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Updated Molecular Knowledge About Histamine Biosynthesis by Bacteria

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Abstract

Histamine poisoning is caused by the ingestion of food containing high levels of histamine, a biogenic amine. Histamine could be expected in virtually all foods that contain proteins or free histidine and that are subject to conditions enabling microbial activity. In most histamine-containing foods the majority of the histamine is generated by decarboxylation of the histidine trough histidine decarboxylase enzymes derived from the bacteria present in food. Bacterial histidine decarboxylases have been extensively studied and characterized in different organisms and two different enzymes groups have been distinguished, pyridoxal phosphate- and the pyruvoyl-dependent. Pyridoxal phosphate-dependent histidine decarboxylases are encountered in Gram-negative bacteria belonging to various species. Pyruvoyl-dependent histidine decarboxylases are found in Gram-positive bacteria and specially in lactic acid bacteria implicated in food fermentation or spoilage. The molecular organization of the genes involved in histamine production have been elucidated in several histamine-producer bacteria. This molecular knowledge has lead to the development of molecular methods for the rapid detection of bacteria possessing the ability to produce histamine. The detection of histamine-producer bacteria is of great importance for its potential health hazard as well as from an economic point of view since products exceeding recommended limits can be refused in commercial transactions.

Keywords

histamine, biogenic amine, histidine decarboxylase, detection methods, food poisoning

INTRODUCTION

Several compounds produced by microorganisms are responsible for several types of food borne diseases. One of these illness, histamine poisoning, can result from the ingestion of food containing unusually high levels of histamine, a biogenic amine. Histamine (or scombroid) fish poisoning, caused by eating spoiled fish, happens throughout the world (Bartholomew et al., 1987; Becker et al., 2001). This poisoning is usually a rather mild illness; however, serious complications, such as cardiac and respiratory manifestations, occur rarely in individuals with preexisting conditions (reviewed by Lehane and Olley, 2000).The implicated fish are mainly of the families *Scomberesocidae* and *Scombridae* (the so-called scombroid fish) and contain large amounts of histamine (Taylor et al., 1989). A hazardous level of histamine is produced by the microbial decarboxylation of the free histidine present in the muscular tissue of fish. The fish are harmless when fresh, and after they have become toxic they may still have a normal appearance and odour. No available method of preparation, including freezing, canning and smoking, will destroy histamine (Etkind et al., 1987).

Histamine fish poisoning is a common form of food poisoning, but many incidents go unreported because of the mildness of the disease, lack of required reporting, and misdiagnosis. Symptoms can be confused with those of "*Salmonella* infection" and food allergy. While the syndrome is that of histamine toxicity, there is individual variation in susceptibility, and clinical signs are more severe in people taking medications, which inhibit enzymes that normally detoxify histamine in the intestine (Stratton et al., 1991).

Histamine is a naturally occurring substance in mammalian physiology. Its biological effects are usually seen only when it is released in large amounts in the course of

allergic and other reactions. Histamine exerts its effects by binding to receptors on cellular membranes in the respiratory, cardiovascular, gastrointestinal and haematological/immunological systems and the skin. Histamine causes dilatation of peripheral blood vessels, causing urticaria, hypotension, flushing and headache. Histamine-induced contraction of intestinal smooth muscle causes abdominal cramps, diarrhoea and vomiting (Attaran and Probst, 2002).

A fairly efficient detoxification system exists in the intestinal tract of mammals, which is capable of metabolising normal dietary intakes of histamine. Under normal conditions in human, exogenous amines absorbed from food are rapidly detoxified by the action of amine oxidases or by conjugation, but in the case of allergenic individuals or if monoamine oxidase (MAO) inhibitors are applied or when too high levels are consumed the detoxification process is disturbed and histamine accumulate in the body. The enzymes monoamine oxidase (MAO), diamine oxidase (DAO), and histamine N-methyltransferase (HMT) play an important role in this detoxification process (Aschenbach et al., 2006).

Several *in vivo* and *in vitro* studies have suggested that the absorption, metabolism, and/or potency of one biogenic amine might be modified in the presence of a second amine. Such potentiators would act to decrease the threshold dose of histamine needed to provoke an adverse reactions. As example, the oral toxicity of histamine in the guinea pig was increased 10 times when it was administered 40 min after oral administration of putrescine and was potentiated by the simultaneous administration of cadaverine (Bjeldanes et al., 1978). Other biogenic amines that may act as potentiators of histamine toxicity include tyramine (a MAO inhibitor that increases blood pressure), tryptamine (which inhibits DAO), and β-phenylethylamine (an inhibitor of both DAO and HMT) (Stratton et al., 1991).

Histamine poisoning is a significant public health and safety concern (Sánchez-Guerrero et al,. 1997; Wu et al., 1997) and a trade issue. Consumers are becoming more demanding, and litigation following food poisoning incidents is becoming more common. Producers, distributors and restaurants are increasingly held liable for the quality of the products they handle and sell. Many countries have set guidelines for maximum permitted levels of histamine. In fish, the European Union set a maximum average content of 100 mg/kg for fresh and canned products (CEE, 1991), whereas the U.S: Food and Drug Administration lowered it in 1995 from 100 to 50 mg/kg (US FDA, 1995).

HISTAMINE AND HISTAMINE-PRODUCER BACTERIA IN FOODS

Prerequisites for the formation of histamine in foods are the availability of free histidine, the presence of histidine decarboxylase-positive microorganisms, conditions that allow bacterial growth, and conditions that favours decarboxylase activity. Free amino acids occurs as such in foods, but may also be liberated from proteins as a result of proteolytic activity. Decarboxylase-positive bacteria may constitute part of the associated population of the food or may be introduced by contamination before, during or after processing of the food. In the case of fermented foods and beverages, the applied starter cultures may also affect the production of histamine.

Histamine could be expected in virtually all foods that contain proteins or free histidine and that are subject to conditions enabling microbial activity. Foodstuffs that can contain particularly high levels of histamine are fish, cheese, beer, wine, sauerkraut and some fermented meat products, such as sausage. As mentioned above, fish of the families *Scombridae* and *Scomberesocidae* are commonly implicated in incidents of histamine

poisoning, thus the term “scombroid fish poisoning” has been used to describe this type of food intoxication (Taylor et al., 1989). The tissues of scombroid fish contain high levels of free histidine, which may be converted partly into histamine by associated bacteria. Fermented fish products and fish paste frequently contain high amounts of histamine. After fish, cheese is the next most commonly implicated food item associated with biogenic amine poisoning. A variety of amines, including histamine, have been found in many different kinds of cheeses (Chang et al., 1985). In wine, histamine and tyramine are, besides putrescine, the most abundant amines in wine (Landete et al., 2005b). In beer, histamine is considered as a indicator of microbial contamination during brewing (Izquierdo-Pulido et al., 1997). Among meat products, although no cases of histamine poisoning have implicated sausage as the causative agent, it is not uncommon for this product to occasionally contain histamine along with other biogenic amines. In conclusion, in virtually all foods that contain proteins and are subject to conditions that enabling microbial activity, histamine can be expected. The total amount of the histamine formed strongly depends on the nature of the food and on the bacteria present.

In most histamine-containing foods the majority of the histamine is generated by decarboxylation of the amino acid histidine through histidine decarboxylase specific enzymes derived from the bacteria present in food. While histamine in fermented foods, such as cheese, wine, beer, etc. is produced by Gram-positive bacteria; histamine in non-fermented food is indicative of undesired microbial activity. Although amino acid decarboxylases are not widely distributed among bacteria, species of many genera are capable of decarboxylating one or more amino acids. However, the ability of bacteria to decarboxylate amino acids is highly variable. It depends not only on the species, but also on the strain and the environmental conditions (Coton et al., 1998b; Marcobal et al., 2006b).

Histidine decarboxylases are enzymes present in bacteria of food concern. In fish, *Enterobacteriaceae*, in particular *Morganella morganii*, have been identified as histamine-producer. It has been suggested that, especially in temperate zones, psychrophilic *Photobacterium* sp. are responsible for histamine production (ten Brink, 1990). Several studies of psychrophilic halophiles (bacteria that thrive in cold, salty water), which occur as part of the normal microbial population of marine fish, revealed their ability to produce high amounts of histamine at temperatures as low as 2.5 ºC. Therefore, low storage temperature are not sufficient to inhibit the formation of toxic amines (Halász et al., 1994). In cheeses, the role *L. buchneri* strains in the formation of histamine has been clearly established (Joosten and Northolt, 1989). In wine, *Oenococcus oeni* and *Lactobacillus hilgardii* strains could synthesize histamine (Rollán et al., 1995; Lucas et al., 2005). The production of histamine in meat has been attributed to the action of several microorganisms. Histamine-producer staphylococcal (Martín et al., 2006; Landeta et al., in press) and enterobacteria (Roig-Sagués et al., 1996) strains have been isolated from fermented meat products.

There are extensive references on the scientific literature about the potential to produce biogenic amines from a wide range of bacteria isolated from many food products. It is well known that some of the histidine decarboxylase detection methods routinely used could produce ambiguous results, as well as that some traditional identification methods could produce erroneous bacterial taxonomical classification, this review is only focused on the histamine-producer bacteria whose histidine decarboxylase proteins or whose corresponding encoding genes have been unequivocally reported so far (Table 1).

HISTAMINE PRODUCTION BY GRAM-POSITIVE BACTERIA

Histidine decarboxylase (HDC, EC 4.1.1.22) converts the amino acid histidine to histamine (Figure 1). Bacterial HDC have been thoroughly studied and characterized in different organisms and two different enzyme families have been distinguished: the pyridoxal phosphate-dependent HDC and the pyruvoyl-dependent HDC, their sequences and characteristics being radically different. Pyridoxal phosphate-dependent HDC are encountered in Gram-negative bacteria belonging to various species, and the pyruvoyl-dependent HDC are found in Gram-positive bacteria and specially in lactic acid bacteria implicated in food fermentation or spoilage (Table 1).

Pyruvoyl-dependent histidine decarboxylase

Most of the amino acid decarboxylases required pyridoxal 5´-phosphate (pyridoxal-P or PLP) as an essential coenzyme. An unexpected development, therefore, was the discovery that in certain of these decarboxylase enzymes covalently bound pyruvate, rather than pyridoxal-P, supplied the carbonyl group essential for the reaction.

Histidine decarboxylase from *Lactobacillus* 30a has been studied most thoroughly, and its perhaps prototypical of this group of enzymes. In 1953 Rodwell reported the isolation of a number of bacteria from horse stomach and sheep rumen with active amino acid decarboxylases. One of these organisms, a homofermentative lactic acid producer designated as *Lactobacillus* 30a, produced high levels of substrate-induced histidine

decarboxylases. Rodwell suggested that the HDC of this organism might not be PLP-dependent. Spectral measurements, chemical analysis of the purified protein showed that this enzyme did not, in fact, contain PLP or any other cofactor known at that time. It did, however, appear to contain a covalently bound pyruvoyl group required for activity.

Pyruvoyl-dependent HDC were later purified to homogeneity from other three Gram-positive bacterial sources: from a *Lactobacillus buchneri* strain isolated from an aged cheese that produced histamine poisoning in man, from *Clostridium perfringens*, and from *Micrococcus* sp. n (Prozorovski and Jörnvall, 1975; Recsei et al., 1983). All four enzymes contain pairs of dissimilar subunits and show considerable amino acid sequence homology, particularly at the C-termini of their smaller β subunits and the N-termini of their α subunits. The HDC $(\alpha\beta)_6$ from *Lactobacillus* 30a is synthesized as an inactive proenzyme (π_6) which undergoes a self-catalyzed serinolysis reaction in which each constituent π subunit cleaves nonhydrolytically between serine residues 81 and 82, with loss of ammonia, to yield a β subunit with Ser-81 at its C-terminus and an α subunit with a catalytically essential pyruvoyl residue (derived from Ser-82) at its N-terminus (Figure 2) (Snell, 1986). A similar proenzyme and activation mechanism have been demonstrated for the HDC from the other organisms.

The four known bacterial HDC (*Lactobacillus* 30a, *Microccocus*, sp., *C. perfringens*, and *L. buchneri*) are structurally similar enzymes organized on common principles. Each contains a pyruvoyl moiety at the catalytic site located on the α subunit. The occurrence of and α and β subunits of similar molecular weights indicates that all are formed from inactive proenzymes similar to that previously described in *Lactobacillus* 30a. The lactobacilli enzymes are immunologically cross-reactive, although not identical, and substantial homology between β chains of the *Lactobacillus* 30a enzyme and the

micrococcal enzyme has been demonstrated by sequence studies. These results suggest that the four enzymes have evolved from a common ancestral protein and the clostridial enzyme, which does not cross-react with antibodies to the *Lactobacillus* 30a enzyme, was the first to diverge from the common lineagen (Recsei et al., 1983). Maintenance of subunit structure may have resulted from requirements of proenzyme activation, enzyme action, or both. Substantial differences in amino acid composition, catalytic behaviour, and physical properties of these enzymes also exist.

Most of the residues implicated in catalysis and substrate binding in the HDC from *Lactobacillus* 30a (Gallagher et al., 1989) are conserved in the four HDC enzymes, indicating that the catalytic mechanism of these enzymes are closely similar (Recsei and Snell, 1985; Huynh and Snell, 1985; Alekseeva et al., 1986). ProHDC from *Lactobacillus* 30a is a protein that autoactivates to HDC by cleaving its peptide chain between serines 81 and 82 and converting Ser-82 to a pyruvoyl moiety (Huynh et al., 1984) (Figures 2 and 3). The pyruvoyl group serves as the prostetic group for the decarboxylation reaction. The conserved residues include those forming the predominantly hydrophobic pocket surrounding the carboxyl group of L-histidine in the *Lactobacillus* 30a enzyme [Ala-80, Ala-153, Ile-180, Phe-195, and Ala-260] and the potential proton donors/acceptors [Glu-197 and Lys-155], the residues that participate in hydrogen bonding to both imidazole nitrogen atoms of the substrate [Asp-63 and Ser-81] and in hydrophobic interactions with the imidazole ring [Ile-59 and Phe-83], and the residues forming a chain of hydrogen bonds near the active site [Ser-81, Tyr-62, and Glu-66] (Figure 3).

Histidine decarboxylase from *Lactobacillus* 30a has been crystallized in a variety of forms (Hackert et al., 1981; Parks et al., 1983; Parks et al., 1985; Gallagher et. al., 1989) (Figure 4 represents a diagram of the pyruvoyl-dependent HDC from *Lactobacillus* 30a).

The 2.5Å X-ray structure of HDC has been solved at pH 4.8. The enzyme is a hexamer of αβ subunits. The central core of the αβ subunit is a β-sandwich which consists of two face-to-face three-stranded antiparallel β-sheets, flanked by α-helices on each side. Three αβ subunits are related by a molecular three-fold symmetry axis to form a trimer whose interfaces have complementary surfaces and extensive molecular interactions. Each of the interfaces contains an active site and a solvent channel that leads from the active site to the exterior of the molecule. The trimers are related by a crystallographic two-fold symmetry axis to form the hexamer with an overall dumbbell shape. The interface between trimers has few molecular interactions (Gallagher et al., 1993). HDC forms a cup-shaped trimer with a deep central cavity containing three active sites. Each active site is formed at the interface between two monomers of the trimers. Two trimers can form weak tail-to-tail interactions to form an hexamer, but the hexamer is unlikely to have any catalytic significance.

Since HDC decarboxylase is derived by post-translational modification of a pyruvate-free proenzyme, histidine decarboxylaseless mutants were isolated with the expectation that mutants blocked in the modification step would synthesize a proenzyme in place of active enzyme. Recsei and Snell have isolated a number of nitrosoguanidine mutants that produce partially active or inactive enzymes. The best studied of these is mutant 3, which produces a full-length protein that, unlike wild type, is inactive when isolated, and must be incubated at pH 7 to activate (Recsei and Snell, 1982) (Figure 3). The resulting protein, although exhibiting chain cleavage and pyruvoyl formation, is catalytically inactive unless the pH is lowered toward the optimum value of 4.8. This mutant has a single amino acid substitution, an Asp for Gly at position 58 (Figure 3) (Vaaler et al., 1982).

Vanderslice et al. (1986) cloned and determined the nucleotide sequence of the proHDC from *Lactobacillus* 30a from a wild type strain and from the mutant with altered autoactivation properties. They concluded that the gene under control of its own promotor and in a high copy number plasmid, was lethal to the *E. coli* host cells. Later, the genes coding for HDC from a wild-type strain and an autoactivation mutant strain of *Lactobacillus* 30a were cloned and expressed in *E. coli* (Copeland et al., 1989). The cloned genes were placed under control of the β-galactosidade promoter and the products were natural length, not fusion proteins. The enzyme kinetics of the proteins isolated from *E. coli* were comparable to those isolated from *Lactobacillus* 30a. Site-directed mutagenesis was used to examine the chemical roles of several active-site residues in HDC from *Lactobacillus* 30a (Figure 3) (Gelfman et al., 1991; Pishko and Robertus, 1993; Pishko et al., 1995).

Similarly, the DNA encoding pyruvoyl-dependent HDC from *Clostridium perfringens* was cloned, sequenced, and overexpressed in *E. coli* (Van Poelje and Snell, 1990). No π subunits of proHDC were observed in crude or purified preparations of the cloned HDC; they appear to undergo rapid cleavage in vivo to the α and β subunits characteristics of this HDC. The rapid and complete conversion of cloned clostridial proHDC to HDC in *E. coli* cells was in contrast to the slow rate of cleavage observed for the cloned proHDC of *Lactobacillus* 30a in the same organisms. To clarify the mechanism of biogenesis and catalysis by the HDC from *C. perfringens*, twelve mutant genes encoding amino acid substitutions at the active site of this enzyme were constructed and expressed in *E. coli*. The resulting mutants proteins were purified to homogeneity, characterized, and subjected to kinetic analysis (Figure 3). Of the residues altered, only Ser-97 was essential for the autocatalytic serinolysis reaction by which this HDC, $(\alpha\beta)_6$, is derived from its

inactive, pyruvate-free precursor, π_6. Proenzyme activation thus seems to be dependent mainly upon the uniquely reactive serine residue Ser-97 and does not involve the obvious participation of other residues as proton donors/abstractors (Van Poelje et al., 1990).

Coton et al. (1998a) purified a histidine decarboxylase from *Oenococcus oeni*, formerly *Leuconostoc oenos*, a wine lactic acid bacteria. Similarly to other Gram-positive bacteria, *O. oeni* HDC comprises two subunits, respectively α and β. The *hdc* gene was cloned and sequenced. The gene encodes a single polypeptide of 316 amino acids, demonstrating that *O .oeni* 9204 HDC was synthesized as a precursor proHDC π_6. A cleavage between Ser-81 and Ser-82 generated the α and β chains, which suggested that the holoenzyme exists as a hexameric structure. The susbtrate specificity was studied with different amino acids which may be precursors of other biogenic amines, and the authors concluded that decarboxylation of histidine by HDC from *O. oeni* is specific. The similarities of these results with those described for other bacterial HDC support the assumption that the pyruvoyl enzymes evolved from a common ancestral protein and have similar catalytic mechanisms.

In 2002, a histidine decarboxylase from *Tetragenococcus muriaticus*, a halophilic histamine-producer bacteria isolated from Japanese fermented squid liver sauce, was purified to homogeneity (Konagaya et al., 2002). The pure enzyme consisted of two polypeptide chains. The optimum pH for the enzyme was 4.5-7.0. This enzyme did not decarboxylate lysine, arginine, tyrosine, tryptophan, and ornithine. Again, the similar psysiological properties of this enzyme and almost identical N-terminal amino acid sequences to those from other Gram-positive bacteria indicated that this enzyme may be evolutionally highly conserved among Gram-positive bacteria.

More recently, in 2005 the HDC sequence from a *Lactobacillus hilgardii* strain isolated from wine was deduced (Lucas et al., 2005). A multiple-amino acid sequence alignment shows that the proenzymes of *L. hilgardii*, *O. oeni*, and *T. muriaticus* are remarkably similar, differing only by two or three residues (Figure 3). The three sequences are also close to the proHDC of *Lactobacillus* strain 30a, sharing 79% sequence identity, and are somewhat more distantly related to the *C. perfringens* proenzyme, with 40% sequence identity. Finally, the HDC sequence from *Staphylococcus capitis* isolated from a cured-dry Spanish ham was elucidated (De las Rivas et al., submitted). The *S. capitis* HDC, 63% identical to the well-known HDC from *Lactobacillus* 30a, was cloned and purified from *E. coli*. The purified HDC protein was in the π chain form. A plausible explanation for this unusual behaviour of the *S. capitis* HDC protein could be deduced from the alignment showed in figure 3. Most of the residues involved in catalysis and substrate binding in the *Lactobacillus* 30a HDC are conserved in the *S. capitis* enzyme, indicating that the catalytic mechanism of these enzymes are closely similar. Mutant 3 from *Lactobacillus* 30a shows only one amino acid replacement at position 58 (Gly to Ala) (Figure 3). The Gly amino acid residue is conserved at this position in all HDC, with exception of *S. capitis* HDC with an Asn residue is present in its place. The Gly-58 to Asn amino acid change in *S. capitis* HDC could be responsible for the slow autoactivation and the appearance of the *S. capitis* HDC in the π chain form. Further work would be needed to demonstrated that this amino acid replacement results in significant changes in physical and catalytical properties of the *S. capitis* HDC protein.

Genetic organization of the histamine biosynthetic locus

Sequence analysis up- and downstream from the *Lactobacillus* 30a *hdcA* gene suggested that HDC was part of a polycistronic message (Vanderslice et al., 1986). The *hdcA* and *hdcB* gene pair is also found in *T. muriaticus*, and *O. oeni*. Remarkably, the sequences of the *hdcA* and *hdcB* genes are almost identical in *L. hilgardii*, *T. muriaticus*, and *O. oeni*, suggesting that the distribution of the genes occurred only recently (Lucas et al., 2005). Since an almost identical *hdcA-hdcB* gene pair is also found in *L. hilgardii*, could suggest that a similar *hdcP-hdcA-hdcB-hisRS* gene cluster may also be present in the former organisms (Figure 5).

In the wine bacteria *L. hilgardii* 0006, the sequence of the *hdcA* locus disclosed a four-gene cluster. The genes *hdcP*, *hdcA*, and *hisRS* code for a histidine/histamine exchanger, a histidine decarboxylase, and a histidyl-tRNA synthetase, respectively, while the function of the *hdcB* product is unknown. Nevertheless, the involvement of the *hdcB* product in the histidine decarboxylation system is strongly suggested by (i) the position of *hdcB* close to the *hdcA* gene, (ii) the conservation of the gene in histamine-producer strains, and (iii) a previous study that showed that *hdcA* and *hdcB* are cotranscribed in *Lactobacillus* strain 30a (Copeland et al., 1989). Given that the *hdcB-hisRS* intergenic region can fold into the terminator-antiterminator structure characteristic of the RNA leader regions of aminoacyl-tRNA synthetase genes, the *hisRS* gene is most likely transcribed from its own promoter. An almost identical gene cluster was found in *Lactobacillus buchneri* B301, an histamine-producer strain isolated from Gouda cheese (Martín et al., 2005) (Figure 5). In *L. buchneri* the expression of *hisRS* depends on the histidine

concentration of the growth medium, and it can be transcribed as monocistronic or *hdcP-hdcA-hisRS* policistronic mRNA.

The HdcP protein showed similarities to integral membrane transporters driving substrate/product exchange. The *L. hilgardii* gene coding for HdcP was cloned and expressed in *Lactococcus lactis*. The recombinant HdcP could drive the uptake of histidine into the cell and the exchange of histidine and histamine. The combination of HdcP and the histidine decarboxylase forms a typical bacterial decarboxylation pathway that may generate metabolic energy or be involved in the acid stress response (Figure 6). Analyses of sequences present in databases suggest that the other two proteins have dispensable functions.

A different genetic organization is observed on the genome of the histamine producer *C. perfringens* strain 13 (Shimuzi et al., 2002). The *hdcA* gene and a homologue of *hdcP* are arranged in the reverse order, and no homologues of *hdcB* and *hisRS* are present. The lack of the latter two indicates that they are not essential to the histidine decarboxylation pathway and are likely to code for accessory functions. As showed in figure 5, *S capitis* IFIJ12 presents a genetic organization similar to *C. perfringens* and different from lactic acid bacteria, as only homologues of *hdcA* and *hdcP* are present (De las Rivas et al., submitted).

An interesting feature in relation to histamine production by Gram-positive bacteria is the location of the HDC encoding gene. Lucas et al. (2005) described that *Lactobacillus hilgardii* strain IOEB 0006 could retain or lose the ability to produce histamine depending on culture conditions. The authors reported that the *hdcA* gene was lost spontaneously during the growth of *L. hilgardii*. They found that the *hdc* gene cluster, responsible for histamine production in *L. hilgardii* IOEB 0006, was located on an 80-kb plasmid that

proved to be unstable. Moreover, it was reported that histamine-producer *O. oeni* strains isolated from wine rapidly lost the capacity to form histamine when they were grown in a synthetic medium. This finding strengthens not only the importance of growth conditions but also the hypothesis that *O. oeni* carries the same 80-kb unstable plasmid as *L. hilgardii* 0006. A plasmid-encoded histidine decarboxylation system could be transferred horizontally, which would be in agreement with the 99 to 100% identical *hdcA*- and *hdcB*-encoded proteins of *L. hilgardii* 0006, *T. muriaticus*, and *O. oeni*. Such a degree of identity strongly suggests that the genes were recently transferred in the three bacteria. It is very likely that *T. muriaticus* and *O. oeni* in fact harbor the same 80-kb unstable plasmid as found in *L. hilgardii* 0006 (Lucas et al., 2005). However, it is remarkable that is spite of *L. buchneri* B301 have a 99-100% identical sequence to *L. hilgardii* in the *hdc* locus, in *L. buchneri* this locus is located in the chromosome.

Similarly to *L. buchneri*, the *hdc* locus in *C. perfringens* and *S. capitis* is located in the chromosome. More interestingly, it was found that as compared to *Staphylococcus epidermidis* ATCC 12228, only the genes involved in histamine production are exclusively present in the *S. capitis* genome (De las Rivas et al., submitted). Sequence data indicated that the nucleotide sequences flanking the histamine biosynthetic genes in *S. capitis*, are located 0.5-Mb apart, and in inverse orientation in the *S. epidermidis* genome, suggesting that a large chromosome reorganization had taken place in *S. capitis* as a consequence of histamine production. Therefore, the acquisition of the genes for histamine production might be associated to a profound reorganization of the genome.

Regulation of histamine production in Gram-positive bacteria

Lactobacillus 30a as well as *C. perfringens* are lactic acid-producing bacteria. As they metabolise, their secretion of acid can reduce the environmental pH to about 4, where growth stops. To counter this effect, the bacteria secrete histamine base. *Lactobacillus buchneri* STA2 was isolated from Swiss cheese implicated in an outbreak of food poisoning (Sumner et al., 1985). This strain vigorously decarboxylates histidine to histamine, which is excreted to the medium. Cells grown in the presence of histidine generate both a trasmembrane pH gradient, inside alkaline, and an electrical potential, inside negative, upon addition of histidine (Figure 6) (Molenaar et al., 1993). The data also suggest that the generation of metabolic energy from histidine decarboxylation results from an electrogenic histidine/histamine exchange and indirect proton extrusion due to the combined action of the decarboxylase and carrier-mediated exchange. Therefore, the primary goal of amino acid decarboxylation may be to prolong the period in which a sufficiently high intracellular pH is present or as a source of metabolic energy. The net effect of the HDC reaction is to remove a proton from the cytoplasm, thereby raising the pH, and to transport the positive charge outside, as histamine, to create an energy-rich electrostatic membrane potential.

By using a proteomic approach, Pessione et al. (2005) demonstrated that HDC biosynthesis in *Lactobacillus* 30a was shown to be closely dependent on the presence of high concentrations of free amino acids in the growth medium and to be modulated by the growth phase.

Lonvaud-Funel and Joyeux (1994) studied histamine production by wine lactic acid-bacteria. They found that histamine levels depended on the quantity of precursors available

and on the presence of yeast lees, which certainly enriched the medium in histidine. Ethanol and pH, which control bacterial growth rate and total population, were also significant factors: pH and low ethanol concentration enhanced histamine production. In synthetic medium *O. oeni* 9204 produced large amount of histamine especially in the poorest nutritional conditions (no glucose, no L-malic acid). Rollan et al. (1995) found that the inhibition of *O. oeni* HDC activity by histamine, the final product of the reaction, in a cell suspension was higher (69%) than that measured in a cell-free extract (48%). Histamine acted as a HDC competitive inhibitor. They also found that up to 10% (v/v) of ethanol increased the histamine production in whole cells of *O. oeni*. They explaining this by an easier transport of histidine inside the cells, owing to the fluidification of the membrane by ethanol.

Landete et al. (2006b) studied the influence of enological factors (organic acids, sugars, ethanol, SO_2, pH and temperature) on the *hdc* gene expression and on the HDC enzymatic activity in wine lactic-acid bacteria strains belonging to different species. They observed that glucose, fructose, malic acid and citric acid diminished *hdc* expression, while ethanol, temperature and pH had effect on HDC activity but not on *hdc* expression. Tartaric acid and L-lactic acid, and SO_2 had no effect on enzyme synthesis and activity. Histamine synthesis was negatively correlated to malic acid, citric acid and glucose content, and positively correlated to ethanol content. Although specific activities were different for the three studied strains, a similar proportional response was obtained for each metabolite and regulation level (expression and activity) in each microorganism. This could mean that a general mechanism rules the synthesis and activity of the histidine decarboxylase in these organisms.

In addition, Landete et al. (2006a) studied the effects of histamine, histidine, and growth phase on histamine production by wine lactic acid bacteria. The authors observed that histidine induces *hdc* expression whereas histamine causes a decrease in its expression. The *hdc* expression is also mediated by the bacterial growth phase, being highest in the exponential phase, while a decrease in *hdc* expression was observed during the stationary phase. Recently, De las Rivas et al. (submitted) studied the regulation of the *S. capitis hdc* promoter. As compared to growth at pH 7.6, the promoter activity showed a 5.2-fold increase at pH 5.5, and a 2.5 and 4.5-fold increase in media containing 0.1% and 0.3% histidine, respectively, however, the highest promoter activity (11.1-fold) was obtained during growth under non-areation conditions.

Experiments done with purified *Lactobacillus* 30a HDC, showed that it have greatly reduced activity at neutral or alkaline pH. This pH regulation is almost certainly the main physiological one for HDC (Pishko et al., 1995). Structural information was obtained by Schelp et al. (2001) that solved the X-ray structure of HDC at pH 8 and revealed the novel mechanism of pH regulation. At high pH an helix is unwound, destroying the substrate binding pocket. At acid pH the helix is stabilized, partly through protonation of Asp-198 and Asp-53 on either side of the molecular interface, acting as a proton trap. pH has a large effect on the tertiary structure of HDC monomers and relatively little or no effect on quaternary structure. This is an example of how pH is used to control enzyme activity through helical stabilization.

HISTAMINE PRODUCTION BY GRAM-NEGATIVE BACTERIA

The occasional occurrence of histamine poisoning in humans establishes the medical importance of these enzymes, which are responsible for histamine formation from histidine in food products. The fact that two very different classes of enzymes have evolved –a pyruvoyl-dependent class apparently restricted to Gram-positive bacteria and a pyridoxal-P dependent class present in Gram-negative bacteria (and mammals) suggests that the reaction has physiological significance that is not yet recognized and provide an unusually clear example of independent evolution of very different proteins to achieve what appears to be a common function.

Pyridoxal-dependent histidine decarboxylase

Tanase et al. (1985) reported without experimental details that histidine decarboxylase from two Gram-negative bacteria, *Morganella morganii* AM-15 and *Raoultella planticola* (formerly *Klebsiella pneumoniae)*, resembled the mammalian enzyme in requiring pyridoxal-P as coenzyme. In 1979, Taylor et al. (1979) reported that histamine-producing *R. planticola* strains T2 (formerly, *Klebsiella pneumoniae* strain T2) was isolated from spoiled tuna sashimi. Several strains of *Morganella* isolated from spoiled fish products that produced histamine poisoning in man were surveyed by Tanase et al. (1985). All the *Morganella* strains analysed produced an inducible HDC, but the authors reported that production was highest in strain AM-15. Approximately 0.64% of the soluble protein in fully induced cells is of this organism was HDC. The *M. morganii* AM-15 HDC was purified, and unlike the pyruvoyl-dependent HDC, the purified enzyme from *M. morganii*

showed a spectrum typical of pyridoxal-P enzymes (Snell and Guirard, 1986). The pyridoxal-P enzyme from *Morganella* and the pyruvoyl enzyme from *Lactobacillus* 30a are equally efficient in catalysing decarboxylation of histidine, but the two enzymes differ in their molecular organization and substrate specificity. The pyridoxal-P enzyme has an M_r of ~170,000 and consists of four identical subunits of M_r ~43,000, whereas the pyruvoyl enzyme has an M_r of ~202,000, and consists of two trimers, $(\alpha_3\beta_3)_2$, with an M_r ~28,000 for α and an M_r ~9,000 for β subunit. In addition, the pyridoxal-P enzyme is comparatively unstable with a pH optimum of 6.5, whereas the pyruvoyl enzyme is relatively stable and has a pH optimum of 4.8. Histamine is a competitive inhibitor of the pyruvoyl enzymes but not of the pyridoxal-P enzymes. Conversely, α-fluoromethylhistidine and α-methylhistidine are excellent inhibitors of the pyridoxal-P enzymes, but not of the pyruvoyl enzymes.

Hayashi et al. (1986) reported that pyridoxal-P HDC from *M. morganii* was inactivated by α-fluoromethylhistidine. Both L-histidine and the competitive inhibitor, L-histidine methyl ester, protected against inactivation. Under optimal conditions a single molecule of α-fluoromethylhistidine inactivates one enzyme subunit. The bound inactivator is released upon denaturation by heat or urea. This released product was not fully characterized. The inactivators binds to a serine residue of the holoenzyme. This residue is not near the lysine residue that binds pyridoxal-P in the primary sequence of the protein, although the two residues must be near one another in the three-dimensional structure to account for these results. Vaaler at al. (1986) reported that the residue that binds pyridoxal-P is Lys-233, and the serine that participates in the binding of α-fluoromethylhistidine is residue 323 (Figure 7). Analyses done assign residues 233 and 323 to non-helical regions of the protein that presumably are in close proximity to each other in the active site of the enzyme. A comparison of the HDC sequence identifies a 9-residue match in 12 amino acids

with the Leu-Ile-Val binding protein from *E. coli*. A conserved sequence Ser-X-His-Lys was described in the reduced active site peptides from pyridoxal-P-dependent glutamate, arginine, and lysine decarboxylases of *E. coli*. The corresponding peptide from the *Morganella* HDC contains a Ser-Gly-His-Lys sequence conforming to the conserved sequence (Figure 7).

The *hdc* gene coding for the *Morganella* HDC was expressed in *E. coli* (Vaaler and Snell, 1989). Fourteen site-directed mutant enzymes were constructed to explore the roles of residues of interest, especially those in the sequence Ser^{230}-X^{231}-His^{232}-Lys^{233} (Figure 7). Ala or Cys in place of Ser-230 yielded mutant enzymes about 7% as active as wild-type, indicating that this serine residue is not essential for catalysis but contributes to activity through conformational or other effects. On the replacements made for His-232 (Asn, Gln, Phe, and Arg), only Gln and Asn gave partially active enzymes (about 12% and 0.2% of wild-type, respectively). The side-chain amide of Gln may act by mimicking the positionally equivalent nitrogen on the imidazole ring of histidine to provide an interaction (e. g., a hydrogem bond) required for efficient catalysis. The Lys-233 residue that interacts with pyridoxal-P appears central to catalytic efficiency since replacing it with Ala yields a mutant protein that is virtually inactive but retains the ability to bind both pyridoxal-P and histidine efficiently. None of four other residues (Met-234, Cys-241, Cys-330, Ser-323) chosen for replacement because of their possible oxidative lability or role in catalysis proved essential for activity, although all replacements reduced the activity of the enzyme significantly. It was reported that wild-type *M. morganii* HDC was crystallized but the crystals were fragmented and cracked upon exposure to air and/or manipulations (Vaaler and Snell, 1989)

HDC from *Raoultella planticola* and *Enterobacter aerogenes* were purified to homogeneity by Guirard and Snell (1987) and were compared with the HDC from *Morganella morganii*. As above mentioned, all three enzymes required pyridoxal-P as a coenzyme, showed optimal activity at pH 6.5, decarboxylated only histidine among the amino acids derived from protein, and were tetramers or dimers of identical subunits. Amino-terminal sequences of the three enzymes showed up to 81% homology through residue 33, but the enzymes differed sufficiently in amino acid composition and sequence so that no cross-reaction occurred between the *R. planticola* or *E. aerogenes* enzymes and antibodies to the decarboxylase from *M. morganii*. All three enzymes are inhibited by carbonyl reagents; by amino-, carboxyl-, and some methyl-substituted histidines; and by α-fluoromethylhistidine. These decarboxylases, all from gram-negative organisms, differed greatly in subunit structure, biogenesis, and other properties from the pyruvoyl-dependent HDC from gram-positive organisms described previously. The *hdc* genes encoding pyridoxal-P-dependent HDC of *R. planticola* and *E. aerogenes* were isolated, sequenced, and expressed in *E. coli*, and the overexpressed enzymes were purified to homogeneity from the recombinant host (Kamath et al., 1991). The two enzymes showed 85% sequence identity and also showed 80% sequence identity with the previously sequenced HDC of *M. morganii*. HDC from all three organisms were specific for L-histidine; arginine, lysine, and ornithine were inactive as substrates. Despite the high degree of sequence identity of these three HDC, the corresponding enzymes from the native hosts differ sufficiently so that polyclonal antisera to the *M. morganii* HDC do not cross-react with the other two enzymes (Guirard and Snell, 1987). This results suggests that those regions most at variance (residues 141-193 and 279-319) are likely to be on the surface of the proteins.

Listonella anguillarum, formerly *Vibrio anguillarum*, an important fish pathogen, is the aetiological agent of the septicaemic disease vibriosis. Most strains of this bacterium carry the 65-kb plasmid pJM1 that encodes an efficient iron uptake system without it bacteria become avirulent. This system includes the siderophore anguibactin. A *hdc* gene is present in pJM1 plasmid, being an essential component of the anguibactin biosynthetic pathway and, therefore of virulence of *L. anguillarum*. A comparison of the *L. anguillarum* HDC with those that contain pyridoxal-P as the essential coenzyme revealed identities of 65.1%, 63.5%, and 61.4%, to HDC from *R. planticola*, *M. morganii*, and *E. aerogenes*, respectively. Among the identical amino acids is Lys-233 that binds the molecule of pyridoxal-P, and Ser-323 that binds the adduct formed between pyridoxal-P and the inhibitor α-fluoromethylhistidine (Hayashi et al., 1986). The conserved sequence, Ser-X-His-Lys, found in other pyridoxal-P-dependent enzymes is also found in these HDC (Figure 7).

Similarly, Mercado-Blanco et al. (2001) studied the biosynthesis of salicilyc acid and pseudomonine by *Pseudomonas fluorescens* WCS374 and found that a HDC is involved. The *P. fluorescens* HDC showed high homology with pyridoxal-P-dependent HDC from *R. planticola* (77% identity), *M. morganii* (76%), *E. aerogenes* (75%), and *L. anguillarum* (62%). In the *P. fluorescens* HDC important features such as the sequence Ser-X-His-Lys, and the residues involved in the binding of pyridoxal-P and α-fluoromethylhistidine are conserved (Figure 7).

In spite that inducible HDC were mainly described, it have been reported that the histamine-forming ability of *Photobacterium phosporeum* was affected by two HDC, a constitutive and an inducible enzyme (Morii and Kasama., 1995; Morii and Kasama. 2004). A database comparison of the deduced amino acid sequence of the inducible HDC from *P.*

phosphoreum displayed high similarity to the five pyridoxal-P-dependent HDC of Gram-negative bacteria (Morii et al., 2006). Neighbor-joining and maximum-parsimony analyses based on the pyridoxal-P-dependent HDC amino acid sequences of Gram-negative bacteria were performed to infer reliable topology. Within the phylogenetic tree, the *P. phosphoreum* isolate and the species of the family *Enterobacteriaceae* formed a phylogenetic clade. The HDC amino acid sequences of *P. phosphoreum* and *L. anguillarum* formed separate phylogenetic branches (Morii et al., 2006).

Genetic organization of the histamine biosynthetic locus

The nucleotide sequence from *Morganella morganii* AM-15 that contains the gene for HDC was determined by Vaaler et al. in 1986, and the *hdc* nucleotide sequence from *R. planticola* and *E. aerogenes* by Kamath et al. (1991). All three HDC are the same length (378 amino acid residues). The high sequence identity exhibited by these three HDC at both the nucleic acid (75%) and amino acid (80%) levels far exceeds the accepted relatedness among these genera of the family *Enterobacteriaceae*. While *Enterobacter* and *Raoultella* have a 30-60% DNA relatedness, *Morganella* is recognized as being more distantly related. Assuming a common parental gene for these three HDC, there has been no addition or deletion of nucleotides during evolution, and of the approximately 20% of amino acid substitutions made there are twice as many nonconservative as conservative changes (Kamath et al., 1991).

Kanki et al. (2002) described that *R. planticola* and *R. ornithinolytica* (formerly *Klebsiella ornithinolytica*) were equal in their histamine-producing capabilities and were determined to possess the *hdc* genes, encoding HDC enzymes. Takahashi et al. (2003)

amplified and sequenced *hdc* gene fragments from Gram-negative bacteria such as *M. morganii*, *R. planticola*, *P. vulgaris*, *Erwinia sp.*, *Photobacterium damselae*, and *P. phosporeum*. The similarities for the nucleotide sequences of 709-bp fragments in these species ranged from 73.8 to 99.2%.

A 2.6-kb DNA region encoding the HDC from *Photobacterium phosphoreum* has been characterized recently (Morii et al., 2006) (Figure 8). The first 563 nucleotides that were located upstream of the *hdc* gene encoded a protein that showed identity to the C-terminal region of amino acid permeases from *Salmonella typhimurium* and *Yersinia pestis*, and to amino acid and/or amine transport protein of *E. coli* (YdjE). A partial ORF that was located downstream of the *hdc* gene encoded 102 amino acid residues protein which N-terminal region had 60% identity to the corresponding region of the histidine-tRNA synthetase of *Y. pestis* (HisS). These are the first data concerning a putative amino acid permease and histidine-t-RNA synthetase in genes flanking regions of an *hdc* gene in Gram-negative bacteria. It is noteworthy that in this Gram-negative bacteria that uses a different HDC, the genetic organization of the histidine decarboxilation pathway is similar to that observed in Gram-positive organisms.

In addition to these HDC, those involved in siderophore biosynthesis should be mentioned. As explained above, Tolmasky et al. (1995) identified and sequenced an *hdc* gene in the *L. anguillarum* plasmid pJM1 which encodes an HDC enzyme and is an essential component for the biosynthesis of anguibactin. The pJM1 plasmid, 65-kb, revealed genes and open reading frames (ORF) encoding iron transporters, nonribosomal peptide enzymes, and other proteins essential for the biosynthesis of the siderophore anguibactin. In pJM1, the gene encoding HDC (*orf13*) is preceded by two transposases, and followed by two putative ABC transporter homologues (Di Lorenzo et al., 2003) (Figure 8).

Mercado-Blanco et al. (2001), in a *Pseudomonas fluorescens* WCS374 gene library, identified a 5-kb chromosomal DNA fragment containing four ORF involved in the biosynthesis of salicylic acid and the siderophore pseudomonine. Products of two ORF (*pmsC* and *pmsB*) showed homologies with chorismate-utilizing enzymes; other ORF (*pmsE*) encoded a protein with strong similarity with enzymes involved in the biosynthesis of siderophores in other bacterial species, and finally, they found an ORF (*pmsA*) that encoded the HDC (Figure 8).

Regulation of histamine production in Gram-negative bacteria

Upstream of each of the *hdc* genes from *M. morganii*, *R. planticola*, and *E. aerogenes* there is a sequence (GGNNNNNNNAAAAAAGGCNANNNNANNCTNTA, where N is any nucleotide) that contains 17 matches among 32 nucleotides. Since all three enzymes are inducible by the addition of histidine to the medium, it have been postulated that it could be a histidine-responsive element. This element was not found upstream of the pJM1 *hdc* suggesting that, if this sequence is proved to be necessary for histidine regulation, the *L. anguillarum hdc* will not be regulated or regulation by histidine will be achieved by different mechanism. Barancin et al. (1998) reported that the fish pathogen *L. anguillarum* secretes histamine when cells harbouring a wild-type HDC gene are cultured in the presence of excess histidine. The secretion of histamine by the wild-type strain, during active secretion of histamine-containing anguibactin siderophore, is also significant since it proves that not all the histamine made by the bacterial cells is used as a precursor for the biosynthesis of anguibactin. The excess histamine released into the extracellular

environment may contribute not only to food poisoning, but also to the pathogenicity of this bacterium.

It was described that histamine-forming ability of *P. phosphoreum* was affected by two HDC, constitutive and inducible (Morii and Kasama, 1995; Morii and Kasama, 2004), and the two HDC activities varied with oxygen tensions for growth and reaction temperatures, pH, and ClNa concentrations. The HDC activity of cell-free extracts was highest in 7 ºC culture. Moreover, percent activity of the constitutive and inducible enzymes was highest for the inducible enzyme in cultures grown at 7 ºC and pH 7.5 and in 5% NaCl. The temperature and pH dependence of HDC was optimum at 30 ºC and pH 6.5 for the inducible enzyme and 40 ºC and pH 6.0 for the constitutive enzyme. On the other hand, HDC activity was opimum in 0% NaCl for the two enzymes (Morii and Kasama, 2004). Morii et al. (2006) found a putative catabolite-repressor-protein (CRP) binding site present upstream of the *P. phosphoreum hdc* gene, the authors suggest that this might indicate that the expression of the *hdc* gene is inducible.

Because freezing greatly decreases the histamine-producer ability of histamine-producer bacteria, especially of *P. phosphoreum*, it has been speculated that histamine fish poisoning is caused by HDC itself from histamine-producer bacterial cells autolyzing during frozen storage, even when these bacteria survive frozen storage. Recently, Kanki et al. (2007) constructed recombinant HDC of *P. phosphoreum*, *P. damselae*, *R. planticola*, and *M. morganii*, and investigated the ability of HDC to produce sufficient histamine to cause histamine fish poisoning. To elucidate the character of these HDC, they examined the specific activity of each recombinant HDC at various temperatures, pH levels, and NaCl concentrations. Further, they also investigated the stability of each HDC under different conditions (in reaction buffer, tuna, and dried saury) at various temperatures. The results

confirm that *P. damselae* HDC readily produced sufficient histamine to cause histamine fish poisoning in fish samples (Kanki et al., 2007).

Additional work need to be done to obtain a deep knowledge about the regulation of histamine production in Gram-negative bacteria.

DETECTION OF BIOGENIC AMINE-PRODUCER BACTERIA

The presence of histamine in foods is of considerable public concern for the food industry and the regulatory agencies, since given the potential health hazard, there is a growing demand from consumers and control authorities to reduce the allowable limits of histamine in foods and beverages. Rapid and simple methods are needed for the analysis of the ability to form histamine by bacteria in order to evaluate the potential risk of bacterial occurring in some food products (reviewed by Marcobal et al., 2006a).

The detection of histamine-producer bacteria using culture techniques is often slow and variable; therefore nowadays methods based in molecular biology are an interesting alternative. These methods provide information about the potential risk of histamine formation.

Non-molecular methods for the detection of biogenic amine-producer bacteria

It is generally extended the use of differential growth media containing a pH indicator, such as bromocresol purple (Niven et al., 1981; Choudhury et al., 1990). When histamine is produced, the positive result is indicated by a change of the medium colour in response of the indicator to a pH shift. The pH change is dependent of the production of the

more alkaline histamine from the amino acid histamine initially included in the medium. Niven's medium have been used for the detection of histamine-producer *Enterobacteriaceae* and other bacteria from fish (Niven et al., 1981; Yoshinaga and Frank, 1982; Chen et al., 1989; Actis et al., 1999; da Silva et al., 2002) and cheese (Marino et al., 2000). Some reports described the occurrence of false-positive reactions in these differential media, due to the formation of other alkaline compounds (Baranowski, 1985; Roig-Sagués et al., 1997). False negative responses as a result of the fermentative activity of some bacteria which produce acid along with histamine, constitute a major problem when screening lactic acid bacteria. Besides this problem, some microorganisms were not able to grow in these relatively simple decarboxylase media. In order to facilitate the growth of meat fastidious bacteria, Maijala (1993) described a decarboxylase agar that, sometimes with slightly modifications, was applied to detect the production of histamine by bacteria isolated from fish (Leisner et al., 1994; da Silva et al., 2002), raw and ripened Salchichón (Roig-Sagués et al., 1996), and wine (Moreno-Arribas et al., 2003; Landete et al., 2005a). Bover-Cid and Holzapfel (1999) improved the screening method and included pyridoxal-5-phosphate in the decarboxylase medium since it is a cofactor for some histidine decarboxylase reactions. Later, Mavromatis and Quantick (2002) modified Niven's medium for the optimised enumeration of histamine-forming bacteria.

Some enzymatic methods were developed for the detection of histamine-producer bacteria. Most of these methods are based in the production of hydrogen peroxide by the action of an oxidase enzyme on the histamine. The enzymatic tests are generally based on the sequential activity of two enzymes: diamine oxidase (DAO), that catalyses the breakdown of histamine in imidazole acetaldehide, ammonia, and hydrogen peroxide; and horseradish peroxidase (HRP) that, in the presence of hydrogen peroxide, produces a

change of colour in the chromogen. Sumner and Taylor (1989) developed an enzymatic method using the chromogen leucocrystal violet (LCV) for detecting histamine-producer, dairy related bacteria. This method is a qualitative method, since it cannot be used for quantification due to the interference caused by the culture media. The limitations indicated by Sumner and Taylor (1989) in the use of this method to quantify histidine decarboxylase activity were solved by López-Sabater et al. (1993) and Rodríguez-Jerez et al. (1994), by filtering the culture medium and, by using a colourless broth. Another attractive alternative for histamine determinations are the biosensors, which combine biological recognition through enzyme specificity with construction simplicity.

Among the different chromatographic techniques recommended for identification and quantification of histamine, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been the most useful. The methods developed to determine the ability of some bacteria to produce histamine based on thin layer chromatography (TLC) constitute a simple alternative to the previous methods. Lin et al. (1977) designed a TLC method for the rapid detection of histamine in tuna. This method was modified by Barancin et al. (1998) to use on bacterial culture supernatants. Briefly, the bacterial culture supernatants were collected by centrifugation and lyophilized. The dried residues were extracted twice with methanol and chromatographed onto precoated silica gel LTC plates. Later, this TLC method was applied by Actis et al. (1999) for the detection of histamine production in bacteria. Recently, García-Moruno et al. (2005) described a method that avoids a prior extraction step of the amines and the centrifuged supernatants are directly derivatised. This method was successfully applied to several biogenic amine-producer strains.

At present, high performance liquid chromatography (HPLC) is the most used technique because of its great versatility, and, therefore, is the official technique for analysing histamine in foods. A large number of reports have been published on the simultaneous detection of multiple amines (Seiler and Knödgen, 1980; Eerola, 1993; Pereiro Monteiro and Bertrand, 1994). Among others, gradient HPLC with pre- or post-column derivatisation and multichannel UV detection (diodearray), subsequent post-column derivatisation with ophthaldialdehyde and 3-mercaptopropionic acid, and fluorescence detection methods have been described (Straub et al., 1993).

Molecular methods for the detection of biogenic amine-producer bacteria

Detection of histamine-producer bacteria by conventional culture techniques is often tedious and unreliable. Several studies describing loss of ability to produce histamine in LAB after cultivation of isolated strains in synthetic media have been reported (Lonvaud-Funel and Joyeux, 1994). As molecular methods are fast, reliable and culture-independent, they are an interesting alternative. Since histamine is produced by the decarboxylation of the amino acid histidine by the enzymatic action of an HDC, it is possible to develop molecular detection methods targeting the genes coding for these histidine decarboxylase enzymes (reviewed by Landete et al., 2007).

Polymerase chain reaction (PCR) has become an important method for the rapid, sensitive, and specific detection of targeted genes. Several oligonucleotide primers have been described to detect histidine decarboxylases encoding genes by PCR. Since the designed oligonucleotides are based on amino acid regions conserved in specific histidine

decarboxylases, pyruvoyl-dependent or pyridoxal-P-dependent, this implies that these primers would allow the detection of all of them.

Pyruvoyl-dependent HDC are present in Gram-positive bacteria. To detect histamine-producer lactic acid bacteria, Le Jeune et al. (1995) designed several oligonucleotide primers (CL1, CL2, JV16HC, and JV17HC) based in the comparison of HDC sequences from *Lactobacillus* 30a, *C. perfringens, L. buchneri,* and *Micrococcus*. Alignment studies showed a high degree of relatedness among the *hdc* gene products of gram-positive bacteria. Primer sets JV16HC/JV17HC, CL1/CL2, and CL1/JV17HC amplify by PCR internal fragments of 370, 150 or 500 pb, approximately, of the *hdc* gene, respectively. In addition, the PCR amplification products of *hdc* genes from *L. buchneri* and *O. oeni* by the CL1/JV17HC primer set, were labelled and used as a probe in DNA hybridization studies. All histidine-decarboxylating lactic acid bacteria gave an hybridization signal with the DNA probes.

The primer sets described by Le Jeune et al. (1995) were used by Torres Alves et al. (2002) to isolate and amplify fragments of histidine-decarboxylase genes (*hdc*) from histamine-producer bacteria frequently found in canned fish. The authors concluded that Le Jeune´s primer set was useful in the detection of histamine-producing bacteria in canned fish, where the DNA is typically very fragmented due to the heating process of sterilization. Marcobal et al. (2005) checked the primers sets described by Le Jeune et al. (1995) in order to choose one of them to be used in a multiplex PCR assay. The assay was useful for the detection of histamine-producer bacteria in control collection strains and in a wine LAB collection.

Since, the previously described PCR and colony hybridization methods (Le Jeune et al., 1995) were used on purified DNA of isolated strains, Coton et al. (1998b) in order to

improve the rapidity of these tests to determine the frequency and distribution of histamine-producer bacteria in wines, applied them directly on wine samples. Landete et al. (2005a) studied the ability of 136 wine LAB to produce histamine. Constantini et al. (2006) used CL1/JV17HC primer set to study the potential to produce histamine in 133 LAB strains isolated from wines of different origins. Only one *L. hilgardii* strain was positive. Since none the *O. oeni* strains analysed gave a positive PCR response, they designed a new primer set, PHDC1/PHDC2 based specifically on the *O. oeni hdc* sequence. The new PCR results confirmed the preceding data, none of the *O. oeni* strains analysed was able to produce histamine.

Coton and Coton (2005) designed a new primer set, HDC3/HDC4, to amplify the *hdc* gene from gram-positive bacteria. The method was used to detect histamine-producer bacteria in smoked salmon. Recently, De las Rivas et al. (2006) in order to design a complete PCR assay for the detection of several decarboxylase genes, designed a new specific primer set, HIS1-F/HIS1-R, to amplify *hdc* genes from gram-positive bacteria. The method was successfully applied to several histamine-producer lactic acid bacteria strains.

Conventional PCR has the drawback of the need to analyze the data by traditional end-point analysis. Therefore, Real-time quantitative PCR (qPCR) is a potential alternative. This would allow continuous monitoring of the PCR amplification process and, under appropriate conditions, quantification of the template. In addition, real-time methods are considerably less time-consuming than regular PCR. Recently, Fernández et al. (2006) proposed a real-time qPCR method for the direct detection and quantification of histamine-producing lactic acid bacteria in culture media, milk, and curd. Primer set Hdc1 and Hdc2 amplifies a 174-bp internal fragment of the histidine decarboxylase gene sequence of different Gram-positive bacteria. The results showed that the proposed procedure was a

rapid, specific and highly sensitive technique for detecting potential histamine-producing strains.

Pyridoxal-P dependent HDC are encountered in Gram-negative bacteria and oligonucleotides to amplify its encoding gene have been designed. Since enteric bacteria have been reported to be the dominant histamine-producer bacteria in fish, Kanki et al. (2002) designed a primer set, KPF2/KPR4, based on the *Raoultella planticola* HDC protein, to identify histamine-producer strains. Based on the same *hdc* sequence, they designed a DNA probe to be used in DNA hybridization experiments. Positive PCR results were obtained in all the histamine-producer strains of *R. planticola* and *R. ornithinolytica*. The results of the PCR tests are correlated with those of the hybridization assays.

Takahashi et al. (2003) developed a molecular method that allows the rapid detection of gram-negative histamine-producers by PCR and the simultaneous differentiation by single-strand conformation polymorphism (SSCP) analysis using the amplification product of the *hdc* gene. By using the PCR primers (hdc-f/hdc-r) almost all gram-negative histamine-producer bacteria, except *Citrobacter brakki*, could be rapidly detected. Recently, newly obtained SSCP patterns from histamine-producer strains were added to establish a more accurate SSCP database (Takahashi et al., 2007).

De las Rivas et al. (2005, 2006) in order to design oligonucleotide primers for the detection of the gram-negative bacteria *hdc* gene, they aligned amino acid sequence of known HDC proteins from *R. planticola*, *M. morgani* and *Pseudomonas fluorescens*, among others, and designed primers sets 106 and 107, and HIS2-F and HIS2-R. Recently, the complete *P. phosphoreum hdc* gene was identified by the use of degenerate PCR primers, DegF/DegR based on the multiple-sequence alignment of the *hdc* genes of gram-negative bacteria (Morii et al., 2006).

In addition, some multiplex PCR reactions have been developed, achieving simultaneous amplification of several amino acid decarboxylases encoding genes including those coding for histidine decarboxylases (Coton and Coton, 2005; Marcobal et al., 2005a; De las Rivas et al., 2005; De las Rivas et al., 2006).

Rapid detection of histamine-producer is important for detecting and preventing food spoilage. The detection of histamine-producer bacteria is of great importance not only for its potential health hazard but also from an economic point of view since products exceeding recommended limits can be refused in some commercial transactions.

CONCLUSIONS

Histamine poisoning is a significant public health and safety concern, and additionally, a trade issue. Consumers are becoming more demanding, and litigation following food poisoning incidents is becoming more common. Producers are increasingly held liable for the quality of the products they sell as any countries have set guidelines for maximum permitted levels of histamine in some food products.

Prerequisites for the formation of histamine in foods are the availability of free histidine, and the presence of histidine decarboxylase-positive microorgansims, conditions that allow bacterial growth, and conditions that favour decarboxylase activity. Histidine-decarboxylase bacteria may constitute part of the associated population of the food or may be introduced by contamination before, during or after processing of the food. In the case of fermented foods and beverages, attention should be done to the histamine-forming ability of the starter culture. While histamine in fermented foods such as cheese, wine, beer, etc. is produced by Gram-positive bacteria; histamine in non-fermented foods is indicative of

undesired microbial activity. Although amino acid decarboxylases are not widely distributed among bacteria, species of many genera are capable of decarboxylating one or more amino acids. However, the ability of bacteria to decarboxylate amino acids is highly variable. It depends not only on the species, but also of the strain.

As mentioned before, there are many references on the scientific literature about the potential to produce histamine by bacteria isolated from food products. However, since some of the histidine decarboxylase detection methods could produce false-positive and negative results, this revision was focused only on the histamine-producer bacteria whose histidine decarboxylases or their corresponding encoding genes have been unequivocally reported so far. Since, the availability of complete genome sequences is increasing every day, in a next future we will be able to have a complete list of the bacterial possessing histidine decarboxylase genes in their genomes. Then, the regulation of the histamine production by this bacteria will become an important research issue. If a general regulation mechanism is present in all these bacteria, it will permit modify food production processes in order to minimize the presence of histamine in food products, and therefore increase their safety.

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FIGURE LEGENDS

Figure 1. Release of the carboxyl group of histidine to form histamine by action of enzyme histidine decarboxylase.

Figure 2. Schematic representation of the relationship of the precursor π subunit sequence of prohistidine decarboxylase to that of the α and β subunits of activated proenzyme.

Figure 3. Comparison of HDC protein sequences from Gram-positive bacteria, such as *Lactobacillus* 30a (L30) (accession P00862), *L. hilgardii* (LHI) (accession Q5DLT9), *L. buchneri* (LBU) (accession Q574E5), *Oenococcus oeni* (OOE) (accession O66000), *S. capitis* (SCA), and *C. perfringens* (CPE) (accession P04194). Clustal W program was used to compare predicted sequences. Residues of *Lactobacillus* 30a involved in catalysis and substrate binding are typed in boldface and are underlined in the *Lactobacillus* 30a

sequence. Residues subjected to site-directed mutagenesis in the *Lactobacillus* 30a or in the *C. perfringens* HDC proteins are indicated (●). Vertical dashed line indicated the autocatalytic π chain cleavage site between Ser-81 and Ser-82 in HDC from *Lactobacillus* 30a, yielding the α and β chains.

Figure 4. Diagram representing the pyruvoyl-dependent HDC from *Lactobacillus* 30a (PDB accession 1PYA).

Figure 5. Genetic organization of the DNA region containing the histidine decarboxylase gene (*hdcA*) in Gram-positive bacteria. 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 *S. capitis* (accession no. AM283479) 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.

Figure 6. Scheme of proton motive force generation by decarboxylation and electrogenic antiport. The combination of HdcP (histidine/histamine antiporter) and the HdcaA (histidine decarboxylase) formed a typical bacterial decarboxylation pathway that may generate metabolic energy or to be involved in the acid stress response. A net charge is transported to the outside, corresponding to the proton consumed by the cell.

Figure 7. Comparison of HDC protein sequences from Gram-negative bacteria, such as *M. morganii* (MMO) (accession A25013), *P. phosphoreum* (PPH) (accession AY223843), *E. aerogenes* (EAE) (accession M62745), *P. fluorescens* (PFL) (accession P95477), and plasmid pJM1 from *L. anguillarum* (LAN) (accession Q56581). Residues of *M. morganii* involved in catalysis and substrate binding are typed in boldface and are underlined in the *M. morganii* sequence. Residues subjected to site-directed mutagenesis in the *M. morganii* HDC protein are indicated (●).

Figure 8. Genetic organization of the DNA region containing the histidine decarboxylase gene in Gram-negative bacteria. The *hdc* region corresponding to *Morganella morganii* AM-15 (accession J02577), *Raoultella planticola* strain T2 (accession M62746), *Enterobacter aerogenes* ATCC 13124 (accession M62745), *Photobacterium phosphoreum* RHE01 (accession AY223843), plasmid pJM1 from *Listonella anguillarum* strain 775 (accession AY312585), and *Pseudomonas fluorescens* WCS374 (accession Y09356) is represented. Arrows indicate ORFs. Genes coding for HDC are represented in black.

Table 1

Table 1. Histamine-producer bacteria

Strains	Accession	Reference
Gram-positive:		
Clostridium perfringens ATCC 13124	DCCLHP	Recsei et al. (1983)
Lactobacillus 30a	DCLBHP	Rodwell (1953)
Lactobacillus buchneri B301	CAG44458	Martín et al. (2005)
Lactobacillus hilgardii 0006*	AAV65956.1	Lucas et al. (2005)
Micrococcus sp.	PN0143	Prozorovski and Jornvall (1983)
Oenococcoccus oeni RM83	AAC38298	Coton et al. (1998)
Staphylococcus capitis IFIJ12	AM283479	De las Rivas et al. (unpublished)
Tetragenococcus muriaticus	BAD51812	Konagaya et al. (2002)
Gram-negative:		
Enterobacter aerogenes ATCC 43176	A40004	Guirard and Snell (1987)
Erwinia sp. MB31	AB083208	Takahashi et al. (2003)
*Listonella anguillarum******	AY312585	Tolmasky et al. (1995)
Morganella morganii	A25013	Hayashi et al. (1986)
Photobacterium damselae JCM8968	BAC45248.1	Takahashi et al. (2003)
Photobacterium phosphoreum RHE01	AAO65983.1	Morii and Kasama (1995)
Proteus vulgaris AU3	BAC20384.1	Takahashi et al. (2003)
Pseudomonas fluorescens WCS374	P95477	Mercado-Blanco et al. (2001)
Raoultella ornytolytica	Q810Z4	Kanki et al. (2002)
Raoultella planticola 4131	BAC20386	Kanki et al. (2002)

*Plasmid encoded histidine decarboxylase

Figure 1

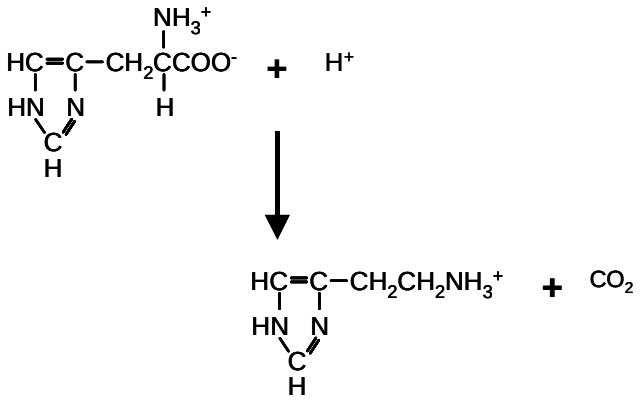
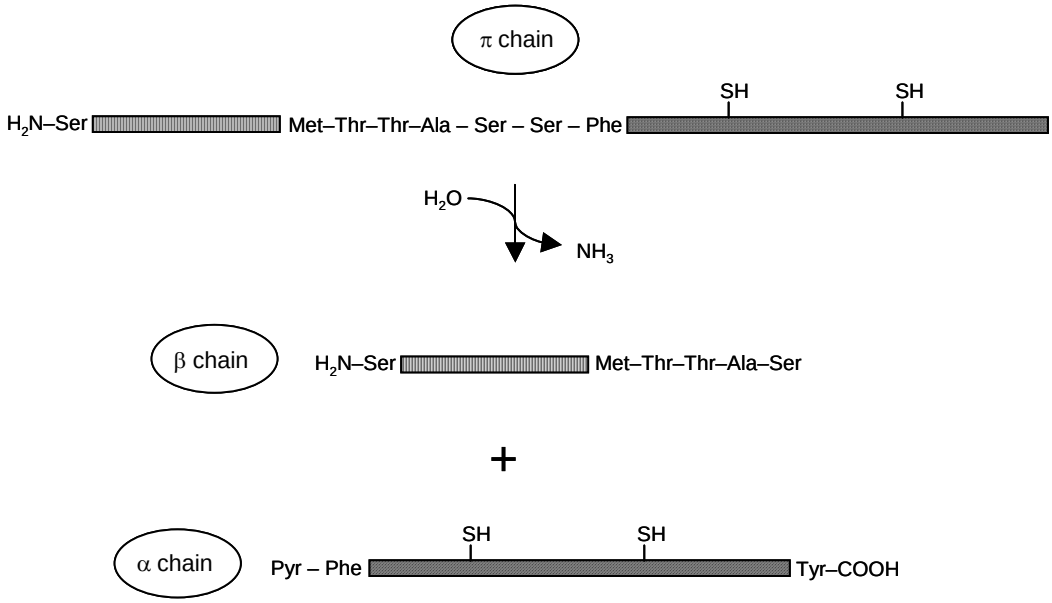


Figure 2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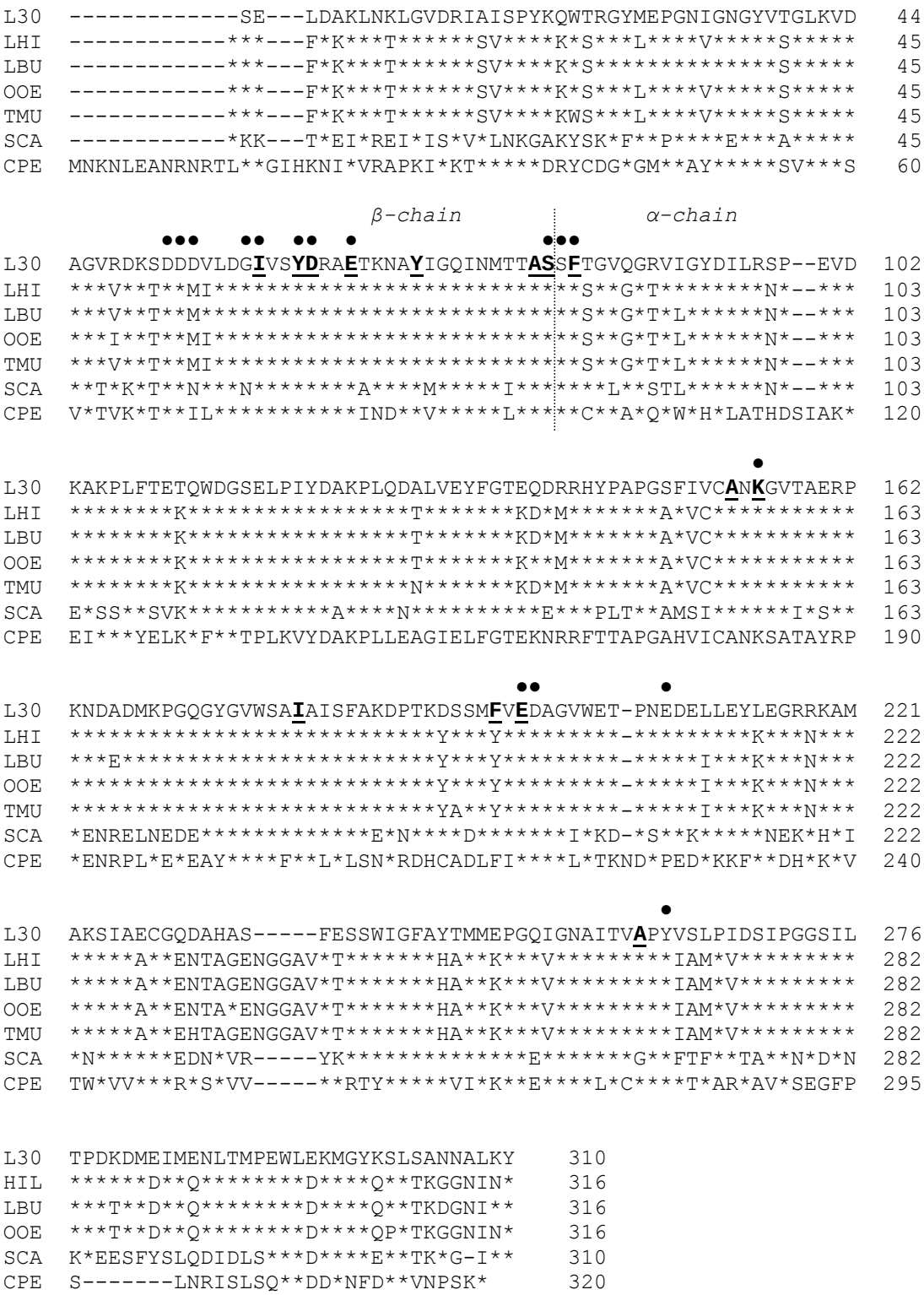
SH
H2N–Ser
Met–Thr–Thr–Ala – Ser – Ser – Phe
H2O
NH3
β chain
H2N–Ser
Met–Thr–Thr–Ala–Ser
+
α chain
SH
SH
Pyr – Phe
Tyr–COOH

Figure 3


β-chain
α-chain
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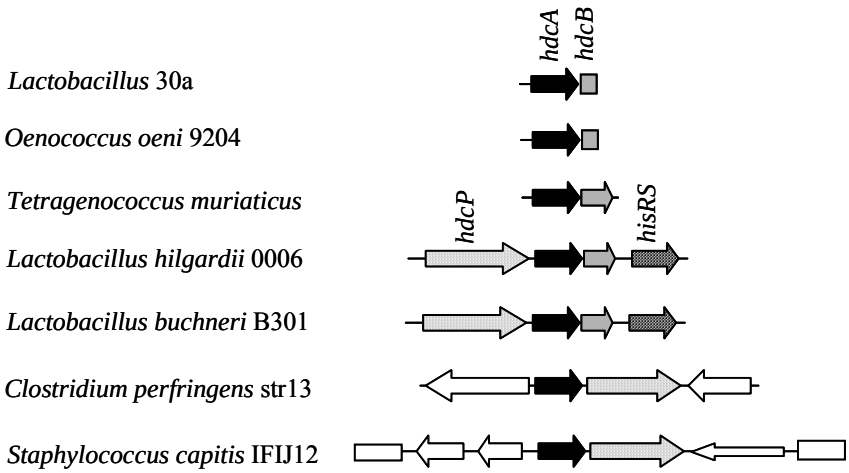
LBU ----------******F*K***T******SV****K*S**********S***** 45
OOE ----------******F*K***T******SV****K*S***L****V*****S***** 45
TMU ----------******F*K***T******SV****KWS***L****V*****S***** 45
SCA ----------*KK---T*EI*REI*IS*V*LNKGAKYSK*F**P****E***A***** 45
CPE MNKNLEANRNRTL**GIHKNI*VRAPKI*KT*****DRYCDG*GM**AY*****SV***S 60
L30 AGVRDKSDDDVLDGIVSYDRAETKNAYIGQINMTTASSFTGVQGRVIGYDILRSP--EVD 102
LHI ***V**T**MI************************S**G*T********N*--*** 103
LBU ***V**T**M************************S**G*T*L******N*--*** 103
OOE ***I**T**MI************************S**G*T*L******N*--*** 103
TMU ***V**T**MI************************S**G*T*L******N*--*** 103
SCA **T*K*T**N***N********A****M*****I*******L**STL******N*--*** 103
CPE V*TVK*T**IL***********IND**V*****L******C**A*Q*W*H*LATHDSIAK* 120
L30 KAKPLFTETQWDGSELPIYDAKPLQDALVEYFGTEQDRRHYPAPGSFIVCANKGVTAERP 162
LHI ********K*****************T*******KD*M*******A*VC********** 163
LBU ********K*****************T*******KD*M*******A*VC********** 163
OOE ********K*****************T*******K**M*******A*VC********** 163
TMU ********K*****************N*******KD*M*******A*VC********** 163
SCA E*SS**SVK**********A****N*********E***PLT**AMSI******I*S** 163
CPE EI***YELK*F**TPLKVYDAKPLLEAGIELFGTEKNRRFTTAPGAHVICANKSATAYRP 190
L30 KNDADMKPGQGYGVWSAIAISFAKDPTKDSSMFVEDAGVWET-PNEDELLEYLEGRRKAM 221
LHI ****************************Y***Y*********-*********K***N*** 222
LBU ***E************************Y***Y*********-*****I***K***N*** 222
OOE ****************************Y***Y*********-*****I***K***N*** 222
TMU ****************************YA**Y*********-*****I***K***N*** 222
SCA *ENRELNEDE*************E*N****D*******I*KD-*S**K*****NEK*H*I 222
CPE *ENRPL*E*EAY****F**L*LSN*RDHCADLFI****L*TKND*PED*KKF**DH*K*V 240
L30 AKSIAECGQDAHAS-----FESSWIGFAYTMMEPGQIGNAITVAPYVSLPIDSIPGGSIL 276
LHI *****A**ENTAGENGGAV*T*******HA**K***V*********IAM*V********* 282
LBU *****A**ENTAGENGGAV*T*******HA**K***V*********IAM*V********* 282
OOE *****A**ENTA*ENGGAV*T*******HA**K***V*********IAM*V********* 282
TMU *****A**EHTAGENGGAV*T*******HA**K***V*********IAM*V********* 282
SCA *N******EDN*VR-----YK**************E*******G**FTF**TA**N*D*N 282
CPE TW*VV***R*S*VV-----**RTY*****VI*K**E****L*C****T*AR*AV*SEGFP 295
L30 TPDKDMEIMENLTMPEWLEKMGYKSLSANNALKY 310
HIL ******D**Q********D****Q**TKGGNIN* 316
LBU ***T**D**Q********D****Q**TKDGNI** 316
OOE ***T**D**Q********D****QP*TKGGNIN* 316
SCA K*EESFYSLQDIDLS***D****E**TK*G-I** 310
CPE S-------LNRISLSQ**DD*NFD**VNPSK* 320

Figure 4

Figure 5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hdcB
Lactobacillus 30a
Oenococcus oeni 9204
Tetragenococcus muriaticus
hdcP
hisRS
Lactobacillus hilgardii 0006
Lactobacillus buchneri B301
Clostridium perfringens str13
Staphylococcus capitis IFIJ12

Figure 6

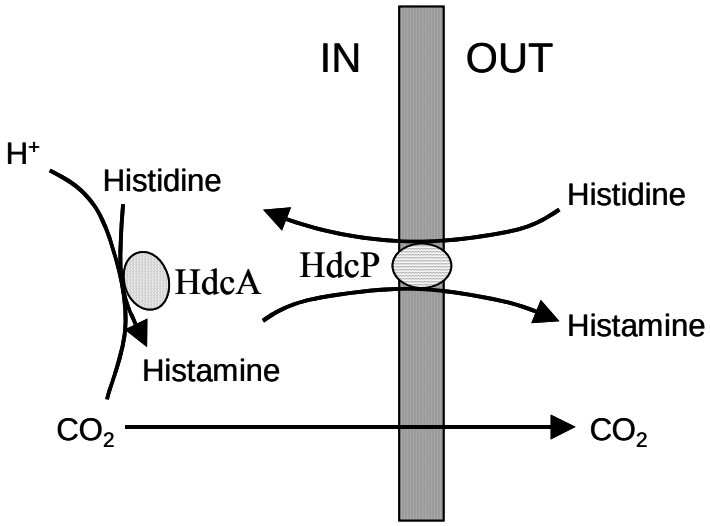
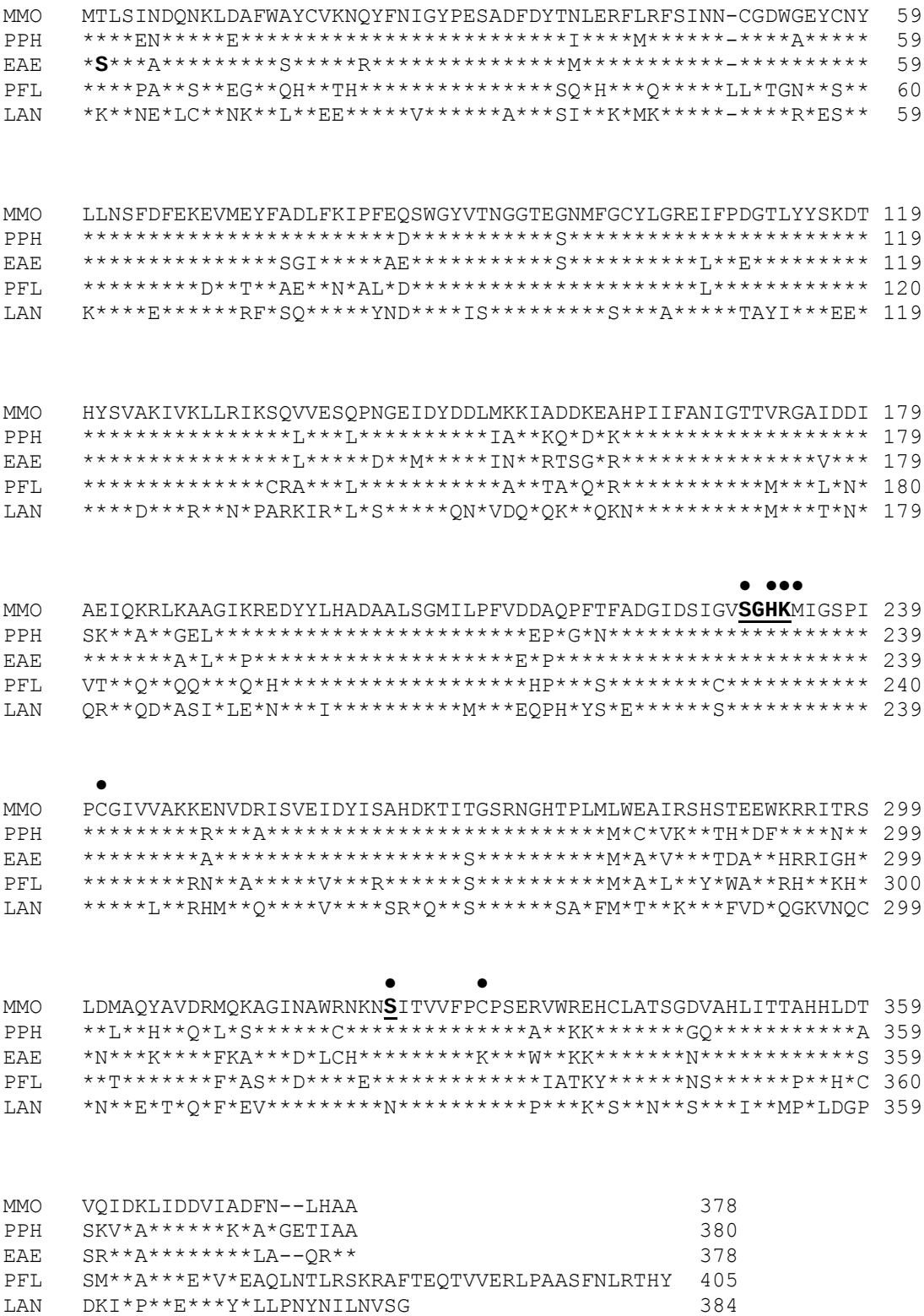

IN
OUT
H+
Histidine
HdcA
HdcP
Histidine
Histamine
Histamine
CO2
CO2

Figure 7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EAE *S***A*********S*****R**********M**********-********** 59
PFL ****PA**S**EG**QH**TH**********SQ*H***Q*****LL*TGN**S** 60
LAN *K**NE*LC**NK**L**EE*****V******A***SI**K*MK*****-****R*ES** 59
MMO LLNSFDFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLYYSKDT 119
PPH ********** D********** S********** 119
EAE **********SGI*****AE**********S**********L**E********* 119
PFL *********D**T**AE**N*AL*D**********L********** 120
LAN K****E******RF*SQ*****YND****IS*********S***A*****TAYI***EE* 119
MMO HYSVAKIVKLLRIKSQVVESQPNGEIDYDDLMKKIADDKEAHPIIFANIGTTVRGAIDDI 179
PPH **********L***L**********IA**KQ*D*K********** 179
EAE **********L*****D**M*****IN**RTSG*R**********V*** 179
PFL **********CRA***L**********A**TA*Q*R**********M***L*N* 180
LAN ****D***R**N*PARKIR*L*S*****QN*VDQ*QK**QKN**********M***T*N* 179
MMO AEIQKRLKAAGIKREDYYLHADAALSGMILPFVDDAQPFTFADGIDSIGVSGHKMIGSPI 239
PPH SK**A**GEL********** EP*G*N********** 239
EAE *******A*L**P********** E*P********** 239
PFL VT**Q**QQ***Q*H********** HP***S********C********** 240
LAN QR**QD*ASI*LE*N***I**********M***EQPH*YS*E******S********** 239
MMO PCGIVVAKKENVDRISVEIDYISAHDKTITGSRNGHTPLMLWEAIRSHSTEEWKRRITRS 299
PPH *********R***A********** M*C*VK**TH*DF****N** 299
EAE *********A********** S**********M*A*V***TDA**HRRIGH* 299
PFL ********RN**A*****V***R****** S**********M*A*L**Y*WA**RH**KH* 300
LAN *****L**RHM**Q****V****SR*Q**S******SA*FM*T**K***FVD*QGKVNQC 299
MMO LDMAQYAVDRMQKAGINAWRNKNSITVVFPCPSERVWREHCLATSGDVAHLITTAHHLDT 359
PPH **L**H**Q*L*S******C**********A**KK*******GQ**********A 359
EAE *N***K****FKA***D*LCH*********K***W**KK*******N**********S 359
PFL **T*******F*AS**D****E**********IATKY******NS******P**H*C 360
LAN *N**E*T*Q*F*EV*********N**********P***K*S**N**S***I**MP*LDGP 359
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PPH SKV*A******K*A*GETIAA 380
EAE SR**A********LA--QR** 378
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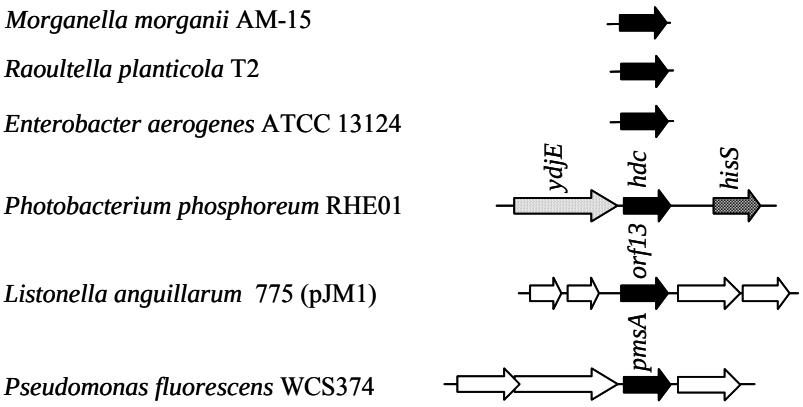
LAN DKI*P**E***Y*LLPNYNILNVSG 384

Figure 8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ydjE
hdc
hisS
orf13
pmsA