Molecular Microbiology (2002) **46**(4), 1123–1133

ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon

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Summary

Expression of the *Pseudomonas aeruginosa* type III secretion system is induced by contact with eukaryotic cells, serum or low Ca^{2+} concentrations. We report that ExsD, a unique protein, is a negative regulator of the type III regulon. Localization studies indicate that ExsD is not secreted by *P. aeruginosa*. To determine the role of *exsD*, a non-polar deletion was returned to the chromosome by allelic exchange. The Δ*exsD* mutant is competent for type III secretion and translocation of the ExoU cytotoxin to eukaryotic host cells. To examine the effect of ExsD on transcription, *lacZ* transcriptional reporter fusions were integrated into the chromosome. Promoters controlling transcription of genes encoding the type III secretory, regulatory and effector proteins demonstrated significant derepression in the Δ*exsD* background. Expression of ExsD from a multicopy plasmid completely repressed transcription of the regulon. Although a mutant in *pscC*, encoding a structural component of the type III translocase, is repressed for expression of the regulon, a Δ*exsD*, *pscC*::Ω double mutant is derepressed. Bacterial two-hybrid data indicate that ExsD binds the transcriptional activator of the regulon, ExsA. We conclude that ExsD is a negative regulator and propose that ExsD functions as an ExsA antiactivator to regulate transcription of the regulon.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen of humans responsible for several infections of clinical importance. Patients at risk of *P. aeruginosa* infection include those with cystic fibrosis, severe burns or compromised immune systems (Giamarellou, 2000). A diverse range of virulence determinants has been implicated in the pathogenesis of *P. aeruginosa*. One that has recently received much attention is the type III secretion system. Type III secretion systems are found in a number of Gram-negative pathogens and function in the delivery of effector proteins to eukaryotic host cells (reviewed by Hueck, 1998). In each of these organisms, there is a highly conserved secretion and translocation machine and a unique set of secreted effector proteins. Ultimately, delivery of these effectors leads to events that promote pathogenesis. These events range from cytoskeletal alterations that promote invasion of *Shigella* to evasion of the host immune response by *Yersinia* sp. (Hueck, 1998).

The *P. aeruginosa* type III secretion system was first shown to play a role in the secretion of exoenzyme S (ExoS) (Yahr *et al*., 1996a). Since the discovery of ExoS, three additional effector proteins (ExoT, ExoU and ExoY) have been identified (Yahr *et al*., 1996b; 1998; Finck-Barbancon *et al*., 1997). ExoS and ExoT stimulate the GTPase activity of Rho signalling molecules resulting in rearrangement of the actin cytoskeleton (Goerhing *et al*., 1999; Krall *et al*., 2000). ExoS is also an ADP-ribosyltransferase of Ras signalling molecules (Barbieri, 2000). *In vivo*, ADP ribosylation of Ras by ExoS disrupts Ras-mediated signalling pathways, ultimately causing cell death (Ganesan *et al*., 1999). ExoY is an adenylate cyclase homologue of the oedema factor component of anthrax toxin and the *Bordetella pertussis* adenylate cyclase toxin (Yahr *et al*., 1998). Translocation of ExoY to eukaryotic host cells results in elevated intracellular cAMP levels and alterations in cell morphology (Yahr *et al*., 1998; Sawa *et al*., 1999). ExoU is an acute cytotoxin, the expression of which has been shown to be a major determinant of mortality in a mouse model of pulmonary infection (Finck-Barbancon *et al*., 1997; Allewelt *et al*., 2000). In addition to the secreted effectors, PcrV, PopB, PopD and PopN are also secreted by the type III system in *P. aeruginosa* (Yahr *et al*., 1997). PcrV, PopB and PopD are required for translocation of the effector proteins to host cells (Frithz-Lindsten *et al*., 1998; Sawa *et al*., 1999). The function of PopN has not been determined in *P. aeruginosa*; however, the PopN homologue in *Yersinia* sp. has a role in regulating the activity of the type III translocation apparatus (Forsberg *et al*., 1991).

Expression of the type III secretion system is highly regulated. Signals known to induce expression include

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contact with eukaryotic host cells, the presence of serum or Ca^{2+} chelation (Iglewski *et al*., 1978; Vallis *et al*., 1999). Any one of these signals results in the co-ordinate transcriptional activation of four operons (*exsD–pscL*, *pscG–popD*, *pcrD–pcrR*, *pscN–pscU*) encoding the type III secretion and translocation machinery. Each of these operons is under the positive transcriptional control of ExsA (Hovey and Frank, 1995; Yahr and Frank, 1995; Yahr *et al*., 1995). ExsA is a member of the AraC family of transcriptional activators and binds to the consensus sequence TxAAAAxA (Hovey and Frank, 1995). ExsA-regulated promoters control expression of the secretion apparatus, translocation machinery and the secreted effector proteins and, therefore, ExsA functions as a central transcriptional regulator of the type III regulon. The mechanisms by which environmental signals are sensed and coupled to ExsA-dependent expression of the type III regulon are unclear.

In addition to the positive regulatory activity of ExsA, a negative regulatory loop has been postulated based on the observation that mutants in components of the secretion apparatus (*pscN*, *pscC or pcrD*) fail to accumulate type III-related exoproducts in the cytoplasm (Yahr *et al*., 1996a; Vallis *et al*., 1999; Hornef *et al*., 2000). The basis for this activity has been unclear. Here, we provide evidence that ExsD is a major component of a negative regulatory pathway controlling expression of the type III regulon.

Results

The exsD m*utant is partially blind to* Ca^{2+}

With few exceptions, the genes encoding the type III secretion machinery in *P. aeruginosa* and *Yersinia* sp. are highly conserved. One of the exceptions is *exsD*, encoding a 32 kDa protein unique to *P. aeruginosa* with no discernible motifs or predicted functions (Frank and Iglewski, 1991; Yahr *et al*., 1996a). To determine the contribution of ExsD to type III secretion, an in frame deletion (codons 4–271) within *exsD* was returned to the chromosome of strain PA103 by allelic exchange. To examine whether ExsD is required for type III secretion, wild-type PA103, PA103*exsA*::Ω, PA103Δ*pcrV* and PA103Δ*exsD* were grown under non-inducing (in the absence of EGTA) or inducing conditions (in the presence of EGTA) for type III secretion. Concentrated culture supernatant fluid and cell-associated fractions were prepared and analysed by SDS-PAGE and immunoblot analyses. Growth of wild-type PA103 in the presence of EGTA induces the expression and secretion of type III-related exoproducts (Fig. 1A–E, lane 1 versus lane 2). An insertion within the transcriptional activator *exsA* prevents expression of the type III regulon regardless of growth conditions (Fig. 1A–E, lanes 3 and 4) (Frank *et al*., 1994). A *pcrV* mutant has been shown previously to possess a Ca^{2+}-blind phenotype (Sawa *et al*., 1999). Mutants with this phenotype exhibit a partial loss of calcium-dependent regulation (Fig. 1A–F, lanes 5 and 6).

Although *exsD* is the first gene of an operon encoding proteins required for secretion, the Δ*exsD* mutation did not block secretion (Fig. 1A, lane 8). Quantification of the total amounts of ExoU and ExoT in the supernatant fluid revealed just the opposite. The Δ*exsD* mutant secretes two- to threefold more ExoU and ExoT (Fig. 1A, lane 2 versus 8). Elevated extracellular levels of ExoU and ExoT were also observed in the Δ*pcrV* mutant. These data suggest that loss of either *exsD* or *pcrV* results in increased expression and/or secretion of ExoU and ExoT.

Although not apparent from the Coomassie-stained gel of culture supernatant fluid, immunoblot analyses indicated that the Δ*exsD* mutant secreted low levels of ExoU and ExoT in the absence of EGTA (Fig. 1B and D, lane 7). In addition, significant accumulation of ExoU and, to a lesser extent, ExoT was seen in the cell-associated fraction when the Δ*exsD* mutant was grown in the absence of EGTA (Fig. 1C and E, lane 7). This accumulation of cell-associated ExoU was largely reversed by growth in the presence of EGTA (Fig. 1C, lane 8). As seen from Fig. 1 and Table 1, expression of ExoU and ExoT in the Δ*exsD* mutant is deregulated, yet secretion is strictly dependent

Table 1. Quantification of the percentage cell-associated and secreted ExoU and ExoT.

	ExoU		ExoT	
Bacterial strain	Cell-associated[a]	Secreted	Cell-associated	Secreted
Wild type –EGTA	ND[b]	ND	ND	ND
Wild type +EGTA	6[c] ± 3	94 ± 3	14 ± 10	86 ± 10
Δ*pcrV* –EGTA	52 ± 11	48 ± 11	22 ± 4	78 ± 4
Δ*pcrV* +EGTA	16 ± 6	84 ± 6	8 ± 2	92 ± 2
Δ*exsD* –EGTA	99 ± 1	1 ± 1	95 ± 2	5 ± 5
Δ*exsD* +EGTA	13 ± 6	87 ± 6	30 ± 11	70 ± 11

a. Cell-associated and secreted fractions were normalized such that each fraction was derived from the same number of cells.
b. ND, Not detected.
c. Values are the average of three separate experiments.

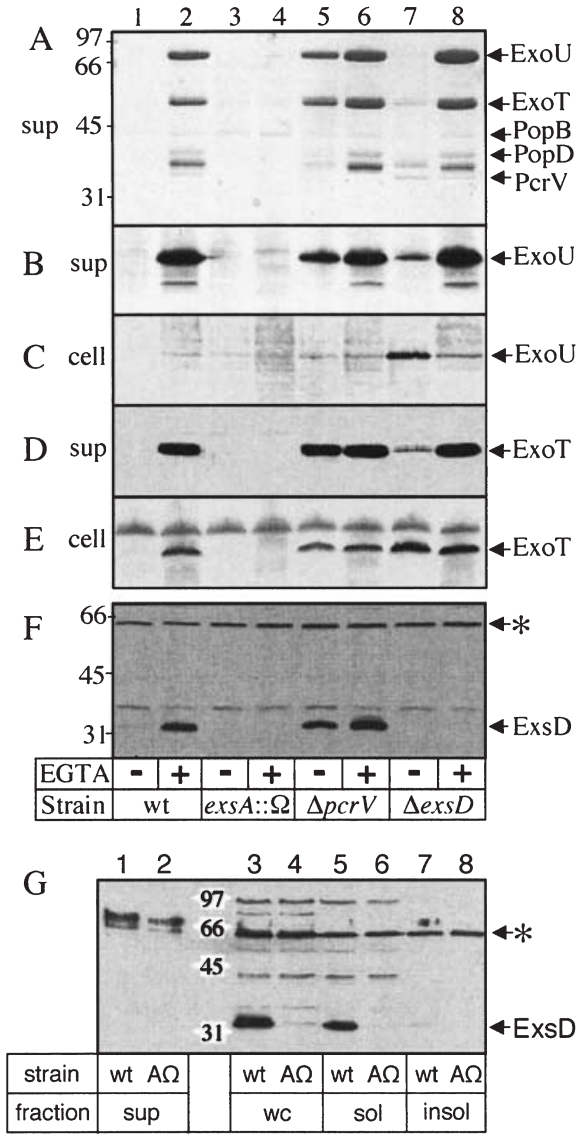

Fig. 1. Characterization of type III secretion in PA103Δ*exsD*.
A–F. Wild-type PA103 (lanes 1 and 2), *exsA*::Ω (lanes 3 and 4), Δ*pcrV* (lanes 5 and 6) and Δ*exsD* (lanes 7 and 8) were grown under non-inducing (– EGTA, lanes 1, 3, 5 and 7) and inducing (+ EGTA, lanes 2, 4, 6 and 8) conditions for type III secretion and subjected to SDS-PAGE and immunoblot analyses.
A. Coomassie-stained gel of concentrated culture supernatants (sup). Type III-related exoproducts are indicated on the right side of the gel.
B–F. Immunoblots with supernatant (B and D) (prepared from 3.6×10^8 cfu) or cell-associated (cell) (equivalent to 3.3×10^7 cfu) fractions (C, E and F). Blots were probed for ExoU (B and C), ExoT (D and E) or ExsD (F).
G. Anti-ExsD immunoblot of supernatant, whole cell (wc), soluble and insoluble (insol) fractions from wild-type and *exsA*::Ω. Molecular weight standards (in thousands) are indicated in (A), (F) and (G). The asterisk in (F) and (G) indicates a cross-reactive band that serves as a loading control.

upon EGTA. These data indicate that chelation of Ca^{2+} by EGTA functions to induce type III secretion rather than expression of the type III regulon. ExoU and ExoT expression was not detected when wild-type PA103 was grown in the absence of EGTA (Fig. 1B–E, lane 1). In the Δ*pcrV* mutant, both expression and secretion of ExoU and ExoT are deregulated and largely independent of EGTA (Fig. 1B–E, lanes 5 versus 6). These results suggest that ExsD is a negative regulator of the type III regulon.

Consistent with earlier transcriptional studies of the promoter (pD) responsible for transcription of the *exsD–pscL* operon, expression of ExsD in wild-type PA103 was dependent upon growth in the presence of EGTA and requires ExsA (Fig. 1F, lanes 1–4) (Yahr and Frank, 1994). Expression of ExsD was independent of EGTA in the Δ*pcrV* mutant (Fig. 1F, lanes 5 and 6) and was not detected in PA103Δ*exsD* (Fig. 1F, lanes 7 and 8). ExsD was found exclusively in the cell-associated soluble fraction, indicating that ExsD is not secreted by *P. aeruginosa* (Fig. 1G).

*PA103*ΔexsD *is translocation competent*

Although competent for type III secretion, *pcrV* or *popD* mutants are defective in translocation of the ExoU cytotoxin to eukaryotic host cells (Sawa *et al*., 1999). To determine whether the Δ*exsD* mutant was still capable of translocating ExoU to eukaryotic cells, a lung epithelia infection model was used. In this system, human donor lungs are dissociated, reconstituted onto permeable membrane supports and maintained under tissue culture conditions (Karp *et al*., 2002). Cells grown in this way are polarized with defined apical and basolateral surfaces, possess tight junctions and mimic the features of native airway epithelia. Epithelia were infected on the apical surface, and *trans*-epithelial resistance was measured as a function of time (Zabner *et al*., 1996). Lung epithelia incubated with PBS alone or with PBS containing PA103 *exsA*::Ω, Δ*pcrV* or Δ*exoU* showed no significant alteration in *trans*-epithelial resistance (Fig. 2). In contrast, incubation with wild-type PA013 or the Δ*exsD* mutant resulted in a rapid loss of *trans*-epithelial resistance (Fig. 2). This is consistent with loss of the tight junctions and is similar in kinetics to ExoU-mediated host cell killing seen in other model systems (Finck-Barbancon *et al*., 1997; Sawa *et al*., 1999). We conclude that the Δ*exsD* mutant is fully capable of translocating ExoU to the cytoplasm of eukaryotic host cells.

Transcription of the type III regulon is derepressed in the absence of ExsD

To examine the effects of ExsD on transcription, *lacZ* transcriptional reporter fusions were constructed using

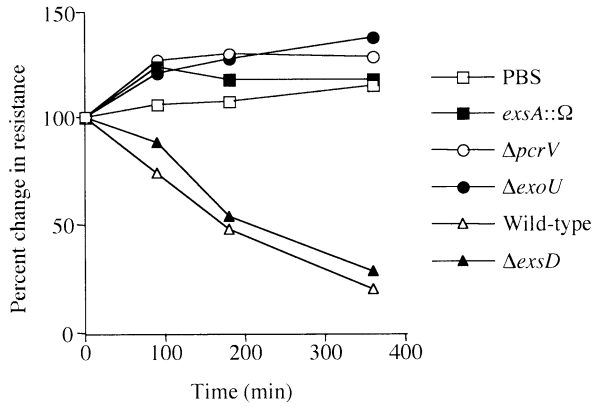

Fig. 2. PA013Δ*exsD* is competent for translocation of the ExoU cytotoxin. Human airway epithelial cells were cultured in 24-well plates. Before infection, cells were washed to remove mucus, and the initial (time 0) *trans*-epithelial resistance was measured with an ohm meter. Cells were infected on the apical surface with a suspension (250 μl) of *P. aeruginosa* (1×10^8 cfu ml^{-1}) in PBS, and *trans*-epithelial resistance was measured at the indicated times after infection. Four wells were infected for each bacterial strain, and the resistance values were averaged and expressed as a percentage of the starting values. The standard error was <15%.

three previously characterized promoters of the regulon. These promoters are responsible for transcription of genes encoding secretory (pD), regulatory (pC) and effector (pS) proteins (Fig. 3A). The pC promoter is located upstream of the *exsCBA* operon, the pD promoter drives transcription of the *exsD–pscB–L* operon, and the pS promoter controls transcription of the exoenzyme S (*exoS*) structural gene (Yahr and Frank, 1994; Yahr *et al.*, 1995; 1996a). Each promoter was cloned upstream of *lacZ* and integrated in single copy into the chromosome of wild-type PA013, *exsA*::Ω, Δ*pcrV* and Δ*exsD*.

Strains bearing the chromosomally integrated promoter fusions were grown to mid-exponential phase under conditions for type III secretion and assayed for β-galactosidase activity. Consistent with earlier studies, the activity of each promoter was induced when wild-type PA103 was grown in the presence of EGTA, and this activity was dependent upon ExsA (Fig. 3B) (Yahr and Frank, 1994; Yahr *et al.*, 1995). When examined in the Δ*exsD* background, the transcriptional activity of each promoter demonstrated a significant derepression regardless of growth conditions (Fig. 3B). To a lesser degree, transcriptional derepression was also observed in the Δ*pcrV* background for each promoter (Fig. 3B). These data demonstrate that the Δ*exsD* mutant is derepressed for transcription at pC, pD and pS regardless of growth conditions. As expression of the type III regulon is coordinately regulated, expression of the entire regulon is most likely derepressed in the absence of *exsD*.

Multiple copies of exsD *in trans inhibit transcription of the regulon*

The data presented thus far are consistent with ExsD being a negative regulator of the type III regulon. To determine whether overexpression of ExsD might lead to repression, *exsD* and its native promoter (pD) were cloned into pUCP18. In strains carrying this construct (pD*exsD*), expression of ExsD was dependent upon the pD promoter, EGTA and *exsA* (data not shown). Wild-type PA103, Δ*pcrV* and Δ*exsD* bearing the chromosomally integrated mini-pD–*lacZ* reporter fusion and carrying either the vector alone or the ExsD expression plasmid were grown under non-inducing and inducing conditions for type III secretion. Cell-associated and supernatant fractions were prepared and analysed by SDS-PAGE and β-galactosidase assays. In each case, strains carrying the pD*exsD* expression plasmid (Fig. 4A–C, lanes 3, 4, 7, 8, 11 and 12) were almost completely repressed for transcription from the pD promoter and for expression of the

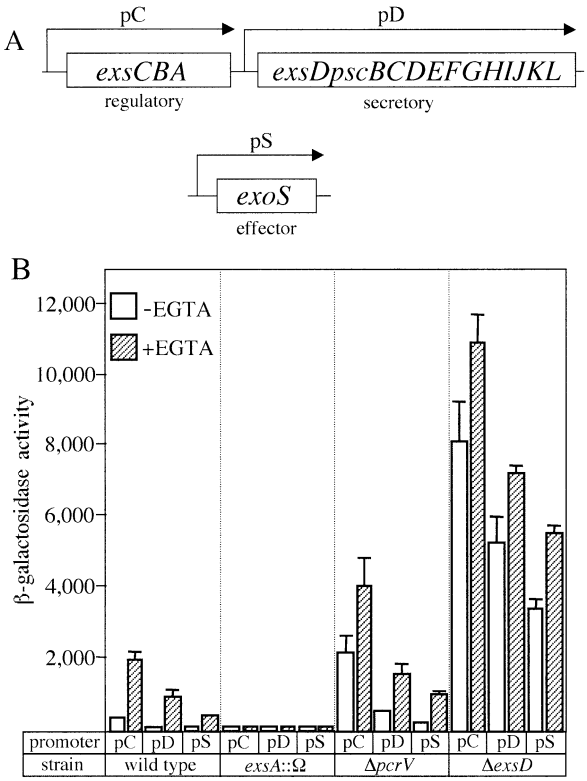

Fig. 3. Transcription of the type III regulon is derepressed in the Δ*exsD* mutant. PA103 wild type, *exsA*::Ω, Δ*pcrV* and Δ*exsD* carrying mini-CTX–*lacZ* transcriptional fusions to the pC, pD or pS promoters were grown under non-inducing (–EGTA, open bars) or inducing (+EGTA, hatched bars) conditions for type III secretion and assayed for β-galactosidase activity using a luminescence assay. β-Galactosidase activity is expressed as relative light units. The values are the average of three separate experiments, and the standard deviation is indicated by error bars.

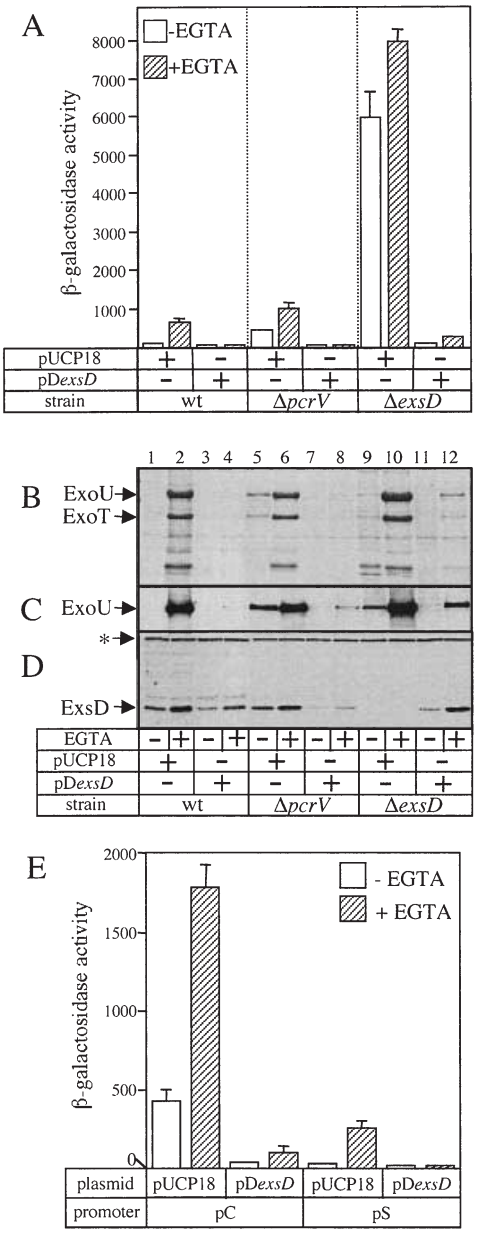

Fig. 4. Expression of ExsD from a plasmid represses transcription from the pD, pC and pS promoters.
A–D. PA103 wild type (lanes 1–4), Δ*pcrV* (lanes 5–8) and Δ*exsD* (lanes 9–12) carrying the mini-pD–*lacZ* transcriptional reporter and either a vector control (pUCP18) or an ExsD expression plasmid (pD*exsD*) were grown under non-inducing (–EGTA, open bars) or inducing (+EGTA, hatched bars) conditions for type III secretion.
A. β-Galactosidase activity from the pD promoter. The values are the average of three separate experiments, and the standard deviation is indicated by error bars.
B–D. Supernatant (prepared from 3.6×10^8 cfu B and C) and cell-associated (equivalent to 3.3×10^7 cfuD) fractions were prepared and analysed by SDS-PAGE and immunoblot analyses.
B. A Coomassie-stained gel of concentrated supernatants.
C and D. Immunoblots probed with antisera against ExoU and ExsD respectively.
E. Wild-type PA103 carrying either the mini-pC–*lacZ* or the mini-pS–*lacZ* reporters and either pUCP18 or pD*exsD* were assayed for β-galactosidase activity as described above.

type III regulon compared with the vector control (Fig. 4A–C, lanes 1, 2, 5, 6, 9 and 10). The percentage reduction in transcription of the β-galactosidase reporter was 98%, 96% and 97% in the wild-type, Δ*pcrV* and Δ*exsD* backgrounds respectively. These data are consistent with ExsD acting as a negative regulator of the type III regulon.

When cells carrying the pD*exsD* expression plasmid were immunoblotted for ExsD, the steady-state level of ExsD was reduced compared with the vector control (Fig. 4D). When expression of ExsD was placed under the transcriptional control of an exogenous promoter (p*lac*), the level of ExsD expression increased >20-fold, and the inhibition of pD transcription was absolute (data not shown). This observation suggests that ExsD autoregulates its own expression. This autoregulation is most likely to be an indirect effect achieved through negative regulation of transcription from the pC promoter. Decreased levels of pC transcription result in less ExsA expression, and this decreases transcription from the pD promoter and reduces ExsD expression levels. These data suggest that modest increases in the expression of ExsD may lead to repression. Consistent with this idea, multiple copies of *exsD in trans* inhibit transcription from the pC and pS promoters to levels similar to those seen for the pD promoter (Fig. 4E).

To eliminate the possibility that multiple copies of the pD promoter titrate ExsA away from other type III promoters, a derivative of pUCP18 carrying only the pD promoter was constructed. This construct had no effect on transcription of the mini-pD–*lacZ* reporter or on expression of the type III regulon compared with a vector control (data not shown). This demonstrates that the negative regulatory effect of pD*exsD* results from expression of ExsD.

ExsD is required for negative regulation in the absence of a functional secretion apparatus

Previous studies have noted the existence of a negative regulatory pathway that prevents expression of the type III regulon in the absence of a functional secretion apparatus (Yahr *et al*., 1996a; Vallis *et al*., 1999). To determine whether ExsD is involved in this regulatory pathway, a polar insertion within *pscC*, encoding the outer membrane secreton component of the type III translocase, was constructed and returned to the chromosomes of wild-type PA103 and the Δ*exsD* mutant bearing the mini-pD–*lacZ* transcriptional reporter. Strains were grown under non-inducing and inducing conditions and analysed for pD transcription and type III secretion. When compared with wild type, the *pscC*::Ω mutant was deficient for type III secretion, and transcription from the pD promoter was repressed (Fig. 5A and B, lanes 2 versus 6). Although low levels of cell-associated ExoU were detected in wild-

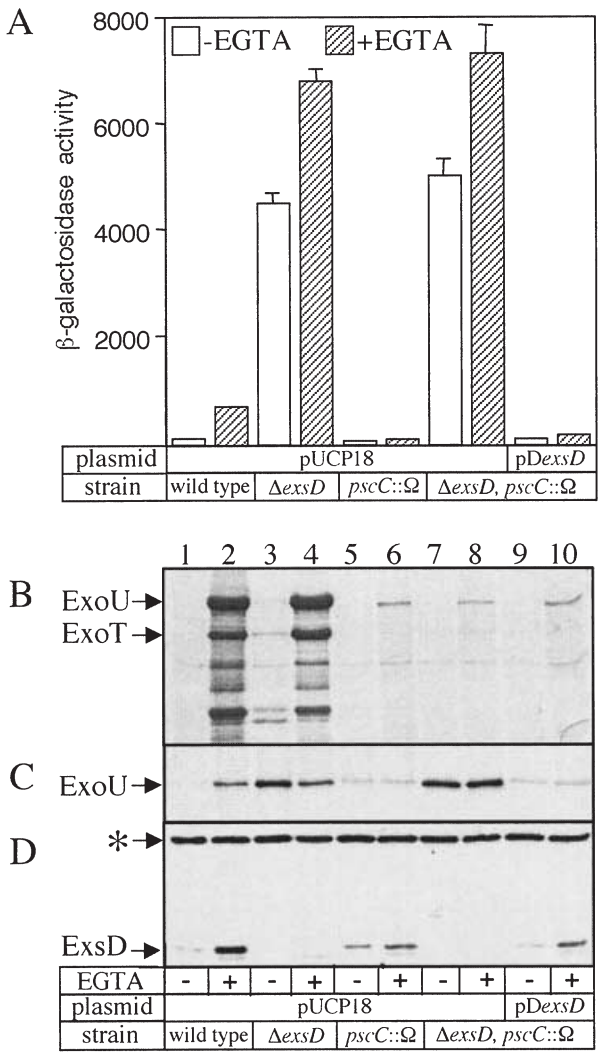

Fig. 5. Repression of the type III regulon in a secretion mutant requires ExsD. PA103 wild type, Δ*exsD*, *pscC*::Ω and the Δ*exsD*, *pscC*::Ω double mutant carrying the mini-pD–*lacZ* transcription reporter and either a vector control (pUCP18) or an ExsD expression plasmid (pD*exsD*) were grown under non-inducing (–EGTA, open bars) or inducing (+EGTA, hatched bars) conditions for type III secretion.
A. β-Galactosidase activity from the pD promoter. The values are the average of three separate experiments, and the standard deviation is indicated by error bars.
Supernatant (prepared from 3.6×10^8 cfu, B) and cell-associated (equivalent to 3.3×10^7 cfu, C and D) fractions were prepared and analysed by SDS-PAGE and immunoblot analyses.
B. A Coomassie-stained gel of concentrated supernatants.
C and D. Immunoblots probed with antisera against ExoU and ExsD respectively.

type cells grown in the presence of EGTA, much less cell-associated ExoU was detected in the *pscC* mutant (Fig. 5C, lanes 2 versus 6). In contrast, with the *pscC*::Ω, Δ*exsD* double mutant, transcription from the pD promoter was derepressed, and significant levels of cell-associated ExoU were detected (Fig. 5A and C, lanes 7 and 8). The accumulation of ExoU in the cell-associated fraction and the derepressed transcription observed with the *pscC*::Ω, Δ*exsD* double mutant were completely reversed by providing cells with the pD*exsD* expression plasmid (Fig. 5A and B, lanes 9 and 10). These data identify ExsD as being essential for repression of the regulon in the absence of a functional secretion apparatus. As noted earlier, comparison of the steady-state levels of ExsD protein between the induced state (Fig. 5D, lane 2) and the repressed state (Fig. 5D, lanes 5, 6, 9 and 10) indicates that there is less ExsD in the latter. This suggests that, once repression has been achieved, maintaining this state requires only low levels of ExsD. As ExsD appears to regulate its own expression indirectly, this may reflect a super-repressed condition.

ExsD interacts with ExsA

An obvious candidate for the negative regulatory activity of ExsD is the ExsA transcriptional activator. To ascertain whether ExsD interacts with ExsA, the bacterial LexA two-hybrid screen was used (Dmitrova *et al*., 1998; Daines and Silver, 2000). In this system, wild-type LexA forms homodimers and binds to a specific operator sequence. Each partner of the homodimer binds to a half-site within the operator. The two-hybrid screen uses a mutant form of LexA (LexA408) with altered DNA binding specificity and a hybrid operator sequence containing a wild-type half-site for LexA and a half-site specific for LexA408. This hybrid operator controls the expression of a chromosomally encoded *lacZ* reporter. Only heterodimers of LexA and LexA408 can bind to the hybrid operator and repress transcription of the *lacZ* reporter. To test for an interaction between ExsD and ExsA, the dimerization domains of wild-type LexA and LexA408 were exchanged for ExsD and ExsA respectively. A productive interaction between LexA–ExsD and LexA408–ExsA is predicted to repress expression of the *lacZ* reporter. When either the LexA–ExsD or LexA408–ExsA expression plasmids were introduced into the reporter strain, no repression of the *lacZ* reporter was observed (Fig. 6). However, the combination of both plasmids resulted in ≈ 80% inhibition of *lacZ* expression. A positive control, containing fusions of LexA and LexA408 to the well-characterized eukaryotic dimerization domains of Fos and Jun, respectively, resulted in 95% inhibition. These data indicate that ExsD binds to ExsA and suggest that the negative regulatory activity of ExsD may function by interfering with ExsA-dependent transcription directly.

Discussion

In the present study, ExsD was identified as the first negative regulator of the *P. aeruginosa* type III regulon. A

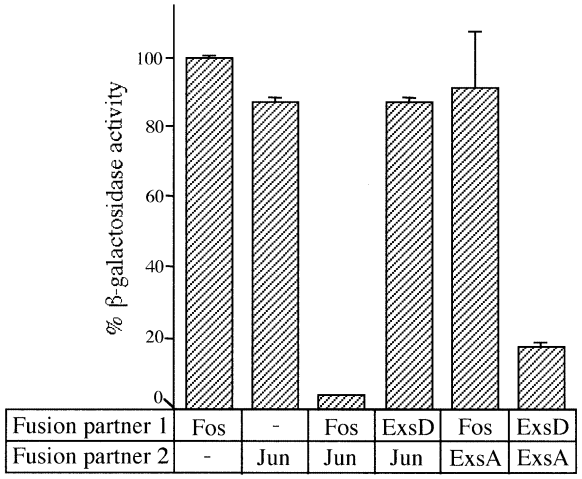

Fig. 6. ExsD interacts with ExsA. *E. coli* strain SU202 expressing the indicated fusions of LexA to either Fos or ExsD (fusion partner 1) or LexA408 to either Jun or ExsA (fusion partner 2) were grown as described in *Experimental procedures* and assayed for β-galactosidase activity. β-Galactosidase activity was normalized to the value obtained with the LexA–Fos expression plasmid. The values are the average of three separate experiments.

mutant in *exsD* is derepressed for transcription of the entire regulon, and multicopy expression of *exsD* from a plasmid results in repression. ExsD was found to be essential for repression when cells were grown in the presence of Ca^{2+} and also in mutants lacking a functional type III secretion apparatus. From these data, we conclude that ExsD is a negative regulator that prevents inappropriate expression of the type III regulon (i.e. in the absence of the appropriate environmental cue or in mutants lacking a functional translocase). Bacterial two-hybrid data indicate that ExsD interacts with the ExsA transcriptional activator and suggest that ExsD may function as an antiactivator to regulate expression of the regulon.

When *exsD* is provided on a multicopy plasmid, transcription of the regulon is repressed and yet the absolute levels of ExsD are reduced. As expression of both chromosomally encoded ExsA and plasmid-encoded ExsD is dependent upon ExsA, we hypothesize that expression of ExsD from a multicopy plasmid disrupts the normal ExsD:ExsA ratio. If one assumes that ExsA has an equal probability of activating transcription from the promoters controlling expression of *exsCBA* (pC) and *exsD–pscL* (pD), expression of plasmid encoded ExsD would be favoured over ExsA. This would effectively lower the concentration of ExsD required to maintain a repressed state. Under this scenario, we would predict that, although the levels of ExsD are relatively unchanged in cells carrying the pD*exsD* expression plasmid, the levels of ExsA would be significantly reduced. This suggests that the ratio of ExsD to ExsA may be an important component of the negative regulatory activity of ExsD.

In both *P. aeruginosa* and *Yersinia* sp., a negative regulatory loop becomes activated in mutants lacking a functional type III secretion system (Plano and Straley, 1993; Allaoui *et al*., 1995; Yahr *et al*., 1996a). In *Yersinia* sp., repression of Yop synthesis is accomplished, at least in part, through LcrQ in *Yersinia pseudotuberculosis* and *Yersinia pestis* or YscM1/YscM2 in *Yersinia enterocolitica* (Rimpilainen *et al*., 1991; Stanier *et al*., 1997). Similar to the phenotype of the *P. aeruginosa* Δ*exsD* mutant, transcription of *yops* is elevated in an *lcrQ* mutant, and overexpression of LcrQ/YscM represses Yops expression (Rimpilainen *et al*., 1992). Although ExsD and LcrQ/YscM have similar regulatory activities, these activities are most probably mechanistically unrelated. First, LcrQ/YscM is a secreted protein, and relief from its negative regulator activity is dependent upon its secretion (Pettersson *et al*., 1996). ExsD is not secreted by *P. aeruginosa*. Secondly, the stability and secretion of LcrQ is dependent upon the SycH chaperone (Wulff-Strobel *et al*., 2002). SycH is also required for secretion of YopH (Cambronne *et al*., 2000). Homologues of SycH and YopH are absent in *P. aeruginosa.* Thirdly, the negative regulatory activity of LcrQ is dependent upon YopD, i.e. overexpression of LcrQ in a YopD mutant has no effect on Yops expression (Williams and Straley, 1998). In *P. aeruginosa*, the activity of ExsD is independent of PopD, a homologue of YopD (T. L. Yahr, unpublished). It is interesting to note that, although ExsD is unique to *P. aeruginosa* and LcrQ is unique to *Yersinia* sp., they are encoded within homologous operons. In *P. aeruginosa*, *exsD* is the first gene of the *pscBCDEFGHIJKL* operon, whereas in *Yersinia* sp., *yscM* is the last gene of the *yscABCDEFGHIJKL* operon. Because ExsD and LcrQ appear to work in part through sensing the functionality of the secretion apparatus, it makes sense that they be co-ordinately regulated and expressed with the secretion apparatus.

Although ExsD has negative regulatory activity, this does not preclude ExsD from playing a role in activation of the type III regulon. On the contrary, inactivation of a negative regulatory pathway in response to environmental cues would have the same effect as activation of a positive regulatory cascade in response to the same stimuli. We propose the following model for the negative regulatory activity of ExsD. Under conditions in which the secretion channel is closed (before contact with eukaryotic host cells, in the absence of EGTA or in mutants lacking a functional secretion apparatus), there is a basal level of ExsA expression. Basal levels of ExsA allow for transcriptional activation of the pD promoter controlling the expression of *exsD*. ExsD functions as a sensor of the secretion channel and samples whether the secretion channel is open or closed. When the channel is closed, ExsD binds

directly to ExsA and prevents further expression of the type III regulon. When the secretion channel is open (either by contact with eukaryotic host cells or in the absence of Ca^{2+}), ExsD no longer interacts with ExsA, resulting in activation of the type III regulon. This would provide a mechanism for co-ordinating expression of the regulon with the activity of the secretion channel.

The co-ordination of gene expression with activation of type III secretion is emerging as a common regulatory theme (Miller, 2002). In *Salmonella typhimurium* and *Shigella flexneri*, expression of the type III regulon requires an interaction between a transcriptional activator (InvF and MxiE respectively) and a co-activator (SicA and IpgC) (Darwin and Miller, 2001; Mavris *et al*., 2002). These co-activators also function as chaperones for type III secreted proteins. When the secretion channel is closed, SicA/IpgC are bound to their chaperone substrates. This prevents them from interacting with InvF/MxiE. Activation of the type III translocase is thought to release the co-activators, allowing them to associate with InvF/MxiE and activate expression of the regulon (Darwin and Miller, 2001; Mavris *et al*., 2002). A second variation on this theme is found in *Yersinia* sp., in which activation of the type III translocase results in the secretion of a negative regulatory protein (LcrQ/YscM) (Pettersson *et al*., 1996). In this case, however, the link between the secretion of LcrQ/YscM and the activation of transcription is unclear. The *P. aeruginosa* system appears to represent a third variation on this theme of co-ordinating gene expression with activation of type III secretion. For *P. aeruginosa*, we hypothesize that activation of the type III translocase suppresses the negative regulatory activity of ExsD (i.e. loss of binding to ExsA). Assuming this to be true, one major question remains. How is the negative regulatory activity of ExsD controlled? As ExsD is not itself secreted, ExsD may sense the presence of free chaperone within the cell. The previously demonstrated regulatory role of chaperones in the *Salmonella*, *Shigella* and *Yersinia* systems lends credence to this model (Cambronne *et al*., 2000; Darwin and Miller, 2001; Mavris *et al*., 2002; Wulff-Strobel *et al*., 2002). One potential mechanism may involve a binding competition between a secreted product and ExsD for binding to chaperone. Under this scenario, activation of type III secretion would release the chaperone from its secretion substrate making it available for binding to ExsD. This might increase the pool of free ExsA and lead to activation of the regulon. Futures studies will be directed towards defining the link between ExsD and the activation of type III secretion.

Experimental procedures

Bacterial strains and culture conditions

Pseudomonas aeruginosa strains were maintained on Vogel Bonner minimal medium with antibiotics as required (400 μg ml^{-1} carbenicillin, 100 μg ml^{-1} tetracycline, 100 μg ml^{-1} gentamicin) (Vogel and Bonner, 1956). For expression of the type III secretion regulon, *P. aeruginosa* was grown at 30°C with vigorous aeration in trypticase soy broth supplemented with 1% glycerol, 100 mM monosodium glutamate and 2 mM EGTA.

*Construction of PA103Δ*exsD *and PA103*pscC::Ω

Primers were used to polymerase chain reaction (PCR) amplify DNA fragments flanking the upstream (5′-TATGG AGCTCATGCAAACGATTGCCGAGCG and 5′-ATCAGGATC CCTGCTCCATTCTCTGCCTTG) and downstream (5′-TATG AAGCTTGTCCCTGGCTCTGCAACAGGGT and 5′-ATACG GATCCTTCTACTGGCAGAGCTGAGCGG) regions of *exsD*. PCR fragments were sequentially ligated into pEX18Tc resulting in an in frame deletion of codons 4–271 (Hoang *et al*., 1998). The resulting plasmid (pEX18TcΔ*exsD*) was mobilized to *P. aeruginosa* by conjugation, and tetracycline-resistant merodiploids were isolated. A merodiploid was resolved as described previously and confirmed by PCR analysis (Sawa *et al*., 1999). The resulting mutant, PA103Δ*exsD*, had no discernible growth defect compared with wild type.

For construction of the polar insertion within *pscC*, primers (5′-TGATAAGCTTCCAGCCTGCCTTACGACTATGTGG and 5′-ACATGAGCTCGCAAGCGCCTGCAGGATCGCTCC) were used to PCR amplify a 2.0 kb fragment of *pscC*. The fragment was digested with *Hin*dIII and *Sst*I and ligated to the corresponding restriction sites of pEX18Tc. A gentamicin Ω cassette was isolated as a *Bam*HI restriction fragment and cloned into the unique *Bam*HI site of the *pscC* fragment (Blondelet-Rouault *et al*., 1997). This insertion results in the disruption of *pscC* at codon 355. The resulting clone (pEX18*pscC*::Ω) was delivered to *P. aeruginosa* by electroporation, and gentamicin-resistant clones were isolated. A gentamicin-resistant, tetracycline-sensitive recombinant was identified by replica plating and shown by PCR analysis to have an insertion within *pscC*.

*Construction of mini-*lacZ *reporter insertions*

Primers were used to PCR amplify promoter fragments corresponding to pC (5′-TGATAAGCTTCCAGCCTGCCTTAC GACTATGTGG) and 5′-TGATGAATTCGCCTCCTAAAGCT CAGCGCATGC), pD (5-ACAGAAGCTTTTCCAGCCAGTC CTATTTCACC and 5′-GACCGAATTCCCTGCTCCATTCTC TGCCTTG), and pS (5′-CTATAAGCTTCTGCAGGCTGAG TACGCTCTCC and 5′-GTAGGAATTCTGATGGTTGCCT TCTCCTGATG). Promoter fragments were digested with *Hin*dIII and *Eco*RI and ligated into the corresponding sites of mini-CTX–*lacZ* (Becher and Schweizer, 2000). Electroporation was used to transform *P. aeruginosa* with these constructs, tetracycline-resistant transformants were selected, and plasmid sequences were excised using the pFLP2 plasmid as described previously (Hoang *et al*., 2000). Insertions were confirmed by Southern blot analysis.

Expression and purification of ExsD

For expression studies in *P. aeruginosa*, primers (5′-ACA

GAAGCTTTTCCAGCCAGTCCTATTTCACC and 5′-GTTAG AGCTCAGTGGCCAATCCGCTTCAACAGATG) were used to PCR amplify a fragment containing the pD promoter and *exsD*. The PCR product was digested with *Hin*dIII and *Sst*I and ligated into pUCP18, resulting in plasmid pD*exsD* (West *et al*., 1994). For purification of ExsD, primers (5′-GAGT CATATGGAGCAGGAAGACGATAAGC and 5′-ATGACTCGA GGCTCTGCCAGTAGAAGTGATCC) incorporating *Nde*I and *Xho*I restriction sites were used to PCR amplify *exsD*. The PCR product was ligated into pET25b (Novagen), resulting in a carboxy-terminal hexahistidine fusion tag. Hexahistidine-tagged ExsD ($ExsD_{His6}$) was purified by Ni^{2+} affinity chromatography as described previously (Yahr *et al*., 1995). Antisera against $ExsD_{His6}$ were produced in a New Zealand White rabbit.

SDS-PAGE, immunoblots and quantitative analyses

For analysis of secreted and cell-associated fractions, cells were grown in trypticase soy broth to an absorbance (A_{540}) of 4.0. Supernatant fractions were prepared by pelleting 1.5 ml of culture (12 500 *g*) for 4 min. Supernatant fractions (1 ml) were transferred to a fresh microfuge tube, and protein was precipitated by the addition of 350 μl of 50% trichloroacetic acid. After a 30 min incubation on ice, precipitated protein was collected by centrifugation (10 min, 12 500 *g*), washed with 1 ml of acetone and suspended in 15 μl of SDS-PAGE sample buffer. Cell-associated fractions were prepared by pelleting 300 μl of cell culture ($A_{540} = 4.0$), suspending in 300 μl of SDS-PAGE sample buffer and sonicating for 10 s. For quantitative analyses, twofold serial dilutions of supernatant and cell-associated fractions were analysed by SDS-PAGE and immunoblot analyses in a single experiment. Immunoblots were scanned and analysed by densitometry using IPLAB gel software (Signal Analytics). Immunoblots were developed using ECL reagents (Amersham Pharmacia Biotech) and anti-rabbit horseradish peroxidase conjugant (Chemicon).

Fractionation

Strains were grown in TSB containing 2 mM EGTA to an absorbance (A_{540}) of 0.75. Supernatant fractions were prepared as described above. For preparation of the soluble and insoluble fractions, 1.7×10^9 cells were sedimented by centrifugation (4 min, 12 500 *g*), washed with 1 ml of 10 mM Tris, pH 8.0, and resedimented. The cell pellet was suspended in 300 μl of 10 mM Tris, pH 8.0, 20 mM EDTA, sonicated for 30 s and centrifuged for 4 min to remove unbroken cells. The cell extract was transferred to a fresh tube and centrifuged at 100 000 *g* for 60 min at 4°C to separate the soluble fraction from the insoluble membrane fraction (pellet).

β-Galactosidase assays

For β-galactosidase assays, cell-associated fractions (*P. aeruginosa* and *Escherichia coli*) were prepared by transferring 1.2×10^8 cells to a microfuge tube. The volume was normalized to 500 μl with TSB, and cells were lysed by the addition of 50 μl of chloroform and vortexing for 20 s. β-Galactosidase activity was measured using a microtitre plate luminescence assay (Applied Biosystems) and a Lucy I luminometer (Anthos) as described previously (Egland and Greenberg, 2000).

Epithelia infection experiments

Human lung epithelial cells were obtained from the Iowa Donor Network and prepared as described previously (Zabner *et al*., 1996; Karp *et al*., 2002). Briefly, cells were enzymatically digested and seeded at a density of 5×10^5 cells cm^{-2} onto collagen-coated permeable membranes and maintained at 37°C (5% CO_2) in a mixture of 49% Dulbecco's modified Eagle medium (DMEM), 49% Ham's F12 medium and 2% Ultraser G. All confluent cell layers had transepithelial electrical resistance values >1200 Ω. Before infection, the apical surface of the epithelial cells was washed extensively with phosphate-buffered saline (PBS) to remove mucus. For infections, *P. aeruginosa* strains were grown under inducing conditions for type III secretion to an absorbance (A_{540}) of 0.75, washed and suspended in PBS (1×10^8 cfu ml^{-1}) containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$, and 250 μl of cell suspension was applied to the apical surface of epithelia cells at a multiplicity of infection (MOI) of 25. Transepithelial resistance was measured using an ohm meter.

Bacterial two-hybrid screen

For construction of the LexA–ExsD fusion, primers (5′-GTGAACCGGTATGGAGCAGGAAGACGATAAGC and 5′-GTTACTCGAGAGTGGCCAATCCGCTCAACAGATG) incorporating *Age*I and *Xho*I restriction sites were used to PCR amplify *exsD*. For construction of the LexA408–ExsA fusion, primers (5′-ATCGGAGCTCATGCAAGGAGCCAAATCTCTT GGC and 5′-TCGTGGTACCGCCGATTCTACTCATGCAGC CGCT) incorporating *Sst*I and *Kpn*I restriction sites were used to PCR amplify *exsA*. PCR products were digested with the indicated restriction enzymes and cloned into the pMS604 (*exsD*) and pSR659 (*exsA*) expression vectors (Dmitrova *et al*., 1998; Daines and Silver, 2000). Clones were confirmed by nucleotide sequence analysis and transformed into the *E. coli* reporter strain SU202 (Dmitrova *et al*., 1998). To assay for β-galactosidase activity, overnight cultures were back-diluted to an A_{600} of 0.05 in LB containing 1 mM IPTG and the appropriate antibiotics. Cultures were incubated at 32°C until the A_{600} reached 1.0. Cells were harvested and assayed for β-galactosidase activity as described earlier.

Acknowledgements

We thank Phoebe Lostroh, Brad Jones, George Stauffer and Pete Greenberg for critical reading of this manuscript, Michael Welsh for lung epithelia, Dara Frank for antisera against ExoU and ExoT, Marcella Card for technical assistance, and Phil Karp and Pary Weber from the Cell Culture Core at the University of Iowa Gene Therapy Center. This work was supported by the Howard Hughes Medical Institute Biomedical Research Support Faculty Start-up Program, the W. M. Keck Microbial Communities and Cell Signaling

Program, and a grant from the Cystic Fibrosis Foundation Research Development Program. G.L. was funded by a Research Undergraduate Experience award (DBI00-97361) from the National Science Foundation.

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