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Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration

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Summary

The microbial decarboxylation of some amino acids leads to the undesirable presence of biogenic amines in foods. One of the most abundant and frequent biogenic amines found in fermented foods is tyramine, which is produced by the decarboxylation of tyrosine. In the present work, transcriptional analysis of tyramine biosynthesis in *Enterococcus durans* IPLA655, a strain isolated from cheese, was studied. The gene coding for the tyrosine decarboxylase (*tdcA*) and that coding for the tyrosine-tyramine antiporter (*tyrP*) form an operon transcribed from the promoter P_{tdcA}, the expression of which is regulated by the extracellular pH and tyrosine concentration. Quantification of gene expression during the log phase of growth showed high concentrations of tyrosine and acidic pH conditions to induce *tdcA-tyrP* polycistronic messenger transcription.

Introduction

Biogenic amines (BA) are natural compounds of low molecular weight, synthesized during the normal metabolism of microorganisms, plants and animals. These compounds are involved in intercellular communication and biosignalling, metabolism and cell proliferation (Medina *et al*., 2003). However, high concentrations of BA in foodstuffs can be toxic (Santos, 1996; Shalaby, 1996). Biogenic amines are mainly formed in foods as a result of microbial decarboxylation of precursor amino acids via substrate-specific enzymes. The enzymatic decarboxylation of tyrosine leads to the undesirable presence of tyramine in fermented foods. Tyramine is the most abundant and frequently detected BA in cheese (Novella-Rodríguez *et al*., 2003), in which it can reach high concentrations (Fernández *et al*., 2006).

Many tyramine producers have been identified, all of which are lactic acid bacteria (LAB). These belong to genera as diverse as *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Tetragenococcus* and *Carnobacterium* (Ten Brink *et al*., 1990; Santos, 1996). Tyrosine is decarboxylated by tyrosine decarboxylase (TdcA) to yield tyramine, which is secreted by a transporter (TyrP) at the same time as further tyrosine is taken in. The amino acid/amine antiport system, coupled with decarboxylation, also functions to generate proton motive force (Molenaar *et al*., 1993; Konings *et al*., 1997; Pereira *et al*., 2009). Furthermore, amino acid decarboxylation has been described as one of the cellular strategies of acid adaptation (Booth *et al*., 2002).

The tyrosine decarboxylase gene (*tdcA*) has been characterized in *Lactobacillus brevis* IOEB 9809 (Lucas and Lonvaud-Funel, 2002; Lucas *et al*., 2003), *Enterococcus faecalis* JH2-2 (Connil *et al*., 2002) and *Enterococcus durans* IPLA655 (Fernández *et al*., 2004). The TyrP encoding gene (*tyrP*) is in all cases downstream and linked to *tdcA*.

It is well known that the amino acid substrate concentration, temperature, pH, a_w, the redox potential and NaCl concentration, among others, all affect the synthesis of amino acid decarboxylases and their activity (Suzzi and Gardini, 2003). Previous studies have noted that in Gram negative bacteria, decarboxylase gene expression requires acidic pH and high concentrations of amino acid substrate (Neely and Olson, 1996; Rhee *et al*., 2005). In our previous work it was observed that an acidic environment and a high tyrosine concentration favour the production of tyramine in *E. durans* IPLA655 (Fernández *et al*., 2007). Similar results have been reported by Marcobal and colleagues (2006) for other LAB strains. However, no data are available on the genetic regulation of tyramine biosynthesis. The present study reports the first transcriptional regulation analysis of the *tdcA* and *tyrP* genes in bacteria.

Results

Transcriptional linkage between tdcA *and* tyrP

To determine the expression profile of *tdcA* and *tyrP*, according to previously obtained physiological data

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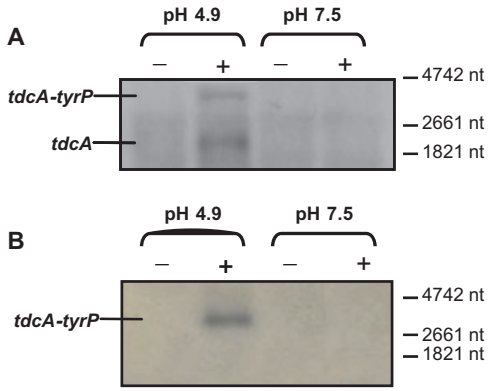

Fig. 1. Expression of *tdcA* and *tyrP* in the presence (+) and absence (–) of 10 mM tyrosine under different pH conditions (pH 4.9 and pH 7.5), determined by Northern blotting using internal probes specific for *tdcA* (A) and *tyrP* (B). Sizes were estimated using RNA Molecular Weight Marker I (Roche Diagnostics).

(Fernández *et al*., 2007), Northern blot experiments were performed with RNA obtained from *E. durans* IPLA655 cultures grown at pH 4.9 and 7.5 in presence and absence of 10 mM tyrosine. The expression of *tdcA* and *tyrP* required a low pH and the presence of tyrosine, which are conditions previously shown to induce tyramine synthesis (Fernández *et al*., 2007) (Fig. 1). Only under these conditions the *tdcA* specific probe revealed a 1.9 kb and a 3.6 kb band (Fig. 1A). The larger fragment was also detected under the same conditions after hybridization with the *tyrP* probe (Fig. 1B), suggesting the existence of a polycistronic mRNA molecule covering both genes. No monocistronic band was detected with the *tyrP* probe. These results indicate that *tdcA* may be transcribed from its own promoter as a monocistronic mRNA, or with *tyrP* as part of a polycistronic mRNA *tdcA-tyrP*. However, *tyrP* did not seem to have its own promoter; transcription was dependent on the *tdcA* promoter.

To confirm that *tdcA* and *tyrP* form an operon, reverse transcription-PCR (RT-PCR) was performed using primer pairs (Table 2) spanning the junction between each of the open reading frames in this region. Parallel reactions without reverse transcriptase (to control for residual genomic contamination in RNA preparations) failed to yield an amplification product. DNA template controls to ensure PCR fidelity for each primer pair uniformly yielded the expected-size PCR product (Fig. 2). A band of the expected size was obtained under tyramine production conditions (pH 4.9 and 10 mM tyrosine). This confirms the prediction that *tdcA* and *tyrP* constitute an operon. Reverse transcription-PCR involving the *tyrS* (the gene upstream *tdcA*) and *tdcA* intergenic region yielded no PCR product (Fig. 2), suggesting that transcription is initiated from the *tdcA* promoter.

Mapping of the tdcA *transcription initiation site*

Primer extension analysis was used to map the precise transcription start point (TSP) for *tdcA*. The reaction was performed using RNA samples extracted under optimal expression conditions. The TSP corresponded to an A located 35 nucleotides 5′ of the ATG codon (Fig. 3). Thirty-four nucleotides upstream of the TSP a putative transcriptional –35 sequence, TTTACA, was found, separated from the –10 region TGTAAT by 15 bp, nearly matching the consensus transcriptional sequence for LAB promoters (bases matching the consensus are underlined). The corresponding RBS region (AGGAGG) was located 8–13 nt 5′ of the ATG codon.

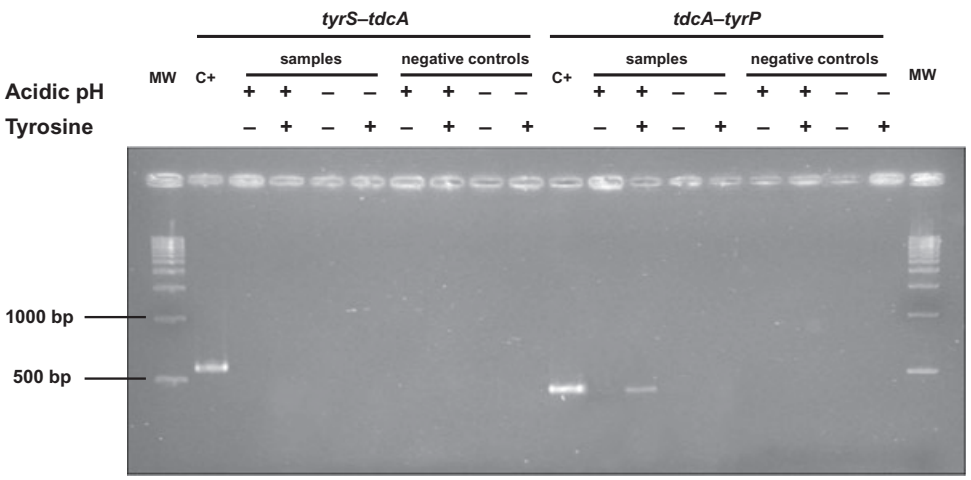

Fig. 2. Reverse transcription-PCR amplification of the intergenic regions *tyrS-tdcA* and *tdcA-tyrP* at acidic (+) and non-acidic pH (–), with (+) and without (–) 10 mM tyrosine; C+, positive control; MW, molecular weight marker.

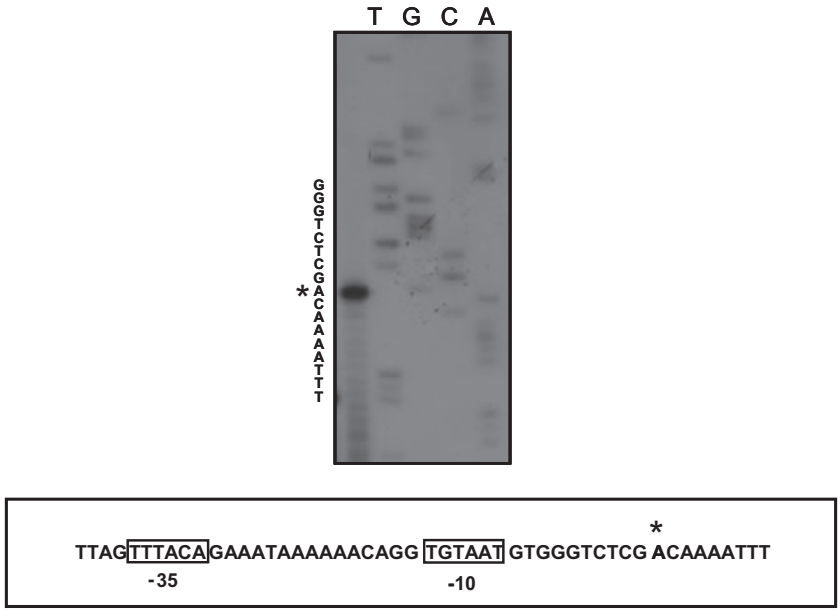

Fig. 3. Primer extension identification of the transcription start site (*) of *tdcA*, and identification of the transcriptional regions –10 and –35 (boxes). Lines T, G, C and A show the DNA sequence (note that the nucleotide sequences, 5′ at the top and 3′ at the bottom, represent the complementary DNA strand, allowing it to be read directly).

As predicted by previous experiments, and even though different reverse primers were used (Table 2), no putative TSP for *tyrP* was identified (data not shown).

tdcA *and* tyrP *transcription is regulated by the extracellular pH and tyrosine concentration*

To confirm and quantify the transcription of the *tdc* operon under different pH and tyrosine concentrations, gene expression was analysed by quantitative real-time PCR (qRT-PCR) in bacteria grown in GM17 and GM17 + T at pH 7.5 and pH 4.9 (Fig. 4). *tdcA* and *tyrP* expression was increased 8- and 13.2-fold respectively when tyrosine was added to the medium at pH 4.9. However, no induction was detected when the pH was 7.5 or when the pH was low in the absence of tyrosine. Therefore, the induction of *tdcA* and *tyrP* transcription requires an acidic environment and the presence of tyrosine.

Quantitative RT-PCR was used to study the effect of different tyrosine concentrations in the growth media on gene expression at optimal pH 4.9. As shown in Fig. 5, *tdcA* and *tyrP* expression increased with increasing concentrations of tyrosine. A concentration of tyrosine of 5 mM was necessary to reach the maximum induction of *tdcA* and *tyrP* expression. However, greater concentrations provoked no further induction of expression.

P_{tdcA} *is induced by acidic pH and tyrosine*

To determine whether *tdcA* and *tyrP* transcription is regulated by the *tdcA* promoter (P_{tdcA}), the transcriptional fusion construct P_{tdcA}-*gusA* was analysed at different pHs and tyrosine concentrations. Figure 6 shows a significant difference in β-glucuronidase activity when the cultures were grown under optimal tyramine production conditions; in fact, this activity was enhanced fivefold. According to the expression profiles for *tdcA* and *tyrP*, β-glucuronidase activity was independent of the tyrosine concentration when the pH of the medium was 7.5.

Discussion

Bacteria respond to changes in the external pH by altering several physiological processes via their corresponding pattern of gene expression. In LAB, several genes are induced when the external pH is acidic in order to ensure survival under such unfavourable – although habitual (because of lactic acid production) – conditions. Many authors have investigated the effect of environmental factors on tyramine formation in LAB, but no information is available on the regulation mechanisms. According to the present results, the extracellular pH and tyrosine concentration are important variables influencing the transcriptional regulation of *tdcA* and *tyrP*. These genes were not expressed at pH 7.5; however, at pH 4.9 they were strongly induced. Under the latter conditions the expression profile was modulated by tyrosine concentration.

Polycistronic mRNA was detected for the *tdcA-tyrP* spanning region under optimal conditions of acidic pH and in the presence of tyrosine, but in smaller amounts than *tdcA* monocistronic mRNA. This inefficient termination may occur due to the presence of a weak terminator

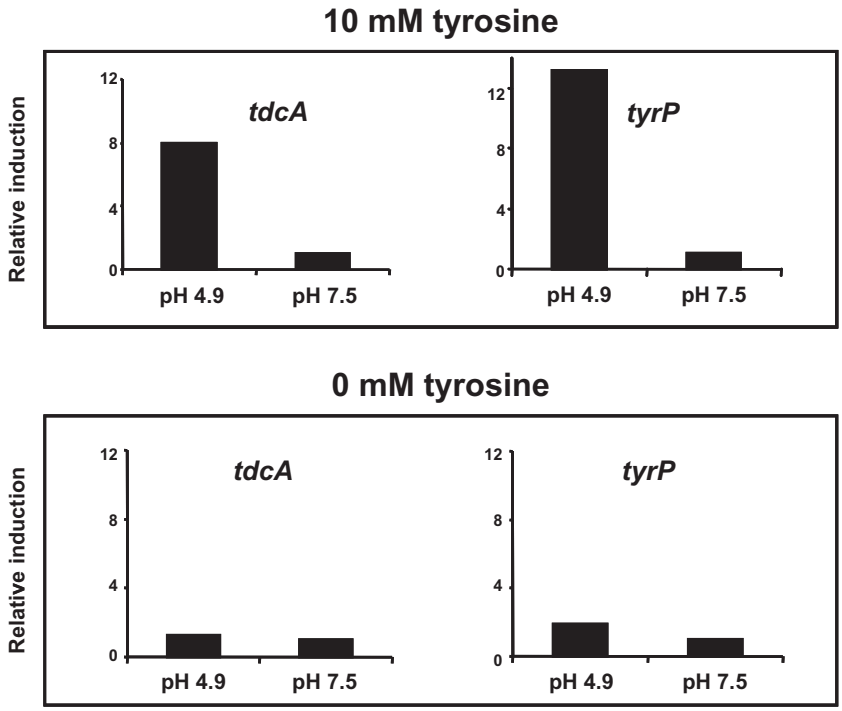

Fig. 4. Relative concentration of *tdcA* and *tyrP* mRNA in presence and absence of 10 mM tyrosine under different pH conditions (pH 4.9 and pH 7.5). The expression level of each gene at pH 7.5 was normalized to 1 and used as reference condition.

($\Delta G = -15$ kcal mol^{-1}) between *tdcA* and *tyrP*. The co-transcription of the gene encoding the amino acid decarboxylase and that encoding the corresponding antiporter has been described in tyramine biosynthesis in *E. faecalis* JH2-2 (Connil *et al*., 2002) and *Lb. brevis* IOEB 9809 (Lucas *et al*., 2003) and in other decarboxylation operons involved in cadaverine and putrescine production in *Escherichia coli* (Meng and Bennett, 1992; Pistocchi *et al*., 1993).

The TSP of *tdcA* was also mapped. Near-consensus –35 and –10 regions were identified. There is not data about consensus regions related to pH promoter control. The absence of conserved sequences in pH-controlled promoters would indicate multiple regulatory mechanisms (Martín-Galiano *et al*., 2005). In some bacteria, the acid tolerance response involves alternative sigma factors (van Schaik and Abee, 2005). It was also proposed that short runs of repeated adenine or thymine residues, which

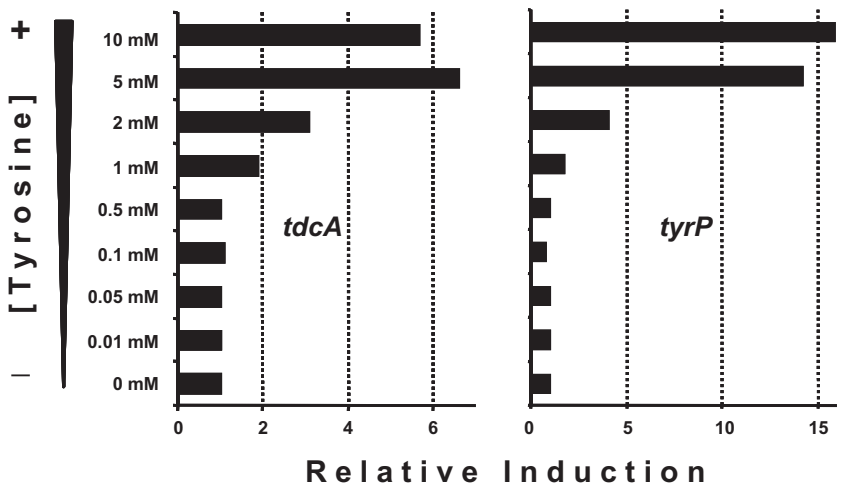

Fig. 5. Effect of different tyrosine concentrations (0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5 and 10 mM) on *tdcA* and *tyrP* expression at pH 4.9. The lowest expression level for both genes (i.e. at 0 mM tyrosine) was normalized to 1 and used as reference condition.

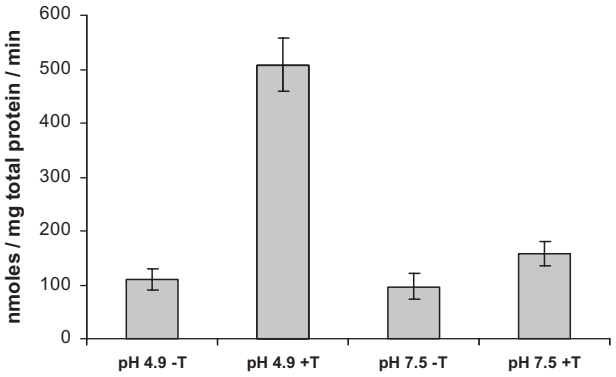

Fig. 6. Average β-glucuronidase activity (expressed in nmoles/mg total protein/min) obtained with the transcriptional fusion construct P_{tdcA}-*gusA* in the presence (+T) and absence (–T) of tyrosine at acidic and neutral pH.

are know to lead intrinsic DNA bending, are related to pH promoter control (Chen *et al*., 2002; Blancato *et al*., 2008). The analysis of P_{tdcA} revealed the presence of a polyA sequence between –10 and –35 boxes (Fig. 3), suggesting that in this case, DNA bending could be involved on pH promoter control.

It is well known that pH is a crucial factor in tyramine synthesis (Marcobal *et al*., 2006; Fernández *et al*., 2007). The present work shows that pH induces the transcription of the *tdc* operon. However, the physiological significance of this process is still under discussion. Many authors have suggested that the decarboxylation of amino acids by microorganisms might fulfil a protective function against extracellular acidification (Gale and Epps, 1942; Booth *et al*., 2002). Tyrosine decarboxylation may play a role in cytoplasmic pH homeostasis and resistance against acid stress through the alkalinizing effect of the decarboxylation reaction (Wolken *et al*., 2006; Pereira *et al*., 2009). In the presence of tyrosine, the tyramine-producing strain *E. durans* IPLA655 provokes an increase in extracellular pH not seen with the non-producing mutant strain *E. durans* IPLA655 *tdcA* (Fernández *et al*., 2007). Different authors prove that BA production depends on amino acid substrate availability (Soksawatmaekhin *et al*., 2004; Fernández *et al*., 2006; Landete *et al*., 2006). To ascertain the regulatory role of tyrosine, the effect of increasing concentrations on gene expression profile were analysed, and it was confirmed that the response to tyrosine concentration is transcriptionally regulated. It is noteworthy that concentrations under 5 mM were insufficient to induce the maximum expression of *tdcA* and *tyrP*, presumably because tyrosine is a substrate amino acid for protein biosynthesis. Therefore, the cells divert it to the decarboxylation pathway only when it is in excess. It is important to indicate that tyrosine concentrations over 5 mM can be found in natural habitats of these bacteria (Fernández *et al*., 2006). In addition to *E. durans* IPLA665 other LAB strains may be adapted to use this resource when available, allowing them to better control their internal pH in acidic environments. This adaptation requires the capacity to sense the pH and the tyrosine concentration and to accordingly regulate the genes coding for the enzymes involved in the decarboxylation mechanism. This work confirms the influence of acidic pH and tyrosine concentration on the regulation of genes encoding the tyramine synthesis pathway in *E. durans* IPLA655.

Experimental procedures

Bacterial strains and media

Table 1 lists the bacterial strains used in this study. *Escherichia coli* was grown with aeration in Luria–Bertani medium at 37°C. *E. durans* IPLA655 and *Lactococcus lactis* NZ9000 were grown at 30°C without aeration in M17 medium (Oxoid) supplemented with 0.005% pyridoxal 5-phosphate and 0.5% glucose (GM17). To analyse the regulatory effect of tyrosine, GM17 was supplemented with 10 mM tyrosine (GM17 + T). When needed, erythromycin (5 μg ml^{-1} for *E. durans* and *L. lactis*), ampicillin (100 μg ml^{-1} for *Escherichia coli*) or chloramphenicol (5 μg ml^{-1} for *E. durans* and *L. lactis*) was added to the culture medium.

DNA manipulation procedures

Cloning was performed according to standard procedures (Sambrook and Russell, 2001). The large scale isolation of

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Characteristics	Source
Strains		
E. coli TOP10		Invitrogen
L. lactis NZ9000	Plasmid-free strain	Kuipers *et al.* (1998)
E. durans IPLA655	Isolated from artisanally made cheese. Tyramine producer	IPLA Collection
Plasmids		
pIL252	Ery^R, origin of replication pAMβ1 (6–9 copies/cell)	Simon and Chopin (1988)
pDA6	Amp^R, pUC18 with P*tdcA* cloned in *SmaI*	This work
pEM172	Ery^R, pIL252 with the *gusA* gene from pNZ273 and terminator from pNZ8048	This work
pDA11	Ery^R, pEM172 including the fusion construct P_{tdcA}-*gusA*	This work

Amp^R, ampicillin resistant; Ery^R, erythromycin resistant.

Table 2. Oligonucleotides used in this study.

Primer	Function	Sequence (5′ to 3′)[a]
Northern blotting		
TDC10	*tdcA* probe amplification (F)	TTCCGTACCATGGCATAATG
RT2	*tdcA* probe amplification (R)	ACCCCATTTTATGTGGGTCGATC
TDC14	*tyrP* probe amplification (F)	GCATCGCCATGGCGTTATGTGCGACAG
T(P)2	*tyrP* probe amplification (R)	ATACCATGAAACTAAAGATGTTCCC
Reverse PCR		
R1	Intergenic region *tyrS-tdcA* (F)	AGTATTAACGGTGACCGCGTTACC
TDC8	Intergenic region *tyrS-tdcA* (R)	GCATGTCCTGGGGCATGTAG
TDC26	Intergenic region *tdcA-tyrP* (F)	CGCGGATCCAAATCTACGCAGATCAATTATTAGC
TDC129	Intergenic region *tdcA-tyrP* (R)	CGTCAGACCGACAAAGGTCC
Primer extension		
TDCA	P_{tdcA} mapping (R)	ATGATTCCATGTGAAACCCTC
TDC126	P_{tyrP} mapping (R)	CTCCGAACTGTCGCACATAACGCCA
TDC128	P_{tyrP} mapping (R)	AATAACCGAAAGCAAAGAGGTTGC
qRT-PCR		
TDC2B	*tdcA* expression analysis (F)	CTGCCGACATTATCGGTGTTGGTC
TDC(P)	*tdcA* expression analysis (R)	GATAGTTGTGATCAACTGGTCAGGG
T2B	*tyP* expression analysis (F)	CCTATCTTGATTTACTGCGATATCC
T(P)	*tyrP* expression analysis (R)	CCCAAGCTGACAACTGTAAAAGTACCCC
16Sf	16SrRNA internal control (F)	AGTACGACCGCAAGGTTGAAACTCA
16Sr	16SrRNA internal control (R)	TGTCAAGACCTGGTAAGGTTCTTC
Promoter clonation		
TDCNdeSalI	P_{tdcA} cloning and sequencing (F)	GGAATTCCATATGTCGACACAAGAAGAAATCGCT (SalI)
TDCEcoRI	P_{tdcA} cloning and sequencing (R)	GGAATTCCAAAAAAAATATTTAAATTTTGTC (EcoRI)

a. Underlined nucleotides indicate restriction cleavage sites not present in the template sequence used to facilitate cloning. The corresponding restriction enzyme is shown in parentheses to the right of the primer sequence.
F, forward; R, reverse; P_{tdcA}, *tdcA* promoter; P_{tyrP}, *tyrP* promoter.

E. coli plasmids for nucleotide sequence analysis was performed using the Plasmid Midi Kit (Qiagen) following the manufacturer's instructions. The plasmid and chromosomal DNA of *E. durans* and *L. lactis* was isolated and transformed as previously described (de Vos *et al*., 1989). All enzymes for DNA technology were used according to the manufacturer's specifications. The oligonucleotides used in PCR were synthesized by Sigma Genosys.

RNA extraction

Total RNA was extracted using the TRI Reagent (Sigma). Cells of *E. durans* were grown in GM17 and GM17 + T at pH 4.9 and pH 7.5 until the mid-log phase of growth (OD_{600} of 0.60). They were then harvested by centrifugation and disrupted using glass beads (diameter up to 50 μm) in a FastPrep FP120 Instrument (Thermo Savant-BIO101/Q-Biogen) at 4°C for 6 × 30 s at power setting 6. The resulting samples were treated as recommended by the manufacturer. Purified RNAs were resuspended in 0.1% diethyl pyrocarbonate-treated water.

RNA samples were electrophoretically checked for rRNA integrity and yield. Total RNA concentrations were determined by UV spectrophotometry by measuring absorbance at 260 nm in a BioPhotometer (Eppendorf).

Northern blot

Total RNA was obtained from *E. durans* IPLA655 grown in GM17 and GM17 + T at pH 4.9 and pH 7.5 as previously described, subjected to electrophoresis in 1.5% agarose gels, blotted, and hybridized following standard protocols (Sambrook and Russell, 2001). Double-stranded DNA probes, amplified using the primers shown in Table 2, were radiolabelled with [α-^{32}P]-dATP by nick translation with DNA polymerase/DNase I (Invitrogen).

Reverse transcription-PCR

RNA samples (2 μg of total RNA) were treated with 2 U of DNase (Fermentas), as described by the manufacturer, to eliminate any DNA contamination. cDNA was then synthesized from total RNA using the iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's recommendations.

Reactions were performed using 2 μl of the cDNA suspension and 0.4 μM of each primer. After the reverse transcription reaction, amplifications were performed for 35 cycles (94°C for 30 s, 55°C for 45 s and 72°C for 1 min) and the resulting fragments electrophoretically analysed in 1.5% agarose gels in TAE buffer (40 mM TRIS/acetate, 1 mM EDTA; pH 8.0). The absence of contaminating DNA in the DNase-treated RNA samples was controlled by PCR performed under the same conditions but without reverse transcriptase. Table 2 shows the primer pairs used.

Quantification of gene expression by reverse transcription real-time PCR

Gene expression was analysed by quantitative real-time PCR (qRT-PCR) as previously described for *Oenococcus oeni* (Desroche *et al*., 2005), using the SYBR® Green PCR Master Mix from 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 of PCR mixture (12.5 μl of SYBR Green Supermix, 1 μl of each primer at 7 μM and 5.5 μl of RNase-free water). All cDNAs were amplified by qRT-PCR using specific primers [Table 2; selected using Primer Express software (Applied Biosystems)] designed to render amplicons of equivalent length (approximately 100 bp). The gene encoding the 16S ribosomal RNA (16S rRNA) was used as an internal control to normalize the RNA concentration. In each run, a negative control was included. Amplifications were performed using the default cycling settings established by Applied Biosystems.

The variable measured for the comparison of gene expression in different environments was the number of PCR cycles required to reach the midpoint of the amplification curve, also known as the threshold cycle (Ct). To relate Ct values to the abundance of mRNA species under different conditions (pH 4.9, pH 7.5, in the presence and absence of tyrosine) they were converted into '*n*-fold' differences (*y*) using the formula $y = 2^{\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

For each gene, qRT-PCR analysis was performed on RNA purified from three independent cultures grown under each of the environmental condition assayed.

Threshold cycle was determined by monitoring the incorporation of SYBR Green into the amplified product by means of fluorescence detection. SYBR Green fluorescence was normalized to a passive reference dye (carboxy-x-rhodamine) included in each reaction.

Primer extension and DNA sequencing

Total RNA isolated during the exponential phase of *E. durans* IPLA655 growth in GM17 + T at pH 4.9 was treated with DNase as already reported and employed as a template for primer extension using the corresponding reverse primer (see Table 2).

One picomole of oligonucleotide, 5′-end-labelled with [γ-^{32}P]-dATP using T4 polynucleotide kinase (New England Biolabs) and purified using the QIAquick Nucleotide Removal Kit (Qiagen), was added to 15 μg of RNA. Elongation of the cDNA strand was performed at 42°C after the addition of 20 U of avian myeloblastosis virus reverse transcriptase (Promega). The resulting cDNA was examined in an 8% denaturing polyacrylamide gel containing 7 M urea, along with a sequence ladder, generated using the same primer and the pDA6 plasmid (Table 1), as a template. The sequence reaction was performed according to the dideoxynucleotide chain termination sequencing method (Sanger *et al*., 1977).

Preparation of the P_{tdcA}-gusA transcriptional fusion construct

A DNA fragment including P_{tdcA} was amplified by PCR using the primers *TDCNdeSalI* and *TDCEcoRI* (Table 2), and cloned into the SmaI site of a pUC18 vector, resulting in the formation of pDA6. To generate a transcriptional fusion construct with the reporter gene *gusA*, a BamHI-SalI pDA6 fragment including P_{tdcA} was cloned into the vector pEM172 digested with the same enzymes to produce the plasmid pDA11. pEM172 is a derivative of the low-copy number vector pIL252 (Simon and Chopin, 1988) and possesses an erythromycin-resistance determinant. The *gusA* gene it carries (from pNZ273) was followed by a terminator from pNZ8048 (Kuipers *et al*., 1998). Plasmid pDA11 was first constructed in *L. lactis* NZ9000 and electroporated into *E. durans* IPLA655. All constructs were sequenced.

Determination of β-glucuronidase activity

To determine β-glucuronidase activity, cells were grown in GM17 and GM17 + T at pH 4.9 and pH 7.5 to an $OD_{600} = 0.60$. They were then disrupted with 0.2 g of glass beads (< 106 μm) (Sigma) and shaken three times at the maximum speed setting of a FastPrep FP120 homogenizer at room temperature for 1 min each time and with a 1 min pause on ice between each pulse. After centrifugation at 12 000 *g* for 10 min at 4°C the resulting supernatant was separated and immediately assayed according to the protocol described by Platteeuw and colleagues (1994), with some modifications.

β-Glucuronidase assays were performed in microtitre plates using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). Fifty microlitres of the supernatants was mixed with 148 μl of GUS buffer [50 mM Na_2HPO_4 (pH 7.0), 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100] and 2 μl of 100 mM *para*-nitro-β-D-glucuronic acid, and immediately incubated at 37°C for 2 h. Light emissions (λ = 405 nm) were measured every 30 s. *E. durans* IPLA655 harbouring plasmid pEM172 was used as a negative control. β-Glucuronidase activity was calculated as the slope of the curve in the linear phase. In each experiment different dilutions of the supernatants in GUS buffer were tested; each condition was assayed independently in triplicate. Values were standardized to protein contents of cell extracts using the BCA Protein Assay Reagent Kit (Pierce).

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