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Short communication

# Site-directed mutagenesis of the *Pseudomonas aeruginosa* type III secretion system protein PscJ reveals an essential role for surface-localized residues in needle complex function

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ABSTRACT

The *Pseudomonas aeruginosa* type III secretion system (TTSS) protein PscJ belongs to the PrgK family of TTSS proteins. These proteins are predicted to form one of the inner membrane localized ring substructures of the TTSS needle complex. To determine which amino acid residues of PscJ are important for its function, the *pscJ* gene was subjected to site-directed mutagenesis. Fifteen individual PscJ amino acid residues that are located in conserved regions of the PrgK family were targeted for mutagenesis. Eight of these residues could be subjected to non-conservative substitution mutagenesis without affecting the function of the resultant mutant protein. Substitution of the other 7 residues (E26, K52, E105, A107, G126, H133, and V189) resulted in either a non-functional protein or the loss of detectable protein. When the essential residues were mapped on to the crystal structure of the *E. coli* PrgK homolog EscJ, the majority appeared to localize to surface-exposed regions of the protein suggesting a role for these regions in the assembly of the PscJ ring structure.

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## 1. Introduction

Type III secretion systems (TTSSs) are important virulence factors for a number of gram-negative bacterial pathogens. These systems serve to deliver effector proteins to the cytoplasm of host cells where they modulate cellular functions in a manner beneficial to the pathogen. One important component of all TTSSs is a membrane-localized protein complex (needle complex) that is believed to act as a conduit through the bacterial cell envelope for secreted TTSS proteins [1,2]. The needle complex was first detected in *Salmonella enterica* serovar Typhimurium and is composed of two separate substructures, a membrane-localized base and an externally located needle that is approximately 50 nm long and is composed of a single protein [3,4]. The base substructure is composed of four ring-shaped structures (two in the outer membrane and two in the inner membrane) connected by a periplasmic-spanning rod. In *S. enterica*, InvG forms the outer membrane rings with PrgK and PrgH forming the remainder of the base [4,5].

The amino acid sequence of the PrgK family of proteins is broadly conserved and is similar to the central domain of the flagella basal body protein FliF. These proteins are predicted to form a multimeric membrane-localized ring structure [6]. Detailed structural analyses have been carried out on the *E. coli* PrgK homolog EscJ, which lacks a carboxyl-terminal section that is present in the other members of the PrgK family. Studies using nuclear magnetic resonance spectroscopy (NMR) showed that EscJ is composed of two domains linked by a flexible linker region [7]. Expression of the two domains from separate vectors in an *escJ* mutant strain failed to complement the secretion defect, suggesting that the linker is required for the function of EscJ. Interestingly, expression of the two domains in the wild-type strain had a dominant negative phenotype with regard to type III secretion. This would suggest that regions in these domains are responsible for the assembly of the multimeric ring. The recent crystal structure of EscJ has provided a much more detailed view of this protein [8]. In agreement with the NMR studies, the crystal structure shows EscJ to be composed of two domains, an amino-terminal domain and a larger carboxyl-terminal domain connected by a linker region. Each domain contains two alpha-helices flanked by a three-stranded beta-sheet. A model of the EscJ ring subcomplex based on the crystal structure has been proposed [8]. In this model, 24 copies of EscJ associate to form a ring-shaped structure with an internal diameter ranging from 73 to 120 Å. Residues in individual monomers were shown to form inter-molecular salt bridges at specific contact points. A functional role for the flexible interdomain linker could not be determined based solely on this model.

The opportunistic pathogen *Pseudomonas aeruginosa* possesses a TTSS that has been shown to be required for the cytotoxicity this

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bacterium displays towards a wide range of host cells [9–13]. Although the needle complex has never been detected in this bacterium, a gene similar to *prgK* (*pscJ*) has been identified. A previous study showed that a mutant of *P. aeruginosa* that was the result of a transposon insertion in the *pscJ* gene was defective in its ability to secrete TTSS proteins [10]. This strain was also shown to be unable to kill both cultured epithelial cells and the social amoeba *Dictyostelium discoideum* [10,12].

In the current study we have used site-directed mutagenesis to discriminate between essential and non-essential PscJ amino acid residues. The results of this study represent the first attempt to systematically examine the structure–function relationship in the PrgK family of TTSS proteins.

## 2. Results and discussion

### 2.1. Mutagenesis and complementation of the pscJ gene

To begin characterizing the *pscJ* gene product, a *pscJ* null strain was created from the wild-type strain OS17. The wild-type strain is a derivative of the *P. aeruginosa* strain PAO *lac* [14] that carries an insertion mutation in the *fleQ* gene [15] and was shown to both lack flagella and be deficient in the production of flagellin (data not shown). A *pscJ* mutant strain was created from the OS17 background by allelic exchange with a *pscJ* deletion mutant as previously described [16]. The mutant and wild-type strains were grown in TTSS-inducing media and the cells were separated from the supernatants by low-speed centrifugation. Proteins present in the culture supernatant were precipitated using trichloroacetic acid and separated by SDS-PAGE as previously described [17]. Following staining with Coomassie Brilliant Blue, it was apparent that several proteins were absent in the culture supernatant of the *pscJ* mutant strain. These proteins were shown by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to be *P. aeruginosa* secreted TTSS proteins (Fig. 1A). When whole cells were subjected to immunoblot analysis using an antiserum specific for PscJ, a band running at approximately 28 kDa was seen to be absent in the mutant in comparison to the wild-type strain (Fig. 1B). The size of this band is in agreement with the predicted size of PscJ indicating that the *pscJ* mutant strain is indeed deficient in the production of this protein.

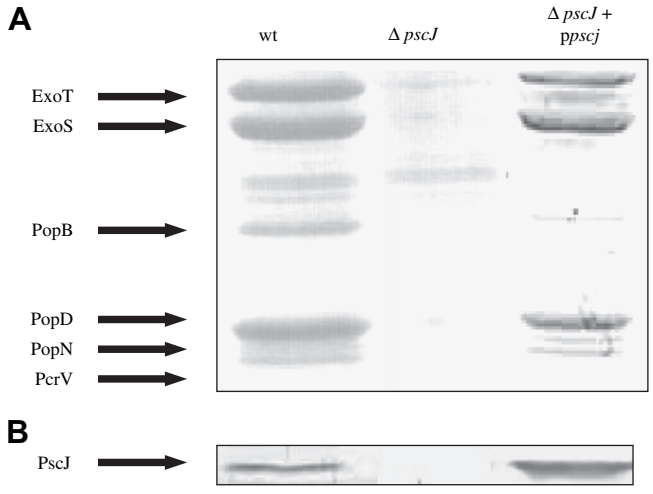


**Fig. 1.** PscJ is required for type III secretion in *P. aeruginosa*. (A) Culture supernatant proteins from the wild-type and the *pscJ* mutant strain were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. A number of proteins were shown to be present in the supernatant of the wild-type strain that were absent from the *pscJ* mutant. These proteins were identified by MALDI-TOF MS to be secreted TTSS proteins. When the mutant strain was transformed with a plasmid containing *pscJ-pscL* the secretion defect was restored. (B) Western immunoblot analysis of whole cells using an anti-PscJ antiserum showed that the wild-type strain expressed a protein with a mobility of approximately 28 kDa that was absent in the mutant. The size of this protein corresponds to the predicted size of PscJ. This band reappeared when the mutant was transformed with the complementing plasmid.

To restore secretion, a plasmid encoding *pscJ* was introduced into the null strain. The secretion defect was not restored by this plasmid (data not shown), suggesting that the *pscJ* mutation may be exerting a polar effect on the expression of genes located downstream. The secretion defect of the *pscJ* null mutant could be restored when the strain was transformed with a plasmid encoding *pscJ* as well as the two downstream genes *pscK* and *pscL* (Fig. 1A). A plasmid encoding *pscK* and *pscL* (without *pscJ*) failed to complement the secretion defect of the mutant (data not shown). These results suggest that the mutation in the *pscJ* gene is indeed polar. However, since a plasmid encoding just *pscK* and *pscL* did not complement the secretion defect of the strain, it can be assumed that the *pscJ* gene is indeed required for secretion and that the *pscJ-pscL* encoding plasmid, pOS37 could be used for studying the roles of individual PscJ amino acid residues.

### 2.2. Non-essential amino acid residues

In the course of this study, 15 amino acid residues in PscJ were subjected to substitution mutagenesis (Table 1). These residues are located in conserved regions of the PrgK/PscJ family of proteins (Fig. 2). Of those 15 residues, eight (K24, K56, E66, K77, K172, D185, S188, and F210) could be subjected to non-conservative substitution without affecting the ability of the resulting proteins ability to complement the secretion defect of the *pscJ* mutant strain (Fig. 3).

Upon natural cleavage of the Sec signal sequence, K24 becomes the second amino acid in the mature form of PscJ. Substitution of K24, which is predicted to be located in the first beta-strand of the protein with a Glu had no effect on the function of PscJ. Both K56 and E66 are predicted to be located in flexible linker regions oriented towards the bottom of the protein. Substitution of either of these residues with an oppositely charged amino acid (K56E and E66K) had no effect on the expression or function of the proteins. In the case of E66, this result is consistent with the fact that the homologous residue in EscJ (K63) could be substituted with an Ala without affecting the function of the protein [8]. Similarly, both K77 (located near the end of helix 2) and K172 (located in the center of

**Table 1**
Expression and function of PscJ site-directed substitution mutants

| PscJ mutant | Expression | Complementation |
|---|---|---|
| K24E | + | + |
| E26K | + | − |
| E26V | + | + |
| K52E | − | − |
| K52A | − | − |
| K56E | + | + |
| E66K | + | + |
| K77E | + | + |
| E105K | + | − |
| E105A | + | +/− |
| A107K | + | − |
| A107E | + | − |
| G126C | +/− | − |
| G126D | + | + |
| G126A | + | + |
| H133D | +[a] | − |
| H133A | + | +/− |
| K172E | + | + |
| D185K | +/− | + |
| S188I | + | + |
| V189E | + | − |
| F210K | + | + |

[a] Altered mobility.

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