Colicin A hybrids: a genetic tool for selection of type II secretion-proficient *Pseudomonas* strains

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The Gram-negative bacterium *Pseudomonas aeruginosa* secretes the majority of its extracellular proteins by the type II secretion mechanism, a two-step process initiated by translocation of signal peptide-bearing exoproteins across the inner membrane. The periplasmic forms are transferred across the outer membrane by a machinery consisting of 12 *xcp* gene products. Although the type II secretion machinery is conserved among Gram-negative bacteria, interactions between the secreted proteins and the machinery are specific. The lack of a selectable phenotype has hampered the development of genetic strategies for studying type II secretion. We report a novel strategy to identify rare events, such as those that allow heterologous secretion or identification of extragenic suppressors correcting *xcp* defects. This is based on creating a host-vector system where the non-secretory phenotype is lethal. The original tool we designed is a hybrid protein containing elastase and the pore-forming domain of colicin A.

INTRODUCTION

In recent years, several mechanisms for extracellular protein targeting across the cell envelope of Gram-negative bacteria have been discovered. Extracellular localization may occur in one step, and both the inner and outer membranes are traversed simultaneously by the secreted proteins in a Sec-independent manner. Alternatively, proteins can be secreted by a two-step process, the first of which is translocation across the inner membrane into the periplasm, mediated by the Sec machinery and the N-terminal signal peptides, followed by their extracellular release. Those pathways are called terminal branches of the general secretory pathway (GSP), from which the type II pathway is the main terminal branch. In *Pseudomonas aeruginosa*, the type II machinery is encoded by 12 *xcp* genes (Filloux *et al.*, 1998), and directs the secretion of several enzymes. The type II system is conserved in Gram-negative bacteria, where

homologues of the individual Xcp components are referred to as Gsp proteins. The components of the Xcp machinery are found associated with (XcpR) or localized in (XcpP, XcpY, XcpZ, XcpA and XcpS) the inner membrane. A group of related proteins, referred to as pseudopilins, are associated with both membranes (XcpT-X). The XcpA/PilD protein is a peptidase that processes and N-methylates the pseudopilins (Strom *et al.*, 1993), which subsequently may assemble into a pilus-like structure (Sauvonnnet *et al.*, 2000). Finally, XcpQ is an outer membrane protein that belongs to a family of proteins designated secretins (Bitter *et al.*, 1998). XcpQ multimers form a complex that probably serves as the channel for the passage of the exoproteins through the outer membrane. The secretion of these exoproteins is specific. For example, pullulanase, which utilizes the type II machinery in *Klebsiella oxytoca*, is not secreted by *P. aeruginosa* despite the sequence similarities between the individual components of these two type II secretion systems (Filloux *et al.*, 1990). This species specificity is proposed to be due to the specific interaction between the targeting signals on each exoprotein and the cognate machinery (Shevchik *et al.*, 1997). It was suggested that the secretion signals carried by the exoproteins are conformational, since these must fold in the periplasm prior to their translocation across the outer membrane (Filloux *et al.*, 1998). Some studies suggest that GspC (XcpP) and GspD (XcpQ) are the likely candidates that recognize the targeting signals of the exoproteins (Lindeberg *et al.*, 1996). The Gsp/Xcp proteins form a macromolecular machinery with many interactions in which species specificity has also been reported (Sandkvist *et al.*, 2000). Several approaches, aimed at defining these different protein-protein interactions, have been employed including cross-linking (Possot *et al.*, 2000), two-hybrid systems (Py *et al.*, 1999), co-immunoprecipitation (Sandkvist *et al.*, 2000) and affinity blotting (Shevchik *et al.*, 1997). Nevertheless, the nature of the specific interactions remains poorly understood. In this

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Fig. 1. Schematic representation of the targeting of ColA and hybrids containing pfColA to the cytoplasmic membrane of *E. coli* (A and B) and *P. aeruginosa* (C and D). (A) ColA is taken up via the OmpF/BtuB-Tol/PAL system. (B) pfColA fused to a signal peptide is transported via the Sec machinery. (C) Anticipated Xcp-dependent secretion of pfColA fused to a *P. aeruginosa* exoprotein in a secretion-proficient strain and (D) its insertion into the cytoplasmic membrane in an *xcp* mutant. The dashed box indicates defective secretion machinery.

study, we developed a new genetic tool to unravel these interactions and to broaden the specificity of the machinery. The basis of this method is the ability of engineered hybrid proteins, consisting of a secreted protein and a toxic polypeptide, to kill cells when accumulating intracellularly, and to provide a selection for the type II pathway-dependent secretion phenotype. The toxic component chosen for testing of the selection method is the pore-forming domain of colicin A (Duché *et al.*, 1999). We demonstrate that the *P. aeruginosa* elastase (LasB) can be used as a vehicle for the secretion of such a domain and that intracellular accumulation of the hybrid protein in an *xcp* mutant kills the cell.

RESULTS

Development of a selection system based on type II secretion pathway-associated lethality

Colicins are plasmid-encoded bacteriocins that are produced by and active against *Escherichia coli*. The cytoplasmic membrane is the target of the pore-forming colicin A (ColA) (Duché *et al.*, 1999). The producing strain is protected from the activity of ColA by the immunity protein, Cai (Espeset *et al.*, 1996). Release of ColA requires the protein Cal, whereas the Sec system is not involved. Uptake of ColA requires outer membrane receptors and the Tol/PAL machinery (Lazdunski *et al.*, 1998) (Figure 1A). The pore-forming domain of ColA (pfColA) could kill the producing strain in a Sec-dependent manner when the coding region for pfColA was cloned in-frame with a signal peptide-coding sequence (sp-pfColA) (Espeset *et al.*, 1996) (Figure 1B). We reasoned that the fate of pfColA could be decided in *P. aeruginosa* by the Xcp machinery, which discriminates between proteins that are secreted across the outer membrane, such as elastase (LasB), and other proteins that are retained in the periplasm. The ability of the Xcp apparatus to sort proteins in the

periplasm is based on the presence of a secretion motif contained within the sequence of the Xcp-dependent exoproteins. Although the nature of this signal is poorly defined, fusing a passenger protein such as β -lactamase to a full-length exoprotein often yields chimeras that are secreted (Lu and Lory, 1996). Because the phenotype of *xcp* mutants is the periplasmic accumulation of exoproteins, we expected that the fusion of pfColA to a secreted protein would result in a lethal effect unless the chimera is efficiently secreted. Survival of the cells should depend entirely on the correct interaction between the secretion signal on the hybrid protein and the functional Xcp machinery (Figure 1C and D).

Is ColA toxic to *P. aeruginosa*?

We initially verified that *P. aeruginosa* is not sensitive to ColA when added externally (data not shown). Next, we cloned the gene fusion encoding sp-pfColA into pMMB67EH, yielding pRV300. While the vector transferred efficiently into PAO1, no transconjugants were recovered when pRV300 was mobilized into the same recipient (data not shown). This result suggested that sp-pfColA is expressed and that *P. aeruginosa* is sensitive to ColA provided that it can reach the periplasmic side of the inner membrane.

LasB–pfColA is toxic for *E. coli*

To investigate whether pfColA can be exported from the periplasm into the extracellular medium by fusing it to a substrate of the Xcp machinery, the signal sequence (sp) of sp-pfColA was replaced by the full-length LasB. The resulting plasmid, pRV700, was transformed into *E. coli* TG1 together with plmTc, which encodes Cai. The transformants were plated on LB agar supplemented with ampicillin and isopropyl- β -D-thiogalactopyranoside (IPTG), but lacking tetracycline. The ampicillin-resistant clones were screened for tetracycline resistance, which identifies cells that carry both plasmids. All the recovered transformants also

Fig. 2. Secretion and elastase activity of LasB–pfColA in *P. aeruginosa*. (A) pRV700 (*lasB*–pfColA) or pMMB67EH was introduced into the the *P. aeruginosa lasB* strain PDO240, and secreted elastase activity was revealed on an elastin-containing plate. (B) Protease activity in the supernatant of PDO240 (*lasB*) containing either pRV700 or pMMB67EH, and PAO1 or PAO7510 (*xcpR54*) containing pMMB67EH. Samples (5 µl) of supernatant harvested from cultures grown to an OD₆₀₀ of 4 were introduced into holes made in a TSA skim milk plate. Plates were incubated overnight at 37°C. (C) Secretion efficiency of LasB–pfColA was analysed by immunoblotting of fractionated cell extracts using an antibody directed against LasB. The positions of molecular size standard proteins (kDa) are indicated on the left and of LasB–pfColA on the right. C, cell extracts; SN, extracellular fractions.

carried plmTc, indicating that *E. coli* cannot survive expression of LasB–pfColA in the absence of Cai, and that the chimeric protein can exert a toxic effect. It should be noted that when the *lasB*–pfColA gene fusion was cloned under control of the *lac* promoter into the low-copy-number plasmid pLAFR3, *E. coli* TG1 could be transformed by the resulting construct, pRV200, without a requirement for plmTc. However, when these cells were grown in liquid medium, a drastic arrest in growth was observed upon IPTG addition (data not shown). This arrest was correlated with higher SDS sensitivity of the cells, which was previously used as a test to demonstrate ColA activity (Cavard and Lazdunski, 1979). LasB is synthesized as a pre-pro-enzyme (Braun *et al.*, 1996) with a propeptide of 17 kDa, which is cleaved off by autoproteolytic processing after folding of the mature domain. The estimated molecular size of the hybrid (56 kDa) indicated that LasB–pfColA did not contain the propeptide (data not shown and Figure 2B). These results indicate that both the pfColA and LasB domains of the fusion protein are active when the protein is expressed in *E. coli*.

LasB–pfColA is secreted by *P. aeruginosa*

The mixed plasmid preparation pRV700/plmTc was electroporated into the *P. aeruginosa lasB* mutant PDO240. The plasmid plmTc was never co-selected since it does not replicate in *P. aeruginosa*. The recovered clones were thus able to tolerate the efficient expression of the LasB–pfColA hybrid without the protection given by Cai. PDO240 carrying pRV700 was patched on an elastin plate, and a halo of elastin hydrolysis was observed after 48 h at 37°C. This result suggested that LasB–pfColA was expressed and secreted, and that the LasB domain had elastinolytic activity (Figure 2A). That elastase activity was indeed detected in the growth medium of *P. aeruginosa* PDO240(pRV700), but not in the medium from a culture of *P. aeruginosa* PDO240(pMMB67EH), was confirmed by checking the proteolytic activity of the extracellular fractions on skim milk plates (Figure 2B). The halo of milk protein degradation is also observed with the supernatant of a *P. aeruginosa* wild-type strain, but not in the *xcpR54* mutant derivative (Figure 2B). The LasB–pfColA fusion protein was detected at the expected position (56 kDa), entirely in the supernatant fraction (Figure 2C). Its secretion was not due to leakage since periplasmic β-lactamase was recovered only in the cell fraction (data not shown). The plasmid encoding LasB–pfColA was isolated from the previous clone and introduced into *E. coli* TG1 by transformation. No colonies could be recovered from the plates containing ampicillin (data not shown), indicating that the plasmid still encodes a LasB–pfColA hybrid that retained its toxic effect. In conclusion, the LasB–pfColA hybrid is specifically and efficiently secreted from *P. aeruginosa*, which explains why, in contrast to *E. coli*, this bacterium survives expression of the chimera without a need for Cai.

Pseudomonas aeruginosa xcp mutants are sensitive to LasB–pfColA

pRV700 was subsequently introduced into the *xcpR54* mutant strain PAO7510. In contrast to the wild-type strain, no survivors could be obtained with the *xcp* mutant after electroporation with pRV700 (Figure 3). This result indicates that periplasmic accumulation of LasB–pfColA in *P. aeruginosa xcp* mutant killed the cells, as observed in *E. coli*.

Selection of secretion-proficient strains from non-secreting *xcp* mutants

The mutant strain PAO7510 was subjected to chemical mutagenesis. pRV700 or pMMB67EH was electroporated into the ethyl methane sulfonate (EMS)-treated bacteria and transformants recovered from plates after 48 h at 30°C. A total of 19 transformants were obtained when the mutagenized PAO7510 strain was electroporated with pRV700, whereas ~600 clones were obtained following electroporation with the vector. In contrast, when the PAO7510 strain was not mutagenized, no transformants were recovered after electroporation with pRV700. The 19 transformants obtained were plated on rhodamine–olive oil plates. A halo was formed on these plates by four of them, indicating that, unlike the parent, they were able to secrete the Xcp-dependent lipase (Figure 4). Moreover, all four clones were

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Fig. 3. Comparison of transfer frequencies of plasmids into wild-type (PAO1) and an *xcp* mutant (PAO7510) of *P. aeruginosa*. Strains were electroporated with pRV700 (*lasB*–*pfColA*) or pMMB67EH. Bacteria were plated on PIA plates containing carbenicillin and IPTG (100 μ M), and the plates incubated at 30°C for 24–36 h.

Fig. 4. Secretory phenotype of EMS-mutagenized PAO7510 (*xcpR54*) clones, selected after expression of *lasB*–*pfColA*. Restoration of secretion was tested on rhodamine–olive oil plates for extracellular lipase activity. Secreting bacteria are characterized by a deep pink halo around the colony. PAO1 containing pMMB67EH or pRV700 (*lasB*–*pfColA*) and PAO7510 containing pMMB67EH were used as controls.

also able to secrete exotoxin A and the endogenous elastase (data not shown), indicating that the mutants recovered an Xcp-dependent secretion phenotype. It should be noticed that eight carbenicillin-resistant clones were recovered when the same mutagenized PAO7510 pool was subjected to electroporation in the absence of any plasmids. These clones, as well as the 15 non-secretory clones described previously, carried an EMS-induced chromosomal mutation conferring carbenicillin resistance; however, they were unable to secrete lipase (data not shown). We concluded that use of the *LasB*–*pfColA* hybrid permits the selection of events that restore a secretory phenotype in an *xcp* mutant. The secretory phenotype of the selected mutants might be the result of an intragenic mutation in *xcpR* (revertants) or a mutation in another gene encoding, for example, a component of the Xcp machinery (second-site suppressors). The nucleotide sequence of the *xcpR* gene, from

the four mutants described previously, revealed, in all cases, a C to T transition that leads to the production of a non-functional XcpR protein (data not shown) with a S355T substitution, and are true revertants of the PAO7510 *P. aeruginosa* strain.

DISCUSSION

The type II secretion pathway in Gram-negative bacteria involves a number of interactions between the secreted proteins and the secretion machinery, as well as between components of the machinery. The secretion apparatus is capable of recognizing the targeting information on the secreted proteins and these are correctly sorted from the large number of proteins that are retained within the cell. This specificity is highlighted since secretion of an exoprotein expressed in a heterologous host is rarely observed. Moreover, individual components of the type II secretion machineries of different hosts could not always be exchanged (Filloux *et al.*, 1990).

The genetic selection approach, reported here, was designed to allow secretion of proteins from bacteria that are naturally unable to secrete these proteins, or that have lost their ability to secrete proteins. The genetic selection requires the secretion process to be efficient in order to avoid the lethal effect of the colicin-containing hybrid protein in the periplasm. We developed our selection procedure in *P. aeruginosa*, which secretes numerous exoproteins via the type II pathway (Filloux *et al.*, 1998). The periplasmic accumulation of these exoproteins in *xcp* mutants does not affect the viability of the bacteria. In order to make this accumulation lethal, we engineered a toxic hybrid protein whose secretion requires the functional Xcp machinery. Xcp-dependent secretion requires the presence of a specific secretion signal on the protein to be transported, which so far has not been identified unequivocally. However, β -lactamase fused as a passenger protein to exotoxin A is secreted in an Xcp-dependent fashion (Lu and Lory, 1996). We hypothesized that fusion of a toxic domain to an exoprotein will result in a genetic tool favouring the secretion of the hybrid rather than its accumulation within the cell, thus rendering a block in protein secretion a lethal event. The toxic domain chosen was the pore-forming domain of colicin A that inserts from the periplasmic side into the cytoplasmic membrane. To carry the toxic domain of *ColA*, we chose *LasB*. *LasB* is synthesized as a pre-pro-protein, the pro-domain being autocatalytically cleaved within the periplasm. The *LasB*–*pfColA* hybrid protein is efficiently secreted by *P. aeruginosa*. Moreover, the hybrid produced showed a clear elastinolytic activity. This indicates that the pro-domain is normally cleaved off and that the *pfColA* domain does not interfere with elastase activity. Therefore, the *LasB*–*pfColA* hybrid protein consists of two independent domains, with the *LasB* domain properly folded, thereby creating the secretion signal that is recognized by the Xcp machinery. It was not possible to introduce the plasmid encoding the *LasB*–*pfColA* hybrid into an *xcp* mutant, indicating that in secretion-defective bacteria the hybrid protein is not secreted and its periplasmic accumulation was lethal. This result shows that *pfColA* retained its pore-forming activity and that *LasB*–*pfColA* is a sensitive tool for discriminating between secreting and non-secreting *P. aeruginosa* strains.

Importantly, we showed that clones capable of Xcp-dependent protein secretion could be recovered from a population of non-secreting mutants. A different type of genetic selection, based on

Table I. Strains and plasmids used in this study

Strains/plasmids	Relevant characteristics ^a	Reference/source
<i>E. coli</i>		
TG1	<i>supE</i> Δ(<i>lac-proAB</i>) <i>thi hsdR</i> Δ5 (F′:: <i>tra</i> Δ36 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q ZΔM15)	laboratory collection
<i>P. aeruginosa</i>		
PAO1	prototroph, <i>chl</i> -2	B. Holloway
PDO240	PAO1, <i>lasB</i> 9Δ Tn501-6	D. Ohman
PAO7510	PAO25, <i>xcpR</i> 54	laboratory collection
Plasmids		
pLAFR3	cosmid, IncP1, Tet ^r	laboratory collection
pMMB67EH	broad host range, Ap ^r , <i>tac</i> promoter	E. Lanka
pRK2013	Km ^r , ColE1 with Tra ⁺ Mob ⁺	Figurski and Helinski (1979)
pImTc	pACYC184 derivative, <i>cai</i> , Tet ^r	V. Géli
pCM1804a	<i>lasB</i> gene in pUC18	B. Iglewski
pCT1	pBR322 derivative, <i>sp-pfColA</i> , Ap ^r	D. Duché
pRV200	<i>lasB</i> – <i>pfColA</i> cloned as an <i>EcoRI</i> – <i>HindIII</i> DNA fragment into pMMB67EH	this study
pRV300	<i>sp-pfColA</i> cloned into pMMB67EH	this study
pRV700	<i>lasB</i> – <i>pfColA</i> cloned as an <i>EcoRI</i> – <i>HindIII</i> DNA fragment into pMMB67EH	this study

^aAp^r, ampicillin resistant; Km^r, kanamycin resistant; Tet^r, tetracycline resistant.

the ability of *P. aeruginosa* to grow on agar plates containing triolein as the sole carbon source if lipase is secreted, was proposed previously (Kagami *et al.*, 1998). This procedure proved to be useful for identifying protein–protein interactions between components of the Xcp machinery. In contrast to this selection method, the toxicity of the colicin-containing hybrid proteins is applicable to secretion of heterologous exoproteins and should unravel the basis of species specificity during the interactions between a secreted substrate and the machinery. We may be able to characterize, on one hand, the nature of the secretion signal contained within the exoprotein, and on the other hand, the Xcp docking protein and its corresponding domain, which will specifically recognize the exoprotein. This can be accomplished by fusion of the *pfColA* domain to a series of non-*P. aeruginosa* exoproteins, and identifying those hybrids that can be secreted by selecting for appropriate mutations. Finally, our selection procedure is highly stringent since the intracellular accumulation of a few toxin molecules is sufficient to lead to cell death. Consequently, this procedure should be useful in identifying hyperefficient secretory machineries that prevent the periplasmic accumulation of overproduced exoproteins. Such mutants may be of biotechnological value, since the secretion efficiency becomes limiting in production processes (Gerritse *et al.*, 1998). In conclusion, because the species-specific recognition of exoproteins appears to be a complex and

subtle process, we believe that by utilizing the colicin-based genetic selection, the bacteria will reveal the finer details of the many unanswered questions about the mechanism of protein secretion and its specificity.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are described in Table I. Cells were grown in L broth at 37°C for *E. coli* and 30°C for *P. aeruginosa*. Tetracycline (15 µg/ml) and ampicillin (50 µg/ml) for *E. coli*, and carbenicillin (500 µg/ml) for *P. aeruginosa* were used. Plasmid transfer in *P. aeruginosa* was performed by using pRK2013 or by electroporation. Transconjugants were selected on PIA (*Pseudomonas* isolation agar). The gene fusion *lasB*–*pfColA* was constructed as follows. The stop codon of the *lasB* gene was replaced by a *NcoI* site using PCR. pCM1804a was used as template for *lasB* gene amplification with reverse primer and *Ela*s4 (5′-AAGGTACCATGGAGGCCAACGCGCTCGGG-3′; *NcoI* and *KpnI* sites in bold and italics, respectively). Amplified DNA was cloned as an *EcoRI*–*KpnI* fragment. The *NcoI* site was used to clone, in-frame with the *lasB* gene, the *pfColA* sequence isolated from pCT1 by *NcoI*–*HindIII* digestion. The resulting gene fusion *lasB*–*pfColA* was cloned under the control of the *tac* promoter into pMMB67EH, yielding pRV700. In order to prevent

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toxicity associated with sp-pfColA and LasB-pfColA, plasmids carrying these gene fusions were prepared in *E. coli* containing plmTc encoding Cai. The nucleotide sequence of the *xcpR* gene was checked by the MWGBiotech company.

Plate assays. Elastase activity was tested by growing colonies on tryptic soy agar (TSA) containing 1% elastin. Proteolytic activity was tested on TSA containing 1.5% skim milk. Lipase activity was tested on plates containing olive oil and rhodamine (Kouker and Jaeger, 1987).

Cell fractionation, SDS-PAGE and immunoblotting. *Pseudomonas aeruginosa* was grown to late exponential phase and 1 OD₆₀₀ unit of cells was harvested by centrifugation. The pellet was solubilized in 50 µl of SDS-PAGE sample buffer. Proteins were precipitated from the supernatant with 12.5% trichloroacetic acid, collected by centrifugation, washed with acetone, and resuspended in 50 µl of SDS-PAGE sample buffer. Samples consisting of cellular and extracellular fractions were heated for 10 min at 95°C, and 5 µl samples were separated by electrophoresis on 11% acrylamide gels. Proteins were blotted onto nitrocellulose membranes and incubated with rabbit antisera directed against LasB or β-lactamase, with peroxidase-conjugated goat anti-rabbit IgG as secondary antibody. Proteins were checked by chemiluminescence (Pierce).

EMS mutagenesis. PAO7510 was grown to an OD₆₀₀ of 6–8. A culture sample was centrifuged, cells were resuspended in M9 medium (Miller, 1972) containing 2% EMS and grown for 3 h at 30°C. Cells were washed with M9 medium and diluted in L broth containing 0.4% glucose to 0.1 OD₆₀₀ unit. The pool of mutagenized bacteria was incubated at 30°C and grown to an OD₆₀₀ of 0.5. Cells were prepared for electroporation and plated on PIA containing carbenicillin and 0.4% glucose.

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