Role of *fimV* in type II secretion system-dependent protein secretion of *Pseudomonas aeruginosa* on solid medium

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Although classical type II secretion systems (T2SSs) are widely present in Gram-negative bacteria, atypical T2SSs can be found in some species. In *Pseudomonas aeruginosa*, in addition to the classical T2SS Xcp, it was reported that two genes, *xphA* and *xqhA*, located outside the *xcp* locus were organized in an operon (*PaQa*) which encodes the orphan *PaQa* subunit. This subunit is able to associate with other components of the classical Xcp machinery to form a functional hybrid T2SS. In the present study, using a transcriptional *lacZ* fusion, we found that the *PaQa* operon was more efficiently expressed (i) on solid LB agar than in liquid LB medium, (ii) at 25 C than at 37 C and (iii) at an early stage of growth. These results suggested an adaptation of the hybrid system to particular environmental conditions. Transposon mutagenesis led to the finding that *vfr* and *fimV* genes are required for optimal expression of the orphan *PaQa* operon in the defined growth conditions used. Using an original culturing device designed to monitor secretion on solid medium, the ring-plate system, we found that T2SS-dependent secretion of exoproteins, namely the elastase LasB, was affected in a *fimV* deletion mutant. Our findings led to the discovery of an interplay between FimV and the global regulator Vfr triggering the modulation of the level of Vfr and consequently the modulation of T2SS-dependent secretion on solid medium.

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INTRODUCTION

The Gram-negative pathogen *Pseudomonas aeruginosa* has evolved a number of sophisticated mechanisms that help it to battle hostile environment and host defences. One of these mechanisms involves secretion of virulence factors such as proteases, phospholipases and toxins that constitute efficient molecular weapons allowing the colonization of host cells by bacteria (Bleves *et al.*, 2010). Six secretion systems have been described so far in Gram-negative bacteria ranging from the type I to the type VI secretion system. *P. aeruginosa* possesses five of the six secretion systems reported, with only the type IV secretion system absent in this bacterium. Such a diverse arsenal shows that it can be a powerful pathogen involved in a wide range of severe and sometimes fatal diseases including cystic fibrosis, nosocomial pneumonia and infections in surgery patients (Bodey *et al.*, 1983).

Most of the main degradative enzymes of *P. aeruginosa* are secreted by the type II secretion system (T2SS), a two-step mechanism involving a transient periplasmic step before

exoenzymes are targeted to the extracellular medium. This system is genetically organized into two divergent operons encoding XcpR-Z and XcpPQ components. The T2SS can be viewed as a molecular nanomachine that consists of an inner-membrane platform, including XcpPRSYZ proteins, and a fibrillar structure (the pseudopilus) composed of the pseudopilins XcpTUVWX that act like a piston using elongation and retraction movements to push exoproteins out of the cell through a channel made by multimers of the secretin XcpQ (Michel & Voulhoux, 2009). The T2SS process was shown to be very specific since components of the machinery cannot be exchanged between distant species (Bouley *et al.*, 2001; Filloux, 2004). In other respects, exoproteins cannot be secreted from one species to another, except for closely related species (de Groot *et al.*, 2001). Such a high specificity was shown to be particularly dependent on XcpP and XcpQ proteins and homologues, which are the gate keepers of the T2SS machinery (Lindeberg *et al.*, 1996; Bleves *et al.*, 1999) and constitute a functional subunit referred as the PQ subunit in *P. aeruginosa*.

Outside the *xcp* locus components of the classical T2SS, we previously reported the presence of two orphan

Abbreviations: QS, quorum sensing; T2SS, type II secretion system.

genes, *xphA* and *xqhA*, organized in an operon, which were found to be homologous to the *xcpP* and *xcpQ* genes, respectively. These genes encode the orphan PaQa subunit which can replace the classical PQ subunit and associate with the XcpR-Z components to make up a functional hybrid T2SS machinery (Michel *et al.*, 2007). The hybrid T2SS was shown to efficiently secrete all the T2SS-dependent exoproteins, except for the aminopeptidase PaAP (PA2939) which accumulated within the cells.

Although the role of this atypical system has not yet been elucidated, we hypothesized that it could (i) either co-exist with the classical T2SS or serve as a rescue system, (ii) only be required in some specific growth conditions such as growth on solid media or in biofilms or (iii) be induced in response to unknown signals occurring during host infection (Michel *et al.*, 2007). In the present work, we aimed to investigate the growth conditions required to optimize functioning of the hybrid T2SS and finding regulators that control expression of the genes encoding the orphan PaQa subunit, in order to better understand the physiological role of this atypical secretory system.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. *P. aeruginosa* strains were grown either in Luria-Bertani (LB) or tryptic soy broth (TSB) at the adequate growth temperature. When required, 150 or 300 µg carbenicillin ml⁻¹ (in liquid or solid culture, respectively) and 75 µg gentamicin ml⁻¹ were added to the growth medium. To generate the *fimV* deletion, 500 bp upstream and 500 bp downstream of the *fimV* gene were amplified by overlapping PCR using dfimV1, dfimV2, dfimV3 and dfimV4 primers (Table 1) and the PCR product was cloned in pKNG101 giving the mutator vector pKNGΔ*fimV*. The *xcpQ xqhA* deletion was generated using pKNGΔ*xqhA* mutation vector in the PAO1ΔQ strain. The resulting strain PAO1ΔQQa was then deleted for *fimV* by using pKNGΔ*fimV* resulting in the PAO1ΔQQaΔ*fimV* strain. The deletion mutants were generated as described by Bleves *et al.* (1999). Wild-type *fimV* gene obtained from plasmid pASM281 was subcloned at the *KpnI/XbaI* sites of pMMB67EH.

Transcriptional fusions. Transcriptional *PPaQa-lacZ* (formerly called *xphA-lacZ* in Michel *et al.*, 2007) and *PPQ-lacZ* fusions were generated in wild-type PAO1 and in mutants by following the procedure of Hoang *et al.* (2000). Expression of the transcriptional fusions was assayed by β-galactosidase activity (Sambrook *et al.*, 1989) using cells grown either in liquid or on solid LB media. In order to standardize growth on solid medium, 2 ml liquid LB medium was first inoculated with frozen cells and grown for 8–10 h at 37 °C. Then, 0.1 OD₆₀₀ equivalent cell unit was loaded on LB agar plates, rigorously streaked in squares of 1.5 × 1.5 cm and incubated at the required temperature for 15 h. Cells were scraped from the plates, resuspended in 1 ml LB and 1 OD₆₀₀ equivalent cell unit was centrifuged (10 000 g, 5 min, 4 °C). β-Galactosidase activity was assayed at 30 °C as described by Sambrook *et al.* (1989) and results were expressed as described by Miller (1972). Statistical analysis of data was performed by paired Student's *t*-test (Excel software).

Construction of mutant library. A mutant library was generated in the strain bearing the *PPaQa-lacZ* transcriptional fusion using the transposon Mariner C9 according to the procedure of Kulasekara *et al.* (2005). The library was screened on selective LB agar medium

containing 75 µg gentamicin ml⁻¹, 25 µg Irgasan ml⁻¹ and 25 µg X-Gal ml⁻¹. Localization of the transposon insertions was carried out by two-round arbitrary PCR using pBT20-1/ARB1D and pBT20-2/ARB2A primer pairs (Table 1). PCR products were sequenced (GATC Company) and mutated genes were identified by BLAST analysis with the sequenced genome of *P. aeruginosa* (<http://v2.pseudomonas.com>).

Protein secretion on solid medium. Protein secretion on solid medium was investigated using the ring-plate culturing system adapted from Levin *et al.* (2007) and modified as follows. Cells were cultured essentially as described above for the study of transcriptional fusions. Cells (0.2 OD₆₀₀ equivalent cell unit) were added on an LB agar disc (1.5–2 mm thick) loaded on a polycarbonate filter (0.05 µm) placed at the centre of the ring-plate. The filter membrane was upheld by the ring compartment of the ring-plate and was in contact with the LB liquid medium filling the rings at the bottom of the device. The ring-plate was then incubated at the required temperature for 5–20 h according to the experiment. During growth, secreted proteins crossed both the agar disc and the filter and were finally recovered in the liquid compartment, while cells were retained by the filter. The liquid filtrate containing extracellular proteins was either directly used for protease activity assays or precipitated on ice with 10 % (w/v) trichloroacetic acid (TCA) and the proteins were treated as previously described for SDS-PAGE or immunodetection analyses (Michel *et al.*, 2007).

Protein analysis and immunodetection. Extracellular proteins were separated from cells by centrifugation (10 000 g, 5 min, 4 °C). Extracellular proteins were precipitated on ice with 10 % (w/v) TCA and treated as described previously (Michel *et al.*, 2007). Cellular and extracellular proteins were solubilized with Laemmli lysis buffer (Laemmli, 1970), separated by SDS-PAGE (12 % w/v) and visualized by either immunodetection as described previously (Michel *et al.*, 2007) or Imperial blue protein stain (Pierce) and silver staining (Swain & Ross, 1995). When indicated, protein quantification was performed using ImageJ software (<http://www.rsweb.nih.gov/ij/>).

Proteolytic activities. Protease activity from liquid extracts was assayed according to Wretling & Pavlovskis (1984) using Azocasein as substrate. Protease activities on solid medium were regularly detected after streaking cells on tryptic soy agar (TSA) containing skimmed milk and incubating plates at 37 or 25 °C. Secretion of active proteases was detected as a clear halo.

RESULTS AND DISCUSSION

Proteolytic activities dependent on hybrid T2SS

As previously reported, reconstitution of the hybrid T2SS constituted by the XcpR-Z components of the classical T2SS and the orphan subunit PaQa restored the secretion of most of the T2SS substrates (Michel *et al.*, 2007). In the present study, using a proteolytic activity assay, a secretion test commonly used in secretion studies, we observed that the ΔPQP_{PaQa} mutant complemented for the *PaQa* mutation exhibited only about 40 % of the total extracellular proteolytic activity recovered in the parent strain (Table 2). These results suggested that restoration of the secretion efficiency by the hybrid T2SS was only partial in liquid cultures. In contrast, the complemented mutant grown on a TSA-milk plate, another typical secretion assay, exhibited the same hydrolytic halo as the parent strain, showing that in these growth conditions, protein secretion was fully restored (Table 2). These results are consistent

Table 1. Strains, plasmids and primers

Strain/plasmid/primer	Description/sequence	Reference or source
Strains		
<i>Escherichia coli</i>		
TG1	<i>supE hsdΔR thiΔ(lac-proAB), F' (traD36 proAB⁺ lacI^A lacΔM15)</i>	Laboratory collection
1047	<i>met supE hsdS recA</i>	Laboratory collection
CC118 (<i>λpir</i>)	<i>Δ(ara-leu) araD ΔlacX74 galE galK phoA-20 thi-1 rpsE rpoB Arg(Am) recA1, Rf^r (λpir)</i>	Herrero <i>et al.</i> (1990)
SM10 (<i>λpir</i>)	<i>tri thr leu tonA lacY SupE recA::RP4 -2-Tc::Mu Km^r λpir</i>	Herrero <i>et al.</i> (1990)
<i>P. aeruginosa</i>		
PAO1	Prototroph, <i>chl-2</i>	B. Holloway
PAO1ΔQ	PAO1 strain with the <i>xcpQ</i> gene deleted	de Groot <i>et al.</i> (2001)
PAO1ΔPaQa	PAO1 strain with the <i>xphA</i> and <i>xqhA</i> genes deleted	Michel <i>et al.</i> (2007)
PAO1ΔPQPqPaQa	PAO1 strain with the <i>xcpP</i> , <i>xcpQ</i> , <i>xphA</i> and <i>xqhA</i> genes deleted	Michel <i>et al.</i> (2007)
PAO1::PPaQa-lacZ	PAO1 strain with transcriptional PPaQa-lacZ fusion	Michel <i>et al.</i> (2007)
PAO1::PPQ-lacZ	PAO1 strain with transcriptional PPQ-lacZ fusion	This study
PAO1ΔfimV	PAO1 strain with the <i>fimV</i> gene deleted	This study
PAO1ΔQQa	PAO1 strain with the <i>xcpQ</i> and <i>fimV</i> genes deleted	This study
PAO1ΔQQaΔfimV	PAO1 strain with the <i>xcpQ</i> , <i>xqhA</i> and <i>fimV</i> genes deleted	This study
PAO1vfr	Mariner transposon mutant of PAO1	This study
PAO1ΔQΔfimV	PAO1ΔQ mutant with the <i>fimV</i> gene deleted	This study
Plasmids		
pRK2013	Km ^r , ColE1, Tra ⁺ Mob ⁺	Figurski & Helinski (1979)
pKNG101	Sm ^r , <i>oriR6K mobRK2 sabBR⁺</i> (suicide vector)	Kaniga <i>et al.</i> (1991)
pKNGΔPaQa	Mutator plasmid for <i>xphA-xqhA</i> deletion	Michel <i>et al.</i> (2007)
pKNGΔfimV	Mutator plasmid for <i>fimV</i> deletion	This study
Mini CTX-lacZ	Tc ^r , Ω-FRT-attP-MCS, <i>ori int oriT</i>	Hoang <i>et al.</i> (2000)
Mini CTX-PPaQa-lacZ	Mini CTX bearing transcriptional fusion <i>xphA'-lacZ</i>	Michel <i>et al.</i> (2007)
Mini CTX-PPQ-lacZ	Mini CTX bearing transcriptional fusion <i>xcpP'-lacZ</i>	This study
pFLP2	Ap ^r , <i>ori1600 oriT rep cI₈₅₇ FLP sacB</i>	Hoang <i>et al.</i> (2000)
pBT20	Ap ^r , Gm ^r , <i>Ptac oriRK6K oriT</i> , Mariner C9	Kulasekara <i>et al.</i> (2005)
pME6032	Tc ^r , <i>lacI^q</i> , <i>Ptac</i> expression vector	Heeb <i>et al.</i> (2002)
pME9603	pME6032 containing wild-type <i>vfr</i>	Fox <i>et al.</i> (2008)
pMMB207	Cm ^r , <i>Ptac</i>	Morales <i>et al.</i> (1991)
pASM281	pMMB207 containing wild-type <i>vfr</i>	Semmler <i>et al.</i> (2000)
pMMB67EH	Ap ^r , RSF 1010 (IncQ), <i>tac</i> promoter	Fürste <i>et al.</i> (1986)
pMMB-fimV	Wild-type <i>fimV</i> cloned at <i>KpnI/XbaI</i> sites of pMMB67EH	This study
pMMB190	Ap ^r , <i>lacUV5</i> , promoter <i>lacZα</i>	Morales <i>et al.</i> (1991)
pMMB-PaQa	pMMB190 bearing <i>xphA-xqhA</i> genes	Michel <i>et al.</i> (2007)
pSW205	Ap ^r , <i>lacZ</i> fusion vector	Gambello <i>et al.</i> (1993)
pTS400	Ap ^r , <i>lasB'-lacZ</i> translational fusion in pSW205	Passador <i>et al.</i> (1993)
Oligonucleotides		
dfimV1	5'-AGCGTATCGGACCGTTGAACGTTACCT-3'	This study
dfimV2	5'-TTCATTTCCGGATCACATAGTGTAATCCCT-3'	This study
dfimV3	5'-AGGGATTACACTATGTGATCCGAAATGAA-3'	This study
dfimV4	5'-ATGGGTGCCGACCAGTACCTGGGCCGC-3'	This study
pBT20-1	5'-AAGCTGTGGTATGGCTGTGCA-3'	Liberati <i>et al.</i> (2006)
ARB1D	5'-GGCCAGGCCTGCAGATGATGNNNNNNNNNGTAT-3'	Liberati <i>et al.</i> (2006)
pBT20-2	5'-AATTGTGAGCGGATAACAATTT-3'	Liberati <i>et al.</i> (2006)
ARB2A	5'-GGCCAGGCCTGCAGATGATG-3'	Liberati <i>et al.</i> (2006)

with those published previously (Michel *et al.*, 2007). Thus, it can be speculated that culture conditions could affect production and/or secretion efficiency of proteolytic exoenzymes. Indeed, the differences observed could be related to a higher efficiency of the hybrid T2SS during growth on solid medium compared with liquid medium, an activation of some of the proteases secreted by the hybrid T2SS or by the secretion of yet unknown proteases. These

hypotheses drove us, as a first step, to study the effect of growth conditions on the expression of the *PaQa* operon.

Expression of the *PaQa* operon during growth on solid medium

In order to compare expression levels of the *PaQa* operon in cells grown in liquid and solid media, we used the

Table 2. Extracellular proteolytic activity and expression of *PPaQa-lacZ* fusion in liquid and solid cultures

Strains were grown in either liquid or solid LB media at 37 °C in the presence of carbenicillin. IPTG (2 mM final concentration) was added as inducer in order to produce the orphan subunit PaQa. Images of the proteolytic assay on TSA milk plates are shown in column three.

Strain	Proteolytic activity on:	
	Liquid LB medium*	Solid medium
PAO1/pMMB	100
PAO1/pPaQa	109 ± 7.5
ΔPQPqPa/pMMB	14.3 ± 3.6
ΔPQPqPa/pPaQa	36.8 ± 1.5

*Total proteolytic activity from LB liquid cultures assayed using Azocasein as substrate. Results are expressed (%) relative to the parent strain bearing the empty vector pMMB190, and are the mean ± SEM of two independent experiments.

transcriptional *PPaQa-lacZ* fusion already generated in our lab in the wild-type strain PAO1 as a tool (Michel *et al.*, 2007). As reported previously, the activity of the fusion was found to be relatively low and constant throughout growth in liquid medium (Michel *et al.*, 2007) in contrast with the increased expression of *PPQ-lacZ* and *PRZ-lacZ* transcriptional fusions (data not shown and Chapon-Hervé *et al.*, 1997). Here, we found that expression of the orphan operon was threefold higher in cells grown on solid LB agar (302.8 ± 15.3 Miller units) than in cells grown in liquid LB (99.3 ± 12 Miller units). This difference was significant compared with the control PAO1 strain ($P < 0.001$ by Student's *t*-test). This result, in agreement with our hypothesis, suggested that the orphan subunit, and thus the hybrid T2SS, could be particularly adapted to growth on solid medium. However, using the transcriptional

fusions *PPQ-lacZ* (monitoring expression of the operon encoding the homologous classical PQ subunit) and *PRZ-lacZ* (monitoring expression of the other T2SS genes) as controls, we found that, similar to the *PaQa* operon, the *PQ* operon was also activated to a greater level on solid medium (3.8-fold) compared with liquid medium. These results could be expected considering that classical and orphan subunits can be exchanged and play the same function in the T2SS secretory machinery. In contrast, the *RZ* operon was almost the same (1.3-fold more than liquid medium) on solid medium (data not shown) showing that not all promoters are similarly activated on solid medium.

Effect of growth temperature and cell ageing on expression of *PaQa*

Recently, using transcriptomic and 2D-PAGE analyses, we reported that gene expression and secretome profiles of *P. aeruginosa* were altered when cells were grown at 25 °C instead of 37 °C (Termine & Michel, 2009). These results suggested that growth temperature may also alter a number of processes related to protein secretion. Therefore, we studied the effect of temperature on the expression of the transcriptional *PPaQa-lacZ* fusion in cells grown on solid medium at various growth temperatures. As shown in Fig. 1(a), expression of the orphan operon correlated inversely with the temperature; the activity of the transcriptional fusion decreased progressively from 25 to 37 °C. In contrast, the activity of the transcriptional *PPQ-lacZ* fusion monitoring expression of the operon encoding the classical PQ subunit (homologous to the orphan subunit) increased from 25 to 37 °C, suggesting a better adaptation to growth at 37 °C. However, it should be emphasized that the transcription level of the *PPQ-lacZ* fusion at 25 °C is comparable to that of the *pPaQa-lacZ* fusion. Thus, it seems likely that hybrid and classical T2SSs could co-exist at the lower growth temperature.

In order to further define the optimal conditions of expression for the orphan genes, the wild-type strain expressing the *PPaQa-lacZ* fusion was grown on solid LB medium at 25 °C and activity of the fusion was assayed for β -galactosidase activity throughout growth. The optimal activity was obtained between 8 and 15 h of growth and decreased after longer incubation (Fig. 1b). This result suggested that the orphan unit could most probably be useful for secretion at an early stage of growth, as proposed previously (Michel *et al.*, 2007).

Therefore, several interesting features can be drawn from our results. (i) The orphan subunit could be particularly dedicated to suboptimal growth temperature, whereas the classical one is better adapted to higher temperatures. (ii) Optimal expression of the orphan operon occurs at an early stage of the bacterial growth. (iii) Orphan and classical subunits can co-exist during growth at suboptimal temperatures but their functioning could be adapted to some specific environmental conditions and controlled by different regulatory mechanisms. From these observations, it can be proposed that the orphan subunit might play an

Fig. 1. Effect of growth temperature and cell ageing on the expression of *PaQa* and *PQ* operons. (a) PAO1 :: *PPaQa-lacZ* and PAO1 :: *PPQ-lacZ* strains were inoculated on solid LB medium as described in Methods, and incubated for 15 h at 25, 30 or 37 °C. β -Galactosidase activities were assayed using 1 OD₆₀₀ cell equivalent. Results are mean $\pm$ SEM of three independent experiments. Significant difference compared with growth at 25 °C is indicated by asterisks (***) $P < 0.001$, Student's *t*-test). (b) The PAO1 :: *PPaQa-lacZ* strain was grown at 25 °C on solid LB medium and cells were withdrawn from the agar plates at the indicated times. The OD₆₀₀ of each culture, calculated as described in Methods, is indicated above each bar. β -Galactosidase activities were assayed using 1 OD₆₀₀ cell equivalent. Light grey bars, *PPaQa-lacZ* fusion; dark grey bars, *lacZ* promoter-less fusion control. Results are mean $\pm$ SEM of three independent experiments.

important role in the environment and not in the host, and could contribute to the establishment of *P. aeruginosa* as a nosocomial pathogen on solid surfaces, for instance in hospitals, while the classical subunit could be more dedicated to direct human infection.

Genes involved in the regulation of expression of the *PaQa* operon

Results obtained from transcriptional studies suggested that expression of the genes encoding the orphan subunit could be under a specific regulatory control. Indeed, our results suggest a different regulatory control of *PaQa* and *PQ* operons, since the latter is known to be regulated by quorum sensing (QS) (Chapon-Hervé *et al.*, 1997; Schuster *et al.*, 2003; Wagner *et al.*, 2003). To identify *PaQa* regulators, a mutant library was engineered by using the properties of the transposon Mariner C9. This transposon was introduced into the wild-type strain bearing the transcriptional *PPaQa-lacZ* fusion, and recombinants were screened on X-Gal plates according to the intensity of blue staining. Seven clones, less blue than the control cells, were further investigated since they could represent mutants affected in putative activators. Transposon insertions in strains were localized by arbitrary PCR amplification and sequencing of the PCR product. Mutations affected PA1370 and PA1547 (hypothetical proteins), PA1549 (predicted ATPase involved in copper transport), PA1554 (cytochrome oxidase), PA0652 (*Vfr*) and finally PA3115 (*FimV*). Two different clones containing a transposon insertion at two distinct positions were mutated in the *vfr* gene. These results are in agreement with microarray studies carried out by Wolfgang *et al.* (2003) showing that expression of *xphA* and *xqhA* genes was highly decreased in a *vfr* mutant.

Fig. 2. Influence of *fimV* and *vfr* genes on the expression of the *PaQa* operon. β -Galactosidase activity of the *PPaQa-lacZ* fusion was assayed in *fimV* and *vfr* mutants and their complemented counterparts grown on LB agar. Results are mean $\pm$ SEM of two independent experiments. Significant difference compared with the control PAO1 strain is indicated by asterisks (***) $P < 0.001$, Student's *t*-test).

Effect of *vfr* and *fimV* mutations on the expression of the *PaQa* operon

We focused on *vfr* and *fimV* genes already reported in the literature. The former gene encodes a well-known global regulator that controls several processes (expression of genes encoding proteases, exotoxin A, twitching motility and QS) (Albus *et al.*, 1997; Beatson *et al.*, 2002; Suh *et al.*, 2002; Kanack *et al.*, 2006; Fox *et al.*, 2008) and the latter encodes a protein involved in type IV pili assembly but has been poorly studied so far (Semmler *et al.*, 2000; Ahn *et al.*, 2004). Both genes were also reported to be required for twitching motility and indeed, as expected, we observed that the selected mutants were impaired for this movement mode (data not shown). As shown in Fig. 2, activity of the *PPaQa-lacZ* fusion was reduced by 18-fold in the *fimV* deletion mutant and less drastically in the *vfr* transposon mutant (fourfold). Complementation of the mutations increased activity of the fusions in these mutants, by 3.5-fold for *fimV* and 1.6-fold for *vfr*. This partial complementation was most probably due to the non-physiological expression level of *fimV* and *vfr* from plasmids. Nevertheless, our results showed that *vfr* and *fimV* genes

are clearly (directly or indirectly) involved in the process that controls expression of the *PaQa* operon.

Effect of the *fimV* deletion on T2SS secretion on solid medium

Having defined the experimental conditions required to optimize expression of the orphan subunit, we investigated protein secretion on solid medium using an original culturing technique adapted for *P. aeruginosa* cells from Levin *et al.* (2007): the ring-plate system. This culturing system was found to be functional, as the secretome of the PAO1 strain exhibited a similar exoprotein profile to that obtained with a classical liquid culture (Fig. 3a, lanes 1 and 2). As the first attempts to detect exoproteins specifically secreted by the hybrid T2SS on ring-plates using a Δ PaQa mutant were unsuccessful, a new approach was investigated. Since we demonstrated a direct relationship between *fimV* and expression of the genes encoding the PaQa subunit, it was tempting to speculate that protein substrates of the hybrid T2SS might be detected by comparing secretome profiles of the parent strain PAO1 and its Δ *fimV* isogenic mutant. However, no exoprotein

Fig. 3. Effect of *fimV* on the T2SS-dependent secretion on solid medium. Cells were grown in ring-plate systems as described in Methods. Cellular and extracellular proteins were analysed by SDS-PAGE and visualized by Imperial blue staining. (a) A control showing the secretome of PAO1 grown in liquid culture is shown to the left. Secretomes of PAO1, Δ *fimV* and complemented Δ *fimV* strains grown in ring-plates are to the right. Molecular weight markers (kDa) are indicated. (b) Immunoblot of extracellular and cellular proteins from cells grown in ring-plates. Proteins were probed with LasB antiserum and revealed by chemiluminescence. Positions of the major exoproteins are indicated by arrows: PmpA, putative metalloprotease; PaAP, aminopeptidase; CbpD, chitin-binding protein; LasB, elastase; PrpL, type IV protease. Experiments were carried out twice (SDS-PAGE) or three times (Western blot) and representative images are shown. LasB secretion has been quantified and results are expressed relative to the wild-type (normalized at 100%).

specific for the hybrid T2SS could be detected in these comparative studies (Fig. 3a, lanes 2 and 3). Interestingly, deletion of the *fimV* gene caused a slight decrease in all the major exoproteins identified in the secretome of PAO1, and complementation of the mutation partially restored the secretion efficiency (Fig. 3a). Indeed, immunodetection of the major exoprotease secreted by PAO1, the elastase LasB, confirmed that the amount of protein was decreased in the *fimV* mutant (Fig. 3b, lane 2) and its level was partially restored to that of the parent strain after complementation of the mutation (Fig. 3b, lane 3). These results suggested that *fimV* could affect secretion via not only the hybrid T2SS but also the classical T2SS. LasB was not found to be accumulated in cells (Fig. 3b, lanes 4–6) in agreement with the cellular protein profiles observed in Fig. 3(a) (lanes 5–7). Furthermore, *P. aeruginosa* mutant strains with the *xcpQ* gene deleted (encoding the secretin of the Xcp machinery, the classical T2SS) retained a residual hydrolytic activity shown on TSA-milk plates (Martínez *et al.*, 1998 and Fig. 4a). This activity is related to the hybrid secretion system functioning with the orphan subunit PaQa. Deletion of both *xcpQ* and *xqhA* (Δ QQa mutant) led to the complete disappearance of the proteolytic halo. We speculated that since the *fimV* gene is required for optimal

expression of the *xphA*–*xqhA* operon, the PaQa-dependent residual hydrolytic activity should be abolished or at least drastically decreased in a mutant with a deletion in both the *xcpQ* and *fimV* genes. To this end, we engineered *fimV* mutants in *xcpQ* and *xcpQ*/*xqhA* backgrounds and tested them for proteolytic activity. As shown in Fig. 4(a) and as expected, the double mutant Δ Q Δ *fimV* was devoid of a proteolytic halo and displayed the same behaviour as the Δ QQa mutant. This result suggests that *fimV* was required for secretion of proteases, mainly of the major exoprotease LasB, by the hybrid system. Immunodetection of LasB confirmed that as already shown in Fig. 3(b), the extracellular LasB level was decreased in PAO1 Δ *fimV* (Fig. 4b, lane 2). This decreased level of LasB could also explain the small hydrolytic halo observed for the PAO1 Δ *fimV* strain in the protease assay on the plate (Fig. 4a). As expected, LasB was faintly or not detected in extracellular extracts of the Δ Q and Δ Q Δ *fimV* mutants (Fig. 4b, lanes 3 and 4), but both strains accumulated large amounts of LasB within the cells showing that they are impaired for LasB secretion (Fig. 4b, lanes 7 and 8).

Influence of *fimV* on *lasB* expression

In contrast with the results obtained with Δ Q and Δ Q Δ *fimV* mutant strains, intracellular accumulation of LasB was not observed in the PAO1 Δ *fimV* mutant (Fig. 3b, lane 5, and Fig. 4b, lane 6). These results could suggest that either intracellularly accumulated LasB was degraded by proteases in this mutant strain or *lasB* expression rather than secretion as such was affected by the *fimV* deletion. In line with the last hypothesis, a translational *lasB*–*lacZ* fusion expressed in the wild-type PAO1 strain and in the *fimV* mutant showed that activity of the fusion was decreased by 70 % in the mutant strain (Fig. 5a). Therefore, it can be concluded that FimV is directly or indirectly required for efficient control of *lasB* expression. Since FimV is thought to be involved in type IV pili assembly, we hypothesized that blocking this process could trigger perturbations of the cell envelope and sensing signals, leading to an alteration of more global regulatory mechanisms, particularly those involved in *lasB* expression. *lasB* expression is known to be dependent on Vfr control via QS regulation (Gambello & Iglewski, 1991; Albus *et al.*, 1997). Moreover, as indicated in Fig. 2, the global regulator Vfr was found to be an activator of the expression of orphan genes that encode the PaQa subunit. In order to test a possible relationship between FimV and the Vfr protein, we checked the level of *vfr* expression in *fimV* mutants. Cellular proteins from PAO1, PAO1 Δ *fimV*, Δ Q and Δ Q Δ *fimV* strains were analysed by Western blotting and probed with Vfr antiserum. Immunodetection revealed that the Vfr level decreased in *fimV* mutants compared with wild-type or Δ Q parent strains (Fig. 5b, c). The relationship between FimV and Vfr appeared to be tight since complementation of the *fimV* mutation fully restored the Vfr level to that of the parent strain (Fig. 5c, lane 3). Thus, these results show that high amounts of Vfr are required for optimal expression of *lasB*. Moreover, it should be pointed out that the level of Vfr

Fig. 4. Influence of *fimV* deletion on the hybrid T2SS secretion and on LasB production on solid medium. (a) Cells were either pricked or streaked on TSA-milk plates and their proteolytic activity was evaluated according to the size of the clear halo zone observed on the plate. (b) Immunoblot of extracellular and cellular proteins from cells grown in ring-plates. Proteins probed with LasB antiserum were detected by chemiluminescence. This experiment was performed twice. LasB secretion has been quantified and the results are expressed relative to the wild-type (normalized at 100 %).

Fig. 5. Factors regulating *lasB* expression. (a) Activity of the *lasB*–*lacZ* translational fusion from PAO1 and its cognate *fimV* mutant grown on solid LB medium. pSW205, empty vector; pTS400, vector bearing the translational *lasB*–*lacZ* fusion. Results are mean $\pm$ SEM of two independent experiments. Significant difference compared with the control PAO1 strain is indicated by asterisks (***) $P < 0.001$ in the Student's *t*-test. (b and c) Effect of *fimV* deletion on Vfr levels. Cellular proteins obtained from PAO1, ΔQ and their cognate *fimV* mutants were analysed by immunoblotting. Proteins were probed with Vfr antiserum and detected by chemiluminescence. Results are representative of two independent experiments. The same protein extracts as those shown in Fig. 4(b) (b) or Fig. 3(b) (c) were used here. The amount of Vfr has been quantified and the results are expressed relative to the wild-type (normalized at 100%).

is not altered in the ΔQ mutant impaired for classical T2SS, suggesting a specific link between the absence of FimV and the decrease in Vfr.

In contrast with Vfr, little is known about the FimV protein of *P. aeruginosa*. Nevertheless, it has been reported recently that a *fimV* mutant was defective in cAMP synthesis (Fulcher

et al., 2010) and that cAMP was required for Vfr activity and *vfr* expression (Fuchs *et al.*, 2010). Vfr has also been shown to control expression of the *lasR* promoter and thus QS in a cAMP-independent manner (Fuchs *et al.*, 2010). However, in contrast with our results, none of these studies indicated a direct relationship between FimV and Vfr proteins.

FimV protein is predicted to contain a LysM domain (<http://v2.pseudomonas.com> and <http://pfam.sanger.ac.uk>) which is thought to have a general peptidoglycan binding function and to be present in a variety of enzymes involved in bacterial cell degradation (Interpro entry IPR018392). As FimV has been shown to be involved in type IV pilus assembly (Semmler *et al.*, 2000), one could imagine that the loss of FimV affects a peptidoglycan breaching step necessary for pilus assembly. It could also be possible that the same peptidoglycan breaching mechanism is required for assembly of the T2SS machinery. Furthermore, in our experimental conditions, the absence of FimV could also cause a particular perturbation of the envelope due to defective pilus assembly and lead to an alteration of sensing signals involved in regulatory mechanisms such as Vfr control. This hypothesis is also supported by the studies of Bayse *et al.* (2005) showing that membrane alterations can modulate QS in *P. aeruginosa*.

Conclusions

In summary, our results showed that high levels of Vfr are required for optimal expression of genes encoding the orphan subunit of the hybrid T2SS and LasB. Although unexpected, FimV was shown to interact with Vfr by a mechanism that acts as a modulator of the Vfr level. Such fine tuning could also modulate both the level of Vfr-dependent exoproteins and the efficiency of hybrid and classical T2SS according to the physiological state of the bacteria. Although no substrate specific for the hybrid T2SS has been identified so far, our results offer new insights into the function of this atypical secretion system. At the current stage of our investigation, it can be suggested that the hybrid T2SS can at least serve as a rescue system in growth conditions where the PQ subunit of the classical T2SS is affected or is poorly efficient. However, it can be reasonably claimed that in contrast with the classical T2SS, the hybrid T2SS could play a particular role in defined environmental conditions such as growth on solid medium at suboptimal growth temperature and at a relatively low cell density. Further work is now needed to improve the ring-plate culturing system in order to identify potential substrates of the hybrid T2SS of *P. aeruginosa* grown on solid medium and to elucidate the physiological role of this atypical secretion system.

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REFERENCES

- Ahn, K. S., Ha, U., Jia, J., Wu, D. & Jin, S. (2004). The *truA* gene of *Pseudomonas aeruginosa* is required for the expression of type III secretory genes. *Microbiology* **150**, 539–547.
- Albus, A. M., Pesci, E. C., Runyen-Janecky, L. J., West, S. E. & Iglewski, B. H. (1997). Vfr controls quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**, 3928–3935.
- Baysse, C., Cullinane, M., Dénervaud, V., Burrowes, E., Dow, J. M., Morrissey, J. P., Tam, L., Trevors, J. T. & O'Gara, F. (2005). Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties. *Microbiology* **151**, 2529–2542.
- Beatson, S. A., Whitchurch, C. B., Sargent, J. L., Levesque, R. C. & Mattick, J. S. (2002). Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 3605–3613.
- Bleves, S., Gérard-Vincent, M., Lazdunski, A. & Filloux, A. (1999). Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 4012–4019.
- Bleves, S., Viarre, V., Salacha, R., Michel, G. P. F., Filloux, A. & Voulhoux, R. (2010). Protein secretion systems in *Pseudomonas aeruginosa*: a wealth of pathogenic weapons. *Int J Med Microbiol* **300**, 534–543.
- Bodey, G. P., Bolivar, R., Fainstein, V. & Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis* **5**, 279–313.
- Bouley, J., Condemine, G. & Shevchik, V. E. (2001). The PDZ domain of OutC and the N-terminal region of OutD determine the secretion specificity of the type II out pathway of *Erwinia chrysanthemi*. *J Mol Biol* **308**, 205–219.
- Chapon-Hervé, V., Akrim, M., Latifi, A., Williams, P., Lazdunski, A. & Bally, M. (1997). Regulation of the *xcp* secretion pathway by multiple quorum-sensing modulons in *Pseudomonas aeruginosa*. *Mol Microbiol* **24**, 1169–1178.
- de Groot, A., Koster, M., Gérard-Vincent, M., Gerritse, G., Lazdunski, A., Tommassen, J. & Filloux, A. (2001). Exchange of Xcp (Gsp) secretion machineries between *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*: species specificity unrelated to substrate recognition. *J Bacteriol* **183**, 959–967.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.
- Filloux, A. (2004). The underlying mechanisms of type II protein secretion. *Biochim Biophys Acta* **1694**, 163–179.
- Fox, A., Haas, D., Reimann, C., Heeb, S., Filloux, A. & Voulhoux, R. (2008). Emergence of secretion-defective sublines of *Pseudomonas aeruginosa* PAO1 resulting from spontaneous mutations in the *vfr* global regulatory gene. *Appl Environ Microbiol* **74**, 1902–1908.
- Fuchs, E. L., Brutinel, E. D., Jones, A. K., Fulcher, N. B., Urbanowski, M. L., Yahr, T. L. & Wolfgang, M. C. (2010). The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and -independent mechanisms. *J Bacteriol* **192**, 3553–3564.
- Fulcher, N. B., Holliday, P. M., Klem, E., Cann, M. J. & Wolfgang, M. C. (2010). The *Pseudomonas aeruginosa* Chp chemosensory system regulates intracellular cAMP levels by modulating adenylate cyclase activity. *Mol Microbiol* **76**, 889–904.
- Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**, 119–131.
- Gambello, M. J. & Iglewski, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000–3009.
- Gambello, M. J., Kaye, S. & Iglewski, B. H. (1993). LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. *Infect Immun* **61**, 1180–1184.
- Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046–1056.
- Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.
- Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59–72.
- Kanack, K. J., Runyen-Janecky, L. J., Ferrell, E. P., Suh, S. J. & West, S. E. (2006). Characterization of DNA-binding specificity and analysis of binding sites of the *Pseudomonas aeruginosa* global regulator, Vfr, a homologue of the *Escherichia coli* cAMP receptor protein. *Microbiology* **152**, 3485–3496.
- Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137–141.
- Kulasekara, H. D., Ventre, I., Kulasekara, B. R., Lazdunski, A., Filloux, A. & Lory, S. (2005). A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. *Mol Microbiol* **55**, 368–380.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Levin, A. M., de Vries, R. P. & Wösten, H. A. (2007). Localization of protein secretion in fungal colonies using a novel culturing technique; the ring-plate system. *J Microbiol Methods* **69**, 399–401.
- Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T. & Ausubel, F. M. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A* **103**, 2833–2838.
- Lindeberg, M., Salmond, G. P. & Collmer, A. (1996). Complementation of deletion mutations in a cloned functional cluster of *Erwinia chrysanthemi* out genes with *Erwinia carotovora* out homologues reveals OutC and OutD as candidate gatekeepers of species-specific secretion of proteins *via* the type II pathway. *Mol Microbiol* **20**, 175–190.
- Martínez, A., Ostrovsky, P. & Nunn, D. N. (1998). Identification of an additional member of the secretin superfamily of proteins in *Pseudomonas aeruginosa* that is able to function in type II protein secretion. *Mol Microbiol* **28**, 1235–1246.
- Michel, G. P. & Voulhoux, R. (2009). The type II secretory system (T2SS) in Gram negative bacteria: a molecular nanomachine for secretion of Sec and Tat-dependent extracellular proteins. In *Bacterial*

Secreted Proteins, vol. 1, pp. 67–92. Edited by K. Wooldridge. Norfolk: Caister Academic Press.

Michel, G. P., Durand, E. & Filloux, A. (2007). XphA/XqhA, a novel GspCD subunit for type II secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **189**, 3776–3783.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Morales, V. M., Bäckman, A. & Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**, 39–47.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. & Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127–1130.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066–2079.

Semmler, A. B., Whitchurch, C. B., Leech, A. J. & Mattick, J. S. (2000). Identification of a novel gene, *fimV*, involved in twitching motility in *Pseudomonas aeruginosa*. *Microbiology* **146**, 1321–1332.

Suh, S. J., Runyen-Janecky, L. J., Maleniak, T. C., Hager, P., MacGregor, C. H., Zielinski-Mozny, N. A., Phibbs, P. V., Jr & West, S. E. (2002). Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. *Microbiology* **148**, 1561–1569.

Swain, M. & Ross, N. W. (1995). A silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecyl sulfate-polyacrylamide gels. *Electrophoresis* **16**, 948–951.

Termine, E. & Michel, G. P. (2009). Transcriptome and secretome analyses of the adaptive response of *Pseudomonas aeruginosa* to suboptimal growth temperature. *Int Microbiol* **12**, 7–12.

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I. & Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**, 2080–2095.

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.

Wretling, B. & Pavlovskis, O. R. (1984). Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. *J Bacteriol* **158**, 801–808.

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