Screening of biogenic amine production by coagulase-negative staphylococci isolated during industrial Spanish dry-cured ham processes

Gerardo Landeta, Blanca de las Rivas, Alfonso V. Carrascosa, 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

The potential to produce biogenic amines was investigated for 56 coagulase-negative staphylococci isolated during industrial Spanish dry-cured ham processes. The presence of biogenic amines from bacterial cultures was determined by thin-layer chromatography. The percentage of strains that decarboxylated amino acid was very low (3.6%). The only staphylococci with aminogenic capacity were an histamine-producer *S. capitis* strain, and a *S. lugdunensis* strain that simultaneously produced putrescine and cadaverine. In both strains, PCR was used to confirm the presence of the genes encoding the amino-acid decarboxylases responsible for the synthesis of these amines. This study reveals that production of biogenic amines is not a widely-distributed property among the staphylococci isolated from Spanish dry-cured hams.

Keyworks: Coagulase-negative staphylococci; Spanish dry-cured ham; *Staphylococcus capitis*; *Staphylococcus lugdunensis*; Biogenic amines; Histamine; Putrescine; Cadaverine

1. Introduction

Spanish dry-cured ham, a valuable meat product in Spain, is a traditional intermediate-moisture meat product which is called Serrano ham when made from various breeds of white hogs, and Iberian ham when made from Iberian (black) hogs (Losantos, Sanabria, Cornejo, & Carrascosa, 2000). Its process manufacturing involves the following stages: salting, postsalting and drying-maturation. Hams are dried-matured at least for 110 days in drying chambers. In the typical elaboration process of Spanish dry-cured ham, the relative humidity during the drying stage, ranges from 50 to 90% and the temperature from 6 to 34 ºC. During this period, water losses and different chemical and biochemical changes occur, resulting into the typical colour, flavour and taste of the ham (Toldrá, 1998).

During ripening of cured hams, protein hydrolysis takes place. Free amino acids are highly correlated with flavour development in aged ham. These amino acids have been reported as precursors of sour, sweet, and bitter taste (Toldrá, 1998; Martín, Antequera, Ventanas, Benitez-Donoso, & Córdoba, 2001). In addition, some of them could contribute to aromatic compounds (Sforza, Galaverna, Schivazappa, Marchelli, Dossens, & Virgili, 2006). On the other hand, amino acid degradation to amines would affect not only the flavour but also the health of the consumer if biogenic amines are formed (Ruíz-Capillas & Jiménez-Colmenero, 2004; Virgili, Saccani, gabba, Tanzi, & Soresi Bordini, 2007). In spite of the relevance to flavour of all these compounds, very little is known about the conditions that rule their production in dry-cured hams. A study that measured the concentration of different free amino acids and amines at different stages of Iberian cured ham processing, revealed that amino acid liberation during ripening of cured ham is not selective and most amino acids increase according

68 to their proportion in porcine skeletal muscle; in addition, amines with higher
69 concentrations were not found in the toxic range (Córdoba, Antequera Rojas, García
70 González, Ventanas Barroso, López Bote, & Asensio, 1994). Later, Hernández-Jover,
71 Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, (1997) reported that
72 dry-cured ham showed, in general, similar levels of amines to those of cooked meat
73 products.

74 The safety of dry-cured hams for consumers could depend partially on the
75 content of biogenic amines, such as histamine, tyramine, putrescine, and cadaverine,
76 which might represent a food poisoning hazard. The production of biogenic amines
77 requires the presence of amino-acid decarboxylating microorganisms which could be
78 detected during the elaboration process (Silla Santos, 1996). Microorganisms have a
79 different ability in synthesizing decarboxylases. The production of biogenic amines in
80 meat products has been attributed to the action of several microorganisms. Within the
81 same species, the presence, the activity, and the specificity of decarboxylases is strain-
82 specific (Suzzi & Gardini, 2003). *Micrococcaceae* are the prevalent microbiological
83 group in the processing of Spanish dry-cured hams. Coagulase-negative staphylococci
84 (CNS) influence technological properties of these cured products. CNS probably play
85 secondary role in the development of aroma as well as flavour and colour, after muscle
86 enzymes.

87 CNS strains isolated during industrial Spanish dry-cured ham processes were
88 selected as possible starter cultures after several in vitro selective tests (ability to reduce
89 nitrates to nitrites, to grow at 4 ºC in media supplemented with 10% of sodium chloride
90 and 100 ppm sodium nitrate) (Cornejo & Carrascosa, 1991; Carrascosa & Cornejo,
91 1991). The aim of this study was to examine the occurrence of amino-acid
92 decarboxylase activity and the presence of the corresponding genes in these CNS strains

93 isolated from Spanish dry-cured ham. This study is of great relevance since food safety
94 is one of the primary concerns of regulatory agencies and consumers. And the study of
95 the microbial quality of meat food products is crucial in order to help industrial and
96 traditional manufacturers to produce safe products.
97
98 **2. Materials and methods**
99
100 *2.1. Strains and growth conditions*
101
102 A total of fifty-six CNS strains were analysed for biogenic amine production.
103 Thirty-five of them were isolated during an industrial Spanish dry-cured ham process
104 using a fast-technology (100 days of ripening). Most of them, 31 strains, were
105 classified, using the API-STAPH system and other tests, as *Staphylococcus xylosus*, one
106 strain was classified as *S. capitis*, and three strains shared taxonomic characteristics
107 with *S. xylosus*, *S. capitis* and *S. sciuri* (Cornejo & Carrascosa, 1991). The additional
108 twenty-one CNS strains were isolated during an industrial Spanish dry-cured ham
109 process following a slow technology (160 days of ripening). Using the API-STAPH
110 system and additional tests, 18 of them were characterized as *S. xylosus* and the other
111 three strains participated in taxonomic characters with *S. xylosus* and *S. capitis*
112 (Carrascosa & Cornejo, 1991).
113 Staphylococcal strains were routinely grown in brain heart infusion (BHI, Difco,
114 France) by incubating at 37 ºC under aerobic conditions. For biogenic amine
115 production, staphylococci were grown in BHI supplemented 0.2% of the corresponding
116 precursor amino acid (L-histidine monohydrochloride, tyrosine disodium salt, L-

ornithine monohydrochloride, and L-lysine monohydrochloride). The cultures were incubated at 37 °C under aerobic conditions during three days.

2.2. Bacterial DNA extraction

Bacterial chromosomal DNA was isolated from overnight cultures using a protocol previously described (Vaquero, Marcobal, & Muñoz, 2004). Precipitated DNA was resuspended in an appropriate volume of TE solution (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) (Sambrook, Fritsch, & Maniatis, 1989).

2.3. PCR amplification and DNA sequencing of the 16S rDNA

16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f and 1387r (Table 1) previously described (Marchesi et al., 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR reaction was 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 $MgCl_2$, 200 µM of each dNTP, primer 63f (1µM), primer 1387r (1µM) and 1 U of Ampli*Taq* Gold DNA polymerase. The reaction was performed in a GeneAmp PCR System 2400 (Perkin Elmer, USA) 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

142 sequencer (Applied Biosystems, USA). Sequence similarity searches were carried out
143 by comparing to sequences from type strains included on the Ribosomal Database.
144
145 *2.4. TLC analysis of biogenic amines from bacterial cultures*
146
147 Staphylococcal strains were grown as described in the "Strains and growth
148 conditions" section. After incubation, the broth media was centrifuged and the
149 supernatant was analysed by thin layer chromatography (TLC) for biogenic amine
150 content as described previously (García-Moruno, Carrascosa, & Muñoz, 2005). Briefly,
151 amines were converted to their fluorescent dansyl derivatives by adding to one volume
152 of the supernatant, one volume of 250 mM disodium phosphate (pH 9.0), 0.1 volume of
153 4 N sodium hydroxide solution, and two volumes of dansyl chloride solution (5 mg/ml
154 of dansyl chloride in acetone). The reaction mixture was thoroughly mixed and
155 incubated in the dark at 55 ºC for 1 h. Then the samples were cooled and stored at 4 ºC
156 until use.
157 The amines were fractionated on precoated silica gel 60 F_{254} TLC plates. The
158 dansylated compounds were separated in chloroform:thriethylamine (4:1), and revealed
159 with propan-2-ol:triethanolamine (4:1). The fluorescent dansyl derivative spots were
160 visualized with the aid of a transiluminator with a suitable UV-light source (312 nm).
161 A standard solution of amines (250 mg/l of tyramine, cadaverine and putrescine)
162 was prepared similarly and used as control.
163
164 *2.5. Presence of amino acid decarboxylase genes*
165

166 Chromosomal DNAs were subjected to PCR amplification to detect the presence
167 of histidine, lysine, and ornithine decarboxylase encoding genes (De las Rivas,
168 Marcobal, Carrascosa, & Muñoz, 2006). Briefly, PCRs were performed in 25 µl
169 amplification reaction mixture as described above. The primers used for the
170 amplification of the histidine decarboxylase encoding gene in Gram-positive bacteria
171 were HIS1-F and HIS-R that amplified a 372-bp DNA fragment (Table 1). To amplify a
172 1185-bp fragment of the lysine decarboxylase gene from Gram-positive bacteria we
173 used primers CAD2-F and CAD2-R (Table 1). As there are two groups of ornithine
174 decarboxylases, we used primers pairs PUT1-F/ PUT1-R and PUT2-F/PUT2-R that
175 amplified 1440 or 624-bp DNA fragments respectively, to amplify both groups of
176 ornithine decarboxylase encoding genes (Table 1). The reactions were performed by
177 using the following cycling parameters: 10 min for enzyme activation at 95 ºC followed
178 by 30 cycles of 30 s at 95 °C, 30 s at 53 °C, and 2 min at 72 °C, and a final extension
179 step of 20 min at 72 °C. PCR products were resolved on a 1% agarose gel (Pronadisa,
180 Spain) and stained with ethidium bromide.
181 The amplification products were gel purified on QUIAquick spin columns
182 (QUIAGEN) for sequencing with the same PCR primers. Sequence similarity searches
183 were carried out using Basic local alignment search (BLAST) (Altschul et al., 1997) on
184 the EMBL/GenBank databases.
185
186 **3. Results and discussion**
187
188 Biogenic amines are compounds implicated in food poisoning incidents.
189 Biogenic amines in meat products are mainly formed by decarboxylation of the
190 corresponding amino acids by microorganisms. Several amino acids can be

191 decarboxylated, as a result biogenic amines are usually found, with histamine, tyramine,
192 putrescine, and cadaverine being the most frequent. Several qualitative and quantitative
193 methods to determine the production of biogenic amines by microorganisms have been
194 described. Most of the screening procedures generally involve the use of a differential
195 medium containing a pH indicator. However, false-positive and false-negative results
196 have been described in these media (Marcobal, de las Rivas, & Muñoz, 2006). A
197 suitable TLC method has been recently described for the detection of biogenic amines
198 from bacterial cultures (García-Moruno, Carrascosa, & Muñoz, 2005).
199 In spite that dry-cured ham showed, in general, a non toxic range of amines, and
200 that they showed similar levels of amines to those of cooked meat products, but taking
201 into account that some CNS strains have been reported as biogenic amine-producers,
202 CNS strains isolated during elaboration of Spanish dry-cured hams were analysed for
203 biogenic amine production. Most of the CNS analysed in this work were not able to
204 produce biogenic amines (Fig. 1). It should be mentioned that the BHI media alone
205 produces an unidentified spot in the TLC plate (Fig. 1). Only two strains out fifty-six
206 strains (3.6%) were found to be strong amine-producers by the TLC method. The two
207 biogenic amine producer strains were the histamine-producer *S. capitis* IFIJ12 strain
208 (Fig. 2A), and *S. xylosus* IFIJ47 that produced simultaneously cadaverine and putrescine
209 (Fig. 2B). In the TLC analysis for biogenic amine-production showed in Fig. 2, the
210 producer CNS strains are compared to the non-producer *S. xylosus* IFIJ46 strain. As
211 showed in Fig. 2B, *S. xylosus* IFIJ47 produces cadaverine and putrescine from the
212 lysine and ornithine, respectively, present in the BHI media (lane 2); when the culture
213 media was supplemented with lysine (lane 4) or ornithine (lane 6), an increase in the
214 corresponding amine could be observed. It might be concluded that even the culture
215 media alone gives a faint spot in the TLC plate (lane M), the amines produced as

consequence of bacterial growth can be easily detected even without amino acid supplementation.

Since the biogenic amine-producer strains were presumptively identified by using the API-STAPH system and other additional tests, we decided to confirm its classification by a molecular technique. To confirm the taxonomical identity of these strains, 1.3 kb DNA fragments coding for the 16S rDNA were amplified. The bacterial isolates identified as being positives for biogenic amine production were then identified using sequences 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. The *S. capitis* IFIJ12 was identical to the sequence from the *S. capitis* type strain (*S. capitis* NCTC 11045^{T}). However, the isolate *S. xylosus* IFIJ47 showed a sequence identical with that of *S. lugdunensis* type strain (*S. lugdunensis* ATCC 43809^{T}) (Freney et al., 1988) (data not shown). Therefore, the IFIJ47 isolate needs to be reclassified as *S. lugdunenesis* IFIJ47.

The strain misidentification of *S. lugdunensis* IFIJ47 could be explained since traditional identification methods, which include biochemical tests, are not easy to perform for the species separation among staphylococci (Rantsiou, Iacumin, Cantoni, Coni, & Cocolin, 2005; Blaiotta, Pennacchia, Villani, Ricciardi, Tofalo, & Parente, 2004). Identification of CNS species using phenotypic methods such as sugar fermentation patterns, may sometimes be uncertain, complicated and time-consuming due to an increasing number of species that vary in few of these characters. Moreover, new species of CNS are continually being classified, making further identification tools necessary. For these reasons, molecular methods have been increasingly used in order to simplify characterization procedures, to provide rapid and reliable identification, or to validate phenotypically determined taxa. The comparison of results obtained by

241 molecular techniques with those of biochemical identification showed the unreliability
242 of the traditional identification methods. As example, the identification of staphylococci
243 from Italian fermented sausages revealed that the same identification was obtained for
244 less than 45% of the isolates analysed (Blaiotta, Pennacchia, Ercolini, Moschetti, &
245 Villani, 2003).

246 In order to correlate the production of biogenic amines with the presence of the
247 corresponding decarboxylase genes, we performed PCR assays for the detection of these
248 genes (De las Rivas et al., 2006). We checked the presence of a gene encoding histidine
249 decarboxylase from Gram-positive bacteria by using HIS1-F and HIS1-R
250 oligonucleotides, a gene encoding lysine decarboxylase from Gram-positive bacteria by
251 using oligonucleotides CAD2-F and CAD2-R. However, for the detection of the
252 ornithine decarboxylase gene, we checked oligonucleotides designed for the detection
253 of the two groups of these proteins, PUT1-F/ PUT1-R and PU2-F/PUT2-R. As showed
254 in Fig. 3, the biogenic-amine producer CNS strains gave the corresponding amplicon of
255 the expected size, so amino acid decarboxylase genes were present on them. The
256 histamine-producer *S. capitis* IFIJ12 strain produces a 372-bp DNA fragment from the
257 histidine decarboxylase encoding gene; cadaverine- and putrescine-producer *S.*
258 *lugdunensis* IFIJ47 strain produces fragments corresponding to the lysine decarboxylase
259 (1185-bp) and only with the primers designed based on the first group of ornithine
260 decarboxylases, primers PUT1-F and PUT1-R, that amplified a 1440-bp fragment (Fig.
261 3). Moreover, the sequence of these DNA fragments showed high identity to amino acid
262 decarboxylase genes. The 372-bp DNA fragment from the *L. capitis* IFIJ12 encoded a
263 fragment 68% identical to the histidine decarboxylase from *Lactobacillus* strain 30a,
264 and more than 66% identity to histidine decarboxylases from other gram-positive
265 bacteria (data not shown). Similarly, the 1440-bp DNA fragment from the *S.*

266 *lugdunensis* IFIJ47 ornithine decarboxylase showed a 69 and 58% identity to ornithine
267 decarboxylases from *Oenococcus oeni* (Marcobal, de las Rivas, Moreno-Arribas, &
268 Muñoz, 2004) and *Lactobacillus* 30a (data not shown). These results indicated that, so
269 far, histidine- and ornithine-decarboxylase sequences from staphylococci are not
270 available. However, the 1185-bp internal DNA fragment from *S. lugdunensis* IFIJ47
271 lysine decarboxylase showed 62 and 59% identity to lysine decarboxylases described
272 previously in other staphylococcal species, such as *S. epidermidis* and *S. haemolyticus*,
273 respectively.

274 Since the sequence of several staphylococcal lysine decarboxylases are available,
275 this implies that cadaverine production seems to be a frequent biochemical property
276 among staphylococcal species. It is noteworthy, that cadaverine production is not
277 included among the characteristics differentiating the species of the genus
278 *Staphylococcus* (Kloos & Schleifer, 1984). However, ornithine decarboxylase activity
279 has been described as a characteristic useful in differentiating *S. lugdunensis* and *S.*
280 *schleiferi* from other novobiocin-susceptible *Staphylococcus* species (Freney et al.,
281 1988).

282 The histamine production by *S. capitis* strains has been reported previously. A *S.*
283 *capitis* strain isolated during the ripening of salted anchovies was able to produce
284 histamine (Hernández-Herrero, Roig-Sagués, Rodríguez-Jérez, & Mora-Ventura, 1999).
285 In addition, histidine decarboxylase activity has been observed in other species
286 belonging to the genera *Staphylococcus*. Histamine production was observed in 76% of
287 the *Staphylococcus xylosus* strains isolated from Spanish sausages (Silla Santos, 1996).
288 However, in our study, the *S. xylosus* strains, 49 out 56 CNS strains analyzed, were no
289 able to produce biogenic amines. Martuscelli, Crudele, Gardinie, & Suzzi (2000) tested
290 51 strains of *S. xylosus* from sausages and found that 21 were able to decarboxylate

291 amino acids in vitro, and only seven produced high amounts of tyramine, spermine and
292 spermidine. Histamine production was never detected.
293 The results obtained in this study are in agreement with those reported recently by
294 Martín, Garriga, Hugas, Bover-Cid, Veciana-Nogués, & Aymerich (2006). They found
295 that concerning the aminogenic potential, it was noteworthy that only 35 out of 240
296 Gram-positive catalase-positive isolates were able to produce biogenic amines, which
297 means that it is not a widely distributed property among staphylococci, especially in
298 comparison with other important bacterial groups for sausage fermentation. Similarly to
299 the *S. lugdunensis* IFIJ47 strain described in this work, they described that the diamines,
300 putrescine and cadaverine, as well as histamine, were produced simultaneously by
301 several strains identified as *S. epidermidis* and by only one strain *of S. xylosus*.
302 Therefore, they concluded that these results confirms that amino acid-decarboxylase
303 activity is a strain dependent characteristic in staphylococci.
304
305 In conclusion, staphylococci are important microorganisms in meat products.
306 Certain species are regularly found in meat products which are produced without the
307 addition of starter cultures. These species could be found in relatively high numbers and
308 could be related to the development of particularly desired flavours. In addition, amino
309 acid decarboxylases are enzymes present in many microorganisms of food concern.
310 Elaboration of Spanish dry-cured ham is a safe process, although occasionally, this meat
311 product could potentially support the accumulation of biogenic amines if a great
312 microbial growth were developed. In fact, the high amounts of proteins present in these
313 products and the proteolytic activity during ripening provide the precursors for
314 decarboxylase activity of wild microorganisms. This study revealed that production of

315 biogenic amine is not a widely-distributed property among the staphylococci isolated
316 during Spanish dry-cured ham processes.
317
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324
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Figure captions

Fig. 1.Thin-layer chromatography (TLC) analysis of non-biogenic amine producer strains. Supernatants of CNS grown in BHI media containing 0.2% ornithine, lysine and histidine were dansylated and separated on a precoated silica gel F_{254} plate. The staphylococcal strains analysed were: *S. xylosus* IFIJ1 (lane 1), *S. xylosus* IFIJ2 (lane 2), *S. xylosus* IFIJ3 (lane 3), *S. xylosus* IFIJ4 (lane 4), *S. xylosus* IFIJ5 (lane 5), *S. xylosus* IFIJ6 (lane 6), *S. xylosus* IFIJ7 (lane 7), *S. xylosus* IFIJ8 (lane 8), and *S. xylosus* IFIJ9 (lane 9). Lane 10, histamine, cadaverine, and putrescine standard solution.

Fig. 2. Thin-layer chromatography (TLC) of biogenic amine-producer staphylococcal strains isolated during Spanish dry-ham elaboration process. (A) TLC detection of histamine production by *S. capitis* IFIJ12. As control, a non producer *S. xylosus* IFIJ46 strain was also shown. The CNS strains were grown in BHI media containing histidine and the histamine produced in the media during growth was derivatized, fractioned and detected. Cultures grown in BHI media containing histidine, *S. xylosus* IFIJ46 (lane 1) and *S. capitis* IFIJ12 (lane 2). Histamine (lane H) standard solution is indicated. (B) TLC detection of cadaverine- and putrescine-production by *S. lugdunensis* IFIJ47. As control, a non-producer *S. xylosus* IFIJ46 strain was also shown. Strains were grown in BHI media or in BHI media supplemented with lysine or ornithine. The cadaverine and putrescine produced in the media during growth was derivatized, fractioned and detected. Cultures grown in BHI media (lane M): *S. xylosus* IFIJ46 (lane 1) and *S. lugdunensis* IFIJ47 (lane 2); in BHI containing 2% lysine: *S. xylosus* IFIJ46 (lane 3) and *S. lugdunensis* IFIJ47 (lane 4); in BHI containing 2% ornithine: *S. xylosus* IFIJ46 (lane

5) and *S. lugdunensis* IFIJ47 (lane 6). Cadaverine (lane C) and putrescine (lane P) standard solutions are also indicated.

Fig. 3. PCR amplifications of amino acid decarboxylases encoding genes. (A) Oligonucleotides HIS1-F and HIS1-R were used to amplify a 372-bp internal fragment of the histidine decarboxylase from *S. capitis* IFIJ12 (lane 1). A 100-bp ladder marker was included in the gel. (B) Oligonucleotides PUT1-F and PUT1-R (lane 1) and PUT2-F and PUT2-R (lane 2) were used to amplify 1440-bp and 624-bp fragments, respectively, from the ornithine decarboxylase encoding gene, and oligonucleotides CAD2-F and CAD2-R to amplify a 1185-bp DNA fragment (lane 3) of the lysine decarboxylase from S. lugdunensis IFIJ47. A DNA marker standard (EcoRI/HindIII digested λ DNA) was included on the right.

Table 1

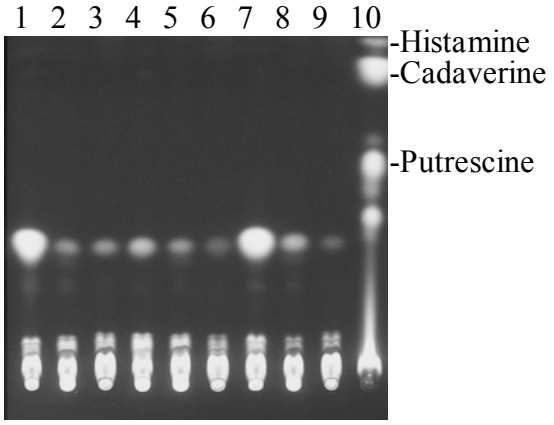
Table 1
Primers used in this study

Primer	Sequence 5´→3´[a]	Reference
63f	CAGGCCTAACACATGCAAGTC	Marchesi et al., 1998
1387r	GGGCGGWGTGTACAAGGC	Marchesi et al., 1998
HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	De las Rivas et al. 2006
HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA	De las Rivas et al. 2006
PUTI-F	TWYMAYGCNGAYAARACNTAYYYTGT	De las Rivas et al. 2006
PUT1-R	ACRCANAGNACNCCNGGNGGRTANGG	De las Rivas et al. 2006
PUT2-F	ATHWGNTWYGGNAAYACNATHAARAA	De las Rivas et al. 2006
PUT2-R	GCNARNCCNCCRAAYTTNCCDATRTC	De las Rivas et al. 2006
CAD2-F	CAYRTNCCNGGNCAYAA	De las Rivas et al. 2006
CAD2-R	GGDATNCCNGGNGGRTA	De las Rivas et al. 2006

[a]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; N = A, C, G, or T.

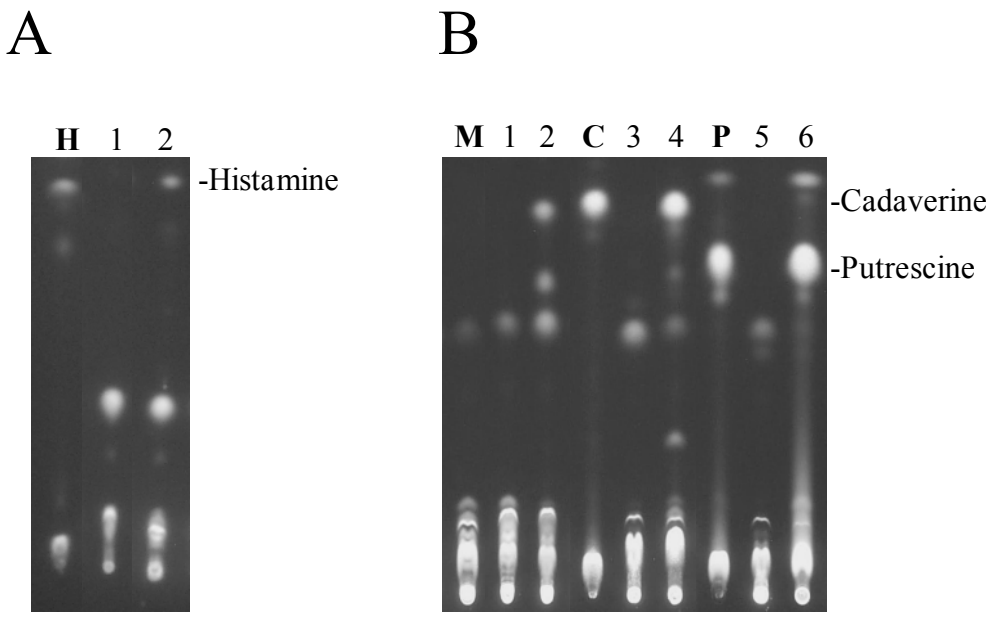
Figure

Figure 1



Figure

Figure 2



Figure

Figure 3