High-cell-density regulation of the *Pseudomonas aeruginosa* type III secretion system: implications for tryptophan catabolites

Da-Kang Shen,^{1,2†} Didier Filopon,^{1‡} Hichem Chaker,¹ Stephanie Boullanger,³ Madiha Derouazi,¹ Benoit Polack¹ and Bertrand Toussaint¹

Correspondence
Bertrand Toussaint
btoussaint@chu-grenoble.fr

¹GREPI, TIMC-IMAG, UMR5525 CNRS/Université Joseph Fourier Faculté de Médecine, Bat. J Roget, Domaine de la Merci, 38700 La Tronche, France

²Department of Microbiology and Parasitology, Shanghai Jiao-Tong University School of Medicine, Shanghai 200025, PR China

³Service Spectrométrie de Masse, CERMAV-CNRS, BP53, 38041 Grenoble cedex 9, France

The *Pseudomonas aeruginosa* type III secretion system (T3SS) is known to be a very important virulence factor in acute human infections, but it is less important in maintaining chronic infections in which T3SS genes are downregulated. *In vitro*, the activation of T3SS expression involves a positive activating loop that acts on the transcriptional regulator ExsA. We have observed that *in vivo* T3SS expression is cell density-dependent in a manner that does not need known quorum-sensing (QS) signals. In addition, stationary-phase culture supernatants added to exponential-phase growing strains can inhibit T3SS expression. The analysis of transposon insertion mutants showed that the production of such T3SS-inhibiting signals might depend on tryptophan synthase and hence tryptophan, which is the precursor of signalling molecules such as indole-3-acetic acid (IAA), kynurenine and *Pseudomonas* quinolone signal (PQS). Commercially available tryptophan-derived molecules were tested for their role in the regulation of T3SS expression. At millimolar concentrations, IAA, 1-naphthalacetic acid (NAA) and 3-hydroxykynurenine inhibited T3SS expression. Inactivation of the tryptophan dioxygenase-encoding *kynA* gene resulted in a decrease in the T3SS-inhibiting activity of supernatants. These observations suggest that tryptophan catabolites are involved in the downregulation of T3SS expression in the transition from a low- to a high-cell-density state.

Received 8 October 2007
Revised 15 May 2008
Accepted 22 May 2008

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes acute and chronic infections in hospitalized individuals, burn victims and cystic fibrosis (CF) patients (Bodey *et al.*, 1983; Lyczak *et al.*, 2002; Pier, 2002). One of the major virulence factors of *P. aeruginosa* is the type III secretion system (T3SS), which consists of 43

coordinately regulated genes encoding type III secretion and translocation machinery, regulatory factors, type III effectors and effector-specific chaperones (Yahr & Wolfgang, 2006). The T3SS is used to deliver a unique set of effectors directly into the cytoplasm of infected host cells that subvert normal host cell physiology in order to promote pathogenesis (Hueck, 1998). Epidemiological studies of *P. aeruginosa* isolated from patients have shown that the presence of a functional T3SS is strongly associated with a higher incidence of systemic spread and a poor clinical outcome (Garau & Gomez, 2003; Hauser *et al.*, 2002; Roy-Burman *et al.*, 2001). To date, four effectors have been described in *P. aeruginosa*, ExoS, ExoT, ExoY and ExoU. Curiously, most strains of *P. aeruginosa* possess either the gene for ExoS or the gene for ExoU, but not both (Feltman *et al.*, 2001). In this study, we have used the CHA strain, whose genome contains the genes encoding ExoS, ExoT and ExoY, but not ExoU (Dacheux *et al.*, 1999; Delic-Attree *et al.*, 1995). T3SS expression is induced by at least

†These authors contributed equally to this work.

‡Present address: URBM-FUNDP University of Namur, rue de Bruxelles 61, B-5000 Namur, Belgium.

Abbreviations: CF, cystic fibrosis; 2,4-D, dichlorophenoxyacetic acid; HSL homoserine lactone; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LC-MS, liquid chromatography-MS; NAA, 1-naphthalacetic acid; PQS, *Pseudomonas* quinolone signal; QS, quorum-sensing; RLU, relative light units; T3SS, type III secretion system.

Two supplementary tables listing the roles of three Trp-derived signalling molecules and *Pseudomonas* spp. genes encoding enzymes involved in IAA biosynthesis are available with the online version of this paper.

three different environmental elements: (i) *in vivo* contact with eukaryotic host cells; (ii) *in vitro* calcium depletion in the medium; and (iii) the presence of serum (Vallis *et al.*, 1999; Yahr & Frank, 1994).

All T3SS genes are coordinately regulated by ExsA, an AraC-like master transcriptional activator, which binds to a consensus sequence in the promoter region of these genes (Hovey & Frank, 1995). In recent years, it has been discovered that a cascade of T3SS proteins, including ExsD, ExsC and ExsE, influences the activity of ExsA (Dasgupta *et al.*, 2004; McCaw *et al.*, 2002; Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). It is interesting to note that *exsA*, *exsC* and *exsE* are located in the same operon, *exsCEBA*, which is positively controlled by ExsA and PsrA (Shen *et al.*, 2006). ExsE is a secreted regulator of T3SS. When the secretion channel is closed, ExsE is complexed with its chaperone ExsC in the cytoplasm, and the transcription of the T3SS genes is repressed due to the sequestration of ExsA by ExsD, an anti-activator. When the secretion channel is opened, ExsE is secreted, leaving ExsC free to interact with ExsD, which releases ExsA, thereby allowing liberated ExsA to activate the transcription of the T3SS genes (including the *exsCEBA* operon). This process represents the positive activation loop of the T3SS that is triggered by cell contact *in vivo* and can be mimicked by calcium depletion *in vitro*. Besides this major regulatory operon, other factors regulate T3SS expression, including cyclic AMP with Vfr, the *Pseudomonas* catabolite repression homologue (Wolfgang *et al.*, 2003), PtrA (Ha *et al.*, 2004) and the RetS/LadS/GacAS two-component regulatory systems (Goodman *et al.*, 2004; Laskowski *et al.*, 2004; Ventre *et al.*, 2006; Zolfaghar *et al.*, 2005). Taken together, these published data indicate that *P. aeruginosa* uses a complex set of signalling pathways both to activate and to repress T3SS expression in response to extracellular and intracellular triggers (Yahr & Wolfgang, 2006).

Several reports suggest that a functional *P. aeruginosa* T3SS is far less common in CF patient isolates than in isolates from patients with acute infections (Dacheux *et al.*, 2000; Roy-Burman *et al.*, 2001). It has been proposed that, following the infection of CF patient airways, *P. aeruginosa* strains evolve to reduce T3SS expression (Lee *et al.*, 2005), or that populations of cells gradually change from a type III protein secretion-positive phenotype to a secretion-negative phenotype (Jain *et al.*, 2004). This pattern seems to be common in bacterial infection, since other T3SSs are downregulated during mammalian cell infection by *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157 and *Shigella flexneri* (Dahan *et al.*, 2004; Eriksson *et al.*, 2003; Faucher *et al.*, 2006; Lucchini *et al.*, 2005). The consistency of these observations suggests that the down-regulation of T3SS expression might be a conserved phenomenon that follows the successful infection of mammalian cells by these Gram-negative pathogens. Presumably, a mechanism exists for the inactivation of T3SS expression once it has served its purpose. This is the case in enterohaemorrhagic and enteropathogenic *E. coli*,

in which maximum T3SS expression occurs during the transition from the late exponential to the stationary phase (Sperandio *et al.*, 1999), but little is known about the mechanisms involved in the arrest of the positive activation loop of the *P. aeruginosa* T3SS.

In this study, we observed that T3SS expression was repressed by stationary-phase culture supernatants, including those from the *P. aeruginosa* wild-type strains CHA or PAO1 and those from mutants that do not produce known quorum-sensing (QS) signals, such as 3-oxo-C12 and C4 acylhomoserine lactone (3-oxo-C₁₂-HSL and C₄-HSL) or the *Pseudomonas* quinolone signal (PQS). We then investigated the nature of the T3SS-inhibiting signal(s) produced in the stationary-phase culture supernatants and showed that the production of such regulatory signal(s) may depend on the catabolism of tryptophan (Trp), which is the precursor of several important signalling molecules, including indole-3-acetic acid (IAA), kynurenine and PQS.

METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strains were maintained on *Pseudomonas* Isolation Agar (Difco) or cultivated in Luria broth (LB) with antibiotics if required [300 µg carbenicillin ml⁻¹, 200 µg gentamicin (Gm) ml⁻¹ and 250 µg tetracycline ml⁻¹]. *E. coli* strains were cultivated in LB with antibiotics if required (100 µg ampicillin ml⁻¹, 10 µg Gm ml⁻¹ and 20 µg tetracycline ml⁻¹). *P. aeruginosa* strains were stored in Protect bacterial preservers (TSC) at -80 °C. *E. coli* strains were maintained at -80 °C in 50% (v/v) glycerol stocks. To induce T3SS expression, *P. aeruginosa* strains were grown in LB supplemented with 5 mM EGTA and 20 mM MgCl₂ (T3SS-inducing conditions). T3SS non-inducing conditions were LB with 5 mM CaCl₂.

Mutant construction. All PCR primers employed in this study are listed in Table 2 and are based on the PAO1 genome sequence (<http://www.pseudomonas.com>) (Stover *et al.*, 2000). All amplifications were carried out with *Pfu* Turbo polymerase (Promega) by using CHA genomic DNA as a template. PCR products were subcloned into pTOPO (Invitrogen) and sequenced to confirm that no mutations had been introduced during amplification. Plasmids were transformed into chemically competent DH5α *E. coli*. All mutant *P. aeruginosa* strains made in this study were derived from wild-type CHA (Toussaint *et al.*, 1993). Unmarked mutants were constructed by removing an internal fragment of coding sequence by means of allelic exchange (Schweizer & Hoang, 1995), using a Cre-lox antibiotic marker recycling method (Quenee *et al.*, 2005). Briefly, the upstream and downstream regions flanking the gene to be deleted were amplified using the primer pairs indicated in Table 2. The amplified regions were subcloned in tandem into the gene replacement vector pEX100Tlink (Quenee *et al.*, 2005). Next, the Gmox resistance marker was inserted between the two fragments to generate the vector pKO-gene as indicated in Table 1. These constructs were then transformed into *E. coli* S17-1 and mobilized into CHA, CHA carrying an *exsCEBA* promoter-*lux* fusion (pC::lux) or CHA carrying an *exoS* promoter-*lux* fusion (pS::lux) (Shen *et al.*, 2006) by mating. Gm-resistant transformants were isolated, the Gm-resistance gene was then excised and gene deletion was confirmed by PCR with the primers indicated in Table 2. The resulting resistance-marker-free mutants, shown in Table 1, had no discernible growth differences when compared with wild-type CHA.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description*	Source or reference
Strains		
<i>E. coli</i> DH5 α	Used for plasmid transformation	Stratagene
<i>E. coli</i> S17-1	Used for transferring constructs into <i>P. aeruginosa</i>	Simon <i>et al.</i> (1983)
CHA	Mucoid CF isolate	Toussaint <i>et al.</i> (1993)
CHA (pC::lux)	lux fusion with <i>exsC</i> promoter integrated at the <i>attB</i> site of the CHA chromosome	Shen <i>et al.</i> (2006)
PAO1	Parental strain	Fletcher <i>et al.</i> (2007)
PAO1 <i>pqsA</i>	<i>pqsA</i> mutant from PAO1 which cannot synthesize AHQs	Fletcher <i>et al.</i> (2007)
CHA (pS::lux)	lux fusion with <i>exoS</i> promoter integrated at the <i>attB</i> site of the CHA chromosome	Shen <i>et al.</i> (2006)
Δ <i>rpoS</i>	CHA containing an in-frame deletion of amino acids 1–207 of <i>rpoS</i> ORF	Shen <i>et al.</i> (2006)
Δ <i>rpoS</i> (pC::lux)	lux fusion with <i>exsC</i> promoter integrated at the <i>attB</i> site of the Δ <i>rpoS</i> chromosome	Shen <i>et al.</i> (2006)
Δ <i>lasI</i>	CHA containing an in-frame deletion of amino acids 1–155 of <i>lasI</i> ORF	This study
Δ <i>lasI</i> (pC::lux)	lux fusion with <i>exsC</i> promoter integrated at the <i>attB</i> site of the Δ <i>lasI</i> chromosome	This study
Δ <i>rhII</i>	CHA containing an in-frame deletion of amino acids 20–201 of <i>rhII</i> ORF	This study
Δ <i>rhII</i> (pC::lux)	lux fusion with <i>exsC</i> promoter integrated at the <i>attB</i> site of the Δ <i>rhII</i> chromosome	This study
Δ <i>lasI rhII</i>	CHA containing two in-frame deletions of both <i>lasI</i> ORF and <i>rhII</i> ORF	This study
Δ <i>lasI rhII</i> (pC::lux)	lux fusion with <i>exsC</i> promoter integrated at the <i>attB</i> site of the Δ <i>lasIrhII</i> chromosome	This study
Δ <i>trpA</i>	CHA containing an in-frame deletion of amino acids 36–247 of <i>trpA</i> ORF	This study
Δ <i>kynA</i>	CHA containing an in-frame deletion of amino acids 17–270 of <i>kynA</i> ORF	This study
Plasmids		
pTOPO	Blunt-end cloning vector; Ap ^r	Invitrogen
pEX100Tlink	Allelic replacement suicide vector; Ap ^r (Cb ^r)	Quenee <i>et al.</i> (2005)
pUCGmlox	pUC18-based vector containing the <i>aacC1</i> gene flanked by <i>lox</i> ; Ap ^r , Gm ^r	Quenee <i>et al.</i> (2005)
pCM157	Cre recombinase; Tc ^r	Marx & Lidstrom (2002)
Mini-CTX-lux	Mini-CTX-lux; Tc ^r	Laskowski <i>et al.</i> (2004)
Mini-CTX- <i>exsCp</i> -lux	Mini-CTX-lux containing <i>exsC</i> promoter; Tc ^r	Laskowski <i>et al.</i> (2004)
mini-CTX- <i>exoSp</i> -lux	Mini-CTX-lux containing <i>exoS</i> promoter; Tc ^r	Laskowski <i>et al.</i> (2004)
pIAPcgfp	pUCP20-derived vector containing <i>gfpmut3</i> fusion with <i>exsC</i> promoter	Dacheux <i>et al.</i> (2001)
pFLP2	Flp recombinase; Ap ^r (Cb ^r)	Hoang <i>et al.</i> (1998)
pUCP20	<i>Escherichia</i> – <i>Pseudomonas</i> shuttle vector; Ap ^r (Cb ^r)	West <i>et al.</i> (1994)
pUC18	<i>Pseudomonas</i> genomic DNA cloning; Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pKO- <i>lasI</i>	pEX100Tlink-derived vector containing up- and downstream regions of <i>lasI</i> and <i>Gmlox</i> ; Ap ^r , Gm ^r	This study
pKO- <i>rhII</i>	pEX100Tlink-derived vector containing up- and downstream regions of <i>rhII</i> and <i>Gmlox</i> ; Ap ^r , Gm ^r	This study
pKO- <i>trpA</i>	pEX100Tlink-derived vector containing up- and downstream regions of <i>trpA</i> and <i>Gmlox</i> ; Ap ^r , Gm ^r	This study
pexsAind	pUCP20-derived vector containing IPTG-inducible <i>exsA</i> ; Ap ^r (Cb ^r)	Filopon <i>et al.</i> (2006)

*Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Gm^r, Gm resistance; Tc^r, tetracycline resistance.

Preparation of the stationary-phase culture supernatants.

Overnight cultures of bacteria were washed twice with LB, reinoculated at 1:100 in 100 ml LB and grown at 37 °C with agitation at 260 r.p.m. to OD₆₀₀ 2.5–3 (stationary phase, high cell density). Bacteria were harvested by centrifugation at 8000 g for 10 min at room temperature, then washed briefly with 10 ml Vogel–Bonner medium (VB) (Vogel & Bonner, 1956) and grown overnight in 100 ml VB at 37 °C with agitation. Supernatants were collected by centrifugation at 15 000 g for 20 min at 4 °C and stored at –80 °C. To obtain concentrated samples, supernatants were prepared normally, lyophilized by freeze dry system/Lyph Lock 4.5 (Labconco), resuspended in a small volume of water, precipitated by incubation in 3 volumes of ethanol for 1 h at –20 °C, and centrifuged at 15 000 g at 4 °C for 45 min to remove salts and proteins. Alcohol was removed by the SpeedVac system (Thermo) or

a Rotavapour evaporator (Buchi), and the precipitate was redissolved in water to obtain the desired concentration, calculated relative to the starting volume of supernatant. This treatment did not change the activity of supernatants.

Reporter gene analysis. Bacterial strains containing (pC::lux) or (pS::lux) were grown in LB with aeration at 37 °C for 14–16 h. Bacteria were washed with LB and then cultured from OD₆₀₀ 0.05 in LB under T3SS-inducing or non-inducing conditions. The stationary-phase culture supernatants and commercial molecules, including Trp (up to 2.5 mM), IAA sodium salt, indole-3-butyric acid (IBA) potassium salt, 1-naphthalacetic acid (NAA) potassium salt, 2,4-dichlorophenoxyacetic acid (2,4-D) potassium salt, and kynurenine sulfate salt or 3-hydroxykynurenine (Sigma), were then added to the culture in order to determine their roles in the regulation of T3SS

Table 2. Primer sequences used in this study

Primer	Sequence*	Description
<i>lasI</i> -U1	5'-ACGGATCCAAGTGGCTATGTCGCCG-3'	Amplification of upstream region flanking <i>lasI</i>
<i>lasI</i> -U2	5'-ACAAGCTTCCGGACCCTTGCTAGGC-3'	
<i>lasI</i> -D1	5'-AGAAGCTTGGCCTGGACGTATCGCG-3'	Amplification of downstream region flanking <i>lasI</i>
<i>lasI</i> -D2	5'-GGGAATTCTCAGCCTGCC-3'	
<i>rhII</i> -U1	5'-TATCTAGACGCGGTGCGCCGCAAGG-3'	Amplification of upstream region flanking <i>rhII</i>
<i>rhII</i> -U2	5'-ATAAGCTTGCTCGGCGATCATGGCG-3'	
<i>rhII</i> -D1	5'-ATAAGCTTGCGTCATCGGGCGTTCC-3'	Amplification of downstream region flanking <i>rhII</i>
<i>rhII</i> -D2	5'-CAGAGCTCTGTCCGAAATCCTCATGC-3'	
<i>trpA</i> -U1	5'-AGAATTCTCAGATCAGTCGTGGCGG-3'	Amplification of upstream region flanking <i>trpA</i>
<i>trpA</i> -U2	5'-TAAGCTTCAGGCGGTCAAGGATGTCCT-3'	
<i>trpA</i> -D1	5'-TAAGCTTCCAGGGAAGACGCGTAGT-3'	Amplification of downstream region flanking <i>trpA</i>
<i>trpA</i> -D2	5'-TGGATCCGCACAAGATCAACAACCTGC-3'	
<i>kynA</i> -U1	5'-AGAGCTCTGCTCAACGGCGGAAAGCGTT-3'	Amplification of upstream region flanking <i>kynA</i>
<i>kynA</i> -U2	5'-TAAGCTTCTCTCCGTCCGTTTCTCCCTG-3'	
<i>kynA</i> -D1	5'-TAAGCTTAGCATGCTCGATACCATCCTC-3'	Amplification of downstream region flanking <i>kynA</i>
<i>kynA</i> -D2	5'-AGGATCCATCGACCAACGCCTGATCAA-3'	
<i>lasI</i> -1	5'-GTGGATGCTCAAGAAGGACTAC-3'	Verification of $\Delta lasI$
<i>lasI</i> -2	5'-CTGATCGGCAACCTTACCC-3'	
<i>rhII</i> -1	5'-CGCGGCGCTGGGTCTCATC-3'	Verification of $\Delta rhII$
<i>rhII</i> -2	5'-GTAACCGCCCTCTCCGTGCGG-3'	
<i>trpA</i> -1	5'-TCAGCGTGCGTTGCGCACGC-3'	Verification of $\Delta trpA$
<i>trpA</i> -2	5'-AAGCATGAGCCGCCTGCAGA-3'	
<i>kynA</i> -1	5'-TACCGTCGCTGCCTACCAGGC-3'	Verification of $\Delta kynA$
<i>kynA</i> -2	5'-CATGGGGCTTTTGCAGCAAGA-3'	
<i>aacC1</i> -F	5'-ATGTTACGCAGCAGCAACGA-3'	Amplification of <i>aacC1</i> probe for Southern blotting
<i>aacC1</i> -R	5'-CAAGTTCCCGAGGTAATCGG-3'	

*Restriction enzyme sites are underlined.

gene expression and toxin secretion. Supernatant was diluted to $1 \times$ from concentrated stationary-phase supernatant, and commercial molecules were used at 1 mM concentration. Relative luminescence units (RLU) of 0.2 ml of culture were measured using a Luminoskan Ascent spectrophotoluminometer (LabSystems) or a TrisStar LB 941 spectrophotoluminometer (Berthold Technologies) for data with PAO1, PAO1*pqsA* and the *kynA* mutant strains. Bacterial quantity was determined by measuring the OD₆₀₀ (OD₆₀₀ of 1 corresponds to 6×10^8 bacteria ml⁻¹ under our growth conditions). The transcription level was expressed as the number of RLU per 6×10^8 bacteria, as indicated. The means and SE presented were obtained from the data for at least three independent experiments each done in triplicate on different days using different starting cultures. To test the possible effects of nutrient deprivation on T3SS activation, CHA (pIApCgfp) was grown from OD₆₀₀ 0.05 in LB (main culture). At different growth times, aliquots were taken and diluted with fresh LB to OD₆₀₀ 0.1, or the OD₆₀₀ of the main culture was maintained and the medium was replaced with fresh LB. Bacteria were then induced to express T3SS genes for 3 h or were left uninduced. The level of *exsCEBA* transcription was measured by fluorescence and expressed as the fold-induction of the pC promoter (induced level/non-induced level). The calculated means and SE were obtained from data from three independent experiments.

To measure the T3SS transcription level in the presence of human AB serum, overnight bacterial cultures were washed with LB, precultured at 1:100 in LB to OD₆₀₀ 0.6–1, washed twice with modified HEPES-buffered saline (mHBS) (Dacheux *et al.*, 1999), and plated at 5×10^5 cells per well in a 96-well culture plate with or without 10% human

serum. RLU readings from the entire culture plate were taken immediately after the addition of bacteria [time (*t*)=0] and at 1 h intervals.

Secretion profile analysis. To examine the effect of IAA and NAA on type III effector secretion, we used wild-type CHA containing the IPTG-inducible plasmid *pexsA*ind (Filopon *et al.*, 2006), which could express exogenous ExsA *in trans*. Bacterial strains were cultivated overnight in LB containing appropriate antibiotics at 37 °C. These cultures were washed twice with LB, reinoculated at 1:100 in LB supplemented with different compounds, and then vigorously shaken at 37 °C for 3 h. Supernatants of the bacterial culture (equivalent to 1 ml at OD₆₀₀ 1) were collected and TCA was added to 13% to precipitate proteins at 4 °C for 30 min. The precipitated proteins were collected by centrifugation at 15 000 *g* for 15 min, washed twice with cold acetone, air-dried, resuspended in SDS-PAGE loading buffer, analysed by 10% SDS-PAGE and visualized by Coomassie staining. Western blots of normalized secreted proteins or of total bacterial lysates were obtained with the use of an anti-ExoS/ExoT serum at 1:1000 dilution.

Transposon mutagenesis, screening and characterization. A library of transposon insertion mutants was generated by mobilizing the suicide plasmid pFAC from *E. coli* S17-1 to *P. aeruginosa* wild-type CHA, as described elsewhere (Wong & Mekalanos, 2000). Briefly, two strains growing at exponential phase were mixed, placed on PIA plates and incubated overnight at 37 °C. Bacteria were resuspended in LB at appropriate dilutions, which were then spread on PIA containing Gm. Approximately 2000 Gm-resistant mutants, from a

library of $\sim 10^6$ clones, were tested. Mutants were grown on microplates and centrifuged, and each supernatant was tested for its ability to inhibit *exoS* expression using the CHA (pC::lux) reporting strain grown in a microplate. To avoid artefacts due to microplate culture conditions, 20 insertion mutants whose supernatants were inactive during the first screen were tested in a second round by conventional culture in 15 ml aerated tubes. Two mutants of the 20 fulfilled the requirement for further analysis (no inhibition of T3SS activation of wild-type control strain). These two mutants were further characterized by cloning all *Pst*I-digested genomic DNA into plasmid pUC18 with Gm selection and then by sequencing the *Pst*I fragments with M13 forward or reverse primers. Southern blot hybridization was performed with a DIG Nucleic Acid Detection kit (Roche) to verify the number of insertion sites in the genomic DNA.

Liquid chromatography-MS (LC-MS) analysis. To assess the level of known QS signals produced by the wild-type strain CHA and its QS mutants, the stationary-phase culture supernatants were quantified by LC-MS analysis of dichloromethane extracts, as described previously (Morin *et al.*, 2003).

RESULTS

T3SS expression is dependent on cell density

Chromosomal transcriptional gene reporters composed of an *exsCEBA* promoter–*lux* fusion (pC::lux), or an *exoS* promoter–*lux* fusion (pS::lux) were used to identify the transcriptional level of T3SS genes (Shen *et al.*, 2006) during bacterial growth. From the results shown in Fig. 1(a) it appears that both *exsCEBA* and *exoS* were expressed in a growth phase-dependent manner when the strains were initially cultured from OD₆₀₀ 0.05 (low cell density) in T3SS-inducing conditions. The expression of *exsCEBA* and *exoS* rapidly attained its maximum levels at OD₆₀₀ ~ 1.7 and ~ 2 , respectively; it then began to decrease with increasing OD₆₀₀ (high cell density). To test for the possible effects of nutrient deprivation during culture growth, i.e. its possible responsibility for *exsCEBA* and *exoS* repression, *in vitro* T3SS activation was tested on cultures at a different cell densities in fresh LB medium to avoid the effects of nutrient deprivation. At different growth times, aliquots of a CHA (pIAPcGfp) culture (main culture) were taken, the medium was replaced with fresh LB and the cell density was maintained or set to 0.1. Bacteria were then induced to express T3SS genes or were left uninduced. The level of *exsCEBA* transcription was measured by fluorescence and expressed as the fold-induction of the pC promoter. T3SS repression was still observed in high-cell-density cultures (OD₆₀₀ > 2) even in fresh media, unlike in bacteria diluted to low cell density before T3SS induction (Fig. 1c). Thus, the effect of nutrient deprivation on the transcription of T3SS genes could not be responsible for the phenotype that we observed at high cell density for the selected T3SS genes. Cell density-dependent repression of this virulence system has been reported elsewhere for *P. aeruginosa* strain PAK but not further investigated (Ha & Jin, 2001). Moreover, such a decline in the expression of *exsCEBA* and *exoS* over time was reminiscent of QS regulation, in which a sufficient number of bacteria (the

bacterial ‘quorum’) initiate the activation or the repression of multiple genes (Fuqua *et al.*, 1994). The fact that QS has an effect on T3SS expression in *P. aeruginosa* (Blevess *et al.*, 2005; Hogardt *et al.*, 2004; Juhas *et al.*, 2005; Schaber *et al.*, 2004) prompted us to examine the possibility that the reduction of *exsCEBA* and *exoS* expression over time was due to QS-based regulation, which controls gene expression in a cell density-dependent manner through the production of different QS-signalling molecules.

Stationary-phase culture supernatants, as opposed to QS signals, inhibit *exsCEBA* expression *in vitro*

We constructed mutants known to be deficient in QS signal generation (Juhas *et al.*, 2005): CHA Δ *lasI*, CHA Δ *rhII* and CHA Δ *lasI-rhII*, and T3SS expression was examined during bacterial growth. First, these mutants showed the same pattern as the wild-type CHA strain: the expression of the *exsCEBA* operon rapidly attained its maximum level at OD₆₀₀ ~ 1.7 and then began to decrease with increasing OD₆₀₀ (Fig. 1b). We also observed, independent of the growth stage, a higher activity from the promoter of the *exsCEBA* operon, pC, in the Δ *rhII* mutant and the Δ *lasI-rhII* double mutant than in the CHA strain. This last result is in agreement with earlier studies showing inhibition of the T3SS by the Rhl QS system (Blevess *et al.*, 2005; Hogardt *et al.* 2004). Second, we examined the ability of the wild-type and mutant strains to produce and secrete a T3SS inhibitory signal at high cell density. To do so, stationary-phase culture supernatants were prepared from the different strains as described in Methods. Then, to evaluate their influence on the expression of *exsCEBA*, each supernatant was added to the CHA (pC::lux) strain, which was cultured from an initial OD₆₀₀ of 0.05 in LB under T3SS-inducing conditions. The transcriptional activity from pC in the presence of supernatants from either the wild-type or the QS mutants was roughly twofold lower than in the absence of supernatants (Fig. 1d). As control, the same volume of VB was added instead of supernatant, and no inhibitory effect on T3SS expression was observed, ruling out a role for VB medium in the repression phenomenon. In order to exclude the role of known *P. aeruginosa* QS signal molecules in T3SS inhibition, stationary-phase culture supernatants were analysed by LC-MS. As shown in Table 3, both 3-oxo-C12-HSL and C4-HSL were produced in the wild-type CHA strain; conversely, the respective acylhomoserine lactone (AHL) was absent in the mutants Δ *lasI* and Δ *rhII* and in the double mutant Δ *lasI-rhII*. Furthermore, extraction experiments on wild-type stationary-phase supernatants using methods known to extract QS signals showed that the T3SS inhibitor(s) was not found in the organic phase, unlike the classical QS molecules (data not shown). In order to study the role of the third known QS signal molecule in *P. aeruginosa*, PQS, strains PAO1 and PAO1 *pqsA* were used. The *pqsA* mutant is not able to produce hydroxyl-alkyl-quinolone signals, including PQS

Fig. 1. Stationary-phase culture supernatants inhibit T3SS expression. (a, b) T3SS expression is dependent on cell density. (a) Wild-type CHA carrying a single copy of $pC::lux$ or a $pS::lux$ transcriptional reporter on its chromosome was grown at 37 °C in LB under T3SS-inducing conditions. At different times during bacterial growth, pC and pS promoter activity was measured by luminescence (RLU) and normalized to bacterial counts (6×10^8 bacteria; bac) (continuous lines, left axis). OD_{600} values of cultures at the selected time points are represented by dashed lines (right axis). Results represent the mean $\pm$ SE of triplicate samples, and are based on at least three independent experiments. $\circ$, CHApClux; $\blacktriangledown$, CHApSlux. (b) pC activity during growth of the wild-type CHA or its isogenic mutants ($\Delta lasI$, $\Delta rhII$ and $\Delta lasI\Delta rhII$) carrying a single copy of $pC::lux$, was measured by luminescence (RLU) and normalized to bacterial count (6×10^8 bacteria; bac). $\circ$, CHApClux; $\square$, $\Delta rhIIpClux$; $\blacksquare$, $\Delta lasI\Delta rhIIpClux$; $\bullet$, $\Delta lasIpClux$. (c) Fold-induction of T3SS from a culture of CHA (pApCgfp) (main culture) at different growth stages, represented by OD_{600} . Before T3SS induction, the OD_{600} of the main culture was kept constant (black bars) or set to 0.1 (white bars), and the medium was replaced with fresh medium. Results represent pC fold-induction. Bars are the mean $\pm$ SE of three independent experiments. (d) Stationary-phase culture supernatants inhibit *exsCEBA* expression independent of QS signals. The wild-type CHA ($pC::lux$) was grown at 37 °C in LB under T3SS-inducing conditions, supplemented or not supplemented with concentrated stationary-phase supernatants from CHA, $\Delta lasI$, $\Delta rhII$, $\Delta lasI\Delta rhII$ or $\Delta rpoS$, as indicated. –, No supernatant, but VB added. The final relative concentration of supernatant was 1 $\times$. RLU were measured at $OD_{600} \sim 1.7$ and normalized to bacterial counts (6×10^8 bacteria; bac). Bars represent the mean $\pm$ SE of triplicate samples and are based on three independent experiments.

(Fletcher *et al.*, 2007). Stationary-phase culture supernatants from these strains were produced as indicated in Methods and tested for T3SS repression activity. Cultures of CHA ($pC::lux$) with the PAO1 or the PQS-production-mutant supernatant had RLU levels (RLU/ OD_{600}) of 247.8 ± 16.08 and 215.2 ± 21.9 , respectively, compared with 1678.7 ± 326.5 without supernatant. Thus, two *P. aeruginosa* strains other than CHA, PAO1 and a PAO1 mutant unable to produce PQS, are also able to secrete the T3SS

inhibitory molecule(s). These results indicate the following: (i) there is/are T3SS-inhibiting signal(s) in the stationary-phase culture supernatants; (ii) known *P. aeruginosa* QS molecules (3-oxo-C12-HSL, C4-HSL and PQS) are not the inhibitory signal; and (iii) AHL synthases LasI, RhII and the AHQ synthesis pathway, which starts with PqsA, are not implicated in the production of such T3SS-inhibiting signal(s). Considering the important role of RpoS, a recognized stationary-phase central regulator (Loewen

Table 3. QS signal production in different *P. aeruginosa* strains

Values are in pmol.

Strain	C4-HSL	3-oxo-C12-HSL
CHA	8399	29.8
$\Delta lasI$	4149	0
$\Delta rhlI$	0	1.6
$\Delta lasI rhlI$	0	0

et al., 1998), which was recently identified as an inhibitor of T3SS expression (Hogardt *et al.*, 2004; Shen *et al.*, 2006), we prepared the stationary-phase culture supernatant from a $\Delta rpoS$ mutant and tested its role in *exsCEBA* expression. As shown in Fig. 1(d), deletion of *rpoS* does not change the inhibitory role of the supernatant on *exsCEBA* expression. Taken together, these results suggest the presence of unknown T3SS-inhibiting signals in the stationary-phase culture supernatants that are distinct from known QS signals, and whose synthesis is not dependent on QS signal synthesis.

Isolation and characterization of mutants impaired in T3SS-inhibiting signal(s) production

To identify the gene(s) involved in the production of the T3SS-inhibiting signal(s) in the stationary-phase culture supernatants, a library of $\sim 10^6$ Gm-resistant mutants was generated with the mariner-based transposon *Himar1::Gm^r* using plasmid pFAC (Diaz-Perez *et al.*, 2004). Roughly 2000 mutants were screened in 96-well plates for the effect of their stationary-phase culture supernatants on the expression of *exoS*. Twenty mutants whose supernatants seemed to lose their ability to inhibit *exoS* expression were selected in the first round of screening. After a second round of close observation, two mutants with a confirmed phenotype were further analysed by cloning their *Pst*I-digested genomic DNA fragments (flanked by the transposon) into the plasmid pUC18 and then sequencing. A Southern blot analysis revealed that the mutation was caused by a single transposition event (data not shown). Sequence comparison against the PAO1 genome (<http://www.pseudomonas.com>; Stover *et al.*, 2000) showed that the first transposon insertion occurred inside the ORF of *trpA* (PA0035), which encodes the Trp synthase chain involved in the biosynthesis of Trp (Hadero & Crawford, 1986), and the second occurred in *leuC* (PA3121), which encodes the 3-isopropylmalate dehydratase large subunit involved in the biosynthesis of leucine and isoleucine. The *trpA* mutant was selected for further investigations in this study because the affected gene is involved in the biosynthesis of Trp, a precursor of several important signalling molecules in plants and bacteria, including IAA (also termed auxin in plants), kynurenine and PQS (Fig. 2, Supplementary Table S1). Furthermore, PAO genome analysis revealed that all the genes required

for the synthesis of these signalling molecules exist in the genome of *P. aeruginosa* (Fig. 2, Supplementary Table S2).

Trp is involved in the production of T3SS-inhibiting signal(s)

To determine the role of *trpA* in the production of T3SS-inhibiting signal(s) during the stationary phase, $\Delta trpA$, a mutant strain with an unmarked in-frame deletion of *trpA*, was constructed through allelic exchange as described in Methods and confirmed by PCR (data not shown). Stationary-phase culture supernatants from this mutant were added to CHA (pC::lux) cultured from an initial OD₆₀₀ of 0.05 in LB under T3SS-inducing conditions. In contrast to the stationary-phase culture supernatants from the wild-type CHA, supernatants from $\Delta trpA$ lost their ability to inhibit *exsCEBA* expression and gave the same profile as a control in which supernatant was replaced by VB, the minimal medium used for the preparation of supernatants (Fig. 3). The addition of 200 μ g Trp ml⁻¹ in VB to the $\Delta trpA$ mutant culture restored the production of T3SS-inhibiting signal(s) from $\Delta trpA$. However, the addition of Trp during the preparation of supernatants from wild-type CHA did not change the ability of the supernatants to inhibit *exsCEBA* expression compared with the non-Trp-amended supernatants (Fig. 3). These results suggest that Trp might be involved in the production of T3SS-inhibiting signal(s) during the stationary phase, but that Trp itself is not the signal.

IAA, NAA and 3-hydroxykynurenine inhibit *exsCEBA* expression

Trp can serve as the precursor of IAA (Cohen *et al.*, 2003), the first plant hormone discovered, which is also termed auxin (Woodward & Bartel, 2005). It was therefore necessary to ascertain whether or not Trp and Trp-derivatives play a direct role in the regulation of T3SS expression. Different concentrations of Trp (up to 2.5 mM) were added to the CHA (pC::lux), cultured from an initial OD₆₀₀ of 0.05 in LB under T3SS-inducing and non-inducing conditions, but no transcriptional differences were observed (data not shown). However, as shown in Fig. 4(a), 1 mM IAA had an evident inhibitory effect on *exsCEBA* expression but did not inhibit bacterial growth. We also tested several commercial auxin-like compounds, including IBA, NAA and 2,4-D (Woodward & Bartel, 2005), but only NAA inhibited *exsCEBA* expression (Fig. 4a). Also, 1 mM 3-hydroxykynurenine, but not kynurenine, inhibited *exsCEBA* expression (Fig. 4a). Furthermore, no transcriptional difference was observed in the presence of up to 20 μ M PQS (data not shown), excluding a role for this QS signal in the T3SS inhibition described. To test the catabolic pathway derived from tryptophan that leads to kynurenine (Fig. 2), we constructed a *kynA* mutant and tested the inhibitory effect of a stationary-phase supernatant from this strain on T3SS activation. Unlike the $\Delta trpA$ strain, which no longer

Fig. 2. Trp is a precursor of several important signalling molecules in *Pseudomonas* spp. (1) Trp-dependent IAA biosynthesis. The two most common routes for IAA biosynthesis in bacteria are the indole-3-acetamide (IAM) pathway and indole-3-pyruvic acid (IPA) pathway (Lambrecht *et al.*, 2000). In *Pseudomonas syringae*, Trp monooxygenase (*laaM*) converts Trp to IAM, and an IAM hydrolase (*laaH*) converts IAM to IAA (Patten & Glick, 1996). *lpdc*, the gene that encodes indolepyruvate decarboxylase and the key gene in the IPA pathway for IAA synthesis, has been isolated from *Pseudomonas putida* (Patten & Glick, 2002). (2) Degradation of Trp to kynurenine. In *P. aeruginosa*, tryptophan 2,3-dioxygenase (TDO), encoded by *kynA* (PA2579), and kynurenine formamidase (KFA), encoded by *kynB* (PA 2081), are involved in this pathway (Kurnasov *et al.*, 2003). (3) PQS biosynthesis. Anthranilate is the precursor of PQS (Calfee *et al.*, 2001), and all enzymes required in the anthranilate pathway for Trp from *P. aeruginosa* have been characterized (Kurnasov *et al.*, 2003). KYN, kynureninase, encoded by *kynU* (PA2080).

Fig. 3. Trp is implicated in the production of T3SS-inhibiting signal(s). Wild-type CHA carrying a single copy of the pC::lux transcriptional reporter on its chromosome was grown at 37 °C in LB in the presence of the stationary-phase culture supernatants (indicated by asterisks). These were prepared in VB (CHA and Δ*trpA*) or VB supplemented with 200 μg Trp ml⁻¹ (CHA200 and Δ*trpA200*). RLU were measured over 4 h of culture and normalized to bacterial counts (6 × 10⁸ bacteria; bac). Bars represent the mean ± SE of triplicate samples and are derived from three independent experiments. White and black bars represent T3SS-inducing and non-inducing conditions, respectively. Neg ctrl and Pos ctrl are negative and positive induction controls, respectively, in which VB (instead of supernatants) was added.

produces a T3SS-inhibiting signal (Fig. 3), the *kynA* mutant still produced some T3SS-inhibiting signal, but much less than the wild-type. Indeed, CHA (pC::lux) grown in the presence of the *kynA* mutant supernatant showed an RLU level, representing T3SS activation, of 689.7 ± 23.7 RLU/OD₆₀₀. Culture grown without supernatant had an RLU level of 959.7 ± 68 RLU/OD₆₀₀, and the CHA supernatant induced a stronger repression (121.8 ± 4.7 RLU/OD₆₀₀). This result suggests that both the kynurenine-derived molecules and the IAA-derived molecules, both of which are Trp-derived molecules, could play a role in the downregulation of T3SS expression. This is indirectly supported by the fact that 3-hydroxykynurenine partially inhibited T3SS expression (Fig. 4a).

In addition to calcium depletion, *in vitro* growth in the presence of serum also leads to generalized T3SS expression and secretion (Vallis *et al.*, 1999). Therefore, we asked whether auxin compounds could inhibit the T3SS expression induced by human serum. As shown in Fig. 4(b), *exsCEBA* transcription was partially inhibited in the presence of 1 mM IAA or NAA, but not with 2,4-D or IBA. No pH change or growth difference was observed to arise from the use of either IAA or IAA-like compounds. Considering the fact that IBA is completely identical to IAA except for two additional methylene groups in the side chain, but has no effect on the expression of the T3SS induced by either calcium depletion or serum, we

Fig. 4. Trp derivatives inhibit T3SS expression. (a) IAA, NAA and 3-hydroxykynurenine inhibit EGTA-induced *exsCEBA* expression. Wild-type CHA carrying a single copy of the pC::lux transcriptional reporter in its chromosome was grown at 37 °C in LB in the absence or presence of 1 mM of the indicated commercial molecules under T3SS-inducing (white bars) or non-inducing conditions (black bars), as described in Methods. RLU were measured after 4 h of culture and related to normalized bacterial counts (6×10^8 bacteria; bac). Bars represent the mean $\pm$ SE of triplicate samples and are derived from three independent experiments. $\blacktriangle$, OD₆₀₀ under T3SS-inducing conditions. Kynu, kynurenine; Ctrl, control condition in which no molecules were added. (b) IAA and NAA inhibit serum-induced *exsCEBA* expression. A culture of the wild-type CHA strain (5×10^5 bacteria) carrying a single copy of the pC::lux transcriptional reporter on its chromosome was incubated with mHBS buffer or 10 % human serum in the absence or presence of 1 mM of the indicated molecules in a 96-well plate. RLU were measured each hour over 4 h of incubation (H1 to H4). Bars represent the mean $\pm$ SE of triplicate samples and are derived from two independent experiments. Ctrl, control condition in which no molecules were added.

concluded that IAA and NAA could inhibit *exsCEBA* expression specifically.

IAA and NAA inhibit type III effector secretion expression downstream of ExsA

The secretion of T3SS effectors strongly correlates with *P. aeruginosa* virulence both in animal models and in studies of human disease (Roy-Burman *et al.*, 2001). To examine whether IAA and NAA are involved in the inhibition of type III effector secretion, culture supernatants were analysed from wild-type CHA containing plasmid *pexsAind*, which can express *exsA* *in trans* via induction by IPTG. When the expression of the *exoS* transcriptional activator is induced using this construct, it allows T3SS gene expression and toxin production even in the presence of calcium (Epaulard *et al.*, 2006; Filopon *et al.*, 2006). As shown in Fig. 5, the growth of bacteria in the presence of EGTA resulted in type III effector secretion. However such secretion was prevented when 1 mM NAA or IAA (data not shown) was added, even when *exsA* was expressed *in trans* at the same time. To distinguish between inhibition of type III secretory activity or inhibition of ExsA-dependent activation, we measured the intrabacterial level of the ExoS and ExoT effectors with an anti-ExoS/T serum.

Fig. 5(b) indicates that even in the presence of *in trans*-expressed *exsA*, the levels of both ExoS and ExoT were dramatically diminished when NAA was added. This result suggests that IAA and NAA could negatively regulate type III effector expression by blocking the activation downstream of ExsA.

DISCUSSION

P. aeruginosa T3SS expression is controlled by multi-component regulatory networks, which integrate a diverse set of environmental cues and whose most likely function is to restrict the energy-consuming expression of 43 genes to the correct place and time (Yahr & Wolfgang, 2006). It would not be surprising if a mechanism exists to arrest T3SS gene expression when not required, since a mechanism exists to activate all of the T3SS operons when necessary through the activation of the ExsA-mediated positive regulatory loop. In this study we showed that the stationary-phase culture supernatants of at least two *P. aeruginosa* strains significantly repress T3SS gene expression (by ~60 % with the tested concentration), and we described our attempts to discover the nature of such T3SS-inhibiting signal(s).

Fig. 5. NAA inhibits T3SS expression even in the presence of *in trans*-expressed ExsA. (a) Wild-type CHA expressing exogenous ExsA from IPTG-inducible plasmid *pexsA*ind was grown in the absence (–) or presence (+) of 1 mM NAA under T3SS-inducing (EGTA+) or non-inducing conditions (EGTA–). IPTG was used at 1 mM concentration. Supernatants were normalized to bacterial counts (6×10^8 per lane) and subjected to TCA precipitation. The extracellular proteins were separated by 10% SDS-PAGE and stained with Coomassie Blue. M, protein marker; sizes in kDa are indicated on the left. (b) Western blot of normalized supernatants or bacterial lysates after separation by 10% SDS-PAGE, transfer onto nitrocellulose membranes and revelation with an anti-ExoS/T serum at 1:1000 dilution.

Considering that QS is a generic regulatory mechanism that controls the expression of virulence genes, including T3SS genes, in a population-density-dependent manner (Juhas *et al.*, 2005), we first ruled out the possibility that such strong T3SS-inhibiting signal(s) are associated with the QS system (Fig. 1d) and showed that neither acyl-HSLs nor PQS are involved in T3SS repression. Transposition mutants allowed us to find evidence that the production of the T3SS-inhibiting signal(s) is Trp-dependent (Fig. 3). Thus tryptophan catabolites accumulate in culture supernatant during growth and act as a QS signal to inhibit T3SS gene expression. In the experiment described in Fig. 1(c), cells from a stationary-phase culture were sedimented, suspended in fresh growth medium and assayed for T3SS gene expression. One would presume that suspending the cells in fresh growth medium would dilute the inhibitory catabolites, and that T3SS gene expression would be restored, contradicting our results. Nevertheless, production of such a signal should not be shut off immediately after medium replacement and should again rapidly reach inhibitory levels in high-cell-density culture due both to the high number of bacteria secreting the catabolites and to a possible auto-induction mechanism to induce their production, similar to the classic QS signalling system. Considering the fact that tryptophan is the precursor of several important signalling molecules, including IAA and

kynurenine, we tested these commercially available molecules in order to investigate their roles in the regulation of T3SS expression.

IAA is critical for plant growth and development, and is often effective at sub-micromolar concentrations in plants (Woodward & Bartel, 2005). Although it is widely accepted that plants use both Trp-dependent and Trp-independent routes to synthesize IAA, the relevant genes, enzymes and intermediates of these pathways have not yet all been identified. Many bacteria are also able to produce IAA, mainly by using a Trp-dependent pathway (Lambrecht *et al.*, 2000), and such a pathway is indeed observed in *Pseudomonas* spp. (Patten & Glick, 2002) (Fig. 2). Recent studies have shown that several strains of *P. aeruginosa* isolated from soil (Bano & Musarrat, 2003) can produce IAA, and a maximum production of IAA of 19.1 mg ml^{-1} (equivalent to 0.1 mM) by strain *P. aeruginosa* FP10 has been observed after 42 h incubation in Dworkin and Foster (DF) salts medium supplemented with $500 \text{ } \mu\text{g Trp ml}^{-1}$ (Ayyadurai *et al.*, 2006).

IAA and a compound similar to IAA, NAA, can indeed inhibit T3SS expression and type III effector secretion at millimolar concentrations (Figs 4 and 5). Although one study has recently shown that IAA can inhibit the expression of genes required for the transfer of T-DNA (type IV secretion) in *Agrobacterium tumefaciens* via VirA, a sensor kinase of a two-component regulatory system (Liu & Nester, 2006), we found that *P. aeruginosa* PA1243 (www.pseudomonas.com) (Stover *et al.*, 2000), which has 49% similarity to VirA, was not involved in the IAA-dependent inhibition of T3SS expression (data not shown). Since the transcription of the T3SS is coupled with secretion (Urbanowski *et al.*, 2005; Rietsch *et al.*, 2005), it is possible that IAA or NAA blocks the secretion channel and thus blocks the activation of ExsA and the transcriptional activation of T3SS genes. Indeed, an equivalent process has been demonstrated for recently identified inhibitory molecules that act as ‘calcium mimetics’ for *Yersinia* type III secretion (Nordfelth *et al.*, 2005). However, in our case we showed clearly that NAA is able to inhibit effector production even in the presence of *in trans*-expressed *exsA*, suggesting that the signal transduction pathway that responds to tryptophan derivatives acts downstream of *exsA* transcription activation of T3SS genes. Recently, one study has shown that RetS/LadS/GacAS, two-component regulatory systems in *P. aeruginosa*, are implicated in the transition between activation of the T3SS (acute infection) and biofilm (chronic infection) via a post-transcriptional regulation of targeted genes after the detection of an unknown signal (Ventre *et al.*, 2006). Therefore, it would be interesting to investigate whether the signals described here are the same as those detected by the RetS/LadS/GacAS pathway.

IAA itself was not detected in our stationary-phase culture supernatants using Salkowski’s reagent (Patten & Glick, 2002). Thus, IAA does not seem to be the natural

T3SS-inhibiting signal, although genes encoding monooxygenase and aminotransferase, enzymes needed in the Trp-dependent biosynthesis of IAA (Supplementary Table S2), do exist in the genome of *P. aeruginosa* PAO1 (www.pseudomonas.com) (Stover *et al.*, 2000). However, we still have evidence that T3SS-inhibiting signals could be Trp catabolites, such as NAA and 3-hydroxykynurenine, because (i) these compounds may be Trp-derived, (ii) they have an inhibitory effect on T3SS expression, and (iii) a *kynA* mutant supernatant loses some ability to induce repression of T3SS genes. Since there are at least seven potential genes involved in the production of IAA (Supplementary Table S2), we have not examined all of their roles; however, a thorough examination of these genes would provide a more precise profile of the role of tryptophan catabolites in the regulation of T3SS expression.

The reason why Trp-derived catabolites, such as IAA, NAA and kynurenin-derived molecules, are directly or indirectly involved in the regulation of T3SS expression remains to be discovered, but we do know that metabolic signals/stresses have a profound influence on T3SS expression. Mutants that lack pyruvate dehydrogenase (*aceA* or *aceB*) (Dacheux *et al.*, 2002) or a glucose transport regulator (*gltR*) (Wolfgang *et al.*, 2003) fail to induce T3SS expression under calcium-depletion conditions. Similarly, overproduction of multi-drug (MDR) efflux pumps (Linares *et al.*, 2005) or overexpression of genes involved in histidine transport and metabolism (Rietsch *et al.*, 2004) prevents T3SS expression. In our study, we have not further examined the *leuC* transposon mutant because its phenotype is less apparent than that of Δ *trpA*. However, the supernatant of the *leuC* transposon mutant did lose some ability to inhibit T3SS expression, further suggesting that metabolic signals/stresses play an important role in the regulation of T3SS expression, or that multiple pathways act synergistically to prevent T3SS activation at high cell density.

A recent study has suggested that one catabolite controlling T3SS expression is derived from acetyl-CoA (Rietsch & Mekalanos, 2006); another study has shown that IAA (0.5 mM) is capable of inducing changes in gene expression, enzyme activity and metabolite level in central metabolic pathways in *E. coli*, with a 2.7-fold increase in the production of acetyl-CoA (Bianco *et al.*, 2006). It follows that the relationship between IAA, acetyl-CoA and T3SS expression needs further investigation. Finally, it appears that a combination of different metabolic or stress signals, either secreted (such as Trp catabolites) or acting directly inside the bacterial cells, is involved in the downregulation of T3SS.

We are convinced that targeting virulence gene expression represents a new method for antibacterial treatment, as shown recently for *Vibrio cholerae*, whereby a small-molecule inhibitor of both toxin and pili expression was shown to protect infant mice from colonization (Hung *et al.*, 2005). Two different small-molecule inhibitors of T3SS

have also been identified as potential drugs in the treatment of *Chlamydia trachomatis*, a Gram-negative bacterium causing sexually transmitted disease and preventable blindness worldwide (Wolf *et al.*, 2006). Thus, the role of Trp catabolites in the acute infection of *P. aeruginosa* and of their receptors on the surface of bacteria remains an interesting area of study. Very recently, another Trp oxidative metabolite, 3-hydroxyoxindole, has been reported to be involved in the regulation of the virulence of *Ralstonia solanacearum* (Delaspre *et al.*, 2007).

Finally, it has also been proven that auxin is the signal for plant-microbe interactions in symbiosis. The question therefore arises concerning the beneficial effects of auxin-like molecules on *P. aeruginosa* during chronic colonization. Interestingly, the signal seen in this study, which is used by *P. aeruginosa* to stop T3SS activity, could at the same time allow the bacteria to become toxic to neutrophils or macrophages, since IAA-like compounds have been shown to be toxic to these cells (Pires de Melo *et al.*, 1998). Furthermore, it has been shown that tryptophan metabolites such as kynurenin, 3-hydroxykynurenin and anthranilic acids suppress the T-cell response in humans (Terness *et al.*, 2002). These compounds act synergistically to induce T-cell cytotoxicity. It has also been demonstrated recently that QS molecules can act as immunomodulators (Pritchard, 2006). It therefore appears essential to investigate the influence of these novel signalling molecules derived from tryptophan in the modulation of host physiological functions and hence in the virulence of *P. aeruginosa*.

ACKNOWLEDGEMENTS

We thank J. Campos-Garcia, Universidad Michoacana de San Nicolás de Hidalgo, Michoacán, Mexico, for providing plasmid pFAC, P. Williams, University of Nottingham, UK, for providing strains PAO1 and PAO1 *pqsA*, and also D. Haras for the quantification of HSL in supernatants. We also thank Gareth Butt for correcting the final draft. S.D.K. was supported by a PhD scholarship from the Agence Universitaire de la Francophonie.

REFERENCES

- Ayyadurai, N., Ravindra Naik, P., Sreehari Rao, M., Sunish Kumar, R., Samrat, S. K., Manohar, M. & Sakthivel, N. (2006). Isolation and characterization of a novel banana rhizosphere bacterium as fungal antagonist and microbial adjuvant in micropropagation of banana. *J Appl Microbiol* **100**, 926–937.
- Bano, N. & Musarrat, J. (2003). Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. *Curr Microbiol* **46**, 324–328.
- Bianco, C., Imperlini, E., Calogero, R., Senatore, B., Pucci, P. & Defez, R. (2006). Indole-3-acetic acid regulates the central metabolic pathways in *Escherichia coli*. *Microbiology* **152**, 2421–2431.
- Blevess, S., Soscia, C., Nogueira-Orlandi, P., Lazdunski, A. & Filloux, A. (2005). Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **187**, 3898–3902.

- Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* 5, 279–313.
- Calfee, M. W., Coleman, J. P. & Pesci, E. C. (2001). Interference with *Pseudomonas* quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 98, 11633–11637.
- Cohen, J. D., Slovin, J. P. & Hendrickson, A. M. (2003). Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. *Trends Plant Sci* 8, 197–199.
- Dacheux, D., Attree, I., Schneider, C. & Toussaint, B. (1999). Cell death of human polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional type III secretion system. *Infect Immun* 67, 6164–6167.
- Dacheux, D., Toussaint, B., Richard, M., Brochier, G., Croize, J. & Attree, I. (2000). *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infect Immun* 68, 2916–2924.
- Dacheux, D., Attree, I. & Toussaint, B. (2001). Expression of ExsA in trans confers type III secretion system-dependent cytotoxicity on noncytotoxic *Pseudomonas aeruginosa* cystic fibrosis isolates. *Infect Immun* 69, 538–542.
- Dacheux, D., Epaulard, O., de Groot, A., Guery, B., Leberre, R., Attree, I., Polack, B. & Toussaint, B. (2002). Activation of the *Pseudomonas aeruginosa* type III secretion system requires an intact pyruvate dehydrogenase *aceAB* operon. *Infect Immun* 70, 3973–3977.
- Dahan, S., Knutton, S., Shaw, R. K., Crepin, V. F., Dougan, G. & Frankel, G. (2004). Transcriptome of enterohemorrhagic *Escherichia coli* O157 adhering to eukaryotic plasma membranes. *Infect Immun* 72, 5452–5459.
- Dasgupta, N., Lykken, G. L., Wolfgang, M. C. & Yahr, T. L. (2004). A novel anti-anti-activator mechanism regulates expression of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* 53, 297–308.
- Delaspre, F., Nieto Penalver, C. G., Saurel, O., Kiefer, P., Gras, E., Milon, A., Boucher, C., Genin, S. & Vorholt, J. A. (2007). The *Ralstonia solanacearum* pathogenicity regulator HrpB induces 3-hydroxyoxindole synthesis. *Proc Natl Acad Sci U S A* 104, 15870–15875.
- Delic-Attree, I., Toussaint, B. & Vignais, P. M. (1995). Cloning and sequence analyses of the genes coding for the integration host factor (IHF) and HU proteins of *Pseudomonas aeruginosa*. *Gene* 154, 61–64.
- Della Chiesa, M., Carlomagno, S., Frumento, G., Balsamo, M., Cantoni, C., Conte, R., Moretta, L., Moretta, A. & Vitale, M. (2006). The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46- and NKG2D-activating receptors and regulates NK-cell function. *Blood* 108, 4118–4125.
- Diaz-Perez, A. L., Zavala-Hernandez, A. N., Cervantes, C. & Campos-Garcia, J. (2004). The *gnyRDBHAL* cluster is involved in acyclic isoprenoid degradation in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 70, 5102–5110.
- Epaulard, O., Toussaint, B., Quenee, L., Derouazi, M., Bosco, N., Villiers, C., Le Berre, R., Guery, B., Filopon, D. & other authors (2006). Anti-tumor immunotherapy via antigen delivery from a live attenuated genetically engineered *Pseudomonas aeruginosa* type III secretion system-based vector. *Mol Ther* 14, 656–661.
- Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* 47, 103–118.
- Faucher, S. P., Porwollik, S., Dozois, C. M., McClelland, M. & Daigle, F. (2006). Transcriptome of *Salmonella enterica* serovar Typhi within macrophages revealed through the selective capture of transcribed sequences. *Proc Natl Acad Sci U S A* 103, 1906–1911.
- Feltman, H., Schuler, G., Khan, S., Jain, M., Peterson, L. & Hauser, A. R. (2001). Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology* 147, 2659–2669.
- Filopon, D., Merieau, A., Bernot, G., Comet, J. P., Leberre, R., Guery, B., Polack, B. & Guespin-Michel, J. (2006). Epigenetic acquisition of inducibility of type III cytotoxicity in *P. aeruginosa*. *BMC Bioinformatics* 7, 272.
- Fletcher, M. P., Diggle, S. P., Cámara, M. & Williams, P. (2007). Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules. *Nat Protoc* 2, 1254–1262.
- Frumento, G., Rotondo, R., Tonetti, M., Damonte, G., Benatti, U. & Ferrara, G. B. (2002). Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med* 196, 459–468.
- Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR–LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* 176, 269–275.
- Garau, J. & Gomez, L. (2003). *Pseudomonas aeruginosa* pneumonia. *Curr Opin Infect Dis* 16, 135–143.
- Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 7, 745–754.
- Ha, U. & Jin, S. (2001). Growth phase-dependent invasion of *Pseudomonas aeruginosa* and its survival within HeLa cells. *Infect Immun* 69, 4398–4406.
- Ha, U. H., Kim, J., Badrane, H., Jia, J., Baker, H. V., Wu, D. & Jin, S. (2004). An *in vivo* inducible gene of *Pseudomonas aeruginosa* encodes an anti-ExsA to suppress the type III secretion system. *Mol Microbiol* 54, 307–320.
- Hadero, A. & Crawford, I. P. (1986). Nucleotide sequence of the genes for tryptophan synthase in *Pseudomonas aeruginosa*. *Mol Biol Evol* 3, 191–204.
- Hauser, A. R., Cobb, E., Bodi, M., Mariscal, D., Valles, J., Engel, J. N. & Rello, J. (2002). Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. *Crit Care Med* 30, 521–528.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP–FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212, 77–86.
- Hogardt, M., Roeder, M., Schreff, A. M., Eberl, L. & Heesemann, J. (2004). Expression of *Pseudomonas aeruginosa* *exoS* is controlled by quorum sensing and RpoS. *Microbiology* 150, 843–851.
- Hovey, A. K. & Frank, D. W. (1995). Analyses of the DNA-binding and transcriptional activation properties of ExsA, the transcriptional activator of the *Pseudomonas aeruginosa* exoenzyme S regulon. *J Bacteriol* 177, 4427–4436.
- Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62, 379–433.
- Hung, D. T., Shakhnovich, E. A., Pierson, E. & Mekalanos, J. J. (2005). Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. *Science* 310, 670–674.
- Hwu, P., Du, M. X., Lapointe, R., Do, M., Taylor, M. W. & Young, H. A. (2000). Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol* 164, 3596–3599.

- Jain, M., Ramirez, D., Seshadri, R., Cullina, J. F., Powers, C. A., Schulert, G. S., Bar-Meir, M., Sullivan, C. L., McColley, S. A. & Hauser, A. R. (2004). Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. *J Clin Microbiol* **42**, 5229–5237.
- Juhas, M., Eberl, L. & Tummli, B. (2005). Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7**, 459–471.
- Kurnasov, O., Jablonski, L., Polanuyer, B., Dorrestein, P., Begley, T. & Osterman, A. (2003). Aerobic tryptophan degradation pathway in bacteria: novel kynurenine formamidase. *FEMS Microbiol Lett* **227**, 219–227.
- Lambrecht, M., Okon, Y., Vande Broek, A. & Vanderleyden, J. (2000). Indole-3-acetic acid: a reciprocal signalling molecule in bacteria–plant interactions. *Trends Microbiol* **8**, 298–300.
- Laskowski, M. A., Osborn, E. & Kazmierczak, B. I. (2004). A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **54**, 1090–1103.
- Lee, V. T., Smith, R. S., Tummli, B. & Lory, S. (2005). Activities of *Pseudomonas aeruginosa* effectors secreted by the type III secretion system in vitro and during infection. *Infect Immun* **73**, 1695–1705.
- Linares, J. F., Lopez, J. A., Camafeita, E., Albar, J. P., Rojo, F. & Martinez, J. L. (2005). Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 1384–1391.
- Liu, P. & Nester, E. W. (2006). Indoleacetic acid, a product of transferred DNA, inhibits *vir* gene expression and growth of *Agrobacterium tumefaciens* C58. *Proc Natl Acad Sci U S A* **103**, 4658–4662.
- Loewen, P. C., Hu, B., Strutinsky, J. & Sparling, R. (1998). Regulation in the *rpoS* regulon of *Escherichia coli*. *Can J Microbiol* **44**, 707–717.
- Lucchini, S., Liu, H., Jin, Q., Hinton, J. C. & Yu, J. (2005). Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen. *Infect Immun* **73**, 88–102.
- Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**, 194–222.
- Marx, C. J. & Lidstrom, M. E. (2002). Broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. *Biotechniques* **33**, 1062–1067.
- McCaw, M. L., Lykken, G. L., Singh, P. K. & Yahr, T. L. (2002). ExsD is a negative regulator of the *Pseudomonas aeruginosa* type III secretion regulon. *Mol Microbiol* **46**, 1123–1133.
- Morin, D., Grasland, B., Vallee-Rehel, K., Dufau, C. & Haras, D. (2003). On-line high-performance liquid chromatography-mass spectrometric detection and quantification of *N*-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. *J Chromatogr A* **1002**, 79–92.
- Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H. & Elofsson, M. (2005). Small-molecule inhibitors specifically targeting type III secretion. *Infect Immun* **73**, 3104–3114.
- Patten, C. L. & Glick, B. R. (1996). Bacterial biosynthesis of indole-3-acetic acid. *Can J Microbiol* **42**, 207–220.
- Patten, C. L. & Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl Environ Microbiol* **68**, 3795–3801.
- Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229–11234.
- Pier, G. B. (2002). CFTR mutations and host susceptibility to *Pseudomonas aeruginosa* lung infection. *Curr Opin Microbiol* **5**, 81–86.
- Pires de Melo, M., Curi, T. C., Miyasaka, C. K., Palanch, A. C. & Curi, R. (1998). Effect of indole acetic acid on oxygen metabolism in cultured rat neutrophil. *Gen Pharmacol* **31**, 573–578.
- Pritchard, D. I. (2006). Immune modulation by *Pseudomonas aeruginosa* quorum-sensing signal molecules. *Int J Med Microbiol* **296**, 111–116.
- Quenee, L., Lamotte, D. & Polack, B. (2005). Combined *sacB*-based negative selection and *cre-lox* antibiotic marker recycling for efficient gene deletion in *Pseudomonas aeruginosa*. *Biotechniques* **38**, 63–67.
- Rietsch, A. & Mekalanos, J. J. (2006). Metabolic regulation of type III secretion gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* **59**, 807–820.
- Rietsch, A., Wolfgang, M. C. & Mekalanos, J. J. (2004). Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. *Infect Immun* **72**, 1383–1390.
- Rietsch, A., Vallet-Gely, I., Dove, S. L. & Mekalanos, J. J. (2005). ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **102**, 8006–8011.
- Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W. & Wiener-Kronish, J. P. (2001). Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* **183**, 1767–1774.
- Schaber, J. A., Carty, N. L., McDonald, N. A., Graham, E. D., Cheluvappa, R., Griswold, J. A. & Hamood, A. N. (2004). Analysis of quorum sensing-deficient clinical isolates of *Pseudomonas aeruginosa*. *J Med Microbiol* **53**, 841–853.
- Schweizer, H. P. & Hoang, T. T. (1995). An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. *Gene* **158**, 15–22.
- Shen, D. K., Filopon, D., Kuhn, L., Polack, B. & Toussaint, B. (2006). PsrA is a positive transcriptional regulator of the type III secretion system in *Pseudomonas aeruginosa*. *Infect Immun* **74**, 1121–1129.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1**, 784–791.
- Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S. & Kaper, J. B. (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* **96**, 15196–15201.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964.
- Terness, P., Bauer, T. M., Rose, L., Dufter, C., Watzlik, A., Simon, H. & Opelz, G. (2002). Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* **196**, 447–457.
- Toussaint, B., Delic-Attree, I. & Vignais, P. M. (1993). *Pseudomonas aeruginosa* contains an IHF-like protein that binds to the *algD* promoter. *Biochem Biophys Res Commun* **196**, 416–421.
- Urbanowski, M. L., Lykken, G. L. & Yahr, T. L. (2005). A secreted regulatory protein couples transcription to the secretory activity of the *Pseudomonas aeruginosa* type III secretion system. *Proc Natl Acad Sci U S A* **102**, 9930–9935.
- Vallis, A. J., Yahr, T. L., Barbieri, J. T. & Frank, D. W. (1999). Regulation of ExoS production and secretion by *Pseudomonas aeruginosa* in response to tissue culture conditions. *Infect Immun* **67**, 914–920.

- Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. & Filloux, A. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**, 171–176.
- Vogel, H. J. & Bonner, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem* **218**, 97–106.
- West, S. E., Schweizer, H. P., Dall, C., Sample, A. K. & Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**, 81–86.
- Wolf, K., Betts, H. J., Chellas-Gery, B., Hower, S., Linton, C. N. & Fields, K. A. (2006). Treatment of *Chlamydia trachomatis* with a small molecule inhibitor of the *Yersinia* type III secretion system disrupts progression of the chlamydial developmental cycle. *Mol Microbiol* **61**, 1543–1555.
- Wolfgang, M. C., Lee, V. T., Gilmore, M. E. & Lory, S. (2003). Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* **4**, 253–263.
- Wong, S. M. & Mekalanos, J. J. (2000). Genetic footprinting with *mariner*-based transposition in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **97**, 10191–10196.
- Woodward, A. W. & Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann Bot (Lond)* **95**, 707–735.
- Yahr, T. L. & Frank, D. W. (1994). Transcriptional organization of the *trans*-regulatory locus which controls exoenzyme S synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 3832–3838.
- Yahr, T. L. & Wolfgang, M. C. (2006). Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* **62**, 631–640.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zolfaghar, I., Angus, A. A., Kang, P. J., To, A., Evans, D. J. & Fleiszig, S. M. (2005). Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms. *Microbes Infect* **7**, 1305–1316.

Edited by: P. Cornelis