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MASTER THESIS

Design and Expression of Recombinant Penicillin V and Aculeacin A Acylases in *E. coli*

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Abstract

Antimicrobial resistance and the search for new therapies is one of the great challenges humanity faces nowadays. In this sense, penicillin V acylase from *Streptomyces lavendulae* ATCC 13664 (*Sl*PVA) and Aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052 (*Au*AAC) are enzymes of great interest. Both of them are important catalysts in the synthesis of semisynthetic antimicrobials as well as they also have been found to potentially act as antimicrobials themselves by quorum quenching processes. Extracellular heterologous production of both enzymes has already been achieved in *S. lividans*. However, production in this organism is cumbersome due to its nature as a filamentous bacterium. The drawbacks include that they are not easy to grow, need long fermentation times and are easily contaminated. In this sense, heterologous protein production in *E. coli* can be regarded as advantageous.

In the course of this work, the synthetic *pva* and *aac* codon-optimized genes, encoding *Sl*PVA and *Au*AAC respectively, were cloned into the commercial plasmid pET-22b for expression in *E. coli*. Furthermore, for potential immobilisation experiments fusion genes with *gfp* gene as well as the substrate binding domain (SBD) sequence of $phaZ_{Sex}$ gene, that encodes the PHB depolymerase from *Streptomyces exfoliatus,* were designed and cloned into pET-22b. Hereafter, production of recombinant *Sl*PVA and *Au*AAC in different *E. coli* strains was attempted. Production medium was optimized and both enzymes were obtained as unprocessed inclusion bodies in *E. coli* BL21 (DE3). Unfortunately, active soluble protein could not be obtained.

However, obtained results can be used for designing further experiments regarding active processed protein as well as refolding of inclusion bodies.

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List of abbreviations

aa	amino acid
aac	*Au*AAC encoding gene
AHL	N-acyl-L-homoserine lactone
ahla	*Au*AHLA encoding gene
6-APA	6-aminopenicillanic acid
***Au*AAC**	aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052
***Au*AHLA**	N-acyl-L-homoserine lactone acylase from *Actinoplanes utahensis* NRRL 12052
B-PER	B-Per Bacterial Protein Extraction Reagent
BSA	bovine serum albumin
CLEA	cross-linking of enzyme aggregates
CLEC	cross-linking of enzyme crystals
DAB	3,3'-diaminobenzidine
DMSO	dimethylsulfoxide
dNTP	desoxyribonucleotide triphosphate
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein from *Aequorea victoria*
gfp	GFP encoding gene
HSL	homoserine lactone
IPTG	β-D-1-thiogalactopyranoside
OD_{600}	optical density at 600 nm
PBP	penicillin binding protein
PBS	Phosphate buffered saline
PDAB	*p*-dimethylaminobenzaldehyd
PGA	penicillin G acylase
PHA	polyhydroxyalkanoate
phaZ$_{Sex}$	*Se*PHB encoding gene
PHB	poly(3-hydroxybutyrate)
PHBV	poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PHV	poly(3-hydroxyvalerate)
PVA	penicillin V acylase
pva	*Sl*PVA encoding gene
QQ	quorum quenching
QS	quorum sensing
***Sl*PVA**	penicillin V acylase from *Streptomyces lavendulae* ATCC 13664
SBD	substrate binding domain of *Se*PHB
sbd	substrate binding domain of *Se*PHB encoding gene
***Se*PHB**	poly(3-hydroxybutyrate) depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
WHO	World Health Organisation

1 Introduction

Antimicrobial resistance has emerged as one of the greatest challenges in public health. Annually over 700,000 deaths around the world, that are estimated to rise to above 10 million by 2050, can be attributed to this crisis (Farha & Brown, 2019). In the last decade a big increase in proportion and absolute numbers of multiresistant strains has been noted (Roca *et al.*, 2015). This has led to the fact that this issue is again on the agenda of international politics and science (Centres for Disease Control Prevention, 2013; World Health Organisation, 2014). The WHO even calls antimicrobial resistance one of the ten threats to global health in 2019. The search for new antimicrobial therapies is of utmost importance in this context.

1.1 β-lactam antibiotics

β-lactam antibiotics, most notably penicillins, are probably publicly the best-known antibiotics. The first antibiotic of this class, penicillin G, was famously discovered by chance by Alexander Fleming in the 1920s (Fleming, 1929). Still, cephalosporins and broad-spectrum penicillins make up major parts of the antibiotics market (Hamad, 2010). Structurally, β-lactam antibiotics can be easily recognized by their 4-membered lactam ring (Figure 1). β-lactam antibiotics target and acylate the same class of transpeptidases, called penicillin binding proteins (PBPs), that are involved in the final assembly of the peptidoglycan of bacterial cell wall. Thus, this results in effectively inhibiting the bacterial cell wall integrity (Walsh & Wencewicz, 2016).

Resistance to β-lactam antibiotics often stems from a group of enzymes called β-lactamases. These hydrolysing enzymes have already been described in the 1940s, before the widespread use of penicillins. Today, over 890 unique representatives of this class have been found in naturally occurring bacterial isolates. Plasmid-encoded β-lactamases pose a great problem for β-lactam antibiotics, as often new specified enzymes arise almost immediately as soon as a new antibiotic is introduced into clinical usage (Bush, 2010).

Important classes of β-lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactams (Figure 1). Penicillins and cephalosporins are the earliest representatives of their class, already having been produced in the 1940s and 1950s. In the late 1960s, carbapenems emerged as a potent and alternative to already existing antibiotics. Still today, they possess the broadest spectrum of activity against Gram-positive and Gram-negative bacteria of their class. Therefore, they are often reserved as “antibiotics of last resort” (Papp-Wallace *et al.*, 2011).

Clavams are only weak antibiotics on their own, however, they are potent time-dependent inactivators of some β-lactamases (Walsh & Wencewicz, 2016).

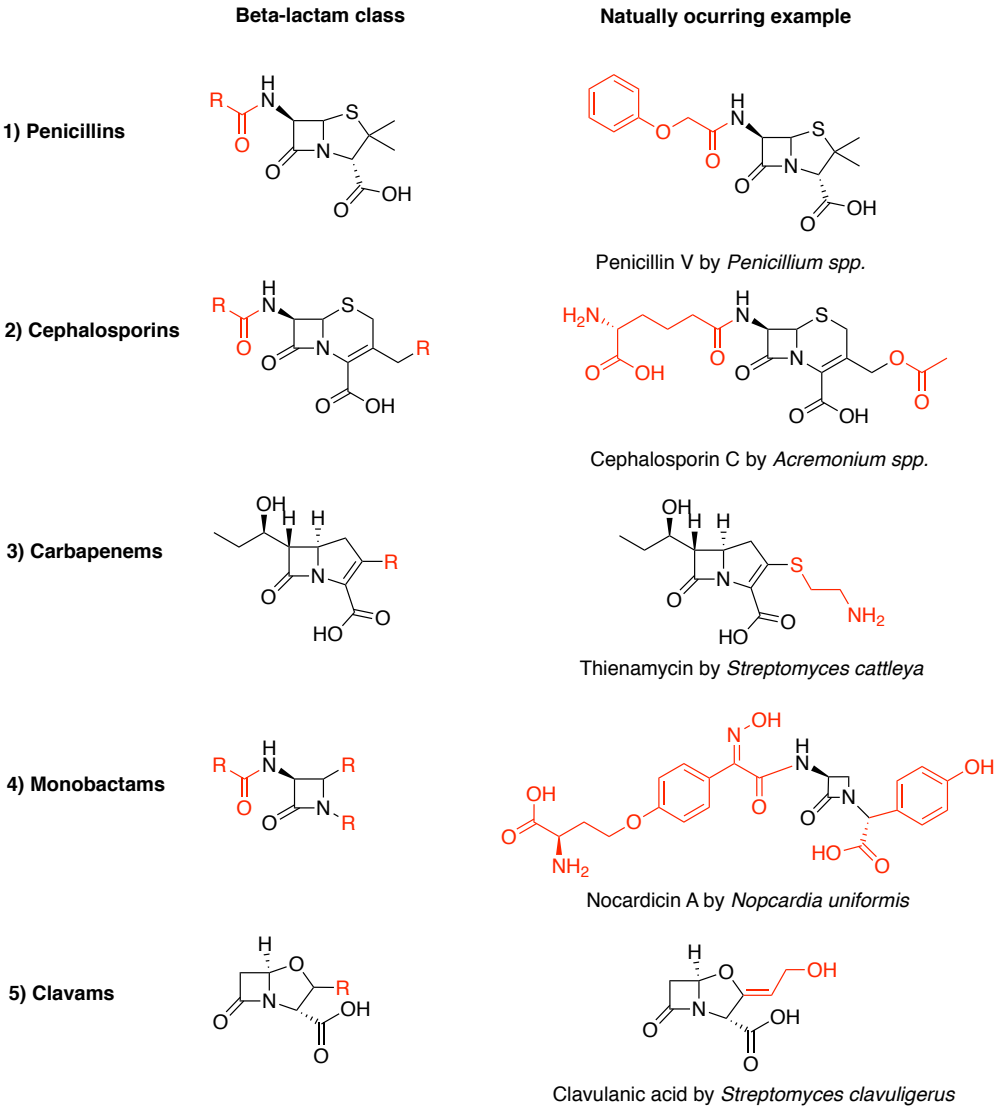


Figure 1: Five important classes of β-lactam antibiotics with a naturally occurring representative. Basic structure is presented in black, whereas side chains are in red. Modified graphic based on Walsh and Wencewicz (2016).

An antibiotic generation can be loosely defined by an incremental improvement in the following parameters: action spectrum, pharmacokinetics of onset and duration, pharmacodistribution in body tissues and sites of infection and lastly, the ability to overcome lactam-resistant microbes. Regarding these criteria, penicillins and cephalosporins, are already in their fifth generation (*e.g.* piperacillin, resp. ceftobiprole), carbapenems in their fourth (*e.g.* razupenem) and monobactams in their second generation (*e.g.* BAL30072) (Walsh & Wencewicz, 2016).

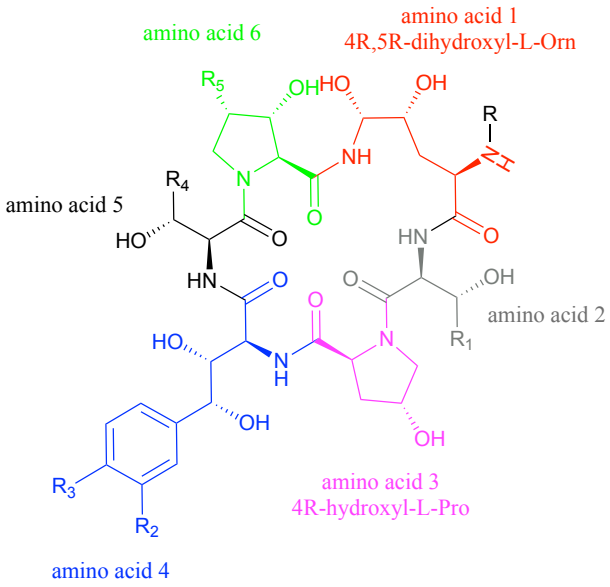
1.2 Echinocandin antifungals

Antifungal therapy has long been left in the shadow behind antibacterial therapy. From the 1960s to 1980s only a few antifungal drugs were available, foremost amphotericin B, a very effective, but toxic antifungal. However, this situation changed from 1980 onwards due to several reasons: a growing incidence of septicaemia caused by invasive fungi and aspergillosis, the rapid appearance of AIDS and as a consequence immunocompromised patients and lastly, the evolution of new opportunistic fungi (Bryskier, 2005).

Antifungals can be divided into three classes: polyene antifungals (*e.g.* amphotericin B), azole antifungals (*e.g.* itraconazole) and echinocandins. Whereas polyene antifungals attack the ergosterol in fungal membranes creating pores and causing cell deaths and azole antifungals generally inhibit ergosterol biosynthesis, echinocandins attack the fungal cell wall by inhibiting the synthesis of β-D-glucan (Bryskier, 2005).

Echinocandins are a state-of-the-art antifungal drug class, however, the first representative, echinocandin B was already described in the 1970s as a secondary metabolite from *Aspergillus nidulans var. echinolatus* (Benz *et al.*, 1974). They excel in low toxicity and rapid fungicidal activity against all *Candida* spp., also showing inhibitory character against *Aspergillus* spp. and *Pneumocystis carinii*. However, they lack in solubility (Denning, 2002). A generalized structure and the respective residues of several naturally occurring echinocandins are presented in Figure 2. The core structure of echinocandins consists out of a hexapeptid scaffold, typically featuring nonproteinogenic amino acids like ornithine, homotyrosine and methyl-proline. A further defining characteristic is the presence of a long-chain fatty acyl amide (Emri *et al.*, 2013).

More than 20 naturally occurring echinocandins have been isolated and described. All of them are produced by Ascomycota fungi, especially by genus *Aspergillus.* Aculeacin A, first isolated from *Aspergillus aculeatus*, strongly inhibits the growth of filamentous fungi and is fungicidal against yeasts and yeast-like fungi except *C. tropicalis* (Mizuno *et al.*, 1977). An important exception is pneumocandin B_o, produced by *Glarea lozoyensis*, first isolated in 1988 and found to be a strong inhibitor of *Pneumocystis carinii*, the causative agent in pneumocystis in immunosuppressed patients (Schwartz *et al.*, 1989).



R: linoleoyl
palmitoyl
myristoyl
10,12-dimethylmyristoyl
R_1: $-CH_3$, -H
R_2: -H, $-HSO_4$, -OH
R_3: -OH, $-HSO_4$
R_4: $-CH_3$, -H, CH_2CONH_2
R_5: $-CH_3$, -H, CH_2OH

Echinocandin	acyl-moiety	ac 2	ac 4	ac 5	aa 6
Echinocandin B	linoleoyl	L-Thr	3S,4S-dihydroxy-L-homoTyr	L-Thr	3S-hydroxyl,4S-methyl-L-Pro
Aculeacin A	palmitoyl	L-Thr	3S,4S-dihydroxy-L-homoTyr	L-Thr	3S-hydroxyl,4S-methyl-L-Pro
Pneumocandin B_0	10,12-dimethylmyristoyl	L-Thr	3S,4S-dihydroxy-L-homoTyr	3R-hydroxyl-L-Gln	3S-hydroxyl- L-Pro

Figure 2: ***above****: generalized structure of naturally occurring echinocandins;: each amino acid (ac) is colour-coded;* ***below****: naturally occurring echinocandins with their respective residues explained* (Emri *et al.*, 2013).

1.3 Bacterial communication as potential drug target by quorum quenching

Bacteria have the ability to work together, influencing their pathogenicity and their resistance to antibiotics. Recently, bacterial communication has been identified as a potential drug target (McBrayer & Tal-Gan, 2019). Even though drugs targeting bacterial communication (see section 1.3.3) might be less effective than traditional antibiotics under 'real life' conditions due to the fact that often they do not interrupt vital functions, literature suggests that they might be less susceptible to bacterial resistance (Grandclément *et al.*, 2015).

1.3.1 Biofilms

Contrary to the planktonic and strictly unicellular way of life of bacteria in many laboratory shake-flask cultures and in the world's largest habitat, the ocean, mostly all bacteria have the ability to form biofilms as a way to provide a mechanically stable and protective environment.

These structures mainly built from extracellular polysaccharides are prevalent on most wet surfaces and provide high stability against environmental stresses like pH or temperature, moreover, they can protect against antibiotics. Their formation is also the basis for improved nutrient transport.

Biofilm-growing bacteria can cause chronic infections that tolerate antibiotic treatment and innate and adaptive immune and inflammatory response. Biofilm growth can occur on the skin (*e.g.* chronic wounds) as well on the inner body surface, such as in the lungs of patients suffering from cystic fibrosis, where *Pseudomonas aeruginosa* is known to produce AHL-dependent biofilms (see section 1.3.2). Furthermore, an increased mutation-frequency and increased horizontal gene transfer can be observed in biofilm-growing bacteria. These circumstances explain the tendency of multi-drug resistance in these organisms. Bacterial cells in biofilms can have the ability to produce antibiotic-degrading enzymes and to have antibiotic targets of low affinity, while overexpressing broad-range efflux-pumps (Høiby *et al.*, 2010).

1.3.2 Quorum sensing

Formation of biofilms is dependent on bacterial communication. For Gram-negative bacteria N-acyl-L-homoserine lactones (AHLs) have been found to play a major role in this process. These molecules play not only a role in biofilm formation, but also in numerous other function such as bioluminescence, production of secondary metabolites, sporulation or competence for DNA uptake (Fetzner, 2015). Often produced in a mixture of several different AHLs, these bacterial pheromones seem to have one species-specific component: the nature of their N-acyl chain (Figure 3). This chain varies in length and can have different oxidation states in the 3-position (*e.g.* a ketone functionality in 3-oxo-C_6-HSL, an AHL produced by *Vibrio fischeri*, that induces bioluminescence in the species (Eberhard *et al.*, 1981)). Due to the fact that significant bacterial response is only triggered by a certain AHL concentration and that they trigger their own biosynthesis (Figure 4), these molecules are also called autoinducers or quorum sensing molecules (Dickschat, 2010).

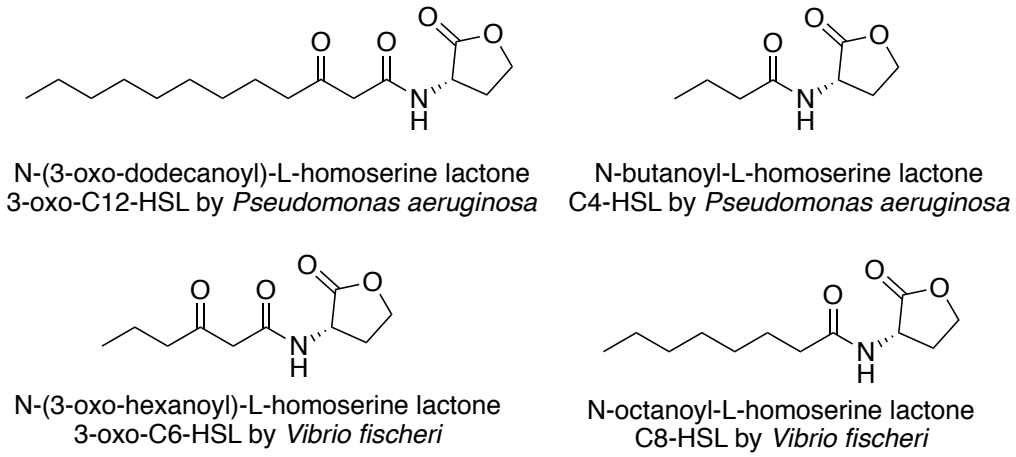


Figure 3: Examples of N-acyl-L-homoserine lactones with their respective producing organism. HSL: homoserine lactone (Dickschat, 2010)

1.3.3 Quorum quenching

Quorum quenching can be understood as interfering with quorum sensing, thereby reducing biofilm production among other processes. In general, three different strategies can be applied (Figure 4): inhibition of signal biosynthesis (*e.g.* methylthioadenosine/S-adenosyl-homocysteine nucleosidase (Parveen & Cornell, 2011)), inhibition of signal detection (*e.g.* LuxR receptor (Koch *et al.*, 2005)) or inactivation of signal molecules. AHLs specifically can be inactivated by reductases, that reduce 3-oxo substituted AHLs, by cytochrome oxidases, that oxidize the acyl chain, by lactonases, that open the homoserine lacton ring, or AHL-acylases, that cleave the acyl group, releasing the homoserine lactone and a fatty acid (Grandclément *et al.*, 2015; Koch *et al.*, 2005). Quorum quenching might be a system less likely to be affected by resistance due to the fact that inhibition biofilm production does not pose strong selective pressure (Defoirdt *et al.*, 2010).

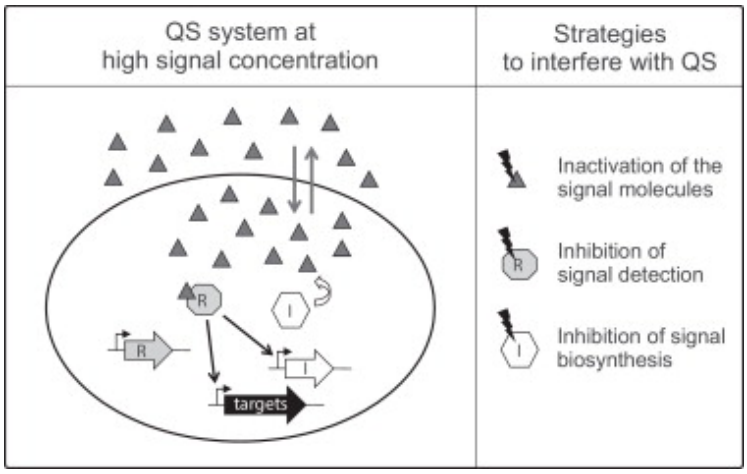


Figure 4: ***Left:*** *quorum sensing system: QS triggers own biosynthesis and induces target expression;* ***right:*** *quorum quenching strategies. Reprinted* from *Fetzner (2015) with Permission from Elsevier.*

1.4 Biotechnological production of β-lactam antibiotics and echinocandins

As mentioned in section 1.1 various antibiotic generations have been developed for β-lactam antibiotics in order to generate better properties. Variation of the side chain of the β-lactam nucleus can lead to preferable attributes like better stability, easier absorption and fewer side effects. The first semisynthetic penicillin examples appeared in the early 1960s exemplified by ampicillin in 1962 and amoxicillin in 1972 (Bruggink, 2011). All semisynthetic penicillins are being synthesized by bulk production of natural occurring penicillins (penicillin G or penicillin V) and subsequent hydrolysis of the acyl group, followed by adding the desired residue (Figure 5).

Traditional industrial chemical synthesis of 6-APA by an one-pot deacylation of penicillin G was first developed in the 1970s. This procedure required the use of hazardous chemicals and solvents. Nowadays, this procedure is largely replaced by enzymatic hydrolysis of penicillin G using immobilized penicillin G acylases (PGAs) as biocatalysts, yielding 100 % enantiomeric pure 6-APA (Arroyo *et al.*, 2017). However, it has been reported that production of penicillin V and subsequent deacylation to 6-APA by penicillin V acylases (PVAs) could be more advantageous (Shewale & Sudhakaran, 1997). Penicillin V shows higher stability in aqueous solution at low pH during extraction from the fermentation broth. Moreover, producer strains of penicillin V are more tolerant towards high concentrations of phenoxyacetic acid than penicillin G producers are towards phenylacetic acid. Therefore, higher yields can be obtained (Hersbach, 1984). Additionally, PVAs withstand high penicillin V concentrations with yields of up to 99 % and show optimal activity at a wide range of pH values (Shewale & Sudhakaran,

1997). Nevertheless, only 15 % of 6-APA produced worldwide stems from penicillin V (Arroyo *et al.*, 2017). During bulk production of natural penicillins by *Penicillium chrysogenum*, about 1-2 % of aliphatic penicillins (*e.g.* penicillin K, F and dihydroF) are created as well. Industrial penicillin acylases struggle with hydrolysis of these by-products, creating problems in subsequent crystallisation of 6-APA (Velasco Bucheli, 2017).

Using enzymes to carry out the synthesis reactions to produce semisynthetic penicillins has the advantage that extra protection-deprotection and purification steps are avoided and allows the use of environmentally friendly solvents. This reaction can be either thermodynamically controlled by shifting the equilibrium (*e.g.* continuously removing product) or kinetically controlled by using activated acyl donors (as amide or ester) (Volpato *et al.*, 2010). In general, large-scale biotechnological production of semisynthetic penicillins is focused on enzymatic acylation of the 6-APA nucleus to an appropriate D-amino acid (Arroyo *et al.*, 2017).

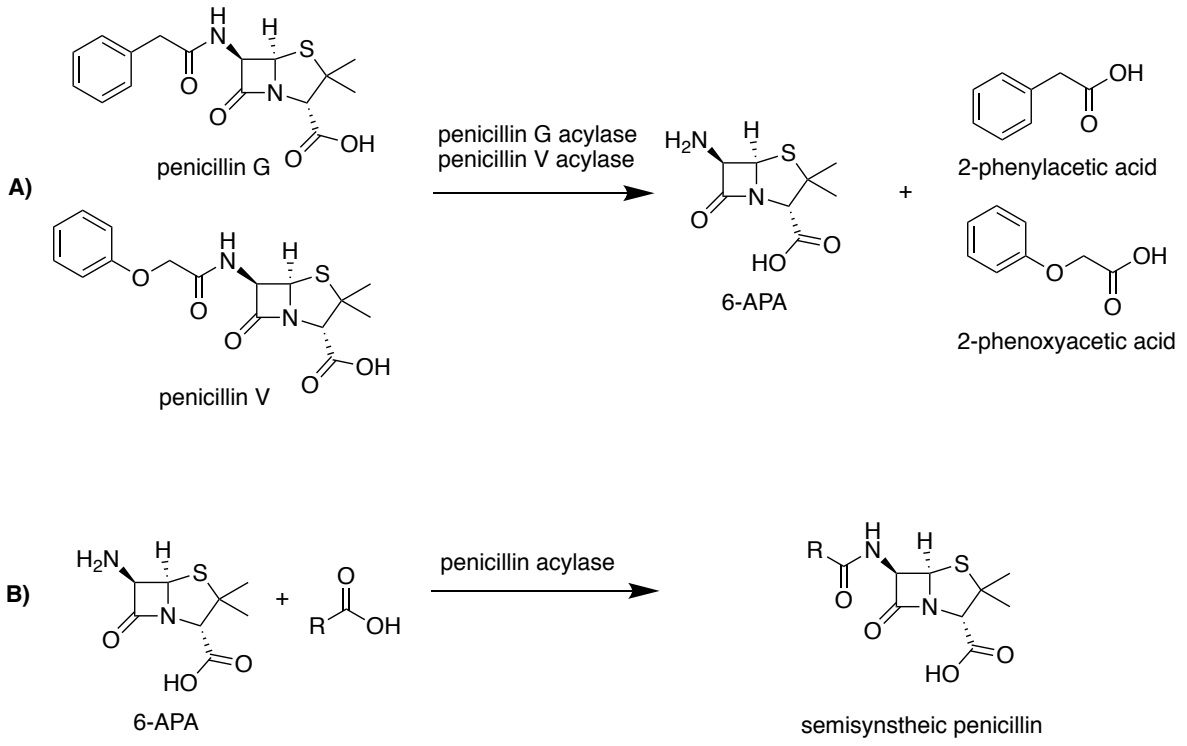


Figure 5: Biotechnological production of semisynthetic penicillins; ***A)*** *enzymatic hydrolysis of penicillin G or penicillin V;* ***B)*** *enzymatic acylation of 6-APA*

Natural echinocandins lack suitable water solubility and often show haemolytic properties. By modifying the molecules, these properties are sought to be improved (Brahmachari, 2016). Similarly to penicillins, the cyclic hexapeptide core of echinocandins can be obtained by enzymatic hydrolysis of the acyl group. Up to now, three semisynthetic echinocandins are FDA-approved: caspofungin, anidulafungin and micafungin. As an example, the semisynthetic

production of anidulofungin and cilofungin is outlined below (Figure 6). Cilofungin was the first semisynthetic echinocandin, however, is not a FDA-approved drug due to its nephrotoxicity (Arroyo *et al.*, 2017). Anidulofungin is produced from echinocandin B or aculeacin A using aculeacin A acylase to obtain the cyclic hexanucleotide core (Figure 6), which in turn is chemically acylated creating anidulofungin. Micafungin is a drug synthesized from the core structure of pneumocandin B_0 and caspofungin from the core structure of echinocandin B (Velasco Bucheli, 2017).

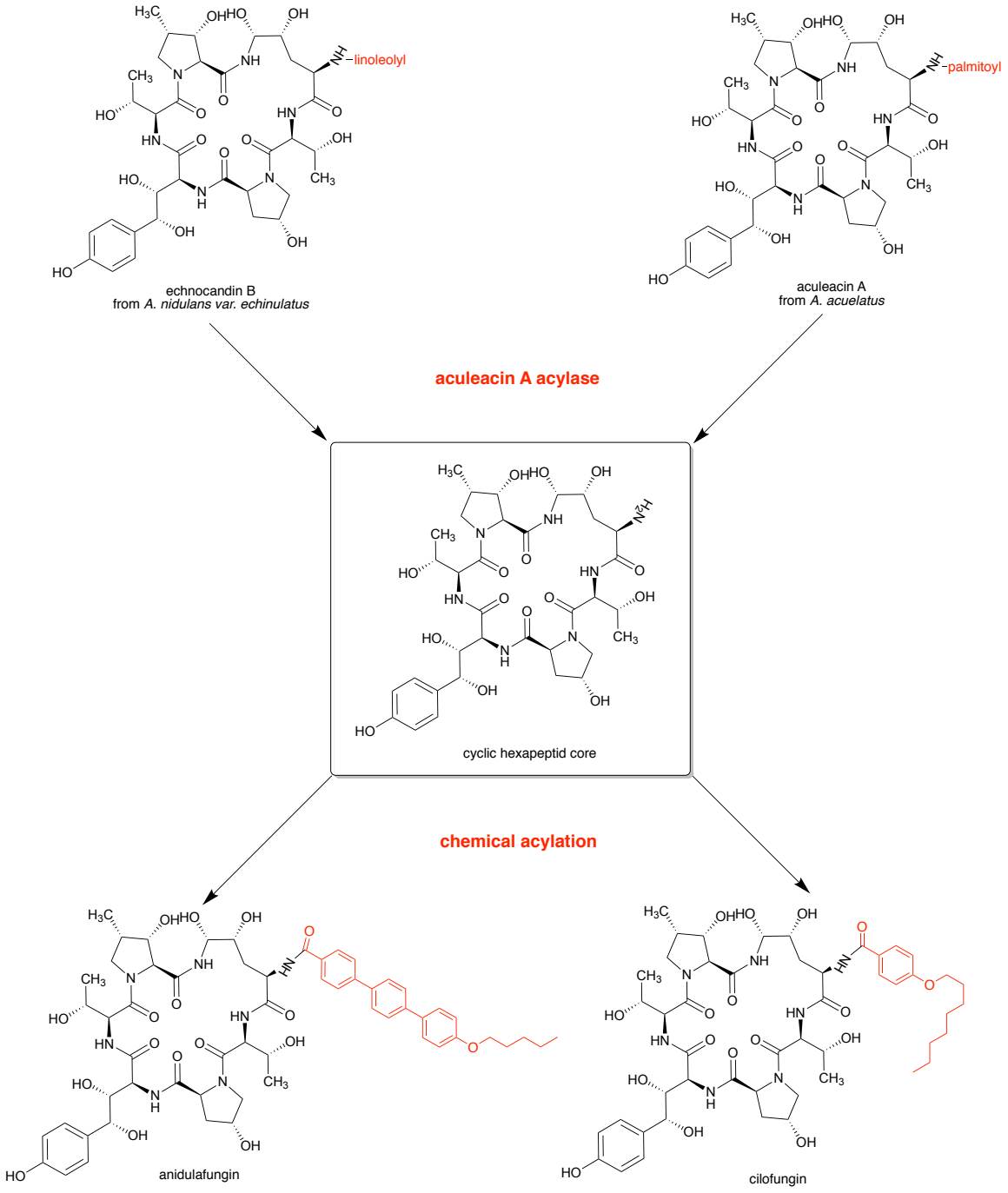


Figure 6: Outline of semisynthetic production of anidulofungin and cilofungin using aculeacin A acylase to produce the cyclic hexapeptide core. Cleaved and added parts of the molecular structure are marked red (Arroyo et al., 2017).

1.5 Biochemical, structural and phylogenetic properties of penicillin, echinocandin and AHL acylases

Penicillin, echinocandin and AHL acylases are aminohydrolases. Penicillin acylases, enzymes that cleave the acyl chain of penicillins yielding 6-APA, can be roughly divided into penicillin G, penicillin V and ampicillin acylases according to the preference of the enzyme to the phenylacetyl (penicillin G), phenoxyacetyl (penicillin V) or 2-amino phenylacetyl (ampicillin) acyl chain. However, this classical distinction has to be expanded by inclusion of enzymes able to hydrolyse aliphatic penicillins (*e.g.* penicillin F, penicillin dihydroF, penicillin K) (Arroyo *et al.*, 2003). Examples for this new group of aliphatic penicillin acylases have been found in *Streptomyces lavendulae* (Torres-Guzmán *et al.*, 2002) and in *Thermus thermophiles* (Torres *et al.*, 2012). Also, aculeacin A acylase from *Actinoplanes utahensis* has been reported to have hydrolytic activity against aliphatic penicillins justifying inclusion to this group (Torres-Bacete *et al.*, 2007).

Penicillin, echinocandin and AHL acylases are part of the N-terminal nucleophile (Ntn) hydrolase superfamily (Hong *et al.*, 2012; Torres-Bacete *et al.*, 2015). Enzymes of this superfamily are activated by intern-mediated autosplicing and contain a single catalytic nucleophilic amino acid, that is located N-terminally. Although no sequence homology exists within the supergroup, structural similarities are evident (Brannigan *et al.*, 1995; Duggleby *et al.*, 1995; Oinonen & Rouvinen, 2000). Typically, the catalytic active centres of these enzymes are arranged in a four-layered αββα-structure, where two antiparallel β-sheets form the core that is surrounded by a layer of α-helices. The single catalytic nucleophile is located at the junction of the β-sheets. However, the nature of this nucleophile differs between enzymes. (Shi *et al.*, 2010). Generally, penicillin, echinocandin and AHL acylases are composed of α- and β-subunits of about 20 kDa and 60 kDa respectively (Torres-Bacete *et al.*, 2007; Velasco Bucheli, 2017). The catalytic mechanism for penicillin G acylase from *E. coli* has already been published and is shown below (Figure 7) (Duggleby *et al.*, 1995). The mechanism proposed resembles the one of a serine protease. Located at the end of the β-subunit, a serine (β-Ser^{1}) acts as the catalytic nucleophile. Its hydroxyl moiety attacks the acyl moiety of the substrate after activation by interaction of water with the α-amino of β-Ser^{1}. A tetrahedral intermediate (T_d1) is formed, which is stabilised by β-Ala^{69} and β-Asn^{241} (oxyanion hole). This transition state collapses leading to deacylation of the substrate. The formed covalent acyl-enzyme intermediate is in turn attacked by water acting as the nucleophile. Again, a tetrahedral

intermediate (T_d2) is formed, which is stabilised by the oxyanion hole. After electron rearrangement the enzyme and the substrate are released.

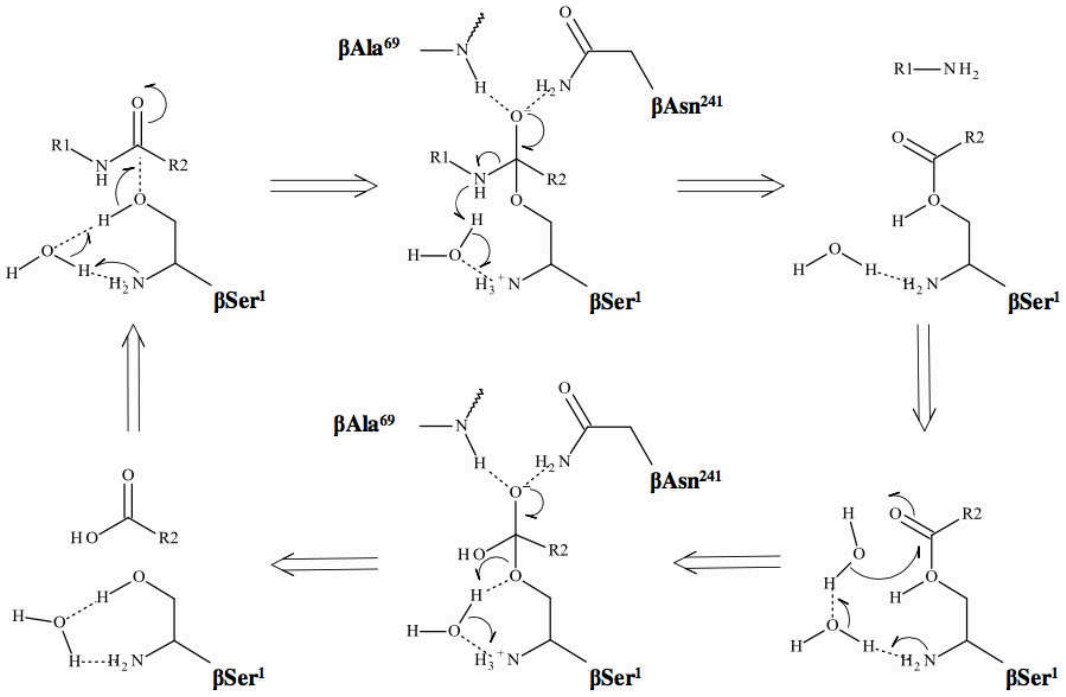


Figure 7: Catalytic mechanism for penicillin G acylase from E. coli proposed by Duggleby et al. (1995). Figure reprinted from Velasco Bucheli (2017).

1.5.1 Phylogenetic analysis of *Sl*PVA and *Au*AAC

In the following, two specific molecules will be closely reviewed: penicillin V acylase from *Streptomyces lavendulae* ATCC 13664 (*Sl*PVA) and aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052 (*Au*AAC). These enzymes are related as shown in Figure 8. *Sl*PVA shares a branch with two enzymes: AHLA from *Streptomyces* sp. M664 and a cyclic lipopeptide acylase from *Streptomyces* sp. FERM BP-5809. The next neighbour to this group is already N-acyl-L-homoserine lacton acylase from *Actinoplanes utahensis* NRRL 12052 (*Au*AHLA), an enzyme also studied in Enzymatic Biotechnology group of UCM. This exemplifyies their close relationship. In turn, all mentioned enzymes share a common ancestor with *Au*AAC (Velasco Bucheli, 2017).

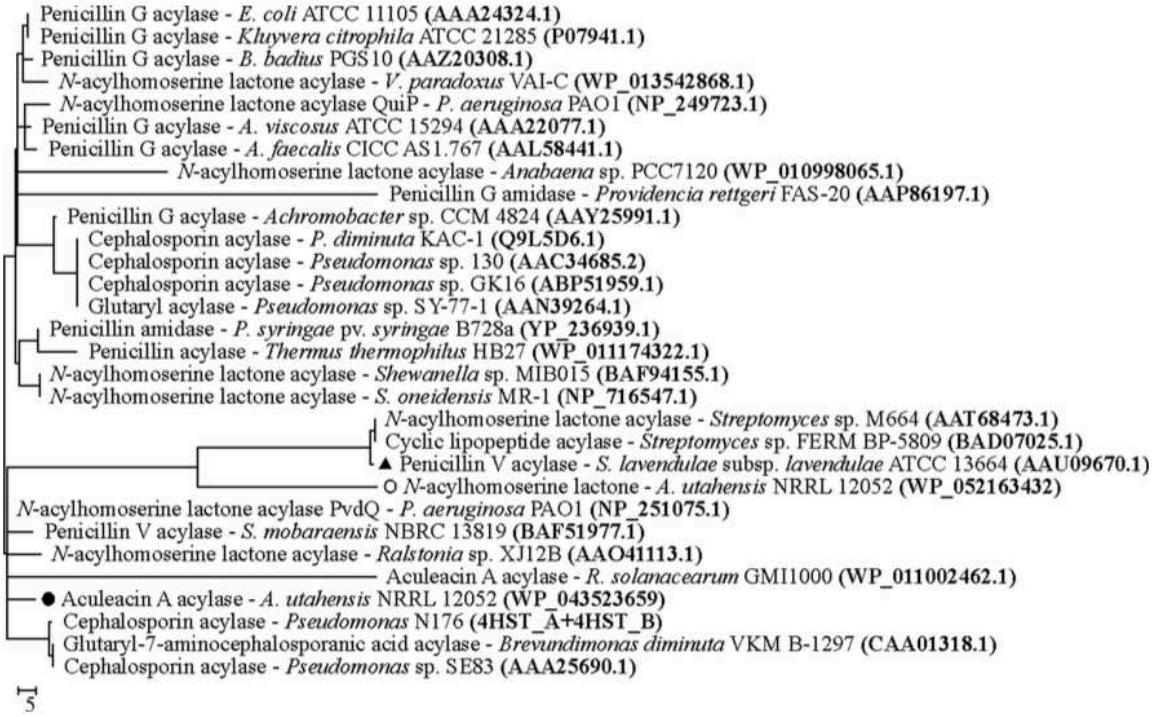


Figure 8: Comparative phylogenetic tree of PVA from Streptomyces lavendulae ATCC 13664 (▲), AAC from Actinoplanes utahensis NRRL 12052 (●) and AHLA from Actinoplanes utahensis NRRL 12052 (O) with other acylases. GenBank is in bold. Figure reprinted from Velasco Bucheli (2017).

1.5.2 Penicillin V acylase from *Streptomyces lavendulae* ATCC 13664

*Sl*PVA was first mentioned as the preferred acylase used for production of 6-APA from penicillin V in a patent from 1961 (Newbolt *et al.*, 1961). It was identified as an extracellular enzyme (Torres *et al.*, 1999) that presents itself in its active form as a heterodimer with an α-subunit of 18.79 kDa and a β-subunit of 60.09 kDa. The *pva* gene encodes an inactive precursor of 806 amino acids, that has a N-terminal signal peptide of 39 amino acids (Figure 9). This signal peptide features typical *Streptomyces* characteristics: a short N-terminal basic sequence followed by hydrophobic amino acids and C-terminally a typical hydrolytic target sequence. Furthermore, a linker peptide of 25 amino acids is proposed between the C-terminus of the α-subunit and the N-terminus of the β-subunit. As outlined in Figure 9, this precursor is probably processed in three steps to achieve its active heterodimer form: the secretion machinery cuts the N-terminal signal peptide followed by autoproteolytic activation by releasing the internal linker peptide and the α-subunit (Torres-Bacete *et al.*, 2015).

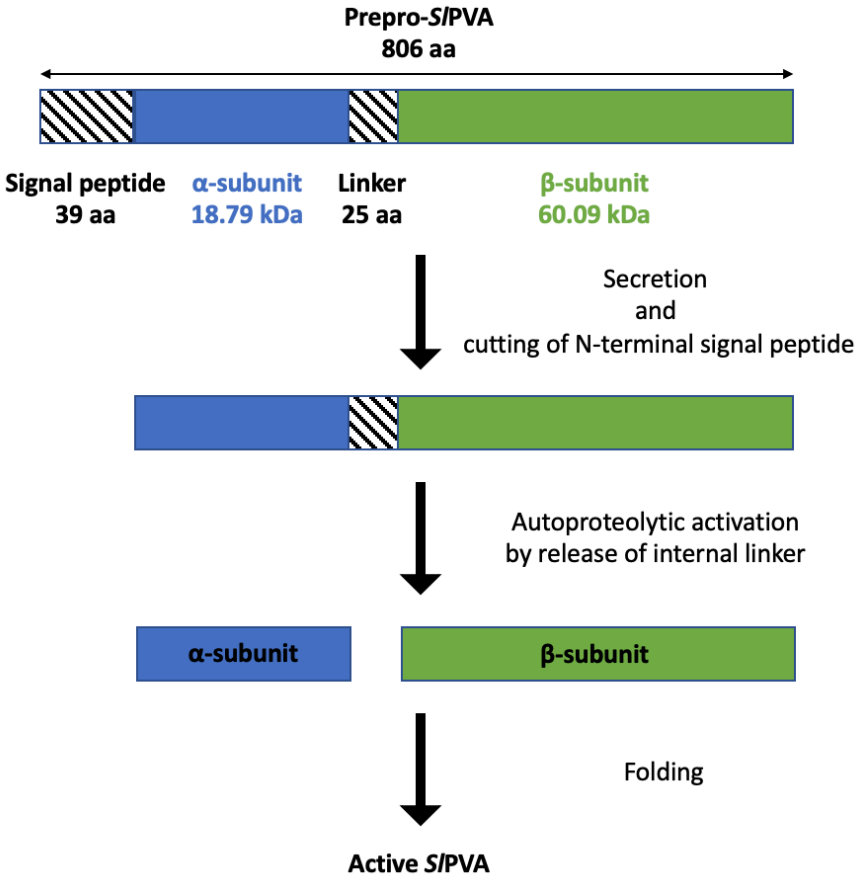


Figure 9: Schematic overview over the preproenzyme encoded by the pva gene and of proposed processing to active enzyme. Lenght of subunits and peptides are not scaled.

The amino acids involved in the catalytic process have been elucidated by site-directed mutagenesis: β-Ser^{1}, β-His^{23}, β-Val^{70} and β-Asn^{272} (Torres-Bacete *et al.*, 2015). Correlating with the mechanism proposed for PVA from *E. coli* (see section 1.5) β-Ser^{1} acts as the nucleophile. Moreover, as 3D-modelling (Figure 10) places β-His^{23} adjacent to β-Ser^{1}, a catalytic dyad is suggested, as it is already known for several serine proteases. The imidazole of β-His^{23} would act as a basic group that enhances the nucleophilicity of β-Ser^{1}. Furthermore, the nitrogens of the main chain of β-Val^{70} and β-His^{23}, as well as the δ-nitrogen of β-Asn^{272} form the oxyanion hole, thus stabilising the tetrahedral intermediates. Amino acids involved in

substrate binding are α-Ala[154], α-Gly[158], β-Tyr[24], β-Arg[31], β-Trp[33], β-Leu[50], β-Ser[53], β-Ser[57], β-Ile[58], β-Ser[161] and β-Val[186] (Perona & Craik, 1995; Torres-Bacete *et al.*, 2015).

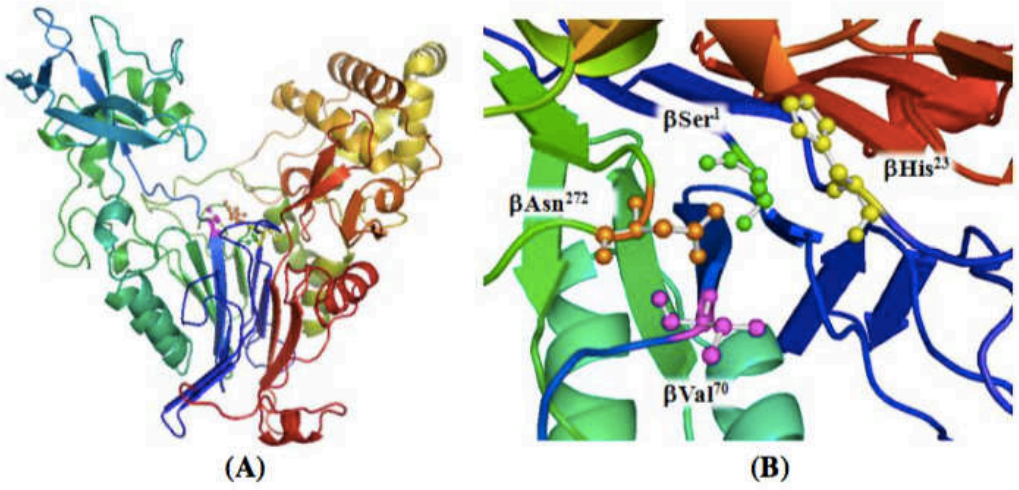


Figure 10: 3D-model of β-subunit of SlPVA. ***(A)*** *Complete model;* ***(B)*** *Detailed model of catalytic site. Reprinted from Torres-Bacete et al. (2015) with permission from the American society for Microbiology.*

*Sl*PVA hydrolyses penicillin V very efficiently. However, interestingly it also shows good activity towards other aliphatic penicillins like penicillin dihydroF, penicillin F and especially penicillin K, which it hydrolyses with the highest catalytic efficiency of all mentioned substrates. These naturally occurring penicillins often are present in the production broths of penicillins G and V and furthermore, hamper with crystallization of the product. *Sl*PVA activity towards penicillin G is only at a rate of 2 – 3 % of the rate for hydrolysis of penicillin V (Torres *et al.*, 1999; Torres-Guzmán *et al.*, 2002). Furthermore, it has been found that *Sl*PVA shows hydrolytic activity towards AHLs. Acylase activity towards aliphatic and β-keto substituted AHLs (C8-, C10-, C12- and C14-HSL) as substrate was confirmed, showing its potential in quorum quenching processes (Velasco Bucheli, 2017).

1.5.3 Aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052

*Au*AAC is an extracellular enzyme that belongs to the group of echinocandin acylases, an enzyme family that hydrolyse naturally occurring echinocandin antifungals like aculeacin A or echinocandin B.

The enzyme exists in its native form as a heterodimer with an α-subunit of 19 kDa and a β-subunit of 55 kDa, where both subunits are integral for its catalytic properties (Takeshima *et al.*, 1989). *Au*AAC keeps 43 % sequence identity with *Sl*PVA (Velasco Bucheli, 2017). It has been suggested, that *Au*AAC is produced as a preproenzyme of 786 amino acids containing a linker sequence of 15 amino acids between the subunits. Furthermore, a leader sequence of 34

amino acids is present that reminds of typical bacterial leader peptides (Junji *et al.*, 1992). Similar to *Sl*PVA, β-Ser^{1}, β-His^{23}, β-Val^{70} and β-Asn^{257} have been reported to form the catalytic centre of the enzyme. Consistent with its classification as part of the Ntn-hydrolases superfamily, β-Ser^{1} acts as the nucleophile attacking the acyl group of the substrate, while β-His^{23} supports the catalysis by enhancing the nucleophilicity of β-Ser^{1} as described in the previous section. The nitrogens of the main chain of β-His^{23} and β-Val^{70} and the δ-nitrogen of β-Asn^{257} form the oxyanion hole that stabilizes the tetrahedral intermediates formed during the catalytic process. Amino acids involved in substrate binding are the following: α-Met^{148}, α-Gly^{152}, β-Phe^{24}, β-Arg^{31}, β-Tyr^{33}, β-Leu^{50}, β-Asp^{53}, β-Glu^{57}, β-Ile^{58}, β-Trp^{157} and β-Val^{182} (Hormigo, 2009).

*Au*AAC not only shows activity towards the antifungal aculeacin A, but also can hydrolyse penicillin V on its own (Torres-Bacete *et al.*, 2007). Remarkably, the enzyme shows the same enzymatic activity towards aliphatic penicillins (penicillin dihydroF, penicillin F and penicillin K) like *Sl*PVA. The before mentioned observations justify the consideration that *Au*AAC is a β-lactam acylase and that *Au*AAC and *Sl*PVA form a new class of β-lactam acylases, that show high specifity towards penicillin K (Torres-Bacete *et al.*, 2007). Moreover, as for *Sl*PVA enzymatic activity towards towards aliphatic and β-keto substituted AHLs with more than 6 carbons has been found, enabling a potential role in quorum quenching processes (Velasco Bucheli, 2017). Furthermore, *Au*AAC is highly thermostable. Enzymatic activity at up to 81.5 °C has been reported (Torres-Bacete *et al.*, 2007).

1.6 Enzyme immobilisation

In industrial processes, enzymes immobilised to a carrier often are necessary to guarantee optimal operational performance. Advantages of enzymes confined to a defined space are numerous: enzyme stability increases, reutilisation of the enzyme is possible and process design and control is facilitated by use of immobilized enzymes. However, it is important to note that limitations exist: enzyme conformation is altered in the immobilized form; the heterogeneity of the enzyme-carrier system that allows different amounts of enzyme bind to the carrier in various fractions; constraints in diffusion of substrate to the carrier and lastly, the economic factor, since enzyme immobilisation represents an additional step. Together, these factors can limit enzyme activity and the effectiveness of the enzyme-carrier system (M. Arroyo, 1998; DiCosimo *et al.*, 2013; Sheldon, 2007).

Enzymes can be immobilized by binding to supports. The nature of the bond can either be physical (hydrophobic or Van-der-Waals interactions), ionic or covalent. Furthermore, entrapment via inclusion in a polymer network is a viable option for immobilization. Furthermore, cross-linking of enzyme aggregates or crystals (CLEA or CLEC) has emerged as new carrierless methods for immobilization (Sheldon, 2007). Lastly, immobilization based on affinity-domain fusion protein is another widely used strategy. It has been reported by Lee *et al.* (2005) that a fusion protein system employing the substrate-binding domain (SBD) of a polyhydroxyalkanoate (PHA) depolymerase as the affinity domain has been successfully used to immobilise proteins to PHA microbeads.

1.6.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are naturally occurring biopolymers that are biodegradable by a wide variety of microorganisms. Their role in bacterial metabolism is defined as carbon and energy storage, supported by uncontrolled growth during fermentation. Accumulation of PHA granules is known to be a microbial survival mechanism. More than 150 different PHAs are known, making them the largest group of natural bioesters. The most important are poly(3-hydroxybutyrate) (PHB), poly(3-hydroxyvalerate) (PHV) and their copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) (Li *et al.*, 2016).

1.6.2 Poly(3-hydroxybutyrate) depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693

Poly(3-hydroxybutyrate) depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693 (*Se*PHB) is encoded by the *phaZ*$_{Sex}$ gene. The gene *phaZ*$_{Sex}$ encodes a 50.35 kDa protein that starts N-terminal with a typical *Streptomyces* signal peptide of 26 amino acids, followed by a catalytic domain containing a lipase box. A substrate-binding domain (SBD), C-terminal, is present, that is homologous with other PHB-depolymerases (Figure 11). Active protein has a molecular weight of 49 kDa (Klingbeil *et al.*, 1996).

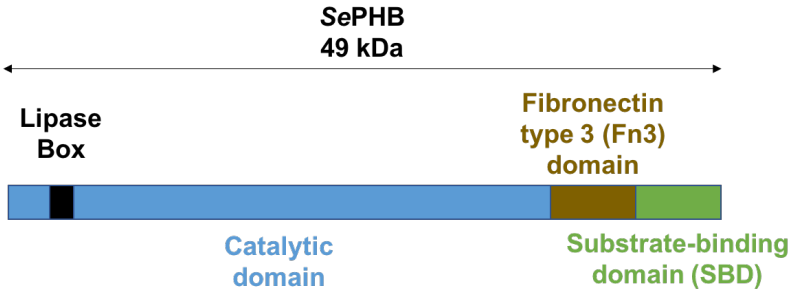


Figure 11: Schematic overview over domains of SePHB. Lenght of domains is not scaled.

It has been reported that the SBDs of several PHB-depolymerases have a very broad range binding specificity, exceeding the catalytic ability of its respective catalytic domain. SBDs of PHB-depolymerases bind to the surface of various PHA (P(3HP), P(3HB), P(2HP)), however, not to the surface of Avicel or chitin, suggesting a molecular recognition between PHAs and the SBD (Kasuya *et al.*, 1999). Furthermore, *Se*PHB shows activity towards *p*NP-acetate and *p*NP-butyrate, showing its ability as lipase and esterase (García-Hidalgo *et al.*, 2012).

2 Objectives

Pathogenic bacteria resistant to current therapies pose one of the biggest challenges nowadays. The global crisis - that the development of new antimicrobials lags behind the rise of new resistances - is a great challenge. The WHO has called for more resources and efforts into R&D, and into the search and development of new antibiotics, antifungals and antimicrobial therapies (World Health Organisation, 2014).

Penicillin V acylase from *Streptomyces lavendulae* ATCC 13664 (*Sl*PVA) and Aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052 (*Au*AAC) are enzymes of great interest. Not only are they important catalysts in bioreactors in the synthesis of semisynthetic antimicrobials and in the search for new molecules, but they also have been found to potentially act as antimicrobials themselves in quorum quenching processes. Extracellular heterologous production of both enzymes by *Streptomyces lividans* has been successfully achieved by the Enzymatic Biotechnology group of UCM, where this work has been carried out (Torres-Bacete *et al.*, 2007; Torres-Bacete *et al.*, 2015). However, this bacterium has drawbacks due to the fact that it is a filamentous microorganism. They are not easy to grow, need long fermentation times and are easily contaminated. In addition, a very limited number of commercial expression vectors are available. Therefore, it is of interest to express both genes in *E. coli*, the microorganism most widely used in heterologous gene expression. However, in previous studies it has not been possible to express them in *E. coli* due to the different use of codons between both bacteria.

Furthermore, in order to apply the enzymes as antimicrobials in quorum quenching processes, immobilization based in affinity-domain fusion protein of both enzymes is also of great interest.

Based on these facts, the main objective of the present work is to optimize the heterologous expression of *Sl*PVA and *Au*AAC by *E. coli* likewise to obtain fusion proteins in order to immobilize both enzymes on biodegradable and biocompatible support. For that, several objectives shall be accomplished in this study:

- Heterologous expression by *E. coli* of *pva* gene codon-optimized and cloned into the commercial expression plasmid pET-28a
- Cloning of *pva* gene codon-optimized into the commercial plasmid pET-22b for production of *Sl*PVA in *E. coli*

- Design and cloning of codon-optimized *pva* and *aac* fusion genes with *gfp*, *sbd* (the substrate binding region of $phaZ_{Sex}$) and *Linker*::*sbd* (the substrate binding region of $phaZ_{Sex}$ connected N-terminally to a linker sequence) into pET-22b for production in *E. coli*
- Heterologous expression of recombinant proteins *Sl*PVA and *Au*AAC by *E. coli*
- Heterologous expression of recombinant fusion proteins *Sl*PVA-GFP, *Sl*PVA-SBD, *Sl*PVA-Linker-SBD, *Au*AAC-GFP, *Au*AAC-SBD and *Au*AAC-Linker-SBD by *E. coli*

3 Material and Methods

3.1 Chemical Reagents and enzymes

Betaine, *D*-sorbitol and Tris(hydroxymethyl)aminomethane (Tris) were supplied by Acros Organics (USA). Coomassie G-250 and ethylenediaminetetraacetic acid (EDTA) were purchased from Fluka (Switzerland). The following chemicals were provided by Fisher Scientifc (USA): acetic acid, agar, agarose, dithiotreitol (DTT), glycerol, glycine, hydrochloric acid, hydrogen peroxide 30 %, kanamycin sulfate, methanol sodium chloride and urea. Acryl amide, bisacryl amide, potassium and sodium phosphates (mono- and dibasic) were purchased from Merck Millipore (Germany). Anhydrous magnesium sulfate and sodium sulfate decahydrate were provided by Probus (Spain). Tryptone and yeast extract were purchased from Pronadisa (Spain). Phenol, potassium hydroxide and sodium hydroxide were purchased from Scharlab (Spain). Sigma Aldrich (USA) supplied the following chemicals: ammonium chloride, ampicillin sodium salt, bromophenol blue, chloramphenicol, 3,3'-diaminobenzidine (DAB), *p*-dimethylaminobenzaldehyd (PDAB), dNTPs, Ficoll 400, *D*-(+)-glucose monohydrate, isopropyl β-D-1-thiogalactopyranoside (IPTG), magnesium chloride hexahydrate, β-mercaptoethanol, sodium dodecyl sulfate (SDS), penicillin V, Ponceau S red, tetracycline, tetramethylethylenediamine (TEMED) and xylene cyanole. Tween-20 was from Acofarma (Spain), ammoniumpersulfate from Bio-Rad (USA), skimmed milk from Centre Lechera Asturiana (Spain) and dimethylsulfoxide (DMSO) from New England Biolabs (USA). Antibióticos S.A. (Spain) gifted 6-aminopenicillanic acid (6-APA).

DNase I and RNase were purchased from Roche (Switzerland). All restriction enzymes and their respective buffers were supplied by New England Biolabs (USA). Lysozyme and bovine serum albumin were from Sigma Aldrich (USA). *Pfu* polymerase, T4 DNA ligase and their respective buffers were provided by Thermo Scientific (USA).

The sole antibody used was a monoclonal anti-polyhistidine-peroxidase antibody produced in mice by Sigma Aldrich (USA).

3.2 Bacterial strains, plasmids and oligonucleotides

All bacterial strains and plasmids used are shown in Table 1. They were stored at -80 °C in 20 % glycerol.

All genes were cloned into pET-22b (5493 bp) purchased from Novagen (USA). This plasmid is aimed for bacterial expression of proteins under the control of strong bacteriophage T7 transcription. Induction is achieved by providing a source T7 RNA polymerase. Since the T7 RNA polymerase gene is under control of the lacUV5 promotor in the used expression hosts (DE3), it can be induced by IPTG. The vector pET-22b specifically carries a His-Tag C-terminally, *bla*, an ampicillin resistance gene, and the *pelB*-leader sequence, a signal sequence for potential periplasmic localization of protein (see Supplemental material for plasmid map).

Furthermore, pET-28a (5369 bp) purchased from Novagen (USA) was used. Similar to pET-22b the promotion of recombinant protein production is achieved by addition of IPTG. However, it carries an N-terminal His-Tag. Moreover, it carries *kan*, a kanamycin resistance gene (see Supplemental material for plasmid map).

Table 1: Bacterial strains used in the course of this work and their characteristics

Strain	Description	Genotype	Reference
E. coli BL21(DE3)	Expression host for recombinant protein	*F', ompT, gal, dcm, lon, hsdSB(r_B- m_B-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	NZYtech, Portugal
E. coli BL21(DE3) pET-28a (*pva*)	Expression host for recombinant *Sl*PVA	Harbouring pET-28a (*pva*)	This work
E. coli BL21(DE3) pET-22b (*pva*)	Expression host for recombinant *Sl*PVA	Harbouring pET-22b (*pva*)	This work
E. coli BL21(DE3) pET-22b (*pva::gfp*)	Expression host for recombinant *Sl*PVA-GFP	Harbouring pET-22b (*pva::gfp*)	This work
E. coli BL21(DE3) pET-22b (*pva::sbd*)	Expression host for recombinant *Sl*PVA-SBD	Harbouring pET-22b (*pva::sbd*)	This work
E. coli BL21(DE3) pET-22b (*aac*)	Expression host for recombinant *Au*AAC	Harbouring pET-22b (*aac*)	This work
E. coli BL21(DE3) pET-22b (*aac::gfp*)	Expression host for recombinant *Au*AAC-GFP	Harbouring pET-22b (*aac::gfp*)	This work
E. coli BL21(DE3) pET-22b (*aac::sbd)*	Expression host for recombinant *Au*AAC-SBD	Harbouring pET-22b (*aac::sbd*)	This work

E. coli BL21 (DE3) (pET-GroEL)	Expression host for recombinant protein; expressing the chaperonin GroEL under IPTG control	*F⁻, ompT, gal, dcm, lon, hsdSB(r_B- m_B-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]); pET-28a (+): GroEL, Cm^R*	(Tan *et al.*, 2015)
E. coli BL21 (DE3) (pET-GroEL) pET-22B (*pva*)	Expression host for recombinant protein; expressing the chaperonin GroEL and recombinant *Sl*PVA under IPTG control	Harbouring pET-22b (*pva*)	This work
E. coli C43 (DE3)	Expression host for recombinant protein	*F⁻, ompT, hsdSB(r_B- m_B-), gal, dcm, DE3*	Lucigen, USA
E. coli C43 (DE3) pET-22b (*pva*)	Expression host for recombinant *Sl*PVA	Harbouring pET-22b (*pva*)	This work
E. coli C43 (DE3) pET-22b (*aac*)	Expression host for recombinant *Au*AAC	Harbouring pET-22b (*aac*)	This work
E. coli Origami	Expression host for recombinant protein	*Δ(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac^++$lacI^q$ pro] (DE3) gor522::Tn10 trxB (Kan^R, Str^R, Tet^R)*	Novagen, USA
E. coli Origami pET-22b (*pva*)	Expression host for recombinant *Sl*PVA	Harbouring pET-22b (*pva*)	This work
E. coli Origami pET-22b (*aac*)	Expression host for recombinant *Au*AAC	Harbouring pET-22b (*aac*)	This work
E. coli NZY5α	Host for cloning experiments	*fhu*A2Δ(*arg*F-*lac*Z)U169 *pho*A *gln*V44 Φ80 Δ(*lac*Z)M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17	NZYtech, Portugal
E. coli NZY5α pET-22b (*pva*)	Host for cloning experiments	Harbouring pET-22b (*pva*)	This work
E. coli NZY5α pET-22b (*pva::gfp*)	Host for cloning experiments	Harbouring pET-22b (*pva::gfp*)	This work
E. coli NZY5α pET-22b (*pva::sbd*)	Host for cloning experiments	Harbouring pET-22b (*pva::sbd*)	This work
E. coli NZY5α pET-22b (*aac*)	Host for cloning experiments	Harbouring pET-22b (*aac*)	This work

E. coli NZY5α pET-22b (*aac::gfp*)	Host for cloning experiments	Harbouring pET-22b (*aac::gfp*)	This work
E. coli NZY5α pET-22b (*aac::sbd*)	Host for cloning experiments	Harbouring pET-22b (*aac::sbd*)	This work
E. coli NZY5α pET-22b (*ahla::gfp*)	Host for cloning experiments	Harbouring pET-22b (*ahla::gfp*)	This work
E. coli NZY5α pET-22b (*ahla::sbd*)	Host for cloning experiments	Harbouring pET-22b (*ahla::sbd*)	This work
E. coli NZY5α pET-22b (*ahla::sbd::linker*)	Host for cloning experiments	Harbouring pET-22b (*ahla::sbd::linker*)	This work

Codon optimization of genes for *E. coli* was performed by GeneScript (USA) with their OptimumGene™ algorithm (Liu *et al.*, 2011). Codon-optimized genes are presented in Table 2. All genes besides *pva*, which was received in pET-28a, were delivered cloned in the commercial plasmid pET-22b.

Table 2: Codon-optimized genes and their characteristics

Codon-optimized gene	Description
pva	Penicillin V acylase from *Streptomyces lavendulae* ATCC 13664
aac	Aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052
ahla::gfp	Fusion gene of N-acyl-L-homoserine acylase from *Actinoplanes utahensis* NRRL 12052 and green fluorescent protein (*gfp*) from *Aequorea victoria*
ahla::sbd	Fusion gene of N-acyl-L-homoserine acylase from *Actinoplanes utahensis* NRRL 12052 and substrate binding domain (*sbd*) of the poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* ($PhaZ_{Sex}$)
ahla::Linker::sbd	Fusion gene of N-acyl-L-homoserine acylase from *Actinoplanes utahensis* NRRL 12052 and substrate binding domain (*sbd*) of the poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* ($PhaZ_{Sex}$). Between the two genes is a short linker sequence

Oligonucleotides used in the course of this work were synthesized by Sigma-Aldrich (USA). They are shown in Table 3.

Table 3: Oligonucleotides used in the course of this work

Gene	Primer	Sequence	T_m [°C]
pva	PVAecNcoI	5'-ATG**CCATGG**GTGGTGGCCTGAGCGC-3'	83.4
	PVA2ecXhoI	5'-ATGC**CTCGAG**ACGACGCTCATGCACAC-3'	79.6
	PVA2ecEcoRI	5'-ATGCG**GAATTC**ACGACGCTCATGCACAC-3'	80.1
	PVA_f719	5'-GCAAACCATTCCGGGTGAAC-3'	68.5
aac	AACecNcoI	5'-ATA**CCATGG**GTGGTTATGCGGCGCTGA-3'	78.9
	AAC2ecEcoRI	5'-ATGTA**GAATTC**ACGACCACGTTGCGCCACA-3'	79.4
	AAC_f715	5'-GCCGTTATGACGTTGAAGGC-3'	66.6
gfp	GFPecEcoRI	5'-ATGCA**GAATTC**ATGAGCAAAGGCGAGGAAC-3'	76.8
	GFPecXhoI	5'-ATGC**CTCGAG**CTTATACAGCTCATCCATGCC-3'	77.2
Linker-sbd	LinkSBDecEcoRI	5'-ATGCA**GAATTC**GGTGGCGGTAGCGGCGGT-3'	84.2
	LinkSBDecXhoI	5'-ATGC**CTCGCG**TTAGCAGGTAACCCAGTAACC-3'	77.1
sbd	SBDecEcoRI	5'-ATGCA**GAATTC**ACCACCGGTGCGGCGGT-3'	84.1
	SBDecXhoI	5'-ACGC**CTCGAG**TTAGCAGGTAACCCAATAACC-3'	75.1
pET-22b	T7p	5'-TAATACGACTCACTATAGGG-3'	55.6
	T7t	5'GCTAGTTATTGCTCAGCGG-3'	60.6

** Restriction sites are highlighted in **bold characters***

3.3 Media composition

3.3.1 Luria-Bertrani (LB) medium

LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCL, pH 7.5) was routinely used for growth of *E. coli* (Sambrook *et al.*, 1989). The broth contained 2 % (w/v) bacto-agar in case of solid medium.

When producing protein using the pET system under the control of the lacUV5 operator, *E. coli* was also grown in LB medium containing 1 % (w/v) glucose (LB+Glucose) to repress basal expression before induction.

In an effort to produce active recombinant protein, *E. coli* cells were as well cultured in LB medium with 1 M *D*-sorbitol and 2.5 mM betaine added (LB+Sorbitol+Betaine) (Ackerley *et al.*, 2004).

3.3.2 Minimal medium 9 (M9)

Minimal medium 9 (M9) was used to grow *E. Coli* in a controlled fashion in a defined medium. The medium was composed of the following (Sambrook *et al.*, 1989):

- 50 mL/L 20X M9 salts (20 g/L NH_4Cl, 60 g/L KH_2PO_4, 120 g/L $Na_2HPO_4*7H_2O$)
- 1 mL/L 1 M $MgSO_4$
- 0.5 g/L NaCl
- 2.5 g/L glucose

3.3.3 B2 medium

In order to optimize the recombinant enzymes production, *E. coli* BL21 (DE3) strains were grown in B2 medium. The composition of this rich medium was the following (Vélez *et al.*, 2014):

- 10 g/L glucose
- 40 g/L glycerol
- 10 g/L tryptone
- 5 g/L yeast extract
- 0.5 g/L $MgCl_2*7H_2O$
- 3.4 g KH_2PO_4
- 9.0 g/L $Na_2HPO_4*12H_2O$
- 2.7 g/L NH_4Cl
- 0.7 g/L Na_2SO_4

3.4 Handling techniques and analysis of DNA

3.4.1 Purification and sequencing of plasmid DNA

Plasmid DNA was obtained using the PureLink Quick Plasmid Miniprep Kit by Invitrogen (USA). Recombinant *E. coli* cells harbouring the plasmids were grown overnight at 37 °C and 250 rpm in a thermostated Multitron Standard incubator (Infors HT, Switzerland) in 5 mL LB medium supplemented with the corresponding antibiotic. After centrifugation (15 min, 3857 x *g*, 4 °C) cells were worked up according to the specifications provided by the supplier. Quantity and purity of the obtained DNA were controlled by agarose gel electrophoresis (see section 3.4.3) and Nanodrop (see section 3.4.5).

Sequencing of plasmid DNA by Sanger-method (Sanger *et al.*, 1977) was performed by Secugen S.L (Spain). DNA analysis and alignment were carried out using the Aliview software (Larsson, 2014).

3.4.2 PCR assays

All PCR protocols were carried out in an Eppendorf Mastercycler Personal (Eppendorf, Germany). Primers (see Table 3) were synthesized by Sigma-Aldrich (USA). The nucleotides were dissolved in the appropriate amount (indicated by Sigma-Aldrich) of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to obtain a 200 mM solution, which was further diluted with Mili-Q water to 5 mM aliquots ready to use. PCR reaction were performed with 0.05 U/µL *Pfu* polymerase, 5 ng/µL template, 0.5 µM primers (forward and reverse) and 200 µM dNTPs in polymerase buffer. Furthermore, if necessary, 10 % (v/v) DMSO was added.

PCR conditions were: 2 min initial denaturation at 96 °C, followed by 30 cycles of 30 s at 96 °C and 276 s annealing/extension at 72 °C and ended by a final extension of 10 min at 72 °C.

Amplified products were observed on 0.8 % agarose gels and controlled for correct size and purity (see section 3.4.3).

3.4.3 DNA agarose gel electrophoresis

DNA agarose gel electrophoresis was performed both for qualitative control of DNA purity and length as well as for DNA purification. The equipment used was a Run One Electrophoresis Cell from EmbiTec (USA) able to apply a constant voltage of 25 V, 50 V and 100 V. As running buffer TAE (40 mM Tris-HCl, 1 mM EDTA, 20 mM acetic acid, pH 8.0) was used. Sample

buffer (Ficoll 400 30 % (w/v), bromophenol blue 0.2 % (w/v), xylene cyanole 0.04 % (v/v) and 40 mM EDTA at pH 7.0) was mixed with samples and Mili-Q water and loaded onto the agarose gels (0.8 % (w/v). For band detection, 1 µL of the intercalant agent GelRed Nucleic Acid Gel Stain (Biotium, USA) was added per 20 mL molten agarose before casting the gel. Bands were detected by UV light on a transilluminator UVIPro V1.0 from UVITec Limited (Spain).

DNA markers used were GeneRuler 1 kb plus DNA Ladder (Thermo Scientific, USA) and Lambda DNA/*Bst*EII (Fermentas, USA).

3.4.4 Isolation and purification of DNA fragments

DNA fragments were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit by GE Healthcare (USA). Therefore, agarose gels were analysed on an UV light transilluminator. Bands of interest were cut out and worked up using the specifications provided by the supplier. Success of the purification and quality of DNA were checked by gel electrophoresis (see section 3.4.3) and spectrophotometrically (see section 3.4.5).

3.4.5 Spectrophotometric analysis of DNA

In order to check DNA purity and quantity, DNA samples were checked by a spectrophotometer NanoDrop-1000 (Thermo Scientific, USA). First, the equipment was equilibrated by measuring the pure buffer as background. After applying 1.5 µL of sample to the equipment, a spectrum in the interval 220 – 350 nm was measured. The concentration of DNA is dependent on its absorbance at 260 nm. The ratio of the absorbance at 280 and 260 nm was used as a measure of purity. A value of 1.8 is considered as optimal.

3.4.6 Colony mini-prep

In order to quickly detect the success of transformation of plasmids, biomass was scraped of a plate, resuspended in 15 µL lysis buffer (0.5 mg/mL lysozyme, 25 mM EDTA, 0.1 mg/mL RNase, 10 % (v/v) glycerol, 0.2 mg/mL bromophenol blue), mixed vigorously by vortex and incubated at room temperature for 15 min. In order to remove proteins, 2 µL of phenol were added, the mixture mixed vigorously by vortex and centrifuged 5 min at 12000 g. The obtained supernatant was used for DNA electrophoresis (see section 3.4.3).

3.4.7 Construction of recombinant plasmids

In order to cut DNA at specific sites, restriction enzymes were used. Concentrations of enzyme, DNA and buffer were chosen according to the specifications of New England Biolabs (USA). Reactions were carried out in a volume of 50 µL containing 5 µL of reaction buffer, 1 µL of each restriction enzyme (XhoI and BamHI: 20 U/µL; EcoRI and NcoI: 10 U/µL) and 1 µg of DNA. The remaining volume was filled up with Mili-Q water. The buffer (NEbuffers 1.1, 2.1, 3.1 and CutSmart) for each reaction was chosen to assure highest possible activity of both restriction enzymes used. In Table 4 all genes treated with restriction enzymes are displayed with the restriction enzymes used and the respective buffer chosen for the reaction. The reaction mixtures were incubated for 3 h at 37 °C. Success of the enzymatic reaction was controlled by gel electrophoresis (see section 3.4.3). Quantitative DNA gel electrophoresis was performed to clean up the fragments. Bands of interest were cut out of the gel and purified as described in section 3.4.4.

Table 4: Genes treated with restriction enzymes and the respective buffer used

Gene	Restriction enzymes	Buffer
NcoI-*pva*-EcoRI	NcoI, EcoRI	2.1
NcoI-*pva*-XhoI	NcoI, XhoI	3.1
NcoI-*aac*-EcoRI	NcoI, EcoRI	2.1
gfp	EcoRI, XhoI	2.1
sbd	EcoRI, XhoI	2.1
Linker::sbd	EcoRI, XhoI	2.1
pET-22b (*ahla::Linker::sbd*)	NcoI, XhoI	3.1
pET-22b (*ahla::gfp*)	NcoI, BamHI, XhoI	3.1
pET-22b *(pva*)	NcoI, XhoI	3.1
pET-22b (*pva*::*gfp*)	NcoI, XhoI	3.1
pET-22b (*pva*::*sbd*)	NcoI, XhoI	3.1
pET-22b (*pva*::*Linker::sbd*)	NcoI, XhoI	3.1
pET-22b (*aac*::*gfp*)	NcoI, XhoI	3.1
pET-22b (*aac*::*sbd*)	NcoI, XhoI	3.1
pET-22b (*aac*::*Linker*::*sbd*)	NcoI, XhoI	3.1

Recombinant plasmids were constructed by ligase reactions. Beforehand, restriction enzyme digestions of the chosen plasmid and the gene to be inserted were carried out as described above. As a ligase a T4 DNA ligase was used. Reaction conditions are provided below. For fusion genes, all parts of the insert were added in equal amounts (20 ng). Finally, DNA purity and quantity were checked spectrophotometrically (see section 3.4.5).

Table 5: Ligase reaction conditions

Plasmid DNA	40 ng
Insert	20 ng
10X Ligation buffer	1X
T4 DNA ligase	1 Weiss U*
Mili-Q water	Fill up to final volume
Reaction volume	40 µL
Temperature	22 °C
Incubation time	1 h

** 1 Weiss U is defined as the amount of enzyme required to convert 1 nmol of ^{32}P-labeled inorganic pyrophosphate into Norit absorbable forml in 20 min at 37 °C, using specified reaction conditions (Weiss et al., 1968). 1 Weiss Unit equals approximately 200 cohesive end ligation units (CEU), which are defined as the amount of enzyme required to give 50 % ligation of HindIII fragments of lambda DNA in 30 min at 16 °C*

3.4.8 Transformation of *Escherichia coli*

Transformation of *Escherichia coli* cells was carried out either by heat shock or electroporation. Heat shock transformation was performed using commercial chemically competent cells by NZYtech (Portugal). Competent cells were thawed on ice. Thereafter, 40 µL of cells were gently mixed by tapping with 5 µL of plasmid DNA (0.2 to 50 ng DNA). This mixture was incubated in an ice bath for 30 min. After a heat shock at 42 °C during 40 s in water bath, the cells were placed on ice for 2 min. Next, 0.9 mL of LB medium was added and the mixture was incubated for 1 h at 37 °C (225 rpm). Then, cells were spun down at 1000 x *g* for 3 min and 700 µL of medium was removed in order to reduce the volume. Afterwards, the cells were resuspended in the remaining volume and spread on LB agar plates containing the required antibiotic. The plates were incubated at 37 °C overnight.

Electrocompetent *E. coli* cells were obtained by growing cells in 100 mL LB medium. After reaching an optical density 0.8 at 600 nm, the cells were centrifuged at 2500 x *g* for 10 min

(4 °C). After removing the supernatant, the pellet was washed 3 times in 10 % (v/v) sterile glycerol by resuspending and centrifuging at the conditions mentioned above. Afterwards, the cells were resuspended in 1 mL 10 % (v/v) sterile glycerol and stored at -80 °C.

Electroporation was performed on a MicroPulser by Bio-Rad (USA). 100 µL of electrocompetent cells were mixed with 100 ng of plasmid DNA in the sterile cuvette gently by gently tapping. After a pulse (2.5 kV*cm^{-1}, 200 Ω, 14 ms) was applied, 0.9 mL of LB medium were added. The cells were allowed to recover for 1 h at 37 °C. The volume was reduced by centrifuging (3 min, 1000 x *g*) and removing 700 µL of media. The rest was resuspended and spread out on LB agar plates containing the required antibiotic and incubated over night at 37 °C.

All plates with transformed cells were inspected the following day, single colonies were picked and isolated to fresh plates containing the selective antibiotic. The presence of plasmid in the selected colonies were controlled by Colony mini-prep (see section 3.4.6).

3.5 Production of recombinant protein

3.5.1 Growth conditions

Recombinant *E. coli* cells were grown at different conditions in order to optimize the heterologous expression. In all cases, precultures of 5 mL LB medium with the appropriate antibiotic added in the right concentration (ampicillin: 100 µg/mL, tetracycline: 10 µg/mL, chloramphenicol: 25 µg/mL, kanamycin: 50 µg/mL) were grown by inoculating with 5 µL of glycerol stocks and incubating overnight at 37 °C and 250 rpm in a Multitron Standard incubator (Infors HT, Switzerland). The next day, 40 mL cultures of desired medium with appropriate antibiotic added were inoculated with the preculture to OD_{600} of 0.005. Then, cells were grown to a specific OD_{600} (0.6 or 1.5) at 37 °C and 250 rpm. Recombinant protein production was induced by addition of IPTG. Two different concentrations of IPTG were used: 1.0 mM and 1.5 mM. When using M9 and LB+Sorbitol+Betaine medium, cells were grown in LB medium up until OD_{600} of 1.5 was reached. Cells were spun down for 5 min at 3857 x *g* and 4°C. After resuspending the biomass in the respective medium, protein induction was carried out as explained above. After induction, cultures were incubated at 20 °C for 20 h in a New Brunswick™ Innova® 40 shaker (Eppendorf, Germany).

Cells were harvested by centrifugation (15 min, 3857 x *g*, 4 °C). After the harvest, biomass was stored at -20 °C.

3.5.2 Cell lysis by sonication

First, recombinant cells were slowly thawed on ice and resuspended in 10 mL (25 % former culture volume) prechilled 20 mM Tris-HCl at pH 8.0. Then, sonication was carried out with 450 Digital Sonifier (Branson, USA) with a tapered microtip (5 mm diameter). Conditions were then following: 8 pulses of 15 s with an amplitude of 30 % followed by 3 pulses of 15 s with an amplitude of 40 %. Between pulses the samples were rested for 15 s. Samples were kept in an ice/water bath during the sonication to avoid heat degradation of proteins.

After cell lysis, the soluble cell extract was separated from solids by centrifugation (15 min, 12000 x *g*, 4 °C). Cell extracts as well as the resulting pellets were tested for target protein presence by western blot (see section 3.6.3) and enzymatic assay (see section 3.6.4).

3.5.3 Inclusion body purification and solubilization

In order to purify inclusion bodies, recombinant cells grown at optimal conditions were thawed on ice. The purification was carried out with the commercial *B-Per Bacterial Protein Extraction Reagent* (Thermo Scientific, USA). The biomass was resuspended in 4 mL *B-PER* per gram biomass. In addition, DNase I and lysozyme were added to a concentration of 5 U/mL and 0.1 mg/mL respectively. The mixture was homogenized and incubated for 15 min at room temperature. Thereafter, the samples were centrifuged at 12000 x *g* during 10 min.

The sediments were recovered and resuspended in 7.5 µL/mg *B-PER*. 0.2 mg/mL lysozyme was added to this suspension and it was mixed vigorously by vortex. After incubation for 5 min at room temperature, the suspension was diluted with 125 µL 1:10 (v/v) *B-PER* per mg sediment. After mixing vigorously by vortex, it was centrifuged (12000 x *g*, 10 min). These steps were repeated 3 times. The resulting white sediments were the purified inclusion bodies, which were stored at -20 °C.

Inclusion bodies were solubilised by the commercial *Inclusion Body Solubilization Reagent* (Thermo Scientific, USA) according to specifications provided by the supplier. Purified inclusion bodies were resuspended in 8 mL of the *Inclusion Body Solubilization Reagent* per gram of wet inclusion body pellet. Moreover, DTT was added to a final concentration of 5 mM. The suspension was homogenized by mixing vigorously by vortex. After 30 min of incubation at room temperature with soft agitation, the remaining cell debris was removed by centrifugation (15000 x *g*, 15 min). The supernatant containing the solubilised inclusion bodies was collected and stored at 4 °C.

3.5.4 Refolding

In order to achieve active protein, solubilised inclusion bodies had to be refolded. Refolding was performed according to specifications provided by the supplier via slow dialysis in a cold room (4 °C). First, samples were dialyzed against 6 M urea for 12 h in a volume of 1 L. Urea acted as a chaotropic agent, which was then slowly removed in order to trigger the refolding process. Thereafter, every 6 – 12 h 250 mL 25 mM Tris-HCl (pH 7.5) was added. When a final volume of 3 L was reached, the dialysis solution was replaced with 2 L of 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Samples were dialyzed for another 6 h. After recovering the samples, they were centrifuged (15 min, 3857 g, 4 °C) in order to remove precipitates. Refolded protein was stored at 4 °C.

3.6 Protein analysis techniques

3.6.1 Quantification of protein content

Overall protein content was determined by Bradford assay (Bradford, 1976). 280 µL Mili-Q water was mixed with 40 µL sample (adequately diluted with Mili-Q water) and 80 µL commercially available *Protein Assay Dye Reagent Concentrate* by Bio-Rad (USA). After incubation of 5 min the resulting absorbance was measured at 595 nm with a Microplate Reader RT-6100 (Rayto, People's Republic of China). A calibration curve with BSA standards ranging from 2.5 to 30 µg/mL was used to interpolate the overall protein concentration of samples.

3.6.2 Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (Laemmli, 1970). Gels with 12.5 % (v/v) acrylamide/bisacrylamide in the running gel and 5 % (v/v) in the stacking gel were prepared.

Samples, liquid or pellets, were diluted or resuspended with sample buffer to a final concentration of 62.5 mM Tris-HCl, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol, 0.005 % (w/v) bromophenol blue at pH 6.8. These mixtures were boiled at 96 °C for 10 min in a thermoblock Digital Dry Bath (Labnet International, USA). After centrifugation (15 min, 12000 g) samples were loaded onto the gel. As a molecular mass standard the *Broad Range Standard* from Bio-Rad (USA) was used

Gels were run with the Mini-protean Tetra Cell by Bio-Rad (USA) at a constant current of 25 mA per gel in the chamber. Running buffer consisted of 250 mM Tris-HCl, 1 % (w/v) SDS and 2 M glycine.

After gel electrophoresis gels were incubated for 20 min in a stain solution consisting of 0.25 % (w/v) Coomassie G-250, 10 % (v/v) acetic acid and 30 % methanol. Gels were destained by soaking in a solution of 10 % acetic acid and 30 % methanol.

3.6.3 Western blot

Detection of recombinant acylases by anti His tag antibodies was performed. After SDS-PAGE (described above), proteins were transferred to a nitrocellulose membrane (NYTRAN®N, Whatman, UK) overnight with a constant voltage of 10 V using a Mini Trans-Blot by Bio-Rad (USA). The electro-transference was performed in the presence of a buffer containing 25 mM Tris-HCl, 0.2 M glycine and 20 % (v/v) methanol at pH 8.3.

Thereafter, the success of the transference was controlled by a Ponceau S red stain. The nitrocellulose was soaked for 5 min in a solution of 1 % (w/v) Ponceau S in 5 % (v/v) acetic acid. Following that, the unbound colorant was washed off the membrane with an abundance of Mili-Q water, until only the protein bands were visible.

After obtaining an image, the membrane continuously washed, until the majority of the colorant was washed away. Then, the membrane was incubated twice for 5 min in PBS buffer (4 mM NaH_2PO_4, 8.4 mM NaH_2PO_4 and 150 mM NaCl at pH 7.4). After that, the membrane was washed twice for 20 min with 4 % (w/v) skimmed milk in PBS, and twice for 5 min with 0.05 % (v/v) Tween-20 in PBS. The anti His tag antibody (5 – 11 mg/mL) was diluted 2000 times in 1.33 % (w/v) skimmed milk in PBS. The membrane was incubated in 10 mL of this solution for 3 h. After the incubation, the membrane was washed again 3 times for 5 min with 0.05 (v/v) Tween-20 in PBS and 3 times for 5 min in PBS. All washing and incubation steps were performed with soft agitation at room temperature.

Bound antibody was detected by the enzymatic reaction of 3,3'-diaminobenzene (DAB) and H_2O_2 catalysed by the peroxidase bound to the antibodies. For that, the membrane was incubated with 0,5 mg/mL DAB in PBS buffer and 3 µL 30 % hydrogen peroxide solution at room temperature for 5 min. Afterwards, the membrane was washed with water and the reaction resulted in brown bands.

3.6.4 Enzymatic activity

Enzymatic activity of penicillin V acylase and acuelacin A acylase was determined by the production of 6-aminopenicillanic acid (6-APA) by hydrolysis of penicillin V. This

colorimetric determination is based on the reaction of produced 6-APA with *p*-dimethylaminobenzaldehyd (PDAB), forming a faintly yellow coloured Schiff base, that absorbs at 415 nm (Bomstein & Evans, 1965).

The activity was measured by mixing 15 µL of sample with 20 µL 1 M phosphate buffer at pH 8.0 and 40 µL of 116 mM penicillin V. After incubating for 20 min at 45 °C and 375 rpm in a DTS-2 thermostatic incubator (ELMI, Latvia), the enzymatic hydrolysis reaction was stopped by adding 150 µL of 30 % (v/v) acetic acid. Produced 6-APA was detected by adding 100 µL of 0.5 % (v/v) PDAB in methanol. Absorbance was immediately measured at 415 nm with a microplate reader DigiScan 340 (Asys Hitech GmBH, Germany). One international activity unit (IU) was defined as the amount of enzyme that produces 1 µmol/min 6-APA under described conditions (Velasco Bucheli, 2017). A calibration curve ranging from 0.05 to 6 mmol 6-APA was used to interpolate produced 6-APA in measured samples.

4 Results

4.1 Production of recombinant *Sl*PVA by *E. coli* BL21(DE3) pET-28a (*pva*)

As mentioned above, the Enzymatic Biotechnology group of UCM, where this work has been carried out, addressed the cloning and expression of *pva* and *aac* genes in *E. coli* without success, due to the different use of codons between genus *Streptomyces* and *Actinoplanes* and *E. coli.* In order to achieve heterologous expression of these genes in *E. coli,* firstly, the *pva* gene encoding *Sl*PVA was chemically synthesized, optimizing the codon usage for *E. coli,* and cloned into the commercial vector pET-28a by GeneScript (USA) (see section 3.2 of Material and Methods). This plasmid is aimed for bacterial protein expression and contains a His-Tag N-terminally. The gene was placed into the vector between the restriction sites NdeI (CATATG) and EcoRI (GAATTC). In addition, the gene contained the stop codon TAA C-terminally before the EcoRI restriction site (see Supplemental material for plasmid map).

E. coli BL21 (DE3) was transformed via heatshock with the recombinant plasmid pET-28a (*pva*) (see section 3.4.8 of Material and Methods) (results not shown). A culture of *E. coli* BL21 (DE3) pET-28a (*pva*) was grown on LB medium infused with kanamycin as selective antibiotic according to section 3.5.1 of Material and Methods. After reaching an OD_{600} of 0.6, recombinant protein production was induced by addition of 1.0 mM IPTG. After 20 h at 20 °C cells were harvested and sonicated according to section 3.5.2 of Material and Methods. A SDS-PAGE with the resulting pellet and supernatant was carried out (see Figure 12). This yielded intense bands in both supernatant and pellet at about 80 kDa. This corresponds well to the size of unprocessed *Sl*PVA. Enzymatic activity assays (see section 3.6.4 of Material and Methods) returned negative and provided proof, that no active enzyme was present in both supernatant and pellet.

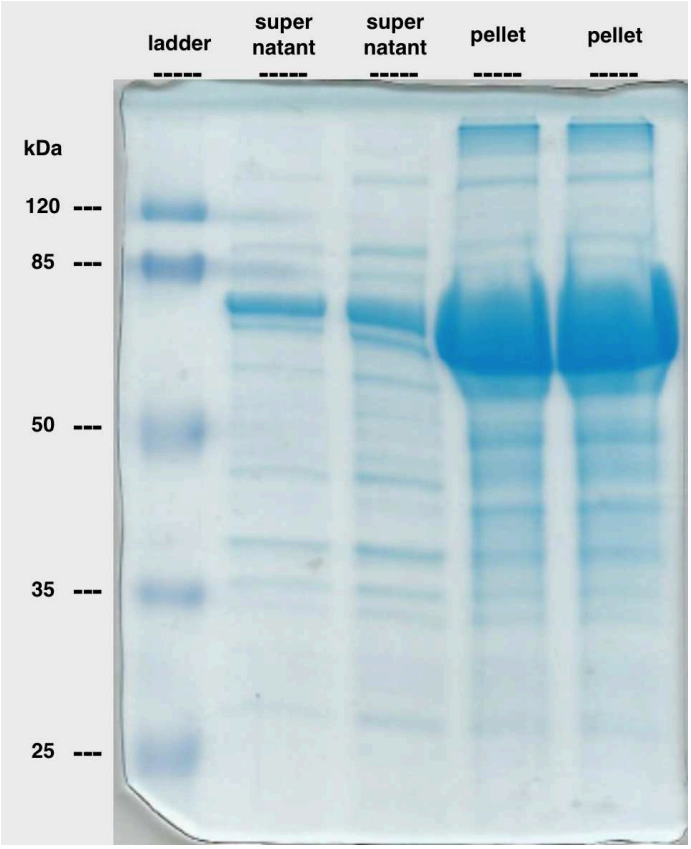


Figure 12: SDS-PAGE of recombinant SlPVA produced by E. coli BL21(DE3) transformed with pET-28a (pva) grown in LB medium after sonication.

4.2 Obtaining and cloning of codon-optimized genes in plasmid pET-22b

The optimized *pva* gene was expressed by *E. coli*, which is a success. However, *Sl*PVA was produced as an inactive unprocessed form. Therefore, another approach was chosen. In order to achieve periplasmic production of processed recombinant enzymes, the codon-optimized *pva* and *aac* genes (see Table 2) were cloned into the expression vector pET-22b, that harbours the N-terminal *pelB*-leader sequence for potential periplasmic location and a C terminal His-Tag (see Supplemental material for vector map). Likewise, fusion genes were obtained and cloned into pET-22b.

All desired recombinant genes are included in (Table 6).

Table 6: Desired recombinant genes and description

Recombinant genes	Description
codon-optimized *pva*	Gene encoding penicillin V acylase from *Streptomyces lavendulae* ATCC 13664, optimized use of codons for *E. coli*
codon-optimized *aac*	Gene encoding aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052, optimized use of codons for *E. coli*
pva::*gfp*	Fusion gene of codon-optimized *pva* and *gfp* gene encoding green fluorescent protein (GFP) from *Aequorea victoria*
pva::*sbd*	Fusion gene of codon-optimized *pva* and *sbd* (DNA sequence encoding substrate binding domain (SBD) of the poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* ($PhaZ_{Sex}$).
pva::*Linker*::*sbd*	Fusion gene of codon-optimized *pva* and *sbd* with a short linker sequence between the two genes.
aac::*gfp*	Fusion gene of codon-optimized *aac* and *gfp* gene encoding green fluorescent protein (GFP) from *Aequorea victoria*
aac::*sbd*	Fusion gene of *aac* and *sbd* (DNA sequence encoding substrate binding domain (SBD) of the poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* ($PhaZ_{Sex}$).
aac::*Linker*::*sbd*	Fusion of codon-optimized gene *aac* and *sbd* with a short linker sequence between the two genes.

4.2.1 Obtaining codon-optimized *pva* and *aac* genes

Regarding *pva*, the goal was, on the one hand, to introduce the restriction sites NcoI (CCATGG) and XhoI (CTCGAG) at 5' and 3' respectively and, on the other hand, NcoI and EcoRI (GAATTC). The first gene (NcoI-*pva*-XhoI) was meant for cloning and expression in pET-22b, while the second one (NcoI-*pva*-EcoRI) was created for obtaining fusion genes *pva*::*gfp, pva*::*sbd and pva*::*Linker*::*sbd* (see Table 6). Furthermore, the C-terminal stop codon TAA present in pET-28a was removed for successful expression in pET-22b, which contains a C-terminal His-Tag (Figure 13). Insertion of genes in pET-22b between NcoI and XhoI

provides expression of the gene directly between the N terminal *pelB*-leader and the C-terminal His-Tag.

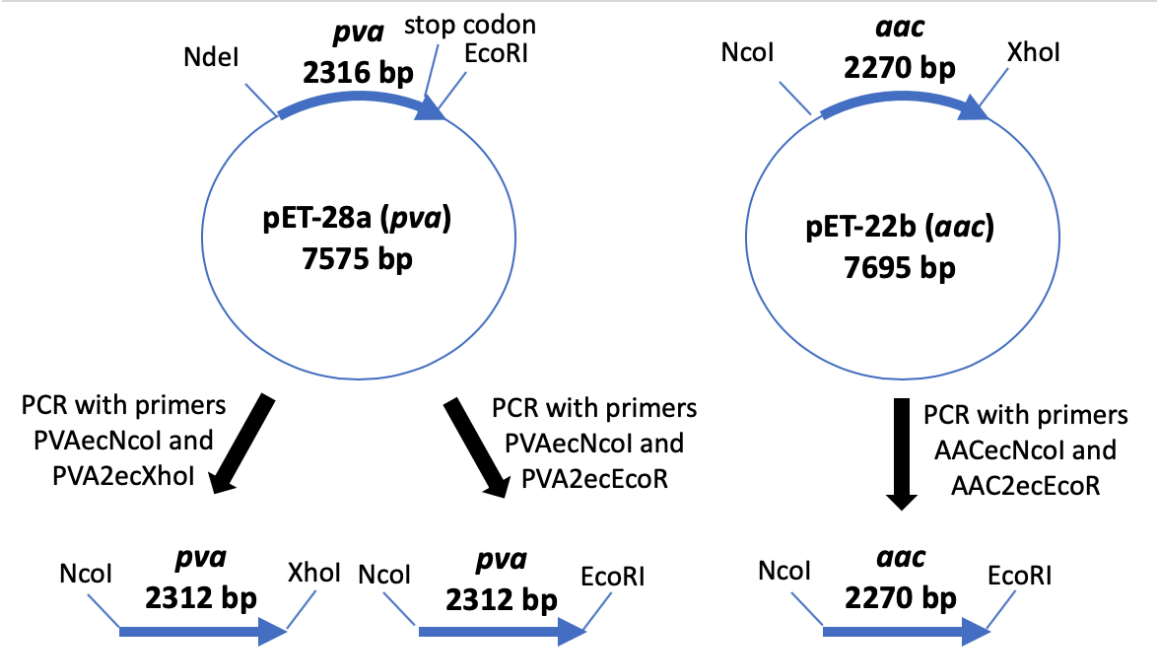


Figure 13: Scheme to obtain codon-optimized pva and aac genes for subsequent cloning

Recombinant *pva* genes were obtained by PCR using the codon-optimized *pva* cloned into pET-28a as template (Figure 13). PCR conditions used are described in section 3.4.2 of Material and Methods , and primers used are listed in Table 3. NcoI-*pva*-XhoI was obtained by using the primers PVAecNcoI and PVA2ecXhoI, whereas NcoI-*pva*-EcoRI was acquired using the primers PVAecNcoI and PVA2ecEcoRI and including 10 % (v/v) DMSO to the reaction mixture. The PCR amplification results are shown in Figure 14. For the NcoI-*pva*-EcoRI gene, a single band just over 2000 bp is visible. For the gene NcoI-*pva*-EcoRI the same strong band just over 2000 bp is present, as is a faint band at about 4000 bp. The desired *pva* gene has a length of 2312 bp including the restriction sites, which corresponds to the most intense band just above 2000 bp, present in both PCR results. The second band present in the PCR mix for NcoI-*pva*-EcoRI represents a side product, which shows the need for further purification. For purification, the entire sample was loaded on an agarose gel, and the supposed NcoI-*pva*-EcoRI band was cut after electrophoresis and cleaned up according to described in section 3.4.4 of Material and Methods. The success of the purification was verified by an agarose gel electrophoresis (Figure 15).

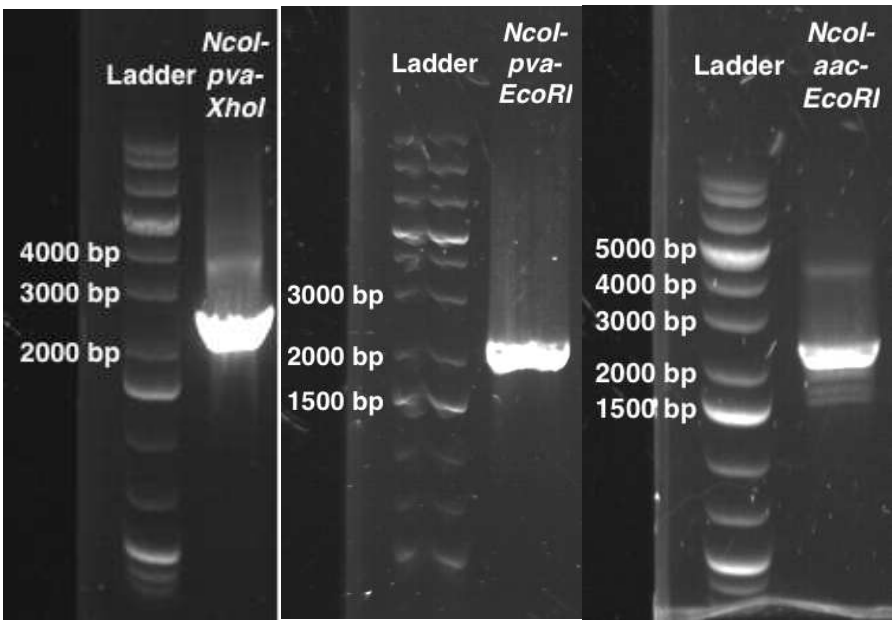


Figure 14: Electrophoresis on agarose gel of PCR amplifications *for* ***Left:*** *NcoI-pva-XhoI;* ***Middle:*** *NcoI-pva-EcoRI;* ***Right:*** *NcoI-aac-XhoI*

In addition, *aac* encoding *Au*AAC was chemically synthesized, optimizing the use of codons for *E. coli,* and cloned into the commercial vector pET-22b by GeneScript (USA) (see Table 2) between the restriction sites NcoI and XhoI (Figure 13). Likewise, in order to obtain the fusion genes *aac::gfp, aac::sbd* and *aac::Linker::sbd* (see Table 6), recombinant *aac* gene with the restriction site XhoI replaced by EcoRI was obtained by PCR, using the codon-optimized *aac* gene cloned into pET-22b as template (Figure 13). The PCR reaction (for conditions see section 3.4.2 of Material and Methods) was performed using the primers AACecNcoI and AAC2ecEcoRI (see Table 3). Successful PCR was assured by adding 10 % (v/v) DMSO to the reaction mix. PCR result was analysed by electrophoresis in agarose gel (Figure 14). The most intense band corresponds to NcoI-*aac*-EcoRI with 2270 bp. Since multiple faint side products are present such as one at about 4500 bp and multiple bands between 1500 bp and 2000 bp further purification of amplified gene was addressed from agarose gel (see section 3.4.4 of Material and Methods). In Figure 15 an agarose gel of the purified product is displayed, showing the success of the purification.

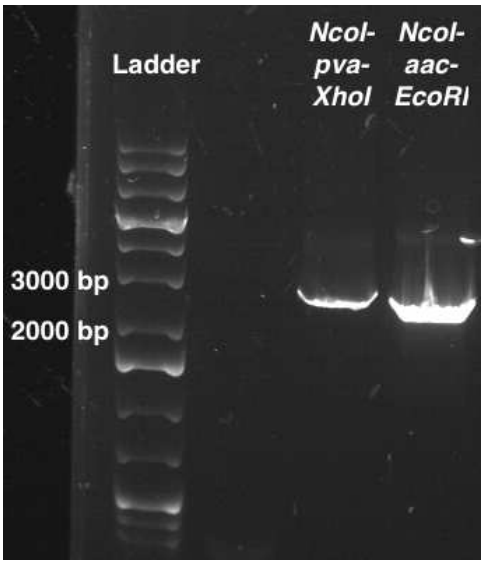


Figure 15: Electrophoresis on agarose gel of NcoI-pva-XhoI and NcoI-aac-EcoRI after purification

4.2.2 Obtaining *gfp*, *sbd* and *Linker::sbd* genes

One of the goal of is this work is to obtain *Sl*PVA and *Au*AAC fusion proteins with GFP from *Aequorea victoria* as well as with the substrate binding domain (SBD) of poly-3-hydroxybutyrate depolymerase from *Streptomyces exfoliatus* (PhaZ$_{Sex}$) and with Linker-SBD. Fusion genes including the SBD of PhaZ$_{Sex}$ serve to immobilise the enzymes on PHB polymers to use in biotechnological applications. Thus, they should not carry the C-terminal His-Tag, as identification and purification can be achieved by immobilisation on the biopolymer PHB. Therefore, the stop codon TAA was included 5' of the His-Tag.

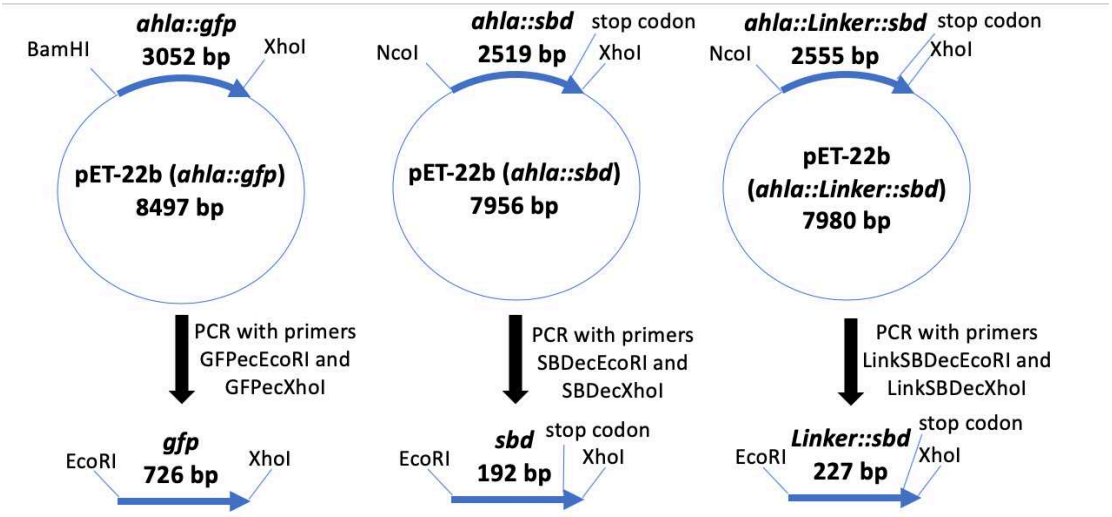


Figure 16: Scheme to obtain gfp, sbd and Linker::sbd genes for subsequent cloning

In simultaneously conducted studies in the Enzymatic Biotechnology Group of UCM, *gfp* gene encoding GFP as well as the DNA sequence encoding SBD from PhaZ$_{Sex}$ without (*sbd*) and with linker (*Linker::sbd*) were chemically synthesized, optimizing the codon usage of *E. coli,* and cloned as fusion genes with *ahla* into the commercial vector pET-22b by GeneScript (USA) (see Table 2). In order to create *Sl*PVA and *Au*AAC fusion proteins with GFP, SBD and Linker-SBD, *gfp, sbd* and *Linker::sbd* were amplified by PCR using the recombinants plasmids pET-22b (*ahla*::*gfp*), pET-22b (*ahla::sbd*) and pET-22b (*ahla::Linker::sbd)* as template (Figure 16). For that, recombinant plasmids were isolated and purified from cultures of recombinant *E. coli* NZY5α pET-22b (*ahla*::*gfp*), *E. coli* NZY5α pET-22b (*ahla::sbd*) and *E. coli* NZY5α pET-22b (*ahla::Linker::sbd*) strains (Figure 17). For pET-22b (*ahla*::*gfp*) (8497 bp) two intensive bands are visible. The band at around 4500 bp represents the supercoiled plasmid DNA, whereas the band at around 20000 bp probably is genomic DNA. There also is a faint band just at around 8000 bp, which could be the linear conformation of the plasmid DNA. For pET-22b (*ahla*::*sbd*) (7956 bp) and pET-22b (*ahla*::*Linker::sbd*) (7980 bp) bands around 4500 bp, that represent supercoiled plasmid DNA, can be observed as the main product.

PCR conditions (see section 3.4.2 of Material and Methods) and primers used (see Table 3) are described in the chapter Material and Methods. In their respective plasmids, *gfp, sbd* and *Linker::sbd* genes were directly cloned as fusion genes to 3' end of *ahla* gene. XhoI restriction sites present to 3' end of the fusion genes *ahla::gfp, ahla::sbd* and *ahla::Linker::sbd* were retained during the PCR. Moreover, to carry out ligase reaction with *pva* and *aac,* EcoRI restriction sites were added to 5' end of *gfp, sbd* and *Linker::sbd* (Figure 16). An agarose gel of the resulting PCR mixtures are displayed below (Figure 17). Bands corresponding to the *gfp* (726 bp), *sbd* (192 bp) and *Linker::sbd* (227 bp) genes can be identified. Furthermore, the band corresponding to the *Linker::sbd* gene lies slightly higher than the one corresponding to the *sbd* gene, that is 35 bp shorter. In addition, in the case of *sbd* an impurity at about 2000 bp is visible which calls for further purification. A quantitative agarose gel electrophoresis was carried out and the band was cut and purified according to section 3.4.4 of Material and Methods.

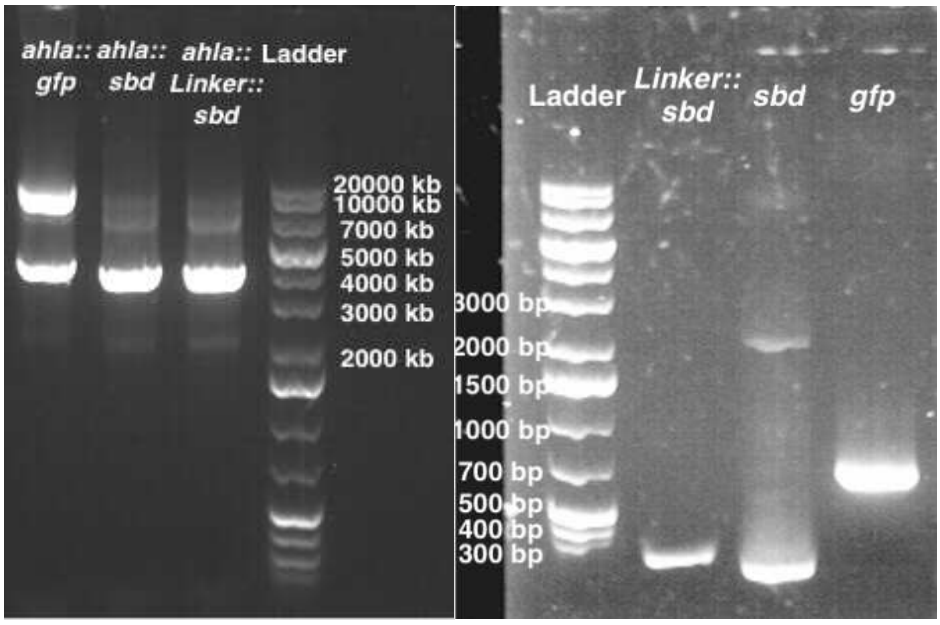


Figure 17: ***Left****: Electrophoresis on agarose gel of purified recombinants plasmids pET-22b (ahla::gfp), pET-22b (ahla::sbd) and pET-22b (ahla::Linker::sbd) cloned into E. coli NZY5α;* ***Right****: Electrophoresis on agarose gel of PCR results for Linker::sbd, sbd and gfp*

4.3 Construction of recombinant genes and cloning into plasmid pET-22b

In order to obtain and clone the desired recombinant genes (Table 6), first, the recombinant genes isolated as described above were subjected to treatment with corresponding restriction enzymes as described in section 3.4.7 of Material and Methods to create sticky ends. The plasmid pET-22b was obtained by restriction digestion of pET-22b (*ahla::gfp*) with NcoI, BamHI and XhoI (the gene ahla::gfp is placed between BamHI and XhoI; digestion with NcoI in order to create correct sticky end for subsequent ligation) or pET-22b (*ahla::Linker::sbd*) with NcoI and XhoI (the gene ahla::Linker::sbd is placed between NcoI and XhoI) (Figure 18).

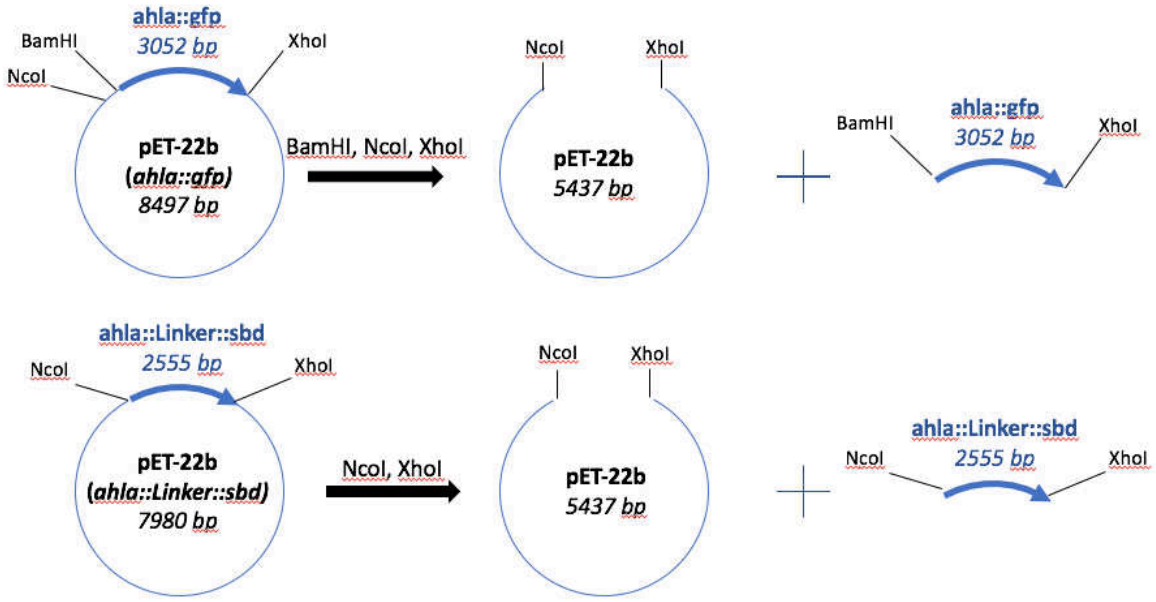


Figure 18: Scheme to obtain the plasmid pET-22b by restriction enzyme digestion

Digestions were analysed by electrophoresis in an agarose gel and digested genes and plasmid were purified as described (see section 3.4.4 of Material and Methods). For all genes, bands corresponding to fragments with correct sizes were found.

Coming up next, purified digested genes were inserted into the vector pET-22b by ligase reaction as described in section 3.4.7 of Material and Methods to create the corresponding desired genes (see Table 6).

4.3.1 Transformation of *E. coli* NZY5α and selection of recombinant plasmids

Aliquots of the ligase reactions were directly used to transform commercial *E. coli* NZY5α by heat shock according to section 3.4.8 of Materials and Methods. Ampicillin resistance was used as selection criterion. After incubating the transformation plates overnight, selected single colonies were picked and transferred to new plates and incubated overnight.

The presence of correct plasmid in recombinant strains was tested by colony mini-prep as described in the section 3.4.6 of Materials and Methods (Figure 19 and Figure 20). This quick-and-dirty method provides a fast insight into the success of a transformation. Genomic DNA (at high sizes over 20000 kb) and RNA residues (at low sizes at the end of the gel) are present in all samples. In general, plasmid DNA was found in the region between 3000 and 5000 bp. Plasmid DNA was supercoiled, hence, is shown at lower sizes on the gel than its actual size. Supercoiled DNA migrates faster in an electrophoresis than equivalent linear DNA due to its topology. In both gels plasmid DNA bands just below 5000 bp and bands below that at about

3000 kDa were found. This suggests, that samples with plasmid DNA just below 5000 bp have recombinant genes inserted into the plasmid, whereas samples with plasmid DNA at about 3000 kDa represent plasmids that have not integrated the desired recombinant genes. According to that criterion, the colony mini-preps can be evaluated. As it can be seen, for all desired recombinant genes, at least one recombinant *E. coli* NZY5α strain harbouring a plasmid with inserted recombinant gene was obtained (Figure 19 and Figure 20).

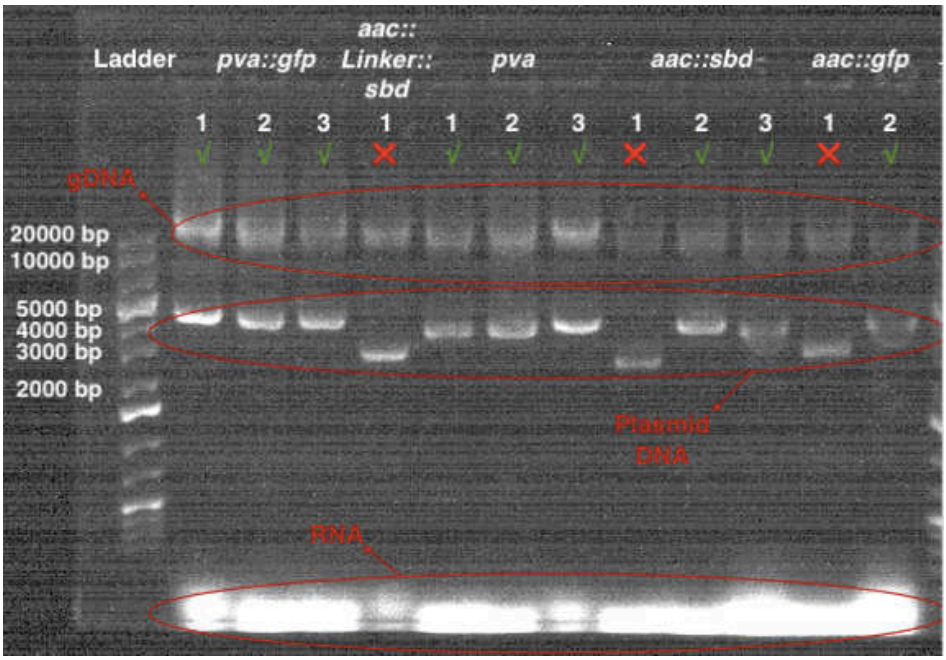


Figure 19: Electrophoresis in agarose gel of colony mini-prep of recombinant E. coli NZY5α pET-22b (pva); pET-22b (pva::gfp), pET-22b (aac::gfp) and pET-22b (aac::sbd) colonies after transformation

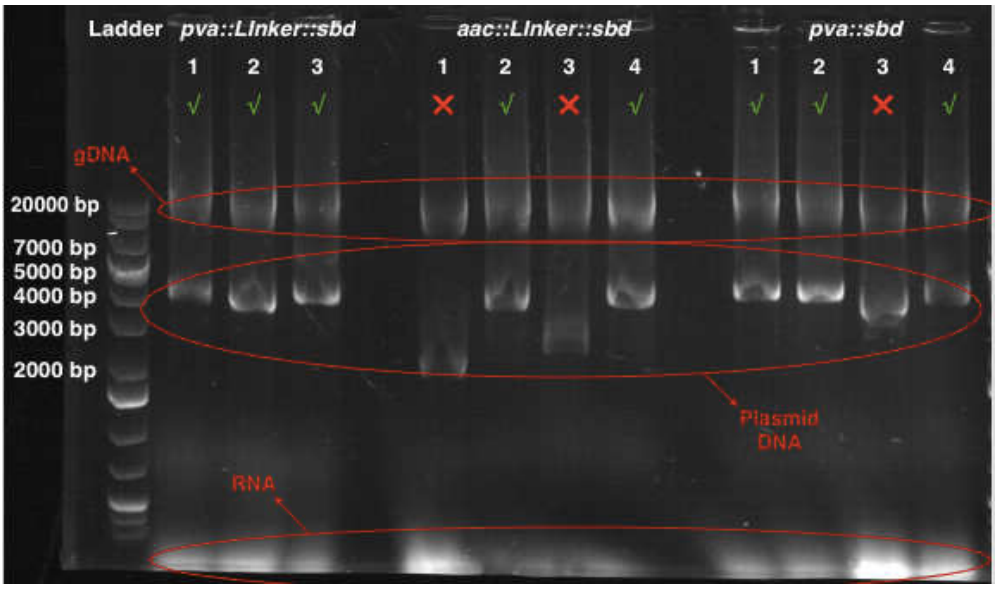


Figure 20: Electrophoresis in agarose gel of colony mini-prep of recombinant E. coli NZY5α pET-22b (pva::Linker::sbd); pET-22b (pva::sbd) and pET-22b (aac::Linker::sbd) colonies after transformation

The presence of genes in the selected recombinant plasmids was verified. For that, plasmids were purified by a mini-prep according to section 3.4.1 of Material and Methods and digested with the restriction enzymes NcoI and XhoI (for procedure see section 3.4.7 of Material and Methods) since all recombinant genes were inserted between these two restriction sites.

Analysis by electrophoresis in agarose gels of reaction mixes are displayed in Figure 21 and Figure 22. In the cases of pET-22b (*pva::gfp)*, pET-22b (*pva*), pET-22b (*aac::sbd*) and pET-22b (*aac::gfp*) plasmids, digestions yielded a visible band just above 5000 bp. This band corresponds well to the plasmid backbone of pET-22b (5437 bp). Regarding genes, in all pET-22b (*pva::gfp*) samples a second band between 3000 and 4000 bp can be observed (Figure 21). This band can be assigned to the fusion gene *pva::gfp* with a total size of 3032 bp, thus, signifying the success of insertion of the correct plasmid. For all pET-22b (*pva*) samples a second band was found between 2000 and 3000 bp. This corresponds well with the size of the *pva* gene at 2312 bp, showing another indication of a successful transformation of correct plasmid. Same can be said for both pET-22b (*aac::sbd*) samples, that feature a second band around 2500 bp, that can be attributed to the *aac::sbd* gene at 2456 bp. Moreover, a second band just above 3000 bp in the pET-22b (*aac::gfp)* sample fits the size of the *aac::gfp* gene at 2990 bp.

In the cases of pET-22b (*aac::Linker::sbd);* pET-22b (*pva::sbd*) and pET-22b *(pva::linker::sbd*) restriction enzyme digestion performed yielded less definitive results. For pET-22b (*pva::sbd*) only one positive colony was obtained, where a band corresponding to the plasmid at about 5500 bp and a faint band at about 2500 bp corresponding to the gene *pva+sbd* (2498 bp) was found (Figure 22). All other transformations of *E. coli* NZY5α with pET-22b (*pva::sbd*) were declared negative as no bands were present in the agarose gel of the restriction enzyme digestions. Furthermore, obtained undigested plasmid DNA of those strains, that were declared negative, already was found at lower sizes (about 3000 bp), whereas the positive clone showed undigested plasmid DNA at about 5000 bp (agarose gel of mini-prep in Figure 23). This also suggests that no correct insertion of the recombinant gene *pva::sbd* into the plasmid had occurred in the samples declared negative. For pET-22b *(pva::Linker::sbd*) bands at about 5500 bp and 2500 bp were obtained which were attributed to the plasmid backbone and the gene *pva::Linker::sbd* (2533 bp) respectively (Figure 22). Also, for pET-22b *(aac::Linker::sbd*) bands at correct sizes could be observed (Figure 22). Bands below the 6369 bp marker representing the plasmid backbone and barely

observable bands just above the 2323 bp marker were found, which were attributed to the gene *aac::Linker::sbd* (2491 bp).

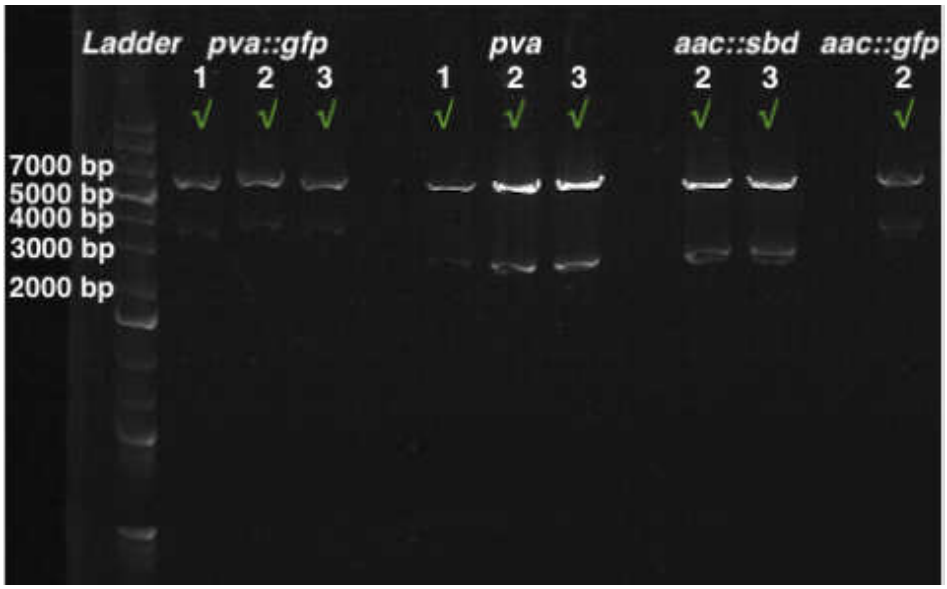


Figure 21: Electrophoresis on agarose gel of restriction enzyme digestion with NcoI and XhoI of pET-22b (pva::gfp), pET-22b (pva), pET-22b (aac::sbd) and pET-22b (aac::gfp) plasmids

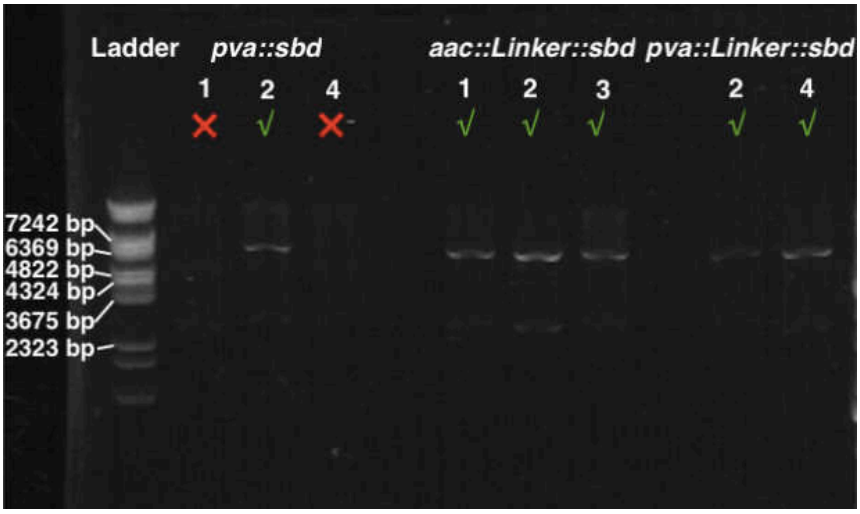


Figure 22: Electrophoresis on agarose gel of restriction enzyme digestion with NcoI and XhoI of pET-22b (pva::sbd), pET-22b (aac::Linker::sbd), pET-22b (pva::Linker::sbd) plasmids

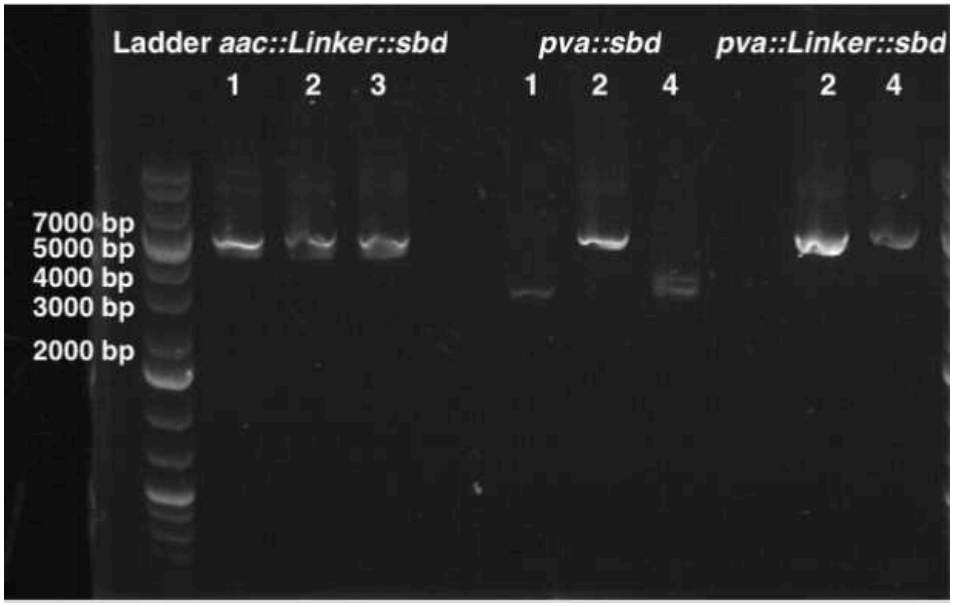


Figure 23: Electrophoresis on agarose gel of mini-prep of E. coli NZY5α colonies transformed with pET-22b (pva::sbd), pET-22b (aac::Linker::sbd) and pET-22b (pva::Linker::sbd)

In all cases, recombinant *E. coli* NZY5α strains were obtained and recombinant plasmids were sequenced in order to verify the correct insertion of the recombinant genes into the plasmid pET-22b and that no mutations had occurred during the cloning process (for conditions see section 3.4.1 of Material and Methods, primers are displayed in Table 3). Full sequence alignments to reference sequences of selected transformants are presented in Supplemental material.

Furthermore, in order to check the correct insertion into the plasmid, sequencing of the genes where carried out using primer T7p for the 5'-region T7 promoter and primer T7t for 3'-region T7 terminator. Consequently, these sequences should include the N-terminal *pelB*-leader (AAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCC CAGCCGGCGATGGCC) placed at the 5' end of the gene and the C-terminal His-Tag (CACCACCACCACCACCAC) at the 3' end.

For recombinant pET-22b (*pva*) plasmid, colony 2 was selected. The sequence obtained completely covers the entire *pva* gene and no mutations are present. Furthermore, the start-codon of *pelB*-leader followed by the NcoI restriction site is present. Also, the full His-Tag and the stop-codon was sequenced.

For recombinant pET-22b (*pva::gfp*) plasmid, colony 1 was selected. Correct cloning of *pva::gfp* was confirmed by the presence of EcoRI restriction site at 2307 bp of the *pva* gene

followed by the *gfp* gene in sequence data. *pelB*-leader, His-Tag and start- and stop-codon were all sequenced confirming the correct insertion of the recombinant fusion gene into the plasmid.

For recombinant pET-22b (*pva::sbd*) plasmid, colony 1 was selected. The complete gene without mutations can be identified in the sequence data. Successful construction of *pva*::*sbd* could be verified by the presence of the EcoRI restriction site at 2307 bp of *pva* gene followed by the *sbd* gene. Correct insertion of the recombinant fusion gene into the plasmid can be corroborated by the presence of the *pelB*-leader and the His-Tag sequences. In addition, the start-codon ATG can be found. The stop codon TAA is present 5' to the His-Tag.

For recombinant pET-22b (*aac::gfp*) plasmids, sequence data shows a good coverage of the reference gene without any mutations present. The fusion gene itself is correctly cloned as the EcoRI restriction site at 2265 bp of *aac* gene is present in sequence data. Moreover, it is well placed between the *pelB*-leader and the His-Tag sequences of the plasmid. Both start- and stop-codon are present as well.

For recombinant pET-22b (*aac::sbd*) plasmid, colony 2 was identified as the best candidate. Sequence data for the full fusion gene was obtained. The fusion gene was well cloned and no mutations are present. Insertion into plasmid can be confirmed by the presence of both the *pelB*-leader and the His-Tag sequences of the plasmid. Furthermore, start-codon and stop-codon can be both observed in the sequence data.

For all recombinant pET-22b (*pva::Linker::sbd*) and pET-22b (*aac::Linker::sbd*) plasmids, colonies no correct sequences were obtained. Sequences suggested that no *Linker::*sbd gene was present as fusion gene 3' to the genes *pva* or *aac*. Instead of the desired *Linker::*sbd gene, an unidentifiable sequence was present. In order to know the failure in the cloning process and to confirm the correct sequence of the *Linker::sbd* gene pre cloning, amplified gene by PCR (see section 4.2.2) was sequenced using the primers LinkSBDecEcoRI and LinkSBDecXhoI. Again, no sequences were obtained confirming that the PCR for the *Linker::sbd* gene has to be optimized.

4.4 Cloning of recombinant genes in *E. coli*

The obtained recombinant plasmids pET-22b (*pva*), pET-22b (*pva::gfp*), pET-22b (*pva::sbd*), pET-22b (*aac::gfp*) and pET-22b (*aac::sbd*) as well as pET-22b (*aac*), which was chemically synthesized by GeneScript (see Table 2), were cloned in several commercial strains of *E. coli* in order to optimize protein production.

4.4.1 Cloning and expression of recombinant genes in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) strain is very common for heterologous expression of recombinant proteins. Therefore, in a first approach, this strain was chosen as an expression system of the recombinant genes. Chemically competent cells were transformed with recombinant plasmids via heat shock, as described in the section 3.4.8 of Materials and Methods. After isolating single colonies to fresh plates containing ampicillin, colony mini-preps were performed to confirm the insertion of recombinant plasmid into the cells (Figure 24). All colonies that contained plasmid DNA were declared as positive. For recombinant pET-22b (*pva*) plasmid two positive clones were obtained with supercoiled plasmid DNA just below 4000 bp. In the case of recombinant pET-22b (*pva::gfp*), transformations yielded one positive clone (clone 1) with supercoiled plasmid DNA at 4000 bp. Likewise, both isolated colonies transformed with pET-22b (*aac::gfp*) and pET-22b (aac::sbd) were found to be positive with supercoiled plasmid DNA at 4000 kb. Of the three picked colonies potentially transformed with pET-22b (*pva::sbd*) only one was declared positive (clone 2). It contained supercoiled plasmid DNA at around 4500 kb. For recombinant pET-22b (*aac),* 4 positive transformants were obtained. The colony mini-prep performed detected supercoiled plasmid DNA just below 3000 kb. Also, a second band was present in all samples at 5000 kb. This band could be interpreted as the open circular conformation of the plasmid DNA.

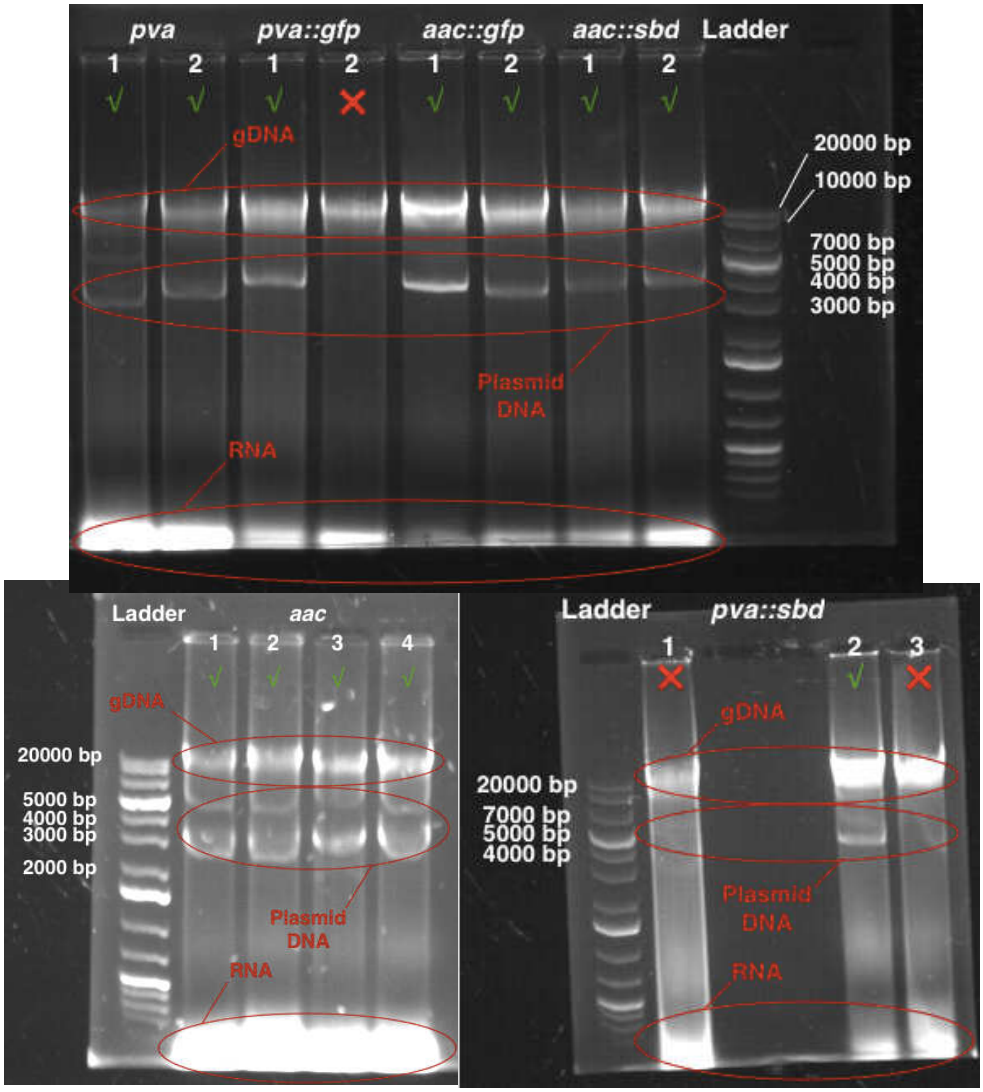


Figure 24: Electrophoresis on agarose gels of colony mini-preps for E. coli BL21 (DE3) colonies transformed with pET-22b (pva), pET-22b (pva::gfp), pET-22b (pva::sbd), pET-22b (aac), pET-22b (aac::gfp) and pET-22b (pva::sbd) after transformation

Expression of *pva* and *aac* by *E. coli* BL21 (DE3) as well as of the fusion genes with *gfp* (*pva::gfp*, *aac::gfp*) was analysed by immunodetection with antibody. For that, cells harbouring the recombinant plasmids were cultured in 100 mL LB medium supplemented with ampicillin according to section 3.5.1 of Material and Methods. Induction with 1.0 mM IPTG was performed at an OD_{600} of 0.6. Cells were harvested after 20 h at 20 °C. After sonication (see section 3.5.2 of Material and Methods) recombinant protein production was determined by western blot (see section 3.6.3 of Material and Methods). The antibody used binds the His-Tag, present C-terminal on the β-subunit, and carries a perioxidase used for detection. Expression of the recombinant genes *pva* and *aac* could be detected. In the western blots, bands corresponding to the unprocessed proteins (α-subunit + β-subunit) at about 80 kDa for *pva* and *aac* could be seen (data not shown). Moreover, expression of the fusion gene *aac::gfp* could be detected. This was revealed by a band in the immunodetection around 120 kDa. This could correspond

to the size of unprocessed fusion protein *Au*AAC-GFP, which has about 110 kDa. Furthermore, multiple bands below 50 kDa were present in the immunodetection. These bands could represent different stages of decomposition of the product. These bands were present mainly in the cell debris suggesting production as inclusion body. However, faint bands in the cell extract were visible at the same sizes as well (Figure 25). For PVA-GFP, no protein could be detected. All recombinant proteins were inactive, as activity assays confirmed (see section 3.6.4 of Material and Methods).

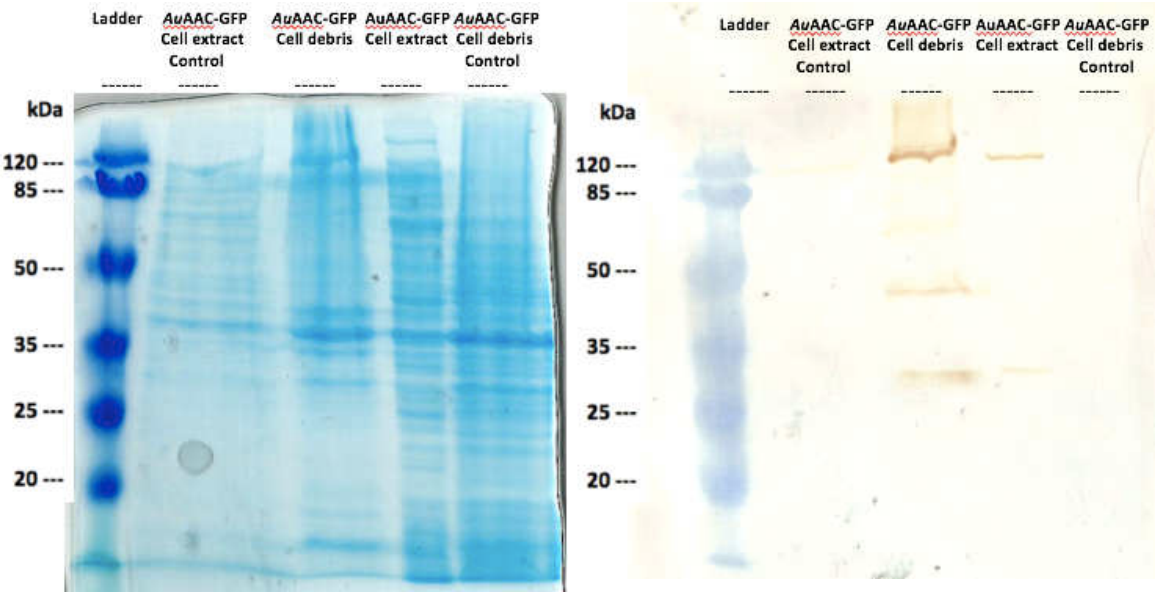


Figure 25: SDS-PAGE and Western blot of recombinant AuAAC-GFP produced by recombinant E. coli BL21 grown in LB medium ***left:*** *Coomassie stain;* ***right****: Immunodetection*

Taken into account the results obtained, the cloning and expression of genes into other *E. coli* strains were addressed.

4.4.2 Cloning and expression of codon-optimized *pva* and *aac* genes in *E. coli* C43 (DE3), *E. coli* Origami and *E. coli* BL21 (DE3) (pET-GroEL)

E. coli C43 (DE3), *E. coli* Origami and *E. coli* BL21 (DE3) (PET-GroEL) strains were chosen to express the recombinant genes. *E. coli* C43 (DE3) is a protein producing strain, that was derived from the *E. coli* BL21 (DE3) strain by adding mutations that reduce cell death caused by overexpression of many toxic proteins (Miroux & Walker, 1996). Second, *E. coli* Origami is K12 derivative carrying mutations in the thioredoxin reductase (*trxB*) and the glutathione reductase (*gor*), thus, enhancing disulphide bond formation in the cytoplasm. Moreover, this strain carries a tetracycline resistance. Lastly, *E. coli* BL21(DE3) (PET-GroEL) is a classic BL21 (DE3) strain already transformed with the commercial plasmid pET-28a carrying the

gene for the chaperonin GroEL. As the strain is already transformed with pET-28a, it carries a kanamycin resistance.

Optimizing the heterologous expression using *E. coli* C43 (DE3), *E. coli* Origami and *E. coli* BL21 (DE3) (PET-GroEL) strains was only addressed with codon-optimized *pva* and *aac* genes. Transformation of pET-22b (*pva*) and pET-22b (*aac*) into the strains was performed by electroporation (see section 3.4.8 of Materials and Methods). For *E. coli* C43 (DE3) cultures, ampicillin resistance was used as the selection marker, whereas tetracycline was used to select for positive *E. coli* Origami cultures. Lastly, both ampicillin and kanamycin resistance were used as selection marker for *E. coli* BL21 (DE3) (pET-GroEL) cultures. After grown cultures were picked and isolated to fresh plates, colony mini-preps were performed (see section 3.4.6 of Materials and Method).

Results of the colony mini-prep for pET-22b (*pva*) in all three strains are displayed in Figure 26. The presence of a band at 4000 kb confirms the integration of plasmid in all 4 colonies of *E. coli* C43 (DE3). Furthermore, faint bands at 5000 kb can be seen. These bands could be another conformation of the plasmid DNA. Transformation of *E. coli* Origami was partially successful. Two positive colonies (colonies 1 and 2) were obtained, signified by a band at around 4000 kb. In the case of *E. coli* BL21 (DE3) (pET-GroEL), only three colonies were obtained after transformation. In two of these (colonies 1 and 2) two distinct bands in the region of plasmid DNA can be detected (4000 kb and 5000 kb). The band at 4000 kb probably represents the recombinant plasmid containing the gene for *Sl*PVA, whereas the bigger band at 5000 kb can be interpreted as the second plasmid containing the gene for the chaperonin GroEL. The third colony displays only barely visible bands and was ruled negative.

The recombinant plasmid pET22-b (*aac*) could only be transformed into *E. coli* C43 (DE3) and *E. coli* Origami. The transformation into *E. coli* BL21 (DE3) (pET-GroEL) failed multiple times, as no colonies could be obtained on agar plates containing ampicillin and kanamycin. This could be explained by the fact that integration of two different plasmids into the same cells is very unlikely. However, colony mini-preps were performed for conformation of plasmid integration in *E. coli* C43 (DE3) and *E. coli* Origami (Figure 27). In agarose gels two positive cultures (cultures 1 and 3) for *E. coli* Origami are displayed with plasmid DNA at just above 3000 kb. Moreover, two positive cultures (cultures 1 and 2) for *E. coli* C43 (DE3) were obtained with plasmid DNA at about 3500 kb.

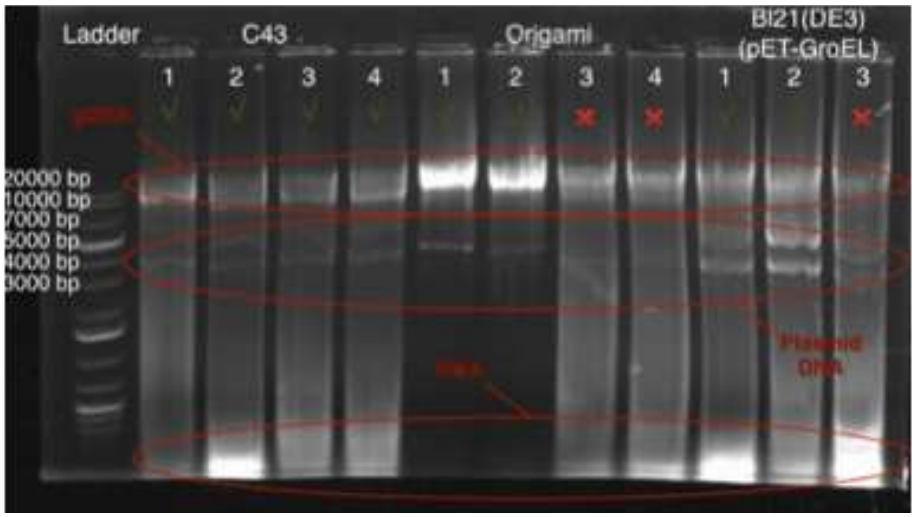


Figure 26: Electrophoresis on agarose gel of colony mini-preps for picked transformants of E. coli C43 (DE3), E. coli Origami and E. coli Bl21 (DE3) (pET-GroEL) strains with pET22b (pva)

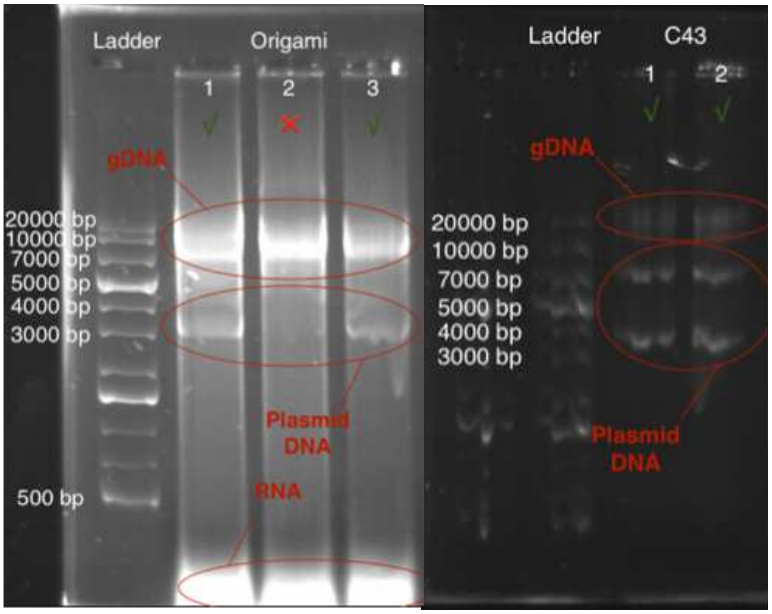


Figure 27: Electrophoresis on agarose gels of colony mini-preps for picked transformants of E. coli C43 (DE3) and E. coli Origami strains with pET22b (aac)

All cells were cultured in LB+Glucose medium and under the conditions described in section 3.5.1 of Materials and Methods. Induction was performed at OD_{600} 1.5 with 1.5 mM IPTG. For each sample, a non-induced control sample was processed the same way. Cells were sonicated according to section 3.5.2 of Materials and Methods. Cell extracts and cell debris were analysed for presence of active enzyme by enzymatic assay (see section 3.6.4 Materials and Methods) and western blot (see section 3.6.3 of Materials and Methods). The antibody used to detect recombinant protein was anti-polyhistidine-peroxidase antibody that binds to the C-terminal His-Tag on the β-subunit.

First, *E. coli* C43 (DE3) transformed with pET-22b (*pva*) and pET-22b (*aac*) were grown. Enzymatic assays performed on broth, cell extract and cell debris returned negative. Furthermore, a Western Blot yielded no antibody band, suggesting that no recombinant protein had been produced (data not shown).

Second, with *E. coli* Origami as host, no positive enzymatic activity was detected. However, a western blot was performed (Figure 28), revealing inactive unprocessed *Au*AAC present in both cell extract and cell debris. This was suggested by a band present in both samples at 80 kDa. For *Sl*PVA, no bands were detected (data not shown).

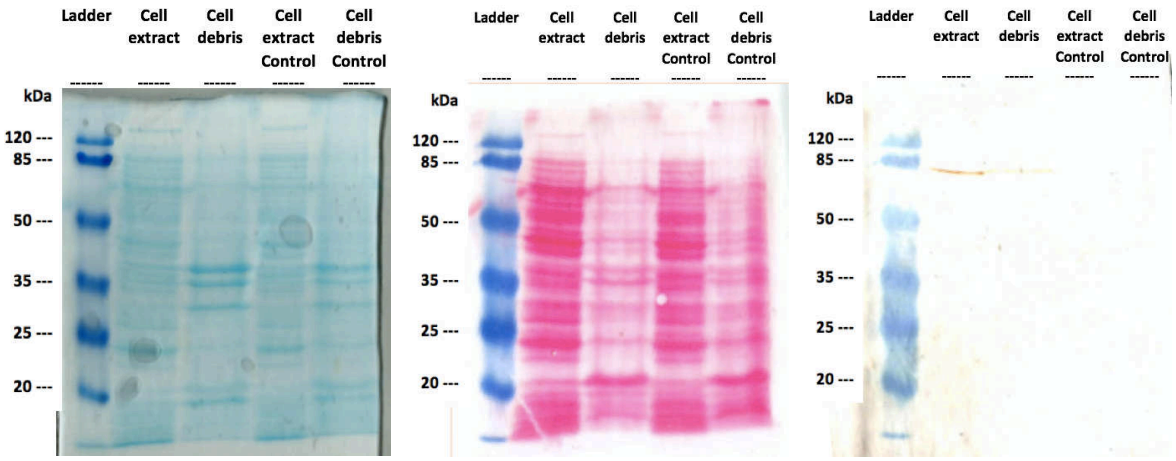


Figure 28: SDS-PAGE and Western blot of recombinant AuAAC produced by recombinant E. coli Origami grown in LB+Glucose medium; ***left:*** *Coomassie stain;* ***middle:*** *Ponceau S stain of membrane;* ***right****: Immunodetection*

At last, *E. coli* BL21 (DE3) (pET-GroEL) transformed with pET-22b (*Sl*PVA) were cultured. Both SDS-PAGE analysis and the membrane stained with Ponceau S suggested strong production of a protein with the size of about 60 kDa. However, no antibody bound to this band confirming it is not the active β-subunit of *Sl*PVA (60.09 kDa). Therefore, this band could be attributed to the chaperonin GroEL, which was produced in this strain as well. Overall, no band binding to the antibody was obtained (data not shown).

4.5 Optimization of recombinant *Sl*PVA and *Au*AAC production by *E. coli* BL21 (DE3)

It was decided that the first goal was to produce active *Sl*PVA and *Au*AAC, before production of recombinant fusion protein was attempted. This decision was due to the fact, that achieving active *Sl*PVA and *Au*AAC was the basis for further experiments with fusion proteins like immobilisation. An optimization of culture conditions as well as expression induction was addressed in order to obtain *Sl*PVA and *Au*AAC in native form.

4.5.1 Selection of the best medium for production of recombinant *Sl*PVA and *Au*AAC by E. coli BL21 (DE3)

E. coli BL21 (DE3) cultures harbouring the plasmids pET-22b (*pva*) and pET-22b (*aac*) were grown according to section 3.5.1 of Material and Methods. In 40 mL shake flask cultures, induction of recombinant protein production was achieved by addition of 1.5 mM IPTG at OD_{600} 1.5. Cells were harvested after 20 h at 20 °C and 250 rpm. These conditions were chosen due to previous success of producing active PGA in *E. coli* BL21 with the described conditions (Vélez *et al.*, 2014). As media selection has proven to be an integral part in producing active recombinant PGA in *E. coli* (Ignatova *et al.*, 2003; Vélez *et al.*, 2014), various media compositions were tested for their ability to enable production of active PVA. Cells were grown in the following media: LB, LB+Glucose, LB+Sorbitol+Betaine, M9 and B2 (see section 3.3 of Material and Methods for exact compositions). After recombinant protein production, cells were harvested and a western blot (see section 3.6.3 of Material and Methods) was performed with obtained biomass. Anti-polyhistidine-peroxidase antibody was used as described above, since in native protein the β subunit carries the His-Tag C-terminal (β-subunit of SlPVA: 60.09 kDa, β-subunit of AuAAC: 55 kDa). In all media, a control sample was grown as well, that was not induced with IPTG was grown as well. SDS-PAGE analysis of all samples as well as immunodetection results are displayed in Figure 29 andFigure 30.

Several bands are shown by immunodetection. In all samples grown in LB a strong band at around 80 kDa is present. This band corresponds well with the size of the unprocessed recombinant proteins. Furthermore, this band is visible as well in the control samples that were not induced with IPTG. This could be explained by the fact that the inducer seems to be leaky, allowing recombinant protein production during the non-induced growth phase. Overall, *Au*AAC production seems to be stronger than *Sl*PVA production, as all bands are more intense. In all samples grown in LB+Glucose medium, especially in *Au*AAC samples, several further bands are visible at lower molecular weights than 80 kDa. These bands could represent different processing stages of the protein carrying the His-Tag. However, it is also possible that they are degradation products of the unprocessed enzymes carrying the C-terminal His-Tag. It is unlikely that they are unspecific bound proteins, because the bands are only present in the induced samples. The control samples show no bands. This means that addition of glucose triggers catabolic repression of the promoter. Moreover, the strongest bands overall were obtained in these samples. Since the western blot revealed possible processed protein, enzymatic assays were performed with all samples in order to check the presence of native

*Sl*PVA and *Au*AAC (see section 3.6.4 of Material and Methods). Unfortunately, no enzyme activity was detected.

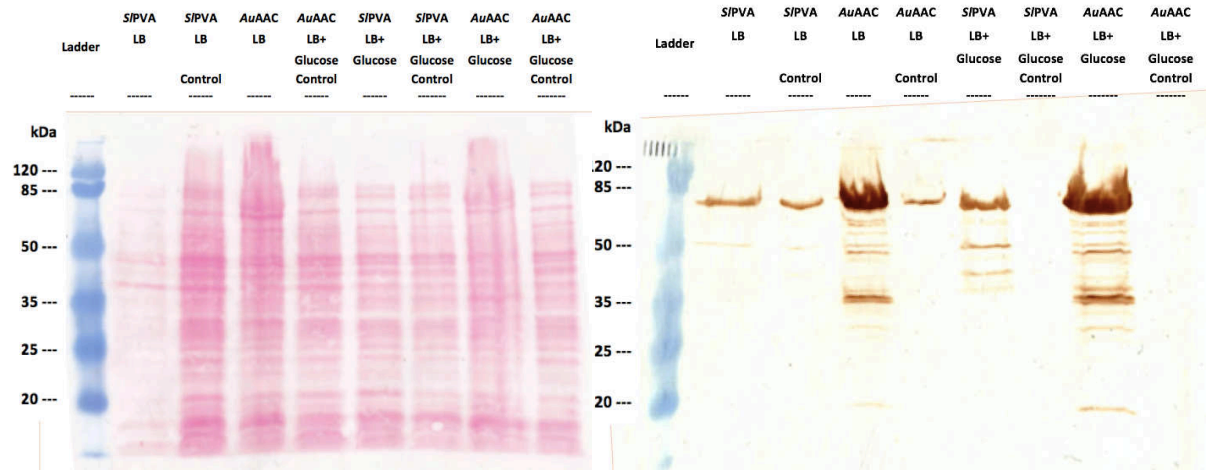


Figure 29: Western blot of recombinant SlPVA and AuAAC produced by E. coli BL21 in different media (LB, LB+Glucose); ***left:*** *Ponceau S stain;* ***right:*** *Immunodetection*

Figure 30 displays pictures of the Ponceau S stain and the immunodetection of the nitrocellulose membrane for all samples grown on the media B2 and LB+Sorbitol+Betaine. Revelation of bound antibody displays similar trends as seen with the other media. *Au*AAC production seems to be stronger than *Sl*PVA. Also, no recombinant protein was found in all control samples. Again, bands at about 80 kDa were obtained, that represent unprocessed recombinant protein. This band is very faint for *Sl*PVA in B2 medium and not visible in LB+Sorbitol+Betaine medium. Furthermore, for *Au*AAC multiple bands below the strong band at 80 kDa are present as in the samples explained above. Again, these bands could be interpreted as different stages of processing of the recombinant protein or degradation products of the preproprotein. In addition, enzyme activity assays were performed for all samples, but no active protein was detected again.

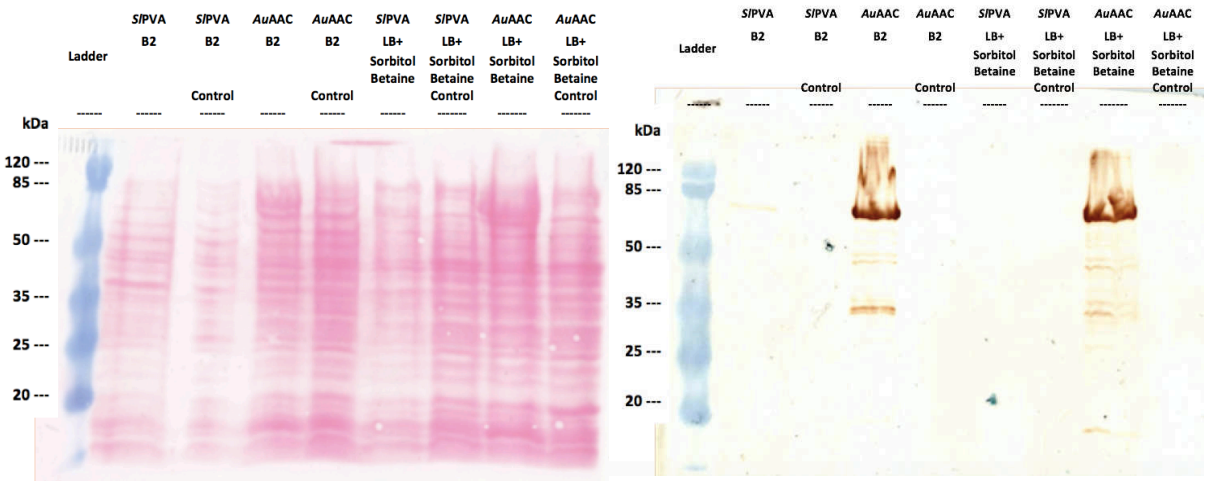


*Figure 30: Western blot of recombinant SlPVA and AuAAC produced in E. coli BL21 in different media (B2, LB+Sorbitol+Glucose)**; left:** Ponceau S; **right:** Immunodetection*

At last, samples grown in M9 medium were analysed (Figure 31). Antibody revelation shows bands at about 80 kDa for *Sl*PVA and *Au*AAC in the induced samples. These can be attributed to inactive recombinant enzyme (α- and β-subunit unprocessed). For *Au*AAC, a faint band at about 35 kDa is visible as well. Again, this band can be interpreted as a degradation product or falsely processed protein carrying the C-terminal His-Tag. Also, *Au*AAC was again produced in higher amounts as *Sl*PVA. No bands are present in control samples. Enzymatic assays yielded no presence of active proteins.

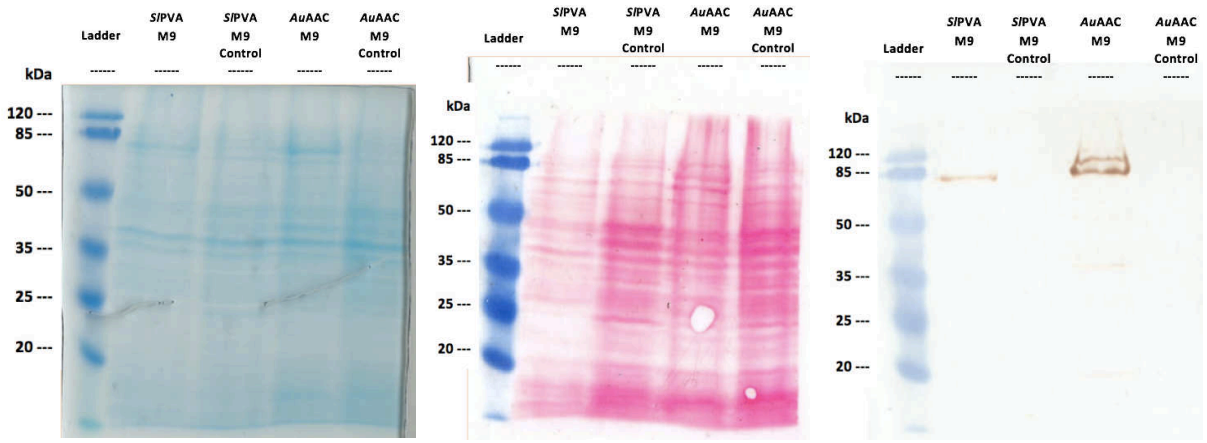


*Figure 31: SDS-PAGE and western blot of recombinant SlPVA and AuAAC produced in E. coli BL21 in M9 medium **left:** Coomassie stain **middle:** Ponceau S stain **right:** Immunodetection*

Even though no active recombinant protein could be detected, LB+Glucose was chosen the best medium to produce recombinant *Sl*PVA and *Au*AAC by *E. coli* BL21 (DE3).

4.6 Purification of recombinant *Sl*PVA and *Au*AAC

Next, it was decided that recombinant *Sl*PVA and *Au*AAC produced by *E. coli* BL21 (DE3) in LB+Glucose as medium should be purified. Furthermore, it is important to know if the recombinant proteins are produced as inclusion bodies or as soluble unprocessed proteins.

4.6.1 Sonication of *E. coli* BL21 (DE3) harbouring pET-22b (*pva*) and pET-22b (*aac*)

E. coli BL21 (DE3) cells harbouring the recombinant plasmids pET-22b (*pva*) and pET-22b (*aac*) were cultured like explained above in section 4.5.1 of Results. After harvesting cells were sonicated (see section 3.5.2 of Material and Methods), obtaining cell extract and a pellet of cell debris for every sample.

First, enzymatic assays (see section 3.6.4 of Material and Methods) were performed on all samples (cell extract, cell debris and cell broth). As expected, all returned negative. Second, western blots (see section 3.6.3 of Material and Methods) with both cell extract and cell debris were performed (Figure 32 and Figure 33). For both western blots, comparison of the Coomassie stain and the Ponceau S stain confirms the correct transference of protein to the nitrocellulose as the same bands are visible on both gels. The immunodetection revealed, that both *Sl*PVA and *Au*AAC are present as preproproteins in the cell debris as indicated by strong bands at 80 kDa. This suggests, that the recombinant proteins are produced as inclusion bodies in the cells. Again, the trend that *Au*AAC seems to be produced stronger than *Sl*PVA can be seen. Furthermore, for *Au*AAC multiple bands below 80 kDa are present. As explained above, these bands could represent either different stages of processing of the protein or degradation products. As no bands are present in the control sample, unspecific binding of the antibody is unlikely.

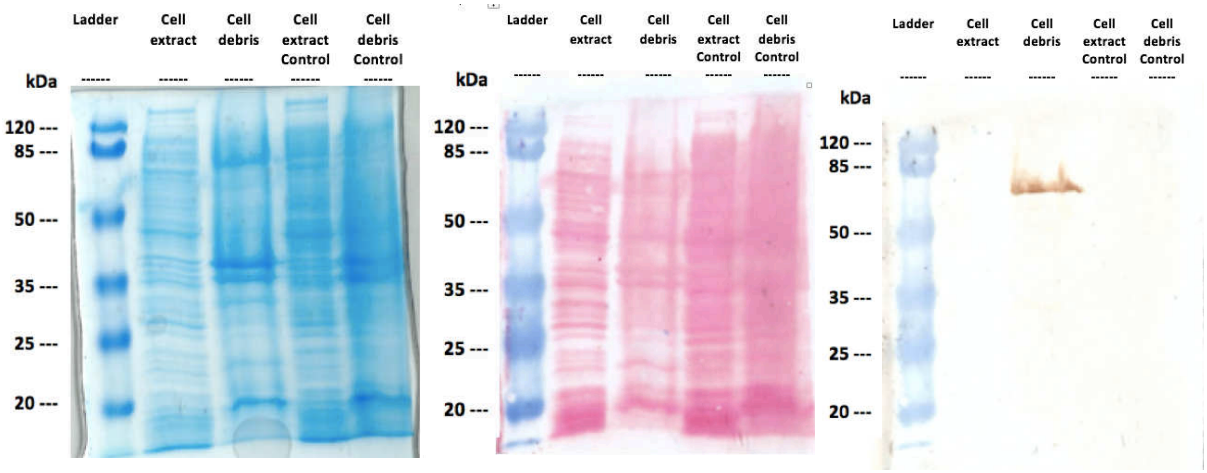


Figure 32: SDS-PAGE and western blot of recombinant SlPVA produced in E. coli BL21 in LB+Glucose medium after sonication; ***left:*** *Coomassie stain;* ***middle:*** *Ponceau S stain;* ***right:*** *immunodetection*

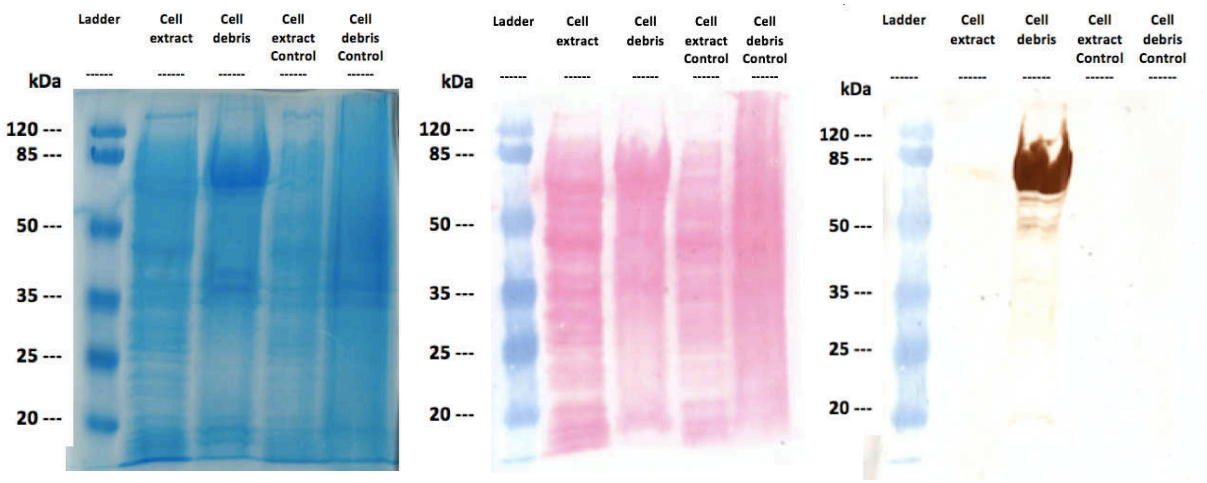


Figure 33: SDS-PAGE and western blot of recombinant AuAAC produced in E. coli BL21 in LB+Glucose medium after sonication; ***left:*** *Coomassie stain;* ***middle:*** *Ponceau S stain;* ***right:*** *immunodetection*

4.6.2 Refolding of inclusion bodies

Recombinant *Sl*PVA and *Au*AAC were produced as explained above (see section 4.6.1 of Results). However, instead of sonicating the biomass, the supposed inclusion bodies were purified and solubilized using a commercial reagents *B-Per Bacterial Protein Extraction Reagent* and *Inclusion Body Solubilization Reagent* (Thermo Scientific, USA) and subsequently refolded (see sections 3.5.3 and 3.5.4 of Material and Methods for protocol). For refolding, as an approach stepwise slow dialysis of the chaotropic agent was chosen. During refolding, a lot of protein went out of solution. According to activity assays (see section 3.6.4 of Material and Methods) no active protein was present before purification, after solubilisation of inclusion bodies and after refolding. Nevertheless, SDS-PAGE and western blots were

performed for solubilized and refolded inclusion bodies. However, as the chaotropic agent present in the commercial *Inclusion Body Solubilization Reagent* would prevent a successful SDS-PAGE, samples of solubilized inclusion bodies were dialysed over night against 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl. During this dialysis, nearly all protein was lost as precipitate. No meaningful information could be gained by the Western Blot as nearly no protein could be loaded onto the SDS-gel. Therefore, only the SDS-gel and the Western Blot of the refolded inclusion body is presented (Figure 34).

When comparing Coomassie stained SDS-PAGE gel and Ponceau S stained nitrocellulose, successful transference of all protein loaded on the gel to the membrane can be confirmed. Bound antibody shows the presence of inactive *Au*AAC at around 60 kDa. Furthermore, various bands at lower sizes (e.g. 40 kDa, 35 kDa) were obtained. These bands probably are different fragments of the protein carrying the C-terminal His-Tag. No *Sl*PVA at all could be detected. The western blot confirmed the failure of the refolding process to produce native recombinant protein.

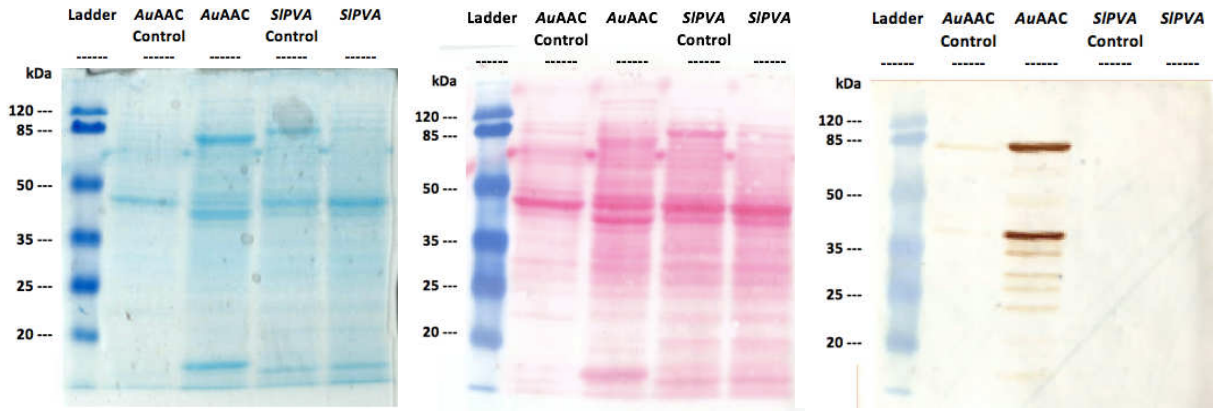


Figure 34: SDS-PAGE and western blot of recombinant AuAAC and SlPVA produced in E. coli BL21 in LB+Glucose medium after refolding; ***left:*** *Coomassie stain;* ***middle:*** *Ponceau S stain;* ***right:*** *immunodetection*

5 Discussion

5.1 Design and construction of recombinant plasmids

The genes used in this study stem from different organisms spread all over the tree of life. GFP is a protein from the eukaryotic jellyfish *Aequorea victorea* and *sbd* codes for the substrate binding domain of the prokaryotic poly-3-hydroxybutyrate depolymerase from the grampositive actinobacterium *Streptomyces exfoliatus*. Furthermore, the genes coding for the acylases *pva* and *aac* stem from the grampositive actinobacteria *Streptomyces lavendulae* and *Actinoplanes utahensis*. Even though, the genetic code is universal, the abundance of each tRNA varies from species to species. This phenomenon called the codon bias leads to the fact that different organisms express degenerate codons differently. Therefore, codon usage always has to be optimized for the host chosen in order to efficiently express recombinant protein (Voet *et al.*, 2016). In the course of this work all genes were codon-optimized for usage in *E. coli* by GeneScript (USA). Recombinant protein, although inactive, could be detected for both acylases *Sl*PVA and *Au*AAC as well as the fusion protein *Au*AAC-GFP meaning that these genes were expressed efficiently. This proves the success of the codon optimization.

All recombinant genes were cloned into the commercial plasmid pET-22b for expression in *E. coli*. This plasmid carries the N-terminal *pelB*-leader sequence and C-terminal a His-Tag. Furthermore, it carries an ampicillin resistance gene *bla* for selection. The recombinant gene itself is under the control of the T7 RNA polymerase, which in turn is under the control of the lacUV5 promotor (see Supplemental material). Thus, expression of recombinant protein can be induced by addition of IPTG. This plasmid was chosen due to different reasons. The C-terminal His-Tag was used for immunodetection. Active protein can be distinguished from unprocessed inactive protein by the size of the protein bound to the anti-polyhistidine-peroxidase antibody. A band at about 80 kDa signifies unprocessed protein, whereas a band at 60 kDa would represent processed β-subunit, on which the C-terminal His-Tag is present. Furthermore, further purification of produced enzyme is facilitated by present His-Tag, as IMAC (immobilised metal affinity chromatography) with Ni^{2+} or Co^{2+} can be performed. The N-terminal *pelB*-leader effectively replaces the leader sequence present in the acylases *pva* and *aac* in their natural hosts *S. lavendulae* and *A. utahensis* (see sections 1.5.2 and 1.5.3 of Introduction). The acylases are produced as preproproteins in their hosts. After secretion into the medium and excertion of the signal peptide the internal spacer between α- and β-subunit is thought to be cut autoproteolytically (see sections 1.5.2 and 1.5.3 of Introduction). The *pelB*-leader sequence

directs protein to the bacterial periplasma, where more favourable conditions for disulfidebonds and thus, correct folding of the protein can be found, which could trigger the activation process. This approach has already successfully been applied with various recombinant proteins including penicillin G acylases from Gram-negative bacteria (Pan, Wang, *et al.*, 2018; Pan, Yu, *et al.*, 2018).

Moreover, gene-optimized *pva* was cloned into the commercial vector pET-28a for expression in *E. coli*. Same as with pET-22b, expression of recombinant protein is controlled addition of IPTG. Furthermore, it contains a kanamycin resistance gene. Contrary to pET-22b, the His-Tag is placed N-terminal (see Supplemental material).

After isolating all genes by PCR, fusion genes and recombinant plasmids were generated by a classic approach using restriction enzyme digestion and subsequent ligation of sticky ends. This process yielded all desired genes but fusion genes with *Linker::sbd*, which was confirmed by restriction enzyme digestion and sequencing (see section 4.3.1 of Results). Subsequent sequencing of the PCR product obtained from the isolation of *Linker::sbd* gene from pET-22b (*ahla::Linker::sbd*) with the primers used for the isolation of the gene (see section 4.2.2 of Results) yielded no sequence. This revealed that no correct PCR product was obtained. Thus, primers must have bound unspecifically and produced the wrong product. In this sense, it is essential to optimize the conditions (e.g. annealing temperature, salt concetration, DMSO content, choice of polymerase) applied in the PCR to isolate *Linker::sbd* gene.

5.2 Cloning and expression of recombinant genes in *E. coli*

Expression of constructed recombinant genes was attempted in different strains of *E. coli*. *E. coli* BL21 (DE3) is a classic T7 expression strain, that is protease deficient. Generally speaking, in all experiments carried out more *Au*AAC was obtained as *Sl*PVA under same growth conditions and cell densities. This could be explained either by the fact, that *aac* is expressed more efficiently as *pva*. This could be caused by the codon optimization performed. Unprocessed *Sl*PVA and *Au*AAC was detected after production in *E. coli* BL21 (DE3). This means that the correct folding of the proteins was not achieved in the cell. Therefore, the autoproteolytic activation process could not happen. Moreover, in the case of the fusion proteins with GFP, only a band representing *Au*AAC-GFP was found on the western blot. However, it is plausible that *Sl*PVA-GFP was produced, however, in an amount too small to detect with the western blot.

E. coli Origami is strain carrying mutations in the thioredoxin reductase (*trxB*) and the glutathione reductase (*gor*), thus, enhancing disulphide bond formation in the cytoplasm. Correct disulphide bonds could trigger proper folding and thus, the autoproteolytic activation process of the proteins. However, this was approach failed to deliver processed recombinant protein. Furthermore, no *Sl*PVA could be detected. The same argument as above could be made that *Sl*PVA was produced in a too small amount to detect on the western blot.

Lastly, an *E. coli* strain already transformed with the commercial plasmid pET-28a carrying the gene for the chaperonin GroEL was used. Overexpression of the chaperonin could help with correct folding of the recombinant proteins. However, transformation of this strain with pET-22b (*aac*) failed. Double transformation is deemed to be a highly unlikely process (Goldsmith *et al.*, 2007). Furthermore, no *Sl*PVA could be detected after culturing *E. coli* BL21(DE3) (pET-GroEL) transformed with pET-22b (*Sl*PVA).

5.3 Obtaining native recombinant *Sl*PVA and *Au*AAC

Recombinant *Sl*PVA and *Au*AAC were obtained as inclusion bodies by *E. coli* BL21 (DE3). A subsequent try to refold the proteins using a standard protocol provided by the manufacturer of the purification and solubilisation agent failed. However, it is worth to say, that refolding is a complicated process, that needs extensive research (Yamaguchi & Miyazaki, 2014). As refolding always is associated with loss of protein and constitutes further process steps, production of active soluble protein often is advantageous.

Obtaining active soluble recombinant protein in *E. coli* is dependent on various factors. It has been reported that temperature at induction plays an important role in producing active PGA in *E. coli*. Generally, low temperatures seem to enhance production of soluble protein. Also, plasmid stability is higher at low temperatures (Vélez *et al.*, 2014). Accordingly, 20 °C was chosen as the optimal induction temperature. Furthermore, applied IPTG concentration for induction affects correct folding of produced recombinant protein in *E. coli*. As PGA from *E. coli* is in the same supergroup, the Ntn-hydrolases, as the desired *Sl*PVA and *Au*AAC, as similar approach was chosen (0.25 mM) as described by Vélez et al. (2014). Moreover, in preliminary experiments, higher IPTG-concentrations were tested (0.4 mM, 1 mM) for their ability to induce active protein production. However, in no case, was recombinant protein production notably affected. At last, choice of medium is reported to heavily influence production of active soluble PGA in *E. coli* (Ignatova *et al.*, 2003; Vélez *et al.*, 2014). According to Vélez *et al.* B2 medium was used to successfully express active penicillin G

acylase in *E. coli* BL21 (DE3) in bioreactor cultures. Furthermore, It has been suggested (Blackwell & Horgan, 1991), that higher internal concentration of osmolytes like betaine increase the yield of active protein. A high concentration of *D*-sorbitol serves to generate a high osmotic pressure, so that betaine is taken up into the cell. However, as presented in section 4.5.1 of Results none of the tested media (LB, LB+Glucose, LB+Sorbitol+Betaine, B2, M9) could yield active protein. As expected, media containing glucose (B2, LB+Glucose) were able to repress basal expression of protein during uninduced phase. The medium LB+Sorbitol+Betaine achieved the same. Choice of LB+Glucose for further experiments can be justified by the fact, that it is the least complex medium, that represses basal expression when not induced. Moreover, when applying the same growth conditions apart from choice of media, the most recombinant protein was obtained.

As no active *Sl*PVA and *Au*AAC could be obtained by production in *E. coli*, for now, fusion genes were not expressed in *E. coli* and accordingly no immobilisation experiments could be performed.

6 Conclusions

Both *pva* gene, encoding penicillin V acylase from *Streptomyces lavendulae* (*Sl*PVA), and *aac* gene, encoding aculeacin A acylase from *Actinoplanes utahensis* (*Au*AAC), were successfully codon-optimized, cloned and expressed in *E. coli.*

Hereafter, fusion genes *pva::gfp, pva::sbd, aac::gfp* and *aac::sbd* were obtained and cloned into the commercial plasmid pET-22b for protein expression in *E. coli.* As the isolation of *linker::sbd* had failed, no fusion genes with *linker::sbd* were prepared.

Recombinant *Sl*PVA and *Au*AAC could be produced as inclusion bodies in *E. coli* BL21 (DE3). An optimization of culture conditions was performed. Usage of LB with 1 % glucose as culture medium, with induction for 20 h at 20 °C with 1.5 mM IPTG at an optical density of 1.5 yielded the highest amount of inclusion bodies. A single attempt to refold the inclusion bodies according to a standard protocol using slow dialysis yielded no active proteins.

Transfromation of *pva* and *aac* into different strains of *E. coli* (C43 (DE3), Origami, BL21 (DE3) (pET-GroEL)) did not accomplish production of active soluble protein.

Further research should focus on the optimization of the culture conditions. However, it seems more likely that *E. coli* is not an appropriate host for expression of these acylases.

7 References

Ackerley, D., Gonzalez, C., Park, C., Blake, R., Keyhan, M., & Matin, A. (2004). Chromate-reducing properties of soluble flavoproteins from Pseudomonas putida and Escherichia coli. *Appl. Environ. Microbiol., 70*(2), 873-882.

Arroyo, M., De la Mata, I., Acebal, C., & Castillón, M. P. (2003). Biotechnological applications of penicillin acylases: state-of-the-art. *Applied microbiology and biotechnology, 60*(5), 507-514.

Arroyo, M., de la Mata, I., García, J.-L., & Barredo, J.-L. (2017). Chapter 17 - Biocatalysis for Industrial Production of Active Pharmaceutical Ingredients (APIs). In G. Brahmachari (Ed.), *Biotechnology of Microbial Enzymes* (pp. 451-473): Academic Press.

Arroyo, M. (1998). Inmovilización de enzimas. Fundamentos, métodos y aplicaciones. *Ars Pharmaceutica, 39*(2), 23-39.

Benz, F., Knüsel, F., Nüesch, J., Treichler, H., Voser, W., Nyfeler, R., & Keller-Schierlein, W. (1974). Stoffwechselprodukte von Mikroorganismen 143. Mitteilung. Echinocandin B, ein neuartiges Polypeptid-Antibioticum aus Aspergillus nidulans var. echinulatus: Isolierung und Bausteine. *Helvetica Chimica Acta, 57*(8), 2459-2477. doi:10.1002/hlca.19740570818

Blackwell, J. R., & Horgan, R. (1991). A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS letters, 295*(1-3), 10-12.

Bomstein, J., & Evans, W. G. (1965). Automated Colorimetric Determination of 6-Aminopenicillanic Acid in Fermentation Media. *Analytical chemistry, 37*(4), 576-578.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254.

Brahmachari, G. (2016). *Biotechnology of microbial enzymes: production, biocatalysis and Industrial applications*: Academic Press.

Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C. E., Smith, J. L., Tomchick, D. R., & Murzin, A. G. (1995). A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature, 378*(6555), 416-419. doi:10.1038/378416a0

Bruggink, A. (2011). *Synthesis of β-lactam antibiotics: chemistry, biocatalysis & process integration*: Springer Science & Business Media.

Bryskier, A. (2005). *Antimicrobial agents: antibacterials and antifungals*: ASM press.

Bush, K. (2010). Bench-to-bedside review: the role of β-lactamases in antibiotic-resistant Gram-negative infections. *Critical Care, 14*(3), 224.

Centres for Disease Control Prevention. (2013). *Antibiotic resistance threats in the United States, 2013*: Centres for Disease Control and Prevention, US Department of Health and ….

Defoirdt, T., Boon, N., & Bossier, P. (2010). Can bacteria evolve resistance to quorum sensing disruption? *PLoS pathogens, 6*(7), e1000989.

Denning, D. W. (2002). Echinocandins: a new class of antifungal. *Journal of Antimicrobial Chemotherapy, 49*(6), 889-891. doi:10.1093/jac/dkf045

Dickschat, J. S. (2010). Quorum sensing and bacterial biofilms. *Natural product reports, 27*(3), 343-369.

DiCosimo, R., McAuliffe, J., Poulose, A. J., & Bohlmann, G. (2013). Industrial use of immobilized enzymes. *Chemical Society Reviews, 42*(15), 6437-6474.

Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G., & Moody, P. C. (1995). Penicillin acylase has a single-amino-acid catalytic centre. *Nature, 373*(6511), 264.

Eberhard, A., Burlingame, A., Eberhard, C., Kenyon, G., Nealson, K., & Oppenheimer, N. (1981). Structural identification of autoinducer of Photobacterium fischeri luciferase. *Biochemistry, 20*(9), 2444-2449.

Emri, T., Majoros, L., Tóth, V., & Pócsi, I. (2013). Echinocandins: production and applications. *Applied Microbiology and Biotechnology, 97*(8), 3267-3284. doi:10.1007/s00253-013-4761-9

Farha, M. A., & Brown, E. D. (2019). Drug repurposing for antimicrobial discovery. *Nature Microbiology, 4*(4), 565-577. doi:10.1038/s41564-019-0357-1

Fetzner, S. (2015). Quorum quenching enzymes. *Journal of Biotechnology, 201*, 2-14. doi:https://doi.org/10.1016/j.jbiotec.2014.09.001

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *British journal of experimental pathology, 10*(3), 226.

García-Hidalgo, J., Hormigo, D., Prieto, M. A., Arroyo, M., & de la Mata, I. (2012). Extracellular production of Streptomyces exfoliatus poly(3-hydroxybutyrate) depolymerase in Rhodococcus sp. T104: determination of optimal biocatalyst conditions. *Applied Microbiology and Biotechnology, 93*(5), 1975-1988. doi:10.1007/s00253-011-3527-5

Goldsmith, M., Kiss, C., Bradbury, A. R. M., & Tawfik, D. S. (2007). Avoiding and controlling double transformation artifacts. *Protein Engineering, Design and Selection, 20*(7), 315-318. doi:10.1093/protein/gzm026

Grandclément, C., Tannières, M., Moréra, S., Dessaux, Y., & Faure, D. (2015). Quorum quenching: role in nature and applied developments. *FEMS Microbiology Reviews, 40*(1), 86-116. doi:10.1093/femsre/fuv038

Hamad, B. (2010). The antibiotics market. *Nature Reviews Drug Discovery, 9*, 675. doi:10.1038/nrd3267

Hersbach, G. (1984). The penicillins: properties, biosynthesis and fermentation. *Biotechnology of Industrial Antibiotics.*, 45-140.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents, 35*(4), 322-332. doi:https://doi.org/10.1016/j.ijantimicag.2009.12.011

Hong, K.-W., Koh, C.-L., Sam, C.-K., Yin, W.-F., & Chan, K.-G. (2012). Quorum quenching revisited—from signal decays to signalling confusion. *Sensors, 12*(4), 4661-4696.

Hormigo, D. (2009). *Caracterización del centro activo de las acilasas de streptomyces lavendulae y actinoplanes utahensis. Búsqueda de nuevas actividades e inmovilización.* (Doctoral dissertation), Universidad Complutense de Madrid.

Ignatova, Z., Mahsunah, A., Georgieva, M., & Kasche, V. (2003). Improvement of posttranslational bottlenecks in the production of penicillin amidase in recombinant Escherichia coli strains. *Appl. Environ. Microbiol., 69*(2), 1237-1245.

Junji, I., Hideo, T., Haruo, I., & Satoshi, O. (1992). Cloning and sequencing of the aculeacin A acylase-encoding gene from Actinoplanes utahensis and expression in Streptomyces lividans. *Gene, 119*(1), 29-35.

Kasuya, K.-i., Ohura, T., Masuda, K., & Doi, Y. (1999). Substrate and binding specificities of bacterial polyhydroxybutyrate depolymerases. *International Journal of Biological Macromolecules, 24*(4), 329-336. doi:https://doi.org/10.1016/S0141-8130(99)00046-X

Klingbeil, B., Kroppenstedt, R. M., & Jendrossek, D. (1996). Taxonomic identification of Streptomyces exfoliatus K10 and characterization of its poly (3-hydroxybutyrate) depolymerase gene. *FEMS microbiology letters, 142*(2-3), 215-221.

Koch, B., Liljefors, T., Persson, T., Nielsen, J., Kjelleberg, S., & Givskov, M. (2005). The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiology, 151*(11), 3589-3602.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature, 227*(5259), 680.

Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics, 30*(22), 3276-3278.

Lee, S. J., Park, J. P., Park, T. J., Lee, S. Y., Lee, S., & Park, J. K. (2005). Selective Immobilization of Fusion Proteins on Poly(hydroxyalkanoate) Microbeads. *Analytical Chemistry, 77*(17), 5755-5759. doi:10.1021/ac0505223

Li, Z., Yang, J., & Loh, X. J. (2016). Polyhydroxyalkanoates: opening doors for a sustainable future. *NPG Asia Materials, 8*(4), e265.

Liu, X., He, Y. U. N., Wang, Z., Wang, C., Liu, Z., Xia, T., . . . Zhang Fang, L. (2011). US Patent No. US 2011/0081708 A1.

McBrayer, D. N., & Tal-Gan, Y. (2019). Deciphering bacterial signalling. *Nature Chemistry, 11*(5), 398-399. doi:10.1038/s41557-019-0265-2

Miroux, B., & Walker, J. E. (1996). Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of molecular biology, 260*(3), 289-298.

Mizuno, K., Yagi, A., Satoi, S., Takada, M., Hayashi, M., Asano, K., & Matsuda, T. (1977). Studies on aculeacin. *The Journal of antibiotics, 30*(4), 297-302.

Newbolt, R. G., Merfyn, R., & Ralph, B. F. (1961). United States Patent No. US3014845.

Oinonen, C., & Rouvinen, J. (2000). Structural comparison of Ntn-hydrolases. *Protein Science, 9*(12), 2329-2337.

Pan, X., Wang, L., Ye, J., Qin, S., & He, B. (2018). Efficient synthesis of β-lactam antibiotics with very low product hydrolysis by a mutant Providencia rettgeri penicillin G acylase. *Applied Microbiology and Biotechnology, 102*(4), 1749-1758. doi:10.1007/s00253-017-8692-8

Pan, X., Yu, Q., Chu, J., Jiang, T., & He, B. (2018). Fitting replacement of signal peptide for highly efficient expression of three penicillin G acylases in E. coli. *Applied Microbiology and Biotechnology, 102*(17), 7455-7464. doi:10.1007/s00253-018-9163-6

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., & Bonomo, R. A. (2011). Carbapenems: Past, Present, and Future. *Antimicrobial Agents and Chemotherapy, 55*(11), 4943-4960. doi:10.1128/aac.00296-11

Parveen, N., & Cornell, K. A. (2011). Methylthioadenosine/S-adenosylhomocysteine nucleosidase, a critical enzyme for bacterial metabolism. *Molecular microbiology, 79*(1), 7-20.

Perona, J. J., & Craik, C. S. (1995). Structural basis of substrate specificity in the serine proteases. *Protein Science, 4*(3), 337-360.

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., . . . Vila, J. (2015). The global threat of antimicrobial resistance: science for intervention. *New Microbes and New Infections, 6*, 22-29. doi:https://doi.org/10.1016/j.nmni.2015.02.007

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*: Cold spring harbor laboratory press.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the national academy of sciences, 74*(12), 5463-5467.

Schwartz, R. E., Giacobbe, R. A., Bland, J. A., & Monaghan, R. L. (1989). L-671, 329, A NEW ANTIFUNGAL AGENT. *The Journal of antibiotics, 42*(2), 163-167.

Sheldon, R. A. (2007). Enzyme immobilization: the quest for optimum performance. *Advanced Synthesis & Catalysis, 349*(8-9), 1289-1307.

Shewale, J. G., & Sudhakaran, V. K. (1997). Penicillin V acylase: its potential in the production of 6-aminopenicillanic acid. *Enzyme and Microbial Technology, 20*(6), 402-410.

Shi, Y.-F., Soumillion, P., & Ueda, M. (2010). Effects of catalytic site mutations on active expression of phage fused penicillin acylase. *Journal of biotechnology, 145*(2), 139-142.

Takeshima, H., Inokoshi, J., Takada, Y., Tanaka, H., & Ōmura, S. (1989). A deacylation enzyme for aculeacin A, a neutral lipopeptide antibiotic, from Actinoplanes utahensis: purification and characterization. *The Journal of Biochemistry, 105*(4), 606-610.

Tan, L., Hu, M., Yu, S., Wang, X., Lu, F., Liu, F., . . . Ding, C. (2015). Characterization of the chaperonin GroEL in Mycoplasma gallisepticum. *Archives of microbiology, 197*(2), 235-244.

Torres, Ferreras, E. R., Cantero, Á., Hidalgo, A., & Berenguer, J. (2012). Functional expression of a penicillin acylase from the extreme thermophile Thermus thermophilus HB27 in Escherichia coli. *Microbial cell factories, 11*(1), 105.

Torres, Ramón, F., de La Mata, I., Acebal, C., & Castillón, M. (1999). Enhanced production of penicillin V acylase from Streptomyces lavendulae. *Applied microbiology and biotechnology, 53*(1), 81-84.

Torres-Bacete, J., Hormigo, D., Stuart, M., Arroyo, M., Torres, P., Castillón, M. P., . . . de la Mata, I. (2007). Newly discovered penicillin acylase activity of aculeacin A acylase from Actinoplanes utahensis. *Appl. Environ. Microbiol., 73*(16), 5378-5381.

Torres-Bacete, J., Hormigo, D., Torres-Gúzman, R., Arroyo, M., Castillón, M. P., García, J. L., . . . de la Mata, I. (2015). Overexpression of penicillin V acylase from Streptomyces lavendulae and elucidation of its catalytic residues. *Appl. Environ. Microbiol., 81*(4), 1225-1233.

Torres-Guzmán, R., de la Mata, I., Torres-Bacete, J., Arroyo, M., Castillón, M. a. P., & Acebal, C. (2002). Substrate specificity of penicillin acylase from Streptomyces lavendulae. *Biochemical and biophysical research communications, 291*(3), 593-597.

Velasco Bucheli, R. (2017). *Directed evolution of penicillin V acylase from" Streptomyces lavendulae" and aculeacin A acylase from" Actinoplanes utahensis".* (Doctoral dissertation), Universidad Complutense de Madrid.

Vélez, A. M., da Silva, A. J., Horta, A. C. L., Sargo, C. R., Campani, G., Silva, G. G., . . . Zangirolami, T. C. (2014). High-throughput strategies for penicillin G acylase production in rE. coli fed-batch cultivations. *BMC biotechnology, 14*(1), 6.

Voet, D., Voet, J. G., & Pratt, C. W. (2016). *Fundamentals of biochemistry: life at the molecular level*: John Wiley & Sons.

Volpato, G., C. Rodrigues, R., & Fernandez-Lafuente, R. (2010). Use of Enzymes in the Production of Semi-Synthetic Penicillins and Cephalosporins: Drawbacks and Perspectives. *Current Medicinal Chemistry, 17*(32), 3855-3873. doi:10.2174/092986710793205435

Walsh, C., & Wencewicz, T. (2016). *Antibiotics: challenges, mechanisms, opportunities*: American Society for Microbiology (ASM).

Weiss, B., Jacquemin-Sablon, A., Live, T. R., Fareed, G. C., & Richardson, C. C. (1968). Enzymatic breakage and joining of deoxyribonucleic acid VI. Further purification and properties of polynucleotide ligase from Escherichia coli infected with bacteriophage T4. *Journal of Biological Chemistry, 243*(17), 4543-4555.

World Health Organisation. (2014). *Antimicrobial resistance: global report on surveillance*: World Health Organization.

Yamaguchi, H., & Miyazaki, M. (2014). Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. *Biomolecules, 4*(1), 235-251. doi:10.3390/biom4010235

8 Supplemental material

8.1 Plasmid map of pET-22b(+)

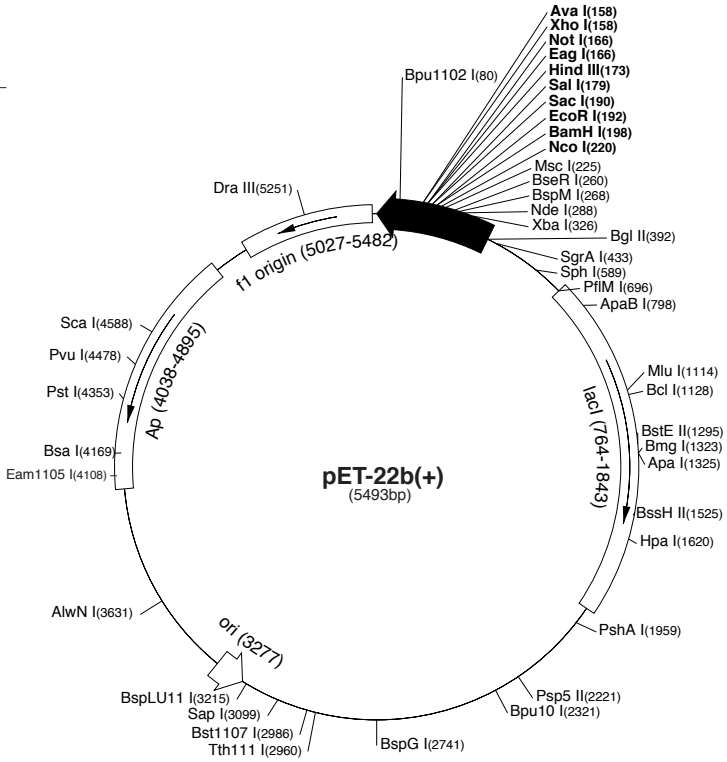
Novagen

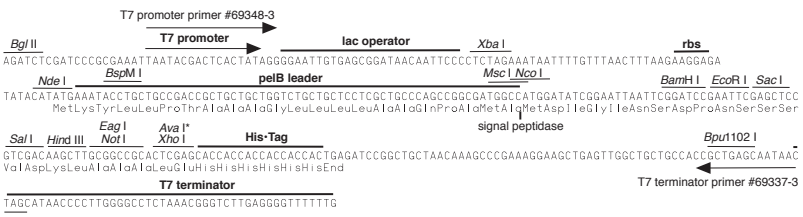
pET-22b(+) Vector

TB038 12/98

The pET-22b(+) vector (Cat. No. 69744-3) carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

pET-22b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
bla coding sequence	4038-4895
f1 origin	5027-5482





pET-22b(+) cloning/expression region

8.1 Plasmid map of pET-28a(+)

Novagen

pET-28a-c(+) Vectors

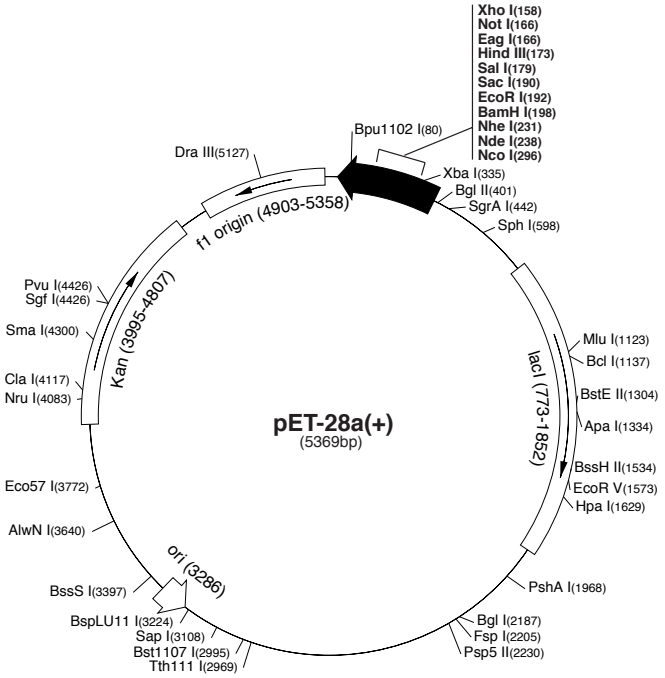
TB074 12/98

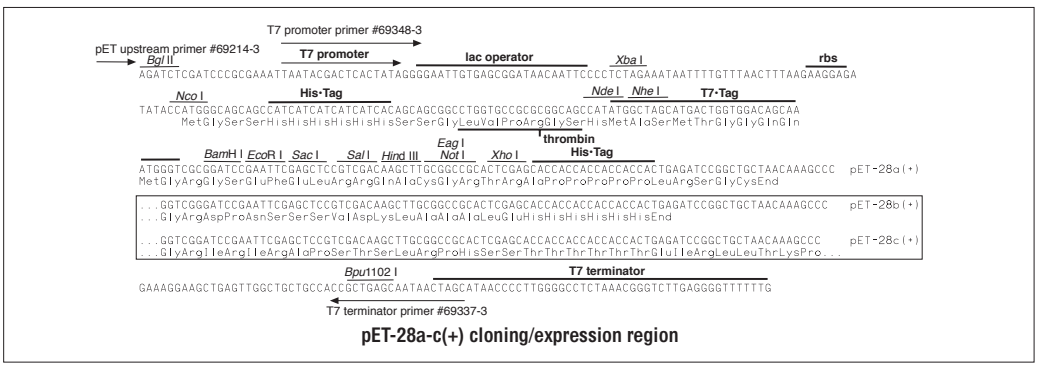
	Cat. No.
pET-28a DNA	69864-3
pET-28b DNA	69865-3
pET-28c DNA	69866-3

The pET-28a-c(+) vectors carry an N-terminal His•Tag®/thrombin/T7•Tag® configuration plus an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.





pET-28a-c(+) cloning/expression region

8.2 Sequence data

In the following the sequence data of the created recombinant genes is displayed. Start- and stopcodons are coloured green, the *pelB*-leader sequence and the His-tag are coloured yellow and restriction sites used for cloning (NcoI (CCATGG), EcoRI (GAATTC) and XhoI (CTGAG)) are coloured grey. Nucleotides not covered during sequencing are red. For a list of all primers used and their respective sequence see Table 3.

8.2.1 pva, pva::gfp and *pva::sbd*

```
pva          1 ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGTGGTGGCCT
pva::gfp     1 ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGTGGTGGCCT
pva::sbd     1 ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGTGGTGGCCT

pva         81 GAGCGCGACCGTTCGTTATACCGAGTACGGCATCCCGCACATCGTGGCGAAGGACTACGCGAATCTGGGCTTTGGCACCG
pva::gfp    81 GAGCGCGACCGTTCGTTATACCGAGTACGGCATCCCGCACATCGTGGCGAAGGACTACGCGAATCTGGGCTTTGGCACCG
pva::sbd    81 GAGCGCGACCGTTCGTTATACCGAGTACGGCATCCCGCACATCGTGGCGAAGGACTACGCGAATCTGGGCTTTGGCACCG

pva        161 GTTGGGCGCAGGCGGCGGACCAAGTTTGCACCCTGGCGGATGGCTTCGTGACCGTTCGTGGTGAGCGTAGCAAATTCTTT
pva::gfp   161 GTTGGGCGCAGGCGGCGGACCAAGTTTGCACCCTGGCGGATGGCTTCGTGACCGTTCGTGGTGAGCGTAGCAAATTCTTT
pva::sbd   161 GTTGGGCGCAGGCGGCGGACCAAGTTTGCACCCTGGCGGATGGCTTCGTGACCGTTCGTGGTGAGCGTAGCAAATTCTTT

pva        241 GGTCCGGATGCGGCGCCGGATTTCAGCCTGAGCAGCGCGGCGAAAAACCTGAGCAGCGACCTGTACTTTCGTGGCGTTCG
pva::gfp   241 GGTCCGGATGCGGCGCCGGATTTCAGCCTGAGCAGCGCGGCGAAAAACCTGAGCAGCGACCTGTACTTTCGTGGCGTTCG
pva::sbd   241 GGTCCGGATGCGGCGCCGGATTTCAGCCTGAGCAGCGCGGCGAAAAACCTGAGCAGCGACCTGTACTTTCGTGGCGTTCG

pva        321 TGATAGCGGTACCGTGGAGAAACTGCTGAAAGTTCCGGCGCCGGCGGGTCCGAGCCGTGATGCGAAGGAAAGCATGCGTG
pva::gfp   321 TGATAGCGGTACCGTGGAGAAACTGCTGAAAGTTCCGGCGCCGGCGGGTCCGAGCCGTGATGCGAAGGAAAGCATGCGTG
pva::sbd   321 TGATAGCGGTACCGTGGAGAAACTGCTGAAAGTTCCGGCGCCGGCGGGTCCGAGCCGTGATGCGAAGGAAAGCATGCGTG

pva        401 GCTTCGCGGCGGGTTATAACGCGTGGCTGCGTCAGAACCGTGACCGTATCACCGATCCGGCGTGCCGTGGCGCGAGCTGG
pva::gfp   401 GCTTCGCGGCGGGTTATAACGCGTGGCTGCGTCAGAACCGTGACCGTATCACCGATCCGGCGTGCCGTGGCGCGAGCTGG
pva::sbd   401 GCTTCGCGGCGGGTTATAACGCGTGGCTGCGTCAGAACCGTGACCGTATCACCGATCCGGCGTGCCGTGGCGCGAGCTGG

pva        481 GTTCGTCCGGTGACCGCGCTGGATGTGGCGGTTCGTGGCTTTGCGCTGGCGGTTCTGGGTGGCCAGGGTCGTGGTATCGA
pva::gfp   481 GTTCGTCCGGTGACCGCGCTGGATGTGGCGGTTCGTGGCTTTGCGCTGGCGGTTCTGGGTGGCCAGGGTCGTGGTATCGA
pva::sbd   481 GTTCGTCCGGTGACCGCGCTGGATGTGGCGGTTCGTGGCTTTGCGCTGGCGGTTCTGGGTGGCCAGGGTCGTGGTATCGA

pva        561 TGGTATTACCGCGGCGCAACCGCCGACCGCGGCTCCGCCGGCGGCGGGCGTTACCCCGAAAGAGGCGGCGGCGGCGGCGC
pva::gfp   561 TGGTATTACCGCGGCGCAACCGCCGACCGCGGCTCCGCCGGCGGCGGGCGTTACCCCGAAAGAGGCGGCGGCGGCGGCGC
pva::sbd   561 TGGTATTACCGCGGCGCAACCGCCGACCGCGGCTCCGCCGGCGGCGGGCGTTACCCCGAAAGAGGCGGCGGCGGCGGCGC
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pva         641 AGCGTCTGCTGAGCACCCAAAACGCGGACATGGGCAGCAACGCGGTGGCGTTTCGTGGTAGCACCACCGCGAACGGTCGT
pva::gfp    641 AGCGTCTGCTGAGCACCCAAAACGCGGACATGGGCAGCAACGCGGTGGCGTTTCGTGGTAGCACCACCGCGAACGGTCGT
pva::sbd    641 AGCGTCTGCTGAGCACCCAAAACGCGGACATGGGCAGCAACGCGGTGGCGTTTCGTGGTAGCACCACCGCGAACGGTCGT

pva         721 GGTCTGCTGCTGGGTAACCCGCACTACCCGTGGGATGGTGGCCGTCGTTTTTGGCAAAGCCAGCAAACCATTCCGGGTGA
pva::gfp    721 GGTCTGCTGCTGGGTAACCCGCACTACCCGTGGGATGGTGGCCGTCGTTTTTGGCAAAGCCAGCAAACCATTCCGGGTGA
pva::sbd    721 GGTCTGCTGCTGGGTAACCCGCACTACCCGTGGGATGGTGGCCGTCGTTTTTGGCAAAGCCAGCAAACCATTCCGGGTGA

pva         801 ACTGAACGTGGCGGGTGGCAGCCTGCTGGGTAGCACCACCGTGAGCATTGGTCACAACGCGGACGTTGCGTGGAGCCACA
pva::gfp    801 ACTGAACGTGGCGGGTGGCAGCCTGCTGGGTAGCACCACCGTGAGCATTGGTCACAACGCGGACGTTGCGTGGAGCCACA
pva::sbd    801 ACTGAACGTGGCGGGTGGCAGCCTGCTGGGTAGCACCACCGTGAGCATTGGTCACAACGCGGACGTTGCGTGGAGCCACA

pva         881 CCGTTGCGACCGGTGTGACCCTGAACCTGCACCAACTGACCCTGGACCCGGCGGATCCGACCGTTTATCTGGTGGATGGC
pva::gfp    881 CCGTTGCGACCGGTGTGACCCTGAACCTGCACCAACTGACCCTGGACCCGGCGGATCCGACCGTTTATCTGGTGGATGGC
pva::sbd    881 CCGTTGCGACCGGTGTGACCCTGAACCTGCACCAACTGACCCTGGACCCGGCGGATCCGACCGTTTATCTGGTGGATGGC

pva         961 AAGCCGCAGCGTATGACCCAACGTACCGTGGCGGTGCCGGTGAAGGGTGCGGCGCCGGTTACCCGTACCCAATGGTGGAC
pva::gfp    961 AAGCCGCAGCGTATGACCCAACGTACCGTGGCGGTGCCGGTGAAGGGTGCGGCGCCGGTTACCCGTACCCAATGGTGGAC
pva::sbd    961 AAGCCGCAGCGTATGACCCAACGTACCGTGGCGGTGCCGGTGAAGGGTGCGGCGCCGGTTACCCGTACCCAATGGTGGAC

pva        1041 CCGTTACGGCCCGGTGGTTACCAGCCTGGGTGCGGCGCTGCCGCTGCCGTGGACCGCGAGCACCGCGTATGCGCTGAACG
pva::gfp   1041 CCGTTACGGCCCGGTGGTTACCAGCCTGGGTGCGGCGCTGCCGCTGCCGTGGACCGCGAGCACCGCGTATGCGCTGAACG
pva::sbd   1041 CCGTTACGGCCCGGTGGTTACCAGCCTGGGTGCGGCGCTGCCGCTGCCGTGGACCGCGAGCACCGCGTATGCGCTGAACG

pva        1121 ACCCGAACGCGGTGAACCTGCGTAGCGCGGATACCAGCCTGGGTTTCAGCAAAGCGCGTAGCACCGCGGGTATTGAACGT
pva::gfp   1121 ACCCGAACGCGGTGAACCTGCGTAGCGCGGATACCAGCCTGGGTTTCAGCAAAGCGCGTAGCACCGCGGGTATTGAACGT
pva::sbd   1121 ACCCGAACGCGGTGAACCTGCGTAGCGCGGATACCAGCCTGGGTTTCAGCAAAGCGCGTAGCACCGCGGGTATTGAACGT

pva        1201 GCGCTGCACCGTAGCCAGGGCCTGCCGTGGGTTAACACCATTGCGGCGGACCGTAGCGGTAACAGCTTCTTTAGCCAGAG
pva::gfp   1201 GCGCTGCACCGTAGCCAGGGCCTGCCGTGGGTTAACACCATTGCGGCGGACCGTAGCGGTAACAGCTTCTTTAGCCAGAG
pva::sbd   1201 GCGCTGCACCGTAGCCAGGGCCTGCCGTGGGTTAACACCATTGCGGCGGACCGTAGCGGTAACAGCTTCTTTAGCCAGAG

pva        1281 CCAAGTGCTGCCGCGTATCACCGATGAGCTGGCGGCGCGTTGCAGCACCCCGCTGGGTCAAGCGACCTACCCGAGCGCGG
pva::gfp   1281 CCAAGTGCTGCCGCGTATCACCGATGAGCTGGCGGCGCGTTGCAGCACCCCGCTGGGTCAAGCGACCTACCCGAGCGCGG
pva::sbd   1281 CCAAGTGCTGCCGCGTATCACCGATGAGCTGGCGGCGCGTTGCAGCACCCCGCTGGGTCAAGCGACCTACCCGAGCGCGG

pva        1361 GCCTGGCGGTTCTGGACGGTAGCACCAGCGCGTGCGCGCTGGGTAGCGACCGTGATGCGGTGCAACCGGGTATTTTCGGC
pva::gfp   1361 GCCTGGCGGTTCTGGACGGTAGCACCAGCGCGTGCGCGCTGGGTAGCGACCGTGATGCGGTGCAACCGGGTATTTTCGGC
pva::sbd   1361 GCCTGGCGGTTCTGGACGGTAGCACCAGCGCGTGCGCGCTGGGTAGCGACCGTGATGCGGTGCAACCGGGTATTTTCGGC
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pva       1441 CCGGGTCGTATGCCGACCCTGAAGAACGCGCCGTACGTTGAGAACAGCAACGACAGCGCGTGGCTGACCAACGCGGATCG
pva::gfp  1441 CCGGGTCGTATGCCGACCCTGAAGAACGCGCCGTACGTTGAGAACAGCAACGACAGCGCGTGGCTGACCAACGCGGATCG
pva::sbd  1441 CCGGGTCGTATGCCGACCCTGAAGAACGCGCCGTACGTTGAGAACAGCAACGACAGCGCGTGGCTGACCAACGCGGATCG

pva       1521 TCCGCTGACCGGCTATGAACGTGTGTTTGGTACCACCGCGACCCAGCGTAGCATCCGTACCCGTGGCGCGATTGAGGACG
pva::gfp  1521 TCCGCTGACCGGCTATGAACGTGTGTTTGGTACCACCGCGACCCAGCGTAGCATCCGTACCCGTGGCGCGATTGAGGACG
pva::sbd  1521 TCCGCTGACCGGCTATGAACGTGTGTTTGGTACCACCGCGACCCAGCGTAGCATCCGTACCCGTGGCGCGATTGAGGACG

pva       1601 TTGCGGCGATGGCGGAACGTGGTCGTCTGCGTGTGACCGATCTGGAGCGTCAGCAACTGGCGAACCGTGCGCCGACCGGT
pva::gfp  1601 TTGCGGCGATGGCGGAACGTGGTCGTCTGCGTGTGACCGATCTGGAGCGTCAGCAACTGGCGAACCGTGCGCCGACCGGT
pva::sbd  1601 TTGCGGCGATGGCGGAACGTGGTCGTCTGCGTGTGACCGATCTGGAGCGTCAGCAACTGGCGAACCGTGCGCCGACCGGT

pva       1681 GACCTGGTTGCGGCGGATGTGGCGAAATGGTGCGCGGCGCTGCCGGGTGGCACCGCGGTGGGCAGCAGCGGTACCCCGGT
pva::gfp  1681 GACCTGGTTGCGGCGGATGTGGCGAAATGGTGCGCGGCGCTGCCGGGTGGCACCGCGGTGGGCAGCAGCGGTACCCCGGT
pva::sbd  1681 GACCTGGTTGCGGCGGATGTGGCGAAATGGTGCGCGGCGCTGCCGGGTGGCACCGCGGTGGGCAGCAGCGGTACCCCGGT

pva       1761 TGATGTGAGCGCGGCGTGCCCGGTGCTGCGTCGTTGGGATCGTAGCGTTGACAGCGATAGCCGTGGTGCGCTGCTGTTCG
pva::gfp  1761 TGATGTGAGCGCGGCGTGCCCGGTGCTGCGTCGTTGGGATCGTAGCGTTGACAGCGATAGCCGTGGTGCGCTGCTGTTCG
pva::sbd  1761 TGATGTGAGCGCGGCGTGCCCGGTGCTGCGTCGTTGGGATCGTAGCGTTGACAGCGATAGCCGTGGTGCGCTGCTGTTCG

pva       1841 ACCGTTTTTGGCGTAAGGCGGCGGCGGTGCCGGCGGCGGAACTGTGGAAAGTTCCGTTTGATGCGGCGGATCCGGTGCGT
pva::gfp  1841 ACCGTTTTTGGCGTAAGGCGGCGGCGGTGCCGG----------------------------------------------
pva::sbd  1841 ACCGTTTTTGGCGTAAGGCGGCGGCGGTGCCGGCGGCGGAACTGTGGAAAGTTCCGTTTGATGCGGCGGATCCGGTGCGT

pva       1921 ACCCCGCGTGGTCTGAACACCGCGGCGCCGGGCGTTGGCAAGGCGCTGGCGGATACCGTGACCGAGCTGAAAGCGGCGGG
pva::gfp  1874 ----------------------------------------------------------------------------
pva::sbd  1921 ACCCCGCGTGGTCTGAACACCGCGGCGCCGGGCGTTGGCAAGGCGCTGGCGGATACCGTGACCGAGCTGAAAGCGGCGGG

pva       2001 TATTGCGCTGAACGCGCCGCTGGGTGAACACCAGTTTGTGGTTCGTAACGGCAAGCGTATTCCGGTTGGTGGCGGTACCG
pva::gfp  1874 ----------------------------------------------------------------------------CCG
pva::sbd  2001 TATTGCGCTGAACGCGCCGCTGGGTGAACACCAGTTTGTGGTTCGTAACGGCAAGCGTATTCCGGTTGGTGGCGGTACCG

pva       2081 AGAGCCTGGGTATCTGGAACAAAATTGAACCGGTTTGGAACCCGGCGGCGGGCGGTTACACCGAGGTGAGCGCGGGCAGC
pva::gfp  1877 AGAGCCTGGGTATCTGGAACAAAATTGAACCGGTTTGGAACCCGGCGGCGGGCGGTTACACCGAGGTGAGCGCGGGCAGC
pva::sbd  2081 AGAGCCTGGGTATCTGGAACAAAATTGAACCGGTTTGGAACCCGGCGGCGGGCGGTTACACCGAGGTGAGCGCGGGCAGC

pva       2161 AGCTATATTCAAGCGGTTGGTTGGGACAACAGCCGTTGCCCGGTGGCGCGTACCCTGCTGACCTACAGCCAGAGCAGCAA
pva::gfp  1957 AGCTATATTCAAGCGGTTGGTTGGGACAACAGCCGTTGCCCGGTGGCGCGTACCCTGCTGACCTACAGCCAGAGCAGCAA
pva::sbd  2161 AGCTATATTCAAGCGGTTGGTTGGGACAACAGCCGTTGCCCGGTGGCGCGTACCCTGCTGACCTACAGCCAGAGCAGCAA

pva       2241 CCCGAACAGCCCGCACTATAGCGATCAAACCCGTCTGTTCAGCGGTGAACGTTGGGTGACCAGCCGTTTTTGCGAAAAGG
pva::gfp  2037 CCCGAACAGCCCGCACTATAGCGATCAAACCCGTCTGTTCAGCGGTGAACGTTGGGTGACCAGCCGTTTTTGCGAAAAGG
pva::sbd  2241 CCCGAACAGCCCGCACTATAGCGATCAAACCCGTCTGTTCAGCGGTGAACGTTGGGTGACCAGCCGTTTTTGCGAAAAGG
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pva       2321 ACATCGCGCGTAGCCCGCAACTGAAGGTGGTTCGTGTGCATGAGCGTCGTCTCGAGCACCACCACCACCACCACTGA---
pva::gfp  2117 ACATCGCGCGTAGCCCGCAACTGAAGGTGGTTCGTGTGCATGAGCGTCGTGAATTCATGAGCAAAGGCGAGGAACTGTTC
pva::sbd  2321 ACATCGCGCGTAGCCCGCAACTGAAGGTGGTTCGTGTGCATGAGCGTCGTGAATTCACCACCGGTGCGGCGGTGTGCGTT

pva            ----------------------------------------------------------------------------
pva::gfp  2197 ACCGGCGTGGTTCCGATCCTGGTTGAACTGGACGGTGATGTGAACGGCCACAAGTTTAGCGTGAGCGGTGAAGGTGAAGG
pva::sbd  2401 ACCGCGAGCAACTATGCGCATACCCAAGCGGGTCGTGCGCACCAAAGCGGTGGCTATACCTACGCGAACGGCAGCAACCA

pva            ----------------------------------------------------------------------------
pva::gfp  2277 TGATGCGACCTATGGTAAACTGACCCTGAAATTCATTTGCACCACCGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTGA
pva::sbd  2481 AAACCTGGGTCTGTGGAATGTTCTGGCGAGCAGCACCATCAAGGAGACCGCGCCGGGTTATTGGGTTACCTGCTAA----

pva            ----------------------------------------------------------------------------
pva::gfp  2357 CCACCTTCAGCTATGGTGTGCAGTGCTTTAGCCGTTACCCGGATCACATGAAGCGTCACGACTTCTTTAAAAGCGCGATG
pva::sbd       ----------------------------------------------------------------------------

pva            ----------------------------------------------------------------------------
pva::gfp  2437 CCGGAGGGTTATGTTCAAGAACGTACCATCTTCTTTAAGGACGATGGCAACTACAAAACCCGTGCGGAAGTGAAGTTCGA
pva::sbd       ----------------------------------------------------------------------------

pva            ----------------------------------------------------------------------------
pva::gfp  2517 AGGTGACACCCTGGTGAACCGTATCGAGCTGAAGGGCATTGATTTTAAAGAAGACGGTAACATTCTGGGCCACAAGCTGG
pva::sbd       ----------------------------------------------------------------------------

pva            ----------------------------------------------------------------------------
pva::gfp  2597 AGTATAACTACAACAGCCACAACGTTTACATCATGGCGGATAAGCAGAAAAACGGTATTAAGGTGAACTTCAAAATCCGT
pva::sbd       ----------------------------------------------------------------------------

pva            ----------------------------------------------------------------------------
pva::gfp  2677 CACAACATTGAAGATGGCAGCGTTCAACTGGCGGACCACTATCAGCAAAACACCCCGATCGGTGATGGCCCGGTTCTGCT
pva::sbd       ----------------------------------------------------------------------------

pva            ----------------------------------------------------------------------------
pva::gfp  2757 GCCGGACAACCACTACCTGAGCACCCAGAGCGCGCTGAGCAAGGACCCGAACGAGAAACGTGACCACATGGTTCTGCTGG
pva::sbd       ----------------------------------------------------------------------------

pva              ------------------------------------------------------------------------
pva::gfp   2837  AGTTTGTGACCGCGGCGGGCATTACCCACGGCATGGATGAGCTGTATAAGCTCGAGCACCACCACCACCACCACTGA
pva::sbd         ------------------------------------------------------------------------
```

8.2.2 *aac::gfp* and *aac::sbd*

```
aac::gfp     1 ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGTGGTTATGC
aac::sbd     1 ATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGGTGGTTATGC

aac::gfp    81 GGCGCTGATTCGTCGTGCGAGCTACGGCGTTCCGCACATTACCGCGGATGATTTCGGCAGCCTGGGTTTTGGCGTTGGTT
aac::sbd    81 GGCGCTGATTCGTCGTGCGAGCTACGGCGTTCCGCACATTACCGCGGATGATTTCGGCAGCCTGGGTTTTGGCGTTGGTT

aac::gfp   161 ATGTGCAGGCGGAGGACAACATCTGCGTTATTGCGGAAAGCGTGGTTACCGCGAACGGCGAACGTAGCCGTTGGTTTGGT
aac::sbd   161 ATGTGCAGGCGGAGGACAACATCTGCGTTATTGCGGAAAGCGTGGTTACCGCGAACGGCGAACGTAGCCGTTGGTTTGGT

aac::gfp   241 GCGACCGGTCCGGATGATGCGGACGTGCGTAGCGACCTGTTCCACCGTAAGGCGATCGACGATCGTGTTGCGGAGCGTCT
aac::sbd   241 GCGACCGGTCCGGATGATGCGGACGTGCGTAGCGACCTGTTCCACCGTAAGGCGATCGACGATCGTGTTGCGGAGCGTCT

aac::gfp   321 GCTGGAAGGTCCGCGTGACGGTGTGCGTGCGCCGAGCGACGATGTTCGTGATCAAATGCGTGGCTTCGTGGCGGGTTACA
aac::sbd   321 GCTGGAAGGTCCGCGTGACGGTGTGCGTGCGCCGAGCGACGATGTTCGTGATCAAATGCGTGGCTTCGTGGCGGGTTACA

aac::gfp   401 ACCACTTTCTGCGTCGTACCGGTGTTCATCGTCTGACCGACCCGGCGTGCCGTGGTAAAGCGTGGGTGCGTCCGCTGAGC
aac::sbd   401 ACCACTTTCTGCGTCGTACCGGTGTTCATCGTCTGACCGACCCGGCGTGCCGTGGTAAAGCGTGGGTGCGTCCGCTGAGC

aac::gfp   481 GAGATCGACCTGTGGCGTACCAGCTGGGATAGCATGGTGCGTGCGGGTAGCGGTGCGCTGCTGGATGGTATTGTTGCGGC
aac::sbd   481 GAGATCGACCTGTGGCGTACCAGCTGGGATAGCATGGTGCGTGCGGGTAGCGGTGCGCTGCTGGATGGTATTGTTGCGGC

aac::gfp   561 GACCCCGCCGACCGCGGCGGGTCCGGCGAGCGCGCCGGAAGCGCCGGACGCGGCGGCGATCGCGGCGGCGCTGGATGGTA
aac::sbd   561 GACCCCGCCGACCGCGGCGGGTCCGGCGAGCGCGCCGGAAGCGCCGGACGCGGCGGCGATCGCGGCGGCGCTGGATGGTA

aac::gfp   641 CCAGCGCGGGTATTGGCAGCAACGCGTATGGTCTGGGTGCGCAGGCGACCGTGAACGGTAGCGGCATGGTTCTGGCGAAC
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aac::gfp   721 CCGCACTTCCCGTGGCAGGGTGCGGAGCGTTTTTACCGTATGCACCTGAAGGTGCCGGGCCGTTATGACGTTGAAGGCGC
aac::sbd   721 CCGCACTTCCCGTGGCAGGGTGCGGAGCGTTTTTACCGTATGCACCTGAAGGTGCCGGGCCGTTATGACGTTGAAGGCGC

aac::gfp   801 GGCGCTGATCGGTGATCCGATCATTGAGATTGGTCACAACCGTACCGTTGCGTGGAGCCACACCGTTAGCACCGCGCGTC
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aac::gfp   881 GTTTCGTGTGGCACCGTCTGAGCCTGGTTCCGGGTGACCCGACCAGCTACTATGTGGATGGCCGTCCGGAACGTATGCGT
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aac::gfp   961 GCGCGTACCGTGACCGTTCAAACCGGTAGCGGTCCGGTGAGCCGTACCTTCCACGACACCCGTTATGGTCCGGTTGCGGT
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aac::gfp 1121 ATGGCTGGCTGCGTATGGGTCAGGCGAAGGACGTGCGTGCGCTGAAAGCGGTTCTGGATCGTCACCAATTTCTGCCGTGG
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