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Gene Cloning, Expression, and Functional Characterization of an Ornithine Decarboxylase Protein from *Serratia liquefaciens* IFI65

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Abstract Putrescine has a negative effect on health and is also used as an indicator of quality on meat products. We investigated the genes involved in putrescine production by *Serratia liquefaciens* IFI65 isolated from a spoiled Spanish dry-cured ham. We report here the genetic organization of its ornithine decarboxylase encoding region. The 5,506-bp DNA region showed the presence of three complete and two partial open reading frames. Putative functions have been assigned to several gene products by sequence comparison with proteins included in the databases. The second gene putatively coded for an ornithine decarboxylase. The functionality of this decarboxylase has been experimentally demonstrated by complementation to an *E. coli* defective mutant. Based on sequence comparisons of some enterobacterial ornithine decarboxylase regions, we have elaborated a hypothetical pathway for the acquisition of putrescine biosynthetic genes in some *Enterobacteriaceae* strains.

Keywords: Putrescine, ornithine decarboxylase, dry-cured ham, *Serratia liquefaciens*, *Enterobacteriaceae*

Biogenic amines are low molecular weight organic bases that possess biological activity. They are generated mainly by the growth of decarboxylase-positive microorganisms under conditions favorable to enzyme activity. As the microbial spoilage of food may be accompanied by the increased production of decarboxylases, the presence of biogenic amines might serve as a useful indicator of food spoilage. Moreover, consumption of foods containing high amounts of these amines can have toxic effects [14]. The most notorious foodborne intoxications caused by biogenic amines are related to histamine and tyramine. Putrescine, although it seems to have a lower pharmacological activity, hampers the detoxification of histamine and tyramine. Moreover, the presence of the amine putrescine is investigated in meat products since it can react with nitrite to form nitrosopyrrolidine, a heterocyclic carcinogenic nitrosamine [18]. In addition, the occurrence of relatively high levels of certain biogenic amines has been reported as indicators of a deterioration process and/or defective elaboration. A combination of putrescine and cadaverine has been suggested as an index of acceptability in fresh meat, because their concentrations increase prior to spoilage and correlate well with the microbial load [13].

Spanish dry-cured ham is a valuable traditional intermediate-moisture meat product, produced from several breeds of white hogs. During ripening of Spanish dry-cured hams, protein hydrolysis takes place. Free amino acids are highly correlated with flavor development in aged hams. These amino acids have been reported as precursors of sour, sweet, and bitter tastes. On the other hand, amino acid degradation to amines would affect not only the flavor but also the health of the consumer if biogenic amines are formed. Córdoba *et al.* [3] studied the evolution of free amino acids and amines during ripening of Spanish dry-cured ham. They described that the larger increases of free amino acids present took place in the drying cycle and the amines with higher concentrations were not in the toxic range.

Marín *et al.* [11] analyzed the growth trends for the family *Enterobacteriaceae* during Spanish dry-cured ham elaboration. The concentration of enterobacteria on the surface underwent a decline during salting; a drop in this count was concomitant with the progressive reduction in water activity during the curing process. The microorganisms involved in alterations taking place during cold storage were cold-tolerant enterobacteria; *e.g.*, "deep putrefaction" defect. Cantoni *et al.* [2] also stated that deep putrefaction was the most common spoilage in Italian dry-cured ham. Losantos *et al.* [10] studied the microbiological and physicochemical

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aspects of spoiled specimens of dry-cured hams affected by deep putrefaction. Taxonomic determination showed that strains of *Enterobacteriaceae* isolated in spoiled Spanish dry-cured hams belonged to the *Serratia liquefaciens* and *Proteus vulgaris* species. Both species are considered to be proteolytic and nonpathogenic [1, 6].

In spite of the relevance of putrescine to both food safety and food spoilage, very little is known about its bacterial production. Herein, we describe the genetic and functional characterizations of a DNA region coding the ornithine decarboxylase responsible for putrescine production in *S. liquefaciens* IFI65 isolated from a spoiled Spanish dry-cured ham. Finally, a hypothetical scenario for the acquisition of this gene in some *Enterobacteriaceae* is proposed.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *S. liquefaciens* and *P. vulgaris* strains used in this study were isolated from spoiled Spanish dry-cured hams [10]. Nutrient agar or nutrient broth media were used for the routine cultivation of these strains. Putrescine production was detected by growing *S. liquefaciens* and *P. vulgaris* in the decarboxylase broth Møller supplemented with histidine, tyrosine, or ornithine (5 g/l). *E. coli* XL1-Blue MRF' and XLOLR were supplied with the ZAP Express Predigested Gigapack Cloning kit (Stratagene, La Jolla, CA, U.S.A.). *E. coli* HT414 (CGSC 6856) was used for expression of ornithine decarboxylase, since it has a deletion in the ornithine decarboxylase gene [16], and was generously provided by the *E. coli* Genetic Stock Center. *E. coli* strains were cultivated in LB or NZY media containing ampicillin, tetracycline, kanamycin, and streptomycin at 100, 12.5, 50, and 50 μg/ml, respectively, when appropriate.

Recombinant DNA Techniques

Standard procedures for restriction endonucleases digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were carried out as previously described [12]. Sequence similarities searches were carried out using the Basic Local alignment search tool (BLAST) on the EMBL/GenBank databases. Signatures were analyzed on the EXPASY site (http://www.expasy.ch) and multiple alignments were done using CLUSTAL W on the EBI site (http://www.ebi.ac.uk) after retrieval of sequences from GenBank and Swiss-Prot.

Construction of a Phage Library

A *S. liquefaciens* IFI65 DNA library was constructed in the ZAP Express vector (Stratagene, La Jolla, CA, U.S.A.). To construct the library, chromosomal DNA was partially digested with Sau3AI restriction enzyme and ligated to the ZAP vector digested with BamHI. 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 supplier. The lambda plaques were screened by hybridization to a digoxigenin-labeled probe and chemiluminescently detected by using the DIG High Prime DNA labeling and detection Starter Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Cloning of the *speF* Gene in *E. coli*

To amplify *speF* from *S. liquefaciens* IFI65, specific oligonucleotides were designed based on the nucleotide sequence determined in this work. The primers used were primer 200 (5'-ACTCTAGAGGGTATTAATAATGCATC-ACCATCATCATCACGAATACCTTTGTCTGAAGAAT GAG) (containing a XbaI site as underlined) and primer 227 (5'-CCCAAGCTTCTAATCATTAATTACTCATGA-CGTAG) (containing a HindIII site as underlined). The gene was first PCR amplified by using *Pfu* DNA polymerase, digested with XbaI and HindIII, and ligated to the expression vector pIN-III(lpp^p-5)A3 [8] digested with the same enzymes. The resulting plasmid was designated pURI4. The absence of mutations within the coding region of *speF* was verified by DNA sequencing.

Enzyme Activity

E. coli strain HT414 carrying pURI4 was grown at 37°C in LB medium containing 100 μg/ml ampicillin and 50 μg/ml streptomycin. When the cultures reached an optical density of 0.6 at 600 nm, the cultures were shifted to 30°C, and gene expression was induced by adding 50 mM IPTG. After 3 h of induction, samples of the cultures were harvested by centrifugation (10,000 ×*g*, 5 min) and washed twice with 50 mM sodium phosphate buffer (pH 6.5). The pelleted bacteria were resuspended in the same buffer and disrupted by sonication. The insoluble fraction was separated by centrifugation (25,000 ×*g*, 15 min), and the supernatant was assayed for ornithine decarboxylase activity.

The assay to determine ornithine decarboxylase activity was performed in 50 mM sodium phosphate buffer (pH 6.5) in the presence of 3.6 mM ornithine and 0.4 mM PLP. The reaction was incubated at 37°C during 1 h. Afterwards, the putrescine formed in the reaction was derivatized and detected by thin-layer chromatography (TLC) as described by García-Moruno *et al.* [5].

Nucleotide Sequence Accession Number

The nucleotide sequence data reported here is available in the GenBank database under the accession number AM117811.

RESULTS AND DISCUSSION

Losantos *et al.* [10] isolated *S. liquefaciens* and *P. vulgaris* strains from spoiled Spanish dry-cured hams, and they concluded that these strains could have caused their deep putrefaction, by growing during the first nonrefrigerated step of the curing process before the decrease of water activity. Some authors have shown that these strains could reach the deep musculature during slaughter and cutting [17]. In addition, Stiles [15] reported that *Serratia* was the genera most commonly present on working surfaces in the meat processing industry.

In order to know the capability of these spoilage strains to produce health hazard compounds, we tested their biogenic amines production. Fifteen *S. liquefaciens* and fifteen *P. vulgaris* strains isolated from spoiled Spanish dry-cured hams were analyzed [10]. From the three amino acids tested, *P. vulgaris* strains did not decarboxylate any of them; however, all the *S. liquefaciens* strains only decarboxylated ornithine in Møller broth. Putrescine production by *S. liquefaciens* IFI65 was confirmed by TLC (Fig. 1). In fact, ornithine decarboxylation is a characteristic differentiating the species of the genus *Serratia*, being *S. liquefaciens*, a positive decarboxylase species [1].

From the fifteen *S. liquefaciens* strains, we amplified a 1.4 kb internal fragment of its ornithine decarboxylase encoding gene (*speF*) by using a PCR method previously described [4] (data not shown). To identify the gene responsible for their ornithine decarboxylase activity, we selected the *S. liquefaciens* IFI65 strain, since it was one of the two strains that were capable of growth at low water activity and also at 4°C [10]. Similarity searches confirmed that the *S. liquefaciens* amplified fragment contains an incomplete ornithine decarboxylase encoding gene. To characterize the complete *speF* gene and the DNA region surrounding it, a phage library of *S. liquefaciens* DNA was

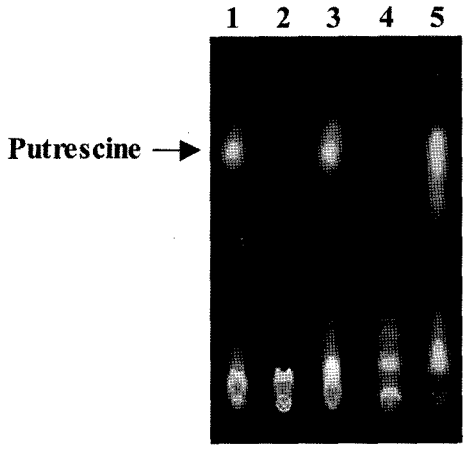

Fig. 1. Putrescine detection by TLC. The putrescine produced was dansylated and separated on a precoated silica gel 60 F_{254} plate.
Lane 1, control putrescine standard solution; lane 2, reaction from *E. coli* HT414 extracts bearing the control plasmid; lane 3, reaction from *E. coli* HT414 extracts bearing the recombinant pURI4 plasmid; lane 4, Møller broth media supplemented with ornithine; lane 5, *S. liquefaciens* IFI65 growing in Møller broth media supplemented with ornithine.

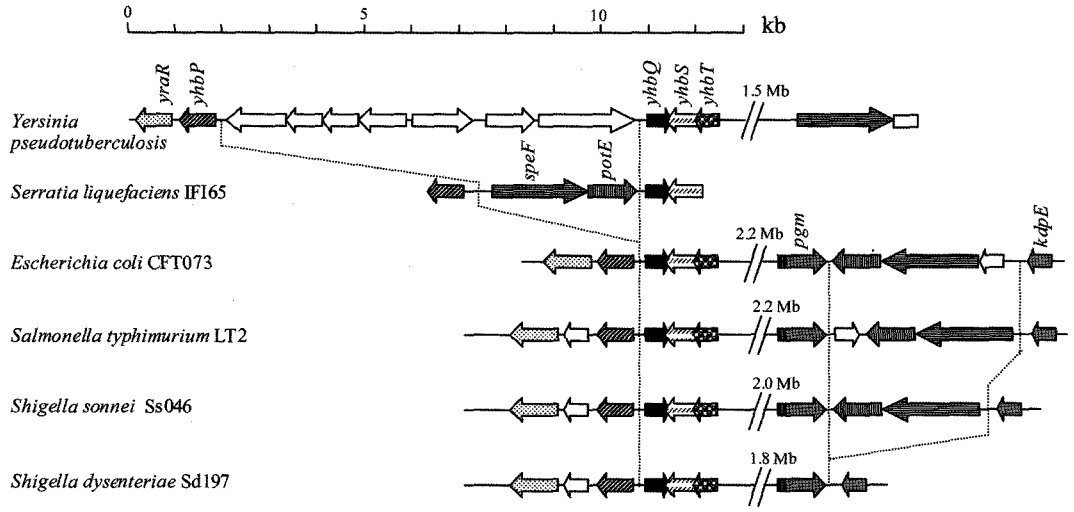

Fig. 2. Genetic organization of the *speF* region from *Serratia liquefaciens* IFI65.
The *speF* regions corresponding to *Yersinia pseudotuberculosis* IP32953 (Accession No. NC_006155; positions 598440-586194 and 3809326-3807164); *Escherichia coli* CFT073 (Accession No. NC_004431; positions 3732448-3734932, and 755903-762468); *Salmonella typhimurium* LT2 (Accession No. NC_003197; positions 3436986-3440056 and 759758-767016); *Shigella sonnei* Ss046 (Accession No. NC_007384; positions 3475024-3478089 and 676497-683074); and *Shigella dysenteriae* Sd197 (Accession No. NC_007606; positions 3088923-3091988 and 588719-591200) are also showed. The sequence from *S. liquefaciens* IFI65 was deposited in GenBank under Accession No. AM117811. Genes are represented by arrows and with arrowheads indicating the direction of transcription. Genes having putative identical functions are represented by identical shading. Some of the genes present in these regions are indicated: *yraR* (hypothetical protein), *yhbP* (hypothetical protein), *yhbQ* (hypothetical protein), *yhbS* (hypothetical acetyltransferase), *yhbT* (hypothetical protein), *speF* (ornithine decarboxylase), *potE* (putrescine-ornithine antiporter), *pgm* (phosphoglucomutase), and *kdpE* (*kdp* operon regulator).

Table 1. *speF* locus-encoded proteins in *S. liquefaciens* IFI65.

Protein	Location in nucleotide sequence	Predicted protein (aa/kDa)	Proposed function	Similar polypeptide (aa)	Database accession no.	Degree of identity (%)	Organism
YhbP	< – 367	–	Hypothetical protein	YP0616 (147)	Q8ZBE9	64% (in a 113 aa overlap)	*Yersinia pestis*
				YPTB0506 (147)	Q66F34	64% (in a 113 aa overlap)	*Yersinia pseudotuberculosis*
				YhbP (147)	Q3YX87	45% (in a 112 aa overlap)	*Shigella sonnei* Ss046
SpeF	1,174–3,336	730/79.4	Ornithine decarboxylase	SpeF (720)	Q8EJZ2	76.2%	*Shewanella oneidensis*
				SpeC (720)	Q666L6	74.5%	*Yersinia pseudotuberculosis*
				SpeC (720)	Q8ZHE0	74.4%	*Yersinia pestis*
PotE	3,394–4,716	454/49.4	Putrescine-ornithine antiporter	PotE (439)	Q3ZYB1	83.1%	*Shigella sonnei* Ss046
				PotE (439)	P0AAF2	83.1%	*Escherichia coli* CFT073
				PotE (439)	Q8ZQW7	82.7%	*Salmonella typhimurium* LT2
YhbQ	4,807–5,115	117 / 12.7	Endonuclease	YPTB0498 (95)	Q66F42	74.7%	*Yersinia pseudotuberculosis*
				YP0608 (95)	Q8ZBE1	73.2%	*Yersinia pestis*
				YhbQ (100)	Q3YX86	72.1%	*Shigella sonnei* Ss046
YhbS	5,102– >	-	Acetyltransferase	YPTB0497 (167)	Q66F43	69% (in a 134 aa overlap)	*Yersinia pseudotuberculosis*
				YP0607 (168)	Q8ZBE0	68% (in a 135 aa overlap)	*Yersinia pestis*
				YhbS (167)	Q7CPQ3	62% (in a 134 aa overlap)	*Salmonella typhimurium* LT2

created. The screening of this library using the 1.4 kb internal DNA fragment as a probe identified two overlapping positive phages. A total 5,506-bp *S. liquefaciens* IFI65 DNA fragment was sequenced. Sequence analysis showed the presence of three complete and two partial open reading frames (ORFs) in the ornithine decarboxylase region (Fig. 2). The first incomplete ORF codes for a protein showing the highest similarities (>60% identity) to the hypothetical protein YhbP from *Yersinia pestis* and *Y. pseudotuberculosis* (Table 1). Contiguous and transcribed in the opposite direction from YhbP, we found the protein predicted to encode a 79.4-kDa ornithine decarboxylase, since the product of this gene is >70% identical to ornithine decarboxylases from several *Enterobacteriaceae*. Protein alignments with known ornithine decarboxylases revealed that the amino acid residues involved in enzymatic activity are conserved in the *S. liquefaciens* protein (data not shown). The third ORF is identified as the *potE* gene. It encodes a 454-amino acid residue protein showing 83.1% identity to the putrescine-ornithine antiporter from *E. coli*. PotE can catalyze both the uptake and excretion of putrescine [7]. The next ORF showed more than 74% identity to *Y. pseudotuberculosis* YhbQ endonuclease. Finally, downstream of YhbQ and divergently transcribed, we found the fifth and uncomplete ORF, showing high protein similarity to YhbS acetyltransferase from *Yersinia* strains.

To confirm that the *speF* gene from *S. liquefaciens* IFI65 encodes a functional ornithine decarboxylase, we expressed this gene in *E. coli* HT414, a defective mutant, by the strategy described in Material and Methods. We prepared cell extract from *E. coli* HT414 harboring the control pIN-III(lpp^p-5)A3 plasmid and the recombinant plasmid pURI4. TLC analysis of supernatant of sonicated cell lysates prepared from *E. coli* HT414 harboring pURI4 showed that this cell lysate was able to decarboxylate the ornithine present in the reaction into putrescine, whereas extracts prepared from control cells containing pIN-III(lpp^p-5)A3 control plasmid did not (Fig. 1). The *speF* gene thus encodes a functional ornithine decarboxylase in *S. liquefaciens* IFI65.

Studies on decarboxylase activity distribution have already provided some valuable chemotaxonomic information within the *Enterobacteriaceae* family [1]. Out of the 63 species of *Enterobacteriaceae* analyzed on the current edition of the

Bergey's Manual of Systematic Bacteriology, half of them were positive for ornithine decarboxylase activity. Nowadays, the complete genome sequences of several *Enterobacteriaceae* have provided information about bacterial chromosomal regions containing ornithine decarboxylase encoding genes. In *Enterobacteriaceae*, in spite of the availability of several of these gene sequences, only the functionality of the *E. coli* ornithine decarboxylase protein has been demonstrated [9].

As showed in Fig. 2, according to their evolutionary relatedness, a different gene organization could be observed in the sequenced *Enterobacteriaceae* strains, suggesting an evolution by module assembly. Based on the genetic organizations shown in Fig. 2, we can propose a hypothetical pathway for the acquisition of putrescine biosynthetic genes in some *Enterobacteriaceae*. It is well known that horizontal gene transfer is an important mechanism for generating genotypic and phenotypic diversity in bacteria, and putrescine production in *S. liquefaciens* could represent an example. Since only half of the *Enterobacteriaceae* are ornithine decarboxylating bacteria, a non-putrescine producer strain, like *Shigella dysenteriae* Sd197, might have acquired *speF* and *potE* genes from an exogenous source and they were introduced between the genes encoding a phosphoglucomutase (*pgm*) and a regulator of the *kdp* operon (*kdpE*), as shown in *S. sonnei* Ss046. Later, additional genes might have been introduced downstream of *potE* (a putative cytoplasmic protein in *S. typhimurium* LT2) or upstream of *speF* (a hypothetical protein in *E. coli* CFT073). Based on the high similarity showed by their proteins responsible for putrescine production, *S. liquefaciens* could have acquired *speF* and *potE* genes by module acquisition from a *Yersinia*, *Salmonella*, *Shigella*, or *Escherichia* strain, or by an independent event from a similar source. In *S. liquefaciens*, both genes were inserted between *yraR*, which encodes a hypothetical protein, and *yhbQ* coding for an endonuclease (Fig. 2). *S. liquefaciens* proteins showed highest identity values to *Y. pseudotuberculosis* proteins. This fact might suggest that the putrescine production module in *S. liquefaciens* was replaced for a seven ORF module of unknown function in *Y. pseudotuberculosis*. However, the origin of putrescine production in *Y. pseudotuberculosis* appears to be more controversial, since a closer inspection of the ornithine decarboxylase region revealed three interesting details: (i) the *potE* gene was absent, (ii) the presence of a defective integrase gene, and (iii) a putative $tRNA^{Phe}$ gene upstream of the ornithine decarboxylase gene. The impact of phages in bacterial evolution is well known and, since tRNA genes may be targeted by prophages, they represented potential targets for insertion of heterologous genes.

In conclusion, we have elucidated the molecular basis for putrescine production in *S. liquefaciens* IFI65, isolated from a spoiled Spanish dry-cured ham. We have demonstrated that *S. liquefaciens* IFI65 possesses a gene that encodes a functional decarboxylase capable of complementing the mutation shown by an *E. coli* ornithine decarboxylase-defective mutant. Based on their amino acid decarboxylase activity, *Enterobacteriaceae* should be considered a microbial quality and safety-related hazard in the development of control systems for dry-cured ham. Additional and further research on ornithine decarboxylase induction and regulation will help to minimize putrescine production and improve the food safety and quality of meat products.

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