Biogenic amine production by bacteria isolated from ice-preserved sardine and mackerel

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The occurrence of \emph{in vitro} production of biogenic amines in bacteria isolated from ice-preserved sardine and mackerel was studied. Biogenic amine-production was investigated by means of amino acid decarboxylation by growth on decarboxylase differential medium, biogenic amine detection by thin-layer chromatography (TLC) and decarboxylase gene detection by PCR. Decarboxylase medium overestimate the number of biogenic amine-producer strains, as the production of amine was confirmed by TLC in only five out the 17 presumptive strains. On the producer strains, PCR was used to confirm the presence of the genes encoding the amino acid decarboxylase responsible for the synthesis of these amines. Moreover, biogenic amine-producer bacteria were molecularly identified by sequencing their 16S rRNA. Form sardine, enterobacteria producing simultaneously several biogenic amines were isolated. A \emph{Kluyvera intermedia} strain producing histamine, putrescine and cadaverine, and an \emph{Enterobacter asburiae} strain producing only the diamines cadaverine and putrescine were identified. From mackerel, lactic acid bacteria from the \emph{Enterococcus durans} species producing tyramine were isolated. This study constitutes the first description of the presence on these putatively harmful species on ice-preserved sardine and mackerel.

\textbf{Keywords}: \emph{Sardina pilchardus}; \emph{Scomber scombrus}; \emph{Enterobacteriaceae}; Histamine; Tyramine; Putrescine; Cadaverine.
The presence of biogenic amines in fish is of concern to researchers, consumers, food companies, and health authorities due to their toxicological effects. Biogenic amines have been classified regarded as potentially hazardous compounds of food that may cause disorders to consumers (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994; Silla, 1996). Moreover, these amines serve as chemical indicators of fish spoilage as these amines are essentially absent or occur at very low levels in fresh fish (Al Bulushi, Poole, Deeth, & Dykes, 2009). Despite a widely reported association between histamine and scombroid food poisoning, histamine alone appears to be insufficient to cause food toxicity. Putrescine and cadaverine have been suggested to potentiate histamine toxicity. With respect to spoilage on the other hand, only cadaverine has been found to be a useful index of the initial stage of fish decomposition (Al Bulushi, Poole, Deeth, & Dykes, 2009). In most early studies on biogenic amine formation in fish, researchers have focused on histamine poisoning and concluded that the families Scombridae and Scomberesocidae are commonly implicated in incidents of histamine poisoning as they contained high levels of free histidine in the muscle. Histamine is produced by bacteria that decarboxylate histidine to histamine as the fish decomposes. Indeed, various scombroids, including mackerel, tunas, saury, bonito, seerfish and butterfly kingfish, have been implicated in cases of histamine poisoning. However, non-scombroid fish which also contained high levels of free histidine in the muscle, such as sardine, pilchards, anchovies, herring and marline, has also been implicated in cases of histamine poisoning (Taylor, 1986).

Biogenic amines accumulation usually results from the decarboxylation of amino acids by enzymes of bacterial origin, which is associated with food hygiene and technology. Therefore, poor hygiene is probably the main factor involved in the formation
of these compounds. Bacterial contamination could be derived from postcatching contamination on board fishing vessels, at the processing plant or in the distribution system. The formation of biogenic amines in fish requires the presence of decarboxylase–producing microorganisms, which may be introduced by contamination. Adequate concentrations of the precursor free amino acids and environmental factors supporting bacterial growth and favouring the synthesis of decarboxylase enzymes are also of critical significance (Halász, Baráth, Simon–Sarkadi, & Holzapfel, 1994).

The presence of bacteria able to produce biogenic amines can be detected by a variety of techniques including HPLC, TLC, GC/MS and enzymatic test (Marcobal, de las Rivas, & Muñoz, 2006). Molecular methods for detection and identification of food-borne bacteria are becoming more widely accepted as an alternative to traditional culture methods. The use of molecular tools for early and rapid detection of biogenic amine bacteria is important for preventing the accumulation of these biogenic amines in fish and other food products. Genetic procedures accelerate getting results and allow the introduction of early control measures to avoid the development of these bacteria. Several studies describing PCR techniques targeting bacterial amino acid decarboxylase genes have been reported (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006; Marcobal, de las Rivas, & Muñoz, 2006; Landete, de las Rivas, Marcobal, & Muñoz, 2007).

Histamine producer strains (Morganella morganii, Proteus vulgaris, Pantoea agglomerans, Enterobacter cloacae) have been identified from mackerel, a scombroid fish (Kim et al., 2001; Tsai et al., 2005). From sardine, a non-scombroid fish which possess a high level of free histidine on the muscle, in spite that microbiological analysis have been done, none specific bacterial species has been related to biogenic amine production (Ababouch, Afilal, Benadeljelil, & Busta, 1991; Erkan & Özden, 2008). Moreover, none of the molecular methods available for bacterial identification and for the detection of
biogenic amine producer bacteria had never been applied in mackerel and sardines samples. Therefore, the present study deals, for the first time, with the molecular characterization of the in vitro biogenic amine-producer microbiota present on ice-preserved sardine and mackerel.

2. Materials and methods

2.1. Sampling procedure

The freshwater fishes, sardine (Sardina pilchardus) and mackerel (Scomber scombrus) were caught in Mediterranean sea (the coast of Tunisia), iced immediately in an ice box and delivered to the laboratory approximately 2 h later. Upon arrival at the lab, sardines and mackerel were placed individually. Whole ungutted fish were stored at 4 °C for 7 days. Samples of dorsal muscle were aseptically homogenised with a pestle in sterile saline solution.

2.2. Evaluation of amino acid-decarboxylase activity

2.2.1. Growth in decarboxylase Niven’s medium

For detection of amino acid decarboxylating bacteria in fish homogenates, samples were serially diluted in saline and spread plated on modified Niven’s agar [0.5% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% CaCO₃, 3% agar, and 0.006% bromocresol purple, pH 5.3] (Mavromatis, & Quantick, 2002) containing L-histidine monohydrochloride, L-
ornithine monohydrochloride, and L-lysine, at 0.25%, and tyrosine disodium salt at 0.2% due to its low solubility. The inoculated plates were incubated at 30 °C during 1–3 days. After the incubation time, the colour around the colonies was reported. Presumptively, the appearance of a purple or slight-purple colour indicated biogenic amine production.

2.2.2. Biogenic amine analysis from bacterial cultures by ion-exchange chromatography

Purple or slight-purple colonies from the Niven’s medium plates were picked out from an appropriate dilution plate. Production of biogenic amines was confirmed by inoculating these individual colonies from Niven’s medium plates directly into tubes containing 5 ml of LB medium containing the same precursor amino acids. After incubation, the broth media was centrifuged and the supernatant was analysed by thin layer chromatography (TLC) for biogenic amine content as described previously (García-Moruno, Carrascosa, & Muñoz, 2005). Briefly, amines were converted to their fluorescent dansyl derivatives by adding to one volume of the supernatant, one volume of 250 mM disodium phosphate (pH 9.0), 0.1 volume of 4 N sodium hydroxide solution, and two volumes of dansyl chloride solution (5 mg/ml of dansyl chloride in acetone). The reaction mixture was thoroughly mixed and incubated in the dark at 55 °C for 1 h. Then the samples were cooled and stored at 4 °C until use.

The amines were fractionated on precoated silica gel 60 F254 TLC plates. The dansylated compounds were separated in chloroform: triethylamine (4:1), and revealed with isopropanol: triethanolamine (4:1). The fluorescent dansyl derivative spots were visualized with the aid of a transiluminator with a suitable UV-light source (312 nm).
A standard solution of amines (5 g/l of histamine and 250 mg/l of tyramine, putrescine, and cadaverine) was prepared similarly and used as control.

2.2.3. Presence of amino acid decarboxylase genes in the biogenic amine-producer strains

For DNA extraction, bacteria were routinely grown in LB broth at 30 °C during 24 h. Bacterial chromosomal DNA was isolated directly from the cultures by using the Wizard genomic DNA purification kit (Promega) according to the manufacturer’s instructions. The DNA concentration was determined by using the Nano-drop Spectrophotometer. Chromosomal DNAs from the biogenic amine-producer strains were subjected to PCR amplification to detect the presence of the corresponding amino acid decarboxylase encoding genes (Table 1) (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006; Landete, de las Rivas, Marcobal, & Muñoz, 2007). These primers were previously described in a complete method for the PCR detection of foodborne bacteria producing biogenic amines (histamine, tyramine, putrescine, and cadaverine) in Gram-positive as well as in Gram-negative bacteria (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006).

PCR reactions were performed in 0.2 ml microcentrifuge tubes in a total volume of 25 µl containing 1µl of template DNA (aprox. 10 ng), 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 200 µM of each dNTP, 1µM of each primer, and 1 U of AmpliTaq Gold DNA polymerase. The reactions were performed in a Mastercycler® Personnall (Epperdorf) using the following cycling parameters: 10 min for enzyme activation at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 53 °C, and 2 min at 72 °C, and a final extension step of 20 min at 72 °C. PCR products were resolved on a 0.7% agarose gel (Pronadisa, Spain) and stained with ethidium bromide.
2.2.4. Analysis of the amino acid decarboxylase genes from the biogenic amine-producer strains

The amplification products were gel purified on QUIA-quick spin columns (QUIAGEN) for sequencing with the same PCR primers used for the amplification. DNA sequencing was carried out by using an Abi Prism 377 DNA™ sequencer (Applied Biosystems). Sequence similarity searches were carried out using Basic Local Alignment Search (BLAST) on the EMBL/GenBank databases (Altschul, Madden, Schaffer, Zhang, Zhang, Miller, & Lipman, 1997). Multiple alignments were done using CLUSTAL W on the EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss-Prot.

2.3. Taxonomical identification of the biogenic amine-producer strains.

Biogenic amine-producer strains were identified by PCR amplification and DNA sequencing of their 16S rDNA. The 16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f (5’-CAGGCCTAACACATGCAAGTC) and 1387r (5’-GGCGGWTGTACAGG) previously described (Marchesi, Sato, Wehtman, Martin, Fry, Hion, & Wade, 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR was performed in 25 μl amplification reaction mixture as described above. The reaction was performed by using the following cycling parameters: initial 10 min for enzyme activation at 95 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1:30 min at 72 °C. Amplified products were resolved on a 0.7%
agarose gel. The amplifications products were purified on QIAquick spin Columns (Quiagen, Germany) for direct sequencing. DNA sequencing was carried out by using an Abi Prism 377™ DNA sequencer (Applied Biosystems, USA). Sequence similarity searches were carried out by comparing to sequences from type strains included on the Ribosomal Database (http://rdp.cme.msu.edu).

3. Results and discussion

Biogenic amines are compounds implicated in food poisoning incidents. Biogenic amines in fish products are mainly produced by bacterial decarboxylation of amino acids. Several amino acids can be decarboxylated and, as a result biogenic amines are usually found, with histamine, tyramine, putrescine, and cadaverine being the most frequent. Many procedures have been proposed to evaluate the decarboxylase activity of microorganisms isolated from foods. Rapid screening methods can have some limitations in terms of sensitivity in detecting biogenic amine production leading to contradictory results. The presence of false-positive and false-negative strains is not negligible. For these reasons, biogenic amine production has to be confirmed by analytical methods such as HPLC. Most of the rapid screening procedures generally involve the use of a differential medium containing a pH indicator. The pH change is dependent on the production of the more alkaline amine form the amino acids initially included in the medium. In order to identify biogenic amine-producer strains, dilutions of sardine and mackerel homogenates were spread on modified Niven’s agar containing histidine, ornitine, lysine and tyrosine. The production of at least one biogenic amine will be recorded by the appearance of a purple or slight-purple colour of the media around the
A total of 17 strains appeared as presumptively biogenic amine-producer in the differential Niven’s medium. In order to confirm the production of biogenic amines by these strains, presumptively positive strains were inoculated in liquid media containing the same amino acids and the production of the amines was confirmed by a chromatographic assay (García-Moruno, Carrascosa, & Muñoz, 2005).

From the 17 strains analyzed, only five strains were found to be strong amine producers (Fig. 1, Table 2). From the sardine homogenate two biogenic amine producer strains were isolated, a putrescine and cadaverine-producer strain (strain 5) (Fig. 1A, Table 2), and a histamine, putrescine and cadaverine-producer strain (strain 9) (Fig. 1B, Table 2). From the mackerel homogenate tyramine-producer strains were only isolated (strains 11, 12 and 13) (Fig. 1C, Table 2). On this study the decarboxylase medium overestimates the number of biogenic amine-producer strains, giving false-positive results. These false results could be due to the production of a substance able to alkalinize the media since when these cultures were analyzed for the presence of biogenic amines by thin-layer chromatography none of them showed amine production. The results obtained in this work confirmed previous results describing that false-positive results could be obtained in decarboxylase growth media (Marcobal, de las Rivas, & Muñoz, 2006). In addition, it has been described in ice-preserved anchovies that ice-storage could hinder the growth of biogenic amine-producer bacteria, since the microorganisms showing this ability are mainly mesophilic bacteria (Pons-Sánchez-Cascado, Bover-Cid, Veciana-Nogués, & Vidal-Carou, 2005). Enteric microorganisms with amine-forming ability constitute a minor proportion of the fish microbiota and are difficult to isolate in high yields.

In order to correlate the production of these amines with the presence of the corresponding decarboxylase genes, we performed PCR assays for the detection of the hdc, tdc, odc and ldc genes, involved in the production of histamine, tyramine, putrescine
and cadaverine, respectively. Since a complete molecular method has been described to
detect biogenic amine producer bacteria, we checked the presence of the corresponding
genes by PCR (De las Rivas, Marcobal, Carrascosa, & Muñoz, 2006). By this method
seventy bacterial decarboxylase encoding genes could be detected (Table 1). The PCR assay
for the seven genes was applied to the biogenic amine-producer strains independently of
the amine produced. As showed in Fig. 2A and Table 2, strain 5, a putrescine and
cadaverine producer strain, amplified a 1440 pb DNA fragment from the ornithine
decarboxylase with PUT1-F and PUT1-R oligonucleotides, and a 1098 pb DNA with
CAD1-F and CAD1-R primers corresponding to lysine decarboxylases from Gram−
negative bacteria. Similarly, strain 9, a histamine−, putrescine− and cadaverine-producer
strain, amplified the same two DNA fragments (1440 and 1098 pb) and one additional 531
bp histidine decarboxylase fragment amplified with oligonucleotides HIS2-F and HIS2-R
(Fig. 2B, Table 2). The tyramine-producer strains (strains 11, 12, and 13) gave only an
amplicon corresponding to the expected size of the tyrosine decarboxylase internal
fragment (825 bp) (Fig. 2C, Table 2).

In order to confirm that these DNA fragments really correspond to internal fragments
of amino acid decarboxylase encoding genes, they were sequenced and similarity searches
were performed. Unfortunately, the sequence of both 1440 bp ornithine decarboxylase
fragments, from strains 5 and 9, could not be obtained as they seem to contain a mix of
two different sequences. This is not an unexpected result as it have been described that
enterobacteria sometimes are able to synthesize two sets of ornithine decarboxylases. One
set, produced during growth on neutral minimal medium, is referred to as the constitutive
(or biosynthetic) ornithine decarboxylase, when cultures are growth at low pH in the
presence of ornithine, an inducible (or degradative) ornithine decarboxylase is induced
(Morris, & Boecker, 1983). In E. coli, the constitutive ornithine decarboxylase bears quite
a striking resemblance to the inducible one, suggesting that they probably share a common evolutionary ancestor (Applebaum, Dunlap, & Morris, 1977). Similarly, *Morganella morganii* possess two closely related ornithine decarboxylases which their encoding genes could be amplified by the same degenerate oligonucleotides (De las Rivas, González, Landete, & Muñoz, 2008). Therefore, strains 5 and 9 could possess two ornithine decarboxylases, constitutive and inducible, being the PCR amplicon a mixture of the two DNA sequences.

As expected, BLAST databases searches of the translated DNA fragments identified high-scoring similarities with amino acid decarboxylases that act on histidine, lysine, and tyrosine. The predicted sequence of the histidine decarboxylase fragment from strain 9 was aligned with some histidine decarboxylases that had the highest overall identity with that of strain 9 (Fig. 3A). The highest sequence identity (99%) was between *Morganella morganii* and strain 9, followed by *Morganella psycrotolerans* (94%), *Enterobacter aerogenes* and *Photobacterium phosphoreum* (84%), and finally, *Photobacterium damselae* and *Raoultella planticola* (82%). These data confirm that strain 9 possess a histidine decarboxylase enzyme, and probably is an enterobacteria.

Similarly, the predicted sequences from the lysine decarboxylase fragments were aligned with lysine decarboxylase proteins included in the databases (Fig. 3B). Lysine decarboxylase from strain 5 showed high identity to lysine decarboxylases from *Citrobacter rodentium* (97%), *Citrobacter koseri* (96%), *Enterobacter cloacae* (94%), *Salmonella enterica* (94%), *Cronobacter sakazaki* (92%) and *Escherichia coli* (92%). Similarly, lysine decarboxylase from strain 9 showed 96% identity to the lysine decarboxylase from *Enterobacter cloacae*, 91% to *Salmonella enterica* and *Citrobacter koseri*, and 90% to the protein from *Citrobacter koseri*. The lysine decarboxylases from
strain 5 and 9 were 91% identical among them. From the similarity showed by their lysine decarboxylase fragments it could be assumed that both strains are enterobacteria. Finally, strains 11, 12, and 13 shared an identical sequence on the tyrosine decarboxylase fragment. This fragment was identical to the tyrosine decarboxylase from Enterococcus faecium, and showed 96% identity to the corresponding protein from E. durans and 94% to the E. hirae protein (Fig. 3C). These results indicated that strain 11 is a lactic acid bacteria, possibly belonging to the Enterococcus genera. Pons-Sánchez–Cascado, Bover-Cid, Veciana-Nogués, & Vidal-Carou (2005) indicated that, as a general rule, lactic acid bacteria were mainly tyramine producers. These results indicated that detection of the production of a biogenic amine in the culture media by TLC is confirmed by the presence of the gene encoding the decarboxylase enzyme by PCR. Therefore, and contrarily to the results obtained in decarboxylase medium, a close relation was observed among the results obtained by TLC and PCR methods. Since the production of biogenic amines was confirmed by chromatographic and molecular methods, we decided to taxonomically identify the bacteria producing amines in this study. Sometimes the phenotypic identification of microbiota is time-consuming and often problematic due to ambiguous biochemical or physiological traits. The development of molecular methods has offered the possibility of accelerating a great deal of bacterial identification. Therefore, the taxonomical identity of the biogenic amine-producer strains was assessed by the amplification of the DNA fragment coding the 16S rDNA. The bacterial isolates were then identified using sequence data from the first 500 bp of the 16S rRNA genes. The sequences obtained were compared to sequences from type strains included on the Ribosomal Database. This molecular analysis identified strain 5 as
belonging to the *Kluyvera intermedia* species, strain 9 to the *Enterobacter asburiae*, and finally, strains 11, 12 and 13 as *Enterococcus durans* strains.

Fish spoilage is mainly caused by bacterial activity and some compounds, such as biogenic amines, can be formed by bacterial action. In fact, biogenic amines should be absent or found at very low levels in fresh fish and their formation is usually associated with spoilage. Therefore, bacterial contamination is probably the main factor involved in the formation of these compounds. Fresh fish can be contaminated by a mixed bacterial population consisting of psychrotrophic Gram-negative bacteria like enterobacteria, and Gram-positive bacteria like lactic acid bacteria. The type of bacteria present in food determines the type and amount of biogenic amine formed. As demonstrated in this study, enterobacteria and enterococci are particularly active. Indeed, enterobacteria have been mostly described as strong producers of histamine and the diamines putrescine and cadaverine in fish. However, lactic acid bacteria have been mainly associated with the formation of tyramine in other fermented food products (Silla, 1996).

*Enterobacteriaceae* are generally considered as microorganisms with a high decarboxylase activity. Møller (1954) studied that the distribution of the decarboxylases of lysine and ornithine differs from the different species of enterobacteria. Diamines, putrescine and cadaverine, are usually common amines often related to the activity of enterobacteria. According to the Bergey’s manual, strains from the *Kluyvera* genera (only the *K. ascorbata* and *K. cryocrescens* species were studied) are putrescine- and cadaverine-producers (Brenner, 1984). As far as we known, this is the first report describing the presence of *Kluyvera* strains in sardine samples. Contrarily, the presence of *Enterobacter* strains had been extensively reported in fish or fish products such as in fresh albacore (Kim, Field, Morrissey, Price, wei, & An, 2001), tuna fish (López Sabater, Rodríguez-Jérez, Roig-Sagués, & Mora–Ventura, 1994), salt-ripened anchovies (Pons
Sanchez-Cascado, Veciana-Nogués, Bover-Cid, Mariné-Font, & Vidal Carou, 2005), ice-preserved anchovies (Pons-Sánchez Casado, Bover-Cid, Veciana-Nogués, & Vidal-Carou, 2005), salted mackerel (Tsai, Lin, Chang, Chen, Kung, Wei, & Hwang, 2005), among others. It had been also described that strains of *E. cloacae* produced simultaneously putrescine and cadaverine, but hot histamine (Pons-Sánchez-Cascado, Bover-Cid, Veciana-Nogués, & Vidal-Carou, 2005). Bjornsdóttir-Butler, Bolton, Jaykus, McClellan-Green, & Green (2010) described that while that *E. cloacae* strains did not produce histamine and did not posses the corresponding gene, strains from the *E. aerogenes* and *E. gergoviae* species produced histamine and contained the gene. Likewise, *E. intermedium* (López-Sabater, Rodríguez-Jérez, Hernández-Herrero, & Mora-Ventura, 1996) and *E. amnigenus* (Takahashi, Kimura, Yoshikawa, & Fujii, 2003) strains were reported as histamine-producers. In this study, a histamine–, putrescine– and cadaverine-producer *E. asburiae* strain, strain 9, had been described.

Among lactic acid bacteria, *Enterococcus* sp. has been frequently isolated from swabs taken from the skin of fish in various processing phases and from work surfaces (Zivkovic, Miokovic, & Sosa, 2001). In this study *E. durans* strains able to produce tyramine were isolated from the mackerel samples. Pons-Sánchez-Cascado, Bover-Cid, Veciana-Nogués, & Vidal-Carou (2005) identified *Enterococcus* strains among the tyrosine decarboxylase positive bacteria isolated from ice-preserved anchovies. In their study, enterococci were found only at the last sampling point, at the end of the ice-preserver storage.

In conclusion, the present study provides information about the bacterial species producing biogenic amines in ice-preserved sardine and mackerel. The results of the study indicated that only a low proportion of strains decarboxylated amino acids in vitro. Enterobacteria were histamine and diamine producers, being strains of *Kluyvera intermedia* and *Enterobacter asburiae* isolated from the first time from sardine.
Enterococci produced tyramine and *E. durans* strains were isolated from ice-preserved mackerel. However, biogenic amine production by decarboxylase positive strains in screening media do not necessarily imply a similar behaviour in fish products. Regardless of strain variation and the effects of environmental parameters, bacterial biogenic amine formation in fish or fish products could represent a hazard for sensitive individuals.

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References


Figure captions

Fig. 1. Thin layer chromatographic (TLC) analysis of biogenic amine producer strains isolated from ice-preserved sardine and mackerel. Supernatants of bacteria grown in LB media containing 0.25% histidine (1), 0.2% tyrosine (2), 0.25% lysine (3) or 0.25% ornithine (4), were dansylated and separated on a precoated silica gel F254 plate. The strains analyzed were the putrescine and cadaverine-producer strain 5 (A), the histamine, putrescine and cadaverine-producer strain 9 (B), and the tyramine-producer strain 11. Cadaverine (C), putrescine (P), and tiramine (T) standard solutions in LB medium are also indicated.

Fig. 2. PCR amplifications of amino acid decarboxylase encoding genes in bacteria isolated from sardine and mackerel and identified as biogenic amine producer by TLC. DNA extracted from strain 5 (A), strain 9 (B) and strain 11 (C) were used to amplify an
internal fragments of amino acid decarboxylase genes. Oligonucleotides HIS1-F and
HIS1-R (1) were used to amplify a 372 bp internal fragment of histidine-decarboxylase from
Gram-positive bacteria; oligonucleotides HIS2-F and HIS2-R (2) to amplify a 531 bp
fragment of histidine decarboxylases from Gram-negative bacteria; primers TDC-F and
TDC-R (3) to amplify an 825 bp fragment of tyrosine decarboxylase encoding genes;
primers PUT1-F and PUT1-R (4) and PUT2-F and PUT2-R (5) to amplify DNA
fragments of 1440 and 624 bp, respectively, coding for ornithine decarboxylases;
oligonucleotides CAD1-F and CAD1-R (6) to amplify a 1098 bp DNA fragment of lysine
decarboxylases from Gram-negative bacteria; and, primers CAD2-F and CAD2-R (7) to
amplify 1185 bp fragments of lysine decarboxylases from Gram-positive bacteria (Table
1). A molecular size standard (EcoRI digested λ DNA) is included in the left of the
agarose gels.

Fig. 3. Alignments of amino acid decarboxylase protein fragments. Clustal W program
was used to compare predicted sequences. Dashes on the alignment indicate identical
residues. At the bottom of the alignment, residues that are identical (*), conserved (.), or
semiconserved (.) in all sequences of the alignments are also indicated. (A) Histidine
decarboxylases from strain 9 (9), Morganella morganii NCIMB 865 (MMO), Morganella
psychrotolerans F39–1 (MPS), Enterobacter aerogenes ATCC 13048 (EAE),
Photobacterium phosphoreum mb36 (PHP), Photobacterium damselae ATCC 33539
(PDA), and Raoultella planticola Y1–1 (RPL). (B) Lysine decarboxylases from strain 5
(5), strain 9 (9), Citrobacter koseri ATCC BAA–895 (CKO), Salmonella enterica serovar
Heidelberg str SL 476 (SEN), Escherichia coli SE15 (ECO), Citrobacter rodentium ICC
168 (CRO), and Enterobacter cloacae SCF1 (ECL), Cronobacter sakazakii ATCC BAA–
Tyrosine decarboxylase from strain 11 (11), *E. faecium* (EFA), *E. durans* (EDR), and *E. hirae* (EHI).
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<th>Gene</th>
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<td>TDC-R</td>
<td>ACRTARTCNACCATRTTRAARTCNGG</td>
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<td>odc</td>
<td>PUT1-F</td>
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<td>1440</td>
<td>Gram-positive/ Gram-negative</td>
</tr>
<tr>
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<td>PUT1-R</td>
<td>ACRCANAGNACNCCNGGNGRTANGG</td>
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<tr>
<td></td>
<td>PUT2-F</td>
<td>AHWGNTYWGGNGAAYACNATHAARAA</td>
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<td>Gram-positive/ Gram-negative</td>
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<td>PUT2-R</td>
<td>GCNARNCCNCCRAAYTTNCCDATRTC</td>
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<td>ldc</td>
<td>CAD1-F</td>
<td>TTYGAYWCGNGNTGGGTNCNTAYACCCRTGDATRTCGNTYTCRAANCCNGG</td>
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<td>CAD1-R</td>
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<tr>
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<td>CAD2-R</td>
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<td>CAD2-F</td>
<td>GGDATNCCNGGNGGRTA</td>
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</tbody>
</table>

$^a$ hdc, histidine decarboxylase; tdc, tyrosine decarboxylase; odc, ornithine decarboxylase; ldc, lysine decarboxylase.

$^b$ Y = C or T; R = A or G; W = A or T; S = C or G; M = A or C; D = A, G, or T; H = A, C, or T; B = C, G, or T; N = A, C, G, or T.
<table>
<thead>
<tr>
<th>No.</th>
<th>Histamine(^a)</th>
<th>Tyramine(^b)</th>
<th>Putrescine(^c)</th>
<th>Cadaverine(^d)</th>
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</thead>
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<tr>
<td></td>
<td>hdc</td>
<td>tdc</td>
<td>odc</td>
<td>ldc</td>
</tr>
<tr>
<td>1</td>
<td>HIS1-F/R (1)</td>
<td>TYR1-F/R (1)</td>
<td>PUT1-F/R (1)</td>
<td>CAD1-F/R (1)</td>
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<tr>
<td>2</td>
<td>HIS2-F/R (2)</td>
<td>TYR2-F/R (2)</td>
<td>PUT2-F/R (2)</td>
<td>CAD2-F/R (2)</td>
</tr>
</tbody>
</table>

\(^a\) Histamine production detected by TLC (HIS) or by PCR (hdc) using oligonucleotides HIS1-F/R (1) or HIS2-F/R (2); \(^b\) Tyramine production detected by TLC (TYR) or by PCR (tdc) using TDC-F/R primers; \(^c\) Putrescine production detected by TLC (PUT) or by PCR (odc) using PUT1-F/R (1) or PUT2-F/R (2) oligonucleotides; \(^d\) Cadaverine production detected by TLC (CAD) or by PCR (ldc) using CAD1-F/R (1) or CAD2-F/R (2) primers.
Figure 2

A

B

C

kb

0.4 - 0.9 - 1.5 - 2.7 - kb