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A putative transport protein is involved in citrulline excretion and re-uptake during arginine deiminase pathway activity by *Lactobacillus sakei*

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Abstract

Arginine conversion through the arginine deiminase (ADI) pathway is a common metabolic trait of *Lactobacillus sakei* which is ascribed to an *arc* operon and which inquisitively involves citrulline excretion and re-uptake. The aim of this study was to verify whether a putative transport protein (encoded by the *PTP* gene) plays a role in citrulline-into-ornithine conversion by *L. sakei* strains. This was achieved through a combination of fermentation experiments, gene expression analysis via quantitative real-time reverse transcription PCR (RT-qPCR) and construction of a *PTP* knock-out mutant. Expression of the *PTP* gene was modulated by environmental pH and was highest in the end-exponential or mid-exponential growth phase for *L. sakei* strains CTC 494 and 23K, respectively. In contrast to known genes of the *arc* operon, the *PTP* gene showed low expression at pH 7.0, in agreement with the finding that citrulline-into-ornithine conversion is inhibited at this pH. The presence of additional energy sources also influenced ADI pathway activity, in particular by decreasing citrulline-into-ornithine conversion. Further insight into the functionality of the *PTP* gene was obtained with a knock-out mutant of *L. sakei* CTC 494 impaired in the *PTP* gene, which displayed inhibition in its ability to convert extracellular citrulline into ornithine. In conclusion, results indicated that the *PTP* gene may putatively encode a citrulline/ornithine antiporter.
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Keywords: *Lactobacillus sakei*; Arginine deiminase; Citrulline; Ornithine

1. Introduction

Lactobacillus sakei is the dominant species in fermented sausages and is therefore often used as an industrial starter culture in meat fermentations (Leroy et al., 2006; Talon et al., 2007). As the meat matrix is a nutrient-rich but carbohydrate-poor environment, the ability of *L. sakei* to survive periods of carbohydrate starvation may depend on its ability to use alternative energy sources present in meat (Chaillou et al., 2005; Champomier-Vergès et al., 2001; Rimaux et al., 2011b). In this sense, the genome sequence of *L. sakei* 23K highlights a specialized metabolic repertoire, suggesting a contribution to competitiveness in, and adaptation to, the meat matrix (Chaillou et al., 2005). Such features include the utilization of nucleosides (Chaillou et al., 2005; Rimaux et al., 2011b) and conversion of arginine, an amino acid abundantly present in meat due to the activity of endogenous proteases, through the arginine deiminase (ADI) pathway (Rimaux et al., 2011a).

In general, the ADI pathway results in the conversion of 1 mol of arginine into 1 mol of ornithine, concomitant with the production of 2 mol of ammonia, 1 mol of carbon dioxide and 1 mol of ATP (Cunin et al., 1986). For *L. sakei*, it has been suggested that arginine conversion may provide improved

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tolerance toward acid stress conditions and generate additional energy, possibly contributing to its adaptation and survival (Champomier-Vergès et al., 1999; Rimaux et al., 2011a). The genes of the ADI pathway in *L. sakei* are clustered into a single *arc* operon (*arcABCTDR*) and are transcribed from a single *arcA* promoter (Rimaux et al., 2012; Zuñiga et al., 1998, 2002). Besides the structural genes (*arcA*, *arcB*, *arcC*, and *arcD*) encoding the ADI pathway enzymes, additional genes are present in the *arc* operon of *L. sakei*. The regulatory gene (*arcR*), possibly acting as an activator, is essential for expression of the *arc* operon (Zuñiga et al., 2002). A putative transaminase gene (*arcT*) is present as well, for which no physiological information is yet available. However, a recent study excludes a contribution of this putative transaminase to flavor formation (Freiding et al., 2011). Furthermore, a gene (*LSA0376*) 139 bp downstream from the *arc* operon of *L. sakei* 23K putatively encodes an uncharacterized transport protein (*PTP* gene) (Zuñiga et al., 1998, 2002), raising questions concerning the exact role of this gene and its possible contribution to the ADI pathway.

Expression of the *arc* operon is tightly regulated in bacteria. For instance, carbon catabolite repression regulates the expression of the *arc* operon in *L. sakei*, whereas the pathway is stimulated by anaerobiosis and arginine (Fernández and Zúñiga, 2006). In addition, the environmental pH and growth phase influence expression of the *arc* operon in *L. sakei* (Rimaux et al., 2012). Although acid stress generally induces expression of the ADI pathway, as described for *Streptococcus gordonii* (Liu et al., 2008), expression of the *arc* operon in *L. sakei* is highest at optimal pH for growth, with lower expression levels toward low pH values (Rimaux et al., 2012). Therefore, the physiological role of ADI pathway activity may vary between species, ranging from acid protection to growth-stimulating effects and survival. Overall, gene expression of the *arc* operon results in a complex transcription pattern in both *Pseudomonas* (Gamper et al., 1991, 1992) and *L. sakei* (Fernández and Zúñiga, 2006; Zuñiga et al., 1998, 2002). In *Pseudomonas*, mRNA processing and partial termination of transcription contribute to differential gene expression of the *arc* operon (Gamper et al., 1992).

A detailed kinetic study of fermentations with *L. sakei* CTC 494 has revealed that the environmental pH has a pronounced effect on the different metabolites of the ADI pathway in this strain (Rimaux et al., 2011a). In summary, arginine conversion occurs in a sequential pattern. Depending on the strain, arginine conversion may start in the mid- (*L. sakei* 23K) or end-exponential growth phase (*L. sakei* CTC 494), hence potentially contributing to growth or survival, respectively. This strain-dependent onset of the ADI pathway suggests that the conversion of this amino acid may confer different physiological benefits upon different strains. Arginine conversion in *L. sakei* always results in an extracellular mixture of both citrulline and ornithine, with the lowest citrulline-to-ornithine ratios at low pH values. When all arginine is depleted, further conversion of citrulline to ornithine takes place intracellularly, but only at pH values below 7.0. This further conversion of citrulline to ornithine has been noted for *Lactobacillus fermentum* IMDO 130101 and *Lactobacillus buchneri* CUC-3, for which the involvement of additional transporters has been suggested (Liu and Pilone, 1998; Liu et al., 1996; Vrancken et al., 2009a). In contrast, one study proposed cell lysis, releasing intracellular enzymes, as the responsible mechanism for extracellular citrulline-into-ornithine conversion (Mira de Orduña et al., 2000).

The aim of this study was to verify whether the *PTP* gene, discovered in *L. sakei* 23K, plays a role in citrulline-into-ornithine conversion by *L. sakei* strains, possibly acting as a transporter through a combined gene expression and fermentation approach.

2. Materials and methods

2.1. Strains, plasmids, media, and growth conditions

Twenty-nine *L. sakei* strains isolated from different fermented food products were used during this study (Table 1). The authenticity of these strains was confirmed through 16S rRNA gene sequencing. All strains were used for screening experiments, whereas *L. sakei* CTC 494 and *L. sakei* 23K were used for fermentation experiments and gene expression studies as well. The strains and plasmids used throughout this study for the construction of a knock-out mutant of *L. sakei* CTC 494 are listed in Table 2. All *L. sakei* and *Escherichia coli* strains were stored at −80 °C in de Man-Rogosa-Sharpe (MRS) medium or Luria-Bertani (LB) medium, respectively, supplemented with 25% (vol vol^{-1}) glycerol as cryoprotectant.

All *L. sakei* strains were grown in MRS medium (Oxoid, Basingstoke, Hampshire, UK) at 30 °C. *E. coli* strains were grown in LB medium consisting of (in g l^{-1}) tryptone (Oxoid), 10; yeast extract (VWR International, Darmstadt, Germany), 5; and NaCl (VWR International), 10, under aerobic conditions in a shaking incubator (Certomat® BS-1, Sartorius AG, Melsungen, Germany) at 37 °C. Solid media were prepared by adding 1.5% (wt vol^{-1}) agar (Oxoid) to the culture media. When required, antibiotics were added to the media, namely erythromycin (Em) and chloramphenicol (Cm) at a final concentration of 5 μg ml^{-1} for *L. sakei* strains and 100 μg ml^{-1} (Em) and 5 μg ml^{-1} (Cm) for *E. coli* strains.

Customized MRS (mMRS) medium, i.e. MRS medium (de Man et al., 1960) without glucose and supplemented with the appropriate energy source consisting of (combinations of) arginine, glucose and ribose, was used for all screening and fermentation experiments. For screening experiments, mMRS medium was supplemented with 3 g l^{-1} of arginine and 5 g l^{-1} of glucose (mMRS1). For gene expression studies, *L. sakei* CTC 494 and *L. sakei* 23K were grown in mMRS medium with 3 g l^{-1} of arginine (mMRS2) as the sole added energy source. Moreover, mMRS medium containing 5 g l^{-1} of glucose (mMRS3) as the sole added energy source was used for control fermentations. Finally, mMRS medium using different combinations of energy sources was used to perform a series of fermentations with *L. sakei* CTC 494: 3 g l^{-1} of arginine and 1 g l^{-1} of glucose (mMRS4); 3 g l^{-1} of arginine, 1 g l^{-1} of glucose, and 1 g l^{-1} of ribose (mMRS5); 3 g l^{-1} of

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