

Ribose Utilization in *Lactobacillus sakei*: Analysis of the Regulation of the *rbs* Operon and Putative Involvement of a New Transporter

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Abstract

A 7-kb DNA fragment of *Lactobacillus sakei*, containing the *rbsD*, *rbsK* and *rbsR* genes was sequenced. The genes responsible for ribose utilization are organized differently from what was previously described for model organisms such as *Escherichia coli* and *Bacillus subtilis*. No gene encoding RbsA, RbsB and RbsC, the subunits of the ribose ABC-transporter, were present in the *rbs* gene cluster. Instead, we found an open reading frame coding for RbsU, a protein similar to GltA, the glucose transporter of *Staphylococcus xylosus*. The disruption of *rbsK*, encoding the ribokinase, impaired growth on ribose. The disruption of *rbsR*, encoding the repressor, had no effect on the ability to grow on ribose, but led to overexpression of a large transcript corresponding to *rbsU*, *rbsD* and *rbsK*, suggesting that RbsU might be involved in ribose utilization. Ribose uptake and phosphorylation assays on the wild type strain and various mutants showed that, in *ptsI* mutants, both ribose uptake and phosphorylation are increased. These increased activities can explain the faster growth rate on ribose that was observed in *ptsI* mutants. The phosphotransferase system is thus involved in the negative regulation of ribose utilization. This regulation might not act at the transcriptional level since the overexpression of the *rbs* genes in the *rbsR* mutant did not lead to the same phenotype. A gene sharing high similarity scores with *ackA* genes, encoding the acetate kinase, was found upstream from the *rbs* gene cluster. The unusual location of this gene is maybe not fortuitous since acetate kinase is involved in ribose catabolism.

Introduction

Lactobacillus sakei is the predominant flora naturally found on meat and meat products. This non-pathogenic lactic acid bacterium is also used for the fermentation of meat in the industrial production of sausages. Meat is a rich substrate allowing the development of several microorganisms, some being detrimental to the hygienic

safety and quality of meat. *L. sakei* is of industrial interest since its ability to develop on meat allows an antagonistic effect on such spoilage or pathogenic organisms. However, meat is a poor source of carbohydrates. Among the few sugars available on fresh meat, ribose and glucose are the main carbon sources that can be used by *L. sakei* to grow in this environment. In order to understand and control the growth of this species we investigated the sugar metabolism. In a previous study, we have shown that glucose is transported by the phosphotransferase system (PTS) and by at least one additional non-PTS permease (Lauret *et al.*, 1996). The cloning of the *ptsHI* operon of *L. sakei*, encoding the two general enzymes of the PTS, allowed the construction and analysis of *ptsI* mutants (Stentz *et al.*, 1997). As expected, the *ptsI* mutations impaired growth on PTS-sugars such as fructose, mannose and sucrose, but the mutants grew faster on ribose, which is not transported by the PTS. This suggested that, in the wild type strain, some components of the PTS negatively regulate ribose utilization. This phenotype is not observed in other bacteria such as *Bacillus subtilis*, where the involvement of the PTS in many regulatory mechanisms is well known (for reviews, see Deutscher *et al.*, 1997; Saier *et al.*, 1996). In order to establish whether the regulation of ribose utilization in *L. sakei* was due to a new regulatory mechanism, we cloned the gene cluster encoding the enzymes responsible for ribose catabolism. In *Escherichia coli* and *B. subtilis*, the *rbs* operon encodes the four subunits of the ribose ABC-transporter, RbsA, RbsB, RbsC and RbsD. RbsA is the nucleotide-binding protein, RbsB the ribose-binding protein, RbsC and RbsD are the membrane components of the permease. The ribokinase and the repressor are encoded by *rbsK* and *rbsR*, respectively, two genes which are also located in the *rbs* operon. The gene order differs in *B. subtilis* (*rbsRKDACB*) and *E. coli* (*rbsDACBRK*). In *B. subtilis*, the results of the chromosome sequencing showed that *ywsB*, an additional open reading frame (ORF) of unknown function, is located downstream from *rbsB*, and might belong to the same operon since the transcription terminator is found downstream from *ywsB* but not between *rbsB* and *ywsB* (Biaudet *et al.*, 1996). In *E. coli*, it was shown that the ABC-transporter is the high affinity transport system for ribose uptake and that a second system, with a lower affinity could also transport the ribose (Lopilato *et al.*, 1984). David and Wiesmeyer (1970) mentioned a constitutive permease, which could transport both glucose and ribose. In *B. subtilis* the regulation of the *rbs* operon is controlled by the RbsR repressor. The presence of a CRE sequence (Catabolite Responsive Element) located between the promoter and the start codon of *rbsR* showed that this operon is also controlled by catabolite repression (Woodson and Devine, 1994). In Gram-positive bacteria it was shown that the CRE sequences are present in many genes regulated by catabolite repression, a mechanism which involves the

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binding of the catabolite control protein CcpA to the CRE elements (Hueck *et al.*, 1994 and references therein). However, CcpA and the repressor RbsR belong to the same GalR/LacI family of transcriptional regulators, and the putative involvement of RbsR in the catabolite repression of the *rbs* operon of *B. subtilis* was mentioned (Woodson and Devine, 1994).

The aim of the present study was to clone the *rbs* operon of *L. sakei* and to investigate the regulation of ribose utilization. The sequencing of a 7-kb DNA fragment encompassing the *rbsDKR* genes showed that *rbsABC* are not present in the *rbs* operon, but instead of these genes, we found a gene encoding a protein similar to a glucose transporter. The transcriptional analysis of this operon showed that it is not regulated by the classical catabolite repression, but rather by a different mechanism.

Results

Molecular Cloning of the *rbsK* and *rbsR* Genes

Part of the *rbsK* gene of *L. sakei* was cloned by hybridization using a heterologous probe corresponding to an internal fragment of the *rbsK* gene of *Lactobacillus pentosus* cloned in the plasmid pXH37A (kindly provided by C. Lokman and P. Pouwels). An *EcoRI/HindIII* fragment of pXH37A was used as a probe in a low stringency hybridization experiment. The amino acid sequence deduced from the *L. pentosus* DNA fragment shared high similarity with the ribokinases of *B. subtilis* and *E. coli* (C. Lokman and P. Pouwels, personal communication). The fragment was thus used as a probe to determine the restriction map of the *rbsK* region on the *L. sakei* chromosome by Southern hybridization experiments (Figure 1B) and to clone by colony hybridization a 699 bp *HincII* DNA fragment of *L. sakei* 23K, leading to plasmid

pRV51 (Figure 1A). The *HincII* fragment was then used as a probe to clone a 1497 bp *HindIII* fragment and a 448-bp *HindIII* fragment leading to pRV53 and pRV54, respectively. Several attempts to clone, by the same hybridization procedure, a larger *ClaI* fragment were unsuccessful. The cloning of a DNA fragment, adjacent to those already cloned, was then performed after PCR amplification. Two primers, derived from the insert cloned in pRV53 and from the pBluescript plasmid, were used in a PCR experiment, using as template a ligation mixture, which contained pBluescript, and 23K chromosomal DNA digested with *SspI* and *ClaI*. The PCR product was then cloned in pBluescript leading to pRV55 (Figure 1A).

A Chromosome Walking Experiment using PCR Allowed the Sequencing of the Upstream Part of *rbsK*

To confirm that one of the cloned genes encodes the ribokinase and to show that only one copy of this gene is present in *L. sakei* 23K chromosomal DNA, we constructed a *rbsK* mutant by chromosomal integration (Figure 2). For this purpose, the *HincII* fragment of pRV51 was cloned in the integrative vector pRV300 leading to pRV52. This plasmid was used to transform the *L. sakei* 23K strain for erythromycin resistance. The integration of pRV52 by single-crossover recombination at the *rbsK* locus was verified by a PCR experiment on chromosomal DNA extracted from transformants, with primers located upstream and downstream from the insert cloned in pRV52. The resulting recombinant strain RV1007 was unable to grow on MCD supplemented with ribose. The RV1007 chromosomal DNA was digested using the *EcoRI* restriction enzyme (Figure 2). The digested DNA was then ligated upon itself. A 4.5 kb DNA fragment was then amplified by PCR using the ligated DNA as template and *rbsA1*, *rbsA2* as primers. *rbsA1* and *rbsA2* were designed from

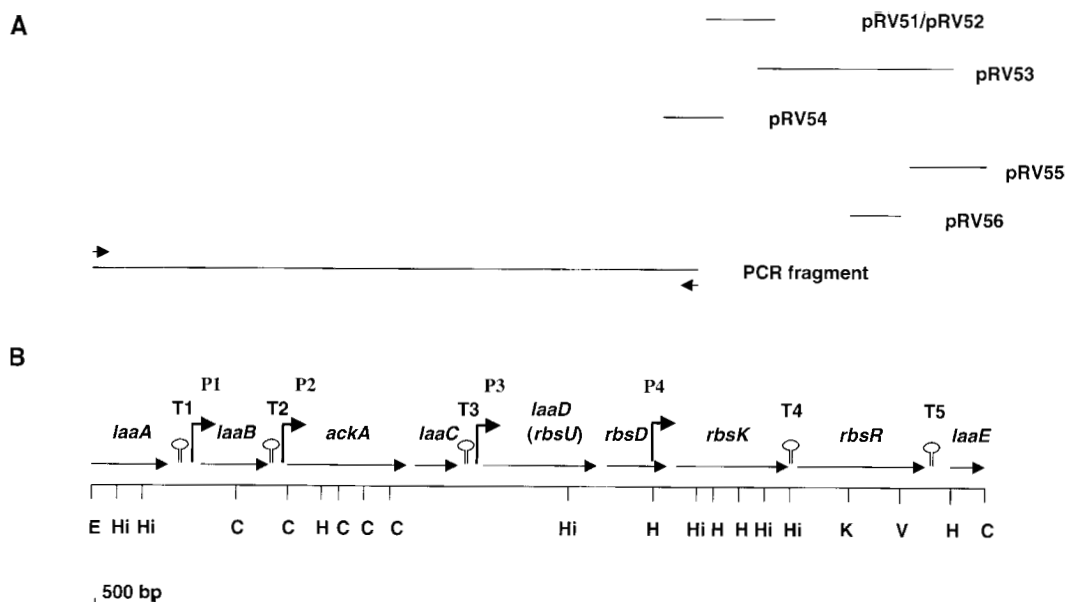


Figure 1. Map of the *rbsK* Region

A. The plasmids used in this study and the position of a large PCR fragment are indicated. PRV51, 53, 54 and 55 contain inserts cloned in pBluescript. PRV52 and pRV56 contain inserts cloned in the integrative vector pRV300. The primers *rbsA2* and *rbsA4* used for the amplification of a PCR fragment are indicated. B. Restriction map of the 7 kb DNA region encompassing the *L. sakei* *rbs* operon. The putative promoters and terminators are indicated as well as the position and orientation of the different ORFs. The restriction sites are indicated for *EcoRI* (E); *HincII* (Hi); *ClaI* (C); *HindIII* (H); *KpnI* (K); *EcoRV* (V).

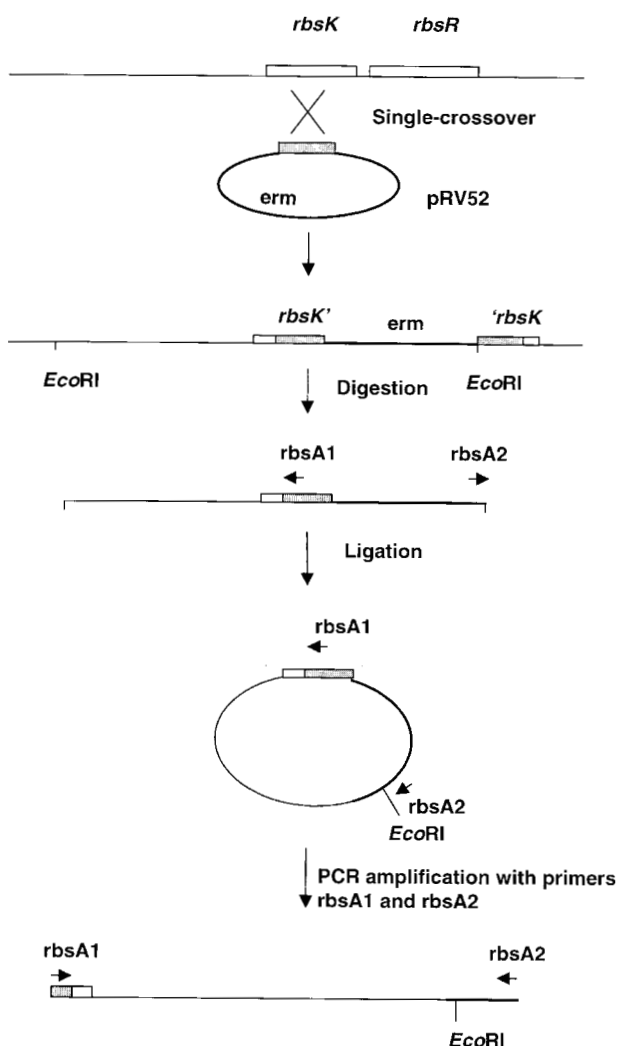


Figure 2. A Chromosome Walking Experiment using PCR Allowed the Sequencing of the Upstream Part of *rbsK*.

sequences of pRV300 and the insert of pRV52, respectively. The sequence of the PCR product extremities allowed us to design the oligonucleotide *rbsA4*. The two primers *rbsA2* and *rbsA4* were then used to amplify a 4.2 kb PCR product directly from the *L. sakei* 23K chromosomal DNA (Figure 1A).

Nucleotide Sequence and Deduced Amino Acid Sequences of the *rbs* Operon Region

The DNA sequence of the different overlapping fragments was determined. The nucleotide sequence of 6,907 bp, starting at a *EcoRI* site and ending at a *Clal* site, was obtained. The size and orientation of the different ORFs as well as putative promoter and terminator sequences are shown Figure 1B. A 396-nucleotide ORF, corresponding to *rbsD* and encoding the 132-amino-acid RbsD protein, was found. Twenty nucleotides downstream from the TAA stop codon is *rbsK*. *rbsK*, which encodes the ribokinase, is 909 nucleotides (303 codons) long and stops at a TAA triplet. Eighty-one nucleotides downstream from the TAA stop codon, a third ORF, *rbsR*, was observed (1008

nucleotides) which encodes a protein identified as RbsR (336 amino acids), the ribose operon repressor protein. *rbsR* probably starts at a GTG triplet, since no other start codon is present at the 5'-end of the gene. Furthermore, a putative ribosome binding site is located six nucleotides upstream from the GTG triplet. A sequence resembling a transcription terminator signal, which consists of a palindromic sequence able to form a stem-loop structure (ΔG° , -6.14 kcal/mol) followed by a stretch of seven T's, was observed 51 nucleotides downstream from the stop codon (T5, Figure 1).

Surprisingly, twenty one nucleotides upstream from the ATG start codon of *rbsD*, there was an ORF (*laaD*) which shared no homology with *rbsA*, *rbsB* or *rbsC*, which are found in *E. coli* and *B. subtilis* *rbs* operons. *laaD* is 885 bp long and encodes a 295 amino-acids protein. A sequence resembling a transcription terminator, which consists of a perfect palindromic sequence able to form a stem-loop structure (ΔG° , -19.37 kcal/mol) followed by a stretch of seven T's, was found 172 nucleotides upstream from *laaD* (T3, Figure 1B). Between this transcription terminator and the start codon of *laaD*, two motifs, resembling a Gram-positive promoter region containing the putative TATAAT -10 box and the putative TTGAAT -35 box, are present (P3, Figure 1B).

A short ORF, *laaC*, was found upstream from *laaD*. *laaC* might encode a 82 amino acid peptide, since a putative ribosome binding site (GGAGTG) is present eight nucleotides upstream from the start codon. Upstream from *laaC*, we found an ORF, which was named *ackA*, since it encodes a 394 amino acid protein sharing high similarity with the acetate kinase of other microorganisms. *ackA* and *laaC* are separated by only 15 bases. Motifs resembling a promoter were found upstream from *ackA* (P2, Figure 1B). It is then probable that these two genes are cotranscribed from the promoter P2, to the terminator T3.

Upstream from this promoter P2, a perfect palindromic structure (ΔG° , -20.43 kcal/mol) followed by a stretch of six T's was observed (T2, Figure 1B). Preceding T2 is *laaB*, encoding a 181 amino acid peptide with a ribosome binding site eight nucleotides upstream from the start-codon. A sequence, which could correspond to a promoter region, was observed 151 nucleotides upstream from the *laaB* start codon. *laaB* might thus be a monocistronic unit.

Further upstream, and to the *EcoRI* site, the 3'-end of an incomplete ORF (*laaA*) with a length of 535 bp was observed. A palindromic structure (ΔG° , -20.41 kcal/mol) followed by a stretch of seven T's was observed between *laaA* and the *laaB* promoter. At the other extremity, and to the *Clal* site, the 5'-end of a 1303 bp incomplete ORF, *laaE*, was observed 98 nucleotides downstream from *rbsR*.

Study of the Amino Acid Sequence Deduced from the Different Genes

The product of the *rbsD* gene shares 43% identity with RbsD of *E. coli* (Bell *et al.*, 1986), 41% identity with RbsD of *Haemophilus influenzae* (GenBank accession N° U32732, gene identification N° HI0501) and 39% identity with RbsD of *B. subtilis* (Woodson and Devine, 1994). Gene *laaD* (located upstream from *rbsD*) and its product is similar (34% identity) to the *gltA* product of *Staphylococcus xylosus* (GenBank accession N° Y14043) which was defined as a glucose uptake protein. *laaD* has been named *rbsU* (for ribose uptake). It shares also high similarity with the product

of *ycxE* of *B. subtilis* (GenBank accession N° Z99106, 39% identity) and with ORF2 of *Bacillus megaterium* (GenBank accession N° D90043, 37% identity). *gltA*, *ycxE* and *ORF2* are all located upstream from the *gdh* gene encoding a glucose dehydrogenase. No homology to any of the ORFs present in the 7-kb DNA could be found with RbsA, RbsB or RbsC, the subunits of the ribose ABC transporter.

Located downstream on the chromosome, the *rbsK* gene of *L. sakei* encodes a protein that shares 42% identity with the ribokinase from *E. coli* (Hope *et al.*, 1986), 41% identity with the one from *H. influenzae* (GenBank accession N° U32732, gene identification N° HI0505), 38%, 36%, and 32% with the ribokinases from *Schizosaccharomyces pombe* (GenBank accession N° AL023554), *Rhizobium etli* (GenBank accession N° AJ001389) and *B. subtilis* (Woodson and Devine, 1994), respectively. The *rbsR* gene encodes a protein that is 32% identical to RbsR from *E. coli* (Mauzy and Hermodson, 1992), 31% identical to the ribose repressor from *H. influenzae* (GenBank accession N° U32732, gene identification N° HI0506) and 30% identical to RbsR from *B. subtilis* (Woodson and Devine, 1994). It also shares 32% identity with PurR of *H. influenzae* (gene identification N° HI1635).

The N-terminal region of the product derived from *laaE*, located downstream from *rbsR* shares no homology with known proteins found in the database. However, a significant similarity (30% identity, 48% similarity) was found between the 83 N-terminal amino-acids of *LaaE* and the N-terminal amino-acids of a protein from *Mycobacterium tuberculosis*, the function of which is unknown (GenBank accession N° Z80775, protein identification N° Rv0047c).

Located upstream from the *rbs* gene cluster, the small ORF *laaC* has no homology with any of the proteins found in the database. The product of *ackA* shares high similarity with the acetate kinase from *B. subtilis* (Grundy *et al.*, 1993) (53% identity) and from other microorganisms. It could thus encode the acetate kinase of *L. sakei*. In several bacteria, both *ackA* and the *rbs* operon have been sequenced but were not shown to be genetically linked. The presence of *ackA* near the *rbs* operon in *L. sakei* might have a physiological significance, since acetate kinase is involved in the heterofermentative pathway of ribose catabolism.

The *laaB* gene product shares 21% identity on 116 amino acids with an hypothetical transcriptional regulator from *B. subtilis* (GenBank accession N° U89914). In the database, this protein is proposed to belong to the MarR family transcriptional regulators. Finally, the sequence of the 178 C-terminal sequence derived from the partial *laaA* ORF are 40% identical to the product of ORF1 from *L. sakei* (GenBank accession N° X98238) described as a putative dipeptidase.

Construction of *L. sakei ptsI* Mutants

In a previous study, *ptsI* mutants have been constructed by insertional mutagenesis (Stentz *et al.*, 1997). The phenotype of these mutants suggested a negative regulation of ribose utilization by the PTS. In order to investigate the role of enzyme I and HPr in this regulation, different mutants were isolated. For this purpose, a new strategy was developed allowing the construction of stable chromosomal mutants by two successive crossovers (Stentz *et al.*, 1999). A deletion of the *ptsI* gene encoding

enzyme I of *L. sakei* was constructed. Since the *ptsHI* operon of *L. sakei* 160X1K was cloned and sequenced (Stentz *et al.*, 1997), oligonucleotides deduced from this sequence were designed in order to amplify two internal fragments corresponding respectively to the 5'-end and 3'-end of *ptsHI*. The first PCR fragment, obtained with the primers delhi3 and delhi4, was 838 bp long and contained the *ptsHI* promoter, *ptsH*, and 413 bp of the *ptsI* 5'-end. The second fragment, obtained with the primers delhi5 and delhi6, was 810 bp long and contained 135 bp of the 3'-end of *ptsI* and the downstream region of the *ptsHI* operon. Each primer contained a restriction site at its 5'-extremity. The two resulting PCR fragments were cloned in the pRV300 integrative vector leading to plasmid pRV22. In pRV22, the *ptsHI* operon contains a 1164 bp deletion in *ptsI*. The plasmid was then used to transform *L. sakei* 23K and the transformants resulting from a single-crossover recombination were selected for erythromycin resistance. The insertion of pRV22 in the *ptsHI* operon was checked by PCR using the delhi3 and delhi6 primers (located at both extremities of the pRV22 insert) and primers from pRV300. Since pRV22 contains the 5'- and the 3'-ends of the *ptsHI* operon, its insertion in the chromosome by a single-crossover in *ptsHI* restored one copy of the wild type operon and was therefore not mutagenic. In order to generate a second crossover, the resulting RV1012 recombinant strain was grown on MRS without erythromycin. After 100 generations, diluted culture aliquots were plated on MRS, and then clones were analyzed for their ability to grow on erythromycin and on MCD medium supplied with fructose. Among 600 clones, 10 clones were erythromycin sensitive including one clone which was unable to grow on MCD supplied with fructose, a PTS-sugar in *L. sakei* (Lauret *et al.*, 1996; Stentz *et al.*, 1997). In this clone, the second crossover should have resulted in the excision of the plasmid and the wild type part of *ptsI*, leading to a single mutated *ptsI* copy on the chromosome. The *ptsI* deletion in the resulting *pts*-recombinant RV2006 strain was checked by a PCR experiment on chromosomal DNA using the primers delhi3 and delhi6. The *ptsI* gene had indeed the expected deletion.

In order to construct a *L. sakei ptsI* point mutation, the primers pairs (mdh190A, mdh190B) and (mdh190C, mdh190D) were used for the amplification of the upstream and downstream region, respectively, of the CAT triplet which encodes His190 of enzyme I, the residue which is phosphorylated by PEP (Alpert *et al.*, 1985). The primers mdh190B and mdh190C are complementary and each contains a GCT triplet encoding an alanine which replaced the CAT triplet. The two resulting fragments of 435 bp and 437 bp had a 26 bp overlapping extremity. They were used as template in a PCR experiment using the primers mdh190A and mdh190D, respectively. A 835-bp fragment was obtained, the sequence of which confirmed the mutation of the CAT triplet to GCT. This fragment was cloned in the pRV300 integrative vector leading to pRV23. This plasmid was used to transform the *L. sakei* 23K strain. The resulting strain, RV1013, was *pts* since the insertion resulted in two partial copies of *ptsI*. By the same procedure as described above, the second crossover was obtained by growing RV1013 without erythromycin. Only 60 generations were necessary to obtain the second

crossover. Among 400 clones, 211 clones were erythromycin sensitive. Five clones were still *pts*⁻ as deduced for their inability to grow on fructose and might thus have kept the mutated copy of *ptsI*. One transformant, RV2007, was used for further experiments. The presence of the expected H190A mutation was confirmed by sequencing the 800 bp surrounding the mutation, on a PCR fragment obtained after amplification on the chromosomal DNA extracted from RV2007.

In order to discriminate between the putative involvement of enzyme I and/or HPr in the negative regulation of ribose utilization, several attempts were made to construct a *ptsH* mutant by the same method as described above. However, no stable plasmid containing inserts with the wild type or mutated *ptsH* gene of *L. sakei* designed to be used in a double crossover experiment could be obtained in *E. coli*. The small size of *ptsH* (264 bp) makes it also impossible to construct a mutant by single crossover, the minimum size for insertion in the *L. sakei* chromosome being estimated to be 300 bp (Leloup *et al.*, 1997). Moreover, such an insertion would have a polar effect and would result in a *ptsHI* mutation. A collection of 2DG resistant mutants was therefore isolated from the 23K strain. Among 33 2DG resistant mutants, 2 were unable to grow on PTS sugars (fructose, sucrose, mannose) and were thus suspected to be mutated in *ptsH* or *ptsI* as was previously observed in 2DG resistant mutants of *L. sakei* (Lauret *et al.*, 1996). In order to determine whether HPr and/or enzyme I were present in those two mutants, western blot experiments were performed on crude extracts of these two mutants, as well as on RV2006, RV2007 and the wild type strain. The results are shown in Figure 3. With antibodies raised against enzyme I from *Staphylococcus carnosus*, we could detect a protein of 67 kDa, which is the expected size of the enzyme I from *L. sakei*. The purified enzyme I was clearly detected by the antiserum, but had an apparent molecular weight slightly larger than the band observed in crude extracts, certainly because of the six additional histidine residues at the N-terminus of the protein. This band was apparent in the wild type strain, and RV2007 which possesses the H190A mutation in *ptsI*, and absent in RV2006 ($\Delta ptsI$) and the two 2DG resistant mutants. A band of smaller size was detected in the extracts of the two 2DG resistant mutants, that might result from proteolysis of enzyme I or in the synthesis of a shorter enzyme I due to the presence of a stop codon responsible for the phenotype of these mutants. The size of the shortened enzyme I was identical in both mutants.

The use of antibodies raised against HPr from *S. carnosus*, allowed the detection of a small band, present in all the mutants and migrating slightly lower than the His-tag purified HPr (Figure 3). This shows that the two mutants selected for their 2DG resistance were very similar and were mutated in *ptsI*, but not *ptsH*. One of the 2DG resistant mutants, RVrib9 was used for further experiments to study the regulation of ribose utilization.

The growth on ribose of all the mutant strains was accelerated as was previously observed in several *ptsI* mutants obtained by single crossover integration (Stentz *et al.*, 1997).

Transcriptional Analysis

Northern experiments were performed with RNA prepared from *L. sakei* 23K, RV2006 and RV1011 in MCD in the

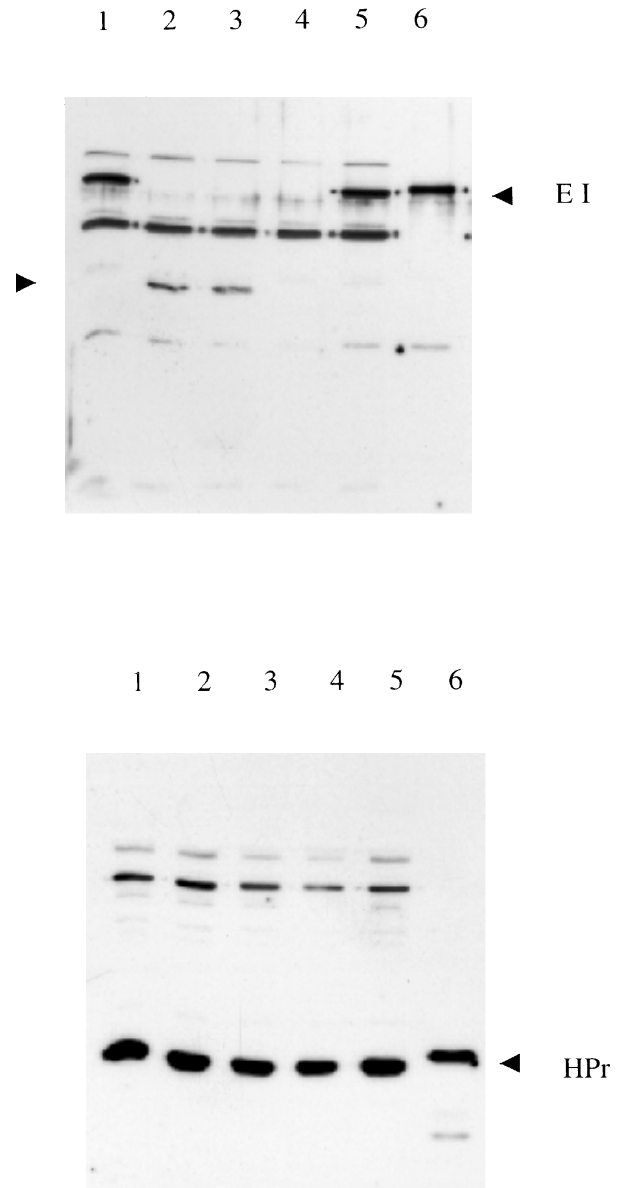


Figure 3. Western-Blot Experiments
Immuno-detection of enzyme I and HPr in crude extracts of 23K (lane 1), RVrib9 (lane 2), RVrib10 (lane 3), RV2006 (lane 4) and RV2007 (lane 5). Arrows at the right indicate the purified His-tag enzymes (lane 6). The left arrow indicates the truncated enzyme I observed in RVrib9 and RVrib10.

presence of glucose or ribose. RV1011 is a *rhsR* mutant constructed by chromosomal integration using the plasmid pRV56. The use of a PCR product containing an internal fragment of *rhsK* revealed several transcripts (Figure 4): a large transcript of 2.6 kb, another of 1.6 kb and a small one of 1.1 kb. When the probe used was a PCR fragment containing an internal fragment of *rhsU*, only one transcript of 2.6 kb could be observed. In both experiments, the amount of the 2.6 kb transcript is much higher for cells of the wild-type strain grown on ribose compared to cells grown on glucose. This 2.6 kb band should correspond to the same transcript, which hybridizes with the *rhsU* and *rhsK* probes, since it is submitted to the same regulation. Moreover, in RV1011, where the repressor RbsR is absent,

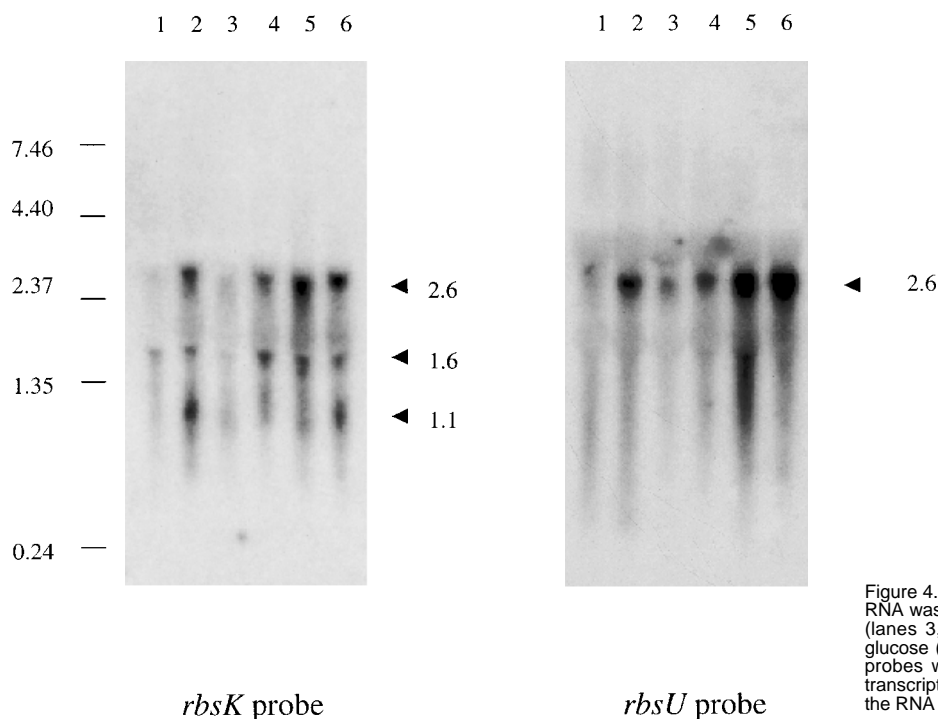


Figure 4. Northern-Blot Experiments
RNA was extracted from 23K (lanes 1, 2), RV2006 (lanes 3, 4) and RV1011 (lanes 5, 6) grown on glucose (lanes 1-3-5) or ribose (lanes 2-4-6). Two probes were used as indicated. The size of the transcripts is indicated with arrows, the position of the RNA ladder is shown.

the transcription level is similar for cells grown on glucose or on ribose. This large transcript should therefore contain *rbsU*, *rbsD* and *rbsK*. Its transcription should start at the P3 promoter and end at a transcription terminator located downstream from *rbsK*. According to the size of this transcript, this terminator should be located within *rbsR*. However, no rho-independent transcription terminator was observed in *rbsR*. The 1.6 kb transcript should contain only *rbsD* and *rbsK*, since it was not observed when an internal fragment of *rbsU* was used as a probe. The small transcript might encode *rbsK* alone. Upstream from *rbsK*, at position 4294 of the nucleotide sequence, a -35 and a -10 box of a putative promoter was observed (P4, Figure 1B). Located between *rbsK* and *rbsR*, there is a palindromic structure (ΔG° , -9.18 kcal/mol) followed by a stretch of five T's which might be a terminator of transcription (T4, Figure 1B). The distance between this promoter and this terminator fits with the 1.1 kb size of the small transcript.

In order to determine whether the low level of transcription of these genes in cells grown on glucose is only due to repression by RbsR or if there is also catabolite repression by glucose, RNA extraction was also performed on RV1011 cells, the *rbsR* mutant. In this mutant, the level of the 2.6 kb transcript and the 1.6 kb transcript is the same whatever sugar is used for growth. The low level of transcription for wild type cells grown on glucose should therefore be due to a repression by RbsR rather than to catabolite repression.

To determine whether the absence of enzyme I in *L. sakei* is responsible for an increase of the *rbs* operon transcription, RNAs extracted from the RV2006 *ptsI* mutant were hybridized in Northern experiments. The level of transcription of *rbs* genes in RV2006 and in the wild type strain grown on ribose is the same suggesting that the accelerated growth of RV2006 on ribose is not due to an increase of transcription of these genes. The amount of

rbs transcripts is low both in the wild type strain and in RV2006 grown on glucose. Surprisingly, the small transcript could not be observed in RV2006, even when grown on ribose. Moreover, it is also not observed in RV1011, the *rbsR* mutant, grown on glucose. These results suggest that the small transcript encoding *rbsK* might be regulated differently from the larger ones.

Ribose and Glucose Uptake and Phosphorylation Measurements

To address the question whether the accelerated growth of *ptsI* mutants is correlated to an increase of the ribose transport or of the ribose phosphorylation, ribose uptake and phosphorylation measurements were performed, in the wild type strain, *ptsI* mutants, and in the RV1011 *rbsR* mutant (Tables 1 and 2). The ribose uptake in cells grown on ribose is similar in all the *ptsI* mutants. Ribose is translocated at an initial rate of 9-10 nmol/min/mg [dry weight]. This rate is slightly higher than in the wild type strain (6.6 nmol/min/mg [dry weight]). For RV1011 (*rbsR*), the uptake is comparable to the level observed in the wild type, although the *rbs* operon is derepressed. Similarly, the ATP-dependent phosphorylation of ribose is identical in the three *ptsI* mutants (720-820 nmol/min/mg protein) whereas it is two fold lower in the wild type strain (381 nmol/min/mg protein). On the other hand, the ribose phosphorylation is lower in RV1011 (191 nmol/min/mg protein) than in the wild type.

Ribose transport and phosphorylation measurements were also investigated for RV1011 grown on glucose. The ribose uptake is three fold increased in the *rbsR* mutant compared to the wild type strain and the rate of ATP-dependent phosphorylation is of 106 nmol/min/mg protein whereas it could not be detected in the wild type strain. These results showed clearly that in the *rbsR* mutant the products of the genes involved in the uptake and

Table 1. Rates of Glucose and Ribose Uptake by Different *L. sakei* Strains Grown on Different Carbon Sources

Carbon source used for growth	Strain	Initial rate of uptake nmol/min/mg [dry wt] of:	
		Glucose	Ribose
Ribose	23K (WT)	-	6.6 ± 0.25
	RV2006 (<i>ptsI</i>)	-	10.1 ± 0.3
	RV2007 (<i>ptsI</i>)	-	10 ± 0.21
	RVrib9 (<i>ptsI</i>)	-	9.4 ± 0.41
	RV1011 (<i>rbsR</i>)	-	6.9 ± 0.31
Glucose	23K (WT)	3.7 ± 0.27	0.7 ± 0.03
	RV2006 (<i>ptsI</i>)	0.5 ± 0.03	-
	RV2007 (<i>ptsI</i>)	0.6 ± 0.04	-
	RVrib9 (<i>ptsI</i>)	0.7 ± 0.05	-
	RV1011 (<i>rbsR</i>)	-	2.2 ± 0.12

(-) = not determined

phosphorylation of ribose are no longer repressed when cells are grown in the absence of ribose.

The uptake of glucose in *ptsI* mutants and in the wild type strain grown on glucose was also investigated. The uptake of glucose in *ptsI* mutants was impaired (0.5-0.7 nmol/min/mg protein) and was seven fold lower than in the wild type strain. These results are supported by previous results where the growth of *ptsI* mutants was impaired on glucose (Stentz *et al.*, 1997). The slow glucose uptake in these mutants is due to a non-PTS glucose permease as previously described (Lauret *et al.*, 1996).

Discussion

As was discussed in a preceding study, the PTS seems to negatively regulate ribose utilization in *L. sakei* (Stentz *et al.*, 1997). In the present study, we have cloned the *rbs* operon involved in the ribose transport and phosphorylation. The analysis of the sequence revealed an unusual organization of the *rbs* operon in *L. sakei*. Indeed, although *rbsK* and *rbsR* are present in this operon, *rbsA*, *rbsB* and *rbsC* are missing. Several attempts to obtain an internal fragment of *rbsB* by PCR amplification, using degenerated oligonucleotides deduced from RbsB from other organisms, were unsuccessful. A PCR fragment of a size larger than expected was obtained, the sequence of which revealed that it was homologous to oligopeptide ABC transporters but not to RbsB. This suggests that at least RbsB is absent in *L. sakei* or that its sequence is different from the other known proteins. The *rbsD* gene is present since upstream from *rbsK* we found an ORF homologous to the *E. coli* and *B. subtilis* RbsD proteins. Upstream from *rbsD*, another ORF sharing similarity with a glucose transporter was found. This might indicate that in *L. sakei*, ribose is not transported as in *B. subtilis* and *E. coli* by an ABC-type transporter. A *rbsK* mutant was constructed. This mutant was unable to grow on ribose demonstrating that the cloned operon corresponds to the *rbs* operon. A *rbsR* mutant was also constructed. The growth of this mutant on ribose was not improved and it overexpressed a large transcript containing *rbsU*, *rbsD* and *rbsK*. No transcription terminator was observed between *rbsU* and *rbsD*. This demonstrates that the transcription of *rbsU* is linked to the transcription of *rbsD* and *rbsK*. The RbsU protein might thus be involved in the transport of ribose.

Table 2. ATP-Dependent Phosphorylation of Ribose in Different *L. sakei* Strains Grown on Different Carbon Sources

Carbon source used for growth	Strain	ATP-dependent phosphorylation of ribose: (nmol/min/mg protein)	
Ribose	23K (WT)	381 ± 13	
	RV2006 (<i>ptsI</i>)	821 ± 10	
	RV2007 (<i>ptsI</i>)	815 ± 9	
	RVrib9 (<i>ptsI</i>)	727 ± 8	
	RV1011 (<i>rbsR</i>)	191 ± 7	
Glucose	23K (WT)	ND	
	RV1011 (<i>rbsR</i>)	106 ± 2	

ND = not detected

Further analysis of ribose uptake and phosphorylation showed that the regulation of ribose utilization is different from what was described in the model bacterium *B. subtilis*. The doubling time of the *L. sakei* wild-type strain grown on ribose is 6 h 30 min ± 30 min and is reduced in all the *ptsI* mutants (2 h 40 min ± 15 min). Ribose uptake and phosphorylation measurements were performed in various *ptsI* mutants and in the wild type strain grown on ribose. Ribose transport is slightly increased in the *ptsI* mutants, but the ATP-dependent phosphorylation of ribose is two fold increased. The increase of the ribose transport might result from a faster phosphorylation. Neither ribose transport or ribose phosphorylation is completely abolished in a wild type background. Therefore, the accelerated growth rate on ribose that was observed in the *ptsI* mutants is not to be due to a complete derepression of ribokinase but rather to a subtle regulation. The PTS regulates thus negatively, by a direct or indirect way, the ribokinase, and possibly the ribose transporter. This regulation acts certainly not at the transcriptional level since the *rbsR* mutant, which overexpressed the *rbs* genes, does not grow faster on ribose and its ribose transport and phosphorylation rates are not increased. It might then be a post-transcriptional regulation or a modulation of the enzyme activities since the transcription in the *ptsI* mutant is not increased whereas the ribose phosphorylation is two fold increased.

In the *rbsR* mutant grown on glucose, the transcription of the *rbs* operon is increased and is comparable to the transcription level that is observed on ribose. The repression of the ribose transport, ribose phosphorylation and *rbs* transcription, observed in the wild type grown on glucose, is thus due to RbsR. This shows that the *rbs* operon of *L. sakei* is not subject to the classical glucose catabolite repression. Indeed, no CRE sequence could be found in the 6,907 bp including the *rbs* operon.

Upstream from the *rbs* operon, an ORF was observed which might encode an acetate kinase. Its location is perhaps not fortuitous and might be linked to the regulation of ribose utilization.

Experimental Procedures

Bacterial Strains and Plasmids

E. coli TG1 (Gibson, 1984), TG90 (Gonzy-Tréboul *et al.*, 1992) and DH5 α (Sambrook *et al.*, 1989) were used for cloning experiments and plasmid propagation. The *E. coli* strain M15[pREP4] (Qiagen) was used for expression and purification of Enzyme I and HPr of *L. sakei*. The *L. sakei* strains used in this study are listed in Table 3. RVrib9 and RVrib10 were isolated as 2-deoxy-D-glucose (2DG)-resistant mutants of *L. sakei* 23K on MRS supplied with ribose 1% and 2DG 50 mM as previously described (Lauret *et al.*, 1996).

Table 3. *L. sakei* Strains

Strain	Genotype or characteristics	Source or reference
23K	Wild-type strain, plasmid cured, used as a recipient strain	Berthier <i>et al.</i> , 1996
RV1007	23K <i>rhsK</i> ::pRV52	This study
RV1011	23K <i>rhsR</i> ::pRV56	This study
RVrib9	23K <i>ptsI</i>	This study
RVrib10	23K <i>ptsI</i>	This study
RV1012	23K <i>ptsI</i> ::pRV22	This study
RV1013	23K <i>ptsI</i> ::pRV23	This study
RV2006	23K Δ <i>ptsI</i>	This study
RV2007	23K <i>ptsIH190A</i>	This study

Inserts from the plasmid pXH37A (Lokman and Pouwels, unpublished results) were used in hybridization experiments. The phagemid pBluescript SK⁺ (Stratagene) was used for cloning experiments in *E. coli* strains. The integrative vector pRV300 (Leloup *et al.*, 1997), a pBluescript derivative containing an erythromycin resistance gene, was used for the construction of *L. sakei* mutants. The expression vector pQE30 (Qiagen) was used to express and purify enzyme I and HPr of *L. sakei*. The other plasmids used in this study are shown Figure 1A. A *HincII* fragment from *L. sakei* chromosomal DNA was cloned in pBluescript and pRV300 leading to pRV51 and pRV52, respectively. Two different *HindIII* fragments were cloned in pBluescript, leading to pRV53 and pRV54, respectively. pRV55 is a plasmid that contains a PCR fragment amplified from *L. sakei* 23K chromosomal DNA. A *KpnI/EcoRV* fragment containing an internal part of *rhsR* was cloned in pRV300 leading to pRV56, and was used to construct a mutant of the *RbsR* repressor. Plasmids pRV22 and pRV23 contain inserts of the *ptsHI* operon cloned in pRV300.

Media, Growth Conditions and Transformations

E. coli strains were grown in LB medium (Sambrook *et al.*, 1989) at 37 °C. *L. sakei* strains were grown at 30 °C in the complex medium MRS (De Man *et al.*, 1960) or in the defined medium MCD (Lauret *et al.*, 1996) supplemented with 0.5% glucose or ribose. *E. coli* and *L. sakei* electrocompetent cells were prepared and transformed by the methods of Dower *et al.* (1988) and Berthier *et al.* (1996), respectively.

Nucleic Acid Extraction and Hybridization

Chromosomal DNA was prepared from *L. sakei* by the method of Anderson and McKay (Anderson and McKay, 1983). Total RNAs were prepared by standard methods as previously described (Stentz *et al.*, 1997). Southern hybridization and colony hybridization were performed using the standard methods (Sambrook *et al.*, 1989). Low stringency hybridization was performed using the conditions described by Sambrook *et al.* (1989) using 25% instead of 50% formamide in the hybridization buffer. DNA probes were labeled by random priming as previously described (Stentz *et al.*, 1997) with ³²P- α -dCTP.

Purification of His-Tag Enzyme I and His-Tag HPr

The oligonucleotides purI1 and purI2 were used to amplify a DNA fragment containing *ptsI* which was cloned at the *Bam*HI and *Kpn*II sites of pQE30. The resulting plasmid was used to transform *E. coli* M15[pREP4]. The oligonucleotides purH1 and purH2 were used to amplify the *ptsH* gene and the same procedure was used. The resulting His-Tag proteins were expressed in *E. coli* and purified on Ni-NTA agarose and eluted by an imidazole gradient in the conditions recommended by the manufacturer (Qiagen). The proteins were used as standard in western-blot experiments.

Western-Blot Experiments

Crude extracts were prepared from bacteria grown in MCD medium to OD₆₀₀ 0.4–0.6. Bacteria from 50 ml cultures were collected by centrifugation and rinsed twice with Tris 100 mM, pH 7.5, and then resuspended in the same buffer at a cell concentration of 25 OD/ml. Cells were broken by sonication for two times 20 seconds at 4 °C, with a 1 min pause at 4 °C. Cellular debris were eliminated by centrifugation. The protein concentration of the resulting crude extract was usually 1–2 mg protein/ml. Proteins (10 μ g) were separated on 12.5% acrylamide (for enzyme I immuno-detection) or 15% acrylamide (for HPr immuno-detection) gels by polyacrylamide-SDS gel electrophoresis (PAGE-SDS). Proteins were electrotransferred on PVDF nylon membranes (Millipore). Antisera raised against the enzyme I and HPr of *S. carnosus* were kindly provided by W. Hengstenberg. Membranes were incubated in TBS buffer (Sambrook *et al.*, 1989) containing 5% milk powder and 0.05% Tween 20. After overnight incubation with antisera, membranes were rinsed and incubated with HRP-protein G (Biorad) and

Table 4. Oligonucleotides Description

Name	Sequence*
rbsA1	5'-TGTA AACGACGCGCCAGTG-3'
rbsA2	5'-GTGTCGACATTGGTTGAAC-3'
rbsA4	5'-ACAGATCCTAGCTTCAGTG-3'
delhi3	5'-CGGGATCCCGAAGCGGTATAGTGTAGAC-3'
delhi4	5'-CGGAATTCGGAAGCGGTATAGTGTAGAC-3'
delhi5	5'-CGGAATTCGCTGTGTACGAGTTCGT-3'
delhi6	5'-CGGGATCCCGAAGCGGTATAGTGTAGAC-3'
mdh190A	5'-CGGAATTCGCTACGAGTGTAGAG-3'
mdh190B	5'-CATAATGGCAGAAGCTGACGTCGGAC-3'
mdh190C	5'-GTCGGACGTCAGCTTCCATTATG-3'
mdh190D	5'-CGGAATTCGCTGCCGTCCATACCTTCTA-3'
purI1	5'-CGGGATCCACTAACTAAGAGGGATT-3'
purI2	5'-GGGGTACCCTAACGATCTTTGATAAAA-3'
purH1	5'-CGGGATCCGAAAACGCGGATTTTCAC-3'
purH2	5'-GGGGTACCCTTATTCAGATAAACCTTC-3'
rib2	5'-CGGAATTCGCTGTTGAACGAGATAGT-3'

* Restriction sites are underlined.

revealed by the use of a western chemiluminescence reagent (NEN) in the conditions described by the manufacturer.

Oligonucleotides and PCR

The oligonucleotides used in this study are listed in Table 4. The reverse PCR amplification was performed on chromosomal DNA of RV1008, previously digested and ligated upon itself as described by Ochman *et al.* (1988). The primers rbsA1 and rbsA2 were used on the ligation mixture for the amplification of a DNA fragment corresponding to the upstream region of the *L. sakei* *rhsK* gene. The PCR amplifications were performed on a Perkin-Elmer 9600 apparatus, with a mix of Taq DNA polymerases from the Expand™ Long Template PCR System used in the conditions described by the manufacturer (Boehringer). The couples of primers delhi3-delhi4 and delhi5-delhi6 were used in a standard PCR experiment in order to generate two fragments of the *ptsHI* region which were used to construct a chromosomal deletion in *ptsI*. The primers mdh190B and mdh190C were used in order to generate two point mutations in *ptsI* changing the CAT triplet, encoding the His 190 amino acid of enzyme I, to the GCT triplet encoding an alanine residue. Rib2 and the Reverse Primer (Stratagene) were used for a PCR reaction on a ligation mixture to generate a DNA fragment corresponding to the downstream part of the *rhs* gene cluster (see the text). Restriction sites added at the 5'-end of primers shown Table 4 are underlined. The standard PCR amplifications were performed as previously described (Stentz *et al.*, 1997).

DNA Sequence Analysis

Double-strand DNA and PCR products were sequenced according to the instructions of the manufacturer (Perkin-Elmer) for cycle sequencing on a GenAmp PCR system 9600 as previously described (Stentz *et al.*, 1997). The sequence was determined on both strands.

Nucleotide Accession Number. The *rhs* sequence has been deposited under GenBank accession N° AF115391.

Carbohydrate Uptake and ATP-Dependent Phosphorylation Measurement

The uptake of ¹⁴C-labelled glucose and ribose was determined using the conditions described (Lauret *et al.*, 1996) using the standard method of rapid cell filtration (Postma, 1977). Preparation of toluenized cells and ATP-dependent phosphorylation assays were performed as described (Lauret *et al.*, 1996). [¹⁴C] glucose (2 MBq/mmol) was purchased from CEA (Saclay, France). [¹⁴C] ribose (8.14 GBq/mmol) was a gift from J. Labarre (CEA Saclay, France).

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