Directed Evolution of a Cephalosporin Acylase

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Changing the substrate specificity of the industrially relevant glutaryl acylase from Pseudomonas *SY-77*

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Bijna alles wat u doet is onbelangrijk,
maar het is erg belangrijk dat u het doet.

Mahatma Gandhi

Paranimfen: Charles Sio
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Chapter 1

General introduction

Antibiotics

Modern healthcare strongly depends on antibiotics. In the past decades the research for new antibiotics has yielded not only many novel compounds, but also insight in their mechanisms of action. Different antibiotics affect several essential processes in the prokaryotic cell. They can interfere with DNA, RNA or protein synthesis, or in the construction of the cell wall or membrane. One of the first identified and still most important classes of antibiotics are the β-lactam antibiotics, which interfere with cell wall synthesis. They account for about 65% of the worldwide antibiotic prescriptions and their total sales are now estimated to be US$ 15 billion [1].

History of β-lactam antibiotics

Although Alexander Fleming already discovered penicillin in 1928 [2], it was not until the second world war that penicillin G (penG) was isolated, characterised and produced at a large scale. This resulted in improved antibacterial therapy around the world. Unfortunately, since this wonderdrug killed a lot of different bacteria, it was used almost unlimited although effects on the natural equilibrium of micro organisms had not been properly investigated. Further research revealed that the instability of penG hampers easy administration and some pathogens are not as sensitive to penG. Moreover, the abundant use has resulted in the occurrence of novel variants of pathogenic bacteria resistant towards this compound. This has created the need for new β-lactam antibiotics that are more effective and more stable [3]. The substitution of the side chain of penG, phenyl acetic acid, with other side chains like D-phenylglycine and D-p-hydroxyphenylglycine, resulted in the semi-synthetic β-lactam antibiotics ampicillin and amoxicillin (Figure 1), respectively, which are more stable and can be orally administered. Substitutions at C2 of the penicillin core resulted in new β-lactam antibiotics to which resistant bacteria are sensitive.

Another step forward in the quest for new antibiotics was the use of derivatives of cephalosporin C (CPC, Figure 1). The isolation of CPC from *Acremonium chrysogenum* (formerly known as *Cephalosporium acremonium*) was first described in 1955 [4]. Although CPC can not be used clinically, since its antibacterial activity is too low in the body, it was an interesting compound because of its *in vitro* activity towards gram negative bacteria. Unfortunately, the production of CPC turned out to be much more difficult than penG since the formation of this compound in the fungus is not uniform. *A. chrysogenum* produces the closely related intermediates deacetylcephalosporin C and penicillin N in considerable amounts as well [5]. These intermediates hardly display any antibiotic action, but are difficult to separate from CPC, resulting in low yields of the desired product.

Since the antibiotic properties of cephalosporins and penicillins were similar *in vitro*, the relationship between them was readily studied and the chemical relationship

between their skeletons was discovered. In 1963 Morin *et al*. described the expansion of the five membered thiazolidine ring of penicillin G to a six membered dihydrotiazine ring, resulting in the cephalosporin core 7-aminodesacetoxycephalosporanic acid (7-ADCA) [6]. This finding was the first step towards the clinically used semi-synthetic variants of cephalosporins, cephalexin and cefadroxil (Figure 1), which have the same side chains as ampicillin and amoxicillin respectively.

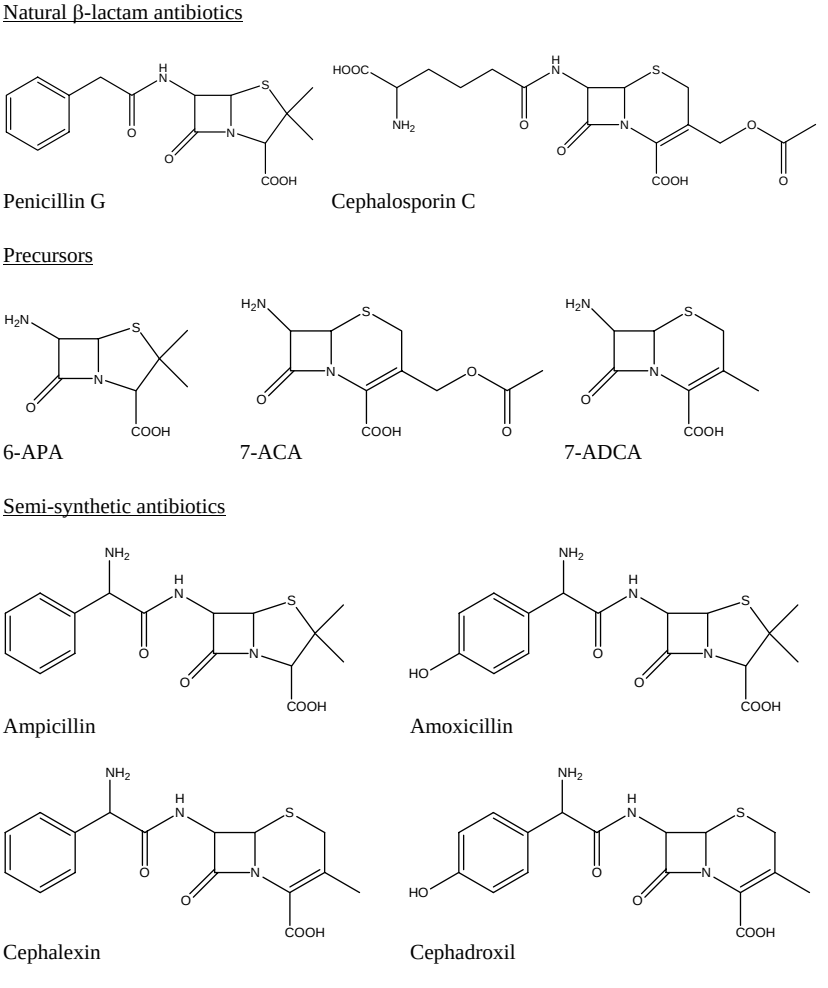

Figure 1. Some (semi-synthetic) β-lactam antibiotics and their precursors

Production of β-lactam antibiotics

The processes for large scale production of β-lactam antibiotics have gone through several drastic changes over the past 50 years. It is therefore a very nice example of the insights and achievements in biotechnology over the past decades [1,5]. Up to the 90's, the only 'bio' component in the production of these β-lactam antibiotics was the fermentation of *P. chrysogenum* and *A. chrysogenum*. All modification were accomplished by chemical synthesis. The phenyl acetyl side chain was cleaved off and the remaining 6-aminopenicillanic acid (6-APA) was protected and coupled to the appropriate side chain. In one variation to obtain cephalosporins, the five membered ring of penG was first expanded chemically to get phenylacetyl-7-ADCA using hazardous chemicals at extremely low temperatures, thereby using a lot of energy. In the early 1960's penicillin G acylase was found to hydrolyse the side chain from the β-lactam core. This process was, however, very inefficient and expensive, since the enzyme was produced at low yields and discarded after each cycle. Consequently, the chemical routes for production of β-lactam antibiotics were further optimised and used until the 1990's. At the same time, research towards biocatalytic routes remained an active field, not in the least due to the fact that the chemical approach produces a lot of waste. The production of cephalexin, for example, which has an annual consumption of about 3000 tons nowadays, generates a waste of 30-40 kg per kg of end product [5]. Since environmental issues have become more important and the cost for waste disposal has increased enormously in the last decade, it is worthwhile to look into more sustainable processes to produce semi-synthetic antibiotics.

The first enzyme to be used in the production of semi-synthetic β-lactam antibiotics was the penG acylase mentioned above. Yields of this enzyme have been highly improved thanks to genetic engineering. It was used for both the hydrolysis of the phenyl acetic side chain from penG and the synthesis of a new aromatic side chain onto 6-APA. Since the deacylation of penG is an equilibrium process, choosing the right conditions will direct the enzymatic reaction towards either hydrolysis or synthesis. A lot of research effort was put into optimising reaction conditions for the enzymatic synthesis of semi-synthetic antibiotics, but this lies outside the scope of this thesis [7-9]. The hydrolytic process became more efficient by screening different bacterial and fungal strains for the acylase and using recombinant DNA technologies in order to get a more efficient, stable and highly produced enzyme. Furthermore, fermentation conditions for the production of penicillin acylase and reaction conditions for hydrolysis of the substrate were optimised [10]. The immobilisation of the enzyme resulted in the possibility of recycling the biocatalyst and thereby reducing enzyme costs [3]. The production of the right stereo-isomer of the side chain was also optimised. By the use of an enzyme which produces only the wanted isomer of the side chain and racemisation of the non-used substrate, conversions of > 95% can be obtained [5].

Optimising production of cephalosporins has been pursued along two different routes, since both 7-aminocephalosporanic acid (7-ACA) and 7-ADCA are precursors for semi-synthetic cephalosporins, like cephalexin, cephaclor, cephuroxim, cephotaxim and cephixim. First, the production of 7-ACA from CPC produced in *A. chrysogenum* was optimised. In order to get 7-ACA, the D-α-amino adipyl side chain has to be hydrolysed from CPC. Since the chemical hydrolysis of this side chain is very expensive, extensive screening of organisms around the world was performed in order to find an enzyme capable of hydrolysing the side chain of CPC. However, this has not resulted in the identification of an efficient enzyme yet. Although different groups claimed to have isolated a cephalosporin acylase, the activities of these enzymes towards CPC are at least a factor 100 slower than towards glutaryl-7-ACA [11-13]. Therefore, a two step approach is used nowadays. First, a D-amino acid oxidase removes the amino group from the side chain. A spontaneous conversion of the resulting ketoadipyl-7-ACA into glutaryl-7-ACA supplies the substrate for the glutaryl acylase. These enzymes are also immobilised and re-used in subsequent cycles of deacylating CPC [14].

The other route towards semi-synthetic cephalosporins starts from 7-ADCA. The production of 7-ADCA out of 6-APA by ring expansion was approached. Since *A. chrysogenum* and other fungi, like *Streptomyces clavuligerus*, produce β-lactam antibiotics with 6-membered rings, the genes for this expansion process were readily discovered and cloned. Unfortunately, the produced enzymes were not capable to expand the 5-membered ring of 6-APA, penG or penicillin V *in vitro* [15]. Mutagenesis and recombination of the expansion genes towards these substrates as well as optimising process conditions were pursued and slowly led to some successes, which lie outside the theme of this thesis [16,17]. In another approach adipic acid instead of phenyl acetic acid was fed to the *P. chrysogenum* strain containing the native gene, resulting in a high production of adipyl-7-ADCA [18]. Cleaving off the adipyl chain results in a cost effective production of 7-ADCA. Although a true adipyl acylase is not available, the cephalosporin acylases mentioned above are capable of hydrolysing the adipic side chain from adipyl-7-ADCA to some extent. In order to make this process economically feasible, enzymatic reaction conditions were optimised [19].

A similar approach was used in *A. chrysogenum*. It was found that the organism produces several different β-lactam compounds because of the double activity of an expandase/hydrolase enzyme encoded by the cefEF gene. To discard the unwanted compounds, the normal route for production of CPC was blocked by inactivating the step from penicillin N to deacetylcephalosporin C, and introducing the cefE gene from *S. clavuligerus*. This gene encodes an expandase enzyme without hydrolytic activity, resulting in a more pure end product, desacetoxycephalosporin C (= amino adipyl-7-ADCA) [20].

β-lactam acylases

All β-lactam acylases mentioned above are part of the family of penicillin amidases (EC 3.5.1.11) and are found in bacteria, yeast and fungi. Several members of this large class of enzymes are widely used in industry, mainly in the production of 6-APA and the synthesis of semi-synthetic β-lactam antibiotics, but also in peptide synthesis and the resolution of racemic mixtures of chiral compounds [10].

All acylases show structural similarity and they are members of the N-terminal nucleophile (Ntn) hydrolase family [21,22]. This family consists of enzymes containing one to four heterodimers. The gene is transcribed into one propeptide which folds and cleaves itself into the heterodimer. Upon cleavage, the N-terminal amino acid of the β-subunit is liberated. This amino acid, a Ser, Thr or Cys, is the active site residue that performs the nucleophilic attack. In cephalosporin and penG acylases the N-terminal residue is a Ser. The OH-group on the side chain of this Ser attacks the peptide bond between the β-lactam core and its side chain. The free terminal NH of the same Ser and a water molecule assist in this nucleophilic attack. The side chain is released and the β-lactam core is covalently attached to the Ser. An attack of a second water molecule releases the core and brings the enzyme back to its original state (Figure 2).

β-lactam acylases are divided into several subfamilies. This classification used to be based on their substrate specificities towards β-lactam antibiotics [23]. In this classification the enzymes are roughly divided into two classes: the penicillin acylases and the cephalosporin acylases. Since the natural function of these enzymes is still not

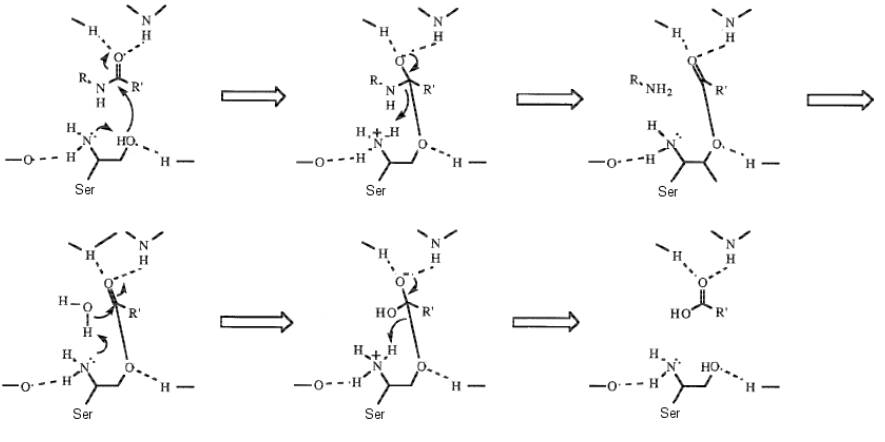

Figure 2. Catalytic mechanism of glutaryl acylase.
The nucleophilic oxygen of Ser199 donates its proton to its own α-amino group and attacks the carbon from the peptide bond of the substrate. The tetrahedral intermediate is stabilised in the oxyanion hole. The core is released upon the donation of the proton of the α-amino group to the nitrogen of the scissile bond. Then, the hydroxyl group of an incoming water attacks the carbonyl carbon of the side chain and donates its proton to the α-amino group again. The reaction is complete upon the donation of this proton to the side chain, to release it from the enzyme.
R = core (β-lactam core or leucine), R'= side chain (glutaryl, adipyl or amino adipyl).

fully understood, all cloned cephalosporin acylases are nowadays classified into five classes according to their gene structure, molecular masses and enzyme properties [11]. The enzymes in the different classes do exhibit different substrate specificities towards glutaryl-7-ACA and CPC. The activity towards CPC is rather low, only 0% to 4% relative to glutaryl-7-ACA hydrolysis. The cephalosporin acylases of class I and III show the highest activity towards CPC [24]. The enzymes of class I are transcribed into a precursor protein of 70 kDa, which comprises a signal peptide, an α-subunit of 16 kDa, a spacer peptide and a β-subunit of 54 kDa (Figure 3). The exact number of amino acids in the signal and spacer peptides differs per enzyme. The acylases that belong to this group are isolated from *Pseudomonas* SY-77 [13], *Pseudomonas* GK-16 [25], *Pseudomonas* C427 [26], *Pseudomonas* sp. 130 [27] and *Pseudomonas diminuta* KAC-1 [12] and show high similarity to each other (> 90% identity). Despite these similarities every enzyme was numbered in a different way. Since this thesis deals with the glutaryl acylase of *Pseudomonas* SY-77, the numbering of all base pairs and amino acids in it are according to the numbering of this enzyme [28]. Crystal structures of both the mature and precursor structure from *Pseudomonas* GK-16 [29] and *P. diminuta* KAC-1 [30,31] have been determined. The structures are almost identical except for the spacer peptide in the precursor structure. These crystal structures revealed that the spacer peptide is most likely autocatalytically cleaved, which was already proposed in biochemical studies. The cleavage of the side chain from glutaryl-7-ACA is a typical Ntn-hydrolysis (Figure 2). During this reaction the amino acids Asn442 and Val268 most likely form the anion hole to stabilise this reaction [30]. The substrate is positioned towards the active site serine by hydrophobic residues around the aliphatic side chain, while charged and hydrophilic residues deeper inside the enzyme interact with the negatively charged head of the substrate. The β-lactam core is positioned outside of the enzyme and seems to have very little interaction with the enzyme [32]. This explains the observation that the enzyme can hydrolyse all kinds of substrates with a glutaryl side chain, like glutaryl-7-ACA and glutaryl-6-APA, but also glutaryl-leucine and glutaryl-serine and esters with glutaryl side chains [28,33]. This feature makes the glutaryl acylase an ideal candidate to be optimised for the sustainable production of the world largest market of intermediates for antibiotics resulting in a totally “green” route by protein engineering.

Figure 3. A glutaryl acylase gene
ss, signal sequence; α, α-subunit; sp, spacer peptide; β, β-subunit

Protein engineering of biocatalysts

Enzymes are able to catalyse all kinds of chemical reactions. They can perform conversions in minutes or even seconds which would take hundreds of years without their interference, which means that they are able to enhance the rate of chemical

reactions with a factor up to 10^{17} [34]. Furthermore, they catalyse reactions which are difficult to perform by chemical methods, like the enantio- or regioselective hydrolysis or addition of chiral groups. All of these features are generally displayed at room temperature under mostly aqueous conditions. Consequently, there is a strong industrial interest in the replacement of traditional chemical processes with bioconversions. The research towards the use of biocatalysts is mainly driven by the exploration of sustainable technologies for the production of chemicals (green routes) and production of more selective and complex active ingredients in a pharmaceutical and agrobiological context [35]. This remains a big challenge, since new biocatalytic processes also have to compete economically with the well-established chemical processes which are optimised for years. Although many complicated chemical reactions can be efficiently performed by biocatalysts, nature demands different properties from enzymes than industry does. In nature most reactions occur at moderate temperatures in aqueous media, while an enzyme in an industrial process usually needs to be as stable as possible in an environment of higher temperatures, high substrate concentrations, sheering forces and organic solvents. Therefore, most enzymes found in soil and water may display the desired activity, but are generally not suited for industrial use [36]. Furthermore, for numerous industrial chemical processes an adequate enzyme can not be readily found in nature, implying that there is a need for novel biocatalysts. Enzymes with the desired activity under industrial conditions can be obtained by optimising process conditions and by protein engineering.

Rational design of new enzyme functions

The most obvious way of generating novel enzyme functions is starting from enzymes with related properties or a small activity towards the desired substrate [37]. The largest effects in protein engineering are obtained by mutating the key residues for enzyme activity, which are often present in the active site where they play a role in the actual enzymatic reaction or may be involved in stabilisation or binding of the substrate. Changing of these residues may improve the enzyme towards the wanted activity. The identification of important active site residues has been speeded up by the crystallisation of numerous enzymes in the past years. The crystal structure of an enzyme usually gives a clue about the residues that are involved in catalysis, especially when the substrate or a similar component is co-crystallised. Interactions between substrate and enzyme or between amino acids in the enzyme itself can be deduced from distances between the linked atoms. Unfortunately, crystal structures show only one possible state the enzyme can accept, which may imply that certain dynamic shapes of the enzyme will never be seen. This makes it difficult to fully comprehend the catalytic process from a single crystal structure and may lead to ignoring important residues. Nevertheless, the crystal structure of an enzyme or a close relative thereof is a good start to search for residues that should be changed in order to modify the activity of an enzyme. Modelling of the altered protein can help a researcher in choosing the right alternatives. The changes in the enzyme can be made at the DNA level by site-directed mutagenesis and the resulting

mutant enzyme can be produced and tested on the desired substrate. A lot of progress has been made in this field as can be seen in the many research articles and reviews about this subject. Unfortunately, rational design predictions do not always result in the desired activities, since the current knowledge about structure-function relation is still not good enough [38]. Crystallisation of mutant enzymes gives the opportunity to compare the new structure with the original enzyme and in an iterative approach the model and prediction of important residues can be improved. Progress is also being made in the field of bio-informatics and the algorithms of computer programs predicting the structure of a mutant enzyme are still becoming better [39,40]. It is, however, obvious that large modifications in enzyme properties can not be achieved by the change of only one or a few residues. The prediction of implications of drastic changes including several amino acids for the enzyme structure and activity, however, go far beyond our present knowledge and computational power.

Directed evolution of proteins

Many enzymes and enzyme classes are still not crystallised yet. This leaves a large number of enzymes unexplorable by rational design. Fortunately, this gap is filled by a newer branch of protein engineering: random mutagenesis. Mother nature has given us a good example how new enzymes with new activities can be evolved from existing ones. In the last decades, research has been focused on mimicking and accelerating this process in the laboratory. Since this evolution process is performed at the DNA level it was first called molecular evolution. But the technique itself is only a means to an end; the evolution of an enzyme is always directed towards a certain wanted activity by the researcher. Therefore, it nowadays is called directed evolution. All directed evolution experiments do have the same general scheme to discover the best mutant enzyme.

Methods of engineering, mutagenesis

In nature, evolution and creation of new functionalities is achieved by mutagenesis, recombination and survival of the fittest. The constantly continuing process of mutagenesis is the easiest one to copy in the lab. Mutations can be introduced at specific places using site-directed or at random mutagenesis throughout the gene. During site-directed mutagenesis an amino acid can be changed into one or more other amino acids (site-saturation mutagenesis). The mutagenesis is generally performed using oligonucleotides with altered bases. Introduction of these oligos is normally PCR based and is a common tool in DNA manipulation [41]. Different variations to this theme have also been described [42-44]. Although most site-directed mutagenesis protocols provide for the creation of only one mutation, it is not limited to this one amino acid change. Different methods to mutagenise more than one amino acid in one experiment have been developed, like QuikChange Multi Site-Directed Mutagenesis [45] and TAMS technology [46]. Although both methods claim to be highly efficient for multiple-site

mutagenesis, the efficiency of mutagenesis decreases drastically with every additional mutational site [45]. Site directed mutagenesis *in situ* can be achieved by transplacement of the mutation from template plasmids to phage DNA using cross over events [47].

Random mutagenesis can be performed *in vivo* or *in vitro*. Mutagenesis *in vivo* is performed by transforming a plasmid containing the gene to be mutagenised to a mutator *E. coli* strain. These strains lack DNA repair mechanisms [48] or contain a modified polymerase with lower fidelity [49] resulting in the introduction of mutations in total DNA during growth. The optimal growth time has to be established, since replicating plasmids for more cycles in the mutator strain will introduce more mutations in the gene of interest, but also in the chromosome, reducing viability of the bacteria. *In vitro* mutagenesis can be divided into two categories. The first strategy is based on modifications of the PCR reaction and called error-prone PCR (epPCR). Mutations are introduced by providing non-optimal conditions for a DNA polymerase, which will lead to more mismatches during extension and thus mutations in the gene of interest. The frequency of mutations introduced by this epPCR can be controlled by the amount of Mg^{2+}, by addition of Mn^{2+}, by using unbalanced or high concentrations of dNTPs, by the amount of template, by the kind of polymerase and finally by varying the extension time of the PCR. The effect of these parameters are different on every template and have to be established for each gene to be mutagenised [50]. To circumvent mutational bias, the original protocol was changed by modifying PCR conditions [51] or using a mutated polymerase which is supposed to give unbiased mutations [52]. In another *in vitro* random mutagenesis approach triphosphate derivatives of nucleoside analogues or universal bases are used in the PCR. These analogues can pair with different bases, thereby introducing all mutations possible. Mutation frequencies can be controlled by the amount and nature of nucleoside analogues and the number of PCR cycles [53,54].

Besides mutational bias there is also bias present in the coding of amino acids, because of the degeneracy in the genetic code [55]. In order to circumvent this bias, methods that are based on the mutagenesis of codons rather than single amino acids were established, like MAX [56] and RID [57]. In the latter approach up to five codons can be randomly deleted and inserted, which is also a well known feature in evolution.

Mutation frequency is an important parameter in directed evolution. More mutations increase the possibility of finding a totally new activity, but also disrupt the overall structure of the enzyme. A mutation frequency of 1-3 mutations per gene, or in case of a large gene per 1000 bps, is commonly regarded as a good rate for finding improvements of the desired activity of an enzyme. Hypermutation is thought to result in too many non-functional mutants and is therefore hardly used, although some nice results have been reported [58,59].

Methods of engineering, recombination

In nature, more radical changes in evolution originate from recombination. The crossovers between chromosomes of ones father and mother in the production of eggs and sperm, and the highly variable parts of antibodies are good examples of this

process. Almost 10 years ago, Pim Stemmer was the first one to recognise this and use it for mimicking evolution [60,61]. The technique is called shuffling and is based on the mixing and subsequent joining of different small DNA fragments in order to form a complete new gene (Figure 4). In the process of shuffling, both recombination frequency and homology are important. A high degree of recombination is important to get all possible combinations of mutations. The homology is needed for the reconstruction of a full-length gene, which will be translated into a protein that should be able to fold into a soluble and active enzyme. Since the small DNA fragments prime each other in order to become a full-length gene, they need to have an overlap of at least 14 bps [61]. This implicates that mutations which are closer together have less chance of being separated.

In order to find the best protein it is important to start from an comprehensive library. This library can be created by random mutagenesis or by using the diversity already present in nature. This last method is called family-shuffling [62] and uses similar genes from the same organism (orthologues) or the same genes from different organisms (paralogues). Since the best results are obtained with a lot of different parents which have an average homology > 80%, family shuffling is more appropriate for eukaryotic gene families, which usually have more members. In prokaryotic genomes appropriate related genes suited for gene shuffling are often absent. Recently, the exploitation of the metagenome has largely expanded the available sequence space of prokaryotic enzymes [63]. This involves the use of randomly picked sequences from soil or water and shuffling the gene of interest with homologous genes with unknown functions.

In the last decade at least 20 different methods were developed to be able to shuffle genes with lower homology, to improve mutant libraries by negative selection for wild type sequences or to get a less biased library [64] (Table 1). Most new methods have been invented to solve drawbacks and failures using the original protocol. Although these procedures aim to solve one problem, they usually appear to create another one. For example, it is difficult to start with highly different parent genes and still reach a high recombination frequency. Methods aiming at higher recombination frequencies start with more homologues parents, thereby introducing fewer mutations. So they have a smaller chance to result in enzymes with totally new activities. On the other hand, methods have been developed to recombine genes without any homology. These processes result mainly in only one crossover, although some methods do generate multiple crossovers at fixed places. Another way of obtaining a high recombination frequency is starting from synthetic oligonucleotides. The advantage of this method is that any mutant can be constructed and thereby the largest possible sequence space can be explored. An additional advantage is the possibility to use other codons than the original ones in order to obtain more homology. Furthermore, the preferred codon usage for the expression host can be applied in the synthetic oligonucleotides. The largest disadvantages of synthetic methods are the high costs and the large size of the library, which quickly exceeds the most elaborate screening and selection methods [65]. Other conflicting parameters seem to be speed and bias. Recombination methods that aim for

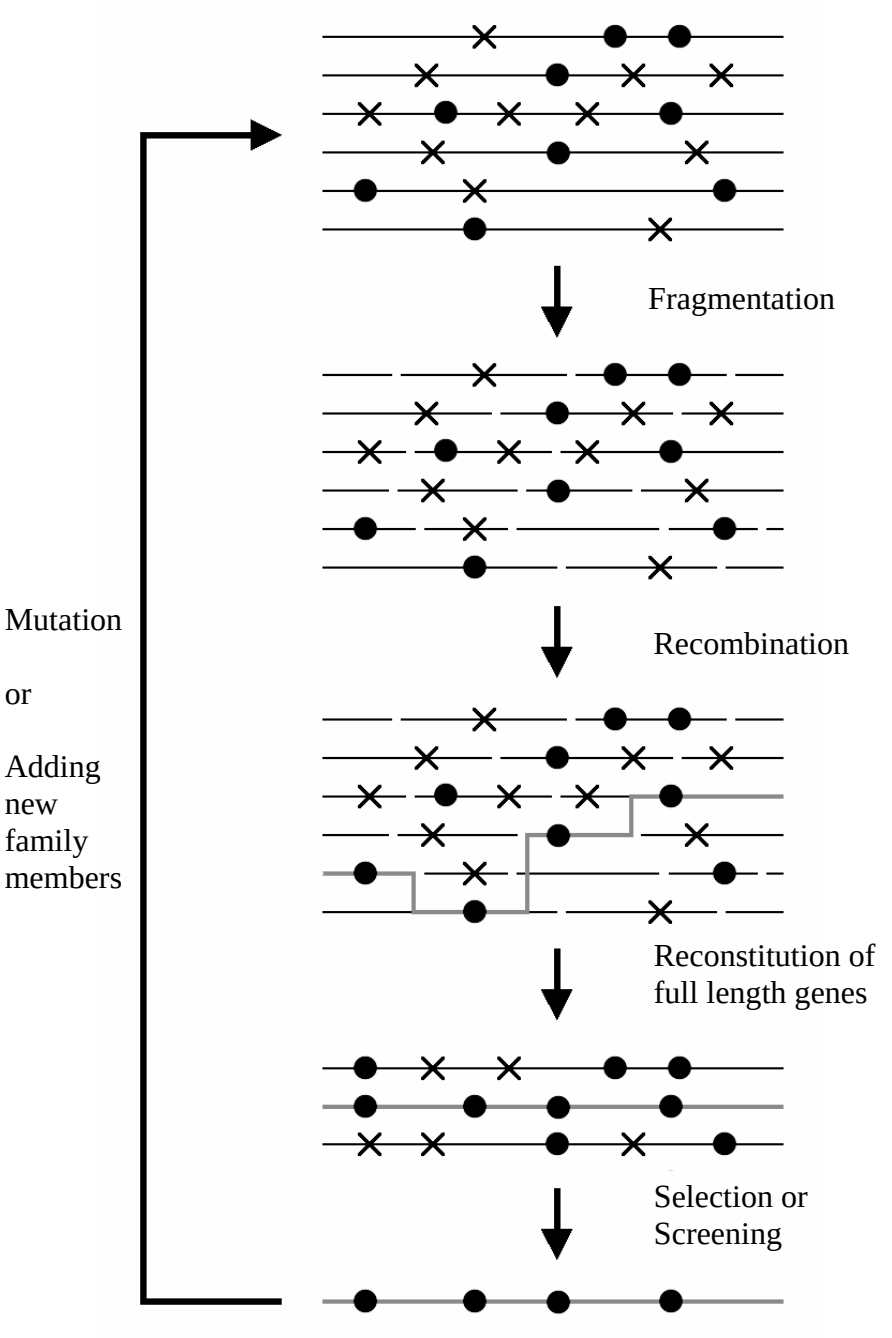

Figure 4. The basic scheme of shuffling.
The starting pool of mutant DNA can be either a mutated gene or a family of related genes.

the generation of unbiased libraries all consist of numerous steps in order to achieve this, resulting in more elaborate procedures, while quick protocols normally result in more wild type background and a biased library. Most of the mentioned methods resulted in good libraries of mutants. Therefore, the choice for one or another strategy is usually led by the size of the protein, the goal of the research, the existence of homologous proteins, the selection and/or screening capacity and practical issues like the equipment and expertise in the research group. Although many different protocols have been described in the last decades, they can be divided into six categories of underlying ideas which each have their own advantages and disadvantages (Table 1).

Table 1. Different recombination techniques with their main advantages and disadvantages

Group	Technique	Members [ref]
Shuffling	Recombination of small fragments based on homology in the sequence between mutations that stem from all kinds of mutagenesis strategies or different family members. Aims for high recombination, but difficult to separate close mutations.	Shuffling [60,61] Family shuffling [62] RE cut shuffling [75] ssDNA shuffling [76] Mn^{2+} DNase cut [77] Endonuclease V cut [78] RPR [79] RETT [80] SCRATCHY [81]
Full length parent shuffling	Recombination of small fragments from different origin using one or more full length parent strands. Higher recombination frequency, but more elaborate.	StEP [82] RACHITT [83]
Single cross over	Recombination of non homologous genes by ligating front and back of two different genes, selection of new genes on size. Recombination possible between low or non-homologous genes, but only one recombination point.	(THIO)ITCHY [84,85] SHIPREC [86] SCRATCHY [81]
Domain swapping	Recombination of structural, functional or less homologous parts of different family members. More active enzymes in the resulting library, but only a few recombination points, which are hard to find.	Exon shuffling [87] DOGS [88] SISDC [89] YLBS [90] SCOPE [91]
In vivo recombination	Recombination using the gap repair system of yeast or de recE/recT system of E. coli. High yield, since no ligation necessary, but specialised vectors and multiple steps necessary.	CLERY [92] ET-recombination [93]
Synthetic shuffling	Recombination of (un)known mutations in synthetic oligonucleotides. Recombination of close mutations possible, but expensive and good selection necessary.	Single step shuffling [94] DHR [95] Synthetic shuffling [96] ADO [97]

Selection of new enzymatic functions

The success of a directed evolution experiment highly depends on the method that is used to select the best mutant enzyme. Since most directed evolution experiments generate a huge mutant library, it is very important to develop an efficient method to search this library for the desired property. Both selection and screening strategies have been developed for all kind of enzyme functions. The big challenge in these strategies is making the improved function quantifiable. Enzymatic assays have to be sufficiently sensitive and specific to identify positive mutants [66].

Selection mimics the natural survival of the fittest strategy and is the most efficient method to find the best mutant, since only mutants of interest will appear. Unfortunately, this approach is not possible for all enzymatic activities. Selection is based on the fact that mutants with the desired enzyme function have an advantage over wild type enzymes. For *in vivo* selection this means that only enzyme activities with a growth or survival advantage can be used. Only a few industrially interesting enzymes are essential for the bacterial cell themselves, so most selection methods are based on enzymatic activities which produce a product that is essential for growth of the expression host. The coupling of the desired enzymatic reaction to survival in the selection step often requires the development of complex, nontrivial and intelligent assays [67]. Sometimes, this means that the substrates in these selection systems are not the desired substrates, but analogues thereof. This may result in the selection of undesired mutants with activity towards the analogue and not towards the wanted substrate. For example, directed evolution of penG acylase towards a glutaryl acylase was performed with a glutaryl-leucine substrate as sole leucine source for the leucine deficient bacteria. Mutants that were selected on this substrate could, however, not hydrolyse the desired substrate glutaryl-7-ACA [68]. It is, therefore, very important to carefully choose the selection substrate, since the first law of directed evolution is: “you get what you select/screen for” [69].

In vitro selection is usually based on the binding of the enzyme to the desired substrate or a transition state analogue, although strategies in which catalytic properties are used for selection are also described [70]. These methods are mostly based on a physical linkage between phenotype and genotype. The first established and most used technique is phage display, which has been successfully used for finding improved enzymes. In this system the enzyme of interest is fused to a coat protein of a filamentous phage and thereby displayed on the outside of the phage, where in principle it is able to retain enzymatic activity. Since the gene encoding the displayed protein is present in the phage particle, the gene of the mutant enzyme with the desired property is linked to its phenotype [71]. Other *in vitro* selection methods with a physical phenotype-genotype linkage are cell-surface display, ribosome display, plasmid display and mRNA-protein fusion [72]. Recently, a different approach was described to maintain a linkage between genotype and phenotype. *In vitro* compartmentalisation is a method in which compartments are formed as aqueous droplets in water-in-oil emulsions which contain

only one gene and a complete transcription/translation machinery [73]. These droplets mimic a bacterial cell by keeping the gene and its product together. The droplets containing an enzyme with the desired activity can be selected by FACS or, when the gene is physically bound to the substrate, by breaking the droplets and fishing out the desired product [74]. The advantages of *in vitro* over *in vivo* selection are the larger sample size of a mutant library and the larger amount of possible enzymatic activities to be tested. A drawback is that making the right water-in-oil emulsions with only one gene per droplet is tricky and *in vitro* translation can be a large problem

Screening for new enzymatic functions

Another way of finding the desired mutant enzyme is by screening. In screening methods all mutants have to be tested for the desired enzymatic reaction, even those that might not be active or accurately folded. The advantage is, however, that almost every enzymatic reaction can be tested, since the activity does not have to be dependent on growth rate or the formation of essential products. This can be done in a qualitative way by relatively simple visual screens such as the formation of coloured or fluorescent products or halos around a colony on a plate. For protein functions such as catalysis of a specific reaction or substrate specificity this is very difficult or even impossible. Quantitative methods are more suited to screen for these enzymatic activities, but are usually more elaborate. This implicates that in a normal time scale only small libraries can be tested (up to 10^5) or high throughput screening (HTS) has to be employed. HTS demands miniaturisation and automation of enzymatic assays. In the past decade a lot of research has been focussed on finding better, cheaper, quicker and more accurate HTS assays [98]. This has made HTS feasible for many laboratories all over the world now, resulting in a lot of smart enzymatic screening methods.

A screening method is used to find the best enzyme out of a large pool of mutants. The resulting amount of clones depends on the accuracy of the assay and should be optimised during the directed evolution experiment. For this optimisation one should consider the size of the library, the amount of (false) positive and false negative colonies that can be allowed, the costs of the assay and the possibility of performing a more accurate assay with the best mutants. Most screening assays are based on spectrophotometric methods in 96 or 384 wells plates [66]. Usually raw cell extracts are incubated with the substrate or an analogue thereof, which will give a coloured product. Another possibility is the use of a discontinuous assay in which the product, but not the substrate, is coloured by another chemical, like fluorescamine [99]. Yet another screening method based on coloured products is a spectroscopic colony screening technology. Colonies are grown on a membrane and transferred to the substrate in the presence of a colouring agent, resulting in blue spots. A digital camera can follow the colour formation as a function of time. Throughputs of ~50.000 micro colonies per membrane have been demonstrated [98]. More HTS methods are developed every day, both for specific enzymatic reactions and general applications, making screening the method of choice for many researchers.

Aims and outline of the thesis

This thesis describes the directed evolution of the cephalosporin acylase from *Pseudomonas* SY-77. The objective of this directed evolution experiment was to change the substrate specificity of the enzyme from hydrolysis of glutaryl-7-ACA towards hydrolysis of adipyl-7-ADCA or even CPC in order to obtain 7-ADCA and 7-ACA, which are key intermediates in the production of semi-synthetic β-lactam antibiotics. In order to become industrially relevant, the hydrolysis activity of the enzyme towards adipyl-7-ADCA or CPC should at least be the same as the activity towards glutaryl-7-ACA of the wild type enzyme.

To achieve this goal, we started with the randomisation of the β-subunit by epPCR to find amino acids important for substrate specificity. For this directed evolution experiment both a new selection strategy and a robotic screening assay had to be developed. The positions of the selected mutant residues were examined in the crystal structure of the highly homologous cephalosporin acylase from *P. diminuta* KAC-1. This pointed our attention towards single residues that are important for the substrate specificity of the enzyme. These amino acids were picked for a site-saturated mutagenesis approach in order to explore their contribution to substrate specificity. As a final approach, multiple mutants were constructed using both rational and random strategies: the best mutant from the α-subunit was combined with several improved mutants of the β-subunit, a library of two totally randomised residues was created and a total randomised library of all five important substrate specificity residues was constructed.

Throughout the thesis both rational and random mutagenesis strategies are used to find an industrial applicable adipyl- or CPC-acylase. The results presented here, point to the conclusion that this is the right way to improve biocatalysts.

Chapter 2

Altering the Substrate Specificity of Cephalosporin Acylase by Directed Evolution of the β-Subunit

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Using directed evolution, we have selected an adipyl acylase enzyme that can be used for a one-step bioconversion of adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA) to 7-ADCA, an important compound for the synthesis of semi-synthetic cephalosporins. The starting point for the directed evolution was the glutaryl acylase from *Pseudomonas* SY-77. The gene fragment encoding the β-subunit was divided into 5 overlapping parts that were mutagenised separately using error-prone PCR. Mutants were selected in a leucine-deficient host using adipyl-leucine as the sole leucine source. In total, 24 out of 41 plate-selected mutants were found to have a significantly improved ratio of adipyl-7-ADCA versus glutaryl-7-ACA hydrolysis. Several mutations around the substrate-binding site were isolated especially in two hotspot positions: residue Phe375 and Asn266. Five mutants were further characterised by determination of their Michaelis-Menten parameters. Strikingly, mutant SY-77^{N266H} shows a nearly 10-fold improved catalytic efficiency (k_{cat}/K_m) on adipyl-7-ADCA, resulting from a 50 % increase in k_{cat} and a 6-fold decrease in K_m, without decreasing the catalytic efficiency on glutaryl-7-ACA. In contrast, the improved adipyl/glutaryl activity ratio of mutant SY-77^{F375L} mainly is a consequence of a decreased catalytic efficiency towards glutaryl-7-ACA. These results are discussed in the light of a structural model of SY-77 glutaryl acylase.

Introduction

Semi-synthetic cephalosporins and penicillins are the most widely used antibiotics. All clinically important semi-synthetic cephalosporins are made from 7-amino-cephalosporanic acid (7-ACA) or 7-aminodesacetoxycephalosporanic acid (7-ADCA). 7-ACA is derived from cephalosporin C (aminoadipyl-7-ACA), which is obtained by fermentation of the fungus *Cephalosporium acremonium*. Deacylation is performed either chemically or by a two-step enzymatic process using a D-amino acid oxidase and a glutaryl acylase. The latter enzyme can be found in several *Pseudomonas* and *Acinetobacter* species [13,26,27,100-103] as well as in some Gram-positive bacteria [104,105]. 7-ADCA is produced from penicillin G made by *Penicillium chrysogenum* involving several polluting chemical steps followed by enzymatic deacylation by penicillin acylase [106]. A first step towards the introduction of a simplified, more environmentally friendly production of 7-ADCA was the development of a genetically modified *P. chrysogenum* that produces adipyl-7-ADCA [18]. For the deacylation of this novel β-lactam, an adipyl acylase is needed. Since the presently identified acylases show little or no activity towards adipyl-7-ADCA, it is of interest to investigate whether a glutaryl acylase can be converted into an adipyl acylase.

In the past few years, directed evolution has been successfully implemented in changing the substrate specificity of several enzymes [107,108], resulting in biocatalysts with novel activities. It has become clear that the success of a directed evolution experiment greatly

depends on the availability of a good selective substrate, which unfortunately is absent for most bioconversions [107]. Artificial substrates that mimic one of the desired catalytic steps may be used for selection; however, it is not clear to what extent the resulting mutants will have lost activity on their natural substrate. Here we describe a strategy to evolve the glutaryl acylase of *Pseudomonas* SY-77 into an adipyl acylase with an improved activity toward adipyl-7-ADCA. The glutaryl acylase from *Pseudomonas* SY-77 has proven to be particularly suitable for developing an industrial process for deacylation [14]. The natural action of the enzyme seems to be directed at hydrolysing di-amino acids with a glutaryl side chain as judged from its high activity on glutaryl-7-A(D)CA. It appears that the enzyme also has a low activity on adipyl-7-ADCA but no activity on cephalosporin C [13].

Since deacylation of β-lactam compounds cannot be used for a growth selection, we took advantage of the di-amino hydrolysing capability of the acylase by replacing the β-lactam moiety with leucine, a compound that can be selected for in a leucine-deficient *Escherichia coli* host strain [109]. In this way, only enzymatic hydrolysis of adipyl-leucine allows for growth on minimal medium. To obtain the desired acylase variant, we have constructed five libraries of overlapping gene fragments of the β-subunit of SY-77 by error-prone PCR (epPCR) and used these libraries separately in the selection procedure. Transformants that prevailed in growth were further characterised and tested for their activity on glutaryl-7-ACA and adipyl-7-ADCA. Mutants with an improved growth capability on the selection substrate also showed an improved activity toward the β-lactam substrate. Mutations were found to accumulate in the proximity of the substrate binding pocket. Frequent mutations were identified at positions Asn266 and Phe375. Crystallographic models have pointed at the role of Phe375 in the determination of substrate specificity, whereas Asn266 was not mentioned before. Strikingly, mutation N266H induces an 8-fold improved catalytic efficiency (k_{cat}/K_m) on adipyl-7-ADCA. The results are discussed on the basis of the three-dimensional structure of SY-77 glutaryl acylase.

Materials and Methods

Bacterial strains and plasmids and DNA manipulations

The plasmid pMcSY-2 was constructed by the introduction of four silent mutations into pMcSY-77 (GenBankTM accession number AF458663) [28], resulting in four additional restriction sites in the gene encoding the SY-77 acylase. The plasmid pMcSY-2 and the leucine-deficient *E. coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the SY-77 acylase. The plasmid pMcSY-YH [28] was used as a positive control for the selection and screening procedure. This plasmid encodes a mutant acylase, carrying the mutation Y178H in the α-subunit, which has a three times better catalytic efficiency (k_{cat}/K_m) than wild type in the hydrolysis of adipyl-7-ADCA.

Molecular DNA techniques were executed following standard protocols [41]. The enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied

according to the instructions of the manufacturer. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen).
Interesting multiple mutants, which were found, were separated in order to pinpoint the mutation responsible for the improved enzymatic activity. In case a suitable restriction site was available, this was used to separate the mutations. Otherwise, single mutants were made by site-directed mutagenesis using PCR. In both cases, the mutated parts of the genes were sequenced on both strands afterwards.

Construction of the random mutant libraries

For the epPCR, the gene fragment encoding the β-subunit of the SY-77 acylase was divided into five partly overlapping regions using seven unique sites in the plasmid (Figure 1). Each region was mutagenised by PCR amplification under error-prone conditions [50]. The concentration of Mg^{2+} and the annealing temperature that gave the highest PCR yield were used. Using these conditions, a pilot experiment on part I was performed in order to determine the mutation rate and distribution under different concentrations of Mn^{2+}.

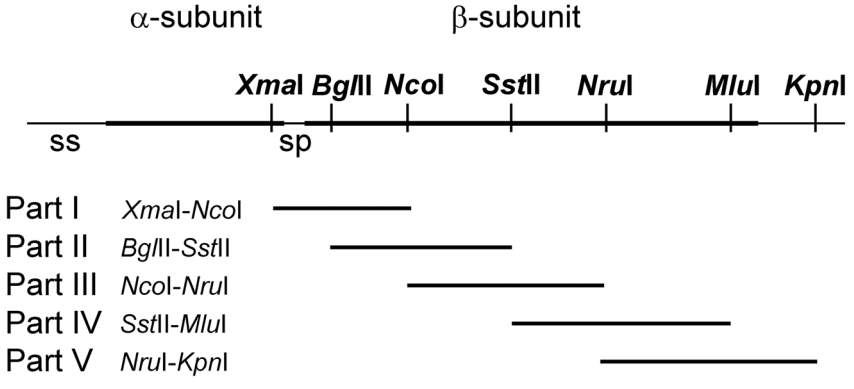

Figure 1. Linear view of the SY-77 acylase gene.
The five parts subjected to epPCR are given below the gene. Unique restriction enzymes used to clone the epPCR parts back into the gene are given above the gene. ss, signal sequence; sp, spacer peptide.

The PCRs were performed in aliquots of 25 µl containing 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 or 5 mM $MgCl_2$, 0.1–0.5 mM $MnCl_2$, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 1 ng of pMcSY-2, 1.25 U recombinant *Taq* DNA polymerase (Invitrogen), and 0.3 µM of each primer. The following primers (from 5' to 3' including underlined restriction sites) were used: part I, tcgcatcgcccgggcgca and ctcgaagaccgggccatgga; part II, cgccggacttcgagatcta and gattggtgaccgcggca; part III, ccgctccagcgtccatggc and tgtagctggtgaactcgcgat; part IV, ggacgatctgccgcgggt and tcggctcgcgacacgcgtt; part V, gcggcgtgggattcgcgag and cagagcgcccggtaccgaaa.
All PCRs were performed in a Mastercycler® gradient thermocycler (Eppendorf) using the following program: 3 min at 95°C, 20 cycles of 45 s at 95°C, 30 s at the annealing temperature, 45 s at 72°C, and a final step of 10 min at 72°C. PCR product and wild type vector were subjected

to a restriction reaction using the suitable enzymes followed by electrophoresis. After the product of the correct size was excised and purified, ligation was performed overnight at 4°C using T4 DNA polymerase (New England Biolabs). 40 µl of ElectroMAX™ DH10B™ Cells (Invitrogen) were transformed with 1 µl of each mutant library, following the protocol of the manufacturer. A 0.1-cm cuvette was used in a Bio-Rad Gene Pulser unit, which was set at 1.8 kV, 25 µF, 200 ohms. A small aliquot of the transformation mixture was plated onto LB agar containing 50 µg/ml chloramphenicol and 0.4% glucose to determine the transformation efficiency. The rest of the transformation mixture was shock-frozen in liquid nitrogen in 10% glycerol and stored at -80°C. Of each library, 20 clones were picked randomly for the analysis of the mutational efficiency.

Selection on agar plates

Cells were slowly thawed on ice and washed twice with 0.9% NaCl. They were starved by incubation in 0.9% NaCl for 2 h at 37°C and plated onto selective minimal medium plates AD-Leu (M9; 50 µg/ml chloramphenicol, 0.1% glycerol, and as a sole leucine source 0.1 mg/ml adipyl-leucine; LGSS Transferbureau Nijmegen, The Netherlands). Approximately 5000 viable *E. coli* DH10B cells containing the mutant plasmids were spread onto each plate. Of each library, a total of 10^5 transformants was plated. Cells were also plated onto control plates: GL-Leu (0.248 mg/ml glutaryl-leucine.2DCHA; Bachem), MLeu (0.04 mg/ml L-leucine; Sigma), and Min (no leucine at all). As a "positive" and a "negative" control, *E. coli* DH10B cells producing SY-77^{Y178H} and SY-77 wild type, respectively, were used. Plates were incubated at 30°C for at least 10 days. Every day new appearing colonies were marked. Colonies were selected on the basis of their date of appearance and size and streaked again on AD-Leu plates to ensure that unique colonies were obtained. Single colonies from these second plates were used to inoculate an overnight culture to determine acylase activity and to isolate plasmid DNA for sequence determination.

Determination of enzyme activity

10 ml of LB containing 50 µg/ml chloramphenicol and 0.1% glycerol was inoculated with 0.1 ml of an overnight culture and incubated at 30°C for 24 h. Cells were harvested (20 min, 3500 rpm) and incubated for 45 min in one-fifth volume of BugBuster (Novagen) at 20°C while vigorously shaking. Cell debris was removed by centrifugation (30 min, 13,000 rpm), and cell free extracts were stored at -20°C.

Enzyme activities were determined on glutaryl-7-ACA and adipyl-7-ADCA using the fluorescamine assay [99] in a 96-well format. All pipetting steps were performed by a Multiprobe II (Canberra Packard). 8 µl of cell free extract was mixed with 92 µl of 20 mM phosphate buffer, pH 7.5, and preheated at 37°C. The reaction was started by the addition of 100 µl of substrate in phosphate buffer, with a final concentration of 2 mM glutaryl-7-ACA or 5 mM adipyl-7-ADCA. After 60 and 120 min incubation at 37°C, an aliquot of 40 µl of reaction mixture was transferred to 140 µl of 0.5 M acetate buffer, pH 4.5, after which 20 µl of 1 mg/ml fluorescamine in acetone was added. After 60 min incubation at room temperature, the A_{380} was measured. Reaction mixtures without substrate or cell free extract were used to correct for absorption of the different reaction mixture components at 380 nm. Activities are given as the ratio between the hydrolysis of adipyl-7-ADCA and glutaryl-7-ACA (AD/GL) to account for variations in acylase concentration in the cell free extracts. The ratios were determined after 60 and 120 min of incubation. Values were accepted if they were consistent, and the average of these two ratios was used in further calculations. The mean and the S.D. of the ratio found in at least three independent

measurements were calculated.
Mutants with an AD/GL ratio of at least 150% of that of the wild type SY-77 acylase were purified as described below. The kinetic parameters of these mutants were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme using the Multiprobe II for all pipetting steps. 140 µl of 20mM phosphate buffer, pH 7.5, with substrate (0.06–2 mM glutaryl-7-ACA or 0.2–10 mM adipyl-7-ADCA) was preheated at 37°C. 40 µl of phosphate buffer containing an appropriate amount of purified enzyme was added, starting the reaction. After 10 min of incubation at 37°C, 40 µl of 2.5 M acetate buffer, pH 4.5, was added to stop the reaction. 20 µl of 1 mg/ml fluorescamine in acetone was added, and the A_{380} was measured after 60 min incubation at room temperature.
Kinetic parameters were obtained by fitting the experimental data from Eadie-Hofstee plots, and the mean and S.D. values of at least four independent measurements were calculated. The k_{cat} was calculated using the theoretical molecular mass of the mature enzyme, 75.9 kDa.

Enzyme characterisation

Protein concentrations of the cell free extracts and purified samples were determined using the DC Protein Assay (Bio-Rad) in a 96-well plate with bovine serum albumin as the reference protein.
The cell free extracts were analysed by SDS-PAGE [110]. The 12.5% gel was stained with Coomassie Brilliant Blue. Identical samples were electroblotted onto a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit antibody against *Pseudomonas* SY-77 glutaryl acylase (Eurogentec S.A.) and subsequently with an alkaline phosphatase-conjugated goat anti-rabbit antibody. Bands corresponding to *Pseudomonas* SY-77 glutaryl acylase were coloured with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.
Mutant and wild type enzyme were purified by three chromatography steps on a Duoflow system (Bio-Rad) using columns from Amersham Biosciences. *E. coli* DH10B containing the plasmids encoding the desired enzymes were grown for 24 h at 30°C in 300 ml of 2*YT medium supplemented with 50 µg/ml chloramphenicol and 0.1% glycerol. Cell free extract was made by sonication (10 min, output 4, 50% duty cycle on a Sonifier 250; Branson) and centrifugation (30 min at 14,000 rpm) and loaded onto a HiTrapQ column. The protein was eluted with a linear gradient of 0-1 M NaCl in 50 mM Tris-HCl, pH 8.8. After analysis on SDS-PAGE, the fractions containing the enzyme were loaded onto a HiTrap phenyl-Sepharose HP column. The protein was eluted with a gradient of 0.7-0 M $(NH_4)_2SO_4$. The pooled fractions containing the enzyme were desalted on a HiPrep 26/10 desalting column and reloaded onto a HiTrapQ column. After rinsing with 220 mM NaCl, the protein was eluted with 330 mM NaCl. The pooled fractions were stored at -20°C. Typical yields were 10 mg of more than 90% pure enzyme per litre of culture.

Results

Constructing the epPCR libraries

The conditions for the construction of the libraries were optimised. The highest PCR yields were obtained using 2.5 mM Mg^{2+} and an annealing temperature of 60°C for parts I, III, and V and 5 mM Mg^{2+} and 66°C for parts II and IV. Part I was subsequently mutagenised in the presence of 0.1-0.5 mM Mn^{2+}. A Mn^{2+} concentration of 0.5 mM

resulted in the desired mutation frequency of 0.7%, corresponding to approximately three base pair mutations per mutant. Since the relatively high GC content (67%) is equally distributed throughout the gene, we used 0.5 mM Mn^{2+} to mutagenise all parts. EpPCR gave a band of the correct size on an agarose gel for all five parts. Although both vector and insert were isolated from an agarose gel, cloning of the epPCR fragments into pMcSY-2 resulted in a significant part of contamination of parts II, III, and V with wild type sequences. Sequencing of at least 400 base pairs of 20 randomly picked clones from each bank revealed that all mutations were equally distributed throughout the gene (Figure 2). The mutational frequency of the different libraries varied from 0.2% for parts III and V to 0.7% for part I, resulting in 1-3 base pair mutations per mutant. The mutational bias was comparable with experiments under similar error-prone conditions as described before [111], although X → C (X ≠ C) changes were lower than expected.

Since the transformation efficiency was not uniform for all five parts and plating of an equal amount of mutants onto each plate was desired, different volumes of the original transformation mixture were plated. All five libraries appeared to contain at least 10^5 transformants, which is enough to harbour every single mutant at least once [74].

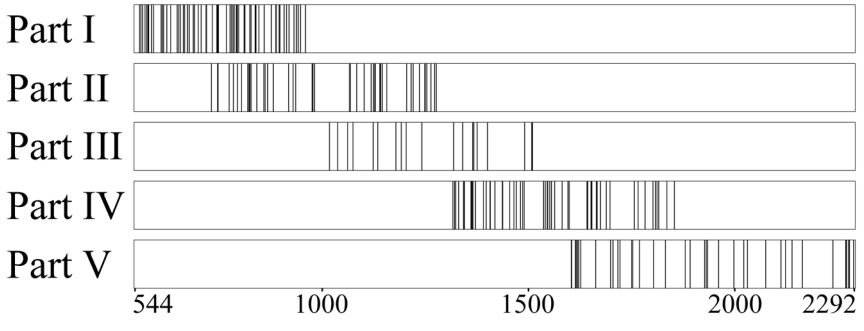

Figure 2. Distribution of mutations over the gene.
Of each epPCR part 20 clones were randomly picked and sequenced. Every identified mutated base is indicated by a line according to its position on the SY-77 gene.

Selection on minimal medium containing adipyl-leucine

Transformation mixtures were plated onto minimal plates in the absence of leucine (Min) or in the presence of leucine (MLeu), adipyl-leucine (AD-Leu), or glutaryl-leucine (GL-Leu). On each plate 5000 mutant plasmid-bearing bacteria were plated. As a reference, *E. coli* DH10B cells producing SY-77 wild type and SY-77^{Y178H} were used. Plates were examined every day, and colonies were marked and closely watched during the next days. After 3-4 days, small colonies appeared on all plates, the ones on Min and AD-Leu plates being somewhat smaller than those on MLeu and GL-Leu plates. On the Min plates, the spots did not grow upon further incubation, whereas colonies on the MLeu and GL-Leu plates increased in size until the 10th day. On the AD-Leu plates,

colonies of the positive mutant producing SY-77^{Y178H} increased in size until the 6th day, whereas the wild type strain colonies grew slowly but steadily until the 10th day. Colonies of the libraries grew at different rates.

On the 10th day, a range of small and large colonies was present on each plate containing leucine of any kind. The average size of the colonies on the AD-Leu plates from *E. coli* DH10B containing the library parts IV and V or the negative control pMcSY-2 was smaller than the average size of the colonies from the other mutant libraries and the bacteria bearing the positive mutant plasmid pMcSY-YH. The size and day of appearance were used as criteria to select colonies of every epPCR part. These colonies were again streaked onto AD-Leu plates to isolate single colonies. Colonies of cells producing SY-77 wild type and SY-77^{Y178H} were also plated onto this medium. Most colonies were selected from the plates of parts I and II. Of the 54 colonies picked, 50 grew on the second AD-Leu plate after five days. Plasmid DNA was isolated from overnight cultures of the putative mutants from the secondary plate and retransformed to *E. coli* DH10B. DNA sequence determination of these plasmids showed that eight plasmids had the wild type sequence. Furthermore, some mutants proved to have identical sequences, leaving 29 unique transformants with mutations throughout the whole gene. However, most mutations occurred in the first 200 amino acids of the β-subunit, which corresponds to the fact that most mutants were selected from the libraries of parts I and II, encoding the first 230 amino acids.

Activity measurements

A 24 hour culture of every mutant was harvested, and cell free extracts were made using BugBuster. Hydrolysis of both adipyl-7-ADCA and glutaryl-7-ACA were measured in triplicate in a medium throughput robotic assay. Several precautions were taken in order to maintain the reliability of the assay. To compensate for differences in acylase concentration in the cell free extracts, the ratio AD/GL was used to characterise the mutants. Furthermore, all measurements were done using the same amount of cell free extract, even those with lower protein concentrations, since BugBuster appeared to affect the hydrolysis of the adipyl substrate more than the glutaryl substrate. In some cases, this led to very low A_{380} values that did not differ significantly from the blank values. In all cases, the production of acylase was verified by blotting the cell free extract (data not shown). Some bacteria proved to produce no acylase at all, whereas other bacteria produced about three times less acylase, resulting in such a low enzyme activity that the ratio was not determinable. Amino acid mutations and activities of all 29 different mutants are given in Table 1.

The results from Table 1 strongly suggest that Asn266 is an important residue for substrate specificity. 22 of the 36 selected and sequenced transformants from parts I and II contain a mutation at this position. The two single mutants N266H and N266S have an AD/GL ratio of 0.19 and 0.14, respectively. Multiple mutants that were found comprising these mutations show the same ratio, implying that the additional mutations

Table 1. The AD/GL ratio of the selected mutants.

Mutants of the five epPCR libraries were selected on minimal medium with adipyl-leucine as sole leucine source. DNA sequences of these selected mutants were determined. Furthermore, cell free extracts were made using BugBuster (Novagen). The activity of the cell free extracts from all mutants on adipyl-7-ADCA and glutaryl-7-ACA was determined in a robotic assay using fluorescamine. The ratio of these values (AD/GL) is used as a measure for the improvement of the enzyme towards adipyl-7-ADCA. The mean +/- S.D. ($n \geq 3$) is given. All selected transformants from Part V appeared to have wild type sequence. Multiple mutants were genetically separated and both DNA sequence and AD/GL ratio were determined.

Part	Mutation	Activity (AD/GL)
I	N266H (2x)	0.19 ± 0.01
	N266H (2x)	N.D.[1]
	N266H, D308N, S320Y	0.19 ± 0.01
	N266H, K314R	0.19 ± 0.00
	N266H, E317V	0.19 ± 0.01
	N266H, S320T	0.19 ± 0.00
	F229L, V237A	0.15 ± 0.01
	F229L[2]	0.15 ± 0.00
	V237A[2]	0.08 ± 0.00
	N266S (2x)	0.14 ± 0.01
	S223P, M271L	0.11 ± 0.00
	M271L[2]	0.12 ± 0.00
	A246V	0.11 ± 0.01
	D193V	0.09 ± 0.00
	Q291L	0.09 ± 0.01
	Q291R	0.09 ± 0.01
	S223P	0.08 ± 0.00
	G207*, Q218L	N.D.[1]
	N266H, S201A	N.D.[1]
	T225S	N.D.[1]
II	N266H (4x)	0.19 ± 0.01
	N266H (1x)	N.D.[1]
	N266H, S301T	0.19 ± 0.01
	N266H, A307E	0.19 ± 0.01
	N266H, E399V, T421K	0.19 ± 0.01
	M271V, Q291K, T374S	0.16 ± 0.01
	M271V, Q291K[2]	0.09 ± 0.00
	T374S[2]	0.12 ± 0.00
	N266S	0.14 ± 0.01
	N266S, F258L, T310S, E399D	0.12 ± 0.03
	M347L	0.12 ± 0.00
	E420V	0.09 ± 0.01
	N226S, F256I, Y426N	N.D.[1]
III	F375L (3x)[3]	0.45 ± 0.02
	W450R	0.09 ± 0.01
IV	M480L, R527L	0.09 ± 0.01
	S639A	0.09 ± 0.01
	V646A	0.08 ± 0.01
pMcSY-77 (WT)		0.09 ± 0.00
pMcSY-YH		0.24 ± 0.00

Notes:

[1] N.D. No activity could be detected; analysis by Western blotting indicated that no acylase was produced.

[2] These mutants were made by site directed mutagenesis in order to determine the mutation responsible for the higher ratio in the double mutant.

[3] F375L was found three times resulting from different base pair substitutions: 2x **C**TC, 1x TT**A**

do not affect the substrate specificity.
Although much less frequently found, mutation F375L results in the best AD/GL ratio found in these epPCR libraries. It has a five times better ratio than the wild type enzyme and even a two times better ratio than our positive control SY-77^{Y178H} [28]. Other clones having an improved AD/GL ratio are the single mutants and SY-77^{A246V} and SY-77^{M347L} and the multiple mutants SY-77$^{F229L+V237A}$, SY-77$^{S223P+M271L}$, and SY-77$^{M271V+Q291K+T374S}$. We genetically separated the multiple mutants and measured the AD/GL activity ratio of the resulting mutants in order to pinpoint the mutation that contributes most to the altered activity. The results as shown in Table 1 show that the single mutants SY-77^{F229L} and SY-77^{M271L} alone give the same activity ratio as their respective double mutants. This suggests that the second mutations have no effect on the activity of the mutants. Separating M271V and Q291K from T374S resulted in a lower total ratio than the triple mutant, indicating a synergistic effect of the mutations. Therefore, the triple mutant was used to determine k_{cat} and K_m.

Characterisation of purified mutants

Mutants with a significantly different enzymatic activity from wild type (i.e. with an AD/GL ratio higher than 0.12) were purified. The K_m and k_{cat} values of these mutants on both adipyl-7-ADCA and glutaryl-7-ACA were determined (Table 2). The ratio of the k_{cat} values of the purified enzymes on adipyl-7-ADCA and glutaryl-7-ACA is in agreement with the AD/GL ratio determined with the cell free extracts. Previously, the protein concentration was determined with the Bradford assay. However, due to precipitation phenomena, it was decided to use the DC protein assay with these purified acylase samples. This resulted in approximately 35% higher protein concentration values and thus in a decreased k_{cat} value for SY-77 wild type compared with an earlier study [28]. SY-77 wild type preparations purified on different days were found to vary up to 20% in enzymatic parameters. Even when taking this variation into account, most mutants show enzymatic properties that are significantly different from SY-77 wild type.

Table 2. K_m and k_{cat} values of purified enzymes with an AD/GL ratio of ≥ 0.12.
Mutant enzymes with an AD/GL ratio of ≥ 0.12 were purified >90%. The V_{max} and K_m on adipyl-7-ADCA and glutaryl-7-ACA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier [28]. WT, wild type enzyme.

	Adipyl-7-ADCA		Glutaryl-7-ACA	
	k_{cat} (s^{-1})	K_m(mM)	k_{cat} (s^{-1})	K_m(mM)
WT	0.46 ± 0.02	0.96 ± 0.03	4.3 ± 0.2	0.033 ± 0.004
N266H	0.55 ± 0.02	0.14 ± 0.02	3.3 ± 0.1	0.016 ± 0.004
N266S	0.34 ± 0.02	0.42 ± 0.04	2.2 ± 0.1	0.094 ± 0.008
F375L	0.67 ± 0.05	0.82 ± 0.04	1.1 ± 0.1	0.27 ± 0.06
F229L	0.47 ± 0.02	0.52 ± 0.04	3.1 ± 0.1	0.048 ± 0.002
M271V + Q291K + T374S	0.61 ± 0.04	0.7 ± 0.1	3.5 ± 0.1	0.043 ± 0.004

The k_{cat} values on adipyl-7-ADCA of SY-77^{F375L} and the triple mutant SY-77$^{M271V+Q291K+T374S}$ are significantly higher than that of wild type, whereas the corresponding K_m values of SY-77^{N266H}, SY-77^{N266S}, and SY-77^{F229L} are significantly lower than that of wild type. The catalytic efficiency of all mutants is increased for adipyl-7-ADCA with an exceptionally high value for SY-77^{N266H}. With respect to the activity on glutaryl-7-ACA, none of the mutants shows an improved k_{cat}, whereas only mutant SY-77^{N266H} exhibits a lower K_m value than SY-77 wild type. Most interestingly, this results in a higher catalytic efficiency of mutant SY-77^{N266H} on both substrates adipyl-7-ADCA and glutaryl-7-ACA (Figure 3).

Discussion

In this paper, we describe the evolution of a glutaryl acylase towards an adipyl acylase, in order to arrive at an efficient hydrolysis of adipyl-7-ADCA into 7-ADCA for the synthesis of semi-synthetic cephalosporins [112]. We performed random mutagenesis on five overlapping parts of the gene, encoding the β-subunit of the acylase. This resulted in five mutant libraries of approximately 10^5 individual clones, which appeared to be large enough to contain every single mutant at least once [74]. Sequencing of 20 randomly picked clones of each part showed that mutation frequencies lie between one and three base pair substitutions per mutant for all parts. Furthermore, the mutations were scattered throughout the gene (Figure 2).

The mutant libraries were transformed into the leucine deficient *E. coli* strain DH10B, after which the transformants were selected for growth capability on minimal medium containing adipyl-leucine. Unfortunately, cells that produced no mutated acylase with activity towards adipyl-leucine appeared to be able to form small colonies, most likely by using the leucine that was liberated into the medium by lysis of dead, mostly non-transformed cells. Consequently, the selection for growth on AD-Leu plates was less clear cut than desired. Nevertheless, we could identify 54 colonies that excelled in early growth and colony size. 50 colonies out of these were able to grow on a second selective plate with adipyl-leucine as a sole leucine source. These transformants were screened for their hydrolysis activity on the substrates glutaryl-7-ACA and adipyl-7-ADCA. All acylase-producing transformants were active on the β-lactam substrates, in contrast to earlier reports describing similar selection procedures [68,113]. This strengthens the hypothesis that the side chain is the most important moiety for substrate binding, as was already suggested from the crystallographic models of penicillin G acylase [114] and cephalosporin acylase [30].

Sixteen unique mutants showed a significantly higher AD/GL activity ratio than SY-77 wild type (Table 1). Five mutants with a 50% increased AD/GL ratio were purified. These mutants have an enhanced catalytic efficiency on adipyl-7-ADCA with respect to wild type (Figure 3), resulting from a significant improvement of either K_m or k_{cat} (Table 2).

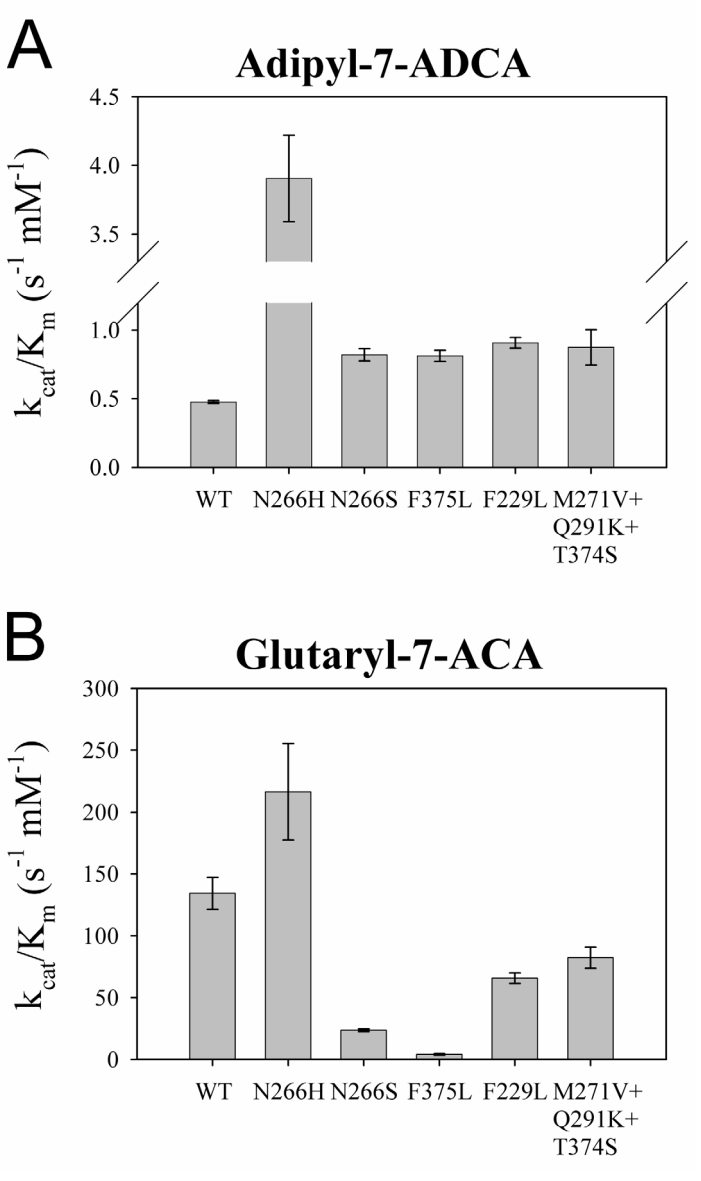

Figure 3. Catalytic efficiency of mutated enzymes on adipyl-7-ADCA and glutaryl-7-ACA.

The catalytic efficiencies of the purified, mutated acylases on adipyl-7-ADCA (A) and glutaryl-7-ACA (B) were calculated from the data given in Table 2. The vertical axe in A is broken to illustrate more clearly the differences in catalytic efficiencies for all mutants.

It thus appears that the selection procedure leads to the isolation of mutants with changes in both kinetic parameters. This seems logical, bearing in mind that the used concentration of adipyl-leucine (0.4 mM) was of the same order of magnitude as the K_m. All mutations that cause a significant increase in AD/GL ratio are localised in close vicinity of the substrate binding site (Figure 4A). Two hot spot positions were found to be mutated with high frequency: Asn266 and Phe375. The latter has been described before as one of the potentially important residues that determine substrate specificity and is thought to interact directly with the substrate [30]. Asn266, however, has not yet been proposed as a target residue for changing substrate specificity and is likely to interact in an indirect manner with the substrate. Its frequent appearance in our collection of selected transformants underscores therefore the strength of random in comparison with structure-based site-directed mutagenesis.

It is striking that mutations in residue Asn266 are found with such high frequency; it was mutated in two-thirds of the selected and sequenced transformants originating from the two parts in which they could be found, parts I and II. This large number was not a consequence of some sequence bias, as proven by sequence determination of randomly picked colonies. Based on mutational efficiency, this residue could have been mutated into several other amino acids (for instance, mutation of one base in the triplet encoding Asn266 can result in Asp, His, Ile, Lys, Ser, Thr, and Tyr). However, apparently only enzymes comprising either of the two different mutations N266H and N266S could pass the selection procedure. The importance of residue Asn266 can be explained with the aid of the reported structures of substrate-bound glutaryl acylase. From these structures, it is clear that Arg255 is crucial for the binding of substrate in the wild type enzyme [32,115]. Arg255 is positioned correctly towards the substrate by a hydrogen bond with residue Asn266 via Tyr351. Replacing Asn266 with His or Ser will alter this network of hydrogen bonds, resulting in an altered positioning of Arg255 toward the substrate. In the case of the N266S mutant, this results in an increased catalytic efficiency for adipyl-7-ADCA and a decreased catalytic efficiency for glutaryl-7-ACA. Mutant SY-77^{N266H} has a unique characteristic: its 8-fold improved catalytic efficiency on adipyl-7-ADCA is accompanied by an improved catalytic efficiency on glutaryl-7-ACA. All other characterised mutants have a decreased catalytic efficiency on glutaryl-7-ACA. Mutant SY-77^{N266H} may accept more substrates and as such be a preferable template for other directed evolution studies.

The enzyme bearing mutation F375L resulted in the best AD/GL ratio found using these epPCR libraries. It was only found in part III and not in part II, which may be due to the prominent presence of the Asn266 mutants in part II. SY-77^{F375L} has a 5-fold higher AD/GL activity ratio compared with the wild type enzyme. This ratio is mainly due to the decreased k_{cat} on glutaryl-7-ACA and not so much the increased k_{cat} on adipyl-7-ADCA. This result emphasises the requirement of enzymatic characterisation in order to be able to identify mutants with the desired properties. The F375L mutation corresponds to the notion that the bulky Phe375 should be changed into a smaller residue in order to provide space for the extra carbon [32]. The enlarged side chain

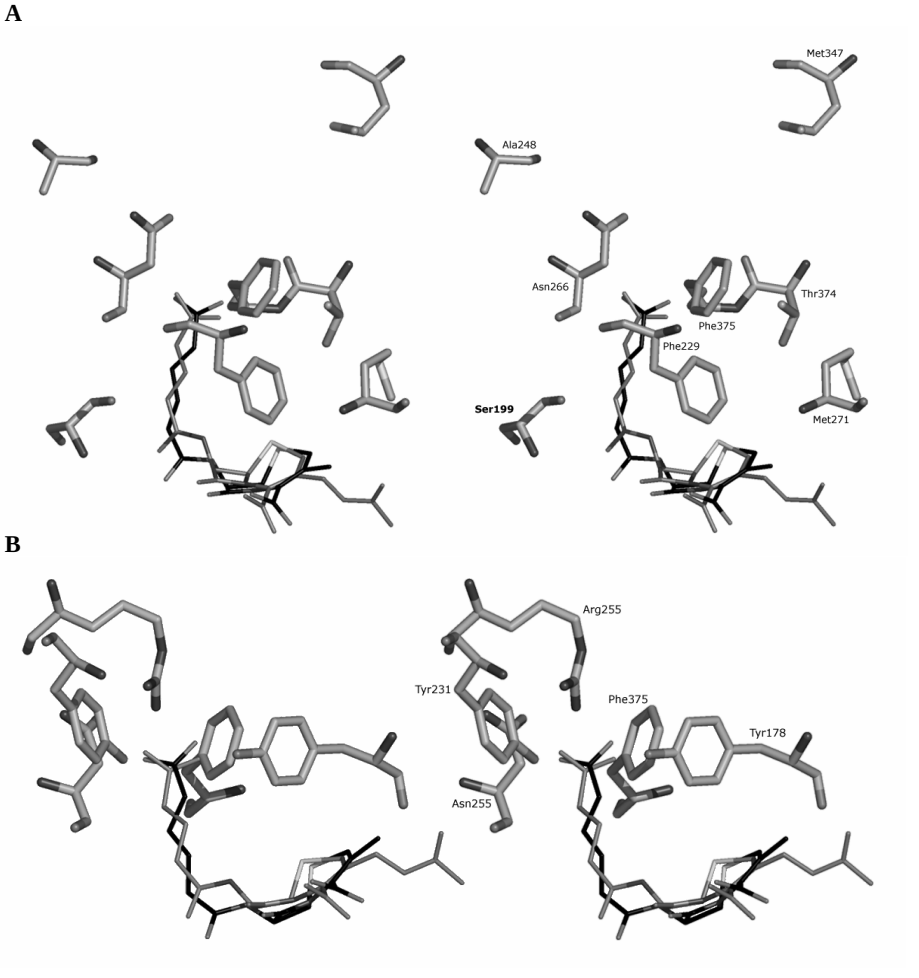

Figure 4. Three-dimensional views on the positions of mutated residues.
A. Three-dimensional view of the mutated residues identified after selection for adipyl activity. The glutaryl acylase SY-77 was modelled with both adipyl-7-ADCA (black) and glutaryl-7-ACA (grey) using the crystal model of the complex with glutaryl-7-ACA. The catalytic residue Ser199 is depicted to illustrate its position relative to the scissile bond of the substrates.
B. Three-dimensional view of five selected residues surrounding the carboxylate group of the side chain. These residues appear to be important to position the carboxylic head of the substrate by direct or indirect interactions.
Both figure 4A and 4B were created using the molecular graphics program PyMOL (W.L. DeLano; available on the World Wide Web at www.pymol.org).
A colour print of this picture can be found on http://www.jbc.org/cgi/content/full/277/44/42121.

binding pocket increases the degrees of freedom of the bound substrate. This may result in a better fit of the alternative adipyl side chain but a decreased binding of the original glutaryl moiety of the substrate.
In the SY-77^{F229L} mutant, the altered residue lies close to the active site residue Tyr231, which was shown to be crucial for the binding of the side chain of glutaryl-7-ACA [32]. Phe229 lies also in close proximity to the β-lactam nucleus. Replacing this amino acid with the smaller leucine will alter the configuration of the substrate binding pocket. A similar effect can be expected for the triple mutation M271V+Q291K+T374S. Met271 lies close to Val268, which has interactions with both the side chain and the nucleus of glutaryl-7-ACA. In addition, Thr374 is the neighbour of Phe375, which interacts with the side chain of glutaryl-7-ACA.

In this study, residues Asn266 and Phe375 of the β-subunit were found to be important targets for changing side chain specificity of glutaryl acylase. In a similar study concerning the α-subunit, Tyr178 was pointed out as an important residue [28]. Mutation of each of these three residues resulted in an improved activity toward adipyl-7-ADCA. Combined with residues Tyr231 and Arg255, which are known to be crucial for the binding of the side chain of glutaryl-7-ACA [32,115], these residues form a pocket in which the carboxylate group of the side chain is embedded (Figure 4B). Residues Tyr178, Tyr231, Asn266, and Phe375 seem to form the corners of a square plane, with the carboxylate group in the centre, which is held in place by the positive charge of Arg255 above this plane. As described before [32], the rest of the aliphatic side chain is positioned tightly in the active site by hydrophobic interactions with several residues. Our results in this and our previous study [28] indicate that the accommodation of larger side chains requires modification in this part of the side chain binding pocket, while keeping the scissile bond properly positioned toward the catalytic Ser199. Therefore, a logical step in evolving this enzyme toward a further improved adipyl acylase or even a cephalosporin C acylase seems to be a combination of mutations of these residues.

Acknowledgments

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Chapter 3

Mutational analysis of a key residue in the substrate specificity of a cephalosporin acylase

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β-lactam acylases are crucial for the synthesis of semi-synthetic cephalosporins and penicillins. Unfortunately, however, there are no cephalosporin acylases known that can efficiently hydrolyse the amino-adipic side chain of this compound. In a previous directed evolution experiment residue Asn266 of the glutaryl acylase from *Pseudomonas* SY-77 was identified as a residue important for substrate specificity. In order to explore the function of this residue in substrate specificity, we performed a complete mutational analysis of position 266. Codons for all amino acids were introduced in the gene and 16 proteins that could be functionally expressed in *Escherichia coli* were purified to homogeneity and their catalytic parameters were determined. The mutant enzymes displayed a broad spectrum of affinities and activities, pointing towards the flexibility of the enzyme at this position. Mutants in which Asn266 was changed into Phe, Gln, Trp and Tyr displayed up to a 2-fold better catalytic efficiency (k_{cat}/K_m) on adipyl-7-aminodesacetoxycephalosporanic acid (adipyl-7-ADCA), due to a decreased K_m. Only mutants SY-77^{N266H} and SY-77^{N266M} showed an improvement of both catalytic parameters, resulting in a 10- and 15-times higher catalytic efficiency on adipyl-7-ADCA, respectively. Remarkably, the catalytic activity (k_{cat}) of SY-77^{N266M} on adipyl-7-ADCA is as high on glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA), and approaches commercially interesting activities. On Cephalosporin C, mutants SY-77^{N266Q}, SY-77^{N266H} and SY-77^{N266M} show a modestly improved hydrolysis. Since these mutants also have a good catalytic efficiency on adipyl-7-ADCA and still activity towards glutaryl-7-ACA, they can be regarded as broader substrate acylases. These results demonstrate that a combination of directed evolution for the identification of important positions and saturation mutagenesis for finding the optimal amino acid is very effective for finding improved biocatalysts.

Introduction

The most widely used antibiotics are semi-synthetic cephalosporins and penicillins. Key intermediates in the synthesis of cephalosporins are 7-aminocephalosporanic acid (7-ACA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA). 7-ACA is obtained by removing the side chain from Cephalosporin C (CPC) produced by the fungus *Acremonium chrysogenum* in a two step enzymatic process. 7-ADCA is produced from penicillin G made by *Penicillium chrysogenum* involving several polluting chemical steps followed by enzymatic deacylation using penicillin acylase [106]. A first step towards the introduction of a simplified, more environmentally friendly production of 7-ADCA was the development of a genetically modified P. chrysogenum strain that produces adipyl-7-ADCA [18]. For the enzymatic deacylation of this novel β-lactam and CPC, novel acylases with activity on adipyl and amino-adipyl side chains, respectively, are needed. As the presently identified acylases show little or no activity towards

adipyl-7-ADCA and CPC [13,25,102], it is of interest to investigate whether they can be created from another acylase, e.g. glutaryl acylase. In a former study we used directed evolution as a tool to identify important residues for the transformation of a glutaryl acylase into an adipyl acylase [116]. Several variants were selected and showed a considerable improvement of the hydrolysis of adipyl-7-ADCA. Sequencing of these variants demonstrated that the mutation of Asn266 into His or Ser occurred frequently. This indicates that position 266 is important for the evolution of the glutaryl acylase into an adipyl acylase, although this was not predicted from the crystal structure [30,32,115].
Since we used a random mutagenesis strategy introducing on average 1-2 point mutations per gene in our directed evolution experiment, it was not possible to obtain all 20 amino acids on this position. Furthermore, the amino acid substitutions obtained by point mutations are usually conservative, while non-conservative mutations often comprise more potential to change the activity of an enzyme [117], e.g. from a glutaryl acylase towards an adipyl acylase or even a CPC acylase. The side chain of residue 266 may influence the enzyme specificity either by a polar interaction with the charged carboxyl group of the substrate side chain or by a better accommodation of the hydrophobic part of this side chain. In order to fully explore the importance of this position for the substrate specificity of the enzyme, we changed the amino acid at position 266 into all other amino acids by a site-directed mutagenesis approach. The mutant enzymes were purified and catalytic parameters were determined on glutaryl-7-ACA, adipyl-7-ADCA and CPC.

Materials and Methods

Bacterial strains and plasmids and DNA manipulations

The plasmid pMcSY-2 [116] and the leucine deficient *Escherichia coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the SY-77 acylase.
Molecular DNA techniques were executed following standard protocols [41]. The enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied according to the instructions of the manufacturer. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (QIAGEN). DNA was extracted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN).
Mutants were made using the megaprimer method described by Landt *et al.* [42]. All PCR reactions were performed in a Mastercycler® gradient thermocycler (Eppendorf) using recombinant *Pfu* polymerase (Stratagene). A 5'-universal primer, CGCCGGACTTCGAGATCTA and a 3'-mutagenic primer, CATGCCGTTGACGGT**SNN**GGTGATGCCCATCCG, were used in the first PCR using the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 30 s at 48°C, 30 s at 72°C and a final step of 10 min at 72°C. The resulting fragment was purified and used as the 5'-primer in a second PCR together with a 3'-universal primer GATTGGTGACCCGCGGCA. In this reaction the following program was used: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 53°C, 1 min at 72°C and a final step of 10 min at 72°C. The second PCR product was purified and cloned into pMcSY-2 using *Bgl*II and *Sst*II. Resulting plasmids were sequenced in order to determine the amino acid at position 266. Amino acids, which were not obtained in the random

primer PCR, were made by using the 3'-mutagenic primer with the specific codon at the NNS position. The cloned DNA fragment was sequenced on both strands afterwards.

Purification of enzymes

Mutant and wild type enzyme were purified in three chromatography steps on a Duoflow system (Bio-Rad) using columns from Amersham Biosciences as described before [116]. Typically, *E. coli* DH10B containing the plasmids encoding the desired enzymes were grown in 2*YT medium (300 ml, 30 hours, 25°C) supplemented with chloramphenicol (50 µg/ml) and glycerol (0.1%). Cell free extract was made by sonication (10 min, output 4, 40% duty cycle on a Sonifier 250, Branson) and centrifugation (30 min, 17000 g). The cell free extract was blotted onto a membrane and stained with polyclonal rabbit antibody against purified SY-77 glutaryl acylase (Eurogentec S.A.) as described before [116]. Mutants that showed a low concentration of acylase were grown again for up to 70 hours at 17°C–20°C in 2 litre of the same medium. Protein concentrations of the purified samples were determined using the DC Protein Assay (Bio-Rad) with bovine serum albumin as the reference protein. The samples were analyzed on a 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue [110].

Enzyme characterization

The kinetic parameters of the purified wild type and mutant enzymes on glutaryl-7-ACA and adipyl-
7-ADCA were tested in an automated fluorescamine assay as described before [116]. Hydrolysis of CPC was determined by the fluorescamine assay, using the highest possible concentration of substrate with respect to background values. 20 µg of enzyme was added to a reaction mixture of 300 µl containing CPC (10 mM) in phosphate buffer (20 mM, pH 7.5). After 20 and 40 hours incubation at 37ºC, an aliquot of 40 µl reaction mixture was transferred to 140 µl acetate buffer (0.5 M, pH 4.5), after which 20 µl of fluorescamine in acetone (1 mg/ml) was added. After incubation at room temperature (60 min) the A_{380} was measured. The enzymes, which showed some hydrolysis in this assay, were used in a more accurate assay. In this assay, 75 µg of enzyme was used in a 300 µl reaction mixture containing CPC (10 mM) in phosphate buffer (20 mM, pH 7.5). Samples of 40 µl were taken from this reaction mixture every hour for five hours. In these samples hydrolysis of CPC was also measured using the automated fluorescamine assay. Data were analysed by linear regression. The slopes were calculated from two experiments with at least three different sets of data points. The significance of deviations in CPC hydrolysis activity was tested by the Student's t-test.

Modelling of the (mutant) enzymes

In order to get a structural impression of the mutational effects we made an overlay of selected active site residues in the structural models of the glutaryl acylase proteins containing mutations N266Q, N266M and N266W. The wild type structure was also refined under the same circumstances to account for differences in crystal structures and modelled structures. The models were constructed on the basis of wild type glutaryl acylase(PDB entry 1FM2) using the molecular graphics and modelling program DS Modeling (Accelrys, San Diego, CA, USA). The structures were refined using the DS CHARMm® module by energy minimisation consisting of 150 steps of steepest descent followed by 5000 iterations of the Adopted Basis-set Newton-Raphson algorithm.

Results

Preparation and purification of the mutant enzymes

The megaprimer method was used in to create the 19 different amino acid mutants on position 266. First, a primer with a randomised codon 266 was used and 96 transformants were sequenced. This resulted in 17 different amino acids at this position in a single mutagenesis round. The missing three mutants were made by site-directed mutagenesis using a specific primer. *E. coli* DH10B cells containing the plasmid encoding the (mutant) enzyme were grown in 300 ml 2*YT-medium at 25°C. After 30 hours the cells were harvested and sonicated. The soluble fraction was used to purify the enzyme in a 3-step protocol. Typically, a yield of more than 10 mg enzyme per litre was reached with a purity of at least 90%.

The enzymes containing mutations N266R, N266I, N266K and N266V were hardly produced under these conditions. Attempts to obtain these mutant enzymes by growing them in different experiments at 17°C-20°C for 30-70 hours in a volume up to two litres failed. At these low temperatures after 70 hours the strains expressing these mutants reached only half the OD_{600} of the strains bearing the wild type gene in 30 hours at 25°C. Furthermore, they still hardly produced any enzyme in the soluble fraction. Western blots of SDS-PAGE gels with cell lysates of these mutants showed that SY-77^{N266R} and SY-77^{N266K} were mainly present in the non-soluble fraction, whereas the enzymes containing mutations N266I and N266V were hardly produced at all (data not shown). Consequently, these four mutants were discarded from further analysis.

In order to determine possible interference of the remaining 16 amino acids at this position with the maturation of the enzymes, 3 µg of purified enzyme was loaded onto an SDS-PAGE gel and stained with Coomassie Brilliant Blue. From these gels (Figure 1) it is clear that mutation N266A results in an impaired processing of the propeptide, while mutations N266D and N266E result in a less efficient cleavage of the spacer from the α-subunit. The other mutations do not seem to affect the processing of the enzyme.

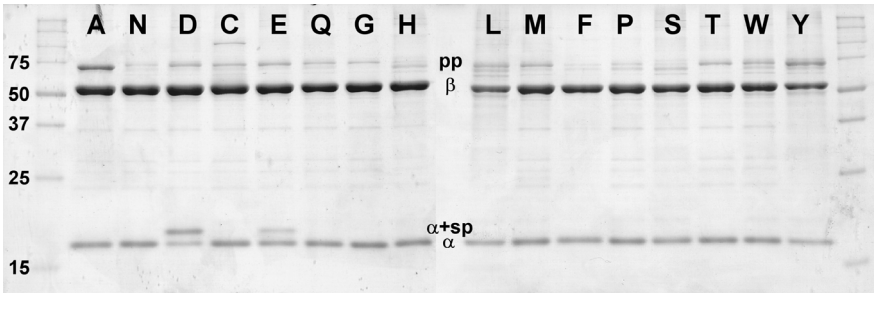

Figure 1. Effects of mutation of Asn266 on the maturation of glutaryl acylase.
The purified enzymes were boiled for 2 minutes in loading buffer and loaded onto a 12.5% SDS-PAGE gel. The amino acid at position 266 is depicted above the lane. pp, propeptide; β, β-subunit; sp, spacer peptide; α, α-subunit.

Activity towards adipyl-7-ADCA and glutaryl-7-ACA

The catalytic parameters of the purified mutant and wild type enzymes were determined on both adipyl-7-ADCA and glutaryl-7-ACA. The activity and affinity constants (k_{cat} and K_m) are listed in Table 1, the catalytic efficiencies (k_{cat}/K_m) are depicted in Figure 2. Interestingly, the library of mutants at position 266 exhibits a considerable diversity of changed catalytic parameters. The most striking mutant is SY-77^{N266M}. Its catalytic efficiency on adipyl-7-ADCA is 15-fold higher than that of wild type. The K_m of this enzyme is 3.5-times lower and the k_{cat} is 4.5-times higher than wild type parameters. The large hydrophobic amino acids Tyr, Trp and Phe at position 266 improve adipyl-7-ADCA hydrolysis by lowering the K_m. Mutation N266H results in an almost 10-fold improved catalytic efficiency on adipyl-7-ADCA, mostly due to a decreased K_m. The amino acid Gln at position 266, which side chain is one C atom longer than the original side chain of Asn, induces a 2-fold decrease of the K_m for adipyl-7-ADCA hydrolysis. Substitution of Asn266 with the small amino acid Cys improves K_m, but decreases k_{cat} on adipyl-7-ADCA resulting in a catalytic efficiency that is similar to wild type. The other amino acids at position 266 have a decreased k_{cat} and/or a higher K_m on adipyl-7-ADCA resulting in a lower catalytic efficiency. Changing Asn266 into any other amino acid in all cases leads to a lower activity towards glutaryl-7-ACA. The extent of this decrease on k_{cat} and/or K_m is distinct for each amino acid.

Table 1. K_m and k_{cat} values on adipyl-7-ADCA and glutaryl-7-ACA of purified enzymes.

The mutant enzymes SY-77^{N266R}, SY-77^{N266I}, SY-77^{N266K} and SY-77^{N266V} did not produce enough enzyme to be purified and catalytic parameters could not be determined. The remaining 16 enzymes were purified >90%. The k_{cat} and K_m on both substrates were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier [116]. N.D., not detectable.

AA at position 266	adipyl-7-ADCA		glutaryl-7-ACA	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Asn (WT)	0.41 ± 0.01	1.2 ± 0.3	4.0 ± 0.3	0.031 ± 0.002
Ala	0.18 ± 0.02	2.2 ± 0.2	1.4 ± 0.1	0.26 ± 0.08
Asp	0.002 ± 0.000	2.2 ± 0.9	N.D.	N.D.
Cys	0.31 ± 0.03	0.80 ± 0.03	2.0 ± 0.1	0.16 ± 0.03
Glu	0.023 ± 0.002	13.8 ± 5.1	0.025 ± 0.002	0.29 ± 0.00
Gln	0.46 ± 0.04	0.62 ± 0.13	2.4 ± 0.2	0.12 ± 0.03
Gly	0.23 ± 0.02	1.4 ± 0.3	2.0 ± 0.4	0.54 ± 0.13
His	0.47 ± 0.01	0.14 ± 0.01	3.1 ± 0.3	0.044 ± 0.008
Leu	0.25 ± 0.02	1.0 ± 0.2	0.082 ± 0.011	0.43 ± 0.04
Met	1.9 ± 0.1	0.33 ± 0.05	1.8 ± 0.1	0.17 ± 0.02
Phe	0.37 ± 0.04	0.6 ± 0.1	0.9 ± 0.2	0.10 ± 0.00
Pro	0.061 ± 0.005	4.4 ± 1.2	0.13 ± 0.01	1.3 ± 0.3
Ser	0.24 ± 0.02	0.99 ± 0.05	1.6 ± 0.1	0.075 ± 0.005
Thr	0.15 ± 0.01	5.7 ± 1.2	0.49 ± 0.14	1.5 ± 0.5
Trp	0.33 ± 0.01	0.59 ± 0.06	1.3 ± 0.1	0.18 ± 0.04
Tyr	0.33 ± 0.01	0.65 ± 0.10	0.61 ± 0.06	0.11 ± 0.01

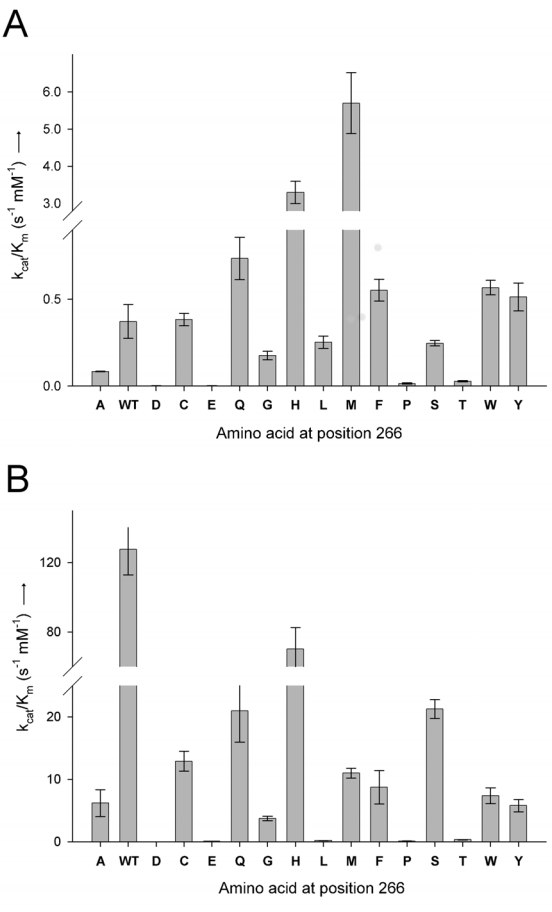

Figure 2. Catalytic efficiencies of purified enzymes on adipyl-7-ADCA (A) and glutaryl-7-ACA (B).
The catalytic parameters were determined by an automated fluorescamine assay performed at different substrate concentrations. The kinetic parameters were calculated from Eadie-Hofstee plots and listed in Table 1. The given values are a mean ± S.D. of at least three independent measurements.

Conversion of Cephalosporin C

For the determination of activity on CPC, 20 µg of all mutant and wild type enzymes were incubated at 37°C with 10 mM CPC. The wild type enzyme and the mutants SY-77^{N266Q}, SY-77^{N266H}, SY-77^{N266M} and SY-77^{N266W} showed hydrolysis after 20 hours. These activities were too low to be able to determine K_m and k_{cat}. In order to avoid long incubation times that may lead to enzyme destabilisation, the conversion rate of the different mutants was calculated from an assay with more enzyme (75 µg). The mean slope of the conversion of CPC in the first five hours was determined and normalised

towards wild type (Figure 3). Mutants SY-77^{N266Q} and SY-77^{N266H} displayed almost a two times improved conversion of CPC over wild type, whereas SY-77^{N266M} has improved 30%. The conversion rate of SY-77^{N266W} is not significantly different from wild type.

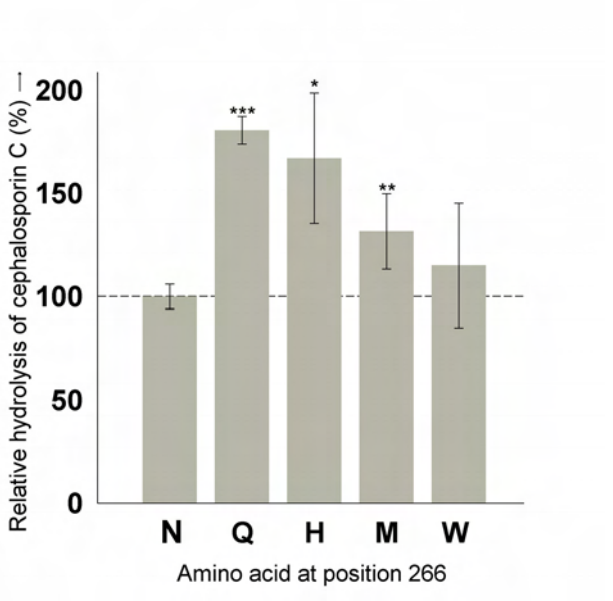

Figure 3. Hydrolysis of 10 mM CPC by a few selected Asn266 mutants in the first five hours.

The slopes of the hydrolysis curves were calculated for at least six different experiments. Conversion of CPC by wild type was set at 100%. Values were tested for statistical significant difference by the Student's t-test; * = $p < 0.01$, ** = $p < 0.005$, *** = $p < 0.0005$, mutant N266W was not significantly different.

Discussion

In a previous study using error prone PCR, amino acid Asn266 of the glutaryl acylase of *Pseudomonas* SY-77 was pointed out as one of the residues defining substrate specificity [116]. This result was surprising since Asn266 was indicated not to be directly involved in substrate-binding, but could interact via Tyr351 with Arg255, which accommodates the carboxylate head of the side chain [32,115]. In order to elucidate the role of Asn266 in substrate specificity, we have mutated Asn266 into the 19 other amino acids and expressed them in *E. coli*. Sixteen of the 20 different enzymes could be expressed in a soluble form and were purified. These 16 acylases were characterised with respect to their activity in the maturation process and the hydrolysis of several substrates. The catalytic properties varied over a broad spectrum (Table 1, Figures 2 and 3) pointing at the active role of position 266 in the enzymatic activities.

Since the maturation of the enzyme is crucial for its activation [118], we first examined the effect of the different mutations on the maturation process. Mutants SY-77^{N266R} and SY-77^{N266K} were only produced as insoluble precursor proteins. Probably, the longer side chains and positive charge result in a non-proper folding of the enzyme. The presence of Ile or Val at position 266 causes a low production of the enzyme, in contrast to other aliphatic residues. It is possible that the branching at the Cβ interferes with the folding process. Mutant SY-77^{N266A} is soluble, but not fully maturated. A significant fraction of this purified protein is in the propeptide form indicating that the first intramolecular cleavage step (between the spacer and the β-subunit) is affected (Figure 1). The second step in the maturation process (cleavage of the spacer peptide from the β-subunit) is supposed to involve a similar catalytic mechanism as the hydrolysis of the substrate [29,118]. Therefore, it would be expected that changes in residues involved in catalysis also interfere with the processing of the enzyme. However, most of the purified mutant proteins do not show the non-cleaved form of α-subunit plus spacer peptide. Obviously, the long period of cell growth and protein purification could give slower processing mutants enough time to mature. Thus, small changes in maturation efficiency would not be detected. Only the change of Asn266 into the charged residues Asp and Glu clearly results in poor cleavage of the spacer peptide from the α-subunit (Figure 1). It seems that these amino acids do not interfere with the folding of the enzyme, but are in competition with the carboxylate group of the Glu188 for the Arg255 [119].

Remarkably, the K_m of the enzyme towards adipyl-7-ADCA has improved in half of the purified mutants, pointing at the flexibility and importance of position 266. Only mutants SY-77^{N266E}, SY-77^{N266P} and SY-77^{N266T} have a strongly increased K_m. Concerning the catalytic activity, merely two mutants, SY-77^{N266H} and SY-77^{N266M}, show a significantly increased k_{cat}. The most striking mutant is SY-77^{N266M}, which shows a 4.5 times higher k_{cat} and a 3.5 times lower K_m, resulting in a 15-times better catalytic efficiency (Figure 2). The k_{cat} on adipyl-7-ADCA is the same as the k_{cat} on glutaryl-7-ACA of this mutant and approaches enzymatic activities that are commercially exploited [14]. Although the side chain of Met is one methyl group longer than Asn, it is flexible and unbranched. Modelling of this amino acid at position 266 in the enzyme suggests that mutation N266M only affects the position of the side chains of Arg255 and Met266 (Figure 4). It is likely that Met266 can have direct hydrophobic interactions with adipyl-7-ADCA. In addition, it clearly displaces the side chain of Arg255, thereby enlarging the substrate binding pocket and creating more space for the longer adipic side chain.

Five other mutants maintain a similar k_{cat} on adipyl-7-ADCA as wild type and show a 1.5 to 2-times decreased K_m. SY-77^{N266H} and SY-77^{N266Q} both have a larger amino acid at position 266 that is able to form hydrogen bonds. In the original crystal structure, the longer side chain of mutant amino acid Gln266 would not fit in the same orientation as Asn. Application of energy minimisation to this mutant enzyme structure bends the Gln side chain towards Arg255, resulting in a more favourable position for hydrogen

bonding with either the substrate or Arg255 (Figure 4). Similar to SY-77^{N266M}, SY-77^{N266Q} appears to displace Arg255, allowing a better accommodation of the adipyl side chain. The catalytic properties on adipyl-7-ADCA of this mutant are however not as good as those from SY-77^{N266M}, probably due to the polar character of the Gln side chain. The large aromatic amino acids in the mutants SY-77^{N266F}, SY-77^{N266W} and SY-77^{N266Y} express similar k_{cat} values and bind adipyl-7-ADCA 2-fold better than wild type. These amino acids are more likely to change the position of the substrate by hydrophobic interactions. Modelling of the mutant with the largest of these three amino acids, SY-77^{N266W}, revealed that the position of Trp266 nearly overlaps with the original position of Asn (Figure 4). Incorporation of this large aromatic side chain does however cause a displacement of the backbone of Arg255 and its closest neighbours away from the substrate. Mutation N266W thus seems to improve the accommodation of the longer adipic side chain by an enlargement of the substrate binding pocket and likely also by increasing the hydrophobic interactions.

None of the mutants show an increased activity or affinity for the preferred substrate glutaryl-7-ACA. Evolution optimised this amino acid, logically resulting in a decreased activity upon changing, as is seen in a lot of mutagenesis studies [108]. Only mutants SY-77^{N266H} and SY-77^{N266S}, which were already found in the directed evolution experiment, do have similar catalytic parameters on the glutaryl substrate as wild type enzyme indicating that these mutations hardly affect the overall structure of the substrate-binding pocket of the enzyme. This is not surprising considering the fact that these amino acids could be created by a single base pair substitution, which usually leads to conservative mutations and preservation of enzyme activity.

Although the importance of residue 266 became evident from a selection experiment using the adipyl side chain, we hypothesised that this residue also might influence the binding of the amino-adipyl side chain of CPC. We found mutants SY-77^{N266H}, SY-77^{N266Q} and SY-77^{N266M} to have a small but significant increase in hydrolysis activity of CPC over wild type (Figure 3). This activity is, however, still two orders of magnitude lower than the hydrolysis of glutaryl-7-ACA. As a consequence of these low activities we could not determine whether these improvements stem from effects on either K_m or k_{cat}.

Interestingly, the same mutants SY-77^{N266Q}, SY-77^{N266H} and SY-77^{N266M} also have an improved activity on adipyl-7-ADCA and SY-77^{N266H} even displays almost the same activity on glutaryl-7-ACA as wild type. Apparently, these three mutated enzymes have expanded their substrate range. These results seem to confirm the hypothesis that a CPC acylase can be made from a glutaryl acylase, but that one amino acid substitution is not sufficient.

In conclusion, we used saturation mutagenesis to complement a previous directed evolution approach in which residue Asn266 was identified as a key residue for substrate specificity. This allowed the analysis of a larger sequence space at this particular position and finding of more radical changes in enzyme activity. The

biochemical and structural effects of the different amino acids at position 266 were analysed and underlined its importance for substrate recognition. This strategy allowed the isolation of a mutant enzyme with a commercial interesting activity towards adipyl-7-ADCA, and appears to make the realisation of an industrial applicable CPC acylase feasible.

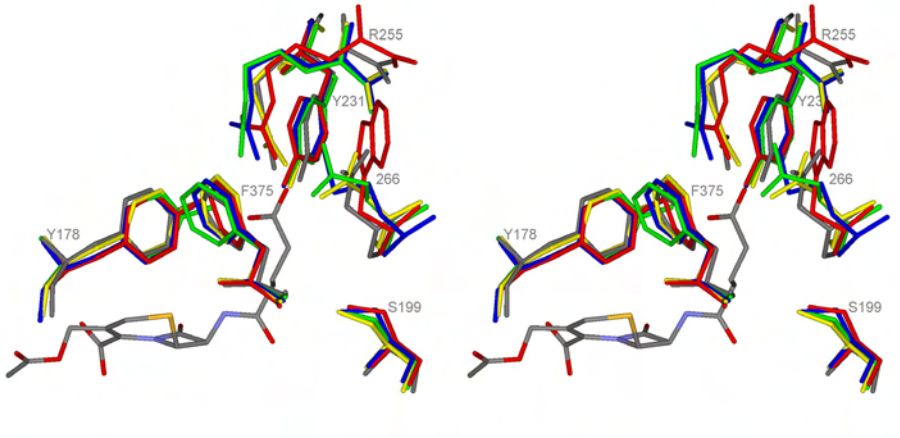

Figure 4. Structural impression of the mutational effects.
Overlay of selected active site residues in the structural models of glutaryl acylase proteins SY-77^{N266Q} (green), SY-77^{N266M} (blue), SY-77^{N266W} (red) and refined wild type (yellow). The models were constructed on the basis of wild type glutaryl acylase (grey; PDB entry 1FM2) using the molecular graphics and modelling program DS Modeling (Accelrys, San Diego, CA, USA). For clarity, glutaryl-7-ACA was added (coordinates from PDB entry 1JVZ).

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Chapter 4

Analysis of a substrate specificity switch residue of cephalosporin acylase

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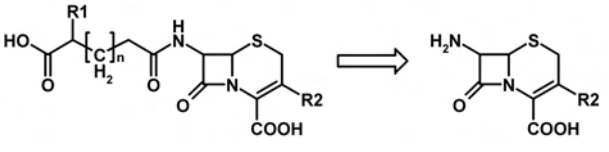
Residue Phe375 of cephalosporin acylase has been identified as one of the residues that is involved in substrate specificity. A complete mutational analysis was performed by substituting Phe375 with the 19 other amino acids and characterising all purified mutant enzymes. Several mutations cause a substrate specificity shift from the preferred substrate of the enzyme, glutaryl-7-ACA, towards the desired substrate, adipyl-7-ADCA. The catalytic efficiency (k_{cat}/K_m) of mutant SY-77^{F375C} towards adipyl-7-ADCA was increased 6-fold with respect to the wild type enzyme, due to a strong decrease of K_m. The k_{cat} of mutant SY-77^{F375H} towards adipyl-7-ADCA was increased 2.4-fold. The mutational effects point at two possible mechanisms by which residue 375 accommodates the long side chain of adipyl-7-ADCA, either by a widening of a hydrophobic ring-like structure that positions the aliphatic part of the side chain of the substrate, or by hydrogen bonding to the carboxylate head of the side chain.

Introduction

Cephalosporin acylase is a crucial enzyme for the development of new enzymatic pathways leading to the production of semi-synthetic cephalosporins. 7-amino-cephalosporanic acid (7-ACA) and 7-aminodesacetoxycephalosporanic acid (7-ADCA) are important intermediates in these pathways. Large quantities of these compounds are needed, but the traditional production routes are time-consuming, expensive and/or polluting [106]. An alternative route to obtain 7-ADCA or 7-ACA is the one-step enzymatic deacylation of either adipyl-7-ADCA [18] or cephalosporin C (CPC) [102]. However, currently there is no biocatalyst available that is capable of performing this task cost-efficiently [106]. A good starting point for the generation of such an adipyl acylase or CPC acylase could be *Pseudomonas* SY-77 glutaryl acylase [13,25], a cephalosporin acylase that is highly active on a similar compound glutaryl-7-ACA (Figure 1).

Crystallisation studies of cephalosporin acylase with and without glutaryl-7-ACA or glutarate showed that Phe375 is part of the substrate binding site of the enzyme [30,32,115]. In an unbiased approach using directed evolution of *Pseudomonas* SY-77 glutaryl acylase, mutant SY-77^{F375L} was found as one of the mutants that significantly increases the activity of the enzyme on adipyl-7-ADCA [116]. However, directed evolution by the introduction of point mutations in the codon for Phe375 allows for the substitution by only six residues (Leu, Ile, Val, Cys, Tyr and Ser) and consequently explores a limited part of the sequence space at this position. Additionally, the mutants were selected on an improved hydrolysis of adipyl-7-ADCA analogues. Consequently, neutral and negative effects and effects on the hydrolysis of other β-lactam compounds that deepen the knowledge of the cephalosporin acylase-substrate interaction could not be observed. Therefore, it was decided to use a saturated mutagenesis approach to analyse the effects of all possible residues at position 375 on the hydrolysis of three β-lactam compounds.

Mutant enzymes with all natural amino acids at position 375 were overproduced and purified to determine their activities in the hydrolysis of glutaryl-7-ACA, adipyl-7-ADCA and CPC, and autocatalytic processing.
Several mutations were shown to improve the kinetic parameters towards adipyl-7-ADCA. The highest increase in k_{cat} was due to the incorporation of a histidine, whereas the largest decrease of the K_m value was found for mutant SY-77^{F375C} that also shows the highest increase in catalytic efficiency. The effects of all mutations on the kinetic parameters are discussed on the basis of the crystal structure of the enzyme, and allowed to elucidate the possible modes by which residues at position 375 can modify substrate specificity.



Compound	n	R1	R2
Glutaryl-7-ACA	1	-H	$-CH_2OCOCH_3$
Adipyl-7-ADCA	2	-H	$-CH_3$
CPC	2	$-NH_2$	$-CH_2OCOCH_3$
7-ACA	-	-	$-CH_2OCOCH_3$
7-ADCA	-	-	$-CH_3$

Figure 1. The production of 7-ACA and 7-ADCA from β-lactam compounds.

Materials and methods

Chemicals

The β-lactam substrates glutaryl-7-ACA, adipyl-7-ADCA and CPC (as sodium salt) were gifts from DSM, The Netherlands. Fluorescamine was from Sigma, BugBuster from Novagen, 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium were from Duchefa, The Netherlands.

Mutagenesis of residue Phe375 of *Pseudomonas* SY-77 glutaryl acylase

Mutagenesis was performed on plasmid pMcSY-2 [116] harbouring the gene encoding *Pseudomonas* SY-77 glutaryl acylase under control of the tac-promoter. The random primer 5'-atg cag gtg ccg acc nng/c aac atc gtc tac gcc g was used to mutate the codon for Phe375 in a megaprimer reaction with *Pfu* DNA polymerase (Stratagene) [42]. The resulting PCR product was ligated into pMcSY-2 by digestion using the *Bgl*II and *Sst*II restriction sites. After transformation into *Escherichia coli* DH10B, cells were plated on LB-agar plates containing 50 µg/ml chloramphenicol. Single colonies were picked for DNA sequence determination. Nine different mutants were obtained using this method, the remaining ten were produced by PCR using the site directed version of the same primer, in which nng/c was replaced with the codon for the desired residue.
A test was conducted to confirm that the transformants were still able to produce acylase. 10 ml 2*YT medium [41] supplemented with 50 µg/ml chloramphenicol and 0.1% glycerol was

inoculated with 0.1 ml of an overnight culture, and grown at 30ºC for 24 h. Cells were harvested from 5 ml of this culture and lysed with the non-ionic detergent BugBuster [116]. The soluble fraction was spotted on a nitrocellulose membrane, which was subsequently incubated with a polyclonal rabbit antibody against purified *Pseudomonas* SY-77 glutaryl acylase (Eurogentec S.A.) and an alkaline phosphatase-conjugated goat anti-rabbit antibody. The appearance of purple spots after incubation with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium confirmed the presence of acylase enzyme in the sample. Transformants that did not produce acylase were discarded.

Characterisation of mutant enzymes

Mutant and wild type enzymes were produced in *E. coli* DH10B in 100 ml cultures and purified by anion exchange and hydrophobic interaction column chromatography as described earlier [116]. The protein concentration in the purified samples was determined using the DC protein assay (Bio-Rad) with BSA as the reference protein. Analysis of the samples by SDS-PAGE [110] was done using a 12.5% gel, which was stained with Coomassie Brilliant Blue.
The kinetic parameters k_{cat}, K_m and k_{cat}/K_m of mutant and wild type enzyme towards glutaryl-7-ACA and adipyl-7-ADCA were determined using the automated fluorescamine assay in 96-well format as described earlier [116]. A concentration range of the β-lactam substrates was incubated with a fixed amount of enzyme in 20 mM phosphate buffer pH 7.5 at 37ºC. The reaction was stopped by the addition of acetate buffer pH 4.5, and the amount of product 7-A(D)CA formed was determined by incubation with fluorescamine and measuring the absorption at 380 nm. The kinetic parameters k_{cat} and K_m were calculated from the rates of reaction at different concentrations of substrate. Activity towards CPC was determined by the fluorescamine assay at a high concentration of substrate [116], using approx. 20 µg of purified enzyme and 5 mM CPC in 200 µl of reaction mixture. Aliquots were taken after 24 and 48 h incubation at 37ºC and incubated with fluorescamine to determine whether CPC had been deacylated.

Results

Production and autocatalytic processing of mutant enzymes

All 19 mutant enzymes and wild type could be expressed and purified from *E. coli* DH10B at a yield of more than 10 mg/l culture. On SDS-PAGE (Figure 2), all purified mutants displayed the bands corresponding to the mature α- and β-subunits, with the exception of mutant SY-77^{F375P}, which showed only the band corresponding to the unprocessed propeptide consisting of the α-subunit, spacer peptide and the β-subunit. Mutants SY-77^{F375I}, SY-77^{F375R}, SY-77^{F375T} and SY-77^{F375V} showed a band corresponding to the unprocessed propeptide in addition to the α and β-subunits, indicating that they underwent partial processing. An impaired processing could also have led to the accumulation of the polypeptide consisting of α-subunit plus spacer peptide, but no band corresponding to this polypeptide was seen in any of the samples. Remarkably, the elution profiles of all mutants were similar to that of wild type enzyme in all column chromatography steps, indicating that the folding of the maturated and the non-maturated enzymes is very similar, which has also been observed for the S199A mutant of cephalosporin acylase [118].

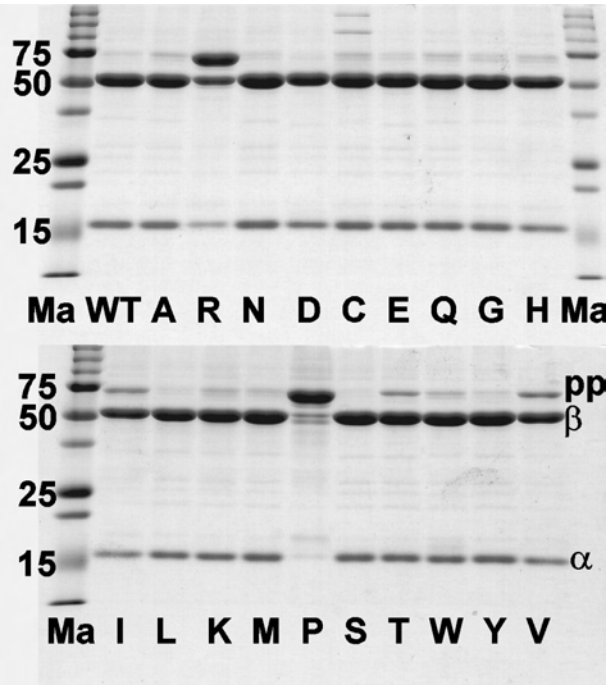


Figure 2. Maturation of wild type and mutant cephalosporin acylases.
Cephalosporin acylase is produced as a propeptide consisting of a signal sequence, α-subunit, spacer peptide and β-subunit. Impairment of the first maturation step leads to the accumulation of propeptide. Impairment of the second maturation step would lead to accumulation of α-subunit + spacer peptide, visible as a band just above the band corresponding to the α-subunit. Samples were incubated at 100ºC for 2 min; each lane contains 3 µg purified protein. The residues in which Phe375 has been mutated are indicated by the one letter code.
Ma = Marker proteins (Bio-Rad); α = α-subunit; β = β-subunit; pp = propeptide.

Hydrolysis of glutaryl-7-ACA

None of the mutations of residue 375 improved the kinetic parameters towards the preferred substrate of the enzyme, glutaryl-7-ACA (Table 1 and Figure 3). The best mutation was F375C, which lowered the catalytic efficiency to about 50% of the value of wild type due to a decrease of k_{cat}. SY-77^{F375C} was the only mutant that did not significantly increase the K_m towards glutaryl-7-ACA. The other sulphur residue Met caused a comparable decrease of k_{cat} accompanied by an 5-fold increase of K_m. The substitution of Phe by Tyr lowered k_{cat} only marginally, but it did cause a 2.5-fold increase of K_m. A similar effect was caused by the F375S mutation, but the other hydroxyl residue Thr had a stronger negative effect on the kinetic parameters. With regard to the aliphatic residues, the smaller residues Gly and Ala caused a less drastic decline of kinetic parameters than the larger residues Val, Leu and Ile. The introduction of all other residues resulted in a drastic decrease of catalytic efficiency.

Hydrolysis of adipyl-7-ADCA

All mutants showed unique kinetic parameters on the desired substrate adipyl-7-ADCA. Five mutants showed an increased catalytic efficiency, three a catalytic efficiency similar to wild type, and eleven a decreased catalytic efficiency (Table 1 and Figure 3). The catalytic efficiency towards adipyl-7-ADCA was increased 6-fold by the introduction of a Cys at position 375, mainly due to a strong reduction of K_m. The other sulphur-containing residue, Met, increased the catalytic efficiency by a factor of 2 by slightly increasing k_{cat} and decreasing K_m. The hydrophilic residues Asn, Gln and His all increased k_{cat}. The catalytic efficiency was increased more than 2-fold by the introduction of Asn and His but not of Gln. In contrast to Asn, Gln and His, the hydrophilic hydroxyl residues Ser and Thr significantly lowered k_{cat} and did not affect K_m. The aliphatic residue Leu increased the catalytic efficiency almost 2-fold by increasing k_{cat} and decreasing K_m. The other aliphatic residues Gly, Ala, Val and Ile all decreased k_{cat}, and only the smallest residues, Gly and Ala, resulted in a lower K_m. The aromatic residue Tyr did not change the kinetic parameters significantly, whereas Trp caused a drastic decrease of k_{cat} accompanied by an increase of K_m. Finally, the introduction of a charged residue, e.g. Arg, Asp, Glu or Lys, lowered the catalytic efficiency to less than 3% of wild type due to both a strong decrease of k_{cat} and a significant increase of K_m.

Table 1. Kinetic parameters of wild type and mutant cephalosporin acylases.
The kinetic parameters were calculated from Eadie-Hofstee plots. Values given are mean ± S.D. of at least three independent measurements. N.D., not detectable.

Residue	adipyl-7-ADCA		glutaryl-7-ACA	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Phe (WT)	0.49 ± 0.04	1.0 ± 0.2	4.0 ± 0.3	0.031 ± 0.002
Ala	0.20 ± 0.01	0.51 ± 0.08	2.35 ± 0.07	0.07 ± 0.01
Arg	0.018 ± 0.002	10 ± 1	0.21 ± 0.05	0.6 ± 0.2
Asn	0.9 ± 0.1	0.83 ± 0.09	1.96 ± 0.05	0.18 ± 0.03
Asp	0.015 ± 0.004	11 ± 3	0.010 ± 0.002	0.22 ± 0.06
Cys	0.55 ± 0.03	0.17 ± 0.03	2.308 ± 0.007	0.038 ± 0.008
Glu	0.08 ± 0.02	12 ± 2	0.008 ± 0.001	0.2 ± 0.1
Gln	0.68 ± 0.05	2.1 ± 0.3	0.80 ± 0.02	0.7 ± 0.2
Gly	0.073 ± 0.004	0.16 ± 0.02	2.46 ± 0.08	0.066 ± 0.008
His	1.16 ± 0.08	0.9 ± 0.2	1.334 ± 0.007	0.11 ± 0.03
Ile	0.097 ± 0.003	2.07 ± 0.03	0.67 ± 0.04	0.10 ± 0.02
Leu	0.67 ± 0.04	0.7 ± 0.1	1.10 ± 0.09	0.26 ± 0.07
Lys	0.036 ± 0.001	2.16 ± 0.07	2.2 ± 0.4	0.7 ± 0.2
Met	0.65 ± 0.02	0.65 ± 0.04	2.02 ± 0.09	0.17 ± 0.02
Pro	N.D.	N.D.	N.D.	N.D.
Ser	0.11 ± 0.01	0.99 ± 0.09	3.1 ± 0.2	0.07 ± 0.02
Thr	0.24 ± 0.04	0.9 ± 0.4	1.8 ± 0.1	0.09 ± 0.03
Trp	0.026 ± 0.001	1.5 ± 0.2	0.47 ± 0.06	0.8 ± 0.1
Tyr	0.43 ± 0.05	0.7 ± 0.1	3.64 ± 0.05	0.08 ± 0.01
Val	0.090 ± 0.003	1.37 ± 0.07	1.02 ± 0.08	0.10 ± 0.02

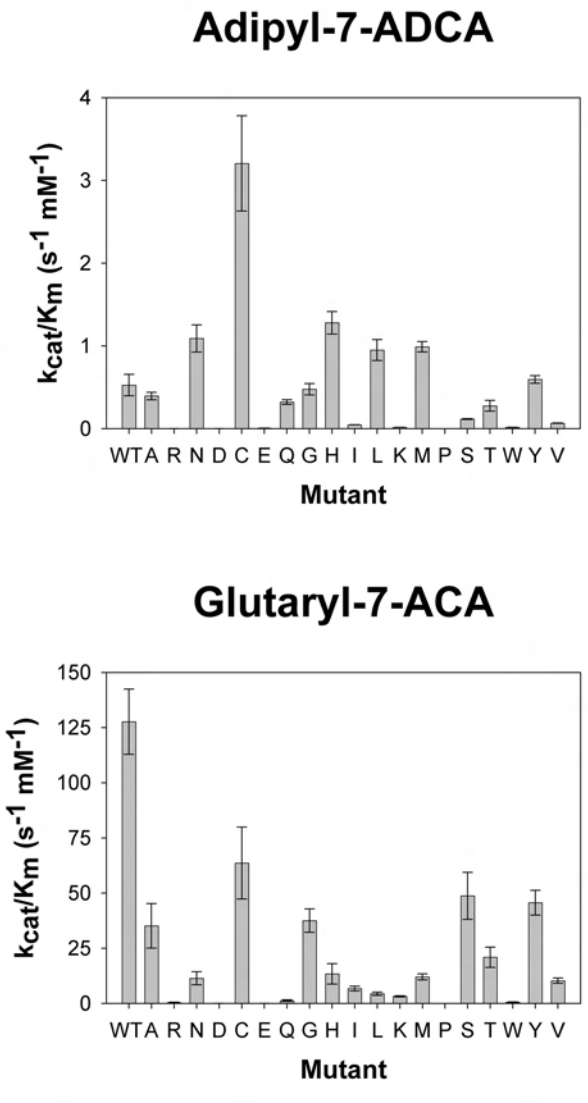


Figure 3. Catalytic efficiency of wild type and mutant cephalosporin acylases.
The kinetic parameters were calculated from Eadie-Hofstee plots. Values given are mean ± S.D. of at least three independent measurements.

Hydrolysis of CPC

Deacylation of CPC by neither wild type *Pseudomonas* SY-77 glutaryl acylase nor any of the mutants could be detected by the fluorescamine assay under the described conditions, in spite of using over three times more enzyme and a 10-fold longer incubation period than in any of the assays using adipyl-7-ADCA and glutaryl-7-ACA (data not shown).

Discussion

In order to investigate the function of position 375 of cephalosporin acylase all 19 mutants of Phe375 were expressed in *E. coli* and purified by column chromatography. Remarkably, all mutants could be produced in high quantities and showed distinctive kinetic parameters on the desired substrate and preferred substrate of the enzyme, adipyl-7-ADCA and glutaryl-7-ACA, respectively. This points at the importance of Phe375 for substrate specificity.

Some mutants did not mature efficiently. The current model for the autocatalytic processing of cephalosporin acylase comprises an intramolecular cleavage followed by an intermolecular cleavage. The side chain binding pocket of the active site is thought to be involved only in the intermolecular cleavage [118,119]. Surprisingly, however, mutagenesis of position 375, which is part of the side chain binding pocket, does not affect the intermolecular cleavage but can affect the intramolecular cleavage. No band corresponding to the polypeptide consisting of α-subunit plus spacer peptide was visible on SDS-PAGE, but five mutants showed a band corresponding to the precursor consisting of α-subunit, spacer peptide and the β-subunit (Figure 2). Additionally, the hydrolysis activity towards glutaryl-7-ACA of the mutants (Table 1) is not related to the capability to perform autocatalytic processing. Our results thus show that intermolecular processing and catalytic activity are not strictly linked.

Until now, the recognised preferred substrate of cephalosporin acylase is glutaryl-7-ACA. It is therefore not surprising that none of the mutations resulted in an improvement of the kinetic parameters using glutaryl-7-ACA, and most mutations resulted in a decline of the kinetic parameters. The hydrolysis of β-lactam substrates by β-lactam acylases proceeds via the formation of an acyl-enzyme intermediate, which is subsequently hydrolysed. In the case of *E. coli* penicillin G acylase, which active site is very similar to that of cephalosporin acylase [30], it has been indicated that the acylation reaction is the rate-limiting step [120]. Assuming this also to be applicable to cephalosporin acylase, k_{cat} would represent the rate of acylation, and K_m the binding of substrate in the active site. In the crystal structure of the enzyme complexed with glutaryl-7-ACA the side chain of the substrate is held in place by various interactions in order to properly position the scissile bond for a nucleophilic attack by Ser199 and form the acyl-enzyme intermediate [32]. The aliphatic part of the side chain protrudes through a ring-like structure of hydrophobic residues consisting of Leu222, Val268 and Phe375, and the carboxylate head of the side chain is positioned in a hydrophilic cavity by electrostatic interactions with Arg255 and hydrogen bonds with Tyr178 and Tyr231. The aromatic ring of Phe375 is positioned alongside the glutaryl side chain (Figure 4). This may explain why the incorporation of the small hydrophobic residues Gly, Ala and Cys causes the smallest negative effects on the kinetic parameters towards glutaryl-7-ACA. The incorporation of some polarity can be overcome, as is demonstrated by the relatively small effects of the F375Y and F375S mutations, but the incorporation of residues with larger side chains results in a poor hydrolysis activity, probably due to

steric hindrance of the side chain.

In order to shift the substrate specificity of the enzyme towards the desired substrate adipyl-7-ADCA, the longer adipyl side chain must be accommodated by the side chain binding pocket while the scissile bond is maintained in a favourable position with respect to Ser199. Two strategies may be used: the hydrophobic ring has to be widened in order to accommodate a twisted aliphatic chain, or the carboxylate head has to be pulled further into the side chain binding pocket, which was proposed to be the mechanism of the improved hydrolysis of adipyl-7-ADCA by mutant SY-77^{Y178H} [28]. The slightly different fit of glutaryl-7-ACA and glutarate in the substrate binding site [32] and the alkylation of Trp202 by substrate analogues [121] indicate that some flexibility in the binding of substrates exists, a prerequisite for these two strategies.

Depending on the nature of the residue that substitutes Phe375 either mechanism may apply. The substitution of Phe375 by the smaller hydrophobic residues Cys, Leu and Met will expand the hydrophobic ring and generate extra space for the longer side chain. Apparently, this is a very delicate procedure, since Val and Ile have a negative influence on the kinetic parameters. The data suggest that a greater expansion of the ring, e.g. via substituting Phe375 by Gly or Ala, increases the ability of the side chain binding pocket to accommodate the adipyl side chain, as is indicated by a strong decrease of K_m. At the same time, the lower k_{cat} values suggest that the positioning of the scissile bond for hydrolysis is less optimal in these mutants.

As for the second mechanism, the increase of k_{cat} upon substituting Phe375 by the hydrogen-bond donors Asn, Gln or His indicates that position 375 can also be used to pull the adipyl side chain further into the side chain binding pocket by means of direct or indirect hydrogen-bonding to the carboxylate head. Again, the structural alterations seem to be very delicate, since the potentially hydrogen bonding Ser and Thr have a negative effect on the k_{cat}, and substitution by Tyr, which is basically a phenylalanine with a hydrogen bonding hydroxyl group, does not alter the kinetic parameters. The effects of these mutations on the hydrolysis of glutaryl-7-ACA are different and suggest that the carboxylate head of the glutaryl side chain cannot use hydrogen bonding to residue 375 to increase binding and hydrolysis. Apparently, the shorter length of the side chain limits the degrees of freedom and thereby prohibits alternative binding modes.

The introduction of a charged residue (Arg, Lys, Glu, Asp) or the bulky Trp at position 375 seems to block the passage of the charged carboxylate head of the side chain through the hydrophobic ring and results in very poor catalytic parameters towards both adipyl-7-ADCA and glutaryl-7-ACA. Surprisingly, most of these mutations do not impair the autocatalytic processing of the enzyme, another indication that processing and catalytic activity are not as closely linked as has been suggested by others.

This study shows that hydrolysis of adipyl-7-ADCA can be increased by improving the hydrophobic interactions between amino acid 375 and the aliphatic side chain of the substrate, as well as by hydrogen bonding of residue 375 to the carboxylate head of the substrate. Furthermore, residue 375 can function as a gatekeeper by blocking passage

through the hydrophobic ring of the substrate binding site by steric or electrostatic means. These results demonstrate that residue 375 is a key amino acid in the protein engineering of cephalosporin acylase. The saturation mutagenesis, in which the effects of all 20 amino acids at this position were analysed, was essential for the discovery of the three different modes by which residue 375 can dictate substrate specificity. Although the catalytic efficiency for adipyl-7-ADCA can be improved 6-fold by mutagenesis of Phe375, no activity on CPC could be detected. Apparently, the amino moiety in the side chain of CPC still comprises an insurmountable problem for an efficient hydrolysis, and additional mutations are required. Mutation F375C decreases the K_m towards adipyl-7-ADCA by a factor six while the K_m towards glutaryl-7-ACA remains identical, indicating that the substrate specificity of this mutant has been extended rather than shifted. Mutant SY-77^{F375C} may thus be regarded as the template of choice for future mutagenesis studies.

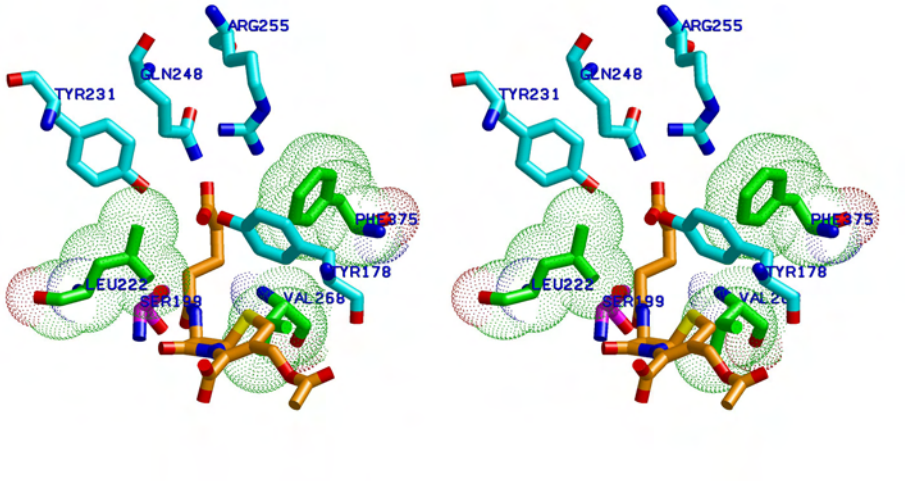


Figure 4. Three-dimensional model of the binding of the side chain of glutaryl-7-ACA by cephalosporin acylase.
Residues Leu222, Val268 and Phe375 (green) form a hydrophobic ring-like structure, through which the aliphatic part of the side chain of glutaryl-7-ACA (gold) protrudes, placing the carboxylate head of the side chain in a hydrophilic cavity consisting of Tyr178, Tyr231, Gln248 and Arg255 (cyan). Ser 199 (magenta) performs the nucleophilic attack on the scissile bond of the substrate. The Van der Waals radii of the residues forming the hydrophobic ring are shown in dots. Phe375 interacts with C4 of the side chain and pushes the hydrophilic head into the cavity (Figure made with RasTop version 2.0.3, www.geneinfinity.org/rastop/, using the co-ordinates of PDB entry 1JVZ).

Acknowledgements

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Chapter 5

Selection of multiple mutated cephalosporin acylases with modified substrate specificity

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Part of this work has been submitted.

Cephalosporin acylases are important enzymes in the production of semi-synthetic β-lactam antibiotics. In the search for improved hydrolysis of cephalosporin C and adipyl-7-ADCA both rational and random mutagenesis approaches have been used. It was concluded that five residues in the active site of the enzyme are important for the binding of the substrate; Tyr178, Tyr231, Arg255, Asn266 and Phe375. Single mutants of residues Tyr178, Asn266 and Phe375 were shown to result in improved activity towards both adipyl-7-ADCA and cephalosporin C. It is, however, likely that a combination of mutations may improve this activity even further. Therefore, we integrated random and rational approaches to change the substrate specificity of a cephalosporin acylase.

We started with the combination of the best mutant in the α-subunit with known improved mutants in the β-subunit. Biochemical analysis of the combinants showed, however, that only one of the mutants, SY-77$^{Y178H+F375L}$, acquired an increased k_{cat} compared to both single parents on adipyl-7-ADCA. All other combinations resulted in a similar or worsened value for both k_{cat} and K_m resulting in a similar or lower catalytic efficiency.

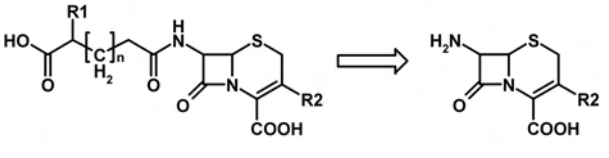
In a second experiment, we created a mutant library in which all possible amino acids at the hot spot positions 266 and 375 were combined. This library was cloned into a background sequence of wild type acylase and with the best α-subunit mutation, Y178H, respectively. In order to avoid extensive screening, the libraries were selected on minimal medium containing adipyl-leucine or amino adipyl-leucine as sole leucine source. No mutants could be selected from the selective plates with amino adipyl-leucine. From the adipyl-leucine plates, however, four different multiple mutants were selected showing a 4-fold improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA. They were purified and catalytic parameters towards glutaryl-7-ACA, adipyl-7-ADCA and cephalosporin C were determined. On adipyl-7-ADCA, k_{cat} values of the multiple mutants are equal or lower than those of the single mutants, while K_m values have improved. Remarkably, only multiple mutants comprising the N266Q mutation display a higher additive catalytic efficiency (k_{cat}/K_m) than the single mutants. Although the mutants were selected on adipyl-leucine, some of them showed improved hydrolytic activity of cephalosporin C compared to wild type acylase. Surprisingly, the single mutant SY-77^{Y178H} was found to have the highest cephalosporin hydrolysis activity. None of the selected combinations of this mutation with mutations at position 266 and/or 375 results in an increased activity towards cephalosporin C.

As a last approach, the randomisation of all five important substrate specificity residues was pursued. In order to prevent a bias towards the original codons, we randomised the amino acids Tyr178, Tyr231, Arg255 and Asn266 by building this part of the gene synthetically. We hybridised and ligated degenerate oligonucleotides containing NNS codons at the positions to be randomised. This synthetic part was cloned into a pool of vectors containing all amino acids on

position 375. The ligation and hybridisation of the synthetic gene resulted in a mutant library that comprises in theory $3*10^6$ different mutants, which covers statistically 94% of the total possible library. Unfortunately, cloning and transformation of the total fragment into the randomised pool of vectors did not yield sufficient transformants. It resulted only in a total of $1.3*10^5$ different colonies, which represents 0.13% of the possible library of the five totally randomised residues. Plating of this small portion of the total library on minimal medium containing amino adipyl-leucine did not result in any active mutants. A new cloning procedure may, however, result in some multiple mutants with an improved activity towards amino adipyl-leucine and consequently towards cephalosporin C.

Introduction

Cephalosporin acylases are important enzymes in the environmentally friendly production of the key intermediates of semi-synthetic cephalosporins, 7-amino-desacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA) (Figure 1). The hydrolytic activity of cephalosporin acylases towards the substrates adipyl-7-ADCA and cephalosporin C (CPC) is, however, very small and industrially not exploitable. In former research we used directed evolution strategies in order to pinpoint important residues of the glutaryl acylase from *Pseudomonas* SY-77 for substrate specificity [28,116]. The results of the directed evolution experiments could be rationalised with the three-dimensional structure of the highly homologous glutaryl acylase from *Pseudomonas diminuta* KAC-1 [32]. Based on these findings, five residues in the active site of the enzyme are considered particularly important for the binding of the substrate; Tyr178, Tyr231, Arg255, Asn266 and Phe375. Four of these residues were also mentioned in rational mutagenesis approaches, but Asn266 was never indicated [24,32,115].



Compound	n	R1	R2
Glutaryl-7-ACA	1	-H	$-CH_2OCOCH_3$
Adipyl-7-ADCA	2	-H	$-CH_3$
CPC	2	$-NH_2$	$-CH_2OCOCH_3$
7-ACA	-	-	$-CH_2OCOCH_3$
7-ADCA	-	-	$-CH_3$

Figure 1: The production of 7-A(D)CA from β-lactam compounds.

In order to see the influence of the five amino acids on substrate specificity, saturation mutagenesis of single residues was performed previously. Within the α-subunit of SY-77 glutaryl acylase, the single mutant SY-77^{Y178H} was found to have the highest hydrolysis activity towards adipyl-7-ADCA [28]. In more detailed studies, positions Asn266 and Phe375 were changed into all 19 other amino acids by site-directed mutagenesis and the activity of the purified mutants was analysed [122,123]. It could be concluded that most amino acids are structurally allowed at either position and that every mutant has its own substrate specificity profile towards glutaryl-7-ACA, adipyl-7-ADCA and CPC. Although up to a 15-fold increase of the catalytic efficiency for hydrolysis of adipyl-7-ADCA was measured, it appeared likely that further improvement towards this compound and activity towards CPC is possible by additional mutations or different combinations of mutations. In spite of being part of the substrate binding site, no mutations were found at positions Tyr231 and Arg255 upon random mutagenesis of the β-subunit. Probably, single mutations at these positions deteriorate acylase activity as Tyr231 is conserved in most acylases and the positive charge of Arg255 is necessary to complement the carboxylic head of the substrate.

The crystal structure of the enzyme reveals a very tight and intricate pattern of interactions among the active site residues and the substrate. It seems logical that several mutations are necessary to significantly improve hydrolysis activity to a desired substrate by creating a new network of interactions with this substrate. Changing only one of the important amino acids will disturb interactions and can result in lower activities towards both the preferred substrate glutaryl-7-ACA and the desired substrates adipyl-7-ADCA and CPC [116,122,123]. Furthermore, the larger side chains of both adipyl-7-ADCA and CPC may need more space and interactions with different amino acids in order to be fully accommodated in the active site of the enzyme. Combinations of single mutations in an enzyme can result in no changes at all or may lead to antagonistic, (partially) additive or synergistic effects, giving insight in the nature and extent of interactions between specific amino acids.

We decided to start with the combination of different pairs of mutations and finish with the total randomisation of all five amino acids mentioned above. Firstly, the best mutation in the α-subunit was combined with mutations in the β-subunit that were known to improve the enzymatic activity. These combinations were screened by biochemical analyses of the purified enzyme. A next step was to randomly combine all amino acids at the two hot spot positions 266 and 375. In order to avoid extensive screening, a library, theoretically comprising all 400 possible mutants, was plated onto selective media containing (amino) adipyl-leucine as sole leucine source. This library was studied in the context of the wild type α-subunit sequence and combined with the Y178H mutation in the α-subunit. Finally, we started with the construction of the library of all five randomised amino acids. Site-saturation mutagenesis is the most straightforward process to create a total randomised library. Maximal randomness is particularly important for larger changes in enzyme activity, like the conversion of a glutaryl acylase into a CPC acylase. Most mutagenesis methods involve a step in which

a mutagenic oligonucleotide is annealed to the wild type template, which may result in a bias towards the original codon as we have observed before [122]. Different modified mutagenesis methods have been developed in order to prevent this bias [124]. The most successful approaches of preventing bias in directed evolution experiments involve the predominant use of synthetic oligonucleotides [65]. Therefore, we decided to randomise the four amino acids Tyr178, Tyr231, Arg255 and Asn266 completely by assembling it from synthetic oligonucleotides. In this way the NNS-codons are present on both strands avoiding bias towards the original codon as was also shown in a degenerate homoduplex shuffling method [95]. Cloning this synthetic part into a pool of vectors comprising all 20 amino acids at position 375 is likely to yield an unbiased library of five totally randomised amino acids, which is not too large to be selected by growth selection. All libraries were plated onto selective media containing adipyl-leucine or amino adipyl-leucine as sole leucine source in order to find better mutants towards both adipyl-7-ADCA and CPC, respectively.

Materials and Methods

Bacterial strains, chemicals, plasmids and DNA manipulations

The plasmid pMcSY-2 [116] and the leucine deficient *Escherichia coli* DH10B (Invitrogen) were used for the cloning of the libraries and the expression of the *Pseudomonas* SY-77 glutaryl acylase. Molecular DNA techniques were executed following standard protocols [41]. The enzymes used for DNA manipulations were purchased from New England Biolabs and Invitrogen and applied according to the instructions of the manufacturer. Isolation and purification of DNA was performed using the QIAprep and QIAquick kits of QIAGEN. The β-lactam substrates glutaryl-7-ACA, adipyl-7-ADCA and CPC (as sodium salt) were gifts from DSM, The Netherlands. Amino adipyl-leucine was synthesised by Syncom B.V., The Netherlands.

Construction and testing of the rationally combined mutants

Improved mutants found in the epPCR library of the β-subunit and saturation mutagenesis of position Phe375 were combined with mutation Y178H in de α-subunit. Initial experiments showed that normal cloning procedures resulted in a significant fraction of wild type sequence in the *Hin*dIII-*Nco*I portion of the gene, probably caused by self-ligation of linearised plasmid that could not be fully separated by gel electrophoresis. To avoid this, the plasmid containing mutation Y178H was truncated by elimination of a small *Stu*I fragment located in the 3' moiety of the gene, encoding part of the β-subunit. The resulting plasmid was named pMcYHtr-Stu. Plating *E. coli* DH10B with this plasmid on minimal plates with glutaryl-leucine, adipyl-leucine or amino adipyl-leucine showed no growth after 14 days of incubation at 30°C. The β-subunits of mutants SY-77^{F229L}, SY-77^{N266H}, SY-77^{N266S}, SY-77$^{M271V+Q291K+T374S}$, SY-77^{F375H} and SY-77^{F375L} were ligated into the truncated plasmid using the appropriate restriction enzymes. Resulting plasmids were sequenced to confirm the presence of the mutations. The mutants were tested for the production of soluble enzyme with a polyclonal antibody by spot blotting as described before [122]. Mutant acylases were purified up to 90% and catalytic parameters on glutaryl-7-ACA, adipyl-7-ADCA and CPC were determined as described before [116].

Construction of the double and triple libraries

To create the library in which both position 266 and 375 are totally randomised, 20 plasmids containing one of the 20 amino acids at position 266 were isolated from *E. coli* DH10B and mixed in equimolar amounts [123]. The same was performed for 20 plasmids containing all different amino acids at position 375 [122]. Fragments containing the 20 F375X mutations were cloned into the backbone of pMcSY-77^{N266X} using the restriction enzymes *Hin*dIII and *Nco*I. Ligation mixtures were transformed to *E. coli* DH10B and plated onto LB plates containing 0.4% glucose and 50 µg/ml chloramphenicol. Ten transformants were picked randomly to determine the DNA sequence of the plasmids. The rest of the transformants was scraped together for further use. This library was named the NF-library.
The fragments from the NF-library containing all 400 mutants were ligated into pMcYHtr-Stu using the restriction enzymes *Xma*I and *Kpn*I. This library was called the YNF-library.

Randomisation of 5 residues using synthetic oligonucleotides

In order to get an unbiased library of five totally randomised residues, synthetic oligonucleotides containing NNS-codons were used (Table 1). The part from the gene between restriction sites *Eco*O109I and *Nco*I was constructed in several steps (Figure 2). The first part, from base 283 until 525, was made by PCR using 0.5 mM of oligonucleotides A1 and B1 in the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 60°C, 1 min at 72°C and a final step of 10 min at 72°C. The resulting PCR product was purified from a 2% agarose gel. The second part of the gene from base 486 until 989 was assembled from synthetic oligonucleotides A2 until A11 and B1 until B10, containing NNS-codons at the codons for amino acids Tyr178, Tyr231, Arg255 and Asn266.
Since synthetic oligonucleotides do not have a phosphate group at the 5'-end, they cannot be joined together by ligase and need to be phosphorylated. In order to get the best possible phosphorylation of the oligonucleotides, they were heated to 100°C for 5 min and immediately put on ice, hereby losing any secondary structures that might block the end of the oligonucleotide from being phosphorylated. After cooling down they were phosphorylated using T4 PolyNucleotideKinase (PNK, Roche) according to the protocol of the supplier. Complementary pairs (e.g. A11 and B1, A2 and B2) were added together and allowed to hybridise by boiling for 5 min in a small water bath, followed by slow ON cooling down in this water bath. Hybridised pairs were ligated together one pair at a time by incubation for 5 min with Ampligase Thermostable DNA ligase (Epicentre). The optimum temperature for this enzyme of 45°C is favourable, since the minimum overhang of 10 bases between the synthetic oligonucleotides has a higher melting temperature then the normal overhang of two to four bases after restriction. The starting temperature of the hybridisation of two double stranded pairs was at least 3°C lower than the melting temperature of each individual pair to prevent the hybridised pairs from melting.

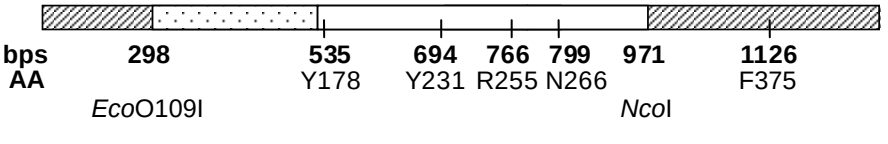


Figure 2. Graphical outline of the strategy in order to get 5 totally randomised residues.

See text for explanation. = vector, = 'normal' PCR, = synthetic gene.

Hybridisation and ligation was performed by lowering temperature 1°C per 2 minutes until 10°C below the hybridising part melting temperature, but not lower than 25°C because of the optimal temperature of Ampligase. The ligation mixtures were kept on ice during which the ligation was checked on a 2% agarose gel. Successfully ligated fragments were used in a following cycle of ligation with Ampligase. After joining all pairs together, the resulting DNA fragments encoding the four randomised amino acids were amplified by PCR using primers A11 and B11 in the following program: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 65°C, 1 min at 72°C and a final step of 10 min at 72°C. In order to calculate the diversity of the amplified PCR product, control PCRs starting from known DNA concentrations were performed. Both parts of the acylase gene were assembled by PCR using 600 ng DNA of each part per 100 µl PCR mix and 0.5 mM of each of the primers A1 and B11 comprising the restriction sites *Eco*O109I and *Nco*I. The following PCR program was used: 3 min at 95°C, 30 cycles of 45 s at 95°C, 1 min at 56°C, 1.5 min at 72°C and a final step of 10 min at 72°C. 160 ng DNA of the resulting library of PCR fragment was ligated into a pool of vectors containing the rest of the acylase gene with codons for all 20 amino acids at position 375.

Table 1. Synthetic oligonucleotides used for randomising 5 residues.
Primers named A are forward primers, B primers are backward primers. NNS codons are given in bold, restriction sites are underlined.

Name	Length	Sequence (5'→3')
A11	29 bps	TTCCGGCGCCGACGTGGTGGCCCACGCCC
A2	32 bps	ACCGCCTGATGAACTTCCTC**NNS**GTCGCGTCG
A3	94 bps	CCCGGGCGCACCCTGGGCGAGGGCGACCCGCCGGACCTGGCATCAA GGATCCAACTCCTGGGCGGTGGCGCCGGGAAAGACGGCGAACGGGA
A4	43 bps	ACGCCCTGCTGCTGCAGAACCCGCACCTGTCCTGGACGACGGA
A5	33 bps	CTACTTCACC**NNS**TACGAGGCGCATCTCGTCAC
A6	39 bps	GCCGGACTTCGAGATCTATGGCGCGACCCAGATCGGCCT
A7	35 bps	GCCGGTCATC**NNS**TTCGCCTTCAACCAGCGGATGG
A8	32 bps	GCATCACC**NNS**ACCGTCAACGGCATGGTGGGG
A9	88 bps	GCCACCAACTATCGGCTGACGCTTCAGGACGGCGGCTATCTGTATGAC GGTCAGGTGCGGCCGTTCGAGCGGCCTCAGGCCTCGTATC
A10	77 bps	GCCTGCGTCAGGCGGACGGGACGACGGTCGACAAGCCGTTGGAGATC CGCTCCAGCGTCCATGGCCCGGTCTTCGAG
B1	39 bps	ATCAGGCGGTGGGCGTGGGCCACCACGTCGGCGCCGGAA
B2	35 bps	GGGTGCGCCCGGGCGACGCGAC**SNN**GAGGAAGTTC
B3	62 bps	CGGCGCCACCGCCCAGGAGTTGGATCCTTGATCGGCCAGGTCCGGCG GGTCGCCCTCGCCCA
B4	51 bps	GGACAGGTGCGGGTTCTGCAGCAGCAGGGCGTTCCCGTTCGCCGTCTT TCC
B5	34 bps	GCGCCTCGTA**SNN**GGTGAAGTAGTCCGTCGTCCA
B6	41 bps	CTGGGTCGCGCCATAGATCTCGAAGTCCGGCGTGACGAGAT
B7	33 bps	GTTGAAGGCGAA**SNN**GATGACCGGCAGGCCGAT
B8	32 bps	CCGTTGACGGT**SNN**GGTGATGCCCATCCGCTG
B9	85 bps	AGGCCGCTCGAACGGCCGCACCTGACCGTCATACAGATAGCCGCCGT CCTGAAGCGTCAGCCGATAGTTGGTGGCCCCCACCATG
B10	90 bps	CTCGAAGACCGGGCCATGGACGCTGGAGCGGATCTCCAACGGCTTGT CGACCGTCGTCCCGTCCGCCTGACGCAGGCGATACGAGGCCTG
A1	30 bps	GGGGGCCGAATACTGGGGCCCGGATTACG
B11	20 bps	CTCGAAGACCGGGCCATGGA

After transformation of these mutant plasmids into the leucine-deficient *E. coli* strain DH10B, transformants were scraped together from LB plates, containing 0.4% glucose and 50 µg/ml chloramphenicol, and frozen as -80°C stocks.

Selection, purification and catalytic parameter determination of mutant enzymes

E. coli DH10B containing the different libraries were taken from -80°C stocks, diluted 5 times in 2x YT-medium and grown at 37°C (250 rpm) for 1 hour, starved for 2 hours in 0.9% NaCl (37°C, 250 rpm), plated onto minimal medium containing 0.1 mg/ml adipyl-leucine or amino adipyl-leucine as sole leucine source and incubated at 30°C. *E. coli* DH10B expressing wild type SY-77 glutaryl acylase and mutant SY-77^{Y178H} were also plated onto the selection plates as a control for the different libraries. Plasmids of transformants that grew faster than control colonies were isolated, retransformed to *E. coli* DH10B and plated onto the same selection medium again. Single colonies appearing on the second plate were tested for the production of enzyme with a polyclonal antibody by spot blotting as described before [122]. Acylase producing mutants were used for an initial activity screen with crude cell extracts. Mutants with an improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA were purified to >90% purity by anion exchange and hydrophobic interaction column chromatography. The kinetic parameters on glutaryl-7-ACA and adipyl-7-ADCA were determined using different substrate concentrations in a fluorescamine assay as described before [116].

Results

Combining the best α- and β-subunit mutations

In previous research we obtained mutants in both the α- and β-subunit which had an improved hydrolysis activity towards adipyl-7-ADCA. Since the multiple mutations of one of the mutants (SY-77$^{M271V+Q291K+T374S}$) appeared to be additive [116], we constructed other combinations which could also have additive or even synergistic effects. Therefore, the best mutation in the α-subunit, Y178H, was combined with six improved enzymes having mutations in the β-subunit, SY-77^{F229L}, SY-77^{N266H}, SY-77^{N266S}, SY-77$^{M271V+Q291K+T374S}$, SY-77^{F375H} and SY-77^{F375L}. All combinations resulted in soluble enzymes and were purified (> 90% pure). Activity towards both glutaryl-7-ACA and adipyl-7-ADCA was measured using the fluorescamine assay. Hydrolysis of glutaryl-7-ACA was decreased in all mutants compared to their single parents (data not shown). On adipyl-7-ADCA only mutant SY-77$^{Y178H+F375L}$ showed an increased k_{cat} compared to both single parents. All other combinations showed similar or worsened k_{cat} and K_m values resulting in a similar or lower catalytic efficiency (Table 2). Remarkably, the high k_{cat} value of single mutant SY-77^{F375H} is reduced 2.6-fold when combined with mutation Y178H.

Construction and selection of libraries randomised at positions Asn266 and Phe375

A library was made by combining gene fragments encoding all 20 amino acids at position 266 with those that encode all 20 amino acids at position 375. Sequence

Table 2. Catalytic parameters of rationally constructed mutants and their parents on adipyl-7-ADCA.

The k_{cat} and K_m on adipyl-7-ADCA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier. The catalytic parameters of mutant SY-77^{Y178H} are mentioned separately, since it is the parent of these mutants.

Mutation(s)	k_{cat} (s^{-1})		K_m (mM)	
	multiple	- Y178H	multiple	- Y178H
Y178H	0.66 ± 0.05	0.38 ± 0.02	0.47 ± 0.05	0.8 ± 0.1
Y178H+F229L	0.71 ± 0.05	0.47 ± 0.02[a]	1.1 ± 0.2	0.52 ± 0.04[a]
Y178H+N266H	0.95 ± 0.04	0.55 ± 0.02[a]	0.19 ± 0.07	0.14 ± 0.02[a]
Y178H+N266S	0.43 ± 0.06	0.34 ± 0.02[a]	0.9 ± 0.3	0.42 ± 0.04[a]
Y178H+M271V+Q291K+T374S	0.44 ± 0.09	0.61 ± 0.04[a]	0.8 ± 0.2	0.7 ± 0.1[a]
Y178H+F375H	0.44 ± 0.03	1.16 ± 0.08[b]	0.56 ± 0.04	0.9 ± 0.2[b]
Y178H+F375L	1.0 ± 0.2	0.67 ± 0.04[b]	0.7 ± 0.1	0.7 ± 0.1[b]

[a] Values were taken from ref. [116]
[b] Values were taken from ref. [122]

analysis of 10 randomly picked clones all revealed different combinations of amino acids at positions 266 and 375, indicating that the random combination of the two positions succeeded. After correction for self-ligation, the plasmids that contain the library randomised at positions Asn266 and Phe375 (NF-library) or this library combined with mutation Y178H (YNF-library) was transformed to *E. coli* DH10B. This resulted in approximately 2000 and 800 discrete colonies for the NF- and YNF-library, which statistically includes all 400 possible mutants with a certainty of 99% and 86%, respectively. The transformants were scraped together and frozen as -80°C stocks.

The frozen stocks of both libraries were thawed, diluted into 2x YT-medium and revived for one hour at 37°C. The cells were washed and starved in 0.9% NaCl in order to drain the internal leucine supply. After another washing step, cells were plated onto minimal medium containing adipyl-leucine or amino adipyl-leucine as a sole leucine source and incubated at 30°C. As a control for the NF- and YNF-library, respectively, both *E. coli* DH10B expressing wild type SY-77 glutaryl acylase and mutant SY-77^{Y178H} were plated. Plates were inspected daily for eight days until very small colonies started growing on both negative control plates and selection plates. At the 9th day, 35 of the largest and earliest spotted clones from both libraries on the adipyl-leucine plates were streaked onto fresh selection plates and incubated at 30°C (Table 3). At the same day the YNF-library showed some very small dots on the selective plates containing amino adipyl-leucine, from which the three largest were selected and streaked onto a fresh selection plate. The NF-library showed no colonies at all on amino adipyl-leucine selection plates. When equal amounts of bacteria of the libraries and wild type were plated with glutaryl-leucine as sole leucine source, a much smaller number of colonies was observed with the libraries.

Plasmids from selected transformants were retransformed to *E. coli* DH10B and production of acylase was tested. Unfortunately, 61% of the transformants did not produce any acylase as shown by spot blotting using a polyclonal rabbit antibody. Crude

extracts of the transformants that did show acylase production were tested for activity towards glutaryl-7-ACA and adipyl-7-ADCA (Table 3). Sequencing of the 15 mutants that produced acylase revealed that some mutants were selected more than once. The mutants SY-77$^{Y178H+N266H}$ and SY-77$^{Y178H+N266M}$ were each selected three times and mutant SY-77$^{N266Q+F375L}$ was selected twice. Of the 12 different mutants only four mutant enzymes showed an improved ratio of adipyl-7-ADCA over glutaryl-7-ACA compared to the wild type enzyme, the other mutants showed hardly any activity at all. All four active enzymes comprised multiple mutations and had an improved ratio of at least four times. Three of these mutants were the ones that were selected multiple times.

Table 3. Selection of transformants of the NF- and YNF-library.
AL, adipyl-leucine; AAL, amino adipyl-leucine; A-7-A, adipyl-7-ADCA; G-7-A, glutaryl-7ACA.

Library and selection medium	# selected mutants	# acylase producers	# different sequences	# better A-7-A/G-7-A	# better on CPC
NF on AL	20	8	7	1	0
NF on AAL	0	-	-	-	-
YNF on AL	15	7	3	3	2
YNF on AAL	3	0	-	-	-

Determining catalytic parameters of mutants found in the NF- and YNF-library

The kinetic parameters k_{cat} and K_m of the purified mutant proteins were determined on glutaryl-7-ACA and adipyl-7-ADCA. Since the hydrolysis of CPC is too low for wild type SY-77 to determine the catalytic parameters k_{cat} and K_m, the conversion rates of the mutants were calculated from the linear slope of hydrolysis in the first four hours (Table 4 and Figure 3). In order to be able to conclude whether the combination of single mutations have additive or even synergistic effects, values from former research of the respective single mutants were added to the table and figure. As seen before, hydrolysis activity of the preferred substrate glutaryl-7-ACA decreases upon improvement towards other substrates. The k_{cat} values of the multiple mutants have decreased compared to the single mutants, whereas the K_m values have increased, resulting in a much lower catalytic efficiency. On adipyl-7-ADCA, the k_{cat} values of multiple mutants are equal or lower than those of the single mutants. The K_m values, however, have improved compared to the associated single mutants. This results in a diverse spectrum of the catalytic efficiency values (Figure 3). Multiple mutants comprising mutations N266Q and F375L/M display a higher catalytic efficiency than the single mutants. The differences are, however, additive and not synergistic. The combination of Y178H with N266H/M does not lead to significantly different catalytic efficiencies on adipyl-7-ADCA than those of the single mutants SY-77^{N266H} or SY-77^{N266M}.

Surprisingly, the single mutant SY-77^{Y178H} shows the highest cephalosporin hydrolysis activity. None of the selected combinations of this mutation with mutations at positions 266 and/or 375 results in an increase of activity towards CPC. Adding N266H to mutation Y178H hardly affects the activity, but the double mutant SY-77$^{Y178H+N266M}$ has

a significantly lower hydrolysis activity than the single mutant. Single mutations at position 375 or combinations with Y178H and/or N266Q do not seem to be beneficial for the enzyme's cephalosporin hydrolysis activity.

Table 4. Catalytic parameters of selected mutants and their single parents on adipyl-7-ADCA and glutaryl-7-ACA.

The k_{cat} and K_m on adipyl-7-ADCA and glutaryl-7-ACA were determined by measuring the initial rate of hydrolysis on a range of substrate concentrations with a fixed amount of enzyme as described earlier. Mutated amino acids are in bold.

Amino Acid at pos.			**adipyl-7-ADCA**		**glutaryl-7-ACA**	
178	266	375	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Tyr	**Gln**	**Leu**	0.48 ± 0.06	0.17 ± 0.02	0.75 ± 0.07	0.17 ± 0.03
His	**His**	Phe	0.95 ± 0.04	0.19 ± 0.07	2.2 ± 0.1	0.08 ± 0.02
His	**Met**	Phe	1.2 ± 0.2	0.17 ± 0.02	0.89 ± 0.02	0.30 ± 0.05
His	**Gln**	**Met**	0.72 ± 0.03	0.28 ± 0.06	0.72 ± 0.06	0.62 ± 0.09
Tyr	Asn	Phe	0.38 ± 0.02	0.8 ± 0.1	4.1 ± 0.3	0.042 ± 0.009
His	Asn	Phe	0.66 ± 0.05	0.47 ± 0.05	2.5 ± 0.2	0.08 ± 0.01
Tyr	**Gln**	Phe[1]	0.46 ± 0.04	0.62 ± 0.13	2.4 ± 0.2	0.12 ± 0.03
Tyr	Asn	**Leu**[2]	0.67 ± 0.04	0.7 ± 0.1	1.10 ± 0.09	0.26 ± 0.07
Tyr	**His**	Phe[1]	0.47 ± 0.01	0.14 ± 0.01	3.1 ± 0.3	0.04 ± 0.01
Tyr	**Met**	Phe[1]	1.9 ± 0.1	0.33 ± 0.05	1.8 ± 0.1	0.17 ± 0.02
Tyr	Asn	**Met**[2]	0.65 ± 0.02	0.65 ± 0.04	2.02 ± 0.09	0.17 ± 0.02

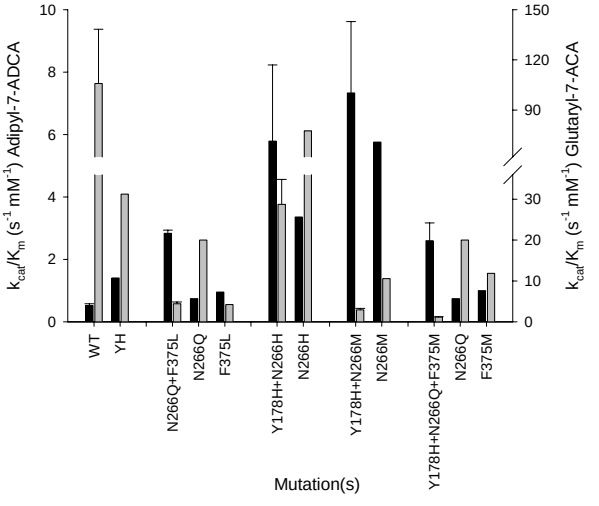
[1] Values were taken from ref. [123]
[2] Values were taken from ref. [122]

Construction and selection of the 5 randomised amino acids library

In order to randomise the four amino acids Tyr178, Tyr231, Arg255 and Asn266 completely, we assembled the part of the gene containing these residues from synthetic oligonucleotides (Table 1). After phosphorylation, complementary pairs were hybridised by heating them for five minutes and cooling down very slowly in order to cover the whole temperature range in which different randomised codons anneal to each other.

First it was tried to assemble all double stranded primer pairs in one tube, but this did not result in any band of the right size. Three PCR rounds of 25 cycles each with primers on the outer ends of the desired product were necessary to get a faint band of the right size. Calculation of the concentration of this band showed that it could only have originated from a pool of DNA that was too small to comprise all possible mutants. Therefore, we decided to connect the different double stranded pairs one at a time. To obtain the best possible hybridisation and ligation without separating the previously hybridised strands, a controlled ligation was performed, using the thermostable Ampligase to ligate the different pairs. Ligation of single and double pairs normally resulted in bands of the right size (Figure 4A and 4B). The ligation of larger fragments resulted, however, in very faint bands, which made it difficult to analyse the success of the experiment. Boosting of the final product with primers at the front and

A



B

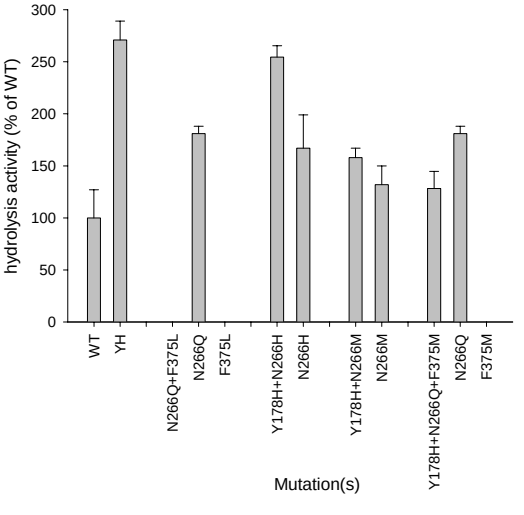


Figure 3. Catalytic efficiencies of selected mutants and their single parents on adipyl-7-ADCA and glutaryl-7-ACA (A) and conversion rates on CPC (B).
Values of earlier described parents were added to the graph in order to compare single and double mutants. Conversion of cephalosporin by mutant SY-77$^{N266Q/F375L}$ was not measurable.

back of the synthetic gene resulted in a band of the right size (Figure 4C). The concentration of this band was calculated and compared with similar PCR products from known start concentrations of DNA. This revealed that the PCR had started from $3*10^6$ molecules which is sufficient to cover all possible combinations ($32^4=1*10^6$). The first part of the synthetic gene was produced by a normal PCR and both PCR products were ligated together by a subsequent PCR step. This resulted in a clear band of the expected size that was cloned into a pool of vectors comprising all 20 amino acids at position 375 (Figure 2). Transformation of the ligation mixture resulted in a total of $1.3*10^5$ different colonies, which comprises only 0.13% of the possible library of five totally randomised residues with 99% certainty. Nonetheless, all transformants were scraped and frozen at -80°C.

The frozen stock of this library was thawed, starved and plated onto minimal medium containing amino adipyl-leucine as sole leucine source. After two weeks 27 colonies were picked, the plasmids were retransformed to *E. coli* DH10B and plated onto minimal medium again. After five days single colonies were picked and acylase production of the 25 remaining colonies was tested. Only eight colonies revealed acylase production, but none of them showed any activity towards glutaryl-7-ACA, adipyl-7-ADCA or CPC.

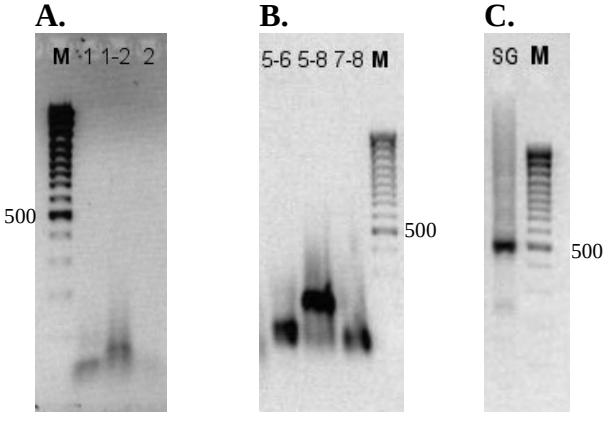


Figure 4. Typical examples of hybridisation of one or two oligonucleotide pairs using Ampligase (A, B) and the resulting synthetic gene (C).

M, Low Range 100 bps Marker, Fermentas MBI

A. Hybridisation of two single pairs: 1, pair A11-B1 (39 bps); 1-2, hybridised pairs 1 and 2 (74 bps); 2, pair A2-B2 (45 bps).

B. Hybridisation of two double pairs: 5-6, ligated oligos A5-B5 and A6-B6 (83 bps); 5-8, ligated pairs 5-6 and 7-8 (152 bps); 7-8, ligated oligos A7-B7 and A8-B8 (77 bps).

C. Synthetic gene (SG) after boosting hybridisation of all 10 pairs (504 bps).

Discussion

Protein engineering of cephalosporin acylases has been pursued for some time in order to find an industrially relevant activity towards adipyl-7-ADCA or CPC. Rational and directed evolution approaches have been used by us and other researchers to achieve this goal [24,28,115,116,122,123]. In this research, we used a combination of both strategies to explore the sequence space of the substrate binding pocket of the enzyme in depth. This was performed in several subsequent experiments.

Rational recombination of improved mutants

First, we rationally combined the mutants in the α- and β-subunit with the highest activity towards adipyl-7-ADCA and determined their catalytic parameters towards glutaryl-7-ACA and adipyl-7-ADCA. Only mutant SY-77$^{Y178H+F375L}$ showed an increased k_{cat} towards adipyl-7-ADCA when compared to both single parents. As described before, His178 may attract the carboxyl group of the adipyl-7-ADCA, forcing the longer adipic side chain to go further inside the active site [28]. The extra space created by mutation F375L can accommodate also part of the side chain, thereby positioning the amide bond even closer to the active site Ser199 [122]. Some other mutations seem to counteract each other, like Y178H and F375H, which both may attract the head of the adipic side chain and thereby decrease the hydrolysis activity towards both glutaryl-7-ACA and adipyl-7-ADCA. So, combinations of improved single mutants may very well distort the active site. Therefore, a more random approach of combining mutations at important positions followed by a selection may result in a better outcome.

Recombination of two site specific random libraries

In former studies it was indicated that amino acids at positions 178, 266 and 375 are important for substrate specificity [28,116]. Single mutations at these positions resulted in improved hydrolysis activity towards adipyl-7-ADCA and in some cases even towards CPC [123]. From the crystal structure it was clear that these amino acids have a direct or indirect interaction with the substrate [32,115]. It therefore seemed likely that combinations of mutations at these positions can increase hydrolysis activity towards the larger side chains of adipyl-7-ADCA and CPC even more than their single mutations. As a first approach in this rationalised random experiment, we decided to combine the 20 different amino acids at position 266 with all 20 amino acids on position 375, and to study this in the context of a wild type sequence and together with the Y178H mutation in the α-subunit. These two libraries were plated onto minimal selective medium containing adipyl-leucine or amino adipyl-leucine as sole leucine source. In this way, we merged the rational approach of changing specific active site residues with the selection method of a directed evolution experiment.

On the minimal medium containing amino adipyl-leucine only a few, very small colonies appeared that did not produce any acylase as determined by spot blotting. Apparently, the hydrolysis activity of the mutants was not adequate to liberate sufficient leucine to allow visible growth. The selection plates containing adipyl-leucine did show small colonies after eight days of incubation. In total 38 colonies were selected and checked for acylase activity (Table 3). Unfortunately, more than half of the colonies did not show any acylase production while amongst the rest only four different mutants were identified with a better hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA compared to the wild type cephalosporin acylase. The mutants that were selected all contained multiple mutations, confirming our hypothesis that multiple mutants do exhibit improved properties towards the desired substrates. Remarkably, single mutants SY-77^{Y178H}, SY-77^{N266H}, SY-77^{N266S} and SY-77^{F375L}, which were selected in the former random mutagenesis experiments, could not be identified in this selection. However, already in previous studies, these mutations could not be isolated in all random mutagenesis approaches, an observation that was assumed to be related to the relative large amount of dead colonies on the selection plates, which may form an alternative leucine source for bacteria that do not show activity towards adipyl-leucine [116]. This part of the selection still needs to be optimised.

The four mutants with an improved hydrolysis ratio of adipyl-7-ADCA over glutaryl-7-ACA were purified and characterised. These mutants expressed a decreased activity towards the preferred substrate glutaryl-7-ACA compared to the wild type enzyme. On adipyl-7-ADCA, the selected mutants show decreased k_{cat} values, but improved K_m values compared to the associated single mutants. Apparently, the optimisation of interactions from residues at the three positions 178, 266 and 375, combined with the used selection procedure, allows for further improvement of the K_m and not the k_{cat} value.

Remarkably, three of the four mutations found on position 266 comprise a more bulky amino acid than the original Asn. Obviously, the indirect interactions of Asn266 with the substrate and neighbouring amino acids make the effects of mutations difficult to predict as observed earlier [123]. Hydrogen bonding and other interactions that for instance may result in a displacement of Arg255, seem to be more important at this side of the active site than creating space for the larger (amino) adipyl side chain. It is noteworthy that in this randomised approach to isolate optimised adipyl-7-ADCA hydrolysing multiple mutants by combining single mutations at positions 178, 266 and 375, the combination of the best single mutants found in an earlier research (SY-77^{Y178H} and SY-77^{N266M} [28,123]) resulted in an improved double mutant displaying the highest catalytic efficiency.

The mutants picked from adipyl-leucine plates show a similar or improved conversion rate of CPC compared to wild type, but they were not found on minimal plates containing amino adipyl-leucine. Similarly, single mutant SY-77^{Y178H} was not isolated from the library, but did show some small colonies when plated as a control on selection plates with amino adipyl-leucine. This mutant displays an almost three times higher

in vitro conversion rate of CPC than wild type acylase, which may be the minimum activity of hydrolysis to allow growth on selective medium containing 0.2 mM amino adipyl-leucine. It should be realised, however, that the *in vitro* activity on CPC was tested at a high substrate concentration (10 mM). Consequently, improved *in vitro* hydrolysis activities are likely to represent changes in k_{cat} values and not so much K_m values, whereas both parameters are related to the capability to grow on selective medium.

None of the selected multiple mutants had a higher CPC hydrolysis rate than SY-77^{Y178H}. Remarkably, the combination with N266M lowers the CPC hydrolysis rate, in contrast to the positive effect on the hydrolysis activity towards adipyl-7-ADCA (Figure 3). The selected combinations with mutations at position 375 show a similar effect. They demonstrate an improved catalytic efficiency towards adipyl-7-ADCA, whereas they appear to negatively affect CPC hydrolysis activity (Figure 3). For further improvement of CPC hydrolysis activity, mutation Y178H may have to be combined to mutations of other (active site) residues than 266 and 375. Alternatively, in a multiple mutant with high CPC hydrolysis activity, an amino acid other than Tyr or His may be needed at position 178. In a recently published research on the cephalosporin acylase from *P. diminuta*, the most improved mutant of in the second round comprised mutations Y149K and Q50βM, which would be Y178K and Q248M in SY-77 acylase [24]. This mutant showed a 6-fold improved activity towards CPC compared to the wild type enzyme, which confirms that other mutations than Y178H may indeed improve hydrolysis activity towards CPC in multiple mutants.

Random mutagenesis of 5 important substrate specificity residues

We randomised the amino acids Tyr178, Tyr231, Arg255 and Asn266 by building this part of the gene synthetically by hybridising 10 degenerate forward and 10 degenerate backward primers separately to form double stranded pairs of synthetic oligonucleotides between 30 and 100 bps in length. Recombining them in one tube did not result in a library large enough to comprise all possible mutants, although the overlap between each double stranded pair was at least 10 bases. To conquer this problem, we attempted to ligate the double stranded pairs one at a time. The first ligations of single and double pairs resulted in the expected bands, but the combination of these pieces to obtain larger fragments was more difficult to achieve. The presence of non-ligated fragments in the latter ligation mixtures may inhibit the hybridisation of the compatible overhangs, as in the one-tube experiment. Moreover, the amount of correctly ligated DNA of the right size becomes less with every step, resulting in even less of the proper product and a more difficult monitoring of the process. Despite these drawbacks, we were able to produce a mutant library which comprises in theory $3*10^6$ different mutants. This amount covers statistically 94% of the total possible library. Sequencing of randomly picked clones should be performed to confirm this number.

Unfortunately, cloning of the mutagenised gene fragments into the randomised pool of vectors was not very efficient. Transformation resulted only in a total of $1.3*10^5$

different colonies, which represent 0.13% of the possible library of the five totally randomised residues. Plating of this small portion of the total library on minimal medium containing amino adipyl-leucine resulted in no active mutants. Although this can be explained by the small sized library, one may expect a few mutants as shown in a synthetic recombination approach, in which only 0.01% of the total library was screened [95]. Recloning of the ligation product will most probably give some multiple mutants, which display an even better hydrolytic activity towards CPC than SY-77^{Y178H}.
In this research, it has become clear that the combination of rational and random procedures leads to improved enzymatic activity towards the industrially valuable substrates adipyl-7-ADCA and CPC. It is, however, important that both the computational design and the experimental methods are properly evaluated beforehand and afterwards to gain more knowledge about each step in the process, resulting in a more general improvement scheme of biocatalysts.

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Chapter 6

Summary, general discussion and perspectives

Summary

This thesis deals with the directed evolution of a cephalosporin acylase. Acylases play an important role in the environmentally friendly production of semi-synthetic β-lactam antibiotics, which are the largest group of antibiotics used world wide. Unfortunately, resistance towards this important class of antibiotics is still rising, stressing the need for new variants of these and other antibiotics. Since the specificity of antibiotics is mainly due to their side chains, semi-synthetic antibiotics with new side chains and thereby new enzymes to produce these compounds are needed. New enzymes can be made by mimicking the evolution in the laboratory while directing it towards the desired activity. This directed evolution technique has resulted in many improved model enzymes in the last decade and promised to be useful for industrial biocatalysts as well. We decided to pursue directed evolution on a industrially applicable enzyme, the cephalosporin acylase of *Pseudomonas* SY-77. A general introduction on this enzyme and directed evolution is given in **chapter 1** of this thesis.

The SY-77 glutaryl acylase is transcribed as a polypeptide which folds and cleaves itself into a heterodimer. This cleavage results in the formation of the N-terminal serine of the β-subunit that is the active site residue performing the nucleophilic attack of the peptide bond of the substrate. Although a lot of different enzymes belong to the so-called cephalosporin acylase family, their activity towards cephalosporin C (CPC) is very low (k_{cat} of < 0.15 s^{-1}) in comparison to the preferred β-lactam substrate glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA). The key intermediates in the production of semi-synthetic cephalosporins are 7-ACA and 7-aminodesacetoxy-cephalosporanic acid (7-ADCA). In the two-step process from CPC to 7-ACA, a D-amino acid oxidase removes the amino group from the side chain, followed by the hydrolysis of the glutaryl side chain by a cephalosporin acylase. In a new production route of 7-ADCA a genetically modified fungus produces adipyl-7-ADCA instead of CPC. Optimisation of a single step production process of 7-ADCA would require a cephalosporin acylase that hydrolyses this compound, adipyl-7-ADCA. Both examples clearly demonstrate that there is need for new cephalosporin acylases which are able to hydrolyse adipyl-7-ADCA or CPC at a high rate.

Directed evolution is a fairly new area of protein engineering. It follows the path of Mother Nature to find new and improved enzyme activities. In nature new enzymes and enzyme activities have evolved by mutation and recombination. In the laboratory, researchers try to direct enzymes towards new activities by mimicking this process, followed by selection for the desired activity. In this process two parts are crucial for the success of an experiment, the quality of the mutant library and the method to search for the best mutant. The perfect mutant library comprises all possible mutants in an equal amount. Several research groups have been working towards this goal in the last decade, resulting in numerous protocols for mutagenesis and recombination, each having their

own advantages and drawbacks[55,64]. The second crucial part of a directed evolution experiment involves the method of finding the best mutant. This can be done by selection or screening. Selection is a process in which the desired activity is coupled to a selectable marker. Since only active enzymes will be sampled, a much larger pool of mutants can be searched. For activities which cannot be coupled to a selectable marker screening is the method of choice. In screening procedures all mutants have to be tested for the desired activity, which decreases the number of searchable mutants. Fortunately, high throughput screening assays have been established for many different enzymes, resulting in the possibility of screening larger libraries.
At the time the work for this thesis was started, the first successful examples of directed evolution were described [125]. These examples, however, were mainly dealing with the exploration of the new techniques. Therefore, model systems were used handling enzymes with easy detectable activities, like improving antibiotic resistance or thermostability of an enzyme, or using substrates that are converted into coloured products. Although these models are very well suited to improve directed evolution techniques, most of them do not lead to useful biocatalysts. Therefore, the aim of this thesis is to use directed evolution in order to find variants of the industrially relevant glutaryl acylase of *Pseudomonas* SY-77 with improved hydrolysis towards specific β-lactam compounds.

Chapter 2 describes a random mutagenesis approach aimed at the finding of amino acids, important for substrate specificity. As the α-subunit of the enzyme was already mutagenised before, we decided to focus on the β-subunit. This part of the gene consists of 1566 bps. Mutagenising this stretch of DNA will result in a library too large to select. Therefore, we divided the gene into five highly overlapping parts of about 550 bps and mutagenised every part separately by epPCR, adjusting the Mn^{2+} concentration in such a way that every mutant comprises one or two mutations on average. The mutagenised parts were cloned back into the rest of the gene and transformed to the leucine auxotroph *Escherichia coli* DH10B. The transformants were plated on minimal medium containing adipyl-leucine as sole leucine source. In this way, only bacteria expressing a mutant enzyme capable of hydrolysing adipyl-leucine to liberate leucine will be able to grow. After two weeks several colonies were picked from the selective plates and crude cell extracts were used to determine the hydrolysis of adipyl-7-ADCA, in order to see if transformants were not only active towards the selection substrate adipyl-leucine, but also towards the desired β-lactam compound. Hydrolysis of glutaryl-7-ACA was also determined and the ratio was calculated to correct for differences in enzyme concentration in the crude cell extracts. Sequencing of the selected mutants showed that most of them comprised a mutation at position 266 or 375. Five mutants had an improved ratio of hydrolysis of adipyl-7-ADCA over glutaryl-7-ACA. These enzymes were purified and catalytic parameters were determined. Mutant SY-77^{N266H} showed an almost ten times improved catalytic efficiency towards adipyl-7-ADCA, while the improvement of the ratio of mutation F375L was merely due to a reduction of the

hydrolytic activity towards glutaryl-7-ACA. The structure of the highly homologous cephalosporin acylase of *Pseudomonas diminuta* KAC-1 revealed that both Asn266 and Phe375 are part of the active site of the enzyme.

In epPCR it is, however, unlikely that two bases within the same codon are mutated. This implies that on average one amino acid may be changed in seven other amino acids, being mainly conservative mutations. This means that the method is highly suited to point out residues that are important for the desired activity, but only a limited part of the sequence space can be explored. **Chapter 3 and 4** describe a complete mutational analysis of positions 266 and 375, respectively, in order to explore the function of these residues in substrate specificity in depth. In each case, all 20 mutant proteins were purified and their catalytic parameters were determined. Most mutant enzymes could be fully functionally expressed in *E. coli* and they displayed a broad spectrum of affinities and activities towards glutaryl-7-ACA, adipyl-7-ADCA and CPC, confirming the importance of these residues for substrate specificity and pointing towards the flexibility of the enzyme at these positions. The mutants SY-77^{N266M} and SY-77^{F375C} appeared to show the most improved activity towards adipyl-7-ADCA, while SY-77^{N266Q} showed a small increase towards cephalosporin C. It should be noted that each of these mutations could only be achieved by a change of two bases in the respective codons, clearly demonstrating how this saturation mutagenesis approach complements the epPCR.

Although up to a 15-fold increase of the catalytic efficiency for hydrolysis of adipyl-7-ADCA was shown in single mutants, it appeared likely that further improvement of the activity on this substrate and activity towards CPC is possible by creating multiple mutations. In **chapter 5** both rational and random approaches are described to find the best combination of mutations in the most important substrate specificity residues. In a rational approach of combining the best mutant in the α-subunit with the most improved mutants found in the β-subunit, only the combination of mutations Y178H and F375L showed an improved hydrolysis activity towards adipyl-7-ADCA compared to both single mutants. Therefore, we approached the combination of two residues in a more random way. The 20 amino acids at position 266 were combined with all 20 amino acids at position 375. This library of β-subunit mutants was cloned into a wild type background and into a plasmid bearing the Y178H mutation in the α-subunit. The resulting libraries were transformed to a leucine deficient *E. coli* strain and selected by growing them on minimal medium with adipyl-leucine or amino adipyl-leucine as sole leucine source. Unfortunately, none of the three transformants picked from the amino adipyl selection plates showed enzyme production. Some transformants from the adipyl selection plates did show enzyme production and were tested for activity towards glutaryl-7-ACA and adipyl-7-ADCA. Four mutants showed improved activity towards the desired substrate, so they were purified and catalytic parameters were determined. Upon sequence analysis it was discovered that the best multiple mutant was a combination of the best single mutants from earlier research (SY-77^{Y178H} and

SY-77^{N266M}) resulting in an additive activity towards adipyl-7-ADCA. With respect to the acylase activity towards CPC, none of the four selected mutant combinations, N266Q+F375L, Y178H+N266H, Y178H+N266M and Y178H+N266Q+F375M, resulted in a higher conversion of this substrate relative to the single mutant SY-77^{Y178H}.
In the search for an even better acylase we started with the generation of a library in which all important residues for substrate specificity discovered until now are totally randomised. Since it was obvious from former research that using oligonucleotides for saturation mutagenesis may result in a biased library towards the original codon, we decided to construct the mutated part of the gene synthetically. The four amino acids Tyr178, Tyr231, Arg 255 and Asn266 were totally randomised using NNS codons on both strands. Although the assembly of ten pairs of oligonucleotides is very easy in theory, the process had to be monitored very closely in order to achieve a library of $3*10^6$ mutants, which statistically comprise 94% of all possible mutants. The cloning of this library, however, was not very successful resulting in only 0.13% of the total possible mutant library of all five residues randomised. Plating this small library onto minimal medium with amino adipyl-leucine as sole leucine source did not result in the selection of any active mutant. Although it is clear that this experiment has to be repeated with a complete library, the results will be part of research that goes beyond this thesis.

General discussion

Directed evolution started as a poor man's protein engineering tool to improve enzymes from which no crystal structures were available. Over the last decades, however, several impressive successes were achieved, turning it into a major protein engineering tool next to rational design. After years of competing, it is clear now that both methods do not exclude each other, but have compatible strengths. Therefore, the two methods of rational design and directed evolution should no longer be used separately, but are to be combined in order to search a larger sequence space [126].

In this thesis both random mutagenesis strategies and more rational methods have been used to develop cephalosporin acylases with improved activity towards adipyl-7-ADCA and CPC. As is clear from chapters 2, 3 and 4, the combination of a directed evolution experiment, comprising of a random mutagenesis strategy followed by selection to find the residues that are important for substrate specificity, with a more detailed saturation mutagenesis of the important residues, to deeply explore sequence space, is a good strategy. However, a reliable and fast screening assay as well as an efficient protein purification method are equally important to grant success.
Mutants were found with improved catalytic efficiency up to 15 times towards adipyl-7-ADCA and up to a 3-fold improved hydrolysis rate of CPC. Although this does not seem to be a very large improvement, the mutant showing the highest activity towards

adipyl-7-ADCA displays a k_{cat} that is only two times lower than the k_{cat} of the wild type enzyme towards glutaryl-7-ACA. So, this mutant enzyme is almost industrially applicable. The enzyme is, however, already a very efficient biocatalyst, which means that an improved activity of five to ten times already is a significant achievement from a scientific point of view. Furthermore, every activity towards CPC is a big achievement, since several decades of screening for this activity did not result in any Cephalosporin C acylase yet [12,13].

The constructed library with all five important substrate specificities randomised was apparently not large enough to permit isolation of even more improved enzymes. It can be stated beyond doubt that several mutations are needed in order to change substrate specificity of an enzyme. As an example, in parallel to the research described in this thesis, Oh *et al*. generated several mutant libraries of the cephalosporin acylase of *P. diminuta* [24]. Guided by the crystal structure, important substrate specificity residue were mutagenised by site directed mutagenesis one at the time. The best mutant was used as a backbone for the total randomisation of a second amino acid and the best mutant out of this library was used for the third round. It was shown that the best single, double and triple mutant had an improved hydrolysis activity towards CPC of respectively 1.8, 6 and 7.9 times the wild type activity. This corroborates with the hypothesis that multiple mutants will improve the activity towards the desired substrate. The combination of new mutants with the best mutant of a former library may, however, not always result in an improvement of activity. The rational combination of two improved mutants in the α- and β-subunit did not result in an improved catalytic efficiency of the multiple mutant in comparison to the single mutants (chapter 5). The mutants selected on adipyl-leucine displayed an improved activity towards adipyl-7-ADCA. However, in none of these multiple mutants the activity towards CPC was increased compared to single mutant SY-77^{Y178H}. This suggests that starting from a fixed mutant backbone may result in the entrapment at a local minimum in the enzyme activity landscape. It is therefore important in protein engineering strategies to keep some randomness upon combination of mutants [127].

Along with the strategy to construct the library, the selection or screening method is a very important part of a directed evolution experiment. The selection method used in this thesis is an elegant method in which the desired enzyme activity is used for the survival of the expression host, resulting in several improved mutants as described throughout the thesis. The advantage of the method is that the selective pressure is on enzyme activity rather than binding of the enzyme to the desired substrate as is described in many other directed evolution experiments [72]. Furthermore, it is a straightforward and technically simple method, since it is an *in vivo* selection, and applicable to numerous peptide cleaving enzymes. Although this method has been used before to change penicillin acylases into cephalosporin acylases [68,128], it only resulted in mutants with improved activity towards the selection substrates and not towards the similar β-lactam compounds. This confirms the first law of directed evolution “you get

what you select/screen for" [69]. However, in our case the mutants selected on adipyl-leucine did show activity towards adipyl-7-ADCA. Perhaps cephalosporin acylases bind differently to the core of the substrate than penicillin acylases. Alternatively, this selection strategy may only be applicable for small changes in enzyme activity and the change from an aromatic penicillin to an aliphatic cephalosporin side chain may be too big a step.

This last hypothesis may also explain why we did not find any active mutants on the selection plate containing amino adipyl-leucine. On the other hand, the selection procedure may also be limiting. For example, the single mutant SY-77^{Y178H}, when plated separately, showed small colonies on amino adipyl-leucine selection plates within nine days, but was not selected in the YNF library (chapter 5). Since the randomness of the library was ensured, this strongly suggests that the selection method is not appropriate to find mutants that show only three times improved hydrolysis activity *in vitro* towards a high concentration of CPC. First of all, the visual identification of small white colonies on the white amino adipyl-leucine selection plates is difficult, but can be trained. Furthermore, the concentration of selection substrate and thereby the leucine concentration in the plate may be limiting. Mutants that are impaired in the expression of acylase have the advantage of producing less protein. So they need less substrate and may grow faster and be selected. In order to improve the selection procedure coloured plates may be used to improve distinguishing a colony from the plate. Another suggestion for improvement of the assay is using a higher concentration of selection substrate in order to minimise the selection pressure on the affinity of the mutants towards the substrate.

The second step in our selection procedure is the ratio of hydrolysis of adipyl-7-ADCA over that of glutaryl-7-ACA. As shown in chapter 2, an improved ratio may be the result of an improvement of activity towards adipyl-7-ADCA or of the decreased hydrolysis of glutaryl-7-ACA. Since the selection procedure is not as unambiguous as we would like it to be and we want to improve enzymes rather towards β-lactam compounds than towards the selection substrates, we do need a second screening step. Purifying all of the selected mutant enzymes is, however, not possible when large libraries are selected, since this process takes several days and only two mutants can be purified per day. Using a method to quantify the amount of active enzyme in the crude cell extract would be a reliable alternative. Unfortunately, all known inhibitors for acylases and peptidases do not affect cephalosporin acylases, which means that an inhibition reaction can not be used for the quantification of the amount of active enzyme. Furthermore, the polyclonal SY-77 antibody, which is used for detection of acylase, appeared to be not suitable for quantification. The absolute values of the crude cell extracts seem, however, to have a predictive value for the hydrolysis activity of the purified mutant. So, both ratio and values should be considered in this second step. Another medium to high throughput screening method would be the use of BIAcore to detect the amount of enzyme binding to the substrate coated on a chip. Although this is an elegant and quantitative method it is a fairly new method and may be difficult to establish.

The last step in the characterisation of the mutant enzymes is the determination of the kinetic parameters by using a range of different substrate concentrations in a discontinuous assay using fluorescamine. The validation of this strategy and the use of the appropriate controls resulted in reproducible kinetic parameters for both adipyl-7-ADCA en glutaryl-7-ACA. However, some aspects should be taken into account. Since the assay is based on the production of primary amines by cephalosporin acylases, it is also prone to background fluorescence of other primary amines. Although the chosen wavelength prevents the detection of primary amines on amino acids (and thus proteins), the presence of the amine group on the side chain of CPC does interfere. This results in a much higher background value and leaves very little space to see small improvements of activity towards the substrate. It is therefore crucial to perform at least two different experiments in triplicate or more for each mutant. As can be seen in chapters 3 and 5, this did generate reproducible hydrolysis rates of CPC.

Although the main theme of this thesis is about the mutagenesis of active site residues and most successful protein engineering strategies aim at these amino acids, there are papers showing that small modifications of an enzyme outside its active site also result in improved enzyme activity [34]. These mutations are usually difficult to explain, but are mostly thought to stabilise conformational changes in the enzyme, which are the result of another transition state with the new substrate or of modified hydrophobic and hydrophilic interactions with the desired solvent. The epPCR of the five parts of the enzyme only resulted in one improved mutant outside the active site, although this residue might be important for the binding of the core of the substrate (chapter 2). Since one mutation, either inside or outside of the active site, might not be enough to improve enzyme activity and structurally close residues are not always closely together in the gene, it would be a good idea to shuffle all five epPCR parts in order to get multiple mutations throughout the whole gene. In this way, mutations that do not improve activity on their own may be discovered.
Another way of obtaining more diversity in an enzyme is by shuffling it with other homologous enzymes. Family shuffling of acylases seems not feasible, since they are mainly homologous in a structural way and not at the amino acid level. Some parts of the enzyme do have more homology to each other than other parts. This might just be enough for shuffling. On the other hand, comparing the differences between several acylases in combination with their substrate specificity might lead to the discovery of other important substrate specificity residues. This approach needs, however, several crystal structures or models of the enzymes to be compared and an extensive calculation capacity. The crystallisation of natural enzymes as well as (multiple) mutants with or without a co-crystallised substrate can increase our knowledge on structure-function relationship and should therefore be a main goal for the coming years.

The cephalosporin acylase of *Pseudomonas* SY-77 appears to be quite amenable to modification of enzymatic activity. A single mutation improved the hydrolytic activity

towards adipyl-7-ADCA already 15-fold, which could be easily selected with a straightforward selection procedure. This may be attributable to the fact that substrate specificity is mainly associated with the side chain and not with the core of the substrate resulting in a broad substrate range. Cephalosporin acylase also shows enantioselective amidase and esterase activity [33]. As cephalosporin acylases are used in the different industrial processes to obtain 7-ACA or 7-ADCA, conditions have already been developed and optimised for its production and quality assurance. Finding another enzyme activity in a mutant cephalosporin acylase only requires the (re)design of a (new) selection or screening strategy, giving it a plethora of industrially interesting and important functions to be explored. This makes the enzyme a perfect adaptable biocatalyst, not only for the production of semi-synthetic cephalosporins, but also in the resolution of racemic mixtures and potentially in the synthesis of peptides [10].

In conclusion, the evolution of a glutaryl acylase into an industrial cephalosporin acylase is most definitely feasible. The foundation has been laid, the house can be build.

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Nederlandse samenvatting

De gerichte evolutie van een cefalosporine acylase

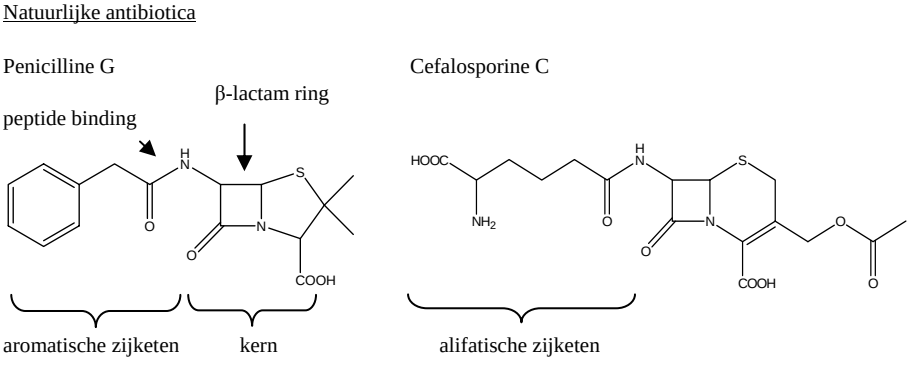
Bijna iedereen heeft wel eens een antibioticumkuur gehad om een infectie te bestrijden. Antibiotica worden voornamelijk door schimmels gemaakt en zijn het verdedigingsmechanisme van deze schimmels tegen bacteriën. De hedendaagse antibiotica zijn meestal varianten van natuurlijke isolaten, ook wel semi-synthetische antibiotica genoemd. Er zijn twee redenen om natuurlijke antibiotica te veranderen. Ten eerste is door veelvuldig en verkeerd gebruik van antibiotica resistentie opgetreden tegen de natuurlijke isolaten. Zo is de MRSA bacterie (Methicilline resistente *Staphylococcus aureus*), die regelmatig in ziekenhuizen de kop opsteekt, alleen nog maar gevoelig voor één bekend antibioticum. Daarnaast zijn de natuurlijke antibiotica niet zo stabiel en is het spectrum van bacteriën die ze kunnen doden beperkt.
Op basis van de chemische structuur zijn verschillende soorten antibiotica te onderscheiden en de antibiotica die op penicilline G lijken, worden de β-lactam antibiotica genoemd (Figuur 1). Penicilline G bestaat uit een kern van een 5-ring plus een 4-ring, een β-lactam groep, met daaraan gekoppeld een zijketen. Een ander, in Nederland minder bekend, β-lactam antibioticum is cefalosporine (Figuur 1). Deze verbinding heeft een 6-ring in plaats van een 5-ring in de kern, waardoor het antibioticum in staat is andere bacteriën te doden dan penicillines. De natuurlijke zijketen van het antibioticum cefalosporine C is niet een aromatische ring, zoals bij penicilline G, maar een lange alifatische keten van zes C-atomen met een carboxyl groep en een amino-groep ook wel aminoadipyl genaamd. Veranderingen in de zijketens veranderen de stabiliteit en werking van β-lactam antibiotica. Zo heeft de zijketen van ampicilline in vergelijking tot penicilline G slechts één extra hydroxyl groep, maar dit antibioticum kan daardoor wel oraal geven worden, terwijl penicilline G direct door het maagzuur wordt afgebroken. Het vervangen van de zijketen van β-lactam antibiotica wordt al jaren industrieel toegepast door eerst de bestaande zijketen bij de peptide binding af te knippen (deacyleren) en er een nieuwe zijketen op dezelfde plek aan te zetten (acyleren). Dit proces wordt tegenwoordig door enzymen gedaan, omdat enzymen heel specifiek bepaalde chemische reacties kunnen katalyseren. Het enzym, dat gebruikt wordt bij het maken van penicilline-achtige antibiotica, wordt penicilline acylase genoemd. Dit enzym heeft een voorkeur voor aromatische zijketens en kan daarom niet de alifatische zijketen van cefalosporines afknippen. Voor deze antibiotica zijn cefalosporine acylases nodig. Helaas zijn er in de natuur nog steeds geen cefalosporine acylases gevonden die de zijketen van cefalosporine C accepteren. Daarom wordt de zijketen van cefalosporine C in twee stappen afgeknipt. In de eerste stap wordt de zijketen één C atoom en één aminogroep korter gemaakt en in de tweede stap wordt de resterende (glutaryl)zijketen door het enzym glutaryl acylase afgesplitst. Dit enzym kan een alifatische zijketen van vijf C-atomen heel goed afsplitsen, zoals de zijketen van glutaryl-7-ACA (Figuur 1) en ook een zijketen met zes C-atomen, zoals adipyl-7-ADCA, wordt langzaam door dit enzym afgesplitst. De aminoadipyl-zijketen van cefalosporine C wordt echter niet door dit enzym herkend en dus ook bijna niet afgeknipt (Figuur 1). Enigszins verwarrend is de naamgeving. Hoewel het enzym nauwelijks activiteit op cefalosporine C vertoont, wordt dit enzym toch tot de familie

der cefalosporine acylases gerekend.
Semi-synthetische cefalosporines kunnen ook uit penicillines worden gemaakt door de 5-ring te vergroten naar een 6-ring en daarna de zijketen eraf te knippen met penicilline acylase. Deze methode is een chemisch ingewikkeld proces dat veel energie kost en waarbij erg veel (chemisch) afval ontstaat. Aangezien dit erg slecht voor het milieu is en afvalverwerking steeds duurder wordt, is men al jaren op zoek naar nieuwe manieren om semi-synthetische cefalosporines te maken. Een stap in de goede richting was de ontdekking van een enzym dat de 5-ring van penicilline om kan zetten in een 6-ring. Helaas bleek dit enzym niet te werken als de penicilline zijketen aan de ring zat. Wel bleek het enzym te werken als je de schimmel in plaats van de aromatische zijketen een alifatische zijketen met zes C-atomen gaf. Dit heeft geleid tot een genetische gemodificeerde schimmel, die adipyl-7-ADCA in plaats van penicilline G produceert. De zijketen van deze stof kan door een cefalosporine acylase langzaam worden afgeknipt. Door de reactie omstandigheden te veranderen kan de reactie versneld worden, waardoor het proces economisch haalbaar wordt. De snelheid is echter nog steeds niet te vergelijken met het penicilline acylase en daarom werd het onderzoek opgestart, dat in dit proefschrift is beschreven. Het doel was om een bestaand cefalosporine acylase zodanig te veranderen dat het de zijketen van adipyl-7-ADCA en misschien zelfs die van cefalosporine C even efficiënt kan afknippen als het originele enzym de zijketen van glutaryl-7-ACA kan hydrolyseren. Voor dit onderzoek werd het glutaryl acylase van de bacterie *Pseudomonas* SY-77 gekozen om mee te beginnen. Het gen, dat voor dit enzym codeert, kan in de veelgebruikte gastheer *Escherichia coli* genetisch worden veranderd, waarna de mutante enzymen door deze bacterie worden geproduceerd.
Er zijn verschillende manieren om enzymen te veranderen. Wij hebben ervoor gekozen om de tactiek van Moeder Natuur af te kijken. Tijdens de evolutie zijn steeds nieuwe enzymen en enzymen met nieuwe activiteiten ontstaan, doordat er continu kleine mutaties en recombinaties optraden. Veel mutaties kunnen een enzym zodanig veranderen dat het een andere activiteit krijgt. Recombinatie is het opnieuw rangschikken van DNA en treedt vooral op bij de productie van ei- en zaadcellen, waarbij de chromosomen van vader en moeder uit elkaar getrokken worden. Soms zijn deze chromosomen zo verstrengeld dat breuken optreden en genen van vader en moeder worden uitgewisseld. Daarnaast zorgen veranderende omstandigheden in de natuur ervoor dat er steeds nieuwe enzymen nodig zijn, waardoor je een survival-of-the-fittest krijgt.
In het lab kan de evolutie versneld nagebootst worden door genen van enzymen te mutageniseren en te recombineren en daarna de gewenste activiteit van één van de mutanten te selecteren. Omdat de onderzoeker het proces heel gericht een bepaalde kant op stuurt, wordt deze methode wel gerichte evolutie genoemd (in het engels directed evolution). In dit proces zijn twee dingen cruciaal, de manier waarop de mutanten gemaakt worden en de methode waarmee het beste enzym uit de groep geselecteerd wordt. Een verzameling van zoveel mogelijk verschillende mutanten wordt een

mutantenbank genoemd. Om de perfecte mutantenbank te maken, moet je met genoeg diversiteit beginnen. Deze diversiteit kun je bereiken door het DNA te mutageniseren of door combinaties van natuurlijke varianten te gebruiken. Er zijn vele wegen die naar Rome leiden, en zo zijn er ook veel verschillende technieken ontwikkeld om goede mutantenbanken te maken. Iedere techniek heeft zijn eigen voor- en nadelen en het hangt van meerdere factoren af welke methode het meest geschikt is voor het doel dat je met een bepaald onderzoek wilt bereiken.

Het tweede belangrijke deel van het gerichte evolutieproces is het vinden van de beste mutant. Dit kan op twee manieren, door middel van selectie of screening. In een selectiemethode wordt ervoor gezorgd dat de gewenste enzymactiviteit de overleving van de bacterie bevordert. Dit lijkt dus het meest op de selectie zoals die in de natuur plaatsvindt. Het voordeel van deze methode is dat alleen de beste mutanten overleven, terwijl bacteriën die enzymen produceren die helemaal niet meer actief zijn, dood gaan. Helaas kan niet voor iedere enzymactiviteit een selectiemethode ontwikkeld worden. Dan is een screeningsmethode het alternatief. In een screening worden alle mutanten individueel getest op de gewenste activiteit. Het nadeel is dus dat je ook alle slechtere en inactieve mutanten moet testen Door het inzetten van robots kunnen steeds meer screeningsactiviteiten snel worden uitgevoerd. Dit wordt High Throughput Screening genoemd.

Het bovenstaande is een samenvatting van **hoofdstuk 1**, waarin een algemene inleiding wordt gegeven over het enzym en de technieken, die tot nu toe zijn ontwikkeld om gerichte evolutie uit te voeren. Bij de start van dit onderzoek waren al verschillende gerichte evolutie experimenten beschreven en uitgevoerd op modelsystemen met makkelijk meetbare enzymactiviteiten. Dit is noodzakelijk om een nieuwe techniek te ontwikkelen, maar levert vaak geen praktisch bruikbaar enzym op. Daarom is besloten om de waarde van deze technieken te testen met een industrieel relevant enzym, dat meteen toegepast kan worden in de productie van semi-synthetische antibiotica. Dit betekende echter wel dat er een goede selectie methode opgezet moest worden en een manier om de activiteit van de nieuwe enzymen te meten.

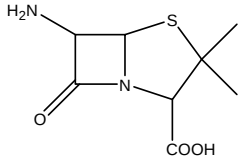


Figuur 1. Structuren van veelvoorkomende antibiotica en hun voorlopers.

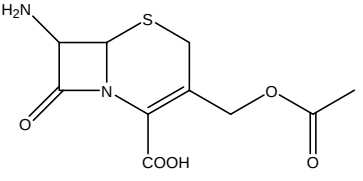
vervolg Figuur 1

Voorlopers en tussenproducten

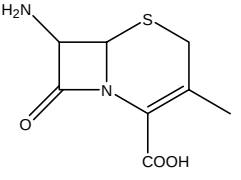
6-APA



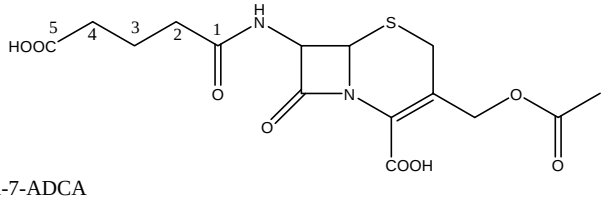
7-ACA



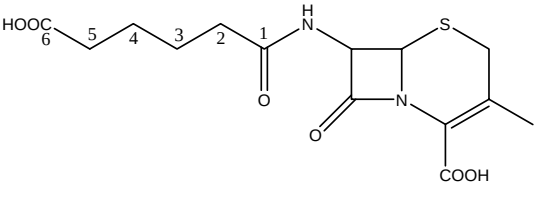
7-ADCA



glutaryl-7-ACA

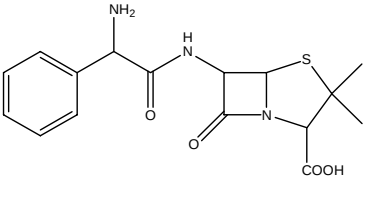


adipyl-7-ADCA

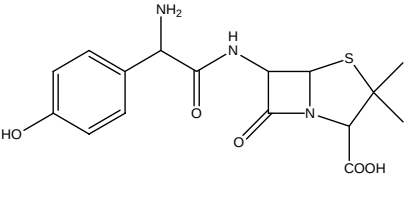


Semi-synthetische antibiotica

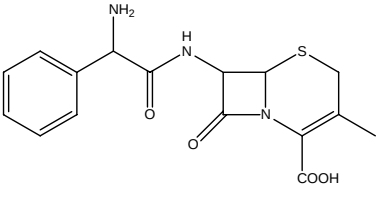
Ampicilline



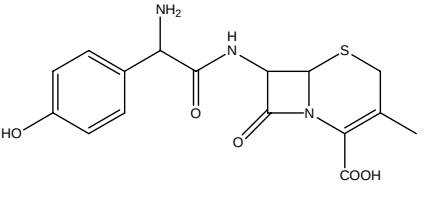
Amoxicilline



Cefalexine



Cefadroxil



In **hoofdstuk 2** wordt een mutagenese methode ontwikkeld om mutanten van het SY-77 glutaryl acylase te maken. Het acylase bestaat uit twee delen (subunits), een kleine α- en een grotere β-subunit. De α-subunit was al eerder gemutageniseerd, daarom hebben we ons op de β-subunit gericht. Uit praktische overwegingen hebben we dit deel van het gen in vijf overlappende delen opgesplitst en elk apart gemutageniseerd. Hiervoor is de random mutagenese strategie error-prone PCR (polymerase kettingreactie) gebruikt. Met deze methode kopieer je DNA in een reageerbuis met behulp van een DNA-polymerase. Door tijdens de reactie een aantal omstandigheden niet optimaal te houden, gaat het polymerase fouten maken. Wij hebben de reactie zodanig verstoord dat er gemiddeld 1-2 fouten per deel werden ingebouwd. Hierdoor ontstaat een mutantenbank met allemaal varianten van het enzym. De varianten in de vijf mutantenbanken zijn vervolgens geselecteerd op hun activiteit om een adipyl-zijketen af te splitsen. Hiertoe zijn de enzymen geproduceerd in *E. coli* DH10B, een bacterie die zelf geen leucine kan maken. Leucine is een aminozuur dat in bijna alle eiwitten voorkomt en is dus noodzakelijk voor de groei van de bacterie. Door nu de bacteriën te laten groeien op een medium met leucine gekoppeld aan de adipyl-zijketen, zullen alleen bacteriën die deze zijketen kunnen afsplitsen, en daarmee leucine vrijmaken, kunnen groeien. We hebben de snelst groeiende kolonies opgepikt en verder geanalyseerd. Daarbij is gekeken of de mutante enzymen in staat waren om het gewenste substraat adipyl-7-ADCA en het oorspronkelijke substraat glutaryl-7-ACA te hydrolyseren. De ratio van deze twee activiteiten werd gebruikt als maat voor de verandering van de enzymactiviteit. De DNA-sequentie, en daarmee de aminozuurvolgorde van de mutanten, werd bepaald en het bleek dat de meest voorkomende mutanten een ander aminozuur hadden op positie 266 of 375. Vijf mutanten hadden een verbeterde adipyl-7-ADCA/glutaryl-7-ACA hydrolyse ratio en deze enzymen werden opgezuiverd uit de bacterie om de enzymatische parameters preciezer te bepalen. Het bleek dat het enzym SY-77^{N266H}, waarin het aminozuur asparagine (N) op positie 266 vervangen is door een histidine (H), het substraat adipyl-7-ADCA bijna 10 keer beter kan hydrolyseren dan het originele SY-77 acylase. De mutant SY-77^{F375L} (fenylalanine op positie 375 naar een leucine), die de beste adipyl/glutaryl ratio had, bleek niet te zijn verbeterd in het afsplitsen van de zijketen van adipyl-7-ADCA, maar verslechterd in de hydrolyse van glutaryl-7-ACA. Het is dus erg belangrijk om de precieze parameters van het gezuiverde enzym te bepalen en niet alleen naar de ratio van activiteit op het ‘oude’ en ‘nieuwe’ substraat te kijken.

Door de aard van de error prone-PCR techniek kan er slechts een gering aantal DNA-mutaties en daarmee ook slechts een deel van alle mogelijke aminozuren ingebouwd worden. Omdat uit de resultaten leek dat posities 266 en 375 belangrijk zijn voor de substraatspecificiteit van het enzym, is ervoor gekozen om deze posities volledig te randomiseren met behulp van de zogenaamde site-saturation mutagenesetechniek. Dit wordt in **hoofdstuk 3 en 4** beschreven. Alle mogelijke varianten zijn opgegroeid om het enzym te zuiveren. Het bleek dat slechts vier mutanten, die een aminozuurverandering

in positie 266 hadden, niet gemaakt konden worden. Van de overige mutanten werd de activiteit voor de hydrolyse van glutaryl-7-ACA, adipyl-7-ADCA en cefalosporine C bepaald. Elke mutant bleek zijn eigen profiel te hebben ten opzichte van deze drie substraten. Dit bevestigt dat de aminozuren op deze twee posities erg belangrijk zijn voor de substraatspecificiteit van het enzym. Bijna alle mutanten zijn verslechterd in de hydrolyse van glutaryl-7-ACA. Dit is logisch, omdat het originele enzym dit substraat heel goed afbreekt. De mutanten die adipyl-7-ADCA het beste kunnen hydrolyseren zijn SY-77^{N266M} (asparagine naar methionine) en SY-77^{F375C} (fenylalanine naar cysteine). De mutant SY-77^{N266Q} (asparagine naar glutamine) bleek cefalosporine C drie keer beter om te zetten dan het originele enzym. Dit is een veelbelovend resultaat, ook al is dit altijd nog een factor 100 lager dan de hydrolyse-activiteit ten opzichte van glutaryl-7-ACA. Al deze aminozuurveranderingen kunnen alleen maar ontstaan door twee of zelfs drie DNA-mutaties en hebben dus een zeer kleine kans aanwezig te zijn in een error-prone PCR mutantenbank. Hieruit blijkt dus dat de gevolgde strategie een goede manier is om een gericht evolutie experiment te doen: eerst zoek je de posities in een enzym die belangrijk zijn voor substraatspecificiteit en daarna verander je de aminozuren op die posities in alle 19 andere aminozuren om te bepalen welk aminozuur op die plek tot de hoogste gewenste activiteit leidt.

Ondanks het feit dat er al behoorlijke verbeteringen van de adipyl-7-ADCA hydrolyse-activiteit was gevonden, is deze activiteit nog steeds niet zo goed als de activiteit van het originele enzym ten opzichte van glutaryl-7-ACA. Het leek ons daarom interessant om te proberen deze activiteit nog verder te verbeteren door meervoudige mutanten te maken. In **hoofdstuk 5** worden dan ook zowel rationele als random mutagenese strategieën beschreven om de beste meervoudige mutant te vinden. Eerst werd de beste mutatie in de α-subunit, histidine in plaats van tyrosine op positie 178 (Y178H), gecombineerd met een aantal van de beste mutaties in de β-subunit. Hieruit bleek dat de combinatie van mutatie Y178H met F375L (fenylalanine naar leucine) een betere hydrolyse-activiteit ten opzichte van de enkele mutanten gaf, terwijl de andere combinaties niet tot een verbetering voerden. Hieruit blijkt dat effecten van mutaties niet altijd additioneel zijn, oftewel één plus één is niet altijd twee.
Omdat combinaties van mutaties niet altijd een voorspelbaar resultaat opleveren, werden de belangrijke posities op een willekeurige manier gecombineerd. Alle mogelijke 20 aminozuren op positie 266 (uit hoofdstuk 3) werden gecombineerd met alle mogelijke aminozuren op positie 375 (uit hoofdstuk 4). Daarnaast werd deze mutantenbank ook nog gecombineerd met mutatie Y178H in de α-subunit. Omdat deze banken uit 20 x 20 = 400 verschillende combinaties bestaan, hebben we de beste mutanten eruit gepikt door de bacteriën te selecteren met de selectiemethode beschreven in hoofdstuk 2. Er werden twee soorten platen gebruikt, één met adipyl-leucine en één met aminoadipyl-leucine. Helaas groeiden er bijna geen kolonies op de platen met aminoadipyl-leucine en leidde deze selectie niet tot nieuwe mutanten. Van de kolonies die van de adipyl-leucine platen waren opgepikt bleken er maar vier verschillende

mutanten te zijn die een verbeterde ratio van adipyl-7-ADCA/glutaryl-7-ACA hydrolyse te hebben. Deze mutanten werden gezuiverd en de enzymatische parameters werden bepaald. De mutant met de hoogste adipyl-7-ADCA hydrolyse activiteit, SY-77$^{Y178H+N266M}$, bleek een combinatie te zijn van de beste enkelvoudige mutanten uit de α- en β-subunit en had dus ook rationeel gemaakt kunnen worden. Cefalosporine C bleek beter omgezet te worden door de enkele mutant SY-77^{Y178H} dan door elk van de vier geselecteerde mutanten. Dit is niet geheel onverwacht, omdat deze mutanten op adipyl-leucine waren geselecteerd en niet op aminoadipyl-leucine.

Als laatste poging om een verbeterd acylase te maken, werden de vijf belangrijke aminozuren voor substraatspecificiteit totaal gerandomiseerd en gecombineerd. Hiertoe werd het stukje gen waarin de aminozuren Tyr178, Tyr231, Arg255 en Asn266 (tyrosine, arginine en asparagine) aanwezig zijn gemaakt met behulp van synthetische stukjes DNA, oligonucleotiden genoemd. De basen die voor deze aminozuren coderen werden gerandomiseerd, zodat codons voor elk aminozuur gevormd kunnen worden. Alle benodigde oligonucleotiden werden aan elkaar geplakt en het zo ontstane genfragment werd vermenigvuldigd met behulp van PCR. Alhoewel dit in theorie heel makkelijk lijkt, was er wel wat optimalisatie nodig om een mutantenbank van $3*10^6$ mutanten te krijgen, die alle mogelijke combinaties bevat. Het synthetische stuk DNA werd teruggeplaatst in een bank van het SY-77 gen waarin op positie 375 alle 20 aminozuren aanwezig waren (uit hoofdstuk 4). Helaas was deze laatste stap niet erg succesvol, waardoor slechts 0.13% van de totaal mogelijke grootte voor de mutantenbank werd verkregen. Hopend op wat geluk (andere onderzoekers hebben al eens verbeterde mutanten gevonden met een bank die slechts 0.01% van de totaal mogelijke populatie omvatte), werd de bank toch op selectieve platen met aminoadipyl-leucine geselecteerd. Helaas leverde dit geen verbeterde mutanten op. Het experiment, en vooral de laatste stap, zal dan ook geoptimaliseerd moeten worden, maar dit kon niet meer in dit proefschrift worden beschreven.

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Linda ☺

Curriculum vitae
Publications
Abbreviations

Curriculum vitae

Linda Otten was born on November 2, 1972 in Zuidwolde (The Netherlands). She started the study Biology at the University of Groningen in 1991. In June 1996 she got a M.Sc. degree in Biotechnology. During her studies she performed a research project at the department of Microbial Physiology (Prof. Dr. L. Dijkhuizen) of the University of Groningen and an internship at AVEBE coop, Foxhol (The Netherlands).
After a few short jobs, she started a research project on directed evolution at the department of Fine Chemicals, Organic Chemistry and Biotechnology at DSM Research, Geleen, The Netherlands in March 1998. On the 1st of July 1999 she started her PhD research at the department of Pharmaceutical Biology (Prof. Dr. W.J. Quax) on the directed evolution of cephalosporin acylases, which is described in this thesis.
Since January 5, 2004 Linda is a post-doctoral researcher in the group of Dr. F. Hollfelder, department of Biochemistry at the Cambridge University (United Kingdom). Her research project is on the evolution of new modules for combinatorial biochemistry.

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Abbreviations

AA	amino acid
7-ACA	7-aminocephalosporanic acid
7-ADCA	7aminodesacetoxycephalosporanic acid
ADO	assembly of designed oligonucleotides
6-APA	6-aminopenicillanic acid
bps	base pairs
CLERY	combinatorial libraries enhanced by recombination in yeast
CPC	Cephalosporin C
DHR	degenerate homoduplex gene family recombination
DNA	deoxyribonucleic acid
DOGS	degenerate oligonucleotide gene shuffling
dNTP	2'-deoxyribonucleotide triphosphate
epPCR	error-prone PCR
ET recombination	recombination using recE and recT
HTS	high throughput screening
ITCHY	incremental truncation for the creation of hybrid enzymes
MAX	maximum efficiency randomisation
NF	mutant library of N266X combined with F375X
PenG	Penicillin G
PCR	polymerase chain reaction
RACHITT	random chimeragenesis on transient templates
RE	restriction enzyme
RETT	recombined extension on truncated templates
RID	random insertion/deletion
RPR	random-priming *in vitro* recombination
SCOPE	structure-based combinatorial protein engineering
SCRATCHY	shuffling combined with ITCHY
SHIPREC	sequence-homology-independent protein recombination
SISDC	sequence-independent site-directed chimeragenesis.
sp	spacer peptide
ss	signal sequence
ssDNA	single stranded DNA
StEP	staggered extension process
THIO-ITCHY	ITCHY with α-phosphothioate deoxynucleotide triphosphates
YLBS	Y-ligation-based block shuffling
YNF	mutant library of Y178H combined with N266X and F375X