Characterization of the tyramine-producing pathway in *Sporolactobacillus* sp. P3J

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A sporulated lactic acid bacterium (LAB) isolated from cider must was shown to harbour the *tdc* gene encoding tyrosine decarboxylase. The isolate belonged to the *Sporolactobacillus* genus and may correspond to a novel species. The ability of the *tdc*-positive strain, *Sporolactobacillus* sp. strain P3J, to produce tyramine in vitro was demonstrated by using HPLC. A 7535 bp nucleotide sequence harbouring the putative *tdc* gene was determined. Analysis of the obtained sequence showed that four tyramine production-associated genes [*tyrosyl-tRNA synthetase (*tyrS*), tyrosine decarboxylase (*tdc*), tyrosine permease (*tyrP*) and Na⁺/H⁺ antiporter (*nhaC*)] were present and were organized as already described in other tyramine-producing LAB. This operon was surrounded by genes showing the highest identities with mobile elements: a putative phage terminase and a putative transposase (downstream and upstream, respectively), suggesting that the tyramine-forming trait was acquired through horizontal gene transfer. Transcription analyses of the *tdc* gene cluster suggested that *tyrS* and *nhaC* are expressed as monocistronic genes while *tdc* would be part of a polycistronic mRNA together with *tyrP*. The presence of tyrosine in the culture medium induced the expression of all genes except for *tyrS*. A clear correlation was observed between initial tyrosine concentration and tyramine production combined with an increase in the final pH reached by the culture. Finally, cloning and expression of the *tyrP* gene in *Lactococcus lactis* demonstrated that its product catalyses the exchange of tyrosine and tyramine.

INTRODUCTION

Biogenic amines (BA) are low-molecular-mass molecules with at least one amine group that can be accumulated in foods (e.g. fish, cheese, wine, sausages, cider) (Chang *et al.*, 1985; ten Brink *et al.*, 1990; Lonvaud-Funel, 2001; Suzzi & Gardini, 2003; Garai *et al.*, 2006) due to the presence of BA-forming microorganisms during elaboration processes or storage. The consumption of foods containing high concentrations of BA may be associated with health effects in sensitive consumers (Ladero *et al.*, 2010). The main BAs studied are histamine and tyramine due to their greater physiological actions and toxicological effects (Caston *et al.*, 2002; Wallace, 2007). In fact, the term ‘cheese reaction’ has been coined to refer to the symptoms induced after ingestion of cheeses with elevated tyramine concentrations. However, the concentration of histamine is regulated for some fish by the USA Food and Drug Administration and EU legislation [Commission Regulation (EC) no. 2073/2005].

BA are produced in food matrices containing free amino acids via intracellular bacterial catabolic pathways that consist of at least a decarboxylase and a transporter responsible for the uptake of the amino acid (i.e. histidine, tyrosine) and the excretion of the corresponding amine (i.e. histamine, tyramine). These decarboxylation reactions have been proposed as metabolic energy-generating pathways via a proton motive force (Molenaar *et al.*, 1993;
Wolken et al., 2006) and/or as stress response mechanisms (Fritz et al., 2009). In order to better understand the origin of BA production in fermented foods, a number of studies have been performed to not only identify the microorganisms able to form these molecules (Burdychova & Komprda, 2007; Bover-Cid et al., 2009; Garai et al., 2007; Coton et al., 2010b; Moon et al., 2010) but also characterize the operons responsible for these activities (i.e. Linares et al., 2009). Over the past few years, BA pathways in lactic acid bacteria (LAB) have been described to be strain-dependent rather than species-specific and horizontal gene transfer was suggested for the acquisition of the gene clusters involved in the production of histamine by Lactobacillus hilgardii (Lucas et al., 2005), tyramine by Lactobacillus brevis (Coton & Coton, 2009) and putrescine by Oenococcus oeni (Marcobal et al., 2006).

Concerning the tyramine pathway, Lucas et al. (2003) described that in L. brevis IOEB 9809, the tyramine decarboxylase (TDC) pathway is encoded by a cluster consisting of four genes. The first gene (tyrS) shows strong similarities with tyrosyl-tRNA synthetase genes, the second gene (tdc) corresponds to the tyrosine decarboxylase, the third gene (tyrP) encodes a tyrosine/tyramine exchanger (Wolken et al., 2006), while the last gene (nhaC) is related to the Na+/H+ antiporter genes. The existence of the same gene organization has been described in other tyramine-producing LAB, Enterococcus faecalis (Connil et al., 2002), Enterococcus hirae (Coton et al., 2004), Enterococcus durans (Fernández et al., 2004) and Enterococcus faecium (GenBank accession no. NZ_AAAK00000000).

In this study, the origin and some functional properties of the tyramine-forming ability of Sporolactobacillus sp. strain P3J, isolated from French cider, were investigated.

METHODS

Bacterial strains and culture conditions. Sporolactobacillus sp. strain P3J was originally isolated on de Man, Rogosa & Sharpe medium (MRS) (AES) from a sample from cider from the Perche area (Orne, France) (Coton et al., 2010b). Sporolactobacillus kofuensis (DSM 17615T), Sporolactobacillus nakayamae subsp. nakayamae (DSM 11696T), Sporolactobacillus terrae (DSM 11697T) and Sporolactobacillus vineae (DSM 21990T) were obtained from DSMZ. All strains were grown in glucose yeast peptone medium (GYP, Chén et al., 2005) adjusted to pH 6.8 and cultures were incubated for 48 h (broth) or 3–5 days (agar). When necessary, GYP was supplemented with tyrosine (1, 5 or 10 mM, Sigma) (GYP + T).

Preparation of template DNA and RNA. Total DNA was extracted from 1 ml overnight bacterial culture with the Nucleospin tissue kit (Macherey-Nagel) according to the manufacturer’s instructions (50–100 ng DNA was used in all PCR experiments).

Total RNA was extracted using the TRI Reagent (Sigma) as follows. Sporolactobacillus sp. cells were grown in GYP and GYP + T at pH 5.0 until OD600 = 0.6. They were then harvested by centrifugation and disrupted using glass beads (diameter up to 106 μm, Sigma) in a Fastprep FP120 Instrument (MP Biomedicals) at 4 °C, six times for 30 s at power setting 6. The resulting samples were treated as recommended by the manufacturer. Purified RNA was resuspended in 0.1 % diethyl pyrocarbonate (DEPC)-treated water. Total RNA concentrations were determined by UV spectrophotometry by measuring absorbance at 260 nm in an Epoch spectrophotometer (Biotek).

PCR amplification. Detection of the tdc gene in the different Sporolactobacillus sp. strains was performed using the PCR method described by Coton et al. (2004). For strain identification, the 16S rRNA gene fragment was obtained after PCR amplification using the universal primers BSF8/BSR1541 (Edwards et al., 1989) as described previously (Coton & Coton, 2005).

Acquisition of unknown sequences adjacent to the tdc gene was performed as described by Coton et al. (2010a). Briefly, the method corresponded to the restriction site-PCR (RS-PCR) described by Sarkar et al. (1993) with a single modification: PCR primers corresponding to the known sequence used for the second PCR were also used for sequencing. All PCR experiments were performed in a Mastercycler Gradient PCR machine (Eppendorf).

Aliquots (18 μl for PCR products and 9 μl for RS-PCR-generated fragments) of each PCR sample were analysed using 0.8 % (w/v) agarose gels (Invitrogen) in 1 x TBE buffer at 130 V for 50 min then visualized with ethidium bromide using a GelDoc2000 and the Quantity One software (Bio-Rad).

DNA sequencing and sequence analysis. The 16S rDNA, tdc and RS-PCR products were purified using the GenElute PCR purification kit (Sigma) and sequenced by Eurofins MWG Operon (Germany). Alignments were performed using the Bionumerics software (Applied Maths). Sequence comparisons against international databases were performed using BLAST (Altschul et al., 1990). Theoretical molecular mass and isoelectrical point were estimated by the Compute pl/Mw tool (http://www.expasy.ch/tools/pi_tool.html), while conserved domains were identified by the NCBI Conserved Domain Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

RT-PCR. RNA samples (2 μg total RNA) were treated with 2 U DNase I (Fermentas) to eliminate any DNA contamination. The absence of contaminating DNA in the DNase-treated RNA samples was controlled by PCR performed under the same conditions but without reverse transcriptase. cDNA was then synthesized from total RNA using the high capacity C-DNA reverse transcription kit (Applied Biosystems). Four different dilutions of cDNA were performed as described by Coton et al. (2010a). Amplifications were performed using the GenEx rt-qPCR kit (Biotek) with the RNA concentration. In each run, a negative and a positive control were included. Amplifications were performed using the default cycling settings established by Applied Biosystems.

The number of PCR cycles required to reach the midpoint of the amplification curve (cycle threshold or Ct) was measured to compare...
gene expression. To relate Cagli to the abundance of mRNA species, they were converted into 'n-fold differences' (y) using the formula $y = 2^{\Delta CT}$ (Livak & Schmittgen, 2001). The condition with the lower level of expression was selected as the calibrator. For each gene, RT-qPCR analysis was performed on RNA purified from three independent cultures grown under each of the environmental conditions assayed.

Cloning and expression of tyrP. The tyrP gene was amplified by PCR from genomic DNA of Sporolactobacillus sp. P3J using primer tyrP-8f (5'GCGAAGACATGGAAGAAACTTTGGCCCAAAAGG-3') and tyrP-8r (5'GGCAATCTAGAAAGGAGGCTGCCGTAG-3'), introducing Ncol and Xbal restriction sites. The 1.6 kb product was ligated into the NICE system expression vector pNZ8048 to yield pNZtyrP-8. Sporolactobacillus sp. P3J was grown on M17 medium supplied with 0.5% glucose and 5 ng/ml nisin (Ruyter et al., 1996). For expression of tyrP, L. lactis NZ9000 pNZtyrP-8 was grown on M17 medium supplied with 0.5% glucose and 5 ng/ml chloramphenicol (m\(^{-1}\)) to mid-exponential growth phase (OD\(_{600}\) 0.6) at 30 °C. Nitin was added to a final concentration of 5 ng ml\(^{-1}\) to induce expression and cells were left to grow for another 1 h.

Tyrosine and tyramine transport assays. L. lactis NZ9000 cells expressing tyrP from pNZtyrP-8 were harvested and washed in 100 mM potassium phosphate (KPi) buffer, pH 6.0 and resuspended in the same buffer to OD\(_{600}\) 2.0. Glucose was added to 10 mM and 100 mM and incubated at 30 °C with constant stirring. After 5 min preincubation, \(^{14}\)C-labelled tyrosine (Amersham Pharmacia) or tyramine (American Radiolabelled Chemicals) was added to a final concentration of 1.15 or 18 μM, respectively. Uptake was stopped by addition of 2 ml ice-cold 0.1 M LiCl followed by filtration through a 0.45 mm pore-size nitrocellulose filter (BA85; Schleicher & Schuell). The filter was washed once with 2 ml ice-cold 0.1 M LiCl and submerged in Emulsifier Scintillator Plus scintillation fluid (Packard Bioscience). Retained radioactivity was counted in a Tri-Carb 2000CA liquid scintillation counter (Packard Instrumentation). In the exchange experiment, unlabelled tyramine was added to cells to a final concentration of 1 mM, 1 min after the addition of \(^{14}\)C-labelled tyrosine. For preloading of cells with tyrosine, cells were resuspended in 100 mM KPi buffer, pH 6.0, containing 5 mM tyrosine and left at room temperature for 1 h. Cells were washed three times with 100 mM KPi buffer, pH 6.0, prior to the uptake experiment at 4 °C.

Detection of biogenic amines by HPLC. Quantitative analysis of tyramine production was carried out by reverse-phase HPLC using a computer-controlled (Millenium 32, Waters) liquid chromatograph (Alliance 2695, Waters). The strain was grown in GYP T medium was also analysed by HPLC. The cultures were centrifuged at 8000 g for 10 min, and the resulting supernatants were filtered using 0.2 μm PTFE membranes (Supor). The resulting samples were derivatized using dabsyl chloride. Separations were carried out on reverse phase/C18 column (XTerra MS C18 5 μm 4.8 × 150 mm, Waters); gradient and detection conditions were as described by Krause et al. (1995).

Statistical analysis. All experiments were repeated at least three times. Mean values ± SD are indicated. All statistical analyses were performed with the SPSS v11.0 software package (IBM).

RESULTS

Strain identification

The cider isolate P3J, obtained on MRS medium, corresponded to a Gram-positive bacterium and exhibited, for some cells, the presence of an endospore in the terminal position. Sequencing of a 1459 bp 16S rRNA gene fragment (GenBank accession no. HQ285998) indicated that the isolate belonged to the Sporolactobacillus genus. High identity levels (96%) were observed with various Sporolactobacillus species [i.e. S. kofuensis (AJ634661), S. inulinus (AB362770) and S. laevolacticus (AB362650)] (Fig. 1). However, the identity levels observed did not allow for presumptive assigning of the P3J isolate to a defined Sporolactobacillus species; therefore, the strain was named Sporolactobacillus sp. P3J.

Genotypic and phenotypic characterization of tyramine production by Sporolactobacillus sp. P3J

Using a multiplex PCR method for the detection of four BA-associated genes (hdc, tdc, odc and agdi), a specific band with a comparable size to the tdc fragment of L. brevis IOEB 9809 (~1100 bp) (data not shown) was amplified from Sporolactobacillus sp. P3J DNA (Coton et al., 2010b). The fragment obtained was sequenced using the T2/5 primer set (Coton & Coton, 2005) and the resulting 1133 bp sequence was compared with international databases using BLAST (Altschul et al., 1990). The highest nucleotidic identities were observed with various tyrosine decarboxylase genes of Gram-positive bacteria, including E. hirae (AY303667), L. brevis ATCC 367 (CP000416) and Lactobacillus curvatus HSCC1737 (AB086652) (75, 75 and 74 %, respectively).

To confirm the ability of this strain to form tyramine in vitro, the supernatant of a 24 h Sporolactobacillus sp. P3J culture in GYP + T medium was also analysed by HPLC. The analysis revealed the production of 8.48 mM tyramine in the tested conditions.
To test the influence of tyrosine (precursor amino acid) on the production of tyramine, GYP medium was supplemented with increasing concentrations of tyrosine (1, 5 and 10 mM). The final OD$_{600}$, pH level and tyramine concentration were measured. The final tyramine concentration was higher as the initial tyrosine concentration in the cultures increased (Pearson coefficient 0.944, $P<0.01$) (Table 1). This tyramine production had no effect on growth, since no differences were observed in the final optical density reached by the cultures. However, a higher final pH was observed as more tyramine was synthesized (Pearson coefficient 0.750, $P<0.01$) (Table 1).

In order to determine if the presence of the tdc operon is exclusive to the Sporolactobacillus sp. P3J strain or if it could be found in members of related species, we evaluated the presence of the tdc operon in the type strains of S. kofuensis (DSM 17615$^T$), S. nakayamae subsp. nakayamae (DSM 11696$^T$), S. terrae (DSM 11697$^T$) and S. vineae (DSM 21990$^T$). None of them allowed for the amplification of the expected specific tdc gene fragment (data not shown).

**Characterization of the tdc region**

In order to determine the complete sequence of the Sporolactobacillus sp. P3J tyramine-production-associated genes, acquisition of the unknown DNA sequences adjacent to the tdc fragment gene, in both the 5' and 3' directions, was performed by using an RS-PCR method based on the one proposed by Sarkar et al. (1993). The repetitive use of this method by the creation of new sets of primers based on each newly determined sequence allowed a 7535 bp nucleotide sequence to be obtained (GenBank accession no. HQ285999) from the original 1133 bp tdc partial sequence. Sequence analysis of the fragment revealed the presence of four complete ORFs flanked by two partial ORFs (Table 2).

The first complete ORF (tyrS) consisted of 1167 bp encoding 388 amino acids, and exhibited strong identities (>77%) with known TyrS proteins, i.e. L. brevis (YP$_{796295}$), E. faecium (ZP$_{06683270}$) and E. durans (CAF33979). Downstream of the tyrS gene, at an intergenic distance of 228 bp, a second ORF (tdc, 1920 bp) was observed. The translated sequence showed identities in the order of 64–76% with the tyrosine decarboxylases of tyramine-producing Gram-positive bacteria, i.e. L. brevis (ABY71221), E. hirae (AAQ73505), Staphylococcus epidermidis (ZP$_{04818142}$) and Tetragenococcus halophilus (BAD93616). The resulting putative protein of the third ORF (tyrP, 1464 bp), situated 65 bp downstream of the tdc gene, shared 80% sequence identity with the functionally characterized tyrosine permease (TyrP) of L. brevis (Wolken et al., 2006). Finally, 208 bp downstream of the putative tyrP gene, a fourth complete ORF (nhaC, 1410 bp) was observed. The putative encoded protein presented the highest level of identity with Na$^+$/H$^+$ antiporters (NhaC) of L. brevis (ABY71223) and E. faecalis (ZP$_{05501989}$) (67 and 58%, respectively).

On the sequenced fragment, the four genes associated with potential tyramine production are flanked upstream by a partial sequence (ter, 773 bp) presenting highest identities (71–75%) in BLASTX analysis with putative phage terminases found in clostridia (i.e. YP$_{001308046}$, YP$_{003843917}$ and ZP$_{02630796}$). However, no start codon was observed and the ter sequence is directly attached to the ATG of the tyrS gene. Downstream, the tdc operon was flanked, at 102 bp of the end of the nhaC gene, by a partial sequence divergently encoding 65 amino acids that showed around 60% identity with a putative transposase found in LAB, i.e. Lactobacillus antri (ZP$_{05746645}$), O. oeni (ZP$_{01544598}$) and L. brevis subsp. gravesensis (ZP$_{03938918.1}$).

**Table 1. Tyramine production according to different initial tyrosine concentrations**

<table>
<thead>
<tr>
<th>Medium</th>
<th>OD$_{600}$</th>
<th>pH</th>
<th>Tyramine concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GYP</td>
<td>0.98 ± 0.02</td>
<td>4.13 ± 0.01$^a$</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>GYP + 1 mM Tyr</td>
<td>0.96 ± 0.26</td>
<td>4.27 ± 0.04$^{ab}$</td>
<td>0.84 ± 0.29$^a$</td>
</tr>
<tr>
<td>GYP + 5 mM Tyr</td>
<td>0.97 ± 0.12</td>
<td>4.36 ± 0.06$^{bc}$</td>
<td>2.48 ± 0.32$^{ab}$</td>
</tr>
<tr>
<td>GYP + 10 mM Tyr</td>
<td>0.87 ± 0.25</td>
<td>4.44 ± 0.05$^c$</td>
<td>8.92 ± 1.56$^b$</td>
</tr>
</tbody>
</table>

($P<0.01$) between the values, analysed by ANOVA and LSD analysis. Only differences between adjacent values are indicated.

**Transcriptional analysis of the TDC operon**

To determine whether tdc was co-transcribed with other genes of the TDC locus, total RNA of Sporolactobacillus sp. P3J was used in RT-PCRs with seven sets of primers designed to amplify individual genes as well as regions spanning gene junctions (Supplementary Table S1). Amplification products were obtained for each of the four targeted genes, while for the primers targeting gene junctions, only the tdc-tyrP couple allowed for amplification, indicating that tdc is cotranscribed with tyrP (Fig. 2).

The individual expression of the cluster genes was measured by RT-qPCR in either the presence (10 mM) or absence of tyrosine. Since the expression of previously characterized tdc clusters requires acidic conditions (Linares et al., 2009), the pH of the medium was adjusted to 5. The results indicated that the expression of tyrS is independent of tyrosine, in contrast, a significant increase (Student's t-test $P<0.01$) in tdc, tyrP and, to a lesser extent, nhaC expression was observed when tyrosine was added to the culture medium (Fig. 3). Tyramine concentration was also analysed by HPLC measurements in all cultures from which RNA was obtained, confirming that tyramine biosynthesis occurred at acidic pH in the presence of tyrosine (data not shown).
Functional expression of tyrP in L. lactis

The tyrP gene was cloned in the NICE system expression vector pNZ8048, yielding pNZtyrP-Sp, for nisin-inducible expression in L. lactis NZ9000 (de Ruyter et al., 1996). Resting cells of L. lactis harbouring pNZtyrP-Sp or the empty vector pNZ8048 were assayed for tyrosine and tyramine uptake using 14C-labelled tyrosine and tyramine, respectively (Fig. 4). At 1.15 mM 14C-labelled tyrosine, there was no increase in tyrosine uptake of cells expressing tyrP compared with control cells showing the background tyrosine uptake mediated by an endogenous tyrosine transport system(s). However, when an approximately 900-fold excess of unlabelled tyramine was added to cells that were allowed to take up 14C-labelled tyrosine for 1 min, cells expressing tyrP rapidly released tyrosine, whereas control cells did not (Fig. 4a), demonstrating tyrosine/tyramine exchange via TyrP.

Control cells harbouring the pNZ8048 vector did not take up significant amounts of 14C-labelled tyramine at 18 mM. Under the same conditions, cells expressing tyrP showed a low, but significant, level of uptake (Fig. 4b). However, tyramine uptake was strongly increased to an initial rate of higher than 100 nmol min⁻¹ mg⁻¹ when cells were incubated for 1 h with a high concentration (5 mM) of tyrosine prior to the uptake experiment. In contrast, control cells did not show a significant increase in uptake. The reverse experiment, preincubation of cells expressing tyrP with tyramine, confirmed the presence of tyrosine/tyramine exchange via TyrP.

Table 2. tdc region putative encoded proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location in nucleotide sequence</th>
<th>G+C (%)</th>
<th>Predicted protein (aa/kDa/pI)*</th>
<th>Conserved domain†</th>
<th>Closest protein (aa)</th>
<th>Proposed function</th>
<th>Identity (%)‡</th>
<th>Accession no.</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcr</td>
<td>0–795</td>
<td>47.0</td>
<td>Partial</td>
<td>Terminase I superfamily</td>
<td>Phage terminase</td>
<td>Phage terminase</td>
<td>73</td>
<td>YP_001308046</td>
<td>C. beijerinckii NCIMB 8052</td>
</tr>
<tr>
<td>tyrS</td>
<td>774–1940</td>
<td>41.6</td>
<td>389/43.6/6.16</td>
<td>Tyrosyl-tRNA synthetase</td>
<td>Tyrosyl-tRNA synthetase</td>
<td>Tyrosyl-tRNA synthetase</td>
<td>78</td>
<td>YP_796295</td>
<td>L. brevis ATCC 367</td>
</tr>
<tr>
<td>tdc</td>
<td>2169–4088</td>
<td>40.7</td>
<td>639/72.1/5.09</td>
<td>Tyrosine decarboxylase</td>
<td>Tyrosine decarboxylase</td>
<td>Tyrosine decarboxylase</td>
<td>76</td>
<td>YP_796294</td>
<td>L. brevis ATCC 367</td>
</tr>
<tr>
<td>tyrP</td>
<td>4154–5617</td>
<td>39.5</td>
<td>487/53.5/9.96</td>
<td>Amino acid</td>
<td>Putative tyrosine</td>
<td>Tyrosine/tyramine antiporter</td>
<td>80</td>
<td>YP_796293</td>
<td>L. brevis ATCC 367</td>
</tr>
<tr>
<td>nhaC</td>
<td>5826–7235</td>
<td>38.7</td>
<td>469/50.4/9.21</td>
<td>Na⁺/H⁺ antiporter</td>
<td>Na⁺/H⁺ antiporter</td>
<td>Na⁺/H⁺ antiporter</td>
<td>68</td>
<td>YP_796292</td>
<td>L. brevis ATCC 367</td>
</tr>
<tr>
<td>rev</td>
<td>7338–7335</td>
<td>42.4</td>
<td>Partial</td>
<td>Putative transposase orfB</td>
<td>Conserved hypothetical protein</td>
<td>Transposase</td>
<td>60</td>
<td>ZP_05746645</td>
<td>L. antri DSM 16041</td>
</tr>
</tbody>
</table>

*Theoretical molecular mass and pI were estimated by using the Compute pl/Mw tool (http://www.expasy.ch/tools/pi_tool.html).
‡Identical amino acid percentage between the predicted sequence and the closest sequence in GenBank using BLASTP.

Fig. 2. RT-PCR amplification with primers designed to amplify the genes and the intergenic regions of tyrS, tyrS-tdc, tdc, tdc-tyrP, tyrP, tyrP-nhaC and nhaC. PCR was carried out on samples treated (+) or not (−) with reverse transcriptase. PCR performed using chromosomal DNA as a template (C+) was only included for the intergenic region analysis.

M, Molecular mass markers (FastRuler low-range DNA ladder; Fermentas); sizes given in bp.
tyrP with a high concentration of tyramine, did not result in a higher uptake of tyrosine (data not shown), suggesting that tyramine did not accumulate in the cells during the preincubation period. Taken together, the results indicate that tyrP was functionally expressed and that its product catalyses the exchange of tyrosine and tyramine.

DISCUSSION

Identification of the cider isolate P3J indicated that it belonged to the Sporolactobacillus genus, thus explaining the observations that it was a Gram-positive catalase-negative bacterium presenting an endospore in terminal position. The identity levels observed did not allow for presumptive assigning of the P3J isolate to a defined species and suggested that it might actually correspond to a novel species within the Sporolactobacillus genus.

Sporolactobacilli represent an interesting genus from a morphological, physiological and evolutionary point of view as they correspond to intermediate forms between the homo-fermentative Lactobacillus group and the Bacillus genus (Kitahara & Suzuki, 1963). Sporolactobacilli have been associated with fermentation starters for Asian alcoholic beverages (Yanagida et al., 1997), wine grapes (Bae et al., 2006) and the associated environments (Yanagida et al., 2005; Chang et al., 2008), as well as spoiled orange juice (Fujita et al., 2010) and the extreme environment of sugar thick juice (Justé et al., 2008). To our knowledge, this study is the first description of a bacterium associated with cider production that belongs to the Sporolactobacillus genus.

Concerning the presence of the TDC pathway in Sporolactobacillus sp. P3J, the genetic and phenotypic results obtained here indicate that the presence of a specific PCR amplification product was correlated with the production of tyramine in the tested conditions. Regarding the Sporolactobacillus sp. P3J TDC pathway, its genetic organization, consisting of four genes [in the following order: tyrosyl-tRNA synthetase gene (tyrS), tyrosine decarboxylase (tdc), tyrosine permease (tyrP) and an Na⁺/H⁺ antiporter (nhaC)] corresponded to that already described in various LAB including L. brevis (Lucas et al., 2003), E. faecalis (Connil et al., 2002), E. hirae (Coton et al., 2004), E. durans (Fernández et al., 2004), E. faecium (GenBank accession no. NZ_AAAK00000000). In T. halophilus, the same type of organization was observed with the exception that the tyrS gene was absent in this species (GenBank accession no. AB059363). TyrS of Sporolactobacillus sp. P3J showed the highest identities (75–78 %) with TyrS associated with tdc pathways supporting the existence of two categories of TyrS, one for general metabolism and one associated with tyramine production as suggested by Coton & Coton (2009) in L. lactis NZ9000 harbouring pNZtyrP-Sp (triangles) and pNZ8048 (circles). Cells were induced with 5 ng nisin ml⁻¹ for 1 h for tyrP expression. ¹⁴C-Labelled tyrosine and ¹⁴C-labelled tyramine were added to final concentrations of 1.15 and 18 μM, respectively. (a) Tyrosine uptake. After 1 min, cells were either left untreated (closed symbols) or unlabelled tyramine was added to a concentration of 1 mM (open symbols). (b) Tyramine uptake was measured in cells preincubated for 1 h in 100 mM KPi buffer, pH 6.0, without (closed symbols) or with (open symbols) 5 mM tyrosine followed by washing with 100 mM KPi buffer, pH 6.0.
associated with clostridial phage terminases, thus suggesting that the integration of the tdc operon was done at this site due to sequence homologies and hence disrupted the phage terminase gene (ter).

It is noteworthy that the genes surrounding the tyramine production operon (ter upstream and rev downstream) correspond to putative mobility genes, suggesting that the tyramine production ability in Sporolactobacillus sp. P3J might have been acquired through horizontal gene transfer (HGT). Some authors have already shown that BA-production-associated pathways are often associated with mobile elements (i.e. plasmids and genomic islands) and therefore have been acquired through HGT, explaining strain to strain variation in the ability to produce BA. On one hand, plasmids harbouring BA production operons have been shown to be responsible for the production of putrescine from ornithine in Staphylococcus epidermidis (Coton et al., 2010a). On the other hand, putative genomic islands harbouring BA production operons have been described as being responsible for production of putrescine (from ornithine) in O. oeni (Marcobal et al., 2006) and of tyramine in L. brevis (Coton & Coton, 2009).

The fact that no amplification of the tdc gene was observed in four strains of the Sporolactobacillus genus suggests that the ability to produce tyramine is rather exclusive to Sporolactobacillus sp. strain P3J, which is also in agreement with trait acquisition through HGT.

Interestingly, the ter gene presented the highest identities with putative plage terminases from sporulated bacteria belonging to the Clostridium and Bacillus genera, while the rev mobility gene presented the highest identities with putative transposases from LAB belonging to the Lactobacillus, Oenococcus and Enterococcus genera. These results were thus in agreement with the fact that sporolactobacilli share common traits with both sporulated and lactic acid bacteria, and suggested that genetic material exchange can occur with both bacterial groups.

Transcription analyses of the tdc gene cluster suggested that tyrS and nhaC are expressed as monocistronic genes, while tdc would be part of a polycistronic mRNA together with tyrP. These results were in agreement with the fact that a terminator (14.9 Kcal) sequence was present on the Sporolactobacillus sp. P3J sequence between tyrP and nhaC. In addition, they were consistent with what was observed in E. durans IPLA655, in which tdc and tyrP were also cotranscribed (Linares et al., 2009) and in L. brevis, in which a strong signal of the tdc-tyrP product indicated that RNAs containing these two genes were probably abundant, although other amplification products (tyrS-tdc and tyrP-nhaC) were also obtained (Lucas et al., 2003).

The expression of the cluster genes measured by RT-qPCR indicated that the expression of tdc and tyrP was induced in the presence of tyrosine. These results were in agreement with the fact that the expression of decarboxylase genes has been linked to the presence of an amino acid substrate in bacteria (Soksawatmaekhin et al., 2004; Linares et al., 2009). Only when there is an excess of amino acid substrate do the bacteria use it for the decarboxylation reaction, thus ensuring its availability for protein biosynthesis. The nhaC gene was also induced by tyrosine, in contrast with tyrS, raising the question about the role of this gene in the tdc operon. However, to date, a specific tyrS gene has always been identified as being linked to the tdc gene clusters described in LAB (Connil et al., 2002; Lucas et al., 2003; Coton et al., 2004; Fernández et al., 2004) except in T. halophilus (GenBank accession no. AB059363). Moreover, in tyramine-producing strains such as E. faecalis V583 or L. brevis ATCC 357 in which the entire genome has been sequenced (GenBank accession nos NC_004668 and NC_008497, respectively), a second tyrS gene can be identified in addition to the tyrS gene of the tdc cluster.

Concerning transport assays, expression of the tyrP gene of Sporolactobacillus sp. P3J in L. lactis identified TyrP as a tyrosine/tyramine exchanger. Precursor/product exchangers of this type function in catabolic amino acid decarboxylation pathways, many of which have been described previously (Molenaar et al., 1993; Kashiwagi et al., 1997; Iyer et al., 2003). TyrP of Sporolactobacillus sp. P3J is the second tyrosine/tyramine exchanger to be cloned and kinetically characterized. The transporter protein shares 80 % amino acid sequence identity with TyrP of L. brevis which was the first one to be characterized (Wolken et al., 2006). L. brevis TyrP showed a much higher initial tyrosine uptake activity than the Sporolactobacillus sp. P3J TyrP in comparable uptake experiments in which 14C-labelled tyrosine was added to resting cells. Nevertheless, tyrosine/tyramine exchange occurred rapidly with both TyrPs (Fig. 4). The reason for the much lower tyrosine uptake via Sporolactobacillus sp. P3J TyrP is unclear. Possible modes of tyrosine uptake via TyrP in these experiments are exchange with intracellular tyrosine or proton/tyrosine symport. The latter is unlikely since no proton motive force-driven tyrosine uptake was observed for L. brevis TyrP. Instead, tyrosine uniport was identified as a second mode of transport in addition to tyrosine/tyramine exchange catalysed by L. brevis TyrP by tyrosine efflux from membrane vesicles loaded with 14C-labelled tyrosine, while the results on tyramine uniport were inconclusive (Wolken et al., 2006). The uniport transport mode was not observed in the present study. Energized cells of L. lactis expressing TyrP did not show much higher levels of accumulation of positively charged 14C-labelled tyrosine than the control cells. Moreover, cells could not be efficiently loaded with unlabelled tyramine, while this was successful for tyrosine (Fig. 4b). Endogenous tyrosine transporters of L. lactis were most likely responsible for loading in the latter case. The low level of tyramine uptake
without tyrosine preloading probably results from exchange with an internal pool of tyrosine, as reported by Kunji et al. (1996).

The physiological role of biogenic amine formation is not yet fully understood although some authors have proposed that decarboxylation pathways could be involved in resistance against acid stress (Azcarate-Peril et al., 2004) and/or generation of energy (Cid et al., 2008; Pereira et al., 2009). In this study, the ability of _Sporolactobacillus_ sp. P3J to produce tyramine under acidic conditions in the presence of tyrosine is demonstrated. These conditions are inherent to fermented products such as cider, from which this strain was isolated. Results obtained in the presence of increasing initial concentrations of tyrosine revealed a correlation with increasing tyramine production with a concomitant increase in the final pH reached by the culture (Table 1), which was expected as the decarboxylation releases an acidic function. This could be in agreement with the proposed role as an acidic resistance mechanism for the TDC pathway. However, the fact that no differences were observed in the final optical density does not demonstrate an increased biological fitness. In this context, the incidence of the presence of the _tdc_ operon on the adaptability and growth of _Sporolactobacillus_ sp. P3J in various environmental conditions, including cider conditions characterized by low pH, should be investigated further.

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