

Expression of ExsA in *trans* Confers Type III Secretion System-Dependent Cytotoxicity on Noncytotoxic *Pseudomonas aeruginosa* Cystic Fibrosis Isolates

DENIS DACHEUX, INA ATTREE, AND BERTRAND TOUSSAINT*

Département de Biologie Moléculaire et Structurale, BBSI, UMR 5092 CNRS,
CEA Grenoble, 38054 Grenoble Cedex 09, France

Received 1 June 2000/Returned for modification 18 August 2000/Accepted 4 October 2000

Twelve *Pseudomonas aeruginosa* cystic fibrosis isolates that are not able to exert a type III secretion system (TTSS)-dependent cytotoxicity towards phagocytes have been further studied. The strains, although possessing TTSS genes and *exsA*, which encodes a positive regulator of the TTSS regulon, showed no transcriptional activation of the *exsCBA* regulatory operon. The expression of *exsA* in *trans* restored the *in vitro* secretion of TTSS proteins and *ex vivo* cytotoxicity.

Pseudomonas aeruginosa is an opportunistic pathogen which affects numerous compromised human hosts, most notably cystic fibrosis (CF) patients. Chronic respiratory infections and inflammatory responses cause progressive pulmonary tissue destruction, which is often fatal for CF patients (5). The pulmonary damage is attributed on the one hand to the uncontrolled release of toxic mediators from an excessive number of necrotic polymorphonuclear neutrophils (PMNs) at the site of infection (17, 19) and on the other to the synthesis and accumulation of bacterial products (4). A number of virulence proteins produced by *P. aeruginosa* that contribute to its pathogenesis have been characterized (11).

A novel virulence mechanism has been recently identified as the type III secretion system (TTSS), which is able to provoke eukaryotic cell intoxication (15, 27). TTSSs are conserved in many gram-negative pathogens and encode on the order of 20 proteins assembled into a complex to secrete and translocate effectors into eukaryotic cell (14). To date, four TTSS-secreted effectors have been identified in *P. aeruginosa* isolates: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are closely related ADP-ribosyltransferases (9, 18, 20–22), ExoY is an adenylate cyclase (29), and ExoU (PepA) is a cytotoxin with unidentified activity (7, 12, 23). We have recently reported that a CF clinical isolate, CHA, is able to induce rapid oncosis of PMNs and macrophages. The cytotoxicity is TTSS dependent but is independent of the toxin ExoU (2, 3). A survey of 29 *P. aeruginosa* CF isolates showed that 6 strains were able to secrete type III proteins *in vitro* and to provoke ExoU-independent oncosis of phagocyte cells. However most of the strains found to be noncytotoxic in an *ex vivo* infection model possess the type III effector genes and the *exsA* gene, which encodes the transcriptional activator of the TTSS regulon (3, 13).

We showed in this study that expression of the activator of the TTSS regulon, ExsA, in *trans* was sufficient to activate *in vitro* secretion and *ex vivo* cytotoxicity toward phagocytes in 9 out of 12 noncytotoxic isolates tested.

The relevant properties of all strains and plasmids used in this study are summarized in Table 1. To measure and visualize the expression of TTSS genes in living *P. aeruginosa*, the promoter of the *exsCBA* operon (8) isolated from the CHA strain was fused with *gfpmut3* (encoding green fluorescent protein [GFP]) (1) in the *P. aeruginosa*-*Escherichia coli* vector pUCP20 (25). First, the *gfpmut3* gene was cloned into *XbaI*-*PstI*-digested pUCP20. *P. aeruginosa* strains transformed with pUCP20-*gfpmut3* showed high GFP fluorescence due to the activity of *plac* present just upstream from the cloned *gfpmut3* gene. To avoid interference with *plac* activity, the –10 region of *plac* was deleted with mutagenic oligonucleotide 5'GCTCACAAT TCCACACACGAGCCGGAAG using the Altered Sites *in vitro* mutagenesis system (Promega, Madison, Wis.) according to the manufacturer's instructions. The sequence of mutagenized plasmid (named pIA101*) was confirmed by sequencing. No background GFP fluorescence could be detected in *P. aeruginosa* strains transformed with pIA101* when cultivated in Luria-Bertani (LB) medium. The *pexsC* (8) region was amplified by PCR using genomic DNA of CHA as the template. Primers 5'TCGGATCCCCCATGAAGGACGTC and 5'AGGGATCCTGCGAACTCGGCAAGCAG were synthesized, with *Bam*HI sites (underlined) incorporated at the 5' ends to facilitate cloning. A 280-bp *pexsC* PCR product was cloned into the *Bam*HI site of pIA101*, giving pIApC, and introduced by electroporation into the CHA strain (6). To test transcriptional fusion, *gfp* expression under control of *pexsC* was measured in the CHA(pIApC) strain grown either in LB or in LB supplemented with 5 mM EGTA and 20 mM MgCl₂, a condition known to activate *pexsC* transcription (26). GFP expression in individual bacteria was visualized by fluorescence microscopy (Zeiss) and quantified with a FluoroImager (Vistra Fluorescens). The expression of *pexsC-gfp* in CHA(pIApC) increased about eightfold during growth under activating conditions (Fig. 1). When the fusion was introduced into the *exsA*-mutated CHA strain CHA-D1 (2), no expression of GFP could be detected under activating growth conditions (Fig. 1). These results showed that the *pexsC-gfp* fusion could be used as an efficient reporter for monitoring transcription of the *exsCBA* operon.

* Corresponding author. Mailing address: DBMS/BBSI, CEA Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex 09, France. Phone: 33.4.76.88.34.83. Fax: 33.4.76.88.51.85. E-mail: btoussaint@cea.fr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype ^a	Source and/or reference
Strains		
<i>E. coli</i> DH5a		Gibco BRL
<i>P. aeruginosa</i>		
CHA	Mucoid CF isolate	24
CHA-D1	<i>exsA</i> ::Gm mutant of CHA	2
CF2, CF3, CF4, CF5, CF9, CF10, CF15, RIE, K569, REN3, REN4	Noncytotoxic CF isolates possessing <i>exsA</i> , <i>exoS</i> , <i>exoT</i> , and <i>exoY</i> genes	3
CF17	Noncytotoxic CF isolate possessing <i>exsA</i> , <i>exoS</i> , <i>exoT</i> , <i>exoY</i> , and <i>exoU</i> genes	3
CF6, CF11, CF12, REN0	Cytotoxic CF isolates possessing <i>exsA</i> , <i>exoS</i> , <i>exoT</i> , and <i>exoY</i> genes	3
Plasmids		
pUCP20	Ap ^r	25
pDD2	pUCP20-derived plasmid containing the <i>exsA</i> gene in the sense orientation	2
pDD3	pUCP20-derived plasmid containing the <i>exsA</i> gene in the inverse orientation	This work
pUCP20- <i>gfpmut3</i>	pUCP20 containing the <i>gfpmut3</i> gene	This work
pIA101*	pUCP20- <i>gfpmut3</i> with deletion of the <i>plac</i> -10 region	This work
pIApC	pIA101* containing <i>pexsC</i>	This work
pIAX12	pIA101*-derived plasmid containing the <i>gfpmut3</i> gene under a constitutive promoter X12	This work (2)

^a Ap^r, ampicillin resistance.

To determine the transcriptional level of the *exsCBA* operon in noncytotoxic *P. aeruginosa* isolates, the *pexsC-gfp* fusion was introduced into strains by electroporation (6). The transformants were selected on Pseudomonas Isolation Agar (Difco) supplemented with 1,000 μ g of carbenicillin per ml. For measurement of *pexsC* activity, the transformed strains were cultivated overnight at 37°C and 300 rpm in LB liquid medium with 300 μ g of carbenicillin per ml. Each culture was diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 and grown to an OD₆₀₀ of 1 under two culture conditions: in LB medium, where *pexsC* was not active (no GFP fluorescence could be detected), and in calcium-depleted LB medium, a condition that activated *pexsC*. GFP fluorescence was quantified in a 96-well plate with a FluoroImager using 2.5×10^8 bacteria per well, washed once with sterile water, and resuspended in 100 μ l of sterile water.

In four selected cytotoxic strains, CF6, CF11, CF12, and REN0, harboring the *pexsC-gfp* fusion, the expression of *pexsC* was increased six- to ninefold under inducing conditions, similar to the activation observed for CHA(pIApC) (Fig. 1). In contrast, strains that were found to be noncytotoxic toward PMNs and macrophages over a period of 6 h in the infection model showed no transcriptional activation of *pexsC* under conditions of calcium depletion (Fig. 1). These results indicate that the absence of secretion of TTSS proteins in vitro and, as a consequence, the absence of a cytotoxic phenotype in the infection model may be due to inefficient transcriptional activation of the *exsCBA* operon (3). The third gene of the operon encodes ExsA, the transcriptional activator necessary for the expression of operons encoding type III secretion complex proteins, translocation proteins, and effectors (26, 28). In order

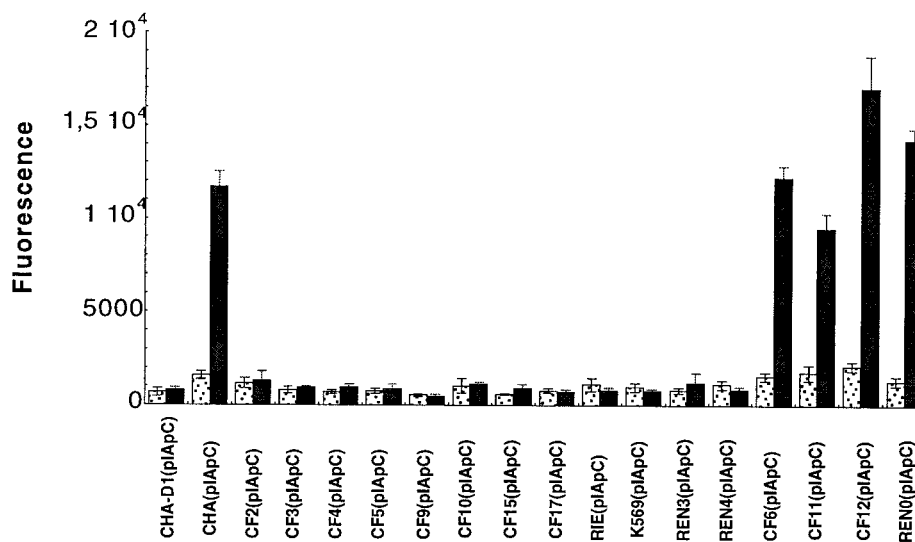


FIG. 1. GFP fluorescence in *P. aeruginosa* isolates transformed with reporter plasmid pIApC, grown in LB medium (stippled bars) or in LB medium supplemented with 5 mM EGTA and 20 mM MgCl₂ (inducing conditions) (solid bars). Error bars represent the standard deviation. Data are from at least three independent experiments.

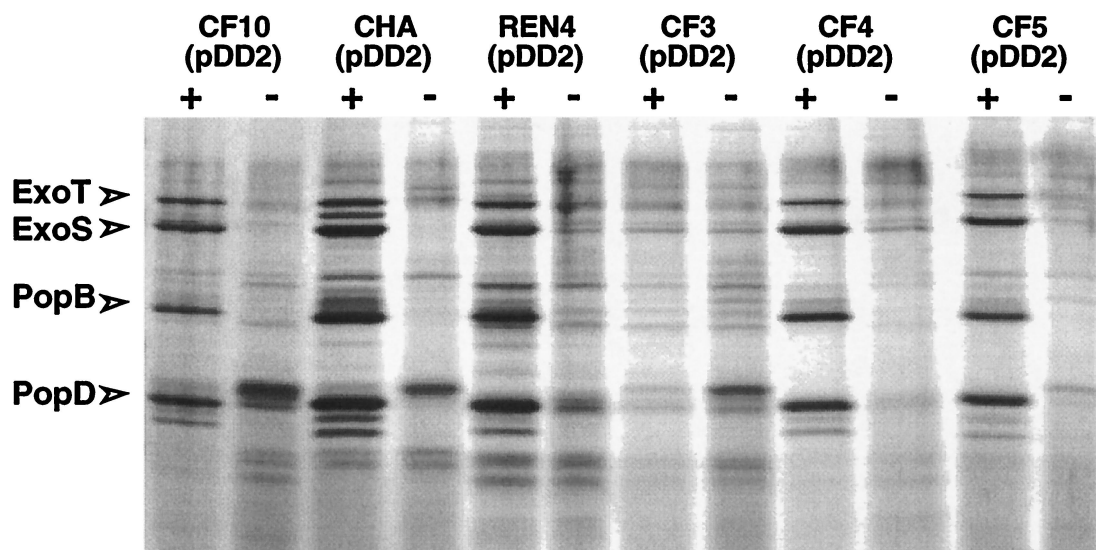


FIG. 2. In vitro TTSS secretion profiles. Silver nitrate-stained 0.1% sodium dodecyl sulfate–12% polyacrylamide gel of supernatants of strains transformed with plasmid pDD2 cultured with (+) or without (–) induction conditions (5 mM EGTA and 20 mM MgCl₂) compared to cytotoxic strain CHA(pDD2). The positions of ExoS, ExoT, PopB, and PopD are shown. The strains CF2, CF9, CF15, CF17, RIE, K569, and REN3 transformed with plasmid pDD2, which are able to secrete the four type III secreted proteins, are not shown.

to see whether the absence of functional ExsA is responsible for the noncytotoxic phenotype, we introduced the functional *exsA* gene, isolated from CHA, into all 12 noncytotoxic strains. The plasmid pDD2, which was previously used to complement the CHA *exsA* mutant strain, CHA-D1, contains the *exsA* gene under the control of a constitutive promoter. The ability of the transformants to secrete in vitro TTSS proteins was first tested. Bacteria were grown in calcium-depleted LB medium, and extracellular proteins were analyzed by 0.1% sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. The protein profiles were compared to the protein profile obtained with the CHA strain, in which secreted ExoS, ExoT, PopB, and PopD were previously identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (2). The results showed that 11 out of the 12 strains harboring pDD2 were able to secrete TTSS proteins (ExoS, ExoT, PopB, and PopD) in vitro (Fig. 2 and data not shown). To check that the in vitro reactivation of the type III secretion was due to the expression of *exsA* in trans, the same strains were electrotransformed with pDD3, in which *exsA* is cloned in the antisense orientation with respect to the promoter. As expected, the strains harboring pDD3 were unable to secrete type III proteins under inducing conditions (data not shown).

Only one isolate, CF3(pDD2), although efficiently transformed, was unable to secrete any of the TTSS proteins in vitro. We considered the possibility that the constitutive promoter isolated from CHA and used to control *exsA* expression in pDD2 might not be recognized in the CF3 strain. To test this, plasmid pIAX12 containing *gfpmut3* placed under the control of the same constitutive promoter as in pDD2 was introduced into the isolates CF3, CF5, and CHA. The fluorescences (in arbitrary units) of CF3(pIAX12), CHA(pIAX12), and CF5(pIAX12) were $26,361 \pm 687$, $31,722 \pm 1588$, and $21,620 \pm 812$, respectively. These similar levels, compared to the low level of fluorescence with transformed strains with

pIA101* as a negative control (data not shown), indicated that the promoter is equally active in the three strains. Thus, the nonreactivation of secretion in CF3(pDD2) is not due to a dysfunction of the promoter but may be to a mutation acting further downstream in the activation process.

P. aeruginosa CF isolates which are cytotoxic to PMNs and macrophages ex vivo demonstrate in vitro secretion of TTSS proteins (3). In order to test whether the reactivation of in vitro secretion of TTSS proteins in the strains transformed with pDD2 was sufficient to reactivate cytotoxicity, infection experiments using PMNs and J774 macrophages were performed as previously described (2, 3). Briefly, PMNs were isolated from whole blood by Percoll gradient centrifugation, and the macrophage cell line J774 was grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco). For infection, bacteria were grown to an OD₆₀₀ of between 1 and 1.2 after dilution of overnight cultures at an OD₆₀₀ of 0.1, washed once and resuspended in the appropriate medium. Infection was carried out in 96-well plates with a multiplicity of infection of 10 in a CO₂ incubator at 37°C. Cytotoxicity was determined by measuring the release of the cytosolic enzyme lactate dehydrogenase into infection supernatant using a Cytotoxicity Detection Kit (Roche Molecular Biochemical, Meylan, France). The cytotoxic CF isolate CHA was able to induce type III secretion-dependent cell death of PMNs (Fig. 3) and macrophages (data not shown), with 80% cell lysis measured at 3 h postinfection. The same phenotype was previously described for cytotoxic CF clinical isolates (2, 3). Untransformed noncytotoxic isolates, similarly to the *exsA* mutant CHA-D1, were unable to provoke cell lysis, even after 6 h postinfection (Fig. 3). The isolates CF5, CF9, CF10, CF15, CF17, RIE, K569, REN3, and REN4 transformed with pDD2 were able to induce 30 to 55% PMN death (Fig. 3) and 30 to 90% macrophage death (data not shown) at 3 h postinfection. The strains harboring pDD3 (*exsA* cloned in the

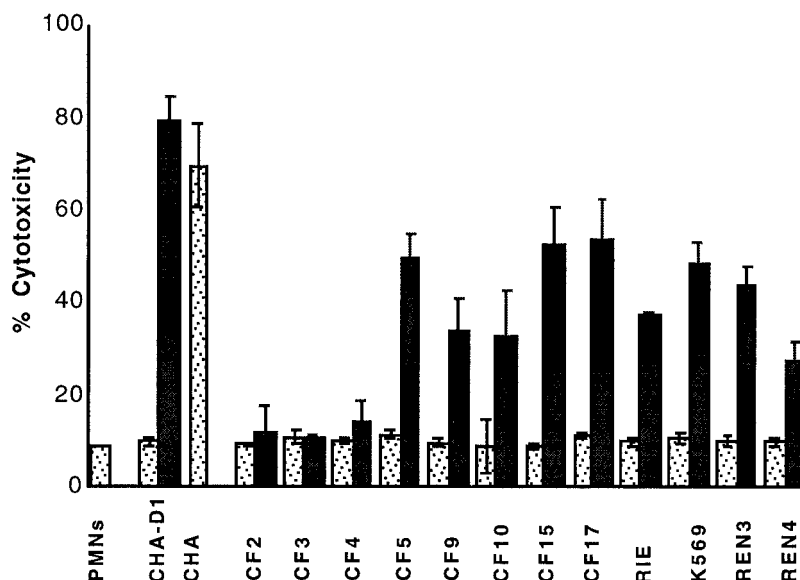


FIG. 3. Cytotoxicity to PMNs in *P. aeruginosa* strains transformed with pDD2. The percentage of cytotoxicity was calculated according to the release of lactate dehydrogenase activity (multiplicity of infection of 10). Stippled bars, PMNs infected by untransformed strains; solid bars, PMNs infected by strains transformed with pDD2. Error bars indicate standard deviations.

inverse orientation) were still noncytotoxic, with values similar to those obtained with untransformed strains (data not shown). The variations of cytotoxicity observed between strains in *ex vivo* infection model (Fig. 3) were not related to the quantity of secreted type III proteins *in vitro* (Fig. 2). We have previously shown that, although cytotoxic, different CF isolates may have different kinetics of cytotoxicity (3). This might be due to differences between strains in cell growth, adhesion, level of activation of the TTSS, and/or resistance to PMNs. Two pDD2-transformed strains (CF2 and CF4) which were capable of secreting TTSS proteins *in vitro* failed to induce cell death of PMNs and macrophages (Fig. 3 and data not shown). Although the secretion complex seemed to be intact, an inefficient translocation process may have caused the lack of cytotoxicity.

In this study, we have shown that 9 out of 12 successfully transformed strains previously characterized as noncytotoxic in a cellular model of infection could be made cytotoxic just by expressing the *exsA* gene in *trans* from a constitutive promoter. ExsA is an activator of TTSS operons, and its expression is autoregulated (26). ExsA is encoded by the last gene in the *exsCBA* operon, which is expressed from the regulated *pexC* promoter. The *exsC* and *exsB* genes are involved in the processing effect on *exsA* mRNA (10). The cytotoxic phenotype conferred by *exsA* in *trans* using a constitutive promoter suggests that the ExsA protein, which is necessary for the expression of the TTSS, is not functional in noncytotoxic strains. This might result either from a mutation(s) in the *exsCBA* operon affecting the coding or regulatory regions or from inappropriate signaling in the regulatory cascade upstream from *pexC*.

The bacterial TTSS is an efficient mechanism for eukaryotic cell intoxication and in many cases a requirement for pathogen survival in the host. However, the synthesis of the system is an energetically expensive process for the bacterial cell, involving more than 20 proteins that make up the secretion complex,

translocation complex, effectors, and regulatory proteins. As is the case for the complex regulatory network controlling the synthesis of the *P. aeruginosa* exopolysaccharide alginate (16), the regulation of the TTSS might involve a signaling pathway upstream from ExsA, which would allow expression of TTSS genes only when appropriate conditions inside the host are met. The environmental stimuli and the signaling pathway upstream from *pexC* affecting expression of the regulatory loci remain to be determined. Our survey of CF clinical isolates showed that about 96% of noncytotoxic strains possess genes capable of encoding proteins of the TTSS (3). Our results suggest that these strains are all potentially cytotoxic.

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