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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

Table 1
Strains of *L. sakei* used in this study.

Strain	Origin	Reference
L. sakei CTC 494	Spanish artisan fermented sausage	(Hugas et al., 1993)
L. sakei 23K	French fermented sausage	(Champomier-Vergès et al., 2001)
L. sakei F2P1	French fermented sausage	IMDO laboratory collection
L. sakei FCP1	French fermented sausage	IMDO laboratory collection
L. sakei 706	German fermented sausage	(Schillinger and Lücke, 1989)
L. sakei SA201-1	Industrial meat starter culture	Danisco, Copenhagen, Denmark
L. sakei SA241-1	Industrial meat starter culture	Danisco, Copenhagen, Denmark
L. sakei Texel Lyoflore 2M	Industrial meat starter culture	Danisco, Copenhagen, Denmark
L. sakei BioAgro SA8-100M	Industrial meat starter culture	Chr. Hansen, Hørsholm, Denmark
L. sakei 2MA4	Unspecified cacciatore starter culture	(Ravyts et al., 2008)
L. sakei CG1	Spontaneous laboratory wheat sourdough fermentation	(Ravyts and De Vuyst, 2011)
L. sakei 329	Spontaneous leek fermentation	IMDO laboratory collection
L. sakei 481	Spontaneous leek fermentation	IMDO laboratory collection
L. sakei 614	Spontaneous leek fermentation	IMDO laboratory collection
L. sakei R366	Romanian mixed-vegetable fermentation	IMDO laboratory collection
L. sakei PTD R9	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei PTD R14	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei DBX R8	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei DBX R1j1	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei PTE R4	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei DFL R6	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei VKE R7	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei DFL R13	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei DBL R6	Spontaneously fermented sausage	IMDO laboratory collection
L. sakei PTE R19	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei VK5 R14	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei VK5 R23	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei VKD R7	Spontaneously fermented sausage	(Janssens et al., 2011)
L. sakei LMG 13558	Sake starter culture	BCCM™/LMG Bacteria Collection, Ghent, Belgium

arginine and 5 g l^{-1} of ribose (mMRS6); and 5 g l^{-1} of arginine and 5 g l^{-1} of glucose (mMRS7). The pH of the media was adjusted to the desired value prior to sterilization. Solid MRS or LB medium was prepared by adding 1.5% (wt vol^{-1}) agar (Oxoid) to the respective media. All chemicals were purchased from VWR International.

2.2. DNA isolation, PCR assays and sequencing of the PTP gene of L. sakei CTC 494

Genomic DNA from *L. sakei* CTC 494 was isolated from an overnight culture in MRS medium at 30 °C, as described previously (Rimaux et al., 2012). Three primer pairs, based on the gene sequence of the *PTP* gene (*LSA0376*) of *L. sakei* 23K (Chaillou et al., 2005), were designed using SNPbox (Weckx et al., 2004) (Table S1), resulting in overlapping PCR amplicons and covering the whole *PTP* gene of *L. sakei* 23K (RefSeq NC_007576; from position 380,081 to 382,203). Further, these PCR amplicons overlapped with and extended the earlier sequenced genomic region of the *L. sakei* CTC 494 *arc* operon (Rimaux et al., 2012). PCR assays were performed, containing 100 ng of genomic DNA of *L. sakei* CTC 494, 200 μM of each dNTP, 20 pmole of each primer, 5 μl of 10× PCR reaction buffer, 1.25 U of *Taq* DNA Polymerase (Roche, Basel, Switzerland) and sterile ultrapure water in a final volume of 50 μl. Following amplification, PCR product sizes (approximately 800 bp) were controlled on 1.0% (wt vol^{-1}) agarose gel and the remaining reaction mixture was purified

Table 2
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli EC1000	Cloning strain harboring a copy of the *repA* gene of pVW01 in its genome, Km^r	(Leenhouts et al., 1996)
L. sakei CTC 494 (pVE6007)	*L. sakei* CTC 494 harboring pVE6007, Cm^r	This study
Plasmids		
pVE6007	*repA*-positive, temperature-sensitive derivative of pWV01, Cm^r	(Maguin et al., 1992)
pORI28	*repA*-negative derivative of pWV01, replicates only with *repA* provided *in trans*, Em^r, 1.7 kb	(Law et al., 1995)
pPTP	pORI28 with a 927-bp internal fragment of the *PTP* gene of *L. sakei* CTC 494, Em^r, 2.7 kb	This study

[a] Cm^r, chloramphenicol-resistant; Em^r, erythromycin-resistant; Km^r, kanamycin-resistant.

using the Wizard® SV Gel and PCR Clean-up system (Promega, Madison, WI, USA). Amplicons were sequenced in the Genetic Service Facility of the Flanders Institute for Biotechnology (VIB, Antwerp, Belgium) and the sequences obtained were assembled using Vector NTI Advance 10 (Invitrogen, Carlsbad, CA, USA). Sequence identity between the *PTP* genes of *L. sakei* CTC 494 and *L. sakei* 23K was determined using the BLASTN algorithm (National Center for Biotechnology Information, NCBI). Furthermore, sequence identity of the protein encoded by the *PTP* gene with protein sequences of other LAB was determined using the BLASTP algorithm and the non-redundant protein sequence database (NCBI). The sequence of the genomic region covering the *arc* operon and the *PTP* gene of *L. sakei* CTC 494 was submitted to the EMBL database (EMBL-EBI, Hinxton, UK) and received accession number FR870226.

2.3. Relative gene expression

All primers for relative gene expression analysis (RT-qPCR) were designed using Primer Express 2.0 (Applied Biosystems, Warrington, UK), based on the corresponding gene sequences of *L. sakei* CTC 494 and *L. sakei* 23K for the target gene (*PTP*) and reference genes (*fusA*, *ileS*, and *pcrA*) (Tables S2 and S3), as described previously (Rimaux et al., 2012). Furthermore, primer pairs spanning the intergenic regions of all genes of the *arc* operon and of the intergenic region between the *arcR* and *PTP* genes of *L. sakei* CTC 494 were designed as well (*arcA–arcB*, *arcB–arcC*, *arcC–arcT*, *arcT–arcD*, *arcD–arcR*, and *arcR–PTP;* Table S2).

Total RNA from growing cultures of *L. sakei* CTC 494 was extracted from samples taken at different time points, representing a continuum from the late-exponential (OD_{600} of 0.3), end-exponential (OD_{600} of 0.5), early-stationary (OD_{600} of 0.9) to the late-stationary growth phase (OD_{600} of 1.0 after 24 h of fermentation). For *L. sakei* 23K, samples for RNA extraction were taken at time points corresponding to the early-exponential (OD_{600} of 0.3), mid-exponential (OD_{600} of 0.5), end-exponential (OD_{600} of 2.5) and stationary growth phases (OD_{600} of 2.7 after 24 h of fermentation). After sampling, cell suspensions (2 ml) were immediately treated with RNAprotect Bacteria Reagent (4 ml) (Qiagen, Hilden, Germany). Sample handling, total RNA extraction, additional DNase treatment, and cDNA synthesis were performed as described previously (Rimaux et al., 2012).

RT-qPCR for analysis of relative gene expression was performed using a 7300 Real-Time PCR System (Applied Biosystems). PCR products were detected using SYBR Green, as described previously (Rimaux et al., 2012). Relative gene expression levels were calculated with DART-PCR (Peirson et al., 2003), taking the amplification efficiency of each primer set into account (Tables S4 and S5). The relative expression levels of the *arc* operon genes at the different growth phases in mMRS2 medium at different pH values versus the control conditions (glucose) were tested for significance by randomization and bootstrapping techniques carried out using the relative expression software tool REST 2005 (Corbett Life Science, Sydney, Australia), using 50,000 random reallocations.

2.4. Construction of a knock-out mutant (PTP^-) of L. sakei *CTC 494*

Plasmid DNA was isolated from *E. coli* and *L. sakei* strains using the Qiagen® Plasmid Mini or Midi kit (Qiagen) with minor modifications for the LAB strains. Cells of a culture in the mid-exponential growth phase (OD_{600} of 0.4–0.6) were harvested by centrifugation (3464 × *g*, 20 min) and washed once with 2 ml of TES buffer [50 mM Tris base, 1 mM EDTA, 6.7% (wt vol^{-1}) sucrose, pH 8.0]. Cells were resuspended in Resuspension Buffer, which was provided with the Qiagen® Plasmid Mini or Midi kit, supplemented with 20 μg μl^{-1} of lysozyme (VWR International) and 0.5 U μl^{-1} of mutanolysin (Sigma–Aldrich). After incubation at 37 °C for 1 h, the further steps were performed according to the supplier's recommendations. Purification of plasmid DNA was carried out with the Qiagen® Plasmid Mini kit (Qiagen).

L. sakei strains were transformed by electroporation, as described previously (Berthier et al., 1996). Briefly, competent cells were prepared by washing cells of a culture in the exponential growth phase (OD_{600} of 0.4–0.6) with an ice-cold 10 mM $MgCl_2$ solution twice and storing the cells in a 0.5 M sucrose solution supplemented with 10% (vol vol^{-1}) glycerol. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) at 1.8 kV, 25 μF and 600 Ω. *E. coli* strains were transformed by heat shock (Sambrook and Russell, 2001). Briefly, competent cells were prepared by washing chilled cells of a culture in the exponential growth phase with a cold 0.1 M $CaCl_2$ solution and storing the cells in a 0.1 M $CaCl_2$ solution supplemented with 15% (vol vol^{-1}) glycerol. Transformation by heat-shock was performed by keeping an ice-cold mixture of competent cells and plasmid at 42 °C for 90 s, followed by placing the mixture back on ice.

A primer pair containing a *Not*I and a *Pst*I restriction recognition site was used to amplify an 800 bp fragment of the *PTP* gene of *L. sakei* CTC 494 (Table S6). PCR assays were performed, containing 100 ng of genomic DNA, 200 μM of each dNTP, 20 pmole of each primer, 5 μl of 10× PCR reaction buffer, 1.25 U of *Taq* DNA Polymerase (Roche) and sterile ultrapure water to obtain a final volume of 50 μl. Following amplification, the PCR product size was controlled on a 1.0% (wt vol^{-1}) agarose gel and the remaining reaction mixture was purified using the Wizard® SV Gel and PCR Clean up system (Promega).

The *PTP* gene fragment was ligated via the *Pst*I/*Not*I sites into the pORI28 plasmid vector with T4 DNA ligase (Fermentas Life Sciences, St. Leon-Rot, Germany), previously cut with the same restriction enzymes. As a result, plasmid pPTP was generated and it was cloned into *E. coli* EC1000. Correct insertion of the *PTP* gene fragment into the pORI28 plasmid vector was verified by PCR, targeting the flanking region of the restriction sites on the pORI28 plasmid (Table S6). Therefore, primers pORI28-PTPF and pORI28-PTPR were used to amplify a 286 bp fragment of the pORI28 plasmid

which, upon correct insertion of the amplified *PTP* gene fragment, should result in a 1213 bp fragment. PCR product sizes were controlled on a 1.0% (wt vol^{-1}) agarose gel and the remaining reaction mixture was purified using the Wizard® SV Gel and PCR Clean up system (Promega) and sequenced in the VIB Genetic Service Facility (Antwerp, Belgium).

For construction of a knock-out mutant, gene inactivation was achieved by site-specific integration of plasmid pPTP into the *PTP* gene of *L. sakei* CTC 494, as described previously (Russell and Klaenhammer, 2001). Briefly, a thermosensitive helper plasmid (pVE6007) was introduced through electroporation into *L. sakei* CTC 494. After selection on MRS agar with Cm, the resulting transformants were picked up and grown overnight in MRS medium with Cm. Next, the pPTP plasmid was transformed into *L. sakei* CTC 494 pVE6007 cells. In this way, through selection on MRS agar supplemented with both Em and Cm, *L. sakei* CTC 494 clones containing both plasmids (pVE6007 and pPTP; Table 2) were obtained. This culture was then submitted to heat shock, resulting in loss of pVE6007 and thus directing pPTP to homologous recombination with genomic DNA. Therefore, *L. sakei* CTC 494 containing pVE6007 and pPTP were transferred [1.0% (vol vol^{-1}) inocula] in 10 ml of pre-warmed (40 °C) MRS medium containing Em. After overnight incubation at 40 °C, 100 µl was plated on pre-warmed (40 °C) MRS agar containing Em and incubated at 40 °C overnight. Transformants were recovered and subcultured three times at 40 °C using alternating pre-warmed (40 °C) liquid MRS medium and MRS agar. The absence of plasmids from Em-resistant and Cm-sensitive strains was confirmed by plasmid extractions followed by agarose gel electrophoresis. The integration of the pPTP plasmid into the *PTP* gene of *L. sakei* CTC 494 was confirmed by PCR using primers flanking the integration site (Table S6). The stability of integration was checked by growing the strains in the absence of antibiotic for 24 h, followed by cultivation on MRS agar containing Em.

2.5. Fermentation experiments with L. sakei CTC 494 and L. sakei 23K

Batch fermentations were set up to study relative expression levels of the intergenic regions between the different genes of the *arc* operon and between the last gene of the *arc* operon (i.e., *arcR*) and the *PTP* gene in *L. sakei* CTC 494. Also, the relative expression level of the *PTP* gene in *L. sakei* CTC 494 was assessed as a function of environmental pH. To account for strain-related effects, the relative expression level of the *PTP* gene was also quantified for *L. sakei* 23K. These fermentations were carried out at 30 °C in 15-L Biostat® C fermentors (Sartorius AG) containing 10 L of the appropriate mMRS medium. Fermentor sterilization and control was performed as previously described (Rimaux et al., 2011a). The inoculum was prepared through three subcultures of the strain under study in the appropriate mMRS medium for 12 h each. The first two subcultures were carried out in 10 ml of the appropriate mMRS medium, followed by a third subculture in 100 ml of this medium. The transfer volumes were always 1% (vol vol^{-1}).

Three fermentations in mMRS2 medium were carried out at different constant pH values (pH 5.0, pH 6.0, and pH 7.0) to investigate the influence of pH on the relative expression levels of the *PTP* gene and the intergenic regions between all *arc* operon genes of *L. sakei* CTC 494. A fermentation with *L. sakei* 23K was performed in mMRS2 medium at pH 6.0 to compare the expression level of the *PTP* gene with that of *L. sakei* CTC 494. Also, control fermentations in mMRS3 medium at pH 6.5 were carried out with both *L. sakei* CTC 494 and *L. sakei* 23K, during which no gene expression of the *arc* operons was assumed due to carbon catabolite repression. Two fermentations using mMRS2 medium were carried out at a constant pH of 6.0 using the wild-type and the mutant strain (PTP^-) of *L. sakei* CTC 494. Furthermore, fermentations with *L. sakei* CTC 494 in mMRS4, mMRS5 and mMRS6 medium were carried out at a constant pH of 6.5 to assess the influence of different energy sources on conversion of citrulline into ornithine by *L. sakei* CTC 494. Finally, a fermentation experiment in mMRS7 medium using *L. sakei* CTC 494 was performed at a constant pH of 6.5 to investigate the arginine conversion capabilities when the strain was grown in the presence of glucose and increased arginine concentrations. Also, screening in 100 ml of mMRS1 medium was set up to investigate the capabilities of all *L. sakei* strains listed in Table 1 to convert arginine when grown in the presence of glucose. All these incubations were carried out in a standard incubator at 30 °C for 72 h.

The fermentations for RT-qPCR were carried out in quadruplicate, whereas all other fermentations were carried out in duplicate; the results and figures presented are representative for the repetitions.

2.6. Analysis of microbial growth and metabolites

During screening and fermentation experiments, samples were regularly withdrawn for determination of bacterial growth and concentrations of all metabolites under study.

Growth was followed by measurement of colony-forming units (CFU) and optical density at 600 nm (OD_{600}). Cell concentrations (in CFU ml^{-1}) were obtained by plating tenfold serial dilutions of the samples in saline [0.85% (wt vol^{-1}) NaCl] solution on MRS agar and incubating at 30 °C for 24 h. Every measurement was performed on three independent samples. The errors on the measurements are represented as standard deviations.

Concentrations of arginine, citrulline and ornithine were determined using a Waters 2695 liquid chromatograph coupled to a Quattro Micro™ mass spectrometer (Waters Corp., Milford, MA, USA), as described previously (Rimaux et al., 2011a). Due to matrix interference, all quantifications were carried out through standard addition; the original sample concentrations with corresponding errors were calculated as described previously (Vrancken et al., 2008).

Concentrations of glucose and ribose were determined through high-performance anion exchange chromatography with pulsed amperometric detection using an ICS3000 chromatograph with a CarboPac™ PA10 column (Dionex, Sunnyvale, CA, USA),

as described previously (Rimaux et al., 2011b; Vrancken et al., 2008). Quantifications were carried out using the method of standard addition (Vrancken et al., 2009b). The concentrations in the original samples were calculated as described previously (Vrancken et al., 2009b).

Concentrations of lactic acid and acetic acid were determined by high-performance liquid chromatography with a Waters chromatograph (Waters Corp.), as described previously (Vrancken et al., 2009b). Samples were analyzed in triplicate and the results are represented as the average of these three independent measurements. The errors on the measurements are represented as standard deviations.

3. Results

3.1. Identification and characterization of the PTP gene in L. sakei CTC 494

The presence of a *PTP* gene in the genome of *L. sakei* CTC 494 was shown by sequencing and assembling PCR amplicons generated with primers designed based on the *PTP* gene (*LSA0376*) of *L. sakei* 23K. This *PTP* gene of 1560 bp, located 139 bp downstream of the *arc* operon of *L. sakei* CTC 494 (Fig. 1), showed 100% sequence identity with the *LSA0376* gene in *L. sakei* 23K and no sequence identity with the *arcD* gene in *L. sakei* CTC 494. Furthermore, the protein encoded by the *PTP* gene was present in several LAB species, displaying about 70–80% identity (data not shown). However, the corresponding genes are often annotated as arginine/ornithine antiporters, putative transporters, or C4-dicarboxylate anaerobic carriers (pfam03606), without further physiological evidence.

3.2. Quantification of the relative expression level of intergenic regions between the different genes of the arc operon as a function of pH in L. sakei CTC 494

For all intergenic regions between the different genes of the *arc* operon and the intergenic region between the *arcR* and *PTP* genes, induction of expression in *L. sakei* CTC 494 was found during fermentation in mMRS2 medium compared to control fermentation in mMRS3 medium. A clear correlation between pH and relative expression levels of the intergenic regions between the different *arc* operon genes was found, with, overall, the highest increase of expression at the end of the exponential growth phase at optimal pH for growth (pH 6.0) (Fig. 2). The highest relative expression level was found for the intergenic region between *arcA* and *arcB*, whereas intergenic regions *arcB–arcC* and *arcC–arcT* displayed the lowest relative expression levels. The relative expression level for the intergenic regions was generally most pronounced at pH 6.0, in particular when compared with pH 5.0 and, to a lesser extent, with pH 7.0. For intergenic region *arcR–PTP*, expression levels at pH 5.0 and pH 7.0 were both much lower than at pH 6.0. The increase in the relative expression level of the *arcR–PTP* intergenic region confirmed that *PTP* is co-expressed with the other genes of the *arc* operon and thus is part of this *arc* operon.

3.3. Quantification of the relative gene expression level of a putative transport protein as a function of pH in L. sakei CTC 494 and L. sakei 23K

The *PTP* gene of *L. sakei* CTC 494 was induced at all pH values and growth phases investigated compared to control fermentation in mMRS3 medium (Fig. 3A). Nevertheless, the relative expression level of this gene was clearly highest when the growing culture reached the end of the exponential growth phase. Furthermore, the environmental pH influenced the expression level of the *PTP* gene in *L. sakei* CTC 494, which showed the highest relative expression level at the end of the exponential growth phase at the optimal pH for growth (pH 6.0).

The expression level of the *PTP* gene in *L. sakei* 23K at optimal pH for growth (pH 6.0) was lower than for *L. sakei* CTC 494 in all growth phases tested (Fig. 3B). The relative expression level of the *PTP* gene was maximal at the first sampling point, suggesting the highest expression level during the early-exponential growth phase.

3.4. Physiological role of the putative transport protein in L. sakei CTC 494

To elucidate the physiological function of the *PTP* gene and its possible contribution to ADI pathway activity in *L. sakei* CTC 494, a knock-out mutant (*PTP*$^-$) of this strain (*L. sakei* CTC 494 PTP$^-$) was successfully constructed using the pVE6007/pORI28 gene disruption system. Fermentation in mMRS2 medium at pH 6.0 using *L. sakei* CTC 494 PTP$^-$ was compared with fermentation with the wild-type strain of *L. sakei* CTC 494 (Fig. 4). For both strains, growth started without a lag phase, reaching final cell counts of approximately 8.2 log (CFU ml^{-1}). Both strains started arginine conversion after approximately 6–8 h of fermentation, resulting in production of both citrulline and ornithine. Once all arginine was depleted (after approximately 16–17 h of fermentation), further conversion of most of the citrulline into ornithine took place in the case of the wild-type strain solely (Fig. 4). In the knock-out mutant, the concentrations of citrulline and ornithine did not change after arginine depletion.

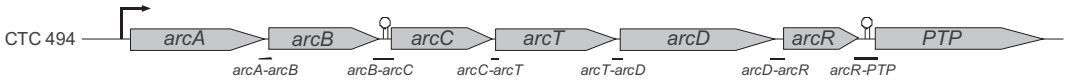

Fig. 1. Organization of the *arc* operon and the *PTP* gene in *L. sakei* CTC 494 (*arcA*, arginine deiminase; *arcB*, ornithine carbamoyltransferase; *arcC*, carbamate kinase; *arcT*, putative transaminase; *arcD*, arginine/ornithine antiporter; *arcR*, regulatory protein; *PTP*, putative transport protein). The different intergenic regions whose expression was targeted by RT-qPCR are indicated by lines representing the corresponding PCR amplicons. For the entire *arc* operon, there are only six sequence variations between *L. sakei* strains CTC 494 and 23K (see also Rimaux et al., 2012): two sequence variation are located in the upstream region of the *arcA* gene and the four remaining sequence variations are located in the *arcA* gene, of which one results in a non-synonymous mutation at nucleotide position 1133.

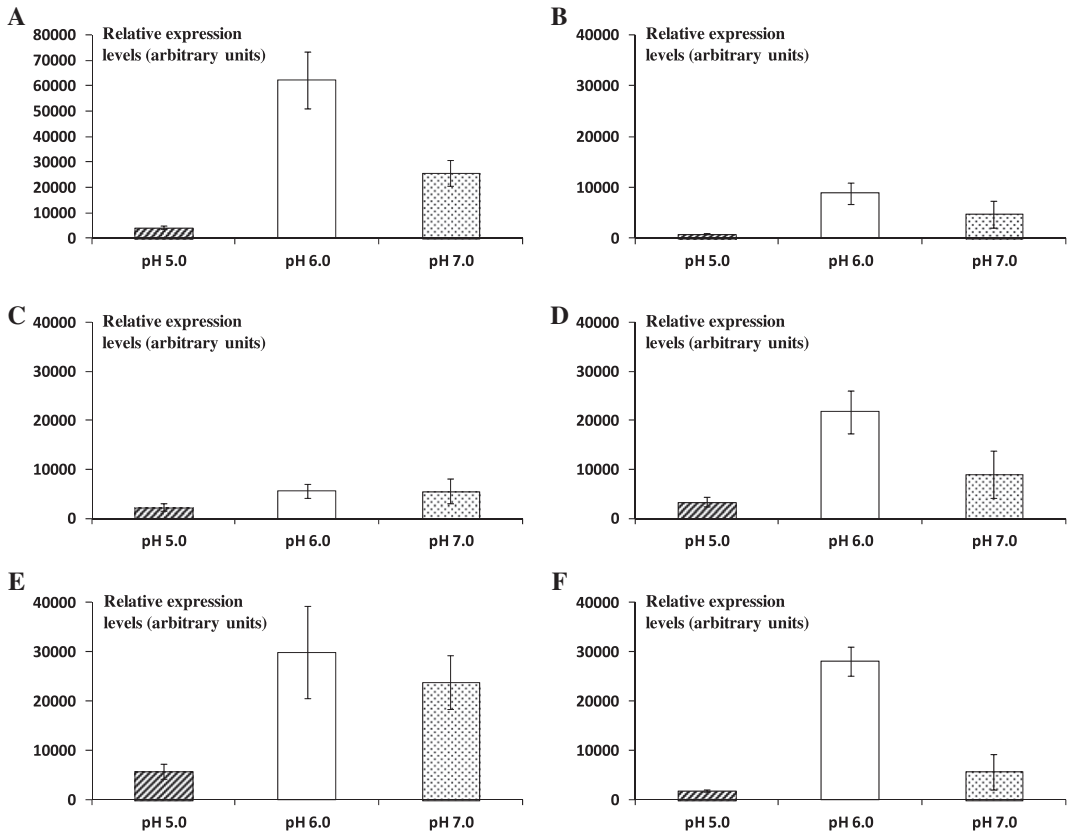

Fig. 2. Relative expression levels of the intergenic regions between all *arc* operon genes and between the *arcR* and *PTP* genes of *L. sakei* CTC 494 [(A) *arcA–arcB*, (B) *arcB–arcC*, (C) *arcC–arcT*, (D) *arcT–arcD*, (E) *arcD–arcR*, and (F) *arcR–PTP*] at the end of exponential growth phase (OD_{600} of 0.5) when grown in mMRS medium supplemented with 3 g l^{-1} of arginine (mMRS2) at different constant pH values (pH 5.0, ▨; pH 6.0, □; and pH 7.0, ▒). Data shown are mean values ± SD from four independent fermentations, where the probability of different expression levels compared to the control fermentation was calculated by the pair-wise fixed reallocation randomization test (50,000 permutations) between samples and control groups ($P < 0.001$).

3.5. Influence of different energy sources on arginine conversion and citrulline-into-ornithine conversion kinetics

Besides high environmental pH, the addition of glucose and/or ribose decreased citrulline-into-ornithine conversion by *L. sakei* CTC 494 at the optimal pH for growth (Fig. 5). For all combinations of energy sources tested, final cell concentrations of approximately 9.0 log (CFU ml^{-1}) were obtained except for fermentation in mMRS2 medium, for which a final cell concentration of 8.0 log (CFU ml^{-1}) was reached (data not shown). In all cases, arginine conversion started after approximately 6–8 h of fermentation and was depleted after

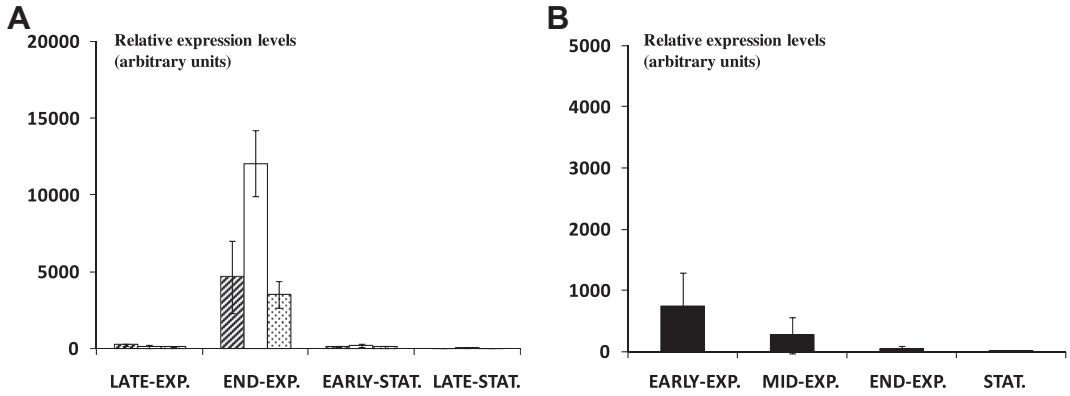

Fig. 3. Relative expression of the *PTP* gene (A) as a function of environmental pH (pH 5.0, ▨; pH 6.0, □; and pH 7.0, ▒) and in different growth phases for *L. sakei* CTC 494 when grown in mMRS medium supplemented with 3 g l^{-1} of arginine (mMRS2), and (B) as a function of growth phase at the optimal pH for growth (pH 6.0, ■) for *L. sakei* 23K when grown in mMRS medium supplemented with 3 g l^{-1} of arginine (mMRS2). The probability of different expression levels compared to the control fermentation was calculated by the pair-wise fixed reallocation randomization test (50,000 permutations) between samples and control groups ($P < 0.001$).

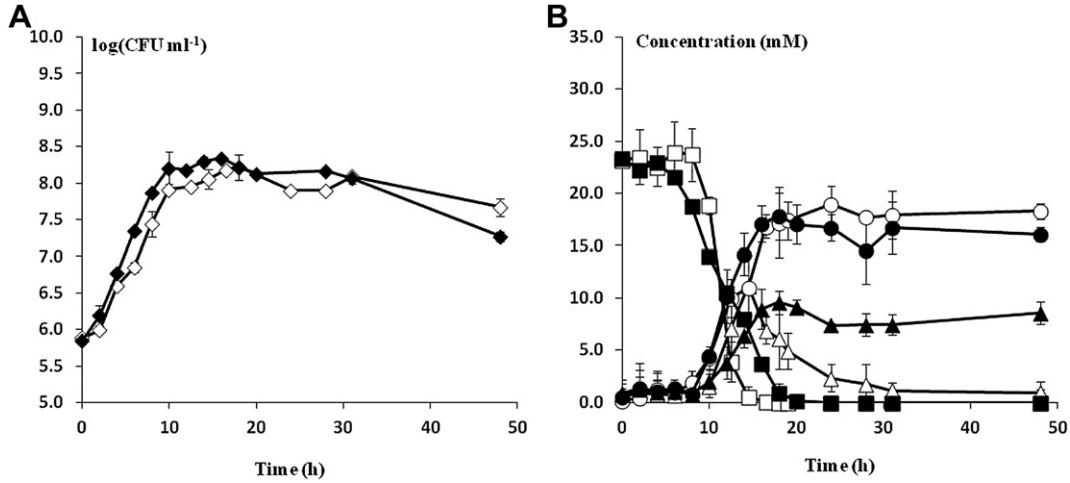

Fig. 4. Growth [log(CFU ml^{-1})] (A) and arginine conversion (B) by *L. sakei* CTC 494 (open symbols) and a knock-out mutant (*PTP*$^-$) of *L. sakei* CTC 494 (closed symbols) when grown in mMRS medium supplemented with 3 g l^{-1} of arginine (mMRS2) at pH 6.0. Extracellular concentrations (in mM) of arginine (squares), citrulline (triangles), and ornithine (circles).

10–14 h, resulting in its complete conversion into both citrulline and ornithine (data not shown). After all arginine was depleted, clear-cut further conversion of citrulline into ornithine took place only for fermentation in mMRS2 medium. Regardless of some variability in the initial citrulline accumulation, further conversion of citrulline into ornithine was somewhat less pronounced (mMRS4 medium) or even strongly reduced (mMRS5 and mMRS6 media) when additional carbohydrates were present, resulting in higher final extracellular citrulline concentrations (Fig. 5).

Moreover, the presence of glucose (mMRS1 medium) reduced the overall kinetics of the ADI pathway in a strain-dependent manner. When testing at a set of 29 *L. sakei* strains with an operational ADI pathway, the arginine conversion kinetics could be divided into three groups (Fig. S1). Whereas no arginine conversion could be found in *L. sakei* CTC 494 after

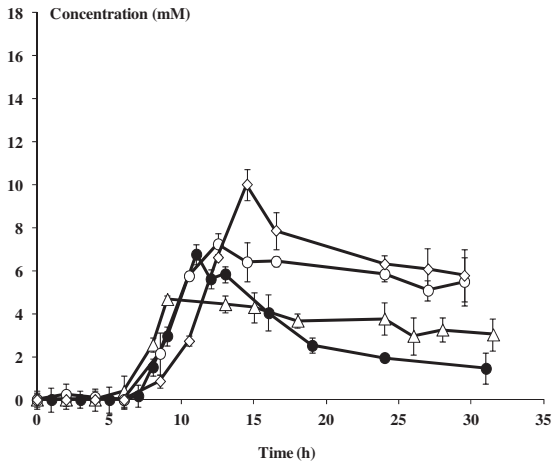

Fig. 5. Evolution of citrulline concentration (in mM) during fermentation of *L. sakei* CTC 494 at 30 °C in mMRS medium lacking glucose (pH 6.5) and supplemented with 3 g l^{-1} of arginine (mMRS2) (●), 3 g l^{-1} of arginine and 1 g l^{-1} of glucose (mMRS4) (△), 3 g l^{-1} of arginine and 1 g l^{-1} of glucose plus ribose (mMRS5) (◇), and 3 g l^{-1} of arginine and 5 g l^{-1} of ribose (mMRS6) (○).

72 h of fermentation, *L. sakei* 23K consumed all arginine after 48 h of fermentation. However, arginine conversion by *L. sakei* CTC 494 occurred when the initial arginine concentration increased from 3 g l^{-1} to 5 g l^{-1} (mMRS7 medium) (Fig. S2).

4. Discussion

Arginine conversion through the ADI pathway by *L. sakei* CTC 494 operates at all pH values allowing growth and results in the production of both citrulline and ornithine (Rimaux et al., 2011a). Interestingly, when all arginine is depleted, pH-dependent citrulline-into-ornithine conversion occurs, albeit not above pH 7.0 at which the ADI pathway is still operational (Rimaux et al., 2011a). During the present study, an additional uncharacterized putative amino acid transporter (encoded by the *PTP* gene), downstream from the *arc* operon, was detected in *L. sakei* CTC 494, which is present in *L. sakei* 23K too (Zuñiga et al., 2002). It was shown that the *PTP* gene was co-expressed with genes of the *arc* operon and thus could be regarded as an additional gene of the *arc* operon in *L. sakei*. Differential expression levels for the intergenic regions of the *arc* operon, with a clear maximum for *arcA*–*arcB*, are possibly related to the presence of a putative rho-independent terminator directly downstream of the *arcB* gene (Rimaux et al., 2012). A knock-out mutant of *L. sakei* CTC 494 (PTP$^-$), impaired in its *PTP* gene, was inhibited in its capacity to convert extracellular citrulline into ornithine after all arginine was depleted. This finding suggests that this gene is a putative citrulline/ornithine antiporter, which will need to be confirmed with direct activity assessments in liposomes, appropriate uptake assays using heterologous expression in defined mutant backgrounds and/or measurements of potential chemiosmotic gradients. Genes displaying sequence identity with this *PTP* gene are present in other LAB species as well (Dong et al., 2004; Tettelin et al., 2001; Vrancken et al., 2009a). Gene expression of the *PTP* gene was highest at the end-exponential or mid-exponential growth phase in the case

of *L. sakei* CTC 494 and *L. sakei* 23K, respectively. This difference in the onset of gene expression between these strains has been found for the other genes of the *arc* operon as well, which may be correlated with differences in the promoter region upstream of the *arc* operon (Rimaux et al., 2012). At pH 7.0, the relative expression level of the *PTP* gene of *L. sakei* CTC 494 was noticeably lower than at the optimum of pH 6.0. This is in contrast to the other genes of the *arc* operon, for which expression at pH 7.0 is only slightly lower than at pH 6.0, but clearly higher than at pH 5.0 (Rimaux et al., 2012).

The presence of additional energy sources in the medium, namely glucose and ribose, decreased the kinetics of arginine and citrulline conversion. The influence of glucose on arginine conversion was strain-dependent. Pronounced differences in metabolic capacity among strains of *L. sakei* are known (Ammor et al., 2005; Guilbaud et al., 2011). A distinct sensitivity toward the presence of glucose might result in the altered metabolic potential of various *L. sakei* strains. Interestingly, similar strain-dependent differences in putative *cre* sites upstream of the *arc* operon in different *L. sakei* strains have been found as well (Rimaux et al., 2012). In the presence of increased amounts of arginine, catabolic repression of ADI activity by *L. sakei* CTC 494 was partially alleviated, which may be related to the fact that the ADI pathway is stimulated by its substrate arginine (Fernández and Zúñiga, 2006). In addition to decreased arginine conversion, the presence of both ribose and glucose decreased citrulline-into-ornithine conversion at the optimal pH for growth, similar to arginine conversion at high pH values. Previous studies also revealed a myriad of differentially expressed genes and proteins when *L. sakei* was grown on ribose instead of glucose (McLeod et al., 2010, 2011). Also, ribose improves survival of *L. sakei* in its stationary growth phase (Champomier-Vergès et al., 1999). Different energy sources lead to different metabolic states, so that it may be assumed that conditions that promote the cellular energy status repress ADI pathway activity, as seen for most bacterial species studied so far (Cunin et al., 1986).

In conclusion, the results obtained in this work indicate that the *arc* operon in *L. sakei* CTC 494 contains an additional previously unknown gene, i.e. a putative citrulline/ornithine antiporter gene (formerly annotated as *PTP*), whose encoded protein is involved in citrulline-into-ornithine conversion through the ADI pathway. Expression of this putative citrulline-into-ornithine transporter gene was influenced by pH and the presence of additional energy sources. Hence, agreement was obtained between fermentation data and *PTP* gene expression levels. Consequently, several regulatory networks may play a role in control of the ADI pathway in *L. sakei* CTC 494. Finally, strain differences within *L. sakei* in ADI pathway activity underline differences in metabolic fitness. Such insights may help in competitiveness-based selection of appropriate starter cultures of *L. sakei* to be used in food fermentation.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resmic.2012.11.004.

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