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Structural Basis of Substrate Binding Specificity Revealed by the Crystal Structures of Polyamine Receptors SpuD and SpuE from *Pseudomonas aeruginosa*

Donghui Wu [1, 2]†, Siew Choo Lim [1, 3]†, Yihu Dong [1], Jien Wu [1], Fei Tao [1], Lian Zhou [1], Lian-Hui Zhang [1, 2]* and Haiwei Song [1, 2]*

[1]*Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos, Singapore 138673*
[2]*Department of Biological Sciences, National University of Singapore, 14 Science Drive, Singapore 117543*
[3]*School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551*

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The type III secretion system (T3SS) of *Pseudomonas aeruginosa* is a key virulence determinant whose expression is induced by polyamine signals from mammalian host. SpuD and SpuE were postulated to be spermidine-preferential binding proteins, which regulate the polyamine content in this bacterial pathogen. In this study, we found that SpuD is a putrescine-preferential binding protein, while SpuE binds to spermidine exclusively. We have determined the crystal structures of SpuD in free form and in complex with putrescine and SpuE in free form and in complex with spermidine. Upon ligand binding, SpuD and SpuE undergo an "open-to-closed" conformational switch with the resultant closed ligand-bound forms, SpuD-putrescine and SpuE-spermidine, similar to their *Escherichia coli* counterparts PotF-putrescine and PotD-spermidine, respectively. Structural comparison suggested that two aromatic residues, Trp271 of SpuE and Phe273 of SpuD in segment II region, are the key structural determinants for putrescine/spermidine recognition specificity. Mutagenesis combined with isothermal titration calorimetry showed that substitution of Trp271 by Phe enabled SpuE to gain substantial binding affinity for putrescine, while replacement of Phe273 by Trp reduced the binding affinity of SpuD toward putrescine by 250-fold. Altogether, these results revealed the molecular mechanism governing polyamine recognition specificity by SpuD and SpuE and provide the basis for further structural and functional studies of polyamine signal importation system in *P. aeruginosa*.

**Corresponding authors.* Institute of Molecular and Cell Biology, 61 Biopolis Drive, Proteos, Singapore 138673.
E-mail addresses: lianhui@imcb.a-star.edu.sg; haiwei@imcb.a-star.edu.sg.

† D.W. and S.C.L. contributed equally to this work.

Abbreviations used: ABC, ATP binding cassette; Mes, 4-morpholineethanesulfonic acid; ITC, isothermal titration calorimetry; MBP, maltodextrin binding protein; NTA, nitrilotriacetic acid; GST, glutathione *S*-transferase; RT-PCR, reverse transcription polymerase chain reaction; SeMet, seleno-L-methionine; RP-HPLC, reverse-phase high-pressure liquid chromatography; PEG, polyethylene glycol; SAD, single anomalous dispersion; ASU, asymmetric unit.

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Introduction

Polyamines (putrescine, spermidine and spermine) are small polycationic molecules that are widely used in nearly all prokaryotic and eukaryotic cells. Polyamines play important roles in many molecular and cellular processes. These roles include stabilization of double-stranded DNA,[1] induction of DNA condensation,[2] regulation of DNA–protein interaction,[3,4] modulation of protein synthesis[5–9] and posttranslational modification,[10,11] regulation of cell proliferation, differentiation and apoptosis.[12–14] Given the importance of polyamines identified at the molecular and cellular levels, it is not surprising to link polyamines with several life events, such as involvement in embryonic development[15] and association with many cancers[16–18] and parasite-related infections.[19]

Periplasmic binding proteins are found in bacteria and act as receptors of a wide spectrum of small-molecule substrates for transport and chemotaxis.[20] Polyamine binding proteins belong to a subfamily of periplasmic binding proteins. In *Escherichia coli*, two polyamine uptake systems have been identified. The spermidine-preferential uptake system is composed of four proteins, PotA, PotB, PotC and PotD, while putrescine-specific uptake system is composed of PotF, PotG, PotH and PotI proteins.[21,22] Both uptake systems belong to the family of ATP binding cassette (ABC) transporters,[23] which comprises one substrate binding protein (PotD or PotF) located within the periplasm, two transmembrane proteins for channel formation (PotB and PotC or PotH and PotI) and one ATPase (PotA or PotG) associated with transmembrane proteins for ATP hydrolysis coupled with polyamine uptake.

Despite extensive studies of polyamine transport in *E. coli*, limited studies have been reported about polyamine transport in *Pseudomonas aeruginosa*. *P. aeruginosa* is a Gram-negative pathogenic bacterium and one of the major pathogens to immunocompromised patients such as those in intensive care units or with cystic fibrosis.[24] *P. aeruginosa* displays strong drug resistance to conventional antibiotic treatments.[24] One gene cluster of *spuABCDEFGH* in *P. aeruginosa* PAO1 was identified by Lu *et al.*[25] Based on the phenotypes of gene knock-out and bioinformatics analysis, it was proposed that SpuDEFGH forms an ABC transporter system for spermidine uptake, in which SpuD and SpuE are the periplasmic spermidine-preferential binding proteins, SpuF is the ATPase and SpuG and SpuH form the transmembrane channel. In addition, the same group found that polyamines could induce resistance to cationic peptide, aminoglycoside and quinolone antibiotics in *P. aeruginosa*.[26] Recently, Zhou *et al.*, found that deletion of *spuE* or *spuEFGH* in *P. aeruginosa* PAO1 significantly down-regulates the transcriptional expression of type III secretion system (T3SS), which is one of the major virulence determinants in bacteria.[27] In addition, mutation of the Spu transporter substantially decreased the T3SS response to mouse liver extracts that contain polyamines and attenuated the cytotoxicity on human cell lines.[27] Given the functional link of polyamine transport system with T3SS in *P. aeruginosa* and induction of antibiotics resistance by polyamines, targeting polyamine transport system may be a new therapeutic intervention strategy for fighting against the increasingly serious antibiotics resistance in *P. aeruginosa* by blocking the T3SS-associated virulence through inhibition of polyamine transport. Therefore, it is critically important to understand the mechanism by which the polyamine is recognized and transported.

Structures of PotD in complex with spermidine and PotF in complex with putrescine have been reported.[28–30] The overall structures of PotD and PotF in ligand-bound forms are similar in that both proteins are composed of two globular domains, that is, the amino-terminal (N) and the carboxy-terminal (C) domains and the ligands are engulfed by the two domains. Recently, the structure of TpPotD, a putrescine-preferential receptor from *Treponema pallidum*, has been solved.[31] In this structure, one 4-morpholineethanesulfonic acid (Mes) molecule included as crystallization buffer was found to be situated in a cleft that overlaps with the polyamine binding site of PotD and PotF. Although these structures provided important insights into the ligand-binding properties, the mechanism by which these proteins confer polyamine recognition specificity remains obscure.

To gain insights into the roles of SpuD and SpuE in polyamine transport and the molecular basis of ligand recognition specificity, we performed genetic, biochemical and X-ray crystallographic analyses on these two proteins. SpuD is found to bind putrescine preferentially, whereas SpuE binds spermidine exclusively. We also determined the crystal structures of SpuD in free form and in complex with putrescine (denoted, respectively, as apo-SpuD and SpuD-putrescine hereafter) and SpuE in free form and in complex with spermidine (denoted, respectively, as apo-SpuE and SpuE-spermidine hereafter). Structural analysis combined with mutational data explains why SpuD prefers to bind putrescine over spermidine, while SpuE only binds to spermidine. Altogether, our data reveal the mechanism governing polyamine recognition specificity by SpuD and SpuE in *P. aeruginosa*. The structural data presented in this study may serve as a guide for designing drugs targeting the polyamine uptake system in *P. aeruginosa*.

Results and Discussion

SpuD and SpuE in the regulation of T3SS

Zhou *et al.* found that knock-out of *spuE* or *spuEFGH* in *P. aeruginosa* PAO1 significantly down-