Catabolism of Serine by *Pediococcus acidilactici* and *Pediococcus pentosaceus*

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Abstract

The ability to produce diacetyl from pyruvate and L-serine was studied in various strains of *Pediococcus pentosaceus* and *Pediococcus acidilactici* isolated from cheese. After being incubated on both substrates, only *P. pentosaceus* produced significant amounts of diacetyl. This property correlated with measurable serine dehydratase activity in cell-free extracts. A gene encoding the serine dehydratase (dsdA) was identified in *P. pentosaceus*, and strains that showed no serine

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dehydratase activity carried mutations that rendered the gene product inactive. A functional dsdA was cloned from *P. pentosaceus* FAM19132 and expressed in *Escherichia coli*. The purified recombinant enzyme catalyzed the formation of pyruvate from L- and D-serine and was active at low pH and elevated NaCl concentrations, environmental conditions usually present in cheese. Analysis of the amino acid profiles of culture supernatants from dsdA⁺ and dsdA⁻ strains of *P. pentosaceus* did not show differences in serine levels. In contrast, *P. acidilactici* degraded serine. Moreover, this species also catabolized threonine and produced alanine and α-aminobutyrate.

1 Introduction

*Pediococcus pentosaceus* and *Pediococcus acidilactici* are homofermentative Gram-positive cocci that produce D- and L-lactate from carbohydrates. These bacteria are found regularly in the non-starter population of raw milk cheeses at the end of the ripening process. Thus, *Pediococcus* spp. were isolated from Cheddar, ewes’ milk cheeses, Comté, AOC Salers, Puone di Moena, and various raw milk cheeses traditionally produced in Switzerland such as Gruyère, Emmental, Appenzeller, and Tilsit (1-8).

Although several reports have indicated that pediococci alone or combined with lactobacilli can accelerate cheese ripening and enhance flavor development, respectively (9-13), knowledge about metabolic activities affecting the maturation of cheese is limited. Studies on physiological and biochemical activities of *Pediococcus* spp. have mainly focused on carbohydrate metabolism and proteolytic and lipolytic activities (14-16). Researchers have also reported that pediococci racemize L-lactate to D-lactate under anaerobic conditions and that pediococci can oxidize lactate to acetate and carbon dioxide under aerobic conditions (17). However, information on
the enzymatic degradation of amino acids, important precursors for flavor compounds, is sparse.

Diacetyl contributes to desirable flavor in fermented dairy products. A pathway well-known for producing diacetyl is through degrading citrate (18). In this pathway, pyruvate is formed as an intermediary compound that is then converted via α-acetolactate to diacetyl. Although pediococci do not appear to utilize citrate, diacetyl formed by this species has been reported (19, 20). Pyruvate, which is derived from the metabolism of carbohydrates or amino acids, is likely used for synthesizing diacetyl.

In this study, the ability of various strains of *P. acidilactici* and of *P. pentosaceus* isolated from cheese to produce diacetyl from pyruvate and serine was analyzed. Furthermore, serine dehydratase activity that releases pyruvate from serine was investigated in detail in these strains. The deamination of serine is of interest since this compound is released by proteolysis during cheese ripening and is a potential precursor for flavor compounds.

2 Material and Methods

2.1 Bacterial strain, media, and growth conditions

The strains used in this study are listed in Table 1. Pediococci which had been identified with 16S rRNA gene sequence analyses were stored at -80 °C in sterile reconstituted skim milk powder (10%, w/v). The bacteria were cultivated at 30 °C in *Lactobacillus plantarum* (22). For our study, the basal broth (pH 7.0) was slightly modified and consisted of KH₂PO₄ 9 g L⁻¹, yeast extract 5 g L⁻¹, casein hydrolysate 1 g L⁻¹, MgSO₄ x 7H₂O 0.1 g L⁻¹, MnCl₂ x 4H₂O 0.1 g L⁻¹, galactose 2 g L⁻¹, and 5 mM L-serine.
Strains of *Escherichia coli* were grown at 37 °C in LB broth (23) supplemented with ampicillin (100 µg mL⁻¹), when necessary.

### 2.2 Determination of diacetyl and acetoin formation

Pediococci were grown in 50 mL of MRS broth for 16 h at 30 °C. Cells were then sedimented at 3000 g for 10 min, washed twice with 50 mM potassium phosphate (pH 5.5), and finally suspended in 1 mL of the same buffer. The cell suspension (0.5 mL) was incubated with either 10 mM pyruvate or 10 mM L-serine in 70 mM potassium phosphate (pH 5.5) containing 1X RPMI 1640 Vitamins Solution (Sigma-Aldrich, Switzerland) and 50 µM pyridoxal 5'-phosphate (PLP) at a final volume of 5 mL in sealed tubes at 30 °C for 24 h.

Diacetyl and acetoin were determined with gas chromatography with flame ionization detection (GC/FID). Therefore, solid sodium chloride was added until the sample was saturated. After the sample was heated at 95 °C for 45 min, 1 mL of the headspace was injected into the GC. The GC was equipped with a capillary column HP-ULTRA 2 cross-linked 5 % phenyl methyl silicone (50 m x 0.32 mm x 0.52 µm). The transfer to the capillary column was performed in split mode (1:8), and helium served as the carrier gas. The temperature program was the following: initial 50 °C (7 min) and then ramp with 40 °C min⁻¹ to 270 °C. Additional operating conditions of the GC were injector temperature of 150 °C and detector temperature of 320 °C.

### 2.3 Preparation of cell-free extract

*Pediococcus* ssp. were grown for 16 h at 30 °C in 50 mL of MRS broth or basal broth. The cells were then harvested by centrifugation (3000g, 10 min, 25 °C), washed twice with 20 mM sodium phosphate (pH 7.4), and finally suspended in 1 mL of the same buffer. After approximately 0.6 g of glass beads (212-300 µm) were added, the cells were disrupted in the Omni Bead Ruptor 24 (Omni International, USA) by
vigorous shaking (one 45-s mixing sequence at a speed of 6 m s\(^{-1}\)). The extract was cleared with centrifugation (17900g, 10 min, 4 °C).

### 2.4 Protein determination

The protein content of the extracts was determined with bicinchoninic acid (BCA) using the micro BCA reagent protocol as described by Smith et al. (24). Bovine serum albumin was used to prepare a standard curve.

### 2.5 Enzyme assay

Serine dehydratase activity was determined as follows: cell-free extract (50 µL) or recombinant enzyme (0.2 µg) was added to 25 mM sodium phosphate (pH 7.4), 50 µM PLP, and various concentrations of L-serine or D-serine, respectively, in a final volume of 200 µL. Cell-free extract was incubated for 30 min and recombinant enzyme for 10 min at 30 °C. The assay was stopped by adding 20 µL of 5 M perchloric acid (Fluka, Switzerland). The solution was centrifuged (17900g, 10 min) and 50 µL of the supernatant was separated by HPLC using an Aminex HPX-87H column (300 x 7.8 mm) and 3.8 mM H\(_2\)SO\(_4\) as the mobile phase. The operating conditions were as follows: column temperature = 65 °C, flow = 0.6 mL min\(^{-1}\), detection at 210 nm. Chromatographic identification of pyruvate was performed by comparing the retention times to those of a pyruvate standard. The amount of pyruvate formed was calculated by referring to a standard curve prepared with various concentrations of sodium pyruvate and normalized for protein content.

The \(K_m\) and \(v_{max}\) values of recombinant enzyme for L-serine and D-serine were determined with variable concentrations of L-serine (0.25 - 10 mM) and D-serine (0.05 - 10 mM), respectively. \(K_m\) and \(v_{max}\) were calculated from Hanes-Woolf plots (the ratio of the substrate and velocity as a function of the substrate) and represent the mean of triplicates.
2.6 Isolation of DNA

Genomic DNA from *Pediococcus* ssp. was extracted by robot extraction (BioRobot EZ1, EZ1 DNA Tissue kit; Qiagen, Switzerland) from 1 mL of culture grown overnight at 30 °C. Before being extracted, cells were treated with 0.05 N NaOH for 15 min at room temperature followed by TES buffer (0.1 M Tris-HCl [pH 8.0], 10 mM EDTA, 25% sucrose) supplemented with lysozyme (1 mg mL\(^{-1}\)) for 1 h at 37 °C.

*E. coli* plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions.

2.7 DNA sequencing

The amplification products obtained with the primer pair PEPE_1745F/PEPE_1745R (Table 1) from the genomic DNA of *P. pentosaceus* strains were sequenced using the BigDye Terminator Cycle Sequencing kit and the ABI Prism 310 Genetic Analyser (Applied Biosystems, Switzerland).

2.8 Construction of plasmids

*DsdA* was amplified with the primer pair PEPE_1745F/PEPE_1745R from genomic DNA of *P. pentosaceus* FAM19132 and *P. pentosaceus* FAM19080 by PCR. The PCR product was cloned into the pEXP5-CT/TOPO expression vector (Invitrogen, Switzerland) according to the manufacturer’s instructions. The cloning was such that the expressed protein had eight extra amino acids including a polyhistidine tag at the C-terminus (KGHHHHHH). The plasmids containing *dsdA* in the proper orientation were confirmed by sequencing and called, depending on the origin of the *dsdA* gene, pEXP5-CT/dsdA[19132] and pEXP5-CT/dsdA[19080]. Restriction, ligation, and calcium-chloride-mediated transformation of *E. coli* cells were performed using standard procedures (23).
2.9 Expression and purification of His-tagged proteins

*E. coli* BL21(DE3) transformed with pEXP5-CT/dsdA[19132] and pEXP5-CT/dsdA[19080] was grown in 200 mL LB broth containing ampicillin (100 µg mL⁻¹) at 37 °C on a shaker. When the optical density at 600 nm reached 0.5, expression of recombinant *dsdA* was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (final concentration). Expression was performed at 27 °C. After 4 h of incubation on a shaker, the bacterial cells were harvested by centrifugation, washed twice with 20 mM sodium phosphate (pH 7.4), and then frozen at -20 °C. Cells were disrupted as described above, and the recombinant proteins were purified with Protino Ni-TED 2000 packed columns (Macherey-Nagel, Switzerland) according to the manufacturer’s instructions. The elution fractions were pooled and immediately applied to PD-10 columns (GE Healthcare, Switzerland) that had been equilibrated with 20 mM sodium phosphate (pH 7.4) to remove imidazole. The eluted protein fraction was concentrated with Ultrace 30K (Millipore, Ireland) and analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by colloidal Coomassie staining. The concentration of the purified protein was determined as described above.

2.10 Size exclusion chromatography

To determine the molecular size under non-denaturing conditions, the purified recombinant proteins were analyzed with size exclusion chromatography on a Superdex 200 HR 10/30 column (GE Healthcare) with 25 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl at a flow rate of 0.5 mL min⁻¹. The column was calibrated with a mixture of proteins of known molecular weight (Gel Filtration Standard, Bio-Rad, Switzerland).
2.11 Two-dimensional thin-layer chromatography

Pediococcus ssp. pre-cultured in MRS broth were grown in 50 mL of MRS broth or 50 mL of a basal broth at 30 °C. To study amino acid metabolism, 1 µL of the culture supernatants was applied spotwise onto HPTLC cellulose plates (Merck, Germany) and separated with two-dimensional thin-layer chromatography (2D-TLC). The plates were developed in the first dimension with n-butanol – acetone – diethylamine - water (10 + 10 + 2 + 5, v/v/v/v) and in the second dimension with 2-propanol – formic acid - water (40 + 2 + 10, v/v/v). To visualize the amino acids, plates were immersed into 0.1 % ninhydrin solution (dissolved in acetone) and heated at 110 °C for 5 min.

3 Results

3.1 Formation of diacetyl and acetoin

The formation of diacetyl and acetoin was studied in resting cell suspensions of nine strains of P. pentosaceus and 10 strains of P. acidilactici (Table 1). Therefore, cells were collected 16 h after inoculation, washed and incubated in phosphate buffer containing pyruvate at pH 5.3. After 24 h incubation at 30 °C, all studied strains of P. pentosaceus produced diacetyl and acetoin (Figure 1A). Only very low levels or no formation of these two flavor compounds were detected in the samples containing the strains of P. acidilactici (data not shown). When the Pediococcus ssp. were incubated in phosphate buffer containing L-serine, diacetyl and acetoin were found in the headspace of the strains of P. pentosaceus (Figure 1B). Four strains, namely, P. pentosaceus FAM17622, P. pentosaceus FAM19080, P. pentosaceus FAM19144, and P. pentosaceus FAM20650, did not produce diacetyl or produced only very low levels. No flavor formation from serine was observed with the strains of P. acidilactici (data not shown).
3.2 Serine dehydratase activity

First, serine dehydratase activity was determined in *P. pentosaceus* FAM19132 which produced diacetyl when incubated with serine. Therefore, cell-free extract was prepared from cells that were collected 12, 24, 48, 72 and 96 h after inoculation of MRS broth. The release of pyruvate from serine was only detected in the samples taken at 12 and 24 h. This showed the presence of a serine dehydratase whose activity was apparently down-regulated in resting cells.

Then, cell-free extracts of all *Pediococcus* ssp. which had been grown for 16 h in MRS were incubated with L-serine. All *P. pentosaceus* strains except *P. pentosaceus* FAM17622, *P. pentosaceus* FAM19080, *P. pentosaceus* FAM19144, and *P. pentosaceus* FAM20650 exhibited serine dehydratase activity (Fig. 1B). On the contrary, no formation of pyruvate was found in the extracts from the *P. acidilactici* strains (data not shown).

3.3 Sequence analysis of *dsdA*

The genome data of *P. pentosaceus* ATCC 25745 (acc. no. CP000422) and the entire genome shotgun sequences of *P. acidilactici* DSM 20284 (acc. no. AEEG01000001-AEEOG1000012) were analyzed for the presence of genes encoding a serine dehydratase. Indeed, the gene *PEPE_1745* from *P. pentosaceus* ATCC 25745 encodes a putative D-serine ammonia-lyase. The translated sequence of this gene showed 49% identity to the well-studied PLP-dependent D-serine dehydratase (DsdA) from *E. coli* (acc. no. P00926). A homologous gene was not found in *P. acidilactici* DSM 20284.

Based on the nucleotide sequence of *PEPE_1745*, the primer pair *PEPE_1745F/PEPE_1745R* was designed and used to amplify and sequence the gene from all studied strains of *P. pentosaceus*. Regarding the four *P. pentosaceus* strains that did not exhibit serine dehydratase activity, a 1-bp deletion and a 19-bp deletion were
Both mutations introduced frameshift mutations (Figure 2). In *P. pentosaceus* FAM20650, a transition from C to T was detected at position 151 leading to a nonsense mutation. By comparing the sequence obtained from FAM19080 with *PEPE_1745*, two missense mutations were identified that substituted a threonine residue with an alanine residue at position 67 and a proline residue with a leucine residue at position 278 in the translated sequence.

### 3.4 Purification and properties of DsdA

The genes homologous to *PEPE_1745* were cloned from FAM19132 and FAM19080 in frame with a C-terminal polyhistidine tag and named *dsdA*[19132] and *dsdA*[19080], respectively. Both proteins were expressed in soluble form in *E. coli* BL21(DE3) and purified to apparent homogeneity with one-step nickel-affinity chromatography (Figure 3). Both proteins migrated with 52 kDa in SDS-polyacrylamide gels (expected 48.5 kDa). The apparent size determined by size exclusion chromatography under non-denaturing conditions was 120 kDa for each protein (data not shown).

The purified recombinant proteins were assayed for serine dehydratase activity. Although no enzyme activity was detected for *DsdA*[19080], *DsdA*[19132] clearly exhibited serine dehydratase activity. This protein formed pyruvate from L-serine and D-serine. No formation of a keto acid was detected when L-threonine was used as substrate. The activity of recombinant *DsdA*[19132] was studied as a function of L-serine and D-serine at 30 °C and pH 7.4. The enzyme followed Michaelis-Menten kinetics, and $K_m = 7.5 \pm 0.3$ mM ($v_{max} = 880.7 \pm 71.3$ µmol min$^{-1}$ mg$^{-1}$) for L-serine and $K_m = 0.5 \pm 0.1$ mM ($v_{max} = 71.3 \pm 4.7$ µmol min$^{-1}$ mg$^{-1}$) for D-serine were calculated.
Sodium phosphate buffer with various pHs and 15 mM L-serine were used to study the pH dependence. DsdA\textsuperscript{[19132]} exhibited optimal activity between pH 6.5 and 7.4 and had about 70\% residual activity at pH 5.5. Furthermore, the influence of NaCl (1\%, 2\%, and 4\%) was studied. Whereas degradation of L-serine was slightly inhibited by increasing the amounts of NaCl, the degradation of D-serine was enhanced (data not shown). To study thermal stability, the enzyme was incubated for 1 h at 43 °C, 48 °C, 57 °C, and 70 °C. The enzyme treated with the three latter temperatures showed strongly decreased serine dehydratase activity (data not shown).

3.5 Serine degradation

To study the bacterial metabolism of serine, the amino acids of culture supernatants were separated with 2D-TLC after 1-, 2- and 3-days fermentation. The amino acid patterns from \textit{P. pentosaceus} FAM19132, \textit{P. pentosaceus} FAM19144, and \textit{P. acidilactici} FAM18098 were compared. No changes in the amino acid profiles were detected when the strains were grown in MRS broth (data not shown). Significant changes were found between the two strains of \textit{P. pentosaceus} and \textit{P. acidilactici} FAM18098 when they were cultured in basal broth. The latter strain completely metabolized serine and threonine, and increased alanine and α-aminobutyrate levels were detected (Figure 4B). In contrast, the serine, threonine, and alanine levels in the culture supernatants of both strains of \textit{P. pentosaceus} were not different from the basal broth, and formation of α-aminobutyrate was not observed (Figure 4C-D).

Moreover, all three pediococci increased the level of ornithine. Further analysis revealed that all studied strains of pediococci produced ornithine, but only the \textit{P. acidilactici} strains degraded threonine and serine (data not shown).
4 Discussion

Non-starter lactic acid bacteria (NSLAB) constitute a significant part of the microbial ecosystem in cheese and influence its maturation and quality (25). Interest in using NSLAB as adjunct cultures in manufacturing cheese to accelerate and enhance flavor formation and to control the adventitious microbiota is increasing. To select appropriate strains, understanding the metabolic activities of the various species present in the NSLAB flora is crucial.

Proteolysis and degradation of amino acids are a major biochemical event during cheese ripening (26). *P. acidilactici* and *P. pentosaceus* are often detected in cheese at the end of the ripening process, and their presence suggests a role during cheese ripening. These species have proteolytic activities (14, 27, 28), but to our knowledge, reports on amino acid use are sparse. Amino acid degradation involves decarboxylases, aminotransferases, deaminases, lyases, and dehydratases (29). Most of the studies on amino acid metabolism by NSLAB have focused on transaminases and lyases. The catabolism of serine by dehydratases is of special interest since these enzymes are active in an anaerobic environment and produce pyruvate, which can be converted not only into lactic acid but also into acetic acid, ethanol, diacetyl, and acetaldehyde. These compounds are aroma active and contribute to the typical flavor of a range of fermented products.

We had observed that the studied strains of *Pediococcus* ssp. isolated from cheese did not metabolize citrate (data not shown). Inspired by reports that *Pediococcus* ssp. produce diacetyl, we first analyzed whether the strains can use pyruvate, an intermediary metabolite in citrate degradation and a precursor of diacetyl formation.

Interestingly, all studied *P. pentosaceus* strains clearly produced diacetyl, a not distinctive property of the *P. acidilactici* strains. When serine was used as precursor, again various *P. pentosaceus* strains produced diacetyl, indicating that serine
dehydratase activity is present that converts serine to pyruvate. In fact, serine
dehydratase activity was measured in the cell-free extracts of strains that produced
significant amounts of diacetyl from serine. This indicated that this enzyme might be
used as a marker for selecting flavor-producing strains.

With the advent of next-generation sequencing technologies, more and more
genome data on lactic acid bacteria are published. These data can be searched for
genes putatively involved in amino acid degradation. In this study, we used the
information to search for serine dehydratases present in pediococci and found that
PEPE_1745 from *P. pentosaceus* ATCC 25745 encodes a putative DsdA.

Interestingly, by sequencing and aligning the nucleotide sequences of the *dsdA* gene
from the *P. pentosaceus* strains (see Figure S1 in the supplemental material),
mutations that clearly affect the functionality of the enzyme were identified in the
strains that did not exhibit serine dehydratase activity. In *P. pentosaceus* FAM17622,
*P. pentosaceus* FAM19144, and *P. pentosaceus* FAM20650, mutations lead to
truncated, non-functional enzymes. In *P. pentosaceus* FAM19080, the open reading
frame of the gene seems to be complete. However, an alignment of DsdA[19080]
with all DsdA sequences from the studied *P. pentosaceus* strains and DsdA from *E.
coli* showed that at position 67 a threonine residue is substituted with an alanine
residue and at position 278 a proline residue is substituted with a leucine residue.
The latter amino acid substitution lies next to the so-called tetrاغlycine loop (residues
G279, G281, G282, and G283 in DsdA from *E. coli*), which is conserved in PLP-
dependent enzymes and is involved in the binding of PLP (30). Therefore, we think
that the binding of PLP is impaired in DsdA from *P. pentosaceus* FAM19080. This
also explains why serine dehydratase activity was not detected in this strain. By
cloning *dsdA* from *P. pentosaceus* FAM19080 and analyzing its gene product, we
confirmed that the enzyme actually is inactive. We suggest that the serine
dehydratase activity detectable in *P. pentosaceus* is provoked by DsdA activity and is
involved in forming diacetyl from serine (Fig. 5). *P. acidilactici* and *P. pentosaceus* are difficult to separate using physiological tests (15); thus, the presence of serine dehydratase activity may be used as a marker for distinguishing both species.

To evaluate whether DsdA from *P. pentosaceus* is active in a cheese environment and plays a role in flavor formation, the recombinant gene product from *P. pentosaceus* FAM19132 was characterized. The recombinant enzyme used L-serine and D-serine, but showed a higher affinity for D-serine. However, L-serine was decomposed more rapidly than D-serine. L-Threonine was not accepted as substrate. The low pH and elevated salt concentrations present in cheese did not have a major impact on the activity. Interestingly, salt seemed to regulate the rate of dehydratation of L-serine and D-serine. That bacterial D-serine dehydratase uses D- and L-serine has been reported for *E. coli*, *Salmonella typhimurium* and *Klebsiella pneumonia* (31, 32, 33). In contrast to the DsdA from *P. pentosaceus*, these enzymes showed more activity toward D-serine and eluted as monomers during size exclusion chromatography. Since the recombinant DsdA[19080] and DsdA[19132] eluted with an apparent molecular weight of 120 kDa, we assume that these proteins formed a dimer. The discrepancy between apparent and calculated molecular weight is most likely explained by a non-globular shape of the protein. The same property was observed for DsdA from *Klebsiella* which showed a molecular weight of 40 and 46 kDa in SDS-polyacrylamide gel and size exclusion chromatography, respectively. The comparable elution behavior of both recombinant proteins indicates that the amino acid substitutions in DsdA[19080] did not affect the protein confirmation.

Although the biochemical properties of DsdA can be well studied, the physiological role remains to be clarified. The strains of *P. pentosaceus* with and without serine dehydratase activity grew in MRS broth and the serine dehydratase activity was detectable in the cell-free extracts of growing dsdA<sup>+</sup> strains, no significant differences in serine levels were found in the culture supernatants. This observation indicates...
that dsdA is expressed during growth in MRS but is not required for growth. In E. coli, dsdA is involved in the detoxification of D-serine, which is toxic to strains of E. coli lacking dsdA. In these strains L-serine and pantothenate synthesis were inhibited by D-serine (34). Moreover, dsdA may also be involved in the generation of energy in P. pentosaceus, since the protein efficiently converted L-serine to pyruvate. Pyruvate is not only a precursor for diacetyl and acetoin but can also be metabolized to acetate by the action of pyruvate oxidase and acetate kinase. The presence of this pathway in pediococci has been reported (35) and a gene encoding a pyruvate oxidase (PEPE_0639) and two genes encoding acetate kinase (PEPE_0336 and PEPE_1822) are present in the genome of P. pentosaceus ATCC 25745. We plan to perform growth studies in chemical defined medium to understand whether D-serine is a growth inhibitor and L-serine can serve as energy source for P. pentosaceus. Since physiological assays with MRS broth do not always reflect the metabolism taking place in a cheese environment, we also analyzed amino acid metabolism in a basal broth previously used for studying serine metabolism of L. plantarum B3089 (22). Lactose was replaced by galactose to avoid glucose repression. Amino acid analysis showed that ornithine was formed by P. acidilactici and P. pentosaceus. This is explained by an active arginine deiminase pathway that converts arginine via citrulline to ornithine and is present in both species (15). Regarding the serine and threonine levels, again no differences were observed between the dsdA⁺ and dsdA⁻ strains of P. pentosaceus. Surprisingly, P. acidilactici FAM18098 degraded serine and threonine and produced significant amounts of alanine and α-aminobutyrate. This metabolism was also observed in all of the other studied P. acidilactici strains (data not shown) indicating that this biochemical activity is generally present in this species. We propose the following pathway: first, serine and threonine are deaminated by a threonine dehydratase to pyruvate and α-ketobutyrate [Fig. 5]. That threonine dehydratases catalyze the deamination of both amino acids was shown for
E. coli (36). Second, pyruvate and α-ketobutyrate are either aminated by the action of an alanine dehydrogenase or transaminated by the action of an aminotransferase. Evidence for the proposed pathway is found in the genome data of P. acidilactici DSM 20284. There, two neighboring genes named HMPREF0623_0054 and HMPREF0623_0055 encode a putative alanine dehydrogenase and threonine dehydratase, respectively. The organization of these genes implies that both genes may form a transcriptional unit and may be involved in the amino acid conversion mentioned above. However, homologous genes (named PEPE_0166 and PEPE_1067) are also present in P. pentosaceus ATCC25745. Gene expression analysis and biochemical characterization of these genes and their gene products are necessary, to understand if these genes are in fact involved in forming alanine and α-aminobutyrate and why this activity is not detected in P. pentosaceus.

The use of P. acidilactici and P. pentosaceus as adjunct cultures in cheese making opens up interesting perspectives for controlling cheese quality and flavor formation. On the one hand, these species have proteolytic activity that can accelerate proteolysis and therefore the maturation of cheese (14). On the other hand, investigations of the amino acid metabolism show the presence of the arginine deiminase pathway. This pathway produces gas and ammonia that can affect pH and eye formation in cheese. Furthermore, the presence of an active serine dehydratase can lead to the production of flavor compounds such as diacetyl and acetate, which contribute to the desirable aroma of various dairy products. However, the role of the serine dehydratase from P. pentosaceus during cheese ripening has to be investigated in the cheese matrix. The observation that P. acidilactici produces alanine, a compound known to confer sweetness to dairy products (37), is promising since these species could be used as natural sweeteners of dairy products. In general, the acceleration of ripening by adding pediococci during cheese manufacturing has been already described (10, 12, 13). However, in these reports...
the composition of amino acids and volatiles has not been analyzed in detail. We plan to perform cheese trials with the strains presented in this report to study their influence on amino acid metabolism and flavor formation in cheese. Understanding the metabolic activity of pediococci in more detail is of interest not only for the dairy industry since these species are already used as starter cultures for various types of sausages and are known to produce undesirable flavor compounds during the manufacture of pickles, wine, and beer (38).

5 Acknowledgments

We thank Monika Haueter for providing technical assistance.

6 References


7 Figures

Figure 1 Formation of diacetyl and acetoin from pyruvate (A.) and L-serine (B.) by various strains of *P. pentosaceus*. A plus sign in parentheses in the right-hand diagram illustrates that the cell-free extract of the strain exhibited serine dehydratase activity; the cell-free extracts of strains lacking serine dehydratase activity are shown with a negative sign.

Figure 2 Schematic illustration of PEPE_1745 showing the mutation sites found in *P. pentosaceus* FAM17622, *P. pentosaceus* FAM19144, *P. pentosaceus* FAM19080, and *P. pentosaceus* FAM20650 that affect function. Additionally, the binding sites of the primers PEPE_1745F and PEPE_1745R used for cloning and sequencing are shown.

Figure 3 SDS-PAGE analysis of the purification of DsdA[19132] and DsdA[19080] by nickel-affinity chromatography. 1: Cell-free extract of *E. coli* after expression, 2: unbound proteins, 3: protein fraction obtained after nickel-affinity chromatography, 4: purified protein after removal of imidazole.

Figure 4 Amino acid patterns of basal broth (A.) fermented with *P. acidilactici* FAM18098 (B.), *P. pentosaceus* FAM19132 (C.) and *P. pentosaceus* FAM19144 (D.) for 3 days. The amino acids were separated with 2D-TLC and visualized with ninhydrin. Thr: threonine; Ser: serine; Ala: alanine; Abu: α-aminobutyrate; Orn: ornithine.

Figure 5 Proposed pathways for the degradation of serine (and threonine) in *P. pentosaceus* and *P. acidilactici*. The synthesis of alanine from pyruvate by *P. acidilactici* can occur by the action of an alanine dehydrogenase (amination) or an aminotransferase (transamination). dsdA: D-serine dehydratase, als: α-acetolactate synthase, ald: α-acetolactate decarboxylase, bdh: acetoin reductase (2,3-butanediol dehydrogenase).
## Tables

### Table 1 Strains, plasmids, primers, and peptides used in this study

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<tr>
<th>Strain</th>
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<td>BL21 (DE3)</td>
<td>F- ompT hsdSB (rB, mB) gal dcm (DE3)</td>
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<td><strong>P. pentosaceus</strong></td>
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<tr>
<td>FAM13073</td>
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<td>Tilsit</td>
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<td>FAM17622</td>
<td>dsdA(^{\text{r}}) (deletion at pos. 107)</td>
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<td>FAM18813</td>
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<td>Tête de moine</td>
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<tr>
<td>FAM19080</td>
<td>dsdA(^{\text{r}}) (amino acid substitutions: T67A, P278L(^{\text{a}}))</td>
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<td>FAM19132</td>
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<td>dsdA(^{\text{r}}) (deletion from pos. 90 to 108)</td>
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<td>FAM20650</td>
<td>dsdA(^{\text{r}}) (nonsense-mutation at pos. 151: C&gt;T)</td>
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<td>DSM 20336</td>
<td>type strain</td>
<td>German Collection of Microorganisms and</td>
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<td><strong>P. acidilactici</strong></td>
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<td>FAM13473</td>
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<td>FAM13881</td>
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<td>FAM17411</td>
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<td>FAM20559</td>
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DSM 20284
type strain

German Collection of Microorganisms and Cell Cultures

Plasmid

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<th>Description</th>
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<tr>
<td>pEXP5-CT/TOPO</td>
<td>E. coli cloning vector, amp'</td>
<td>Invitrogen</td>
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<td>pEXP5-CT/dsdA[19132]</td>
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<td>This study</td>
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<td>CT/dsdA[19080]</td>
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<td>This study</td>
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Primer Sequence (5' to 3')

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<th>Primer</th>
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<td>PEPE_1745F</td>
<td>ATGATTGATGTTGATGCTCTA</td>
<td>cloning and sequencing of dsdA</td>
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<tr>
<td>PEPE_1745R</td>
<td>TTAACTTGATGCTCTTCAG</td>
<td>cloning and sequencing of dsdA</td>
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3 a compared to PEPE_1745 from *P. pentosaceus* ATCC 25745