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# Improved Multiplex-PCR Method for the Simultaneous Detection of Food Bacteria Producing Biogenic Amines

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**Abstract**

This study describes a simple and rapid multiplex-PCR method to determine the ability to produce histamine, tyramine and putrescine by bacteria. The assay is an improved method based on an assay designed by lactic acid bacteria. This improved method includes a pair of primers based on sequences from histidine decarboxylases from gram-negative bacteria. Under the optimised conditions, the assay yielded a 367-bp DNA fragment from histidine decarboxylases of gram-positive bacteria, 534-bp fragment from histidine decarboxylases of gram-negative bacteria, 924-bp from bacterial tyrosine decarboxylases, and 1446-bp fragment from bacterial ornithine decarboxylases. The method was successfully applied to several biogenic amine-producing bacterial strains, even when DNAs of several target organisms were included in the same reaction. This simple method could be easily incorporated in food control laboratories to detect potentially biogenic amine-producing bacteria in foods.

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## 1.Introduction

Biogenic amines are organic bases of low molecular weight, possessing biological activity. Although biogenic amines such histamine, tyrosine and putrescine are needed for many critical functions in man and animals, consumption of foods containing high amounts of these amines can have toxicological effects. A typical phenomenon is the "cheese reaction", usually caused by high levels of tyramine in cheese. However, the most notorious food-borne intoxications caused by biogenic amines are related to histamine. Histamine poisoning is often referred to as "scombroid fish poisoning" because of the frequent association of this illness with the consumption of scombriod fish, such as tuna, mackerel and sardines [1]. Putrescine, although seems to have a lower pharmacological activity than histamine and tyramine, hampers the detoxification of histamine and/or tyramine. Moreover, putrescine can react with nitrite to form carcinogenic nitrosamines. For this reason it is particularly important to prevent the accumulation of biogenic amines in cured food products [2].

Biogenic amines are present in a wide range of food products, including fish products, meat products, dairy products, wine, beer, vegetables, fruits, and nuts [3]. In non-fermented foods, the presence of biogenic amines is indicative of undesired microbial activity [4]. In most amine-containing foods the majority of the amines are generated by decarboxylation of the corresponding amino acids through substrate-specific enzymes derived from microorganisms present in the food. Although amino acid decarboxylases are not widely distributed among bacteria, species of many genera are capable of decarboxylating one or more amino acids. However, the ability of microorganims to decarboxylate amino acids is highly variable. It depends not only on the species, but also on the strain and the environmental conditions [5, 6].

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Early detection of biogenic amine-producing bacteria is important in the food industry because there are cause of food poisoning. Therefore, the use of methods for the early and rapid detection of these bacteria is important for preventing biogenic amine accumulation in food products. Molecular methods for detection and identification of food-borne bacteria are becoming widely accepted as an alternative to traditional culture methods. PCR has become an important method for the rapid, sensitive, and specific detection of targeted genes. PCR techniques targeting bacterial amino acid decarboxylase genes have been previously designed. Le Jeune et al. [7] developed three primer sets for the detection of histamine-producing lactic acid bacteria; similarly, a primer set for the detection of histamine-producing gram-negative bacteria was described [8]. Recently, Coton et al. [9] described a primer set for the detection of tyramine-producing lactic acid bacteria. All these methods detect bacteria producing only one biogenic amine; therefore, a multiplex-PCR method for the simultaneously detection of lactic acid bacteria producing histamine, tyramine and putrescine was developed in our laboratory recently [10].

While histamine in fermented products is produced by gram-positive lactic acid bacteria, histamine in raw fish products is caused mostly by gram-negative enteric bacteria such as *Morganella morganii*, *Klebsiella* spp., etc. Similarly, most Enterobacteriaceae, gram-negative bacteria, are capable of producing putrescine in food products. Therefore, our previously described multiplex-PCR method should be improved in order to detect also gram-negative biogenic amine-producing bacteria in foods.


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## 2. Materials and methods

### *2.1. Strains and growth conditions*

The following pure cultures of histamine-producing bacterial strains were purchased from the Spanish Type Culture Collection (CECT): *Klebsiella planticola* CECT 843, *Proteus vulgaris* CECT 484, *Photobacterium phosphoreum* CECT 4192. The CECT also provided *Morganella morganii* CECT 173, histamine and putrescine producing, and the tyramine-producing *Lactobacillus brevis* CECT 5354. *Lactobacillus* 30a (ATCC 33222), an histamine- and putrescine-producing strain was purchased from the American Type Culture Collection (ATCC). An histamine producing *Staphylococcus* sp. strain and a putrescine-producing *Serratia liquefaciens* strain, were kindly provided by Dr. A. V. Carrascosa; both strains were isolated from meat products and belong to his bacterial collection. *Escherichia coli* DH5α, a laboratory K-12 strain, was used as putrescine-producing gram-negative strain.

Lactic acid bacteria strains were grown in MRS broth (Difco, France) and incubated at 30 ºC. Gram-negative bacteria were growth in nutritive agar (Difco, France) and incubated at the temperature recommended by the CECT.

Production of biogenic amines was tested by growing each strain in the decarboxylase medium described by Maijala [11]. The medium contained the corresponding precursor amino acid (L-histidine monohydrochloride, tyrosine disodium salt, and L-ornithine monohydrochloride) at a 0.5% final concentration. The precursor amino acids were purchased from Sigma (St. Louis, MO, USA). A positive result is indicated by a change to purple in response of the indicator to a pH shift.


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1 *2.2. DNA manipulations and PCR procedures*

2

3 Chromosomal DNA extraction was carried out as described elsewhere [12]. PCR
4 products were carried out in 1.5 % agarose gels and stained with ethidium bromide as
5 described [13]. PCR amplifications were performed as described previously [10].
6 Briefly, the PCR was performed in 25-µl amplification reaction mixture containing 12.5
7 ng of template DNA, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 mM
8 of each dNTP, and 1 U of AmpliTaq Gold™ DNA polymerase. The relative
9 concentration of several primers was optimised previously [10] being 0.3 µM of primer
10 set JV16HC-JV17HC, 1 µM of primer set 3-16, and 2 µM of primer set P1-rev-P2-for.
11 Primers for the amplification of the histidine decarboxylase (*hdc*) gene of gram-negative
12 bacteria were used in a final concentration of 1 µM. The two primers sets tested for the
13 amplification of the *hdc* gene in gram-negative bacteria were: primer hdc-f and hdc-r,
14 previously described [8], and primers 106 (5´-
15 AAYTCNTTYGAYTTYGARAARGARG) coding for NSFDFEKEV and 107 (5´-
16 ATNGGNGANCCDATCATYTTRTGNCC) coding for GHKMIGSPI, that amplified
17 DNA fragments of 709 and 534 bp, respectively.
18 The reactions were performed in a GeneAmp PCR System 2400 (Perkin-Elmer)
19 using the following cycling parameters: 10 min for enzyme activation at 95 ºC followed
20 by 30 cycles of 30 sec at 95 ºC, 30 sec at 52 ºC, and 2 min at 72 ºC; and a final
21 extension step of 10 min at 72 ºC [10].

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## 3. Results and discussion

### *3.1. Amplification of the* hdc *gene in gram-negative bacteria*

We have previously described a multiplex-PCR method for the simultaneous detection of lactic acid bacteria producing histamine, tyramine and putrescine [10]. It is well know that gram-negative bacteria are able to produce these compounds in foods. In order to extend the scope of our method by detecting also gram-negative bacteria, and taking as starter method our previous multiplex-PCR assay, we planned to develop a new multiplex-PCR method.

First of all, we need to select new oligonucleotide primers for the amplification of amino acid decarboxylase genes in gram-negative bacteria. Since the most frequent foodborne intoxications caused by biogenic amines involves histamine, first, we checked oligonucleotide primers to amplify the *hdc* gene in gram-negative bacteria. Since histamine is the decarboxylation product of histidine, catalysed specifically by the enzyme histidine decarboxylase (HDC), it is possible to develop a molecular detection method that detects the gene responsible for production of this enzyme. There are two distinct classes of HDCs enzymes, pyridoxal 5´-phosphate (PLP)-dependent in gram-negative bacteria [14] and pyruvoil-dependent in gram-positive bacteria [15, 16]. Uniplex-PCR methods for the detection of genes encoding one type of HDC coding genes have been developed. For gram-positive bacteria, Le Jeune et al. [7] described primer set JV16HC and JV17HC that were successfully used to detect histamine-producing *Clostridium perfringens* strains [17]. Our multiplex-PCR method described for lactic acid bacteria includes this primer set. For gram-negative bacteria, Takahashi et al. [8] described primers hdc-f and hdc-r that amplify a 709-bp fragment of the *hdc* gene in a high number of histamine-producing strains. Therefore, in order to select primers

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for the amplification of the *hdc* gene in gram-negative bacteria, our first approach was to use Takahashi´s primers in a uniplex-PCR experiment and by using the PCR program optimized for the multiplex assay. As shown in Fig. 1 A, histamine-producing bacteria gave an amplicon of the expected size, however, in some strains, additional unspecific amplicons could be observed. This is not surprising, since Takahashi et al. [8] performed their uniplex-PCR assay at higher annealing temperature (58 ºC) than the optimised for the multiplex assay (52 ºC).

In order to design a new pair of oligonucleotide primers for the detection of gram-negative bacteria *hdc* gene to be used in the multiplex-PCR assay, we aligned amino acid sequences of known *hdc* proteins from *Raoultella planticola* (accession number P28578), *Morganella morganii* (P05034), and *Pseudomonas fluorescens* (P95477), among others. Two conserved domains were selected to design the synthetic primers 106 and 107, that amplified a 534-bp *hdc* DNA fragment. When these primers were used in a uniplex-PCR reaction by using the PCR program optimised for the multiplex assay, only the expected 534-bp fragment was obtained (Fig. 1, B). It is noteworthy that psycrophilic *Photobacterium* spp. could be easily detected by this primer set since it has been shown to be a more dominant histamine producing than *Morganella morganii* in fish samples stored at temperatures lower than 15 ºC [18].

As deduced from these results, primers 106 and 107 might constitute a valid uniplex-PCR method for the successful detection of histamine-producing gram-negative bacteria. These primers seems to be more specific than Takahashi´s primers, since at the same annealing temperature they gave only the specific amplicon.


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*3.2. Amplification of the* odc *gene in gram-negative bacteria*

Similarly, oligonucleotide primers need to be designed for the detection of gram-negative bacteria *odc* gene to be used in the multiplex-PCR assay. Primers 3 and 16 were designed previously based on amino acid sequences of well-known or putative ornithine decarboxylases from gram-positive (*Lactobacillus* 30a, *Lactobacillus jhonsonii* NCC533) and gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, and so on). We have previously demonstrated that this primer set amplified successfully lactic acid bacteria producing putrescine. However, we did not tested them in gram-negative bacteria. In order to test the ability of primers 3 and 16 to amplify *odc* genes in gram-negative bacteria, we performed uniplex-PCR experiments in putrescine-producing strains belonging to *Morganella morganii*, *Escherichia coli*, and *Serratia liquefaciens* species. As expected, and by using the optimised PCR program for the multiplex assay, primer set 3-16 gave only the expected amplicon of 1.4 kb (data not shown).

As far as we known, primers 3 and 16 are the only primers described in the literature to be applied in a PCR method for the molecular detection of putrescine-producing bacteria. These primers were successfully used in the detection of the putrescine-producing lactic acid bacteria, and, by using them, we identified the ornithine decarboxylase gene in *Oenococcus oeni* BIFI-83, a putrescine-producing lactic-acid bacteria [19].

Moreover, in this study we have demonstrated that putrescine-producing gram-negative bacteria could be also successfully detected by primer set 3-16.

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*3.3. PCR-multiplex detection of biogenic amine-producing bacteria*

As mentioned above, we have previously described a multiplex-PCR method for the detection of lactic acid bacteria producing histamine, tyramine and putrescine [10]. In order to extend the scope of this multiplex-PCR method to gram-negative biogenic amine producing strains, we need to incorporate primers sets to amplify their histidine, tyrosine and ornithine decarboxylase genes.

As far as we known, only gram-positive bacteria have been described to produce tyramine [9, 20, 21]. However, gene sequences of gram-negative bacteria sharing similarity with plant tyrosine/L-DOPA decarboxylases have been discovered in the complete genomes of *Pseudomonas putida* KT2440 (accession number NP_744697) and *Yersinia pestis* (NP_404801). These proteins showed also high similarity to a decarboxylase from the gram-negative bacteria *Sorangium cellulosum* So ce90 that only decarboxylates L-dopa [22]; therefore, it is possible that similar proteins in gram-negative bacteria might possess the same specific function.

In order to detect gram-negative putrescine-producing bacteria, we have demonstrated above that primer set 3-16 are also useful for their detection and, being an advantage since they are already included in the multiplex-PCR method. Similarly, primer set 106-107 have been shown to be useful for the detection of the histamine producing gram-negative bacteria; however, they need to be included in the multiplex-PCR assay.

We performed PCR-experiments by including the four oligonucleotide pairs in the same reaction, and by using several DNAs from biogenic amine producing strains as DNA template. In a first attempt, primer set 106-107 was included in the multiplex reaction at 1 μM final concentration. At this concentration, their amplicon does not interfere with other amplicons, and originates a specific 534 bp *hdc* DNA fragment only

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in the putrescine-producing strains. Table 1 summarizes the results obtained by the application of the improved multiplex-PCR method to biogenic amine-producing non-lactic acid bacteria. Figure 2 showed some of the DNA fragment obtained. All the biogenic amine producing bacteria amplified DNA products of the expected sizes only in presence of respective DNA template.

In order to test the resolution of the multiplex-PCR method to amplify several amplicons simultaneously, we performed PCR experiments by adding a DNA mixture of *Serratia liquefaciens* strain (gram-negative and putrescine-producing strain), *Lacobacillus brevis* CECT 5354 (gram-positive and tyramine-producing strain), *Proteus vulgaris* CECT 484 (gram-negative and histamine-producing strain) and *Staphylococcus* sp. (gram-positive and histamine-producing strain) (Fig. 2). The four amplicons could be easily identified in the agarose gel. Therefore, we establish this conditions as the optimal for the improved multiplex assay since all the amplicons presented strong intensity, the fragments sizes showed a good discrimination on agarose gel, and non-specific amplicons could be observed.

In summary, we have developed an improved multiplex-PCR method for the routine detection of harmful bacteria which are potential producers of histamine, tyramine and putrescine in food substrates. The use of this method for the early and rapid detection of biogenic-amine producing bacteria is important for preventing the accumulation of histamine in food products.

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## Figure legends

Fig. 1. Uniplex-PCR amplification of the histidine decarboxylase gene (*hdc*) in gram-negative bacteria by using primers hdc-f and hdc-r that give an amplicon of 709 bp (A) or primers 106 and 107 giving an amplicon of 534 bp (B). PCR was performed by using the conditions described in the Material and methods section by using DNA from *Photobacter phosphoreum* CECT 4192 (lane 1), *Proteus vulgaris* CECT 484 (lane 2), *Klebsiella planticola* CECT 843 (lane 3) or *Morganella morganii* CECT 173 as DNA template. The molecular sizes (in kilobases) of some standards (*Eco*RI and *Hin*dIII digested λ DNA) are indicated on the left.

Fig. 2. Multiplex-PCR detection of bacteria producing histamine, tyramine and putrescine. The multiplex-PCR assay was performed by including simultaneously the following primers pairs JV16HC and JV17HC at 0.3 μM final concentration (amplicon size 367 bp), 106 and 107 at 1 μM (amplicon size 534 bp), P1-rev and P2-for at 2 μM (amplicon size 924 bp) and 3 and 16 at 1 μM (amplicon size 1446 bp) in the reaction. The reaction was performed by using chromosomal DNA from *Serratia liquefaciens* (lane 1), *Staphylococcus* sp. (lane 2), *Lactobacillus brevis* CECT 5354 (lane 3), *Lactobacillus* 30a (lane 4), *Proteus vulgaris* CECT 484, and a DNA mixture of four DNAs extracted from *Serratia liquefaciens*, *Lactobacillus brevis* CECT 5354, *Proteus vulgaris* CECT 484 and *Staphylococcus* sp. (lane 6). The molecular sizes (in kilobases) of some standards (*Eco*RI and *Hin*dIII digested λ DNA) are indicated on the left. A 50 bp leader marker was also included on the right.

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**Table 1.** Application of the multiplex-PCR method to biogenic amine-producing non-lactic acid bacteria.

| | Target gene and amplicon size (bp)[a] | | | |
|---|---|---|---|---|
| | *hdc* | | *tdc* | *odc* |
| Bacterial species | 367 | 534 | 924 | 1446 |
| *Klebsiella planticola* | - | + | - | - |
| *Proteus vulgaris* | - | + | - | - |
| *Photobacterium phosphoreum* | - | + | - | - |
| *Morganella morganii* | - | + | - | + |
| *Escherichia coli* | - | - | - | + |
| *Serratia liquefaciens* | - | - | - | + |
| *Staphylococcus* sp. | + | - | - | - |

[a]*hdc*, histidine decarboxylase gene amplified with primer set JV16HC-JV17HC (367 bp) or primer set 106-107 (534 bp); *tdc*, tyrosine decarboxylase gene amplified with primer set P1-rev-P2-for; and, *odc*, ornithine decarboxylase gene amplified with primers 3 and 16.

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Figure 1

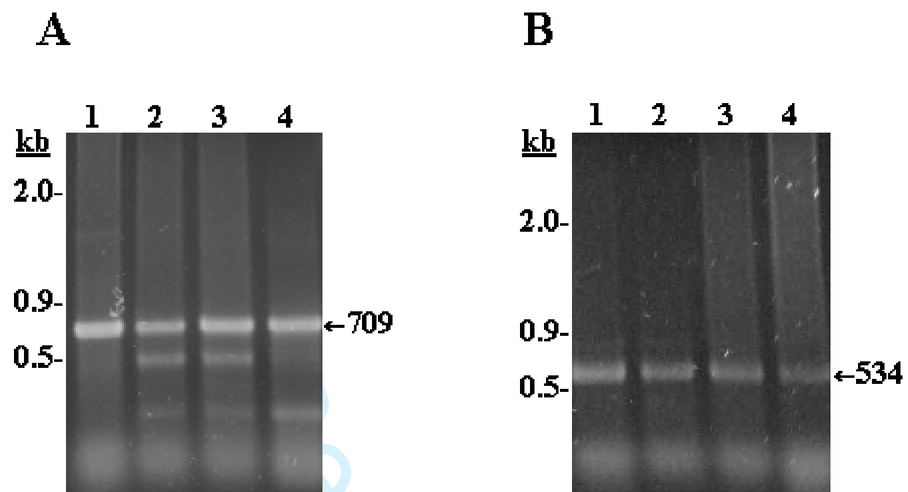


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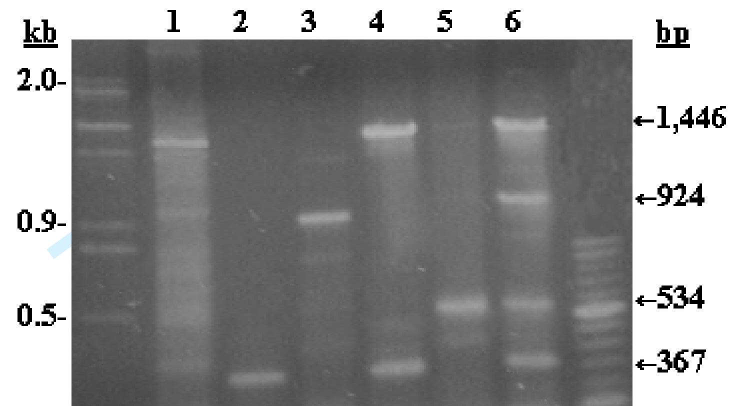
Figure 2

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