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ExsB Is Required for Correct Assembly of the *Pseudomonas aeruginosa* Type III Secretion Apparatus in the Bacterial Membrane and Full Virulence *In Vivo*

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***Pseudomonas aeruginosa* is responsible for high-morbidity infections of cystic fibrosis patients and is a major agent of nosocomial infections. One of its most potent virulence factors is a type III secretion system (T3SS) that injects toxins directly into the host cell cytoplasm. ExsB, a lipoprotein localized in the bacterial outer membrane, is one of the components of this machinery, of which the function remained elusive until now. The localization of the *exsB* gene within the *exsCEBA* regulatory gene operon suggested an implication in the T3SS regulation, while its similarity with *yscW* from *Yersinia* spp. argued in favor of a role in machinery assembly. The present work shows that ExsB is necessary for full *in vivo* virulence of *P. aeruginosa*. Furthermore, the requirement of ExsB for optimal T3SS assembly and activity is demonstrated using eukaryotic cell infection and *in vitro* assays. In particular, ExsB promotes the assembly of the T3SS secretin in the bacterial outer membrane, highlighting the molecular role of ExsB as a pilotin. This involvement in the regulation of the T3S apparatus assembly may explain the localization of the ExsB-encoding gene within the regulatory gene operon.**

Pseudomonas aeruginosa is a Gram-negative bacterium that thrives in a variety of environments (1, 2) and can colonize diverse hosts, from invertebrates to humans (3, 4). It represents a real threat to human health, being responsible for the most frequent hospital-acquired infections along with *Escherichia coli* and *Staphylococcus aureus* in France (5). Moreover, it is the first cause of mortality and morbidity for people suffering from cystic fibrosis (6). As most clinical isolates are multiresistant to antibiotics, it is imperative to find new antibacterial strategies against this pathogen (7, 8). In this perspective, a better understanding of the virulence mechanisms of *P. aeruginosa* turned out to be a valuable approach to identify therapeutic targets and eventually develop new and more specific drugs.

One major virulence factor of *P. aeruginosa* is the type III secretion system (T3SS). This system is well conserved among bacterial pathogens, such as *Yersinia pestis*, the causative agent of plague, and *Salmonella* species or *Shigella flexneri*, responsible for intestinal diseases (9). It has been shown that the T3SS of *P. aeruginosa* is particularly active during acute infections and is associated with poor clinical outcomes (10). Indeed, the presence of active T3SS is correlated with an increased mortality (11).

The T3SS allows the bacterium to inject toxins directly into the host cell cytoplasm in order to hijack several cellular pathways, leading to disruption of the actin cytoskeleton and to cell death. This injection machinery consists of more than 20 proteins, assembled in a syringe-like structure and composed of three complexes: the basal body, the needle, and the translocon (12). In the cytoplasm, a proteinaceous ring makes the connection between the basal body and other soluble proteins. The basal body spans the two bacterial membranes and is made of three major proteins: PscD and PscJ in the inner membrane and the secretin PscC in the outer membrane. Structural studies on T3SS from different bacteria indicate that the outer membrane components oligomerize into rings of 12 or 15 subunits while the inner membrane proteins form 24-mer assemblies (13). The needle is anchored to the basal body and forms an 80-nm long narrow channel that protrudes from the bacterial surface. It is assembled by polymerization of the small protein PscF. Finally, the translocon, consisting of the tip protein PcrV and the pore proteins PopB and PopD, forms a pore at the extremity of the needle, into the eukaryotic membrane (14).

The presence of the secretin ring in the outer membrane is essential for the T3SS activity. Proteins belonging to the secretin family are found in several protein transport systems, such as the type II secretion system or the type IV pili (15). T3SS secretins assemble in oligomeric rings of 12 or 15 subunits, with a 5-nm-diameter pore, that were observed by electron microscopy (16–22). The T3SS secretin oligomers are very stable, are heat and SDS resistant, and have a high molecular mass of 985 kDa in *Yersinia enterocolitica* (17). The N-terminal domains of secretins display

Received 14 January 2015 **Accepted** 6 February 2015

Accepted manuscript posted online 17 February 2015

Citation Perdu C, Huber P, Bouillot S, Blocker A, Elsen S, Attrée I, Faudry E. 2015. ExsB is required for correct assembly of the *Pseudomonas aeruginosa* type III secretion apparatus in the bacterial membrane and full virulence *in vivo*. Infect Immun 83:1789–1798. doi:10.1128/IAI.00048-15.

Editor: A. J. Bäumler

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00048-15.

doi:10.1128/IAI.00048-15

low similarity and are proposed to have a role in specific interactions in each system. In contrast, the C-terminal domains are conserved and involved in the oligomerization and pore formation. However, a small nonconserved stretch at their C-terminal end was found, in some studies, to interact with small lipoproteins, named pilotins (23, 24). Indeed, the secretins often require other proteins, in particular pilotins, for a correct targeting and/or assembly in the outer membrane (25).

Pilotins are usually low-molecular-weight lipoproteins that were previously classified into three structural classes (25), before a fourth class was more recently characterized (26). The study of some pilotins from different secretion systems showed that they are involved in the stability, localization, and/or oligomerization of secretins. For instance, the well-studied pilotins OutS from the T2SS of *Dickeya dadantii* and PilW and PilF from the type IV pili of *Neisseria* sp. and *P. aeruginosa*, respectively, were shown to be required for the localization in the outer membrane and/or assembly and/or stability of the oligomers of OutD and PilQ secretins (27–30). Some T3SS pilotins were also characterized. In the case of *S. flexneri*, two lipoproteins, MxiM and MxiJ, participate in secretin oligomerization and stabilization (31–33). In *Salmonella enterica* serovar Typhimurium and *Y. enterocolitica*, deletions of the lipoprotein genes *invH* and *yscW*, respectively, lead to a decreased quantity of secretin oligomers InvG and YscC, respectively, and a mislocalization of the secretin to the inner membrane instead of the outer membrane (34, 35). Moreover, a phenotypic analysis of *Yersinia* and *Salmonella* strains lacking the YscW or InvH pilotin revealed a drastic decrease in protein secretion as well as in apoptosis of infected cells (36–40).

The ExsB protein from *Pseudomonas aeruginosa* displays 26% sequence identity with the YscW pilotin and is also predicted to be a lipoprotein with a low molecular mass of 15.6 kDa and a high isoelectric point of 9.4 (36, 41). It was thought for more than a decade that this protein was not produced in *P. aeruginosa* (42), suggesting an important evolutionary divergence between *P. aeruginosa* and *Yersinia* spp. (35). However, our previous work indicated that ExsB is expressed and located in the bacterial outer membrane, and its three-dimensional structure was determined. It folds in seven β-strands and one α-helix and exhibits two charged zones that could be involved in the interaction with lipids and other proteins (41). Nevertheless, this fold was functionally uninformative, especially since pilotins display very different ones (25, 26). Moreover, the role of ExsB in *P. aeruginosa* was not investigated previously, due to the position of the gene within the regulatory gene operon, which makes genetic manipulations difficult. Indeed, its gene is localized immediately downstream from *exsE*, encoding a component of the T3SS regulatory cascade, and upstream from the gene encoding ExsA, the main transcriptional activator of the system.

The aim of this study was to establish the role of ExsB in *P. aeruginosa* general toxicity and more specifically in the functioning of its T3SS. In this perspective, a comparative study of the phenotypes of a mutant strain lacking *exsB* gene, the corresponding complemented strain, and the wild-type strain was carried out. *In vivo* and *in vitro* analyses demonstrated the requirement of ExsB for *P. aeruginosa* complete virulence and for T3SS optimal activity. Moreover, the investigation of the molecular role of ExsB revealed its function in the secretin assembly.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial cultures were grown in Luria-Bertani (LB) broth at 37°C and 300 rpm. The antibiotics were added at the appropriate concentrations: 300 mg/liter for carbenicillin, 25 mg/liter for kanamycin, and 10 mg/liter for tetracycline. For *in vitro* T3SS induction in *P. aeruginosa* strains, the culture medium was depleted in calcium with 5 mM EGTA and 20 mM $MgCl_2$, and bacterial suspensions were used at an optical density at 600 nm (OD_{600}) of 1, which corresponds to the exponential growth phase.

The *exsB* deletion mutant, CHAΔ*exsB*, was constructed in several steps with splicing by overhang extension (SOE) PCR. Briefly, the two flanking regions of *exsB* were amplified from the pIA60 plasmid (Table 1) with the primer pairs Delta_exsB_1/Delta_exsB_2 (Table 2) and Delta_exsB_3/Delta_exsB_4, respectively. The two resulting DNA fragments were used together for another PCR step with the primers Delta_exsB_1 and Delta_exsB_4, leading to a DNA fragment containing only 4% of the *exsB* sequence (GTGAGGTGCTGGATGTGA). Then, this DNA fragment was cloned into the SmaI restriction sites of the pEX100T suicide plasmid containing the counterselectable *sacB* marker. The plasmid was introduced into *P. aeruginosa* strain CHA by triparental mating, using the conjugative properties of the helper plasmid pRK2013. Cointegration events were selected on *Pseudomonas* isolation agar (PIA) plates containing carbenicillin. Single colonies were then plated on PIA medium containing 5% sucrose to select for the loss of plasmid: the resulting strains were checked for carbenicillin sensitivity, and the gene deletion was confirmed by PCR.

The DNA fragments needed for introducing a TGA codon in the *exsB* sequence were generated by SOE PCR. A 295-bp fragment encompassing the upstream and 5′ regions of *exsB* was amplified using CHA genomic DNA and primers ExsB-TGA-H-F1 (EcoRI site at its 5′ end)/ExsB-TGA-H-R1. The ExsB-TGA-H-F2/ExsB-TGA-H-R2 (BamHI site at its 5′ end) primers were used to amplify the downstream 337-bp fragment of *exsB*. The overlapping primers generated a modification of the 12th codon, replacing TGC by a TGA stop codon, and converted the downstream GT CAGC sequence into a GTCGAC HindII site helpful for further mutant identification. The two fragments were used for a third PCR step using ExsB-TGA-H-F1/ExsB-TGA-H-R2 primers. The resulting 602-bp fragment was cloned into pCR-Blunt II-TOPO vector and sequenced. After cleavage with EcoRI-BamHI, the SOE PCR fragment was inserted into the suicide plasmid pEXG2 (containing *sacB*), giving pEX-ExsB TGA-H. The plasmid was introduced into *P. aeruginosa* strain CHA by triparental mating as described above, using gentamicin instead of carbenicillin. The mutation was first identified by the presence of the HindII site inserted contiguous to the TGA stop codon and then confirmed by sequencing of the CHA-B_{TGA} strain.

For the chromosomic complementation of the mutants, *exsB* gene with its own ribosome binding site (RBS) was amplified and inserted into the pIApC plasmid using the restriction sites XbaI and HindIII. The resulting DNA fragment containing *exsB* fused to *pC*, the promoter of the *exsCEBA* operon, was then extracted and cloned into the miniCTX integrative vector. Finally, the complemented strains CHAΔ*exsB*/*exsB* and CHA-B_{TGA}/*exsB* were obtained after transfer of the construct into the mutant strains, transformation with the pFLP2 plasmid to excise the miniCTX backbone, and selection on medium containing 5% sucrose to select for the loss of pFLP2.

Electrophoresis and Western blotting. T3SS-induced cultures of *P. aeruginosa* (1 ml) were centrifuged at 13,000 × *g* for 5 min to separate cells from supernatants. Cells were resuspended in 100 μl of 1× Laemmli loading buffer to measure the levels of intracellular PopB, PcrV, ExsB, and PscC, while 100 μl of supernatants was mixed with 25 μl of 5× Laemmli loading buffer to monitor secreted PopB and PcrV. ExsA, ExsD, and PscG, were detected in soluble extracts corresponding to 50× cell suspensions lysed by sonication and cleared from membrane and cellular debris by ultracentrifugation. Proteins were separated by SDS-PAGE, using 15% or 18% acrylamide gels, and transferred onto nitrocellulose membranes. For

TABLE 1 Plasmids used in this study

Name of plasmid	Construction and characteristics[a]	Source or reference
pIA60	Plasmid derived from pUC18, containing the *pcrGVH-popBD* and *exsCEBA* operons from strain CHA; Ap^r Cb^r	66
pCR-Blunt II-TOPO	PCR product cloning vector; *lacZ*α *ccdB*; Km^r	Invitrogen
pTOPO Δ*exsB*	pCR-Blunt II-TOPO containing the SOE PCR fragment Δ*exsB* amplified using Delta_exsB_1/4 primers; Km^r	This work
pEX100T	Allelic exchange vector; *sacB mobRK2*; Ap^r Cb^r	67
pEX100T Δ*exsB*	pEX100T containing the SOE PCR fragment Δ*exsB*; Ap^r Cb^r	This work
pRK2013	Tra^+ Mob^+ ColE1; Km^r	68
pTOPO-ExsB-TGA-H	pCR-Blunt II-TOPO containing the SOE PCR fragment *exsB*-TGA-H amplified using ExsB-TGA-H-F1/R2 primers; Km^r	This work
pEXG2	Allelic exchange vector, *colE1* origin, *oriT sacB*; Gm^r	69
pEXG2-ExsB-TGA-H	pEXG2 containing the SOE PCR fragment *exsB*-TGA-H; Gm^r	This work
pIA *pC-gfp*	pUCP20 derivative containing the transcriptional fusion of *gfp* with *pC*, the promoter of the *exsCEBA* operon; Ap^r Cb^r	66
pTOPO *RBS exsB*	pCR_Blunt II-TOPO containing the PCR fragment RBS *exsB* amplified with Xba_RBS_ExsB/ExsB_Hind; Km^r	This work
pIApC *RBS exsB*	pIApC containing the PCR fragment RBS *exsB*; Ap^r Cb^r	This work
miniCTX	Integrative vector; Ω-FRT-attP-MCS *ori int oriT*; Tcr	70
miniCTX *pC RBS exsB*	miniCTX containing the transcriptional fusion *pC-exsB*; Tc^r	This work
pFLP2	Plasmid expressing the Flp recombinase; Ap^r Cb^r	71
$pExoS_{GAP}Bla$	pUC20 derivative containing the translational fusion $exoS_{GAP}$-*bla*; Ap^r Cb^r	48
pDD2	pUCP20 derivative constitutively overexpressing *exsA*; Ap^r Cb^r	51

[a] Ap^r/Cb^r, resistance to ampicillin for *E. coli* and carbenicillin for *P. aeruginosa*; Km^r, resistance to kanamycin; Tc^r, resistance to tetracycline; Gm^r, resistance to gentamicin.

the analysis of secretin oligomers, 8% acrylamide gels were used. The membranes were saturated in 5% nonfat milk and incubated with primary antibodies raised against PopB (43), ExsB (41), PcrV (44), PscC (our laboratory), ExsA and ExsD (45), PscG (46), XcpY (kind gift of R. Voulhoux), RpoA (from NeoClone), and OprI-F (given by G. E. Gilleland). Secondary horseradish peroxidase-conjugated antibodies were then added, followed by chemiluminescence detection (Luminata Classico, Millipore).

Mouse infection assays. For each *P. aeruginosa* strain, 10 BALB/c mice (males weighing 20 g; from Harlan breeding) were infected by inhalation of 2×10^6 bacteria in order to induce pneumopathy. The survival was monitored every 2 h. For the statistical analysis, Kaplan-Meier curves were established and the log rank test was applied. All protocols in this study were conducted in strict accordance with the French guidelines for the care and use of laboratory animals. The protocol for mouse infection was approved by the animal research committee of the institute (project license number CETEA No 12-021).

Cell retraction assay. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord and cultured at 37°C, 5% CO_2 in endothelial basal medium 2 (EBM2) containing 2% fetal bovine serum. The infection was set up at a multiplicity of infection (MOI) of 1 by *P. aeruginosa* strains at an OD_{600} of 1. As formerly described (47), cells were fixed with cold methanol at 3 h postinfection. They were then labeled with a β-actin antibody and with a fluorescent secondary antibody. Under these conditions, the primary antibody detects free actin along with actin fibers, thus providing a uniform cell staining. For each condition, the field surface covered by the cells on three distinct pictures was measured with ImageJ software. The percentage of cellular retraction was calculated by normalizing the infected conditions to the noninfected conditions. One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was performed to compare the mean percentages of cellular retraction.

Injection of chimeric toxin. The injection of the chimeric toxin ExoS-β-lactamase was assayed as previously described (48). BJAB cells were grown at 37°C, 5% CO_2 in RPMI medium containing 10% fetal bovine serum. After 3 h of infection by *P. aeruginosa* strains producing the chimeric toxin, BJAB cells were incubated with 1 mM CCF2/AM (Invitrogen) during 30 min, and their fluorescence was then analyzed by flow cytometry (fluorescence-activated cell sorter [FACS] MoFlo). The intact CCF2 probe emits a green fluorescence, but its cleavage by the β-lactamase domain of the chimeric toxin leads to the formation of a product emitting a blue fluorescence. Dead cells were removed based on forward

TABLE 2 Oligonucleotides used in this study

Name	Sequence[a]	Use
Delta_exsB_1	5′-CCCGGGGATGTGCTGGGCGAGGGG	SOE PCR to delete the gene coding for ExsB
Delta_exsB_2	5′-GGCAATGTATCACATCCAGCACCTCACCTGCG	
Delta_exsB_3	5′-GTGCTGGATGTGATACATTGCCTGCTGTTTCGGA	
Delta_exsB_4	5′-CCCGGGCCGAAGCGCTGGACGAAGC	
ExsB-TGA-H-F1	5′-CCGAATTCGCCGGTGCAGCCGTCCC	SOE PCR to insert a stop codon within the *exsB* gene
ExsB-TGA-H-R1	5′-GGCGCGGGCTGGTCGACTCAACCGCCGAGCAGCAACGC	
ExsB-TGA-H-F2	5′-GCTCGGCGGTTGAGTCGACCAGCCCGCGCCGATGTCG	
ExsB-TGA-H-R2	5′-CCGGATCCGAACCAGCCGGACCCGCTCA	
Xba_RBS_ExsB	5′-TCTAGAACGCCGCTGGAGGC	PCR amplification of RBS *exsB* for complementation studies
ExsB_Hind	5′-AAGCTTTCAATCGTTGCCAGATCTTTC	

[a] Restriction sites are underlined.

scatter/side scatter (FSC/SSC) parameters, and the blue fluorescence of the remaining cells was measured. A gate in the histogram plot allows the definition of the highly blue fluorescent population corresponding to the injected cells.

Quantification of T3SS needles on bacterial surface. After induction of the T3SS, 500 μl of the *P. aeruginosa* suspension was collected and centrifuged at 13,000 × *g* for 5 min at 4°C. Bacteria were concentrated 10 times in PBS. Three microliters of this suspension was dropped onto glow discharge, copper 200 mesh, carbon- and Formvar-coated grids. After 3 washes with filtered water, a negative staining was accomplished by incubation with 1% uranyl acetate during 1 min. The samples were observed by electron microscopy, with an FEI Tecnai 12 fitted with an FEI Eagle 4k by 4k charge-coupled-device (CCD) camera at a magnification of ×23,000 using the FEI Tecnai Imaging Analysis (TIA) software. The T3S needles were identified by their size, with a diameter of about 6.5 nm and a length of 70 nm. The number of needles present at the surface of the bacteria and that of detached needles were determined on 60 pictures for both the *exsB* mutant and the complemented strains.

Inner and outer membrane separation. Membrane separation was achieved on the three *P. aeruginosa* strains overexpressing the T3SS activator ExsA. A sucrose gradient was used to separate the membranes according to their density, as previously described (49). The main steps of this procedure are recapitulated here. First, total membranes from 500 ml of culture were prepared in 500 μl of a buffer containing 8 mM Tris–HCl at pH 7.4, 20% sucrose (wt/vol), 4 mM EDTA, and protease inhibitor cocktail complete (Roche). They were then loaded on a 35 to 55% sucrose gradient and subjected to 72 h of ultracentrifugation at 190,000 × *g*. Finally, 15 fractions of 750 μl were collected and analyzed by SDS-PAGE and Western blotting. For illustration, representative fractions of the inner and outer membranes were also analyzed by SDS-PAGE and Western blotting. NADH oxidase activity, a marker of inner membranes, was measured as previously described (50).

RESULTS

Deletion of *exsB* reduces *P. aeruginosa* virulence during mouse infection. The highly virulent strain CHA featuring an active T3SS (51) was used throughout this work. In this genetic background, both a strain lacking *exsB* gene (Δ*exsB*) and the complemented mutant harboring a chromosomally carried copy of the wild-type *exsB* gene (Δ*exsB*/*exsB*) were constructed in order to study the role of ExsB in *P. aeruginosa*. This mutation and its complementation, as well as genetic manipulation described further in this work, did not alter the growth rates of the strains (data not shown).

In order to evaluate the involvement of ExsB in *P. aeruginosa* virulence *in vivo*, mice were infected by inhalation of the different bacterial strains to induce acute pneumonia, and animal survival was monitored during 36 h (Fig. 1). The mean survival times for animals infected by the wild-type and the complemented strains are 17.1 and 19.4 h, respectively, while the survival time increases to 26.9 h for mice infected by the Δ*exsB* mutant strain. This difference is statistically significant (*P* value, <0.01 with the log rank test between wild-type and Δ*exsB* strains). Therefore, this result indicates that ExsB may be involved in *P. aeruginosa* virulence during acute pneumonia infection.

ExsB is required for T3SS optimal activity toward eukaryotic cells. To assess the role of ExsB in T3SS activity toward eukaryotic cells, a cytotoxicity assay on human primary endothelial cells (HUVEC) was employed. This assay relies on the disruption of the actin cytoskeleton by T3SS toxins, leading to a progressive cellular retraction that is directly correlated to ExoS/ExoT effects on cofilin activation (47). The Δ*exsB* strain displayed 40% lower cytotoxicity in this assay than that displayed by the parental and complemented strains (Fig. 2A) ($P < 0.05$).

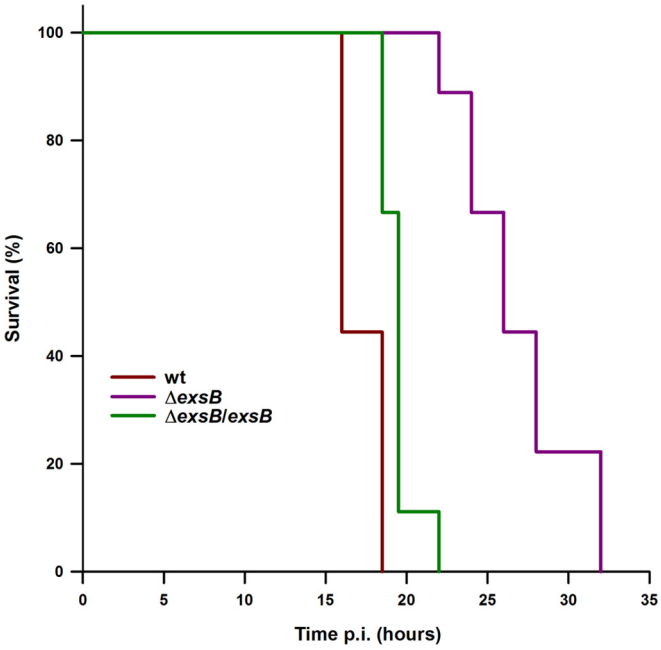


FIG 1 *exsB* deletion reduces *P. aeruginosa* virulence in a mouse pneumonia model. Survival curves of mice infected by wild-type (wt), mutant lacking *exsB* (Δ*exsB*), and complemented (Δ*exsB*/*exsB*) strains of *P. aeruginosa*. Mice were infected with 2 × 10^6 bacteria, and survival was monitored every 2 h. p.i., postinfection.

Because the decrease in cytotoxicity observed in the absence of *exsB* suggests a decrease in the amount of effectors translocated into the host cell cytoplasm, the injection of a chimeric toxin, ExoS-β-lactamase, was assessed in the presence or absence of ExsB. In this assay, cells are loaded with a fluorescent reporter molecule (CCF2) emitting a green fluorescence when no chimeric toxin is injected. Upon injection, cleavage of the fluorescent probe by the β-lactamase results in blue fluorescence (48). Analysis of cell fluorescence showed a decrease in the percentage of blue-fluorescent cells upon infection by the mutant strain lacking *exsB*, in comparison with the wild-type and the complemented strains (Fig. 2B), showing the requirement of ExsB for optimal toxin injection. This reduced toxin injection in the absence of ExsB was not due to an uncontrolled secretion and a leakage in the extracellular medium, as no β-lactamase activity could be detected in the extracellular medium (data not shown), indicating that ExoS-Bla is not secreted in the extracellular medium in the absence of ExsB.

The absence of *exsB* expression causes a decrease in *in vitro* secretion of T3SS proteins and affects the regulatory gene operon. Taken together, the previous experiments revealed that *exsB* gene deletion leads to a decrease in T3SS activity toward eukaryotic hosts. These observations could be explained by a decrease in the secretion activity. Indeed, the secretion of two chimeric exotoxin-β-lactamase proteins, ExoS-Bla and ExoY-Bla, was reduced in the Δ*exsB* mutant (see Fig. S1 in the supplemental material). Furthermore, T3SS secretion was assessed *in vitro* by analyzing the expression and secretion of two T3SS substrates, PopB and PcrV, by immunoblotting. Clearly, the *exsB* deletion almost abolished T3SS secretion as the two proteins were barely detected in the supernatants but accumulated inside the bacterial cytoplasm (Fig. 3A). However, complementation of the mutant

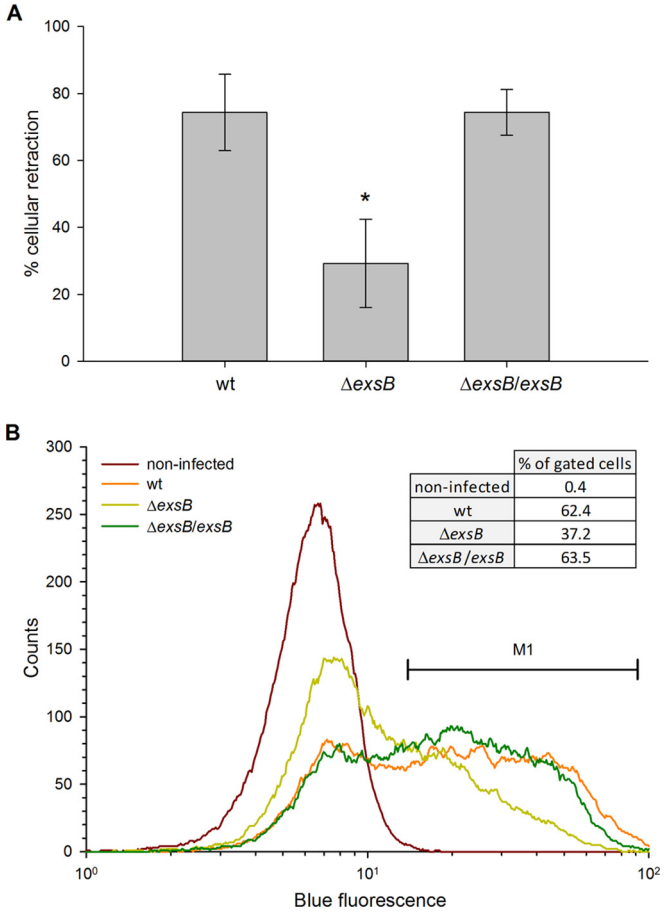


FIG 2 *exsB* deletion reduces T3SS activity toward eukaryotic cells. (A) Cytotoxicity assay on HUVEC. The percentage of cellular retraction was measured after 3 h of infection by the wild-type (wt), mutant (Δ*exsB*), and complemented (Δ*exsB/exsB*) strains. One-way ANOVA was performed (overall *P* value, 0.003). The star indicates a statistically significant difference of cytotoxicity caused by Δ*exsB* compared to wt and Δ*exsB/exsB* strains (Tukey's *post hoc* test, $P < 0.01$). (B) Injection of the chimeric toxin ExoS-β-lactamase into the BJAB cells. After 3 h of infection by wild-type (wt), mutant (Δ*exsB*), and complemented (Δ*exsB/exsB*) strains, the injection of ExoS-β-lactamase was examined by FACS analysis. The blue fluorescence of the cells reveals the cleavage of the CCF2 fluorescent probe by the injected chimeric toxins. Dead cells were removed based on forward scatter/side scatter (FSC/SSC) parameters. The table in the inset indicates the percentage of blue cells selected in the M1 gate.

did not restore a wild-type phenotype, as the Δ*exsB/exsB* complemented strain exhibited higher secretion than the wild-type strain. This indicates that *exsB* deletion likely has a polar effect on the surrounding genes of the regulatory gene operon, probably on the downstream gene *exsA* encoding the key transcriptional activator of the T3SS genes (Fig. 3B).

To explore this possibility, the effect of *exsB* deletion on *exsA* expression was assessed by monitoring ExsA levels in the bacterial extracts by Western blotting. An overproduction of this protein was observed both in the mutant and complemented strains (Fig. 3C, compare ΔB and ΔB/B to the wild type [wt]). This means that the deletion of 96% of the *exsB* gene affects ExsA production, probably by influencing mRNA stability or mRNA recognition by regulatory elements. This increase in the ExsA level could explain the observed increase of PopB and PcrV secretion in the complemented strain.

In order to reduce the impact of *exsB* inactivation on *exsCEBA* mRNA, we constructed a new mutant in which the 12th codon of *exsB* was replaced by the TGA stop codon (Fig. 3B). However, this also provoked a polar effect on ExsA expression, with a smaller amount of ExsA in the stop codon mutant (B_{TGA}) and the complemented strain (B_{TGA}/B) than in the wild-type strain (Fig. 3C). This reduction of ExsA levels correlated with a lower expression and secretion level of PopB and PcrV (data not shown). This ExsA decrease is also responsible for the dramatic low level of ExsB in the B_{TGA}/B strain. Although it is not visible in the presented immunoblot, intended to compare ExsB levels in all the strains, ExsB could be detected in the complemented strain (B_{TGA}/B) upon prolonged exposure (not shown).

We considered that the differences in the ExsA expression could mask subtle phenotypic differences between the wild-type and mutant strains. For this reason, strains were transformed with the pDD2 plasmid, which moderately overproduces ExsA (51), in order to restore similar levels of expression of this T3SS activator in all the strains (Fig. 3C, right). This allowed investigation of the effect of ExsB absence *per se*. Under these conditions, the secretion of T3SS substrates was strikingly reduced in the strain lacking *exsB* in comparison to both the wild-type and Δ*exsB/exsB* strains, and the substrate accumulation inside the bacteria was confirmed (Fig. 3D). This was also observed in the B_{TGA} mutant when the ExsA level was restored with the pDD2 plasmid (see Fig. S2 in the supplemental material). In conclusion, the absence of ExsB did not lead to a defective biosynthesis of secreted proteins but to a decrease of *in vitro* secretion.

The number of T3SS needles decreases in the absence of ExsB. The cytoplasmic accumulation of T3SS substrates could be explained by an incorrect assembly of the export apparatus, and defects in T3SS needle assembly are known to preclude the secretion of translocators and exotoxins (52). Quantification of the needle subunit PscF by Western blotting showed that the absence of ExsB did not lead to a decrease of PscF levels (data not shown). Thus, we investigated by direct observation whether the absence of ExsB caused a defect in needle assembly by counting the number of apparatus found on the bacterial surface. Transmission electron microscopy (TEM) images of individual bacteria from the Δ*exsB* and Δ*exsB/exsB* strains were taken, and T3SS needles were quantified (Fig. 4A). In this experiment, the mutant strain was compared to the complemented strain rather than to the wild-type strain, to avoid artifacts due to the regulatory effect of the Δ*exsB* mutation. Analysis of 60 images for each strain revealed that in the absence of ExsB, a significant decrease in the number of T3SS needles was observed, both at the bacterial surface and in the extracellular medium (Fig. 4B and C). It is known that manipulation of concentrated bacterial suspensions results in the detachment of surface-exposed PscF needles (52). In conclusion, the diminution of T3SS needles in the *exsB* mutant shows that ExsB participates in the T3SS assembly.

ExsB promotes outer membrane targeting of the secretin PscC. If ExsB is a pilotin for the PscC secretin, it should participate in the stability and/or localization of the secretin. Analysis of PscC levels by immunoblotting revealed a clear decrease in strains lacking ExsB (Fig. 5). To rule out any regulatory effect of ExsB depletion, we examined the levels of ExsD and PscG, encoded by the same operon upstream and downstream from *pscC*, respectively.

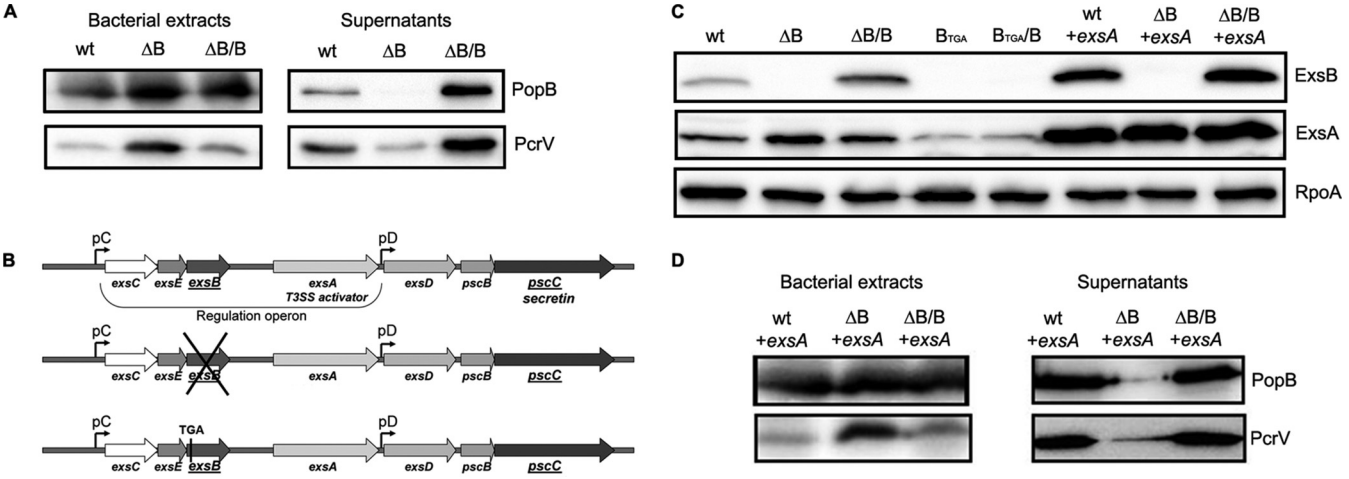


FIG 3 T3SS activity *in vitro* is impaired in the absence of ExsB, and *exsB* deletion has a polar effect on the T3SS regulatory gene operon *exsCEBA*. (A) Two T3SS proteins, PopB and PcrV, were detected by Western blotting in bacterial extracts and in the corresponding supernatants. The strains assessed are the wild-type strain (wt), the mutant strain lacking *exsB* (Δ*B*), and the corresponding complemented strain (Δ*B/B*). (B) Genetic organization of T3SS regulatory operon and downstream genes in the wild-type strain (top), in the Δ*exsB* mutant strain (middle), and in the stop codon *exsB* mutant (bottom). (C) Detection of ExsB and T3SS transcriptional activator ExsA by Western blotting in various strains: wild type; mutant lacking *exsB* and the corresponding complemented strain; stop codon mutant (B_{TGA}) and the corresponding complemented strain (B_{TGA}/B); wild-type, mutant lacking *exsB*, and complemented strains overexpressing *exsA* (wt+*exsA*, ΔB+*exsA*, ΔB/B+*exsA*). RpoA is the α-subunit of the RNA polymerase that is used as a loading control. (D) Two T3SS proteins, PopB and PcrV, were detected by Western blotting in bacterial extracts and supernatants. The strains assessed are the wild-type, mutant lacking *exsB*, and complemented strains. All these strains overproduce the T3SS activator ExsA to the same level.

Again, the polar effect on ExsA expression (see Fig. 3C) altered the expression of ExsD and PscG. However, a striking decrease of PscC was observed in strains lacking ExsB, in a pattern clearly different from those of ExsD and PscG, in particular with the use of additional copies of *exsA* (Fig. 5, +*exsA* strains).

In the absence of their pilotins, secretins are commonly found to be degraded or not correctly targeted/inserted in the outer membrane (25). The stability of the secretin was assessed by quantifying PscC by Western blotting in extracts prepared from bacteria incubated in the presence of chloramphenicol for up to 3 h, to prevent protein neosynthesis. Despite a reduced amount of PscC at time point zero (t_0) in the absence of ExsB, the stability of PscC followed similar kinetics in the wild-type, *exsB*-deleted, and complemented strains, reaching a 40% degradation after 3 h (data not shown). To further investigate the pilotin role of ExsB, the localization of the secretin was studied by analyzing the protein content of the inner and outer membranes separated on a sucrose gradient according to their density (Fig. 6).

The amount of the PscC secretin in the outer membrane of the mutant strain was drastically reduced in comparison to that in the wild-type and complemented strains. Moreover, in the absence of ExsB, the secretin partially accumulated in the inner membrane. These results show that ExsB participates in the assembly/targeting of PscC into the outer membrane, thereby characterizing ExsB as a pilotin for the secretin PscC.

DISCUSSION

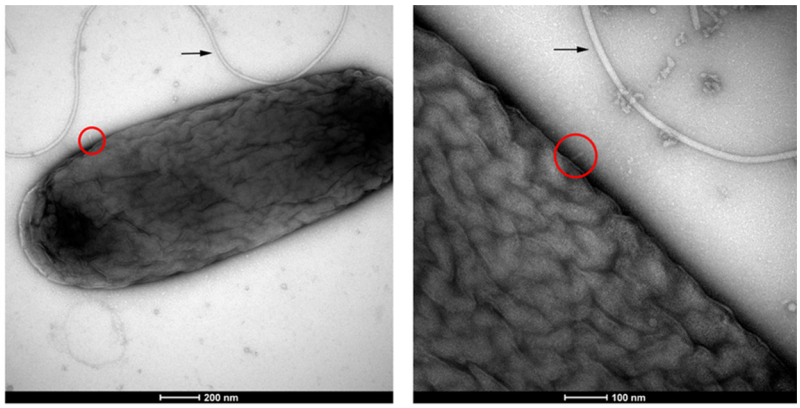
The first attempts to detect ExsB in *P. aeruginosa* failed, and this protein was then thought not to be expressed (42). More recently, the use of antibodies raised against this protein allowed the demonstration of its presence in *P. aeruginosa* outer membrane fractions, and its crystallographic structure was solved (41). However, its role remained to be determined.

Our *in vivo* data in the mouse model of acute pneumonia demonstrated that ExsB has an important role in *P. aeruginosa* virulence, as a significant delay in animal mortality was observed in the absence of ExsB. Furthermore, the characterization of T3SS-dependent toxicity in cellular models showed a decrease in the secretion of T3SS substrates in the Δ*exsB* strain and a decrease in the injection of toxins inside host cell cytoplasm. Taken together, we demonstrate that ExsB is an important determinant of T3SS function.

The examination of PscC's status in the Δ*exsB* strain indicated that the diminution of T3SS activity following *exsB* deletion is related to an ExsB "pilotin" role toward the secretin PscC. Indeed, membrane separation on a sucrose gradient showed that the amount of secretin in the outer membrane is considerably reduced in the absence of ExsB. Moreover, the production of proteins from the *pscC*-containing operon was similar in all strains, indicating that the reduction of PscC level was not due to a defective genetic regulation (see ExsD and PscG in Fig. 5). In conclusion, ExsB is required for the optimal targeting and/or assembly of PscC, in agreement with the activities of previously characterized T3SS pilotins from *Y. enterocolitica*, *S. flexneri*, and *S.* Typhimurium (23, 31, 32, 34, 35, 53). This clarification of the role of ExsB is important because the failure in detecting this protein (42) led to the hypothesis that *P. aeruginosa* could have evolved a different pilotin or a secretin that does not require a pilotin (35).

ExsB is an outer membrane lipoprotein displaying a lipobox allowing the covalent addition of the lipid moiety that permits the attachment to the bacterial membranes (41). In Gram-negative bacteria, the Lol system is responsible for lipoprotein sorting to the outer membrane (54). By extension, this system could be involved in driving ExsB, in complex with the PscC secretin, to the outer membrane, as it was already suggested for the pilotin from *S. flexneri* (32, 33). This hypothesis is supported by the fact that PscC levels are considerably affected by LolB depletion (55). If ExsB

A



B

Strain \ Number of needles	Needles present on the bacterial surface	Detached needles	Total needles
Δ*exsB*	2	3	5
Δ*exsB*/*exsB*	11	24	35

C

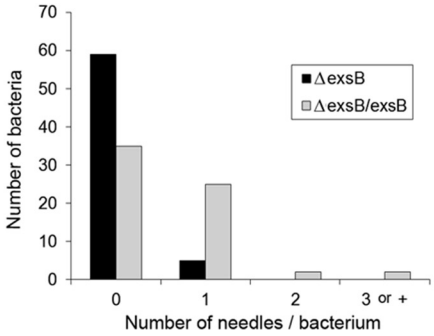


FIG 4 Involvement of ExsB in T3SS needle assembly. (A) Representative images of *P. aeruginosa* bacteria exhibiting a T3SS needle observed by transmission electronic microcopy (TEM) with negative staining. The needles are indicated by a red circle, and the flagellum is shown by a black arrow. (B) Number of T3SS needles quantified for the mutant strain lacking *exsB* (Δ*exsB*) and the complemented strain (Δ*exsB*/*exsB*). (C) Number of bacteria possessing 0, 1, 2, or >3 T3SS needles both at their surface and in their close extracellular environment.

worked as a shuttle for PscC, in concert with the Lol system, then the interaction between ExsB and the PscC would be transient. In agreement with this hypothesis, we were not able to detect any interaction between ExsB and PscC despite several attempts using far-Western overlay, pulldown, and immunoprecipitation, with or without prior cross-linking, followed by immunoblot or mass spectrometry analysis. Similar difficulties in detecting the interaction between other pilotin and secretin proteins were also re-

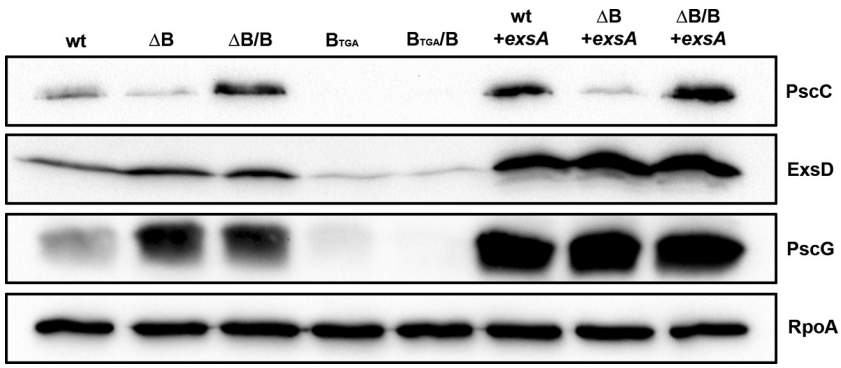


FIG 5 *exsB* deletion induces a reduction of the T3SS secretin PscC levels. PscC was detected by immunoblotting in the following: wild type (wt); mutant lacking *exsB* (ΔB) and the corresponding complemented strain (ΔB/B); stop codon mutant (B_{TGA}) and the corresponding complemented strain (B_{TGA}/B); wild-type, mutant lacking *exsB*, and complemented strains overexpressing *exsA* (wt+*exsA*, ΔB+*exsA*, ΔB/B+*exsA*). ExsD and PscG proteins are encoded by genes located in the same operon as *pscC* and are used as expression controls. RpoA is the α-subunit of the RNA polymerase that is used as a loading control.

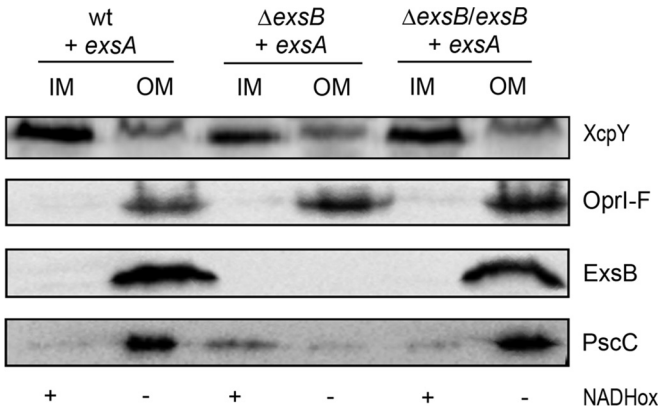


FIG 6 *exsB* deletion leads to a reduction of PscC targeting the outer membrane. The inner membrane (IM) and outer membrane (OM) of wild-type (wt), mutant lacking *exsB* (Δ*exsB*), and complemented (Δ*exsB*/*exsB*) strains overexpressing the activator gene *exsA* were separated according to their density on a sucrose gradient. The separated fractions were analyzed by immunoblotting. Marker proteins identify inner and outer membrane fractions. The XcpY protein is an inner membrane component of the T2SS, and the OprI-F porins are outer membrane proteins. NADH oxidase activity (NADHox) is a marker of inner membranes.

ported in T4SS (56), for example, and other T3SSs. Indeed, the use of a cross-linker was essential to identify this interaction in *Shigella* and *Salmonella* species (23, 32, 33, 53), and in *Yersinia* spp., the two proteins did not copurify (17). However, the authors of the last study showed, by using *Yersinia* strains lacking all the other T3SS genes, that the pilotin directly acts on the secretin (17).

Intriguingly, in the absence of ExsB a small amount of secretin oligomers is still found in the outer membrane of *P. aeruginosa* and the T3SS is still partially active, as shown by secretion and infection assays. This suggests that another T3SS protein could participate in PscC assembly. Indeed, in *S. flexneri* T3SS, *mxiM* and *mxiJ* deletions have similar effects on the secretin MxiD (31). MxiJ and its homolog in *P. aeruginosa* PscJ are lipoproteins attached to the periplasmic side of the inner membrane and forming a ring-like structure facing the secretin. From this position, it is possible that PscJ works in concert with ExsB to promote complete assembly and targeting of PscC secretin. To confirm this hypothesis of two-player pilotins, future studies should analyze T3SS activity and the PscC status of *P. aeruginosa* mutants still to be generated: a strain lacking *pscJ* and a double mutant, Δ*exsB* Δ*pscJ*. Indeed, like in *Yersinia*, PscJ is an essential component of the T3SS (57, 58), but its role in the secretin assembly has never been investigated.

The C-terminal domain of the secretin is predicted to harbor the interaction site for the pilotin, as for the T2SS of *D. dadantii* and *Klebsiella oxytoca* or for the T3SS of *S. flexneri* (23, 24, 29, 32, 59), and the folding of this domain could also be responsible for the specificity of the secretin-pilotin interaction (60). However, in the case of *Yersinia*, the pilotin can act on the secretin in the absence of this domain (35). Based on the methodology of the present work, mutations of the C-terminal domain of PscC and on ExsB should be performed in order to gain insights into the secretin-pilotin interactions based on the variety of pilotin structures (32, 41, 60, 61). In parallel, obtaining the recombinant PscC secretin would allow new attempts to decipher these interactions.

Deletion of *exsB*, but also the introduction of a stop codon point mutation in the *exsB* sequence, resulted in a modification of the expression level of the T3SS activator ExsA, in both mutant and complemented strains. This polar effect is probably due to a modification of endonuclease recognition of the *exsCEBA* sequence that affects the stability of the entire *exsCEBA* operon mRNA. Alternatively, yet-uncharacterized regulatory features in this operon can be envisaged. Notably, a mechanism of posttranscriptional regulation of this operon was recently established, targeting the *exsA* gene (62). The operon region upstream of *exsA* appears thus to be extremely important for regulation and sensitive to sequence modifications.

In preliminary cell infection experiments on J774 mouse macrophages, the phenotypic difference between the wild-type strain and the isogenic mutant lacking *exsB* was tiny, probably because ExsA overproduction masked the T3SS defect induced by *exsB* deletion. As a confirmation, the differences in phenotypes were clearly enhanced by using a plasmid restoring similar levels of ExsA in all the strains (see Fig. S3 in the supplemental material). Interestingly, the use of this plasmid was not necessary to observe a strong decrease in cytotoxicity toward endothelial cells in the absence of ExsB (Fig. 2A). However, after introduction of the plasmid into the strains, the defective phenotype of the deletion mutant was confirmed with a stronger statistical significance (see Fig. S4 in the supplemental material). This difference between infection assays is likely due to the very high sensitivity of macrophages to T3SS-induced membrane permeabilization. In this assay, the low residual T3SS activity in the *exsB* mutant could induce large membrane damage depending on the multiplicity of infection (MOI) used. Indeed, the presence of the *exsA* overexpression plasmid was necessary to observe a significant effect of the *exsB* deletion at an MOI of 1, but this effect was masked at an MOI of 10 while it was observed at an MOI of 0.2 even in the absence of the plasmid (data not shown). These results confirm the previous suggestion that an excessive ratio of bacteria can mask differences in toxic activities between strains (63).

This work allowed the characterization of the role of *P. aeruginosa* ExsB toward the secretin PscC in an intact T3SS context, while previous studies on the *Yersinia* and *Shigella* orthologs were performed in a virulence plasmid-cured background, i.e., in bacteria devoid of other T3SS components (31, 35). ExsB appeared to be critical for the correct targeting and assembly of PscC, which is in turn the crucial initial docking platform of the whole T3SS machinery (64, 65). Thus, the localization of the gene encoding ExsB in the regulatory gene operon may reflect its involvement not in the genetic regulation but in the regulation of the T3SS assembly.

ACKNOWLEDGMENTS

We thank François Cretin for help with the CCF2 technique, Véronique Collin-Faure (LCBM, CEA Grenoble) for flow cytometry analysis, Michel Ragno, Laura Pascalon, and Alexandra Crété for technical help, and Viviana Job and Andrea Dessen (Bacterial Pathogenesis Group, Grenoble Structural Biology Institute) for helpful discussions.

C.P. received a fellowship from the IRTELIS Ph.D. program from CEA.

REFERENCES

1. **Green SK, Schroth MN, Cho JJ, Kominos SK, Vitanza-jack VB.** 1974. Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. Appl Microbiol **28:**987–991.
2. **Remold SK, Brown CK, Farris JE, Hundley TC, Perpich JA, Purdy ME.**

2011. Differential habitat use and niche partitioning by *Pseudomonas* species in human homes. Microb Ecol **62:**505–517. http://dx.doi.org/10.1007/s00248-011-9844-5.

3. **Young VM.** 1977. *Pseudomonas aeruginosa*: ecological aspects and patient colonization. Books on Demand, Stoughton, WI.
4. **Fotedar R, Nayar E, Samantray JC, Shriniwas Banerjee U, Dogra V, Kumar A.** 1989. Cockroaches as vectors of pathogenic bacteria. J Commun Dis **21:**318–322.
5. **Thiolet JM, Lacave L, Jarno P, Metzger MH, Tronel H, Gautier C, L'heriteau F, Coignard B.** 2007. Prevalence des infections nosocomiales, France, 2006. Bull Epidemiol Hebd **51-52:**429–432.
6. **Govan JR, Deretic V.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol Rev **60:**539–574.
7. **Strateva T, Yordanov D.** 2009. *Pseudomonas aeruginosa*-a phenomenon of bacterial resistance. J Med Microbiol **58:**1133–1148. http://dx.doi.org/10.1099/jmm.0.009142-0.
8. **de Bentzmann S, Plesiat P.** 2011. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. Environ Microbiol **13:**1655–1665. http://dx.doi.org/10.1111/j.1462-2920.2011.02469.x.
9. **Coburn B, Sekirov I, Finlay BB.** 2007. Type III secretion systems and disease. Clin Microbiol Rev **20:**535–549. http://dx.doi.org/10.1128/CMR.00013-07.
10. **Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, Rello J.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit Care Med **30:**521–528. http://dx.doi.org/10.1097/00003246-200203000-00005.
11. **Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, Sawa T, Frank DW, Wiener-Kronish JP.** 2001. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J Infect Dis **183:**1767–1774. http://dx.doi.org/10.1086/320737.
12. **Blocker A, Jouihri N, Larquet E, Gounon P, Ebel F, Parsot C, Sansonetti P, Allaoui A.** 2001. Structure and composition of the *Shigella flexneri* "needle complex," a part of its type III secreton. Mol Microbiol **39:**652–663. http://dx.doi.org/10.1046/j.1365-2958.2001.02200.x.
13. **Chatterjee S, Chaudhury S, McShan AC, Kaur K, De Guzman RN.** 2013. Structure and biophysics of type III secretion in bacteria. Biochemistry **52:**2508–2517. http://dx.doi.org/10.1021/bi400160a.
14. **Mattei PJ, Faudry E, Job V, Izore T, Attree I, Dessen A.** 2011. Membrane targeting and pore formation by the type III secretion system translocon. FEBS J **278:**414–426. http://dx.doi.org/10.1111/j.1742-4658.2010.07974.x.
15. **Korotkov KV, Gonen T, Hol WG.** 2011. Secretins: dynamic channels for protein transport across membranes. Trends Biochem Sci **36:**433–443. http://dx.doi.org/10.1016/j.tibs.2011.04.002.
16. **Bergeron JR, Worrall LJ, Sgourakis NG, DiMaio F, Pfuetzner RA, Felise HB, Vuckovic M, Yu AC, Miller SI, Baker D, Strynadka NC.** 2013. A refined model of the prototypical *Salmonella* SPI-1 T3SS basal body reveals the molecular basis for its assembly. PLoS Pathog **9:**e1003307. http://dx.doi.org/10.1371/journal.ppat.1003307.
17. **Burghout P, van Boxtel R, Van Gelder P, Ringler P, Muller SA, Tommassen J, Koster M.** 2004. Structure and electrophysiological properties of the YscC secretin from the type III secretion system of *Yersinia enterocolitica*. J Bacteriol **186:**4645–4654. http://dx.doi.org/10.1128/JB.186.14.4645-4654.2004.
18. **Heinz DW.** 2013. Secrets of a secretin. Structure **21:**2098–2099. http://dx.doi.org/10.1016/j.str.2013.11.002.
19. **Hodgkinson JL, Horsley A, Stabat D, Simon M, Johnson S, da Fonseca PC, Morris EP, Wall JS, Lea SM, Blocker AJ.** 2009. Three-dimensional reconstruction of the *Shigella* T3SS transmembrane regions reveals 12-fold symmetry and novel features throughout. Nat Struct Mol Biol **16:**477–485. http://dx.doi.org/10.1038/nsmb.1599.
20. **Koster M, Bitter W, de Cock H, Allaoui A, Cornelis GR, Tommassen J.** 1997. The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. Mol Microbiol **26:**789–797. http://dx.doi.org/10.1046/j.1365-2958.1997.6141981.x.
21. **Kowal J, Chami M, Ringler P, Muller SA, Kudryashev M, Castano-Diez D, Amstutz M, Cornelis GR, Stahlberg H, Engel A.** 2013. Structure of the dodecameric *Yersinia enterocolitica* secretin YscC and its trypsin-resistant core. Structure **21:**2152–2161. http://dx.doi.org/10.1016/j.str.2013.09.012.
22. **Schraidt O, Marlovits TC.** 2011. Three-dimensional model of Salmonella's needle complex at subnanometer resolution. Science **331:**1192–1195. http://dx.doi.org/10.1126/science.1199358.
23. **Lario PI, Pfuetzner RA, Frey EA, Creagh L, Haynes C, Maurelli AT, Strynadka NC.** 2005. Structure and biochemical analysis of a secretin pilot protein. EMBO J **24:**1111–1121. http://dx.doi.org/10.1038/sj.emboj.7600610.
24. **Gu S, Rehman S, Wang X, Shevchik VE, Pickersgill RW.** 2012. Structural and functional insights into the pilotin-secretin complex of the type II secretion system. PLoS Pathog **8:**e1002531. http://dx.doi.org/10.1371/journal.ppat.1002531.
25. **Koo J, Burrows LL, Howell PL.** 2012. Decoding the roles of pilotins and accessory proteins in secretin escort services. FEMS Microbiol Lett **328:**1–12. http://dx.doi.org/10.1111/j.1574-6968.2011.02464.x.
26. **Dunstan RA, Heinz E, Wijeyewickrema LC, Pike RN, Purcell AW, Evans TJ, Praszkier J, Robins-Browne RM, Strugnell RA, Korotkov KV, Lithgow T.** 2013. Assembly of the type II secretion system such as found in *Vibrio cholerae* depends on the novel pilotin AspS. PLoS Pathog **9:**e1003117. http://dx.doi.org/10.1371/journal.ppat.1003117.
27. **Carbonnelle E, Helaine S, Prouvensier L, Nassif X, Pelicic V.** 2005. Type IV pilus biogenesis in *Neisseria meningitidis*: PilW is involved in a step occurring after pilus assembly, essential for fibre stability and function. Mol Microbiol **55:**54–64. http://dx.doi.org/10.1111/j.1365-2958.2004.04364.x.
28. **Koo J, Tammam S, Ku SY, Sampaleanu LM, Burrows LL, Howell PL.** 2008. PilF is an outer membrane lipoprotein required for multimerization and localization of the *Pseudomonas aeruginosa* type IV pilus secretin. J Bacteriol **190:**6961–6969. http://dx.doi.org/10.1128/JB.00996-08.
29. **Shevchik VE, Condemine G.** 1998. Functional characterization of the *Erwinia chrysanthemi* OutS protein, an element of a type II secretion system. Microbiology **144**(Part 11)**:**3219–3228.
30. **Szeto TH, Dessen A, Pelicic V.** 2011. Structure/function analysis of *Neisseria meningitidis* PilW, a conserved protein that plays multiple roles in type IV pilus biology. Infect Immun **79:**3028–3035. http://dx.doi.org/10.1128/IAI.05313-11.
31. **Schuch R, Maurelli AT.** 2001. MxiM and MxiJ, base elements of the Mxi-Spa type III secretion system of *Shigella*, interact with and stabilize the MxiD secretin in the cell envelope. J Bacteriol **183:**6991–6998. http://dx.doi.org/10.1128/JB.183.24.6991-6998.2001.
32. **Okon M, Moraes TF, Lario PI, Creagh AL, Haynes CA, Strynadka NC, McIntosh LP.** 2008. Structural characterization of the type-III pilot-secretin complex from *Shigella flexneri*. Structure **16:**1544–1554. http://dx.doi.org/10.1016/j.str.2008.08.006.
33. **Derrick J.** 2008. A pilot sheds light on secretin assembly. Structure **16:**1441–1442. http://dx.doi.org/10.1016/j.str.2008.09.001.
34. **Crago AM, Koronakis V.** 1998. *Salmonella* InvG forms a ring-like multimer that requires the InvH lipoprotein for outer membrane localization. Mol Microbiol **30:**47–56. http://dx.doi.org/10.1046/j.1365-2958.1998.01036.x.
35. **Burghout P, Beckers F, de Wit E, van Boxtel R, Cornelis GR, Tommassen J, Koster M.** 2004. Role of the pilot protein YscW in the biogenesis of the YscC secretin in *Yersinia enterocolitica*. J Bacteriol **186:**5366–5375. http://dx.doi.org/10.1128/JB.186.16.5366-5375.2004.
36. **Allaoui A, Scheen R, Lambert de Rouvroit C, Cornelis GR.** 1995. VirG, a *Yersinia enterocolitica* lipoprotein involved in Ca2+ dependency, is related to exsB of *Pseudomonas aeruginosa*. J Bacteriol **177:**4230–4237.
37. **Lodge J, Douce GR, Amin II, Bolton AJ, Martin GD, Chatfield S, Dougan G, Brown NL, Stephen J.** 1995. Biological and genetic characterization of TnphoA mutants of *Salmonella typhimurium* TML in the context of gastroenteritis. Infect Immun **63:**762–769.
38. **Watson PR, Paulin SM, Bland AP, Jones PW, Wallis TS.** 1995. Characterization of intestinal invasion by *Salmonella typhimurium* and *Salmonella dublin* and effect of a mutation in the invH gene. Infect Immun **63:**2743–2754.
39. **Bi Y, Du Z, Yang H, Guo Z, Tan Y, Zhu Z, Yang R.** 2009. Reduced apoptosis of mouse macrophages induced by yscW mutant of *Yersinia pestis* results from the reduced secretion of YopJ and relates to caspase-3 signal pathway. Scand J Immunol **70:**358–367. http://dx.doi.org/10.1111/j.1365-3083.2009.02297.x.
40. **Pati NB, Vishwakarma V, Jaiswal S, Periaswamy B, Hardt WD, Suar M.** 2013. Deletion of invH gene in *Salmonella enterica* serovar Typhimurium

limits the secretion of Sip effector proteins. Microbes Infect **15:**66–73. http://dx.doi.org/10.1016/j.micinf.2012.10.014.
41. **Izore T, Perdu C, Job V, Attree I, Faudry E, Dessen A.** 2011. Structural characterization and membrane localization of ExsB from the type III secretion system (T3SS) of *Pseudomonas aeruginosa*. J Mol Biol **413:**236–246. http://dx.doi.org/10.1016/j.jmb.2011.07.043.
42. **Goranson J, Hovey AK, Frank DW.** 1997. Functional analysis of exsC and exsB in regulation of exoenzyme S production by *Pseudomonas aeruginosa*. J Bacteriol **179:**1646–1654.
43. **Goure J, Pastor A, Faudry E, Chabert J, Dessen A, Attree I.** 2004. The V antigen of *Pseudomonas aeruginosa* is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. Infect Immun **72:**4741–4750. http://dx.doi.org/10.1128/IAI.72.8.4741-4750.2004.
44. **Goure J, Broz P, Attree O, Cornelis GR, Attree I.** 2005. Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. J Infect Dis **192:**218–225. http://dx.doi.org/10.1086/430932.
45. **Thibault J, Faudry E, Ebel C, Attree I, Elsen S.** 2009. Anti-activator ExsD forms a 1:1 complex with ExsA to inhibit transcription of type III secretion operons. J Biol Chem **284:**15762–15770. http://dx.doi.org/10.1074/jbc.M109.003533.
46. **Ple S, Job V, Dessen A, Attree I.** 2010. Cochaperone interactions in export of the type III needle component PscF of *Pseudomonas aeruginosa*. J Bacteriol **192:**3801–3808. http://dx.doi.org/10.1128/JB.00117-10.
47. **Huber P, Bouillot S, Elsen S, Attree I.** 2013. Sequential inactivation of Rho GTPases and Lim kinase by *Pseudomonas aeruginosa* toxins ExoS and ExoT leads to endothelial monolayer breakdown. Cell Mol Life Sci **71:**1927–1941. http://dx.doi.org/10.1007/s00018-013-1451-9.
48. **Verove J, Bernarde C, Bohn YS, Boulay F, Rabiet MJ, Attree I, Cretin F.** 2012. Injection of *Pseudomonas aeruginosa* Exo toxins into host cells can be modulated by host factors at the level of translocon assembly and/or activity. PLoS One **7:**e30488. http://dx.doi.org/10.1371/journal.pone.0030488.
49. **Viarre V, Cascales E, Ball G, Michel GP, Filloux A, Voulhoux R.** 2009. HxcQ liposecretin is self-piloted to the outer membrane by its N-terminal lipid anchor. J Biol Chem **284:**33815–33823. http://dx.doi.org/10.1074/jbc.M109.065938.
50. **Aubert D, MacDonald DK, Valvano MA.** 2010. BcsKC is an essential protein for the type VI secretion system activity in *Burkholderia cenocepacia* that forms an outer membrane complex with BcsLB. J Biol Chem **285:**35988–35998. http://dx.doi.org/10.1074/jbc.M110.120402.
51. **Dacheux D, Attree I, Schneider C, Toussaint B.** 1999. Cell death of human polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional type III secretion system. Infect Immun **67:**6164–6167.
52. **Pastor A, Chabert J, Louwagie M, Garin J, Attree I.** 2005. PscF is a major component of the *Pseudomonas aeruginosa* type III secretion needle. FEMS Microbiol Lett **253:**95–101. http://dx.doi.org/10.1016/j.femsle.2005.09.028.
53. **Daefler S, Russel M.** 1998. The *Salmonella typhimurium* InvH protein is an outer membrane lipoprotein required for the proper localization of InvG. Mol Microbiol **28:**1367–1380. http://dx.doi.org/10.1046/j.1365-2958.1998.00908.x.
54. **Okuda S, Tokuda H.** 2011. Lipoprotein sorting in bacteria. Annu Rev Microbiol **65:**239–259. http://dx.doi.org/10.1146/annurev-micro-090110-102859.
55. **Hoang HH, Nickerson NN, Lee VT, Kazimirova A, Chami M, Pugsley AP, Lory S.** 2011. Outer membrane targeting of *Pseudomonas aeruginosa* proteins shows variable dependence on the components of Bam and Lol machineries. mBio **2**(6):e00246–11. http://dx.doi.org/10.1128/mBio.00246-11.
56. **Koo J, Tang T, Harvey H, Tammam S, Sampaleanu L, Burrows LL, Howell PL.** 2013. Functional mapping of PilF and PilQ in the *Pseudomonas aeruginosa* type IV pilus system. Biochemistry **52:**2914–2923. http://dx.doi.org/10.1021/bi3015345.
57. **Burns RE, McDaniel-Craig A, Sukhan A.** 2008. Site-directed mutagenesis of the *Pseudomonas aeruginosa* type III secretion system protein PscJ reveals an essential role for surface-localized residues in needle complex function. Microb Pathog **45:**225–230. http://dx.doi.org/10.1016/j.micpath.2008.05.002.
58. **Pukatzki S, Kessin RH, Mekalanos JJ.** 2002. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci U S A **99:**3159–3164. http://dx.doi.org/10.1073/pnas.052704399.
59. **Daefler S, Guilvout I, Hardie KR, Pugsley AP, Russel M.** 1997. The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIVf1 function. Mol Microbiol **24:**465–475. http://dx.doi.org/10.1046/j.1365-2958.1997.3531727.x.
60. **Tosi T, Nickerson NN, Mollica L, Jensen MR, Blackledge M, Baron B, England P, Pugsley AP, Dessen A.** 2011. Pilotin-secretin recognition in the type II secretion system of *Klebsiella oxytoca*. Mol Microbiol **82:**1422–1432. http://dx.doi.org/10.1111/j.1365-2958.2011.07896.x.
61. **Korotkov KV, Hol WG.** 2013. Crystal structure of the pilotin from the enterohemorrhagic *Escherichia coli* type II secretion system. J Struct Biol **182:**186–191. http://dx.doi.org/10.1016/j.jsb.2013.02.013.
62. **Intile PJ, Diaz MR, Urbanowski ML, Wolfgang MC, Yahr TL.** 2014. The AlgZR two-component system recalibrates the RsmAYZ posttranscriptional regulatory system to inhibit expression of the *Pseudomonas aeruginosa* type III secretion system. J Bacteriol **196:**357–366. http://dx.doi.org/10.1128/JB.01199-13.
63. **Sun Y, Karmakar M, Taylor PR, Rietsch A, Pearlman E.** 2012. ExoS and ExoT ADP ribosyltransferase activities mediate *Pseudomonas aeruginosa* keratitis by promoting neutrophil apoptosis and bacterial survival. J Immunol **188:**1884–1895. http://dx.doi.org/10.4049/jimmunol.1102148.
64. **Diepold A, Amstutz M, Abel S, Sorg I, Jenal U, Cornelis GR.** 2010. Deciphering the assembly of the *Yersinia* type III secretion injectisome. EMBO J **29:**1928–1940. http://dx.doi.org/10.1038/emboj.2010.84.
65. **Diepold A, Wiesand U, Cornelis GR.** 2011. The assembly of the export apparatus (YscR,S,T,U,V) of the *Yersinia* type III secretion apparatus occurs independently of other structural components and involves the formation of an YscV oligomer. Mol Microbiol **82:**502–514. http://dx.doi.org/10.1111/j.1365-2958.2011.07830.x.
66. **Dacheux D, Goure J, Chabert J, Usson Y, Attree I.** 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. Mol Microbiol **40:**76–85. http://dx.doi.org/10.1046/j.1365-2958.2001.02368.x.
67. **Schweizer HP, Hoang TT.** 1995. An improved system for gene replacement and xylE fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15–22.
68. **Figurski DH, Helinski DR.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A **76:**1648–1652.
69. **Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ.** 2005. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **102:**8006–8011. http://dx.doi.org/10.1073/pnas.0503005102.
70. **Hoang TT, Kutchma AJ, Becher A, Schweizer HP.** 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72. http://dx.doi.org/10.1006/plas.1999.1441.
71. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.