The tyrosine decarboxylation test does not differentiate *Enterococcus faecalis* from *Enterococcus faecium*

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Running title

Tyrosine decarboxylation test in enterococci

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Summary

According to the current edition of the Bergey's Manual of Systematic Bacteriology [11] the tyrosine decarboxylation test allows the differentiation of enterococci. Tyrosine is decarboxylated to the biogenic amine tyramine by *E. faecalis* and not by *E. faecium* strains. In the present study we sequenced the 16S rDNA of two tyramine-producing strains, BIFI-56 and BIFI-58, presumptively classified as *E. faecalis*. Their 16S rDNA were identical to the same fragment from the *E. faecium* type strain. Several *E. faecium* strains were then checked for their ability to decarboxylate tyrosine and also a putative tyrosine decarboxylase-coding gene was PCR amplified from these strains. All the strains confirmed as *E. faecium* produced tyramine and possessed a DNA fragment coding for a putative tyrosine decarboxylase. The concordance of the two methods allows us to conclude that the tyrosine decarboxylase test cannot be used in the differentiation of *E. faecalis* from *E. faecium* since at least some *E. faecium* strains are tyramine producers.

Key words: Tyramine production – biogenic amines – food safety – *Enterococcus faecium* – *Enterococcus faecalis* – Tyrosine decarboxylase – taxonomy

Main text

Biogenic amines, as histamine and tyramine, have been implicated in food poisoning incidents, usually from the consumption of fermented foods like cheese, meat, fish products and wine. Biogenic amines in food are mainly formed by decarboxylation of the corresponding amino acids by microorganisms. Enterococci have been implicated in cases of food poisoning, e.g. by production of biogenic amines, based on their isolation in high numbers from suspect foods [19]. Enterococci are ubiquitous microorganisms, but have a predominant habitat in the gastrointestinal tract of humans and animals. Because of their heat tolerance and survival under adverse environmental conditions, enterococci can colonise diverse niches and may then serve as indicators of the sanitary quality of food. Indeed, enterococci commonly occur in large numbers in vegetables, plant material and foods, especially those of animal origin such as fermented sausages and cheeses. In processed meats, enterococci are generally not desirable because they cause spoilage [6]. On the contrary, enterococci have important implications in the dairy industry. The beneficial role of enterococci in the development of cheese aroma has led to the inclusion of enterococcal strains in certain starter cultures. *Enterococcus faecium* strain K77D has been considered acceptable for use as a starter culture in fermented dairy products by the UK Advisory Committee on Novel Foods and Processes [16]. Some food-borne enterococci also share a number of useful biotechnological traits (e.g. bacteriocin production, probiotic properties), which led to earlier applications in fermented foods [6].

The identification of the enterococci has always been problematic. It is difficult to unequivocally categorise isolates into one of the *Enterococcus* species by physiological tests because heterogeneity in phenotypic features is very high, regardless of the origin of the isolates [5, 13, 14]. The precise differentiation of enterococcal species has taken on additional importance because of the acquisition of antibiotic resistance traits among strains [17].

Enterococci are Gram-positive catalase negative cocci that in many food microbiology laboratories are classified according to the scheme proposed in the current edition of the Bergey's Manual of Systematic Bacteriology, 1st ed. [11]. In this scheme, on the basis of sugar fermentations, tetrazolium reduction and tyrosine decarboxylation it is possible to differentiate *Enterococcus faecalis* from *E. faecium*. The amino acid

tyrosine is reported to be decarboxylated to the biogenic amine tyramine by *E. faecalis* and not by *E. faecium*.

The purpose of the present work was to assess the usefulness of the decarboxylase test to differentiate *E. faecalis* from *E. faecium* strains given that the production of biogenic amines is a relevant property related to the technological performance of enterococci in some fermented foods.

Material and Methods

Two tyramine-producing strains were isolated in a wide screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine [10]. These strains, BIFI-56 and BIFI-58, belonging to the bacterial culture collection from the Instituto de Fermentaciones Industriales (BIFI), showed the following characteristics: both were gram-positive cocci, catalase negative and bile esculin positive, did not produce gas from MRS broth [4], grew in culture broth containing 6.5% NaCl, and grew at 10 °C and 45°C. Both strains can be identified presumptively as belonging to the genus *Enterococcus*. Since both strains produced ammonia from arginine, according to the Bergey's scheme they should be classified as *E. faecalis* or *E. faecium*. Taking into account that they were tyramine producers, both were considered *E. faecalis* strains. In order to complete the identification of these strains we sequenced the 16S rDNA. Chromosomal DNA from both strains was isolated by using a protocol previously described [2]. By using chromosomal DNA, 16S rDNAs were PCR amplified using the pair of primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') (W = A or T) (numbering is based on the *Escherichia coli* 16S rRNA gene) previously described [9]. The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR amplifications were performed as described previously [12]. Amplified DNA fragments were sequenced. DNA sequencing was carried out by using an Abi 377^{TM} DNA sequencer (Applied Biosystems, Inc). Sequence data for the first 620 pb of the 16S rRNA amplified gene from both strains were identified by database comparison using Basic local alignment search (BLAST) [1] on the EMBL/GenBank nucleotide databases.

Results and discussion

These sequences of strains BIFI-56 and BIFI-58 showed a 100% identity to the same fragment of the 16S rRNA sequence from the *E. faecium* type strain (ATCC 19434, DSM 20477). Therefore the 16S rRNA sequence revealed that both strains were *E. faecium* isolates.

In order to know if both strains were atypical for the tyramine production test, three additional *E. faecium* strains available from the Spanish Type Culture Collection were tested for the tyrosine decarboxylation test. The *E. faecium* strains CECT 410 (type strain, ATCC 19434) CECT 964, and CECT 4102 and the tyramine-producers BIFI-56 and BIFI-58 were grown in MRS broth (Difco, France) containing 0.5% of the precursor amino acid, tyrosine di-sodium salt (Sigma, USA). The *E. faecium* strains were incubated at 30 °C for 7 days. After the incubation time, the tyramine present in the supernatants of the bacterial cultures was determined by thin-layer chromatography (TLC). Tyramine was converted to its fluorescent dansyl derivative using a modification of the method of Rosier and Petergham [15]. The fractionation and detection of the amines was performed by the method of Shakila et al. [18]. The fluorescent dansyl derivative zone was visualized with a UV-light source (312 nm). A standard tyramine solution was prepared by the same protocol. Figure 1 shows the TLC separation of tyramine from bacterial extracts and the tyramine standard control. Extracts prepared from the *E. faecium* strains CECT 410, CECT 4102, BIFI-56 and BIFI-58 showed tyramine spots, whereas the spot was absent in extracts obtained from *E. faecium* CECT 964. In order to assess the taxonomic classification of *E. faecium* CECT 964, chromosomal DNA was extracted and the 16S rDNAs were PCR amplified, sequenced and analyzed as described above. The sequence was identical to the same fragment of the 16S rRNA sequence from the *E. flavescens* type strain (DSM 7370). *E. faecium* CECT 964 (ATCC 14432, NCIMB 9645) was originally isolated from grass silage [8] and deposited in the ATCC as *Streptococcus faecium* ssp. *mobilis*. Later, it was reclassified as *E. casseliflavus* and provided by the CECT as *E. faecium*. This strain was originally described as lacking an active tyrosine decarboxylase system [8] in concordance to the results described in this study.

Recently, Connil et al. [3] reported the identification of the *E. faecalis* tyrosine decarboxylase operon involved in tyramine production. The tyrosine decarboxylase is a 620-amino acid residue protein having homologies with amino acid decarboxylases.

Searching in the databases for proteins similar to the *E. faecalis* tyrosine decarboxylase, a protein from the uncompleted *E. faecium* genome shows a 85% identity. Preliminary sequence data of the *E. faecium* genome were obtained from the DOE Joint Genome Institute at http://genome.jgi-psf.org/draft_microbes/entfa/entfa.home.html. This protein codes for a putative tyrosine decarboxylase in *E. faecium*. In order to check the presence of the gene coding for this protein in the *E. faecium* strains under study, two oligonucleotides were designed. We used primer 57 (5'-ATGAGTGAATCATTGTCG-3') and 58 (5'-TTATTTTGCTTCGCTTGCC-3') to amplify a 1.9 kb DNA fragment including the complete gene coding for the putative tyrosine decarboxylase. PCR amplifications were performed as above and the resulting DNA fragments were analyzed in an agarose gel (Figure 2). All the tyramine producer strains gave a 1.9 kb DNA fragment. The strain CECT 964 did not amplify the DNA fragment. These results agreed with those obtained from the chromatographic analysis.

In summary, all the *E. faecium* strains analysed were unexpectedly able to produce tyramine from tyrosine. In a recent study, Sarantinopoulos et al. [16] reported that 124 of the 129 enterococci analyzed produced tyramine from tyrosine, the producers strains belonged to both, *E. faecalis* and *E. faecium*, species. Previously, Giraffa et al. [7] described that 92% of the *E. faecalis* and 84% of *E. faecium* strains analyzed, produced tyramine. However, in both cases the production of biogenic amines was only evaluated in a decarboxylase agar medium where, sometimes, false-positive reactions have been described [10]. The results of our study demonstrate that the *E. faecium* strains analyzed actually possess a gene coding for a putative tyrosine decarboxylase possibly involved in the tyramine production which in turn is unequivocally detected by TLC.

It is important to bear in mind that the production of biogenic amines is a relevant property in food technology. In order to evaluate the potential risk of enterococci occurring in some food products and their possible use as starters for cheese production, detailed information about the biochemical properties of lactic acid bacteria is the first step in strain selection for technological applications in the food industry. Concerning food technology, tyrosine decarboxylase activity has long been considered a taxonomically important characteristic used for the identification of enterococci.

The number of the *E. faecium* strains analyzed in this study is too low to conclude that tyrosine decarboxylation is a general feature of this species but enough to demonstrate the uselessness of the tyrosine-decarboxylase test to distinguish *E. faecalis*

from *E. faecium*. The inclusion of tyrosine decarboxylase-positive strains within *E. faecium* will necessitate a revision in the species identification scheme for the enterococci. Bergey's test scheme for the identification of enterococcal species should be modified to include tyramine producer *E. faecium* strains.

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Figure legends

Figure 1. TLC separation of tyramine produced by *Enterococcus faecium* strains. Enterococci were grown in MRS broth containing 0.5% tyrosine. The tyramine produced in the media during growth was derivatized, fractionated and detected. Tyramine present initially in the growth media (1), tyramine produced by *E. faecium* CECT 410 (2), CECT 964 (3), CECT 4102 (4), BIFI-56 (5) and BIFI-58 (6), and tyramine standard solution (7).

Figure 2. PCR amplification of a putative tyrosine decarboxylase coding gene from *E. faecium* strains. Appropriate nucleotides were used to amplify a 1.9 kb fragment containing the complete tyrosine decarboxylase gene. DNA was prepared from the following *E. faecium* strains: CECT 410 (1), CECT 964 (2), CECT 4102 (3), BIFI-56 (4) and BIFI-58 (5). The molecular size of the standard (kb) are indicated at the left.