Molecular cloning and functional characterization of a histidine decarboxylase from *Staphylococcus capitis*

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RUNNING HEADLINE: *HISTAMINE PRODUCTION BY STAPH. CAPITIS*

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ABSTRACT

Aims: Histamine intoxication is probably the best known toxicological problem of food-borne disease. A histamine-producing *Staphylococcus capitis* strain has been isolated from a cured meat product. The aim of this study was gain deeper insights into the genetic determinants for histamine production in *Staph. capitis*.

Methods and Results: The nucleotide sequence of a 6446-bp chromosomal DNA fragment containing the *hdcA* gene encoding histidine decarboxylase has been determined in *Staph. capitis* IFIJ12. This DNA fragment contains five complete and two partial open reading frames. Putative functions have been assigned to gene products by sequence comparison with proteins included in the databases. The *hdcA* gene has been expressed in *E. coli* resulting in histidine decarboxylase activity. The presence of a functional promoter (P*hdc*) located upstream of *hdcA* has been demonstrated. Insertion of the histamine biosynthetic locus in *Staph. capitis* seems to be associated to a noticeable genome reorganization.

Conclusions: Among the staphylococcal species analysed in this study only *Staph. capitis* strains produce histamine. The *hdcA* gene cloned from *Staph. capitis* encodes a functional histidine decarboxylase that produce histamine from the amino acid histidine.

Significance and Impact of the study: The identification of the DNA region involved in histamine production in *Staph. capitis* will allow further work in order to avoid histamine production in foods.

Keywords: Biogenic amines; Histamine, Histidine; Coagulase negative-staphylococci

INTRODUCTION

Coagulase negative staphylococci are important microorganisms in fermented and cured meat products. While lactic acid bacteria ensure the safety of products by reducing the pH, staphylococci influence sensorial properties of meat products. They play important role in the development of aroma as well as flavour and colour of fermented products (Hugas and Monfort, 1997).

Certain staphylococcal species are regularly found in "naturally" processed meat products, which are produced without addition of starter cultures. However, in order to exercise greater control over the fermentations, common practices therefore involve inoculation with selected starter cultures (Miralles *et al*. 1996). To select a suitable strain to use as starter culture not only sensorial properties are important but also the safety aspects need to be considered. Safety of these products for consumers depends on the content of biogenic amines, which might represent a food poisoning hazard (Silla, 1996). Histamine is the most important biogenic amine in relation to food-borne intoxications, showing the highest biological activity and causing hypertension, hypotension, headache, nausea and vomiting (Bodmer *et al.* 1999).

Biogenic amines may appear due to bacterial decarboxylation reactions from precursor amino acids. Histamine is produced by enzymatic decarboxylation of the histidine present in foods. The enzyme histidine decarboxylase (HDC) catalyses the conversion of histidine into histamine by removing the α-carboxylate group of the substrate. Two separate cofactors have been utilized to perform this decarboxylation. In some bacterial HDC the cofactor is a covalently attached pyridoxal 5-phosphate, while others utilize a covalently attached pyruvoyl moiety. Bacterial histidine decarboxylases (HDC) have been studied and characterized in different organisms. Two enzyme families have been distinguished based on the cofactor used: the pyridoxal phosphate-

dependent and the pyruvoyl-dependent, being their sequences and characteristics radically different. Pyridoxal phosphate-dependent HDC are encountered in Gram-negative bacteria belonging to various species (i.e., *Raoultella planticola*, *Enterobacter aerogenes*, *Photobacterium phosphoreum*, etc.) (Takahashi *et al*. 2003; Morii *et al*., 2006). Pyruvoyl-dependent HDC are associated with Gram-positive bacteria and specially lactic acid bacteria implicated in food fermentation or spoilage, and they use a covalently bound pyruvoyl moiety as a prosthetic group. This pyruvoyl-dependent enzyme was reported in *Lactobacillus* 30a (Chang and Snell, 1968), *Clostridium perfringens* (Recsei *et al*. 1983), *Oenococcus oeni* (Coton *et al*. 1998), *Tetragenococcus muriaticus* (Konagaya *et al*. 2002), *Lactobacillus buchneri* (Martín *et al*. 2005), and *Lactobacillus hilgardii* (Lucas *et al*. 2006).

The gene encoding HDC (*hdcA*) has been identified in several Gram-positive bacteria. In lactic acid bacteria this gene is part of a cluster composed of four genes, and at least in *L. hilgardii*, and presumably in *O. oeni*, the *hdcA* gene was located on an 80-kb plasmid. However, *C. perfringens* showed a different genomic organization and in this bacteria the *hdcA* gene is located in the chromosome.

Screening of a collection of staphylococci isolated during an industrial Spanish dry-cured ham process, allowed the identification of *Staphylococcus capitis* IFIJ12, a strain able to decarboxylate histidine to produce histamine (Landeta *et al*. in press). The purpose of the present work was to gain deeper insight into genetic determinants for histamine production by *Staph. capitis* given that the production of histamine is a relevant property related to food quality and safety. In this work we have used different genetic and biochemical approaches to characterize histamine production in *Staph. capitis* IFIJ12.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The histamine-producer *Staph. capitis* IFIJ12 strain was isolated during an industrial Spanish dry-cured ham process following a slow technology (Carrascosa and Cornejo, 1991). The following staphylococcal type strains were purchased from the Spanish Type Culture Collection (CECT): *Staph. epidermidis* CECT 232^T (ATCC 14990^T), *S. hominis* CECT 234^T (ATCC 27844^T), *Staph. saprophyticus* ssp. *saprophyticus* CECT 235^T (ATCC 15305^T), *Staph. warneri* CECT 236^T (ATCC 27836^T), *Staph. xylosus* CECT 237^T (ATCC 29971^T). The *Staph. capitis* type strain NCTC 11045^T (ATCC 27840^T) was provided by T. Boquete (Staphylococcus Reference Laboratory, Spain). *E. coli* XL1-Blue MRF´ and *E. coli* XLOLR were supplied with the ZAP Express Predigested Gigapack® Cloning kit (Stratagene) and used in the construction of a *Staph. capitis* IFIJ12 DNA library. *E. coli* DH5αF´ (Promega) was used for all DNA manipulations. *E. coli* JM109 (DE3) (Promega) was used for expression in pT7-7 vector. Plasmid pT7-7 (USB) is an expression vector carrying isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter used for the expression in *E. coli*.

Staphylococci were routinely grown in BHI broth (Difco) at 37 ºC with shaking. Histamine production was detected by TLC (García-Moruno *et al*. 2005) by growing staphylococci in BHI supplemented with 0.2% histidine. *E .coli* cells were incubated in Luria-Bertani (LB) medium (Sambrook *et al*. 1989) for bacterial streak and glycerol stocks. For bacterial cultures prior to phage attachment LB medium was supplemented with maltose and magnesium for optimal lambda phage receptor expression on the surface of the host cell. The medium used for agar plates and top agar for plaque

formation was NZY (Sambrook *et al*. 1989). When required ampicillin, tetracycline, kanamycin, and streptomycin were added to the medium at 100, 12.5, 50, and 50 μg ml^{-1}, respectively.

For the analysis of the translational P*hdc::lacZ* fussions, *E. coli* DH5α was growth in LB media (Sambrook *et al.* 1989) and in LB buffered to 7.6 and 5.5. LB-7.6 medium was LB buffered at pH 7.6 with a final concentration of 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), and LB-5.8 was LB buffered at pH 5.5 with a final concentration of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Slonczewski *et al.* 1987). LB media was supplemented with 0.1 or 0.3% histidine and the cells were grown at 37 ºC in aeration. To study the effect of aeration, LB cultures were also growth under aeration (shaken culture) and non-aeration (static culture) conditions.

DNA manipulations and hybridization

Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were carried out as described (Sambrook *et al*. 1989). Chromosomal DNA and plasmid preparation were carried out as described elsewhere (Muñoz *et al*. 1999). Sequence similarities searches were carried out using Basic Local alignment search tool (BLAST) on the EMBL/GenBank databases. Computer prokaryotic promoter predictions were carried out at the Internet site http://www.fruitfly.org/seq_tools/promoter.html. RNA secondary structure predictions were carried out by the GeneBee program (http://www.genebee.msu.su/genebee.html). Multiple alignments were done using CLUSTAL W on EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GeneBank and Swiss-Prot. The nucleotide sequence

149 data reported here is available in the GenBank database under the accession number
150 AM283479.
151 A *Staph. capitis* IFIJ12 DNA library was constructed in the ZAP Express®
152 vector (Stratagene). To construct the library, chromosomal DNA was partially digested
153 with *Sau*3AI restriction enzyme and ligated to the ZAP vector digested with *Bam*HI.
154 The packaging and titering of the recombinant lambda phages, the amplification of the
155 library, and the *in vivo* excision of the pBK-CMV phagemid vector from the ZAP
156 Express vector, were performed according to the recommendations of the supplier. The
157 lambda plaques were screened by hybridisation to a digoxigenin-labelled probe and
158 chemiluminescent detected by using the DIG High Prime DNA labelling and detection
159 Starter Kit (Roche) according to the manufacturer´s instructions. The probe was a 372-
160 bp *Staph. capitis* IFIJ12 DNA fragment previously PCR amplified using the degenerate
161 oligonucleotides HIS1-F and HIS1-R based on alignments of Gram-positive bacterial
162 histidine decarboxylase sequences (De las Rivas *et al*. 2006).
163
164 **Heterologous expression of *hdcA* in *E. coli***
165 To amplify *hdcA* from *Staph. capitis* IFIJ12, specific oligonucleotides were designed
166 based on the nucleotide sequence previously determined. The primers used were primer
167 198 (5´-
168 GGGAATTCCATATG*CATCACCATCATCATCAC*AAAAAAACGGATGAAATCTTA
169 AGG) (a *Nde*I recognition site is underlined and a six-poli-His tag is written in italics)
170 and primer 199 (5´-CCCAAGCTTCACTCAGAATTAATATTTAATTCC) (an *Hin*dIII
171 site is underlined). The gene was first PCR amplified by using *Pfu* DNA polymerase,
172 digested with *Nde*I and *Hin*dIII, and ligated to the expression vector pT7-7 digested
173 with the same enzymes. The resulting plasmid was designated pAM28. The pAM28

plasmid was constructed and amplified in the *E. coli* DH5α strain and then transferred, for protein production, to the host JM109(DE3) (pLysS) *E. coli* strain.

Histidine decarboxylase activity

E.coli JM109(DE3) (pLysS) cells carrying pAM28 were grown at 37 ºC in LB medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. When the cultures reached an optical density of 0.6 at 600 nm, the cultures were shifted to 30 ºC, and gene expression was induced by adding 50 mM IPTG. After 3 h of induction, samples of the cultures were harvested by centrifugation (10000 *g*, 5 min) and washed twice with 50 mM sodium phosphate buffer (pH 6.5). The pelleted bacteria were resuspended in the same buffer and disrupted by sonication. The insoluble fraction was separated by centrifugation (25000 *g*, 15 min), and the supernatant was assayed for histidine decarboxylase activity.

The assay to determine histidine decarboxylase activity was performed in 50 mM sodium phosphate buffer (pH 6.5) in the presence of 3.6 mM histidine. The reaction was incubated at 37 ºC during 1 h. Afterwards, the histamine formed in the reaction was derivatized and detected by thin-layer chromatography (TLC) as described previously (García-Moruno *et al*. 2005).

Histidine decarboxylase purification

Soluble cell free extracts prepared as described above were applied to a His-Trap™-FF crude chelating affinity column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole, to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0,

100 mM NaCl containing 10 mM imidazole to 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 500 mM imidazole. Fractions containing the eluted HDC were pooled and the protein was then dialyzed against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. Histidine decarboxylase activity of the highly purified HDC protein was determined as explained above.

For the DHC autoactivation assay, highly purified HDC protein was incubated in presence of 0.8 M potassium phosphate pH 7.6 at 37 ºC. Aliquots were removed at intervals over time and the autoactivation was stopped by adding SDS to a final concentration of 1% followed by freezing (Copeland *et al*. 1987).

Construction of translational P*hdc::lacZ* fussion

To construct a translational fusion of the *hdc* promoter region of *hdcA* (P*hdc*) and the *lacZ* reporter gene, a 325-bp DNA fragment covering this promoter region was amplified by PCR using 10 ng of plasmid pUJ9, a promoterless *lacZ* vector (De Lorenzo *et al*. 1990), as template and the following primers: 336 (5´-AAAGAATTCATAAAAATATCCCAG; an engineered *Eco*RI site is underlined) and 337 (5´-GGGGATC**CAT**AACTAA**CCCTTC**TTTTAG; the start codon and the original RBS of the *hdcA* gene are indicated in boldface letters, and an engineered *Bam*HI site is underlined). To create plasmid pHDCP, the PCR-amplified fragment was cut with *Eco*RI and *Bam*HI endonucleases and ligated to the *Eco*RI and *Bam*HI double-digested promoterless *lacZ* vector pUJ9. Plasmid pHDCP contains the promoter and RBS from the *S. capitis hdcA* gene in a translational *lacZ* fussion.

The activity of the *hdc* promoter was monitored by assaying β-galactosidase accumulation in cells harbouring P*hdc::lacZ* fusion. P*hdc* expression was induced by

223 growing cells in different conditions. β-galactosidase activity was measured as
224 described by Miller (Miller, 1972) and expressed in Miller units.
225
226 **RESULTS**
227
228 **Sequence analysis of the *hdcA* locus in *Staphylococcus capitis***
229 Thirty five staphylococcal strains isolated during an industrial Spanish dry-cured ham
230 process were examined for the ability to produce the biogenic amine histamine by
231 growing them in BHI media containing histidine. Only the *Staph. capitis* IFIJ12 strain
232 was able to form histamine (Landeta *et al*., in press). Amino acid decarboxylase
233 encoding genes have not been described so far in staphylococci, therefore we decided to
234 characterize it. Firstly, in order to identify the gene responsible for this HDC activity,
235 we amplified a 372-bp internal fragment of the HDC encoding gene by using the
236 degenerate oligonucleotides HIS1-F and HIS1-R based on conserved domains of HDC
237 proteins (De las Rivas *et al*. 2006). This fragment was sequenced and similarity
238 searches showed that it contains an incomplete *hdcA* gene sequence. To clone the
239 complete *hdcA* gene a phage library of *Staph. ca*pitis IFIJ12 genomic DNA was created.
240 The screening of this library using the 372-bp internal *hdcA* fragment as a probe
241 rendered two positive phages. A total of 6446 bp *Staph. capitis* DNA fragment was
242 sequenced from plasmids derived from positive phages (Fig. 1).
243 Sequence analysis of this DNA fragment showed the presence of five complete
244 and two partial open reading frames (ORFs), which properties and similarities to
245 proteins in the databases are showed in Table 1. Six putative promoters were detected
246 upstream all ORFs, with exception of the sixth ORF. Putative transcription terminators
247 followed the stop codon of the second and fifth ORFs. The analysis of this nucleotide

sequence suggested that only the fourth and fifth genes are organized as a single operon (Fig. 1).

Functional expression of *hdcA*

To confirm that the *hdcA* gene from *Staph. capitis* IFIJ12 encodes a functional HDC, we expressed this gene in *E. coli* following the strategy described in Material and Methods section, consisting in amplifying the genes by PCR and cloning the products under the control of the T7 RNA polymerase-inducible ϕ10 promoter.

Cell extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. Control cells containing the pT7-7 vector plasmid alone did not show expression over the 3-h time course analysed, whereas expression of additional 34.2-kDa protein was apparent with cells harbouring pAM28 (Fig. 2, a1). In addition, cells extracts from *E.coli* JM109(DE3) (pLysS) cells harbouring the recombinant plasmid pAM28 were able to decarboxylate the histidine present in the reaction to histamine, whereas extracts prepared from control cells containing the vector plasmid alone did not. Fig. 2 (b1) showed a TLC analysis of the enzymatic reaction. Thus, we could prove experimentally that the *hdcA* gene encodes a functional HDC.

As the protein was cloned containing a purification poli-His tag, HDC was purified on a His-Trap^{TM}- FF crude chelating column and eluted with a stepwise gradient of imidazole. Highly purified HDC protein was obtained from pAM28 (Fig. 2, a2). The eluted HDC protein was dialysed to eliminate the imidazole, and checked for HDC activity. TLC analysis demonstrated that highly purified HDC protein was able to decarboxylate histidine to form histamine (Fig. 2, b2).

The predicted sequence of the HDC was aligned with HDC proteins from Gram-positive bacteria (supplementary file). As deduced from the HDC aligment, most of the

residues implicated in catalysis and substrate binding in the HDC from *Lactobacillus* 30a (Gallagher *et al.* 1989) are conserved in the *Staph. capitis* enzyme. However, the residue Ala-260, forming the hydrophobic pocket, is not conserved in the *Staph. capitis* HDC protein, and a Gly residue is present in its place.

In addition to the wild type HDC enzymes, a number of mutants that produce partially active or inactive enzymes have been isolated. More interestingly, mutant 3 of HDC from *Lactobacillus* 30a, which produces a full-length protein that is slowly autoactivated, shows only one amino acid replacement at position 58 (G58A), the Gly amino acid residue is conserved at this position in all HDC, with exception of *Staph. capitis* HDC with a Asn residue present in its place. An autoactivation assay was performed in order to know if *S. capitis* HDC follows a similar slow autoactivation. The result showed that along incubation, *Staph. capitis* HDC seems to be degraded instead to be autocleaved into an α chain (23 kDa) and a β chain (11.5 kDa) (Fig. 3).

Regulation of the P*hdc* promoter

Sequence analysis of the *Staph. capitis* DNA fragment showed the presence of a putative promoter upstream the *hdcA* gene (from nucleotide 2254 to 2281). To determine whether the proposed sequence actually represents P*hdc*, the corresponding region was PCR amplified, cloned into pUJ9, which is a promoter-probe plasmid containing the promoterless *lacZ* gene (De Lorenzo *et al*. 1990), and the resulting recombinant plasmid, pHDCP was introduced by transformation into *E. coli*. The promoter activity was tested by assaying β-galactosidase activity. *E. coli* cells harbouring pHDCP showed β-galactosidase activity, however activity was not found in pUJ9 cells. These results demonstrate the presence of a functional promoter in the cloned fragment.

Table 2 shows the expression of β-galactosidase from the P*hdc*::*lacZ* protein fussion at different growth conditions. Taking into account the limitation of the experimental assay used, the results indicated that as compare to growth at pH 7.6, pHDCP showed a 5.2-fold increase of β-galactosidase expression at pH 5.5, a 2.5 and 4.5-fold increase in media containing 0.1% and 0.3% histidine, respectively, and 11.1-fold increase during growth under non-aeration conditions.

Genomic reorganization associated to histamine production in *Staph. capitis*

The screening of the staphylococcal strains isolated from Spanish-dry cured ham revealed that histamine production is a rare feature in this genera (Landeta *et al*., in press). Since most of the strains analysed belonged to the *Staph. xylosus* species, we decided to check type strains of six staphylococcal species routinely isolated from food products for histamine production. Strains were growth in media containing 0.2% histidine and their supernatants were analysed by TLC. Only type strain from *Staph. capitis* was able to produce histamine (data not shown). This result confirms that histamine production is not a widely distributed property in staphylococcal species.

In order to know if the genetic organization showed by *Staph. capitis* IFIJ12 was shared by *Staph. capitis* NCTC 11045, type strain, several DNA amplification experiments spanning all the 6.4 kb *hdc* chromosomal region were performed. The *S. capitis* NCTC 11045 strain amplified PCR fragments that were apparently identical among them (data not shown). In addition, the 930 pb *hdcA* gene from *Staph. capitis* NCTC 11045 was amplified and completely sequenced. The result confirmed that the *hdcA* gene from both *Staph. capitis* strains were nearly identical as only two conservative nucleotide changes were found.

As proteins flanking HdcA-HdcP in *Staph. capitis* showed the highest identities scores to proteins from other staphylococcal species, mainly *Staph. epidermidis*, we revised the genetic organization and the nucleotide sequence around these genes on these staphylococcal strains whose genomes have been completely sequenced. Surprisingly a high degree of nucleotide identity was found with *Staph. epidermidis* ATCC 12228 (NC_004461). The leftmost 1156 nucleotides of *Staph. capitis* IFIJ12 sequence and nucleotides 103018 to 104191 in *Staph. epidermidis* ATCC 12228 genome, showed 93% nucleotide identity; this region comprises up to the putative transcription terminator located upstream *argR* (Fig. 1). In addition, nucleotide position from 4879 to 6446 in *Staph. capitis* and nucleotides 571120 to 569548 in *Staph. epidermidis* ATCC 12228, showed 98% identity between both strains; this region comprises from the putative transcription terminator located downstream *hdcP*, to the end of the *Staph. capitis* sequence (Fig. 1). So, only *orf3*, *hdcA* and *hdcP* genes are exclusively present in the *Staph. capitis* genome. In addition, these data indicate that the nucleotide sequences flanking the histamine biosynthetic genes in *Staph. capitis*, are located almost 0.5-Mb apart, and in inverse orientation in the *Staph. epidermidis* genome.

DISCUSSION

The presence of histamine in food is of concern in relation to both food safety and food spoilage since it has been implicated in food poisoning incidents. Therefore information about histamine biosynthesis by food bacteria is relevant. We have found that *Staph. capitis* strains are able to produce histamine. Since the gene responsible for histamine production in this species has not been genetically characterized, we decided to elucidate it. By using a previously described PCR method to detect histamine producing

bacteria (De las Rivas *et al*. 2006), and by the construction of a genomic library, we have determined the sequence of a 6446-bp chromosomal DNA fragment containing the *hdcA* gene encoding histidine decarboxylase. Moreover, the *hdcA* gene was hyperexpressed in a *E. coli* vector, and the recombinant HDC protein was purified. By using the highly purified *Staph. capitis* HDC protein we proved experimentally that *hdcA* encodes a functional pyruvoyl-dependent decarboxylase capable of producing histamine from the amino acid L-histidine.

Pyruvoyl-dependent HDCs uses a covalently bound pyruvoyl moiety as cofactor in the decarboxylation reaction. The pyruvate cofactor is formed as HDC undergoes and autoactivation serinolysis reaction in which an inactive π chain is cleaved to produce an α- and β-chain. It is noteworthy that the highly purified HDC protein from *Staph. capitis* is in the π chain form (34.2-kDa); however, no π subunits were observed in crude or purified preparations of the cloned HDC from *C. perfringens* (van Poelje and Snell, 1990). In *C. perfringens*, HDC appear to undergo rapid cleavage in vivo to the α (24.9-kDa) and β (10.5-kDa) subunits characteristics of this pyruvoyl HDCs. Pyruvoyl-dependent HDC have been purified to homogeneity from six gram-positive bacterial sources (*Lactobacillus* 30a, *L. buchneri*, *C. perfringens*, *Micrococcus* sp.n., *O. oeni*, and *T. muriaticus*) and all the six enzymes contain pairs of dissimilar subunits (van Poelje and Snell, 1990).

To explain the unusual behaviour of the *Staph. capitis* HDC protein, the predicted sequence of the HDC was aligned with HDC proteins from Gram-positive bacteria. Most of the residues implicated in catalysis and substrate binding in the HDC from *Lactobacillus* 30a (Gallagher *et al.* 1989) are conserved in the *Staph. capitis* enzyme, indicating that the catalytic mechanism of these enzymes are closely similar. More interestingly, mutant 3 of HDC from *Lactobacillus* 30a shows only one amino

372 acid replacement at position 58 (G58A), the Gly amino acid residue is conserved at this
373 position in all HDC, with exception of *Staph. capitis* HDC with a Asn residue present in
374 its place (supplementary file). The G58N amino acid change in *Staph. capitis* HDC
375 could be responsible for the slow autoactivation and the appearance of the *Staph. capitis*
376 HDC in the π chain form.

377 Activation of wild type *Lactobacillus* 30a proenzyme occurs more rapidly than
378 that of mutant 3 proenzyme. The mutant protein, although exhibiting chain cleavage and
379 pyruvoyl formation, is catalytically inactive unless the pH is raised to an optimum value
380 of 7.6 (Copeland *et al*. 1987). Fig. 3 showed that along incubation in these activation
381 conditions, *Staph. capitis* HDC seems not to be autoactivated into an α-chain (23 kDa)
382 and a β-chain (11.5 kDa). The mechanism followed and the conditions needed for the
383 *Staph. capitis* proenzyme autocleavage remains unknown.

384 Some reports have been described the influence of physicochemical factors on
385 histamine production. HDC has been shown to have greatly reduced activity at neutral
386 or alkaline pH (Schelp *et al*. 2001). In order to known the regulation of the P*hdc*, *E. coli*
387 cells bearing pHDCP were incubated in different growth conditions (Table 2). P*hdc*
388 seems to be induced in media containing histidine, and mainly, during growth under
389 non-areation conditions. Similar conditions have been described previously to induce
390 other bacterial amino acid decarboxylases. As far as we known, pH or presence of
391 histidine in the media have been reported to induce pyruvoyl-dependent HDC (Coton *et*
392 *al*. 1998), however, this result constitutes the first report describing that the most
393 important induction of the HDC protein is obtained under non-aeration growth
394 condition.

395 As reported previously, *L. hilgardii* and *L. buchneri* shared a common genetic
396 organization in the HDC region (Fig. 1). The gene cluster *hdcP-hdcA-hdcB-hisRS* codes

for a histidine/histamine exchanger (*hdcP*), a histidine decarboxylase (*hdcA*), and a histidyl-tRNA synthetase (*hisRS*), while the function of the *hdcB* product is unknown (Lucas *et al*. 2005; Martín *et al*. 2005). As noticed by Lucas *et al*. (2005) the *hdcA-hdcB* gene pair is also found in *Lactobacillus* strain 30a, *T. muriaticus*, and *O. oeni*, suggesting that all the four gene cluster may be also present in these organisms. A different organization is observed on the genome of *C. perfringens* strain 13. The *hdcA* gene and a homologue of *hdcP* are arranged in the reverse order, and no homologues of *hdcB* and *hisRS* are present. As showed in Fig. 1, *Staph. capitis* IFIJ12 presents a genetic organization similar to *C. perfringens* and different from lactic acid bacteria. A detailed comparison of HDC proteins reveals that proteins from lactic acid bacteria and *Staph. capitis* showed a similar identity degree to the HDC clostridial protein (42 to 47% identity) (data not shown). Interestingly, the staphylococcal HDC protein is more similar to lactic acid bacterial proteins (59 to 61% identity) than to the clostridial enzyme (47%). The knowledge of more histamine biosynthetic clusters would help to the elucidation of the possible evolution of these genes in bacteria.

The data present in this work provide insights into the origin of a new histamine biosynthetic locus. Taking into account that i) *Staph. capitis* is closely related to species of the *Staph. epidermidis* species group (Kloos and Schleifer, 1984), and ii) *Staph. capitis* is the only staphylococcal histamine-producer species among the staphylococcal species analysed in this study, the data might suggests that a chromosome reorganization had taken place in *Staph. capitis* as a consequence of histamine production. Therefore, the acquisition of the genes for histamine production might be associated to a reorganization of the genome. Comparison of closely related genome sequences can provide a clue as to how macroscopic genome polymorphisms were formed through various events of recombination (Kawai *et al.* 2006). The availability of

the *Staph. capitis* complete genome and their comparison to the closely related *Staph. epidermidis* genome will lead to find genome rearrangements and polymorphisms among these species.

In summary, food safety control is one of the more critical aspects when it comes to consumer safety of fermented products. Proper assessment of strains regularly found in fermented products is crucial for quality control. The production of histamine is a relevant property related to food safety. We have elucidated the molecular basis for this property in *Staph. capitis*. We have demonstrated that *Staph. capitis* possess a gene that encodes a functional histidine decarboxylase capable of producing histamine from the amino acid histidine. Experiments demonstrated that this gene is induced in media containing histidine, and mainly, during growth under non-areation conditions. Additional and further research on histidine decarboxylase induction and regulation will help to prevent histamine formation and accumulation in fermented meat products.

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FIGURE LEGENDS

Fig. 1. Genetic organization of the *Staph. capitis* IFIJ12 6446-bp DNA region containing the histidine decarboxylase gene (*hdcA*). The sequence from *Staph. capitis* was deposited in GenBank under the accession no. AM283479. The *hdc* region corresponding to *Oenococcus oeni* 9204 (accession U58865), *Lactobacillus* sp. strain 30a (accession J02613), *Tetragenococcus muriaticus* (accession AB04078), *Lactobacillus hilgardii* 0006 (accession AY651779), *Lactobacillus buchneri* B301 (accession AJ749838), *Clostridium perfringens* str13 (accession NC_003366, positions 1890669-216493) and a chromosomal region from *Staphylococcus epidermidis* ATCC 12228 (accession NC_004461, positions 103018-571120) are represented. Arrows indicate ORFs. Thick and thin arrows represent complete and interrupted ORFs, respectively. Genes having putative identical functions are represented by identical shading. The location of putative and functional promoters (vertical bent arrow) and transcription terminator regions (ball and stick) are also indicated.

Fig. 2. (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. (1) SDS-PAGE of soluble cells extracts of IPTG-induced cultures of *E. coli* JM109 (DE3) (pLysS) bearing pT7-7 (lane 1) or recombinant plasmid pAM28 (lane 2). (2) SDS-PAGE of the affinity purification of HDC protein. Fractions eluted from His-affinity column with 125 mM imidazol are showed (lanes 1 to 9). The arrow indicated the overproduced HDC protein. The gels were stained with Coomassie blue. Molecular mass markers are indicated at the left (SDS-PAGE Standards, Bio-Rad). (b) Thin-layer chromatography (TLC) detection of histamine. (1) Histamine produced by soluble cells

extracts of IPTG-induced cultures of *E. coli* JM109 (DE3) (pLysS) bearing pT7-7 (lane 1) or recombinant plasmid pAM28 (lane 2). (2) Histamine produced by the highly purified HDC protein (lane 1). Lane H, control histamine standard solution. The histamine produced during the enzymatic reactions was dansylated and separated on a precoated silica gel 60 F_{254} plate. The arrow indicates the histamine produced.

Fig. 3. Autoactivation chain cleavage assay of the highly purified HDC from *Staph. capitis*. The figure shows a 10% polyacrylamide gel stained with Coomassie blue. Highly purified HDC protein was eluted with 125 mM imidazol, dialyzed and incubated in 0.8 M potassium phosphate pH 7.6 during 0h (lane 1), 1h (lane 2), 4h (lane 3), 8h (lane 4) and 16h (lane 5).

Table 1
hdc region-encoded proteins: properties and similarities to proteins in the databases

Gene	Location in nucleotide sequence	G+C (%)	Predicted protein (aa/kDa)	Similar Polypeptide(s) (aa)	Proposed function	Database accession no.	Degree of identity (%)	Organism
arcA	< - 454/c	31.3	-	SAUSA300_0065(411)	Arginine deiminase	Q2FKJ4	100 (in 151 aa overlap)	*S. aureus* USA300
				SE_0106 (411)	Arginine deiminase	ARCA1	99.3 (in 151 aa overlap)	*S. epidermidis* ATCC 12228
argR	702-1151/c	25.1	149 / 16.8	SE_0107 (148)	Arginine repressor	Q8CQG4	93.9	*S. epidermidis* ATCC 12228
				SAUSA30_0066(148)	Arginine repressor	Q2FKJ3	93.2	*S. aureus* USA300
orf3	1368-2024/c	25.9	218 / 24.3	BH0427 (301)	Cation transporter	Q9KFQ1	34.4	*B. halodurans* JCM 9153
				CPE1621 (268)	Hypothetical protein	Q8XJY2	33.3	*C. perfringens* strain 13
hdcA	2350 - 3282	34.9	310 / 34.2	HdcA (310)	Histidine decarboxylase	P00862	63.2	*Lactobacillus* sp. strain 30a
				LreuDRAFT_1190(311)	Histidine carboxylase	Q2BWG1	61.7	*L. reuteri* JCM1112
hdcP	3299 - 4786	32.0	495 / 52.2	CPE0389 (481)	Putative amino acid transporter	P30818	50.7	*C. perfringens* strain 13
				LreuDRAFT_1191(481)	Probable amino acid permease	Q2BWG0	50.1	*L. reuteri* JCM1112
orf6	4931 – 5275/c	28.7	114 /	SERP0460 (114)	Hypothetical protein	Q5HQT7	96.5	*S. epidermidis* ATCC 35984
				SE_0573 (114)	Hypothetical protein	Q8CTC8	94.7	*S. epidermidis* ATCC 12228
orf7	5502 - >	32.9	-	SE_0571 (435)	Na+-transporting ATP synthase	Q8CPX8	89.9 (in 326 aa overlap)	*S. epidermidis* ATCC 12228
				SERP0458 (435)	Na+ -transport family protein	Q5HQT9	89.9 (in 326 aa overlap)	*S. epidermidis* ATCC 35984

Table 2

Staphylococcus capitis P*hdc* induction measured as β-galactosidase activity

Culture condition	Miller units	Increase (x fold)
pH 7.6	630	1
pH 5.5	3,267	5.2
0.1% His	1,550	2.5
0.3% His	2,844	4.5
Aeration	1,070	1.7
Non-aeration	7,000	11.1

Figure 1

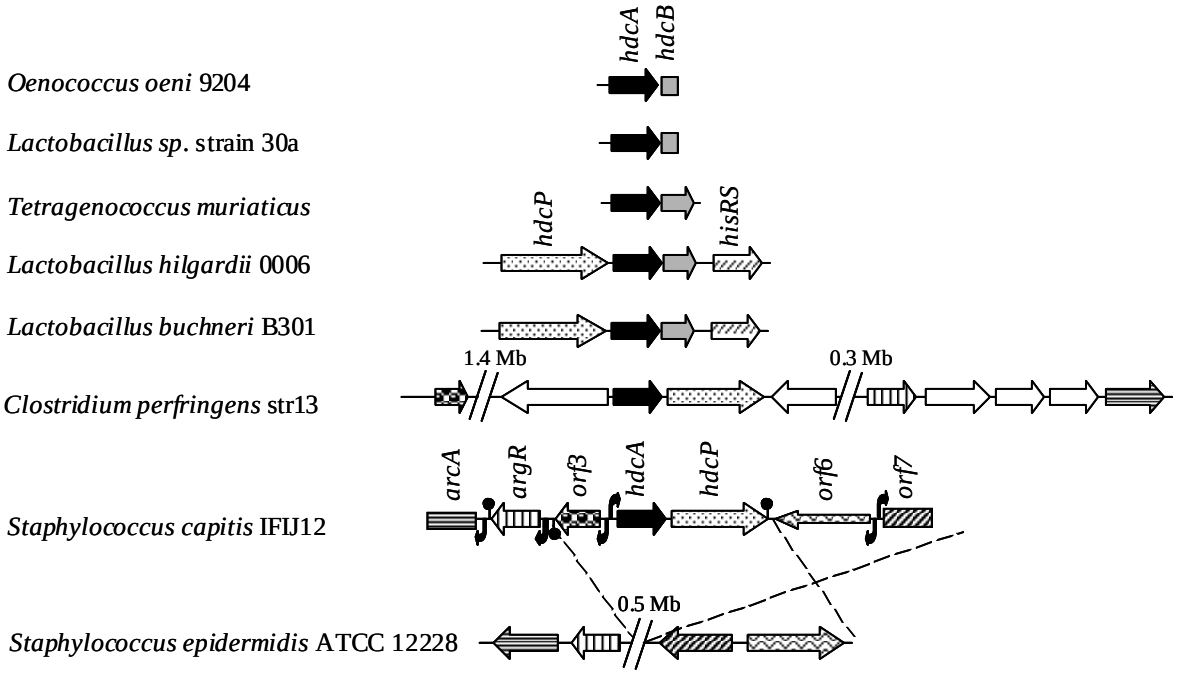
hdcA
hdcB
Oenococcus oeni 9204
Lactobacillus sp. strain 30a
Tetragenococcus muriaticus
hdcP
hisRS
Lactobacillus hilgardii 0006
Lactobacillus buchneri B301
1.4 Mb
0.3 Mb
Clostridium perfringens str13
arcA
argR
orf3
hdcA
hdcP
orf6
orf7
Staphylococcus capitis IFIJ12
0.5 Mb
Staphylococcus epidermidis ATCC 12228

Figure 2

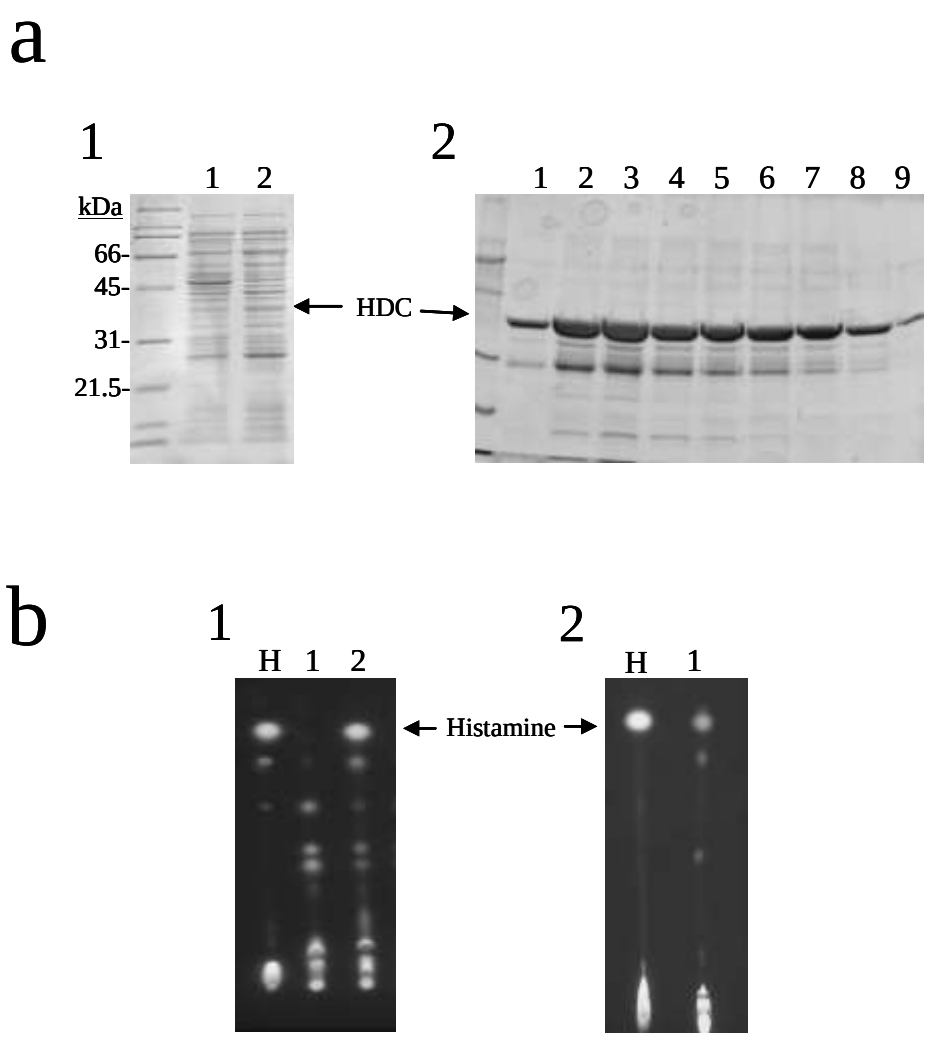

Figure 3