# Evidence for Involvement of at Least Six Proteins in Adaptation of *Lactobacillus sakei* to Cold Temperatures and Addition of NaCl

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*Lactobacillus sakei* is a lactic acid bacterium widely represented in the natural flora of fresh meat. The aim of this study was to analyze the differences in protein expression during environmental changes encountered during technological processes in which *L. sakei* is involved in order to gain insight into the ability of this species to grow and survive in such environments. Using two-dimensional electrophoresis, we observed significant variation of a set of 21 proteins in cells grown at 4°C or in the presence of 4% NaCl. Six proteins could be identified by determination of their N-terminal sequences, and the corresponding gene clusters were studied. Two proteins belong to carbon metabolic pathways, and four can be clustered as general stress proteins. A phenotype was observed at low temperature for five of the six mutants constructed for these genes. The survival of four mutants during stationary phase at 4°C was affected, and surprisingly, one mutant showed enhanced survival during stationary phase at low temperatures.

*Lactobacillus sakei*, formerly *Lactobacillus sake* (49), is a lactic acid bacterium belonging to the natural flora of fresh meat and is the predominant flora of vacuum-packed meat stored at low temperatures. Moreover, some *L. sakei* strains produce bacteriocins active against various bacterial species, including *Listeria monocytogenes* (43). For these reasons, it is one of the main starters used in Europe, in association with micrococci, for the production of fermented sausage (18). Current knowledge about this species has recently been reviewed (5). A genetic map of the chromosome of *L. sakei* 23K, our model strain, has been established (10), and its sequence is being determined (4).

During technological processes, *L. sakei*, like many other lactic acid bacteria involved in food technology, is exposed to several stress conditions such as the presence of salt or cold temperatures. Little is known about its ability to respond to such environmental parameters, but recent studies have been focused on the impact of these factors on bacteriocin production (27, 28). The heat shock genes *hrcA*, *groEL*, *dnaK*, and *dnaJ* have also been identified, and their transcription has been shown to be induced by heat shock, salt, and ethanol (44). The 5′ end of a gene putatively encoding a ClpX protein has been described, but no experimental data showing its function has been reported (45).

Proteomics has been revealed to be a powerful tool for investigation of the responses of microorganisms to various environmental changes. For lactic acid bacteria, cold adaptation and salt stress challenge have been illuminated by this approach (6). Generally, proteins induced under stress conditions are clustered into three groups: (i) proteins involved in

DNA or protein repair, referred to as chaperons, (ii) proteins of the general metabolism, and (iii) proteins specific for a given stress. In a proteomic analysis study of stress response in *Enterococcus faecalis*, 27 spots have been characterized (14). These proteins could be classified into three categories: proteins known as stress proteins with a protective function such as DnaK and GroEL; proteins involved in various metabolic pathways, such as enzymes of catabolic pathways or proteins under carbon-catabolite repression; and proteins of unknown function.

The induction of general and specific stress response proteins, but mainly that of heat shock proteins, has been well studied in *Escherichia coli* and *Bacillus subtilis* (20, 54). Large protective mechanisms induced by various stress conditions have been described for *Lactococcus lactis* (38), and some proteins are cross-induced by different stresses. When carbohydrate starved during stationary phase, *L. lactis* IL1403 becomes resistant to acid, ethanol, heat, oxidative, and osmotic-stresses (19). In *L. lactis* MG1363, an overlap between the heat and salt stress responses has been reported, demonstrating that all salt stress-induced proteins were also induced by heat-stress (23). In *Streptococcus mutans*, 52 proteins were overexpressed by salt but only 10 were specifically induced by salt stress (47). In the group of cold-induced proteins are also found low-molecular-weight proteins called cold shock proteins, induced in a wide range of bacteria including lactic acid bacteria. These proteins are involved in mRNA folding during protein synthesis (53).

No detailed study of cold or salt adaptation in *L. sakei* is yet available. The aim of the present study was to determine by a proteomic approach (29) the main proteins which are affected either by the presence of salt or by growth at suboptimal temperatures. Among 21 spots whose intensity varied on two-dimensional (2D) gels, 6 were identified and further studied. One was previously identified as 6-phosphate-fructokinase (PFK) (30), and five were identified by data mining against the

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TABLE 1. Oligonucleotides used to amplify internal fragments of genes for mutant construction

| Primer | Sequence[a] |
|---|---|
| 1 | 5′-GG<u>GGTACC</u> GCA AGA TTT TGC TGA AGG-3′ (KpnI) |
| 1rev | 5′-GG<u>GGTACC</u> GAC GAA GTT TGT CCG ACG-3′ |
| 17 | 5′-CG<u>GAATTC</u> AAG ATG GAT CCA ATT ACG-3′ (EcoRI) |
| 17rev | 5′-CG<u>GAATTC</u> CAT GAC GCC TTC TGC CAA-3′ |
| 5 | 5′-CG<u>GAATTC</u> CAG GTG GGT GTT TCT GGT-3′ |
| 5rev | 5′-CG<u>GAATTC</u> GCT CGC CAA GGT TCC TTT-3′ |
| 20 | 5′-CG<u>GAATTC</u> AAT GGA TAA GAT GAT GGA-3′ |
| 20rev | 5′-CG<u>GAATTC</u> AGT CAA CAA GTC AGA AAC-3′ |
| 7 | 5′-CG<u>GAATTC</u> AAC GAT GAT TAA TGA AGG-3′ |
| 7rev | 5′-CG<u>GAATTC</u> TTC GTG GGC TTT TTC GAG-3′ |
| 3 | 5′-CG<u>GAATTC</u> TTT AGT TGG ATT AGA CGG-3′ |
| 3rev | 5′-CG<u>GAATTC</u> ATG AGC AAT CGT TCA ATA-3′ |

[a] Restriction sites added to the 5′ ends of primers are underlined.

partial DNA sequence of the *L. sakei* 23K chromosome. One, glycerol-3-phosphate dehydrogenase (G3PDH; encoded by *glpD*), is involved in glycerol metabolism, and four can be classified as general stress proteins by their similarity to Asp, Usp, MsrA, or Ohr proteins described in other bacterial species. Analysis of mutants with mutations in the corresponding genes revealed that five mutants had a modified survival rate during stationary phase at 4°C, suggesting their involvement in adaptation to growth at low temperatures in *L. sakei*.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* TGI (42) was used for plasmid propagation. Cloning and sequencing experiments were performed with the pBluescript phagemid SK(+) (Stratagene). *L. sakei* mutants were constructed with the integrative vector pRV300 (26) and were all derived from the wild-type strain *L. sakei* 23K (2).

**Media, growth conditions, and electrotransformation.** *E. coli* strains were grown on Luria-Bertani medium at 37°C (42). The complex MRS medium (Difco) described by De Man et al. (7) was used for *L. sakei* propagation at 30°C under aerobiosis without shaking. Physiological studies concerning growth and survival under various conditions were performed in the chemically defined medium MCD (25) supplemented with 0.5% glucose. *E. coli* and *L. sakei* electrocompetent cells were prepared and transformed by the methods of Dower et al. (9) and Berthier et al. (2), respectively. Electroporation was carried out with a Gene Pulser and a Pulse controller apparatus (Bio-Rad). Ampicillin (50 mg liter$^{-1}$ for *E. coli*) or erythromycin (5 mg liter$^{-1}$ for *L. sakei*) was added to the medium for the selection and propagation of transformants.

Cell growth and viability were monitored by measuring the optical density at 600 nm on a Novaspec II visible spectrophotometer (Pharmacia) and by determination of CFU per milliliter. Viable cell counts were determined by serial dilutions of samples and plating on MRS agar. Plates were incubated under aerobiosis at 30°C for 30 h. All the measurements were performed in at least three independent assays.

**2D electrophoresis conditions and N-terminal sequencing.** Bacterial-extract preparation, electrophoresis, and N-terminal protein sequencing were performed by standard methods as previously described (29). Gels were repeated three times and analyzed by Image Master software (Amersham Pharmacia Biotech) after silver staining. N-terminal sequences were determined on spots extracted from Coomassie-stained gels after transfer to a polyvinylidene difluoride membrane and were then searched against the coding sequences (CDS) deduced from genome sequencing of the *L. sakei* chromosome. Amino acid sequencing was performed by the automated P. Edman method on an Applied Biosystems 494 Procise Protein Sequenator (Foster City, Calif.).

**Nucleic acid manipulation and DNA sequence analysis.** DNA extractions and manipulations, and PCR amplification reactions, were performed by standard methods as previously described (45). PCR experiments were performed on a Perkin-Elmer 9600 apparatus using *Taq* DNA polymerase from Fermentas. PCR fragments corresponding to an internal part of the genes were amplified and cloned into the integrative plasmid pRV300 (26). The primers used to amplify internal fragments of genes to be mutated are listed in Table 1. EcoRI or KpnI restriction sites were added at the 5′ ends of primers in order to facilitate further

cloning. Recombinant plasmids were then integrated into the chromosome of *L. sakei* 23K by homologous recombination, leading to knockout mutants. Mutants with mutations in *glpD*, *pfk*, *msrA*, *asp-2*, *ohr*, and *usp* were designated RV4047, RV4043, RV4048, RV4045, RV4044, and RV4046, respectively. The genetic structure of mutated genes was checked by PCR in all mutants.

The genes and their surrounding regions were annotated with the Artemis annotation tool from the Sanger Center (41; http://www.sanger.ac.uk/Software/Artemis). DNA sequences were analyzed with GCG programs (Wisconsin package, version 10.0; Genetics Computer Group, Madison, Wis.) via the genome server from the Institut National de la Recherche Agronomique (INRA).

**Nucleotide sequence accession numbers.** GenBank accession numbers corresponding to genes described in this article are AY58823, AY588238, AY588239, AY588240, AY588241, and AY588242.

## RESULTS

**Identification of the main proteins affected by growth at 4°C or in the presence of NaCl.** In a recent study, we demonstrated that *L. sakei* was able to grow and survive at low temperatures (4°C) or in the presence of 4% NaCl. Both the doubling time and the final cell density were affected, but long-term survival was enhanced under these two conditions (31). In order to detect proteins putatively involved in the response of *L. sakei* to cold temperatures or NaCl, we compared protein patterns of cells harvested in mid-log phase during growth at 4°C or with 4% NaCl. The optimal growth temperature, 30°C, with omission of NaCl in the culture medium, was used as the standard condition.

Twenty-one proteins whose intensities were affected by one of these two parameters were detected (Table 2). Seventeen proteins were affected during growth at 4°C, of which 14 were overexpressed and only 3 were detected in lower quantities at low temperatures. Among 11 spots whose intensities varied with NaCl concentration, 9 were induced and 2 were detected in smaller amounts. Finally, 7 spots were affected by both conditions, 5 being overexpressed and 2 being underexpressed.

Among these 21 spots, only 6 N-terminal sequences could be determined. One polypeptide was blocked at its N-terminal extremity, and the remainder were present in amounts too small to obtain an N-terminal sequence long enough to be used. The six proteins whose N-terminal sequences were determined were further studied.

**Analysis of gene clusters encoding the proteins involved in low-temperature or salt adaptation.** Because the genome sequence of *L. sakei* 23K was partially determined in our laboratory, we used the N-terminal sequences of the proteins to

TABLE 2. Quantitative variation of protein expression levels during growth at 4°C or at 4% NaCl[a]

| Fold change in expression level | | pI[b] | Molecular mass (kDa)[b] | Spot no. | N-terminal sequence |
|---|---|---|---|---|---|
| 4°C vs 30°C | 4% NaCl vs 30°C | | | | |
| >10 | — | 5.18 | 73.32 | 1 | TFSLESRQQAIENLK |
| >10 | — | 5.8 | 42.60 | 2 | Blocked |
| >10 | >5–10 | 6.4 | 15.80 | 3 | MLEYHNILVGLDGSELADSA |
| >5–10 | — | 6.13 | 42.48 | 4 | ND |
| >5–10 | — | 5.20 | 22.58 | 5 | ATETAIFA |
| >5–10 | — | 5.74 | 24.85 | 6 | ND |
| >5–10 | >5–10 | 5.08 | 14.48 | 7 | MAKQYETTMINEGGRNXFVYDPEKS |
| >2–5 | — | 5.18 | 58.59 | 8 | ND |
| >2–5 | — | 4.73 | 47.29 | 9 | ND |
| >2–5 | — | 6.35 | 41.83 | 10 | ND |
| >2–5 | — | 5.04 | 21.29 | 11 | ND |
| >2–5 | >2–5 | 5.03 | 67.63 | 12 | ND |
| >2–5 | >2–5 | 4.92 | 44.79 | 13 | ND |
| >2–5 | >2–5 | 4.92 | 44.11 | 14 | ND |
| <0.4–0.5 | — | 5.25 | 31.14 | 15 | ND |
| <0.4–0.5 | <0.4–0.5 | 4.93 | 27.40 | 16 | ND |
| <0.4–0.5 | <0.4–0.5 | 5.63 | 39.57 | 17 | MKRIGILT |
| — | >2–5 | 4.71 | 21.24 | 18 | ND |
| — | >2–5 | 5.10 | 40.52 | 19 | ND |
| — | >2–5 | 4.61 | 20.74 | 20 | MDXPNQXMDKEMD |
| — | >2–5 | 4.98 | 39.57 | 21 | ND |

[a] Twenty-one proteins showed changes in their expression levels depending on temperature or NaCl concentration. Results are means from at least three independent gels. —, no detectable effect. ND, not determined (not enough material).
[b] Values determined on 2D gels.

identify the corresponding genes in order to gain information on their genetic neighborhood. This allowed us to identify spot 17 as the PFK by its identity score to PFKs of other lactobacilli (30). A PCR fragment was cloned, sequenced, and used to construct a mutant and to clone the *pfk* gene. Further BLAST searches against the partial sequence of the *L. sakei* chromosome allowed the identification of a contig encompassing *pfk* and its surrounding region. Downstream from *pfk*, a *pyk* gene encoding pyruvate kinase (PYK) was observed (Fig. 1A). The two genes are separated by only 31 bp, and a rho-independent transcription terminator was detected downstream from *pyk* but not between the two genes. Moreover, no clear-cut promoter sequence could be detected upstream from *pyk*. This suggests that both genes might be expressed in a polycistronic mRNA. Since we observed that PFK was underexpressed at 4°C and in the presence of salt, we carefully examined whether a protein similar to PYK was similarly regulated. No spot in the pI and molecular mass range of PYK whose intensity was varying could be observed. Furthermore, measurement of PYK activity in cells grown under various conditions did not show PYK activity variations (data not shown), in contrast to what was previously observed for PFK activity (30). It seems, thus, that if protein expression level and PFK activity could be correlated to growth at low temperatures or in the presence of salt, this could not be observed for PYK, indicating either that the two genes do not belong to the same operon or that PFK regulation by temperature and salt acts at a posttranscriptional level.

The sequence of the 15 N-terminal amino acids of spot 1 did not match that of any protein from the databases; however, it could be aligned with amino acids 2 to 16 of one CDS deduced from the *L. sakei* chromosome partial sequence. This CDS was 608 codons long, and the pI (5.01) and molecular mass (66.7

kDa) deduced from the DNA sequence were in accordance with those measured for spot 1 on 2D gels (Table 2). The protein, deduced from the DNA sequence, was 61% identical to the G3PDH of *Lactobacillus plantarum* (Q88ZF0) and also showed significant homology scores with other bacterial G3PDH proteins. Upstream from *glpD*, encoding G3PDH, we observed the glycerol kinase gene *glpK*, which was previously mapped onto the *L. sakei* chromosome (10). Downstream from *glpD*, a gene putatively encoding the putative glycerol facilitator GplF was observed. GlpF was 66% identical to GlpF of *L. plantarum* (Q88ZE9). A single transcription terminator was observed downstream from *glpF*, and several putative promoters were observed (Fig. 1B). The presence of these three genes in *L. sakei*, their organization, and evidence for expression of GlpD demonstrate that this species is able to use glycerol as a carbon source, as was previously reported (46). The gene order *glpK*, *glpD*, *glpF* in *L. sakei* is similar to the organization observed in *L. plantarum* but different from that of other gram-positive bacteria. In *L. lactis*, for example, the gene order is *glpF*, *glpD*, *glpK* (3). In *B. subtilis*, GlpP (absent in the *L. sakei* cluster), whose gene is located upstream from *glpK*, regulates the transcription and stability of the *glpD* transcript (21, 40, 16). Moreover, transcription terminators involved in gene regulation were observed between *glpK* and *glpD* (22). We cannot yet conclude that such a complex regulation of the *glp* operon exists in *L. sakei* also.

The 8-amino-acid N-terminal sequence from spot 5 matched a CDS encoding a protein 76% identical to *L. plantarum* MsrA2 (TrEMBL accession number Q88W34) and 72% identical to *L. plantarum* MsrA4 (TrEMBL accession number Q88VQ9), both of which are described as putative MsrA, a peptide methionine sulfoxide reductase catalyzing the reduction of methionine sulfoxide to methionine. This enzyme is
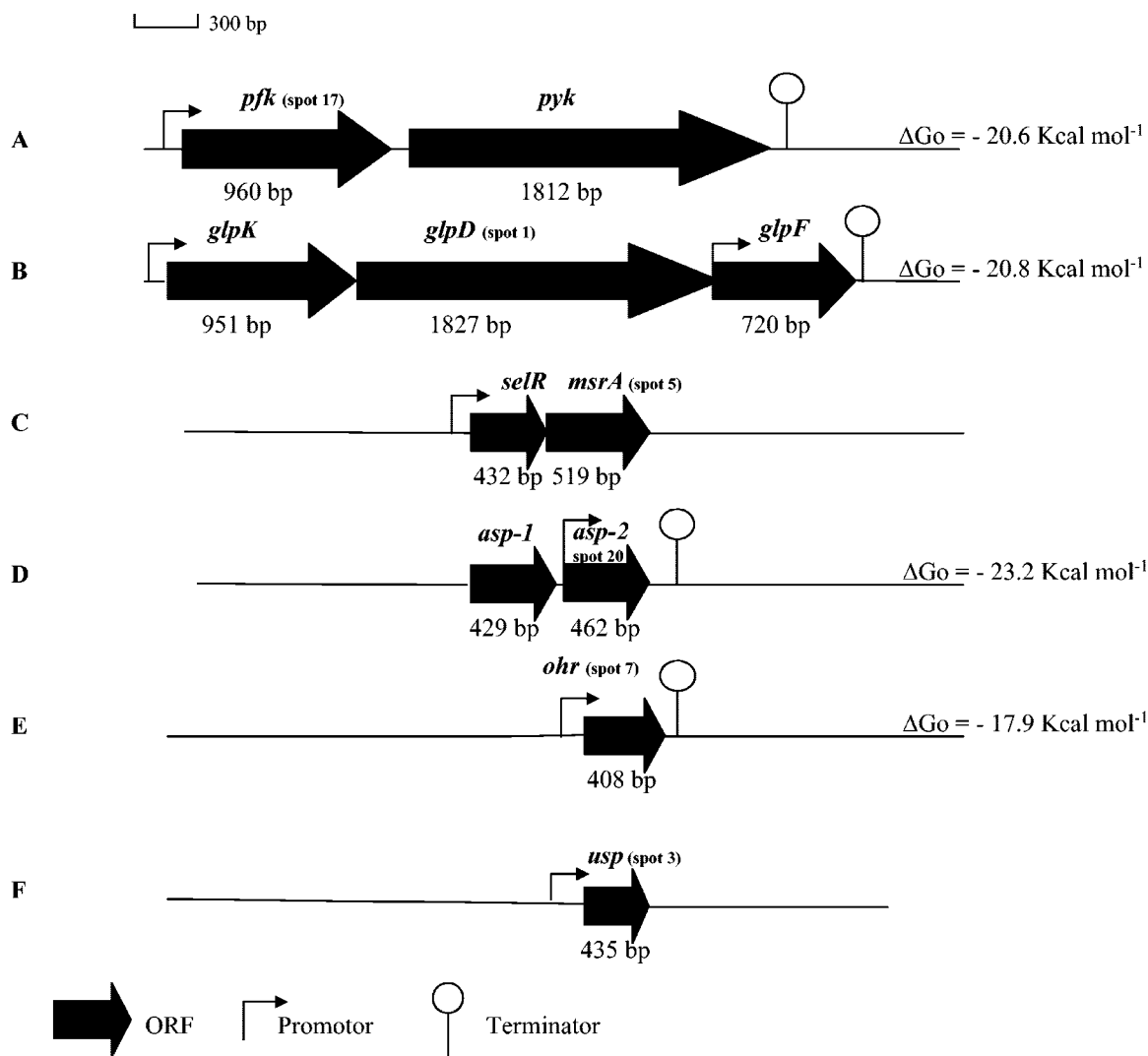
FIG. 1. Schematic organization of genes encoding the proteins whose expression is regulated by salt or low temperatures in *L. sakei*. Spots 17, 1, 5, 20, 7, and 3 are encoded by the *pfk*, *glpD*, *msrA*, *asp-2*, *ohr*, and *usp* genes, respectively.

present in all living organisms and is involved in the repair of damage caused by protein oxidation. Analysis of the nucleotide sequence showed that the putative *L. sakei msrA* gene encodes a 172-amino-acid protein with a calculated molecular mass of 19.5 kDa and a pI of 5.12, both in accordance with values measured for spot 5 on the gels (Table 2). Only 3 bp upstream from the start codon of *msrA*, we observed a 143-codon CDS encoding a protein 71% identical to the MsrA3 protein (MsrB) of *L. plantarum* (TrEMBL accession number Q88W33) and 74% identical to *E. faecalis* MsrB (TrEMBL accession number Q9XB38). All these proteins contain a *selR* domain previously known as DUF25. No Rho-independent transcription terminator could be detected between the two *L. sakei* genes (Fig. 1C). Two kinds of Msr proteins have been described in the literature: the MsrB domain (*selR* domain) is stereospecific for the *R* isomer of the sulfoxide of methionine sulfoxide, whereas the MsrA domain is stereospecific for the *S* isomer. In some organisms these two proteins are fused in a single protein. This is the case for *Neisseria meningitidis* (TrEMBL accession num-

ber Q9JWMS) and *Campylobacter fetus* (TrEMBL accession number Q93KF3). In *L. sakei* the two domains are encoded by two distinct genes, certainly belonging to the same operon. This organization is similar to that observed for *L. plantarum*, in which genes were named *msrA3* and *msrA4*, while the paralogs *msrA1* and *msrA2* were located elsewhere on the chromosome. The two *L. sakei* genes were named *selR* and *msrA*. Because *selR* and *msrA* seem to be transcribed from a polycistronic mRNA, one could expect that the expression level of SelR should also vary on 2D gels. Nevertheless, we could not observe such a variation in the relevant zone of 2D gels.

N-terminal sequencing of spot 20 gave information on 13 amino acids. This sequence matched a CDS in the *L. sakei* genome database encoding a protein 47% identical to *L. plantarum* Asp-1 (TrEMBL accession number Q88Y61), 33.6% identical to *Staphylococcus aureus* Asp-23 (TrEMBL accession number Q53485), induced by alkaline shock (24), and 31% identical to the general stress protein Gls-24 of *E. faecalis* (TrEMBL accession number O32593), which is induced by
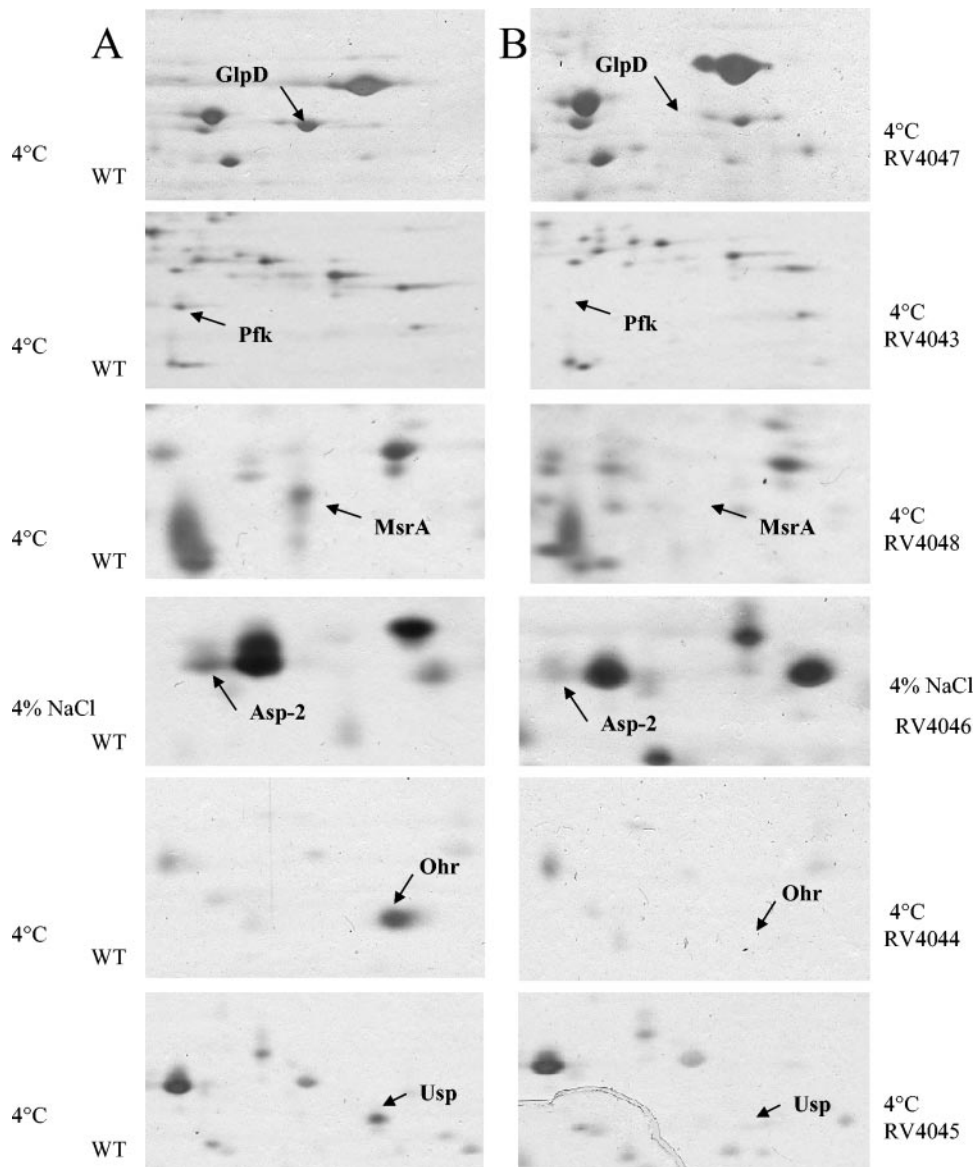
FIG. 2. Portions of 2D gels showing proteins expressed in the wild type (WT) at 4°C or with 4% NaCl (A) and lack of protein expression in mutants at 4°C or with 4% NaCl (B).

glucose starvation and various stresses. The putative *L. sakei* Asp protein contains 153 residues, and its calculated molecular mass (17 kDa) and pI (4.68) are in accordance with those measured for spot 20 (Table 2). Upstream from this *L. sakei asp* gene, we observed a paralog, encoding a protein also similar to Asp (Fig. 1D). The second protein is 41 and 37% identical to *L. plantarum* Asp-1 and *E. faecalis* Gls-24, respectively. In *E. faecalis*, a similar genetic organization with two paralog operons was also observed in the *gls-24* region (13). The presence of three *gls-24* paralogs (Ef0604, *ymG*, and *ytgH*) was recently demonstrated in *E. faecalis* (15), and analysis of the complete *L. lactis* IL1403 genome also revealed the presence of several paralog genes encoding proteins from the same family (3). Some of these genes encode proteins induced under several stress conditions, and a common origin has been argued for three paralogs. Proteins encoded by the two adjacent

genes of *L. sakei* are 44% identical and were therefore named Asp-1 and Asp-2 as in *L. plantarum* (Fig. 1D).

The 25 N-terminal amino-acids of spot 7 matched a CDS of the *L. sakei* chromosome, encoding a protein sharing 60% identity with Lin2302 of *L. monocytogenes* (TrEMBL accession number Q929H4). Lin2302 is a protein belonging to the Ohr/ OsmC family. Phylogenetic analysis revealed that proteins encoded by *ohr* and *osmC* homologs cluster into two related subfamilies that are functionally and structurally distinct, each defined by a specific motif. The conserved amino acid motif VCPY around position 125, specific for the Ohr family and absent in members of the OsmC family (1), was present at position 117 in the *L. sakei* protein identified as spot 7. The corresponding gene was thus named *ohr*. The *L. sakei* Ohr protein is 135 amino acids long, with a calculated molecular mass of 14.8 kDa and a pI of 5.13, both in agreement with the
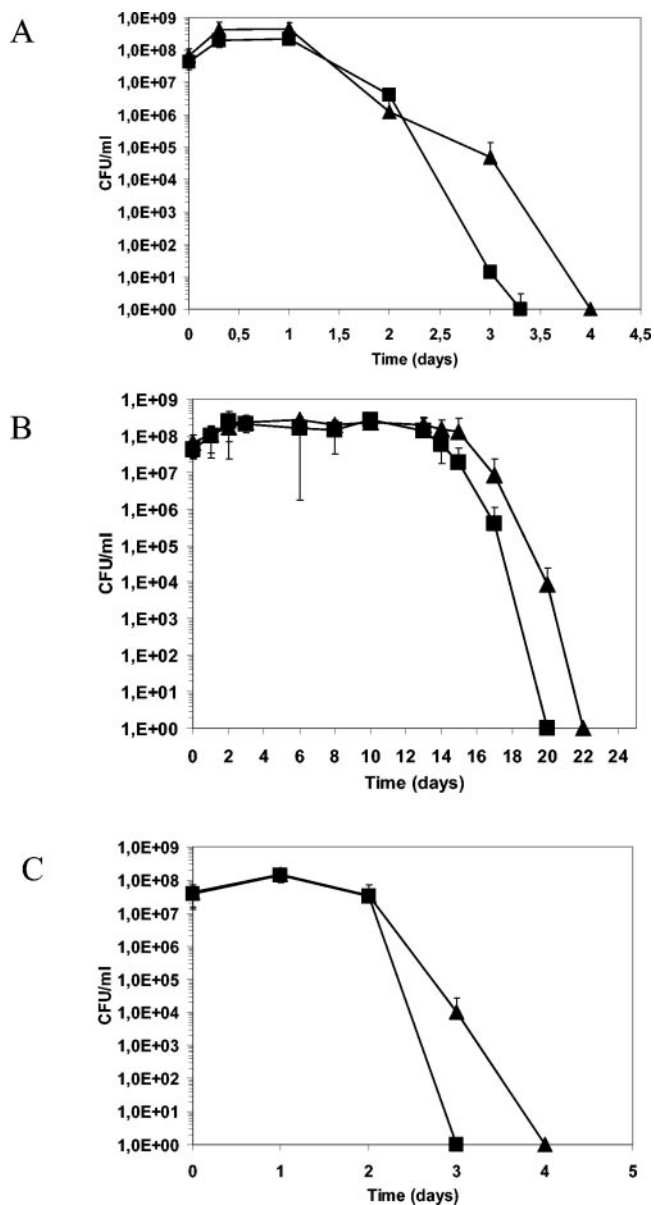
FIG. 3. Survival of the wild-type strain (triangles) and RV4043 (squares) at 30°C (A), at 4°C (B), and with 4% NaCl (C) in MCD with 0.5% ribose. Values are means from three independent experiments. Error bars, standard deviations.

values of spot 20 (Table 2). Putative promoter and terminator motifs were observed upstream and downstream from *ohr* (Fig. 1E), suggesting a monocistronic organization as has been observed for *Pseudomonas aeruginosa* and *Deinococcus radiodurans* (1).

Finally, analysis of the *L. sakei* CDS matching the first 21 amino acids from the N terminus of spot 3 showed identity scores with proteins belonging to the universal stress protein (USP) family. The highest score (36% identity) was obtained with the conserved hypothetical protein YxbE of *L. lactis* (TrEMBL accession number Q9CDI4). Several genes encoding USP-like proteins, the physiological and molecular assignment of which have not yet been determined, are present in the

*L. lactis* genome. The calculated molecular mass (15.6 kDa) and pI (5.93) were in accordance with those of spot 3, whose size was 144 amino acids (Table 2). Analysis of the nucleotide sequence of the region surrounding the *L. sakei* gene, named *usp* (Fig. 1F), showed that the putative *usp* gene did not belong to an operon, as was described for the monocistronic *uspA* gene of *E. coli* (33).

**Construction of mutants.** Mutants were constructed for each of the six genes described above, coding for proteins whose expression was modified by growth at 4°C or with NaCl, and disappearance of the corresponding spots was verified by 2-D electrophoresis (Fig. 2). Mutants were grown in MCD medium under the standard condition, at 4°C, or in the presence of 4% NaCl. As mentioned above, growth of the wild-type strain *L. sakei* 23K at 4°C or with NaCl was impaired, but long term survival was enhanced. Thus, the mutants were tested for their ability to grow and survive under these conditions.

**The survival of the *pfk* mutant is affected at 30°C, at 4°C, and with 4% NaCl.** The *pfk* mutant (RV4043) is not able to grow with glucose as the carbon source. Thus, the study was performed with MCD medium containing ribose, which is not used by the glycolytic pathway. Under these conditions, the growth of the mutant is identical to that of the wild-type strain both at 30 and at 4°C. At 30°C, with or without NaCl, and at 4°C, survival of the mutant was affected (Fig. 3). Thus, PFK could play a role, direct or not, in cell survival.

**The long-term survival of the *glpD* mutant (RV4047) is enhanced.** At 30°C, with or without NaCl, and at 4°C, growth of the mutant was not affected in glucose-containing MCD. However, at 4°C, the mutant showed enhanced survival during stationary phase compared to the wild-type strain (Fig. 4A).

**Long-term survival at low temperatures is affected in *msrA* (RV4048), *asp-2* (RV4045), and *usp* (RV4046) mutants.** Growth of the three mutants was not modified. However, long-term survival was reduced after growth at 4°C (Fig. 4B, C, and D), indicating that the putative proteins MsrA, Asp-2, and Usp play a role in adaptation of *L. sakei* to low temperatures. Such a phenotype was not observed in cells grown in the presence of NaCl.

**No phenotype could be observed for the *ohr* mutant (RV4044).** Mutant RV4044 grew and survived at 30°C, at 4°C, and in the presence of 4% NaCl, as did the wild-type strain (data not shown).

## DISCUSSION

By 2D electrophoresis we detected 21 proteins, expression of which was modified during growth at 4°C or with 4% NaCl. In *S. mutans*, 52 proteins, 10 of which were specific proteins, were induced by NaCl stress (47). In *E. faecalis*, 13 proteins were induced by salt (37). In *L. lactis*, analysis of 2D gels allowed identification of the general stress proteins DnaK, GroEL, and GroES, but no specifically NaCl-induced protein could be detected (23). After a cold stress, the number of proteins affected differs with the species but also with the methods that are used. In fact, in *L. lactis* IL1403, 12 proteins are induced even though 22 proteins are induced in *L. lactis* MG1363 (35, 50, 51, 52). In *Streptococcus thermophilus*, 24 versus 2 proteins were induced in strains CNRZ302 and PB, respectively (50, 36). By 2D electrophoretic analysis, 11 proteins were shown to be induced
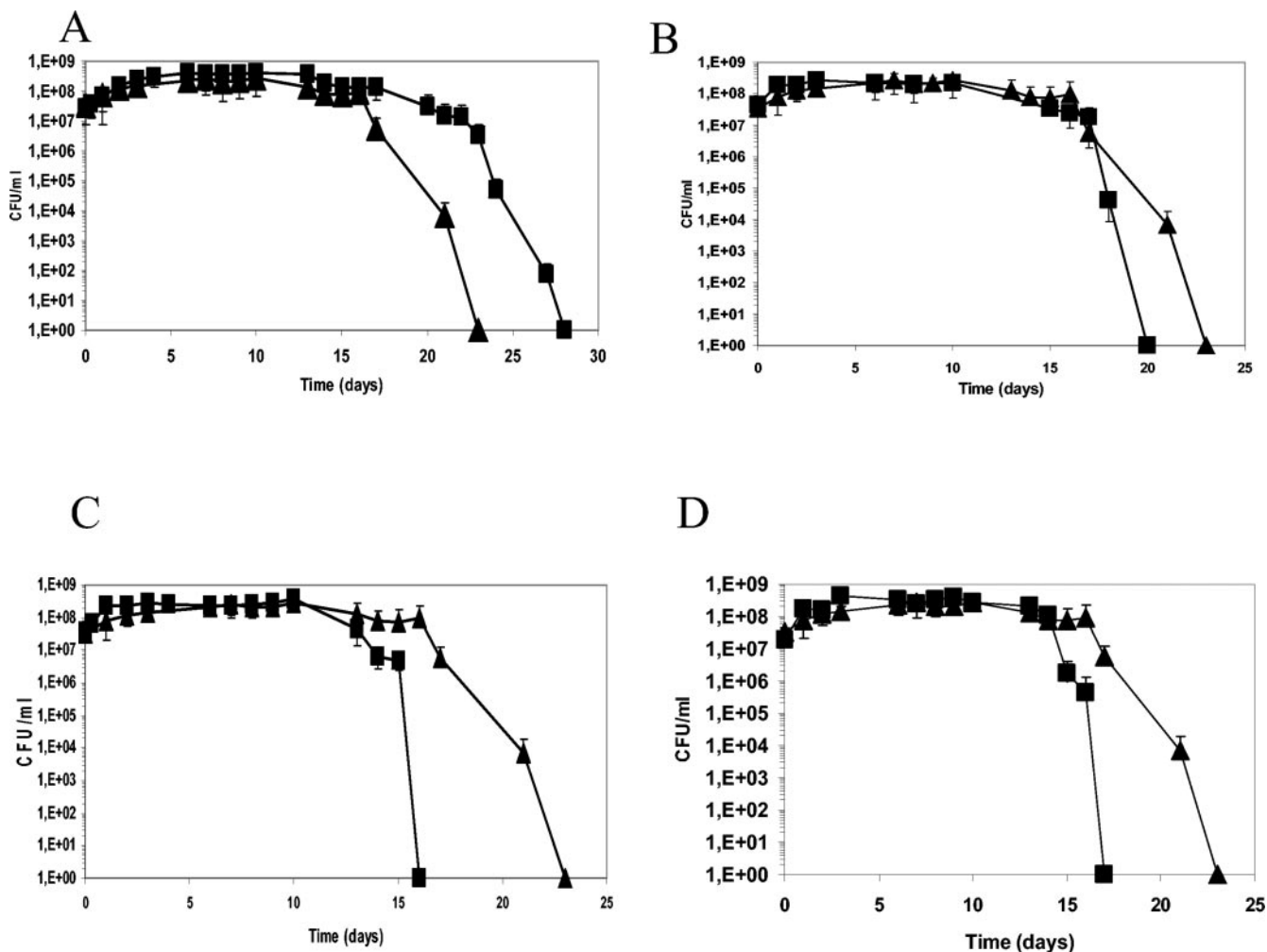
FIG. 4. Growth and survival of the wild-type strain (triangles) and various mutants (squares) at 4°C in MCD with 0.5% glucose. (A) RV4047 (Δ*glpD*); (B) RV4046 (Δ*usp*); (C) RV4045 (Δ*asp-2*); (D) RV4048 (Δ*msrA*). Values are means from three independent experiments. Error bars, standard deviations.

after a cold stress in *E. faecalis* (35). Concerning repressed proteins, in *L. lactis* MG1363, most of them are repressed by salt and about 30 are repressed after a cold shock (53). In *L. sakei* only three proteins are repressed, two by salt and one by low temperatures only.

In the present study, we analyzed six proteins which are affected during growth of *L. sakei* at low temperatures or in the presence of salt. Two are proteins belonging to carbon metabolic pathways, and four are general stress proteins.

One of the two proteins involved in the metabolic pathway is the PFK. It is underexpressed either at low temperatures or in the presence of NaCl. PFK catalyzes the conversion of fructose-6-phosphate into fructose-1,6-bisphosphate in the glycolytic pathway, the central and constitutive route of carbohydrate catabolism, and its role for catabolic repression is known (8). Our results suggest that the *L. sakei* PFK activity, and therefore the flux through the glycolytic pathway, decreases at 4°C or in the presence of NaCl. In *B. subtilis*, low temperatures induce glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzing one step of glycolysis, and HPr, involved both in

sugar uptake and in catabolite repression (17). However, it has been demonstrated for *L. lactis* that GAPDH is not induced, whereas both HPr and CcpA are induced, by low temperatures (52). It is possible that in *L. sakei*, CcpA and HPr are induced, as in *B. subtilis*, by glucose 6-P (11) and thus can control the transcription of genes involved in cold or salt adaptation in *L. sakei*.

The second protein for which expression varied with temperature or salt, and which is also involved in carbon metabolism, is G3PDH, belonging to the glycerol catabolic pathway. It is known that glycerol metabolism is linked to membrane properties. G3P can be converted to phosphatidic acid, which leads to membrane phospholipid synthesis. In the *glpD* mutant, G3P catabolism into dihydroxyacetone phosphate is no longer possible, and all glycerol metabolism might then be redirected into phosphatidic acid synthesis, which becomes available for phospholipid biosynthesis, used to maintain membrane integrity. This might explain the enhanced survival of the mutant at 4°C. However, it is contradictory to the fact that GlpD is overexpressed at low temperatures. It has been shown for *E.*

*coli* that membrane permeability was reduced in a *glpF* mutant, leading to better resistance to ethanol (48). We can hypothesize that modification of glycerol metabolism in the *glpD* mutant might confer such modified membrane properties, leading to the enhanced survival observed. Mutants with mutations in the other four genes encoding general stress proteins (*msrA*, *asp-2*, *usp*, and *ohr*), like the *glpD* mutant, reveal no difference during growth at 30°C, at 4°C, or with 4% NaCl. Nevertheless, three of the four proteins seem to play a role during survival, since the survival of mutants was affected. These results demonstrate that proteins expressed during growth can play an important role in survival during stationary phase.

It is known that in other bacteria, MsrA is involved in the repair of oxidative damage of proteins, and its function seems to be related to protection of the cell against oxidative stress. Our results demonstrate that in *L. sakei*, MsrA is induced at low temperatures and is necessary for optimal survival during stationary phase.

One of the proteins induced by cold or NaCl presents homology to Asp-23 of *S. aureus* and Gls-24 of *E. faecalis*. In *S. aureus*, Asp-23 is induced by alkaline shock and is under the control of $\sigma^B$ (12). In *E. faecalis*, Gls-24 is induced by different stresses and by glucose starvation. As in *E. faecalis*, the *L. sakei gls-24* gene is duplicated. In *E. faecalis*, a *gls-24* mutant showed a longer doubling time than the wild-type strain, but its survival was identical under conditions of glucose starvation or heat, hydrogen peroxide, CdCl$_2$, acid pH, basic pH, or ethanol shock. A reduction in survival was observed only in the presence of 0.3% NaCl. Such results were not observed for the mutant with a mutation of the second copy of *gls-24*, a phenotype which was similar to that of the wild-type strain (15). In *L. sakei*, the growth of the *asp-2* mutant is not affected by salt, but survival is reduced at 4°C. In that case we cannot exclude a polar effect of the mutation. The *asp-1* and *asp-2* genes are probably both involved in the stress response.

We also identified an Usp homolog protein. In *E. coli*, UspA has been proposed to have a general protective function related to the growth arrest state. Its induction results from transcriptional activation of a single $\sigma^{70}$-dependent promoter (33). Growth of an *uspA* mutant of *E. coli* is not affected, but increased sensitivity to complete and prolonged starvation conditions, or to various stresses, including osmotic shock, has been reported (34). The phenotype of the *L. sakei usp* mutant is similar to that observed for the *E. coli usp* mutant with regard to survival, except that it does not seem to be involved in an osmotic stress response.

The last protein identified was Ohr. Ohr is known to be involved in organic peroxide protection (1, 32). Osmotic stress has not yet been reported to induce expression of the *ohr* homologs. In *L. sakei*, the homologous Ohr protein is induced by low temperatures and salt. In *E. faecalis*, an *ohr* gene encodes a general stress protein named Gsp-65, induced by different stresses (heat, pH, SDS, bile salts, ethanol, NaCl, tBOOH [*tert*-butylhydroperoxide]) (39). The *gsp-65* mutant had no clear phenotype relative to the wild-type strain regarding response to these stresses. Only a higher sensitivity to tBOOH, an oxidative agent, was observed (39). Survival of the *gsp-65* mutant was not significantly affected by NaCl, heat shock, pH, detergent, or H$_2$O$_2$, but the mutant was less resistant to the oxidative stress generated with tBOOH and etha-

nol. This might be explained by the involvement of another protein(s) which compensates for the Ohr deficiency. Like those of the *E. faecalis gsp-65* mutant, the growth and survival of *L. sakei* RV4044 are not affected in the presence of salt or low temperatures.

This work provides an initial insight into the mechanisms of adaptation of *L. sakei* to its environment and illuminates the role of key proteins indispensable for survival of this species. This complex regulation should be further elucidated by analysis of protein patterns in the mutant strains and further identification of other proteins.

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