A Novel Putrescine Exporter SapBCDF of Escherichia coli

Recent research has suggested that polyamines (putrescine, spermidine, and spermine) in the intestinal tract impact the health of animals either negatively or positively. The concentration of polyamines in the intestinal tract results from the balance of uptake and export of the intestinal bacteria. However, the mechanism of polyamine export from bacterial cells to the intestinal lumen is still unclear. In Escherichia coli, PotE was previously identified as a transporter responsible for putrescine excretion in an acidic growth environment. We observed putrescine concentration in the culture supernatant was increased from 0 to 50 μM during growth of E. coli under neutral conditions. Screening for the unidentified putrescine exporter was performed using a gene knock-out collection of E. coli. Complementation of the deletion mutant with the sapBCDF genes restored putrescine levels in the culture supernatant. Additionally, the ΔsapBCDF strain did not facilitate uptake of putrescine from the culture supernatant. Quantification of stable isotope-labeled putrescine derived from stable isotope-labeled arginine supplemented in the medium revealed that SapBCDF exported putrescine from E. coli cells to the culture supernatant. It was previously reported that SapABCDF of Salmonella enterica sv. typhimurium and Haemophilus influenzae conferred resistance to antimicrobial peptides; however, the E. coli ΔsapBCDF strain did not affect resistance to antimicrobial peptide LL-37. These results strongly suggest that the natural function of the SapBCDF proteins is the export of putrescine.

Polyamines (putrescine, spermidine, and spermine) are alicyclic amines possessing two or more amino groups. Polyamines are widely distributed in eukaryotic and prokaryotic cells (1–4). They are found at high concentrations in proliferating cells, for example cancer cells and bacteria in the exponential growth phase (5, 6).

Polyamines play an important role as growth factors in animals, plants, and bacteria, because polyamines bind to intracellular polyanions such as nucleic acids and promote syntheses of protein and nucleic acid (3, 4). When the biosynthesis of polyamines is disrupted, cell growth is inhibited, whereas exogenous polyamine supplementation reduces the growth inhibitory effects of polyamine deficiency.

In Escherichia coli cells, putrescine is synthesized from ornithine by ornithine decarboxylase (SpeC/SpeF) or from arginine by the sequential actions of arginine decarboxylase (SpeA) and agmatinase (SpeB). Putrescine is converted to spermidine, another polyamine, by the addition of an aminopropyl group derived from decarboxylated S-adenosylmethionine by spermidine synthase (SpeE).

We previously reported that polyamines in the intestinal tract are derived from the gut microbiota (7), and it has been recently reported that polyamines in the intestinal tract impact the health of animals either negatively (8–10) or positively (11–13). Briefly, polyamine catabolism contributes to enterotoxigenic Bacteroides fragilis-induced colon tumorigenesis (8), and levels of rectal mucosal polyamines are increased in colorectal adenoma (9). However, up-regulation of colonic luminal polyamines produced by the intestinal microbiota delays senescence in mice (11, 12). At physiological pH, polyamines are positively charged and hydrophilic, and therefore, they cannot pass through hydrophobic cytoplasmic membranes. Consequently, polyamine transporters are required for their uptake and export in the intestinal bacteria, and the concentration of polyamines in the intestinal tract results from the balance of uptake by polyamine importers and export by polyamine exporters of the intestinal bacteria.

In E. coli, which is a model organism of the intestinal bacteria, five putrescine importers have been experimentally identified. PotFGBHI has been identified as an ATP-dependent putrescine transporter of the ATP-binding cassette

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**This article contains supplemental Fig. S1 and Table S1.

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Novel Putrescine Exporter SapBCDF

(ABC)2 transporter family (14). PotABCD is a spermidine transporter of the ABC transporter family that takes up putrescine with lower affinity (15). PuuP was discovered as a putrescine importer dependent on proton-motive force (16) and is indispensable when E. coli grows on putrescine as a sole carbon or nitrogen source (16). PlaP is a proton-dependent putrescine importer that is important when E. coli exhibits surface motility (17). PotE is responsible for both excretion and uptake of putrescine (18, 19). PotE is a proton-dependent putrescine importer at neutral pH, but at acidic pH PotE is a putrescine-ornithine antiporter (19). An acid-inducible ornithine decarboxylase is encoded by speF, which is located in the same operon as potE (20). SpeF converts ornithine to putrescine with consumption of a proton, and PotE exports putrescine with uptake of ornithine (18). Through this process, E. coli adapts to the acidic environment. In addition, at neutral pH, E. coli excretes putrescine into the environment independently of PotE (21), suggesting that there are other unidentified putrescine exporters in E. coli.

Considering the importance of gut microbes as the source of polyamines in intestine, obtaining a better understanding of polyamine export is clearly of interest. In this study, we performed a genome-wide screening for novel putrescine exporters of E. coli and demonstrated biochemically that the sapBCDF operon contributes to putrescine export from the cell to the environment.

Results

Screening for a Putrescine Exporter—Based on the hypothesis that the putrescine concentration in the culture supernatant of strains with a deletion of the gene encoding a putrescine exporter is lower than that of the parental strain, the putrescine concentration was measured in the culture supernatant of 123 strains with deletions of genes involved in or annotated as transport systems (Fig. 1 and supplemental Table S1). The deletion strains were obtained from the Keio collection, which is an E. coli single gene deletion mutant library that has been described previously (22). The screening indicated that the putrescine concentration of the culture supernatant of E. coli ΔsapF strain (JW1283) was the lowest (18.6 μM) of the tested strains, and the second lowest putrescine concentration of the culture supernatant was 25.5 μM observed in ΔsapD strain (JW1284). These values were significantly lower than those of the parental strain (BW25113, 48.8 μM) or the median (48.7 μM) of the strains tested (Fig. 1A). These results suggested that sapD and sapF contribute to putrescine export from the cell.

Putrescine Concentration of the Culture Supernatant Is Not Influenced by ΔsapA but Is Affected by ΔsapBCDF—An in silico analysis predicts that sapD and sapF are located in the sapAB-CDF operon (Fig. 1B), but the function of sapABCDF has not been experimentally determined. From in silico annotation, SapA is predicted as a periplasmic binding protein of an ABC transporter, and SapB and SapC are predicted as integral membrane proteins of an ABC transporter; furthermore, SapD and SapF are predicted to be ATP-binding proteins of an ABC transporter (Fig. 1B). Based on the hypothesis that sapABCDR encodes a novel putrescine exporter, putrescine concentrations of culture supernatants of ΔsapA (JW1287), ΔsapB (JW1286), and ΔsapC (JW1285) strains were measured. Unexpectedly, the concentration of putrescine in the culture supernatant of ΔsapA (48.3 μM) was almost equivalent to that of the parental strain BW25113. In contrast, the putrescine concentrations of the culture supernatant of ΔsapB and ΔsapC strains were 37% (18.2 μM) and 47% (23.4 μM) that of the parental strain BW25113, respectively. These results indicate that the decrease of the putrescine concentration of culture supernatant came from the deletion of sapB, sapC, sapD, and sapF genes but that sapA was not involved in the decrease of putrescine.

SapBCDF Does Not Contribute to Resistance against Antimicrobial Peptide LL-37—Previous studies have reported that SapABCDF proteins of Salmonella enterica sv. typhimurium (23) and Haemophilus influenzae (24) contribute to resistance

The abbreviations used are: ABC, ATP-binding cassette; S.I.Put, stable isotope-labeled putrescine; S.I.Arg, stable isotope-labeled arginine.

FIGURE 1. Putrescine concentrations of culture supernatant of screened strains and depiction of putative sapABCD operon. A, putrescine concentrations of the culture supernatant of the screened strains. Bacterial strains were grown for 6 h at 37°C with reciprocal shaking at 140 rpm in 5 ml of M9 + tryptone + succinate medium in a 20-ml test tube. Culture supernatant was harvested and subjected to HPLC analysis. Dots in the box plot indicate putrescine concentration of culture supernatant of tested mutants. The concentrations of putrescine of culture supernatant of wild type strain (BW25113, WT), ΔsapD (JW1284), and ΔsapF (JW1283) are indicated as solid dots. B, location of putative sapABCD operon and its deleted or subcloned regions in this study. Locations and directions of genes are indicated by arrows, and the annotations of genes are indicated below the arrows. Locations of puuP encoding putrescine importer (16) and genes encoding enzymes for putrescine degradation pathway, the Puu pathway (16, 36 – 40), are indicated by arrows. Locations of predicted promoters are shown by arrowheads; gray arrowheads indicate α promoters, and black arrowheads indicate α promoters. The deleted regions of the chromosome in the ΔsapBCDF strains and cloned regions in the pACYC184 vector are shown in the illustration.
against antimicrobial peptides by uptake of these peptides followed by intracellular degradation of the peptide bonds. To examine the contribution of sapBCDF of E. coli to resistance against an antimicrobial peptide, the susceptibility of the E. coli MG1655 (wild type) and YS40 (MG1655 ΔsapBCDF) to the antimicrobial peptide LL-37 was analyzed (Fig. 2). E. coli was killed by LL-37 in a manner dependent on the concentration of the antimicrobial peptide; however, susceptibility to the LL-37 was not significantly different in MG1655 and YS40 (ΔsapBCDF) (Fig. 2). These results demonstrate that SapBCDF does not contribute to resistance against the antimicrobial peptide LL-37.

**sapBCDF Increases the Concentration of Putrescine in Culture Supernatant**—To elucidate the role of sapBCDF in the regulation of putrescine concentration in culture supernatant, YS111 (pACYC184/wild type), YS112 (pACYC184/ΔsapBCDF), and YS113 (pACYC184-sapBCDF) were constructed, and the cell density (A_{600}), putrescine concentrations of culture supernatant normalized by the cell density (µM/ A_{600}), and putrescine concentration in the cells (nanomoles/mg of protein) were measured (Fig. 3). Cell growth of YS111 (parental strain) and YS112 (sapBCDF-deleted strain) was not significantly different, although that of YS113 (sapBCDF-complemented strain) was slightly increased compared with the YS111 and YS112 (Fig. 3A). Putrescine concentrations of culture supernatants of YS111 (parental strain) and YS113 (sapBCDF-complemented strain) peaked at 4 h after inoculation, reached 41.9 and 38.4 µM/A_{600}, respectively (Fig. 3B), and decreased to zero at 12 h. In contrast, the peak putrescine concentration of culture supernatant of YS112 (sapBCDF-deleted strain) was 26.2 µM/A_{600} (63% of parental strain) at 4 h after inoculation (Fig. 3B). The difference in the putrescine concentration of culture supernatant between sapBCDF strains SK627 (pACYC184/ΔpuuP, parental strain), SK628 (sapBCDF-deleted strain), and SK634 (sapBCDF-comple-
Novel Putrescine Exporter SapBCDF

ment strain) were constructed in the puuP deletion background. Cell growth of SK627 (parental strain) and SK634 (sapBCDF-complemented strain) was almost identical; however, cell growth of SK628 (sapBCDF-deleted strain) was inhibited compared with SK627 and SK634 (supplemental Fig. S1A). Putrescine concentrations of culture supernatant of SK627 (parental strain) and SK634 (sapBCDF-complemented strain) peaked at 8–10 h, respectively, after inoculation and reached 103.4 and 83.6 μM, respectively (supplemental Fig. S1B). In contrast, the maximum putrescine concentration of culture supernatant of SK628 (sapBCDF-deleted strain) was only 33.6 μM (32% of parental strain) at 12 h after inoculation (supplemental Fig. S1B). Putrescine concentration of culture supernatant normalized by cell growth (μM/A600) showed a similar trend where putrescine concentration of the culture supernatant depended on the presence of sapBCDF (supplemental Fig. S1C). These results demonstrate that sapBCDF plays an important role in increasing putrescine concentration of the culture supernatant.

ΔsapBCDF Does Not Stimulate Putrescine Uptake—To eliminate the possibility that putrescine uptake was facilitated by deletion of sapBCDF, YS233 (pACYC184/ΔpuuP ΔspeB ΔspeC parental strain), YS234 (sapBCDF-deleted strain), and YS235 (sapBCDF-complemented strain) were grown in M9 + tryptone medium supplemented with 100 μM putrescine, and the concentration of putrescine of the culture supernatant was measured. In this experiment, to facilitate comparison of decreases of putrescine in the culture supernatant, export of putrescine from E. coli cells was abolished by deletion of speB and speC genes encoding enzymes for putrescine biosynthesis. Cell growth of YS235 (sapBCDF-complemented strain) was considerably inhibited compared with that of YS233 (parental strain); furthermore, cell growth of YS234 (sapBCDF-deleted strain) was considerably decreased compared with that of YS235 (sapBCDF-complemented strain) (Fig. 4A). Putrescine concentrations of the culture supernatant of tested strains were decreased gradually, but no significant differences of the putrescine concentration of culture supernatants were observed (Fig. 4B). Decrease of the concentration of putrescine normalized by the cell growth (μM/A600) was not significantly different at 8 h after inoculation of the tested strains (Fig. 4C), suggesting that deletion and complementation of sapBCDF did not influence uptake of putrescine from the medium. Taken together, the decrease of putrescine concentration of the culture supernatant by the deletion of sapBCDF (Fig. 3 and supplemental Fig. S1) did not result from increased putrescine uptake but from decreased putrescine export from E. coli cells.

Export of Putrescine by SapBCDF—To demonstrate clearly that the increase of putrescine in the culture supernatant resulted from transport of putrescine from E. coli cells into the environment mediated by SapBCDF, an assay using stable isotope-labeled arginine (S.I.Arg) was performed. In this experiment (Fig. 5A), S.I.Arg is imported into E. coli cells by an arginine transporter and metabolized to stable isotope-labeled putrescine (S.I.Put) via stable isotope-labeled agmatine through sequential reactions catalyzed by SpeA (arginine decarboxylase) and SpeB (agmatine ureohydrolase). If the resultant S.I.Put is exported from the E. coli cells to the medium by SapBCDF, the concentration of S.I.Put in the culture supernatant will be influenced by deletion and complementation of sapBCDF. In the culture supernatant of SK627 (pACYC184/ΔpuuP, parental strain), concentration of S.I.Put was 21.8 μM/A600. In contrast, the concentration of S.I.Put in culture supernatant of SK628 (sapBCDF-deleted strain) was 8.3 μM/A600 and this value was a 62% decrease (p < 0.01, Tukey’s test) from the value of parental strain SK627. In the complementation strain SK634 (sapBCDF-complemented strain), the concentration of S.I.Put in culture supernatant was restored to 77% (16.9 μM/A600) of the value of the parental strain SK627 (Fig. 5B). Total putrescine concentration (Fig. 5C) showed similar trends to S.I.Put concentration in culture supernatant (Fig. 5B), and the ratio of stable isotope-labeled and -unlabeled putrescine was almost same in the three strains used in the study (Fig. 5D), suggesting the stable isotope labeling affected neither arginine metabolism...
nor putrescine export from E. coli cells. These results demonstrated that SapBCDF is responsible for putrescine export.

Discussion
This study has revealed that SapBCDF of E. coli exports putrescine from cells to the extracellular environment. In previous studies, MdtJI of E. coli (25) and Blt of Bacillus subtilis (26) were reported as spermidine exporters. Additionally, in Shigella flexneri it was reported that MdtJI was a putrescine exporter (27). However, in these three reports the strains over-expressing genes of polyamine exporters were used for assays of polyamine export. Furthermore, none of these previous studies analyzed the decreased polyamine export activity of the mutant strains with the deletion of genes encoding polyamine exporters nor measured the polyamine concentration of the culture supernatant (25–27). It was previously reported that PotE is a spermidine exporter (28). However, there are many reports where these methods were not used because of the technical difficulty of the procedure (29). In this study, because inside-out membrane vesicles and the reconstituted proteoliposomes were not used, the kinetic parameters were not determined; however, this study clearly revealed that S.I.Put metabolized from S.I.Arg in E. coli cells was exported from cells to the extracellular environment by SapBCDF (Fig. 5).

SapABCDF is specifically distributed within γ-proteobacteria. Previous studies reported that SapABCDF contributes to resistance of bacteria against cationic antimicrobial peptides as follows: LL-37, β-defensin, and protamine, produced by mam-mals (23, 30). Parra-Lopez et al. (23) reported that S. enterica sv. typhimurium ΔsapABCDF strain was more sensitive to prota-mine than the parental strain, and they hypothesized that S. enterica sv. typhimurium took up antimicrobial peptides followed by the degradation in the cell by peptidases (23). This hypothesis was experimentally confirmed in H. influenzae using LL-37 and β-defensin (30). Furthermore, H. influenzae sapA mutant exhibited attenuated survival in a chinchilla model of otitis media (24). The amino acid identity of SapABCDF in E. coli and S. enterica sv. typhimurium is very high (SapA, 90%; SapB, 92%; SapC, 95%; SapD, 96%; and SapF, 98%). Nonetheless, to date there has been no study showing that SapABCDF of E. coli contributes to resistance against antimicrobial peptides. In this study, it was shown that SapABCDF of E. coli did not contribute to resistance against an antimicrobial peptide LL-37 (Fig. 2). In E. coli, there is no report describing experimentally the function of SapA, SapB, SapC, or SapF, and there has been only one report that SapD (also known as TrkE) of E. coli plays a role as an ATPase for potassium transporters TrkH and TrkG (31). Similarly to E. coli, it was reported previously that the uptake of potassium by the H. influenzae ΔsapD strain decreased, suggesting that SapD is involved in the uptake of potassium (32). In previous reports, it was described that in plants and animals intracellular polyamine inhibited the uptake of potassium from the extracellular environment (33–35).

Therefore, it is possible that in E. coli potassium uptake by TrkH and TrkG driven by ATPase activity of SapD has some relationship to putrescine export by SapABCDF.

Polymamines are important for cell proliferation, and therefore, the intracellular concentrations of polymamines in bacteria are high at exponential growth phase and lower at stationary phase (Fig. 3C) (6), and both degradation and export of polymamines may consume the intracellular pool of polymamines. The Puu pathway is the putrescine degradation pathway (16, 36–40) expressed at early stationary phase. If the regulation of sapABCDF, mediating putrescine export, and the puu gene cluster, responsible for putrescine degradation, is executed in a coordinate manner, the putrescine level effectively decreases from exponential growth phase to stationary phase in E. coli. Because the sapABCDF gene cluster is located immediately adjacent to the puu gene cluster on the E. coli chromosome (Fig. 1B), it is possible that genes of this region are coordinately regulated. Therefore, it is probable that these two co-localized gene clusters, sapABCDF and the puu gene cluster, function to decrease putrescine levels at the end of the exponential growth phase.
**Novel Putrescine Exporter SapBCDF**

**TABLE 1**

| Strain, plasmid, or oligonucleotide | Characteristic or sequence | Source or Ref. |
|------------------------------------|---------------------------|---------------|
| Strain | Characteristic or sequence | Source or Ref. |
| BW25113 | rmB3 ΔlacZΔ4787 | 22, 46 |
| MG1655 | F’ prototrophic | Laboratory stock |
| SK614 | MG1655 but ΔpuuP::kan’ FRT | This study |
| SK623 | MG1655 but ΔpuuP::kan’ FRT | This study |
| SK626 | MG1655 but ΔpuuP::FRT ΔsapBCDF::kan’ FRT | This study |
| SK627 | pACYC184/SK626 | This study |
| SK628 | pACYC184/SK626 | This study |
| SK634 | pSK607/ SK626 | This study |
| YS40 | MG1655 but ΔsapBCDF::kan’ FRT | This study |
| YS111 | pACYC184/MG1655 | This study |
| YS112 | pACYC184/YS40 | This study |
| YS113 | pSK607/YS40 | This study |
| YS226 | MG1655 but ΔpuuP::FRT ΔspeB::FRT ΔspeC::FRT | This study |
| YS227 | MG1655 but ΔpuuP::FRT ΔspeB::FRT ΔspeC::FRT ΔsapBCDF::FRT | This study |
| YS233 | pACYC184/YS226 | This study |
| YS234 | pACYC184/YS227 | This study |
| YS235 | pSK607/YS227 | This study |

| Plasmid | pACYC184 | p15A replicon cat’ tet’ | New England Biolabs |
|---------|----------|-------------------------|---------------------|
| pCP20 | oriR101 bla’ cat’ d857 XPR | 42 |
| pKD3 | oriRy bla’ FRT cat’ FRT | 42 |
| pKD13 | oriRy bla’ FRT cat’ FRT | 42 |
| pSK607 | p15A replicon cat’ sapB’ C’ D’ F’ | This study |

| Oligonucleotide | TTT_HindIII_sapBCDF_start_side | This study |
|-----------------|---------------------------------|-----------|
| AAA_SphI_sapBCDF_term_side | AAAACATGCTTGTTGGGACCCCACACGTCGCA | This study |

In this study, export of putrescine was not inhibited by the deletion of sapA (supplemental Table S1). It is logical that SapA is not involved in the export of putrescine from the cytosol to the extracellular environment because SapA is annotated as a periplasmic substrate-binding protein of an ABC transporter. Furthermore, it was previously reported that sapABCDF of *S. enterica sv. typhimurium* is expressed polycistronically (23) in *E. coli*; however, the predicted promoter of sapA is located independently from that of sapBCDF (Fig. 1B), and the predicted σ factor for sapA (σ70) is different from that for sapBCDF (σ25). Therefore, it is quite possible that sapA and sapBCDF are expressed separately, suggesting that SapBCDF has a function independent of SapA. In this study, as the first report identifying the functions of SapB, SapC, and SapF, it was shown that SapBCDF of *E. coli* exported putrescine from cells to the extracellular environment (Figs. 3 and 5 and supplemental Fig. S1B) but did not contribute to resistance against an antimicrobial peptide LL-37 (Fig. 2). Therefore, it is very probable that SapBCDF is a novel putrescine exporter functioning in the neutral environmental conditions. However, ~30 μM putrescine was detected in the culture supernatant of a ΔpuuP ΔsapBCDF double mutant (supplemental Fig. S1B), suggesting the existence of additional putrescine exporters other than SapBCDF in *E. coli*. Now, we are further investigating putrescine export in *E. coli*, a model organism of intestinal bacteria, to identify additional export mechanisms.

**Experimental Procedures**

**Strains and Plasmids**—Strains used in this study are listed in Table 1; however, the Keio gene knock-out collection (22) used for initial screening for the putrescine exporter is listed in supplemental Table S1. P1 transduction (41) was used to transfer the chromosomal deletion of genes as follows: ΔpuuP (JW1289) in the Keio collection (22), into MG1655 (wild-type background), generating SK614 (ΔpuuP::FRT-kan’-FRT). Plasmid pCP20 (42) was introduced to eliminate the kanamycin resistance gene, generating SK623 (ΔpuuP::FRT). Gene disruptions of speB, speC, and sapBCDF were performed employing a previously described method using pKD3 or pKD13 (42). pSK607 (pACYC184-sapB’ C’ D’ F’) was constructed as follows. The 4,142-bp DNA fragment, including sapBCDF and 500-bp of the upstream region of sapB on the chromosome of *E. coli* MG1655, was amplified by PCR using KOD-plus-polymerase (Toyobo, Osaka, Japan), “TTT_HindIII_sapBCDF_start_side” and “AAA_SphI_sapBCDF_term_side” as primers, and genomic DNA of *E. coli* MG1655 as template. The amplified fragment was cloned into pACYC184 digested by HindIII and SphI, and the cloned region was sequenced to confirm there was no mutation.

**Media and Growth Conditions**—M9 + tryptone medium (M9 minimal medium, except that 1% Bacto-tryptone was used instead of 0.2% glucose) (36) was employed for the bactericidal assay (43) and for analysis of putrescine concentration of the culture supernatant of strains with a deletion of puuP encoding a putrescine importer previously described (16). Because puuP is negatively regulated by succinate (39), 0.2% of sodium succinate was supplemented to the M9 + tryptone medium for analysis of putrescine concentration of culture supernatant of strains with puuP’ backgrounds. One millimolar stable isotope-labeled arginine was supplemented to the M9 + tryptone medium for analysis of stable isotope-labeled putrescine concentrations of the culture supernatant of strains. In screening for putrescine exporters, strains were grown in 5 ml of M9 + tryptone + succinate medium in 20-ml test tubes at 37 °C, with reciprocal shaking at 140 rpm for 6 h. In the other experiments, strains were grown at 37 °C with reciprocal shaking at 140 rpm in 60 ml of media in 300-ml Erlenmeyer flasks.
Bactericidal Assay—To assess the susceptibility of E. coli MG1655 and YS40 (MG1655 except ΔsapBCDF) strains to an antimicrobial peptide LL-37, the experiment was performed according to Harwig et al. (43) with some modifications. Briefly, E. coli MG1655 and YS40 (MG1655 except ΔsapBCDF) were grown in M9 + tryptone medium at 140 rpm at 37 °C for 4 h. An assay medium was prepared by adding 100 μL of LB medium to 6.9 ml of 10 mM sodium phosphate buffer (pH 7.4) and warmed to 37 °C prior to use. Cells were washed with ice-cold 10 mM sodium phosphate buffer (pH 7.4) and resuspended in the same buffer to a concentration of 5 × 10^6 cells/ml. A reaction mixture containing 10 μL of cell suspension, 5 μL of LL-37, and 35 μL of assay medium was incubated for 2 h at 37 °C. The reaction was stopped by adding 450 μL of ice-cold 150 mM sodium chloride to the reaction mixture. After the reaction, the reaction mixture was serially diluted with ice-cold 10 mM sodium phosphate buffer (pH 7.4) and plated on LB medium. Plates were incubated at 37 °C for 22 h, and the numbers of colonies were counted to quantify cell viability in the reaction mixture. Cell viability in the reaction mixture was quantified by counting colony formations. Survival ratios were calculated by dividing the colony-forming units of the cells treated with LL-37 by those of the cells without LL-37 treatment.

Quantification of Polyamines—Polyamine concentrations were quantified by HPLC. HPLC analysis and sample preparation were performed as described previously (44). Briefly, a normal phase HPLC system (Chromaster, Hitachi Ltd., Tokyo, Japan) equipped with a cation-exchange column (catalog no. 2619PH, 4.6 × 50 mm; Hitachi) was used for separation of polyamines. Eluted polyamines were derivatized with o-phthalaldehyde using the post-column method and were detected using a fluorescence detector (λ_ex 340 nm and λ_em 435 nm). The concentration of each polyamine was calculated based on a standard curve created using standards of known concentrations. In the preparation of culture supernatant samples, 500 μL of culture was centrifuged (18,700 × g, 4 °C, 5 min), and the supernatant was collected. To remove proteins, 1/10th volume of 100% trichloroacetic acid was added and mixed using a vortex machine followed by centrifugation (18,700 × g, 4 °C, 30 min). After the centrifugation, polyamines in the supernatant were analyzed by HPLC. In the preparation of whole-cell samples, 500 μL of A_600 = 0.5 (samples cultured for 2 h) or A_600 = 1 (samples cultured for 4–24 h) culture was centrifuged, and the pellet was washed with 1 ml of ice-cold M9 minimal medium without glucose. The washed pellet was resuspended in 300 μL of 5% (w/v) trichloroacetic acid and boiled for 15 min to rupture the cells. The suspension was centrifuged (21,500 × g, 4 °C, 15 min); the supernatant was applied to the HPLC column after filtration using Cosmonice filter W (Nacalai Tesque, Kyoto, Japan), and the precipitated protein was dissolved in 300 μL of 0.1 N NaOH. Protein concentration of the solution was quantified by the Bradford method using a protein assay kit (Bio-Rad). The resulting concentration of putrescine was expressed as nanomoles/mg of total cell protein.

Detection of Stable Isotope-labeled Putrescine by Gas Chromatography-Mass Spectrometry—The amount of stable isotope-labeled putrescine was determined by gas chromatography-mass spectrometry (GC-MS) using a modified version of the methods described in Chen et al. (45). E. coli strains were cultured for 8 h in M9 + tryptone medium supplemented with stable isotope-labeled L-[13C6,15N4]arginine (Wako Pure Chemicals, Osaka, Japan) at a final concentration of 1 mM. The culture supernatant of the strain grown in S.l.Arg was mixed with 10% (v/v) of 100% (w/v) trichloroacetic acid to precipitate proteins. The sample was then clarified by centrifugation at 18,700 × g for 35 min at 4 °C, and 600 μl of the supernatant was extracted by vortexing for 1 min in 2 ml of diethyl ether. The emulsion was then separated by centrifugation at 15,000 × g for 5 min at 4 °C, and the ether layer containing lipids, carbohydrates, and other potential contaminants was discarded, and the aqueous layer was extracted in the same manner once more. A 500-μl aliquot was supplemented with 10 μL of 0.01% (w/v) 1,6-hexanediamine (Kanto Chemical, Tokyo, Japan) as internal standard and was adjusted to pH 11.5 ± 0.5 with 5 M NaOH. To carry out the N-ethoxycarbonylation of the amines, 1 ml of diethyl ether containing 50 μL of ethylchloroformate (Kanto Chemical) was added to the sample solution. The reaction mixture was shaken at room temperature for 30 min and then centrifuged at 15,000 × g for 5 min at 4 °C. The ether layer containing the polyamine N-ethoxycarbonyl derivatives was transferred to a glass tube with a screw cap. This derivatization reaction was repeated by re-extracting the aqueous phase with 1 ml of diethyl ether containing 50 μL of ethylchloroformate. The ether layers from the two extractions were combined and dried under a dry nitrogen stream. Dried N-ethoxycarbonyl polyamine derivatives were taken up in 100 μL of ethyl acetate to which 200 μL of trifluoroacetic acid anhydride (Sigma) was added. The mixture in sealed glass tubes was placed on a 75 °C heating block for 1 h to complete the trifluoroacetylation reaction and then completely dried under a dry nitrogen stream. Derivatives were reconstituted in 200 μL of ethyl acetate, and 2 μL of derivatized samples were injected into a GC-MS. Analysis was carried on an Equity-5 capillary column (30 × 0.25 mm, 0.25-μm film thickness, Sigma) using helium as a carrier gas. Temperatures of injector and source were 260 and 150 °C, respectively. The GC oven was programmed from 140 to 190 °C at 8 °C/min, followed by a 4-min hold, and then to 300 °C at 20 °C/min, followed by a 4-min hold. A final temperature increase to 320 °C at 20 °C/min was held as a bake out for 4 min. Fragment ions were monitored in selected ion monitoring mode, and the ion with m/z 355 was used as the basic fragment for putrescine. Extraction and derivatization rates were standardized using 1,6-hexanediamine, and putrescine was quantified using external calibration curves.

Author Contributions—Y. S. conducted most of the experiments, analyzed the results, and wrote the paper. A. N., A. K., M. S., K. H., and S. K. conducted the experiments. Y. S., A. N., and S. K. wrote the paper. Y. S., M. M., K. I., T. K., H. S., and S. K. edited the manuscript.

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Novel Putrescine Exporter SapBCDF

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