CHAPTER 5

Heterologous production of *Escherichia coli* penicillin G acylase in *Pseudomonas aeruginosa*

Joanna Krzeslak, Peter Braun, Rome Voulhoux, Robbert H. Cool, and Wim J. Quax

Submitted for publication

Heterologous production of *Escherichia coli* Penicillin G Acylase in *Pseudomonas aeruginosa*

Penicillin G acylase (PGA) is a widely studied bacterial enzyme of great industrial importance. Since its overproduction in the original organisms is mostly limited to the intracellular bacterial spaces which may lead to aggregation and cell toxicity, we have set out to explore the host organism *Pseudomonas aeruginosa* for secretion of folded proteins to the extracellular medium. We have made fusion proteins, consisting of *Pseudomonas* Sec- or Tat-specific signal peptides, the elastase propeptide and the mature penicillin G acylase. With all constructs we obtained production of PGA in *P. aeruginosa*, but we observed that processing of PGA was temperature dependent and that the active enzyme could only be found after growth at 25°C or lower temperatures. Remarkably, the mature protein, expressed from a TatProPGA hybrid, was not only found in the extracellular medium and the periplasm, but also in the cytoplasm as assessed by comparison to the reporter beta-lactamase protein. The unusual cytoplasmic localization of the mature protein strongly suggests that processing of PGA can also occur in the cytoplasm of *P. aeruginosa*. The extracellular localization of the TatProPGA hybrid was found not to be dependent on the *tatABC*-genes. The elastase signal sequence/propeptide combination appeared to be an inadequate carrier for transporting penicillin G acylase across the outer membrane of *P. aeruginosa*.

Introduction

Transport of proteins out of the cytoplasm is a powerful and fundamental attribute of living organisms. This phenomenon has been exploited by industry to manufacture biologically active proteins for easy product purification. In bacteria, the transport across the cytoplasmic membrane is mainly via the Sec machinery[1-3], but folded proteins are assumed to be handled via the Tat pathway (twin-arginine translocation)[4-6]. In Gram-negative bacteria, extracellular translocation requires an additional machinery as the Sec- and Tat-dependent pathways translocate proteins only across the inner membrane (IM). For the transport via the outer membrane (OM) cells utilize additional translocation components, such as the type II secretion system of a general secretory pathway (GSP) which is conserved among Gram-negative bacteria. In *Pseudomonas aeruginosa*, the type II secretion system, the Xcp machinery composed of at least 12 Xcp proteins, is responsible for secretion of a subset of extracellular proteins including elastase, lipases, alkaline phosphatase, exotoxin A, and phospholipases[7]. Yet, no common secretion signal for exported proteins could be identified. Therefore, it remains unclear what this secretion motif is. There are reports underlining the significance of linear amino acid stretches that would be properly exhibited on the folded protein surface[8,9]. For instance, a pectate lyase fusion protein composed of two homologous forms from *Erwinia chrysanthemi* and *Erwinia carotovora* revealed the importance of certain loop regions for species-specific secretion[10]. In addition, a putative secretion domain of exotoxin A was successfully used to secrete a beta-lactamase hybrid to the extracellular medium in *P. aeruginosa*[9]. Also, a pullulanase-beta-lactamase hybrid protein with an N-terminal pullulanase segment was efficiently and completely transported to the cell surface in *E. coli*[8]. Yet, many

attempts to identify a secretion signal, like the one of cholera toxin by creating hybrid proteins (B-subunit pentamer with either beta-lactamase or alkaline phosphatase)[11], were ineffective. Overall, it is likely that the final protein conformation defines whether it is a suitable target for a general secretory pathway or not. Specifically, the secretory signal would be a composition of residues from distal parts of the protein polypeptide chain which if brought together during folding may be recognized by the secretion apparatus. Even small changes in the protein may influence the fate of the protein. For example, mutations within the cellulose Cel5 from *E. chrysanthemi* secreted via the type II secretion pathway led to a stable, however non secretable mutant[12]. Also, mutated aerolysin from *Aeromonas salmonicida* was affected in transport across the outer membrane[13].

Interestingly, many secreted proteases are synthesized as an inactive precursor polypeptide that possesses a unique propeptide sequence. These bacterial propeptides, transcribed as a one product with their substrate protein, form in the periplasm after self-processing a non-covalent complex with their protein. The propeptides are required for proper folding and are not essential for the final activity of protein. They are cleaved off autoproteolitically at the specific site of the precursor protein[14]. Elastase, a secreted thermolysin-like metalloprotease from *P. aeruginosa*, has a 18 kDa propeptide that is required for proper folding and secretion of elastase[15]. The propeptide has been found to be associated with the mature elastase during transport via the secretin[16].

The transport across the cytoplasmic membranes takes place mainly by signal peptides, sophisticated N-terminal domains consisting of the N-, H-, and C-regions that carry essential information about protein destination[17]. The Sec-type signal peptides are composed of a hydrophobic H-region of at least nine amino acids that can be preceded by positively charged residues within the first few amino acids (N-region), and a more polar C-region containing peptidase cleavage site[18]. In comparison, the typical Tat-type signal peptides are characterized by elongation of an N-region with a twin-arginine motif (RR), a modestly hydrophobic H-region and positively charged residues at the C-region[19,20]. Even tiny differences within the signal peptide among various proteins may target proteins to different secretion pathways[6,21,22].

Beta-lactam acylases from *E. coli* and *Pseudomonas* play an important role in the semi-synthesis of existing and novel antibiotics. In particular, penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) has significant industrial potential since it hydrolysis Penicillin G to 6-aminopenicillanic acid (6-APA), the beta-lactam nucleus of semi-synthetic antibiotics[23]. The most common with respect to industrial use is penicillin G acylase from *E. coli*[24]. The physiological function of this enzyme remains unclear but it is likely involved in the metabolism of aromatic compounds as a carbon source[25]. Its native expression profile was shown to be dependent on temperature, oxygen levels, and the presence of phenylacetic acid[26-28]. It is synthesized as a precursor polypeptide pre-pro-PGA (ppPGA) of 94 kDa that after translocation across the IM as a pro-PGA (pPGA), undergoes an unusual posttranslational processing to eventually yield, in the periplasmic space, a mature enzyme consisting of two subunits, α (23 kDa) and β (62 kDa)[29,30]. Figure 1 presents schematically PGA synthesis and processing in *E. coli* cells. The catalytic residue, serine Ser-290, is located at the amino-terminus of the β-subunit[31,32]. Replacing this residue with cysteine results in a processed but inactive enzyme, associating the serine residue as a catalytic nucleophile[33,34]. Moreover,

substitution with arginine, threonine, or glycin impairs enzyme processing, indicating the significance of the serine residue for autocatalytic cleavage[33].

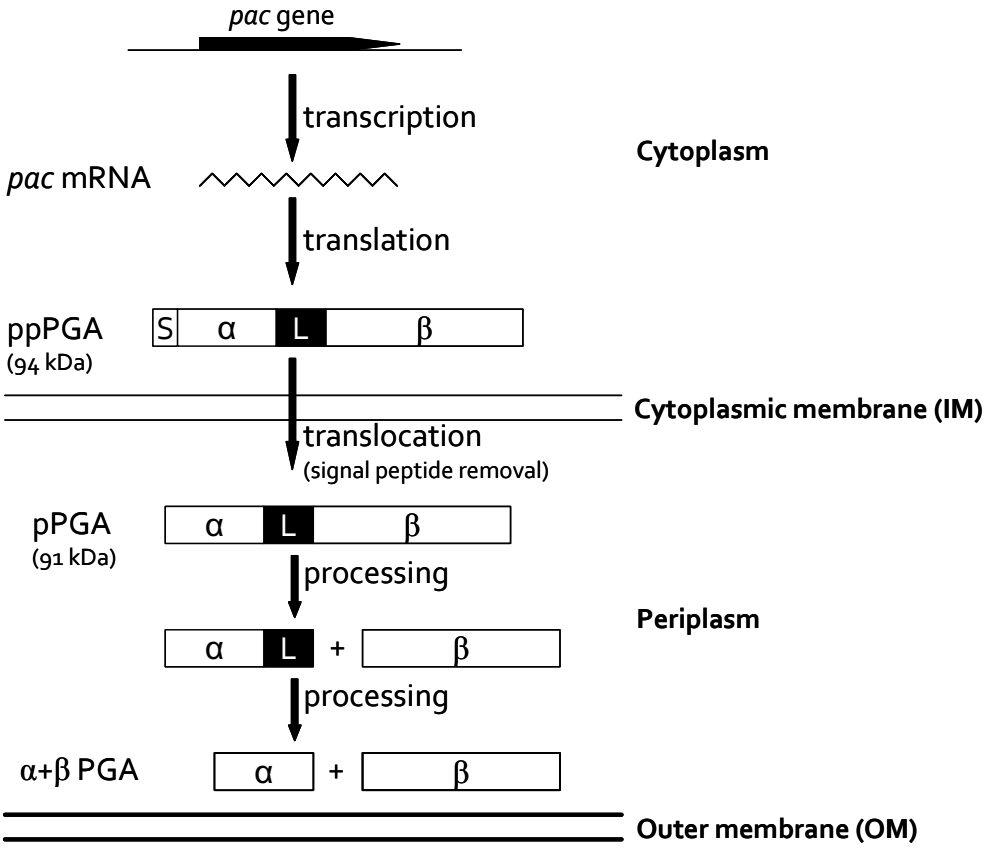


Figure 1. General scheme of PGA synthesis and processing in *E. coli.*
The *pac* gene encodes a 94 kDa polypeptide precursor (ppPGA) consisting of a signal peptide (S), an alfa-subunit (α), a linker (L), and a beta-subunit (β). The signal peptide is removed from the ppPGA during translocation into the periplasm. In the periplasm pPGA undergoes posttranslational modification that results in active PGA.

It has been reported that the *E. coli* penicillin acylase is functionally transported in *E. coli* across the inner membrane via the Tat pathway[35], which has only a limited capacity. In addition, *E. coli* is unable to secrete the enzyme into the extracellular medium as it lacks the GSP pathway. Hence, the attempts for overproduction have led to undesired protein accumulation and aggregation impairing protein maturation and activity[36-38], thus posing limitations to industrial development. The possible approach to solve this issue would be to find an alternative translocation mechanism for the delivery of this enzyme class to the periplasm and subsequently to the extracellular medium. Therefore, it is of key importance to develop a secretion system in an appropriate host, capable of producing and secreting correctly folded acylases. As *Pseudomonas* species are effective secretion hosts, with relatively rapid growth and ability to accumulate proteins in the extracellular medium, they are

attractive candidates for studying secretion, and production of commercially relevant, heterologous proteins. Since penicillin acylases can be folded in the periplasm, the type II secretion system may be recruited for their secretion. The pore (secretin) of the *Pseudomonas* type II secretion pathway has been shown by electron microscope analysis to be sufficiently large to allow for the transport of bulky proteins[39]. Moreover, several successful and encouraging approaches of directing heterologous proteins to the Tat pathway by combining them with a Tat signal peptide have been reported[20,40-43].

In this study, we investigated if *E. coli* PGA can, in *P. aeruginosa*, be functionally expressed, produced and transported from the cytoplasm to the periplasm with known *Pseudomonas* Sec-type or Tat-type signal peptides. Ultimately we have tested whether the elastase propeptide can be used as a secretion signal to target PGA to the extracellular medium via the Xcp machinery.

Materials and methods

Bacterial strains, plasmids and media

The plasmids and bacterial strains used in this study are listed in Table 1. Bacterial cells were propagated at 37°C in L-broth (LB)[44], unless otherwise indicated. Plasmids were maintained by addition of ampicillin (100 mg/l) for *E. coli* strains and carbenicillin (200 mg/l) for *P. aeruginosa*.

DNA manipulations

Amplification reactions with high-fidelity *Phusion* polymerase, digestions with restriction enzymes, ligations with T_4 DNA ligase were performed according to standard conditions[44] or as recommended by the manufacturer. Isolation of genomic DNA from *P. aeruginosa* was done using a GeneElute Bacterial Genomic DNA kit from Sigma-Aldrich, according to manufacturer instructions. The PGA-based fusion proteins are schematically depicted in Figure 2. The principle of an overlap extension PCR method[45], based on assembling of few DNA fragments simultaneously, was used to make fusion proteins. During the first conventional PCR rounds fragments of a target sequence were amplified separately. Subsequently, purified intermediate products with complementary extremities from the first PCR were fused and amplified with the help of two universal primers, in a second PCR reaction. Thus, the final products were built up of the different amplified PCR fragments. For amplification of *lasB* and *plcN* genes chromosomal DNA of *P. aeruginosa* PAO1 strain was used as a template. Amplification of the *pac* gene was done on genomic DNA of the *E. coli* ATCC9637 strain[46]. **(i) Construction of pMMB-PGA.** The complete *pac* gene of 2.54 kbps was amplified with a forward primer pac-F (5′ CCG GCG GAA TTC ATG AAA AAT AGA AAT CGT 3′) and a backward primer pac-R (5′ GCT GAC AAG CTT TTA TCT CTG AAC GTG CAA 3′) carrying *EcoRI* and *HindIII* restriction sites (underlined), respectively. After digestion, this PCR product was ligated into the broad-host range vector pMMB67EH (8.77 kbps) which was also cleaved with these two enzymes, resulting in pMMB-PGA. **(ii) Construction of pMMB-SecProPGA.** In the first PCR round two fragments for a protein hybrid were amplified separately. The fragment of 0.93 kbps, including the promoter, the signal peptide, and the propeptide sequence of the *lasB* gene, was amplified with a lasBProm-F primer (5′ CAG GAA ACA GAA TTC CCA GAA AGC GTG CAA 3′, *EcoRI* restriction site is underlined) and a lasBProp-R primer (5′ CAC TTG ACG ACT GCT CT G CAG GGG CCA GGC CTT CCC A 3′). The second fragment of 2.46 kbps, corresponding to the *pac* gene sequence (without its native signal peptide), was amplified with the pacAlfa-F (5′ TGG GAA GGC CTG GCC CCT GCA GAG CAG TCG TCA AGT G 3′) and pac-R primers. Resulting DNA fragments were annealed and amplified using the lasBProm-F and pac-R primers in the second PCR. The final product was digested with *EcoRI* and *HindIII* restriction enzymes and cloned into the pMMB67EH vector, yielding pMMB-SecProPGA. **(ii) Construction of pMMB-TatProPGA.** In order to construct the pMMB-TatProPGA, four fragments were amplified independently in the first PCR round. The first part of the hybrid corresponding to the promoter of the *lasB* gene was amplified with the lasBProm-F forward primer and the lasBProm-R reverse primer (5′ CTG CTT TTC GAA ATC ATC TTG TTC AGT TCT CCT 3′). The second fragment, the *plcN* gene signal peptide, was generated with the following primers: plcNSP-F (5′ GGA GAA CTG AAC AAG ATG ATT TCG AAA AGC A 3′) and plcNSP-R (5′

CGT CGA TCA GGT CGG CCG CCA GGG CCG CCT GGA T 3′). The third part of 0.53 kbps corresponding to the elastase propeptide *lasB* was amplified with the lasBProp-F (5′ CCA GGC GGC CCT GGC GGC CGA CCT GAT CGA CGT 3′) and lasBProp-R primers. The fourth fragment harboring the *pac* gene devoid the signal peptide sequence was amplified with the pacAlfa-F and pac-R primers. During the second PCR, four independent fragments from the first PCRs were annealed and the final product was amplified with the lasBProm-F and pac-R primers. Finally, a purified and *EcoRI-HindIII* digested PCR product of 3.1 kbps was ligated into the corresponding sites of the pMMB67EH vector.

Table 1. Strains and plasmids used in this study.

Strains	Properties	Reference
E. coli		
DH5α	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Invitrogen
ATCC9637	W strain	[46]
P. aeruginosa		
PAO1	Prototroph, wild type	B. Holloway collection
PAO1Δ*tatABC*	Chromosomal *tatABC* deletion in PAO1	[47]
PAO25	PAO1 *leu arg*	[48]
PAN8	PAO25 *lasB::Km*^{r} *aprE*	[16]
PAN9	PAO25 xcpQ::Gm^r *lasB::Km*^{r} *aprE::Hg*^{r}	[16]
PAN11	PAO25 *lasB::Km*^{r} *xcpR*	[16]
PAO7510	EMS-induced *xcpR* mutant of PAO25	[49]
Plasmids	**Properties**	**Reference**
pMMB67EH	Amp^R, RSF replicon (IncQ), tac promoter	[50]
pMMB-PGA	Native complete *E. coli pac* gene in pMMB67EH vector	This study
pMMB-SecProPGA	Fusion protein consisting of *P. aeruginosa lasB* promoter, *lasB* signal peptide, *lasB* propeptide and *E. coli pac* α-, β-subunits in pMMB67EH	This study
pMMB-TatProPGA	Fusion protein consisting of *P. aeruginosa lasB* promoter, *plcN* signal peptide, *lasB* propeptide, and *E. coli pac* α-, β-subunits in pMMB67EH	This study

Growth conditions, cell fractionation, SDS-PAGE, and immunoblotting

Overnight cultures of various *P. aeruginosa* strains were 100x diluted in fresh LB and grown with agitation at 37°C to an OD_{600} of 0.5-0.7. Next, the temperature was lowered to 22°C and cells were grown for about 16 h (stationary phase). Cells carrying the pMMB-PGA construct were induced with 0.5 mM IPTG at OD_{600} 0.5-0.7. Cells (equivalent to 1 OD_{600} unit) were removed by centrifugation (10 min, 5,000 rpm), supernatants of growth medium were collected, transferred to new tubes and centrifuged again (10 min, 13,000 rpm) to remove all remaining cells. Next, proteins from growth medium fractions were precipitated with 10% TCA or used in the activity assay. Pelleted total cells were analyzed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) or were resuspended in 10 mM Tris-HCl, pH 7.4. After centrifugation (10 min, 4,500 rpm), cells were resuspended in 200 µl buffer A containing 10 mM Tris-HCl, pH 8.0, 25% sucrose, 2 mM EDTA, and 0.5 mg/ml lysozyme. After incubation on ice for 25 min, 50 µl of buffer B containing 10 mM Tris-HCl, pH 8.0, 20% sucrose and 125 mM $MgCl_2$ was added. The suspension was centrifuged (15 min, at 4°C, 13,000 rpm) and the supernatant, representing the periplasmic fraction, was used directly in the activity assay or after TCA precipitation analyzed on SDS-PAGE. Furthermore, the pellet resuspended in 250 µl of 50 mM Tris-HCl, pH 8.0, 2 mM EDTA buffer was sonicated and cell debris was removed by centrifugation (30 min, 4°C, 13,000 rpm). The supernatant, representing the cytoplasmic fraction, was used directly in the enzymatic assay or was analyzed by SDS-PAGE. SDS-PAGE was performed on NuPAGE 4-12% Bis-Tris gels (Invitrogen). The same OD_{600} equivalents were loaded on SDS-PAGE. After electrophoresis proteins were transferred to nitrocellulose membranes which were subsequently incubated

with the appropriate primary antisera (rabbit antiserum raised against PGA or beta-lactamase). Detection was performed by enhanced chemiluminescense (ECL) with HRP-conjugated goat antibody against rabbit.

Enzymatic activity assay
The enzymatic activity assay was based on the conversion of 6-nitro-3-phenylacetamide benzoic acid (NIPAB) to 3-amino-6-nitrobenzoic acid (NABA). The single reaction was performed at 37°C in 200 µl containing 50 mM phosphate buffer pH 7.0, 3 mM NIPAB with 10-30 µl enzyme/proteins solution. The growth medium (30 µl), periplasmic (15 µl), cytoplasmic (10 µl), and soluble cell-free extract (10 µl) fractions gained after fractionation of proteins from different strains were used as enzyme/protein solutions in the assay. The release of NABA in the assay mixtures was followed by measuring the changes in absorbance at 405 nm. Purified PGA was used as a positive enzyme control. The protein concentrations of fractions used in the activity assay were determined by a Bradford assay using BSA (bovine serum albumin) as a standard (Pierce, Rockford, Illinois, USA). All reactions were performed in triplicates in 96-well plates.

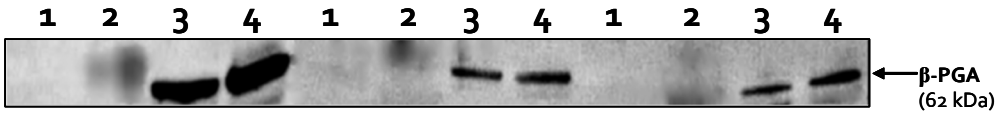
Results

P. aeruginosa produces processed and active native *E. coli* PGA

To determine whether the putative signal peptide of a *pac* gene promotes expression of penicillin G acylase (PGA) in *P. aeruginosa*, the complete *pac* gene was cloned into the pMMB67EH shuttle vector. Subsequently, the *pac*-containing plasmid pMMB-PGA and the empty vector pMMB67EH were introduced into PAO1 and PAO1*ΔtatABC* strains. Expression of PGA was initially studied at 37°C and 30°C, however no protein production was observed. Expression experiments carried out at lower temperatures (25°C and lower) revealed functional PGA production and processing. In our final experiments bacterial cells were grown at 22°C, using 0.5 mM IPTG for induction. The cytoplasmic, periplasmic and extracellular protein profiles were analyzed by Western blotting and immunodetection with specific polyclonal antibody against PGA. As shown in Figure 2A, β-PGA (62 kDa) was detectable in the cytoplasmic, periplasmic and growth medium fractions of pMMB-PGA containing strains, and not in the strains with the empty vector. Detection of the α-subunit of PGA appeared to be difficult especially if quantities of protein are low, forcing us to judge the effectiveness of PGA processing solely by detection of β-PGA. Both strains, PAO1 and PAO1*ΔtatABC*, were able to produce processed PGA at comparable levels. The majority of the protein was found in the growth medium fractions (Figure 2A). However, detection of the periplasmic marker protein, beta-lactamase (Bla), in these medium fractions, strongly suggested that the presence of PGA in the medium fractions was not due to secretion but to cell lysis. Specifically, the ratio of beta-lactamase found in the extracellular medium versus periplasmic beta-lactamase was approximately 1:0.9 for strains carrying the pMMB-PGA vector and approximately 1:6 for strains carrying the empty vector (data not shown). In agreement, growth medium fractions of strains carrying pMMB-PGA in contrast to the ones with the empty vector contained more cellular proteins (data not shown) indicating that PGA overexpression facilitates cell lysis. Also, strains with pMMB-PGA had a slower growth rate. Interestingly, only β-PGA (Figure 2A), and no precursor protein was found in the cytoplasmic fraction of both PAO1 and PAO1*ΔtatABC* strains. This suggests that either (i) PGA may undergo processing already in the cytoplasm or (ii) the transport to the periplasm and processing is efficient and the cytoplasmic fraction is simply contaminated with periplasmic proteins. We found some beta-lactamase in the

cytoplasmic fractions (data not shown) thus supporting the second alternative. The fact that PGA processing was not impaired in the PAO1*ΔtatABC* strain suggests that PGA does not take the Tat route in *P. aeruginosa* if guided by the native *E. coli* signal sequence.

(A)



(B)

Specific PGA activity (U mg^{-1})											
Medium				Periplasm				Cytoplasm			
1	2	3	4	1	2	3	4	1	2	3	4
1.4 ±0.4	1.5 ±0.5	134 ±0.6	261 ±0.7	1.4 ±1.4	2.0 ±1.9	100 ±0.8	54 ±0.2	0.4 ±0.1	0 ±0	76 ±0.6	82 ±1.1

Figure 2. Localization and activity of PGA overexpressed from the pMMB-PGA construct.
Cells of: 1- PAO1 pMMB67EH, 2- PAO1*ΔtatABC* pMMB67EH, 3- PAO1 pMMB-PGA, and 4- PAO1*ΔtatABC* pMMB-PGA strains were separated from the growth medium fraction and fractionated further to the periplasm and cytoplasm. (**A**) Detection of β-PGA (62 kDa) by SDS-PAGE, Western blotting and immunodetection with a rabbit antiserum against PGA. Equimolar amounts (based on original OD600) were loaded. (**B**) Hydrolysis of 6-nitro-3-phenylacetamide benzoic acid (NIPAB) by PGA present in protein fractions. The assay conditions and fractionation procedure are described in materials and methods.

To examine the enzymatic PGA activity in the cytoplasmic, periplasmic and growth medium protein fractions of *P. aeruginosa* PAO1 and PAO1*ΔtatABC* strains carrying pMMB67EH or pMMB-PGA, we employed the activity assay that measures the release of NABA. All fractions, with and without PGA, were incubated at 37°C in 50 mM phosphate buffer pH 7.0, containing 3 mM NIPAB as a substrate. Enzymatic PGA activity expressed in U/mg was found for all the fractions containing PGA (Figure 2B). Whereas, the fractions from strains harboring the empty vector did not show substrate hydrolysis (the values between 0-2 U/mg activity are considered to be an insignificant background values of the protein fractions) (Figure 2B), the highest PGA activity was found in the growth medium fractions of strains overexpressing PGA (Figure 2B). Since the enzymatic activity of PGA is highly dependent on the correct spatial arrangement of the α and β subunits we conclude that the enzyme must be correctly folded.

Taken together, these results demonstrate that production of *E. coli* PGA carrying its native signal peptide in *P. aeruginosa* results in a mature and active protein. Our results demonstrate that cell lysis takes place, yet they do not exclude PGA secretion.

Construction of fusion proteins

To investigate the effect of the elastase (*lasB*) propeptide, which has been implicated in Xcp secretion, on expression, export and secretion of *E. coli* PGA by *P. aeruginosa*, plasmids carrying PGA fused to the propeptide sequence of the elastase gene (*lasB*) were constructed. Both Sec- and Tat-specific signal peptides are known to couple transport over the inner membrane to the Xcp machinery. Therefore, the *pac* signal peptide was replaced with the sequences encoding signal peptides of *P. aeruginosa* elastase (*lasB*) or *P. aeruginosa* phospholipase (*plcN*) (Table 2). The fusion proteins were designed to keep the propeptide covalently attached to the PGA moiety during secretion. The constructs were expressed from the *lasB* quorum sensing regulated promoter (Figure 2) and the resulting plasmids were introduced into *P. aeruginosa* strains. By addition of the propeptide sequence we could examine whether it can serve as a secretion signal for PGA and/or may be essential to prevent cellular PGA proteolysis.

Table 2. Signal peptides used to study *E. coli* PGA expression and export in *P. aeruginosa*.

Signal peptide	Sequence (aas)	Export pathway
spPGA *E. coli*	MKNRNRMIVNCVTASLMYYWSLPALA	Tat
spLasB *P. aeruginosa*	MKKVSTLDLLFVAIMGVSPAAFA	Sec
spPlcN *P. aeruginosa*	MISKS**RR**SFIRLAAGTVGATVATSMLPSSIGAALA	Tat

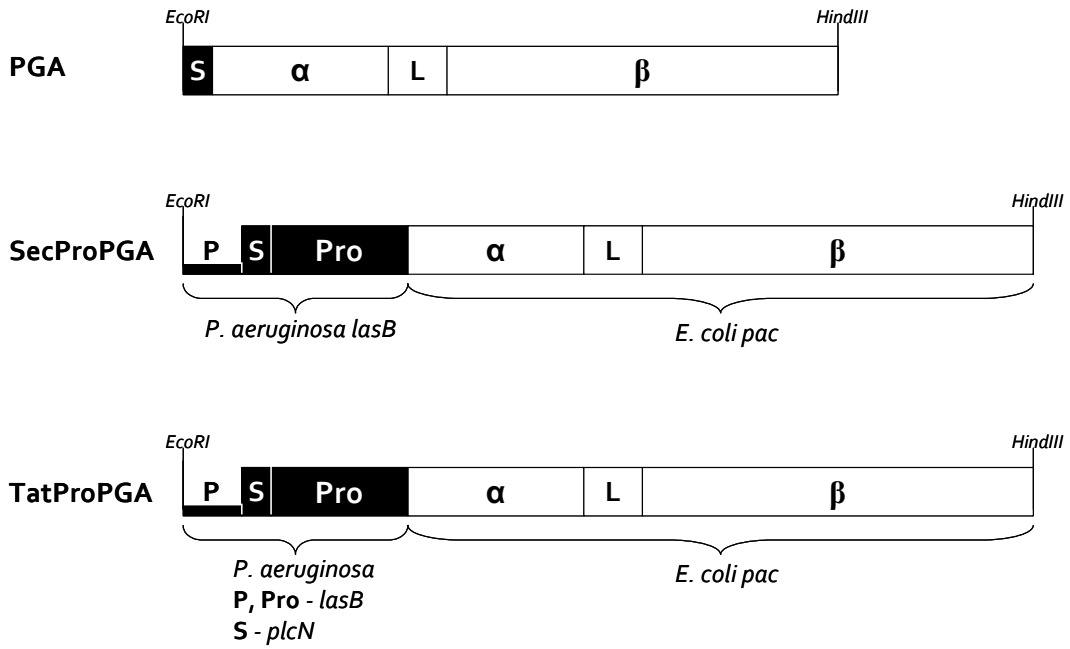


Figure 3. Schematic representation of the PGA fusion proteins.
PGA fusion proteins were build up with DNA sequences encoding the *P. aeruginosa* elastase *lasB* promoter (P), a signal peptide (S), the *P. aeruginosa* elastase *lasB* propeptide (Pro), the alpha-subunit of PGA (α), a linker (L), and the beta-subunit of PGA (β). The SecProPGA hybrid has the signal sequence from *lasB*, the Tat-PGA hybrid has the signal peptide from the *P. aeruginosa plcN* gene. Restriction sites (*EcoRI*, *HindIII*) used for cloning are indicated.

Expression of the SecProPGA hybrid

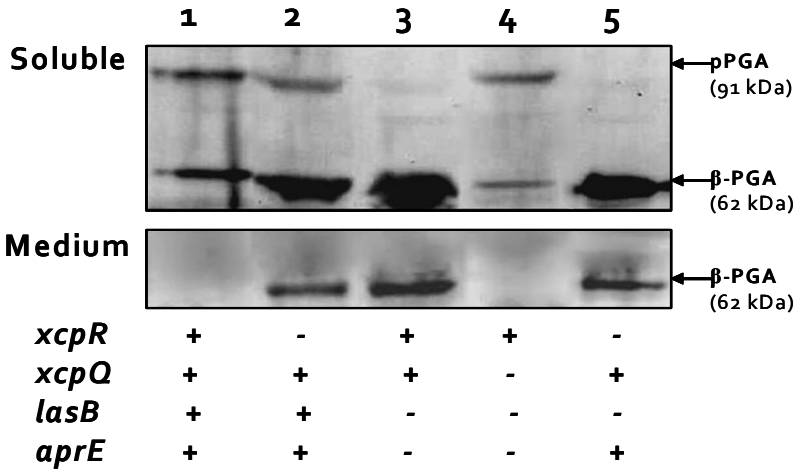
A construct (SecProPGA) consisting of the elastase signal peptide and propeptide followed by the *pac* gene sequence devoid from its native signal peptide sequence was made (Figure 3) and cloned into the broad-host range vector pMMB67EH, yielding pMMB-SecProPGA. In order to examine the potential of SecProPGA in export and secretion, the vector was transformed to different *P. aeruginosa* background strains including the Xcp machinery impaired strain (PAO7510), the proteases deficient strain (PAN8) or a combination of both (PAN9, PAN11). The growth temperature was set up at 22°C. The expression profile of SecProPGA in the cell-free protein extracts (soluble), comprising cytoplasmic and periplasmic proteins, and the growth medium fractions, was inspected by immunodetection with anti-PGA. Figure 4A shows, in the cellular soluble protein fractions (soluble) from the PAO25, PAO7510 (*ΔxcpR*), and PAN9 (Δ*lasB, ΔaprE, ΔxcpQ*) strains carrying pMMB-SecProPGA, two bands corresponding to unprocessed pro-PGA (higher band) and to β-PGA, indicating inefficient protein maturation. There is only a single band corresponding to unprocessed pro-PGA visible and the size markers do suggest that is runs around 90 kDa. This may correspond to unprocessed mature PGA (theoretical 91 kDa) and not to the complete fusion protein with an elastase propeptide (theoretical 112 kDa). Furthermore, SecProPGA in strains PAN8 (Δ*lasB, ΔaprE*) and PAN11 (Δ*lasB, ΔxcpR*) was efficiently produced and processed to the β-subunit (Figure 4A, soluble). The mutant strain, deficient in *lasB* and *aprE* proteases and with the intact Xcp apparatus (PAN8), gave the highest expression levels of processed PGA when compared with other strains (Figure 4A, soluble). Also, the PAN11 strain (Δ*lasB, ΔxcpR*) was able to produce completely processed PGA at high levels (Figure 4A, soluble). Importantly, PGA could be found in the extracellular medium of both the Xcp intact and Xcp impaired strains (Figure 4A, medium), excluding the Xcp machinery as the responsible PGA secretion motor. In addition, beta-lactamase could be found in the soluble and growth medium fractions, indicating cell lysis (data not shown). There was greater degree of lysis for strains carrying pMMB-SecProPGA in comparison to the strains carrying the empty pMMB67EH vector (data not shown). Overall, these results implicate that neither the presence of the intact Xcp machinery nor lack of it has any obvious influence on SecProPGA expression profile.

The soluble and growth medium fractions, carrying pMMB-SecProPGA, were screened in the enzymatic activity assay to examine if produced PGA was able to hydrolyze the NIPAB substrate. Figure 4B reveals that samples from cellular soluble fractions from strains with processed PGA (Figure 4A; soluble) were able to hydrolyze NIPAB with activities between 34-75 U/mg (Figure 4B). The low activity of 16 U/mg found in the growth medium fraction of the PAO7510 strain could suggest that the band seen on a picture (Figure 4A, lane 2 of supernatant) reflects inactive, improperly folded or degraded PGA. The medium samples from PAN8 and PAN11 strains exhibited the highest PGA enzymatic activity of 280 and 407 U/mg, respectively (Figure 4B).

Above results revealed that for the efficient SecProPGA fusion protein processing, stability, and activity there is no lucid correlation with the presence or absence of the components of the Xcp machinery and presence or absence of extracellular proteases. Yet, the lack of extracellular proteases (*lasB, aprE*), or at least one of them, may have a protective effect on PGA activity and stability once it is efficiently produced and processed, as the growth

medium fractions of PAN8 and PAN11 strains displayed the highest enzymatic activity (Figure 4A, 4B).

(A)



(B)

	Specific PGA activity (U mg^{-1})				
	1	**2**	**3**	**4**	**5**
Soluble	75 ±0.3	42 ±1	42 ±0.5	34 ±0.7	74 ±1.5
Medium	10 ±0.8	16 ±6.5	280 ±5	70 ±1.5	407 ±9.7

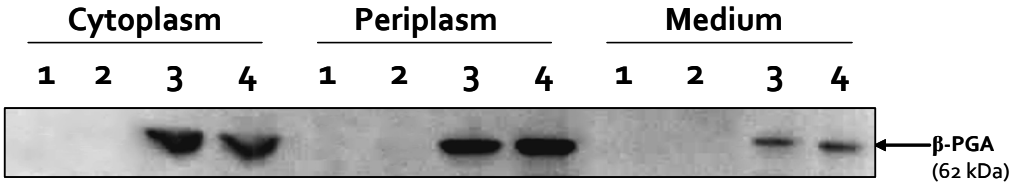
Figure 4. Detection and activity of PGA overexpressed from the pMMB-SecProPGA construct. Immunoblot showing a detection of pPGA (91 kDa) and β-PGA (62 kDa) with anti-PGA (**A**) and PGA activity on NIPAB (**B**) of proteins from the growth medium (**medium**) and cell-free protein extracts (**soluble**) from 1- PAO25 , 2- PAO7510, 3- PAN8, 4- PAN9, and 5- PAN11 strains carrying the pMMB-SecProPGA construct. Growth medium fractions were collected after two-step centrifugation to remove all cells. Proteins from medium fractions were precipitated with 10% TCA for SDS-PAGE or used directly in the activity assay. Pelleted total cells were resuspended and sonicated in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA buffer, cell debris was removed by centrifugation. The supernatant, representing the soluble (periplasm and cytoplasm) fraction was used directly in the enzymatic assay or was analyzed by SDS-PAGE. Equimolar amounts (based on original OD600) were loaded.

The fate of PGA expressed from the TatProPGA fusion protein containing the Tat-specific signal sequence and the elastase propeptide

To examine the effects of replacement of the *E. coli* PGA native signal peptide with a *P. aeruginosa* Tat-specific signal peptide on PGA expression, processing and transport we constructed a fusion protein TatProPGA. The TatProPGA construct consisting of the Tat signal peptide from a *P. aeruginosa plcN* gene, the elastase (*lasB*) propeptide and the *pac* gene sequence lacking its signal peptide (Figure 3) was cloned into the pMMB67EH vector. The

pMMB-TatProPGA construct was introduced to *P. aeruginosa* strains (PAO1 and PAO1*ΔtatABC*). For expression and protein fractionation, bacterial cultures were grown and treated as indicated in the methods section. Immunodetection analysis of the cytoplasmic, periplasmic and growth medium fractions with anti-PGA showed the band corresponding to β-PGA (62 kDa) for the pMMB-TatProPGA carrying strains (Figure 5A). Moreover, no unprocessed protein product could be detected designating an efficient TatProPGA processing. Expression levels of TatProPGA in PAO1 and PAO1*ΔtatABC* strains were alike (Figure 5A). The Tat deficiency of PAO1*ΔtatABC* did not impair or affect PGA expression, designating that TatProPGA is not exported in *P. aeruginosa* in a Tat-dependent manner. The unusual presence of β-PGA in the cytoplasm (Figure 5A) was further investigated by checking the localization of the normally periplasmic beta-lactamase. The majority of this protein was found in the periplasm and medium, and only in very modest amounts in the cytoplasm (Supplemental Figure 1). The fact that TatProPGA PAO1 and especially TatProPGA PAO1*ΔtatABC* strains gave in the cytoplasm very low beta-lactamase levels with considerably high levels of β-PGA could indicate that PGA processing, may take place already in the cytoplasm. Furthermore, the growth medium fractions of TatProPGA carrying strains versus the strains with the empty vector had more cellular proteins (data not shown), again indicating cell lysis caused by PGA overexpression. In addition, another construct consisting of the mature PGA sequence and the *plcN* Tat-specific signal peptide without the elastase propeptide sequence, was unable to produce PGA under tested conditions (data not shown).

(A)



(B)

Specific PGA activity (U mg^{-1})

Cytoplasm				Periplasm				Medium			
1	2	3	4	1	2	3	4	1	2	3	4
1.7 ±0.3	0.9 ±0.2	98 ±0.9	82 ±3.9	8.5 ±0.6	1.0 ±0	143 ±1.6	142 ±2.4	0.9 ±0.1	0.6 ±0.4	237 ±1.5	253 ±9.9

Figure 5. Localization and activity of PGA overexpressed from the pMMB-TatProPGA construct.
Cells of PAO1 pMMB67EH (1), PAO1*ΔtatABC* pMMB67EH (2), PAO1 pMMB-TatProPGA (3), and PAO1*ΔtatABC* pMMB-TatProPGA (4) strains were separated from the growth medium (medium) and fractionated further to the periplasm and cytoplasm. (**A**) Detection of β-PGA (62 kDa) by SDS-PAGE, Western blotting and immunodetection with a rabbit antiserum against PGA. (**B**) Hydrolysis of 6-nitro-3-phenylacetamide benzoic acid (NIPAB) by PGA present in protein fractions. The assay conditions and fractionation procedure are described in materials and methods.

The enzymatic assay was engaged to scrutinize the TatProPGA enzymatic activity in the cytoplasmic, periplasmic and growth medium fractions of *P. aeruginosa* PAO1 and PAO1*ΔtatABC* strains. Reactions were prepared as described in materials and methods. All fractions containing PGA, in contrast to the fractions without PGA, were able to hydrolyze the NIPAB substrate (Figure 5B). The highest enzymatic activity (237 and 253 U/mg) was recorded in the growth medium and the lowest (98 and 82 U/mg) in the cytoplasmic fractions of TatProPGA overexpressing strains (Figure 5B). Evidently, the deficiency of the Tat machinery did not affect the compartmental distribution of PGA activity when compared to the wild type strain PAO1.

Altogether, our present findings demonstrate that in *P. aeruginosa* the TatProPGA fusion protein does not require a functional Tat machinery for translocation across the IM, for efficient posttranslational maturation as well as for proper folding essential for enzymatic activity.

Discussion

The ability of bacteria to efficiently secrete high amounts of proteins into the extracellular medium is commonly used in industry for high-level protein production as it simplifies the purification process. The Gram-negative bacterium, *P. aeruginosa*, secrets a number of enzymes and toxins. Therefore, it is a potent candidate for the efficient transport of heterologous fusion proteins. The type II secretion system (T2SS or secreton) of these bacteria is able to differentiate the exported proteins from the entire pool of periplasmic and membrane proteins, and it recognizes either the Tat- or Sec-exported proteins. Up till now, no true secretion signals governing this process could be defined as *P. aeruginosa* secretes many dissimilar proteins[51], with no common secretion motif. Yet, current knowledge highlights the importance of a conformational secretion signal of folded proteins essential for translocation[7]. Overall, the phenomenon of secretion is complex. Thus, for successful secretion certainly several factors, together and independently, play a key role. Here, we have investigated whether the type II secretion system of *P. aeruginosa* allows the transport to the medium of *E. coli* penicillin G acylase equipped with Tat- and Sec-specific signal peptides and the elastase propeptide.

Our data illustrate that the *pac* gene of *E. coli* can be successfully expressed in an active form in *P. aeruginosa* and that the produced enzyme is partially released to the extracellular medium due to cell lysis (Figures 2, 4, and 5). We demonstrated no significant differences in the expression profiles of PGA with the native *E. coli* signal peptide or the *Pseudomonas* Tat-specific signal peptide with propeptide, the enzyme could be effectively produced in all cases. Unexpectedly, switching off the Tat apparatus in the PAO1*ΔtatABC* pMMB-TatProPGA strain did not affect production, processing, activity and transport of PGA when compared to the parental strain with the intact Tat system (Figure 5). The signal peptide of the TatProPGA hybrid was expected to direct the propeptide protein to the Tat apparatus as its signal peptide comprises the conserved twin-arginine motif S-**R**-**R** and belongs to the *Pseudomonas* phospholipase gene (*plcN*) which is exported in a Tat-dependent manner[52]. Perhaps, the *plcN* gene signal peptide is not simply a "Tat-only" signal sequence but the destination of a fusion protein is also determined by the sequence of the fusion protein itself. Furthermore, *E. coli*

PGA was shown to have an unusual Tat signal peptide[35]. Therefore, our results with processed and active PGA in PAO1*ΔtatABC* pMMB-PGA strain are not totally unexpected since we could not tell in advance if the native *E. coli* Tat-specific signal peptide would be also recognized by the *Pseudomonas* Tat machinery. Since the expression of PGA from pMMB-PGA and pMMB-TatProPGA was not impaired in PAO1*ΔtatABC* strain, this protein most probably is exported across the inner membrane via the alternative route, the Sec pathway.

The expression and maturation of PGA from the SecProPGA hybrid was impaired in PAO25 and PAN9 strains (Figure 4A). Our experiments in a proteinase-deficient strain did not result in obviously higher protein productivity. Yet, the strains with efficiently processed PGA (PAN8, PAN11) were lacking one or two of the proteases genes and showed the highest specific PGA activity in the growth medium fractions (Figure 4A, 4B). Interestingly, we observed a thicker band of about 60 kDa visible on Coomassie stained SDS-PAGE of the soluble fraction of PAN9 pMMB-SecProPGA (data not shown). The band was extracted from the gel, digested with trypsin, and analyzed with matrix-assisted laser desorption ionization-time of flight mass spectrometry. Protein identification based on mass spectra verified overexpression of the 60 kDa GroEL protein, a chaperon protein involved in folding and stabilization of proteins[53]. Actually, in *E. coli* the GroEL chaperon has been successfully coexpressed with a *pac* gene lacking the signal peptide, and it was shown to prevent intracellular proteolysis of PGA[54]. After a series of expression experiments we have also observed that for optimal PGA expression, cells had to be cultured at maximal 25°C. For example, at 37°C there was no detectable PGA production. This is in agreement with previous studies on PGA expression where the temperature was shown to be an important factor for PGA efficient expression and processing[28,37]. Cell lysis was mostly observed in strains producing PGA, which might have caused the physiological burden and probably explains the partial liberation of PGA into the extracellular medium. Active PGA could be found in the medium fraction of the Xcp-deficient strain (PAN11, Figure 4), indicating no dependence of the Xcp-mediated secretion. These results plus the detection of the periplasmic marker (beta-lactamase) in the growth medium fractions indicated that cell lysis rather than active secretion explained the presence of PGA in the medium fraction.

Maturation of penicillin acylase is considered to take place in the periplasmic space after translocation across the inner membrane and cleavage of the signal peptide[29]. However, it was shown that the active enzyme can also be produced by coexpression of the alpha- and beta-subunits from separate plasmids in the cytoplasm of *E. coli*[55]. In accordance with this observation, *Xu et al.* has demonstrated that the expression of the *pac* gene lacking its signal peptide in *E. coli* occurred in the cytoplasm[54]. These data support our observation with the pMMB-TatProPGA construct that suggest processing of PGA in *P. aeruginosa* - at least in part - to take place in the cytoplasm (Figure 5A). Noteworthy, the separation of the periplasmic space from the cytoplasm is a technical challenge. From what we have observed, PGA overexpression seems to lead to cellular lysis. The burden of PGA overexpression may be explained by the cytoplasmic and periplasmic accumulation of PGA. The cytoplasmic accumulation of PGA precursor proteins could be a result of hampered translocation through the inner membrane and a jamming of the translocation machinery (Tat or Sec). Subsequently, the accumulation of precursor proteins could make their signal peptides susceptible towards cytoplasmic proteases that would degrade the signal peptide leaving the mature protein intact

in the cytoplasm. It was shown that the signal peptide of TorA precursor protein was sensitive to proteolysis in the cytoplasm in the absence of its chaperon protein TorD[56]. Perhaps, in *E. coli* PGA has such a general cytoplasmic chaperon protein that is not present at all or in limiting amounts in *P. aeruginosa*. Moreover, impaired transport of PGA to the periplasm could possibly be also promoted by the lack or limitation of a cofactor needed for membrane translocation. In agreement, *E. coli* and *Alcaligenes faecalis* penicillin G acylase expressed in *E. coli* were shown to require Ca^{2+} for the efficient membrane transport and maturation[57,58].

The possible role of bacterial propeptides in targeting to the secretion machinery has not been extensively studied. At present it is known that propeptides are important for correct protein folding and for prevention from protein degradation and/or intracellular activity[14,59,60]. A relatively well studied peculiar enzyme from *P. aeruginosa* elastase *lasB*, possessing a propeptide sequence, is formed as a pre-pro-enzyme (53 kDa) consisting of a signal peptide, a propeptide (18 kDa) and a mature protein (33 kDa)[16,61]. The elastase propeptide, exhibiting an intramolecular chaperone role, was shown to be essential in correct folding and secretion of elastase[15]. The elastase mutant lacking the propeptide sequence was inactive and prone to proteolytic degradation, thus non secretable[15]. Whilst, the propeptide expressed itself could be successfully secreted in *P. aeruginosa* to the surrounding medium in the Xcp-dependent manner[16]. Moreover, *Mclever et al.* identified residues within the propeptide important for elastase secretion[62]. The latter results are very encouraging, however as yet there is not sufficient knowledge whether the elastase propeptide can be used as a carrier for secretion of heterologous proteins. Our approach was aimed to engineer SecProPGA and TatProPGA in such a way that they would be overexpressed as complete fusion proteins with the propeptide attached. For this purpose the elastase propeptide native cleavage site was modified during a cloning procedure. The western blot results with SecProPGA and TatProPGA (Figure 4 and Figure 5) indicate however, that the elastase propeptide is cleaved off. It's plausible that the cleavage is associated with the proteolytic activity present in protein extracts. Noteworthy, the western blot analysis, with the antibody against the elastase propeptide, of some SecProPGA samples, revealed a band that could correspond to the complete fusion protein, SecProPGA, of theoretical molecular weight 112 kDa (data not shown). The propeptide susceptibility to proteolityc cleavage/degradation can perhaps explain the lack of secretion of the fusion proteins.

We have shown that mature PGA can be produced in *P. aeruginosa* even when it is not actively secreted into the medium. Our efforts to secrete the protein by the *Pseudomonas* strains using Sec or Tat signal peptides and the elastase propeptide did not result in real secretion. Even though several aspects of the production and translocation of acylases have been elucidated, further experiments are needed to fully explore the potential of *Pseudomonas* to secrete β-lactam acylases.

References

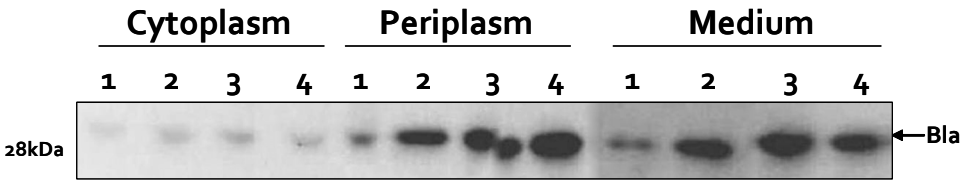
1. Driessen,A.J., Fekkes,P., & van der Wolk,J.P. The Sec system. *Curr. Opin. Microbiol.* 1, 216-222 (1998).
2. Muller,M., Koch,H.G., Beck,K., & Schafer,U. Protein traffic in bacteria: multiple routes from the ribosome to and across the membrane. *Prog. Nucleic Acid Res. Mol. Biol.* 66, 107-157 (2001).
3. Pugsley,A.P. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev.* 57, 50-108 (1993).

4. Berks,B.C., Sargent,F., & Palmer,T. The Tat protein export pathway. *Mol. Microbiol.* 35, 260-274 (2000).
5. Voulhoux,R., Ball,G., Ize,B., Vasil,M.L., Lazdunski,A., Wu,L.F., & Filloux,A. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. *EMBO J.* 20, 6735-6741 (2001).
6. Weiner,J.H., Bilous,P.T., Shaw,G.M., Lubitz,S.P., Frost,L., Thomas,G.H., Cole,J.A., & Turner,R.J. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* 93, 93-101 (1998).
7. Filloux,A., Michel,G., & Bally,M. GSP-dependent protein secretion in gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* 22, 177-198 (1998).
8. Kornacker,M.G. & Pugsley,A.P. The normally periplasmic enzyme beta-lactamase is specifically and efficiently translocated through the *Escherichia coli* outer membrane when it is fused to the cell-surface enzyme pullulanase. *Mol. Microbiol.* 4, 1101-1109 (1990).
9. Lu,H.M. & Lory,S. A specific targeting domain in mature exotoxin A is required for its extracellular secretion from *Pseudomonas aeruginosa*. *EMBO J.* 15, 429-436 (1996).
10. Lindeberg,M., Boyd,C.M., Keen,N.T., & Collmer,A. External loops at the C terminus of *Erwinia chrysanthemi* pectate lyase C are required for species-specific secretion through the out type II pathway. *J. Bacteriol.* 180, 1431-1437 (1998).
11. Hirst,T.R., Sanchez,J., Kaper,J.B., Hardy,S.J., & Holmgren,J. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A* 81, 7752-7756 (1984).
12. Chapon,V., Czjzek,M., El Hassouni,M., Py,B., Juy,M., & Barras,F. Type II protein secretion in gram-negative pathogenic bacteria: the study of the structure/secretion relationships of the cellulase Cel5 (formerly EGZ) from *Erwinia chrysanthemi*. *J. Mol. Biol.* 310, 1055-1066 (2001).
13. Wong,K.R. & Buckley,J.T. Site-directed mutagenesis of a single tryptophan near the middle of the channel-forming toxin aerolysin inhibits its transfer across the outer membrane of *Aeromonas salmonicida*. *J. Biol. Chem.* 266, 14451-14456 (1991).
14. Braun,P. & Tommassen,J. Function of bacterial propeptides. *Trends Microbiol.* 6, 6-8 (1998).
15. Braun,P., Tommassen,J., & Filloux,A. Role of the propeptide in folding and secretion of elastase of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 19, 297-306 (1996).
16. Braun,P., de Groot,A., Bitter,W., & Tommassen,J. Secretion of elastinolytic enzymes and their propeptides by *Pseudomonas aeruginosa*. *J. Bacteriol.* 180, 3467-3469 (1998).
17. von Heijne,G. Life and death of a signal peptide. *Nature* 396, 111, 113 (1998).
18. von Heijne,G. The signal peptide. *J. Membr. Biol.* 115, 195-201 (1990).
19. Bogsch,E., Brink,S., & Robinson,C. Pathway specificity for a delta pH-dependent precursor thylakoid lumen protein is governed by a 'Sec-avoidance' motif in the transfer peptide and a 'Sec-incompatible' mature protein. *EMBO J.* 16, 3851-3859 (1997).
20. Cristobal,S., de Gier,J.W., Nielsen,H., & von Heijne,G. Competition between Sec- and TAT-dependent protein translocation in *Escherichia coli*. *EMBO J.* 18, 2982-2990 (1999).
21. Berks,B.C. A common export pathway for proteins binding complex redox cofactors? *Mol. Microbiol.* 22, 393-404 (1996).
22. Ng,D.T., Brown,J.D., & Walter,P. Signal sequences specify the targeting route to the endoplasmic reticulum membrane. *J. Cell Biol.* 134, 269-278 (1996).
23. Sio,C.F. & Quax,W.J. Improved beta-lactam acylases and their use as industrial biocatalysts. *Curr. Opin. Biotechnol.* 15, 349-355 (2004).
24. Vandamme,E.J. & Voets,J.P. Microbial penicillin acylases. *Adv. Appl. Microbiol.* 17, 311-369 (1974).
25. Merino,E., Balbas,P., Recillas,F., Becerril,B., Valle,F., & Bolivar,F. Carbon regulation and the role in nature of the *Escherichia coli* penicillin acylase (pac) gene. *Mol. Microbiol.* 6, 2175-2182 (1992).
26. Valle,F., Balbas,P., Merino,E., & Bolivar,F. The role of penicillin amidases in nature and in industry. *Trends Biochem. Sci.* 16, 36-40 (1991).
27. Roa,A. & Garcia,J.L. New insights into the regulation of the pac gene from *Escherichia coli* W ATCC 11105. *FEMS Microbiol. Lett.* 177, 7-14 (1999).
28. Keilmann,C., Wanner,G., & Bock,A. Molecular basis of the exclusive low-temperature synthesis of an enzyme in *E. coli:* penicillin acylase. *Biol. Chem. Hoppe Seyler* 374, 983-992 (1993).

29. Schumacher,G., Sizmann,D., Haug,H., Buckel,P., & Bock,A. Penicillin acylase from *E. coli*: unique gene-protein relation. *Nucleic Acids Res.* 14, 5713-5727 (1986).
30. Bruns,W., Hoppe,J., Tsai,H., Bruning,H.J., Maywald,F., Collins,J., & Mayer,H. Structure of the penicillin acylase gene from *Escherichia coli*: a periplasmic enzyme that undergoes multiple proteolytic processing. *J. Mol. Appl. Genet.* 3, 36-44 (1985).
31. Daumy,G.O., Danley,D., & McColl,A.S. Role of protein subunits in *Proteus rettgeri* penicillin G acylase. *J. Bacteriol.* 163, 1279-1281 (1985).
32. Duggleby,H.J., Tolley,S.P., Hill,C.P., Dodson,E.J., Dodson,G., & Moody,P.C. Penicillin acylase has a single-amino-acid catalytic centre. *Nature* 373, 264-268 (1995).
33. Choi,K.S., Kim,J.A., & Kang,H.S. Effects of site-directed mutations on processing and activities of penicillin G acylase from *Escherichia coli* ATCC 11105. *J. Bacteriol.* 174, 6270-6276 (1992).
34. Slade,A., Horrocks,A.J., Lindsay,C.D., Dunbar,B., & Virden,R. Site-directed chemical conversion of serine to cysteine in penicillin acylase from *Escherichia coli* ATCC 11105. Effect on conformation and catalytic activity. *Eur. J. Biochem.* 197, 75-80 (1991).
35. Ignatova,Z., Hornle,C., Nurk,A., & Kasche,V. Unusual signal peptide directs penicillin amidase from *Escherichia coli* to the Tat translocation machinery. *Biochem. Biophys. Res. Commun.* 291, 146-149 (2002).
36. Chou,C.P., Yu,C.C., Tseng,J.H., Lin,M.I., & Lin,H.K. Genetic manipulation to identify limiting steps and develop strategies for high-level expression of penicillin acylase in *Escherichia coli*. *Biotechnol. Bioeng.* 63, 263-272 (1999).
37. Scherrer,S., Robas,N., Zouheiry,H., Branlant,G., & Branlant,C. Periplasmic aggregation limits the proteolytic maturation of the *Escherichia coli* penicillin G amidase precursor polypeptide. *Appl. Microbiol. Biotechnol.* 42, 85-91 (1994).
38. Sriubolmas,N., Panbangred,W., Sriurairatana,S., & Meevootisom,V. Localization and characterization of inclusion bodies in recombinant *Escherichia coli* cells overproducing penicillin G acylase. *Appl. Microbiol. Biotechnol.* 47, 373-378 (1997).
39. Brok,R., Van Gelder,P., Winterhalter,M., Ziese,U., Koster,A.J., de Cock,H., Koster,M., Tommassen,J., & Bitter,W. The C-terminal domain of the *Pseudomonas* secretin XcpQ forms oligomeric rings with pore activity. *J. Mol. Biol.* 294, 1169-1179 (1999).
40. Ize,B., Gerard,F., Zhang,M., Chanal,A., Voulhoux,R., Palmer,T., Filloux,A., & Wu,L.F. In vivo dissection of the Tat translocation pathway in *Escherichia coli*. *J. Mol. Biol.* 317, 327-335 (2002).
41. Stanley,N.R., Sargent,F., Buchanan,G., Shi,J., Stewart,V., Palmer,T., & Berks,B.C. Behaviour of topological marker proteins targeted to the Tat protein transport pathway. *Mol. Microbiol.* 43, 1005-1021 (2002).
42. Santini,C.L., Bernadac,A., Zhang,M., Chanal,A., Ize,B., Blanco,C., & Wu,L.F. Translocation of jellyfish green fluorescent protein via the Tat system of *Escherichia coli* and change of its periplasmic localization in response to osmotic up-shock. *J. Biol. Chem.* 276, 8159-8164 (2001).
43. Thomas,J.D., Daniel,R.A., Errington,J., & Robinson,C. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. *Mol. Microbiol.* 39, 47-53 (2001).
44. Sambrook,J.F.E.F.a.M.T. Molecular cloning: a laboratory manual. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 1989).
45. Yolov,A.A. & Shabarova,Z.A. Constructing DNA by polymerase recombination. *Nucleic Acids Res.* 18, 3983-3986 (1990).
46. White,P.J. The regulation of diaminopimelate decarboxylase activity in *Escherichia coli* strain w. *J. Gen. Microbiol.* 96, 51-62 (1976).
47. Voulhoux,R., Filloux,A., & Schalk,I.J. Pyoverdine-mediated iron uptake in *Pseudomonas aeruginosa*: the Tat system is required for PvdN but not for FpvA transport. *J. Bacteriol.* 188, 3317-3323 (2006).
48. Haas,D. & Holloway,B.W. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. *Mol. Gen. Genet.* 144, 243-251 (1976).
49. Filloux,A., Murgier,M., Wretlind,B., & Lazdunski,A. Characterization of two *Pseudomonas aeruginosa* mutants with defective secretion of extracellular proteins and comparison with other mutants. *FEMS Microbiol. Lett.* 40, 159-163 (1987).
50. Furste,J.P., Pansegrau,W., Frank,R., Blocker,H., Scholz,P., Bagdasarian,M., & Lanka,E. Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. *Gene* 48, 119-131 (1986).

51. Lazdunski,A., Guzzo,J., Filloux,A., Bally,M., & Murgier,M. Secretion of extracellular proteins by *Pseudomonas aeruginosa*. *Biochimie* 72, 147-156 (1990).
52. Snyder,A., Vasil,A.I., Zajdowicz,S.L., Wilson,Z.R., & Vasil,M.L. Role of the *Pseudomonas aeruginosa* PlcH Tat signal peptide in protein secretion, transcription, and cross-species Tat secretion system compatibility. *J. Bacteriol.* 188, 1762-1774 (2006).
53. Farinha,M.A., Mockett,R., Went,C.J., Jardine,S., Naczynski,L.M., & Kropinski,A.M. Physical mapping of several heat-shock genes in *Pseudomonas aeruginosa* and the cloning of the mopA (GroEL) gene. *Can. J. Microbiol.* 42, 326-334 (1996).
54. Xu,Y., Hsieh,M.Y., Narayanan,N., Anderson,W.A., Scharer,J.M., Moo-Young,M., & Chou,C.P. Cytoplasmic overexpression, folding, and processing of penicillin acylase precursor in *Escherichia coli*. *Biotechnol. Prog.* 21, 1357-1365 (2005).
55. Burtscher,H. & Schumacher,G. Reconstitution in vivo of penicillin G acylase activity from separately expressed subunits. *Eur. J. Biochem.* 205, 77-83 (1992).
56. Genest,O., Seduk,F., Ilbesr,M., Meean,V., & Iobbi-Nivol,C. Signal peptide protection by specific chaperone. *Biochem. Biophys. Res. Commun.* 339, 991-995 (2005).
57. Ignatova,Z., Wischnewski,F., Notbohm,H., & Kasche,V. Pro-sequence and Ca2+-binding: implications for folding and maturation of Ntn-hydrolase penicillin amidase from *E. coli*. *J. Mol. Biol.* 348, 999-1014 (2005).
58. Kasche,V., Ignatova,Z., Markl,H., Plate,W., Punckt,N., Schmidt,D., Wiegandt,K., & Ernst,B. Ca2+ is a cofactor required for membrane transport and maturation and is a yield-determining factor in high cell density penicillin amidase production. *Biotechnol. Prog.* 21, 432-438 (2005).
59. Wetmore,D.R., Wong,S.L., & Roche,R.S. The role of the pro-sequence in the processing and secretion of the thermolysin-like neutral protease from *Bacillus cereus*. *Mol. Microbiol.* 6, 1593-1604 (1992).
60. Ohta,Y., Hojo,H., Aimoto,S., Kobayashi,T., Zhu,X., Jordan,F., & Inouye,M. Pro-peptide as an intramolecular chaperone: renaturation of denatured subtilisin E with a synthetic pro-peptide [corrected]. *Mol. Microbiol.* 5, 1507-1510 (1991).
61. Thayer,M.M., Flaherty,K.M., & McKay,D.B. Three-dimensional structure of the elastase of *Pseudomonas aeruginosa* at 1.5-A resolution. *J. Biol. Chem.* 266, 2864-2871 (1991).
62. McIver,K.S., Kessler,E., & Ohman,D.E. Identification of residues in the Pseudomonas aeruginosa elastase propeptide required for chaperone and secretion activities. *Microbiology* 150, 3969-3977 (2004).

Supplementary material



Supplemental Figure 1. Localization of beta-lactamase (Bla) overexpressed from the pMMB-TatProPGA plasmid. Cells of PAO1 pMMB67EH (**1**), PAO1*ΔtatABC* pMMB67EH (**2**), PAO1 pMMB-TatProPGA (**3**), and PAO1*ΔtatABC* pMMB-TatProPGA (**4**) strains were separated from the culture medium (medium) and fractionated further to the periplasm and cytoplasm. Detection of the beta-lactamase (31.5 kDa) by SDS-PAGE, Western blotting and immunodetection with a rabbit antiserum against beta-lactamase.