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## Physiology, metabolism

### Adaptation of *Lactobacillus sakei* to meat: a new regulatory mechanism of ribose utilization?

Régis STENTZ\*\*, Monique CORNET, Stéphane CHAILLOU,  
Monique ZAGOREC\*

Laboratoire de Recherches sur la Viande, INRA-CRJ, Domaine de Vilvert,  
78350 Jouy-en-Josas, France

**Abstract** — *Lactobacillus sakei* is a lactic acid bacterium commonly found on meat and meat products. Meat is a rich substrate but contains small amounts of sugars, mainly glucose and ribose. The phosphotransferase system (PTS) is a multienzymatic complex responsible for sugar uptake. It is also involved in the regulation of metabolism through various mechanisms (catabolite activation and repression, inducer expulsion and exclusion). The *ptsHI* operon of *L. sakei*, encoding the general enzymes of the PTS, was studied and mutants were constructed. On ribose, these mutants grow twice as fast as the wild-type strain. This phenotype was never described in other bacteria and suggests that the PTS regulates ribose utilization. When compared to what is known from the regulations involving the PTS in other bacteria, this mechanism might be new. In *Bacillus subtilis* and *Escherichia coli*, in which ribose catabolism was investigated, ribose is transported by an ABC transporter, encoded by *rbsABCD* genes and then phosphorylated by the *rbsK* encoded ribose kinase. Ribose-5P is then metabolized through the pentose-P pathway involving xylulose-5P phosphoketolase and acetate kinase. Whereas phosphoketolase and acetate kinase activities remained unchanged in *L. sakei ptsI* mutants, ribose kinase activity and uptake were increased by a factor of 2.5 and 1.5, respectively. The target of the PTS regulation would thus be transport and/or phosphorylation of ribose. The gene cluster encoding a ribose transporter, ribose kinase and a regulator was cloned and sequenced. In *L. sakei* no gene encoding RbsA, RbsB or RbsC could be found. However, *rbsD* was present as well as a new gene *rbsU*, encoding a protein homologous to a glucose transporter responsible for facilitated diffusion of glucose. The *rbsUDK* operon is induced by ribose via the regulator RbsR encoded by *rbsR* located downstream of *rbsUDK*. In *ptsI* mutants, this operon was not overexpressed on ribose. This shows that the regulation of ribose utilization is not a transcriptional regulation. Upstream from the *rbs* operon, a gene encoding acetate kinase (*ackA*) was found. In other bacteria in which these genes were identified, *ackA* and the *rbs* operon are not linked. Moreover, in *B. subtilis*, *ackA* is regulated by catabolite activation whereas the *rbs* operon is repressed by catabolite repression, two mechanisms involving the PTS. In *L. sakei*, *ackA* and the *rbs* operon are adjacent on the

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\* Correspondence and reprints

Tel.: (33) 1 34 65 22 89; fax: (33) 1 34 65 21 05; e-mail: zagorec@biotec.jouy.inra.fr

\*\* Present address: Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK.

chromosome and they are not regulated as described above. We propose that in this species, ribose and glucose utilization is regulated in a different way, allowing *L. sakei* to catabolize both glucose and ribose, the sole sugars present in meat.

**sugar / PTS / fermentation / glucose**

**Résumé — Adaptation de *Lactobacillus sakei* au milieu carné : un nouveau mécanisme de régulation de l'utilisation du ribose ?** *Lactobacillus sakei* est une bactérie lactique communément trouvée sur la viande et les produits carnés. Le milieu carné est un substrat particulièrement riche mais contient peu de sucres, essentiellement du glucose et du ribose. Le système des phosphotransférases (PTS) est un complexe enzymatique responsable de l'internalisation de certains sucres, et jouant un rôle régulateur du métabolisme par différents mécanismes (répression et activation cataboliques, exclusion et expulsion d'inducteur). L'opéron *ptsHI* de *L. sakei*, qui code pour les enzymes générales du PTS, a été étudié et des mutants ont été construits. Les mutants de ce système poussent 2 fois plus vite sur ribose que la souche sauvage. Ce phénotype n'avait jamais été décrit dans d'autres bactéries et suggère que le PTS régule l'utilisation du ribose. Par rapport à ce qui est connu des différentes régulations dans lesquelles est impliqué le PTS chez d'autres bactéries, ce mécanisme semblait nouveau. Chez *Bacillus subtilis* et *Escherichia coli* où le métabolisme de ce pentose a été étudié, il a été montré que le ribose est internalisé par un transporteur de type ABC, codé par les gènes *rbsABCD* puis phosphorylé par la ribokinase codée par *rbsK*. Le ribose-5P est alors métabolisé via la voie des pentoses-P impliquant la xylulose-5P phosphocétolase et l'acétate kinase. Chez les mutants *ptsI* de *L. sakei* qui poussent plus rapidement sur ribose, les activités phosphocétolase et acétate kinase sont inchangées alors que l'activité ribokinase est augmentée d'un facteur 2,5 et le transport du ribose d'un facteur 1,5. La cible de la régulation par le PTS résiderait donc au niveau du transport et/ou de la phosphorylation du ribose. L'opéron codant pour le transporteur du ribose, la ribokinase et un régulateur a été cloné et séquencé. Aucun gène équivalent à *rbsA*, *rbsB* ou *rbsC* n'a pu être mis en évidence. Par contre, *rbsD* est présent, et un nouveau gène *rbsU*, codant pour une protéine similaire à un transporteur du glucose, agissant par diffusion facilitée a été observé. L'opéron *rbsUDK* est induit par le ribose via le répresseur RbsR, dont le gène est situé en aval de *rbsUDK*. Dans les mutants *ptsI*, cet opéron n'est pas surexprimé en présence de ribose. Ceci montre que la régulation de l'utilisation du ribose par le PTS n'agit pas au niveau transcriptionnel. En amont de l'opéron *rbs*, un gène codant pour une acétate kinase (*ackA*) a été observé. Chez d'autres bactéries où ces gènes sont connus, *ackA* et l'opéron *rbs* ne sont pas liés génétiquement. Enfin, chez *B. subtilis*, le gène *ackA* est régulé par activation catabolique alors que l'opéron *rbs* est soumis à la répression catabolique, deux mécanismes faisant intervenir le PTS. Chez *L. sakei*, *ackA* et l'opéron *rbs* sont regroupés sur le chromosome et ne sont pas soumis à ces régulations. Nous proposons donc que l'utilisation du ribose et du glucose soit régulée chez cette bactérie d'une manière différente, lui permettant de métaboliser au mieux à la fois le glucose et le ribose, les deux seuls sucres présents sur la viande.

**sucres / PTS / fermentation / glucose / viande**

## 1. INTRODUCTION

*Lactobacillus sakei* is a lactic acid bacterium naturally occurring on meat and meat products [9]. On vacuum-packed meat, *L. sakei* represents the main bacterial flora.

This species is also found on fish products [12]. These raw materials contain many nutrients allowing bacterial growth but their carbohydrate content is relatively poor. Among the few sugars found in meat and raw fish, glucose and ribose are the only

sugars that *L. sakei* can utilize for its growth. In bacteria, sugars can be transported by various systems including the phosphotransferase system (PTS) [13] and ATP Binding Cassette (ABC) transporters [10]. The PTS also plays a central regulatory role on metabolism. In several Gram-positive bacteria, HPr, a phosphocarrier protein of the PTS has been shown to be involved in catabolite repression, catabolite activation, inducer exclusion and inducer expulsion [6, 14, 16]. HPr can be phosphorylated at the His-15 residue by enzyme I, another phosphocarrier of the PTS, at the expense of phosphoenolpyruvate (PEP) [13]. In *Enterococcus casseliflavus* and *Enterococcus faecalis*, P-His-HPr can then activate the glycerol kinase, enhancing facilitated diffusion of glycerol [3]. In *ptsI* mutants lacking enzyme I, glycerol cannot be transported anymore [15]. Consequently the growth of these mutants is abolished both on PTS-transported sugars and on glycerol which is not transported by the PTS. HPr is also phosphorylated at the Ser-46 residue by the HPr kinase HPrK, at the expense of ATP. P-Ser-HPr plays, then, a role in the transcription control of genes that are repressed by catabolite repression [6] or induced by catabolite activation [14, 19]. In *Bacillus subtilis ptsH* mutants lacking P-Ser-HPr, the catabolite repression is released [5]. In *L. sakei*, the *ptsHI* operon encoding HPr and enzyme I was previously cloned and sequenced [18]. Several mutants of *ptsI* were constructed. In these mutants the doubling time of growth on ribose is reduced 2–3 times, suggesting that the PTS regulates ribose utilization. The *rbsUDKR* operon of *L. sakei* was cloned [17]. In several bacteria, ribose is transported by an ABC transporter and in *B. subtilis*, the *rbsKDACB* operon is regulated by catabolite repression [20]. Surprisingly, no genes encoding the ABC transporter for ribose could be found in *L. sakei*. Only *rbsD*, putatively encoding one of the ribose ABC transporter subunits was found. Upstream from *rbsD*, a gene named *rbsU* is present, which encodes a protein homologous to a

glucose transporter of *Staphylococcus xylo-sus* responsible for glucose transport by facilitated diffusion [7]. The genes encoded by *rbsUDK* are cotranscribed, suggesting that RbsU is indeed involved in ribose transport. The transcription of *rbsUDK* is not increased in *ptsI* mutants of *L. sakei*. Furthermore, in a *rbsR* mutant overexpressing *rbsUDK*, the growth of ribose is not enhanced [17]. This data suggests that in *L. sakei*, ribose utilization is regulated by a new mechanism, which does not act at the transcriptional level and has not yet been described in other bacteria in which the role of the PTS and ribose metabolism were investigated. This new mechanism is as yet unknown and several questions must be answered: what is the regulator? What is the target? What is the regulatory mechanism? What is the signal?

## 2. MATERIALS AND METHODS

*L. sakei* 23K [1] is the wild-type strain used as recipient strain for the construction or isolation of mutants. RV2006 is a  $\Delta ptsI$  mutant, RV2007 contains the *ptsI* H190A point mutation. Both mutants were constructed by double-crossover [17]. RVrib9, RVrib10 and RV52 have been isolated as 2 deoxy-D-glucose (2DG) resistant mutants. RVrib9 and RVrib10 are mutated in *ptsI* and RV52 contains a mutation in the genes encoding enzyme II<sup>Man</sup>. *L. sakei* strains were propagated in the complex MRS medium [4] and cultivated in the MCD chemically defined medium [11] for physiological studies. Carbon sources were added at 0.5%. Bacteria were grown at 30 °C and bacterial growth was followed by optical density at 600 nm.

For the measurement of enzymatic activities, bacteria were grown until mid log phase, collected, rinsed and resuspended in Tris 0.1 mol·L<sup>-1</sup>, pH 7.0 and either broken with glass beads or toluenized. Activities were measured by standard methods.

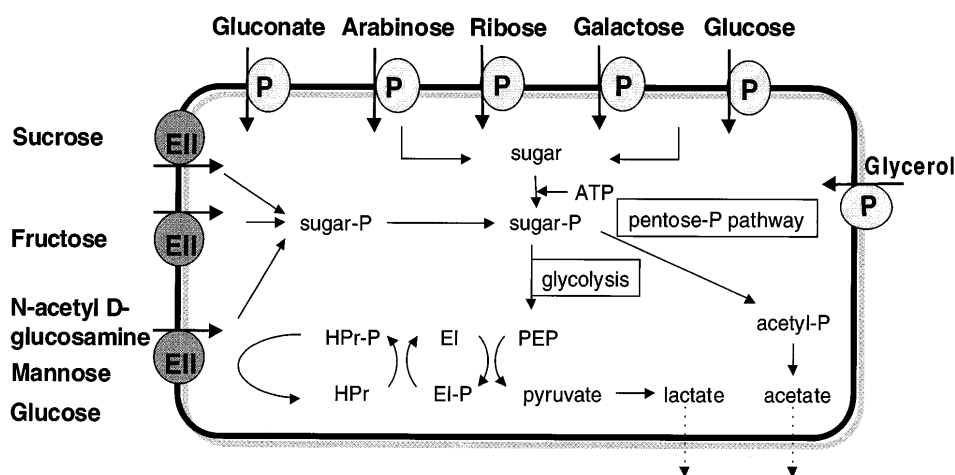
### 3. RESULTS AND DISCUSSION

#### 3.1. Energy sources utilized by *L. sakei*

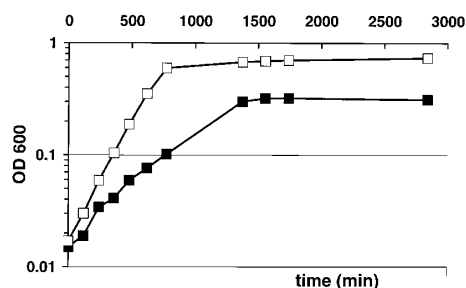
Although *L. sakei* can use arginine via the arginine deiminase pathway, leading to ATP synthesis, this amino acid does not allow growth of *L. sakei* but rather survival during stationary phase [2]. Instead, this species uses other energy sources to ensure its growth on meat. In a previous study, we have shown that fructose, sucrose, N-acetyl-D-glucosamine, mannose and glucose can be used as PTS sugars [11]. Among these sugars, glucose, mannose and N-acetyl-D-glucosamine are transported by a common system, the PTS-dependent enzyme  $\text{II}^{\text{Man}}$ . Additionally, glucose is transported by at least one uncharacterized non-PTS permease. Galactose, gluconate, ribose and arabinose are transported by non-PTS permeases (Fig. 1). *L. sakei* can also grow poorly on glycerol, and this growth is totally abolished in *ptsI* mutants, suggesting that the regulation of glycerol kinase described in enterococci and *B. subtilis* might also exist in *L. sakei*.

#### 3.2. The PTS in *L. sakei*

The *ptsHI* operon was previously cloned and sequenced [18]. Several mutants in *ptsI*, encoding enzyme I were isolated. Two mutants, RVrib9 and RVrib10, isolated as 2DG resistant mutants contain a mutation in *ptsI* leading to the synthesis of a truncated enzyme I. Two other mutants were constructed by double recombination, leading to a deletion in *ptsI* (RV2006) or to the replacement of the phosphorylatable histidine 190 residue by an alanine (RV2007) [17]. All these mutants had lost the ability to grow on the PTS-transported sugars fructose, mannose and sucrose. When grown on ribose, the *ptsI* mutants had a doubling time reduced to 2–3 h whereas the doubling time of the wild-type strain was 6–7 h (Fig. 2). This suggests that in the wild-type, the PTS regulates negatively ribose utilization. This phenotype was never described in any bacterial *ptsI* mutant. Indeed, in a *B. subtilis* *ptsI* mutant, the growth rate on ribose was unchanged when compared to the wild-type (not shown). This unusual observation suggests that ribose utilization in *L. sakei* might



**Figure 1.** Schematic representation of carbon sources utilization by *Lactobacillus sakei*. P: non-PTS permeases, EII: enzyme  $\text{II}^{\text{sugar}}$  of the PTS.

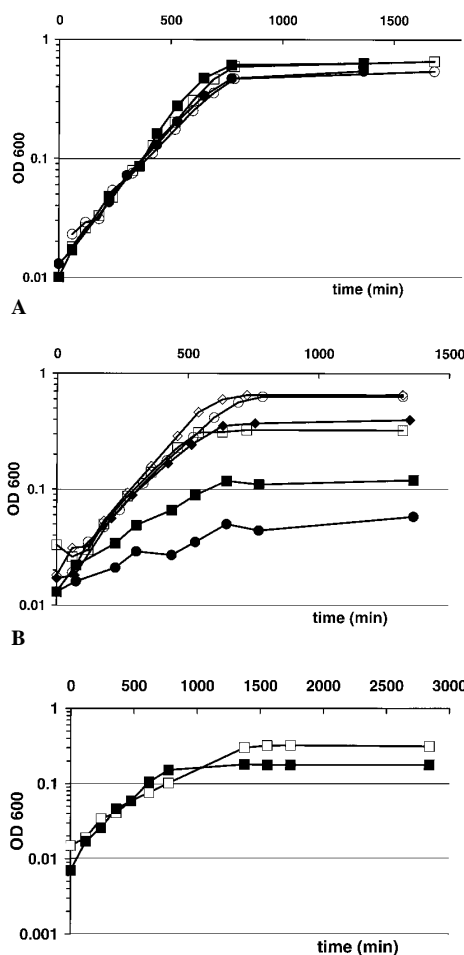


**Figure 2.** Growth of *L. sakei* 23K (wild type, bold symbols) and RV2006 ( $\Delta ptsI$ , open symbols) on MCD containing 0.5% ribose.

be regulated by a new regulatory mechanism. In these mutants, all harboring the same phenotype on ribose, enzyme I was either missing, truncated or not phosphorylatable. Consequently, HPr, as well as enzymes II were not phosphorylated. Thus this phenotype can result from a modification in the phosphorylation state of enzyme I, HPr or some enzyme II. In order to verify the involvement of enzyme I and/or HPr in the regulation of ribose catabolism, a 2DG resistant mutant, affected in the PTS but not in *ptsHI* was isolated.

2DG is a glucose toxic analog transported by enzyme II<sup>Man</sup>. *L. sakei* mutants in the genes encoding enzyme II<sup>Man</sup> can be isolated as resistant to this toxic analog [11]. Such a mutant, RV52, was isolated from the *L. sakei* 23K strain. Its growth on sucrose and fructose, two PTS sugars, is not affected (Fig. 3A). RV52 cannot grow on mannose transported by enzyme II<sup>Man</sup>. On glucose, transported by both a non-PTS permease and enzyme II<sup>Man</sup>, RV52 can grow but less faster than the parent strain (Fig. 3B). This data strongly suggests that RV52 is affected in enzyme II<sup>Man</sup>. The growth of RV52 on ribose was similar to what was observed with 23K (Fig. 3C). Thus enzyme II<sup>Man</sup> is not involved in the PTS-mediated regulation of ribose metabolism.

In order to obtain more information on ribose utilization, the *rbs* operon was cloned.



**Figure 3.** Growth of *L. sakei* 23K (wild type, open symbols) and RV52 (mannose mutant, bold symbols) on (A) fructose (circles) or sucrose (squares); (B) mannose (circles), glucose 0.1% (squares) or glucose 0.5% (diamonds); (C) ribose. Bacteria were grown in MCD medium containing 0.5% sugars (except glucose 0.1%).

### 3.3. The ribose operon

The *rbs* operon of *L. sakei* was cloned and sequenced [17]. Surprisingly, *rbsA*, *rbsB* and *rbsC* were not present in this operon. Several attempts to clone them were unsuccessful. The *rbsUDKR* gene cluster encodes

RbsU, a protein homologous to a glucose transporter of *S. xylosus*, RbsD, one of the membrane-bound subunits of the ribose ABC transporter, ribose kinase, and RbsR the repressor. *rbsUDK* are cotranscribed and induced by ribose (repressed by RbsR in the absence of ribose). This suggests that RbsD and RbsU are involved in ribose utilization. In a *rbsR* mutant which overexpresses *rbsUDK*, the growth on ribose is not accelerated. In *ptsI* mutants, the transcription of *rbsUDK* is not modified, but growth on ribose is accelerated [17]. Thus the regulation of ribose utilization by the PTS is not a transcriptional regulation.

Upstream from *rbsU*, in a separate transcription unit, a gene encoding a putative acetate kinase (*ackA*) was observed. In several other bacteria, the genome of which has been sequenced, *ackA* is not genetically linked to the genes responsible for ribose uptake and phosphorylation. In *B. subtilis*, transcription of *ackA* is regulated by catabolite activation through a catabolite responsive element (*cre*) [8] whereas the *rbs* operon is regulated by catabolite repression, also through a *cre* sequence. In *L. sakei* no *cre* sequence could be found in the upstream part of *ackA* or *rbsU*, suggesting that the transcription of both genes is not under global catabolite regulatory control. Since

acetate kinase catalyses an energetically-important step of ribose catabolism, yielding one additional ATP per ribose molecule, the question arises whether the genetic link between *ackA* and the *rbs* operon might have a physiological significance in the regulation of ribose utilization by the PTS.

### 3.4. Regulation of ribose utilization by the PTS

The activity of several enzymes involved in sugar catabolism was measured in a *ptsI* mutant and its parent strain grown on glucose or on ribose (Tab. I). Activities of the glycolytic enzymes aldolase and phosphofructokinase were slightly higher on glucose than on ribose, and the levels were similar between the wild-type and the mutant. Similar results were observed with glucose-6P dehydrogenase and 6P-gluconate dehydrogenase. For both acetate kinase and phosphoketolase, a higher activity was observed in the wild-type strain grown on ribose than on glucose (factor 2.5 and 26, respectively). This suggests that these two enzymes are repressed in the presence of glucose. In the *ptsI* mutant, both activities became constitutive since no glucose repression could be observed. Acetate

**Table I.** Enzymatic activities of aldolase, phosphofructokinase, acetate kinase, xylulose-5P phosphoketolase, glucose-6P dehydrogenase and gluconate-6P dehydrogenase. Activities were determined on crude extracts of bacteria grown on MCD medium supplemented with 0.5% glucose or ribose. Activities are expressed in Units·min<sup>-1</sup>·mg protein<sup>-1</sup>. Values are the mean of at least two independent experiments. Standard deviations were less than 20%.

Enzyme	WT		<i>ptsI</i>	
	Glucose	Ribose	Glucose	Ribose
Aldolase	1.01	0.23	1.03	0.44
Phosphofructokinase	2.49	1.05	2.60	1.34
Acetate kinase	1.70	4.13	1.78	1.89
Phosphoketolase	0.036	0.95	0.107	0.97
Glucose-6P dehydrogenase	0.079	0.096	0.065	0.069
Gluconate-6P dehydrogenase	0.067	0.101	0.074	0.101



kinase and phosphoketolase activities were identical in the wild-type strain and in the mutant strain grown on ribose. The accelerated growth of the mutant on ribose should thus not result from an increase of these activities. Moreover, ribose uptake and ATP dependent phosphorylation were increased 1.5 and 2.5 times respectively in the mutant grown on ribose as compared to the parent strain [17]. Thus ribose uptake and/or phosphorylation is/are the target(s) of the regulation by the PTS. As this regulation does not act on the transcription of the *rbsDKU* operon [17], this strongly suggests that regulation acts on the activity of the corresponding proteins.

#### 4. CONCLUSION

Sugars present in meat are mainly glucose and ribose but are both present in small amounts. *L. sakei* is particularly well adapted to this environment since it is very commonly found on meat and is the main flora on vacuum-packed meat. The study of the PTS and ribose metabolism in this bacterium revealed that: (i) organization of genes responsible for ribose catabolism is so far unique; (ii) ribose utilization is not repressed by glucose at the transcriptional level; (iii) however, ribose utilization is regulated by the PTS by a mechanism that has not yet been described in other bacteria; (iv) phosphoketolase, the key enzyme of pentose-P pathway, as well as acetate kinase activities are not increased in *ptsI* mutants, whereas ribose uptake and phosphorylation are increased, as is the ability to grow on ribose. These specific features might result in a co-metabolism of glucose and ribose during growth of *L. sakei* on meat. Indeed, since glucose and ribose are the sole sugars available for growth of *L. sakei* on meat, the absence of catabolite regulation of ribose utilization should lead to the possibility of using both sugars simultaneously instead of a sequential utilization. Whether this metabolism would lead to an advantage

toward other microbial flora found on meat is not yet demonstrated. Glucose and ribose are present on meat in almost constant concentrations since glucose is derived from glycogen and ribose from ATP. Other microbial flora might use only glucose, whereas *L. sakei* might take an advantage in using both sugars.

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