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, Faudy 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. Baumler
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00048-15.
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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 PilQ 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 apoposis 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 MgCl2, and bacterial suspensions were used at an optical density at 600 nm (OD600) of 1, which corresponds to the exponential growth phase.

The exsB deletion mutant, CHAΔexsB, was constructed in several steps with splicing by overlap extension (SOE) PCR. Briefly, the two flanking regions of exsB were amplified from the pLAc60 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 (GTTAGGGTCTGGAAGTG). Then, this DNA fragment was cloned into the Smal restriction site 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 *P. aeruginosa* genomic DNA and primers ExsB-TGA-H-F1 (EcoRI site at its 5′ end)/ExsB-TGA-H-R1. The ExsB-TGA-H-R2/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 upstream GT sequence into an ATGACG HindIII 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 marker). The resulting DNA fragment containing *exsB* fused to *pc* encoding a T1000 2.5-kb fragment of the miniCTX integrative vector. Finally, the complemented strains CHAΔexsB/exsB and CHA-BTGA/exsB were obtained after transfer of the construct into the mutant strains, transformation with the *pF2P* plasmid to excise the miniCTX backbone, and selection on medium containing 5% sucrose to select for the loss of *pF2P*.

**Electrophoresis and Western blotting.** T3SS-induced cultures of *P. aeruginosa* (1 ml) were centrifuged at 13,000 *g* for 5 min to separate extracellular and intracellular proteins. 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 supernatant was mixed with 25 μl of 5× Laemmli loading buffer to monitor secreted PopB and PcrV. Lysates 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
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. Voul-
houx), RpoA (from NeoClone), and Oprl-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 × 10³ bacteria in order to induce pneumonia. 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 li-
cense 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% CO₂ in endothelial basal medium 2 (EBM2) containing 2% fetal bovine serum. After 3 h of infection by *P. aeruginosa* strains producing the chimera toxin, BJAB cells were incubated with 1 mM CCF2/AM (Invitro-
gen) 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, 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 retrac-
tion.

Infection 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% CO₂ in RPMI medium containing 10% fetal bovine serum. After 3 h of infection by *P. aeruginosa* strains producing the chimera toxin, BJAB cells were incubated with 1 mM CCF2/AM (Invitro-
gen) 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-lacta-
mase domain of the chimeric toxin leads to the formation of a product emitting a blue fluorescence. Dead cells were removed based on forward

### Table 1 Plasmids used in this study

| Name of plasmid | Construction and characteristics | Source or reference |
|----------------|---------------------------------|---------------------|
| plA60          | Plasmid derived from pUC18, containing the pcrGVH-popBD and exsCEBA operons from strain CHA; Ap⁶ Cb⁶ | 66 |
| pCR-Blunt II-TOPO | PCR product cloning vector; lacZα ccdB; Km⁷ | Invitrogen |
| pTOPO ΔexsB    | pCR-Blunt II-TOPO containing the SOE PCR fragment ΔexsB amplified using ΔexsB_exsB_1/4 primers; Km⁷ | This work |
| pEX100T        | Alleric exchange vector; sae mobRR2; Ap⁶ Cb⁶ | 67 |
| pEX100T exsB   | pEX100T containing the SOE PCR fragment ΔexsB; Ap⁶ Cb⁶ | This work |
| pRKR2013       | Tra¹ Mob¹ ColE1; Km⁷ | 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; Km⁷ | This work |
| pEXG2          | Alleric exchange vector, colE1 origin, oriT saeB; Gm⁷ | 69 |
| pEXG2-ExsB-TGA-H | pEXG2 containing the SOE PCR fragment exsB-TGA-H; Gm⁷ | This work |
| pIA pC-gfp     | pUCP20 derivative containing the transcriptional fusion of gfp with pC, the promoter of the exsB operon; Ap⁶ Cb⁶ | 66 |
| pTOPO RBS exsB | pCR-Blunt II-TOPO containing the PCR fragment RBS exsB amplified with Xba_RBS_ExsB/ExsB_Hind; Km⁷ | This work |
| pApC RBS exsB  | pApC containing the PCR fragment RBS exsB; Ap⁶ Cb⁶ | 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; Tc⁷ | This work |
| pFLP2          | Plasmid expressing the Flp recombinase; Ap⁶ Cb⁶ | 71 |
| pExsAexsBBl    | pUC20 derivative containing the translational fusion exsAexsBBl; Ap⁶ Cb⁶ | 48 |
| pDD2           | pUCP20 derivative constitutively overexpressing exsA; Ap⁶ Cb⁶ | 51 |

*Ap⁶/Cb⁶, resistance to ampicillin for *E. coli* and carbenicillin for *P. aeruginosa*; Km⁷, resistance to kanamycin; Tc⁷, resistance to tetracycline; Gm⁷, resistance to gentamicin.*

### Table 2 Oligonucleotides used in this study

| Name of oligonucleotide | Sequence | Use |
|-------------------------|----------|-----|
| Delta_exsB_1            | 5’-CCCGGGGATATGCTGACGAGGGG | SOE PCR to delete the gene coding for ExsB |
| Delta_exsB_2            | 5’-CCGCGAGAACTTACCGAGCGAGCG | SOE PCR to insert a stop codon within the exsB gene |
| Delta_exsB_3            | 5’-GTGCTTGATGCTATGACGAGCGG | PCR amplification of RBS exsB for complementation studies |
| Delta_exsB_4            | 5’-CCCGGGGCAAGGGCGCTGACGAGG | |
| ExsB-TGA-H-F1           | 5’-CCGAGGCGGTCAGCGGAGCGGAGCG | |
| ExsB-TGA-H-R1           | 5’-GGGCGCGGATACCGAGCGGAGCGG | |
| ExsB-TGA-H-F2           | 5’-GGTACGGGGTACCGAGCGGAGCGG | |
| ExsB-TGA-H-R2           | 5’-GGTACGGGGTACCGAGCGGAGCGG | |
| Xba_RBS_ExsB            | 5’-TGCTACGCGGATACCGAGCGGAGCGG | |
| ExsB_Hind               | 5’-AACTTTAAACTGCTGGCCAGATCTTC | |

*a Restriction sites are underlined.*
activation (47). The

The highly virulent strain CHA featuring an active T3SS was monitored during 36 h (Fig. 1). The mean survival times for both the exsB mutant and the complemented strains.

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 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 (ΔexsB) and the complemented mutant harboring a chromosomally carried copy of the wild-type exsB gene (ΔexsB/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 ΔexsB mutant strain. This difference is statistically significant (P value, <0.01 with the log rank test between wild-type and ΔexsB 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 cofillin activation (47). The ΔexsB strain displayed 40% lower cytotoxicity in this assay than that displayed by the parental and complemented strains (Fig. 2A) (P < 0.05).

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 ΔexsB 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
did not restore a wild-type phenotype, as the \(\Delta exsB/exsB\) 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 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 \(B_{TCA}/B\) 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 \(B_{TCA}/B\) 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 \(B_{TCA}/B\) 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 \(\Delta exsB/exsB\) 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 \(\Delta exsB\) and \(\Delta exsB/exsB\) 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 \(\Delta exsB\) 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 PscD 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 the corresponding supernatants. The strains assessed are the wild-type strain (wt), the mutant strain lacking exsB (ΔB), and the corresponding complemented strain (ΔB/B). (B) Genetic organization of T3SS regulatory operon and downstream genes in the wild-type strain (top), in the ΔexsB 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 (BexsA); and the corresponding complemented strain (BexsA/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 pilots, 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 (t0) 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 ΔexsB 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 ΔexsB 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 pilots 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 (35). If ExsB
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 4 Involvement of ExsB in T3SS needle assembly. (A) Representative images of P. aeruginosa bacteria exhibiting a T3SS needle observed by transmission electronic microscopy (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 (ΔexsB) and the complemented strain (ΔexsB/exsB). (C) Number of bacteria possessing 0, 1, 2, or >3 T3SS needles both at their surface and in their close extracellular environment.

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_{PSCA}) and the corresponding complemented strain (B_{PSCA}/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.
T3SS genes, that the pilotin directly acts on the secretin (17). In parallel, obtaining the recombinant PscC should be performed in order to gain insights into the secretion and activity of the T3SS (56), for example, and other T3SSs. Indeed, 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).

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 deletion 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 Ragon, Laura Pascalon, and Alexandra Crété for technical help, and Véronique Remold SK, Brown CK, Farris JE, Hundley TC, Perpich JA, Purdy ME.

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