Gene organization of the ornithine decarboxylase encoding region in *Morganella morganii*

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RUNNING HEADLINE: *ORNITHINE DECARBOXYLASE IN M. MORGANII*

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ABSTRACT

Aims: The production of putrescine is a relevant property related to food quality and safety. *Morganella morganii* is responsible for putrescine production in fresh fish decomposition. The aim of this study was gain deeper insights into the genetic determinants for putrescine production in *M. morganii*.

Methods and Results: The 6972 bp DNA region showed the presence of three complete and two partial open reading frames all transcribed in the same direction. The second and third genes putatively coded for an ornithine decarboxylase (SpeF) and a putrescine-ornithine antiporter (PotE), respectively, and constituted an operon. The *speF* gene has been expressed in *E. coli* HT414, an ornithine decarboxylase defective mutant, resulting in ornithine decarboxylase activity. The genetic organization of the SpeF-PotE encoding region in *M. morganii* is different to that of *E. coli* and several *Salmonella* species.

Conclusions: The *speF* gene cloned from *M. morganii* encodes a functional ornithine decarboxylase involved in putrescine production. Phylogenetic tree based on 16S rDNA showed that ornithine decarboxylase activity is not related to a specific phylogenetic tree branch in Enterobacteriaceae.

Significance and Impact of the study: The identification of the DNA region involved in putrescine production in *M. morganii* will allow additional research on their induction and regulation in order to minimize putrescine production in foods.

Keywords: Biogenic amines; Putrescine; Ornithine; Quality index; Enterobacteriaceae; *Morganella morganii*.

INTRODUCTION

Biogenic amines in foods are of concern in relation to both food safety and food spoilage since they have been implicated in food poisoning incidents, usually from the consumption of fermented foods and, in addition, they have been reported as indicators of a deterioration process and/or defective elaboration (Silla, 1996).

Biogenic amine production has been most extensively studied with respect to histamine and tyramine, probably the two most important biogenic amines of bacterial origin in food, due to their toxicological effects (Halász *et al*. 1994). Putrescine and cadaverine are also investigated since they may potentiate the toxicity of the above amines, and they even might serve as indicators of poor hygienic quality in some food substrates (Bardócz, 1995). Biogenic amines do not usually represent any health hazard to individuals unless large amounts are ingested, or the natural mechanism for the catabolism of the amines is inhibited or genetically deficient. Typical symptoms may be observed in certain individuals, and include nausea, sweating, headache and hyper- or hypotension (Halász *et al*. 1994).

The usefulness of biogenic amines as a quality index will depend on the nature of the product; results tend to be more satisfactory in fresh and heat-treated products than in fermented products (Ruiz-Capillas and Jiménez-Colmenero, 2004). Biogenic amines have been used as quality indexes and indicators of unwanted microbial activity in fish and meat products. A combination of putrescine and cadaverine has been suggested as an index of acceptability in fresh meat, because their concentration increase prior to spoilage and correlate well with the microbial load (Ruiz-Capillas and Jiménez-Colmenero, 2004).

Biogenic amines are mainly formed by decarboxylation of the corresponding amino acids by microorganisms through substrate-specific decarboxylase enzymes. The

occurrence and distribution of amino acid decarboxylase activity within different genera and species of the Enterobacteriaceae, mainly isolated from fish, has been extensively reported. The distribution of the decarboxylase of lysine, arginine, and ornithine differs for the groups of Enterobacteriaceae, and this distribution generally characterized the individual groups (Møller, 1954). Therefore, studies on decarboxylase activity distribution have already provided some valuable chemotaxonomic information within the Enterobacteriaceae family (Brenner, 1984). Out the 63 species of Enterobacteriaceae analysed on the current edition of the Bergey´s Manual of Systematic Bacteriology, 47.6% were positive for ornithine decarboxylase activity. Among these species, *Morganella morganii* strains decarboxylate ornithine to produce putrescine. Putrescine-producing *M. morganii* strains were prominent in decomposed seafood (Benner *et al.* 2004) and fish (Veciana-Nogués *et al*. 2004).

The purpose of the present work was to gain deeper insight into genetic determinants for putrescine production by *M. morganii* given that the production of putrescine is a relevant property related to food quality and safety. We reported here the genetic characterization of a DNA region containing the gene coding for an ornithine decarboxylase in *M. morganii* ATCC 25830. This gene has been expressed in an ornithine decarboxylase defective mutant of *Escherichia coli* and biochemically characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

M. morganii ATCC 25830 (CECT 173), type strain, was purchased from the Spanish Type Culture Collection (CECT). *M. morganii* DSM 46262 and DSM 30117 (ATCC

8019) were purchased from the DSMZ collection. *E. coli* XL1-Blue MRF´ [*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F´proAB lacIqZΔM15 Tn10 (Tet^r)*] and *E. coli* XLOLR [*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 thi-1 recA1 gyrA96 relA1 lac [F´proAB lacI^{q}ZΔM15 Tn10 (Tet^r) Su$^-$ λ^r*] were supplied with the ZAP Express Predigested Gigapack® Cloning kit (Stratagene) used in the construction of a *M. morganii* DNA library. *E. coli* DH5αF´ [F´/*end A1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1 Δ*(*lacIZYA-argF)U169 deoR* (*Φ80dlacΔ(lacZ)M15;* Promega] was used for all DNA manipulations. *E. coli* HT414 [*thr-1, fhuA21, lacY1, glnV44*(AS), λ$^-$, *Δ*(*speB-speA)97, Δ*(*speC-glcB)63, rplL9*(L?), *thi-1, hsdS1*] was used for expression of ornithine decarboxylase since it has a deletion in the ornithine decarboxylase gene (Tabor *et al*. 1981). This strain was generously provided by the *E. coli* Genetic Stock Center as CGSC strain # 6856. Plasmid pIN-III(lpp^p-5)A3 is an expression vector carrying isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter used for the expression in *E.coli* (Inouye and Inouye, 1985).

M. morganii was routinely grown in nutrient agar or nutrient broth (Difco) at 37 ºC with shaking. Putrescine production was detected by growing *M. morganii* in the decarboxylase broth Møller with ornithine (Difco). *E .coli* cells were incubated in Luria-Bertani (LB) medium (Sambrook *et al*. 1989) for bacterial streak and glycerol stocks. For bacterial cultures prior to phage attachment LB medium was supplemented with maltose and magnesium for optimal lambda phage receptor expression on the surface of the host cell. The medium used for agar plates and top agar for plaque formation was NZY (Sambrook *et al*. 1989). When required ampicillin, tetracycline, kanamycin, and streptomycin were added to the medium at 100, 12.5, 50, and 50 μg ml^{-1}, respectively.

DNA manipulations and hybridisation.

Chromosomal DNA and plasmid preparation were carried out as described elsewhere (Muñoz *et al*. 1999). Restriction endonuclease *Sau*3A1 (Roche) was used according to the recommendations of the suppliers. Gel electrophoresis of plasmids, restriction fragments, and PCR products were carried out in agarose gels as described (Sambrook *et al*. 1989).

To construct the *M. morganii* DNA library, chromosomal DNA was partially digested with *Sau*3AI restriction enzyme and ligated to the ZAP Express® vector (Stratagene) digested with *Bam*HI. The ZAP Express vector can accommodate inserts ranging from 0 to 12 kbp. The packaging and titering of the recombinant lambda phages, the amplification of the library, and the in vivo excision of the pBK-CMV phagemid vector from the ZAP Express vector, were performed according to the recommendations of the suppliers. The lambda plaques were screened by hybridisation to a digoxigenin-labelled probe and chemiluminescently detected by using the DIG High Prime DNA labelling and detection Starter Kit (Roche) according to the manufacturer´s instructions.

The probe was a 1.4 kbp *M. morganii* DNA fragment previously PCR amplified by using the degenerate oligonucleotides 3 (5´-GTNTTYAAYGCNGAYAARACNTAYTTYGT) and 16 (5´-TACRCARAATACTCCNGGNGGRTANGG). Oligonucleotides 3 and 16 were based on alignments of bacterial ornithine decarboxylases sequences, and were designed to amplify genes coding for ornithine decarboxylases in gram-positive and gram-negative bacteria (Marcobal *et al*. 2005; De las Rivas *et al*. 2005). PCR amplifications were performed as previously described (Marcobal *et al*. 2005; De las Rivas *et al*. 2005).

DNA sequencing was carried out by using an Abi Prism 3700^{TM} DNA sequencer (Applied Biosystems). Sequence similarities searches were carried out using Basic

Local alignment search tool (BLAST) (Altschul *et al*. 1997) on the EMBL/GenBank databases. Computer promoter predictions carried out at the Internet site http://www.fruitfly.org/seq_tools/promoter.html. Signatures, pI/MW, etc. were analysed on EXPASY (http://www.expasy.ch) site and multiple alignments were done using CLUSTAL W on EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss-Prot. Phylogenetic trees and RNA secondary structure predictions were carried out by the GeneBee program (http://www.genebee.msu.su/genebee.html).

RNA isolation and RT-PCR

Total RNA was isolated from exponentially growing *M. morganii* ATCC 25630 cultures by the RNeasy Mini kit (Quiagen) according to the protocol supplied by the manufacturers. The RNA integrity was checked by standard denaturing agarose gel electrophoresis. To remove any contaminating DNA, RNA was treated for 2 h at 37 ºC with rDNase1 from the DNA-free kit (Ambion).

DNase-treated RNA was subjected to reverse transcription into cDNA with SuperScript™ Reverse Transcriptase (Invitrogene). For RT-PCR, ten percent of the total cDNA was then PCR amplified with *Taq* DNA polymerase (Applied Biosystems) with three sets of primers designed to amplify internal regions of *speF* (primers 110 and 338) and *potE* (primers 195 and 340), and the region spanning the *speF-potE* gene junction (primers 114 and 339). The oligonucleotides primers used were: 110 (5´-CAACTGGGTACGTATGACGG), 114 (5´-GGTATACCATCCGTCAGTTATGTCAGG), 195 (5´-CGCTGGGTATCTGTATTGC), 338 (5´- CAGGATTTCAGCAGCAACTTACG), 339 (5´- CAAAGATAAGACAGTACCGAACACTG), and 340 (5´-CGGAGCAGGACATAATCATCAG). The PCR products were run on a 1% agarose

gel. As a control, PCR of DNase-treated RNA was performed with the same primers to check for any DNA contamination.

Heterologous expression of *speF* in *E. coli*

To clone the *Morganella morganii speF* gene in the absence of its own promoter, the gene was first PCR amplified from *M. morganii* ATCC 25830 DNA by using *Pfu* DNA polymerase and oligonucleotides based in the nucleotide sequence previously determined. To amplify *speF* we used primer 294 (5´-ACTCTAGAGGGTATTAATAATGAAAAGTTATAAAATCGCTGTCAGC) and 229 (5´- CCCAAGCTTTTAACTAACTCAGTGTTTAATTAC) (the underlined sequences indicate restriction sites for *Xba*I in primer 294, and *Hin*dIII in 229). The amplified fragment was digested with *Xba*I and *Hin*dIII and ligated to the expression vector pIN-III(lpp^{p}-5)A3 previously digested with the same enzymes. The ligation mixture was introduced by transformation into the strain *E. coli* DH5αF´. The recombinant plasmid pURI5 obtained from *E. coli* DH5αF´, was later used to transform the ornithine decarboxylase defective strain *E. coli* HT414.

Ornithine decarboxylase activity determination

Cell extracts for SpeF enzymatic assay were obtained from induced cultures. Briefly, *E. coli* HT414 cells harbouring the control or the recombinant plasmid pURI5 were grown in LB broth supplemented with ampicillin (100 μg ml^{-1}) and streptomycin (50 μg ml^{-1}) at 37 ºC to an optical density at 600 nm of 0.6, the cultures were shifted to 30 ºC, and gene expression was induced by adding IPTG to 0.05 mol l^{-1} (final concentration). After 3 h of induction, samples of the cultures were harvested by centrifugation and the pelleted bacteria were resuspended in 0.05 mol l^{-1} phosphate buffer (pH 6.5) and

disrupted by sonication. The insoluble fractions were separated by centrifugation (15,000 x g, 15 min), and the supernatants were used for enzyme assay.

The assay to determine ornithine decarboxylase activity was performed similarly to the tyrosine decarboxylase assay described previously (Moreno-Arribas and Lonvaud-Funel, 2001) in the presence of 0.0036 mol l^{-1} ornithine and 0.4 mM PLP, the cofactor of both decarboxylase reactions, but in 0.050 mol l^{-1} phosphate buffer (pH 6.5) instead of in 0.2 mol l^{-1} sodium acetate buffer (pH 5.0). The reaction was incubated at 37 ºC during 1 h. After the incubation time, the putrescine formed in the reaction was derivatized, and fractionated and detected by thin-layer chromatography by the method described by García-Moruno *et al*. (2005).

RESULTS

Gene organization of the *speF* locus in *Morganella morganii* ATCC 25830

We used the decarboxylase broth Møller with ornithine to confirm that *Morganella morganii* ATCC 25830 was a putrescine producing strain. As expected, a clear positive reaction was obtained (data not shown). In order to identify the gene responsible of this decarboxylase activity, we amplified an 1.4 kbp internal fragment of the ornithine decarboxylase encoding gene by using oligonucleotides 5 and 16 (De las Rivas *et al*. 2005). This fragment was sequenced and similarity searches showed that it contains an incomplete ornithine decarboxylase encoding gene (*speF*) sequence. In order to clone the complete *speF* gene a phage library of *M. morganii* ATCC 25830 genomic DNA was created. The screening of this library using the 1.4 kbp internal DNA fragment as a probe rendered two positive phages. A total of 6972 bp *M. morganii* DNA fragment was sequenced from pRIV5 and pRIV9 plasmids, derived from the positive phages (Fig. 1).

The sequence from *M. morganii* ATCC 25830 was deposited in GenBank under the accession no. AM087552.

Sequence analysis of this DNA fragment showed the presence of three complete and two partial open reading frames (ORFs) all transcribed in the same direction (Fig. 1). Only one putative promoter was detectable upstream of the second ORF. Computer promoter predictions showed the sequence (nucleotide 1556) TTGAgg-15 pb-TATgAa (1582) that could function as a promoter and also predicted the putative transcription start site at 9 nucleotides after the –10 consensus sequence. Putative transcription terminators followed the stop codon of the first, third and fourth ORFs. The analysis of this nucleotide sequence suggested that the second and third genes are organized as a single operon.

The first ORF, incomplete, possibly codes for a lysyl-tRNA synthetase (or lysine-tRNA ligase) since the 118 amino acid overlapping is more than 90% identical to lysyl-tRNA synthetases from other Enterobacteriaceae (Table 1). The second ORF is predicted to encode a 78.5-kDa ornithine decarboxylase since the product of this gene is 76% identical to pyridoxal phosphate-dependent ornithine decarboxylases (SpeF) from several species of Enterobacteriaceae. The third ORF (1298 bp) was identified as the *potE* gene. It encodes a 432-amino acid residue, 45.7-kDa, protein showing 82.3% identity to putrescine-ornithine antiporters (PotE) of several *Salmonella* species. The 633 bp ORF dowstream of *potE*, and separated by a putative transcription terminator, encodes a 22.8-kDa (210-amino acid residue) protein. The identity observed between this protein and TetR family transcriptional regulators from *Idiomarina loihiensis* and *Geobacter sulfurreducens* suggests that this ORF codes for a TetR protein (Table 1). Finally, homology searches carried out with the fifth and uncomplete ORF, ORF1 in

Table 1, only reveals similarity to hypothetical membrane proteins from *Fusobacterium nucleatum* subsp. *vincentii* and *Fusobacterium nucleatum* subsp. *nucleatum* (Table 1).

In order to know if the genetic organization showed by *M. morganii* ATCC 25830 was shared by all *M. morganii* strains, several DNA amplification experiments spanning all the *speF* chromosomal region were performed in *M. morganii* DSM 46262 and DSM 30117 strains. DNA amplifications were done by PCR using oligonucleotides designed on the basis of the ornithine decarboxylase encoding region from *M. morganii* ATCC 25830. All the three *M. morganii* strains analysed amplified PCR fragments that were apparently identical among them (data not shown). In addition, the *speF* genes from *M. morganii* DSM 46262 and DSM 30117 were PCR amplified by using primers 294 and 229, were completely sequenced and deposited in GenBank under accession numbers AM397058 and AM397059, respectively. The results confirmed that the three *M. morganii* strains were nearly identical since only one nucleotide change was found in the nucleotide triplet coding for Leu-644, which was a CTG codon in *M. morganii* DSM 46262 and a TTG codon in *M. morganii* ATCC 25830 and DSM 30117 strains.

The *speF* and *potE* genes in *M. morganii* ATCC 25830 are cotranscribed.

As mentioned above, the sequence analysis of the ornithine decarboxylase encoding region in *M. morganii* suggested that the *speF* and *potE* genes are organized as a single operon (Fig. 1). To confirm this hypothetical operonic organization, RT-PCR was carried out with mRNA prepared from *M. morganii* ATCC 25830. RT-PCR using primers 114 and 339 (Fig. 3) confirmed the cotranscription of *speF* and *potE* genes. As showed in Figure 3, when primers 110 and 338, 114 and 339, and 195 and 340 were used, the expected amplimers were obtained. However, PCR products were not observed with primers designed to amplify regions spanning *lysS-speF* or *potE-tetR*

gene junctions (data not shown). No PCR products were detected in control reactions that were designed to detect chromosomal DNA contamination. The transcriptional analysis results are consistent with the DNA sequence study.

Enzymatic analysis of the *speF* gene

The *speF* gene of *M. moganii* was expressed in *E. coli* following the strategy described in Materials and Methods section. The correct sequence and insertion of *speF* into recombinant plasmid pURI5 was verified by restriction analysis and DNA sequencing.

Cells extracts were prepared from *E. coli* HT414 harbouring the control and the recombinant plasmid pURI5 as described above. The extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. None of the cell extracts analysed showed an apparent protein band of the expected size, 78.5 kDa (data not shown). Even not obvious protein hyperproduction was achieved, the supernatant of sonicated cell lysates prepared from *E. coli* HT414 harbouring the recombinant plasmid pURI5 was able to decarboxylate the ornithine present in the reaction to putrescine, whereas extracts prepared from control cells containing the vector plasmid alone did not Fig. 4 showed a TLC analysis of the enzymatic reactions. Thus, we could prove experimentally that the *speF* gene encodes an ornithine decarboxylase.

Structure of the *speF-potE* region in Enterobacteriaceae

Since *M. morganii* SpeF and PotE proteins showed the highest identities scores to SpeF and PotE proteins from different *Salmonella* strains (Table 1), we revised the genetic organization of the *speF-potE* region on these strains. Fig. 1 showed that although *M. morganii* SpeF and PotE proteins possessed high identity to *Salmonella* proteins, they showed a different genetic organization on their encoding locus.

In the sequenced *Salmonella* strains, the genes encoding the two-component regulatory system, KdpD and KdpE, that regulates the *kdp* operon which encodes a high-affinity potassium translocating ATPase, are localized upstream *speF*. Downstream *potE* was found a gene encoding a putative cytoplasmic protein, annotated as possible 5´-nitroimidazole antibiotic resistance in *S. paratyphi*, and it was followed by a phosphoglucomutase encoding gene (*pgm*) (Fig. 1). This common genetic organization was not conserved in *E. coli*. A 51-amino acid residues hypothetical protein was located immediately upstream *E. coli speF*. Moreover, the putative cytoplasmic protein located downstream *potE* was absent in the *E. coli speF* region (Fig. 1).

In order to know if the ability to decarboxylate ornithine was associated to a specific branch in a Enterobacteriaceae evolutive tree, some of the members of this family, from which ornithine decarboxylase activity was previously described, were used to construct a phylogenetic tree based on the 16S rRNA gene sequences (Fig. 5). As showed in Fig. 5, tree based on 16S rDNA shows that *Proteus* spp., *Morganella morganii* and *Providencia alcalifaciens* formed a coherent clade which rooted other Enterobacteriaceae. However, as showed in Fig. 5 the ability to decarboxylate ornithine is not associated with a specific branch of the phylogenetic tree since putrescine-producer strains could be found in all the tree branches.

DISCUSSION

The occurrence of relatively high levels of certain biogenic amines has been reported as indicators of a deterioration process and/or defective elaboration. Benner et al. (Benner *et al*. 2004) reported that *M. morganii* was responsible for putrescine production in shrimp decomposed at 24 ºC and 36 ºC. Gingerich et al. (1999) reported that fresh

bluefish did not contain detectable levels of putrescine; however, putrescine levels increased with time, reaching a maximum level in *M. morganii*-inoculated samples stored at 10 ºC. Typically, *Morganella morganii* (formerly, *Proteus morganii*) strains contains a strong ornithine decarboxylase activity (Møller, 1954). Since the gene responsible for putrescine production in this species has not been genetically characterized, we decided to elucidate it. Firstly, we used the decarboxylase broth Møller with ornithine to confirm that *Morganella morganii* ATCC 25830 was a putrescine producing strain as previously described (Gingerich *et al*. 1999). By using a previously described PCR method to detect putrescine producing gram-negative bacteria (De las Rivas *et al*. 2005), and by the construction of a genomic library, we have determined the sequence of a 6972 bp *M. morganii* DNA fragment containing *speF*, the ornithine decarboxylase encoding gene. Moreover, by expressing the *speF* gene in a *E. coli* ornithine decarboxylase mutant we proved experimentally that *speF* encodes a functional decarboxylase capable of producing putrescine from the amino acid L-ornithine.

The alignment of *M. morganii* SpeF to some SpeF proteins included in the databases showed an absolute conservation among amino acid residues involved in cofactor and substrate-binding domains (Fig. 2). Based on the three-dimensional structure of the *Lactobacillus* 30a ornithine decarboxylase (Momany *et al*. 1995b) the sequence ^{318}FDSAW^{322} in *M. morganii* SpeF forms part of a β-strand and turns immediately behind the cofactor and is conserved among the decarboxylases. In *Lactobacillus* 30a, the phosphate of the PLP interacts with a cluster of hydroxyls from serine and threonine residues, as well as main-chain amide nitrogens. Charge stabilization of the PLP is provided by the dipole moment arising from helices plus the presence of H^{355} in the classic decarboxylase fingerprint sequence, SXHL. In *M.*

morganii SpeF, all these residue are conserved, with exception of the residue corresponding to S^{199} which is substitute by a threonine residue (T^{201}) able to provide the hydroxyl group (Momany *et al*. 1995a). A critical amino acid residue that is also conserved is H^{226} which is involved in cofactor binding and is a possible proton donor in decarboxylases.

Kashiwagi et al. (1991) described that in *E. coli* the gene coding for PotE (*potE*) together with the gene for ornithine decarboxylase (*speF*) constitutes an operon (Kashiwagi *et al*. 1991). A similar organization occurs in *M. morganii*. PotE protein encodes a putrescine-ornithine antiporter and, can catalyse both the uptake and excretion of putrescine (Igarashi and Kashiwagi. 1999). PotE are proteins containing 12 transmembrane segments linked by hydrophilic segments of variable length with the N- and C-termini located in the cytoplasm (Momany *et al*. 1995a; Igarashi and Kashiwagi. 1999). Site-directed mutagenesis in *E. coli* PotE was used to identify the amino acids involved in the transport activity of PotE. Three glutamic acid residues located at the cytoplasmic side of PotE, are involved in both, the uptake and the excretion of putrescine (Kashiwagi *et al.* 1997). In *M. morganii* PotE, only E^{76} and E^{206} residues are conserved (data not shown).

Although *M. morganii* SpeF and PotE proteins showed the highest identities scores to SpeF and PotE proteins from different *Salmonella* strains, they showed a different genetic organization on their encoding locus. We assumed that Figure 1 might reflects evolutionary relationship between these Enterobacteriaceae species. Phylogenetic studies on Enterobacteriaceae and other prokaryotes have used rRNA sequences. Figure 5 showed a phylogenetic tree based on the 16S rRNA gene sequences and revealed that the ability to decarboxylate ornithine was not associated to a specific branch in the Enterobacteriaceae evolutive tree. Several authors have suggested that

sometimes there is no correlation between 16S rRNA gene and protein-coding genes when the latter is more dependent on selection pressure or had been horizontally transferred across groups.

Figure 5 showed that *Proteus* spp., *M. morganii* and *Providencia alcalifaciens* formed a coherent tree clade. The phylogenetic position of these species was identical in the phylogenetic trees based on *gyrB*, RNA polymerase β-subunit (*rpoB*) and translational initiation factor 2 (*infB*) sequences (Møller *et al*. 1997; Hedergaard *et al*. 1999). Until recently, the members of the *Morganella* were classified as *Proteus* and were thus considering in the light of their membership in that genus rather than as a separate group.

The obtained phylogenetic tree also confirms the close position between *E. coli* and *S. enterica*, this relationship might justified the high similarity observed between their *speF* regions (Fig. 1). Moreover, the relatively distant position of *M. morganii* is coherent with a different genetic organization of the *speF* region.

In summary, the production of putrescine is a relevant property related to food quality and safety. Since *M. morganii* is responsible for putrescine production in fresh fish decomposition, we have elucidated the molecular basis for this property. We have demonstrated that *M. morganii* ATCC25830 possess a gene that encodes a functional decarboxylase capable of producing putrescine from the amino acid L-ornithine. We have found that the genetic organization of the ornithine decarboxylase encoding region in *M. morganii* is different to that of *E. coli* and several *Salmonella* species, according to their relatively distant phylogenetic position. Additional and further research on ornithine decarboxylase induction and regulation will help to improve the food safety

and/or quality of fish products in order to prevent putrescine formation and accumulation.

ACKNOWLEDGEMENTS

This work was supported by grant RM03-002 (INIA), AGL2005-00470 (CICYT), and S-0505/AGR/000153 (CAM). We thank E. Díaz and B. Galán for their help with the RNA experiments. We thank R. González and A.V. Carrascosa for their critical reading of the manuscript. The technical assistance of M. V. Santamaría and A. Gómez is greatly appreciated.

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Table 1
speF locus-encoded proteins: properties and similarities to proteins in the databases

Protein	Location in nucleotide sequence	Predicted protein (aa*/kDa)	Proposed function	Similar polypeptide (aa)	Database accession no.	Degree of identity (%)	Organism
LysS	< - 360	-	Lysyl-t-RNA synthetase	LysS (504)	NP_930765.1	93% (in a 118 aa overlap)	*Photorhabdus luminescens*
				LysS (505)	NP_670571.1	91% (in a 118 aa overlap)	*Yersinia pestis* KIM
SpeF	1647-3815	722 / 78.5	Ornithine decarboxylase	SpeF (732)	Q8ZQW6	76.7%	*Salmonella typhimurium* LT2
				SpeF (732)	Q57RN4	76.6%	*Salmonella enterica*
				SpeF (732)	Q5PCJ3	76.3%	*Salmonella paratyphi* A
PotE	3879-5177	432 / 45.7	Putrescine-ornithine antiporter	PotE (439)	Q8ZQW7	82.3%	*Salmonella typhimurium* LT2
				PotE (439)	Q57RN5	82.3%	*Salmonella enterica*
				PotE (439)	Q5PCJ2	82.3%	*Salmonella paratyphi* A
TetR	5329-5961	210 / 22.8	Transcriptional regulator	Hypothetical (162)	Q881C5	47.5%	*Pseudomonas syringae*
				TetR (208)	Q5R0E4	36.9%	*Idiomarina loihiensis*
				TetR (196)	Q74F39	35.6%	*Geobacter sulfurreducens*
ORF1	6046- >	-	Hypothetical membrane protein	FNV1001 (427)	ZP_00144255	29% (in a 260 aa overlap)	*Fusobacterium nucleatum*

*aa, amino acid residues

FIGURE LEGENDS

Fig. 1 Genetic organization of the *M. morganii* ATCC 25830 7-kbp DNA region containing the ornithine decarboxylase gene (*speF*). The sequence from *M. morganii* ATCC 25830 was deposited in GenBank under the accession no. AM087552. The *speF* region corresponding to *E. coli* ATCC 700928 (accession NC_004431, positions 762821-755849), *S. typhimurium* LT2 (accession NC_003197, positions 767224-760252), *S. enterica* (accession NC_006905, positions 809116-816088), and *S. paratyphi* ATCC 9159 (accession NC_006511, positions 2123320-2130292). Arrows indicate genes. Genes having putative identical functions are represented by identical shading. The localization of putative promoter (vertical bent arrow) and transcription terminator regions (ball and stick) are also indicated. The plasmids used in this study are also shown.

Fig. 2 Comparison of protein sequence SpeF from *M. morganii* (MMO), *E. coli* (ECO) (accession Q8FJV8), *S. typhimurium* (STY) (accession Q8ZQW6), *S. enterica* (SEN) (accession Q57RN4), and *S. paratyphi* (SPA) (accession Q5PCJ3). Clustal W program was used to compare predicted sequences. Residues involved in PLP-binding (▼) are typed in boldface and are underlined in the *M. Morganii* sequence. Asterisks indicated amino acid identity, and dashed, gaps introduced to maximize similarities.

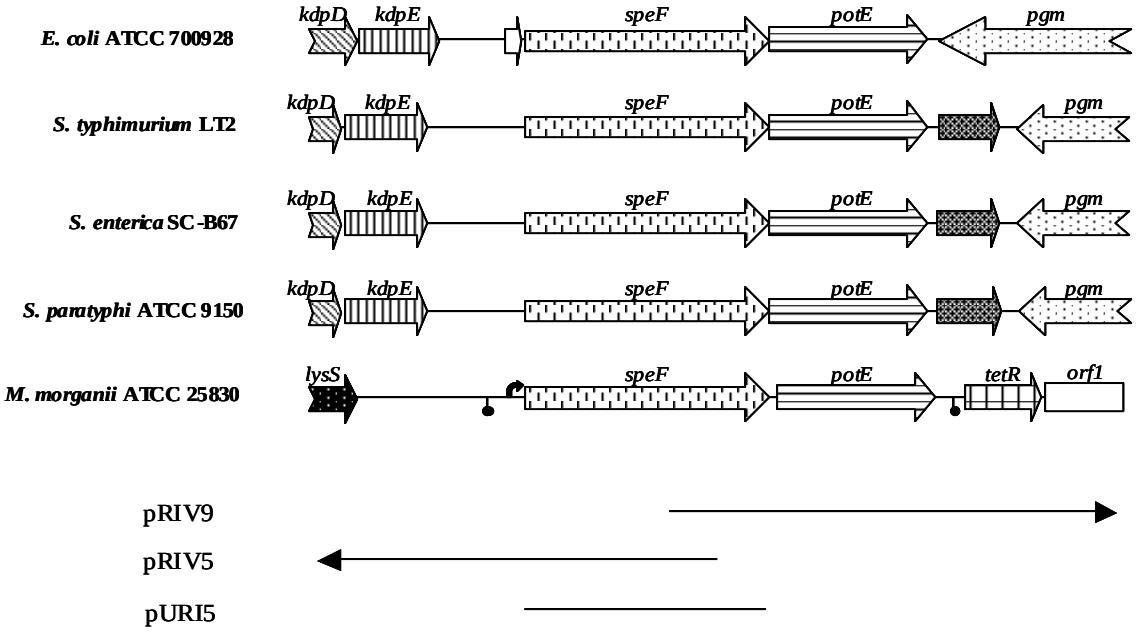
Fig. 3. RT-PCR amplification with three sets of primers designed to amplify internal or intergenic regions: 0.45 kb internal *speF* region, primers 110 and 338 (lane 1); 0.62 kb *speF-potE* intergenic region, primers 114 and 339 (lane 2); 0.53 kb internal *potE* region, primers 195 and 340 (lane 3). Negative controls were conducted without reverse

transcriptase (lanes 4, 5, and 6, respectively). The 100-bp DNA Ladder was used as a molecular weight marker.

Fig. 4 TLC detection of putrescine produced by soluble cells extracts of IPTG-induced cultures of *E. coli* HT414 bearing recombinant plasmid pURI5. The putrescine produced during the enzymatic reactions was dansylated and separated on a precoated silica gel 60 F_{254} plate. Lane 1, control putrescine standard solution; lane 2, reaction from *E.coli* HT414 bearing the control pIN-III(lpp^{p}-5)A3 plasmid; lane 3, reaction from *E.coli* HT414 bearing the recombinant pURI5 plasmid. The arrow indicates the putrescine produced.

Fig. 5 Neighbour-joining phylogenetic tree obtained from 16S rRNA gene sequences and ornithine decarboxylase activity. The 16S rRNA sequences were obtained from GenBank and EMBL databases: *Citrobacter freundii* (accession no. M59291), *Enterobacter aerogenes* (AB004750), *Enterobacter cloacae* (AJ251469), *Escherichia coli* (X80725), *Hafnia alvei* (M59155), *Klebsiella pneumoniae* (X87276), *Morganella morganii* (AJ301681), *Proteus mirabilis* (AJ301682), *Proteus vulgaris* (AJ301683), *Providencia alcalifaciens* (AJ301684), *Salmonella enterica* (AF332600), *Serratia ficaria* (AJ233428), *Serratia fonticola* (AJ233429), *Serratia marcescens* (AJ233431), *Serratia odorifera* (AJ233432), *Serratia plymuthica* (AJ233433), and *Serratia rubidaea* (AJ233436).The scale bar represents an estimated 5 base substitutions per 1000 nt positions. The ornithine decarboxylase data were obtained from the Bergey´s Manual of Systematic Bacteriology (Brenner *et al*. 1984). The Møller media was used for the decarboxylase test and symbols represents: +, 90-100% of strains are positive; d, 26-75% positive; [-], 11-25% positive; -, 0-10% positive.

Figure 1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kdpE
speF
potE
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S. typhimurium LT2
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kdpE
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potE
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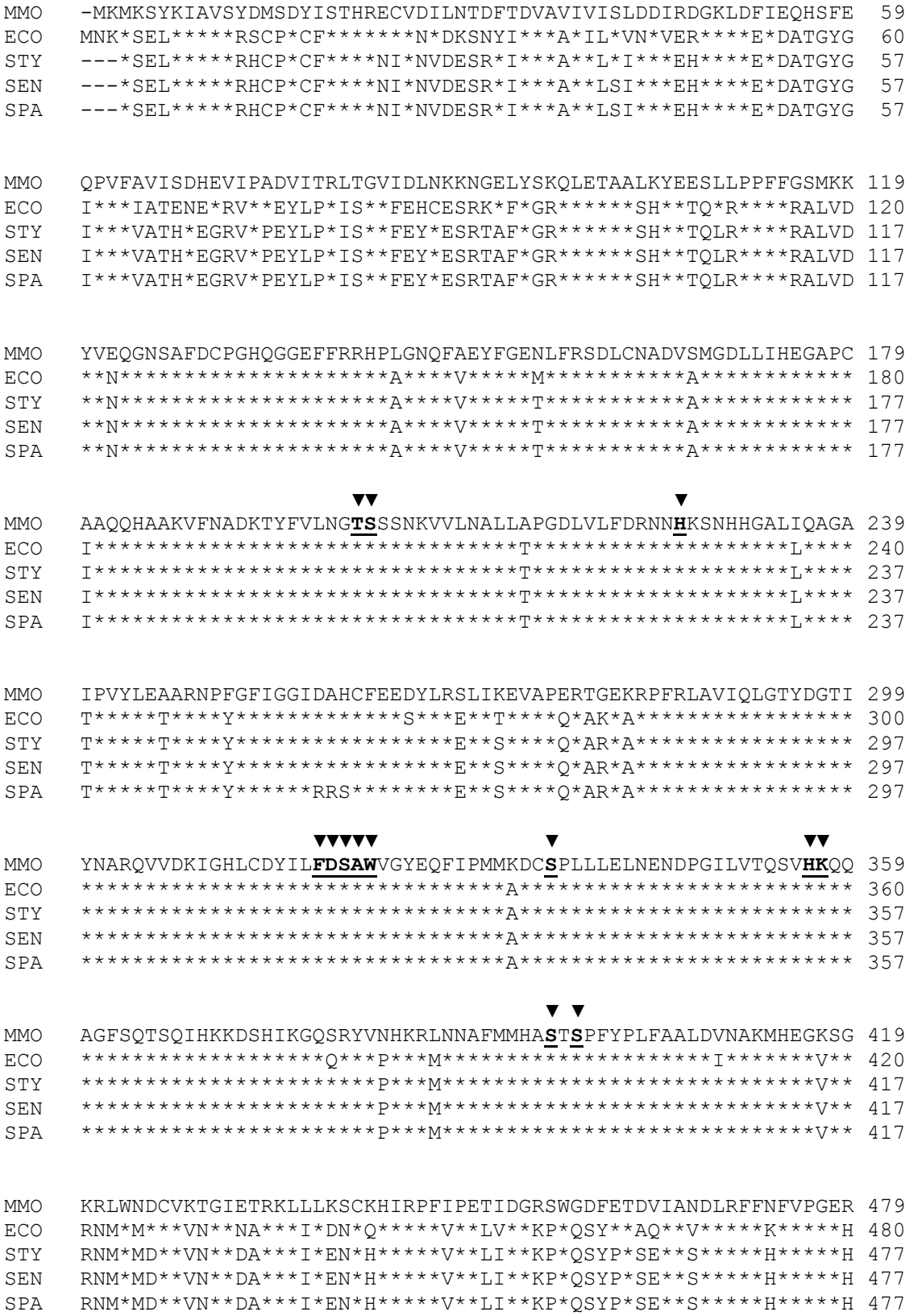
S. enterica SC-B67
kdpD
kdpE
speF
potE
pgm
S. paratyphi ATCC 9150
kdpD
kdpE
speF
potE
pgm
M. morganii ATCC 25830
lysS
speF
potE
tetR
orf1
pRIV9
pRIV5
pURI5

Figure 2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ECO MNK*SEL*****RSCP*CF*******N*DKSNYI***A*IL*VN*VER****E*DATGYG 60
STY ---*SEL*****RHCP*CF****NI*NVDESR*I***A**L*I***EH****E*DATGYG 57
SEN ---*SEL*****RHCP*CF****NI*NVDESR*I***A**LSI***EH****E*DATGYG 57
SPA ---*SEL*****RHCP*CF****NI*NVDESR*I***A**LSI***EH****E*DATGYG 57
MMO QPVFAVISDHEVIPADVITRLTGVIDLNKKNGELYSKQLETAALKYEESLLPPFFGSMKK 119
ECO I***IATENE*RV**EYLP*IS**FEHCESRK*F*GR******SH**TQ*R****RALVD 120
STY I***VATH*EGRV*PEYLP*IS**FEY*ESRTAF*GR******SH**TQLR****RALVD 117
SEN I***VATH*EGRV*PEYLP*IS**FEY*ESRTAF*GR******SH**TQLR****RALVD 117
SPA I***VATH*EGRV*PEYLP*IS**FEY*ESRTAF*GR******SH**TQLR****RALVD 117
MMO YVEQGNSAFDCPGHQGGEFFRRHPLGNQFAEYFGENLFRSDLCNADVSMGDLLIHEGAPC 179
ECO **N*********************A****V*****M**********A********** 180
STY **N*********************A****V*****T**********A********** 177
SEN **N*********************A****V*****T**********A********** 177
SPA **N*********************A****V*****T**********A********** 177
MMO AAQQHAAKVFNADKTYFVLNGTSSSNKVVLNALLAPGDLVLFDRNNHKSNHHGALIQAGA 239
ECO I**********************************T*******************L**** 240
STY I**********************************T*******************L**** 237
SEN I**********************************T*******************L**** 237
SPA I**********************************T*******************L**** 237
MMO IPVYLEAARNPFGFIGGIDAHCFEEDYLRSLIKEVAPERTGEKRPFRLAVIQLGTYDGTI 299
ECO T*****T****Y*************S***E**T****Q*AK*A***************** 300
STY T*****T****Y*****************E**S****Q*AR*A***************** 297
SEN T*****T****Y*****************E**S****Q*AR*A***************** 297
SPA T*****T****Y******RRS********E**S****Q*AR*A***************** 297
MMO YNARQVVDKIGHLCDYILFDSAWVGYEQFIPMMKDCSPLLLELNENDPGILVTQSVHKQQ 359
ECO ********************************A************************** 360
STY ********************************A************************** 357
SEN ********************************A************************** 357
SPA ********************************A************************** 357
MMO AGFSQTSQIHKKDSHIKGQSRYVNHKRLNNAFMMHASTSPFYPLFAALDVNAKMHEGKSG 419
ECO ******************Q***P***M*********************I*******V** 420
STY ***********************P***M*****************************V** 417
SEN ***********************P***M*****************************V** 417
SPA ***********************P***M*****************************V** 417
MMO KRLWNDCVKTGIETRKLLLKSCKHIRPFIPETIDGRSWGDFETDVIANDLRFFNFVPGER 479
ECO RNM*M***VN**NA***I*DN*Q*****V**LV**KP*QSY**AQ**V*****K*****H 480
STY RNM*MD**VN**DA***I*EN*H*****V**LI**KP*QSYP*SE**S*****H*****H 477
SEN RNM*MD**VN**DA***I*EN*H*****V**LI**KP*QSYP*SE**S*****H*****H 477
SPA RNM*MD**VN**DA***I*EN*H*****V**LI**KP*QSYP*SE**S*****H*****H 477

Fig.2 cont.

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MMO   WHAFEGYEENQYFVDPCKLLLTTPGIDAKTGNYEAFGVPATILANYLRENNIIPEKCDLN 539
ECO   **S****A*******************RN*E************F****G********* 540
STY   **A****A*H****************N*AS*E**DFGV*******F****GVV******* 537
SEN   **A****A*H****************N*AS*E**DFGV*******F****GVV******* 537
SPA   **A****A*H****************N*AS*E**DFGV*******F****GVV******* 537

MMO   SILFLLTPAESMAKMQHLVAQIARFEQLLEQDAPLKEVLPSVYHAHEARYQGYTIRQLCQ 599
ECO   **********D***L*Q***LLV***K***A****A*****I*KQ**E**A***L***** 600
STY   **********D***L*Q***LL****K***A****A*****I*KQ*****A***L***** 597
SEN   **********D***L*Q***LL****K***A****A*****I*KQ*****AD**L***** 597
SPA   **********D***L*Q***LL****K***A****A*****I*KQ*****AD**L***** 597

MMO   EMHDMYVKFNVKQLQKEMFRKSHFPQVSMLPQAANIAFVRGNAELVAVDQIEGRIAAEGA 659
ECO   ****L*A**************E***R***N**E**Y*YL**EV***RLPDA********* 660
STY   ****L*ARH****************K***N**E**Y*YL**EV***RLPEA********* 657
SEN   ****L*ARH****************K***N**E**Y*YL**EV***RLPEA********* 657
SPA   ****L*ARH****************K***N**E**Y*YL**EV***RLPEA********* 657

MMO   LPYPPGVLCVVPGEIWGGSVQQYFLALEEGINLFPGFSPELQGVYIRCDEDGRQRAYGYV 719
ECO   ******************A*LR**S********L***A********EEH-***KQVWC** 719
STY   ********************LR**S********L***A********EEH-***KQVWC** 716
SEN   ********************LR**S********L***A********EEH-***KQVWC** 716
SPA   ********************LR**S********L***A********EEH-***KQVWC** 716

MMO   IKH------------- 732
ECO   **PRDAQSTLLKGEKL 735
STY   **PRDAQRSLLKEEKL 732
SEN   **PRDAQRSLLKEEKL 732
SPA   **PRDAQRSLLKEEKL 732
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Figure 3

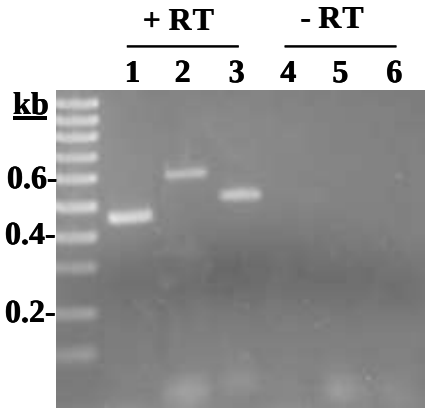


Figure 4

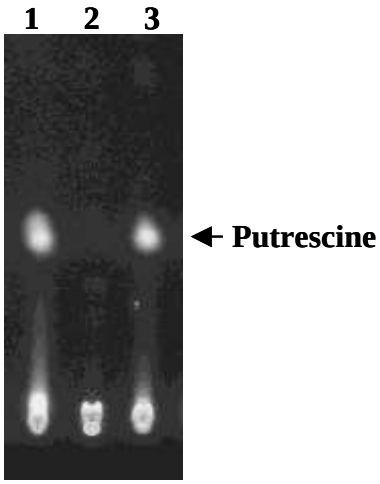
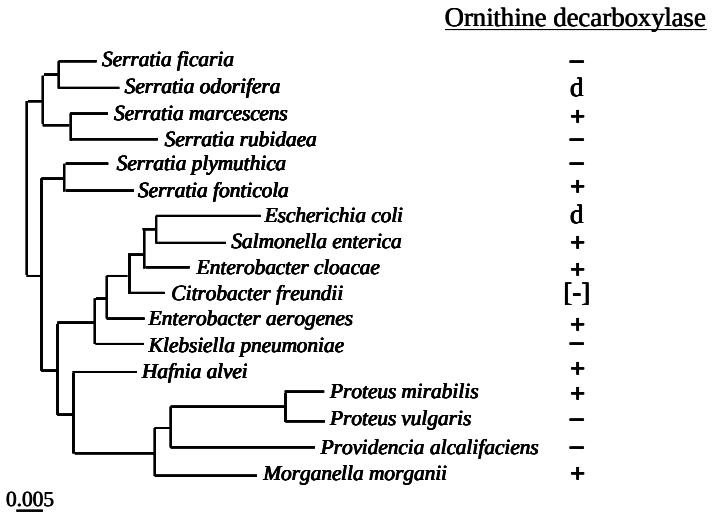


Figure 5


Ornithine decarboxylase
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Serratia odorifera
Serratia marcescens
Serratia rubidaea
Serratia plymuthica
Serratia fonticola
Escherichia coli
Salmonella enterica
Enterobacter cloacae
Citrobacter freundii
Enterobacter aerogenes
Klebsiella pneumoniae
Hafnia alvei
Proteus mirabilis
Proteus vulgaris
Providencia alcalifaciens
Morganella morganii
–
d
+
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+
d
+
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