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Recommended Citation
Kurihara, Shin; Sakai, Yumi; Suzuki, Hideyuki; Muth, Aaron; Phanstiel, Otto IV; and Rather, Philip N., "Putrescine Importer PlaP Contributes to Swarming Motility and Urothelial Cell Invasion in Proteus mirabilis" (2013). Faculty Bibliography 2010s. 4247.
https://stars.library.ucf.edu/facultybib2010/4247
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Putrescine Importer PlaP Contributes to Swarming Motility and Urotheilial Cell Invasion in Proteus mirabilis

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Background: Polyanines play roles in bacterial cell-to-cell signaling processes.

Results: In Proteus mirabilis, PlaP is important for putrescine uptake, swarming motility, and urothelial cell invasion, and the putrescine transport inhibitor Triamide-44 inhibits these processes.

Conclusion: PlaP is the primary putrescine transporter in P. mirabilis.

Significance: This research suggests that novel drug cocktails that target both microbial putrescine uptake and biosynthesis can be developed.

Previously, we reported that the speA gene, encoding arginine decarboxylase, is required for swarming in the urinary tract pathogen Proteus mirabilis. In addition, this previous study suggested that putrescine may act as a cell-to-cell signaling molecule (Sturgill, G., and Rather, P. N. (2004) Mol. Microbiol. 51, 437–446). In this new study, PlaP, a putative putrescine importer, was characterized in P. mirabilis. In a wild-type background, a plaP null mutation resulted in a modest swarming defect and slightly decreased levels of intracellular putrescine. In P. mirabilis speA mutant with greatly reduced levels of intracellular putrescine, plaP was required for the putrescine-dependent rescue of swarming motility. When a speA plaP double mutant was grown in the presence of extracellular putrescine, the intracellular levels of putrescine were greatly reduced compared with the speA mutant alone, indicating that PlaP functioned as the primary putrescine importer. In urothelial cell invasion assays, a speA mutant exhibited a 50% reduction in invasion when compared with wild type, and this defect could be restored by putrescine in a PlaP-dependent manner. The putrescine analog Triamide-44 partially inhibited the uptake of putrescine by PlaP and decreased both putrescine-stimulated swarming and urothelial cell invasion in a speA mutant.

Polyamines are aliphatic amines that have two or more amino groups. The basic polyamines are putrescine, spermidine, and spermine, and it has been reported that several plants (1) and bacteria (2) produce a variety of unusual polyamines such as norspermidine. Polyamines are widely distributed from prokaryotic cells (3) to higher plants and animals (4), and polyamines play important roles in a variety of cellular functions, including translation, transcription, and chromatin remodeling.

Recent studies suggest that polyamines play important roles in cell-to-cell signaling in bacteria. In Yersinia pestis, the etiological agent of bubonic and pneumonic plague, SpeA and SpeC, which include the synthetic pathways for putrescine generation, are indispensable in forming normal biofilms. Indeed, the ΔspeA ΔspeC mutant could form biofilms only when the medium was supplemented with putrescine (5). In Vibrio cholerae, a human intestinal pathogen, norspermidine activates biofilm formation in a norspermidine sensor NspS-dependent manner (6) and a mutant in the biosynthetic pathway of norspermidine exhibited severe defects in biofilm formation (7). In Bacillus subtilis, self-produced norspermidine mediates biofilm disassembly by targeting exopolysaccharides (8), and biofilm formation of a speA mutant is dependent on spermidine (9). In Escherichia coli, polyamines are important for both biofilm formation (10) and surface motility (11, 12).

In addition, a growing body of evidence indicates that polyamines are important for bacterial virulence. For example, in Salmonella enterica sv. typhimurium, polyamines are required for virulence (13); in Legionella pneumophila, intracellular growth is enhanced by polyamines derived from host cells (14), and in Streptococcus pneumoniae, polyamine biosynthetic pathways and the spermidine transporter PotABCD are important for infection (15, 16).

In E. coli, putrescine is synthesized from ornithine by ornithine decarboxylase (SpeC and SpeE) or from arginine by the sequential actions of arginine decarboxylase (SpeA) and agmatinase (SpeB) (17). Spermidine can be formed from putrescine by the addition of an aminopropyl group catalyzed by an aminopropyltransferase (SpeE) (18). The source of the propylamine group is decarboxylated S-adenosyl-L-methionine, which is produced by the action of the adenosylmethionine decarboxylase (SpeD) (19). In addition, there are four putrescine transporters, PotE (20), PotFGHI (21), PuuP (22), and
PlaP (12), and one spermidine transporter PotABCD (23) in 

E. coli.

The Gram-negative bacterium Proteus mirabilis is well 

known for its ability to cause urinary tract infections in 

humans (24). A prominent feature of P. mirabilis is the ability to carry 

out a highly coordinated multilayered migration on solid media 
termed swarming (25–27). Swarming involves a complex 

repeating cycle of differentiation between two cell types, vegetative 

tative and swarmer cells. The vegetative form predominates in 

liquid and is a typical Gram-negative rod. When vegetative cells 

are placed on solid surfaces such as agar, the cell differentiates 

into a swarmer cell after a period of 3–4 h. Swarmer cells 

express levels of flagellin, encoded by the flaA locus, that are 

10-fold higher than vegetative cells (28). The process of swarming 

requires that swarmer cells align together to form multilayered 

rafts that translocate across solid surfaces (29). Differentiated 

swarmer cells are more invasive for urothelial cells than 

the vegetative cells (30). P. mirabilis also produces several virulence factors that are coordinately regulated with swarmer cell differentiation, such as urease, IgA protease, and hemolysin (31–33).

It has previously been reported that putrescine is required for 

swarmer cell differentiation in P. mirabilis, possibly by acting as a 
cell-to-cell signaling molecule (34). When extracellular polyamines 

are used as signaling molecules, a polyamine sensor or importer is required because the hydrophilic nature of polyamines prevents their transfer across membranes. Consistent with this, we previously reported in E. coli that spermidine and putrescine induce bacterial surface motility on semisolid media in a spermidine importer PotABCD- (11) and putrescine importer PlaP (12)-dependent manner, respectively.

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(K_m) of PlaP (155 μM) is 40–300 times higher than that 

of other importers reported previously (12). The low affinity of 

PlaP for putrescine may allow E. coli to sense the cell density 

depending on the concentration of extracellular putrescine 

(12). Orthologs of PlaP are distributed among Enterobacteriaceae such as Yersinia, Serratia, Photobacterium, and Morganella. In P. mirabilis, a putative PlaP ortholog (PMI0843) is 459 amino acids and is predicted to be localized to the cytoplasmic membrane. In this study, we report that PlaP is important for putrescine uptake, swelling motility, and urothelial cell invasion of P. mirabilis. In addition, the inhibition of putrescine uptake using the putrescine transport inhibitor Triamide-44 decreased swarming and urothelial cell invasion.

**EXPERIMENTAL PROCEDURES**

**Media and Growth Conditions—**E. coli and P. mirabilis were grown in modified Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% yeast extract, 0.5% NaCl) with reciprocal shaking at 250 rpm at 37 °C or on LB plates (containing 1.5% agar) at 37 °C. For construction of P. mirabilis mutants, LSW plates (1% tryptone, 0.5% yeast extract, 0.5% glycerol, 2% agar) were used. For sucrose selection, 10% sucrose was added to LSW plates. For the analysis of intracellular putrescine concentrations, overnight cultures of P. mirabilis strains were inoculated to the same initial cell density (A_600 of 0.03) into 2 ml of LB or LB + 0.25% NaCl (1% tryptone, 0.5% yeast extract, 0.25% NaCl) broth in test tubes (13 × 100 mm) and were grown at 37 °C with reciprocal shaking at 250 rpm. When the absorbance (A_600) reached 0.2 (~1.5 h after inoculation), putrescine (Sigma) and/or Triamide-44 (35, 36) were added in the concentrations shown in the figure legends. Cells were then harvested after 40 min (when the concentration of putrescine was 25 μM) or 1 h (when the concentration of putrescine was 50 μM or media used was LB + 0.25% NaCl). For cell invasion assays, overnight cultures of P. mirabilis strains were inoculated to the same initial cell density (A_600 of 0.03) into 2 ml of LB broth in test tubes (13 × 100 mm) and were grown at 37 °C with reciprocal shaking at 250 rpm. When the A_600 reached 0.2 (~1.5 h after inoculation), cells were harvested and subjected to the infection assay.

Antibiotics were used for selection at concentrations of 25 μg/ml for both chloramphenicol and streptomycin and 100 μg/ml for ampicillin for E. coli. Antibiotic concentrations for the selection of P. mirabilis were 100 μg/ml for chloramphenicol, 35 μg/ml for streptomycin, and 15 μg/ml for tetracycline.

**Strain and Plasmid Constructions**—For cloning purposes, E. coli strains XL1-Blue and CC118 were used (Table 1). For conjugal matings described previously (37), E. coli strain either SK680 (for introducing ΔplaP) or SK707 (for complementation of plaP^+) was used as the donor strains, and either P. mirabilis PM7002 or PM437 was used as the recipients. Exconjugants were selected on tetracycline and streptomycin to select for a Campbell-type insertion into the plaP region. The resulting strains, SK685 and SK732 harboring both the wild-type allele and the mutated plaP alleles, were subjected to sucrose selection to select for the second recombination event, which results in excision of the vector leaving either the wild-type allele or the plaP:Cm mutation. The plaP:Cm mutation in the resulting strains SK694 and SK738 was confirmed by Southern blot analysis and PCR (supplemental Fig. S1). For complementation of the plaP mutant, the wild-type plaP gene was inserted into the chromosome of the plaP mutant via pKNG101 integration using SK707 as the donor strain and either SK694 or SK738 as the recipient strains. The complemented allele of the resulting strains (SK713 and SK739) was confirmed by Southern blotting and PCR (supplemental Fig. S1). To construct plasmid pUT::mini-Tn5Cm was digested using EcoRI, yielding a 3-kb CmR fragment that was subcloned into pBluescriptII SK(−) digested with EcoRI. To construct pKNG101-plaP^+, the plaP (PMI0843) gene and 500-bp upstream and downstream region of plaP was amplified by PCR using Finnzyme (Thermo scientific) as polymerase, “plaP.up” and “plaP.down” as primers, and P. mirabilis H14320 genomic DNA as template. The resulting 2,520-bp fragment was cloned into pBluescriptII SK(−) digested with EcoRI. To construct pKNG101-plaP^+, the plaP gene was amplified by PCR using the primers “plaP.up” and “plaP.down” and the wild-type plaP gene was inserted into the vector using KpnI and SpeI, yielding a 5.9-kb vector fragment. The resulting vector fragment was subcloned into pBluescriptII SK(−) digested with EcoRI. To construct pKNG101-plaP^+, the plaP gene was amplified by PCR using the primers “plaP.up” and “plaP.down” and the wild-type plaP gene was inserted into the vector using KpnI and SpeI, yielding a 5.9-kb vector fragment. The resulting vector fragment was subcloned into pBluescriptII SK(−) digested with EcoRI.
including the plaP upstream region and 30 bp of the plaP gene, was amplified by PCR using Finnzyme polymerase, “plaP up 1,000 bp” and “plaP 30-bp down ATG” as primers, and H4320 genomic DNA. Amplified 1-kbp fragment was digested with HindIII (in plaP 30-bp down ATG) and BamHI (in “plaP up 1000 bp”). The resulting fragment was ligated to pQ50 digested by HindIII and BamHI. To construct pQ50-plaP-lacZ” (SmR), pQ50-plaP-lacZ” (AmpR) was partially digested using Scal. The resulting 7.8-kbp fragment (digested only once) was ligated to the 2.1-kbp SmR fragment generated by the digestion of pKNG101 by EcoRV and Smal.

**Southern Blot Analysis**—To confirm the appropriate plaP gene disruption or integration of the wild-type plaP gene for complementation in single copy, chromosomal DNA from the plaP mutants (SK694 and SK738) or the complemented strains (SK713 and SK732) were extracted and separately digested with BamHI, HindIII, PstI, and EcoRI before being transferred to a nitrocellulose membrane and probed with a plaP-specific digoxigenin-labeled probe (supplemental Fig. S1).

**Swarm Assays**—To examine swarming phenotypes, overnight cultures of *P. mirabilis* strains were diluted in LB to an *A*600 of 0.4, and 3 μl of the diluted culture was spotted onto the center of LB or LB + 0.25% NaCl (w/v) plates containing 1.5% agar. After incubation at 37°C, the picture was taken or the diameter of swarming was measured.

**β-Galactosidase Assays**—β-Galactosidase assays were carried out as described previously (38). Overnight cultures of *P. mirabilis* strains were inoculated to the same initial cell density (*A*600 of 0.03) into 2 ml of LB or LB + 0.25% NaCl broth in test tubes (13 × 100 mm) and were grown at 37°C with reciprocal shaking at 250 rpm, and cells were harvested at different growth phases. Putrescine (50 μM) or T443 (100 μM) were added to LB broth when the *A*600 reached 0.2 (~1.5 h after inoculation), and cells were harvested after 40 min (when T44 was added) or 1 h (when putrescine was added). *Semi-quantitative RT-PCR*—The cDNA synthesis reaction on total RNA from PM7002 harvested at different growth phases was performed using iScript™ cDNA synthesis kit (Bio-Rad) and the gene-specific primer “plaPcDNA,” following manufacturer’s instructions. The expression of plaP gene was monitored by semi-quantitative RT-PCR using the cDNA product as template and primers “plaP RT for” and “plaP RT rev.” As an internal control, expression of the 16S rRNA gene was also examined from the same RNA samples after random cDNA synthesis using primers “QRT 16S FWD” and “QRT 16S REV.” The absence of contaminating DNA from both samples was confirmed by the inability to generate PCR products in the absence of cDNA synthesis.

**Analysis of Intracellular Putrescine Concentration**—The concentration of putrescine in the cells was measured as described previously (39). Briefly, an HPLC system equipped

### Table 1

| Strains, plasmids, and oligonucleotides in this study | Characteristic or sequence | Source or Ref. |
|--------------------------------------------------------|-----------------------------|---------------|
| Strains (E. coli)                                      |                             |               |
| SK680                                                  | SM10 (Apri)/pKNG101ΔplaP     | This study    |
| SK707                                                  | SM10 (Apri)/pKNG101-plaP     | This study    |
| SM10 (Apri)                                            | thl-1 thr leu tonA lacY supE recA RP4-2-Tc::Mu Km R Apri | V. de Lorenzo |
| **Strains (P. mirabilis)**                             |                             |               |
| H4320                                                  | Wild type, complete genome  sequence was performed | 51, 52        |
| PM7002                                                 | Wild type                   | ATCC          |
| PM437                                                  | speA::mini-Tn5 (KmR) lacZ   | 34            |
| SK685                                                  | speA::mini-Tn5 (KmR) lacZ1, plaP ΔplaP::CmR* | This study    |
| SK694                                                  | speA::mini-Tn5 (KmR) lacZ1 plaPΔplaP::CmR* | This study    |
| SK713                                                  | speA::mini-Tn5 (KmR) lacZ1 plaPΔplaP::CmR*plaP* complemented | This study    |
| SK732                                                  | plaPΔplaP::CmR*             | This study    |
| SK738                                                  | ΔplaP::CmR*                 | This study    |
| SK739                                                  | ΔplaP::CmR* plaP* complemented | This study    |
| SK750                                                  | pQ500-plaP-lacZ (SmR)       | This study    |
| **Plasmids**                                           |                             |               |
| pBluescriptII SK(-)                                    | ColE1 replicon lacZa AmpR    | Stratagene    |
| pKNG101                                                | R6K replicon lacZa AmpR SmR  | 37            |
| pSK                                                    | ColE1 replicon lacZa AmpR SmR | Laboratory stock |
| pKNG101-ΔplaP*                                         | R6K replicon mobB sacB R+ SmR | This study    |
| pKNG101-ΔplaP*                                         | R6K replicon mobB sacB R+ ΔplaP::CmR* SmR | This study    |
| pQ50-ΔplaP-lacZ” (AmpR)                                | pRO1600 replicon plaP-lacZ” AmpR | This study    |
| pQ50                                                   | pRO1600 replicon plaP-lacZ” SmR | This study    |
| pUT::mini-Tn5Cm                                        | R6K replicon mini-Tn5Cm AmpR | 45            |
| **Oligonucleotides**                                   |                             |               |
| plaP.up                                                | 5′-CCACTAGTGGTTTGGATTAATGTGTAATGA-3′ |               |
| plaP.down                                              | 5′-AAGCCCGTCAAGAAGGCGATTACA-3′ |               |
| plaP.cDNA                                              | 5′-CGTGTGTTGTGATCTAATACTA-3′ |               |
| plaP RT for                                            | 5′-GGCACTAGCGAGCGAACCATCA-3′ |               |
| plaP RT rev                                            | 5′-ATGGGGTTGCGATTTTATTACA-3′ |               |
| QRT 16S FWD                                            | 5′-GCTCAGATAGCAGCTGGC-3′    |               |
| QRT 16S REV                                            | 5′-GCGAGACCCCTGCTGTTG-3′    |               |
| M13For                                                 | 5′-GTAACAGCGGCGCACT-3′      |               |
| M13Rev                                                 | 5′-CAACACCAGAAGCACTGATGAC-3′ |               |
| plaP up 1,000 bp                                       | 5′-TTTGAACCTTCACCACTTGAAGAGGCCGT-3′ |               |
| plaP 30-bp down ATG                                     | 5′-CCCAAGCAGTGTGAGGAGATGAC-3′ |               |
with a cation exchange column, TSKgel PolyaminePak (Toso, Tokyo, Japan), was used for separation, identification, and quantification of putrescine. α-Pthalaldehyde was used as the detection reagent, and fluorescence was detected with a fluorescence detector. Standard putrescine was purchased from Sigma.

Urothelial Cell Invasion Assays—Cell invasion assays were performed as described previously (40), with some modifications. UMUC-3 urothelial cells (ATCC) were cultured in EMEM supplemented with 10% fetal calf serum (FCS) in 24-well plates until reaching confluency. Prior to being infected, monolayers were washed twice with serum-free EMEM and resuspended in serum-free EMEM. Harvested bacterial cells, the monolayer was washed twice with 500 μl of 0.1% Triton X-100. A 1:10 dilution series was made with PBS and plated on LB plates containing 3% agar and incubated at 37 °C for 24 h, and viable cells were determined by colony counts.

In P. mirabilis, a putative PlaP homolog (PMI0843) is present, exhibiting 60% amino acid identity to PlaP of E. coli. To investigate the role of the P. mirabilis PlaP homolog, a plaP null mutant was constructed (see under “Experimental Procedures”). The plaP::Cm mutation was first constructed in a P. mirabilis speA::Km mutant deficient in putrescine biosynthesis, as the lower intracellular levels of putrescine in this background would emphasize a role for PlaP in putrescine import. The speA::Km mutant was previously shown to exhibit a severe swarming defect, which could be rescued by the addition of putrescine to agar plates (34). Three strains, PM437 (speA::Km), SK694 (speA::Km, plaP::Cm), and SK713 (speA::Km, plaP::Cm/plaP+ complemented), did not swarm on an LB plate without putrescine because they share the speA::Km mutation and are deficient in putrescine synthesis (Fig. 2B) (34). In contrast, PM437 (speA::Km) and SK713 (speA::Km, plaP::Cm/plaP+ complemented) strains swarmed normally on an LB plate supplemented with putrescine (Fig. 2C). However, SK694 (speA::Km, plaP::Cm) did not swarm on the LB agar supplemented with putrescine (Fig. 2C), indicating that plaP is required for the response to extracellular putrescine.

**RESULTS**

**PlaP Is Required for the Response to Extracellular Putrescine in P. mirabilis**—In P. mirabilis, putrescine is likely synthesized from ornithine by ornithine decarboxylase (SpeF) or from arginine by the sequential actions of arginine decarboxylase (SpeA) and agmatinase (SpeB) (Fig. 1) (34). Interestingly, P. mirabilis lacks the spermidine biosynthetic pathway composed of SpeD and SpeE. In addition, genes encoding enzymes forming alternative pathways for spermidine and norspermidine synthesis are not predicted to be encoded in P. mirabilis (7). Also, in contrast with E. coli having numerous putrescine importers, P. mirabilis appears to have only two, PotE and PlaP, and one spermidine transporter, PotABCD, according to bioinformatic analysis (see Fig. 1). Previously, an E. coli mutant deficient in putrescine synthesis (ΔspeAB and ΔspeC) and missing the polyamine importers PotABCD and YdcSTUV was dependent on both putrescine and the putrescine importer PlaP for surface motility on semisolid media (12).

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Putrescine Importer Adds to Invasiveness of P. mirabilis

PlaP is the Main Putrescine Importer in P. mirabilis—
To directly test the role of PlaP in putrescine import, the intracellular putrescine concentrations for PM437 (speA::Km), SK694 (speA::Km, plaP::Cm), and SK713 (speA::Km, plaP::Cm/plaP+ complemented) were determined after growth in media supplemented with or without 50 μM putrescine (Fig. 2D). The intracellular concentration of putrescine in PM437 (speA::Km) and SK713 (speA::Km, plaP::Cm/plaP+ complemented) grown in the broth supplemented with putrescine (dark gray bars in Fig. 2D) were 7-fold higher than in media without putrescine (white bars in Fig. 2D). However, this increase in the levels of intracellular putrescine was not observed in the plaP::Cm mutant (SK694) (Fig. 2D).

Role of plaP in a Wild-type Background—
To assess the role of PlaP in a wild-type (speA+) genetic background, strains PM7002 (wild type), SK738 (plaP::Cm), and SK739 (plaP::Cm/blaP+ complemented) were subjected to swarming assays, and the intracellular levels of putrescine were determined in each strain. Relative to wild-type PM7002, strain SK738 (plaP::Cm) exhibited a swarming defect with a migration distance that was reduced to 60% of either PM7002 or SK739 (plaP::Cm/blaP+ complemented) on LB + 0.25% NaCl plates (Fig. 3A). The intracellular concentrations of putrescine concentration in PM7002, SK738 (plaP::Cm), and SK739 (plaP::Cm/blaP+ complemented) were not significantly different when P. mirabilis strains were grown in LB + 0.25% NaCl broth (Fig. 3B). When normal LB (containing 0.5% NaCl) was used, neither the swarming defect nor the decrease in intracellular putrescine associated with the deletion of plaP gene was observed.

Regulation of PlaP—
To assay the regulation of plaP, a plaP-lacz transcriptional fusion was constructed in the plasmid pQF50 (41) and introduced into wild-type PM7002. The expression of plaP-lacZ decreased from lag phase to early exponential phase, and then increased in a density-dependent manner with levels of β-galactosidase ~2–3-fold higher at late exponential phase and stationary phase (Fig. 4). Semi-quantitative RT-PCR using total RNA of PM7002 harvested in the different growth phases showed a similar trend, where plaP expression in stationary phase was greater than in exponential phase (data not shown). The addition of putrescine (50 μM) or decreasing the NaCl concentration had no effect on plaP-lacZ expression (data not shown).

Putrescine and the Putrescine Importer PlaP Are Important for the Invasion of Urothelial Cells—
To assess the role of PlaP and putrescine in the invasion of urothelial cells by P. mirabilis,
invasion assays were performed using the urothelial cell line UMUC-3. Exogenously added putrescine at the concentration of 25 μM resulted in a 2-fold increase in the invasion ability of P. mirabilis strains PM437 (speA::Km) and SK713 (speA::Km, plaP::Cm/plaP⁺ complemented) (white and checked bars in Fig. 5B), but putrescine did not increase the invasion ability of SK694 (speA::Km, plaP::Cm) (white and checked bars in Fig. 5B). These results show that extracellular putrescine and its importer PlaP contribute to the ability of a P. mirabilis speA::Km mutant to invade urothelial cells. However, in a speA⁻ background, the plaP mutation did not effect the invasion of strains grown in either LB or LB + 0.25% NaCl (Fig. 6A).

Putrescine Importer Adds to Invasiveness of P. mirabilis

**FIGURE 5.** PlaP-dependent invasion of urothelial cells by P. mirabilis, and inhibition by the putrescine analog T44 on invasion ability, swarming motility, and putrescine uptake of a putrescine-deficient speA mutant. A, chemical structure of T44 is shown. B, strains PM437 (speA::Km), SK694 (speA::Km, plaP::Cm), and SK713 (speA::Km, plaP::Cm/plaP⁺ complemented) were tested for the ability to invade UMUC-3 urothelial cells by the gentamicin protection assay. Wells were supplemented with 25 μM putrescine (checked bars), with 100 μM T44 (black bars), and with both 25 μM putrescine and 100 μM T44 (horizontal striped bars) or without additives (white bars). Data are expressed as the fold increase in invasion over that of PM7002 (wild type) without additives. The assays were performed three times, and values were expressed as the mean ± S.D. *p < 0.01. C, swarming diameter of PM437 (speA::Km), SK694 (speA::Km, plaP::Cm), and SK713 (speA::Km, plaP::Cm/plaP⁺ complemented) is shown during growth on an LB plate supplemented with 25 μM putrescine (checked bars), 100 μM T44 (black bars), and with both 25 μM putrescine and 100 μM T44 (horizontal striped bars) or without additives (white bars). D, intracellular putrescine concentrations of PM437 (speA::Km), SK694 (speA::Km, plaP::Cm), and SK713 (speA::Km, plaP::Cm/plaP⁺ complemented) are shown during growth in LB broth supplemented with 25 μM putrescine (checked bars), 100 μM T44 (black bars), and with both 25 μM putrescine and 100 μM T44 (horizontal striped bars) or without additives (white bars). The assays were performed three times, and values were expressed as the mean ± S.D.

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**DISCUSSION**

In this report, PlaP in *P. mirabilis* was shown to be important for swarming motility and to act as a putrescine importer (Fig. 2). These results are similar to those found in *E. coli*, where PlaP encodes a putrescine importer that is important for surface motility (12). Putrescine can potentially act as a signaling molecule to regulate swarmer cell differentiation in *P. mirabilis* (34). In addition, studies in other bacteria suggest that polyamines can act as signaling molecules for bacterial communication (5–12). It should be noted that these previous studies were performed using polyamine-deficient strains, and former results could stem from severe polyamine deficiency itself (5, 7, 9–12, 34). In this report, however, the *plaP* mutation also resulted in a statistically significant reduction in swarming in a wild-type background (Fig. 3A), indicating that response to extracellular putrescine is required for normal swarming. This further supports the possibility that *P. mirabilis* uses putrescine as a cell-cell signaling molecule.

In this study, the role of PlaP in putrescine uptake and the resulting defects in swarming and invasion were more pronounced in a *speA* mutant due to the very low levels of putrescine present in this strain (Fig. 2D). There are two predicted pathways for putrescine synthesis in *P. mirabilis*, the SpeA/B-dependent pathway that utilizes arginine and the ornithine decarboxylase-dependent pathway that utilizes ornithine (Fig. 1). *E. coli* has two genes encoding ornithine decarboxylase (*speC* and *speF*). Among them, it is thought that *speF* is inactive when bacteria grow at near-neutral pH (42) and ∆speAB ∆speC *E. coli* strains completely lose putrescine inside the cell (12). Additionally, in *E. coli*, *speF* forms an operon with *potE*, encoding a putrescine-ornithine antiporter. In *P. mirabilis*, the gene encoding ornithine decarboxylase is also located next to *potE*, suggesting this is not *speC* but *speF*. The results obtained in this study suggest that the contribution of SpeF to putrescine biosynthesis is insignificant when compared with SpeA because the putrescine concentration in PM437 *speA::Km* is decreased by 90% compared with PM7002 wild type (compare white bar of PM437 in Fig. 2C with white bar of PM7002 in Fig. 3B). In addition, it was previously reported that a *speF* mutant does not exhibit a swarming defect, and a *speB/speF* double mutant is further reduced for swarming (43). These results suggest that putrescine is predominantly made from arginine in *P. mirabilis*. Additional studies using difluoromethylornithine (DFMO), a selective ornithine decarboxylase inhibitor, support this hypothesis. In short, DFMO at 1 mM had no effect on growth or invasion ability (Fig. 6B). In addition to lacking orthologs of genes involved in putrescine import (20, 22, 44) and the spermidine synthetic pathway (19), which was discovered in *E. coli*, *P. mirabilis* lacks degradation pathways (39, 46–50) for putrescine. Therefore, it is thought that *P. mirabilis* has a simple system for the transport and metabolism of putrescine, i.e., a functional synthetic pathway (SpeA and SpeB), a putative exporter (PotE), and an importer (PlaP). This would facilitate *P. mirabilis* in using putrescine as a signaling molecule because the putrescine concentration inside the cell could easily change in response to the concentration of extracellular putrescine.

In *P. mirabilis*, the ability to invade human urothelial cells is coupled to motility and swarmer cell differentiation (30). The polyamine analog T44 partially inhibited the uptake of putrescine mediated by PlaP (Fig. 5D), and reduced the ability of putrescine to rescue swarming in the *speA* mutant (Fig. 5C). Furthermore, the ability of the *P. mirabilis* *speA* mutant to invade human urothelial cells was stimulated by putrescine imported by PlaP and decreased by putrescine transport inhibitor T44 (Fig. 5B). However, deletion of *plaP* did not have an effect on the invasion activity of *speA*+ strains (Fig. 6A), and DFMO, a selective inhibitor for ornithine decarboxylase encoded by *speF*, did not have an effect on either PM7002 (wild-type) or SK738 (∆plaP) (Fig. 6B). These results suggest that
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concurrent use of putrescine transport inhibitor and future inhibitors, which target the arginine to putrescine pathway (SpeA and/or SpeB), may be very effective agents against P. mirabilis.
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J. Biol. Chem. 2013, 288:15668-15676.
doi: 10.1074/jbc.M113.454090 originally published online April 9, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.454090

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