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.

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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 micro-organisms 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;

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Abbreviations: BA, biogenic amines; LAB, lactic acid bacteria; RS-PCR, restriction site-PCR; RT-qPCR, real-time RT-PCR; TDC, tyramine decarboxylase.

The GenBank/EMBL/DBJ accession number for the 7535 bp RS-PCR-generated sequence obtained in this study is HQ285999.

A supplementary table of primer sequences is available with the online version of this paper.

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 micro-organisms 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_AAAK000000000).

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 17615^T), *Sporolactobacillus nakayamae* subsp. *nakayamae* (DSM 11696^T), *Sporolactobacillus terrae* (DSM 11697^T) and *Sporolactobacillus vineae* (DSM 21990^T) were obtained from DSMZ. All strains were grown in glucose yeast peptone medium (GYP, Chen *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 × 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 pI/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) following the manufacturer's recommendations. PCRs were performed using 2 µl cDNA suspension and 0.4 µM each primer (Supplementary Table S1, available with the online version of this paper). The amplifications were performed for 35 cycles (94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min) and the resulting fragments were electrophoretically analysed in 1.5 % agarose gels in TAE buffer (40 mM TRIS/acetate, 1 mM EDTA, pH 8.0).

Quantification of gene expression by real-time RT-PCR (RT-qPCR). Gene expression was analysed by RT-qPCR as previously described for *O. oeni* (Desroche *et al.*, 2005), using the SYBR Green PCR master mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems). Four different dilutions of cDNA were tested. Five microlitres of each solution was added to 20 µl PCR mixture (12.5 µl SYBR Green Supermix, 1 µl each primer at 7 µM and 5.5 µl RNase-free water). All cDNAs were amplified by RT-qPCR using specific primers (Supplementary Table S1) selected using Primer Express software (Applied Biosystems) and designed to produce amplicons of similar length (approx. 100 bp). The gene encoding the 16S rRNA was used as an internal control to normalize 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 C_T) was measured to compare

gene expression. To relate C_T values to the abundance of mRNA species, they were converted into 'n-fold differences' (y) using the formula $y=2^{\Delta\Delta C_T}$ (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-Sp-fw (5'-GCGAGACCATGGAAGAAGTGGCCCAAGG-3') and tyrP-Sp-rv (5'-GCGAAATCTAGATCAAAGAGGGCTGCCCGTAG-3'), introducing *NcoI* and *XbaI* restriction sites. The 1.61 kb product was ligated into the NICE system expression vector pNZ8048 to yield pNZtyrP-Sp, which was transformed to *Lactococcus lactis* NZ9000 (de Ruyter *et al.*, 1996). For expression of *tyrP*, *L. lactis* NZ9000 pNZtyrP-Sp was grown on M17 medium supplied with 0.5 % glucose and 5 ng chloramphenicol ml^{-1} to mid-exponential growth phase (OD_{600} 0.6) at 30 °C. Nisin 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-Sp 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 μl samples were 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 analyser (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 (Millennium 32, Waters) liquid chromatograph (Alliance 2695, Waters). The strain was grown in GYP+T for 24 h. 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 $\times$ 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 $\pm$ 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 TD2/TD5 primer set (Coton & Coton, 2005) and the resulting 1133 bp sequence was compared with international databases using BLAST (Altschul *et al.*, 1990). The highest nucleotide 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.

Fig. 1. Phylogenetic tree comparing partial 16S rRNA gene sequences of *Sporolactobacillus* species described to date. The tree was constructed using the Pearson correlation and UPGMA (unweighted pair group method with arithmetic mean); bootstrap values are indicated at branch points (percentage of 1000 replicates).

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 OD₆₀₀, 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).

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).

Table 1. Tyramine production according to different initial tyrosine concentrations

The same letter within a column indicates a statistically significant difference ($P<0.01$) between the values, analysed by ANOVA and LSD analysis. Only differences between adjacent values are indicated.

Medium	OD ₆₀₀	pH	Tyramine concentration (mM)
GYP	0.98 ± 0.02	4.13 ± 0.01 ^a	0.09 ± 0.03
GYP + 1 mM Tyr	0.96 ± 0.26	4.27 ± 0.04 ^{a,b}	0.84 ± 0.29 ^a
GYP + 5 mM Tyr	0.97 ± 0.12	4.36 ± 0.06 ^{b,c}	2.48 ± 0.32 ^{a,b}
GYP + 10 mM Tyr	0.87 ± 0.25	4.44 ± 0.05 ^c	8.92 ± 1.56 ^b

Table 2. *tdc* region putative encoded proteins

Gene	Location in nucleotide sequence	G + C (%)	Predicted protein (aa/kDa/pI)*	Conserved domain†	Closest protein (aa)	Proposed function	Identity (%)‡	Accesssion no.	Organism
<i>ter</i>	0–795	47.0	Partial	Terminase 1 superfamily	Phage terminase	Phage terminase	73	YP_001308046	<i>Clostridium beijerinckii</i> NCIMB 8052
<i>tyrS</i>	774–1940	41.6	389/43.6/6.16	Tyrosyl-tRNA synthetase catalytic core	Tyrosyl-tRNA synthetase	Tyrosyl-tRNA synthetase	78	YP_796295	<i>L. brevis</i> ATCC 367
<i>tdc</i>	2169–4088	40.7	639/72.1/5.09	Tyrosine decarboxylase family	Tyrosine decarboxylase	Tyrosine decarboxylase	76	YP_796294	<i>L. brevis</i> ATCC 367
<i>tyrP</i>	4154–5617	39.5	487/53.5/9.96	Amino acid permease	Putative tyrosine permease	Tyrosine/tyramine antiporter	80	YP_796293	<i>L. brevis</i> ATCC 367
<i>nhaC</i>	5826–7235	38.7	469/50.4/9.21	Na ⁺ /H ⁺ antiporter	Na ⁺ /H ⁺ antiporter	Na ⁺ /H ⁺ antiporter	68	YP_796292	<i>L. brevis</i> ATCC 367
<i>rev</i>	7338–7535	42.4	Partial	Putative transposase <i>orfB</i>	Conserved hypothetical protein	Transposase	60	ZP_05746645	<i>L. antri</i> DSM 16041

*Theoretical molecular mass and pI were estimated by using the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

†Identified by the NCBI Conserved Domain Search tool (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

‡Identical amino acid percentage between the predicted sequence and the closest sequence in GenBank using BLASTP.

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 μM 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 μM. 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

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.

Fig. 3. Effect of tyrosine on the expression level of *tyrS*, *tdc*, *tyrP* and *nhaC* from *Sporolactobacillus* sp. cells grown in GYP medium either in the absence (white bars) or presence (grey bars) of 10 mM tyrosine. Each value is the mean $\pm$ SD of three independent experiments. *** $P < 0.01$.

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.*

Fig. 4. Tyrosine (a) and tyramine (b) uptake 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. ^{14}C -labelled tyrosine and ^{14}C -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.

brevis. Interestingly, the analysis of *tyrS* indicated that the reverse complement sequence of the first 22 bases was 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 histamine in *L. hilgardii* (Lucas *et al.*, 2005) and *T. halophilus* (Satomi *et al.*, 2008) as well as, recently, 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 phage 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 tyramine 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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