UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA
Departamento de Microbiología II

TESIS DOCTORAL

Directed evolution of penicillin V acylase from *Streptomyces lavendulae* and aculeacin A acylase from *Actinoplanes utahensis*
Evolución dirigida de penicilina V acilasa de *Streptomyces lavendulae* y aculeacina A acilasa de *Actinoplanes utahensis*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Rodrigo Velasco Bucheli

Directores

Mª Isabel de la Mata Riesco
Miguel Arroyo Sánchez

Madrid, 2017

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Complutense University of Madrid
Faculty of Pharmacy
Department of Microbiology II and Parasitology

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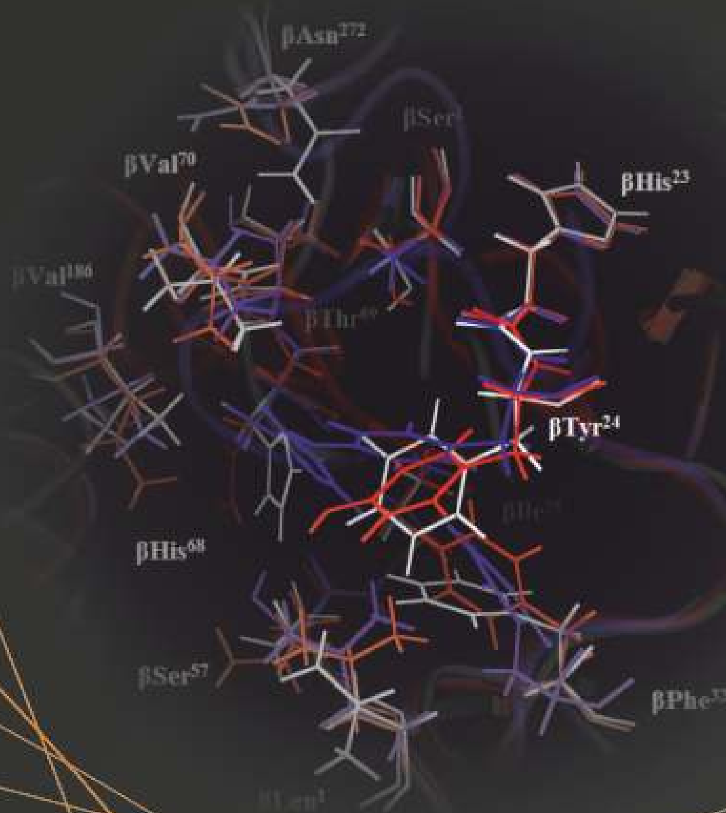

RODRIGO VELASCO BUCHELI

Supervisors
Mª Isabel de la Mata Riesco
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Presentado por

Rodrigo Velasco Bucheli

Dirección

Dra. Mª Isabel de la Mata Riesco
Dr. Miguel Arroyo Sánchez

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FACULTY OF PHARMACY
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DIRECTED EVOLUTION OF PENICILLIN V ACYLASE FROM *Streptomyces lavendulae* AND ACULEACIN A ACYLASE FROM *Actinoplanes utahensis*

Ph.D. Thesis
by

RODRIGO VELASCO BUCHELI

Supervisors
Mª Isabel de la Mata Riesco, Ph.D.
Miguel Arroyo Sánchez, Ph.D.

Madrid, 2016

UNIVERSIDAD COMPLUTENSE DE MADRID
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Tesis Doctoral
por

RODRIGO VELASCO BUCHELI

Dirección
Mª Isabel de la Mata Riesco, Ph.D.
Miguel Arroyo Sánchez, Ph.D.

Madrid, 2016

TABLE OF CONTENTS

LIST OF ABBREVIATIONS xi
LIST OF FIGURES xiii
LIST OF TABLES xvii
LIST OF PUBLICATIONS OBTAINED DURING THIS STUDY xix
THESIS ABSTRACT (English) 1
RESUMEN DE LA TESIS (Spanish) 5
I INTRODUCTION 9
1. β-LACTAM ANTIBIOTICS 11
2. ECHINOCANDINS, STATE OF THE ART OF ANTIFUNGALS 14
3. BIOTECHNOLOGICAL PRODUCTION OF SEMISYNTHETIC β-LACTAM ANTIBIOTICS AND ECHINOCANDINS 15
4. PENICILLIN AND ECHINOCANDIN ACYLASES 18
4.1. Biochemical and structural characteristics of penicillin and echinocandin acylases 21
4.2. Penicillin and echinocandin acylases in pharmaceutical industry 24
5. BACTERIAL COMMUNICATION AND THE POTENTIAL BIOLOGICAL ROLE OF PENICILLIN AND ECHINOCANDIN ACYLASES 24
5.1. *Quorum sensing* system 25
5.2. *Quorum quenching* system 26
6. PENICILLIN V ACYLASE FROM *Streptomyces lavendulae* ATCC 13664 . 28
7. ACULEACIN A ACYLASE FROM *Actinoplanes utahensis* NRRL 12052 ... 30
8. DIRECTED MOLECULAR EVOLUTION 31
8.1. Creation of libraries 32
8.1.1. Mutator strains 33
8.1.2. Error-prone PCR 35
8.1.3. DNA shuffling 36
8.1.4. Gene assembling mutagenesis 36
8.2. Host selection 37
8.3. Screening and selection 38
8.3.1. Genetic selection 38
8.3.2. Screening for enzymes 38
8.4. Statistical analysis 39

8.4.1. Statistical tools 39
8.4.2. Assessment of assay quality 40
8.4.3. Statistically-guided selection of hits in HTS 40
9. COMPUTATIONAL INTELLIGENCE: BIOINFORMATICS 40
9.1. Current computational intelligence 41
9.2. Genome Analysis 41
9.3. Protein engineering: computational tools 42
9.4. Docking 42
II OBJECTIVES 45
III MATERIALS AND METHODS 49
1. CHEMICALS REAGENTS 51
2. BACTERIAL STRAINS, PLASMIDS AND OLIGONUCLEOTIDES 52
3. MEDIA AND CULTURE CONDITIONS 53
4. HANDLING TECHNIQUES AND ANALYSIS OF DNA 54
4.1. Purification and sequencing of DNA 54
4.2. PCR assays 55
4.3. DNA electrophoresis 56
4.4. Isolation of DNA fragments 56
4.5. Colony mini-prep 56
4.6. Colony PCR 56
5. SEQUENCING OF GENOMES FROM *Streptomyces lavendulae* ATCC 13664 AND *Actinoplanes utahensis* NRRL 12052 56
6. TRANSFORMATION OF BACTERIAL STRAINS 57
6.1. Methodologies employed with *E. coli* DH5α 57
6.2. Methodologies employed with *Rhodococcus* sp. T104 57
7. OBTAINING OF A LIBRARY OF MUTANT PLASMIDS 58
8. HIGH-THROUGHPUT SCREENING OF THE LIBRARY OF MUTANTS BY ASSAYING ENZYMATIC ACTIVITY 59
8.1. EVOware optimization 59
8.2. Penicillin V acylase activity 60
8.3. *N*-acyl-L-homoserine lactone (AHL) acylase activity 60
8.3.1. Synthesis and purification of aliphatic AHLs 60
8.3.2. Spectrophotometric assays 61
9. STATISTICAL ANALYSIS 61

9.1. Mutant clones selection ... 61
9.2. Distribution models of the spot densities ... 62
10. PROTEIN ANALYSIS TECHNIQUES ... 62
10.1. Quantification of protein content ... 62
10.2. Polyacrylamide gel electrophoresis (PAGE) in presence of sodium dodecyl sulfate (SDS) ... 62
10.3. Heterologous expression of proteins ... 63
10.3.1. Protein expression of recombinant enzymes by *Streptomyces lividans* 1326 ... 63
10.3.2. Protein expression of recombinant enzymes by *Rhodococcus* sp. T104 ... 63
10.4. Isolation and purification of acylases ... 64
10.4.1. Isolation and purification of enzymes derived from *Sl*PVA ... 64
10.4.2. Isolation and purification of enzymes derived of *Au*AAC ... 65
10.5. Determination of kinetic parameters ... 65
11. BIOINFORMATIC ANALYSIS OF RECOMBINANT ENZYMES ... 66
11.1. Identification of recombinant clones sequences ... 66
11.2. Sequence alignments ... 66
11.3. Prediction of protein characteristics ... 67
11.4. Homology modelling of protein ... 67
12. ESTIMATION OF CHELATING PROPERTIES IN ECHINOCANDINS ... 67
IV RESULTS ... 69
1. SHOTGUN SEQUENCING OF GENOMES ... 71
1.1. Draft genome sequence of *Streptomyces lavendulae* ATCC 13664 ... 71
1.2. Draft genome sequence of *Actinoplanes utahensis* NRRL 12052 ... 77
1.3. Estimation of the chelating properties of echinocandins ... 84
2. NOVEL AHL-ACYLASE FROM *Actinoplanes utahensis* ... 84
3. DETERMINATION OF KINETIC PARAMETERS OF *Sl*PVA AND *Au*AAC USING AHLs AS SUBSTRATES ... 85
4. DIRECTED MOLECULAR EVOLUTION OF *Sl*PVA AND *Au*AAC ... 90
4.1. Obtaining of *Sl*PVA and *Au*AAC mutant libraries ... 90
4.2. High-Throughput Screening (HTS) ... 91
4.2.1. Screening of mutants of penicillin V acylase from *Streptomyces* sp. with improved acylase activities ... 92
4.2.2. Screening of aculeacin A acylase mutants from *Actinoplanes utahensis* NRRL 12052 with improved acylase activities ... 96

4.2.3. Statistical analysis ... 101
4.2.4. Distribution model ... 103
4.3. Expression of recombinant clones in *Rhodococcus* sp. T104 employing pENS shuttle vector ... 105
5. ANALYSIS OF MUTANTS WITH EVOLVED ACYLASE BY DIRECTED EVOLUTION ... 107
5.1. Changes throughout enzymes sequences ... 107
5.2. Prediction of the three-dimensional structure ... 108
5.3. Characteristics of the residues involved in the binding pocket ... 113
5.4. Alteration of residues involved in binding pocket ... 115
5.5. Characteristics of residues mutated by directed molecular evolution ... 117
V DISCUSSION ... 121
1. DRAFT GENOME SEQUENCES OF *Actinoplanes utahensis* NRRL 12052 AND *Streptomyces lavendulae* ATCC 13664 ... 123
2. MULTIPLE SPOTLIGHTS OF *Sl*PVA, *Au*AAC AND *Au*AHLA ... 124
2.1. Comparative study with other acylases ... 125
2.2. Potential biological role of *Sl*PVA, *Au*AAC and *Au*AHLA ... 127
2.3. Estimation of kinetic parameters by fluorometric measurements ... 129
2.4. Reaction of postulated mechanism in *Sl*PVA, *Au*AAC and *Au*AHLA ... 131
3. DIRECTED MOLECULAR EVOLUTION OF *Sl*PVA AND *Au*AAC ... 132
3.1. Recombinant acylase 2pva1,21(25) of *Sl*PVA ... 135
3.2. Recombinant acylase 2pva1,2 (55) of *Sl*PVA ... 136
3.2.1. Deletion in the C-terminal end ... 137
3.2.2. Deletion in the N-terminal end ... 138
3.2.3. Deletion in both ends ... 138
3.3. Recombinant acylase 2pva1,2(149) of *Sl*PVA ... 139
3.4. Recombinant acylase a(292) of *Au*AAC ... 140
4. IMPROVEMENT OF ENZYME PERFORMANCES BASED ON THERMODYNAMICS ... 142
4.1. Entropy effect ... 142
4.2. Surface effect ... 143
4.3. Comparative study of recombinant acylases similar to *Sl*PVA and *Au*AAC .. 144
4.3.1. Penicillin G acylase from *Kluyvera citrophila* ATCC 21285 ... 145
4.3.2. Penicillin G acylase from *Escherichia coli* ATCC 11105 ... 146

4.3.3. Glutaryl acylase from *Pseudomonas* SY-77-1 147
4.3.4. Cephalosporin C acylase from *Pseudomonas* SE83 148
4.3.5. Cephalosporin C acylase from *Pseudomonas* N176 149
4.3.6. Cephalosporin C acylase from *Pseudomonas* sp. 130 151
4.3.7. Cephalosporin C acylase from *Pseudomonas diminuta* KAC-1 151
VI CONCLUSIONS 153
VII REFERENCES 157
VIII SUPPLEMENTARY MATERIAL 199
S.1. Acyl-homoserine lactones implicated in *QS* 201
S.2. Sequence of vector pENV19 207
S.3. Sequence of vector pENS 208
S.4. Nucleotide and amino acid sequences of *Sl*PVA in addition to p*ermE** 209
S.5. Nucleotide and amino acid sequences *Au*AAC in addition to of p*ermE** 211
S.6. Residues related with the performance of acylases 213
S.7. Chromophores employed to quantify primary amines 217
S.8. Relative coordinates between residues in *Sl*PVA, *Au*AAC and *Au*AHLA 218
S.9. Similar studies of directed evolution in other acylases 219
S.10. Alteration of the predicted solvent accessibility in recombinant acylases 222

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LIST OF ABBREVIATIONS

a.u.	Arbitrary units
aa	Amino acids
aac	Aculeacin A acylase-encoding gene
AAC	Aculeacin A acylase
AHL	*N*-Acyl-L-homoserine lactone
ahla	Acyl-homoserine lactone acylase-encoding gene
***Au*AAC**	Aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052
***Au*AHLA**	Acyl-homoserine lactone acylase from *Actinoplanes utahensis* NRRL 12052
Ap	Ampicillin
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDS	Coding DNA Sequence
cfu	Colony forming unit
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMBL-EBI	European Molecular Biology Laboratory - European Bioinformatic Institute
HPLC	High-performance liquid chromatography
HSL	L-Homoserine lactone
HTS	High-throughput screening
IU	International unit
k_{cat}	Catalytic constant
k_{cat}/K_M	Catalytic efficacy
K_M	Michaelis-Menten constant
Kn	Kanamycin
LB	Luria-Bertani Medium
Mb	Megabases
MCS	Multiple cloning site
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
nt	Nucleotide
NRPS	Non-ribosomal peptide synthetase
OPA	*o*-Phthalaldehyde
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
oxo-C_n-HSL	*N*-(3-oxo-alkanoyl)-L-homoserine lactone with n carbons in the side chain
PCR	Polymerase chain reaction
PDAB	*p*-Dimethylaminobenzaldehyde
p*ermE**	Promoter mutated of the erythromycin resistance gene from *Saccharopolyspora erithraea*
PGA	Penicillin G acylase
$PhaZ_{Sex}$	PHB depolymerase from *Streptomyces exfoliatus* DSMZ 41693
PHB	Poly(3-hydroxybutyrate)
PKS	Polyketide synthase

PPV	Positive predictive value
PV	Penicillin V
pva	Penicillin V acylase-encoding gene
PVA	Penicillin V acylase
QQ	*Quorum quenching*
QS	*Quorum sensing*
RBS	Ribosomal binding site
rRNA	Ribosomal ribonucleic acid
SDS-PAGE	Polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate
SFM	Soya flour mannitol agar
***Sl*PVA**	Penicillin V acylase from *Streptomyces lavendulae* ATCC 13664
SP	Signal peptide
TAE	Tris-Acetate-EDTA buffer
TE	Tris-EDTA buffer
TES	Tris-EDTA-Sucrose buffer
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
tRNA	Transfer ribonucleic acid
TSB	Tryptic Soy Broth
Tsr	Thiostrepton
UV	Ultraviolet
$\mathbf{V_{max}}$	Maximal velocity
YEME	Yeast extract malt extract supplemented with glycine
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
2xYT	Yeast extract tryptone medium
2xYT+G	Yeast extract tryptone medium supplemented with glucose
5’-MTA	5’-methylthioadenosine
6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
7-ADCA	7-aminodesacetoxycephalosporanic acid
8-oxodGTP	8-oxo-2’-deoxyguanosine-5’-triphosphate

LIST OF FIGURES

Figure 1. Most common natural and semisynthetic penicillins
Figure 2. Most common natural and semisynthetic cephalosporins
Figure 3. Biotechnological process to produce semisynthetic penicillins
Figure 4. Biotechnological production of the building-block of semisynthetic echinocandins
Figure 5. Semisynthetic echinocandins
Figure 6. Genetic organization of the *pac* gene in *E. coli* and the steps involved in the synthesis of penicillin G acylase
Figure 7. Crystal structures of β-lactam acylases shown in line ribbon representation
Figure 8. Catalytic mechanism proposed of penicillin G acylase from *E. coli*
Figure 9. Major *N*-acyl-L-homoserine lactones in *quorum sensing* from Gram-negative bacteria
Figure 10. *QQ* mechanism displayed by *V. fischeri* as an illustrative model
Figure 11. 3D-structure model of the β-subunit of *Sl*PVA obtained by Phyre2
Figure 12. General scheme of directed evolution
Figure 13. General scheme of directed evolution by a mutator strain
Figure 14. General scheme of directed evolution by error-prone PCR
Figure 15. General scheme of directed evolution by DNA shuffling
Figure 16. General scheme of directed evolution by gene assembling mutagenesis
Figure 17. Scheme to obtain recombinant plasmids by *E. coli* XL1-Red
Figure 18. Tecan Freedom EVO 75 workstation employed in the protocol of enzymatic activity determination of the mutant clones of *Sl*PVA and *Au*AAC
Figure 19. Construction of shuttle vector pENS and pENV19 from pNV19 and pEM4
Figure 20. Phylogenetic tree of *S. lavendulae* ATCC 13664 elaborated from the sequence of 16S rRNA employing BlastN as the alignment tool
Figure 21. Phylogenetic tree of *S. lavendulae* ATCC 13664 elaborated from whole genome sequence employing JSpecies as the alignment tool
Figure 22. Representative coding sequences upstream and downstream of the gene of penicillin V acylase from *Streptomyces* sp.
Figure 23. Distribution of subsystem categories in *Streptomyces* sp. according to RAST
Figure 24. Phylogenetic tree of *A. utahensis* NRRL 12052 elaborated from the sequence of 16S rRNA employing BlastN as the alignment tool
Figure 25. Clusters 3 and 5 within a fragment of the contig 8 containing each one acylases genes, and the rough prediction of core scaffolds produced by those clusters based on assumed PKS/NRPS collinearity without considering tailoring reactions
Figure 26. Predicted signal peptide of *Au*AHLA
Figure 27. Amino acid and nucleotide sequences of *Au*AHLA
Figure 28. Representative coding sequences upstream and downstream of the gene of aculeacin A acylase from *A. utahensis* NRRL 12052
Figure 29. Distribution of subsystem categories in *A. utahensis* NRRL 12052 according to RAST
Figure 30. Nonlinear hyperbolic regression of the activity at different concentrations of several AHLs by *Sl*PVA at 45ºC and pH 8.0
Figure 31. Nonlinear hyperbolic regression of the activity at different concentrations of several AHLs by *Au*AAC at 45ºC and pH 8.0

Figure 32. Penicillin V acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 33. C_6-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 34. C_8-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 35. C_{10}-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 36. oxo-C_6-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 37. oxo-C_8-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 38. oxo-C_{10}-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■)
Figure 39. Penicillin V acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 40. C_6-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 41. C_8-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 42. C_{10}-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 43. oxo-C_6-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 44. oxo-C_8-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 45. oxo-C_{10}-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■)
Figure 46. Acylase activity of mutant clones from *Sl*PVA and *Au*AAC at different operational conditions and substrates
Figure 47. Plots of predicted signal peptide and cleavage site
Figure 48. Chromatographic profile in *Au*AAC purification
Figure 49. Amino acid alignment of the α- and β-subunit from *Sl*PVA and *Au*AAC, and their recombinant proteins
Figure 50. Predicted templates employed by several services to model 3D-structures of *Sl*PVA, *Au*AAC, their recombinant proteins and *Au*AHLA
Figure 51. Prediction of 3D-structure of *Sl*PVA
Figure 52. Prediction of 3D-structure of *Au*AAC
Figure 53. Prediction of 3D-structure of *Au*AHLA
Figure 54. Prediction of 3D-structure of the recombinant clone of *Sl*PVA named 2pva1,21(25)
Figure 55. Prediction of 3D-structure of the recombinant clone of *Sl*PVA named 2pva1,2(55)
Figure 56. Prediction of 3D-structure of the recombinant clone of *Sl*PVA named 2pva1,2(149)
Figure 57. Prediction of 3D-structure of the recombinant clone of *Au*AAC named a(292)
Figure 58. Comparative study of *Sl*PVA, *Au*AAC and *Au*AHLA with others acylases
Figure 59. Superposition of the predicted catalytic pocket of *Sl*PVA, *Au*AAC and *Au*AHLA

Figure S.2.1. Shuttle vector pENV19
Figure S.3.1. Shuttle vector pENS
Figure S.4.1. Nucleotide and amino acid sequences of *Sl*PVA in addition to p*ermE** sequence
Figure S.5.1. Nucleotide and amino acid sequences of *Au*AAC in addition to p*ermE** sequence
Figure S.7.1. Reactions involved in the quantification of primary amines

LIST OF TABLES

Table 1. Main classes of antifungals and their action mechanisms and implications for their efficacy
Table 2. Core structure of common natural echinocandins
Table 3. Substrate specificity of several β-lactam acylases and other related amidohydrolases
Table 4. Alternative methodologies to introduce variations in gene sequences by directed molecular evolution
Table 5. Bacterial strains
Table 6. Vectors
Table 7. Primers employed in DNA sequencing
Table 8. Clusters predicted by antiSMASH within the genome of *S. lavendulae* ATCC 13664
Table 9. Clusters predicted by antiSMASH within the genome of *A. utahensis* NRRL 12052
Table 10. Apparent kinetic parameters and specific parameters of *Sl*PVA
Table 11. Apparent kinetic parameters and specific parameters of *Au*AAC
Table 12. Apparent kinetic parameters of parental *Sl*PVA and *Au*AAC assayed on different natural penicillins at optimal conditions
Table 13. Library of obtained mutant clones from *Sl*PVA and *Au*AAC by directed evolution with acylase activities in the first and second screening
Table 14. Set of mutant clones from *Sl*PVA and *Au*AAC available to catalyze different substrates
Table 15. Best fit of statistic models to the experimental results with each substrate
Table 16. Excess kurtosis and skewness from each operational condition
Table 17. Predicted signal peptide and cleavage parameters of *Sl*PVA, *Au*AAC and $PhaZ_{Sex}$
Table 18. Modifications throughout sequences in selected mutant clones
Table 19. Templates for 3D-structure prediction of *Sl*PVA, *Au*AAC, their recombinant proteins and *Au*AHLA
Table 20. Predicted ligand binding residues
Table 21. Characteristics of the residues involved in the binding pocket from *Sl*PVA, *Au*AAC and *Au*AHLA
Table 22. Distances in Angstroms between amino acids in 2pva1,21(25) according to the I-Tasser model
Table 23. Distances in Angstroms between amino acids in 2pva1,2(55) according to the I-Tasser model
Table 24. Distances in Angstroms between amino acids in 2pva1,2(149) according to the I-Tasser model
Table 25. Distances in Angstroms between amino acids in a(292) according to the I-Tasser model
Table 26. Characteristics of the residues modified in the recombinant clones
Table 27. Location of the last catalytic residue of the β-subunit in acylases
Table 28. Spacer peptides reported in several acylases
Table S.6.1. Predicted and reported residues involved in the catalytic pocket of acylases
Table S.8.1. Distances in Angstroms between amino acids in *Sl*PVA according to the I-Tasser model

Table S.8.2. Distances in Angstroms between amino acids in *Au*AAC according to the I-Tasser model
Table S.8.3. Distances in Angstroms between amino acids in *Au*AHLA according to the I-Tasser model
Table S.9.1. Spatial distribution of residues related with the performance in some acylases
Table S.10.1. Alteration of the predicted solvent accessibility in recombinant acylases

LIST OF PUBLICATIONS OBTAINED DURING THIS STUDY

Velasco-Bucheli, R.; del Cerro, C.; Hormigo, D.; Acebal, C.; Arroyo, M.; García, J. L.; de la Mata, I. (2015) "Draft genome sequence of *Actinoplanes utahensis* NRRL 12052, a microorganism involved in industrial production of pharmaceutical intermediates". *Genome Announc*., **3**(1): e01411-14.

Velasco-Bucheli, R.; Hormigo, D.; del Cerro, C.; Acebal, C.; Arroyo, M.; García, J. L.; de la Mata, I. "New *N*-acyl-homoserine lactone acylase activities of penicillin V acylase from *Streptomyces lavendulae* and aculeacin A acylase from *Actinoplanes utahensis* and their implication in *quorum quenching*". (*forthcoming*).

Velasco-Bucheli, R.; Hormigo, D.; García-Hidalgo, J.; Acebal, C.; Arroyo, M.; de la Mata, I. "Ultimate shuttle vector for extracellular overexpression of Gram-positive proteins in *Rhodococcus* sp." (*forthcoming*).

Velasco-Bucheli, R; Hormigo, D; Acebal, C; Arroyo, M; de la Mata, I. "Improved and new biocatalytic activities of two acylases by coupling of directed molecular evolution techniques and high-throughput screening". (*forthcoming*).

THESIS ABSTRACT (English)

Nowadays sequencing and consequent deposit in public data bases of bacterial genomes have been increased exponentially and constitutes an inevitable tool in basic and applied research. However, the elucidation of the encrypted information along their coding sequences and the particularities of each microorganism are barriers to be overcome by the researcher. Thus, bioinformatic studies integrated with experimental evidences are inescapably addressed in the laboratory. In particular, it is important to mention the versatility that holds the Gram-positive bacteria and its implications that transcends natural environments and interferes time after time in biotechnological processes. For this reason, in this study the genomes of the bacterial strains *Streptomyces lavendulae* ATCC 13664 and *Actinoplanes utahensis* NRRL 12052 were sequenced, and thanks to this information, it was possible to determine several features from those microorganisms. In this sense, the analysis of the 16S rRNA sequence as well as the comparison of the whole genome against a local database of genomes suggests that the strain of *S. lavendulae* is misassigned and should be assigned as a new specie, because despite the fact that it was detected phylogenetically close to other strains of *S. lavendulae*, it was located closer to other *S. griseus* species.

Likewise, within the genome of *A. utahensis* highlights the presence of acyl-homoserine lactone acylase (*Au*AHLA), which is reported here for the first time. The bioinformatic analyses developed emphasizes that this enzyme had similar characteristics with respect to aculeacin A acylase (*Au*AAC) from *A. utahensis* and penicillin V acylase (*Sl*PVA) from *S. lavendulae*. Surprisingly, it is noteworthy to mention that the transmembrane echinocandin B (ECB) deacylase was not detected within the genome of *A. utahensis*. Information about ECB deacylase reported by other authors and its sequence differs slightly with respect to *Au*AAC. Although the sequence of ECB deacylase has not been deposited, the authors reported the amino-terminus of each subunit. Thus, the present study suggests that this ECB deacylase should be reassigned. Likewise, it is important to mention that in both genomes clusters related with the biosynthesis of NRPS (non-ribosomal peptide-synthase) and PKS (polyketide synthase) were detected. Specifically, both *Au*AAC as *Au*AHLA were located within a cluster associated with the biosynthesis of siderophores (*i.e.* predicted gobichelin and laspartomycin, respectively). These molecules are employed by the bacteria as iron chelating compounds, and humans use their biological activity. In contrast, although the platform employed did not predict any cluster containing *Sl*PVA, further studies might indicate that *Sl*PVA could be implicated in the biosynthesis of some siderophore, similarly to that exposed with the acylases from *A. utahensis*.

On the other hand, the capacities of *Sl*PVA and *Au*AAC to hydrolyze natural β-lactamic antibiotics, echinocandins, *N*-acyl-homoserine lactones, among other molecules with amide bond have been previously determined and widely reported. Both enzymes have properties that make them suitable for pharmaceutical industrial applications in the production of β-lactam antibiotics and semisynthetic antifungics. In fact, thanks to the capability to hydrolyze effectively several *N*-acyl-homoserine lactones (AHLs), both enzymes have been postulated to be employed in antimicrobial therapy by *quorum quenching* processes or interruption of *quorum sensing*, which is a complex intercellular communication that many bacteria use to regulate several biological functions. This communication between microorganisms is carried out by molecular signaling, and is based on the production, diffusion and recognition of small molecules, being AHLs the

most widely studied in Gram-negative bacteria. Both academic and industrial sectors have been interested in the capacity of these bacteria. They are capable of fighting against pathogenic microorganisms and/or against those that could affect biotechnological processes. In addition, their versatility to hydrolyze several molecules is remarkable. In the best scenario the activity, selectivity and stability of these enzymes could be improved. They could then be employed in industrial bioprocesses, such as the production of building blocks and intermediaries (*e.g.* 6-aminopenicillanic acid), the synthesis of new antibiotics or their direct use as antimicrobial agents. Thus, directed molecular evolution or *in vitro* enzyme evolution combined with high-throughput screening (HTS) to analyze mutants emerges as a biotechnological technique to obtain improved enzymes with new characteristics. These include enhanced activity, selectivity and stability at different temperature or pH values, substrate specificity, apart from gaining a mutant library available to their application in different processes and conditions.

For the development of this HTS study, mutants from both *Sl*PVA and *Au*AAC were obtained initially by employing the mutator strain *Escherichia coli* XL-1 Red, which introduces low rate of random mutations along the sequence. This collection of mutants was submitted to a screening with the help of a robotic platform supplied by Tecan. Hence, we evaluated the acylase activity of more than 2,000 recombinant clones of each enzyme against penicillin V, as well as the acylase activity of about 1000 of those clones against several AHLs with aliphatic and β-keto substituted aliphatic side-chains of different length (*i.e.* C_6-HSL, C_8-HSL, C_{10}-HSL, oxo-C_6-HSL, oxo-C_8-HSL and oxo-C_{10}-HSL). The directed molecular evolution was carried out by assays performed at two temperatures (30ºC and 45ºC) and at two pH values (6.0 and 8.0), and under each scenario each protein was tested employing each substrate. In this sense, 3993 recombinant clones able to express both acylases were evaluated under 56 possible scenarios, generating almost 210,000 colorimetric assays, which were divided in more than 190,000 tests in a preliminary screening to detect improved activities, and almost 27,000 tests in a second screening with the aim to corroborate those clones with improved characteristics known as hits. During the corroboration protocol four replicates for each recombinant clone were performed. Due to this screening and employing a statistical criterion of the Anderson-Darling test a collection of 158 mutant clones from both enzymes were finally obtained, and 14 of them had several improved activities. The Anderson-Darling test gives more weight to the tails of the distribution of samples than to the spots located in the middle of the Gauss bell. Further analyses of the results have been performed by statistical and bioinformatic tools, which demonstrate that the proposed methodology in the exposed HTS constitutes a successful means to detect improved acylase activities.

Thereafter, the analysis of the sequences and the spatial distribution of the residues in those hits detected the mutations took place in the residues located on the surface of the protein. In this sense, four of these mutant clones were selected to further analysis, since they showed a wide variety of mutations along their sequence. Thus, two of those recombinant clones from *Sl*PVA, named 2pva1,21(25) and 2pva1,2(149), displayed mutations in the β-subunit (*i.e.* βR373W) and in α-subunit (*i.e.* αA169T, in addition to a silent mutation), respectively. However, it is important to mention that the recombinant clone named 2pva,2(55) displayed a deletion at the end of the β-subunit (*i.e.* ΔG1215), which generates the modification of the open reading frame and the truncation of the length of the enzyme. It must be pointed out that in this recombinant clone only one residue was altered among those implied in the catalysis (*i.e.* βAsn^{272}), and the new amino acid that substitutes this function (*i.e.* βArg^{236}), is almost located at the same distance as the original residue. The

validity of this argument was corroborated by the alignment of both *Sl*PVA and *Au*AAC sequences with 174 sequences of acylases. Additionally, the analysis of the recombinant clone of *Au*AAC named a(292), displayed a mutation close to the spacer peptide (*i.e.* αI184F). This leaves again the door open for the discussion previously exposed by other authors about the role of this fragment of the sequence during enzyme maturation, as well as the effect on folding, stability and activity of the acylase.

Likewise, just as mentioned above, the alignment was performed with 174 available sequences of acylases, which allowed determining that those mutations were located in residues with high entropic values. The contact with the solvent was then altered, a fact that is likely due to the properties of the amino acid side-chain (*e.g.* βR373W and αA169T in *Sl*PVA), to the particular location of the mutations, and their steric hindrance (*e.g.* βV204F in *Sl*PVA and αI184F in *Au*AAC), among other options. Similar analyses were carried out with seven acylases that show similar features to *Sl*PVA and *Au*AAC, and were subjected to mutation processes equivalent to those exposed in this study, such as penicillin G acylases from *Kluyvera citrophila* ATCC 21285 and *Escherichia coli* ATCC 11105, glutaryl acylase from *Pseudomonas* SY-77-1, and cephalosporin acylases from *Pseudomonas* SE83, *Pseudomonas* N176 and *P. diminuta* KAC-1. As a consequence, almost every mutation described in these acylases displays residues on the enzyme surface with medium to high entropy values, whereas some of those mutated amino acids were located in the catalytic pocket as expected (*i.e.* a conserved residue implies a low entropy value). Similarly to that shown by *Sl*PVA and *Au*AAC, these particular modifications altered the spatial disposition of those amino acids, as deduced from the changes observed in the closest distance between the active groups of the catalytic residues and the hydroxyl of βSer^1 (*i.e.* main catalytic residue), which in turn means the location with respect to the substrate. In the same manner, a detailed study of the residues located on the surface of *Sl*PVA, *Au*AAC, the above-mentioned seven acylases, as well as their recombinant clones allowed to make out the extension of the alteration on the surface of the enzymes, and their interactions with the solvent. This was evidenced by the reduction or the increment in the exposure of each amino acid to the solvent, and even by the change in the orientation of some residues with respect to the solvent.

Equally, further bioinformatic analysis permitted the study of the structure-function relationship in these enzymes, which demonstrated the importance of the residues located in the surface of the acylases and their apparent roles. This information was correlated with statistical values (*i.e.* abundance of residues and the representativity of each cluster of them according to the side-chain), in addition to thermodynamic interpretation (*i.e.* entropy), with the aim of informing about their importance on protein stability, selectivity and activity. However, because most of the studies report information about the conservation of the residues (*e.g.* catalytic pocket and substrate binding pocket), further studies about thermodynamic parameters are essential to clarify the role of the surface in enzyme performance.

Finally, it is noteworthy to mention that the study developed and the results obtained with the solution of *o*-phthalaldehyde in order to quantify the kinetic parameters of the parental enzymes and their recombinant clones were crucial to estimate their activity against AHLs with acyl side-chains of different length. It is important to highlight that this is the first successful report about kinetic parameters from acylases in the catalysis of several AHLs, which in turn avoids the drawbacks related to the solubility of the substrates that has been reported by other authors. In this sense, the experimental design helped to determine the

biochemical constants from both enzymes and their recombinant clones by fluorometric assays, which was possible by a fast and easy methodology that could be employed with high reliability in the quantification of primary amines in solution.

RESUMEN DE LA TESIS (Spanish)

Actualmente la secuenciación y el consecuente depósito en bases de datos públicas de genomas bacterianos han incrementado de manera exponencial y constituye una herramienta inevitable en la investigación básica y aplicada. Sin embargo, la elucidación de la información encriptada en sus secuencias codificantes aunado a las particularidades de cada microorganismo, constituyen las barreras a ser superadas por parte de los investigadores, para lo cual estudios bioinformáticos integrados con evidencias experimentales son ineludibles de abordar en el laboratorio. En particular, es menester reconocer la versatilidad que ostentan las bacterias Gram-positivas y sus implicaciones que trascienden los entornos naturales y se inmiscuyen cada vez más en procesos biotecnológicos. Por tal motivo, en el presente estudio se secuenciaron los genomas de las cepas bacterianas *Streptomyces lavendulae* ATCC 13664 y *Actinoplanes utahensis* NRRL 12052, gracias a lo cual se logró determinar múltiples características de dichos microorganismos. En este sentido, el presente estudio logró determinar con base en la secuencia del 16S rRNA, al igual que con fundamento en una comparación de todo el genoma frente a una base de datos local de genomas, que la cepa de *S. lavendulae* se encuentra mal asignada y por lo tanto debería ser reasignada como una nueva especie, dado que fue detectada filogenéticamente cerca a otras especies de *S. lavendulae*, y en contraste dicha cepa se localiza aún más cerca de otras especies de *S. griseus*.

Igualmente, dentro del genoma de *A. utahensis* resalta la detección de una acil-homoserin lactona acilasa (*Au*AHLA) putativa, la cual es documentada por primera vez en este estudio. Los análisis bioinformáticos desarrollados destacaron que dicha enzima presenta características similares a la aculeacin A acilasa (*Au*AAC) de *A. utahensis* y a la penicilina V acilasa (*Sl*PVA) de *S. lavendulae*. Igualmente, cabe mencionar que no fue detectada la equinocandina B (ECB) deacilasa transmembrana dentro del genoma de *A. utahensis*, la cual se había descrito previamente por otros autores y que solo difiere ligeramente en su secuencia con respecto a *Au*AAC (aunque no se ha depositado la secuencia completa de la ECB deacilasa, si se ha informado sobre fragmentos del amino-terminal de cada subunidad), lo cual permite proponer que la ECB deacilasa debe ser reasignada. Asimismo, es de resaltar que en los dos microorganismos secuenciados fueron detectados clúster relacionados con la biosíntesis de NRPS (de su sigla en inglés *non-ribosomal peptide-synthase*) y PKS (de su sigla en inglés *polyketide synthase*). Específicamente, tanto *Au*AAC como *Au*AHLA fueron localizadas dentro de clústeres relacionados con la biosíntesis de sideróforos (*i.e.* gobichelina y laspartomicina, respectivamente según la predicción realizada), moléculas que son empleadas por las bacterias como compuestos quelantes del hierro, y que los seres humanos aprovechan gracias a su actividad biológica. En contraste, a pesar de que la plataforma empleada no predijo ningún clúster que contenga *Sl*PVA, estudios adicionales permitieron que el presente estudio no descarte que *Sl*PVA esté implicada en la biosíntesis de algún sideróforo, tal y como fue el caso de las acilasas de *A. utahensis*.

Por otro lado, previamente se ha determinado y difundido ampliamente acerca de las capacidades que poseen las enzimas *Sl*PVA y *Au*AAC para hidrolizar antibióticos β-lactámicos naturales, equinocandinas, *N*-acil-homoserin lactonas, entre otras moléculas que contienen un enlace amida. Las dos enzimas presentan una serie de propiedades que las hacen muy adecuadas para su empleo en la industria farmacéutica en la producción de antibióticos β-lactámicos y antifúngicos semisintéticos. De hecho, gracias a esa capacidad

para hidrolizar eficientemente varias *N*-acil-homoserin lactonas (AHLs), se ha postulado que las dos acilasas pueden utilizarse en terapia antimicrobiana mediante su implicación en procesos *quórum quenching* o interrupción del *quórum sensing*, el cual es un complejo proceso de comunicación intercelular que numerosas bacterias utilizan para regular una gran variedad de funciones biológicas. Esta comunicación celular bacteriana se lleva a cabo mediante señalización molecular basada en la producción, difusión y reconocimiento de pequeñas moléculas, siendo el caso de las AHLs el sistema más ampliamente estudiado en bacterias Gram-negativas. En este sentido, esa capacidad que poseen estas bacterias para combatir contra microorganismos patógenos y/o que afectan procesos biotecnológicos, además de su versatilidad para hidrolizar diversas moléculas, respalda el interés para ser empleadas a nivel industrial y académico, y en el mejor de los casos mejorar la actividad, selectividad y estabilidad, en aras de ser empleadas en bioprocesos industriales para la obtención de precursores e intermediarios (*e.g.* ácido 6-aminopenicilánico), en la síntesis de nuevos antibióticos, así como en su utilización directa como agentes antimicrobianos. Así, la evolución molecular dirigida o evolución *in vitro* de enzimas, junto al análisis de los mutantes mediante la técnica de cribado de alto rendimiento (HTS, de su sigla en inglés *High-Throughput Screening*) surgen como una potente técnica biotecnológica para obtener enzimas con mejores y nuevas características, como es el caso de potenciar la actividad, la estabilidad y la selectividad a valores de pH y temperaturas diferentes, especificidades de sustrato, así como conseguir una colección de mutantes disponible, para ser implementados en distintos procesos y condiciones.

En aras del desarrollo de este estudio de HTS, inicialmente se obtuvieron diferentes mutantes tanto de *Sl*PVA como de *Au*AAC empleando la cepa mutagénica *Escherichia coli* XL-1 Red, la cual introduce bajo número de mutaciones aleatorias a lo largo de la secuencia; dicha librería fue sometida a un cribado con la ayuda de una plataforma robotizada de Tecan. De este modo, se logró evaluar la actividad acilasa de cerca de 2,000 clones recombinantes con cada enzima en contra de penicilina V, así como la actividad acilasa de alrededor de 1,000 de esos clones contra diversas AHLs con cadenas laterales alifáticas y β-ceto sustituidas de diferente longitud (*i.e.* C_6-HSL, C_8-HSL, C_{10}-HSL, oxo-C_6-HSL, oxo-C_8-HSL y oxo-C_{10}-HSL). La evolución molecular dirigida se efectuó mediante ensayos llevados a cabo a dos temperaturas (30ºC y 45ºC) y a dos valores de pH (6.00 y 8.00), escenarios utilizados con cada una de las proteínas y empleando cada uno de los sustratos. En este sentido, 3993 clones recombinantes capaces de expresar las dos acilasas fueron evaluados en los 56 escenarios posibles, de tal manera que fueron generados aproximadamente 210,000 ensayos colorimétricos, divididos en más de 190,000 ensayos en un cribado preliminar para la detección de actividades mejoradas, y alrededor de 27,000 ensayos en una segunda criba con el objetivo de corroborar dichos clones con características mejoradas (conocidos en inglés como *hits*), para lo cual fue necesario realizar pruebas con cuatro réplicas por cada clon recombinante. Gracias a este cribado y empleando un criterio estadístico de Anderson-Darling, que da mayor peso estadístico a las colas de las distribuciones de puntos sobre los valores localizados en la mitad de la campana de Gauss, fue posible conseguir una colección de 158 clones mutantes de las dos enzimas, y 14 de ellos poseen múltiple actividades mejoradas. Análisis adicionales de los resultados se han efectuado mediante herramientas estadísticas y bioinformáticas, lo cual demuestra que la metodología propuesta en el presente HTS reviste un éxito para la detección de actividades acilasas mejoradas.

Posteriormente, el análisis de las secuencias y la distribución espacial de los residuos en los clones recombinantes más importantes acorde al criterio estadístico del presente

estudio, permitieron detectar que las mutaciones se efectuaron en residuos localizados en la superficie de las proteínas. En este sentido, se seleccionaron cuatro clones mutantes para posteriores análisis, los cuales muestran una gran variedad de mutaciones a lo largo de su secuencia. Así, dos de los clones recombinantes de *Sl*PVA, denominados 2pva1,21(25) y 2pva1,2(149), presentaron mutaciones en la subunidad β (*i.e.* βR373W) y en la subunidad α (*i.e.* αA169T, además de una mutación silenciosa), respectivamente. Sin embargo, es de destacar que otro de sus clones recombinantes, denominado 2pva,2(55), presentó una deleción al final de la subunidad β (*i.e.* ΔG1215), lo cual generó que se modificara parte de su marco de lectura y se truncara la longitud de la enzima. Es de resaltar que este clon recombinante solamente alteró uno de sus residuos implicados en la catálisis (*i.e.* βAsn^{272}), y ahora un nuevo amino ácido sustituye dicha función (*i.e.* βArg^{236}), el cual se encuentra localizado prácticamente a la misma distancia que se encontraba el residuo original; dicho postulado fue contrastado con el alineamiento con 174 secuencia de acilasas en aras de comprobar la validez de dicho argumento. Adicionalmente, se analizó un clon recombinante de *Au*AAC, denominado a(292), en el que se detectó una mutación cercana al péptido espaciador (*i.e.* αI184F), lo cual abre la puerta una vez más a la discusión previamente expuesta por otros autores acerca del papel de este fragmento de la secuencia en la maduración de la enzima, así como su efecto en el posterior plegamiento, estabilidad y actividad de la acilasa.

Asimismo, tal y como se mencionó previamente, fue llevado a cabo un alineamiento de secuencias con 174 acilasas disponibles en las bases de datos, lo cual permitió determinar que dichas mutaciones estaban localizados en residuos con alto valor entrópico y que su vez alteraron el contacto con el solvente, lo cual es debido probablemente a las propiedades de la cadena lateral de los residuos (*e.g.* βR373W y αA169T en *Sl*PVA), a la ubicación particular de las mutaciones y su impedimento estérico (*e.g.* βV204F en *Sl*PVA y αI184F en *Au*AAC), entre otras opciones. Este tipo de análisis se ha realizado de forma similar con siete acilasas que ostentan características análogas a *Sl*PVA y *Au*AAC, y que han sido sometidas a procesos de mutaciones equivalentes a los expuestos en el presente estudio, tal es el caso de las penicilina G acilasas de *Kluyvera citrophila* ATCC 21285 y *Escherichia coli* ATCC 11105, la glutaril acilasa de *Pseudomonas* SY-77-1, y las cefalosporina acilasas de *Pseudomonas* SE83, *Pseudomonas* N176 y *P. diminuta* KAC-1. De este modo, prácticamente todas las mutaciones descritas en dichas acilasas muestran residuos con valor de entropía medio a alto en la superficie de las enzimas, y como era de esperar algunos de esos amino ácidos mutados fueron localizados dentro del bolsillo catalítico (*i.e.* residuos más conservados implican menores valores de entropía). De modo análogo a lo evidenciado en el caso de *Sl*PVA y *Au*AAC, dichas modificaciones puntuales alteraron la disposición espacial de todos los amino ácidos, la cual fue deducida a partir de las modificaciones en la distancia más cercana entre los grupos activos de los residuos implicados en la catálisis en relación al grupo hidroxilo de βSer^{1} (*i.e.* principal residuo catalítico), lo cual a su vez significa su localización respecto al sustrato. Asimismo, un estudio detallado de los residuos localizados en la superficie de *Sl*PVA, *Au*AAC, y de las siete acilasas comparadas, al igual que sus respectivos clones recombinantes, permitió vislumbrar la extensión de la alteración en la superficie de las enzimas y su interacción con el solvente, lo cual fue evidenciado mediante la reducción o el incremento del porcentaje de exposición al solvente de cada amino ácido, e incluso por el cambio de orientación de algunos residuos con respecto al disolvente.

Igualmente, análisis bioinformáticos adicionales permitieron estudiar la relación estructura-función en estas enzimas, lo cual demostró la importancia de los residuos

localizados en la superficie de las acilasas y los posibles papeles que ellos desempeñan. Esta información fue correlacionada con valores estadísticos (*i.e.* abundancia de los residuos y la representatividad de cada uno de sus clúster de acuerdo a las propiedades de la cadena lateral), al igual que con interpretación termodinámica (*i.e.* entropía), con el objetivo de informar la importancia acerca de la estabilidad, selectividad y la actividad de estas proteínas. Sin embargo, dado que la mayoría de los estudios reportan información acerca de residuos conservados (*e.g.* bolsillo catalítico y sitio de unión del sustrato), son esenciales estudios adicionales acerca de parámetros termodinámicos para clarificar el papel de la superficie en el desempeño de las acilasas.

Finalmente, es de destacar el estudio desarrollado y los resultados obtenidos con la solución de *o*-ftalaldehído para la cuantificación de los parámetros cinéticos de las enzimas parentales y de sus clones recombinantes, la cual fue crucial para estimar su actividad empleando AHLs con cadenas acilo de distinta longitud como sustratos. Es importante mencionar que este es el primer informe exitoso sobre parámetros cinéticos de acilasas catalizando varias AHLs y que evita los problemas implícitos de solubilidad de los sustratos que ha sido descrito por otros autores. En este sentido, el diseño experimental ayudó a determinar las constantes bioquímicas de ambas enzimas y sus clones recombinantes mediantes ensayos fluorimétricos, empleando una metodología rápida y fácil, la cual puede ser empleada en la cuantificación de aminas primarias en solución con alta fiabilidad.

I INTRODUCTION

In spite of the efforts in searching new and powerful antibiotics and antifungals, pathogenic microorganisms develop new resistance systems against antimicrobial agents. In addition, many of those new compounds cannot be employed due to their toxicity and/or allergenic capacity. Likewise, the lack of new molecules during last years is another drawback to control infectious diseases. Particularly, the scenario of fungal infections is complicated because only a few antimicotic compounds are available on the market. In this sense, echinocandins are the most powerful antifungals, despite the fact that there are only three drugs available. Therefore, it is imperative to have as many of antifungals of this kind as possible.

Recently, the WHO report 2014 (World Health Organization 2014) warned about the problem of resistance displayed by pathogenic microorganisms against antimicrobials, which make difficult the control of severe common infections They indicated that this is an actual threat nowadays for public health nowadays. Although the estimated sales of antibiotics reached 42,000 millions of dollars in 2009 (Hamad, B. 2010), the trend has lagged during the last years, with long periods of time without obtaining new effective molecules (Brogan, D. M. *et al.* 2013). In this sense, the WHO has called for more resources and efforts in R&D to search and develop new antibiotic and antifungal compounds as well as antimicrobial agents (World Health Organization 2014).

1. β-LACTAM ANTIBIOTICS

Since the discovery of the first antibiotic penicillin by Sir Alexander Fleming, β-lactam antibiotics (both penicillins and cephalosporins) are considered the most employed antimicrobials worldwide (Hamad, B. 2010; Brogan, D. M. *et al.* 2013). Some of the main penicillins are summarized in Figure 1, whereas Figure 2 listed some of the most well-known echinocandins (Russell, A. D. 2004). Despite the huge number of this kind of compounds, there is a clamour for new molecules as well as biocatalytic processes for their production (Marešová, H. *et al.* 2014).

The pharmacological use of these drugs has been widely extended thanks to the possibility to prepare semisynthetic penicillins and cephalosporins from those natural penicillins and cephalosporins achieved by fermentation processes (*e.g.* penicillin G or V, and cephalosporin C).

High consumption of β-lactam antibiotics has lead to the accelerated emergence of bacteria that are resistant to the action of these drugs. The most common mode of bacterial resistance to β-lactams is the presence of β-lactamases, which hydrolyses the β-lactam ring. Up to now, several hundreds of such β-lactamases have been identified, threatening seriously the continued use of all β-lactam antibiotics. Plasmid-encoded β-lactamases especially contribute to bacterial resistance spreading as plasmids can easily be transferred to other bacteria and to other species. In addition, point mutations of such β-lactamases may occur so rapidly that bacteria obtain resistance to new β-lactam antibiotics. Currently, semisynthetic β-lactam antibiotics have been introduced in response to the evolution of β-lactamases and to the demand for antibiotics with broader antibacterial spectrum and/or improved pharmacological properties. Up to now, several hundreds of such β-lactamases have been identified, threatening seriously the continued use of all β-lactam antibiotics. Plasmid-encoded β-lactamases especially contribute to bacterial resistance spreading as plasmids can easily be transferred to other bacteria and to other species. In addition, point mutations of such β-lactamases may occur so rapidly that bacteria obtain resistance to new

β-lactam antibiotics. Currently, semisynthetic β-lactam antibiotics have been introduced in response to the evolution of β-lactamases and to the demand for antibiotics with broader antibacterial spectrum and/or improved pharmacological properties.

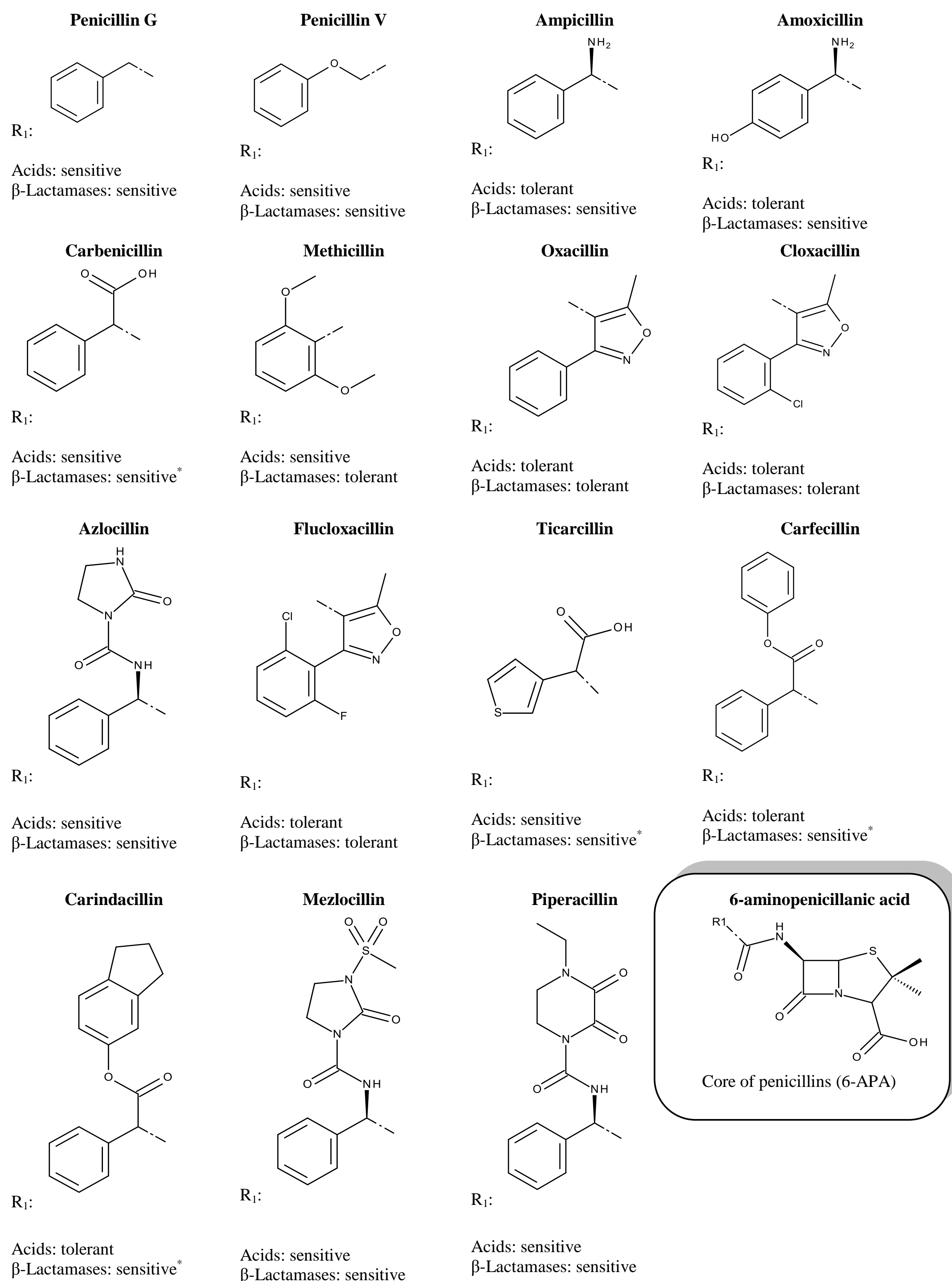


Figure 1. Most common natural and semisynthetic penicillins. * Stability to β-lactamases from Gram-negative bacteria but not against β-lactamases from *Staphylococcus aureus*

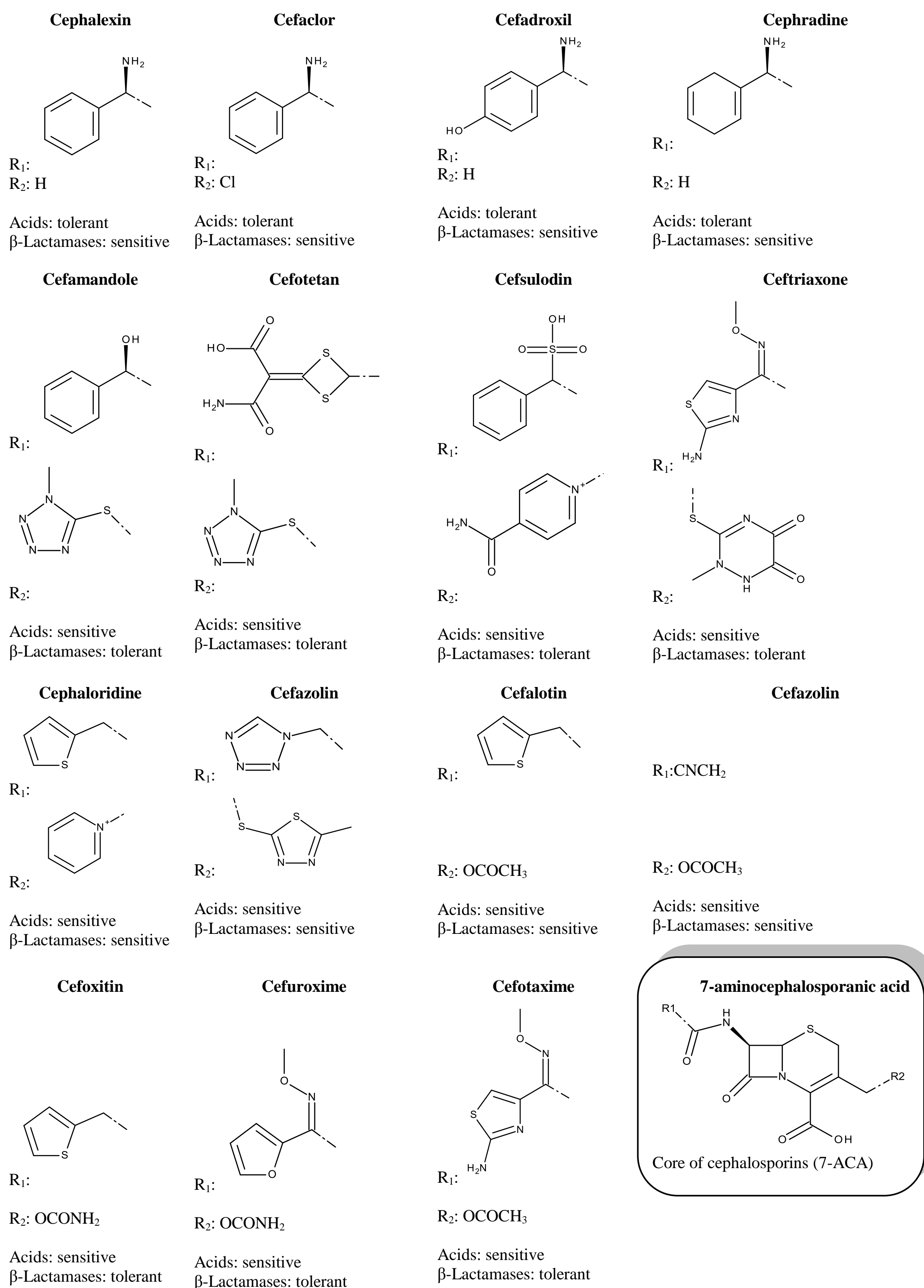


Figure 2. Most common natural and semisynthetic cephalosporins

β-Lactam antibiotics are chemically characterized by the presence of a common four-membered heterocyclic ring, so-called β-lactam nucleus, which is responsible of their antibacterial activity. The β-lactam ring is fused to a five-membered thiazolidine ring in

penicillins and to a six-membered dihydrothiazine ring in cephalosporins. β-Lactam antibiotics exert their antibacterial action by inactivating D-Ala-D-Ala-transpeptidases (penicillin-binding proteins, PBPs) which are membrane-bound enzymes involved in the biosynthesis of the bacterial cell wall (Walsh, C. 2000; Ritter, T. K. *et al.* 2001; Arroyo, M. *et al.* 2005; Sauvage, E. *et al.* 2008).

2. ECHINOCANDINS, STATE OF THE ART OF ANTIFUNGALS

With regard to antifungals, the main classes of drugs comprises of polyenes (*e.g.* amphotericin B), azoles (*e.g.* itraconazole, fluconazole, voriconazole, and the new posaconazole, among others), as well as echinocandins (Table 1).

Table 1. Main classes of antifungals and their action mechanisms and implications for their efficacy. Taken and modified from (Diomedi P., A. 2004)

	Target	**Activity**	**Clinical implications**
Polyenes	Membrane	Interaction with ergosterol, causing cell death	Potent antifungal activity and broad spectrum
Azoles	Membrane	Synthesis of ergosterol is avoided by the inhibition of CYP450, which damages the citoplasmatic membrane	Potent antifungal activity and variable spectrum
Echinocandins	Cell wall	Fungal lysis is caused due to inhibition of the synthesis of glucan	Potent antifungal activity and broad spectrum. Potentially synergic in combined therapies

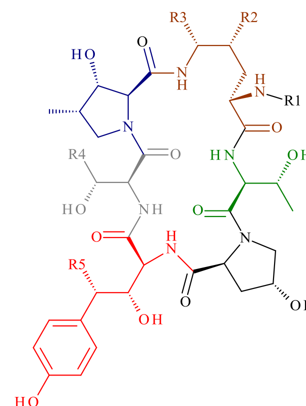
Resistance against azoles has emerged due to the extensive use of antifungals, which have been mainly employed in agriculture (*e.g.* azoles), making difficult the control of infections and enhancing the mortality and morbility of pathogenic fungi. As a matter of fact, this is a serious problem, especially due to the alarming increase in fungal infections associated with immunocompromised situations associated with many diseases such as cancer, allergies and other pathologies, as well as to the result of the administration of potent drugs, including broad spectrum antibiotics (BCC Research 2014a).

In this sense, echinocandins are the latest and most powerful class of antifungals available in the market. Echinocandins are produced mainly by fungi, mainly those from the genus *Aspergillus* (Mizuno, K. *et al.* 1977; Satoi, S. *et al.* 1977; Boeck, L. D. *et al.* 1989). Some important exceptions are pneumocandin B_0 produced by the fungus *Glarea lozoyensis* (Leonard, W. R. J. *et al.* 2002), and WF11899A produced by *Coelomycetes* (Hino, M. *et al.* 2001), which have the peculiarity of a sulfate residue linked to a benzene ring, giving name to the group of sulfo-echinocandins. The mechanism of action of echinocandins is based on a non-competitive inhibition of the enzyme UDP-glucose β-(1,3)-D-glucan-β-(3D-glycosyltransferase, which is essential for the synthesis of 1,3-β-D glucan, an important component of the cell wall of several fungi (Sucher, A. J. *et al.* 2009). In this sense, semisynthetic echinocandins try to avoid the resistance acquired by the bacteria against natural echinocandins (Table 2).

Table 2. Core structure of common natural echinocandins*

Name	R_1	R_2	R_3	R_4	R_5
Aculeacin A	Palmitic	OH	OH	CH_3	OH
Echinocandin B	Linoleic	OH	OH	CH_3	OH
Echinocandin C	Linoleic	H	OH	CH_3	H
Echinocandin D	Linoleic	H	H	CH_3	H
A-30912-H	Linoleic	OH	OCH_3	CH_3	OH
S-31794 F/I	Myristic	OH	OH	CH_2CONH_2	OH
Pneumocandin B_0	(10*R*,12*S*)-dimethylmyristic	OH	OH	CH_2CONH_2	OH

* Structure of the basic cyclohexapeptide in echinocandins. Each amino acid is represented in different color and R_1 represents the fatty acid linked by an amide bond to the cyclohexapeptide



3. BIOTECHNOLOGICAL PRODUCTION OF SEMISYNTHETIC β-LACTAM ANTIBIOTICS AND ECHINOCANDINS

The discovery of methods to produce the nucleus of the penicillin molecule, the 6-aminopenicillanic acid (6-APA), and that of the cephalosporin molecule, 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) has enabled the production of semisynthetic penicillins and cephalosporins on a large scale. In fact, coupling of different acyl moieties (or side chains) to free 6-APA, 7-ACA and 7-ADCA nuclei has allowed the synthesis of new clinically valuable antibiotics with fewer side effects, diminished toxicity, greater selectivity against pathogens, broader antimicrobial range, and improved pharmacological properties. For instance, some clinically valuable semi-synthetic penicillins such as ampicillin (with broader activity spectrum against Gram-negative bacteria) and amoxicillin (high oral absorption) have been obtained by coupling phenylglycine and hydroxyphenylglycine to 6-APA (Fig. 1). On the other hand, combination of different side chains in the 7-amino position and variations of substituents in the 3-acetoxy position of the cephalosporin nucleus has resulted in four generations of cephalosporins with different pharmacological properties (Fig. 2) (Neu, H. C. 1992). First generation cephalosporins (such as cefazolin, cephalexin and cephadroxyl) are usually active *in vitro* against Gram-positive cocci but have limited activity against Gram-negative bacteria. Second generation cephalosporins (such as cefaclor and cefamandol) are more active against Gram-negative bacteria than first generation cephalosporins. Third generation cephalosporins (v.g. cefotaxime, ceftriaxone) have an expanded spectrum of activity, and fourth generation drugs (v.g. cefepime, cefpirome) show higher resistance to inactivation by chromosomally and plasmid encoded β-lactamases than the other semi-synthetic cephalosporins.

Semisynthetic penicillins can be obtained from 6-APA by different methods. Chemical methods for producing 6-APA are polluting and expensive, requiring the use of hazardous chemicals and organic solvents. In contrast, enzymatic cleavage of natural penicillins to obtain the β-lactam nuclei has demonstrated to have a positive impact on both, the economics of the process and the environment (Bruggink, A. *et al.* 2001). In a first instance, natural penicillins (mainly penicillin G and V) are produced in bulk by fermentation of *Penicillium chrysogenum*.

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penicillins to obtain the β-lactam nuclei has demonstrated to have a positive impact on both, the economics of the process and the environment. In a first instance, natural penicillins (mainly penicillin G and V) are produced in bulk by fermentation of *Penicillium chrysogenum*. Then, these fermented products are deacylated enzymatically to generate 6-APA (Fig. 3). The enzymes that are able to cleave the amide bond between the β-lactam nucleus from penicillins and the carboxylic acid functionality, leaving the cyclic β-lactam amide bond intact, are grouped under the general name of penicillin acylases (also known as penicillin amidohydrolases or penicillin amidases, EC 3.5.1.11). Thus, enzymatic reactions catalyzed by penicillin acylases are regio-, and enantioselective yielding 100% pure enantiomeric 6-APA. In addition, biotechnological processes for the large-scale production of semisynthetic penicillins are focused on the condensation of the appropriate D-amino acid derivative with 6-APA catalyzed by penicillin acylases (Bruggink, A. *et al.* 1998; Wegman, Margreth A. *et al.* 2001; Arroyo, M. *et al.* 2003; Deng, S. *et al.* 2016; Xue, P. *et al.* 2016).

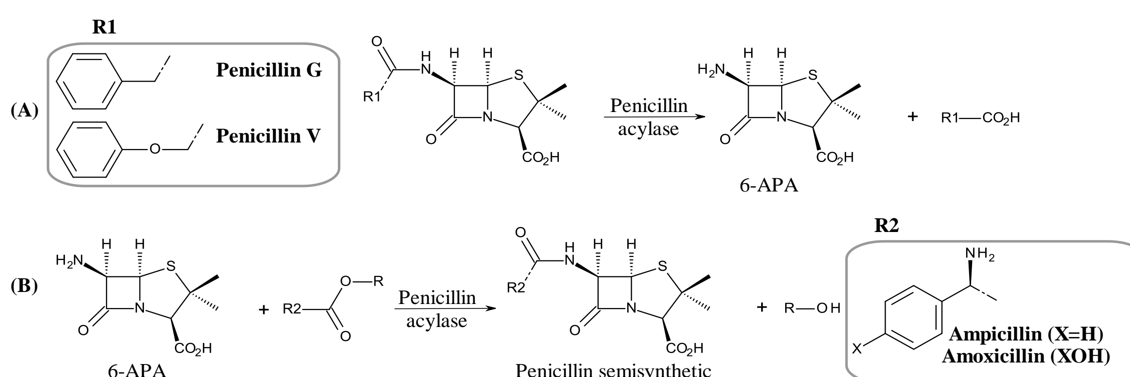

Figure 3. Biotechnological process to produce semisynthetic penicillins. **(A)** Enzymatic deacylation of natural penicillins and **(B)** enzymatic acylation of 6-APA

These processes are environmentally friendly since they are performed in aqueous media and under mild conditions of temperature and pH, entailing a greater sustainability due to reduced use of organic solvents, reactants and energy, as well as less production of pollutant residues. Those features are included within the 12 principles of green chemistry (Anastas, P. T. *et al.* 1998).

During the fermentative production of penicillin G or V by *Penicillium chrysogenum*, other natural aliphatic penicillins are also synthesized (*e.g.* penicillin K, F and dihydroF). These byproducts represent only 1-2 % of the total penicillin in fermentation broth, but they are not hydrolyzed by industrial penicillin G or V acylases hampering the crystallization of 6-APA. Although both facts imply a lower yield of this building block and then a significative economic loss, these problems unfortunately persist in the industry.

Another important aspect in the biotechnological production of semisynthetic β-lactam antibiotics is the availability of versatile penicillin acylases to carry out efficient biocatalytic processes, since antibiotics have complex structures with heterocycles substituents in positions 3 and 7 of the β-lactam nucleus, complicating the enzymatic synthesis.

Likewise, searching and production of antifungic agents like echinocandins are based on the same approach applied in the production of semisynthetic penicillins. By using

echinocandins and echinocandin acylases as precursors and biocatalysts, respectively, it is possible to get a cyclic hexapeptide core which is the building block to obtain semisynthetic echinocandins (Fig. 4) (Gandolfi, R. *et al.* 2012; Yao, J. *et al.* 2012).

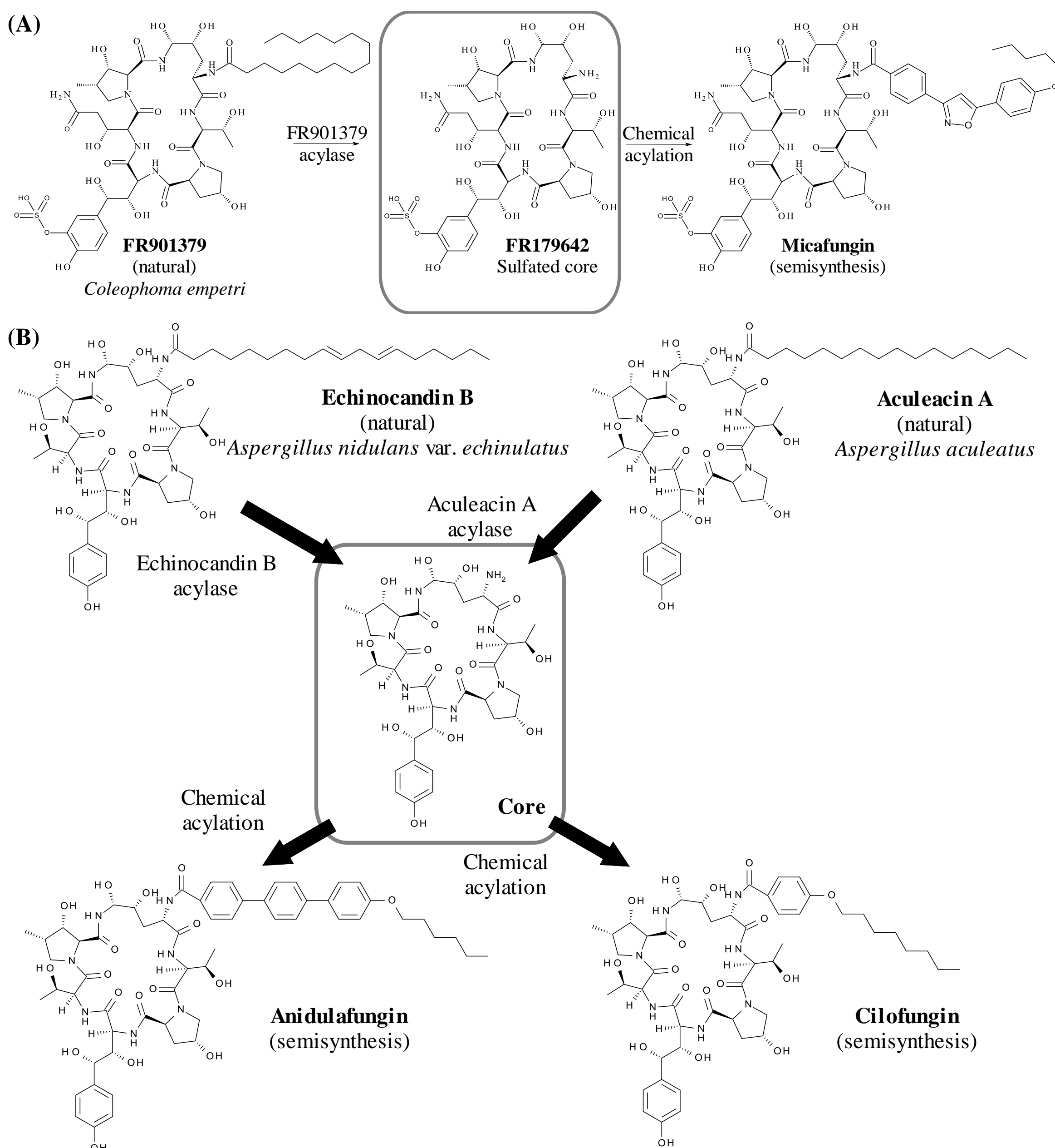

Figure 4. Biotechnological production of the building-block of semisynthetic echinocandins. Taken and modified from (Arroyo, M. *et al.* 2016). Synthesis of **(A)** micafungin, **(B)** anidulafungin and cilofungin

Some of those natural echinocandins (Table 2) are employed to prepare the cyclic hexapeptide cores, which are the building blocks to obtain new antifungals with improved activities. In this sense, echinocandin B was employed to synthesize cilofungin, which was the first semisynthetic derivative evaluated in clinical trials, but it was discarded due to associated nephrotoxicity. Nevertheless, further modifications to the core led to anidulafungin (LY303366), commercialized as Eraxis® by Pfizer since 2006 (Fig. 5.B) (Lattif, A. A. *et al.* 2010). However, the first synthetic echinocandin available in the

market was caspofungin (MK991) in 2001, which is obtained from echinocandin B_0 (Lattif, A. A. *et al.* 2010), and commercialized as Cancidas® by Merck (Fig. 5.A). Thereafter, in 2004 micafungin was obtained (FK463) from pneumocandin A_0, which is available on the market as Mycamine® (Lattif, A. A. *et al.* 2010), and is commercialized by Fujisawa (Fig. 5.C). It is worth mentioning that these compounds can also be obtained from FR901379 (Fig. 4) (Ueda, S. *et al.* 2011a). Finally, in 2008 Novexel announced that the molecule named aminocandin (HMR3270) is under reformulation studies after initiating Phase I trials (FRX, N. 2008). Such a molecule was obtained from deoxymulundocandin (Butler, M. S. *et al.* 2014), but unfortunately this antifungal compound is no longer in development (Moriyama, B. *et al.* 2014). Up to now, only three molecules derived from natural echinocandins are available on the market to fight against pathogenic fungi.

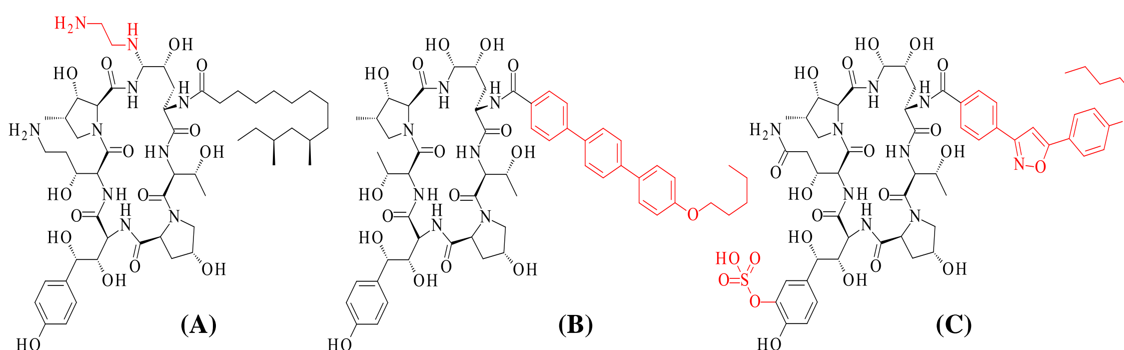

Figure 5. Semisynthetic echinocandins. The modifications with respect to the natural echinocandins are highlighted in red. **(A)** Caspofungin, **(B)** anidulafungin and **(C)** micafungin

Due to problems associated with resistance development, numerous and constant efforts are carried out in the pharmaceutical industry in order to search for new and powerful antibiotics and antifungals, as well as novel and newfangled approaches to control and eliminate pathogenic microorganisms more efficiently and safely (Tan, Y.-T. *et al.* 2000; Martinez, J. L. 2009). One strategy is based on searching for new penicillin or echinocandin acylases with improved properties, which might be used in the synthesis of new compounds or be employed directly as antimicrobial agents as enzybiotics.

4. PENICILLIN AND ECHINOCANDIN ACYLASES

Penicillin acylases as well as echinocandin acylases are amidohydrolases, which are able to cleave the amide bond between the amino group from a core structure, and an acyl group that determines the specificity of the enzyme. In this sense, Table 3 summarizes the main classes of acylases according to their substrate specificity. According to their side chain preference, penicillin acylases are mainly divided in penicillin G acylases, which preferentially catalyze the hydrolysis and synthesis of β-lactam antibiotics with a phenylacetyl side chain; and penicillin V acylases, which catalyze the hydrolysis and synthesis of phenoxyacetyl substituted β-lactam antibiotics. Nevertheless, this classical classification has been broadened with a new group of aliphatic penicillin acylases that preferentially hydrolyse penicillin F, penicillin dihydroF and penicillin K rather than penicillin G and V (Avinash, V. S. *et al.* 2016). Representative examples of aliphatic penicillin acylases would include those reported from *Streptomyces lavendulae* (Torres-Guzmán, R. et al. 2002), *Streptomyces mobaraensis* (Koreishi, M. *et al.* 2007; Zhang, D. *et al.* 2007), and *Thermus thermophilus* (Torres, L. et al. 2012). Aculeacin A acylase from *Actinoplanes utahensis* should also be included in this group due to its ability to hydrolyze

aliphatic penicillins apart from the aliphatic acyl-chain from aculeacin A (Torres-Bacete, J. *et al.* 2015). The availability of chromogenic analogues of aliphatic penicillins (synthetic amides of 2-nitro-5-amino-benzoic acid) could allow a more detailed classification of this group of enzymes (Arroyo, M. *et al.* 2002).

Table 3. Substrate specificity of several β-lactam acylases and other related amidohydrolases

Source (location)	Substrates	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (mM)	Reference
Penicillin G acylases				
Escherichia coli (periplasmic)	Penicillin G Cephalexin Ampicillin Cephaloridine Cephalotin Benzylpenicilloic acid 7-PADCA	$1×10^7$ $2.6×10^4$ $2.2×10^3$ $3.3×10^5$ $0.6×10^6$ $2.0×10^4$ $5.0×10^6$	$4.6×10^{-3}$ 2.1 5.2 0.1 $4.2×10^{-2}$ 2.0 $1.0×10^{-2}$	(Margolin, A. L. *et al.* 1980)
Kluyvera citrophila	Penicillin G Penicillin V Penicillin dihydroF NIPAB C_6-HSL oxo-C_6-HSL	* * * $2.3×10^6$ $0.67×10^3$ $0.11×10^3$	* * * $1.1×10^{-2}$ 0.28 0.10	(Roa, A. *et al.* 1994; Mukherji, R. *et al.* 2014)
Alcaligenes faecalis	Penicillin G NIPAB	$1.3×10^7$ $2.1×10^7$	$4.2×10^{-3}$ $4.5×10^{-3}$	(Švedas, V. *et al.* 1997)
Providencia rettgeri	Penicillin G NIPAB	* *	* $1.97×10^{-2}$	(Ševo, M. *et al.* 2002)
Arthrobacter viscosus (extracellular)	Penicillin G	*	0.42	(Ohashi, H. *et al.* 1988)
Bacillus megaterium	Penicillin G	*	1.83	(de Souza, V. R. *et al.* 2005)
B. badius (intracellular)	Penicillin G Penicillin V Cephalexin Ampicillin Cephalotin Carbenicillin NIPAB	$1.03×10^6$ $1.13×10^3$ $5.82×10^4$ $2.1×10^4$ $8.6×10^4$ $1.83×10^3$ $8.05×10^5$	$3.9×10^{-2}$ 39.0 0.79 1.0 1.0 47.59 $4.1×10^{-2}$	(Rajendhran, J. *et al.* 2007)
Achromobacter xylosoxidans (periplasmic)	Penicillin G NIPAB	$8.2×10^6$ $2.6×10^6$	$8.9×10^{-3}$ $2.7×10^{-2}$	(Cai, G. *et al.* 2004)
Achromobacter sp. (cytoplasmic)	Penicillin G Penicillin V Ampicillin Amoxicillin Cephalexin NIPAB	$1.5×10^7$ * * * * $4.2×10^6$	$1.9×10^{-3}$ * * * * $4.5×10^{-3}$	(Škrob, F. *et al.* 2003)
PAS2 (periplasmic)	Penicillin G Cephalexin Ampicillin Amoxicillin Cefadroxil NIPAB	$2.08×10^6$ $1.54×10^4$ $2.78×10^4$ $3.76×10^4$ $4.58×10^4$ $6×10^6$	$1.2×10^{-2}$ 1.3 0.58 0.40 0.28 $4×10^{-3}$	(Gabor, E. M. *et al.* 2005)
ACPGA001	Penicillin G Penicillin V Ampicillin Amoxicillin Carbenicillin Cephalothin Cephaloridine NIPAB	$1.4×10^7$ $1.23×10^6$ $0.52×10^6$ $0.53×10^6$ $1.23×10^6$ $5.02×10^6$ $4.08×10^6$ $7.02×10^6$	$4.6×10^{-3}$ $3.93×10^{-2}$ $9.44×10^{-2}$ 0.11 $5.19×10^{-2}$ $1.56×10^{-2}$ $1.64×10^{-2}$ $8.3×10^{-3}$	(Zhang, Q. *et al.* 2014)
Penicillin V acylase				
B. sphaericus (cytoplasm)	Penicillin V	*	140	(Olsson, A. *et al.* 1985b)
B. subtilis	Penicillin V	*	*	(Rathinaswamy, P. *et al.* 2005)
Aeromonas sp. ACY95 (intracellular)	Penicillin G Penicillin V Cephalosporin C	* * *	* * *	(Deshpande, B. S. *et al.* 1996)
Fusarium oxysporum	Penicillin V	*	5.2	(Lowe, D. A. *et al.* 1986)

Source (location)	Substrates	k_{cat}/K_M ($M^{-1}s^{-1}$)	K_M (mM)	Reference
Erwinia aroideae	Penicillin G Penicillin V Cloxacillin Methicillin	* * * *	* * * *	(Vandamme, E. J. *et al.* 1975)
Rudolfiella aurantiaca	Penicillin V	0.24×10^3	*	(Kumar, A. *et al.* 2008)
Cephalosporin acylases				
Pseudomonas sp. C427 (periplasmic)	Glutaril 7-ACA	*	*	(Nagao, K. *et al.* 2004)
Brevundimonas diminuta	Glutaril 7-ACA	3.7×10^4	0.36	(Khatuntseva, S. A. *et al.* 2008)
Acyl-homoserine lactone acylases				
Pseudomonas aeruginosa - PvdQ (periplasmic)	C_{11}-HSL to C_{14}-HSL oxo-C_{12}-HSL to oxo-C_{14}-HSL OH-C_{12}-HSL to OH-C_{14}-HSL	* * *	* * *	(Sio, C. F. *et al.* 2006)
P. aeruginosa - QuiP	C_8-HSL to C_{14}-HSL	*	*	(Huang, J. J. *et al.* 2006)
P. aeruginosa PA0305	Penicillin G Penicillin V C_8-HSL oxo-C_{12}-HSL C_6-HSL to C_{14}-HSL oxo-C_6-HSL to oxo-C_{14}-HSL	* * 0.14×10^4 7.8×10^4 * *	* * * * * *	(Wahjudi, M. *et al.* 2011)
Ralstonia sp. XJ12B	oxo-C_8-HSL to oxo-C_{12}-HSL	*	*	(Lin, Y.-H. *et al.* 2003)
Streptomyces sp. M664	Penicillin G C_4-HSL to C_{12}-HSL oxo-C_6-HSL to oxo-C_8-HSL	* * *	* * *	(Park, S. Y. *et al.* 2005)
P. syringae - HacA	C_6-HSL to C_{12}-HSL oxo-C_6-HSL	* *	* *	(Shepherd, R. W. *et al.* 2009)
P. syringae - HacB (intracellular)	C_8-HSL to C_{12}-HSL	*	*	(Shepherd, R. W. *et al.* 2009)
R. solanacearum GMI1000	C_8-HSL to C_{10}-HSL oxo-C_8-HSL	* *	* *	(Chen, C.-N. *et al.* 2009)
Anabaena sp. PCC7120	C_4-HSL to C_{14}-HSL oxo-C_4-HSL to oxo-C_{14}-HSL OH-C_4-HSL to OH-C_{14}-HSL	* * *	* * *	(Romero, M. *et al.* 2008)
Variovorax paradoxus VAI-C	C_4-HSL to C_{12}-HSL oxo-C_6-HSL	* *	* *	(Leadbetter, J. R. *et al.* 2000)
Comamonas sp. D1	C_4-HSL to C_{16}-HSL oxo-C_4-HSL to C_{16}-HSL OH-C_4-HSL to OH-C_{16}-HSL	* * *	* * *	(Uroz, S. *et al.* 2007)
Delftia sp. VM4 (intracellular)	C_6-HSL oxo-C_6-HSL oxo-C_8-HSL	2.66×10^4 4.56×10^4 3.63×10^4	1.25 0.5 0.67	(Maisuria, V. B. *et al.* 2015)
Streptomyces sp. LPC029	C_6-HSL to C_{12}-HSL oxo-C_6-HSL to oxo-C_8-HSL	* *	* *	(Chankhamhaengdecha, S. *et al.* 2013)
Pseudomonas sp. 1A1	C_6-HSL to C_{12}-HSL oxo-C_6-HSL to oxo-C_{10}-HSL	* *	* *	(Cheong, W.-S. *et al.* 2013)
Pseudomonas sp. PAI-A	C_6-HSL to C_{12}-HSL oxo-C_6-HSL to oxo-C_{10}-HSL	* *	* *	(Huang, J. J. *et al.* 2003)
Aliphatic penicillin acylase				
S. lavendulae (extracellular)	Penicillin K Penicillin F Penicillin dihydroF Penicillin G Penicillin V Aculeacin A	1.65×10^5 6.41×10^3 2.19×10^4 0.75×10^4 3.89×10^4 *	0.14 1.2 0.69 60.20 2.05 *	(Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2015)
A. utahensis (extracellular)	Penicillin K Penicillin F Penicillin dihydroF Penicillin G Penicillin V Aculeacin A	3.48×10^4 0.13×10^3 0.75×10^3 0.01×10^3 4.55×10^3 *	1.0 15.1 5.6 155.8 15.4 6.3	(Torres-Bacete, J. *et al.* 2007; Hormigo, D. *et al.* 2010)
S. mobaraensis	Penicillin G Penicillin V NIPOAB	2.1×10^3 5.19×10^4 6.65×10^4	12.0 5.2 1.6	(Zhang, D. *et al.* 2007)
Thermus thermophilus (membrane anchored)	Penicillin K Penicillin F Penicillin dihydroF Penicillin G Penicillin V	1.61×10^4 0.96×10^3 1.49×10^3 1×10^4 0.77×10^3	0.32 0.94 0.98 2.9 0.74	(Torres, L. *et al.* 2012)

*Activity detected but not quantified

These enzymes are produced by a wide variety of microorganisms such as bacteria, actinomycetes, yeasts and fungi (Sudhakaran, V. K. *et al.* 1985b; Sudhakaran, V. K. *et al.* 1985a). Despite the industrial relevance of these enzymes, the biological role of β-lactam acylases remains unclear, but several postulates have been made according to their catalytic activity (Avinash, V. S. *et al.* 2016). Initially, several studies linked these enzymes to penicillin G biosynthesis (Arnstein, H. R. V. *et al.* 1960; Wolff, E. C. *et al.* 1960; Flynn, E. H. *et al.* 1962; Cole, M. *et al.* 1963), whereas other authors considered their connection with antibiotic resistance (Claridge, C. A. *et al.* 1960; English, A. R. *et al.* 1960; Rolinson, G. N. *et al.* 1960; Huang, H. T. *et al.* 1963; Uri, J. *et al.* 1963; Holt, R. J. *et al.* 1964a; Holt, R. J. *et al.* 1964b; Cole, M. *et al.* 1966). At the same time, other studies suggested that penicillin acylase activity was involved in the production of phenylacetic acid (Kameda, Y. *et al.* 1961; Szentirmai, A. 1964), and the degradation of aromatic compounds, which might be used as a carbon and energy source (Valle, F. *et al.* 1991; Merino, E. *et al.* 1992; Prieto, M. A. *et al.* 1993). Even more, some studies suggested that the presence of such activity could be accidental and non-specific (Hamilton-Miller, J. M. T. 1966; Vandamme, E. J. *et al.* 1975).

β-lactam acylases (such as aculeacin A acylase as another aliphatic penicillin acylase) (Torres-Bacete, J. *et al.* 2015) are members of the *N*-terminal nucleophile (Ntn) hydrolase superfamily, which has been described to possess a single-amino-acid catalytic centre (Brannigan, J. A. *et al.* 1995; Duggleby, H. J. *et al.* 1995; Oinonen, C. *et al.* 2000). Other members of this superfamily include *N*-acyl-L-homoserine lactone (AHL) acylases, whose activities work as a type of censorship to block interbacterial communication (Dong, Y. H. *et al.* 2005; Hong, K.-W. *et al.* 2012). AHL acylases catalyze the hydrolytic cleavage of the amide bond from different *N*-Acyl-L-homoserine lactones to form homoserine lactone and the corresponding free fatty acid (Table 3). A wide variety of AHLs with different acyl chains are found in Nature. Chain lengths from C_4 to C_{18} have been observed (for further information, see Supplement S.1 in the Supplementary Material Chapter); a ketone or a hydroxyl group are frequently found in β-position, and in some cases the acyl chain is branched or unsaturated (Dickschat, J. S. 2010).

4.1. Biochemical and structural characteristics of penicillin and echinocandin acylases

The majority of β-lactam acylases and other related amidohydrolases such as echinocandin acylases are heterodimers composed of a low molecular weight α-subunit of about 20 kDa and a high molecular weight β-subunit, of approximately 60 kDa (Sudhakaran, V. K. *et al.* 1992; García, J. L. *et al.* 1995). Except tetrameric and monomeric enzymes from *Bacillus sphaericus* and *Rudolfiella aurantiaca*, respectively, most of β-lactam acylases are synthesized as a single polypeptide chain with four differentiated segments, including a signal peptide, the α-subunit, a spacer peptide and the β-subunit (Oinonen, C. *et al.* 2000). The signal sequence of this preproenzyme is removed upon transport to the periplasmic space where the spacer peptide is autocatalitically cleaved from the N-terminal side of the β-subunit and subsequently from the C-terminal side of the α-subunit (Kasche, V. *et al.* 1999b; Hewitt, L. *et al.* 2000). This process yields the two separate subunits of the mature enzyme, which are held together by hydrophobic and non-covalent bonding (Fig. 6). As reported, α-subunit is involved in substrate binding and hence in enzyme specificity, whereas β-subunit contains those residues involved in catalysis (Sudhakaran, V. K. *et al.* 1992; Kasche, V. *et al.* 1999a; Kim, Y. *et al.* 2002).

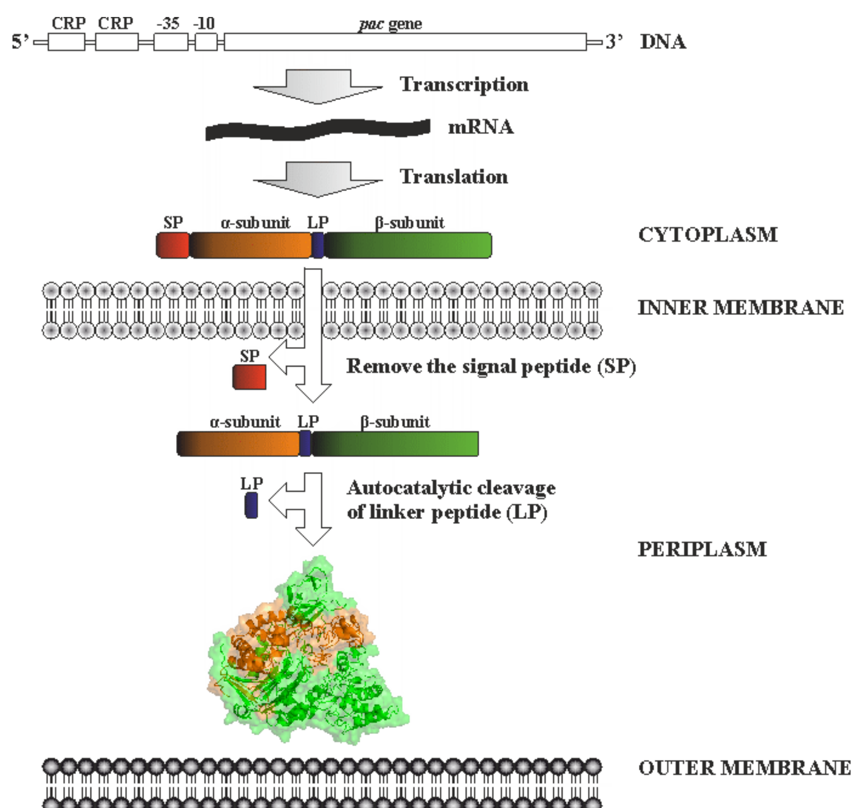

Figure 6. Genetic organization of the *pac* gene in *E. coli* and the steps involved in the synthesis of penicillin G acylase. Taken and modified from (Sizmann, D. *et al.* 1990)

As mentioned above, β-lactam acylases are members of the Ntn-hydrolase superfamily, which are characterized by a catalytic residue in the N-terminal position. Another common feature of this superfamiliy is an enzyme activation via an autoproteolytic process catalyzed by a residue that is further located at the N-terminus of the β-chain. Even though the nucleophilic residue differs (*e.g.* serine for penicillin G acylase from *E. coli* (Duggleby, H. J. *et al.* 1995) and glutaryl-7-ACA acylase from *Pseudomonas diminuta* (Kim, Y. et al. 2000), and cysteine for penicillin V acylase from *B. sphaericus* (Suresh, C. G. et al. 1999), all β-lactam acylases share a strikingly similar arrangement in the catalytic environment (Fig. 7). The typical fold of the Ntn-hydrolase superfamiliy consists of a four-layered catalytically active αββα-core structure (Oinonen, C. *et al.* 2000). This core is formed by two antiparallel β-sheets packed against each other, and these β-sheets are covered by a layer of α-helices on one side. The active N-terminal nucleophile is at the junction of the two anti-parallel β-sheets as single catalytic residue, which is produced from an inactive precursor followed by an intermediate auto-splicing (Shi, Y.-F. *et al.* 2010).

Resolution of the crystal structures of several β-lactam acylases indicated that the catalytic mechanism of these enzymes involves the formation of an acyl-enzyme intermediate (Duggleby, H. J. *et al.* 1995; McVey, C. E. *et al.* 2001). In fact, the proposed mechanism for the hydrolysis of penicillin G by *E. coli* penicillin G acylase (Fig. 8) is very similar to the mechanism of serine proteases. The hydroxyl group of the serine that is located at the N-terminal end of the β-subunit (βSer^1) is activated through a bridging water molecule by its own α-amino group. The serine oxygen attacks the acyl carbon atom of the substrate, forming an oxyanion tetrahedral intermediate (T_d1), which is stabilised via hydrogen bonds to βAsn^{241} and βAla^{69} (oxyanion hole). Rearrangement of electrons leads to the collapse of the intermediate, resulting in the release of the leaving group and a covalent acyl-enzyme

intermediate. The enzyme is subsequently deacylated by a nucleophile, which leads via a similar tetrahedral intermediate (T_d2) to the free enzyme and the acylation product of the nucleophile. Structural comparison of Ntn-hydrolases has revealed similar machinery among them with partial variations in the substrate binding and the oxyanion hole (Shi, Y.-F. *et al.* 2010).

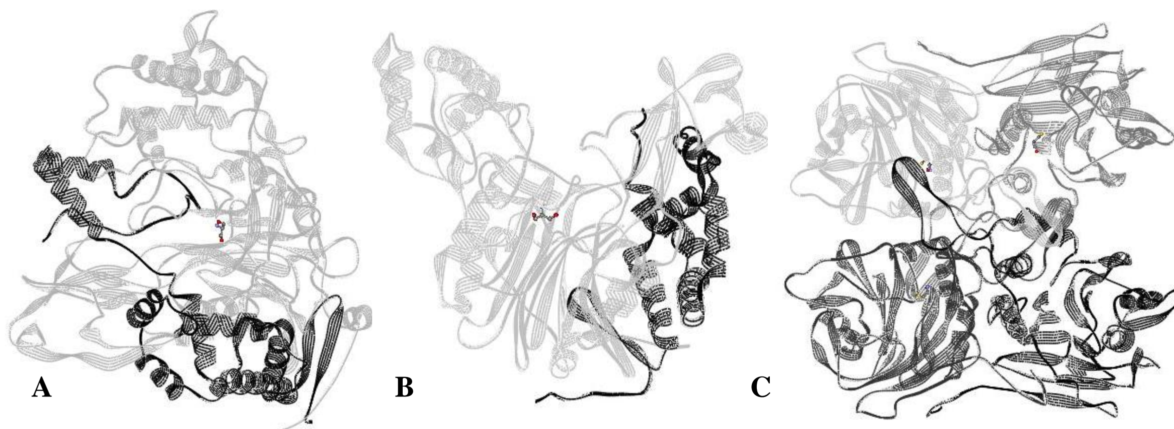

Figure 7. Crystal structures of β-lactam acylases shown in line ribbon representation. **(A)** penicillin G acylase from *E. coli* (PDB 1FXV) and **(B)** glutaryl-7-ACA acylase from *Pseudomonas diminuta* (PDB 1FM2) showing their α-subunit (black) and β-subunit (grey) with the N-terminal catalytic serine in ball-and-stick; **(C)** penicillin V acylase from *B. sphaericus* (PDB 3PVA) showing its four subunits with each N-terminal catalytic cysteine in ball-and-stick.

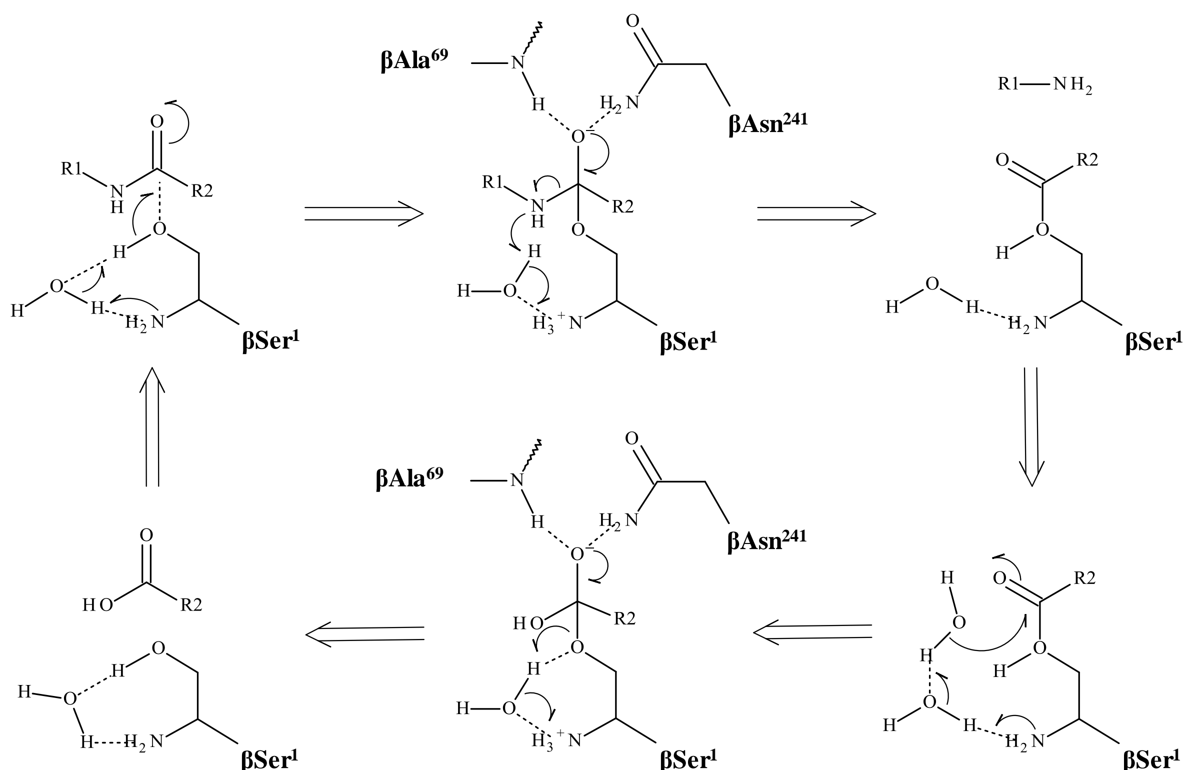

Figure 8. Catalytic mechanism proposed of penicillin G acylase from *E. coli*. Taken and modified from (Duggleby, H. J. *et al.* 1995)

4.2. Penicillin and echinocandin acylases in pharmaceutical industry

The main industrial application of penicillin acylases is 6-APA production by enzymatic deacylation of natural penicillins obtained by fermentation, avoiding contaminant chemicals (Fig. 3.A) (Marešová, H. *et al.* 2014).

Also, pharmaceutical companies claim for novel biocatalysts to be used in biotechnological processes for the large-scale production of semisynthetic penicillins with fewer side effects, diminished toxicity, greater selectivity against pathogens, broader antimicrobial range, and improved pharmacological properties (Giordano, R. C. *et al.* 2006). This process consists in the condensation of the appropriate carboxylic acid derivative with 6-APA as β-lactam core (Fig. 3.B) (Kasche, V. 1986; Kasche, V. *et al.* 1987b). Although the reactions could be carried out by enzymatic or chemical procedures, penicillin acylase-catalyzed reactions are preferred at industrial scale. Such biotransformations can be performed either by thermodynamically or kinetically controlled synthesis. The thermodynamically controlled synthesis involves the direct acylation of 6-APA by free acids at low pH values, whereas the kinetically controlled synthesis involves an acyl group transfer reaction where activated acids, esters, amides, among others, are used as the acylating agents. Additionally, penicillin acylases can be employed to resolve racemic mixtures due to their stereospecificity towards L-amino acids (*e.g.* D-phenylglycine) (Shewale, J. G. *et al.* 1990). Other applications of penicillin acylases include the protection of reactive groups in the synthesis of peptides (Waldmann, H. 1988) as well as protection or unprotection of sugars (Waldmann, H. *et al.* 1991) thanks to their hydrolytic and synthetic capacity. Likewise, Liu and co-workers developed a biosensor for penicillin G quantification (Liu, J. *et al.* 1998), and some authors have even described an application of penicillin acylases in pro-drug activation (Kerr, D. E. *et al.* 1990; Bignami, G. S. *et al.* 1992; Vrudhula, V. M. *et al.* 1993; Gopin, A. *et al.* 2006; Zhang, W. G. *et al.* 2006).

Similarly, industrial application of echinocandin acylases is based on their capability to hydrolyze natural echinocandins (*e.g.* aculeacin A, echinocandin B and FR901379), which are obtained by fermentative processes. The aim is to yield a cyclic hexapeptide that can be employed as a building block for new semisynthetic echinocandins (Takeshima, H. *et al.* 1989) with improved pharmacological properties (Fujie, A. 2007; Yao, J. *et al.* 2012). In conclusion, both penicillin and echinocandin acylases are key enzymes for the industrial production of semisynthetic antibiotics (Fig. 3) and antifungal (Fig. 4) compounds.

5. BACTERIAL COMMUNICATION AND THE POTENTIAL BIOLOGICAL ROLE OF PENICILLIN AND ECHINOCANDIN ACYLASES

As briefly mentioned above, *N*-acyl-L-homoserine lactone acylases is another group of amidohydrolases (Table 3) that plays an important role in *quorum quenching* (*QQ*) or interference of the *quorum sensing* (*QS*), which is a complex intercellular communication system employed by several microorganisms (Fuqua, W. C. *et al.* 1994). In Gram-negative bacteria, *N*-acyl-L-homoserine lactones (AHLs) (Fig. 9) are involved in the regulation of a variety of biological functions, such as antibiotic production, plasmid transference, motility, virulence and biofilm formation (Roche, D. M. *et al.* 2004; Dong, Y.-H. *et al.* 2005; Park, S. Y. *et al.* 2005), in addition to the adaptability to the environmental conditions (Soares, J. A. *et al.* 2011).

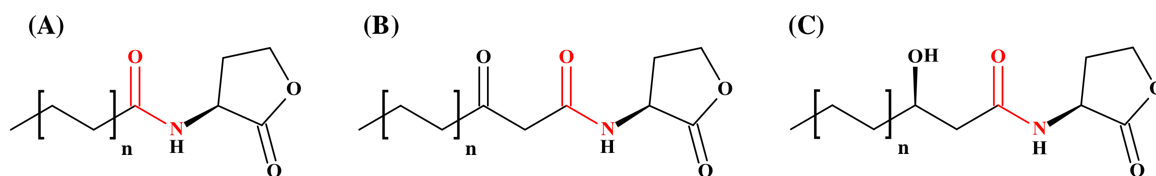

Figure 9. Major *N*-acyl-L-homoserine lactones in *quorum sensing* from Gram-negative bacteria. **(A)** *N*-acyl-L-homoserine lactones, n=1-6, **(B)** *N*-(3-oxo-acyl)-L-homoserine lactones, n=0-8, **(C)** *N*-(3-hydroxy-acyl)-L-homoserine lactones, n=0-6. The amide group which is susceptible to be hydrolyzed by AHL acylases is highlighted in red

AHLs are subjected to biological inactivation either by lactone ring cleavage catalyzed by lactonases, or amide bond hydrolysis performed by AHL acylases. In this sense, *QQ* based on AHL acylases (Raina, S. *et al.* 2009) is the most interesting strategy to interrupt *QS* mediated by AHLs (Dickschat, J. S. 2010).

Cell communication in bacteria is performed by the action of several molecules, and these compounds work like recognition signals. The secretion, detection and interruption of *QS* pheromones and microbial hormones allow the regulation of important genes that are necessary for microbial survival (Yajima, A. 2014). There are different microbial signaling molecules, which are classified in acyl-homoserine lactones, oligopeptide autoinducers, AI-2 family, alkyl quinolones, terpenoids, CAI-1 family, ComX pheromones, diffusible signal factors, diffusible extracellular factor, fatty acids and mating hormones, and so forth (Waters, C. M. *et al.* 2005; Yajima, A. 2014). However, it is worth mentioning that another kind of interaction between cells has been reported, such as physical signals (Reguera, G. 2011).

5.1. *Quorum sensing* system

One of the main topics of the present study has been the enzymatic amide bond cleavage of AHLs, molecules involved in *QS* as mentioned above. As a matter of fact, both *Sl*PVA and *Au*AAC are able to hydrolyze these bonds. In addition, the biological importance of these molecules has been estimated taking into account the number of microorganisms involved with these compounds. Recently, Dickschat (Dickschat, J. S. 2010) has published an interesting review that collects the main AHLs implicated in *QS,* the chemistry of bacterial biofilm (upstream and downstream of the AHLs biosynthesis), as well as their role during cell communication. Supplement S.1 in the Supplementary the Material Chapter, summarizes the main updated AHLs until December 2015, collecting the usual and unusual structures of AHLs, and covering molecules with aliphatic, *p*-phenylpropenoid (such as *p*-coumaroyl-HSL) (Schaefer, A. L. *et al.* 2008), and branched side chains (Thiel, V. *et al.* 2009; Lindemann, A. *et al.* 2011) as well as dienoyl-HSL (Thiel, V. *et al.* 2009). Thus, it is expected that new structural variants (*e.g.* with insaturations, with oxidized side chains at different positions, and with heteroatoms different to oxygen) may be discovered (Yajima, A. 2014). Due to the complexity of these structures, many analytical tools have been employed and combined to elucidate each AHL, such as chromatographic techniques (*i.e.* thin-layer chromatography biosensor assay, HPLC, gas chromatography), capillary electrophoresis, and different spectroscopy techniques (*i.e.* mass spectrometry, nuclear magnetic resonance, infrared spectroscopy).

5.2. *Quorum quenching* system

Just as mentioned before, communication between bacteria (*QS*) is an important signalling mechanism, which allows the interaction, reaction and in general, the survival of the microorganisms from the same species, or even from different genera. Therefore, a biofilm represents a polymicrobial community where some bacteria interact with neighboring cells by *QS*, whereas others interrupt this communication by *QQ* (Hong, K.-W. *et al.* 2012). In this context, *QQ* can take place in three different scenarios. Firstly, inhibiting those enzymes related with the biosynthesis of signal molecules (*e.g.* *S*-adenosylmethionine synthase and LuxI homolog proteins) (Parveen, N. *et al.* 2011). A second mechanism would be based on the degradation of the *QS* signal molecules by enzymatic reactions mainly catalyzed by AHL-lactonases and AHL-acylases. However, sequestration, signal competition and environmental conditions can be considered as external perturbation (Whitehead, N. A. *et al.* 2001; Hong, K.-W. *et al.* 2012). Finally, similarly to the inhibition of those enzymes involved in biosynthesis of AHLs, the third mechanism would be based on the inhibition of LuxR homolog proteins considered as *QS* receptors (Koch, B. *et al.* 2005; Chen, G. *et al.* 2011). All these apparent *QQ* alternatives are depicted in Figure 10, where an illustrative model of the system of *Vibrio fischeri* is represented (Schaefer, A. L. *et al.* 1996; Fuqua, C. *et al.* 2002). However, a similar analysis may be inferred in some other microorganisms thanks to homologous signalling systems (*i.e.* inducer and receptor) detected in their genomes, which seems to reflect both duplication and horizontal transfer, in different cases (Lerat, E. *et al.* 2004).

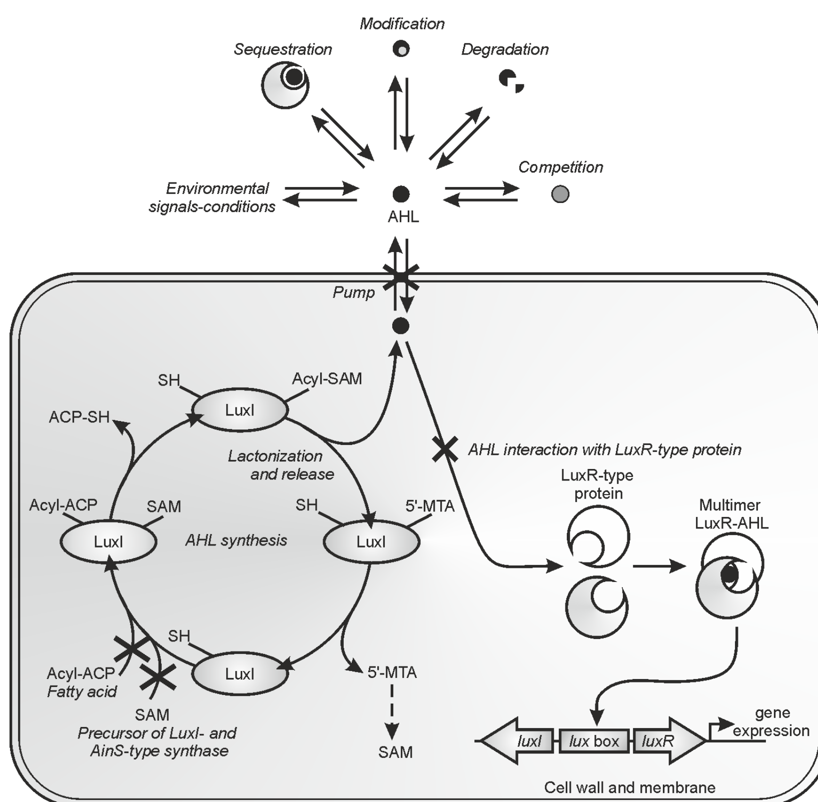

Figure 10. *QQ* mechanism displayed by *V. fischeri* as an illustrative model

If one-fold alteration of the AHL structure is considered as an alternative to interrupt *QS*, five different changes might be accomplished through degradation and modification reactions. Dong and Zhang suggested four theoretical ways to achieve such purpose

mediated by lactonases, acylases, decarboxylases and deaminases. Nevertheless, only the first two enzymes have been found (Dong, Y. H. *et al.* 2005). In addition, oxidoreductases reacting on 3'-substituents located in the side chain of AHLs have also been reported (Uroz, S. *et al.* 2005; Chan, K.-G. *et al.* 2011), and they have been considered as a fifth mechanism of modifying but not destroying AHLs (Hong, K.-W. *et al.* 2012).

Although this interaction system is performed in bacteria, there are conserved enzymes present in mammals such as PON1, PON2 and PON3, which are involved in the hydrolysis and inactivation of several compounds by employing their lactonase/esterase activity (Draganov, D. I. *et al.* 2005; Camps, J. *et al.* 2011). These enzymes carry out important physiological functions, such as drugs metabolism and detoxification of organophosphorus compounds (Ng, C. J. *et al.* 2005).

Although several *QQ* enzymes have been postulated to interrupt *QS*, surprisingly no experiment to date has corroborated the link between *QQ* and the ability of gaining competitive advantage (Hibbing, M. E. *et al.* 2010). In this sense, there is a considerable number of bacteria displaying *QQ* enzymes, which should be further investigated in order to gain insight into their enigmatic roles (Hong, K.-W. *et al.* 2012).

A) *Quorum quenching* in pharmacology

The production of virulence factors is regulated by *QS* in bacteria (Bassler, B. L. 2002), a mechanism that is not limited to the communication between the same species, but reported especially in polymicrobial biofilms (Riedel, K. *et al.* 2001). Thereby, *QQ* enzymes (*e.g.* lactonases, acylases and oxidoreductases) are suitable candidates to be used as drugs against virulent microorganisms, because they inactivate signaling molecules without interfering with the enzymatic mechanism inside the bacterial cell (Hong, K.-W. *et al.* 2012). It is worth mentioning that *QS* interference does not prevent biofilm formation, but it becomes more susceptible to be attacked by both antimicrobial compounds and immune responses from the host (Estrela, A. B. *et al.* 2010). Likewise, *QQ* shows several drawbacks for controlling virulence since not all pathogenic bacteria use this system (Hong, K.-W. *et al.* 2012).

B) *Quorum quenching* in anti-biofouling

The undesirable accumulation of biological material on structures immersed in the marine environment, such as microorganisms, plants, algae and invertebrate animals is named biofouling (Hong, K.-W. *et al.* 2012), which has a severe impact on several industries (*e.g.* shipping, fishing and aquaculture) as well as in oceanographic sensors (Callow, J. A. *et al.* 2011). Many techniques for biofouling inhibition have been proposed, such as employing *QQ* compounds (Fusetani, N. 2011) or using anti-biofouling surfaces (Schumacher, J. F. *et al.* 2007). However, none of these technologies have been successful (Callow, J. A. *et al.* 2011).

C) *Quorum quenching* in aquaculture

The presence of antibiotic-resistant bacteria is a common problem in aquaculture industry, due to the indiscriminate use of antibiotics and its ubiquitous presence in water (Grigorakis, K. *et al.* 2011). Thus, *QQ* compounds produced by aquatic organisms have arisen as an alternative to combat pathogens (Natrah, F. M. I. *et al.* 2011).

D) *Quorum quenching* in agriculture

This is one of the most important and relevant topics in order to win the fight against pathogens involving human food and animal feed. In this field, natural bacteria expressing *QQ* compounds or enzymes might be used as biocontrol agents (Hong, K.-W. *et al.* 2012). Transgenic cultures expressing *QQ* enzymes could be employed as well (Dong, Y.-H. *et al.* 2001), but this last option is not universally accepted due to biosafety and legal regulations (Hong, K.-W. *et al.* 2012).

E) *Quorum quenching*, advantages and perspectives

Risk of *QQ* resistance (Defoirdt, T. *et al.* 2010) might be reduced by employing *QQ* enzymes with a broad specificity against different AHLs (*e.g.* lactonases and acylases). Despite the fact that this strategy could inactivate beneficial microbial *QS* pathways, this approach avoids manipulating or targeting the receptors (Hong, K.-W. *et al.* 2012). Another alternative mentioned above was the administration of *QQ* compounds, but novel compounds are needed towards resistant bacteria. For this reason, the efficacy of the procedure could be improved by combining *QQ* approaches with other treatments thanks to a synergistic effect (Estrela, A. B. *et al.* 2010), although the consequences are still unknown. Finally, the last proposal is related to target essential functions in infectious bacteria, such as virulence factors, with the aim to conserve endogenous microflora while imposing a relative mild selective pressure (Clatworthy, A. E. *et al.* 2007).

Penicillin V acylase from *Streptomyces lavendulae* ATCC 13664 (EC 3.5.1.11) and aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052 (E.C. 3.5.1.70) could be considered as versatile enzymes, which have the capability to hydrolyze the amide bond between an aliphatic side chain (with the carboxylic acid functionality) and a nucleus containing an amino group present in many substrates (*e.g.* aliphatic penicillins, aculeacin A and *N*-acyl-L-homoserine lactones), in addition to catalyze the acylation to obtain new β-lactam antibiotics (Torres-Bacete, J. *et al.* 2007; Hormigo, D. 2009; Torres-Bacete, J. *et al.* 2015).

6. PENICILLIN V ACYLASE FROM *Streptomyces lavendulae* ATCC 13664

In 1961 *S. lavendulae* ATCC 13664 culture broth was described to show penicillin V acylase activity for first time (Rolinson, G. N. *et al.* 1961). After verifying that this microorganism had an extracellular penicillin V acylase (Torres, R. *et al.* 1999), Torres-Guzmán and co-workers established that the purified enzyme was a preproenzyme with a signal peptide and a linker peptide (Torres-Guzmán, R. 2004). Further studies from the same group revealed that the *pva* gene encodes an inactive precursor protein containing a secretion signal peptide that is activated by two internal autoproteolytic cleavages that release a 25-amino-acid linker peptide and two large domains that render the heterodimeric structure, a small one α-subunit of approximately 18.8 kDa, and a big β-subunit with 60.1 kDa (Torres-Bacete, J. *et al.* 2015).

It is worth mentioning that the sequence of the encoding gene of this enzyme has no homology to that described for penicillin V acylase from *Bacillus sphaericus* (Olsson, A. *et al.* 1985a), neither to those reported for the acylases from *E. coli* and *P. diminuta* (Olsson, A. *et al.* 1985b; Kim, Y. *et al.* 2000). On the contrary, it was quite similar to other acylases, such as penicillin G acylase and glutaryl-7-ACA acylase from different

microorganisms, with aculeacin A acylase from *Actinoplanes utahensis* NRRL 12052 (Takeshima, H. *et al.* 1989; Inokoshi, J. *et al.* 1992), *N*-acyl-homoserine lactone acylase from *Streptomyces* sp. M664 (Park, S. Y. *et al.* 2005) and lipopeptide acylase from *Streptomyces* sp. FERM 5809 (Shibata, T. *et al.* 2002).

From an industrial point of view, only 12 % of the global 6-APA production employ penicillin V and penicillin V acylase, whereas the remaining 88 % is obtained by hydrolysis of penicillin G catalyzed by penicillin G acylase (Shewale, J. G. *et al.* 1997). In spite of that, the advantages of a process with penicillin V and a penicillin V acylase (such as *Sl*PVA) to obtain 6-APA are quite clear:

- Penicillin V displays higher stability at low pH values than penicillin G, facilitating its extraction from culture broths (Herschbach, G. J. H. *et al.* 1984).
- The strains producers of penicillin V tolerate higher concentrations of phenoxyacetic acid than penicillin G producers, and therefore fermentation yields can be improved (Herschbach, G. J. H. *et al.* 1984).
- Penicillin V acylases can stand high concentrations of penicillin V during the hydrolysis process, which enhances 6-APA production, even with yields of 99 % (Shewale, J. G. *et al.* 1997).
- Penicillin V acylases show an optimal activity at a wide range of pH values (Shewale, J. G. *et al.* 1997) that avoids an exhaustive pH control, and thus the expenses of the process are lower

In this sense, a comparative analysis of the kinetic parameters of *Sl*PVA against several substrates is shown in Table 3. As observed, *Sl*PVA is able to hydrolyze aliphatic and aromatic acyl groups of different penicillins, as well as chromogenic substrates such as NIPOAB, NIHAB and NIOAB (Torres-Guzmán, R. *et al.* 2002). In the case of *Sl*PVA, the amino acids involved directly in the catalysis were determined by site-directed mutagenesis, and they are βSer^{1}, βHis^{23}, βVal^{70} and βAsn^{272} (Torres-Bacete, J. *et al.* 2015), and probably βSer^{1} and βHis^{23} form a catalytic diad similar to that described for other serine proteases (Duggleby, H. J. *et al.* 1995; Perona, J. J. *et al.* 1995); likewise, within the substrate binding pocket are αAla^{154}, αGly^{158}, βTyr^{24}, βArg^{31}, βTrp^{33}, βLeu^{50}, βSer^{53}, βSer^{57}, βIle^{58}, βSer^{67} and βVal^{186} (Fig. 11) (Torres-Bacete, J. *et al.* 2015).

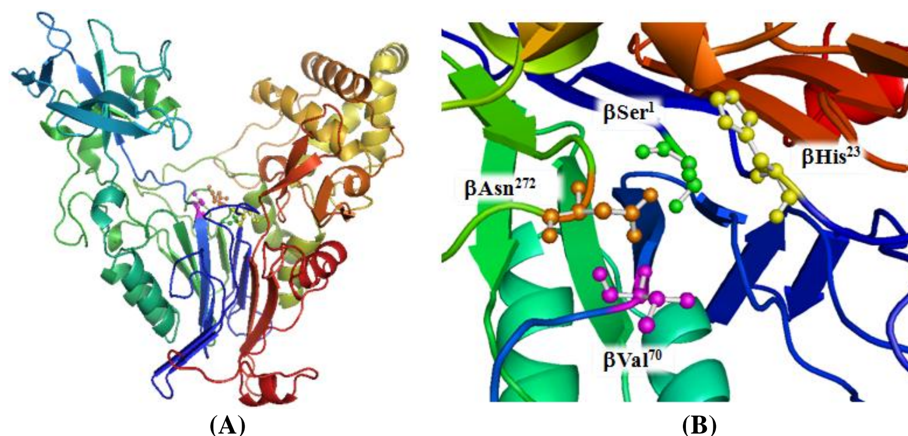

Figure 11. 3D-structure model of the β-subunit of *Sl*PVA obtained by Phyre2. **(A)** Full model and **(B)** detail of the active-site catalytic amino acids. Taken and modified from Torres-Bacete (Torres-Bacete, J. *et al.* 2015)

On the other hand, deconvolution of the far-UV circular dichroism spectrum of the enzyme (Torres-Bacete, J. *et al.* 2015) revealed the presence of 20-30 % of α-helix, 16 % of β-sheet, 15-20 % of β-turns and 30 % of random coil.

As described, *Sl*PVA has been thoroughly studied and characterized by the research group of Enzymatic Biotechnology of the Complutense University of Madrid (Torres, R. *et al.* 1998; Torres, R. *et al.* 1999; Torres-Bacete, J. *et al.* 2000; Torres-Guzmán, R. *et al.* 2001a; Torres-Guzmán, R. *et al.* 2001b; Torres-Guzmán, R. *et al.* 2002; Hormigo, D. 2009; Torres-Bacete, J. *et al.* 2015). These investigations showed that *Sl*PVA displays interesting properties to be employed for the industrial production of 6-APA in enzymatic bioreactors.

7. ACULEACIN A ACYLASE FROM *Actinoplanes utahensis* NRRL 12052

Aculeacin A acylase belongs to the echinocandin acylases family, a group of microbial enzymes which are able to hydrolyze natural echinocandins (*e.g.* aculeacin A, echinocandin B and FR901379) (Takeshima, H. *et al.* 1989; Kreuzman, A. J. *et al.* 2000; Ueda, S. *et al.* 2011b). Echinocandins are produced by fermentation processes and further deacylated in order to obtain the cyclic hexapeptide, which is employed as a building block for the preparation of semisynthetic echinocandins with enhanced antifungal activity and fewer side-effects (Fig. 4). New semisynthetic antifungals should be available since a small number of therapeutic agents are ready nowadays to treat systemic resistant infections produced by pathogenic fungus (*e.g. Candida* spp. and *Aspergillus* spp.) (Sucher, A. J. *et al.* 2009). In this sense, aculeacin A acylase from *Actinoplanes utahensis* may be considered as an interesting biocatalyst for the preparation of the building block of semisynthetic echinocandins. In addition, this enzyme shows penicillin acylase activity (Torres-Bacete, J. *et al.* 2007), as well as activity against some AHLs (Hormigo, D. 2009).

Similarly to penicillin V acylase, aculeacin A acylase is produced as a preproenzyme, and is secreted extracellularly as a heterodimer with an α-subunit of 19 kDa and a β-subunit of 55 kDa (Inokoshi, J. *et al.* 1992). An initial analysis of aculeacin A acylase-encoding gene sequence revealed that this enzyme is dissimilar to other aculeacin acylases, but shows high identity with different acylases, mainly with *Sl*PVA (identity of 41 %), and acyl-homoserine lactone acylase from *Ralstonia* sp. XJ12B (identity of 41 %) (Torres-Bacete, J. *et al.* 2015). Besides, this enzyme may be also considered as a potential tool in *QQ* of Gram-negative bacteria due to its similarity with the acyl-homoserine lactone acylase from *Ralstonia solanacearum* GMI1000 (Chen, C.-N. *et al.* 2009).

Table 3 shows the kinetic parameters of *Au*AAC employing several substrates. Once more, it is worth mentioning that *Au*AAC is also able to hydrolyze both aliphatic as aromatic acyl side chains of different substrates. In addition, site-directed mutagenesis indicated that the amino acids directly involved in catalysis were βSer^{1}, βHis^{23}, βVal^{70} and βAsn^{257} (Hormigo, D. 2009). Likewise, *Au*AAC has also been studied and characterized exhaustively by the research group of Enzymatic Biotechnology of the Complutense University of Madrid (Torres, R. *et al.* 1998; Torres, R. *et al.* 1999; Torres-Bacete, J. *et al.* 2000; Torres-Guzmán, R. *et al.* 2001a; Torres-Guzmán, R. *et al.* 2001b; Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007; Hormigo, D. 2009; Hormigo, D. *et al.* 2010; Velasco-Bucheli, R. *et al.* 2015), showing attractive properties to be employed in the industry too, in addition to the synthesis of antibiotics and semisynthetic antifungals.

8. DIRECTED MOLECULAR EVOLUTION

Development of new biocatalysts has been consolidated as a new initiative to improve the efficiency of chemical processes at an industrial level. During the last years, directed molecular evolution or evolution *in vitro* of enzymes has emerged as a powerful technique which imitates natural evolution on a temporal scale in the lab (Valetti, F. *et al.* 2004; Bershtein, S. *et al.* 2008). Directed evolution implies the generation of genetic heterogeneity into a cell population by introducing random mutations, and then selecting recombinant enzymes with the desired feature (Wang, M. *et al.* 2012). Thus, this procedure integrates gene mutagenesis, expression, and selection (hereafter named screening) of recombinant genes libraries, consisting typically of 10^3 to 10^6 transformants (Feng, X. *et al.* 2012).

The advantages of this technique in comparison to rational methods in protein engineering are sometimes limited as a consequence of the scarce knowledge about the structure-function relationship (Antikainen, N. M. *et al.* 2005; Gerlt, J. A. *et al.* 2009). Furthermore, several studies have revealed that those changes affecting enzyme selectivity are located usually far away from the catalytic pocket, which means that small differences throughout the protein could have an unpredictable impact on the activity (Wada, M. *et al.* 2003; van Loo, B. *et al.* 2004; Yuan, L. *et al.* 2005; Zumárraga, M. *et al.* 2007). Thus, random mutagenesis is useful not only to achieve enzymes with improved properties, but also to study their structure-function relationship.

The key step in a directed evolution process is the imposition of a strong selective pressure by the researcher, where beneficial mutations should prevail against those that can be harmful or deleterious (Valetti, F. *et al.* 2004).

Massive screening (Reymond, J.-L. 2006) followed by analyses of generated mutants is the real bottleneck of this methodology. This is the reason why the design of a good protocol of massive screening is crucial to achieving good outputs, since selection is accomplished not by selective pressure of the medium but through acquisition of an useful characteristic displayed by the enzyme in a specific application (Cohen, N. *et al.* 2001; Aharoni, A. *et al.* 2005). Taking this consideration into account, the implementation of the so-called High-Throughput Screening (HTS) allows the fast and reproducible evaluation of random mutations, avoiding punctual focused or unidirectional changes. Thus, modifications generated throughout the protein structure are taken into consideration in the study, whereas it is unlikely considered by directed mutations.

Modifications are introduced in the gene sequence of interest in a reiterative manner, followed by additional rounds of random mutations in the target gene (Fig. 12), and screening of those colonies whose enzyme activity and/or stability have been improved. Directed evolution is a powerful tool to obtain improved enzymes, but furthermore it offers the advantage of generating enzyme variants that could clarify the relationship between protein sequence, structure and function (Cirino, P. C. *et al.* 2003).

It is important to highlight that a good strategy of directed molecular evolution requires (i) a good protocol to introduce mutations throughout the gene sequence, (ii) a host to express an active enzyme, and (iii) a screening procedure endorsed by statistical criteria (Alcalde, M. 2012).

β-Lactam acylases have been altered and improved by additional rounds of random mutations (Arroyo, M. *et al.* 2005). In this sense, the activity and/or stability of many of these enzymes have been improved by directed evolution or side-directed mutagenesis, such as penicillin G acylase from *K. citrophila* ATCC 21285 (Martín, J. *et al.* 1990; Roa, A. *et al.* 1994) and *E. coli* ATCC 11105 (del Río, G. *et al.* 1995; Lee, H. *et al.* 2000; Morillas, M. *et al.* 2003; Balci, H. *et al.* 2014), glutaryl 7-ACA acylase from *Pseudomonas* SY-77-1 (Otten, L. G. *et al.* 2002; Sio, C. F. *et al.* 2002; López-Gallego, F. *et al.* 2008), as well as cephalosporin C acylases from *Pseudomonas* SE83 (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012; Xiao, Y. *et al.* 2014; Zhang, J. *et al.* 2014), *Pseudomonas* N176 (Ishii, Y. *et al.* 1995; Pollegioni, L. *et al.* 2005; Golden, E. *et al.* 2013; Conti, G. *et al.* 2014) and *P. diminuta* KAC-1 (Mei, T. *et al.* 2015).

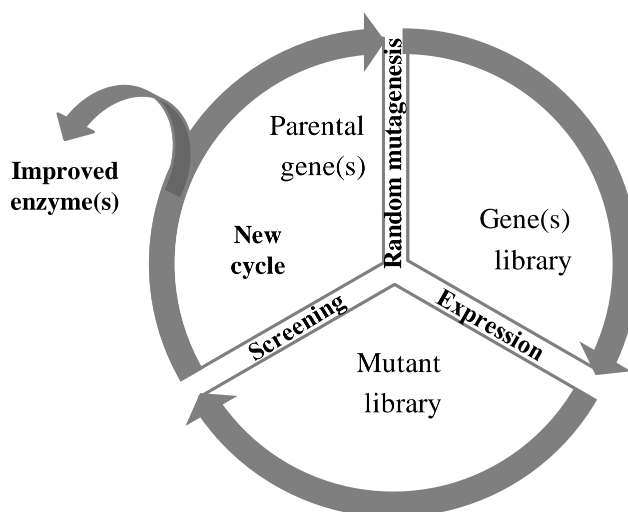

Figure 12. General scheme of directed evolution

8.1. Creation of libraries

As an iterative algorithm, directed evolution is useful to diversify and select from a molecular level to a whole ecosystem, becoming a centerpiece for industrial biocatalyst development in both academia and biotech companies. The trend to draw on directed evolution is mainly due to its straightforward concept, and these studies can be developed with facilities located in many labs. Additionally, it frequently gives useful solutions, some of them unanticipated by the researcher (Arnold, F. H. *et al.* 2003). In this sense, several strategies have emerged with the aim to generate random mutations, and the difference of the rate of mutations within the sequence, and the particular advantages and drawbacks of each one of them makes the difference towards different targets, then favouring the size of the libraries, and consequently the success of the screening. The alternatives to introduce variations in gene sequences by directed molecular evolution can be developed by recombinant (also known as sexual) or non-recombinant (or asexual) methodologies. Within the first option, error-prone PCR is a well-known random mutagenesis protocol, in addition to saturation mutagenesis techniques. On the other hand, DNA shuffling has been developed within several *in vivo* and *in vitro* alternatives, whereas new techniques emerge everyday as opportunities to create libraries of recombinant clones (Table 4).

Table 4. Alternative methodologies to introduce variations in gene sequences by directed molecular evolution. Adapted and modified from (Alcalde, M. 2012)

Non-recombinant (asexual)	Random mutagenesis		epPCR (error-prone PCR) (Cirino, P. C. *et al.* 2003)
			Mutagenic strains (Cox, E. C. 1976)
			Physical and chemical mutagenesis (Kodym, A. *et al.* 2003)
			RID (Random Insertion and Deletion mutagenesis) (Murakami, H. *et al.* 2000)
			SeSaM (Sequence Saturation Mutagenesis) (Wong, T. S. *et al.* 2004)
	Saturation mutagenesis		Site-saturation mutagenesis (Urban, A. *et al.* 1997)
			Max randomization (Hughes, M. D. *et al.* 2003)
Recombinant (sexual)	*In vitro*	DNA homologous recombination	StEP (Staggered Extension Process) (Zhao, H. *et al.* 1998)
			RACHITT (Random Chimeragenesis on Transient Templates) (Coco, W. M. *et al.* 2001)
			Gene assembly mutagenesis (Bessette, P. H. *et al.* 2003)
			RPR (Random Primer *in vitro* Recombination) (Shao, Z. *et al.* 1998)
		Homology superior to 60 % (family shuffling)	DOGS (Degenerate Oligonucleotides Gene Shuffling) (Bergquist, P. L. *et al.* 2005)
			Single-Stranded DNA Family Shuffling (Kikuchi, M. *et al.* 2000)
			Restriction-enzyme-based method (Kikuchi, M. *et al.* 1999)
		Homology inferior to 60 %	ITCHY (Incremental Truncation for the Creation of Hybrid enzYmes) (Ostermeier, M. *et al.* 1999b)
			THIO-ITCHY (Lutz, S. *et al.* 2001a)
			SCRATCHY (combination of ITCHY and DNA shuffling) (Lutz, S. *et al.* 2001b)
			SHIPREC (Sequence Homology-Independent Protein RECombiantion) (Sieber, V. *et al.* 2001)
			Exon shuffling *in vivo* (Kolkman, J. A. et al. 2001)
	In vivo		CLERY (Combinatorial Libraries Enhanced by Recombination in Yeast) (Abécassis, V. *et al.* 2000)
			Random chimeragenesis by heteroduplex (Volkov, A. A. *et al.* 1999)
			IVOE (*in vivo* Overlap Extension) (Alcalde, M. *et al.* 2006)
			IVAM (*in vivo* Assembly of Mutant with different mutational spectra) (Zumárraga, M. *et al.* 2008)

8.1.1. Mutator strains

Mutate a gene sequence can be achieved by employing mutator strains. In particular, bacteria are used due to their unusually high rates of spontaneous DNA mutagenesis. The accessibility of this methodology to create random libraries makes unnecessary the use of specialized equipment or cloning techniques, and the whole process is developed in a few days with minimal endeavour and with little knowledge of DNA recombination (Nguyen, A. W. *et al.* 2003). Relatively stable mutator strains are capable to introduce diverse modification throughout the plasmid which contains the gene, providing transformants with a broad spectrum of modifications in the sequence, such as base substitutions, insertions and deletions (Cox, E. C. 1976). Thus, genetic deficiencies in DNA proofreading and editing mechanism in mutator strains are suitable to perform modifications in a specific sequence, and these deficiencies are generally related to mutations in *mutD*, *mutS* and *mutT* genes (Nguyen, A. W. *et al.* 2003).

Modifications in *mutD* affect the repairing of incorrect base incorporation, because it can interfere with DNA polymerase III (*i.e.* activity 3'-5' exonuclease) (Miller, J. H. 1998). Most of the obtained mutations are transitions (85 %), and in lower extent transversions (10 %) and frameshifts (5 %) (Cox, E. C. 1976; Schaaper, R. M. 1988). Additionally, *mutD* strains display a mutation rate from 10 to 100 times higher, whereas cultures in rich media exhibit a mutation rate up to 10^3-10^5-folds with respect to the wild-type (Cox, E. C. 1976). On the other hand, the very short patch system specifically repairs G/T mismatches associated with *dcm* methylation sites to G/C, and its main role is thought to be the rectification of mismatches caused by the deamination of 5-methylcytosine to thymine.

Particularly, this system is dependent on DNA polymerase I, *mutS* and *mutL* (Harfe, B. D. *et al.* 2000). *mutS* gene codifies an ATPase that binds as a homodimer to DNA, displaying *in vitro* specificity for base-base mispairs and for insertion/deletion of up to 4 nucleotides (Parker, B. O. *et al.* 1992). Thus, *mutS* is a protein that affects mismatch recognition (Harfe, B. D. *et al.* 2000), and the presence of mutation throughout this gene disables DNA mismatch repair, triggering transitions and transversions (Hsieh, P. 2001). In a wild-type strain 5 % of the total mutation events are related to single-base frameshift mutations but approximately 25 % accounts for mismatch repair of defective strains, with almost all of these events in the last strains occurring in a 5N mononucleotide run (Harfe, B. D. *et al.* 2000). Likewise, errors during DNA synthesis are more susceptible to escape from polymerase proofreading in long mononucleotide runs (Kroutil, L. C. *et al.* 1996; Tran, H. T. *et al.* 1997).

Finally, *mutT* mutations prevent the degradation of 8-oxodGTP in mismatches involving A:G (Fowler, R. G. *et al.* 1997), resulting mainly in AT-CG transversions (Cox, E. C. 1976). Likewise, Schaaper and Dunn (Schaaper, R. M. 1988) demonstrated that *mutT* indeed prevents A/G rather than T/C mispairings. Thus, *mutT* prevents the A/8-oxodGTP mispairings originated by misincoporation of the oxidized triphosphate 8-oxodGTP from template A. However, *mutT* is part of a huge sophisticated defense system dedicated to avoid mutagenic consequences of 8-oxodGTP at both the dNTP and DNA levels (Fowler, R. G. *et al.* 1997). In consequence, the combination of all these mutated genes (*i.e. mutD*, *mutS* and *mutT*) elevates mutagenesis rates to 0.5 mutations per 1 kb on average after 30 generations of growth in *E. coli* XL1-Red (Fig. 13) (Greener, A. *et al.* 1997). Although these characteristics provide significant advantages in some applications, this technique also has some disadvantages that are inherent to the random mutations. These drawbacks can be summarized in three important aspects, such as (i) instability of the mutator strain, (ii) rate of mutation within the gene sequence, and (iii) selectivity of the modifications in the gene against other changes in the plasmid.

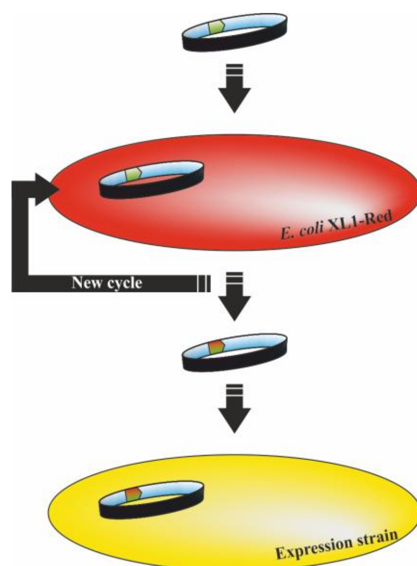

Figure 13. General scheme of directed evolution by a mutator strain

The first drawback is that the mutator phenotype is intrinsically unstable and must be monitored. Likewise, since higher frequency of modifications causes slow growth rate of the strain, those clones with reduced mutator phenotype will prevent further mutagenesis due to culture overgrowth. The second inconvenient is associated with the mutation rate

throughout the sequence, since many days of growth are necessary if more than a couple mutations are desired or a faster evolutionary improvement is required (Daugherty, P. S. *et al.* 2000). In such cases, PCR-based mutagenesis is recommended to consume less time and achieve a higher amount of modifications (Greener, A. *et al.* 1997; Nguyen, A. W. *et al.* 2003). The last problem is related to modifications outside the sequence of the target gene, such as the promoter, replication origin, or resistance marker. In this case, mutations within the target gene are not achieved during evolution, resulting in the selection of false positives or loss of those improved mutant clones (Greener, A. *et al.* 1997).

8.1.2. Error-prone PCR

One of the most employed and preferred methodology to generate random mutations is error-prone PCR. This protocol modifies standard PCR methods to enhance the rate and frequency of errors inherent to the polymerase (Cadwell, R. C. *et al.* 1992). Although *Taq* polymerase (Keohavong, P. *et al.* 1989) has been widely employed due to its high error frequency associated toward AT to GC changes, nowadays there are procedures that include different polymerases whose biases allow to increase a particular mutation type, such as a GC to AT change (Cirino, P. C. *et al.* 2003).

Likewise, error-prone PCR protocols usually modify the $MgCl_2$ content with respect to usual PCR procedures in order to increment the error-rate (Lin-Goerke, J. L. *et al.* 1997). For example, a higher $MgCl_2$ concentration stabilizes non-complementary pairs (Ling, L. L. *et al.* 1991; Cadwell, R. C. *et al.* 1994). In this sense, our group has established that the presence of an adequate concentration of $MnCl_2$ and low amount of $MgCl_2$ is crucial to obtain mutant clones. Similarly, changes in the ratio of nucleotides (Nishiya, Y. *et al.* 1994; Fromant, M. *et al.* 1995; Shafikhani, S. *et al.* 1997), or the inclusion of nucleotide analogs (*e.g.* 8-oxo-dGTP or dITP) (Spee, J. H. *et al.* 1993) can cause mutations. Other options to generate mutations throughout the sequence are increasing/decreasing the number of cycles, or altering the initial template concentration (Cirino, P. C. *et al.* 2003). However, it is worth mentioning that the same error-prone PCR protocol likely exhibits different mutation frequencies among different genes, because this methodology is dependent on the length and base composition of the template (Fig. 14). Thus, an appropriate level of mutations can be successfully found by checking representative samples obtained by different error-prone PCR protocols (Cirino, P. C. *et al.* 2003).

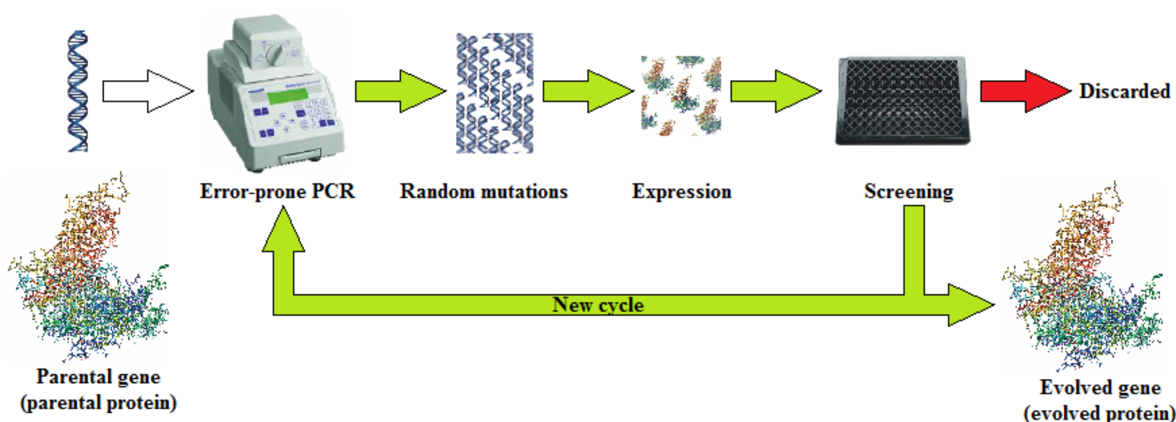

Figure 14. General scheme of directed evolution by error-prone PCR

8.1.3. DNA shuffling

Some years ago, Stemmer developed a new methodology for *in vitro* recombination of homologous genes named DNA shuffling (Stemmer, W. P. 1994). In this technique genes are digested randomly by DNase I, and thereafter the obtained fragments are amplified by PCR. Later, some fragments with a specific size are purified from the whole pool, and then reassembled by normal PCR without primers (Fig. 15).

Thus, recombination of fragments from different parents tends to be in regions of high sequence identity, sites that anneal and reassemble a new sequence. Thereafter, a PCR protocol is performed with the primers to generate full-length chimeras suitable for cloning into an expression plasmid (Joern, J. M. 2003). Like other random mutations protocols, this approach may yield either enzyme with improved activity and/or stability (Brühlmann, F. *et al.* 1999; Chang, C.-C. J. *et al.* 1999; Christians, F. C. *et al.* 1999; Ness, J. E. *et al.* 1999), or too many mutations (Zhao, H. *et al.* 1997) or crossovers (Kikuchi, M. *et al.* 1999) to be useful.

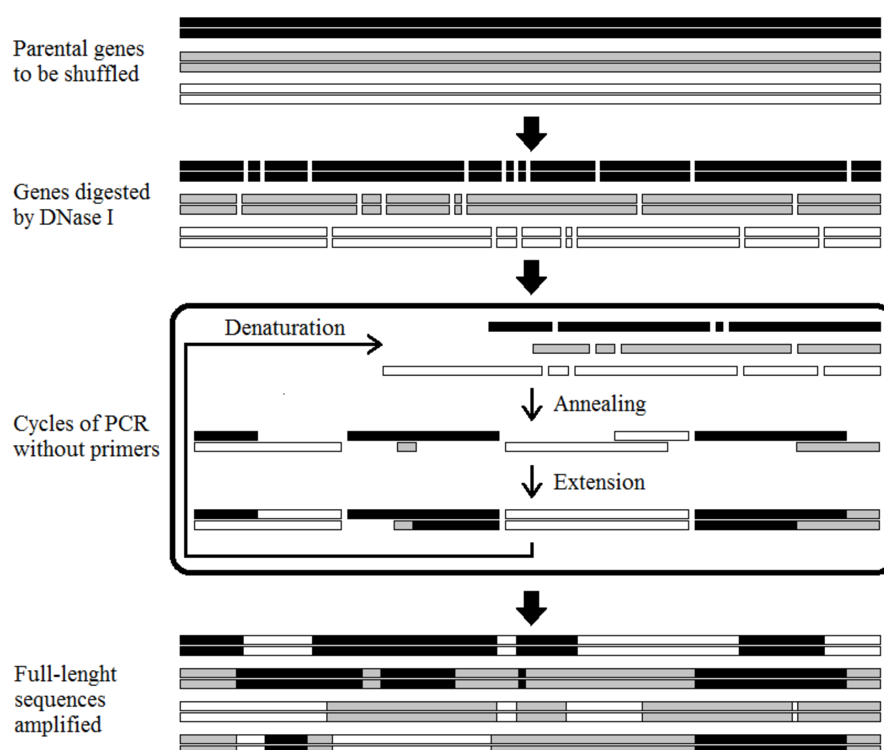

Figure 15. General scheme of directed evolution by DNA shuffling

8.1.4. Gene assembling mutagenesis

PCR protocols have been employed to assemble whole genomes, plasmids, and even viral genomes from relative short, synthetic, overlapping oligonucleotides (Stemmer, W. P. C. *et al.* 1995; Cello, J. *et al.* 2002). Thus, a set of primers spanning the length of a particular gene might be used to modify its sequence by replacing primers and reassembling the gene (Fig. 16). These primers (*i.e.* degenerate primers for both strands) encoding new amino acid residues produce libraries of mutants where multiple mutations can be obtained in a single step and with no limitations on their proximity (Bessette, P. H. *et al.* 2003).

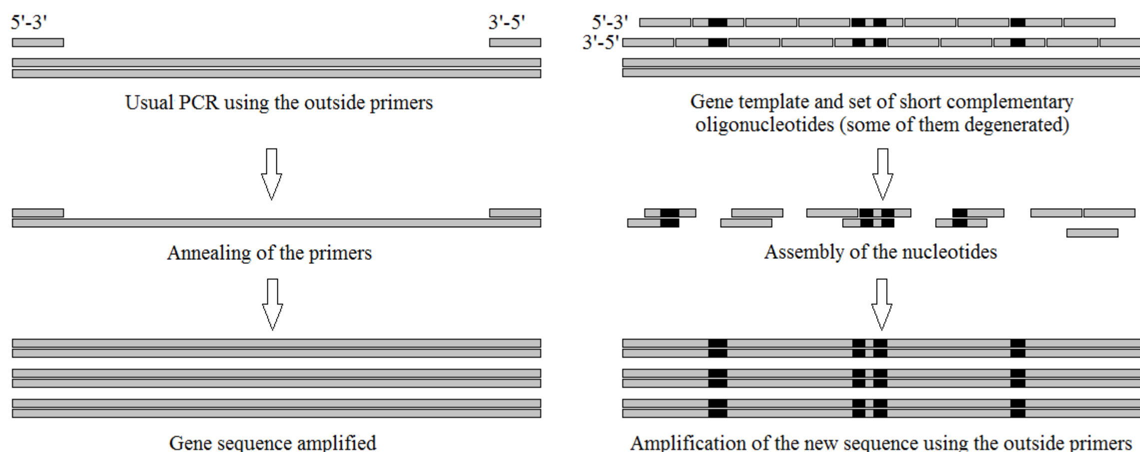

Figure 16. General scheme of directed evolution by gene assembling mutagenesis

This technique is recognized for its simplicity and possibility to achieve multiple mutated sites. Nevertheless a final product without template contamination is sometimes difficult to be obtained due to the presence of non-mutated sequences, or sequences with other modifications due to the error associated to the polymerases. Thereby, the characteristics of the oligonucleotides (*e.g.* length and number), the number of cycles (Bessette, P. H. *et al.* 2003), and the employed PCR protocol influence the success of the technique.

8.2. Host selection

According to BCC Research (BCC Research 2014b), the global market for enzymes in industrial applications was nearly $4.5 billion in 2012 and $4.8 billion in 2013, whereas it is expected to reach around $7.1 billion by 2018, which means a 8.2 % increment in 2018 with respect to 2013. Although protein homologous expression can be carried out in the parental microorganism to obtain enzymes at industrial scale, several companies employ heterologous expression in their processes.

Adequate heterologous expression of those encoding genes of interest is the main drawback to be overcome (Alcalde, M. 2012). In this sense, *Escherichia coli, Saccharomyces cerevisiae* and *Bacillus subtilis* are the main microorganisms employed for this purpose (Neidleman, S. L. 1997). The host microorganism should be adequate for correct protein expression, codon usage, desired product yield, among others (Alcalde, M. 2012).

Thus, advantages of genetically engineered microorganisms in the expression of foreign proteins are (i) increased availability and yield, (ii) bioprocess control, (iii) well-known microorganisms are employed, and (iv) cost reduction (Neidleman, S. L. 1997). In this sense, hundreds of bioproducts have been launched on the multibillion dollar industrial enzyme market, and most of them are recombinant proteins (Demain, A. L. *et al.* 2016). However, many inconvenients should be solved: (i) the integrity and purity of enzymes must be tested and assured, (ii) the quality control requires enough yield, (iii) continuous monitoring to avoid protein instability, (iv) sufficient knowledge about the host microorganism, and (v) worldwide government regulation framework related to recombinant proteins (Neidleman, S. L. 1997).

8.3. Screening and selection

HTS was introduced at the end of 80's (Pereira, D. A. *et al.* 2007) and has allowed researchers from academia and industry to improve the efficiency and speed of directed evolution during these last decades. In this endeavour, some bottlenecks have emerged as real obstacles to be surpassed in HTS studies, such as reagents, sensitivity and efficiency of the technique liquid handling platform, costs, and so forth. Most of these aspects must be set up in early stages of the study in order to avoid false positives, false negatives, wasting time and unnecessary expense of resources.

Thus, an adequate expression system and a satisfactory high-throughput assay should be settled before the generation of the library of enzyme variants, based on an accurate methodology, which must be very sensitive to detect low activity levels of the parental enzyme at the beginning of the directed evolution experiment (Cirino, P. C. *et al.* 2003).

8.3.1. Genetic selection

Genetic selection is considered a powerful instrument to screen protein sequences submitted to directed molecular evolution. This approach has been used to analyze the function of enzymes involved in DNA metabolism, to study the mutability of protein domains, and to generate mutant proteins possessing properties different from those selected by natural evolution (Sneeden, J. L. *et al.* 2003). Genetic selection can be accomplished by different techniques.

Firstly, a genetic selection can be carried out by growing the microorganism under restrictive conditions (Camps, M. *et al.* 2003), taking into account different aspects such as the presence of inducers, substrate supplementation, temperature or antibiotic resistance. Likewise, complementation emerges as another useful approach when the mutant strain is lacking or deficient in a specific gene (Sneeden, J. L. *et al.* 2003). These techniques are usually employed and no special skills are needed to accomplish the genetic manipulation.

Other sophisticated methods are based on the upstream or downstream manipulation of the gene sequence, as well as the interaction of the protein structure with exogenous material. On the other hand, the so-called autogene selection (Chelliserrykattil, J. *et al.* 2003) is based on the ability of a protein to amplify its own gene. Similarly, genetic selection can be carried out by protein fusion with a reporter protein (*e.g.* green fluorescent protein) (Waldo, G. S. *et al.* 1999) and chloramphenicol acetyltransferase (Maxwell, K. L. *et al.* 1999)) or by the Proside technique (PRotein Stability Increased by Directed Evolution), which is an *in vitro* selection technique that links the proteolytic resistance of the protein to be stabilized with the infectivity of a filamentous phage (Martin, A. *et al.* 2001). Finally, the approach towards protein epitopes allows finding small molecules or even small polypeptides (named mini-proteins) that retain the ability to bind to a target protein (Rudgers, G. W. *et al.* 2001).

8.3.2. Screening for enzymes

After genetic selection, screening is obviously necessary in any HTS process. Once the mutant library is achieved, the search of the desired mutant is the first challenge to beat. This stage in HTS is usually carried out by spectrophotometric or fluorescence techniques (Salazar, O. *et al.* 2003), although some screenings are performed directly in agar plates

(*e.g.* different morphology and colour, presence/absence), where changes can be directly detected by simple visualization (López-Camacho, C. *et al.* 1996) or digital image analysis (Joo, H. *et al.* 1999). Taking into account these considerations, directed evolution can be sometimes thwarted by experimental limitations because a huge amount of mutants must be screened (Salazar, O. *et al.* 2003).

The most difficult part in designing a HTS study is to set up a screening that (i) captures the desired feature, and (ii) can be repeated thousands of times with different samples. In this stage, efforts are focused on the continuous development, validation, implementation (Salazar, O. *et al.* 2003) and improvement of the method, ensuring that a relevant and robust screening is established (Macarrón, R. *et al.* 2011). Once the screening has been accomplished, data are shown as a cloud of spots (where each spot corresponds to an assay), which must be analyzed to determine if the experiment has been successful. In a directed evolution experiment, thousands of data points are generated every day and contain information about library quality, evolvability of the parental enzyme, and precision of the high-throughput method (Salazar, O. *et al.* 2003). In this sense, statistics guarantees the adequate compilation, manipulation, processing and interpretation of all data information.

Summarizing, the assay must prove low variability and high sample signal compared to background signal, in order to minimize the presence of false negatives and false positives as much as possible. In addition, screening must have sufficient throughput and low cost to handle large sample collections (Macarrón, R. *et al.* 2011).

8.4. Statistical analysis

Selection of "hits" (considered as those spots which show better results compared to the parental strain) is the final and most important decision in a screening study. However, the common fluctuation and variability of the system complicate the correct selection among all the results that are obtained as a graphic representation from the experimental data. In this context, reproducibility of experiments gives confidence to the researcher, but statistics is the ultimate tool to make decisions in science, especially when randomness is not hazard. Thus, statistical skills are necessary for the analysis of experiments performed during the screening in order to facilitate the correct interpretation of data, and thus the acquisition of valuable information to make further decisions

Some authors have addressed the importance of statistical analysis in HTS workflow (Coma, I. *et al.* 2009), emphasizing that key decisions have to be made at least at three crucial stages: (i) assay development (reliability, reproducibility and sensitivity), (ii) HTS process monitoring (in order to avoid bias and misleading hit identification), and (iii) data analysis of preliminary HTS data (proper identification of hits).

8.4.1. Statistical tools

There are several approaches to evaluate the information flow during HTS experiments. The Z-factor (commonly written as Z') is widely employed as a measure of statistical effect size, and it has been proposed to be used in HTS in order to judge whether the response in a particular assay is large enough to warrant further attention (Zhang, J.-H. *et al.* 1999). It is an attempt to quantify the suitability of a particular assay for use in a full-scale, high-throughput screen. On the other hand, the z-score (also called standard score, z-value or

normal score) is the signed number of standard deviations an observation or datum is above the mean. It is a dimensionless quantity which is obtained by subtracting the population mean from an individual raw score, and then dividing the difference by the population standard deviation (Coma, I. *et al.* 2009).

Similarly, new statistical approaches have recently emerged to improve the quality of the screening such as MSR (Minimum Significant Ratio) (Eastwood, B. J. *et al.* 2006), that considers statistical significance, as well as B-score, *BZ*-scores and R-score (Wu, Z. *et al.* 2008; Coma, I. *et al.* 2009) that analyze temporal or spatial patterns (*i.e.* well, row and edge effects on plates), background signals and digital images. No matter the statistical tool, the acquired information should be revised in order to identify abnormal patterns and early dead-ends.

8.4.2. Assessment of assay quality

HTS experiments obviously provide a huge amount of raw data and require effective automatic procedures to select real hits. Thus, systematic error sources must be identified (Gagarin, A. *et al.* 2006) in order to set up appropriate error limits according to quality management (Westgard, J. O. 2006). With the aim to guarantee the process quality and its cost implications, type I errors (probability of a false error rejection) and type II errors (probability of not rejecting an error) must be avoided (Coma, I. *et al.* 2009).

Reduction of spatial and temporal variability is usually very difficult to maintain throughout HTS. Searching errors by inspection of plates using either software programs, digital images, or even at first sight, can help to detect these abnormal systematic patterns (Gunter, B. *et al.* 2003; Gribbon, P. *et al.* 2005).

8.4.3. Statistically-guided selection of hits in HTS

In order to make the screening valuable, the huge quantity of spots should be expressed as meaningful information and knowledge after being processed, with the purpose to interpret and understand the tendencies of the study. Although the limit between false negatives and false positives could be interpreted as an ambiguous assumption, the threshold will be used to discriminate. Although type I errors are unacceptable, type II errors are annoying, but sometimes those weak hits would be better chosen, assuming the risk to repeat the assays and unveil their relevance in subsequent stages (Coma, I. *et al.* 2009).

9. COMPUTATIONAL INTELLIGENCE: BIOINFORMATICS

Nowadays, computational intelligence (CI) is a consolidated research area which is employed worldwide, and it is expanding every day. Compared to conventional methods, many difficult tasks can be easily performed by CI (Hassanien, A. E. *et al.* 2013). In particular, bioinformatics employs computational resources to handle biological data, and this has spawned a new research area, that is currently focused on expediting, ameliorating, and diversifying research information (Holton, T. A. *et al.* 2013). Integration of available CI resources has vastly facilitated research in biotechnology as well.

9.1. Current computational intelligence

During the last decades the huge amount of available information about genomes, protein and gene sequences, and gene expression data has claimed for effective and efficient computational tools for its storage, analysis and interpretation (Hassanien, A. E. *et al.* 2013). In this sense, bioinformatics encompasses different areas of informatics, applied mathematics, statistics, computer science, artificial intelligence, chemistry, and biochemistry, trying to explain biological events, even at molecular level (Gusfield, D. 2004; Cios, K. J. *et al.* 2005; Kelemen, A. *et al.* 2008; Smolinski, T. G. *et al.* 2008b; Smolinski, T. G. *et al.* 2008a; Valentini, G. *et al.* 2009). Application of computational methods in biological research has allowed many advances in sequence alignment (*e.g.* gene and protein structure), gene finding, genome assembly, molecular predictions (*e.g.* protein structure, gene expression and protein-protein interactions), and modelling of evolution (Smolinski, T. G. *et al.* 2008b; Smolinski, T. G. *et al.* 2008a). Recent efforts have been focused to a new era of CI, where principles, theoretical aspects, and design methodology of algorithms mimic Nature (Hassanien, A. E. *et al.* 2013).

9.2. Genome Analysis

Haemophilus influenzae (Fleischmann, R. D. *et al.* 1995) and *Mycoplasma genitalium* (Fraser, C. M. *et al.* 1995) were the first microorganisms whose genomes were sequenced. That was in 1995, and only two years later the genome sequences of two prototype of bacteria, *Escherichia coli* (Blattner, F. R. *et al.* 1997) and *Bacillus subtilis* (Kunst, F. *et al.* 1997) were available. By the beginning of 2000, 23 genomes of different unicellular organisms had been reported, and at least 70 microbial genomes were partially sequenced (Galperin, M. Y. *et al.* 2001). Nowadays, genome annotation is no longer the limiting step in research, due to faster sequencing techniques and analysis. However, the lack of knowledge about the encoded information of genes in each genome might be considered a challenge to be beaten in the future (Galperin, M. Y. *et al.* 2001).

In this sense, the structural-function annotation of unknown proteins identified in many genomes (indicated as “putative”) can be performed using several software packages which can:

1. search for sequence similarities, through BlastN (Altschul, S. F. *et al.* 1997) from NCBI (National Center for Biotechnology Information), or Clustal W and Clustal X (Larkin, M. A. *et al.* 2007) from EMBL-EBI (European Molecular Biology Laboratory - European Bioinformatic Institute);
2. compare the protein sequence in order to identify functional motifs and structural domains through BlastP (Altschul, S. F. *et al.* 1997) from NCBI, SMART (Schultz, J. *et al.* 1998; Letunic, I. *et al.* 2014) from EMBL-EBI, Prosite (Sigrist, C. J. A. *et al.* 2012) in ExPASy (Expert Protein Analysis System) bioinformatic resource portal (Artimo, P. *et al.* 2012) from SIB (Swiss Institute of Bioinformatics), or Pfam (Finn, R. D. *et al.* 2014) from EMBL-EBI;
3. perform structural prediction from sequences with low complexity, such as probable signal peptides, transmembrane segments, or coiled-coil regions through in ExPASy bioinformatic resource portal (Artimo, P. *et al.* 2012) from SIB, or PredictProtein (Yachdav, G. *et al.* 2014);
4. predict the secondary and even the tertiary structure of the protein through Phyre2 (Protein Homology/analogY Recognition Engine) (Kelley, L. A. *et al.* 2015) SwissModel accessible via ExPASy (Arnold, K. *et al.* 2006; Guex, N. *et al.* 2009;

Kiefer, F. *et al.* 2009; Biasini, M. *et al.* 2014), Robetta (Raman, S. *et al.* 2009; Song, Y. *et al.* 2013), I-Tasser (Zhang, Y. 2008; Roy, A. *et al.* 2010; Yang, J. *et al.* 2015), or Lomets (LOcal MEta-Threading-Server) (Wu, S. *et al.* 2007).

Frequently, some software programs for comparing sequences may fail, and then other approaches can be employed taking into consideration all other available data from the genome (Huymen, M. A. *et al.* 1997). Availability of many complete genomes offers new opportunities to predict gene functions in each of these genomes and in new ones, which rely on the basic premise that genetic information organization in each particular genome reflects a long history of mutations. From an evolutionary point of view, those annotations are quite important and help to understand and analyze transference of functional information, phylogenetic patterns and their use for differential genome, examination of gene fusions and conserved gene strings, and so forth (Galperin, M. Y. *et al.* 2001).

9.3. Protein engineering: computational tools

Limitations shown by wild-type enzymes have been overcome thanks to protein engineering, which facilitates the design of mutant libraries in directed evolution studies based on bioinformatic tools. In addition, advanced high-throughput methods and liquid handling platforms have allowed the tailor-made design of biocatalysts for industrial applications (Davids, T. *et al.* 2013). Years ago an enzyme-catalyzed process was conditioned to enzyme limitations, but nowadays many of these biocatalysts fit perfectly to process specifications (Bornscheuer, U. T. *et al.* 2012).

Protein crystallography or nuclear magnetic resonance (NMR) are techniques that have allowed the elucidation of many proteins structures and they have been employed to identify potential sites for mutagenesis or “hot spots” (Damborsky, J. *et al.* 2014). However, several chemical and physical properties of enzymes (*e.g.* substrate specificity, stability, chemo- and enantioselectivity) are difficult to optimize by rational design (Fox, R. J. *et al.* 2007). For these reasons, many computational tools are very useful guide further structural modifications to alter enzyme properties (Damborsky, J. *et al.* 2009). Some widely used examples are represented by software packages such as HotSpot Wizard (Pavelka, A. *et al.* 2009), ProSAR (Fox, R. J. *et al.* 2007) and SCHEMA (Meyer, M. M. *et al.* 2003). Likewise, other recent improved proposals are ASRA (Feng, X. *et al.* 2012), ZEBRA (Suplatov, D. *et al.* 2013), and JANUS (Addington, T. A. *et al.* 2013), among others. Particularly interesting for the present work is the recent new alternative for the computational design of penicillin acylases developed by the same creators of the ZEBRA web server (Suplatov, D. *et al.* 2014).

9.4. Docking

Elucidation of those mechanisms describing biomolecular recognition has become so important that it has contributed to push forward research in many fields during the last decades (Boehr, D. D. *et al.* 2009; Csermely, P. *et al.* 2010; Changeux, J.-P. *et al.* 2011). More than a half century ago, the rigid idea of binding based on lock-and-key model was changed to a sophisticated theory based on a dynamic and flexible mechanism (Koshland, D. E. 1958; Monod, J. *et al.* 1965). Such concept introduced more complexity for explaining a biomolecular recognition process, which was described as an intricate combination of orchestrated and random motions (Feixas, F. *et al.* 2014).

In this sense, computational docking is the predicting process to estimate the best orientation and conformation of a small molecule when bound to a target larger receptor (usually a protein) in order to form a stable complex molecule (Khamis, M. A. *et al.* 2015).

Elucidation of the role played by the ligand throughout binding is one of the main challenges in research, trying whether the ligand stabilizes specific pre-existent conformational states displayed by the protein, or in contrast it is responsible for inducing a conformational change in the receptor upon binding (Feixas, F. *et al.* 2014). Recent studies have shown that conformational selection (*i.e.* first option) is usually followed by a conformational adjustment (*i.e.* second option) (Wlodarski, T. *et al.* 2009).

Docking tools emerge in order to predict whether and how a small molecule binds to a target (Ain, Q. U. *et al.* 2015) according to a mathematical predictive model, which represents binding free energy and hence stability of resulting complex molecule (Khamis, M. A. *et al.* 2015). The docking reliability depends on the accuracy of the selected scoring function of the predictive model. Improving the accuracy of those scoring functions over the years, for structure-based binding affinity prediction or virtual screening has proven to be a challenge in any class of method (Ain, Q. U. *et al.* 2015), especially because although current docking methods deal with flexible ligands, managing receptor flexibility has been difficult (B-Rao, C. *et al.* 2009).

Likewise, allosteric effects have always been strongly linked to biomolecular recognition, which induces a protein conformational change that affects activity of another site and alters protein function (Feixas, F. *et al.* 2014). However, identification of allosteric sites and allosteric mechanisms has never been a straightforward task due to the flexibility, plasticity and wide spectrum of protein motions that gives them ability to adopt multiple conformations. In fact, any conformational states (*i.e.* bounded or unbounded) might be more or less populated, and this can hardly be captured by X-ray crystallography (Feixas, F. *et al.* 2014). During the last few years, protein dynamics has advanced noticeably with techniques such as NMR, allowing that enzyme motion and generation of conformational ensembles to be understood (Dyson, H. J. *et al.* 2004; Mittermaier, A. *et al.* 2006; Fenwick, R. B. *et al.* 2011). In addition, specialized computer hardware and software have answered many questions about protein folding at atomic level (Lindorff-Larsen, K. *et al.* 2011), biomolecular recognition (Shan, Y. *et al.* 2011), as well as design and development of screenings (Jorgensen, W. L. 2004; Nichols, S. E. *et al.* 2012). Therefore, combination of experimental techniques and theoretical simulations have allowed a better comprehension about interactions and mechanisms in biomolecular recognition (Markwick, P. R. L. *et al.* 2007; van Gunsteren, W. F. *et al.* 2008; Candotti, M. *et al.* 2013; Nygaard, R. *et al.* 2013).

II OBJECTIVES

Pharmaceutical industry constantly focuses its efforts on searching new drugs (including new antimicrobials) and improved manufacturing processes to increase its benefits. Therefore, development of new enzyme-catalyzed processes for the production of molecules with therapeutic activity, as well as the application of enzymes as enzybiotics is of great interest.

Despite these efforts, pathogenic microorganisms keep on acquiring new resistance mechanisms against these antimicrobial agents. In addition, many of those new powerful compounds cannot be employed due to their toxic and allergenic capacities. Likewise, it is worth emphasizing that these difficulties are coupled with low benefits, which in turn reduce R&D investments, and then obviously a lower number of new molecules are discovered, becoming a worldwide vicious circle. The problem is more dramatic in the case of fungal infections, because there are very few compounds available on the market. Thus, nowadays it is an important challenge obtaining of newfangled molecules to control and eliminate pathogenic microorganisms more effectively and safely, as well as alternative processes which help to this aim.

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 both enzymes of great interest to be used in industrial bioreactors for the synthesis of β-lactam antibiotics and semisynthetic antifungals, as well as antimicrobial agents or enzybiotics whose mechanism is based on *quorum quenching* processes. Molecular directed evolution of these enzymes will provide a library of enzymes with improved catalytic activities to be employed in many different processes. Thus, high-throughput screening (HTS) is an essential tool to perform the massive analysis of thousands of mutant clones, and for the development of a protocol which will allow the selection of those recombinant clones displaying better acylase activity with respect to the parental strains under the tested conditions.

Based on these facts, the main objective of the present Thesis is to obtain libraries of mutant enzymes from *Sl*PVA and *Au*AAC by directed molecular evolution, which display improved catalytic capacities and could be employed in many processes. Likewise, the development of this objective will allow establishing an alternative platform to perform directed molecular evolution in Gram-positive bacteria, and set up a system integrating the available technologies, the expression microorganism *Rhodococcus* sp. T104, and the shuttle vector pENV19. In particular, the specific objectives addressed in the present study include:

1. the analysis of the genome of both microorganisms and the proposal of *Sl*PVA and *Au*AAC roles in bacteria;
2. the generation of random mutations of the penicillin V acylase-encoding gene from *S. lavendulae* ATCC 13664 and aculeacin A acylase-encoding gene from *A. utahensis* NRRL 12052, and the selection of recombinant enzymes by high-throughput screening capable to catalyze different reactions under specific conditions:
    - 2.1. selection of recombinant enzymes with improved catalytic activity against the natural substrate penicillin V;
    - 2.2. selection of recombinant enzymes with acylase activity against different AHLs involved in *quorum sensing* processes;

   2.3. selection of acylases with improved activities at low pH values or low temperature values, or at both conditions, with respect to the optimal conditions shown by the parental enzymes;
   2.4. selection of acylases with improved activities against β-keto substituted aliphatic AHLs at different operational conditions;
3. prediction and analysis of the operational conditions and behavior of some of the recombinant enzymes by bioinformatic tools.

III MATERIALS AND METHODS

1. CHEMICALS REAGENTS

Organic solvents such as ethanol, methanol, chloroform, acetic acid, acetone and 2-propanol were purchased from Scharlab (Spain). Likewise, glycerol and DMSO were supplied by Sigma-Aldrich (USA). EDTA and SDS were purchased from Merk (Germany). HPLC grade solvents were obtained from Scharlab (Spain).

Bacto agar, malt extract and bacto tryptone were from Difco (Spain). Yeast extract, soy peptone and agarose (D1 Media EEO) were purchased from Pronadisa (Spain). Antibiotics (*i.e.* kanamycin, ampicillin, tobramycin, tetracycline, nalidixic acid and thiostrepton) were supplied by Sigma-Aldrich (USA). Potassium phosphate (mono- and dibasic), sodium chloride, sucrose and D-(+)-glucose were supplied by Sigma-Aldrich (USA).

Plasmid isolation and PCR products purification kits were from Roche (Germany). DNA purification kit from agarose gel slices was from GE Healthcare (United Kingdom). Solution of phenol saturated with 0.1 M citrate buffer pH 4.3 and isoamyl alcohol were from Sigma-Aldrich (USA). Restrictions enzymes and dNTPs were supplied by New England BioLabs (USA). *Taq* DNA polymerase and *Pfu* DNA polymerase were purchased from Fisher Scientific (USA). T4 DNA ligase was from Roche (Germany). RNase was from Sigma-Aldrich (USA). DNA markers for electrophoresis were GeneRuler 1 Kb DNA ladder from Thermo Scientific (USA), and Lambda DNA/*Bst*EII from Fermentas (Germany). Fluorescent nucleic acid dye employed in agarose gel was GelRed nucleic acid gel stain from Biotium (USA). All oligonucleotides were synthesized by Sigma-Aldrich (Germany). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and β-mercaptoethanol were purchased in Sigma-Aldrich (USA). Agarose D-1 low EEO for DNA electrophoresis was supplied by Pronadisa (Spain).

Likewise, butyryl chloride, hexanoyl chloride, octanoyl chloride, capric acid chloride, lauroyl chloride, myristoyl chloride, *p*-dimethylaminobenzaldehyde (PDAB), *o*-phthalaldehyde solution (OPA), bovine serum albumin (BSA), and lysozyme were from Sigma-Aldrich (USA). Fluram and L-homoserine lactone chloride were purchased from Santa Cruz Biotechnology (USA). *N*-(β-ketocaproyl)-L-homoserine lactone, *N*-(3-oxo-octanoyl)-L-homoserine lactone, *N*-(3-oxo-dodecanoyl)-L-homoserine lactone and *N*-(3-oxo-myristoyl)-L-homoserine lactone were from Santa Cruz Biotechnology (USA), whereas *N*-(3-oxo-decanoyl)-L-homoserine lactone was from Sigma-Aldrich (USA). 6-aminopenicillanic acid (6-APA) was a gift from Antibíóticos S.A. (León, Spain). Penicillin V, aculeacin A and caspofungin diacetate were purchased from Sigma-Aldrich (USA), and pneumocandin B was gift from Merk (USA). QuantiChrom^{TM} Iron Assay Kit (DIFE-250) was from BioAssay Systems LLC (USA).

Chromatographic cartridges Bio-Scale Mini UNOsphere S were from Bio-Rad (USA). Likewise, protein ladder of electrophoresis and reagents to assess protein content were from Bio-Rad (USA). Those products employed in proteins electrophoresis, such as acrylamide, bis-acrylamide, 2-propanol and Coomassie brilliant blue G250 were provided by Fluka (Switzerland). Acids and bases were obtained from Scharlab (Spain). Furthermore, Sigma-Aldrich (USA) provided PEG 35000. Bromophenol blue, tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS) and acetic acid were acquired from Sigma-Aldrich (USA).

2. BACTERIAL STRAINS, PLASMIDS AND OLIGONUCLEOTIDES

Bacterial strains employed in this study are indicated in Table 5, whereas vectors are shown in Table 6. Penicillin V acylase encoding gene (*pva*), aculeacin A acylase encoding gene (*aac*) and acyl-homoserine lactone acylase encoding gene (*ahla*) were cloned within the shuttle vector pENV19 (for further information, see Supplement S.2 in the Supplementary Material Chapter) and generated the recombinant plasmids pENV19*pva*, pENV19*aac* and pENV19*ahla*. Similarly, recombinant clones obtained during this study were hosted in pENV19, and only the selected hits were cloned and expressed in pENS (for further information, see Supplement S.3 in the Supplementary Material Chapter). Genes were cloned between *Xba*I and *Eco*RI restriction sites. Shuttle vector pENV19 and pENS were based in previous articles (García-Hidalgo, J. *et al.* 2012; García-Hidalgo, J. *et al.* 2013; Torres-Bacete, J. *et al.* 2015) and employed to express the enzymes and host genes. Shuttle vector pENS was designed as an alternative expression system in Gram-positive bacteria, which is derived from pENV19, and contains the signal peptide of PHB depolymerase from *S. exfoliatus* DSMZ 41693 between *Pst*I and *Sal*I.

Table 5. Bacterial strains

Bacterial strain	Relevant description (genotype)	Reference
S. lavendulae ATCC 13664	Presence of native penicillin V acylase	(Batchelor, F. R. *et al.* 1961; Torres, R. *et al.* 1998)
A. utahensis NRRL 12052	Presence of native aculeacin A acylase and native acyl-homoserine lactone acylase	(Takeshima, H. *et al.* 1989; Inokoshi, J. *et al.* 1992; Velasco-Bucheli, R. *et al.* 2015)
S. lividans 1326	Expression system (*Tsr*^{S} SLP2$^+$ SLP3$^+$)	(Hopwood, D. A. *et al.* 1983)
S. lividans 1326 pEM4*pva*	Presence of recombinant penicillin V acylase (*Tsr*^{R} *Apr*^{R})	(Torres-Bacete, J. *et al.* 2015)
S. lividans 1326 pEM4*aac*	Presence of recombinant aculeacin A acylase (*Tsr*^{R} *Apr*^{R})	(Torres-Bacete, J. *et al.* 2007)
S. exfoliatus DSMZ 41693	Containing signal peptide of poly(3-hydroxybutyrate) depolymerase	(Klingbeil, B. *et al.* 1996; García-Hidalgo, J. *et al.* 2012)
Rhodococcus sp. T104	Expression system and host genes (*Kn*^{s})	(Hernández-Jústiz, O. *et al.* 1999)
E. coli XL1-Red (Agilent Technologies)	For random mutagenesis (*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* Tet^R)	(Greener, A. *et al.* 1997)
E. coli DH5α	For hosting recombinant plasmids (F$^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1)	(Hanahan, D. 1983)
E. coli ET12567	For transforming *S. lividans* by conjugation (*dam13*::Tn*9 dcm6 hsdM hsdR recF143 zjj201*::Tn*10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44* F$^-$)	(MacNeil, D. J. *et al.* 1992)

Recombinant plasmids containing *ahla*, *pva* and *aac* genes are derived from pENV19, which is a small bifunctional vector derived from two plasmids: (i) pNV19 (Chiba, K. *et al.* 2007) kindly provided by Dr. Gerben Zylstra (Rutgers University, USA), and (ii) pEM4 (Quirós, L. M. *et al.* 1998) gently provided by Dr. José Antonio Salas (Oviedo University, Spain). Thus, shuttle vector pENV19 contains replication origin for *E. coli* and *Nocardia* spp., *neo* gene (kanamycin/neomycin resistance gene), and multiple cloning sites (MCS) from pEM4. Likewise, the strong promoter of the erythromycin resistance gene (p*ermE**) from *Saccharopolyspora erithraea* (Bibb, M. J. *et al.* 1985) is located within *Hind*III and *Pst*I restriction sites. pENV19 vector has been previously developed in the Enzymatic Biotechnology research group of Complutense University of Madrid in collaboration with the group of Dr. Gerben Zylstra of the Biotechnology Center for Agriculture and the Environment of the University of Rutgers, New Brunswick, New Jersey, USA (Hormigo, D. 2009).

Table 6. Vectors

Plasmid	Description (relevant genotype)	Reference
pEM4	Contains constitutive p*ermE** promoter (*Apr*^{R}, *Tsr*^{R}, permE*)	(Quirós, L. M. *et al.* 1998)
pNV19	*E. coli-Rhodococcus* shuttle vector (4.4 Kb, *Kn*^{R} pAL5000ori ColE1ori *lacZ*)	(Chiba, K. *et al.* 2007)
pENV19	*E. coli-Rhodococcus* shuttle vector with the constitutive p*ermE** promoter (5.1 Kb, *Kn*^{R} pAL5000ori p*ermE** ColE1ori)	(García-Hidalgo, J. *et al.* 2012; García-Hidalgo, J. *et al.* 2013; Torres-Bacete, J. *et al.* 2015)
pENS	*E. coli-Rhodococcus* shuttle vector with the constitutive p*ermE** promoter and the signal peptide of PHB depolymerase from *S. exfoliatus* DSMZ 41693 (5.1 Kb, Kn^R pAL5000ori p*ermE** ColE1ori)	This study
pEM4	Allow transference of DNA information to *Streptomyces* (7.9 Kb, Tsr^R Apr^R p*ermE** promoter)	(Quirós, L. M. *et al.* 1998)

The sequences of *pva* and *aac* as well as the location of the primers are described in Supplements S.4 and S.5 in the Supplementary Material Chapter, respectively. Additionally, acyl-homoserine lactone acylase (*ahla*) sequence detected in *A. utahensis* genome (Velasco-Bucheli, R. *et al.* 2015) was cloned similarly to the other genes. Primer sets were designed using primer-Blast software (Altschul, S. F. *et al.* 1997), and sequences are shown in Table 7.

Table 7. Primers employed in DNA sequencing[1]

	Primer	Sequence	Tm
pva	PVAatg	5'-TGC**TCTAGA***GGAGG*AACACCC*ATG*ACCTTCCGTAACCGCCTCAGACTG-3'	85
	αPVA	5'-GC**TCTAGA**GGCGGCGGCCTCT-3'	74
	N	5'-CGAACCCGTCGGCCAGCGTGCACA-3'	81
	NCsacI	5'-GCCGCCGGGTACAACGCCTGGC-3'	68
	NCsac3	5'-CTGCCACCCGCCGCAGCCTCC-3'	75
	NCsac4	5'-GCCGCCAGCTCGTCCGTGATCC-3'	72
	C	5'-GGCCGATCCCACGGTCTATCTGGT-3'	77
	CsacII	5'-CCG**GAATTC**CCGCGGGCACCGCCGCCGCC-3'	80
	PVAstop	5'-CAACAGCCGGTGCCCGGTGG-3'	70
	PVA-F	5´-CCG**GAATTC**CCTACCGCCGCTCGTGCACCCG-3´	108
aac	AACgtg	5'-TGC**TCTAGA***GGAGG*TGCCGCC*GTG*ACGTCCTCGTACATGCGCC-3'	89
	αAAC	5'- GC**TCTAGA**GGCGGCTATGCGGCCCTGAT -3'	76
	AAC-Nt	5'-GCTCACCGTTGGCCGTCACCACGCTCTCGGC-3'	89
	AAC-4	5'-GTCGCCGCGACGCCACCGACAGCC-3'	90
	AAC-5	5'-CACCTTCGACTGGACGCCGGC-3'	73
	AAC-6	5'-CGTCGCGGACGAGTTCGGCGCCG-3'	86
	AAC-Ct	5'-TCGTGCCGGGCGTGGGATACCCGC-3'	80
	AAC-2	5'-CCG**GAATTC**CTCAGCGTCCCCGCTGTGCCAC-3'	104
ahla	AHLA1	5'-GC**TCTAGA***GGAGG*TGCCGCC*GTG*GCCCGTCCGTTCA-3'	90
	AHLA-ab	5'-GC**TCTAGA**TCCGGCGCCGACCGTCCGCACGCCGT-3'	92
	AHLA-Nt	5'-GGTTGTACCCGGTGACGTAG-3'	64
	AHLA-3	5'-CGTACTACAGCGGCATCCAG-3'	66
	AHLA-4	5'-ATGGTGTCCAGGGAGAACCC-3'	67
	AHLA-5	5'-AACCAGGTGTTCAGCATCCG-3'	67
	AHLA-6	5'-CCCAGCAGACCATCGTGTC-3'	67
	AHLA-Ct	5'-GTTCGTTCCGCCTCGACA-3'	68
	AHLA2	5'- CG**GAATTC**CTCACCGCGGCGCTCGCTCGGTCAGTCTGAT-3'	91
SP *pha*	SEPS1	5'-GC**CTGCAG***GGAGG*TGCGC*ATG*AAGATCAGACAACTCCTCGTC-3'	89
	SEPS2	5'-GC**GTCGAC**CGCCGTCGCACCGCCGACCGTCGTCGCGGCGATTCC-3'	75

[1] The restriction sites *Xba*I and *Eco*RI are shown in bold, ribosomal binding site sequence for *Streptomyces* is shown in italics, the start codons are shown in italics and underlined, and the stop codons are shown underlined

3. MEDIA AND CULTURE CONDITIONS

Luria-Bertani (LB) medium for growing *E. coli* was composed by yeast extract 5 g/L, bacto tryptone 10 g/L and NaCl 5 g/L (Sambrook, J. *et al.* 2001). *Rhodococcus* sp.T104

was inoculated in Yeast extract Tryptone (2xYT) medium (yeast extract 10 g/L, bacto tryptone 16 g/L and NaCl 5 g/L) supplemented with glucose 5 g/L and hereafter named 2xYT+G (Sambrook, J. *et al.* 2001). Likewise, Yeast Extract Malt Extract (YEME) medium (yeast extract 3 g/L, malt extract 3 g/L, bacto peptone 5 g/L, glucose 10 g/L, sucrose 340 g/L, 5 mM $MgCl_2 \cdot 6H_2O$) (Kieser, T. *et al.* 2000) supplemented with glycine 0.5 % was employed for cultivation and sporulation of *S. lavendulae*, *S. lividans* and *A. utahensis*. Solid media contained agar at 2 % concentration (Sambrook, J. *et al.* 1989). Solid medium for *Streptomyces* and *Actinoplanes* was R2YE, which was made with 80 mL of solution A (103 g sucrose, 5 g yeast extract, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 0.1 g casamino acids, 800 mL distilled water), 11 mL of solution B (1 mL KH_2PO_4 0.5 %, 8 mL 0.25 M $CaCl_2 \cdot 6H_2O$, 1.5 mL L-proline 20 %, 0.5 mL 1 M NaOH), 10 mL of TES buffer 5.73 %, adjusted at pH 7.2 (25 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, sucrose 10.3 %) and 0.2 mL of trace element solution ($ZnCl_2$ 40 mg/L, $FeCl_3 \cdot 6H_2O$ 200 mg/L, $CuCl_2 \cdot 2H_2O$ 10 mg/L, $MnCl_2 \cdot 4H_2O$ 10 mg/L, $Na_2B_4O_7 \cdot 10H_2O$ 10 mg/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10 mg/L) (Thompson, C. J. *et al.* 1980).

SFM medium was employed for *Streptomyces* sporulation, and its composition was mannitol 20 g/L, soy fluor 20 g/L, 10 mM $MgCl_2$ and agar 20 g/L. Recombinant strains containing recombinant plasmids with the acylases-encoding genes were cultured in TSB medium (tryptic soy 30 g/L) in baffle shake flasks.

When required, antibiotic concentration was adjusted to different values: ampicillin (Ap) at 100 µg/mL or kanamycin (Kn) at 50 µg/mL when *E. coli* DH5α was employed, and Kn at 100 µg/mL when *Rhodococcus* sp. T104 was used (García-Hidalgo, J. *et al.* 2012). *S. lividans* strains employed antibiotic thiostrepton (Tsr) at 5 mg/mL.

4. HANDLING TECHNIQUES AND ANALYSIS OF DNA

4.1. Purification and sequencing of DNA

Purification of genomic DNA from *Streptomyces lavendulae* ATCC13664 and *Actinoplanes utahensis* NRRL 12052 was performed as previously described (Kieser, T. *et al.* 2000). In each case, cells obtained from solid media were inoculated in 50 mL of YEME with glycine 0.5 % and incubated during 72 h at 250 rpm and 30ºC. Cells were harvested at 3000×*g* during 15 minutes and 4ºC, and then resuspended in 10 mL of sucrose 10 %. Thereafter, cells were harvested by centrifugation under the same conditions, and the pellet was resuspended in 10 mL of lysis buffer (25 mM Tris-HCl, 25 mM EDTA and sucrose 10 %, pH 8.0). This mixture was incubated at 37ºC during one hour in the presence of lysozyme at a final concentration of 10 mg/mL. Thereafter SDS was added at a final concentration of 2 % (p/v), and samples were gently stirred and further incubated for 10 min at 37ºC.

Thus, samples were mixed 6 consecutive times with phenol solution equilibrated with 1 M Tris-HCl pH 8.0, in presence of 8-hydroxyquinoline 0.1 %. Aqueous phase was separated by centrifugation at 3000×*g* each time. The last extraction with phenol solution was made in presence of potassium acetate 0.3 M.

Afterwards, samples were mixed six consecutive times with a phenol solution saturated with 1 M Tris-HCl pH 8.0 in presence of 8-hydroxyquinoline 0.1 %. Every time, aqueous phase was discarded after centrifugation at 3000×*g*. Last extraction was carried out with a

phenol solution saturated with 0.3 M potassium acetate. Phenolic phase was removed and DNA was precipitated adding 10 mL of isopropanol. Genomic DNA was collected with a glass rod, and washed with cold ethanol at 70 % (v/v). Once dried, DNA was dissolved in 2 mL of TE (10 mM Tris-HCl pH 7.0 and 1 mM EDTA pH 7.0). Then, samples were treated with RNase at a final concentration of 40 µg/mL and incubated at 37ºC during 3 h. Later, potassium acetate was added up to a final concentration of 0.3 M, and total DNA was precipitated with 4 mL of isopropanol. DNA was resuspended in TE buffer. Protein bound to DNA was discarded after treatment with proteinase K 0.5 mg/mL during 4 h at 37ºC in SET buffer (100 mM NaCl, 10 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.5, freshly prepared when employed) supplemented with SDS at 1 % (p/v).

Plasmid purification was performed using the High Pure Plasmid Isolation Kit from Roche (Germany) according to the specifications from the supplier. Purification of plasmids from *E. coli* was carried out as follows: cells were inoculated in 5 mL of LB medium supplemented with the corresponding antibiotic, and then cultivated at 37ºC and 250 rpm. In the case of *Rhodococcus,* cells of recombinant clones of *Rhodococcus* sp. T104 were incubated in 5 mL of 2xYT+G supplemented with kanamycin at 30ºC and 250 rpm during 48 h. In this case, the kit protocol was slightly changed: before employing the supplied lysis buffer, cells were resuspended in the corresponding buffer and incubated for 2 h at 37ºC in presence of lysozyme at 2.5 mg/mL concentration. After that, the procedure was exactly the same as described by the manufacturer.

Plasmidic DNA was sequenced according to Sanger (Sanger, F. *et al.* 1977) by BigDye Terminator v3.1 with an automatic DNA sequencer ABI Prism 3730 (Applied Biosystems Inc, USA) from Secugen S. L. (Centro de Investigaciones Biológicas, CIB-CSIC). Nucleotide analysis was carried out with Chromas 2.4 software (Technelysium Pty Ltd, Australia).

4.2. PCR assays

PCR protocols were performed in an Eppendorf Mastercycler Personal 5332. Primers employed in PCR protocols (Table 7) were synthesized by Sigma-Aldrich. Each PCR was performed in 25 µL final volume containing 50 ng of template, 4 µL of each primer at 5 µM (reverse and forward), 2.5 µL DMSO, 2.5 µL buffer 10X, 2.5 µL of 2.5 mM dNTPs, and 0.5 µL of *Pfu* polymerase (Fisher).

PCR conditions for full gene amplification were set depending on the template employed. For *ahla*, *aac* and *pva* PCR amplification, the following “touchdown” program was used: 2 min at 96ºC, followed by 5 cycles of 1 min at 96ºC, 2 min at 70ºC, 8 min at 72ºC, thereafter 5 cycles of 1 min at 96ºC, 2 min at 68ºC, 8 min at 72ºC, then 5 cycles of 1 min at 96ºC, 2 min at 63ºC, 8 min at 72ºC, followed for 20 cycles of 1 min at 96ºC, 2 min at 60ºC, 8 min at 72ºC, and a final extension of 20 min at 72ºC. Those amplified fragments were observed in 0.8 %.agarose gels.

PCR conditions for amplification of small fragments were as follow: 5 min at 95ºC, 35 cycles of 1 min at 95ºC, 30 s at 60ºC, 1 min at 72ºC, and finally an extension of 10 min at 72ºC. Those amplified fragments were observed 1.5 % agarose gels. PCR fragments were purified by High Pure PCR Product Purification kit from Roche (Germany).

4.3. DNA electrophoresis

DNA electrophoresis was employed to analyze and isolate DNA samples in agarose gels. Electrophoresis was performed in a Run One Electrophoresis Cell from EmbiTec (USA) at 100 V constant current, and using TAE (40 mM Tris-HCl, 2 mM EDTA, 20 mM acetic acid pH 8.0) as running buffer. Loading buffer contained Ficoll 400 30 %, bromophenol blue 0.2 %, xylene cyanol 0.2 % and 40 mM EDTA pH 7.0. Band detection in gels was carried out by mixing agarose with GelRed Nucleic Acid Gel Stain (Biotium), and the signal was detected by UV light in a transilluminator UVIPro V1.0 from UVItec Limited (UK). DNA markers employed were GeneRuler 1 Kb DNA Ladder from Thermo Scientific (USA), and Lambda DNA/*Bst*EII from Fermentas (Germany).

4.4. Isolation of DNA fragments

All products derived from PCR amplifications and DNA digestions were isolated by the GeneClean Turbo Kit from MP Biomedicals LLC (USA) according to the instructions from the supplier. DNA products were obtained from bands which were separated by agarose electrophoresis according to their fragment size in 0.8 % and 1.5 % agarose gels.

4.5. Colony mini-prep

This technique was useful for detecting the presence of plasmids with and without inserted foreign DNA. Each colony was included in 15 µL of lysis buffer (500 µL of lysozyme at 10 mg/mL, 500 µL of 0.5 M EDTA, 250 µL of 1.0 M Tris-HCl pH 7.5, 100 µL of RNase at10 mg/mL, 1.15 mL of glycerol at 87 % (v/v), 7.5 mL of water, and bromophenol blue at a final concentration of 2 mg/mL), and the mixture was incubated at room temperature for 15 min. Later, 2 µL of chloroform was added, and then vortexed. Finally, the mixture was centrifuged at 3000×*g* for 2 min, and the supernatant was used to confirm the presence of plasmid by DNA electrophoresis.

4.6. Colony PCR

This technique was used for the detection of plasmids containing specific inserted foreign DNA. Thus, each colony was resuspended in 50 µL of water and this suspension was employed in PCR instead of the amount of water necessary in a normal PCR protocol. Thereafter, the procedure employed was according to the Section 4.2 in the Materials and Methods Chapter.

5. SEQUENCING OF GENOMES FROM *Streptomyces lavendulae* ATCC 13664 AND *Actinoplanes utahensis* NRRL 12052

S. lavendulae and *A. utahensis* genomes were obtained from a shotgun library constructed and sequenced by a Titanium kit in a 454 GS-FLX instrument (Roche Diagnostic, Brandford, CT). *S. lavendulae* genome was obtained at Lifesequencing S.L. (Spain), and *A. utahensis* genome was obtained at Fundación Parque Científico de Madrid (Spain). The procedure was followed according to the manufacturer, but the emulsion PCR (emPCR) methodology was changed in the case of *A. utahensis* replacing all the water content in the live amplification mixture by emPCR additive. Also, an additional sequencing was carried out with *A. utahensis* genome in order to improve the quality of the draft. Such manual

assembly was performed in collaboration with the research group of Dr José Luis García at Centro de Investigaciones Biológicas (CIB-CSIC, Madrid).

The software employed to assemble the reads was Newbler 2.5.3, whereas RAST server (Aziz, R. *et al.* 2008) was used to predict open reading frames (ORFs) and RNA genes. These whole genomes shotgun projects have been deposited at DDBJ/EMBL/GenBank under accession JPNY00000000 (version JPNY01000000) and JRTT00000000 (version JRTT01000000) for *S. lavendulae* and *A. utahensis*, respectively. Analysis of genes by MEGA6 (Tamura, K. *et al.* 2013) was employed for conducting sequence alignment and inferring phylogenetic trees, employing Maximum Likelihood as statistical method, Jones-Taylor-Thornton as substitution model and Nearest-Neighbor-Interchange as Maximum Likelihood heuristic method. In addition, alignment of genomes sequences from both microorganisms against some genomes in local databases were made employing JSpecies (Richter, M. *et al.* 2009). Finally, biosynthesis clusters were identified using antiSMASH analysis (Antibiotics & Secondary Metabolite Analysis Shell) (Medema, M. H. *et al.* 2011).

6. TRANSFORMATION OF BACTERIAL STRAINS

Transformation of bacterial strains was carried out according to the genus, and taking into account the mechanism of transference of DNA information involved. Thus, plasmid transfer was carried out following three different alternative protocols, which included heat pulse, electric shock or mating. In the same sense, four species of microorganisms were employed: *E. coli* DH5α and *Rhodococcus* sp. T104. In this sense, competent and electrocompetent cells of these microorganisms were prepared as described below.

6.1. Methodologies employed with *E. coli* DH5α

E. coli DH5α was employed according to different objectives, since it was used as host for plasmids and constructions. Thus, *E. coli* DH5α transformation was carried out by heat pulse. A single colony from a fresh culture was employed to inoculate 10 mL of LB, and then incubated overnight at 37ºC and 250 rpm orbital shaking. This pre-culture was inoculated into 100 mL of LB and incubated under the same conditions to achieve an OD_{600} = 0.3. Cells were harvested at 3000×*g* and 4ºC during 10 min, and the pellet was resuspended in 15 mL of 100 mM $CaCl_2$ at 4ºC. After 30 min of incubation in ice, cells were harvested under the same described above conditions, and resuspended in 2 mL of a solution of 100 mM $CaCl_2$ and 15 % (v/v) glycerol (and finally the obtained competent cells were kept at -80ºC.

The procedure for cell transformation consisted in mixing 200 µL of competent cells with 2 µg of plasmidic DNA, and keeping the mixture in ice during 10 min. Then, the mixture was subjected to a heat pulse at 42ºC for 30 s in a water bath, and afterwards cells were returned to ice during 2 min. After that, 950 µL of LB was added, and transformants were incubated at 37ºC for 1 h under agitation. Finally, samples were plated in LB agar supplemented with the antibiotic.

6.2. Methodologies employed with *Rhodococcus* sp. T104

Rhodococcus sp. T104 was transformed by electroporation and this bacterium was employed as expression system. In this case, bacteria was inoculated into 10 mL of

2xYT+G by duplicate, under enough aeration (20 % liquid volume into the flask), and incubated at 30ºC and 250 rpm orbital shaking during 12-24 h to reach an OD_{600} = 1.0. Then, cells were harvested and washed three times with 10 mL of pre-chilled glycerol 10 % (v/v), and centrifuged at 3000×*g* and 4ºC for 7 min. Finally, the pellets obtained in each duplicate were resuspended together in glycerol 10 % (v/v) up to a 1 mL final volume, and kept at -80ºC.

Electrocompetent cells were thawed in ice and employed to transform the bacteria (Dower, W. J. *et al.* 1988). An amount of 0.1 µg of plasmidic DNA was added to 200 µL of *Rhodococcus* cell suspension in an electroporation cuvette of 0.2 cm. The mixture was subjected to an electric pulse (2.5 kV cm^{-1}, 200 Ω, 25 µF, for 14 ms), using a GenePulser II (Bio-Rad, Richmond, USA). Thereafter, 500 µL of 2xYT+G medium were added immediately and transformants were incubated for 3 h at 30 ºC without agitation. Finally, cells were plated on 2xYT+G agar supplemented with the adequate antibiotic and incubated for 72 h, or until colonies appear (Hormigo, D. 2009).

7. OBTAINING OF A LIBRARY OF MUTANT PLASMIDS

Libraries of mutant enzymes from *Sl*PVA and *Au*AAC was obtained by employing the recombinant pENV19*pva* plasmid (containing the cloned *pva* gene from *S. lavendulae* ATCC 13664 which codifies for PVA) and recombinant pENV19*aac* plasmid (containing the cloned *aac* gene from *A. utahensis* NRRL 12052 which codifies for AAC). These recombinant plasmids were transfered to *E. coli* XL1-Red mutator strain (Agilent Technologies) according to instructions indicated by the manufacturer.

Thus, 100 µL of cells and 1.7 µL of β-mercaptoethanol (25 mM final concentration) were mixed in a pre-chilled polypropylene round-bottomed tube (15 mL size). Reaction mixture was gently stirred and then incubated in ice during 10 min, swirling the tubes every 2 min. Later, 50 ng of recombinant plasmid DNA was added into each aliquot of cells, and the mixture was incubated during 30 min in ice. A heat pulse at 42ºC for 45 s was carried out, followed by incubation in ice during 2 min. Finally, 0.9 mL of LB, which was pre-heated at 42ºC, was added to each tube and further incubated at 37ºC and 250 rpm orbital shaking for 1 h. The final mixture was divided in aliquots (*i.e.* 200, 300 and 500 µL) which were inoculated into LB agar solid medium supplemented with kanamycin at 50 µg/mL final concentration. Petri dishes were incubated at 37ºC during 30 h.

According to the manufacturer protocol, an amount of 200 cfu of *E. coli* XL1-Red recombinant cells (harbouring the recombinant plasmids for each gene) were inoculated into 10 mL of LB supplemented with kanamycin 50 µg/mL final concentration, and the culture medium was incubated at 37ºC for 24 hours. Thereafter, 100 µL of this broth was inoculated into 9.9 mL of freshly prepared LB supplemented with the antibiotic, and incubated again at 37ºC during 24 hours. The obtained culture medium was centrifuged at 3500×*g* and 4ºC on a Hettich Universal 30 RF centrifuge, and cells were harvested in order to isolate the recombinant plasmid. Plasmid purification was carried out with the High Pure Plasmid Isolation kit from Roche. The step of cell inoculation in freshly prepared medium was repeated four consecutive days, obtaining a library of five mutant plasmids. The procedure was performed twice using both recombinant plasmids for each gene (Fig. 17).

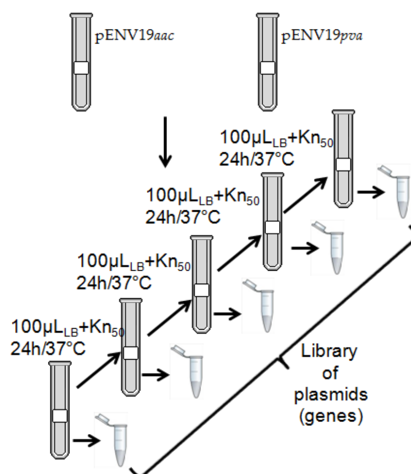

Figure 17. Scheme to obtain recombinant plasmids by *E. coli* XL1-Red

8. HIGH-THROUGHPUT SCREENING OF THE LIBRARY OF MUTANTS BY ASSAYING ENZYMATIC ACTIVITY

The screening employed colorimetric enzymatic assays which were slightly similar to those previously described by Hormigo (Hormigo, D. 2009), in order to measure the acylase activity of *Sl*PVA and *Au*AAC. During HTS, hydrolase activity of 96 mutant recombinant clones could be tested simultaneously against each substrate (*i.e.* penicillin V, oxo-C_6-HSL, oxo-C_8-HSL, oxo-C_{10}-HSL, C_6-HSL, C_8-HSL and C_{10}-HSL) at two temperature values and two pH values, which allowed the analysis of 56 different biocatalytic scenarios. Manipulation and automatic liquid handling were carried out by a Freedom EVO 75 platform (Tecan). This equipment was an important and essential tool for the massive analysis of mutants during this study. In this sense, an operation algorithm was designed to work simultaneously with 96 microwell plates at two pH values (6.0 and 8.0) and two temperatures (30ºC and 45ºC).

Rhodococcus sp. T104 recombinant clones were grown in 200 µL of 2xYT+G medium supplemented with kanamycin 100 µg/mL final concentration into 96-well microplates for 72 h at 30ºC and 250 rpm orbital shaking on a digital thermostatic shaker DTS-2 Elmi Skyline (Latvia). Samples were centrifuged at 3000×*g* for 10 min at room temperature, and supernatants were assayed for activity. The first screening was performed without replica, and the obtained hits were further cultivated in 200 µL of 2xYT+G medium supplemented with kanamycin 100 µg/mL final concentration for 72 h at 30ºC and 250 rpm orbital shaking, ensuring that initial OD_{600} was 0.1. The protocol was the same as the first screening, but each sample was evaluated four times.

8.1. EVOware optimization

As mentioned previously, handling and dispensation of liquids for the reaction mixture (*i.e.* substrate and samples) was performed with the help of the platform Freedom EVOware from Tecan (Fig. 18). HTS dynamics has been incorporated in research laboratories thanks to the efficiency of this workstation and its adequate manipulation. Additionally, results reproducibility is guaranteed, since mistakes due to manual liquid handling are avoided. Thus, results are obtained faster with such automated protocols, making the most of economic and personal lab resources.

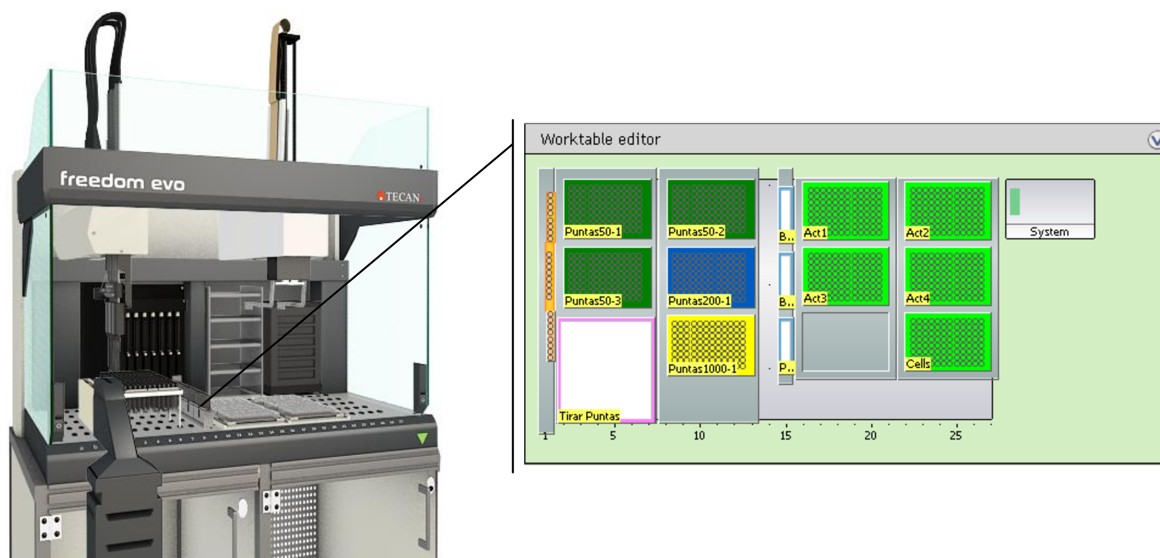

Figure 18. Tecan Freedom EVO 75 workstation employed in the protocol of enzymatic activity determination of the mutant clones of *Sl*PVA and *Au*AAC

8.2. Penicillin V acylase activity

In the screening, penicillin acylase activity was determined by quantifying the released 6-APA during the hydrolysis with PDAB (Bomstein, J. *et al.* 1965; Balasingham, K. *et al.* 1972). *Rhodococcus* sp. T104 recombinant clones were cultured in 200 µL of 2YT+G for 72 h at 30ºC and 375 rpm; thereafter, samples were centrifugated at 3000×*g* for 10 min at 4ºC (Eppendor centrifuge 5810R). The activity of the supernatant coming from the culture broth of a single colony was determined for 20 min and 375 rpm at a fixed temperature (30ºC or 45ºC) employing 75 µL incubation mixture that contained 15 µL of supernatant, 20 µL of 1 M phosphate buffer (pH 6.0 or 8.0) and 40 µL of 116 mM penicillin V. Later, 150 µL of acetic acid 20 % (v/v) were added to stop the reaction. Finally, produced 6-APA was detected by adding 100 µL of PDAB 0.5 % (v/v) dissolved in methanol, and the absorbance was immediately registered at 414 nm and room temperature in a microplate reader DigiScan 340 (Asys Hitech GmbH, Germany). One international activity unit (IU) was defined as the amount of enzyme producing 1 µmol/min of 6-APA under the described assay conditions.

8.3. *N*-acyl-L-homoserine lactone (AHL) acylase activity

Homoserine lactone acylase activity was estimated according to the reaction of fluram (fluorescamine) with L-homoserine lactone (Udenfriend, S. *et al.* 1972). The syntheses of aliphatic AHLs were necessary during these experiments, since only the pure L-enantiomer of β-keto substituted aliphatic AHLs are available on the market. Nevertheless, the racemic mixture of aliphatic AHLs are available, whereas a very few L-enantiomers have been recently offered by some companies such as Santa Cruz Biotechnology, but at very expensive prices.

8.3.1. Synthesis and purification of aliphatic AHLs

All aliphatic AHLs (C_4-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL and C_{14}-HSL) were synthesized according to the procedure described elsewhere (Thomas, P. W. *et al.* 2005).

In every case, 10 mmol of L-homoserine lactone hydrochloride was dissolved in 25 mL of cold dimethylformamide in addition to 23 mmol of cold trimethylamine. Afterwards, 14 mmol of the correspondent acyl chloride was added dropwise under agitation. Reaction mixture was kept for 2 h at room temperature under constant agitation. Then, the solvent was removed via rotary evaporator, and later the samples were dissolved in 15 mL of dichloromethane and the products were washed three times with an equal volume of 1 M Na_2SO_4, and NaCl saturated (200 g/L) in order to eliminate salts. Finally, the solvent was removed and the products were dried with anhydrous $MgSO_4$.

Once synthesized, the isolation and purification of the AHLs were carried out by a silicagel 60 (Merck) chromatography (1.7 mm inner diameter, 20 cm length). Elution was performed with a *n*-hexane-EtOAc gradient system (100:0; 90:10; 80:20; 70:30; 60:40; 0:100, v/v). Compounds in the fractions were observed by thin layer chromatography (TLC), using an aqueous solution of potassium permanganate (3 g $KMnO_4$, 20 g Na_2CO_3, 5 mL of NaOH 5 %, gauged till 300 mL with water) to spray the TLC plates which were further heated briefly (*ca.* 1 min at 100ºC). Fractions with pure AHLs were united and concentrated and the solvent was removed via rotary evaporator. Structures elucidation was determined by ^1H and ^{13}C NMR analysis.

8.3.2. Spectrophotometric assays

A reaction volume of 75 µL (composed by 15 µL of the supernatant of the culture broth coming from a single colony, 50 µL of 1 M phosphate buffer (pH 6.0 or 8.0), and 10 µL of AHLs at an adequate concentration and dissolved in DMSO) was incubated for 20 min and 375 rpm at the required temperature (30ºC or 45ºC). Afterwards, reaction was stopped with 150 µL of 0.5 M sodium acetate pH 4.5, and product formation was quantified by adding 20 µL of fluram 0.1 % dissolved in acetone. Once this mixture was incubated for 40 min, absorbance was measured at 405 nm. Substrate concentration employed for the evaluation of each AHL reached saturation, being 50, 25 and 10 mM for C_6-HSL, C_8-HSL and C_{10}-HSL, respectively. Since parental enzymes lack oxo-AHL-acylase activities according to Hormigo (Hormigo, D. 2009), substrate concentrations adjusted to 8 mM in all cases. One international activity unit (IU) was defined as the amount of enzyme producing 1 µmol/min of homoserine lactone under the described assay conditions.

9. STATISTICAL ANALYSIS

Colonies were selected according to the enzymatic activity of their culture broths (for further information about the enzymatic assays, see Section 8 in the Materials and Methods Chapter). Those colonies considered as hits (*i.e.* improved activities, new hydrolytic spectra, or both) were selected to carry out a new screening in order to corroborate initial results. The statistical criterion selected these mutant clones located over the third quartile in each combination of pH and temperature conditions. Likewise, the percentages of colonies with higher enzymatic activity compared to the wild-type strains were quantified in each scenario.

9.1. Mutant clones selection

Selection was performed based on the activities displayed by each mutant clone under every operational condition. The analysis of results did not contemplate the spatial dependence of the raw data within the microplate (*e.g.* row, column, edges), but the

temporal pattern was actually considered giving weight only to the effect of the assay, microplate by microplate. Considering a standard normal distribution of samples (Efron, B. 2004), *z*-score criterion was fulfilled as the statistical significance. In this sense, z-score is considered as ($z_i = (x_i - \bar{x})/S_x$), where x_i is the sample absorbance, $\bar{x}$ is the mean of sample population without controls in the microplate, and S_x is the standard deviation (Brideau, C. *et al.* 2003; Prummer, M. 2012). *z*-Score values (z_i) higher or equal to 3 were selected for a further second screening. Likewise, Positive Predictive Value (PPV) for every evaluated condition in this study was considered as the criterion to validate the screening, and it was defined as the ratio of clones corroborated in the second screening with respect to those clones selected in the first screening.

9.2. Distribution models of the spot densities

Fitting of densities was carried out by Mathwave EasyFit data analysis software version 5.5 professional. Every operational condition was tested at 56 different probability distribution models. Adjusting of the obtained result to an expected cumulative distribution function (goodness of fit) was developed according to Anderson-Darling test, giving more weight to tails than other tests (spots far away from means). Anderson-Darling statistic (A^2) is defined as:

$$A^2 = -n - \frac{1}{n}\sum_{i=1}^{n}(2i-1)\cdot\left[ln\,F(X_i) + ln\left(1 - F(X_{n-i+1})\right)\right]$$

where *n* is the number of samples evaluated and *F(X)* is the cumulative distribution function. Anderson-Darling test has a significance level of 0.01 which correspond to critical value of 3.9074 (EasyFit uses the same critical values for all distribution models), and without rejection of null hypothesis. Thus, this analysis considered top five fitted distributions at each condition where A^2 was lower than critical value.

10. PROTEIN ANALYSIS TECHNIQUES

10.1. Quantification of protein content

Quantification of protein concentration was carried out according to the coomassie blue method (Bradford, M. M. 1976) employing commercial protein coomassie reagent from Bio-Rad. Each assay was determined by mixing 40 µL of sample (adequately diluted in water), with 80 µL of Bio-Rad reagent, and 280 µL of water. The mixture was mixed vigorously and then incubated at room temperature for 10 min. Absorbance was measured at 595 nm, and the protein concentration was calculated interpolating in a calibration curve prepared with BSA standards whose concentrations ranged from 2.5 to 30.0 µg/mL).

10.2. Polyacrylamide gel electrophoresis (PAGE) in presence of sodium dodecyl sulfate (SDS)

Enzyme presence and purity in every fraction obtained during chromatographic purification (for further information, see Section 10.4 in the Materials and Methods Chapter) was carried out by SDS-electrophoresis. Thereby, samples with acylase activity were mixed with cold acetone (1:10 v/v), and kept in ice during 30 min; then, samples were centrifuged at 9000×*g* and 4ºC for 30 min. Supernatant was removed and pellet was dried. Thereafter, pellet resuspended in 20 µL of sample buffer (62.5 mM Tris-HCl pH 6.8, glycerol 10 % (v/v), SDS 2 % (v/v), β-mercaptoethanol 5 % (v/v) and bromophenol blue

0.005 % (p/v)) and incubated at 95ºC for 10 min. Each sample was subjected to electrophoresis in a polyacrylamide gel under denaturing conditions in presence of SDS 0.1 % (SDS-PAGE).

Gels were prepared with acrylamide/bis-acrylamide in the running gel and stacking gel at 12.5 % (v/v) and 5 % (v/v) concentration, respectively (Laemmli, U. K. 1970). Electrophoresis was carried out at room temperature in a Mini-Protean Tetra cell from Bio-Rad (USA) at 25 mA each gel, and the running buffer employed was 25 mM Tris-HCl pH 8.8, 192 mM glycine and SDS 0.1 %. Protein fixation on gel was performed at room temperature with a solution containing 2-propanol 25 % (v/v) and acetic acid 10 % (v/v) under continuous shaking during 15 min. Protein gel staining was carried out in an acetic acid 10 % (v/v) solution containing 20 mg of comassie blue G-250 for 1 h, and the excess of dye was removed with acetic acid 10 % (v/v) under continuous shaking.

10.3. Heterologous expression of proteins

10.3.1. Protein expression of recombinant enzymes by *Streptomyces lividans* 1326

Enzymes were obtained by fermentation of the recombinant *Streptomyces lividans* 1326 strain (harbouring the corresponding encoding gene) in TSB medium supplemented with thiostrepton using baffled shake flasks (Torres-Bacete, J. *et al.* 2007; Torres-Bacete, J. *et al.* 2015). Thus, 2×10^6 spores/mL were inoculated into 500 mL of broth, and culture was kept at 250 rpm orbital shaking and 30ºC for 96 h. Then, cells were removed by centrifugation at 5000×*g* for 30 min at 4ºC. During chromatographic purification (for further information, see Section 10.4 in the Materials and Methods Chapter), enzyme activity of each fraction was assayed following a protocol which was slightly similar to the one described in Section 8.2 in the Materials and Methods Chapter. In this case, the incubation mixture contained 20 µL of each fraction and 40 µL of 116 mM penicillin V, and further incubated for 20 min and 375 rpm at 45ºC. Thereafter the procedure was exactly as described in Section 8.2, in the Materials and Methods Chapter.

10.3.2. Protein expression of recombinant enzymes by *Rhodococcus* sp. T104

Alternatively to the protein expression by *Streptomyces lividans* 1326, heterologous expression of *Sl*PVA and *Au*AAC was carried out following the methodology employed by García-Hidalgo and co-workers (García-Hidalgo, J. *et al.* 2012; García-Hidalgo, J. *et al.* 2013). Thus, a fragment of 227 bp containing p*ermE** and MCS obtained from pEM4 (Quirós, L. M. *et al.* 1998) was cloned between *Hind*III and *Eco*RI restriction sites in pNV19 (Chiba, K. *et al.* 2007), and this shuttle vector was named pENV19. In addition, the signal peptide (SP) of PHB depolymerase from *Streptomyces exfoliatus* DSMZ 41693 was obtained by PCR amplification using the primers SEPS1 and SEPS2, and the insert was cloned downstream of p*ermE** between *Pst*I and *Sal*I restriction sites; this plasmid hereafter was named pENS (Fig. 19).

Recombinant genes encoding *Sl*PVA and *Au*AAC without their own signal peptide were cloned downstream of the signal peptide of PHB depolymerase from *S. exfoliatus* between *Xba*I and *Eco*RI restriction sites (Fig. 19). Recombinant *Sl*PVA clones were amplified by PCR using the primers αPVA and PVA-F, and those of *Au*AAC employing αAAC and AAC-2 (Table 7). These PCRs amplified only α- and β-subunits in addition to the linker peptide. In this sense, those hits with their own signal peptide were cloned in pENV19,

whereas those recombinant genes expressed in pENS were amplified by PCR to remove their own signal peptide in the insert.

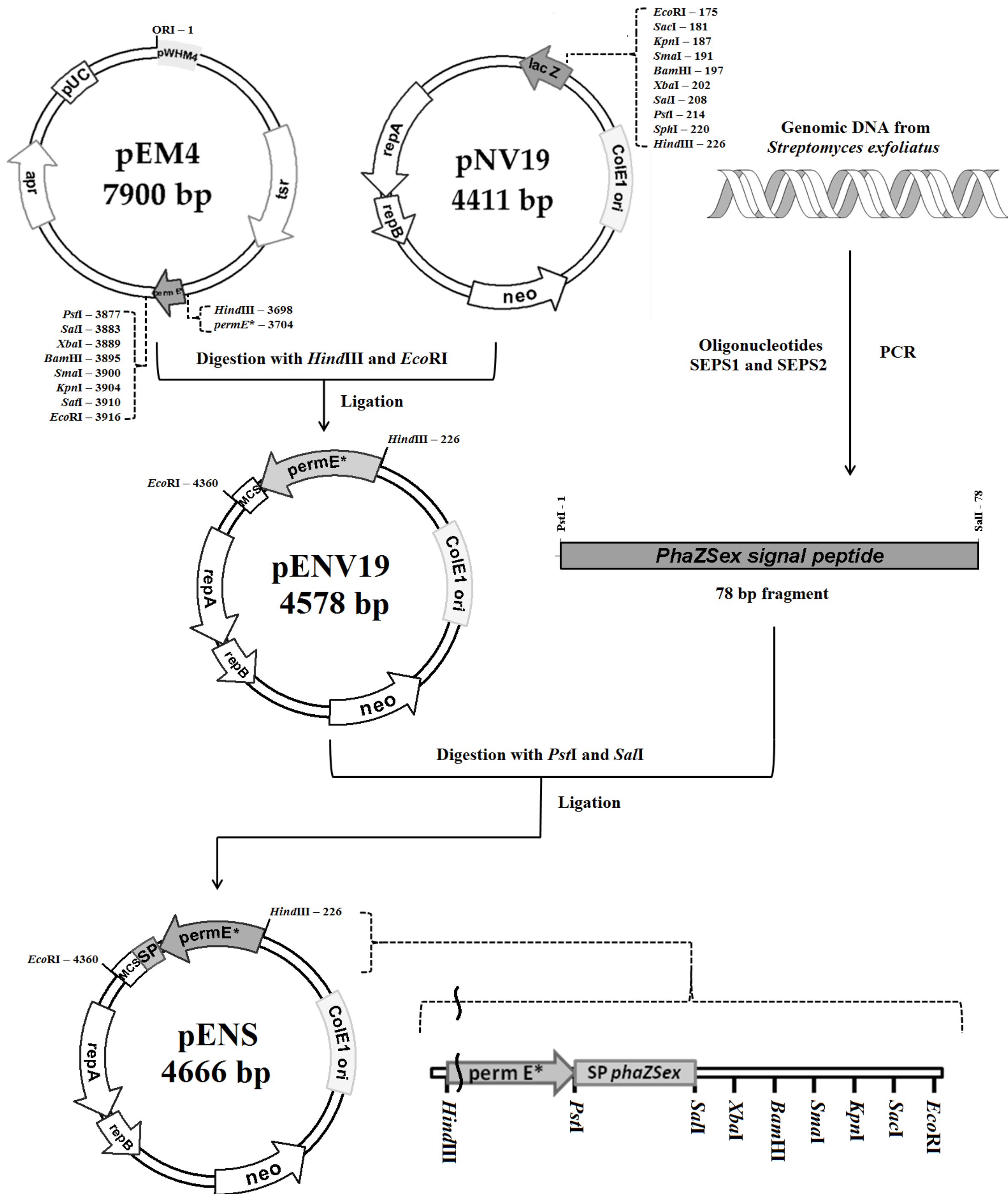

Figure 19. Construction of shuttle vector pENS and pENV19 from pNV19 and pEM4

10.4. Isolation and purification of acylases

10.4.1. Isolation and purification of enzymes derived from *Sl*PVA

Chromatographic cartridges Bio-Scale Mini UNOsphere S (Bio-Rad, USA) were used for isolation of purification of recombinant-*Sl*PVA-derived acylases from culture broths. Enzyme purification was carried out in only one ionic exchange chromatographic step. The

broth (200 mL) was adjusted at pH 7.0, and then loaded in the chromatographic column (2 x 5 mL) previously equilibrated with 10 mM potassium phosphate buffer pH 7.0. An isocratic flow was performed with 30 mL of the same buffer, and then the protein was eluted with 85 mL of 10 mM potassium phosphate pH 7.0, by a continuous gradient of NaCl from 0.0 to 1.0 M. Later, the column was washed with 30 mL of 10 mM potassium phosphate pH 7.0 containing 1.0 M NaCl. Chromatography was performed at room temperature in a BioLogic LP system (low-pressure chromatography) from Bio-Rad at a flow of 1.0 mL/min, and each fraction contained 1.5 mL.

10.4.2. Isolation and purification of enzymes derived of *Au*AAC

Chromatographic cartridges Bio-Scale Mini UNOsphere S (Bio-Rad, USA) were used for isolation of purification of recombinant *Au*AAC-derived acylases from culture broths. Enzyme purification was carried out in only one ionic exchange chromatographic step. The broth was adjusted at pH 6.5, and then loaded in a chromatographic column (2 x 5 mL) previously equilibrated with 5 mM potassium phosphate buffer pH 6.5. An isocratic flow was performed with 30 mL of the same buffer, and then the protein was eluted with 85 mL of 5 mM potassium phosphate pH 6.5, by a continuous gradient of NaCl from 0.0 to 1.0 M. Later, the column was washed with 30 mL of 5 mM potassium phosphate at pH 6.5 containing 1.0 M NaCl. Likewise, chromatography was performed at room temperature in a BioLogic LP system from Bio-Rad at a flow of 1.0 mL/min, and each fraction contained 1.5 mL.

10.5. Determination of kinetic parameters

In contrast to HTS assays (for further information, see Section 8 in the Materials and Methods Chapter), determination of kinetic parameters was carried out by a fluorometric assay which was previously standardized. Firstly, enzyme activity was measured by triplicate employing all substrates with six different amounts of enzyme (ranging from 0.02 to 0.80 µg), ensuring that substrate concentration was at saturation (*i.e.* C_4-HSL 8.0 mM, C_6-HSL 50.19 mM, C_8-HSL 25.08 mM, C_{10}-HSL 10.18 mM, C_{12}-HSL 80.00 mM, C_{14}-HSL 15.00 mM, oxo-C_6-HSL 8.21 mM, oxo-C_8-HSL 8.29 mM, oxo-C_{10}-HSL 7.23 mM, oxo-C_{12}-HSL 8.40 mM and oxo-C_{14}-HSL 7.68 mM) (Hormigo, D. 2009) in order to establish the adequate amount of enzyme to be employed throughout the evaluation. Reaction was carried out in 100 µL at 45ºC and pH 8.0 for 10 min. Thereafter, reactions assayed at low substrate concentration (*i.e.* C_4-HSL 0.41 mM, C_6-HSL 0.41 mM, C_8-HSL 1.26 mM, C_{10}-HSL 0.51 mM, C_{12}-HSL 0.40 mM, C_{14}-HSL 0.75 mM, oxo-C_6-HSL 0.41 mM, oxo-C_8-HSL 0.42 mM, oxo-C_{10}-HSL 0.37 mM, oxo-C_{12}-HSL 0.84 mM and oxo-C_{14}-HSL 0.77 mM) were stopped at eight different reaction times (1, 2, 3, 5, 9, 10, 20 and 30 min) in order to establish the adequate reaction time. As a consequence, enzyme concentration and reaction time were established for further experiments.

Apparent kinetic parameters (Michaelis-Menten constant or K_M and maximal velocity or V_{max}) were calculated for both parental and recombinant enzymes by a nonlinear regression. The kinetic parameters values were roughly estimated with a set of eight experimental results using Hyper32 program (available on http://homepage.ntlworld.com/john.easterby/hyper32.html). Taking into account the results of this preliminary evaluation, sixteen concentrations of each substrate were again assayed, and the results fitted to a hyperbola using Hyper32 program. In this sense, Michaelis-Menten equation describes the variation of reaction velocity with substrate concentration:

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}$$

The fluorometric assay was based on the detection of the primary amines which are present in the released reaction products, as reported elsewhere (Wahjudi, M. *et al.* 2011; Clevenger, K. D. *et al.* 2013; Mukherji, R. *et al.* 2014). Kinetic studies were performed at specific pH and temperature values which ensured that the enzyme displayed catalytic activity (for further information, see Section 8 in the Materials and Methods Chapter). The reaction was carried out in 100 µL final reaction mixture (10 µL of enzyme solution, 70 µL of 1 M phosphate buffer, and 20 µL of substrate dissolved in DMSO). Reaction mixtures were kept in ice before incubation and after reaction time, in order to start and stop reaction on time, respectively.

The reagent *o*-phthalaldehyde (OPA) was employed for the detection and quantification of those reaction products which contained a primary amine such as HSL. OPA in presence of β-mercaptoethanol at a high pH value reacts rapidly with primary amines to form intense and highly stable fluorescent derivatives (Švedas, V.-J. K. *et al.* 1980). Once the reaction was stopped in ice, each sample containing the complete reaction mixture was mixed directly with 100 µL of OPA commercial solution, which allowed the quantification of the product concentration and thus the determination of acylase activity. The final mixture was incubated for 2 min at 25ºC to yield the fluorescent derivative whose fluoresce intensity was estimated employing a FLUOstar Omega (BMG Labtech). A calibration curve prepared with pure HSL standards was employed to calculate the HSL amount released after enzymatic hydrolysis. Operational conditions of the fluorometer were set at 25ºC, excitation/emission at 355 nm/460 nm, gain of 500, and a required value of 60 %, with a positioning delay of 0.2 s. Measurements started immediately, employing 5 flashes per well and 1 multichromatic; intensity of signal due to excitation and emission was measured on the top of the well.

11. BIOINFORMATIC ANALYSIS OF RECOMBINANT ENZYMES

11.1. Identification of recombinant clones sequences

Nucleotide sequences of recombinant clones were determined and analyzed by BlastN from NCBI. Alignments of sequences amplified with the corresponding primers in DNA sequencing were employed to elucidate modifications in those recombinant clones, whereas parental enzymes were employed as targets. Those modifications detected in genes sequences were translated to amino acids by the ExPASy translate tool.

11.2. Sequence alignments

Alignment of amino acids sequences of the recombinant clones with the NCBI database was carried out by COBALT (constraint-based multiple protein alignment tool) (Papadopoulos, J. S. *et al.* 2007). Those alignments were performed taking into account *Au*AAC as the reference, and performing only the alignment of two proteins simultaneously, in order to find similarities with the parental enzymes and detect those residues implicated in the catalytic process according to literature (*i.e.* catalytic amino acids and residues forming the substrate binding pocket) (Zhang, D. *et al.* 2007), as well as those amino acids mutated by directed molecular evolution in this study. Previously to this comparison, all enzymes were submitted to the web server SignalP 4.1 (Petersen, T. N. *et*

al. 2011), in order to identify and delete the presence of a signal peptide in the sequence which could interfere in the amino acid numeration of the α-subunit.

11.3. Prediction of protein characteristics

Parental and recombinant proteins were analyzed by several platforms. Presence of a signal peptide was carried out using SignalP 4.1 server (Petersen, T. N. *et al.* 2011), BPROM was used to detect promoters (Solovyev, V. *et al.* 2010), and subunits identification was estimated by ProtParam (Gasteiger, E. *et al.* 2005). Several predictions were performed online by PredictProtein (Yachdav, G. *et al.* 2014), such as the presence of transmembrane domains (Rost, B. *et al.* 1996; Reeb, J. *et al.* 2015) and disulfide bridges (Ceroni, A. *et al.* 2006), the expected solvent accessibility in residues (Bigelow, H. R. *et al.* 2004; Rost, B. *et al.* 2004), the biological process ontology (Hamp, T. *et al.* 2013), and the composition of secondary structure (Bigelow, H. R. *et al.* 2004; Rost, B. *et al.* 2004).

11.4. Homology modelling of protein

Parental enzymes as well as recombinant proteins were submitted to several webservers to predict their protein 3D-structure, by using single template homology modelling. In particular, five different platforms were employed in order to make a statistical consensus of the spatial location of specific residues within the enzymes. Specifically, Phyre2, SwissModel, Robetta, I-Tasser and Lomets were employed in this modelling.

Phyre2 (Kelley, L. A. *et al.* 2015) is an on-line web service for protein structure prediction and it is considered one of the most popular method in this field. SwissModel is a fully automated protein structure-modelling server accessible via ExPASy (Arnold, K. *et al.* 2006; Guex, N. *et al.* 2009; Kiefer, F. *et al.* 2009; Biasini, M. *et al.* 2014). Robetta server provides both *ab initio* and comparative models of protein domains, and its performance compared to other servers improves as the difficulty of target is increased (Raman, S. *et al.* 2009; Song, Y. *et al.* 2013). I-Tasser is a server for protein and function predictions, which has been recently considered the best server for protein structure prediction where 3D models are built based on multiple-threading alignments and iterative assembly simulations (Zhang, Y. 2008; Roy, A. *et al.* 2010; Yang, J. *et al.* 2015). Finally, Lomets is an on-line web service for protein structure prediction generated by ranking and selecting models from nine state-of-the-art threading programs (*i.e.* FFAS-3D, HHsearch, MUSTER, pGenTHREADER, PPAS, PRC, PROSPECT2, SP3, and SPARKS-X) (Wu, S. *et al.* 2007). Protein visualization was performed by PyMol program (Schrödinger, L. L. C. 2014).

12. ESTIMATION OF CHELATING PROPERTIES IN ECHINOCANDINS

Chelating properties of echinocandins (*i.e.* aculeacin A, caspofungin and pneumocandin B_0) were evaluated using QuantiChrom™ Iron Assay Kit (DIFE-250) according to the instructions of the manufacturer. A calibration curve was prepared to quantify iron concentration (*i.e.* Fe^{3+}, ranging from 1.43 to 14.32 pmol). Chelating potential of each echinocandin was determined as follows: 2 pmol of each echinocandin previously dissolved in DMSO were added to an aqueous solution containing 11.46 pmol Fe^{3+}. The mixture was incubated at 25ºC and 375 rpm orbital shaking for 10 min. Thereafter, 200 µL of a solution containing ascorbic acid 0.1% (p/v), 2,4,6-tris(2-pyridyl)-*S*-triazine 0.1% (p/v), sodium acetate 0.1% (p/v) and acetic acid 1.8% (p/v) was added to 50 µL withdrawn

from the mixture mentioned above, and then incubated for 40 min at 25ºC and 375 rpm orbital shaking. Optical density was finally read at 595 nm, and concentration quantified by the calibration curve.

IV RESULTS

1. SHOTGUN SEQUENCING OF GENOMES

Organisms must be grouped according to a hierarchical structure within a general group in order to understand their great diversity. Taxonomy is responsible for this mission and deals with the classification of living beings in categories, such as order, family and genus. Identification is the practical side of taxonomy, establishing if an organism belongs to a recognized taxon. Phylogenetic analysis is based on the study of certain features that are supposed to be important for evolution of taxonomic groups. This information is really useful to establish phylogenetic relationships and evolutive stages of organisms, allowing the construction of phylogenetic trees. In the past, identification, characterization and classification of microorganisms was only performed by morphological criteria and biochemical properties, but nowadays criteria based on molecular biology are used as well and have allowed the re-assignation of many species.

Original identification, characterization and classification of *Streptomyces lavendulae* ATCC 13664 and *Actinoplanes utahensis* NRRL 12052, both Gram-positive filamentous bacteria, were carried out by classical morphological and biochemical criteria (Rolinson, G. N. *et al.* 1961; Abbott, B. J. *et al.* 1981). However, in previous studies the gene-sequence of 16S rRNA from *S. lavendulae* ATCC 13664 was amplified, and the authors established that the strain location was far from other species of *S. lavendulae* (Anzai, Y. *et al.* 1997; Torres-Bacete, J. 2005) Instead, this strain is located close to other *S. griseus* species. This fact is important since the strain ATCC 13664 is the only *S. lavendulae* strain that possesses penicillin acylase activity within its natural enzymatic system, although many species of *Streptomyces* have been reported with acylase activity (Brenda enzyme database, accessed 11th may 2016). Thus, the genome assembly is necessary to classify correctly this strain. Similarly, the knowledge of *Actinoplanes* NRRL 12052 genome is very interesting since this strain is the only one with penicillin acylase activity. Likewise, the relevance of this microorganism is based on its enzymatic plasticity to hydrolyze several compounds with pharmaceutical relevance.

Moreover, the knowledge of genomes allows a deeper knowledge of these microorganisms and the importance of their enzymes in natural environments, as well as their biological role, such as penicillin acylase and aculeacin acylase. Indeed, both acylases are key biocatalysts for the generation of building blocks to synthesize therapeutic antimicrobials, but their physiological roles remain unknown.

1.1. Draft genome sequence of *Streptomyces lavendulae* ATCC 13664

Sequencing of *S. lavendulae* genome was carried out as described in the Materials and Methods Chapter, Section 5. Nucleotide sequence from *S. lavendulae* ATCC 13664 has been deposited at GenBank under accession number JPNY00000000. The version described here is the first one, deposited under accession number JPNY01000000. This *S. lavendulae* draft genome contains 7.8 Mb with a GC content of 71.5 %. A total of 1.1×10^6 reads were assembled by Newbler 2.5.3 software, generating 650 large contigs and providing 9.4-fold coverage.

As shown in Figure 20, this sequence was compared with 24 sequences displayed by BlastN. Despite *S. lavendulae* ATCC 13664 is located evolutionarily close from other *S. lavendulae* strains such as *S. lavendulae* subsp. *lavendulae* IFO 13709 (identity 98 %), its location in the phylogenetic tree demonstrates that it is closer to *S. griseus* strain

ABRIINW 13 (identity 99 %). It is important to point out that *S. lavendulae* subsp. *lavendulae* IFO 13709 has been employed as the out-group reference. This phylogenetic misallocation of *S. lavendulae* ATCC 13664 had been previously discussed according to gene-sequence of 16S rRNA amplified by PCR (Anzai, Y. *et al.* 1997; Torres-Bacete, J. 2005). It is worth mentioning that the size of 16S rRNA fragments amplified in those studies were different to the gene sequence detected in the genome, in addition that the fiability of a genome project is higher in comparison to usual PCR protocols.

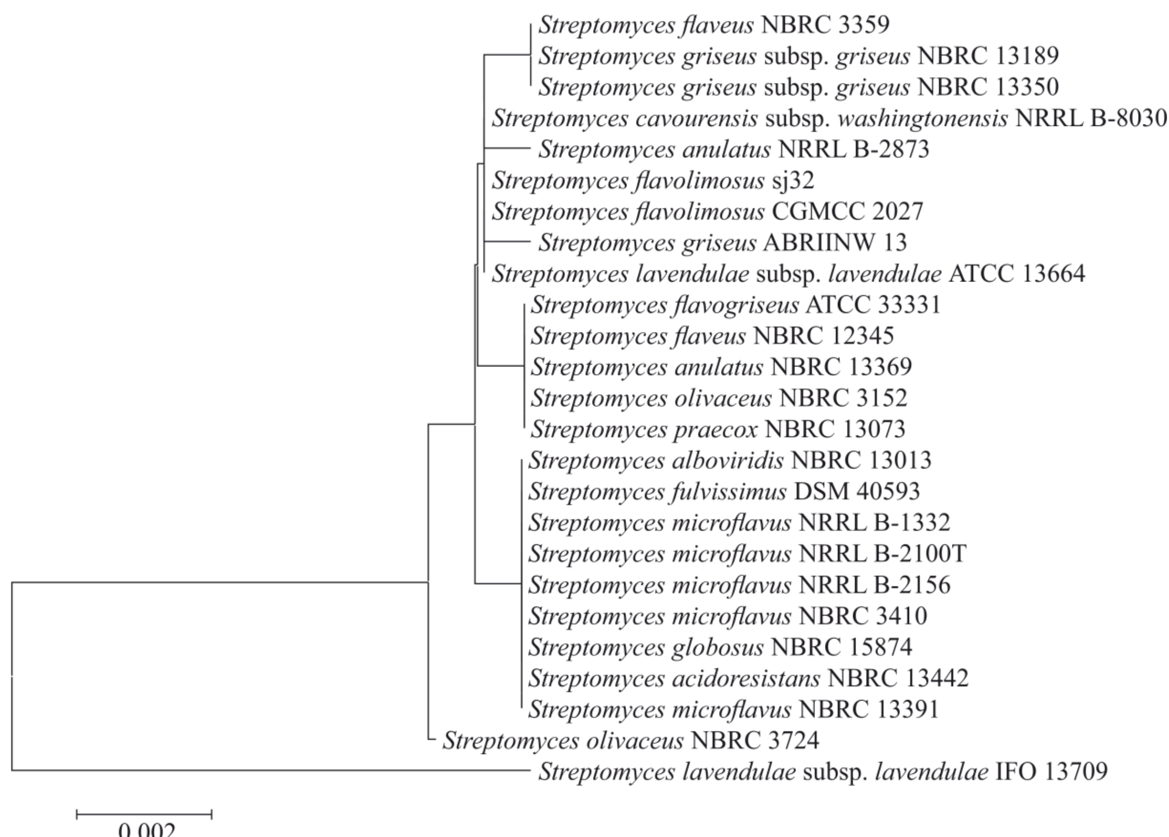

Figure 20. Phylogenetic tree of *S. lavendulae* ATCC 13664 elaborated from the sequence of 16S rRNA employing BlastN as the alignment tool. *S. lavendulae* subsp. *lavendulae* IFO 13709 was considered as the out-group reference

Thereby, a deep analysis of this strain was carried out by JSpecies software, which allows the comparison of the whole genome with available genomes in public databases (Fig. 21). This algorithm compares the alignment of conserved sequences throughout the genome (*e.g.* 16S rRNA and 5S rRNA, among others) with other conserved sequences in databases, generating the highest reliability in sequence comparisons, and allowing the accurate location of the strain within the genus. Thus, the selection of those genomes to be compared with *S. lavendulae* ATCC 13664 was done (i) according to partial alignment performed by BlastN employing several conserved sequences in its RNA gene-sequence, and (ii) considering only those alignments carried out with complete genomes available in databases. Once again, the strain ATCC 13664 was not located close to other *S. lavendulae* species, but it was closer to other *S. griseus* strain one more time (*i.e.* 97 % of identity with the strain NBRC 13350), corroborating the previous analysis (Fig. 20). However, 16S rRNA gene-sequence is not enough operative in this case, so whole genome comparison should be employed.

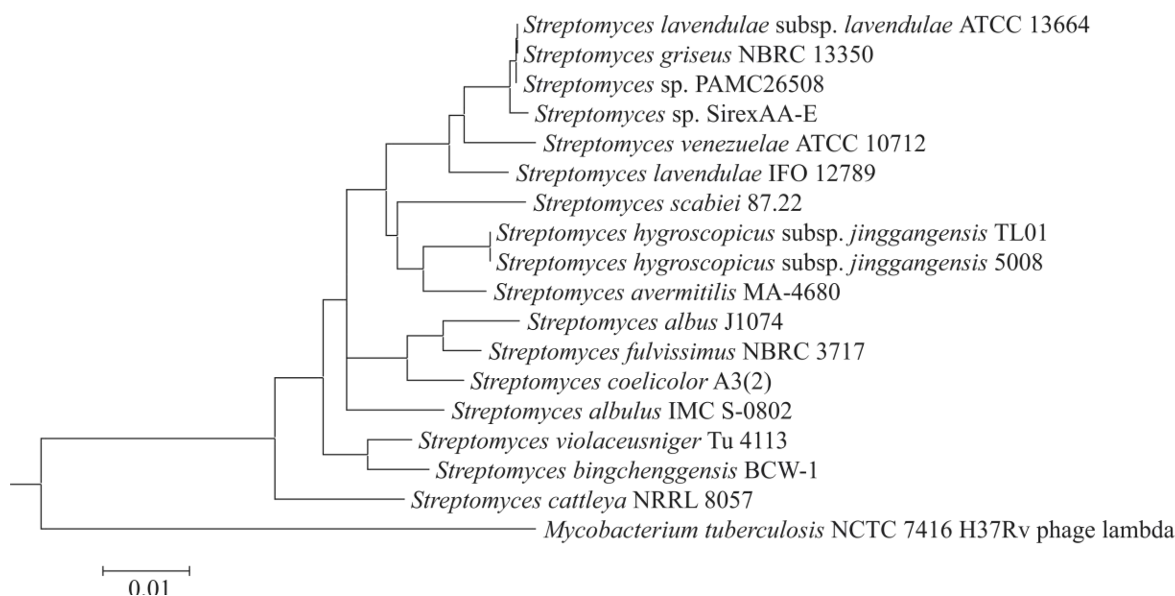

Figure 21. Phylogenetic tree of *S. lavendulae* ATCC 13664 elaborated from whole genome sequence employing JSpecies as the alignment tool. *Mycobacterium tuberculosis* NCTC 7416 H37Rv phage lambda was considered as the out-group reference

However, despite the fact that *S. griseus* is a well-known streptomycin producer (Distler, J. *et al.* 1992), this biosynthetic cluster was not detected in *S. lavendulae* ATCC 13664. Likewise, penicillin amidases have been detected in *S. griseus* but no in *S. lavendulae* according to BlastP. All these results suggest that *Streptomyces lavendulae* ATCC 13664 should be re-assigned, and here is proposed *Streptomyces complutensis*. However, further analysis should be performed to classify this strain a as a new *Streptomyces* sp.

In addition, analysis by RAST allowed the location of the encoding DNA sequence (CDS) for penicillin V acylase (PVA) within the contig 70, showing a size of 2421 bp (GenBank: AY611030.1). Likewise, some representative CDSs upstream and downstream of the *pva* gene from *S. lavendulae* ATCC 13664 (hereafter named *Streptomyces* sp.) are highlighted in Figure 22. Analysis was carried out with those available genomes in the NCBI database containing enzymes with similar sequences as PVA from *Streptomyces* sp. In total, 16 genomes within the *Streptomyces* genus were compared considering the genome of *Streptomyces* sp. as reference.

Thus, this analysis showed the presence of one acylase encoding gene throughout the *Streptomyces* genus, which probably could be employed by this bacterium in its natural metabolism or as a self-defense mechanism against xenobiotics. In addition, the presence and proximity of genes encoding for a β-lactamase and an acylase in some species of this genus could implicate these enzymes in *quorum quenching* (*QQ*) processes. Furthermore, thanks to this analysis it was possible to identify similarities and differences among sequences of this region within each genome (*ca.* 20,000 bp were analyzed up- and downstream of the gene). Once more, it is evident that this strain was misassigned to *S. lavendulae* and should be re-assigned as *Streptomyces* sp. Analysis performed by BPROM (Solovyev, V. *et al.* 2010) did not display the presence of promoters close enough to PVA or β-lactamase, which could initiate the transcription of those enzymes in the microorganism.

Likewise, it is important to remark that some genes related with ABC-transporter system (Fig. 22) were detected close to *pva*. This system is usually related with secretion of siderophores, molecules involved in iron metabolism in bacteria (Cézard, C. *et al.* 2015).

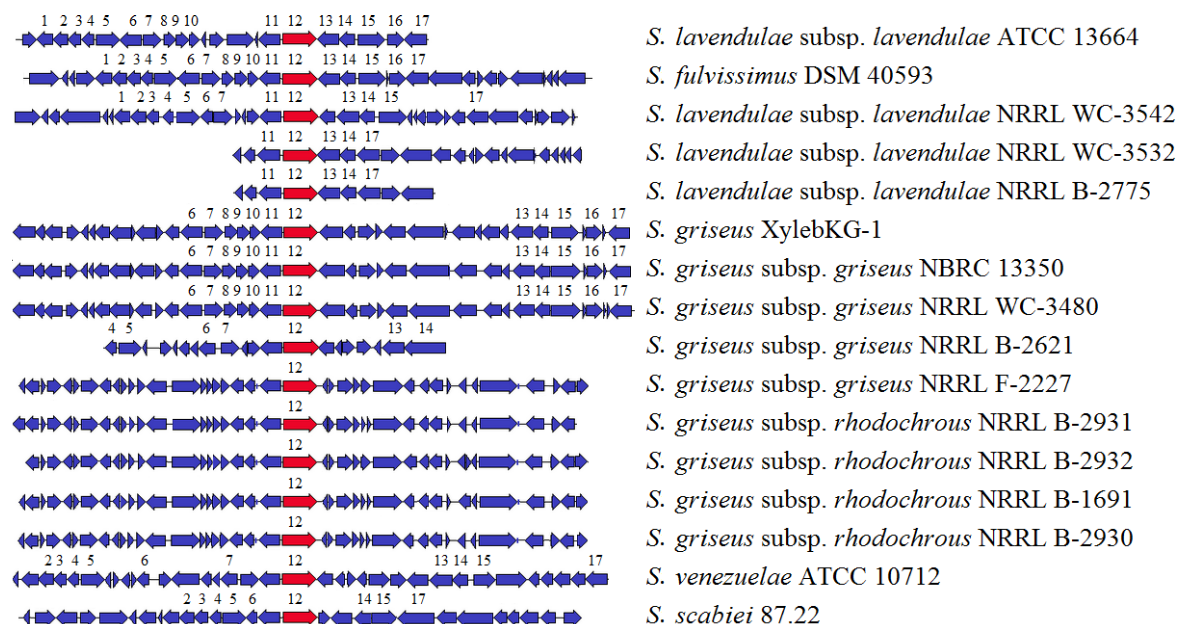

Figure 22. Representative coding sequences upstream and downstream of the gene of penicillin V acylase from *Streptomyces* sp. **(1)** 3-ketoacyl-CoA thiolase, **(2)** lipid-transfer protein, **(3)** DNA-binding protein, **(4)** enoyl-CoA hydratase, **(5)** long-chain-fatty-acid-CoA ligase, **(6)** hydrolase, **(7)** phenylacetate-CoA ligase, **(8)** amino acid ABC-transporter, **(9)** polar amino acid ABC-transporter, **(10)** ABC-type polar amino acid transport system, **(11)** long-chain-fatty-acid-CoA ligase, **(12)** penicillin V acylase, **(13)** long-chain-fatty-acid-CoA ligase, **(14)** enoyl-acyl-carrier-protein reductase, **(15)** β-lactamase (cephalosporinase), **(16)** putative secreted protein, **(17)** β-carotene ketolase

Analysis performed by antiSMASH pipeline (Blin, K. *et al.* 2013) allowed the location of 66 clusters within the genome. *In silico* prediction is based on the consensus of multiple methods. In particular, our interest was focused in PKS (polyketide synthase) and NRPS (non-ribosomal peptide-synthetase). In this sense, PKS at domain specificities are predicted by a sequence of amino acid signature sequence of the active site (Yadav, G. *et al.* 2003), as well as by comparison against a collection of profile hidden Markov models based on Minowa and co-workers method (Minowa, Y. *et al.* 2007). In the NRPS case, both signature sequence method and support-vector machines-based method of NRPSPredictor2 (Rausch, C. *et al.* 2005; Röttig, M. *et al.* 2011), and Minowa and co-workers method (Minowa, Y. *et al.* 2007) were employed and those domains were detected by BlastP. In our case, NRPS and PKS clusters were detected in addition to bacteriocin and terpenes clusters, among other types (Table 8). Detection and location of NRPS and PKS clusters throughout this genome should be considered a success, since they could potentially encode for new antibiotics or bioactive compounds, or both.

Table 8. Clusters predicted by antiSMASH within the genome of *S. lavendulae* ATCC 13664

Cluster	Type	Contig (location), length	Monomer prediction[1]
1	Terpene	2 (17606-44513), 26908 bp	
2	Putative	2 (69671-77939), 8269 bp	
3	NRPS - trans-AT PKS	3 (46686-75594), 28909 bp	(gly)
4	Siderophore	5 (52241-61529), 9289 bp	
5	Siderophore	6 (37086-58940), 21855 bp	
6	Terpene	12 (21524-48620), 27097 bp	
7	Putative	13 (119-9731), 9613 bp	
8	Putative	19 (31436-39907), 8472 bp	
9	NRPS - type I PKS	24 (1-42060) 42060 bp	(pk-orn)
10	Putative	51 (163-33070), 32908 bp	
11	Putative	57 (17673-29808), 12136 bp	
12	Putative	59 (4865-12187), 7323 bp	
13	Type III PKS	61 (8930-30326), 21397 bp	
14	Putative	62 (12048-28571), 16524 bp	
15	Thiopetide - lanthipeptide	63 (7357-30115), 22759 bp	
16	Putative	66 (17364-29584), 12221 bp	
17	Putative	75 (559-25870), 25312 bp	
18	Putative	79 (14242-26282), 12041 bp	
19	Type III PKS	85 (1-26002), 26002 bp	
20	Type I PKS - butyrolactone	89 (1-25268), 25268 bp	(mal-mal-mal)
21	Butyrolactone	93 (1569-25509), 23941 bp	
22	NRPS	94 (1-25446), 25446 bp	(nrp-val)+(val)
23	Putative	96 (4727-25145), 20419 bp	
24	Putative	97 (14570-24969), 10340 bp	
25	Terpene	99 (1-24778), 24778 bp	
26	Putative	105 (604-9050), 8447 bp	
27	Melanin	105 (16008-23412), 7405 bp	
28	Putative	113 (1389-20392), 19004 bp	
29	Bacteriocin	115 (1-21857), 21857, bp	
30	Putative	123 (1558-19722), 18165 bp	
31	NRPS	126 (1-19842), 19722 bp	(nrp)+(cys)
32	Melanin	136 (926-18863), 17938 bp	
33	Type I PKS	139 (1-18430), 18430 bp	(nrp)
34	Butyrolactone	143 (4842-17983), 13142 bp	
35	Type II PKS	144 (1-17902), 17902 bp	
36	NRPS	148 (1-17472), 17472 bp	(thr)
37	NRPS	149 (1-17456), 17456 bp	(orn)
38	Bacteriocin	151 (1-17288), 17288 bp	
39	Ectoine	153 (5703-17114), 11412 bp	
40	Terpene	156 (1-16858). 16858 bp	
41	Putative	159 (37-11604), 11568 bp	
42	Putative	161 (101-16034), 15934 bp	
43	Thiopeptide - lanthipeptide	162 (1-16213), 16213 bp	
44	Putative	166 (2283-15918), 13636 bp	
45	Putative	169 (4333-14496), 10134 bp	
46	Putative	174 (8440-15450), 7011 bp	
47	Bacteriocin	205 (5889-13572), 7684 bp	
48	Putative	231 (560-10823), 10264 bp	
49	Other	233 (1-12041), 12041 bp	
50	Putative	244 (69-11137), 11069 bp	
51	Other	255 (1-11005), 11005 bp	
52	Terpene	300 (1-8379), 8379 bp	
53	Trans-AT PKS - hglks	336 (1-7059), 7059 bp	
54	Terpene	339 (1-6982), 6982 bp	
55	Type I PKS	348 (1-6666), 6666 bp	(pk-mal)

Cluster	Type	Contig (location), length	Monomer prediction[1]
56	Bacteriocin	350 (1207-6606), 5400 bp	
57	Terpene	355 (1-6378), 6378 bp	
58	NRPS	363 (1-6294), 6294 bp	(orn)
59	Putative	365 (757-6089), 5333 bp	
60	Terpene	415 (1-4898), 4898 bp	
61	Terpene	431 (1-4533), 4533 bp	
62	Type I PKS	465 (1-3573), 3573 bp	(mmal)
63	Type I PKS	492 (1-2872), 2872 bp	
64	Type I PKS	505 (1-2472), 2472 bp	
65	Other	546 (1-1904), 1904 bp	
66	Bacteriocin	556 (1-1630), 1630 bp	

[1] cys: cysteine; gly: glycine; hglks: unusual PKS hglE-like or hglD-like; mal: malonyl-CoA; mmal: methoxymalonyl-CoA; non-ribosomal peptide; orn: ornithine; nrp: pk: polyketide; thr: threonine; val: valine; other: cluster containing a secondary metabolite-related protein that does not fit into any other category

Unfortunately *pva* gene was detected within the contig 70 which is a short sequence with only 28867 bp, and probably this is the reason why no clusters were located within this contig according to antiSMASH. In fact, only 64 contigs have clusters from 650 original sequenced contigs from the genome shotgun project. It is worth mentioning that all those strains (Fig. 22) were analyzed by antiSMASH and all of them have an average value of 68 clusters, which means that the number of predicted clusters in *Streptomyces* sp. is similar to other species of this genus (data not shown). Likewise, the analysis estimated that 69 % of those strains had their homologous acylase-encoding genes outside of the clusters, 19 % were located within putative clusters, 6 % within a terpene cluster, and 6 % within a t1PKS-transatPKS-NRPS cluster. In addition, those available genomes in databases had an average value of 105 contigs, which means that regardless of the number of contigs in the present genome shotgun project, the information obtained in this platform is even similar to those genomes with less contigs (*e.g. S. griseus* subsp. *griseus* NBRC 13350 have 67 clusters, and the homologous gene is outside of the clusters).

Finally, several subsystems were identified in the genome by RAST (Fig. 23), which proposed a set of their possible functional roles. Therefore, 27 subsystems were grouped, and those related with (i) carbohydrates, amino acids and derivatives, (ii) protein metabolism and cofactors, (iii) vitamins, prosthetic groups and pigments, are the larger ones.

It is worth mentioning that some genes involved in virulence, disease and defense were also detected. The presence of genes related with tetracycline and fluoroquinolones resistance, as well as 18 diverse secondary metabolism genes could also be observed. In addition, the presence of 10 β-lactamases is remarkable in this microorganism, just as mentioned above. All those subsystems were clustered in three main groups named (i) metabolism, (ii) cellular processes and signaling, and (iii) information and others (Fig. 23).

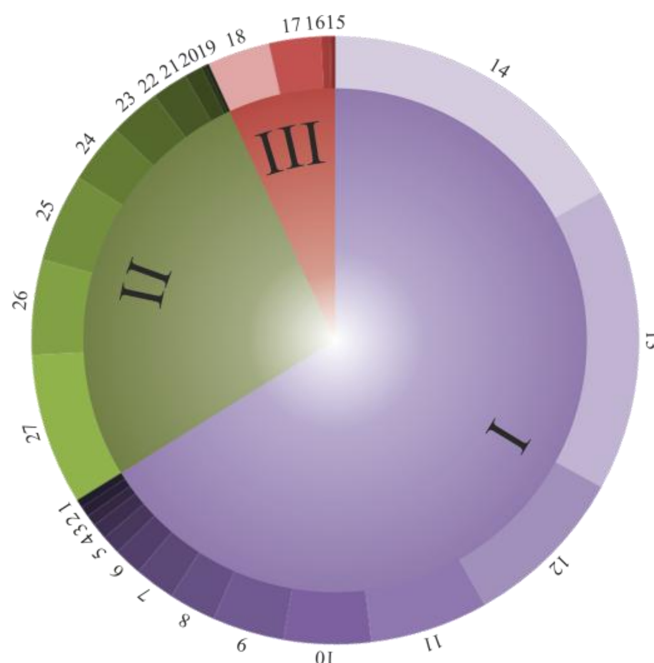

Figure 23. Distribution of subsystem categories in *Streptomyces* sp. according to RAST. **(1)** Potassium, **(2)** DNA metabolism, **(3)** secondary metabolism, **(4)** nitrogen, **(5)** aromatic compounds, **(6)** phosphorous, **(7)** iron acquisition and metabolism, **(8)** sulfur, **(9)** nucleosides and nucleotides, **(10)** respiration, **(11)** fatty acids, lipids and isoprenoids, **(12)** protein, **(13)** amino acids and derivatives, **(14)** carbohydrates, **(15)** phages, prophages, transposable elements, plasmids, **(16)** miscellaneous, **(17)** RNA, **(18)** DNA, **(19)** motility and chemotaxis, **(20)** dormancy and sporulation, **(21)** cell division and cell cycle, **(22)** regulation and cell signaling, **(23)** virulence, disease and defense, **(24)** membrane transport, **(25)** cell wall and capsule, **(26)** stress response, **(27)** cofactor, vitamins, prosthetic groups and pigments

1.2. Draft genome sequence of *Actinoplanes utahensis* NRRL 12052

Actinoplanes utahensis NRRL 12052 is a Gram-positive bacteria able to hydrolyze the amide bond of aliphatic side chains which are present in many antibiotics, such as penicillins (Torres-Bacete, J. *et al.* 2007; Hormigo, D. *et al.* 2010), lipopeptides (Boeck, L. D. *et al.* 1988; Boeck, L. D. *et al.* 1989; Debono, M. *et al.* 1989; Takeshima, H. *et al.* 1989; Kreuzman, A. J. *et al.* 2000; Borders, D. B. *et al.* 2007; Hormigo, D. *et al.* 2010), glycopeptides (Snyder, N. J. *et al.* 1998), glycolipodepsipeptides (Gandolfi, R. *et al.* 2012), as well as capsaicin (Romano, D. *et al.* 2011), among others (Hormigo, D. 2009). The genome of *A. utahensis* NRRL 12052 has been sequenced and deposited at GenBank under accession number JRTT00000000, and the version described here was deposited under accession number JRTT01000000 (Velasco-Bucheli, R. *et al.* 2015). The *A. utahensis* draft genome comprises of 9.5 Mb with a 71.2 % G+C content, obtaining 396 large contigs from 2.1×10^6 reads by Newbler 2.5.3, which were reduced up to 141 contigs by manual assembly providing 39.4-fold coverage.

According to RAST, there are 77 RNA genes which are divided in 6 rRNA genes and 71 tRNA genes. In addition, the genome contains 8744 CDSs, 730 of them associated with putative functions, and 3010 are hypothetical proteins. A sequence of 1520 bp was located within the contig 81, and it was assigned to 16S rRNA and employed to build the phylogenetic tree by BlastN (Fig. 24). Unfortunately, JSpecies analysis was unsuccessful

since the obtained results were not satisfactory to the software threshold, and none of them reached enough identity with *A. utahensis* NRRL 12052.

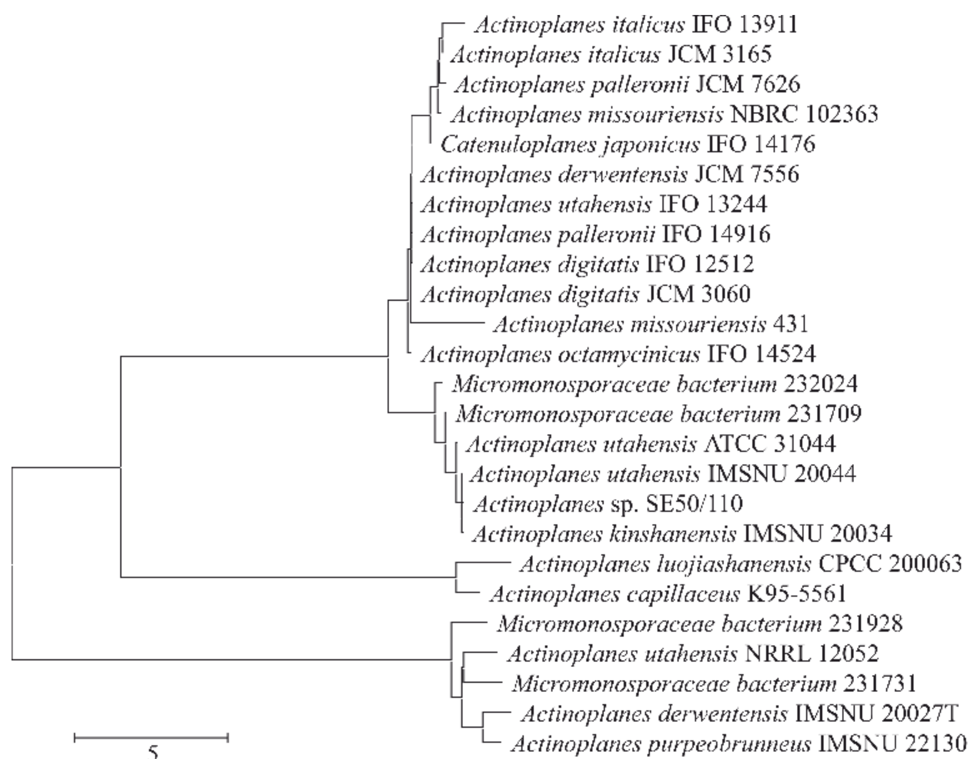

Figure 24. Phylogenetic tree of *A. utahensis* NRRL 12052 elaborated from the sequence of 16S rRNA employing BlastN as the alignment tool. *Catenuloplanes japonicus* IFO 14176 was considered as the out-group reference

CDS of aculeacin A acylase from *A. utahensis* were localized in the genome by RAST analysis. *Au*AAC encoding gene is located within the contig 8, showing a size of 2363 bp (GenBank: NZ_JRTT01000008.1). Once more, antiSMASH platform allowed the location of 24 clusters in the genome, which are described in Table 9.

Some of those clusters were related with NRPS, PKS, as well as bacteriocin, among others. Similarly, the presence of three clusters within the contig 8 should be remarked, as well as the presence of the aculeacin A acylase-encoding gene (*aac*). In particular, *aac* gene is located within the third cluster that is implicated in NRPS synthesis. This cluster is involved in the biosynthesis of a polyketide with predicted monomer (gly-nrp-nrp-nrp)+(nrp-nrp), (Fig. 25). Likewise, the presence of ABC-transporter coding sequences close to *aac* (Fig. 25) must be pointed out; such sequences are similar to the ones detected near *pva* (Fig. 22), and involved as well in the secretion of siderophores (*i.e.* iron metabolism in microorganism, and antibiotics used by humans) (Hider, R. C. *et al.* 2010).

Table 9. Clusters predicted by antiSMASH within the genome of *A. utahensis* NRRL 12052

Cluster	Type	Contig (location), length	Monomer prediction[1]
1	Type I PKS cluster - NRPS	5 (1-62527), 62527 bp	(nrp)+(mal)+(mal)+(gly)+(gly)+(mal)
2	Bacteriocin	7 (68537-80324), 11788 bp	
3	NRPS	8 (100740-162815), 62076 bp	(gly-nrp-nrp-nrp)+(nrp-nrp)
4	Oligosaccharide	8 (157125-193342), 36218 bp	
5	NRPS	8 (184153-213911), 29759 bp	(nrp)+(tyr-pro)
6	Aminoglycoside/aminocyclitol	12 (125216-146424), 21209 bp	
7	NRPS	13 (1-48635), 48635 bp	(ala)+(nrp)+(nrp)
8	Bacteriocin - NRPS	15 (71001-140693), 69693 bp	(nrp)+(nrp)+(asn-asn)+(nrp)+(pk)+(mal)
9	Aminoglycoside/aminocyclitol	15 (151867-177638), 25772 bp	
10	Bacteriocin	19 (60685-71217), 10533 bp	
11	NRPS	23 (1-52437), 52437 bp	(nrp-nrp)+(nrp-nrp)+(thr-nrp)
12	NRPS	26 (1-29783), 29783 bp	(nrp)
13	Melanin	28 (35099-46127), 11029 bp	
14	Oligosaccharide	33 (5152-33114), 27963 bp	
15	Melanin	43 (14442-25378), 10937 bp	
16	Terpene	46 (1909-24662), 22754 bp	
17	NRPS	50 (1-33715), 33715 bp	(pk)+(pro-gly-nrp)
18	Terpene	55 (10136-25353), 15218 bp	
19	NRPS	76 (1-2653), 2653 bp	(bht)
20	Melanin	129 (6966-28720), 21755 bp	
21	NRPS	129 (27727-98013), 70287 bp	(nrp-nrp-ser)+(nrp-nrp-nrp-ser-nrp)
22	Siderophore	130 (140344-159012), 18669	
23	NRPS - Type I PKS	131 (46577-98594), 52018 bp	(nrp-mal-nrp)
24	Type III PKS	137 (208941-250014), 41074 bp	

[1] nrp: non-ribosomal peptide; mal: malonyl-CoA; gly: glycine; tyr: tyrosine; pro: proline; ala: alanine; asn; asparagine; pk: polyketide; thr: threonine; bht: β-hydroxy-tyrosine; ser: serine

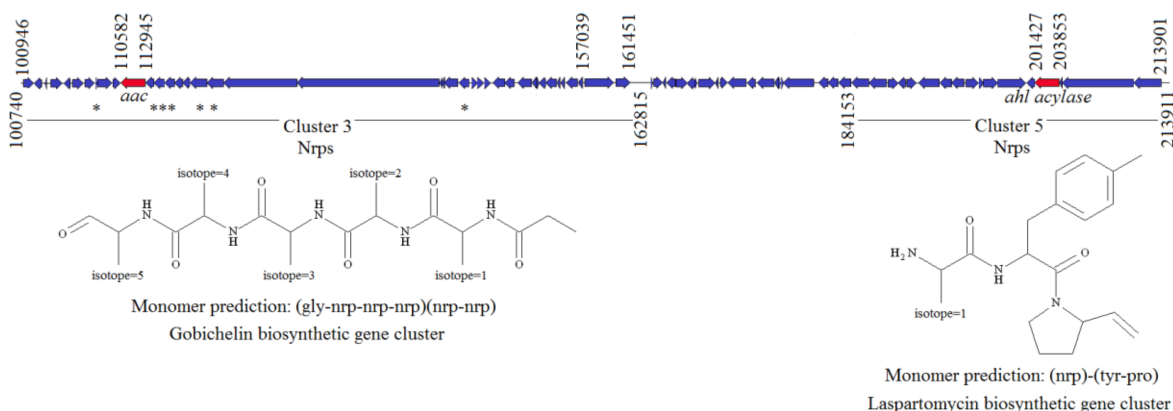

Figure 25. Clusters 3 and 5 within a fragment of the contig 8 containing each one acylases genes, and the rough prediction of core scaffolds produced by those clusters based on assumed PKS/NRPS collinearity without considering tailoring reactions. Those genes highlighted with asterisks are related with ABC-transporter systems, and *aac* and *ahla* genes are highlighted in red

It is remarkable that a putative AHL acylase was detected within the contig 8 in addition to the *aac* gene. Hereafter, this putative enzyme will be referred as *Au*AHLA (GenBank: NZ_JRTT01000008.1), which is reported for first time in this microorganism. Bioinformatic analysis of this novel AHL acylase indicated the presence of conserved essential amino acids involved in catalysis (*i.e.* βSer^{1}, βHis^{23}, βVal^{70} and βAsn^{282}) (Duggleby, H. J. *et al.* 1995) and substrate binding (*i.e.* αVal^{227}, αGly^{231}, βTyr^{24}, βArg^{31}, βTrp^{33}, βLeu^{50}, βPhe^{53}, βLeu^{57}, βIle^{58}, βSer^{67} and βLeu^{188}) (Zhang, D. *et al.* 2007; Torres-

Bacete, J. *et al.* 2015) with respect to other acylases (for further information, see Section 2 in the Results Chapter). Likewise, this gene is located at 88 kb upstream of *aac* gene and it was detected within the fifth cluster which codifies for the synthesis of another NRPS, with predicted monomers (nrp)+(tyr-pro). In addition, despite the fact that genes related with ABC-transporter system were not detected around *ahla* gene, the relative proximity of cluster 5 with those encoding sequences along cluster 3 could be interpreted as an alternative to siderophore secretion. Moreover, it was not discarded the presence of similar genes upstream of the contig 8 and within cluster 3 (*i.e.* the cluster is located at the end of this contig and probably it is truncated).

antiSMASH catalogues the genes contained in each cluster according to their function as: (i) biosynthetic, (ii) transport-related, (iii) regulatory and (iv) others. Thus, both acylase-encoding genes (i.e. *aac* and *ahla*) were catalogued as other genes, so the presence of those genes within NRPS clusters (Fig. 25) apparently could be a coincidence, at least with the present evidence. In addition, the analysis by BPROM (Solovyev, V. *et al.* 2010) did not indicate the presence of promoters, close enough to *aac* gene, which would initiate transcription of this gene in the bacterium.

Likewise, this study demonstrated that the putative AHL acylase gene (*ahla*) encodes for a protein of 802 amino acids (aa) that shows a modular organization composed by: (i) a predicted signal peptide of 27 aa (Fig. 26) according to SignalP predictor (Petersen, T. N. *et al.* 2011), (ii) an α-subunit of 21.6 kDa, and (iii) a β-subunit of 59.8 kDa (Fig. 27) considering the prediction of ExPASy (Gasteiger, E. *et al.* 2005). No transmembrane domain was predicted in the enzyme according to PredictProtein server (Rost, B. *et al.* 1996; Reeb, J. *et al.* 2015). In this case, the prediction performed by BPROM allowed the detection of a promoter upstream of *ahla*. Similarly to *Streptomyces* sp, RAST identified the presence of 16 β-lactamases in this microorganism suggesting a probable mechanism of *QQ*.

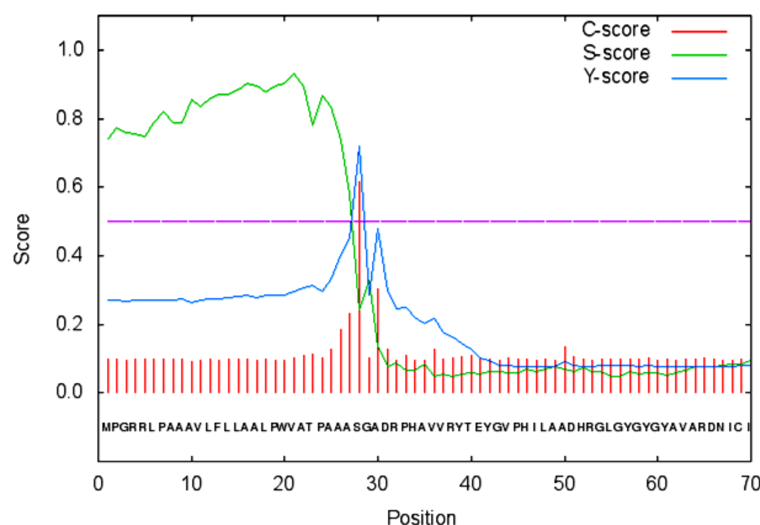

Figure 26. Predicted signal peptide of *Au*AHLA. C-score (probability of a cleavage site), S-score (probability of the presence of a SP), and Y-score (geometric average of C-score and S-score)

```
                                                          tctagaggaggtgccgcc      20
                                                          XbaI RBS consensus
                                AHLA1
gtg gcc cgt ccg ttc acc ctg ccc ggc cgc cgc ctc ccc gcg gcg gcc      68
 M   A   R   P   F   T   L   P   G   R   R   L   P   A   A   A       16
gtg ctg ttc ctg ctc gcc gcg ctg ccc tgg gtg gcg aca ccc gcc gcc     116
 V   L   F   L   L   A   A   L   P   W   V   A   T   P   A   A       32
                                        AHLA-ab
gcg tcc ggc gcc gac cgt ccg cac gcc gtc gtc cgc tac acc gag tac     164
 A   S α G   A   D   R   P   H   A   V   V   R   Y   T   E   Y       48
ggt gtc ccg cac att ctc gcc gcc gac cac cgt ggc ctc gga tac ggc     212
 G   V   P   H   I   L   A   A   D   H   R   G   L   G   Y   G       64
tac gga tat gcc gtc gcg cgg gac aac atc tgc att ctc gcc gac agt     260
 Y   G   Y   A   V   A   R   D   N   I   C   I   L   A   D   S       80
tat gtc acc gtc tcg gcg cag cga tcg ctg ttc cac ggc ccg gcc gcg     308
 Y   V   T   V   S   A   Q   R   S   L   F   H   G   P   A   A       96
ccg acc cgt tcc gat ttc ggc cgg gtc acc acc gcg ctg aac agc gac     356
 P   T   R   S   D   F   G   R   V   T   T   A   L   N   S   D      112
ctg tat ttc cag aac ctc aac gac agc ggt gtg gtg gaa cgg ctg atg     404
 L   Y   F   Q   N   L   N   D   S   G   V   V   E   R   L   M      128
agc ctg ccc gcg ccg ctg ggc ccg gag cgc gag gtc acc gac atg gtg     452
 S   L   P   A   P   L   G   P   E   R   E   V   T   D   M   V      144
 AHLA-Nt
cgc ggc tac gtc acc ggg tac aac cgc tac ctg cgc gac acc ggg gtg     500
 R   G   Y   V   T   G   Y   N   R   Y   L   R   D   T   G   V      160
gac gcc ctc acc gac ccg gcc tgc cgg ggc gcc gac tgg gtg cgg ccg     548
 D   A   L   T   D   P   A   C   R   G   A   D   W   V   R   P      176
atc acc gag ctc gac gtg ttc cgt gcc atg cac gcc ttc gtg atc ggc     596
 I   T   E   L   D   V   F   R   A   M   H   A   F   V   I   G      192
tcc ggc agc ggc tcg gtg atc gac ggc ctg gtc acc ccg cag ccg ccg     644
 S   G   S   G   S   V   I   D   G   L   V   T   P   Q   P   P      208
gcc ggc ccg gtc ccg ccg acg gcc acc ccc gcg gtg tcg gcc gag cag     692
 A   G   P   V   P   P   T   A   T   P   A   V   S   A   E   Q      224
gcc cgc acc gtg cgt gac agc ggc gcc gac cgg ggc agc aac gcc gtc     740
 A   R   T   V   R   D   S   G   A   D   R   G   S β N   A   V      240
gcc gcc ggg cgg gac ggc acc tcc ggg gcg cgc agc gtg ctg ctc ggc     788
 A   A   G   R   D   G   T   S   G   A   R   S   V   L   L   G      256
aac ccg cac tac ggc tgg tcc ggg gtg gcc cgg ttc tgg cag agc cag     836
 N   P   H   Y   G   W   S   G   V   A   R   F   W   Q   S   Q      272
ctc acc atc ccg gga cgg ctc aac gtc tcc ggc gcc ggg ctg ctc ggc     884
 L   T   I   P   G   R   L   N   V   S   G   A   G   L   L   G      288
ttc ccg gcc gtg ctg atc ggc cac aac cgc gac gtg gcc tgg tcc cac     932
 F   P   A   V   L   I   G   H   N   R   D   V   A   W   S   H      304
acg gtg tcc aca ccc acc acg tac ggc ctg ttc gag ctg acc acc gac     980
 T   V   S   T   P   T   T   Y   G   L   F   E   L   T   T   D      320
ccc gcc gac ccg acc cgg tac ctg cgc gac ggc gtg tcc acg ccg atg    1028
 P   A   D   P   T   R   Y   L   R   D   G   V   S   T   P   M      336
acc tcc cgg acc gtc acg gtg cgg gcg aag cag gcg gac ggc tcg gtc    1076
 T   S   R   T   V   T   V   R   A   K   Q   A   D   G   S   V      352
acc ccg gtc tcc cgg acg ctg tgg tcg acc gaa ctg ggc ccg gtg atc    1124
 T   P   V   S   R   T   L   W   S   T   E   L   G   P   V   I      368
ggc gcg atc ccc ggc gtc acc ctg ccg tgg ggc gcc acc gtg cac gtc    1172
 G   A   I   P   G   V   T   L   P   W   G   A   T   V   H   V      384
                                AHLA-5
ttc cgg gac gcc aac gcg ggc aac ctg cgg atg ctg aac acc tgg ttc    1220
 F   R   D   A   N   A   G   N   L   R   M   L   N   T   W   F      400
ggg ttc ggc gcc gcc cgt agc acg tcc gag gtg gac cag gtg ctg cgc    1268
 G   F   G   A   A   R   S   T   S   E   V   D   Q   V   L   R      416
cgc acc cag ggc gtc ccg tgg ctc aac acc gtc gcg tcc gac cgt gcc    1316
 R   T   Q   G   V   P   W   L   N   T   V   A   S   D   R   A      432
                                    AHLA-3
ggg aac gcg tac tac agc ggc atc cag gtg gtc ccg cac gtc acc gac    1364
 G   N   A   Y   Y   S   G   I   Q   V   V   P   H   V   T   D      448
gag cgg ctc gcc gcg tgc agc acc gcg ctg ggg cag cag ctg ttc cgg    1412
 E   R   L   A   A   C   S   T   A   L   G   Q   Q   L   F   R      464
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atc aac ggg acg ccg gtg ctg gac ggc agc cgc gcc gcg tgt gcc tgg   1460
 I   N   G   T   P   V   L   D   G   S   R   A   A   C   A   W     480
ggc acc gac ccg gac gcg gtg gag agc ggc ctg ttc ggg ccg tcc cgg   1508
 G   T   D   P   D   A   V   E   S   G   L   F   G   P   S   R     496
ctg ccc acc ctg acc cgg gcc gac tac gtc gcc aac gcc aac gac agc   1556
 L   P   T   L   T   R   A   D   Y   V   A   N   A   N   D   S     512
gcc tgg ctg acc aac ccg gcc cag ccg ctg acc ggc tac ccg gcg gtc   1604
 A   W   L   T   N   P   A   Q   P   L   T   G   Y   P   A   V     528
ctc ggc gcg gcc ggc acc cag cgg tcg gcg cgg acc cag cag acc atc   1652
 L   G   A   A   G   T   Q   R   S   A   R   T   Q   Q   T   I     544
          AHLA-6
gtg tcg gcg cag cgg cgg ctg gcg ggc gcc gac ggc ctg ccc ggc gcc   1700
 V   S   A   Q   R   R   L   A   G   A   D   G   L   P   G   A     560
                            AHLA-4
ggg ttc tcc ctg gac acc atg agc cgg gtg ctg ttc tcc gac gac agc   1748
 G   F   S   L   D   T   M   S   R   V   L   F   S   D   D   S     576
cgg gtc gcc gag ctc acc gcc gcg gac gcc gcc gcc atg tgc gcc gcg   1796
 R   V   A   E   L   T   A   A   D   A   A   A   M   C   A   A     592
ttc ccg gac ggt gtc gcc gcc ggc gca gcc ggg ccg gtc gac gtc acg   1844
 F   P   D   G   V   A   A   G   A   A   G   P   V   D   V   T     608
gag gcg tgc ccg gtg ctg gcc gcc tgg gat cgt tcg ttc cgc ctc gac   1892
 E   A   C   P   V   L   A   A   W   D   R   S   F   R   L   D     624
AHLA-Ct
agc cgc gga tcg ctg ctg ttc gcc cgg ttc gcc acc cgc ctc ggc gcg   1940
 S   R   G   S   L   L   F   A   R   F   A   T   R   L   G   A     640
gtg ccc ggc ggg ccg tgg gcc acc ccg ttc gac ccg gcc gac ccg atc   1988
 V   P   G   G   P   W   A   T   P   F   D   P   A   D   P   I     656
ggc acg ccg gcc ggg ctg gcc acc gcc aaa ccg gcg gtg cag cgg gcg   2036
 G   T   P   A   G   L   A   T   A   K   P   A   V   Q   R   A     672
ttc gcc gac gcg gtc gcc gaa ctg cgg tcg gcg ggc atc gcg ctg gac   2084
 F   A   D   A   V   A   E   L   R   S   A   G   I   A   L   D     688
gcg ccg ctc ggc gac cac cag agc gtc acc cgc gcc ggc gag acg atc   2132
 A   P   L   G   D   H   Q   S   V   T   R   A   G   E   T   I     704
ccg gtg cac ggc gcg ccg cac gcg ctg ggc gtg ctc aac gtg atc acc   2180
 P   V   H   G   A   P   H   A   L   G   V   L   N   V   I   T     720
ccg acg tgg cgg gcc ggc gcg ggc aac gtc gac gtc gtc cac ggc tcc   2228
 P   T   W   R   A   G   A   G   N   V   D   V   V   H   G   S     736
agc ttc atc cag gtg gtg gag ttc ggc gcg acc ggc gcg ccg cgg gcc   2276
 S   F   I   Q   V   V   E   F   G   A   T   G   A   P   R   A     752
cgc acc ctg ctg acg tac tcc cag tcg gcc gac ccc acc tcc ccg cac   2324
 R   T   L   L   T   Y   S   Q   S   A   D   P   T   S   P   H     768
cac gcc gac cag acc cgg ctg ttc tcc cgc tcc acg tgg gtg acc agc   2372
 H   A   D   Q   T   R   L   F   S   R   S   T   W   V   T   S     784
                                                        AHLA-2
cgc ttc acc gag cgc gag atc gcg gcg tcg ccg gtg ctg agc cag atc   2420
 R   F   T   E   R   E   I   A   A   S   P   V   L   S   Q   I     800
aga ctg acc gag cga gcg ccg cgg tga ggaattc                       2456
 R   L   T   E   R   A   P   R   -   EcoRI                         808
```

Figure 27. Amino acid and nucleotide sequences of *Au*AHLA. GenBank: WP_052163432.1). In the nucleotides sequence: start codon (gtg) is highlighted in green, end codon (tga) is highlighted in red, primer sequences are under green arrows. In the amino acids sequence: catalytic residues are highlighted in blue, residues forming substrate binding pocket in orange, α and β represent the beginning of corresponding subunit.

In Figure 28 the presence of CDSs up- and downstream of the *aac* gene is illustrated. In this case, 4 genomes of the genus *Actinoplanes* available in the NCBI database were compared against the sequence up- and downstream of the *aac* gene. In contrast to the similarities shown by *pva* gene, the genomes of this genus do not display conserved CDSs up- nor downstream of *aac*, which could be due to the low number of available genomes. Thus, according to BlastP outputs, this analysis was extended to the genus *Solinispora* (*i.e.* 34 species), due to the sequence similarity of this gene between both genera.

Unfortunately, only a couple of genes are conserved between both genera in this region of both genomes, and some of them are related with ABC-type transport systems and putative membrane proteins (data not shown).

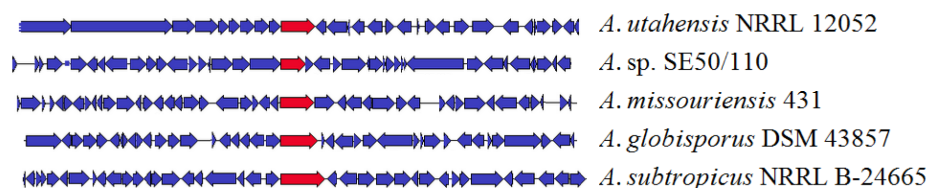

Figure 28. Representative coding sequences upstream and downstream of the gene of aculeacin A acylase from *A. utahensis* NRRL 12052

Alike in *Streptomyces* sp. genome, RAST analysis identified subsystems in *A. utahensis* which are illustrated in Figure 29. Similarly, 27 subsystems, clustered in three main groups, were detected, and the more abundant were those related with; (i) carbohydrates, amino acids and derivatives, (ii) protein metabolism, and (iii) cofactor, vitamins, prosthetic groups and pigments (Fig. 23). Likewise, genes involved in virulence, disease and defense were detected. It is also remarkable the presence of genes related with tetracycline, fluoroquinolones and vancomycin resistances, and 8 diverse secondary metabolism genes were identified as well.

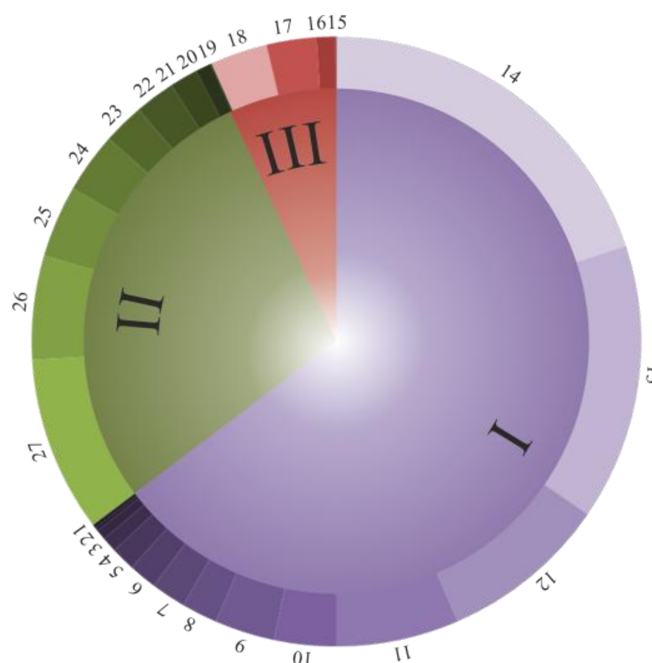

Figure 29. Distribution of subsystem categories in *A. utahensis* NRRL 12052 according to RAST. **(1)** Iron acquisition and metabolism, **(2)** secondary metabolism, **(3)** potassium, **(4)** DNA metabolism, **(5)** nitrogen, **(6)** sulfur, **(7)** aromatic compounds, **(8)** phosphorous, **(9)** nucleosides and nucleotides, **(10)** respiration, **(11)** fatty acids, lipids and isoprenoids, **(12)** protein, **(13)** amino acids and derivatives, **(14)** carbohydrates, **(15)** phages, prophages, transposable elements, plasmids, **(16)** miscellaneous, **(17)** DNA, **(18)** RNA, **(19)** dormancy and sporulation, **(20)** cell division and cell cycle, **(21)** regulation and cell signaling, **(22)** motility and chemotaxis, **(23)** virulence, disease and defense, **(24)** membrane transport, **(25)** cell wall and capsule, **(26)** stress response, **(27)** cofactor, vitamins, prosthetic groups and pigments

Surprisingly, a single *aac* gene was detected in this microorganism, suggesting that the reported membrane-associated echinocandin B deacylase gene (Kreuzman, A. J. *et al.* 2000; Arnold, F. *et al.* 2003; Shao, L. *et al.* 2013) which only differs from soluble *Au*AAC form (Takeshima, H. *et al.* 1989; Inokoshi, J. *et al.* 1992) by two additional N-terminal amino acids is encoded by the same gene that *Au*AAC, and do not displayed transmembrane domain in the gene sequence according to PredictProtein (Yachdav, G. *et al.* 2014).

1.3. Estimation of the chelating properties of echinocandins

In order to understand the biological role of some echinocandins (*i.e.* aculeacin A, pneumocandin B_0 and caspofungin), the chelating properties of these molecules were tested, which should be interpreted as the possibility that these compounds may capture iron. Despite the fact that these bioactive molecules have been described as antifungal compounds and they are available on the market, during this study it was suggested that these compounds could play a similar biological role to siderophores.

For instance, the biological role of siderophores is related with iron metabolism in microorganisms, although they are employed as antibiotics in human therapy (Hider, R. C. *et al.* 2010). In this sense, the hypothesis of a similar role of echinocandins during their biosynthesis was discarded, because no chelating properties were detected in these compounds during this study.

2. NOVEL AHL-ACYLASE FROM *Actinoplanes utahensis*

During this study the presence of a putative *Au*AHLA in *A. utahensis* genome was detected (Fig. 27). Whilst *Au*AAC and *Sl*PVA identity is 43 %, *Au*AHLA sequence keeps 44 % and 51 % of identity with *Au*AAC and *Sl*PVA, respectively. This information was considered during this study, and although kinetic parameters of *Au*AHLA were not estimated, a set of preliminary assays was performed with several substrates in order to corroborate the bioinformatic predictions.

In this sense, recombinant *Rhodococcus* sp. T104 with pENV19*ahla* harbouring AHLA-encoding gene was obtained as described in Section 2 in the Materials and Methods Chapter. Supernatant broth of this recombinant strain was evaluated to detect acylase activity employing two penicillins (*i.e.* penicillin V 116 mM and penicillin G 116 mM), ten AHLs (*i.e.* C_6-HSL 50.19 mM, C_8-HSL 25.08 mM, C_{10}-HSL 10.18 mM, C_{12}-HSL 80.00 mM, C_{14}-HSL 15.00 mM, oxo-C_6-HSL 8.21 mM, oxo-C_8-HSL 8.29 mM, oxo-C_{10}-HSL 7.23 mM, oxo-C_{12}-HSL 8.40 mM and oxo-C_{14}-HSL7.68 mM), and three echinocandins (*i.e.* pneumocandin B_0, caspofungin and aculeacin A, 0.5 mM all of them) as substrates. Despite optimal operational conditions were unknown up to date, the supernatant was adjusted at pH 7.0 and pH 8.0 and the reactions were carried out at those pH values. Reaction mixture containing 80 μL of the supernatant and 20 μL of substrate was incubated at 45ºC during 20 min, and thereafter each reaction was stopped with 150 μL of acetic acid 20 % (v/v). In this sense, time reaction was enough to catalyze substrate hydrolysis under the optimal conditions established for *Sl*PVA and *Au*AAC. Enzyme activities were measured after the addition of 100 μL of PDAB 0.5 % in methanol, and subsequent recording of the absorbance at 414 nm and room temperature.

Surprisingly, *Au*AHLA had similar catalytic behavior as *Sl*PVA and *Au*AAC, and penicillin V was the best substrate so far in contrast to other substrates under the established conditions. In addition, this enzyme was apparently active employing echinocandins as substrates. Furthermore, this enzyme showed activity using long side-chain aliphatic AHLs as substrates, and β-keto substitutions in the side-chain slightly affected such activity. However, further studies are needed to clarify this catalytic behavior (data not shown).

3. DETERMINATION OF KINETIC PARAMETERS OF *Sl*PVA AND *Au*AAC USING AHLs AS SUBSTRATES

Although a kinetic characterization of the parental enzymes was carried out previously employing a spectrophotometric method (Hormigo, D. 2009) , *Sl*PVA and *Au*AAC kinetic parameters were evaluated once more but using a fluorometric in order to compare the activities between native and recombinant enzymes. Parental enzymes were tested with each substrate at the described optimal conditions (*i.e.* 45ºC and pH 8.0) (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). It should be mentioned that this fluorometric technique is more sensitive than spectrophotometry, detecting smaller changes in the concentration of primary amino groups.

As a matter of fact, this is the first study in which apparent kinetic parameters of several AHLs are determined without problems of solubility of these compounds at concentrations reaching substrate saturation (Xu, F. *et al.* 2003; Hormigo, D. 2009; Wahjudi, M. *et al.* 2011; Clevenger, K. D. *et al.* 2013; Mukherji, R. *et al.* 2014) and employing pure enzymes instead of soil samples (Wang, Y.-J. *et al.* 2005). This methodology allowed the quantification of homoserine lactone (HSL) released after enzymatic hydrolysis of short and long chained AHLs (*i.e.* aliphatic and β-keto substituted aliphatic) catalyzed by the acylases. The detection range of HSL was between 150 pmol to 46 nmol, which reduced the amount of AHL required in the enzymatic reaction, and therefore diminishing those problems related to the solubility of these compounds at concentrations reaching substrate saturation. Likewise, fluorescence measurements were not interfered by the presence of precipitates within the well, in contrast to absorbance measurements that were employed before (Hormigo, D. 2009). It is important to point out that the standardized procedure used in this study measured the intensity of fluorescence due to excitation and emission on the top of the well (for further information, see Section 8.3 in the Materials and Methods Chapter).

Standardization of the reaction conditions was carried out before the estimation of the kinetic parameters of both enzymes. Firstly, several assays were performed in order to calculate the adequate amount of enzyme to be employed in the reaction. Parental enzymes were tested with each substrate, increasing the enzyme concentration at a fixed reaction time of 10 min, and a substrate concentration that reached enzyme saturation. Likewise, standardization included the estimation of the optimal reaction time for every assay (see Section 10.5 in the Materials and Methods Chapter) that was 10 min. Although β-keto substituted aliphatic AHLs were reported not to be hydrolyzed by the parental enzymes (Hormigo, D. 2009), displayed activity of both *Sl*PVA and *Au*AAC with these molecules at 45ºC and pH 8.0 was significant. Therefore, the fluorometric method to assay acylase activity employing β-keto substituted aliphatic AHLs should be considered more suitable and sensitive than the spectrophotometric method that was employed previously.

After assay standardization, an amount of 0.19 µg of *Sl*PVA and 0.23 µg of *Au*AAC were the optimal to measure the activity employing both kinds of AHLs (*i.e.* aliphatic and β-keto substituted aliphatic AHLs) as substrates. It is worth mentioning that the affinity of *Sl*PVA and *Au*AAC increase with the length of the aliphatic side-chain of the AHL, whereas acylase activity is slightly affected by the β-keto substitution. In contrast, enzymes displayed very low affinity for those aliphatic AHLs with a small side-chain (*i.e.* C_4-HSL and C_6-HSL).

It is important to remark that those assays for acylase activity determination based on PDAB and fluorescamine (fluram) were performed at low pH since the reaction is stopped by addition of an acid (for further information, see Sections 8.2 and 8.3 in the Materials and Methods Chapter). On the other hand, Schiff base formation between 6-APA and PDAB is affected at high pH values among other factors (Deshpande, B. S. *et al.* 1993) Likewise, primary amines such as HSL show a strong fluorescence with fluorescamine in between pH 7 to 9, and the sensitivity of the technique decreases with pH (Baker, W. L. 1985). As an interesting alternative, the protocol based on the reaction of primary amines with *o*-phthalaldehyde (OPA) at high pH values (for further information, see Section 10.5 in the Materials and Methods Chapter) allows the adequate conditions for fluorescence emission (Švedas, V.-J. K. *et al.* 1980). Thus, OPA offers several advantages over fluram and PDAB, such as (i) much higher solubility in water and stability than the other chromophores that must be dissolved in organic solvents (for further information, see Sections 8.2 and 8.3 in the Materials and Methods Chapter), and (ii) greater fluorescent quantum yields (Benson, J. R. *et al.* 1975). Indeed, fluram sensitivity has been established between 5 and 10-fold lower with respect to OPA (Benson, J. R. *et al.* 1975), and PDAB is almost equally sensitive to fluram (data not shown).

It is important to point out that isoindoles formed by OPA are unfortunately unstable in presence of light, attacked by acids, and air oxidation (White, J. D. *et al.* 1969). Therefore, reaction with OPA had to be carried out as fast as possible, and kept at 25ºC in the chamber of the FLUOstar microplate fluorometer for 2 min in order to avoid those drawbacks. Likewise, OPA was kept at 4ºC in presence of β-mercaptoethanol to evade its oxidation (Švedas, V.-J. K. *et al.* 1980). Thereby, enzymatic assays based on OPA have taken advantage of such properties, in addition to higher sensitivity of fluorometric methods than spectrophotometric techniques (Fricker, L. D. *et al.* 1990; Olojo, R. O. *et al.* 2005; El-Enany, N. *et al.* 2007; Mahmoud, A. M. *et al.* 2009; Önal, A. 2011). Higher reactivity of OPA in comparison with PDAB and fluram can be deduced from their reactions with primary amines (for further information, see Supplement S.7 in the Supplementary Material Chapter).

A preliminary study of the effect of substrate concentration on enzyme activity was carried out with eight concentrations of each substrate (data not shown), in order to estimate the most appropriate concentrations that should be employed for an accurate determination of K_M and k_{cat} values under each condition. The values of velocity were expressed as IU/mg of enzyme, where one international activity unit was defined as the amount of enzyme producing 1 µmol/min of homoserine lactone under the assay conditions. Acylase activity displayed at different substrate concentrations by *Sl*PVA (Fig. 30) and *Au*AAC (Fig. 31) at 45ºC and pH 8.0 were fitted to nonlinear regressions, allowing the determination of kinetic parameters (Tables 10 and 11). However, acylase activity of both enzymes employing AHLs with the smallest side-chains (*i.e.* C_4-HSL and C_6-HSL) as substrates, both aliphatic and β-keto substituted, were not quantified since very low activities were measured under

the reaction conditions (for further information, see Section 8.3 in the Materials and Methods Chapter).

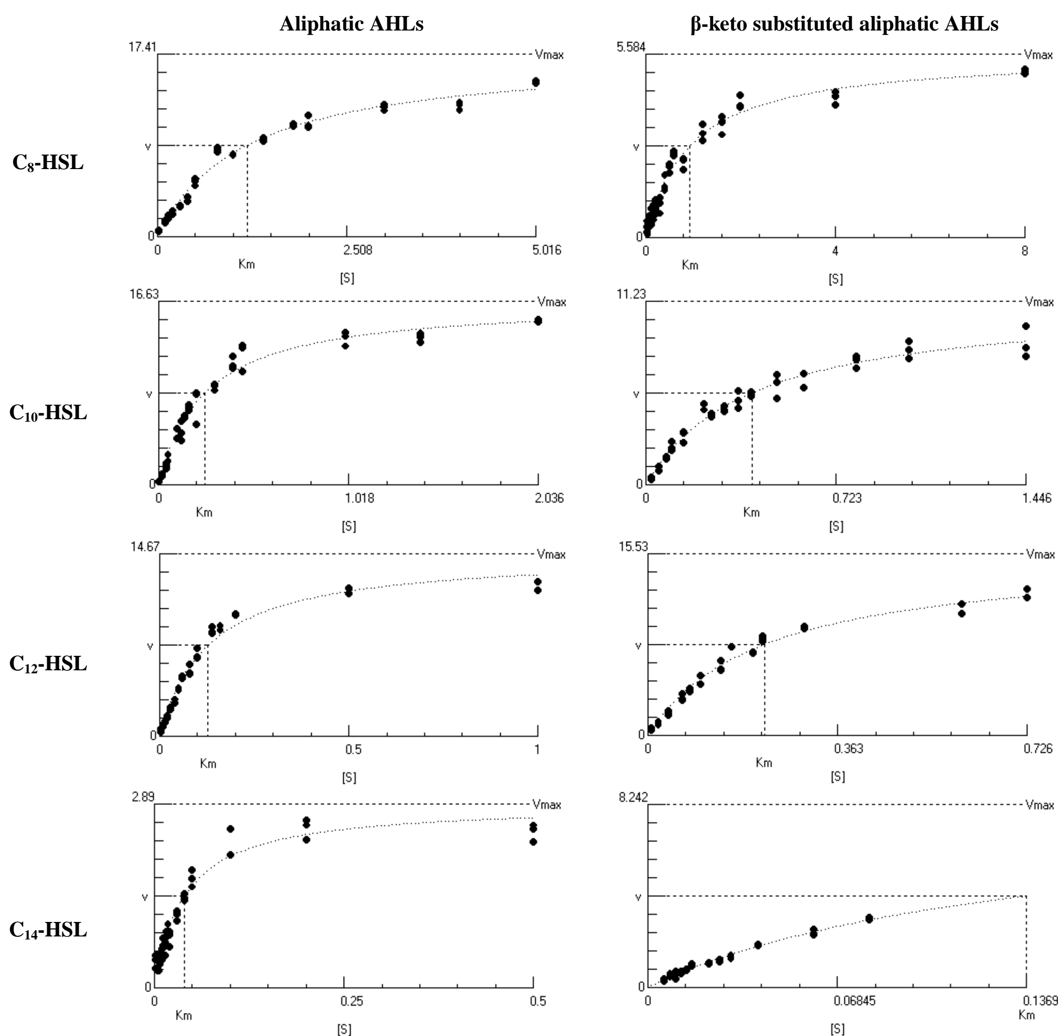

Figure 30. Nonlinear hyperbolic regression of the activity at different concentrations of several AHLs by *Sl*PVA at 45ºC and pH 8.0. The x-axis represents substrate concentration (mM) and the y-axis represents reaction velocity (IU/mg)

Table 10. Apparent kinetic parameters and specific parameters of *Sl*PVA

	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M ($M^{-1}s^{-1}$)	
	30ºC*	45ºC	30ºC*	45ºC	30ºC*	45ºC
C_8-HSL	1.114±0.35	1.186±0.156	3.63	22.89	3.26	19.30
oxo-C_8-HSL	0.313±0.169	0.934±0.137	1.91	7.34	6.10	7.86
C_{10}-HSL	0.034±0.018	0.247±0.035	2.63	21.86	77.50	88.44
oxo-C_{10}-HSL	0.259±0.059	0.403±0.065	4.69	14.76	18.11	36.63
C_{12}-HSL	0.033±0.011	0.127±0.017	2.23	19.29	66.78	149.97
oxo-C_{12}-HSL	0.069±0.016	0.223±0.031	4.91	20.42	70.96	91.56
C_{14}-HSL	**	0.040±0.007	**	3.80	**	96.26
oxo-C_{14}-HSL	0.026±0.006	0.137±0.032	2.23	10.84	85.21	79.15

* K_M, k_{cat} and k_{cat}/K_M at 30ºC are considered specific parameters, whereas those values at 45ºC are considered kinetic parameters

** Residual acylase activity was detected but apparent kinetic parameters could not be estimated under the assay conditions

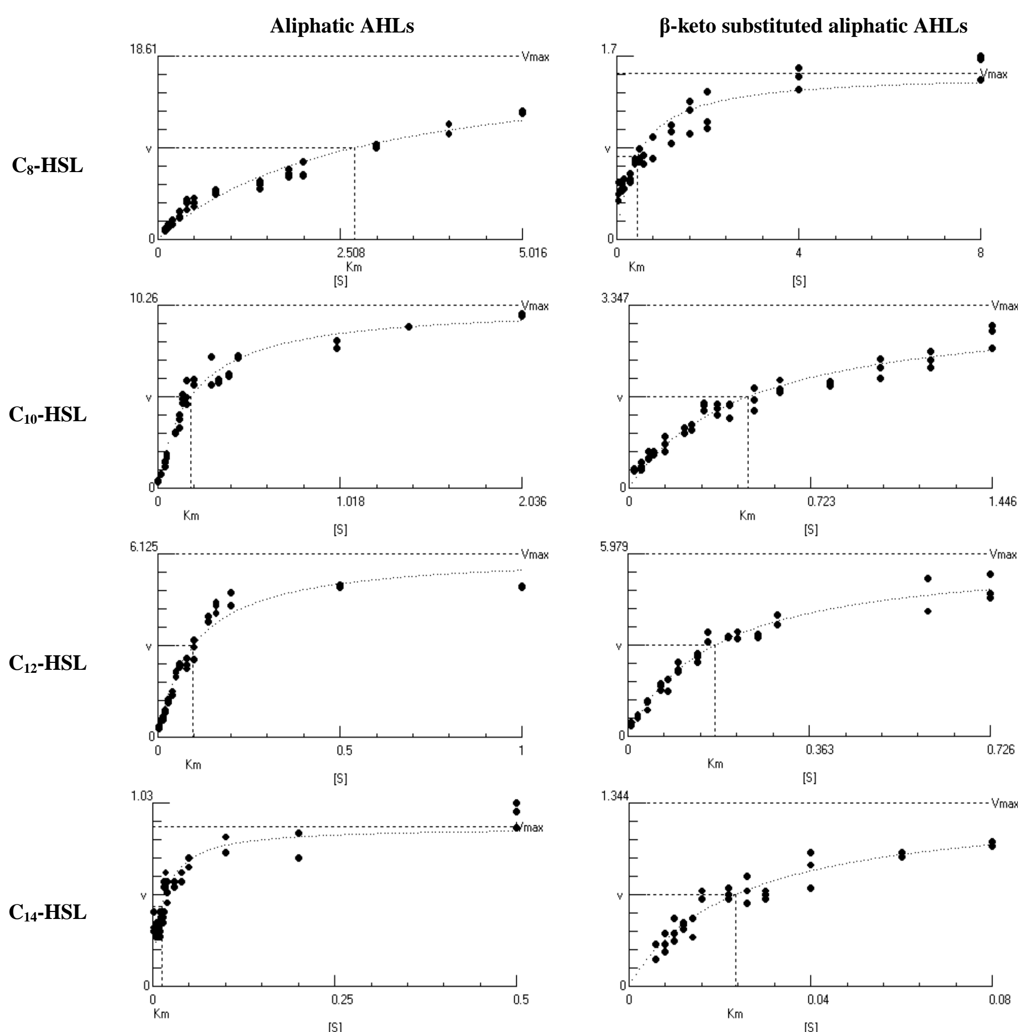

Figure 31. Nonlinear hyperbolic regression of the activity at different concentrations of several AHLS by *Au*AAC at 45ºC and pH 8.0. The x-axis represents substrate concentration (mM) and the y-axis represents reaction velocity (IU/mg)

Table 11. Apparent kinetic parameters and specific parameters of *Au*AAC

	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M ($M^{-1}s^{-1}$)	
	30ºC*	45ºC	30ºC*	45ºC	30ºC*	45ºC
C_8-HSL	0.303±0.172	2.700±0.758	2.23	24.75	7.36	9.17
oxo-C_8-HSL	**	0.449±0.142	**	2.05	**	4.55
C_{10}-HSL	0.014±0.012	0.186±0.029	1.86	13.65	137.02	73.24
oxo-C_{10}-HSL	0.043±0.013	0.473±0.095	1.43	4.45	33.22	9.41
C_{12}-HSL	**	0.097±0.016	**	8.15	**	83.65
oxo-C_{12}-HSL	0.010±0.003	0.175±0.030	1.68	7.95	166.15	45.36
C_{14}-HSL	**	0.013±0.003	**	1.19	**	91.40
oxo-C_{14}-HSL	**	0.024±0.006	**	1.79	**	76.16

* K_M, k_{cat} and k_{cat}/K_M at 30ºC are considered specific parameters, whereas those values at 45C are considered kinetic parameters
** Residual acylase activity was detected but apparent kinetic parameters could not be estimated under the assay conditions

It is highly remarkable that these results must be considered the first reported apparent kinetic parameters against several AHLs that have been determined by a single technique without problems of solubility of these compounds at concentrations reaching substrate saturation (Xu, F. *et al.* 2003; Hormigo, D. 2009; Wahjudi, M. *et al.* 2011; Clevenger, K.

D. *et al.* 2013; Mukherji, R. *et al.* 2014) and employing pure enzymes instead of soil samples (Wang, Y.-J. *et al.* 2005).

As discussed before, optimal operational conditions for both enzymes were established at 45ºC and pH 8.0 (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). However, similar analyses were carried out keeping optimal pH value but reducing temperature (*i.e.* pH 8.0 and 30ºC), as well as keeping optimal temperature but lowering pH value (*i.e.* pH 6.0 and 45ºC). Those additional analyses were carried out with the only purpose to establish and quantify the influence of those conditions in the activities of the mutant clones, and not with the intention to determine kinetic parameters at these uncommon conditions (*i.e.* hereafter named apparent kinetic parameters at optimal operational conditions, and named specific parameters at other conditions).

Despite to the technique sensitivity, no catalytic activity was detected at a low pH value (data not shown), whereas specific activities were found at a low reaction temperature. Specific parameters of *Sl*PVA (Table 10) and *Au*AAC (Table 11) in such conditions indicate that activities displayed by both enzymes at 30ºC and pH 8.0 are slightly similar to those reported at 45ºC and pH 8.0. Likewise, it is important to mention that basal acylase activity was displayed by both enzymes employing aliphatic AHLs with the shortest side-chains (*i.e.* C_4-HSL, C_6-HSL and oxo-C_6-HSL), hampering the determination of apparent kinetic parameters and specific activities under the assay conditions.

Recently, Torres-Bacete and co-workers (Torres-Bacete, J. *et al.* 2015) reported the kinetic parameters of recombinant *Sl*PVA employing different natural penicillins as substrates, which were quite similar to those previously reported for its wild-type counterpart (Torres-Guzmán, R. *et al.* 2002). Likewise, kinetic parameters of *Au*AAC were estimated employing the same substrates (Torres-Bacete, J. *et al.* 2007). In fact, these previous studies allowed the determination of the apparent kinetic parameters employing aromatic and aliphatic penicillins taking into account their side-chain Table 12. Likewise, both enzymes demonstrated to display hydrolytic activity against aculeacin A (Torres-Bacete, J. *et al.* 2007; Torres-Bacete, J. *et al.* 2015), which is a lipopetide with antifungal activity. This echinocandin was characterized by Mizuno and co-workers (Mizuno, K. *et al.* 1977) and contains a molecule of palmitic acid in the side-chain of a cyclohexapeptide (Takeshima, H. *et al.* 1989).

Table 12. Apparent kinetic parameters of parental *Sl*PVA and *Au*AAC assayed on different natural penicillins at optimal conditions (Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007)

Substrate	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M (mM^{-1} s^{-1})	
	*Sl*PVA	*Au*AAC	*Sl*PVA	*Au*AAC	*Sl*PVA	*Au*AAC
Penicillin V (C_6H_6-O-CH_2-CO-)	2.05	15.4	60.25	70.3	38.88	4.55
Penicillin K (C_7H_{15}-CO-)	0.14	1.0	22.71	33.3	165.30	34.79
Penicillin dihydro-F (C_5H_{11}-CO-)	0.69	5.6	15.19	4.2	21.85	0.75
Penicillin F (C_5H_9-CO-)	1.20	15.1	7.69	1.9	6.41	0.13
Penicillin G (C_6H_6-CH_2-CO-)	60.20	155.8	3.40	2.2	0.075	0.01

It is important to remark that enzymatic activity with those substrates containing the shortest aliphatic side-chains (such as penicillin dihydro-F and penicillin F) could be detected and quantified (Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007), whereas estimation of apparent kinetic parameters with C_6-HSL or C_4-HSL and their correspondent β-keto substituted AHL was impossible by the adopted fluorometric assay in

this study. This fact could be related to the low affinity of enzymes for these substrates, as well as the assay conditions employed in this study (low amount of enzyme to catalyze a few nanomoles in a small reaction volume).

Additionally, the relevance of *Sl*PVA and *Au*AAC have been ascribed to the plasticity of their catalytic pocket that is able to accommodate and catalyze penicillin V in addition to aliphatic penicillins, showing an increased catalytic efficiency when increasing the length of the side-chain of the substrate (Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2005; Torres-Bacete, J. *et al.* 2007). The results shown in Tables 10 and 11 confirm that both enzymes displayed catalytic activities against a wide spectrum of different substrates, corroborating the original hypothesis about their preference to hydrolyze substrates with a long side-chain. Nevertheless, it is necessary to clarify whether both enzymes should be classified as a new acylase subfamily, presumably involved in *QQ* processes.

4. DIRECTED MOLECULAR EVOLUTION OF *Sl*PVA AND *Au*AAC

As mentioned above, penicillin V acylase from *S. lavendulae* (*Sl*PVA) and aculeacin A acylase from *A. utahensis* (*Au*AAC) are enzymes with great potential to be employed for industrial applications. However, it is worth emphasizing that new and more powerful antibiotics are searched worldwide, as well as improved biocatalysts to make current processes more efficient. In this sense, this study employed a mutator strain as an approach to obtain recombinant enzymes with enhanced activity at optimal conditions, as well as altered substrate specificity and improved operational conditions.

4.1. Obtaining of *Sl*PVA and *Au*AAC mutant libraries

E. coli XL1-Red (Stratagene) was employed in order to introduce random mutations within the acylase genes. As mentioned in the Section 8.1.1 in the Introduction Chapter, this bacterium is deficient in three of the primary DNA repair pathways, *mutS* (error-prone mismatch repair), *mutD* (deficient in 3'-to 5'-exonuclease of DNA polymerase III), and *mutT* (unable to hydrolyze 8-oxo-GTP), which generate transcriptional mismatches, allowing the accumulation of mutations that apparently could encode enzymes with enhanced activities, stabilities, and/or wide spectrum of substrates. Additionally, transformation efficiency of this bacterium is higher than 1.0×10^6 cfu/µg, which increases the probability of mutations.

Random mutagenesis mechanism includes modifications divided into the next five categories: (i) transitions, which involve substitution of a purine nucleotide by another purine, or a pyrimidine by a second pyrimidine, (ii) transversions, which involve substitution of a purine nucleotide by a pyrimidine, or vice versa, (iii) deletions, in which one or more nucleotides are deleted from a gene, (iv) insertions, in which one or more extra nucleotides are incorporated into a gene, and (v) inversions, which involve the 180º rotation of a double-stranded DNA segment of two base pairs or longer (Salazar, O. *et al.* 2003).

Transformations of mutator strain with recombinant plasmids from pENV19 (Fig. 19) with harbouring each gene (*i.e. pva* and *aac*) yielded ten mutant genes libraries after five days of incubation (for further information, see Section 7 in the Materials and Methods Chapter). Due to its high mutagenicity, long culture periods of *E. coli* XL1-Red must be avoided (incubations should not be longer than 5 or 7 days) otherwise too many mutations

could be incorporated throughout the gene sequence, giving useless enzymes (*e.g.* deleterious activities, possible ORFs modification). Therefore, a PCR-based method is recommended if multiple mutations are desired (Greener, A. *et al.* 1997). The obtained collections of recombinant plasmids were used to transform *Rhodococcus* sp. T104 cells by electroporation. Transformation efficiency was 2.48×10^5 cfu/μg_{DNA} and 3.57×10^5 cfu/μg_{DNA} for *Sl*PVA and *Au*AAC, respectively, which means that recombinant strains were obtained successfully under the established conditions. In addition, this high transformation efficiency let obtain a library of 1,917 mutant clones from *aac* and a library 2,076 from *pva*. Selection of mutant clones was carried out in solid medium supplemented with kanamycin as resistance marker (for further information, see Section 3 in the Materials and Methods Chapter). Colonies were kept in 2xYT+G agar supplemented with kanamycin at room temperature, and further employed in the high-throughput screening (HTS).

4.2. High-Throughput Screening (HTS)

As mentioned in Section 8 in the Introduction Chapter, analysis and massive screening of mutant clones is the true bottleneck of the selection of mutant strains with improved behaviour, since directed evolution tries to simulate natural evolution but shortening the time of the process to just a few days in the lab. Establishment of a satisfactory methodology is crucial for obtaining good results in a huge screening. . In this sense, HTS can handle the analysis of many hundreds or even thousands of mutants under a specific condition. In the case of acylases from *Streptomyces* sp. and *A. utahensis*, the main objective was to obtain two libraries of both enzymes able to work under reaction conditions that were different to the optimum pH and temperature previously reported (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). In particular, such conditions were pH 6.0, (a harsh condition to the stability of the parental enzymes), and 30ºC which could let them be employed in different processes, such as decrease or inhibition of cellular communication between bacteria by *QQ* in membrane biological reactors or wastewater treatment plants. Thus, those colonies previously isolated were employed to carry out the massive screening taking into account their acylase activities that were measured according to Section 10.5 in the Materials and Methods Chapter. 96-wells microplates were employed in the evaluation, and handling and dispensing of samples, substrates and buffers were carried out by the Freedom EVOware platform from Tecan.

As a result, around 4,000 mutant clones (*ca.* 2,000 of each enzyme) and their penicillin V acylase activities at two temperatures and two pH values were tested. Similarly, nearly 2,000 of these mutant clones were tested in order to detect acyl-homoserine lactone acylase activities (*ca.* 1,000 strains per each enzyme). The established algorithm allowed simultaneous evaluation of the ability of one colony to hydrolyze one substrate at two pH values (6.0 and 8.0) and two temperatures (30ºC and 45ºC). This protocol saved time and resources, due to the high throughput of the process, and it was also friendly to the environment. Similarly, the liquid handling design, multiple pipetting and dispensing, guaranteed null standard deviation, and this value was corroborated periodically to certify the correct operation of the robot and the results reliability of the platform. At the end of this study 219,912 assays were carried out, 192,456 in the first screening (without replicates), and 27,456 in the second one (each sample was tested four-fold at the same conditions).

4.2.1. Screening of mutants of penicillin V acylase from *Streptomyces* sp. with improved acylase activities

Seven substrates under different reaction conditions were tested in this study (for further information, see Section 8 in the Materials and Methods Chapter). In addition to a natural substrate (*i.e.* penicillin V), acylase activities of mutant clones from *Sl*PVA were also evaluated employing AHLs as substrates. These molecules are involved in *quorum sensing* processes since the amide bond hydrolysis that releases their side-chains triggers *quorum quenching*, a mechanism that could be considered as an alternative approach to fight against pathogenic Gram-negative bacteria. Thanks to this methodology, a mutant library was ready to be evaluated employing different substrates under different reaction conditions (two pH values and two temperatures). It is important to remark that 30ºC and pH 6.0 can be considered as reaction conditions closer to those detected in the environment, instead of 45ºC and pH 8.0, optimal conditions for *Sl*PVA.

4.2.1.1.Screening of acylase activity employing penicillin V as substrate

In this HTS 2,076 mutant clones of *Sl*PVA were analyzed. Firstly, analysis was accomplished employing penicillin V as substrate (for further information, see Section 8.2 in the Materials and Methods Chapter), and the enzymatic activities of each mutant clone (A_C) with respect to the parental strain (A_0) were compared (Fig. 32). Similarly, representation of the fraction of mutant densities are illustrated as histograms in front of each axis of every plot (*i.e.* in top and right side of the plot), and the densities from each relative activity at a specific conditions. As observed from these histograms, many spots are represented by a few samples at the same position as a consequence of the scale of the plots.

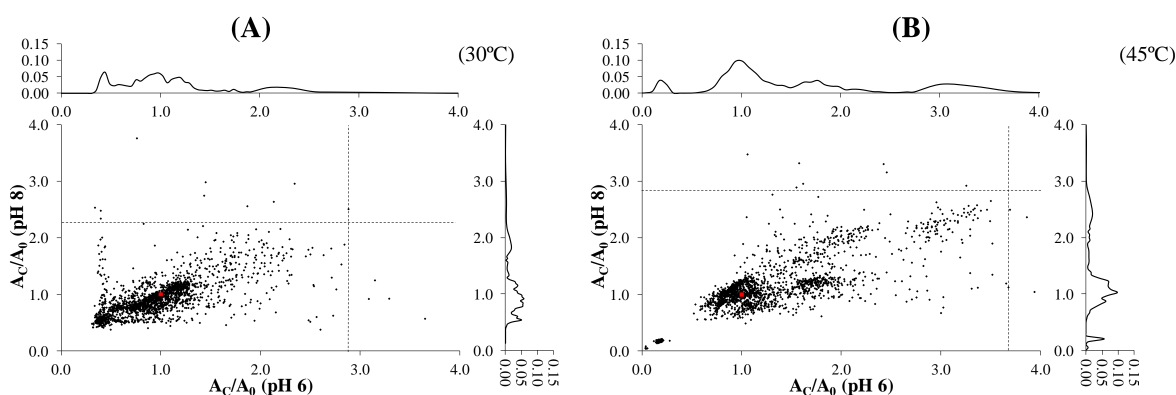

Figure 32. Penicillin V acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

As mentioned, HTS allowed the comparison of the activity (A_C) of each mutant clone with respect to the parental strain (A_0). These assays permitted to reflect a favorable or detrimental modification of acylase performance in a plot to any colony with respect to parental enzyme. The dispersion of spots clearly indicates randomness of mutations, which could be beneficial, deleterious or even void for the enzyme. The loss of activity might be due to many modifications introduced throughout the gene sequence. In the case of penicillin V acylase activity at 30ºC (Fig. 32), the presence of clones showing

approximately four-fold the activity of the parental clone at both pH values were observed. The enhanced activity at this temperature is an important success, since it is a lower temperature than the optimal shown by the parental one (*i.e.* 45ºC), and at a pH value in which parental enzyme shows no activity and is unstable (Torres-Bacete, J. *et al.* 2001). Penicillin V acylase activity at 45ºC is barely affected by the mutations, and this is the reason why most of the spots are concentrated around the wild-type (red spots in Fig. 32). Also, deleterious mutations were detected and those spots are located close to the corner of the plot, which indicates presence of mutants displaying negligible acylase activity. In contrast, dispersion at 45ºC and both pH values shows that most clones were generated with higher acylase activity, which could be interpreted that sequence modification at the optimal temperature condition needs to overcome an adverse condition (*i.e.* pH) or just improve the existing one. In contrast, the improvements at 30ºC were obtained in addition to the temperature, because the pH could be dissimilar to the optimum (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). Mutants obtained at these conditions had four-fold higher activity at both pH values (similar to 30ºC). It is worth mentioning that those clones with improved activity at different scenarios are dissimilar among them, and this means the successful availability of a collection of enzymes able to be applied in different types of reactions. In particular, the percentage of spots located above of the ratio A_C/A_0 = 1.0 (*i.e.* parental *Sl*PVA) at 30ºC and pH 6.0 were 39 %, whereas 33 % corresponded to those spots at 30ºC and pH 8.0. Likewise, 56 % were spots at 45ºC, and 57 % of spots at pH 6.0 and pH 8.0, respectively (Fig. 32). In these Figures, dot lines represent the selection criterion to keep the best clones in each evaluated condition, which was based on the *z*-score (for further information, see Section 9 in the Materials and Methods Chapter).

4.2.1.2. Tests of acylase activity employing aliphatic N-acyl-L-homoserine lactones as substrates

Additional to the assays with penicillin V as substrate, some of these mutant clones were tested to catalyze the main *N*-acyl-L-homoserine lactones that are involved in *quorum sensing QS* processes (*i.e.* C_6-HSL, C_8-HSL, C_{10}-HSL, oxo-C_6-HSL, oxo-C_8-HSL and oxo-C_{10}-HSL according to Supplement S.1 in the Supplementary Material Chapter). The acylase activity of these mutant clones employing aliphatic AHLs displayed similar behavior to that previously described with penicillin V. Herein, dispersion of spots clearly indicates randomness of mutations, which could be beneficial (high ratio), deleterious (low ratio) or even null (high density of mutants around parental clone) to enzyme in all the evaluated scenarios. Thus, Figure 33 shows the evaluation of 1,018 mutant clones from *Sl*PVA against C_6-HSL. In this occasion, percentage of improved activity of mutant clones against C_6-HSL (*i.e.* ratio A_C/A_0 higher than 1.0) at 30ºC were 19 % at pH 6.0 and 36 % at pH 8.0, whereas the results obtained at 45ºC were 38 % at pH 6.0 and 33 % at pH 8.0. One again, the enhanced activity that was achieved at 30ºC and pH 6.0 is a successful result, since these conditions are inadequate for a correct performance of both enzymes (Torres-Bacete, J. *et al.* 2000; Hormigo, D. *et al.* 2010). Similarly, 1,020 mutants were evaluated to quantify C_8-HSL acylase activity, as well as 1,018 mutant clones were employed to detect acylase activity using C_{10}-HSL as substrate. Results obtained with both substrates are shown in Figures 34 and 35, respectively. Percentage of *Sl*PVA mutants with improved activity against C_8-HSL, at 30ºC were 41 % at pH 6.0 and 35 % at pH 8.0. In the same sense, this percentage at 45ºC were 47 % at pH 6.0 and 44 % at pH 8.0 (Fig. 34), whereas these mutants with enhanced activities using C_{10}-HSL as substrate at 30ºC were 55 % at pH 6.0 and 27 % at pH 8.0, and at 45ºC were 44 % at pH 6.0 and 25 % at pH 8.0 (Fig. 35).

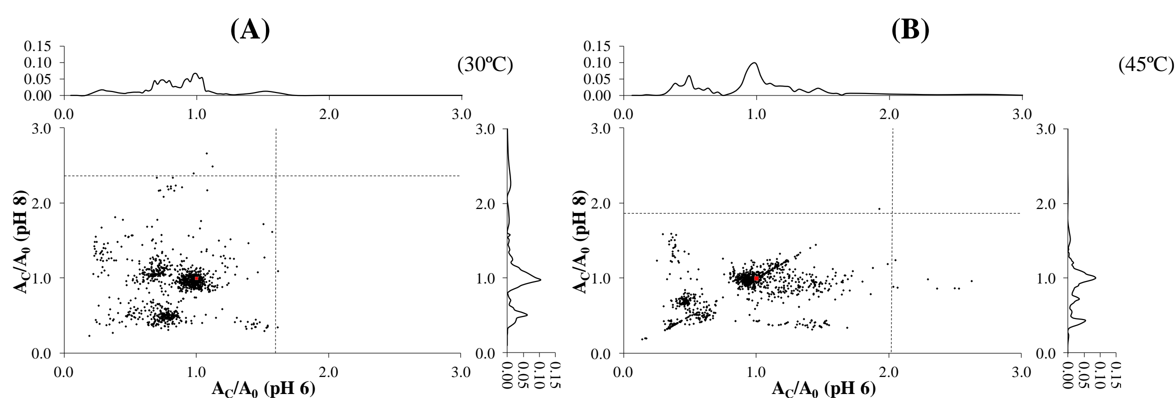

Figure 33. C_6-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

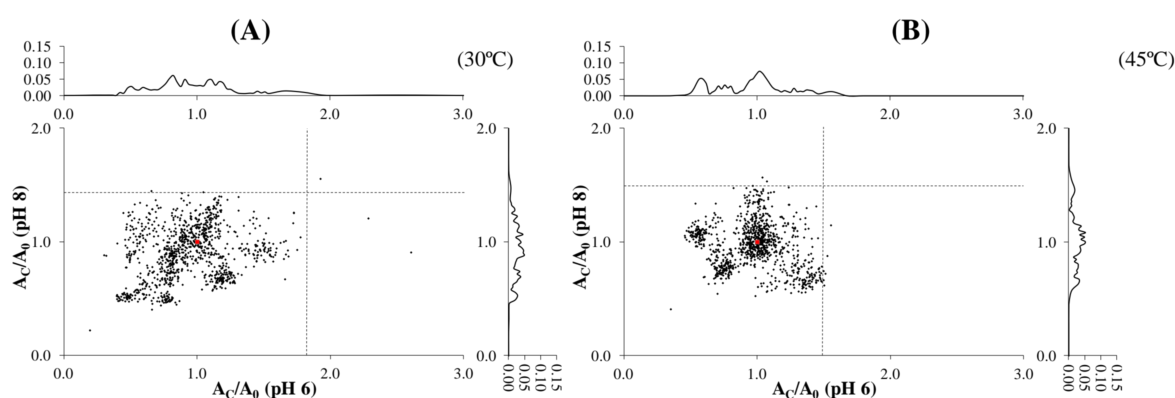

Figure 34. C_8-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

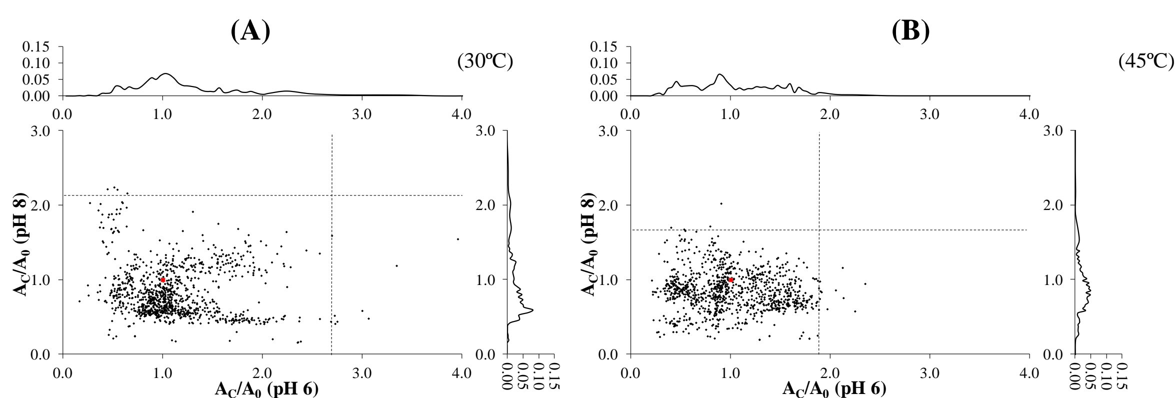

Figure 35. C_{10}-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

4.2.1.3.Tests of acylase activity employing β-keto N-acyl-L-homoserine lactones as substrates

Recently, AHLs with aliphatic side chains and β-keto substituted side-chains with 6, 8, and 10 atoms of carbon have been described to participate in most of the *QS* systems (for further information, see Supplement S.1 in the Supplementary Material Chapter) (Dickschat, J. S. 2010). Those substrates were considered during the HTS in order to improve the catalytic performance of *Sl*PVA, although wild-type enzyme was reported to display null activity against β-keto substituted aliphatic AHLs, or at least this activity was not detected with fluram (for further information, see Section 8.3 in the Materials and Methods Chapter) (Hormigo, D. 2009).

As exposed, this aspect was contrasted here (for further information, see Section 3 in the Results Chapter), and acylase activity measurement by fluorometric assays was possible to detect. However, spectrophotometric assays were employed during the HTS study (for further information, see Section 8.3.2 in the Materials and Methods Chapter), so that acylase activity (IU/mL) of each clone is hereafter represented in the axis of each plot, instead of the ratio with respect to parental strain that was represented with aliphatic AHLs. Thus, the assays were carried out with oxo-C_6-HSL, oxo-C_8-HSL and oxo-C_{10}-HSL.

Figure 36 shows oxo-C_6-HSL-acylase activity of 1,017 mutant clones from *Sl*PVA. As expected, most of mutant clones displayed similar activity employing this substrate under the tested scenarios in comparison to parental strain (*i.e.* spots located close to the histograms corner). Nevertheless, the presence of some mutants separated from the mean displaced to the right the criterion for the selection of the the best mutant clones (for further information, Section 9 in the Materials and Methods Chapter). The proportion of mutants with enhanced activity at 30ºC employing oxo-C_6-HSL was 33 % at pH 6.0 and 41 % at pH 8.0, whereas at 45ºC such proportion was 38 % at pH 6.0, and 40 % at pH 8.0 (Fig. 36).

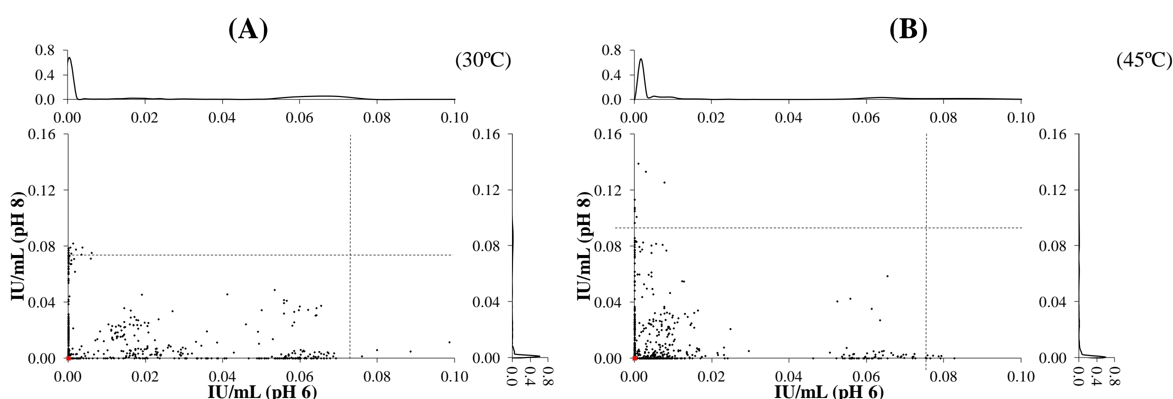

Figure 36. oxo-C_6-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

Finally, mutant clones from *Sl*PVA were tested against oxo-C_8-HSL and oxo-C_{10}-HSL-acylase (1,053 and 745, respectively), and the activities are represented in Figures 37 and 38, respectively. Although some of the spots are located far with respect to parental strain

(*i.e.* β-keto-acylase activity), most of them are situated close to the corner. In particular, the proportion of mutants with enhanced acylase activity at 30ºC employing oxo-C_8-HSL was 47 % at pH 6.0 and 44 % at pH 8.0, whereas such proportion at 45ºC was 29 % at pH 6.0, and 35 % at pH 8.0 (Fig. 37).

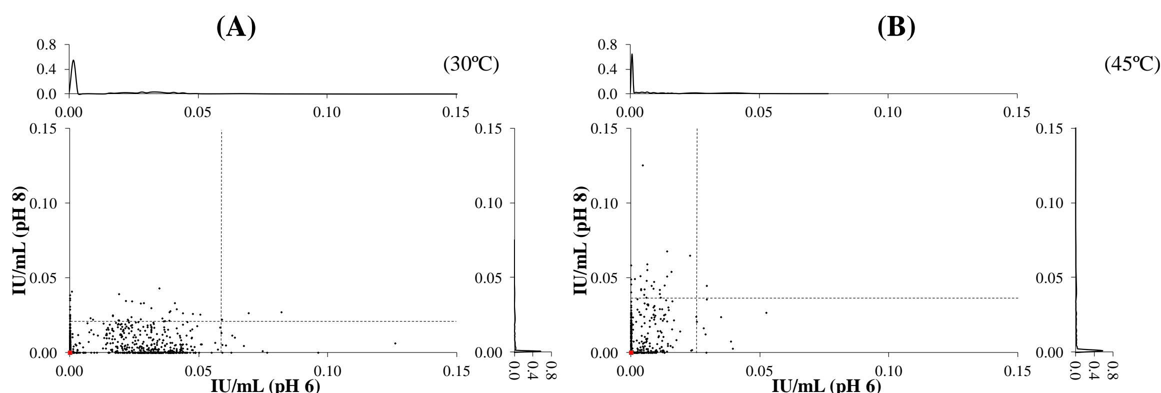

Figure 37. oxo-C_8-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

On the other hand, the percentage of mutant clones with improved activities at 30ºC employing oxo-C_{10}-HSL as substrate was 40 % at pH 6.0 and it was 18 % at pH 8.0, whereas such percentage at 45ºC was 31 % at pH 6.0 and 18 % at pH 8.0 (Fig. 38). Although of the percentage of mutant clones with enhanced activities employing β-keto substituted AHLs as substrates is rather low, it is noteworthy that this protocol allows the detection of mutant clones that are able to hydrolyze such molecules at different conditions by spectrophotometric assays.

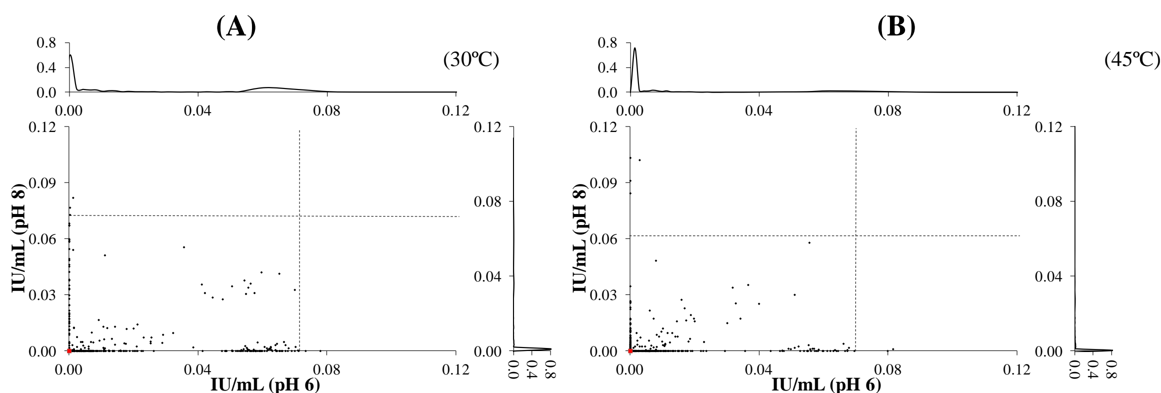

Figure 38. oxo-C_{10}-HSL-acylase activity displayed by *Sl*PVA mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

4.2.2. Screening of aculeacin A acylase mutants from *Actinoplanes utahensis* NRRL 12052 with improved acylase activities

As described for *Sl*PVA above, mutant clones of *Au*AAC were evaluated employing the same substrates. Once again, it is worth mentioning the relevance of this enzyme due to its

promiscuity to accept several types of molecules, which could be an opportunity to obtain improved enzymes.

4.2.2.1.Screening of acylase activity employing penicillin V as substrate

Similarly to the mutant library of *Sl*PVA, 1,917 mutant clones form *Au*AAC were analyzed by HTS following the same methodology (for further information, see Section 8 in the Materials and Methods Chapter). Regarding to penicillin V acylase activity, the dispersions were quite similar to those detected with *Sl*PVA (Fig. 39).

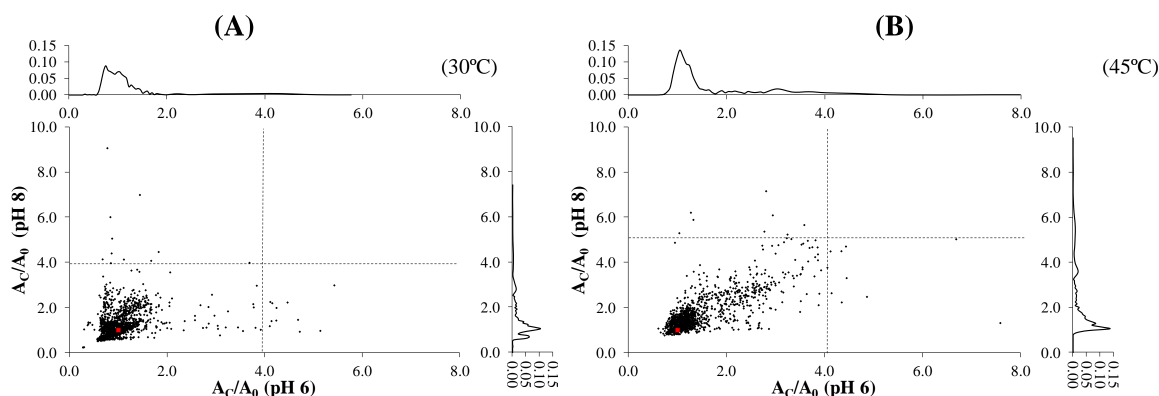

Figure 39. Penicillin V acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

Again, a comparison between activities of each mutant clone (A_C) versus parental strain (A_0) was plotted, and the dispersion of spots represents the randomness of the genetic modifications, observing a high density of spots around the native enzyme. However, improved clones with higher activity were detected at pH 6.0 and pH 8.0 at 30ºC, and some of them displayed approximately 5-fold (pH 6.0) and 9-fold (pH 8.0) increased activity compared to the parental enzyme, whereas at 45ºC the enzyme displayed approximately 8-fold increased activity at both pH values tested (Fig. 39). The percentage of mutants that displayed higher activities at 30ºC employing penicillin V as substrate was 36 % at pH 6.0 and 52 % at pH 8.0, whereas at 45ºC such percentage was 64 % at pH 6.0 and 75 % at pH 8.0. The obtained results are quite interesting, since *Au*AAC behaves similarly to *Sl*PVA (Fig. 32).

4.2.2.2.Screening of acylase activity employing aliphatic N-acyl-L-homoserines lactone as substrates

Analysis performed with *Au*AAC was identical to the study carried out with *Sl*PVA. Some of these mutant clones were selected to catalyze the same aliphatic *N*-acyl-L-homoserine lactones implied in *QS* processes. In this case, 1,070 mutant clones from *Au*AAC were evaluated employing C_6-HSL as substrate (Figure 40), The percentage of mutants with enhanced activities at 30ºC employing C_6-HSL was 20 % at pH 6.0 and 43 % at pH 8.0, whereas such percentage at 45ºC was 38 % at pH 6.0 and 30 % at pH 8.0.

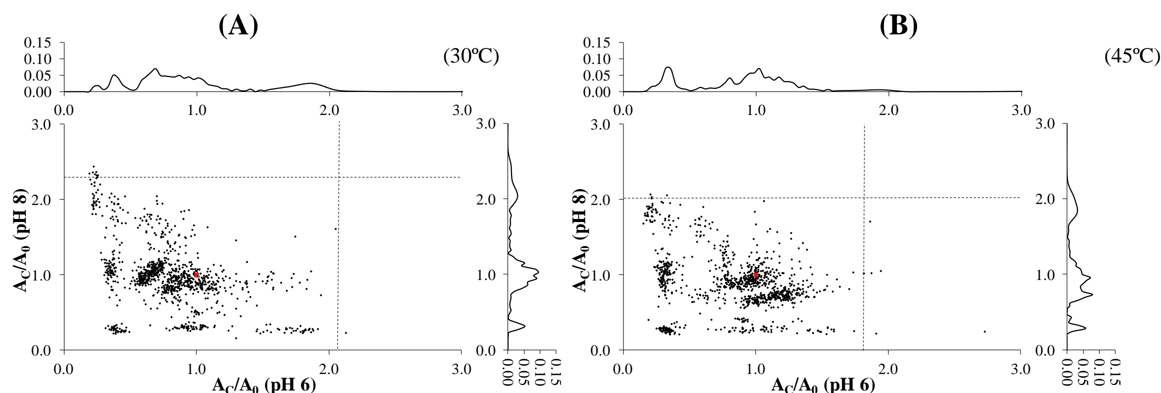

Figure 40. C_6-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

Like *Sl*PVA clones, the same statistical criterion was applied to *Au*AAC mutant clones (for further information, Section 9 in the Materials and Methods Chapter). Likewise, 1,083 mutant clones were analyzed to determined C_8-HSL acylase activity (Fig. 41) whereas 1,070 were evaluated in the case of C_{10}-HSL activity (Fig. 42). In the case of C_8-HSL (Fig. 41), the percentage of mutant clones with improved activities at 30ºC was 50 % at pH 6.0 and 24 % at pH 8.0, whereas such percentage at 45ºC was 31 % at pH 6.0 and 57 % at pH 8.0. In the case of C_{10}-HSL (Fig. 42), the percentage of mutant clones with enhanced activity at 30ºC was 59 % at pH 6.0 and 49 % at pH 8.0, whereas such percentage at 45ºC was 61 % at pH 6.0 and 44 % at pH 8.0.

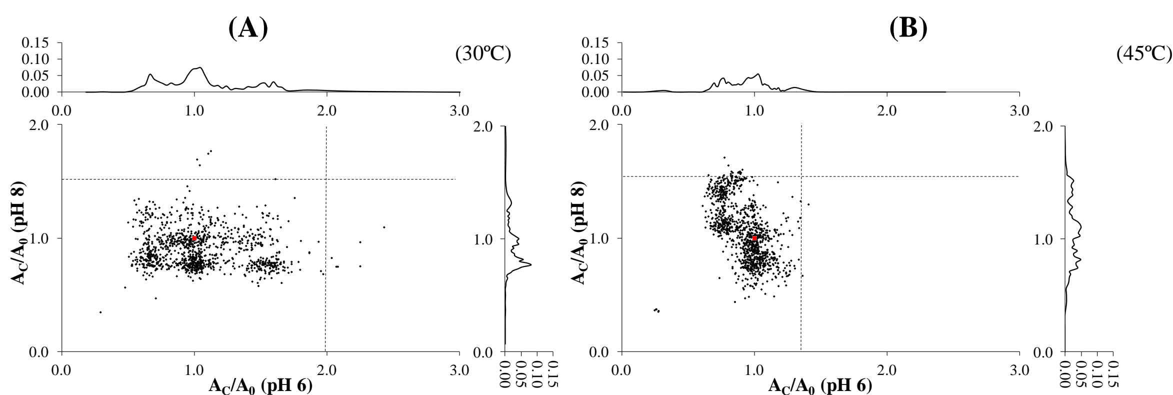

Figure 41. C_8-AHL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

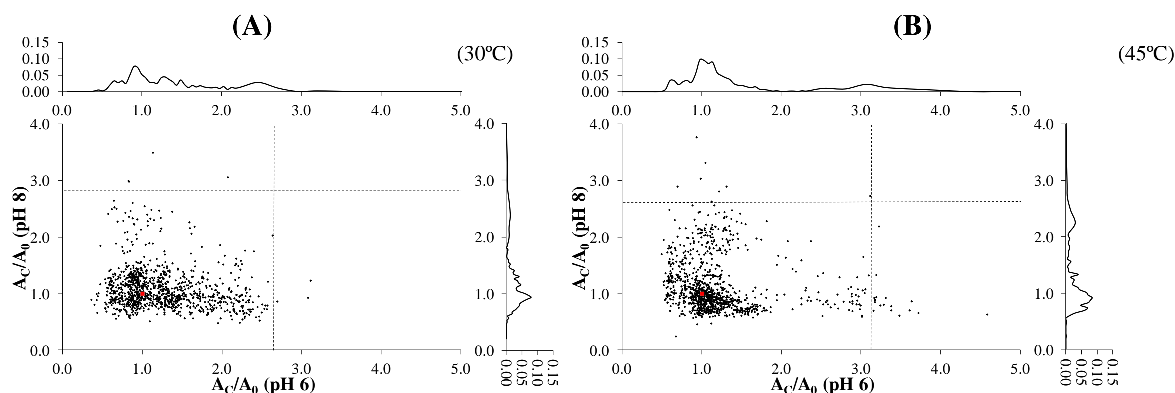

Figure 42. C_{10}-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

4.2.2.3.Screening of acylase activity employing β-keto N-acyl-L-homoserine lactones as substrates

Likewise, the activity of *Au*AAC mutant clones was evaluated employing β-keto substituted aliphatic AHLs as substrates. The oxo-C_6-HSL acylase activity of 1,070 mutant clones from *Au*AAC is represented in Figure 43, and similarly to the case of *Sl*PVA, most of mutant clones displayed similar activity employing this substrate under these operational conditions in comparison to the wild-type strain (Hormigo, D. 2009). The percentage of *Au*AAC mutant clones Figure 43 with improved activities at 30ºC employing oxo-C_6-HSL as substrate was 65 % at pH 6.0 and 65 % at pH 8.0, whereas such percentage at 45ºC was 44 % at pH 6.0 and 59 % at pH 8.0.

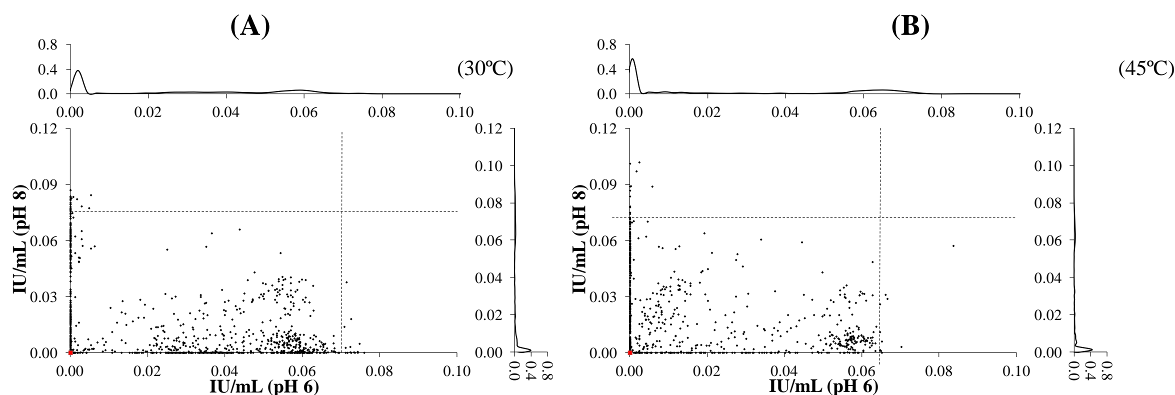

Figure 43. oxo-C_6-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

Likewise, the screening of *Au*AAC clones with oxo-C_8-HSL acylase and oxo-C_{10}-HSL acylase activities yielded 1,037 and 844 mutant clones, respectively, as shown in Figures 44 and 45. Similarly to the case of oxo-C_8-HSL acylase activity displayed by *Sl*PVA mutant clones Figure 37, few *Au*AAC mutant clones are located far from the wild-type strain, whereas most of them are situated practically in the corner. However, these raw data

from the screening have demonstrated the possibility of achieving recombinant enzymes with improved acylase activities against β-keto substituted aliphatic AHLs, even at rather different operational conditions than the optimum of native enzymes. The percentage of mutant clones with improved activities at 30ºC employing oxo-C_8-HSL as substrate was 33 % at pH 6.0 and 37 % at pH 8.0, whereas at 45ºC such percentage was 38 % at pH 6.0 and 48 % at pH 8.0 (Fig. 44). Likewise, the percentage of mutant clones with enhanced activity at 30ºC employing oxo-C_{10}-HSL as substrate was 29 % at pH 6.0 and 14 % at pH 8.0, whereas at 45ºC such percentage was 25 % and 13 % at pH 6.0 and pH 8.0.

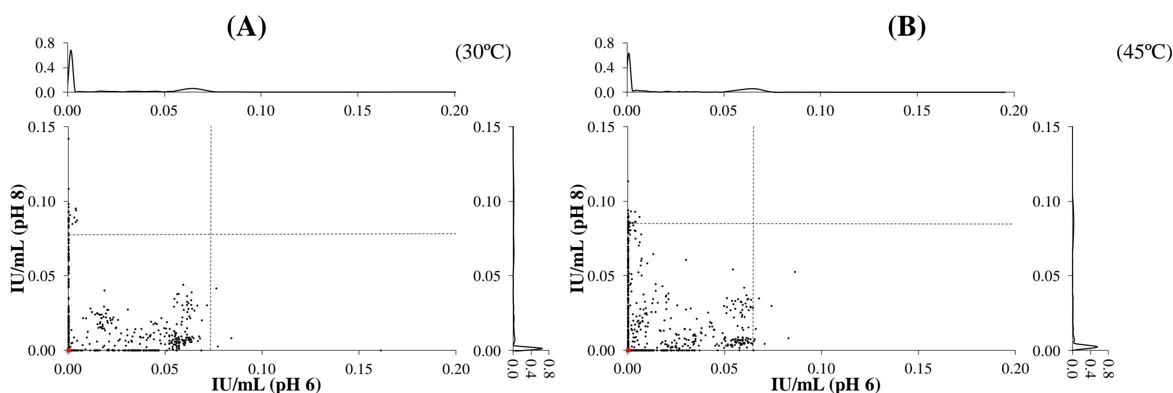

Figure 44. oxo-C_8-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

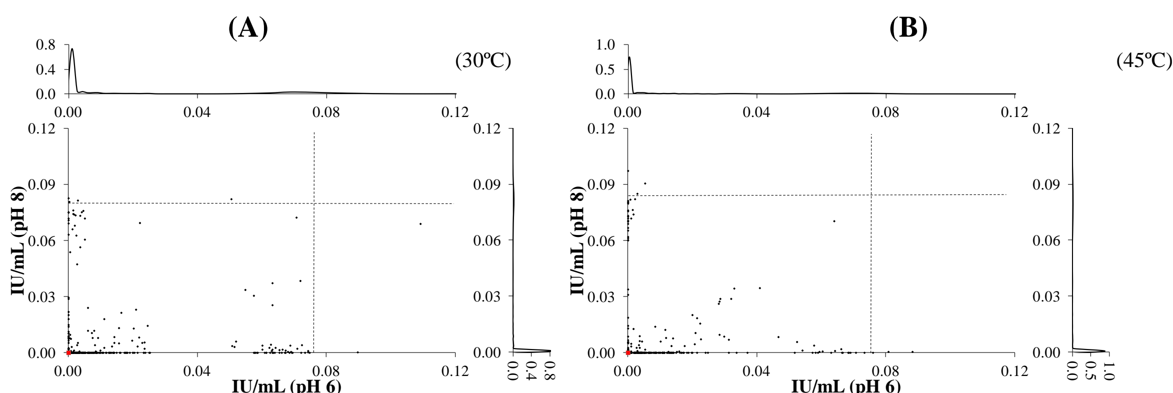

Figure 45. oxo-C_{10}-HSL-acylase activity displayed by *Au*AAC mutant clones (■) with respect to parental strain (■). Representation of the ratio of activity of mutant clone (A_C) with respect to parental clone (A_0) at **(A)** 30ºC and both pH values, and **(B)** 45ºC and both pH values. Histograms of spot densities are located in the periphery of each figure

The amount of assays under each evaluated biocatalytic scenario (temperature, pH, substrate and type of enzyme) is shown in Figure 46, which summarizes the results for both *Sl*PVA and *Au*AAC mutant clones, respectively. The analysis of both recombinant enzymes indicates the percentage of mutant clones expressing higher penicillin V acylase activity than parental strains, which range between 35-60 % in the case of *Sl*PVA, and it is between 42-89 % in the case of *Au*AAC. Likewise, about 1,000 mutant clones were tested at each condition employing AHLs as substrates with both enzymes, and represented the percentage of mutants with changes in their catalytic activity is also shown. One again, results obtained with β-keto substituted aliphatic AHLs are highly remarkable since this

catalytic activity was not detected by using the assays with fluram methodology (Hormigo, D. 2009). In particular, there were even mutant clones that were able to catalyze these substrates under harsh operational conditions (*e.g.* lower pH and/or temperature than the optimum) (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007).

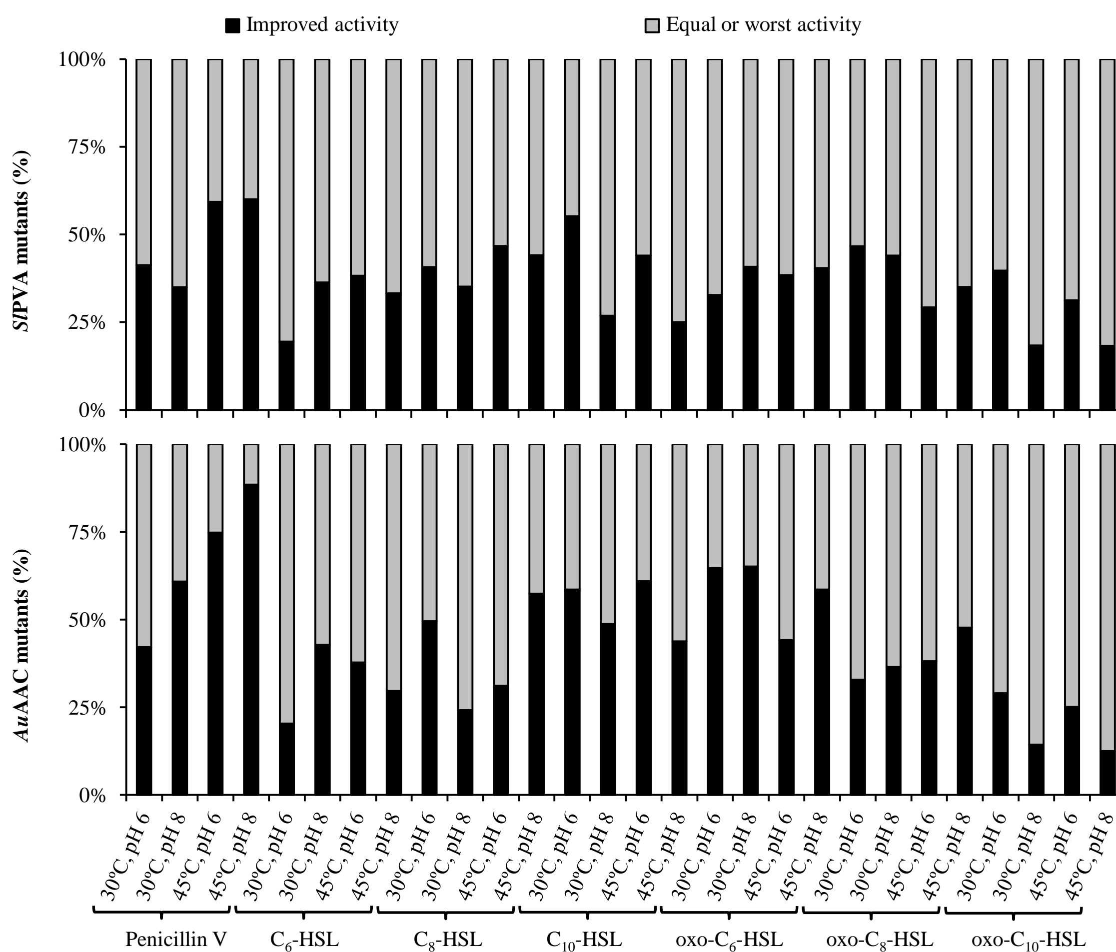

Figure 46. Acylase activity of mutant clones from *Sl*PVA and *Au*AAC at different operational conditions and substrates

In the case of aliphatic AHLs, the worst scenario for a good performance of both enzymes coincided in 20 % at 30ºC and pH 6.0 with C_6-HSL, whereas the best situations were 55 % for *Sl*PVA and 61 % for *Au*AAC employing C_{10}-HSL as substrate. This tendency is in agreement with the preference of these acylases to accept substrates containing long side-chains in their acyl moiety. In the case of β-keto substituted aliphatic AHLs, once again the worst scenario with both enzymes is observed employing the same substrate and the same operational condition (*i.e.* oxo-C_{10}-HSL, 30ºC and pH 8.0), in which less than 20 % of these mutant clones displayed apparent activity. Curiously, the best conditions were oxo-C_8-HSL at 30ºC and pH 6.0 with *Sl*PVA (reaching 47 %), and oxo-C_6-HSL at 30ºC and pH 8.0 with *Au*AAC (achieving 65 %). However, directed evolution is caused by random mutations, which means that nopredictable tendency could be inferred.

4.2.3. Statistical analysis

In any study, the importance of statistical analysis is more evident when the number of samples or raw data is huge. In particular, here 219,912 assays were carried out, 192,456 of

them in the first screening (*i.e.* scanning for new activities and operational conditions), and the remaining 27,456 assays in the second screening (*i.e.* corroborating those hits). Thereby, the development of an exhaustive data analysis was performed with these results, in order to find potential recombinant enzymes (*i.e. Sl*PVA and *Au*AAC) with different and improved acylase activities. Thus, statistical information was employed to analyze genetic modification obtained by directed evolution in combination with HTS, paving the correct way to create a library of mutant clones, and therefore to establish this technique as an important alternative for directed evolution of acylases.

As mentioned above, selection of these mutant clones was performed according to their activities under every operational condition (Fig. 32-45), and temporal pattern was considered as the only important effect during the evaluations. In this sense, exhaustive statistical analysis such as *B*-score, *BZ*-score or *R*-score was not considered here (Wu, Z. *et al.* 2008; Coma, I. *et al.* 2009). As shown in Figures 32 to 45, all histograms could be considered as normal distributions (Efron, B. 2004), and only those spots within the third quartile in the plots and with z_i values higher or equal to 3 were selected for a second screening (*i.e.* validation of those hits) (Brideau, C. *et al.* 2003; Prummer, M. 2012). Some authors suggest the selection of nearly 1 % of mutants to be analyzed in a second screening (Malo, N. *et al.* 2006). In fact, this is the base of *z*-score criterion for values equal or higher than 3 (Coma, I. *et al.* 2009). However, sometimes the results oscillation among microplate did not allow the comparison between activities analyzed at different times.

For this reason, in some cases the selection of samples for the second screening was higher (*e.g.* oxo-C_8-HSL-acylase activity with *Sl*PVA), but always guaranteeing that the total number to be analyzed in the second screening was less than 10 %, in order to avoid false negatives (Table 13) (Efron, B. 2004). In fact, this decision was supported later by the feedback of the Positive Predictive Value (PPV) for almost every condition evaluated in this study (Table 13), which quantifies the ratio of clones corroborated in the second screening with respect to those clones selected in the first screening. In particular, despite the fact that C_6-HSL hydrolysis catalyzed by recombinant clones of *Sl*PVA, and the C_8-HSL hydrolysis catalyzed by recombinant clones of *Au*AAC were not good enough predictions in the first screening, in the remaining scenarios the evaluations reached good PPV values. Thus, the correlation of percentage of false positives (*i.e.* false hits discarded in the second screening) with respect to all mutants is presented as percentage of error in each evaluated scenario (Table 13). This error indicates that the designed HTS here was satisfactory, because in all the cases the error reached values lower than 5 %.

Table 13. Library of obtained mutant clones from *Sl*PVA and *Au*AAC by directed evolution with acylase activities in the first and second screening

	***Sl*PVA**					***Au*AAC**				
	1st	2nd	Library	PPV	Error (%)	1st	2nd	Library	PPV	Error (%)
Penicillin V	2,076	24	5	0.208	0.91	1,917	25	15	0.600	0.52
C_6-HSL	1,018	13	1	0.077	1.18	1,070	19	7	0.368	1.12
C_8-HSL	1,020	25	5	0.200	1.96	1,083	47	1	0.021	4.25
C_{10}-HSL	1,018	39	14	0.359	2.46	1,070	32	18	0.563	1.31
oxo-C_6-HSL	1,017	25	16	0.640	0.88	1,070	53	38	0.717	1.40
oxo-C_8-HSL	1,053	79	28	0.354	4.84	1,037	38	7	0.184	2.99
oxo-C_{10}-HSL	745	6	2	0.333	0.54	844	4	1	0.250	0.36

The rejection achieved by the second screening allowed the conformation of a library available to be employed in further studies. As mentioned above, the number of replicates in the second part of the HTS was four (for further information, see Section 9 in the

Materials and Methods Chapter). The amount of replicates influences the scope of precision of the test; thus, by four replicates permitted the reduction to only 50 % of the ambiguity related with only one measure carried out initially (Malo, N. *et al.* 2006). The number of mutant clones from every enzyme available to carry out each biocatalytic process is listed in Table 13. Thus, taking into account the second screening was possible to keep 172 mutant clones from 429 initial potential improved acylases (*i.e.* 40 %). However, it is important to point out that 14 of them have multiple enhanced activities, so finally the mutant library has 158 mutant clones, containing recombinant enzymes with improved activities. Thus, it is worth pointing out that the relevance of these sets of mutants able to hydrolyze amide bonds under environmental conditions dissimilar to the wild-type strains, and even capable to catalyze substrates with no apparent acylase activity by the native enzymes, at different values of pH and temperature (Table 14). In the second screening, those clones selected initially that do not complied at least one of the following criteria were discarded: (i) to enhance acylase activity at least 50 % in the hydrolysis of aliphatic AHLs, and (ii) to achieve a minimum value of 100 IU/mL with those β-substituted aliphatic AHLs according to fluram assay.

Table 14. Set of mutant clones from *Sl*PVA and *Au*AAC available to catalyze different substrates

	***Sl*PVA**				***Au*AAC**				**TOTAL**
	(6.0, 30)[1]	(8.0, 30)	(6.0, 45)	(8.0, 45)	(6.0, 30)	(8.0, 30)	(6.0, 45)	(8.0, 45)	
Penicillin V	0	1	0	2	1	1	8	10	23
C_6-HSL	1	0	0	0	0	4	0	0	5
C_8-HSL	1	1	1	0	0	0	1	0	4
C_{10}-HSL	6	3	7	1	0	2	10	7	36
oxo-C_6-HSL	1	1	2	2	9	23	18	19	75
oxo-C_8-HSL	3	12	1	8	0	0	1	0	25
oxo-C_{10}-HSL	0	1	1	1	0	0	0	1	4
TOTAL	12	19	12	14	10	30	38	37	172

[1] Values within parentheses represents: (pH, temperature)

4.2.4. Distribution model

Table 15 shows the fitting densities considering Anderson-Darling test, which determined that the top five distributions at each condition evaluated in this HTS were (in parenthesis, percent of times proposed): Dagum (64.3), Johnson SB (50.0), Generalized extreme value (46.4), Burr (39.3) and Frechet 3P (30.4).

The model of analysis of global distribution for all the operational conditions evaluated in this HTS study established that Dagum (78.6 %), Johnson SB (50.0 %) and Burr (48.2 %) were the best distribution functions proposed by the software, as well as Quantile-Quantile plots corroborated these results (data not shown). It is noteworthy that Johnson SB model fits perfectly to aliphatic AHLs, but not to penicillin V and oxo-AHLs (only 25-75 %). Despite the fact that Johnson SB model fits half of the time to this HTS, this is described as a complex probability density function (formula not shown) in contrast to Dagum, which is considerably straightforward to handle, and its simplicity describes really good our study.

Table 15. Best fit of statistic models to the experimental results with each substrate

	Best models	Main models[1]
Penicillin V	Dagum (75.0), Frechet 3P (50.0), Weibull, (50.0), Log-Pearson 3 (37.5), Burr (37.5)	Dagum (75.0), Pearson (75.0), Weibull, (62.5), Frechet (62.5), Burr (50.0)
C_6-HSL	Johnson SB (75.0), Burr (62.5), Generalized extreme value (62.5), Dagum (50.0), Error (50.0)	Johnson SB (75.0), Dagum (75.0), Burr (62.5), Generalized extreme value (62.5), Error (50.0)
C_8-HSL	Johnson SB (75.0), Beta (62.5), Generalized gamma (62.5), Dagum 4P (50.0), Error (50.0)	Johnson SB (75.0), Dagum (75.0), Beta (62.5), Generalized gamma (62.5), Error (50.0)
C_{10}-HSL	Burr (87.5), Generalized extreme value (75.0), Johnson SB (62.5), Dagum (50.0), Nakagami (37.5)	Burr (75.0), Generalized extreme value (75.0), Dagum (75.0), Johnson SB (62.5), Nakagami (37.5)
oxo-C_6-HSL	Dagum (75.0), Johnson SB (62.5), Frechet 3P (37.5), Lognormal 3P (37.5), Fatigue life 3P (25.0)	Dagum (75.0), Johnson SB (62.5), Pearson (50.0), Frechet (37.5), Lognormal (37.5)
oxo-C_8-HSL	Dagum (87.5), Generalized extreme value (87.5), Frechet 3P (75.0), Burr (62.5), Johnson SB (37.5)	Dagum (87.5), Generalized extreme value (87.5), Frechet (75.0), Burr (75.0), Johnson SB (37.5)
oxo-C_{10}-HSL	Dagum (87.5), Lognormal 3P (62.5), Frechet 3P (50.0), Inverse Gaussian 3P (50.0), Log-logistic 3P (50.0)	Dagum (87.5), Lognormal (62.5), Pearson (62.5), Frechet (50.0), Inverse Gaussian (50.0)

[1] Fit models which differ only in the number of parameters (*e.g.* Dagum and Dagum 4P)

Moreover, Dagum and Burr distribution functions (both with three parameters) adjusted good to selection criterion in all conditions (A^2 lower than critical value for α=0.01). It is important to remark that Dagum is defined over all positive real numbers ($0 < x \leq +\infty$), whereas Burr is for non-negative values ($0 \leq x \leq +\infty$), which is important for oxo-AHLs due to the apparently null activity by the parental enzymes under the assay condition. As shown, the mathematical functions from these models (Dagum and Burr with three parameters), density function practically is the same equation with only one difference in the presence or absence of the shape parameter κ, which multiplies the shape parameter α in the exponential function from the numerator in Dagum and Burr, respectively. The following equations defined the probability density functions of these models with three parameters, two of them defined as continuous shape parameter (*i.e.* κ and α), and the other one as continuous scale parameter (β), which are linked with the statistical and the shape of the dispersion.

Dagum distribution

$$f(x) = \frac{\alpha\kappa\left(\frac{x}{\beta}\right)^{\alpha\kappa-1}}{\beta\left(1+\left(\frac{x}{\beta}\right)^{\alpha}\right)^{\kappa+1}}$$

Burr distribution

$$f(x) = \frac{\alpha\kappa\left(\frac{x}{\beta}\right)^{\alpha-1}}{\beta\left(1+\left(\frac{x}{\beta}\right)^{\alpha}\right)^{\kappa+1}}$$

Supported by these statistics fits, the analysis allowed to find that the skewness was positive in all the conditions evaluated except for the hydrolysis of C_8-HSL with *Sl*PVA at 45ºC and both pH values, which indicates that the majority of mutant clones are located to the right of the mean (*i.e.* enhanced activities are more evident than detrimental activities). With regard to excess kurtosis, Table 16 shows clearly the tendencies from every operational condition. Herein the contrast between these mutants with improvement in their aliphatic AHL-acylase activities with respect to those samples improved in their acylase activities against β-keto substituted aliphatic AHLs (except to C_{10}-HSL and oxo-C_6-HSL with *Au*AAC) is observed. Likewise, a leptokurtic tendency (L) represents higher density of samples close to the media, whereas a platykurtic (P) refers to a great variation in the location of the spots (Table 16).

Table 16. Excess kurtosis and skewness from each operational condition[1]

	***Sl*PVA**				***Au*AAC**			
	(6.0, 30)[2]	(8.0, 30)	(6.0, 45)	(8.0, 45)	(6.0, 30)	(8.0, 30)	(6.0, 45)	(8.0, 45)
Penicillin V	L+	L+	L+	L+	L+	L+	L+	L+
C_6-HSL	P+	P+	P+	P+	P+	P+	P+	P+
C_8-HSL	P+	P+	P-	P-	P+	L+	P+	P+
C_{10}-HSL	P+	P+	P+	P+	P+	L+	L+	L+
oxo-C_6-HSL	L+	L+	L+	L+	P+	P+	P+	L+
oxo-C_8-HSL	L+	L+	L+	L+	L+	L+	L+	L+
oxo-C_{10}-HSL	P+	L+	L+	L+	L+	L+	L+	L+

°[1] Excess kurtosis: “L” leptokurtic, “P” platykurtic. Skewness: “+” positive, “-” negative
[2] Values within parentheses represents: (pH, temperature)

Whereas the majority of random mutations were benign (*i.e.* skewness) to both enzymes, oscillation (*i.e.* kurtosis) was higher with aliphatic AHLs than penicillin V and β-keto substituted aliphatic AHLs. That means that most of them gained activity with respect to parental enzymes in spite of the dispersion of the spots. The kurtosis might indicate that the same structural modifications of these recombinant enzymes make them more susceptible to changes in their acylase activities when employing aliphatic AHLs in contrast to the other substrates evaluated in this study (*i.e.* penicillin V and oxo-AHLs) that do not show important alterations in their acylase activities.

4.3. Expression of recombinant clones in *Rhodococcus* sp. T104 employing pENS shuttle vector

Expression of recombinant enzymes obtained by random mutagenesis was evaluated employing the shuttle vector pENS as described in Section 10.3.2 in the Materials and Methods Chapter. This option was tested as an alternative to obtain enough amounts of proteins employing *Rhodococcus* sp. T104, circumventing the possibility that the signal peptides of each enzyme were not recognized correctly by this host. The comparison of the predicted signal peptides and cleavage sites of *Sl*PVA, *Au*AAC and $PhaZ_{Sex}$ in Figure 47 demonstrates that the best recognition of this sequence among these Gram-positive bacteria was obtained with $PhaZ_{Sex}$ signal peptide, which contains 26 aa (García-Hidalgo, J. *et al.* 2012).

The evaluation of the best predicted signal was carried out by the SignalP 4.1 server (Petersen, T. N. *et al.* 2011), which results in three scores: C-score (probability of a cleavage site), S-score (probability of the presence of a signal peptide), and Y-score (geometric average of C-score and S-score). In particular, the values of *Sl*PVA, *Au*AAC and $PhaZ_{Sex}$ are shown in Table 17.

Theoretical values obtained by SignalP should be interpreted as the probability of the presence of a signal peptide. Thus, the higher values of both C-score and S-score were obtained for $PhaZ_{Sex}$, and as a consequence Y-score was also higher. These results demonstrated that $PhaZ_{Sex}$ theoretically had the best chances to be expressed in Gram-positive bacteria. It is important to highlight that this assertion was corroborated experimentally by the quantification of the protein secreted to the broth. Thus, clones with *Sl*PVA, *Au*AAC and $PhaZ_{Sex}$ were cloned in pENV19, and results revealed that the best expression was observed with $PhaZ_{Sex}$ (data not shown). These results supported the construction of the shuttle vector pENS that could release the desired enzymes extracellularly in *Rhodococcus* sp. T104 fermentation broths (Fig. 19).

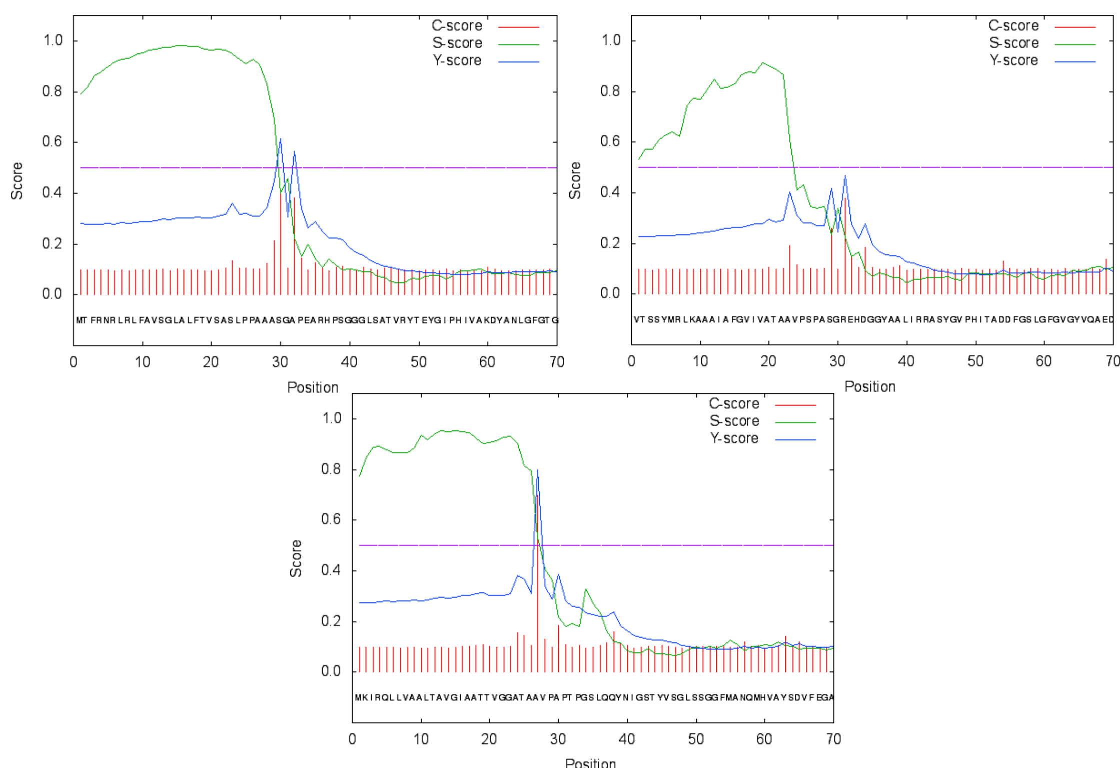

Figure 47. Plots of predicted signal peptide and cleavage site. In the top-left the predicted signal peptide of *Sl*PVA, top-right the predicted signal peptide of *Au*AAC and bottom the predicted signal peptide of PHB depolymerase from *Streptomyces exfoliatus* DSMZ 41693. C-score (probability of a cleavage site), S-score (probability of the presence of a SP), and Y-score (geometric average of C-score and S-score)

Table 17. Predicted signal peptide and cleavage parameters of *Sl*PVA, *Au*AAC and $PhaZ_{Sex}$

	C-score	S-score	Y-score
***Sl*PVA**	0.403	0.769	0.605
***Au*AAC**	0.378	0.543	0.467
$PhaZ_{Sex}$	0.699	0.836	0.797

For instance, Figure 48. shows the chromatographic profiles obtained during the purification of *Au*AAC from the fermentation broths of different recombinant strains. In this sense, purification profile obtained from a culture of 400 mL of *Rhodococcus* sp. T104 transformed with the shuttle vector pENV19*aac* (Fig. 48.A) showed lower amount of acylase that was bound to S-sepharose in comparison to other producer strains. On the other side, the best yield during *Au*AAC isolation was obtained from 400 mL of culture broth inoculated with *S. lividans* 1326 with pEM4*aac* (Torres-Bacete, J. *et al.* 2007) (Fig. 48.C). However, despite the fact that similar incubation conditions were performed with mutant clones of *Rhodococcus* sp. T104 transformed with pENS*aac* (Fig. 48.B), the amount of *Au*AAC isolated from this recombinant clone was not significant with respect to *S. lividans*. However, it is remarkable that purified *Au*AAC yield employing pENS*aac* vector was higher than the amount obtained with pENV19*aac* vector, using the same bacterium. Assays of recombinant enzymes for activity determination were carried out under the same conditions (*i.e.* pH, temperature, enzyme amount and time).

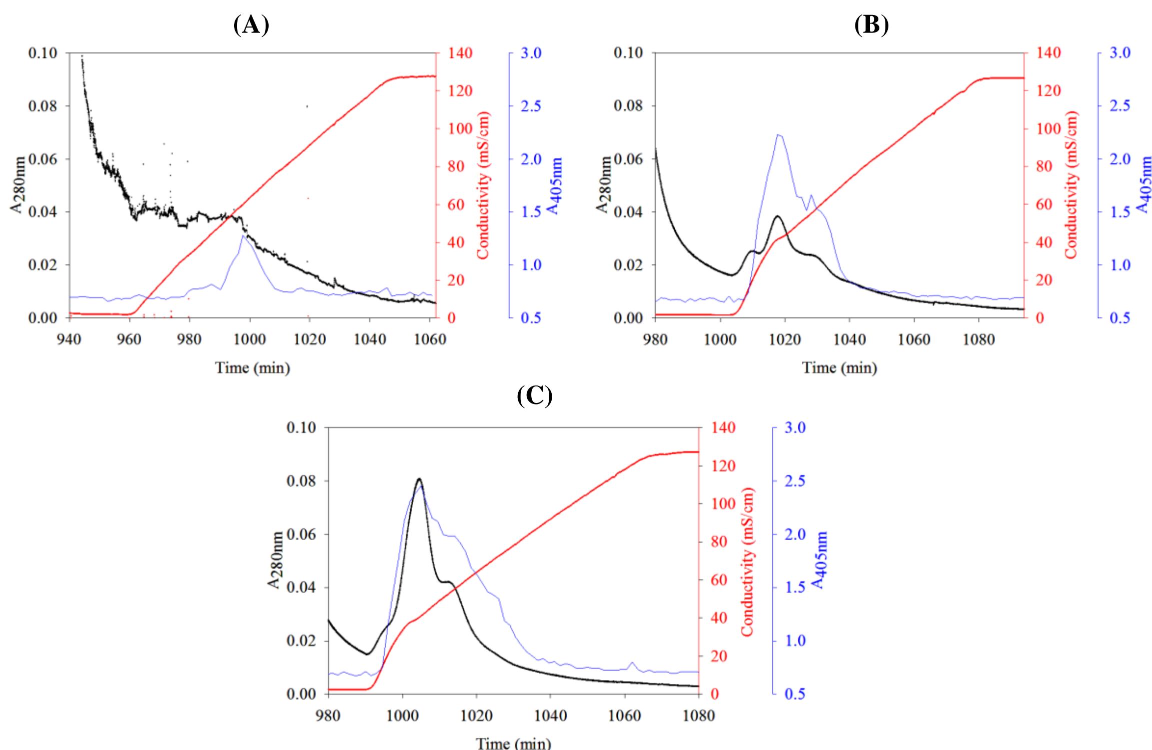

Figure 48. Chromatographic profile in *Au*AAC purification. Enzyme profile obtained from culture broths of **(A)** *Rhodococcus* sp. T104 and pENV19*aac* as shuttle vector, **(B)** *Rhodococcus* sp. T104 and pENS*aac* as shuttle vector, **(C)** *S. lividans* 1326 carried *aac* gene and pEM4*aac*

5. ANALYSIS OF MUTANTS WITH EVOLVED ACYLASE BY DIRECTED EVOLUTION

Although the library obtained by the present HTS study contained 158 recombinant proteins, only the most relevant clones were sequenced. In order of importance, the criteria to select those strains were: (i) new biocatalytic activity, (ii) alteration of their optimal operational conditions, (iii) significant enhancement of the activity, and (iv) wider activity spectrum, such as substrate promiscuity and broad range of operational conditions.

5.1. Changes throughout enzymes sequences

In addition to the changes in residues throughout the gene, some of these mutant clones had silent mutations or modification in restriction sites (data not shown). The modifications introduced by the mutator strain *E. coli* XL1-Red in the plasmid affected both the sequences of the gene and the promoter (Table 18), although the sequence of kanamycin resistance cassette could have been modified as well, which would have hampered the detection of improved enzymes. This fact means that random mutations were introduced by the mutator strain throughout the plasmid, and some of them were tested at 56 different scenarios (*i.e.* 7 substrates at four operational conditions).

Only those recombinant enzymes with mutations throughout the amino acid sequence within α- or β-subunit are represented in Figure 49. These mutant clones were selected mainly by their capacity to hydrolyze β-keto substituted aliphatic HSLs (Table 18), as well as by the location of their mutations. Thus, the recombinant clone named 2pva1,25(25) have a mutation in β-subunit (*i.e.* where catalytic residues are located). Likewise, the

recombinant clone named 2pva1,2(55) codifies a peculiar recombinant acylase that has been truncated in the C-terminal end (*i.e.* modification of the ORF). The last recombinant clone from *Sl*PVA was named 2pva1,2(149), which have a mutation in the α-subunit. Finally, it is important to mention the case of the last clone selected in this study, named a(292), because the mutation surprisingly was present within the linker peptide.

Table 18. Modifications throughout sequences in selected mutant clones

Parental enzyme	Recombinant clones	Modifications	Substrate						
			Penicillin V	oxo-C_6-HSL	C_6-HSL	oxo-C_8-HSL	C_8-HSL	oxo-C_{10}-HSL	C_{10}-HSL
*Sl*PVA	2pva1,21(25)*	βR373W	X						X
	2pva1,21(30)	c127g; t148g**	X		X		X		
	2pva1,2(55)	ΔG1215***, ORF modified		X				X	
	2pva1,2(149)	αA169T; *βG487G*	X	X					
*Au*AAC	a(25)	g27c					X		
	a(27)	g152a					X		
	a(292)	αI184F	X			X			
	aac(11)	g151a, *βV70V*	X				X		
	aac(59)	g151a	X		X				
	4aac(127)	g20a**	X						X

* The name corresponds with the numeration of recombinant clones during the experiments
** Some modifications in restrictions sites were detected
*** The nucleotide 1215 was deleted, and here the initial nucleotide in α-subunit was considered as number 1

```
SlPVA         1   G(166)AAQ(234)QVLPRITDELAARCSTPLGQATYPSAGLAVLDGSTSACALGSDRDAVQPGIFGPGRMPTL(109)RRW(191)
2pva1,21(25)  1   ·(166)···(234)······························································(109)·W·(191)
2pva1,2(55)   1   ·(166)···(234)·FCRGSRTSWRHAARPRWARPPTRRPGSRCWTDRRRPARWGATGTRYSRGSSGRAGCRR
2pva1,2(149)  1   ·(166)·T·(234)······························································(109)···(191)

AuAAC         1   G(181)AIA(568)
a(292)        1   ·(181)·F·(568)
```

Figure 49. Amino acid alignment of the α- and β-subunit from *Sl*PVA and *Au*AAC, and their recombinant proteins

5.2. Prediction of the three-dimensional structure

Tertiary structures of enzymes were determined by various web-server predictors in order to obtain a statistical consensus of the spatial location of residues within the enzymes. The study employed Phyre2, SwissModel, Robetta, Lomets and I-Tasser on-line platforms. It is important to mention that the first four servers employed here performed very fast enzyme modelling in contrast to other predictors, and Robetta only displayed information about the closest domain prediction and PDB information of the predicted template. For instance, the fastest predictors employed less than 4 h per simulation, but I-Tasser prediction lasted for more than 72 h per each simulation. Presumably, this is the reason why the predicted template employed by SwissModel and Robetta was 1KEH (*i.e.* cephalosporin acylase from *P. diminuta*) with almost all recombinant proteins (Table 19). Despite their fast simulation speed, Phyre2 and Lomets agreed with the predicted template adopted by I-Tasser (*i.e.* 3S8R, glutaryl 7-aminocephalosporanic acid acylase from *Pseudomonas* sp. GK16) which has recently been considered the best server for protein structure prediction. In particular, Lomets generates 3D-models by collecting high-scoring target-to-template alignments from 9 locally-installed threading programs (for further information, see

Section 11.4 in the Materials and Methods Chapter). Although the template in nearly all those cases was the same (*i.e.* 3S8R), it is important to point out that this structure was considered as a starting model employed by the servers, which in turn was adjusted by further intramolecular considerations according to each recombinant protein.

Table 19. Templates for 3D-structure prediction of *Sl*PVA, *Au*AAC, their recombinant proteins and *Au*AHLA

	Phyre2	SwissModel	Robetta	Lomets	I-Tasser
***Sl*PVA**	3S8R	1KEH	1KEH	3S8R	3S8R
***Au*AAC**	3S8R	1KEH	1KEH	3S8R	3S8R
***Au*AHLA**	3S8R	1KEH	4E56	3S8R	4WKU
2pva1,21(25)	3S8R	3S8R	1KEH	3S8R	3S8R
2pva1,2(55)	3S8R	-	1KEH	3S8R	3S8R
2pva1,2(149)	3S8R	1KEH	1KEH	3S8R	3S8R
a(292)	3S8R	1KEH	1KEH	3S8R	3S8R

3S8R: Glutaryl 7-aminocephalosporanic acid acylase (Kim, J. K. *et al.* 2003)
1KEH: Cephalosporin acylase (Kim, Y. *et al.* 2002)
4E56: Cephalosporin acylase mutant (to be published)
4WKU: Acyl-homoserine lactone acylase (Clevenger, K. D. *et al.* 2014)

Both 1KEH (Kim, Y. *et al.* 2002) and 3S8R (Kim, J. K. *et al.* 2003) are proteins that folds into a compact structure containing the αββα motif (Murzin, A. G. *et al.* 1995), as previously described as the typical fold of Ntn-hydrolases (Brannigan, J. A. *et al.* 1995; Oinonen, C. *et al.* 2000). This αββα-core structure is formed by two antiparallel β-sheets packed against each other, which in turn are covered by a layer of antiparallel α-helices on one side (Artymiuk, P. J. 1995; Brannigan, J. A. *et al.* 1995). Figure 50 illustrates the most popular predicted templates contemplated by the servers employed here (biological assembly). As expected and observed in those models, the templates employed are practically the same in all cases. Proteins are plotted with the solvent-accessible surface area, which means that this shadow should be interpreted as cut-off on the surface of the protein, and only those residues that form this shell are in contact and interacting with water molecules at all possible positions of protein (Schrödinger, L. L. C. 2014).

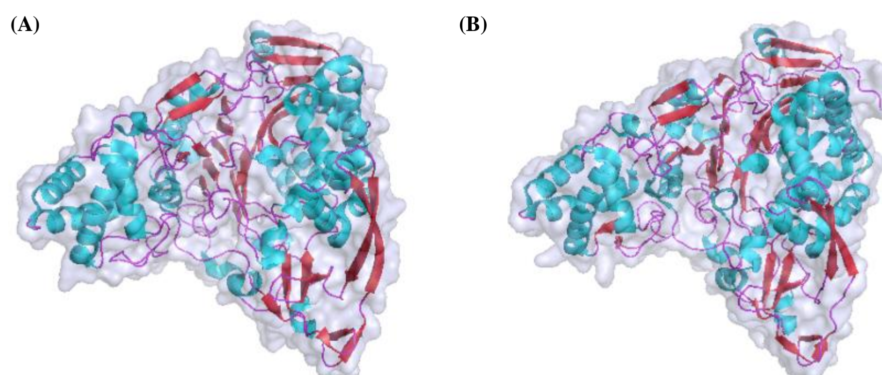

Figure 50. Predicted templates employed by several services to model 3D-structures of *Sl*PVA, *Au*AAC, their recombinant proteins and *Au*AHLA. **(A)** 1KEH and **(B)** 3S8R

Moreover, 3-D models obtained for parental *Sl*PVA and *Au*AAC are shown in Figures 51 and 52. Additionally, 3D-models obtained with their recombinant enzymes are depicted in Figures 53 to 57. An overview of all these models indicates that they are very similar with the exception of the model of 2pva1,2(55), whose structure is drastically altered due to the deletion.

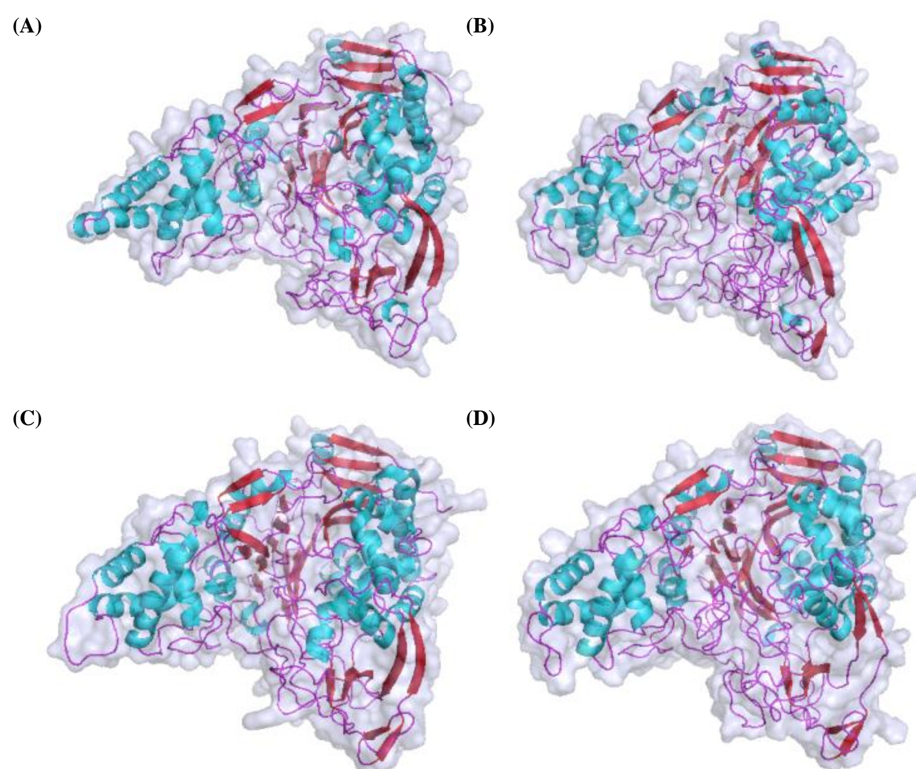

Figure 51. Prediction of 3D-structure of *Sl*PVA. Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

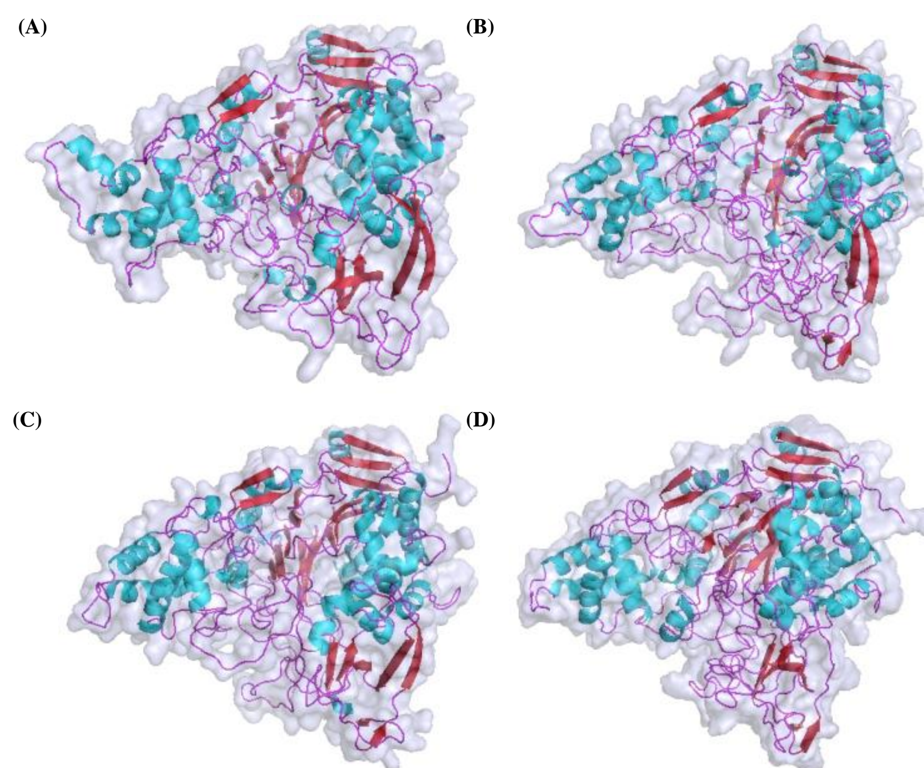

Figure 52. Prediction of 3D-structure of *Au*AAC. Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

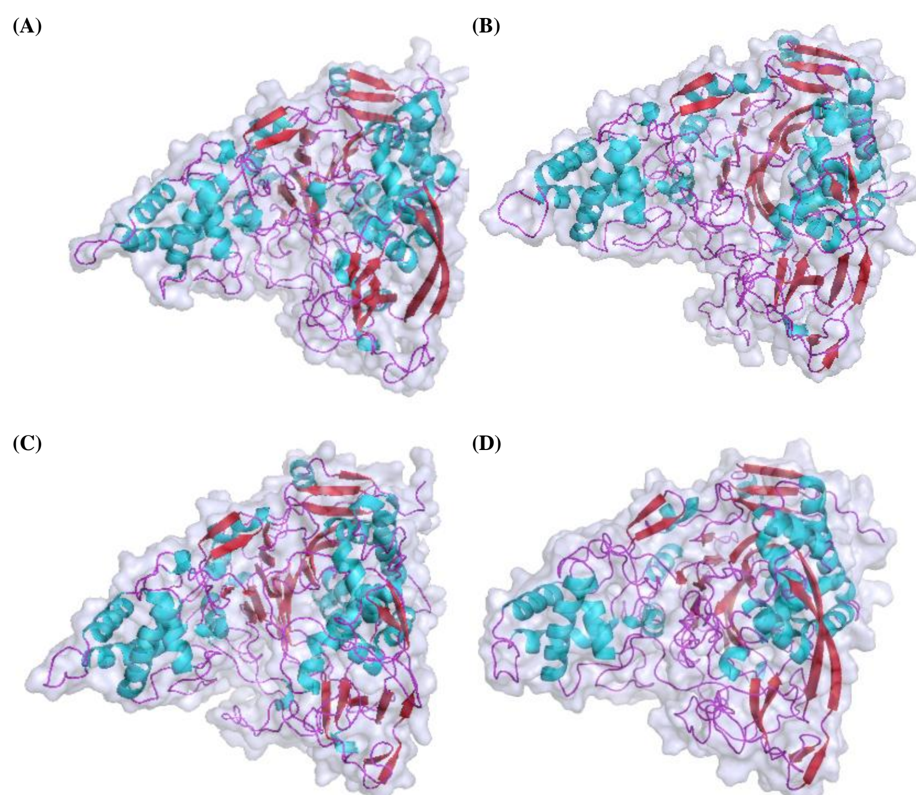

Figure 53. Prediction of 3D-structure of *Au*AHLA. Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

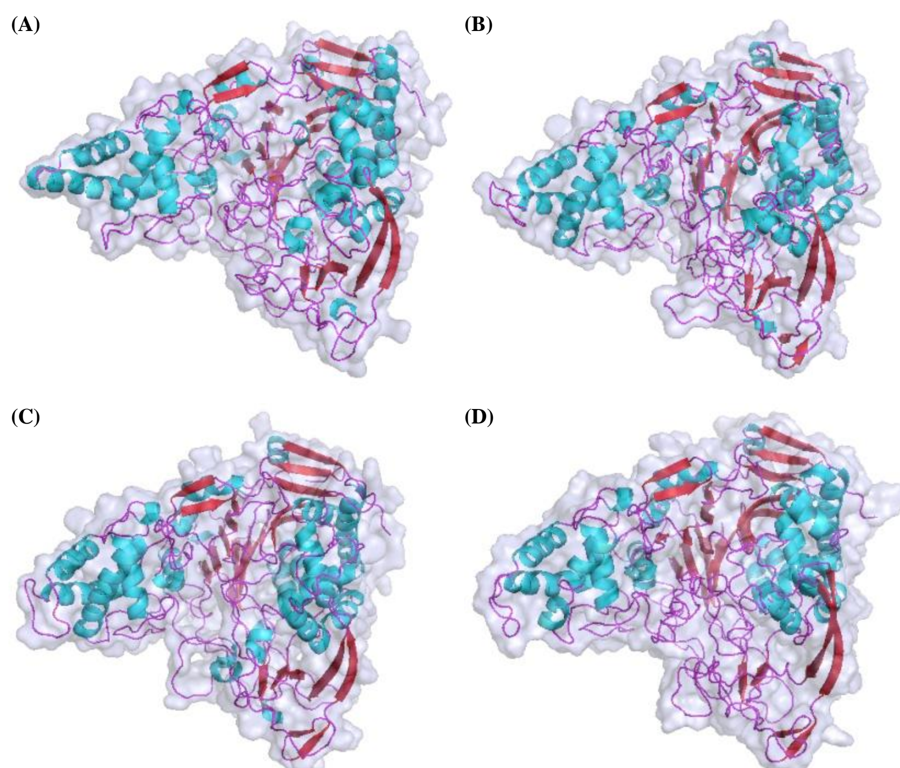

Figure 54. Prediction of 3D-structure of the recombinant clone of *Sl*PVA named 2pva1,21(25). Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

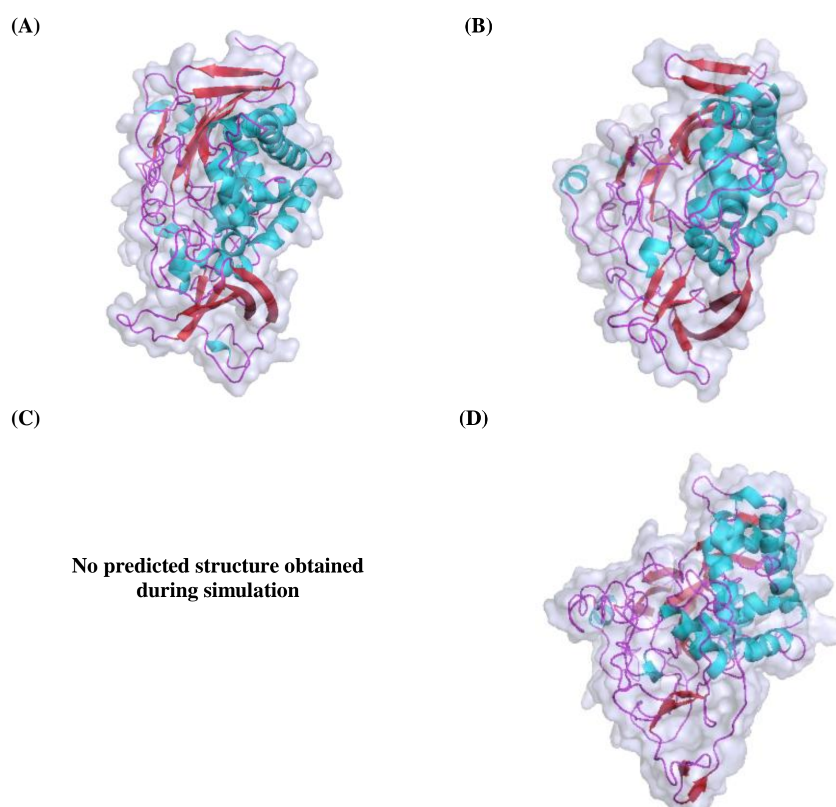

Figure 55. Prediction of 3D-structure of the recombinant clone of *Sl*PVA named 2pva1,2(55). Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

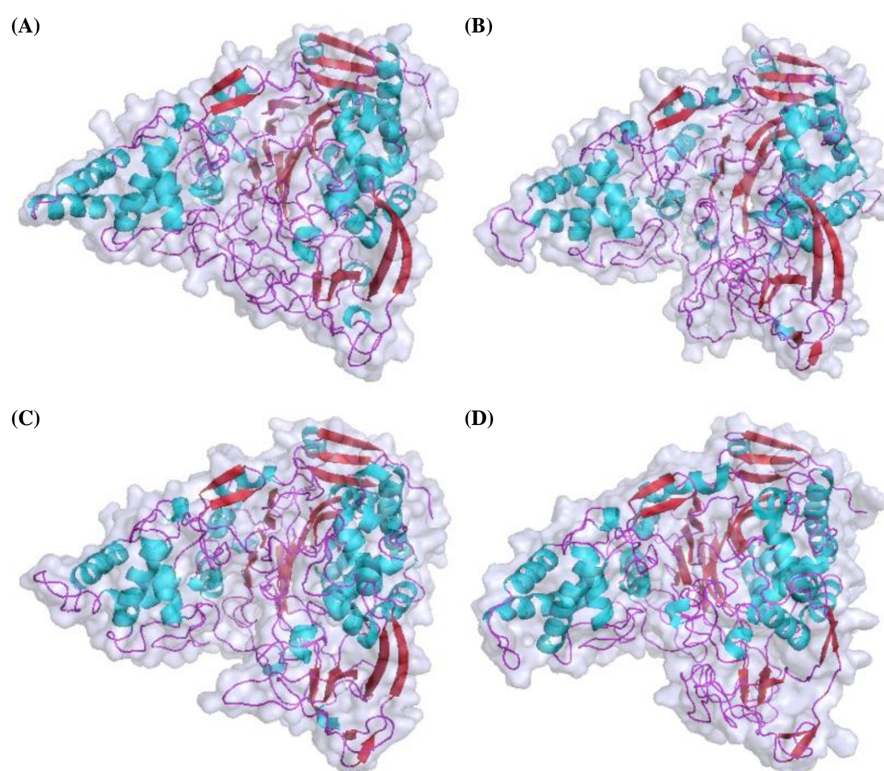

Figure 56. Prediction of three-dimensional structure of the recombinant clone of *Sl*PVA named 2pva1,2(149). Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

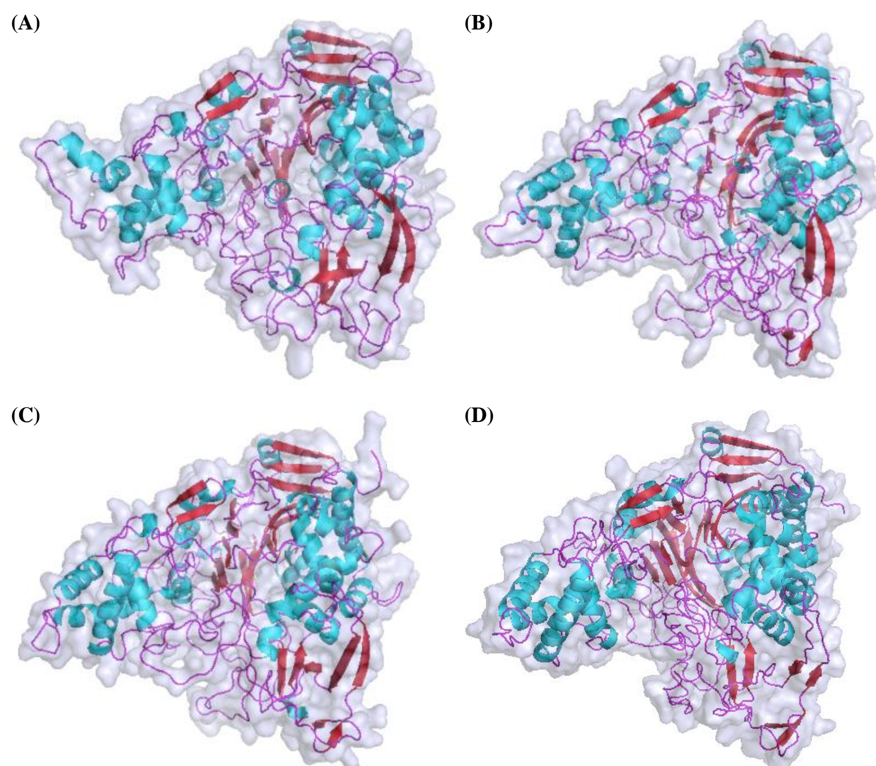

Figure 57. Prediction of three-dimensional structure of the recombinant clone of *Au*AAC named a(292). Models obtained by the servers **(A)** Phyre2, **(B)** SwissModel, **(C)** Lomets, and **(D)** I-Tasser

5.3. Characteristics of the residues involved in the binding pocket

Binding pockets of *Sl*PVA, *Au*AAC and *Au*AHLA were aligned by COBALT (Papadopoulos, J. S. *et al.* 2007) using 174 sequences of those acylases reported until December 2015. Likewise, I-Tasser predicted the location of amino acids involved in the binding pocket. Those residues estimated by this server as ligand binding sites are listed in Table 20, where those ones in bold are amino acids predicted by both I-Tasser and COBALT, in addition to other residues determined exclusively by COBALT according to the alignment.

Table 20. Predicted ligand binding residues

Enzyme	Ligand binding site
*Sl*PVA	**βSer^{1}**, **βHis^{23}**, **βTyr^{24}**, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, βVal^{186}, **βAsn^{272}**
*Au*AAc	**βSer^{1}**, **βHis^{23}**, **βPhe^{24}**, βPhe^{32}, βLeu^{50}, βGlu^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, **βVal^{182}**, **βAsn^{268}**
*Au*AHLA	**βSer^{1}**, **βHis^{23}**, **βTyr^{24}**, βPhe^{32}, βLeu^{50}, βLeu^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, βLeu^{188}, **βAsn^{274}**
2pva1,21(25)	**βSer^{1}**, **βHis^{23}**, **βTyr^{24}**, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, βVal^{186}, **βAsn^{272}**
2pva1,2(55)	**βSer^{1}**, **βHis^{23}**, **βTyr^{24}**, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, βVal^{186}, **βArg^{236}**
2pva1,2(149)	**βSer^{1}**, **βHis^{23}**, **βTyr^{24}**, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, βVal^{186}, **βAsn^{272}**
a(292)	**βSer^{1}**, **βHis^{23}**, **βPhe^{24}**, βPhe^{32}, βLeu^{50}, βGlu^{57}, βIle^{58}, **βHis^{68}**, **βThr^{69}**, **βVal^{70}**, **βVal^{182}**, **βAsn^{268}**

It is important to remark that most of these predicted amino acids (Table 20) are in agreement with those reported for several acylases in literature (Zhang, D. *et al.* 2007; Hormigo, D. 2009; Torres-Bacete, J. *et al.* 2015) as well as with those ones previously estimated by COBALT and reported elsewhere (Papadopoulos, J. S. *et al.* 2007) (for further information, see Section 5.2 in the Results Chapter). In addition, some important features about the residues related with the binding pocket from *Sl*PVA, *Au*AAC and *Au*AHLA are indicated in Table 21. Likewise, an overall and exhaustive comparison about

location, properties, abundance, and entropy (among other factors) of those amino acids is included in Supplement S.6 in the Supplementary Material Chapter.

Table 21. Characteristics of the residues involved in the binding pocket from *Sl*PVA, *Au*AAC and *Au*AHLA

Subunit	Residue			Secondary structure	Conserved residue and side-chain	Most common	
	*Sl*PVA	*Au*AAC	*Au*AHLA			Residue	Side-chain properties
β (catalytic residues)	βSer^{1}	βSer^{1}	βSer^{1}	Coil	S (100 %) Polar (100 %)	S (100 %)	Polar (100 %)
	βHis^{23}	βHis^{23}	βHis^{23}	Coil	H (84 %) Basic (87 %)	H (84 %)	Basic (87 %)
	βVal^{70}	βVal^{70}	βVal^{70}	Coil	V (33 %) Nonpolar (63 %)	V (33 %)	Nonpolar (63 %)
	βAsn^{272}	βAsn^{268}	βAsn^{274}	Coil	N (99 %) Polar (100 %)	N (99 %)	Polar (100 %)
α (substrate binding pocket)	αAla^{154}	αMet^{149}	αVal^{157}	Helix	A (22 %) Nonpolar (66 %) M (6 % Nonpolar (66 %) V (12 % Nonpolar (66 %)	A (22 %)	Nonpolar (66 %)
	αGly^{158}	αGly^{153}	αGly^{161}	Coil	G (34 %) Polar (55 %)	G (34 %)	Polar (55 %)
	αThr^{167}	αVal^{162}	αVal^{170}	Coil^{SlPVA}, Helix$^{AuAAC,\ AuAHLA}$	T (9 %) Polar (25 %) V(10 %) Nonpolar (60 %)	F (14 %)	Nonpolar (60 %)
β (substrate binding pocket)	βGly^{8}	βAla^{8}	βArg^{8}	Coil	G (28 %) Polar (36 %) A (5 %) Nonpolar (47 %) R (4 %) Basic (17 %)	P (41 %)	Nonpolar (47 %)
	βThr^{10}	βAla^{10}	βGly^{10}	Coil	T (2 %) Polar (8 %) A (5 %) Nonpolar (13 %) G (2 %) Polar (8 %)	K (41 %)	Basic (80 %)
	βTyr^{24}	βPhe^{24}	βTyr^{24}	Coil	Y (4 %) Polar (12 %) F (24 %) Nonpolar (77 %)	L (43 %)	Nonpolar (77 %)
	βArg^{31}	βArg^{31}	βArg^{31}	Helix^{SlPVA}, Strand$^{AuAAC,\ AuAHLA}$	R (24 %) Basic (24 %)	R (24 %)	Nonpolar (53 %)
	βPhe^{32}	βPhe^{32}	βPhe^{32}	Helix^{SlPVA}, Strand$^{AuAAC,\ AuAHLA}$	F (24 %) Nonpolar (72 %)	W (42 %)	Nonpolar (72 %)
	βTrp^{33}	βTyr^{33}	βTrp^{33}	Helix^{SlPVA}, Strand$^{AuAAC,\ AuAHLA}$	W (14 %) Nonpolar (31 %) Y (62 %) Polar (69 %)	Y (62 %)	Polar (69 %)
	βLeu^{50}	βLeu^{50}	βLeu^{50}	Coil	L (40 %) Nonpolar (86 %)	L (40 %)	Nonpolar (86 %)
	βSer^{53}	βAsp^{53}	βPhe^{53}	Coil	S (6 %) Polar (25 %) D (1 %) Acidic (1 %) F (2 %) Nonpolar (75 %)	L (27 %)	Nonpolar (75 %)
	βSer^{57}	βGlu^{57}	βLeu^{57}	Strand	S (9 %) Polar (26 %) E (1 %) Acidic (1 %) L (18 %) Nonpolar (59 %)	V (21 %)	Nonpolar (59 %)
	βIle^{58}	βIle^{58}	βIle^{58}	Strand	I (40 %) Nonpolar (93 %)	I (40 %)	Nonpolar (93 %)
	βSer^{67}	βSer^{67}	βSer^{67}	Strand	S (20 %) Polar (98 %)	G (38 %)	Polar (98 %)
	βHis^{68}	βHis^{68}	βHis^{68}	Strand	H (26 %) Basic (26 %)	H (26 %)	Nonpolar (49 %)
	βThr^{69}	βThr^{69}	βThr^{69}	Coil	T (94 %) Polar (99 %)	T (94 %)	Polar (99 %)
	βThr^{72}	βThr^{72}	βThr^{72}	Helix	T (23 %) Polar (54 %)	T (23 %)	Polar (54 %)
	βVal^{186}	βVal^{182}	βLeu^{188}	Strain	V (16 %) Nonpolar (54 %) L (15 %) Nonpolar (54 %)	Q (20 %)	Nonpolar (54 %)

Thereby, those residues predicted by COBALT and I-Tasser are depicted in Table 21, as well as those ones reported and related with *Sl*PVA and *Au*AAC (Hormigo, D. 2009; Torres-Bacete, J. *et al.* 2015). In addition, there are other residues that have been considered crucial in significant improvements by directed molecular evolution or site-directed mutagenesis with the acylases from *E. coli* ATCC 11105 (del Río, G. *et al.* 1995; Lee, H. *et al.* 2000; Morillas, M. *et al.* 2003; Balci, H. *et al.* 2014) and *Pseudomonas* SY-77-1 (Otten, L. G. *et al.* 2002; Sio, C. F. *et al.* 2002).

In particular, the residues in Table 21 have been postulated as the most common amino acids among acylases, and the side-chain properties in this part of the enzymes are practically conserved. On the other hand, those residues related with the binding pocket

showed several options. In this sense, half of those residues detected and the nature of their side-chain are highly conserved in *Sl*PVA, *Au*AAC and *Au*AHLA (*i.e.* amino acids aligned with αAla^{154}, αGly^{158}, βPhe^{32}, βLeu^{50}, βIle^{58}, βSer^{67}, βThr^{69}, βThr^{72} and βVal^{186} in *Sl*PVA). In contrast, the other half of residues related with the binding pocket does not exhibit a plausible pattern of interpretation (*i.e.* amino acids aligned with αAla^{167}, βGly^{8}, βThr^{10}, βTyr^{24}, βArg^{31}, βTrp^{33}, βSer^{53}, βSer^{57} and βHis^{68} in *Sl*PVA).

5.4. Alteration of residues involved in binding pocket

Additionally, alteration of the catalytic pocket has been estimated in some selected mutant clones. Thus, relative coordinates among residues inside *Sl*PVA, *Au*AAC and *Au*AHLA has been documented in Supplement S.8 in the Supplementary Material Chapter, which should be interpreted as the closest distance between active groups of residues within three-dimensional structure of each acylase. Thus, the location of residues inside enzymes (*i.e.* relative coordinates) fluctuates similarly in all acylases that were simulated (3.4-15.0 Å in *Sl*PVA, 3.4-14.6 Å in *Au*AAC, and 3.6-15.2 Å in *Au*AHLA). Those lengths were interpreted as relative coordinates between ligand binding residues, which in turn are related with the location of residues within the catalytic pocket with respect to the substrate.

Those scenarios represent the alteration of binding pocket likely due to the strengthening, weakening, or breaking of the hydrogen bonds that are originally present in the native enzymes, or even a new bond formation. Thus, the length of hydrogen bonds depends of the characteristics of functional groups that are involved. The lower average length estimated in literature in this kind of interaction is 2.6±0.1Å and the higher value is 3.1±0.1Å (Kyte, J. 1995). In this study, four scenarios were considered according to the displacement predicted by the I-Tasser models of the spatial location of each residue with respect to other amino acids: (n) no displacement, (-) less than 2.5 Å, (+) between 2.5-3.2 Å (both included) and (++) more than 3.2 Å. Nevertheless, this is only a qualitative estimation of the alteration of hydrogen bonds inside catalytic pocket, but not a confirmation of the strengthening, weakening, breaking or formation of hydrogen bonds within the binding pocket. Thus, Tables 22 to 25 summarize the relative coordinates among residues inside the recombinant proteins 2pva1,21(25), 2pva1,2(55), 2pva1,2(149) and a(292), respectively, as well as the displacement of those locations with respect to the parental enzymes.

In particular, in the case of the recombinant acylase 2pva1,21(25) (Table 22), the alteration of ligand binding residues is basically focalized in βAsn^{272}. The change of relative location of this residue is drastically altered with respect to βHis^{23} and βVal^{70} and slightly modified with respect to βTyr^{24}, βSer^{57} and βHis^{68}. In contrast, variations of its location with respect to other amino acids of catalytic pocket are less than 2.5 Å, but no additional information could be obtained from this information.

Table 22. Distances in Angstroms between amino acids in 2pva1,21(25) according to the I-Tasser model

aa	βSer^{1}	βHis^{23}	βTyr^{24}	βPhe^{32}	βLeu^{50}	βSer^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{186}	βAsn^{272}
βSer^{1}		5.7(-)	8.9(-)	8.1(-)	10.7(-)	11.8(-)	5.9(-)	8.3(-)	5.3(-)	7.2(-)	8.4(-)	8.0(-)
βHis^{23}	5.7(-)		11.2(-)	10.1(-)	12.9(-)	16.1(-)	10.3(-)	13.5(-)	9.5(-)	9.4(-)	12.8(-)	6.2(++)
βTyr^{24}	8.9(-)	11.2(-)		4.4(-)	4.1(-)	7.9(-)	7.9(-)	7.5(-)	10.0(-)	5.2(-)	6.2(-)	12.3(+)
βPhe^{32}	8.1(-)	10.1(-)	4.4(-)		3.5(-)	6.2(-)	4.1(-)	6.7(-)	11.5(-)	9.1(-)	9.3(-)	13.8(-)
βLeu^{50}	10.7(-)	12.9(-)	4.1(-)	3.5(-)		5.5(-)	7.3(-)	7.8(-)	13.1(-)	9.2(n)	8.6(-)	15.6(-)
βSer^{57}	11.8(-)	16.1(-)	7.9(-)	6.2(-)	5.5(-)		5.8(-)	4.5(-)	12.9(-)	11.4(-)	7.8(-)	18.2(+)
βIle^{58}	5.9(-)	10.3(-)	7.9(-)	4.1(-)	7.3(-)	5.8(-)		4.2(-)	9.2(-)	9.6(-)	8.5(n)	13.5(-)
βHis^{68}	8.3(-)	13.5(-)	7.5(-)	6.7(-)	7.8(-)	4.5(-)	4.2(-)		8.7(-)	8.9(-)	4.6(-)	14.8(+)
βThr^{69}	5.3(-)	9.5(-)	10.0(-)	11.5(-)	13.1(-)	12.9(-)	9.2(-)	8.7(-)		5.4(-)	6.0(-)	7.4(-)
βVal^{70}	7.2(-)	9.4(-)	5.2(-)	9.1(-)	9.2(n)	11.4(-)	9.6(-)	8.9(-)	5.4(-)		5.2(-)	7.3(++)
βVal^{186}	8.4(-)	12.8(-)	6.2(-)	9.3(-)	8.6(-)	7.8(-)	8.5(n)	4.6(-)	6.0(-)	5.2(-)		12.0(-)
βAsn^{272}	8.0(-)	6.2(++)	12.3(+)	13.8(-)	15.6(-)	18.2(+)	13.5(-)	14.8(+)	7.4(-)	7.3(++)	12.0(-)	

Similar analysis was performed on the relative locations of residues in the catalytic pocket of recombinant acylase 2pva1,2(55) (Table 23). As expected, the alterations of coordinates of those residues were more significant as well as the modification of the last residues in the β-subunit. In this case, the amino acids βTyr^{24} and βHis^{68} were drastically modified whereas βVal^{70} was barely affected. Likewise, as predicted by the I-Tasser model, βArg^{236} could replace effectively βAsn^{272} of *Sl*PVA according to the location (for further information, see Table 20), conserving the nature of both residues (*i.e.* βArg^{236} and βAsn^{272} are both polar amino acids). In this case, the alteration of the catalytic pocket was unexpected, since the sequence of residues that cover the catalytic pocket as a lid was removed in this recombinant protein, so its expected changes in relative location were higher.

Table 23. Distances in Angstroms between amino acids in 2pva1,2(55) according to the I-Tasser model

aa	βSer^{1}	βHis^{23}	βTyr^{24}	βPhe^{32}	βLeu^{50}	βSer^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{186}	βArg^{236}
βSer^{1}		7.6(-)	10.9(++)	8.5(-)	9.6(-)	9.5(-)	6.0(-)	8.2(-)	5.0(-)	4.8(+)	7.5(-)	8.5(-)
βHis^{23}	7.6(-)		10.0(-)	11.8(-)	13.5(-)	15.4(-)	11.4(-)	15.6(++)	10.8(-)	10.5(-)	14.9(-)	10.0(-)
βTyr^{24}	10.9(++)	10.0(-)		3.4(+)	8.2(+)	11.1(++)	9.8(+)	15.5(++)	15.9(++)	12.9(++)	15.6(++)	16.3(++)
βPhe^{32}	8.5(-)	11.8(-)	3.4(+)		4.9(-)	4.8(-)	4.1(-)	9.6(++)	12.8(-)	10.5(-)	10.7(-)	15.9(-)
βLeu^{50}	9.6(-)	13.5(-)	8.2(+)	4.9(-)		5.2(-)	8.3(-)	10.1(++)	13.5(-)	8.9(-)	9.2(-)	14.5(-)
βSer^{57}	9.5(-)	15.4(-)	11.1(++)	4.8(-)	5.2(-)		4.6(-)	6.5(-)	12.5(-)	10.0(-)	7.9(-)	16.4(-)
βIle^{58}	6.0(-)	11.4(-)	9.8(+)	4.1(-)	8.3(-)	4.6(-)		6.0(+)	9.4(n)	9.1(-)	8.2(-)	14.4(-)
βHis^{68}	8.2(-)	15.6(++)	15.5(++)	9.6(++)	10.1(++)	6.5(-)	6.0(+)		8.2(-)	8.4(-)	3.6(-)	14.2(-)
βThr^{69}	5.0(-)	10.8(-)	15.9(++)	12.8(-)	13.5(-)	12.5(-)	9.4(n)	8.2(-)		5.4(-)	6.8(-)	7.9(-)
βVal^{70}	4.8(+)	10.5(-)	12.9(++)	10.5(-)	8.9(-)	10.0(-)	9.1(-)	8.4(-)	5.4(-)		5.3(-)	5.5(-)
βVal^{186}	7.5(-)	14.9(-)	15.6(++)	10.7(-)	9.2(-)	7.9(-)	8.2(-)	3.6(-)	6.8(-)	5.3(-)		11.5(-)
βArg^{236}	8.5(-)	10.0(-)	16.3(++)	15.9(-)	14.5(-)	16.4(-)	14.4(-)	14.2(-)	7.9(-)	5.5(-)	11.5(-)	

Likewise, as shown in Table 24 recombinant enzyme 2pva1,2(149) showed similar alterations within its binding pocket. In this case, only βAsn^{272} was altered considerably in contrast to the moderate modifications in βTyr^{24}, or no plausible alterations in other residues. Finally, information of relative coordinates from recombinant enzyme a(292) is shown in Table 25. In this case, relative positions of βHis^{68} were drastically altered, and βGlu^{57} was hardly modified, whereas the rest of amino acids remained practically in the same relative position within the catalytic pocket.

Table 24. Distances in Angstroms between amino acids in 2pva1,2(149) according to the I-Tasser model

aa	βSer^{1}	βHis^{23}	βTyr^{24}	βPhe^{32}	βLeu^{50}	βSer^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{186}	βAsn^{272}
βSer^{1}		6.7(-)	10.0(-)	8.1(-)	10.9(-)	12.2(-)	5.8(-)	5.2(-)	5.3(-)	7.2(-)	8.4(-)	8.4(-)
βHis^{23}	6.7(-)		11.6(-)	9.4(n)	12.0(-)	16.3(-)	9.7(-)	10.3(-)	11.2(-)	10.2(-)	14.1(-)	8.8(-)
βTyr^{24}	10.0(-)	11.6(-)		6.9(-)	4.4(-)	9.9(-)	10.0(+)	7.5(-)	11.1(+)	7.2(+)	8.2(-)	12.6(+)
βPhe^{32}	8.1(-)	9.4(n)	6.9(-)		3.6(-)	6.8(-)	4.0(n)	5.1(-)	11.6(-)	10.9(-)	9.4(-)	14.5(-)
βLeu^{50}	10.9(-)	12.0(-)	4.4(-)	3.6(-)		6.2(-)	8.1(-)	7.1(-)	12.9(-)	10.5(-)	8.7(-)	16.0(-)
βSer^{57}	12.2(-)	16.3(-)	9.9(-)	6.8(-)	6.2(-)		6.6(-)	7.1(-)	13.3(-)	8.1(-)	8.1(-)	19.1(++)
βIle^{58}	5.8(-)	9.7(-)	10.0(+)	4.0(n)	8.1(-)	6.6(-)		3.9(-)	9.4(n)	10.9(-)	8.3(-)	14.1(-)
βHis^{68}	5.2(-)	10.3(-)	7.5(-)	5.1(-)	7.1(-)	7.1(-)	3.9(-)		6.9(-)	7.6(-)	4.9(-)	12.2(-)
βThr^{69}	5.3(-)	11.2(-)	11.1(+)	11.6(-)	12.9(-)	13.3(-)	9.4(n)	6.9(-)		5.4(-)	5.9(-)	7.6(-)
βVal^{70}	7.2(-)	10.2(-)	7.2(+)	10.9(-)	10.5(-)	8.1(-)	10.9(-)	7.6(-)	5.4(-)		6.6(-)	5.7(-)
βVal^{186}	8.4(-)	14.1(-)	8.2(-)	9.4(-)	8.7(-)	8.1(-)	8.3(-)	4.9(-)	5.9(-)	6.6(-)		13.0(+)
βAsn^{272}	8.4(-)	8.8(-)	12.6(+)	14.5(-)	16.0(-)	19.1(++)	14.1(-)	12.2(-)	7.6(-)	5.7(-)	13.0(+)	

Table 25. Distances in Angstroms between amino acids in a(292) according to the I-Tasser model

aa	βSer^{1}	βHis^{23}	βPhe^{24}	βPhe^{32}	βLeu^{50}	βGlu^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{182}	βAsn^{268}
βSer^{1}		6.8(-)	7.1(-)	8.4(-)	11.2(-)	7.3(+)	6.5(-)	8.9(+)	4.1(-)	7.1(-)	8.0(-)	5.4(-)
βHis^{23}	6.8(-)		7.6(-)	9.7(-)	13.1(-)	12.1(-)	10.1(-)	15.2(++)	9.7(-)	10.5(-)	13.4(-)	7.4(-)
βPhe^{24}	7.1(-)	7.6(-)		3.8(-)	4.3(-)	6.1(-)	7.0(-)	10.1(++)	8.4(-)	5.4(n)	7.1(-)	8.0(-)
βPhe^{32}	8.4(-)	9.7(-)	3.8(-)		3.7(-)	3.3(-)	3.7(-)	10.0(++)	11.0(-)	9.3(n)	9.1(-)	11.5(-)
βLeu^{50}	11.2(-)	13.1(-)	4.3(-)	3.7(-)		4.3(-)	7.1(-)	10.4(-)	12.8(-)	9.7(-)	9.0(-)	13.1(-)
βGlu^{57}	7.3(+)	12.1(-)	6.1(-)	3.3(-)	4.3(-)		3.3(+)	4.9(-)	8.7(+)	8.5(-)	5.6(-)	10.3(-)
βIle^{58}	6.5(-)	10.1(-)	7.0(-)	3.7(-)	7.1(-)	3.3(+)		6.9(-)	9.6(-)	10.3(-)	8.8(-)	11.0(-)
βHis^{68}	8.9(+)	15.2(++)	10.1(++)	10.0(++)	10.4(-)	4.9(-)	6.9(-)		8.7(-)	10.0(++)	3.5(-)	11.0(+)
βThr^{69}	4.1(-)	9.7(-)	8.4(-)	11.0(-)	12.8(-)	8.7(+)	9.6(-)	8.7(-)		4.9(-)	6.5(-)	3.0(-)
βVal^{70}	7.1(-)	10.5(-)	5.4(n)	9.3(n)	9.7(-)	8.5(-)	10.3(-)	10.0(++)	4.9(-)		5.3(-)	3.7(-)
βVal^{182}	8.0(-)	13.4(-)	7.1(-)	9.1(-)	9.0(-)	5.6(-)	8.8(-)	3.5(-)	6.5(-)	5.3(-)		7.7(-)
βAsn^{268}	5.4(-)	7.4(-)	8.0(-)	11.5(-)	13.1(-)	10.3(-)	11.0(-)	11.0(+)	3.0(-)	3.7(-)	7.7(-)	

5.5. Characteristics of residues mutated by directed molecular evolution

Analysis of the modifications observed in *Sl*PVA and *Au*AAC sequences during this study could be considered as a starting point to possibly improve other acylases. In this sense, Table 26 shows the alignment performed with 174 sequences of reported acylases, which allowed the comparison about location, properties, abundance, entropy, among other factors, of these mutated residues (for further information, see Supplement S.9 in Supplementary Material Chapter). It is important to point out that these mutations altered the three-dimensional structure all the recombinant acylases analyzed here, although modified residues (Table 18) were not located in ligand binding pocket (Table 20). In this sense, the main features of those recombinant enzymes are summarized in Table 26. Interestingly, the location of modified residue was on the protein surface in all those cases. Additionally, relative locations of all those amino acids were far away from βSer^{1}, which is the main catalytic residue in Ntn-hydrolases. In the case of the truncated enzyme 2pva1,2(55), the benzyl group of βPhe^{204} (*i.e.* first residue altered in sequence) was considered as the reference to measure the length with respect to hydroxyl group of βSer^{1}.

As shown Supplement S.9 in the Supplementary Material Chapter, as well as Tables 22 to 25, relative coordinates of mutated residues was significant. In the case of 2pva1,21(25), the distance of β373 with respect to βSer^{1} was decreased in 6.6 Å and the the rest of the binding pocket remained almost intact. In the case of 2pva1,2(55), the binding pocket was altered and deletion of a segment close to the C-terminal was observed. In the case of

2pva1,2(149), the binding pocket was slightly altered and evident changes were concentrated in the last part of the α-subunit. Finally in the case of a(292), the distance of α184 with respect to βSer[1] was reduced 5.1 Å, in addition to important alteration of other residues at the end of the α-subunit as well as the catalytic pocket). Curiously, the location of all amino acids mutated in these recombinant acylases were located on the protein surface, and in the particular case of 2pva1,2(55) a segment of the last part of β-subunit was removed. This segment might play the role of a lid, which could be similar to the lid that participates in interfacial activation of lipases (Rehm, S. *et al.* 2010).

Table 26. Characteristics of the residues modified in the recombinant clones

Clone[1]	Residue	Position	r[3]	SS[4]	$H_i(l)$		Conservation of residue				Most common	
							Parental		Mutation		Residue	Acyl[5]
					i=1	i=2	Residue	Acyl[5]	Residue	Acyl[5]		
(A)	βR373W	Surface	31.2	Helix	1.90	1.25	Arg (8.6 %)	Basic (15.5 %)	Trp (0.0 %)	Nonpolar (46.6 %)	Ala (45.4%)	Nonpolar (46.6 %)
(B)[2]	ORF	Surface	18.3	Coil	2.24	1.11	Val (16.1%)	Nonpolar (36.8 %)	Phe (1.7 %)	Nonpolar (36.8 %)	Arg (29.9%)	Basic (40.2 %)
(C)	αA169T	Surface	23.4	Coil	2.18	0.93	Ala (43.1%)	Nonpolar (69.0 %)	Thr (4.6 %)	Polar (20.1 %)	Ala (43.1%)	Nonpolar (69.0 %)
(D)	αI184F	Surface	19.4	Helix	2.34	1.09	Ile (9.2 %)	Nonpolar (64.4 %)	Phe (2.9 %)	Nonpolar (64.4 %)	Leu (28.7%)	Nonpolar (64.4 %)

[1] (A): 2pva1,21(25), (B): 2pva1,2(55), (C): 2pva1,2(149), (D): a(292)
[2] Modification of ORF alter amino acids closest to catalytic pocket, and distance from βSer[1] considers the first residue changed in sequence (*i.e.* βPhe[204])
[3] Distance from functional group to hydroxyl of βSer[1] in Angstroms (Å)
[4] Secondary structure where is located mutation
[5] Side-chain properties of residue described

In this sense, Supplement S.6 in the Supplementary Material Chapter highlights the respective abundance of each residue and the representativity of each cluster of amino acids according to the side-chain. This proportion is represented as uncertainty, also called entropy. Entropy was calculated according to Shannon definition (Shannon, C. E. 1948),

$$\mathrm{H_i}(l) = -\sum f(b,l) \ln f(b,l)$$

where $\mathrm{H_i}(l)$ is the entropy at position l, b represents the residue, and $f(b,l)$ is the frequency that a residue b is found at a position l (Paramesvaran, J. *et al.* 2009). The subscript "i" could be 1 if entropy was calculated considering residue abundance (*i.e.* 20 amino acids and gaps), whereas 2 correlates cluster of amino acids abundance (*i.e.* polar, nonpolar, acidic, basic and gaps). Despite the fact that entropy was calculated with $\log_{(\text{base } 2)}$ and expressed as binary digits (*i.e.* bits) according to the original definition of Shannon, in the present study entropy was calculated with natural logarithm and expressed in natural unit (*i.e.* nats). In this sense, entropy at position l considered 21 options of substituents (*i.e.* 20 amino acids and gaps), then the maximum value possible would be 3.045:

$$-21 * (1/21) * \ln(1/21) = 3.045$$

and the minimum value is zero, which represents a fully conserved residue:

$$-21 * (21/21) * \ln(21/21) = 0$$

A similar analysis was carried out with the amino acid nature at position l considered 5 options of residues (*i.e.* polar, nonpolar, acidic, basic and gaps). Then, the maximum value possible was 1.609, whereas the minimum value was zero again.

It is important to remark that both factors are correlated, but abundance is a statistical representation, whereas entropy is a thermodynamic measurement (Table 26). In particular, this statistic study correlates numerical information available about acylases, but entropy should be considered as the most useful parameter for understanding protein stability, since the randomness of the system increases when a protein molecule is deactivated, and such event is in turn a direct measurement of entropy (Gummadi, S. N. 2003). Although the entropy values were calculated for those residues mutated in several studies (for further information, see Supplement S.6 in Supplementary Material Chapter), important information about alterations on the surface of those recombinant clones is given in Supplement S.10 in the Supplementary Material Chapter.

V DISCUSSION

1. DRAFT GENOME SEQUENCES OF *Actinoplanes utahensis* NRRL 12052 AND *Streptomyces lavendulae* ATCC 13664

The importance of the knowledge of genome sequences in science has turned in one of the main aims in several fields, such as medicine as well as biotechnology. In particular, *Streptomyces lividans* ATCC 13664 (named as *Streptomyces* sp. in this study) and *Actinoplanes utahensis* NRRL 12052 have been considered biotechnologically relevant due to their ability to produce building blocks and semisynthetic bioactive compounds by hydrolysis (Boeck, L. D. *et al.* 1988; Boeck, L. D. *et al.* 1989; Debono, M. *et al.* 1989; Takeshima, H. *et al.* 1989; Snyder, N. J. *et al.* 1998; Torres, R. *et al.* 1998; Kreuzman, A. J. *et al.* 2000; Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007; Hormigo, D. *et al.* 2010; Romano, D. *et al.* 2011; Gandolfi, R. *et al.* 2012) and acylation (Debono, M. *et al.* 1989; Hormigo, D. 2009).

Genomes sequences allow the understanding and elucidation of many gene functions, the identification of phylogenetic relationships, biosynthesis clusters, and the consolidation of useful databases, among others. Although sometimes the interpretation of the biological function of all genes is difficult, this information highlights the importance of the DNA fingerprint information in each microorganism. In this way, almost two decades ago, Anzai and co-workers (Anzai, Y. *et al.* 1997) amplified 1516 bp belonging to 16S rRNA from *S. lavendulae* subsp. *lavendulae* IFO 12341 (*i.e.* also known as *Streptomyces lavendulae* ATCC 13664), and they concluded that this strain should not be assigned to *S. lavendulae*, which was corroborated later (Torres-Bacete, J. 2005). It is remarkable that those studies carried out earlier were based on PCR amplifications. The original identification and characterization were performed by biochemistry assays (Rolinson, G. N. *et al.* 1961), whereas the analysis exposed here was based on genome sequencing. Thus, genome comparison of this microorganism allows locating this strain close to other *S. lavendulae* species, but it was closer to *S. griseus* species (see Figures 20 and 21). Also, despite the fact that *S. griseus* is a well-known streptomycin producer (Distler, J. *et al.* 1992), this biosynthetic cluster was not detected in *S. lavendulae* ATCC 13664. Likewise, penicillin acylases have been detected in *S. griseus* but not in *S. lavendulae*. It is hence proposed here that *S. lavendulae* ATCC 13664 should be assigned as *Streptomyces* sp. Nevertheless, it is important to point out that a second strain, considered as a contaminant of *S. lavendulae* subsp. *lavendulae* ATCC 13664 has been reported, although only one of them displayed acylase activity and typical pigmentation of this strain (Torres-Guzmán, R. 2004). This isolated strain has been employed to obtain the wild-type *Sl*PVA (Torres-Bacete, J. 2005), which in turn was employed to obtain the recombinant clone (Torres-Bacete, J. *et al.* 2015) that was employed in this study.

On the other hand, *A. utahensis* NRRL 12052 was not located close to other *A. utahensis* strains according to a DNA fragment that belonged to 16S rRNA. This comparison resulted in high similarities with several species of other genera, such as *Micromonospora* and *Salinispora* (Fig. 24), which was expected since all of them belong to *Micromonosporaceae* family (Thawai, C. *et al.* 2006). This comparison is analogous to the report by Schwientek and co-workers (Schwientek, P. *et al.* 2012) with another *Actinoplanes* strain, which was not classified within the existing species of this genus. Unfortunately, comparison with genomes available in databases did not prove an evolutionary bond with other genera because the threshold of JSpecies was not reached. The precise location of this strain was probably difficult to achieve because only a few strains of this genus have been sequenced.

In the same manner, the presence of β-lactamases is noteworthy, in addition to other encoding-genes related with antibiotics resistance (Velasco-Bucheli, R. *et al.* 2015). Both the proposed role of these genes in *QQ* mechanisms and the importance of NRPS and PKS presence might be considered as a defense alternative from these microorganisms. These mechanisms of defense could be important for the behavior of the bacteria within its environment.

Furthermore, another putative acylase-gene encoding (*ahla*) was detected in the genome of *A. utahensis* NRRL 12052 (Velasco-Bucheli, R. *et al.* 2015). Moreover, *ahla* gene has been cloned and its expression yielded an extracellular amidohydrolase denominated *N*-acyl-homoserine lactone acylase. *Au*AHLA shows similar residues in the catalytic pocket to other acylases, and high identity with *Au*AAC (44 %) and *Sl*PVA (51 %), as well as with several reported *N*-acyl-homoserine lactone acylases. Likewise, *Au*AHLA had similar catalytic performance as *Sl*PVA and *Au*AAC, and penicillin V was the best substrate so far in contrast to other substrates, but it was apparently active against a few echinocandins and several AHLs.

On the other hand, the presence of a membrane-associated echinocandin B (ECB) deacylase in *A. utahensis* NRRL 12052 has been previously reported (Kreuzman, A. J. *et al.* 2000). Surprisingly, the amino acid sequence of ECB deacylase is similar to the *Au*AAC sequence and only differs from the soluble *Au*AAC form by two additional N-terminal amino acids in the α-subunit (Kreuzman, A. J. *et al.* 2000; Arnold, F. *et al.* 2003; Shao, L. *et al.* 2013). In fact, ECB deacylase-encoding gene was not detected in the genome and *aac* and *ahla* were the only acylases-encoding genes detected. This fact suggests that ECB deacylase and the soluble *Au*AAC are encoded by the same gene (Velasco-Bucheli, R. *et al.* 2015), and they could be produced by different post-translational processing or by different maturation forms. For this reason, it is proposed here that ECB deacylase and *Au*AAC should be reassigned as the same enzyme.

Finally, as expected, nucleotide sequence of *Sl*PVA and *Au*AAC were detected within the corresponding genomes, allowing the identification of full sequences of both enzymes that share the described sequences (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). *Sl*PVA-encoding gene was detected in contig 70, and it is important to remark the presence of one β-lactamase downstream of this sequence (Torres, R. *et al.* 1998; Torres-Bacete, J. 2005; Torres-Bacete, J. *et al.* 2007; Hormigo, D. 2009). On the other hand, *aac* and *ahla* genes were located along contig 8 and within clusters involved in the biosynthesis of siderophores. However, *aac* and *ahla* were not related with any function of the biosynthesis of these compounds by antiSMASH (Velasco-Bucheli, R. *et al.* 2015).

2. MULTIPLE SPOTLIGHTS OF *Sl*PVA, *Au*AAC AND *Au*AHLA

As described in the The Introduction Chapter, both *Au*AAC and *Sl*PVA are synthesized as preproenzymes and secreted as heterodimers, with an α-subunit of 18.8 kDa and 19 kDa, and a β-subunit of 60.1 kDa and 55 kDa, respectively (Inokoshi, J. *et al.* 1992; Torres-Bacete, J. *et al.* 2015). Bioinformatic simulations of both enzymes performed by PredictProtein detected neither transmembrane domains nor disulfide bridges despite of the presence of multiple cysteines throughout the sequences (8 in *Sl*PVA, and 6 in *Au*AAC). The first aspect was expected because they are preproenzymes, and the eventual presence of disulfide bridges indicates the plasticity and flexibility of the secondary structure. This feature allows the molding of its spatial disposition according to the surrounding and the

substrate, but the susceptibility to lose its folding and its catalytic performance is inherent. Similarly, a signal peptide as well as the conserved residues shared with other acylases, among other typical features of acylases, were detected in *Au*AHLA by bioinformatic approaches and then experimentally validated. This means that this acylase is a preproenzyme, and belongs to Ntn-hydrolases like *Sl*PVA and *Au*AAC.

Likewise, in both cases the expected solvent accessibility was estimated over 45 % for the exposed residues with more than 16 % on the surface, and more than 52 % in all other residues (Bigelow, H. R. *et al.* 2004; Rost, B. *et al.* 2004), demonstrating the importance of aqueous systems to carry out enzymatic catalysis.

On the other hand, the estimation of the secondary structure was 30.5 % as helix, 14.1 % as strand and 55.3 % as loops in *Sl*PVA and 28.2 % as helix, 15.9 % as strand and 55.9 % as loops in *Au*AAC (Bigelow, H. R. *et al.* 2004; Rost, B. *et al.* 2004). Alike, experimental values of circular dichroism have revealed that *Sl*PVA contains 20-30 % of α-helix, 16 % of β-sheet, 15-20 % of β-turns and 30 % of random coil (Torres-Bacete, J. *et al.* 2015), in contrast to *Au*AAC that contains 29 % of α-helix, 18 % of β-sheet, 17 % of β-turns and 36 % of random coil (Torres-Bacete, J. *et al.* 2007).

In addition, the signal peptide prediction was performed by SignalP 4.1 server (Petersen, T. N. *et al.* 2011) and it have 29 aa in *Sl*PVA and 30 aa in *Au*AAC (Fig. 47), as well as 27 aa in *Au*AHLA (Fig. 26).

2.1. Comparative study with other acylases

Amino acid sequences of *Sl*PVA, *Au*AAC and *Au*AHLA were compared with sequences of known acylases as well as among themselves (Altschul, S. F. *et al.* 1997). The identity of *Au*AAC and *Sl*PVA is 43 %, whereas *Au*AHLA sequence has a 44 % identity with *Au*AAC and 51 % with *Sl*PVA. Similarly, *Sl*PVA showed a high sequence identity (*i.e.* 86 %) with cyclic lipopeptide acylase from *Streptomyces* sp. FERM BP-5809 (Ueda, S. *et al.* 2011b), whereas it shows 44 % identity with *Au*AAC and 50 % with *Au*AHLA. *Au*AAC, *Au*AHLA and *Sl*PVA share significant identities (87, 50 and 43 %, respectively) with AHL acylase from *Streptomyces* sp. M664 (Park, S. Y. *et al.* 2005).

Furthermore, aculeacin A acylase from *Ralstonia solanacearum* GMI1000 (Chen, C.-N. *et al.* 2009) and *N*-acyl-homoserine lactone acylase from *Ralstonia* sp. XJ12B (Lin, Y.-H. *et al.* 2003), displays 42 % of identity with *Au*AAC and 41 % with *Sl*PVA, respectively, whereas *Au*AHLA has 40 %. In addition, *N*-acyl-homoserine lactone acylases from *Pseudomonas aeruginosa* PAO1 (Sio, C. F. *et al.* 2006), *Shewanella* sp. MIB015 (Morohoshi, T. *et al.* 2008b), *Shewanella oneidensis* MR-1 (Elias, D. A. *et al.* 2005) and *Variovorax paradoxus* VAI-C (Leadbetter, J. R. *et al.* 2000), display identity between 33-39 %, 31-38 % and 32-36 % with respect to *Au*AAC (39, 33, 33 and 35 %, respectively), *Au*AHLA (38, 32, 31 and 38 %, respectively) and *Sl*PVA (36, 32, 33 and 35 %, respectively), respectively. However, *Au*AAC, *Au*AHLA and *Sl*PVA share low sequence identity with most of the β-lactam acylases characterized so far.

*Sl*PVA and *Au*AAC are able to cleave off penicillins with hydrophobic acyl-chains (Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007) and also they are able to hydrolyze aculeacin A (Torres-Bacete, J. *et al.* 2007; Torres-Bacete, J. *et al.* 2015). Equally, *Au*AAC hydrolyzes amide bonds with long and hydrophobic acyl-chains as

palmitic acid from a cyclic hexapeptide core of an echinocandin (Takeshima, H. *et al.* 1989; Hormigo, D. *et al.* 2010). Both *Sl*PVA and *Au*AAC are able to hydrolyze AHLs and prefer aliphatic long chains in the substrates. This trend has been detected in this study with *Au*AHLA as well, although its activity was scarcely explored and further studies are needed to clarify its catalytic properties. Similarly, AHL acylase from *Streptomyces* sp. FERM BP-5809 hydrolyzed a cyclic lipopeptide composed of a hexapeptide moiety and acyl-chains longer than C_{16} (Ueda, S. *et al.* 2011b), whereas AHL from *Streptomyces* sp. M664 effectively hydrolyzed C_8-HSL, C_{10}-HSL and 3-oxo-C_{12}-AHLs (Park, S. Y. *et al.* 2005). Finally, penicillin V acylase from *S. mobaraensis* shows high affinity and hydrolytic activity for capsaicin, which consists of an 8-methyl-6-nonene side chain connected to a vanillyl core through an amide bond (Zhang, D. *et al.* 2007).

A phylogenetic study based on amino acid sequences of several acylases was carried out by BlastP with further analysis developed by MEGA6 (Fig. 58). It was shown that *Sl*PVA, AHL acylase from *Streptomyces* sp. M664 and cyclic lipopeptide acylase from *Streptomyces* sp. FERM BP-5809 are close together in the phylogenetic tree. This group in turn is close to *Au*AAC and AAC from *Ralstonia solanacearum* GMI1000. Moreover, *Au*AHLA is located close to *Sl*PVA as expected and corroborated in their sequences.

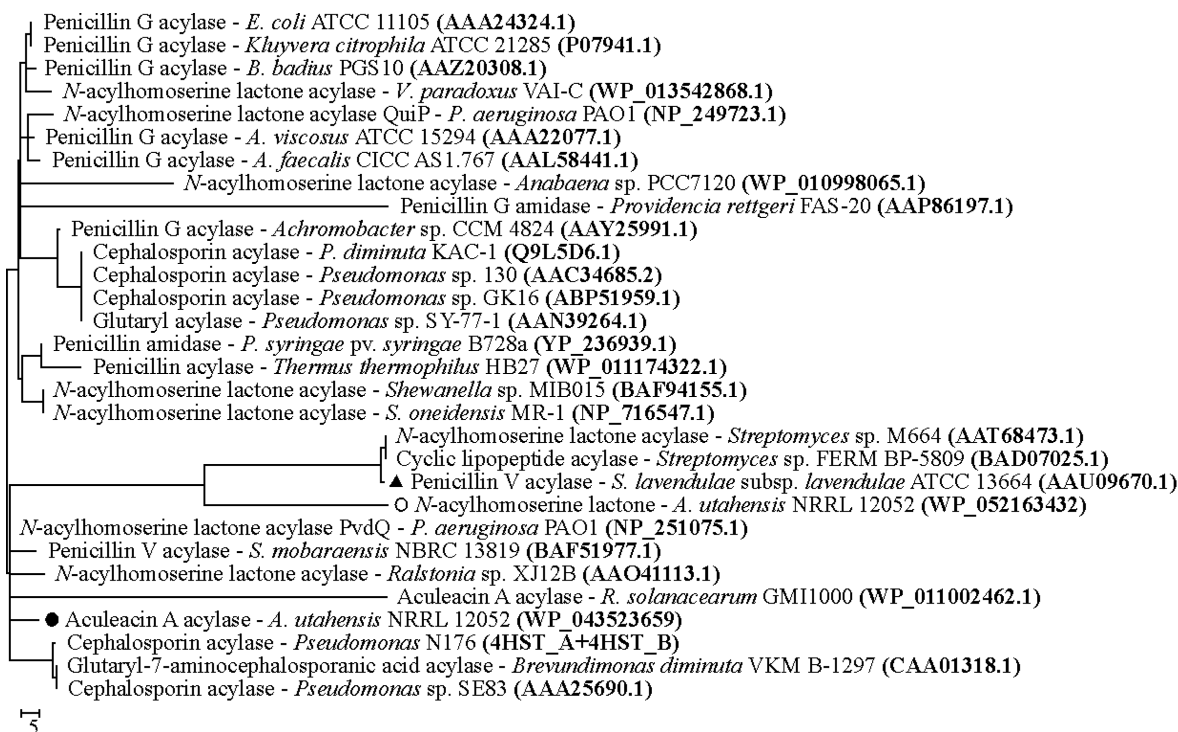

Figure 58. Comparative study of *Sl*PVA, *Au*AAC and *Au*AHLA with others acylases. GenBank is between parentheses in bold

Furthemore, the biological process ontology prediction linked up the enzymes with antibiotic biosynthetic process (44 % and 60 % for *Sl*PVA and *Au*AAC), *QS* (26 % and 25 % for *Sl*PVA and *Au*AAC) and with response to antibiotic (9 % in both cases) (Hamp, T. *et al.* 2013), all of them presumably related with self-defense systems.

2.2. Potential biological role of *Sl*PVA, *Au*AAC and *Au*AHLA

Penicillin acylases have been recognized as enzymes of tremendous industrial importance for more than 50 years now (Mukherji, R. *et al.* 2014). In contrast, echinocandin acylases have been explored approximately since the last decade of the past century (Boeck, L. D. *et al.* 1988). Interestingly, these enzymes are vital for industrial production of semisynthetic antibiotics and antifungal compounds due to their capability to hydrolyze amide bonds. The specificity of those enzymes change according to acyl group and class of substrate catalyzed (*e.g.* *N*-acyl-homoserine lactone acylase, penicillin acylase, and echinocandin acylase).

Regardless their utilities in the industry, several biological roles have been postulated to this kind of acylases. Initially, several studies linked up these enzymes with benzylpenicillin biosynthesis (Arnstein, H. R. V. *et al.* 1960; Wolff, E. C. *et al.* 1960; Flynn, E. H. *et al.* 1962; Cole, M. *et al.* 1963), whereas other authors considered their connection with resistance to antibiotics (Claridge, C. A. *et al.* 1960; English, A. R. *et al.* 1960; Rolinson, G. N. *et al.* 1960; Huang, H. T. *et al.* 1963; Uri, J. *et al.* 1963; Holt, R. J. *et al.* 1964b; Holt, R. J. *et al.* 1964a; Cole, M. *et al.* 1966). Some studies, in contrast, suggested that their amidase activity could be an accidental enzymatic activity and non-specific (Hamilton-Miller, J. M. T. 1966; Vandamme, E. J. *et al.* 1975). Other options emerged simultaneously as alternative considerations, such as employing phenylacetic acid as a carbon source by bacteria (Kameda, Y. *et al.* 1961; Szentirmai, A. 1964), and penicillin G acylase from *E. coli* was involved within clusters related with aromatic compounds and their utility as a carbon and energy source (Valle, F. *et al.* 1991; Merino, E. *et al.* 1992; Prieto, M. A. *et al.* 1993).

Likewise, over the past two decades several authors have suggested the connection with *QQ* processes (Lin, Y.-H. *et al.* 2003; Dong, Y. H. *et al.* 2005; Park, S. Y. *et al.* 2005; Sio, C. F. *et al.* 2006; Chen, C.-N. *et al.* 2009; Hong, K.-W. *et al.* 2012; Mukherji, R. *et al.* 2014) and this role has been connected to *Au*AAC (Torres-Bacete, J. *et al.* 2007; Hormigo, D. 2009; Velasco-Bucheli, R. *et al.* 2015) and *Sl*PVA (Hormigo, D. 2009) due to its activity against some aliphatic and β-keto substituted aliphatic AHLs (see Tables 10 and 11).

Notwithstanding the evidence about the catalytic performance of enzymes employing several substrates, such as penicillins, echinocandins and AHLs, the real role of *Sl*PVA and *Au*AAC in these microorganisms is unknown nowadays. However, it is important to highlight the relationship postulated in other genera between acylases and NRPS such as pyoverdine, gobichelin and laspartomycin, all of them siderophores related with the microbial iron metabolism.

Recently, the knowledge about siderophores (*i.e.* high-affinity iron chelating compounds) and its relationship with acylases in *Pseudomonas* genus has been deeply studied (Fuchs, R. *et al.* 2001; Cézard, C. *et al.* 2015), and the evidence about the connection of virulence and the iron metabolism in this genus has been closely related (Lamont, I. L. *et al.* 2002; Cornelis, P. *et al.* 2013; Cézard, C. *et al.* 2015). In particular, *P. aeruginosa* regulates the production of at least three virulence factors, which are major contributors to cause diseases by this microorganism (Lamont, I. L. *et al.* 2002). One of those siderophores is known as pyoverdine and within its biosynthetic cluster, the *pvdQ* gene is located (Lamont, I. L. *et al.* 2003), which has 36, 39 and 38 % identity with *Sl*PVA, *Au*AAC and *Au*AHLA,

respectively. It is noteworthy to mention that PvdQ removes the fatty acid in pyoverdine maturation within the periplasm (Miethke, M. *et al.* 2007; Drake, E. J. *et al.* 2011; Cézard, C. *et al.* 2015), whereas *pvdQ* mutant does not (Hannauer, M. *et al.* 2012). This means that a nucleophilic attack performed by PvdQ leads to ferribactin (*i.e.* precursor of pyoverdine), and thereafter is modified at least by three enzymes before being released outside of the cell, as pyoverdine. Despite the fact that its capability to remove fatty acid chains from AHLs has been described (Sio, C. F. *et al.* 2006; Bokhove, M. *et al.* 2010), it has also been a topic of discussion, because the relationship of PvdQ with pyoverdine maturation is indisputable (Drake, E. J. *et al.* 2011; Hannauer, M. *et al.* 2012). For further information, a few interesting reviews (Miethke, M. *et al.* 2007; Hider, R. C. *et al.* 2010; Schalk, I. J. *et al.* 2013; Cézard, C. *et al.* 2015) describe and summarize the role of PvdQ in pyoverdine biosynthesis.

Even though most of the studies about siderophore biosynthesis are related with *P. aeruginosa* PAO1, similar studies have been performed with *P. syringae* pv. phaseolicola NPS3121 (González-Villanueva, L. *et al.* 2014), *P. syringae* pv. phaseolicola 1448a, which has an orthologous gene to *pvdQ* (gene number 1937, according to annotation in the *Pseudomonas* genome database) (Owen, J. G. *et al.* 2011), and *P. syringae* pv. tabaci 6605 (Taguchi, F. *et al.* 2006a; Taguchi, F. *et al.* 2010). In particular, siderophores biosynthesis and AHLs have been correlated, and the implication of this relationship with *QS* has been proposed (Fong, K. P. *et al.* 2003; Taguchi, F. *et al.* 2006a; Taguchi, F. *et al.* 2006b; Taguchi, F. *et al.* 2010). Likewise, regulation of siderophore production by *QS* has been described in other genera and it was well summarized by Sandy and co-workers (Sandy, M. *et al.* 2009). Thus, the regulation of siderophore production by *QS* has been described in select Gram-negative bacteria (Stintzi, A. *et al.* 1998; Lilley, B. N. *et al.* 2000; Fong, K. P. *et al.* 2003; James, C. E. *et al.* 2006; Wang, Q. *et al.* 2007; Harrison, F. *et al.* 2009; Sandy, M. *et al.* 2009) and the correlation of its biosynthesis with AHLs has been widely studied (Lewenza, S. *et al.* 1999; Lewenza, S. *et al.* 2001; Taguchi, F. *et al.* 2006a; Taguchi, F. *et al.* 2006b; Taguchi, F. *et al.* 2010). Indeed, siderophores have been catalogued as *QS* molecules (Cézard, C. *et al.* 2015) as well as AHLs.

The analysis of the *A. utahensis* genome identified 24 clusters, 3 of them in the same contig where *aac* (NZ_JRTT01000008.1) and *ahla* (NZ_JRTT01000008.1) are located. In particular, both genes are within the predicted biosynthetic clusters of gobichelin and laspartomycin, respectively, but no related function with this biosynthesis was proposed in both cases (Fig. 25) (Velasco-Bucheli, R. *et al.* 2015).

In this sense, two gobichelin structures have been described in *Streptomyces* sp. NRRL F-4415 (Chen, Y. *et al.* 2013). Chen and co-workers found that the biosynthetic mechanism for gobichelin A shows some similarity with the mechanism exhibited by *Amycolatopsis* sp. AA4 in amychelin biosynthesis (Seyedsayamdost, M. R. *et al.* 2011). Whereas gobichelin A is directly released from the NRPS assembly line by a thioesterase, the NRPS for amychelin biosynthesis lacks a C-terminal thioesterase domain for peptide chain release. This fact suggests that amychelin biosynthesis requires an external α/β hydrolase such as AmcB present in the cluster. Similar situation happens with the siderophore named albachelin from *A. alba* JCM 10030 (Kodani, S. *et al.* 2015), and once more an α/β hydrolase-encoding gene similar to AmcB from *Amycolatopsis* sp. AA4 was detected, so the same role was proposed in this mechanism.

Notwithstanding laspartomycin was originally isolated and characterized almost half-century ago from *Streptomyces viridochromogenes* var. *komabensis* ATCC 29814 (Naganawa, H. *et al.* 1968), its laspartomycin biosynthetic gene cluster was detected only until 2011. In this cluster the locus Orf29 is located (*i.e.* a predicted α/β hydrolase-coding sequence), which apparently activates and modifies a precursor lipid chain (Wang, Y. *et al.* 2011) and might be involved in either an acyltransferase reaction or a thioesterase type II function (Müller, C. *et al.* 2007). In turn, Müller and co-workers detected an α/β hydrolase-coding gene *lipE* within the biosynthetic cluster of friulimicin in *Actinoplanes friuliensis* HAG010964 (Müller, C. *et al.* 2007).

It is also noteworthy that enzymatic extracts from *A. utahensis* NRLL 12052 (*i.e.* enzymes with acylase activity) have been employed in order to obtain the core of laspartomycin (Borders, D. B. *et al.* 2007). This data together with the bioinformatic analysis reported here suggests that at least *Au*AAC and *Au*AHLA could be involved with siderophores and *QQ* processes.

On the contrary, *Sl*PVA was not detected within this kind of cluster, which could be a consequence of the short length of contig 8 where the *pva* gene was detected. Although this contig has 28,867 bp and the average size of NRPS clusters in *Streptomyces* sp. and *A. utahensis* are 20,945 bp (Table 8) and 46,689 bp (Table 9), respectively, the probability to locate similar clusters in this contig is ephemeral. However, the possibility that most of those clusters are partial lengths is not discarded in this study. For instance, the predicted biosynthesis clusters of gobichelin (*i.e.* 62075 bp) and laspartomycin (*ca.* 29758 bp, probably this is a partial size) are bigger than the length of contig 8 in *Streptomyces* sp. Several clusters were detected within *Streptomyces* sp. genome and not one of them contains this acylase. Nonetheless, it is not ruled out here that this enzyme is contained in some clusters and that its role could be similar to those previously exposed to *Au*AAC and *Au*AHLA, as well as in other acylases and hydrolases.

Thereby, ABC-transporter coding sequences were detected close to *pva* (Fig. 22) and *aac* (Fig. 25). Also, an alternative to the secretion of laspartomycin through the intervention of *ahla* was proposed. It is important to remember that the ABC-system is involved in secretion of some siderophores (*i.e.* iron metabolism in microorganism and used as antibiotics in human therapy) (Hider, R. C. *et al.* 2010).

2.3. Estimation of kinetic parameters by fluorometric measurements

The methodology employed to quantify kinetic parameters was a successful and innovative tool developed in the present study. Hence, the repercussion of the implementation of this alternative is beyond the measurement of HSL, because by this technique the quantification of other primary amines is possible. Thus, just as mentioned in Section 8.3 in the Materials and Methods Chapter, *o*-phthalaldehyde (OPA) was employed to quantify kinetic parameters of *Sl*PVA and *Au*AAC, as well as their recombinant clones, employing AHLs as substrates.

Although previous studies described that *Sl*PVA and *Au*AAC hydrolyze aliphatic AHLs, activity using β-keto substituted aliphatic AHLs was not detected (Hormigo, D. 2009). In contrast, fluorescent derivatives from the reaction of primary amines with OPA in presence of β-mercaptoethanol at a high pH value have been effectively measured here. β-Mercaptoethanol facilitates a rapid reaction to form intensely fluorescent derivatives and

simultaneously gives high and fast stability to OPA (Švedas, V.-J. K. *et al.* 1980). Therefore, kinetic parameters have been quantified by fluorometric measurements, which have shown a similar trend in the activity against both kinds of AHLs evaluated with *Sl*PVA and *Au*AAC (Tables 10 and 11, respectively). Thus, acyl-homoserine lactone acylase activity is significant in both acylases. This allows intuiting that the technique based on spectrophotometric measurements is less sensitive than spectrofluorometric analysis employed here, which is well-known and has been demonstrated (Fricker, L. D. *et al.* 1990; Olojo, R. O. *et al.* 2005; El-Enany, N. *et al.* 2007; Mahmoud, A. M. *et al.* 2009; Önal, A. 2011).

The analysis shown in Section 3 in the Results Chapter states that spectrophotometric assays performed with PDAB and fluram were developed at low pH values (for further information, see Sections 8.2 and 8.3 in the Materials and Methods Chapter). In particular, the assays to detect primary amines (*e.g.* 6-APA and HSL) performed with fluorescamine show strong fluorescence at pH values among 7 and 9, but sensitivity decreases with pH (Baker, W. L. 1985). Quite the opposite is observed in PDAB reactions with 6-APA and the consequent formation of its Schiff base, which is affected at basic pH values (Deshpande, B. S. *et al.* 1993). In contrast, OPA methodology at high pH value (for further information, see Sections 10.5 in the Materials and Methods Chapter) propitiates suitable conditions for fluorescence emission (Švedas, V.-J. K. *et al.* 1980).

In addition, OPA offers several advantages over fluram and PDAB, such as high solubility and stability in aqueous solutions, which is distinctly superior to other chromophores that require organic solvents to be dissolved. In addition, OPA exhibits greater fluorescent quantum yield (Benson, J. R. *et al.* 1975). Indeed, OPA sensitivity has been established between 5 and 10-fold higher in comparison to fluram (Benson, J. R. *et al.* 1975), which in turn has been almost equally sensitive to PDAB according to the experiments performed here (data not shown). In this sense, reactions with OPA were developed rapidly at basic pH value, and the mixture was kept at 25ºC during 2 min in order to avoid drawbacks such as isoindoles instability formed by light, attack by acids, and air oxidation (White, J. D. *et al.* 1969). Likewise, OPA solution was kept at 4ºC in the presence of β-mercaptoethanol before being employed in the assays with the objective of evading its oxidation (Švedas, V.-J. K. *et al.* 1980).

To sum up, Supplement S.7 in the Supplementary Material Chapter let deduce the high reactivity of OPA for detecting primary amines in comparison with PDAB and fluram. The complex mechanism involving fluram have been scarcely studied, although a tentative reaction of amine group with α,β-unsaturated ketone at dihydrofuranone ring by 1,4-addition (*i.e.* known nucleophilic attack of Michael addition) causes the consequent amine in *trans* to phenyl ring (*i.e.* lower steric hindrance) and a hemiketal. This in turn originates a diketone after a keto-enol tautomerism and acid group formation. Thereafter, 1,2-addition by a nucleophilic attack of amine to ketone generates a β-amino-α,β-unsaturated ketone.

On the other hand, PDAB mechanism involves its aldehyde group with the primary amine released after enzymatic reaction (*e.g.* 6-APA or HSL) to condense a water molecule and form a Schiff base. Similarly, those aldehyde groups in OPA condense with primary amines, but in this case the double condensation is performed in two steps. Firstly, one of those aldehydes implicated in the reaction is involved in a nucleophilic attack and liberates a water molecule (*i.e.* similar to PDAB mechanism). Then, due to the proximity of this amino group with the second aldehyde and the stabilization of this intermediate through β-

mercaptoethanol, a second nucleophilic attack takes place, and propitiates the releasing of a second water molecule simultaneously to the reordering of the structure to form an isoindole.

The complexity of those mechanisms might be visualized in terms of time (*i.e.* incubation) and stability of product (*i.e.* measurement). In particular, according to the methodologies employed in this study, fluram requires 40 min of incubation, OPA needs 2 min, whereas PDAB reacts immediately (for further information, see Sections 8.3, 10.5 and 8.2 in the Materials and Methods Chapter, respectively). It is important to remark that PDAB absorbance increases for a while and thereafter decreases with time, in contrast to fluram that increases slowly until reaching an asymptotic trend after 40 min and thereafter reduces the intensity (data not shown). Even though no consensus about optimal time to incubate OPA with amines was found in literature, the incubation after a short period of time was adopted here in order to allow reaching 25ºC to the reaction mixture. In fact some authors suggest immediate product formation (Benson, J. R. *et al.* 1975), whereas another proposes that a maximum takes place after 2 min (Roth, M. *et al.* 1973), in contrast to 5-6 min proposed lately (Švedas, V.-J. K. *et al.* 1980). Regardless of time, the presence of β-mercaptoethanol in OPA solution do not allow the loss of sensitivity or increases in the fluorescent background (Benson, J. R. *et al.* 1975), and the stability of the product was contrasted and it was almost intact over time (data not shown).

2.4. Reaction of postulated mechanism in *Sl*PVA, *Au*AAC and *Au*AHLA

Recently, the putative oxyanion hole residues from *Sl*PVA was estimated by site-directed mutagenesis (Torres-Bacete, J. *et al.* 2015). Thus, the spatial proximity of βSer^{1}, βHis^{23}, βVal^{70} and βAsn^{72} within the hydrophobic pocket, in addition to enzymes inactivation (*i.e.* mutations of either βSer^{1} or βHis^{23}) or drastic activity reductions (*i.e.* mutations of either βVal^{70} or βAsn^{272}) were considered as a starting point to postulate those residues as crucial in catalysis. Likewise, αAla^{154}, αGly^{158}, βTyr^{24}, βArg^{31}, βTrp^{33}, βLeu^{50}, βSer^{53}, βSer^{57}, βIle^{58}, βSer^{61}, and βVal^{186}, have been estimated as amino acids involved in the substrate binding pocket of *Sl*PVA. Most of those residues are in agreement with those predicted here (*i.e.* Table 20, βSer^{1}, βHis^{23}, βTyr^{24}, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, βHis^{68}, βThr^{69}, βVal^{70}, βVal^{186}, βAsn^{272}), and the discrepancies exhibited in some amino acids could be associated to their proximity to the catalytic pocket. Similar analyses have been carried out by several authors with other acylases (Brannigan, J. A. *et al.* 1995; Duggleby, H. J. *et al.* 1995; Kim, Y. *et al.* 2000; Oinonen, C. *et al.* 2000; Kim, Y. *et al.* 2001; Pollegioni, L. *et al.* 2005; Bokhove, M. *et al.* 2010; Pollegioni, L. *et al.* 2013; Suplatov, D. *et al.* 2014; Mollica, L. *et al.* 2015), and some of their postulates were employed to interpret the information obtained here. In spite in fact that similar analysis was carried out recently with *Au*AAC, Isogai and co-workers mistakenly predicted the spacer peptide (*i.e.* cleavage between αAla^{174}-αSer^{175} and αGly^{197}-βSer^{1}) (Isogai, Y. *et al.* 2016), which is longer to that detected experimentally (*i.e.* cleavage between αAla^{181}-αAla^{182} and αGly^{197}-βSer^{1}) (Inokoshi, J. *et al.* 1992). This ambiguity was employed thereafter in their docking simulations. Thus, Isogai and co-workers did not detect docking poses between *Au*AAC and β-lactam substrates, arguing the narrow space within binding pocket and the lack of space to accommodate the bulky benzyl group of penicillin G side-chain (Isogai, Y. *et al.* 2016). This computational approach, however, is in contradiction with experimental quantification of kinetic parameters (Torres-Bacete, J. *et al.* 2007) and does not match with the evidence that *Au*AAC is capable of hydrolyzing penicillin V (*i.e.* phenoxyacetic acid is a bulkier side-chain) (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). In fact, although

Isogai and co-workers based their study on some mutations reported in glutaryl acylase from *Pseudomonas* SY-77-1 (Otten, L. G. *et al.* 2002; Sio, C. F. *et al.* 2002; López-Gallego, F. *et al.* 2008) (for further information, see Supplement S.9 in the Supplementary Material Chapter), they detected discrepancy between their computational and experimental approaches (Isogai, Y. *et al.* 2016).

Just as mentioned in Table 20, predicted residues in *Sl*PVA, *Au*AAC and *Au*AHLA were similar, which was expected because the predicted template employed by several servers was 1KEH in almost all proteins (Table 19). Superpositions of these predicted models are illustrated in Figure 59, and as can be observed, the difference between them apparently is minimum, and only slight changes in the orientation within the catalytic pocket and among the ligand binding residues have been observed. Thus, a tentative catalytic mechanism implied in hydrolysis of aliphatic side-chains, in particular in *Sl*PVA, *Au*AAC and *Au*AHLA, could be similar in all of them. However, further studies such as molecular dynamics, crystallography, NMR and docking are necessary in order to understand the actual molecular mechanism involved in their catalysis, and in this way open the way for understanding their biological role.

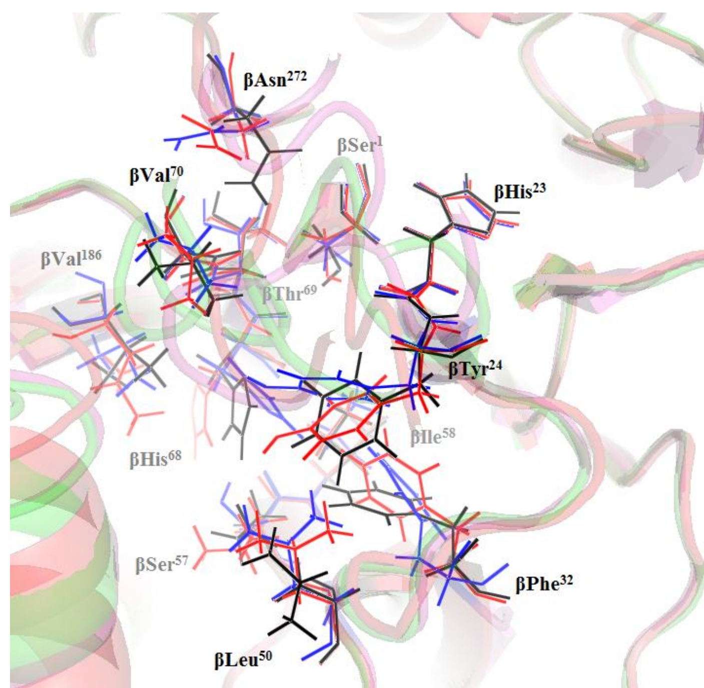

Figure 59. Superposition of the predicted catalytic pocket of *Sl*PVA, *Au*AAC and *Au*AHLA. The sequences are represented in blue, black and red to illustrate *Sl*PVA, *Au*AAC and *Au*AHLA, respectively. The numeration and residue correspond to *Sl*PVA

3. DIRECTED MOLECULAR EVOLUTION OF *Sl*PVA AND *Au*AAC

The substrate promiscuity, stability, performance and industrial applications of *Sl*PVA and *Au*AAC have been reported (Torres, R. *et al.* 1998; Torres, R. *et al.* 1999; Torres-Bacete, J. *et al.* 2000; Torres-Guzmán, R. *et al.* 2001a; Torres-Guzmán, R. *et al.* 2001b; Torres-Guzmán, R. *et al.* 2002; Torres-Bacete, J. *et al.* 2007; Hormigo, D. 2009; Hormigo, D. *et al.* 2010; Torres-Bacete, J. *et al.* 2015; Velasco-Bucheli, R. *et al.* 2015). In the present study, further analysis and improvements have been achieved in order to understand and improve their catalytic properties, their behavior during catalysis and their biological role

as well. Some authors may suggest the requirement to employ structural analysis of enzymes rather than evolutionary stochastic approaches and the screening of large random libraries (Suplatov, D. *et al.* 2014). However, the connection and interaction of both tendencies with other theoretical and empirical approaches will facilitate the improvement of enzymes performance.

Directed evolution mimics Darwinian selection on a laboratory level (Cobb, R. E. *et al.* 2013) and has been consolidated as a powerful biotechnological technique developed at temporal scale in the lab (Valetti, F. *et al.* 2004; Bershtein, S. *et al.* 2008). This innovative technology has the advantage that it does not require ample knowledge of the interest gene and offers fast and effective means of generating mutants with improved traits (Stephens, D. E. *et al.* 2007). Equally, directed evolution coupled with the development and strengthening of powerful HTS have been successfully employed to solve problems in protein engineering (Labrou, N. E. 2010). Thus, significant strides have been obtained in several fields that have allowed to achieve improvements in many aspects such as activity and stability of enzymes, the engineering of biosynthetic pathways, the tuning of functional regulatory systems, and the development of specific phenotypes (Cobb, R. E. *et al.* 2013).

Thus, in order to understand these enzymes, Supplement S.9 in the Supplementary Material Chapter the location of residues in protein structures of *Sl*PVA and *Au*AAC is summarized. This shows the distance to βSer^1, which should be interpreted as the closest distance between the active groups of each residue within the structure of every acylase (those cells with text in red highlight the positions that altered their relative coordinates in more than 2.5 Å with respect to βSer^1). Most of the residues described here and in previous studies have been detected on the surface of recombinant enzymes, and as expected, the positions β50, β57, β58, β68, β70, β186 and β272 (according to *Sl*PVA numeration) were located within the catalytic pocket.

Directed evolution using random mutagenesis is a versatile tool for developing novel enzymes. It has been used to (i) improve the catalytic activity and efficiency of the enzyme, (ii) to modulate (limit or expand) substrate specificity, (iii) to alter cofactor specificity, and (iv) to improve stability over a wider range of conditions, and so forth. Even though random mutagenesis uncovers a diversity in a very small region of sequence space, it has been proven to be sufficiently efficient to allow the wholesale changes required, for example, to evolve a novel activity in a target gene (Altreuter, D. H. *et al.* 1999; Reetz, M. T. *et al.* 2000; Zhao, H. *et al.* 2003; Callanan, M. J. *et al.* 2007).

Directed evolution studies in the case of *Sl*PVA and *Au*AAC are highly promising for obtaining libraries of enzymes with improved properties, which could be employed in different processes, such as the synthesis of antibiotics and antifungals (*e.g.* semisynthetic echinocandins), or be directly used as antimicrobial agents. Therefore, in the present study, directed evolution of *Sl*PVA and *Au*AAC has been performed by random mutagenesis employing the mutator strain *E. coli* XL1-Red. This procedure was carried out by the platform Freedom EVO 75 (Tecan) for liquid handling, in combination with the expression strain *Rhodococcus* sp. T1014 and recombinant plasmids from pENV19. Results of these directed evolution processes have allowed the attainment of a considerable library of mutant clones (*i.e.* 1917 from *aac* and 2076 from *pva*). Such mutant clones secrete recombinant enzymes with higher acylase activity with respect to parental enzymes at different pH values (6.0 and 8.0) and temperatures (30ºC and 45ºC).

Yet, as mentioned above, the analysis of the library of the generated mutants is the rate-determining step to achieve mutants with improved properties by directed evolution. With this purpose and in first place, it is important to have an adequate expression system. In this case, due to the high G+C content in the genomes of these Actinomycetes (i.e. *Streptomyces* sp. and *A. utahensis*) and different codon usage, gene expression in *E. coli* is difficult and complicated as well. Therefore, it was necessary to employ a homologous expression system here, such as *Rhodococcus* sp. T104 in combination with a bifunctional vector pENV19, which is derived from the plasmids pEM4 and pNV19 developed in the research group of Enzymatic Biotechnology of the Complutense University of Madrid, in collaboration with the research group of Dr. Gerben Zylstra of Rutgers University (Hormigo, D. 2009; García-Hidalgo, J. *et al.* 2012; García-Hidalgo, J. *et al.* 2013).

There is no method of HTS that suits for every enzyme, but there must be one HTS protocol that suits for one enzyme in directed evolution (Zuo, Z.-Y. *et al.* 2007). Thus, *Rhodococcus* sp. T104 harbouring recombinant pENV19 is an easy handling strain, in addition that it is capable of producing extracellular acylases. Consequently, this system can be consolidated as an useful tool in directed evolution for this kind of enzymes. Similarly, robotic autonomous work equipments allow addressing massive studies of samples. Thereby, platforms similar to Tecan, with a friendly and versatile interface allow better administration of both economic and human resources.

Directed evolution studies generally involve multiple cycles of mutation and analysis of many mutants (Wang, M. *et al.* 2012). However, the results obtained in this study have been a success, as long as the recombinant enzymes obtained here were achieved in only one mutation cycle. This is exactly the part of the process where the enhancements with respect to the parental enzyme are less sensitive, precisely because the mutations are performed on the parental enzyme. In addition, it is important to emphasize that *E. coli* XL1-Red is a strain associated with a low rate of mutations (Daugherty, P. S. *et al.* 2000; Rasila, T. S. *et al.* 2009; Badran, A. H. *et al.* 2015) in comparison with PCR-based mutagenesis protocols that consume less time and accomplish a higher amount of mutations (Greener, A. *et al.* 1997; Nguyen, A. W. *et al.* 2003). It can hence be expected that significant improvements will be achieved by additional cycles.

Despite the fact that most of the studies with the *E. coli* XL1-Red mutator strain are related with the improvement or modification of the functions in proteins (Blagodatski, A. *et al.* 2011; Packer, M. S. *et al.* 2015), this technique has been employed to observe the functions of a viral gene by mutation of the whole plant viral genome (Lu, X. *et al.* 2001), and the interactions with DNA of 16S rRNA (Bélanger, F. *et al.* 2005). The aim was to increase the antibodies production (Coia, G. *et al.* 1997), to enhance and understand resistance mechanism (Dai, G. *et al.* 2000; Farrow, K. A. *et al.* 2002; Johnson, M. D. *et al.* 2014), and to understand membrane protein assembly (Hatahet, F. *et al.* 2015), and so forth.

Obtaining random mutant clones is a consolidated biotechnological tool (Chatterjee, R. *et al.* 2006), and it has been demonstrated that it is highly efficient to improve the properties in almost any kind of proteins. It can easily be foreseen that further advances developed in this field will strengthen this technique both on an industrial level and in the academic realm (Wang, M. *et al.* 2012).

Thus, it is remarkable to obtain recombinant enzymes from *Sl*PVA and *Au*AAC with enhanced properties with respect to the parental enzymes. These enzymes with improved

activities at 30ºC or at pH 6.0, or both, have been obtained, under conditions where the parental enzymes do not display activity or they are not stable (Torres, R. *et al.* 1998; Torres-Bacete, J. *et al.* 2007). The selection of recombinants clones was based on the Anderson-Darling test, which gives more weight to tails (*i.e.* spots far away from means) than other tests in a distribution of samples.

So, recombinant acylases from *Sl*PVA and *Au*AAC with improved catalytic performance under uncommon conditions were obtained, employing some molecules involved in *QS* of Gram-negative bacteria. Thus, the performance of these acylases was improved by employing aliphatic and β-keto substituted aliphatic AHLs as substrate (Table 13). By this methodology, it was possible to improve the enzyme performance without erasing their original acylase activities. Also, some of these mutant clones simultaneously displayed higher activities under different operational conditions, such as pH and temperature. Selected recombinant clones of *Sl*PVA were 2pva1,21(25) (*i.e.* βR373W), 2pva1,2(55) (*i.e.* ΔG1215, which modified the ORF since βV204F), 2pva1,2(149) (*i.e.* αA169T in addition to one silent mutation), whereas a(292) (*i.e.* αI184F) was obtained from *Au*AAC. Those clones were submitted to further analyses. It is remarkable that some usual drawbacks presented with XL1-Red were evaded here, such as the accumulation of mutations in the strain, which causes useless cultures and low mutation frequency under standard conditions (0.5 mutations per kilo base) (Labrou, N. E. 2010), except in the case of 2pva1,2(55).

Finally, the next Sections explain information about statistic values (*i.e.* the abundance of each residue and the representation of each cluster of amino acids according to the side-chain), in addition to thermodynamic interpretation (*i.e.* entropy), among other factors (for further information, see Supplements S.6, S.9 and S.10 in the Supplementary Material Chapter). Likewise, the importance and correlation of both values give numerical information about acylases and its protein stability, respectively. However, this Section does not pretend to argue the information about improvements obtained with punctual mutations. Table 18 shows that the improvements were biased to short chains, long chains, aliphatic chains or β-keto substituted aliphatic chains, but the knowledge about this topic should be verified by further techniques in the laboratory. In this direction, results obtained here hopefully will allow undertaking new evolutionary cycles with the purpose of generating new mutant libraries from both enzymes.

3.1. Recombinant acylase 2pva1,21(25) of *Sl*PVA

The amino acid sequence alignments (for further information, see Supplement S.6 in the Supplementary Material Chapter) have shown that the mutated residues in this clone are not necessarily highly conserved according to $H_1(l)$. In particular, βArg^{373} was located on the enzyme surface, where a basic residue (15.5 %) is mutated by a nonpolar residue (46.6 %) such as tryptophan (*i.e.* βR373W). In spite of the abundance of this kind of amino acids at this position in acylases, this is the only one with a tryptophan there. In fact, most acylases have an alanine (45.4 %) and more than one fourth of them have a polar residue, which explains the entropy values. It is remarkable that no evidence of importance at this position in the performance of this kind of enzyme has been reported, and it is impossible to deduce this from previous studies carried out with similar acylases (further information, see Sections 4.3 in this Chapter). In particular, despite the fact that $H_1(\beta 373)$ has the lowest entropy value according to the mutated residues analyzed here, this value is relatively high and it reveals a low rate of conservation of this residue in acylases. It similarly highlights that at least four residues located on enzyme surface were drastically affected by this

mutation, including βTrp[373] itself. Likewise, the orientation of αAla[131] was altered with respect to the solvent, which in turn aligns with αGly[140] in *Pseudomonas* SE83. The residue mutated simultaneously with other amino acids in similar studies of directed evolution (for further information, see Section 4.3.4 in this Chapter) (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012). It is also important to mention that 3.9 % of residues changed the orientation with respect to the solvent, and an additional 1.0 % of them now are in contact with the solvent (for further information, see Supplement S.10 in the Supplementary Material Chapter).

3.2. Recombinant acylase 2pva1,2 (55) of *Sl*PVA

The analysis of the recombinant enzyme named 2pva1,2(55) constitutes a particular case, because one nucleotide was deleted in *pva* gene sequence. Thus, a considerable alteration was achieved in the C-terminal end of the β-subunit, which shortened the ORF and produced a truncated *Sl*PVA. Hence, the location of a new stop codon reduced the size of the acylase in 302 aa, and the truncated *Sl*PVA has 261 residues in the β-subunit whilst the parental enzyme has 565 aa. It should be mentioned that the truncated enzyme is able to hydrolyze β-keto substituted aliphatic AHLs more efficiently than the parental enzyme, and most of the ligand binding residues predicted by I-Tasser were conserved (Table 19). The fragment of sequence that was deleted in the C-terminal end of β-subunit could be considered as a lid that plays a role in the steric hindrance; it limits the molecules in the catalytic pocket (Fig. 54). It is worth noticing that βAsn[272] in *Sl*PVA or related residues are present in all acylases compared in this study (for further information, see Supplement S.9 in the Supplementary Material Chapter), and this recombinant clone constitutes the first acylase without residue there. However, the function in the catalysis of βAsn[272] in parental *Sl*PVA probably is replaced by βArg[236] in this truncated acylase, and its location with respect to βSer[1] is almost the same in other acylases. Even though further analyses by additional techniques are required in order to clarify this new molecular mechanism, several acylases have shown this conserved residue between β240-β250. This means that the new position is not a far-fetched idea (Table 27). Likewise, this study allows intuiting that the C-terminal end is not involved in the catalysis nor in the stability of the enzyme, at least in this recombinant clone.

Table 27. Location of the last catalytic residue of the β-subunit in acylases

Range	Percent	Bacteria with relevant acylases* (location in β-subunit, GenBank accession number of the acylase)
β230-β239	4.6 %	
β240-β249	52.9 %	*K. citrophila* ATCC 21285 (240, P07941.1), *E. coli* ATCC 11105 (241, AAA24324.1), *A. faecalis* CICC ASI.767 (241, AAL58441.1), *P. rettgeri* FAS-20 (241, AAP86197.1), *Achromobacter* sp. CCM 4824 (241, AAY25991.1), *Pseudomonas* N176 (242, 4HST_A+4HST_B), *B. diminuta* VKM B-1297 (242, CAA01318.1), *Pseudomonas* SE83 (242, AAA25690.1), *T. thermophilus* HB27 (242, WP_011174322.1), *Pseudomonas* sp. 130 (244, AAC34685.2), *P. diminuta* KAC-1 (244, Q9L5D6.1), *Pseudomonas* sp. GK16 (244, ABP51959.1), *Pseudomonas* sp. SY-77-1 (244, AAN39264.1), *A. viscosus* ATCC 15294 (245, AAA22077.1), *B. badius* PGS10 (245, AAZ20308.1), *P. syringae* B728a (246, YP_236939.1)
β250-β259	12.1 %	*P. aeruginosa* PAO1 (253, NP_249723.1)
β260-β269	9.8 %	*Anabaena* sp. PCC7120 (261, WP_010998065.1), *Au*AAC (268, WP_043523659), *P. aeruginosa* PAO1 (269, NP_251075.1)
β270-β279	19.0 %	*Shewanella* sp. MIB015 (270, BAF94155.1), *S. oneidensis* MR-1 (270, NP_716547.1), *Streptomyces* sp. M664 (272, AAT68473.1), *Streptomyces* sp. FERM BP-5809 (272, BAD07025.1), *Sl*PVA (272, AAU09670.1), *Au*AHLA (274, WP_052163432), *R. solanacearum* GMI1000 (274, WP_011002462.1), *Ralstonia* sp. XJ12B (274, AAO41113.1), *S. mobaraensis* NBRC 13819 (276, BAF51977.1), *V. paradoxus* VAI-C (278, WP_013542868.1)
β280-β290	1.7 %	

* Phylogenetic study of acylases with high identity to *Sl*PVA, *Au*AAC and *Au*AHLA reported in Figure 58

Despite the fact that a truncated enzyme could be an abstract concept to understand, several studies with truncated enzymes have been developed, and even some of them are marketed nowadays by the main commercial companies (*e.g.* DNA polymerase, T4 RNA ligase 2, serine/threonine-protein kinase).

In this way, enzyme truncation has been detected in the N-terminal end, in the C-terminal end, or in both ends simultaneously (Ostermeier, M. *et al.* 1999a; Lutz, S. *et al.* 2001a; Lutz, S. *et al.* 2001b; Ostermeier, M. *et al.* 2002). Thus, following Sections will try to illustrate those scenarios with a few studies performed with some enzymes, but those Sections do not intend to delve into these issues, and those approaches are presented here in order to demonstrate that a truncated enzyme is not as strange as probably can be considered. Thus, it is speculated here that related bacterial enzymes may be amenable to similar engineered enhancements.

3.2.1. Deletion in the C-terminal end

Similar to the case of 2pva1,2(55), the deletion of a considerable segment in the carboxyl-terminus have been detected in other enzymes. For instance, more than 20 % of sequence of α 1,6-fucosyltransferase from *Rhizobium* sp. NG234R was deleted and its activity was not affected (Bastida, A. *et al.* 2002), as well as almost 23% of the keratanase-II sequence from *B. circulans* was deleted achieving improved activity and specificity (Steward, M. *et al.* 2015). Likewise, it is worth mentioning the modification carried out in rhoptry-associated protein 1 from *Babesia bigemina* (Boonchit, S. *et al.* 2006). This protein has a molecular weight of 84 kDa in contrast to 36 kDa of the recombinant clone (*i.e.* the size was reduced in almost 57 %), and it leads to a higher specificity.

Two interesting examples detected in humans are related with a 48-kDa human T-cell protein-tyrosinephosphatase, which is entirely specific for phosphotyrosine residues. In first instance, a truncated 37-kDa protein (*i.e.* missing 23 % in the C-terminal segment) altered the synchrony of mitosis (*i.e.* results in a form of cellular anarchy) (Cool, D. E. *et al.* 1992), as well as a 33-kDa protein displayed lower specific activity (Zander, N. F. *et al.* 1991).

Similarly, a truncated glucanase showed improved thermal stability and higher specific enzymatic activity than the wild-type after the reduction of its molecular weight in 10 kDa (Shyur, L. F. *et al.* 2006).

In order to understand this kind of manipulation, Li and co-workers established that a metalloprotease from tomato (*i.e.* 1-aminocyclopropane-1-carboxylic acid synthase) is active at multiple cleavage sites within the hypervariable C-terminus (Li, J.-F. *et al.* 2005).

However, most of these studies revealed slight changes in those sequences before the loss of enzyme activity. In particular, the performance of cathecol 2,3-dioxygenases from *Planococcus* sp. S5 was improved after the deletion of 18 aa (*e.g.* pH stability, a high substrate affinity, and reduced K_M) (Hupert-Kocurek, K. *et al.* 2014). Equally, several truncations have been performed on the C-terminal end of human lecithin (416 aa), but only a deletion of 18 aa did not affect the performance, despite the fact that further deletions (22 up to 111 aa) led to the loss of activity (Francone, O. L. *et al.* 1996).

3.2.2. Deletion in the N-terminal end

Although the recombinant clone 2pva1,2(55) truncated the C-terminal end, similar changes have also been detected in the N-terminal end. For instance, the amino-terminus in ethanolamine ammonia-lyase has been considered as not essential for catalysis. Based on this concept, a truncated enzyme with similar catalytic properties to the wild-type was designed, but this recombinant clone is highly soluble, resistant to aggregation and oxidative inactivation (Akita, K. *et al.* 2010).

Impressive truncation has also been detected in other enzymes, such as the deletion of 112-residues in glycogen branching enzyme. This explained that the truncation of the amino-terminal domain has altered the branching pattern of the enzyme (Binderup, K. *et al.* 2002). However, the most impactful case was probably detected in human xylosyltransferase 1. Müller and co-workers (Müller, S. *et al.* 2006) established that as many as 260 residues from 959 original residues could be truncated without affecting its catalytic activity, although further truncation caused drastic damages on its catalytic activity.

As mentioned above, the presence of truncated enzymes has been detected frequently as isoforms in Nature. For instance, an active N-terminal truncated cytochrome P450 was detected in human livers (Jeon, S. *et al.* 2008), and some isoforms displayed the typical spectral characteristics and functional properties with respect to its the full-length counterpart (Park, H.-G. *et al.* 2014).

The extent and advantages of the manipulation in the N-terminal end has promoted the generation of six theoretical CALB from *Candida antarctica*, which is one of the most studied and widely applied enzyme in the industry. One of those recombinant clones (*i.e.* deletion of 34 residues) is expressed in greater amount and displayed enhanced activity as well (Hughes, S. R. *et al.* 2012).

However, it is important to mention that sometimes those manipulations are not the best option. For instance, although sometimes the truncated enzyme is active, and probably with enhanced performance, occasionally its overexpression leads to a complete loss of activity (Thapar, N. *et al.* 2000).

3.2.3. Deletion in both ends

Finally, simultaneous deletions of residues located in both ends of the sequences have been studied scarcely, but the transcendence of these approaches is indisputable. In first instance, a recombinant clone of TEM β-lactamase truncated in both ends was distinguished. This recombinant enzyme increased its melting temperature, displayed significantly higher catalytic activity, whereas it also delayed the guanidine-induced denaturation. It is thus proven that iterative truncation-optimization cycles can exploit stability-trait linkages in proteins (Speck, J. *et al.* 2012). In fact, the deletion of fragments in both ends of the human HPTPβ caused little effect on substrate affinity, whereas further deletions in both extremes caused the drop of its activity. This supports the interpretation that N- and C-terminal ends can be defined without affecting the catalytic domain (Wang, Y. *et al.* 1992).

A similar finding shows that a truncated ADP-glucose pyrophosphorylase is highly active in absence of activators, and its performance is improved in their presence. In contrast, the deletion of only the N-terminal or the C-terminal ends displayed similar properties to the doubly truncated or the intact enzyme, respectively. It can be concluded that the amino-terminus is required for enzyme allosteric regulation (Wu, M.-X. *et al.* 2001).

3.3. Recombinant acylase 2pva1,2(149) of *Sl*PVA

On the other hand, 2pva1,2(149) has changed a nonpolar residue (69.0 % of prevalence in acylases) to a polar one (20.1 %) in the position α169. Although alanine is the most common residue in acylases (43.1 %) at this position (*i.e.* present in *Sl*PVA and *Au*AAC as well), this recombinant clone had the mutation αA169T in addition to a silent mutation. In this case, H_2(α169) has the lowest entropy value in comparison to the residues mutated here.

Although most acylases subjected to genomic manipulation have this residue on the surface (for further information, see Section 4.3 in this Chapter), it is important to mention three of those cases. First, αAla^{169} aligns with the superficial αIle^{201} in cephalosporin C acylase from *Pseudomonas* SE83, and only after several mutations (*i.e.* seven in total) αIle^{201} turned around to the interior of the folding of the enzyme (distinguished in cells with red borders in Supplement S.9 in the Supplementary Material Chapter) (Wang, Y. *et al.* 2012). A similar process happened after the deletion of flanking residues around the spacer peptide in *Pseudomonas* SE83 (for further information, see Section 4.3.4 in this Chapter) (Zhang, J. *et al.* 2014), as well as in αIle^{211} in penicillin G acylase from *Kluyvera citrophila* ATCC 21285 (for further information, see Section 4.3.1 in this Chapter) (Roa, A. *et al.* 1994).

The second case is the strategic location of αCys^{200} in cephalosporin C acylase from *Pseudomonas* N176, which is closest to the gate toward the catalytic pocket after the mutation (for further information, see Section 4.3.5 in this Chapter) (Ishii, Y. *et al.* 1995), and probably plays an important role in catalysis due to steric hindrance.

The final but no less important fact is related by the alignment with glutaryl acylase from *Pseudomonas* SY-77-1 (for further information, see Section 4.3.3 in this Chapter). Supplement S.9 in the Supplementary Material Chapter shows that αAla^{169} in *Sl*PVA could play a similar role to αAla^{151} in glutaryl acylase from *Pseudomonas* SY-77-1, which in turn is at two amino acids from αTyr^{149}. The importance of this residue stems from its location in the tunnel toward the catalytic pocket, and αTyr^{149} has been mutated two times in order to improve the enzyme performance in the catalysis of cephalosporin C (Sio, C. F. *et al.* 2002; López-Gallego, F. *et al.* 2008). The relative location of the residues within the catalytic pocket is similar in both enzymes according to the I-Tasser models. Unfortunately, due to current evidence and the lack of enzyme crystals, it could not be established that the tunnel in fact leads toward the catalytic pocket in *Sl*PVA. The only fact proven in this study was the importance of αAla^{169} in the catalytic performance of *Sl*PVA.

In the same way, as shown Supplement S.10 in the Supplementary Material Chapter, the interaction of the overall residues with the solvent was increased, since 1.2 % additional of total amount of amino acids are in contact with the solvent in a(292) in contrast to the parental enzyme, and only 0.5 % of them reduced the interaction on the surface.

3.4. Recombinant acylase a(292) of *Au*AAC

In this case, a(292) mantain a nonpolar residue on the protein surface (αI184F). The alignment of this residue displays that acylases from *Pseudomonas* sp. 130, SY-77-1 and *P. diminuta* KAC-1 do not have this amino acid, which in turn is in agreement with 17.2 % of the aligned acylases (for further information, see Supplement S.6 in the Supplementary Material Chapter). Likewise, in several cases analyzed in Supplement S.9 in the Supplementary Material Chapter, those residues aligned with αPhe^{184} were displaced in more than 2.5 Å in relation to βSer^{1}.

The particular location of αPhe^{184} at 12 residues to βSer^{1} demonstrates the relevance of amino acids located close to the linker peptide. The spacer peptide of *Au*AAC has 15 residues and the cleavage has been determined at first instance between αGly^{196}-βSer^{1}, and the second cleavage is located between αAla^{181}-αAla^{182} (Inokoshi, J. *et al.* 1992). This implies that the mutated residue is located at two residues of the second cleavage within the spacer peptide (it is highlighted in bold and italic letter in Table 28).

Several studies deal with spacer peptides in acylases, as well as their sequences, flanking residues and their length are depicted in Table 28. However, there is only some consensus among these sequences in acylases: most of them only show similarities within a certain genus or according to the closeness between the different genera. Unfortunately, a conclusion based on this information is unclear. Four cases are brought up in order to argue the possible roles performed by the residues located around βSer^{1}: (i) cephalosporin C acylase from *Pseudomonas* SE83, (ii) penicillin G acylase from *E. coli* ATCC 11105, (iii) acyl-homoserine lactone acylase from *P. aeruginosa* PAO1, and (iv) cephalosporin acylase from *Pseudomonas* sp. 130.

Table 28. Spacer peptides reported in several acylases

Enzyme	Flank*	Length	Spacer peptide	Reference
P. aeruginosa PAO1	-	0	–	(Wahjudi, M. *et al.* 2011)
Pseudomonas SE83	αA^{232}	7	SDAAGGG	(Zhang, J. *et al.* 2014)
Pseudomonas C427	αD^{190}	8	PPDLADQG	(Ishii, Y. *et al.* 1994)
Pseudomonas sp. GK16	αG^{169}	9	DPPDLADQG	(Kim, J. K. *et al.* 2006)
Pseudomonas sp. 130**	αE^{159}	10	GDPPDLADQG	(Li, Y. *et al.* 1999)
Pseudomonas SY-77-1	αE^{159}	10	GDPPDLADQG	(Sio, C. F. *et al.* 2002)
P. diminuta KAC-1	αG^{160}	11	EGDPPDLADQG	(Kim, Y. *et al.* 2002)
Thermus thermophilus HB27	αR^{246}	9	MAPPRFMEA	(Torres, L. *et al.* 2012)
A. utahensis NRRL 12052	αA^{181}	15	AA***I***AAA (...) TSAGIG	(Inokoshi, J. *et al.* 1992)
Arthrobacter viscous ATCC 15294	αS^{210}	31	SAVIKA (...) LPLKIG	(Konstantinović, M. *et al.* 1994)
Bacillus badius PGS10	αS^{212}	31	SAVIDA (...) LPLKIG	(Rajendhran, J. *et al.* 2007)
Alcaligenes faecalis ATCC 19018	αA^{228}	37	QAGTQD (...) DFAPKA	(Verhaert, R. M. *et al.* 1997)
K. citrophila ATCC 21285	αA^{209}	54	ALLVPR (...) AGYPTT	(Barbero, J. L. *et al.* 1986)
E. coli ATCC 11105***	αA^{209}	54	ALLPRY (...) AGYPTT	(Schumacher, G. *et al.* 1986)
Providencia rettgeri ATCC 31052	αS^{228}	56	QLNQIS (...) AGYPTT	(Klei, H. E. *et al.* 1995)
Ralstonia sp. XJ12B	αT^{197}	15	VALDRE (...) RDMPIG	(Zhang, L. H. *et al.* 2003)
Deinococcus radiodurans R1	αA^{168}	24	STAAPR (...) NDLPIG	(Koch, G. *et al.* 2014)
S. mobaraensis NBRC 13819	αR^{184}	28	TVGGGK (...) HDTGMG	(Zhang, D. *et al.* 2007)

* All residues are located within this amino acid in α-subunit and βSer^{1}

** Further studies demonstrates that the specific cleavage site is movable in the spacer range (Yin, J. *et al.* 2011)

*** An additional cleavage also forms a mature and active enzyme (Kasche, V. *et al.* 1987a; Kasche, V. *et al.* 1999b)

As can be seen above, the spacer peptide length in cephalosporin C acylase from *Pseudomonas* SE83 is integrated by 7 aa and it is located between αAla^{232} and βSer^{1} (Zhang, J. *et al.* 2014). The catalytic efficiency was enhanced in this study and no effects by deletions of flanking residues to the spacer peptide were detected on its maturation process, activity and stability. The first recombinant clone deleted αAla^{227} and αMet^{228}, and the second one αAla^{212}, αAsp^{213}, αLeu^{214} and αAla^{215}, all of them close to the spacer peptide. Additionally, several options of deletions around the second cleavage of the linker peptide were evaluated in this study. Similarly, cephalosporin acylase from *Pseudomonas* sp. GK16 releases a spacer peptide with 9 aa that is located between αGly^{169} and βSer^{1} (Kim, J. K. *et al.* 2006). This study also reported that some mutations close to the spacer peptide caused serious defects in the autocleavage. In particular, the modification in the mature sequence of the recombinant enzymes and their implications in the acylase performance were not explained, but probably the folding of the enzyme or the spacer peptide length was altered.

Similarly, extensive studies about autocleavage of the spacer peptide in penicillin G acylase from *E. coli* ATCC 11105 were carried out (Kasche, V. *et al.* 1987a; Kasche, V. *et al.* 1999b). The presence of two optional cleavages was detected, one of them yielding a dominant enzyme with a isoelectric point 7.0 after hydrolysis of αAla^{209}-αAla^{210}, in addition to an acylase with inferior isoelectric point (*i.e.* 6.7) obtained by hydrolysis of αPhe^{201}-αAsn^{202}, which slightly altered its kinetic properties. In this case, the length of the spacer peptide in the dominant enzyme is 55 aa, and after a "third" cleavage it produces the release of 8 additional residues (*i.e.* 62 aa in total).

Surprisingly no spacer peptide has been reported in the acyl-homoserine lactone acylase from *P. aeruginosa* PAO1 (Wahjudi, M. *et al.* 2011), which converted this enzyme in the only Ntn-hydrolase without this linker. It is important to remark that the spacer peptide connects both subunits, and by an autocatalytic mechanism leads to the formation of a free and catalytically active N-terminal (Oinonen, C. *et al.* 2000).

The final case is the most curious among the previous examples. Although cephalosporin acylase from *Pseudomonas* sp. 130 has a defined spacer peptide of 10 aa (Li, Y. *et al.* 1999), the specific cleavage site has been reported as movable in the spacer range. This finding reveals that the spacer sequence may contribute to the hydrolytic activity (Yin, J. *et al.* 2011). Nevertheless, it is necessary to recognize that the spacer peptide completely blocks the active site (Chand, D. *et al.* 2015).

Additionally, several studies highlight the importance of βSer^{1} in the catalysis of substrates and during the autocleavage in acylases (Brannigan, J. A. *et al.* 1995; Mao, X. *et al.* 2004; Yin, J. *et al.* 2011; Varshney, N. K. *et al.* 2013; Chand, D. *et al.* 2015). This in turn is also a well-known mechanism in the autocatalytic processes of other enzymes (Little, J. W. 1993; Bron, S. *et al.* 1998; Guan, C. *et al.* 1998; Lee, H. *et al.* 2000). However, the participation of different residues during the autocatalytic mechanism is recognized nowadays. For instance, the motif integrated by βSer^{1}, βHis^{23} and βGlu^{455} in cephalosporin C acylase from *Pseudomonas* sp. 130 was postulated as crucially important for efficient autoproteolysis (Mao, X. *et al.* 2004), whereas βSer^{1}, αLys^{168} and βLys^{214} might have a role in the spacer peptide cleavage in penicillin G acylase from *K. citrophila* ATCC 21285 (Chand, D. *et al.* 2015), or βSer^{1} and αLys^{10} play this role in penicillin G acylase from *E. coli* ATCC 11105 by (Lee, H. *et al.* 2000). Thus, the randomness and lack of consensus about this topic is in opposition to the well-conserved amino acids involved in catalysis,

which is presumably due to the difference among the spacer peptides among acylases (Table 28).

In this sense, further studies should be employed in order to clarify this aspect, but it is not discarded here that the mutation present in a(292) probably affected the spacer peptide length (*i.e.* the alteration of the second cleavage), or could have modified the folding of the enzyme during the maturation.

4. IMPROVEMENT OF ENZYME PERFORMANCES BASED ON THERMODYNAMICS

The relevance of entropy among other thermodynamic parameters in the protein performance has been highlighted in several studies. The first approach was carried out almost 40 years ago describing important contributions of free energy, enthalpy and entropy rather than using only the concept of Arrhenius activation energy (Low, P. S. *et al.* 1973). Thereafter, accurate predictions of free energies, enthalpies and entropies in enzymes have become an invaluable tool to understand their biocatalytic activity and stability, and their interconnections and relationships as well. For this reason, computer simulations in connection with experimental data have started to fill this hole of knowledge in enzymology.

4.1. Entropy effect

The first study of directed evolutions who tried to explain thermostability and flexibility by entropy arguments was carried out by mutations in a loop of subtilisin E, which in turn was located on the surface of the protein (Zhao, H. *et al.* 1999).

Thereafter, the connection between thermal stabilization and directed mutagenesis was explained, either (i) with the increasing thermodynamic stability of a protein (*i.e.* increase free-energy difference between the unfolded and the folded state), or (ii) because these mutations decrease the rate of unfolding (*i.e.* increasing free energy difference between the folded state and the transition state of unfolding) (Lehmann, M. *et al.* 2001). Although this fact was argued and widely exemplified (Steipe, B. 1999), this concept was originally linked to the introduction of a disulfide bridge, which means that the concept and its implication are appropriate just in a limited number of cases, specifically where the introduction of additional disulfide bridges takes place.

Similarly, the role of the mutation X→Pro to reduce the entropy of the unfolded state was explained in several studies. The first theoretic report about this effect was carried out based on the side-chains of amino acid for the α-helix-to-random coil transitions (Némethy, G. *et al.* 1966). This concept was successfully applied to T4 lysozyme (Matthews, B. W. *et al.* 1987; Nicholson, H. *et al.* 1992). Initially it was considered that the combination of several mutations that improve the stability could be additive and should be a strategy to be employed (Matthews, B. W. *et al.* 1987). However, the numbers of sites seems to be limited in phage T4 lysozyme, which is probably influenced by the solvent accessibility (Nicholson, H. *et al.* 1992). Similar mutations of residues have been carried out on the surface of subtilisin S41 from *Bacillus* TA41, which clarified that this widespread loss of conformational entropy is probably offset by the increase in stability that takes place due to (i) buried hydrophobic residues, (ii) the creation of new hydrogen bonds and (iii) the restriction of solvent accessibility (Arnold, F. H. *et al.* 2001).

In contrast to the methodology of calculating the entropy proposed by Sahnnon (and employed here) (Shannon, C. E. 1948), Villà and co-workers debated that the value of entropic effects in biocatalysis has been frequently deduced from the trend in the rates of intramolecular cyclization reaction (Villà, J. *et al.* 2000). Thus, entropic contributions to accelerate enzymatic reactions were smaller than previous estimations, and they considered that electrostatic effects were the major factor in enzyme catalysis (Russell, A. J. *et al.* 1987). As the reactants were free at the transition state, many movements were originally free. The binding to the enzyme did not completely freeze the movement of the reacting fragments. Thus, their entropic contribution is eliminated and the binding entropy is not necessarily equal to the activation entropy. Whereas this concept was widely accepted and verified, it is important to remark that it was created to explain the catalysis of enzymes but not their stability, which obviously could affect the protein performance. Sometimes the tradeoff among activity and stability is difficult to predict and obtain simultaneously, but it does not mean the impossibility to reach this prospect, and the balance of both aspects is the real challenge in directed evolution (Feng, S. *et al.* 2007; Couñago, R. *et al.* 2008; Studer, R. A. *et al.* 2014; Niu, C. *et al.* 2015).

Several concepts and theories to improve enzyme stability have been described in literature (Iyer, P. V. *et al.* 2008), and some of them have illustrated the implications of entropic mechanisms of enzymes by bioinformatic methodologies (Bae, E. *et al.* 2008; Moon, S. *et al.* 2014) as well as its immediate proteomic implications (Berezovsky, I. N. *et al.* 2005). In fact, bioinformatic approaches carried out by local structural entropy (*i.e.* extent of conformational heterogeneity) have been employed to introduce mutations with fewer conformational states (*i.e.* more stable structure), but the limitations of this process are likely due to the ignorance of other interactions that bridge distant regions in a given sequence (Moon, S. *et al.* 2014)

For instance, mutations in orthologous proteins from thermophile and mesophile, contribute notwithstanding to a decrease of vibrational entropy (Frappier, V. *et al.* 2015). This positive correlation between the abundance of amino acids and the difference of vibrational entropy implies that residues that increase rigidity are detected more often in enzymes of thermophiles. Those recognized by increase flexibility are frequently found in proteins of mesophiles. Likewise, computer simulations have demonstrated that the “softness” of the protein-solvent surface regulates the activation of the enthalpy-entropy balance. Similarly, there is no doubt that punctual mutations are able to affect thermodynamic activation parameters significantly (Isaksen, G. V. *et al.* 2014), as well as quite the reverse knowledge about those parameters, and their implications to predict the interaction of protein with other molecules is a challenge (Estrada, J. *et al.* 2015).

4.2. Surface effect

Regarding the protein surface, several studies have focused around the role of the protein surface and its influence in enzyme performance, mainly in stability and activity fields. The rules postulated originally in order to improve enzyme stability based on modifications on their surface were: (i) negative or positive charges on the protein surface could increase or reduce pK_a values of the groups, respectively; (ii) maximize the changes of pH-activity at low ionic strength; (iii) significant changes will be manifested at ionic strengths as high as 0.1 M if multiple-charged counterions are avoided; and (iv) the modifications should avoid concentrations of counterions in the active site cavity (Russell, A. J. *et al.* 1987).

Based on the surface of subtilisin (model system), these implications were formulated for electrostatic interactions as cumulative, dielectric constant, counterion binding and kinetic constants. However, only a couple years later the contribution to proteins stability was refuted by exposed residues on the enzymes surface (Šali, D. *et al.* 1991).

Both trends have been debated for a long time, but nowadays it is undoubted that small changes in sequence influence the overall protein structure, regardless the location of the mutation, to the extent that has been postulated that single mutations are able to affect thermodynamic activation parameters significantly (Isaksen, G. V. *et al.* 2014). In particular, this theoretical concept was adapted to engineer acylases through mutations of superficial residues by six rules: (i) mutate basic residues outside of fatal structure elements (*i.e.* α-helix, β-sheet and β-turn); (ii) mutate non-conservative amino acids; (iii) mutate residues with only a few interactions (*i.e.* no salt bridge and less than 3 hydrogen bonds to other amino acids); (iv) change amino acids far away from enzyme active site; (v) change basic by neutral amino acids to enhance stability in non-aqueous medium; and (vi) change amino acids to smaller ones, to minimize conformation (Yang, S. et al. 2002).

To sum up, several factors could be altered by specific mutations, such as electrostatic interactions, structure elements (*i.e.* α-helix, β-sheet, β-turn and random coils), side-chain properties of residue (*i.e.* polar, nonpolar, basic and acidic), length of the side-chain, hydrogen bond (Fersht, A. R. *et al.* 1993), disulfide bond (Matsumura, M. *et al.* 1989; Fersht, A. R. *et al.* 1993), anisotropic interactions (Quang, L. J. *et al.* 2014; Lund, M. 2016), presence of counterion (Russell, A. J. *et al.* 1987; Horovitz, A. *et al.* 1990), as well as free energy of ionizable groups (Yang, A.-S. *et al.* 1993). In fact, the extent of punctual modifications could affect buried interactions, which have been described as important factors for the stability (Waldburger, C. D. *et al.* 1995; Sindelar, C. V. *et al.* 1998; Kajander, T. *et al.* 2000), and activity of an enzyme (Sindelar, C. V. *et al.* 1998).

Undoubtedly, sometimes achieving improvements simultaneously in both activity and stability is a challenge in protein engineering as well as during natural evolution, because the trend for conserving residues is stronger in relation to activity, but not to stability (Schreiber, G. *et al.* 1994). Several strategies to overcome drawbacks in protein engineering have been postulated in order to avoid early dead-ends (Dalby, P. A. 2011), However, the coordination across the bioinformatic, theoretical and experimental field still presents a challenging task. The objective is to mimic Nature, which in turn with its multitude of complex functions, has been far more successful (Socha, R. D. *et al.* 2013).

In the next Section a comparative study of mutant acylases related to *Sl*PVA and *Au*AAC have been addressed to analyze the role of thermodynamic parameters and surface in catalysis.

4.3. Comparative study of recombinant acylases similar to *Sl*PVA and *Au*AAC

As mentioned above, the main objective in directed evolution is to obtain enzymes with improved or new properties, including enhancement or modification of activity, stability or substrate specificity, or their combinations. The approach of this technique can be carried out by several methodologies. Thus, many studies have been carried out to improve performance, activity or stability in acylases by directed evolution or side-directed mutagenesis, such as in the cases of penicillin G acylase from *K. citrophila* ATCC 21285 (Martín, J. *et al.* 1990; Roa, A. *et al.* 1994) and *E. coli* ATCC 11105 (del Río, G. *et al.*

1995; Lee, H. *et al.* 2000; Morillas, M. *et al.* 2003; Balci, H. *et al.* 2014), as well as performed with glutaryl acylase from *Pseudomonas* SY-77-1 (Otten, L. G. *et al.* 2002; Sio, C. F. *et al.* 2002; López-Gallego, F. *et al.* 2008), and cephalosporin C acylases from *Pseudomonas* SE83 (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012; Xiao, Y. *et al.* 2014; Zhang, J. *et al.* 2014), *Pseudomonas* N176 (Ishii, Y. *et al.* 1995; Pollegioni, L. *et al.* 2005; Golden, E. *et al.* 2013; Conti, G. *et al.* 2014) and *P. diminuta* KAC-1 (Mei, T. *et al.* 2015). Thus, the comparison with other studies reveals more insight (for further information, see Supplement S.9 in the Supplementary Material Chapter). Those studies were performed with acylases located among the most similar enzymes to *Sl*PVA and *Au*AAC (Fig. 58). Similarly, the improved enzymes obtained in other studies have been contrasted with *Sl*PVA and *Au*AAC.

4.3.1. Penicillin G acylase from *Kluyvera citrophila* ATCC 21285

It is remarkable that these mutants obtained from this acylase have mutations only in buried residues (*i.e.* far away from the catalytic pocket as well as from the surface, which avoids interaction with substrate or solvent). Penicillin G acylase from *K. citrophila* ATCC 21285 (Barbero, J. L. *et al.* 1986) was the first acylase subjected to side-directed mutagenesis (Martín, J. *et al.* 1990). This caused the modification of the substrate specificity and enhanced the thermostability through the mutation αM142A. In fact, this mutation was detected in a non-conserved residue in acylases (e.g, aligned with αArg^{124} in *Sl*PVA), as well as this amino acid lacks in almost one fourth of them, such as in the case of *Au*AAC. This fact is corroborated with entropy values at this position (*i.e.* $H_1(\alpha 124)=2.357$ and $H_2(\alpha 124)=1.509$), which is the highest entropy value among the residues compared here according to their side-chain properties (selection and analysis of residues considered those located in the catalytic pocket, forming substrate binding pocket or mutated in related studies). It is important to remember that although both residues are nonpolar, the size difference between methionine and alanine is considerable, and the presence of a sulfide group might alter the spatial disposition inside of the enzyme. This mutation has caused that 0.4 % of residues are buried, in addition to the original amino acids inside of the parental enzyme, and reduced the interaction of the residues located on surface with the solvent by 1.6 % (for further information, see Supplement S.10 in the Supplementary Material Chapter). Two of those residues are αThr^{150} and βPhe^{71}. The first of them in turn aligns with αGly^{140} in cephalosporin C acylase from *Pseudomonas SE83* (for further information, see Section 4.3.4 in this Chapter), which was modified simultaneously with other residues by directed evolution (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012). The second one aligned with βPhe^{71} in penicillin G acylase from *E. coli* ATCC 11105 (for further information, see Section 4.3.2 in this Chapter), which was mutated by site-directed mutagenesis (Morillas, M. *et al.* 2003). It is important to point out that these mutations improved the specific activities in both cases.

Later, the first study of random mutations in acylases reported in Spain (Roa, A. *et al.* 1994) was carried out by a mutator strain (Greener, A. *et al.* 1997). In this study, the substrate specificity was modified by βF78V mutation in a buried residue (*i.e.* βHis^{79} in *Sl*PVA and *Au*AAC, and it is 4.0 % of the time in acylases). Despite the fact that this residue is apparently conserved (*i.e.* tyrosine 68.4 % versus phenylalanine 23.6 %) and the mutation kept a nonpolar amino acid, it is remarkable that valine is so far an uncommon residue there and the size and anisotropy effect is quite different between both amino acids. Likewise, βF78V altered the enzyme structure considerably in contrast to αM142A (red letters in Supplement S.9 in the Supplementary Material Chapter). This mutation increases

the interaction with the solvent by 3.2 %, whereas it buries the same amount of residues exposed in αM142A (one of them aligned with αThr[169] and mutated in 2pva1,2(149)). It is worth mentioning that βHis[79] in *Sl*PVA is highly conserved according to the entropy value (*i.e.* lowest value among residues analyzed in Supplement S.9 in the Supplementary Material Chapter), even more than some predicted residues forming the substrate binding pocket (*i.e.* β24, β32, β50, β57, β58, β68 and β186, according to *Sl*PVA) or within the catalytic residues (*i.e.* βVal[70], according to *Sl*PVA) (Table 20).

4.3.2. Penicillin G acylase from *Escherichia coli* ATCC 11105

Penicillin G acylase from *E. coli* ATCC 11105 (Oh, S. J. *et al.* 1987) has also been mutated in several studies. The first approach has enhanced the stability at alkaline pH by the mutation βW431R in a predicted buried residue (del Río, G. *et al.* 1995), which could be attributed to the basicity of arginine instead of the nonpolar characteristic of tryptophan, as well as to the higher anisotropic effects of tryptophan. In addition, this residue is highly conserved according to entropy values, even more than some residues predicted as amino acids forming the substrate binding pocket (*e.g.* β57, β68, and β186, according to *Sl*PVA) or within the catalytic residues (*i.e.* βVal[70], according to *Sl*PVA). Similarly, the necessity of a basic residue at the superficial position β10 in this acylase has been determined (Lee, H. *et al.* 2000). The presence of lysine or arginine displayed similar performance, but it is improved if the residue is histidine; also, a polar residue there practically makes the enzyme activity disappear, which is in agreement with most of the acylases but not with *Sl*PVA, *Au*AAC and *Au*AHLA (*i.e.* βAla[10], βThr[10] and βGly[10], respectively).

Thereafter, two important recombinant clones have been obtained by directed molecular evolution (Morillas, M. *et al.* 2003). The mutation βF71C (*i.e.* change a nonpolar by a polar residue) and βF71L (*i.e.* keep a nonpolar residue) have increased in both cases k_{cat}/K_M with respect to the parental enzyme. The residue β71 is on the protein surface and within the tunnel toward the catalytic pocket, which could play a role in catalysis like a shutoff valve (similar situation is described in Section 4.3.3 in this Chapter). This mutation probably has decreased steric hindrance for substrate binding, which implies a decrease in conformational entropy in the bound substrate, although there is no direct contact between the residue and the molecule. Supplement S.6 in the Supplementary Material Chapter shows that the most common residue there is threonine (more than half of acylases have a polar residue there, and *Sl*PVA, *Au*AAC and *Au*AHLA are among them), although more than one third have nonpolar residues at this position in acylases. This fact is appreciated considering the low entropy values according to side-chain properties. Surprisingly, this is the only acylase with cysteine at this position among those enzymes aligned here. It highlights that a polar residue there has altered the relative coordinates drastically (*i.e.* more than 2.5 Å) in 78.1 % of those residues established as crucial in the protein performance, whereas the presence of a nonpolar residue has affected just 15.6 % of them (*i.e.* α221> β471>α222> β241>α234).

Finally, activity improvement and the stability of this acylase were achieved by error-prone PCR (Balci, H. *et al.* 2014). Thus, several silent mutations in addition to βK8I were detected in this recombinant clone. It was determined that the change of a basic by a nonpolar residue on the surface propitiates an improvement of the activity and the pH stability. Although this recombinant clone enhanced the activity 4-fold and displayed stability at a higher pH value with respect to the parent enzyme, both enzymes showed similar substrate specificity under standard assay conditions. This fact is important because

the potential to catalyze a wide spectrum of substrates is not necessarily altered, whereas improvements in its performance were achieved. In fact, nonpolar residues is the most common residue at this position among acylases (*i.e.* 47.1 %), although no consensus was detected among *Sl*PVA, *Au*AAC and *Au*AHLA (*i.e.* βGly^{8}, βAla^{8} and βArg^{8}, respectively). Curiously, in contrast to previous studies with this enzyme (del Río, G. *et al.* 1995; Lee, H. *et al.* 2000), pH stability was improved with a polar residue instead of a basic amino acid on the surface, which in fact was the original amino acid there. In contrast to studies carried out with other acylases, almost all the crucial residues that were detected in the α-subunit were displaced more than 2.5 Å with respect to βSer^{1} in this acylase (for further information, see Supplement S.9 in the Supplementary Material Chapter). Supplement S.10 in the Supplementary Material Chapter indicates that these alterations were achieved through βK8I, βF71C, and that βW431R reduced the residue interactions on the surface with the solvent considerably, whereas βF71C practically was not altered on the enzyme surface (*i.e.* this is the recombinant clone that displayed lower effect on the surface among those analyzed here).

4.3.3. Glutaryl acylase from *Pseudomonas* SY-77-1

Studies of directed evolution of glutaryl acylase from *Pseudomonas* SY-77-1 have determined that the mutation αY149H (*i.e.* change a polar by a basic residue, both of them on surface and close to the gate toward the catalytic site) improved the catalytic efficiency 3-fold (Sio, C. F. *et al.* 2002). Likewise, those mutations in the residues within the catalytic pocket, βN68H (*i.e.* change a polar by a basic residue) and βF177L (*i.e.* keep a nonpolar residue), have altered the substrate specificity of the enzyme (Otten, L. G. *et al.* 2002). In particular, the recombinant clone with the mutation βN68H has improved the catalytic efficiency 10-fold (k_{cat}/K_M) on adipyl-7-ADCA without decreasing the catalytic efficiency on glutaryl-7-ACA. Finally, a double mutant (*i.e.* αY149F/βF177H) has achieved a recombinant clone that increased the catalytic efficiency 6.5-fold and K_M decreased 3-fold (López-Gallego, F. *et al.* 2008).

It highlights that some of those recombinant clones (*i.e.* βN68H and βF177L) conserve a similar amino acids nature to *Au*AAC and *Sl*PVA (*i.e.* histidine and valine, respectively). Likewise, the positive control employed by Otten and co-workers (*i.e.* recombinant clone αY149H previously obtained) does not have consensus in this residue with *Sl*PVA (αThr^{167}), *Au*AAC (αVal^{162}), neither with their wild-type nor with their recombinant clones. The alteration of the relative coordinates alteration with the mutation αY149F reduces its distance in 4.0 Å, in addition to some slight variations in binding pocket residues (for further information, see Supplement S.6 in the Supplementary Material Chapter). Curiously, βAsn^{68} alters drastically its location within the catalytic pocket as well as the spatial disposition of other residues within it. In addition, this mutation modifies the orientation βGly^{75} (for further information, see Supplement S.9 in the Supplementary Material Chapter). This residue aligns with βThr^{75} in cephalosporin C acylase from *Pseudomonas* SE83, which in turn has been mutated to improve its performance (a similar situation is described in Section 4.3.4 in this Chapter) (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012). The alteration of the interaction with the solvent of those residues on the surface is not so obvious, presumably due to their location (*i.e.* binding pocket and the tunnel toward it). The mutation βF177L also alters the catalytic pocket location considerably. Probably, the place of βAsn^{68} and βPhe^{177} play a crucial role in substrate specificity because both amino acids are located within the catalytic pocket. Thereafter, double mutant αY149F/βF177H slightly changes the relative coordinates of the residues analyzed here,

and this recombinant clone could be deducted based on previous studies carried out by the same research group (López-Gallego, F. *et al.* 2008).

It is remarkable that the alignment with αTyr^{149} from SY-77-1 displayed a polar residue (*i.e.* αThr^{167}) in *Sl*PVA and a nonpolar residue in *Au*AAC and *Au*AHLA (*i.e.* αVal^{162} and αVal^{170}, respectively), which in fact is the most common kind of residue among acylases (*i.e.* 59.8 % of nonpolar versus 25.3 % of polar amino acids). Similarly, βHis^{68} is a conserved residue in *Sl*PVA, *Au*AAC and *Au*AHLA as well as in recombinant clones of *Pseudomonas* SY-77-1. In the same sense, the residues aligned with α149 in *Sl*PVA, *Au*AAC and *Au*AHLA are detected on the surface of the first two, but it is buried in the last one, whereas β68 and β177 were located within the catalytic pocket, just as detected in the strain SY-77-1 and its recombinant clones. According to the thermodynamic parameters of these positions, it is important to mention the relative low values of entropies related with side-chain properties of residue (*i.e.* nature conserved), despite the fact that the residues aligned with α149 are distributed among 19 of 20 possible amino acids.

Supplement S.9 in the Supplementary Material Chapter shows that the studies related with this strain have displaced more than 2.5 Å only some amino acids located in the tunnel toward the catalytic pocket (*i.e.* α149) or within it (*i.e.* β50, β57 and β68). In contrast, other studies altered several residues (*i.e.* at least, this explanation is adjusted to the relative coordinates of the residues described here, which have demonstrated a direct implication in the performance of enzymes).

Usually the aim of directed molecular evolution studies is to achieve enzymes with improved activities or stabilities, or both (Arnold, F. H. *et al.* 2001). However, sometimes the interest is biased to obtain different performances, such as substrate specificity, which should be understood as enantiomeric selectivity of enzymes or new specificity, where the last one remains a major challenge (Zhao, H. *et al.* 2002).

4.3.4. Cephalosporin C acylase from *Pseudomonas* SE83

In particular, the mutation of βHis^{57} within its binding pocket (*i.e.* position β57 in *Sl*PVA, *Au*AAC and *Au*AHLA) increased specific activity whereas the enzyme surface was not affected considerably (for further information, see Supplement S.10 in the Supplementary Material Chapter) (Xiao, Y. *et al.* 2014). It is noteworthy that this recombinant clone was displaced by more than 2.5 Å in several residues, including βAla^{57}, βArg^{24} (data not shown) and βHis^{70} within the binding pocket, which in turn was mutated later to obtain the double mutant βH57A/βH70Y (Xiao, Y. *et al.* 2014) and displays increased specific activity with no substrate inhibition nor important modification on the surface. In this case, the presence of both mutations caused the displacement of βPhe^{58} and βArg^{24} in more than 2.5 Å (data not shown) within the catalytic pocket, and no significant effect on the surface was detected.

Likewise, an additional interesting mutant obtained by Xiao and co-workers (Xiao, Y. *et al.* 2014) (*i.e.* βH57A/βH70Y/βI176N) showed a k_{cat} 3.26-fold and a product inhibition constant (K_{IP}) 3.08-fold of the wild type. The mutation βI176N is of particular interest here, because this amino acid is buried in the enzyme, in addition that curiously this residue is absent in *K. citrophila* ATCC 21285 (Barbero, J. L. *et al.* 1986) and *E. coli* ATCC 11105 (Oh, S. J. *et al.* 1987), and in more than one fourth of acylases as well (for further information, see Supplement S.9 in the Supplementary Material Chapter). Despite

this fact, the low entropy value of H_2(β183) demonstrates the bias to nonpolar residues or their absence of this one (for further information, see Supplement S.6 in the Supplementary Material Chapter), which might explain the importance of the surface in this recombinant clone (for further information, see Supplement S.10 in the Supplementary Material Chapter).

Similarly, an earlier study carried out with native cephalosporin C acylase obtained a recombinant clone with increased specific activity and K_{IP} after the introduction of several mutations throughout the sequence (*i.e.* αV122A/αG140S/βF58N/βI75T/βI176V/βS471C) (Shin, Y. C. *et al.* 2009). Thus, there are three important facts in this study. Firstly, one of those modifications was performed in αGly^{140}, which is absent in *Au*AAC. The second aspect is the simultaneous mutation of six amino acids, two of them on the protein surface, one within the catalytic pocket and the rest in the residues buried inside of the enzyme. This consideration stands out in comparison with other studies, because these modifications were performed in residues buried inside the biomolecule.. Presumably, however, the real effect was due to the overall alteration of the sequence rather than specific mutations. Further studies will be needed to clear up this condition. The last important fact is the highly conserved side-chain properties of β58 among acylases (*i.e.* lowest entropy value among residues analyzed in Supplement S.9 and S.6 in the Supplementary Material Chapter), even more than some residues involved in the substrate binding pocket (*i.e.* β24, β32, β57, β68 and β186, according to *Sl*PVA) and within the catalytic residues (*i.e.* βVal^{70}, according to *Sl*PVA) (for further information, see Table 20).

Thereafter, this mutant clone with multiple mutations was considered as a template to obtain a new acylase with an additional mutation located within the substrate transport tunnel (*i.e.* βGly^{436}) (Wang, Y. *et al.* 2012). This residue was catalogued as important in the enzyme performance and probably influences the binding and anchoring of substrate by steric hindrance (Kim, S. *et al.* 2001). This recombinant acylase increased once more specific activity, and reduced even more the predicted solvent accessibility even more. Both studies altered the orientation toward the solvent of βIle^{75} (which in turn was mutated), and makes them the only studies with different orientations in one mutation.

Even though the deletion of flanking residues around the spacer peptide enhanced the catalytic efficiency and the maturation process, the activity and stability of enzyme were not affected (for further information, see Section 3.4 in this Chapter) (Zhang, J. *et al.* 2014). It is important to mention that all these studies carried out with acylase from this strain affected the crucial residues located in α-subunit considerably (at least those reported up to now, and listed in Supplement S.9 in the Supplementary Material Chapter), and most of them were displaced considerably with respect to βSer^{1}. In addition, the alterations of the relative coordinates of the residues involved in the binding pocket in those studies were appreciable (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012; Xiao, Y. *et al.* 2014). At least once most of these residues were displaced by more than 2.5 Å with respect to βSer^{1}. Thus, βHis^{23} and βHis^{178} were affected once, βHis^{57} and βHis^{70} two times, βArg^{24} four times and βPhe^{58} five times (data not shown).

4.3.5. Cephalosporin C acylase from *Pseudomonas* N176

On the other hand, cephalosporin C acylase from *Pseudomonas* N176 (Aramori, I. *et al.* 1991) was mutated in first instance by Ishii and co-workers (Ishii, Y. *et al.* 1995). The mutation βM31F on the surface (keeps a nonpolar residue, which in turn is the most

abundant among acylases) increased specific activity against cephalosporin C. This mutation occasioned modifications throughout the sequence and the relative coordinates within the binding pocket was altered by more than 2.5 Å in some residues (*i.e.* β57, β70 and β242), and some of them were modified later in related studies (for further information, see Supplement S.9 in the Supplementary Material Chapter). This recombinant clone was considered as a template to obtain a "double mutant" with increased activity (βM31F/βH57S/βH70S) (Pollegioni, L. *et al.* 2005), which was interpreted through the crystal structures, both as a parental and a recombinant clone (Golden, E. *et al.* 2013). The "double mutant" changed basic residues to a polar residue in both positions within the catalytic pocket despite the fact that most of the acylases have nonpolar amino acids there. Also, this "double mutant" caused that βSer^{70} and βAsn^{242} increase their distances to βSer^{1} (reduced the difference caused previously) but keeps closer βPhe^{58} to βSer^{1} within the catalytic pocket (*i.e.* displaced in more than 2.5 Å); further modifications maintained these amino acids outside and inside of its original locations, respectively. It is remarkable that the mutations of those residues within the binding pocket (*i.e.* β57 and β70 in *Sl*PVA) were mutated later in cephalosporin C acylase from *Pseudomonas* SE83 (Xiao, Y. *et al.* 2014), just as exposed above (a similar situation is described in Section 4.3.4 in this Chapter).

Similarly, a recombinant clone named "triple mutant" was obtained by error-prone PCR (four mutations in total, αA215Y/βM31F/βH57S/βH70S) with higher V_{max} value and increased kinetic efficiency on cephalosporin C (Pollegioni, L. *et al.* 2005). The additional mutation was detected in a superficial residue with a relatively high entropy value (*i.e.* $H_1(\alpha 187)=2.413$ and $H_2(\alpha 187)=1.431$). It is important to mention that this is the only acylase with a tyrosine at this position, whereas alanine is the most common one. Likewise, one fifth of the time the amino acid nature is polar, versus 44.3 % are nonpolar and 15.5 % lack this residue. This extra mutation caused that βIle^{75} changed its orientation with respect to the solvent. It highlights that this residue was aligned with βThr^{75} in cephalosporin C acylase from *Pseudomonas* SE83, which was mutated by directed evolution (similar situation is described in Section 4.3.4 in this Chapter) (Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012). Additionally, the sequence modifications performed after Ishii and co-workers caused that αCys^{200} gets away from βSer^{1} and reduced steric hindrance within the tunnel toward the catalytic pocket, just as mentioned in Section 4.3.3 in this Chapter.

In addition, the recombinant clone αM165S/βM31F/βH57S/βH70S exhibited higher activity on both cephalosporin C and glutaryl-7-ACA than the "double mutant" and structural details suggest that this modification avoids steric hindrance to cephalosporin C (Golden, E. *et al.* 2013). Again, in this case the alteration of amino acid nature was performed from nonpolar to polar and it was kept buried within the enzyme but the distance to βSer^{1} was increased by 3.1 Å. Once more it is important to consider the overall change of structure instead of punctual mutations.

Likewise, a recombinant clone βM31F/βH57S/βH70S/βL154Y improved the conversion of cephalosporin C into 7-ACA under conditions resembling those used on an industrial level (Conti, G. *et al.* 2014). According to the authors, this variant may be suitable for industrial applications of the mono-step process for cephalosporin C conversion. Once more, the residue mutates a nonpolar to a polar residue and βTyr^{154} was moved far away with respect to βSer^{1} (*i.e* 70.7 % of the time this amino acid is nonpolar in acylases) (Golden, E. *et al.* 2013),.

4.3.6. Cephalosporin C acylase from *Pseudomonas* sp. 130

A similar study was performed with cephalosporin acylase from *Pseudomonas* sp. 130 (Yang, Y. L. *et al.* 1991). A recombinant clone (αY149F) was obtained that shows 2-fold the catalytic efficiency of the parental enzyme, as well as two additional recombinant clones (βQ50N and βQ50N/βK198A) with 3-fold the catalytic efficiency of the parental enzyme (Zhang, W. *et al.* 2005). In addition, these mutants have 50 % of the parental K_M. Worth noting is the discussion about the importance of the residues located on the enzyme surface and their implications in surface charge, which in turn affect the isoelectric point of the protein and its stability. This aspect was contrasted with the first approach carried out with penicillin G acylase from *Bacillus megaterium* (Yang, S. et al. 2002) by combining the known functional and structural properties of penicillin G acylase from *E. coli*. Despite the fact that the initial discussion was developed almost three decades ago (Russell, A. J. *et al.* 1987), unfortunately no obvious stability enhancement or improvement was obtained.

It is important to mention that this acylase could be processed to an active enzyme without signal peptide (Li, Y. *et al.* 1998), demonstrating that the lack of this section of sequence had no influence on the folding and maturation of this acylase, and that the N-terminal end of the α-subunit was not important to enzyme activity (Li, Y. *et al.* 1999). Curiously, those studies performed with this enzyme reduced the solvent accessibility after the mutations.

4.3.7. Cephalosporin C acylase from *Pseudomonas diminuta* KAC-1

A recombinant clone with three mutations throughout the sequence (*i.e.* αY151W/βQ50G/βF177Y) was obtained by saturation mutagenesis, and this is the most recent study performed with acylases in this field (Mei, T. *et al.* 2015). However, the relevance of these positions in this enzyme were possibly concluded based on related studies with other acylases from this genus (Fig. 58). Thus, the alignment showed that αTyr^{151} and βPhe^{177} in *P. diminuta* KAC-1 corresponds with αHis^{149} and βPhe^{177} in glutaryl acylase from *Pseudomonas* SY-77-1, which have been mutated to αTyr^{149} (Sio, C. F. *et al.* 2002) and βLeu^{177} (Otten, L. G. *et al.* 2002) to improve the catalytic efficiency toward cephalosporin C (for further information, see Section 4.3.3 in this Chapter). In addition, previous studies have established that the position β50 integrates the catalytic pocket in several acylases (*i.e.* βGln^{50} in cephalosporin acylase from *P. diminuta* KAC-1) (Zhang, D. *et al.* 2007; Hormigo, D. 2009; Torres-Bacete, J. *et al.* 2015), as well as the mutation βT149F have already been performed (*i.e.* βY151W carried out by Mei and co-workers) (Zhang, D. *et al.* 2007).

Summarizing, the selection of hot spots in this study is feasible to formulate processes according to previous studies. Surprisingly, the alteration of residues throughout the sequence have been lower in comparison with the improvements in other acylases by similar techniques. Thus, although the rational design of the enzymes has been focalized on the residues located close to the catalytic pocket (*i.e.* amino acids forming the substrate binding pocket and catalytic residues), with the obvious idea that is more plausible to affect the biocatalyst by altering the disposition of the active site, the studies have veered to alter new positions throughout the enzyme. However, the utility of those residues outside of the cavity where the reaction takes place is very difficult to demonstrate. In fact, probably most of the mutations within the catalytic pocket would trigger in inactive enzymes, because they are the most highly conserved residues in proteins (Zuo, Z.-Y. *et al.* 2007). Thus, directed evolution is a versatile tool for developing novel enzymes. It has

been used to improve catalytic activity of enzyme and its efficiency, modulate (limit or expand) substrate specificity, alter cofactor specificity, and improve stability over a wider range of conditions, among others (Altreuter, D. H. *et al.* 1999; Reetz, M. T. *et al.* 2000; Zhao, H. *et al.* 2003; Callanan, M. J. *et al.* 2007). Thus, the parental enzyme evolvability and the precision of the HTS method are a challenge in the creation of an appropriate mechanism to obtain the desired properties (Salazar, O. *et al.* 2003).

Studies of directed molecular evolution of *Sl*PVA and *Au*AAC have been carried out in order to improve catalytic performance against several substrates under different operational conditions. Thus, 3,993 recombinant clones from both acylases have been tested at two pH values and two temperatures against seven substrates (*i.e.* 56 scenarios and around 210,000 assays) by a colorimetric high-throughput screening in order to evaluate their acylase activity. By this screening, it has been possible to keep a mutant library of 158 mutant clones, containing recombinant enzymes with improved activities, and 14 of them have multiple enhanced activities.

Sequence analysis and spatial distribution of residues in the selected clones have localized the mutations on the protein surface. Thereafter, 174 sequences of acylases available on databases have been aligned and it has established that these mutations were detected on residues with high entropy values. These mutations in turn alter the contact with the solvent probably due to the properties of the residue side-chain (*e.g.* βR373W and αA169T in *Sl*PVA), the particular location of the mutation or its steric hindrance (*e.g.* βV204F in *Sl*PVA, and αI184F in *Au*AAC), among others. Similar analyses have been done with seven related acylases subjected to mutations (*i.e. K. citrophila* ATCC 21285, *E. coli* ATCC 11105, *Pseudomonas* SY-77-1, *Pseudomonas* SE83, *Pseudomonas* N176, *Pseudomonas* sp. 130 and *P. diminuta* KAC-1), and practically all of them displayed residues with middle to high entropy values on the surface of the enzymes, and as expected, some of these residues have been located within the catalytic pocket. Those specific modifications altered the overall spatial disposition of the residues, such as changing their orientation with respect to the solvent or the relative location of some residues, which should be interpreted as the closest distance between active groups of residues in relation to hydroxyl of βSer^1 (*i.e.* main catalytic residue), which in turn means the location with respect to the substrate.

Bioinformatic analysis has allowed studying the function-structure relationship, showing the importance of residues on acylase surfaces and their potential roles. This information has been correlated with statistic values and thermodynamic interpretation, in order to inform the importance about protein stability and activity.

Finally, despite the fact that most of the studies report information about residues conservation, further studies about thermodynamic parameters are essential to clarify the surface role in protein performance.

VI CONCLUSIONS

The results of this Doctoral Thesis provide in-depth details about analysis of genomes, directed evolution and bioinformatic approaches related to microbial acylases. In particular, this work is focused on aculeacin A acylase (*Au*AAC) from *Actinoplanes utahensis* NRRL 12052 and penicillin V acylase (*Sl*PVA) from *Streptomyces lavendulae* ATCC 13664, enzymes with unequivocal industrial interest. The present study has been developed as a consequence of a constant search for biotechnological resources, ideal biocatalysts and improved operational conditions for a specific biocatalytic process, paving the way for the search of improved enzymes and providing new alternatives of *quorum quenching*. The main conclusions of the present Thesis are:

1. The genomes of *Streptomyces lavendulae* ATCC 13664 and *Actinoplanes utahensis* NRRL 12052 have been sequenced and their analyses have allowed the detection of several important clusters within each microorganism. The presence of clusters related with NRPS, PKS, and other important genes involved in antibiotic resistance have been detected, and the phylogenetic evolution analysis of each strain has been carried out.
2. The phylogenetic evolution analysis and the study of evolutionary relationships have demonstrated that *Streptomyces lavendulae* subsp. *lavendulae* ATCC 13664, described as a penicillin V acylase producer, has been misassigned within the genus, and a new assignment might be proposed as *Streptomyces* sp.
3. The putative gene *ahla* of *Actinoplanes utahensis* NRRL 12052 has been described and demonstrated that encodes for a novel *N*-acyl-homoserine lactone acylase (*Au*AHLA) with penicillin V acylase activity, suggesting the participation of this microorganism in *quorum quenching* processes.
4. In contrast to previous reports, the encoding gene of echinocandin B deacylase has not been detected within the genome of *A. utahensis*, and only the *aac* gene was located in the genome. Moreover, no transmembrane domain has been detected in this sequence, indicating that the reported echinocandin B deacylase must be a form of *Au*AAC resulting from a partial processing and needs its reassignment.
5. For the first time, the roles of *Au*AAC and *Au*AHLA have been associated to siderophore biosynthesis. In this sense, the *aac*-coding sequence has been detected within a predicted cluster related with gobichelin biosynthesis, whereas the *ahla*-encoding gene has been located in the predicted cluster of laspartomycin biosynthesis.
6. The development of a highly sensitive fluorometric method has allowed the determination of the kinetic parameters of both *Au*AAC and *Sl*PVA employing several *N*-(acyl)-L-homoserine lactones (AHLs) as substrates. For the first time, hydrolytic activity with *N*-(3-oxo-acyl)-L-homoserine lactones (AHLs) has been detected for these enzymes. Furthermore, this fluorometric method was slightly modified from the protocols reported previously in literature, and allowed the fast acquisition of results, as well as high reproducibility and high sensitivity. With respect to previous techniques, the high sensitivity of this method has overcome problems of solubility at saturated substrate concentrations during enzymatic assays. In consequence, the fluorometric method might be proposed as a standard method to estimate the presence of primary amines (*e.g.* in homoserine lactone) with high confidence in enzyme-catalyzed reactions.
7. A methodology to carry out directed molecular evolution of acylases from Actinomycetes has been developed. This methodology is based on the expression of acylases by the system composed by *Rhodococcus* sp. T104 and the shuttle vector pENV19. The mutant libraries of *Sl*PVA and *Au*AAC have been obtained by the mutator strain *E. coli* XL1-Red, and the high-throughput screening (HTS) has been

performed by the robotic platform Freedom EVOware 75. This procedure have allowed the evaluation of approximately 2,000 recombinant strains of each enzyme employing penicillin V as substrate, and nearly 1,000 of those clones catalyzed both *N*-acyl-L-homoserine lactones as *N*-(3-oxo-acyl)-L-homoserine lactones (AHLs, molecules involved in *quorum sensing*) under different operational conditions. In total, approximately 220,000 assays have been carried out by this alternative methodology at 56 different operational scenarios.

8. As a result of directed evolution, 156 recombinant mutant clones of *Sl*PVA and *Au*AAC with higher penicillin V acylase activities than the parental enzymes at the tested conditions (*i.e.* pH values of 6.0 and 8.0, and at 30ºC and 45ºC) have been obtained. It is worth mentioning that nine recombinant clones of *Au*AAC displayed improved activity at pH 6.0, whereas one recombinant clone of *Sl*PVA and two of *Au*AAC showed improved activity at 30ºC. The clones with activity at pH 6.0 constitute a successful result of this study, since parental *Au*AAC does not display activity at this pH value, in addition to their instability under these conditions. Likewise, those mutant clones obtained with activities at 30ºC are another achieved goal since parental enzymes do not display activity at this temperature either.
9. Recombinant clones from both *Sl*PVA and *Au*AAC with improved activities employing aliphatic and β-keto substituted aliphatic AHLs as substrates at the two pH values and the two temperatures have been assayed. It should be highlighted that 24 recombinant clones of *Sl*PVA and 39 recombinant clones of *Au*AAC showed improved activity at pH 6.0, whereas 30 recombinant clones of *Sl*PVA and 38 recombinant clones of *Au*AAC showed improved activity at 30ºC. Once again, it is noteworthy that those recombinant strains are capable of hydrolyzing amide bonds at lower pH and temperature values, where parental enzymes do not show apparent activities or they are not stable. This fact could implicate these acylases in the interruption of cell communication, and supports that they could be involved in alternative *quorum quenching* processes.
10. The criteria to select recombinant clones in this study have been basically the presence of activity using aliphatic and β-keto substituted aliphatic AHLs as substrates, the presence of activity at pH 6.0 and/or 30ºC, and the presence of activity employing AHLs containing short side-chains as substrates. The recombinant clones of *Sl*PVA named 2pva1,25(25), 2pva1,2(55) and 2pva1,2(149), and the recombinant clone of *Au*AAC named a(292) were selected, and their analyzed sequences displayed mutations in the β-subunit, a modification of the ORF, mutations in the α-subunit, and mutations within the linker peptide, respectively.
11. The presence of a truncated acylase of *Sl*PVA in the present study might indicate that the C-terminal end is not involved in the catalysis, nor in the enzyme stability. However, further studies are needed to understand the real role of this sequence segment in acylases.
12. The analysis of mutations detected in the recombinant clones 2pva1,25(25), 2pva1,2(55) and 2pva1,2(149) of *Sl*PVA, and a(292) of *Au*AAC, as well as other mutant enzymes from seven acylases related with native *Sl*PVA and *Au*AAC, have shown the potential role of entropy of those residues (*i.e.* interpreted as conservation of those amino acids among acylases) and the implication of the surface, in the improvement of the performance of these enzymes. Moreover, sequence comparisons and bioinformatic approaches have allowed the determination of βSer^{1}, βHis^{23}, βTyr^{24}, βPhe^{32}, βLeu^{50}, βSer^{57}, βIle^{58}, βHis^{68}, βThr^{69}, βVal^{70}, βVal^{186}, βAsn^{272} in *Sl*PVA, and βSer^{1}, βHis^{23}, βTyr^{24}, βPhe^{32}, βLeu^{50}, βLeu^{57}, βIle^{58}, βHis^{68}, βThr^{69}, βVal^{70}, βLeu^{188}, βAsn^{274} in *Au*AAC, as part of the ligand binding residues in these acylases.

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VIII SUPPLEMENTARY MATERIAL

S.1. Acyl-homoserine lactones implicated in *QS*

$C_{4:0}$-HSL (86 microorganisms)[1]
P. aeruginosa PAO1 (Pearson, J. P. *et al.* 1995; Winson, M. K. *et al.* 1995), *Serratia liquefaciens* MG1 (Eberl, L. *et al.* 1996), *Aeromonas hydrophila* A1, *A. salmonicida* NCIMB1102 (Swift, S. *et al.* 1997), *P. fluorescens* 2-79, *Erwinia carotovora* pv. *atroseptica* SR8, *E. carotovora* pv. *carotovora* DM2105, *Xanthomonas oryzae* pv *oryzicola* BLS303, *Rhizobium meliloti* Rm41 (Cha, C. *et al.* 1998), *Serratia* sp. ATCC39006 (Thomson, N. R. *et al.* 2000), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000), *P. chlororaphis* PCL1391 (Chin-A-Woeng, T. F. C. *et al.* 2001), *Burkholderia cepacia* ATCC17765 (Conway, B.-A. D. *et al.* 2002), *Vibrio vulnificus* (Morin, D. *et al.* 2003), *A. hydrophila* ATCC7966, *A. hydrophila* 96-3-35, *A. salmonicida* NCIMB1110, *A. salmonicida* 02-9-1 (Bruhn, J. B. *et al.* 2005), *Halomonas eurihalina* F2-7, *H. eurihalina* F9-6, *H. eurihalina* M4, *H. maura* S-30, *H. maura* S-31, *H. maura* X-2, *H. maura* B-100, *H. anticariensis* FP-34, *H. anticariensis* FP-35, *H. anticariensis* FP-36 (Llamas, I. *et al.* 2005), *Enterobacteriaceae* MRa46-1, *Vibrionaceae* MRa42 (Rasch, M. *et al.* 2005), *P. aeruginosa* (Cataldi, T. R. I. *et al.* 2007), *P. chlororaphis* 30-84 (Khan, S. R. *et al.* 2007), *P. chlororaphis* GP72 (Liu, H. *et al.* 2007), *S. plymuthica* HRO-C48 (Liu, X. *et al.* 2007), *Yersinia pseudotuberculosis* YPIII (Ortori, C. A. *et al.* 2007), *Pantoea* sp. CBMAI732 (Pomini, A. M. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *P. agglomerans* pv. *gypsophilae* (Chalupowicz, L. *et al.* 2008), *P. chlororaphis* 449 (Veselova, M. A. *et al.* 2008), *Pseudomonas* sp. M18 (Lu, J. *et al.* 2009), *P. aeruginosa* PA14 (Venkataraman, A. *et al.* 2010), *P. aeruginosa* CGMCC 1.860 (Yong, Y.-C. *et al.* 2010), *P. aeruginosa* PUPa3 (Babić, F. *et al.* 2010), *S. plymuthica* G3 (Liu, X. *et al.* 2011), *A. salmonicida* subsp. *achromogenes* Keldur265-87 (Schwenteit, J. *et al.* 2011), *A. aquarium* B2M05, *A. hydrophila* B015, *A. hydrophila* B1M18, *A. jandaei* B087, *A. media* Bi11, *A. media* B026, *A. media* B1M53, *A. salmonicida* B079 (Huang, Y. *et al.* 2012), *Proteus mirabilis* O18 (Stankowska, D. *et al.* 2012), *A. hydrophila* YJ-1, *A. hydrophila* HAE-1, *A. hydrophila* HAE-2, *A. hydrophila* B-1, *A. hydrophila* B-2, *A. hydrophila* S-1, *A. hydrophila* S-2, *A. hydrophila* M-13, *A. hydrophila* NL-1, *A. hydrophila* NL-2, *A. hydrophila* TPS-30, *A. hydrophila* TPS-49, *A. hydrophila* X-1, *A. hydrophila* BJ, *A. hydrophila* BC, *A. hydrophila* GML, *A. hydrophila* BX-50, *A. hydrophila* BH-50, *A. hydrophila* AN-1, *A. hydrophila* LS-4, *A. hydrophila* J-1 (Chu, W. *et al.* 2013), *Ochrobactrum* sp., *Vibrio* sp. 11-6DEP, *Shewanella* sp., *V. harveyi*, *Alteromonas* sp. (Cuadrado-Silva, C. T. *et al.* 2013), *A. hydrophila* P9, *A. hydrophila* P43, *P. fluorescens* P71, *P. fluorescens* B44, *P. fluorescens* B45, *P. fluorescens* B48 (Haslan, E. *et al.* 2013), *A. veronii* biovar sobria strain 159 (Chan, X. Y. *et al.* 2015)

oxo-$C_{4:0}$-HSL (1 microorganism)
Y. pseudotuberculosis YPIII (Ortori, C. A. *et al.* 2007)

OH-$C_{4:0}$-HSL (2 microorganisms)
V. harveyi MAV (Cao, J. G. *et al.* 1989), *Xenorhabdus nematophilus* 19061 (Dunphy, G. *et al.* 1997)

$C_{5:0}$-HSL (5 microorganisms)
Erwinia carotovora SCC1 (Brader, G. *et al.* 2005), *P. chlororaphis* 30-84, *P. chlororaphis* PCL1391, *P. fluorescens* 2-79 (Khan, S. R. *et al.* 2007), *S. plymuthica* G3 (Liu, X. *et al.* 2011)

$C_{iso\text{-}5:0}$-HSL (1 microorganism)
Bradyrhizobium japonicum USDA110 (Lindemann, A. *et al.* 2011)

oxo-$C_{5:0}$-HSL (1 microorganism)
E. carotovora SCC1 (Brader, G. *et al.* 2005)

$C_{6:0}$-HSL (193 microorganisms)
Agrobacterium tumefaciens K794 (Zhang, L. *et al.* 1993), *V. fiseheri* MJ-1 (Kuo, A. *et al.* 1994), *P. aeruginosa* PAO1 (Pearson, J. P. *et al.* 1995; Winson, M. K. *et al.* 1995), *Y. enterocolitica* 10460 (Throup, J. P. *et al.* 1995), *S. liquefaciens* MG1 (Eberl, L. *et al.* 1996), *Chromobacterium violaceum* ATCC31532 (McClean, K. H. *et al.* 1997), *A. hydrophila* A1, *A. salmonicida* NCIMB1102 (Swift, S. *et al.* 1997), *P. aureofaciens* 30-84 (Wood, D. W. *et al.* 1997), *E. chrysanthemi* (Nasser, W. *et al.* 1998), *Y. pseudotuberculosis* YPIII (Atkinson, S. *et al.* 1999), *R. leguminosarum* bv. viciae (Rodelas, B. *et al.* 1999), *P. aeruginosa* SH1 (Geisenberger, O. *et al.* 2000), *R. leguminosarum* 8401 (Lithgow, J. K. *et al.* 2000), *P. fluorescens* F113 (Laue, B. E. *et al.* 2000), *Serratia* sp. ATCC39006 (Thomson, N. R. *et al.* 2000), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000), *B. cepacia* DSM50180, *B. cepacia* ATCC25416 (Andersen, J. B. *et al.* 2001), *B. cepacia* LMG6963, *B. cepacia* LMG1222, *B. cepacia* LMG14087, *B. cepacia* LMG14095, *B. cepacia* LMG16663, *B. cepacia* LMG18821, *B. cepacia* LMG6981, *B. cepacia* LMG6988, *B. cepacia* LMG6993, *B. cepacia* LMG12615, *B. cepacia* LMG14271, *B. cepacia* LMG16654, *B. cepacia* LMG16657, *B. cepacia* LMG16659, *B. cepacia* LMG6997, *B. cepacia* LMG7000, *B. cepacia* LMG14291, *B. cepacia* LMG18941, *B. cepacia* LMG18942, *B. cepacia* LMG18943, *B. cepacia* LMG18944, *B. cepacia* LMG18946, *B. cepacia* LMG16670, *B. cepacia* R-6108, *B. cepacia* R-6274, *B. cepacia* R-6282, *B. cepacia* R-6285, *B. cepacia* R-136, *B. cepacia* R-3338, *B. cepacia* R-6270, *B. cepacia* R-6272, *B. cepacia* R-6273, *B. cepacia* R-6276, *B. cepacia* R-6279, *B. cepacia* R-6280, *B. cepacia* R-6281, *B. cepacia* R-10033, *B. cepacia* R-3976, *B. vietnamiensis* LMG6998, *B. vietnamiensis* LMG6999, *B. vietnamiensis* LMG10929, *B. vietnamiensis* LMG16232, *B. vietnamiensis* LMG18835, *B. vietnamiensis* LMG18836, *B. vietnamiensis* R-128, *B. vietnamiensis* R-723, *B. vietnamiensis* R-921 (Gotschlich, A. *et al.* 2001), *B. cepacia* K56-2 (Gotschlich, A. *et al.* 2001; Lewenza, S. *et al.* 2001), *B. cepacia* Pc715j (Lewenza, S. *et al.* 2001), *R. leguminosarum* 248, *R. leguminosarum* 300, *R. leguminosarum* TOM, *R. leguminosarum* 8002 (Lithgow, J. K. *et al.* 2001), *V. anguillarum* NB10 (Milton, D. L. *et al.* 2001), *B. cepacia* G4 (Park, J.-H. *et al.* 2001), *P. aeruginosa* SH38 (Riedel, K. *et al.* 2001), *B. vietnamiensis* G4 (Conway, B.-A. *et al.* 2002; Malott, R. J. *et al.* 2007), *S. marcescens* SS-1 (Horng, Y.-T. *et al.* 2002), *V. vulnificus* (Morin, D. *et al.* 2003), *Sinorhizobium meliloti* AK631 (Teplitski, M. *et al.* 2003), *B. glumae* BGR1 (Kim, J. *et al.* 2004), *Edwardsiella tarda* NUF251 (Morohoshi, T. *et al.* 2004), *B. thailandensis* DW503 (Ulrich, R. L. *et al.* 2004c), *E. carotovora* SCC1, *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *A. salmonicida* 02-9-1, *V. salmonicida* NCIMB2262, *V. salmonicida* 289 (Bruhn, J. B. *et al.* 2005), *Nitrosomonas europea* Schmidt (Burton, E. O. *et al.* 2005), *vitis* F2/5 (Li, Y. *et al.* 2005), *H. eurihalina* F2-7, *H. eurihalina* F9-6, *H. eurihalina* M4, *H. maura* S-30, *H. maura* S-31, *H. maura* X-2, *H. maura* B-100, *H. anticariensis* FP-34, *H. anticariensis* FP-35, *H. anticariensis* FP-36 (Llamas, I. *et al.* 2005), *E. psidii* R. IBSBF435 (Pomini, A. M. *et al.* 2005), *Enterobacteriaceae* C2JM, *Enterobacteriaceae* MRa45, *Enterobacteriaceae* MRa46-2, *Enterobacteriaceae* MRa48, *Enterobacteriaceae* MRa49, *Enterobacteriaceae* MRa50, *Enterobacteriaceae* MRa51 (Rasch, M. *et al.* 2005), *Y. enterocolitica* 90/54 (Atkinson, S. *et al.* 2006), *Y. pestis* KIM6+ (Kirwan, J. P. *et al.* 2006), *Methylobacterium extorquens* AM1 (Nieto Penalver, C. G. *et al.* 2006), *B. plantarii* ATCC43733 (Solis, R. *et al.* 2006), *Azospirillum lipoferum* B510, *A. lipoferum* B518 (Vial, L. *et al.* 2006), *Y. enterocolitica* (Cataldi, T. R. I. *et al.* 2007), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316

(Kastbjerg, V. G. *et al.* 2007), *P. chlororaphis* 30-84, *P. chlororaphis* PCL1391, *P. fluorescens* 2-79 (Khan, S. R. *et al.* 2007), *P. corrugata* CFBP5454, *P. corrugata* CFBP10532, *P. corrugata* CFBP10950, *P. corrugata* CFBP10058, *P. corrugata* 421, *P. corrugata* 717, *P. corrugata* par8 (Licciardello, G. *et al.* 2007), *P. chlororaphis* GP72 (Liu, H. *et al.* 2007), *S. plymuthica* HRO-C48 (Liu, X. *et al.* 2007), *B. vietnamiensis* PC259, *B. vietnamiensis* FC466, *B. vietnamiensis* FC441, *B. vietnamiensis* C2822, *B. vietnamiensis* FC369, *B. vietnamiensis* DB01 (Malott, R. J. *et al.* 2007), *P. ananatis* SK-1 (Morohoshi, T. *et al.* 2007), *Pantoea* sp. CBMAI732 (Pomini, A. M. *et al.* 2007), *Burkholderia* CBMB40, *Burkholderia* CBPB-HOD, *Burkholderia* CBPB-HIM (Poonguzhali, S. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *B. mallei* ATCC23344 (Duerkop, B. A. *et al.* 2008), *P. chlororaphis* 449 (Veselova, M. A. *et al.* 2008), *Pseudomonas* sp. M18 (Lu, J. *et al.* 2009), *A. culicicola* 3249 (Thiel, V. *et al.* 2009), *P. aeruginosa* CGMCC 1.860 (Yong, Y.-C. *et al.* 2010), *B. cenocepacia* J2315 (Fang, K. *et al.* 2011), *S. plymuthica* G3 (Liu, X. *et al.* 2011), *Salmonella enterica* subsp. *enterica* sv. Typhimurium (Nesse, L. *et al.* 2011), *C. aquaticum* PRAA4-1 (Rekha, P. D. *et al.* 2011), *Acinetobacter tjernbergiae* B017, *A. hydrophila* B015, *S. adhaerens* 5D19, *Ensifer adhaerens* ATCC 31499, *E. adhaerens* ORE 529 (Huang, Y. *et al.* 2012), *Mortierella alpina* A-178 (Kai, K. *et al.* 2012a), *Gluconacetobacter diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012), *Pseudomonas* sp. HF-1 (Wang, M.-Z. *et al.* 2012), *A. hydrophila* YJ-1, *A. hydrophila* HAE-1, *A. hydrophila* HAE-2, *A. hydrophila* B-1, *A. hydrophila* B-2, *A. hydrophila* S-1, *A. hydrophila* S-2, *A. hydrophila* M-13, *A. hydrophila* NL-1, *A. hydrophila* NL-2, *A. hydrophila* TPS-30, *A. hydrophila* TPS-49, *A. hydrophila* X-1, *A. hydrophila* BJ, *A. hydrophila* BC, *A. hydrophila* GML, *A. hydrophila* BX-50, *A. hydrophila* BH-50, *A. hydrophila* AN-1, *A. hydrophila* LS-4, *A. hydrophila* J-1 (Chu, W. *et al.* 2013), *Ochrobactrum* sp., *Vibrio* sp. 23-6PIN, *V. campbellii*, *O. pseudogringnonense*, *Shewanella* sp., *V. harveyi* (Cuadrado-Silva, C. T. *et al.* 2013), *A. hydrophila* P9, *A. hydrophila* P43, *A. hydrophila* B96, *P. fluorescens* P71, *P. fluorescens* B52 (Haslan, E. *et al.* 2013), *V. variabilis* T01 (Mohamad, N. I. *et al.* 2015)

oxo-$C_{6:0}$ (75 microorganisms)
Photobacterium fischeri MJ-1 (Eberhard, A. *et al.* 1981), *E. carotovora* ATCC39018 (Bainton, N. J. *et al.* 1992), *V. fiseheri* MJ-1 (Kuo, A. *et al.* 1994; Shaw, P. D. *et al.* 1997), *Y. enterocolitica* 10460 (Throup, J. P. *et al.* 1995), *E. stewartii* DC283 (Beck von Bodman, S. *et al.* 1995), *P. aeruginosa* PAO1 (Winson, M. K. *et al.* 1995; Shaw, P. D. *et al.* 1997), *P. syringae* pv. *tabaci* 2024 (Shaw, P. D. *et al.* 1997), *P. syringae pv. syringae* B728a, *P. syringae* pv. *savastanoi* Olive 1670, *P. syringae* pv. *coronofaciens* PC27, *P. fluorescens* 2-79, *E. carotovora* pv. *atroseptica* SR8, *E. carotovora* pv. *carotovora* DM2105 (Cha, C. *et al.* 1998), *E. chrysanthemi* (Nasser, W. *et al.* 1998), *Y. pseudotuberculosis* YPIII (Atkinson, S. *et al.* 1999), *Hafnia alvei*, *E. agglomerans, S. liquefaciens* (Gram, L. *et al.* 1999), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000), *B. cepacia* ATCC25416 (Andersen, J. B. *et al.* 2001), *P. putida* IsoF, *P. putida* Z2D, *Rahnella aquatilis* T13, *R. aquatilis* TAA (Steidle, A. *et al.* 2001), *B. cepacia* ATCC17765 (Conway, B.-A. D. *et al.* 2002), *S. marcescens* SS-1 (Horng, Y.-T. *et al.* 2002), *Y. enterocolitica* WA-314, *Y. enterocolitica* 8081 (Jacobi, C. A. *et al.* 2003), *S. proteamaculans* B5a, *Enterobacter agglomerans* B6a (Flodgaard, L. R. *et al.* 2003), *P. putida* WCS358 (Bertani, I. *et al.* 2004), *V. campbellii* (Taylor, M. W. *et al.* 2004), *E. amylovora* IPV-BO 1077/7 (Venturi, V. *et al.* 2004), *E. carotovora* SCC1, *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *A. salmonicida* 02-9-1, *V. salmonicida* NCIMB2262, *V. salmonicida* 289 (Bruhn, J. B. *et al.* 2005), *Enterobacteriaceae* A2JM, *Enterobacteriaceae* A9JM, *Enterobacteriaceae* A10JM, *Enterobacteriaceae* B4JM, *Enterobacteriaceae* C2JM, *Enterobacteriaceae* MRa45, *Enterobacteriaceae* MRa51, *Enterobacteriaceae* MRa54, *Pseudomonas* C1JM (Rasch, M. *et al.* 2005), *Y. enterocolitica* 90/54 (Atkinson, S. *et al.* 2006), *P. putida* PCL1445 (Dubern, J.-F. *et al.* 2006), *Y. pestis* KIM6+ (Kirwan, J. P. *et al.* 2006), *A. lipoferum* B510, *A. lipoferum* B518 (Vial, L. *et al.* 2006), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *Pectobacterium atrosepticum* 6276 (Latour, X. *et al.* 2007), *P. corrugata* CFBP5454, *P. corrugata* CFBP10532, *P. corrugata* CFBP10950, *P. corrugata* CFBP10058, *P. corrugata* 421, *P. corrugata* 717, *P. corrugata* par8 (Licciardello, G. *et al.* 2007), *S. plymuthica* HRO-C48 (Liu, X. *et al.* 2007), *P. ananatis* SK-1 (Morohoshi, T. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *E. chrysanthemi* pv. zeae (Hussain, M. B. B. M. *et al.* 2008), *A. thiooxidans* DSMZ 11478 (Ruiz, L. M. *et al.* 2008), *P. chlororaphis* 449 (Veselova, M. A. *et al.* 2008), *S. plymuthica* G3 (Liu, X. *et al.* 2011), *Pseudomonas* sp. HF-1 (Wang, M.-Z. *et al.* 2012)

OH-$C_{6:0}$-HSL (10 microorganisms)
P. fluorescens 2-79 (Shaw, P. D. *et al.* 1997; Khan, S. R. *et al.* 2007), *V. anguillarum* NB10 (Milton, D. L. *et al.* 2001; Buchholtz, C. *et al.* 2006), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *S. plymuthica* IC1270 (Ovadis, M. *et al.* 2004), *V. anguillarum* 90-11-287 (Buchholtz, C. *et al.* 2006), *P. chlororaphis* 30-84, *P. chlororaphis* PCL1391 (Khan, S. R. *et al.* 2007), *A. culicicola* 3249 (Thiel, V. *et al.* 2009), *S. plymuthica* G3 (Liu, X. *et al.* 2011), *P. chlororaphis* B001 (Huang, Y. *et al.* 2012)

$C_{7:0}$-HSL (14 microorganisms)
R. leguminosarum 8401 (Lithgow, J. K. *et al.* 2000), *R. leguminosarum* 248, *R. leguminosarum* 300, *R. leguminosarum* TOM, *R. leguminosarum* 8002 (Lithgow, J. K. *et al.* 2001), *S. marcescens* SS-1 (Horng, Y.-T. *et al.* 2002), *E. tarda* NUF251 (Morohoshi, T. *et al.* 2004), *E. psidii* R. IBSBF435 (Pomini, A. M. *et al.* 2005), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *Burkholderia* CBMB40, *Burkholderia* CBPB-HOD, *Burkholderia* CBPB-HIM (Poonguzhali, S. *et al.* 2007), *S. plymuthica* G3 (Liu, X. *et al.* 2011), *M. alpina* A-178 (Kai, K. *et al.* 2012a)

$C_{iso\text{-}7:0}$-HSL (1 microorganism)
A. culicicola 3249 (Thiel, V. *et al.* 2009)

$C_{iso\text{-}7:1}$-HSL (1 microorganism)
A. culicicola 3249 (Thiel, V. *et al.* 2009)

oxo-$C_{7:0}$-HSL (10 microorganisms)
E. carotovora SCC1, *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *S. plymuthica* G3 (Liu, X. *et al.* 2011)

OH-$C_{7:0}$-HSL (3 microorganisms)
P. chlororaphis 30-84, *P. chlororaphis* PCL1391, *P. fluorescens* 2-79 (Khan, S. R. *et al.* 2007)

OH-$C_{iso\text{-}7:0}$-HSL (1 microorganisms)
A. culicicola 3249 (Thiel, V. *et al.* 2009)

$C_{8:0}$-HSL (173 microorganisms)
V. fiseheri MJ-1 (Kuo, A. *et al.* 1994), *Rhodocbacter sphaeroides* 2.4.1 (Shaw, P. D. *et al.* 1997), *R. solanacearum* K60 (Shaw, P. D. *et al.* 1997; Cha, C. *et al.* 1998), *P. fluorescens* 2-79, *R. solanacearum* AW1, *R. leguminosarum* bv. *trifolii* 162E8, *R. meliloti* Rm41, *R.*

leguminosarum bv. *phaseoli* 14482 (Cha, C. *et al.* 1998), *B. cepacia* K56-2 (Lewenza, S. *et al.* 1999; Gotschlich, A. *et al.* 2001; Lewenza, S. *et al.* 2001), *Y. pseudotuberculosis* YPIII (Atkinson, S. *et al.* 1999), *R. leguminosarum* bv. viciae (Rodelas, B. *et al.* 1999), *P. aeruginosa* SH1(Geisenberger, O. *et al.* 2000), *R. leguminosarum* 8401 (Lithgow, J. K. *et al.* 2000), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000),*B. cepacia* DSM50180 (Andersen, J. B. *et al.* 2001), *B. cepacia* ATCC25416 (Andersen, J. B. *et al.* 2001; Conway, B.-A. D. *et al.* 2002), *P. chlororaphis* PCL1391 (Chin-A-Woeng, T. F. C. *et al.* 2001), *B. cepacia* LMG6963, *B. cepacia* LMG1222, *B. cepacia* LMG14087, *B. cepacia* LMG14095, *B. cepacia* LMG16663, *B. cepacia* LMG6981, *B. cepacia* LMG6988, *B. cepacia* LMG6993, *B. cepacia* LMG12615, *B. cepacia* LMG14271, *B. cepacia* LMG16657, *B. cepacia* LMG6997, *B. cepacia* LMG14291, *B. cepacia* LMG18941, *B. cepacia* LMG18942, *B. cepacia* LMG18943, *B. cepacia* LMG18944, *B. cepacia* LMG18946, *B. cepacia* LMG16670, *B. cepacia* R-6108, *B. cepacia* R-6274, *B. cepacia* R-6282, *B. cepacia* R-6285, *B. cepacia* R-136, *B. cepacia* R-3338, *B. cepacia* R-6270, *B. cepacia* R-6272, *B. cepacia* R-6273, *B. cepacia* R-6276, *B. cepacia* R-6279, *B. cepacia* R-6280, *B. cepacia* R-6281, *B. cepacia* R-10033, *B. cepacia* R-3976, *B. vietnamiensis* LMG6998, *B. vietnamiensis* LMG6999, *B. vietnamiensis* LMG10929, *B. vietnamiensis* LMG16232, *B. vietnamiensis* LMG18835, *B. vietnamiensis* LMG18836, *B. vietnamiensis* R-128, *B. vietnamiensis* R-723, *B. vietnamiensis* R-921 (Gotschlich, A. *et al.* 2001), *B. cepacia* CEP509, *B. cepacia* J415, *B. cepacia* C1394, *B. cepacia* LMG7000 (Gotschlich, A. *et al.* 2001; Conway, B.-A. D. *et al.* 2002), *B. cepacia* Pc715j (Lewenza, S. *et al.* 2001), *R. leguminosarum* 248, *R. leguminosarum* 300, *R. leguminosarum* TOM, *R. leguminosarum* 8002 (Lithgow, J. K. *et al.* 2001), *B. cepacia* G4 (Park, J.-H. *et al.* 2001), *B. cepacia* C9139, *B. cepacia* ATCC17759, *B. cepacia* ATCC10856, *B. cepacia* ATCC17616, *B. cepacia* C1576, *B. cepacia* C1962, *B. cepacia* C1257, *B. cepacia* C4455, *B. cepacia* BC7, *B. cepacia* K56-2, *B. cepacia* PC184, *B. cepacia* ATCC35254, *B. cepacia* LMG14294, *B. cepacia* LMG14086, *B. cepacia* C2822, *B. cepacia* C9178, *B. cepacia* PC259, *B. cepacia* FC441, *B. cepacia* LMG16232 (Conway, B.-A. D. *et al.* 2002), *B. vietnamiensis* G4 (Conway, B.-A. *et al.* 2002; Malott, R. J. *et al.* 2007), *S. marcescens* SS-1 (Horng, Y.-T. *et al.* 2002), *S. meliloti* Rm1021 (Marketon, M. M. *et al.* 2002), *B. cepacia* LA3 (Frommberger, M. *et al.* 2003), *S. proteamaculans* B5a, *E. agglomerans* B6a (Flodgaard, L. R. *et al.* 2003), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *Mesorhizobium huakuii* 93 (Zhu, J. *et al.* 2003), *B. cepacia* JA-7, *B. cepacia* LA-10 (Cataldi, T. R. I. *et al.* 2004), *B. pseudomallei* DD503 (Ulrich, R. L. *et al.* 2004a), *B. mallei* ATCC23344 (Ulrich, R. L. *et al.* 2004b; Duerkop, B. A. *et al.* 2007; Duerkop, B. A. *et al.* 2008), *B. thailandensis* DW503 (Ulrich, R. L. *et al.* 2004c), *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *Y. ruckeri* NCIMB1316, *Y. ruckeri* 88-6-44 (Bruhn, J. B. *et al.* 2005; Kastbjerg, V. G. *et al.* 2007), *N. europea* Schmidt (Burton, E. O. *et al.* 2005), *A. vitis* F2/5 (Li, Y. *et al.* 2005), *H. eurihalina* F2-7, *H. eurihalina* F9-6, *H. eurihalina* M4, *H. maura* S-30, *H. maura* S-31, *H. maura* X-2, *H. maura* B-100, *H. anticariensis* FP-34, *H. anticariensis* FP-35, *H. anticariensis* FP-36 (Llamas, I. *et al.* 2005), *Vibrionaceae* MRa39 (Rasch, M. *et al.* 2005), *B. pseudomallei* KHW (Song, Y. *et al.* 2005; Chan, Y. Y. *et al.* 2007), *Dinoroseobacter shibae* DFL16, *Roseobacter litoralis* DSM7001 (Wagner-Döbler, I. *et al.* 2005), *Y. pestis* KIM6+ (Kirwan, J. P. *et al.* 2006), *B. pseudomallei* pp844 (Lumjiaktase, P. *et al.* 2006), *M. extorquens* AM1 (Nieto Penalver, C. G. *et al.* 2006), *B. plantarii* ATCC43733 (Solis, R. *et al.* 2006), *A. lipoferum* TVV3, *A. lipoferum* B52, *A. lipoferum* B510, *A. lipoferum* B518 (Vial, L. *et al.* 2006), *A. hydrophila, A. salmonicida*, *Y. enterocolitica*, *S liquefasciens* (Cataldi, T. R. I. *et al.* 2007), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113 (Kastbjerg, V. G. *et al.* 2007), *P. atrosepticum* 6276 (Latour, X. *et al.* 2007), *B. ubonensis,* AB030584 (Li, X. *et al.* 2007), *P. corrugata* CFBP5454, *P. corrugata* CFBP10532, *P. corrugata* CFBP10950, *P. corrugata* CFBP10058, *P. corrugata* 421, *P. corrugata* 717, *P. corrugata* par8 (Licciardello, G. *et al.* 2007), *B. vietnamiensis* PC259, *B. vietnamiensis* FC466, *B. vietnamiensis* FC441, *B. vietnamiensis* C2822, *B. vietnamiensis* FC369, *B. vietnamiensis* DB01 (Malott, R. J. *et al.* 2007), *Burkholderia* CBMB40, *Burkholderia* CBPB-HOD, *Burkholderia* CBPB-HIM (Poonguzhali, S. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *C. violaceum* ATCC12472 (Morohoshi, T. *et al.* 2008a), *Gloeothece* PCC6909 (Sharif, D. I. *et al.* 2008), *Y. pestis* CO92 (Gelhaus, H. C. *et al.* 2009), *A. culicicola* 3249 (Thiel, V. *et al.* 2009), *B. cenocepacia* J2315 (Fang, K. *et al.* 2011), *S. enterica* subsp. *enterica* sv. Typhimurium (Nesse, L. *et al.* 2011), *S. fredii* SMH12, *S. fredii* ISP42, *S. fredii* IS123 (Pérez-Montaño, F. *et al.* 2011), *C. aquaticum* CC-SEYA-1 (Rekha, P. D. *et al.* 2011), *A. media* B1M53, *P. panipatensis* B1M30 (Huang, Y. *et al.* 2012), *M. alpina* A-178 (Kai, K. *et al.* 2012a), *G. diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012), *P. fluorescens* B44, *P. fluorescens* B45, *P. fluorescens* B48 (Haslan, E. *et al.* 2013)

oxo-$C_{8:0}$-HSL (65 microorganisms)

A. tumefaciens K794 (Zhang, L. *et al.* 1993), *P. aeruginosa* PAO1, *V. fischeri* MJ1, *P. syringae* pv. *tabaci* 2024 (Shaw, P. D. *et al.* 1997), *Ralstonia solanacearum* K60, *P. fluorescens* 2-79 (Shaw, P. D. *et al.* 1997; Cha, C. *et al.* 1998), *P. syringae* pv. *savastanoi* Olive 1670, *P. syringae pv. coronofaciens* PC27, *R. solanacearum* AW1, *A. tumefaciens* Bo542, *E. carotovora* pv. *atroseptica* SR8, *E. carotovora* pv. *carotovora* DM2105, *X. campestris*, pv. *pelargoni* X-5, *X. campestris* pv. *campestris* 4546, *X. oryzae* pv. *oryzicola* BLS303, *R. leguminosarum* bv. *trifolii* 162E8, *R. meliloti* Rm41, *R. leguminosarum* bv. *trifolii* 14480, *R. leguminosarum* bv. *phaseoli* 14482, *A. tumefaciens* C58 (Cha, C. *et al.* 1998), *Hafnia alvei*, *E. agglomerans, S. liquefaciens* (Gram, L. *et al.* 1999), *P. aeruginosa* 6294 (Charlton, T. S. *et al.* 2000), *P. aeruginosa* SH1(Geisenberger, O. *et al.* 2000), *P. putida* IsoF, *P. putida* Z2D, *R. aquatilis* TAA (Steidle, A. *et al.* 2001), *Rhizobium* sp. NGR234 (He, X. *et al.* 2003), *V. vulnificus* (Morin, D. *et al.* 2003), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *R. etli* CFN42 (Tun-Garrido, C. *et al.* 2003), *P. putida* WCS358 (Bertani, I. *et al.* 2004), *B. glumae* BGR1 (Kim, J. *et al.* 2004), *E. carotovora* SCC1, *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *Y. ruckeri* NCIMB1316, *Y. ruckeri* 88-6-44 (Bruhn, J. B. *et al.* 2005; Kastbjerg, V. G. *et al.* 2007), *A. vitis* F2/5 (Li, Y. *et al.* 2005), *P. putida* PCL1445 (Dubern, J.-F. *et al.* 2006), *Y. pestis* KIM6+ (Kirwan, J. P. *et al.* 2006), *B. pseudomallei* pp844 (Lumjiaktase, P. *et al.* 2006), *A. lipoferum* TVV3, *A. lipoferum* B52, *A. lipoferum* B510, *A. lipoferum* B518 (Vial, L. *et al.* 2006), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113 (Kastbjerg, V. G. *et al.* 2007), *P. atrosepticum* 6276 (Latour, X. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *A. thiooxidans* DSMZ504, *A. thiooxidans* DSMZ 9463, *A. thiooxidans* DSMZ11478 (Ruiz, L. M. *et al.* 2008), *Pseudoalteromonas* sp. 520P1 (Wang, Y. *et al.* 2008), *Y. pestis* CO92 (Gelhaus, H. C. *et al.* 2009), *Acidovorax avenae* subsp. *citrulli* XLJ12 (Tao, C. *et al.* 2009), *S. fredii* SMH12, *S. fredii* IS123 (Pérez-Montaño, F. *et al.* 2011), *A. veronii* B1M14, *P. alcaligenes* B2M20, *S. adhaerens* 5D19, *E. adhaerens* ATCC 31499, *E. adhaerens* ORE 529 (Huang, Y. *et al.* 2012), *Pseudomonas* sp. HF-1 (Wang, M.-Z. *et al.* 2012)

OH-$C_{8:0}$-HSL (33 microorganisms)

R. meliloti YA2 (Shaw, P. D. *et al.* 1997), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *R. etli* CFN42 (Tun-Garrido, C. *et al.* 2003), *B. pseudomallei* DD503 (Ulrich, R. L. *et al.* 2004a), *S. plymuthica* IC1270 (Ovadis, M. *et al.* 2004), *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *P. fluorescens* 5064 (Cui, X. *et al.* 2005), *Acidithiobacillus ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *Photobacterium phosphoreum* P100 (Flodgaard, L. R. *et al.* 2005), *Burkholderia phytofirmans* sp. nov. (Sessitsch, A. *et al.* 2005), *V. anguillarum* 90-11-287, *V. anguillarum* NB10 (Buchholtz, C. *et al.* 2006), *B. pseudomallei* pp844 (Lumjiaktase, P. *et al.* 2006), *A. lipoferum* B518 (Vial, L. *et al.* 2006), *B. pseudomallei* KHW (Chan, Y. Y. *et al.* 2007), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *P. chlororaphis* 30-84, *P. chlororaphis* PCL1391, *P. fluorescens* 2-79 (Khan, S. R. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. A. *et al.* 2007), *B. mallei* ATCC23344 (Duerkop, B. A. *et al.* 2008), *C. violaceum* ATCC12472 (Morohoshi, T. *et al.* 2008a), *A. culicicola* 3249 (Thiel, V. *et al.* 2009), *S. fredii* ISP42, *S. fredii* IS123 (Pérez-Montaño, F. *et al.* 2011), *A. media* B1M53, *P. alcaligenes* B2M20, *P. panipatensis* B1M30 (Huang, Y. *et al.* 2012)

$C_{9:0}$-HSL (1 microorganism)
E. carotovora SCC3193 (Brader, G. *et al.* 2005)

***$C_{iso\text{-}9:0}$*-HSL (2 microorganisms)**
A. culicicola 3249 (Thiel, V. *et al.* 2009), *M. alpina* A-178 (Kai, K. *et al.* 2012a)

oxo-$C_{9:0}$-HSL (8 microorganisms)
E. carotovora SCC3193 (Brader, G. *et al.* 2005), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007)

OH-$C_{iso\text{-}9:0}$-HSL (1 microorganism)
A. culicicola 3249 (Thiel, V. *et al.* 2009)

***p*-Coumaroyl-HSL (3 microorganisms)**
Rhodopseudomonas palustris CGA009, *Bradyrhizobium* BTAi1, *Silicibacter pomeroyi* DSS-3 (Schaefer, A. L. *et al.* 2008)

Cinnamoyl-HSL (1 microorganism)
R. palustris CGA814 (Ahlgren, N. A. *et al.* 2011)

$C_{10:0}$-HSL (38 microorganisms)
E. chrysanthemi (Nasser, W. *et al.* 1998), *P. fluorescens* F113 (Laue, B. E. *et al.* 2000), *B. vietnamiensis* LMG6998, *B. vietnamiensis* LMG6999, *B. vietnamiensis* LMG10929, *B. vietnamiensis* R-128 (J1697), *B. vietnamiensis* R-723 (PC30), *B. vietnamiensis* R-921 (Gotschlich, A. *et al.* 2001), *B. cepacia* G4 (Park, J.-H. *et al.* 2001; Conway, B.-A. D. *et al.* 2002), *B. cepacia* LMG10929 (Conway, B.-A. D. *et al.* 2002), *B. vietnamiensis* G4 (Conway, B.-A. *et al.* 2002; Malott, R. J. *et al.* 2007), *B. cepacia* LA3 (Frommberger, M. *et al.* 2003), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *B. cepacia* JA-7, *B. cepacia* LA-10 (Cataldi, T. R. I. *et al.* 2004), *B. pseudomallei* DD503 (Ulrich, R. L. *et al.* 2004a), *B. mallei* ATCC23344 (Ulrich, R. L. *et al.* 2004b), *B. thailandensis* DW503 (Ulrich, R. L. *et al.* 2004c), *B. pseudomallei* 008 (Valade, E. *et al.* 2004), *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *A. salmonicida* 02-9-1 (Bruhn, J. B. *et al.* 2005), *N. europea* Schmidt (Burton, E. O. *et al.* 2005), *Pseudomonas* C1JM, *Vibrionaceae* MRa39 (Rasch, M. *et al.* 2005), *A. lipoferum* TVV3 (Vial, L. *et al.* 2006), *A. salmonicida*, *P. aeruginosa*, *Y. enterocolitica* (Cataldi, T. R. I. *et al.* 2007), *B. vietnamiensis* PC259, *B. vietnamiensis* FC369, *B. vietnamiensis* DB01 (Malott, R. J. *et al.* 2007), *Burkholderia* CBMB40 (Poonguzhali, S. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *G. intermedius* NCI1051 (Iida, A. *et al.* 2008), *C. violaceum* ATCC12472 (Morohoshi, T. *et al.* 2008a), *A. baumannii* M2 (Niu, C. *et al.* 2008), *M. alpina* A-178 (Kai, K. *et al.* 2012a), *G. diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012)

$C_{10:1}$-HSL (1 microorganism)
M. alpina A-178 (Kai, K. *et al.* 2012b)

oxo-$C_{10:0}$-HSL (32 microorganisms)
P. aeruginosa PAO1 (Shaw, P. D. *et al.* 1997), *V. anguillarum* NB10 (Milton, D. L. *et al.* 1997; Buchholz, F. *et al.* 1998), *A. tumefaciens* Bo542, *E. carotovora* pv. *atroseptica* SR8 (Cha, C. *et al.* 1998), *P. aeruginosa* 6294 (Charlton, T. S. *et al.* 2000), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000), *B. vietnamiensis* R-921 (Gotschlich, A. *et al.* 2001), *P. putida* IsoF, *P. putida* Z2D (Steidle, A. *et al.* 2001), *B. vietnamiensis* G4 (Conway, B.-A. *et al.* 2002), *V. vulnificus* (Morin, D. *et al.* 2003), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *P. putida* WCS358 (Bertani, I. *et al.* 2004), *E. carotovora* SCC3193 (Brader, G. *et al.* 2005), *Vibrionaceae* MRa39 (Rasch, M. *et al.* 2005), *Y. enterocolitica* 90/54 (Atkinson, S. *et al.* 2006), *V. anguillarum* 90-11-287 (Buchholtz, C. *et al.* 2006), *P. putida* PCL1445 (Dubern, J.-F. *et al.* 2006), *Y. pestis* KIM6+ (Kirwan, J. P. *et al.* 2006), *A. lipoferum* TVV3 (Vial, L. *et al.* 2006), *B. pseudomallei* KHW (Chan, Y. Y. *et al.* 2007), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *Mesorhizobium* sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007), *P. atrosepticum* 6276 (Latour, X. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *G. diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012), *V. variabilis* T01 (Mohamad, N. I. *et al.* 2015)

oxo-$C_{10:1}$-HSL (1 microorganism)
S. liquefaciens ATCC27592 (Cataldi, T. R. I. *et al.* 2008)

OH-$C_{10:0}$-HSL (16 microorganisms)
S. meliloti AK631 (Teplitski, M. *et al.* 2003), *A. ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *Enterobacteriaceae* C3-1JM, *Enterobacteriaceae* MRa50, *Vibrionaceae* MRa39 (Rasch, M. *et al.* 2005), *R. gallaeciencis* T5 (Wagner-Döbler, I. *et al.* 2005), *V. anguillarum* 90-11-287, *V. anguillarum* NB10 (Buchholtz, C. *et al.* 2006), *B. pseudomallei* pp844 (Lumjiaktase, P. *et al.* 2006), *A. lipoferum* TVV3 (Vial, L. *et al.* 2006), *B. pseudomallei* KHW (Chan, Y. Y. *et al.* 2007), *Acidovorax* sp. N35 (Fekete, A. *et al.* 2007), *P. chlororaphis* 30-84, *P. chlororaphis* PCL1391(Khan, S. R. *et al.* 2007), *P. fluorescens* 2-79 (Shaw, P. D. *et al.* 1997; Khan, S. R. *et al.* 2007), *C. violaceum* ATCC12472 (Morohoshi, T. *et al.* 2008a)

oxo-$C_{11:0}$-HSL (1 microorganism)
Y. pseudotuberculosis YPIII (Ortori, C. *et al.* 2007)

oxo-$C_{11:2}$-HSL (1 microorganism)
S. liquefaciens ATCC27592 (Cataldi, T. R. I. *et al.* 2008)

$C_{12:0}$-HSL (25 microorganisms)
R. meliloti L5-30, *R. meliloti* YA2 (Shaw, P. D. *et al.* 1997), *P. aeruginosa* PAO579 (Wu, H. *et al.* 2000), *B. vietnamiensis* R-921 (Gotschlich, A. *et al.* 2001), *B. cepacia* G4 (Park, J.-H. *et al.* 2001), *B. vietnamiensis* G4 (Conway, B.-A. *et al.* 2002), *S. meliloti* Rm1021 (Marketon, M. M. *et al.* 2002), *B. cepacia* JA-7, *B. cepacia* LA-10 (Cataldi, T. R. I. *et al.* 2004), *A. ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *H. anticariensis* FP-36 (Llamas, I. *et al.* 2005), *A. hydrophila, A. salmonicida*, *P. aeruginosa*, *Y. enterocolitica*, *P. fluorescens*, *S liquefasciens* (Cataldi, T. R. I. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *G. intermedius* NCI1051 (Iida, A. *et al.* 2008), *A. baumannii* M2 (Niu, C. *et al.* 2008), *Brucella melitensis* 16M (Weeks, J. *et al.* 2010), *diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012), *P. aeruginosa* MW3A (Wong, C.-S. *et al.* 2012), *V. variabilis* T01 (Mohamad, N. I. *et al.* 2015)

$C_{12:1}$-HSL (2 microorganisms)
Mesorhizobium sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007), *G. intermedius* NCI1051 (Iida, A. *et al.* 2008)

oxo-$C_{12:0}$ (24 microorganisms)
P. aeruginosa PAO1 (Pearson, J. P. *et al.* 1994; Winson, M. K. *et al.* 1995; Shaw, P. D. *et al.* 1997), *Rhodobacter sphaeroides* 2.4.1 (Puskas, A. *et al.* 1997), *P. aeruginosa* 6294 (Charlton, T. S. *et al.* 2000), *P. aeruginosa* SH1(Geisenberger, O. *et al.* 2000), *P. putida* IsoF, *P. putida* Z2D (Steidle, A. *et al.* 2001), *V. vulnificus* (Morin, D. *et al.* 2003), *P. putida* WCS358 (Bertani, I. *et al.* 2004), *A. ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *Y. enterocolitica* 90/54 (Atkinson, S. *et al.* 2006), *V. anguillarum* 90-11-287, *V. anguillarum* NB10 (Buchholtz, C. *et al.* 2006), *P. putida* PCL1445 (Dubern, J.-F. *et al.* 2006), *Y. ruckeri* 150, *Y. ruckeri* 88-6-32, *Y. ruckeri* 88-6-44, *Y. ruckeri* 89-4-77, *Y. ruckeri* 89-5-113, *Y. ruckeri* NCIMB1316 (Kastbjerg, V. G. *et al.* 2007), *Mesorhizobium* sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *P. aeruginosa* PUPa3 (Babić, F. *et al.* 2010), *P. aeruginosa* PA14 (Venkataraman, A. *et al.* 2010), *diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012)

oxo-$C_{12:1}$-HSL (1 microorganism)
Mesorhizobium sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007)

OH-$C_{12:0}$-HSL (6 microorganisms)
A. ferrooxidans ATCC23270 (Farah, C. *et al.* 2005), *B. pseudomallei* pp844 (Lumjiaktase, P. *et al.* 2006), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *V. scophthalmi* A089, *V. scophthalmi* A102 (García-Aljaro, C. *et al.* 2008), *A. baumannii* M2 (Niu, C. *et al.* 2008)

$C_{13:0}$-HSL (1 microorganism)
Y. pseudotuberculosis YPIII (Ortori, C. *et al.* 2007)

oxo-$C_{13:0}$-HSL (1 microorganism)
Y. pseudotuberculosis YPIII (Ortori, C. *et al.* 2007)

oxo-$C_{13:2}$-HSL (1 microorganism)
S. liquefaciens ATCC27592 (Cataldi, T. R. I. *et al.* 2008)

$C_{14:0}$-HSL (22 microorganisms)
B. vietnamiensis R-921 (Gotschlich, A. *et al.* 2001), *Rhodobacter capsulatus* SB1003 (Schaefer, A. L. *et al.* 2002), *S. meliloti* 1021 (Teplitski, M. *et al.* 2003; Gao, M. *et al.* 2005), *B. cepacia* JA-7, *B. cepacia* LA-10 (Cataldi, T. R. I. *et al.* 2004), *A. ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *R. tolerans* EL52, *R. tolerans* EL78, *R. tolerans* EL83, *R. tolerans* EL90, *R. tolerans* EL164, *R. tolerans* EL171, *R. tolerans* EL172, *R. tolerans* EL222 (Wagner-Döbler, I. *et al.* 2005), *A. hydrophila, A. salmonicida*, *Y. enterocolitica* (Cataldi, T. R. I. *et al.* 2007), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008), *A. baumannii* M2 (Niu, C. *et al.* 2008), *Herbaspirillum frisingense* Mb11 (Rothballer, M. *et al.* 2008), *Pseudoalteromonas* sp. 520P1 (Wang, Y. *et al.* 2008), *G. diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012)

$C_{14:1}$-HSL (16 microorganisms)
A. vitis F2/5, *A. vitis* CG506 (Li, Y. *et al.* 2005), *Roseovarius mucosus* DFL24, *R. tolerans* EL52, *R. tolerans* EL78, *R. tolerans* EL83, *R. tolerans* EL90, *R. tolerans* EL164, *R. tolerans* EL171, *R. tolerans* EL172, *R. tolerans* EL222, *Jannaschia helgolandensis* HEL10, *J. helgolandensis* HEL26, *J. helgolandensis* HEL43 (Wagner-Döbler, I. *et al.* 2005), *M. extorquens* AM1 (Nieto Penalver, C. G. *et al.* 2006), *H. frisingense* Mb11 (Rothballer, M. *et al.* 2008)

$C_{14:2}$-HSL (1 microorganism)
M. extorquens AM1 (Nieto Penalver, C. G. *et al.* 2006)

oxo-$C_{14:0}$-HSL (13 microorganisms)
P. aeruginosa 6294 (Charlton, T. S. *et al.* 2000), *P. aeruginosa* SH1(Geisenberger, O. *et al.* 2000), *S. meliloti* Rm1021 (Marketon, M. M. *et al.* 2002), *V. vulnificus* (Morin, D. *et al.* 2003), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *S. meliloti* 1021 (Teplitski, M. *et al.* 2003; Gao, M. *et al.* 2005), *A. ferrooxidans* ATCC23270 (Farah, C. *et al.* 2005), *Y. enterocolitica* 90/54 (Atkinson, S. *et al.* 2006), *B. pseudomallei* KHW (Chan, Y. Y. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *S. fredii* ISP42 (Pérez-Montaño, F. *et al.* 2011), *diazotrophicus* PAL5 (Nieto-Peñalver, C. G. *et al.* 2012), *P. aeruginosa* MW3A (Wong, C.-S. *et al.* 2012)

oxo-$C_{14:1}$-HSL (3 microorganisms)
A. ferrooxidans ATCC23270 (Farah, C. *et al.* 2005), *R. tolerans* EL78, *R. tolerans* EL90 (Wagner-Döbler, I. *et al.* 2005)

OH-$C_{14:0}$-HSL (3 microorganisms)
Mesorhizobium sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007), *Y. pseudotuberculosis* YPIII (Ortori, C. *et al.* 2007), *H. frisingense* Mb11 (Rothballer, M. *et al.* 2008)

OH-$C_{14:1}$-HSL (7 microorganisms)
R. leguminosarum 8401 (Gray, K. M. *et al.* 1996; Lithgow, J. K. *et al.* 2000), *R. leguminosarum* bv. *viciae* 248 (Schripsema, J. *et al.* 1996), *Rhodobacter sphaeroides* 2.4.1 (Puskas, A. *et al.* 1997), *P. fluorescens* F113 (Laue, B. E. *et al.* 2000), *R. leguminosarum* 300, *R. leguminosarum* TOM, *R. leguminosarum* 8002 (Lithgow, J. K. *et al.* 2001)

$C_{15:0}$-HSL (1 microorganism)
Y. pseudotuberculosis YPIII (Ortori, C. *et al.* 2007)

OH-$C_{15:0}$-HSL (1 microorganisms)
Mesorhizobium sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007)

$C_{16:0}$-HSL (8 microorganisms)
R. capsulatus SB1003, *Paracoccus denitrificans* ATCC177441 (Schaefer, A. L. *et al.* 2002), *S. meliloti* 1021 (Teplitski, M. *et al.* 2003; Gao, M. *et al.* 2005), *R. tolerans* EL164, *R. tolerans* EL171, *Staleya guttiformis* LM09 (Wagner-Döbler, I. *et al.* 2005), *Mesorhizobium* sp. R8-Ret-T53-13d (Krick, A. *et al.* 2007), *A. baumannii* M2 (Niu, C. *et al.* 2008)

$C_{16:1}$-HSL (9 microorganisms)
S. meliloti Rm1021 (Marketon, M. M. *et al.* 2002), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *S. meliloti* 1021 (Teplitski, M. *et al.* 2003; Gao, M. *et al.* 2005), *A. vitis* F2/5, *A. vitis* CG506 (Li, Y. *et al.* 2005), *R. tolerans* EL164, *S. guttiformis* LM09, *J. helgolandensis* HEL10, *J. helgolandensis* HEL26 (Wagner-Döbler, I. *et al.* 2005)

$C_{16:2}$-HSL (4 microorganisms)
S. guttiformis LM09, *J. helgolandensis* HEL10, *J. helgolandensis* HEL26, *J. helgolandensis* HEL43 (Wagner-Döbler, I. *et al.* 2005; Thiel, V. *et al.* 2009)

oxo-$C_{16:0}$-HSL (1 microorganism)
S. meliloti 1021 (Gao, M. *et al.* 2005)

oxo-$C_{16:1}$-HSL (5 microorganisms)
S. meliloti Rm1021 (Marketon, M. M. *et al.* 2002), *S. meliloti* AK631 (Teplitski, M. *et al.* 2003), *S. meliloti* 1021 (Teplitski, M. *et al.* 2003; Gao, M. *et al.* 2005), *A. vitis* F2/5, *A. vitis* CG506 (Li, Y. *et al.* 2005)

OH-$C_{16:0}$-HSL (2 microorganisms)
A. ferrooxidans ATCC23270 (Farah, C. *et al.* 2005), *S. liquefaciens* ATCC27592 (Cataldi, T. R. I. *et al.* 2008)

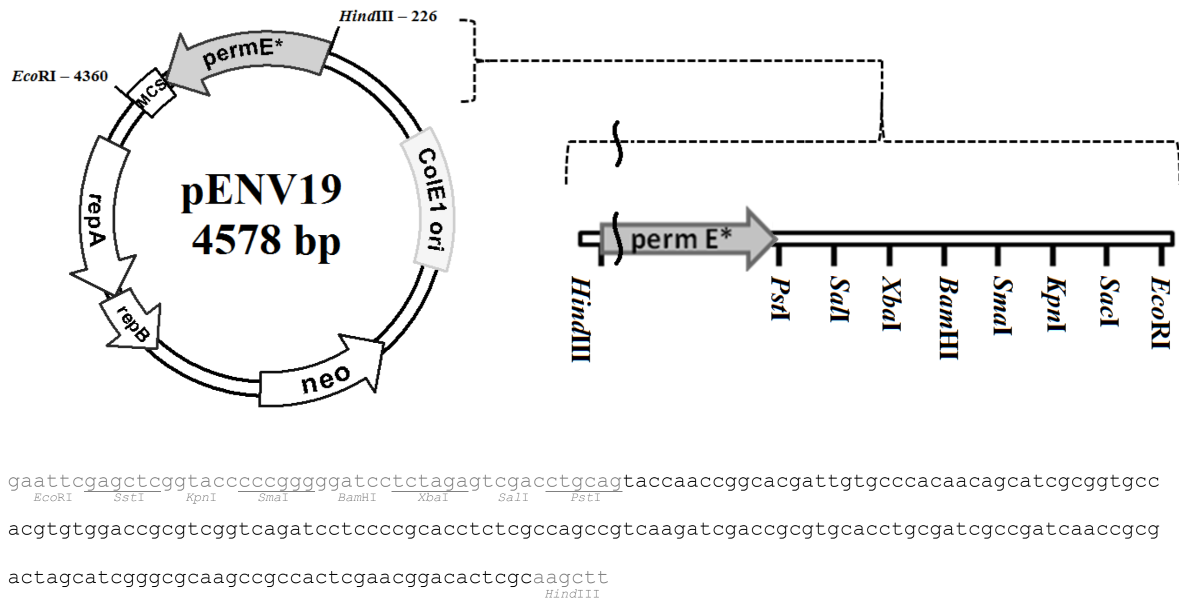
$C_{18:0}$-HSL (1 microorganism)
S. meliloti Rm1021 (Marketon, M. M. *et al.* 2002)

$C_{18:1}$-HSL (6 microorganisms)
D. shibae DFL27, *D. shibae* DFL30, *D. shibae* DFL31, *R. mucosus* DFL35, *R. mucosus* DFL24, *R. gallaeciencis* T5 (Wagner-Döbler, I. *et al.* 2005)

$C_{18:2}$-HSL (5 microorganisms)
D. shibae DFL16, *D. shibae* DFL27, *D. shibae* DFL30, *D. shibae* DFL31, *D. shibae* DFL36 (Wagner-Döbler, I. *et al.* 2005)

[1] The usage of $C_{n:m}$-HSL descriptors indicates m double bonds in an *N*-alkanoyl chain with n carbons, and in parentheses is displayed the number of strains with $C_{n:m}$-HSL involved in *quorum sensing*.

S.2. Sequence of vector pENV19



gaattcgagctcggtacccggggatcctctagagtcgacctgcagtaccaaccggcacgattgtgcccacaacagcatcgcggtgcc
acgtgtggaccgcgtcggtcagatcctccccgcacctctcgccagccgtcaagatcgaccgcgtgcacctgcgatcgccgatcaaccgcg
actagcatcgggcgcaagccgccactcgaacggacactcgcaagctt

*Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Hind*III

Figure S.2.1. Shuttle vector pENV19

S.3. Sequence of vector pENS

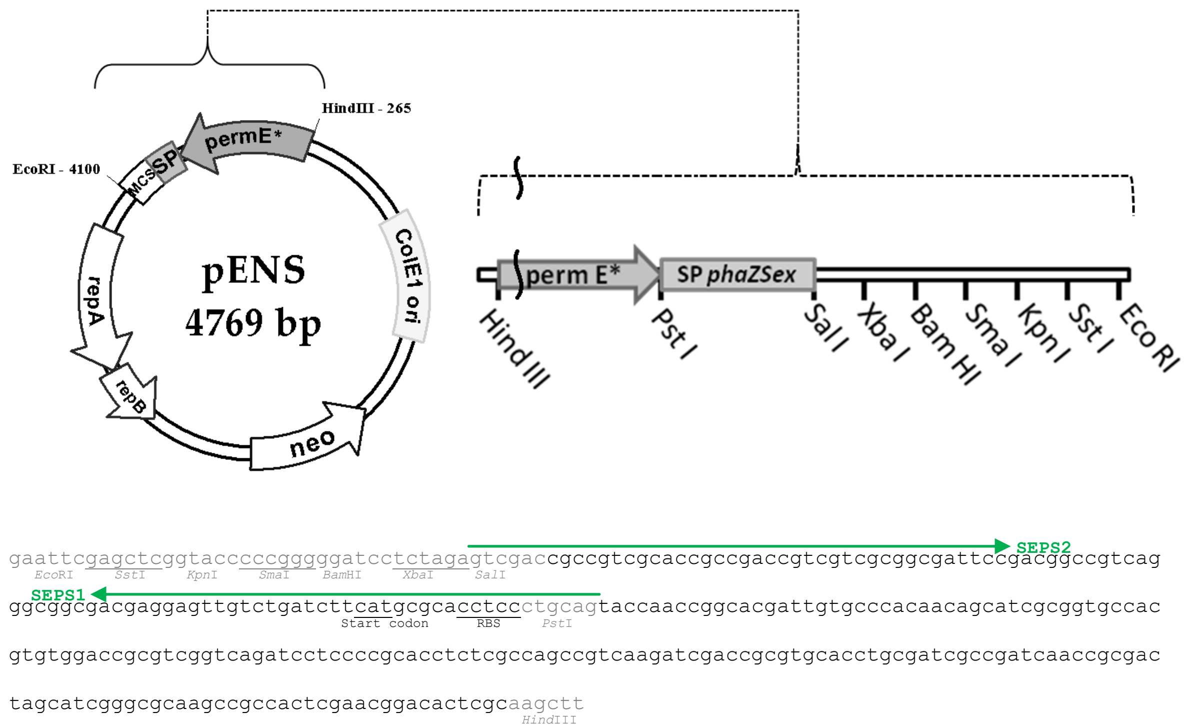


Figure S.3.1. Shuttle vector pENS

S.4. Nucleotide and amino acid sequences of *Sl*PVA in addition to p*ermE**

```
                                 aagcttgcgagtgtccgttcgagtggcggc     30
                                   HindIII
ttgcgcccgatgctagtcgcggttgatcggcgatcgcaggtgcacgcggtcgatcttgacggc     93
tggcgagaggtgcggggaggatctgaccgacgcggtccacacgtggcaccgcgatgctgttgt    156
gggcacaatcgtgccggttggtaggatccagacctgcaggtcgactctagaggaggaacaccc    219
                        BamHI     PstI    SalI     XbaI RBS consensus
                                      PVAatg
atg acc ttc cgt aac cgc ctc aga ctg ttc gcg gtc tcc ggt ctc gcc    267
 M   T   F   R   N   R   L   R   L   F   A   V   S   G   L   A     16
                                                          NCsac3
ctg ttc acc gtg tcg gcg tcg ctg cca ccc gcc gca gcc tcc gga gcg    315
 L   F   T   V   S   A   S   L   P   P   A   A   A   S   G   A     32
                                             αPVA
ccg gag gcc cgg cat ccg tcg ggc ggc ggc ctc tcg gcc acc gtc cgg    363
 P   E   A   R   H   P   S   G α G   G   L   S   A   T   V   R     48
                                                      RTP1
tac acg gag tac ggc att ccg cac atc gtg gcg aag gac tac gcg aac    411
 Y   T   E   Y   G   I   P   H   I   V   A   K   D   Y   A   N     64
                                                    N
ctg ggc ttc ggc acc ggc tgg gca cag gcc gcc gac cag gtg tgc acg    459
 L   G   F   G   T   G   W   A   Q   A   A   D   Q   V   C   T     80
ctg gcc gac ggg ttc gtc acg gta cgc ggc gag cgg tcg aag ttc ttc    507
 L   A   D   G   F   V   T   V   R   G   E   R   S   K   F   F     96
ggc ccg gac gcg gcc ccg gac ttc tcc ctc tcc tcg gcg gcg aag aac    555
 G   P   D   A   A   P   D   F   S   L   S   S   A   A   K   N    112
  RTP2
ctc tcc agc gat ctg tac ttc cgg ggc gtc cgg gac agc ggc acc gtg    603
 L   S   S   D   L   Y   F   R   G   V   R   D   S   G   T   V    128
gag aag ctg ctg aag gtc ccg gct ccg gcg ggt ccg agc cgg gac gcc    651
 E   K   L   L   K   V   P   A   P   A   G   P   S   R   D   A    144
                                                         NCsacI
aag gag tcg atg cgc gga ttc gcc gcc ggg tac aac gcc tgg ctc cgg    699
 K   E   S   M   R   G   F   A   A   G   Y   N   A   W   L   R    160
cag aac cgc gac cgc atc acc gac ccc gcc tgc cgg ggc gcc tcc tgg    747
 Q   N   R   D   R   I   T   D   P   A   C   R   G   A   S   W    176
gtg cgc ccg gtc acc gcg ctg gac gtg gcg gta cgg ggc ttc gcc ctg    795
 V   R   P   V   T   A   L   D   V   A   V   R   G   F   A   L    192
gcc gtg ctc ggc ggc cag ggg cgc ggc atc gac ggc atc acc gcc gcc    843
 A   V   L   G   G   Q   G   R   G   I   D   G   I   T   A   A    208
cag ccc ccg acg gcc gca ccg ccc gcc gcc ggg gtc acc ccg aag gag    891
 Q   P   P   T   A   A   P   P   A   A   G   V   T   P   K   E    224
gcg gca gcg gcg gcc cag cgg ctt ctg tcc acg cag aac gcc gac atg    939
 A   A   A   A   A   Q   R   L   L   S   T   Q   N   A   D   M    240
ggc tcc aac gcg gtc gcc ttc cgg ggg tcc acc acg gcg aac ggg cgc    987
 G   S β N   A   V   A   F   R   G   S   T   T   A   N   G   R    256
ggg ctg ctc ctc ggc aac ccg cac tat ccg tgg gac ggc ggc cgc cgc   1035
 G   L   L   L   G   N   P   H   Y   P   W   D   G   G   R   R    272
ttc tgg cag tcg cag cag acg atc ccg ggc gag ctg aac gtg gcg ggc   1083
 F   W   Q   S   Q   Q   T   I   P   G   E   L   N   V   A   G    288
gga tcc ctg ctc ggc tcg acg acc gtc tcg atc ggg cac aac gcg gac   1131
 G   S   L   L   G   S   T   T   V   S   I   G   H   N   A   D    304
gtg gcc tgg agc cac acg gtg gcg acc ggc gtc acg ctg aac ctg cac   1179
 V   A   W   S   H   T   V   A   T   G   V   T   L   N   L   H    320
                                                    C
cag ctg acc ctg gat ccg gcc gat ccc acg gtc tat ctg gtg gac ggg   1227
 Q   L   T   L   D   P   A   D   P   T   V   Y   L   V   D   G    336
aag ccg cag cgg atg acg cag cgc acg gtc gcc gtg ccg gtg aag ggc   1275
 K   P   Q   R   M   T   Q   R   T   V   A   V   P   V   K   G    352
gcc gcc ccg gtg acc cgg acc cag tgg tgg acc cgg tac ggc ccg gtg   1323
 A   A   P   V   T   R   T   Q   W   W   T   R   Y   G   P   V    368
gtc acc tcg ctg ggg gcg gcg ctg ccg ctg ccc tgg acg gcg agc acc   1371
 V   T   S   L   G   A   A   L   P   L   P   W   T   A   S   T    384
gcg tac gcg ctg aac gac ccg aac gcg gtg aac ctg cgc agc gcc gac   1419
 A   Y   A   L   N   D   P   N   A   V   N   L   R   S   A   D    400
acc tcg ctc ggc ttc agc aag gca cgc tcc acc gcc ggg atc gag cgg   1467
 T   S   L   G   F   S   K   A   R   S   T   A   G   I   E   R    416
```

```
gcg ctc cac cgg tcc cag ggg ctg ccc tgg gtg aac acg atc gcc gcc   1515
 A   L   H   R   S   Q   G   L   P   W   V   N   T   I   A   A     432
                                                         NCsac4
gac cgg tcg ggg aac tcc ttc ttc tcc cag tcg cag gtt ctg ccg agg   1563
 D   R   S   G   N   S   F   F   S   Q   S   Q   V   L   P   R     448
atc acg gac gag ctg gcg gca cgc tgc tcg acc ccg ctg ggc cag gcc   1611
 I   T   D   E   L   A   A   R   C   S   T   P   L   G   Q   A     464
acc tac ccg tcg gcc ggg ctc gcg gtg ctg gac gga tcg acg tcg gcc   1659
 T   Y   P   S   A   G   L   A   V   L   D   G   S   T   S   A     480
tgc gcg ctg ggg agc gac cgg gac gcg gta cag ccg ggg atc ttc ggg   1707
 C   A   L   G   S   D   R   D   A   V   Q   P   G   I   F   G     496
ccg ggc cgg atg ccg acg ctg aag aac gcc ccg tac gtc gag aac tcc   1755
 P   G   R   M   P   T   L   K   N   A   P   Y   V   E   N   S     512
aac gac agc gcc tgg ctg acc aac gcc gac cgc ccg ctg acc ggt tac   1803
 N   D   S   A   W   L   T   N   A   D   R   P   L   T   G   Y     528
gag cgg gtc ttc ggc acg acc gcc acc cag cgg tcg atc cgg acc cgg   1851
 E   R   V   F   G   T   T   A   T   Q   R   S   I   R   T   R     544
ggc gcg atc gag gat gtc gcg gcg atg gcg gag cgc ggg cgg ctg cgt   1899
 G   A   I   E   D   V   A   A   M   A   E   R   G   R   L   R     560
gtg acg gac ctg gag cgc cag cag ctc gcc aac cgg gcg ccg acc ggg   1947
 V   T   D   L   E   R   Q   Q   L   A   N   R   A   P   T   G     576
gat ctg gtc gcg gcc gac gtg gcg aag tgg tgc gcc gcc ctg ccc ggc   1995
 D   L   V   A   A   D   V   A   K   W   C   A   A   L   P   G     592
ggg acc gcc gtg ggc agc agc ggt acg ccg gtc gac gtg tcg gcg gcc   2043
 G   T   A   V   G   S   S   G   T   P   V   D   V   S   A   A     608
tgc ccg gtg ctg cgg cgg tgg gac cgg agc gtg gac agc gac agc cgg   2091
 C   P   V   L   R   R   W   D   R   S   V   D   S   D   S   R     624
                                     CsacII
ggc gcg ctg ctc ttc gac cgg ttc tgg cgc aag gcg gcg gcg gtg ccc   2139
 G   A   L   L   F   D   R   F   W   R   K   A   A   A   V   P     640
gcg gcc gag ctg tgg aag gta ccg ttc gac gcg gcc gat ccg gta cgg   2187
 A   A   E   L   W   K   V   P   F   D   A   A   D   P   V   R     656
acc ccg cgc ggc ctc aac acc gcc gca ccc ggc gtg ggg aag gcg ctg   2235
 T   P   R   G   L   N   T   A   A   P   G   V   G   K   A   L     672
gcc gac acg gtg acg gag ctg aag gcg gcg ggc atc gcg ctc aac gcg   2283
 A   D   T   V   T   E   L   K   A   A   G   I   A   L   N   A     688
cct ttg ggt gag cac cag ttc gtc gta cgg aac ggg aag cgc atc ccg   2331
 P   L   G   E   H   Q   F   V   V   R   N   G   K   R   I   P     704
gtc ggc ggc ggc acg gag tcg ctc ggc atc tgg aac aag atc gag ccg   2379
 V   G   G   G   T   E   S   L   G   I   W   N   K   I   E   P     720
gtg tgg aac ccg gcg gcg ggc ggc tac acc gag gtg tcg gcc ggg tcc   2427
 V   W   N   P   A   A   G   G   Y   T   E   V   S   A   G   S     736
                             PVA Stop
agc tac atc cag gcg gtc ggc tgg gac aac agc cgg tgc ccg gtg gcc   2475
 S   Y   I   Q   A   V   G   W   D   N   S   R   C   P   V   A     752
cgg acg ctg ctc acg tac tcc cag tcc tcg aac ccg aac tcg ccg cac   2523
 R   T   L   L   T   Y   S   Q   S   S   N   P   N   S   P   H     768
tac agc gac cag acg cgg ctg ttc tcg ggt gag cgc tgg gtg acg tcc   2571
 Y   S   D   Q   T   R   L   F   S   G   E   R   W   V   T   S     784
cgg ttc tgc gag aag gac atc gcc cgc tcg ccg cag ctg aag gtg gtg   2619
 R   F   C   E   K   D   I   A   R   S   P   Q   L   K   V   V     800
                                   PVA-F
cgg gtg cac gag cgg cgg tag gaattc                                2646
 R   V   H   E   R   R   -    EcoRI                                806
```

Figure S.4.1. Nucleotide and amino acid sequences of *Sl*PVA in addition to p*ermE** sequence. GenBank: AAU09670.1. Throughout nucleotides sequence: Restriction sites are highlighted in yellow, RBS consensus from *Streptomyces* is highlighted in gray, start codon (atg) is highlighted in green, end codon (tag) is highlighted in red, primer sequences are under green arrows. Throughout amino acids sequence: signal peptide in red, catalytic residues in blue, residues forming substrate binding pocket in orange, α and β represent the beginning of corresponding subunit.

S.5. Nucleotide and amino acid sequences *Au*AAC in addition to of p*ermE**

```
                                            aagcttgcgagtgtccgttcgagtggcggc      30
                                             HindIII

ttgcgcccgatgctagtcgcggttgatcggcgatcgcaggtgcacgcggtcgatcttgacggc      93
tggcgagaggtgcggggaggatctgaccgacgcggtccacacgtggcaccgcgatgctgttgt     156
gggcacaatcgtgccggttggtaggatccagacctgcaggtcgactctagaggaggtgccgcc     219
                        BamHI     PstI    SalI   XbaI RBS consensus
                                       AACgtg
gtg acg tcc tcg tac atg cgc ctg aaa gca gca gcg atc gcc ttc ggt     267
 V   T   S   S   Y   M   R   L   K   A   A   A   I   A   F   G      16
gtg atc gtg gcg acc gca gcc gtg ccg tca ccc gct tcc ggc agg gaa     315
 V   I   V   A   T   A   A   V   P   S   P   A   S   G   R   E      32
                   RTA1            αAAC
cat gac ggc ggc tat gcg gcc ctg atc cgc cgg gcc tcg tac ggc gtc     363
 H   D   G α G   Y   A   A   L   I   R   R   A   S   Y   G   V      48
ccg cac atc acc gcc gac gac ttc ggg agc ctc ggt ttc ggc gtc ggg     411
 P   H   I   T   A   D   D   F   G   S   L   G   F   G   V   G      64
                      RTA2                AAC-Nt
tac gtg cag gcc gag gac aac atc tgc gtc atc gcc gag agc gtg gtg     459
 Y   V   Q   A   E   D   N   I   C   V   I   A   E   S   V   V      80
acg gcc aac ggt gag cgg tcg cgg tgg ttc ggt gcg acc ggg ccg gac     507
 T   A   N   G   E   R   S   R   W   F   G   A   T   G   P   D      96
gac gcc gat gtg cgc agc gac ctc ttc cac cgc aag gcg atc gac gac     555
 D   A   D   V   R   S   D   L   F   H   R   K   A   I   D   D     112
cgc gtc gcc gag cgg ctc ctc gaa ggg ccc cgc gac ggc gtg cgg gcg     603
 R   V   A   E   R   L   L   E   G   P   R   D   G   V   R   A     128
ccg tcg gac gac gtc cgg gac cag atg cgc ggc ttc gtc gcc ggc tac     651
 P   S   D   D   V   R   D   Q   M   R   G   F   V   A   G   Y     144
aac cac ttc cta cgc cgc acc ggc gtg cac cgc ctg acc gac ccg gcg     699
 N   H   F   L   R   R   T   G   V   H   R   L   T   D   P   A     160
tgc cgc ggc aag gcc tgg gtg cgc ccg ctc tcc gag atc gat ctc tgg     747
 C   R   G   K   A   W   V   R   P   L   S   E   I   D   L   W     176
cgt acg tcg tgg gac agc atg gtc cgg gcc ggt tcc ggg gcg ctg ctc     795
 R   T   S   W   D   S   M   V   R   A   G   S   G   A   L   L     192
                                           AAC-4
gac ggc atc gtc gcc gcg acg cca ccg aca gcc gcc ggg ccc gcg tca     843
 D   G   I   V   A   A   T   P   P   T   A   A   G   P   A   S     208
gcc ccg gag gca ccc gac gcc gcc gcg atc gcc gcc gcc ctc gac ggg     891
 A   P   E   A   P   D   A   A   A   I   A   A   A   L   D   G     224
acg agc gcg ggc atc ggc agc aac gcg tac ggc ctc ggc gcg cag gcc     939
 T   S   A   G   I   G   S β N   A   Y   G   L   G   A   Q   A     240
acc gtg aac ggc agc ggg atg gtg ctg gcc aac ccg cac ttc ccg tgg     987
 T   V   N   G   S   G   M   V   L   A   N   P   H   F   P   W     256
cag ggc gcc gaa cgc ttc tac cgg atg cac ctc aag gtg ccc ggc cgc    1035
 Q   G   A   E   R   F   Y   R   M   H   L   K   V   P   G   R     272
tac gac gtc gag ggc gcg gcg ctg atc ggc gac ccg atc atc gag atc    1083
 Y   D   V   E   G   A   A   L   I   G   D   P   I   I   E   I     288
ggg cac aac cgc acg gtc gcc tgg agc cac acc gtc tcc acc gcc cgc    1131
 G   H   N   R   T   V   A   W   S   H   T   V   S   T   A   R     304
cgg ttc gtg tgg cac cgc ctg agc ctc gtg ccc ggc gac ccc acc tcc    1179
 R   F   V   W   H   R   L   S   L   V   P   G   D   P   T   S     320
tat tac gtc gac ggc cgg ccc gag cgg atg cgc gcc cgc acg gtc acg    1227
 Y   Y   V   D   G   R   P   E   R   M   R   A   R   T   V   T     336
gtc cag acc ggc agc ggc ccg gtc agc cgc acc ttc cac gac acc cgc    1275
 V   Q   T   G   S   G   P   V   S   R   T   F   H   D   T   R     352
                                                            AAC-5
tac ggc ccg gtg gcc gtg gtg ccg ggc acc ttc gac tgg acg ccg gcc    1323
 Y   G   P   V   A   V   V   P   G   T   F   D   W   T   P   A     368
acc gcg tac gcc atc acc gac gtc aac gcg ggc aac aac cgc gcc ttc    1371
 T   A   Y   A   I   T   D   V   N   A   G   N   N   R   A   F     384
gac ggg tgg ctg cgg atg ggc cag gcc aag gac gtc cgg gcg ctc aag    1419
 D   G   W   L   R   M   G   Q   A   K   D   V   R   A   L   K     400
gcg gtc ctc gac cgg cac cag ttc ctg ccc tgg gtc aac gtg atc gcc    1467
 A   V   L   D   R   H   Q   F   L   P   W   V   N   V   I   A     416
```

```
gcc gac gcg cgg ggc gag gcc ctc tac ggc gat cat tcg gtc gtc ccc   1515
 A   D   A   R   G   E   A   L   Y   G   D   H   S   V   V   P     432
cgg gtg acc ggc gcg ctc gct gcc gcc tgc atc ccg gcg ccg ttc cag   1563
 R   V   T   G   A   L   A   A   A   C   I   P   A   P   F   Q     448
ccg ctc tac gcc tcc agc ggc cag gcg gtc ctg gac ggt tcc cgg tcg   1611
 P   L   Y   A   S   S   G   Q   A   V   L   D   G   S   R   S     464
gac tgc gcg ctc ggc gcc gac ccc gac gcc gcg gtc ccg ggc att ctc   1659
 D   C   A   L   G   A   D   P   D   A   A   V   P   G   I   L     480
ggc ccg gcg agc ctg ccg gtg cgg ttc cgc gac gac tac gtc acc aac   1707
 G   P   A   S   L   P   V   R   F   R   D   D   Y   V   T   N     496
tcc aac gac agt cac tgg ctg gcc agc ccg gcc gcc ccg ctg gaa ggc   1755
 S   N   D   S   H   W   L   A   S   P   A   A   P   L   E   G     512
ttc ccg cgg atc ctc ggc aac gaa cgc acc ccg cgc agc ctg cgc acc   1803
 F   P   R   I   L   G   N   E   R   T   P   R   S   L   R   T     528
cgg ctc ggg ctg gac cag atc cag cag cgc ctc gcc ggc acg gac ggt   1851
 R   L   G   L   D   Q   I   Q   Q   R   L   A   G   T   D   G     544
ctg ccc ggc aag ggc ttc acc acc gcc cgg ctc tgg cag gtc atg ttc   1899
 L   P   G   K   G   F   T   T   A   R   L   W   Q   V   M   F     560
             AAC-6
ggc aac cgg atg cac ggc gcc gaa ctc gtc cgc gac gac ctg gtc gcg   1947
 G   N   R   M   H   G   A   E   L   V   R   D   D   L   V   A     576
ctc tgc cgc cgc cag ccg acc gcg acc gcc tcg aac ggc gcg atc gtc   1995
 L   C   R   R   Q   P   T   A   T   A   S   N   G   A   I   V     592
gac ctc acc gcg gcc tgc acg gcg ctg tcc cgc ttc gat gag cgt gcc   2043
 D   L   T   A   A   C   T   A   L   S   R   F   D   E   R   A     608
gac ctg gac agc cgg ggc gcg cac ctg ttc acc gag ttc gcc ctc gcg   2091
 D   L   D   S   R   G   A   H   L   F   T   E   F   A   L   A     624
ggc gga atc agg ttc gcc gac acc ttc gag gtg acc gat ccg gta cgc   2139
 G   G   I   R   F   A   D   T   F   E   V   T   D   P   V   R     640
acc ccg cgc cgt ctg aac acc acg gat ccg cgg gta cgg acg gcg ctc   2187
 T   P   R   R   L   N   T   T   D   P   R   V   R   T   A   L     656
gcc gac gcc gtg caa cgg ctc gcc ggc atc ccc ctc gac gcg aag ctg   2235
 A   D   A   V   Q   R   L   A   G   I   P   L   D   A   K   L     672
gga gac atc cac acc gac agc cgc ggc gaa cgg cgc atc ccc atc cac   2283
 G   D   I   H   T   D   S   R   G   E   R   R   I   P   I   H     688
ggt ggc cgc ggg gaa gca ggc acc ttc aac gtg atc acc aac ccg ctc   2331
 G   G   R   G   E   A   G   T   F   N   V   I   T   N   P   L     704
                             AAC-Ct
gtg ccg ggc gtg gga tac ccg cag gtc gtc cac gga aca tcg ttc gtg   2379
 V   P   G   V   G   Y   P   Q   V   V   H   G   T   S   F   V     720
atg gcc gtc gaa ctc ggc ccg cac ggc ccg tcg gga cgg cag atc ctc   2427
 M   A   V   E   L   G   P   H   G   P   S   G   R   Q   I   L     736
acc tat gcg cag tcg act aac ccg aac tca ccc tgg tac gcc gac cag   2475
 T   Y   A   Q   S   T   N   P   N   S   P   W   Y   A   D   Q     752
acc gtg ctc tac tcg cgg aag ggc tgg gac acc atc aag tac acc gag   2523
 T   V   L   Y   S   R   K   G   W   D   T   I   K   Y   T   E     768
                                                AAC-2
gcg cag atc gcg gcc gac ccg aac ctg cgc gtc tac cgg gtg gca cag   2571
 A   Q   I   A   A   D   P   N   L   R   V   Y   R   V   A   Q     784
cgg gga cgc tga ggaattc                                           2589
 R   G   R   -    EcoRI                                            787
```

Figure S.5.1. Nucleotide and amino acid sequences of *Au*AAC in addition to p*ermE** sequence. GenBank: WP_043523659. Throughout nucleotides sequence: restriction sites are highlighted in yellow, RBS consensus from *Streptomyces* is highlighted in gray, start codon (gtg) is highlighted in green, end codon (tga) is highlighted in red, primer sequences are under green arrows. Throughout amino acids sequence: signal peptide in red, catalytic residues in blue, residues forming substrate binding pocket in orange, α and β represent the beginning of corresponding subunit.

S.6. Residues related with the performance of acylases

Table S.6.1. Predicted and reported residues involved in the catalytic pocket of acylases. Text in red showing mutated amino acids here or in related studies. Cells with gray background represents ligand binding residues

		Catalytic residues				Mutations				Residues involved in the performance of acylases						
Enzyme	*Sl* PVA	βSer^{1}	βHis^{23}	βVal^{70}	βAsn^{272}	αAla^{169}	αAla^{189}	βVal^{204}	βArg^{373}	αAla^{113}	αArg^{124}	αAla^{131}	αArg^{133}	αAla^{154}	αGly^{158}	αThr^{167}
	Au AAC	βSer^{1}	βHis^{23}	βVal^{70}	βAsn^{268}	αAla^{164}	αIle^{184}	βVal^{200}	βArg^{373}	αVal^{107}	αGly^{118}	-	αArg^{128}	αMet^{149}	αGly^{153}	αVal^{162}
	Au AHLA	βSer^{1}	βHis^{23}	βVal^{70}	βAsn^{274}	αPro^{172}	αAla^{192}	βVal^{206}	βAla^{380}	αVal^{115}	αGly^{126}	αAla^{134}	αArg^{136}	αVal^{157}	αGly^{161}	αVal^{170}
Nonpolar	Met	0.0%	0.0%	6.9%	0.0%	1.1%	3.4%	1.7%	0.0%	0.0%	0.6%	0.6%	0.0%	0.6%	4.6%	0.0%
	Ala	0.0%	0.0%	11.5%	0.0%	43.1%	9.2%	9.8%	45.4%	6.3%	4.6%	28.2%	27.6%	28.7%	9.8%	5.2%
	Leu	0.0%	0.0%	1.1%	0.0%	6.3%	28.7%	5.7%	1.1%	13.8%	6.9%	17.2%	6.9%	6.3%	23.6%	0.0%
	Pro	0.0%	0.0%	0.0%	0.0%	2.9%	2.3%	0.6%	0.0%	26.4%	4.6%	3.4%	4.6%	2.9%	8.6%	41.4%
	Ile	0.0%	0.0%	1.1%	0.0%	8.6%	9.2%	1.1%	0.0%	0.0%	1.7%	1.1%	0.6%	3.4%	2.9%	0.6%
	Trp	0.0%	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.1%	1.7%	0.0%	0.6%	0.0%
	Phe	0.0%	0.0%	9.2%	0.0%	1.7%	2.9%	1.7%	0.0%	0.0%	0.6%	0.6%	0.6%	0.6%	2.9%	0.0%
	Val	0.0%	0.0%	32.8%	0.0%	5.2%	8.6%	16.1%	0.0%	2.3%	2.3%	5.2%	2.3%	4.6%	8.0%	0.0%
	Total	**0.0%**	**0.0%**	**63.2%**	**0.0%**	**69.0%**	**64.4%**	**36.8%**	**46.6%**	**48.9%**	**21.3%**	**57.5%**	**44.3%**	**47.1%**	**60.9%**	**47.1%**
Polar	Gly	0.0%	0.0%	0.0%	0.0%	2.9%	0.6%	2.3%	4.6%	4.0%	3.4%	5.7%	8.0%	2.3%	1.7%	27.6%
	Thr	0.0%	0.0%	1.7%	0.6%	4.6%	0.6%	11.5%	3.4%	4.0%	2.9%	2.3%	4.0%	8.6%	1.1%	1.7%
	Ser	100.0%	0.0%	4.0%	0.6%	6.9%	1.1%	4.0%	6.3%	4.6%	6.3%	5.2%	4.6%	4.6%	4.0%	6.3%
	Cys	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	Tyr	0.0%	0.0%	2.3%	0.0%	0.0%	0.6%	0.6%	0.0%	0.0%	5.2%	1.7%	0.6%	1.1%	2.3%	0.0%
	Asn	0.0%	0.0%	23.6%	98.9%	1.1%	1.1%	1.1%	5.7%	1.7%	1.1%	1.7%	1.1%	0.0%	1.1%	0.0%
	Gln	0.0%	12.6%	0.0%	0.0%	1.1%	4.0%	2.3%	5.7%	7.5%	8.6%	8.6%	1.7%	3.4%	1.1%	0.0%
	Total	**100.0%**	**12.6%**	**31.6%**	**100.0%**	**20.1%**	**8.0%**	**21.8%**	**25.9%**	**21.8%**	**27.6%**	**25.3%**	**20.1%**	**20.1%**	**11.5%**	**35.6%**
Acidic	Asp	0.0%	0.6%	0.0%	0.0%	1.7%	4.0%	1.1%	6.9%	8.0%	20.7%	1.1%	5.7%	1.7%	0.6%	0.0%
	Glu	0.0%	0.0%	0.0%	0.0%	2.9%	1.1%	0.0%	5.2%	4.0%	16.7%	1.1%	5.7%	5.2%	3.4%	0.0%
	Total	**0.0%**	**0.6%**	**0.0%**	**0.0%**	**4.6%**	**5.2%**	**1.1%**	**12.1%**	**12.1%**	**37.4%**	**2.3%**	**11.5%**	**6.9%**	**4.0%**	**0.0%**
Basic	His	0.0%	83.9%	5.2%	0.0%	0.0%	0.6%	2.3%	0.0%	2.3%	2.9%	0.0%	0.0%	0.0%	0.6%	0.0%
	Lys	0.0%	0.0%	0.0%	0.0%	1.7%	1.1%	8.0%	6.9%	4.0%	1.7%	2.3%	6.9%	0.6%	0.6%	13.2%
	Arg	0.0%	2.9%	0.0%	0.0%	0.0%	3.4%	29.9%	8.6%	3.4%	2.9%	5.2%	1.1%	6.9%	4.6%	4.0%
	Total	**0.0%**	**86.8%**	**5.2%**	**0.0%**	**1.7%**	**5.2%**	**40.2%**	**15.5%**	**9.8%**	**7.5%**	**7.5%**	**8.0%**	**7.5%**	**5.7%**	**17.2%**
Others	-	0.0%	0.0%	0.0%	0.0%	4.0%	16.7%	0.0%	0.0%	6.9%	5.7%	6.9%	15.5%	17.8%	17.2%	0.0%
	nd	0.0%	0.0%	0.0%	0.0%	0.6%	0.6%	0.0%	0.0%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.0%
	Total	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**4.6%**	**17.2%**	**0.0%**	**0.0%**	**7.5%**	**6.3%**	**7.5%**	**16.1%**	**18.4%**	**17.8%**	**0.0%**
	$H_1(l)$	**0.000**	**0.540**	**1.930**	**0.071**	**2.180**	**2.348**	**2.241**	**1.904**	**2.437**	**2.594**	**2.412**	**2.413**	**2.341**	**2.485**	**1.545**
	$H_2(l)$	**0.000**	**0.414**	**0.807**	**0.000**	**0.932**	**1.096**	**1.118**	**1.250**	**1.361**	**1.423**	**1.141**	**1.431**	**1.369**	**1.152**	**1.025**

Table S.6.1. Predicted and reported residues involved in the catalytic pocket of acylases. Text in red showing mutated amino acids here or in related studies. Cells with gray background represents ligand binding residues (continuation)

		Residues involved in the performance of acylases														
Enzyme	*Sl* PVA	αGly^{180}	αVal^{181}	αThr^{182}	αPro^{183}	αAla^{187}	αArg^{192}	αLeu^{193}	βGly^{8}	βThr^{10}	βTyr^{24}	βArg^{31}	βPhe^{32}	βTrp^{33}	βLeu^{50}	βSer^{53}
	Au AAC	αAla^{175}	αPro^{176}	αGlu^{177}	αAla^{178}	αAla^{182}	αAla^{187}	αLeu^{188}	βAla^{8}	βAla^{10}	βPhe^{24}	βArg^{31}	βPhe^{32}	βTyr^{33}	βLeu^{50}	βAsp^{53}
	Au AHLA	αAla^{183}	αThr^{184}	αPro^{185}	αAla^{186}	αGlu^{190}	αVal^{195}	αArg^{196}	βArg^{8}	βGly^{10}	βTyr^{24}	βArg^{31}	βPhe^{32}	βTrp^{33}	βLeu^{50}	βPhe^{53}
Nonpolar	Met	5.7%	0.0%	0.6%	0.6%	0.0%	0.6%	4.6%	17.2%	2.9%	1.7%	0.6%	6.9%	0.0%	0.0%	4.6%
	Ala	22.4%	6.3%	4.6%	28.2%	27.6%	28.7%	9.8%	5.7%	6.3%	5.7%	1.7%	5.2%	0.6%	1.7%	6.9%
	Leu	5.2%	13.8%	6.9%	17.2%	6.9%	6.3%	23.6%	27.0%	18.4%	13.8%	0.0%	9.2%	0.0%	20.1%	4.6%
	Pro	10.9%	26.4%	4.6%	3.4%	4.6%	2.9%	8.6%	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	4.0%
	Ile	1.1%	0.0%	1.7%	1.1%	0.6%	3.4%	2.9%	5.2%	6.9%	40.2%	0.0%	2.3%	0.0%	1.7%	0.0%
	Trp	0.0%	0.0%	0.0%	1.1%	1.7%	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	25.9%	0.0%
	Phe	2.9%	0.0%	0.6%	0.6%	0.6%	0.6%	2.9%	2.3%	2.3%	28.2%	0.0%	6.3%	0.0%	13.8%	13.8%
	Val	4.0%	2.3%	2.3%	5.2%	2.3%	4.6%	8.0%	17.2%	21.3%	3.4%	0.0%	19.0%	0.6%	6.9%	0.0%
	Total	**52.3%**	**48.9%**	**21.3%**	**57.5%**	**44.3%**	**47.1%**	**60.9%**	**74.7%**	**58.6%**	**93.1%**	**2.3%**	**48.9%**	**1.1%**	**70.1%**	**33.9%**
Polar	Gly	6.9%	4.0%	3.4%	5.7%	8.0%	2.3%	1.7%	0.0%	1.7%	1.1%	37.9%	0.0%	0.0%	5.7%	17.8%
	Thr	2.9%	4.0%	2.9%	2.3%	4.0%	8.6%	1.1%	6.3%	0.0%	0.0%	31.6%	0.6%	93.7%	0.6%	23.0%
	Ser	9.8%	4.6%	6.3%	5.2%	4.6%	4.6%	4.0%	6.3%	9.2%	2.3%	19.5%	10.9%	5.2%	4.0%	4.0%
	Cys	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.6%	0.0%	0.0%	8.6%	0.0%	0.0%	0.0%	0.6%
	Tyr	0.6%	0.0%	5.2%	1.7%	0.6%	1.1%	2.3%	9.2%	0.6%	0.0%	0.0%	1.7%	0.0%	17.8%	2.9%
	Asn	1.7%	1.7%	1.1%	1.7%	1.1%	0.0%	1.1%	1.1%	13.2%	1.1%	0.0%	12.1%	0.0%	0.0%	2.9%
	Gln	1.7%	7.5%	8.6%	8.6%	1.7%	3.4%	1.1%	1.1%	1.1%	1.1%	0.0%	0.0%	0.0%	0.0%	2.9%
	Total	**23.6%**	**21.8%**	**27.6%**	**25.3%**	**20.1%**	**20.1%**	**11.5%**	**24.7%**	**25.9%**	**5.7%**	**97.7%**	**25.3%**	**98.9%**	**28.2%**	**54.0%**
Acidic	Asp	0.6%	8.0%	20.7%	1.1%	5.7%	1.7%	0.6%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	Glu	5.2%	4.0%	16.7%	1.1%	5.7%	5.2%	3.4%	0.0%	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%	3.4%
	Total	**5.7%**	**12.1%**	**37.4%**	**2.3%**	**11.5%**	**6.9%**	**4.0%**	**0.6%**	**1.1%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**3.4%**
Basic	His	0.6%	2.3%	2.9%	0.0%	0.0%	0.0%	0.6%	0.0%	4.6%	1.1%	0.0%	25.9%	0.0%	0.0%	1.7%
	Lys	3.4%	4.0%	1.7%	2.3%	6.9%	0.6%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.3%
	Arg	6.3%	3.4%	2.9%	5.2%	1.1%	6.9%	4.6%	0.0%	9.8%	0.0%	0.0%	0.0%	0.0%	0.0%	4.6%
	Total	**10.3%**	**9.8%**	**7.5%**	**7.5%**	**8.0%**	**7.5%**	**5.7%**	**0.0%**	**14.4%**	**1.1%**	**0.0%**	**25.9%**	**0.0%**	**0.0%**	**8.6%**
Others	-	7.5%	6.9%	5.7%	6.9%	15.5%	17.8%	17.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	0.0%
	nd	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	Total	**8.0%**	**7.5%**	**6.3%**	**7.5%**	**16.1%**	**18.4%**	**17.8%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**1.7%**	**0.0%**
	$H_1(l)$	**2.597**	**2.437**	**2.594**	**2.412**	**2.413**	**2.341**	**2.485**	**2.094**	**2.276**	**1.639**	**1.362**	**2.080**	**0.274**	**1.970**	**2.395**
	$H_2(l)$	**1.283**	**1.361**	**1.423**	**1.141**	**1.431**	**1.369**	**1.152**	**0.593**	**0.993**	**0.282**	**0.109**	**1.047**	**0.063**	**0.676**	**1.027**

Table S.6.1. Predicted and reported residues involved in the catalytic pocket of acylases. Text in red showing amino acids mutated here or in related studies. Cells with gray background represents ligand binding residues (continuation)

	Enzyme	Residues involved in the performance of acylases															
Enzyme	*Sl*PVA	βSer[53]	βSer[57]	βIle[58]	βSer[67]	βHis[68]	βThr[69]	βThr[72]	βThr[75]	βHis[79]	βSer[161]	βLeu[183]	βVal[186]	βArg[207]	βGly[450]	βTyr[453]	βTyr[497]
	*Au*AAC	βAsp[53]	βGlu[57]	βIle[58]	βSer[67]	βHis[68]	βThr[69]	βThr[72]	βArg[75]	βHis[79]	βTrp[157]	βLeu[179]	βVal[182]	βArg[203]	βGly[443]	βPhe[446]	βPhe[489]
	*Au*AHLA	βPhe[53]	βLeu[57]	βIle[58]	βSer[67]	βHis[68]	βThr[69]	βThr[72]	βThr[75]	βPhe[79]	βTrp[163]	βVal[185]	βLeu[188]	βHis[209]	βGly[456]	βPhe[459]	βPhe[502]
Nonpolar	Met	17.2%	2.9%	1.7%	0.6%	6.9%	0.0%	4.6%	1.7%	0.0%	0.0%	2.9%	0.0%	0.0%	0.6%	0.6%	3.4%
	Ala	5.7%	6.3%	5.7%	1.7%	5.2%	0.6%	6.9%	1.7%	0.6%	2.3%	8.0%	0.0%	1.7%	8.0%	1.1%	0.6%
	Leu	27.0%	18.4%	13.8%	0.0%	9.2%	0.0%	4.6%	2.3%	0.0%	19.5%	6.9%	14.9%	8.0%	0.0%	4.0%	5.7%
	Pro	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%	4.0%	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.6%	1.1%
	Ile	5.2%	6.9%	40.2%	0.0%	2.3%	0.0%	0.0%	8.0%	1.1%	1.7%	20.7%	8.0%	14.9%	0.6%	2.9%	1.7%
	Trp	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.6%	26.4%	0.0%	0.6%	0.0%	0.6%	6.3%	25.3%
	Phe	2.3%	2.3%	28.2%	0.0%	6.3%	0.0%	13.8%	0.6%	23.6%	13.8%	0.0%	14.4%	0.6%	0.0%	0.6%	16.1%
	Val	17.2%	21.3%	3.4%	0.0%	19.0%	0.6%	0.0%	20.1%	1.7%	6.9%	29.3%	16.1%	8.6%	0.0%	6.3%	2.3%
	Total	**74.7%**	**58.6%**	**93.1%**	**2.3%**	**48.9%**	**1.1%**	**33.9%**	**35.6%**	**27.6%**	**70.7%**	**67.8%**	**54.0%**	**33.9%**	**9.8%**	**22.4%**	**56.3%**
Polar	Gly	0.0%	1.7%	1.1%	37.9%	0.0%	0.0%	17.8%	14.4%	0.0%	5.2%	0.6%	0.0%	6.9%	73.0%	5.2%	2.3%
	Thr	6.3%	0.0%	0.0%	31.6%	0.6%	93.7%	23.0%	6.9%	0.0%	0.0%	2.9%	2.9%	0.6%	0.6%	1.7%	0.0%
	Ser	6.3%	9.2%	2.3%	19.5%	10.9%	5.2%	4.0%	0.6%	0.0%	4.0%	1.1%	1.1%	8.6%	4.6%	12.6%	8.6%
	Cys	0.6%	0.0%	0.0%	8.6%	0.0%	0.0%	0.6%	0.0%	0.0%	0.0%	1.1%	0.0%	0.0%	0.0%	0.0%	1.1%
	Tyr	9.2%	0.6%	0.0%	0.0%	1.7%	0.0%	2.9%	1.1%	68.4%	18.4%	0.0%	0.6%	0.6%	0.0%	1.1%	17.8%
	Asn	1.1%	13.2%	1.1%	0.0%	12.1%	0.0%	2.9%	11.5%	0.0%	0.0%	0.6%	10.3%	17.2%	0.6%	10.9%	1.1%
	Gln	1.1%	1.1%	1.1%	0.0%	0.0%	0.0%	2.9%	1.1%	0.0%	0.0%	0.0%	20.1%	1.7%	0.0%	21.3%	2.3%
	Total	**24.7%**	**25.9%**	**5.7%**	**97.7%**	**25.3%**	**98.9%**	**54.0%**	**35.6%**	**68.4%**	**27.6%**	**6.3%**	**35.1%**	**35.6%**	**78.7%**	**52.9%**	**33.3%**
Acidic	Asp	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	8.6%	0.0%	0.0%	0.0%	0.0%	9.8%	2.9%	1.7%	2.3%
	Glu	0.0%	1.1%	0.0%	0.0%	0.0%	0.0%	3.4%	0.6%	0.0%	0.0%	0.0%	1.7%	1.1%	4.6%	0.0%	1.7%
	Total	**0.6%**	**1.1%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**3.4%**	**9.2%**	**0.0%**	**0.0%**	**0.0%**	**1.7%**	**10.9%**	**7.5%**	**1.7%**	**4.0%**
Basic	His	0.0%	4.6%	1.1%	0.0%	25.9%	0.0%	1.7%	10.3%	4.0%	0.0%	0.0%	9.2%	1.7%	0.0%	20.1%	0.6%
	Lys	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%	0.0%	0.0%	12.6%	0.6%	0.0%	0.0%
	Arg	0.0%	9.8%	0.0%	0.0%	0.0%	0.0%	4.6%	9.2%	0.0%	0.0%	0.0%	0.0%	4.6%	2.3%	2.3%	5.2%
	Total	**0.0%**	**14.4%**	**1.1%**	**0.0%**	**25.9%**	**0.0%**	**8.6%**	**19.5%**	**4.0%**	**0.0%**	**0.0%**	**9.2%**	**19.0%**	**2.9%**	**22.4%**	**5.7%**
Others	-	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	25.9%	0.0%	0.6%	1.1%	0.6%	0.6%
	nd	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	Total	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**1.7%**	**25.9%**	**0.0%**	**0.6%**	**1.1%**	**0.6%**	**0.6%**
	$H_1(l)$	**2.094**	**2.276**	**1.639**	**1.362**	**2.080**	**0.274**	**1.970**	**2.372**	**0.910**	**1.949**	**1.789**	**2.119**	**2.407**	**1.134**	**2.337**	**2.324**
	$H_2(l)$	**0.593**	**0.993**	**0.282**	**0.109**	**1.047**	**0.063**	**0.676**	**1.274**	**0.744**	**0.670**	**0.788**	**0.990**	**1.321**	**0.763**	**1.107**	**1.013**

Sequences alignment for *Sl*PVA, *Au*AAC and *Au*AHLA considered 174 acylase. Strain name is followed by the GenBank number of the acylase between brackets: *S. lavendulae* ATCC 13664 {AAU09670.1} (Torres-Bacete, J. *et al.* 2015), *A. utahensis* NRRL 12052 {KHD77856.1, KHD77917.1} (Velasco-Bucheli, R. *et al.* 2015) *S. mobaraensis* NBRC 13819 {BAF51977.1} (Zhang, D. *et al.* 2007), *Streptomyces* sp. M664 {AAT68473.1} (Park, S. Y. *et al.* 2005), *Streptomyces* sp. FERM BP-5809 {BAD07025.1} (Ueda, S. *et al.* 2011b), *P. diminuta* KAC-1 {Q9L5D6.1} (Kim, D.-W. *et al.* 1999; Mei, T. *et al.* 2015), *E. coli* ATCC 11105 {AAA24324.1}(Oh, S. J. *et al.* 1987; del Río, G. *et al.* 1995; Lee, H. *et al.* 2000; Morillas, M. *et al.* 2003; Balci, H. *et al.* 2014), *P. aeruginosa* PAO1 {NP_251075.1, NP_248996.1} (Sio, C. F. *et al.* 2006; Wahjudi, M. *et al.* 2011), *Cupriavidus metallidurans* sp. CH34 {WP_011516313.1} (Janssen, P. J. *et al.* 2010), *Ralstonia* sp. XJ12B {AAO41113.1} (Lin, Y.-H. *et al.* 2003), *P. syringae* Psyr_1971

{YP_235052.1}, *P. syringae* Psyr_4858 {YP_237923.1} (Shepherd, R. W. *et al.* 2009), *P. aeruginosa* PA1032 {NP_249723.1} (Huang, J. J. *et al.* 2006), *T. thermophilus* HB27 {WP_011174322.1} (Torres, L. *et al.* 2012), *R. solanacearum* GMI1000 {WP_011002462.1} (Chen, C.-N. *et al.* 2009), *Anabaena* sp. PCC7120 {WP_010998065.1} (Romero, M. *et al.* 2008), *V. paradoxus* VAI-C {WP_013542868.1} (Leadbetter, J. R. *et al.* 2000), *B. badius* PGS10 {AAZ20308.1} (Rajendran, K. *et al.* 2013), *K. citrophila* ATCC 21285 {P07941.1}(Barbero, J. L. *et al.* 1986; Martín, J. *et al.* 1990; Roa, A. *et al.* 1994), *Achromobacter* sp. CCM 4824 {AAY25991.1}(Škrob, F. *et al.* 2003), *Pseudomonas* N176 {4HST_A, 4HST_B} (Aramori, I. *et al.* 1991; Ishii, Y. *et al.* 1995; Pollegioni, L. *et al.* 2005; Golden, E. *et al.* 2013; Conti, G. *et al.* 2014), *Arthrobacter viscosus* ATCC 15294 {AAA22077.1} (Konstantinović, M. *et al.* 1994), *Nocardioides* sp. JS614 {ABL80313.1} (Cobzaru, C. *et al.* 2011), *Streptomyces* sp. Mg1 {EDX24783.1} (Hoefler, B. C. *et al.* 2013), *S. ambofaciens* ATCC 23877 {CAJ89876.1} (Choulet, F. *et al.* 2006), *S. griseus* subsp. *griseus* NBRC 13350 {YP_001825806.1, WP_012380445.1} (Ohnishi, Y. *et al.* 2008), *S. sviceus* ATCC 29083 {EDY53465.1} (Fischbach, M. *et al.* 2009), *Symbiobacterium thermophilum* IAM 14863 {WP_011194204.1} (Ueda, K. *et al.* 2004), *Archaeoglobus fulgidus* DSM 4304 {AAB90749.1} (Klenk, H.-P. *et al.* 1997), *Brevundimonas diminuta* VKM B-1297 {CAA01318.1} (Khatuntseva, S. A. *et al.* 2007), *Caulobacter crescentus* CB15 {NP_419938.1} (Nierman, W. C. *et al.* 2001), *Erythrobacter* sp. NAP1 {EAQ29218.1} (Koblížek, M. *et al.* 2011), *Erythrobacter* sp. SD-21 {EDL48749.1} (Anderson, C. R. *et al.* 2009), *Candidatus Solibacter usitatus* Ellin6076 {ABJ85895.1} (Ward, N. L. *et al.* 2009), *Chloroflexus aggregans* DSM 9485 {WP_015941778.1} (Hanada, S. *et al.* 1995), *C. aurantiacus* J-10-fl {YP_001635920.1} (Tang, K. H. *et al.* 2011), *Herpetosiphon aurantiacus* ATCC 23779 {WP_012192514.1} (Kiss, H. *et al.* 2011), *Roseiflexus* sp. RS-1 {WP_011956053.1} (Klatt, C. G. *et al.* 2007), *Cyanothece* sp. ATCC 51142 {YP_001806020.1}, *Cyanothece* sp. CCY0110 {EAZ88162.1} (Welsh, E. A. *et al.* 2008), *Cyanothece* sp. PCC 7424 {WP_012599730.1} (Bandyopadhyay, A. *et al.* 2011), *R. castenholzii* DSM 13941 {WP_012120173.1} (Copeland, A. *et al.* 2007e), *Deinococcus radiodurans* R1 {NP_285578.1} (White, O. *et al.* 1999), *T. aquaticus* Y51MC23 {EED11161.1} (Plessis, E. M. *et al.* 2010), *Bacillus* sp. B14905 {EAZ85866.1} (Edwards, R. *et al.* 2007), *B. anthracis* str. A0488 {EDR21250.1} (Dodson, R. J. *et al.* 2007), *B. badius* {AAZ20308.1} (Rajendhran, J. et al. 2007), *Oceanobacillus iheyensis* HTE831 {WP_011067700.1} (Takami, H. *et al.* 2002), *B. thuringiensis* serovar *konkukian* str. 97-27 {YP_037354.1} (Han, C. S. *et al.* 2006), *Cyanothece* sp. PCC 7822 {WP_013321804.1} (Aryal, U. K. *et al.* 2013), *Cyanothece* sp. PCC 8801 {WP_012595712.1} (Lucas, S. *et al.* 2008), *Cyanothece* sp. PCC 8802 {WP_015784822.1} (Bandyopadhyay, A. *et al.* 2011), *Gloeobacter violaceus* PCC 7421 {NP_924934.1} (Nakamura, Y. *et al.* 2003), *Microcoleus chthonoplastes* PCC 7420 {EDX78436.1} (Tandeau de Marsac, N. *et al.* 2008), *Nostoc punctiforme* PCC 73102 {WP_012407707.1} (Copeland, A. *et al.* 2008d), *Synechocystis* sp. PCC 6803 {BAA10098.1} (Kaneko, T. *et al.* 1995), *Algoriphagus* sp. PR1 {EAZ79782.1} (Alegado, R. A. *et al.* 2011), *Microscilla marina* ATCC 23134 {EAY25007.1} (Haygood, M. *et al.* 2007), *Pedobacter* sp. BAL39 {EDM38775.1} (Hagstrom, A. *et al.* 2007), *Polaribacter irgensii* 23-P {EAR11760.1} (Gosink, J. J. *et al.* 1998), *Robiginitalea biformata* HTCC2501 {WP_015753069.1} (Oh, H.-M. *et al.* 2009b), *Salinibacter ruber* DSM 13855 {YP_445269.1} (Mongodin, E. F. *et al.* 2005), *E. litoralis* HTCC2594 {WP_011413253.1} (Oh, H.-M. *et al.* 2009a), *Hoeflea phototrophica* DFL-43 {EDQ33592.1} (Biebl, H. *et al.* 2006), *Hyphomonas neptunium* ATCC 15444 {YP_758954.1} (Badger, J. H. *et al.* 2006), *Parvibaculum lavamentivorans* DS-1 {WP_012109814.1} (Schleheck, D. *et al.* 2011), *Rhodospirillum rubrum* ATCC 11170 {YP_426927.1} (Munk, A. C. *et al.* 2011), *Sphingopyxis alaskensis* RB2256 {ABF54097.1} (Lauro, F. M. *et al.* 2009), *Cellulophaga lytica* DSM 7489 {WP_013622576.1} (Pati, A. *et al.* 2011), *A. avenae* subsp. *citrulli* AAC00-1 {WP_011794286.1} (Copeland, A. *et al.* 2006a), *Alcaligenes faecalis* CICC AS1.767 {AAL58441.1} (Zhou, Z. *et al.* 2003), *Burkholderia* sp. H160 {WP_008918997.1} (Ormeño-Orrillo, E. *et al.* 2012), *P. cepacia* BY21 {AAX49566.1} (Khang, Y.-H. et al. 2000), *C. algicola* DSM 14237 {WP_013552350.1} (Abt, B. *et al.* 2011), *Comamonas testosteroni* KF-1 {EED68172.1} (Lucas, S. *et al.* 2009), *C. taiwanensis* STM6070 {WP_018005626.1} (Kyrpides, N. *et al.* 2015), *Delftia acidovorans* SPH-1 {WP_012206604.1} (Schleheck, D. *et al.* 2004), *Leptothrix cholodnii* SP-6 {WP_012346936.1} (Copeland, A. *et al.* 2008b), *Limnobacter* sp. MED105 {EDM84756.1} (Pinhassi, J. *et al.* 2007), *Methylibium petroleiphilum* PM1 {ABM94781.1} (Kane, S. R. *et al.* 2007), *R. eutropha* H16 {YP_726387.1} (Pohlmann, A. *et al.* 2006), *R. eutropha* JMP134 {YP_296050.1} (Copeland, A. *et al.* 2005), *R. pickettii* OR214 {WP_004627477} (Utturkar, S. M. *et al.* 2013), *A. baumannii* AB0057 {WP_000935120.1} (Adams, M. D. *et al.* 2008), *A. baumannii* ACICU {WP_000935122.1} (Iacono, M. *et al.* 2008), *A. baumannii* AYE {YP_001713604.1} (Vallenet, D. *et al.* 2008), *A. macleodii* 'Deep ecotype' {WP_012518383.1} (Ivars-Martinez, E. *et al.* 2008), *Azotobacter vinelandii* DJ {WP_012701109.1, WP_012700554.1} (Setubal, J. C. *et al.* 2009), *Cellvibrio japonicus* Ueda107 {WP_012487479.1} (DeBoy, R. T. *et al.* 2008), *Congregibacter litoralis* KT 71 {EAQ95987.1} (Fuchs, B. M. *et al.* 2007), *Hahella chejuensis* KCTC 2396 {WP_011399225.1} (Jeong, H. *et al.* 2005), *Idiomarina baltica* OS145 {EAQ30875.1} (Brettar, I. *et al.* 2003), Gammaproteobacterium HTCC2143 {EAW33100.1} (Oh, H.-M. *et al.* 2010), Gammaproteobacterium HTCC2207 {EAS48178.1} (Stingl, U. *et al.* 2007), *Marinobacter algicola* DG893 {EDM49623.1} (Green, D. H. *et al.* 2006), *Myxococcus xanthus* DK 1622 {ABF89234.1} (Goldman, B. S. *et al.* 2006), *Nitrococcus mobilis* Nb-231 {EAR21801.1} (Waterbury, J. *et al.* 2006), *Oceanobacter* sp. RED65 {EAT11539.1} (Pinhassi, J. *et al.* 2006), *Photorhabdus luminescens* subsp. *laumondii* TTO1 {WP_011147707.1} (Duchaud, E. *et al.* 2003), *Providencia rettgeri* FAS-20 {AAP86197.1} (Ljubijankić, G. *et al.* 1992), *P. stuartii* ATCC 25827 {EDU61018.1} (Sudarsanam, P. *et al.* 2012), *Pseudomonas* sp. 130 {AAC34685.2}(Yang, Y. L. *et al.* 1991), *Pseudomonas* sp. GK16 {ABP51959.1} (Lee, Y.-S. *et al.* 1996), *Pseudomonas* sp. SY-77-1 {AAN39264.1} (Otten, L. G. *et al.* 2002; Sio, C. F. *et al.* 2002), *Pseudomonas* sp. THA1 {AAP68796.1}, *Pseudomonas* sp. THA2 {AAP68797.1}, *Pseudomonas* sp. THA3 {AAP68798.1} (Luo, H. *et al.* 2003), *P. aeruginosa* 2192 {EAZ57728.1}, *P. aeruginosa* C3719 {EAZ51610.1} (Mathee, K. *et al.* 2008), *P. aeruginosa* PA7 {ABR83040.1} (Roy, P. H. *et al.* 2010), *P. aeruginosa* UCBPP-PA14 {YP_792238.1} (Lee, D. *et al.* 2006), *P. entomophila* L48 {WP_011532537.1} (Vodovar, N. *et al.* 2006), *P. fluorescens* Pf0-1 {ABA72956.1} (Silby, M. *et al.* 2009), *P. fluorescens* Pf-5 {AAY90553.1} (Paulsen, I. T. *et al.* 2005), *P. putida* F1 {WP_012051410.1} (Copeland, A. *et al.* 2007a), *P. putida* GB-1 {YP_001670529.1} (Copeland, A. *et al.* 2008a), *P. putida* KT2440 {NP_743269.1} (Nelson, K. E. *et al.* 2002), *P. putida* W619 {WP_012312248.1}, *S. proteamaculans* 568 {ABV42242.1} (Taghavi, S. *et al.* 2009), *P. syringae* pv. *phaseolicola* 1448A {YP_273648.1} (Joardar, V. *et al.* 2005), *P. syringae* pv. *syringae* B728a {YP_236939.1}, *P. syringae* pv. *tomato* str. DC3000 {NP_793894.1} (Feil, H. *et al.* 2005), *Psychrobacter cryohalolentis* K5 {WP_011514746.1} (Bakermans, C. *et al.* 2006), *Shewanella* sp. ANA-3 {WP_011718166.1} (Copeland, A. *et al.* 2013), *Shewanella* sp. MIB015 {BAF94155.1} (Morohoshi, T. *et al.* 2008b), *Shewanella* sp. MR-4 {WP_011621555.1} (Copeland, A. *et al.* 2006c), *Shewanella* sp. MR-7 {WP_011627127.1} (Copeland, A. *et al.* 2006d), *Shewanella* sp. W3-18-1 {WP_011790574.1} (Qiu, D. *et al.* 2013), *S. amazonensis* SB2B {WP_041410504.1} (Copeland, A. *et al.* 2006e), *S. baltica* OS155 {WP_011845873} (Copeland, A. *et al.* 2007c), *S. baltica* OS185 {WP_012090114.1} (Copeland, A. *et al.* 2007d), *S. frigidimarina* NCIMB 400 {WP_011636250.1} (Copeland, A. *et al.* 2006b), *S. oneidensis* MR-1 {NP_716547.1} (Elias, D. A. *et al.* 2005), *S. putrefaciens* 200 {WP_014609960.1} (DiChristina, T. J. *et al.* 2002), *S. putrefaciens* CN-32 {WP_011918743.1} (Copeland, A. *et al.* 2007b), *S. woodyi* ATCC 51908 {WP_012322786.1} (Copeland, A. *et al.* 2008c), *Desulfatibacillum alkenivorans* AK-01 {WP_015949378.1} (Callaghan, A. V. *et al.* 2012), *Plesiocystis pacifica* SIR-1 {EDM74924.1} (Iizuka, T. *et al.* 2003), *Stigmatella aurantiaca* DW4/3-1 {WP_002615283.1} (Huntley, S. *et al.* 2011), *Zobellia galactanivorans* DsiJT {CAZ98735.1} (Thomas, F. et al. 2012), *Nitrosomonas* sp. Is79A3 {WP_013966225.1} (Lucas, S. *et al.* 2011), *Pseudomonas* sp. SE83 {AAA25690.1} (Matsuda, A. *et al.* 1987; Shin, Y. C. *et al.* 2009; Wang, Y. *et al.* 2012; Xiao, Y. *et al.* 2014; Zhang, J. *et al.* 2014), uncultured bacterium ACPGA001 with signal peptide {ADG60270.1} (Zhang, Q. *et al.* 2014), and *B. megaterium* ATCC 14945 {CAA85774.1} (Martín, L. *et al.* 1995).

S.7. Chromophores employed to quantify primary amines

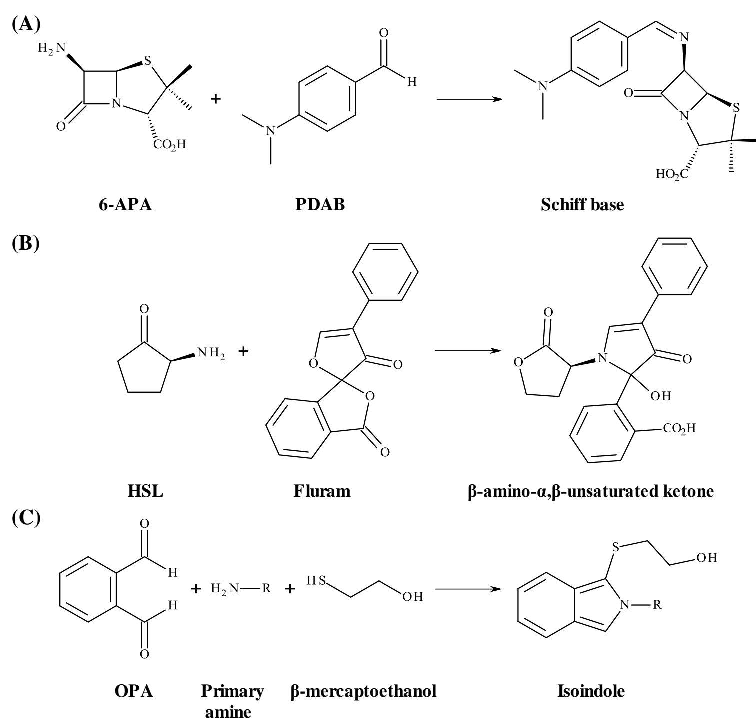


Figure S.7.1. Reactions involved in the quantification of primary amines. Reactions performed during this study were carried out with **(A)** PDAB, **(B)** Fluram, and **(C)** OPA

S.8. Relative coordinates between residues in *Sl*PVA, *Au*AAC and *Au*AHLA

Table S.8.1. Distances in Angstroms between amino acids in *Sl*PVA according to I-Tasser model

aa	βSer^{1}	βHis^{23}	βTyr^{24}	βPhe^{32}	βLeu^{50}	βSer^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{186}	βAsn^{272}
βSer^{1}		6.9	7.6	8.9	11.1	10.9	6.1	6.8	5.1	7.7	9.3	6.3
βHis^{23}	6.9		9.8	9.4	12.1	14.9	9.6	11.1	11.0	10.4	14.5	10.5
βTyr^{24}	7.6	9.8		5.9	5.1	7.5	7.3	5.1	8.2	4.4	6.6	9.5
βPhe^{32}	8.9	9.4	5.9		3.7	7.0	4.0	5.4	12.0	10.7	10.4	14.3
βLeu^{50}	11.1	12.1	5.1	3.7		5.9	7.9	6.7	12.8	9.2	8.4	14.4
βSer^{57}	10.9	14.9	7.5	7.0	5.9		5.4	5.3	11.1	10.5	6.1	15.0
βIle^{58}	6.1	9.6	7.3	4.0	7.9	5.4		3.4	9.4	10.2	8.5	13.7
βHis^{68}	6.8	11.1	5.1	5.4	6.7	5.3	3.4		7.9	7.9	5.4	12.0
βThr^{69}	5.1	11.0	8.2	12.0	12.8	11.1	9.4	7.9		6.0	6.7	5.8
βVal^{70}	7.7	10.4	4.4	10.7	9.2	10.5	10.2	7.9	6.0		6.8	3.7
βVal^{186}	9.3	14.5	6.6	10.4	8.4	6.1	8.5	5.4	6.7	6.8		10.1
βAsn^{272}	6.3	10.5	9.5	14.3	14.4	15.0	13.7	12.0	5.8	3.7	10.1	

Table S.8.2. Distances in Angstroms between amino acids in *Au*AAC according to I-Tasser model

aa	βSer^{1}	βHis^{23}	βPhe^{24}	βPhe^{32}	βLeu^{50}	βGlu^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βVal^{182}	βAsn^{268}
βSer^{1}		6.7	7.0	8.7	12.8	10.1	5.9	5.7	5.4	6.8	8.3	5.8
βHis^{23}	6.7		7.5	10.7	14.4	13.9	10.0	11.0	11.2	10.4	14.0	7.5
βPhe^{24}	7.0	7.5		3.5	4.8	5.2	6.5	5.2	9.7	5.4	7.8	8.4
βPhe^{32}	8.7	10.7	3.5		4.7	3.4	4.0	6.0	12.1	9.3	9.0	12.3
βLeu^{50}	12.8	14.4	4.8	4.7		3.4	8.8	8.7	14.6	9.6	10.0	13.9
βGlu^{57}	10.1	13.9	5.2	3.4	3.4		6.0	5.0	11.5	8.2	6.3	12.2
βIle^{58}	5.9	10.0	6.5	4.0	8.8	6.0		4.8	9.5	9.1	8.2	10.7
βHis^{68}	5.7	11.0	5.2	6.0	8.7	5.0	4.8		6.6	5.0	3.7	7.8
βThr^{69}	5.4	11.2	9.7	12.1	14.6	11.5	9.5	6.6		5.6	6.2	4.3
βVal^{70}	6.8	10.4	5.4	9.3	9.6	8.2	9.1	5.0	5.6		5.2	3.9
βVal^{182}	8.3	14.0	7.8	9.0	10.0	6.3	8.2	3.7	6.2	5.2		8.4
βAsn^{268}	5.8	7.5	8.4	12.3	13.9	12.2	10.7	7.8	4.3	3.9	8.4	

Table S.8.3. Distances in Angstroms between amino acids in *Au*AHLA according to I-Tasser model

aa	βSer^{1}	βHis^{23}	βTyr^{24}	βPhe^{32}	βLeu^{50}	βLeu^{57}	βIle^{58}	βHis^{68}	βThr^{69}	βVal^{70}	βLeu^{188}	βAsn^{274}
βSer^{1}		6.7	7.7	7.5	10.6	10.5	5.8	8.3	5.2	6.6	8.2	7.9
βHis^{23}	6.7		10.8	8.9	12.4	14.5	9.8	14.7	11.2	11.0	14.6	10.7
βTyr^{24}	7.7	10.8		4.7	3.8	5.5	7.2	10.1	10.4	5.9	7.8	10.7
βPhe^{32}	7.5	8.9	4.7		4.4	4.8	4.0	9.6	11.5	9.6	9.3	13.2
βLeu^{50}	10.6	12.4	3.8	4.4		3.9	8.3	11.7	13.7	9.1	9.0	14.2
βLeu^{57}	10.5	14.5	5.5	4.8	3.9		4.5	6.9	12.2	10.1	5.9	15.2
βIle^{58}	5.8	9.8	7.2	4.0	8.3	4.5		6.0	9.4	10.0	7.8	13.1
βHis^{68}	8.3	14.7	10.1	9.6	11.7	6.9	6.0		7.6	10.1	3.6	13.3
βThr^{69}	5.2	11.2	10.4	11.5	13.7	12.2	9.4	7.6		5.7	6.3	6.2
βVal^{70}	6.6	11.0	5.9	9.6	9.1	10.1	10.0	10.1	5.7		5.3	3.7
βLeu^{188}	8.2	14.6	7.8	9.3	9.0	5.9	7.8	3.6	6.3	5.3		10.0
βAsn^{274}	7.9	10.7	10.7	13.2	14.2	15.2	13.1	13.3	6.2	3.7	10.0	

S.9. Similar studies of directed evolution in other acylases

Table S.9.1. Spatial distribution of residues related with the performance in some acylases. Protein structure modeled by I-Tasser (B: Buried, S:Surface, BP: Binding Pocket and G: Gate toward catalytic pocket). Distances to βSer[1] in parentheses in Angstroms. Gray background represent parental enzyme. Black background shows mutated amino acids. Text in red highlights those positions altered in more than 2.5 Å their relative coordinates. Cells with red borders reveal residues that changed their orientation toward or from the solvent

	RESIDUES AND THEIR RELATIVE COORDINATES											
***Sl*PVA** (Torres-Bacete, J. et al. 2015)	αAla113 B (28.5)	αArg124 S (40.4)	αAla131 B (29.4)	αArg133 S (38.4)	αThr167 S (28.4)	αAla169 S (22.1)	αGly180 S (19.4)	αVal181 S (21.6)	αThr182 S (22.0)	αPro183 S (24.8)	αAla187 S (27.8)	αAla189 S (29.1)
***Sl*PVA** 2pva1,21(25)	αAla113 B (28.6)	αArg124 S (38.5)	αAla131 S (30.1)	αArg133 S (38.5)	αThr167 S (26.2)	αAla169 S (22.7)	αGly180 S (18.1)	αVal181 S (20.2)	αThr182 S (22.6)	αPro183 S (24.7)	αAla187 S (24.9)	αAla189 S (28.4)
***Sl*PVA** 2pva1,2(55)	αAla113 B (27.4)	αArg124 S (38.3)	αAla131 B (27.8)	αArg133 S (38.7)	αThr167 S (25.4)	αAla169 S (20.3)	αGly180 S (13.3)	αVal181 S (9.1)	αThr182 S (10.6)	αPro183 S (14.4)	αAla187 S (18.7)	αAla189 S (17.4)
***Sl*PVA** 2pva1,2(149)	αAla113 B (28.3)	αArg124 S (40.7)	αAla131 B (29.1)	αArg133 S (38.4)	αThr167 S (27.0)	αThr169 S (23.4)	αGly180 S (16.4)	αVal181 S (14.6)	αThr182 S (20.4)	αPro183 S (21.7)	αAla187 S (25.3)	αAla189 S (23.7)
***Au*AAC** (Torres-Bacete, J. et al. 2007)	αVal107 B (28.6)	αGly118 S (36.2)	- -	αArg128 S (33.8)	αVal162 S (26.4)	αAla164 S (23.7)	αAla175 S (19.4)	αPro176 S (19.6)	αGlu177 S (22.9)	αAla178 S (24.7)	αAla182 S (23.8)	αIle184 S (24.5)
***Au*AAC** a(292)	αVal107 B (28.0)	αGly118 S (36.3)	- -	αArg128 S (35.6)	αVal162 S (26.9)	αAla164 S (24.5)	αAla175 S (17.4)	αPro176 S (18.2)	αGlu177 S (18.2)	αAla178 S (20.9)	αAla182 S (26.6)	αPhe184 S (19.4)
***Au*AHLA** (Velasco-Bucheli, R. et al. 2015)	αVal115 B (26.3)	αGly126 S (35.8)	αAla134 S (28.8)	αArg136 S (38.3)	αVal170 B (26.7)	αPro172 S (22.8)	αAla183 S (18.1)	αThr184 S (18.7)	αPro185 S (19.2)	αAla186 S (20.3)	αGlu190 S (25.9)	αAla192 S (27.1)
ATCC 21285 (Barbero, J. et al. 1986)	αGly123 S (30.7)	αMet142 B (16.7)	αThr150 S (23.3)	αGlu152 B (27.2)	αAla209 S (35.7)	αLeu211 S (32.0)	αMet222 S (32.8)	αLeu223 S (33.9)	αAsp224 S (37.4)	αArg225 S (37.2)	αAla233 S (27.8)	αLeu235 S (21.3)
ATCC 21285 (Martín, J. et al. 1990)	αGly123 S (31.3)	αAla142 B (15.9)	αThr150 B (22.3)	αGlu152 B (23.3)	αAla209 S (35.7)	αLeu211 S (33.5)	αMet222 S (32.9)	αLeu223 S (32.9)	αAsp224 S (37.5)	αArg225 S (39.1)	αAla233 S (24.8)	αLeu235 S (21.0)
ATCC 21285 (Roa, A. et al. 1994)	αGly123 S (29.7)	αMet142 B (12.3)	αThr150 S (21.9)	αGlu152 B (17.5)	αAla209 S (31.6)	αLeu211 B (22.4)	αMet222 S (33.6)	αLeu223 S (30.1)	αAsp224 S (34.2)	αArg225 S (31.7)	αAla233 S (26.6)	αLeu235 S (19.9)
ATCC 11105 (Oh, S. J. et al. 1987)	αLeu115 B (31.0)	αMet142 B (12.3)	αVal168 S (36.8)	αGln170 S (37.1)	αThr208 S (35.0)	αAla210 S (35.7)	αMet221 S (37.5)	αLeu122 S (28.1)	αAsp223 S (36.7)	αArg224 S (37.8)	αGly228 S (31.5)	αAsp230 S (29.0)
ATCC 11105 (del Rio, G. et al. 1995)	αLeu115 B (32.4)	αMet142 B (12.2)	αVal168 S (39.5)	αGln170 S (39.5)	αThr208 S (36.8)	αAla210 S (37.2)	αMet221 S (26.1)	αLeu122 S (27.9)	αAsp223 S (34.8)	αArg224 S (33.2)	αGly228 S (29.4)	αAsp230 S (21.2)
ATCC 11105 (Morillas, M. et al. 2003)	αLeu115 B (32.6)	αMet142 B (16.2)	αVal168 S (46.7)	αGln170 S (44.7)	αThr208 S (21.1)	αAla210 S (23.5)	αMet221 S (27.7)	αLeu122 S (23.8)	αAsp223 S (18.9)	αArg224 S (23.6)	αGly228 S (21.2)	αAsp230 S (23.0)
ATCC 11105 (Morillas, M. et al. 2003)	αLeu115 B (30.6)	αMet142 B (12.7)	αVal168 S (37.9)	αGln170 S (37.2)	αThr208 S (35.2)	αAla210 S (35.4)	αMet221 S (28.4)	αLeu122 S (32.3)	αAsp223 S (35.4)	αArg224 S (35.4)	αGly228 S (30.2)	αAsp230 S (29.9)
ATCC 11105 (Balci, H. et al. 2014)	αLeu115 B (34.0)	αMet142 B (14.2)	αVal168 S (39.4)	αGln170 S (41.8)	αThr208 S (31.8)	αAla210 S (27.7)	αMet221 S (33.3)	αLeu122 S (36.8)	αAsp223 S (35.6)	αArg224 S (37.9)	αGly228 S (31.2)	αAsp230 S (33.7)
SY-77-1 (Shibuya, Y. et al. 1981)	αAla108 B (27.8)	- -	αIle122 B (29.8)	αPro124 S (33.1)	αTyr149 S,G (8.2)	αAla151 S (19.3)	αPro162 S (18.7)	αPro163 S (17.4)	αAsp164 S (18.2)	αLeu165 S (13.8)	- -	- -
SY-77-1 (Sio, C. F. et al. 2002)	αAla108 B (28.4)	- -	αIle122 B (30.7)	αPro124 S (33.9)	αHis149 S,G (12.2)	αAla151 S (20.9)	αPro162 S (20.1)	αPro163 S (18.5)	αAsp164 S (19.2)	αLeu165 S (16.2)	- -	- -
SY-77-1 (Otten, L. G. et al. 2002)	αAla108 B (29.7)	- -	αIle122 B (31.8)	αPro124 S (35.1)	αTyr149 S,G (8.6)	αAla151 S (19.6)	αPro162 S (18.9)	αPro163 S (17.6)	αAsp164 S (17.3)	αLeu165 S (13.7)	- -	- -
SY-77-1 (Otten, L. G. et al. 2002)	αAla108 B (29.7)	- -	αIle122 B (31.4)	αPro124 S (35.3)	αTyr149 S,G (9.6)	αAla151 S (20.1)	αPro162 S (19.6)	αPro163 S (18.2)	αAsp164 S (18.8)	αLeu165 S (14.6)	- -	- -
SY-77-1 (López-Gallego, F. et al. 2008)	αAla108 B (28.4)	- -	αIle122 B (30.2)	αPro124 S (34.0)	αPhe149 S,G (10.0)	αAla151 S (20.8)	αPro162 S (20.0)	αPro163 S (18.6)	αAsp164 S (19.2)	αLeu165 S (14.4)	- -	- -
SE83 (Matsuda, A. et al. 1987)	αVal122 B (28.0)	αGly132 S (34.8)	αGly140 S (32.2)	αMet165 B (14.8)	αLeu199 S (15.6)	αIle201 S (17.4)	αAla212 S (20.0)	αAsp213 S (21.6)	αLeu214 S (21.9)	αAla215 S (19.0)	αAsp222 S (27.0)	αLeu224 S (19.3)
SE83 (Shin, Y. C. et al. 2009)	αAla122 B (28.2)	αGly132 S (34.9)	αSer140 S (30.2)	αMet165 B (16.5)	αLeu199 S (16.5)	αIle201 S (22.7)	αAla212 S (24.8)	αAsp213 S (27.5)	αLeu214 S (27.9)	αAla215 S (26.0)	αAsp222 S (28.8)	αLeu224 S (28.7)
SE83 (Wang, Y. et al. 2012)	αAla122 B (28.6)	αGly132 S (35.2)	αSer140 S (31.0)	αMet165 B (18.6)	αLeu199 S (25.3)	αIle201 B (22.1)	αAla212 S (26.9)	αAsp213 S (28.2)	αLeu214 S (28.7)	αAla215 S (26.3)	αAsp222 S (29.7)	αLeu224 S (25.7)
SE83 (Xiao, Y. et al. 2014)	αVal122 B (27.3)	αGly132 S (34.8)	αGly140 S (32.1)	αMet165 B (17.3)	αLeu199 S (17.1)	αIle201 S (14.7)	αAla212 S (13.3)	αAsp213 S (15.7)	αLeu214 S (17.8)	αAla215 S (19.7)	αAsp222 S (23.4)	αLeu224 S (24.3)
SE83 (Xiao, Y. et al. 2014)	αVal122 B (27.1)	αGly132 S (35.0)	αGly140 S (32.4)	αMet165 B (16.9)	αLeu199 S (24.8)	αIle201 S (22.8)	αAla212 S (18.3)	αAsp213 S (23.6)	αLeu214 S (22.9)	αAla215 S (20.2)	αAsp222 S (25.1)	αLeu224 S (23.5)
SE83 (Xiao, Y. et al. 2014)	αVal122 B (28.1)	αGly132 S (35.6)	αGly140 S (32.8)	αMet165 B (16.8)	αLeu199 S (13.7)	αIle201 S (15.6)	αAla212 S (24.6)	αAsp213 S (26.0)	αLeu214 S (26.6)	αAla215 S (23.8)	αAsp222 S (30.6)	αLeu224 S (30.1)
SE83 (Zhang, J. et al. 2014)	αVal122 B (27.1)	αGly132 S (35.2)	αGly140 S (32.0)	αMet165 B (16.1)	αLeu199 S (22.8)	αIle201 S (23.0)	αAla212 S (29.7)	αAsp213 S (27.9)	αLeu214 S (24.0)	αAla215 S (30.4)	αAsp222 S (26.8)	αLeu224 S (24.8)
SE83 (Zhang, J. et al. 2014)	αVal122 B (28.3)	αGly132 S (36.2)	αGly140 S (33.6)	αMet165 B (17.2)	αLeu199 B (17.1)	αIle201 B (15.1)	- -	- -	- -	- -	αAsp222 S (26.1)	αLeu224 S (25.8)
N176 (Aramori, I. et al. 1991)	αVal122 B (28.2)	αGly162 B (15.4)	αLeu163 B (19.6)	αMet165 B (17.0)	αAsp197 S (26.1)	αCys200 S (17.7)	αArg209 S (23.4)	αLeu210 S (25.0)	αGlu211 S (21.5)	αAla212 S (20.6)	αAla215 S (19.8)	αLeu217 S (27.0)
N176 (Ishii, Y. et al. 1995)	αVal122 B (27.4)	αGly162 B (16.0)	αLeu163 B (20.2)	αMet165 B (19.5)	αAsp197 S (25.9)	αCys200 S (18.6)	αArg209 S (24.2)	αLeu210 S (23.6)	αGlu211 S (27.6)	αAla212 S (22.0)	αAla215 S (20.8)	αLeu217 S (28.4)
N176 (Pollegioni, L. et al. 2005)	αVal122 B (28.0)	αGly162 B (15.8)	αLeu163 B (20.2)	αMet165 B (18.6)	αAsp197 S (29.3)	αCys200 S (23.6)	αArg209 S (24.7)	αLeu210 S (23.4)	αGlu211 S (24.5)	αAla212 S (19.5)	αAla215 S (25.4)	αLeu217 S (28.7)
N176 (Pollegioni, L. et al. 2005)	αVal122 B (27.9)	αGly162 B (15.5)	αLeu163 B (19.3)	αMet165 B (17.9)	αAsp197 S (28.2)	αCys200 S (21.3)	αArg209 S (17.3)	αLeu210 S (23.3)	αGlu211 S (23.2)	αAla212 S (19.4)	αTyr215 S (18.7)	αLeu217 S (27.2)
N176 (Golden, E. et al. 2013)	αVal122 B (26.2)	αGly162 B (14.4)	αLeu163 B (18.5)	αSer165 B (13.9)	αAsp197 S (24.2)	αCys200 S (20.1)	αArg209 S (12.9)	αLeu210 S (15.5)	αGlu211 S (17.0)	αAla212 S (19.9)	αAla215 S (19.2)	αLeu217 S (25.9)
N176 (Conti, G. et al. 2014)	αVal122 B (27.7)	αGly162 B (15.7)	αLeu163 B (20.1)	αMet165 B (18.6)	αAsp197 S (29.1)	αCys200 S (22.5)	αArg209 S (24.6)	αLeu210 S (23.7)	αGlu211 S (22.0)	αAla212 S (20.7)	αAla215 S (21.8)	αLeu217 S (27.6)
130 (Yang, Y. L. et al. 1991)	αAla108 B (28.3)	- -	αIle122 B (30.2)	αPro124 S (33.6)	αTyr149 S (10.5)	αAla151 S (20.8)	αPro162 S (19.9)	αPro163 S (18.4)	αAsp164 S (19.1)	αLeu165 S (14.9)	- -	- -
130 (Zhang, W. et al. 2005)	αAla108 B (29.0)	- -	αIle122 B (31.1)	αPro124 S (34.7)	αPhe149 S (10.1)	αAla151 S (20.9)	αPro162 S (20.3)	αPro163 S (18.9)	αAsp164 S (17.9)	αLeu165 S (15.8)	- -	- -
130 (Zhang, W. et al. 2005)	αAla108 B (28.4)	- -	αIle122 B (30.1)	αPro124 S (33.6)	αTyr149 S (9.7)	αAla151 S (20.8)	αPro162 B (19.9)	αPro163 S (18.5)	αAsp164 S (17.1)	αLeu165 S (15.2)	- -	- -
130 (Zhang, W. et al. 2005)	αAla108 B (27.8)	- -	αIle122 B (29.7)	αPro124 S (33.3)	αTyr149 S (8.8)	αAla151 S (19.5)	αPro162 S (18.9)	αPro163 S (17.4)	αAsp164 S (18.4)	αLeu165 S (13.9)	- -	- -
KAC-1 (Kim, D.-W. et al. 1999)	αAla110 B (28.6)	- -	αIle124 B (30.2)	αPro126 S (33.9)	αTyr149 S (10.0)	αAla153 S (21.1)	αPro164 S (20.2)	αPro165 S (18.7)	αAsp166 S (18.9)	αLeu167 S (15.0)	- -	- -
KAC-1 (Mei, T. et al. 2015)	αAla110 B (27.8)	- -	αIle124 B (29.8)	αPro126 S (33.2)	αTrp149 S (10.6)	αAla153 S (19.3)	αPro164 S (18.7)	αPro165 S (17.3)	αAsp166 S (17.8)	αLeu167 S (13.7)	- -	- -

Table S.9.1. Spatial distribution of residues related with the performance in some acylases. Protein structure modeled by I-Tasser (B: Buried, S:Surface, BP: Binding Pocket and G: Gate toward catalytic pocket). Distance to βSer[1] in parentheses in Angstroms. Gray background represent parental enzyme. Black background shows mutated amino acids. Text in red highlights those positions altered in more than 2.5 Å their relative coordinates. Cells with red borders reveal residues that changed their orientation toward or from the solvent (continuation)

	RESIDUES AND THEIR RELATIVE COORDINATES										
***Sl*PVA** (Torres-Bacete, J. et al. 2015)	αArg192 S (22.6)	αLeu193 B (24.0)	βGly8 S (19.8)	βArg31 B (18.9)	βLeu50 BP (11.1)	βSer57 BP (10.9)	βIle58 BP (6.1)	βHis68 BP (6.8)	βVal70 BP (7.7)	βThr72 S,G	βThr75 B (17.4)
***Sl*PVA** 2pva1,21(25)	αArg192 S (26.9)	αLeu193 B (24.5)	βGly8 S (21.5)	βArg31 B (16.6)	βLeu50 BP (10.7)	βSer57 BP (11.8)	βIle58 BP (5.9)	βHis68 BP (8.3)	βVal70 BP (7.2)	βThr72 S,G	βThr75 B (17.8)
***Sl*PVA** 2pva1,2(55)	αArg192 S (22.1)	αLeu193 S (16.5)	βGly8 S (23.7)	βArg31 B (16.5)	βLeu50 BP (9.6)	βSer57 BP (9.5)	βIle58 BP (6.0)	βHis68 BP (8.2)	βVal70 BP (4.8)	βThr72 S,G	βThr75 S (15.9)
***Sl*PVA** 2pva1,2(149)	αArg192 S (29.5)	αLeu193 B (22.3)	βGly8 S (21.4)	βArg31 B (17.5)	βLeu50 BP (10.9)	βSer57 BP (12.2)	βIle58 BP (5.8)	βHis68 BP (5.2)	βVal70 BP (7.2)	βThr72 S,G	βThr75 B (17.3)
***Au* AAC** (Torres-Bacete, J. et al. 2007)	αAla187 S (24.9)	αLeu188 S (17.9)	βAla8 S (22.6)	βArg31 B (18.6)	βLeu50 BP (12.8)	βGlu57 BP (10.1)	βIle58 BP (5.9)	βHis68 BP (5.7)	βVal70 BP (6.8)	βThr72 S,G	βThr75 B (17.6)
***Au* AAC** a(292)	αAla187 S (23.1)	αLeu188 S (21.9)	βAla8 S (22.5)	βArg31 B (18.4)	βLeu50 BP (11.2)	βGlu57 BP (7.3)	βIle58 BP (6.5)	βHis68 BP (8.9)	βVal70 BP (7.1)	βThr72 S,G	βThr75 S (16.9)
***Au* AHLA** (Velasco-Bucheli, R. et al. 2015)	αVal195 S (17.7)	αArg196 S (18.0)	βArg8 S (26.5)	βArg31 B (17.6)	βLeu50 BP (10.6)	βLeu57 BP (10.5)	βIle58 BP (5.8)	βHis68 BP (8.3)	βVal70 BP (6.6)	βThr72 S,G	βThr75 B (16.6)
ATCC 21285 (Barbero, J. et al. 1986)	αThr238 S (28.1)	αAla239 S (30.3)	βLys8 S (24.1)	βTyr31 B (13.4)	βPro49 BP (12.5)	βVal56 BP (9.7)	βPhe57 BP (6.1)	βSer67 BP (6.8)	βAla69 BP (7.2)	βPhe71 B (13.3)	βAsp74 B (18.4)
ATCC 21285 (Martín, J. et al. 1990)	αThr238 S (27.4)	αAla239 S (30.0)	βLys8 S (23.3)	βTyr31 B (13.6)	βPro49 BP (12.4)	βVal56 BP (9.9)	βPhe57 BP (4.9)	βSer67 BP (6.4)	βAla69 BP (7.5)	βPhe71 S (12.1)	βAsp74 B (17.5)
ATCC 21285 (Roa, A. et al. 1994)	αThr238 S (22.8)	αAla239 S (25.3)	βLys8 S (27.5)	βTyr31 B (14.7)	βPro49 BP (11.3)	βVal56 BP (9.3)	βPhe57 BP (5.2)	βSer67 BP (8.0)	βAla69 BP (5.5)	βPhe71 B (11.7)	βAsp74 B (15.7)
ATCC 11105 (Oh, S. J. et al. 1987)	αLeu233 S (24.0)	αLeu234 S (25.2)	βLys8 S (25.3)	βTyr31 B (17.2)	βPro49 BP (11.8)	βVal56 BP (8.5)	βPhe57 BP (5.4)	βSer67 BP (6.4)	βAla69 BP (6.2)	βPhe71 S (11.4)	βAsp74 B (16.4)
ATCC 11105 (del Rio, G. et al. 1995)	αLeu233 S (23.3)	αLeu234 S (23.3)	βLys8 S (24.8)	βTyr31 S (17.3)	βPro49 BP (11.5)	βVal56 BP (8.5)	βPhe57 BP (4.5)	βSer67 BP (5.9)	βAla69 BP (6.1)	βPhe71 S (11.5)	βAsp74 B (15.4)
ATCC 11105 (Morillas, M. et al. 2003)	αLeu233 S (24.1)	αLeu234 S (16.1)	βLys8 S (25.8)	βTyr31 S (11.9)	βPro49 BP (14.4)	βVal56 BP (12.9)	βPhe57 BP (6.8)	βSer67 BP (11.4)	βAla69 BP (9.7)	βCys71 S (15.0)	βAsp74 B (19.8)
ATCC 11105 (Morillas, M. et al. 2003)	αLeu233 S (25.1)	αLeu234 S (21.8)	βLys8 S (25.2)	βTyr31 S (18.8)	βPro49 BP (12.3)	βVal56 BP (9.0)	βPhe57 BP (5.2)	βSer67 BP (6.2)	βAla69 BP (5.6)	βLeu71 S (10.2)	βAsp74 B (15.4)
ATCC 11105 (Balci, H. et al. 2014)	αLeu233 S (24.6)	αLeu234 S (21.3)	βIle8 S (21.9)	βTyr31 S (17.5)	βPro49 BP (12.5)	βVal56 BP (9.9)	βPhe57 BP (4.8)	βSer67 BP (5.9)	βAla69 BP (7.5)	βPhe71 S (10.2)	βAsp74 B (16.6)
SY-77-1 (Shibuya, Y. et al. 1981)	- -	- -	βPro8 S (21.7)	βThr32 B (15.3)	βGln50 BP (10.1)	βArg57 BP (6.8)	βPhe58 BP (4.8)	βAsn68 BP (8.2)	βVal70 BP (5.6)	βGly72 S,G	βGly75 B (18.1)
SY-77-1 (Sio, C. F. et al. 2002)	- -	- -	βPro8 S (20.6)	βThr32 B (16.1)	βGln50 BP (11.2)	βArg57 BP (8.5)	βPhe58 BP (5.1)	βAsn68 BP (7.9)	βVal70 BP (7.0)	βGly72 S,G	βGly75 B (19.4)
SY-77-1 (Otten, L. G. et al. 2002)	- -	- -	βPro8 S (21.4)	βThr32 B (17.5)	βGln50 BP (12.6)	βArg57 BP (9.4)	βPhe58 BP (6.9)	βHis68 BP (10.8)	βVal70 BP (5.6)	βGly72 S,G	βGly75 S (17.8)
SY-77-1 (Otten, L. G. et al. 2002)	- -	- -	βPro8 S (20.8)	βThr32 B (17.6)	βGln50 BP (10.7)	βArg57 BP (10.3)	βPhe58 BP (6.4)	βAsn68 BP (9.0)	βVal70 BP (6.1)	βGly72 S,G	βGly75 B (18.2)
SY-77-1 (López-Gallego, F. et al. 2008)	- -	- -	βPro8 S (20.5)	βThr32 B (15.9)	βGln50 BP (12.0)	βArg57 BP (8.5)	βPhe58 BP (4.7)	βAsn68 BP (7.5)	βVal70 BP (7.1)	βGly72 S,G	βGly75 B (19.4)
SE83 (Matsuda, A. et al. 1987)	αAla227 S (29.4)	αMet228 S (31.2)	βPro8 S (20.3)	βMet31 B (17.7)	βVal49 BP (11.8)	βHis57 BP (10.4)	βPhe58 BP (4.7)	βVal68 BP (6.1)	βHis70 BP (8.4)	βPhe72 S (11.0)	βIle75 S (17.1)
SE83 (Shin, Y. C. et al. 2009)	αAla227 S (29.6)	αMet228 S (30.5)	βPro8 S (20.8)	βMet31 B (17.1)	βVal49 BP (11.3)	βHis57 BP (12.5)	βAsn58 BP (10.1)	βVal68 BP (5.5)	βHis70 BP (6.6)	βPhe72 S (10.4)	βThr75 B (16.8)
SE83 (Wang, Y. et al. 2012)	αAla227 S (30.7)	αMet228 S (31.5)	βPro8 S (20.1)	βMet31 B (17.8)	βVal49 BP (12.2)	βHis57 BP (9.3)	βAsn58 BP (8.7)	βVal68 BP (5.6)	βHis70 BP (10.7)	βPhe72 S (10.6)	βThr75 B (17.4)
SE83 (Xiao, Y. et al. 2014)	αAla227 S (28.9)	αMet228 S (31.2)	βPro8 S (20.4)	βMet31 B (17.7)	βVal49 BP (11.3)	βAla57 BP (7.8)	βPhe58 BP (4.0)	βVal68 BP (6.8)	βHis70 BP (5.9)	βPhe72 S (10.5)	βIle75 S (16.3)
SE83 (Xiao, Y. et al. 2014)	αAla227 S (29.7)	αMet228 S (23.8)	βPro8 S (20.1)	βMet31 B (17.8)	βVal49 BP (11.9)	βAla57 BP (8.4)	βPhe58 BP (8.3)	βVal68 BP (6.5)	βTyr70 BP (7.7)	βPhe72 S (11.0)	βIle75 S (15.8)
SE83 (Xiao, Y. et al. 2014)	αAla227 S (30.0)	αMet228 S (30.6)	βPro8 S (20.4)	βMet31 B (17.7)	βVal49 BP (12.3)	βAla57 BP (9.2)	βPhe58 BP (8.3)	βVal68 BP (6.1)	βTyr70 BP (8.0)	βPhe72 S (11.1)	βIle75 S (15.7)
SE83 (Zhang, J. et al. 2014)	- -	- -	βPro8 S (20.7)	βMet31 B (15.1)	βVal49 BP (11.6)	βHis57 BP (10.4)	βPhe58 BP (4.4)	βVal68 BP (6.2)	βHis70 BP (11.0)	βPhe72 S (13.1)	βIle75 S (16.2)
SE83 (Zhang, J. et al. 2014)	αAla227 S (29.0)	αMet228 S (31.0)	βPro8 S (20.1)	βMet31 B (18.6)	βVal49 BP (12.5)	βHis57 BP (7.2)	βPhe58 BP (9.8)	βVal68 BP (6.8)	βHis70 BP (9.9)	βPhe72 S (10.9)	βIle75 S (15.9)
N176 (Aramori, I. et al. 1991)	αAla220 S (19.8)	αVal221 S (16.3)	βPro8 S (21.4)	βMet31 S (18.3)	βVal49 BP (12.4)	βHis57 BP (11.5)	βPhe58 BP (11.2)	βVal68 BP (6.2)	βHis70 BP (9.4)	βPhe72 S (11.4)	βIle75 S (16.6)
N176 (Ishii, Y. et al. 1995)	αAla220 S (22.7)	αVal221 S (17.6)	βPro8 S (20.3)	βPhe31 S (14.2)	βVal49 BP (11.9)	βHis57 BP (8.8)	βPhe58 BP (9.0)	βVal68 BP (6.3)	βHis70 BP (6.0)	βPhe72 S (10.4)	βIle75 S (16.3)
N176 (Pollegioni, L. et al. 2005)	αAla220 S (22.5)	αVal221 S (16.3)	βPro8 S (20.4)	βPhe31 S (15.2)	βVal49 BP (11.7)	βSer57 BP (8.2)	βPhe58 BP (8.5)	βVal68 BP (4.7)	βSer70 BP (7.3)	βPhe72 S (10.2)	βIle75 S (16.4)
N176 (Pollegioni, L. et al. 2005)	αAla220 S (20.8)	αVal221 S (16.1)	βPro8 S (21.2)	βPhe31 S (16.5)	βVal49 BP (12.2)	βSer57 BP (8.9)	βPhe58 BP (8.2)	βVal68 BP (6.7)	βSer70 BP (7.2)	βPhe72 S (11.1)	βIle75 B (16.2)
N176 (Golden, E. et al. 2013)	αAla220 S (20.3)	αVal221 S (18.5)	βPro8 S (22.0)	βPhe31 S (14.5)	βVal49 BP (10.5)	βSer57 BP (8.3)	βPhe58 BP (5.1)	βVal68 BP (5.8)	βSer70 BP (7.2)	βPhe72 S (9.6)	βIle75 S (15.6)
N176 (Conti, G. et al. 2014)	αAla220 S (22.0)	αVal221 S (17.7)	βPro8 S (20.3)	βPhe31 S (15.9)	βVal49 BP (11.6)	βSer57 BP (8.4)	βPhe58 BP (8.5)	βVal68 BP (6.4)	βSer70 BP (8.6)	βPhe72 S (10.7)	βIle75 S (16.6)
130 (Yang, Y. L. et al. 1991)	- -	- -	βPro8 S (20.5)	βThr32 B (16.1)	βGln50 BP (9.3)	βArg57 BP (10.0)	βPhe58 BP (4.7)	βAsn68 BP (8.4)	βVal70 BP (7.0)	βGly72 S (12.4)	βGly75 B (19.3)
130 (Zhang, W. et al. 2005)	- -	- -	βPro8 S (20.0)	βThr32 B (16.6)	βGln50 BP (11.3)	βArg57 BP (10.4)	βPhe58 BP (5.1)	βAsn68 BP (8.2)	βVal70 BP (7.2)	βGly72 S (12.2)	βGly75 B (19.2)
130 (Zhang, W. et al. 2005)	- -	- -	βPro8 S (20.4)	βThr32 B (16.0)	βAsn50 BP (10.4)	βArg57 BP (9.9)	βPhe58 BP (4.9)	βAsn68 BP (7.5)	βVal70 BP (7.0)	βGly72 S (12.5)	βGly75 B (19.4)
130 (Zhang, W. et al. 2005)	- -	- -	βPro8 S (21.6)	βThr32 B (15.7)	βAsn50 BP (9.7)	βArg57 BP (7.9)	βPhe58 BP (4.8)	βAsn68 BP (8.0)	βVal70 BP (5.7)	βGly72 S (11.2)	βGly75 B (18.1)
KAC-1 (Kim, D.-W. et al. 1999)	- -	- -	βPro8 S (20.4)	βThr32 B (15.9)	βGln50 BP (11.9)	βArg57 BP (8.9)	βPhe58 BP (4.7)	βAsn68 BP (8.5)	βVal70 BP (7.1)	βGly72 S (12.7)	βGly75 B (19.6)
KAC-1 (Mei, T. et al. 2015)	- -	- -	βPro8 S (21.8)	βThr32 B (15.3)	βGly50 BP (12.5)	βArg57 BP (7.8)	βPhe58 BP (4.9)	βAsn68 BP (8.0)	βVal70 BP (5.7)	βGly72 S (11.1)	βGly75 B (18.0)

Table S.9.1. Spatial distribution of residues related with the performance in some acylases. Protein structure modeled by I-Tasser (B: Buried, S:Surface, BP: Binding Pocket and G: Gate toward catalytic pocket). Distance to βSer[1] in parentheses in Angstroms. Gray background represent parental enzyme. Black background shows mutated amino acids. Text in red highlights those positions altered in more than 2.5 Å their relative coordinates. Cells with red borders reveal residues that changed their orientation toward or from the solvent (continuation)

	RESIDUES AND THEIR RELATIVE COORDINATES										
***Sl*PVA** (Torres-Bacete, J. et al. 2015)	βHis79 B (31.1)	βSer161 B (16.7)	βLeu183 B (14.2)	βVal186 BP (9.3)	βVal204 S (19.8)	βArg207 S (30.2)	βAsn272 BP (6.3)	βArg373 S (37.8)	βGly450 S (24.2)	βGln453 B (20.2)	βTyr497 B (12.2)
***Sl*PVA** 2pva1,21(25)	βHis79 B (30.4)	βSer161 B (16.1)	βLeu183 B (12.7)	βVal186 BP (8.4)	βVal204 S (19.4)	βArg207 S (27.6)	βAsn272 BP (8.0)	βTrp373 S (31.2)	βGly450 S (24.5)	βGln453 B (20.4)	βTyr497 B (10.8)
***Sl*PVA** 2pva1,2(55)	βHis79 B (29.5)	βSer161 B (15.6)	βLeu183 B (12.5)	βVal186 BP (7.5)	βPhe204 S (18.3)	βArg206 S (25.5)	βArg236 S (8.5)	-	-	-	-
***Sl*PVA** 2pva1,2(149)	βHis79 B (29.3)	βSer161 B (16.0)	βLeu183 B (14.2)	βVal186 BP (8.4)	βVal204 S (18.8)	βArg207 S (30.7)	βAsn272 BP (8.4)	βArg373 S (36.6)	βGly450 S (24.6)	βGln453 B (20.6)	βTyr497 B (12.9)
***Au*AAC** (Torres-Bacete, J. et al. 2007)	βHis79 B (29.9)	βTrp157 B (12.7)	βLeu179 B (13.5)	βVal182 BP (8.3)	βVal200 B (20.1)	βArg203 S (26.9)	βAsn268 BP (5.8)	βArg373 S (38.4)	βGly443 B (24.7)	βHis446 B (22.1)	βPhe489 B (9.0)
***Au*AAC** a(292)	βHis79 B (30.1)	βTrp157 B (13.5)	βLeu179 B (12.8)	βVal182 BP (8.0)	βVal200 B (20.0)	βArg203 S (26.9)	βAsn268 BP (5.4)	βArg373 S (37.3)	βGly443 B (24.6)	βHis446 B (22.4)	βPhe489 B (9.0)
***Au*AHLA** (Velasco-Bucheli, R. et al. 2015)	βPhe79 B (27.6)	βTrp163 B (12.6)	βVal185 B (12.8)	βLeu188 BP (8.2)	βVal206 B (18.4)	βHis209 S (29.7)	βAsn274 BP (7.9)	βAla380 S (33.2)	βGly456 S (24.9)	βGln459 B (20.3)	βPhe502 B (9.2)
ATCC 21285 (Barbero, J. et al. 1986)	βPhe78 B (29.4)	βTrp154 B (13.5)	-	βIle177 BP (8.2)	βAla195 S (20.0)	βAsp198 S (28.1)	βAsn240 BP (8.4)	βSer323 S (35.0)	βAsp426 S (35.0)	βTrp429 B (37.7)	βArg469 B (17.5)
ATCC 21285 (Martín, J. et al. 1990)	βPhe78 B (29.4)	βTrp154 B (12.9)	-	βIle177 BP (7.3)	βAla195 S (19.4)	βAsp198 S (28.5)	βAsn240 BP (9.6)	βSer323 S (34.5)	βAsp426 S (33.7)	βTrp429 S (38.0)	βArg469 B (18.5)
ATCC 21285 (Roa, A. et al. 1994)	βVal78 B (29.3)	βTrp154 B (13.6)	-	βIle177 BP (6.7)	βAla195 S (18.7)	βAsp198 S (28.5)	βAsn240 BP (7.7)	βSer323 S (34.2)	βAsp426 S (38.0)	βTrp429 S (36.4)	βArg469 B (18.6)
ATCC 11105 (Oh, S. J. et al. 1987)	βPhe78 B (28.1)	βTrp154 B (13.4)	-	βIle177 BP (8.2)	βAla195 B (18.8)	βAsp198 S (27.2)	βAsn241 BP (8.6)	βArg325 S (35.5)	βGlu428 S (39.7)	βTrp431 B (36.9)	βArg471 B (18.1)
ATCC 11105 (del Río, G. et al. 1995)	βPhe78 B (28.5)	βTrp154 B (12.6)	-	βIle177 BP (7.2)	βAla195 B (18.6)	βAsp198 S (27.1)	βAsn241 BP (6.6)	βArg325 S (38.5)	βGlu428 S (38.6)	βArg431 B (38.1)	βArg471 B (18.9)
ATCC 11105 (Morillas, M. et al. 2003)	βPhe78 B (33.3)	βTrp154 B (15.6)	-	βIle177 BP (11.8)	βAla195 B (22.9)	βAsp198 S (31.4)	βAsn241 BP (10.1)	βArg325 S (34.6)	βGlu428 S (37.1)	βTrp431 B (34.0)	βArg471 B (14.3)
ATCC 11105 (Morillas, M. et al. 2003)	βPhe78 B (28.3)	βTrp154 B (14.2)	-	βIle177 BP (7.4)	βAla195 B (18.3)	βAsp198 S (26.7)	βAsn241 BP (4.6)	βArg325 S (37.9)	βGlu428 S (39.7)	βTrp431 B (38.4)	βArg471 B (12.2)
ATCC 11105 (Balci, H. et al. 2014)	βPhe78 B (29.7)	βTrp154 B (12.7)	-	βIle177 BP (8.8)	βAla195 B (19.8)	βAsp198 S (29.1)	βAsn241 BP (7.9)	βArg325 S (31.7)	βGlu428 S (40.2)	βTrp431 B (37.2)	βArg471 B (10.2)
SY-77-1 (Shibuya, Y. et al. 1981)	βTyr79 B (27.6)	βTyr153 B (10.3)	βVal174 B (12.7)	βPhe177 BP (7.4)	βVal195 B (18.4)	βLys198 S (30.7)	βAsn244 BP (4.7)	βAla333 S (33.4)	βGly411 B (24.9)	βSer414 B (22.0)	βTrp457 B (10.2)
SY-77-1 (Sio, C. F. et al. 2002)	βTyr79 B (29.4)	βTyr153 B (11.0)	βVal174 B (13.5)	βPhe177 BP (8.5)	βVal195 B (19.5)	βLys198 S (27.3)	βAsn244 BP (5.8)	βAla333 S (33.9)	βGly411 B (24.5)	βSer414 B (22.0)	βTrp457 B (9.7)
SY-77-1 (Otten, L. G. et al. 2002)	βTyr79 B (26.9)	βTyr153 B (13.3)	βVal174 B (13.4)	βPhe177 BP (8.0)	βVal195 B (18.7)	βLys198 S (29.5)	βAsn244 BP (2.9)	βAla333 S (33.6)	βGly411 B (25.1)	βSer414 B (21.7)	βTrp457 B (11.4)
SY-77-1 (Otten, L. G. et al. 2002)	βTyr79 B (27.6)	βTyr153 B (12.1)	βVal174 B (14.5)	βLeu177 BP (8.0)	βVal195 B (18.8)	βLys198 S (30.5)	βAsn244 BP (4.2)	βAla333 S (33.5)	βGly411 B (25.1)	βSer414 B (21.9)	βTrp457 B (11.1)
SY-77-1 (López-Gallego, F. et al. 2008)	βTyr79 B (28.8)	βTyr153 B (10.5)	βVal174 B (13.4)	βHis177 BP (8.4)	βVal195 B (19.5)	βLys198 S (28.0)	βAsn244 BP (5.9)	βAla333 S (33.8)	βGly411 B (24.5)	βSer414 B (21.3)	βTrp457 B (9.2)
SE83 (Matsuda, A. et al. 1987)	βTyr79 B (30.5)	βLeu154 B (13.4)	βIle176 B (15.6)	βHis178 BP (9.4)	βArg196 B (21.8)	βSer199 S (28.0)	βAsn242 BP (8.5)	βAla321 S (34.0)	βAla436 S (16.2)	βAsn439 S (12.0)	βSer471 B (8.6)
SE83 (Shin, Y. C. et al. 2009)	βTyr79 B (30.6)	βLeu154 B (13.4)	βVal176 B (15.9)	βHis178 BP (8.0)	βArg196 B (22.8)	βSer199 S (27.8)	βAsn242 BP (7.7)	βAla321 S (34.1)	βAla436 S (13.3)	βAsn439 S (8.7)	βCys471 B (10.0)
SE83 (Wang, Y. et al. 2012)	βTyr79 B (31.1)	βLeu154 B (13.2)	βVal176 B (15.9)	βHis178 BP (10.0)	βArg196 B (23.8)	βSer199 S (27.9)	βAsn242 BP (8.6)	βAla321 S (33.8)	βGly436 S (17.5)	βAsn439 S (7.8)	βCys471 B (7.6)
SE83 (Xiao, Y. et al. 2014)	βTyr79 B (30.5)	βLeu154 B (13.0)	βIle176 B (16.0)	βHis178 BP (9.9)	βArg196 B (22.3)	βSer199 S (28.3)	βAsn242 BP (6.2)	βAla321 S (33.6)	βAla436 S (15.0)	βAsn439 S (14.7)	βSer471 B (9.8)
SE83 (Xiao, Y. et al. 2014)	βTyr79 B (30.5)	βLeu154 B (13.0)	βIle176 B (16.1)	βHis178 BP (10.4)	βArg196 B (23.2)	βSer199 S (28.1)	βAsn242 BP (6.9)	βAla321 S (33.7)	βAla436 S (15.5)	βAsn439 S (13.8)	βSer471 B (9.5)
SE83 (Xiao, Y. et al. 2014)	βTyr79 B (29.3)	βLeu154 B (13.1)	βAsn176 B (14.8)	βHis178 BP (9.7)	βArg196 B (23.2)	βSer199 S (27.6)	βAsn242 BP (7.8)	βAla321 S (33.9)	βAla436 S (19.1)	βAsn439 S (16.7)	βSer471 B (8.5)
SE83 (Zhang, J. et al. 2014)	βTyr79 B (29.0)	βLeu154 B (13.0)	βIle176 B (16.7)	βHis178 BP (10.8)	βArg196 B (23.8)	βSer199 S (28.3)	βAsn242 BP (9.4)	βAla321 S (34.0)	βAla436 S (19.2)	βAsn439 S (20.1)	βSer471 B (8.8)
SE83 (Zhang, J. et al. 2014)	βTyr79 B (28.5)	βLeu154 B (13.7)	βIle176 B (15.4)	βHis178 BP (12.3)	βArg196 B (22.7)	βSer199 S (27.5)	βAsn242 BP (8.2)	βAla321 S (33.9)	βAla436 S (22.6)	βAsn439 S (13.8)	βSer471 B (8.5)
N176 (Aramori, I. et al. 1991)	βTyr79 B (28.3)	βLeu154 B (14.5)	βIle176 B (15.8)	βHis178 BP (9.3)	βArg196 B (21.6)	βSer199 S (26.7)	βAsn242 BP (6.7)	βAla321 S (33.8)	βGly436 S (17.8)	βAsn439 S (14.5)	βSer471 B (9.6)
N176 (Ishii, Y. et al. 1995)	βTyr79 B (29.0)	βLeu154 B (13.2)	βIle176 B (16.6)	βHis178 BP (10.3)	βArg196 B (23.2)	βSer199 S (28.3)	βAsn242 BP (9.3)	βAla321 S (33.5)	βGly436 S (17.4)	βAsn439 S (12.4)	βSer471 B (8.3)
N176 (Pollegioni, L. et al. 2005)	βTyr79 B (30.0)	βLeu154 B (13.0)	βIle176 B (15.8)	βHis178 BP (10.1)	βArg196 B (22.8)	βSer199 S (27.9)	βAsn242 BP (8.6)	βAla321 S (33.9)	βGly436 S (15.8)	βAsn439 S (20.1)	βSer471 B (7.8)
N176 (Pollegioni, L. et al. 2005)	βTyr79 B (28.4)	βLeu154 B (15.7)	βIle176 B (16.1)	βHis178 BP (12.3)	βArg196 B (22.0)	βSer199 S (27.1)	βAsn242 BP (7.5)	βAla321 S (33.8)	βGly436 S (20.8)	βAsn439 S (14.9)	βSer471 B (9.9)
N176 (Golden, E. et al. 2013)	βTyr79 B (27.2)	βLeu154 B (12.8)	βIle176 B (14.8)	βHis178 BP (9.0)	βArg196 B (21.4)	βSer199 S (27.2)	βAsn242 BP (6.6)	βAla321 S (34.7)	βGly436 S (17.4)	βAsn439 S (15.6)	βSer471 B (12.0)
N176 (Conti, G. et al. 2014)	βTyr79 B (29.9)	βTyr154 B (10.9)	βIle176 B (14.5)	βHis178 BP (10.7)	βArg196 B (22.7)	βSer199 S (28.1)	βAsn242 BP (8.0)	βAla321 S (34.0)	βGly436 S (18.3)	βAsn439 S (15.9)	βSer471 B (8.6)
130 (Yang, Y. L. et al. 1991)	βTyr79 B (28.7)	βTyr153 B (11.3)	βVal174 B (13.4)	βPhe177 BP (7.1)	βVal195 S (19.4)	βLys198 S (27.3)	βAsn244 BP (5.4)	βAla333 S (34.0)	βGly411 B (24.5)	βSer414 B (21.6)	βTrp457 B (9.7)
130 (Zhang, W. et al. 2005)	βTyr79 B (29.0)	βTyr153 B (11.1)	βVal174 B (13.4)	βPhe177 BP (7.9)	βVal195 S (19.1)	βLys198 S (27.6)	βAsn244 BP (5.1)	βAla333 S (33.7)	βGly411 B (24.8)	βSer414 B (21.7)	βTrp457 B (10.1)
130 (Zhang, W. et al. 2005)	βTyr79 B (29.7)	βTyr153 B (10.5)	βVal174 B (13.5)	βPhe177 BP (8.4)	βVal195 B (19.6)	βLys198 S (27.5)	βAsn244 BP (5.5)	βAla333 S (33.8)	βGly411 B (24.5)	βSer414 B (21.8)	βTrp457 B (9.3)
130 (Zhang, W. et al. 2005)	βTyr79 S (28.1)	βTyr153 B (10.1)	βVal174 B (12.6)	βPhe177 BP (6.0)	βVal195 B (18.3)	βAla198 S (26.7)	βAsn244 BP (4.5)	βAla333 S (34.4)	βGly411 B (25.0)	βSer414 B (22.4)	βTrp457 B (10.4)
KAC-1 (Kim, D.-W. et al. 1999)	βTyr79 B (29.5)	βTyr153 B (11.1)	βVal174 B (13.7)	βPhe177 BP (7.4)	βVal195 S (19.6)	βLys198 S (32.0)	βAsn244 BP (5.8)	βAla333 S (33.8)	βGly411 S (24.3)	βSer414 B (21.5)	βTrp457 B (9.5)
KAC-1 (Mei, T. et al. 2015)	βTyr79 B (27.7)	βTyr153 B (10.2)	βVal174 B (12.6)	βTyr177 BP (9.4)	βVal195 S (18.4)	βLys198 S (25.9)	βAsn244 BP (4.4)	βAla333 S (34.5)	βGly411 B (24.9)	βSer414 B (21.8)	βTrp457 B (10.3)

S.10. Alteration of the predicted solvent accessibility in recombinant acylases

Table S.10.1. Alteration of the predicted solvent accessibility in recombinant acylases. Buried: residues that changed the orientation respect to the solvent (originally buried, or after mutation); Surface: residues with increased or reduced interaction with the solvent. The recombinant 2pva1,2(55) is not represented here because it was truncated and obviously the alterations are notorious

			BURIED									SURFACE								
PARENTAL	**RECOMBINANT CLONE**	**aa**	-30%	-20%	-10%	%	10%	20%	30%	%	Difference	-30%	-20%	-10%	%	10%	20%	30%	%	Difference
Sl PVA	2pva1,21(25)	767	0.0%	0.0%	1.4%	1.4%	2.3%	0.1%	0.0%	2.5%	1.0%	0.0%	0.3%	5.0%	5.2%	4.3%	0.0%	0.0%	4.3%	-0.9%
	2pva1,2(149)	767	0.0%	0.0%	0.8%	0.8%	1.8%	0.1%	0.0%	2.0%	1.2%	0.0%	0.1%	4.0%	4.2%	3.5%	0.1%	0.0%	3.7%	-0.5%
Au AAC	a(292)	753	0.0%	0.0%	1.3%	1.3%	0.3%	0.0%	0.0%	0.3%	-1.1%	0.0%	0.0%	4.0%	4.0%	3.9%	0.0%	0.0%	3.9%	-0.1%
K. citrophila ATCC 21285	αM142A	818	0.0%	0.0%	1.5%	1.5%	1.1%	0.0%	0.0%	1.1%	-0.4%	0.0%	0.1%	4.9%	5.0%	3.3%	0.1%	0.0%	3.4%	-1.6%
	βF78V	818	0.0%	0.0%	1.8%	1.8%	1.2%	0.1%	0.1%	1.5%	-0.4%	0.0%	0.0%	1.6%	1.6%	4.6%	0.1%	0.0%	4.8%	3.2%
E. coli ATCC 11105	βW431R	820	0.0%	0.0%	1.2%	1.2%	0.7%	0.0%	0.0%	0.7%	-0.5%	0.0%	0.2%	3.8%	4.0%	2.6%	0.0%	0.0%	2.6%	-1.5%
	βF71C	820	0.0%	0.0%	0.5%	0.5%	0.7%	0.0%	0.0%	0.7%	0.2%	0.0%	0.0%	4.9%	4.9%	2.8%	0.0%	0.0%	2.8%	-2.1%
	βF71L	820	0.0%	0.0%	0.7%	0.7%	1.0%	0.0%	0.0%	1.0%	0.2%	0.0%	0.0%	2.2%	2.2%	2.2%	0.1%	0.0%	2.3%	0.1%
	βK8I	820	0.0%	0.7%	0.7%	1.5%	0.7%	0.0%	0.0%	0.7%	-0.7%	0.0%	0.0%	5.1%	5.1%	2.2%	0.0%	0.0%	2.2%	-2.9%
Pseudomonas sp. SY-77-1	αY149H	691	0.0%	0.0%	1.2%	1.2%	0.9%	0.0%	0.0%	0.9%	-0.3%	0.0%	0.3%	4.1%	4.3%	4.2%	0.0%	0.0%	4.2%	-0.1%
	βN68H	691	0.0%	0.0%	0.7%	0.7%	1.7%	0.0%	0.0%	1.7%	1.0%	0.0%	0.3%	3.9%	4.2%	4.1%	0.3%	0.0%	4.3%	0.1%
	βF177L	691	0.0%	0.0%	0.7%	0.7%	1.9%	0.0%	0.0%	1.9%	1.2%	0.0%	0.0%	3.6%	3.6%	3.8%	0.3%	0.0%	4.1%	0.4%
	αY149F/βF177H	691	0.0%	1.0%	1.0%	2.0%	1.3%	0.3%	0.0%	1.6%	-0.4%	0.0%	0.1%	3.6%	3.8%	3.3%	0.0%	0.0%	3.3%	-0.4%
Pseudomonas sp. SE83	αV122A/αG140S/βF58N/βI75T/βI176V/βS471C	774	0.0%	0.0%	1.4%	1.4%	1.3%	0.0%	0.0%	1.3%	-0.1%	0.0%	0.1%	3.5%	3.6%	2.7%	0.0%	0.0%	2.7%	-0.9%
	αV122A/αG140S/βF58N/βI75T/βI176V/βA436G/βS471C	774	0.0%	0.0%	1.6%	1.6%	1.3%	0.0%	0.0%	1.3%	-0.3%	0.0%	0.1%	4.1%	4.3%	3.0%	0.0%	0.0%	3.0%	-1.3%
	βH57A	774	0.0%	0.0%	0.9%	0.9%	1.0%	0.0%	0.0%	1.0%	0.1%	0.0%	0.0%	1.3%	1.3%	0.9%	0.0%	0.0%	0.9%	-0.4%
	βH57A/βH70Y	774	0.0%	0.0%	0.9%	0.9%	1.3%	0.0%	0.0%	1.3%	0.4%	0.0%	0.1%	1.8%	1.9%	1.9%	0.0%	0.0%	1.9%	0.0%
	βH57A/βH70Y/βI176N	774	0.0%	0.0%	1.0%	1.0%	1.9%	0.0%	0.0%	1.9%	0.9%	0.0%	0.1%	2.7%	2.8%	1.6%	0.0%	0.0%	1.6%	-1.3%
	αΔA227/αΔM228	772	0.0%	0.0%	0.8%	0.8%	1.0%	0.3%	0.0%	1.3%	0.5%	0.0%	0.3%	4.0%	4.3%	2.7%	0.1%	0.0%	2.8%	-1.4%
	αΔA212/αΔD213/αΔL214/αΔA215	770	0.0%	0.0%	0.8%	0.8%	1.6%	0.1%	0.0%	1.7%	0.9%	0.0%	0.4%	4.4%	4.8%	2.9%	0.3%	0.0%	3.1%	-1.7%
Pseudomonas N176	βM31F	772	0.0%	0.0%	0.5%	0.5%	1.3%	0.0%	0.0%	1.3%	0.8%	0.0%	0.0%	2.2%	2.2%	2.1%	0.0%	0.0%	2.1%	-0.1%
	βM31F/βH57S/βH70S	772	0.0%	0.0%	0.4%	0.4%	1.0%	0.0%	0.0%	1.0%	0.6%	0.1%	0.0%	2.5%	2.6%	2.3%	0.0%	0.0%	2.3%	-0.3%
	αA215Y/βM31F/βH57S/βH70S	772	0.0%	0.0%	0.9%	0.9%	1.3%	0.0%	0.0%	1.3%	0.4%	0.1%	0.0%	3.0%	3.1%	3.4%	0.0%	0.0%	3.4%	0.3%
	αM165S/βM31F/βH57S/βH70S	772	0.0%	0.1%	0.6%	0.8%	1.8%	0.0%	0.0%	1.8%	1.0%	0.1%	0.0%	3.5%	3.6%	3.5%	0.1%	0.0%	3.6%	0.0%
	βM31F/βH57S/βH70S/βL154Y	772	0.0%	0.0%	0.6%	0.6%	1.9%	0.0%	0.0%	1.9%	1.3%	0.1%	0.0%	3.5%	3.6%	3.0%	0.1%	0.0%	3.1%	-0.5%
Pseudomonas sp. 130	αY149F	691	0.0%	0.3%	1.3%	1.6%	0.4%	0.3%	0.0%	0.7%	-0.9%	0.0%	0.3%	2.9%	3.2%	2.3%	0.0%	0.0%	2.3%	-0.9%
	βQ50N	691	0.0%	0.0%	1.2%	1.2%	0.6%	0.0%	0.0%	0.6%	-0.6%	0.0%	0.0%	3.0%	3.0%	2.0%	0.0%	0.0%	2.0%	-1.0%
	βQ50N/βK198A	691	0.0%	0.0%	1.2%	1.2%	0.4%	0.0%	0.0%	0.4%	-0.7%	0.0%	0.0%	3.6%	3.6%	2.2%	0.1%	0.0%	2.3%	-1.3%
P. diminuta KAC-1	αY150W/βQ50G/βF177Y	693	0.1%	0.0%	0.6%	0.7%	1.9%	0.1%	0.0%	2.0%	1.3%	0.1%	0.1%	5.1%	5.3%	4.9%	0.3%	0.0%	5.2%	-0.1%
	AVERAGE	759	0.0%	0.1%	1.0%	1.1%	1.2%	0.1%	0.0%	1.3%	0.2%	0.0%	0.1%	3.5%	3.6%	3.0%	0.1%	0.0%	3.0%	-0.6%
	STANDARD DEVIATION	47	0.0%	0.2%	0.4%	0.4%	0.5%	0.1%	0.0%	0.5%	0.7%	0.1%	0.1%	1.1%	1.1%	1.0%	0.1%	0.0%	1.0%	1.1%

Complutense University of Madrid
Faculty of Pharmacy
Department of Microbiology II and Parasitology