Molecular methods for the detection of biogenic amine-producing bacteria on foods

José María Landete, Blanca de las Rivas, Angela Marcobal, Rosario Muñoz*

Departamento de Microbiología, Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid

*Corresponding author. Tel.: +34-91-5622900; fax: +34-91-5644853

E-mail address: rmunoz@ifi.csic.es (R. Muñoz)

Abstract

Biogenic amines are low molecular weight organic bases that can be detected in raw and processed foods. Several toxicological problems resulting from the ingestion of food containing biogenic amines have been described. Biogenic amines are mainly produced by the decarboxylation of certain amino acids by microbial action. Since the ability of microorganisms to decarboxylate amino acid is highly variable, being in most cases strain-specific, the detection of bacteria possessing amino acid decarboxylase activity is important to estimate the risk of biogenic amine food content and to prevent biogenic amine accumulation in food products. Molecular methods for the early and rapid detection of these producer bacteria are becoming an alternative to traditional culture methods. PCR methods offer the advantages of speed, sensitivity, simplicity and specific detection of amino acid decarboxylase genes. Moreover, these molecular methods detect potential biogenic amine risk formation in food before the amine is produced. The aim of the present review is to give a complete overview of the molecular methods proposed in the literature for the detection of biogenic amine-producing bacteria. These genetic procedures allow the introduction of early control measures to avoid the development of these bacteria.

Contents

1. Introduction

Biogenic amines (BA) are organic bases endowed with biological activity, which are frequently found in fermented foods and beverages. Although BA are found in a wide range of foods and drinks, their concentration vary extensively (Silla, 1996). Foods likely to contain high levels of these compounds are dairy products, fish and fish products, meat and meat products, fermented vegetables and soy products, and alcoholic beverages such as wine and beer (Ten Brink et al. 1990; Halàsz et al. 1994; Suzzi and Gardini, 2003).The main BA encountered in foods and beverages include histamine, tyramine, putrescine, and cadaverine (Silla, 1996). Consumption of foods containing

66 high amounts of these amines can have toxicological effects (Shalaby, 1996). These
67 problems may be more severe in sensitive consumers having a reduced mono- and
68 diamine oxidase activity (Bodmer et al., 1999), the enzyme responsible for its
69 detoxification. Moreover, alcohol ingestion increase the undesirable effects produced by
70 the presence of BA (Maynard and Schenker, 1996). More specifically, histamine is
71 known to cause headaches, low blood pressure, heart palpitations, edema, vomiting,
72 diarrhea, etc. Tyramine can produce hypertension, and the diamines putrescine and
73 cadaverine, although not toxic themselves, aggravate the adverse effects of histamine
74 and tyramine, as they compete for some of the mechanisms involved on its
75 detoxification (ten Brink et al., 1990; Bardócz, 1995; Straub et al., 1995).
76 Scombroid poisoning is a type of food poisoning that results from eating
77 mishandled tuna and related scombroid fish containing elevated levels of histamine
78 (Lehane and Olley, 2000). Histamine intoxication is a sanitary problem of food-borne
79 disease associated with eating fish (Lehane and Olley, 2000). It has been suggested that
80 the lack of relationship between histamine content and sensory attributes explains the
81 high incidence of scombroid toxicity (Priebe, 1984). Tyramine is usually the most
82 frequently encountered BA in fermented products. Consumption of foods containing
83 high amounts of tyramine can have toxicological effects. A typical phenomenon is the
84 “cheese reaction”, usually caused by high levels of tyramine in cheese. The term
85 “cheese reaction” refers to the food and drug interaction occurring between tyramine
86 containing food and classical MAO inhibitors drugs, which are irreversible and non-
87 selective. The BA putrescine, although it seems to have a lower pharmacological
88 activity, hampers the detoxification of histamine and tyramine. Moreover, the presence
89 of the amine putrescine is investigated in meat products since it can react with nitrite to
90 form nitrosopyrrolidine, an heterocyclic carcinogenic nitrosamine (Wathersen et al.,

1975). Cadaverine is a BA not considered toxic, although it can potentiate the toxicity of histamine. In foods, the presence of BA is indicative of undesired microbial activity. In addition, the occurrence of relatively high levels of certain BA has been reported as indicators of a deterioration process and/or defective elaboration. The amounts of histamine, putrescine and cadaverine usually increase during spoilage of fish or meat (Karmas et al., 1981). A combination of putrescine and cadaverine has been suggested as an index of acceptability in fresh meat, because their concentration increases prior to spoilage and correlates well with the microbial load (Ruíz-Capillas and Jimenez-Colmenero, 2004).

BA in foods are mainly generated by decarboxylation of the corresponding amino acids through substrate-specific decarboxylase enzymes derived from microorganisms present in the food (Silla, 1996). 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 microorganisms to decarboxylate amino acids is highly variable. It depends not only on the species, but also on the strain and the environmental conditions (e.g. Coton et al. 1998a; Marcobal et al., 2006c).

During the last two decades, methods for the detection of BA-producing bacteria have been developed (reviewed by Marcobal et al. 2006b). Several detection method are based on differential growth media signalling the increase of the pH upon BA formation (e.g. Maijala, 1993; Bover-Cid and Holzapfel, 1999). Enzymatic methods, specific for histamine-producing bacteria, are based in the production of hydrogen peroxide by the action of an oxidase enzyme on the histamine (e.g. Sumner and Taylor, 1989). Among the different chromatographic techniques recommended for identification and quantification of BA, thin layer chromatography (e.g. García-Moruno et al. 2005) and

high performance liquid chromatography (e.g. Maijala, 1993; Straub et al., 1995; Bover-Cid and Holzapfel, 1999) have been the most useful. However, the detection of BA-producing bacteria by conventional culture techniques is often tedious and unreliable, exhibiting disadvantages such as lack of speed, appearance of false positive/negative results, low sensibility, requirements for costly and sophisticated equipment, as HPLC, or that only one BA is detected.

Early detection of BA-producing bacteria is important in the food industry because it could be a cause of food poisoning. Therefore, the use of methods for the early and rapid detection of these bacteria is important for preventing BA accumulation in food products. Molecular methods for detection and identification of food-borne bacteria are becoming an alternative to traditional culture methods. PCR and DNA hybridization have become important methods and offer the advantages of speed, sensitive, simplicity and specific detection of targeted genes. Genetic procedures accelerate getting results and allow the introduction of early control measures to avoid the development of these bacteria. Several studies describing loss of ability to produce BA in lactic acid bacteria (LAB) after prolonged storage or cultivation of isolated strains in synthetic media have been reported (Lonvaud-Funel and Joyeux, 1994; Izquierdo-Pulido et al., 1997). Since molecular methods are fast, reliable and culture-independient, they are an interesting alternative to solve the shortcomings of traditional methods. Moreover, molecular methods detect potential BA risk formation in food before the amine is produced. Although, an intrinsic disadvantage of PCR is the detection of non-viable cells. The ability to distinguish between viable and nonviable organisms is crucial when PCR is used for risk assessment of BA accumulation such as in food processing plant.

Since during the last decade several molecular methods have been described for the unambiguous detection of bacteria capable to produce one or several BA, this article aims to provide complete information about the PCR and DNA hybridization methods proposed in the literature for the detection of BA-producing bacteria.

2. Detection of histamine-producing bacteria

Histamine forms in a variety of foods, including raw fish, wine, cheese, fermented meat and fish products (Straub et al., 1995; Silla, 1996). While histamine in fermented products, such as wine, cheese, and fish sauce, is produced by gram-positive LAB; histamine in raw fish products is caused mostly by gram-negative enteric bacteria. During the decomposition of fish such as tuna and mackerel, histamine forms in significant amounts due to bacterial decarboxylation of histidine present in the muscle tissue. Therefore, rapid-detection of histamine-producing bacteria is important for detecting and preventing microbial contamination and high histamine levels during processing of fish products.

Since histamine is the decarboxylation product of histidine catalysed specifically by the enzyme histidine decarboxylase (HDC; EC 4.1.1.22), it is possible to develop a molecular detection method that detects the presence of the gene encoding this enzyme. Bacterial HDC have been thoroughly studied and characterized in different organisms and two enzyme families have been distinguished, being their sequences and characteristics radically different. Pyridoxal phosphate-dependent HDC are encountered in gram-negative bacteria belonging to various species (i.e., *Morganella morganii*, *Enterobacter aerogenes, Photobacterium phosphoreum*, *Raoultella planticola*, etc.) and are associated with fish products spoilage. Pyruvoyl-dependent HDC are present in

gram-positive bacteria and especially LAB implicated in food fermentation or spoilage, such as *Clostridium perfringens*, *Tetragenococcus muriaticus*, *Oenococcus oeni*, *Lactobacillus hilgardii*, and *Lactobacillus buchneri*, among others.

2.1. Gram-positive bacteria

Several gram-positive bacteria, and especially LAB, implicated in food fermentation or spoilage are able to produce histamine. To detect histamine-producing LAB, Le Jeune et al. (1995) designed several oligonucleotide primers (CL1, CL2, JV16HC, and JV17HC) (Table 1) based in the comparison of the nucleotide sequences of the histidine decarboxylase genes (*hdc*) of *Lactobacillus* strain 30a and *C. perfringens*, and the amino acid sequences of these HDC and those of *L. buchneri* and *Micrococcus* (Figure 1). Alignment studies showed a high degree of relatedness among the *hdc* gene products of gram-positive bacteria. Primer sets JV16HC/JV17HC, CL1/CL2, and CL1/JV17HC amplify by PCR internal fragments of 370, 150 or 500 pb, approximately, of the *hdc* gene, respectively (Table 2). JV16HC/JV17HC primer set was shown to be suitable for the detection of all histamine-producing LAB analysed. The authors demonstrated that all strains identified as histamine-producers gave a positive PCR result. Moreover, strains which did not exhibit HDC activity failed to give a signal in the PCR assay. The PCR amplification products of *hdc* genes from *L. buchneri* and *O. oeni* by the CL1/JV17Hc primer set, were labelled and used as a probe in DNA hybridisation studies.

The primer sets described by Le Jeune et al. (1995) were used by Torres Alves et al. (2002) to isolate and amplify fragments of histidine-decarboxylase genes (*hdc*) of histamine-producing bacteria frequently found in canned fish. The authors reported that

190 by using the PCR conditions described previously (Le Jeune et al., 1995) (Table 2),
191 JV16HC/JV17HC primer set also amplified non-expected fragments of 100, 150 and
192 250 bp. Some of the expected PCR amplified fragments were sequenced automatically,
193 and presented high similarity to the *C. perfringens hdc* gene. The authors concluded that
194 Le Jeune´s primer set was useful in the detection of histamine-producing bacteria in
195 canned fish, where the DNA is typically very fragmented due to the heating process of
196 sterilization.

197 Marcobal et al. (2005) checked the primers sets described by Le Jeune et al.
198 (1995) in order to choose one of them to be used in a multiplex PCR assay. Since the
199 JV16HC/JV17HC set gave a stronger intensity amplicon than CL1/JV17HC set, it was
200 used in the multiplex PCR assay.

201 Since, the previously described PCR and colony hybridization methods (Le
202 Jeune et al. 1995) used purified DNA of isolated strains, seemed to be convenient for
203 rapidly detecting histamine-producing bacteria, Coton et al. (1998b) in order to improve
204 the rapidity of these tests to determine the frequency and distribution of histamine-
205 producing bacteria in wines, applied them directly on wine samples. They used primers
206 CL1 and JV17, a slightly modificated version of JV17HC primer (Coton et al 1998a)
207 (Table 1). They used CL1/JV17 primers to analyze the presence of histamine-producing
208 bacteria directly on wine samples.

209 Landete et al. (2005) studied the ability of 136 wine LAB to produce histamine.
210 They found that some LAB positive for histamine production were not amplified with
211 JV16HC/JV17HC primers under the conditions originally described by Le Jeune et al.
212 (1995). Therefore, they modified the PCR amplification programme (Table 2). By using
213 the modified programme, histamine-producing lactobacilli, pediococci, and
214 leuconostocs strains showed positive amplification by the JV16HC/JV17HC primers.

215 Nevertheless, only 56% of the *O. oeni* histamine-producing strains showed
216 amplification for *hdc*. Therefore, they modified the original CL1 primer sequence (Le
217 Jeune et al., 1995) and designed the CL1mod primer (Table 1). By using
218 CL1mod/JV17HC primer set, all histamine-producing *O. oeni* strains were positive in
219 the PCR test.

220 Alignment of partial *hdc* gene sequences (Figure 1) allows Coton and Coton
221 (2005) to design a new primer set, HDC3/HDC4 (Table 1), to amplify the *hdc* gene
222 from gram-positive bacteria. The method was used to detect histamine-producing
223 bacteria in smoked salmon.

224 Constantini et al. (2006) used CL1/JV17HC primer set to study the potential to
225 produce histamine in 133 LAB strains isolated from wines of different origins. Only one
226 *L. hilgardii* strain was positive. Histamine production by *L. hilgardii* was confirmed
227 trough TLC and HPLC analysis of the broth medium enriched with histidine. Since
228 none the *O. oeni* strains analysed gave a positive PCR response, they designed a new
229 primer set, PHDC1/PHDC2 (Table 1) based specifically on the *O. oeni hdc* sequence
230 (Figure 1). The new PCR results confirmed the preceding data, none of the *O. oeni*
231 strains analysed was able to produce histamine.

232 Recently, De las Rivas et al. (2006a) in order to design a complete PCR assay
233 for the detection of several decarboxylase genes, designed a new specific primer set,
234 HIS1-F/HIS1-R (Table 1, Figure 1), to amplify *hdc* genes from gram-positive bacteria.
235 The method was successfully applied to several histamine-producing LAB strains.
236 Moreover, none of the non histamine-producing strains gave a PCR product of the
237 expected size.

238 Conventional PCR methods have the drawback of data analysis by traditional
239 end-point analysis. Therefore, Real-time quantitative PCR (qPCR) is a potential

alternative. This would allow continuous monitoring of the PCR amplification process and, under appropriate conditions, quantification of the template. In addition, real-time methods are considerably less time-consuming than regular PCR. Recently, Fernández et al. (2006) proposed a real-time qPCR method for the direct detection and quantification of histamine-producing LAB in culture media, milk, and curd. Primer set Hdc1 and Hdc2 (Table 1) amplifies a 174-bp internal fragment of the histidine decarboxylase gene sequence of different Gram-positive bacteria. The results showed that the proposed procedure was a rapid, specific, and highly sensitive technique for detecting potential histamine-producing strains.

2.2. Gram-negative bacteria

The use of molecular tools for early and rapid detection of gram-negative histamine producing bacteria is important for preventing the accumulation of histamine in fish products and other food products. Since enteric bacteria have been reported to be the dominant histamine-producing bacteria in fish, Kanki et al. (2002) designed a primer set, KPF2/KPR4 (Table 1), based on the *Raoultella planticola* HDC protein (Figure 2), to identify histamine-producing strains. Based on the same *hdc* sequence, they designed a DNA probe to be used in DNA hybridization experiments. Positive PCR results were obtained in all the histamine-producing strains of *R. planticola* and *R. ornithinolytica*. The results of the PCR tests are correlated with those of the hybridization assays.

Takahashi et al. (2003) developed a molecular method that allows the rapid detection of gram-negative histamine-producers by PCR and the simultaneous differentiation by single-strand conformation polymorphism (SSCP) analysis using the

amplification product of the *hdc* gene. The method was tested in a collection of 37 strains of histamine-producing bacteria (8 reference strains from culture collections and 29 isolates from fish) and 470 strains of non histamine-producing bacteria isolated from fish. Histamine production of bacteria was determined by paper chromatography and confirmed by HPLC. The PCR primers (hdc-f/hdc-r) amplify the *hdc* gene of gram-negative histamine-producing bacteria in fish samples and other sources. Strains of *M. morganii*, *R. planticola*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *P. damselae*, *P. phosphoreum*, *Hafnia alvei*, *Erwinia* sp, *Proteus vulgaris* were positive with the PCR method proposed by Takahashi et al. (2003). All the histamine-producing gram-negative bacteria produced a PCR product of the expected size, except for a strain of *Citrobacter braakii*. In contrast, none of the non histamine-producing strains (470 strains) produced an amplification product. Histamine accumulation occurred when PCR amplification of *hdc* was positive in all of fish samples tested and the presence of powerful histamine-producers was confirmed by subsequent SSCP identification. SSCP analysis was performed to differentiate the *hdc* genes amplified from fresh fish. Although multiple banding patterns were obtained within the same species in pure cultures experiments, repeatability in PCR-SSCP was observed in 100% of the time within the same strain. Thus, the variation in band patterns was due to the minute variation of *hdc* sequences within the same species. The results of the Takahashi´s study support the practical application of PCR-SSCP analysis of *hdc* gene.

De las Rivas et al. (2005) found that the primers designed by Takahashi et al. (2003) gave unspecific amplicons in the conditions optimized for a multiplex-PCR assay. In order to design a new pair of oligonucleotide primers for the detection of the gram-negative bacteria *hdc* gene, they aligned amino acid sequence of known HDC proteins from *R. planticola*, *M. morgani* and *Pseudomonas fluorescens*, among others

290 (Figure 2). Two conserved domains were selected to design the 106 and 107 synthetic
291 primers (Table 1) that were more specific than Takahashi´s primers in the assay
292 conditions. Recently, De las Rivas et al. (2006a) designed a new primer set, HIS2-F and
293 HIS2-R primers (Table 1), in order to design a complete PCR assay for the detection of
294 several decarboxylase genes. The new designed primers allowed the amplification of a
295 specific 531 bp DNA fragment from gram-negative histamine-producing bacteria
296 including *P. phosphoreum* strains, the dominant histamine-producing species in
297 refrigerated fish samples. The complete *P. phosphoreum hdc* gene was identified
298 recently by the use of degenerate PCR primers, DegF/DegR (Table 1) based on the
299 multiple-sequence alignment of the *hdc* genes of gram-negative bacteria (Morii et al.,
300 2006).
301
302 **3. Detection of tyramine-producing bacteria**
303
304 As far as we know, only gram-positive bacteria have been described to produce
305 tyramine. Many LAB involved in food processing can decarboxylate tyrosine to
306 produce tyramine. These bacteria belong to genera as diverse as *Lactobacillus*,
307 *Enterococcus* or *Carnobacterium* (ten Brink et al., 1990). The comparison of the
308 tyrosine decarboxylase (*tdc*) gene clusters from different producing microorganisms
309 revealed a high similarity either in gene sequence and organization (Fernández et al.,
310 2004).
311 Concerning tyrosine decarboxylases (TDC; EC 4.1.1.25), only enzymes using
312 pyridoxal phosphate as a cofactor have been described. Purification and
313 microsequencing of the TDC of *Lactobacillus brevis* IOEB 9809 allowed Lucas and
314 Lonvaud-Funel (2002) to design a degenerate primer set (P2-for/P1-rev) (Table 3,

315 Figure 3) that was used to detect *tdc* gene fragments in three other *L. brevis* strains out
316 of six screened.

317 Marcobal et al. (2005) checked the P2-for/P1-rev primer set and a new designed
318 primer set (41/42) in order to choose one of them to be used in a multiplex PCR assay
319 (Table 3). Since 41/42 set produced an unspecific fragment, the P2-for/P1-rev set was
320 used in the multiplex PCR assay. However, due to competitive reaction between the
321 primer sets, the primer concentration was optimised for the multiplex assay (Table 2).
322 The assay was useful for the detection of tyramine-producing bacteria in control
323 collection strains and in a wine LAB collection.

324 Constantini et al. (2005) also used the P2-for/P1-rev primer set to amplify the
325 *tdc* gene of 133 strains isolated from wine and must. They also designed a new primer
326 set, Pt3/Pt4 (Table 3), based on the *tdc L. brevis* and *E. faecalis* nucleotide sequences.
327 The results obtained with both set of primers were the same. Only four positive strains
328 were found, all belonging to the *L. brevis* species. The tyramine produced by these
329 strains was quantified by HPLC, thus confirming the results observed by PCR.

330 Recently, P1-rev primer was used in combination with p0303 primer (Lucas et
331 al. 2003) to analyse by PCR the presence of the *tdc* gene in 150 LAB strains isolated
332 from wine (Landete et al., 2006). All the 32 strains that gave a positive PCR
333 amplification were tyramine producers.

334 The identification of the *Carnobacterium divergens* 508 TDC protein and
335 comparison with known TDC sequences from *E. faecalis*, *E. faecium* and a partial
336 sequence from *L. brevis* (Figure 3) allowed Coton et al. (2004) to design a set of
337 consensual primers, TD2 and TD5 (Table 3), for the detection of the *tdc* gene. These
338 primers were used in PCR experiments and allowed for the detection of tyramine-
339 producing bacteria. They were validated in PCR experiments using DNA extracted from

five known tyramine-producing LAB strains (*L. brevis*, *C. divergens*, *C. piscicola*, *E. faecalis*, and *E. faecium*) and from *L. buchneri* DSMZ 5987 as a negative control. This primer set was later used by Coton and Coton (2005) in a multiplex assay. They amplified, from purified DNA, the *tdc* 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.

Marcobal et al. (2004a) designed the 57/58 primer set (Table 3) to amplify the complete *tdc E. faecium* gene. This primer set was used to detect the *tdc* gene in tyramine-producing *E. faecium* strains.

Fernández et al. (2004) designed TDC1/TDC2 primers (Table 3) to detect tyramine-producing strains by PCR. Reactions were initially performed by the use of total DNA from 17 different tyramine-producing LAB strains. The analysis of the PCR products showed a specific 720 pb single fragment. No PCR product was detected when DNA from 14 non-producing strains belonging to different genera, was used as template. Similarly, the internal fragment of the *tdc* 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.

Recently, De las Rivas et al. (2006a) designed a new primer set, TDC-F/TDC-R primers (Table 3), to design a complete PCR assay for the detection of several

365 decarboxylase genes. They amplified the *tdc* gene from *E. faecalis*, *E. faecium*, *C.*
366 *divergens* and *L. brevis*. Since the designed oligonucleotides are based on regions
367 conserved in other gram-positive bacteria, this implies that these primers would also
368 allow for the detection of tyramine-producing bacteria belonging to species such as *L.*
369 *lactis*, *L. curvatus* and *T. halophilus*.

370 It have been demonstrated that enterococcal TDC is also able to decarboxylate
371 phenylalanine, an amino acid structurally related to tyrosine, originating the BA
372 phenylethylamine (Marcobal et al., 2006a). Therefore, the oligonucleotide primers
373 described for the detection of the *tdc* gene, are useful for the detection of
374 phenylethylamine-producing enterococci. Landete et al. (2006) demonstrates that
375 phenylethylamine production is always associated with tyramine production in LAB.
376 However, purified tyrosine decarboxylase from *Lactobacillus brevis* does not
377 demonstrate the ability to decarboxylate L-phenylalanine; instead it is L-tyrosine
378 specific (Moreno-Arribas and Lonvaud-Funel, 2001)

379

380 **4. Detection of putrescine-producing bacteria**

381

382 Ornithine decarboxylase (ODC, EC 4.1.1.17) is a PLP-dependent enzyme which
383 catalyses the conversion of ornithine to putrescine at the beginning of the polyamine
384 pathway. De las Rivas et al. (2006a) observed that bacterial ODC alignments showed
385 two separated groups, one of them includes several proteins from gram-positive
386 bacteria, such as from some *Lactobacillus* strains, and gram-negative bacteria (*E. coli*,
387 *Haemophilus influenzae*, *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio cholerae*,
388 *Vibrio parahaemoliticus*, and *Yersinia pestis* strains, among others). The second, and
389 minoritary, group includes ODC from *Brucella and Pseudomonas* strains (Figure 4).

390 Marcobal et al. (2004b) reported the identification of an ornithine decarboxylase
391 gene (*odc*) in the putrescine-producing *Oenococcus oeni* RM83 strain by using 3/16
392 primer set (Table 4). These primers were designed based on two conserved domains
393 showed by alignment of amino acid sequences of ODC proteins (Figure 4). The *odc*
394 gene appears to be rarely present in the genome of *O. oeni*, since in a PCR screening for
395 the presence of this gene in 42 oenococcal strains none of the strains possessed and *odc*
396 gene copy. Granchi et al. (2005) described a PCR protocol to specifically detect
397 putrescine-producing *O. oeni* strains.
398 The 3 and 16 primers were checked by Marcobal et al. (2005) to be used in a
399 multiplex assay. In addition, they designed two new primers, 4 and 15 (Table 4), based
400 in conserved domains of amino acid sequences from the first mentioned group of
401 bacterial ODC. These four primers could be combined resulting in four primer sets, 3/4,
402 15/16, 3/16, and 4/15 (Table 2). Based on the size of its amplicon, 3/16 primer set was
403 choosen for the multiplex assay. The method was useful for the detection of putrescine-
404 producing bacteria in control collection strains and in a wine LAB collection.
405 Primers 3 and 16 were designed based on ODC from gram-positive and gram-
406 negative bacteria. It was previously demonstrated that this primer set amplified
407 successfully putrescine-producing LAB strains (Marcobal et al., 2004b; Marcobal et al,
408 2005). However, to test the ability of 3/16 primers to amplify *odc* genes from gram-
409 negative bacteria, De las Rivas et al. (2005) performed PCR experiments in putrescine-
410 producing strains belonging to *Morganella morganii*, *Serratia liquefaciens*, and *E. coli*
411 species. As expected, 3/16 primer set gave only the specific amplicon. In addition, 3/16
412 primer set has been also used to locate and characterize *odc* genes from gram-negative
413 bacteria, such as *M. morganii* (De las Rivas et al., 2006b) as well as *S. liquefaciens* (De
414 las Rivas et al., 2007).

In a study of the ability of 133 strains of LAB isolated from wines to produce BA, for the detection of putrescine-producing LAB strains, Constantini et al. (2006) designed two new primers, AODC1 and AODC2 (Table 4), which were chosen by aligning nucleotide sequences of *odc* from *Lactobacillus* strain 30a and *O. oeni*. PCR assays were performed with various combinations of the four primers 3, 16, AODC1, and AODC2. The PCR results, also confirmed by TLC and HPLC analysis, showed that none wine LAB strain was *odc* positive.

Recently, De las Rivas et al. (2006a) designed a new primer set, PUT1-F/PUT1-R (Table 4), based in sequences from some enterobacteria and LAB. These primers allowed to detect *odc* genes from bacteria included in the first ODC group above mentioned.

Since the second, and minor, ODC group includes ODC proteins from important food bacteria, such as strains belonging to the *Pseudomonas* genera, and given the importance of detecting putrescine-producing strains of *Pseudomonas*, De las Rivas et al. (2006a) also designed a primer set based on this group of ODC proteins. The designed primer set, PUT2-F/PUT2-R (Table 4), amplifies a 624-bp DNA fragment of ODC from *Pseudomonas*.

5. Detection of cadaverine-producing bacteria

Cadaverine is derived from the amino acid lysine by decarboxylation. Its synthesis is catalyzed by the lysine decarboxylase enzyme (LDC, EC 4.1.1.18). De las Rivas et al. (2006a) reported that, similarly to ODC, LDC enzymes can be aligned in two groups according to its Gram classification (Figure 5). The first LDC group comprises proteins from Gram-negative bacteria, mainly *Enterobacteriaceae* (such as *E.*

coli, *S. enterica*, *H. alvei*, *S. typhimurium*, *S. flexneri*, *Vibrio cholerae*, *V. vulnificus*, and *V. parahaemolyticus*). The second group includes lysine decarboxylases from Gram-positive bacteria such as *Bacillus halodurans*, *B. subtilis*, *C. acetobutylicum*, *C. perfringens*, *Listeria innocua*, *L. monocytogenes*, *Staphylococcus aureus,* and *S. epidermidis*.

On each LDC protein group, two conserved domains were selected by de las Rivas et al. (2006a) to design degenerate synthetic primers to amplify the corresponding *ldc* gene. CAD1-F /CAD1-R primer set (Table 5) amplifies a specific DNA fragment of LDC from Gram-negative bacteria. CAD2-F/CAD2-R primer set (Table 5) was designed in order to detect the *ldc* gene from Gram-positive bacteria. Both primer sets were used for the amplification of *ldc* genes from control cadaverine-producing strains.

6. Simultaneous detection of biogenic amine-producing bacteria

Even though the detection protocols of BA producing-bacteria are simplified by PCR techniques, they remain tedious since they are designed as uniplex amplification, thus allowing for the detection of a single species per PCR. Therefore some multiplex PCR assays based on primers targeting amino acid decarboxylase gene sequences have been developed. The multiplex PCR assay provides a technique that can be successfully used for the routine detection of strains that are potential producers of histamine, tyramine, putrescine and cadaverine in foods. All (two, three or four) target amines can be detected at one time in a multiplex PCR assay. Therefore, the multiplex PCR assays reduce reagent quantities and labor costs.

A multiplex PCR assay for the detection of histamine and tyramine-producing LAB was developed by Coton and Coton (2005). A third set of primers targeting the

16S rRNA gene of eubacteria was also used as an internal control. To detect the *hdc* gene of LAB they used HDC3/HDC4 primer set (Table 1), and TD2/TD5 primer set (Table 3) to detect the *tdc* gene. The three primers sets were chosen to allow for easy distinction of the amplified fragments on agarose gel thanks to their respective sizes (*hdc*, 440 bp; *tdc*, 1110 bp; and, internal control 1530 bp) (Table 2).The multiplex PCR was performed on extracted DNA as well as directly on cell colonies. This multiplex PCR method was applied to LAB isolated from smoked salmon

Marcobal et al. (2005) selected three pairs of primers for a multiplex PCR assay for the simultaneous detection of LAB strains, which potentially produce histamine, tyramine, and putrescine on fermented foods. The primer sets were JV16HC/JV17HC (Table 1), P1-rev/P2-for (Table 3), and 3/16 (Table 4) for the detection of the *hdc, tdc,* and, *odc* genes, respectively. As mentioned before, these primers were based on sequences from HDC, TDC, and ODC from LAB. Previously, the authors checked several primer sets to detect each gene. To establish the optimal set of primers for the multiplex assay, they took into account the strong intensity of the amplicons for the target DNA and good discrimination of fragment sizes on the agarose gels, as well as the absence of non-specific amplicons. Under the optimized conditions, the assay yielded DNA fragments of 367, 924, and 1446-bp DNA of *hdc*, *tdc*, and *odc* genes, respectively (Table 2). For multiplex PCR, conditions were as described for the uniplex reaction except that the relative concentration of the primers was optimized by checking increasing or decreasing primer concentration (Table 2). When the DNA of several target organisms was included in the same reaction, two or three corresponding amplicons of different sizes were observed. This assay was useful for the detection of amine-producing bacteria in control collection strains and in a wine LAB collection. No amplification was observed with DNA from non BA-producing LAB strains.

In order to extend the scope of this multiplex-PCR method by detecting also gram-negative BA-producing strains, and taking as starter method the previous multiplex PCR assay, De las Rivas et al. (2005) only incorporates a primer set to amplify the *hdc* gene from gram-negative bacteria since only gram-positive bacteria have been described to produce tyramine, and the primer set 3/16 successfully detected most of the *odc* genes from gram-negative bacteria. Under the optimised conditions, the assay yielded a 367-bp DNA fragment of the *hdc* gene from gram-positive bacteria (JV16HC/JV17HC primer set), 534-bp fragment from *hdc* of gram-negative bacteria (106/107 primer set) (Table 1), 924-bp from *tdc* (P2-for/P1-rev primer set), and a 1446-bp fragment from bacterial *odc* (3/16 primer set). The method was successfully applied to several BA-producing bacterial strains, even when DNAs of several target organisms were included in the same reaction. The authors performed PCR experiments by adding a DNA mixture of *S. liquefaciens* strain (a gram-negative and putrescine-producing strain), *L. brevis* (a gram-positive and tyramine-producing strain), *P. vulgaris* (a gram-negative and histamine-producing strain) and *Staphylococcus* sp. (a gram-positive and histamine-producing strain). The four amplicons presented strong intensity, the fragments sizes showed a good discrimination on agarose gel, and non-specific amplicons could be observed.

Recently, De las Rivas et al. (2006a) described a complete method useful for the specific detection of a wide range of gram-positive and gram-negative food bacteria producing the four most important BA, histamine, tyramine, putrescine, and cadaverine. This method is based on PCR primers targeting amino acid decarboxylase genes. Under the conditions used in the study, the assay yielded fragments of 372 and 531-bp from *hdc* genes (HIS1-F/HIS1-R and HIS2-F/HIS2-R primers), 825-bp fragment from *tdc* (TDC-F/TDC-R primers), fragments of 624 and 1440 bp from *odc* (PUT1-F/PUT1-R

515 and PUT2-F/PUT2-R primers), and 1098- and 1185-bp fragments from *ldc* genes
516 (CAD1-F/CAD1-R and CAD2-F/CAD2-R primers). Because several BA-producing
517 bacteria could be found in the same food substrate, the authors decided to check the
518 usefulness of the PCR method to detect specific decarboxylase genes in a complex
519 bacterial population. Therefore, they used a mixture of seven DNAs obtained from BA-
520 producing bacteria as DNA template in the PCR reaction. By using each specific primer
521 set, only the corresponding specific amplicon was obtained. Moreover, the seven
522 specific amplicons were clearly resolved from each other in an agarose gel. Many
523 strains have been tested using this assay; moreover, because the designed
524 oligonucleotides are based on conserved domains, the described method should allow
525 BA detection in a wide range of bacteria.
526
527 **7. Conclusions**
528
529 Although amino acid decarboxylases are not widely distributed among bacteria,
530 species of many genera are capable of decarboxylating one or more amino acids.
531 However, the ability of microorganisms to decarboxylate amino acids is highly variable.
532 It depends not only on the species, but also on the strain and the environmental
533 conditions.
534 The molecular techniques offer fast, easy, and reliable methods for analysing
535 food samples (at any step in the elaboration process) for the presence of BA-producing
536 bacteria. PCR assays provide methods that can be successfully used for the routine
537 detection of bacterial strains potentially producers of histamine, tyramine, putrescine,
538 and cadaverine in foods. These procedures are highly specific method, and their results
539 are easy to interpret compared to others conventional methods.

Acknowledgments

This work was supported by grant RM03-002 (INIA), AGL2005-00470 (CICYT), and S-0505/AGR/000153 (CAM). The technical assistance of M. V. Santamaría and A. Gómez is greatly appreciated.

References

Bardócz, S., 1995. Polyamines in food and their consequences for food quality and human health. Trends in Food Science and Technology 6, 341-346.

Bover-Cid, S., Holzapfel, W., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. International Journal of Food Microbiology 53, 33-41.

Bodmer, S., Imark, C., Kneubühl, M., 1999. Biogenic amines in food. Histamine and food processing. Inflammation Research 48, 296-300.

Constantini, A., Cersosimo, M., del Prete, V., Garcia-Moruno, E., 2006. Production of biogenic amine by lactic acid bacteria, screening by PCR, thin layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. Journal of Food Protection 69, 391-396.

Coton, E., Coton, M., 2005. Multiplex PCR for colony direct detection of gram-positive histamine- and tyramine-producing bacteria. Journal of Microbiological. Methods 63, 296-304.

564 Coton, M., Coton, E., Lucas, P., Lonvaud, A., 2004. Identification of the gene encoding
565 a putative tyrosine dacarboxylase of *Carnobacterium divergens* 508.
566 Development of molecular tools for the detection of tyramine-producing
567 bacteria. Food Microbiology 21, 125-130.
568 Coton, E., Rollan, G., Bertrand, A, Lonvaud-Funel, A., 1998a. Histamine-producing
569 lactic acid bacteria in wines: early detection, frequency and distribution.
570 American Journal of Enology and Viticulture 49, 199-204.
571 Coton, E., Rollan, G.C., Lonvaud-Funel., A., 1998b. Histidine decarboxylase of
572 *Leuconostoc oenos* 9204: purification, kinetic properties, cloning and nucleotide
573 sequence of the *hdc* gene. Journal of Applied Microbiology 84, 143-151.
574 De las Rivas, B., Marcobal, A., Muñoz, R., 2005. Improved multiplex-PCR method for
575 the simultaneous detection of food bacteria producing biogenic amines. FEMS
576 Microbiology Letters 244, 367-272.
577 De las Rivas, B., Marcobal, A., Carrascosa, A., Muñoz, R., 2006a. PCR detection of
578 food bacteria producing the biogenic amines histamine, tyramine, putrescine and
579 cadaverine. Journal of Food Protection 69:2509-2514.
580 De las Rivas, B., Marcobal, A., Muñoz, R., 2006b. Gene organization of the ornithine
581 decarboxylase encoding region in *Morganella morganii.* Journal of Applied
582 Microbiology doi:10.1111/j.1365-2672.2006.03188.
583 De las Rivas, B., Carrascosa, A.V., Muñoz, R. 2007.Gene cloning, expression and
584 functional characterization of a ornithine decarboxylase protein from *Serratia*
585 *liquefaciens* IFI65. Journal of Microbiology and Biotechnology (in press).
586 Fernández, M., Liñares, D.M., Álvarez, M.A., 2004. Sequencing of the tyrosine
587 decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a

588 PCR method for detecting tyrosine decarboxylating lactic acid bacteria. Journal
589 of Food Protection 67, 2521-2529.
590 Fernández, M., del Rio, B., Linares, D. M., Martín, M. C., Álvarez, M. A., 2006. Real-
591 time polymerase chain reaction for quantitative detection of histamine-producing
592 bacteria: use in cheese production. Journal of Dairy Science 89, 3763-3769.
593 Fernández, M., Flórez, A.B., Liñares, D.M., Mayo, B., Álvarez, M.A., 2006. Early PCR
594 detection of tyramine-producing bacteria during cheese production. Journal of
595 Dairy Research 30, 1-4.
596 García-Moruno, E., Carrascosa, A., Muñoz, R., 2005. A rapid and inexpensive method
597 for the determination of biogenic amines from bacterial cultures by thin-layer
598 chromatography. Journal of Food Protection 68, 625-9.
599 Granchi, L.G., Talini, D., Rigacci, S., Guerrini, S., Berti., A, Vincenzini, M., 2006.
600 Detection of putrescine-producer *Oenococcus oeni* strains by PCR. 8th
601 Symposium on lactic acid bacteria, The Netherlands.
602 Halàsz, A., Barath, A., Simon-Sarkadi, L., Holzapfel, W., 1994. Biogenic amines and
603 their production by microorganisms in food. Trends in Food Science and
604 Technology 5, 42-49.
605 Izquierdo-Pulido, M., Carceller-Rosa, J.M., Mariné-Font, A., Vidal-Carou, M.C., 1997.
606 Tyramine formation by *Pediococcus* spp. during beer fermentation. Journal of
607 Food Protection 60, 831-836
608 Kanki, M., Yoda, T., Tsukamoto, T., Shibata, T., 2002. *Klebsiella pneumoniae* produces
609 no histamine: *Raoultella planticola* and *Raoultella ornithinolytica* strains are
610 histamine producers. Applied and Environmental Microbiology 68, 3462-3466.
611 Karmas, E., 1981. Biogenic amines as indicators of seafood freshness. Lebensmittel-
612 Wissenschaft and Technologie 14, 273-275.

613 Landete, J.M., Ferrer, S., Pardo, I., 2005. Which are the lactic acid bacteria responsible
614 for histamine production in wine?. Journal of Applied Microbiology 99, 580-
615 586.
616 Landete, J.M., Pardo, I., Ferrer, S., 2006. Tyramine and phenylethylamine production
617 among lactic acid bacteria isolated from wine. International Journal of Food
618 Microbiology (DOI: 10.1016/j.ijfoodmicro.2006.10.051).
619 Lehane, L. Olley, J., 2000. Histamine fish poisoning revisited. International Journal of
620 Food Microbiology 58, 1-37.
621 Le Jeune, C., Lonvaud-Funel, A., Ten Brink., Hofstra, H., van der Vossen. J.M.B.M.,
622 1995. Development of a detection system for histidine decarboxylating lactic
623 acid bacteria on DNA probes, PCR and activity test. Journal of Applied
624 Bacteriology 78, 316-326.
625 Lonvaud-Funel, A., Joyeux, A., 1994. Histamine production by wine lactic acid
626 bacteria: Isolation of a histamine-producing strain of *Leuconostoc oenos*. Journal
627 of Applied Bacteriology 77, 401-407.
628 Lucas, P., Lonvaud-Funel, A., 2002. Purification and partial gene sequence of the
629 tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. FEMS Microbiology
630 Letters 211, 85-89.
631 Lucas, P., Landete, J., Coton, M., Coton, E., Lonvaud-Funel, A., 2003. The tyrosine
632 decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and
633 conservation in tyramine-producing bacteria. FEMS Microbiology Letters 229,
634 65-71.
635 Maijala, R. L., 1993. Formation of histamine and tyramine by some lactic acid bacteria
636 in MRS-broth and modified decarboxylation agar. Letters in Applied
637 Microbiology 17, 40-43.

638 Maynard, L. S., Schenker, V. J., 1996. Monoamine-oxidase inhibition by ethanol in
639 vitro. Nature 196, 575-576.
640 Marcobal, A., de las Rivas, B., García-Moruno, E., Muñoz, R., 2004a. The tyrosine
641 decarboxylation test does not differentiate *Enterococcus faecalis* from
642 *Enterococcus faecium*. Systematic and Applied Microbiology 27, 423-426.
643 Marcobal., A., de las Rivas, B., Moreno-Arribas, M.V., Muñoz, R., 2004b.
644 Identification of the ornithine decarboxylase gene in the putrescine-producer
645 *Oenococcus oeni* BIFI-83. FEMS Microbiology Letters 239, 213-220.
646 Marcobal, A., de las Rivas, B., Moreno-Arribas, M.V., Muñoz, R., 2005. Multiplex
647 PCR method for the simultaneous detection of histamine-, tyramine-, and
648 putrescine-producing lactic acid bacteria in foods. Journal of Food Protection
649 68, 874-878.
650 Marcobal, A., de las Rivas, B., Muñoz, R., 2006a. First genetic characterization of a
651 bacterial beta-phenylethylalanine biosynthetic enzyme in *Enterococcus faecium*
652 RM58. FEMS Microbiology Letters 258, 144-149.
653 Marcobal, A., de las Rivas, B., Muñoz, R., 2006b. Methods for the detection of bacteria
654 producing biogenic amines on foods: a survey. Journal für Verbraucherschutz
655 und Lebensmittelsicherheit 1, 187-196.
656 Marcobal, A., Martín-Álvarez, P.J., Moreno-Arribas, M.V., Muñoz, R., 2006c. A
657 multifactorial design for studying factors influencing growth and tyramine
658 production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and
659 *Enterococcus faecium* BIFI-58. Research in Microbiology 157, 417-424.
660 Moreno-Arribas, M. V., Lonvaud-Funel, A. 2001. Purification and characterization of
661 tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine.
662 FEMS Microbiology Letters 195, 103-107.

663 Morii, H., Kasama, K., Herrera-Espinoza, R., 2006. Cloning and sequencing of the
664 histidine decarboxylase gene from *Photobacterium phosphoreum* and its
665 functional expression in *Escherichia coli*. Journal of Food Protection 69, 1768-
666 1776.
667 Priebe, K., 1984. Is the histamine concentration a criterion for the spoilage of fish?.
668 Archiv fur Lebensmittelhygiene 35, 123-128.
669 Ruíz-Capillas, C., Jiménez-Colmenero, F., 2004. Biogenic amines in meat and meat
670 products. Critical Reviews in Food Science and Nutrition 44, 489-499.
671 Shalaby, A.R., 1996. Significance of biogenic amines in food safety and human health.
672 Food Research International 29, 675– 690.
673 Silla, M.H., 1996. Biogenic amines: their importance in foods. International Journal of
674 Food Microbiology 29, 213, 231.
675 Straub, B. W., Kicherer, M., Schilcher, S. M., Hammes, W. P., 1995. The formation of
676 biogenic amines by fermentation organisms. Zeitschrift fur Lebensmittel-
677 Untersuchung und-Forschung 201, 79-82.
678 Sumner, S.S. and Taylor, S.L., 1989. Detection method for histamine- producing, dairy-
679 related bacteria using diamine oxidase and leucocrystal violet. Journal of Food
680 Protection 52, 105-108.
681 Suzzi, G., Gardini, F., 2003. Biogenic amines in dry fermented sausages: a review.
682 International Journal of Food Microbiology 88, 41-51.
683 Takahashi, H., Kimura, B., Yoshikawa, M., Fujii, T., 2003. Cloning and sequencing of
684 the histidine decarboxylase genes of gram-negative, histamine-producing
685 bacteria and their application in detection and identification of these organisms
686 in fish. Applied and Environmental Microbiology 69, 2568-2578.

687 Ten Brink, B., Damink, C., Joosten, H.M.L.J., Huis In't Veld. J.H.J., 1990. Ocurrence
688 and formation of biologically active amines in foods. International Journal of
689 Food Microbiology 11, 73-84.
690 Torres Alves, dos Santos, A.T., Martins, A.F., 2002. Detection of histamine-producing
691 bacteria using polymerase chain reaction techniques and DNA probes. European
692 Food Research and Technology 214, 178-180.
693 Wathersen, J.J., Scanlan, R.A., Bills, D.D., Libbey, L.M., 1975. Formation of
694 heterocyclic N-nitrosamines from the reaction of nitrite and selected primary
695 diamines and amino acids. Journal of Agricultural and Food Chemistry 23, 898-
696 902.
697
698
699 **Figure captions**
700
701 Figure 1. Partial alignment of conserved HDC protein regions from gram-positive
702 bacteria where oligonucleotide primers have been designed. The represented sequences
703 are from *Lactobacillus buchneri* B301 (accession CAG44458) (LBU), *Oenococcus oeni*
704 9204 (AAC38298) (OOE), *Lactobacillus* sp. 30a ATCC 33222 (DCLBHP) (L30),
705 *Lactobacillus hilgardii* 0006 (AAV65956.1) (LHI), *Clostridium perfringens* ATCC
706 13124 (DCCLHP) (CPE), *Tetragenococcus muriaticus* (BAD51812) (TMU), and a
707 partial sequence from *Micrococcus* sp. (PN0143) (MIC). The positions of the nucleotide
708 primers are indicated by arrows.
709
710 Figure 2. Partial alignment of conserved HDC protein regions from gram-negative
711 bacteria where oligonucleotide primers have been designed. The represented sequences

712 are from *Enterobacter aerogenes* ATCC 43176 (A40004) (EAE), *Escherichia coli* MA-
713 A (BAC20387) (ECO), *Morganella morganii* (A25013) (MMO), *Photobacterium*
714 *phosphoreum* RHE01 (AA065983.1) (PPH), and *Pseudomonas fluorescens* WCS374
715 (P95477) (PFL); and partial sequences from *Erwinia* sp. MB31 (AB083208) (ERW),
716 *Raoultella planticola* 4131 (BAC20386) (RPL), *Raoultella ornytolytica* (Q8l0Z4)
717 (RPL), *Proteus vulgaris* AU3 (BAC20384.1) (PVU), and *Photobacterium damselae*
718 JCM8968 (BAC45248.1) (PDA).The position of the nucleotide primers are indicated by
719 arrows.
720
721 Figure 3. Partial alignment of conserved bacterial TDC protein regions where
722 oligonucleotide primers have been designed. The represented sequences are from
723 *Lactococcus lactis* IPLA 655 (CAF33980) (LLA), *Carnobacterium divergens* 508
724 (AAQ73505) (CDI), *Enterococcus faecalis* (AAM46082) (EFA), *Enterocoocus faecium*
725 BIFI-58 (CAH04395) (EFI), *Lactobacillus brevis* IOEB 9809 (AAN77279) (LBR),
726 *Lactobacillus curvatus* HSCC1737 (BAE02560) (LCU), and *Tetragenococcus*
727 *halophilus* (BAD93616) (THA). The position of the nucleotide primers are indicated by
728 arrows.
729
730 Figure 4. Partial alignment of conserved bacterial ODC protein regions where
731 oligonucleotide primers have been designed. Bacterial ODC alignments showed two
732 separated groups, one of them (A) includes several proteins from gram-positive bacteria
733 such as *Lactobacillus* sp. 30a (P43099) (L30), *Lactobacillus johnsonii* (NP_965822.1)
734 (LJO) and *Oenococcus oeni* (CAG34069) (OEN) and from gram-negative bacteria such
735 as *Escherichia coli* MG1655 (P21169) (P24169) (ECO), *Haemophilus influenzae*
736 ATCC 51907 (P44317) (HAE), *Pasteurella multocida* (Q9CMC3) (PMU), *Salmonella*

typhimurium LT2 (NP_462030) (NP_459686) (STY), *Shigella flexneri* 2a str. 301 (NP_708736) (SFL), *Vibrio cholerae* N16961 (NP_233445) (VCH), *Vibrio parahaemolyticus* RIMD 2210633 (NP_801145) (VPA), *Vibrio vulnificus* (Q8D4Q4) (VVU), and *Yersinia pestis* KIM (NP_670646) (YPE). (B) The second ODC protein group includes *Brucella melitensis* 16M (NP_542111) (BME), *Pseudomonas aeruginosa* PAO1 (AAG07907) (PAE), and *Pseudomonas putida* KT2440 (NP_743025) (PPU). The position of the nucleotide primers are indicated by arrows.

Figure 5. Partial alignment of conserved bacterial LDC protein regions where oligonucleotide primers have been designed. Bacterial LDC are aligned in two groups. (A) The first group comprises proteins from Gram-negative bacteria, mainly *Enterobacteriaceae* [*Escherichia coli* W3110 (P52095) (ECO), *Salmonella enterica* subsp. *enterica* serovar Typhi Saty-USM-130 (AAQ16557) (SEN), *Hafnia alvei* (P05033) (HAL), *Eikenella corrodens* ATCC 23824 (AAD18126) (ECR), *Salmonella typhimurium* LT2 (AAL19198) (STY), *Shigella flexneri* 2a str. 301 (NP_706131) (SFL), *Vibrio cholerae* 01 biovar *eltor* str. N16961 (NP_229937) (VCH), *Vibrio vulnificus* YJ016 (NP_935174) (VVU), and *Vibrio parahaemolyticus* RIMD 2210633 (NP_799269) (VPA)]. (B) The second LDC group includes lysine decarboxylases from Gram-positive bacteria, such as *Bacillus halodurans* C-125 (BAB03760) (BHA), *Bacillus subtilis* (P12885) (BSU), *Clostridium acetobutylicum* ATCC 824 (NP_346938) (CAC), *Clostridium perfringens* str. 13 (NP_563369) (CPE), *Listeria innocua* (NP_472170) (LIN), *Listeria monocytogenes* EGD-e (NP_466216) (LMO), *Staphylococcus aureus* subsp. *aureus* N315 (NP_373691) (SAU), and *Staphylococcus epidermidis* ATCC 12228 (NP_765857) (SEP).

Table 1

Table 1.
Primers designed for the detection of histamine-producing bacteria

Primer	5´→3´sequence	Coding for	Described by
Gram-positive bacteria :			
CL1	CCWGGWAAWATWGGWAATGGWTA	PGNIGNGY	Le Jeune et al. 1995
CL2	GAWGCWGTWGTCATATTWATTTGWCC	GQINMTTAS	Le Jeune et al. 1995
JV16HC	AGATGGTATTGTTTCTTATG	DGIVSY	Le Jeune et al. 1995
JV17HC	AGACCATACACCATAACCTT	QGYGVWS	Le Jeune et al. 1995
JV17	AGACCATACACCATAACCTTG	QGYGVWS	Coton et al. 1998
CL1mod	CCAGGWAACATTGGTAATGGATA	PGNIGNGY	Landete et al. 2005
HDC3	GATGGTATTGTTTCKTATGA	DGIVSY	Coton and Coton, 2005
HDC4	CAAACACCAGCATCTTC	EDAGVW	Coton and Coton, 2005
PHDC1	CCGTGCGGAAACAAAGAAT	RAETKN	Constantini et al. 2006
PHDC2	CCAAACACCAGCATCTTCA	EDAGVW	Constantini et al. 2006
HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	GIVSYDRAE	De las Rivas et al. 2006
HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA	YGVWSAIAI	De las Rivas et al. 2006
Hdc1	TTGACCGTATCTCAGTGAGTCCAT	DRIAISPY	Fernández et al. 2006
Hdc2	ACGGTCATACGAAACAATACCATC	DGIVSYDR	Fernández et al. 2006
Gram-negative bacteria :			
KPF2	AAAGCTGGGGGTATGTGACC	ESWGYVT	Kanki et al. 2003
KPR4	GTGATGGAGTTTTTGTTGC	RNKNSIT	Kanki et al. 2003
hdc-f	TCHATYARYAACTGYGGTGACTGGRG	SISNCGDW	Takahashi et al. 2003
hdc-r	CCCACAKCATBARWGGDGTRTGRCC	RNGMTPLMM	Takahashi et al. 2003
106	AAYTCNTTYGAYTTYGARAARGARG	NSFDFEKEV	De las Rivas et al. 2005
107	ATNGGNGANCCDATCATYTTRTGNCC	GHKMIGSPI	De las Rivas et al. 2005
HIS2-F	AAYTSNTTYGAYTTYGARAARGARGT	NSFDFEKEV	De las Rivas et al. 2006
HIS2-R	TANGGNSANCCDATCATYTTRTGNCC	GHKMIGSPI	De las Rivas et al. 2006
DegF	GGYGGIACIGARGGNAANATG	GGTEGNM	Morii et al. 2006
DegR	GGRAAIACICANGTNWTGGAG	NSITVVFP	Morii et al. 2006

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.

Table 2

Table 2
PCR conditions used to detect biogenic amine-producing bacteria by conventional PCR methods
[a]ND, No data available

Gene	Primer set	Amplicon size (pb)	Primer (µM)	PCR Step 1	PCR Step 2	PCR Step 3	Cycle number	Reference
hdc	CL1/CL2	150	ND[a]	96 °C, 30 s	42 °C, 30 s	72°C, 2 min	4	Le Jeune et al. 1995
				95 °C, 1 min	48 °C, 1 min	72 °C 1min	36	
	CL1/JV17HC	458	ND	95 °C, 1 min	48 °C, 1 min	72 °C, 1 min	30	Le Jeune et al. 1995
	JV16HC/JV17HC	367	ND	95 °C, 1 min	48 °C, 1 min	72 °C, 1 min	30	Le Jeune et al. 1995
			0.3	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
			ND	95 °C, 1 min	39.6 °C, 1 min	72 °C, 1 min	40	Landete et al. 2005
	JV17/CL1	458	ND	94 °C, 30 s	48 °C, 30 s	72 °C, 2 min	40	Coton et al. 1998
	CL1mod/JV17HC	458	0.05	94 °C, 30 s	39.4 °C, 30 s	72 °C, 2 min	40	Landete et al. 2005
	HDC3/HDC4	435	0.02	95 °C, 45 s	48 °C, 45 s	72 °C, 2 min	35	Coton and Coton, 2005
	PHDC1/PHDC2	497	0.4	94 °C, 1 min	50 °C, 1 min	72 °C, 2 min	30	Costantini et al. 2006
	HIS1-F/HIS1-R	372	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
	KPF2/KPR4	685	0.025	98 °C, 5 s	62 °C, 5 s	72 °C, 5 s	30	Kanki et al. 2003
	hdc-f/hdc-r	709	0.02	94 °C, 1 min	58 °C, 1 min	72°C, 1 min	35	Takahashi et al. 2003
	106/107	534	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2005
	HIS2-F/HIS2-R	531	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
	DegF/DegR	712	0.2	94 °C, 1 min 30 s	45 °C, 1 min	50 °C, 1 min	35	Morii et al. 2006
tdc	P2-for/P1-rev	924	ND	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min	35	Lucas and Lonvaud-Funel, 2002
			2	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
			0.4	94 °C, 1 min	50 °C, 1 min	72 °C, 2 min	30	Constantini et al. 2006
	41/42	213	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
	Pt3/Pt4	560	0.4	94 °C, 1 min	50 °C, 1 min	72 °C, 2 min	30	Constantini et al. 2006
	P1-rev/p0303	370	0.05	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min	35	Landete et al. (submitted)
	TD5/TD2	1100	ND	95 °C, 45 s	48 °C, 45 s	72 °C, 1 min	35	Coton et al. 2004
	TDC1/TDC2	720	0.2	95 °C, 45 s	50 °C, 1min	72 °C, 1 min	35	Fernández et al. 2004
	TDC-F/TDC-R	825	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
odc	3/4	1056	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
	3/16	1446	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
	16/15	1353	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
	4/15	972	1	95 °C, 30 s	52 °C, 30 s	72 °C, 2 min	30	Marcobal et al. 2005
	AODC1/AODC2	1500	1	94 °C, 1 min	50 °C, 1 min	72 °C, 2 min	30	Constantini et al. 2006
	PUT1-F/PUT1-R	1440	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
	PUT2-F/PUT2-R	624	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
ldc	CAD1-F/CAD1-R	1098	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006
	CAD2-F/CAD2-R	1185	1	95 °C, 30 s	53 °C, 30 s	72 °C, 2 min	30	De las Rivas et al. 2006

Table 3
Primers designed for the detection of tyramine producing-bacteria

Primer	5´→3´sequence	Coding for	Described by
P2-for	GAYATIATIGGIATIGGIYTIGAYCARG	DIIGIGLDQ	Lucas and Lonvaud-Funel, 2002
P1-rev	CCRTARTCIGGIATIGCRAARTCIGTRTG	HTDFAIPDY	Lucas and Lonvaud-Funel, 2002
41	CAYGTNGAYGCNGCNTAYGGNGG	HVDAAYGG	Marcobal et al. 2005
42	AYRTANCCCATYTTRTGNGGRTC	DPHKMMGY	Marcobal et al. 2005
Pt3	TACACGTAGATGCTGCATATG	MVDYVLKE	Constantini et al. 2006
Pt4	ATGGTTGACTATGTTTTAAAAGAA	HVDAAYGG	Constantini et al. 2006
p0303	CCACTGCTGCATCTGTTTG	ATAASW	Lucas et al. 2003
TD5	CAAATGGAAGAAGAAGTAGG	QMEEEVG	Coton et al., 2004
TD2	ACATAGTCAACCATRTTGAA	FNMVDYV	Coton et al., 2004
57	ATGAGTGAATCATTGTCG	MSESL	Marcobal et al. 2004a
58	TTATTTTGCTTCGCTTGCC	ASEAK	Marcobal et al. 2004a
TDC1	AACTATCGTATGGATATCAACG	NYRMDIN	Fernández et al. 2004
TDC2	TAGTCAACCATATTGAAATCTGG	PDFNMVDY	Fernández et al. 2004
TDC-F	TGGYTNGTNCCNCARACNAARCAYTA	WLVPQTKHK	De las Rivas et al. 2006
TDC-R	ACRTARTCNACCATRTTRAARTCNGG	PDFNMVDYV	De las Rivas et al. 2006

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.

Table 4
Primers designed for the detection of putrescine producing-bacteria

Primer	5´→3´sequence	Coding for	Described by
3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	VFNADKTYF	Marcobal et al. 2004b
16	TACRCARAATACTCCNGGNGGRTANGG	PYPPGVFCV	Marcobal et al. 2004b
4	ATNGARTTNAGTTCRCAYTTYTCNGG	PEKCDLNSI	Marcobal et al. 2005
15	GGTAYTGTTYGAYCGGAAWAAWCAYAA	VLFDRNNHK	Marcobal et al. 2005
AODC1	GMTCGTGAAATYAARCKG		Constantini et al. 2006
AODC2	KGRGTTCMGCYGGRGTAT		Constantini et al. 2006
PUT1-F	TWYMAYGCNGAYAARACNTAYYYTGT	FNADKTYFV	De las Rivas et al. 2006
PUT1-R	ACRCANAGNACNCCNGNGGRTANGG	PYPPGVLCV	De las Rivas et al. 2006
PUT2-F	ATHWGNTWYGGNAAYACNATHAARAA	ISYGNTIKK	De las Rivas et al. 2006
PUT2-R	GCNARNCCNCCRAAYTTNCCDARTC	DIGKFGGLA	De las Rivas et al. 2006

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.

Table 5
Primers designed for the detection of cadaverine producing-bacteria

Primer	5´→3´sequence	Coding for	Described by
CAD1-F	TTYGAYWCNGCNTGGGTNCCNTAYAC	PGFETDIHG	De las Rivas et al. 2006
CAD1-R	CCRTGDATRTCNGTYTCRAANCCNGG	FDSAWVPYT	De las Rivas et al. 2006
CAD2-F	CAYRTNCCNGGNCAYAA	HVPGHK	De las Rivas et al. 2006
CAD2-R	GGDATNCCNGGNGGRTA	YPPGIP	De las Rivas et al. 2006

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 1

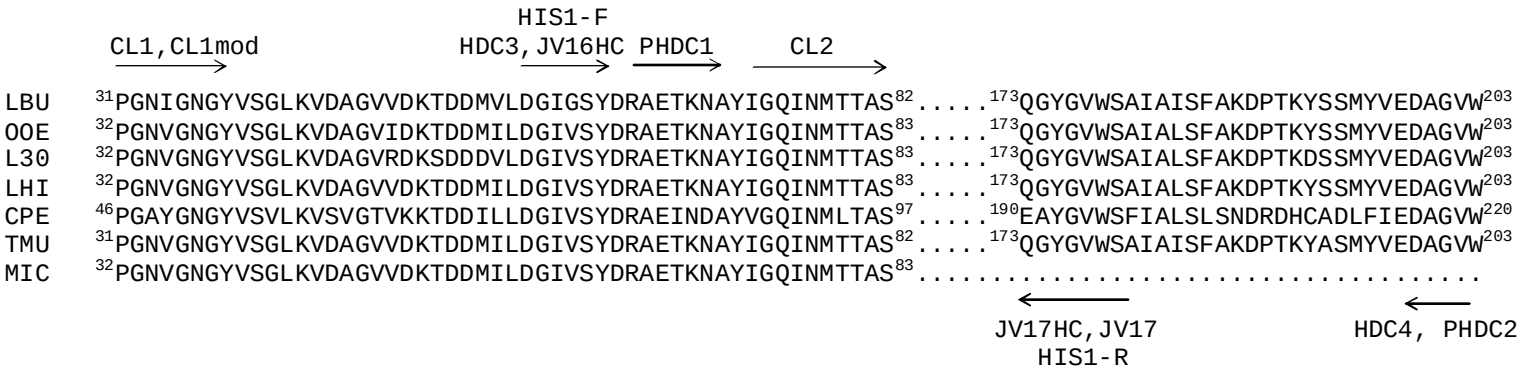


Figure 2

Figure 2

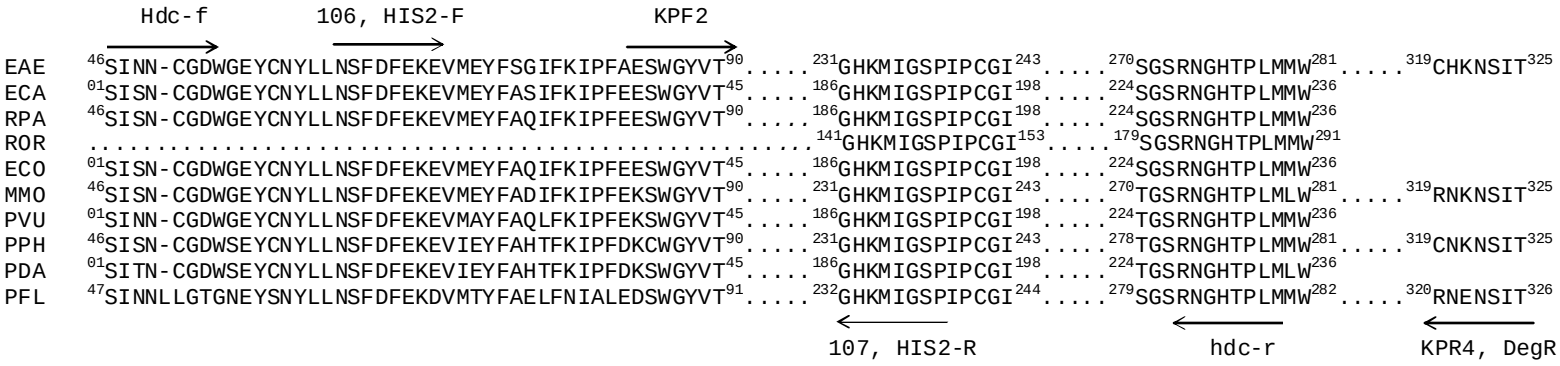


Figure 3

Figure 3

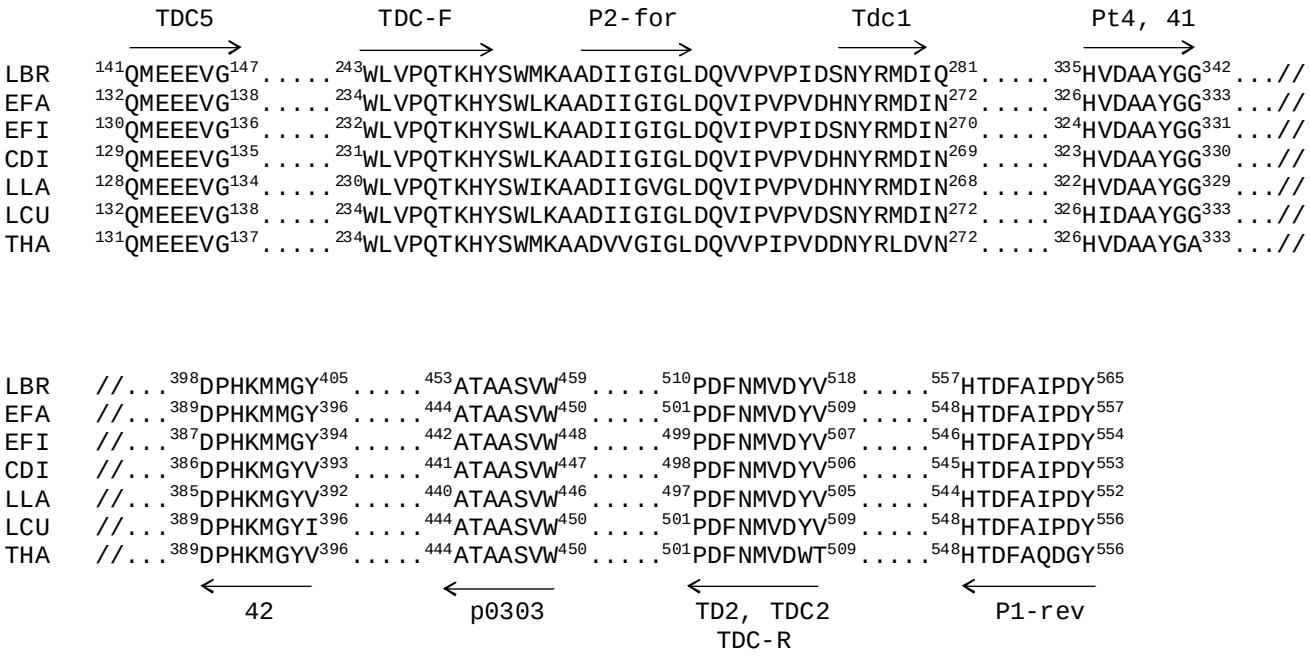
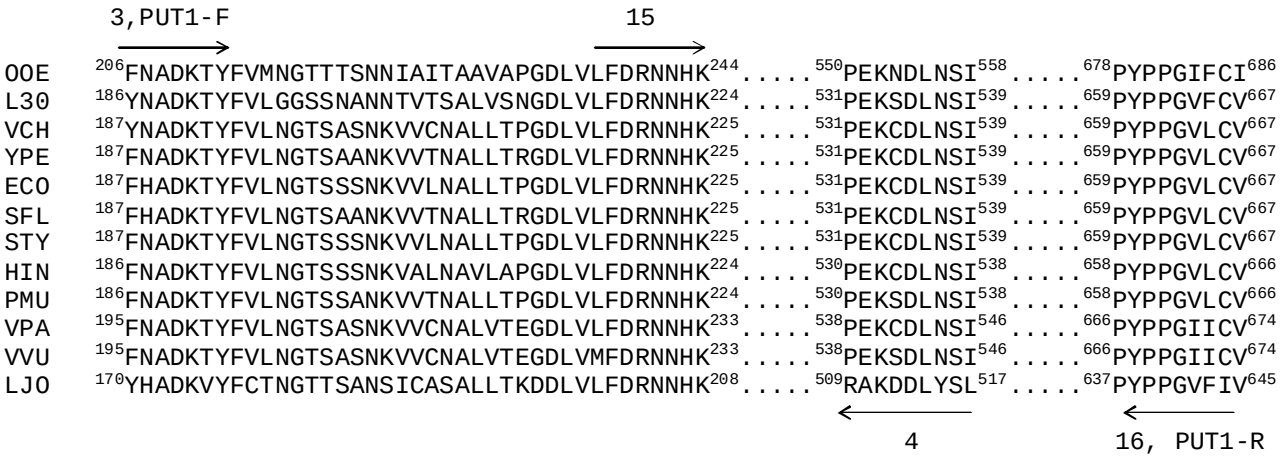


Figure 4

Figure 4

A



B

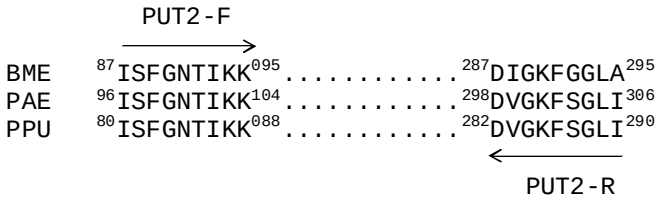


Figure 5

Figure 5