Pseudomonas as a microbial enzyme factory

Krzeslak, Joanna Kamila

CHAPTER 7

Summary, general discussion and future perspectives

Summary, general discussion and future perspectives

Pseudomonads are Gram-negative bacteria occupying various ecological niches, often acting as animal and plant pathogens, or biocontrol, bioremedation, and spoilage agents. Typically, they possess a periplasmic space and are capable of secreting a large variety of endogenous proteins into the growth medium. Therefore, in recent years, more interest has been drawn towards Gram-negative hosts, such as *Pseudomonas* species, for the production of commercially and pharmaceutically interesting enzymes like lipases, penicillin acylases, and other biocatalysts which require periplasmic folding. These enzymes can not be efficiently produced by traditional hosts, such as *Bacillus* or *Aspergillus*, due to the lack of a periplasmic folding compartment and adequate secretion machinery. To tackle this problem it is rational to develop a Gram-negative secretion host possessing a nano-machine for the secretion of folded proteins and a periplasm suitable for the assembly and folding of complex biocatalysts. *Pseudomonas alcaligenes* serves as an ideal candidate as it is an industrially validated non-pathogenic strain. In addition, the available knowledge about the closely related and well-studied model organism, *Pseudomonas aeruginosa*, can be applied to the industrial *P. alcaligenes* strain. Yet, before the successful enzyme production on industrial scale can take place a solid fundamental knowledge about the protein to be produced and the expression host needs to be gained. In this context, the research presented in this thesis explores Pseudomonads as microbial enzyme factories. *Pseudomonas* can serve as a potential source of industrially potent enzymes and the host for nanotechnological production of biocatalysts. In particular, the fundamental issues, such as the regulation of lipase expression in *P. alcaligenes*, secretion of heterologous proteins and a search for industrially potent enzymes within *P. aeruginosa*, were studied here. **Chapter 1** provides background information introducing to the subjects presented in this thesis.

Lipase gene expression regulation in *P. alcaligenes*

The data presented in **Chapter 3** demonstrate that the lipase gene expression of *P. alcaligenes* can be switched on by the addition of certain medium components such as soybean oil. The induction mechanism of lipase expression was elucidated by screening for a lipase hyper-producing strain from the pool of *P. alcaligenes* strains with a cosmid library of *P. alcaligenes* genomic DNA fragments. As a result, a fragment comprising two previously unidentified genes, *lipQ* and *lipR*, was found. Their encoded proteins belong to the NtrBC family of regulators that regulate gene expression via binding to a specific upstream activating sequence (UAS). For *P. alcaligenes*, such an UAS region upstream the lipase promoter was identified in a previous study[1], strongly suggesting that LipR acts as a positive regulator of lipase expression. Our results showed that inactivation of a *lipR* gene down-regulates lipase expression and overexpression of the *lipQR* operon increases lipase yield threefold. Also, we observed that cell-free protein extracts of a LipR-overexpressing strain caused a retardation of the lipase promoter fragment in the electrophoretic mobility shift assay (EMSA) indicating that lipase expression in *P. alcaligenes* is under the control of a LipQR two-component system. Further studies, in **chapter 4**, demonstrated direct LipR binding to the lipase promoter in the electrophoretic mobility shift assay and surface plasmon resonance (SPR) experiment. The

experiments performed with the *lipA-lacZ* transcriptional fusion in the *P. alcaligenes lipR* and *rpoN* mutant strains demonstrated clearly that LipR and RpoN are essential for the lipase promoter activation. Moreover, the ATP hydrolytic activity of LipR has been shown to be stimulated by DNA and LipR phosphorylation.

Bacterial cells, as means of survival and environmental adaptation, evolved sophisticated regulatory strategies for gene regulation in response to specific conditions. The transcription of numerous genes may be controlled by several regulators and may be followed by posttranscriptional regulatory processes. Thus, universally the regulation events may take place at the different molecular levels, in a direct or indirect manner. Here, we have identified a LipQR two-component system involved in direct regulation of lipase expression in *P. alcaligenes* (**chapters 3** and **4**). The LipQR system bears remarkable resemblance to the CbrAB two-component system from *P. aeruginosa*[2,3]. Yet, the CbrB protein, in contrast to LipR, has not been shown or indicated to be involved in regulation of lipase expression. For *P. aeruginosa*, the CbrAB two-component system was shown to play a role in the utilization of carbon and nitrogen sources[2,4]. However, no reports demonstrating the direct binding of CbrB to any promoter sequence have been shown. Furthermore, the LipQR system in *P. alcaligenes*, on the contrary to the CbrAB system, has not been indicated to be important in carbon-nitrogen utilization. Thus, taken together it appears that the LipQR and CbrAB two-component systems perform different regulatory tasks under physiological conditions. Also, the precise localization and nature of the direct stimulus activating LipQ and CbrA, needs to be still recognized. It is noteworthy, however, that we established that the presence of particular substrates in the growth medium of *P. alcaligenes*, such as soybean oil, stimulated lipase expression, indicating the potential stimuli triggers for LipQ[5]. Generally, the activity of bacterial response regulators, such as NtrC-like proteins, is controlled by phosphorylation and dephosphorylation of an aspartate residue at their N-terminus by the histidine protein kinase. The aspartyl-phosphate linkage is known to be very labile in nature[6,7]. Phosphorylation of the N-terminal receiver domain stimulates an essential for transcriptional regulation oligomerization of the central domain. In our studies (**chapter 4**), we observed in the gel retardation assay that LipR *in vitro* phosphorylation did not displayed better LipR binding activity to the lipase promoter. In contrast, our SPR experiment demonstrated that LipR binding ability and affinity was triggered by LipR phosphorylation. Possibly, the quite fast off-rate of LipR/LipR-P at the lipase promoter causes the detection of increased binding between LipR and LipR-P to the lipase promoter in the gel retardation assay difficult. Also, the unstable LipR-DNA complexes may dissociate prior to, or during, the running of the gel. It implicates that analyzing the binding of labile response regulators by use of advanced technologies such as SPR is preferable. It would be of great interest to study further the LipQR mode of action on lipase expression regulation. In particular, the basic question still to be answered involves the effect of phosphorylation on LipR oligomeric state. Also, the exact lipase promoter region interacting with LipR could be verified by use of footprint experiments. The overexpression and purification of LipQ could provide valuable information on the stimuli signal and the partner relationship between LipR and LipQ within the regulatory cascade of lipase expression. Lastly, this knowledge could be transferred to improve the lipase production levels at large scale.

Probing the type II pathway of *P. aeruginosa* for the export of heterologous proteins

A number of hydrolytic enzymes such as elastase, lipase, phospholipases or exotoxin A are secreted by the type II secretory pathway in *P. aeruginosa*. These proteins carry an N-terminal signal peptide that is cleaved off during translocation across the cytoplasmic membrane via the Sec pathway[8] or via the Tat pathway[9]. Next, in the periplasm the folded proteins are recognized by the Xcp machinery which transports them to the extracellular medium. Over past years, many researchers have thought of applying such bacterial, secretion nano-machineries for the export of heterologous proteins. Especially, for proteins of which expression and secretion in a native host is somewhat limited. For example, the overproduction of penicillin G acylase is mostly limited to the intracellular bacterial spaces which may lead to protein aggregation and cell toxicity. In an attempt to overcome this problem, in **chapter 5**, we investigated the host organism *P. aeruginosa* for production of penicillin G acylase (PGA). For this purpose, we assessed the effect of the Sec- and Tat-specific signal peptides, and the elastase propeptide on the translocation of PGA across the inner and the outer membrane. We obtained production of active protein, however, our approach to arrive at an effective secretion with the help of the signal peptide/propeptide turned out to be not successful. Moreover, the mature protein, expressed from a TatPGA hybrid, was found in the periplasm as well as in the cytoplasm. The unusual cytoplasmic localization of the mature protein strongly suggests that processing can also occur in the cytoplasm of *P. aeruginosa*. In agreement with our observation, Xu *et al.* has demonstrated that the expression of the leaderless *pac* gene in *E. coli* occurred in the cytoplasm[10]. An alternative explanation for the cytoplasmic localization of PGA involves the cytoplasmic accumulation of the precursor protein. The signal peptide of the aggregated precursor protein might be susceptible towards cytoplasmic proteases that would degrade the signal peptide leaving the mature protein intact in the cytoplasm. For instance, the signal peptide of a TorA precursor protein was sensitive to proteolysis in the cytoplasm in the absence of its chaperon protein TorD[11]. Furthermore, the elastase propeptide appeared to be an insufficient passenger to actively secrete penicillin acylase (SecPGA and TatPGA) to the extracellular medium. Perhaps, PGA and a propeptide are not able to form a secretable complex that would be recognized and secreted by the Xcp machinery. Also, we observed that cell lysis caused by PGA overexpression led to its presence in the extracellular medium. Possibly, the secretion might succeed when other signal peptides and/or secretion motifs will be employed. Despite the fact that several issues of the production and translocation of acylases have been elucidated, additional experiments are required to fully explore the potential of *Pseudomonas* to secrete β-lactam acylases. This research is just a starting and challenging point for future studies.

Potential of bacterial acylases

The first reports about penicillin acylases, of the β-lactam acylases class, have appeared almost 50 years ago. With the course of time these intriguing enzymes have drawn significant academic and industrial attention. Importantly, β-lactam acylases are widely used in industry for production of semi-synthetic penicillins and cephalosporins. This class of enzymes is relatively well-characterized and it displays high selectivity towards the acid side chain of the substrate. One of the best characterized enzymes is bacterial penicillin G acylase with already well established industrial importance. Acylases are produced by a variety of organisms, such

as bacteria, yeast, and fungi. Their physiological function most of the time remains unclear. Yet, due to their biotechnological potential they attract lots interest. For instance, many putative acylases of the N-terminal nucleophile (NTN) family can be identified among the genomes of *Pseudomonas* species. Significantly, at least four putative acylases have been identified in the *P. aeruginosa* PAO1 genome. Two of which (PvdQ, QuiP) were found to hydrolyse acyl homoserine lactones (AHLs) indicating a role in quorum quenching. These enzymes degrade long-chain AHLs and not AHLs with short side chains. Notably, the overexpression of PvdQ or QuiP in *P. aeruginosa* remarkably inhibits or delays the accumulation of 3-oxo-C12HSL signal molecule and thereby decreases expression of several virulence factors such as elastase and pyocyanin, important in *P. aeruginosa* pathogenicity[12-14]. For *Ralstonia* strain XJ12B, AiiD acylase inactivating both long- and short-chain AHLs was identified[15]. Clearly, finding and isolating new members of NTN-hydrolase family, such as quorum quenching AHL acylases, tempts to consider these enzymes as potential antimicrobial agents. **Chapter 2** outlines the current knowledge about the quorum quenching acylases and discusses, in particular, the acylases from *P. aeruginosa*.

In **chapter 6**, we report on cloning, overexpression, and characterization of a putative β-lactam acylase, PA1893, from *P. aeruginosa*. The PA1893 gene belongs to a cluster of genes (from PA1897 to PA1891) that may perhaps form a transcription unit. The recombinant PA1893 protein with a C-terminal 6xHis tag was overexpressed in *E. coli*, purified and the mature protein was found to be consisting of the α- and β-subunit. We showed that the β-subunit possesses a conserved N-terminal serine that is known to act as the catalytic nucleophile. Moreover, purified PA1893His did not display any hydrolytic activity on short and long acyl chain AHLs, neither on various β-lactam compounds. Our experiments showed that the wild type PAO1 *P. aeruginosa* strain and the mutant strain lacking PA1893 were able to grow equally well on penicillin G, penicillin V, cephalosporin C, and cephalexin indicating that PA1893 is not involved in the metabolism of tested antibiotics. Interestingly, we observed increased elastase production during the mid-logarithmic phase for the PA1893 deletion mutant (PAO1*ΔPA1893*). Also, addition of PA1893His to the growth medium of PAO1 and PAO1*ΔPA1893* reduced the production of elastase at the mid-logarithmic stage. These results indicate that PA1893 is somewhat a part of the quorum sensing-related regulon.

The absence of any measurable activity of PA1893His suggests that PA1893 displays activity on other substrates and the real substrate(s) has not been tested yet. The number of potential substrates for penicillin acylases is huge. Hence, in search for enzymatic activity of an uncharacterized enzyme it's rational to employ a high throughput screening against a range of available natural and synthetic substrates. The gained data could point to the physiological function as well as the industrial potential of a candidate enzyme. The biological role of β-lactam acylases has been proposed to be involved in the degradation of phenylacetylated compounds to generate a phenylacetic acid (PA) for a carbon and energy source, yet it remains largely unclear[16,17]. Generally, microorganisms can utilize various aromatic compounds as sole carbon and energy sources thanks to the presence of sophisticated catabolic pathways, composed of a set of genes, involved in biodegradation. Although diverse microorganisms are able of aerobic degradation of many naturally occurring or human-made compounds, *Pseudomonas* species particularly, are able to degrade a broad number of compounds[18-20]. In this context, the remarkable localization of PA1893 within the PA1897-PA1891 cluster might

suggest that proteins of this cluster are involved in energy source metabolism at some stage. Yet, additional studies are required to verify this hypothesis. Perhaps, experiments such as growth experiments on minimal media supplemented with different carbon sources and further characterization of PA1893 adjacent proteins, would elucidate this possibility. Furthermore, the PA1897-PA1891 genes were shown to be quorum-controlled by the transcriptome analysis of *P. aeruginosa*[21,22]. Also, the elevated levels of PA1893[23] and PA1897-PA1892[24] were detected during biofilms development. Our results of decreased elastase production at the mid-logarithmic phase after PA1893His addition to the PAO1 and PAO1*ΔPA1893* growing cultures also suggest PA1893 involvement in the quorum sensing circuit. The latter results shall inspire further studies essential to unravel the role and value of PA1893.

In summary, the research presented in this thesis investigates, broadly speaking, *Pseudomonas* for the production of pharmaceutically relevant proteins. As a result, we demonstrated at the molecular level that the LipRQ two-component system of *P. alcaligenes* governs the regulation of lipase expression by direct LipR binding to the lipase promoter. We also analyzed conditions inducing lipase expression. These findings are certainly important for the optimization of lipase production on industrial scale. Furthermore, the observations presented here, concerning PA1893, should stimulate future work extended by the list of PA1893 potential substrate compounds to begin with. Finally, a study with *P. aeruginosa* as a host for heterologous protein expression and secretion suggests that for each and every individual heterologous protein to be expressed a number of different factors determines the production levels and the fate of the protein. It appears that the periplasmic space of *P. aeruginosa* can serve as a potent folding compartment for various proteins, yet achieving the successful secretion will require further studies before the somewhat universal secretion signal can be defined. Also, the efficient *Pseudomonas* factory might have to be, ultimately, engineered in a protein-specific manner to fulfill the requirements of a particular heterologous protein. Thus, it would be of great value to pursue on this broad and challenging research in near future to further identify the key factors important for protein expression and secretion within the *Pseudomonas* enzyme factories.

References

1. Cox,M., Gerritse,G., Dankmeyer,L., & Quax,W.J. Characterization of the promoter and upstream activating sequence from the *Pseudomonas alcaligenes* lipase gene. *J. Biotechnol.* 86, 9-17 (2001).
2. Nishijyo,T., Haas,D., & Itoh,Y. The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 40, 917-931 (2001).
3. Stover,C.K., Pham,X.Q., Erwin,A.L., Mizoguchi,S.D., Warrener,P., Hickey,M.J., Brinkman,F.S., Hufnagle,W.O., Kowalik,D.J., Lagrou,M., Garber,R.L., Goltry,L., Tolentino,E., Westbrock-Wadman,S., Yuan,Y., Brody,L.L., Coulter,S.N., Folger,K.R., Kas,A., Larbig,K., Lim,R., Smith,K., Spencer,D., Wong,G.K., Wu,Z., Paulsen,I.T., Reizer,J., Saier,M.H., Hancock,R.E., Lory,S., & Olson,M.V. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 406, 959-964 (2000).
4. Li,W. & Lu,C.D. Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa*. *J. Bacteriol.* 189, 5413-5420 (2007).
5. Krzeslak,J., Gerritse,G., van Merkerk,R., Cool,R.H., & Quax,W.J. Lipase expression in *Pseudomonas alcaligenes* is under the control of a two-component regulatory system. *Appl. Environ. Microbiol.* 74, 1402-1411 (2008).

6. Keener,J. & Kustu,S. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino-terminal domain of NTRC. *Proc. Natl. Acad. Sci. U. S. A* 85, 4976-4980 (1988).
7. Weiss,V. & Magasanik,B. Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A* 85, 8919-8923 (1988).
8. Arts,J., van Boxtel,R., Filloux,A., Tommassen,J., & Koster,M. Export of the pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the signal recognition particle-Sec pathway. *J. Bacteriol.* 189, 2069-2076 (2007).
9. 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).
10. 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).
11. 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).
12. Huang,J.J., Han,J.I., Zhang,L.H., & Leadbetter,J.R. Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* 69, 5941-5949 (2003).
13. Huang,J.J., Petersen,A., Whiteley,M., & Leadbetter,J.R. Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* 72, 1190-1197 (2006).
14. Sio,C.F., Otten,L.G., Cool,R.H., Diggle,S.P., Braun,P.G., Bos,R., Daykin,M., Camara,M., Williams,P., & Quax,W.J. Quorum quenching by an N-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infect. Immun.* 74, 1673-1682 (2006).
15. Lin,Y.H., Xu,J.L., Hu,J., Wang,L.H., Ong,S.L., Leadbetter,J.R., & Zhang,L.H. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol. Microbiol.* 47, 849-860 (2003).
16. 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).
17. 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).
18. Bartolome-Martin,D., Martinez-Garcia,E., Mascaraque,V., Rubio,J., Perera,J., & Alonso,S. Characterization of a second functional gene cluster for the catabolism of phenylacetic acid in *Pseudomonas* sp. strain Y2. *Gene* 341, 167-179 (2004).
19. Jimenez,J.I., Minambres,B., Garcia,J.L., & Diaz,E. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4, 824-841 (2002).
20. Luengo,J.M., Garcia,J.L., & Olivera,E.R. The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. *Mol. Microbiol.* 39, 1434-1442 (2001).
21. Schuster,M., Lostroh,C.P., Ogi,T., & Greenberg,E.P. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* 185, 2066-2079 (2003).
22. Wagner,V.E., Bushnell,D., Passador,L., Brooks,A.I., & Iglewski,B.H. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185, 2080-2095 (2003).
23. Southey-Pillig,C.J., Davies,D.G., & Sauer,K. Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 187, 8114-8126 (2005).
24. Hentzer,M., Elberl,L., & Givskov,M. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. *Biofilms* 2, 37-61 (2005).