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Identification of an Iron-Binding Protein of the Dps Family Expressed by *Streptococcus thermophilus*

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Abstract. *Streptococcus thermophilus* PB18 can grow between 20° and 52°C and is resistant to various stresses such as heat, acidic or cold shock. During cold shock, a protein of 21.5 kDa was previously shown to be induced in *S. thermophilus*. In addition to its cold-shock induction, 2D-PAGE revealed that the 21.5-kDa protein was also expressed during the stationary phase of growth. The recent access to the genome sequence of *S. thermophilus* LMG18311 allowed the identification of a 173-amino acid protein displaying a strong homology between the 21.5-kDa protein and members of the Dps family of proteins. Specific staining of non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) followed by two-dimensional PAGE (2D-PAGE) showed that the 21.5-kDa protein was an iron-binding protein.

Lactic acid bacteria (LAB) are commonly used in the dairy industry. Within the LAB group, *S. thermophilus* is usually found in dairy fermentation as a starter in the yoghurt-and cheese-making industries. Low temperature is commonly used during processing and storage of industrial dairy products. Bacteria have developed specific responses to survive under stress conditions. The cold-shock response is characterized by physiological (growth rate) and biochemical changes including membrane modifications to maintain membrane fluidity and inhibition of DNA, RNA, and synthesis of most housekeeping proteins [22]. Those deleterious effects of cold shock are overcome by a rapid induction of cold-induced proteins (CIPs). These proteins play a role in DNA replication, transcription, and repair, in RNA structure and translation. Among the CIPs, the cold shock proteins (CSPs) have the highest induction level. CSPs are low-molecular mass proteins (approximately 7 kDa) [22]. CspA, a major cold shock protein, was first identified in *Escherichia coli* and was then observed in a variety of Gram-negative and Gram-positive bacteria [7, 15]. CspA is a trancriptional activator of at least two genes coding for CIPs, the nucleoid-associated DNA-binding protein H-NS and the GyrA subunit of the topoisomerase DNA gyrase [4, 16]. CspA is also an RNA chaperone, which helps the initiation of translation [14].

The cold shock response of *Streptococcus thermophilus* has been studied [20, 21, 24]. After exposure to a cold stress, *S. thermophilus* PB18 overexpressed a protein of 7.5 kDa with sequence homology to the CSPs and a protein of 21.5 kDa. The first 20 amino acid residues of the amino-terminal region of the 21.5-kDa protein were previously determined by Edman degradation. However, similarity searches against various public databases did not allow identification of this protein [20].

This work was undertaken to characterize the 21.5-kDa protein induced in *S. thermophilus* during a cold shock.

Materials and Methods

Bacterial strain and culture media. The strain *S. thermophilus* PB18, originating from traditional cheese, was preserved in reconstituted skimmed milk [10% (wt/vol)] at −80°C. Studies in cold shock conditions were performed at 20°C as previously described [20]. Studies in early stationary phase ($O.D._{650nm}$: 5.5) were realized with cells grown for 4 h at 42°C in M17 medium [10] supplemented with 10 $g \cdot L^{-1}$ lactose.

Non-denaturing-PAGE and iron-binding protein detection. Whole-cell soluble proteins were obtained as described by Gonzalez-Marquez

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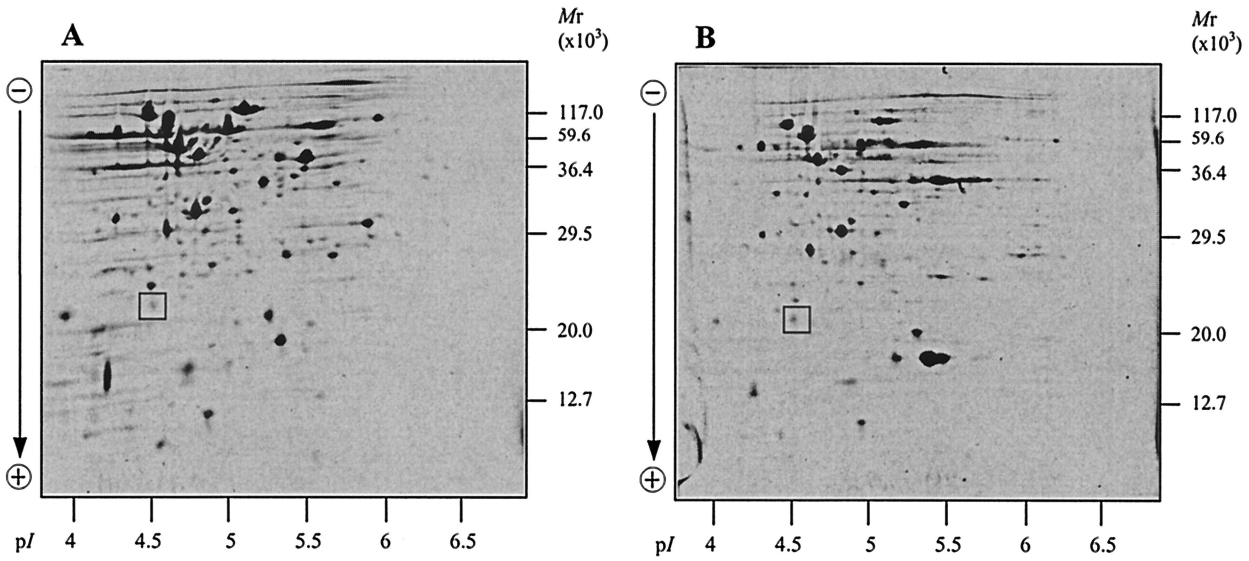

Fig. 1. Bidimensional PAGE of the whole-cell soluble proteins (20 μg) of *S. thermophilus* PB18 in cold shock at 20°C (A) and in stationary phase at 42°C (B). Proteins were revealed by Coomassie blue staining. Position of the spot corresponding to the 21.5-kDa protein is indicated by a box.

et al. [8]. Before electrophoresis, protein samples (60 μg) were incubated for 2 h at room temperature in a ferrous iron solution of 1 mM $FeSO_4$. Equine ferritine (Sigma, St-Louis, MO, USA) was used as a FereneS staining standard. Incubation with iron solution was not necessary as ferritine was already iron complexed. Protein samples (1.5 mg · mL^{-1}) were prepared in 375 mM Tris-HCl buffer, pH 8.9, containing 10% (vol/vol) glycerol and 0.01% (wt/vol) bromophenol blue. Non-denaturing(ND)-PAGE separation was performed in 7.5% (wt/vol) polyacrylamide gel (10 × 7 cm) containing 375 mM Tris-HCl buffer, pH 8.9. The electrode buffer (pH 8.3) consisted of 25 mM Tris, with 192 mM glycine. Iron-binding protein detection was performed using FereneS reagent according to Chung, [6].

2D-PAGE. For the first dimension (IEF), whole-cell soluble proteins (100 μg) were dissolved in a mixture of 8 M urea, 4% (wt/vol) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate), 40 mM Tris, 0.2% (vol/vol) ampholytes 3–10 (Bio-lytes 3–10; Bio-Rad, Hercules, CA, USA), 2 mM tributyl phosphine, and 0.001% (wt/vol) bromophenol blue. IEF was performed by using a pre-cast immobilized pH gradient (IPG) strip of 7 cm at 4% (wt/vol) polyacrylamide with a linear pH gradient range of 4–7 (IPG ReadyStrip pH 4–7, length 7 cm, Bio-Rad). The strip was rehydrated with the protein solution (0.8 mg · mL^{-1}) in the protean IEF cell (Bio-Rad) for 15 h at 20°C under active conditions (constant voltage of 50 V). The focusing was performed at 20°C in three steps. First, a low voltage (250 V for 15 min) was required. Secondly, a voltage ramp from 250 to 4000 V was applied. Thirdly, the voltage was kept at a constant value of 4000 V for 5 h. The IPG strip was then equilibrated for 15 min in 0.125 M Tris-HCl buffer pH 8.6 containing 6 M urea, 2% (wt/vol) SDS, 20% (vol/vol) glycerol, and 5 mM tributyl phosphine. The IPG strip was embedded on the top of a SDS-PAGE stacking gel by using molten 1% (wt/vol) agarose in electrode buffer [25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS; pH 8.3]. The second dimensional electrophoretic separation was realized in 15% polyacrylamide gels (10 × 7 cm) containing 375 mM Tris-HCl buffer, pH 8.9, with 0.1% (wt/vol) SDS by using the 2-D Mini-Protean Electrophoresis System (Bio-Rad), during 1 h at 200 V.

Proteins were revealed by Coomassie blue staining or silver staining by Morrissey's method with modifications [18].

DNA and protein analysis. 2D gels were scanned, and their images were analyzed with the PDQuest 2D analyzer software (Bio-Rad).

The open reading frame search was performed with the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Homology searches were performed with the Blast computational database (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment of proteins was realized with the ClustalW program (http://www.npsa-pbil. ibcp.fr/cgi-bin/npsa_automat.pI?page=npasa_clustalw.html).

Theoretical protein parameters (isoelectric point and molecular mass) were calculated with the ProtParam program (http://www.expasy.ch/tools/protparam.html).

Results and Discussion

A previous work showed that *S. thermophilus* PB18 induced a 21.5-kDa protein under cold shock. The 21.5-kDa protein has two isoelectric variants with apparent isoelectric points (p*I*app) of 4.4 and 4.5. This protein is not produced during the exponential phase of growth [20]. In this study, whole-cell soluble proteins of *S. thermophilus* were analyzed by 2D-PAGE after a cold shock (20°C) and in early stationary phase of growth at 42°C. Analysis of electrophoretic profiles revealed the presence of the 21.5-kDa protein with a p*I*app of 4.5 for the two growth conditions (Fig. 1). Thus, the 21.5-kDa protein is not only a cold-shock protein, since it was also expressed during the early stationary phase of growth. Its isoelectric variant with a p*I*app of 4.4 is not revealed by Coomassie staining because of too low quantity.

The nucleotidic sequence corresponding to the amino-terminal region (TDSIKETVNHQAEWPYN) of the 21.5-kDa protein was identified by a blastN search against the genome of *S. thermophilus* LMG18311 (P.

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AGAGGTACCAAGAAAAAAATATAAAATACTAAGCATACCTAACTATTCGAAAAAAGACAGAAAAAGCGTTTTACT

                                                                        T
TGTATCTTATTTATAAATATTTTAAAATATAAACATAAACAATAATAGTTCTAAATAAGGAGAAAGAATATGACA
                                                                      M  T
                                                                      1

 D  S  I  K  E  T  I  K  E  T  V  N  H  Q  A  E  W  P  Y  N
GATTCAATTAAAGAAACAATCAAGGAAACAGTTAACCATCAAGCGGAGACACCAACAAACACAAAAACCAAAGCA
 D  S  I  K  E  T  I  K  E  T  V  N  H  Q  A  E  T  P  T  N  T  K  T  K  A
                        10                                   20

GTATTAAATCAAGCGGTTGCCGATTTGTCTGTAGCAGCTTCTATTGTGCATCAAGTTCATTGGTATATGCGTGGT
 V  L  N  Q  A  V  A  D  L  S  V  A  A  S  I  V  H  Q  V  H  W  Y  M  R  G
          30                                   40                        50

CCTGTTTTCCTTTATCTTCACCCAAAAATGGATGAATTAATGGATAGTTTGAATTCCTATCTTGATAAGATTAGT
 P  V  F  L  Y  L  H  P  K  M  D  E  L  M  D  S  L  N  S  Y  L  D  K  I  S
                        60                                   70

GAGCGTTTGATTACCATTGGTGGTGAACCCTACTCAACTTTGGTAGAGTTTTCATCTAATTCAGGTTTGACTGAA
 E  R  L  I  T  I  G  G  E  P  Y  S  T  L  V  E  F  S  S  N  S  G  L  T  E
       80                                   90                        100

ACTACTGGTACATTTGATCAACCAATGTCTGATCGAATTCAGCTATTGGTTGATATATACAAATACTTGTCTGTC
 T  T  G  T  F  D  Q  P  M  S  D  R  I  Q  L  L  V  D  I  Y  K  Y  L  S  V
                        110                                  120

TTGTTCCAAGTTGGCTTGGATATCACAGATGAAGAAGGAGATGTTCCTTCAAATGATATCTTTACGGATGCAAAA
 L  F  Q  V  G  L  D  I  T  D  E  E  G  D  V  P  S  N  D  I  F  T  D  A  K
          130                                  140                       150

TCAGAAATTGATAAGACGATCTGGATGTTGACTGCAGAACTTGGACAAGCATCAGGCTTGAAATAATCAAATTTT
 S  E  I  D  K  T  I  W  M  L  T  A  E  L  G  Q  A  S  G  L  K
                        160                        170       173

GAGTTAAGATGATTGTCAAGAGTTCCTTTAAAAGTGTATAATAGATACGAAATACTTTTAGAAGAAGAGAGGCAG

CGAT
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Fig. 2. Comparison of the amino-terminal sequence of the 21.5-kDa protein (in italic) with the complete nucleotidic (in bold) and amino acid sequences of the putative protein of *S. thermophilus* LMG18311. The ORF of the putative protein of *S. thermophilus* LMG18311 is underlined.

Hols, unpublished data). It allowed one to have the theoretical complete sequence of the protein. The complete gene codes for a 173-amino acid protein (Fig. 2). This putative protein, displayed a calculated molecular mass of 19.3 kDa and a calculated isoelectric point of 4.52, which are similar to the apparent molecular mass of 21.5 kDa and the p*I*app of 4.5 of the 21.5-kDa protein of *S. thermophilus* PB18.

Comparison of the complete amino acid sequence of the hypothetical protein of *S. thermophilus* LMG18311 with protein and nucleic acid sequence databases indicated that it had similarities with the Dps (DNA-binding Protein from Starved cells) family of protein [1] (Fig. 3). Alignment of the amino acid sequences shows that the Dps protein family signature (PROSITE access number PS00818, http://www.npsa-pbil.ibcp.fr/cgi-bin/pattern_prosite.pl) was also present in the hypothetical protein (amino acid residues from H47 to D63). It also showed that it was strongly homologous with the Dpr protein of *Streptococcus mutans* [25], with 68% of identity (Fig. 3). It should be noted that the amino-terminal sequences of the proteins of the Dps family display high variability

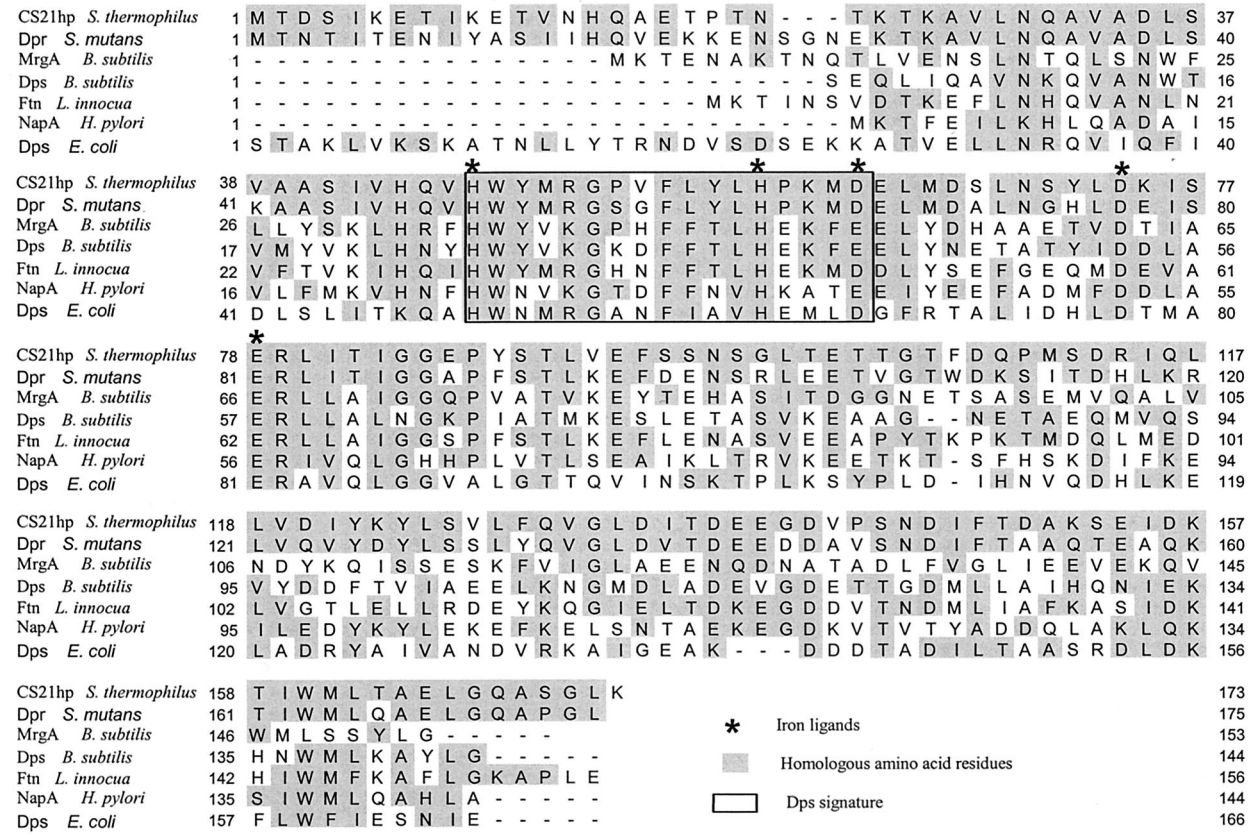

Fig. 3. Sequence alignment of homologous proteins with the hypothetical cold shock protein of *S. thermophilus* LMG18311 (CS21hp). Dpr: Dps-like peroxide resistance; Dps: DNA-binding protein from starved cells; Ftn: ferritine; MrgA: Metallo regulated geneA; Nap A: "Neutrophil activating protein A". Dps protein family signature is a pattern from PROSITE database (http://npsa-pbil.ibcp.fr/cgi-bin/pattern_prosite.pl).

(Fig. 3). This might explain why the amino-terminal sequence of the 21.5-kDa protein of *S. thermophilus* PB18 did not allow identification of homologous proteins in relevant databases. In *Escherichia coli*, Dps is strongly expressed during stationary phase and during growth under oxidative stress. It binds DNA to protect it from oxidative damage [1, 9, 17]. Numerous proteins with sequence homology to Dps have been found in distantly related bacteria. Some are iron-binding proteins like ferritins, some others are DNA-binding proteins, [1–3, 5, 13, 19, 23, 25, 26].

Iron binding ability was investigated to determine whether the 21.5-kDa protein of *S. thermophilus* PB18 had the same property as its counterpart from *S. mutans* [25, 26]. Whole-cell soluble proteins of *S. thermophilus* PB18 grown in cold shock and in stationary phase conditions were separated by ND-PAGE. Iron binding proteins were detected with the specific reagent FereneS. Only one protein band was revealed for each condition (Fig. 4A). This band was excised from the ND-PAGE gel and placed under the denaturing conditions used for 2D-PAGE. The 2D-PAGE analysis revealed two spots with same molecular masses of 21.5 kDa and respective p*I*app of 4.4 and 4.5 (Fig. 4C), which correspond to the 21.5-kDa protein [20]. The variant with a p*I*app of 4.4 was seen because of silver staining. Combination of ND- and 2D-PAGE shows that the 21.5-kDa protein could bind ferrous iron.

Proteins of the Dps family are multimeric. They are constituted of approximately 20-kDa subunits. The iron-binding ability shared by members of the Dps family exists only when the proteins are in their multimeric form. The amino acid residues involved in the iron-binding capacity of the ferritin of *Listeria innocua* (H31, H43, D47, D58, and E62) are present in the putative protein of *S. thermophilus* LMG18311 (H47, H59, D63, D74, and E78) (Fig. 3). Since the 21.5-kDa protein bound iron, it might then form a dodecamer as the ferritin of *L. innocua* and Dpr of *S. mutans* [13, 26].

Recently, a protein homologous to the ferritin of *L. innocua* was found in the psychrotrophic bacteria *Listeria monocytogenes* [12] in cold-shock conditions. Binding of iron ions during a cold stress seems to be a strategy to protect the cells. Ferrous iron (Fe^{2+}) has a deleterious

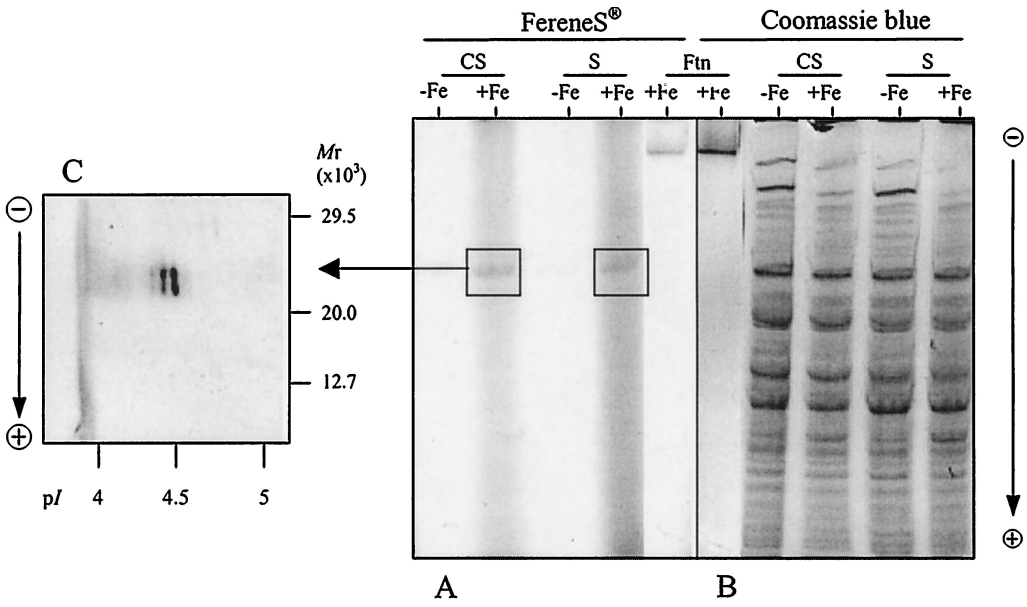

Fig. 4. Identification of iron binding proteins in whole-cell soluble proteins of *S. thermophilus* PB18. Whole-cell soluble proteins (60 μg) extracted of *S. thermophilus* PB18 in cold chock at 20°C (CS) or in stationary phase at 42°C (S) were incubated for 2 h at room temperature with ($+Fe^{2+}$) or without ($-Fe^{2+}$) incubation in 1 mM $FeSO_4$. Proteins were separated by ND-PAGE. Iron-binding proteins were revealed with FereneS® (A), and total proteins were revealed with Coomassie blue staining (B). Ftn was commercial equine ferritin complexed with iron. The iron-binding protein extracted from the ND-PAGE gel (cold shock conditions) was characterized by bidimensional PAGE (C). Proteins were revealed by silver staining.

effect in cells growing aerobically, since it can provide reactive oxygen species [11], while ferric iron (Fe^{3+}) is not toxic for bacteria. When the temperature decreases, the time required for completion of the oxidation of Fe^{2+} into Fe^{3+} is increased. Thus, it can be hypothesised that, during a cold shock, *S. thermophilus* overexpressed the iron-binding protein of 21.5 kDa to sequester toxic ferrous iron ions.

In conclusion, a nucleotidic sequence coding for a putative protein similar to the 21.5-kDa cold shock protein of *S. thermophilus* PB18 was found in *S. thermophilus* LMG18311. The putative protein of *S. thermophilus* LMG18311 was identified as an iron-binding protein of the Dps family. The 21.5-kDa cold shock protein of *S. thermophilus* PB18 bound iron and was expressed during the stationary phase of growth as the protein of the Dps family. The proteins of the Dps family, which are widely found in bacteria, probably protect the cell, in particular DNA, from damaging agents. They appeared to be general stress proteins induced in response to different stresses.

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