university of groningen

University Medical Center Groningen

University of Groningen

Pseudomonas as a microbial enzyme factory

Krzeslak, Joanna Kamila

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Krzeslak, J. K. (2009). *Pseudomonas as a microbial enzyme factory: the source of industrially potent enzymes and the host for heterologous enzyme production*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 10-12-2024

CHAPTER 2

Quorum quenching acylases in *Pseudomonas aeruginosa*

Joanna Krzeslak, Mariana Wahjudi, and Wim J. Quax

Pseudomonas. *Volume 5: A Model System in Biology*, *Chapter 15 (2007)*

Quorum quenching acylases in *Pseudomonas aeruginosa*

The β-lactam acylases, mainly found by screening samples from natural sources, represent a unique family of heterodimeric N-terminal nucleophile hydrolases. Interestingly, most of the strains found to be producing β-lactam acylases are *Pseudomonas* species. We have shown that these enzymes show high selectivity towards the acid side chain, but are far more promiscuous with regard to the amine moiety of the substrate. It is therefore highly unlikely that the industrially relevant deacylation of β-lactam compounds has evolved specifically in nature. Notably, no less than four putative acylases of the N-terminal nucleophile family have been identified in the *Pseudomonas aeruginosa* PAO1 genome. We have investigated these four putative acylases of which one could be expressed in *Escherichia coli.* Interestingly, the enzyme was found to catalyse the hydrolysis of acyl homoserine lactones as reported for *Ralstonia* acylase suggesting a role in quorum quenching. In a bioassay, the purified acylase was shown to degrade AHL signal molecules with side chains ranging from 11 to 14 carbons at physiologically low concentrations confirming quorum quenching activity. The discovery of quorum quenching AHL acylases in the genome of *P. aeruginosa* POA1 may explain infection progression and offer new targets for anti-bacterial therapy.

NTN-hydrolases as β-lactam acylases

In 1960, while searching for an enzyme capable of hydrolyzing penicillin G, an enzyme was described which afterward was used extensively for the commercial production of 6-aminopenicillanic acid (6-APA), the most important intermediate for the industrial production of semisynthetic penicillins[1]. This first enzyme was named penicillin acylase (EC 3.5.1.11) and later on numerous bacterial species have been described as penicillin G acylase-producing strains, including *E. coli*, *Kluyvera citrophila*, and *Alcaligenes faecalis*[2-4]. Recombinant DNA methods have been applied to not only increase the yields of commercially used penicillin G acylases[5], but also to decipher the complex processing of these enzymes[6]. The penicillin G acylase of *E. coli* ATCC11105, a paradigm for this enzyme class, was found to be produced as a large precursor protein, which is transported into the periplasm and further processed to the mature protein constituting a small (α) and a large (β) subunit. Not only this heterodimeric structure, but also the role of the N-terminal serine residue as a nucleophile for the deacylation reaction is evolutionarily preserved resulting in renaming these enzymes to N-terminal nucleophile (NTN) hydrolases[7] comprising the much larger family of β-lactam acylases.

Whereas the conversion of penicillin G requires an enzyme with specificity for the aromatic phenyl-acetate side chain, the processing of the second largest β-lactam fermentation product, cephalosporin C, would require the cleavage of amino-adipyl, an aliphatic side chain, from the β-lactam nucleus. Since no enzyme capable of performing a one-step deacylation was found[8], a two enzyme-mediated reaction has been introduced to produce 7-aminocephalosporanic acid (ACA). In this process, D-amino acid oxidase converts amino-adipyl into glutaryl and glutaryl acylase performs the enzymatic deacylation to cephalosporin C. The glutaryl acylases (EC 3.5.1.-) can be obtained from several *Pseudomonas* species[8-13].

Interestingly, the specificity of the β-lactam acylases is mainly directed towards the acyl side chain and the enzyme is not selective to the β-lactam moiety at all. Penicillin G acylase can also hydrolyse phenylacetyl-leucine[4] and cephalosporin acylase can also hydrolyse glutaryl-leucine[14]. This has further raised the question on the natural role of NTN-hydrolases in Gram-negative bacteria as the hydrolysis of β-lactam can not be considered as an evolutionary advantage. 6-APA and 7-ACA are for most Gram- negative bacteria more bactericidal as the non-hydrolysed precursors. The discovery that one of the members of this NTN-hydrolase family can hydrolyse acyl homoserine lactone compounds has shed some light on the possible physiological role of these enzymes[13-15]. The presence of no less than four related genes encoding putative NTN-hydrolases in the genome of *P. aeruginosa*[16] has encouraged us to investigate the function of acylases in quorum sensing.

Bacterial acylases (and lactonases) as quorum sensing interfering enzymes

P. aeruginosa and most Gram-negative bacteria use acyl-homoserine lactones (AHLs) as signal molecules. In general, AHL consists of a homoserine lactone moiety and an acyl chain. The length and substitutions of acyl side-chains vary amongst the species. Two signal molecules, 3-oxo-C12HSL and C4HSL have been studied in great detail in *P. aeruginosa*. The common acyl groups of AHLs identified so far vary from 4 to 18 carbons in length; they may be saturated or unsaturated, and with or without a C-3 substitution (usually hydroxy- or oxo-)[17].

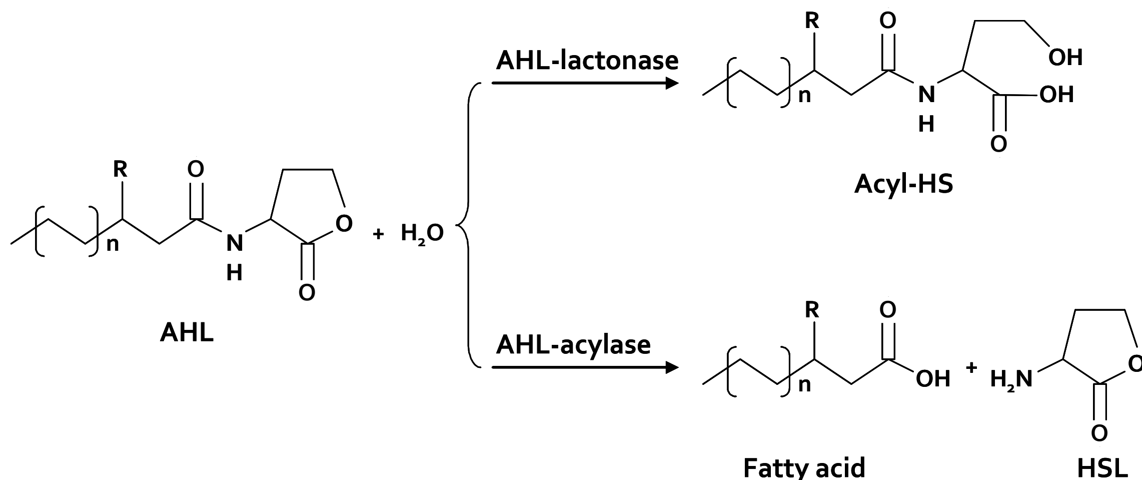

Figure 1. Degradation of homoserine lactone molecules via two alternative enzymatic systems: lactonase (top) and acylase (bottom).

As quorum sensing has a contribution to the modulation of some target genes, including virulent factors, it is of great interest to study the means of interference with quorum sensing systems. Interruption of quorum sensing is also called quorum quenching. Since AHLs may also act directly as virulence factors in mammalian hosts (review by ref[18,19]) degradation of

AHLs may be interesting not only to stop the quorum sensing system, but also to directly reduce the virulence effects.

Quorum quenching can be accomplished in several ways (i) by blockage of AHLs production, (ii) by degradation of AHL signal molecules, or (iii) by interference with activation of the signal receptor. Several enzymes have already been described to be competent to degrade signal molecules. Lactonases inactivate AHLs by hydrolyzing the ester bond of the lactone ring of the molecules to yield acyl-homoserine. Acylases cleave the amide bond that connects the lactone moiety and acyl side-chain (Figure 1). All of the resulting breakdown products are not active as signal molecules.

Some of the enzymes degrading AHL were shown to act as quorum quenching enzymes under physiological conditions[13,20-22]. Constitutive expression of the AHL lactonase and acylase in quorum sensing bacteria was shown to abolish or dramatically reduce AHLs accumulation[23-25]. Additionally, degradation of AHL signal molecules by lactonases or acylases was shown to have impact on expression of quorum sensing regulated genes.

Several bacteria were found to produce AHLs lactonases. AiiA of *Bacillus sp*. strain 240B1 is the first AHL lactonase found[23]. Other members of the *Bacillus* genus were also confirmed to produce AiiA homologues, i.e. *Bacillus cereus*[26], *Bacillus mycoides* and *Bacillus thuringiensis*[27], and *B. thuringiensis* subsp. *kyushuensis*[28]. In addition, several AiiA homologues were produced by other bacteria, AhlD (acyl homoserine lactone degradation) by *Arthrobacter sp*. and AhlK by *Klebsiella pneumoniae* KCTC2241[29]. *Agrobacterium tumefaciens* was found to produce two AiiA homologues, AttM and AiiB[20,22].

Although lactone opening of AHLs was shown to interfere with quorum sensing, there may be a complication in practice. The breakdown product of AHL lactonase, that is a ring-opened AHL molecule, spontaneously undergoes ring formation in acidic environment (review by ref[30]). Therefore, the deacylation process might be preferred, since fatty acid generated by the process is metabolized and the reaction can not revert[31]. In addition, the other acylase-cleavage product, homoserine lactone (HSL), is a potential growth inhibitor for several bacteria. It suppresses for instance the growth of *E. coli* and *Arthrobacter* strain VAI-A[32,33] giving an additional effect against bacterial growth.

AHLs cleaved by strains producing acylases give homoserine lactone and fatty acid. The latter one is afterward metabolized. Thus, AHLs can be used as a sole carbon and energy source. *Variovorax paradoxus* was reported to degrade 3-oxo-C6HSL, C4HSL, C6HSL, C8HSL, C10HSL, and C12HSL through deacylation process and to use these as sources of carbon, energy, and nitrogen[31]. An acylase originating from *Ralstonia* strain XJ12B shows homology to a number of other acylases and N-terminal hydrolases of the NTN-hydrolase super family. This acylase, AiiD, deactivates both long- and short-chain AHLs[24]. Other acylases that showed activity toward both short- and long-chain AHLs were produced by *Rhodococcus erythropolis* W2 and *Streptomyces* sp. strain M664. *R. erythropolis* degrades AHL through both oxidoreductase and amidolytic activities[34]. *Streptomyces* sp. produces AhlM, an AHL acylase, which exhibits broad substrate specificity, including activity on penicillin G[21]. Furthermore, the two acylases from *P. aeruginosa*, PvdQ (PA2385) and QuiP (PA1032), were shown as well to have potential as quorum sensing interfering enzymes. These enzymes degrade long acyl-chain-HSLs and not ASLs with short side chains[13,15,35]. PvdQ and QuiP are discussed in more detail later in the chapter.

Acylases in the Pseudomonadaceae family

So far, five sequences of characterized AHL acylases have been deposited to the National Center for Biotechnology Information (NCBI) database. They include *Ralstonia sp*. strain XJ12B AiiD ([24]; AAO41113), *R. erythropolis* W2 QsdA ([34]; AAT06802), *P. aeruginosa* PAO1 PvdQ ([13,15]; NP_251075), *P. aeruginosa* PAO1 QuiP ([35]; NP_249723), and *Streptomyces sp*. strain M664 AhlM ([21]; AAT68473). Two of them belong to the Pseudomonadaceae family. To investigate the distribution of homologous to AHL acylase sequences genes amongst Pseudomonadaceae genomes, the AiiD amino acid sequence of *Ralstonia* sp. was used as a query against the complete genomes of Pseudomonadaceae members available in the NCBI database. AiiD belongs to NTN cephalosporin acylases and shows highest similarity to PvdQ and QuiP in comparison to other AHL acylases (data not reported here). Subsequently, it was used as a blast request sequence using the Blastp program (http://www3.ncbi.nlm.nih.gov/BLAST/). The alignments for all of sequences were created using the ClustalV method in MegAlign 5.07 (DNA Star, USA).

The analysis identifies at least 27 amino acid sequences distributed over *Pseudomonadaceae* members deduced from open reading frames (ORFs) with significant identity scores to AiiD (July 22, 2006). Many of them are deduced protein sequences with undefined functions yet and classified as members of the penicillin acylase family or proteins related to penicillin acylase. Most of them have been tentatively annotated as amidases or acylases.

The sequences of the three AHL acylases of non-Pseudomonadaceae members included show an identity in range of 11-39% to all of the homologues sequences. Each genome of Pseudomonadaceae members has at least two distinct *aiiD* homologues genes. One homologue from each strain exhibits identity with *Ralstonia* AiiD ranging from 20 to 39%. Moreover, the conserved structural elements of these homologues show that they all belong to the NTN-hydrolase cephalosporin acylase family (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). In the *P. aeruginosa* PAO1 genome, there are four AiiD homologues, including PvdQ and QuiP. Two other distinct putative AiiD homologues in *P. aeruginosa* PAO1 genome, PA1893 and PA0305, will be discussed in the next part of the chapter.

A phylogenetic analysis of the AiiD homologues shows that they fall into three clusters (Figure 2). The first cluster covers *Ralstonia* AiiD, *Streptomyces* AhlM, and PvdQ. Moreover, a homologue of each representative strain that shows similarity with one or more of the three AHL acylases emerges in the first cluster. They are envisaged to be prospective AHL acylases.

The second and the third cluster are only distantly related to cluster 1 and they harbour a number of cephalosporin and penicillin acylases. Interestingly, QuiP which resides in cluster 3 also hydrolyses AHLs. In conclusion, it appears that in the genome of every Pseudomonadaceae member there might be at least one putative AHL acylase.

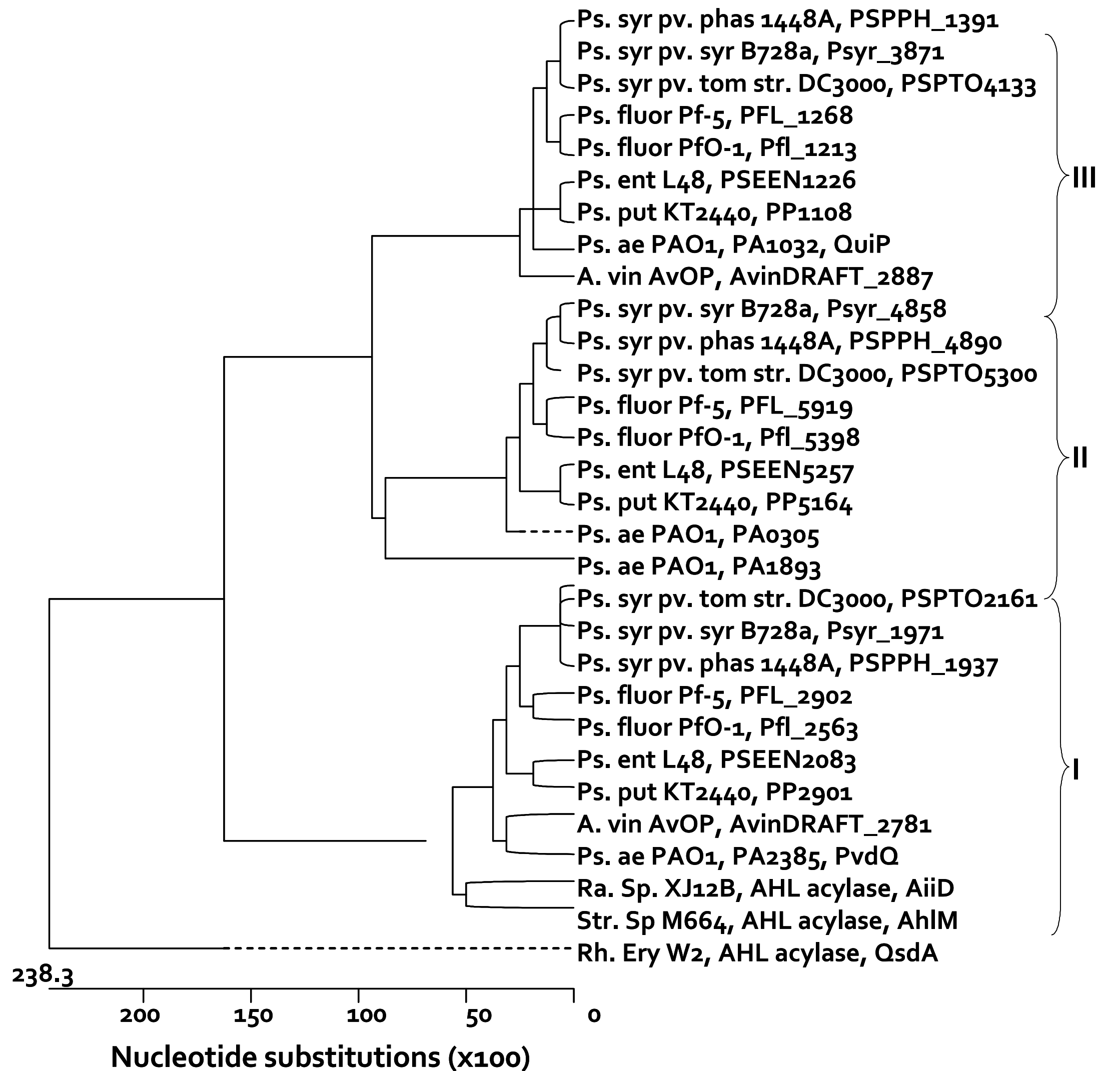

Figure 2. Phylogenetic tree of Pseudomonadaceae AHL acylases and AHL acylases homologues of known function based on the protein alignment of *Ralstonia* strain XJ12B AiiD. The phylogenetic tree was made with the DNASTAR sequence analysis software (DNASTAR Inc.). Distances are shown below the tree. Abbreviations: Ps = *Pseudomonas*; pv = *pathovar*; syr = *syringae*; phas = *phaseolitica*; tom = *tomato*; fluor = *fluorescens*; ent = *entomophila*; put = *putida*; ae = *aeruginosa*; A vin = *Azotobacter vinelandii*; Ra sp = *Ralstonia* sp.; Str sp = *Streptomyces* sp.; and Rh ery = *Rhodococcus erythropolis.* The numbers after the strain name refer to the locus tag.

Acylases in *Pseudomonas aeruginosa*

When searching for acylases homologues (NTN-hydrolases) within *Pseudomonas aeruginosa* PAO1 genome, one can find four candidates: *pvdQ* (PA2385), *quiP* (PA1032), PA0305, and PA1893 (see Figure 5). The identification numbers of the genes come from the *Pseudomonas* genome project (www.pseudomonas.com). The products of the *pvdQ* and *quiP* genes were shown to encode AHL acylases[13,15,35]. PA0305 and PA1893 encode hypothetical proteins of unknown function (www.pseudomonas.com). The PvdQ precursor shares 37% amino acid

identity with aculeacin A acylase (AAC) from the high GC Gram-positive organism, *Actinoplanes utahensis*[36,37] and 39% with AHL acylase AiiD from *Ralstonia* XJ12B[24]. PvdQ and PA1893 precursors also share a significant match (25%) at the peptide level with *Pseudomonas sp. SY-77* glutaryl acylase SY-77[14], for PA0305 it is 22%, and for QuiP it is 20%. Moreover, QuiP and PA0305 share 27-26% amino acid identity with cephalosporin acylase acyII from *Pseudomonas sp. SE83* and 22-21% with aculeacin A acylase from *A. utahensis*[36,37]. Table 1 presents an overview of amino acids identity of *P. aeruginosa* acylases homologues with known acylases.

Table 1. Amino acid sequence identity of *P. aeruginosa* PAO1 acylases homologues with known acylases. Protein sequences were aligned using the SECentral (Clone Manager, version 6.00 and Align Plus 4, version 4.10, Scientific and Educational Software, Cary, NC).

Amino acid identity in %	PvdQ (PA2385)	QuiP (PA1032)	PA0305	PA1893
SY-77 glutaryl acylase *Pseudomonas sp.* SY-77	25	20	22	25
AiiD acyl-homoserine lactone acylase *Ralstonia* XJ12B	39	20	21	23
AAC aculeacin A acylase *Actinoplanes utahensis*	37	22	21	23
PGA penicillin G acylase *Escherichia coli*	18	22	24	22
Cephalosporin acylase (acyII) *Pseudomonas sp.* SE83	23	27	26	29

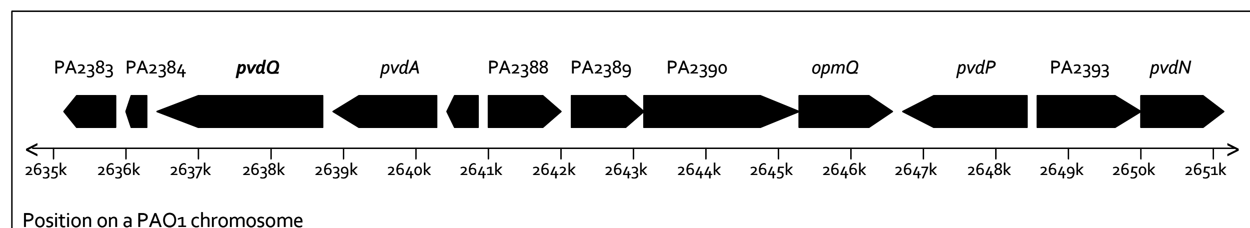

Figure 3. Schematic representation of a *pvdQ* gene localization within the *P. aeruginosa* chromosome.

PvdQ (PA2385)

PvdQ is the first described N-terminal nucleophile hydrolase, with biochemical properties similar to β-lactam acylases, found in *P. aeruginosa* PAO1. Figure 3 shows the *pvdQ* gene localization on a *P. aeruginosa* chromosome. The PvdQ enzyme was found to have AHL acylase activity towards long AHLs[13,15]. The earliest report about microbial AHL acylase with quorum quenching potential can be found in a publication of *Lin et al.*[24]. An identified enzyme, *aiiD*, found in *Ralstonia* strain XJ12B was cloned and expressed in *E. coli* and *P. aeruginosa*. The

expression of *aiiD* in *P. aeruginosa* resulted in reduced elastase and pyocyanin production, and decreased ability to swarm suggesting quorum quenching activity of AiiD[24]. This finding encouraged a search for other acylases with quorum quenching properties. The second bacterial AHL acylase described is *pvdQ* (PA2385), which was found in *P. aeruginosa*[13,15,35,38]. AiiD and PvdQ share a significant amino acid identity of 39%.

```
                             spacer                       β-subunit
AiiD         232 ----------------G-SNGWAFGADATANRRGVLLGNPHFPWTTTN-RFYQVHLTVPG 273
AAC          229 ----------------G-SNAYGLGAQATVNGSGMVLANPHFPWQGAE-RFYRMHLKVPG 270
SY-77        198 ----------------G-SNSWAVAPGKTANGNALLLQNPHLSWTTDYFTYYEAHLVTP- 239
CA           223 ALLKAMGGDASDAAGGG-SNNWAVAPGRTATGRPILAGDPHRVFEIPG-MYAQHHLAC-D 279
PGA          289 ----------------T-SNMWVIGKSKAQDAKAIMVNGPQFGWYAPA-YTYGIGLHGAG 330
PvdQ PA2385  216 ----------------G-SNAIAVGSERSADGKGMLLANPHFPWNGAM-RFYQMHLTIPG 257
QuiP PA1032  263 ----------------AASNNWAIAPQRSRSGKSLMANDTHLPLSMPS-VWNYVQIRSP- 304
PA0305       244 QFE-------------G-SNAWVVAGSRTASGKPLLAGDPHIRFAAPA-VWYEAQLSAPG 288
PA1893       253 ----------------G-SNNWVVSASRSATGKPLLANDPHLRLTNPA-AFYLASLKIPG 294
Conserved residues               G S                    H           Y
```

Figure 4. Sequence alignment of the conserved regions of several acylases and their homologues. Acylases homologous used in the alignment include: AiiD acyl homoserine lactone acylase (794 aas) from *Ralstonia* strain XJ12B[24]; AAC aculeacin A acylase (786 aas) from *A. utahensis*[36,37]; SY-77 glutaryl acylase (720 aas) from *Pseudomonas sp.* SY-77[14]; CA cephalosporin acylase (774 aas) from *Pseudomonas sp.* SE-83[11] ; PGA penicillin G acylase (846aas) from *E. coli*[6]; PvdQ (762 aas)[38,39], QuiP (847 aas)[35], PA0305 (795 aas), and PA1893 (809 aas) from *P. aeruginosa* PAO1. The boxed residues represent the important residues for the autocatalytic processing and activity of known acylases.

The *pvdQ* gene is translated as a precursor consisting of four parts: a putative signal sequence, the α-subunit, a spacer peptide, and the β-subunit, whereas the active enzyme consists of only the α- and β-subunits of 18 and 60kDa, respectively. PvdQ is a periplasmic protein. The β-subunit of PvdQ starts with a serine (Ser) residue, at position 217(aas), which is characteristic for β-lactam acylases[13]. Figure 4 presents an alignment of PvdQ with other acylases within the conserved residues region. It is known that the N-terminal residue (Ser) of the β-subunit in NTN-hydrolases plays an important role in enzyme autocatalytic processing as well as in enzymatic activity. Rational randomization at this position resulted in leucine, valine, lysine, arginine or aspartic acid mutant. All the PvdQ mutants could be expressed in *E. coli* from the plasmid, as the wild type PvdQ. However, for the mutant proteins the processing was impaired (only precursor polypeptide was present). This result confirms the importance of 217Ser residue for PvdQ autocatalytic processing and supports the fact that PvdQ is the NTN-hydrolase (unpublished data, Krzeslak *et al*.).

The *pvdQ* gene lies in a pyoverdin operon[38,39]. Pyoverdin is one of *P. aeruginosa* siderophores and it is important in iron uptake. For growth, *P. aeruginosa* needs iron as an essential cofactor. The bioavailability of iron is limited by its low solubility in nature[40,41]. *P. aeruginosa* overcomes this problem by synthesizing high affinity iron-chelating molecules,

siderophores that provide the organism with iron under the most nutritionally dilute conditions[42]. *P. aeruginosa* produces two chemically unrelated siderophores, pyoverdins (PVD)[43,44] and to a limited extent pyochelin[45,46]. These siderophores function as powerful iron chelators, solubilizing and transporting iron through the bacterial membranes via specific receptor proteins at the outer membrane level[47-49,49]. It makes pyoverdin essential for *P. aeruginosa* growth and virulence. In general, pyoverdins consist of three distinct structural parts, a dihydroxyquinoline chromophore responsible for their fluorescence and colour, a peptide chain comprising 7-8 amino acids bound to the carboxyl group of the chromophore, and a small dicarboxylic acid connected via an amide bond to the amino group of the chromophore[50]. Pyoverdines of a single strain have the same peptide but may differ in the nature of the acyl group. The acyl chain of pyoverdin is made of a dicarboxylic acid residue, which can be either succinate, or its amide form, or α-ketoglutarate or glutamate, depending on the producing strain or growth conditions[51]. The mechanism for formation of the pyoverdin chromophore was illustrated by *Dorrestein et al.*[52]. For the biosynthesis of pyoverdin two regions of the *P. aeruginosa* chromosome are important: the *pvd* locus and *pvc* locus. The *pvc* locus (*pvcA*, *pvcB*, *pvcC and pvcD* genes) is involved in the synthesis of the chromophore part of pyoverdin and the *pvd* locus (*pvdQ, pvdA, fpvI, fpvR, pvdP, pvdO, pvdN, pvdM, pvdF, pvdE, fpvA, pvdIJ, pvdD, pvdH, pvdL, pvdG, pvdS, pvdY, and pvdX* genes) in the synthesis of the peptide chain. The genes involved in the acyl chain formation are not characterized yet[53-55]. The function of *pvdQ*, *pvdP*, *pvdY*, *pvdO*, *and pvdX* genes in the pyoverdin biosynthesis pathway still remains unknown. Nevertheless, it was shown that their expression occurs under iron limiting conditions[56] in concert with the onset of pyoverdin synthesis under these conditions.

The *pvdQ* presence in the *pvd* locus suggests an involvement in the pyoverdin biosynthesis. *Lamont et al.* indicated that *pvdQ* plays a role in pyoverdin synthesis as pyoverdin is not produced in the *pvdQ* negative strain[38]. The *pvdQ* gene has no PvdS-dependent promoter but it has an iron starvation box implying that transcription of this gene takes place under iron restriction[51]. Both, *pvdQ* and *pvdA*, were found in *P. aeruginosa* strains producing type I, type II, and type III pyoverdin[38], suggesting that they are required in biosynthesis of all three types of pyoverdin.

Initially, it was speculated that PvdQ could be involved in degradation of AHLs as a carbon source[15] as *Huang et al.* indicated that PAO1 can utilize its quorum sensing molecules (AHLs) as a sole energy source essential for growth. Additional experiments demonstrated that the *pvdQ* knockout mutant strains were still able to grow in a medium supplemented with the long AHL (3-oxo-C12HSL) as a sole energy source[15]. In addition, the examination of *pvdQ* mRNA expression levels showed no increase in mRNA levels when cultures utilized different carbon sources[35] suggesting that AHL utilization is not solely dependent on PvdQ and therefore, it was proposed that another enzyme is involved in AHLs utilization. Extensive investigation of PvdQ done by *Sio et al.*[13], proved that PvdQ degrades efficiently AHLs with side chains ranging from 11 to 14 carbons but not short-chain AHLs. The substituent at the 3` position of the side chain did not affect its activity. In addition, *Sio et al*. found that overexpression of PvdQ in *P. aeruginosa* or exogenous addition of PvdQ to *P. aeruginosa*-growing cultures inhibits or delays the accumulation of signal molecules (3-oxo-C12HSL and 2-heptyl-3-hydroxy-4(1H)-quinolone) and thereby decreases expression of several virulence

factors such as elastase and pyocyanin, important in *P. aeruginosa* pathogenicity. Hence, it can act as a quorum quencher *in vitro*[13]. It was also demonstrated that PvdQ has no activity towards β-lactams[13].

Up to now, our knowledge is still incomplete to define the physiological role of PvdQ. Several experiments showed that *pvd*Q is needed for biosynthesis of pyoverdin and *pvdQ* expression is up-regulated under iron limiting conditions[39]. There is a possibility that one of the precursors in the pyoverdin biosynthetic pathway may serve as a substrate for PvdQ. However, this has not been shown yet. The difficulty in determining the physiological role of PvdQ also comes from the diversity in *P. aeruginosa* bacterial forms (biofilm, planktonic), the variations in growth conditions, nutrients availability and other environmental factors.

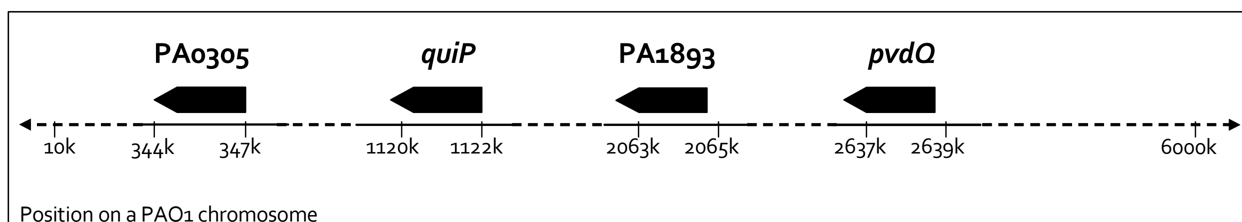

Figure 5. Chromosomal localisation of four genes encoding (putative) NTN-hydrolases on the *P. aeruginosa* genome.

QuiP (PA1032)

PA1032, recently named as *quiP* (for quorum signal utilization and inactivation protein), is a second *P. aeruginosa* acylase homologue identified up to now[35]. In Figure 6 the localization of *quiP* on genomic DNA of *P. aeruginosa* is shown. Protein sequence analysis predicts QuiP to have a significant similarity to acylases; QuiP shows 20-27% identity on a peptide level with other acylases (Table 1). As is characteristic for NTN-hydrolases, the gene encodes a polypeptide that undergoes post-translational maturation to result in a heterodimeric mature protein[57]. However, the post-translational processing of the QuiP precursor has not been demonstrated yet. An alignment of the conserved region of known acylases with QuiP shows at position 297 asparagine (N), whereas for most acylases there is tyrosine, a strongly conserved residue (Figure 4). The *quiP* gene was cloned into an inducible plasmid and expressed in *E. coli*, and only unprocessed form of the protein could be detected, the separate α and β-subunits were not visible. Visible, unprocessed QuiP was a 90-kDa protein. When analyzed by LC-MS/MS, the generated peptides matched with *in silico* prediction[35]. Signal peptide prediction indicates that protein will be transported to the periplasm, as observed for other acylases.

Phenotypic analysis of a *quiP* transposon mutant (mutant ID33050; Washington collection www.genome.washington.edu/UWGC/) showed that a strain carrying the transposon insertion was impaired in growth on decanoyl-HSL when compared to the parental strain. QuiP complementation studies showed that cells could restore AHL degrading potential when QuiP was constitutively expressed from a plasmid. It was also observed that constitutive *quiP* expression in *P. aeruginosa* resulted in a remarkable decrease of 3-oxo-C12HSL

accumulation. An analogous observation was made when *quiP* was expressed in *E. coli* from the inducible plasmid; cell extracts were able to degrade AHLs. In addition, the study of *quiP* mRNA expression levels for cultures grown on C10HSL, decanoate or succinate as a carbon source demonstrated an increase in mRNA level for cultures grown on C10HSL. These findings indicate that QuiP is involved in AHL utilization[35]. In particular, QuiP displays specificity towards long (C7HSL, C8HSL, C10HSL, 3-oxo-C12HSL, and C14HSL) but not short AHLs. Furthermore, *quiP* has not been picked up in microarray analysis as a gene regulated by quorum sensing[58,59].

Concluding, recent results show that QuiP has AHL activity, thus implying its involvement in signal decay in *P. aeruginosa*. However, it is not known yet under which physiological conditions *quiP* is expressed and what the biological function of QuiP is.

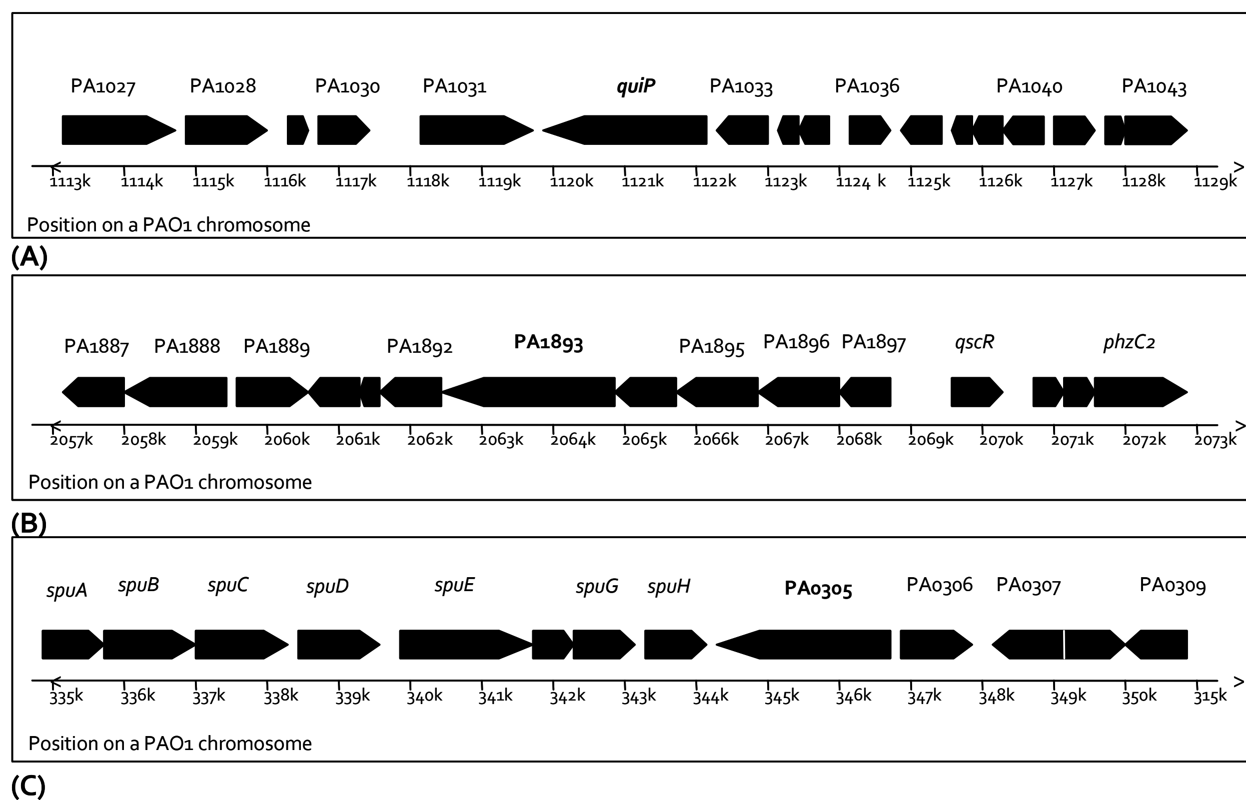

Figure 6. Detailed schematic representation of *quiP* (A), PA1893 (B), and PA0305 (C) gene localization within the *P. aeruginosa* chromosome.

PA1893

PA1893 is the third acylase homologue within the *P. aeruginosa* PAO1 genome. Its localization on the chromosome is shown in Figure 5 and in detail in Figure 6. The PA1893 ORF shares 29% amino acid identity with cephalosporin acylase (acyII) from *Pseudomonas sp.* SE83[11,59] and 25% with *Pseudomonas sp.* SY-77 glutaryl acylase[14,59]. The PA1893 peptide sequence was partially aligned with other acylases (Figure 4). As can be seen from this alignment, PA1893 has

conserved residues known to be important for autocatalytic processing and enzymatic activity of known NTN-hydrolases. According to the *in silico* prediction, PA1893 is a polypeptide of 809aa (89.7kDa) that has, at the N-terminus, a hydrophobic stretch of 29 amino acids encoding a signal peptide. The predicted β-subunit of 556aa (61.8kDa) starts with a serine (Ser) residue at position 254. PA1893 is predicted to undergo post-translational modification and to consist of the α and β-subunit as a mature protein[57,60]. However, protein maturation and subunit identification have not been shown yet, due to problems with protein solubility[35].

The PA1893 transposon mutant (mutant ID7831; Washington collection www.genome.washington.edu/UWGC/) was grown on decanoyl-HSL to study its ability to degrade AHLs. Results of this experiment showed no significant growth differences in comparison to the parental strain[35]. In addition, it was observed that mRNA expression level of PA1893 was up-regulated when *Pseudomonas* cultures were grown in medium supplemented with C10HSL as a carbon source in comparison to decanoate and succinate[35]. Microarray experiments provide information that PA1893 is a quorum activated gene[58,59]. PA1893 is discussed in more detail in chapter 6 of the thesis.

PA0305

The fourth acylase homologue in *P. aeruginosa* genome is PA0305. Figure 6 presents the PA0305 localization on the genome. Similarly to the three other acylase homologues, PA0305 also is predicted to encode a NTN-hydrolase that undergoes post-translational modification[57,60]. PA0305 shows 26% amino acid similarity to cephalosporin acylase (acyII) from *Pseudomonas sp.* SE83[11,59] and 24% to penicillin G acylase from *E. coli*[6]. The degree of PA0305 identity on a peptide level to other known acylases is deciphered in Table 1. Sequence alignment of the β-subunit shows that PA0305 has at the position 248-serine (S), at the position 270-histidine (H), and at the position 280-tyrosine (Y), residues highly conserved among NTN-hydrolases (Figure 4). *In silico* analysis predicts that the gene is transcribed as a precursor peptide consisting of a signal sequence, a α-subunit, a spacer peptide, and a β-subunit, while the active enzyme consists of just the α- and β-subunit. PA0305 is a polypeptide of 795aas (87.3kDa) that has a signal peptide of 25aas at the N-terminus. The predicted β-subunit starts with a serine residue (S) at the position 248 and consists of 548aas (59.9kDa).

There is little known about the physiological role or enzymatic activity of this enzyme. So far, the PA0305 ORF was picked up by PCR from PAO1 chromosomal DNA and expressed in *E. coli*[35]. However, only an unprocessed PA0305 polypeptide could be seen on SDS-PAGE gel. To investigate a possible involvement of PA0305 in AHLs utilization, a PA0305 transposon mutant (mutant ID32876; Washington collection www.genome.washington.edu/UWGC/) was examined for its ability to grow on decanoyl-HSL as a growth substrate. The growth phenotype of the PA0305 transposon mutant did not show any significant difference when compared to the wild type strain[35]. Therefore, PA0305 is not implicated in direct AHLs utilization in *P. aeruginosa*. Moreover, no increase in the PA0305 mRNA expression level was observed when cultures were grown utilizing C10HSL as a carbon source, relatively to growth on decanoate and succinate[35].

The physiological role of PA0305 is unidentified. Therefore, additional studies have to be performed to unravel the function of PA0305 in *P. aeruginosa*.

What for *Pseudomonas* may need acylases?

Although many NTN-hydrolases have been identified and characterized in different microorganisms over last decade, their physiological role most of the time remains unclear. Regarding the abundance of acylases, it is undoubtedly evident that there is a rationale for bacteria to have these types of enzymes. In general, acylases are responsible for deacylation of antibiotics (penicillins, cephalosporins), AHL compounds or cyclic lipopeptides. As acylases hydrolyze an amide bond that links a ring-like nucleus to a side chain (aromatic or aliphatic), their substrates are molecules with an amide bond. Yet, acylases can be quite specific acting on a very few substrates only. Interestingly, it was observed that penicillin G acylase can also hydrolyse cephalosporin C and even phenylacetic-leucine[61]. Also glutaryl cephalosporin acylase SY-77 was found to be very specific for the acyl side chain, only glutaryl, and very promiscuous for the acid moiety hydrolyzing even glutaryl-serine and glutaryl-leucine. From the 3D structure it becomes obvious that the acyl side chain is fully buried within the active site cleft, whereas the acid moiety has evolved limited interaction with the enzyme[62] (see Figure 7). This raises the possibility that the NTN-hydrolases are broad-spectrum enzymes towards the acid moiety and small-spectrum with respect to the acyl side chain. Within β-lactam acylases there are some indications that penicillin acylases could act as scavengers for nutrient compounds[63], whereas there are no hints on such a role for cephalosporin acylases. QuiP and PvdQ are indicated to be involved in AHL scavenging and whether the primary function of PvdQ is in pyoverdin biosynthesis is not completely clear yet. Results from studies with a PvdQ negative *P. aeruginosa* strain that is impaired in pyoverdin production[38] may suggest a possible direct involvement of PvdQ in pyoverdin biosynthesis; however, until now there are no clear-cut leads for this. The observed pyoverdin negative phenotype of the *ΔpvdQ* PAO1 strain could simply be the indirect effect of *pvdQ* deletion on pyoverdin production. The fundamental question is whether *P. aeruginosa* needs acylases to be able to perform its physiological processes or to regulate its physiological processes. For example, the disruption of *P. aeruginosa* acylases genes, separately in each gene, doesn't have a lethal effect on bacteria. Nevertheless, mutants display some phenotypic changes[13,35]. As mentioned earlier, two *P. aeruginosa* acylases (PvdQ, QuiP) were shown to have AHL activity, but is their primary biological role in *P. aeruginosa* to degrade 3-oxo-C12HSL signal molecule as a sole energy source or to regulate its own quorum sensing activity? Some bacteria break down AHLs and metabolize them, where other strains might just degrade them so that other bacterial partners in a community can utilize these products[32]. Perhaps, these enzymes also control interactions within microbial communities, like between quorum sensing bacteria and AHL-degrading bacteria. Does *P. aeruginosa* need acylases for bacterial networking, for virulence, to be able to compete with other bacteria for limited resources or for environmental adaptation? What does stimulate acylases' expression? Is the level of acylases expression different for the planktonic form than for the biofilm form? Does *P. aeruginosa* need acylases to degrade its own signal molecules or signal molecules of other bacteria? PvdQ and QuiP are quite specific towards long chain AHLs[13,35], so would that be the best strategy for bacteria to utilize these enzymes as communication interferers - quorum quenchers - between bacteria? Could acylases play a dual role: termination of other bacteria and utilization of disrupted signal molecules as a carbon source? It is known that quorum sensing plays an important role in the virulence of many

pathogenic bacteria, therefore it serves as a target for anti-pathogenic treatments[64]. In *P. aeruginosa* it was shown that inactivation of quorum sensing systems dramatically reduces virulence[65]. Thus, while investigating the physiological role of acylases, from *P. aeruginosa* and other species in their natural environment there is a huge temptation to look at them as enzymes with a potential for antimicrobial therapies. As one of the possibilities to interfere with bacterial quorum sensing is to employ AHL acylases or lactonases capable to degrade quorum signal molecules. Few simple experiments, with a promising outcome, have been already done to examine the quorum quenching activity of AHL-inactivating bacteria[23,25,29,66]. At present, the role that acylases play in their natural ecological environment still remains unclear, and the physiological function of PvdQ and QuiP in AHL degradation remains to be verified. Possibly, acylases have assigned more complex tasks than just simple degradation. Therefore, there are many questions to be answered in order to understand what *Pseudomonas*, as well other species, needs acylases for. This knowledge could give as a deeper insight into the role of acylases and into their potential role in antibiosis.

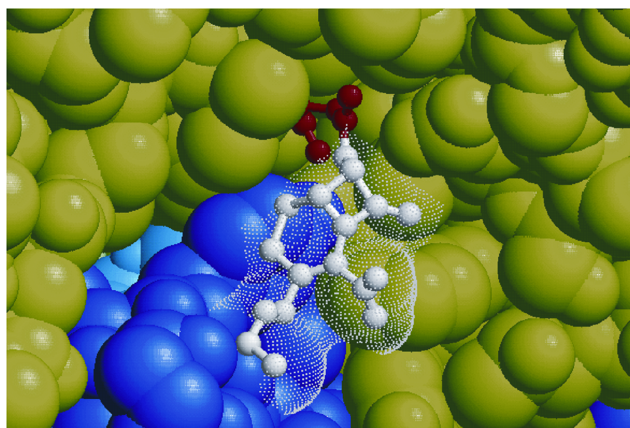

Figure 7. Solvent accessibility of glutaryl cephalosporin in complex with *Pseudomonas* SY-77 NTN-hydrolase. It can be observed that the acyl side chain (red) is fully covered by the β-chain (green) and α-chain (blue) residues, whereas the cephalosporin moiety (white) is readily accessible to the solvent (small dots).

Conclusions

Naturally occurring acylases within the *P. aeruginosa* genome, as well as within other microbial genomes, are intriguing enzymes with puzzling biological functions, yet with a clear biotechnological and pharmaceutical potential. Interfering with quorum sensing opens up to start a new era of antimicrobial therapies as an alternative to traditional antibiotics. In particular, their strength lies in a possibility to design targeted enzymes for degrading specific HSL molecules. Clearly, still more studies need to be performed in order to understand and realize what these enzymes mean for *P. aeruginosa,* and consequently what usefulness they can bring for us. The more we know about these enzymes, the more we understand about physiology of *P. aeruginosa* and the broader range of possible applications it provides. Identification and further characterization of *P. aeruginosa* acylases represent an interesting research topic for the near future and may lead us to novel therapies to fight infections.

References

1. Rolinson,G.N., Batchelor,F.R., Butterwoth,D., Cameron-Wood,J., Cole,M., Eustace,G.C., Hart,M.V., Richards,M., & Chain,E.B. Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. *Nature* 187, 236-237 (1960).
2. Balasingham,K., Warburton,D., Dunnill,P., & Lilly,M.D. The isolation and kinetics of penicillin amidase from *Escherichia coli*. *Biochim. Biophys. Acta* 276, 250-256 (1972).
3. Barbero,J.L., Buesa,J.M., Gonzalez,d.B., Mendez,E., Pez-Aranda,A., & Garcia,J.L. Complete nucleotide sequence of the penicillin acylase gene from *Kluyvera citrophila*. *Gene* 49, 69-80 (1986).
4. Verhaert,R.M., Riemens,A.M., van der Laan,J.M., van Duin,J., & Quax,W.J. Molecular cloning and analysis of the gene encoding the thermostable penicillin G acylase from *Alcaligenes faecalis*. *Appl. Environ. Microbiol.* 63, 3412-3418 (1997).
5. 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).
6. 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).
7. Brannigan,J.A., Dodson,G., Duggleby,H.J., Moody,P.C., Smith,J.L., Tomchick,D.R., & Murzin,A.G. A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature* 378, 416-419 (1995).
8. Aramori,I., Fukagawa,M., Tsumura,M., Iwami,M., Ono,H., Kojo,H., Kohsaka,M., Ueda,Y., & Imanaka,H. Cloning and nucleotide sequencing of a novel 7 beta-(4-carboxybutanamido)cephalosporanic acid acylase gene of *Bacillus laterosporus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *J. Bacteriol.* 173, 7848-7855 (1991).
9. Ishiye,M. & Niwa,M. Nucleotide sequence and expression in *Escherichia coli* of the cephalosporin acylase gene of a *Pseudomonas* strain. *Biochim. Biophys. Acta* 1132, 233-239 (1992).
10. Li,Y., Jiang,W., Yang,Y., Zhao,G., & Wang,E. Overproduction and purification of glutaryl 7-amino cephalosporanic acid acylase. *Protein Expr. Purif.* 12, 233-238 (1998).
11. Matsuda,A., Toma,K., & Komatsu,K. Nucleotide sequences of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. *J. Bacteriol.* 169, 5821-5826 (1987).
12. Matsuda,A., Matsuyama,K., Yamamoto,K., Ichikawa,S., & Komatsu,K. Cloning and characterization of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. *J. Bacteriol.* 169, 5815-5820 (1987).
13. 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).
14. Sio,C.F., Riemens,A.M., van der Laan,J.M., Verhaert,R.M., & Quax,W.J. Directed evolution of a glutaryl acylase into an adipyl acylase. *Eur. J. Biochem.* 269, 4495-4504 (2002).
15. 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).
16. 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).
17. Fuqua,C., Parsek,M.R., & Greenberg,E.P. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* 35, 439-468 (2001).
18. Gardiner,S.M., Chhabra,S.R., Harty,C., Williams,P., Pritchard,D.I., Bycroft,B.W., & Bennett,T. Haemodynamic effects of the bacterial quorum sensing signal molecule, N-(3-oxododecanoyl)-L-

homoserine lactone, in conscious, normal and endotoxaemic rats. *Br. J. Pharmacol.* 133, 1047-1054 (2001).
19. Smith,R.S. & Iglewski,B.H. *Pseudomonas aeruginosa* quorum sensing as a potential antimicrobial target. *J. Clin. Invest* 112, 1460-1465 (2003).
20. Carlier,A., Uroz,S., Smadja,B., Fray,R., Latour,X., Dessaux,Y., & Faure,D. The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, *aiiB*, also encoding N-Acyl homoserine lactonase activity. *Appl. Environ. Microbiol.* 69, 4989-4993 (2003).
21. Park,S.Y., Kang,H.O., Jang,H.S., Lee,J.K., Koo,B.T., & Yum,D.Y. Identification of extracellular N-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. *Appl. Environ. Microbiol.* 71, 2632-2641 (2005).
22. Zhang,H.B., Wang,L.H., & Zhang,L.H. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U. S. A* 99, 4638-4643 (2002).
23. Dong,Y.H., Xu,J.L., Li,X.Z., & Zhang,L.H. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. U. S. A* 97, 3526-3531 (2000).
24. 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).
25. Reimmann,C., Ginet,N., Michel,L., Keel,C., Michaux,P., Krishnapillai,V., Zala,M., Heurlier,K., Triandafillu,K., Harms,H., Defago,G., & Haas,D. Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* 148, 923-932 (2002).
26. Dong,Y.H., Wang,L.H., Xu,J.L., Zhang,H.B., Zhang,X.F., & Zhang,L.H. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* 411, 813-817 (2001).
27. Dong,Y.H., Gusti,A.R., Zhang,Q., Xu,J.L., & Zhang,L.H. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. *Appl. Environ. Microbiol.* 68, 1754-1759 (2002).
28. Lee,S.J., Park,S.Y., Lee,J.J., Yum,D.Y., Koo,B.T., & Lee,J.K. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 68, 3919-3924 (2002).
29. Park,S.Y., Lee,S.J., Oh,T.K., Oh,J.W., Koo,B.T., Yum,D.Y., & Lee,J.K. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. *Microbiology* 149, 1541-1550 (2003).
30. Rasmussen,T.B. & Givskov,M. Quorum sensing inhibitors: a bargain of effects. *Microbiology* 152, 895-904 (2006).
31. Leadbetter,J.R. & Greenberg,E.P. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. *J. Bacteriol.* 182, 6921-6926 (2000).
32. Flagan,S., Ching,W.K., & Leadbetter,J.R. *Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*. *Appl. Environ. Microbiol.* 69, 909-916 (2003).
33. Zakataeva,N.P., Aleshin,V.V., Tokmakova,I.L., Troshin,P.V., & Livshits,V.A. The novel transmembrane *Escherichia coli* proteins involved in the amino acid efflux. *FEBS Lett.* 452, 228-232 (1999).
34. Uroz,S., Chhabra,S.R., Camara,M., Williams,P., Oger,P., & Dessaux,Y. N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology* 151, 3313-3322 (2005).
35. 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).
36. Inokoshi,J., Takeshima,H., Ikeda,H., & Omura,S. Cloning and sequencing of the aculeacin A acylase-encoding gene from *Actinoplanes utahensis* and expression in *Streptomyces lividans*. *Gene* 119, 29-35 (1992).
37. Takeshima,H., Inokoshi,J., Takada,Y., Tanaka,H., & Omura,S. A deacylation enzyme for aculeacin A, a neutral lipopeptide antibiotic, from *Actinoplanes utahensis*: purification and characterization. *J. Biochem. (Tokyo)* 105, 606-610 (1989).

38. Lamont,I.L. & Martin,L.W. Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*. *Microbiology* 149, 833-842 (2003).
39. Ochsner,U.A., Wilderman,P.J., Vasil,A.I., & Vasil,M.L. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. *Mol. Microbiol.* 45, 1277-1287 (2002).
40. Neilands,J.B. Microbial iron compounds. *Annu. Rev. Biochem.* 50, 715-731 (1981).
41. Neilands,J.B. Siderophores. *Arch. Biochem. Biophys.* 302, 1-3 (1993).
42. Braun,V. & Killmann,H. Bacterial solutions to the iron-supply problem. *Trends Biochem. Sci.* 24, 104-109 (1999).
43. Briskot,G., Taraz,K., & Budzikiewicz,H. [Pyoverdin-type siderophores from *Pseudomonas aeruginosa*]. *Z. Naturforsch. [C]* 41, 497-506 (1986).
44. Meyer,J.M., Neely,A., Stintzi,A., Georges,C., & Holder,I.A. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* 64, 518-523 (1996).
45. Cox,C.D., Rinehart,K.L., Jr., Moore,M.L., & Cook,J.C., Jr. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A* 78, 4256-4260 (1981).
46. Cox,C.D. & Adams,P. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. *Infect. Immun.* 48, 130-138 (1985).
47. Liu,P.V. & Shokrani,F. Biological activities of pyochelins: iron-chelating agents of *Pseudomonas aeruginosa*. *Infect. Immun.* 22, 878-890 (1978).
48. Meyer,J.M., Hohnadel,D., Khan,A., & Cornelis,P. Pyoverdin-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdin receptor. *Mol. Microbiol.* 4, 1401-1405 (1990).
49. Heinrichs,D.E., Young,L., & Poole,K. Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *Infect. Immun.* 59, 3680-3684 (1991).
50. Budzikiewicz,H. Siderophores of fluorescent pseudomonads. *Z. Naturforsch. [C]* 52, 713-720 (1997).
51. Meyer,J.M. Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch. Microbiol.* 174, 135-142 (2000).
52. Dorrestein,P.C., Poole,K., & Begley,T.P. Formation of the chromophore of the pyoverdine siderophores by an oxidative cascade. *Org. Lett.* 5, 2215-2217 (2003).
53. Stintzi,A., Cornelis,P., Hohnadel,D., Meyer,J.M., Dean,C., Poole,K., Kourambas,S., & Krishnapillai,V. Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa* PAO. *Microbiology* 142 (Pt 5), 1181-1190 (1996).
54. Stintzi,A., Johnson,Z., Stonehouse,M., Ochsner,U., Meyer,J.M., Vasil,M.L., & Poole,K. The pvc gene cluster of *Pseudomonas aeruginosa*: role in synthesis of the pyoverdine chromophore and regulation by PtxR and PvdS. *J. Bacteriol.* 181, 4118-4124 (1999).
55. Tsuda,M., Miyazaki,H., & Nakazawa,T. Genetic and physical mapping of genes involved in pyoverdin production in *Pseudomonas aeruginosa* PAO. *J. Bacteriol.* 177, 423-431 (1995).
56. Smith,E.E., Sims,E.H., Spencer,D.H., Kaul,R., & Olson,M.V. Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. *J. Bacteriol.* 187, 2138-2147 (2005).
57. Hewitt,L., Kasche,V., Lummer,K., Lewis,R.J., Murshudov,G.N., Verma,C.S., Dodson,G.G., & Wilson,K.S. Structure of a slow processing precursor penicillin acylase from *Escherichia coli* reveals the linker peptide blocking the active-site cleft. *J. Mol. Biol.* 302, 887-898 (2000).
58. 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).
59. 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).
60. 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).
61. Sio,C.F. & Quax,W.J. Improved beta-lactam acylases and their use as industrial biocatalysts. *Curr. Opin. Biotechnol.* 15, 349-355 (2004).

62. Kim,Y. & Hol,W.G. Structure of cephalosporin acylase in complex with glutaryl-7-aminocephalosporanic acid and glutarate: insight into the basis of its substrate specificity. *Chem. Biol.* 8, 1253-1264 (2001).
63. Galan,B., Garcia,J.L., & Prieto,M.A. The PaaX repressor, a link between penicillin G acylase and the phenylacetyl-coenzyme A catabolon of *Escherichia coli* W. *J. Bacteriol.* 186, 2215-2220 (2004).
64. Hentzer,M. & Givskov,M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J. Clin. Invest* 112, 1300-1307 (2003).
65. Pearson,J.P., Feldman,M., Iglewski,B.H., & Prince,A. *Pseudomonas aeruginosa* cell-to-cell signaling is required for virulence in a model of acute pulmonary infection. *Infect. Immun.* 68, 4331-4334 (2000).
66. Uroz,S., D'Angelo-Picard,C., Carlier,A., Elasri,M., Sicot,C., Petit,A., Oger,P., Faure,D., & Dessaux,Y. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology* 149, 1981-1989 (2003).