	5.70	IVa
9	C17.0	5.83	Ib
10	C18.0	5.79	Ia
11	C19.8	5.92	Ib
12	C20.0b	5.74	IVa
13	C20.3	5.68	IVa
14	C26.0	5.77	IVa
15	C27.0	5.73	III
16	C28.5	5.71	IVa
17	C28.7	5.70	IVa
18	C35.0	5.80	IVb
19	C39.0	5.52	IVa
20	C39.5	5.71	IVb
21	D19.2	6.03	Ia
22	D19.8	6.04	IVc
23	D24.5	6.08	IIb
24	D30.2	6.09	Ib
25	E7.0	6.63	Ia
26	E8.0	6.50	Ia
27	E24.5	6.74	III
28	E26.5	6.80	IVa
29	E31.0	6.65	Ib



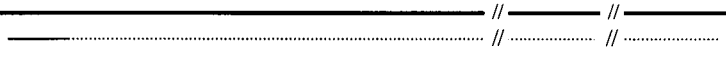
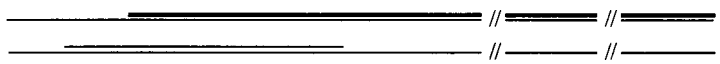
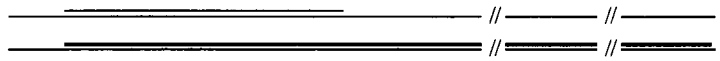
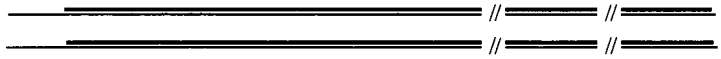
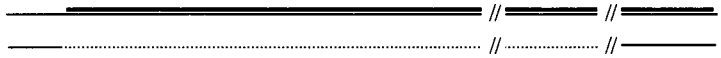
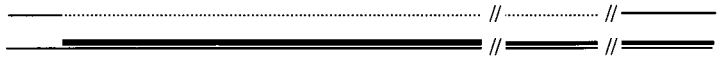
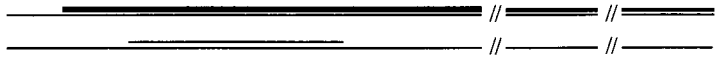
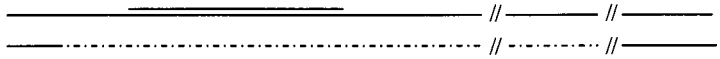
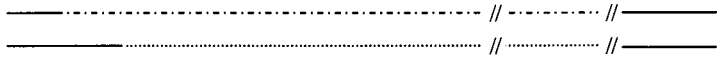
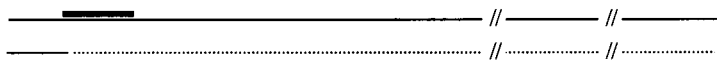
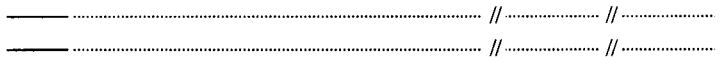
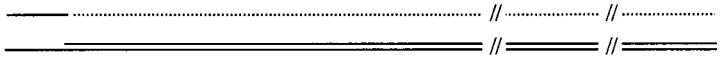
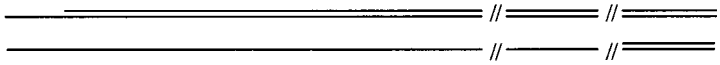
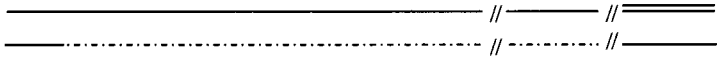
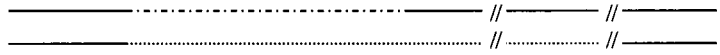
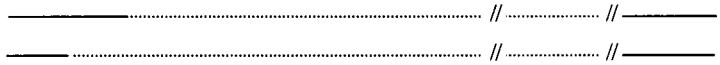
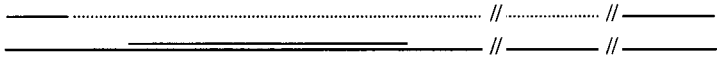
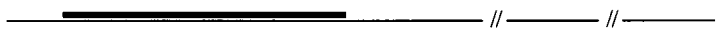
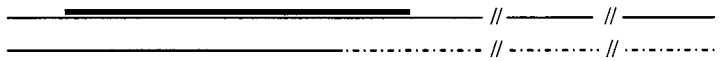
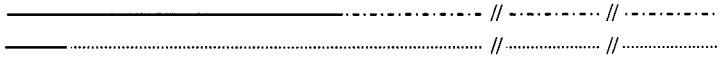
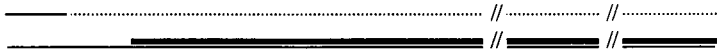
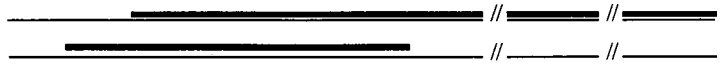
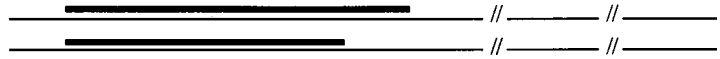
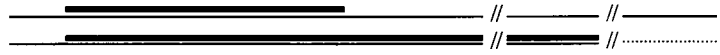
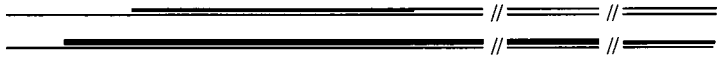
low (═══), moderate (═══) and high (═══) over-expression of the protein compared to their relative level (——) before the downshift ; moderate (·-·-·-) or pronounced (·····) under-expression .
(a) : numbers refer to the spots on fig. 1. **(b)** : see Results for the meaning of the different groups.
* : time (h) after the cold shock.

to 5°C, respectively. The relative level of synthesis of each protein at the preshift temperature (either 20°C [Table 1] or 30°C [Table 2]) was used as reference to estimate the postshift variations.

Whatever the cold shock applied, gene expression seemed not to be affected in the aggregate. Indeed, among a mean of about 500 detectable spots on autoradiograms, less than 8% showed variations in intensity after the stress. Adjustments of protein synthesis occurred in 29 for the 15°C and 37 polypeptides for the 25°C downshifts. In both downshifts, 12 proteins were underexpressed, while there were 25 overexpressed proteins with the larger downshift and only 17 with the smaller downshift. Twenty proteins showed common variations in both cold shocks: B31.0, B47.0, C7.0, C8.0, C17.0, D19.2, D24.5, D30.2, E7.0, E8.0, E24.5, and E31.0 were overexpressed, whereas C15.9, C16.0, C20.0b, C20.3, C28.5, C35.0, C39.0, and D19.8 were underexpressed. However, the levels and kinetics of their repression or induction could be different.

The majority of the variations occurred during the first hour following the downshifts, i.e., 15 of 29 and 24 of 37 for the 20- and 30-to-5°C shocks, respectively. This was particularly the case for the repressed proteins because the synthesis of 9 of 12 and 8 of 12 had already decreased by the first labeling after the cold shocks from 20 and 30°C, respectively. In the same way, 16 of the 25 overexpressed proteins after the largest shift were noticed in the first hour postshift, compared with only 6 of 17 for the smallest downshift. In the later case, the majority (9 of 17) of the positive variations took place preferentially during the second or third hour postshock. It was also interesting that the relative rate of synthesis of a few polypeptides increased in

TABLE 2. Proteins whose relative rates of synthesis varied following cold shock from 30 to 5°C.

Protein number (a)	Estimated coord.	pI	30°C 5°C CS lag-period // growth // 5°C 0 1 2 3 4 5 6 9 10 20 21*	Group (b)
1	A26.5	4.95		IVa
2	B7.9	5.05		IIb
3	B21.8	5.31		Ia
4	B31.0	5.15		IIa
5	B47.0	5.27		IIa
6	B74.0	5.33		IVb
7	C7.0	5.93		IIa
8	C8.0	5.86		IIa
9	C15.0	5.77		Ib
10	C15.9	5.92		IVb
11	C16.0	5.70		IVb
12	C17.0	5.83		Ib
13	C19.0	5.48		Ia
14	C20.0a	5.63		Ia
15	C20.0b	5.74		IVa
16	C20.3	5.68		IVa
17	C23.0	5.97		IIa
18	C25.0	5.80		III
19	C28.5	5.71		IVb
20	C35.0	5.80		IVb
21	C39.0	5.52		IVb
22	C55.0	5.55		IVb
23	D7.2	6.07		Ib
24	D8.0	6.02		Ib
25	D16.8	6.10		IIb
26	D18.0	6.48		Ia
27	D19.2	6.03		Ia
28	D19.8	6.04		IVc
29	D20.0	6.04		IVa
30	D24.5	6.08		IIb
31	D30.2	6.09		Ia
32	D34.8	6.46		Ia
33	E7.0	6.63		Ia
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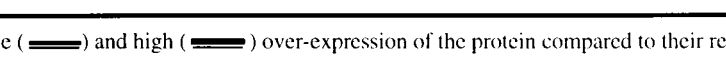
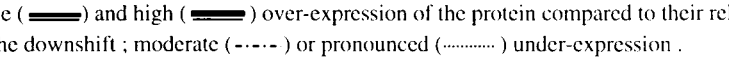
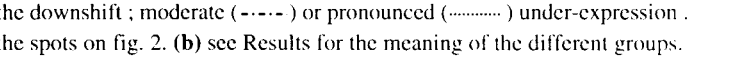
35	E24.5	6.74		IIb
36	E31.0	6.65		IIa
37	E42.0	6.67		Ia

low (═══), moderate (═══) and high (═══) over-expression of the protein compared to their relative level (———) before the downshift ; moderate (- - - -) or pronounced (.....) under-expression .

(a) numbers refer to the spots on fig. 2. **(b)** see Results for the meaning of the different groups.

* : time (h) after the cold shock.

the last hour of the lag phase. Finally, the increased expression of a minority of proteins was detected only in the last labeling.

The variable levels of proteins indicated that their overexpression or repression was either transient or maintained until the end of the experiment. The middle of the lag phase corresponded to the maximum intensity of variations for most of the proteins. We have distinguished five groups of proteins according to their level of expression and kinetics of variation (Table 1 and 2).

Group I included the temporarily overexpressed proteins (i.e., 9 and 14 polypeptides for the cold shocks from 20 and 30°C, respectively). This group was subdivided into subgroups

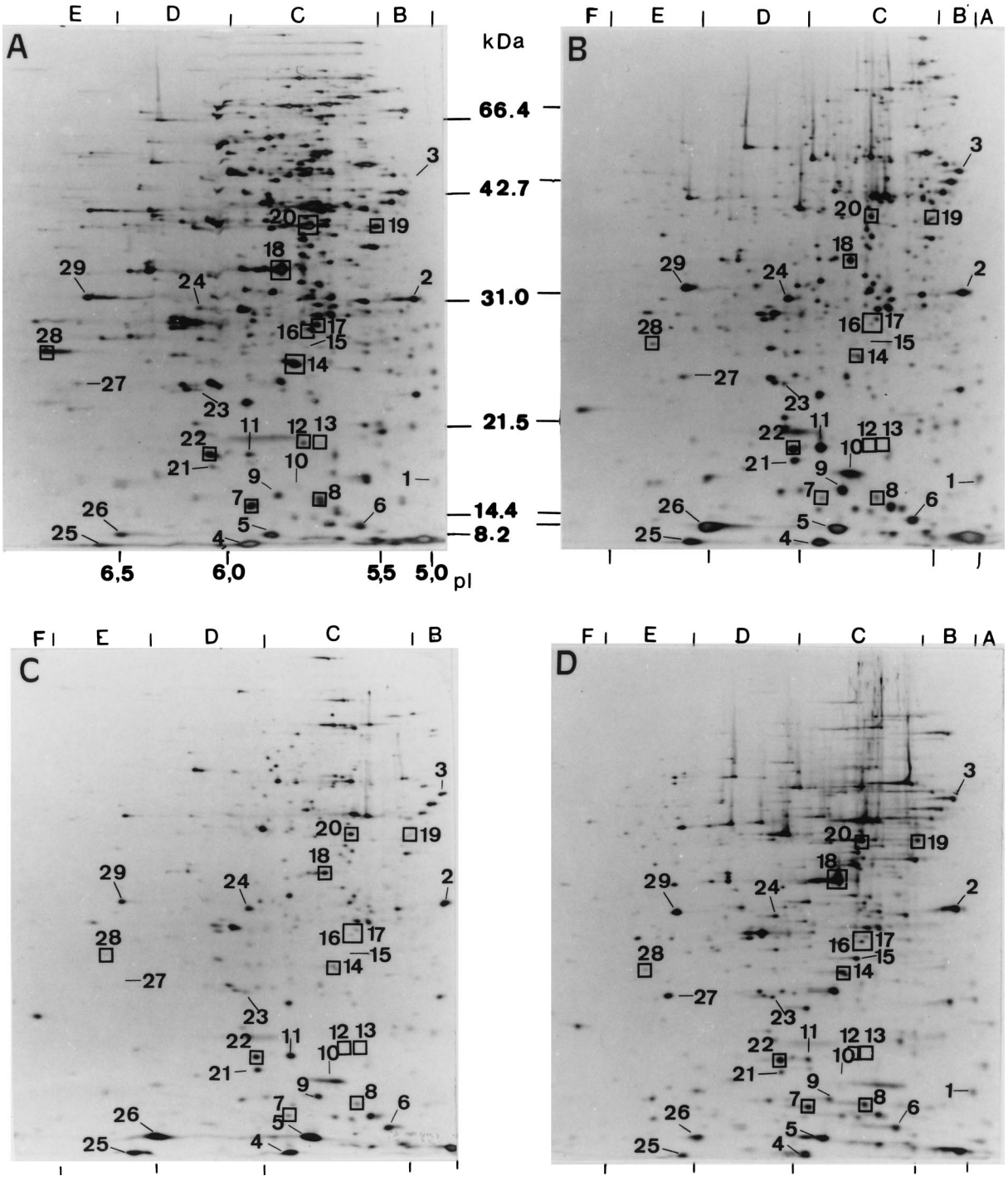


FIG. 1. 2-DE of *P. fragi* newly synthesized proteins labeled with L-[^{35}S]methionine. Labelings were carried out during one generation time at 20°C (A) and then from 2 to 3 h (B), 3 to 4 h (C), and 9 to 10 h (D) following the downshift to 5°C. —, overexpressed proteins; □, underexpressed proteins.

Ia and Ib according to the beginning of the overexpression. Proteins of group Ia were induced immediately after the shift, whereas the synthesis of proteins of group Ib increased 1 h after the shift (C15.0 and D8.0) or later during the lag period, such as was the case for C13.0 and C17.0 in the 20-to-5°C downshift. Group II contained proteins whose relative rate of synthesis was still high at the end of the experiments. Six and ten proteins belonged to this group for the 20- and 30-to-5°C shifts, respectively. As for group I, we have differentiated two subgroups of polypeptides in regard to the beginning of the induction. Members of group IIa were immediately overproduced, whereas those of subset IIb were induced a few hours after the shift. Group III comprised the few proteins whose relative rate of synthesis increased 9 h (C27.0 and E24.5) or 20 h (C25.0) after the downshift from 20 or 30°C. The fourth group contained the proteins which were repressed after the

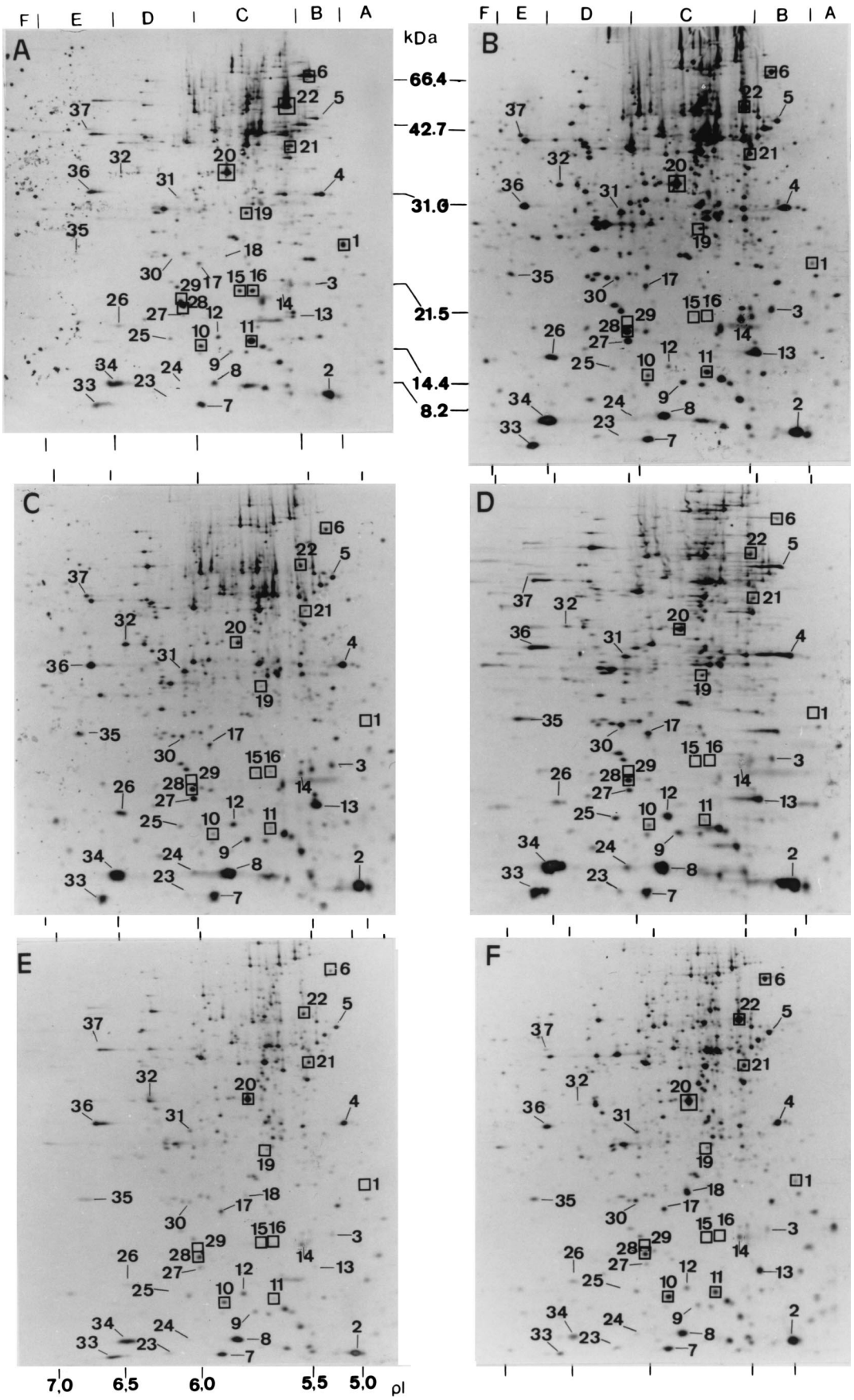


FIG. 2. 2-DE of *P. fragi* newly synthesized proteins labeled with L-[^{35}S]methionine. Labelings were carried out during one generation time at 30°C (A) and then from 0 to 1 h (B), 1 to 4 h (C), 5 to 6 h (D), 9 to 10 h (E), and 20 to 21 h (F) following the downshift to 5°C. —, overexpressed proteins; □, underexpressed proteins.

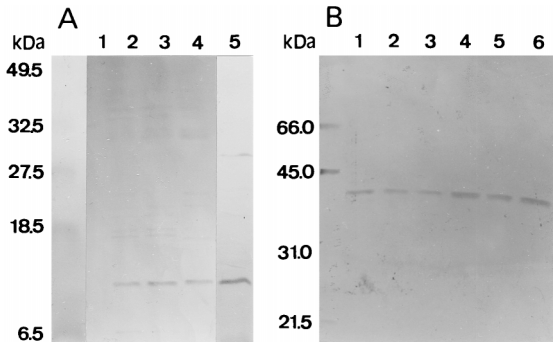


FIG. 3. (A) Western blot analysis after SDS-PAGE of protein extracts from *P. fragi* and *E. coli* with antiserum raised against *E. coli* H-NS. The *P. fragi* extracts were recovered at 30°C (lane 1) and 1 h (lane 2), 4 h (lane 3), and 17 h (lane 4) after a downshift to 5°C. *E. coli hns*$^+$ extracts (5) were obtained from a culture submitted to a 4-h-long transfer from 37 to 10°C. Molecular masses are indicated on the left. (B) Western analysis performed on *P. fragi* cell extracts, using *E. coli* anti-RpoS serum. Proteins extracts were recovered from *P. fragi* grown at 30°C (lane 1) and transferred at 5°C for 1 h (lane 2), 3 h (lane 3), 5 h (lane 4), 9 h (lane 5), and 20 h (lane 6). Molecular masses are indicated on the left.

downshifts. Three subgroups were discerned: group IVa contained the polypeptides repressed immediately after the shift and during the 9 or 20 h of the experiments (e.g., A26.5, C20.0b, and C20.3); group IVb consisted of transiently underexpressed proteins such as C15.9, C35.0; and group IVc included only D19.8, the synthesis of which decreased when bacterial growth resumed.

Immunochemical analysis. Total protein extracts of *P. fragi* were recovered from cultures grown at 30°C and at different times after their transfer to 5°C. Protein samples were separated by SDS-PAGE and submitted to Western blot detection with either the *E. coli* anti-H-NS serum (Fig. 3A) or the *E. coli* anti-RpoS serum (Fig. 3B). A 16-kDa protein band was recognized in some *P. fragi* extracts by the antiserum raised against the *E. coli* H-NS Csp. This protein, not detected at 30°C (lane 1), was induced during the first hour postshift (lane 2). Its level of expression remained almost constant several hours after the 25°C downshift (lane 3 and 4). The *P. fragi* H-NS-like protein was similar in molecular mass to the *E. coli* H-NS (15.6 kDa [lane 5]).

The antiserum raised against the *E. coli* RpoS stationary-phase sigma factor recognized a protein band with a molecular mass estimated at 37 kDa (Fig. 3B). The relative expression of this protein decreased slightly in the first few hours postshift, but later, at 5 h, it increased to reach a level higher than that before the downshift.

Unfortunately, it was not possible to link accurately the H-NS- and RpoS-immunorelated proteins with spots on 2-DE autoradiograms.

Microsequencing of four low-molecular-mass Csps. The four low-molecular-mass proteins C7.0, C8.0, E7.0, and E8.0 showed the highest levels of induction among all of the cold-induced proteins. We attempted to determine their amino acid sequences from their 2-DE spots blotted onto a polyvinylidene difluoride membrane. The N-terminal sequences of E7.0 (TapA) and E8.0 (TapB) were then obtained (Table 3), while C7.0 and C8.0 were blocked. So, after an in situ enzymatic digestion with the endoprotease Lys-C and purification of the resulting peptides by high-pressure liquid chromatography, we determined the amino acid sequences of some of the peptides from C7.0 (CapA) and C8.0 (CapB) (Table 3). The partial sequences of the four small proteins of *P. fragi* showed high homologies with CspA, the main *E. coli* Csp.

PCR amplifications of the genes encoding CapA, CapB, TapA, and TapB. PCR experiments, carried out at 55°C with PFW25 and PFG23 as primers, amplified a main product of about 160 bp and a minor one of about 550 bp. Amplified fragments were cloned in the pCRII cloning vector and sequenced. The 160-bp fragment corresponded to a mixture of the three coding sequences for CapA, CapB, and TapA. The 550-bp PCR product, limited by PFW25 and PFG23 at the 5′ and 3′ ends, respectively, contained the *tapA* gene. The sequence upstream of the *tapA* gene showed no homology with any sequence from databases. A 194-bp product was amplified at 60°C by using PFMS20 and PFG23 as primers. The cloned fragments encoded either CapA or TapB. The nucleotide sequences of the PCR products are compiled in Fig. 4A. Comparison of the four *P. fragi* amino acid sequences, deduced from PCR products and peptide microsequencing, with the sequence of *E. coli* CspA showed identities of about 60% (Fig. 4B). Sequence identities between the four *P. fragi* peptides were 77 to 83% except for TapA-TapB (97%) and CapA-CapB (88%). The two nucleic acid-binding motifs RNP-1 and RNP-2 of this family of proteins, corresponding to the octapeptide KGFGFITP and to the pentapeptide VFVHF, respectively, in *E. coli* CspA, are also conserved in the *P. fragi* proteins. There is only one conservative substitution, V/L, in the RNP-2 motif of the *P. fragi* peptides.

Cloning and sequencing of genomic DNA. The *capB* PCR-amplified gene was used in Southern blot experiments carried out with *P. fragi* genomic DNA digested by several restriction enzymes. Three main fragments, a 5.1-kb *Bam*HI-*Hin*dIII fragment and two *Eco*RI fragments of 3.2 and 2.5 kb, were recognized by the *capB* probe and cloned in the pUC18 cloning vector. The recombinant plasmids from 400 transformants were screened in dot blot experiments using the same *capB* probe. The positive clones contained either the 5.1-kb *Bam*HI-*Hin*dIII or the 2.5-kb *Eco*RI insert, one but any of them contained the 3.2-kb *Eco*RI fragment. Southern blotting carried out with restriction fragments of each clone led to subcloning of a 1.9-kb *Bam*HI-*Eco*RI digest from a 5.1-kb *Bam*HI-*Hin*dIII fragment and a 0.75-kb *Sph*I-*Eco*RI fragment from a 2.5-kb *Eco*RI insert (Fig. 5A). Their sequences revealed that the 0.75-kb *Sph*I-*Eco*RI fragment was within the 1.9-kb *Bam*HI-*Eco*RI fragment. The inserts contained the *capB* gene (Fig. 5B), and no other coding sequence for a cold shock gene was identified in the downstream region. *capB* had a GC con-

TABLE 3. N-terminal (E7.0 and E8.0) and internal (C7.0 and C8.0) amino acid sequences of the four main cold-induced proteins

Protein	Amino acid sequence[a]
C7.0 (CapA)	WFND; GFGFITPQGG; AIESDGF; EGQTVS; GMQAAQV
C8.0 (CapB)	WFND; GFGFITPQSG; AIQSDGF; EGQQVSFIATRGQ; GMQAEEVQ
E7.0 (TapA)	XNRQTGTVKWFNDEKGFGF
E8.0 (TapB)	SQRQSGTVKWFNDEKGFGFITPESGXDLF

[a] X, undetermined amino acid.

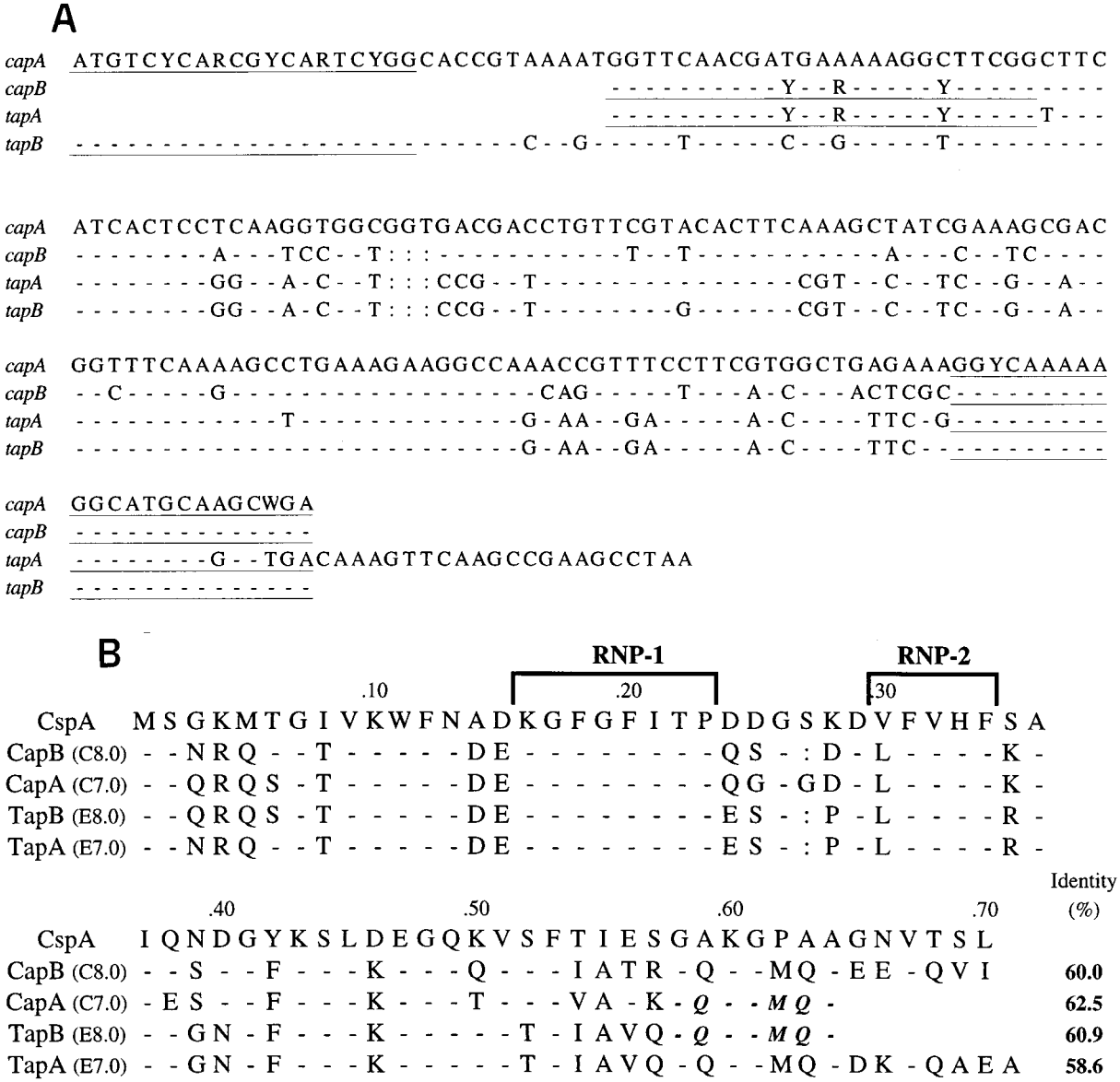


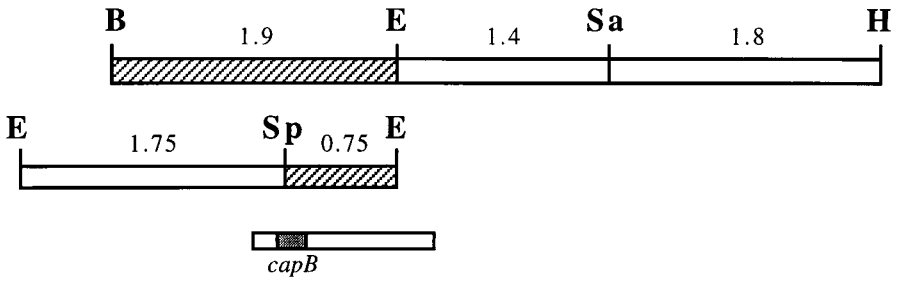
FIG. 4. (A) Nucleotide sequences of PCR-amplified fragments cloned in the pCRII cloning vector. Primers are underlined, and only different residues are indicated. Colons indicate missing nucleotides. The percentage of identity of each gene with *cspA* is given at the end of each sequence. R = A or G, Y = C or T, W = A or T. (B) Comparison of amino acid sequences, deduced from the PCR-amplified fragments and from peptide microsequencing, with the sequence of CspA protein of *E. coli*. The amino acids of the C-terminal sequences of CapA and TapB (italicized) correspond to the deduced sequences from the primers. Dashes indicate identical residues; colons indicate missing residues.

tent of 47%, and its sequence showed identities of 63% with the *E. coli cspA* gene sequence and 83, 77, and 74% with the partial sequences of *P. fragi capA*, *tapA*, and *tapB*, respectively.

DISCUSSION

A rapid change from high to low temperature, applied to mid-exponential-phase cells of *P. fragi*, induced a lag period whose duration increased with the magnitude of the shift. After this lag phase, growth resumed with a characteristic 5°C growth rate. Such results have been observed with the mesophile *E. coli* (18), the psychrotrophic yeast *Trichosporon pullulans* (22), and *Arthrobacter globiformis* (3), *Listeria monocytogenes*, and *L. innocua* (38). On the contrary, other microorganisms such as the psychrotroph *Vibrio* sp. (1) and the mesophile *Lactococcus lactis* subsp. *lactis* (37) or *B. subtilis* (29) had no lag phase consequent to a cold treatment. Their growth continued at an intermediate rate followed by a growth rate characteristic of final low temperature. Thus, the time to readapt to the low temperature is not directly dependent on the temperature range of growth of the bacterium, i.e., of its psychrophilic, psychrotrophic, or mesophilic character. In the same way, this criterion of classification according to temperature range of growth fails to predict the consequences of cold shocks on protein synthesis. Indeed, after cold shock treatments, the relative rate of synthesis of most cellular proteins was maintained in *P. fragi*, as for other psychrotrophs such as *B. psychrophilus* (49) or *A. globiformis* (3), but also for mesophiles such as *B. subtilis* (29). On the contrary, the mesophile *E. coli* (18), but also the psychrotrophs *L. monocytogenes* and

A



B

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                                                CTCCAGCGTCACGTGGCCTTCCCACTCTGGCTCAAGCGGTGTCAC  45
GTTAACGATGATGCCGCAACGCGCGTAGGTGCTTTTACCCAGGCAGATGGTCAGCACGTT  105
GCGTGGAATACGGAAATATTCAACGGTGCGCGCCAGGGCGAAGGAGTTGGGCGGGATGAT  165
GCACACATCGCTGGTGACATCGACAAAGCTGCCTTCATCGAAGTTTTTCGGATCAACGGT  225
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GCTCGACACGCCGTAAGAAATCAGACGATCAGCCCCTTCGCCGCGTACCTGACGCTCAAC  345
GAAAGGTTCGATCATGCCGTGCTCTTGCGCCATGCGGCGAATCCACTTGTCTGATTTGAT  405
GCTCATGGCGGGGGTGTCCTGAATAGCGAGGTGGAAAAATTCTGTTCGGCATCTTACCGG  465
GGCCGGGGTTTCGGTTCAAAGGCTGGGGGGAAATTCTTGAAAATAGCTGTAGGCGCACTG  525
AATACAGGCTGTACGGGCAACCATCAGTCACGTTTTTCGAGAAACCTTCGCAAAGACCCT  585
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CTCTACACGATATGTTGAATTTCGATCCAACCATCTCCAAGAATTTTTCCTGCTCTTTGC  705
ACTCAGTCTCGGCCAGGGCTCTTCCTGCGTCGCAGTTATCTTTGTTCAAGGAGTTACACC  765
ATGTCTAATCGCCAAACTGGTACCGTTAAGTGGTTCAACGATGAAAAAGGCTTCGGCTTC  825
 M  S  N  R  Q  T  G  T  V  K  W  F  N  D  E  K  G  F  G  F   20
ATCACTCCACAATCCGGTGACGACCTGTTTGTTCACTTCAAAGCAATCCAATCCGACGGC  885
 I  T  P  Q  S  G  D  D  L  F  V  H  F  K  A  I  Q  S  D  G   40
TTCAAGAGCCTGAAAGAAGGCCAACAGGTTTCTTTCATCGCTACTCGCGGTCAGAAAGGC  945
 F  K  S  L  K  E  G  Q  Q  V  S  F  I  A  T  R  G  Q  K  G   60
ATGCAAGCTGAGGAAGTTCAAGTTATCTAACTTGTACTGATCCAGTAAAAAACCCCGCCC 1005
 M  Q  A  E  E  V  Q  V  I  *                                  69
TTAAAAGCGGGGTTTTTTTATGCCTGTCGTTTAAAGACCTTGTAGGAGCGAGCTTGCCTC 1065
GCGATCTTTTGGTCTTTAAAAAGATCGCGCGACAATCTCGCTCCTACAGGGGTTTACGGG 1125
GTATCAGTCGTCGCT                                               1140
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FIG. 5. (A) Restriction map of the 5.1-kb *Bam*HI-*Hind*III and 2.5-kb *Eco*RI DNA fragments and the 1.14-kb sequenced fragment with the region encoding *capB*. The 1.9-kb *Bam*HI-*Eco*RI and 0.75-kb *Sph*I-*Eco*RI restriction fragments, recognized by the *capB* probe and subcloned in pUC18, are hatched. Abbreviations: B, *Bam*-HI; E, *Eco*RI; H, *Hind*III; Sa, *Sal*I; Sp, *Sph*I. (B) Nucleotide sequence of the *P. fragi capB* gene including flanking 5′ and 3′ regions. The sequence reported begins 252 bp downstream the *Eco*RI site (in bold) and ends 168 bp upstream the *capB* coding sequence. The putative Shine-Dalgarno site is underlined, and inverted repeats (putative ρ-independent terminator sequences) are underlined with arrows. Numbers on the right give the nucleotide position (in italics) and the corresponding location of the deduced amino-acid (in bold).

L. innocua (38), have a dramatic decrease in protein synthesis immediately after a downshift in temperature. Nevertheless, all of these bacteria responded to this stress by the overexpression of a subset of proteins called Csps. In general, their number and the magnitude of their variation increase with the magnitude of the shift. In *P. fragi*, among about 500 spots detectable by 2-DE, there was a modified level of synthesis of 37 and 29 proteins after the transfers from 30 and 20 to 5°C, respectively. Among these variations, 25 and 17, respectively, involved Csps, while the 12 other variations observed for each of the two cold shocks involved underexpressed proteins. Twenty proteins varied in the same way upon both downshifts, but the variations

were generally amplified in the greater shift. Indeed, the variations were more important (C16.0 and D24.5) and/or happened earlier (C7.0 and C8.0) and/or lasted longer (C7.0, C8.0, C17.0, D24.5, D30.2, E7.0, E8.0, and E31.0) in the 30-to-5°C cold shock. This relationship between the downshift amplitude and protein variations has been observed for other bacteria (1, 3, 18, 31, 37) and the psychrotrophic yeast *T. pullullans* (22). This finding indicates that to cope with cold shock, microorganisms regulate protein expression and particularly a subset of polypeptides which probably play an essential role in the adaptation to low temperature.

In this study, we monitored the kinetics of protein variations during the first hours following the downshifts in temperature, i.e., during the lag phase, but also long after growth resumption. This analysis led us to classify the proteins in four groups according to their kinetics and ways of variations and to derive at least two classes of proteins involved in cold adaptation, the well-known Csps and the cold acclimation proteins (Caps). The Csps included polypeptides whose relative level of synthesis increased transiently upon a temperature downshift, whereas the Caps had optimal expression during balanced growth at low temperature (13). Among the characterized proteins from the 20- and 30-to-5°C downshifts, 9 and 14, respectively, belonged to group I of the Csps. These Csps were subdivided into early Csps (subset Ia), overexpressed immediately after the shift, and late Csps (subset Ib), overproduced only 1 or 2 h postshift. Most Csps were optimally induced during the lag phase, and their synthesis decreased with growth resumption. Csps are undoubtedly involved in the adaptation of cells to the new cold environment. As demonstrated for *E. coli*, the adaptative response is greatly involved in translation. Indeed, among the *E. coli* Csps are the initiation factors 2α and 2β (18) and also two ribosome-associated proteins, CsdA (21) and RbfA (17), recently identified.

Some of the *P. fragi* Csps could also be considered Caps (subgroup IIa). Indeed, as Csps, they were rapidly induced, but their relative rate of expression remained high for a number of hours, i.e., when growth had resumed for at least one generation time. Among this subgroup of proteins, spots C7.0, C8.0, and C23.0 have been previously characterized by their continuous overexpression in cells adapted to low temperatures (13). It was also the case for the Caps A17.8 and D16.8 of subgroup IIb, whose increased synthesis took place later during the lag phase. The distinction between these two subgroups was not always obvious and sometimes depended on the amplitude of the downshifts (e.g., spots C7.0 and C8.0). However, the characteristic common to proteins of group II was their higher level of synthesis at 5°C than at the preshift temperature, even several hours after growth resumption. These proteins therefore may be involved in cellular processes allowing continuous growth in a cold environment. Some polypeptides could operate in maintaining metabolic pathways or membrane fluidity at low temperatures. Others could be involved in cold-specific proteolysis of denaturated proteins as described for *A. globiformis* (39). Moreover, it is tempting to suggest that the rapidly induced proteins of subgroup IIa could be involved in similar adaptative mechanisms as Csps. The variabilities in the kinetics and levels of induction of some Csps and Caps according to the cold shock amplitude indicate common targets for these two groups of proteins. Such overlapping of the conditions of expression of some Caps which are rapidly and strongly overexpressed upon cold shocks, such as Csps, have been described for other microorganisms, both psychrophiles (1, 41) and psychrotrophs (3, 49). In *B. psychrophilus*, for example, eight of the nine Csps overexpressed in response to a shift from 20 to 0°C were major proteins in cells grown at 0°C (49). These proteins were produced at lower levels during continuous growth at 0°C compared to their maximum level of synthesis reached after a downshift to 0°C.

The 16-kDa protein of *P. fragi*, immunorelated to the H-NS histone-like Csp of *E. coli*, behaves like a Cap of group IIa. In *E. coli*, H-NS represses the transcription of genes belonging to different operons, including its own gene. H-NS prevents the transcription of rRNA by binding to the *rrn* promoter (47). It also regulates negatively *lysU*, which codes for lysyl-tRNA synthetase (14). The growth temperature is an important environmental determinant regulating the expression of *hns*. Besides, H-NS, which is required for optimal continuous growth at suboptimal temperatures (8), is overexpressed upon cold shock under the positive control of the main Csp, CspA (25). Unlike *E. coli* H-NS, the *P. fragi* H-NS-like protein was not synthesized at the optimal growth temperature (30°C). Nevertheless, its induction after the cold shock treatments suggests a similar way of regulation by the CspA-homolog protein(s). Thus, the H-NS-like protein could be induced by one or two of the four *P. fragi* CspA-like proteins, CapA (C7.0) and/or CapB (C8.0). Indeed, the overexpression of these peptides after cold shock treatments and their very low expression at continuous optimal growth temperature (13) correlate with the levels of expression of the H-NS-like protein. Some relatively high levels of CapA and CapB could be necessary for the cells to induce the synthesis of the H-NS-like protein.

The last labelings carried out in each experiment revealed the overexpression of three polypeptides, C27.0 and E24.5 in the 20-to-5°C downshift and C25.0 in the 30-to-5°C shift. These proteins were classified in the independent group III because their induction was obviously not correlated with the temperature downshift. Their overexpression took place in the 5°C mid-exponential growth phase (9 h postshift) or later (20 h postshift), when the growth rate reduced.

In *P. fragi*, we have identified, by Western blot analysis, a homolog of the RpoS protein of *E. coli*. The increased expression of this 37 kDa-protein from the fifth hour after a shift from 30°C could be correlated with the induction of group III proteins. Indeed, the onset of stationary phase of *E. coli* is connected with the induction of RpoS, the stationary-phase sigma factor (σ^s), which controls positively the expression of more than 30 genes whose products are involved in starvation survival and stress resistance (28). Moreover, the induction of the *P. aeruginosa* RpoS, a 38 kDa-protein, was associated with stationary-phase cells but began from the second half of the mid-logarithmic phase (9, 46). Otherwise, the detection of low quantities of the σ^s-like protein in *P. fragi* preshift cells may be compared with the low level of σ^s protein observed in actively growing cells of *P. aeruginosa* (9). In the same way, the decreased synthesis of σ^s-like protein in the first hours postshift could be correlated with the decreased level of ppGpp (guanosine tetraphosphate). As a matter of fact, in *E. coli*, the ppGpp level decreases following a downshift (19), and it has been suggested that ppGpp may be a positive regulator of σ^s synthesis at the level of *rpoS* transcription (28).

A fourth group has been created for the polypeptides the synthesis of which decreased after the cold shocks. Differences in the kinetics of their variations formed three subgroups. Among these repressed proteins, some (A26.5, B74.0, C15.9, C16.0, C20.0b, C20.3, C55.0, and D19.8) were previously designated Haps, for heat acclimation proteins, because of their optimal expression in cells grown at steady-state high temperature (25 to 34°C) (13, 32). Michel et al. (32) subsequently characterized A26.5, B74.0, C55.0, and D19.8 as Hsps because of transient overexpression upon heat shock treatments. B74.0 and C55.0 were found to be immunorelated to the *E. coli*

DnaK and GroEL Hsps, respectively. Thus, these two molecular chaperones are repressed by downshifts in temperature as in the mesophilic *E. coli* (18) and *B. subtilis* (29). Their synthesis was derepressed long after growth resumption, as it was for *E. coli* shifted from 37 to 15°C (21). This derepression could indicate that cells have recovered steady-state physiological conditions. In fact, these proteins are produced at all temperatures, but in *E. coli* (48) as well as in *P. fragi* (13, 32), their level of synthesis decreases with temperature decreasing.

The level and kinetics of expression of cellular proteins of *P. fragi*, according to the temperature and its variations, have led to their designation as Csps, Caps, Hsps, and Haps (references 13 and 32 and this work). However, four proteins, C23.0, E7.0, E8.0, and E24.5, appeared to belong to both Csps and Hsps. Except for E24.5, these proteins had been earlier characterized as Haps (13) and thus could be designated Taps (temperature adaptation proteins) in a broad sense. Such proteins have been observed only in *L. monocytogenes*, *L. innocua* (38), and *B. subtilis* (29). Their nature is unknown, but they could be involved in the maintenance of fundamental cellular processes such as transcription or translation. In *P. fragi*, the identity of C23.0 and E24.5 remains to be determined. On the contrary, the microsequencing and PCR results confirm the previous immunochemical study (13) showing that E7.0 and E8.0, also called TapA and TapB, respectively, belong to a family of peptides whose first described member was CspA, the major *E. coli* Csp (10). Otherwise, these results also confirm that CapA (C7.0) and CapB (C8.0) belong to this family of low-molecular-mass proteins.

Obviously, these proteins occur in a wide range of eubacteria, gram negative and gram positive, whatever their growth range of temperature. Some of these bacteria harbor several peptides of this family, but except for *P. fragi* and *B. subtilis* (12), they are not all cold shock inducible. In *E. coli* (16, 33) only three of seven, and in *B. cereus* (30) two of six, CspA-like proteins are involved in the cold shock response. To date, the functions of the other peptides remain unknown. However, all of these peptides are characterized by the presence of two nucleic acid-binding motifs, RNP-1 and RNP-2, allowing RNA and single-stranded DNA interactions. Brandi et al. (5) have shown that the *E. coli* CspA can play a positive role in translation by favoring the interaction of its CspA transcript with ribosomes. Moreover, an mRNA chaperone role with low sequence specificity is suspected at low temperature for CspA, which could prevent the formation of secondary structures (15). On the other hand, *E. coli* CspA (26, 34) and *B. subtilis* CspB (11) have been shown to bind single-stranded DNA containing the Y-box motif, a sequence containing the inverted core pentanucleotide CCAAT. Numerous eukaryotic transcription factors are known to bind specifically to the Y-box motif located in gene enhancers and promoters (24). In the same way, an ATTGG sequence is present upstream of the *B. subtilis cspB* and the *E. coli cspA* genes, as well as in two CspA-activated genes, *hns* (4, 25) and *gyrA* (20). This demonstrates an autoregulation of these two nucleic acid-binding Csps by their product and its functioning as a transcriptional activator on other cold shock genes for CspA. In *B. subtilis*, such a function could be assumed by a dimer of CspB which can bind both strands of the DNA at the AATTG and CCAAT sequences (11) and can indeed present a dimer structure stabilized by hydrogen bonds (44). This dimerization would be impossible for the *E. coli* CspA, due to the presence of three extra N-terminal amino acids (34). For the same reason, we can speculate that the four *P. fragi* peptides cannot act as a dimer. In other respects, no Y-box motif has been recovered in the upstream sequence of *P. fragi capB*, indicating that the gene is not autoregulated by its own product and/or that another promoter(s) is involved in its induction. Moreover, Brandi et al. (4) have shown that the Y-box motif was not strictly required for the cold shock induction. Indeed, they demonstrated that the insertion in *E. coli* of an *hns-cat* fusion, containing the *Proteus vulgaris* promoter devoid of CAATT sequence, was cold shock inducible.

Considering sequence homologies, conservation of nucleic acid-binding domain, and mode of expression, we can reasonably assume similar functions for the four small *P. fragi* Csps to those described for the *E. coli* CspA in the adaptative response of cells to cold shocks. However, considering their expression according to temperature (13) and its variations (reference 32 and this work), the four *P. fragi* peptides also probably have essential roles in temperature adaptation in a broad sense. This implies that their regulation in this psychrotroph could be slightly different from that in *E. coli*. Indeed, in spite of strong similarities between peptides belonging to the family of small nucleic acid-binding proteins, our studies pointed out several fundamental facts which are specific to the four *P. fragi* peptides. First, the four proteins are cold shock overexpressed, TapA and TapB transiently and CapA and CapB definitively. Second, in cultures at steady-state temperature, CapA and CapB are optimally expressed at low temperatures (4 to 10°C) whereas TapA and TapB are optimally expressed at temperatures around the optimal growth temperature (25 to 30°C) (13). Third, TapA and TapB are heat shock inducible. None of these properties have been described for the *E. coli* CspA or other members of this family, and their involvement in the heat and cold shock responses is interesting. The fact that either of these protein pairs is overexpressed according to the growth temperature means there is a subtle way of regulation with the probable involvement of temperature-sensitive molecules. Finally, the persistence of the expression of CapA and CapB long after cold shocks, when cells had recovered a balanced growth at 5°C, could be one of the key factors in the ability of *P. fragi* to grow at temperatures not suitable for mesophilic bacteria.

There are clearly more functions of the four small nucleic acid-binding proteins of *P. fragi* than of *E. coli* or *B. subtilis* proteins. According to the literature, the *E. coli* CspA acts at both transcriptional and translational levels. Indeed, we can assume some differences and perhaps more complex mechanisms of regulation and roles for the *P. fragi* peptides. Numerous questions regarding their regulation, function(s), and mode of action remain. Moreover, the characterization of the other Csps would be also essential in order to understand the adaptative response to cold shocks. In the same way, the particular role of Caps, which seem to be specific to psychrotroph bacteria, would be very interesting to elucidate. The physiological and molecular basis of growth of psychrotrophs and psychrophiles at very low temperatures could be partly explained by the expression of such a group of proteins.

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