Secretin channel-interactors prevent antibiotic influx during type IV pili assembly in
*Pseudomonas aeruginosa.*

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Abstract

Type IV pili (T4P) are important virulence factors involved in host attachment and other aspects of bacterial pathogenesis. In Gram-negative bacteria, the T4P filament is polymerized from pilin subunits at the platform complex in the inner membrane (IM) and exits the outer membrane (OM) through the OM secretin channel. Although essential for T4P assembly and function, the OM secretin complexes can potentially impair the permeability barrier function of the OM and allow the entry of antibiotics and other toxic molecules. The mechanism by which Gram-negative bacteria prevent secretin-mediated OM leakage is currently not well understood. Here, we report a discovery of SlkA and SlkB (PA5122 and PA5123) that prevent permeation of several classes of antibiotics through the secretin channel of Pseudomonas aeruginosa type IV pili. We found these periplasmic proteins interact with the OM secretin complex and prevent toxic molecules from entering through the channel when there is a problem in the assembly of the T4P IM subcomplexes or when docking between the OM and IM complexes is defective. Thus, our results indicate that the secretin channel-interacting proteins play an important role in maintaining the OM permeability barrier, suggesting they may be attractive targets for potentiaters that sensitize Gram-negative pathogens to antibiotics that are normally ineffective at penetrating the OM.

Key words: type IV pili, secretin, outer membrane, permeability barrier, antibiotics, drug resistance, macrolide
Introduction

Gram-negative bacterial pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacteriaceae* are often multidrug-resistant because their cell envelope functions as an effective permeability barrier against a variety of antibiotics (Nikaido, 2003; Delcour, 2009). Their cell envelope is a multi-layered structure consisting of the cytoplasmic (inner) membrane (IM), the outer membrane (OM), and the peptidoglycan layer in the periplasmic space between the two membranes. Among these layers, the OM plays a crucial role in the barrier function of the envelope due to its asymmetric bilayer structure consisting of two types of membrane lipids: lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet. The long glycan chains of the LPS limits the diffusion of hydrophobic compounds and the lipid bilayer prevents the entry of hydrophilic compounds across the OM. In addition, RND type efflux pumps that span the entire envelope efficiently remove various toxic molecules from both the cytoplasm and periplasm, reinforcing the barrier function of the OM (Silver, 2011; Raetz & Whitfield, 2002; Krishnamoorthy *et al*, 2017; Li *et al*, 2015).

The permeability barrier formed by the OM is necessarily imperfect because it must allow the entry of nutrients required for bacterial growth. To promote diffusion across the OM, Gram-negative bacteria assemble a variety of integral outer membrane proteins (OMPs) to transport molecules across the OM and the pore structure of these OMPs compromise the permeability barrier. General porins and substrate-specific channels responsible for nutrient transport have been shown to facilitate the diffusion of small hydrophilic compounds into the periplasm through their open pore structure with hydrophilic inner rim (Pagès *et al*, 2008; Prajapati *et al*, 2021). In *Enterobacteriaceae*, loss of the general porin OmpF results in elevated resistance to small hydrophilic antibiotics such as beta-lactams (Delcour, 2009; Sugawara *et al*, 2016; Choi & Lee, 2019; Vergalli *et al*, 2020). In *P. aeruginosa*, OprD, which is normally responsible for the transport of basic amino acids and peptides, functions as a
carbapenem entry channel and an oprD mutation is frequently observed in carbapenem-resistant clinical isolates (Fukuoka et al., 1993; Lee & Ko, 2012; Kim et al., 2016; Wolter et al., 2004).

Besides the OMPs whose pore structure is formed from a single polypeptide, Gram-negative pathogens also assemble multimeric channels in the OM as part of large transenvelope complexes like pili and secretion systems, many of which are critical for virulence and survival in the host (Nikaido, 2003). A group of homologous proteins called secretins form 12- to 15-meric OM channel complexes to accommodate protein substrates in several envelope-spanning virulence systems: type II secretion systems (T2SS), type III secretion systems (T3SS), and type IV pili assembly systems (T4PS) (Majewski et al., 2018; Korotkov et al., 2011). Cryo-electron microscopy (cryo-EM) of the secretin complexes revealed that these proteins share a similar architecture. A less-conserved N-terminal domain forms a periplasmic vestibule that interacts with the IM components of the virulence system, and a well-conserved C-terminal domain forms an OM channel with one or two gate structures (Weaver et al., 2020; Hu et al., 2018; D'Imprima et al., 2017; Naskar et al., 2021). Although secretin channels form much larger pore structures than the channels formed by a single polypeptide, it has been assumed that the gate structures of secretins prevent leakage of periplasmic contents and permeation of extracellular chemicals when they are not in use for protein secretion or pilus assembly (Majewski et al., 2018; Korotkov et al., 2011). However, in vitro studies have suggested that secretin channels are not completely sealed in their resting state and that diffusion of small compounds occurs through these channels (Disconzi et al., 2014). In addition, mutations in the secretin gene pilQ of T4P have been reported to increase the MIC of several antibiotics in pathogenic Neisseria species (Tzeng et al., 2019; Zhao et al., 2005; Nandi et al., 2015). These results indicate that the secretin complex can function as a conduit that allows the entry of antibiotics across the OM although mechanistic details of drug entry have remained largely unclear.
To uncover factors important for maintaining the OM permeability barrier in *P. aeruginosa*, we employed transposon sequencing (Tn-seq). A transposon mutant library was challenged with the hydrophobic antibiotic erythromycin at a concentration that normally does not cause a growth defect due to the barrier function of the OM. Comparison of the transposon profiles between treated and untreated samples identified two orthologous genes *PA5122* and *PA5123*, encoding putative periplasmic proteins that are required for normal erythromycin resistance. We discovered that these proteins prevent secretin leakiness and therefore have renamed them SlkA and SlkB (pronounced “slick-A” and “slick-B”). Genetic and microscopic analyses suggested that the Slk proteins interact with the secretin complex of the T4PS to prevent the diffusion of drugs across the OM when the OM secretin complex is not docked with the IM complex. Overall, our results indicate that the gate structure found in secretin channels is insufficient to maintain the OM permeability barrier and that additional partner proteins are required to prevent the entry of toxic compounds during the vulnerable stages of T4P assembly.
Results

PA5122 and PA5123 (SlkA and SlkB) are periplasmic proteins involved in maintenance of permeability barrier function

To identify factors important for maintaining OM barrier function in P. aeruginosa against hydrophobic antibiotics, we employed a Tn-seq approach with erythromycin treatment (Fig. 1A). Wild-type P. aeruginosa PAO1 cells were mutagenized by random insertion of a mariner transposon from pBTK30 that was transferred via conjugation. The resulting mutant library was grown for roughly ten generations in LB lacking or containing 10 μg/mL of erythromycin, which normally does not cause a growth defect in P. aeruginosa due to the permeability barrier function of the OM and the activity of RND efflux pumps. Comparison of the transposon insertion profile between erythromycin-treated and untreated samples revealed marked reduction of transposon insertions in oprM and PA5122 in the drug-treated sample, which suggested that these genes play important roles for survival under erythromycin treatment (Fig. 1B). OprM is an outer membrane channel component of major RND efflux systems MexAB-OprM and MexXY-OprM that are responsible for extrusion of various toxic compounds and antibiotics from both periplasm and cytoplasm (Li et al, 2015, 1995; Tsutsumi et al, 2019). Thus, the oprM mutant is likely to be hypersensitive to erythromycin due to defective efflux activity. The function of PA5122 has not been documented yet. We therefore focused our efforts on revealing its cellular function.

PA5122 is predicted to encode a periplasmic protein, suggesting that the function of PA5122 is likely to be related to maintaining the envelope barrier rather than inhibiting erythromycin action on the ribosome. PA5122 constitutes an operon with a downstream gene PA5123, which also encodes a putative periplasmic protein. Interestingly, PA5122 and PA5123 encode homologous proteins with 35.5% sequence identity and 50.8% similarity, suggesting that the cellular role of PA5123 might be similar to that of PA5122. Thus, we made individual mutants and a mutant deleted for both genes, and compared their susceptibility to
erythromycin with a wild-type strain. In accordance with the Tn-seq data, the ΔPA5122 and
the ΔPA5122-5123 strains showed erythromycin susceptibility, but the ΔPA5123 strain did
not (Fig. 1C). To see if this difference arises due to a polar effect of PA5122 mutation on
PA5123 expression, we individually expressed PA5122 and PA5123 in the ΔPA5122-5123
strain and tested for the suppression of erythromycin susceptibility. Expression of either
PA5122 or PA5123 suppressed the erythromycin susceptibility (Fig. 1D), suggesting that
PA5123 has a similar function to PA5122 in maintaining the permeability barrier of the P.
aeruginosa envelope. Based on our study presented below, we discovered that PA5122 and
PA5123 prevent chemical influx through the T4P secretin channel and will henceforth refer to
these proteins as SlkA and SlkB for prevention of secretin leakiness.

To test if inactivation of SlkA and SlkB causes a general defect in envelope barrier function,
we examined the sensitivity of this mutant to various classes of antibiotics. The ΔslkAB strain
showed increased sensitivity to several antibiotics of the macrolide and aminoglycoside
classes, such as azithromycin, gentamicin, and tobramycin when compared with the parental
PAO1 strain (Fig. 1E and Fig. S1). It also showed increased sensitivity to trimethoprim
(TMP)-sulfamethoxazole (SMX). As macrolides are hydrophobic and large, we also tested if
the ΔslkAB strain becomes generally sensitive to other lipophilic or bulky antibiotics such as
rifampicin, fusidic acid, and vancomycin, but inactivation of SlkA and SlkB did not noticeably
increases the sensitivity to these drugs (Fig. S1).

We also compared the antibiotic sensitivity of the ΔslkAB strain with that of the ΔoprM strain
to test if the function of Slk proteins is related to the MexAB-OprM and MexXY-OprM efflux
pumps. However, the antibiotic sensitivity profile of the ΔslkAB strain was quite different from
that of the ΔoprM strain. The ΔslkAB strain was much more susceptible to macrolides than
the ΔoprM strain, whereas the ΔoprM strain showed higher sensitivity to aminoglycosides
and TMP/SMT than the ΔslkAB strain. In addition, the MIC of beta-lactam drugs did not
change much in the ΔslkAB strain but was increased in the ΔoprM strain (Fig. S1). The
difference in the antibiotic sensitivity profile between the ΔslkAB and ΔoprM strains suggested that Slk proteins are likely to have a cellular function independent of the MexAB-OprM and MexXY-OprM efflux pumps.

**Slk proteins prevent permeation of antibiotics through the OM secretin complex of the type IV pili**

To gain insight into the cellular function of the Slk proteins, we performed a genetic selection for suppressors of the erythromycin sensitivity phenotype of mutants lacking these proteins. The ΔslkAB strain was mutagenized by random insertion of a mariner transposon, and the resulting mutant library was selected on LB plates containing 50 μg/mL erythromycin to obtain suppressors. Then, transposon insertion sites of thirteen suppressors were mapped by arbitrarily primed PCR. Among them, nine had insertions in pilQ, two had insertions upstream of pilQ, and one each were mapped in pilM and pilP (Fig. 2A). For the remaining suppressors, one had an insertion in pilF and the other was mapped 124 bases upstream of the fimV coding sequence. PilQ is a component of *P. aeruginosa* T4P that assembles into a secretin channel in the OM through which a pilus exits the cell. Mutations upstream of pilQ were reported to significantly lower PilQ protein levels and inhibit PilQ multimer assembly (Ayers *et al.*, 2009). PilF is an OM lipoprotein known as a pilotin that promotes localization and multimer assembly of the secretin PilQ in the OM (Koo *et al.*, 2008). FimV is an IM protein that is also required for PilQ secretin assembly in the OM (Wehbi *et al.*, 2011). Overall, the identified suppressors all had mutations that inhibit assembly of the secretin complex of the T4P. Thus, assembly of the T4P secretin complex in the absence of Slk proteins seemed responsible for the susceptibility of ΔslkAB mutants to erythromycin. Indeed, erythromycin sensitivity of the ΔslkAB strain was suppressed when we introduced a pilQ null mutation, and the suppression was abrogated when pilQ was expressed from an ectopic site on the chromosome (the Tn7 integration locus) in the ΔpilQ ΔslkAB strain (Fig. 2B). Deletion of
pilMNOPQ also suppressed the erythromycin sensitivity of the ΔslkAB strain, but pilQ expression alone was sufficient for abolishing the suppression, indicating that mutations upstream of pilQ suppressed the erythromycin sensitivity of the ΔslkAB strain via a polar effect on pilQ expression. Although a pilQ mutation suppressed macrolide sensitivity of the ΔslkAB strain, it did not increase the resistance of the wild-type PAO1 strain to macrolides (Fig. 2C and Fig. S2), suggesting that assembly of the secretin channel does not cause a permeability defect with normal expression of the slkAB genes.

Incomplete assembly of the T4P causes antibiotic permeation through the secretin channel in the absence of SlkA and SlkB.

As pilQ deletion suppressed the erythromycin susceptibility of the ΔslkAB strain, we wondered if Slk proteins were previously unidentified components of the T4P and tested if they were required for T4P-driven twitching motility. Unexpectedly, even the wild type PAO1 strain used for the Tn-seq experiment and the initial characterization of the SlkAB phenotype was twitching-defective (Fig. 3A). As genetic variability among PAO1 strains has been widely recognized (Klockgether et al., 2009; Sidorenko et al., 2017; Chandler et al., 2019), we obtained a twitching-proficient PAO1 strain to examine phenotypes related to twitching motility. The twitching-proficient PAO1 strain will be referred to as PAO1\textsuperscript{tw} henceforth to distinguish it from the twitching-defective PAO1 strain. We introduced a slkAB mutation in several twitching-proficient \textit{P. aeruginosa} strains PAO1\textsuperscript{tw}, PA14, and PAK, and compared the twitching motility and erythromycin sensitivity between the WT strains and their ΔslkAB derivatives. A slkAB deletion did not cause an obvious defect of the twitching motility in any of the \textit{P. aeruginosa} strains, showing that Slk proteins are not essential components of T4P (Fig. 3A). Surprisingly, however, deletion of slkAB did not cause erythromycin sensitivity in the twitching-proficient strains unlike in the twitching-defective PAO1 strain (Fig. 3B and Fig. S3). Although puzzling at first, this result led us to hypothesize that the erythromycin
sensitivity of slkAB mutants might be related to the twitching motility defect of our original parent strain.

To investigate the relationship between erythromycin sensitivity and the twitching motility defect, we looked for mutations that can affect twitching motility by resequencing the genomes of the two PAO1 strains using the PAO1 reference genome (NC_002516) (Stover et al, 2000). The two genomes showed differences at a few genetic loci, including a change at the pilC locus (Table S1-S2). PilC is the platform protein that coordinates the polymerization and depolymerization of T4P (Takhar et al, 2013). Thus, the mutation near pilC seemed responsible for the twitching motility defect. After aligning pilC coding sequences of PAO1, PAO1tw, PA14, and PAK, we realized that the pilC start codon was misannotated in the PAO1 reference genome and that there is actually a frameshift mutation in pilC of the reference genome and our twitching-defective PAO1 strain by the insertion of four bases (ACTG) after the 27th codon (Fig. S4). Accordingly, ectopic expression of the pilC of the PAO1tw strain restored the twitching motility in the twitching-defective PAO1 strain and its ΔslkAB derivative (Fig. S5A).

Next, we examined the effect of pilC expression on the erythromycin susceptibility of the ΔslkAB derivative of the twitching-defective PAO1 strain. To our amazement, pilC expression completely suppressed the erythromycin susceptibility, suggesting that the pilC mutation is indeed responsible for the difference in the erythromycin susceptibility phenotype between strains (Fig. S5B). To further examine the requirements for antibiotic susceptibility, we tested the effect of combining pilC and slkAB mutations in the twitching-proficient PAO1tw, PA14, and PAK strains. As expected, the ΔpilC ΔslkAB strains became susceptible to erythromycin (Fig. 3B and Fig. S3). Moreover, the erythromycin sensitivity of the ΔpilC ΔslkAB strains was suppressed by a pilQ mutation. These results suggested that the PilQ secretin channel functions as a pore through which antibiotics are translocated when Slk proteins are inactivated and type IV pili assembly is not completed because of a PilC defect.
We next sought to determine if other mutations that cause a defect in T4P assembly also result in antibiotic permeation through the PilQ secretin in the ΔslkAB strain. The T4P assembly system is composed of four subcomplexes: the platform protein PilC and cytoplasmic motor proteins PilBTU; the pilus shaft consisting of the major pilin PilA, minor pilins FimU-PilVWXE, and the adhesin PilY1; the alignment complex made up of PilMNOP; and the secretin complex consisting of PilQ, PilF, and TsaP (Burrows, 2012; McCallum et al, 2019). We first tested if the lack of the pilus shaft also causes antibiotic permeation in the absence of Sik proteins similar to a pilC mutation. Assembly of the pilus shaft begins with formation of a priming complex consisting of an adhesin and minor pilins (Treuner-Lange et al, 2020), and the pilus elongates by addition of major pilins at the base of the pilus in the IM (Jacobsen et al, 2020). We hypothesized that the loss of the pilus shaft resulting from deletion of the major pilin pilA or the adhesin pilY1 would leave the PilQ secretin pore unoccupied and cause antibiotic permeation through the secretin pore if the Sik proteins are not present. Accordingly, introduction of pilA or pilY1 mutation also caused erythromycin sensitivity in the ΔslkAB derivative of the PAO1tw strain, and the sensitivity was suppressed by pilQ deletion as observed for pilC mutants (Fig. 3C).

To test the effect of mutations in the alignment complex genes on antibiotic permeation through the PilQ secretin, we used a ΔpilMNOPQ mutant strain that ectopically expresses pilQ from an inducible promoter. This configuration was required because mutations in the pilMNOP genes appeared to be polar on the expression of downstream pilQ (Ayers et al, 2009) (Fig. 2A). The ΔslkAB ΔpilMNOPQ mutant strain became susceptible to erythromycin treatment upon induction of pilQ expression from an ectopic locus in the chromosome, but showed erythromycin resistance without pilQ induction (Fig. 3D). Thus, mutations in genes encoding components of the T4P subcomplexes assembling at the IM seemed to generally cause a PilQ-mediated defect in the OM permeation barrier upon inactivation of the Sik proteins.
Loss of TsaP also causes PilQ-mediated antibiotic diffusion in the absence of Slk proteins

In contrast to the mutations of the IM subcomplexes, mutations of the genes required for assembly of the OM secretin channel such as pilF and fimV were identified as suppressors of the erythromycin susceptibility along with pilQ mutations (Fig. 2A). TsaP is a component of the T4P secretin complex that forms peripheral spikes surrounding the complex (Siewering et al, 2014; Chang et al, 2016; McCallum et al, 2021). It was shown to be important for T4P surface assembly and twitching motility in Neisseria gonorrhoeae and Myxococcus xanthus (Siewering et al, 2014). However, the PilQ secretin complexes are still formed in the ΔtsaP strain irrespective of the twitching motility defect (Siewering et al, 2014; Chang et al, 2016).

Moreover, in P. aeruginosa, a tsaP mutation did not cause a twitching motility defect as severe as in N. gonorrhoeae or M. xanthus (McCallum et al, 2021). Thus, we suspected that the phenotype related to antibiotic permeation might be different between the ΔtsaP strain and other mutant strains of the T4P secretin complex. Indeed, inactivation of TsaP did not suppress the erythromycin susceptibility of the ΔpilCΔslkAB strain (Fig. S6).

TsaP was proposed to function in anchoring the OM secretin complex to the peptidoglycan layer and/or aligning the secretin complex with the IM complex (Siewering et al, 2014). We reasoned that the alignment problem would cause a significant fraction of PilQ secretin pores to remain unoccupied in the ΔtsaP mutant. If so, a tsaP mutation might also cause an OM permeability defect in the absence of Slk proteins. Accordingly, the ΔtsaPΔslkAB mutant became susceptible to erythromycin, and the erythromycin sensitivity was suppressed by pilQ mutation (Fig. 3E). An alternative explanation for the permeability defect of the ΔtsaP ΔslkAB strain seemed to be that TsaP and the Slk proteins function redundantly for preventing permeation through the T4P secretin complex. However, unlike the ΔslkAB ΔpilC double mutant, the ΔtsaP ΔpilC double mutant did not exhibit a noticeable increase in
erythromycin susceptibility (Fig. S7), suggesting that TsaP does not have a major role in preventing permeation through the PilQ secretin channel but is instead important to prevent the formation of disengaged pores.

**Slk proteins interact with the PilQ secretin complex.**

The above results led us to hypothesize that the Slk proteins prevent antibiotic permeation by interacting with the PilQ secretin channel when the channel is not docked with the IM complex. As PilQ localizes to the poles independently of the Slk proteins or the IM complex assembly (Fig. S8) (Carter et al., 2017), we reasoned that the interaction between Slk proteins and the PilQ channel can be assessed by examining the polar localization of Slk proteins in the PAO1^tw^ strain and its T4P mutant derivatives. To monitor SlkA localization, we used a SlkA-mScarlet fusion protein produced from its native chromosomal locus. As SlkB might interfere with SlkA-mScarlet localization by competing for interaction with the PilQ channel, we deleted **slkB** when we introduced **slkA-mScarlet** fusion at the native locus. As for SlkB localization, **slkB-mScarlet** was expressed from an ectopic locus in the chromosome (the Tn7 integration site) in the Δ**slkAB** strain background to avoid competition between SlkB-mScarlet and untagged Slk proteins. SlkA-mScarlet and SlkB-mScarlet are partially functional in that the strains expressing the fusion proteins do not show a permeability defect when combined with Δ**pilC** that is as severe as the Δ**pilC ΔslkAB** strain (Fig. S9).

In accordance with our hypothesis, SlkA-mScarlet and SlkB-mScarlet localized to the poles in a PilQ-dependent fashion, albeit weakly, indicating that Slk proteins interact with the PilQ secretin complex (Fig. 4 and Fig. S10). Strikingly, the polar localization of Slk proteins was dramatically enhanced when assembly of the IM complex was impaired by Δ**pilC**, suggesting that Slk proteins interact with the PilQ secretin channel more strongly when the IM complex assembly is inhibited. These results imply that the Slk proteins and the IM complex compete for interaction with the PilQ secretin channel and that the Slk proteins are displaced when
the IM complex assembles with the secretin channel. Overall, the localization pattern of Slk proteins in wild-type and T4P mutant strains is consistent with our hypothesis that Slk proteins interact with the PilQ secretin channel to prevent the diffusion through the OM when the IM complex is not properly assembled with the secretin channel.

**Slk proteins are likely to function specifically for the T4PS in *P. aeruginosa***

In *P. aeruginosa*, there are three more virulence systems that assemble OM channels comprised of secretin homologues: two T2SS (Xcp and Hxc) and a T3SS (Psc). As the secretin complexes of these systems are likely to be assembled independently of the subcomplexes in the IM (Diepold & Wagner, 2014; Thomassin *et al.*, 2017; Douzi *et al.*, 2012), we examined if the Slk proteins are also involved in preventing drug permeation through these secretin channels. The platform protein gene of each system, xcpS, hxcS, or pscJ, was deleted to inhibit the assembly of the IM subcomplexes of the secretin-containing systems in the ΔslkAB strain of the PAO1tw strain. The antibiotic sensitivity of the resulting strains was then compared with that of the ΔslkAB strain in growth conditions that support the production and assembly of the secretin complexes of each virulence system. For assessing the antibiotic susceptibility of the ΔhxcS ΔslkAB strain, a phosphate-limiting proteose peptone medium was used to induce the expression of Hxc T2SS (Ball *et al.*, 2002). The effect of a pscJ mutation was examined in the strains that overproduce the master regulator ExsA to induce T3SS expression. However, we did not observe increased sensitivity of these strains to macrolides compared with that of the PAO1tw and its ΔslkAB derivative (Fig. S11), which suggested that SlkA and SlkB are likely to function specifically with the T4PS.
The cell envelope of Gram-negative pathogens functions as an effective permeability barrier against antibiotics because the OM efficiently restricts the influx of various toxic compounds and efflux pumps expel the toxic molecules from the periplasm as well as from the cytoplasm. Nevertheless, diffusion of antibiotics across the OM occurs inevitably because Gram-negative bacteria assemble channel structures in the OM to carry out various cellular functions important for survival and virulence. General porins and substrate-specific channels required for nutrient transport have been implicated as a major route for diffusion of small and hydrophilic drugs such as beta-lactams (Vergalli et al., 2020; Zgurskaya et al., 2015). Secretin complexes assembled in the OM as part of protein secretion machineries are another type of channel structures that can potentially serve as a conduit for drug diffusion, but mechanistic details related to the permeation of compounds through these structures have remained unclear. In this study, we found that defects of the IM complex assembly or alignment between the IM and OM complexes of *P. aeruginosa* T4P assembly systems can cause diffusion of drugs through the OM secretin channel. We also discovered that in these situations, drug diffusion through the secretin channel is prevented by periplasmic proteins SlkA and SlkB that interact specifically with the channel complex until the T4PS is fully assembled.

Although secretin channels form large pores to accommodate protein substrates, diffusion through the channels is thought to be minimized by the gate structure of the channels that are closed unless proteins are secreted through the channel. However, our results indicate that the gate structure does not effectively seal the T4P secretin channel against certain classes of antibiotics such as macrolides and aminoglycosides in *P. aeruginosa* when the IM complex is not docked to the channel. It has been assumed that there are “plug” proteins that help seal secretin channels more tightly when the channels are not engaged in their function. Supporting this idea, electron densities for plug-like proteins have been visualized.
in several EM studies of secretin complexes, but the identity of the plug proteins has not been determined yet (Chami et al, 2005; Chernyatina & Low, 2019; Ghosal et al, 2019; McCallum et al, 2021). A recent cryo-electron tomography analysis of T4P in *Myxococcus xanthus* suggested that PilY1 is likely to function as a plug at the entry to the PilQ secretin vestibule based on the tomograms taken in the cells lacking specific domains of T4P components (Treuner-Lange et al, 2020). Thus, after the assembly of T4PS, the proteins that are located at the top of the pilus shaft structure such as PilY1 appear to function as a plug that seals the secretin channel in the retracted pili, while the channel is likely to be sealed by the pilus shaft itself in the extended pili (Fig. 5). However, the plug structure was only visible in the fully assembled T4PS, suggesting that the plug is installed when the IM complex assembles with the secretin channel. Thus, it remained unclear whether there are plug proteins that seal the secretin channel when the transenvelope complex is not fully assembled.

Results from our genetic and microscopic analyses indicate that the Slk proteins function as a plug for the secretin channels that are not docked with the IM complexes. The *slkAB* mutant showed an OM barrier defect, but the defect only became obvious when the IM complex could not be assembled or aligned with the OM secretin channel. This result suggested that either the Slk proteins or the IM complex can prevent the diffusion through the secretin channel, and thus the activity of the Slk proteins is not critical when the T4PS is efficiently assembled. In addition, when we assessed the interaction of Slk proteins with the secretin complex using the PilQ-dependent polar localization as an interaction proxy, inhibition of the IM complex assembly strongly enhanced the polar localization of Slk proteins (Fig. 4 and Fig S10). Moreover, Slk proteins were completely delocalized in the *pilQ* mutant background irrespective of the IM complex mutation, indicating that the enhanced polar localization is also dependent on PilQ. Thus, while it is formally possible that Slk proteins are recruited to the poles via interactions with other proteins associated with the PilQ secretin channel, these results strongly suggested that they engage with the secretin
channel before the IM complex docks to the channel and are displaced from the channel upon full T4PS assembly. SlkA (PA5122) was also serendipitously detected in a sample of *P. aeruginosa* PilQ secretin complex prepared for cryo-EM (McCallum *et al.*, 2021). Although the role of SlkA was not appreciated by the authors at the time, their result provides independent support for the specific interaction between SlkA and the PilQ secretin channel. Taken together, all the available data are consistent with the idea that the Slk proteins function as a plug that help seal the secretin channel to maintain the OM permeability barrier until the T4PS is fully assembled (Fig. 5).

SlkAB homologues are only found in gamma-proteobacteria and not widely conserved among bacterial species that have the T4PS or other secretin-containing virulence systems (Fig. S12-13), but we speculate that different types of plug proteins for secretin channels are likely to exist among bacteria that lack SlkAB homologues. In many secretin-reliant systems, assembly of secretin complexes occurs independently of the IM complex assembly (Korotkov *et al.*, 2011; Diepold & Wagner, 2014; Carter *et al.*, 2017; Friedrich *et al.*, 2014; Douzi *et al.*, 2012; Thomassin *et al.*, 2017). Thus, defective assembly of the IM complex or improper alignment between the OM and IM complexes in the secretin-dependent systems might also cause a breach in the permeability barrier function of the OM. Proteins that help seal the secretin pores are therefore also likely to exist among other secretin-dependent virulence systems. In this regard, it is noteworthy that other important secretin interacting proteins are also divergent in sequence. For example, the pilotins that deliver secretins to the OM and/or aid in secretin assembly belong to several groups of proteins that are unrelated in their sequence and structure even though they serve a similar function (Silva *et al.*, 2020; Koo *et al.*, 2012). We thus suspect that proteins that prevent diffusion through secretin channels might also be diverse among secretin-dependent systems.

Because we identified the OM permeability defect of the *slk* mutant with macrolides that are lipophilic, we initially suspected that the T4P secretin channel might preferentially facilitate
the diffusion of hydrophobic drugs. However, hydrophobicity did not seem to be a general property of the chemicals that permeate through the secretin channel. Instead, this channel increased susceptibility of *P. aeruginosa* to several classes of drugs with different properties. Thus, although the loss of *Slk* protein function combined with defects in T4P assembly clearly creates a PilQ-dependent OM permeability defect, the precise chemical properties that promote permeation through the secretin channel remain to be determined. Understanding these and other chemical properties that promote OM permeation will be critical for the development of future antibiotic therapies effective against Gram-negative pathogens.

OM secretin channels are essential components of many virulence systems and thus have been considered as attractive targets for development of antivirulence drugs (Baron, 2010; Korotkov *et al*, 2011). Our discovery that the PilQ channel functions as a pore for drug diffusion in the *slkAB* mutant indicates that secretin channels can also be exploited as a conduit that facilitate drug delivery into Gram-negative pathogens. Thus, understanding how secretin-reliant systems prevent diffusion of toxic compounds through secretin channels is a promising avenue for developing effective strategies that broaden the spectrum of several approved antibiotics to include activity against Gram-negative infections.
Materials and Methods

**Media, Bacterial Strains, and Plasmids.**

Cells were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl), VBMM, minimal M9 medium supplemented with 0.2 % glucose and 0.2 % casamino acids, or proteose peptone medium (Cheng et al., 1970). The strains and plasmids that were used for the study are listed in *SI Appendix*, Table S3 and Table S4. Detailed procedures for strain and plasmid construction are also provided in *SI Appendix*.

**Transposon sequencing**

*P. aeruginosa* PAO1 was mutagenized with the mariner transposon from pTBK30 (Goodman et al., 2004). Genomic DNA from each cell pellet was extracted, fragmented, poly-C tailed, and sequenced as previously described (Lai et al., 2017). Detailed procedure is provided in *SI Appendix*.

**Antibiotic susceptibility testing – spot dilution assay**

Overnight grown strains of interests were diluted to OD_{600}=2.0, serially diluted 10-fold, and 5 μl of each dilution was spotted on LB agar supplemented with indicated antibiotics. The plates were photographed after incubation for 20 hours at 37 °C, unless otherwise specified.

**Antibiotic susceptibility testing – agar diffusion assay**

Freshly saturated cultures were diluted to OD_{600} = 2.0, and 125 μL of the normalized cultures was mixed with 5 mL molten H-top soft agar (1% tryptone, 0.8% NaCl, 0.7% agar) and spread on 10 centimeter diameter LB agar plates. Then, MIC Test Strips (Liofilchem) were applied to the solidified soft agar surface. Alternatively, antibiotics were serially diluted and 5
μL of each dilution was spotted on the soft agar surface. The plates were incubated for 24 hours at 37 °C before being photographed.

**Microscopic image acquisition and analysis**

Growth conditions prior to microscopy are described in the figure legends. Prior to imaging, cells were immobilized on 2% agarose pads containing 1X M9 salts and covered with #1.5 coverslips. Micrographs were obtained using a Leica DM2500 LED microscope equipped with a Leica DFC7000 GT camera, Fluo Illuminator LRF 4/22, HC PL APO 100x/1.40 Oil Ph3 objective lens, and Leica Las X acquisition software. Images in the red channel were obtained using N2.1 filter cube. Automated cell segmentation and identification as well as measurements of fluorescence signal at the single cell level were carried out using Oufti (Paintdakhi et al, 2016). For demographics, custom-written MATLAB code was used to arrange cells from top to bottom according to their length as previously described (Sher et al, 2020).

**ACKNOWLEDGMENTS.**

We thank the members of Cho lab at Sungkyunkwan University for helpful comments and discussion. This study was supported by the National Research Foundation of KOREA (NRF-2019R1A2C1002648) and the Basic Science Research Program of the Ministry of Education (NRF-2019R1A6A1A10073079).
References

Ayers M, Sampaleanu LM, Tammam S, Koo J, Harvey H, Howell PL & Burrows LL (2009) PilM/N/O/P Proteins Form an Inner Membrane Complex That Affects the Stability of the Pseudomonas aeruginosa Type IV Pilus Secretin. *J Mol Biol* 394: 128–142

Ball G, Durand É, Lazdunski A & Filloux A (2002) A novel type II secretion system in Pseudomonas aeruginosa. *Mol Microbiol* 43: 475–485

Baron C (2010) Antivirulence drugs to target bacterial secretion systems. *Curr Opin Microbiol* 13: 100–105

Burrows LL (2012) Pseudomonas aeruginosa Twitching Motility: Type IV Pili in Action. *Annu Rev Microbiol* 66: 493–520

Carter T, Buensuceso RNC, Tammam S, Lamers RP, Harvey H, Howell PL & Burrows LL (2017) The Type IVa Pilus Machinery Is Recruited to Sites of Future Cell Division. *Mbio* 8: e02103-16

Chami M, Guilvout I, Gregorini M, Rémy HW, Müller SA, Valerio M, Engel A, Pugsley AP & Bayan N (2005) Structural Insights into the Secretin PulD and Its Trypsin-resistant Core*. *J Biol Chem* 280: 37732–37741

Chandler CE, Horspool AM, Hill PJ, Wozniak DJ, Schertzer JW, Rasko DA & Ernst RK (2019) Genomic and Phenotypic Diversity among Ten Laboratory Isolates of Pseudomonas aeruginosa PAO1. *J Bacteriol* 201

Chang Y-W, Rettberg LA, Treuner-Lange A, Iwasa J, Søgaard-Andersen L & Jensen GJ (2016) Architecture of the type IVa pilus machine. *Science* 351: aad2001

Cheng K-J, Ingram JM & Costerton JW (1970) Release of Alkaline Phosphatase from Cells of Pseudomonas aeruginosa by Manipulation of Cation Concentration and of p H. *J Bacteriol* 104: 748–753

Chernyatina AA & Low HH (2019) Core architecture of a bacterial type II secretion system. *Nat Commun* 10: 5437

Choi U & Lee C-R (2019) Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in Escherichia coli. *Front Microbiol* 10: 953

Delcour AH (2009) Outer membrane permeability and antibiotic resistance. *Biochimica Et Biophysica Acta - Proteins Proteom* 1794: 808–816

Diepold A & Wagner S (2014) Assembly of the bacterial type III secretion machinery. *Fems Microbiol Rev* 38: 802–822
D’Imprima E, Salzer R, Bhaskara RM, Sánchez R, Rose I, Kirchner L, Hummer G, Kühlbrandt W, Vonck J & Averhoff B (2017) Cryo-EM structure of the bifunctional secretin complex of Thermus thermophilus. Elife 6: e30483

Disconzi E, Guilvout I, Chami M, Masi M, Huysmans GHM, Pugsley AP & Bayan N (2014) Bacterial Secretins Form Constitutively Open Pores Akin to General Porins. J Bacteriol 196: 121–128

Douzi B, Filloux A & Voulhoux R (2012) On the path to uncover the bacterial type II secretion system. Philosophical Transactions Royal Soc B Biological Sci 367: 1059–1072

Friedrich C, Bulyha I & Søgaard-Andersen L (2014) Outside-In Assembly Pathway of the Type IV Pilus System in Myxococcus xanthus. J Bacteriol 196: 378–390

Fukuoka T, Ohya S, Narita T, Katsuta M, Iijima M, Masuda N, Yasuda H, Trias J & Nikaido H (1993) Activity of the carbapenem panipenem and role of the OprD (D2) protein in its diffusion through the Pseudomonas aeruginosa outer membrane. Antimicrob Agents Ch 37: 322–327

Ghosal D, Kim KW, Zheng H, Kaplan M, Truchan HK, Lopez AE, McIntire IE, Vogel JP, Cianciotto NP & Jensen GJ (2019) In vivo structure of the Legionella type II secretion system by electron cryotomography. Nat Microbiol 4: 2101–2108

Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS & Lory S (2004) A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas aeruginosa. Dev Cell 7: 745–754

Hu J, Worrall LJ, Hong C, Vuckovic M, Atkinson CE, Caveney N, Yu Z & Strynadka NCJ (2018) Cryo-EM analysis of the T3S injectisome reveals the structure of the needle and open secretin. Nat Commun 9: 3840

Jacobsen T, Bardiaux B, Francetic O, Izadi-Pruneyre N & Nilges M (2020) Structure and function of minor pilins of type IV pili. Med Microbiol Immun 209: 301–308

Kim CH, Kang HY, Kim BR, Jeon H, Lee YC, Lee SH & Lee JC (2016) Mutational inactivation of OprD in carbapenem-resistant Pseudomonas aeruginosa isolates from Korean hospitals. J Microbiol 54: 44–49

Klockgether J, Munder A, Neugebauer J, Davenport CF, Stanke F, Larbig KD, Heeb S, Schöck U, Pohl TM, Wielhmann L, et al (2009) Genome Diversity of Pseudomonas aeruginosa PA01 Laboratory Strains † †. J Bacteriol 192: 1113–1121

Koo J, Burrows LL & Howell PL (2012) Decoding the roles of pilotins and accessory proteins in secretin escort services. Fem Microbiol Lett 328: 1–12

Koo J, Tammam S, Ku S-Y, Sampaleanu LM, Burrows LL & Howell PL (2008) PilF Is an Outer Membrane Lipoprotein Required for Multimerization and Localization of the Pseudomonas aeruginosa Type IV Pilus Secretin * †. J Bacteriol 190: 6961–6969

Korotkov KV, Gonen T & Hol WGJ (2011) Secretins: dynamic channels for protein transport across membranes. Trends Biochem Sci 36: 433–443
Synergy between Active Efflux and Outer Membrane Diffusion Defines Rules of Antibiotic Permeation into Gram-Negative Bacteria. *Mbio* 8: e01172-17

Lai GC, Cho H & Bernhardt TG (2017) The mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis in *Escherichia coli*. *Plos Genet* 13: e1006934

Lee J-Y & Ko KS (2012) OprD mutations and inactivation, expression of efflux pumps and AmpC, and metallo-β-lactamases in carbapenem-resistant *Pseudomonas aeruginosa* isolates from South Korea. *Int J Antimicrob Ag* 40: 168–172

Li XZ, Nikaido H & Poole K (1995) Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Ch* 39: 1948–1953

Li X-Z, Plésiat P & Nikaido H (2015) The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. *Clin Microbiol Rev* 28: 337–418

Majewski DD, Worrall LJ & Strynadka NC (2018) Secretins revealed: structural insights into the giant gated outer membrane portals of bacteria. *Curr Opin Struc Biol* 51: 61–72

McCallum M, Burrows LL & Howell PL (2019) The Dynamic Structures of the Type IV Pilus. *Microbiol Spectr* 7

McCallum M, Tammam S, Rubinstein JL, Burrows LL & Howell PL (2021) CryoEM map of *Pseudomonas aeruginosa* PilQ enables structural characterization of TsaP. *Structure* 29: 457–466.e4

Nandi S, Swanson S, Tomberg J & Nicholas RA (2015) Diffusion of Antibiotics through the PilQ Secretin in *Neisseria gonorrhoeae* Occurs through the Immature, Sodium Dodecyl Sulfate-Labile Form. *J Bacteriol* 197: 1308–1321

Naskar S, Hohl M, Tassinari M & Low HH (2021) The structure and mechanism of the bacterial type II secretion system. *Mol Microbiol* 115: 412–424

Nikaido H (2003) Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol Mol Biol R* 67: 593–656

Pagès J-M, James CE & Winterhalter M (2008) The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat Rev Microbiol* 6: 893–903

Paintdakhi A, Parry B, Campos M, Irnov I, Elf J, Surovtsev I & Jacobs-Wagner C (2016) Outfi: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. *Mol Microbiol* 99: 767–777

Prajapati JD, Kleinekathöfer U & Winterhalter M (2021) How to Enter a Bacterium: Bacterial Porins and the Permeation of Antibiotics. *Chem Rev* 121: 5158–5192

Raetz CRH & Whitfield C (2002) LIPOPOLYSACCHARIDE ENDOTOXINS. *Biochemistry-us* 71: 635–700
Sher JW, Lim HC & Bernhardt TG (2020) Global phenotypic profiling identifies a conserved actinobacterial cofactor for a bifunctional PBP-type cell wall synthase. *Elife* 9: e54761

Sidorenko J, Jatsenko T & Kivisaar M (2017) Ongoing evolution of *Pseudomonas aeruginosa* PAO1 sublines complicates studies of DNA damage repair and tolerance. *Mutat Res Fundam Mol Mech Mutagen* 797: 26–37

Siewering K, Jain S, Friedrich C, Webber-Birungi MT, Semchonok DA, Binzen I, Wagner A, Huntley S, Kahnt J, Klingl A, et al (2014) Peptidoglycan-binding protein TsaP functions in surface assembly of type IV pili. *Proc National Acad Sci* 111: E953–E961

Silva YR de O, Contreras-Martel C, Macheboeuf P & Dessen A (2020) Bacterial secretins: Mechanisms of assembly and membrane targeting. *Protein Sci* 29: 893–904

Silver LL (2011) Challenges of Antibacterial Discovery. *Clin Microbiol Rev* 24: 71–109

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, et al (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406: 959–964

Sugawara E, Kojima S & Nikaido H (2016) Klebsiella pneumoniae Major Porins OmpK35 and OmpK36 Allow More Efficient Diffusion of β-Lactams than Their *Escherichia coli* Homologs OmpF and OmpC. *J Bacteriol* 198: 3200–3208

Takhar HK, Kemp K, Kim M, Howell PL & Burrows LL (2013) The Platform Protein Is Essential for Type IV Pilus Biogenesis*. *J Biol Chem* 288: 9721–9728

Thomassin J, Moreno JS, Guilvout I, Nhieu GTV & Francetic O (2017) The trans-envelope architecture and function of the type 2 secretion system: new insights raising new questions. *Mol Microbiol* 105: 211–226

Treuner-Lange A, Chang Y-W, Glatter T, Herfurth M, Lindow S, Chreifi G, Jensen GJ & Søgaard-Andersen L (2020) PilY1 and minor pilins form a complex priming the type IVa pilus in *Myxococcus xanthus*. *Nat Commun* 11: 5054

Tsutsumi K, Yonehara R, Ishizaka-Ikeda E, Miyazaki N, Maeda S, Iwasaki K, Nakagawa A & Yamashita E (2019) Structures of the wild-type *MexAB-OprM* tripartite pump reveal its complex formation and drug efflux mechanism. *Nat Commun* 10: 1520

Tzeng Y, Berman Z, Toh E, Bazan JA, Turner AN, Retchless AC, Wang X, Nelson DE & Stephens DS (2019) Heteroresistance to the model antimicrobial peptide polymyxin B in the emerging Neisseria meningitidis lineage 11.2 urethritis clade: mutations in the pilMNOPQ operon. *Mol Microbiol* 111: 254–268

Vergalli J, Bodrenko IV, Masi M, Moynié L, Acosta-Gutiérrez S, Naismith JH, Davin-Regli A, Ceccarelli M, Berg B van den, Winterhalter M, et al (2020) Porins and small-molecule translocation across the outer membrane of Gram-negative bacteria. *Nat Rev Microbiol* 18: 164–176

Weaver SJ, Ortega DR, Sazinsky MH, Dalia TN, Dalia AB & Jensen GJ (2020) CryoEM structure of the type IVa pilus secretin required for natural competence in *Vibrio cholerae*. *Nat Commun* 11: 5080
Wehbi H, Portillo E, Harvey H, Shimkoff AE, Scheurwater EM, Howell PL & Burrows LL (2011) The Peptidoglycan-Binding Protein FimV Promotes Assembly of the Pseudomonas aeruginosa Type IV Pilus Secretin. *J Bacteriol* 193: 540–550

Wolter DJ, Hanson ND & Lister PD (2004) Insertional inactivation of oprD in clinical isolates of Pseudomonas aeruginosa leading to carbapenem resistance. *Fems Microbiol Lett* 236: 137–143

Zgurskaya HI, López CA & Gnanakaran S (2015) Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It. *Acs Infect Dis* 1: 512–522

Zhao S, Tobiason DM, Hu M, Seifert HS & Nicholas RA (2005) The penC mutation conferring antibiotic resistance in Neisseria gonorrhoeae arises from a mutation in the PilQ secretin that interferes with multimer stability. *Mol Microbiol* 57: 1238–1251
Figure 1. SlkA and SlkB play an important role in maintaining the envelope permeability barrier. (A) Scheme for identifying mutations that cause an envelope barrier defect. Mutants that have a defect in the permeability barrier function become susceptible to erythromycin treatment and mutations that cause erythromycin susceptibility are identified by a Tn-seq approach. (B) Transposon insertion profiles for oprM and PA5122-PA5123 (slkAB) loci. Lines above the locus map represent transposon insertion sites and the height of the lines reflects the number of sequencing reads at each site. The regions that show significantly fewer reads in the erythromycin-treated sample compared with the untreated sample are indicated with red dotted lines. (C) Spot dilution assay for PAO1 (WT), OKP5 (ΔslkA), OKP6 (ΔslkB), and OKP7 (ΔslkAB) strains on LB agar only or supplemented with 50 μg/mL erythromycin. The strains were grown overnight in LB at 37 °C and normalized for cell density (OD₆₀₀ = 2). Normalized cultures were then serially diluted 10-fold and 5 μL of each dilution (10⁻¹-10⁻⁶) was spotted onto LB agar lacking or containing 50 μg/mL erythromycin. The plates were incubated at 37 °C and photographed after 20 hours. (D) Spot dilution assay for PAO1 harboring pJN105 (empty vector) and OKP7 strains harboring pJN105, pOKP17 (slkA), or pOKP18 (slkB) on LB agar supplemented with 50 μg/mL erythromycin. (E) Comparison of the sensitivity of ΔslkAB and ΔoprM strains to various antibiotics. Cells of the PAO1, OKP7 (ΔslkAB), and OKP62 (ΔoprM) strains were mixed with molten soft agar and overlaid on LB plates. Test strips impregnated with indicated antibiotics in a concentration gradient were then placed on the lawn of cells and the plates were incubated for 24 hours at 37 °C before being photographed.

Figure 2. Assembly of the PilQ secretin complex of the T4PS causes the envelope barrier defect of the ΔslkAB strain. (A) A diagram of the transposon insertion sites of the
suppressors isolated from the selection of the OKP7(ΔslkAB) strain on LB agar containing
50 μg/mL erythromycin. The triangles above the locus map represent the insertion for which
the direction of transcription in the gentamicin resistance cassette of the transposon is in the
same orientation as the disrupted gene; the triangles below, opposite direction. (B) OKP15
(ΔslkAB ΔpilQ) and OKP50 (ΔslkAB ΔpilMNOPQ) strains harboring a chromosomally
integrated expression construct attTn7::pOKP121 [Ptoplac-uv5::pilQ] or an empty vector control
attTn7::pKHT105 were grown in LB containing 1mM IPTG, serially diluted, and spotted onto
indicated LB agar supplemented with 1mM IPTG to keep inducing pilQ expression. PAO1
and OKP7 (ΔslkAB) strains were also grown and spotted for comparison of growth
phenotype. (C) Erythromycin sensitivity of PAO1, OKP7 (ΔslkAB), OKP14 (ΔpilQ), OKP15
(ΔslkAB ΔpilQ) strains was compared using erythromycin test strips as in Fig. 1E.

Figure 3. Effect of type IV pili mutations on the drug diffusion through the PilQ
complex in the ΔslkAB strain. (A) Comparison of twitching motility among PAO1, PAO1Tw,
PA14, PAK, and their ΔslkAB derivatives. A single colony of each strain was stab-inoculated
through LB agar to the polystyrene dish. After incubation for 48 hours at 30 °C, LB agar was
removed and the cells attached to the polystyrene dish were stained with crystal violet and
photographed. The diagram on the right shows a frameshift mutation in pilC of the PAO1
strain used for the Tn-seq and initial characterization. (B-E) Spot dilution assays to test the
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complex (pilMNOP), and tsaP genes were introduced in the PAO1Tw strain and its ΔslkAB
and ΔslkAB ΔpilQ derivatives. The resulting strains were grown in LB, serially diluted,
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inactivated T4P gene products in each spot dilution assay, pilC for (B), pilA or pilY1 for (C),
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cartoons on the right. Minor pilins are not designated and PilBTU is not drawn for simplicity.

(D) For testing the effect of alignment complex inactivation, pilQ was expressed from an ectopic locus as in Fig. 2B to avoid the polar effect on pilQ expression by pilMNOP mutation. The LB and LB agar contained 1 mM IPTG to induce pilQ expression.

**Figure 4. PilQ-dependent polar localization of SlkA and its enhancement upon inactivation of the T4P platform PilC.** (A) A PAO1\textsuperscript{tw} derivative that expresses slkA\textsuperscript{-mScarlet} from its native locus (OKP59) and its ΔpilQ, ΔpilC, and ΔpilC ΔpilQ derivatives (OKP60, OKP61, and OKP120) were grown overnight in LB. The overnight cultures were diluted 1:100 in M9-glucose (0.2%) medium, grown to exponential phase (OD\textsubscript{600} = 0.2~0.3), and imaged on 2% agarose pads containing 1X M9 salts. Shown on top of each panel are representative fluorescent images showing the localization of SlkA\textsuperscript{-mScarlet} in each strain.

Below the micrographs are demographs that reflect SlkA\textsuperscript{-mScarlet} localization throughout a population of 500 cells arranged according to cell length. Single-cell fluorescence quantification was performed using Oufti (59). (B) The mean SlkA\textsuperscript{-mScarlet} signal intensity profile per unit length was calculated for the 500 single-cells of each strain shown in (A). Each plot displays the mean signal every 0.0648 microns for 11 points, spanning from the outermost cell tips inward. The cell pole is defined by the yellow highlighted region corresponding to the outermost 0.324 microns. (C) Quantification of SlkA\textsuperscript{-mScarlet} signal at the poles. Shown are mean and standard deviation of the SlkA\textsuperscript{-mScarlet} fluorescent signal intensity at the cell poles, defined in (B), for each of the indicated strains.

**Figure 5. Model for Slk proteins functioning as a plug that prevent leakage through the OM secretin complex of the T4P.** (A) The large, cylindrical figure represents the multimeric OM channel consisting of PilQ secretin and TsaP proteins. The gate structure of the secretin channel is omitted for simplicity. Slk proteins indicated as a red circle prevent
diffusion of compounds through the channel until the IM complex docks with the secretin channel. (B) Slik proteins are displaced when the T4P is fully assembled. After the full assembly of T4P, some components of the IM complexes are likely to function as a plug in the complexes with retracted pili, while the pili itself seals the channel in the complexes with extended pili.
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SUPPLEMENTAL MATERIAL FOR:

Secretin channel-interactors prevent antibiotic influx during type IV pili assembly in *Pseudomonas aeruginosa*.

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Figure S1. Comparison of the sensitivity of the PAO1, OKP7 (ΔslkAB), and OKP62 (ΔoprM) strains to various antibiotics. (A) Lawns of the PAO1 and its indicated mutant derivatives were plated in soft agar, incubated with indicated antibiotic test strips for 24 hours at 37 °C, and imaged as in Figure 1E. (B) Lawns of the same P. aeruginosa strains were spotted with 5 μL aliquots of tobramycin solutions at the indicated concentrations and imaged after incubation for 24 hours at 37 °C.
Figure S2. Azithromycin sensitivity of the ΔslkAB strain is suppressed by ΔpilQ.

Lawns of PAO1 and its indicated mutant derivatives, OKP7(ΔslkAB), OKP14(ΔpilQ), and OKP15 (ΔslkAB ΔpilQ), were plated in soft agar, incubated with azithromycin test strips for 24 hours at 37 °C, and imaged as in Figure 2C.
Figure S3. Inactivation of the IM complex assembly causes an OM secretin channel-dependent permeability barrier defect in the ΔslkAB mutant of PA14 (A) and PAK (B) strains. (A) PA14 and its mutant derivatives, OKP46 (ΔslkAB), OKP64 (ΔpilC), OKP65 (ΔpilC ΔslkAB), and OKP80 (ΔpilC ΔslkAB ΔpilQ), were grown overnight in LB, serially diluted, spotted onto LB agar lacking or containing 50 μg/mL erythromycin, and imaged after incubation for 20 hours at 37 °C. (B) Erythromycin susceptibility of PAK, and its mutant derivatives, OKP47 (ΔslkAB), OKP48 (ΔpilC), OKP57 (ΔpilC ΔslkAB), and OKP82 (ΔpilC ΔslkAB ΔpilQ), was examined in the same way.
Figure S4. The PAO1 strain used for the Tn-Seq and initial characterization has a
frameshift mutation by 4 base insertion in the coding sequence of pilC. Alignment of
the pilC coding sequences of several P. aeruginosa strains revealed that the PAO1 strain we
initially used has a frameshift mutation in the pilC gene by insertion of 4 bases (indicated in
red) after the 27th codon. The same frameshift mutation was present in the PAO1 reference
genome (NC_002516), but was not recognized as a frameshift mutation because an internal
ATG codon (indicated in blue) was misannotated as the start codon. Twitching proficient
strains, PAO1tw, PA14, and PAK, do not have this insertion. Conserved bases among the
pilC coding sequences of different P. aeruginosa strains are indicated as * at the bottom.
Figure S5. Expression of the WT pilC gene not only restores the twitching motility of PAO1 and its ΔslkAB derivative, but also suppresses erythromycin sensitivity of the ΔslkAB derivative. (A) The twitching-defective PAO strain and its ΔslkAB derivative (OKP7) harboring pJN105 (empty vector) or pOKP139 expressing pilC from the twitching-proficient strain were tested for twitching motility on the polystyrene dish as described in Fig. 3A. (B) The same strains were grown overnight in LB supplemented with 10 μg/mL gentamicin, serially diluted in LB, spotted onto LB agar lacking or containing 50 μg/mL erythromycin, and imaged after incubation for 20 hours at 37 °C.
**Figure S6.** Inactivation of TsAP does not suppress the synthetic envelope defect of the ΔpilC ΔslkAB strain. PAO1^tw^ (WT) and its mutant derivatives, OKP24 (ΔpilC ΔslkAB), OKP51 (ΔpilC ΔslkAB ΔpilQ), OKP52 (ΔpilC ΔslkAB ΔfimV), and OKP53 (ΔpilC ΔslkAB ΔtsaP), were grown overnight, serially diluted, spotted onto LB agar lacking or containing 50 μg/mL erythromycin, and imaged after incubation for 20 hours at 37 °C.
Figure S7. Inactivation of TsaP does not cause a synthetic envelope defect in the ΔpilC strain. (A) Mutant derivatives of PAO1<sup>bw</sup>, OKP23 (ΔpilC), OKP24 (ΔpilC ΔslkAB), and OKP74 (ΔpilC ΔtsaP), were grown overnight, serially diluted, spotted onto LB agar lacking or containing 50 μg/mL erythromycin, and imaged after incubation for 20 hours at 37 °C. (B) Lawns of the same <i>P. aeruginosa</i> strains were plated in soft agar, incubated with erythromycin test strips for 24 hours at 37 °C, and photographed.
Figure S8. PilQ localizes to the poles. PAO1<sup>tw</sup> and its ΔslkAB and ΔpilC mutant derivatives that express pilQ-mScarlet<sup>SW</sup> from its native locus (OKP20, OKP21, and OKP29) were grown overnight in LB. The overnight cultures were diluted 1:100 in M9-glucose (0.2%) medium, grown at 37 °C to an exponential phase (OD600 = 0.2~0.3), and imaged on 2% agarose pads containing 1X M9 salts. Bar equals 3 µm.
**Figure S9. SlkA-mScarlet and SlkB-mScarlet are partially functional.**

**(A)** OKP23 [PAO1\textsuperscript{ΔpilC}], OKP24 [PAO1\textsuperscript{ΔslkAB ΔpilC}], and OKP61 [PAO1\textsuperscript{ΔpilC slkA-mScarlet ΔslkB}] strains were grown overnight in LB, serially diluted, and spotted on LB agar lacking or containing 50 μg/mL erythromycin. The plates were photographed after incubation for 28 hours at 37 °C to visualize suppression of the barrier defect by slkA-mScarlet expression.

**(B)** OKP23 (attTn7::pKHT104, empty vector), OKP24 (attTn7::pKHT104), and OKP24 (attTn7::pOKP154, P\textsubscript{toplac-dn1}::slkB-mScarlet) strains were grown overnight in LB, serially diluted, and spotted on LB agar supplemented with 100 μM IPTG or 100 μM IPTG and 50 μg/mL erythromycin. The plates were photographed after incubation for 20 hours at 37 °C.
Figure S10. PilQ-dependent polar localization of SlkB and its enhancement upon inactivation of T4P platform PilC. PAO1<sup>tw</sup> derivatives that express slkB-mScarlet under an IPTG-inducible toplac-dn1 promoter, HJP1[PAO1<sup>tw</sup>ΔslkB](attTn7::pKOP154), OKP15[PAO1<sup>tw</sup>ΔslkB ΔpilQ](attTn7::pKOP154), OKP24[PAO1<sup>tw</sup>ΔslkB ΔpilC], and OKP51[PAO1<sup>tw</sup>ΔslkB ΔpilC ΔpilQ](attTn7::pKOP154) were grown overnight in LB. The overnight cultures were diluted 1:100 in M9-glucose (0.2%) medium supplemented with 100 μM IPTG, grown to an exponential phase (OD600 =
0.2~0.3), and imaged on 2% agarose pads containing 1X M9 salts. The images were processed and presented in the same way as described for Figure 4.
Figure S11. Slk proteins are likely to function specifically for T4PS in *P. aeruginosa*.

(A) Mutant derivatives of PAO1\(^{\text{tw}}\), HJP1 (PAO1\(^{\text{tw}}\) ΔslkAB), OKP24 (PAO1\(^{\text{tw}}\) ΔslkAB ΔpilC), and OKP26 (PAO1\(^{\text{tw}}\) ΔslkAB ΔxcpS) were grown overnight in LB, serially diluted, and spotted on LB agar lacking or containing 50 μg/mL erythromycin. The plates were photographed after incubation for 20 hours at 37 °C. (B) HJP1, OKP24, and OKP115 (PAO1\(^{\text{tw}}\) ΔslkAB ΔhxcS) were grown overnight in LB, diluted on phosphate-limiting proteose peptone medium, and spotted on proteose peptone agar lacking or containing 50 μg/mL erythromycin for induction of Hxc T2SS genes. (C) HJP1, OKP24, and OKP28 (PAO1\(^{\text{tw}}\) ΔslkAB ΔhxcS) were grown overnight in LB, diluted on phosphate-limiting proteose peptone medium, and spotted on proteose peptone agar lacking or containing 50 μg/mL erythromycin for induction of Hxc T2SS genes.
ΔslkAB ΔpscJ) harboring pBO48 that express exsA for induction of T3SS genes were grown overnight in LB supplemented with 10 μg/mL gentamicin, serially diluted in LB, and spotted on LB agar lacking or containing 50 μg/mL erythromycin.
**Figure S12.** Phylogenetic distribution of SlkA and SlkB homologues. Shown is a phylogenetic tree depicting the occurrence of SlkA (red) and SlkB (blue) homologues among bacteria. The phylogenetic tree was constructed using phyloT (http://phylot.biobyte.de/) and visualized and annotated using iTOL (http://itol.embl.de/).
Figure S13. Phylogenetic tree showing the distribution of SlkA and SlkB homologues among gamma-proteobacteria
| Strain     | Genotype                                                                 | Source/Reference                      |
|------------|---------------------------------------------------------------------------|--------------------------------------|
| PAO1       | Wild type [twitching-defective due to a frameshift mutation in pilC]      | Dove lab, HMS                        |
| PAO1<sup>tw</sup> | Wild type                                                              | Cho lab, CHA Univ.                   |
| PA14       | Wild type                                                                | Cho lab, CHA Univ.                   |
| PAK        | Wild type                                                                | Cho lab, CHA Univ.                   |
| DH5α(λpir) | F− endA1 hsdR17 (rm<sup>+</sup>) supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)<sub>Δ189</sub> Φ80lacZΔM15 λpir | (Pal et al, 2005)                    |
| Sm10(λpir) | Kan<sup>R</sup> thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu attλ::pir | (Lorenzo & Timmis, 1994)             |
| PAO1/pJN105 | PAO1 / pJN105 P<sub>ara</sub>::*empty                                  | This study                           |
| PAO1<sup>tw</sup> (attTn7::pKHT105) | PAO1<sup>tw</sup> attTn7::P<sub>topac-uv5</sub>::*empty | This study                           |
| HJP1       | PAO1<sup>tw</sup> ΔslkAB                                                | This study                           |
| HJP1/pBO048 | PAO1<sup>tw</sup> ΔslkAB / pJN105 Para::exsA                             | This study                           |
| HJP1 (attTn7::pOKP154) | PAO1<sup>tw</sup> ΔslkAB attTn7::P<sub>toplac-dn1</sub>:: slkB-mScarlet | This study                           |
| OKP5       | PAO1 ΔslkA                                                                | This study                           |
| OKP6       | PAO1 ΔslkB                                                                | This study                           |
| OKP7       | PAO1 ΔslkAB                                                                | This study                           |
| OKP7/pJN105 | PAO1 ΔslkAB / pJN105 P<sub>ara</sub>::*empty                              | This study                           |
| OKP7/pOKP17 | PAO1 ΔslkAB / pJN105 P<sub>ara</sub>::*slkA                               | This study                           |
| OKP7/pOKP18 | PAO1 ΔslkAB / pJN105 P<sub>ara</sub>::*slkB                               | This study                           |
| OKP7/pOKP139 | PAO1 ΔslkAB / pJN105 P<sub>ara</sub>::*pilC                               | This study                           |
| OKP12      | PAO1 ΔpilQ                                                                | This study                           |
| OKP13      | PAO1 ΔslkAB ΔpilQ                                                         | This study                           |
| OKP13      | PAO1 ΔslkAB ΔpilQ                                                         | This study                           |
This study

OKP13
PAO1 ΔslkAB ΔpilQ
(attTn7::pOKP121)
attTn7::P<sub>toplac-uv5</sub>::empty
This study

OKP14
PAO1<sup>tw</sup> ΔpilQ
This study

OKP15
PAO1<sup>tw</sup> ΔslkAB ΔpilQ
This study

OKP15
PAO1<sup>tw</sup> ΔslkAB ΔpilQ
(attTn7::pOKP121)
attTn7::P<sub>toplac-uv5</sub>::RBS<sub>Φ10</sub>-pilQ
This study

OKP15
PAO1<sup>tw</sup> ΔslkAB ΔpilQ
(attTn7::pOKP154)
attTn7::P<sub>toplac-dn1</sub>:: slkB-mScarlet
This study

OKP20
PAO1<sup>tw</sup> pilQ-mScarlet<sup>SW</sup>
This study

OKP21
PAO1<sup>tw</sup> ΔslkAB pilQ-mScarlet<sup>SW</sup>
This study

OKP23
PAO1<sup>tw</sup> ΔpilC
This study

OKP23
PAO1<sup>tw</sup> ΔpilC
(attTn7::pKHT104)
attTn7::P<sub>toplac-dn1</sub>::empty
This study

OKP24
PAO1<sup>tw</sup> ΔslkAB ΔpilC
This study

OKP24
PAO1<sup>tw</sup> ΔslkAB ΔpilC
(attTn7::pKHT104)
attTn7::P<sub>toplac-dn1</sub>::empty
This study

OKP24
PAO1<sup>tw</sup> ΔslkAB ΔpilC
(attTn7::pOKP154)
attTn7::P<sub>toplac-dn1</sub>:: slkB-mScarlet
This study

OKP24
/pBO048
PAO1<sup>tw</sup> ΔslkAB ΔpilC / pJN105 Para::exsA
This study

OKP26
PAO1<sup>tw</sup> ΔslkAB ΔxcpS
This study

OKP28
PAO1<sup>tw</sup> ΔslkAB ΔpscJ
This study

OKP28
/pBO048
PAO1<sup>tw</sup> ΔslkAB ΔpscJ / pJN105 Para::exsA
This study

OKP29
PAO1<sup>tw</sup> ΔpilC pilQ-mScarlet<sup>SW</sup>
This study

OKP44
PAO1<sup>tw</sup> ΔpilC ΔpilQ
This study

OKP45
PAO1<sup>tw</sup> ΔslkAB ΔpilA
This study

OKP46
PA14 ΔslkAB
This study

OKP47
PAK ΔslkAB
This study

OKP48
PAK ΔpilC
This study

OKP49
PAO1<sup>tw</sup> ΔpilMNOPQ
This study
OKP49  PAO1<sup>tm</sup> ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::empty  This study
OKP49  PAO1<sup>tm</sup> ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::RBS<sub>Φ10</sub>-pilQ  This study
OKP50  PAO1<sup>tm</sup> ΔslkAB ΔpilMNOPQ  This study
OKP50  PAO1<sup>tm</sup> ΔslkAB ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::empty  This study
OKP50  PAO1<sup>tm</sup> ΔslkAB ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::RBS<sub>Φ10</sub>-pilQ  This study
OKP51  PAO1<sup>tm</sup> ΔslkAB ΔpilC ΔpilQ  This study
OKP51  PAO1<sup>tm</sup> ΔslkAB ΔpilC ΔpilQ  attTn7::P<sub>toplac-dn1</sub>:: slkB-mScarlet  This study
OKP52  PAO1<sup>tm</sup> ΔslkAB ΔpilQ ΔfimV  This study
OKP53  PAO1<sup>tm</sup> ΔslkAB ΔpilC ΔtsaP  This study
OKP57  PAK ΔslkAB ΔpilC  This study
OKP59  PAO1<sup>tm</sup> slkA-mScarlet ΔslkB  This study
OKP60  PAO1<sup>tm</sup> ΔpilQ slkA-mScarlet ΔslkB  This study
OKP61  PAO1<sup>tm</sup> ΔpilC slkA-mScarlet ΔslkB  This study
OKP62  PAO1 ΔoprM  This study
OKP64  PA14 ΔpilC  This study
OKP65  PA14 ΔslkAB ΔpilC  This study
OKP68  PAO1 ΔslkAB ΔpilMNOPQ  This study
OKP68  PAO1 ΔslkAB ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::empty  This study
OKP68  PAO1 ΔslkAB ΔpilMNOPQ  attTn7::P<sub>toplac-uv5</sub>::RBS<sub>Φ10</sub>-pilQ  This study
OKP69  PAO1<sup>tm</sup> ΔslkAB Δpila ΔpilQ  This study
OKP73  PAO1<sup>tm</sup> ΔslkAB ΔtsaP  This study
OKP74  PAO1<sup>tm</sup> ΔpilC ΔtsaP  This study
OKP77  PAO1<sup>tm</sup> ΔslkAB ΔpilY1  This study
OKP78  PAO1<sup>tm</sup> ΔslkAB ΔpilY1 ΔpilQ  This study
OKP80  PA14 ΔslkAB ΔpilC ΔpilQ  This study
| Strain   | Mutations                        | Notes   |
|----------|----------------------------------|---------|
| OKP82    | PAKΔslkAB ΔpilC ΔpilQ            | This study |
| OKP86    | PAO1<sup>tm</sup> ΔtsaP          | This study |
| OKP87    | PAO1<sup>tm</sup> ΔslkAB ΔtsaP ΔpilQ | This study |
| OKP96    | PAO1<sup>tm</sup> ΔpilA          | This study |
| OKP97    | PAO1<sup>tm</sup> ΔpilY1         | This study |
| OKP115   | PAO1<sup>tm</sup> ΔslkAB ΔhxcS   | This study |
| OKP120   | PAO1<sup>tm</sup> ΔpilC ΔpilQ slkA-mScarlet ΔslkB | This study |
| Plasmid   | Genotype*                                                      | Origin | Source/Reference         |
|----------|---------------------------------------------------------------|--------|--------------------------|
| pBTK30   | aacC1 bla oriT mariner C9                                     | R6K    | (Goodman et al, 2004)    |
| pEXG2    | aacC1 sacB oriT [facilitates allelic exchange in P. aeruginosa] | ColE1  | (Rietsch et al, 2005)    |
| pKHT104  | aacC1 bla Tn7 lacI\(^v\)\(\text{P}_{\text{toplac-dn1}}\) [facilitates insertion of \(\text{P}_{\text{toplac-dn1}}\)-regulated sequences into the attTn7 attachment site of P. aeruginosa] | pUC18  | Dove lab HMS              |
| pKHT105  | aacC1 bla Tn7 lacI\(^v\)\(\text{P}_{\text{toplac-uv5}}\) [facilitates insertion of \(\text{P}_{\text{toplac-uv5}}\)-regulated sequences into the attTn7 attachment site of P. aeruginosa] | pUC18  | Dove lab HMS              |
| pJN105   | aacC1 araC \(\text{P}_{\text{ara}}\) [replicating arabinose-inducible expression vector for P. aeruginosa] | pBBR1  | (Newman & Fuqua, 1999)   |
| pTNS3    | bla oriR6K tnsABCD from P1 and \(\text{P}_{\text{lac}}\) [Tn7 transposase expression] | R6K    | (Choi et al, 2007)       |
| pOKP10   | pEXG2 \(\Delta\text{slkA}(4-501)\)                           | ColE1  | This study               |
| pOKP11   | pEXG2 \(\Delta\text{slkB}(4-552)\)                          | ColE1  | This study               |
| pOKP12   | pEXG2 \(\Delta\text{slkAB}\)                                 | ColE1  | This study               |
| pOKP16   | aacC1 araC \(\text{P}_{\text{ara}}\)::Empty [BamHI site in the araI removed in pJN105] | pBBR1  | This study               |
| pOKP17   | pJN105 \(\text{P}_{\text{ara}}::\text{slkA}\)                | pBBR1  | This study               |
| pOKP18   | pJN105 \(\text{P}_{\text{ara}}::\text{slkB}\)                | pBBR1  | This study               |
| pOKP23   | pEXG2 \(\Delta\text{pilQ}(4-1845)\)                          | ColE1  | This study               |
| pOKP26   | pOKP16 \(\text{P}_{\text{ara}}::\text{mScarlet}\)            | pBBR1  | This study               |
| pOKP28   | pOKP16 \(\text{P}_{\text{ara}}::\text{pilQ}(1-396)-\text{mScarlet}\) | pBBR1  | This study               |
| pOKP29   | pOKP16 \(\text{P}_{\text{ara}}::\text{pilQ}(1-396)-\text{mScarlet-pilQ}(397-2145)\) | pBBR1  | This study               |
| pOKP35   | pEXG2 pilQ(1-396)-mScarlet-pilQ(397-827)                      | ColE1  | This study               |
| pOKP42   | pEXG2 \(\Delta\text{pilC}(4-1039)\)                          | ColE1  | This study               |
| pOKP43   | pEXG2 \(\Delta\text{xcpS}(4-996)\)                           | ColE1  | This study               |
| pOKP44   | pEXG2 \(\Delta\text{pscJ}(4-612)\)                           | ColE1  | This study               |
| pOKP57 | pOKP16 P<sub>ara</sub>::slkA-mScarlet | pBBR1 | This study |
|--------|--------------------------------------|--------|------------|
| pOKP82 | pEXG2 ∆pillA (50-368)                | ColE1  | This study |
| pOKP88 | pEXG2 ∆pillMNOPQ                     | ColE1  | This study |
| pOKP92 | pEXG2 PA14 ∆slkAB (ΔPA14_RS27590-PA14_RS27595) | ColE1  | This study |
| pOKP93 | pEXG2 PAK ∆slkAB (ΔY880_RS24175-Y880_RS24180) | ColE1  | This study |
| pOKP94 | pEXG2 PA14 ∆pill (4-1102) (ΔPA14_RS23960) | ColE1  | This study |
| pOKP95 | pEXG2 PAK ∆pill (4-1119) (ΔY880_RS20950) | ColE1  | This study |
| pOKP98 | pEXG2 ∆fimV (22-2705)                | ColE1  | This study |
| pOKP99 | pEXG2 ∆tsaP (10-970)                 | ColE1  | This study |
| pOKP101| pEXG2 slkA-mScarlet-∆slkB(1-57)      | ColE1  | This study |
| pOKP103| pEXG2 ∆oprM (4-1135)                 | ColE1  | This study |
| pOKP107| pEXG2 ∆hxcS (4-994)                  | ColE1  | This study |
| pOKP115| pEXG2 ∆pillY1 (4-3173)               | ColE1  | This study |
| pOKP116| pEXG2 PA14 ∆pillQ (4-1831) (ΔPA14_RS27185) | ColE1  | This study |
| pOKP117| pEXG2 PAK ∆pillQ (4-1845) (ΔY880_RS23755) | ColE1  | This study |
| pOKP121| pKHT105 RBS<sub>φ10</sub>·pillQ      | pUC18  | This study |
| pOKP139| pJN105 Para::pillC                   | pBBR1  | This study |
| pOKP154| pKHT104 slkB-mScarlet                | pUC18  | This study |
| pBO37  | pKHT104 dsbAss-mScarlet              | pUC18  | This study |
| pBO48  | pJN105 Para::exsA                    | pBBR1  | This study |

* Numbers in pEXG2 derivatives represent the base numbers of each open reading frame.
SUPPLEMENTAL MATERIALS AND METHODS

Media, Bacterial Strains, and Plasmids

Cells were grown in either LB [1% tryptone, 0.5% yeast extract, 0.5% NaCl], Vogel-Bonner minimal medium (VBMM) [3.42 g/L trisodium citrate dihydrate, 2 g/L citric acid, 10 g/L K$_2$HPO$_4$, 3.5 g/L NaNHPO$_4$-4H$_2$O, pH7, 1 mM MgSO$_4$, 0.1 mM CaCl$_2$], minimal M9 medium supplemented with glucose [6 g/L Na$_2$HPO$_4$ anhydrous, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 1 g/L NH$_4$Cl, 0.2% glucose, 2 mM MgSO$_4$, 0.1 mM CaCl$_2$], or proteose peptone medium [20 mM NH$_4$Cl, 20 mM KCl, 120 mM Tris-HCl, pH7.4, 0.5% glucose, 0.5% proteose peptone (Difco), and 1.6 mM MgSO$_4$] (Cheng et al., 1970). The strains and plasmids that were used for the study are listed in Table S3 and Table S4. Detailed procedures for strain and plasmid construction are also provided in the Supporting Information.

Plasmid construction

**pBO37** – To make a vector for expression of periplasmic C-terminal mScarlet fusion proteins, an 838 bp g-block DNA fragment containing φ10 RBS-NcoI site—the signal sequence of PAO1 dsbA-BamHI site-PstI site-5 amino acid linker-mScarlet coding sequence between BglII and KpnI sites was synthesized from IDT. The g-Block DNA was digested with BglII and KpnI and ligated with pKHT4 digested with BamHI and KpnI, which make the BamHI site between dsbA signal sequence and mScarlet unique for later use.

**pBO48** – To clone exsA under arabinose promoter, exsA gene with native RBS was amplified using a primer pair 5’- GCTAGAATTGCAGACGGGAAGTGTTGG -3’ and 5’- GCTATCTAGACGTCAGTTATTTTTAGCCCGG-3’. The resulting PCR product was digested with EcoRI and XbaI and ligated with pJN105 digested with the same enzymes to generate pBO48.
pOKP10 – To construct a plasmid for deletion of slkA (PA5122), a 700bp region upstream of slkA was amplified using 5’-GCTAAAGCTTCTGGGAAAGCGCTCCTCGAG-3’ and 5’-TTTCTTCCCGAGGTGACGGTCTCCGGGTGA-3’. A 701 bp region downstream of slkA was amplified using 5’-CGGAGACCGTCCATGCACCTCGGGAAGAAACGATG-3’ and 5’-GCTATCTAGAGTGACCAATCTGGTG-3’ . The resulting PCR products were spliced using overlap extension PCR (OE) using the primers 5’-GCTAAAGCTTCTGGGAAAGCGCTCCTCGAG-3’ and 5’-GCTATCTAGAGTGACCAATCTGGTG-3’. The OE product was digested with HindIII and XbaI and ligated with pEXG2 digested with the same enzymes to generate pOKP10.

pOKP11 – To construct a plasmid for deletion of slkB (PA5123), a 702bp region upstream of slkB was amplified using 5’-GCTAAAGCTTCTGGGCAAGGAGGTTC-3’ and 5’-CTCGCTTGGCTCTCATGGACGGTCTCCGGGTGA-3’. A 701 bp region downstream of slkB was amplified using 5’-CGGGAAGAAACGATGATAGAGCCAAGCGAGCGCTA-3’ and 5’-GCTATCTAGAGTGACCAATCTGGTG-3’ . The PCR products were spliced by OE using the primers 5’-GCTAAAGCTTCTGGGCAAGGAGGTTC-3’ and 5’-GCTATCTAGAGTGACCAATCTGGTG-3’. The OE product was cloned between HindIII and XbaI sites of pEXG2 as described above to generate pOKP11.

pOKP12 – To construct a plasmid for deletion of slkAB (PA5122-P A5123), a 700bp region upstream of slkA was amplified using 5’-GCTAAAGCTTCTGGGAAAGCGCTCCTCGAG-3’ and 5’-CTCGCTTGGCTCTCATGGACGGTCTCCGGGTGA-3’. A 701 bp region downstream of slkB was amplified using 5’-
CGGAGACCGTCCATGATAGGCAAGCGAGCGCTAG-3' and 5'-
GCTATCTAGACCCGAGCGCTAG-3'. The PCR products were spliced by OE using the primers 5'- GCTAAGGCTCCTCGAGGCAAGCGAGCGCTAG-3' and 5'-
GCTATCTAGACCCGAGCGCTAG-3'. The OE product was cloned between HindIII and Xbal sites of pEXG2 as described above to generate pOKP12.

pOKP17-pOKP18 – To clone slkA under arabinose promoter, slkA gene with native RBS was amplified using a primer pairs 5'- GTCAGAATTCGGTACCCGGAGACCGTCATAGGCAAGCGAGCGCTAG-3' and 5'-
GTCATCTAGACCCGAGCGCTAG-3'. For slkB cloning, slkB with native RBS was amplified with 5'-
GTCAGAATTCGGTACCCGGAGACCGTCATAGGCAAGCGAGCGCTAG-3' and 5'-
GTCATCTAGACCCGAGCGCTAG-3'. Each PCR product was digested with EcoRI and Xbal and ligated with pJN105 digested with the same enzymes to generate pOKP17 (slkA) and pOKP18 (slkB).

pOKP23 – To construct a plasmid for a pilQ deletion, a 620 bp DNA upstream of pilQ was amplified using 5'- GCTAAGGCTCCTCGAGGCAAGCGAGCGCTAG-3' and 5'-
CACCTGACTGTCGATACCGAGCCGCCTCGAGGCAAGCGAGCGCTAG-3'. A 620 bp region including a 300 bp pilQ sequence and a 320 bp region downstream of pilQ was amplified using 5'-
CGGAGTCGGACGACGTCGATACCGAGCCGCCTCGAGGCAAGCGAGCGCTAG-3' and 5'-
GCTATCTAGACCCGAGCGCTAG-3'. The OE product was cloned into pEXG2 using HindIII and Xbal sites to generate pOKP12.
pOKP26 – To clone GS linker-\textit{mScarlet} under arabinose promoter, \textit{mScarlet} gene with a GS linker was amplified with a primer pair 5’-
\[
\text{GTCA}\GG\text{ATCCGGTAGGTCCTAAAGGTGAAGCAGTTATC-3’ and 5’-}
\]
\[
\text{GTCA}\text{TCTAGATTACCTTACAGTCCATACCCATACCTC-3’} \text{ using pBO37 as a template. The resulting PCR product was digested with BamHI and XbaI and ligated with pOKP16 digested with the same enzymes to generate pOKP26.}
\]

pOKP28-pOKP29 – A PilQ-\textit{mScarlet} sandwich fusion was constructed as described in Carter et al (2017) with \textit{mScarlet} located between the first and second AMIM domains of PilQ. The 416 bp region including 20 bp upstream of \textit{pilQ} and 396 bp \textit{pilQ} coding sequence was amplified using a primer pair 5’-\text{GTCA\text{AGAT}CTGACCACTACAGCTGGCGCCGACG-3’} and 5’-\text{TTACCTTTAGAAACGCTACCGACGCTGGCGCCG-3’}. \textit{mScarlet} sequence was amplified with 5’-\text{GCCAGCTCGGTAGGTCCTAAAGGTGAAGCAGTTATC-3’} and 5’-\text{GTCA\text{AGAT}CTGACCACTACAGCTGGCGCCGACG-3’} \text{ using pBO37 as a template. The PCR products were spliced by OE with a primer pair 5’-
\[
\text{GTCA}\GG\text{ATCCGGTAGGTCCTAAAGGTGAAGCAGTTATC-3’ and 5’-
\]
\[
\text{GTCA}\GG\text{ATCCGGTAGGTCCTAAAGGTGAAGCAGTTATC-3’}. \text{ The overlap extension product was cloned into pOKP16 using EcoRI and BamHI sites to generate pOKP28 that express a truncated pilQ-\textit{mScarlet} fusion gene.}
\]

The 1,749 bp coding sequence of \textit{pilQ} at the 3’ end was amplified with a primer pair 5’-
\[
\text{GTCA}\GG\text{ATCCGGTAGGTCCTAAAGGTGAAGCAGTTATC-3’ and 5’-
\]
\[
\text{GTCA}\text{TCTAGATTACGACGCGGATCGGATGG-3’}. \text{ The PCR product was digested with BamHI and XbaI and ligated with pOKP28 digested with the same enzymes to generate pOKP29 that expresses pilQ-\textit{mScarlet} sandwich fusion under arabinose promoter.}
pOKP35 – To construct a plasmid for generating pilQ-mScarlet sandwich fusion at the native pilQ locus, a 1552 bp region of pilQ-mScarlet sandwich fusion was amplified with a primer pair 5'-GCTAAGCTTCTGTACAACGGAGTCGGACG-3' and 5'-GCTATCTAGATTGTCTTTCTTGCGCCGCTC-3' using pOKP29 as the template. The PCR product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP35.

pOKP42 – To construct a plasmid for a pilC deletion, a 707 bp sequence upstream of pilC from the pilC start codon was amplified using 5'-

GCTATCTAGATATCGCTATAACCCGGCCTG -3' and 5'-

ATCGAGCGAACCAGGACATGGATATTCTTGGTCAC-3'. A downstream 717 bp region, including 186 bp pilC coding sequence at the 3' end was amplified using 5'-

AAGGATTAATCCATGTCCGGTTCGCTCGATGAGATG -3' and 5'-

GCTAGGATCCGAAGAGGCCGAATGTTGGG-3'. The PCR products were spliced by OE using the primers 5'- GCTATCTAGATATCGCTATAACCCGGCCTG -3' and 5'-

GCTAGGATCCGAAGAGGCCGAATGTTGGG-3'. The OE product was cloned into pEXG2 using BamHI and XbaI.

pOKP82 – To construct a plasmid for a pilA deletion, a 689 bp region upstream of pilA including the 49 bp pilA sequence at the 5' end was amplified using a primer pair 5'-

GCTAAAGCTTCAAGCTTCTGTCTTCTCGACC-3' and 5'-

GCAGTACGTTCAAGAAACCACGATCATCAGTTTCG-3'. A 647 bp region downstream of pilA including 82 bp pilA coding sequence at the 3' end was amplified with a primer pair 5'-

TGATGATCGTGTTGTCTGAACCGTACTGCGGATG-3' and 5'-

GCTATCTAGACCTACGATGCCTTCTG-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAAGCTTCAAGCTTCTGTCTTCTCGACC-3' and 5'-

GCTATCTAGACCTACGATGCCTTCTG-3'. The resulting OE product was cloned into
pEXG2 using BamHI and XbaI.

**pOKP88** – To construct a plasmid for deletion of *pilMNOPQ* operon, a 620 bp upstream region from *pilM* start codon was amplified using a primer pair 5’-GCTAAAGCTTTCTTCGAGAAGCCTCTTTC-3’ and 5’-ACCTTGACTTCGACCACGACCAATTCCCTATTAGC-3’. A 620 bp downstream region including 300 bp *pilQ* coding sequence at the 3’ end was amplified using 5’-AGGGAATTGGTCGTGGTCGAAGTGCAAGGTACCA-3’ and 5’-GCTATCTAGACGAGGCATGCAGGTACAC-3’. The PCR products were spliced by a primer pair 5’-GCTAAAGCTTTCTTCGAGAAGCCTCTTTC-3’ and 5’-GCTATCTAGACGAGGCATGCAGGTACAC-3’. The resulting OE product was clone into pEXG2 using BamHI and XbaI.

**pOKP92** – To construct a plasmid for a *slkAB* (*PA14_RS27590*-*PA14_RS27595*) deletion in PA14, a 700 bp upstream region from the start codon of *PA14_RS27590* (*slkA*) was amplified using 5’-GCTAAAGCTTTCTGGGAAAGCGCTCCTCGAG-3’ and 5’-GCTAGCGCTCGCTCATGGACGGTCTCCGGGTG-3’. A 691 bp downstream region including 12 bp *PA14_RS27595* (*slkB*) coding sequence at the 3’ end was amplified using 5’-GGAGACCGTCCATGAGCGGACGTAGCGCAAC-3’ and 5’-GCTATCTAGACGAGGCAGCAGCGTGGGTC-3’. The PCR products were spliced by a primer pair 5’-GCTAAAGCTTTCTGGGAAAGCGCTCCTCGAG-3’ and 5’-GCTATCTAGACGAGGCAGCAGCGTGGGTC-3’. The resulting OE product was clone into pEXG2 using BamHI and XbaI.

**pOKP93** – To construct a plasmid for a *slkAB* (*Y880_RS24175*-*Y880_RS24180*) deletion in
PAK, a 699 bp upstream region from the start codon of Y880_RS24175 (slkA) was amplified using 5'-GCTAAAGCCTTTTGAAAAGCGCTCCTCGAG-3' and 5'
CTCGCTTGGCTCTATCATGGACGGTCTCCGGGTG-3'. A 700 bp downstream region including 21 bp Y880_RS24180 (slkB) coding sequence at the 3' end was amplified using 5'
CGGAGACCCTCATGATAGAGCCAAGCGAGCGCTAG-3' and 5'
GCTATCTAGAGCACCAGCAGCTTGGTGTC-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAAGCCTTTTGAAAAGCGCTCCTCGAG-3' and 5'
GCTATCTAGAGCACCAGCAGCTTGGTGTC-3'. The resulting OE product was clone into pEXG2 using BamHI and XbaI.

pOKP94 – To construct a plasmid for a pilC deletion in PA14, a 234 bp region upstream of PA14_RS23960 (pilC) was amplified using 5'-GCTAAAGCCTTTGCGCTGCAGCATTGC-3' and 5'
TTGTCAGGTTGTCGACATGGATTAGTCCTTGGTCAC-3'. A 306 bp downstream region, including 116 bp PA14_RS23960 (pilC) sequence was amplified using 5'
AAGGACTAATCCATGTCGACAACCTGACAACGTTG-3' and 5'
GCTATCTAGAGGTGTCGCTGCAGCATTGC-3'. PCR products were spliced by OE using a primer pair 5'-GCTAAAGCCTTTGCGCTGCAGCATTGC-3' and 5'
GCTATCTAGAGGTGTCGCTGCAGCATTGC-3'. The OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP94.

pOKP95 – To construct a plasmid for a pilC deletion in PAK, a 615 bp region upstream of Y880_RS20950 (pilC) was amplified using 5'
GCTAAAGCCTTTGCGCTGCAGCATTGC-3' and 5'
ATCGGTTCATGAGCATGGATTACATCCTTGGTCACG-3'. A 633 bp downstream region including a 102 bp Y880_RS20950 sequence at the 3' end was amplified using 5'
AGGATTAATCCATGTCCTGGAACCGATGATCATG-3' and 5'
The PCR products were spliced by OE using the primers 5'-GCTAAGCTTAAACCAGGCTAACCCTG-3' and 5'-
GCTAAGCTTAAACCAGGCTAACCCTG-3'. The resulting OE product was cloned into pEXG2 using HindIII and BamHI sites to generate pOKP93.

**pOKP98** – To construct a plasmid for a *fimV* deletion, a 750 bp region upstream of *fimV* was amplified using a primer pair 5'-GCTAAGCTTAAACCAGGCTAACCCTG-3' and 5'-
TGGCTGTCATTACCTCAGTGAAGCCGAACC-3'. A 719 bp downstream region including a 55 bp *fimV* sequence at the 3' end was amplified using a primer pair 5'-
CGGCTTCGTACACTGAGGTAATGACAGCCAGG-3' and 5'-
GCTATCTAGATGCGCACCATATGGGAAGG-3'. The PCR products were spliced by OE using the primers 5'-GCTAAGCTTAAACCAGGCTAACCCTG-3' and 5'-
GCTAAGCTTAAACCAGGCTAACCCTG-3'. The OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP98.

**pOKP99** – To construct a plasmid for a *tsaP* deletion, a 653 bp region upstream of *tsaP* was amplified using 5'-GCTAAGCTTAAACCAGGCTAACCCTG-3' and 5'-
TGGCCATCAGAACCATATGGGAAGG-3'. A 536 bp downstream region, including a 56 bp *tsaP* sequence at the 3' end was amplified using a primer pair 5'-
CACTGCATGAGGAAATGTTCTGATGGCCAGG-3' and 5'-
GCTATCTAGAGTTCCAGAGTCTCCGGAG-3'. The PCR products were spliced by OE using the primers 5'-GCTAAGCTTAAACCAGGCTAACCCTG-3' and 5'-
GCTAAGCTTAAACCAGGCTAACCCTG-3'. The OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP99.
pOKP101 – To construct a plasmid for generating slkA-mScarlet with a slkB deletion at the
native slkAB locus, a 1262 bp slkA-mScarlet sequence was amplified with a primer pair 5’-
GCTAAAGCTTGTGCAACGCTTGGCAGGTTC-3’ and 5’-
TAGGTGTAGACCCCTTTACTTATACTAGTTCATCCATCACCTC-3’ using pOKP57 a template. A
516 bp region of 5’ end-truncated slkB was amplified using 5’-
GAACGTATAAGTAAGGGGTCTACCTACACCTAC-3’ and 5’-
GCTACTAGACTAGCGCTGGCTTCG-3’. PCR products were spliced by overlap
extension PCR using the primers 5’-GCTAAAGCTTGTGCAACGCTTGGCAGGTTC-3’ and
5’-GCTACTAGACTAGCGCTGGCTTCG-3’. The overlap extension product was cloned
into pEXG2 using HindIII and XbaI sites to generate pOKP101.

pOKP103 – To construct a plasmid for an oprM deletion, a 700 bp region upstream of oprM
was amplified using 5’-GCTAAAGCTTGTGCAACGCTAATGGCGT-3’ and 5’-
GGAACGTGCTGGCATATCATTTGGCCTTTCT-3’. A 628 bp downstream region
including a 323 bp oprM sequence at the 3’ end was amplified using 5’-
AGGGGCAATGATATGTCCAGACGGCGTTCCAG-3’ and 5’-
GCTACTAGACGGGCTCGAGAATGC-3’. The PCR products were spliced by OE using
the primers 5’-GCTAAAGCTTGTGCAACGCTAATGGCGT-3’ and 5’-
GCTACTAGACGGGCTCGAGAATGC-3’. The OE product was cloned into pEXG2 using
HindIII and XbaI sites to generate pOKP103.

pOKP107 – To construct a plasmid for a hxcS deletion, a 721 bp region upstream of hxcS
was amplified using 5’-GCTAAAGCTTGTGCAACGCTTGGCAGGCAGCCTGACCTAC-3’ and 5’-
GGGGGTGAGGATCGCATCAGGCGTCCCGGGTC-3’. A 698 bp downstream region
including a 221 bp hxcS sequence at the 3’ end was amplified with a primer pair 5’-
CCGGGACGCCGCTGATGCGATCCTCACCCACCTGATC-3’ and 5’-
GCTATCTAGAGGAAGTCGTCTTGCATGAGG-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAAGCTTTGGACAACCTGATCCGCGCCAG-3' and 5'-GCTATCTAGAGGAAGTCGTCTTGCATGAGG-3'. The resulting OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP107.

pOKP115 – To construct a plasmid for a pilY1 deletion, a 514 bp region upstream of pilY1 was amplified using 5'-GCTAAAGCTTTGCATCGCGAACTGACATCGTCCGATCAG-3' and 5'-CAGGGTCATCTTCATCGCCAGGCTCGATCAG-3'. A 509 bp downstream region including a 313 bp pilY1 sequence at the 3' end was amplified using 5'-ATCGAGCTCGCATCGCAACGATGCACCTGTGC-3' and 5'-GCTATCTAGACTTGACCTGGAAACAGGCTG-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAAGCTTTGCATCGCGAACTGACATCGTCCGATCAG-3' and 5'-GCTATCTAGACTTGACCTGGAAACAGGCTG-3'. The OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP115.

pOKP116 – To construct a plasmid for a pilQ deletion in PA14, a 234 bp region upstream of PA14_RS27185 (pilQ) was amplified using 5'-GCTAAAGCTTTACTTTATAGCGCTTGCGATCCGACC-3' and 5'-CCTTCACCTCAACGACATCGTCCGACTCCGTTTGA-3'. A 622 bp downstream region including a 302 bp PA14_RS27185 sequence at the 3' end was amplified using 5'-CGGAGTCGGACATGTCGTTTGGTGAGTGAAGTGACCC-3' and 5'-GCTATCTAGACCGAGGCATCGAGTAC-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAAGCTTTACTTTATAGCGCTTGCGATCCGACC-3' and 5'-GCTATCTAGACCGAGGCATCGAGTAC-3'. The resulting OE product was cloned into pEXG2 using HindIII and XbaI sites to generate pOKP116.
To construct a plasmid for a pilQ deletion in PAK, a 620 bp region upstream of Y880_ RS23755 (pilQ) was amplified using 5'-GCTAAGCTTGCCGAAGACCTATCGCTAC-3' and 5'-CACCCTTGACCTTGACCATTCTCGGACTCCGGTTGTA-3'. A 620 bp downstream region, including a 300 bp Y880_ RS23755 sequence at the 3' end was amplified using 5'-CGGAGTCGGACGATGTCGACCTACAAGGTGACCAA-3' and 5'-GCTAAGCTTGCCGAAGACCTATCGCTAC-3'. The PCR products were spliced by OE using a primer pair 5'-GCTAAGCTTGCCGAAGACCTATCGCTAC-3' and 5'-GCTAAGCTTGCCGAAGACCTATCGCTAC-3'. The resulting OE product was cloned into pEXG2 using HindIII and BamHI sites to generate pOKP117.

For constructing a plasmid that expresses pilQ under an IPTG-inducible promoter, pilQ was cloned in pKHT5 using a SLIC (sequence and ligation independent cloning) method (Jeong et al., 2012) because pilQ has a KpnI site in the coding sequence. Briefly, pilQ with a Φ10 RBS was amplified using a primer pair 5'-GCTTAGTCGACAGCTAGCCGGATCCTTAAGAAGGAGATATACATATGAACAGTGGCCTC-3' and 5'-TTATGCTAAGCTTGCCGAAGACCTATCGCTAC-3'. The PCR product was mixed with pKHT105 digested with KpnI and BamHI, and processed with T4 DNA polymerase (NEB) for 2 min at RT, and transformed into E.coli competent cells to generate pOKP121.

To clone pilC under arabinose promoter, PAO1<sup>lw</sup> pilC with native RBS was amplified using a primer pair 5'-GCTACTGCAGCGTGACCAGGAATATCC-3' and 5'-GCTACTGAGTTATCCGACGACGTTGC-3'. The PCR product was digested with EcoRI and XbaI and ligated with pJN105 digested with the same enzymes to generate pOKP139.
pOKP154 – To construct a plasmid that expresses slkB-mScarlet under a weak IPTG-inducible promoter ($P_{toplac-dn1}$), slkB was amplified using a primer pair 5’-GCTACCATGGCTATCCCCGCATTCTC-3’ and 5’-GCTACTGCAAGGCGCTCGTTGGCTC-3’. The PCR product was digested with NcoI and PstI and ligated with pBO037 digested with the same enzymes to generate pOKP154.

P. aeruginosa strain construction

Gene knockout and chromosomal gene fusion

Plasmids for gene knockout or gene fusion were transferred into P. aeruginosa by conjugation from and E. coli donor [Sm10(λpir)] on LB plates. Transconjugants were selected on Vogel-Bonner minimal medium (VBMM) agar supplemented with 30 μg/mL gentamicin and purified on LB agar supplemented with 30 μg/mL gentamicin. For gene deletions in the slkAB mutants, 10 μg/mL gentamicin was used to prevent enrichment of random mutations that suppress the barrier defect. To remove the integrated plasmid for allele exchange, purified transconjugants was grown overnight in LB with 10 μg/mL gentamicin at 37 °C. The overnight culture was diluted 1:100 in LB and grown for 4hrs at 30 °C to allow plasmid recombination to take place. The resulting culture was diluted 1:20 and 100 μL of the dilution was spread on LB agar supplemented with 5% sucrose. The plates were incubated overnight at 30 °C to select for colonies that lost the plasmid via double crossover. Sucrose-resistant colonies were patched on LB agar supplemented with either 5% sucrose or 30 μg/mL gentamicin. Gentamicin-sensitive colonies were further screened for the desired gene alteration by PCR with a primer pair that flank the altered chromosomal region.

Transformation of P. aeruginosa strains
All transformations of *P. aeruginosa* strains were performed by electroporation using electrocompetent cells made with 0.3 M sucrose solution. Briefly, 6 mL overnight culture *P. aeruginosa* strains were washed with 0.3 M sucrose solution 4 times at room temperature. After the final wash, the cell pellets were resuspended in 200 µL of 0.3 M sucrose solution. The resulting competent cells (50 µL) were electroporated with 50 ng of plasmid DNA.

Integration of plasmids for gene expression at the Tn7 attachment site.

Plasmids for gene expression from the Tn7 attachment site were introduced into the target cell by electroporation following a previously described method (Choi & Schweizer, 2006). The recipient strain was co-electroporated with a plasmid for gene expression and pTNS3 [bla oriR6K tnsABCD] that expresses Tn7 transposase. Transformants were selected on LB agar supplemented with 30 µg/mL gentamicin. For gene deletions in the strains that can show an envelope permeability defect, 10 µg/mL gentamicin was used to prevent enrichment of random mutations that suppress the permeability defect. Gentamicin-resistant colonies were subject to diagnostic PCR with primer pairs 5’-CACAGCATAACTGGACTGATTTC-3’ and 5’-GCACATCGGCGACGTGCTCTC-3’ to verify the integration of the transposon at the Tn7 site.

OKP5 (∆slkA), OKP6 (∆slkB), OKP7 (∆slkA B), and OKP12 (∆pilQ) – To delete slkA, slkB, slkAB, or pilQ in PAO1, pOKP10, pOKP11, pOKP12, or pOKP23 was transferred to PAO1 (the twitching-defective strain) using Sm10(λpir). Candidates for the deletion strains were obtained by selection with gentamicin and counter-selection with sucrose as described above. Colonies with the desired gene deletion were screened with primer pairs 5’-TTCGGATGCTGCGGCAACTC-3’ and 5’-CAAACCAGGCACCCGAAAAGG-3’ for ∆slkA, 5’-GATGATCGGCACGAAAGG-3’ and 5’-CTTCTTCGATGACGTTG-3’ for ∆slkB, 5’-
TTCGGATGCTGCGGCAACTC-3' and 5'-CTTCTTCGATGATGACGTTG-3' for ΔslkAB, 5'-ACGACTTGGGACCTTCGTC-3' and 5'-ATTTCGCGGTAGAGCGGTC-3' for ΔpilQ.

OKP13 – To generate OKP13 (∆pilQ ∆slkAB), pilQ was deleted in OKP7(∆slkAB) by basically using the same procedure for gene deletion except that 10 μg/mL gentamicin was used for selection of transconjugants. As OKP7 is much more susceptible to aminoglycosides than the wild type P. aeruginosa strains due to its defective OM barrier function, a lower concentration of gentamicin was used to avoid selection of strains with random mutations that suppress the barrier defect.

OKP13(attTn7::pKHT105) and OKP13(attTn7::pOKP121) – pOKP121 that express pilQ from an IPTG-inducible toplac-uv5 promoter and its empty vector plasmid were integrated into OKP13 by using the procedure described above.

HJP1 – slkAB was deleted in PAO1tw by following the same procedure described above after transferring pOKP11.

OKP14 and OKP15 – pilQ was deleted in PAO1tw and HJP1 by following the same procedure described above after transferring pOKP23 to generate OKP14 (PAO1tw ΔpilQ) and OKP15(PAO1tw ΔslkAB ΔpilQ)

OKP15(attTn7::pOKP121) – pOKP121 that express pilQ from an IPTG-inducible toplac-uv5 promoter was integrated into OKP15 (PAO1tw ΔslkAB ΔpilQ) by using the procedure described above.
OKP20 and OKP21 – Strains that express pilQ-mScarlet sandwich fusion at the native pilQ locus in the PAO1<sup>tw</sup> and HJP1 strain backgrounds were generated by using the procedure described above after transferring pOKP35 by conjugation.

OKP23 – pilC was deleted in PAO1<sup>tw</sup> by using the procedure described above with pOKP42.

OKP24 – To generate OKP24 (ΔpilC ΔslkAB), pilC was deleted in HJP1 (ΔslkAB) by basically using the same procedure used to produce OKP23 except that 10 μg/mL gentamicin was used for selection of transconjugants to avoid enrichment of random mutations that suppress the barrier defect.

OKP23(attTn7::pKHT104) and OKP24(attTn7::pKHT104) – To integrate P<sub>toplac-dn1</sub>-regulated empty vector into the Tn7 attachment site, pKHT104 was co-electroporated with pTNS3 into OKP23 and OKP24. A lower concentration of gentamicin (10 μg/mL) was used for selection to avoid enrichment of random mutations that suppress the barrier defect.

OKP26, OKP28, and OKP115 – xcpS, pscJ, or hxcS was deleted in HJP1 by using pOKP43, pOKP44, or pOKP107 to generate double mutant strains in PAO1<sup>tw</sup>, ΔslkAB ΔxcpS (OKP26), ΔslkAB ΔpscJ (OKP28), ΔslkAB ΔhxcS (OKP115).

OKP29 – pilQ-mScarlet sandwich fusion at the native pilQ locus was introduced in OKP23 (ΔpilC) using pOKP35 to generate OKP29 (PAO1<sup>tw</sup> ΔpilC pilQ-mScarlet<sup>SW</sup>).
OKP44 – To generate a ΔpilC ΔpilQ double mutant in the PAO1 tw strain background, pilC was deleted in OKP14 (ΔpilQ) by using pOKP42.

OKP45 and OKP96 – pilA was deleted in HJP1 and PAO1 tw by using pOKP82 to generate OKP45 (PAO1 twΔslkAB ΔpilA) and OKP96 (PAO1 tw ΔpilA).

OKP46 – slkAB was deleted in PA14 by using pOKP92 to generate a PA14 ΔslkAB strain.

OKP47 – slkAB was deleted in PAK by using pOKP93 to generate a PAK ΔslkAB strain.

OKP64 – pilC was deleted in PA14 by using pOKP94 to generate a PA14 ΔpilC strain.

OKP48 – pilC was deleted in PAK by using pOKP95 to generate a PAK ΔpilC strain.

OKP49 and OKP50 – pilMNOPQ was deleted in PAO1 tw and HJP1 using pOKP88 to generate OKP49 (PAO1 twΔpilMNOPQ) and OKP50 (PAO1 twΔslkAB ΔpilMNOPQ), respectively.

PAO1 tw(attTn7::pKHT105), OKP49(attTn7::pKHT105), OKP49(attTn7::pOKP121), OKP50(attTn7::pKHT105), OKP50(attTn7::pOKP121) – pOKP121 (Ptoplac-uv5-regulated pilQ) or its empty vector plasmid pKHT105 were integrated into the Tn7 attachment site by co-
electroporation with pTNS3. Candidates with the integrated plasmids were obtained by selection with 10 µg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP51 – *pilQ* was deleted in PAO1™ Δ*slkAB ΔpilC* strain by using pOKP23 to generate OKP24 (PAO1™Δ*slkAB ΔpilC ΔpilQ*). A lower concentration of gentamicin (10 µg/mL) was used for selection of transconjugants to avoid enrichment of random mutations that suppress the barrier defect.

HJP1(attTn7::pOKP154), OKP15(attTn7::pOKP154), OKP24(attTn7::pOKP154), OKP51 – To integrate a plasmid expressing *slkB-mScarlet* from P<sub>toplac-dn1</sub> (pOKP154) into the Tn7 attachment site, pOKP154 was co-electroporated with pTNS3 into HJP1, OKP15, OKP24, OKP51. Candidates for the integrated strains were obtained by selection with 10 µg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP52 and OKP53 – *fimV* or *tsaP* was deleted in OKP24 (PAO1™Δ*slkAB ΔpilC*) by using pOKP98 (*fimV* deletion) or pOKP99 (*tsaP* deletion) to generate OKP52 and OKP53, respectively. A lower concentration of gentamicin (10 µg/mL) was used for selection of transconjugants to avoid enrichment of random mutations that suppress the barrier defect.

OKP57 – *pilC* was deleted in OKP47 (PAK Δ*slkAB*) by using pOKP95.

OKP59, OKP60, OKP61, and OKP120 – *slkA-mScarlet* with a *slkB* deletion was introduced
at the slkAB locus of PAO1\textsuperscript{tw}, OKP14 (PAO1\textsuperscript{tw} ΔpilQ), OKP23 (PAO1\textsuperscript{tw} ΔpilC), and OKP44 (PAO1\textsuperscript{tw} ΔpilC ΔpilQ) strains by using pOKP101.

OKP62 – oprM was deleted in PAO1 by using pOKP103.

OKP65 – pilC was deleted in OKP46 (PA14 ΔslkAB) by using pOKP94. Candidates for the deletion strain were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP68 – pilMNOPQ was deleted in HJP1 (PAO1 ΔslkAB) using pOKP88.

OKP69 – pilQ was deleted in OKP45 (PAO1\textsuperscript{tw} ΔslkAB ΔpilA) by using pOKP23. Candidates for the deletion strain were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP68(attTn7::pKHT105) and OKP68(attTn7::pOKP121) – A plasmid expressing pilQ from $P_{\text{toplac}-uv5}$ (pOKP121) and its empty vector plasmid (pKHT105) were integrated at the Tn7 attachment site of OKP68 by co-electroporation with pTNS3. Candidates for the integrated strains were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP73, OKP74, and OKP86 – tsaP was deleted was in PAO1\textsuperscript{tw}, HJP1 (PAO1\textsuperscript{tw} ΔslkAB), or OKP23 (PAO1\textsuperscript{tw} ΔpilC) by using pOKP99. Candidates for the deletion strains were obtained
by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP80 – pilQ was deleted in OKP65 (PA14 ΔslkAB ΔpilC) by using pOKP116. Candidates for the deletion strains were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP82 – pilQ was deleted in OKP57 (PAK ΔslkAB ΔpilC) by using pOKP57. Candidates for the deletion strains were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP87 – pilQ was deleted in OKP73 (PAO1 ΔslkAB ΔtsaP) by using pOKP23. Candidates for the deletion strain were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

OKP77, OKP78, and OKP97 – pilY1 was deleted in PAO1, HJP1 (PAO1 ΔslkAB), and OKP15 (PAO1 ΔslkAB ΔpilQ) by using pOKP115. Candidates for the deletion strains were obtained by selection with 10 μg/mL gentamicin to avoid enrichment of random mutations that suppress the barrier defect.

**Generation of the transposon insertion library**

*P. aeruginosa* strains were mutagenized with a mariner transposon delivery vector, pBTK30, (Goodman *et al*, 2004) using the following mating protocol. The donor strain SM10(λpir) carrying pBTK30 was grown in LB supplemented with 50 μg/mL ampicillin at 37 °C and *P.*
aeruginosa strains were grown in LB at 42 °C overnight. The next morning, cultures were concentrated and adjusted to an OD600 of 5 for the donor and 10 for the recipient. Equal volumes of donor and recipient were mixed together and 50 μL aliquots were spotted on prewarmed LB plates. Mating was allowed to proceed at 37 °C for one hour prior to resuspension in VBMM supplemented with 30 μg/mL gentamicin. Transconjugants were selected on VBMM agar supplemented with 30 μg/mL gentamicin at 30 °C for 24 hours. Donor-only and recipient-only controls were performed in parallel to ensure proper selection of P. aeruginosa transconjugants. More than 5 million transconjugant colonies were collected from agar plates by suspension in VBMM broth supplemented with 30 μg/mL gentamicin and frozen at -80 °C.

Transposon sequencing

A transposon mutant library of PAO1 was thawed and incubated in LB for roughly two doublings. The resuscitated mutant library culture was diluted in LB either lacking or containing 10 μg/mL erythromycin and incubated for 10 doublings to a final OD600 of 0.5 at 37 °C. After the incubation, the cells were pelleted and frozen. Genomic DNA from each cell pellet was extracted, fragmented, and poly-C tailed as previously described (Lai et al., 2017). The transposon-chromosome junctions in the resulting DNA were amplified by using Easy-A Hi-Fi Cloning System (Agilent Technologies). The primers used were the poly-C tail-specific primer 5'- GTGACTGGAGTTCCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGGG-3' and the transposon-specific primer 5'-GGTTCTGGACCACTGTTGAG-3'. The transposon-chromosome junctions were further amplified in a second, nested PCR with the primers that add sequencing barcodes to each mutant library, NEBNext Multiplex Oligos for Illumina (NEB), and the transposon-specific primer 5'- AATGATACGGCGACCACCGAGATCTACACTCTTTTTCTGGAAGCGAGCGATCGTTT G-3'. The final PCR products were quantified and equal amounts of each barcoded library
were mixed. The pooled sequencing library was run on a 2% agarose gel, and DNA fragments ranging from 200 and 500 bp were excised and purified using QIAquick Gel Extraction Kit (Qiagen). The resulting library was sequenced using a MiSeq reagent kit V3 (150-cycle) (Illumina) with the custom primer 5’-CTAGAGACCGGGACTTATCAGCCAACCTGTTA-3’. Sequencing reads were trimmed using trimmomatic (Bolger et al., 2014) to remove adaptor sequences, and mapped to chromosomal TA dinucleotides (mariner insertion sites) on the P. aeruginosa PAO1 genome (NC_002516) using bowtie 1.0.0 (Langmead et al., 2009). Differences in the total number of reads at any given TA site between untreated and erythromycin-treated samples were determined using a Mann-Whitney U test. Transposon insertion profiles were visualized using the Sanger Artemis Genome Browser and Annotation tool.

**Whole genome sequencing**

The genomic DNA of PAO1 and PAO1™ strains was extracted from overnight grown cultures using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s manual. Purification of the extracted genomic DNA was performed using Genomic DNA Clean & Concentrator™-25 (Zymo Research) according to the manufacturer’s manual. The genomic DNA samples were sequenced by Macrogen Inc. using an Illumina HiSeq platform. Sequencing reads were analyzed with Geneious Prime software using PAO1 genome (NC_002516) as a reference.

**Suppressor selection**

To select for suppressors of the erythromycin susceptibility phenotype of OKP7 (PAO1 ΔslkAB), the strain was mutagenized by random insertion of a mariner-based transposon from pBTK30, as described (Goodman et al., 2004). The mutant library was spread on LB
agar supplemented with 50 μg/mL erythromycin to select for mutants that can suppress the OM permeability defect. The transposon insertion sites of the suppressors were determined by arbitrarily primed PCR. The first round was performed with a primer pair 5’-
GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT-3’ and 5’-
GGTTCTGGACCAGTTGCGTGAG-3’. The second, nested PCR was performed with 5’-
GGCCACGCGTCGACTAGTAC-3’ and 5’- CGAACCGAACAGGCTTATGTCAATTG-3’ to increase specificity and sensitivity. The resulting PCR products were sequenced with the primer 5’-CGAACCGAACAGGCTTATGTCAATTG-3’ that anneal to the transposon sequence to identify the transposon-chromosome junctions.

**Twitching motility assay**

To examine type IV pili-mediated twitching motility, a single colony from a freshly streaked LB agar plate was picked with a toothpick and stab-inoculated through LB agar (1% agar) to the polystyrene dish. After incubation for 48 hours at 30 °C, the LB agar was removed and the cells attached to the polystyrene dish were stained with 1% crystal violet for 5 min. The dish was then rinsed with water to remove excess stain and the stained zone was photographed after air drying.

**Phylogenetic tree generation**

To generate the phylogenetic tree, the amino acids sequence of PA5122 and PA5123 were input into BLASTp and searched against the NCBI “non redundant” (nr) database with an e-value cutoff of 1e-6 for each protein (Pruitt et al, 2005). We used a complex and diverse set of 1773 bacterial taxa called ‘Representative Genomes’ that is available on NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/, Representative_Genomes.00.tar.gz). The phylogenetic tree was constructed using phylot (http://phylot.biobyte.de/) and BLASTp results were
plotted against the tree. The tree was visualized and annotated using iTOL 
(http://itol.embl.de/) (Letunic & Bork, 2016).
References

Bolger AM, Lohse M & Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120

Cheng K-J, Ingram JM & Costerton JW (1970) Release of Alkaline Phosphatase from Cells of Pseudomonas aeruginosa by Manipulation of Cation Concentration and of pH. J Bacteriol 104: 748–753

Choi K-H, Mima T, Casart Y, Rholl D, Kumar A, Beacham IR & Schweizer HP (2007) Genetic Tools for Select-Agent-Compliant Manipulation of Burkholderia pseudomallei ✓. Appl Environ Microb 74: 1064–1075

Choi K-H & Schweizer HP (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa. Nat Protoc 1: 153–161

Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS & Lory S (2004) A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas aeruginosa. Dev Cell 7: 745–754

Jeong J-Y, Yim H-S, Ryu J-Y, Lee HS, Lee J-H, Seen D-S & Kang SG (2012) One-Step Sequence- and Ligation-Independent Cloning as a Rapid and Versatile Cloning Method for Functional Genomics Studies. Appl Environ Microb 78: 5440–5443

Lai GC, Cho H & Bernhardt TG (2017) The mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis in Escherichia coli. Plos Genet 13: e1006934

Langmead B, Trapnell C, Pop M & Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25

Letunic I & Bork P (2016) Interactive tree of life (iTOl) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 44: W242–W245

Lorenzo V de & Timmis KN (1994) [31] Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol 235: 386–405

Newman JR & Fuqua C (1999) Broad-host-range expression vectors that carry the l-arabinose-inducible Escherichia coli araBAD promoter and the araC regulator. Gene 227: 197–203

Pal D, Venkova-Canova T, Srivastava P & Chattoraj DK (2005) Multipartite Regulation of rctB, the Replication Initiator Gene of Vibrio cholerae Chromosome II. J Bacteriol 187: 7167–7175
Pruitt KD, Tatusova T & Maglott DR (2005) NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* 33: D501–D504

Rietsch A, Vallet-Gely I, Dove SL & Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in Pseudomonas aeruginosa. *Proc National Acad Sci* 102: 8006–8011