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**The general secretory pathway (GSP) is a two-step process for the secretion of proteins by Gram-negative bacteria. The translocation across the outer membrane is carried out by the type II system, which involves machinery called the secreton. This step is considered to be an extension of the general export pathway, i.e. the export of proteins across the inner membrane by the Sec machinery. Here, we demonstrate that two substrates for the *Pseudomonas aeruginosa* secreton, both phospholipases, use the twin-arginine translocation (Tat) system, instead of the Sec system, for the first step of translocation across the inner membrane. These results challenge the previous vision of the GSP and suggest for the first time a mosaic model in which both the Sec and the Tat systems feed substrates into the secreton. Moreover, since *P.aeruginosa* phospholipases are secreted virulence factors, the Tat system appears to be a novel determinant of bacterial virulence.**

*Keywords*: phospholipase/*Pseudomonas aeruginosa*/
Sec machinery/Tat system/type II secretory pathway

## Introhhction

*PPeuPomoPaP aerugiPoPa* is ai opportuiistic humai pathogii aii ai importait causi of hospital-acquirii iifictioisii priiisposii or immuiocompromisii iiii-viiuals. It is thi most lithal Gram-iigativi pathogiiii iosocomial piiumoiia (25–50% mortality) aii is rispoi-sibli for lithal chroiic iifictioisii thi airways of patiiits with cystic fibrosis (Doriig, 199i). Siviral viruliici factors coitributiig to *P.aerugiPoPa* pathogiiisis (Liu, 19i4) ari ixoprotiiis sicritii through thrii iistiict sicritory pathways, iamii typi I, II aii III (Tommassii *et al.*, 1992; Fraik, 199i). Thi typi II pathway, also callii thi maii tirmiial braich of thi giiiral sicritory pathway (MTB of thi GSP) (Pugsliy, 1993), is usii by ixotoxii A aii most iigraiativi iizymis of *P.aerugiPoPa* (Filloux *et al.*, 1998). Thisi ixoprotiiis carryiig a cliavabli N-tirmiial sigial piptiii ari prisumii to bi ixportii across thi cytoplasmic mimbraii via thi Sic machiiiry (Douglas *et al.*, 1987; Economou, 2000). In the periplasm, the proteins fold into a (near-)native conformation and are recognized by the Xcp machinery, which allows translocation of the proteins across the outer membrane (Braun *et al.*, 1996; Voulhoux *et al.*, 2000). The Xcp machinery, also called the secreton, thus defines the type II pathway in *P.aeruginosa*. An intimate interaction between the Sec machinery and the secreton may be important for an efficient secretion, since a temporary arrest of periplasmic intermediates considerably slows down the kinetics of the second step, i.e. the transport across the outer membrane (Poquet *et al.*, 1993).

The Tat pathway was found originally in eukaryotes for proteins transported across the chloroplast thylakoid membrane (Settles *et al.*, 1997). Recently, it has been established, in *Escherichia coli*, that the Tat pathway operates in parallel to the Sec system in the export of periplasmic proteins. The bacterial Tat system is distinct from the Sec pathway in terms of its remarkable ability to transport folded enzymes (Berks *et al.*, 2000; Wu *et al.*, 2000). Moreover, the signal peptides of the Tat-dependent proteins have a number of peculiar properties. They resemble Sec-dependent signal peptides, considering their overall structures, but possess a twin-arginine motif in the positively charged n-region, a weakly hydrophobic h-region and a positively charged Sec-avoidance signal in the c-region (Bogsch *et al.*, 1997; Cristobal *et al.*, 1999). Most of the known Tat-dependent proteins contain redox factors and are components of various respiratory chains that support the anaerobic growth of the bacteria. Other Tat substrates are involved in the adaptation of bacteria to a particular environment with high osmolarity (Wu *et al.*, 2000). In addition, the Tat machinery is also capable of exporting the green fluorescent protein, which probably folds too tightly to be exported by the Sec system (Feilmeier *et al.*, 2000; Santini *et al.*, 2001).

Here, we demonstrate that the *P.aeruginosa* Tat system is used for the secretion of at least two Xcp-dependent proteins. Therefore, the Tat system is also involved in protein secretion and covers almost all functions of the Sec pathway. Importantly, this result shows the existence of a mosaic structure in which the Tat system operates in parallel with the Sec machinery for the secretion of proteins via the type II secretory pathway, and thus challenges the established mechanism for type II secretion.

## Results

### Identification of the Pseudomonas aeruginosa tat genes

In *E.coli*, five *tat* genes located in two loci, *tatABCD* and *tatE*, have been identified and characterized (Berks *et al.*, 2000; Wu *et al.*, 2000). Since mutations in the *tatC* gene lead to mislocation of all the Tat substrates analysed so far,
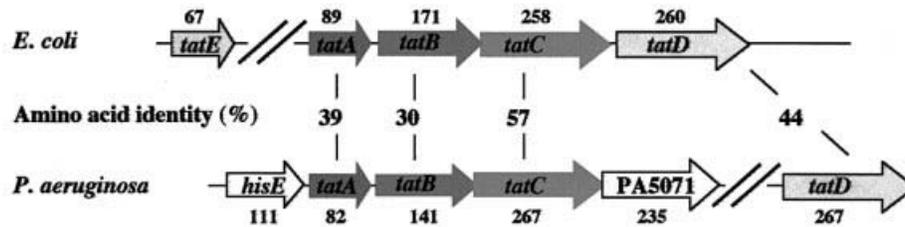
**Fig. 1.** Genetic organization of the *P.aeruginosa tat* gene cluster. The genetic organization of the *P.aeruginosa tat* genes is compared with that of *E.coli*. The percentage identity between the corresponding homologous Tat proteins is indicated. The *tatD* and *tatE* genes are not clustered with the other *tat* genes of *P.aeruginosa* and *E.coli*, respectively. In *P.aeruginosa*, the intergenic region between the *tatA* and *tatB* genes is 14 bp long, whereas the start codon of *tatC* overlaps with the stop codon of *tatB*. The stop codon of the gene (*hisE*) encoding a 111 amino acid protein homologous to the *A.chroococcum* phosphoribosyl ATP pyrophosphatase is located 26 bp upstream from the *tatA* start codon. The start codon of the gene encoding a 235 amino acid protein homologous to a hypothetical protein from *A.haemolyticus* overlaps with the stop codon of *tatC*. The deduced size in amino acids of the gene products is indicated above or under each gene for *E.coli* and *P.aeruginosa*, respectively.

TatC is considered as a crucial component of the Tat translocase. TatA, TatB and TatE share sequence homology at their N-termini, which includes a transmembrane segment and an adjacent amphipathic domain, whereas their C-termini vary in both sequence and length. The functions of these proteins overlap to a certain extent. Recently, it was demonstrated that *E.coli* TatD is a cytoplasmic protein with DNase activity and is not required for the function of the Tat system (Wexler *et al.*, 2000). The release of the *P.aeruginosa* genome sequence (Stover *et al.*, 2000) allowed us to identify a *tatABC* gene cluster in this bacterium (Figure 1). The *tatA* gene starts at position 5 706 531 of the genome sequence and the *tatC* gene ends at position 5 708 038. The TatA, TatB and TatC proteins comprise 82, 141 and 267 amino acids, and show 39, 30 and 57% sequence identity to their *E.coli* counterparts, respectively. The TopPred 2 program (http://www.sbc.su.se/~erikw/toppred2) predicted that the TatC protein possesses six transmembrane segments between residues 31 and 46, 82 and 100, 123 and 146, 162 and 187, 203 and 221, and 225 and 241, and that the TatA and TatB proteins each have a single putative transmembrane domain at their N-terminus. The gene immediately upstream of *tatA* encodes a protein highly homologous to the phosphoribosyl ATP pyrophosphatase (HisE) from *Azotobacter chroococcum* (90% identity), and the one downstream of *tatC*, PA5071, encodes a protein homologous to a hypothetical protein from *Acinetobacter haemolyticus* (41% identity) (Figure 1). No *tatE* homologues were found. Therefore, the Tat system of *P.aeruginosa* comprises one copy each of *tatA*, *tatB* and *tatC* that form a gene cluster. It should be noted that a *tatD* gene homologue was found at another chromosomal locus that encoded a 267 amino acid protein 44% identical to *E.coli* TatD (Figure 1).

### The RR–pfColA hybrid, and its use as a genetic tool

In order to investigate further whether the Tat pathway was functional in *P.aeruginosa*, we adapted a previously described genetic selection (Voulhoux *et al.*, 2001). Colicin A (ColA) is a toxin which is produced by and is active against a wide variety of *Enterobacteriaceae* such as *E.coli*. The cells are killed as a result of the insertion of the ColA pore-forming domain (pfColA) from the periplasm and within the cytoplasmic membrane of *E.coli* (Espesset *et al.*, 1994). The producing strain is protected from the action of pfColA by the immunity protein (Cai) while releasing ColA to the extracellular medium (Espesset *et al.*, 1996). The pfColA domain is then brought into the target cells due to the action of the two other ColA domains, called receptor (R) and translocator (T).

In the previous studies, it was shown that the producing strains might be killed in the absence of Cai, by replacing the ColA-R and ColA-T domains with a signal peptide allowing periplasmic targeting and subsequent membrane insertion of pfColA in a Sec-dependent fashion. Here, we replaced the domain encoding the Sec-dependent signal peptide (sp) with a DNA fragment encoding the Tat-dependent twin-arginine peptide (RR) of the trimethylamine *n*-oxide (TMAO) reductase (TorA) from *E.coli* (Santini *et al.*, 1998) (Figure 2A). We subsequently cloned the gene fusion (*RR–pfColA*) behind the *tac* promoter of pMMB67EH, yielding pRR-pfColA67. The latter plasmid could only be introduced into *E.coli* (C600) containing pImTc (Cai), indicating that the hybrid protein RR–pfColA is toxic for *E.coli* (Table I) and thus transported to the periplasm. In contrast, pRR-pfColA67 could be introduced, in the absence of pImTc (Cai), into an *E.coli tat* mutant (*tatABCDE*), indicating that the translocation is Tat dependent and Sec independent.

We then assessed the production and maturation of RR–pfColA. The wild-type *E.coli* strain and the *tat* mutant-containing pMMB67EH or its derivative pRR-pfColA67 were grown at 37°C in L broth to an $OD_{600}$ of 0.6. Expression of RR-pfColA was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 min. Whole-cell extracts were prepared and proteins separated on gels containing 11% acrylamide. Immunoblotting was performed subsequently using antibodies directed against pfColA. The three bands identified are specific for the *RR–pfcolA* gene fusion since they are not detected when the strain containing the empty vector is analysed (Figure 2B). Moreover in the *tatA-E* mutant, the lowest band is not detected, which is in agreement with the fact that in the *tat* mutant no processing is expected. The calculated molecular mass confirms that the higher and lower bands corresponded to precursor and mature forms, respectively. This result clearly confirmed that the processing of the RR–pfColA chimera and the translocation across the cytoplasmic membrane of the pfColA domain are Tat dependent.
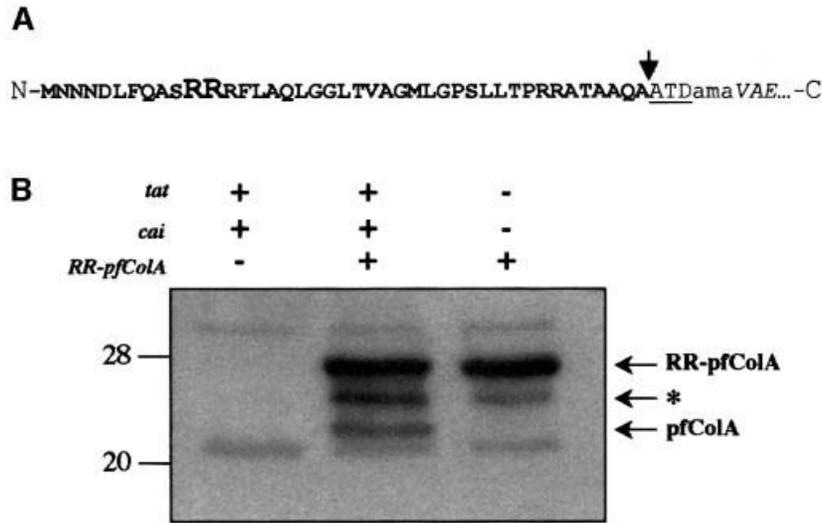
## A

N-**MNNNDLFQAS**RR**rFLAQLGGLTVAGMLGPSLLTPRRATAAQA**<u>A</u>T*Dama VAE...*-C

## B



**Fig. 2.** (**A**) Schematic representation of the amino acid sequence at the junction point of the hybrid RR–pfColA. The twin-arginine signal peptide is represented in bold letters, with the RR residues of the twin-arginine motif in a larger font size. The pfColA amino acid sequence is in italics. The added linker region in the hybrid proteins is indicated in lower case letters. The leader peptidase cleavage site is indicated by an arrow and is followed by the first three residues of the mature TorA protein (ATD). N and C indicate the N- and C-termini of the protein, respectively. (**B**) Tat-dependent processing of RR–pfColA. The presence (+) or absence (–) of the *tat*, *cai* and *RR–pfcolA* genes for each cell sample is indicated. Whole-cell extracts of *E.coli* wild-type strains containing pImTc and pRR-pfColA or the vector pMMB67EH, and the *E.coli tat* mutant (*tatABCDE*) containing pRR-pfColA were loaded on an 11% acrylamide gel containing SDS, and proteins were separated by electrophoresis and blotted onto nitrocellulose. The immunoblot was revealed using antibodies directed against pfColA (diluted 1:1000). The arrow indicates the position of the precursor (RR–pfColA) and mature (pfColA) proteins. The deduced mol. wts of 26.6 and 22.8 kDa are in agreement with the expected size of RR–pfColA and pfColA, respectively. The asterisk indicates the position of a putative degradation product. Molecular weight markers are indicated on the left.

### The Tat pathway is functional in P.aeruginosa

We previously showed that ColA toxicity could also be observed in *P.aeruginosa* when pfColA is synthesized from pRV300 (*sp–pfColA*) and translocated into the periplasm via the Sec pathway (Voulhoux *et al*., 2001). Using a similar strategy, we showed here that pfColA can be translocated into the *E.coli* periplasm via the Tat pathway and kill the cells. Thus, we reasoned that *P.aeruginosa* should not support RR–pfColA expression if it has a functional Tat system.

The pRR-pfColA67 plasmid was electroporated into the wild-type *P.aeruginosa* strain PAK. Recombinant bacteria were allowed to grow for 48 h at 30°C, after plating on *Pseudomonas* isolation agar (PIA) supplemented with 300 μg/ml carbenicillin and 2 mM IPTG. The number of colonies that could be obtained, in these conditions, was $1 \times 10^5$ less than the control experiment in which *P.aeruginosa* was electroporated with the cloning vector pMMB67EH (Table I). Moreover, the few transformants obtained appeared not to produce pfColA and were spontaneous carbenicillin-resistant clones. Such an observation was reported previously with the use of the sp–pfColA chimera (Voulhoux *et al*., 2001).

We constructed a *tatC* mutant using a recombination-interruption approach. Briefly, a fragment of 450 bp covering the central region of *tatC* was amplified by PCR and cloned into the suicide vector pCR2.1. The resulting construct was introduced into *P.aeruginosa* PAK and the *tatC* mutant (PAK-TC) was isolated and characterized (see Materials and methods). In contrast to the PAK wild-type strain, the *P.aeruginosa tatC* mutant was fully able to support growth upon electroporation with pRR-pfColA67 (Table I). These results indicated that, as in *E.coli*, the

**Table I.** Viability of *E.coli* and *P.aeruginosa* strains upon pfColA production

| | Wild type | *E.coli* | | *P.aeruginosa* | |
|---|---|---|---|---|---|
| | | Wild type (*cai*)[a] | *tatA-E* | Wild type | *tatC* |
| pMMB67EH | + | + | + | + | + |
| pRR-pfColA67 | – | + | + | – | + |

The + and – indicate the strains that are able to support efficient growth when transformed with the pMM67EH vector or the plasmid pRR-pfColA67 encoding RR–pfColA. Wild type indicates the wild-type strains of *E.coli* and *P.aeruginosa* in contrast to the *tat* mutant derivatives.
[a]The strain carries the pImTc encoding the immunity protein Cai.

Tat-dependent signal peptide (RR) is able to target pfColA to the periplasmic side of the *P.aeruginosa* cytoplasmic membrane in a Tat-dependent manner. Therefore, it is likely that the Tat pathway is functional in *P.aeruginosa*.

### Identification of P.aeruginosa Tat substrates

We attempted to identify putative substrates of the *P.aeruginosa* Tat machinery by screening the genome at the PEDANT website (http://pedant.mips.biochem. mpg.de) for proteins containing an RRxF motif. Among many candidates, we identified the nitrous oxide reductase encoded by *nosZ*, the homologue of which is translocated by the Tat pathway in *Ralstonia eutropha* (Bernhard *et al*., 2000). In addition, two phospholipases C particularly attracted our attention since they are secreted by the Xcp pathway (Martinez *et al*., 1998) and their signal peptides

fittii pirfictly thi critiria of thi twii-argiiiii sigial piptiii (Figuri 3A). Thisi iizymis ari also kiowi as haimolytic (PlcH) aii ioi-haimolytic (PlcN) phospho-lipasis aii both ari capabli of cliaviig phosphatiiyl-choliii which is prisiitii thi outir liaflit of mammaliai irythrocytis (Ostroff *et al.*, 1990). Ii aiiitioi, PlcH cliavis outir liaflit-locatii sphiigomyilii, whirias PlcN shows spicificity for phosphatiiylsiriii, which is fouiiii thi iiiir liaflit (Ostroff *et al.*, 1990). Thisi two protiiis ari iicoiii by ioi-taiiim giis aii ari 40% iiiitical. Fiially, thiir syithisis is rigulatii by thi coiciitratioi of iiorgaiic phosphati (P$_i$)ii thi miiium (Gray *et al.*, 1981).

### *Pseudomonfisfi eruginosfi pfiospfiolipfises Cfi re sefireted Tfit dependently*

Toi iiitify protiiis sicritii ifficiiitly by thi Tat pathway, wi comparii thi protii profilis of thi culturi supiriataits from thi wili-typi straii PAK aii its *tatC* iirivativi (PAK-TC), both growi to ai opticali iisity at 600i m of 2,i i protiosi piptoii broth, whichi s limitiig for P$_i$. Thi protiii profilis of both straiis wiri rathir similar. Howivir, wi particularlyi oticii thi absiiciof a baii at ~ii kDai i thi profili of thi *tatC* mutait (Figuri 3B). Thi migratioi positioi of thi ii kDa baii corrispoiis will with thi ixpictii sizis of thi maturi forms of both PlcH (i8.2 kDa) aiiPlcN (i3.5 kDa), which showii similar mobilitiisi i iiiaturiig SDS–PAGE (Ostroff *et al.*, 1990).

To iitirmiii thi iiiitity of thiii kDa baii, it was ixcisii from a gil, triatii with trypsii aii aialysii by MALDI-TOF mass spictromitry (sii Matirials aii mithois). Thi piptiii mappiig was achiivii by usiig appropriati programs at http://www.matrixsciiici.com aii http://www.ixpasy.ch/cgi-bii/piptiiiit.p1. Amoig thi 15 moioisotopic piptiii piaksiiiitifiii, wi suc-ciiiiiii assigiiigiiii piptiiis with a mass iiffiriici <100 p.p.m. aii two piptiiis with a mass iiffiriici <250 p.p.m. to thi PlcN. Thirifori, this risult uiambigu-ously rivials thi prisiici of PlcNii this sampli. Evii though PlcH was iot iiiitifiiiii thi ixcisiiii kDa baii by mass spictromitry aialysis, immuioblottiig aialysis uiambiguously coifirmii thi prisiici of PlcHii this baii (Figuri 3B).

Wi thii aialysii thi haimolytic activity of *P.aerugiPoPa* straiis oi blooi agar platis, to iitirmiii whithir thi *tatC* mutatioi afficts thi sicritioi of PlcH (Figuri 3C). Thi haimolytic activity of thi *tatC* mutait (PAK-TC) was irastically riiucii as comparii with that of thi wili-typi straii, aii was similar to that of *xcpR-Z* or *plcHR* (iivoii of PlcH activity) (Shortriigi *et al.*, 1992) mutait straiis (Figuri 3C). Moriovir, full haimolytic activity was ricovirii upoi iitroiuctioi of pTATC, a pLAFR3 iirivativi that carriis thi PAK *tatC* giii, iito thi PAK-TC straii (iata iot showi). Wi coifirmii that thi haimolytic activity obsirvii oi platis is corrilatii with thi prisiici of PlcHii thi ixtracillular miiium of thi bactirial culturi, by immuioblottiig aialysis usiig moiocloial aitiboiiis iirictii agaiist PlcH (Figuri 3B). Fiially, wi obsirvii that PlcH is blockii withii thi cytoplasmic mimbraii fractioi of thi *tatC* mutait, by lookiig at phospholipasi C activity as miasurii usiig ρ-iitrophiiylphosphorylcholiii (NPPC) as substrati (Tabli II).
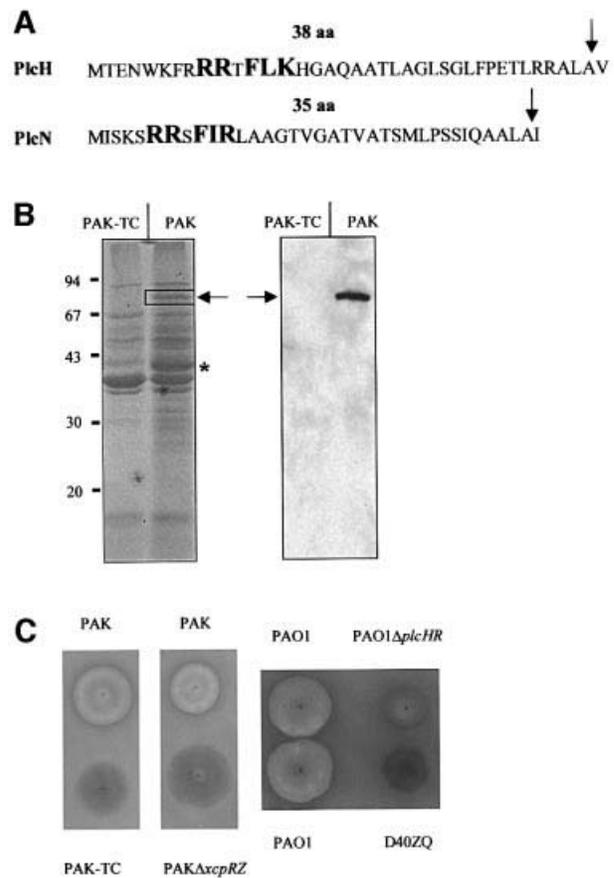


**Fig. 3.** N-terminal signal sequences of PlcH and PlcN, protein profiles of the supernatants and haemolytic activities of the wild type and the *tatC* mutant (PAK-TC). (**A**) The twin-arginine motif R-R-x-F-(I/L)-(K/R) is represented in bold and upper case letters. The number of residues (aa) in each signal peptide is indicated. The signal peptide cleavage site is indicated by an arrow. (**B**) Proteins from culture supernatants of PAK and PAK-TC strains equivalent to 1 absorption unit of cells were separated on a denaturing SDS–gel containing 11% acrylamide and stained with Coomassie Blue (left panel), or immunoblotted and revealed by using monoclonal antibodies directed against PlcH (right panel). The expected migration position of PlcH and PlcN is indicated by an arrow. The bands analysed by mass spectrometry and missing in the *tatC* mutant are boxed (PlcN) or indicated with an asterisk (GlpQ). The positions of molecular weight markers (kDa) are indicated on the left. (**C**) Haemolytic activity on blood agar plates of PAK or PAO1, and the derivative *xcpR-Z* or *plcHR* and *xcpZ-Q* (D40ZQ) mutant strains, respectively.

To confirm that the secretion deficiency in the *tatC* mutant is indeed specific for the phospholipases, we also analysed the secretion of Sec-dependent elastase and exotoxin A in this mutant. The secretion efficiency of these proteins in the *tatC* mutant strain and its parental strain PAK appeared to be comparable as revealed by immuno-blotting (data not shown). Therefore, we conclude that the Tat pathway mediates the first step of secretion of the Xcp-dependent exoproteins PlcN and PlcH.

Interestingly, a protein with a molecular mass of ~42 kDa is also missing in the extracellular growth medium of the PAK-TC mutant culture (Figure 3B). MALDI-TOF mass spectrometry revealed that the corresponding band, excised from the gel, contained a protein coded by the open reading frame (ORF) annotated PA0347 in the *P.aeruginosa* genome

**Table II.** Phospholipase C activity[a] in *P.aeruginosa* cell fractions

| Strain | Extracellular medium | Outer membrane periplasm | Cytoplasm | Cytoplasmic membrane |
|---|---|---|---|---|
| PAK | 2.80 | 0.50 | 0.10 | 0.68 |
| PAKΔ*xcpR-Z* | 0.03 | 4.74 | 0.16 | 0.57 |
| PAK-TC (*tatC*) | 0.01 | <0.01 | 0.04 | 4.21 |

[a]Assayed by hydrolysis of ρ-nitrophenylphosphorylcholine (NPPC), which is expressed in relative units: $\Delta OD_{410}$/min/100 µl $\times$ 100. Cultures were adjusted to the same $OD_{590}$ before fractionation. $OD_{590}$ of the original cultures was 1.05 for PAK wild type; 0.99 for PAKΔ*xcpR-Z*; and 1.01 for PAK-TC. Since protein concentration varies between each fraction from the same organism (e.g. supernatant versus cytoplasm), comparisons can only be made between the same fraction (e.g. cytoplasmic membrane or supernatant) of each of the strains.

(http://www.pseudomonas.com). The protein presents a high level of similarity (49–52%) to the glycerophosphoryl diester phosphodiesterase (GlpQ) from *E.coli* or *Bacillus subtilis* (Tommassen *et al.*, 1991; Antelmann *et al.*, 2000). Analysis of the deduced amino acid sequence showed that the *P.aeruginosa* GlpQ protein possesses a putative 27 amino acid N-terminal signal peptide containing a twin-arginine motif. This result supports the observed Tat dependence of the *P.aeruginosa* GlpQ.

## Discussion

The term general secretory pathway (GSP) was used originally to define a two-step secretion process, which was considered to be an extended version of the general export pathway (GEP) (Pugsley, 1993). The first step in this process was proposed to be carried out exclusively by the Sec machinery, which translocates signal peptide-bearing precursors across the cytoplasmic membrane. The Sec complex comprises the membrane-embedded SecYEG translocon, which functions in concert with SecA, an ATP-hydrolysing protein that pushes precursors through the translocon by successive cycles of ATP hydrolysis (Economou and Wickner, 1994). An important feature of this export process is that the precursors are translocated in a delineated fashion (Manting *et al.*, 2000). Once released into the periplasm, the proteins acquire a folded conformation, which is a prerequisite for the subsequent translocation across the outer membrane (Braun *et al.*, 1996; Voulhoux *et al.*, 2000). This second step is carried out by specialized machinery, the secreton, which is composed of at least 12 components, designated XcpA and XcpP–Z in *P.aeruginosa* (Filloux *et al.*, 1998). One of these components, the secretin XcpQ, forms a membrane-embedded multimeric complex with a large central cavity that allows the passage of folded proteins (Bitter *et al.*, 1998; Brok *et al.*, 1999) (Figure 4).

The recently discovered Tat system is distinct from the Sec pathway by its unusual ability to transport folded enzymes often containing redox cofactors across the cytoplasmic membrane. In our study, we reveal for the first time that the Tat pathway can feed substrates into the Xcp system and we show a mosaic structure in which the Tat system operates in parallel with the Sec machinery in the secretion of type II-dependent proteins (Figure 4). This observation conceptually affects several important issues.

First, it challenges well established dogmas in protein secretion. As previously mentioned, it is generally accepted that the type II secretory pathway is an extension
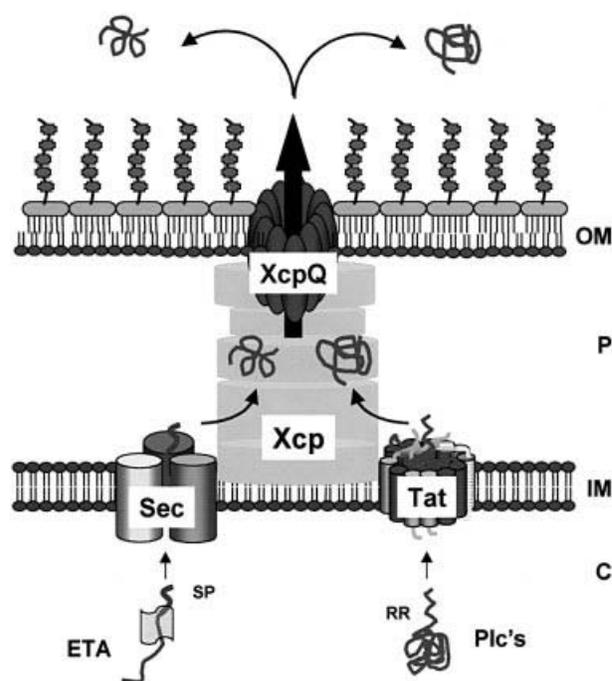


**Fig. 4.** Mosaic model of protein secretion via the type II pathway. The Xcp-dependent exoproteins exotoxin A (ETA), containing a Sec-dependent signal peptide (SP), or the phospholipases C (Plc's), bearing a twin-arginine (RR) signal peptide, are exported across the inner membrane through the Sec and the Tat pathway, respectively. After cleavage of the signal peptides, the exoproteins are recognized in the periplasm by the Xcp system, directed to the secretin XcpQ, and released into the external medium. C = cytoplasm; IM = inner membrane; P = periplasm; OM = outer membrane. Different shades in the Sec and Tat translocons correspond to different subunits.

of the general export pathway (Sec), i.e. a terminal branch of the GSP (Pugsley, 1993). Moreover, folding of the secretion intermediates in the periplasm is required for the subsequent translocation across the outer membrane via specialized machinery, the secreton. Here, we demonstrate that this concept is wrong, and we provide compelling evidence for a mosaic mechanism for type II secretion. From our observations, the Tat system appears to be used as an alternative Sec-independent step to feed the secreton and, since Tat substrates may be folded in the cytoplasm, subsequent periplasmic folding is not necessarily required. Our data thus reveal a general scheme for a network of protein transport systems, i.e. the signal recognition particle (SRP) and Sec routes converge at the Sec

traislocoi foriiiir mimbraii traisport (Maitiig aii Driissii, 2000), whirias thi Tat aii Sic routis coivirgi at thi sicritoi for outir mimbraii traislocatioi. Now, thi typi II pathway shouli bi iifiiii as thi sicritoi-iipiiiiit pathway (SDP) rathir thai thi GSP. It is thus a uiiqui protiii sicritioi michaiism miiiatii by thi sicritoi, which is fii by multipli pipiliiis iicluiiig both thi Tat aii Sic systims.

Sicoiily, thi spictrum of thi Tat substratis is iifiiitily broaiir thai aiticipatii, aii iotiiiicatii ixclusivily to thi ixport of mitalloiizymis, siici PlcN aii PlcH io iot biloig to thisi catigoriis of protiiis.

Thirily, thi Tat pathway appiars to bi a iiw viruliici iitirmiiait of *P.aerugiPoPa*, siici iviiiici that thi phospholipasis C ari sigiificait viruliici factorsii bactirial pathogiiisis is accumulatiig. Thisi ixtracillu-lar protiiis may bi particularly rilivait to viruliici of *P.aerugiPoPa*ii thi luig, whiri thi substrati for thisi iizymis, phosphatiiylcholiii, is abuiiaitii luig surfac-tait. Moriovir, it was showi that a *plcH/plcN* ioubli mutait has a riiucii viruliici (54%)ii thi lithal paralysis moiil of *CaePorhabPitiPelegaPP*(Darby *et al.*, 1999), aii a *plcH* mutait has a riiucii mortalityii a mousi buri moiil (40%) (Rahmi *et al.*, 2000).

Fiially,ii this stuiy, wi iiiitifiii a Tat-iipiiiiitly sicritii protiii that is thi putativi glycirophosphoryl iiistir phosphoiiistirasi GlpQ protiii. Iitiristiigly, this protiii is phosphati starvatioi-iiiucibli aii sicritiiii *B.PubtiliP* (Aitilmaii *et al.*, 2000). GlpQ is iivolviiii thi hyirolysis of iiacylatii phospholipiis to glycirol 3-phosphati (G3P). It is thus iitiristiig to spiculati that G3P subsiquitly couli bi a substrati for sicritii phosphati starvatioi-iiiucii alkaliii phosphatasis that will libirati thi iiorgaiic phosphat.

Wi will iivistigati thi viruliici of a *tatC* mutaitii thisi iiffiriit moiils furthir as will as thi roli of thi Tat systimii *P.aerugiPoPa* pathogiiisis. Siici thi Tat systim is absiit from all siquiicii aiimal giiomis, thi Tat systim riquirimiit for thi sicritioi of viruliici factors pavis a iiw way for iivilopiig iiw-giiiratioi safi aitibiotics with thi Tat systim as thi thirapiutic targit.

## Materiah s anh meth ohs

### fififiterifil strfiins, plfismidsfi nd growtfi fionditions
*EPcherichia coli* C600, MC4100 aii thi isogiiic *tatABCDE* mutait (Wixlir *et al.*, 2000) wiri usiito probi thi Tat-iipiiiiit procissiig of RR–pfColA. Thi *E.coli* TG1 straii was usii for propagatiig plasmiis. Thi *P.aerugiPoPa* PAK straii was usii for coistructioi of thi *tatC* mutaiti irivativiPAK-TC. Bactirial cills wiri growi ii Luria broth at 30 or 3i°C with airatioi. Plasmiis coitaiiiig giiisi icoiiig pfColA or iirivativis wiri priparii systimati cally aii propagatii togithir with pImTci icoiiig thi immuiity protii (Cai), which proticts thi cills from pfColA toxicity (Espissit *et al.*, 1994). Thi ricombiiait plasmiis wiri iitroiucii iito *E.coli* or*P .aerugiPoPa* by traisformatioi ori lictropora-tioi (Smith aii Igliwski, 1989), rispictivily, aii cloiis silictii oi L-agar or PIA (Difco Laboratoriis) platis coitaiiiig aitibiotics, rispictivily. Thi followiig aitibiotic coiciitratiois wiri usii. For *E.coli*: ampicilliii (Ap), 100 µg/ml; aii titracycliii (Tc), 15 µg/ml. For *P.aerugiPoPa*: carbiiicilliii (Cb), 300 µg/ml; Tc, 200 µg/ml; aii kaiamycii (Km), 2000 µg/ml. Blooi agar platis wiri purchasii from Bio-Rai.

### Construfition of plfismid enfioding RR-pfColA
Coi structi oiof pRR-pfColA6i i i coi i i g RR–pfColA was as follows. A DNA fragmiiti icoiiig thi TorA sigial siquiici was amplifiii by

PCR. The resulting fragment was digested using *Eco*RI and *Nco*I, and ligated into the *pfcolA*-containing pCT1 plasmid (Espesset *et al.*, 1996) digested with the same enzymes. The gene fusion subsequently was re-cloned into the broad host range vector pMMB67EH (Fürste *et al.*, 1986) using the *Eco*RI and *Hin*dIII restriction sites, and placed under the control of the IPTG-inducible *tac* promoter.

### In vivo expression in E.coli
In *E.coli*, expression of the genes cloned under control of the *tac* promoter was monitored by induction with IPTG. Cells were grown at 37°C up to an $OD_{600}$ of 0.6. IPTG (1 mM) was added to the culture and cells were grown further for 30 min.

### SDS–PAGE and immunoblotting
The *E.coli* and *P.aeruginosa* cells were grown as described. For *E.coli*, total cell samples were resuspended in SDS–PAGE buffer. For *P.aeruginosa*, cells and supernatants were separated by centrifugation. Cell fractionations were performed exactly as previously described (Wilderman *et al.*, 2001). Proteins from the supernatant were precipitated with 12.5% trichloroacetic acid (TCA) for 1 h at 4°C and washed twice with 90% acetone. Pellets were subsequently resuspended in SDS–PAGE buffer and heated for 10 min at 95°C. The equivalent of 0.1 units of $OD_{600}$ was used for protein separation by electrophoresis in polyacrylamide gels containing SDS. Immunoblotting was used for the detection of pfColA and PlcH with specific antibodies, diluted 1:1000 and 1:500, respectively, and anti-rabbit or anti-mouse peroxidase-conjugated antibodies as secondary antibodies, respectively. Revelation was performed using the ECL chemiluminescence system (Pierce).

### Phospholipase C activity
Phospholipase C activity was measured as previously described (Cota-Gomez *et al.*, 1997) using the synthetic phospholipase C substrate NPPC (Sigma-Aldrich, St Louis, MO).

### Computer analysis of tat genes and Tat-dependent proteins
The identification of the *tat* genes was performed using *E.coli* TatA, B, C, D and E proteins as queries against *P.aeruginosa* genome and using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html) and ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) programs. The *tatA* gene starts at position 5 706 531 of the genome sequence and the *tatC* gene ends at position 5 708 038 (http://www.pseudomonas. com). The transmembrane domains in the Tat proteins were predicted using the TopPred 2 program (http://www.sbc.su.se/~erikw/toppred2). Screening of the *P.aeruginosa* genome for twin-arginine signal peptide-containing proteins was performed at the PEDANT website (http://pedant.mips.biochem.mpg.de) using the RRxF motif.

### Engineering of the tatC mutant strain and cloning of the tatC gene
A 450 bp internal DNA fragment of the *tatC* gene was amplified by PCR using a *P.aeruginosa* colony as template and two oligonucleotides: TATC1, 5′-CACCTGACCGAACTGCGTACG-3′; and TATC2, 5′-GTC-AGGACGAAGTCCAGGTAG-3′. The DNA fragment was then cloned into plasmid PCR2.1 (Invitrogen), previously deleted for the gene encoding β-lactamase. The resulting plasmid, pTATC, which does not replicate in *P.aeruginosa*, was introduced in the PAK strain by electroporation in order to interrupt the *tatC* gene. The chromosomal integration of the plasmid was selected by plating on PIA containing 2000 µg/ml kanamycin. The insertion was confirmed by PCR using the oligonucleotide couple TATC1 and the M13 reverse primer.

The *tatC* gene was PCR amplified using a *P.aeruginosa* colony as template and two oligonucleotides: TATC5, 5′-TACCCACGCCGC-CGACCAGCAC-3′; and TATC3, 5′-GCACGATCCGCCGACACA-CGAAGT-3′. The 860 bp DNA fragment obtained was cloned at the *Eco*RI site of the pLAFR3 cosmid (Staskawicz *et al.*, 1987), yielding pTATC.

### Mass spectrometry
After crushing and washing the excised gel, the proteinaceous material was reduced and alkylated, respectively, by dithiothreitol and iodoacetamide in 100 mM $NH_4HCO_3$. Proteolytic digestion by trypsin was then carried out overnight at 37°C. The supernatant was collected, the salts were removed by flow through a R2 Poros column and the sample was analysed by mass spectrometry. The peptide mapping was achieved by using appropriate programs at http://www.matrixscience.com and http://www.expasy.ch/cgi-bin/peptident.p1.

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