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Analysis of Secretin-Induced Stress in *Pseudomonas aeruginosa* Suggests Prevention Rather than Response and Identifies a Novel Protein Involved in Secretin Function▽

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Secretins are bacterial outer membrane proteins that are important for protein export. However, they can also mislocalize and cause stress to the bacterial cell, which is dealt with by the well-conserved phage shock protein (Psp) system in a highly specific manner. Nevertheless, some bacteria have secretins but no Psp system. A notable example is *Pseudomonas aeruginosa*, a prolific protein secretor with the potential to produce seven different secretins. We were interested in investigating how *P. aeruginosa* might deal with the potential for secretin-induced stress without a Psp system. Microarray analysis revealed the absence of any transcriptional response to XcpQ secretin overproduction. However, transposon insertions in either *rpoN*, *truB*, PA4068, PA4069, or PA0943 rendered *P. aeruginosa* hypersensitive to XcpQ production. The PA0943 gene was studied further and found to encode a soluble periplasmic protein important for XcpQ localization to the outer membrane. Consistent with this, a PA0943 null mutation reduced the levels of type 2 secretion-dependent proteins in the culture supernatant. Therefore, this work has identified a novel protein required for normal secretin function in *P. aeruginosa*. Taken together, all of our data suggest that *P. aeruginosa* lacks a functional equivalent of the Psp stress response system. Rather, null mutations in genes such as PA0943 may cause increased secretin-induced stress to which *P. aeruginosa* cannot respond. Providing the PA0943 mutant with the ability to respond, in the form of critical Psp proteins from another species, alleviated its secretin sensitivity.

In gram-negative bacteria the process of protein export often requires proteins and complex structures to be assembled in, or passed through, the cell envelope. This in turn has the potential to induce so-called extracytoplasmic stress responses (ESRs), typified by the RpoE and Cpx responses of *Escherichia coli* and its relatives (46, 47). These ESRs lead to increased synthesis of many proteins, some of which are proteases and folding factors that help to clear the cell envelope of mislocalized and/or misfolded proteins. In addition to critical roles in general physiology, ESRs can also be important during host infection and biofilm formation (2, 13, 45, 48).

A specialized family of pore-forming outer membrane proteins, known as secretins, is especially important for protein export in gram-negative bacteria (16). Secretins are components of type 2 and type 3 secretion systems and are also involved in the biogenesis of type 4 pili and in filamentous phage export. In those bacteria where it has been studied, secretin production does not induce general ESRs such as the RpoE and Cpx systems (27, 32, 50). Instead secretins, especially when they mislocalize within the cell envelope, specifically induce a specialized ESR known as the phage shock protein (Psp) system (reviewed in reference 10). Secretin mislocalization can be artificially induced by overproduction, but it probably also occurs to some extent when secretins are produced at their normal physiological level, at least in the gastrointestinal pathogen *Yersinia enterocolitica* (11). However, the full extent to which endogenous secretin production might cause stress has not been tested in any species.

The Psp system has been extensively studied in *E. coli* K-12 and *Y. enterocolitica*. It is linked to important phenotypes, including virulence and biofilm formation (2, 11). Many different stressors, all of which have the potential to damage the cell envelope, can activate the Psp system. These stimuli may all cause a reduction in the proton motive force, which might be the signal that activates the Psp response. Mislocalized secretins may reduce the proton motive force by inserting into the inner membrane to form an aberrant pore (18). The Psp system may play a role in maintaining the proton motive force by an unknown mechanism (24). The most critical components of the Psp system are the PspF, PspA, PspB, and PspC proteins, encoded by adjacent genes. These proteins are involved both in regulating *psp* gene expression and in mediating the poorly characterized physiological response (10).

Many of the stimuli that activate the Psp system, such as extreme heat or osmotic shock, simultaneously induce other stress responses (38). However, secretin overproduction is an exquisitely specific modulator of gene expression. Global transcriptional profiling in three different bacterial genera has revealed that secretin overproduction/mislocalization induces the *psp* genes without significantly affecting the expression of any others (27, 50). It is also especially striking that in *Y. enterocolitica* only Psp system null mutants have the phenotype of being specifically and highly sensitive to stress induced by secretin overproduction (50). All of these observations show a highly specific relationship between secretin-induced stress and the Psp system. This suggests that the Psp system plays a

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critical and unique role to allow survival during secretin-induced stress. Furthermore, the Psp system is widely conserved in the gammaproteobacteria and is also found in some non-gammaproteobacteria (10). Therefore, it may play an essential role in responding to secretin-induced stress in many gram-negative bacterial species.

The preceding hypothesis is somewhat confounded by the observation that some bacteria produce secretins but their genomes do not encode a homolog of the Psp system. There is more than one example of this, but we were particularly intrigued by *Pseudomonas aeruginosa*, a widely studied species that is an important opportunistic pathogen. It is an unusually prolific protein secretor that relies heavily on secretins (3). These include the XcpQ secretin of a type 2 secretion system (14), the PscC secretin of a type 3 secretion system (57), and the PilQ secretin of a type 4 pilus (29). However, sequenced *P. aeruginosa* genomes do not encode any obvious homolog of the Psp system. Therefore, we were curious to know how *P. aeruginosa* might deal with the potential for secretin-induced stress. The results described here suggest that this species does not have a specific secretin-induced stress response functionally analogous to the Psp system. However, transposon mutagenesis identified some novel *P. aeruginosa* genes that may be particularly important to prevent secretin-induced stress from occurring in the first place. Among these was PA0943, which we showed to encode a soluble periplasmic protein important for XcpQ localization to the outer membrane and for the normal functioning of the Xcp protein export system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth conditions. Bacterial strains and plasmids are listed in Table 1. Bacteria were routinely grown in Luria-Bertani-Miller (LB) broth or on LB agar plates. For analysis of *P. aeruginosa* culture supernatant proteins and alkaline phosphatase activities, proteose peptone medium was used (8). The antibiotics used were ampicillin (200 μg ml^{-1}), irgasan (25 μg ml^{-1}), streptomycin (200 μg ml^{-1}), spectinomycin (500 μg ml^{-1}), carbenicillin (150 μg ml^{-1}), and gentamicin (75 μg ml^{-1}).

Strain and plasmid constructions. Transposon insertions were backcrossed to wild-type *P. aeruginosa* by high-efficiency electroporation of wild-type cells, as previously described (9). Backcrossed strains were confirmed by colony PCR and Southern hybridization analysis.

P. aeruginosa deletion mutants were constructed using the suicide vector pEX18Ap (22). Two ~500-bp fragments corresponding to the regions immediately upstream and downstream of the deletion site were amplified by PCR. The PCR primers incorporated a common restriction site at one end of each fragment. The fragments were ligated at this common site and cloned into plasmid pEX18Ap, and the DNA sequence was confirmed. The plasmids were integrated into the *P. aeruginosa* chromosome following conjugation from *E. coli*, and then sucrose-resistant segregants were isolated. Deletion mutations were confirmed by colony PCR and/or Southern hybridization analysis.

P. aeruginosa trkA and PA3731 mutants were constructed by suicide plasmid insertion mutagenesis. In both cases an ~300-bp fragment corresponding to the central region of each gene was amplified by PCR, cloned into suicide vector pAJD1, and verified by DNA sequencing. pAJD1 is a derivative of pGY2 (58) with the *bla*$^+$ *cat*$^+$ BamHI fragment deleted. The plasmid was integrated into the *P. aeruginosa* chromosome following conjugation from *E. coli*. Correct integration was confirmed by Southern hybridization analysis.

Single-copy complementation strains were made by integration into the *attB* site of the *P. aeruginosa* chromosome using the backbone of pmini-CTX-T7 as the integration delivery vector and subsequent pFLP2-mediated excision of vector sequences as described previously (23). Integrations were confirmed by colony PCR analysis (23).

tacp-xcpQ (pAJD942) and *tacp-oprF* (pAJD1164) expression plasmids were constructed by amplifying the *xcpQ* and *oprF* genes, respectively, from *P. aeruginosa* PAK genomic DNA and cloning them into plasmid pVLT35. The *araBp-pspBC* expression plasmid pAJD1365 was constructed by transferring the *Y. enterocolitica pspBC* genes from plasmid pAJD1011 (33) as an EcoRI-XbaI fragment into plasmid pJN105. All PCR-generated fragments were verified by DNA sequencing.

RNA preparation and microarray analysis. RNA was isolated from wild-type *P. aeruginosa* containing either the empty vector plasmid pVLT35 or its *xcpQ* expression derivative pAJD942. In both cases duplicate cultures were grown in LB broth with aeration to an optical density (at 600 nm) of 0.4 to 0.6 (mid-log phase). The growth medium contained 75 μM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce a nontoxic level of *xcpQ* expression from plasmid pAJD942, which ensured that both strains were growing at the same rate. Total RNA was extracted, reverse transcribed, fragmented, and labeled exactly as described previously (54). The samples were hybridized to Affymetrix GeneChip *P. aeruginosa* microarrays, and the chips were washed and scanned according to the manufacturer's protocol. Data from the probe sets specific to strain PAK (54) were filtered for statistically significant differences (Student's *t* test *P* value of ≤0.05) and signal above the noise level (present [detectable] call in at least one sample) using commercially available software.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype/features	Reference or source
P. aeruginosa strains[a]		
PAK	Wild type strain	51
AJDP65	PA0943::*mariner*-Gm^r	This study
AJDP69	*rpoN*::*mariner*-Gm^r	This study
AJDP124	PA4068::*mariner*-Gm^r	This study
AJDP168	PA4069::*mariner*-Gm^r	This study
AJDP250	*truB*::*mariner*-Gm^r	This study
AJDP267	*trkA*::pAJD1	This study
AJDP270	PA3731::pAJD1	This study
AJDP279	Δ*algU*	This study
AJDP388	ΔPA0943	This study
AJDP392	ΔPA4068	This study
AJDP419	ΔPA4069	This study
AJDP422	Δ(PA4069-PA4068)	This study
AJDP504	Δ*purMN*	This study
AJDP511	*attB*::[ΔPA4069-PA4068$^+$]	This study
AJDP512	*attB*::[PA4069$^+$]	This study
AJDP513	*attB*::[PA4069$^+$-PA4068$^+$]	This study
AJDP514	ΔPA4068 *attB*::[ΔPA4069-PA4068$^+$]	This study
AJDP515	ΔPA4068 *attB*::[PA4069$^+$-PA4068$^+$]	This study
AJDP518	ΔPA4069 *attB*::[PA4069$^+$]	This study
AJDP519	ΔPA4069 *attB*::[PA4069$^+$-PA4068$^+$]	This study
AJDP522	Δ(PA4069-PA4068) *attB*::[ΔPA4069-PA4068$^+$]	This study
AJDP523	Δ(PA4069-PA4068) *attB*::[PA4069$^+$]	This study
AJDP524	Δ(PA4069-PA4068) *attB*::[PA4069$^+$-PA4068$^+$]	This study
AJDP553	*attB*::[Δ*purMN*-PA0943$^+$]	This study
AJDP556	ΔPA0943 *attB*::[Δ*purMN*-PA0943$^+$]	This study
AJDP605	Δ(*xcpZ-Q*) Δ(*xphA-xqhA*)	This study
Plasmids		
pBTK30	*mariner*-Gm^r delivery plasmid, R6K *ori*	17
pEX18Ap	Amp^r, pMB1 *ori*, *oriT*, *sacB*$^+$	22
pFLP2	Amp^r, pMB1 *ori*, pRO1600 *ori*, *oriT*, *sacB*$^+$, FLP$^+$	22
pJN105	Gm^r, *araBp* expression vector, pBBR *ori*	39
pmini-CTX-T7	Tet^r, pMB1 *ori*, *oriT*, *int*$^+$, *attP*$^+$	23
pVLT35	Sm^r, Sp^r, *tacp* expression vector, RSF1010 *ori*	12
pAJD1	Sm^r, Sp^r, R6K *ori*	This study
pAJD942	*tacp-xcpQ* in pVLT35	This study
pAJD1164	*tacp-oprF* in pVLT35	This study
pAJD1365	*araBp-pspBC* in pJN105	This study

[a] All *P. aeruginosa* strains are derivatives of strain PAK.

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Transposon mutagenesis. *P. aeruginosa* containing *tacp-xcpQ* expression plasmid pAJD942 was mutagenized with a *mariner*-based element encoded by plasmid pBTK30 (17) that was delivered by conjugation from *E. coli* SM10 λ*pir* (37). Mutants were isolated on LB agar containing irgasan, streptomycin, and gentamicin. XcpQ-sensitive transposon insertion mutants were identified as described in Results. Southern hybridization using the gentamicin resistance gene of the transposon as a probe was used to confirm a single transposon insertion. Insertion sites were determined by arbitrary primed PCR and DNA sequencing (43).

Growth curves. Saturated cultures were diluted into 5 ml LB broth in 18-mm test tubes at an optical density (at 600 nm) of approximately 0.1. The medium contained various concentrations of IPTG and glutamine or arabinose, as noted. Cultures were grown at 37°C with agitation, and a 0.1-ml aliquot was removed at hourly intervals for optical density determination. Data in each figure are from a single experiment in which all strains were tested simultaneously. Each experiment was also done at least twice to ensure reproducibility.

Biofilm assay. Early biofilm formation in vitro was assessed essentially as described previously (43). Briefly, saturated cultures were diluted (1:100) into M63 medium supplemented with 0.2% glucose and 0.5% Casamino Acids in individual 13-mm-diameter borosilicate glass tubes or in the wells of a 96-well polyvinyl chloride microtiter dish. Following incubation without agitation at 30°C for 10 h, the culture medium was removed by aspiration and the vessels were rinsed thoroughly with water. A 0.1% solution of crystal violet in water was added, followed by incubation at room temperature for 30 min. The crystal violet solution was removed, and the vessels were rinsed again before being photographed. To address the concern that surface attachment defects might be due to growth defects, the growth of each transposon insertion mutant in biofilm formation medium was determined as described previously (44).

Preparation of polyclonal antisera and immunoblotting. A fragment of the *xcpQ* gene (encoding amino acid residues 12 to 100 of the mature protein) was amplified from *P. aeruginosa* PAK genomic DNA by PCR and cloned into plasmid pET-32a (+) (Novagen) to encode a TrxA-His$_6$-XcpQ-His$_6$ fusion protein (TrxA = thioredoxin). The PA0943, PA4068, and PA4069 genes were amplified from *P. aeruginosa* PAK genomic DNA and cloned into plasmid pET-24b (+) (Novagen) to encode proteins with a C-terminal His$_6$ fusion. All expression plasmids were transferred into *E. coli* strain ER2566 (NEB), and the strains were grown to mid-exponential phase at 37°C in LB broth containing 1 mM IPTG. Total cell lysates were prepared, and the proteins were purified under denaturing conditions by nickel-nitrilotriacetic acid affinity chromatography as described by the manufacturer (Qiagen). Polyclonal rabbit antisera were raised against the purified fusion proteins at Covance Research Products, Denver, PA.

For immunoblot analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose or polyvinylidene fluoride (PVDF) by electroblotting. Chemiluminescent detection followed sequential incubation with a diluted polyclonal antiserum and then goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad) used at a dilution of 1 in 5,000 or 1 in 10,000. The anti-XcpQ, anti-PA0943, anti-PA4068, and anti-PA4069 polyclonal antisera were used at a dilution of 1 in 500. The anti-SecY (21) and anti-OprF (28) antisera were used at a dilution of 1 in 5,000.

Preparation of bacterial cell fractions. *P. aeruginosa* cells were separated into soluble, inner membrane, and outer membrane fractions based on published procedures (21, 41). Cultures (25 ml) of strains containing either the pVLT35 or pAJD942 plasmid were grown at 37°C with aeration in LB broth supplemented with 25 μM IPTG until the optical density at 600 nm reached approximately 1.0. Cultures were placed on ice, and cells were collected by centrifugation at 4°C. The cells were resuspended in 5 ml of 0.2 M Tris-HCl (pH 7.5)–0.2 mM EDTA containing complete protease inhibitors (Roche) and lysed by two passages through a French pressure cell. Unbroken cells were collected by centrifugation at 12,000 × *g* for 2 min at 4°C. The supernatant was transferred to a new tube, and the membrane pellet was collected by centrifugation at 100,000 × *g* for 60 min at 4°C (supernatant was the soluble fraction). The membrane pellet was resuspended in 1.25 ml 20 mM Tris-HCl (pH 7.6), and then 2% Sarkosyl (final concentration) was added to a 500-μl aliquot. The sample was incubated at 26°C for 20 min on a roller drum, and then centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant fraction containing the solubilized inner membrane was retained. The pellet (outer membrane) was resuspended in 500 μl 2% SDS in 20 mM Tris-HCl (pH 7.6).

A *P. aeruginosa* periplasmic extract was prepared by cold osmotic shock. The PAK strain was grown at 37°C in 100 ml LB broth with aeration until the optical density (at 600 nm) was approximately 1.5. Cells were harvested by centrifugation at 4°C and resuspended in 8 ml per 0.1 g (wet weight) of 20% sucrose–30 mM Tris-HCl (pH 8). A 1/500 volume of 0.5 M EDTA (pH 8) was added, and the suspension was incubated at room temperature for 10 min with slow inversion on a rocking platform. Cells were collected by centrifugation at 8,000 × *g* for 10 min at 4°C and resuspended in 10 ml ice-cold 5 mM $MgSO_4$. The suspension was incubated in an ice-water bath for 10 min with slow inversion on a rocking platform. Spheroplasts were collected by centrifugation at 8,000 × *g* for 10 min at 4°C. Periplasmic proteins in the supernatant were concentrated by adding trichloroacetic acid to a final concentration of 10% (vol/vol) and incubating on ice for 1 h. The proteins were collected by centrifugation at 16,000 × *g* for 30 min at 4°C, washed with ice-cold acetone, and collected again by centrifugation. The periplasmic and spheroplast pellets were each resuspended in SDS-PAGE sample buffer.

Detection of XcpQ multimers. Samples were mixed with SDS-PAGE sample buffer and incubated at either 50°C to maintain multimers or at 100°C to dissociate multimers (30). They were then separated by SDS-PAGE on a 4 to 12% polyacrylamide gradient gel, transferred to a PVDF membrane, and detected with polyclonal antiserum against XcpQ.

Analysis of culture supernatant proteins. A single colony was used to inoculate 3 ml proteose peptone broth in an 18-mm test tube. Cultures were incubated at 37°C with shaking until the optical density (at 600 nm) was approximately 2. Bacterial cells were removed by centrifugation at 6,000 × *g* for 15 min at room temperature. The supernatant was passed through a 0.2-μm-pore-size low-protein-binding filter (Millipore). Proteins from a volume of supernatant equivalent to that derived from 1 ml of a culture at an optical density at 600 nm of 1.0 (i.e., one optical density [600 nm] unit) were precipitated by trichloroacetic acid precipitation as described above. Pellets were resuspended in SDS-PAGE sample buffer containing β-mercaptoethanol and heated at 100°C for 10 min. Proteins were separated by SDS-PAGE (10% polyacrylamide) and visualized by staining with BioSafe Coomassie blue (Bio-Rad).

Alkaline phosphatase assays. Cultures were grown as described above for analyzing culture supernatant proteins. Bacterial cells were separated from supernatants by centrifugation at 6,000 × *g* for 15 min. The supernatant was passed through a 0.2-μm-pore-size low-protein-binding filter (Millipore) and stored at −20°C. The pellets were washed with phosphate-buffered saline and resuspended in cell disruption buffer (0.5 M Tris-HCl [pH 8.0], 0.1% Triton X-100, 10 μg/ml DNase-RNase mix, 10 μg/ml lysozyme). Resuspended pellets were frozen at −70°C overnight to disrupt the cells. Cells and supernatant were thawed at 37°C for 30 min before use. To measure alkaline phosphatase activity, 100 to 200 μl of each sample was added to 900 to 800 μl of 1 M Tris-HCl (pH 8.0) buffer, and then 400 μl of a 4-mg/ml solution of *p*-nitrophenyl phosphate hexahydrate (Amresco) in 1 M Tris-HCl (pH 8.0) was added to start the reaction. The reaction mixture was incubated at room temperature until visibly yellow, at which point 1 M K_2HPO_4 was added to stop the reaction and absorbance at 405 nm was determined. Activities were determined according to the formula described by Miller (36), which included normalization for the cell density of the cultures at harvest and for sample volumes used in the assay. Each individual culture was assayed in duplicate, and the activities reported are averages of results for three independent cultures. Percent secretion values were calculated as the fraction of alkaline phosphatase activity in the supernatant versus the total activity in both fractions.

RESULTS

Rationale. The Psp system is essential for *Y. enterocolitica* and *E. coli* to survive secretin-induced stress (11, 50). We have also found the same to be true for *Salmonella enterica* serovar Typhimurium (data not shown). Therefore, we hypothesize that the Psp system may be essential for survival during secretin-induced stress in most, if not all, species that have it. This raises the intriguing question of how the potential for secretin-induced stress might be dealt with in a species without a Psp system, such as *P. aeruginosa*. We eliminated the trivial possibility that *P. aeruginosa* secretins inherently cannot cause the same stress as those from Psp$^+$ species because overexpressing the *P. aeruginosa xcpQ* secretin gene was toxic to a *Y. enterocolitica psp* null strain but not to a *psp*$^+$ strain (data not shown).

***P. aeruginosa* genes encoding AlgU (RpoE), TrkA, and a protein related to PspA are not required for growth during secretin-induced stress.** Besides the Psp system, defects in the RpoE system and the TrkA potassium transporter caused

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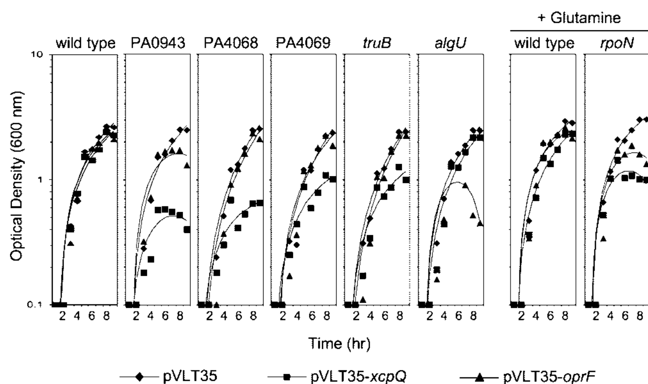

FIG. 1. Effect of XcpQ or OprF overproduction on the growth of *P. aeruginosa* strains. Strains with the *tac* promoter expression plasmid pVLT35 or derivatives encoding XcpQ or OprF were grown in LB broth containing 150 μM IPTG, and optical density was measured at hourly intervals. Gene names above each graph indicate the location of a null mutation. For the experiment shown in the right-hand two panels, 0.2% glutamine was included in the medium.

some sensitivity to secretin-induced stress in *Y. enterocolitica* (50). Therefore, we constructed *P. aeruginosa* strains with *algU* (PA0762; *rpoE* ortholog) or *trkA* (PA0016) null mutations. However, neither caused hypersensitivity to a *tacp-xcpQ* expression plasmid (data not shown and Fig. 1).

The PA3731 gene encodes a protein with similarity to PspA. However, it is not linked to homologs of other *psp* genes and is more similar to *E. coli* K-12 *yifJ* (b4182) than *pspA*. Nevertheless, we constructed a *P. aeruginosa* strain with a PA3731 null mutation. However, it was not hypersensitive to XcpQ production (data not shown).

Microarray analysis suggests no transcriptional response to secretin-induced stress in *P. aeruginosa*. Microarrays revealed that secretin overproduction in *E. coli*, *S. enterica* serovar Typhimurium, and *Y. enterocolitica* induces *psp* gene expression without significantly affecting the expression of any other genes (27, 50). This demonstrates the importance of the Psp system and that secretin overproduction is an extremely specific modulator of gene expression. Therefore, the transcriptome of *P. aeruginosa* during *xcpQ* overexpression was compared to that in the absence of *xcpQ* overexpression. Among the almost 6,000 genes surveyed, only the overexpressed *xcpQ* gene had significantly (≥2-fold) altered expression between the two conditions (data not shown). This suggests that *P. aeruginosa* does not have a transcriptional response to secretin induced-stress that would be analogous to *psp* gene induction.

Isolation of secretin-sensitive *P. aeruginosa* mutants. At this point we formulated two hypotheses. First, *P. aeruginosa* may have a functional equivalent of the Psp response that is not recognizable by homology and is not transcriptionally regulated in the same manner as the Psp system. Alternatively, *P. aeruginosa* might prevent significant secretin stress from occurring, which would negate the need for a stress response system. To investigate these hypotheses, we screened for *P. aeruginosa* transposon insertion mutants hypersensitive to XcpQ production.

Approximately 12,000 random transposon insertion mutants, each containing an IPTG-inducible *xcpQ* expression plasmid, were patched onto LB agar with 250 μM IPTG. Several grew poorly in the presence of IPTG. Sensitivity to XcpQ was screened further by growth analysis in LB broth with or without IPTG. Some mutants had only slight sensitivity to *xcpQ* overexpression and/or significant growth defects even in the absence of IPTG, and these were eliminated. This led to the identification of eight secretin-sensitive mutants. The transposon insertions were backcrossed into the wild-type strain (see Materials and Methods), which reproduced the secretin-sensitive phenotypes. The phenotypes were not XcpQ specific, because the mutants were also sensitive to overproduction of the *Y. enterocolitica* YsaC secretin (data not shown).

The transposon insertion sites in the eight mutants, all of which arose from independent transposition events (data not shown), were determined. Three had insertions in *truB* (PA4742), which encodes a pseudouridine-55 synthase that modifies tRNA molecules. Two had insertions in *rpoN* (PA4462), encoding σ^{54}. The remaining three mutants had insertions in previously uncharacterized genes. Two were in the same putative operon, one insertion in PA4068 and one in PA4069. The final mutant had an insertion in PA0943.

Phenotypes of secretin-sensitive *P. aeruginosa* mutants. For the five genes identified in the screen, a single transposon insertion mutant was selected. The *tacp-xcpQ* expression plasmid or the empty *tacp* vector control was introduced, and we compared growth rates/yields in LB broth containing IPTG. The PA0943 and PA4068 mutants were most sensitive to XcpQ in terms of growth yield (Fig. 1). Note that the *rpoN* mutant was assessed in medium supplemented with glutamine to correct a general growth deficiency (Fig. 1).

To probe specificity, we also tested for sensitivity to the outer membrane porin OprF (56), which is not a secretin. OprF overproduction was confirmed by immunoblotting (data not shown). The PA4068, PA4069, and *truB* mutants were insensitive to OprF (Fig. 1). The PA0943 mutant showed some sensitivity to OprF, but it was much less pronounced than the sensitivity to XcpQ. The *rpoN* mutant was almost as sensitive to OprF as it was to XcpQ. An *algU* (*rpoE* ortholog) null mutant was also analyzed. It was not sensitive to XcpQ, but it was sensitive to OprF (Fig. 1). This further validates the efficacy of the *oprF* expression plasmid and is consistent with activation of the *algU* regulon by some extracytoplasmic stresses (55).

Most *P. aeruginosa* secretin-sensitive mutants have surface attachment defects. *E. coli* ESRs, including the Psp system, are important for biofilm formation (2, 13). We reasoned that if any of our secretin-sensitive mutants were defective in an ESR, they might have a biofilm defect. Alternatively, the transposon insertions might cause secretin hypersensitivity due to an altered cell envelope, which might also manifest as a biofilm defect. Therefore, we tested the mutants for altered biofilm formation.

We used a surface attachment assay to test the secretin-sensitive mutants (without the *tacp-xcpQ* expression plasmid) for defects in early stages of simple biofilm formation on borosilicate glass (44). Transposon insertions in four of the five genes identified in our screen caused a defect in this assay (Fig. 2). These four mutants were also defective in attaching to polyvinyl chloride plastic (data not shown). At the 10-hour assay point, all five mutants reached an optical density similar to that of the wild type in the medium used for these assays (data not shown).

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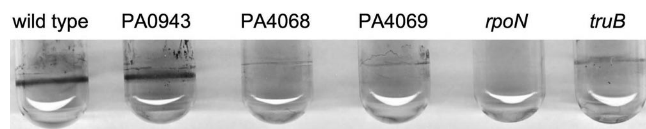

FIG. 2. Surface attachment phenotypes of secretin-sensitive mutants. Attached bacteria were stained with crystal violet after 10 h of static incubation in borosilicate glass tubes and appear as a dark ring. Gene names indicate the location of a *mariner* insertion.

Molecular genetic analysis of the PA4069-PA4068 locus. *rpoN* and *truB* have been studied before in *P. aeruginosa*. RpoN is required for the expression of many genes, and both it and a *truB* mutant have pleiotropic phenotypes (see, e.g., reference 49). We did not study these two loci further. In contrast, PA4068, PA4069, and PA0943 have not been studied before. PA4068 and PA4069 overlap to form a predicted two-gene operon (Fig. 3A). We used deletion and complementation analysis to investigate whether both genes are required for tolerance to secretin-induced stress.

We compared the growth of ΔPA4068, ΔPA4069, and Δ(PA4069-PA4068) deletion mutants containing the *tacp-xcpQ* expression plasmid or the empty vector control. The ΔPA4068 and Δ(PA4069-PA4068) mutants were similarly sensitive to XcpQ, whereas a ΔPA4069 in-frame deletion mutant was insensitive (Fig. 3A). Furthermore, the XcpQ sensitivity of the Δ(PA4069-PA4068) mutant was complemented by PA4068 alone but not by PA4069 (Fig. 3A). Therefore, only PA4068 is required for resistance to XcpQ overproduction. We have also found the same for the surface attachment defect (data not shown). PA4068 is annotated as a putative nucleoside diphosphate-sugar epimerase. PA4069 has predicted dTDP-4-dehydrorhamnose reductase activity. Both activities are often involved in the biosynthesis of cell surface polysaccharides and lipopolysaccharide (LPSs). Therefore, the XcpQ sensitivity and surface attachment defects may be due to an altered cell envelope component (see Discussion).

Molecular genetic analysis of the PA0943 locus. PA0943 is predicted to be the final gene of a three-gene operon, with its coding region overlapping the upstream gene (Fig. 3B). The two upstream genes are *purMN*, which encode enzymes essential for purine biosynthesis in many species. However, the PA0943 protein is not similar to proteins of known function. Furthermore, although the *purMN* operon is well conserved, database searches suggest that PA0943 has limited conservation (data not shown). The PA0943 transposon insertion was close to the 3′ end of the gene, and we were concerned that the phenotype might require the production of a truncated protein. Therefore, we used in-frame deletion and complementation analysis to investigate the locus further.

Both PA0943 transposon insertion and in-frame deletion mutants were highly sensitive to overproduced XcpQ (Fig. 3B). Furthermore, the PA0943 gene complemented both mutations. We also constructed a *purMN* in-frame deletion mutant, which failed to grow in minimal medium and grew poorly in LB medium unless the media were supplemented with adenine (data not shown). In contrast, the ΔPA0943 in-frame deletion mutant grew normally on both media, indicating that PA0943 is not essential for purine biosynthesis. In medium supplemented with adenine, the Δ*purMN* mutant was insensitive to

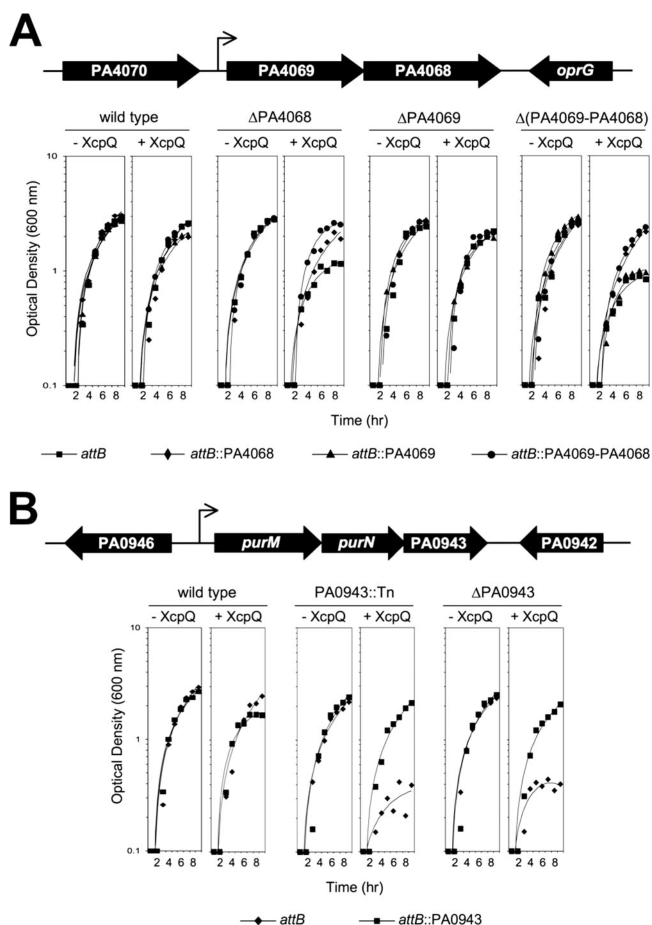

FIG. 3. Molecular genetic analysis of the PA4069-PA4068 and PA0943 loci. (A) Complementation analysis of ΔPA4068, ΔPA4069, and Δ(PA4069-PA4068) deletion mutants. A representation of the chromosomal organization of the PA4069-PA4068 locus and flanking genes is shown at the top (the thin arrow indicates the putative location of a promoter). The *tac* promoter expression plasmid pVLT35 (− XcpQ) or a derivative encoding XcpQ (+ XcpQ) was transferred into various strains (genotypes are indicated above the graphs). Strains had either no integration or PA4068, PA4069, or PA4069 and PA4068 integrated into the *attB* site of the chromosome as indicated by the key. Strains were grown in LB broth containing 150 μM IPTG, and optical density was measured at hourly intervals. (B) Complementation analysis of PA0943 null mutants. A representation of the chromosomal organization of PA0943 and its flanking genes is shown at the top (the thin arrow indicates the putative location of a promoter). The *tac* promoter expression plasmid pVLT35 (− XcpQ) or a derivative encoding XcpQ (+ XcpQ) was transferred into various strains (genotypes are indicated above the graphs). Strains had either no integration or PA0943 integrated into the *attB* site of the chromosome as indicated by the key. Strains were grown in LB broth containing 150 μM IPTG, and optical density was measured at hourly intervals.

xcpQ overexpression, whereas the ΔPA0943 remained sensitive (data not shown). Therefore, only PA0943 plays a role in resistance to XcpQ overproduction, and a deficiency in purine biosynthesis is not involved in this phenotype.

A PA0943 null mutation causes aberrant subcellular localization of XcpQ. In the course of characterizing our mutants, we used immunoblotting to confirm the presence of XcpQ in whole-cell lysates following induction of the *tacp-xcpQ* expression plasmid. We noticed that the steady-state level of XcpQ in

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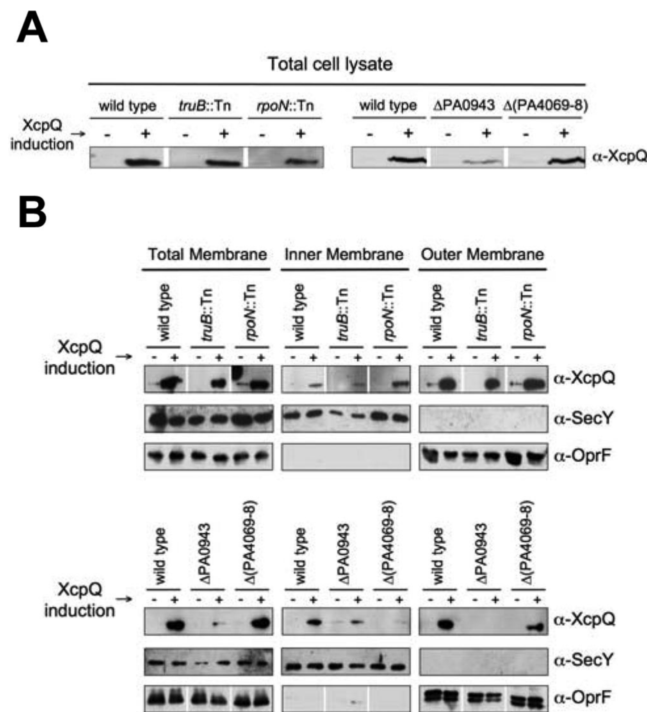

FIG. 4. A PA0943 null mutation causes increased XcpQ mislocalization. (A) Immunoblot analysis of XcpQ in whole-cell lysates. Strains containing a *tacp-xcpQ* expression plasmid were grown to mid-log phase in LB broth containing either no IPTG (−) or 25 μM IPTG (+). Total cell lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and detected with a polyclonal antiserum against XcpQ. The four mutant strains were analyzed in two separate experiments. The first, on the left, was with the *truB* and *rpoN* mutants along with a wild-type control. The second, on the right, was with the ΔPA0943 and Δ(PA4069-PA4068) mutants and a repeat of the wild-type control. For each experiment, samples were analyzed on a single gel. However, positions where lanes between samples have been cropped out are indicated by horizontal white spaces. Optical density equivalents were loaded in each lane, and approximately equal protein loading was confirmed by total protein staining of the nitrocellulose membrane (data not shown). (B) Subcellular localization analysis of XcpQ. Strains with the *tac* promoter expression plasmid pVLT35 (−) or a derivative encoding XcpQ (+) were grown to mid-log phase in LB broth containing 25 μM IPTG. Membrane fractions were prepared, separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and detected with polyclonal antiserum against XcpQ, SecY, or OprF. The four mutant strains were analyzed in two separate experiments. The first, at the top, was with the *truB* and *rpoN* mutants along with a wild-type control. The second, at the bottom, was with the ΔPA0943 and Δ(PA4069-PA4068) mutants and a repeat of the wild-type control. For each boxed panel, the samples were analyzed on a single gel. However, positions where empty lanes between samples have been cropped out are indicated by horizontal white spaces. Samples derived from an equivalent amount of whole cells were loaded for each fraction.

the PA0943 null mutant was strikingly lower than that in the wild type (Fig. 4A). This reduced XcpQ level was restored by reintroduction of the PA0943 gene, and the phenomenon was not specific to XcpQ because the steady-state level of an overproduced YsaC-His_6 protein was also lower in this mutant (data not shown).

The mislocalization of secretins often coincides with their decreased stability (see, e.g., references 7, 19, and 20). Therefore, we hypothesized that the reduced level of XcpQ in the PA0943 mutant might reflect increased XcpQ mislocalization. To test this, we grew wild-type and mutant strains containing either the *tacp-xcpQ* plasmid or its empty vector control in LB broth with IPTG. Cells were harvested in late exponential phase, and subcellular fractions were prepared. We then attempted to detect XcpQ protein in each fraction by immunoblotting. For all strains the large majority of XcpQ was found in the total membrane fraction (Fig. 4B) rather than the soluble fraction (data not shown). Therefore, we focused our analysis on the membranes. Wild-type, *truB*, *rpoN*, and PA4069-PA4068 null mutants had similar levels of XcpQ in the total membrane fraction. However, the ΔPA0943 mutant had a much lower XcpQ level in the total membrane (Fig. 4B), which is consistent with the lower level in whole cells (Fig. 4A). When the inner and outer membranes of wild-type, *truB*::Tn, *rpoN*::Tn, and Δ(PA4069-PA4068) strains were separated, the majority of the XcpQ was in the outer membrane as expected. However, a significant amount fractionated with the inner membrane, which probably reflects some mislocalization due to *xcpQ* overexpression. The results for the ΔPA0943 mutant provided a striking contrast. XcpQ was undetectable in the outer membrane, such that it was all associated with the inner membrane (Fig. 4B). However, total protein staining of the outer membrane fraction following SDS-PAGE revealed largely indistinguishable profiles for wild-type and ΔPA0943 strains (data not shown). This suggests that there are no global problems with outer membrane protein localization in the mutant. Control immunoblot experiments with antisera against inner membrane (SecY) and outer membrane (OprF) proteins validated the membrane fractions and also served as loading controls (Fig. 4B).

A PA0943 null mutation reduces the export of type 2-secreted proteins. The preceding experiments showed that PA0943 was important for the correct localization of overproduced XcpQ. We could not reliably and reproducibly detect endogenously produced XcpQ by immunoblotting (Fig. 4 and data not shown). This problem has been reported by others (4), probably due to the very low level of native XcpQ (6). However, we reasoned that if PA0943 is important for the correct localization of endogenous XcpQ, then there should be a defect in the export of Xcp substrates. Therefore, we investigated culture supernatant proteins.

Wild-type and ΔPA0943 strains were grown in proteose peptone broth, and cell-free culture supernatants were examined by SDS-PAGE. As a control we included a Δ*xcp* Δ*xqh* strain with a deletion of the *xcp* locus and also the *xphA-xqhA* locus, which can function as part of the Xcp system (30, 35). The secreted protein profiles of wild-type and ΔPA0943 strains were similar (Fig. 5A). However, a few specific protein bands were reduced in intensity in the ΔPA0943 mutant. Moreover, most bands with reduced intensity in the PA0943 mutant were completely absent in the Δ*xcp* Δ*xqh* mutant, which suggested that they were Xcp substrates. The three most prominent representatives were excised from a gel and identified by electrospray ionization-liquid chromatography-tandem mass spectrometry (Protein Core Facility at Columbia University Medical Center). They were PA0572, PA3296/PhoA, and PA0347/GlpQ (Fig. 5A). PhoA is a known substrate of the Xcp system (15). PA0572 and GlpQ have also been characterized as

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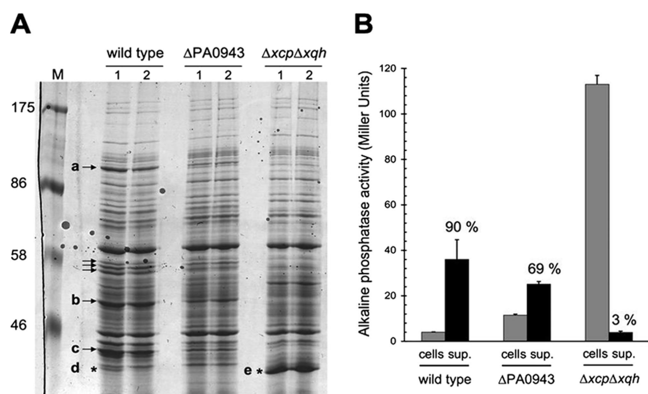

FIG. 5. Deletion of PA0943 reduces the export of type 2 secretion-dependent proteins. (A) Cell-free culture supernatants from two independent cultures (lanes 1 and 2) of strains PAK (wild type), AJDP388 (ΔPA0943), and AJDP605 (Δ*xcp* Δ*xqhA*) were separated on a 10% SDS-polyacrylamide gel that was stained with Coomassie blue. Black arrows indicate proteins with reduced intensity in the ΔPA0943 mutant and absent in the Δ*xcp*Δ*xqhA* mutant. Three of these were identified by mass spectrometry as PA0572 (a), PA3296/PhoA (b), and PA0347/GlpQ (c). Asterisks indicate a protein with reduced intensity in the ΔPA0943 mutant and increased intensity in the Δ*xcp* Δ*xqhA* mutant. It was identified by mass spectrometry as PA0688/LapA from both wild-type (d) and Δ*xcp* Δ*xqhA* (e) strains. Molecular mass standards (in kDa) are indicated on the left. (B) Cell-associated (cells) and culture supernatant (sup.) alkaline phosphatase activities of strains PAK (wild type), AJDP388 (ΔPA0943), and AJDP605 (Δ*xcp* Δ*xqhA*). Error bars indicate the positive standard deviations from the means. The percentage of alkaline phosphatase secretion for each strain is shown (calculated as the fraction of supernatant activity versus the total activity in both compartments).

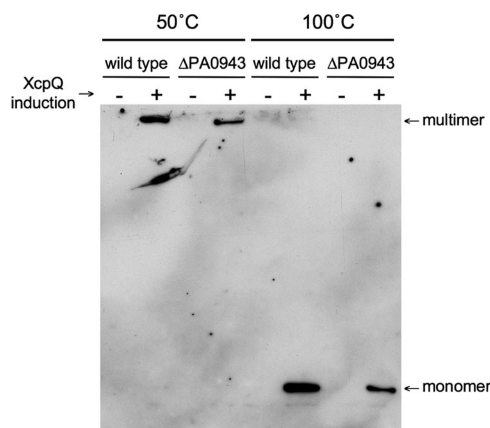

FIG. 6. Deletion of PA0943 does not affect XcpQ multimerization. Inner membrane fractions of the wild-type and ΔPA0943 strains used for Fig. 4 were heated at either 50°C or 100°C for 15 min, separated by SDS-PAGE on a 4 to 12% polyacrylamide gradient gel, transferred to a PVDF membrane, and detected with polyclonal antiserum against XcpQ. The positions of slowly migrating XcpQ multimers and faster-migrating XcpQ monomers are labeled.

extracellular proteins (40, 53). Their absence in the supernatant of the Δ*xcp* Δ*xqh* mutant indicates that they are probable Xcp substrates.

One protein was absent from the ΔPA0943 strain but apparently overproduced/secreted in the Δ*xcp* Δ*xqh* mutant (Fig. 5A). Others have also reported a protein of the same size to be present in large amounts in the supernatant of an *xcp* null strain (1). Those authors identified the protein as PA0688/LapA, which is a substrate of the Hxc type 2 secretion system. To determine if the protein missing from the PA0943 mutant and overproduced in the Δ*xcp* Δ*xqh* strain was also LapA, we excised the band from both wild-type and Δ*xcp* Δ*xqh* strains and identified it by electrospray ionization-liquid chromatography-tandem mass spectrometry analysis. In both cases the protein was identified as PA0688/LapA. Therefore, the ΔPA0943 mutant is apparently defective in exporting the substrates of two different type 2 secretion systems (Xcp and Hxc).

We also quantified alkaline phosphatase activities in culture supernatants. Consistent with the analysis of supernatant protein profiles, the ΔPA0943 mutant had reduced alkaline phosphatase secretion, whereas it was almost abolished in the Δ*xcp* Δ*xqh* strain. Taken together, all of these data suggest that type 2 secretion efficiency is reduced in a ΔPA0943 mutant.

A PA0943 null mutation does not affect XcpQ multimerization. Inner membrane accumulation of XcpQ cannot explain the sensitivity of the PA0943 mutant to XcpQ production, because even more XcpQ is found in the inner membrane of the wild type (Fig. 4B). However, in common with other secretins, XcpQ forms a large SDS-stable multimeric complex (4). We wondered if there might be a difference between the wild type and the PA0943 mutant with respect to the oligomerization of the inner membrane-accumulated XcpQ. To test this, the inner membrane fractions of the wild-type and ΔPA0943 strains used for Fig. 4B were heated at 50°C or 100°C in the presence of SDS, which maintains or dissociates XcpQ multimers, respectively (30). The samples were then analyzed by SDS-PAGE and immunoblotting with XcpQ antiserum. After heating to only 50°C, XcpQ appeared as a slowly migrating multimer that was indistinguishable in the two strains (Fig. 6). Therefore, the PA0943 mutation does not affect XcpQ multimerization.

PA0943 encodes a soluble periplasmic protein. A PA0943 null mutation affects secretin localization and type 2 protein secretion. Therefore, we were interested to know if the PA0943 protein was located in the cell envelope, which would be consistent with a direct role in secretion-associated processes. A polyclonal antiserum was raised against a PA0943-His_6 fusion protein and used to detect the native PA0943 protein in the soluble and total membrane fractions generated as part of the experiment in Fig. 4B. This showed that PA0943 was a soluble protein (Fig. 7A). To address whether it was cytoplasmic or periplasmic, we released periplasmic proteins by cold osmotic shock. The PA0943 protein was detected in the periplasmic extract (Fig. 7B). As a control, PA4068 and PA4069 proteins, which are also soluble but predicted to be cytoplasmic (data not shown), were not released by cold osmotic shock. Together, these data show that PA0943 is a soluble periplasmic protein. This is consistent with the predicted presence of an N-terminal *sec*-dependent signal sequence and the fact that an active PA0943-alkaline phosphatase fusion protein was isolated in a genome-wide screen (26).

The *Y. enterocolitica* PspBC proteins rescue the secretin sensitivity of a *P. aeruginosa* PA0943 null mutant. The final experiment was motivated by the fact that it seems unlikely that the PA0943 mutation inactivates a stress response. Instead, the

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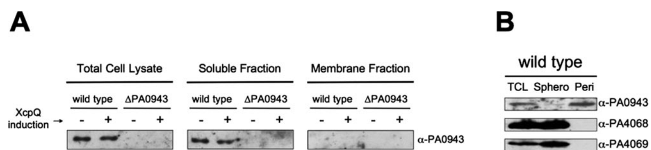

FIG. 7. PA0943 is a soluble periplasmic protein. (A) Total cell lysate, soluble (cytoplasm and periplasm), and membrane (inner and outer) fractions generated from the wild-type strain during the experiment in Fig. 4 were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and detected with polyclonal antiserum against PA0943. (B) Total cell lysate (TCL), spheroplast (Sphero), and periplasmic (Peri) fractions were generated from a wild-type strain, separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and detected with polyclonal antisera against PA0943, PA4068, and PA4069.

mutation may simply cause more stress to occur by mislocalizing overproduced XcpQ within the cell envelope. Toxicity could result because *P. aeruginosa* cannot respond to the increased secretin stress. If so, we hypothesized that providing the PA0943 mutant with the ability to respond, in the form of critical components of the Psp system, might reverse its secretin sensitivity. In *Y. enterocolitica*, overproduction of PspBC in the absence of all other Psp proteins (including PspA) is sufficient to allow tolerance of secretin-induced stress (33). Therefore, we tested whether the *Y. enterocolitica pspBC* genes could rescue the secretin sensitivity of a *P. aeruginosa* ΔPA0943 mutant.

We monitored the growth of *P. aeruginosa* wild-type and ΔPA0943 strains containing pJN105-$pspBC^{+}$, or the empty pJN105 control, either with or without *xcpQ* overexpression (Fig. 8). Production of the PspBC proteins in *P. aeruginosa* was confirmed by immunoblotting (data not shown). When *pspBC* were absent, the ΔPA0943 mutant was hypersensitive to XcpQ overproduction (Fig. 8). However, when *pspBC* were expressed, the wild-type and ΔPA0943 strains grew similarly when XcpQ was overproduced (Fig. 8). Immunoblot analysis showed that *pspBC* expression did not interfere with XcpQ overproduction, which would be a trivial explanation for the phenotype (data not shown). Therefore, PspBC from *Y. enterocolitica* can rescue the secretin sensitivity of the *P. aeruginosa* ΔPA0943 mutant.

If our hypothesis explained at the beginning of this section is correct, then PspBC do not rescue the secretin sensitivity of the ΔPA0943 mutant because they have the same function as PA0943. Consistent with this, expression of PA0943 in *Y. enterocolitic*a did not correct the secretin sensitivity of a *psp* null strain (data not shown; the successful production of the PA0943 protein in *Y. enterocolitica* was confirmed by immunoblotting). Instead, PspBC may alleviate the increased secretin stress in *P. aeruginosa* that is a downstream consequence of the ΔPA0943 mutation, with the original deficiency of the mutant still remaining. This appears to be true, because although expression of *pspBC* rescued the secretin sensitivity of the *P. aeruginosa* ΔPA0943 mutant (Fig. 8), it did not restore the XcpQ steady-state level to that of the wild type (data not shown).

Taken together these data suggest that that the ΔPA0943 mutation may cause increased secretin stress to occur in *P. aeruginosa*, to which it is apparently unable to mount a response. Providing the ability to respond, in the form of the *Y.*

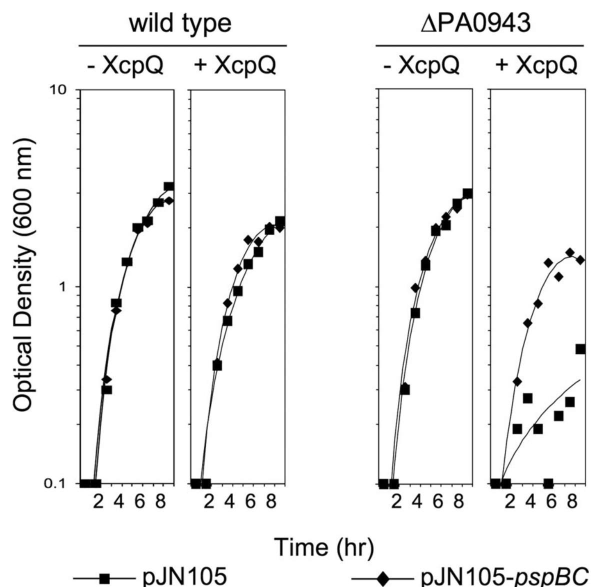

FIG. 8. The *Y. enterocolitica* PspBC proteins rescue the XcpQ sensitivity of a *P. aeruginosa* ΔPA0943 mutant. The *tac* promoter expression plasmid pVLT35 (− XcpQ) or a derivative encoding XcpQ (+ XcpQ) was transferred into wild-type and ΔPA0943 strains as indicated above each graph. The strains also contained either the *araB* promoter expression plasmid pJN105 or a derivative encoding PspBC as indicated by the key at the bottom of the figure. Strains were grown in LB broth containing 150 μM IPTG and 0.02% arabinose.

enterocolitica PspBC proteins, can alleviate this increased secretin stress without correcting the primary deficiency caused by the mutation.

DISCUSSION

The Psp system is a widely conserved stress response triggered by events that can affect the integrity of the cytoplasmic membrane and perhaps cause aberrant transmembrane ion gradients (reviewed in reference 10). Many stimuli that induce *psp* gene expression, such as heat, ethanol, osmotic shock, or proton ionophores, also cause numerous other transcriptional responses. However, a notable exception is the mislocalization of a secretin, which induces only the *psp* genes (27, 50). Furthermore, in *Y. enterocolitica* it may be that only *psp* null mutations cause severe and specific sensitivity to secretins (50). We certainly do not contend that the only role of the Psp system is to deal with secretin-induced stress. Rather, it may have evolved as a more general response to stresses that can compromise cytoplasmic membrane integrity, with secretin mislocalization being just one example. Nevertheless, there is a highly specific and important relationship between the Psp system and secretin-induced stress. The Psp system is essential for survival during secretin-induced stress in at least three different genera. This, coupled with its conservation, provided the question that motivated this study: if the Psp system is essential for bacteria to survive secretin stress, then what about bacteria that have secretins but no Psp system? *P. aeruginosa* is the example that we chose to study. Specifically, we hypothesized that *P. aeruginosa* might have a functional equivalent of the Psp stress response or that it might not need a response

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because significant secretin-induced stress does not normally occur. Our findings supported the latter hypothesis.

We identified transposon insertions in four *P. aeruginosa* loci that caused significant secretin sensitivity. However, unlike the Psp system, we do not think that these genes are involved in responding to secretin stress (discussed below). Among these mutations were two insertions in the PA4069-PA4068 locus. Only PA4068 was involved in the secretin-sensitive phenotype (Fig. 3). Both genes are predicted to encode proteins with activities often important for modifying cell surface polysaccharides (www.pseudomonas.com). Inactivation of PA4068 also caused a surface attachment defect, which would be consistent with an altered cell surface. Other indirect evidence of an altered cell envelope is that a PA4068 mutant is more sensitive to EDTA and NaCl than the wild type and has an altered cell shape (J. Seo and A. J. Darwin, unpublished data). We considered that altered LPS might explain increased sensitivity to XcpQ, because modification of LPS affects the function of the Xcp type 2 secretion system (5, 34). However, we have not detected any differences in the staining pattern following SDS-PAGE separation of purified LPS (Seo and Darwin, unpublished data), although that does not rule out a change that evades detection by this method.

Another gene identified in the screen was PA0943. A PA0943 null mutant had the most severe secretin-sensitive phenotype in terms of growth yield (Fig. 1). PA0943 appears to be in the *purMN* operon (Fig. 3). However, PA0943 is much less widely conserved than *purMN*, and it is not essential for purine biosynthesis. In fact, the incorporation of PA0943 into the *purMN* operon might not be important for function. For example, in the marine bacterium *Idiomarina loihiensis* L2TR, a PA0943 homolog (IL-1664) is separated from *purN* by a transposase gene and is in the opposite orientation to *purMN*.

As we began investigating PA0943 in more detail, the first thing we uncovered was that a PA0943 null mutation reduced the steady-state level of overproduced XcpQ and prevented it from associating with the outer membrane fraction (Fig. 4). It is likely that the increased XcpQ mislocalization causes the toxicity, although the exact reason is unclear. In *E. coli* mislocalized secretins may be toxic due to their increased interaction with the inner membrane (18). However, the level of overproduced XcpQ associated with the inner membrane was actually lower in the ΔPA0943 mutant than in the wild type (Fig. 4). Perhaps the nature or kinetics of the interaction between XcpQ and the inner membrane is different in wild-type and ΔPA0943 cells, with the latter interaction being a more toxic event.

It was also important to show that a PA0943 null mutant had an XcpQ-associated phenotype that did not depend on artificial XcpQ overproduction. We did this by finding that a ΔPA0943 mutant was deficient in the export of Xcp secretion system substrates. This suggests that the function of endogenously produced XcpQ is also compromised. Interestingly, although Xcp-dependent secretion was reduced in the ΔPA0943 mutant, it was not abolished (Fig. 5). We speculate that some XcpQ may still successfully insert in the outer membrane at a level that is well below our limit of detection. Therefore, although the PA0943 protein may be important for XcpQ outer membrane localization, it might not be essential for it.

The effect of a PA0943 null mutation on XcpQ concentration and localization calls to mind pilot proteins, which are required for the outer membrane insertion of some secretins (see, e.g., reference 19). The only known pilot protein in *P. aeruginosa* is PilF, which is an outer membrane lipoprotein that pilots the PilQ secretin (25). PilF is required for PilQ multimerization (25), whereas we have shown that PA0943 is not required for XcpQ multimerization (Fig. 6). However, this does not rule PA0943 out as a pilot protein for XcpQ. The PulD secretin of *Klebsiella oxytoca* still multimerizes without its pilot protein, but the multimer inserts into the inner membrane (18). XcpQ behaves similarly in the absence of PA0943 (Fig. 5 and 7).

Although some of the phenotypes of the null mutant suggest that PA0943 could be a pilot protein for XcpQ, some of our other observations lead us to speculate that PA0943 might not be a classic pilot protein. First, we found that PA0943 is a soluble periplasmic protein (Fig. 7), whereas most characterized pilot proteins are outer membrane lipoproteins. Second, the effects of the PA0943 null mutation may not be limited to XcpQ, because there is also a defect in export of the LapA substrate of the Hxc type 2 secretion system (Fig. 5), and like XcpQ, an overproduced YsaC secretin from *Y. enterocolitica* also has a lower steady-state level in a *P. aeruginosa* ΔPA0943 strain. This suggests that PA0943 has a more general effect. This is in contrast to all characterized pilot proteins, which are specific for a single secretin.

The HHpred tertiary structure prediction and alignment tool (http://toolkit.tuebingen.mpg.de/hhpred) predicts a β-sheet-rich C-terminal domain for the PA0943 protein with structural similarity to regions of uncharacterized members of the LppX and LolA family of proteins (over 90% confidence probability) (data not shown). LppX of *Mycobacterium tuberculosis* is involved in cell envelope lipid trafficking (52). LolA binds lipoproteins and is involved in their transport from the inner to the outer membrane (31). The MxiM pilot protein also shares structural similarity with LolA, which has been incorporated into a model for its mechanism of action (42). Therefore, if the structural prediction for PA0943 is correct, it leaves open the possibility that it does act similarly to at least one pilot protein.

The original motivation for this work was to investigate how *P. aeruginosa* might deal with the potential for secretin-induced stress without a Psp system. In particular, we had considered the possibility that *P. aeruginosa* could have proteins that serve a function similar to that of the Psp system in responding to secretin-induced stress. However, taken together our data argue against this. First, there was no transcriptional response to secretin overproduction in *P. aeruginosa*. Second, rather than inactivating stress response proteins, the transposon insertions we identified might simply cause secretin stress to occur. Essentially, this would mean that *P. aeruginosa* prevents rather than responds to secretin stress. For example, the PA0943 mutation caused XcpQ to mislocalize within the cell envelope, which may be directly toxic to the cell. Secretin mislocalization is an event that induces the Psp response in Psp^+ species (10). Of course, *P. aeruginosa* has no Psp response to induce. However, when we provided the PA0943 mutant with the critical PspBC components from *Y. enterocolitica*, the secretin sensitivity phenotype was reversed (Fig. 8). Although the precise

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physiological function of PspBC is not yet known, this result supports the hypothesis that *P. aeruginosa* lacks an analogous secretin stress response of its own. It also suggests that the *Y. enterocolitica* PspBC proteins can function in *P. aeruginosa*, which provides more evidence to support our previous conclusion that PspBC may be able to exert their physiological effects in isolation (50).

Studies of *Enterobacteriaceae* have shown that secretin overproduction is a highly specific modulator of bacterial gene expression (27, 50), and an extremely restricted number of genes are needed to survive these conditions (50). In this study we have found the same to be true for *P. aeruginosa*, although our screen of approximately 12,000 transposon mutants has not yet reached full saturation. Investigating the phenomenon of secretin-induced stress tolerance in *P. aeruginosa* is important for several reasons. First, we have been able to associate phenotypes with genes that, to our knowledge, have not been studied before. Second, we have identified a novel periplasmic protein that is important for XcpQ localization, but not multimerization, and for the efficient functioning of important protein export systems. Third, long-term studies of the genes we found could provide insight into secretin trafficking to the outer membrane in *P. aeruginosa*, a process that is not well understood in most cases (3).

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