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Full Paper

Isolation of the high polyamine-producing bacterium *Staphylococcus epidermidis* FB146 from fermented foods and identification of polyamine-related genes

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It has been reported that the intake of polyamines contributes to the extension of healthy life span in animals. Fermented foods contain high concentrations of polyamines thought to be derived from fermentation bacteria. This suggests that bacteria that produce high levels of polyamines could be isolated from fermented foods and utilized as a source of polyamines for human nutrition. In this study, *Staphylococcus epidermidis* FB146 was isolated from miso, a Japanese fermented bean paste, and found to have a high concentration of putrescine in its culture supernatant (452 µM). We analyzed the presence of polyamines in the culture supernatants and cells of the type strains of 21 representative *Staphylococcus* species in addition to *S. epidermidis* FB146, and only *S. epidermidis* FB146 showed high putrescine productivity. Furthermore, whole-genome sequencing of *S. epidermidis* FB146 was performed, and the ornithine decarboxylase gene (*odc*), which is involved in putrescine synthesis, and the putrescine:ornithine antiporter gene (*potE*), which is thought to contribute to the release of putrescine into the culture supernatant, were present on plasmid DNA harbored by *S. epidermidis* FB146.

Key words: polyamine, fermented foods, miso, *Staphylococcus epidermidis*

INTRODUCTION

Polyamines are hydrocarbon compounds with two or more amino groups in their molecular structures and are found in the cells of almost all organisms, from prokaryotes to higher plants and animals [1]. They have been reported to contribute to the stabilization of genomic DNA [1] and inhibition of abnormal methylation [2], as well as to promote gene transcription and translation [3, 4] and cell differentiation [5].

The relationship between polyamines and cancer has been studied for many years [6–8]. Inhibition of host polyamine biosynthesis has been reported to inhibit cancer growth [9] and is still being studied, but there are no reports of a contribution to cancer initiation. Clinical trials have reported the safety of orally administered 2-(difluoromethyl)-DL-ornithine hydrochloride (DFMO), an ODC inhibitor, but there was no statistically significant difference in cancer prevention [10]. On the other hand, more recent phase II and III studies where DFMO was given both as a single agent and in combination with PCV therapy (procarbazine, lomustine, and vincristine) showed statistically significant improvements in survival in patients with anaplastic astrocytomas and gliomas [11].

In contrast, polyamines have begun to attract attention in recent years as factors that extend the healthy life spans of animals. For example, polyamines have been reported to reduce the risk of cardiovascular disease [12], to inhibit inflammation in blood vessels [13–17], to increase life span [14, 18, 19], to improve brain function [20, 21], and to enhance the intestinal barrier [22]. There are three sources of polyamines for the human body: oral intake [23], intestinal microbiota [24, 25], and biosynthesis in human cells. It is known that the capacity to biosynthesize polyamines declines with age [23], but the manipulation of polyamine biosynthesis is currently difficult. Therefore, in order to control the concentration of polyamines in the body, it would be effective to control the amount of polyamines derived from food and intestinal microbiota. Some fermented foods contain

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high concentrations of polyamines [23], and these polyamines are thought to be derived from fermentation bacteria. If a bacterium that produces high concentrations of polyamines is screened for from these food-related fermentation bacteria, it may be possible to develop supplements containing high concentrations of polyamines from its culture supernatant, and ingestion of the identified high-polyamine bacterium as a probiotic is expected to result in polyamine production in the gastrointestinal tract.

Staphylococcus epidermidis is found mainly in the epidermis and nasal cavity of humans. It is usually nonpathogenic and is known to act as a barrier to protect the skin from other pathogens [26]. However, it can become pathogenic when it enters the human bloodstream through catheters and other artifacts [27]. *Staphylococcus* spp. include food poisoning bacteria such as *Staphylococcus aureus*, but there are also fermented foods containing *S. epidermidis* [28, 29]. Furthermore, *Staphylococcus xylosus* have been used as a starter for fermented foods [30].

In this study, *S. epidermidis* FB146, which is capable of producing high concentrations of putrescine, was isolated from miso, a fermented food (Japanese fermented bean paste). The polyamine-producing ability of *S. epidermidis* FB146 was compared with that of type strains of representative *Staphylococcus* spp. Furthermore, polyamine metabolism and transport genes of *S. epidermidis* FB146, whose whole genome was sequenced in this study, were compared with those of type strains of representative *Staphylococcus* spp.

MATERIALS AND METHODS

Bacterial strains used

Strains used in this study are shown in Table 1. A total of 21 species, comprising 18 and 15 species (some of which overlapped) isolated from foods [29–31] and clinical specimens [32–34], respectively, were purchased from JCM (Japan Collection of Microorganisms, Ibaraki, Japan) and DSMZ (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

Isolation of bacteria from fermented foods

Sterile phosphate buffered saline (PBS) was added to sample containers containing 1 to 2 g of the fermented food samples shown in Supplementary Table 2 to bring their volumes to 10–20 mL in order to obtain 10% (w/v) solutions. Samples were completely suspended by vortexing and stirring. Then, 10^2-, 10^3-, 10^4-, and 10^5-fold stepwise dilutions were prepared using sterile PBS. One hundred microliters of each dilutions were spread onto MRS plates and incubated under anaerobic conditions using AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 120 hr. Colonies were then picked, streaked onto MRS plates, and incubated under anaerobic conditions using AnaeroPack at 37°C for 48 hr. The colonies of 81 strains were each inoculated into 500 µL of MRS broth, incubated under anaerobic conditions using AnaeroPack at 37°C for 48 hr, and stored as frozen glycerol stock.

High-throughput quantification of putrescine in culture supernatants of bacteria derived from fermented foods

Bacterial cells were incubated under anaerobic conditions using AnaeroPack in MRS broth at 37°C for 48 hr. The culture was centrifuged (1,900×g, 20 min), and the culture supernatant was subjected to putrescine quantification by a simple quantification method (PuO-POD-4AA-TOPS method) described previously [35].

Table 1. Bacterial strains used in this study and times at which cultures were sampled during growing and stationary phases

Species	Literature	Sampling time (hr)	
		Growing phase	Stationary phase
S. aureus subsp. *aureus* JCM 20624^T	[28, 29, 32, 33, 34]	7.5	20
S. capitis subsp. *capitis* JCM 2420^T	[28, 29, 32, 33, 34]	12	33
S. caprae DSM 20608^T	[28, 32, 33, 34]	9	20
S. carnosus DSM 20501^T	[28, 32, 33, 58]	9	26
S. cohnii subsp. *cohnii* JCM 2417^T	[29, 31, 32, 53, 58]	20	46
S. epidermidis JCM 2414^T	[32, 33, 34, 58]	15	33
S. epidermidis FB146	n/a	12.5	47
S. equorum DSM 20674^T	[32]	15	33
S. gallinarum DSM 20610^T	[29, 58]	12.5	47
S. haemolyticus JCM 2416^T	[58]	12	33
S. hominis subsp. *hominis* JCM 31912^T	[31, 33]	15	46
S. intermedius JCM 2422^T	[32, 33, 34]	9	20
S. kloosii DSM 20676^T	[32, 33]	47	77
S. lentus JCM 2426^T	[29, 32, 33, 34]	15	33
S. lugdunensis DSM 4804^T	[32, 58]	12	20
S. pasteuri DSM 10656^T	[29, 58]	9	20
S. pettenkoferi DSM 19554^T	[32, 58]	12.5	47
S. saprophyticus subsp. *saprophyticus* JCM 2427^T	[58]	26	47
S. sciuri JCM 2425^T	[32]	9	26
S. simulans JCM 2424^T	[32, 33, 34]	7.5	20
S. warneri JCM 2415^T	[29, 32]	9	20
S. xylosus JCM 2418^T	[32]	12	20

n/a: not applicable.

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Growth curve of S. epidermidis *FB146 and type strains of representative* Staphylococcus *spp.*

S. epidermidis FB146 and type strains of representative *Staphylococcus* spp. were incubated for 96 hr under anaerobic conditions using AnaeroPack in DSMZ 92 medium at 37°C. Determination of the growing and stationary phases was performed as follows. First, the OD_{600} value of the *Staphylococcus* sp. culture was measured over time. When the OD_{600} values of the culture became almost the same in two consecutive readings, the culture was judged to have entered the stationary phase and used as the stationary phase culture. When the OD_{600} value of the culture medium was about half that of the stationary phase culture, the culture was used as the growing phase culture.

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from bacteria obtained from 1 mL of culture using a Wizard® Genomic DNA Purification Kit (Promega, Southampton, Hants, UK). Before DNA extraction using the kit, 60 µL of 10 mg/mL lysozyme and 60 µL of 10 mg/mL lysostaphin were added to the cell pellet, and this was followed by incubation at 37°C for 1 hr to weaken the cell wall of *S. epidermidis* to allow for efficient cell lysis. Then, the pellet was obtained by centrifugation (18,700×g, 1 min).

Plasmid DNA extraction

Plasmid DNA was extracted from bacteria obtained from 1 mL of culture using a Wizard® Plus SV Minipreps DNA Purification System (Promega). Before DNA extraction using the kit, 250 µL of 20 mg/mL lysozyme, 300 µL of 1.5 M sucrose, and 10 µL of 100 U mutanolysin (Sigma) were added, and this was followed by incubation at 37°C for 1 hr to weaken the cell wall of *S. epidermidis* to allow for efficient cell lysis. Then, the cells were pelleted by centrifugation (18,700×g, 1 min) and subjected to plasmid extraction.

Quantification of polyamines by high performance liquid chromatography (HPLC)

The cultures of *Staphylococcus* spp. in DSMZ 92 medium were sampled in the growing and stationary phases. They were centrifuged (18,700×g, 1 min), and the cells and culture supernatant were collected. For measurement of polyamine concentration in the cells, the collected cells were washed twice with PBS (18,700×g, 5 min). They were then resuspended in 300 µL of 5% (v/v) trichloroacetic acid and incubated in boiling water for 15 min. After centrifugation (18,700×g, 5 min), the supernatant was filtered through Cosmonice filter W (Merck, Darmstadt, Germany), and the polyamines in the cells were analyzed by HPLC. Cell debris was dissolved in 300 µL of 0.1 N NaOH. The protein concentration in the NaOH solution was measured by Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercule, CA, USA) and standardized by the protein concentration in the cells.

For the measurement of the polyamine concentration in the culture supernatant, 20 µL of 100% TCA was added to 200 µL of culture supernatant to treat the protein in the culture supernatant. Centrifugation (18,700×g, 5 min) was performed, and the supernatant was filtered with Cosmonice filter W. Polyamines in the filtered supernatants were analyzed by HPLC. An HPLC system (Chromaster, Hitachi Ltd., Tokyo, Japan) equipped with a cation-exchange column (#2619PH, 4.6×50 mm; Hitachi) maintained at 67°C was used to measure polyamine concentrations. Polyamines were eluted by mobile phase A (45.2 mM tri-sodium citrate, 63.3 mM sodium chloride, and 60.9 mM citric acid) and mobile phase B (200 mM tri-sodium citrate, 2 M sodium chloride, 5% ethanol, and 5% 1-propanol). The concentration of mobile phase B was linearly increased from 50 to 85% during minutes 0–6, maintained at 85% during minutes 6–12, increased to 100% during minutes 12–18, maintained at 100% during minutes 18–45, and then returned to 50% for minutes 45–60. Eluted polyamines were derivatized with o-phthalaldehyde using the post-column method and detected using a fluorescence detector (λ_{ex} 340 nm, λ_{em} 435 nm). For the derivatization of polyamines, reaction solution 1 (0.4 N NaOH) and reaction solution 2 (234 mM boric acid, 0.05% Brij-35, 5.96 mM *o*-phthalaldehyde, 0.2% 2-mercaptoethanol) were mixed with the eluate at 67°C. The concentration of each polyamine was calculated based on a standard curve created using standards of known concentrations. The standards used and their retention times were as follows: agmatine, 34.8 min; cadaverine, 20.6 min; carboxyspermidine, 8.4 min; putrescine, 15.2 min; spermidine, 26.0 min; and spermine, 39.1 min.

Culture with L*-ornithine, a precursor of putrescine*

S. epidermidis FB146 was cultured in MRS medium supplemented with up to 1 M L-ornithine and incubated in air at 37°C for 48 hr. The concentration of putrescine in the culture supernatant was then measured by HPLC.

Whole-genome sequencing and de novo assembly of S. epidermidis *FB146*

One microgram of extracted *S. epidermidis* FB146 gDNA was subjected to library construction using a 1D Genomic DNA Ligation Sequencing Kit (SQK-LSK109, Oxford Nanopore Technologies, Oxford, UK) and sequenced on a MinION sequencer (Mk1B, Oxford Nanopore Technologies) according to the manufacturer's protocol. The resulting reads were corrected and assembled into contigs with Canu version 1.9 [36] using the default configuration with the option for a total contig size of 3 Mbp. The raw reads were registered in DDBJ (accession number: PRJDB12978).

Conservation analysis of polyamine biosynthetic enzymes and transporters

Using the amino acid sequence described previously as a query sequence for a tBlastn search [25], the sequence conservation of polyamine biosynthetic enzymes and transporters was analyzed in the genomes of *Staphylococcus* spp. (Table 1). Reference sequences used in the tBlastn analysis are shown in Supplementary Table 3. When the blast score was more than 500 bits, we determined that the sequence was likely to be conserved.

RESULTS

Isolation of bacteria from fermented foods

Thirty-eight, 5, 18, 7, and 13 strains were isolated from narezushi and misos A–D, respectively (Supplementary Table 1). All 81 of the above strains were inoculated into MRS medium and incubated under anaerobic conditions using an AnaeroPack at 37°C for 48 hr, and their growth (OD_{600}) was observed (Supplementary Table 1).

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High-throughput quantification of putrescine in culture supernatants of bacteria derived from fermented foods and determination of a high-polyamine-producing bacterium by 16S rDNA analysis

The concentrations of putrescine in culture supernatants from the 81 isolated strains were estimated (Supplementary Table 1) using the high-throughput quantification method (PuO-POD-4AA-TOPS method) as described previously [35]. The concentrations of putrescine detected in the 81 supernatants ranged from 0 to 206 µM; the culture supernatant of FB146, which was isolated from miso D, had the highest level of putrescine (206 µM; Supplementary Table 1). The PuO-POD-4AA-TOPS method [35] detected 76 µM putrescine in the MRS medium used in this screening; however, an HPLC analysis showed that the MRS medium was almost free of putrescine. Therefore, under the conditions of this experiment, the PuO-POD-4AA-TOPS method was considered unreliable when putrescine was detected at concentrations below approximately 100 µM. Further analyses focused on FB146, the only strain in which more than 100 µM putrescine was detected in the culture supernatant. The concentration of putrescine in the culture supernatant of FB146 was 436 µM, as determined by HPLC analysis, whereas the concentrations of spermidine and spermine were below the detection limit (data not shown).

A 16S rDNA analysis of FB146 was performed to estimate the bacterial species. The following primers were used: 7F, AGAGTTTGATYMTGGCTCAG; 518R, GTATTACCGCGGCTGCTGG; 907R, CCGTCAATTCMTTTGAGTTT; 1492R, GGCTACCTTGTTACGACTT; and 1510R, ACGGYTACCTTGTTACGACTT. A BLAST search [37] using the obtained 16S rDNA sequence, which was composed of 1,442 bp and included the V1–V9 region of isolate FB146, showed a perfect match with the corresponding region of the 16S rDNA of *Staphylococcus epidermidis* JCM 2414^T.

Growth curves of representative* Staphylococcus *spp. and* S. epidermidis *FB146

In this study, 22 species, comprising 18 *Staphylococcus* spp. isolated from foods [29–31], 15 *Staphylococcus* spp. isolated from clinical specimens [32–34] (some of which overlapped with those isolated from foods), and *S. epidermidis* FB146, were cultured in DSMZ 92 medium. Of the 22 strains, the growing phase ended within 15 hr in 9 strains but continued for up to 30 hr in 10 strains. In 3 strains (*Staphylococcus cohnii* subsp. *cohnii* JCM 2417^T, *Staphylococcus hominis* subsp. *hominis* JCM 31912^T, and *Staphylococcus kloosii* DSM 20676^T), growth continued for up to 75 hr (Fig. 1). The culture times for the bacteria sampled during the growing and stationary phases are shown in Table 1.

Polyamine concentrations in culture supernatants of* Staphylococcus *spp.

The concentrations of polyamines in the culture supernatants of *Staphylococcus* spp. during the growing and stationary phases were analyzed by HPLC. Because there are no reports proving that putrescine is extracellularly degraded, an increase in the putrescine level in the culture supernatant is thought to result from the release of putrescine by the cultured bacteria. Increases in the putrescine and cadaverine concentrations in culture supernatants were observed in the growing and stationary phases of two of the tested strains (*S. epidermidis* FB146, *Staphylococcus lugdunensis* DSM 4804^T) among the 22 *Staphylococcus* spp. tested (Fig. 2A, 2B). The culture supernatants of *S. epidermidis* FB146 had putrescine concentrations of 43.4 µM and 452 µM in the growing and stationary phases, respectively (Fig. 2A). They also had cadaverine concentrations of 46.7 µM and 1,207 µM in the growing and stationary phases, respectively (Fig. 2B). The culture supernatants of *S. lugdunensis* DSM 4804^T had putrescine concentrations of 44.6 µM and 47.8 µM in the growing and stationary phases, respectively (Fig. 2A). They also had cadaverine concentrations of 96.0 µM and 1,270 µM in the growing and stationary phases, respectively (Fig. 2B). Spermidine and spermine were not detected in the culture supernatants of any *Staphylococcus* spp. analyzed, either in the growing or stationary phase.

Polyamine concentrations in the cells of* Staphylococcus *spp.

Polyamine concentrations in the cells of *Staphylococcus* spp. were normalized to cellular protein levels and expressed as nmol/mg protein. Cells of *S. epidermidis* FB146 had putrescine concentrations of 2.3 nmol/mg and 48.0 nmol/mg in the growing and stationary phases, respectively (Fig. 3A). They did not contain cadaverine in the growing phase but did have a cadaverine concentration of 113 nmol/mg in the stationary phase (Fig. 3B). Cells of *S. lugdunensis* DSM 4804^T did not contain putrescine in the growing phase but did have a putrescine concentration of 3.5 nmol/mg in the stationary phase (Fig. 3A). They also had cadaverine concentrations of 18.3 nmol/mg and 180 nmol/mg in the growing and stationary phases, respectively (Fig. 3B). Among the 22 *Staphylococcus* spp. tested, spermidine was present in the cells of 17 *Staphylococcus* spp.: in particular, the spermidine concentrations in the cells of *S. intermedius* JCM 2422^T (47.1 nmol/mg) in the stationary phase, *S. aureus* subsp. *aureus* JCM 20624^T (40.9 nmol/mg) in the stationary phase, and *Staphylococcus pasteuri* DSM 10656^T (27.5 nmol/mg) in the growing phase were relatively high (Fig. 3C).

Sequencing of the genome of* S. epidermidis *FB146

According to the next-generation sequencing results, *S. epidermidis* FB146 was predicted to have a 2.5 Mbp chromosome and 11, 25, and 37 kbp linear plasmids. It is noteworthy that the 25 kbp plasmid encodes *odc* (an ornithine decarboxylase gene) and *potE* (the putrescine:ornithine antiporter gene). Since it is relatively uncommon for *Staphylococcus* spp. to have linear plasmids, Sanger sequencing was performed on the 25 kbp plasmid. The results showed that it was not a 25 kbp linear plasmid but was instead a 12.5 kbp circular plasmid (named plasmid A; Fig. 4) and that *odc* and *potE* were 2,175 bp and 1,311 bp long, respectively. In addition, plasmid A encodes for hypothetical proteins, an major facilitator superfamily (MFS) transporter, tryptophan:tRNA ligase, a type I toxin-antitoxin system, a cation transporter, the RepB family plasmid replication initiator protein, and a plasmid recombination enzyme.

Comparative genomics using the chromosome of *S. epidermidis* JCM 2414^T as the reference sequence (accession ID: GCA_006094375.1) and chromosome of *S. epidermidis* FB146 revealed that most of the chromosomes of the two bacteria were nearly homologous, but some regions were completely nonhomologous (Supplementary Fig. 1).

The results of a tBlastn search for the *odc* and *potE* sequences on plasmid A of *S. epidermidis* FB146 showed that the *odc* and

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potE sequences were 88% and 89% identical to those on the plasmid DNA of putrescine-producing *S. epidermidis* 2015B [38], respectively. Phylogenetic trees were constructed using previously reported ClustalW alignments of ODC and PotE proteins (Supplementary Fig. 2); they showed that the ODC (Supplementary Fig. 2A) and PotE (Supplementary Fig. 2B) of *S. epidermidis* FB146 were closer in evolutionary distance to those of *S. epidermidis* 2015 B than to those of any other bacteria included in the trees. Moreover, the positions of each bacterium in the phylogenetic trees of these two proteins were nearly identical.

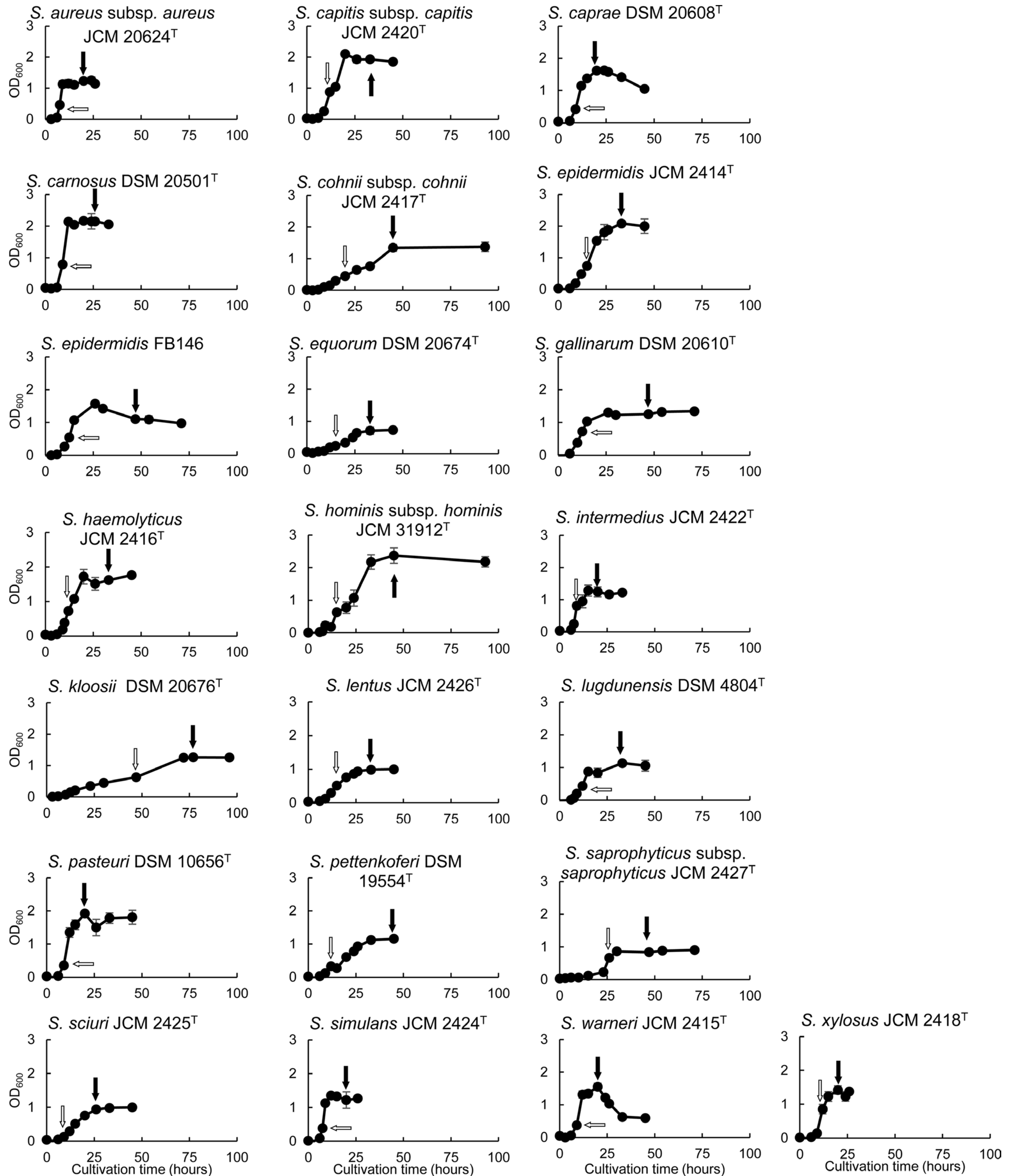

Fig. 1. Growth curves of *Staphylococcus* spp.

The vertical and horizontal axes show the optical density at 600 nm and cultivation time, respectively. White and black arrows indicate the time of sampling of the growing and stationary phase cultures, respectively. Culturing was performed three times independently, and the results are presented as the mean and standard deviation of the measurements.

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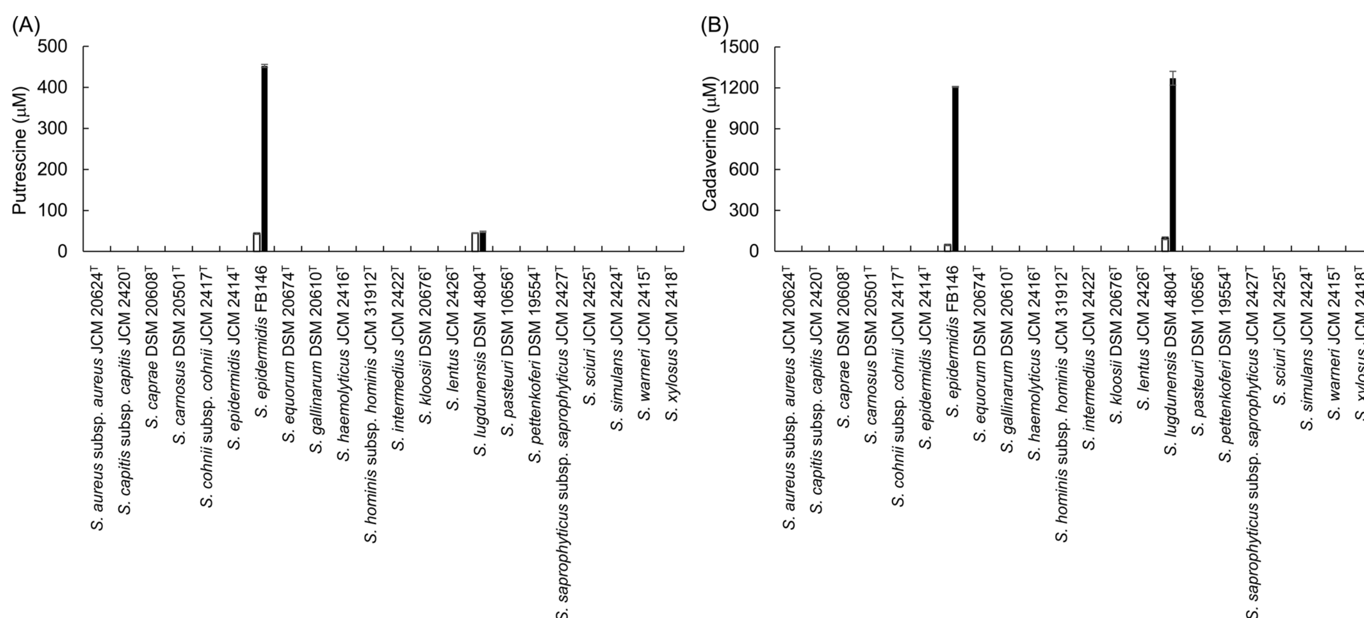

Fig. 2. Polyamine concentrations of culture supernatants of *Staphylococcus* spp.

Putrescine (A) and cadaverine (B) concentrations of culture supernatants of the tested *Staphylococcus* spp. in the growing and stationary phases were quantified by HPLC. The white and black bars indicate the polyamine concentrations of the culture supernatants in the growing and stationary phases, respectively. Data are presented as the mean ± SD (n=3).

Ideally, bacteria used as probiotics should not produce any histamine. No homologs of the *S. capitis hdcA* gene encoding histamine decarboxylase (Supplementary Table 3) were found in the genome of *S. epidermidis* FB146 (data not shown). *S. epidermidis* FB146 did not produce any histamine when it was cultured in MRS medium, but it produced a low concentration of histamine (0.3 µM) when it was cultured in MRS medium supplemented with 10 mM histidine (Supplementary Fig. 3).

Known polyamine biosynthesis, degradation, and transport proteins in tested **Staphylococcus** *spp.*

The results of our tBlastn analysis on the 22 *Staphylococcus* spp. revealed the presence of a single coding region homologous to AdiA (arginine decarboxylase), SpeC (constitutive ornithine decarboxylase), and SpeF (ornithine decarboxylase) on plasmid A of *S. epidermidis* FB146 (from 4,879 to 7,035 nt) and on the chromosome of *S. lugdunensis* DSM 4804^T (from 641,223 to 639,022 nt of NZ_PPPV01000016.1; Fig. 5). Similarly, a coding region (the region from 7,095 to 8,405 nt of plasmid A and the region from 639,043 to 637,730 nt of NZ_PPPV01000016.1) homologous to PotE was detected only on plasmid A of *S. epidermidis* FB146 and on the chromosome of *S. lugdunensis* DSM 4804^T among the 22 *Staphylococcus* spp. (Fig. 5). On the chromosomes of *Staphylococcus gallinarum* DSM 20610^T and *S. kloosii* DSM 20676^T, a single coding region (the region from 8,550 to 9,851 nt of NZ_RXWT01000393.1 and the region from 1,322,496 to 1,323,797 nt of NZ_CP027846.1) homologous to both PlaP (low affinity putrescine importer) [39] and PuuP (putrescine importer) [40] was found (Fig. 5). Homologs of putrescine transcarbamylase (AguB) [41] were found in 21 of the *Staphylococcus* spp. screened, excluding *S. lugdunensis* DSM 4804^T (Fig. 5).

Among the components of the putrescine ABC transporter (PotFGHI) [42], PotG and PotH were found in all tested *Staphylococcus* spp., while PotF and PotI were found in 21 and 20 of the 22 screened species (Fig. 5), respectively. Furthermore, homologs of the ATP-binding protein of the spermidine/putrescine transporter (PotABCD) [43] were found in all *Staphylococcus* spp. (Fig. 5). However, since PotA (ATP-binding protein), PotB (permease), PotC (permease), and PotD (periplasmic binding protein) are highly homologous to PotG, PotH, PotI, and PotF, respectively, each component with homologous function comprising PotABCD and PotFGHI was determined by tBlastn analysis to be homologous to a single coding region on the chromosome of *Staphylococcus* spp. (for example, a single coding region of *Staphylococcus* spp. was determined to be homologous to both PotA and PotG).

Among the components of the putrescine exporter (SapBCDF) [44], SapD and SapF were found in all the *Staphylococcus* spp., while SapB and SapC were found in 16 and 11 species (Fig. 5), respectively. No homologs of *N*-carbamoylputrescine amidohydrolase (NCPAH) [45], arginine decarboxylase (SpeA) [46], or agmatine:putrescine antiporter (AguD) [47] were found in the tested *Staphylococcus* spp. (Fig. 5). A homolog of spermidine *N*-acetyltransferase (SpeG) [48] was found in 16 of the 22 screened *Staphylococcus* spp. (Fig. 5).

Putrescine concentration in the culture supernatant when **S. epidermidis** *FB146 was cultured in a medium supplemented with ornithine*

When *S. epidermidis* FB146 was cultured in MRS medium with ornithine, a precursor of putrescine, putrescine production increased as the amount of supplemented ornithine increased. This increase in putrescine production continued up to 250 mM of supplemented ornithine, resulting in 279 mM putrescine in the culture supernatant of *S. epidermidis* FB146 (Fig. 6).

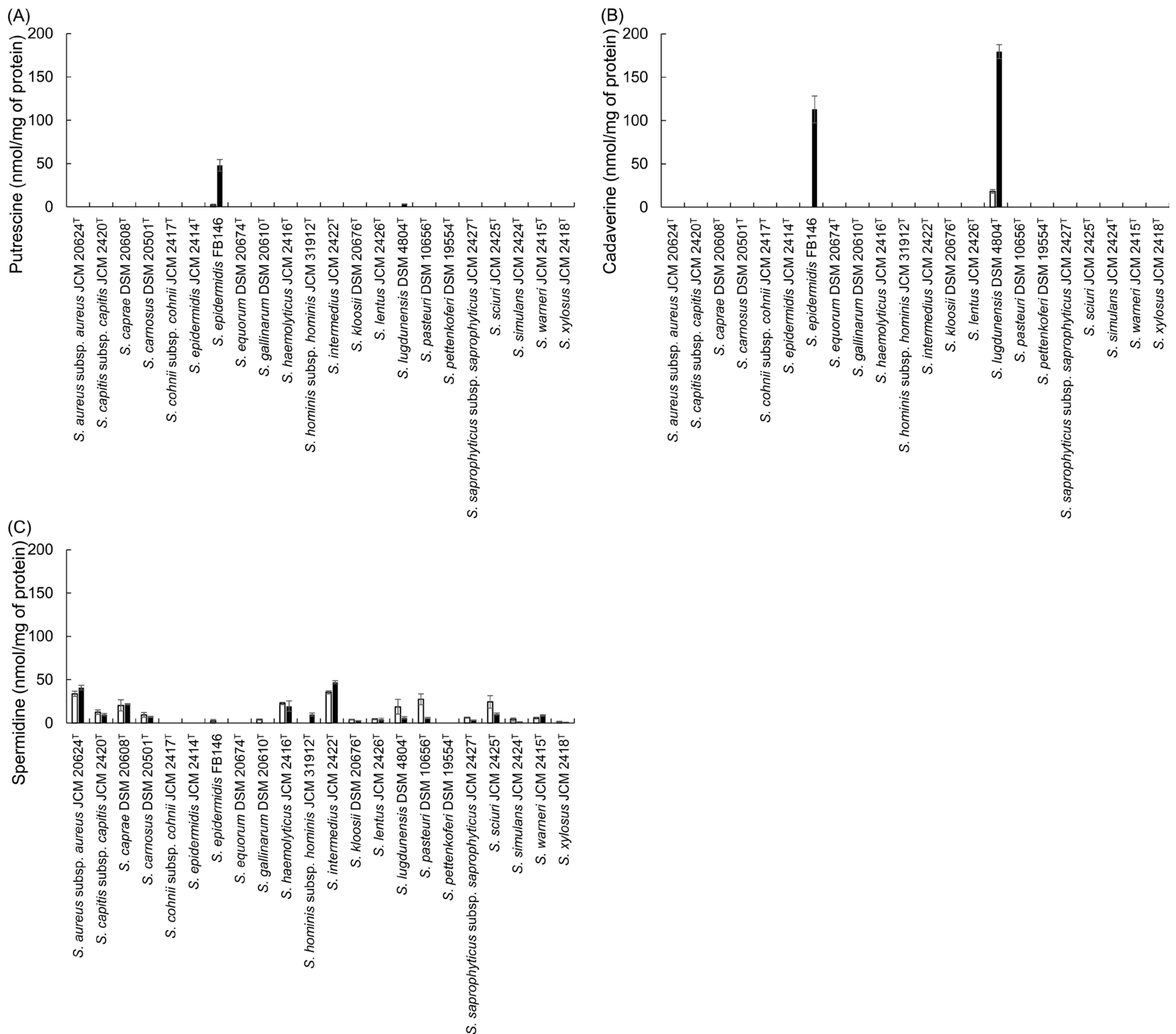

Fig. 3. Polyamine concentrations in cells of *Staphylococcus* spp.

Intracellular putrescine (A), cadaverine (B), and spermidine (C) concentrations of the tested *Staphylococcus* spp. in the growing and stationary phases are shown. The amount of polyamines in the cell was quantified by HPLC and normalized to the cellular protein concentration. The white and black bars indicate the polyamine concentrations of cells in the growing and stationary phases, respectively. Data are presented as the mean ± SD (n=3).

DISCUSSION

In this study, our simple high-throughput quantification method for putrescine (PuO-POD-4AA-TOPS method [35]) was used for high-throughput quantification of putrescine in culture supernatants of 82 strains isolated from fermented foods. The results showed that 206 µM putrescine was present in the culture supernatant of *S. epidermidis* FB146. Given that PuO, one of the enzymes used in this high-throughput quantification method, catalyzing reactions involving not only putrescine but also cadaverine [35], the concentration of putrescine in the culture supernatant might have been overestimated. Therefore, *S. epidermidis* FB146 was grown again, and the putrescine concentration of the culture supernatant was measured by high performance liquid chromatography (HPLC) and compared with those of type strains of representative *Staphylococcus* spp. Among the 22 *Staphylococcus* species tested, the culture supernatants of *S. epidermidis* FB146 and *S. lugdunensis* DSM 4804^{T} contained 452 and 48.1 µM putrescine, respectively, and the other strains did not produce putrescine. Therefore, it could be concluded that *S. epidermidis* FB146 is a high-putrescine-producing *Staphylococcus* derived from fermented foods.

Genus *Staphylococcus* includes *S. aureus*, strains of which cause food poisoning and nosocomial infections. On the other hand, *Staphylococcus* species include *Staphylococcus saprophyticus* and *Staphylococcus carnosus* [29], which are present in the cheese brining process, and *S. xylosus* [49], which is used as a starter for fermented sausage. Therefore, if its safety can be guaranteed, the high-polyamine-producing bacterium *S. epidermidis* FB146 isolated in this study could be used for food

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Fig. 4. Diagram of plasmid A where *odc* and *potE* are encoded.

applications. A previous study identified a polyamine-producing strain of *S. epidermidis* among 15 strains isolated from a fish basket, dry sausages, and milk and cheese [38]. In addition, 2 of the 22 strains in this study were found to be polyamine-producing *Staphylococcus*. However, polyamine-producing *Staphylococcus* was not found in 5 isolates from sausages [50] or 9 isolates from traditional slightly dry fermented and smoked meat products [51]. These results suggest that polyamine-producing *S. epidermidis* strains are rare.

In a previous study, it was reported that 16 mM putrescine was present in the culture supernatant of *S. epidermidis* 2015B derived from mahi-mahi fish cages when the strain was cultured in a medium [52] supplemented with 14.8 mM of ornithine [38]. It was also reported that 7.4 mM putrescine was present in the culture supernatant when sausage-derived *Lactobacillus curvatus* was cultured in MRS medium supplemented with 5.9 mM ornithine [51]. In this study, there was 279 mM putrescine in the culture supernatant when *S. epidermidis* FB146 was cultured in MRS medium supplemented with 250 mM ornithine (Fig. 6), but it is unclear why the putrescine concentration increased by about 30 mM to 279 mM as a result of supplementation with 250 mM ornithine; this was likely due to an experimental error.

Fig. 5. Occurrence of homologous proteins responsible for the biosynthesis, degradation, and transport of polyamines in the genomes of tested *Staphylococcus* spp.

Homologous proteins responsible for the biosynthesis, degradation, and transport of putrescine and spermidine in the tested *Staphylococcus* spp. A tBlastn analysis was performed against the genomes of the tested *Staphylococcus* spp. using query proteins involving polyamine biosynthesis, transport, and degradation pathways.

AdiA: arginine decarboxylase; AguA: agmatine deiminase; AguB: putrescine carbamoyltransferase; NCPAH: *N*-carbamoylputrescine amidohydrolase; SpeA: arginine decarboxylase; SpeB: agmatinase; SpeC: constitutive ornithine decarboxylase; SpeF: inducible ornithine decarboxylase; AguD: agmatine:putrescine antiporter; PlaP: putrescine importer; PotE: putrescine:ornithine antiporter; PotF: putrescine ABC transporter periplasmic binding protein; PotG: ATP-binding protein of putrescine transporter PotFGHI; PotH: permease of putrescine transporter PotFGHI; PotI: permease of putrescine transporter PotFGHI; PuuP: putrescine importer; SapB: transmembrane protein of putrescine exporter SapBCDF; SapC: transmembrane protein of putrescine exporter SapBCDF; SapD: ATP-binding protein of putrescine exporter SapBCDF; SapF: ATP-binding protein of putrescine exporter SapBCDF; AAT: agmatine aminopropyltransferase; APAUH: aminopropylagmatine ureohydrolase; CASDC: carboxyspermidine decarboxylase; CASDH: carboxyspermidine dehydrogenase; SpeD: *S*-adenosylmethionine decarboxylase, proenzyme; SpeE: spermidine synthase; MdtI: multidrug efflux system transporter; MdtJ: multidrug efflux system transporter; PotA: ATP-binding protein of spermidine/putrescine transporter PotABCD; PotB: permease of spermidine/putrescine transporter PotABCD; PotC: permease of spermidine/putrescine transporter PotABCD; PotD: spermidine/putrescine ABC transporter. The color of each column indicates the bit score from the tBlastn analysis: (black) > 500, (dark gray) 300–500, (gray) 200–300, (light gray) 100–200, and (white) <100.

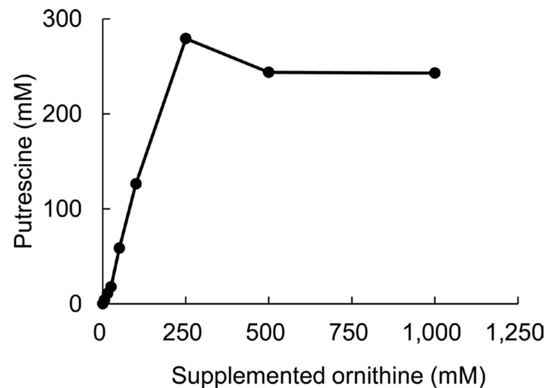

Fig. 6. Putrescine concentrations in culture supernatants when *Staphylococcus epidermidis* FB146 was cultured in MRS medium supplemented with L-ornithine.

S. epidermidis FB146 was grown aerobically for 48 hr in MRS medium supplemented with 5, 10, 14.8, 25, 50, 100, 250, 500, or 1,000 mM L-ornithine, respectively, and the putrescine concentrations of the culture supernatants were analyzed.

An *Escherichia coli* strain genetically engineered to inactivate the pathway for degradation of putrescine produced 275 mM putrescine when cultured in a R/2 medium containing 2,898 mM glucose and 67 mM magnesium sulfate [53]. On the other hand, *S. epidermidis* FB146 is capable of producing putrescine as well as or better than *E. coli*, which is encouraging for industrial production of putrescine, even without genetic modification.

S. epidermidis FB146 produced only 0.3 µM histamine, even in the medium supplemented with 10 mM histidine, which conforms to the limit suggested by the U.S. Food and Drug Administration (Fish and Fishery Products Hazards and Controls Guidance Fourth Edition–June 2021), which is <450 µM. In addition, *S. epidermidis* FB146 does not harbor *hdcA*. Thus, it can be concluded that this bacterium is a low-histamine-producing bacterium similar to the putrescine-producing bacterium *S. epidermidis* 2015 B, which has been reported to be a low-histamine-producing bacterium [54].

The results of a tBlastn analysis showed that AdiA, SpeC, and SpeF were homologous to a single coding region in both the *S. epidermidis* FB146 and *S. lugdunensis* DSM 4804^T strains. Of these three proteins, AdiA, which had the lowest homology, seemed the most unlikely to confer physiological function on *Staphylococcus*. Two types of ODCs have been reported, including biosynthetic (constitutive) SpeC [55] and degradative (induced) SpeF [56]. The expression of *speF* is induced by acidic pH in the environment [57]. The ODC encoded on plasmid A of *S. epidermidis* FB146 is likely SpeF. This is because *potE*—which encodes the putrescine:ornithine antiporter employed to neutralize the pH of the environment by releasing putrescine produced by decarboxylation of ornithine by SpeF—is located next to *speF* on the plasmid and because the sequence of the ODC from *S. epidermidis* FB146 has a higher homology to SpeF than to SpeC. This suggests that plasmid A may confer acid resistance to *S. epidermidis* FB146 through the conversion of ornithine to putrescine, catalyzed by SpeF, followed by putrescine export by PotE. In both *S. gallinarum* DSM 20610^T and *S. kloosii* DSM 20676^T, a single coding region was homologous to PlaP (low affinity putrescine importer) [39] and PuuP (putrescine importer) [40] (Fig. 5). Based on their homology, these two bacterial species were thought to contain PuuP rather than PlaP. Both PotFGHI and PotABCD are ABC transporters, and PotFGHI transports putrescine as a substrate, whereas PotABCD transports putrescine and spermidine as substrates but transports spermidine more efficiently. In most of the *Staphylococcus* spp. tested, both PotFGHI and PotABCD were detected by tBlastn search, but in all of the tested species, only a single polyamine ABC transporter was encoded in the *Staphylococcus* genome. In the genomes of all of the *Staphylococcus* spp. tested, homology in periplasmic binding proteins involved in substrate binding was higher for PotD than for PotF (Fig. 5), suggesting that the *Staphylococcus* spp. tested have PotABCD rather than PotFGHI.

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