MAJOR ARTICLE

Genotypic and Phenotypic Analysis of Type III Secretion System in a Cohort of *Pseudomonas aeruginosa* Bacteremia Isolates: Evidence for a Possible Association between O Serotypes and *exo* Genes

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The type III secretion system (TTSS) of *Pseudomonas aeruginosa* was characterized genetically and phenotypically in 92 epidemiologically unrelated bacteremic strains. Four groups of strains (TTSS types) were defined according to the level of type III protein secretion and kinetics of cytotoxicity. Type 1 strains ($n = 26$) were highly and rapidly cytotoxic and secreted ExoU, type 2 strains ($n = 48$) exhibited slower cytotoxic rates and expressed ExoS but not ExoU, type 3 strains ($n = 14$) were poorly cytotoxic, and type 4 strains ($n = 4$) were not cytotoxic. Type 3 and 4 strains did not have detectable secretion phenotype; however, some type 4 strains were able to reach a level of cytotoxicity similar to that of type 1 and type 2 strains when complemented in trans by a functional *exsA* gene. A statistically significant association ($P < .001$) was found between TTSS types and detection of the mutually exclusive *exoU* and *exoS* genes. In addition, 24 of 25 serotype O:1, O:10, and O:11 strains contained *exoU*, whereas 54 of 55 serotype O:3, O:4, O:6, O:12, and O:16 strains contained *exoS* ($P < .001$). Our results demonstrate correlations among *exoU* or *exoS* genotype, TTSS phenotype, and O serotype in bacteremic *P. aeruginosa* isolates.

Pseudomonas aeruginosa is an opportunistic pathogen that is notorious for its resistance to antibiotics and is responsible for up to 11% of nosocomial infections. Although colonization usually precedes infection, the exact source and mode of transmission of this pathogen are often unclear because of its ubiquitous presence in the hospital environment. *P. aeruginosa* is responsible for 3% of catheter-associated bloodstream infections [1]. The case-fatality rate of bacteremia due to *P. aeruginosa* is severe, ranging from 32% to 73%, with attributable mortality estimated to be ~30% [2]. These infections affect patients with severe immunosuppression or underlying diseases, such as cystic fibrosis or severe burns. The pathogenesis of *P. aeruginosa* infection is multifactorial, as suggested by a wide array of virulence determinants expressed by the bacterium: lipopolysaccharide, pili, pyocyanin, phospholipases, hemolysins, alkaline proteases, elastase, and exotoxin A [3, 4].

The type III secretion system (TTSS) is an important, albeit recently recognized, virulence determinant of *P. aeruginosa*. This cell contact–dependent apparatus directs secretion and translocation into a target cell of

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several bacterial proteins, named exoenzymes (Exo) U, S, T, and Y [5]. It is believed that these exoproducts are injected directly into the cytosol of target cells via a translocation complex composed of proteins coded by the *pcrGVHpopBD* operon. One of these proteins, PcrV, which is homologous to the LcrV antigen found at the cell surface of *Yersinia* species [5], plays a key role, because antibodies raised against PcrV are able to block the TTSS-dependent cytotoxicity ex vivo and in a mouse model of infection [6, 7]. This cytotoxicity is cell type and effector dependent [8].

Many clinical *P. aeruginosa* strains are able to induce the oncosis (necrosis) of phagocytes, presumably via the pore-forming activity of the "translocation" proteins PopB/D and PcrV [9, 10]. Conversely, epithelial cells infected by the same strains become round in shape without significant loss of viability; this morphological change is attributed to the activity of effectors ExoT and ExoS [8]. These 2 closely related proteins exert both GTPase-activating and ADP-ribosyltransferase activities on small GTP-binding proteins of the Ras and Rho families [11, 12]. *P. aeruginosa* strains that are capable of inducing rapid, nonapoptotic epithelial cell death have been found to produce an exotoxin, ExoU, the activity of which is still unknown [13]. Of interest, in a murine model of acute pneumonia, such strains were more virulent than non–ExoU-producing strains [14]. Another TTSS-associated exotoxin, ExoY, has been described in *P. aeruginosa;* however, the contribution of this adenylate cyclase to the virulence of the pathogen remains unclear [15]. Expression of the 4 exoproteins (ExoS, ExoT, ExoU, and ExoY) and of the components of the secretion apparatus are controlled by ExsA, a central transcriptional regulator of the XylS/AraC family [16]. ExsA is required for the induction of in vitro type III secretion and cytotoxicity [17].

Recent studies have investigated the presence and functionality of TTSS genes in *P. aeruginosa* strains involved in human diseases, such as cystic fibrosis [18, 19] and pneumonia [19, 20]. The role of *exo* genes in *P. aeruginosa* bacteremia is uncertain; 2 previous studies, each examining a small number of isolates, demonstrated discordant results [21, 22]. To gain insight into the virulence mechanisms associated with bloodstream infection, we examined a large panel of *P. aeruginosa* isolates collected in 6 French university hospitals during a 1-year period.

MATERIALS AND METHODS

Study design. The bacteremic strains of *P. aeruginosa* described here were collected throughout 1999 by the Groupe d'Etudes des Septicémies à *Pseudomonas aeruginosa,* a group of microbiologists from 6 French university hospitals (Besançon, Lille, Paris, Reims, Saint-Etienne, and Toulouse). The data recorded prospectively from infected patients included demographic characteristics, diagnosis at admission, prior colonization by *P. aeruginosa,* suspected portal of entry, antibiotic treatment, and clinical outcome.

Bacterial identification. Isolation and identification of *P. aeruginosa* strains were performed according to conventional microbiology methods. All the *P. aeruginosa* strains were kept frozen at −80°C in one laboratory until analysis. The O serotyping of isolates was performed by slide agglutination with specific antiserum from Biorad.

Genotypic characterization of isolates. Strains were compared by arbitrarily primed–polymerase chain reaction (AP-PCR), using the ERIC-2 primer (5′-AAGTAAGTGACTGGGGTGAGCG-3′), according to the procedure described by Mahenthiralingam et al. [23]. The reproducibility of profiles was tested in at least 2 independent experiments. Strains were considered to be identical or closely related if they showed identical banding profiles or if minor differences in the intensity of 1 or 2 bands were not confirmed in repetitive experiments. Strains were also compared by pulsed-field gel electrophoresis (PFGE) with macrorestriction endonuclease *Dra*I. The protocol used was described elsewhere [24], except that *P. aeruginosa* cells were embedded in 2% agarose (Seaplaque; FMC Bioproducts) blocks and treated by the rapid lysis procedure of Matushek et al. [25]. The DNA inserts then were digested at 37°C for 3 h with 40 U of *Dra*I (Roche Diagnostics), and DNA fragments were separated by PFGE in 1% agarose gel with a CHEF-DrII apparatus (Biorad). According to the criteria of Tenover et al. [26], 2 strains of *P. aeruginosa* were considered to be distinct genotypically if their PFGE profiles differed by >2 bands.

Detection of* exoU *and* exoS *genes by real-time PCR. Strains were grown overnight in Luria-Bertani (LB) broth. DNA was extracted from bacterial cells by use of the Qiamp DNA blood kit (Qiagen). The LightCycler PCR system (Roche Diagnostics) was used for detection of *exoU* and *exoS*. Primers *exoU* 2998 (5′-GCTAAGGCTTGGCGGAATA-3′) and *exoU* 3182 (5′-AGATCACACCCAGCGGTAAC-3′) were designed from the GenBank sequence U97065 to amplify a 250-bp fragment of the *exoU* gene. Primers *exoS* 1783 (5′-GGAGCTGGATGCGGGACA-3′) and *exoS* 2135 (5′-GGCCGCCTCTTCGAGAAC- 3′) were designed from the GenBank sequence L27629 to amplify a 370-bp fragment of the *exoS* gene. Amplification of the 2 genes was performed in separate experiments. The PCR mixture contained 5 μL of template DNA, 0.7 μL of LightCycler master mixture (Roche Diagnostics), 10 pmol of each pair of primers, 4 mmol/L $MgCl_2$, and PCR-grade sterile water (final volume, 20 μL). The same parameters were used for amplification of both genes: an initial denaturation step of 8 min was followed by 45 cycles of denaturation (15 s at 97°C), annealing (7 s at 63°C), and polymerization (14 s at 72°C). The temperature transition rate was 20°C/s in all segments. The

double-stranded PCR product was quantified during the annealing step by measuring the fluorescence generated by the binding of SYBR green dye (Roche Diagnostics) to the template amplified DNA. The specificity of the signal was controlled by comparing the melting curve obtained for each PCR amplicon to the curve produced with a positive control strain. Strains PA103 and PAO1 (which are positive for *exoU* and *exoS*, respectively) and negative controls were included in all PCRs.

Cytotoxicity assays on macrophages. Cytotoxicity assays were done using the murine macrophage line J774.A1 (ATCC TIB-67) grown in Dulbecco's modified Eagle medium (Gibco) supplemented with heat-inactivated fetal calf serum (Gibco) at 10%. For infection experiments, the macrophages were seeded in culture plates 20 h before the addition of the bacteria. Each *P. aeruginosa* strain was grown in 3 mL of LB medium and adjusted to an A_{600} of 1.0–1.5, after dilution of an overnight culture. Bacteria then were centrifuged, resuspended in eukaryotic medium, and added to cells at an MOI of 5. Cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) released into culture supernatants, by use of a cytotoxicity detection kit (Roche Diagnostics), as described elsewhere [10]. The 100% value represented the LDH released from cells lysed by 0.1% TritonX-100. Noncytotoxic or poorly cytotoxic strains were electroporated by the plasmid pDD2, which expresses the *exsA* gene from a constitutive promoter [16]. Transformed strains, selected on plates of *Pseudomonas* isolation agar (Difco) supplemented with carbenicillin, were tested by use of the cytotoxicity assay described above.

In vitro secretion of type III system proteins. The secretion of type III proteins was induced in vitro by chelating Ca^{2+} ions in the medium. Overnight cultures of *P. aeruginosa* strains were diluted to an A_{600} of 0.1 and grown further for 4 h in LB broth with or without 5 mmol/L EGTA and 20 mmol/L $MgCl_2$. The cultures were centrifuged at 12,000 *g* for 10 min to eliminate bacteria. Forty microliters of the supernatants were submitted directly to 0.1% SDS–12% PAGE, and the secreted proteins were revealed by silver staining of the gel. The protein profiles were compared with that obtained with the cystic fibrosis isolate CHA, for which secreted ExoS, ExoT, PopB, and PopD have been identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) [17]. The ExoU protein was localized by MALDI-TOF, using several ExoU-positive strains.

Statistical analysis. Data were analyzed by use of Epi Info

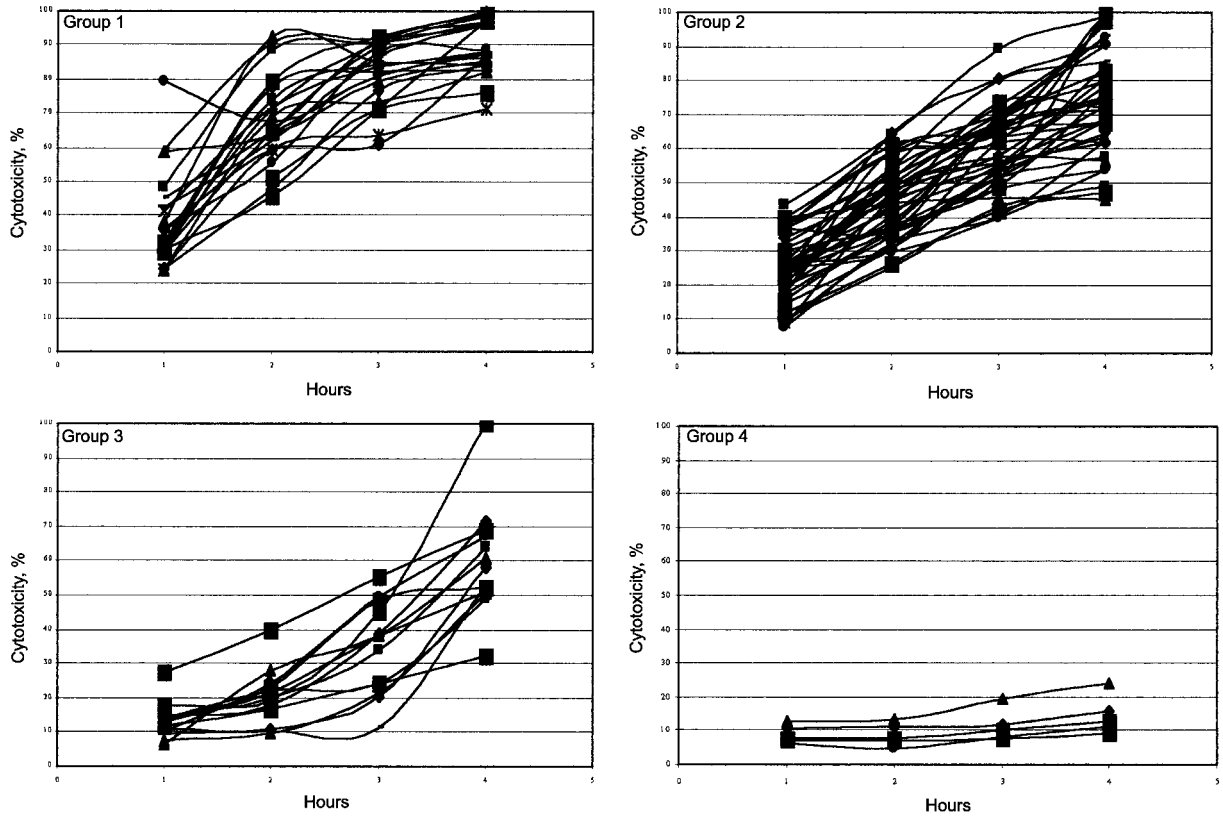

Figure 1. Kinetics of cytotoxicity of *Pseudomonas aeruginosa* strains from the Groupe d'Etudes des Septicémies à *Pseudomonas aeruginosa* collection. Macrophages J774.A1 were infected with bacteria at an MOI of 5. The release of lactate dehydrogenase was determined hourly in 30 μL of infected supernatants by use of a cytotoxicity detection kit. The strains were tested under the same conditions in 4 independent experiments. Each curve represents a separate isolate.

Table 1. Correlation between the presence (+) or absence (−) of *exoU* or *exoS* genes and in vitro type III secretion system (TTSS) type or O serotype of bacteremic *Pseudomonas aeruginosa* strains.

Characteristic	Gene pattern		
	exoU$^+$/*exoS*$^-$	*exoU*$^-$/*exoS*$^+$	*exoU*$^+$/*exoS*$^+$
TTSS type			
1	24	0	2
2	0	48	0
3	3	11	0
4	0	4	0
O serotype			
1	4	1	0
3	1	7	0
4	0	9	1
6	0	23	0
7	1	0	0
8	1	0	0
9	0	1	0
10	2	0	0
11	17	0	0
12	0	7	0
15	0	1	0
16	0	6	0
Nonagglutinable	1	7	0
Polyagglutinable	0	1	1
Total	27	63	2

NOTE. Data are no. of isolates. $P < .001$, for the distribution of the 3 gene patterns among both TTSS types and O serotypes (Fisher's exact test).

(version 6.04b; CDC–World Health Organization) and SPSS (version 10.0; SPSS) software. Fisher's exact and Student's *t* tests were used for univariate and bivariate analyses, respectively. $P < .05$ was considered to be significant.

RESULTS

Collection and selection of bacteremic strains. During the study period, 109 bacteremic episodes due to *P. aeruginosa* were recorded for 105 hospitalized patients, and 104 *P. aeruginosa* isolates were collected. Because *P. aeruginosa* can cause hospital outbreaks involving multiple patients, we typed these isolates by AP-PCR and PFGE to eliminate replicates that may overrepresent epidemic clones in further analyses. Eleven epidemiologically related profiles were observed in 23 patients from 5 hospitals, 10 of them shared by 2 patients and 1 shared by 3 patients (data not shown). Patients with identical isolates were in the same hospital. The redundant isolates were subtracted from the collection, and 92 genotypically distinct strains were kept for further investigations. The case-fatality rate of *P. aeruginosa* bacteremia was 32.6%.

Characterization of TTSS proteins and cytotoxicity. Each strain of the collection was characterized for in vitro secretion of TTSS proteins ExoU, ExoS, ExoT, PopB, and PopD. In parallel, each strain was analyzed for its cytotoxic phenotype toward the J774.A1 macrophage line. The 92 strains fell into 4 TTSS types on the basis of 2 criteria: the profiles of TTSS proteins secretion obtained by SDS-PAGE and the kinetics of cytotoxicity. Detailed results are presented in figure 1 and summarized in table 1.

TTSS type 1 strains ($n = 26$; 28.3% of all isolates) exhibited rapid kinetics of cytotoxicity, with 60% lethality of test cells within 1 h after infection (figure 1). All TTSS type 1 strains secreted ExoU and ExoT, except 1 strain that produced ExoU alone. Two strains were found to secrete ExoS also. Comparable amounts of PopB and PopD were detected in all strains.

TTSS type 2 strains ($n = 48$; 52.2% of all isolates) displayed kinetics of cytotoxicity that were slower than those of type 1 strains (figure 1) and comparable to those reported elsewhere for the CHA isolate [8]. All these strains secreted ExoS and ExoT, but none produced ExoU. In addition, motile strains from this group pack swarmed around the cells during infection of macrophages (data not shown), as does the CHA strain [10].

TTSS type 3 strains ($n = 14$; 15.2% of all isolates) were able to induce some cell death (50% LDH release) 3–4 h after infection, whereas type 4 strains ($n = 4$; 4.3% of all isolates) exhibited no cytotoxicity, even 4 h after infection (figure 1). No protein secretion was detected in vitro in either of these groups (figure 2*A*).

Additional experiments were performed on 7 strains that showed either poor (type 3) or no (type 4) cytotoxicity but that harbored either the *exoS* or *exoU* gene (see below). We postulated that the defect in secretion and cytotoxicity could be bypassed by introducing a functional *exsA* gene in trans, because it was already observed for some strains isolated from patients with cystic fibrosis [17]. Four isolates could be electroporated successfully with the cloned *exsA* gene. Once transformed with *exsA*, 1 strain of group 3 having *exoU* recovered a TTSS type 1 pattern, and 3 strains of group 4 having *exoS*, a TTSS type 2 pattern at both levels of secretion and cytotoxicity (figure 2*A* and 2*B*).

Detection of* exoU *and* exoS *genes and association with O serotypes. Detection of the *exoU* and *exoS* genes in the 92 *P. aeruginosa* isolates was done by use of real-time PCR. Twenty-nine (31.5%) and 65 (70.7%) strains harbored *exoU* and *exoS*, respectively. Only 2 strains were found to possess both genes. The correlation between the TTSS types and the presence or the absence of *exoU* and *exoS* was statistically significant ($P < .001$; table 1).

The presence or absence of *exoU* strongly correlated with O serotype ($P < .001$). All *P. aeruginosa* strains belonging to O serotypes 1, 10, or 11 (5, 2, and 17 isolates, respectively), except

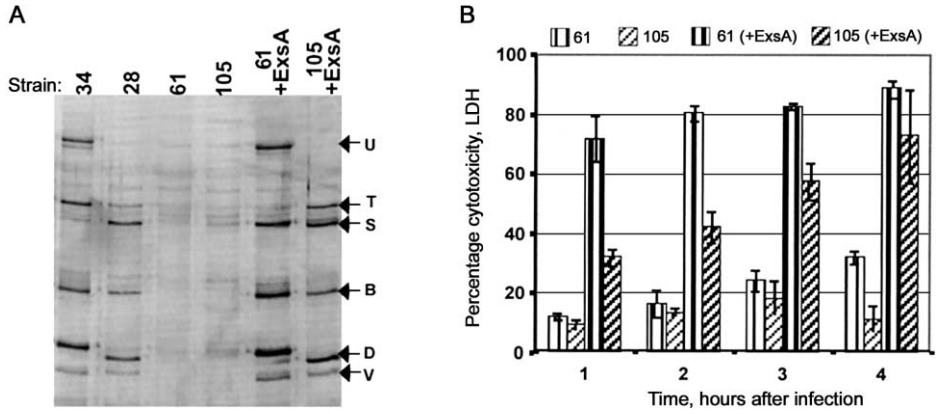

Figure 2. Phenotypic characterization of representative *Pseudomonas aeruginosa* strains from the Groupe d'Etudes des Septicémies à *Pseudomonas aeruginosa* collection. *A,* Analysis of the secretion capacity by silver-stained denaturing polyacrylamide gels: strain 34 (ExoU positive, type III secretion system [TTSS] type 1), strain 28 (ExoS positive, TTSS type 2), strain 61 (exhibiting both *exoU* and *exoS* genes and a low level of secretion of TTSS proteins, TTSS type 3) and strain 105 (exhibiting both *exoS* and *exoT* genes and no secretion of TTSS proteins, TTSS type 4). The induction of TTSS secretion was obtained by culturing the strains in calcium-depleted Luria-Bertani medium. Proteins ExoU, ExoT, ExoS, PopB, PopD, and PcrV are indicated by the letters U, T, S, B, D, and V, respectively. *B,* Cytotoxicity of strains 61 and 105. Both isolates were electroporated with a plasmid expressing *exsA*. Data are mean ± SD of triplicate determinations. LDH, lactate dehydrogenase.

1 (O:1 serotype), harbored the *exoU* gene. In contrast, all strains belonging to O serotypes 3, 4, 6, 12, or 16 (8, 10, 23, 7 and 6 isolates, respectively), except 1 (O:3 serotype), harbored the *exoS* gene. Detailed results are presented in table 1.

No statistical correlation was found between the portal of entry of bacteremia and the *exoU* or *exoS* genotype, except for strains initially recovered from urine, in which *exoS* was strongly predominant (11/12 strains; $P = .11$). Moreover, no correlation could be established between the presence of *exoU* or *exoS* and the outcome of the bacteremia (data not shown).

DISCUSSION

To avoid bias in the phenotypic and genotypic analysis of the TTSS type of bacteremic *P. aeruginosa* strains, we first screened our collection to retain only epidemiologically unrelated strains. Evidence of cross-infection was shown in 5 hospitals (23 related profiles in a cohort of 104 bacteremic strains), thereby underlining the necessity of comparing the strains with powerful molecular typing methods before analyzing their phenotypic and genotypic traits. The same precautions were taken by Feltman et al. [22] to study the prevalence of TTSS genes in 115 isolates (11 of them exhibiting a clonal origin).

In the present study, the presence of *exoT* and *pcrV* genes was not investigated, because previous reports documented the presence of these 2 markers in nearly all *P. aeruginosa* strains from both clinical and environmental sources [22, 27]. All strains possessed either *exoU* or *exoS,* except 2 strains that harbored both genes. The proportion of strains responsible for bacteremia that exhibited *exoU* (31.5%) was higher than that reported by Hirakata et al. [21] (10.8%) but close to that observed by Feltman et al. [22] in a small series of 20 blood strains (40%). Our data confirm that the *exoU* and *exoS* genes are almost mutually exclusive [22, 28, 29] and that *exoS*-positive strains are predominant among urinary tract isolates [22, 28, 30].

Of importance, we found that most of the bacteremic strains (80%) were strongly cytotoxic for macrophages and that the ExoU-secreting isolates killed the phagocytic cells more rapidly. These observations reinforce the conclusions of other studies showing that ExoU is an important virulence factor in *P. aeruginosa* [8, 13] and possibly is involved in the invasiveness of the pathogen [14]. As previously demonstrated by our group [10], the death of macrophages infected with ExoU-defective strains occurs by a mechanism of TTSS-dependent pore formation. It was proposed that, in this case, cell death was due to an unknown cytotoxin [8] or to the TTSS translocation apparatus itself [10].

Despite a good overall correlation between the genotypic and phenotypic approaches used to characterize the strains and the presence of *exo* genes in all the strains tested, a defect in the expression of TTSS (no detectable secretion of predominant type III proteins and poor or absence of cytotoxicity) was observed in a notable proportion (19.6%) of isolates (types 3 and 4). This finding indicates that the TTSS system is not essential for virulence in humans, that there may be compensatory virulence factors not yet discovered, or that underlying host condition may affect the type of *P. aeruginosa* infection that can occur. Furthermore, complementation of those strains by the transcriptional activator ExsA was sufficient to restore the secretion of type III proteins in vitro and, in consequence, the cytotoxic phenotype toward macrophages. These results show the direct positive relationship between the level of secretion

and cytotoxicity. Because ExsA plays a pivotal role in the transcription of TTSS genes [17], variations in the intracellular levels of this regulator logically could account for differences in the ex vivo, and possibly in vivo, cytotoxicity of clinical strains. It remains unclear whether the absence of in vitro TTSS secretion may reliably predict the inability of *P. aeruginosa* to secrete in vivo, because no data on the in vivo cytotoxicity of type 3 or 4 strains are presently available. This information would be essential to understanding the in vivo potential virulence of such strains.

Another interesting finding of this study was the differential distribution of *exoU* and *exoS* genes among the O serotypes. The *exoU* gene was detected in serotypes frequently involved in human infections (O:1, O:10, and O:11) [31]. Lomholt et al. [28] found a correlation between the presence of *exoU* and some electrophoretic types of *P. aeruginosa* isolated from keratitis; unfortunately, the O serotypes of those strains were not reported. Although type III effector genes appear to be similarly distributed in *P. aeruginosa* strains isolated from both clinical and environmental sources [22], some recent data show that *exoS* is essential for the survival of the bacterium in the soil but not in clinical settings [32]. This observation indicates that *exoS* existed before the evolutionary transition from the soil organism to the human pathogen. Other lines of evidence suggest that the *exoU* gene has been acquired by *P. aeruginosa* through horizontal transfer from another bacterial species. This notion is supported by the fact that the guanine plus cytosine content of *exoU* is 59%, compared with 67.2% for the *P. aeruginosa* chromosome as a whole [33, 34]. By sequence alignment, we noted that *exoU* is located immediately adjacent to an insertion-like element that exhibits 70%–80% homology with an insertion sequence (IS 1209, located in serotype O:5 *P. aeruginosa* strains between the *wbpL* and *wbpM* genes) that controls the differentiation of serotype of the bacterium [35]. Recently, Raymond et al. [36] suggested that additional genes not linked to the O antigen–B band could contribute to assembly of the coat polysaccharide of *P. aeruginosa* strains and be responsible for serotypes differences. In light of these data, one could well imagine that the association observed between the *exoU* gene and some O serotypes could be due to the insertion at multiple sites of a sequence linking *exoU* and elements involved in the control of the expression of these O serotypes. Additional studies on strains originating from various geographic areas are now required to confirm the high prevalence of *exoU* in some specific O serotypes. In France, most bacteremic strains belonging to serotypes O:1, O:10, or O:11 can already be considered to be strongly cytotoxic because of their production of ExoU.

From a clinical point of view, detection of *exoU* and *exoS* genes in hospital isolates of *P. aeruginosa* could be useful, because these genes were strongly correlated with different TTSS types. The real-time PCR method developed here proved to be rapid, specific, and cost effective for such a detection. Once validated, this technique could be used to detect TTSS genes of *P. aeruginosa* directly in clinical samples.

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