Phenotypic and genotypic evaluation of biogenic amine production by lactic acid bacteria isolated from fish and fish products

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Abstract

In this work, production of the biogenic amines histamine, tyramine and putrescine by a collection of 74 lactic acid bacteria (LAB) of aquatic origin has been investigated by means of three different methodologies: agar plate assay in decarboxylase differential growth medium, thin–layer chromatography (TLC) and PCR. None of the evaluated LAB strains showed neither production of histamine and putrescine, nor presence of the genetic determinants encoding the corresponding decarboxylase activities. However, the tyrosine decarboxylase gene (*tyrDC*) was present in all the enterococcal strains, and tyramine production was detected by TLC in all of them but in *E. faecium* BCS59 and MV5. Analysis of the tyrosine decarboxylase operon of these strains revealed the presence of an insertion sequence upstream *tyrDC* that could be responsible for their lack of tyrosine decarboxylase activity.

Keywords: fish probiotics, lactic acid bacteria, enterococci, biogenic amines, tyramine

1. Introduction

Biogenic amines (BAs) are low molecular weight organic bases which exert different biological activities (ten Brink et al., 1990). The presence of BAs in foods is mainly due to amino acid decarboxylation by substrate–specific enzymes of microbial origin (ten Brink et al., 1990; Halász et al., 1994). In non–fermented foods (mainly fish), BAs are produced by undesirable microorganisms and are indicative of food spoilage; on the other hand, many lactic acid bacteria (LAB) might be involved in BAs production, and thus LAB starter cultures devoid of amino acid decarboxylase activity should be carefully selected to avoid the presence of BAs in fermented foods (ten Brink et al., 1990; Silla et al., 1996). Notwithstanding the physiological functions of BAs in living cells (Halász et al., 1994; Silla et al., 1996), their presence at high levels in foods represents a public health issue due to the risk of food intoxication. In this context, the two BAs most frequently involved in food intoxication are histamine and tyramine, produced from the precursor amino acids histidine and tyrosine by the histidine decarboxylase (HDC) and tyrosine decarboxylase (TDC) enzymes, respectively. Histamine, with an upper limit for human consumption of 100 mg/kg in some fishery products in the European Union [Commission Regulation (EC) No 1441/2007] is responsible for the so–called scombroid poisoning. This food intoxication is associated with the fish families *Scombridae* and, to a lesser extent, *Clupeidae* and *Engraulidae*, which contain high levels of free histidine in their tissues. It is characterized by headache, low blood pressure, heart palpitations, edema, etc. (Lehane et al., 2000; Kim et al., 2009). On the other hand, tyramine, with a suggested threshold value for human consumption of 100–800 mg/kg, is most frequently present in fermented foods, mainly cheese, and has been associated with food–induced migraines and hypertensive crisis

75 (ten Brink et al., 1990). Other BAs, such as putrescine, produced from ornithine by the
76 action of the ornithine decarboxylase (ODC) enzyme, may potentiate the toxicity of the
77 BAs mentioned above due to interference with their detoxification systems. Moreover,
78 some BAs are potential carcinogen precursors, since they can react with nitrites present
79 in food and give rise to nitrosamines (Halász et al., 1994).
80 LAB belonging to different genera are being increasingly employed as probiotics to
81 improve human and animal health (Verschuere et al., 2000; Kalliomäki et al., 2008). In
82 this respect, our research group is currently investigating the suitability of a collection
83 of LAB with antimicrobial activity isolated from fish and fish products as probiotics for
84 aquaculture (Gómez–Sala, 2004; Muñoz–Atienza, 2009). According to the *Guidelines*
85 *for the Evaluation of Probiotics in Food* (FAO/WHO, 2002), a careful safety evaluation
86 of the strains intended for this use should be performed. This includes, among other
87 aspects, the evaluation of the presence of undesirable metabolic activities such as the
88 production of BAs. Here, we present the results of the evaluation of histamine, tyramine
89 and putrescine production by a collection of 74 LAB of aquatic origin by using three
90 different methodologies, namely an agar plate assay in decarboxylase differential
91 growth medium, thin–layer chromatography (TLC) and PCR, and describe a genetic
92 event which may be responsible for the lack of tyramine production by two enterococcal
93 strains containing the gene encoding the tyrosine decarboxylase enzyme (*tyrDC*).
94
95 2. Materials and methods
96
97 *2.1 Bacterial strains and growth conditions*
98 The LAB strains of aquatic origin used in this study (Table 1) were isolated and
99 taxonomically identified in previous works (Gómez et al., 2004; Muñoz–Atienza, 2009).

100 *Lactobacillus brevis* CECT (Colección Española de Cultivos Tipo) 4121 was used as
101 positive control for tyramine production, and *Lactobacillus* sp. 30a (ATCC [American
102 Type Culture Collection] 33222) was used as positive control for histamine and
103 putrescine production (García–Moruno et al., 2005). *Escherichia coli* DH5α was used as
104 host for recombinant plasmids. Unless otherwise stated, LAB strains were grown in
105 MRS broth (Oxoid Ltd., Basingstoke, England) at 30ºC. *Escherichia coli* cells were
106 incubated in Luria Bertani (LB) medium (Sambrook et al., 1989) at 37ºC with shaking.
107 When required, ampicillin (100 μg/ml) was added to the LB medium.
108
109 *2.2 Decarboxylase differential growth medium for the screening of HDC, TDC and*
110 *ODC activities in LAB*
111 The presence of these enzymatic activities in the selected LAB strains was evaluated
112 by an agar plate assay using the improved decarboxylase differential growth medium
113 (from this point, plate assay) described by Bover–Cid and Holzapfel (1999). Briefly, the
114 strains were subcultured 5 times in MRS broth containing 0.1% (wt/vol) of the
115 corresponding amino acid precursor (Sigma–Aldrich, St. Louis, MO, USA) and 0.005%
116 (wt/wol) of pyridoxal–5–phosphate (Sigma–Aldrich) at 30ºC overnight in order to
117 promote the corresponding enzyme induction. Subsequently, cultures were streaked in
118 duplicate on decarboxylase differential growth medium agar (1.5% wt/vol) plates with
119 or without (negative control) the corresponding amino acid precursor, and incubated at
120 37ºC for 4 days under aerobic and anaerobic (Gas–Pack, Oxoid) conditions. After
121 incubation, BAs production was detected by a yellow to violet color change of the
122 decarboxylase medium due to the alkalinisation produced by the decarboxylation of the
123 corresponding amino acid precursor.
124

2.3 TLC analysis of histamine, tyramine and putrescine production by LAB

LAB strains were grown in MRS broth containing 1% (wt/vol) of the corresponding amino acid precursor and incubated at 30ºC for 7 days. Non–inoculated media processed under the same conditions were used as negative controls. After incubation, the cell–free culture supernatants were obtained by centrifugation (12,000 × *g* at 4ºC for 10 min) and analysed by TLC as described by García Moruno et al. (2005). Stock solutions of amines (250 mg/l of histamine, tyramine, or putrescine) in 40% ethanol were prepared and further diluted 1:10 (vol:vol) in distilled water to be used as controls.

2.4 PCR detection of histidine, tyrosine and ornithine decarboxylase encoding genes in LAB

Total genomic DNA from LAB strains was isolated from overnight cultures using the Wizard DNA Purification kit (Promega, Madison, WI, USA). The isolated DNA was subjected to PCR amplification to detect the presence of the histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tyrDC*) and orthithine decarboxylase (*odc*) genes by using the primers CL1–JV17HC, TD2–TD5 and 3–16, respectively (reviewed by Landete et al. [2007]). Reactions were carried out in a total volume of 25 μl containing 150–250 ng of template DNA, 0.2 mM of each dNTP, 35 pmol of each primer, 1.5 mM $MgCl_2$ and 0.75U of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) in the buffer provided by the manufacturer, and by using the amplification conditions reviewed by Landete et al. (2007). The resulting PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels containing ethidium bromide as staining agent.

149 *2.5 Nucleotide sequence and functional analysis of* tyrDC *in the enterococcal strains*
150 *lacking tyramine production*
151 Total genomic DNA from overnight cultures of *Enterococcus faecium* BCS59 and
152 MV5, containing *tyrDC* (*tyrDC*$^{+}$) but lacking production of tyramine when analysed by
153 plate assay and TLC (TDC^{-}), was isolated with the Wizard DNA Purification kit and
154 subjected to PCR as described above using primers 57 (5´–ATGAGTGAATC
155 ATTGTCG) and 58 (5´–TTATTTTGCTTCGCTTGCC) designed by Marcobal et al.
156 (2004). The PCR–generated products were extracted from the agarose (1–1.5% wt/vol)
157 gels and purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden,
158 Germany) for sequencing with the same primers. ORF finding and similarity searches
159 were performed with the ORF finder and the Basic Local Alignment Search tools
160 (Altschul et al., 1997), respectively, available at the National Center for Biotechnology
161 Information (NCBI; http://www.ncbi.nlm.nih.gov/). Computer promoter predictions
162 were carried out at http://www.fruitfly.org/seq_tools/promoter.html.
163 In order to determine if the *tyrDC* variant found in *E. faecium* BCS59 and MV5
164 encoded a functional TDC enzyme, an amino acid substitution was introduced into
165 plasmid pAM3, carrying *tyrDC* from *E. faecium* RM58 cloned into pIN–III–A3 vector
166 under control of the lpp^{P}–5 and lac^{po} promoters (Marcobal et al., 2006a). The *tyrDC*
167 variant was constructed by the site–directed mutagenesis PCR technique using plasmid
168 pAM3 as template. The mutagenic primers designed in this work to introduce the amino
169 acid change (Ala–186–Thr) were 613 (5´–CTGAGCTAGTTACTGGAAAAAGCGA)
170 and 614 (5´–TCGCTTTTTCCAGTA ACTAGCTCAG). Briefly, the corresponding pair
171 of primers was used as homologous primer pair in a PCR reaction using pAM3 plasmid
172 as template DNA and *Pfu* DNA polymerase (Promega). The PCR product was digested
173 with *Dpn*I, that exclusively restricts methylated DNA (Geiser et al., 2001). Competent

E. coli DH5α cells were transformed directly with the digestion product. The resulting pAM3$_{A-T}$ plasmid, expressing the *tyrDC* variant, was sequenced to verify the absence of unwanted mutations. Subsequently, cell extracts were obtained from *E. coli* DH5α cells harboring either pAM3$_{A-T}$ or the original pAM3 plasmid after induction with IPTG (0.5 mM final concentration) as described by Marcobal et al. (2006a), and analysed for tyramine production by TLC as described above.

2.6 Analysis of the nucleotide sequence surrounding tyrDC *in* tyrDC^{+}*/TDC*$^{-}$ *enterococcal strains*

In order to determine the nucleotide sequence located upstream and downstream *tyrDC* in *E. faecium* BCS59 and MV5, lacking TDC production, total genomic DNA of these strains obtained with the Wizard DNA purification kit was subjected to PCR as described above using the degenerated primers 599 (5´–TGGMGRGRYG CNATHAAYCARCARAC) and 602 5´–GWYTKNSWNGGNCCNGTNACCCA). These primers were designed in this work based on the conserved motifs WRDAINQQ and WVTGPSQ found in the sequence of *tyrS* and *tyrP*, respectively, of different LAB deposited at the NCBI. New primers based on the nucleotide sequences of the PCR products obtained were subsequently designed for chromosome walking experiments until the sequence of the intergenic regions *tyrS–tyrDC* and *tyrDC–tyrP* was completed. ORF finding and similarity searches of the nucleotide sequences were performed as described above. The nucleotide sequence obtained in this work was deposited in GenBank under accession number HM921050.

199 3. Results

200

201 *3.1 Screening of HDC, TDC and ODC activities in LAB*

202 The results of the screening of decarboxylase activities by the plate assay are shown
203 in Table 1. HDC and ODC activities were not detected in any of the LAB strains tested;
204 however, TDC activity was detected in all the *Enterococcus faecalis* and *E. faecium*
205 strains, except in *E. faecium* BCS59, CV1 and MV5. The results obtained under aerobic
206 and anaerobic incubation did not differ, except in the case of *E. faecium* SMA101, which
207 only yielded a clear positive reaction when grown on anaerobiosis.

208

209 *3.2 TLC-analysis of histamine, tyramine and putrescine production by LAB*

210 Table 1 shows the results of the TLC screening of the presence of histamine, tyramine
211 and putrescine in the supernatans from the LAB cultures. Histamine and putrescine were
212 not produced by any of the strains; in contrast, tyramine was detected in the supernatants
213 from all the enterococcal strains, except in *E. faecium* BCS59 and MV5, but was not
214 produced by any of the non-enterococcal strains.

215

216 *3.3 PCR detection of* hdc, tyrDC *and* odc *in LAB*

217 The results of the PCR screening of the presence of decarboxylase genes in LAB are
218 shown in Table 1. While *hdc* and *odc* were not present in any of the strains, *tyrDC* was
219 found in the 34 enterococcal strains analysed in this study. The presence of *tyrDC* was
220 not detected in any of the non-enterococcal strains.

221

222 *3.4 Nucleotide sequence and functional analysis of* tyrDC *in* E. faecium *BCS59 and*
223 *MV5*

As shown in Table 1, *E. faecium* BCS59 and MV5 did not display a positive result when their tyramine production was analysed by the plate assay or TLC; however they yielded a positive result in the PCR assay. Alignment of the nucleotide sequences of the PCR products obtained with primers 57–58 in these strains showed that their *tyrDC* genes were identical. ORF finding and subsequent BLAST analysis of these sequences revealed that *tyrDC* encodes a 625 amino acid protein identical to the tyrosine decarboxylase of *E. faecium* E1039 (accession number ZP_06675610.1). Besides, the protein is highly similar (99% identity) to pyridoxal–dependent decarboxylases of *E. faecium* DO (accession number ZP_00602894) and other *E. faecium* strains, and to a phenylalanine and tyrosine decarboxylase of *E. faecium* RM58 (accession number AJ783966), whose functional activity has been already demonstrated (Marcobal et al., 2006a). Interestingly, compared to the latter, TDCs of E. *faecium* BCS59 and MV5 contain a single amino acid variation at position 186 (alanine to threonine substitution). TLC analysis of *E. coli* pAM3$_{A-T}$ expressing this *tyrDC* variant showed that, similarly to the control *E. coli* (pAM3), the recombinant strain was able to produce tyramine (Fig. 1), thus indicating that this amino acid substitution is not responsible for the lack of tyramine production observed in these *tyrDC*$^{+}$/TDC^{-} enterococcal strains.

3.5 Analysis of the nucleotide sequence surrounding tyrDC *in* E. faecium *BCS59 and MV5*

ORF finding and subsequent BLAST analysis of a nucleotide region of 5,335 bp containing *tyrDC* in *E. faecium* BCS59 and MV5 (Fig. 2) showed that this region also encodes the following proteins: (i) upstream *tyrDC*, the 375 C–terminal amino acid residues of a protein identical to a tyrosyl–tRNA synthetase from *E. faecium* DO (accession number ZP_00602895); and (ii) downstream *tyrDC*, the 279 N–terminal amino

249 acid residues of a protein with the highest homology (99% identity) with an amino acid
250 permease from *E. faecium* TC6 (accession number ZP_05921494) and other *E. faecium*
251 strains, an amino acid permease–associated region of *E. faecium* DO (accession number
252 ZP_00602893), and an APC family amino acid–polyamine organocation transporter of *E.*
253 *faecium* TX1330 (accession number ZP_03981092). Moreover, a protein of 319 amino
254 acid residues (36,867 Da) with a theoretical pI of 9.72, identical and highly similar (99%
255 identity) to integrases from *E. faecalis* T2 (accession number ZP_05426872) and *E.*
256 *faecium* DO (accession number ZP_00603339.1), respectively, was found to be encoded
257 by an ORF located in the intergenic region (1,200 bp) located between the end of the
258 tyrosyl–tRNA synthetase and the beginning of the pyridoxal–dependent tyrosine
259 decarboxylase genes of *E. faecium* BCS59 and MV5.
260
261 4. Discussion
262
263 Several qualitative and quantitative methods have been described to determine the
264 production of BAs by bacteria (García Moruno et al., 2005; Coton and Coton, 2005;
265 Landete et al., 2007; Costantini et al., 2006). In this work, the ability to produce BAs of
266 74 LAB previously isolated from fish and fish products (Gómez–Sala et al., 2004;
267 Muñoz–Atienza, 2009) has been evaluated by using phenotypic (agar plate assay using
268 the improved decarboxylase differential growth medium and TLC) and genotypic (PCR)
269 methods. None of the evaluated strains, identified as *Pediococcus pentosaceus*,
270 *Lactobacillus sakei*, *Weissella cibaria*, *Leuconostoc mesenteroides*, *Lactococcus lactis*,
271 *Lactobacillus curvatus*, *E. faecium* and *E. faecalis* (Gómez–Sala et al., 2004; Muñoz–
272 Atienza, 2009), showed neither production of histamine and putrescine, nor presence of
273 the genetic determinants encoding the corresponding decarboxylase activities. However,

274 *tyrDC* was present in all the enterococcal strains, and tyramine production was detected
275 by TLC in all of them but in *E. faecium* BCS59 and MV5. Despite the fact that the ability
276 of microorganisms to decarboxylate amino acids is highly variable, depending not only
277 on the species but also on the strain and the environmental conditions (Mazzoli et al.,
278 2009; Fernández et al., 2007; Marcobal et al., 2006b), tyramine production seems to be a
279 widely distributed trait in enterococci (Bonetta et al., 2008; Torriani et al., 2008; Latorre–
280 Moratalla et al., 2010). The lack of detection of tyramine production by *E. faecium* CV1
281 (*tyrDC*$^+$, TDC$^+$ by TLC) in the plate assay might be due to its reduced ability to
282 decarboxylate tyrosine, yielding tyramine concentrations below the detection threshold
283 for this method, estimated around 350 mg/l (Bover–Cid and Holzapfel, 1999). This is in
284 accordance with previous works describing false–negative results from the differential
285 decarboxylase medium (de las Rivas et al., 2008). Taking into account the false–negative
286 (*i.e.*, *E. faecium* CV1) and false–positive (*i.e.*, *E. faecium* BCS59 and MV5) results
287 obtained by the plate assay and PCR, respectively, we favor the use of the specific and
288 sensitive TLC method for reliable determination of BA production. In this context, TLC
289 has a lower detection level (*i.e.*, 10 mg/l for tyramine and putrescine) than the plate assay
290 and is an inexpensive method that does not require costly and sophisticated equipment or
291 specially trained staff (García–Moruno et al., 2005). Nevertheless, according to our
292 results, PCR could also be performed as a fast, preliminary screening tool for the
293 detection of potential BA producers, which should be further confirmed by TLC.
294 Additionally, it is possible to use multiplex PCR assays allowing for the simultaneous
295 detection of several decarboxylase genes in a single reaction (Coton and Coton, 2005; de
296 las Rivas et al., 2005; Marcobal et al., 2005).
297 The amino acid sequence of TDC from *E. faecium* BCS59 and MV5, which did not
298 produce tyramine according to the plate assay and TLC, but gave a positive result in the

299 PCR, were identical to that of the TDC from *E. faecium* 1039, a fecal isolate collected
300 during a community surveillance program, whose genome has been recently sequenced
301 (van Schaik et al., 2010). Although there is no information available about the
302 functionality of the TDC from this strain, the corresponding enzyme from *E. faecium*
303 RM58, which only differs by a single amino acid at position 186 (alanine to threonine
304 substitution), has been shown to be active (Marcobal et al., 2006a). Therefore, TDC from
305 *E. faecium* RM58 was used as a template to study the functional significance of the
306 amino acid substitution found in TDC from *E. faecium* BCS59 and MV5. In this context,
307 it is known that TDC enzymes, which belong to the aspartate aminotransferase
308 superfamily (fold type I) of pyridoxal phosphate–dependent enzymes, are characterized
309 by the presence of a pyridoxal 5´–phosphate binding pocket and a catalytic lysine residue
310 [*Conserved domains* tool, NCBI database (Marchler–Bauer et al., 2009)]. The observation
311 that the TDC variant found in this study is similarly active to that of *E. faecium* RM58
312 could be explained by the fact that the alanine to threonine substitution is present at a
313 position not included in any of these conserved domains.
314 The operon encoding the tyrosine decarboxylation pathway has been described in
315 several LAB and contains, besides *tyrDC*, at least two additional genes: (i) *tyrS*, usually
316 situated upstream *tyrDC* and encoding a tyrosyl–tRNA synthetase; and (ii) *tyrP*, generally
317 located downstream *tyrDC* and encoding a tyrosine permease (Connil et al., 2002; Lucas
318 et al., 2003; Coton et al., 2004; Coton and Coton, 2009). Comparison of the nucleotide
319 sequence upstream *tyrDC* in *E. faecium* BCS59 and MV5 with that of *E. faecium* DO
320 revealed differences in their intergenic *tyrS–tyrDC* regions, which consist of 1,199 and
321 290 bp, respectively. In this respect, a fragment of 143 bp, located between coordinate
322 positions 124 and 266 of the intergenic *tyrS–tyrDC* region of *E. faecium* DO, is missing in
323 *E. faecium* BCS59 and MV5, and has been replaced by a 1,052 bp sequence. However,

the sequence (24 bp) located between the end of this 1,052 bp sequence and the *tyrDC* translation initiation codon is identical in the strains analysed in this study and *E. faecium* DO. Analysis of the 1,199 bp sequence revealed that it contains an *orf* on its complementary strand, which has been termed *int*, encoding a putative protein of 319 amino acid residues identical to enterococcal integrases, as well as the repeated motif CATTTGT located at positions 117–123 (direct repeat left, DRL) and 1,080–1,086 (direct repeat right, DRR). In this respect, the location of DRs at both sides of the 1,052 bp sequence and the presence of an *orf* encoding a putative protein of the integrase family are common features of insertion sequences (ISs). Interestingly, Coton and Coton (2009) described the presence of a sequence of similar length (1,046 bp) downstream *tyrS* in *Lb. brevis* NS77, which is flanked by the repeated motif GTTTGG and encodes a putative protein of 230 amino acids showing high identities with IS30 family transposases. Based on this observation, and on the GC content of the IS, the authors suggested that the tyrosine decarboxylase pathway in *Lb. brevis* is located in a putative genomic island which would only be present in some strains, thus explaining why tyramine production is a strain–related feature in this species. However, this does not seem to be the case for the IS found in our study, since its GC content (39.1%) is similar to that reported for *E. faecium* DO (37.9%), and the predicted 319 amino acid integrase is only weakly similar (17% identity) to the 230 amino acid transposase of *Lb. brevis* NS77. On the other hand, the 1,052 bp sequence described in this study is also present in the *E. faecium* DO genome sequence in a location not related to the *tyrDC* operon (coordinate positions 57,590–58,549 in contig. 654, EfaeDRAFT_2307; available at http://genome.ornl.gov/microbial/efae/)

According to previous works reporting the presence of transcription initiation sites located upstream *tyrDC* in the tyramine operons of *Lb. brevis* (Lucas et al., 2003) and *E.*

faecalis (Connil et al., 2002), computer predictions also suggests the existence of a *tyrDC* promoter in *E. faecium* DO (Fig. 2B). However, this region is not present in *E. faecium* BCS59 and MV5 due to the integration of an IS which does not provide an alternative promoter. This event could explain the lack of TDC activity in these *tyrDC*$^{+}$/TDC^{-} strains, and could also be related to the strain-dependent tyramine production ability observed in other studies (Ladero et al., 2009).

In summary, we have investigated a collection of 74 LAB of aquatic origin for their ability to produce histamine, tyramine and putrescine, and demonstrated that enterococci were the only BA (tyramine) producers. Two out of 33 enterococci did not produce tyramine despite of containing *tyrDC*, which is likely due to the integration of an IS in the promoter region of this gene. The lack of BA production by 43 potential probiotic LAB strains is a desirable trait; however, the stability of the TDC^{-} phenotype in *E. faecium* BCS59 and MV5 remains to be determined. A comprehensive safety evaluation of these LAB strains exerting antimicrobial activity against fish pathogens is currently under progress in order to fully exploit their potential use as probiotics in aquaculture.

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Figure captions

Figure 1. TLC detection of tyramine in cell extracts of *E. coli* DH5α harboring pAM3$_{A-T}$ and expressing the variant *tyrDC*. Lane 1, tyramine standard solution; lane 2, *E. coli* DH5α (pIN-III-A3); lane 3, *E. coli* DH5α (pAM3); and lane 4, *E. coli* DH5α (pAM3$_{A-T}$). The position of tyramine is indicated by an arrow.

Figure 2. (A) Schematic representation of the nucleotide region involved in tyramine production in *E. faecium* DO (5,089 bp) (upper figure) and *E. faecium* BCS59 and MV5 (5,335 bp) (lower figure). The relative positions and sizes of the genes are represented by arrows. The position of the *E. faecium* DO putative *tyrDC* promoter is indicated by a small flag. Directs repeats (DRL and DRR) are represented by black vertical bars. Numbering refers to the intergenic *tyrS–tyrDC* region. (B) Partial nucleotide sequence located upstream *tyrDC* in *E. faecium* DO (upper sequence), and *E. faecium* BCS59 and MV5 (lower sequence). Putative ribosome–binding sites are indicated in boldface. Putative –35 and –10 consensus promoter sequences are underlined, start codons are indicated by boldface italics and *tyrS* stop codon is marked with an asterisk. The DRR is enclosed in a box. The 143 bp intergenic *tyrS–tyrDC* region present in *E. faecium* DO but absent in *E. faecium* BCS59 and MV5, and a fragment of 316 nucleotides of the 1,199 bp IS of *E. faecium* BCS59 and MV5 are shadowed in light and dark gray, respectively.

Figure 1 in PPT format

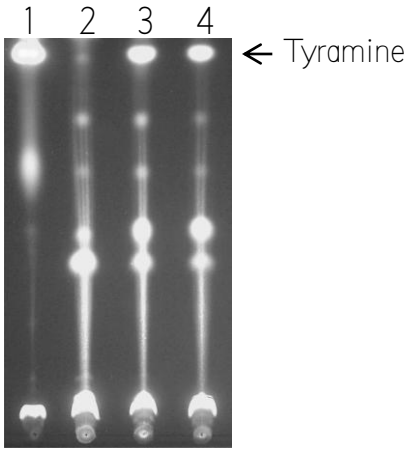
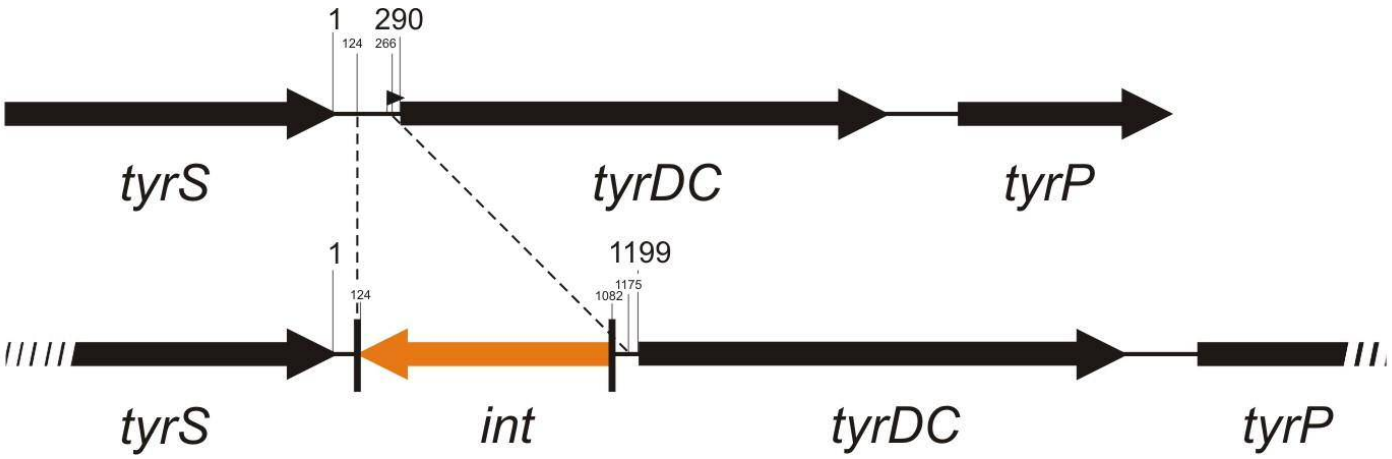


Figure 1

Figure 1 in PPT format

A



B

***E. faecium* DO**

```
1208  TTATTCGAAAAGGTAAGAAAAATTACTTTTTGGCCAAAGTAATTGATTAG
      (tyrS)                                          *
1258  AATTTAGTAAGTTTTTACCTTGAAAAAGGGAAATACTATTACAACATTCC

1308  AATTTTGAAACCCATCCTAAAATAGAACGCTGGTTGTCGTTAATACAATC

1358  TTTGCGAGGGAGTTTACATTTGTAAATTCCCTCGTTTTTTTTAATTATTT

1408  CAAAAGTGTGAACAATTCGGATAATTGATACATATTAAAATATGATTAAA

1458  AATCAGGCCAAAACTTAGTTTACAGAATAGAAGAACAGGTGTAATGTAGG
                          -35                   -10
1508  GCTCGACAAAATTTAAATATTTTTTTAGGAGGGTTTCACTATGAGTGAAT
                                 RBS          tyrDC →
```

***E. faecium* BCS59 and MV5**

```
2012  CCTCAGGTAAAACAAGCGGGCGTCTACCACAGTTTGATTTGTTTTTCTTA

2062  TACTGTTGATAGTACTCTAAGGCTGATTTTCCTTGCTTGAAGAACAGGTA

2112  TACTTTATGGATCGTTTGTCTTGAACGATTCAAGCAATGCGCAGTTTTCG

2162  CAACAGATTGATTTATTTTGAAATAAGACTCTATCATTACAAGTTCATCC

2212  GTTGTAAGATGGGTATAGGTCATTTGTACTCACTCCTTATAATTCTTTGG
                      ← int          RBS
2262  ACGGAACTATTTTGAGTGTAGCACAAATGACTTTTTTAGTTGTCTAGCTT

2312  AATTTTACAATCGGCGATATTTTTTTAGGAGGGTTTCACTATGAGTGAAT
                                 RBS          tyrDC →
```

Figure 2

Table

Table 1. Evaluation of BAs production and of the presence of histidine, tyrosine and ornithine decarboxylase genes in LAB of aquatic origin

Bacterial strain	Origin	Histamine			Tyramine			Putrescine		
		Plate assay	TLC	PCR	Plate assay	TLC	PCR	Plate assay	TLC	PCR
Enterococcus faecalis GM22	*Lepidorhombus boscii*	-	-	-	+	+	+	-	-	-
E. faecalis GM26	*L. boscii*	-	-	-	+	+	+	-	-	-
E. faecalis GM33	*L. boscii*	-	-	-	+	+	+	-	-	-
Enterococcus faecium BCS59	*Gadus morhua*	-	-	-	-	-	+	-	-	-
E. faecium BCS971	*G. morhua*	-	-	-	+	+	+	-	-	-
E. faecium BCS972	*G. morhua*	-	-	-	+	+	+	-	-	-
E. faecium BNM58	*Thunnus alalunga*	-	-	-	+	+	+	-	-	-
E. faecium CV1	*Loligo vulgaris*	-	-	-	-	+	+	-	-	-
E. faecium CV2	*L. vulgaris*	-	-	-	+	+	+	-	-	-
E. faecium CGM171	*Nephrops norvegicus*	-	-	-	+	+	+	-	-	-
E. faecium CGM172	*N. norvegicus*	-	-	-	+	+	+	-	-	-
E. faecium GM23	*L. boscii*	-	-	-	+	+	+	-	-	-
E. faecium GM29	*L. boscii*	-	-	-	+	+	+	-	-	-
E. faecium GM351	*L. boscii*	-	-	-	+	+	+	-	-	-
E. faecium GM352	*L. boscii*	-	-	-	+	+	+	-	-	-
E. faecium LPP29	*Dicentrarchus labrax*	-	-	-	+	+	+	-	-	-
E. faecium MV5	*Molva molva*	-	-	-	-	-	+	-	-	-
E. faecium NV50	*Necora puber*	-	-	-	+	+	+	-	-	-
E. faecium NV51	*N. puber*	-	-	-	+	+	+	-	-	-

Table 1 (cont.)

E. faecium NV52	*N. puber*	-	-	-	+	+	+	-	-	-
E. faecium NV54	*N. puber*	-	-	-	+	+	+	-	-	-
E. faecium NV56	*N. puber*	-	-	-	+	+	+	-	-	-
E. faecium P623	*Octopus vulgaris*	-	-	-	+	+	+	-	-	-
E. faecium SMA101	*Salmo salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA102	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA310	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA320	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA361	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA362	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA7	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMA8	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium SMF8	*S. salar*	-	-	-	+	+	+	-	-	-
E. faecium TPM76	*Oncorhynchus mykiss*	-	-	-	+	+	+	-	-	-
E. faecium TPP2	*O. mykiss*	-	-	-	+	+	+	-	-	-
Lactococcus lactis cremoris SMF110	*S. salar*	-	-	-	-	-	-	-	-	-
L. lactis cremoris SMF161	*S. salar*	-	-	-	-	-	-	-	-	-
L. lactis cremoris SMF166	*S. salar*	-	-	-	-	-	-	-	-	-
Lactobacillus curvatus curvatus BCS35	*G. morhua*	-	-	-	-	-	-	-	-	-
Lactobacillus. sakei carnosus B43	*Cerastoderma edule*	-	-	-	-	-	-	-	-	-
Lb. sakei carnosus SMA17	*S. salar*	-	-	-	-	-	-	-	-	-
Lb. sakei carnosus SMM73	*S. salar*	-	-	-	-	-	-	-	-	-
Lb. sakei sakei B11	*C. edule*	-	-	-	-	-	-	-	-	-
Lb. sakei sakei SMA14	*S. solar*	-	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides cremoris BCS251	*G. morhua*	-	-	-	-	-	-	-	-	-

Table 1 (cont.)

Lc. mesenteroides cremoris BCS252	*G. morhua*	-	-	-	-	-	-	-	-	-
Lc. mesenteroides cremoris SMM69	*S. salar*	-	-	-	-	-	-	-	-	-
Pediococcus pentosaceus B5	*C. edule*	-	-	-	-	-	-	-	-	-
P. pentosaceus B260	*C. edule*	-	-	-	-	-	-	-	-	-
P. pentosaceus BCS46	*G. morhua*	-	-	-	-	-	-	-	-	-
P. pentosaceus LPM78	*D. labrax*	-	-	-	-	-	-	-	-	-
P. pentosaceus LPM83	*D. labrax*	-	-	-	-	-	-	-	-	-
P. pentosaceus LPP32	*D. labrax*	-	-	-	-	-	-	-	-	-
P. pentosaceus LPV46	*D. labrax*	-	-	-	-	-	-	-	-	-
P. pentosaceus LPV57	*D. labrax*	-	-	-	-	-	-	-	-	-
P. pentosaceus P621	*O. vulgaris*	-	-	-	-	-	-	-	-	-
P. pentosaceus SMF120	*S. salar*	-	-	-	-	-	-	-	-	-
P. pentosaceus SMF130	*S. salar*	-	-	-	-	-	-	-	-	-
P. pentosaceus TPP3	*O. mykiss*	-	-	-	-	-	-	-	-	-
Weissella cibaria B41	*C. edule*	-	-	-	-	-	-	-	-	-
W. cibaria B4620	*C. edule*	-	-	-	-	-	-	-	-	-
W. cibaria BCS50	*G. morhua*	-	-	-	-	-	-	-	-	-
W. cibaria BNM69	*T. alalunga*	-	-	-	-	-	-	-	-	-
W. cibaria P38	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P50	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P61	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P622	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P63	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P64	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P69	*O. vulgaris*	-	-	-	-	-	-	-	-	-

Table 1 (cont.)

W. cibaria P71	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria P73	*O. vulgaris*	-	-	-	-	-	-	-	-	-
W. cibaria SDM381	*Sardina pilchardus*	-	-	-	-	-	-	-	-	-
W. cibaria SDM389	*S. pilchardus*	-	-	-	-	-	-	-	-	-
W. cibaria SMA25	*S. salar*	-	-	-	-	-	-	-	-	-