Origin of the Putrescine-Producing Ability of the Coagulase-Negative Bacterium Staphylococcus epidermidis 2015B

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A multiplex PCR method, aimed at the detection of genes associated with biogenic amine production, identified the odc gene encoding ornithine decarboxylase in 1 of 15 strains of Staphylococcus epidermidis. The ability of the positive strain, S. epidermidis 2015B, to produce putrescine in vitro was demonstrated by high-performance liquid chromatography (HPLC). In this strain, the odc gene was detected on plasmid DNA, suggesting that the ability to form putrescine is carried by a mobile element, which explains the fact that the trait is strain dependent within the S. epidermidis species. A 6,292-bp nucleotide sequence harboring the putative odc gene was determined. S. epidermidis ornithine decarboxylase (ODC) showed 60 to 65% sequence identity with known ODCs of Gram-positive as well as Gram-negative bacteria. Downstream of the odc gene, a gene encoding a putative amino acid transporter was found that shared 59% sequence identity with the ornithine/putrescine exchanger (PotE) of Escherichia coli. Cloning and expression of the potE gene of S. epidermidis 2015B in Lactococcus lactis demonstrated that the gene product transported ornithine and putrescine into the cells and efficiently exchanged putrescine for ornithine. Analysis of the flanking regions showed high identity levels with different S. epidermidis plasmid sequences, which would confirm the plasmidic location of the odc operon. It follows that the odc and potE gene pair encodes a putrescine-producing pathway in S. epidermidis 2015B that was acquired through horizontal gene transfer.

Coagulase-negative staphylococci (CNS) are often present in food-related samples and especially in fermented products (cheese or dry sausage) (4, 19, 23, 39). They are considered positive flora involved in the development of organoleptic characteristics of end products. Some strains are even used as starters (11, 41). However, although CNS are generally recognized as safe microorganisms, questions regarding presumption of safety have been raised for some CNS species which are common to human and food environments, such as Staphylococcus epidermidis and S. saprophyticus (responsible for nosocomial and urinary tract infections, respectively). One of the main risk factors identified corresponds to the ability to produce biogenic amines (BA) (33, 34, 43, 44) because of the toxicological implication of these molecules in food products (42). Histamine and tyramine, the main studied amines, can lead to more or less severe intoxications when ingested in great quantities (27) or by people with some catabolic deficiency (9). Putrescine and cadaverine are considered potentiators of the histamine and tyramine effect (21, 31). Most studies concerning biogenic amine production by staphylococci have been performed with fermented meat products (6, 33, 34, 35, 43). Other authors identified CNS strains that produced biogenic amines in other foodstuffs and especially fish-related samples (18, 22).

Biogenic amines (BA) are produced in food matrices containing free amino acids via intracellular bacterial catabolic pathways that consist of a decarboxylase and a transporter responsible for the uptake of the amino acid and the excretion of the amine. The pathways convert histidine to histamine, tyrosine to tyramine, ornithine to putrescine, etc. Alternatively, putrescine can be formed by agmatine deimination, which itself is the decarboxylation product of arginine. These two pathways may participate in the generation of metabolic energy and/or resistance against acid stress. The decarboxylation pathways are secondary metabolic-energy-generating pathways that generate proton motive force, whereas the deiminase pathways produce ATP at substrate level and ammonia (28).

The genes encoding the various decarboxylation pathways responsible for BA formation have been extensively studied in different bacterial groups and especially in lactic acid bacteria (10, 14, 15, 26, 29, 30, 32, 33). Little work has been done on the corresponding genes in CNS. Recently, histidine decarboxylase genes have been characterized in S. capitis (16) and S. epidermidis (K. Yokoi and K. Kodaira, unpublished data; GenBank accession no. AB378754), while a fragment of a putative tyrosine decarboxylase gene was described in an S. epidermidis strain (46). Genome sequencing has demonstrated that putrescine production by ornithine decarboxylation is a trait that is abundantly found in the Gram-negative Gammaproteobacteria but rarely in Gram-positive bacteria. The pathway in Escherichia coli is an example of a well-characterized pathway at both the genetic and physiological levels (24).

In this study, the origin of the ability of S. epidermidis 2015B to form putrescine and the genetic basis of strain-to-strain variation of this trait in the S. epidermidis species were investigated.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. epidermidis* strain 2015B was originally isolated from a mahimahi fish basket (1) and kindly provided by D. Green from the Center of Marine Sciences and Technology, Food Science Department, of the North Carolina State University. Six *S. epidermidis* strains related to dry sausage samples (VIII10B3, VIII20B2, OB2, VIII 10B1, SV1, and S2) were obtained from the collection of the Association pour le Développement de l’Institut de la Viande (ADIV) (Clermont-Ferrand, France), while eight milk (S42) and cheese (S115, S118, and S119 from Camembert cheeses and S147, S148, S149, and S152 from Pont-l’Évêque cheeses) isolates came from the ADI-RNA Normandie collection (Villers-Bocage, France). Strains were grown in tryptic soy broth yeast extract using a GelDoc2000 and then visualized with ethidium bromide staining using a GelDoc2000 and X-ray film. Preparations of DNA. Bacterial cultures were first grown to an optical density at 600 nm (OD600) of 0.6. Total DNA was then extracted from bacterial cultures using 1.5 ml of culture with the Nucleoprep tissue kit (Macherey-Nagel) according to manufacturer’s instructions (50 to 100 ng of DNA was used in all PCR experiments). The DNA was then purified using the Plasmid Miniprep Kit (Qiagen) according to a user-developed protocol (http://www1.qiagen.com/literature/protocols/pdf/rapidPl.pdf). Plasmid restrictions were performed using the AvaI and XhoI restriction enzymes (Fermentas) according to the manufacturer’s instructions.

PCR amplification. The detection of four BA-associated genes (hdc, odc, and the AgDI and TyrDC genes) was performed using a multiplex PCR method. This method corresponded to a modification of the multiplex PCR method previously described by Coton and Cotillon (12) targeting the TyrDC and hdc genes with the specific primers TTD/TDS and HSC/HDC4 as well as a PCR internal control (16S rDNA gene). Two new primer sets were added for detection on the one hand of *ode* genes, ODC1 (5’ NCAYAACARACCAAGYNGG 3’) and ODC2 (5’ GRTANGGNTNCGGCACTTCTC 3’), and, on the other hand, of *AgDI* genes, AgDI1 (5’ CAYGTNGAYGGHSAAGG 3’) and AgDI2 (5’ TGTTGNGTRATR CAGTGTAAT 3’). Primer concentrations were 0.8 μM for ODC1, ODC2, AgDI1, and AgDI2, 0.2 μM for TTD and TDS, 0.12 μM for HSC and HDC4, and 0.05 μM for BFS8 and BSR1541. All multiplex experiments were carried out in the presence of 200 μM deoxynucleoside triphosphate (dNTP) (Invitrogen), 10 μg/ml bovine serum albumin (BSA) (Amersham), and 1 U HotMaster Tag polymerase (5 PRIME GmbH) in a final volume of 50 μl. The amplification program was as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min.

Cloning and expression of *potE*. The sequence encoding the putative ornithine/putrescine exchange was amplified by PCR from total DNA with primer potEFw (5’-GCAGAAAACCATGGTCAAGAAGAAAAAAGGTAGTTACCTC-3’) and primer potERv (5’-GCAGAATGCTAGAAGTTGCAAGTTAATCAGATTATCAGATTACAAA-3’), introducing NcoI and XbaI restriction sites. The 1.47-μb product was ligated in the NICE expression system vector pNZ8048 to yield pNZpotE-Se, which was transformed to Lactococcus lactis NZ9000 (17). For expression of *potE*, L. lactis NZ9000 cells containing pNZpotE-Se were grown in M17 medium supplemented with 0.5% glucose and induced with nisin (5 ng/ml final concentration) in mid-exponential phase. Cells were harvested after 1 h of induction.

Putrescine and putrescine transport assays. After 1 h of nisin-induced *potE* expression, cells were washed in 100 mM potassium phosphate (KP) buffer, pH 6.0, and resuspended in the same buffer to an optical density at 600 nm (OD600) of 2. Glucose was added to 0.2% (wt/vol), and 100-μl samples were incubated at 30°C with constant stirring. After 5 min of preincubation, 14C-labeled ornithine (PerkinElmer) or putrescine (Amersham) was added to a final concentration of 17.5 μM or 4.5 μM, respectively. Uptake was stopped at the indicated time points by the addition of 2 ml of ice-cold 0.1 M LiCl solution, immediately followed by filtration through a 0.45-μm-pore-size nitrocellulose filter (BA 85; Schleicher & Schuell GmbH). The filter was washed once with 2 ml ice-cold 0.1 M LiCl and submerged in Emulsol Scintillator Plus scintillation fluid (Packard BioScience), and the retained radioactivity was measured in a Tri-Carb 2000CA liquid scintillation analyzer (Packard Instrumentation). In the exchange experiment, 5 μl of 1 M unlabeled ornithine was added to the cells, 1 min after the addition of 14C-labeled putrescine.

Nucleotide sequence accession number. The nucleotide sequence determined in this work has been deposited in GenBank (accession no. GU799625).

RESULTS

Genotypic and phenotypic characterization of putrescine production by *Staphylococcus epidermidis* strains. The ability to produce biogenic amines, including histamine, tyramine, and putrescine (from either ornithine or agmatine), was evaluated in *S. epidermidis*. Total DNA isolated from 15 strains of food-related *S. epidermidis* was assayed for the presence of genes corresponding to histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tyrD*), and agmatine decarboxylase (*AgDI*) using a multiplex PCR method.

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The ability of the 15 strains to form biogenic amines in vitro was evaluated on the improved detection medium (5) supplemented with six precursor amino acids (histidine, lysine, ornithine, phenylalanine, tryptophan, and tyrosine) and measured by HPLC. The S. epidermidis 2015B culture supernatant was the only one to contain significant amounts of biogenic amines, namely, putrescine (1,460.85 μg/ml) and, to a lesser extent, cadaverine (146.92 μg/ml). Remarkably, while S. epidermidis 2015B (isolated from a mahimahi fish basket) was described as a histamine-forming strain (1), only a very minor amount of this amine (4.03 μg/ml) was produced. Two strains, namely, OB2 and S42, produced minor amounts of tryptamine (6.01 and 14.00 μg/ml, respectively) and phenylethylamine (26.10 and 26.34 μg/ml, respectively).

To localize the putrescine production pathway, plasmid DNA was extracted from the S. epidermidis 2015B strain followed by digestion using the restriction enzyme AvaI or XhoI. After electrophoresis, a PCR using the odc primer set was performed on each of the obtained bands by directly targeting each band in the gel as DNA matrix. For both the AvaI and XhoI restrictions, the top band allowed for the amplification of the odc fragment (Fig. 2). The results strongly suggest that a catabolic ornithine decarboxylation pathway encoded on a plasmid is responsible for putrescine formation by S. epidermidis 2015B.

**Characterization of the odc region.** The obtained PCR fragment identifying the odc gene in S. epidermidis 2015B was sequenced and consisted of 832 bp. In order to determine the complete sequence of the S. epidermidis 2015B odc gene as well as of its flanking regions, a multiplex restriction site-PCR (RS-PCR) method, based on the one proposed by Weber et al. (47), was used. The method allows for rapid acquisition of unknown DNA sequences adjacent to a known segment in both the 5’ and 3’ directions. The repetitive use of this method by the creation of new sets of primers based on each newly determined sequence allowed us to obtain a 6,292-bp-long nucleotide sequence (GenBank accession no. GU799625) from the original 832-bp odc partial sequence. Sequence analysis of the fragment revealed the presence of three complete open reading frames (ORFs) flanked by two partial ORFs (Table 1).

The odc gene consisted of 2,175 bp encoding a 724-amino-acid protein exhibiting an ornithine decarboxylase family conserved domain. Sequence identities on the order of 60 to 65% were observed with the known ornithine decarboxylases of Gram-positive bacteria, i.e., Oenococcus oeni (AJ746165) and Lactobacillus 30a (LSU11816), as well as ODCs of Gram-negative bacteria, including Haemophilus influenzae (L42023) and Dichelobacter nodosus (CP000513). Comparison of the translated sequence to the ODC sequence of Lactobacillus 30a, for which the crystallographic structure has been determined (37), indicated that the residues involved in enzymatic activity were conserved (31, 38), including the pyridoxal-5-phosphate binding domain and the residue involved in the association of dimers into dodecamers. Although the S. epidermidis 2015B ODC showed the highest percent identity with the O. oeni ODC (GenBank accession no. CAG34069), it lacks the N-terminal extension of the latter (31).

Immediately downstream of the odc gene at an intergenic distance of only 22 bp, and therefore certainly cotranscribed, an open reading frame encoding a 442-amino-acid-long protein was found. The putative protein shares 59% sequence identity with the functionally characterized putrescine-ornithine antiporter (PotE) of E. coli. Homologues of PotE are found downstream of the ODC-encoding genes of O. oeni (CAM07323) and various Gram-negative bacteria as well (Fig. 3). It is likely that the two genes form a single transcriptional unit encoding the ornithine decarboxylation pathway. The same operon structure is not found around the odc genes found in a group of lactobacilli. Rather, an amino acid trans-
### Functional expression of potE in Lactococcus lactis.

The potE gene of *S. epidermidis* was cloned in the NICE expression system vector pNZ8048 (plasmid named pNZotSotE-Sε) for nisin-inducible expression in *L. lactis* NZ9000 (17). At a concentration of 17.5 μM ^14^C-ornithine, the host cells containing the empty vector pNZ8048 took up ornithine at an initial rate of 2.7 nmol min^−1^ mg^−1^, demonstrating the presence of an endogenous ornithine transport system in *L. lactis* NZ9000 (Fig. 4A). *L. lactis* cells expressing the *S. epidermidis* transporter gene showed a higher initial rate of ornithine uptake of 5.0 nmol min^−1^ mg^−1^, demonstrating that potE encoded an ornithine transport protein. Even more convincing was the uptake of putrescine at an initial rate of 7.8 nmol min^−1^ mg^−1^ at a concentration of 4.5 μM by the recombinant strain, while the host strain did not take up significant amounts of ^14^C-labeled putrescine until a plateau was reached, after which an approximately 200-fold excess of unlabeled ornithine was added. The result was a rapid release of ^14^C-labeled putrescine from

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Functional expression of *S. epidermidis* PotE in the ornithine decarboxylation pathway is the combined uptake of ornithine and excretion of putrescine. To demonstrate that the transporter catalyzes ornithine/putrescine exchange, cells were allowed to take up ^14^C-labeled putrescine until a plateau was reached, after which an approximately 200-fold excess of unlabeled ornithine was added. The result was a rapid release of ^14^C-labeled putrescine from

### TABLE 1. odc region putative encoded proteins

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the cells, showing the exchange mode of transport (Fig. 4B, triangles).

**DISCUSSION**

Concerning biogenic amine production by staphylococci, several studies have been performed in various foodstuffs. In fermented meat products, the results obtained are contrasted; while the ability of strains of *S. carnosus* or *S. xylosus* to form BA was reported in dry sausages (34, 35, 43), other studies indicated that no or few BA producers were found among CNS strains in the same type of products (6, 33). In fish-related samples, BA-producing CNS strains were also observed and belonged mainly to the *S. epidermidis* and *S. capitis* species in salted anchovies (22) and *S. hominis* in temperature-abused tuna (18). BA production by CNS in dairy products does not seem to constitute a safety problem so far (25), although Straub et al. (44) reported that an *S. epidermidis* strain isolated from a cheese surface was able to produce BA.

In this study, the genotypic and phenotypic characterization of food-related *S. epidermidis* isolates for biogenic amine production indicated that only 1 out of 15 strains was able to produce significant amounts of biogenic amines (putrescine and, to a lesser extent, cadaverine), thus indicating the existence of a strain-to-strain variation in the ability to form BA in this species.

Some authors have already shown the existence of a strain-dependent ability to form BA in various bacterial species. For instance, the ability to form histamine was shown to be strain dependent in *O. oeni* (14) and *Lactobacillus hilgardii* (30). In the latter case, the amine-producing pathway was harbored by an unstable plasmid (30), whereas for tyramine production, the presence of a putative genomic island harboring the complete *TyrDC* operon was shown to be associated with the ability to form tyramine in *L. brevis* (13). Concerning putrescine production, Marcobal et al. (32) recently demonstrated that the ability to form putrescine in *O. oeni* was a strain-dependent trait and that the involved genes, the ornithine decarboxylase, *odc*, and the putrescine-ornithine antporter, *potE*, were situated at the chromosomal level in a region that was certainly acquired through horizontal gene transfer (HGT).

For *S. epidermidis* 2015B, the presence of a sequence corresponding to a mobilization protein (MobC) was observed upstream of the *odc* gene. In *S. aureus*, MobC has been shown to correspond to a nicking accessory protein belonging to the relaxosome complex, also constituted by the MobA (DNA relaxase) and MobB (mobilization accessory protein) proteins, that allows for the preparation of plasmid transfer between bacterial cells by conjugation. MobC proteins have been shown to possess binding specificity for the origin of transfer (*oriT*) and therefore contribute to the site- and strand-specific cleavage at a unique nick site (*nic*) within the *oriT* performed by the relaxosome (8). The obtained *mobC* sequence, also found on various *S. epidermidis* plasmids, suggested that the putative putrescine production pathway could be carried by a plasmid. Comparison to an international data bank of the 628-bp intergenic sequence situated between the putative *mobC* and *odc* genes indicated that the 380 leftmost base pairs presented high identity levels (from 91 to 96%) with regions of *S. epidermidis* plasmids (i.e., GenBank accession no. SEU40259 and GQ900505). For the intergenic sequence situated between the putative cation efflux protein and IS*431mec*-like transposase gene, the 306 leftmost base pairs showed a high degree of identity (91%) with *S. epidermidis* plasmidic fragments (i.e., GenBank accession no. GQ900505 and AF045240). Therefore, the obtained sequences as well as experiments performed on the plasmid extraction confirmed that the putrescine production pathway, consisting at least of the *odc* and *potE* genes, is carried by a plasmid in *S. epidermidis* strain 2015B and was thus acquired through HGT. This localization explains the fact that the putrescine production trait is observed to be strain dependent in the *S. epidermidis* species.

Remarkably, as already observed for *O. oeni* (32), the ge-

![FIG. 3. Phylogenetic tree comparing the protein sequences of amino acid transporters associated with ornithine decarboxylases. The tree was constructed using the Pearson correlation; unweighted-pair group method using average linkages (UPGMA) and bootstrap values are indicated at branch points (percentages of 1,000 replications).](image-url)
The role of biogenic formation by amino acid decarboxylation is believed to protect against acid stress and/or to generate metabolic energy (48). The pathways that consist of a decarboxylase and an amino acid/biogenic amine exchanger generate proton motive force through proton consumption in the cytoplasmic decarboxylation reaction and membrane potential generation through electrogenic exchange of the precursor amino acid and the corresponding biogenic amine (36). In this context, Azcarate-Peril et al. (3) identified the odc gene to be involved in acid tolerance in *L. acidophilus* NCFM by challenging mutants with a variety of acidic conditions, and Pereira et al. (40) demonstrated the involvement of ODC in proton motive force generation. Bover-Cid et al. (7) indicated that ornithine decarboxylase in *Lactobacillus curvatus* CTC273 would not seem to be a mechanism to neutralize the acid environment but may rather play a role in supplying metabolic energy.

Finally, the presence, downstream of the putrescine production pathway, of a cation efflux protein-encoding gene involved in detoxification action towards toxic metallic ions raises the question of the existence of a resistance island (combining the putrescine production pathway for acid stress resistance and the cation efflux protein for tolerance to toxic metallic ions), harbored by a plasmid, in *S. epidermidis* 2015B. The effect of the presence of these genes on the adaptability and growth of *S. epidermidis* 2015B in various environmental conditions should be further investigated.

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