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Tyramine and Phenylethylamine Biosynthesis by Food Bacteria

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Abstract

*Tyramine poisoning is caused by the ingestion of food containing high levels of tyramine, a biogenic amine. Any food containing free tyrosine are subject to tyramine formation if poor sanitation and low quality foods are used or if the food is subject to temperature abuse or extended storage time. Tyramine is generated by decarboxylation of the tyrosine trough tyrosine decarboxylase (TDC) enzymes derived from the bacteria present in the food. Bacterial TDC have been only unequivocally identified and characterized in Gram-positive bacteria, especially in lactic acid bacteria. Pyridoxal phosphate (PLP)-dependent TDC encoding genes (*tyrDC*) appeared flanking by a similar genetic organization in several especies of lactic acid bacteria, suggesting a common origin by a single mobile genetic element. Bacterial TDC are also able to decarboxylate phenylalanine to produce phenylethylamine (PEA), another biogenic amine. The molecular knowledge of the genes involved in tyramine production has lead to the development of molecular methods for the detection of bacteria able to produce tyramine and PEA. These rapid and simple methods could be used for the analysis of the ability to form tyramine by bacteria in order to evaluate the potential risk of tyramine biosynthesis in food products.*

Keywords

tyramine, phenylethylamine, biogenic amine, tyrosine decarboxylase, detection methods

INTRODUCTION

Since the identification of the “cheese reaction” hypertensive crisis induced by dietary tyramine among individuals on monoamine oxidase inhibitor (MAOI) drugs (Davies et al., 1963; Blackwell et al., 1963) numerous articles have addressed the tyramine content on foods. The action of the vasoactive amines, largely tyramine, is the underlying cause of the hypertensive crisis that may occur in individuals on MAOI drugs. The physiological effects of tyramine include: peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose, and release of norepinephrine (Shalaby, 1966). While tyramine is normaly present in small amounts, the healthy gut readily detoxifies it through the action of monoamine oxidase (MAO). Two primary isoforms of monoamine oxidase selectively deaminate neurotransmitters, and one isoform will predominate in various body tissues. MAO-A deaminates serotonin in the central nervous system and dietary monoamines in the gastrointestinal system. MAO-B is found predominatly in liver and muscle and deaminates dopamine and phenyletylamine (PEA). Physiologic active amines may also be deaminated by diamine oxidase (DAO) in the gut that provide protection from small amounts normally presented by foods (Ten Brink et al., 1990; McCabe-Sellers et al., 2006). MAOI drugs are used to inhibit the actions of monoamine oxidase, especially in the central nervous system. The first generation of MAOI drugs are nonspecific, inhibit both isoforms of MAO and the potential for a hypertensive crisis increases.

A definition of a clinically significant tyramine level relates to the severity of the blood pressure rise. The presence of 6 mg in one or two usual servings is thought to be sufficient to cause a mild adverse event while 10-25 mg will produce a severe adverse event in those using MAOI drugs (Da Prada et al., 1988). For unmedicated adults, 200-800

mg of dietary tyramine is needed to induce a mild rise (30 mm Hg) in blood pressure. A limit of 200-800 mg in one or two usual serving has been proposed for tyramine in foods (Da Prada et al., 1988). Tyramine is a vasopressor amine responsible for some food-induced migraines and hypertensive crisis in sensitive individuals (McCabe-Sellers et al., 2006).

The biogenic amine β-phenylethylamine (PEA) is a physiological constituent in the mammalian brain acting as mood elevator in the central nervous system (Szabo et al., 2001). PEA may function primarily as an "endogenous amphetamine", being an enhancer substance that facilitates the release of the neurotransmitters catecholamine and serotonin, which has been linked to the regulation of mood, physical energy and attention (Shimazu and Miklya, 2004). MAO-B selectively metabolises PEA to phenylacetic acid. However, in individuals with reduced MAO detoxifying activity, PEA ingestion has sometimes been associated with symptoms such as headache, dizziness and discomfort (Lüthy and Schlatter, 1983; Premont et al., 2001; Millichap and Yee, 2003). Treshold values of 30 mg/kg for PEA have been reported (ten Brink et al., 1990)

Although the toxicity of individual biogenic amines in general is beyond all doubt, it is very difficult to determine the exact toxicity threshold of these compounds. The toxic dose is strongly dependent on the efficiency of detoxification, which may vary considerably between different individuals. The effects of tyramine may be potentiated by other consumed compounds, including other biogenic amines. It is quite conceivable that simultaneously consumption of a number of food products (such as wine, cheese, sauerkraut and fermented sausage) results in biogenic amine poisoning whereas consumption of each of these products alone does not present any problem.There is renewed interest in the study of the ocurrence of biogenic amines in fermented foods

because of recent food legislation, consumer demand for healthier foods, and increasing attention to sensitive groups.

TYRAMINE AND TYRAMINE-PRODUCER BACTERIA IN FOODS

As for histamine, prerequisites for the formation of tyramine in foods are the availability of free tyrosine, the presence of tyrosine decarboxylase (TDC)-positive microorganisms, conditions that allow bacterial growth and conditions that favour decarboxylase activity (Halasz et al., 1994; Landete et al., 2008). Free amino acids occurs as such in foods, but may also be liberated from proteins as a result of proteolytic activity. Decarboxylase-positive bacteria may constitute part of the associated population of the food or may be introduced by contamination before, during or after processing of the food. In the case of fermented foods and beverages, the applied starter cultures may also affect the production of tyramine (Straub et al., 1995).

Tyramine could be expected in virtually all foods that contain proteins or free tyrosine and that are subject to conditions enabling microbial activity. Most foods can be safely consumed if bought fresh, cooked fresh, and consumed fresh in modest quantities. In cheeses, the food industry has striven to develop new processes using different microbial strains to reduce the development of tyramine in cured or aged cheeses. Numerous studies described the formation of tyramine by different microorganism during the elaboration and ripening of several cheeses (Joosten and Northolt, 1989; Stratton et al., 1991; Roig-Sagués et al., 2002). The milk quality and the length of the ripening or storage appear to be dominant factors in the production of tyramine in cheeses (Novella-Rodríguez et al., 2002).

Starting with high quality fresh meat and using good manufacturing practices greatly reduces the risk of tyramine formation in processed meat products (Rice et al., 1975; Masson et al., 1996; Suzzi and Gardini, 2003; Ruíz-Capillas et al., 2004). Most case reports of tyramine in fresh meat have been from meats stored at or beyond the end of the recommendation storage time (Boulton et al., 1970). Vegetables processed in brine from high-quality raw materials do not develop high levels of tyramine unless contaminated or abused by temperature and storage time (Moret et al., 2005). Alcoholic beverages, particularly wines, do not contain high levels of tyramine when consumed in modest quantities (Zee et al., 1983; Landete et al., 2005).

In tyramine-containing foods the majority of the tyramine is generated by decarboxylation of the amino acid tyramine through tyrosine decarboxylase (TDC) specific enzymes derived from the bacteria present in food. Tyramine often is the main amine found in fermented products, especially cheeses and fermented sausages. This amine is produced through tyrosine decarboxylation by a variety of lactic acid bacteria (including lactobacilli, enterococci, and carnobacteria), all of which are actively present during the manufacture of most fermented food products. The ability of bacteria to decarboxylate tyramine is highly variable. It depends not only on the species, but also on the strain and the environmental conditions (Gardini et al., 2001, 2008; Marcobal et al., 2006c; Coton and Coton, 2008).

In relation to PEA, its origin in the human diet is diverse, and the contribution of PEA of microbial origin is unknown. In foods, PEA levels of 100 mg/kg in chocolate and cheese have been reported (Halász et al., 1994). PEA production has been described during food fermentations (Santos et al., 2003) and it was suggested to be formed as a result of the activity of tyrosine decarboxylating bacteria towards this structurally related amino acid, L-phenylalanine (Millichap and Yee, 2003).

This review is focused on the tyramine-producer bacteria whose TDC proteins or whose corresponding encoding genes have been unequivocally reported so far (Table 1).

TYRAMINE PRODUCTION BY GRAM-POSITIVE BACTERIA

Tyrosine decarboxylase (TDC, EC 4.1.1.25) converts the amino acid tyrosine to the biogenic amine tyramine (Figure 1). Bacterial TDC have been only thoroughly studied and characterized in Gram-positive bacteria and, especially, in lactic acid bacteria implicated in food fermentation or spoilage. Although pyridoxal phosphate (PLP)-dependent TDC are encountered in Gram-positive bacteria, putative TDC from Gram-negative bacteria remain uncharacterized (Table 1).

Pyridoxal-dependent tyrosine decarboxylase

As early as 1940, Gale described the production of tyramine from tyrosine by washed suspensions of *Enterococcus faecalis* (formerly *Streptococcus faecalis*) and describes the properties of the tyrosine decarboxylase (TDC) enzyme responsible of its biosynthesis (Gale, 1940). He found that i) the decarboxylase involved is optimally active at pH 5.0; ii) cultures grown at 27 ºC have the same activity as those grown at 37 ºC; iii) the activity of the washed suspension varies with the age of the culture; iv) the activity of the washed suspension depends upon the pH of the growth medium; v) the decarboxylation is quantitative; vi) of 7 strains of *E. faecalis* investigated, six decarboxylated tyrosine; and, vii) in no case was any other amino-acid decarboxylated to form an amine under the

experimental conditions described. Therefore, Gale concluded that the tyrosine decarboxylase is thus strictly specific (Gale, 1940).

Later, Epps (1944) described the isolation, partial purification and some properties of the TDC from crude bacterial *E. faecalis* extracts. In 1970, Chabner and Livingston purified the enzyme 12-times to a specific activity of 12.6 U/mg protein by use of ammonium sulphate fractionation and preparative Sephadex G-200 chromatography. Gel filtration of this preparation resulted in a molecular weight above 200 kDa. No indication of the purity of the final preparation was given by the authors. In order to construct a suitable scheme for the purification of the enzyme and to obtain more information with respect to its electrophoretic and chromatographic behaviour and general, chemical properties, Allenmark and Servenius (1978) employed preparative isoelectric focusing, molecular sieving on agarose gels, and hydrophobic interaction chromatography on octyl-, phenyl-, and butyl-substituted Sepharose. Isoelectric focusing demonstrated two separate fractions possessing enzyme activity that had pI values of 4.5 and 3.2. In the chromatographic methods, however, the activity was obtained in a single peak. It was found that the hydrophobic interaction chromatography on phenyl-Sepharose was particularly suitable for purification purposes. The enzyme was very firmly bound to octyl-Sepharose CL-4B but retained most of its activity even in the bound state. They also demonstrated that the TDC obtained from freeze-dried cells of specially grown cultures of *Enterococcus faecalis* and used in all purification experiments contained very little pyridoxal phosphate. In order to solve some problems concerning the purification and characterization of the *E. faecalis* TDC, fast polymer liquid chromatography (FPLC) methods were used (Borresen et al., 1989). TDC was isolated from *E. faecalis* by FPLC anion exchange chromatography, yielding a 11-times purification, 72% recovery and 23.2 U/mg protein. FPLC on Phenyl-

Superose resulted in purification to 115 U/mg protein. By FPLC chromatofocusing, TDC eluted at pH 4.3; isolectric focusing gave two bands (pI 4.4 and 4.5). With pyridoxal 5´-phosphate removed by ultrafiltration, only one band (pI 4.4) appeared, and SDS polyacrylamide electrophoresis confirmed the purity. FPLC gel filtration resulted in 143 kDa of molecular weight; in SDS electrophoresis, TDC had the monomer weight 75 kDa, showing a dimmer structure for the enzyme.

Previous work by Joosten et al. (1995) resulted in the isolation of *E. faecalis* tyrosine decarboxylaseless mutants after the treatment with a mutagenic agent (ethyl methanosulfonate), as the screening of approximately 2,000 colonies dit not revealed the presence of any spontaneous mutant. However, in order to identify the gene encoding TDC in *E. faecalis*, Connil et al. (2002a) screened a library of *E. faecalis* insertional mutants that allow the isolation of a mutant affected in tyramine production. The growth of this mutant was similar to that of the wild-type *E. faecalis* strain in Maijala broth (Maijala, 1993), whereas HPLC analyses showed that tyramine production was completely abolished. Genetic characterization of the integration locus revealed a unique integration locus corresponding to the central region of a 1,860-bp ORF. This ORF encodes a decarboxylase (TDC) which had homology with amino acid decarboxylases from prokaryotes and tyrosine decarboxylase from parsley (*Pisum crispum*). Furthermore, the TDC sequence of *E. faecalis* contained the consensus pattern observed for pyridoxal phosphate-dependent decarboxylases (also shown as group II decarboxylases, S-[LIVMFYW]-X(5)-K-[LIVMFYWGH]-[LIVMFYWG]-X(3)-[LIVMFYW]-X-[CA]-X(2)-[LIVMFYWQ]-X(2)-[RK] (Figure 2), where lysine (K) is the attachment site for the cofactor pyridoxal phosphate (Sandmeier, 1994). However, in the *E. faecalis* TDC, the ultimate amino acid of the consensus pattern would not be arginine (R) or lysine but glutamine (Q) (Figure 2).

Furthermore, *E. faecalis* TDC has 20% identity with the TDC II sequence from parsley (*P. crispum*), including the HVDAAY motif (Figure 2), particularly conserved in pyridoxal phosphate-dependent decarboxylases. The description of the *E. faecalis* TDC constituted the first description of a TDC protein in prokaryotes.

According to previous editions of the Bergey´s Manual of Systematic Bacteriology, the tyrosine decarboxylation test allows to differentiate *E. faecalis* from *E. faecium* (Mundt et al., 1984). However, by searching in the databases from proteins similar to the *E. faecalis* TDC, a protein from the uncompleted *E. faecium* genome was found. This protein codes for a putative TDC in *E. faecium*. Marcobal et al. (2004) analysed the production of tyramine by some *E. faecalis* strains, and described that all the *E. faecium* strains analysed were able to produce tyramine from tyrosine. In addition, the strains analyzed possess a gene coding for a putative TDC involved in tyramine production, as demonstrated later by the same authors (Marcobal et al., 2006b). The *tyrDC* gene from *E. faecium* was expressed in *E. coli*, resulting in TDC activity. This activity was lost when a truncated protein lacking 84 amino acids at its C-terminus was expressed in *E. coli*. In spite that the number of *E. faecium* strains analysed was low to conclude that tyrosine decarboxylase is a general feature of *E. faecium* species but the number was enough to demonstrate the uselessness of the tyrosine-decarboxylase test to distinguish *E. faecalis* from *E. faecium* (Marcobal et al., 2004). Recently, the use of TDC from *E. faecalis* and *E. faecium* has been described for the synthesis of aromatic amino alcohols (Steinreiber et al., 2007).

Carnobacterium strains are psychrotroph and halotolerant bacteria able to grow under harsh growing conditions (Mauguin and Novel, 1991). Most *Carnobacterium* strains could produce high levels of tyramine (Bover-Cid and Holzapfel, 1999). The sequence of *Carnobacterium divergens* TDC was determined (Coton et al., 2004). It encoded for a 624

amino acid protein with a theoretical pI of 5.10 and a molecular weight of 70.1 kDa. This TDC protein showed the highest similarity to *E. faecium* TDC (94% identity) and *E. faecalis* (85% identity) and exhibited conserved domains characteristics of the group II (PLP-dependent) decarboxylase family (Figure 2). *C. divergens* TDC also contained the consensus pattern observed for pyridoxal phosphate-dependent decarboxylases, including the highly conserved HVDAAY motif and the PLP attachment site with lysine being the ultimate amino acid of the consensus pattern (Figure 2). The PLP attachment site consensus pattern for both *C. divergens* and *E. faecium* were identical whereas those of *E. faecalis* and *L. brevis* differ by two amino acids including the ultimate amino acid of the consensus pattern, glutamine (Coton et al., 2004) (Figure 2).

Several *Lactobacillus brevis* tyramine-producing strains were isolated from wines (Moreno-Arribas et al., 2000). TDC from *L. brevis* was purified by a rapid procedure involving anion exchange chromatography, ultrafiltration and hydrophobic interaction chromatography (Moreno-Arribas and Lonvaud-Funel, 2001). The molecular mass of the native form of *L. brevis* TDC, as estimated by gel filtration chromatography, was approximately 140 kDa, consisting apparently of two subunits of approximately 70 kDa according to SDS-PAGE. Kinetic studies on pure enzyme confirmed 5.0 as the pH optimum for activity. Enzyme activity was dependent on exogenously supplied pyridoxal phosphate. Later, purification and microsequencing of the TDC of *L. brevis* allowed determining a partial sequence of the *tyrDC* gene encoding 264 amino acid of the enzyme (Lucas and Lonvaud-Funel, 2002). A total of 7.9-kb DNA sequence of the *L. brevis tyrDC* locus was determined by Lucas et al. (2003). The *tyrDC* gene encoded a protein of 626 amino acids with a calculated molecular mass of 70.5 kDa, in agreement with previous experimental measurements. Recently, Coton and Coton (2008) reported the sequence of

12.1-kb on the TDC pathway encoding operon region in *L. brevis* NS77 as compared to the 11.1-kb equivalent region in the complete genome sequenced *Lb. brevis* ATCC 367.

In addition, strains belonging to other lactobacilli species have been described to produce tyramine (Straub et al., 1994). In a study carried out to evaluate the safety properties of lactic acid bacteria isolated from slightly fermented sausages, the authors found that tyramine were produced by 14.4% of the isolates, all belonging to the species *L. curvatus* (Aymerich et al., 2006). Partial *tyrDC* gene sequencing of two different isolates of *L. curvatus* was achieved. Comparison of the sequenced fragments between both *L. curvatus* strains gave a 99% sequence similarity. Highest similary (74%) was obtained with the *tyrDC* gene sequences from *E. faecalis* and *L. brevis* (Aymerich et al, 2006; Torriani et al., 2008). More recently, in order to know the ability of wine *L. plantarum* strains to form tyramine, Arena et al. (2007), found that only one strain isolated from red wine was able to produce tyramine. Furthermore, the gene encoding for the TDC was partially cloned form the tyramine-producer strain. The similarity observed between the amino acid sequence of the amplified PCR product and the TDC proteins previously identified in *L. brevis* and *L. curvatus* confirmed that the fragment was an internal fragment of the *tyrDC* gene.

Torriani et al. (2008), by using degenerate PCR primers based on *tyrDC* gene sequences, determined the 1.0 kb sequence of the *tyrDC* gene from several tyramine-producer strains. Among them, this gene was detected in a *L. fermentum* strains, and the nucleotide sequence of its amplification product was 100% identical to that of *L. brevis* IOEB 9809. In addition a *tyrDC* DNA fragment was amplified from a tyramine-producer *Staphylococcus epidermidis* strain. This sequence revealed a high level of similarity (99%) with *tyrDC* sequences from *E. faecalis* (Torriani et al., 2008).

Genetic organization of the tyramine biosynthetic locus

The *E. faecalis* region was the first TDC locus described in prokaryotes (Connil et al., 2002a) (Figure 3). In *E. faecalis* upstream of the *tyrDC* gene, it could be found an ORF (*tyrS*) corresponding to a 47.3 kDa protein having high similarity with tyrosyl tRNA synthetases (54% identity with the tyrosyl tRNA synthetase of *Bacillus subtilis*) (Figure 3).Tyrosyl tRNA synthetases are aminoacyl-tRNA synthetases of class I characterized by the HIGH and KMSKS amino acid motifs involved in an ATP-dependent activation of tyrosine by forming an enzyme-bound tyrosyl-adenylate intermediate (Xin et al., 2000). The HIGH motif is perfectly conserved in *E. faecalis* tyrosyl tRNA synthetase, while the KMSKS motif is represented by the KFGKT sequence. The ORF located downstream of *tyrDC, tyrP*, encoded a 51.6 kDa protein with 12 hydrophobic fragments. Homologies were found between the amino acid sequence deduced from this ORF and proteins encoding amino acid antiporters (28% identity with an amino acid antiporter of *Chlamydia pneumoniae*). Reverse transcription-PCR analyses demonstrated that the gene (*tyrDC*) encoding the putative tyrosine decarboxylase of *E. faecalis* is cotranscribed with the downstream gene encoding a putative tyrosine-tyramine antiporter and with the upstream tyrosyl-tRNA synthesase gene (Connil et al., 2002a) (Figure 3).

In *Enterococcus durans* IPLA 655 (formerly classified as *Lactococcus lactis* IPLA 655) the genes encoding a putative tyrosyl tRNA synthetase (*tyrS*), a tyrosine decarboxylase (*tyrDC*), and a tyrosine-tyramine antiporter (*tyrP*) were also sequenced. The disruption of the *tyrDC* gene yielded a strain unable to produce tyramine (Fernández et al., 2004) (Figure 3).

The region encoding a putative TDC in *Carnobacterium divergens* and *Carnobacterium piscicola* were also identified (Coton et al., 2004) (Figure 3). A putative tyrosyl-tRNA synthetase gene (*tyrS*) was identified from a partial gene sequence upstream of the *tyrDC* gene and another partial ORF potentially coding for an amino acid transporter was identified downstream (*tyrP*). Comparison to the TDC operon of *E. faecalis* showed that these three genes are probably part of an operon involved in tyramine production in carnobacteria (Figure 3).

As in enterococci and in *C. divergens*, analyses of the *L. brevis tyrDC* genetic organization revealed the presence of four complete ORF transcribed in the same direction (Figure 3) (Lucas et al., 2003). The first ORF showed strong similarities with genes encoding tyrosyl-tRNA synthetases (*tyrS*). The next ORF, the *tyrDC*, encoded a protein with a 70.5 kDa similar to amino acid decarboxylases. Downstream of *tyrDC* were found two ORF related to genes of amino acid permeases (*tyrP*) and Na+/H+ antiporters (*nhaC-2*). Among these genes, it could be found short intergenic regions, suggesting that these four genes could be cotranscribed. However, putative transcription termination hairpins were identified in the *tyrS-tyrDC* and *tyrP-nhaC-2* regions. The transcription start point was located 34-35 nucleotides upstream of the start codon, suggesting that *tyrS*, preceding the *tyrDC* gene, was transcribed separately. By reverse transcription-polymerase chain reaction it was showed that the genes *tyrS*, *tyrDC*, *tyrP* and *nhaC-2* are cotranscribed (Lucas et al., 2003). The authors also showed that *tyrDC* transcripts could be initiated at two different promoters: one upstream of *tyrDC* and the other upstream of *tyrS*. Their results suggest that the TDC operon would be made up of the four genes and that transcription regulation could occur and exclude *tyrS* and /or *nhaC-2* from a part of the transcripts. Comparison to the *L. brevis* IOEB 9800 TDC operon sequence to databanks

allowed the identification of the operon enconding the TDC pathway in the recently available *L. brevis* ATCC 367 complete genome sequence (Figure 3) (Coton and Coton, 2008). The genetic organisation of the TDC operon flanking regions is as follows: upstream of the TDC operon, a gene coding for a putative permease of the drug/metabolite transporter superfamily (*dtp*) is followed by a gene encoding for a putative acetyl-CoA acetyltransferase (*act*) while downstream of the TDC operon, a gene coding for a putrescine transcarbamylase gene (*aguB*) followed by an agmatine/putrescine exchanger gene (*aguD*) are found (Lucas et al., 2007), therefore agmatine deiminase pathways genes in *L. brevis* are linked to the tyrosine decarboxylation operon. For *L. brevis* NS77, analysis of the TDC pathway encoding operon region, spanning from the end of the *dtp* gene to the beginning of the *aguD* gene, as compared to the corresponding region in *L. brevis* ATCC 367, showed the presence of an extra ORF between the *act* and *tyrS* genes. This ORF (*tra*) encodes for a putative protein of 230 residues similar to transposases.

Lucas et al. (2003) found the same TDC gene clusters in the available genome sequences of *E. faecalis* and *E. faecium*. They suggest that *tyrDC* and *tyrP* are elements of a single operon because they were cotranscribed in both *L. brevis* IOEB 9808 and *E. faecalis*, and genes of bacterial amino acid decarboxylases are generally associated to genes of corresponding amino acid permeases. In agreement, phylogenetic analyses of *tyrS* and *nhaC*-2 encoded proteins supported the hypothesis of a co-evolution of *tyrS*, *tyrDC*, *tyrP* and *nhaC-2*. These analyses also suggested that the four genes were transferred in bacteria as a single mobile genetic element.

It is generally established the existence of a strain-dependent ability to form biogenic amines. Concerning the ability of *L. brevis* to form tyramine, as mentioned above, Lucas et al. (2003) showed for the strain IOEB 9809, that this trait was encoded at the

chromosomal level. Moreover, Lucas et al. (2007) also indicated that the presence of the *tyrDC* gene was a strain-dependent trait in *L. brevis* strains. In order to evaluate if the observed differences in the tyramine-producing ability were strain-dependent or if they could be correlated to the existence of a new species or subspecies within the *Lb. brevis* species, Coton and Coton (2008) confirmed that all the strains, tyramine-producers and non-producers, belonged to the *L. brevis* species. By using primers designed on the sequence of the *tyrDC* operon, Coton and Coton (2008) demonstrated that a large portion of the *tyrDC* operon was lacking on the non-tyramine producer *L. brevis* strains, as *L. brevis* NS25 (Figure 3). This strain showed an intergenic region of only 413 bp between the *atc* and *aguB* genes. The presence of the TDC region (*tyrS*, *tyrDC*, *tyrP*, and *nhaC-2* genes) in *L. brevis* ATCC 367 and NS77 and its absence in *L. brevis* NS25 indicates either that the presence of this region results from a horizontal transfer event, either that its absence corresponds to the complete loss of the region in *L. brevis* NS25. The fact that region encodes a complete metabolic pathway, the presence of a mobility gene (*tra*) in *L. brevis* NS77 strain, as well as an atypical GC content, are features corresponding to a genomic island definition. The involvement of a genomic island as the origin of an biogenic amine biosynthetic locus was firstly reported by Marcobal et al. (2006d) in a putrescine-producer *Oenococcus oeni* strain.

Regulation of tyramine production in Gram-positive bacteria

Factors affecting tyramine production in various lactic acid bacteria producer strains have been studied. Tyramine production by *Carnobacterium divergens* was tested in

relation to different conditions of pH, temperature, glucose, oxygen availability, potassium nitrate and chloride content, using an experimental design (Masson et al., 1997). The results obtained showed that maximal tyramine production ocurred during the stationary phase in acidic conditions obtained by low initial pH (<5) or addition of glucose (0.6%) to the medium. Tyramine production was slower at 5 ºC than at 23 ºC, was inhibited by 10% sodium chloride, however, was not affected by the presence of potassium nitrate or oxygen availability. Later, several studies were done to determine if *C. divergens* strains producing tyramine in laboratoty medium were able to produce this amine in meat (Masson et al., 1999) or in a cold-smoked salmon extract (Connil et al., 2002b). In the fisrt assay, the strain was inoculated into a sterile meat-fat mixture of initial pH 5.3 or 4.9 and incubated at 15 ºC or 25 ºC. Maximum tyramine and PEA production was observed in the sample of initial pH 5.3 incubated at 25 ºC. The authors concluded that the amount of tyramine produced correlated with the growth of the strain, and the amount of lactate and acetate produced (Masson et al., 1999). In a sterile cold-smoked salmon extract, a complete factorial design 2^3 was used to study *C. divergens* tyramine production at varying temperatures, NaCl levels and glucose concentrations (Connil et al., 2002b). The results showed that temperature, NaCl, and glucose variations did not greatly affect tyramine production by *C. divergens* under the experimental conditions used. The authors reported that maximal tyramine production occurred during the stationary growth phase in acidic conditions obtained by low initial pH (≤5) or by the addition of glucose (0.6%) to the medium.

In order to know if two different tyramine-producer lactic acid bacteria species showed a similar behaviour, a multifactorial design was used for studying factors influencing growth and tyramine production in *L. brevis* CECT 4669 and *E. faecium* RM58 (Marcobal et al., 2006c). The effects of five physicochemical factors (incubation

temperature and time, environmental pH, added tyrosine concentration, and pyridoxal phosphate (PLP) supplementation) on cell growth and tyramine production were analyzed under aerobic and anaerobic conditions. Incubation time was found to be the most important variable influencing growth in *L. brevis*, while pH showed the highest contribution in *E. faecium*. The production of tyramine was dependent on the added tyrosine concentration and incubation time. In both strains, the proposed model predicted that the anaerobic condition at acidic pH (4.4) in the presence of a high tyrosine concentration favors tyramine production. The model that predicted optimum conditions for tyramine production confirms earlier results showing that amino acid decarboxylases are markedly induced by growth under anaerobic conditions at low pH in rich medium containing excess amino acids (Gale, 1946).

The tyramine-producer *E. durans* IPLA 655 strain was grown in a bioreactor under different conditions to determine the influence of carbon source, tyrosine and tyramine concentrations, and pH on tyramine production (Fernández et al., 2007a). The carbon source appeared to have no significant effect on the production of tyramine. In contrast, tyrosine was necessary for tyramine production, while the presence of tyramine itself in the growth medium inhibited such production. pH showed by far the greatest influence on tyramine synthesis; tyramine was produced in the greatest quantities at pH 5.0, although this was accompanied by a reduced growth rate. These data confirmed that pH is a crucial factor in tyramine synthesis.

The influence on TDC activity of several compounds present in wine was studied in tyramine-producer *L. brevis* strains (Moreno-Arribas and Lonvaud-Funel, 1999). Citric acid an lactic acid inhibited TDC activity. Results obtained with ethanol (0-10%) showed that TDC activity was unaffected. In a tyramine-producer *L. plantarum* strain isolated from

wine, the influence of factors such as nutrients (glucose, fructose, L-malic acid, etc.), pH and ethanol content in cultures was also studied (Arena et al., 2007). A negative effect of sugars such as glucose and fructose, and L-malic acid on TDC activity was observed.

An exhaustive study on tyramine production by *Enterococcus faecalis* EF37 have been carried out. Firstly, Gardini et al. (2001) studied the combined effects of temperature, pH, and NaCl concentration on the growth dynamics of *E. faecalis* EF37 on tyramine production by using a Central Composite Design. The production of tyramine, under the conditions assayed, was found to be mainly dependent on the extend of growth of *E. faecalis*. Later, a quantitative RT-PCR assay was applied to quantify *tyrDC* and its transcript in pure cultures and in meat and meat products (Torriani et al., 2008). This assay allowed estimation of the influence of different variables (pH, temperature, and NaCl concentration) on the *tyrDC* expression of the tyraminogenic strain *E. faecalis* EF37 after 72 h of growth. Data obtained suggest that stressful conditions could induce greater TDC activity. In model system, a temperature around 30 ºC minimizes *tyrDC* expression; in contrast, the intermediate concentration of NaCl (about 4.5%) allowed the maximum level of *tyrDC* expression after 72 h. A comparison of the results presented by Gardini et al. (2001) and those obtained by Torriani et al. (2008), revealed that the copies of *tyrDC* transcript were negatively correlated with growth after 72 h, as measured by optical density. The conditions less favorable for rapid growth within 72 h resulted in a relatively higher *tyrDC* expression at this time, suggesting that stress or suboptimal environmental conditions could induce a higher tyrosine decarboxylase activity not necesssarily associated with higher cell production. This increased TDC activity could be explained by the fact that the production of biogenic amines is the result of biochemical pathways associated with membrane transport phenomena such as energy transduction by secondary transport

processes and proton motive force, which are important for the survival of lactic acid bacteria in hostile enviroments.

The same real time-PCR assay was used to monitor the presence of *tyrDC* and its transcript in a meat model system with salt and/or sugar after inoculation with *E. faecalis* EF37 (Torriani et al., 2008). The expression of *tyrDC* differed depending on the composition of the meat model. The authors concluded that, from the preliminary results obtained, it is difficult to determine the precise effect of environmental factors on bacterial *tyrDC* expression in a real food system. Later, Gardini et al. (2008) monitored TDC activity of *E. faecalis* EF37 during fermentation and ripening of a traditional dry fermented sausage by means of microbiological, chemical, and molecular approaches in relation to three technological factors: fermentation temperature, sodium chloride concentration, and amount of glucose added to the meat mixture. Besides the analytical detemination of tyramine and PEA accumulation, the presence and quantification of the *tyrDC* gene and its mRNA transcript were also investigated by using RT-PCR. According to the mathematical models obtained, sodium chloride concentration was the most determinant factor of the final tyramine and PEA accumulation and also of the levels of *tyrDC* present in the final product. In contrast, an effect of glucose concetration of *tyrDC* expression was observed in the last period of ripening. Moreover, increasing amounts of sodium chloride and decreasing fermentation temperature resulted in a reduced *tyrDC* expression. In this work, for the first time, a bacterial TDC potential is directly examined through a molecular approach in a fermented meat.

Recently, Makhzami et al. (2008) applied transcriptional approaches in cheese to characterize food *E. faecalis* isolates during cheese processing. The authors compared gene expression of *E. faecalis* cultivated in the cheese matrix versus BHI medium, using RT-

PCR and microarrays. Semi-quantitative analysis of transcript levels of the *tyrDC* gene were performed in the strain cultivated in both conditions, by a two-step semi-quantitative RT-PCR approach. For the *E. faecalis* strain, level of the *tyrDC* transcript was clearly reduced in cheese compared to BHI medium. Its repression in cheese may reflect the unfavourable conditions for biogenic amine production in the cheese model used.

Apart from TDC, identification of the TDC operons raised questions about the role of its encoded proteins. Based on similarity to other bacterial amino acid decarboxylation systems, the operon could supply energy and participate in pH regulation (Landete et al., 2008). The putative tyrosine transporter gene of *L. brevis* (*tyrP*) was expressed in *Lactococcus lactis* and functionally characterized (Wolken et al., 2006). The transporter TyrP catalyzes homologous tyrosine-tyramine exchange and heterologous exchange between tyrosine and its decarboxylation product tyramine (Figure 4). It is proposed that tyrosine decarboxylation in *L. brevis* results in proton motive force generation by an indirect proton pumping mechanism (Wolken et al., 2006). The transport properties of the *tyrP* gene product in the TDC operon of *L. brevis* IOEB 9809 support the idea of a proton motive pathway, as both tyrosine and its decarboxylation product, tyramine, are substrates of TyrP; TyrP catalyzes exchange much more efficiently than unidirectional transport, and the tyrosine-tyramine exchange is electrogenic (Figure 4). The pathway consists of the tyrosine-tyramine exchange TyrP and the tyrosine decarboxylase TDC. Tyrosine is taken up form the medium by TyrP and, subsequently, decarboxylated by TDC, yielding tyramine that is excreted by TyrP coupled to the uptake of tyrosine (Figure 4). The physiological function of the pathway is generation of proton motive force, membrane potential and pH gradient, are generated separately in the two steps of the pathway. Turnover of TyrP results in membrane potential of physiological polarity (positive out), since monovalent, positively

charged tyramine is exchanged for uncharged tyrosine. The decarboxylation of tyrosine catalyzed by TDC consumes a proton which alkalinizes the cytoplasm relative to the external medium. For each turnover of the pathway in which one external tyrosine molecule is converted to one external tyramine molecule, one positive charge is translocated across the membrane and one proton is removed from the cytoplasm, which is equivalent to the pumping of one proton across the membrane (Figure 4). Hence, the pathway is an indirect proton pump. Alternatively, the pathway may play a role in cytoplasmic pH homeostasis and resistance against acid stress by virtue of the alkanizing effect of the decarboxylation reaction (Molenaar et al., 1993).

The role of tyrosine decarboxylation pathway in the response to *E. faecium* E17 cells to an acid challenge have been investigated (Pereira et al., 2009). It was found that 91% of the cells were able to remain viable in the presence of tyrosine when they were incubated for 3 h in a complex medium at pH 2.5. This effect was shown to be related to the TDC pathway. Therefore, the role of tyrosine decarboxylation in pH homeostasis was studied. The results showed that the TDC pathway generates a proton motive force compsed of a pH gradient formed due to proton consumption in the decarboxylation reaction and by a membrane potential which results from electrogenic transport to tyrosine in exchange for the corresponding biogenic amine tyramine (Figure 4). Similarly to *L. brevis*, *E. faecium* transporter catalyzes homologous tyrosine/tyramine antiport, as well as electrogenic heterologous tyrosine-tyramine exchange (Pereira et al., 2009). Therefore, the TDC pathway contributes to an acid response mechanism in *E. faecium* E17(Figure 4) . This decarboxylation pathway gives the strain a competitive advantage in nutrient-depleted conditions, as well as in harsh acidic environments, and a better chance of survival, which contributes to higher cell counts in food fermentation products.

TYRAMINE PRODUCTION BY GRAM-NEGATIVE BACTERIA

Sandmeier et al. (1994) compared the amino acid sequences of different eukaryotic and prokaryotic pyridoxal phosphate dependent amino acid decarboxylases. Their results indicated that they could be subdivided into four different groups that seem to be evolutionarily unrelated to each other. Group II comprises eukaryotic and prokaryotic glutamate and histidine decarboxylases, and eukaryotic tyrosine and aromatic L-amino acid decarboxylases. Therefore, when the deduced amino acid sequence of a bacterial protein reveals strong similarity to eukaryotic tyrosine, L-dopa and aromatic-L-amino acid decarboxylases, it is annotated as putative TDC or aromatic-L-amino acid decarboxylase. This is the case for a group of proteins from Gram-negative bacteria (Table 1, Figures 5 and 6) which appeared in the databases as putative TDC. There are proteins having aprox. 450 amino acid residues, showing highest similarity to eukaryotic TDC, and containing on its central portion of the protein a motif which matches the pyridoxal phosphate-dependent decarboxylase group II consensus pattern (Figure 5). However, as suggested by Sandmeier et al. (1994) the evolutionary pedigree of group II decarboxylases indicated that the common ancestor of the eukaryotic enzymes did not diverge into substrate-specific enzymes until after the divergenence of prokaryotic and eukaryotic. For instance, eukaryotic histidine decarboxylase are more closely related to eukaryotic aromatic-L-amino acid decarboxylase (33-55% identity) than to their bacterial counterparts (18-20% identity) (Sandmeier et al., 1994). Therefore, caution should be taken when assuming bacterial TDC proteins similar to eukaryotic TDCs. From the proteins included in Figure 5, only the

activity of the decarboxylase from *Sorangium cellulosum* So ce90 has been biochemically demostrated, being a L-dopa decarboxylase and not a tyrosine decarboxylase (Müller et al., 2000). In addition, results obtained in our laboratory showed that the decarboxylase gene from *P. putida* KT2440 (Figure 4 and Figure 5) did not encoded a functional tyrosine decarboxylase (unpublished results)

In relation to the genetic organization of the chromosomal region surrounding the putative decarboxylase (DC) encoding genes, in contrast to Gram-positive bacteria, these bacteria did not show a similar genetic organization (Figure 6). Only the related bacteria *P. putida* and *P. entomophila* possessed a homologous gene upstream the DC gene, a gene encoding for a LysR family transcriptional regulator. A similar regulator encoding gene could be found donwstream and divergently transcribed from the decarboxylase gene in *G. bethesdensis* (Figure 6).

In summary, so far the production of tyramine by Gram-negative bacteria have not been unequivocally reported, therefore, caution should be taken when assuming their proteins as having TDC activity.

PHENYLETHYLAMINE PRODUCTION BY BACTERIA

As early as 1940, Gale tested washed suspensions of *E. faecalis* (formerly *Streptococcus faecalis*) for the decarboxylation of sixteen amino acids, including phenylalanine, and conclude that the cell suspension only decarboxylated tyrosine (Gale, 1940). Later, in 1944, Epps found that in an acetone powder of *E. faecalis* cell extract, tyrosine and dihydrophenylalanine were decarboxylated to their corresponding amines

(Epps, 1944). Later, in the course of his studies, McGilvery and Cohen (1948) proved that an *E. faecalis* extract also showed a concomitant decarboxylation of phenylalanine by the decarboxylase preparation. The low activity with phenylalanine, approximately 0.01 that obtained with tyrosine, coupled with the inability to saturate the enzyme with tyrosine, made it difficult to conclude whether tyrosine decarboxylase also decarboxylates phenylalanine, or whether a second decarboxylase is present in the *E. faecalis* extract (McGilvery and Cohen, 1948). In 2006, this long-standing question regarding the specificity of tyrosine decarboxylation in enterococci was solved (Marcobal et al., 2006b). Marcobal et al. (2004) described that *E. faecium* strains were able to decarboxylate tyrosine and they also contain a putative tyrosine-decarboxylase encoding gene. A gene from an *E. faecium* strain coding for a 625 amino acid residues protein that shows 85% identity to *E. faecalis* TDC was expressed in *E. coli*, resulting in phenylalanine and tyrosine decarboxylase activity. Both activities were lost when a truncated protein lacking 84 amino acids at its C-terminus was expressed in *E. coli*. Thus, it was prove experimentally that the *tyrDC* gene in *E. faecium* encodes a functional and dual decarboxylase which is able to perform phenylalanine as well as tyrosine decarboxylation, resulting in PEA or tyramine production (Figure 7).

There are many reports referring the simultaneous production of both biogenic amines, tyramine and PEA. Beutlig and Walter (2002) studied the ability of 22 enterococcal strains to form tyramine and PEA. They found that most of the enterococcal strains were able to form large amounts of PEA and, simultaneosuly, tyramine. This ability was observed for all the examined strains of the especies *E. faecalis* and *E. hirae*. In a typical Italian goat cheese, Bonetta et al. (2008) described that 93.5% of the tyrosine

decarboxylating strains, mainly *E. faecalis*, were also able to decarboxylate phenylalanine, giving to tyramine and PEA production.

Aymerich et al. (2006) reported that more than half of the *L. curvatus* strains isolated form slightly fermented sausages showed a strong tyrosine-decarboxylase activity, being most of them also able to simultaneously produce considerable amounts of PEA.

The ability of wine LAB to produce tyramine and PEA was investigated by biochemical and genetic methods (Landete et al., 2007a). All strains possessing the *tyrDC* gene were shown to produce tyramine and PEA. The strains that produced tyramine also produced PEA, but the levels of PEA were 4 to 5 fold lower. Wine containing high quantities of tyramine and PEA were found to contain *L. brevis* strains. The authors concluded that the abilities to form tyramine and PEA are not common among wine LAB.

Gardini et al. (2001) in a study of the combined effects of temperature, pH and NaCl concentration on biogenic amine production in *E. faecalis* EF37, reported that quantitatively, the most important biogenic amine produced was PEA but substantial amount of tyramine were detected in all the samples. Later, Gardini et al. (2008) in a study of the aminogenic potential of the same *E. faecalis* strain in dry fermented sausage described that the dual decarboxylase activity of TDC, on tyrosine as well as on phenylalanine, was revealed on the latter period of ripening in which PEA accumulated in detectable amounts. They also reported that, even if the amounts of PEA were lower that those of tyramine, the shapes of the surface response graphics are similar to those obtained for the *tyrDC* mRNA transcripts after the same ripening time. Joosten (1988) suggested that, at the end of ripening, tyrosine could become a limiting substrate for the activity of decarboxylase enzyme and was substituted by phenylalanine that is decarboxylated to PEA, even with a reduced efficiency.

OTHER BACTERIAL AROMATIC L-AMINO ACID DECARBOXYLASES

Bacterial decarboxylation of aromatic amino acids to their corresponding amines has been demonstrated in a number of different microbial strains. In spite of the many descriptions of decarboxylases of tyrosine, histidine, etc. the enzymes which catalyze the decarboxylation of tryptophan, 5-hydroxy-tryptophan and phenylalanine have not been extensively investigated.

In Gram-positive bacteria, though Perley and Stowe (Perley and Stowe, 1966) reported that cell-free extracts from *Bacillus cereus* catalyzed the formation of tryptamine from tryptophan, apart for its optimum pH (8.0) the properties of this bacterial tryptophan decarboxylase remain unknown. Recently, Kato et al. (2007) purified and partially characterized an N-hydroxy-L-phenylalanine decarboxylase from *Bacillus* sp. OxB-1. However, this enzyme does not accept L-aromatic amino acids, such as phenylalanine, tyrosine, tryptophan, and L-dopa as substrates. Nakazawa et al. (1977) studied aromatic L-amino acid decarboxylase activities in 26 strains from type cultures and in 300 strains isolated from soil. These strains were assayed by measuring the amount of aromatic amines formed from the aromatic L-amino acids: 3, 4-dihydroxy-L-phenylalanine, tyrosine, phenylalanine, tryptophan, and 5-hydroxy-L-tryptophan. They reported that these activities were widely distributed in various bacteria belonging to the genera *Acinetobacter*, *Micrococcus*, and *Staphylococcus*. Bacterial strains belonging to the *Micrococcaceae* showed the highest decarboxylase activity toward tryptophan, 5-hydroxy-L-tryptophan and phenylalanine. *Micrococcus percitreus* AJ 1065 strain showed the highest decarboxylase

activity toward tryptophan and, therefore, was selected as a source of aromatic L-amino acid decarboxylase. Results of experiments with this bacterium showed that the aromatic amine formed from tryptophan was tryptamine. The aromatic L-amino acid decarboxylase was crystallized from cell-free extract of this strain (Nakazawa et al., 1981). The purified enzyme catalyzed the stoichiometric conversion of tryptophan to tryptamine in the presence of pyridoxal phosphate. The enzyme also catalyzed decarboxylation of 5-hydroxy-L-tryptophan, phenylalanine, tyrosine, 3,4-dihydroxy-L-phenylalanine (L-DOPA), L-kynurenine and their α-methyl amino acid derivatives. Crystalline aromatic L-amino acid decarboxylase from *M. percitreus* was inactive in the absence of pyridoxal phosphate (PLP) (Nakazawa et al., 1983). Nakazawa et al. (1987) studied the inhibition of aromatic L-amino acid decarboxylase from *M. percitreus* by substrate analogs. They found that phenylalanine and tyrosine are good substrates of the enzyme and strongly inhibit tryptophan decarboxylation, but both show uncompetitive inhibition indicating the binding site of the substrate tryptophan and that of the inhibitors are different. In addition, decarboxylation of tryptophan by the enzyme was also inhibited by aromatic amines such as PEA, tyramine, and dopamine, among others, which corresponds to or are related to the substrates of the enzyme. The genetic characterization of this aromatic L-amino acid decarboxylase have not been still carried out.

In Gram-negative bacteria, it has been identified an L-dopa decarboxylase gene (*ddc*) in *Polyangium cellulosum* So ce90 (formerly, *Sorangium cellulosum* So ce90) (Müller et al., 2000). The postulated *ddc* gene encodes a DDC protein with a calculated M_r of 57 kDa (Figure 4). Crude extracts prepared from cells of *P. cellulosum* were assayed for the ability to convert tyrosine or L-dopa to tyramine or dopamine, respectively. Unexpectedly, no such activity was detected using various enzyme assay conditions. A

possible reason for the absent enzyme activity may be the culture conditions used for growing the *P. cellulosum* cells. However, in order to examine the functionality of DDC, it was heterologously expressed in *E. coli*. Enzyme assays with L-dopa, tyrosine, tryptophan, 5-hydroxytryptophan and with phenylalanine and phenylpyruvate as substrates showed that cell extracts were only able to convert L-dopa to dopamine (Müller et al., 2000). Due to their lifestyle, it has been postulated that this microorganism could have acquired the *ddc* gene in horizontal transfer process (Bode and Müller, 2003).

DETECTION OF TYRAMINE-PRODUCER BACTERIA

Similarly to the presence of histamine in foods due to its toxicological effects, the presence of tyramine in foods is of considerable public concern for the food industry and the regulatory agencies, since given the potential health hazard, there is a growing demand from consumers and control authorities to reduce the allowable limits of tyramine in foods and beverages. Rapid and simple methods are needed for the analysis of the ability to form tyramine by bacteria in order to evaluate its potential risk.

Non-molecular methods for the detection of tyramine-producer bacteria

The simplest methods for the detection of tyramine-producer bacteria are those based on differential growth media. Generally differential growth media consist of a basal composition (peptone, yeast or meat extract, salt and/or glucose) to which the precursor

amino acids are added. These differential media contained a pH indicator, such as bromocresol purple. When biogenic amines are produced, the positive result is indicated by a change of the medium colour in response of the indicator to a pH shift. The pH change is dependent of the production of the more alkaline amine from the amino acids initially included in the medium (Marcobal et al., 2006a). Although these media produce adequate results in *Enterobacteriaceae*, due to their composition they had only limited value for the study of lactic acid bacteria. The occurrence of false-positive reactions, due to the formation of other alkaline compounds, or false negative responses, as a result of the fermentative activity of some bacteria which produce acid along with biogenic amines, has been described.

These differential media produce adequate results in *Enterobacteriaceae*, but due to their composition they had only limited value for the study of lactic acid bacteria. Besides this problem, some microorganisms, such as fastidious lactic acid bacteria, were not able to grow in these relatively simple decarboxylase media. To avoid these difficulties some modifications were done in order to adapt and improve the method, e. g. include the presence of pyridoxal phosphate as enhancer of the decarboxylase activity expression (Bover-Cid and Holzapfel, 1999; Roig-Sagués et al., 1997).

Some of these differential growth media were used for the detection of tyramine producing bacteria. Da Silva et al (2002) in a screening of tyramine-producing bacteria isolated from Portuguese vacuum-packed cold-smoked fish described that tyrosine-agar was shown to be a good indicator medium for the detection of bacteria that produce high levels of tyramine, since typical colonies surrounded by a translucent halo were easily distinguished. Also, a modified decarboxylation agar proposed by Maijala (1993) was

shown to be more promissory for use on detection of tyramine lactic acid bacteria strains, probably due to the medium composition (Da Silva et al., 2002).

Landete et al. (2007a) described a Tyramine Production Medium (TPM) to test bacterial TDC activity. TPM media contained meat and yeast extracts, tryptone, glucose, fructose, several salts, pyridoxal phosphate, Tween 80 and tyrosine. Strains streaked on solid TPM plates were considered tyramine positve if a clear zone below the grown cells developed because of solubilisation of tyrosine. The suitability of the TPM plates was evaluated by comparison with results obtained form the media of Maijala (1993) and Bover-Cid and Holzapfel (1999), and also by an HPLC assay. About 15% of false positives were observed in the media of Maijala (1993) and Bover-Cid and Holzadfel (1999), not in TPM. These false positives produced a colour change in the media plates of both media, but were negative in TPM plates, negative in HPLC determination of tyramine synthesis in TPM broth, and negative for the PCR test. A correlation of 100% was observed between the results obtained on TPM plates, in TPM broth, and the presence of a *tyrDC* gene by PCR. In TPM there is no interference with the acidification produced by the fermentation of sugars. All the tested strains grew well in TPM. Non false positives or negatives were observed neither on TPM plates nor in TPM liquid medium. Therefore, the TPM plate medium is an easy, conventional and suitable method to screen lactic acid bacteria for TDC activity in laboratories lacking sophisticated equipment. Similarly, the production of PEA was assayed in differential based media (MDA, Maijala, 1993) supplemented with 10 g/L of phenylalanine and 0.25 g/L of pyridoxal 5-phosphate and in the improved medium of Bover-Cid and Holzapfel (1999) using 1 g/L of phenylalanine. Some strains gave a positive reaction, but the production of PEA was not confirmed in liquid medium by HPLC (Landete et al., 2007a).

Fluorimetric methods have been also described for the analysis of tyramine. Tyramine could be extracted from bacterial culture supernatants by a weak cation exchange resin; then, tyramine could be fixed on the resin with 50 mM phosphate buffer (pH 6.5) and eluted with 2 N HCl (Eitenmiller et al., 1978), and, finally tyramine is quantified by the 1-nitroso-2-naphtol fluorimetric method (Santos-Buelga et al., 1981).

Phan et al. (1983) developed a highly sensitive and rapid spectrophotometric assay for tyramine determination. In the assay, tyramine reacts with 2, 4 ,6-trinitrobenzenesulfonic acid to give a product soluble in toluene whereas tyrosine does not. The amount of tyramine produced enzymatically could be determined by reading the absorbance at 340 nm of a toluene extract of the reaction mixture.

Among the different chromatographic techniques recommended for identification and quantification of tyramine, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have been the most useful (Önal, 2007). TLC methods constitute a simple solution to the reports describing false-positive or negative reactions in routine screening procedures generally involving the use of a differential medium. TLC methods have many advantages, including simplicity of operation and cost effectiveness. Recently, García-Moruno et al. (2005) developed a TLC method for the separation and identification of tyramine and PEA from bacterial culture supernantans, which are only centrifuged and directly derivatized. This method has been successfully applied to several BA-producer strains (Marcobal et al., 2006b; Landeta et al., 2007).

HPLC with pre- or post-column derivatisation is by far the mostly frequently reported technique for tyramine separation and quantification. Although HPLC techniques required costly and sophisticated equipment, careful maintenance, expensive solvents,

accessories and specially trained staff; however, HPLC methods are reported to be more efficient, sensitive and reproducible compared to other analytical methods (Önal, 2007).

Molecular methods for the detection of tyramine-producer bacteria

In spite that bacterial PEA formation has been less analyzed, it could be assumed that the oligonucleotide primers described for the detection of the *tyrDC* gene (Table 2), are also useful for the detection of tyramine and phenylethylamine-producing bacteria, as it has been shown that TDC is also able to decarboxylate phenylalanine, to originate PEA. Tyramine production has been more studied, therefore a clear relationship between the presence of the *tyrDC* gene and the ability to produce tyramine has been determined (Aymerich et al., 2006; Constantini et al., 2006; Fernández et al., 2006; Arena et al., 2007), and suitable PCR methods for the detection of tyramine-producer strains have been proposed (Landete et al., 2007b). As far as we know, only gram-positive bacteria have been described to produce tyramine. Many lactic acid bacteria involved in food processing could decarboxylate tyrosine to produce tyramine. These bacteria belong to genera as diverse as *Lactobacillus*, *Enterococcus* or *Carnobacterium* (ten Brink et al., 1990). As explained previously (Figure 3), the comparison of the TDC gene clusters from different producing microorganisms revealed a high similarity either in gene sequence and organization.

Purification and microsequencing of the TDC of *Lactobacillus brevis* IOEB 9809 allowed Lucas and Lonvaud-Funel (2002) to design a degenerate primer set (P2-for/P1-rev) (Table 2) that was used to detect *tyrDC* gene fragments in three other *L. brevis* strains out of six screened.

Marcobal et al. (2005) checked the P2-for/P1-rev primer set and a new designed primer set (41/42) (Table 2) in order to choose one of them to be used in a multiplex PCR assay. The proposed assay was useful for the detection of tyramine-producing bacteria in control collection strains and in a wine LAB collection (Marcobal et al., 2005; De las Rivas et al., 2005). Constantini et al. (2005) also used the P2-for/P1-rev primer set to amplify the *tyrDC* gene of 133 strains isolated from wine and must. They also designed a new primer set, Pt3/Pt4 (Table 2), which produce identical results that primers P2-for/P1-rev. Only four positive strains were found, all belonging to the *L. brevis* species. The tyramine produced by these strains was quantified by HPLC, thus confirming the results observed by PCR. Recently, P1-rev primer was used in combination with p0303 primer (Lucas et al. 2003) to analyse by PCR the presence of the *tyrDC* gene in 150 LAB strains isolated from wine (Landete et al., 2007a). All the 32 strains that gave a positive PCR amplification were tyramine producers.

The identification of the *Carnobacterium divergens* 508 TDC protein allowed Coton et al. (2004) to design a set of consensual primers, TD2 and TD5, for the detection of the *tyrDC* gene (Table 2). These primers were used in PCR experiments and allowed for the detection of tyramine-producing bacteria. This primer set was later used by Coton and Coton (2005) in a multiplex assay. They amplified, from purified DNA, the *tyrDC* gene from 28 known tyramine-producing bacteria (7 carnobacteria, 1 lactobacilli, and 20 enterococci). The method was also applied to DNA prepared from cell colonies. No differences in results were observed between extracted DNA or cell colony DNA. De las Rivas et al. (2006) designed a new primer set, TDC-F/TDC-R primers (Table 2), to design a complete PCR assay for the detection of several decarboxylase genes. They amplified the *tyrDC* gene from *E. faecalis*, *E. faecium*, *C. divergens* and *L. brevis*.

Fernández et al. (2004) designed TDC1/TDC2 primers to detect tyramine-producing strains by PCR (Table 2). Reactions were initially performed by the use of total DNA from 17 different tyramine-producing LAB strains. Similarly, the internal fragment of the *tyrDC* gene was successfully amplified when colonies were used directly as template in the PCR reaction. The usefulness of the PCR technique was evaluated in milk, curd, and commercial cheese samples. Recently, the method was used to detect the presence of tyramine-producing bacteria during cheese manufacture and ripening of six different batches of a farmhouse blue cheese (Fernández et al., 2006). The authors found that the presence of tyramine-producing microorganisms in the early stages of manufacture correlated with a high concentration of tyramine in mature cheese samples. Later, Fernández et al (2007b) used TDC1/TDC2 primers to detect the presence or tyramine-producers strains in 61 cheese samples and the results were compared with HPLC tyramine detection results. PCR positive results were obtained with 41 samples. In nine of the samples, tyramine was not detected by HPLC. In three cases, tyramine-producer strains were not detected by PCR although tyramine was detected by HPLC. The results show an acceptable correlation between PCR and HPLC results. The three cheeses in wich tyramine was detected by HPLC, yet no amplification product was obtained, were all made from raw milk. The tyramine-producing strains responsible may have been Gram-negative bacteria, which would not have been detected by the primers used. The low number of samples with these characteristics appears to indicate that the main tyramine-producer strains in cheeses are LAB, which are easily detected by PCR.

Aymerich et al. (2006) reported the partial sequence of the *tyrDC* gene from *L. curvatus*. To specifically detect *L. curvatus* strains carrying the *tyrDC gene*, new primers tdcC1/tdcC2 from the partial *tyrDC* gene sequence of *L. curvatus* were designed (Table 2).

Isolates of *L. curvatus* from slightly fermented sausages were submitted to the assay with these specific primers. Positive results were perfectly correlated only with the tyramine-producer *L. curvatus* strains (Aymerich et al., 2006). Therefore, the PCR assay developed to detect the *tyrDC* gene of *L. curvatus* could be used as a rapid method to characterize potential tyramine-producer strains within this species.

Recently, Torriani et al (2008) aligned TDC sequences from *E. faecalis*, *L. brevis* and the enterobacteria *Yersinia pestis*, for design the degenerate primers DEC2 and DEC3 (Table 2). The primer set DEC2/DEC3 produced an approximately 1 kb amplification product with DNA extracted from all the tyramine-positive enterococci, including strains of *E. faecium*, *E. faecalis*, and *E. gallinarum*. Many staphylococci also produced a positive DNA reaction with these primers. The amplification product was obtained for all *S. epidermidis* strains and a tyramine-producing strain of *Staphylococcus xylosus*. However, some other *S. xylosus* strains that had produced small amounts of tyramine had a negative result with the DEC2/DEC3 primers. None of the DNA extracted from the lactobacilli was amplified by these primers, despite the fact that these strains were considered tyraminogenic according to the results of conventional analysis. To improve the reliability of the PCR assay to detect *tyrDC*-positive strains, especially strains of lactobacilli, the DEC5 new primer was designed based on a highly conserved region from enterococci and lactobacilli (Table 2). Primers DEC5/DEC3 confirmed all the positive results obtained with the DEC2/DEC3 primers, and also amplified a 350-bp DNA fragment from tyramine-producer lactobacilli.

A real-time PCR assay for the detection of *tyrDC* was also developed by Torriani et al. (2008). Sequences present in databases were used to design primers TYR3f/TYR4r for the real-time PCR assay (Table 2). This assay was applied to DNA extracted from

tyramine-producers and non-producers bacteria. The correct amplification product and the absence of nonspecific PCR products were verified through the examination of the dissociation curve. A single product was formed in the PCRs, and no false-positive or false-negative results were obtained. This RT-PCR method was used for the quantitative determination of *tyrDC* and its transcript in model systems and in a meat model system after inoculation with a tyramine-producer strain. The authors concluded that the expression of *tyrDC* differed depending on the composition of the meat model and this is difficult to determine the precise effect of environmental factors on bacterial *tyrDC* expression in a real food system.

The products of gene expression (mRNA) have a high turnover rate, and therefore their quantification is indicative of the metabolic activity of the bacteria expressing the *tyrDC* potential. Gardini et al. (2008) indicates that the quantification of the *tyrDC* transcripts (from the RNA extracts) seems to be less sensitive that that of *tyrDC* (from the DNA extracts), since, in fermented meat, the data could be modeled only after 19 days of ripening, and they did not correlated with the tyramine content. However, it is the result of the expression of the corresponding gene at a given time, which is unstable, meaning that it does not accumulate in the foof matrix. Moreover, RNA extraction usually results in a lower recuperation in comparison with that of DNA, even when all the precautions are taken into account.

Another quantitative PCR method for the detection of tyramine producer bacteria was recently developed by Nannelli et al. (2008). This method was applied to detect and quantify the bacteria producing tyramine in wine.

The advantages deriving from the application of molecular protocols directly to food products is emphasized by their capacity to estimate TDC activities of microorganisms

associated with specific manufacturing technology. This should prove to be a rapid and universal method for the technological evaluation of the safety of the processing conditions. The detection of *tyrDC* transcripts, even if less sensitive that the quantification of the relative gene, can help to elucidate the critical steps during food manufacturing at which the environmental conditions allow that bacterial decarboxylase enzyme activities, resulting in the progressive accumulation of tyramine and PEA.

CONCLUSIONS

There is a renewed interest on the study of the occurrence of biogenic amines in foods because of recent food legislations, consumer demand for healthier foods, and increasing attention to senstitive groups.

A fundamental requisite for the formation of tyramine in foods is the presence of TDC-postitive bacteria. The ability of bacteria to decarboxylate tyramine is highly variable. It depends not only on the species, but also on the strain. There are many references on the scientific literature about the potential to produce tyramine by bacteria isolated from food products. However, since some of the detection methods used could produce false-positive and negative results, this revision was focused only on the tyramine-producer bacteria whose tyramine decarboxylases or their corresponding encoding genes have been unequivocally reported so far. In all the bacteria analyzed, the presence of the same four genes TDC cluster support the hypothesis of a co-evolution, and suggests that the four genes were transferred in bacteria by a horizontal transfer event. The knowledge of the genes involved in tyramine production, allowed the development of molecular detection

methods for the detection of the producer bacteria. In addition, molecular techniques are an important tool in the study of the factors affecting tyramine production during food elaboration. The quantification of *tyrDC* and its transcript can help to elucidate the critical steps and factors during food manufacturing at which bacterial aminogenesis is possible, thus allowing researchers to propose technological measures to control decarboxylase activities. However, sometimes minimizing tyramine accumulation by changing the production conditions are not possible. Rather, the solution is to prevent the appearance of tyramine-producing bacteria.

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REFERENCES

Allenmark, S., and Servenius, B. (1978). Characterization of bacterial L(-)-tyrosine decarboxylase by isoelectric focusing and gel chromatography. *J. Chromatogr*. **153**: 239-245.

Arena, M. E., Fiocco, D., Manca de Nadra, M. C., Pardo, I., and Spano. G. (2007). Characterization of a *Lactobacillus plantarum* strain able to produce tyramine and partial cloning of a putative tyrosine decarboxylase gene. *Curr. Microbiol*. **55**: 205-210.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., and Hugas, M. (2006). Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J. Appl. Microbiol*. **100**: 40-49.

Beutling, D. M., and Walter, D. (2002). 2-Phenylethylamine formation by enterococci in vitro. *Eur. Food Res Technol*. **215**: 240-242.

Blackwell, B. (1963). Hypertensive crisis due to monoamine-oxidase inhibitors. *The Lancet* **2**: 849.

Bode, H. B., and Müller, R. (2003). Possibility of bacterial recruitment of plant genes associated with the biosynthesis of secondary metabolites. *Plant Physiol*. **132**: 1153-1161.

Bonetta, S., Bonetta, S., Carraro, E., Coïsson, J. D., Travaglia, F., and Arlorio, M. (2008) Detection of biogenic amine producer bacteria in a typical Italian goat cheese. *J. Food Prot.* **71**: 205-209.

Borresen, T., Klausen, N. K., Larsen, L. M., and Sorensen, H. (1989). Purification and characterization of tyrosine decarboxylase and aromatic-L-amino-acid decarboxylase. *Biochim. Biophys. Acta* **993**: 108-115.

Boulton, A. A., Cookson, B., and Paulton, R. (1970). Hypertensive crisis in a patient on MAOI antidepressants following a meal of beef liver. *Can. Med. Assoc. J*. **102**: 1394-1395.

Bover-Cid, S. and Holzapfel, W. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol*. **53**: 33–41.

Chabner, B., and Livingston, D. (1970). A simple enzymic assay for pyridoxal phosphate. *Anal. Biochem*. **34**: 413-423.

Connil, N., Le Breton, Y., Dousset, X., Auffray, Y., Rincé, A., and Prévost, H. (2002a). Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. *Appl. Environ. Microbiol*. **68**: 3537-3544.

Connil, N., Plissoneau, L., Onno, B., Pilet, M. F., Prevost, H., and Dousset, X. (2002b). Growth of *Carnobacterium divergens* V41 and production of biogenic amines and divercin V41 in sterile cold-smoked salmon extract at varying temperatures, NaCl levels, and glucose concentrations. *J. Food Prot*. **65**: 333-338.

Costantini, A., Cersosimo, M., del Prete, V., and García-Moruno, E. (2006). Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. *J. Food Protect.* **69**: 391–396.

Coton, M. and Coton, E. (2005). Multiplex PCR for colony direct detection of Gram positive histamine- and tyramine-producing bacteria. *J. Microbiol. Methods* **63**: 296–304.

Coton, E. and Coton, M. (2009). Evidence of horizontal transfer as origin to strain variation of the tyramine production trait in *Lactobacillus brevis*. *Food Microbiol*. **26**: 52-57.

Coton, M., Coton, E., Lucas P., and Lonvaud, A. (2004). Identification of the gene encoding a putative tyrosine decarboxylase of *Carnobacterium divergenes* 508. Development of molecular tools for the detection of tyramine-producing bacteria. *Food Microbiol*. **21**: 125-130.

Da Prada, M., Zurcher, G., Wuthrich, I. (1988). On tyramine, food, beverages: the reversible MAO inhibitor monoclobemide. *J. Neural Trans*. **26**: 31-36.

Da Silva, M. V., Pinho, O., Ferreira, I., Plestlova, L., and Gibbs, P. A. (2002). Production of histamine and tyramine by bacteria isolated from Portuguese vacuum-packed cold-smoked fish. *Food Control* **13**: 457–461.

Davies, E. B. (1963). Tranylcypromine and cheese. *The Lancet* **2**: 691-692.

De las Rivas, B., Marcobal, A., and Muñoz, R. (2005). Improved multiplex- PCR method for the simultaneous detection of food bacteria producing biogenic amines. *FEMS Microbiol. Lett*. **244**: 367–372.

De las Rivas, B., Marcobal, A., Carrascosa, A. V., and Muñoz, R. (2006). PCR detection of foodborne bacteria producing the biogenic amines histamine, tyramine, putrescine, and cadaverine. *J. Food Prot*. **69**: 2509-2514.

Eitenmiller, R. P., Koehler, P. F., Reagan, J. O. (1978). Tyramine in fermented sausages: factors affecting formation of tyramine and tyrosine decarboxylase. *J. Food Sci*. **43**: 689-693.

Epps, H. M. R. (1944). Studies on bacterial amino-acid decarboxylase. 2. L (-)-tyrosine decarboxylase from *Streptococcus faecalis*. *Biochem J*. **38**: 242-249.

Fernández, M., Linares, D. M., Alvarez, M. A. (2004). Sequencing of the tyrosine decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. *J. Food Prot*. **67**: 2521-2529.

Fernández, M., Flórez, A. B., Linares, D. M., Mayo, B., and Alvarez, M. A. (2006). Early PCR detection of tyramine-producing bacteria during cheese production. *J. Dairy Res*. **73**: 318-321.

Fernández, M., Linares, D. M., Rodríguez, A., Alvarez, M. A. (2007a). Factors affecting tyramine production in *Enterococcus durans* IPLA 655. *Appl. Microbiol. Biotechnol*. **73**: 1400-1406.

Fernández, M., Linares, D. M., Del Rio, B., Ladero, V., and Alvarez, M. A. (2007b). HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. *J. Dairy Res*. **74**: 276-282.

Gale, E. F. (1940). The production of amines by bacteria. 2. The production of tyramine by *Streptococcus faecalis*. *Biochem J*. **34**: 846-852.

Gale, E. F. (1946). The bacterial amino acid decarboxylases. *Adv. Enzymol*. **6**:1-32.

García-Moruno, E., Carrascosa, A. V., and Muñoz, R. (2005). A rapid and inexpensive method for the determination of biogenic amines from bacterial cultures by thin layer chromatography. *J. Food Protect*. **68**: 625–629.

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E., and Suzzi, G. (2001). Effects of pH, temperature and ClNa concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *Int. J. Food Microbiol*. **64**: 105-117.

Gardini. F., Bover-Cid, S., Tofalo, R., Belletti, N., Gatto, V., Suzzi, G., and Torriani, S. (2008). Modeling the aminogenic potential of *Enterococcus faecalis* EF37 in dry fermented sausages through chemical and molecular approaches. *Appl. Environ. Microbiol*. **74**: 2740-2750.

Halász, A., Baráth, A., Simon-Sarkadi, L., and Holzapfel, W. (1994). Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol*. **5**: 42-48.

Joosten H. M. L. J. (1988). Conditions allowing the formation of biogenic-amines in cheese. 3. Factors influenecing the amounts formed. *Neth. Milk Dairy J.* **41**: 329-357.

Joosten, H. M. L. J., and Northolt, M. D. (1989). Detection, growth and amine-producing capacity of lactobacilli in cheese. *Appl. Environ. Microbiol*. **55**: 2356–2359.

Joosten, H. M. L. J., Gaya, P., and Nuñez, M. (1995). Isolation of tyrosine decarboxylaseless mutants of a bacteriocin-producing *Enterococcus faecalis* strain and their application in cheese. *J. Food Prot*. **58**: 1222-1226.

Kato, Y., Tsuda, Y., Asano, Y. (2007). Purification and partial characterization of N-hydroxy-L-phenylalanine decarboxylase/oxidase from *Bacillus* sp. strain OxB-1, an enzyme involved in aldoxime biosynthesis in the "aldoxime-nitrile pathway". *Biochim. Biophys. Acta*. **1774**: 856-865.

Landeta, G., de las Rivas, B., Carrascosa, A. V., and Muñoz, R. (2007). Screening of biogenic amine production by coagulase-negative staphylococci isolated during industrial Spanish dry-cured ham processes. *Meat Sci*. **77**: 556-561.

Landete, J. M., Ferrer, S., Polo, L., and Pardo, I. (2005). Biogenic amines in wines from three Spanish regions. *J. Agric. Food Chem*. **53**: 1119-1124.

Landete, J. M., Pardo, I., and Ferrer, S. (2007a). Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. *Int. J. Food Microbiol*. **115**: 364-368.

Landete, J. M., de las Rivas, B., Marcobal, A., and Muñoz, R. (2007b). Molecular methods for the detection of biogenic amine-producing bacteria on foods. *Int. J. Food Microbiol*. **117**: 258-269.

Landete, J. M., de las Rivas, B., Marcobal, A., Muñoz, R. (2008). Updated molecular knowledge about histamine biosynthesis by bacteria. *Crir. Rew. Food Sci. Nutr*. **48**: 697-714.

Lucas, P., and Lonvaud-Funel, A. (2002). Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. *FEMS Microbiol. Lett*. **211**: 85-89.

Lucas, P., Landete J., Coton, M., Coton, E., and Lonvaud-Funel, A. (2003). The tyrosine decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation in tyramine-producing bacteria. *FEMS Microbiol. Lett*. **229**: 65-71.

Lucas, P. M., Blancato, V. S., Claisse, O., Magni, C., Lolkema, J. S., Lonvaud-Funel, A. (2007). Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. *Microbiology*, **153**: 2221-2230.

Lüthy, J., Schlatter, C. (1983). Biogene amine in lebensmitteln: Zur wirkung von histamine, tyramin und phenylethylamin auf den Menschen. *Z. Lebesm-Unters-Forsch*. **177**: 439-443.

Maijala, R. L. (1993). Formation of histamine and tyramine by some lactic acid bacteria in MRS broth and modified decarboxylation agar. *Lett. Appl. Microbiol*. **17**: 40–43.

Makhzami, S., Quénée, P., Akary, E., Bach, C., Aigle, M., Delacroix-Buchet, A., Ogier, J. –C., Serror, P. (2008). *In situ* gene expression in cheese matrices: Application to a set of enterocccal genes. *J. Microbiol. Methods* **75**: 485-490.

Marcobal, A., de las Rivas, A., García-Moruno, E., and Muñoz, R. (2004). The tyrosine decarboxylation test does not differentiate *Enterococcus faecalis* from *Enterococcus faecium*. *System. Appl. Microbiol*. **27**: 423-426.

Marcobal, A., de las Rivas, B., Moreno-Arribas, M. V., and Muñoz, R. (2005). Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. *J. Food Prot*. **68**: 874-878.

Marcobal, A., de las Rivas, B., and Muñoz, R. (2006a). Methods for the detection of bacteria producing biogenic amines on foods: A survey. *J. Verbr. Lebensm*. **1**: 187-196.

Marcobal, A., de las Rivas, B., and Muñoz, R. (2006b). First genetic characterization of a bacterial β-phenylethylamine biosynthetic enzyme in *Enterococcus faecium* RM58. *FEMS Microbiol Lett*. **258**: 144-149.

Marcobal, A., Martín-Alvarez, P. J., Moreno-Arribas, M. V., and Muñoz, R. (2006c). A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. *Res. Microbiol*. **157**: 417-424.

Marcobal, A., de las Rivas, B., Moreno-Arribas, M. V., Muñoz, R. (2006d). Evidence for horizontal gene transfer as origin of putrescine production in *Oenococcus oeni* RM83. *Appl. Environ. Microbiol*. **72**: 7954-7958.

Masson, F., Johansson, G., Montel, M. C. (1999). Tyramine production by a strain of *Carnobacterium divergens* inoculated in meat-fat mixture. *Meat Sci*. **52**: 65-69.

Masson, F., Talon, R., Montel, M. C. (1996). Histamine and tyramine production by bacteria from meat products. *Int. J. Food Microbiol*. **32**: 199-207.

Masson, F., Lebert, A., Talon, R., Montel, M. C. (1997). Effects of physico-chemical factors influencing tyramine production by *Carnobacterium divergens*. *J. Appl. Microbiol*. **83**: 36-42.

Mauguin, S., Novel, G. (1991). Characterization of lactic acid bacteria isolated from seafood. *J. Appl. Microbiol*. **83**: 36-42.

McCabe-Sellers, B., J., Staggs, C. G., Bogle, M. L. (2006). Tyramine in foods and monoamine oxidase inhibitors drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. *J. Food Compos. Anal*. **19**:S58-S65.

McGilvery, R. W., and Cohen, P. P. (1948). The decarboxylation of L-phenylalanine by *Streptococcus faecalis* R. *J. Biol. Chem*. **174**: 813-816.

Millichap, J. G., Yee, M. M. (2003). The diet factor in pediatric and adolescent migraine. *Pediatr. Neurol*. **28**: 9-15.

Molenaar, D., Bosscher, J. S., ten Brink, B., Driessen, J. M., Konings, W. N. (1993). Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *J. Bacteriol*. **175**: 2864-2870.

Moreno-Arribas, V., Lonvaud-Funel, A. (1999). Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. *FEMS Microbiol. Lett*. **180**: 55-60.

Moreno-Arribas, V., and Lonvaud-Funel, A. (2001). Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine. *FEMS Microbiol. Lett*. **195**: 103-107.

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., and Lonvaud-Funel, A. (2000). Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. *J. Appl. Microbiol*. **88**: 584-593.

Moret, S., Smela, D. Populin, T., Conte, L. S. (2005). A survey on free biogenic amine content of fresh and preserved vegetables. *Food Chem*. **89**: 355-361.

Müller, R., Gerth, K., Brandt, P., Blöcker, H., Beyer, S. (2000). Identification of an L-dopa decarboxylase gene from *Sorangium cellulosum* So ce90. *Arch. Microbiol*. **173**: 303-306.

Mundt, L. J. O. Enterococci, pp. 1063-1065. In Bergey´s Manual of Systematic Bacteriology, vol 1, Krieg N. R. and Holt J. G. (ed), Baltimore, Williams and Wilkins (1984).

Nakazawa, H., Sano, K., Kumagai, H., and Yamada, H. (1977). Distribution and formation of aromatic L-amino acid decarboxylase in bacteria. *Agric. Biol. Chem*. **41**: 2241-2247.

Nakazawa, H., Kumagai, H., and Yamada, H. (1981). Aromatic L-amino acid decarboxylase from *Micrococcus percitreus*. Purification, crystallization and properties. *Agric. Biol. Chem*. **45**: 2543-2552.

Nakazawa, H., Kumagai, H., and Yamada, H. (1983). Cofactor requirements of constitutive aromatic L-amino acid decarboxylase from *Micrococcus percitreus*. *Agric. Biol. Chem*. **47**: 1605-1609.

Nakazawa, H., Kumagai, H., and Yamada, H. (1987). Inhibition of aromatic L-amino acid decarboxylase from *Micrococcus percitreus* by substrate analogs. *Agric. Biol. Chem*. **51**: 2531-2537.

Nannelli, F., Claisse, O., Gindreau, E., de Revel, G., Lonvaud-Funel, A., and Lucas, P. M. (2008). Determination of lactic acid bacteria producing biogenic amines in wine by quantitative PCR methods. *Lett. Appl. Microbiol*. **47**: 594-599.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Trillo-Mesa, A. J., Vidal-Carou, M. C. (2002). Profile of biogenic amines in gota cheese made from pasteurizad and pressurized milk. *J. Food Sci*. **67**: 2940-2944.

Önal, A. (2007). A review: current analytical methods for the determination of biogenic amines in foods. *Food Chem*. **103**: 1475-1486.

Pereira, C. I., Matos, D., San Romáo, M. V., Barreto Crespo, M. T. (2009). Dual role for the tyrosine decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge and generation of a proton motive force. *Appl. Environ. Microbiol*. **75**: 345-352.

Perley J. E., and Stowe B. B. (1966). The production of tryptamine from tryptophan by *Bacillus cereus* (KTV). *Biochem. J*. **100**: 169-174.

Phan, A. P. H., Ngo, T. T., and Lenhoff, H. M. (1983). Tyrosine decarboxylase. Spectrophotometric assay and application in determining pyridoxal-5′-phosphate. *Appl. Biochem. Biotechnol*. **8**: 127-133.

Premont, R. T., Gainetdinov, R. R., and Caron, M. G. (2001). Folllowing the trace of elusive amines. *Proc. Natl. Acad. Sci. USA*. **98**: 9474-9475.

Rice, S., Eitenmiller, R. R., Koehler, P. E. (1975). Histamine and tyramine content of meat products. *J. Milk Food Technol*. **38**: 256-258.

Roig-Sagués, A. X., Hernández-Herrero, M. M., López-Sabater, E. I., Rodríguez-Jerez, J. J., and Mora-Ventura, M. T. (1997). Evaluation of three decarboxylating agar media to detect histamine and tyramine-producing bacteria in ripened sausages. *Lett. Appl. Microbiol*. **25**: 309–12.

Roig-Sagués, A. X., Molina, A. P., Hernández-Herrero, M. (2002). Histamine and tyramine-forming microorganisms in Spanish tradicional cheeses. *Eur. Food Res. Technol*. **215**: 96-100.

Ruiz-Capillas, C., Jiménez-Colmenero, F. (2004). Biogenic amines in meat and meat products. *Crit. Rew. Food Sci. Nutr*. **44**: 489-499.

Sandmeier, E., Hale T. I., and Christen, P. (1994). Multiple evolutionary origin of pyridoxal 5´-phosphate-dependent amino acid decarboxylases. *Eur. J. Biochem*. **221**: 997-1002.

Santos, W. C., Souza, M. R., Cerqueira, M. M. O. P., Glória, M. B. A. (2003). Bioactive amines formation in milk by *Lactococcus* in the presence or not of rennet and NaCl at 20 and 32 ºC. *Food Chem*. **81**: 595-606.

Santos-Buelga C., Nogales-Alarcon, A., and Mariné-Font, A. (1981). A method for the analysis of tyramine in meat products: its content in some samples. *J. Food Sci*. **46**:1794-

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Res. Int*. **27**: 675-690.

Shimazu, S., Miklya, I. (2004). Pharmacological studies with endogenous enhancer substances: beta-phenylethylamine, tryptamine, and their synthetic derivatives. *Prog. Neuropsychopharmacol Biol. Psychiatry* **28**: 421-427.

Steinreiber, J., Schürmann, M., van Assema, F., Wolberg, M., Fesko, K., Reisinger, C., Mink, D., and Griengl, H. (2007). Synthesis of aromatic 1,2-amino alcohols utilizing a bienzymatic dynamic kinetic asymmetric transformation. *Adv. Synth. Catal*., **349**: 1379-1386.

Stratton, J. E., Hutkins, R. W., and Taylor, S. L. (1991). Biogenic amines in cheese and other fermented foods. A review. *J. Food Prot*. **54**: 460-470.

Straub, B., Kicherer, M., Schilcher, S. M., and Hammes, W. P. (1994). Formation of tyramine by *Lactobacillus curvatus* LTH 972. *Z. Lebensm Unters Forsch*. **199**: 9-12.

Straub, B. W., Kicherer, M., Schilcher, S. M., Hammes, W. P. (1995). The formation of biogenic amines by fermentation organisms. *Z. Lebensm. Unters Forsch*. **201**: 79-82.

Suzzi, G., and Gardini, F. (2003). Bigenic amines in dry fermented sausages: a review. *Int. J. Food Microbiol*. **88**: 41-54.

Szabo, A., Billet, E., and Turner, J. (2001). Phenylethylamine, a possible link to the antidepressant effects of exercise. *Br. J. Sports Med*. **35**: 342-343.

Ten Brink, B., Damink, C., Joosten, H. M. L. J., and Huis in`t Veld, J. H. J. (1990). Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol*. **11**: 73-84.

Torriani, S., Gatto, V., Sembeni, S., Tofalo, R., Suzzi, G., Belletti, N., Gardini, F., Bover-Cid, S. (2008). Rapid detection and quantification of tyrosine decarboxylase gene (*tdc*) and its expression in Gram-positive bacteria associated with fermented foods using PCR-based methods. *J. Food Prot*. **71**: 93-101.

Wolken, W. A. M., Lucas, P. M., Lonvaud-Funel, A., Lolkema, J. S. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis*. *J. Bacteriol*. **188**: 2198-2206.

Xin, Y., Li, W., Dwyer, D. S., and First, E. A. (2000). Correlating amino acid conservation with function in tyrosyl-tRNA synthetase. *J. Mol. Biol*. **303**: 287-298.

Zee, J. A., Simard, R. E., L´Hereux, L., Tremblay, J. (1983). Biogenic amines in wines. *Am. J. Enol. Vitic*. **34**: 6-9.

FIGURE LEGENDS

Figure 1. Release of the carboxyl group of tyrosine to form tyramine by action of enzyme tyrosine decarboxylase (TDC).

Figure 2. Comparison of TDC protein sequences from Gram-positive bacteria, such as *Lactobacillus brevis* IOEB 9809 (LBE) (accession AAN77279), *Enterococcus durans* IPLA 655 (EDU) (accession CAF33980), *E. faecalis* JH2-2 (EFS) (accession AAM46082), *E. faecium* RM58 (EFM) (accession CAH04395), *Lactobacillus curvatus* HSCC1737 (LCU) (accession BAE02560), *Carnobacterium divergens* V41 (CDI) (accession ABC68277), and *Tetragenococcus halophilus* (THA) (accession BAD93616). Clustal W program was used to compare predicted sequences. Residues of the HVDAAY motif (●) and those involved in the pyridoxal-phosphate binding (♦) are indicated. Asteriks and dashes indicated amino acid identity and gaps introduced to maximize similarity, respectively.

Figure 3. Genetic organization of the DNA region containing the tyrosine decarboxylase gene (*tyrDC*) in Gram-positive bacteria. The TDC region corresponding to *Lactobacillus brevis* NS25 (accession EU195890), *L. brevis* NS77 (accession EU195891), *L. brevis* ATCC 367 (accession NC_008497), *Enterococcus faecalis* V583 (accession AE016830), *E. durans* IPLA 655 (accession AJ630043), *E. hirae* (accession AY303667), *Carnobacterium*

divergens 508 (accession DQ336701), and *Tetragenococcus halophilus* (accession AB059363) are represented. Arrows indicate ORFs. Genes having putative identical functions are represented by identical shading. The ORFs are *dtp* (permease of the drug/metabolite transporter superfamily), *act* (acetyl-CoA acetyltransferase), *tra* (transposase), *tyrS* (tyrosyl-tRNA synthetase), *tyrDC* (tyrosine decarboxylase), *tyrP* (tyrosine permease), *nhaC-2* (Na+/H+ antiporter), *aguB* (putrescine transcarbamylase), and *aguD* (agmatine/putrescine exchanger).

Figure 4. Scheme of proton motive force generation by decarboxylation and electrogenic antiport. The combination of TyrP (tyrosine/tyramine antiporter) and the TDC (tyrosine decarboxylase) formed a typical bacterial decarboxylation pathway that may generate metabolic energy or to be involved in the acid stress response. A net charge is transported to the outside, corresponding to the proton consumed by the cell.

Figure 5. Comparison of pyridoxal-dependent decarboxylase protein sequences from Gram-negative bacteria, annotated as tyrosine decarboxylases or aromatic-L-amino acid decarboxylases, such those from *Pseudomonas putida* KT2440 (PPU) (accession NP_744697) annotated as putative TDC, *P. putida* W619 (PPU*) (accession ACA72723) annotated as aromatic-L-amino acid decarboxylase, *Pseudomonas entomophila* L48 (PEN) (accession YP_607981) annotated as putative TDC, *Yersinia pestis* CO92 (YPE) (accession NP_404801) annotated as putative pyridoxal-dependent decarboxylase, *Sorangium cellulosum* So ce90 (SCE) (accession CAB71551) an aromatic-L-amino acid decarboxylase, *Granulibacter bethesdensis* CGDNIH1 (GBE) (accession YP_744539) putative TDC, and *Gluconoacetobacter dizotrophicus* Pal 5 (GDI) (accession CAP55834),

also a putative TDC. Clustal W program was used to compare predicted sequences. Residues involved in the binding of pyridoxal-phosphate, including the lysine (K) at the attachment site, are indicated (♦). Asteriks and dashes indicated amino acid identity and gaps introduced to maximize similarity, respectively.

Figure 6. Genetic organization of the DNA region containing the pyrydoxal-dependent decarboxylase protein sequences from Gram-negative bacteria, annotated as tyrosine decarboxylases or aromatic-L-amino acid decarboxylases. The decarboxylase region corresponding to *Pseudomonas putida* KT2440 (accession NC_002947.3), *Pseudomonas entomophila* L48 (accession NC_008027.1), *Yersinia pestis* CO92 (accession NC_003143.1), *Sorangium cellulosum* SoCe90 (accession CAB71551), *Granulibacter bethesdensis* CGDNIHI (accession NC_008343.1), and *Gluconoacetobacter diazotrophicus* Pal 5 (accession AM 889285.1) is represented. Arrows indicate ORFs. Genes coding for decarboxylases (*DC*) are represented in black. Apart from DC, the only common ORF in some of these sequences is a LysR family trasncriptional regulator, represented in grey.

Figure 7. Release of the carboxyl group of phenylalanine, structurally analog to tyrosine, to form phenylethylamine (PEA) by action of the enzyme tyrosine decarboxylase (TDC).

Table 1

Table 1. Tyramine-producer bacteria

Strains	Accession	Reference
Gram-positive:		
Bacillus thuringiensis ATCC 35646[1]	ZP_00743696	
Carnobacterium divergens[1]	ABC68277	
Enterococcus durans IPLA 655[2]	CAF33980	Fernández et al., 2004, 2007
Enterococcus hirae	AAQ73505	Coton et al., 2004
Enterococcus faecalis	AAM46082	Connil et al., 2002
Enterococcus faecium[3]	CAH04395	Marcobal et al., 2006
Lactobacillus brevis IOEB 9809	AAN77279	Lucas et al., 2003
Lactobacillus curvatus	BAE02560	
Tetragenococcus halophilus	BAD93616	
Gram-negative:		
Pseudomonas entomophila L48[1]	YP_607981	Vodovar et al., 2006
Pseudomonas putida KT2440[1]	NP_744697	Nelson et al., 2002
Pseudomonas putida W619[4]	ACA72723	
Gluconacetobacter diazotrophicus PAI 5[1]	EDU29841	
Granulibacter bethesdensis CGDNIH1[1]	ABI61616	Greenberg et al., 2007

[1] Putative
[2] Previously identified as *Lactococcus lactis* IPLA 655
[3] Showed also phenylethylamine production
[4] Annotated as aromatic-L-amino acid decarboxylase

Table 2

Table 2. Primers designed for the PCR detection of bacterial *tyrDC*genes

Primer	5′→3′sequence	Reference
P2-for	GAYATIATIGGIATIGGIYTIGAYCARG	Lucas and Lonvaud-Funel, 2002
P1-rev	CCRTARTCIGGIATIGCRAARTCIGTRTG	Lucas and Lonvaud-Funel, 2002
41	CAYGTNGAYGCNGCNTAYGGNGG	Marcobal et al. 2005
42	AYRTANCCCATYTTRTGNGGRTC	Marcobal et al. 2005
Pt3	TACACGTAGATGCTGCATATG	Constantini et al. 2006
Pt4	ATGGTTGACTATGTTTTAAAAGAA	Constantini et al. 2006
p0303	CCACTGCTGCATCTGTTTG	Lucas et al. 2003
TD5	CAAATGGAAGAAGAAGTAGG	Coton et al., 2004
TD2	ACATAGTCAACCATRTTGAA	Coton et al., 2004
57	ATGAGTGAATCATTGTCG	Marcobal et al. 2004
58	TTATTTTGCTTCGCTTGCC	Marcobal et al. 2004
TDC1	AACTATCGTATGGATATCAACG	Fernández et al. 2004
TDC2	TAGTCAACCATATTGAAATCTGG	Fernández et al. 2004
TDC-F	TGGYTNGTNCCNCARACNAARCAYTA	De las Rivas et al. 2006
TDC-R	ACRTARTCNACCATRTTRAARTCNGG	De las Rivas et al. 2006
tdcC1	CTGGTGGGATTGCTATTC	Aymerich et al., 2006
tdcC2	TTCGTTCATTTCCACAAGG	Aymerich et al., 2006
DEC2	GAAMCTARATGCASTAKTCAT	Torriani et al., 2008
DEC3	CCGCCAGCAGAATATGGAAYRTANCCCAT	Torriani et al., 2008
DEC5	CGTTGTTGGTGTTGTTGGCACNACNGARGARG	Torriani et al., 2008
TYR3f	CGTACACATTCAGTTGCATGGCAT	Torriani et al., 2008
TYR4r	ATGTCCTACTTCTTCTTCCATTTG	Torriani et al., 2008
tdcf	CAAATGGAAGAAGAAGTTGG	Nannelli et al., 2008
tdcr	GAACCATCAGCAACAATGTG	Nannelli et al., 2008

K = G or T; R = A or G; W = A or T; Y = C or T; S = C or G; M = A or C; D = A, G, or T; N = A, G, C, or T.

Figure 1

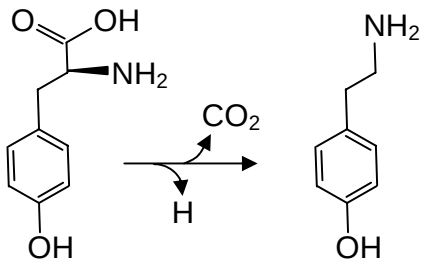


Figure 2

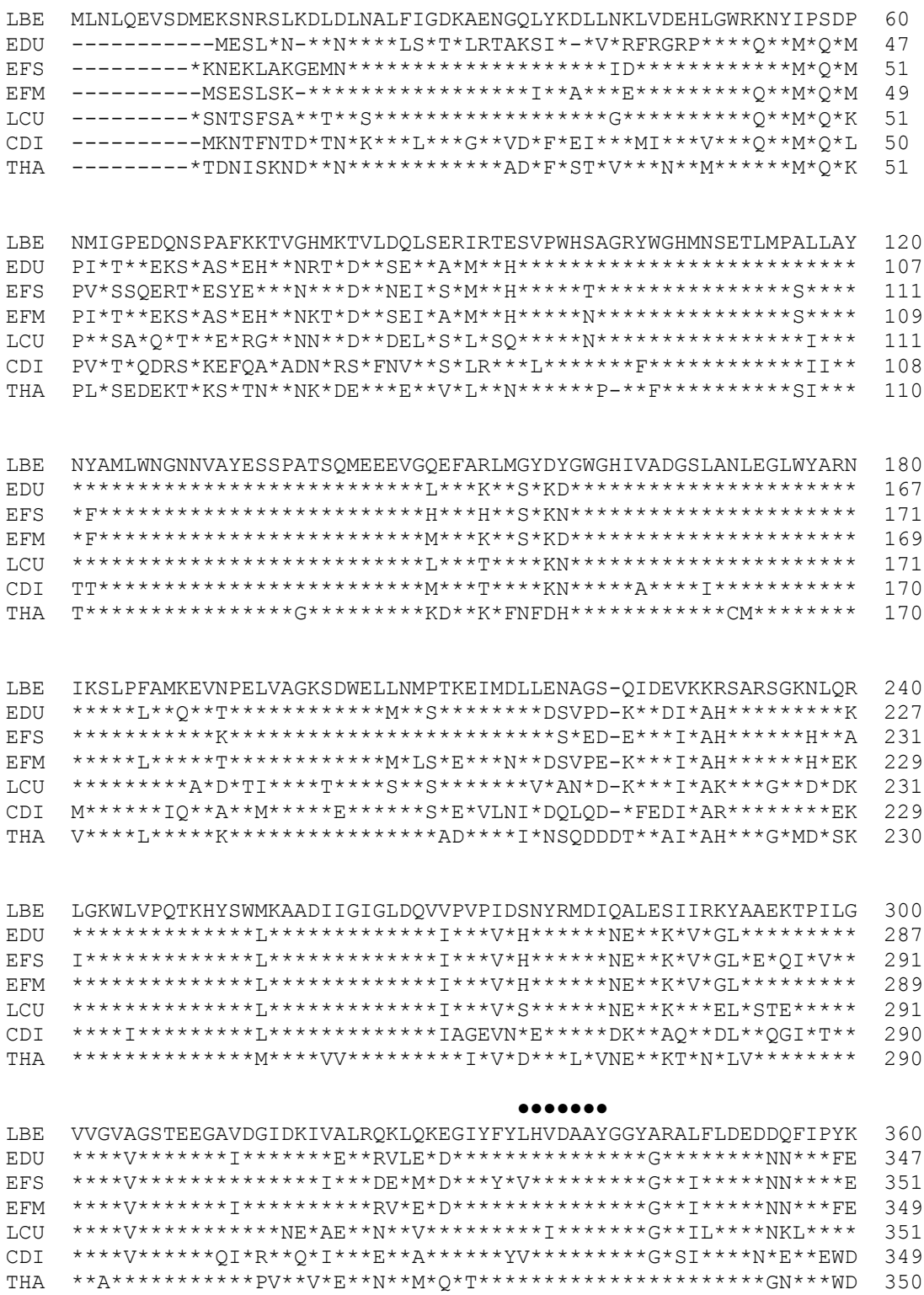

LBE MLNLQEVSDMEKSNRSLKDLDLNALFIGDKAENGQLYKDLLNKLVDEHLGWRKNYIPSDP 60
EDU ----------MESL*N-**N****LS*T*LRTAKSI*-*V*RFRGRP****Q**M*Q*M 47
EFS ---------*KNEKLAKGEMN*******************ID***********M*Q*M 51
EFM ----------MSESLSK-*****************I**A***E*********Q**M*Q*M 49
LCU ---------*SNTSFSA**T**S*****************G*********Q**M*Q*K 51
CDI ----------MKNTFNTD*TN*K***L***G**VD*F*EI***MI***V***Q**M*Q*L 50
THA ---------*TDNISKND**N***********AD*F*ST*V***N**M******M*Q*K 51
LBE NMIGPEDQNSPAFKKTVGHMKTVLDQLSERIRTESVPWHSAGRYWGHMNSETLMPALLAY 120
EDU PI*T**EKS*AS*EH**NRT*D**SE**A*M**H************************ 107
EFS PV*SSQERT*ESYE***N***D**NEI*S*M**H*****T**************S**** 111
EFM PI*T**EKS*AS*EH**NKT*D**SEI*A*M**H*****N**************S**** 109
LCU P**SA*Q*T**E*RG**NN**D**DEL*S*L*SQ*****N***************I*** 111
CDI PV*T*QDRS*KEFQA*ADN*RS*FNV**S*LR***L*******F***********II** 108
THA PL*SEDEKT*KS*TN**NK*DE***E**V*L**N******P-**F**********SI*** 110
LBE NYAMLWNGNNVAYESSPATSQMEEEVGQEFARLMGYDYGWGHIVADGSLANLEGLWYARN 180
EDU **************************L***K**S*KD********************** 167
EFS *F*************************H***H**S*KN********************** 171
EFM *F*************************M***K**S*KD********************** 169
LCU **************************L***T****KN********************** 171
CDI TT************************M***T****KN*****A****I********** 170
THA T****************G*********KD**K*FNFDH***********CM******** 170
LBE IKSLPFAMKEVNPELVAGKSDWELLNMPTKEIMDLLENAGS-QIDEVKKRSARSGKNLQR 240
EDU *****L**Q**T***********M**S********DSVPD-K**DI*AH*********K 227
EFS **********K************************S*ED-E***I*AH******H**A 231
EFM *****L*****T***********M*LS*E***N**DSVPE-K***I*AH******H*EK 229
LCU *********A*D*TI****T****S**S*******V*AN*D-K***I*AK***G**D*DK 231
CDI M******IQ**A**M*****E******S*E*VLNI*DQLQD-*FEDI*AR********EK 229
THA V****L*****K***************AD****I*NSQDDDT**AI*AH***G*MD*SK 230
LBE LGKWLVPQTKHYSWMKAADIIGIGLDQVVPVPIDSNYRMDIQALESIIRKYAAEKTPILG 300
EDU **************L*************I***V*H******NE**K*V*GL********* 287
EFS I*************L*************I***V*H******NE**K*V*GL*E*QI*V** 291
EFM **************L*************I***V*H******NE**K*V*GL********* 289
LCU **************L*************I***V*S******NE**K***EL*STE***** 291
CDI ****I*********L*************IAGEVN*E*****DK**AQ**DL**QGI*T** 290
THA **************M****VV*********I*V*D***L*VNE**KT*N*LV******** 290
LBE VVGVAGSTEEGAVDGIDKIVALRQKLQKEGIYFYLHVDAAYGGYARALFLDEDDQFIPYK 360
EDU ****V*******I*******E**RVLE*D**************G********NN***FE 347
EFS ****V***************I***DE*M*D***Y*V*********G**I*****NN****E 351
EFM ****V*******I**********RV*E*D***************G**I*****NN***FE 349
LCU ****V***********NE*AE**N**V*********I*******G**IL****NKL**** 351
CDI ****V******QI*R**Q*I***E**A******YV*********G*SI****N*E**EWD 349
THA **A***********PV**V*E**N**M*Q*T**********************GN***WD 350

Figure 2 (cont.)

```
                                                ♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦
LBE  NLQKVHAENHVFTEDKEYIKPEVYAAYKAFDQAESITIDPHKMGYVPYSAGGIVIQDIRM  420
EDU  **KD**FKHN****N*N**LE**HS****IEE***V******************K*V**  407
EFA  D**D**E*YG**K*K**H*SR***D****IEL***V*********I**************  411
EFC  D*KD**YKYN****N*D**LE**HS****IEE***V******************K****  409
LCU  D**SKF**YN****EENLVSEHT*N**A**PE***V*********I*******A******  411
CDI  QIEA*Y*K*GI*M*KNDWLTR***ESF**ISL***V*********I******VV*K****  409
THA  K**E**QKYGD*Q*N****SKDT*N*F**ISE**TV*****************A*RN*T*  410

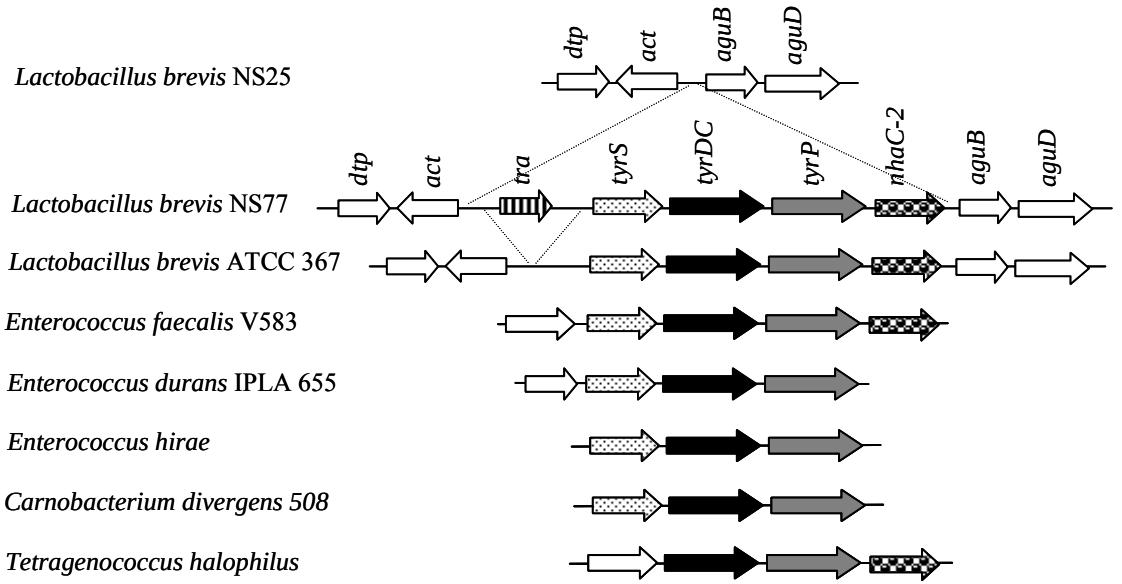
LBE  RDTISYFATYVFEKGADIPALLGAYILEGSKAGATAASVWAAHHTLPLNVTGYGKLEGAS  480
EDU  **V*****************************************V**********M***  467
EFA  **V*****************************************V*****A*****I***  471
EFC  **V*****************************************V**********M***  469
LCU  **V*****************************************K***********V***  471
CDI  **V*********************************A**T**K***********M***  469
THA  *NI*********D*RSKA*SM***************A**T**RV*****A*****I*R*  470

LBE  IEGAHRYYDFLKNLKFEVAGKRISVHPLISPDFNMVDYVLKEDGNDDLIEMNRLNHAFYE  540
EDU  ******F*N**N**S*K*GD*E*E****TY*********F**K*****VA**K***DV*D  527
EFS  ***S*HF****ND*T*K*GD*E*E**T*TH*********F**K*****VA**K***DV*D  531
EFM  ******F*N**ND*S*K*GD*E*E****TY*********F**K*****VA**K***DV*D  529
LCU  ****R*F*N**SG*E*K*GD*T*E****TD*********FQ*K**NN*V***E***E**N  531
CDI  ****YHF*H*IDGKE*K*GD*T*EL*A*TK*********FN*K**T**VK**K***D**D  529
THA  **A*N*F*D**NN*S*NINGQE*E*N**TK*******WTFNIK****FAK**D**L***Q  530

LBE  QASYVKGSLYGKEYIVSHTDFAIPDYGDSPLAFAESLGFSEVEWRHAGKVTIIRASVMTP  600
EDU  YS******I**N*FLT**********N***Q*VNQ****DE**NR*****VL*******  587
EFS  Y******NI*NN*F*T**********N***K*VNS****DE**NR*****VL**A****  591
EFM  YS******I**N*FLT**********N***Q*VNQ****DE**NR*****VL*******  589
LCU  ********I**N*YLT**********N**FK*VNN**VTEEG*KE*****VL**A****  591
CDI  Y***A**G**NN*F*T*******EE**H**FE*VNG****RK**ER*D****L***A*S*  589
THA  E**F***PI*NNDFIT******QDG*****K*YVK****TDAD*QKEKA*G****C*L**  590

LBE  YMNQRENFDYFAPRIKKAIQADLEKVYASVNQKENV-  636
EDU  ***KE*H*EEY*EK**A*L*EK***I**DQLLASEAK  624
EFS  ***DK*E***Y**K*QA*L*EK**QI*DVK-------  621
EFM  ***KE*H*EEY*EK**A*L*EK***I**DQLLASEAK  626
LCU  ***KA********K**Q*M*EK**AI*EDRK------  622
CDI  ***DK*V**EY*AK*EA***SK**AI**EEN------  620
THA  LLYDKDN*AEY*DK**S*MTEK*T*IVDEKVLQT*--  624
```

Figure 3


dtp
act
aguB
aguD
Lactobacillus brevis NS25
dtp
act
tra
tyrS
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tyrP
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aguB
aguD
Lactobacillus brevis NS77
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Carnobacterium divergens 508
Tetragenococcus halophilus

Figure 4

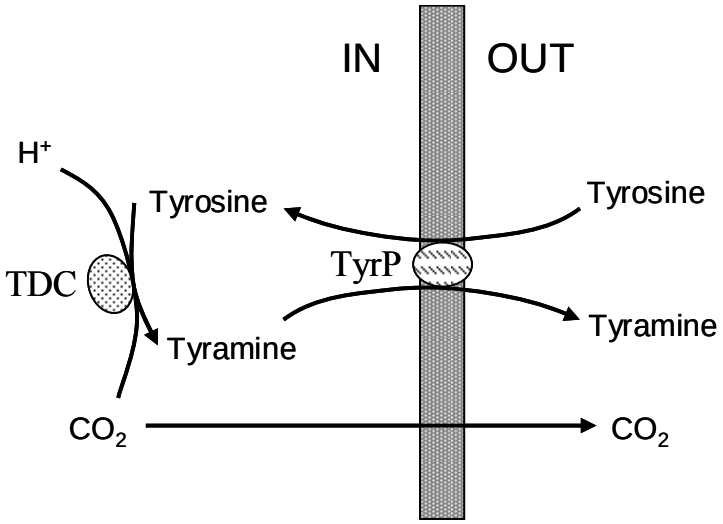
IN
OUT
H+
Tyrosine
Tyrosine
TDC
TyrP
Tyramine
Tyramine
CO2
CO2

Figure 5


PPU ----------MTPEQFRQYGHQLIDLIADYRQTVGERPVMAQVEPGYLKAALPATAP 47
PPU* ----------**G** 47
PEN ----------**********************A*****************DM** 47
YPE ----------****E**RH*YAM*********NIEL*G*NPTTA**EI*SR*SLN** 47
SCE --MSDPRKERLGD*DL*E**RI*MRI**WA***LGHPDRY**FPAIR**DV*GR*AP*P* 58
GBE MSETPCVADGLD*DDWDAL*SL**RML*DMF*HLAGARSGA*WRPLPH-QVRDVFKTP** 59
GDI -------MTGLD*DNWDDL*SL**RMV*DMI*RLA*LRDG**WRPMPP-EIRTA*HTDL* 52
PPU QQGEPFAAILDDVNNLVMPGLSHWQHPDFYGYFPSNGTLSSVLGDFLSTGLGVLGLSWQS 107
PPU* **************T** 107
PEN **A***E***K**DQ*L** 107
YPE EKA***EH*IS*IEE*I****L*******F******VE*******C*******I****** 107
SCE VEP**MD*V*T*FEQIIL**IT**N**R*FA**ANTASGPGI**EL*AAC*N*NVML*RT 118
GBE RD*SSLESVYEQFR*D*Q*YATGNI**R*M*WVHGG**AVGM*A*M*AAG*NAN--LGGR 117
GDI AAPT*PE*LY*RFRDQIA*YATGNT**G*M*WVHGG**GVGM*AEL*AG**NAN--CGGR 110
PPU SPALSELEETTLDWLRQLLGLSGQWSGVIQDTASTSTLVALISARERATDYALVRGGLQA 167
PPU* **************V*******E******************C***************** 167
PEN **********************S******************C****T************ 167
YPE ****T*I**VATN**LKM****VA*******S***A*******G***TSN***MNK*M*N 167
SCE ***AT****LV*S****M*D*DAGLH*A*M*****A*M**IAA**DS*EPTIRL**--M* 176
GBE DH*PV*V*RQVIR*SAE***MPSET**ILVTGS*MANFIGILV*-RL*ALGPQ**HE-GV 175
GDI DH*PV*I*R*VIG*AAEIM*FPADAT**LVTGS*MANMI*V*T*SRAVA*GITL*QR-GV 169
PPU EPKPLIVYVSAHAHSSVDKAALLAGFGRDNIRLIPTDERYALRPEALQAAIEQDLAAGNQ 227
PPU* *A*************************A*****A***QF*M*****L********D**L 227
PEN QAR**V*********************************QF*M*****R***DK******* 227
YPE SDA*****T*SE***G*N*********HN*T**V****HN*MS*L**KKIIQ***VK**R 227
SCE GQRRMRLYA*EQA***IE***ITL*I*QEGV*K****PAFRMV****R**VVE**G**LR 236
GBE GQSGFTA*AG*N**GCIPR*MDM****S*AL*SV*L*DAHCM*LD**A*MVAE*R*Q*RL 235
GDI GGRQ*VG*AA*T**GCIAR*FD***L*T*AL*I**V*STHRMDIQ**R**V*SDRQQGFE 229
PPU PCAVVATTGTTTTTALDPLRPVGEIAQANGLWLHVDSAMAGSAMILPECRWMWDGIELAD 287
PPU* **********A********Q*******Q****************************** 287
PEN ********************AI************************************** 287
YPE **I********AA**M***DAI*D*T*RY**********************HL*C***K** 287
SCE *FC*A**V***S**SV**IPAIVSVCREH*******A*Y**M*A*V**H*DVLA*C*G** 296
GBE *AL*I**AGSVD*G*I*D*DALATFCA*E*MR****G*FGAMLALS*VL*HRVN*LAR** 295
GDI *FI*IG*A**VD*GSI*D*GAIAD**GQEDI*F***G*FGAL**LSRTL*PHLR*LDR** 289
♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦
PPU SVVVNAHKWLGVAFDCSIYYVRDPQHLIRVMSTNPSYLQSA---VDGEVKNLRDWGIPLG 344
PPU* **---********** 344
PEN **---********** 344
YPE ***I*P*****A*****V*C*Q*SE****I******F***D---A**K*R*Y******** 344
SCE *L***PH***FTPM***VL****ADR*K*AF*LV*E*LR-----TEGD*T*YM****Q** 351
GBE *IAFDF***PQ*PY*AGCVL****VLHRQAFAQDLA**RRDIRGLS*GAPWPCDL*PD*S 355
GDI *LAFDF***AQ*PY*AGCIL**E*GRQAAAFAQSLA**SREDRGLA*NAPWFCDL*PD*S 349

Figure 5 (cont.)

```
PPU  RRFRALKLWFMLRSEGVDALQARLRRDLDNAQWLAGQVEAAAEWEVLAPVQ----LQTLC 400
PPU* ****************E***Q*********R*****I***G**V******----***** 400
PEN  ******************N**Q*********R***E**Q*SG***L******----***** 400
YPE  **********LI*EQG*EG********IA**N***E*IKNSDD*KLV***V----***** 400
SCE  *********MIV*YF*HEG*A**I*EH*RLG*Q**QW*D*DPD**R***TP----FSTV* 407
GBE  *G*****V*M**KTY*ADR*G*VVETCC*V*TH**AR*A*EP*L*L***LPG*NG*NVV* 415
GDI  *G*****V*MT*GTY*TVR*GQMVDECCAV*AH**RR*DREPLL*R****----G*N*V* 405

PPU  IRHRPAGLEG--------------EALDAHTKGWAERLNASGAAYVTPATLDGRWMVRVS 446
PPU* **********--------------********A*********E********N******** 446
PEN  *****D****----------------********R***D******D********E******** 446
YPE  E*G**MGD**----------------****NY*RS***K**Q**KG*******G******** 446
SCE  F*M**SA*ACIMRSADEAERESIERE**RLNEALLDEV*K**RVFLSHTR*HG*YTI**A 467
GBE  FRFIAA-----------------PGD**RLNIELVADVQE**V*VPSTTH*R*TLAI*AA 475
GDI  FRVRVP-----------------DVE**WLNGELVKD*HE**I*APSTTMVG*VKAIRAA 465

PPU  IGALPTERGDVQRLWARLQDVIKG---------------------- 470
PPU* V*******EH*EQ**Q***A*VN*---------------------- 470
PEN  V*******EH*E***Q***T*VA---------------------- 469
YPE  I*T*G***HH*EK**LL**SLV*ER----------------------471
SCE  I*NIRSDEVA*REA*EC*RAAGARLCADERFVSCSRSADEGRGKS 512
GBE  *VNHR*R*E*ADI*VNAVLKAGRCRVSQLVATG------------ 491
GDI  *VNHRTVAAD*DLMVDAVLRLAQERQGLGQRA------------- 480
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Figure 6

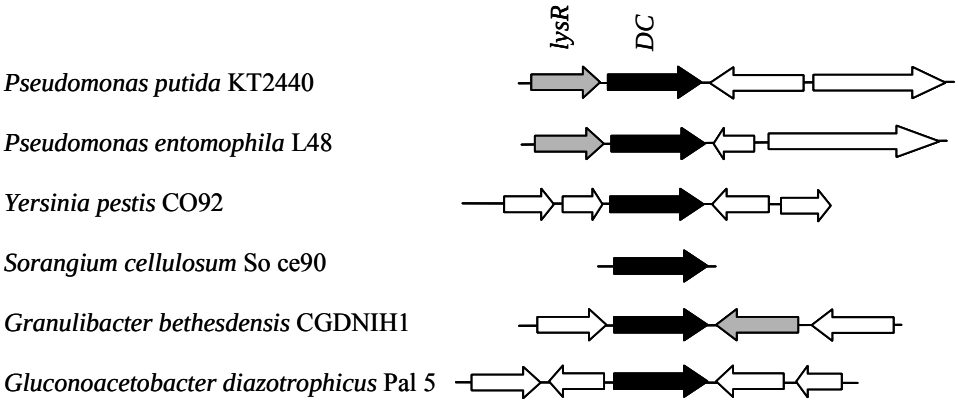
lysR
DC
Pseudomonas putida KT2440
Pseudomonas entomophila L48
Yersinia pestis CO92
Sorangium cellulosum So ce90
Granulibacter bethesdensis CGDNIH1
Gluconoacetobacter diazotrophicus Pal 5

Figure 7