Chapter 3: Directed evolution of a glutaryl acylase into an adipyl acylase

Charles F. Sio, Anja M. Riemens, Jan-Metske van der Laan,
Raymond M.D. Verhaert & Wim J. Quax
European Journal of Biochemistry 2002, **269**: 4495-4504

Chapter 3

Summary

Semi-synthetic cephalosporin antibiotics belong to the top ten of most sold drugs, and are produced from 7-aminodesacetoxycephalosporanic acid (7-ADCA). Recently new routes have been developed which allow for the production of adipyl-7-ADCA by a novel fermentation process. To complete the biosynthesis of 7-ADCA a highly active adipyl acylase is needed for deacylation of the adipyl derivative. Such an adipyl acylase can be generated from known glutaryl acylases.
The glutaryl acylase of *Pseudomonas* SY-77 was mutated in a first round by exploration mutagenesis. For selection the mutants were grown on an adipyl substrate. The residues that are important to the adipyl acylase activity were identified, and in a second round saturation mutagenesis of this selected stretch of residues yielded variants with a three-fold increased catalytic efficiency. The effect of the mutations could be rationalized on hindsight by the 3D structure of the acylase.
In conclusion, the substrate specificity of a dicarboxylic acid acylase was shifted towards adipyl-7-ADCA by a two-step directed evolution strategy. Although derivatives of the substrate were used for selection, mutants retained activity on the β-lactam substrate. The strategy herein described may be generally applicable to all β-lactam acylases.

Introduction

Cephalosporin antibiotics belong to the most used drugs world-wide. The total global market for this class of β-lactams is included in the top ten of most sold therapeutics, surpassing the penicillin class of β-lactams [117]. Semi-synthetic cephalosporins are industrially produced from the β-lactam nuclei 7-aminocephalosporanic acid (7-ACA) and 7-ADCA. The methods by which these intermediates are obtained have changed drastically over the past two decades (Figure 1). The original process for 7-ACA consisted of chemical deacylation of the mother compound cephalosporin C (CPC, α-D-aminoadipyl-7-ACA) from *Cephalosporium acremonium*, a costly and polluting method [23]. More recently enzymatic deacylation has been introduced. Although a one-step enzymatic deacylation [36] is not yet feasible, the combination of two enzyme-mediated reactions produces 7-ACA in a cheaper and more environmentally friendly manner. In this process D-amino acid oxidase and a glutaryl acylase perform an enzymatic deacylation of CPC (Figure 1, left, steps A and B). The other intermediate 7-ADCA is produced with penicillin G from *Penicillium chrysogenum* as the starting compound, which is converted into cephalosporin G (cephG) by an expensive and laborious chemical ring expansion reaction. Subsequent deacylation is achieved enzymatically by a penicillin G acylase (Figure 1, middle, steps C and D) [15]. The latest development in the field is the use of a genetically modified *P. chrysogenum*, transformed with an expandase gene from *Streptomyces clavuligerus* to produce adipyl-7-ADCA upon fermentation with adipate feed [16]. Deacylation of adipyl-7-ADCA cannot be done with penicillin acylases, but requires an enzyme with affinity towards the adipate side chain (Figure 1, right, step E). The currently known deacylating enzymes, however, have a low activity on this substrate.

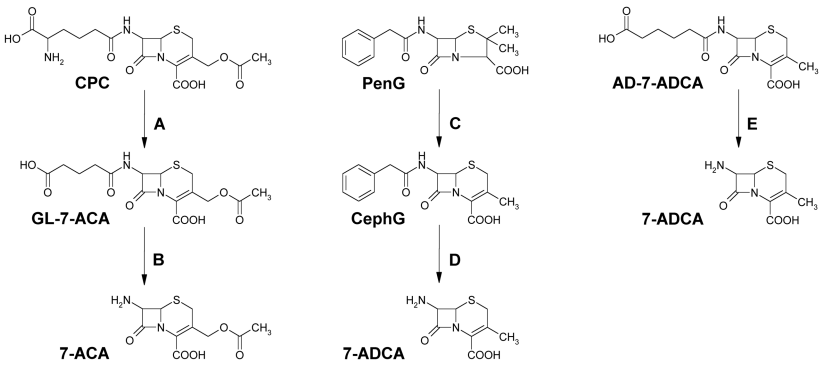

Figure 1. Production of 7-A(D)CA from various fermentation products.
7-ACA is produced from CPC by the action of D-amino acid oxidase (step A) and a glutaryl acylase (step B). 7-ADCA is produced from penicillin G by a chemical ring expansion (step C) and the action of a penicillin G acylase (step D). An alternative way to produce 7-ADCA is the bioconversion of adipyl-7-ADCA by an adipyl acylase (step E).

Hence there is a strong need for an enzyme with high substrate specificity for adipyl-7-ADCA to provide the catalyst for this novel process [60,118].
Enzymes of the β-lactam acylase family (EC 3.5.1.11) are capable of catalyzing the deacylation reaction needed to produce the β-lactam nucleus from naturally occurring β-lactams. The β-lactam acylases have traditionally been subdivided into penicillin acylases and cephalosporin acylases [119]. This classification, however, has become irrelevant since substrate specificity is determined primarily by the side chain, not by the β-lactam nucleus [15,51,120]. In our opinion, a categorization based on the side chains that are a substrate for the enzyme is to be preferred. The accepted substrates fall into one of two distinct groups: those with hydrophobic aromatic side chains and those with aliphatic dicarboxylic acid side chains. The dicarboxylic acid acylases can be subdivided into succinyl [36] and glutaryl acylases [22,26,28,31,32,35,36]. The activity of the glutaryl acylases on substrates with adipyl and α-aminoadipyl side chains varies greatly. As the glutaryl, adipyl and α-aminoadipyl side chains are all very similar, it can be envisaged that a glutaryl acylase is a good starting point for directed evolution of an adipyl acylase. Subtle changes in structure may be sufficient to allow the enzyme to better accommodate adipyl side chains, while maintaining the activity on the β-lactam substrates.
The gram-negative bacterium *Pseudomonas* SY-77, isolated from soil in 1981 [22], produces a dicarboxylic acid acylase with high activity on glutaryl-7-ACA, but low activity on adipyl-7-ADCA and no activity on CPC. The enzyme was found to be transported into the periplasm allowing a straightforward purification also at an industrial scale. It was the first dicarboxylic acid acylase to be isolated and cloned, under the name of *Pseudomonas* GK-16 glutaryl acylase [24,25]. Due to the attractive potential for industrial use, the *Pseudomonas* SY-77 glutaryl acylase was chosen to be the subject of our studies. The enzyme shows a high similarity (>90% identity) to the glutaryl acylases of *Pseudomonas* C427 [26], *Pseudomonas* sp.130 [28] and *Pseudomonas diminuta* KAC-1 [29]. The crystal structure of the latter enzyme has recently been published [51].

In this report we describe the cloning and characterization of the gene encoding *Pseudomonas* SY-77 glutaryl acylase and the characterization of the corresponding enzyme expressed in *E. coli*. A two-step directed evolution approach was developed to enhance the activity of the enzyme on adipyl-7-ADCA. It consists of exploration mutagenesis to locate the residues of the enzyme that are important to the adipyl activity, followed by saturation mutagenesis of these residues to fully explore all possible variants. Mutants were initially selected on a derivative of the substrate and later tested on the original β-lactam substrate. The strategy has led to the finding of mutants that are better catalysts for the hydrolysis of adipyl-7-ADCA. The selected mutants have been rationalized on hindsight with the aid of the crystal structure of the substrate binding site.

Materials and methods

Isolation and cloning of the gene encoding Pseudomonas *SY-77 glutaryl acylase*

Traditional cloning vectors such as the pUC series contain a β-lactamase gene, which interferes with β-lactam acylase assays. Therefore, plasmid pUNN1, which contains a neomycin resistance marker, was constructed as follows: pUB110 was digested with *Sna*BI and *Taq*I, and the 1.3 kb fragment containing the neomycin resistance gene was cloned into pUC19, which had been opened with *Sma*I and *Acc*I. A fragment of 1.3 kb was removed from the resulting plasmid by digestion with *Eco*RI and *Sca*I, and was substituted for the 1.0 kb *Eco*RI-*Sca*I fragment of pUC18. This plasmid was cut with *Pst*I, and the 1.3 kb fragment was cloned in the *Pst*I site of pUN121 [121]. This altered pUN121 plasmid was digested with *Kpn*I and *Xba*I and treated with nuclease S1 to remove the overhangs. Self-ligation yielded plasmid pUNN1.
Chromosomal DNA extracted from *Pseudomonas* SY-77 [22] was digested with *Hpa*I and *Sma*I and ligated to *Sma*I linearized pUNN1. *E. coli* HB101 cells transformed with this vector were probed with the oligonucleotide 5'-ATG CTG AGA GTT CTG CAC CGG GCG GCG TCC GCC TTG, derived from the partial sequence of the gene of *Pseudomonas* GK16 [25]. The plasmid was isolated from hybridizing colonies and partially digested with *Bam*HI and *Sma*I. Fragments of 2.6 kb were ligated into *Bam*HI-*Sal*I opened pUC18, and *E. coli* HB101 cells transformed with the resulting plasmid pUCGL-7A showed acylase activity. The 2.6 kb fragment was cloned in pTZ19R (Amersham Pharmacia, Sweden). An *Nde*I site was introduced at the ATG start codon of the open reading frame by annealing the oligonucleotide 5'-CAG AAC TCT CAG CAT ATG TTT CCC CTC TCA. The 2.5 kb *Nde*I-*Hind*III fragment was cloned in *Nde*I-*Hind*III-opened pMcTNde, a derivative of pMc-5 [122] containing a *tac* promoter [123] followed by a ribosome binding site and an *Nde*I site. This yielded plasmid pMcSY-77.

DNA sequencing and sequence analysis

The DNA sequence of the complete gene was determined in pTZ19R. For the mutants the entire DNA fragment subjected to mutagenesis has been sequenced in pMcSY-77 (Cycle sequencing [124] on a Alf Express II using ThermoSequenase fluorescent primer cycle kit, Amersham Pharmacia, Sweden). The gene encoding *Pseudomonas* SY-77 glutaryl acylase has received GenBank accession number AF458663. DNA and protein sequences were

analyzed using the software package Lasergene (DNAstar, USA). The GenBank accession numbers for the sequences used are M11436 (GK16), AF085353 (Sp.130) and AF251710 (KAC-1). The sequence of the C427 enzyme was taken from reference [26].

Mutagenesis of the gene encoding **Pseudomonas** *SY-77 glutaryl acylase*

Silent mutations yielding restriction sites in the acylase gene were introduced by the phasmid pMa/c system [122], using suitable gapped duplexes that were annealed to specific mismatch oligonucleotides.
Region directed mutagenesis of the α-subunit was performed by annealing the gapped duplex with five spiked oligonucleotides [125] of about 80 basepairs long. The oligonucleotides corresponded to the bases encoding amino acids 50-80, 81-108, 109-136, 137-164 and 165-192. Analysis of a representation of the mutant libraries showed that each transformant contained on average 0.79 point mutations in the acylase gene. It was found that on average 33% of the transformants contained 1 mutation and 14% 2 mutations. Therefore a full set of all possible single mutants requires a library size of $80x4x(100/33) = 0.97x10^3$ mutants. For each spiked oligonucleotide a library of more than $1x10^4$ colonies was plated on selective media, accounting for a >10 times representation of the single mutant library.
Saturation mutagenesis was performed by PCR with the primer 5'-GCC CAG GGT GCG GCC GGG CGA CGC NNG/C NNG/C NNG/C GAA GTT CAT CAG GCG GTG GGC GTG GGC. This resulted in the mutagenesis of amino acids 177 to 179 into all 20 possible amino acids. A library representing the full set of all possible mutants and combinations consists of $32^3 = 3.3x10^4$ mutants. Of this library $>1x10^6$ mutants were plated on selective media.

Selection of mutants on adipyl-serine

Selective media were prepared by the method of Garcia *et al* [126]. Mutated genes were cloned in the pMcTNde vector and transformed to *E. coli* PC2051 (F-; *thy*A; *ser*A; *his*; *met*G; *gal*K; *rps*L; *deo*B; λ-, obtained from NCCB, Utrecht, the Netherlands). Cells were plated on M9 minimal medium [127] containing 0.1 mg/ml adipyl-serine (LGSS, Transferbureau Nijmegen, the Netherlands), 0.2 mM IPTG, 1 μg/ml thiamine, 50 μg/ml chloramphenicol, 20 μg/ml L-histidine, 20 μg/ml L-methionine and 10 μg/ml thymine. The plates were incubated at 30ºC, and colonies emerged after 7 to 14 days. Cells growing exclusively in the presence of adipyl-serine were considered to have an acylase gene with the desired specificity on adipyl side chains. Cells expressing the WT acylase gene did not form colonies within 14 days.

Purification of SY-77 glutaryl acylase and mutants

Plasmids containing WT and desired mutated acylase genes were isolated (Plasmid Midi Kit, Qiagen Germany) and transformed to *E. coli* DH5α by standard methods [127]. 0.5-liter fermentations were done in 2xTY medium [127] containing 0.4% glucose and 50 μg/ml chloramphenicol, with the addition of 0.2 mM IPTG after 7 hours of incubation, at which time OD_{600} was approximately 1. Cells were incubated in a rotary air heated shaker at 250 rpm at 30ºC. At 24 h intervals the acylase activity of a small sample was assayed to

determine whether a sufficient amount of active enzyme had been produced. Cells were harvested after 72 or 96 h incubation, at which time the OD_{600} of the fermentation culture was approximately 7. Two 0.5-liter fermentations were combined and cells were harvested by centrifugation (10 min, 3000xg, 4ºC, RC-5B centrifuge, Sorvall-DuPont USA) and washed with 100 ml of 50mM Tris-HCl 2mM EDTA pH 8.8 ($T_{50}E_2$). The pellet was resuspended in 30 ml $T_{50}E_2$, sonicated (15 min, 40% duty cycle, output 3, 3.25 mm micro tip on a Sonifier 250, Branson USA) and the membrane fraction was removed by centrifugation (30 min, 22000xg, 4ºC). The enzyme was purified to homogeneity using ammonium sulfate precipitation and three chromatography steps. The periplasmic and cytoplasmic fraction was diluted 2-fold with $T_{50}E_2$ and ammonium sulfate was added to 35% saturation. The precipitate was discarded and ammonium sulfate was added to the supernatant to 55% saturation. The resulting precipitate containing the acylase was resuspended in 20 ml 50mM Tris-HCl pH 8.8 (T_{50}) and dialyzed (Servapor, Serva Germany) against the same buffer. The solution was then loaded on a Q-sepharose Fast Flow column (Amersham Pharmacia, Sweden) in an Econo system (Bio-Rad, USA) and eluted with a gradient of 0-0.4 M NaCl. Fractions were pooled on basis of enzyme activity and SDS-PAGE, ammonium sulfate was added to a final concentration of 0.7 M and the sample was loaded on a phenyl sepharose CL-4B column (Amersham Pharmacia, Sweden) in the Econo system. Fractions were eluted with a linear gradient of 0.7-0 M ammonium sulfate, pooled on basis of enzyme activity and SDS-PAGE, and dialyzed against T_{50}. The final purification and concentration was performed on a HiTrapQ column (Amersham Pharmacia, Sweden) on a Duoflow system (Bio-Rad, USA). Sample was eluted in a step gradient of 0, 0.25, 0.35 and 1 M NaCl. All enzyme activity was found in the 0.35 M NaCl fraction, and enzyme purity was analyzed by SDS-PAGE. The concentration of protein in all samples was determined by both the Bradford and Lowry method, since mutated tyrosine residues might interfere with the result. However, both methods gave the same protein concentrations.

N-terminal sequencing

The N-termini of both subunits were determined as follows. Purified protein was loaded on an SDS-PAGE gel with 0.4 mM thioglycolic acid (Sigma, USA) supplemented to the separating gel. After electrophoresis the protein bands were electroblotted to a polyvinylidenedifluoride membrane (Schleicher & Schuell, USA). The membrane was stained with Brilliant Blue G (Aldrich, USA) and the bands representing the α and β subunits were cut out. The amino acid sequence of the N-terminus was determined by an automated Edman degradation reaction on a Perkin Elmer / Applied Biosystems 476A system (Perkin Elmer, USA).

Enzyme assay and kinetics

Primary amino groups can be detected by fluorescamine [128]. An assay for the detection of 7-A(D)CA generated by the hydrolysis of glutaryl-7-ACA and adipyl-7-ADCA was performed essentially as described in the literature [129]. Reaction was carried out in 20 mM phosphate buffer pH 7.5 at 37ºC. Aliquots of the reaction mixture were transferred to a 0.2 M acetate buffer pH 4.5, which stopped the enzyme reaction. A stock solution of 1 mg/ml fluorescamine in water-free acetone was added to a final concentration of 0.1 mg

fluorescamine per ml detection mixture, and A_{378} was measured after 60 min on a Uvikon 923B spectrophotometer (Kontron, Italy). Values were corrected for absorption by both substrate and sample and compared to a calibration curve of 7-ACA or 7-ADCA, respectively. 1.2 mM Glutaryl-7-ACA was used as substrate for the analysis of fractions during the purification. For the determination of v_{max} and K_m on glutaryl-7-ACA concentrations of 2, 1, 0.6, 0.4, 0.2, 0.15, 0.12, 0.10, 0.08 and 0.06 mM of glutaryl-7-ACA were used. 1.5 µg of purified protein was incubated in 500 µl reaction mixture for 5 min, after which 200 µl of the reaction mixture was transferred to 520 µl of acetate buffer and 80 µl of 1 mg/ml fluorescamine solution was added. For the determination of v_{max} and K_m on adipyl-7-ADCA concentrations of 3, 1.5, 0.8, 0.6 and 0.4 mM of adipyl-7-ADCA were used. 5 µg of purified protein, or 2.5 µg of purified Y178H mutant protein, was incubated in 500 µl reaction mixture for 30 min, after which detection was performed as described for glutaryl-7-ACA. Kinetic parameters were obtained from Eadie-Hofstee plots, and the mean and standard deviation of values of at least 4 independent measurements were calculated. Values were tested for statistical significant difference by a one-sided Student's t-test with pooled variance. The k_{cat} was calculated using the theoretical molecular weight of the mature enzyme, 75.9 kDa.

Results

Isolation and characterization of the gene

The gene encoding *Pseudomonas* SY-77 glutaryl acylase was cloned into pMcTNde, and *E. coli* DH5α transformed with the resulting plasmid pMcSY-77 was shown to produce the active enzyme. The open reading frame of 2163 bases encodes a 720 amino acid protein (Figure 2). The N-terminal part of the protein matches the partial sequence of SY-77 acylase previously published by Matsuda *et al* [25] in all but 2 of 311 amino acids. The full sequence of the enzyme shows high similarity with the deduced amino acid sequences of *Pseudomonas* sp.130, *P. diminuta* KAC-1 and *Pseudomonas* C427. Notably, the similarity with the glutaryl acylase of *Pseudomonas* C427 is strongly reduced in a fragment of 91 amino acids (see Figure 2), which is solely the result of frame-shifts caused by 6 deletions scattered in a stretch of 273 basepairs in the DNA sequence of this gene. Interestingly, this frame-shift does not seem to influence the activity and range of substrates of the enzyme. We suggest that this gene should be resequenced before drawing any conclusions.

Characterization of the enzyme

A total of 2.5 mg *Pseudomonas* SY-77 glutaryl acylase was purified from 1 liter fermentation broth of *E. coli* DH5α::pMcSY-77. The purified enzyme shows two bands on SDS-PAGE, one of approximately 55 kDa and another of approximately 17 kDa (Figure 3 lane A). Some small extra bands are visible only in the boiled samples. Since they are not separated in the non-boiled sample we conclude that these are probably degradation products of the enzyme. In the non-boiled sample also a band of approximately 70 kDa shows up, which is probably the non-denatured enzyme consisting

Signal Peptide

```
SY-77 1    MLRVLHRAASALVMATVIGLAPAVAFA                                                           27
GK16  1    ···························LA                                                         29
Sp130 1    ···························                                                           27
C427  1    ········T·······A·····G··L·                                                           27
KAC-1 1    ······················G····LA                                                         29
```

α-subunit

```
SY-77 28   LAEPTSTPQAPIAAYKPRSNEILWDGYGVPHIYGVDAPSAFYGYGWAQARSHGDNILRLYGEARGKGAEYWGPDYEQTTVWL   109
GK16  30     ·················································Q······························   109
Sp130 28   LA································································································   109
C427  28   LA································································································   109
KAC-1 30     ································································································   109

SY-77 110  LTNGVPERAQQWYAQQSPDFRANLDAFAAGINAYAQQNPDDISPEVRQVLPVSGADVVAHAHRLMNFLYVASPGRTLGE      188
GK16  110  ············································D·································EG     189
Sp130 110  ············································D·································E      188
C427  110  ······························································································EGD    190
KAC-1 110  ······························································································       187
```

Spacer peptide*

```
SY-77 189   GDPPDLADQG                                                                           198
GK16  190    D········                                                                           198
Sp130 189   GD········                                                                           198
C427  191     ········                                                                           198
KAC-1 188  EGD········                                                                           198
```

β-subunit

```
SY-77 199  SNSWAVAPGKTANGNALLLQNPHLSWTTDYFTYYEAHLVTPDFEIYGATQIGLPVIRFAFNQRMGITNTVNGMVGATNYRLT   280
GK16  199  ··································································································   280
Sp130 199  ··································································································   280
C427  199  ··································································································   280
KAC-1 199  ··································································································   280

SY-77 281  LQDGGYLYDGQVRPFERPQASYRLRQADGTTVDKPLEIRSSVHGPVFERADGTAVAVRVAGLDRPGMLEQYFDMITADSFDD   362
GK16  281  ·······························                                                      311
Sp130 281  ·····································Y············································   362
C427  281  ··GD·············R···········S····················································   362
KAC-1 281  ·················R···········S·······························································H····   362

SY-77 363  YEAALARMQVPTFNIVYADREGTINYSFNGVAPKRAEGDIAFWQGLVPGDSSRYLWTETHPLDDLPRVTNPPGGFVQNSNDP   444
Sp130 363  ··································································································   444
C427  363  ····M·······················TAWR·NGPRATSPSGR·SAGPFLAL-·······WTICRASPIRRAASCRTPMIR   443
KAC-1 363  ····M········································N····································   444

SY-77 445  PWTPTWPVTYTPKDFPSYLAPQTPHSLRAQQSVRLMSENDDLTLERFMALQLSHRAVMADRTLPDLIPAALIDPDPEVQAAA   526
Sp130 445  ··········································R·······································   526
C427  444  RGRRPG·SPTR·RTS·PIWR·RRALPA·SAKRASDV-·············································   524
KAC-1 445  ············R·H····································F······························   526

SY-77 527  RLLAAWDREFTSDSRAALLFEEWARLFAGQNFAGQAGFATPWSLDKPVSTPYGVRDPKAAVDQLRTAIANTKRKYGAIDRPF   608
Sp130 527  ··································································································   608
C427  525  ··································································································   606
KAC-1 527  ····································A·············································   608

SY-77 609  GDASRMILNDVNVPGAAGYGNLGSFRVFTWSDPDENGVRTPVHGETWVAMIEFSTPVRAYGLMSYGNSRQPGTTHYSDQIER   690
Sp130 609  ··································································································   690
C427  607  ···········I······································································   688
KAC-1 609  ·····································I············································   690

SY-77 691  VSRADFRELLLRREQVEAAVQERTPFNFKP                                                        720
Sp130 691  ····························KP                                                        720
C427  689  ····························KP                                                        718
KAC-1 691  ····························                                                          718
```

Figure 2. Sequence alignment of the deduced amino acid sequence of the glutaryl acylases from *Pseudomonas* SY-77 (SY-77), *Pseudomonas* GK16 (GK16), *Pseudomonas* sp.130 (Sp130), *Pseudomonas* C427 (C427) and *P. diminuta* KAC-1 (KAC-1).

The important residues for the adipyl acylase activity are underlined (L177-V179), the active site cleft residues are double underlined. Only the first 311 amino acids of the sequence of *Pseudomonas* GK16 glutaryl acylase are known. A dot marks identity with the SY-77 acylase sequence.

*: The length of the spacer peptide is derived from C-terminal sequencing of the α-subunit and N-terminal sequencing of the β-subunit. For the SY-77 enzyme the C-terminal sequence of the α-subunit is derived from comparison.

of the $\alpha_1\beta_1$ complex (Figure 3 lane E). The N-termini of the α- and β-subunit were determined to be Leu-Ala-Glu-Pro-Thr and Ser-Asn-Ser-Trp-Ala, respectively. These observations combined with the deduced amino acid sequence and the characteristics of the known homologous acylases [26,48,49,51] indicate that the enzyme has the typical β-lactam acylase structure. The first stretch of 27 amino acids has the properties of a Sec-type signal peptide [130,131] and is absent in the mature protein. Removal of the spacer peptide of 10 amino acids leaves a catalytically active enzyme consisting of an α-subunit of 161 amino acids weighing 17.7 kDa and a β-subunit of 522 amino acids weighing 58.2 kDa, in accordance with the experimental data. No bands at the mobility of unprocessed polypeptide were seen on SDS-PAGE.

The kinetic parameters of the purified WT acylase were determined on glutaryl-7-ACA and adipyl-7-ADCA, since these are the substrates of industrial interest. The activity of *Pseudomonas* SY-77 glutaryl acylase is independent of the substitution at position 3 of the dihydrothiazine ring of the cephalosporin nucleus, i.e. activity on and affinity towards glutaryl-7-ACA and glutaryl-7-ADCA are comparable [22], which was also shown for *Pseudomonas* sp.130 [49]. Kinetic parameters were obtained by varying substrate concentration and measuring the initial rate of hydrolysis. The enzyme deacylated

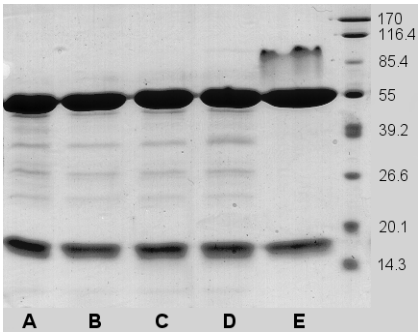

Figure 3. SDS-PAGE of purified WT and mutant *Pseudomonas* SY-77 glutaryl acylase enzymes.

Lanes: A, WT enzyme; B, mutant Y178H; C, mutant V179G; D, mutant L177I+Y178W+V179M; E, WT enzyme in sample buffer without dithiothreitol (DTT), not boiled. Each lane contains 7.5 μg sample. Marker proteins from Roche.

Table 1. Mutants obtained by exploration mutagenesis.
The number of independent isolates and the DNA sequence of all mutations are given. The data show that all codons have a single basepair mutation.

No. of mutants	Mutation in gene	Mutation in enzyme
13	**T**AT - **C**AT	Y178H
4	T**A**T - T**T**T	Y178F
4	G**T**C - G**G**C	V179G
1	**G**TC - **C**TC, **T**AT - **C**AT	V62L+Y178H
1	**C**TC**T**AT - **A**TC**C**AT	L177I+Y178H

glutaryl-7-ACA with a catalytic constant k_{cat} of 8.1 s^{-1} and with a Michaelis constant K_m of 0.08 mM. Adipyl-7-ADCA is deacylated at a lower k_{cat} of 0.65 s^{-1} and a higher K_m of 1.2 mM (Figure 4). These large differences indicate that the enzyme has a much lower specificity for the adipyl side chain, although this differs by just one CH_2 group from glutaryl.

Exploration mutagenesis of the α-subunit of the enzyme

A complete randomization of the acylase would require the construction of 20^{720} = $5.5x10^{936}$ mutants. Consequently, a two-step strategy is required in which first those residues are identified that are important to the adipyl acylase activity and, secondly, selected residues are subjected to full randomization allowing the most effective exploration of sequence space.
In order to find improved adipyl acylases this strategy was applied to the α-subunit of *Pseudomonas* SY-77 glutaryl acylase, since the α-subunit is known to be involved in the substrate specificity of β-lactam acylases [132,133]. Exploration mutagenesis was executed by inserting in total five spiked oligonucleotides into the gene by the gapped duplex method. The spiked oligonucleotides were constructed to harbor on average one point mutation each. Combined, the five oligonucleotides spanned most of the α-subunit. To select mutants that were capable of hydrolyzing adipyl substrates the mutant library was cloned in the high expression vector pMcTNde and transformed to the serine auxotrophic bacterium *E. coli* PC2051. Transformants were plated on selective plates of M9 minimal medium supplemented with adipyl-serine (Figure 5) and incubated at 30ºC. Control bacteria expressing WT enzyme could not set free serine and did not form colonies within 14 days. However, several variants in the mutant library had acquired the ability to set free serine, since the mutant library did form colonies, first visible after 7 days. All 34 colonies visible after 14 days were plated on M9 medium lacking adipyl-serine to check for amino acid revertants, which were not found. Extracted plasmid DNA was retransformed to fresh *E. coli* PC2051, and transformants were plated on M9 + adipyl-serine. The transformants of 11 mutants (32%) were unable to grow on the selective medium, indicating that they were partial revertants or that the hydrolyzing capability was located on the chromosome. These mutants were discarded. The transformants of 23 mutants (68%) did grow on the medium, indicating that the ability to hydrolyze adipyl-serine was plasmid-bound. The acylase genes of these mutants were sequenced and found to contain mutations of the following codons: Leu177, Tyr178 and

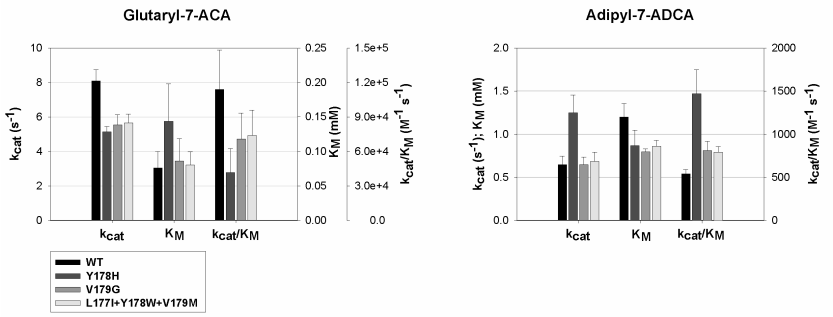

Figure 4. Kinetic parameters of WT and mutant *Pseudomonas* SY-77 glutaryl acylase on glutaryl-7-ACA and adipyl-7-ADCA.
Shown are values of mean ± S.D. of at least 4 independent measurements. Color figure at end of thesis.

adipyl substrate as is indicated by the lower K_m. The mutant Y178H in addition has a two-fold increased catalytic constant k_{cat} on adipyl-7-ADCA. On the other hand, the mutants are not improved in catalysis of glutaryl-7-ACA as shown by the lower k_{cat} of all mutants and the lower affinity of mutant Y178H for the glutaryl substrate. A parameter to compare enzymes is given by the catalytic efficiency k_{cat} / K_m. The specificity for the adipyl substrate is improved for all mutants as is indicated by the increased k_{cat} / K_m value on adipyl-7-ADCA. In contrast, the preference for the glutaryl substrate is decreased.
The catalytic efficiency of the Y178H mutant of *Pseudomonas* SY-77 glutaryl acylase has shifted from β-lactam substrates with a glutaryl side chain towards β-lactam substrates with an adipyl side chain. Both the activity on and the affinity for adipyl-7-ADCA of the Y178H-mutant enzyme have improved. The mutants V179G and L177I + Y178W + V179M have an improved affinity for adipyl-7-ADCA, however the activity is unchanged. In the selection plates the concentration of adipyl-serine is 0.4 mM, well below the determined K_m for adipyl-7-ADCA. Therefore it was possible to select mutants on basis of k_{cat} / K_m rather than just k_{cat}.

Discussion

The production of cephalosporin antibiotics requires a cost-effective process for 7-ADCA production. The fermentation product adipyl-7-ADCA can be the source of this 7-ADCA provided that a good catalyst is available for the deacylation reaction. This article describes for the first time a successful strategy for the directed evolution of such an adipyl acylase. We have been able to select several variants of *Pseudomonas* SY-77 glutaryl acylase with a two- to three-fold increased catalytic efficiency on adipyl-7-ADCA. With the creation of a good adipyl acylase a completely "green" production of cephalosporin antibiotics will become feasible, resulting in reduced pollution and lower costs. In such a process a transgenic *P. chrysogenum* produces adipyl-7-ADCA [16], which is hydrolyzed by an adipyl acylase to 7-ADCA (this study), and converted into clinically used antibiotics by a penicillin acylase [15].

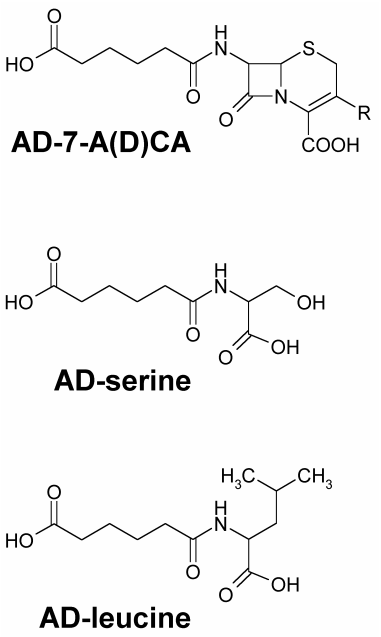

Figure 5. Structures of the adipyl-7-A(D)CA and the selection substrates.
R is methyl (7-ADCA) or acetoxymethyl (7-ACA).

We have obtained the mutants of *Pseudomonas* SY-77 glutaryl acylase by employing the very powerful combination of exploration mutagenesis and saturation mutagenesis. Exploration of limited sequence space of the complete α-subunit has lead to the identification of those residues that are important to the adipyl acylase activity. Subsequently, the complete sequence space of the selected region was explored. This yielded two improved single mutants and an improved triple mutant. The latter contains four basepair substitutions in two consecutive codons, a combination that would have been impossible to create by other mutagenesis methods.
Furthermore, this article describes a successful selection method for acylase mutants based on the growth of serine auxotrophic host bacteria on minimal medium containing adipyl-serine as the sole source of serine. A similar method was reported to be used for the selection of dicarboxylic acid acylases using leucine derivatives. However, the selected mutants had lost the activity on β-lactam substrates [80,136]. Our results prove that it is possible to select acylase mutants on derivatives while retaining the activity on the β-lactam substrate. Moreover, the mutants could also grow on adipyl-leucine (data not shown) confirming that substrate specificity is determined primarily by the side chain (Figure 5). It may well be that any amino acid linked to adipyl can be used as the selection substrate for mutant genes when using appropriate auxotrophic bacteria. Our strategy is the first working directed evolution method applicable to the β-lactam acylase family, and it can in our opinion be extended to obtain other dicarboxylic acid acylases such as an acylase for CPC.

The high similarity between the glutaryl acylases of *Pseudomonas* SY-77 and *P. diminuta* KAC-1, for which the crystal structures of the native enzyme [51] and the complexes with glutaryl-7-ACA and glutarate [59] were recently solved, allows for a structural interpretation of the changed functional properties of the mutants. The amino acids 177-179, which were selected in the exploration mutagenesis round, are the only residues of the α-subunit that are a part of the side chain binding pocket. In the structure of the enzyme complexed with glutaryl-7-ACA (Figure 6) the scissile bond of the substrate is placed at a favorable position with respect to the catalytically active serine by various interactions with the side chain and, to a lesser extent, the β-lactam nucleus. The negative charge of the carboxylate group of the glutaryl side chain is compensated for by the positive charge on the arginine Arg255. In addition, hydrogen bonds are formed with the amino groups of Arg255 and with the hydroxyl groups of Tyr178 and Tyr231. The carbon atoms of the side chain make hydrophobic interactions with residues Leu222, Val268 and Phe375. This vast network of interactions with the side chain results in a very specific side chain binding pocket, which may explain the limited substrate specificity. Whereas glutaryl-7-ACA could be accommodated quite well by the enzyme using molecular modeling, adipyl-7-ADCA could not be properly fitted due to the longer side chain (Figure 6). This could explain the observed lower activity and affinity for the adipyl substrate (see Figure 4).

In the model of the mutant Y178H the tyrosine is substituted by the smaller and more hydrophilic histidine. This expands the side chain binding pocket, allowing the scissile bond to be orientated much better with respect to the catalytically active serine, as shown in the model of the complex of the Y178H mutant and adipyl-7-ADCA (Figure 6). In this binding mode the adipyl carboxylate group can be accommodated in the generated extra space and be stabilized by hydrogen bonds with His178 and Arg255. Consequently, activity and affinity for the adipyl substrate increase. In the triple mutant L177I+Y178W+V179M the tyrosine is replaced by the more bulky tryptophan residue. It is possible to position the tryptophan side chain in such a way that the five-membered pyrrole ring more or less superimposes onto the His178 ring while the six-membered benzene ring points to the exterior. This will create additional space to accommodate the adipyl side chain, but the bulky nature of the tryptophan side chain hampers the positioning of the nitrogen with respect to the adipyl carboxylate group and prevents hydrogen bonding. In the third mutant, V179G, the introduction of a glycine at position 179 might increase the flexibility of the backbone as well as generate space for a conformational change, which may facilitate the binding of the longer adipyl chain. Such conformational changes have been observed in penicillin G acylase, in which the flexibility of the residues corresponding to Leu177 and Tyr178 plays a key role in substrate binding [137-139].

Whereas the catalytic efficiency for the adipyl substrate is increased, the catalytic efficiency of all mutants for the glutaryl substrate is decreased. This can be explained by the loss of the hydrogen bond to Tyr178 in the case of mutants Y178H and L177I+Y178W+V179M. For the V179G mutant the decreased catalytic efficiency can be explained by an altered positioning of glutaryl-7-ACA as a result of the decreased rigidity of the substrate binding pocket.

In conclusion, we could demonstrate that the introduction of a smaller, highly hydrophilic hydrogen bond donor at position 178 facilitates the processing of substrates with longer

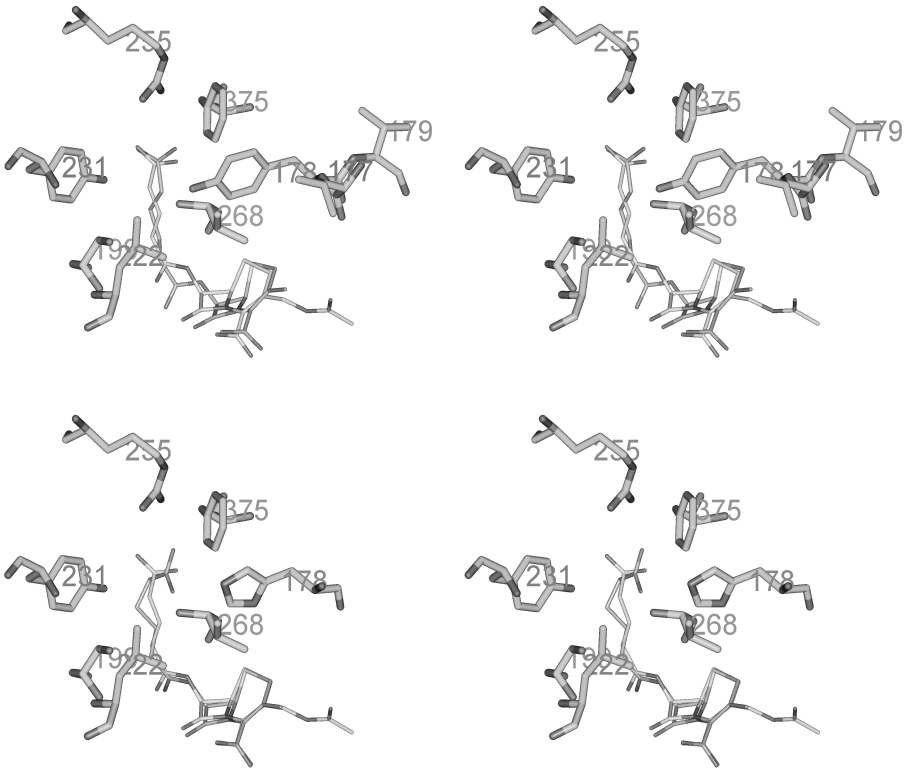

Figure 6. Models of the active site of native and mutated glutaryl acylase with bound substrates.

Modeling was performed using InsightII&Discover (Accelrys, USA) on a Silicon Graphics Octane. At the time of writing only the atomic coordinates of the free *P. diminuta* KAC-1 were available (PDB ID 1FM2). Hydrogens were added automatically and the environment of the acylase was modeled as vacuum. Models of the substrates were constructed and energy minimized using the CVFF forcefield [140]. Energy minimization was done using a dielectric constant of 1 and a non-bonded cut-off distance of 10 Å. Initially the glutaryl acylase was fixed and the atoms of the substrate were allowed to move. In subsequent rounds of minimization the constraints on the amino acids forming the active site were gradually removed and replaced by distance restraints which were based on the reported distances observed in the complex with glutaryl-7-ACA [59] . Mutations in the glutaryl acylase were modeled with Insight. Color picture at back of thesis.

Top: WT glutaryl acylase in complex with glutaryl-7-ACA (turquoise) and adipyl-7-ADCA (ochre). The nucleophile, Oγ of Ser199, is located close to the carboxyl function of the scissile peptide bond of glutaryl-7-ACA. The scissile bond of adipyl-7-ADCA is forced away from the catalytically active serine.

Bottom: The model of the Y178H mutant glutaryl acylase in complex with adipyl-7-ADCA (ochre). The structure of glutaryl-7-ACA (turquoise) is superimposed. Because of the mutation, the scissile bond of adipyl-7-ADCA is placed at a much more favorable position with respect to Ser199.

side chains. Seemingly in contrast, substitution of this residue for a small [59] or an acidic amino acid [52] was suggested to generate an α-aminoadipyl acylase from glutaryl acylase. We suggest that position 178 is needed to bind the carboxylate group of CPC, whereas the generation of extra space for the longer aliphatic chain and the binding of the amino group need to be accomplished by additional mutations. From the structural information it is clear that the active site is constituted by various regions from the α-subunit and from the β-subunit. This implies that for further improvements of the acylase on either adipyl substrates or other β-lactam side chains the β-subunit should also be subjected to exploration mutagenesis, followed by saturation mutagenesis. In order to combine the best mutations from both subunits, recombinatorial techniques for mutagenesis will be required. These experiments will be subject of further investigation.

Table 2. Five mutants of *Pseudomonas* SY-77 glutaryl acylase with improved adipyl acylase activity.
The mutants were obtained after one round of exploration mutagenesis, with selection on adipyl-serine.

Single mutants	Y178H
	V179G
Double mutant	L177I + Y178H
Triple mutants	L177I + Y178H + V179I
	L177I + Y178W + V179M

Val179 (Table 1). The double mutant V62L+Y178H was discarded, since the single mutant V62L, made from the double mutant, was found to be unable to grow under selective pressure. Apparently, amino acids 177 to 179 are important residues for the side chain specificity of acylases.

Saturation mutagenesis of the selected area

In order to obtain the best possible adipyl acylase, saturation mutagenesis was performed on the bases encoding amino acids 177-179. The mutant library, cloned in pMcTNde and transformed to *E. coli* PC2051, was grown on the selective medium containing adipyl-serine. The fastest growing mutants were checked for revertants and ten mutant acylase genes were sequenced. The already known single and double mutants, Y178H and L177I+Y178H, were found, in addition to two new mutants, L177I + Y178H + V179I and L177I + Y178W + V179M. Crude enzyme preparations of all mutants obtained in the two mutagenesis rounds were made by sonication and ammonium sulfate precipitation and assayed on glutaryl-serine using the fluorescamine assay. The activity was used to dose the sample in the assay on adipyl-serine. All mutants with the exception of Y178F showed an increased activity on the adipyl substrate. Consequently, the Y178F mutant was discarded. In total, five unique mutants with an increased activity on adipyl-serine were found after the two mutagenesis rounds (Table 2). The multiple mutants L177I + Y178H and L177I + Y178H + V179I and the single mutant Y178H had the same increase of activity on adipyl-serine. The mutants V179G and L177I + Y178W + V179M showed a different level of increase. Therefore three mutants were selected for further detailed analysis: Y178H, V179G and L177I + Y178W + V179M. Milligrams of mutant enzymes were purified from 0.5-liter fermentations of *E. coli* DH5α by the same protocol as was previously used for the WT enzyme. The purified mutants show bands on SDS-PAGE that match the pattern of the WT acylase exactly (Figure 3 lane B, C, and D). Apparently, these mutations do not affect the autocatalytic processing of the enzyme. This is surprising, since an altered processing is often observed in mutants of β-lactam acylases [134], although such mutants may still show acylase activity [135].

Substrate specificity of the selected mutants

The substrate specificity of the mutant acylases was analyzed by determining the kinetic parameters on glutaryl-7-ACA and adipyl-7-ADCA, and comparing them to the kinetic parameters of the WT enzyme (Figure 4). All mutants have an improved affinity for the