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Quorum Sensing Negatively Controls Type III Secretion Regulon Expression in *Pseudomonas aeruginosa* PAO1

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A systematic analysis of the type III secretion (T3S) genes of *Pseudomonas aeruginosa* strain PAO1 revealed that they are under quorum-sensing control. This observation was supported by the down-regulation of the T3S regulon in the presence of RhlR-C_4HSL and the corresponding advanced secretion of ExoS in a *rhlI* mutant.

Pseudomonas aeruginosa is an opportunistic bacterial pathogen, responsible for infections in immuno-compromised people and individuals with cystic fibrosis. The type III secretion system (T3S) is a major virulence determinant of *P. aeruginosa* and is correlated with the severity of human infections (17). It allows direct delivery of several toxic proteins, called effectors, into the cytosol of the eukaryotic target cell. The T3S is induced under low-Ca^{2+} conditions (8) or upon contact between the bacterium and the eukaryotic cell (24). The T3S regulon is controlled by the transcriptional activator ExsA (8), a member of the AraC/XylS family, which binds a consensus sequence located within the target gene promoter (12). The ExsD protein was shown to be an antiactivator that counteracts the positive effect of ExsA (16), whereas ExsC, which interacts with ExsD, could be an anti-anti-activator (5). Moreover, a novel regulatory pathway, which is dependent on cyclic AMP and the cyclic AMP-binding protein Vfr, activates the T3S regulon (27).

In *P. aeruginosa*, synthesis and secretion of a number of virulence factors are controlled by quorum sensing (QS). QS is crucial in the pathogenesis of *P. aeruginosa* infections (18) and controls virulence factor gene expression in the lungs of cystic fibrosis patients (6). QS is a regulatory mechanism whereby bacteria sense the environment and coordinate the expression of various genes within the bacterial population (10, 15). It involves an interaction between a small diffusible molecule, an acylhomoserine lactone, and a transcriptional activator. Two QS systems, LasR/I-3OC_{12}-homoserine lactone (HSL) and RhlR/I-C_4-HSL, have been well characterized in *P. aeruginosa* (9, 14). In the QS hierarchy, the Las system controls expression of *rhlR* (13). The Las and Rhl systems have been shown to activate the expression of over 200 genes (20, 25). In this report, the activity of T3S gene promoters from the PAO1 strain, whose genome has been sequenced (23), was systematically checked upon standard T3S induction before studying the relationship with QS regulation.

ExsA-dependent and Ca^{2+}-independent expression of *exsA* and *psc* secretion genes. All transcriptional *lacZ* fusions used in this study (Table 1) were constructed using PCR-amplified promoter regions of PAO1 T3S genes, containing −10/−35 RNA polymerase-binding boxes and the ExsA-binding consensus sequence. The DNA fragments were cloned in pMP220 upstream of the promoterless *lacZ* gene. The strains containing the pMP220-derived constructs were grown at 37°C under noninducing (LB) or inducing (LB, 5 mM EGTA, 20 mM $MgCl_2$) T3S conditions. The β-galactosidase activity was measured during cell growth as previously described (1).

Analyses of the *exsCBA* operon, encoding regulatory components, and of the *exsD–pscA-L* operon, encoding the Psc components of the PAO1 T3S machinery, indicated that their respective promoters, *pC* and *pD*, were activated in an ExsA-dependent manner (eightfold and threefold decrease in an *exsA* mutant), independently of Ca^{2+} limitation (Fig. 1A and B). In an *exsA* mutant, both *pC* and *pD* activities were slightly higher than the control strain carrying the pMP220 empty vector, suggesting a basal level of expression that is ExsA independent.

We confirmed the Ca^{2+}-independent expression of *psc* secretion genes from *pD* by testing the presence of PscF within cell extracts of *P. aeruginosa* strain PA103 grown in a medium containing Ca^{2+}, or not, and using anti-PscF antibodies. PscF was produced in the presence of Ca^{2+}, even though at a markedly reduced level compared to a strain grown in a Ca^{2+}-depleted medium (Fig. 1D). This suggests that the secretion apparatus might assemble before the contact with the eukaryotic cell.

ExsA- and Ca^{2+}-dependent expression of effector genes. The expression analysis of effector genes of PAO1, namely, *exoS*, *-T*, and *-Y* genes, showed that they were all strictly regulated by ExsA in a Ca^{2+}-dependent manner (Table 2). As described by Wolfgang and collaborators (27), we observed that expression of each effector gene was greatly induced upon ExsA overproduction, even in a Ca^{2+}-rich medium (Table 2). To test whether the massive expression of *exoY* could lead to

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
P. aeruginosa strains		
PAO1	Wild-type prototroph	B. Holloway
PAO1*exsA*	*exsA* mutant of PAO1, Cb^r	19
PAO1*pscC*	*pscC* mutant of PAO1, Cb^r	19
PAO1R	*lasR* mutant of PAO1	13
PDO100	*rhlI* mutant of PAO1, Hg^r	2
PA103	Cytotoxic respiratory clinical isolate, Fla^-	7
E. coli strains		
TOP10 F′	ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697* (F′ *lacI*^{q} Tn*10 mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Invitrogen
TG1	*supE* Δ(*lac-proAB*) *thi hsdR*Δ*5* (F′ *tra*Δ*36 proA*$^+$*B*$^+$*Z*Δ*M15*)	Laboratory collection
Plasmids		
pRK2013	Tra^+ Mob^+ Km^r	Laboratory collection
pCR2.1	TA cloning vector, Ap^r	Invitrogen
pMP220	Broad-host-range *lacZ* transcriptional fusion, Tc^r	Laboratory collection
pMMB190	Broad host range, *tac* promoter, Ap^r	Laboratory collection
pSBC6	1.2-kb DNA fragment carrying *exsA*, cloned in pMMB190	This work
pSB307	Broad host range, 275-bp-containing *pS*, *pS-lacZ* reporter, Tc^r	This work
pSB302	Broad host range, 211-bp-containing *pT*, *pT-lacZ* reporter, Tc^r	This work
pSB303	Broad host range, 191-bp-containing *pY*, *pY-lacZ* reporter, Tc^r	This work
pSB305	Broad host range, 219-bp-containing *pD*, *pD-lacZ* reporter, Tc^r	This work
pSB308	Broad host range, 320-bp-containing *pG*, *pG-lacZ* reporter, Tc^r	This work
pSB313	Broad host range, 552-bp-containing *pN*, *pN-lacZ* reporter, Tc^r	This work
pPP4	Broad host range, 249-bp-containing *pC*, *pC-lacZ* reporter, Tc^r	This work
pBAD/*Myc*-HisA	Expression vector, *araBAD* promoter, addition of a poly(His) tag at the C-terminal part of the protein	Invitrogen
pPP8	1,145-bp DNA fragment (SBO25-26) containing *exoY* from PAO1, digested with PstI and HindIII, cloned in pBAD/*Myc*-HisA digested with PstI and HindIII	This work
pSBC4	270-bp DNA fragment (SBO9-10) containing *pscF* from PAO1, digested with NcoI and HindIII, cloned in pBAD/*Myc*-HisA digested with NcoI and HindIII	This work

increased in vitro secretion, the occurrence of extracellular ExoY was monitored using anti-ExoY antibodies. Whereas no ExoY effector was detected in the supernatant of an *exsA* mutant, ExsA overproduction led to a dramatic increase in the extracellular level of ExoY under T3S-inducing conditions (data not shown). Interestingly, ExoY neither could be found in the supernatant nor was accumulated in the cytoplasm (data not shown) when strains were grown in the presence of Ca^{2+}, indicating that overproduction of ExsA did not override Ca^{2+} regulation in terms of global function of the T3S. Similar results were obtained for ExoS secretion (data not shown).

ExsA- and Ca^{2+}-dependent expression of the "translocation" and the "plug" operons. Lastly, we studied activity of the *pG* and *pN* promoters. *pG* controls expression of the *pcrGVH-popBD* operon, which encodes components required for effector translocation across the eukaryotic cell membrane (4). *pN* controls expression of the *popN-pcr1234DR* operon, which encodes PopN and Pcr1, the YopN (also called plug) and TyeA homologues, two proteins involved in the control of Yop effectors release in *Yersinia* (3). *pG* and *pN* activities were observed only under T3S-inducing conditions (without Ca^{2+}) in the PAO1 strain and were dependent on ExsA (Table 2).

Our data revealing the Ca^{2+} independency and marginal ExsA dependency of T3S regulatory and secretion operons in PAO1 corroborate a DNA microarray study done with the PAK strain (27). The data are partially in disagreement with earlier studies done in other *P. aeruginosa* backgrounds, which showed a strict ExsA and Ca^{2+} chelation dependency for all T3S regulons (4, 5, 16, 28). Ca^{2+} independency and marginal VirF dependency were previously described for *Yersinia* T3S regulatory and secretion operons (3). The *Yersinia* T3S and the *P. aeruginosa* T3S were classified in the same T3S subfamily, according to their conserved genetic organization and homologies. Our observation describing the similarity in T3S regulation between these bacteria supports this classification.

T3S genes are negatively controlled by QS. We examined the effect of QS on expression of the T3S regulon. Firstly, we verified that expression of the *lasR* and *rhlR* genes, encoding the two QS regulators, was not modified under T3S-inducing conditions (data not shown). Secondly, we used the same set of T3S promoter fusions, which were introduced in either a *lasR* (PAOR) (13) or a *rhlI* (PDO100) (2) mutant. In the *lasR* mutant, expression levels of *pD* ("secretion" operon), *pS*, *pT*, *pY*, *pG* ("translocation" operon), and *pN* ("plug" operon) were fairly similar to expression levels obtained in the PAO1 strain (data not shown). Interestingly, each of these promoter fusions was 1.5- to 4.3-fold up-regulated in the *rhlI* genetic background (Fig. 1C and 2A to E). The inactivation of a gene encoding a homoserine lactone (HSL) synthase, such as *rhlI*, can be phenotypically restored by addition of the corresponding HSL

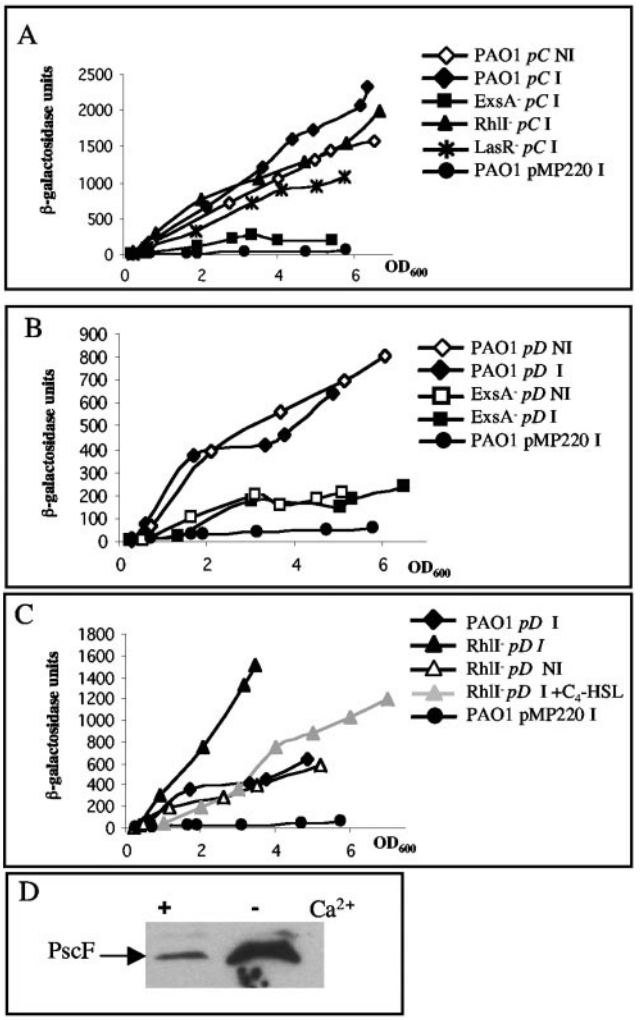

FIG. 1. Expression of the transcriptional fusions *pC-lacZ* from pPP4 (A) and *pD-lacZ* from pSB305 (B and C) in *P. aeruginosa* strain PAO1 (diamonds), PAO1*exsA* (squares), PAO1*rhlI* (triangles), or PAO1*lasR* (crosses). Cultures were grown at 37°C in the absence (closed symbols, I) or presence (open symbols, NI) of Ca^{2+} and with exogenously added 10 μM C_4-HSL (grey symbols). The results represent a single experiment, which was repeated three times without variations. The level of β-galactosidase activity from PAO1/pMP220 (circles) was recorded after growth under T3S-inducing conditions. (D) Intracellular immunodetection of PscF in *P. aeruginosa* strain PA103 grown under T3S-inducing ($-Ca^{2+}$) or noninducing ($+Ca^{2+}$) conditions. The equivalent of an OD_{600} of 0.4 was loaded in each case.

(26). Addition of C_4-HSL in the *rhlI* mutant culture medium reduced activities of *pD*, *pS*, *pT*, and *pY* to PAO1 levels (Fig. 1C and 2A to C).

Our observation corroborates a preliminary study showing QS-dependent control of *exoS* (11) and presents this control as a global mechanism on the T3S regulon, since we concluded that *pD*, *pS*, *pT*, *pY*, *pG*, and *pN* are all submitted to a negative RhlR-C_4-HSL-dependent control. Interestingly, *exsCBA* is the only T3S operon that is not controlled by a QS component, since *pC* activity is affected by neither a *lasR* nor a *rhlI* mutation (Fig. 1A).

More interestingly, we noticed that the effect of the *rhlI* mutation on secretion genes is observed only in Ca^{2+} limitation. In the presence of Ca^{2+}, these genes are indeed expressed at a wild-type level in the *rhlI* mutant (Fig. 1C). This suggests that the effect of *rhlI* on secretion gene expression is probably indirect and goes through an intermediate component whose expression is Ca^{2+} regulated. This component cannot be encoded by *exsA*, *exsC*, or *exsD*, since we showed that their expression is Ca^{2+} independent. In agreement with this hypothesis is our observation that *exsCBA* is not regulated by QS. Previous work indicated that two adenylate cyclases, CyaA and CyaB, are produced in a Ca^{2+}-dependent manner and have been identified as T3S regulators (27). These proteins might be possible candidates for the link between T3S and QS.

Influence of the *rhlI* mutation on ExoS secretion. We also studied the effect of the *rhlI* mutation on the in vitro secretion of ExoS (Fig. 3). At an early stage during the exponential growth phase (optical density at 600 nm [OD_{600}] = 0.35), ExoS was significantly secreted by the *rhlI* mutant. By contrast, efficient ExoS secretion in the PAO1 supernatant was found only at later growth stages (OD_{600} = 0.7) (Fig. 3). Thus, ExoS secretion is advanced during the growth of an *rhlI* mutant. Moreover, the addition of exogenous C_4-HSL to the culture medium of a *rhlI* mutant delayed ExoS secretion and thus mimicked the behavior observed in PAO1 (Fig. 3). These results strictly corroborate our data obtained with the promoter gene fusions.

Our study clearly identified the T3S regulon, except for the regulatory operon *exsCBA*, as a negative target for QS in *P. aeruginosa*. This is the first *P. aeruginosa* virulence factor for which a negative regulation by RhlR/I-C_4-HSL has been demonstrated. The QS repression of the T3S regulon suggests that the associated virulence functions are likely to be required

TABLE 2. T3S promoter gene activities

Promoter operon or gene	Fold induction in Ca^{2+} depletion[a]	ExsA dependency	Feedback in secretion mutant[b]	Fold induction upon ExsA overproduction[c]	Fold induction in *rhlI* mutant[c]
pS	8.3	Strict	1.9	6.5	2
pT	5.6	Strict	1.4	21.7	4.2
pY	3.9	Strict	2.9	37.8	4.2
pC	Blind	Marginal			No change
pD	Blind	Marginal			2.5
pG	3	Strict			4.3
pN	3.2	Strict			1.5

[a] Fold induction corresponds to the β-galactosidase activity ratio observed in early stationary phase and due to T3S induction.
[b] Fold repression in β-galactosidase activity ratio due to *pscC* mutation.
[c] β-Galactosidase activity ratio.

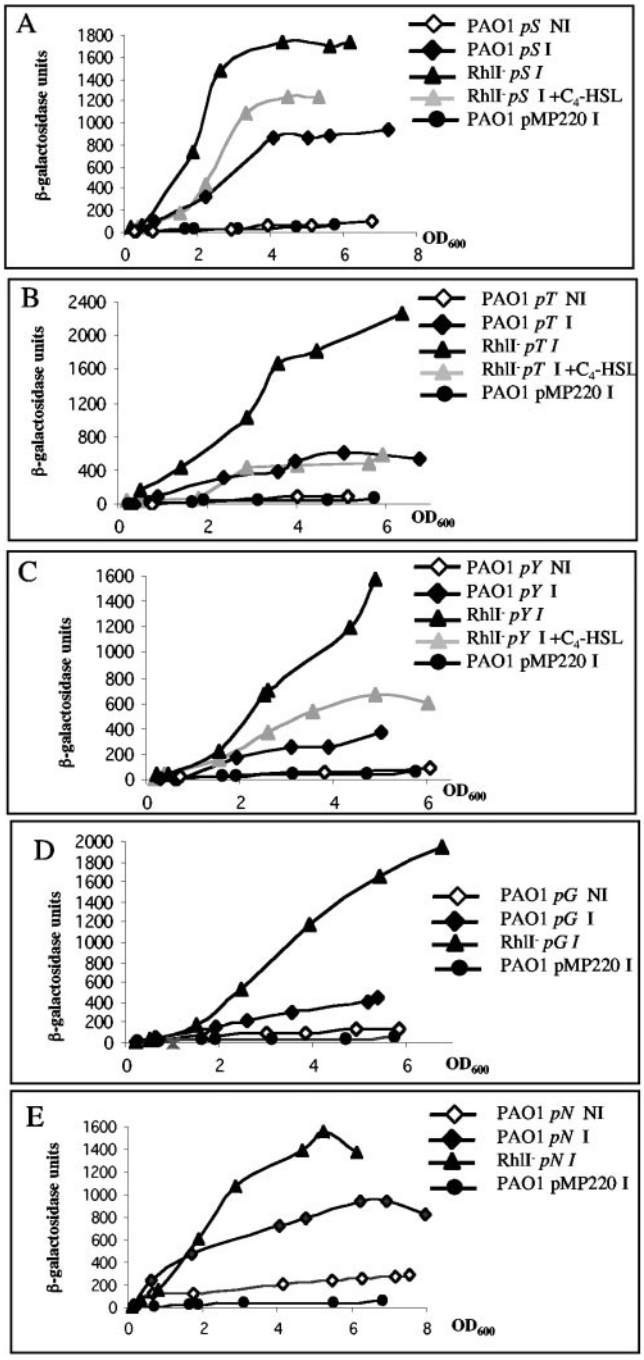

FIG. 2. Expression of the transcriptional fusions *pS-lacZ* from pSB307 (A), *pT-lacZ* from pSB302 (B), *pY-lacZ* from pSB303 (C), *pG-lacZ* from pSB308 (D), and *pN-lacZ* from pSB313 (E) in *P. aeruginosa* strains PAO1 (diamonds) and PAO1*rhlI* (triangles). See the legend of Fig. 1 for more information.

at early stages of bacterial infection (colonization and dissemination), prior to the development of a chronic infection and the establishment of a high-cell-density bacterial population.

A relationship between QS and T3S has previously been proposed for enteropathogenic and enterohemorrhagic *Escherichia coli*. In those bacteria, T3S is required for the production of attaching and effacing lesions on epithelial cells. However, in this case, and in contrast to the *P. aeruginosa* T3S, QS activates enteropathogenic and enterohemorrhagic *E. coli* T3S genes via a LuxS protein and its cognate autoinducer, called AI-3, which is different from the HSL system (21, 22).

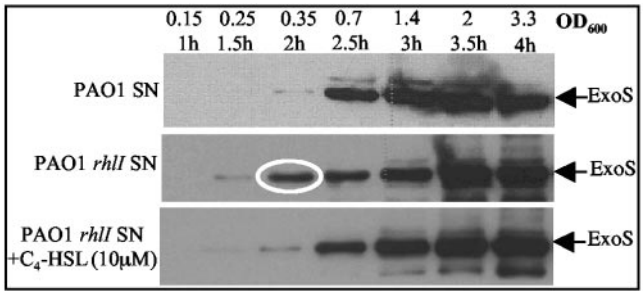

FIG. 3. Immunodetection of ExoS in culture supernatants of PAO1 or PAO1*rhlI* grown at 37°C under T3S-inducing conditions and with exogenously added 10 μM C_4-HSL where indicated. Samples were taken each 30 min over a 4-h growth period. The equivalent of an OD_{600} of 1 was loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel.

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