**Isolation and typification of histamine-producing *Lactobacillus vaginalis* strains
from cheese**

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Abstract

In food, the biogenic amine (BA) histamine is mainly produced by histidine decarboxylation catalyzed by microbial histidine decarboxylase. The consumption of foods containing high concentrations of histamine can trigger adverse neurological, gastrointestinal and respiratory reactions. Indeed, histamine is one of the most toxic of all BAs, and is often detected in high concentration in cheese. However, little is known about the microorganisms responsible for its accumulation in this food. In the present work, 25 histamine-producing *Lactobacillus vaginalis* strains were isolated from a blue-veined cheese (the first time that histamine-producing strains of this species have been isolated from any food). The restriction profiles of their genomes were analysed by PFGE, and seven lineages identified. The presence of the histidine decarboxylase gene (*hdcA*) was confirmed by PCR. The nucleotide sequence and genetic organization of the histamine biosynthesis gene cluster (HDC) and its flanking regions are described for a representative strain (*L. vaginalis* IPLA11050).

Keywords

Biogenic amines, histamine, cheese, *Lactobacillus vaginalis*, PFGE, *hdcA*

1. Introduction

Histamine is a biogenic amine (BA) produced by certain microorganisms via the enzymatic decarboxylation of histidine. If large amounts of histamine are ingested with food, the detoxifying enzymes of the intestinal epithelium may be unable to cope; some may then enter the systemic circulation and trigger neurological, gastrointestinal and respiratory symptoms (Ladero, 2010; Maintz and Novak, 2007; Stratta and Badino, 2012). Histamine is, in fact, one of the most toxic and most commonly found BAs in foods. The adverse reactions it induces are together described as histamine intolerance or scombroid poisoning (a reference to the frequent presence of histamine in fish of the family Scombroidae) (Stratta and Badino, 2012).

Foods that may contain high concentrations of histamine include fish, fish products, fermented meat, vegetables, dairy products and alcoholic beverages (Ladero et al., 2011; Landete et al., 2005). Despite its toxicological effects, histamine levels are only regulated in fresh fish (limited to 100 mg kg⁻¹ by European Union Commission Directives 2073/2005 and 1019/2013, and to 50 mg kg⁻¹ by the Food and Drug Administration USA [FDA, 2001]) and cured fish products (limited to 200 mg kg⁻¹ by the European Commission). Some upper limits for histamine in other foods and beverages have been suggested, e.g., 100 mg kg⁻¹ in food in general and 2 mg L⁻¹ in alcoholic beverages (Halasz et al., 1994). However, no regulations are in force, even for cheese, in which concentrations of histamine may exceed 1000 mg kg⁻¹ (Fernandez et al., 2007). Although a number of microorganisms in cheese, including moulds, yeasts and Gram negative bacteria, are able to produce histamine (Linares et al., 2012), lactic acid bacteria (LAB) are thought to be mainly responsible for its accumulation

(Fernandez et al., 2007). LAB may form part of the milk microbiota, can be introduced by contamination before, during or after processing, or may even be part of the starter culture. However, few histamine-producing LAB -*Lactobacillus buchneri*, *Lactobacillus curvatus* and *Lactobacillus helveticus*- have actually been isolated from cheese itself (Burdychova and Komprda, 2007; Ladero et al., 2008; Linares et al., 2011). Research is therefore needed to identify which bacterial species are responsible for the accumulation of this BA, and to determine the factors involved in its biosynthesis. Only then can appropriate preventive measures be put in place.

Much effort has already been invested in characterizing the bacterial genes involved in the biosynthesis of histamine, and in their regulation. The sequencing of these genes would allow the development and optimization of new and efficient histamine-producer detection methods (Linares et al., 2014). In Gram positive bacteria, the genes responsible for the biosynthesis of histamine are usually grouped in a cluster: the histidine decarboxylase cluster (HDC). The HDC cluster of histamine-producing lactobacilli generally involves three genes typically orientated in the same direction: *hdcP*, *hdcA*, and *hdcB* (Lucas et al., 2005; Martin et al., 2005). The first gene of the cluster, *hdcP*, encodes the histidine/histamine antiporter (HdcP). This is followed by *hdcA*, which codes for histidine decarboxylase (HdcA). The *hdcA* gene is then followed by *hdcB*, which encodes a protein that catalyses the maturation of HdcA (Trip et al., 2011). In some *Lactobacillus* species, a fourth gene (*hisS*), which encodes a protein similar to histidyl-tRNA synthetases, is located downstream of *hdcB* (Martin et al., 2005).

In the present work, histamine-producing *Lactobacillus vaginalis* strains were isolated from a blue-veined cheese. To our knowledge, this is the first work in which histamine-

producing *L. vaginalis* strains have been isolated from food. The isolates were genetically characterised by pulsed-field gel electrophoresis (PFGE) and checked for the presence of the HDC cluster. Further, the nucleotide sequence and the genetic organization of the HDC cluster and flanking regions of a representative *L. vaginalis* strain were determined.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus parabuchneri DSM 5987 was used as a histamine-producing positive control. All the lactobacilli used or isolated in all experiments were grown in MRS (Oxoid, Basingstoke, England) at 37°C without aeration. Where indicated, the medium was supplemented with 10 mM histidine (Sigma-Aldrich, Madrid, Spain).

2.2. Isolation of histamine-producing bacteria from cheese samples

One gram of Cabrales cheese (a Spanish, raw-milk, blue-veined cheese) was homogenised for 2 min in 9 ml of an enrichment medium using a Lab-Blender 400 Stomacher (Seward Ltd., London, UK). This enrichment medium was MRS broth supplemented with 50 µg/mL cycloheximide (Sigma-Aldrich) to inhibit yeast and mould growth, 5 mM histidine, and 0.005% (w/v) pyridoxal phosphate to promote the growth of histamine-producing bacteria. Enrichment cultures were grown for 24 h at 37°C under anaerobic conditions (10% H₂, 10% CO₂ and 80% N₂) in a Mac 500

anaerobic workstation (Don Whitley Scientific, Shipley, UK). After two rounds of enrichment, serial dilutions of the cultures were spread on MRS agar plates supplemented with 5mM histidine and 0.005% (w/v) pyridoxal phosphate. These plates were then incubated for 48 h at 37°C under anaerobic conditions. Isolated colonies were inoculated into microtitre plates containing liquid decarboxylase medium (MDA) (Bover-Cid and Holzapfel, 1999) supplemented with 50 mM Histidine, and incubated under anaerobic conditions for 72 h at 37°C. The isolates that caused alkalization of the medium (indicated by its change to a purple colour) were selected for further analysis.

2.3. Quantification of histamine by ultra-high performance liquid chromatography

To test the capacity of each isolate to produce histamine, they were grown in MRS broth supplemented with 50 mM histidine for 24 h at 37°C under anaerobic conditions. The cells were then removed by centrifugation at 8000 g for 10 min, and 100 µL of the supernatant were derivatised and the histamine content quantified by ultra-high performance liquid chromatography (UHPLC) using an H-Class AcquityUPLC™ system (Waters, Milford, MA, USA) as previously described (Redruello et al., 2013). Separation was performed at 35°C in a Waters AcquityUPLC™ BEHC18 1.7 µm column (2.1 x 100 mm). Data were acquired and analysed using Empower 2 software (Waters). To determine the amount of histamine present in cheese, 1 g samples were processed to extract the BA as previously described (Herrero-Fresno et al., 2012). One hundred µL were then derivatised and analysed as described above.

2.4. DNA manipulation procedures

Total DNA was isolated from 2 ml of bacterial cultures grown in MRS supplemented with 1% (w/v) glycine (USB Corporation, Cleveland, OH, USA) using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions.

PCR reactions were performed using an iCycler™ thermocycler (Bio-Rad, Spain) and DreamTaq™ DNA Polymerase (Fermentas, Vilnius, Lithuania). The PCR products were purified using the ATP™ Gel/PCR Extraction Kit (ATP™ Biotech Inc., Taipei City, Taiwan) and sequenced by Macrogen Inc. (Seoul, Republic of Korea).

2.5. Histamine-producing isolates: identification at the species level by 16S rRNA sequencing

Histamine-producing isolates were identified at the species level by amplification of part of the *16S rRNA* gene using total DNA as a template and the universal primers 27F and 1942R (Lane, 1991) (Table 1). The PCR program involved 5 min at 95°C followed by 35 cycles of amplification (30 s at 95°C, 30 s at 50°C, and 90 s at 68°C) and a final extension step of 10 min at 68°C. The resulting sequences were aligned and compared with the eubacterial *16S rRNA* gene sequences available in the GenBank and EMBL databases using BLAST software (Altschul et al., 1997). Sequences with a percentage similarity of 98% or higher were considered the same species (Stackebrandt and Goebel, 1994).

2.6. PCR amplification of the hdcA gene

The *hdcA* gene was sought in the histamine-producing isolates by PCR, using the primers *hdc3* and *hdc4* (Coton and Coton, 2005) (Table 1). One nanogram of total DNA

was used as a template for PCR amplification under previously described conditions (Coton and Coton, 2005). *L. parabuchneri* DSM 5987 was used as a histamine-producing positive control.

2.7. Macrorestriction of genomic DNA and analysis by pulsed-field gel electrophoresis

Twenty five histamine-producing *L. vaginalis* isolates were analysed by PFGE as previously described (Herrero-Fresno et al., 2012), with the exceptions that the agarose blocks were treated with *Sfi*I, *Not*I and *Apa*I endonucleases (Takara, Shuzo Co., Ltd., Japan), and the pulse ramps used were 0.1-2 s for 12 h and 2-20 s for 6 h.

2.8. Southern analysis

To map the *hdc* gene, the *Apa*I and *Not*I PFGE gels were transferred to Amersham HybondTM-N nylon membranes (GE Healthcare, Little Chalfont, UK) (Sambrook et al., 1989). The probe was obtained by PCR (as described in paragraph 2.6) from a *L. vaginalis* isolate. DNA hybridizations were performed using the non-radioactive DNA Labelling and Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions.

2.9. Sequencing and analysis of the histidine decarboxylase cluster and flanking regions of *Lactobacillus vaginalis*

The sequences of the complete HDC cluster and its flanking regions were obtained by overlapping PCR and subsequent sequencing of the PCR products. The primer pairs

(Table 1) were designed based on the genome sequence of *L. vaginalis* ATTC 49540 (GenBank Accession No ACGV01000000). The PCR program consisted of 5 min at 95°C followed by 35 cycles of amplification (30 s at 95°C, 30 s at 57°C, and 90 s at 72°C) and a final extension step of 10 min at 72°C. The PCR product sequences were assembled using Vector NTI Advance v.9.1 software (Invitrogen, Carlsbad, CA, USA). The BLAST and BLASTP programs were used to determine the similarities of the deduced amino acid sequences to those present in the databases. Analysis of the transmembrane elements was performed using the TMHMM tool (<http://www.cbs.dtu.dk/services/TMHMM/>; Danish Technical University, Lyngby, Denmark). Conserved regions within proteins in the Conserved Domain Database (CDD) were identified using CD-Search software (Marchler-Bauer et al., 2003) developed by the National Center for Biotechnology Information (NCBI, Rockville, MD, USA). Multiple alignment was performed using ClustalW software (Larkin et al., 2007), and the phylogenetic tree constructed using MEGA v.4 software, employing the Neighbour-Joining clustering method.

3. Results

3.1. Isolation of histamine-producing strains

A sample of cheese that contained 566.41 mg/kg histamine was used as the starting material for screening for histamine-producing bacteria. As described above, the sample was homogenised and inoculated into enrichment media. After two rounds of enrichment, colonies were isolated on solid MRS supplemented with histidine and

pyridoxal phosphate. A total of 960 colonies were then picked off and subjected to functional selection in microtitre plates containing MDA decarboxylase medium with 50 mM histidine. On the basis of colour change in the medium, 221 isolates were classified as potential histamine-producing bacteria. UPLC analysis of cell-free supernatants confirmed the production of histamine by 68 of these 221 isolates. All isolates were able to produce more than 11 mM histamine after 24 hours of incubation, except one, which produced 3.5 mM histamine.

3.2. Histamine-producing isolates: identification at the species level by 16S rRNA sequencing

Forty two histamine-producing isolates were identified as *L. reuteri* (100% identical), 1 as *L. parabuchneri* (100% identical), and (surprisingly) 25 as *L. vaginalis* (100% identical). These *L. vaginalis* isolates were named as strains IPLA11140, IPLA11141, IPLA11142, IPLA11143, IPLA11144, IPLA11145, IPLA11147, IPLA11050, IPLA11051, IPLA11052, IPLA11053, IPLA11054, IPLA11055, IPLA11056, IPLA11057, IPLA11058, IPLA11060, IPLA11062, IPLA11064, IPLA11065, IPLA11067, IPLA11068, IPLA11069, IPLA11070 and IPLA11075.

3.3. Verification of the presence of hdcA in the genome of histamine-producing L. vaginalis isolates

The presence of *hdcA* (the gene encoding histidine decarboxylase) was sought by PCR in the genomes of the histamine-producing *L. vaginalis* isolates, using total DNA from each isolate as a template. All isolates yielded a PCR product of the expected size (440 bp) (data not shown). The obtained amplicons were sequenced and compared with

sequences for microbial genomes in the mentioned databases. The sequences were identical to a gene annotated as *hdcA* in the partially sequenced genome of *L. vaginalis* ATCC 49540 (GenBank Accession No ACGV01000000), a species that to our knowledge has never been described as a histamine producer.

3.3. Macrorestriction analysis of genomic DNA of *L. vaginalis* and mapping of *hdcA*

To further characterise the 25 histamine-producing *L. vaginalis* isolates, their genomic DNA were subjected to PFGE analysis. The genomes were first analysed by *SfiI* macrorestriction. All were resistant to digestion by this enzyme, preventing the proper comparison of isolates (data not shown). *ApaI*-macrorestriction PFGE analysis (Fig. 1A) differentiated the isolates into three *ApaI* profiles (A1-A3). All except two of the isolates tested were assignable to the A2 profile (92%). Macrorestriction analysis of *L. vaginalis* DNA with *NotI* yielded 8-10 fragments ranging in size from 20 to 400 kb (Fig. 1C). Six different *NotI* profiles (N1-N6) were noted, with most isolates (80%) belonging to N2. The other five profiles (N1 and N3-N6) were represented by only one isolate each. Taken together, these results allowed seven lineages to be distinguished - L1: A2N2, L2: A2N3, L3: A1N4, L4: A2N1, L5: A2N6, L6: A3N2 and L7: A2N5. Lineage L1 was the most common (76%); the other lineages (L2-L7) were represented only by one isolate each (Table 2).

To locate the *hdcA* gene in the *ApaI* and *NotI* genomic profiles of *L. vaginalis*, Southern hybridization was performed using a specific probe. The results are shown in Fig. 1B and 1D. The *hdc* probe mapped to the same *ApaI*-fragment of c.20 kb in all strains, and to *NotI* fragments of two sizes, c.230 kb (N1, N2, N3, N5, N6) and c.400 kb (N4).

3.5. Sequencing and analysis of the HDC cluster of *L. vaginalis* IPLA 11050

Since the nucleotide sequence of the *hdc3/hdc4* PCR product was 100% identical in all 25 *L. vaginalis* isolates, a representative strain (*L. vaginalis* IPLA11050), belonging to the most common lineage (L1) was selected as a model for further genetic characterization of the HDC cluster. As well as an annotation for *hdcA*, the genome sequence of *L. vaginalis* ATCC 49540 also has annotations for the putative *hdcP*, *hdcB* and *hisS* genes (GenBank Accession No ACGV01000000). This sequence allowed the design of a set of primers used in overlapping PCR to sequence the complete HDC cluster in *L. vaginalis* IPLA1105. The assembly of the overlapping PCR reactions rendered a 6,268 bp fragment (GenBank Accession No LN828720) carrying four open reading frames (ORFs) in the same orientation (Fig. 2). The first gene encoded a predicted protein of 485 amino acid residues with a molecular mass of 52.7 kDa and a pI of 7.16. This protein showed 83% similarity to the basic amino acid/polyamine antiporter (APA) family of *L. reuteri* (Gene Bank Accession No WP_003669317), 73% similarity to the first-described histidine/histamine antiporter, i.e., that of *L. buchneri* (Gene Bank Accession No CAG44460) (Martin et al., 2005; Molenaar et al., 1993), and 73% similarity to the histidine/histamine antiporter of *L. hilgardii* (Gene Bank Accession No WP_032491231) (Lucas et al., 2005). Analysis of the deduced amino acid sequence, performed using the TMHMM tool, predicted the presence of 13 transmembrane elements (data not shown). Therefore, the first gene of the cluster must encode the putative histidine/histamine antiporter, and was named *hdcP*.

Downstream of *hdcP* an ORF was detected that encodes a protein of 311 amino acid residues with a molecular mass of 34.2 kDa and a pI of 4.65. Analysis of its amino acid

sequence revealed it to belong to the group of bacterial histidine decarboxylases (whose members use a covalently bound pyruvoyl residue as a prosthetic group (Riley and Snell, 1968)). The protein showed 95% amino acid similarity to the histidine decarboxylase of *L. reuteri* (Gene Bank Accession No WP_003669317), as well as 90% similarity to that of *L. saerimneri* 30A (Gene Bank Accession No WP_009552067), i.e., the first pyruvoyl type histidine decarboxylase described (Riley and Snell, 1968). Consequently this gene was referred to as *hdcA*.

The following ORF encoded a protein of 174 amino acid residues with a molecular mass of 19.9 kDa and a pI of 5.96. The comparison of its deduced amino acid sequence with those present in databases revealed 74% similarity to the HdcB protein of *L. reuteri* (Gene Bank Accession No WP_003669316). An orthologous protein has been shown to act as an activator of the HdcA proenzyme in *S. thermophilus* (Trip et al., 2011). The protein encoded by this ORF in *L. vaginalis* showed 32% similarity to that of *S. thermophilus* (Gene Bank Accession No CBK46768). It was designated as *hdcB*.

The ORF downstream of *hdcB* encoded a protein of 433 amino acid residues, with a predicted molecular mass of 49.1 kDa and a pI of 5.01. It showed a Class II Histidyl-tRNA synthetase (HisRS)-like catalytic core domain (as determined from its structure and the presence of three characteristic sequence motifs). The comparison of the deduced amino acid sequence with those present in databases revealed it to be 94% similar to the histidyl-tRNA synthetase of *L. reuteri* (accession number WP_003669315). It also showed 73% similarity to the histidyl-tRNA synthetase (HisRS) of the HDC cluster in *L. buchneri* (Gene Bank Accession No CBK46768). This ORF was called *hisS*.

The HDC cluster of *L. vaginalis* IPLA11050 showed the typical genetic organization of histamine clusters in other *Lactobacillus* species (Fig. 2). However, the nucleotide

sequence of the *hdcA* gene (Fig. 3) does not appear to be as conserved as the organization of the cluster. This might have important implications for the design of molecular tools used in the detection of histamine producers in food.

3.6. Determination of the nucleotide sequence of the flanking regions of the HDC cluster of *L. vaginalis* IPLA 11050

The ability of some LAB to produce certain BAs has been described as a strain-specific trait acquired through horizontal gene transfer (Coton and Coton, 2009; Marcobal et al., 2006), while in others it is considered a species-level feature (Ladero et al., 2012). To determine which might be the case in *L. vaginalis*, the flanking regions of the HDC cluster were sequenced by overlapping PCR. The assembly of the overlapping PCR reactions, together with the HDC cluster, rendered a 16,075 bp fragment (GenBank Accession No LN828720).

The region upstream of the HDC cluster showed the sequence of a histidyl-tRNA and two complete ORFs; the deduced amino acid sequences were a C4 dicarboxylate anaerobic carrier and a histidine kinase (Fig. 4). Downstream of the HDC cluster, the deduced amino acid sequences of five complete ORFs included a major facilitator superfamily (MFS) transporter, a LysR family regulator, a 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase, methylenetetrahydrofolate reductase, and the Na⁺/H⁺ antiporter NhaC (Fig. 4).

Two primer pairs were designed to PCR-amplify the flanking regions of the HDC cluster in the 25 *L. vaginalis* isolates. Primer pair 1 (Table 1) was used to amplify the region located between the C4-dicarboxylate anaerobic carrier and the *hdcP* genes (Fig.

4). All the isolates showed the same nucleotide sequence in the flanking region upstream of the HDC cluster. Primer pair 13 (Table 1) was used to amplify the region between *hisS* and the MFS gene (Fig. 4). All the *L. vaginalis* isolates showed the same nucleotide sequence in the flanking region downstream of the HDC cluster. Two different direct repeats of 15 (a': GGCGAACACGTTTTT) and 18 (b': CTTGAGGGAGCTTAGGCT) bp were found flanking the HDC cluster and the genes encoding the histidine-tRNA and the putative MFS transporter (Fig. 4). MFS is the largest superfamily of secondary active transporters, which target a wide spectrum of substrates including ions, carbohydrates, lipids, amino acids and peptides, nucleosides and other molecules (Yan, 2013). Although the presence of this gene was analysed, it was not found next to the cluster HDC of other histamine-producing species.

4. Discussion

BAs mainly accumulate in food via microbial decarboxylation of the corresponding substrate amino acids. Histamine has been described as the most common cause of food-borne intoxications (Bodmer et al., 1999). It is often present in fermented foods, especially cheese, in which it may far exceed the recommended (though not legislatively enforced) value of 100 mg kg⁻¹ (Fernandez et al., 2007). Despite the importance of this BA, only a few histamine-producing bacteria have ever been isolated from cheese. The aim of the present study was to isolate, identify and type the histamine-producing LAB in Cabrales cheese, which frequently has high concentrations of histamine (Roig-Sagues et al., 2002).

Of the 68 isolates with the ability to synthesise histamine, one was identified as *L. parabuchneri* and 42 as *L. reuteri*, which have previously been described as histamine producers (Fröhlich-Wyder et al., 2013; Thomas et al., 2012). Surprisingly, the

remaining 25 histamine-producing isolates were all identified as *L. vaginalis*, a species that to our knowledge has never been reported as a histamine producer, nor indeed has ever been isolated from cheese. In fact, it has only been found in raw milk (Baruzzi et al., 2011), traditional Chinese dairy products and fermented vegetables (Nawaz et al., 2011), sourdough (Mihhalevski et al., 2011) and buckwheat (Moroni et al., 2011). *L. vaginalis* shows phenotypic similarities to *Lactobacillus fermentum* and genetically is most closely related to *Lactobacillus oris* and *L. reuteri* (Embley et al., 1989).

The 25 *L. vaginalis* isolates were classified into seven lineages based on the results of PFGE analysis with the restriction enzymes *ApaI* and *NotI*. The presence of seven lineages shows that the isolates do not belong to a clonal group, and demonstrates that the presence of *L. vaginalis* was not due to contamination.

Since *L. vaginalis* has never been described as a histamine producer, the genes involved in the production of this BA were analysed. Fortunately, the putative HDC cluster genes are annotated in the draft genome sequence of *L. vaginalis* ATTC 49540 (GenBank Accession No ACGV01000000). This allowed a simple strategy to be designed for sequencing the homologous genes of a representative *L. vaginalis* strain. Subsequent analysis and sequence comparison established that these genes were indeed responsible for the synthesis of histamine.

Not all of the sequences of the HDC cluster of all *Lactobacillus* members deposited in databases are complete, but the organization of the examined *L. vaginalis* genes was the same as that described for *L. reuteri* (Thomas et al., 2012), *L. buchneri* (Martin et al., 2005) and *L. hilgardii* (Lucas et al., 2005). The same organization also appears in the HDC cluster of LAB genera such as *Oenococcus* and *Tetragenococcus*. This organization is different to that of other Gram positive bacteria such as *S. thermophilus*,

Staphylococcus epidermidis and *Staphylococcus capitis*, which show the *hdcP* gene downstream of the *hdcA* gene. The clusters of these bacteria also lack the *hisS* gene.

Nucleotide sequence comparisons of the whole *L. vaginalis* HDC cluster with sequences deposited in the databases showed the strongest similarity with that of *L. reuteri* (82%). The *hdcA* gene was strongly conserved among some LAB, with greater than 70% nucleotide sequence similarity (Fig. 3). These results suggest that the HDC cluster may have a common origin in LAB. Some authors suggest that, in LAB, horizontal gene transfer of BA-related loci occurs given their sequence homology and the fact that, in some species, they are located on plasmids (Ladero et al., 2012; Lucas et al., 2008; Satomi et al., 2008). In the present work, the localization of the *hdcA* gene was determined in two different sized *NotI*-fragments (230 kb and 400 kb) from *L. vaginalis* strains representative of each of the seven lineages detected. Given their large size, these fragments would appear to form part of the bacterial chromosome. Moreover, no plasmids were observed in S1-PFGE analysis or alkaline lysis plasmid isolation (data not shown). The HDC locus is also localised in the chromosome in species such as *L. buchneri* (Martin et al., 2005) and *L. reuteri* (Thomas et al., 2012), although it is found in a plasmid in *L. hilgardii* (Lucas et al., 2005) and *O. oeni* (Lucas et al., 2008). The fact that the HDC cluster is located in the chromosome, and that the flanking regions are the same in all 25 histamine-producing *L. vaginalis* strains, suggests that histamine production is a species-level trait. However, the presence of two direct DNA repeat sequences flanking the HDC cluster, together with the genes coding for a histidine-tRNA and a putative MFS transporter, suggests that the origin of this region in *L. vaginalis* might have involved horizontal gene transfer.

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6. Figure legends

Figure 1: Macrorestriction genomic profiles for *L. vaginalis*, and mapping of *hdc*. A and C: PFGE profiles generated by *Apa*I and *Not*I digestions respectively. B and D: Hybridization of (A) and (C) with an *hdc* probe. Lanes MW1 and MW2: low range PFG marker and the lambda ladder PFG marker (New England Biolabs, Ipswich, MA, USA).

Figure 2. Comparison of the genetic organization of the HDC clusters in different Gram positive bacteria. The organization of the HDC cluster of *L. vaginalis* IPLA11050 (LN828720) was compared with those of *L. reuteri* (NC009513), *L. saerimneri* 30A (Romano et al., 2013), *L. buchneri* (AJ749838), *L. hilgardii* (AY651779), *L. sakei* (DQ132888), *O. oeni* (DQ132887), *T. halophilus* (AB362339), *S. capitis* (AM283479), *S. epidermidis* (AB583189) and *S. thermophilus* (FN686790). Dashed arrow, *hdcP* gene; black arrow, *hdcA* gene; grey arrow *hdcB* gene; white arrow, *hdcS* gene. Nucleotide sequence accession numbers are indicated in parentheses.

Figure 3. Phylogenetic tree of the *hdcA* genes from *L. vaginalis* IPLA11050 (LN828720), *L. reuteri* (NC009513), *L. saerimneri* 30A (Romano et al., 2013), *L. buchneri* (AJ749838), *L. hilgardii* (AY651779), *L. sakei* (DQ132888), *O. oeni* (DQ132887), *T. halophilus* (AB362339), *S. capitis* (AM283479), *S. epidermidis* (AB583189) and *S. thermophilus* (FN686790). Nucleotide sequences were aligned

using ClustalW software (Larkin et al., 2007). The phylogenetic tree was constructed using MEGA v.4 software, employing the Neighbour-Joining clustering method.

Figure 4. Genetic organization of the *hdc* cluster and flanking regions of *L. vaginalis* IPLA11050. a: histidine kinase, b: C4 dicarboxylate anaerobic carrier, c: histidyl-tRNA, d: *hdcP*, e: *hdcA*, f: *hdcB*, g: *hisS*, h: MFS transporter, i: *lysR*, j: *metE*, k: *metF*, l: *nhaC*. Primers: 1a: hisLvC4F, 1b: hisPLv4R, 13a: HisSdsLvF, 13b: HisSdsLvR. Direct repeats: a' and b'.

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Table 1. Primers used in this study

Primer	Pair	Nucleotide sequence (5'-3')	Function	Reference
hdc3		GATGGTATTGTTTCKTATGA	<i>hdcA</i> internal fragment amplification	Coton and Coton, 2005
hdc4		CAAACACCAGCATCTTC	<i>hdcA</i> internal fragment amplification	Coton and Coton, 2005
27F		AGAGTTTGATYMTGGCTCAG	<i>16S rRNA</i> gene sequencing	Lane, 1991
1942R		TACGGYTACCTTGTTACGACTT	<i>16S rRNA</i> gene sequencing	Lane, 1991
<u>Sequencing of <i>HDC</i> cluster</u>				
hisLvC4F	1	TTTCATTGTGCCATCTTCTTCCG	<i>hdcP</i> amplification	This study
hisPLv4R	1	AACTTCAATTACGATTGTTGCG	<i>hdcP</i> amplification	This study
hisPLv2F	2	CTAAGGTACAGCAAGTTGCTACGG	<i>hdcP</i> amplification	This study
hisALv2R	2	GCATCTTGACCACACTCTGC	<i>hdcA</i> amplification	This study
hisLr2F	3	TGCTGAGTGTGGTCAAGATGC	<i>hdcA</i> amplification	This study
hisLr8R	3	CGACCACCACCACAAATTGTCG	<i>hdcS</i> amplification	This study
hisLr5F	4	CAATTTACTCAAATCGGTTGTGAAGC	<i>hisS</i> amplification	This study
hisLv1	4	CCCTACATTAAATTACATGTCC	<i>hisS</i> downstream region amplification	This study
<u>Sequencing of flanking regions</u>				
RR-F	5	CGTCCGCCCCGCATCTCACTGTTTTG	Partial response regulator amplification	This study
C4-R2	5	CACGTTGAGCTTCTTCAGAGTTGCC	C4-dicarboxylate anaerobic carrier amplification	This study

C4-F	6	GGATGCAACTGCTAATCAGCCAAAG	C4-dicarboxylate anaerobic carrier amplification	This study
C4-R	6	CCAGAATTCCACCACCGAAGATGAG	C4-dicarboxylate anaerobic carrier amplification	This study
MTS-F	7	AAGAGAACTGTTAATAGGAAGATAAC	MTS amplification	This study
lysS-R	7	CGACTCTACCTTACCCAACCCAGCC	<i>lysS</i> amplification	This study
lys-F	8	CTCACCAATAATTTTGAATCCGTAATC	<i>lysS</i> amplification	This study
metE-R	8	GATCAAGTCGTCATAAATATCCCG	<i>metE</i> amplification	This study
metE-F	9	TTTATTAATGACGCTGTAAGTACC	<i>metE</i> amplification	This study
metE-R2	9	CCCGTTAGCTTCTAGGTCAAGGAC	<i>metE</i> amplification	This study
metE-F2	10	TAAGGGGATGCTGACAGGACCAG	<i>metE</i> amplification	This study
metF-R	10	CAATGTTTTGCTTAACCTGTCGGG	<i>metF</i> amplification	This study
metF-F	11	TGGCAAAATATCATGATCATCCCC	<i>metF</i> amplification	This study
nhaC-R	11	TCTCCGTTATCTGATTCTACCAAC	<i>nhaC</i> amplification	This study
nhaC-F	12	CCCGCTACCGCTTTGACCAATAATC	<i>nhaC</i> amplification	This study
HP-R	12	GGAGCAAAAATGAAAAATTGTGGTGG	<i>nhaC</i> amplification	This study
HisSdsLvF	13	CTGAAGCGGACAGCAGTGAGAAAGG	<i>hisS</i> -MTS amplification	This study
HisSdsLvR	13	TTGGGGATGGGATCTGTGCTTCC	MTS amplification	This study

Table 2. PFGE profiles of the *L. vaginalis* strains.

Lineages (n° strains)	Profiles		Isolates
	ApaI	NotI	
L1 (19)	A2	N2	IPLA11050, IPLA11051, IPLA11052, IPLA11053, IPLA11055, IPLA11056, IPLA11057, IPLA11058, IPLA11062, IPLA11065, IPLA11067, IPLA11069, IPLA11075, IPLA11140, IPLA11141, IPLA11142, IPLA11143, IPLA11145, IPLA11146
L2 (1)	A2	N3	IPLA11054
L3 (1)	A1	N4	IPLA11070
L4 (1)	A2	N1	IPLA11064
L5 (1)	A2	N6	IPLA11144
L6 (1)	A3	N2	IPLA11060
L7 (1)	A2	N5	IPLA11068

Figure 1

L. vaginalis

L. reuteri

L. saerimneri 30A

L. buchneri

L. hilgardii

L. sakei

O. oeni

T. halophilus

S. capitis

S. epidermidis

S. thermophilus

Figure 2

Figure 3

Figure 4