# STUDIES ON KEY STEPS CONTROLLING BIOSYNTHESIS OF ANTIBIOTICS THIOMARINOL AND MUPIROCIN

**by**


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# Abstract

The modular polyketide synthase responsible for biosynthesis of the antibiotic mupirocin occupies 75 kb of *Pseudomonas fluorescens* NCIMB 10586, while a hybrid of PKS/NRPS is responsible for biosynthesis of the antibiotic thiomarinol located on a 97 kb plasmid pTML1 in *Pseudoalteromonas* spp SANK 73390. Biosynthesis of the acyl side chains in mupirocin and thiomarinol are thought to be either through esterification of the fully synthesised fatty acid ($C_9$ or $C_8$) or through extension of the PK derived ester starter unit which is predicted to be carried out on MmpB and TmpB. *mupU/O/V/C/F* and *macpE* are proposed to be sufficient for the conversion of pseudomonic acid B to pseudomonic acid A. Mupirocin is regulated via quorum sensing, while regulation of thiomarinol was not identified.

Production of thiomarinol was determined to occur after 8 hours of growth, while acidic conditions and use of acetone with ethyl acetate improved the extraction. TmlU, the thiomarinol amide ligase, did not complement a *mupU* mutant in mupirocin, and was found to block the biosynthesis of 9-hydroxynonanoic acid, causing truncation of 9-HN. This suggests that MupU, prevents MmpB from being an iterative PKS. KS-B2/ACP-B2 was shown to be involved in the removal of C8-OH from thiomarinol. Genetically manipulated *mupU* increased the production of mupirocin to 3 to 4 fold without abolishing PA-B production. Fused *mupU-macpE* complemented the NCIMB10586Δ*mupU*Δ*macpE* double mutant. However, insertion of this fusion into MmpB blocked the biosynthesis of mupirocin, while insertion after MmpA did not changed the pathway. Attempts to mobilise pTML1 revealed that a hybrid plasmid of RK2-R6K *γ-ori* was integrated into pTML1 but recovery of this cointegrate has not yet been recovered in *E. coli*.

**To the soul of my parents, especially my father**

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## List of abbreviations

| | |
|---|---|
| 3-HP | 3-hydroxypropionate |
| 6-dEB | 6-deoxyerythronolide B |
| 9-HN | 9-hydroxynonanoic acid |
| (A) | adenylation |
| A, C, G, T | nucleotides: adenine, cytosine, guanine, thymine |
| aa | amino acid |
| ACP | acyl carrier protein |
| AH | acyl hydrolase |
| AHL | acyl-homoserine lactone |
| Amp | ampicillin |
| AMP | adenosine monophosphate |
| $Ap^R$ | ampicillin resistant |
| AROS | aromatases |
| Asn | asparagin |
| AT | acyl transferase |
| ATP | adenosine triphosphate |
| BHR | broad host range |
| C | condensation |
| CHS | chalcone synthase |
| CLF | chain length factor |
| Cm | chloramphenicol |
| $Cm^R$ | chloramphenicol resistant |
| CoA | coenzyme A |
| CsCl | cesium chloride |
| CYCS | cyclases |
| Cys | cysteine |
| DEBS | deoxyerythronolide B synthase |
| DH | dehydratase |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| E | epimerisation |
| EDTA | ethylene-diamine-tetraacetic acid |
| ELSD | evaporative light scattering detector |
| ER | enoyl reductase |
| FASs | fatty acid synthases |
| GTs | glycosyltransferases |
| HCS | HMG-CoA synthase |

| | |
|---|---|
| His | histidine |
| HMG | 3-hydroxy-3-methyl glutaric acid |
| HPLC | high performance liquid chromatography |
| HR | homologous recombination |
| Ile | isoleucine |
| IleRS | isoleucyl tRNA synthase |
| IPTG | isopropyl- β-D-thiogalactopyranoside |
| Junk DNA | non-essential DNA segment from pTML1 |
| Kan | kanamycin |
| $Km^R$ | kanamycin resistant |
| KR | β-ketoacyl reductase |
| KS | β-ketoacyl synthase |
| L-agar | Luria-Bertani agar |
| L-broth | Luria-Bertani broth |
| LC | liquid chromatography |
| LM | loading module |
| M-broth | Marine broth |
| M-agar | Marine agar |
| MA | monic acid |
| MAT/MCAT | malonyl-CoA:ACP transacylase |
| MCS | Multiple cloning site |
| MIC | Minimum inhibitory concentration |
| Mmp | mupirocin multifunctional protein |
| MPM | mupiroocin production media |
| mRNA | messenger RNA |
| MRSA | methicillin resistance *Staphylococcus aureus* |
| MS | mass spectrometry |
| MT | methyl transferase |
| NMR | nuclear magnetic resonance |
| NRPS | non-ribosomal peptide synthetase |
| OD | optical density |
| ORF | open reading frame |
| *oriT* | origin of conjugal DNA transfer |
| *oriV* | origin of vegetative replication |
| PA | pseudomonic acid |
| Par | partitioning |
| PCP | peptidyl carrier protein |
| PCR | polymerase chain reaction |
| Phe | phenylalanine |
| *pir* | Rep protein π-producer |
| PKSs | polyketide synthases |
| *polA1* | DNA polymerase I |
| PPtase | phosphopantetheinyl transferase |
| QS | quorum sensing |
| qsc | quorum sensing controlled |
| rbs | ribosomal binding site |
| RNase | ribonuclease |
| SAM | *S*-adenosylmethionine |
| SDS | sodium dodecyl sulphate |
| SDW | sterile distilled water |

| | |
|---|---|
| SSM | secondary stage medium |
| T | thiolation |
| $Tc^R$ | tetracycline resistant |
| TE | thioesterase |
| Tet | tetracycline |
| THP | tetrahydropyran ring |
| TM | thiomarinol |
| TNE | tris-sodium-EDTA |
| *trfA* | *trans*-acting replication function |
| Tris | tris(hydroxymethyl) aminomethane |
| tsp | transcriptional start point |
| TTC | 2, 3, 5-triphenyltetrazolium chloride |
| UV | ultraviolet |
| WT | wild type |
| X-gal | 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside |
| π | *trans*-acting Rep protein |

# CHAPTER 1

## INTRODUCTION

# 1. Introduction

## 1.1 Polyketide natural products

The worldwide distribution of antibiotic resistance, especially the emergence of diverse clones of methicillin resistant *Staphylococcus aureus* (MRSA) is threatening the scientific revolution of antibiotic therapy (Gould, 2009). Therefore, to overcome such problems, many scientists are involved in the search for new antibiotics effective against multiply drug resistant bacteria. Effective biological activities have been investigated with several natural products of novel structure (Sujatha *et al*., 2005). Among the most important microbial secondary metabolites in medicine are polyketides, represented in clinical uses by antibiotics (erythromycin A, rifamycin S), antifungals (amphotericin B), antiparasitics (avermectin), as agents lowering blood cholesterol (lovastatin), and rapamycin as an example of immunosuppressants (Weissman and Leadlay, 2005). Structurally, polyketides include a very diverse family of natural products (Hranueli *et al*., 2001), and have been divided into two groups: those that contain one to six aromatic rings designated as aromatic polyketides, while the second group is subdivided according to their chemical diversity into macrolides and ansamycins (having both lactone and lactam rings), polyethers and polyenes, designated as complex polyketides (O'Hagan, 1991). Structures of a small selection of polyketides with biological activites and pharmacological properties are presented (Figure 1.1). The size of polyketide gene clusters ranges from 20 to more than 100 kb and DNA sequencing of many of these clusters has shown substantial homology, suggesting that they must have emerged by evolution from a common ancestor (Hranueli *et al*., 2001). Moreover, bioinformatic studies on modular PKSs in actinomyctes revealed that a single cross over by recombination between modules of PKSs could be the major driving force for the evolution of a new PKS with chemical diversity (Zucko *et al*., 2012).

Actinomycetes genera and particularly *Streptomyces* species are the group of organisms that have been manifested to produce the largest number of secondary metabolites: actinomycetes synthesize 40% of the total antibiotically active substances, and 40% of that total consists of polyketides (Hranueli *et al*., 2001). In particular, *Streptomyces* is widely known as an important industrial microorganism, since they produce many biologically active metabolites, including antibiotics (Williams *et al*., 1983).

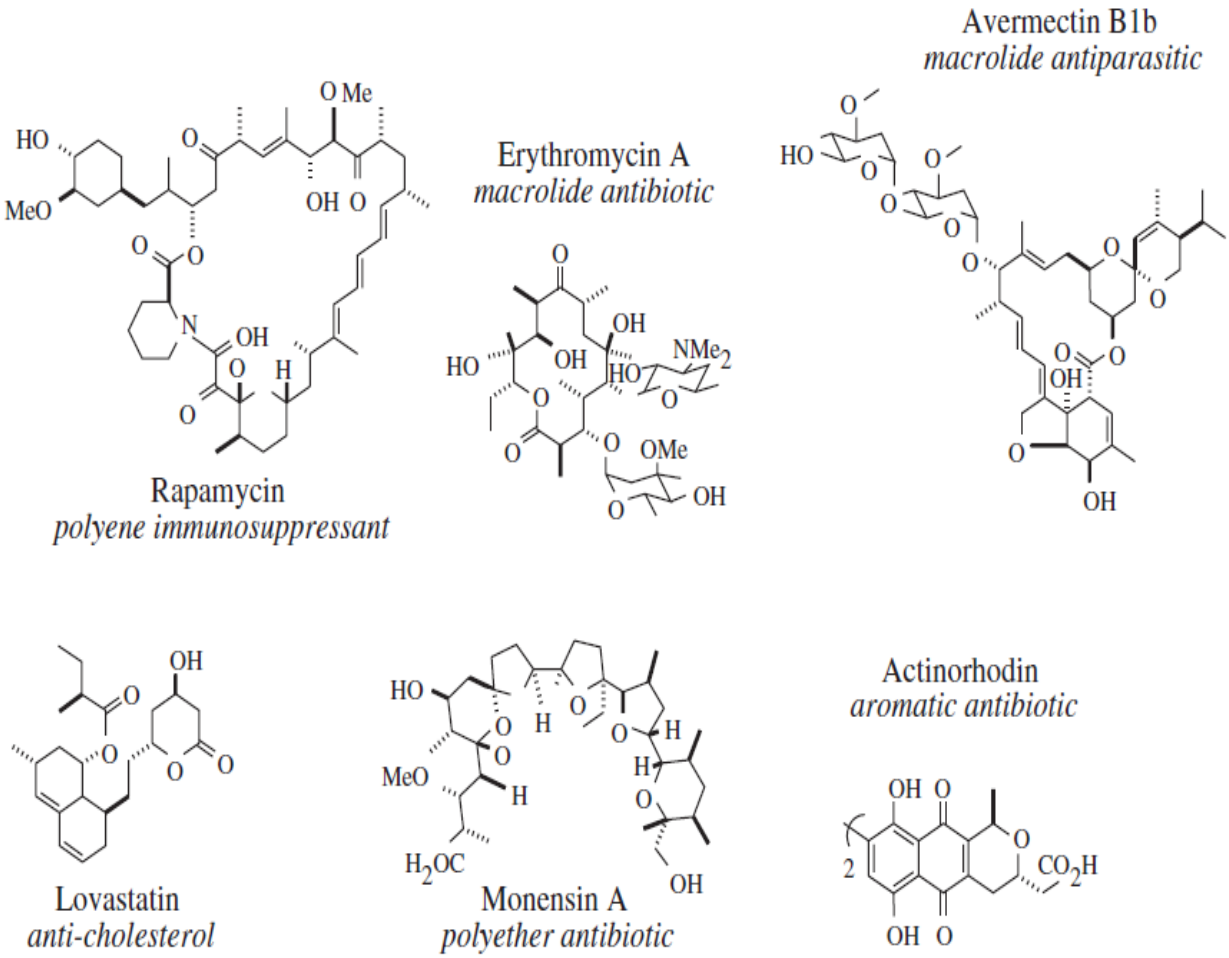



**Figure 1.1 Examples of polyketides with pharmacological properties** (adapted from Weissman, 2004).

Mupirocin (Figure 1.11), as an example of a polyketide product, is produced as a mixture of pseudomonic acids that exhibit immense structural and functional diversity and possess great pharmaceutical potential (Pfeifer and Khosla 2001; Nicolaou *et al*., 2009). It has been used clinically as Bactroban in the UK since 1985 (Cookson, 1990). The main use of mupirocin is to control *Staphylococcus aureus* (especially MRSA) (Wilcox *et al*., 2003). Low toxicity to human and animals made possible the use of this product for various kinds of skin, ear and nose infections (Szell *et al*., 2002). However, mupirocin is unstable in vivo, because of the hydrolytic inactivation of the ester-bond between monic acid and 9-hydroxynonanoic acid (Sutherland *et al*., 1985).

Thiomarinol (Figure 1.18), an interesting example in the class of naturally hybrid molecules (Mancini *et al*., 2007) with a strong antimicrobial activity, is produced by the marine bacterium, *Alteromonas rava* sp. nov. SANK 733390 (Shiozawa *et al*., 1993). Deduction based on chemical configuration confirmed that is a hybrid of two types of antibiotics, pseudomonic acids and pyrrothines (Shiozawa *et al*., 1995). As for mupirocin, the mode of action of thiomarinol is to inhibit bacterial isoleucyl–tRNA synthetase, and the inhibition is stronger by thiomarinols A, B and D (Shiozawa *et al*., 1997). Two other antibiotics belonging to the group of pyrrothine antibiotics, holomycin and *N*-propionylholothin are produced by a mutant strain of cephamycin C producer, and were identified on the basis of activity against a β-lactamase-producing strain of *Serratia marcescens* (Okamura *et al*., 1977; Kenig and Reading, 1979). Approximately 35% of strains of surface associated marine bacteria, in addition to a diverse range of free-living and sediment-inhabiting organisms were observed to produce compounds with antimicrobial properties (Burgess *et al*., 1991, 1999).

Polyketide chain building and assembly are catalyzed by polyketide synthases (PKSs), which are homologues to fatty acid synthase (FASs) required for the biosynthesis of fatty acids (Staunton and Weissman, 2001). Although, all the reactive enzymatic steps involved in chain initiation, condensation steps and termination are homologous, PKSs need to be highly programmed (Hopwood and Sherman, 1990). However, the insights into the enzymatic process of fatty acid assembly will of course help to illuminate the relevant steps carried out during polyketide biosynthesis (Staunton and Weissman, 2001).

## 1.2 Enzymology of fatty acid biosynthesis

Biosynthesis of fatty acids involves a sequence of reactions, beginning with a starter acyl group, and the extender units that comes from the activated acyl-CoA precursors which are assembled by head-to-tail linkage into the growing chain, until the chain reaches the required length (Staunton and Weissman, 2001; Smith and Tsai, 2007). The starter unit, which is often an acetyl group derived from acetyl-CoA, is condensed with the extender units which are acetyl groups derived from malonyl-CoA which is made by carboxylation of acetyl-CoA at the α-position so that it can undergo concerted decarboxylation, allowing chain elongation. The β-keto ester that is formed is reduced successively to a hydroxy, dehydrated alkene and another reduction to give a final alkane chain that is fully reduced (Figure 1.2) (Staunton and Weissman, 2001; Kwan and Schulz, 2011).

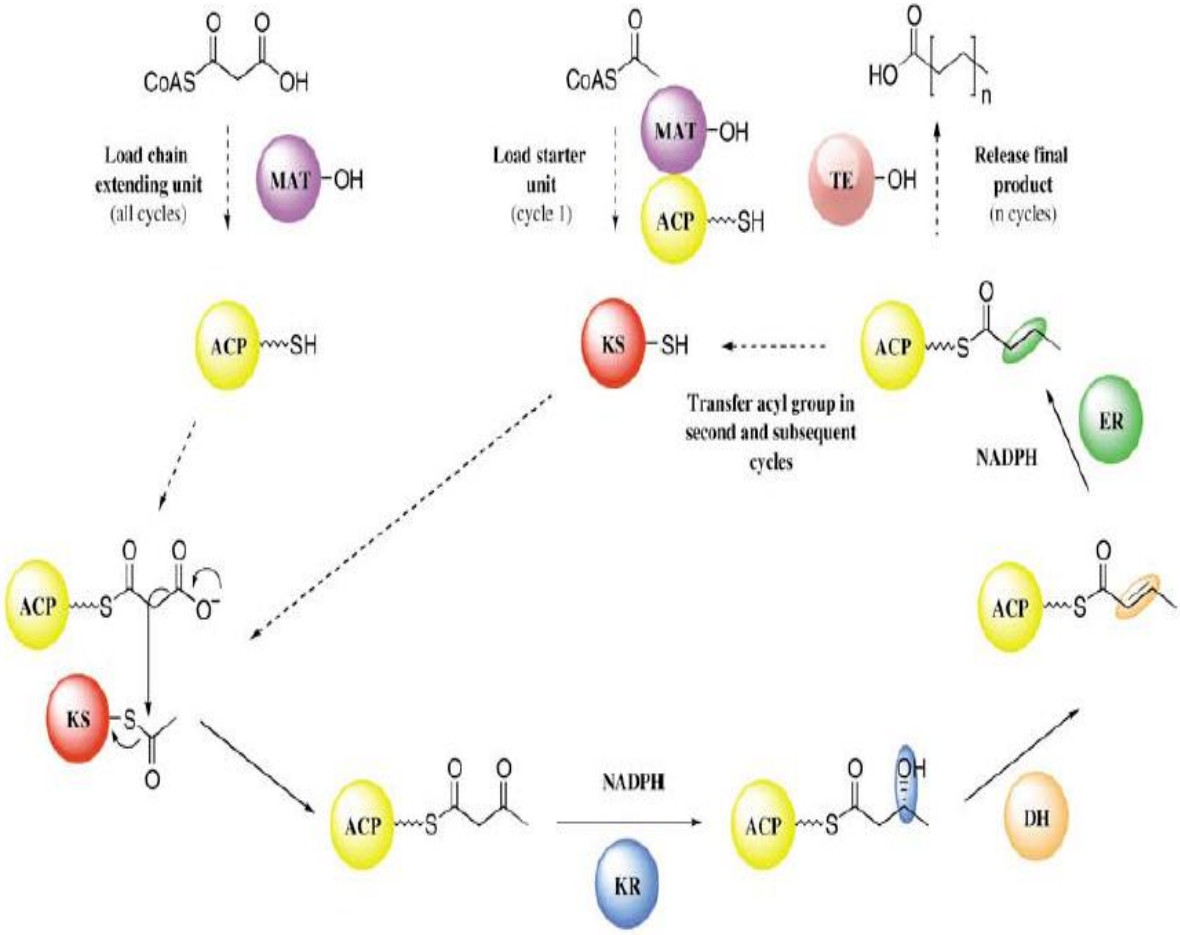



**Figure 1.2 Biosynthetic cycle of fatty acid.** Malonyl-acetyl transferase (MAT), domains presented by acyl carrier protein (ACP); ketosynthase (KS); ketoreductase (KR); dehydratase (DH), and enoyl reductase (ER) (Source: Staunton and Weissman, 2001).

The set of enzymes that are involved in chain extension usually carry the acyl units that participate in these chemical reactions as thioesters. The starter acyl unit is tethered to the thiol of the cysteine residue of the ketosynthase (KS), the enzyme involved in Claisen condensation. Malonate, the chain extender unit is attached to a thiol residue of a protein designated as an acyl carrier protein (ACP) (Kwan and Schulz, 2011). This last thiol is the terminus of the phosphopantetheine residue that is added to the unique serine residue of the ACP by post-translational modification, and it is not part of the primary protein (ACP) chain (Figure 1.3). The function of the long flexible phosphopantetheine arm is to carry the

growing chain and pass it to the other enzymes that are responsible for chain extension (Staunton and Weissman, 2001). Chain elongation is the process of condensation catalysed by ketosynthase (KS) which results in an extended acyl chain in the form of a β-ketoacyl intermediate that is bound to the ACP. In subsequent steps, the β-ketoacyl thioester intermediate can then be reduced at the β-carbon by the ketoreductase (KR-catalysing the reduction of the keto ester to a hydroxyl), dehydrated by a dehydratase (DH-catalysing dehydration of the hydroxyl group to form a double bond), and then reduction of the double bond to a saturated methylene by the enoyl reductase (ER) (Staunton and Weissman, 2001; Kwan and Schulz, 2011). The previous sequence of reactions ensures the completion of the first round of chain extension. After the transfer of the saturated extended acyl chain intermediate from ACP to the KS the cycle is repeated. A number of identical cycles of chain extension using the same enzymes successively, eventually lead to a chain of the required length (usually consist of 14, 16 or 18 carbons). Then the chain is usually released either as a free acid or as an acyl ester after it has been passed to the thioesterase (TE). The set of the domains (KS, ACP, KR, DH, and TE) are present as components in all fatty acid synthases (FASs) (Staunton and Weissman, 2001) and are available in the active form (Kwan and Schulz, 2011). Malonyl-acetyl transferase (MAT), a protein as a seventh partner, has a role in the transfer of the building blocks of the fatty acid chain represented by acetate (to start) and malonate (to extend) from the particular coenzyme A to the active thiol resides of the appropriate domains (Staunton and Weissman, 2001).

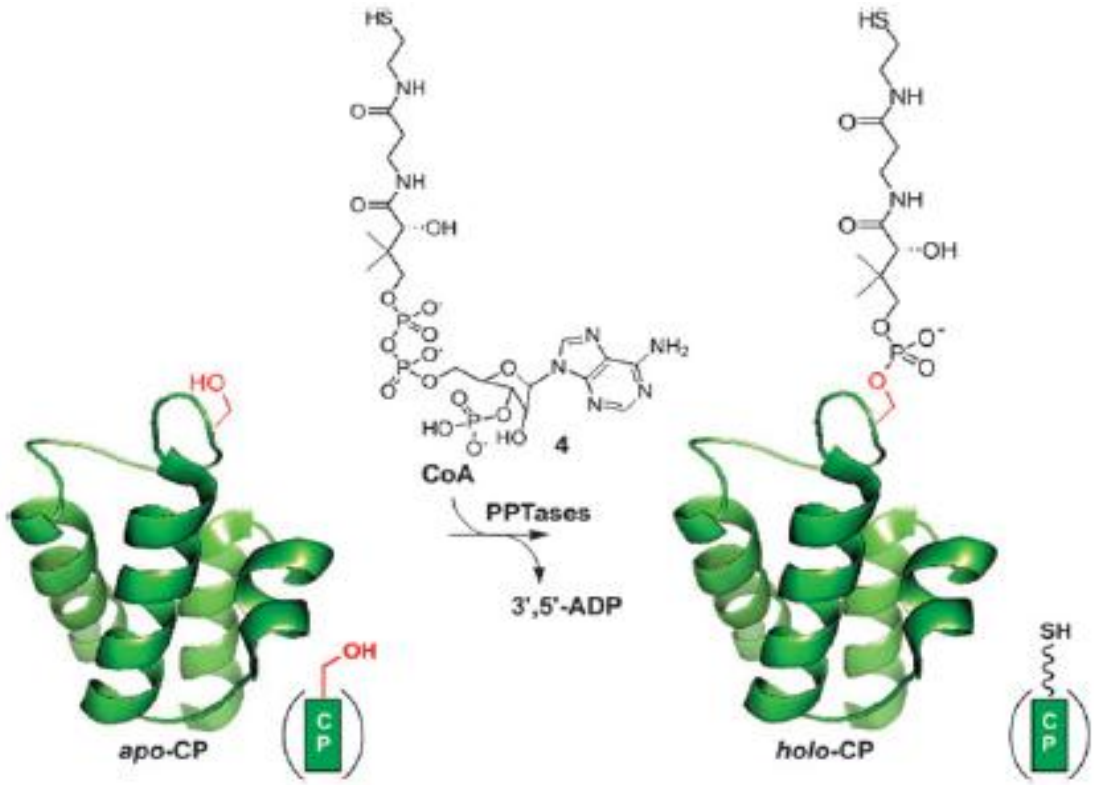



**Figure 1.3 Post-translational modification of the carrier protein mediated by coenzyme A.** The carrier protein is shown as a helical bundle, with cartoon representation in parentheses. Transfer of the 4′-phosphopantetheine prosthetic group to the conserved serine residue of the carrier protein (CP) is mediated by PPtase, converting the CP from *apo* to *holo* form. The thiol at the terminus of the protein can then be used to carry intermediates during FAS, PKS, and NRPS biosynthesis (adapted from Meier and Burkart, 2009).

## 1.3 Biosynthesis of polyketides

Despite the chemical diversity of polyketides and their classification into two main types (type I with multiple catalytic activities contained within large multienzyme complexes that are joined covalently and type II with multiple catalytic activities located on discrete, separated enzymes) they are synthesized by a uniform biochemistry (Hopwood and Sherman,

1990). However, although biosynthesis of type III PKSs, the chalcone synthase superfamily (Austin and Noel, 2003) that were identified as the simplest known PKSs (Kwan and Schulz, 2011), resembles the biosynthesis of type I and type II PKSs superficially, it has a number of important differences (Staunton and Weissman, 2001).

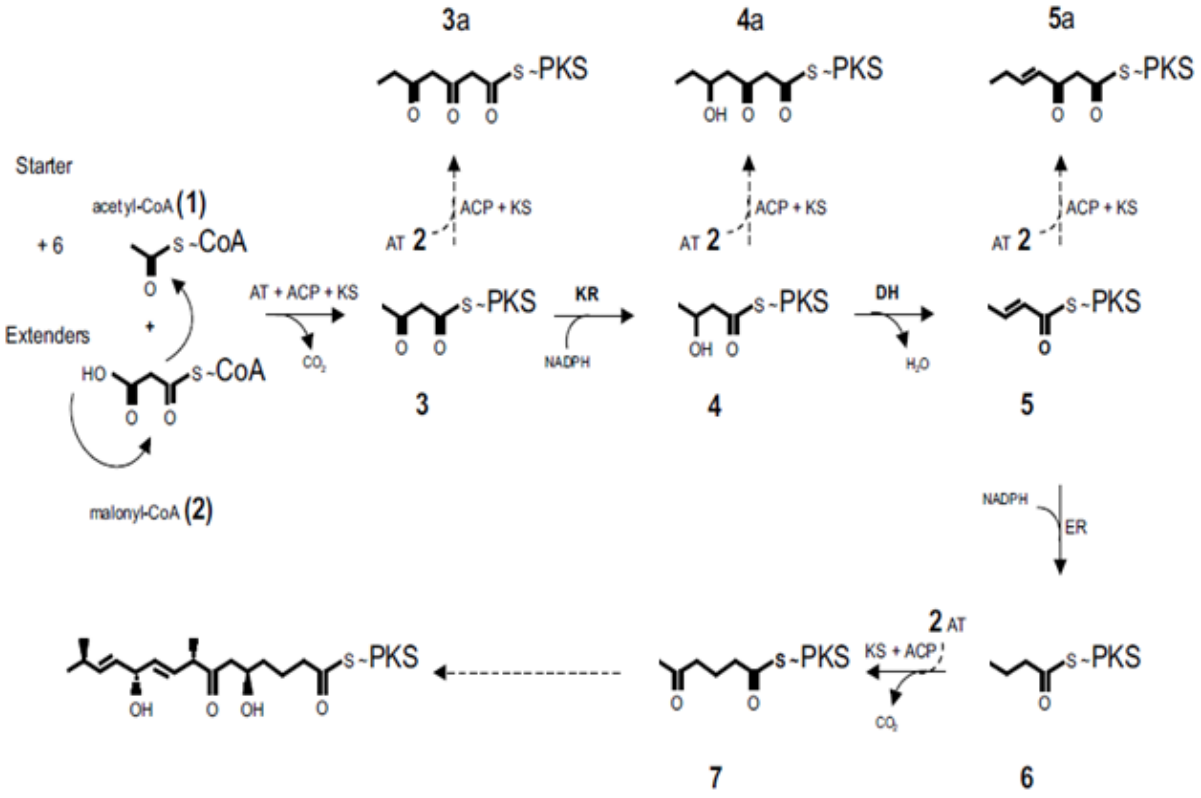



**Figure 1.4 Sequence of events in the biosynthesis of polyketides.** Polyketide synthase (PKS) carries two thiol groups on two separate enzymes; the first one on the condensing enzyme (KS) and the second on the phosphopantetheinylated acyl carrier protein (ACP). Successive steps of reactions are labelled: acyl transferase (AT); ketosynthase (KS); ketoreductase (KR); dehydratase (DH); enoylreductase (ER) (source: Hranueli *et al*., 2001).

Condensation of the starter with an extender unit is the initiation point that ensures the growth of the carbon chain of the polyketide. Both the starter and the extender units are available in the host and are derived from the acyl-CoA precursor. Polyketides usually use

acetyl-CoA and propionyl-CoA as the starter units, and malonyl-CoA and methylmalonyl CoA as extender units (Kwan and Schulz, 2011; Ridley *et al*., 2008). In addition, PKSs can utilize the CoA derivatives of some other carboxylic acids as starters or extender units as well.

Ketosynthase (KS) is the enzyme responsible for the Claisen condensation by decarboxylation of the extender unit. Subsequently, the fate of the diketide that is produced depends on whether it undergoes any reduction by the presence of one or more of the reductive enzymes (KR, DH, and ER) (Kwan and Schulz, 2011). Condensation of another extender unit with the diketide will be the next step, and will end up with the unreduced triketide if the keto group of the diketide has not been reduced. For this reason this type of synthase is known as polyketide. However, the availability of the essential enzyme activities, one or more of the following sequential reactions will take place; β-ketoreduction, dehydration and enoylreduction. The consequence of this is the formation of compounds having a β-hydroxyl group, unsaturated double bond or completely reduced β-carbon (Hranueli *et al*., 2001). Acyltransferase (AT) is particularly responsible for loading the acyl carrier protein (ACP) in the PKS complex with the extender unit for each condensation, and the specificity of the AT determines the extender unit (Smith *et al*., 2003). The triketide formed may then pass through a new round of reduction in the β-keto group, depending on the presence of the reductive enzymes that are actively able to act on triketides. The level of the reduction of that triketide, whether it is full, partial or left unreduced does not correlate with the reductive fate of diketide formed in the prior condensation, nor does it depend on what will happen to the keto group represented by tetra-, penta-, and hexaketides formed in the next accompanying condensation cycles (Figure 1.4) (Hranueli *et al*., 2001).

### 1.3.1 Modular Type I polyketide synthase

Modular type I PKSs are arguably the most important group of polyketide antibiotics, that are large multi-modular enzymes ranging from 100- to 10,000 kDa, and each catalytic site located on a discrete protein domain, which are optimally arranged to be used only once (Hopwood, 1997; McDaniel *et al*., 2005). The functional units responsible for each condensation are named 'modules'. Each module consists of a set of domains, the core of which is represented by ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP), which are linked together covalently and responsible for single chain-extension. It also includes any reductive domains; ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) if the keto group formed by the chain-extension requires any modification before handing the intermediate onto the downstream module. Hence, these multienzyme complexes have been designated as modular polyketide synthases (PKSs) (Weissman, 2004; Weissman and Leadlay, 2005). These modules are further organised into a more complex multimodular subunits. For example, in the biosynthesis of erythromycin, three polypeptides of the 6-dEB synthases (DEBS) are present, designated as DEBS 1, DEBS 2, and DEBS 3, respectively (Weissman and Leadlay, 2005). Although a typical subunit consists of two to three modules, in other PKSs, such as the synthase that produces the polyketide toxin mycolactone, a single polypeptide unusually incorporates nine modules (Smith *et al*., 2003). The individual subunits within the PKSs must recognise the correct partners and it must resist making any incorrect associations that might end up with wrong products. Docking domains located at the extremity of the PKS proteins, which are present in the form of folded structures, act to ensure that such interactions between the subunits occur correctly (Broadhurst *et al*., 2003).

Modular PKSs are preceded by a loading module, which functions in the transfer of the starter unit from the CoA enzymes pool to the KS of module 1, while the last module ends with a thioesterase (TE), which is responsible for the essential process of removing the completed polyketide chain (Hill and Staunton, 2010). The growing PK chains are normally tethered on the acyl carrier protein by the long flexible 'swinging arm' all the way through the synthase, which ensures that they are correctly shepherded when transfered between diverse active centres and not allowed to 'wander off' into the cell (Weissman *et al*., 2004). Domains within the natural modules are normally joined by linkers. These linkers consist of sequences of amino acids ranging from 20 up to more than 250 (Weissman, 2004), thought to play an important role in maintaining the correct architecture of the activites along the biosynthetic process (Staunton and Weissman, 2001). These linkers may work like rigid connectors or may act by providing some sort of flexibility, which will allow and facilitate the movement of the multienzymes (Bevitt *et al*., 1992; Staunton and Wilkinson, 1997). The modular biosynthetic arrangement of type I PKS system suggests the possibility of mixing and splicing domains and even modules within and between various PKS systems, which could create recombinant enzymes that are capable of producing novel, 'unnatural' polyketide natural products (Kuščer *et al*., 2005).

### 1.3.1.1 Erythromycin biosynthetic pathway

The biosynthetic gene cluster of erythromycin (*ery* genes) was discovered after the identification of the resistance gene, *ermE* (Cortes′ *et al*., 1990). The expectation that synthase genes would cluster around the genes that confer self-resistance to the antibiotic increased the probability of the expression of both set of genes at the same time (Hill and Staunton, 2010). The biosynthetic core of erythromycin A, 6-deoxyerythronolide B (6-dEB)

(Figure 1.5), provided the basis for understanding the function and modularity of PKSs that are responsible for the assembly of complex polyketides over the last 15 years (Weissman and Leadlay, 2005). Three successive open reading frames (ORFs), *eryAI*, *eryAII* and *eryAIII* were identified as genes responsible for biosynthesis of the aglycone core (the intermediate 6-dEB) of this macrolide antibiotic. These genes encode three giant proteins that dock together forming a gigantic multienzyme polypeptide, designated 6-deoxyerythronolide B synthase (DEBS) 1, (DEBS) 2, and (DEBS) 3, in the putative enzymatic order that they function in the synthetic operations (Figure 1.5) (Staunton and Weissman, 2001; Hill and Staunton, 2010). It is obvious from the diagram that each of the DEBS proteins consists of two functional units or modules, therefore named as 'modular' PKS (Staunton and Weissman, 2001). The core component of each module includes KS, acyltransferase (AT), and ACP domains. These domains together extend the growing polyketide chain by two carbon atoms. In addition, it generates a β-keto group that can be modified through a variable set of reductive domains including ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains, which are typically attached to the core module (Donadio *et al*., 1991; Ridley *et al*., 2008). DEBS 1 at the front of the synthase has a didomain ($AT_L$-$ACP_L$), which was formerly known as the loading module (LM), whose function is to accept the starter unit propionate from the external pool of propionyl-CoA and to transfer it to the KS of module 1 (Hill and Staunton, 2010). Co-operation between the three essential domains KS, AT and ACP will catalyse one cycle of chain extension through the formation of carbon-carbon bond by Claisen condensation, which will end up in a β-ketoacyl thioster intermediate (Staunton and Weissman, 2001). The structure of domains in module 1 ensures that the keto group undergoes only a partial reduction to hydroxyl intermediate by the KR due to the absence of other reductive domains DH and ER. Transfer of this hydroxyl intermediate to the

downstream KS of module 2 within DEBS 1 which consists of an equivalent set of reactions would generate another hydroxyl acyl intermediate (Hill and Staunton, 2010).

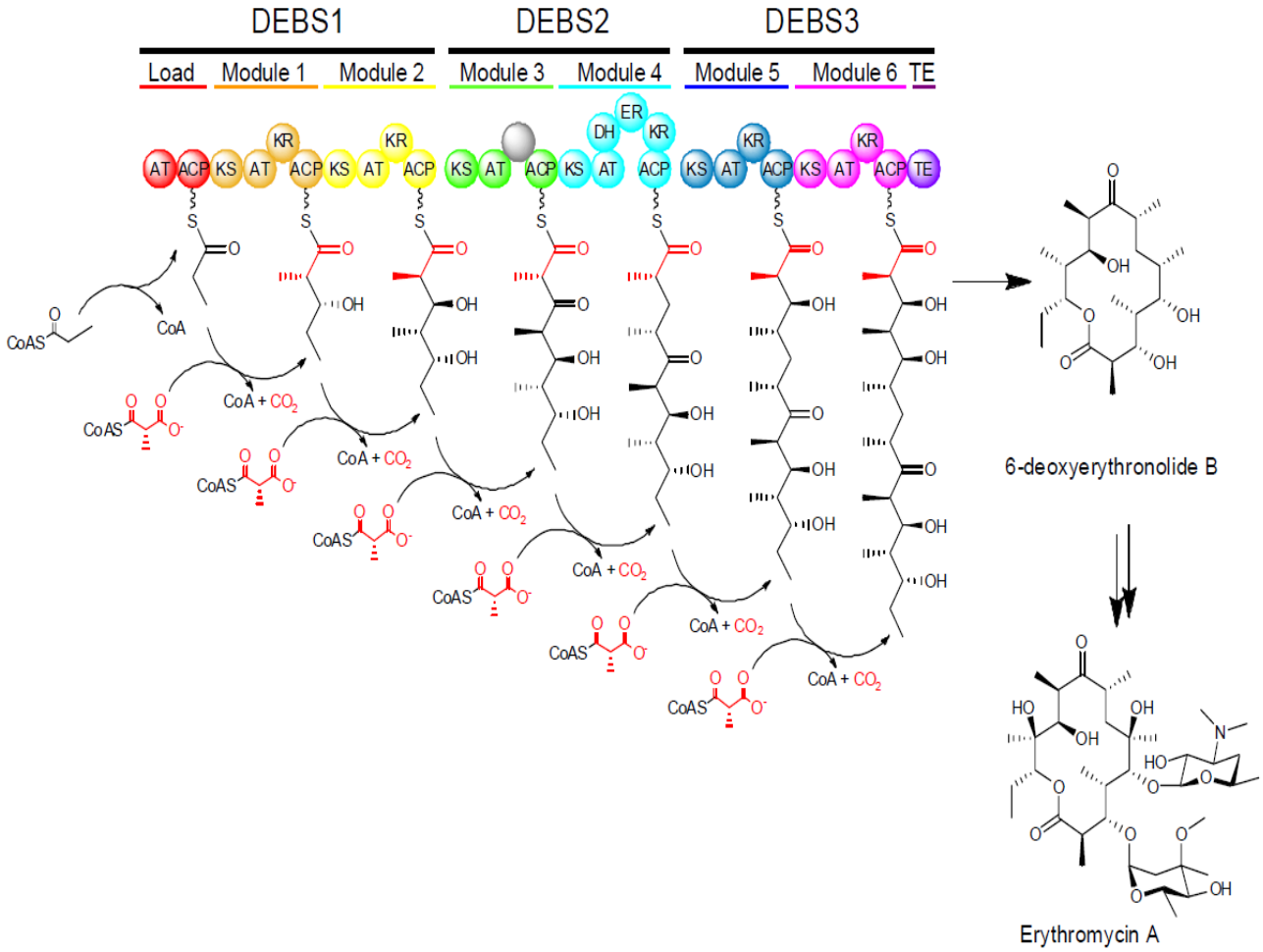



**Figure 1.5 Biosynthesis of erythromycin A.** 6-deoxyerythronolide B synthase (DEBS) is responsible for the biosynthesis of the aglycone, which subsequently converts to erythromycin A by post-translational modifications (source: Kwan and Schulz, 2011).

Subsequently, the growing chain is passed from DEBS 1 to the KS of module 3 in DEBS 2, and a new round of chain extension will take place. However, as no reductive domains are

present in this module, the keto group should survive intact. Following the transfer of the acyl intermediate to module 4, the β-keto group undergoes full reduction as in FAS, and it the passes through all the modifications catalysed by the reductive domains (KR, DH and ER), resulting in a methylene moiety (Hill and Staunton, 2010). Transfer of the growing chain to the KS of module 5 in DEBS 3 is delivered by interprotein transfer. In the two further rounds of condensation by modules 5 and 6, the keto groups are partially reduced to hydroxyl, and lead to the completed PK chain. TE is the last domain of module 6 within DEBS 3 instead of KS of a downstream module. The activity of TE is thought to be through catalysing the removal and cyclisation of the fully formed heptaketide (PKS) intermediate to give 6-dEB (the aglycone). Then, non-PKS enzymes, encoded by genes on either sides of the resistance gene, *ermE*, act at the late stage of the PKS pathway through the conversion of 6-dEB to the active antibiotic erythromycin A (Staunton and Weissman, 2001).

### 1.3.1.2 Structural model for type I PKS

Determining the structural organisation of modular PKSs is valuable for engineering applications. Descriptive structural models should consider two very important points. The first is the ability of the ACP domain in each module to interact with other domains. The second is the interaction between successive modules in a successful fashion to ensure chain transfer to the next module (Weissman and Leadlay, 2005). A structural model for the erythromycin DEBS (subunits) proteins has been proposed, which proposes that the identical PKS subunits are wrapped around each other to form a double helical homodimer associated head-to-head and tail-to tail (Figure 1.6) (Staunton *et al*., 1996). In this model, each protein subunit is in the form of a homodimer, and the individual domains, especially the KS-AT didomain and the thioesterase (TE) activities remain dimeric even when they have been

released as separate proteins from the PKS. On the other hand, the reductive domains represented by KR, DH and ER are monomeric. During chain extension, a preferential interaction between a particular KS within the ACP domain on the opposite subunit was observed (Weissman and Leadlay, 2005). This sort of interaction is facilitated by the angle of the coil which brings the ACP of one chain over the KS of the opposite subunit. However, no such interaction was detectable between the reductive domains of the complementary chains (Staunton and Weissman, 2001). This 'double helical' model places the KS (and possibly the AT as well) at the core of the helix, while the reductive domains tend to protrude out of the loop at the periphery of the structure. Moreover, it appears that all the available domains within each module are able to interact with the ACP domain, and the closest such interaction that is facilitated by the subunit interface occurs between the KS and ACP domains of the same module (Weissman and Leadlay, 2005). According to the crystallographic structure, the thioesterases (TEs) have been identified as homodimers and belong to the family of the α/β hydrolase (Staunton *et al*., 1996).

NMR was used by Weissman and co-workers to elucidate the structure of the docking domains in the DEBS system that brings all the multienzymes together to act in collaboration (Broadhurst *et al*., 2003). Since DEBS consist of three multienzymes and the nascent PK chain must transfer to the downstream multienzyme, there must be some mechanism that brings them together. Therefore, it was suggested that there must be a docking motif to ensure that the multienzymes in charge come together at least for long time enough to permit the transfer of the growing PK chain from the terminal ACP of one multienzyme to the first KS domain of the next multienzyme. In addition, the docking process requires it to be carried out with a highly effective level of discrimination so as to ensure that correct pairs of multienzymes become associated. Furthermore, the C-terminal residues of the ACP partner

and the N-terminal residue of the KS partner are considered to be the most likely candidates for the position where the docking domains are situated (Hill and Staunton, 2010).

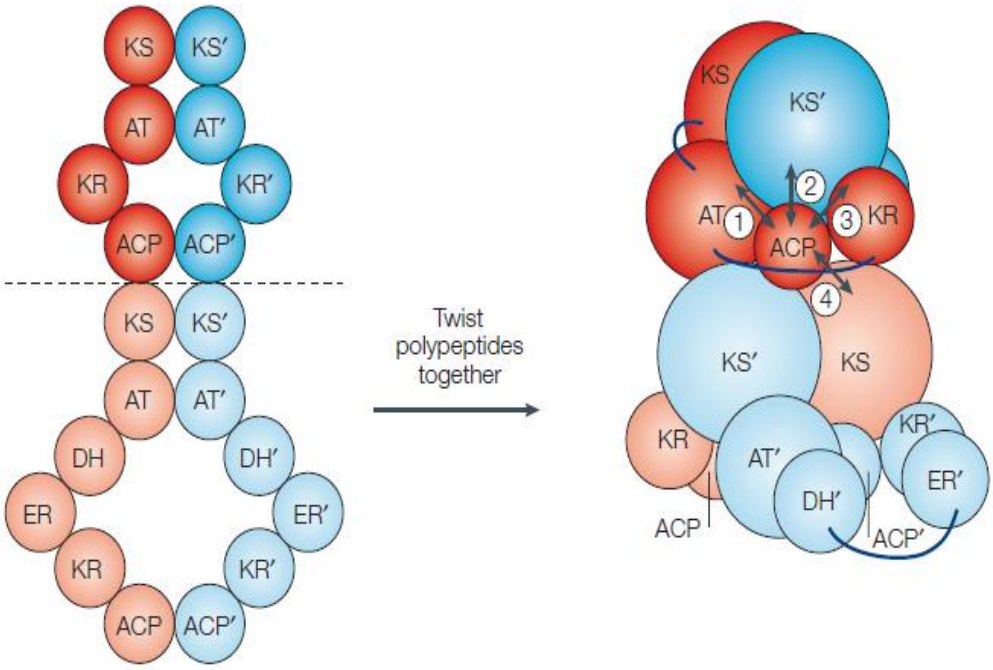



**Figure 1.6 Double helical model represents the arrangement of domains of modular polyketide synthases** (adapted from Weissman and Leadlay, 2005).

Experimentally, artificial covalent links were used to join DEBS 1 with DEBS 2 and DEBS 2 with DEBS 3. These new systems that are artificially joined retained the ability to take part in the biosynthesis of the 6-dEB as a normal metabolite of erythromycin (Squire *et al*., 2003; McDaniel *et al*., 1997). Exploiting genetic engineering, fragments containing the C-terminal part of the ACP from the last module of the upstream multienzyme, were joined covalently by artificial linkage with fragments from the N-terminal end of the KS at the start of the downstream multienzyme to prepare a short protein. It was anticipated that the linkage

must give a higher chance to these isolated fragments to fold properly so as to give domains which in turn may associate together in such a way that allows them to be responsible for docking (Broadhurst *et al*., 2003). The success of this strategy was confirmed by multidimentional NMR which showed that the docking domain takes the dimerization form of a rigid rod-like coiled-coil, which is positioned at the N-terminal end of DEBS3 and DEBS2 as well (Figure 1.7). Helping the subunits to associate together, and providing them with assistance to become a dimer and to be able to maintain the dimeric state in the first place, are two critical and valuable roles played by the docking domains (Weissman, 2004). Their role in dimerization has been proved by the successful use of elements from the structutre of the docking domains as a dimerziation motif in another system. The portion of the prepared protein derived from the C-terminus of the upstream ACP formed three helical regions, 1, 2, and 3. A striking structural motif that consists of an intertwined four α-helix bundle was formed by the first two helices (1 and 2), which may form the dimerzation motif for the tail end of the upstream multienzyme. A mobile linker region is stretched from the bundle leading to helix 3, which in turn docks with the rod-like docking domain of the downstream KS. Therefore, this manner of association between helices of adjacent multenzymes would be regarded as the docking motif.

The coiled-coil that is formed at the N-terminal docking domain of DEBS 3, is a well-known motif that is utilised by proteins to associate together and stabilise each other (Branden and Tooze, 1999). The particular hydrophobic residues which hold helices 1, 2, 3, and 4 together are shared between several different synthases, which suggests that they may use their docking domains for the stabilization of the dimer (Weissman, 2004). Further experiments conducted to investigate different aspects of the recognition processes between helices 3 and 4, ensure that the correct pairs of multienzymes associate together (Weissman, 2006a and 2006b).

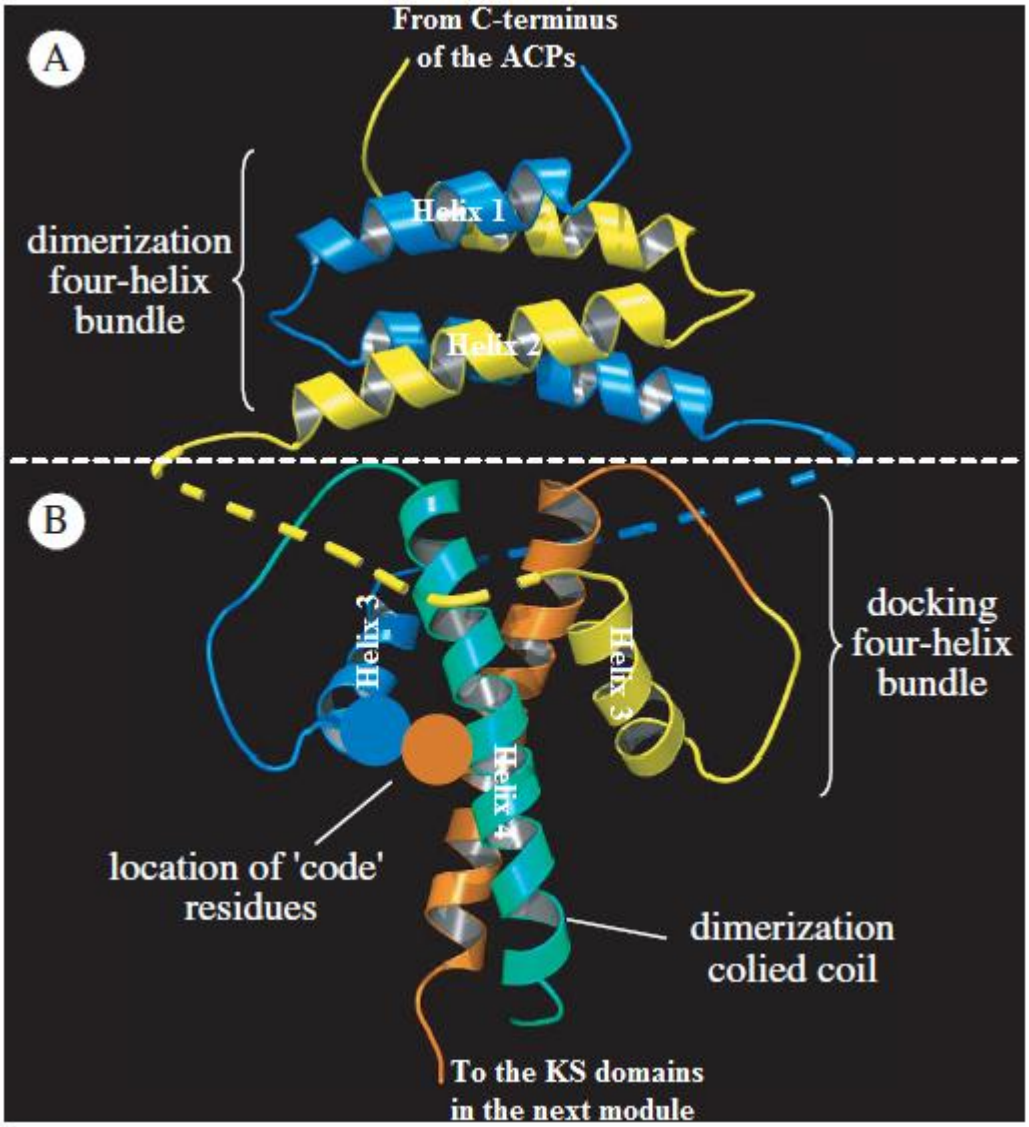



**Figure 1.7 Structure of 'Dock 2-3' by NMR.** A model of docking between DEBS 2 and DEBS 3. The structure comprises two separate, non-interacting domains, DEBS 2-ACP (A) and DEBS 3-KS (B). The dimerization motif consists of helices 1 and 2, while the docking domains include helix 3 of ACP and helix 4 of KS. Coloured spheres represent the two residues that possibly define a 'code for docking' (Sources: Weissman, 2004; Hill and Staunton, 2010).

### 1.3.2 The iterative type II PKSs

Aromatic PKSs are iterative type II systems consisting of multienzymes in which the active sites are located on discreet polypeptide subunits. The catalytic centres are anticipated to work several times in a repetitive manner during the iterative cycles of the polyketide chain extension and assembly (Kuščer *et al*., 2005). The polyfunctional aromatic antibiotics such as actinorhodin, tetracycline, doxorubicin, and frenolicin, are among the most widely studied type II iterative polyketides (Ridley *et al*., 2008). Phenolic aromatic compound are the characteristic products of this type of PKS. Biosynthetic reactions include the formation of polyketone chains during cycles of condensation of the building blocks, which then undergo cyclisation and aromatisation processes to produce aromatic compounds (Staunton and Weissman, 2001). The heterodimeric ketosynthase (KS), chain length factor (CLF), and acyl carrier protein (ACP) in addition to a malonyl-CoA:ACP transacylase (MAT), which are believed to be recruited from bacterial fatty acid synthases, all together are considered as the core set of enzymes of these types of polyketide synthases (Figure 1.8A). Cooperatively, the minimal PKS proteins essential for biosynthesis of a polyketide are the four previously mentioned proteins (Hutchinson, 1999; Ridley *et al*., 2008). Chain extension (and often initiation) using malonate as a chain extender unit is cataylzed by KS-CLF to form a poly-β-ketide intermediate by decarboxylative condensation of malonyl building blocks that are delivered by ACP, while MAT provides malonyl groups to the PKS (Hutchinson, 1999; Reeves, 2003). A highly reactive polyketide chain of defined length and strength is produce by the action of these proteins collectively, that is under the control of a deep pocket in KS-CLF (Ridley *et al*., 2008).

Subsequently, the poly-β-ketone intermediates are changed to compounds containing an aromatic ring by the action of unique tailoring enzymes such as ketoreductases (KRs),

aromatases (AROS), and cyclases (CYCS). This is consistent with what has been shown by Hranueli *et al*., (2001) about the implication of ketosynthase (KS) α and β, acyl carreir protein (ACP), cyclase/aromatase and cyclase (Figure 1.8B) as a set of six proteins required for the biosynthesis of actinorhodin by *Streptomyces coelicolor*.

Although that it is still believed that the ACP in this type of PKS needs assistance for loading building blocks, it has been observed by Simpson and coworkers that the ACP is able to accept a malonate and any other acyl groups from the respective CoA thioesters without the need for external assistance (Matharu *et al*., 1998). Moreover, since no specific gene identifed in the actinorhodin (act) cluster encodes a malonyl-CoA: holo-acyl carrier protein transacylase (MCAT), there is no clear mechanism for selection of the starter or the extender unit within this cluster. Arthur *et al*., (2005) also proved the ability of ACP in this PKS system of self- malonylation by an *in vitro* study. However, more recently, hexanoate in benastatin (Xu *et al*., 2007), and malonamate in oxytetracyline (Zhang *et al*., 2006) have been identified as examples of building blocks other than acetate units derived from malonyl-CoA by decarboxylation.

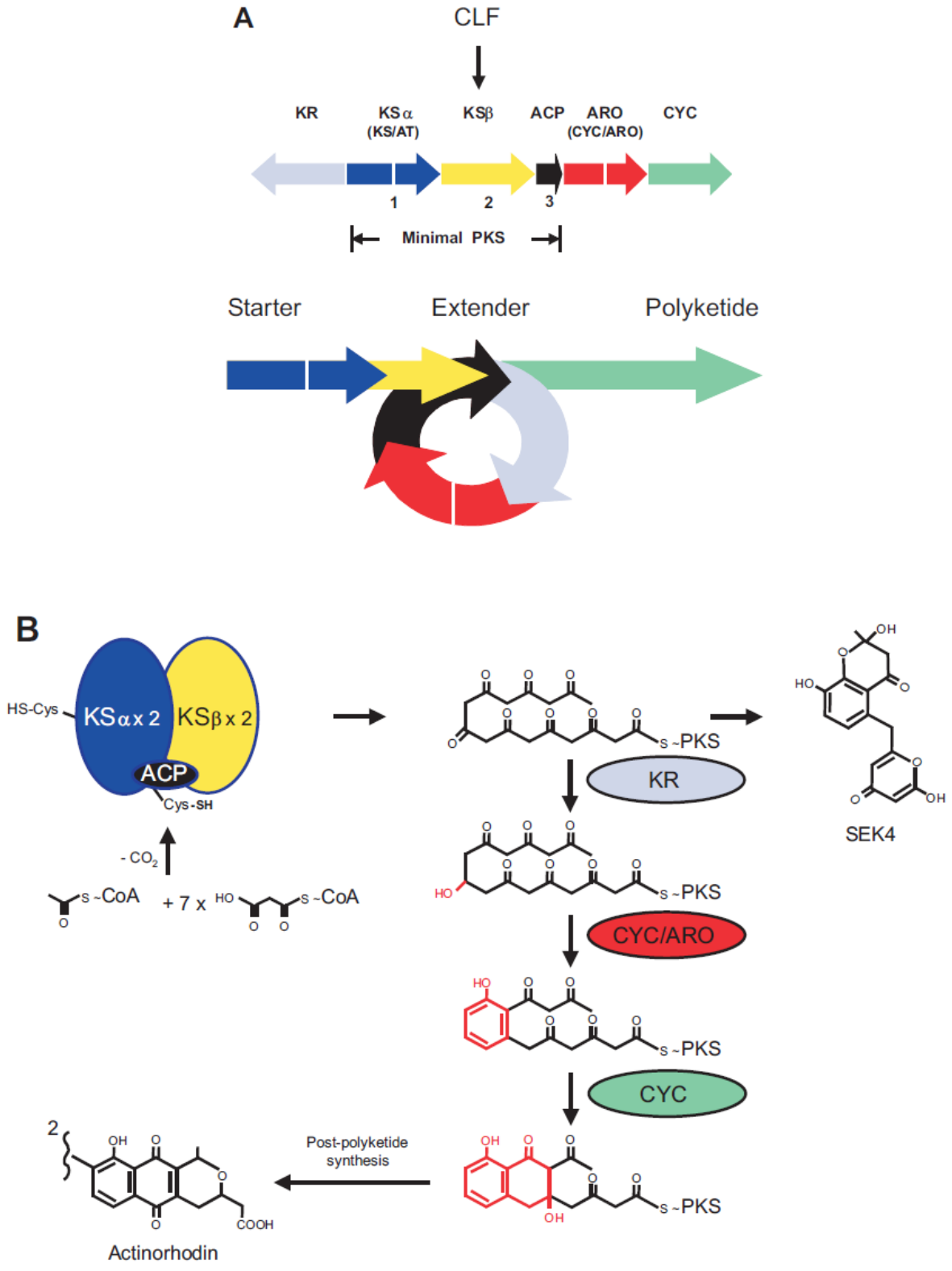



**Figure 1.8 General scheme of genes encoding for actinorhodin, as an example of type II polyketide synthase.** (A) The conserved minimal PKS for all type II PKS gene cluster consist of β-ketoacyl synthase (KS) α and β, and acyl carrier protein (ACP). (B) mono- or bifunctional proteins that are used iteratively for the assembly of the polyketide backbone (source: Hranueli *et al.*, 2001).

The mechanistic actions of PKS enzymes in type II are quite different from similar ones in type I, as they generate cyclic and aromatic compounds that do not require further extensive rounds of reduction alone, or with dehydration. Consequently, there are several principal challenges faced in studying type II PKS, which include trying to determine what controls the number of times such chain extensions occur, how the poly-β-ketone intermediates are folded into the correct orientation for cyclization, and how to select which carbonyl and methylene groups react to form the six-membered rings of the final cyclic compounds (Hutchinson and Fujii, 1995). However, the diversity of aromatic polyketides discovered so far could be due to the presence or abscence of accessory (tailoring) enzymes which add additional modifications to the product (Ridley *et al*., 2008). Since type II PKS's are structurally very similar, it is impossible to predict the nature of the polyketide synthesized from the structure and the architecture of the gene cluster, which is different from other type of PKS's in some of which there is a one-to-one correspondence between active sites and product structure (Hranueli *et al*., 2001).

### 1.3.3 Type III polyketide synthases

This type of polyketide synthases is responsible for the biosynthesis of chalcones and stilbenes in plants, as well as polyhydroxyphenols in bacteria. Enzymes of this system, which are self contained, are able to form homodimers. Flavonoids are plant-derived natural products considered to be derived from an important branch metabolite, 2', 4, 4', 6'-tetrahydroxychalcone which is the product of the chalcone synthase (CHS) (Reimold *et al*., 1983). Chalcone synthase (Figure 1.9A) was the first type III enzyme discovered in the biosynthetic pathway of flavonoids, followed by the discovery of stilbene synthase a short while later as a second enzyme leading to these type III PKSs being collectively termed the

'chalcone synthase/stilbene synthase superfamily' (Schröder, 1999; Austin and Noel, 2003). Chalcone synthases provide the starting materials for a variety of secondary metabolites with important roles including defence against pathogenic micro-organisms, protection against the damage by UV light, and further additional specificities (Schröder, 1999). The wide distribution of CHS-like proteins in bacteria and fungi has been revealed recently by rapid expansion in genome sequence data. For example, they have been found in *Saccharomyces cerevisiae*, *Azotobacter oryaze* and some other fungi, and even type III PKSs have been characterised in some of them (Yu *et al*., 2012). Nevertheless, the sequence identity is only 25% between bacterial and plant CHSs, indicating that bacterial type III PKSs may possibly be able to perform reactions different from those of classical CHSs (Figure 1.9B) (Li and Müller, 2009).

Comparatively, type III PKSs, includes a wider and more diverse range of starter molecules than those involved in type I and type II PKSs. These consist of different aromatic and aliphatic CoA thioesters, for example coumaryl-CoA and methyl-anthraniloyl-CoA, used by the classical plant CHSs. In addition, the most recently identified, medium and long-chain fatty acyl-CoA esters, which are used by specific bacterial type III enzymes, which are involved in the biosynthesis of phenolic lipids (Schröder, 1999; Funa *et al*., 1999). Although, chalcone synthases are the most extensively studied type III enzymes, both chalcone and stilbene are generated by a similar chain-elongation reaction, which includes a starter unit from 4-coumaroyl-CoA followed by three successive decarboxylative condensation reactions using malonyl-CoA as the extender. However, bacterial type III PKS is able to utilise other acyl-CoAs than malonyl-CoA as extender units (Li and Müller, 2009). Differential aldol-cyclization and aromatization of the produced linear polyketide intermediate (either C6→C1 or C2→C7) (Tsai, 2004) occurs within the same enzyme active site to yield 2', 4, 4', 6' –

tetrahydroxychalcone as a final product for the synthesis of chalcone or stilbene (Baerson and Rimando, 2007).

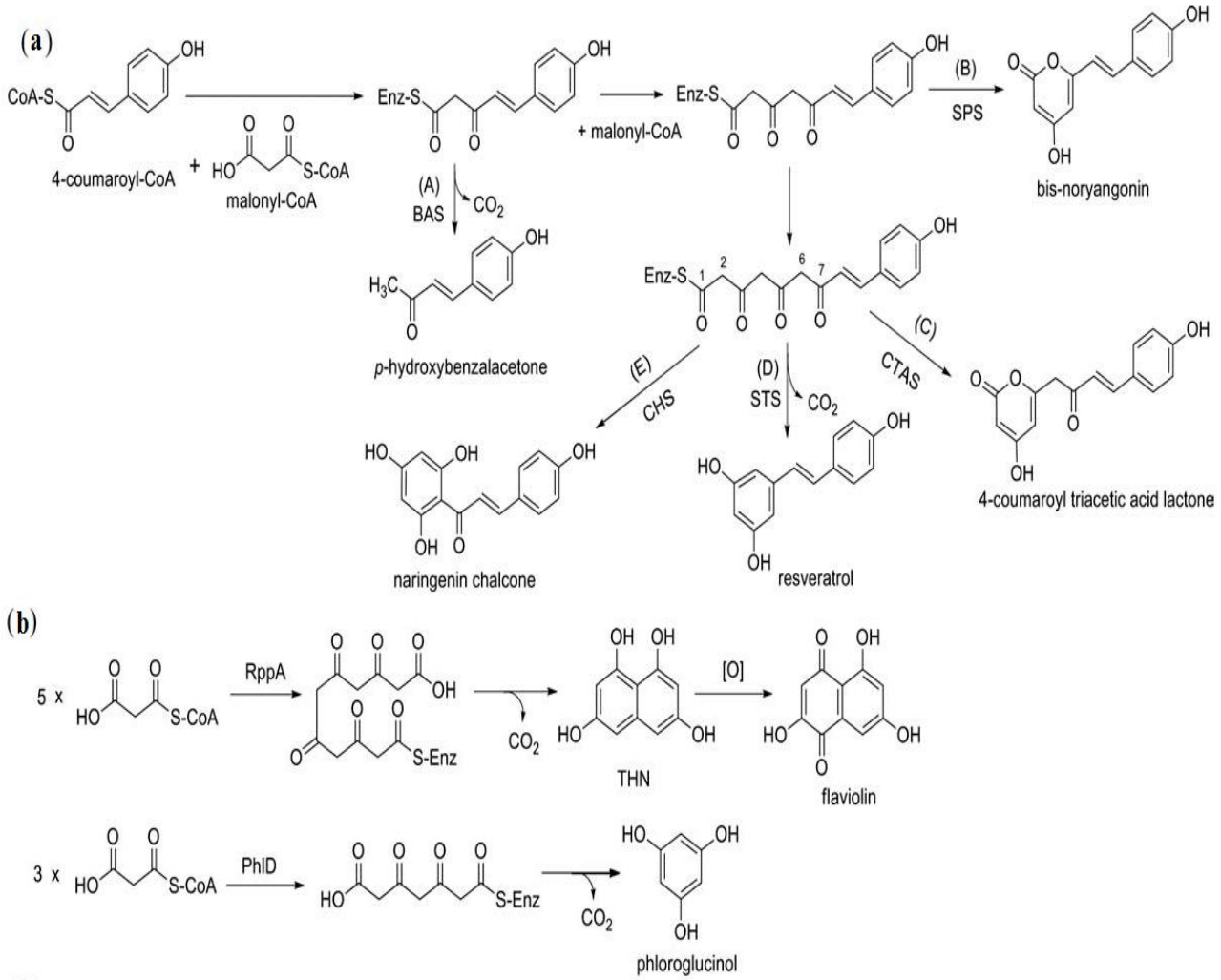



**Figure 1.9 Representative reactions catalyzed by type III PKSs.** (a) The use of 4-coumaroyl-CoA as the starter unit and malonyl-CoA as the extender unit by plant type III PKSs: BSA, benzalacetone synthase; SPS, styrylpyrone synthase; CTAS, triacetic acid synthase; STA, stilbene synthase; CHS, chalcone synthase. (b) RppA, 1, 3, 6, 8-tetrahydroxynaphthalene synthase, and PhID, phloroglucinol synthase, as examples of bacterial type III PKSs (source: Yu *et al*., 2012).

Architecturally, the plant and bacterial type III PKSs are considered the simplest among the three types of PKSs, as they consist of homodimeric enzymes that possess subunits between 40-45 KDa in size (Tsai, 2004; Baerson and Rimando, 2007). Similar to type II

PKSs, type III PKSs catalyse iterative decarboxylative condensation reactions, usually using malonyl-CoA as the extender units. Moreover, based on the homodimeric form, the single active site cysteine in each monomer catalyses a complex series of reactions iteratively, consists of priming, extension, and intramolecular cyclisation to form polyketide products (Austin and Noel, 2003; Tsai, 2004; Yu *et al*., 2012). The crystal structure of CHS2 from alfalfa determines four residues encompassing Cys164, Phe215, His303, and Asn336 which are conserved between all the CHS2 related enzymes, and these are suggested as the possible elements of a single active site to catalyze all the decarboxylative condensation reactions collectively (Ferrer *et al*., 1999). Non-involvement of the acyl carrier proteins and the use of free CoA thioesters directly as substrates are considered as the unique character of this family of PKS (Baerson and Rimando, 2007). However, bacterial alkylpyrone synthases, such as ArsB and ArsC synthase from *Azotobacter vinelandii*, which are responsible for the biosynthesis of phenolic lipids and use a long chain acyl-CoA as starter units rather than malonyl-CoAs, transfer the C22-C26 acyl chain starter units directly from the ACP domains of the type I FAS to the type III synthase (ArsB and ArsC) (Miyanaga *et al*., 2008).

## 1.4 Post –PKS modifications

The already synthesized and cyclised polyketides can undergo some modifications through hydroxylation, glycosylation, methylation and/or acylation. It was believed that these tailoring enzymes, or post PKs modifications, were crucial for the addition of special necessary and functional groups to the polyketides skeleton that are important for their structural diversity and biological activity (Rix *et al*., 2002). Using gene knockouts, post-PKs enzyme function can be typically investigated, and then checking the characteristics of the resulting metabolites, allows a conclusion about the sequence of modification events to be

established (Rix *et al*., 2002; Hong *et al*., 2004). Changes catalyzed by oxidoreductases, which includes oxygenases, peroxidases, oxidases, reductases (i.e ketoreductases), and dehydrogenases, are among the most frequently found post-PKS modifications. Introducing oxygen-containing functionalities, exemplified by hydroxyl groups (hydroxylases), epoxides (epoxidases), aldehyde or keto groups, or modifying such functionalities by either addition or removal of hydrogen atoms, such as changing an aldehyed into a carboxylic acid, are general reactions carried out by oxidoreductases (Kim and Park, 2008).

Transferases are a group of enzymes that act by introducing novel functional groups, which subsequently alter the product profiles relative to the substrate. Several important enzymes are included in this group including alkyl (usually methyl) transferases, aminotransferases, acyl (usually acetyl) transferases, gylcosyltransferases (GTs) and kinases. Both methyl- and glycosyltransferses are considered as the most significant post-PKS tailoring enzymes (Kim and Park, 2008). GTs functions in the attachment of sugar moieties, most often deoxysugars, which confer important and valuable characteristics to a molecule, since they play an essential and profound role in the biological activity of many PKs and other natural product-based drugs (Mendez and Salas, 2001). Methyl-transferases can methylate O, N or C-atoms, using *S*-adenosylmethionine (SAM) as co-factor. Methylation reactions take place either at the polyketide-derived aglycone moiety or on the sugar residues, either before or following the glycosyl transfer (Rix *et al*., 2002). The following examples illustrate the power of post-PKS engineering.

In the erythromycin producer *Saccharopolyspora erythraea*, all the required information about the order of the occurrence of the post-polyketide modifications and methodologies for gene transfer is available. The deduction that the lactone ring carrying the 6,7 unsaturation is a substrate for mycarosyl- and desosaminyltransferases, and C-12 hydroxylase as a post-polyketide modification enzymes, is the formation of $\Delta^{6,7}$-anhydroerythromycin C (Donadio

*et al*., 1993). In a genetically modified *Saccharopolyspora erythraea*, the three previously mentioned reactions have thus far been observed within numerous different cyclic structures, even when the alterations were quite close to the site of modification (Donadio *et al*., 1991; McAlpine *et al*., 1987; Weber *et al*., 1991).

Studies on the nystatin (antifungal polyene macrolide) gene cluster identified two genes (*NysDiii* and *NysDii*) that putatively participate in the biosynthesis of mycosamine, and (*NysDi)* responsible for the mycosamine attachment to the nystatin aglycone. The suggested functions of these genes are as mannose dehydratase, aminotransferase and a glycosyltransferase, respectively. Furthermore, the deoxysugar mycosamine is suspected to have a significant function for nystatin bioactivity (Nedal, 2007).

A specific gene disruption of the complex macrocyclic polyketide TA antibiotic produced by *Myxococcus xanthus* identified several genes necessary for post-modification processes during its biosynthesis, and in the subsequent production of biologically active TA antibiotic. These chemical modifications involve the addition of several carbon atoms originating from acetate at C-13, C-17 and C-32, three C-methylations at C-2, C-4 and C-8, O-methylation at C-34 and a specific hydroxylation at C-20 (Paitan *et al*., 1999b). A cytochrome P-450 hydroxylase is responsible for the unique hydroxylation at C-20 within TA biosynthetic gene cluster, which is essential for the production of an active TA molecule (Paitan *et al*., 1999a).

## 1.5 Non-ribosomal peptide synthetase (NRPS)

This vast family of compounds mainly synthesized by micro-organisms constitute the peptide based natural products. They exhibit a wide range of biological activities with different functions ranging from defending against other micro-organisms like vancomycin,

to iron sequestration (yersiniabactin), or cell wall components (gylcopeptidolipids). Furthermore, some of these peptides like the antibiotic vancomycin, or the antitumour agent bleomycin, have proved to have an important role in medicine. Although some peptides, for example lantibiotics are synthesized by the ribosomal machinery, the vast majority are confirmed to be biosynthesized by large multifunctional modular enzymes which are known as nonribosomal peptide synthetases (NRPSs) (Lautru and Challis, 2004). Molecules produced by NRPS are often cyclic, and the amino acids are connected by peptide bonds (Challis and Naismith, 2004). The basic difference between PKS and NRPS systems is in the selection of the monomers, in which PKSs utilise activated fatty acids monomers (Acyl CoA thioesters), while NRPSs consume ATP to activate amino acids in the form of AMP esters (Keating, and Walsh, 1999).

NRPSs exhibit a modular architecture as large multifunctional enzymes, similar to that in the PKSs. Therefore, they consist of repeats of modules as the basic peptide synthetase units that are responsible for the activation, covalent attachment as a thioester, and optional modification of a single amino acid (Lautru and Challis, 2004). Thus one amino acid is incorporated within every single step of peptide synthesis (Kopp and Marahiel, 2007). Moreover, the number and the order of the modules within the NRPS depend upon the number of the amino acids and the length or the sequence of the peptide being synthesized, respectively. Every module consists of structurally different and independent domains, in which each fulfills a specific function, and is normally specific to a particular amino acid substrate (Challis and Naismith, 2004). A minimal chain elongation module contains three core domains: the 50 kDa adenylation (A) domain, the peptidyl carrier protein (PCP) domain (or thiolation [T] domain) and the 50 kDa condensation (C) domain (Lautru and Challis, 2004). However, in some cases the first module has a three-domain organization, which occurs in the assembly lines that form *N*-acylated peptides, for example plipastatin (Steller *et*

*al*., 1999), in which the C-A-T (PCP) is the typical order of the domains. The adenylation domain firstly is responsible for selecting the amino acid monomer and activating it via the formation of an aminoacyl adenylate, and then through covalently binding of the activated amino acid via a thioester to the 4´-phoshopantetheinyl (4´Ppant) cofactor of the next peptidyl carrier domain (Figure 1.10). Phosphopantetheinyl transferase is responsible for the post-translational attachement of the mentioned cofactor to the conserved serine of PCP prior to binding of the activated amino acid. The C domain is the last essential domain, which is responsible for the formation of the peptide bond between the aminoacyl thioester attached to the PCP domain of the module in charge and that of the previous module (Lautru and Challis, 2004; Fischbach and Walsh, 2006).

Extra optional or alternative domains can be seen within some modules which introduce changes or modifications to the amino acid that has been incorporated. For example, an epimerisation (E) domain which catalyzes racemisation (L and D equilibration) of the Cα of the amino acid, a reaction which occurs either before or after the formation of the peptide bond. This domain is normally located after the PCP domain of the C terminus of some modules. Since in several NRPSs the cyclisation (Cy) domain substitutes for the condensation (C) domain (Konz and Marahiel, 1999; Lautru and Challis, 2004), the Cy domain catalyses both condensation and intramolecular heterocyclisation of serine, cysteine or threonine residues to make the corresponding thiazoline or oxazaline ring. The *N*-methylation (transfer of $CH_3$ group from *S*-adenosylmethionine (SAM) to the nitrogen of the amine) is an extra reaction catalyzed by methyltransferase (MT) domain which is found in NRPSs with C-A-MT-T modules (Challis and Naismith, 2004).

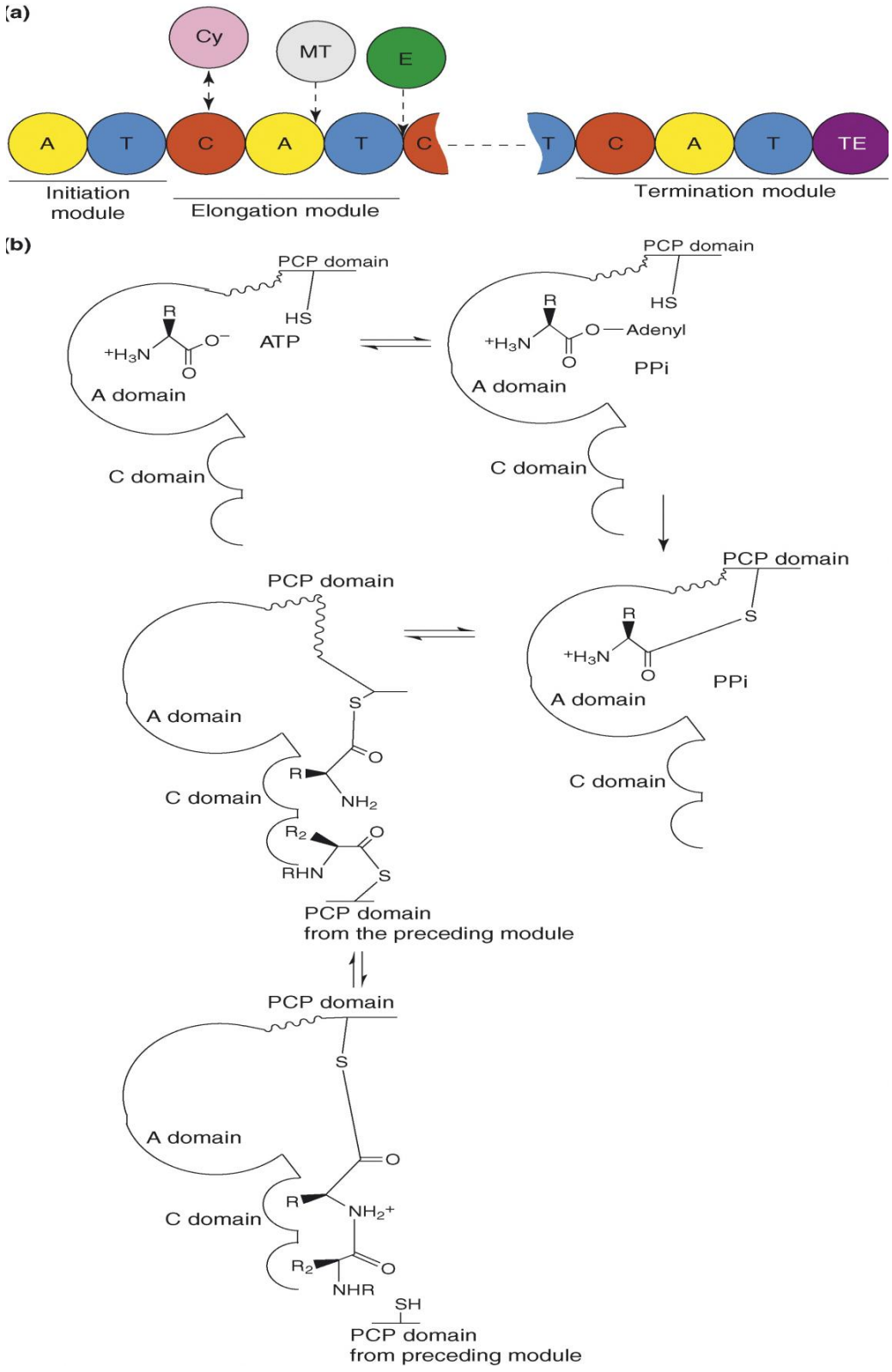



**Figure 1.10 Typical representation of non-ribosomal peptide biosynthesis (NRPSs)**. **(a)** Modules and domains included in NRPS. **(b)** Biosynthetic processes represented by the scheme. (Source: Challis and Naismith, 2004).

The final step is release of the assembled peptide from the NRPS into the solution from the termination module of the NRPS, which is mostly accomplished by a thioesterase domain (TE) which belongs to the α,β-hydrolase fold family and is found at the C-terminus of the last module which can be hydrolytic or cyclising (Keating *et al*., 2001; Kohli and Walsh, 2003). However, in myxochelin biosynthesis, a reductase catalysed thioester reduction yields an aldehyde as an alternative TE-independent manner of chain release (Lautru and Challis, 2004; Gaitatzis *et al*., 2001).

A study on the congocidine biosynthetic gene cluster from *Streptomyces ambofaciens* revealed an unusual organisation of NRPS genes which do not encode large multimodular enzymes. Only four NRPS genes code for discrete modules or domains. A typical NRPS module (A-PCP-C) is encoded by *cgc18*, while two genes (*cgc2* and *cgc16*) encode two stand-alone C domains, and only *cgc19* codes for a stand-alone PCP domain (Juguet *et al*., 2009). Similarly, one typical NRPS module (Cy-A-T), a free-standing C domain, and two thioesterases in addition to other genes responsible for holomycin production, were identified in *Steptomyces clavuligerus* (Huang *et al*., 2011). Furthermore, the same single NRPS module is encoded by *holA* in the pyrothine (holomycin) part of thiomarinol, the hybrid (PKS-NRPS) antibiotic produced by *Pseudoalteromonas* spp SANK 73390, whose logical interpretation suggests that this NRPS (HolA) may act as an iterative NRPS for the formation of the cysteinyl-cysteine dipeptide (Fukuda *et al*., 2011).

## 1.6 Mupirocin

### 1.6.1 Structure and indication (uses)

Mupirocin is a polyketide antibiotic produced as a mixture of pseudomonic acids by a soil bacteria *Pseudomonas fluorescens* NCIMB 10586. Pseudomonic acids (Figure 1.11) consists of a $C_{17}$ unit, monic acid, esterified to a $C_9$ saturated fatty acid, 9-hydroxynonanoic acid (9-HN) via an *α,β*-unsaturated ester linkage, and pseudomonic acid A is the major component, which accounts for about 90% of the mixture (Fuller *et al*., 1971; Chain and Mellows, 1974; Chain and Mellows, 1977). Pseudomonic acid A is responsible for most of the antibacterial activity (Fuller *et al*., 1971). Attack of the 7-hydroxyl group on the carbons 10,11 (the epoxide) outside the pH range of 4-9 irreversibly change and results in the production of two cyclic ethers (Clayton *et al*., 1979), represented by compounds 1 and 2 in Figure 1.11. Pseudomonic acid B constitutes about 8% of the total mupirocin, and has an additional hydroxyl group at the position $C_8$ (Chain and Mellows, 1977). The remaining 2% consist of two minor metabolites, pseudomonic acids C and D, respectively. Pseudomonic acid C has a low polarity and a double bond in the place of the epoxide group between $C_{10}$ and $C_{11}$, also it is more stable than pseudomonic acid A under mild acidic conditions (Clayton *et al*., 1980)., while pseudomonic acid D has unsaturated fatty acid moiety with an alkene group at C4′-C5′ (O'Hanlon *et al*., 1983).

Mupirocin (marketed as Bactroban) has been clinically used in the UK since 1985 (Cookson, 1990). The main use of mupirocin is to control infections by *Staphylococcus aureus*, especially methicillin resistant *S. aureus* (MRSA), and some other Gram positive bacteria, such as *Streptococcus pyogenes* and *Strep. pneumonia*, as well as some Gram negative bacteria (*Haemophilus influenzae, Neisseria gonorrhoeae*), while other antibiotics showed ineffectivness (Shiozawa *et al*., 1995; Szell *et al*., 2002). Because of its low toxicity to human and animals, it is used for the treatment of various kinds of skin, ear and nose

infections (Szell *et al*., 2002). Mupirocin is unstable in vivo, because of the hydrolytic inactivation of the ester-bond between MA and 9-HN, and therefore, many attempts have been made to improve and develop derivatives with more stability, that can be useful systemically (Sutherland *et al*., 1985; Kim *et al*., 2003).

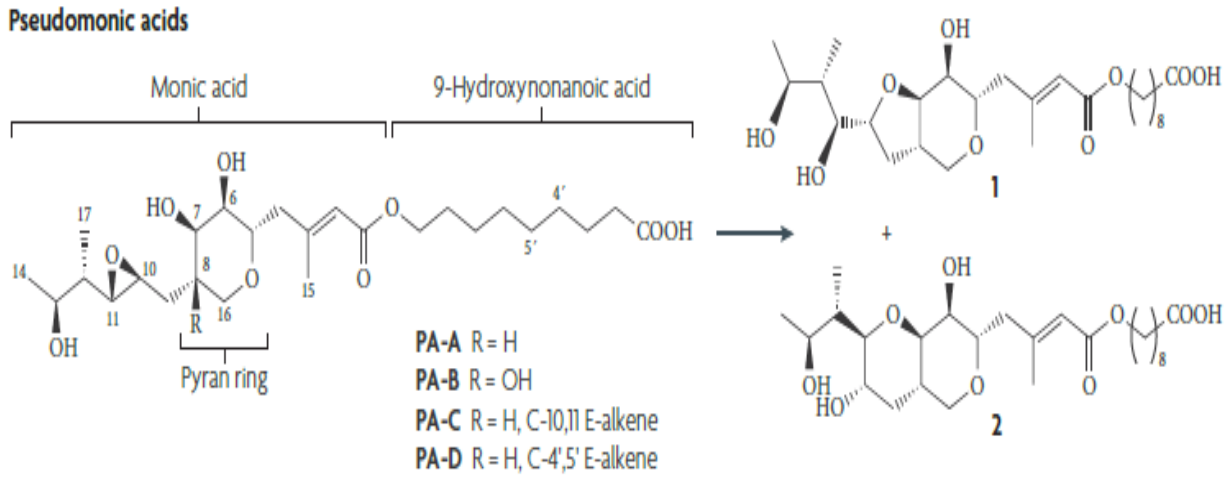



**Figure 1.11 Chemical structure of mupirocin (pseudomonic acids (PAs) represented by PA-A, B, C and D).** Compounds 1 and 2 are derivatives of PA-A produced under acidic or basic conditions (adapted from Thomas, *et al*., 2010).

## 1.6.2 Mechanism of mupirocin inhibition

Mupirocin, and more specifically pseudomonic acid A binds reversibly to the catalytic centre of bacterial isoleucyl-tRNA synthetase (IleRS) (Figure 1.12), and this inhibition results in the depletion of the charged tRNA which eventually blocks both protein synthesis and the growth in the pathogenic bacteria (Hughes and Mellows, 1978a, b, 1980). Structural elucidation of mupirocin by Chain and Mellows, (1977), revealed that the tail portion containing the 14-methyl terminus closely resembles the isoleucyl moiety of the isoleucyl-adenylate reaction intermediate (Ile-AMS) (Nakama *et al*., 2001; Silvian *et al*., 1999).

Mupirocin acts by inhibiting IleRS as a bifunctional entity, reacting with and inhibiting both the isoleucine and the ATP binding (Yanagisawa *et al*., 1994**)**. X-ray crystal structures of IleRS bound with either mupirocin or Ile-AMS separately confirmed the previous observation (Nakama *et al*., 2001; Hurdle *et al*., 2004). Mupirocin and the intermediate Ile-AMS binds to the same regions of the isoleucyl-tRNA synthetase. Because of the same carbon skeleton of the epoxide-containing side chain of PA-A and the hydrophobic side chain of the L-isoleucine, mupirocin binds to the same hydrophobic pocket of the IleRS that the L-isoleucine binds to normally (Hughes and Mellows, 1978b; Hurdle *et al*., 2004). Mupirocin interacts with this isoleucine-specific binding pocket, which consist of Pro-46, Trp-518, and Trp-558 via Van der Waals interactions. The pyran ring and the region around C1-C3 fit into the ATP-binding pocket of IleRS, which is similar to what Ile-AMS binds (Nakama *et al*., 2001; Hurdle *et al*., 2004). Since the models described above show that mupirocin (PA) binds in a similar fashion as Ile-AMS binding to the IleRS, it is predicted that mupirocin binds to the same architecture that is essential for the binding of both the L-isoleucine and the ATP (Brown *et al*., 2000; Hurdle *et al*., 2004) mimicking the effect of ribose and adenine.

Although all the inhibitory effects occur through the MA part of pseudomonic acid revealed by the X-ray models, the effect of the 9-HN has also been discussed. Brown *et al*., (2000) showed that the 9-HN fits into the hydrophobic pocket of the IleRS where valine 588 is, and an interaction between the carboxylic end of mupirocin (9-HN) and the amide of valine through a hydrogen bond is important for stabilising mupirocin within the structure of IleRS.

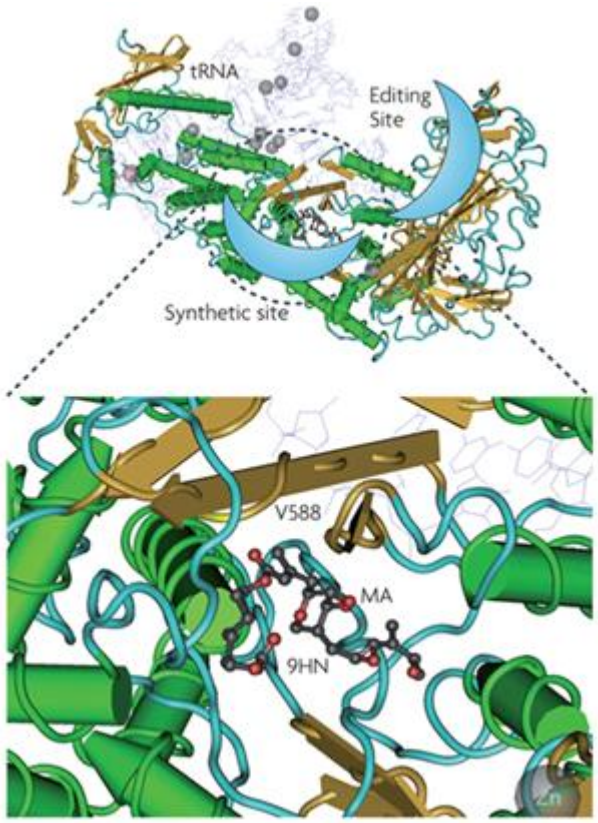



**Figure 1.12 Mechanistic model of mupirocin binding to its target enzyme, isoleucyl-tRNA synthetase, from *Staphylococcus aureus*.** Two separate active sites are present within the Isoleucyl-tRNA synthetase (IleRS); synthetic site, responsible for the synthesis of $tRNA^{Ile}$ through an Ile–AMS intermediate, and the editing site for editing of the $tRNA^{Ile}$. Synthetic site, is the binding site of mupirocin, which results in mimicking the influence of ribose and adenine. Structure of mupirocin is shown with carbons in grey and oxygens in red (source: Thomas *et al*., 2010).

## 1.6.3 Mechanisms of resistance to mupirocin

Although mupirocin shows excellent selectivity for the bacterial isoleucyl-tRNA synthetase, resistance generally emerges shortly after it enters clinical use. *Pseudomonas fluorescens* NCIMB 10586, the mupirocin producer is resistant to the antibiotic due to the

presence of an IleRS enzyme with lower affinity for mupirocin rather than that of *Escherichia coli* B, and an outer cell membrane preventing re-entry of the antibiotic to the cell (Hughes *et al.*, 1980). Analysis revealed that the mupirocin producer has two distinct IleRS (IleRS-R1 and IleRS-R2) encoded by two separate *ileS* genes, whose products share only 29.6% identical residues, indicating that they are distinct and differ in their recent origin. *Pseudomonas fluorescens* is able to grow and survive in a medium containing in excess of 1000 µg/ml of mupirocin, and *E. coli* showed the same result when expressing both of the *ileS* genes. In addition, *ileS* encoding IleRS-R2 is the more important gene for the survival of the producer (Yanagisawa and Kawakami, 2003). Within the biosynthetic gene cluster, *mupM* encodes MupM (IleRS-R2), which is a eukaryotic like IleRS and confer resistance to mupirocin (El-Sayed *et al*., 2003), while the other is located elsewhere in the chromosome.

The majority of Gram negative bacteria are relatively resistant to mupirocin, which is possibly due to poor permeability or impermeability of the outer membrane, or through an inability to reach the specific intracellular target, that binds isoleucyl-tRNA (Capobinco *et al*., 1989). Depending on the level of resistance, the mupirocin-resistant strains are classified into groups; those which exert low-level resistance (resistance to 8-256 µg/ml of mupirocin), which is related to mutation in the host enzyme (IleRS), while the second group shows high-level of mupirocin resistance (resistance to > 512 µg/ml of mupirocin) resulting from an increase in the copy number, which is due to introduction of exogenous enzymes (Kim *et al*., 2003; Antonio *et al*., 2002; Yanagisawa and Kawakami, 2003; Hudgson *et al*., 1994). The appearance of *Staphylococcus aureus* with low level mupirocin resistance due to mutation in a single amino acid (V588F, V631F and G593V) residue within the sequence of IleRS (Hurdle *et al*., 2004; Antonio *et al*., 2002; Farmer *et al*., 1992) showed that the residues which alter the enzyme and confer resistance to mupirocin are located near the KMSKS conserved motif (Antonio *et al*., 2002). High level resistance is due to the presence of IleRS

which has similarities to eukaryotic IleRS proteins, and may have been acquired through horizontal gene transfer (Yanagisawa and Kawakami, 2003; Hudgson *et al*., 1994; Rangaswamy *et al*., 2002). For high level resistance, bacteria like *Staphylococcus aureus* aquire the *mupA* gene which is often plasmid borne and encodes a eukaryotic like IleRS, which confers high level of resistance (Cookson, 1998; Hudgson *et al*., 1994).

### 1.6.4 The mupirocin biosynthetic (*mup*) gene cluster and pathway

Transposon mutagenesis of *Pseudomonas fluorescens* NCIMB 10586 and subsequent chromosomal mapping of the sites of the mutations, identified a > 60 kb region involved in the biosynthesis of mupirocin (Whatling *et al*., 1995). The entire region of chromosomal DNA was analysed by sequencing in both directions and showed that the *mup* cluster occupied over 74 kb, and consists of thirty five open reading frames (ORFs), including six large ORFs (*mmpA-F*) containing domains similar to multifunctional proteins of type I polyketide synthase and fatty acid synthase systems, and 29 genes (*mupA-X* and *macpA-E*), in which (*mupB*, *mupD*, *mupG*, and *mupS*) are similar to type II systems (El-Sayed *et al.*, 2003). Therefore, it is thought that mupirocin is synthesized through a combination of type I and type II PKS system. All the genes included in the biosynthesis of mupirocin with their respective function are shown in Figure 1.13 and Table 1.1, respectively. The order of the PKS genes involved in the biosynthesis of mupirocin is not colinear with the proposed biosynthetic pathway (Figure 1.14). *mmpD* and *mmpA* encode two large multifunctional proteins (MmpD and MmpA) which together encode the first four and the last two modules of elongation steps, respectively, in addition to a single transfer/non-elongating module. They consist of suitable required domains; ketosynthase (KS), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH) and methyl transferase (MT) as well, that are

collectively responsible for the biosynthesis of the MA (PKS backbone) part of mupirocin. *mmpC* encodes MmpC, which consist of two acyltransferse (AT) domains and a putative enoyl reductase (ER) domain (El-Sayed *et al.*, 2003; Gurney and Thomas, 2011). AT domains are predicted to function in loading the biosynthetic modules with the starter and the extender units for chain initiation and elongation (El-Sayed *et al.*, 2003). However, AT2, but not AT1, exhibited malonyl transferase activity which functions in loading all the ACPs with malonyl units (Gurney, PhD thesis, University of Birmingham, 2013). Since in Pederin, PedC, a member of AT2 group does not display detectable AT activity, but rather was confirmed to act as a proofreading enzyme by removing the stalled acyl units from blocked modules, but not malonyl-ACP, it is proposed to rename this enzyme as acyl hydrolase (AH) (Jensen *et al*., 2012). This suggests that AT1 in mupirocin might works as acyl hydrolase as well. MmpB is encoded by *mmpB*, which consists of a single KS, KR, DH domains, and a triple ACP domain, which is believed to be responsible for the biosynthesis of the fatty acid (9-HN) side chain of mupirocin, as well as a thioesterase (TE) domain, located at the end of the cluster, suggested to work as a hydrolase in the final step of the pathway for the release of mupirocin. It is also obvious that the *mup* cluster lacks a loading domain for the attachment of an activated starter unit to the first KS in the system (El-Sayed *et al.*, 2003).

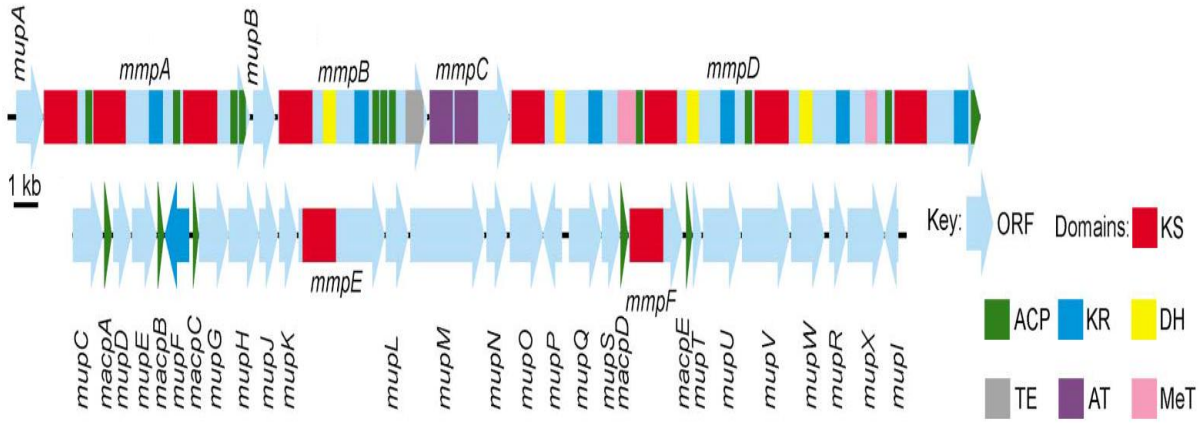



**Figure 1.13 Organization of the mupirocin biosynthesis (*mup*) genes cluster** (source: El-Sayed *et al*., 2003).

**Table 1.1 Enzymatic functions encoded by orfs of the mupirocin biosynthesis cluster** (source: Thomas *et al*., 2010).

| ORF | Deduced functions of the encoded protein |
|---|---|
| *mupA* | Reduced flavin mononucleotide ($FMNH_2$) oxygenase |
| *mmpA* | Polyketide synthase (ketosynthase, acyl carrier protein and ketoreductase) |
| *mupB* | 3-Oxo-acyl carrier protein synthase |
| *mmpB* | Polyketide synthase (ketosynthase, dehydratase, ketoreductase, acyl carrier protein and thioesterase) |
| *mmpC* | Acyltransferase and enoyl reductase |
| *mmpD* | Polyketide synthase (ketosynthase, dehydratase, ketoreductase, methyltransferase and acyl carrier protein) |
| *mupC* | Dienoyl CoA reductase |
| *macpA* | Acyl carrier protein |
| *mupD* | 3-Oxo-acyl carrier protein reductase |
| *mupE* | Enoyl reductase |
| *macpB* | Acyl carrier protein |
| *mupF* | Ketoreductase |
| *macpC* | Acyl carrier protein |
| *mupG* | 3-Oxo-acyl carrier protein synthase |
| *mupH* | β-hydroxyl-β-methyl glutarate CoA synthase |
| *mupJ* | Enoyl CoA hydratase |
| *mupK* | Enoyl CoA hydratase |
| *mmpE* | Polyketide synthase (ketosynthase and hydroxylase) |
| *mupL* | Hydrolase |
| *mupM* | Isoleucyl-tRNA synthetase |
| *mupN* | Phosphopantetheinyl transferase |
| *mupO* | Cytochrome P450 |
| *mupP* | Unknown |
| *mupQ* | Acyl CoA synthase |
| *mupS* | 3-Oxo-acyl carrier protein reductase |
| *macpD* | Acyl carrier protein |
| *mmpF* | Polyketide synthase (ketosynthase) |
| *macpE* | Acyl carrier protein |
| *mupT* | Ferrodoxin dioxygenase |
| *mupU* | Acyl CoA synthase |
| *mupV* | Oxidoreductase |
| *mupW* | Dioxygenase |
| *mupR* | Transcriptional activator |
| *mupX* | Amidase |
| *mupI* | *N*-Acyl homoserine lactone synthase |

*macp*, mupirocin acyl carrier protein gene; *mmp*, mupirocin multifunctional polypeptide gene.

### 1.6.4.1 Pathway for monic acid assembly

The monic acid moiety of mupirocin consists of a $C_{17}$ heptaketide intermediate, which is synthesised by six rounds of chain extension (Claisen condensation). The catalytic reactions are carried out on the multifunctional proteins MmpD and MmpA, respectively. The activated starter unit (acetyl-coenzyme A intermediate) is transferred by AT2 of MmpC acting *in trans* to the 4′-phosphopantetheine arm of ACP-D1 of the first module of MmpD, which is then transferred to the thiol group of the active site cysteine of the KS-D1. Then loading of the extender unit (normally malonate or methyl malonate) by the action of AT2 to the unbound ACP-D1, which then followed by decarboxylative condensation catalysed by the KS-D1. The nascent PK chain then undergoes ketoreduction by KR-D1 but not dehydration, since the dehydration domain in this module is predicted to be non-functional because of genetic alteration in its active site, and the presence of the hydroxyl group at the β-position of C-13 in the structure of the intermediate after module 1 is considered as an indicator. The growing chain is then transferred to the three successive modules within MmpD where chain-extension occurs catalysed by ACP/KS-D2, D3 and D4, respectively, to give a $C_{12}$ pentaketide intermediate. The β-keto group of the PK chain intermediate within each module (round of chain-extension) undergoes reduction to different levels depending on the activity of the reductive domains present in each module (El-Sayed *et al*., 2003). The methyltransferase (MT) domains in module 1 and 3, are responsible for the addition of the two methyl groups (C-16 and C17), which are present in the final structure of mupirocin, and incorporated from *S*-adenosylmethionine (SAM) (Feline *et al*., 1977). The polyketide chain ($C_{12}$ pentaketide) which is produced by four cycles of condensation (Figure 1.14) is then transferred to the first module of MmpA. The ketosynthase (KS-A1) of the first module (module 5 in the pathway) of MmpA is unusual, since one of the two histidine residues in the active site is changed, so it is more likely to function in the transfer of the PK chain to the

downstream module in line, than to act in elongation. However, experiments with deletion of that KS or even active site mutations, abolished production of mupirocin, confirming that it is essential for the biosynthetic pathway. The chain intermediate undergoes two further rounds of condensation on MmpA to give the $C_{17}$ heptaketide precursor to monic acid (El-Sayed *et al.*, 2003).

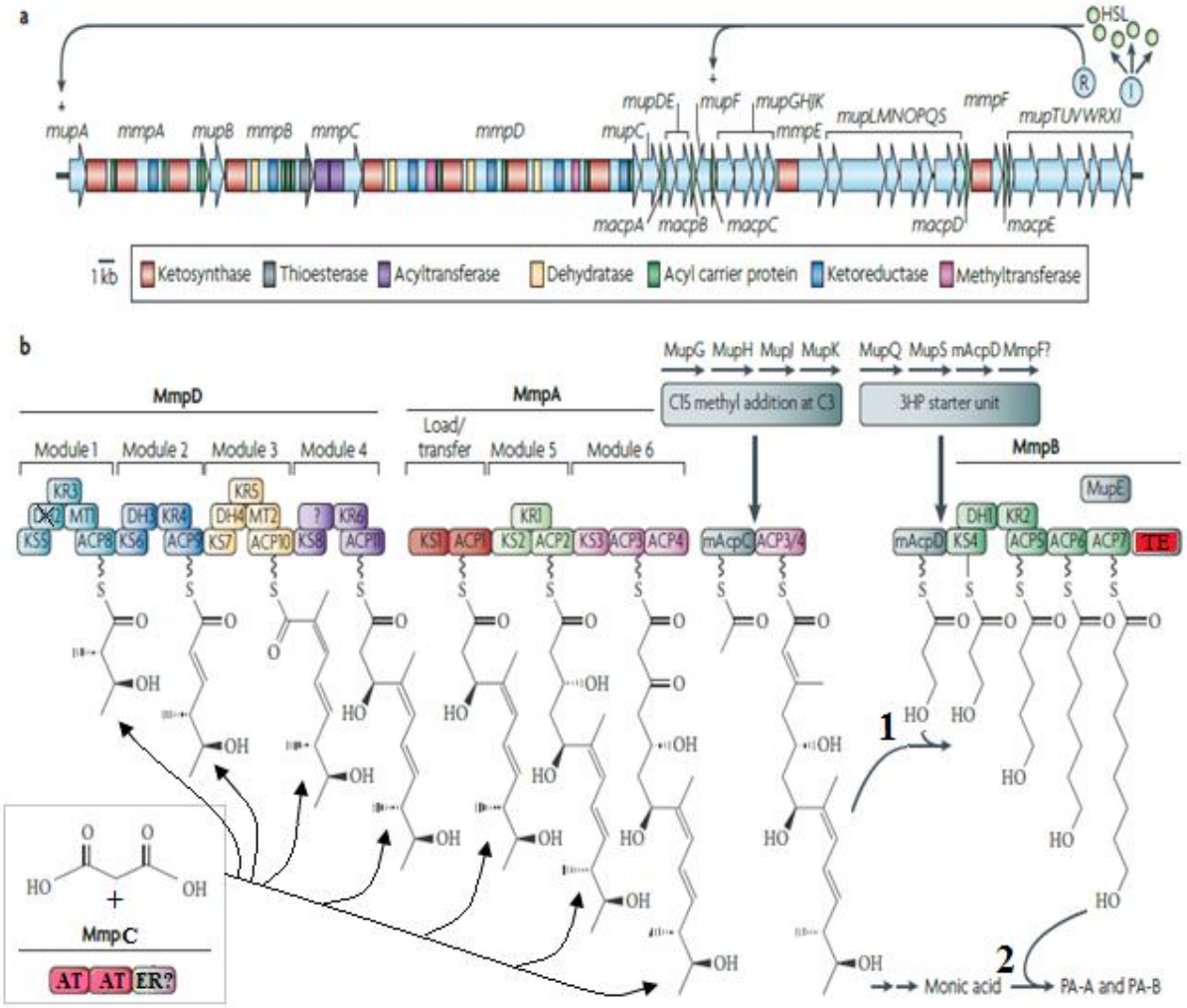



**Figure 1.14 Biosynthetic genes of mupirocin, and a scheme representing the proposed pathway for its production. a.** Genetic organization of the *mup* cluster and the proposed system of regulation of mupirocin expression. **b.** Schematic representation of the proposed pathway for biosynthesis of mupirocin. Six modules of MmpD and MmpA are responsible for biosynthesis of the monic acid (MA) backbone via condensation of extender units with further modifications. 3-hydroxypropionate is proposed as a starter unit for biosynthesis of 9-HN through iterative condensations, ketoreduction and dehydration catalysed by MmpB. Successive elongations from the MA that is already esterified to 3-HP (pathway 1); or, separate synthesis accompanied by esterification (pathway 2), are two possible pathways for the addition of 9-HN to the MA (PKS) backbone (source: Thomas *et al*., 2010).

### 1.6.4.2 Pathway for biosynthesis of 9-Hydroxynonanoic acid (9-HN)

The mupirocin biosynthetic pathway currently thought to be most likely is presented in Figure 1.14. This shows the involvement of MmpD and MmpA, respectively, in the biosynthetic pathway of the MA moiety described in the previous section, so it is reasonable to predict that MmpB is responsible for the biosynthesis of the 9-HN part. Feeding experiments suggests that 3-hydroxypropionate is the most acceptable starter for the biosynthesis of 9-HN (El-Sayed *et al*., 2003). It is proposed that mAcpD (an ACP), MupS (a putative reductase) and MupQ (similar to acyl-CoA ligases) work together in the biosynthesis of 3-hydroxypropionate (Figure 1.15). This suggestion is built on sequence homology and mutational analysis. One of the homologies is the polyketide difficidin (Chen *et al*., 2006): as part of the synthase gene cluster it shows genes encoding for proteins related to the previously mentioned mupirocin genes, since the probable starter of that system is derived for 3-hydroxypropionate (Thomas *et al.*, 2010). MmpB provides essential domains required for the biosynthesis of the fatty acid (9-HN), through iterative use of the domains and condensation using three malonate molecules. The triplicate ACP domains (ACP/Ba, Bb and Bc) within MmpB could be a good indicator of its capability to incorporate three malonate molecules though three successive elongation steps. The ATs of the MmpC acting *in trans* are the possible candidates for loading MmpB with the extender malonate molecules. Since there is no enoylreductase (ER) domain in MmpB, the putative enoylreductase (MupE) most possibly works *in trans* to provide that function to give fully saturated 9-HN (El-Sayed *et al*., 2003). However, inactivating MupE shows the production of compounds (PAs derivatives) with 6´-7´ enoyl bond, but not derivatives with completely unsaturated fatty acids. Further experimental results revealed the involvement of MupD with MupE in the enoyl bond reduction (Hothersall *et al*., 2007; Macioszek, PhD thesis, University of Birmingham, 2009). Within MmpC, a third domain downstream of the ATs, is predicated to work like an ER

which might participate in the essential reductive steps during the biosynthesis of 9-HN. The only thioesterase (TE) domain in the whole mupirocin system is located at the end of MmpB, which is responsible for the release of final product (El-Sayed *et al*., 2003). Until now there are two possible suggestions related to the release of mupirocin as a final product. The first, is that both the MA moiety and the 9-HN are synthesised separately, then esterifed together to give the complete structure of mupirocin. The second suggestion is that the 9-HN elongated through using a $C_3$ starter unit (3-HP) esterifed with the MA (Figure 1.14) (Murphy *et al*., 2011).

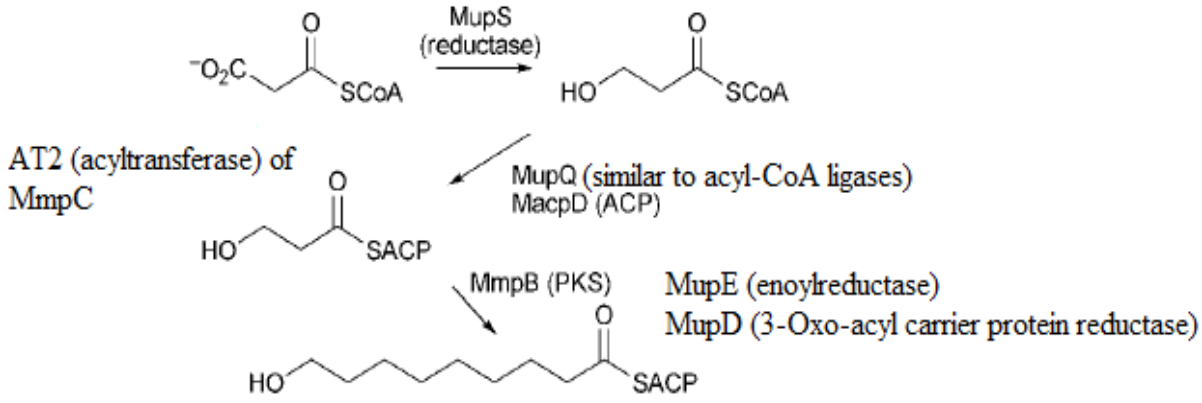



Figure 1.15 **Proposed mechanism of biosynthesis of 9-hydroxynonanoic acid from 3-hydroxypropionate.** Synthesis of 9-HN from 3-HP starter unit is proposed to be catalysed by multifunctional proteins MmpB and MmpC along with MupE, MupD, and other reductive activity acting *in trans* (Piel, 2010 based on data of El-Sayed *et al*., 2003; Cooper *et al*., 2005ab; Hothersall *et al*., 2007; Wu *et al*., 2007 and 2008).

## 1.6.5 Post-PKS tailoring

Immediately after the production of the polyketide synthase derived core, there are further modifications of the backbone by tailoring enzymes. Therefore, determining the role of every gene that encodes different proteins within the biosynthetic *mup* cluster may provide

significant information about the biosynthesis pathway (Cooper *et al*., 2005a). In-frame deletion of *mupO*, *mupU*, *mupV*, and *macpE* abolished the production of PA-A, but lead to a significant increase in the production of PA-B (Cooper *et al.*, 2005a). This result suggests the role of these proteins in the key reduction steps which thus control the flux of the PK intermediates down this channel. It was expected previously that PA-B is not simply produced by hydroxylation of PA-A, but rather it appear to be either an intermediate or may be a shunt product. However, recent observation in Bristol indicates that PA-B is a precursor of PA-A (Gao *et al*., unpublished data). For more details see also Chapter 5. Another two mupirocin analogues, mupirocin C and mupirocin F, are released by strains of *P. fluorescens* with deletions in *mupC* and *mupF*, respectively. Based on the structure of those two compounds, MupC is proposed to reduce the C8, C9 double bond, while MupF should be responsible for catalysing the reduction of the C-7 ketone. Further double deletions of *mupC* with either of *mupO*, *mupU*, *mupV*, or *macpE* lead to the production of PA-B, indicating that the role of MupC and MupF comes after the role of MupO, MupU, MupV, and mAcpE, respectively. Therefore, for the biosynthesis of PA-A, MupU is proposed to mediate the transfer of the intermediate to mAcpE, and then MupO (cytochrome $P_{450}$) would activate the molecule through catalyzing the oxidation of the C-7 hydroxyl to the ketone, and dehydration by MupV to give dienoyl-ketone (C-8,9 olefin). This would then undergo reduction by MupC, accompanied by C-7 ketone reduction catalyzed by MupF, which would then be converted to PA-A (Hothersall *et al*., 2007). A novel metabolite designated mupirocin W, lacking the tetrahydropyran ring (THP) produced, but having the attached 9-HN as a result of *mupW* and *mupT* deletion, points to their role in the oxidative activation of the 16-methyl group, essential for the formation of THP (Figure 1.16). It also indicates that the 16-hydroxylation and the following THP formation happens as the last stage in the biosynthesis of PA-A, since the other proposed late stages of modifications including 5-hydroxylation,

10,11 epoxidation and esterification with the 9-HN moiety were not affected (Cooper *et al*., 2005b).

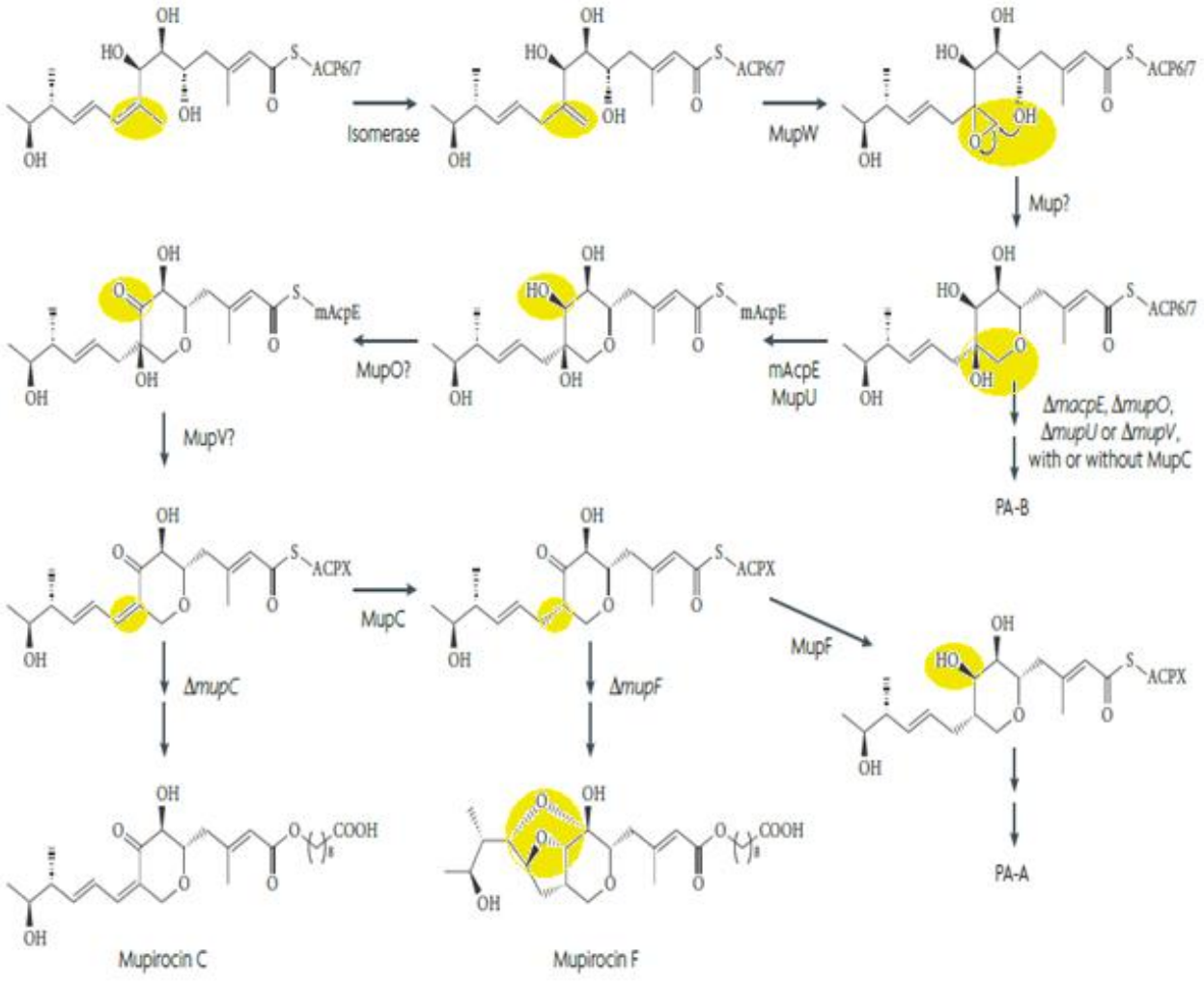



**Figure 1.16 Formation of the pyran ring of the monic acid.** The postulated roles of the key tailoring enzymes that are identified as responsible for the formation of the tetrahydropyran ring of the monic acid moiety and determining the condition of oxidation around the ring. Production of pseudomonic acid (PA) A but not pseudomonic acid B was abolished when *mupO*, *mupU*, *mupV* and the mupirocin acyl carrier protein E genes (*macpE*) were knocked out (Δ) either singly or in combinations, indicating that PA-A is not acting as a precursor to PA-B. Mupirocin C is the product of *mupC* knockout, while mupirocin F is the product of *mupF* knockout (source: Thomas *et al*., 2010).

Hexaketide mupirocin H, a novel metabolite produced as a result of mutating *mupH*, an HMG-CoA synthase (HCS), which encodes for β-hydroxy-β-methyl-glutaryl CoA, as a first member of PAs family with truncation in the PKS backbone (MA) (Figure 1.17). It appears that *mupH* is part of a cassette which consists of five genes: *macpC*, *mupG*, *H*, *J* and *K*, respectively whose functions are presented in Table 1.1. These act at module A3 and functions in the incorporation of acetate at C-3 to generate C-15 methyl group. The proposed mechanism starts with the transfer of malonate to mAcpC (also known as mAcp14) by the aid of MmpC, decarboxylation of the acetate derived from malonate catalyzed by MupG to make acetyl-MacpC. Condensation of the β-ketothiolester moiety of the PK chain with the acetyl-mAcpC catalysed by MupH makes the glutaryl thiolester. A glutaconate intermediate would then be generated by dehydration catalyzed by MupJ and this undergoes decarboxylation by MupK to produce the β-methylthioester in the MA precursor (Wu *et al.*, 2008; Hothersall *et al*., 2007). Further analysis through mutating any of the previous components reveals the same phenotype, in which the truncated hexaketide mupirocin H and the tetraketide mupiric acid were the two major isolable metabolites. In addition, mutating some of the other genes including *mupB*, *mupL*, *mmpB*, and *mmpF*, and of particular interest *mupQ*, *mupS* and *macpD* (proposed to be involved in the biosynthesis of 3-HP) showed production of mupirocin H and mupiric acid and with the same level as has been produced by the *mupH* cassette mutations. Therefore, a 'leaky hosepipe' mechanism was proposed, which considers the predominant release of those metabolites as a result of the presence of spontaneous cleavage mechanisms or 'labile points' in the pathway. Those labile points take place at significant levels whenever there are mutations that block the biosynthetic pathway either in proximity or far from those points (Wu *et al*., 2008).

Repeated ACPs, duplicate ACPs in module 3 of MmpA (ACPs-A3a, and -A3b) and triplicates in MmpB (ACPs-Ba, -Bb and –Bc, respectively) within the biosynthetic pathway

have been studied functionally. Those ACP domains examined using different ways of mutations. Single deletion of the ACPs of MmpA, result in the reduction of mupirocin production to about 60% compare to the wild type, and those ACPs were proposed to work in parallel (Rahman *et al*., 2005). Strains with single or double deletion of the acyl carrier proteins included in MmpB are still able to produce mupirocin, but with a lower efficiency compared to the wild type strain, while deletion of all the three ACPs blocks mupirocin production. Thus, the ACPs function by increasing the flow through the biosynthetic pathway as if they work in parallel. However, inactivating the ACPs-Bb and -Bc with point mutation reduced the production, in contrast to point mutation with ACP-Ba, which blocks the pathway completely, indicating that not all ACPs of MmpB work in parallel (Rahman *et al*., 2005).

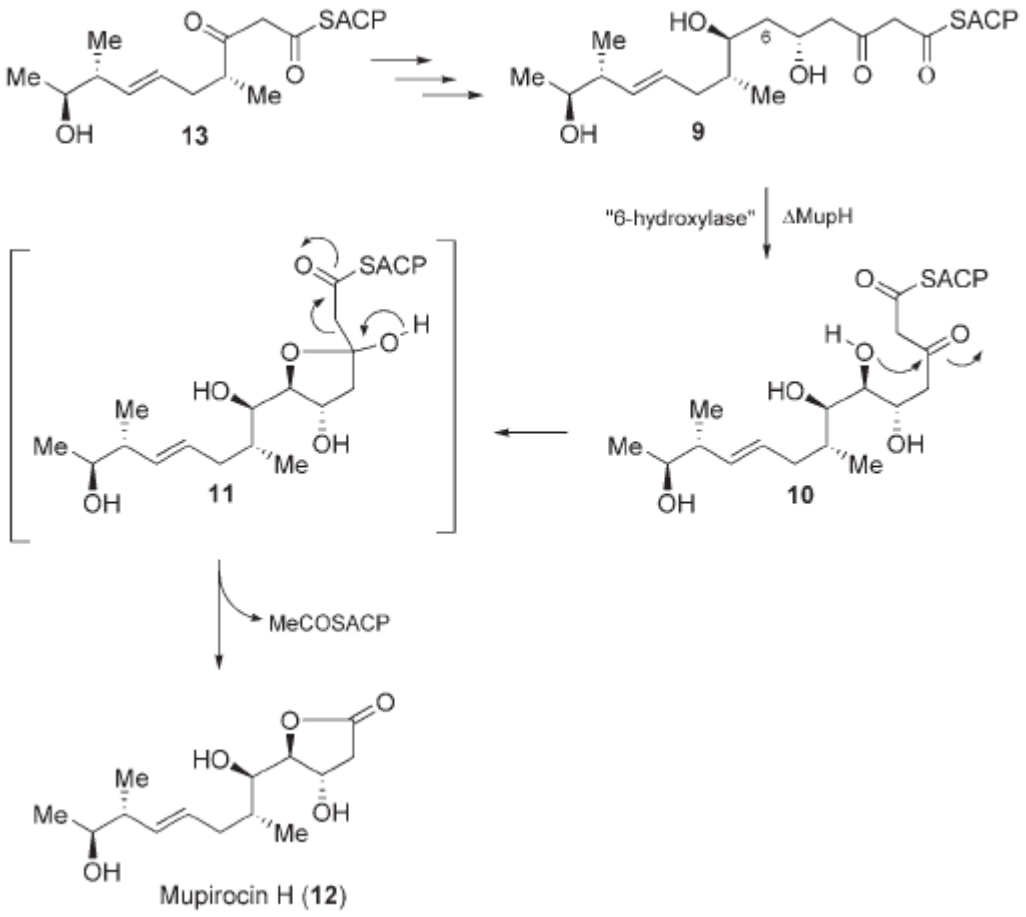



**Figure 1.17. Proposed mechanism for biosynthesis of mupirocin H** (Wu *et al*., 2008).

### 1.6.6 Regulation of mupirocin biosynthesis pathway

The mupirocin biosynthesis cluster of *Pseudomonas fluorescens* NCIMB 10586 was revealed to be regulated through an AHL (acyl-homoserine lactone) dependent quorum sensing (QS) system. Quroum sensing (QS) is defined as a mechanism that enables the bacteria to control the expression of specific target genes when responding to a critical concentration of signal molecules, which represents a way of sensing the cell density of a bacterial population. For example, QS in bacteria regulates gene expression of virulence factors, sporulation, biofilm formation, swarming, biosynthesis of antibiotic and bioluminescence. Although a critical concentration of AHL-signal molecules is essential for the majority of the genes that are AHL-quorum regulated in Gram-negative bacteria, it may be not enough (sufficient) for their expression. One possible explanation is that the bacterial growth phase could play a significant and valuable role in preventing the expression of genes at early stages of the growth even if the concentrations of AHLs are at a quorum regulated level. Numerous regulatory controls were identified to be present and act by ensuring the timely expression of QS-controlled genes (qsc), in accordance with both the concentration of AHL and metabolic status of the bacterial cell. However, there are some degrading enzymes that target the AHL molecules themselves, thus affecting both the total concentration of the available AHL and hence QS response (Venturi, 2006). Marine symbiotic bacteria *Vibrio fischeri* and the related free-living *Vibrio harveyi* are the first studied, which showed QS while studying the expression of bioluminescence which is density-dependent (Nealson *et al*., 1970; Nealson and Hastings, 1979). Those two species produce and respond to the signalling molecules in the form of *N*-acyl-homoserine lactones (AHL) as autoinducers (Eberhard *et al*., 1981; Cao and Meighen, 1989). AHL QS is simply based on the molecular system which is mediated by two specific proteins of the LuxI-LuxR families (Fuqua *et al.*, 2001; Whitehead *et al*., 2001). Synthesis of AHL is the responsibility of LuxI protein which is one of the major

cytoplasmic enzymes. AHL then accumulates intra- and extra-cellularly in proportion to the cell density. When the AHL reaches the QS concentration, it interacts directly and sufficiently strongly with LuxR-type proteins to form LuxR-AHL complexes which can then bind at specific promoter DNA sequence termed as *lux*-boxes of QS regulated genes, and subsequently affecting their expression (Venturi, 2006). Within the mupirocin gene cluster, *mupR* and *mupI* genes were shown to encode proteins with great similarity to LuxR/LuxI of *Vibrio fischeri* and LasR/LasI of *P. aeruginosa* (Gambello and Aglewski, 1991). In frame deletion of *mupI* and *mupR* separately abolished mupirocin production, indicating that they plays an essential role in the biosynthesis. MupI was identified as an AHL synthase that activates *mupR*, the transcriptional activator. Inactivation of the *mup* cluster at different points by using catechol 2,3-dioxygenase (*xylE*) as a reporter gene, abolished production of AHL, but this could be overcome by the addition of 3-oxo-C10 *N*-acyl homoserine lactone exogenously, demonstrating that *mup* cluster is regulated via a quorum signal (El-Sayed *et al*., 2001). *N*-(3-oxodecanoyl) was identified as the AHL signal produced by *mupI* (Hothersall *et al*., 2011).

Similar to the 'Lux boxes' regulatory region that is bound by LuxR, the entire regulatory region that is controlled by MupR was identified. Potential regulatory regions are located upstream of the *mupA* promoter (El-Sayed *et al*., 2001), and between the divergence of *mupF* and *macpC*. Therefore, it is predicted that transcription of the *mup* cluster may include one very long transcript with more additional internal start points (Hothersall *et al*., 2011). Deletion of the *mupX* gene reduces the production of mupirocin by 40%, without blocking the overall biosynthesis of mupirocin, and is predicted to function as amidase/hydrolase. Therefore, this gene may not produce enzymes involve directly in the biosynthetic pathway, but may be needed for the optimum expression of the other genes within the cluster (Cooper *et al*., 2005a). The role of *mupX* gene, encoding for amidase/hydrolase was suggested to

regulate the expression of the biosynthetic genes cluster indirectly via two ways. The first one is by degrading the AHL (cleaving the amide bond) so as to keep the level in balance within the system, and the second is by degrading the minor AHL signals that are produced by the mupirocin producer, which act as inhibitors of MupR (Hothersall *et al*., 2011). Strikingly, expression of *mupR* (which encodes the positive transcriptional regulator, MupR) appears to act as a rate-limiting step during mupirocin production, as its overexpression in-trans both in the wild type *P. fluorescens* NCIMB 10586, 10586Δ*mupC* and 10586Δ*mupF*, increased the production of mupirocin, mupirocin C and F, and the minor shunt products, mupirocin H and mupiric acid, respectively up to 17 fold (Hothersall *et al*., 2011).

## 1.7 Thiomarinol (PKS-NRPS hybrid)

### 1.7.1 Structure and mode of action

Thiomarinol, a hybrid of two independent antibiotics; a pseudomonic acid analogue (PKS moiety) and a pyrrothine (NRPS part), isolated from a marine bacterium *Alteromonas rava* SANK 73390, with antimicrobial activity against both Gram positive and Gram negative bacteria stronger than either separately (Shiozawa *et al*., 1993). Pseudomonic acid C is most similar to thiomarinol, especially with regard to the stereochemistry of the six-membered ether ring in the monic acid moiety (Shiozawa and Takahashi, 1994). However, thiomarinol is different from pseudomonic acids (Figure 1.18) by the presence of 8-hydroxyoctanoic acid instead of 9-hydroxynonanoic acid, which is combined with the chromophore part (holothin) (Shiozawa *et al*., 1995). Pyrrothine is among a group of antibiotics produced by *Streptomyces clavuligerus* (Kenig and Reading, 1979), such as holothin, pyrrothine (*N*-methylholothin),

holomycin (acetylholothin), thiolutin (acetylpyrrothine), and others (Korzybski *et al*., 1978), that have a yellowish chromophore containing a disulfide bond. Pyrrothine antibiotics showed a broad spectrum activity, in which they differ depending upon the length of their acyl side chain (Korzybski *et al*., 1978; Mcinerney *et al*., 1991). The pyrrothine part is attached through an amide linkage to the 8-hydroxyoctanoic acid moiety of marinolic acid (pseudomonic acid like analogue) of thiomarinol (Fukuda *et al*., 2011).

Thiomarinol includes a series of related molecules with the different forms being designated A-to-G (Shiozawa *et al*., 1997; 1993; 1995; Shiozawa and Takahashi, 1994). The pigmented thiomarinol (thiomarinol A) producer appeared to be *Pseudoalteromonas*, which was previously classified incorrectly as *Alteromonas rava* (Abraham, 2004; Anderson *et al*., 1974; Ballester *et al*., 1977; Barja *et al*., 1989). Comparison of the molecular architecture of thiomarinol compounds relative to thiomarinol A (TMA), revealed that TMB possess an additional two oxygen atoms with the chromophore part (holothin), and TMC is considered as its monodeoxy derivative (4-deoxythiomarinol A) (Shiozawa *et al*., 1995). Also, the structure of TMD was deduced as 14-homothiomarinol A, TME as having two more methylenes in the acylchromophore part than thiomarinol A, TMF as ketothiomarinol A, and TMG which has the same molecular formula and the same acylchromophore as TMC (Shiozawa *et al*., 1997).

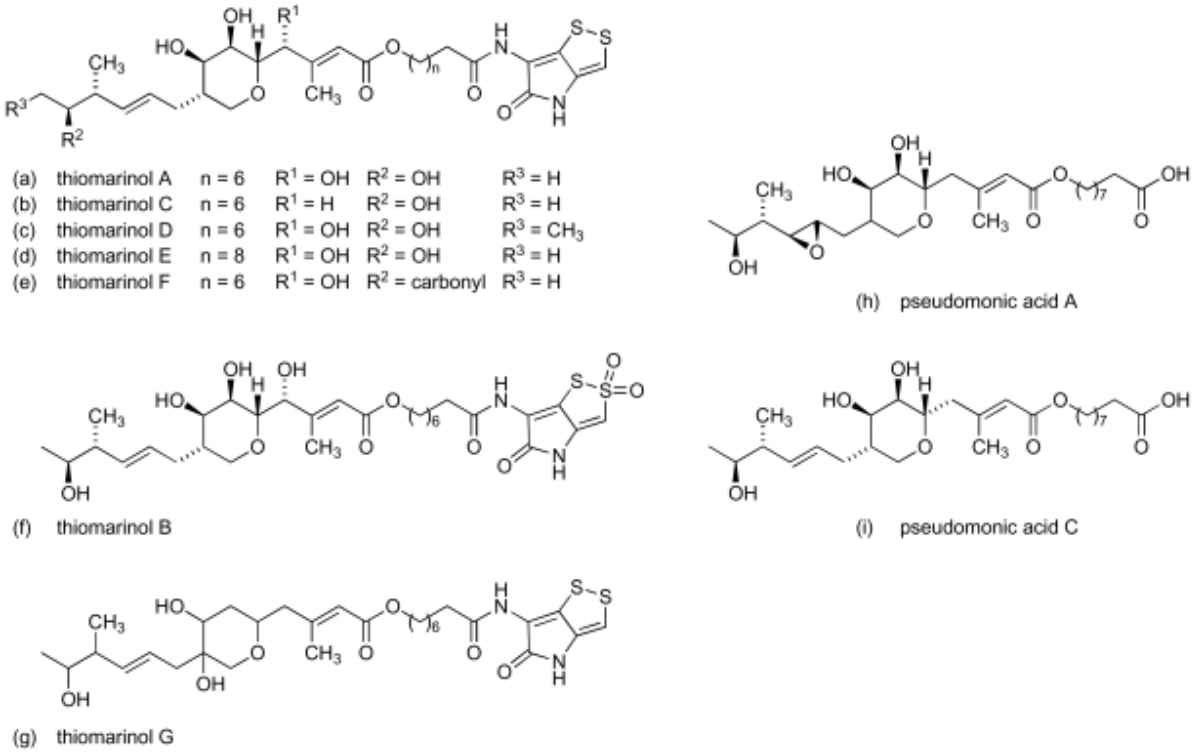



**Figure 1.18.** Chemical structure of thiomarinols A-G and the related pseudomonic acids A and C (source: Rahman *et al*., 2010).

Among all thiomarinol derivatives, TMA, TMB and TME were shown to have a similar potency with pronounced activity against Gram positive bacteria including MRSA, and slightly less activity with TMC, while TMG is reported to be the weakest one (Shiozawa *et al*., 1997). Specific inhibition of bacterial isoleucyl–tRNA synthetase is the mode of action of pseudomonic acids (Hughes and Mellows, 1980), and the inhibition is stronger by thiomarinols A, B and D, as they have a similar homoisoleucine type structure as pseudomonic acid A, whereas no inhibition of leucyl-tRNA, valyl-tRNA, and phenylalanyl-tRNA synthetases was observed by thiomarinol (Shiozawa *et al*., 1997). However, although it is obvious that the pyrrothine part improves the inhibitory activity of thiomarinol (Fukuda *et al*., 2011), it is not yet known whether the pyrrothine part imparts any additional mechanism of antibacterial activity (Murphy *et al*., 2011). Moreover, the broad spectrum as well as the

strong *in vitro* antimicrobial activity exhibited by thiomarinol, raises the possibility that inhibition of RNA synthesis may be its other target (Shiozawa *et al*., 1993). This fact raises the possibility of an increase in the uptake of thiomarinol since the bacterial outer membrane is thought to work as a barrier that protects Gram-negative bacteria (Fukuda *et al*., 2011).

### 1.7.2 The genetic organization of the thiomarinol biosynthesis (*tml*) cluster

Sequencing of the whole genome of *Pseudolteromonas* spp SANK 73390 (thiomarinol producer) performed to identify the biosynthetic genes of thiomarinol (*tml*). Results leads to the identification of approximately 97 kb of DNA contained all the genes required for thiomarinol biosynthesis (Figure 1.19). This was shown to consist of a trans-AT PKS including essential biosynthetic genes and associated reductive enzymes with great similarity to the mupirocin (*mup*) cluster, connected with the NRPS part comprising a set of accessory enzymes exhibiting considerable similarity to that which has been shown to control the biosynthesis of holomycin in *Streptomyces clavuligerus* (Huang *et al*., 2011). These observations emerged while investigating the 273 contigs from a whole genome sequencing project, in which part of one of them was found to encode many genes with great similarity to those in the *mup* cluster. Amplification of thiomarinol KS sequences using degenerate primers lead to the identification of a number of PKS genes, including the largest gene (*tmpD*), with similarity to *mmpD* in the *mup* cluster. Using suicide mutagenesis, the KS of the *tmpD* gene was mutated, and the mutants showed a reduction in the antibacterial activity. LCMS analysis confirmed that the mutants had lost the ability to produce thiomarinol, while they were still able to produce pyrrothine (Fukuda *et al*., 2011).

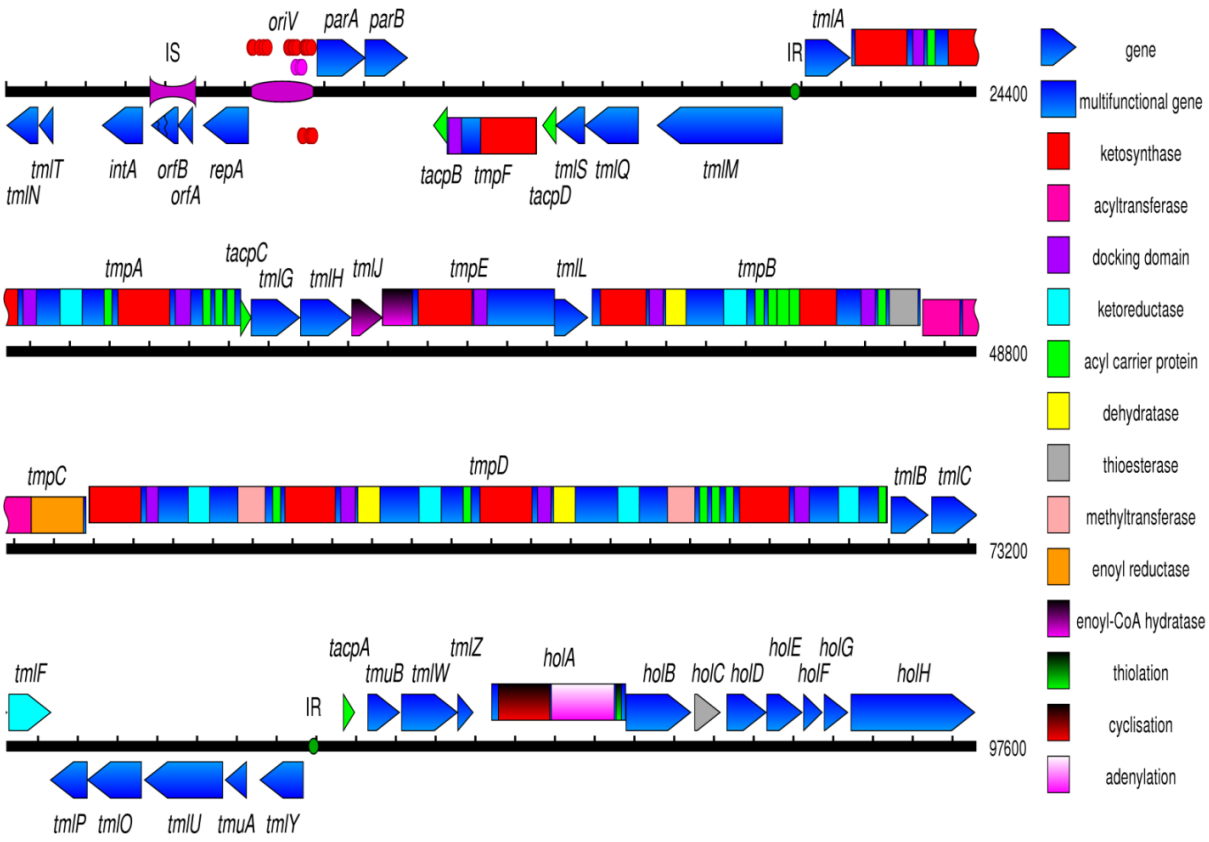



**Figure 1.19. The thiomarinol biosynthesis (*tml*) gene cluster located on a plasmid pTML1** (source: Fukuda *et al*., 2011).

Subsequent productive analysis of the contig evidentally showed that it constitutes a circular plasmid which was named pTML1. The following evidence confirmed that it is a circular plasmid. One orf was identified based on the annotation of the contig, which was predicted to encode for a protein (Rep), that initiates DNA replication (Fukuda *et al*., 2011), and whose most similar sequence match is the Rep protein encoded by the megaplasmid-like chromosome II of *Pseudomonas haloplanktis* TAC125 (CR945247: 635,328 bp) (Medigue *et al*., 2005). Also two other adjacent orfs were identified as encoding partitioning proteins ParA/ParB equivalent to the plasmid partitioning proteins from the same source. In addition, experimental data confirmed that this contig is a circle, since the incomplete ends of the contig, were similar to the front and back of a single gene (the oxido-reductase gene that have

been designated as *holA*), compatible with the possibility that they should make a circle by joining the ends together (Fukuda *et al*., 2011). The suicide plasmid pAKE604: *tmpD* carries the *sacB* gene which encodes levansucrase that confers sucrose sensitivity in Gram-negative bacteria and so the *tmpD* knockout was used to verify whether pTML1 and thiomarinol production could be lost. A mutant with white colonies which lost the yellow pigment (indicating the loss of the pyrrothine moiety of thiomarinol) was obtained and the whole element was confirmed by using multiple PCR reactions across different DNA segments from the annotated contig including the *rep-par* region which confirmed that pTML1 has been lost. Therefore, since SANK 73390 could still survive, it is deduced that this contig is a plasmid rather than a chromosome (as a chromosome by definition carries at least one essential gene and cannot be displaced without the loss of the viability) (Fukuda *et al*., 2011).

### 1.7.3 Thiomarinol biosynthesis pathway

The final sequence of pTML1 is 97600 bp with a G+C content of 43.2%, which is slightly higher than the G+C content (40.6%) of the summed contigs representing chromosomal DNA. This is in contrast with the G+C content of the mupirocin biosynthetic cluster of *Pseudomonas fluorescens* which is 56% (El-Sayed *et al*., 2003). Forty five orfs were identified in the final annotation (Figure 1.19), five of which are predicted to have typical mobile DNA functions (replication, partitioning, transposition and integration), although none of them have been identified which might be involved in either the transfer or mobilisation through conjugation. An essentially complete mupirocin gene cluster has been identified consisting of twenty seven orfs, which should encode the biosynthesis of the moiety termed marinolic acid which is the pseudomonic acid analogue (Figure 1.20) (Fukuda *et al*., 2011). The type I polyketide synthase (PKS) genes (*tmpA-tmpD)* have a similar order

to that of *mup* cluster (El-Sayed *et al*., 2003). The minor dissimilarities in the proteins being the presence of additional (extra) ACPs, which have been shown generally to increase the overall pathway flow (Rahman *et al*., 2005), in module 3 of TmpD, module 6 in TmpA and in TmpB with two extra ACPs, one included in a group of four and one precedes an extra KS (creating an extra module in TmpB) (Fukuda *et al*., 2011). It is predicted that the extra KS-ACP within TmpB may have a role in the final processing (tailoring steps) to thiomarinol production before its release by TE (see also Chapter 5) (Fukuda *et al*., 2011). There are at least five transcriptional units (*tmlT* to *tmlN*, *tmlM* to *tacpB*, *tmlA* to *tmlF*, *tmlY* to *tmlP* and *tacpB* to *holH*) within thiomarinol genes cluster. These are differentiated by the difference in the direction of transcription and their interruption by the plasmid backbone functions of replication/partitioning and transposition functions (Fukuda *et al*., 2011).

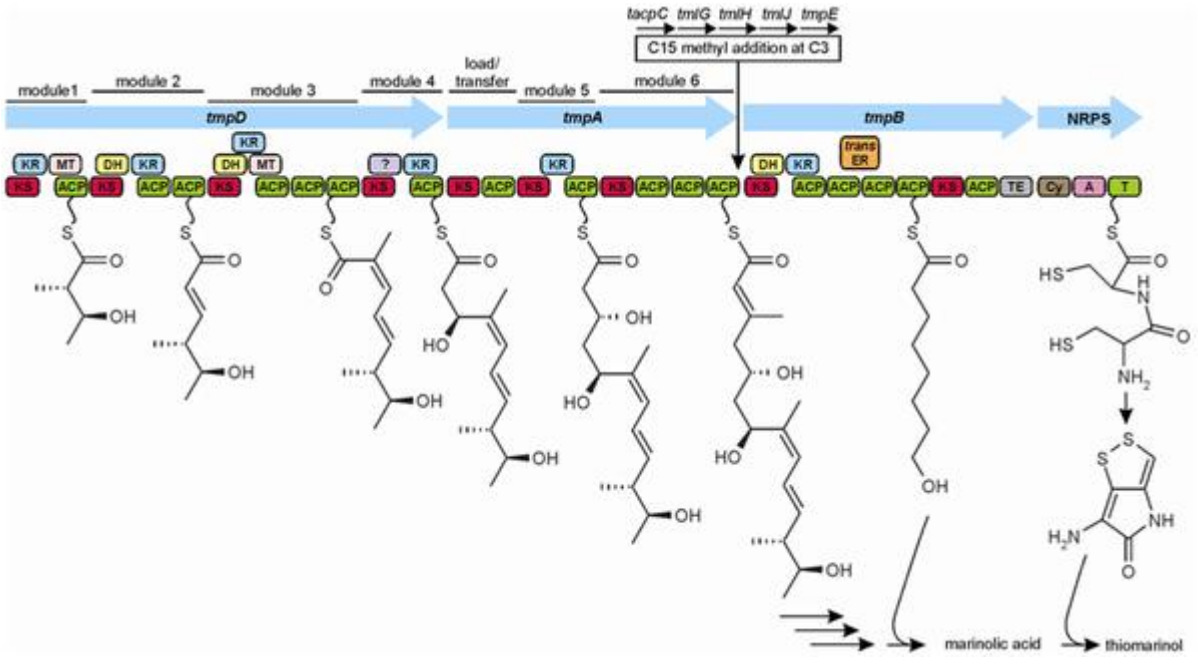



**Figure 1.20 Schematic representation of the predicted biosynthesis pathway of thiomarinol explaining the critical roles of both TmpD (modules 1 to 4) and TmpA (modules 5 and 6), respectively for monic acid, TmpB for the fatty acid (8-hydroxyoctanoic acid) side chain and HolA (NRPS) for pyrrothine** (source: Fukuda *et al*., 2011).

Although the PKS and the associated parts of biosynthetic genes of mupirocin and thiomarinol are conserved, a high degree of divergence with regard to the sequence of the predicted products of the identified orfs was observed. The amino acid sequence alignments revealed sequence similarities ranging from approximately 40% to 65%, although the functionally constrained regions showed a higher degree of identity. These bioinformatics data in accordance with the predicted products raised questions about the possibility of functional cross-complementation between the two pathways, which requires more experimental evidence (see also Chapter 3) (Fukuda *et al*., 2011).

Fukuda *et al*., 2011 described seven orfs (designated as *holA* to *holH*) in the annotated sequence of the thiomarinol cluster which showed valuable similarity to the non-ribosomal peptide synthase (NRPS) genes cluster from the holomycin producer *Streptomyces clavuligerus* (Okamura *et al*., 1977; Huang *et al*., 2011), and *Photorabdus assymbiotica* (Wilkinson *et al*., 2009) that generate related compounds (Bode, 2009). Briefly the orfs are designated as follows: *holA* (NRPS), *holB* (oxidoreductase), *holC* (thiolesterase), *holD* (dehydrogenase), *holE* (acyltransferase), *holF* and *holG* as oxygenase and decarboxylase, respectively. However, the role of *holH* is not known, which is currently under investigation. The most important part is *holA* (the NRPS) which encodes for a single adenylation (A), thiolation (peptidyl carrier protein, PCP or T), and cyclisation/condensation (C) domains, which is hypothesized to function as a dimer for adding and joining the two cysteine molecules for the formation of a cysteinyl-cysteine dipeptide (Fukuda *et al*., 2011).

An internal segment of *holA* was used to generate a mutation within the pyrrothine genes to confirm their identity. HPLC and further LCMS analysis performed for the detection of the product released by *holA* (NRPS) and the *tmpD* (PKS) mutants in comparison to the wild type thiomarinol producer. Thiomarinol A (640 Dalton) was produced both in the whole cells and the supernatant of the wild type. Marinolic acid (486 Da) was produced by the strain with

*holA* (NRPS) mutation (lost the yellow pigment of pyrrothine), while only the yellow pigmented pyrrothine was produced by the *tmpD* (PKS) mutant. These observations indicate that it is possible for both marinolic acid and pyrrothine to be made independently. *tmlU* encodes TmlU, a protein that shows similarity to SimL in *Streptomyces antibioticus* that is responsible for the creation of the amide bond during simocyclinone biosynthesis pathway (Luft *et al*., 2005; Pacholec *et al*., 2005), and NovL in *Streptomyces spheroids* having the same function in the biosynthesis of novobiocin (Steffensky *et al*., 2000). A mutant strain with in-frame deletion in *tmlU* shows the ability to produce both marinolic acid and pyrrothines but lost thiomarinol production, implicating TmlU in linking the two parts of thiomarinol together (Fukuda *et al*., 2011).

Further analysis confirmed the two antibiotics in the biosynthetic pathway of thiomarinol and provided strong evidence for the possibility of performing mutasynthesis and creating new families of hybrids. This consisted of restoring thiomarinol production via co-fermentation of both the Δ*tmpD* and Δ*holA* strains, and feeding Δ*tmpD* with marinolic acid. Two novel pyrrothine amides (Figure 1.21) created while feeding Δ*tmpD* with pseudomonic acid A (Fukuda *et al*., 2011). Further feeding experiments showed that alternative substrates including pseudomonic acids could give alternative metabolites which made by *P. fluorescens* or amines, such as anhydroornithine could be incorporated by the two mutants (Δ*tmpD* and Δ*holA* respectively) (Murphy *et al*., 2011).

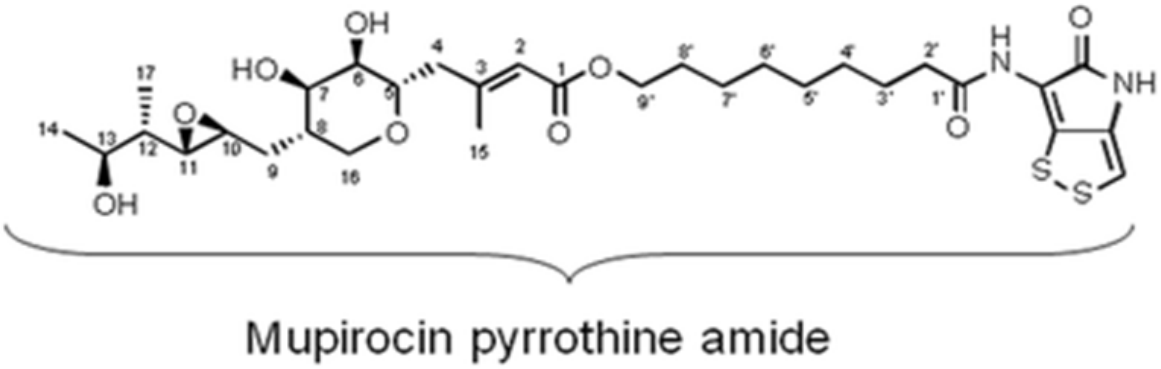



**Figure 1.21. Structure of the hybrids (mupirocin-pyrrothine amide) created by joining pseudomonic acid A with pyrrothine via an amide bond** (source: Fukuda *et al*., 2011).

## 1.7.4 Resistance to thiomarinol

Since thiomarinol has not been commercialised yet, this section will discuss the potency of thiomarinol and the gene responsible for conferring self-resistance to the thiomarinol producer. A mutant strain of SANK 73390 which was confirmed as having lost the pTML1 plasmid shows sensitivity to thiomarinol, indicating that pTML1 is essential for resistance and *tmlM* was found to be responsible for the putative isoleucyl tRNA synthetase (Fukuda *et al*., 2011), similarly to the *mupM* gene in mupirocin producer that acts by preventing the suicide (El-Sayed *et al*., 2003; Yanagisawa and Kawakami, 2003). Therefore, *tmlM* may confer resistance to the marinolic acid part, while no gene is identified to confer resistance to the pyrrothine part. *In-trans* expression of both *mupM* and *tmlM* separately in *E. coli* performed under the control of *tac* promoter showed that *mupM* confers partial resistance to thiomarinol (MIC=1 µg/ml), in contrast to *tmlM* which gives higher level of resistance (MIC

≥16 µg/ml) (Fukuda *et al*., 2011). This observation suggests that isoleucyl-tRNA synthetase is the sole target of thiomarinol. Investigation of the activity of thiomarinol against methicillin resistant *Staphylococcus aureus* (MRSA) was carried out by plate bioassay and minimum inhibitory concentration (MIC) which showed that the growth of MRSA without and with the plasmid-borne *mupA* gene (encoding an IleRS which confers high level of resistance to mupirocin) was inhibited by thiomarinol. The same context described above was used for cloning and expression of this *mupA* gene (not the *mupA* gene from mupirocin biosynthetic cluster) in *E. coli* and the data showed that it also confers on *E. coli* resistance to mupirocin (MIC=50 µg/ml), but gives partial resistance (MIC=2 to 4 µg/ml) to thiomarinol, which was slightly higher than that for the *mupM* gene. However, the growth of *E. coli* was clearly inhibited at concentrations of thiomarinol that were not completely inhibitory to bacterial strains carrying SANK 73390 specific *ileS* gene (*tmlM*). Therefore, this means that both *mupM* and *mupA* (encodes mupirocin resistant *ileS*) are only able to confer partial resistance to thiomarinol, while for complete resistance it is essential for the strain to carry an altered *ileS* gene (*tmlM*) (Fukuda *et al*., 2011).

The antimicrobial activity of the novel compounds including mupirocin pyrrothine amides created by mutasynthesis (Murphy *et al*., 2011) were examined in comparison to thiomarinol, via using *E. coli* and *S. aureus* (MRSA) as indicator strains. Results indicated that the growth of both *E. coli* and *S. aureus* (more sensitive MIC≤30 ng/ml) inhibited by thiomarinol and the novel mupirocin pyrrothine amides (Fukuda *et al*., 2011). However, thiomarinol showed slightly more activity (MIC= 16-32 µg/ml) against MRSA carrying the *mupA* gene than the pyrrothine amides (MIC= 8 µg/ml). Marinolic acid showed less activity than mupirocin against *S. aureus*, which confirmed that the pyrrothine part is responsible for the increased potency of thiomarinol (Fukuda *et al*., 2011).

### 1.7.5 Modification enzymes

Considering tailoring enzymes of thiomarinol biosynthetic pathway, works are currently underway to characterise them. However, because there is great similarity between thiomarinol (especially marinolic acid part) and mupirocin, the function of several genes with their encoded enzymes could be deduced based on comparison with their analogues in mupirocin system.

Since sequence alignment of TmlA showed identity to MupA (in mupirocin), in addition to OmnC and PedJ proteins from the biosynthetic pathway of onnamide and pederin, respectively, and as they are proposed to have a role in the addition of hydroxyl groups at $C_7$ (Piel, 2002; Piel *et al*., 2004), same function proposed to TmlA as well (Fukuda *et al*., 2011). Moreover, the conserved position of *tmlA/mupA* directly upstream of *tmpA/mmpA* in both mupirocin and thiomarinol cluster, suggests the responsibility of *tmpA/mmpA* in the $C_6$ hydroxylation (Fukuda *et al*., 2011) of the MA. This possibly happens at the time of its attachment to the non-elongating module positioned at the beginning of TmpA/MmpA (El-Sayed *et al*., 2003).

In thiomarinol, it is not known which gene encodes for the second hydratase (homologue of MupK in mupirocin) (Fukuda *et al*., 2011), which is responsible for decarboxylation, the last step in the pathway of HMG-CoA synthase (HCS) cassette which is responsible for the addition of $C_{15}$ methyl group (Wu *et al*., 2007). However, since the N-terminus of TmpE showed identity with MupK, it is expected that TmpE might have the ability to provide this function (Fukuda *et al*., 2011). The lack of MupK analogue in thiomarinol is predicted to be as a result of gene fusion, since in the *mup* cluster both *mupK* and *mmpE* are located one after the other, so, a simple fusion could end up with such possible arrangement seen in *tml* cluster. This explains the possibility of the HCS cassette working where TmpE is positioned (Fukuda

*et al*., 2011). The idea which is supported by the hypothesis that the HCS cassette in kalamanticin/batumin cluster functions at the positions (Mattheus *et al*., 2010) which showed some degree of similarity to that of TmpE in the thiomarinol cluster with regard to the protein fusion, which could be taken as evidence (Fukuda *et al*., 2011).

In the *mup* cluster, *macpE*, is one of the genes located away from the multifunctional enzymes (Cooper *et al*., 2005a), which does not have an equivalent in the thiomarinol biosynthetic cluster. During the biosynthesis of mupirocin, MupU is proposed to play an essential role in the transfer of the intermediates to mAcpE, which undergoes further processing through MupO, V, C, and F for the production of pseudomonic acid A as a final product (Hothersall *et al*., 2007). However, the presence of an extra KS-B2 and ACP-B2 in TmpB which is absent in MmpB, while TmlU which has weak similarity to MupU but must have a different or additional role to that of MupU (see also Chapter 3), suggests that all the final processing steps for thiomarinol biosynthesis might possibly takes place on TmpB before its release by the TmpB thioesterase (TE). This suggestion minimizes the possibility that leakiness results from bottlenecks (see section 1.6.4.3) in the biosynthetic pathway, the reason which might explain why thiomarinol G (PA-B equivalent with $C_9$ hydroxylation), is hardly detectable (released in a tiny amount) (Fukuda *et al*., 2011).

Considering the pyrrothine part of thiomarinol, work is currently underway to investigate the role of *holH* in the biosynthesis of thiomarinol via an in-frame deletion, in addition to other genes as well.

### 1.7.6 Regulation of thiomarinol biosythesis

The sequence analysis of the thiomarinol genes cluster revealed inverted repeats in the divergent promoter regions, which have been identified as putative operator sequences through which the genes of thiomarinol (pTML1 plasmid) are regulated. These inverted repeats are located in the regions between the following divergent transcriptional units; *tmlM* to *tacpB/tmlA* to *tmlF* and *tmlY* to *tmlP/tacpA* to *holA* (Figure 1.19) units, respectively. However, there are no clear cut evidences of candidates to function like regulatory proteins in the plasmid, and neither of the inverted repeats showed identity or any similarity to the Lux box that are found in the *mup* cluster, which is regulated through QS regulation. Therefore, it is expected that for the regulation of the plasmid, including thiomarinol biosynthetic genes cluster, it might require some chromosomal genes. This could explain why the plasmid faces problems if it were to transfer to an heterologous host which is different from the orginial host by not being able to support the plasmid with the essential requirement that it functions in plasmid regulation and thiomarinol production (see also Chapter 6). The fact that no evidence is available for the conjugation transfer function on the plasmid makes it more complicated, although there is evidence that thiomarinol biosynthetic genes cluster appear likely to be regulated so as to give expression at late exponential or early stationary phase of the growth of the producer *Pseudoalteromonas* spp SANK 73390 (see also Chapter 2).

## 1.8 This project: context and objectives

This study includes several subprojects investigating the two closely related antibiotics, mupirocin and thiomarinol, both active against MRSA and the former in clinical use. The *mup* cluster has been sequenced, and putative enzymes encoded by most of the genes have been identified. This has enabled the specifc role of various enzymes in the biosynthesis of

mupirocin to be determined (Whatling *et al*., 1995; El-Sayed *et al*., 2001; El-Sayed *et al*., 2003). In particular and relevant to this study further analysis revealed the successive roles of MupO, MupU, MupV and mAcpE in processing the intermediates for the synthesis of pseudomonic acid A (major compound), and the significant role of MupW and MupT in pyran ring formation. MupX (Cooper *et al*., 2005a, b) has also been identified to have an interesting role with MupI and MupR (rate-limiting step) in the regulation of mupirocin biosynthesis (Hothersall *et al*., 2011). Chapter 2 of this thesis focuses on control of gene expression by the thiomarinol producer *Pseudoalteromonas* spp. SANK 73390. This was carried out through studying the growth curve, and determining the timing of thiomarinol production relative to the growth while optimizing the conditions for the growth of the producer, production and extraction of thiomarinol. This data indicates that the thiomarinol gene cluster appears to be regulated so as to give expression in late exponential or early stationary phase. For increased production of thiomarinol industrially we need to understand how the biosynthetic gene cluster is controlled and how expression of the genes is regulated. Therefore, Chapter 3 focuses on determining the role of the gene which encodes a critical function in the pathway and which should be strategic for this purpose. Based on the observation that inactivation of *tmlU* prevented thiomarinol production (Fukuda *et al*., 2011), it was hypothesised that this gene controls the production of thiomarinol by catalysing the amide bond formation that brings together the two components of this antibiotic-marinolic acid and pyrrothine. We hoped that further studying *tmlU* should therefore test this hypothesis as well as allowing the possibility of creating novel hybrid antibiotics between analogues of pyrrothine (part of thiomarinol) and mupirocin that could be joined by the enzyme encoded by *tmlU*. Therefore, the first step was by over-expression of *tmlU* in the mutant NCIMB10586Δ*mupU* (cross-complementation), then in the wild type *P. fluorescens* NCIMB 10586 and other mutants described in Chapter 3. The presence of extra KS and ACP

in TmpB of thiomarinol biosynthetic cluster and the absence of a mAcpE homologue which is responsible for accepting the polyketide intermediate from MupU where it is processed by MupO, V, C, and F in the mupirocin system (Hothersall *et al*., 2011) raises the possibility of the involvement of TmpB in the last tailoring steps of thiomarinol biosynthesis (Fukuda *et al*., 2011). Therefore, the aim of thiomarinol subproject in Chapter 4 was to investigate the role of this extra non-elongating (KS-B2/ACP-B2) module of TmpB in the removal of the $C_8$-hydroxyl group, through construction of gene –knockout, which would provide information about the structure of the late-stage intermediate in the biosynthetic steps in the pathway.

In mupirocin, the proposed mechanism of intermediate transfer by MupU to mAcpE, and further processing by additional tailoring enzymes (MupO, MupV, MupC, and MupF) for the production of PA-A (Hothersall *et al*., 2007), prompted Macioszek to attempt to shut down PA-B production in favour of PA-A via *in-trans* expression of these genes together in *P. fluorescens* NCIMB 10586 (Macioszek, PhD thesis, University of Birmingham, 2009). One of the aims of this study was to continue that work by different strategies. The first one was by increasing the expression of *mupU* (transferring the intermediates) via genetic manipulation to insert a new promoter upstream *mupU*, and the second was by the fusion of *mupU* and *macpE* so that they would be expressed and translated into a single functional polypeptide. Since the last one was also successful, and as MupU/mAcpE is expected to work before the thioesterase (TE) of MmpB, the fused *mupU-macpE* inserted into two different positions within mupirocin cluster of strain with *mupU* and *macpE* knocked out, constructed during this study, described in Chapter 5.

Knowing the regulatory system and how the expression of thiomarinol biosynthetic genes cluster is regulated, may reveal ways to increase the production of this antibiotic industrially. Since thiomarinol biosynthetic genes cluster in *Pseudoalteromonas* spp. SANK 73390 is located on plasmid pTML1 (Fukuda *et al*., 2011), and data in Chapter 2 showed that the

thiomarinol gene cluster appears to be regulated so as to give expression in late exponential or early stationary phase, it would not make sense for genes to be on a plasmid and able to move between bacteria if they could not carry their regulatory system with them. Therefore, it was proposed to transfer pTML1 to a new host to see if the genes are expressed and can be regulated in the same way, or whether it needs one or more chromosomal genes from *Pseudoalteromonas* spp. SANK 73390. For this purpose, attempts to transfer pTML1 plasmid out of SANK 73390 were carried out in Chapter 6 using different strategies including the use of different vectors constructed with different specificites, such as RK2 vector, R6K replicon, and a vector which depends on an *E. coli* strain with chromosomally integrated *pir* gene for the replication.

Overall, the aim of these investigations was to cover more aspects of the critical steps that control thiomarinol biosynthesis, and to give better understanding about various enzymes in the synthesis of mupirocin and thiomarinol which have functional and specific roles in switching on the system to a specific compound. Also to make a link between those two antibiotics, which would serve in two most useful and powerful ways, the first one is to provide more valuable understanding about synthesis context of every part (i.e fatty acid side chain), and second to generate more powerful tools for creating a new hybrid with higher potency.

# CHAPTER 2

## OPTIMAL CONDITIONS FOR THIOMARINOL EXTRACTION AND DETECTION FROM THE PRODUCER *Pseudoalteromonas* STRAIN SANK 73390

## 2.1 Introduction:-

Thiomarinol, the naturally hybrid antibiotic of pseudomonic acid (PKS part) and holomycin (NRPS part) has occuring much attention in recent years due to its antibacterial activity against both Gram-positive and Gram-negative stronger than either of the two separate antibiotics (Shiozawa *et al*., 1993), which offers important pharmaceutical and medicinal benefits. Thiomarinol is produced by Gram-negative marine bacterium *Pseudoalteromonas* spp SANK 73390. Holomycin belongs to the group of pyrrothine antibiotics with a yellowish chromophore containing a disulphide bond (Korzybski *et al*., 1978), and so the holomycin component means that thiomarinol has a yellowish colour. It has been hypothesised that only pigmented species of the genus *Pseudoalteromonas*, such as the thiomarinol producer, are able to produce natural products (Egan *et al*., 2002), and they seem to have more restricted growth requirements, such as the need for a special amino acids (Bowman, 2007). *Pseudoalteromonas* spp SANK 73390 grows at temperatures between 4$^{o}$C to 32$^{o}$C, with optimum growth temperature falling between 17$^{o}$C to 26$^{o}$C. However, temperatures not falling within this range are also applicable for strains adapted to grow at lower/higher temperatures. Furthermore, growth at temperatures ranging between 20$^{o}$C to 26$^{o}$C, and maintaining the pH of the culture medium between pH 5 to pH 8 is preferable for the production of thiomarinol B and/or C but without stopping the growth of the microorganism or having any irreversible influence on the quality of the final product (Takahashi *et al*., 1995).

Acetone and ethyl acetate were used for the isolation and extraction of thiomarinol from broth cultures of SANK 73390 which was then concentrated in *vacuo* in order to give the final yellowish oily substance (Shiozawa *et al*., 1993). Moreover, extraction of thiomarinol B and/or C has been performed according to their physico-chemical properties and by using

water-immiscible organic solvents, for example ethyl acetate or ethylene chloride under either neutral or acidic conditions, then purified and applied on preparative HPLC, using a normal or reverse phase column (Senshu-Park, ODS, H4251, 10 x 250 mm) and 40% acetonitrile at a flow rate of 5 ml/minutes (Takahashi *et al*., 1995).

The present study illustrates several different attempts searching for the best way of sampling, extracting and then profiling production of thiomarinol from the producer micro-organism marine *Pseudoalteromonas* spp SANK 73390. Different protocols were implemented to monitor thiomarinol with regard to both the quality and the quantity of production.

## 2.2 Materials and Methods:-

### 2.2.1 Bacterial strains

Marine bacterium *Pseudoalteromonas* spp SANK 73390 was used as the wild type thiomarinol producer. *Bacillus subtilis* 1604 was used as the test organism in the bioassay to detect the antibacterial activity of thiomarinol.

### 2.2.2 Growth and culture conditions

*Pseudoalteromonas* spp SANK 73390 was grown at 23$^{o}$C in M-broth consisting of peptone (5.0 g/l), yeast extract (1.00 g/l), ferric citrate (0.10 g/l), sodium chloride (19.45 g/l), magnesium chloride (5.9 g/l), magnesium sulfate (3.24 g/l, calcium chloride (1.80 g/l), potassium chloride (0.55 g/l), sodium bicarbonate (0.16 g/l), potassium bromide (0.08 g/l),

strontium chloride (0.034 g/l), boric acid (0.022 g/l), sodium silicate (0.004 g/l), sodium fluoride (0.0024 g/l), ammonium nitrate (0.0016 g/l), and disodium phosphate (0.008 g/l), and M-agar (M-broth, except magnesium chloride with (8.8 g/l), sodium sulfate (3.24 g/l) instead of magnesium sulfate, and (15.0 g/l) agar). *Bacillus subtilis* 1604 was grown at 37$^{o}$C in L-broth medium consisting of yeast extract (10 g/l), tryptone (5 g/l), NaCL (10 g/l), and Glucose (1 g/l). All medium was sterilised by autoclaving (121$^{o}$C at a pressure of 15 pounds per square inch for 15 min).

### 2.2.3 Determination of the growth curve of *Pseudoalteromonas* spp SANK 73390

Seed cultures of *Pseudoalteromonas* spp SANK 73390 were prepared by inoculating 25 ml marine broth in 250 ml Erlenmeyer flask (Takahashi *et al*., 1995) with a single colony from the young culture of SANK 73390, followed by overnight incubation at 23$^{o}$C with shaking at 200 rpm. Triplicates of three separate 500 ml flasks each containing 100 ml marine broth were inoculated with the freshly prepared seed culture as follows respectively; 1 ml washed bacterial cells (100X), 1 ml (100X) and 2 ml (50X) without washing. The process of washing involves spinning down the culture, discarding the supernatant, and then re-suspending in 1 ml of fresh marine broth. Cultures were incubated at 23$^{o}$C with shaking at 200 rpm for up to 12 h, 21 h and 48 h separately at different times, and 1 ml samples were taken either every 3 h or every h for determining the optical density at 600 nm from time zero.

### 2.2.4 Optimal conditions for thiomarinol extraction

Samples of 800 µl or 2 ml from the broth cultures of SANK 73390 strain were used for the extraction of thiomarinol. To stabilise thiomarinol compounds the pH of the samples was adjusted to pH 6.0 (acidic condition). An equal volume (800 µl or 2 ml) of acetone was added to each sample and mixed well for 5 minutes using a vortex mixer, and then left in the fridge for at least 1 h to allow complete decomposition of bacterial cell wall. Evaporation of acetone was then carried out using a speed vacuum pump for 1 h, followed by the addition of an equal volume of ethyl acetate and mixed well for at least 5 min. At this stage the mixture of thiomarinol, ethyl acetate, and cell debris left for enough time (at least 30-60 min) to ensure that the whole mixture separated into two phases. The yellow compound (thiomarinol) moved to the upper solvent (ethyl acetate) phase, while cell debris settled in the bottom (water) phase. The upper phase of ethyl acetate and thiomarinol was transferred to a new 2 ml microfuge tube, while the bottom phase was poured off. The ethyl acetate was dried out completely by speed vac. pump to obtain dried thiomarinol. The dried out samples were then dissolved in either 80 µl or 200 µl of methanol in the case of preparing antibiotic discs or dimethyl sulfoxide (DMSO) for HPLC analysis.

### 2.2.5 Sample analysis by HPLC

HPLC analysis was performed to detect the presence of thiomarinol in samples prepared in the previous section from cultures of *Pseudoalteromonas* spp SANK 73390. Two solvents were used; solvent A (HPLC grade water); and solvent B (HPLC grade acetonitrile), and both were supplemented with 0.01% formic acid to adjust the pH of the mobile phase. Both solvents were degassed using an aspirator in order to completely release all the bubbles from the solvents before use. HPLC was performed using Uni-point LC system software, reverse

phase C18 column (15 cm x 4.6 mm), UV detector was used at a sensitivity of 0.002 absorbance units at 385 nm. 10 µl, 20 µl or 100 µl of each sample was injected into the HPLC machine. Thiomarinol and other intermediates were eluted using a mobile phase gradient (40% or 35%) of acetonitrile, and $H_2O$ (60% or 65%) at a flow rate of 1 ml $min^{-1}$.

### 2.2.6 Paper disc assay for thiomarinol production

Cultures of the indicator strain were prepared by inoculating 5 ml of L-broth with *Bacillus subtilis* 1604 and incubated overnight at 37$^{o}$C with shaking at 200 rpm. The bottom agar media was prepared the next day by pouring 15 ml of L-agar into a plastic petridish and left for 1 h to solidify. Then the upper soft-agar media was prepared by pouring 20 ml of melted L-agar and 20 ml of pre-warmed L-broth into a Falcon tube and mixed well. To this was added 0.4 ml of the indicator broth and the Falcon tube inverted to ensure complete mixing. 10 ml of the soft-agar mixture was poured onto the bottom agar media and left on the bench for at least 30 min to solidify. While waiting for the upper soft-agar to solidify with the bottom one, paper discs from the extracted samples of thiomarinol were prepared by first marking the paper discs with a pencil. The discs were placed on an aluminium surface and 40 µl of the samples in methanol dissolved were added onto the paper discs slowly and left for 10 min to dry out. Antibiotic discs were then placed onto the prepared antibacterial indicator plate and incubated overnight at 37$^{o}$C. The size of the clearance zone around the antibiotic discs was then taken as an estimate of the antibacterial activity of thiomarinol present in the antibiotic discs.

## 2.3 Results

### 2.3.1 The importance of washing bacterial inocula for cultures to profile antibiotic production

Preliminary studies on production of thiomarinol indicated that even 100-fold diluted cultures of SANK73390 gave a significant level of antibacterial activity by plate bioassay indicating either production of thiomarinol in low density cultures or significant carryover of antibiotic from the stationary phase cultures. It was therefore decided that bacteria from stationary phase cultures should be washed to remove existing antibiotic before inoculation of the fresh, dilute culture. Since bacterial cultures are not ready instantly to be used from agar plates, they need to be ensured that they are growing very well and are presented as young and fully active before using them for investigation. This could be done be transferring the cultures several times into another media (broth is preferable), which leads to increase in cell number and increase in cell population mass, in addition to ensuring the uniformity. Researchers normally follow bacterial growth as populations instead of following the growth of individual cells, and this is due to that the characteristics of individual cells usually is preferable to be studied in the context of populations of cells. Therefore, cultures with and without washing and with different densities of inoculation were investigated (section 2.2.3). From the growth curves with washed inocula diluted 100x, unwashed inocula diluted 100x and 50x (Figure 2.1 A, B and C) incubated for 48 h, 21 h and 12 h, respectively, it can be seen that the logarithmic phase occurred between 3-9 h of incubation followed by a stationary phase until the end of the incubation period (48 hr). Washing the bacterial cell inocula prior of the inoculation had no negative effect on the growth profile. When the growth curved of washed cells was repeated in triplicate it can be seen that the growth profile is highly

reproducible (Figure 2.2). Also it is of significance that the growth curves were repeated several times and the cell densities measured either every hour or every 3 h intervals.

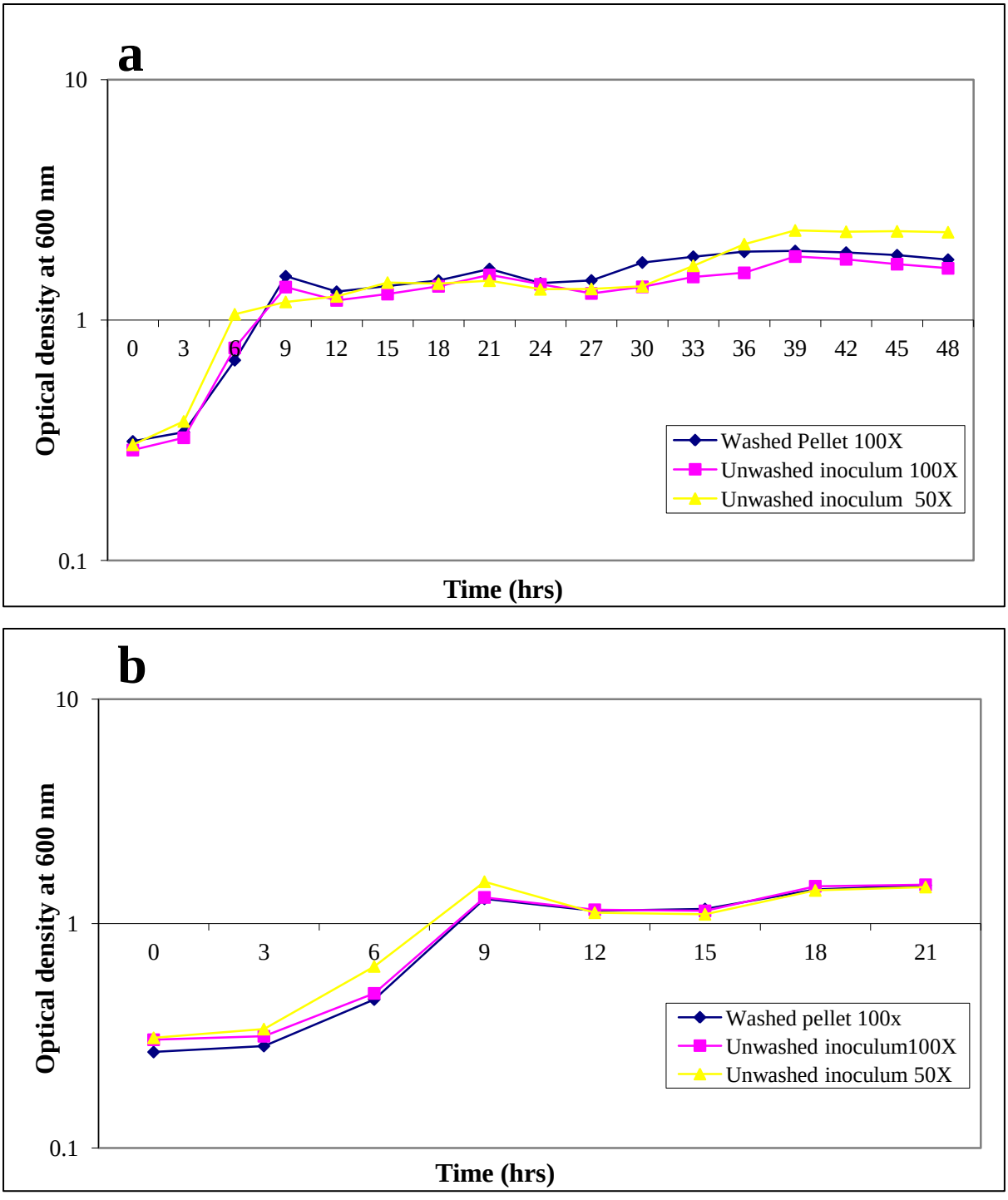
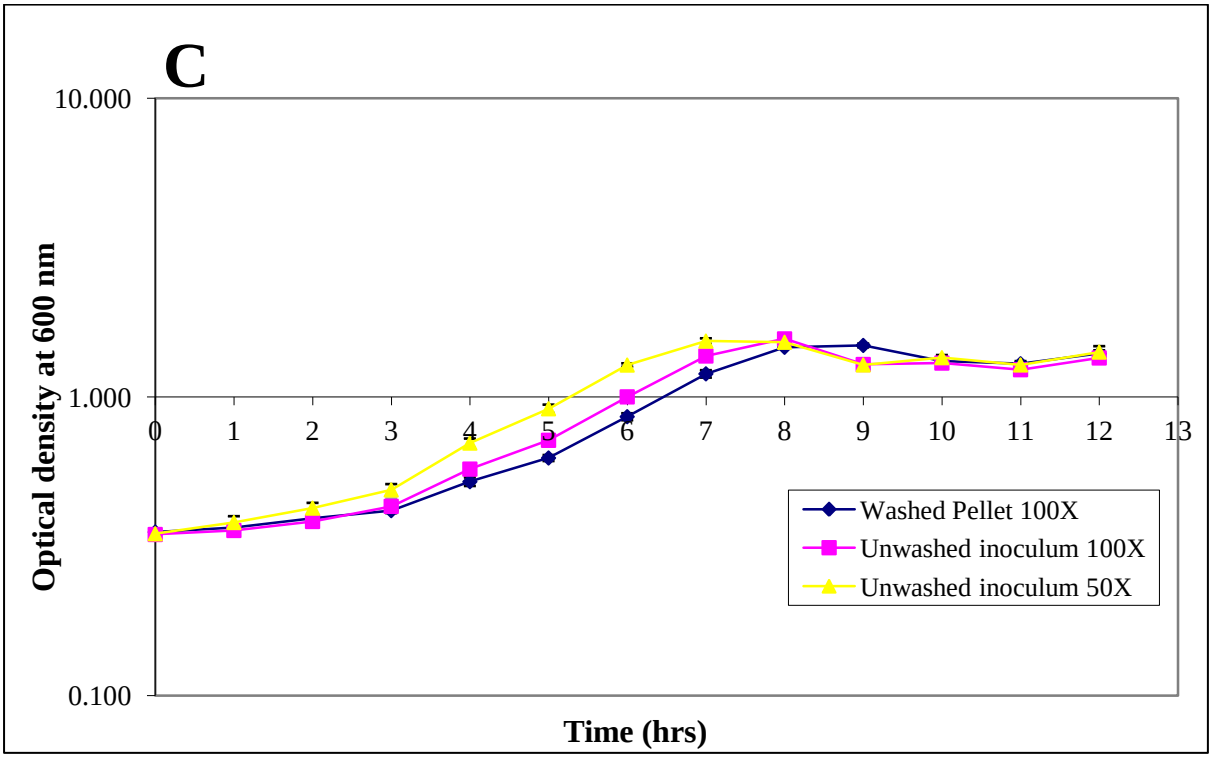



**Figure 2.1 Growth curve of *Pseudoalteromonas* spp. SANK 73390.** Growth checked every 3 hrs over 48 hour (a), over 21 hr (b) and every hour for 12 hr (c). Blue line (washed pellet 100X); Pink line (Unwashed inoculum 100X); and Yellow line (Unwashed inoculum 50X).

**(Figure 2.1 continued)**



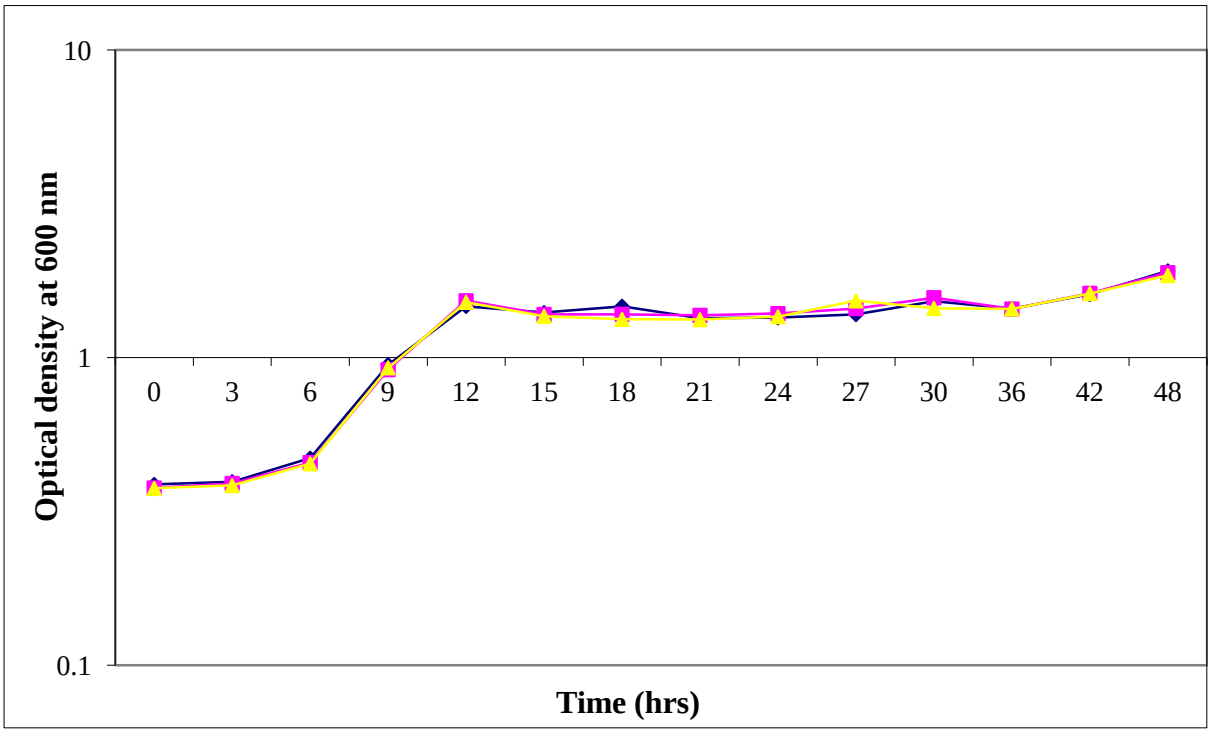




**Figure 2.2 Growth curve of *Pseudoalteromonas* sp. SANK 73390 using washed bacterial pellet as inocula with triplicate cultures.**

### 2.3.2 Efficient solvent for concentrating thiomarinol, sample volume and optimal concentration of acetonitrile for thiomarinol detection

The extracted samples were primarily dissolved in 40 µl or 80 µl of methanol, and 10 µl or 20 µl subjected to HPLC analysis for 1 h or 25 min using 40% acetonitrile and formic acid (0.01%), with $H_2O$ 60% and formic acid (0.01%), at a flow rate of 1 ml $min^{-1}$. HPLC chromatograms showed that the thiomarinol peak could not be separated from the other unknown peaks due to the hydrophobic property of thiomarinol. Also, it is expected that the unknown peaks might possibly belong to some other materials that had been adsorbed and detected by the HPLC. However, when the extracted samples were dissolved in 200 µl of DMSO, and 100 µl used for HPLC analysis for 25 min with solvent concentrations 35% acetonitrile and formic acid (0.01%), with $H_2O$ 65% and formic acid (0.01%), at a flow rate of 1 ml $min^{-1}$, typical and separated thiomarinol peak was observed as a result of change in the retention time.

### 2.3.3 Disc bioassay does not correlate completely with the amount of thiomarinol produced in comparison with that of HPLC analysis

Paper disc bioassay were performed to investigate the presence of antibiotic (thiomarinol) in the extracts obtained from using cultures with and without washing and with different densities of inoculation over 48 h of incubation at 23$^{o}$C with shaking at 200 rpm. The results of bioassay (Figure 2.3) did show the presence of thiomarinol in the paper discs prepared from extracts after 9 h incubation and onward from cultures with unwashed inoculation. However, there was also a sign of slight inhibition zones even with paper discs prepared from extracts at time zero and after 3, and 6 h of incubation, but there was not enough to give a

clear zone of inhibition, remaining cloudy and rather indeterminate. However, the presence of thiomarinol in paper discs prepared from extracts of culture with washed inoculation after 12 h and up to 48 h incubation could be determined, and there was no observable sign of antibiotic around paper discs prepared from samples extracted at other times (0, 3, 6, and 9 h) of incubation. The size of the clearance zone around the antibiotic discs was then taken as an estimate of the amount of thiomarinol present in the antibiotic discs.

Although the profile of thiomarinol production with incubation time up to 48 h using the disc bioassay showed reasonable similarity to the amount of thiomarinol determined with that of HPLC analysis, it did not correlate completely.

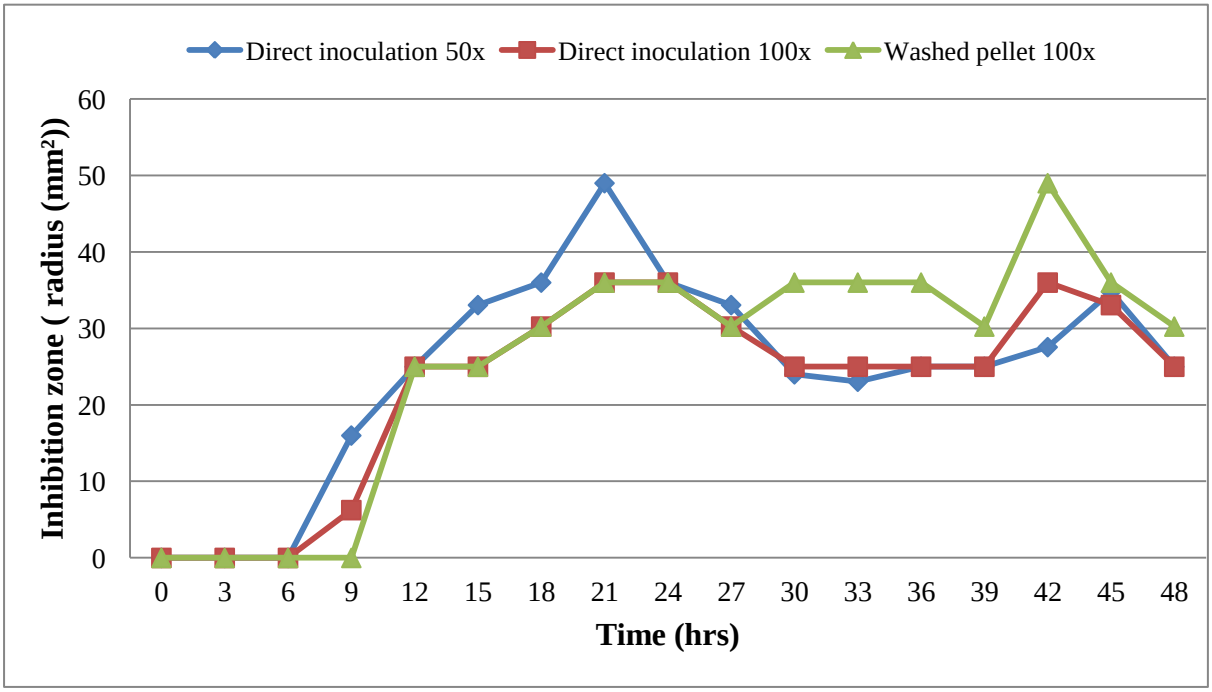



**Figure 2.3 Disc bioassay activity of thiomarinol produced by SANK 73390.**

### 2.3.4 Addition of HCl improved thiomarinol extraction

In order to investigate the effect of acidic condition on thiomarinol extraction, samples in triplicates with two different volumes (800 µl and 2 ml) were used for the extraction of thiomarinol. Samples were then separated into two groups. HCl was added to the first group to adjust the pH to the acidic condition, while the second group was left without HCl addition. Thiomarinol was extracted following the procedure described in section 2.2.4, and the extracted samples were then subjected to HPLC analysis (section 2.2.5). HPLC chromatograms significantly showed the importance of pH adjustment of the samples for thiomarinol extraction, as the yield increased two fold compared to the samples without pH adjustment (Table 2.1 and Figure 2.4). In addition, the yield was significantly higher in samples of 2 ml than 0.8 ml. This result indicates the role of pH adjustment of samples to the acidic condition in improving the yield of thiomarinol which might be due to the role of HCl (acidic pH) in stabilising thiomarinol compounds while using acetone in combination with ethyl acetate for thiomarinol extraction.

**Table 2.1 Effect of pH adjustment on the yield of thiomarinol.**

| **Sample description** | **Concentration µg/ml** | |
|---|---|---|
| | **Sample volume** | |
| | 800 µl | 2 ml |
| + HCl | 4.85 ± 0.44 µg/ml | 4.91 ± 0.59 µg/ml |
| -HCl | 1.66 ± 0.29 µg/ml | 1.63 ± 0.09 µg/ml |

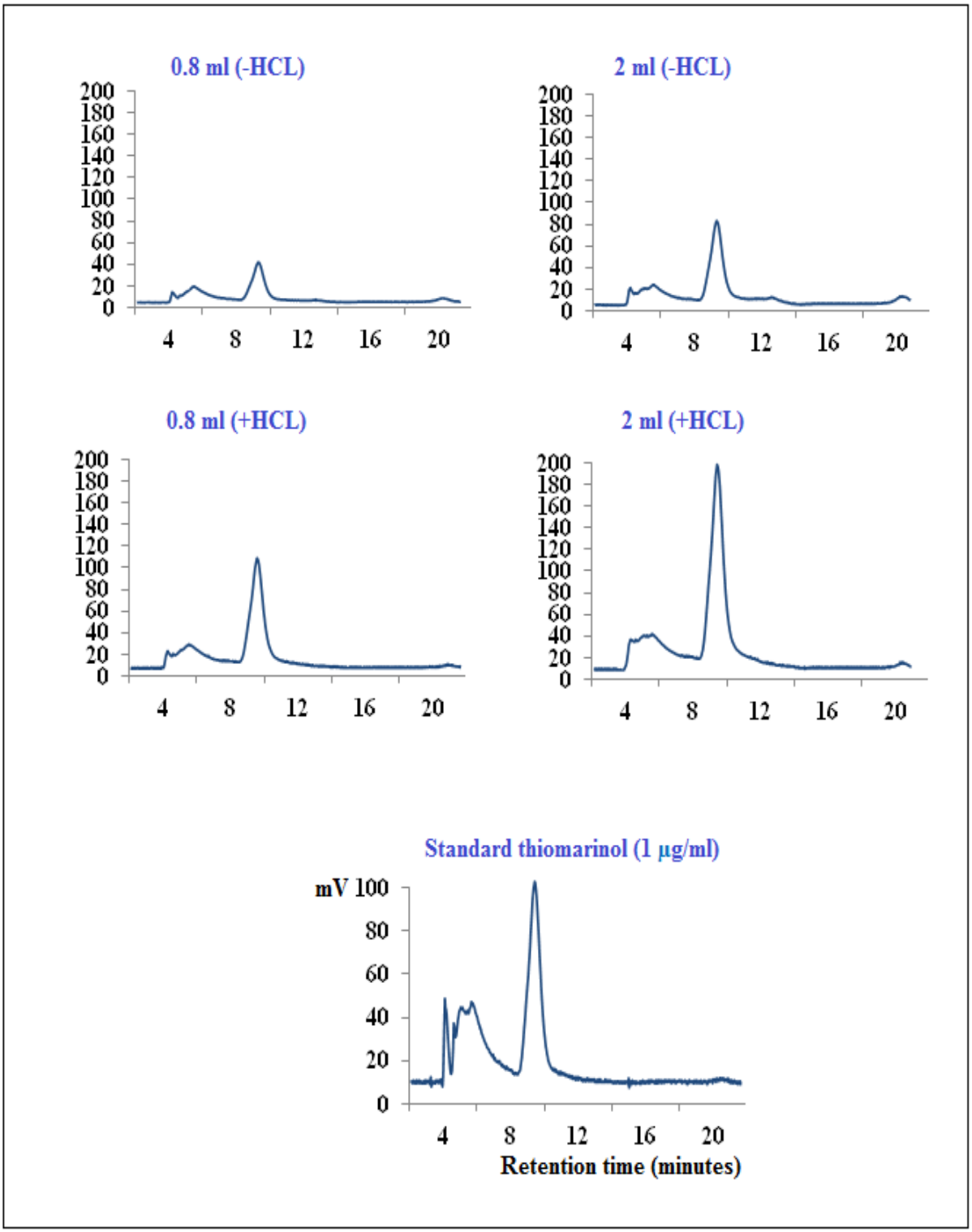



**Figure 2.4 HPLC chromatogram at 385 nm of thiomarinol from *Pseudoalteromonas* spp SANK 73390 showing the effect of HCl addition on thiomarinol extraction in different volumes of samples.**

### 2.3.5 In-efficiency of ethyl acetate alone to extract thiomarinol attached to the bacterial cells unless used with acetone

Samples of 800 µl and 2 ml were used for extraction of thiomarinol by using ethyl acetate alone. Extraction was carried out separately in total sample, supernatant and bacterial cell pellet. The pellet was re-suspended in fresh marine broth (same volume of the total sample), and then HCl was added to each part separately to adjust the pH to acidic conditions. Then thiomarinol extraction was carried out using ethyl acetate as well as ethyl acetate with acetone (section 2.2.4). The amount of thiomarinol extracted using ethyl acetate alone from each part of the broth culture of SANK 73390 was measured separately by HPLC chromatogram (Table 2.2 and Figure 2.5). These data showed that some thiomarinol remains attached to the pellet when using ethyl acetate alone for the extraction. However, when acetone was used with ethyl acetate for the extraction, all thiomarinol could be determined, illustrating the critical role of acetone in decomposing the bacterial cell pellet so that extraction detected as much thiomarinol as possible, although the difference is not highly significant which mostly due to changes while the baseline of the peaks was adjusted. (Table 2.3 and Figure 2.6).

**Table 2.2 Extraction of thiomarinol using ethyl acetate alone.**

| Sample description | Concentration µg/ml | |
|---|---|---|
| | Sample volume | |
| | 800 µl | 2 ml |
| Total culture | 7.59 | 7.82 |
| Bacterial cells pellet | 1.01 | 0.61 |
| Supernatant | 4.10 | 4.98 |

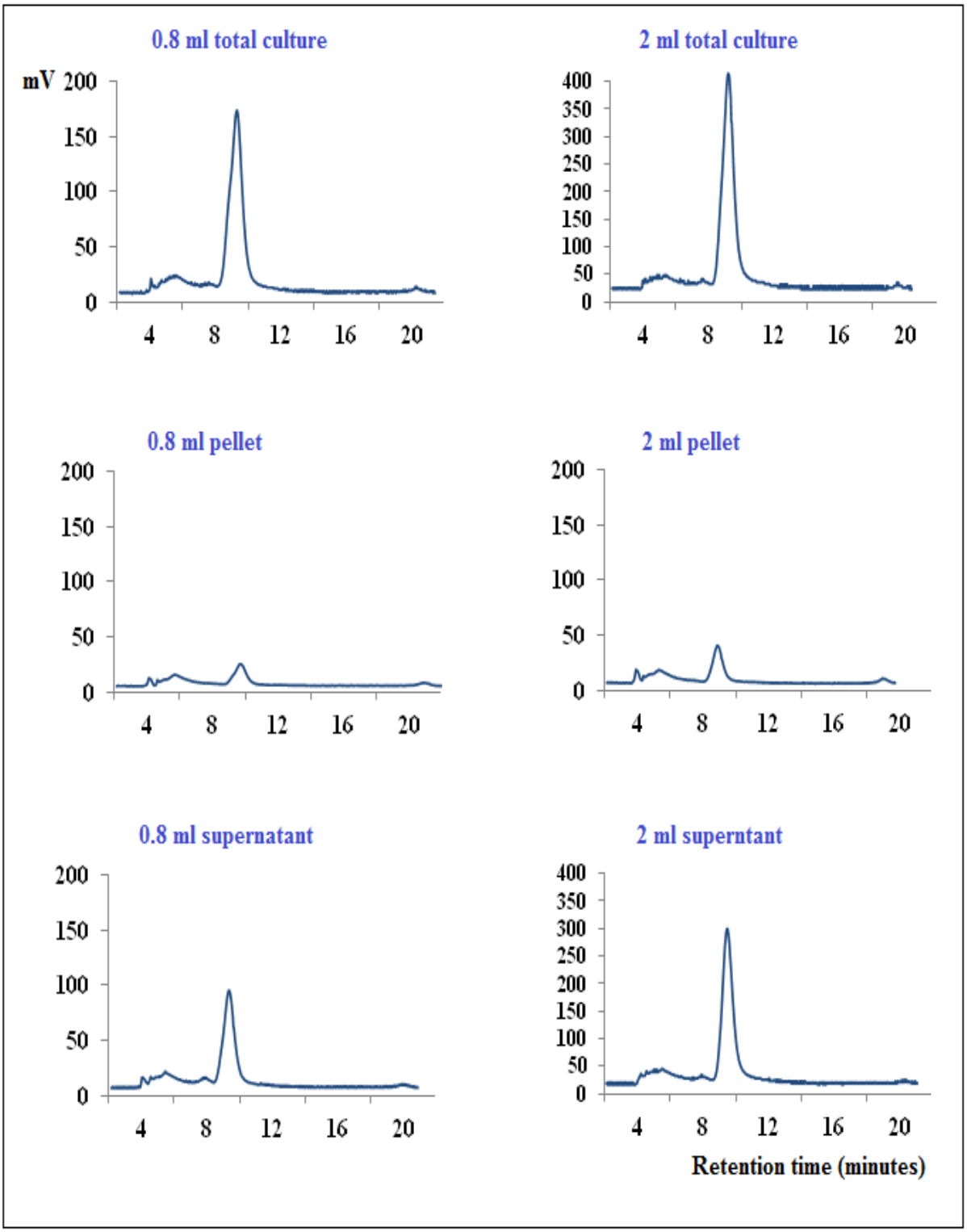



**Figure 2.5 HPLC analysis at 385 nm of thiomarinol extracted from *Pseudoalteromonas* spp SANK 73390 in different volumes (0.8 ml and 2 ml) and using solely ethyl acetate.**

**Table 2.3 Effect of using acetone in combination with ethyl acetate for thiomarinol extraction.**

| Sample volume | Concentration µg/ml | |
|---|---|---|
| | Acetone + ethyl acetate | Ethyl acetate |
| 800 µl | 5.95 ± 0.12 µg/ml | 5.86 ± 1.17 µg/ml |

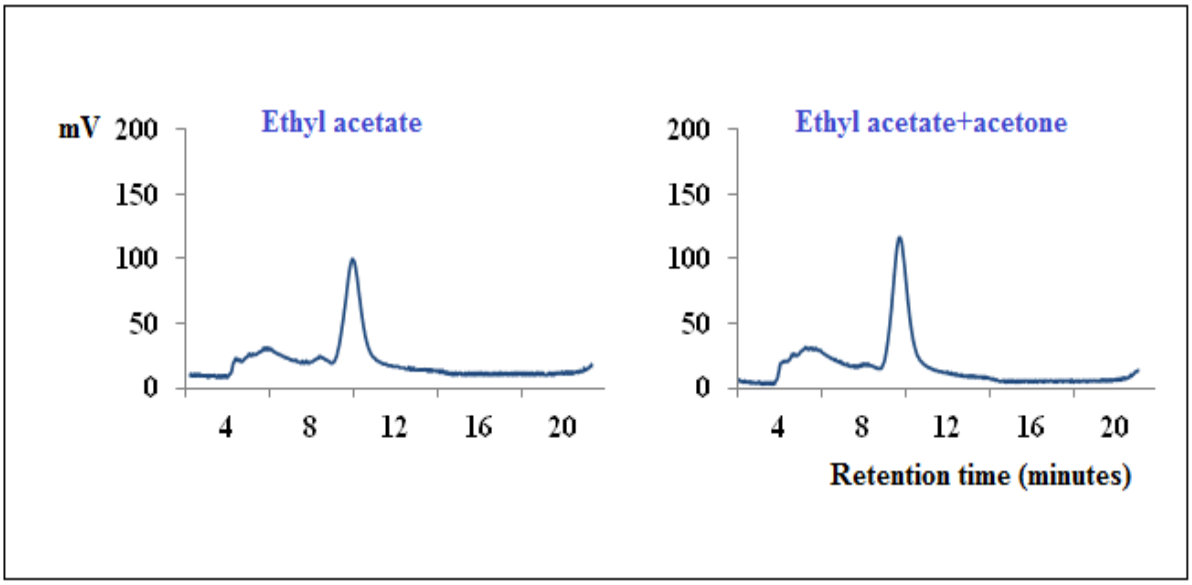



**Figure 2.6 HPLC analysis at 385 nm of thiomarinol extracted from *Pseudoalteromonas* spp SANK 73390 in 0.8 ml of culture samples using ethyl acetate alone and in combination with acetone.**

### 2.3.6 Best sample volume for extraction of thiomarinol at early stages of the growth (0, 6, 12 and 18) h.

Since we had demonstrated that acetone with ethyl acetate improved thiomarinol extraction, therefore, we wanted to determine whether 800 µl or 2 ml was better for sampling from the broth cultures of SANK 73390 at early stages of the growth (0, 6, 12, and 18 h). Samples of 800 µl and 2 ml were used from cultures at the growth times specified, and thiomarinol extracted by adjusting samples pH to acidic condition and following the acetone-ethyl acetate protocol. HPLC chromatograms revealed the usefulness of both 800 µl and 2 ml for sampling at early stages since both showed no detectable thiomarinol in the samples collected after 6 h of the growth of SANK 73390, while thiomarinol was observed in the samples of both volumes after 12 h (Table 2.4 and Figure 2.7).

**Table 2.4 Best sample volume for thiomarinol extraction.**

| Sampling time (hr.) | Concentration µg/ml | |
|---|---|---|
| | Sample volume | |
| | 800 µl | 2 ml |
| 0 | No tml | No tml |
| 6 | No tml | No tml |
| 12 | 2.05 | 1.92 ± 0.08 µg/ml |
| 18 | 6.32 | 6.38 ± 0.84 µg/ml |

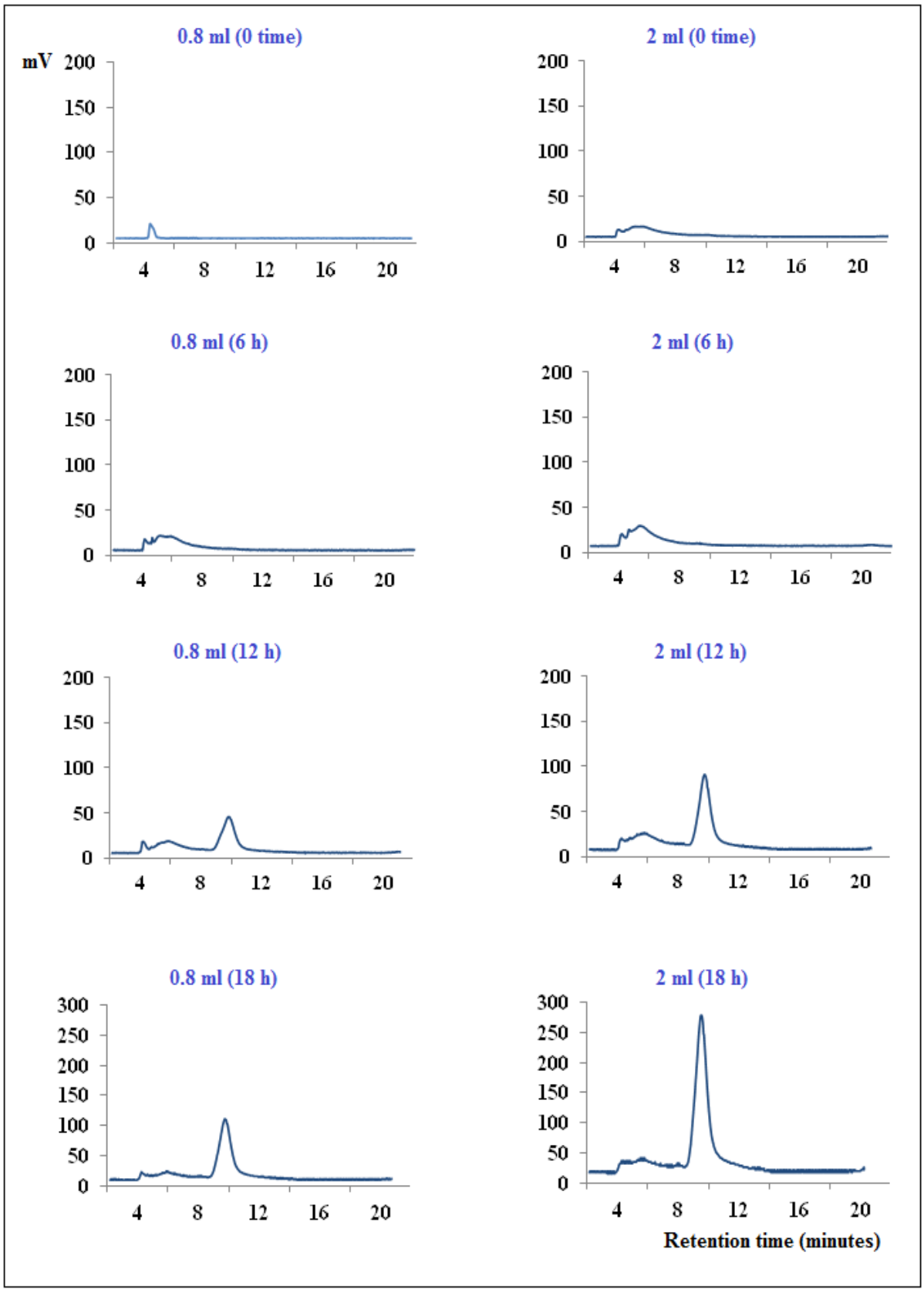



**Figure 2.7 HPLC analysis at 385 nm of thiomarinol extracted from 0.8 ml and 2 ml of samples of *Pseudoalteromonas* spp SANK 73390 at early stages of the growth using ethyl acetate and acetone**.

### 2.3.7 Time course of thiomarinol production

For the purpose of analysing the production profile of thiomarinol by HPLC over a long period of the growth incubation time, triplicate cultures with washed inocula of SANK 73390 were incubated for 48 h at 23$^{o}$C with shaking at 200 rpm. Sampling commenced from 0 time up to 48 hr of incubation. 2 ml samples were taken out from broth cultures every 3 h up to 30 h, followed by sampling every 6 h. The acetone-ethyl acetate protocol was followed for the extraction of thiomarinol (Materials and Methods section 2.2.4). The extracted thiomarinol dissolved in 200 µl DMSO and 100 µl used for HPLC analysis for 25 min with solvent concentrations 35% acetonitrile and formic acid (0.01%), with $H_2O$ 65% and formic acid (0.01%), at a flow rate of 1 ml $min^{-1}$. HPLC chromatograms (Figure 2.8) showed a typical peak of thiomarinol in samples of 12 h and following samples, but not in the samples before 12 h of incubation (0, 3, 6 and 9 h). To identify precisely the starting point of thiomarinol production, all the steps mentioned from culturing to HPLC analysis were repeated twice with duplicates, first for samples at 7, 8, 9 and 10 h of incubation, and then for samples at 9, 10, 11 and 12 h of incubation. The HPLC analysis confirmed that thiomarinol production started after 9 h of incubation. Then the concentration of thiomarinol in all the samples with thiomarinol peak was estimated and the concentration plotted against the incubation time (Figure 2.9). Results indicated that production of thiomarinol increased quickly after the starting point (9 h) until 12 h, then increased slightly to reach a maximum production at 18 h within the first 24 h of incubation. Production of thiomarinol then fluctuated and decreased at 27 h but then dramatically increased to 36 h as the maximum point of thiomarinol production, followed by a slight reduction until the last point of incubation (48 h).

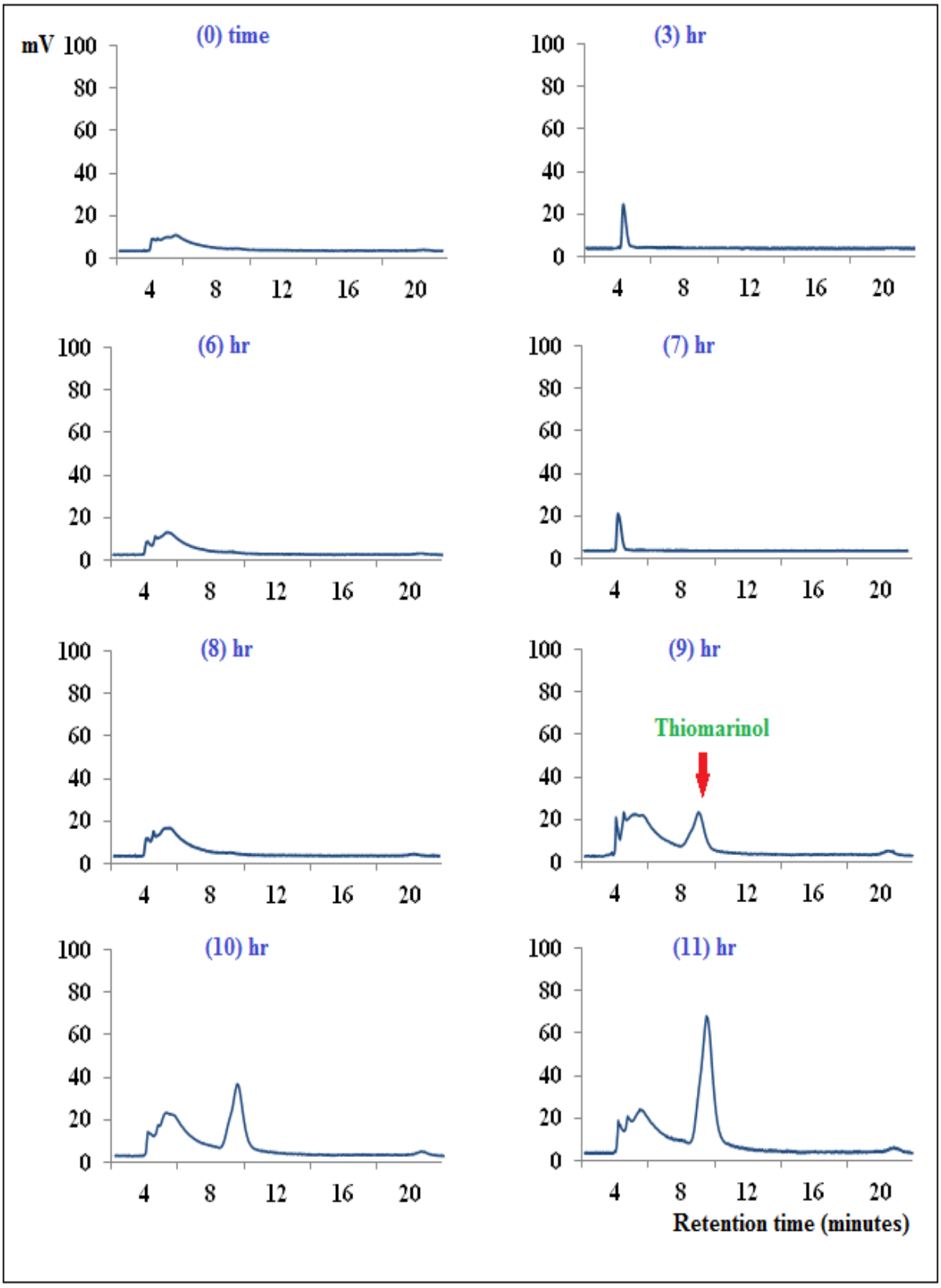
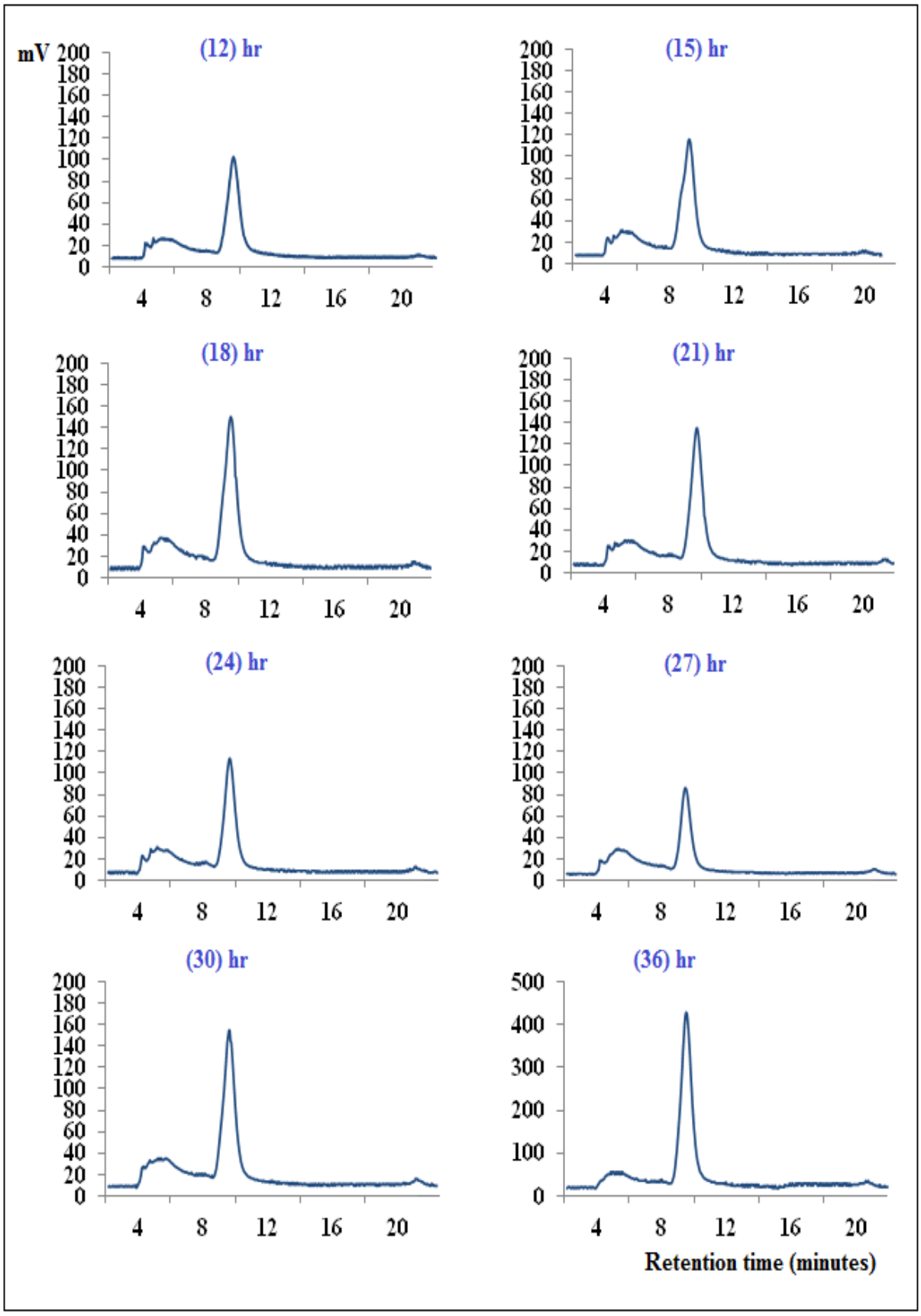
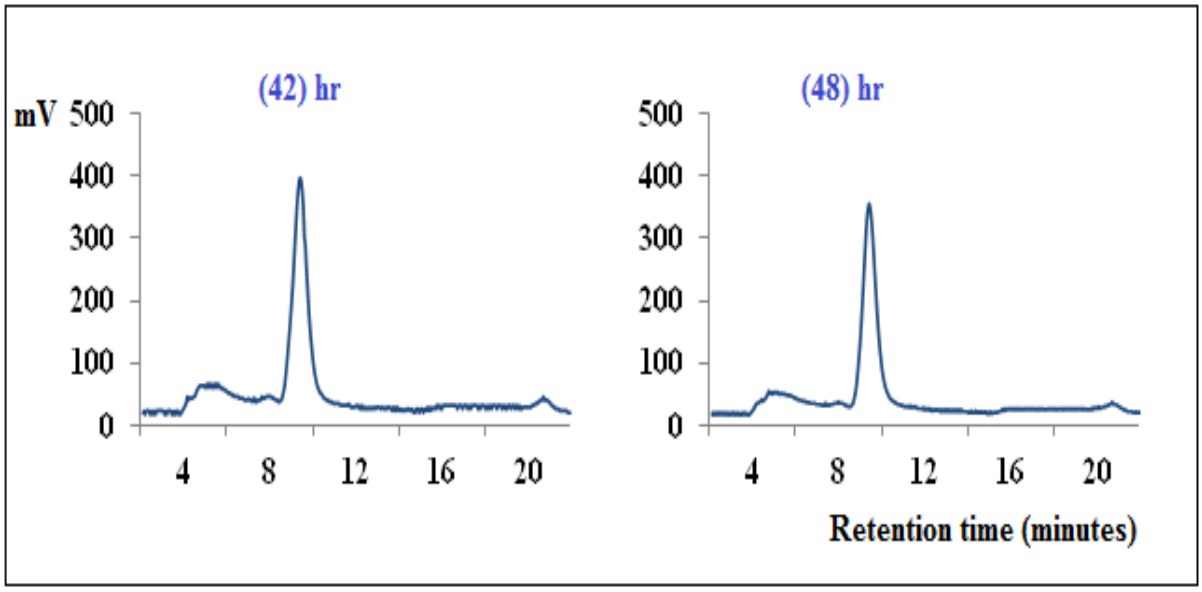



**Figure 2.8 Time course of thiomarinol production from *Pseudoalteromonas* spp SANK 73390 analysed by HPLC at 385 nm on 2 ml samples at different times of the growth up to 48 h using triplicate cultures, and using acetone-ethyl acetate for the extraction.**

**(Figure 2.9 continued)**



**(Figure 2.9 continued)**



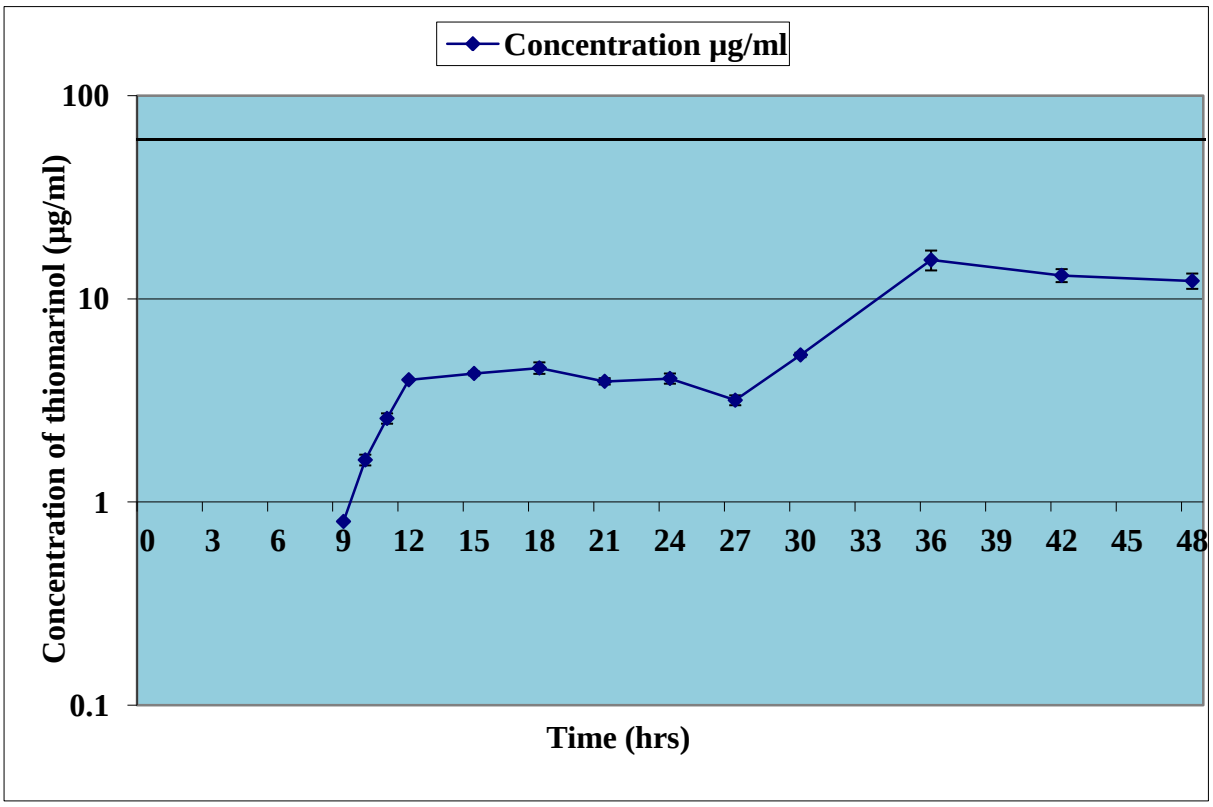




**Figure 2.9 Time course production of thiomarinol.**

## 2.4 Discussion

Thiomarinol has a strong and broad spectrum antibacterial activity, and more specifically against MRSA. Thus over the last decade there has been research into the production and extraction of thiomarinol for use as a new interesting antibiotic. However, these studies did not address the critical role of HCl, particularly in improving the extraction process or a specific role of each solvent in the protocol. In addition, there is no indication about the timing of thiomarinol expression. In this study the onset and maximum point of thiomarinol production has been determined relative to the growth phases of the producer *Pseudoalteromonas* spp SANK 73390, while optimising the parameters of metabolite extraction and HPLC analysis.

Typically, secondary metabolites and more specifically antibiotics are expected to be produced during stationary phase, indicating a strong association between growth and antibiotic production. We therefore initially determined the growth phases of *Pseudoalteromonas* spp SANK 73390 to help us determine at which time point we might expect to start detecting thiomarinol production. We found that under our standard conditions the logarithmic phase occurred between 3-9 h of the growth time, followed by a stationary phase which lasts for the rest of the incubation time. Although the pH of the culture broth of SANK 73390 was adjusted to acidic pH with HCl by Shiozawa *et al*., (1993) during the isolation of thiomarinol, they have not stated clearly the reason, or the usefulness of the adjustment. It might have been used to stop the fermentation, or to start the extraction, since it is preferable to extract the thiomarinol (i.e thiomarinol B and/ or C) that are existing in the filtrate or in the supernatant with water-immiscible organic solvent including ethyl acetate under neutral or acidic conditions (Takahashi *et al*., 1995). However, HPLC analysis showed a significant improvement in thiomarinol extraction after adjusting pH of the samples to the

acidic condition, which might be due to the role of acidic condition in stabilising thiomarinol compounds. We have also showed the inability of ethyl acetate alone to extract all the thiomarinol present in the culture, since our data confirmed that some thiomarinol is still trapped intracellularly or attached to the bacterial pellet after extraction. Therefore, to overcome this problem, acetone was used in combination with ethyl acetate for the purpose of releasing and extracting the trapped thiomarinol as well. As a consequence, there was a small increase in the yield of thiomarinol. These findings extend those of Shiozawa *et al*., (1993), and are in good agreement with those of Takahashi *et al*., (1995), that aqueous acetone or methanol with a concentration of 50-90% may purify intracellular thiomarinol B and/or C. More recently acetone was effectively used for the extraction of antifungal metabolite KB425796-A from the fermentation broth of *Paenibacillus* sp. 530603 (Kai *et al*., 2013).

Compounds elute at different time points when analysed by HPLC according to their chemical properties such as whether hydrophilic or hydrophobic, and the condition of both of the mobile phase and the stationary phase (column). Detection of thiomarinol using 40% acetonitrile was not successful to separate thiomarinol peak from other unknown compound peaks. Therefore, and since thiomarinol appears to be hydrophobic, when the concentration of acetonitrile was changed in the mobile phase to 35%, thiomarinol peak was eluted and separated from the other unknown peaks. Moreover, we found that 100 µl of samples as a sufficient amount to be analysed accurately by HPLC, which gave a typical thiomarinol peak. The dried extracts were first dissolved in methanol, since as a crystal solvent methanol forms two hydrogen bonds to thiomarinol (especially TH-B) while recrystallizing with methanol-$H_2O$ solution, which play a pronounced role in stabilizing the crystal structure of the antibiotic (Shiozawa *et al*., 1995). However, as methanol evaporates quickly, and so consequently affected the sample volumes in uncapped tubes prepared for HPLC analysis,

DMSO was used as alternative solvent. When the best method of extraction was established, it appeared that culture samples of 800 µl is sufficient for the extraction and quantification of thiomarinol even at early stages of the growth. We monitored the amount of thiomarinol produced by batch culture of *Pseudoalteromonas* spp SANK 73390 over 48 hours using HPLC. Thiomarinol production was detected from the $9^{th}$ hour of incubation and was maximal from 18 to 36 hours and it then dropped after the 36 hour. These observations tend to be in agreement with Takahashi *et al*., (1995), that the amount of thiomarinol reaches maximum level after 19 hour of incubation and onward when they monitored production of thiomarinol B and C. However, they did not specify the initial point of the production. If we compare the timely expression of thiomarinol relative to the growth of the producer, we can see that SANK 73390 grew firstly up to the formation of a considerable amount of growth (population density), and then followed by the formation of thiomarinol. Therefore, these results are consistent with those observed by Pirt and Righelato, (1967), and Lurie *et al*., (1975), as they reported the observation of two phases during the propagation of antibiotic producers. The ideal characteristic of the first phase (trophophase) is the rapid growth of the producer to reach a population density (biomass production), while a slow growth and maximal productivity of the antibiotics is the characteristic of the second phase (idiophase). However, although the amount of thiomarinol produced by disc bioassay did show some degree of similarity with that of HPLC analysis they were not correlated completely.

Generally, it could be concluded that this work has demonstrated that 3 to 9 h is the time of the logarithmic phase of *Pseudoalteromonas* spp SANK 73390, and that 9 h (late exponential phase) is the initial point of thiomarinol production, which is an important indicator of the regulated expression of thiomarinol gene cluster. Therefore, future work should include follow-up work designed to quantify level of gene expression by using the available techniques such as dot/slot blotting or possibly by Northern blotting. We have also shown

that acidic conditions and using acetone with ethyl acetate improves thiomarinol extraction, while dissolving the extracted thiomarinol in methanol for disc bioassay is preferable. Injection of 100 µl of the extracted thiomarinol into HPLC is better and using a solvent gradient of 35% acetonitrile and 65% water separates a typical peak of thiomarinol.

# CHAPTER THREE

## INVESTIGATING THE INHIBITORY EFFECT OF THE THIOMARINOL AMIDE SYNTHETASE, TmlU, ON 9HN ELONGATION IN MUPIROCIN SYNTHESIS

## 3.1 Introduction

Much research in recent years has focused on the growing family of ATP-dependent amide synthetases involved in antibiotic biosynthesis, due to their potentially useful role in combinatorial biosynthesis of new analogues. One of the best–known group of antibiotics are the aminocoumarins (Figure 3.1), produced by several different *Streptomyces* strains, which include novobiocin, clorobiocin and coumermycin $A_1$ as three classical aminocoumarins, in addition to the structurally more complex simocyclinones and rubradirins (Heide, 2009). The amino group of the 3-amino-4,7-dihydroxyaminocoumarin moiety of all aminocoumarin antibiotics is combined with an acyl moiety *via* an amide bond (Figure 3.1). Biochemical investigations revealed the catalytic enzymes responsible for the formation of this amide bond in the novobiocin, clorobiocin, coumermycin and simocyclinone biosynthesis clusters which are named NovL (Steffensky *et al*., 2000), CloL (Galm *et al*., 2004), CouL (Schmutz *et al*., 2003; Caren *et al*., 2004), and SimL (Luft *et al*., 2005; Pacholec *et al*., 2005), respectively. Although there is just one amide synthetase gene (*couL*) in the biosynthetic gene cluster of coumermycin $A_1$ as revealed by sequence analysis (Wang *et al*., 2000), two aminocoumarin moieties have been determined to be connected to the acyl component 3-methyl-pyrrole-2,4-dicarboxylic acid via amide bonds (Schmutz *et al*., 2003).

In the thiomarinol biosynthetic cluster, *tmlU* has been identified as encoding a putative amide synthetase required for the attachment of the pyrrothine moiety to an 8-hydroxyoctanoic acid side chain in the mupirocin-like component, designated marinolic acid on the basis of its similarity with the more generic name of mupirocin, pseudomonic acid (Figure 3.2) (Fukuda *et al*., 2011). It was named TmlU on the basis of weak amino acid similarity to MupU, the acyl CoA synthase in the *mup* cluster proposed to be responsible for

loading an intermediate in the pathway, PA-B, to mAcpE, where it is further processed by MupO, V, C and F for the production of PA-A (Hothersall *et al*., 2007).

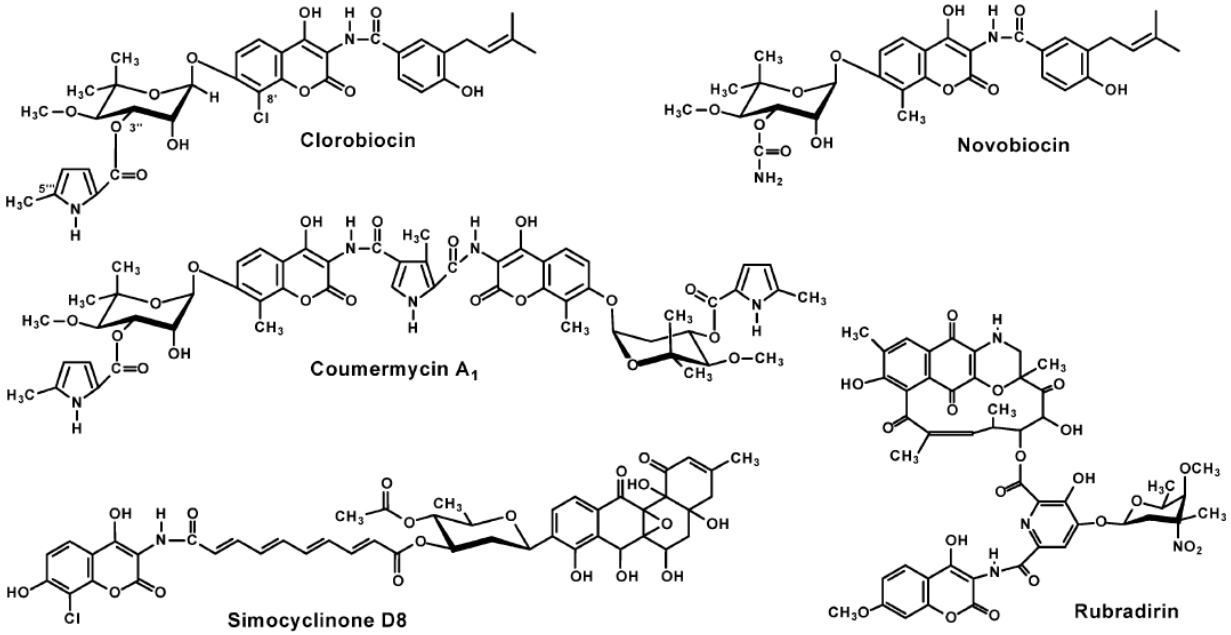



**Figure 3.1 Structures of the aminocoumarin antibiotics clorobiocin, novobiocin, coumermycin $A_1$, simocyclinone D8 and rubradirin (adapted from Heide, 2009).**

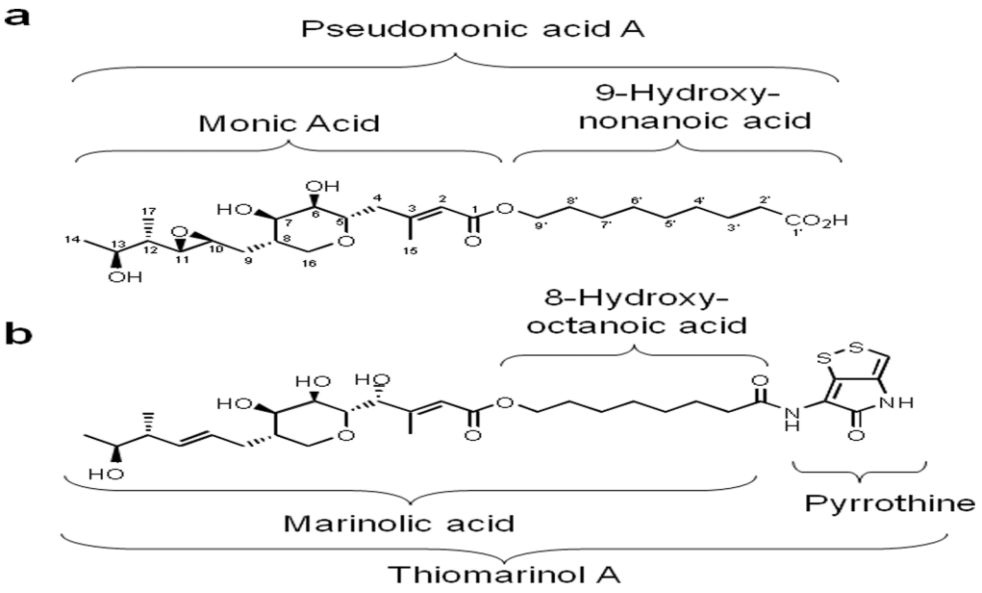



**Figure 3.2 Chemical structures of: (a) pseudomonic acid A (mupirocin), and (b) thiomarinol A. Holomycin is the amide *N*-acetylpyrrothine. In addition to thiomarinol, producer bacteria generate a variety of fatty acyl pyrrothine analogues (adapted from Fukuda *et al*., 2011).**

Incorporation of synthetic analogues of the acyl moieties into mutants of aminocoumarin antibiotic producers, defective in production of the acyl component, depends upon their acceptance by the producers amide synthetases, CloL, NovL, and CouL, respectively (Galm *et al*., 2004). The yields from such experiments with the clorobiocin system through feeding synthetic analogue of different acyl moieties in vivo has been confirmed to depend directly upon the *in vitro* conversion rates of the acyl moieties by the amide synthetase, as well as substrate specificity of the amide synthetase (Galm *et al*., 2004; Luft *et al*., 2005). Two novel pyrrothine amides (Figure 3.3) have been gained through feeding pseudomonic acid A to the Δ *tmpD* mutant of thiomarinol producer *Pseuoalteromonas sp* SANK 73390 (Fukuda *et al*., 2011). The possibility of creation of new families of hybrid antibiotics increased after successful incorporation of a range of alternative substrates such as different pseudomonic acid derivatives produced by *Pseudomonas fluorescens* or amines, such as anhydroornithine and anhydrolysine into mutants defective in *tmpD* and *holA* respectively (Murphy *et al*., 2011). However, although it is obvious that TmlU is responsible for the formation of the amide bond between marinolic acid and pyrrothine, and we know very little about its substrate specificity (Murphy *et al*., 2011), little attention has been paid to the possibility of it having additional roles.

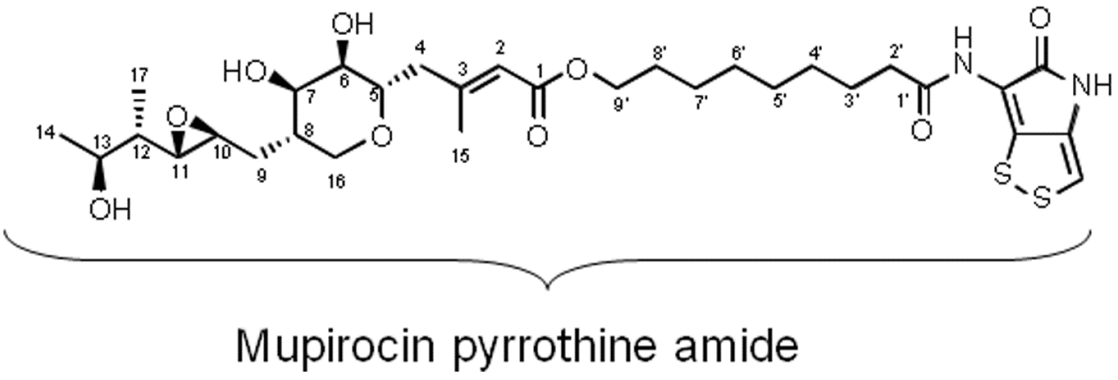



**Figure 3.3 Mupirocin pyrrothine amide, a new hybrid created consisting of pseudomonic acid A joined with pyrrothine via an amide bond (source: Fukuda *et al*., 2011).**

The purpose of this study is to characterise the controlling role of TmlU in linking marinolic acid and pyrrothine, and to determine whether TmlU can complement NCIMB10586Δ*mupU*. Truncated products released when TmlU was expressed *in trans* in the NCIMB10586 WT and different mutants including NCIMB10586Δ*mupU* indicated that TmlU cannot complement NCIMB10586Δ*mupU*, and significantly interferes with normal mupirocin biosynthesis.

## 3.2 Materials and methods

### 3.2.1 Bacterial strains and plasmids

Competent DH5α, an *Escherichia coli* strain was used for plasmid transformation and propagation. Young culture of *Pseudomonas fluorescens* NCIMB10586 was used as a standard wild type mupirocin-producing bacterium. *E. coli* strain S17-1 was used to mobilise expression vectors into NCIMB10586 WT and mutant strains by bacterial conjugation. The mupirocin-sensitive organism *Bacillus subtilis* 1064 was used in biological assays to detect the antibacterial activity of mupirocin. Bacterial strains and plasmids used in this study are listed in Table 3.1, and 3.3, respectively.

### 3.2.2 Growth media and culture conditions

L-broth consisting of yeast extract (10 g/l), tryptone (5 g/l), NaCl (10 g/l), and glucose (1 g/l) or L-agar (L-broth with 15 g/l agar) supplemented with either 100 μg/ml ampicillin or 15 μg/ml tetracycline (Table 3.2) was used as culture media for the growth of NCIMB10586 WT and mutants with or without the presence of vectors. Same media used for the growth of

*Escherichia coli* strains, either supplemented with 15 µg/ml tetracycline or without. All medium was sterilised by autoclaving (121$^{o}$C for 15 min under pressure of 15 bar) before adding antibiotics or IPTG. *Pseudomonas fluorescens* strains were grown at 30$^{o}$C, while *Escherichia coli* strains were grown at 37$^{o}$C.

**Table 3.1 Bacterial strains used during this study.**

| Bacterial strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli* DH5α | F⁻ *Φ80dlacZΔM15, recA1, endA1, gyrA86, thi-1, HsdR17(r$_{k}^{-}$, m$_{k}^{+}$), supE44, relA1, deoR, Δ(lacZYA-ArgF)U169* | Hanahan, 1983 |
| *Pseudomonas fluorescens* NCIMB10586 | Wild type (WT) mupirocin producer | Whatling *et al*., 1995 |
| *Escherichia coli* S17-1 | *RecA pro hsdR RP4-2-Tc::Mu-km::Tn7* | Simon *et al*., 1983 |
| *Bacillus subtilis* 1604 | *Trp*C2 | Moir *et al*., 1979 |
| NCIMB10586 Δ*mupU* | *P. fluorescens* with deletion of *mupU* gene | Cooper *et al*., 2005a |
| NCIMB10586 Δ*mupX* | *P. fluorescens* with deletion of *mupX* gene | Cooper *et al*., 2005a |
| NCIMB10586 Δ*TE* | *P. fluorescens* with deletion of *TE* gene | El-Sayed *et al*, 2003 |
| NCIMB10586 Δ*ACP5* (ACP-Ba) (*mmpB*) | *P. fluorescens* with *ACP5* (*mmpB*) deletion | Rahman *et al*., 2005 |
| NCIMB10586 Δ*ACP6* (ACP-Bb) (*mmpB*) | *P. fluorescens* with *ACP6* (*mmpB*) deletion | Rahman *et al*., 2005 |
| NCIMB10586 Δ*ACP7* (ACP-Bc) (*mmpB*) | *P. fluorescens* with *ACP7* (*mmpB*) deletion | Rahman *et al*., 2005 |
| NCIMB10586 *ACP5* (ACP-Ba) pm (*mmpB*) | *P.fluorescens* with *ACP5* active site mutation (Serine to Alanine) | Rahman *et al*., 2005 |

| | | |
|---|---|---|
| NCIMB10586 *ACP6* (ACP-Bb) pm (*mmpB*) | *P.fluorescens* with *ACP6* active site mutation (Serine to Alanine) | Rahman *et al*., 2005 |
| NCIMB10586 *ACP7* (ACP-Bc) pm (*mmpB*) | *P.fluorescens* with *ACP7* active site mutation (Serine to Alanine) | Rahman *et al*., 2005 |
| NCIMB10586 Δ*ACP5/6* (*mmpB*) | *P. fluorescens* with *ACP5/6* (*mmpB*) deletion | Rahman *et al*., 2005 |
| NCIMB10586 Δ*ACP6/7* (*mmpB*) | *P. fluorescens* with *ACP6/7* (*mmpB*) deletion | Rahman *et al*., 2005 |
| NCIMB10586 Δ*ACP5/7* (*mmpB*) | *P. fluorescens* with *ACP5/7* (*mmpB*) deletion | Rahman *et al*., 2005 |

**Table 3.2 Antibiotics used in this study.**

| Antibiotic | Stock solution (mg/ml) | Working solution (µg/ml) |
|---|---|---|
| Ampicillin | 100 | 100 |
| Tetracycline hydrochloride | 15 | 15 |

**Table 3.3 Plasmids used and constructed during MupU and TmlU protein study.**

| plasmid | Size (Kb) | Genotype Description | Reference |
|---|---|---|---|
| pET28a | 5.3 | $Kan^R$, His-Tag,*T7p*,*lacI* | Novagen |
| pET28a-*tmlU* | 7.2 | 1.9 kb *Eco*RI-*Sal*I fragment containing *tmlU* Cloned into pET28a | Fukuda *et al*., 2011 |
| pJH10 | 13.76 | IncQ replicon, multiple cloning site (*Eco*RI-*Sac*I), $Tet^R$ | EL-Sayed *et al*., 2001 |
| pJH10-*Xho*1-linker | 13.76 | IncQ replicon, multiple cloning site (*Eco*RI, *Nhe*I, *Xho*I, *Kpn*I, *Cla*I, *Xba*I, *Sac*I), $Tet^R$ with *Xho*1-linker | Hothersall *et al*., 2007 |
| pSCCU | 15.36 | 1.6 kb *Eco*RI-*Sac*I fragment containing *mupU* Cloned into pJH10 | Cooper *et al*., 2005a |
| pAMH1 | 15.72 | 1.96 kb *Eco*RI-*Sal*I fragment containing *tmlU* Cloned into pJH10-*Xho*1-linker | This study |

## 3.2.3 Isolation of plasmid DNA

### 3.2.3.1 Large scale production of high quality DNA (maxi-prep)

The method for the plasmid DNA preparation below is essentially the large scale preparation of plasmid DNA according to the alkaline-SDS method of Birnboim and Doly, (1979) with modifications described by Smith and Thomas, (1983).

Usually 400 ml L-broth supplemented with appropriate antibiotics for plasmid selection was used for growing overnight culture of bacteria carrying plasmids. This O/N culture was then transferred to two 200 ml pots, balanced equally. The culture was spun down at 10,000 *g* for 10 minutes at 4$^{o}$C. The supernatant was then discarded and the bacterial pellet re-suspended in 25 ml of ice-cold lysis buffer (solution 1), starting from the first pot then transferred to the other pot to re-suspend the whole pellet and mix them together. 50 ml of freshly prepared NaOH-SDS mix (solution 2) was added to the re-suspended bacterial pellet, and the pot was inverted several times to ensure the complete lysis of the bacterial cells. The pot was then incubated on ice for 5 minutes, followed by the addition of 37.5 ml of 3 M sodium acetate, pH 5.0 (solution 3) and carefully mixing the contents by inverting the pot for 5 times, then placed back on ice for 5 more minutes. To pellet proteins and cell debris, the pot was centrifuged at 10,000 *g* for 15 minutes at 4$^{o}$C. Either a pot of another preparation which was balanced with sterile distilled water, or a pot with just sterile distilled water used for keeping the rotor balance during centrifugation. To remove any remaining cell debris, supernatant was filtered through Whatman 1 mm paper, and the cell free filtrate collected in another clean centrifuge pot containing 100 ml isopropanol. At this stage it is possible to store the pots at -20$^{o}$C.

The pots were then centrifuged at 14,000 *g* for 10-15 minutes at 4$^{o}$C to precipitate plasmid DNA. The supernatant discarded and the pellet dried by leaving the pots for at least 15-30

minutes to ensure that all the isopropanol dried out completely, which is essential for the next step. DNA pellet was then dissolved in 3 ml of 1X TNE buffer, pH 8.0. Volume of DNA solution measured accurately, and then caesium chloride added in a ratio of 4.75 gm: 4.5 ml plasmid DNA in clean universal bottle. The salt dissolved by gently shaking the bottle, then the volume made up to 4.5 ml with 1X TNE buffer. Further mixing to ensure that the salt dissolved, and then 0.5 ml Ethidium Bromide solution (10 mg/ ml) was added. After mixing, the solution transferred to two Beckman ultracentrifuge tubes, balanced, then filled up to the frosted line using 1X TNE buffer. The tubes were sealed using the cap heating machine, and then centrifuged at 100,000 rpm for overnight at $20^{o}C$ using a Beckman TL 100 ultracentrifuge.

After overnight centrifugation, the plasmid DNA bands in the Beckman tubes visualised with UV light, and by using a syringe with a wide bore the extracted plasmid DNA was collected and transferred to a clean tube, followed by the addition of an equal volume of $CsCl/H_2O$ saturated isopropanol with gentle mixing to remove Ethidium Bromide. The top pink layer of Ethidium Bromide was removed by Pasteur pipette, and this step repeated until no more pink layer collected at the top of the DNA solution indicating that the Ethidium bromide had virtually all been removed. 400 µl of the plasmid DNA solution was placed into 1.5 ml microfuge tube, and to this 500 µl of distilled water, 100 µl of 3M sodium acetate, and 530 of µl isopropanol were added sequentially. The tube was mixed well, and then centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was poured off, and the pellet re-dissolved in 200 µl of 1X TNE. To this, 25 µl of 3M sodium acetate, and 500 µl of ethanol were added, followed by centrifugation at 10,000 rpm for 10 minutes at room temperature to pellet the precipitate. The pellet was then dried well and re-dissolved in 100 µl of $1/10^{th}$ TNE.

### 3.2.3.2 Bioneer kit for isolation of plasmid DNA

Plasmid DNA with high purity isolated using *AccuPrep* plasmid extraction kit (Bioneer), and the protocol described in the manual was followed accurately. To collect bacteria cell pellet 5 ml of overnight culture of bacteria spun down for 3 minutes at 14,000 *g*. The supernatant was poured off and the pellet re-suspended in 250 µl of re-suspension buffer by vortex mixing to ensure that the pellet completely re-suspended. 250 µl of the lysis buffer was added to the bacterial cells suspension and mixed by inverting the tube gently for 5 times, followed by incubation at room temperature for 5 minutes. To this, 350 µl of neutralising solution was added, and then centrifuged at 14,000 *g* for 10 minutes at 4$^{o}$C. Supernatant transferred to the assembled filter column with collection tube and spun down at 14,000 *g* for 1 minute at room temperature. The flow-through discarded, and 500 µl of nuclease inhibitor was then added to the top of the filter and spun at 14,000 *g* for 1 minute at room temperature after incubation for 5 minutes at room temperature. Again the flow-through was poured off. The filter was then washed with 700 µl of wash buffer and centrifuged at 14,000 *g* for 1 minute at room temperature, followed by discarding the flow-through. Centrifugation was repeated one more time to ensure that the ethanol on the filter had completely dried off. Then the filter was transferred to a sterilised micro-centrifuge tube, and 50-100 µl of elution buffer was added generally 50 µl was used to obtain concentrated DNA. The tube was incubated for 1 minute at room temperature, and plasmid DNA was eluted by centrifugation at 14,000 *g* for 1 minute at 4$^{o}$C. Plasmid DNA was then stored at -20$^{o}$C.

## 3.2.4 Manipulation of plasmid DNA

### 3.2.4.1 Restriction digestion analysis

Plasmid DNA was digested using specific and suitable restriction enzymes purchased from New England Biolabs (NEB), MBI Fermentas, or Invitrogen. Usually, total digestion

reactions were carried out either in 20 µl or 40 µl with incubation for 2 h at 37$^{o}$C. 10X Bovine Serum Albumin (BSA) prepared as a stock for the digestion reaction, was kept at -20$^{o}$C. Optimum reaction mixture contained a desired amount of DNA (up to 1 µg), 1/10 volume of 10x restriction enzyme buffer, 1-10 units of restriction enzyme and distilled water to adjust the reaction volume. However, 1/10 volume of 10x BSA was added to the reaction whenever it was recommended for typical enzyme function. In case of double digestions where the two enzymes required the same buffer, enzymes were added simultaneously.

### 3.2.4.2 Agarose gel electrophoresis

Plasmid DNA, PCR, and restriction enzyme digestion were analysed using agarose gel electrophoresis. 1.0-1.5% agarose was mixed with 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8) and 0.2 µg/µl ethidium bromide for gels preparation. DNA samples were mixed in 1x DNA loading dye (6x stock solution contains 15% ficoll, 0.25% bromophenol blue and 0.25% xylene cyanol). Prepared samples were run against 5 µl 1 kb ladder (NEB of Fermentas). Gels were typically run at 100-140 V in 1x TAE buffer.

### 3.2.4.3 Purification of DNA from agarose gels

A sterilised scalpel was used for the excision of DNA fragments out of the gel, and purified using the Gel Band Purification Kit (GE Healthcare). 300 mg in a single batch is the maximum allowed weight of gel slice that could be purified using this method in a single tube. 300 µl of Capture Buffer (GE Healthcare) was added to the 300 mg gel slice. After closing the lid, the tube was mixed by vortex mixing and incubated at 55$^{o}$C in a water bath while mixing the tube every 3 minutes until the agarose was completely dissolved (15-30

min). When the agarose was dissolved the sample was centrifuged briefly to collect the whole sample, then it was transferred to the column assembled with the collection tube and incubated for 1 min at room temperature followed by centrifugation at room temperature for 30 s at 12,000 g. The flow through was discarded and followed by washing the column with 500 µl of wash buffer, then centrifugation at room temperature for 30 s at 12,000 g. The column was transferred into a sterilised 1.5 ml tube and 15-20 µl of elution buffer was applied to the top of the column, followed by incubation for 1 min at room temperature, and then centrifuged at room temperature for 1 min at 12,000 g to recover the purified DNA. This last step was repeated twice to ensure the DNA was eluted completely, and to make the total volume as 30-40 µl.

### 3.2.4.4 Ligation

The enzyme T4 DNA ligase, purchased from Invitrogen was used for performing the ligations, which carried out usually in a total of 20 µl reaction mixture. The amount of DNA of both the insert and the vector was measured in 1 µl of each using nanodrop, and then mixed together in a ratio of approximately 3:1, respectively to make up no more than 100 ng of DNA in total. The required quantities of both of the vector and insert DNA were calculated using the following formula:

$$\frac{\text{ng of vector } x \text{ size of insert (kb)}}{\text{size of vector (kb)}} \; x \; \frac{3 \text{ insert}}{1 \text{ vector}} = \text{ng of insert DNA}$$

The ligation reaction was usually incubated overnight at 4$^{o}$C.

### 3.2.5 Preparation of competent cells

Competent cells of *E. coli* strain DH5α, S17-1 or GM2163 were prepared using the calcium chloride method (Cohen *et al*., 1972). 1 ml of the overnight (starter) cultures incubated at 37$^{o}$C, 200 rpm was used for the inoculation of 100 ml of LB, and then incubated at 37$^{o}$C, 200 rpm until to mid-log phase ($OD_{600}$~0.4-0.6). The young cultures were then harvested by centrifugation for 7 minutes at 5000 g and at 4$^{o}$C. Supernatant was discarded and the cells were re-suspended in ice-cold 100 mM $CaCl_2$ and incubated on ice for 30-45 minutes. Bacterial cells were pelleted again using the same parameters as before, and re-suspended in 5 ml of 100 mM $CaCl_2$. Fresh competent cells were ready at this stage and could be stored at 4$^{o}$C if used within two weeks. However, cells were re-suspended in 5 ml of 100 mM $CaCl_2$ with 20% glycerol solution for longer storage. Liquid nitrogen was used for freezing 100 µl of competent cells in a sterilised microfuge tubes and stored at -80$^{o}$C.

### 3.2.6 Transformation of bacteria

For bacterial transformation, plasmid DNA (300-200 ng) or 10 µl of ligation mixture were added to 100 µl of competent cells and placed on ice for 30-60 min. The cells were then heat-shocked at 42$^{o}$C for 2 min using a water bath. After incubation, 0.5 ml of fresh LBB was added and the cells were incubated at 37$^{o}$C for at least 1 hr with gentle mixing every 10 min. After incubation, 200 µl and 50 µl of the cell suspension were spread on two selective agar plates separately, and incubated overnight at 37$^{o}$C.

### 3.2.7 Transfer of plasmid DNA by conjugation

*E. coli* S17-1 was used for mobilising plasmids (for example pJH10 with the insert) into *P. fluorescens* via bacterial conjugation. 1 ml of overnight culture of each strain (donor and

recipients) was mixed in a sterile universal bottle. The mixture was then filtered using a sterile filter, and the filter transferred on to L-agar without any antibiotic selection to support the conjugation, followed by overnight incubation of the plate at 30$^{o}$C. The filter was then removed from the plate, and transferred into 1 ml of saline solution (0.85%) and vortex mixed to re-suspend the cells. Serial dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ of bacterial suspension were prepared using saline solution. Then 100 µl of the dilutions was spread on M9 minimal media supplemented with tetracycline or another appropriate antibiotic and then incubated at 30$^{o}$C for 2-3 days. A single colony was then picked up and streaked again on the same media mentioned before and incubated at 30$^{o}$C for 24 h to obtain pure cultures with isolated colonies. Single colonies from the pure culture were then streaked on L-agar plates supplemented with tetracycline and ampicillin.

### 3.2.8 Bioassay for mupirocin production

Single colonies of trans-conjugant, mutants and wild type strains were inoculated into 5 ml LB containing appropriate antibiotics. The cultures were incubated overnight at 30$^{o}$C with shaking at 200 rpm. The $OD_{600}$ of each culture was measured and the cultures were then diluted to the $OD_{600}$ of the lowest reading using fresh LBB so that each patch started at the same density. 10 µl of each culture was spotted onto L-agar plates without IPTG as well as with 0.5 mM IPTG. The plates were incubated overnight at room temperature. Overnight cultures of *Bacillus subtilis* 1064 (sensitive organism) were grown in L-broth without any antibiotic selection at 37$^{o}$C with shaking at 200 rpm. The plates with spot culture were overlaid with a mixture of 100 ml L-agar (molten and cooled to 45$^{o}$C) with 4 ml of *Bacillus subtilis* 1064 culture and 0.5 ml of 2, 3, 5-triphenyltetrazolium chloride (5% TTC) and left on the bench for about 1 hr, then incubated overnight at 37$^{o}$C. TTC is used as a reagent that is

reduced to give a red precipitate in the presence of bacterial cells. Therefore, the area in which the *B. subtilis* 1064 were growing turned red and where the bacteria had been killed by antibiotic production were seen as a clear zone. The size of the clearance zone around the spot culture was taken as an estimate of the amount of mupirocin produced.

### 3.2.9 Culture preparation for HPLC

In order to obtain the seed culture of trans-conjugant strains, mutants, and the wild type *P. fluorescens* NCIMB10586, a single bacterial colony was inoculated into 25 ml of LB with appropriate antibiotic (tetracycline or ampicillin) in a 250 sterile conical flask, and incubated overnight at 25$^{o}$C, 200 rpm. 1.25% ml (5%) of the seed culture was then used to inoculate 25 ml of fresh MPM (Mupirocin Production Media: Yeast extract, 2.3 g/l; $NaHPO_4$, 2.6 g/l; $KH_2PO_4$, 2.4 g/l; $(NH_4)_2SO_4$, 5 g/l). 550 µl of 40% glucose was added as supplementary material to each 200 ml MPM after autoclaving, and 0.5 mM IPTG was added to the production media in case of induction. The new culture was then shaken overnight at 22$^{o}$C, 200 rpm, for 40 hours. 1 ml of each culture was spun down by centrifugation at 15,000 g for 10 minutes. Before injection into the HPLC, the supernatant was filtered through 0.2 µm filter (Pall-Gelman, labs).

It should be noted that a new production media (SSM) was used for $ACP_S$ mutants expressing *tmlU in trans* and the following experiments, which prepared as follows:-

(Soya flour 25 g/l; spray dried corn liquor 2.5 g/l; $(NH_4)_2SO_4$ 5.0 g/l, $MgSO_4.7H_2O$ 0.5 g/l; $Na_2HPO_4$ 1.0 g/l; $KH_2PO_4$ 1.5 g/l; KCL 1.0 g/l; $CaCO_3$ 6.25 g/l) with pH adjusted to 7.5, then 10 ml of separately autoclaved 40% glucose was added to each 200 ml of the media before using.

### 3.2.10 Sample analysis by HPLC

HPLC analysis was performed to detect antibiotic production by wild type, mutant, and trans-conjugant strains of NCIMB10586. Two solvents were used; solvent A (HPLC grade water); and solvent B (HPLC grade acetonitrile), and both were supplemented with 0.01% formic acid to adjust the pH of the mobile phase. Both solvents were degassed using an aspirator in order to completely release all the bubbles from the solvents before using. HPLC was performed using Uni-point LC system software, reverse phase C18 column (15 cm x 4.6 mm), UV detector was used at a sensitivity of 0.002 absorbance units at 233 nm. 100 µl of filtered supernatant of each sample was injected into the HPLC machine. Mupirocin and other intermediates were eluted using a solvent gradient (5—70%) of water/acetonitrile over 1 hr at 1 ml/min flow rate.

### 3.2.11 Sample analysis by ES-MS and NMR

Samples were subjected to ES-MS analysis followed by NMR in the School of Chemistry-University of Bristol by Dr Zhongshu Song.

## 3.3 Results

### 3.3.1 Putting *tmlU* under the control of *tac*-promoter

For the purpose of in *trans* expression of *tmlU* using a broad host range vector pJH10, the *tmlU* expression plasmid was constructed. The *tmlU* gene was cut out on an *Eco*RI-*Sal*I fragment from pET28a *tmlU* and inserted into the expression vector pJH10-*Xho*I linker, digested with *Eco*RI-*Xho*I enzyme. This yielded an expression plasmid designated pAMH1 as shown in Figure 3.4. However, pSCCU is a pJH10 vector for *in trans* expressing of *mupU*, that was constructed previously (Table 3.3).

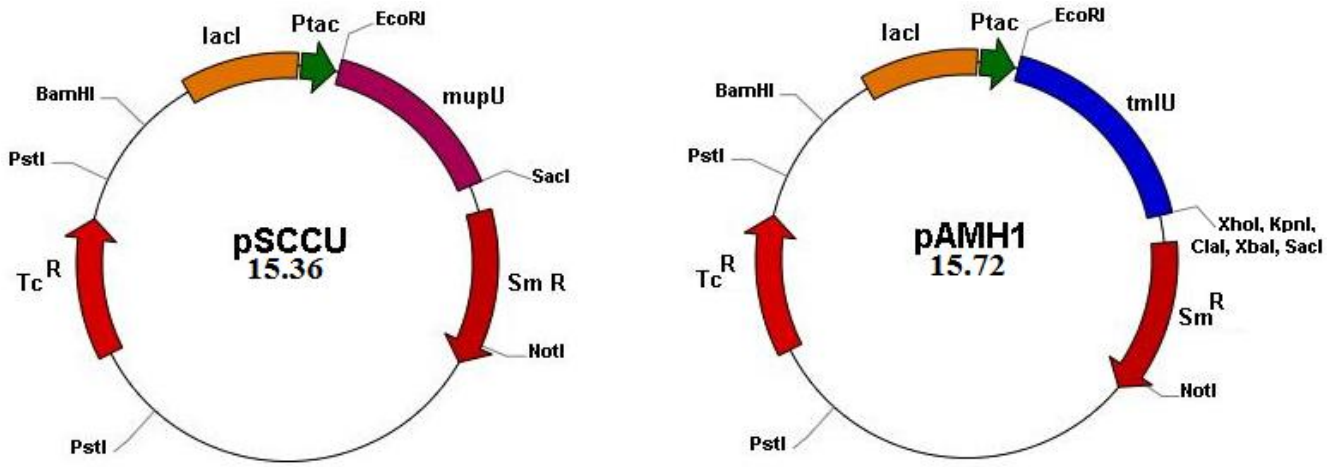



**Figure 3.4 pJH10 based plasmids containing *mupU* and *tmlU* genes.**

### 3.3.2 Bioassay to check the effect of TmlU and MupU

Although there is only weak similarity between TmlU, the putative amide synthetase in thiomarinol cluster, and MupU, the acyl CoA synthase in mupirocin system, we wanted to

investigate whether the *in trans* over-expression of both *mupU*, and *tmlU*, each separately, could complement NCIMB10586Δ*mupU* strain. According to our hypothesis over-expression of *tmlU in trans* might complement NCIMB10586Δ*mupU*, which could be of significance for mutasynthesis experiments through feeding other compounds, mostly analogues of pyrrothine to NCIMB 10586 WT. The presence of TmlU might allow join to mupirocin to produce new hybrids. Also, it might give extra details about the role of TmlU in joining marinolic acid with the pyrrothine in thiomarinol system. Therefore, to test the formulated hypothesis, both *mupU*, and *tmlU* were over-expressed *in trans* separately, in NCIMB10586Δ*mupU* strain, using pSCCU plasmid (encoding *mupU*), and pAMH1 plasmid (encoding *tmlU*), respectively. On bioassay plates (Figure 3.5 and 3.6), it was observed that pSCCU plasmid provided in *trans* did complement NCIMB10586Δ*mupU*, and the strain exhibited mupirocin production like the wild type with production being increased slightly with IPTG induction. However, over-expression of *tmlU* from pAMH1 not only did not complement the NCIMB10586Δ*mupU* strain, but also blocked the normal biosynthesis of mupirocin. This demonstrates that TmlU is different from MupU and has a different functionality.

In order to determine specifically where TmlU is interacting in mupirocin cluster, we predicted that TmlU will block the biosynthesis in the NCIMB10586 WT as well, through modifying PA-A. In addition, since one of the predicted activities of MupX is to hydrolyse amides, perhaps it can counteract the action of TmlU. Therefore, putting TmlU into NCIMB10586 Δ*mupX* strain might not need to be induced so much to inactivate mupirocin, because the enzyme that works in the opposite direction will not be there. Therefore, to test this hypothesis, TmlU was over-expressed *in trans* from pAMH1 plasmid in both of the NCIMB10586 WT, and NCIMB10586Δ*mupX* strains, respectively. The results from bioassay (Figures 3.5 and 3.6) showed a similar effect with over-expression of *tmlU* in the NCIMB10586 WT, and NCIMB10586Δ*mupX* strains, through affecting the production of

mupirocin. The effect was stronger with IPTG induction and this blocked the normal biosynthesis of mupirocin. This indicates that TmlU interference with mupirocin biosynthesis is not affected by the presence or the absence of MupX, and that MupX is not affecting the normal function of TmlU as an amide synthetase.

Furthermore, and to further explore what TmlU is doing to the mupirocin cluster, we proposed that TmlU may either compete or interact with thioesterase (TE) to promote the release of pseudomonic acids (PAs). Therefore, by using pAMH1 plasmid, over-expression of *tmlU* in NCIMB10586Δ*TE* strain was carried out. On bioassay plates (Figure 3.5 and 3.6) no observable difference could be seen in NCIMB10586Δ*TE* with and without the presence of TmlU, even when the expression was induced with IPTG, indicating that TmlU is not working like an abnormal thiolesterase or hydrolase. It should also be mentioned that in all the previous bioassay data presented, both NCIMB10586 WT, and WT with empty pJH10 vector provided *in trans*, were taken as controls.

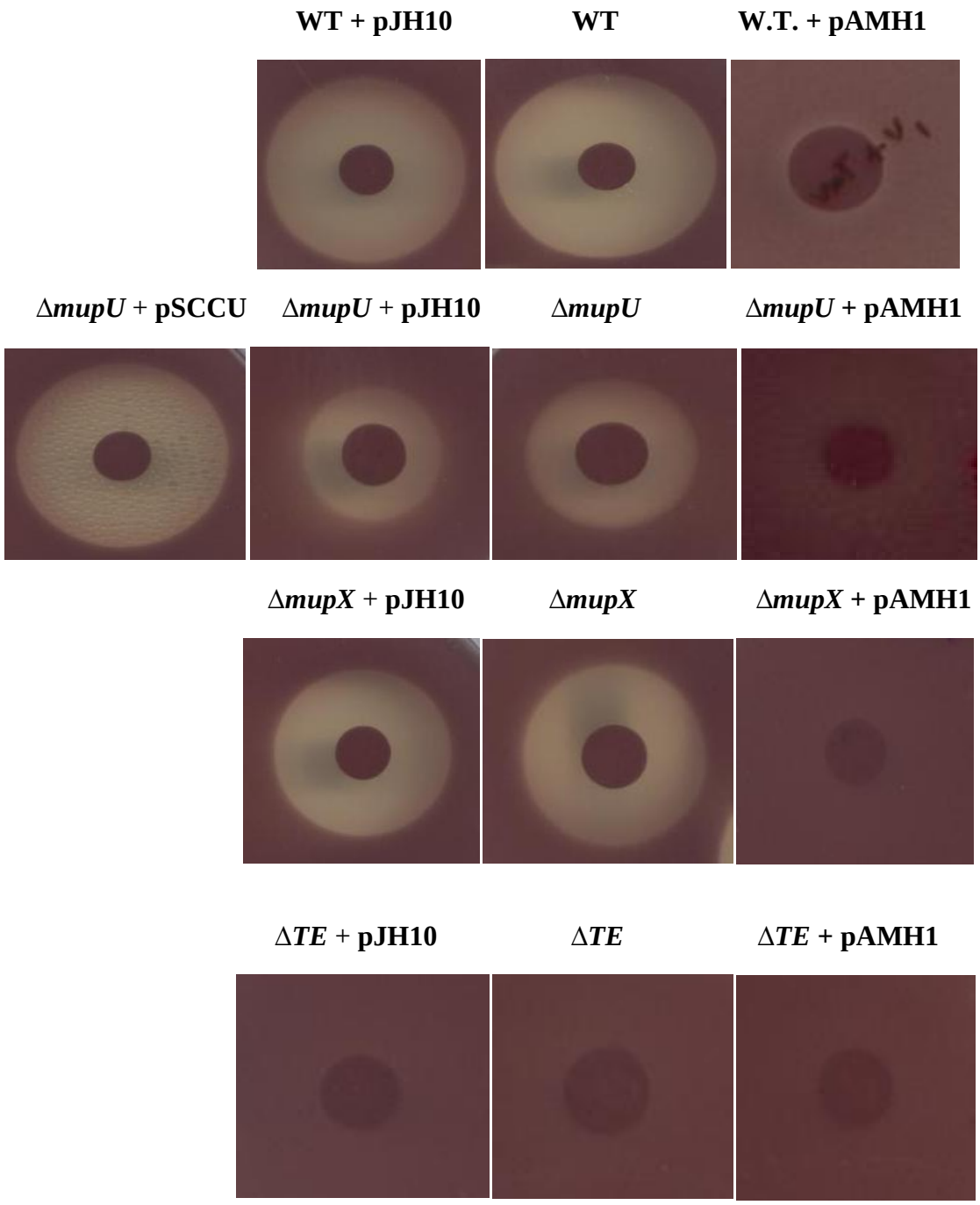
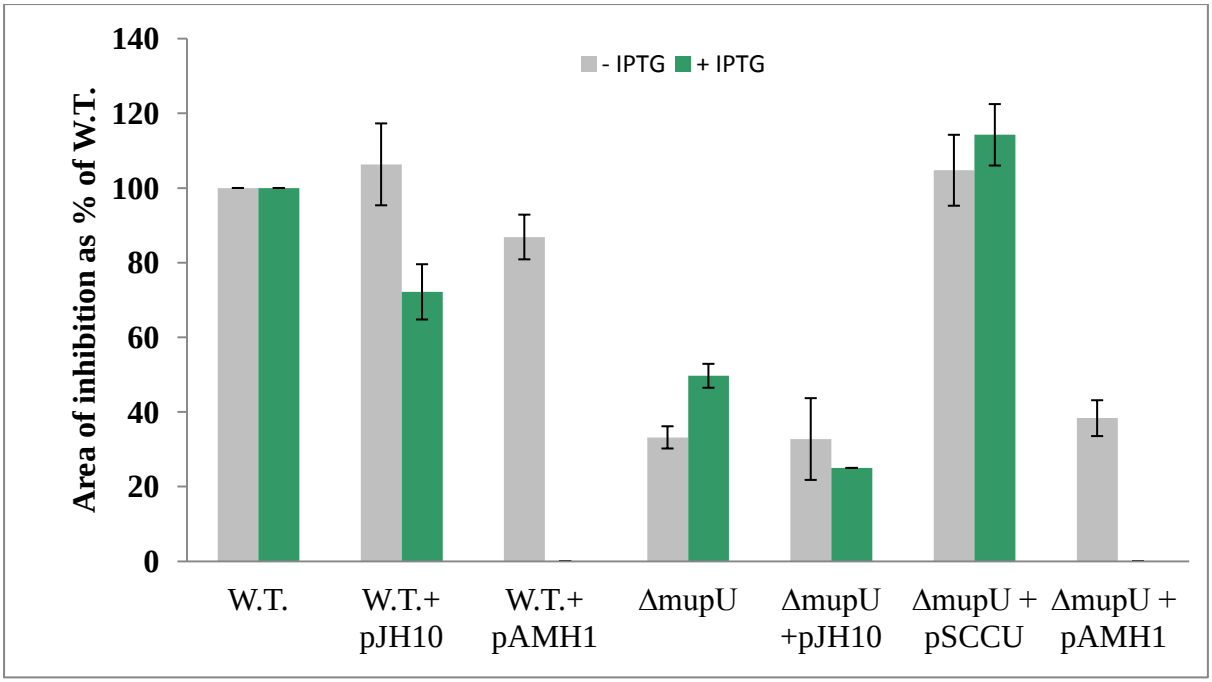



**Figure 3.5 Complementation analysis of the NCIMB10586Δ*mupU* with pSCCU (pJH10-*mupU*) and pAMH1 (pJH10-*tmlU*) plasmids *in trans*; and the effect of *in trans* expression of pAMH1 plasmid in the NCIMB10586 WT, NCIMB10586Δ*mupX* and NCIMB10586Δ*TE*.** The zone of clearance around the central spot culture is taken as an estimate of antibacterial activity.



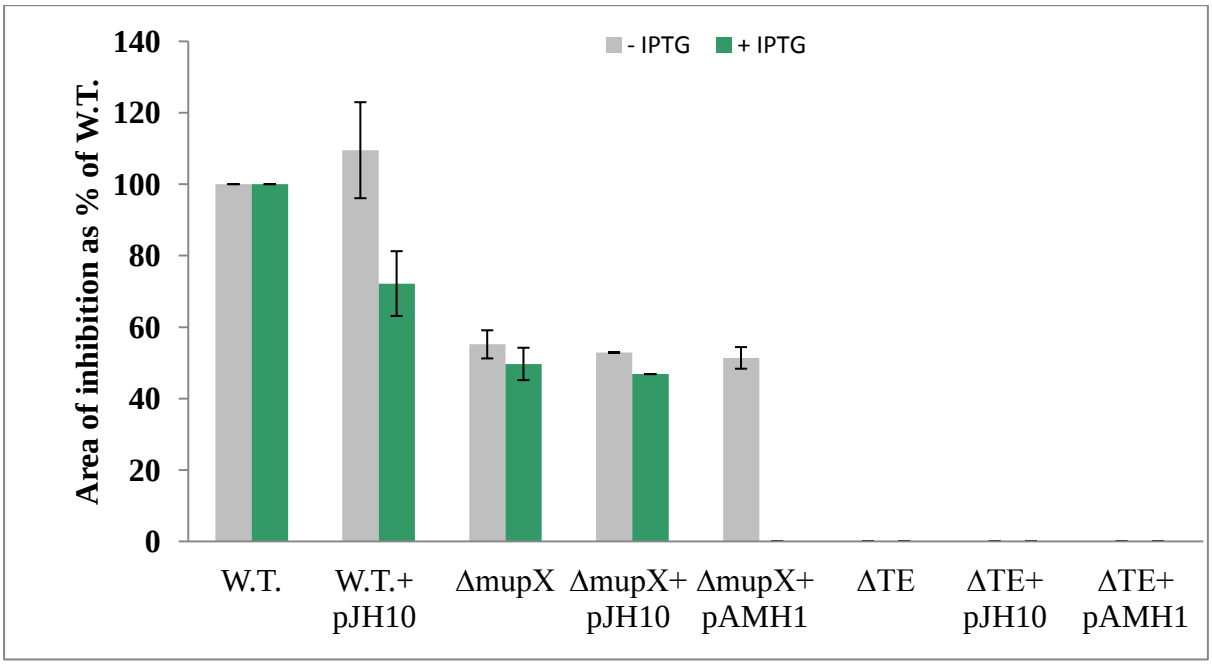




**Figure 3.6 Quantitative bioassay of the NCIMB10586 WT, NCIMB10586Δ*mupX* and NCIMB10586Δ*TE* mutants expressing TmlU protein from pAMH1 plasmid, and NCIMB10586Δ*mupU* expressing MupU protein from pSCCU plasmid, also triplicates were used for each case.**

### 3.3.3 HPLC analysis of pseudomonic acids produced by WT NCIMB10586, NCIMB10586Δ*mupU*, NCIMB10586Δ*mupX* and NCIMB10586Δ*TE* expressing *tmlU* in *trans*; PA-A and PA-B production by NCIMB10586Δ*mupU* expressing *mupU* in *trans*

HPLC analysis was used for investigating pseudomonic acids produced by NCIMB10586Δ*mupU*, over-expressing both pSCCU plasmid (encoding *mupU*), and pAMH1 plasmid (encoding *tmlU*), separately, and also NCIMB10586 WT, NCIMB10586Δ*mupX*, and NCIMB10586Δ*TE* expressing pAMH1 plasmid (encoding *tmlU*) only. As controls, both NCIMB10586 WT and WT with empty pJH10 vector provided *in trans* were used. HPLC analysis showed that over-expression of *mupU* from pSCCU did complement NCIMB10586Δ*mupU*, and the strain restored mupirocin production (PA-A and PA-B) like the wild type strain even without IPTG induction. However, over-expression of *tmlU* from pAMH1 modified most of PA-B in NCIMB10586Δ*mupU* to a new compound that was eluted earlier (with retention time between 13 to 14 min) than the normal pseudomonic acid compounds, rather than complementing it. Moreover, over-expression of *tmlU* both in the NCIMB10586 WT and NCIMB10586Δ*mupX* modified most of the PA-A to the same compound that had been observed in NCIMB10586Δ*mupU* strain with *tmlU*, but without any observable effect on NCIMB10586Δ*TE*.

More precisely, there was still release of some PA-B in the case of *tmlU* expression in NCIMB10586Δ*mupU*, and release of less PA-A when *tmlU* was expressed in both of the NCIMB10586 WT and NCIMB10586Δ*mupX* without induction, but when expression was induced with 0.5 mM IPTG, all remaining PA-B and PA-A was modified to the new compound as well (Figure 3.7 and 3.8).

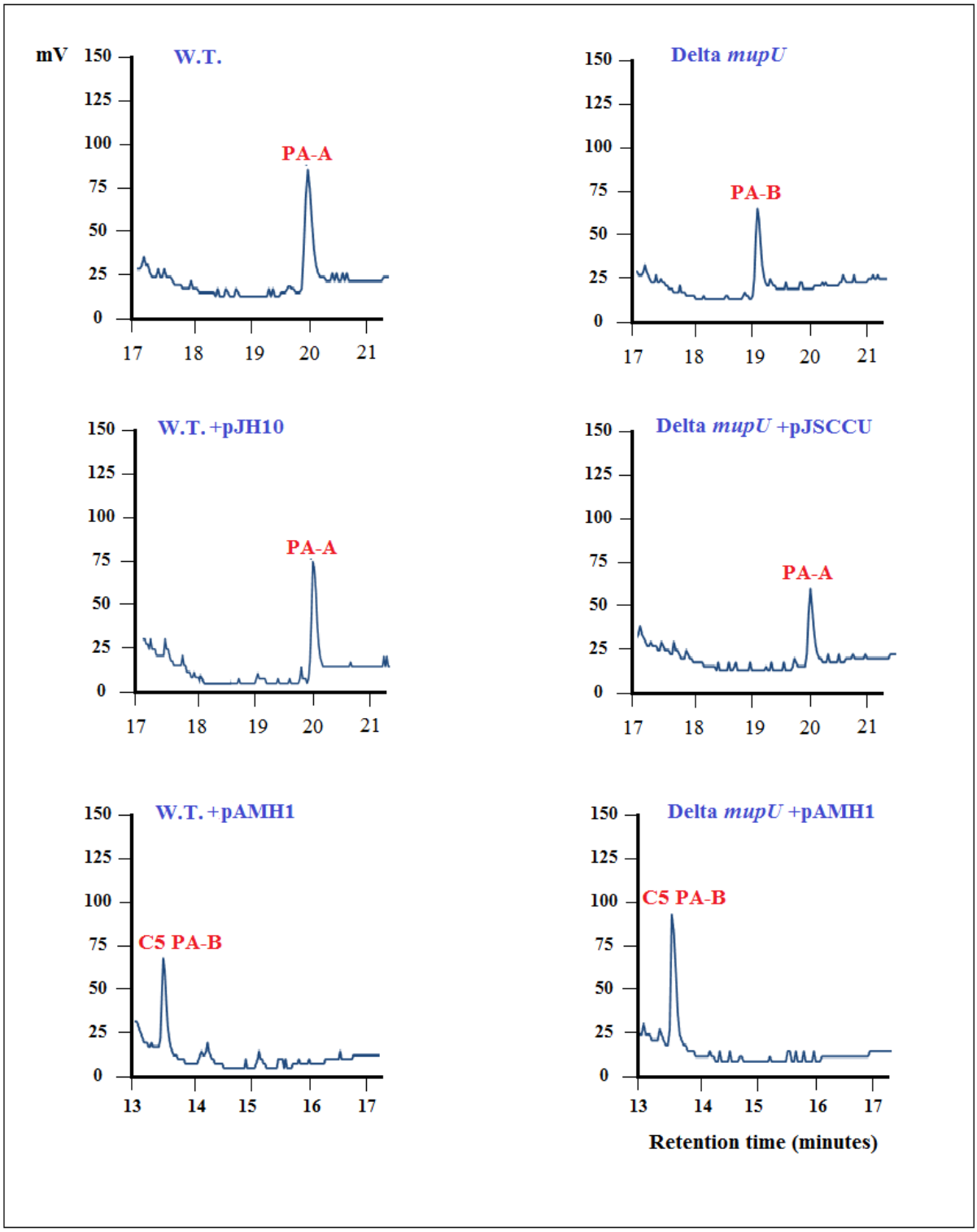



**Figure 3.7 HPLC chromatograms of NCIMB10586 WT with pAMH1 plasmid, and NCIMB10586Δ*mupU* first with pSCCU plasmid and second with pAMH1 plasmid expressed *in trans*. WT and WT+pJH10 strains are controls.**

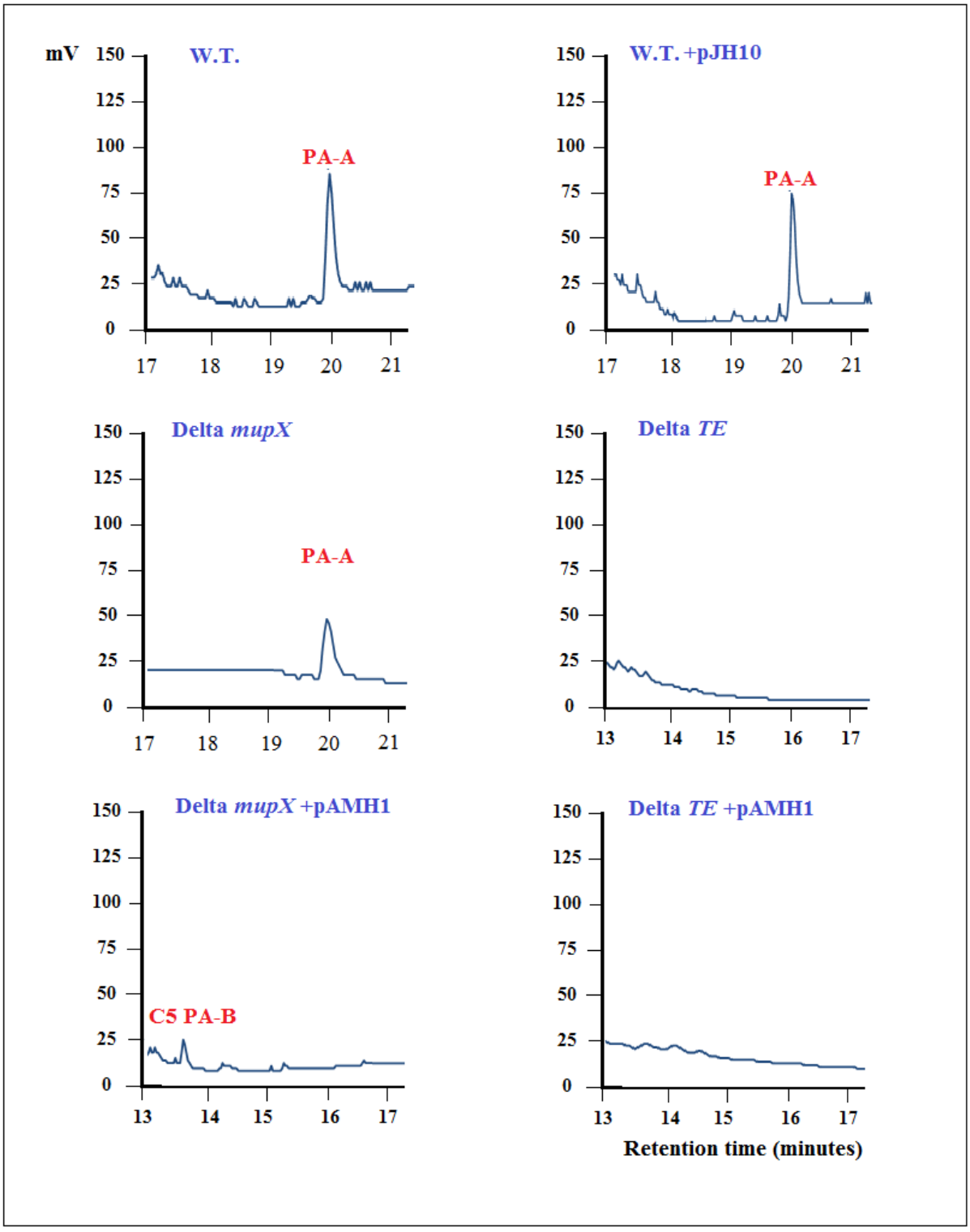



**Figure 3.8 HPLC chromatograms of IPTG induced NCIMB10586Δ*mupX* and NCIMB10586Δ*TE* strains with pAMH1 plasmid expressed *in trans*. WT and WT+pJH10 strains are controls.**

### 3.3.4 ES-MS analysis of pseudomonic acids produced by NCIMB10586 WT, NCIMB10586Δ*mupU,* NCIMB10586Δ*mupX*, and NCIMB10586Δ*TE* expressing *tmlU* in *trans,* and PA-A and PA-B production by NCIMB10586Δ*mupU* strain expressing *mupU* in *trans*

HPLC analysis of NCIMB10586 WT, NCIMB10586Δ*mupU,* NCIMB10586Δ*mupX*, and NCIMB10586Δ*TE* expressing *tmlU in trans*, was followed by ES-MS analysis and NMR to confirm and to provide extra evidence about the changes in PAs production from the effect of TmlU. As a control NCIMB10586 WT showed the production of both PA-A and PA-B, respectively, and NCIMB10586 WT with empty vector (pJH10) did not have any negative effect on PAs production. Production of mupirocin (PA-A and PA-B) was confirmed in the NCIMB10586Δ*mupU* when *mupU* was expressed *in trans*. However, conversion of PA-B to truncated $C_5$ and $C_7$ version of PA-B was observed when *tmlU* was over-expressed *in trans*, although all PA-B was converted to $C_5$ PA-B with IPTG induction (Figure 3.9a).

Conversion of the $C_9$ version of PA-A to $C_5$ and $C_7$ version was observed when *tmlU* was over-expressed *in trans* both in the NCIMB10586 WT and NCIMB10586Δ*mupX*, and the effect was stronger with induction, which results in the conversion of all PA-A to only $C_5$ (PA-A and PA-B), respectively. Surprisingly, we could see the release of a smaller amount of $C_5$ PA-A and B when *tmlU* was over-expressed in NCIMB10586Δ*TE*, although release of a very tiny amount of $C_9$ PA-A was observed in NCIMB10586Δ*TE*, with and without *tmlU* (3.9b).

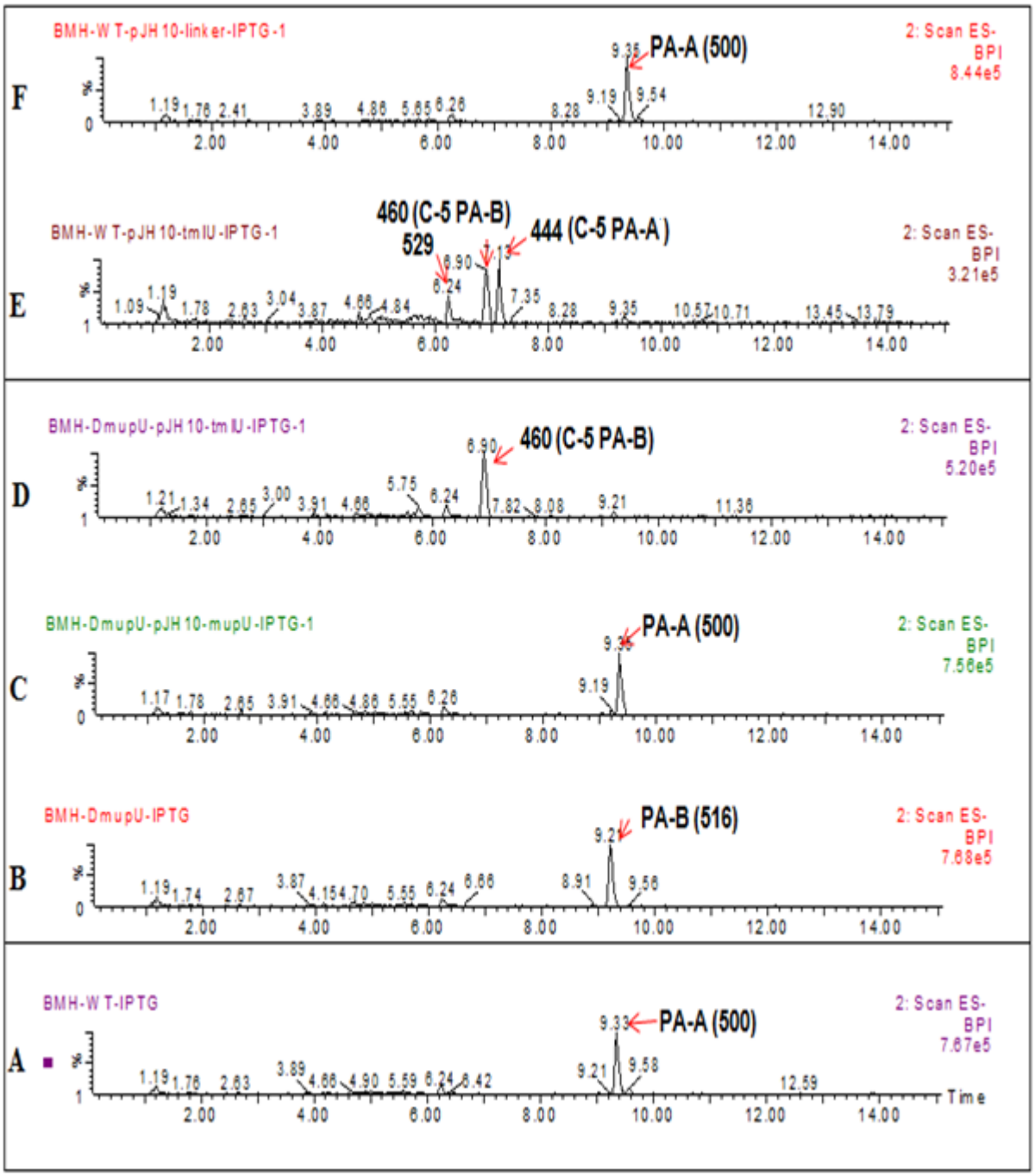



**Figure 3.9a Detection of various pseudomonic acids produced by NCIMB10586 WT and NCIMB10586Δ*mupU* strains expressing pAMH1 plasmid *in trans*, with the sole expression of pSCCU plasmid in NCIMB10586Δ*mupU* with induction using Electrospray Ionisation (ES-MS). WT and WT+pJH10 strains are taken as controls.** NCIMB10586Δ*mupU* complementation with pSCCU restored PA-A production. However, pAMH1 plasmid expressing *tmlU* in NCIMB10586Δ*mupU* strain produced $C_5$ PA-B only, while NCIMB10586 WT produced equal amounts of $C_5$ PA-A and B; *tmlU* in NCIMB10586Δ*mupU* did not produce $C_5$ PA-A at all (data from Bristol).

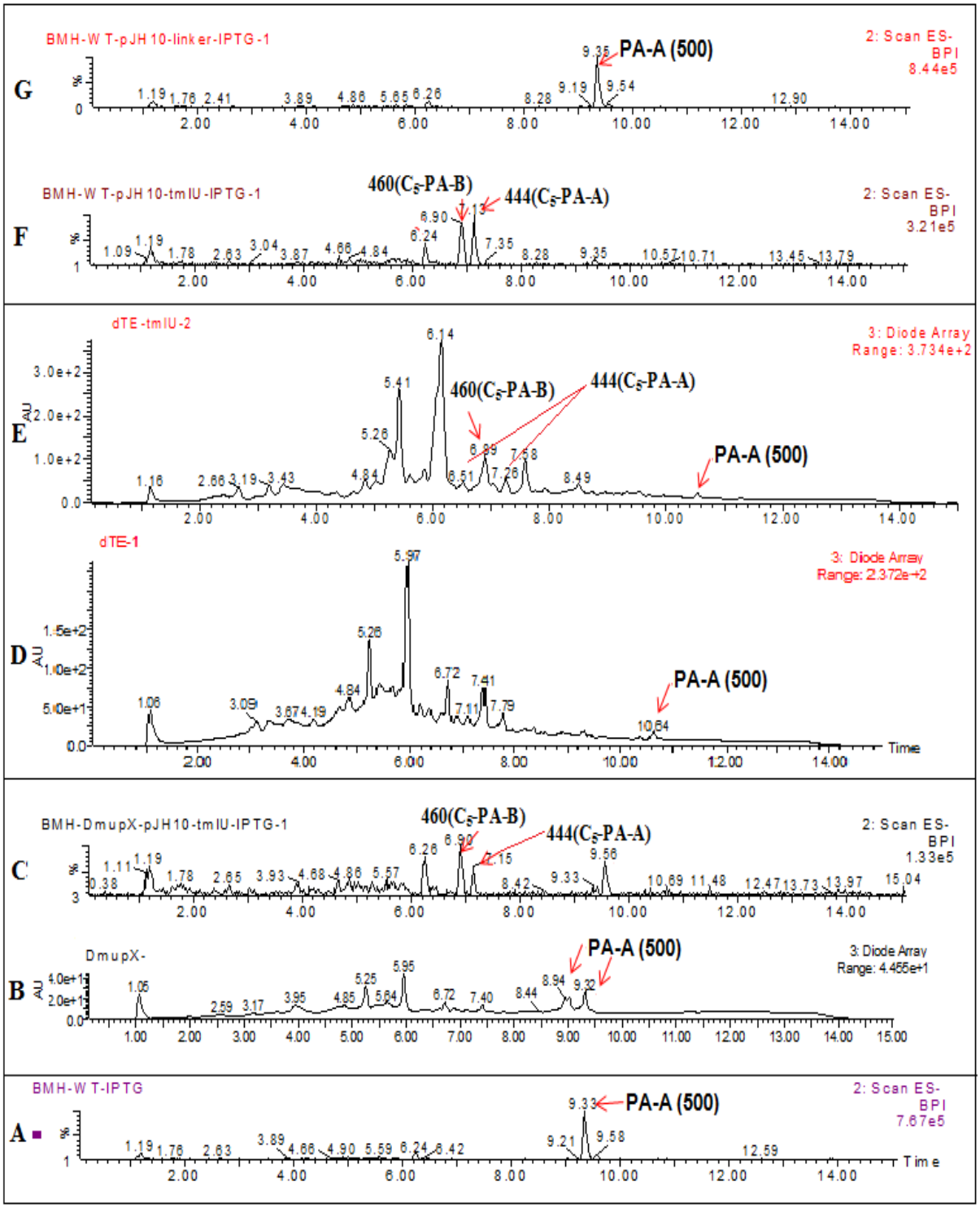



**Figure 3.9b Detection of various pseudomonic acids produced by NCIMB10586 WT, NCIMB10586Δ*mupX* and NCIMB10586Δ*TE* strains, expressing pAMH1 plasmid *in trans* (B-F) using Electrospray Ionisation (ES-MS). WT and WT+pJH10 strains are taken as controls (A and G).** pAMH1 plasmid expressing *tmlU* in NCIMB10586 WT, NCIMB10586Δ*mupX* and NCIMB10586Δ*TE* strains produced $C_5$ PA-B at the same ratio; *tmlU* in the WT produced equal amount of $C_5$ PA-A and B; *tmlU* expression in NCIMB10586Δ*mupX* produced half amount of $C_5$ PA-A (data from Bristol).

### 3.3.5 Effect of TmlU on tandem ACPs of MmpB

The data described above showed that TmlU interferes with mupirocin biosynthesis, and more specifically with the biosynthesis of 9HN (9-Hydroxynonanoic acid), which is predicted by El-Sayed *et al*., (2003), to be the product of MmpB (with tandem $ACP_S$ 5 (Ba), 6 (Bb), and 7 (Bc)) on the basis that it is the most like candidate for an iterative fatty acid synthase working via three condensation using malonate as extender unit. The results showed that TmlU blocks the $2^{nd}$ ($C_5$-$C_7$) and the $3^{rd}$ ($C_7$-$C_9$) round of condensation, but not the $1^{st}$ one ($C_3$-$C_5$). Although MmpB can function with just one ACP (Rahman *et al*., 2005) it is possible that there may be differential involvement in successive condensations so it seemed reasonable to determine the effect of over-expressing *tmlU* in NCIMB10586 with mutations in $ACP_S$ of MmpB.

#### 3.3.5.1 TmlU and $ACP_S$ (5 (Ba), 6 (Bb), and 7 (Bc)) point mutation

*tmlU* was expressed in NCIMB10586 with point mutations (pm) in single $ACP_s$(5, 6, or 7), where the active site serine was mutated to alanine. The purpose of this experiment was to see whether TmlU is targeting the active site of $ACP_s$ for its interference or not. Bioassay performed to confirm that expression of *tmlU* from pAMH1 plasmid by *tac* promoter is functional. Bioassay did not show any detectable clearance zone of inhibition around the culture spot of ACP5 pm with *in trans* expression of *tmlU*, neither with nor without induction, which means it was quite similar to ACP5 pm itself which produces no biologically active compounds as previously observed by Ayesha Rahman (Rahman *et al*., 2005). By contrast, there was still a significant clearance zone of antibacterial activity around culture spot of both strains of ACP6 and ACP7 point mutation with *tmlU* expression in *trans*,

without induction, but that activity disappeared when induced with 0.5 mM IPTG (Figure 3.10 and 3.11). Indicating that TmlU was likely to interfering with 9-HN elongation in these mutants as well.

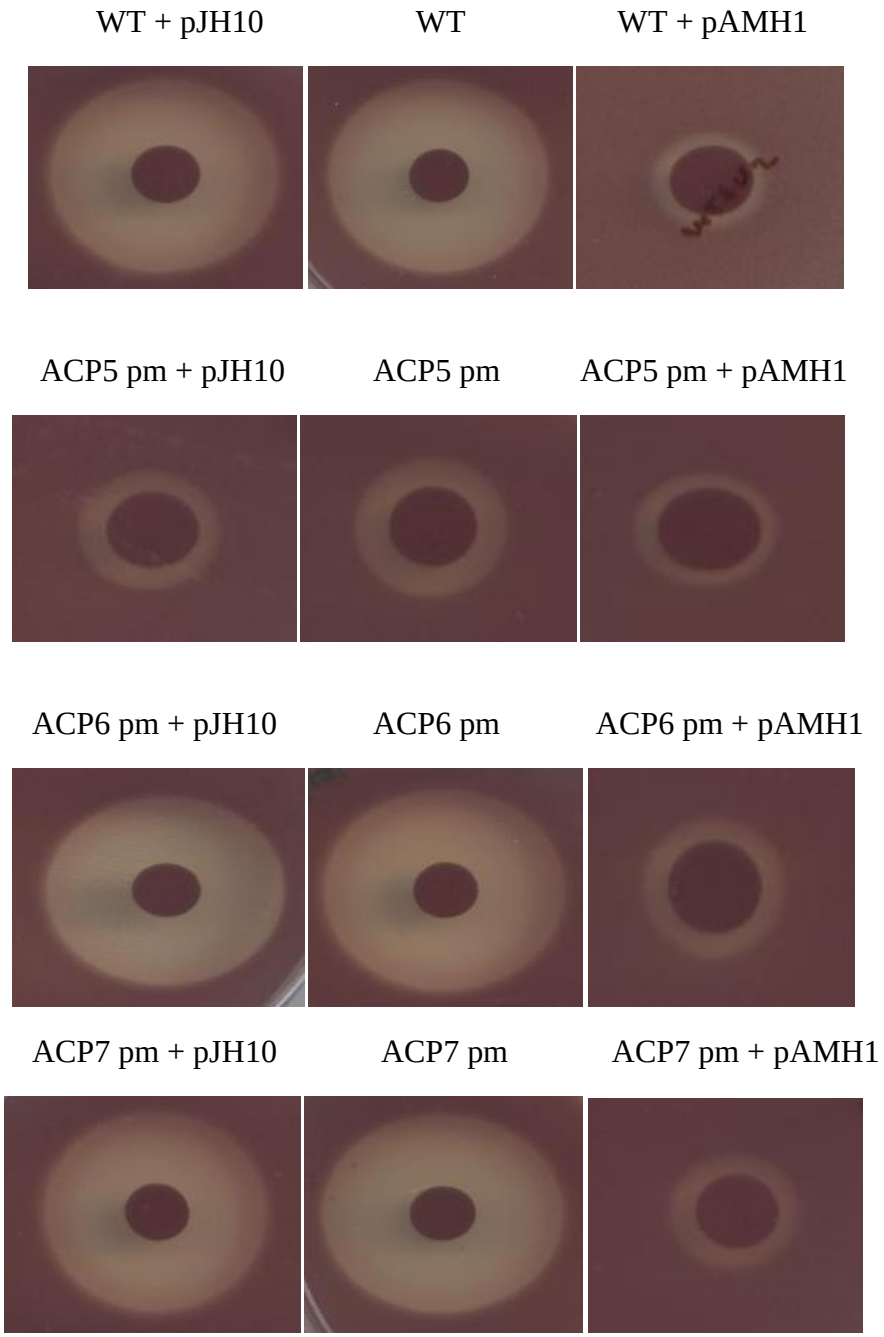



**Figure 3.10 Bioassay to show the expression of *tmlU*, and its influence on strains of NCIMB10586 with active site mutation in one of the $ACP_s$ (ACP5, ACP6, or ACP7) of MmpB, separately. W.T, W.T with pAMH1 plasmid, and W.T with pJH10 vector were taken as controls.**

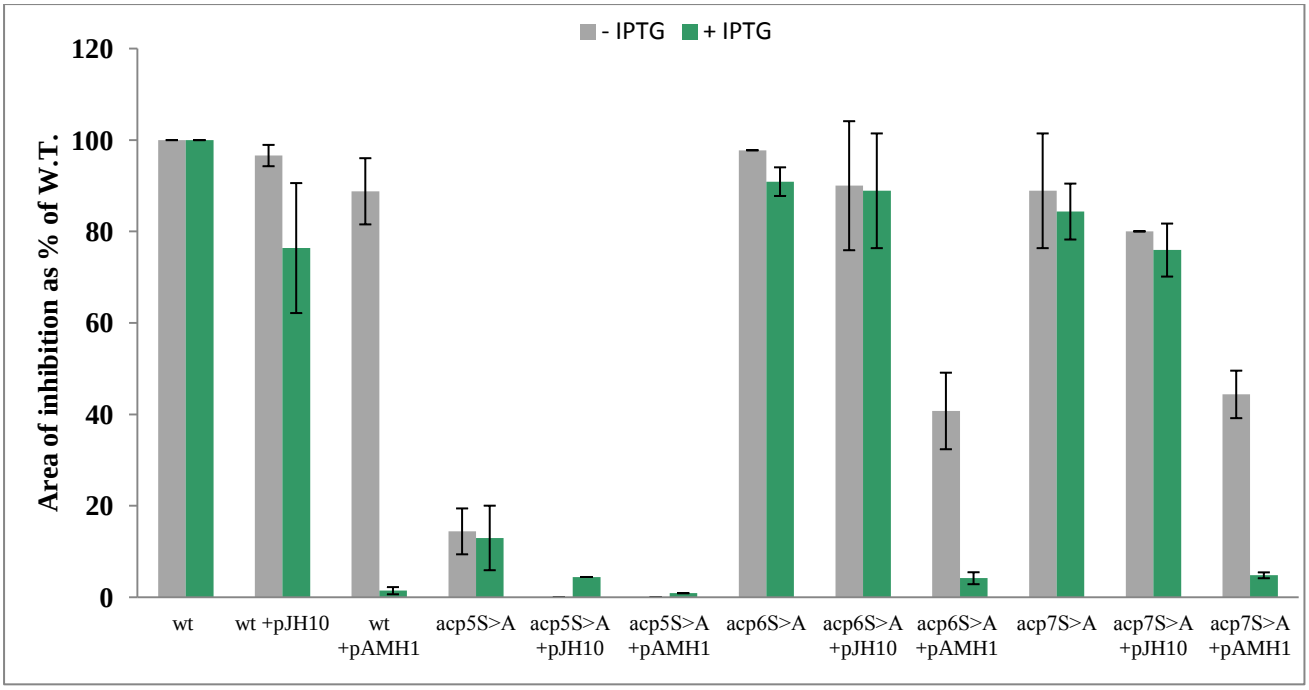



**Figure 3.11 Quantitative bioassay of NCIMB10586 strains with active site mutation in one of the ACP$_S$ (ACP5, ACP6, or ACP7) of MmpB, separately, expressing *tmlU* from pAMH1 plasmid. NCIMB 10586 WT, NCIMB10586 WT with pAMH1 plasmid, and NCIMB10586 WT with pJH10 vector were taken as controls. Triplicates were used.**

HPLC analysis was performed to give clear evidence about the product released after *tmlU* expression *in trans* from pAMH1 plasmid in NCIMB10586 strains with a point mutation in one of the ACP$_S$ (5, 6, or 7). NCIMB10586 WT with pAMH1 (*in trans* expression of *tmlU*), and NCIMB10586 WT with pJH10 vector used as controls. There was no detectable compound in strain with ACP5 pm when *tmlU* was expressed *in trans*. This result was the same as the mutant strain (ACP5 pm) even without *in trans* expression of any gene. In contrast, a compound eluted earlier in strains of either ACP6 or ACP7 pm with *tmlU* expression *in trans*, and this appears to be the same compound that has been detected and analysed in the WT with *in trans* expression of *tmlU* (Figure 3.12). That compound was identified in previous experiments as a shorter pseudomonic acid, the $C_5$ version of either PA-B or PA-B or both. This result may rule out the possibility that TmlU interacts with the phosphopantetheinylated version of the active site of the MmpB ACP$_S$.

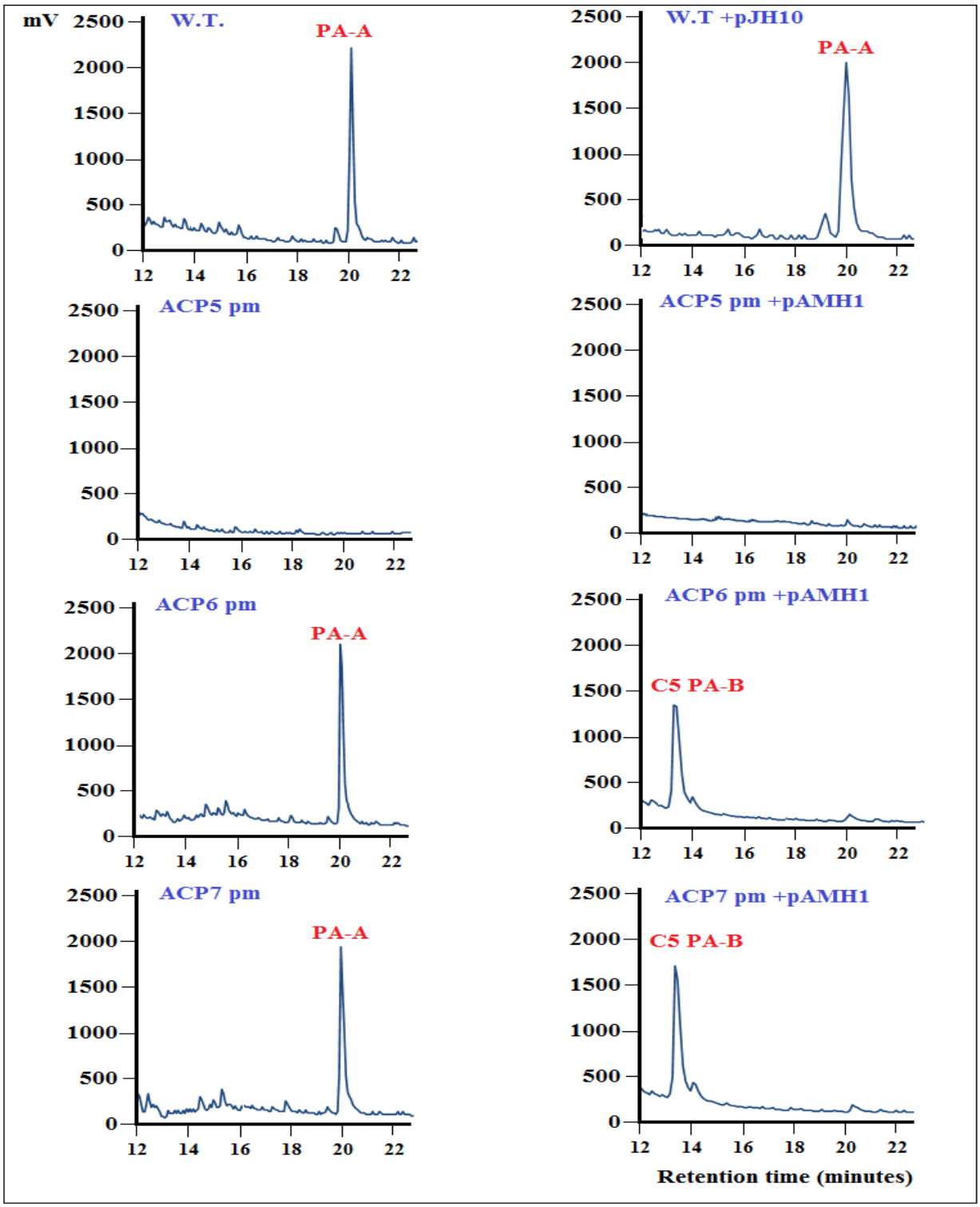



**Figure 3.12 HPLC chromatograms of IPTG induced NCIMB10586 strains with $ACP_S$ (either 5, 6, or 7) active site mutations, with pAMH1 plasmid expressed *in trans*. NCIMB10586 WT and WT+pJH10 strains are controls.**

### 3.3.5.2 TmlU effect in NCIMB10586 strains with $ACP_S$ (5, 6, or 7) single deletions

Expression of *tmlU* in NCIMB10586 strains with single deletions in one of the $ACP_S$ of MmpB carried out using the expression vector pAMH1. The purpose of this experiment was to see whether we are able to link the *tmlU* effect to one of those $ACP_S$. Bioassay was performed using *Bacillus subtilis* 1064 as sensitive bacteria, and plates of both LA and LA supplemented with 0.5 mM IPTG. Clearance zone around the spot of the culture was measured from the disc diameter as zone of antibacterial activity, and all the calculations made to work out the final result using WT once as 100 %. Data from this quantitative bioassay showed a stronger effect of TmU on the biosynthesis of mupirocin in NCIMB10586ΔACP7, than on NCIMB10586ΔACP5 without induction. Moreover, this effect was much stronger when *tmlU* expression was induced with 0.5 mM IPTG that blocks biosynthesis of mupirocin. However, this effect was less on NCIMB10586ΔACP6 even with induction (Figure 3.13 and 3.14).

In order to be more confident about any particular compound that is being released as a result of *tmlU* expression in NCIMB10586 strains with single deletion in one of the $ACP_S$ (5, 6, or 7) of MmpB, HPLC analysis performed. NCIMB10586 WT, and NCIMB10586 WT with pJH10 vector used as controls. The HPLC chromatograms showed that TmlU blocks mupirocin biosynthesis and again causes the formation of the new compound, which has a retention time between 13 to 14 min which is the same compound as observed previously with *tmlU* expression in NCIMB10586 WT, and strains with active site mutation in ACP6 and ACP7. However, a small peak of PA-A was observed in the strain with the ACP6

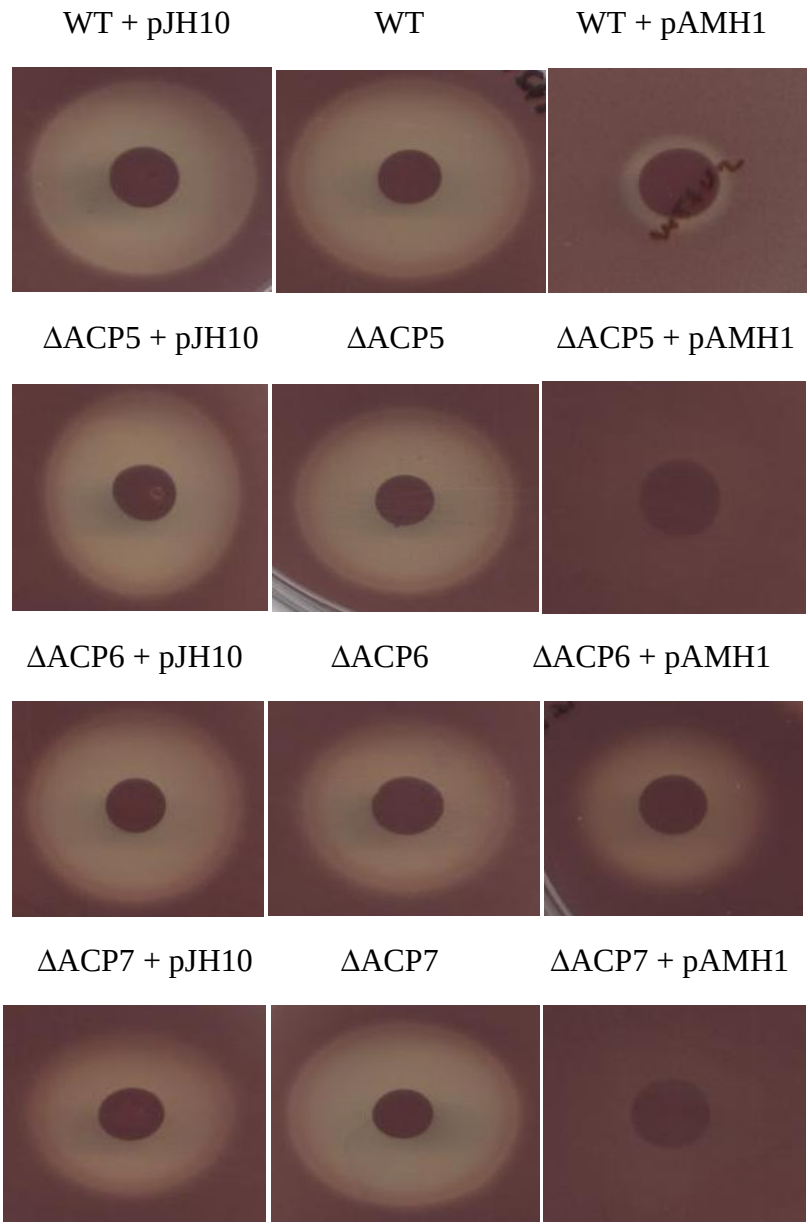



**Figure 3.13 Bioassay to show the expression of *tmlU*, and its influence on NCIMB10586 strains with deletion in one of the $ACP_S$ (ACP5, ACP6, or ACP7) of MmpB, separately. NCIMB10586 WT, WT with pAMH1 plasmid, and WT with pJH10 vector were taken as controls.**

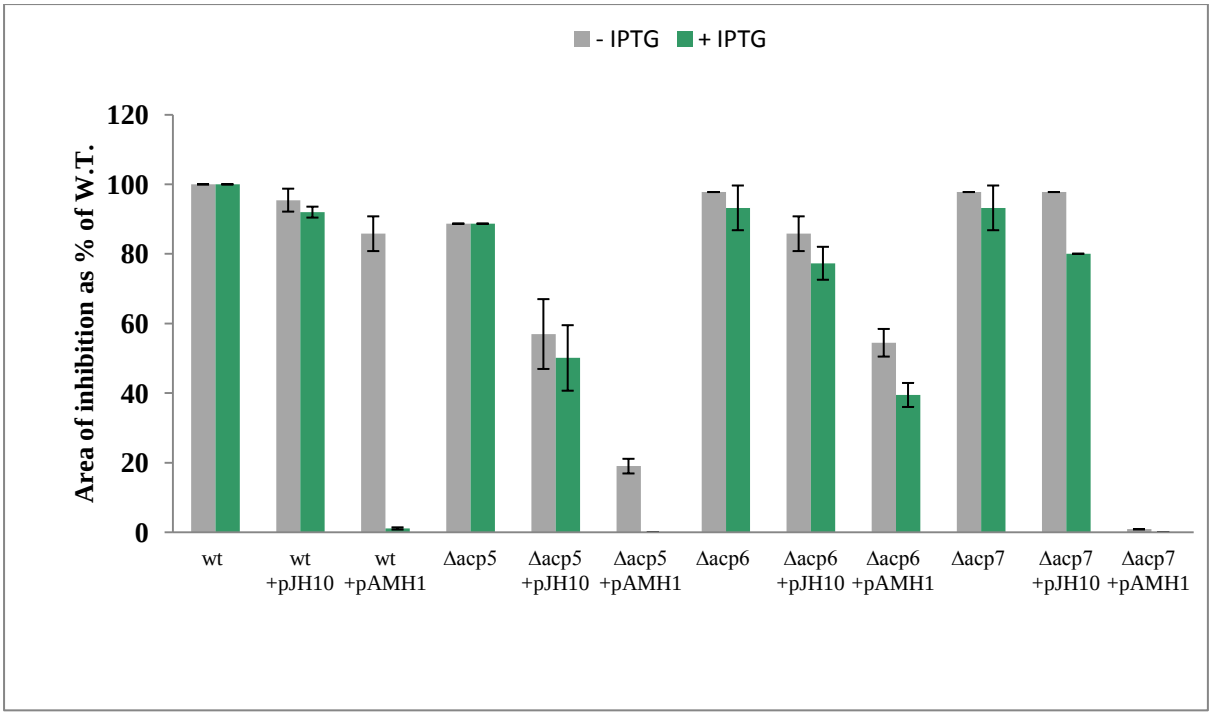



**Figure 3.14 Quantitative bioassay of NCIMB10586 strains with deletion in one of the ACP$_S$ (ACP5, ACP6, or ACP7) of MmpB, separately, expressing *tmlU* from pAMH1 plasmid. NCIMB10586 WT, WT with pAMH1 plasmid, and WT with pJH10 vector were taken as controls. 5 replicates were used for each case.**

deletion, in addition to the new compound that has been seen in strains with deletion in other ACP$_S$ (Figure 3.15). The observation that deletion of ACP6 appears to reduce the sensitivity to the negative effects of *tmlU* expression is very significant since it provides the first direct evidence that MmpB is involved in 9HN production. It suggests that normal ACP configuration is needed for sensitivity and the fact that NCIMB10586ΔACP6 is still sensitive to *tmlU* expression may indicate that *tmlU* interacts with all the ACP$_S$ in the tandem ACP of MmpB.

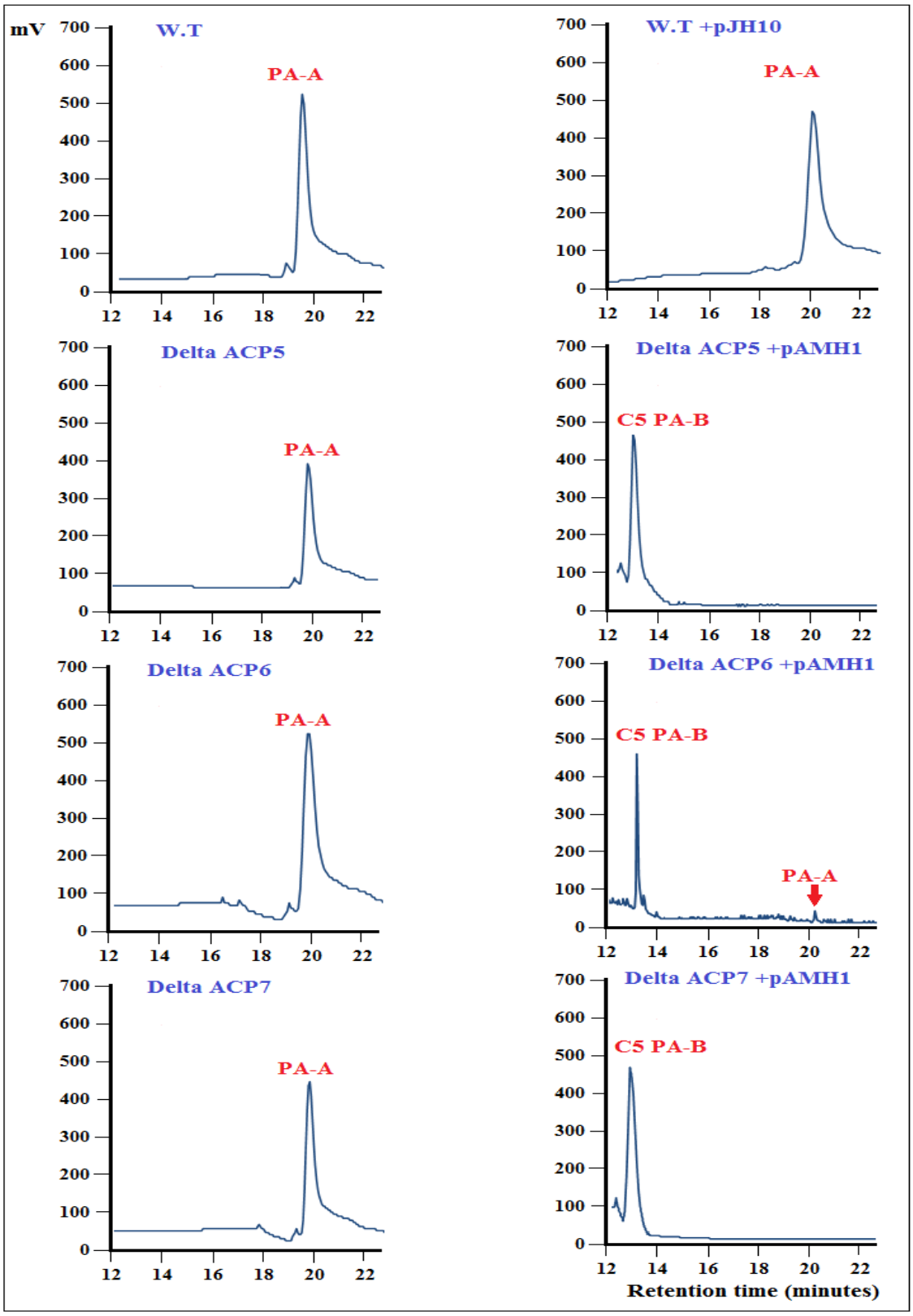



**Figure 3.15 HPLC chromatograms of IPTG induced NCIMB10586 strains with single deletion in one of the $ACP_S$ (5, 6, or 7) of MmpB with pAMH1 plasmid expressed *in trans*. NCIMB10586 WT and WT+pJH10 strains are controls.**

### 3.3.5.3 TmlU and $ACP_S$ (5, 6, or 7) double deletion

As the results of *tmlU* expression in NCIMB10586 strains with single deletion in $ACP_S$ (5, 6, or 7) suggested that TmlU may interact with the $ACP_S$, and to obtain more specific details about that interaction, *tmlU* was expressed in NCIMB10586 strains with double deletions of those $ACP_S$. Quantitative bioassay showed that the effect of *tmlU* was very similar, in which there was a very small inhibition zone with all the three double deletions of $ACP_S$ when *tmlU* is being expressed, in a way that is negligible, since NCIMB10586 WT showed the same zone, and it confirms that TmlU is interacting with all those $ACP_S$ (Figure 3.16 and 3.17). As expected, there was no observable negative effect of pJH10 vector on any of those strains with double deletion of $ACP_S$.

HPLC analysis was carried out to confirm the data obtained by bioassay, and to make sure whether the same compound is being released by *tmlU* expression in those strains with double $ACP_S$ deletion. NCIMB10586 WT and WT with pJH10 vector were used as controls. The HPLC chromatograms showed the release of the same compound that has been seen with previous experiments about TmlU interaction. This compound eluted earlier than the standard mupirocin and with a different level in each strain with double $ACP_S$ deletion (Figure 3.18). Although of presence of a very small peak that looks to be PA-A (based on retention time) in NCIMB10586ΔACP6/7 and NCIMB10586ΔACP5/7 with *tmlU* expression, and a very less clear one with NCIMB10586ΔACP5/6, however, since it is totally different from the one that has been detected in NCIMB10586ΔACP6 with *tmlU*, it could not be consider as PA-A. This exclude the possibility of the release of even a tiny amount of PA-A, since there is only one ACP in NCIMB10586 strains with double $ACP_S$ deletion which *tmlU* interact with.

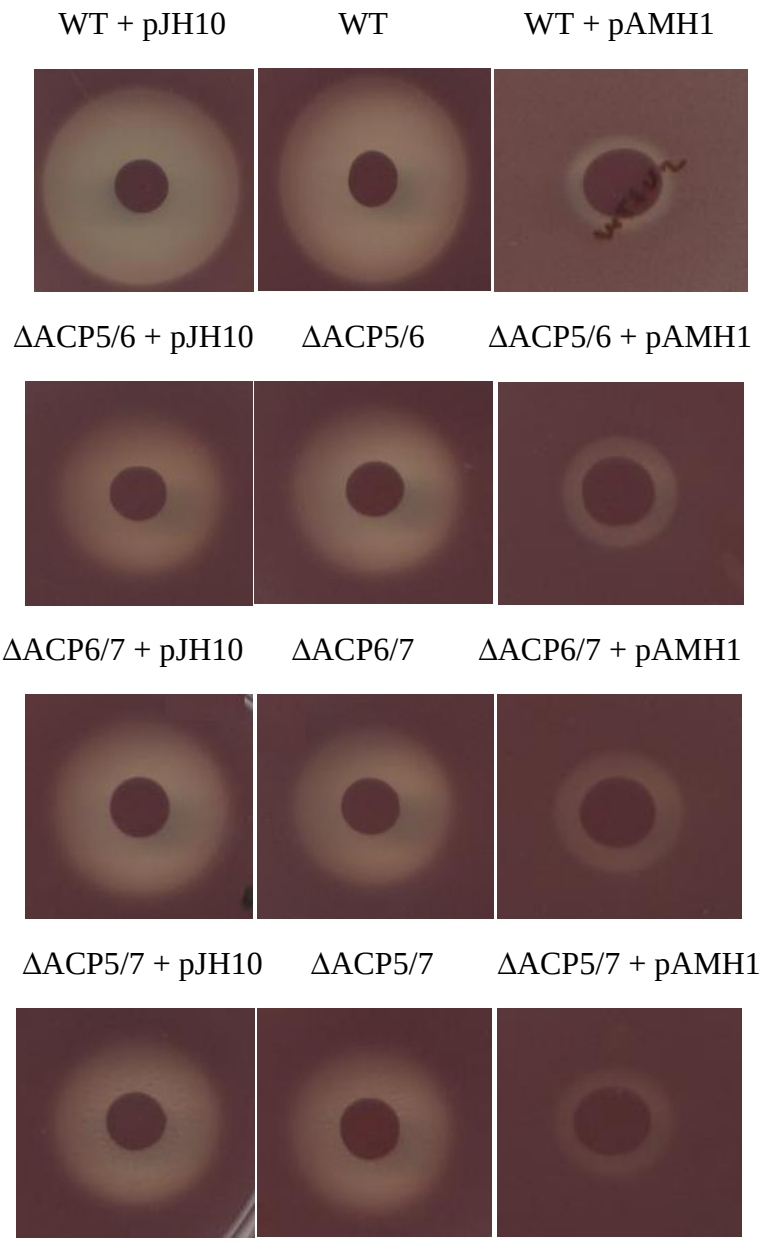



**Figure 3.16 Bioassay to show the expression of *tmlU*, and its influence on NCIMB10586 strains with double deletion of the $ACP_S$ (ACP5, ACP6, or ACP7) of MmpB, separately. NCIMB10586 WT, WT with pAMH1 plasmid, and WT with pJH10 vector were taken as controls.**

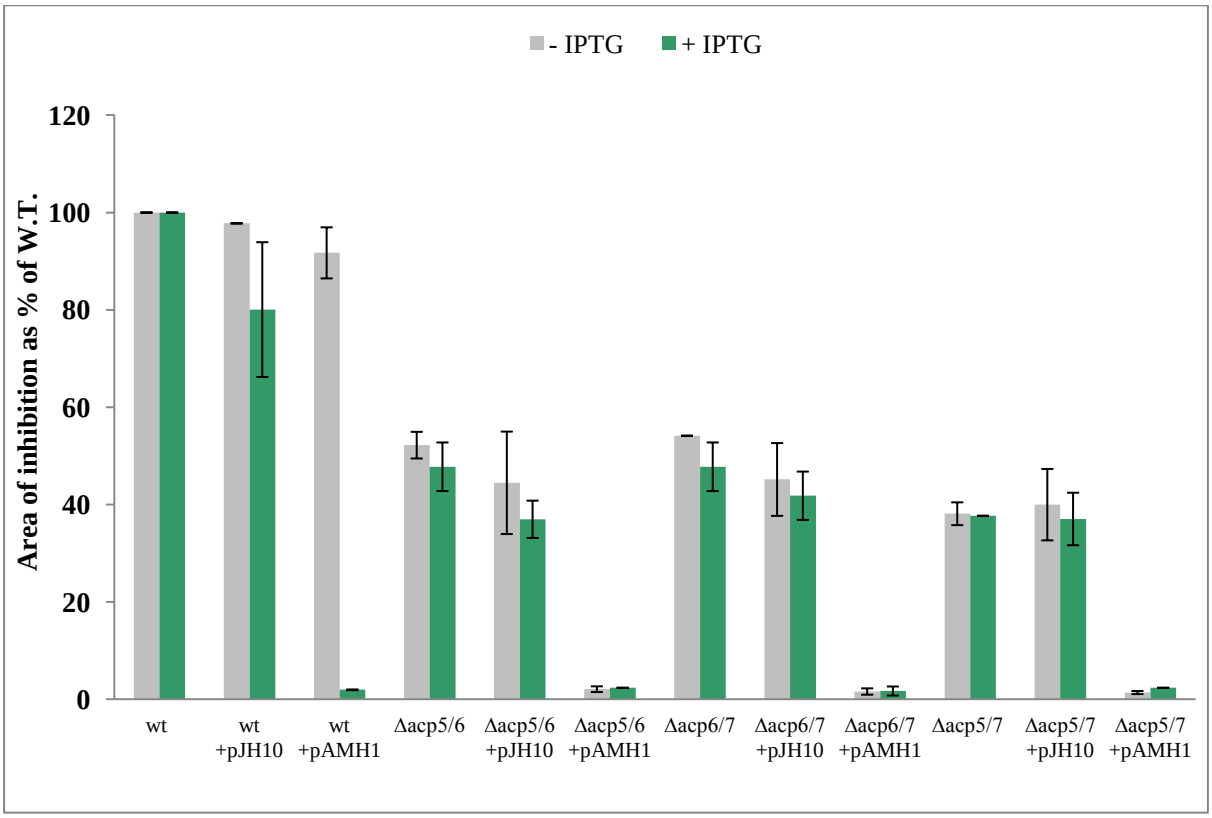



**Figure 3.17 Quantitative bioassay of NCIMB10586 strains with double deletion of the $ACP_S$ (ACP5, ACP6, or ACP7) of MmpB, separately, expressing *tmlU* from pAMH1 plasmid. NCIMB10586 WT, WT with pAMH1 plasmid, and WT with pJH10 vector were taken as controls, also triplicates samples were used.**

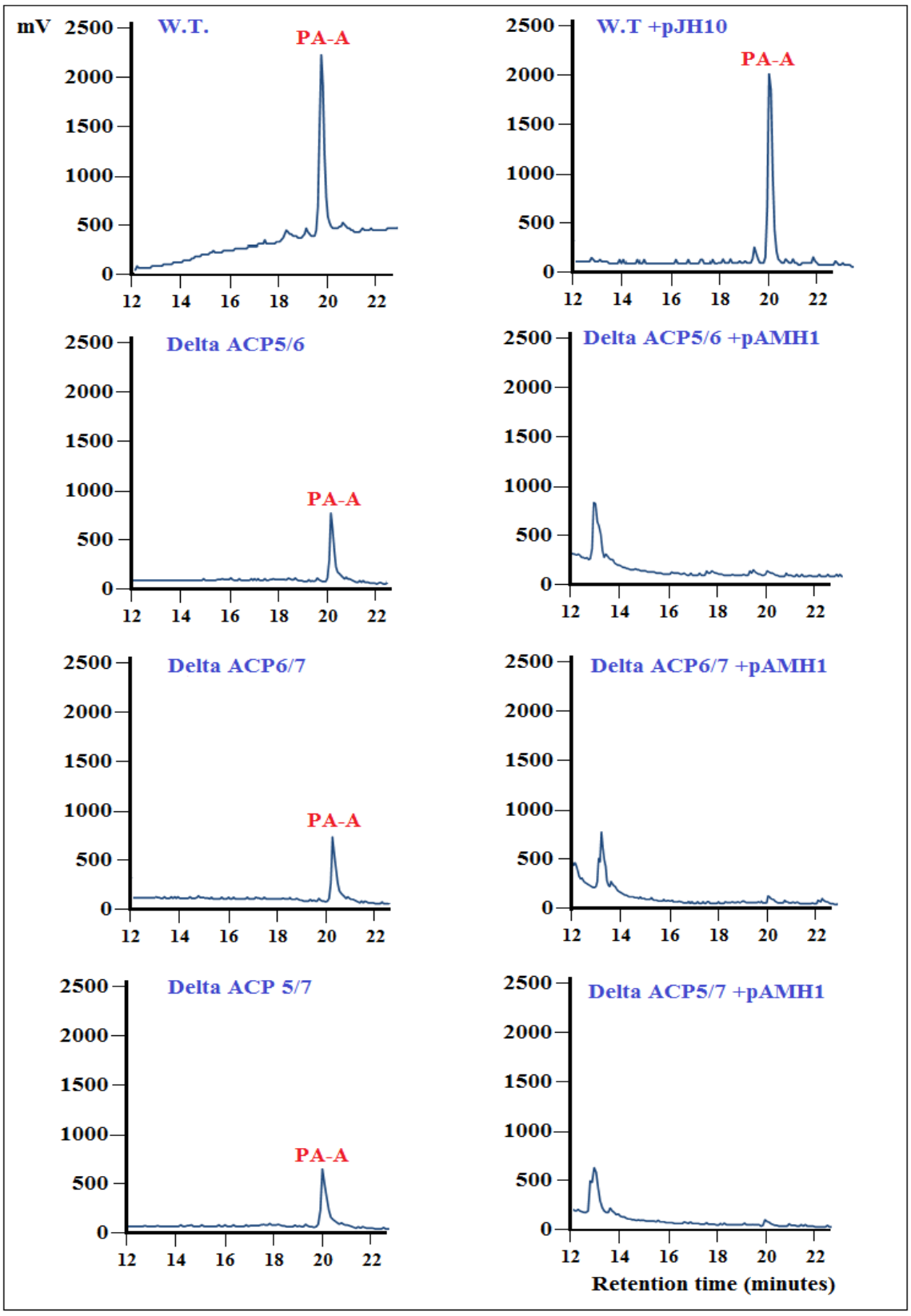



**Figure 3.18 HPLC chromatograms of IPTG induced NCIMB10586 strains with double deletion of the $ACP_S$ (5, 6, or 7), with pAMH1 plasmid expressed *in trans*. NCIMB10586 WT and WT+pJH10 strains are controls.**

## 3.4 Discussion

Prior work has documented that amide synthetase implicated in additional role to the ligase activity during the biosynthesis of antibiotics. In aminocoumarins as an example, reports indicated that, although CouL from coumermycin $A_1$ is active in the form of a monomer, it is able of catalysing two adenylation and two acyl transfer reactions, results in the formation of two amide bonds (Schmutz *et al*., 2003). Recent investigations from rubradirin biosynthesis revealed more than one gene responsible for the aminocoumarin acyl ligases, and that RubC1 is a bi-functional enzyme that might catalyse both the activation of tyrosine as well as the aminocoumarin acyl ligation (Boll *et al*., 2011). However, these studies have just focused on the role of amide ligases within the family of amincoumarin antibiotics rather than being expanded to other antibiotics with similar ligases. In this study the putative amide synthetase TmlU from the biosynthesis pathway of thiomarinol was tested for the possibility of complementing the defect in MupU from mupirocin biosynthetic pathway, the most related antibiotic to thiomarinol. We also wished to investigate any additional interesting role that TmlU might have which may open a strategic way to the development of novel compounds with improved characteristics.

The results indicated that TmlU cannot complement NCIMB10586Δ*mupU*, and the effect was stronger when induced with IPTG which prevented any observable biological activity, and this means that TmlU cannot promote the movement of the intermediate from MmpB to mAcpE assuming that the scheme proposed for the role of MupU and mAcpE in mupirocin biosynthesis (Hothersall *et al*., 2007) is correct. Most striking was the fact that not only did TmlU not complement MupU, but it even seemed to reduce the antibacterial activity remaining in the NCIMB10586Δ*mupU*, and this effect of TmlU was seen when expressed *in trans* in the NCIMB10586 WT and NCIMB10586Δ*mupX* as well. However, data from both

HPLC analysis and LC-MS followed by NMR experiment showed that TmlU promotes premature release of intermediates, which were confirmed to be homologues of PA-A and PA-B with truncated $C_5$ and $C_7$ side chains, while only the $C_5$ version instead of the $C_9$ side chain was observed as a major product in the case of IPTG induction (Figure 3.19). These indicate that the increased level of TmlU allows it to compete better with the normal processes happening on MmpB, and that essentially most of the intermediates are released after the first or the second round of condensation with a corresponding reduction in the release of specific PA-A with $C_9$ side chain. This indicates that in the NCIMB10586 WT, TmlU at low levels blocks elongation but does not block the conversion to PA-A. These findings tend to be in a good agreement with those of Hothersall *et al*., (2011), in which they observed related metabolites to pseudomonic acid A released in trace quantities while up-regulating NCIMB10586 WT via *in trans* expression of *mupR*, the transcriptional activator. Moreover, these results are consistent with Murphy's suggestion that both in mupirocin and thiomarinols the saturated acyl side chains does not essentially have to be produced by the ligation of the fully assembled ($C_9$ or $C_8$, respectively) hydroxy acids but are more likely to be built up by successive elongations from the PKS-derived product (Murphy *et al*., 2011).

It was hypothesised that TmlU might compete with TE to capture the activated thiolester intermediate in order to create an amide, but release it if there is no appropriate amino-donor, or probably it associates with TE and blocks its normal function. Therefore, to test the formulated hypothesis, *tmlU* was expressed *in trans* in the NCIMB10586ΔTE, and both bioassay and the HPLC data did not show the release of any product with biological activity neither with nor without IPTG induction. However, LC-MS results revealed the release of low amounts of truncated $C_5$ PA-A and PA-B with a very faint amount of $C_9$ PA-A. This result might indicate that TmlU could release some intermediates but with a less efficiency than in the presence of TE, in addition to affecting the elongation processes. However, in the

rifamycin biosynthesis cluster, the amide synthase encoded by *rifF* gene was found to function as a chain terminator by displacing the thioester linkage of the assembled polyketide chain, which means it is substituting for the function of TE (August *et al*., 1998). Comparable to the current findings, Yu *et al*., (1999), showed the release and accumulation of a series of linear polyketides from rifamycin B mutants, even when *rifF* gene was inactivated which results in the disruption of rifamycin B synthesis. Additionally, repositioning of the TE domain within *eryPKS* downstream of DEBS1 resulted in the production of a triketide lactone rather than the normal 14-member macrolide (Cortes *et al*., 1995). More surprisingly, polyketides of virtually all the elongation stages have been produced by Bacillaene (*bae*), the *trans*-AT PKS, in mutants with an inactivated TE domain (Moldenhauer *et al*., 2007, 2010). With the exception in rifamycin (Yu *et al*., 1999), this phenomenon has not been observed in *cis*-AT $PK_S$ and suggests the existence of a proofreading mechanism that releases the stalled acyl intermediates (unites) from the enzyme, which has not been characterised yet (Jensen *et al*., 2012). Later on, the same group proved in Pederin, that PedC, a member of $AT_2$ group does not display detectable AT activity (loading $ACP_S$ with malonyl units), but rather acts as a proofreading enzyme by removing the stalled acyl units from blocked modules. Therefore, they proposed to rename this enzyme as acyl hydrolase (AH) than PedC or $AT_2$, and suggested that to enhance polyketide yields, addition of enzymes in this group ($AH_S$) to *tran*-AT PKS pathways lacking these enzymes could have a meaningful result. Concomitantly, in the mupirocin system, AT2 was found to be responsible for loading $ACP_S$ with malonyl extender units while not AT1 (Gurney, PhD thesis, University of Birmingham, 2013), which might work as a proofreading function in the system, and presumably, might explain the reason why we are getting the release of a tiny amount of $C_9$ PA-A in mutant with TE deletion, as well as the release of stalled oligoketides ($C_5$ PA-A and PA-B) when TmlU is present.

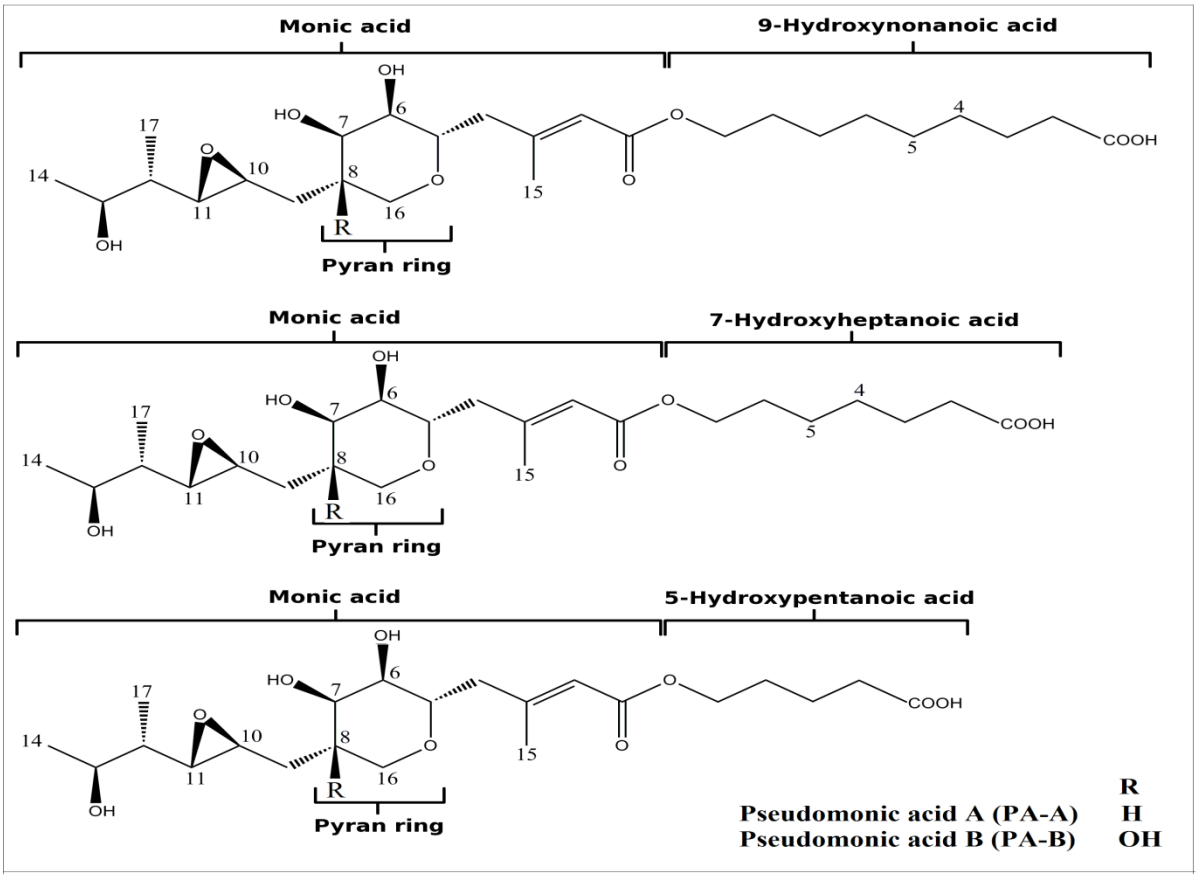



**Figure 3.19 Chemical structure of shorter pseudomonic acids ($C_5$ and $C_7$) version of PA-A and PA-B, compare to normal pseudomonic acid (mupirocin) with $C_9$ fatty acid side chain.**

Consequently, and as predicted by El-Sayed et al., (2003), that MmpB (with tandem $ACP_S$ 5, 6, and 7) is more likely to be responsible for the biosynthesis of 9HN (9-Hydroxynonanoic acid) by three condensations using malonate molecule, and our results showed that TmlU blocks the 2nd ($C_5$-$C_7$) and the 3rd ($C_7$-$C_9$) round of condensation, but not the 1st one ($C_3$-$C_5$). Therefore, this might indicate that the 2nd and the 3rd round of condensation are more likely happening on MmpB, while the 1st condensation ($C_3$-$C_5$) might happen outside MmpB on another protein within the mupirocin system since at low levels of TmlU only the 3rd ($C_7$-$C_9$) round of condensation is blocked, followed by the 2nd ($C_5$-$C_7$) round when the levels of TmlU

increased, but without affecting the 1st (the $C_5$ version) condensation. Moreover, Rahman *et al*., (2005) showed that inactivating the $ACP_5$ (ACP-Ba) domain by Ser-to-Ala mutation at the acyl binding site, prevents other $ACP_S$ ($ACP_s$-Bb and Bc) from access to the machinery, suggesting they work in parallel. Therefore, we predicted that TmlU might interact with either the 2nd or the 3rd $ACP_S$ of MmpB. To evaluate this possibility, *tmlU* was expressed in NCIMB10586 strains with point mutation in the active site of MmpB $ACP_S$. Bioassay data revealed blocking the production of biologically active product in all three strains with point mutation when TmlU was available at high levels. This result was consistent with the HPLC chromatograms, that showed production of truncated shorter pseudomonic acids when *tmlU* expressed in $ACP_6$ (Bb) and $ACP_7$ (Bc) point mutants but not in the strain with $ACP_5$ (Ba) point mutation, as already there is no product released from this mutant. This indicates that TmlU is not targeting the active site serine residue of the tested $ACP_S$ since it is still able to block the biosynthetic pathway and modify PA-A. To convince ourselves about the possibility of any protein-protein interaction between TmlU and either one or more $ACP_S$ of MmpB, *tmlU* expression in NCIMB10586 mutants with both single and double deletion in those $ACP_S$ was carried out. Plate bioassay revealed blocking of biosynthesis, and there was no production of biologically active product of significance in mutants with double ACP deletions and even in NCIMB10586 mutants with single deletion of ACP5 (Ba) and $ACP_7$ (Bc), respectively, when *tmlU* induced. However, a reasonable amount of antibacterial activity was observed in $ACP_6$ (Bb) mutant, even in presence of high levels of TmlU. Subsequent HPLC analysis confirmed modification of $C_9$ PA-A to the truncated $C_5$ version in all the deletion mutants mentioned, with the exception of production of observable amount of $C_9$ PA-A in $ACP_6$ (Bb) mutant in addition to the truncated compound. Therefore, these data did not give support to the idea of protein-protein interaction between TmlU and MmpB-

$ACP_{S,}$ and at present, we do not have such a model to explain that, the fact that needs more experiments (Figure 3.20).

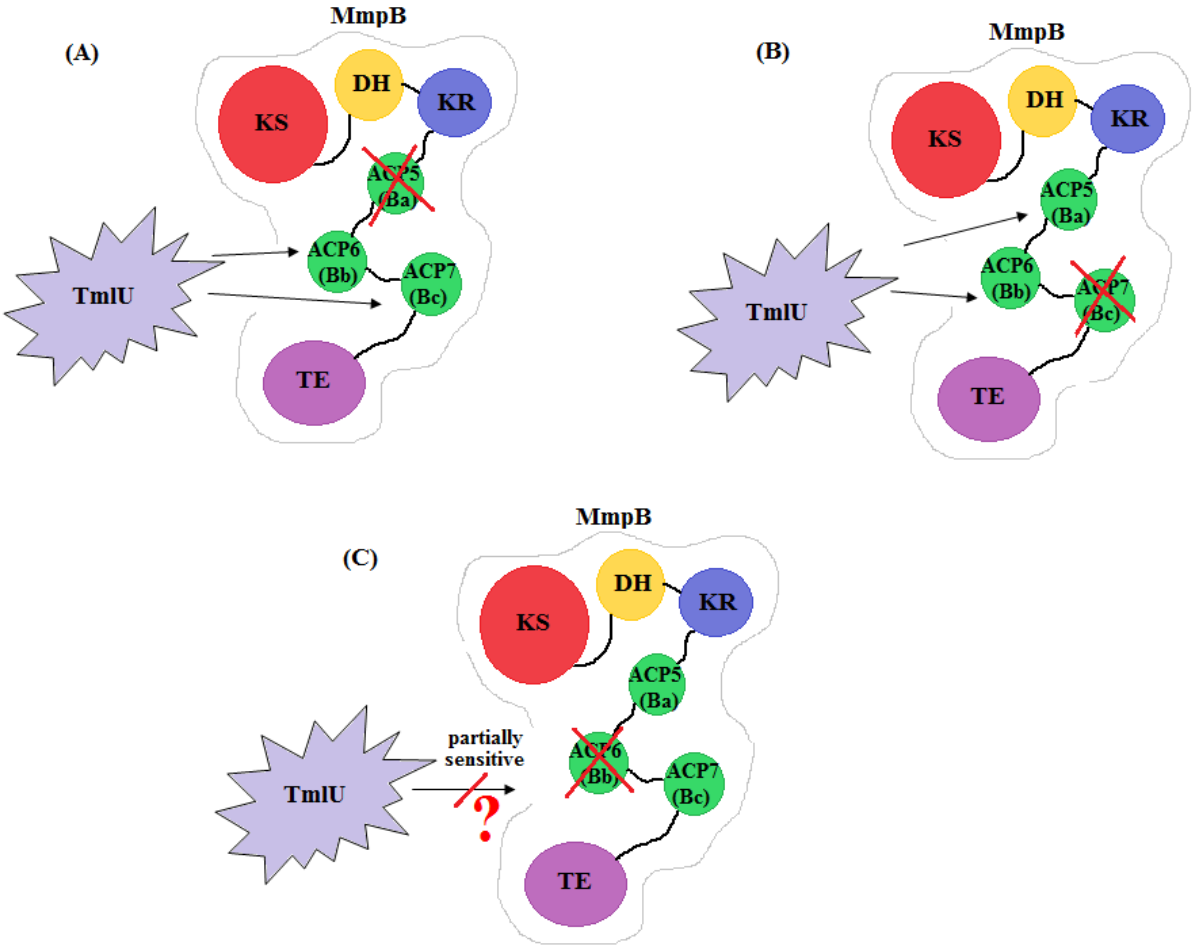



**Figure 3.20 Primary proposed interaction of TmlU (thiomarinol amide ligase) with MmpB of mupirocin biosynthetic pathway.** (A), and (B) interference of TmlU with mupirocin system with either ACP5 (Ba) or ACP7 (Bc) deletion of MmpB, and partial sensitivity with ACP6 (Bb) deletion in (C).

Recently, Keatinge-Clay describes the control of the flow of polyketide intermediates in modular type I PKS through the pathway as one of the biggest mysteries in the enzymology of this group of polyketide (Keatinge-Clay, 2012). This review proposed a theory supported by accumulating evidence about how $ACP_S$ interacts, in which apparently it interact with the

KS of the same module through a specific surface, while it interacts with the KS of the downstream module using a different surface, and that is either due to surface complementarity or geometric restraint imposed by the linker. However, and in order to come out with mechanisms that explains precisely the manner that control the flow of intermediates during polyketide synthesis, extra experimental data is needed (Kapur *et al*., 2010; Charkoudian *et al*., 2011; Kapur *et al*., 2012). Nevertheless, all cases of intermediate release, as a phenomenon involving in *trans*-AT $PKS_S$, for example, in Bacillaene (Moldenhauer *et al*., 2007), mupirocin (Wu *et al*., 2008), and rhizoxin (Kusebauch et al., 2009), have been reported in systems containing AH-type enzymes within the assembly line (Jensen *et al*., 2012). In accordance to this, Piel and co-workers proposed the significance of this coincidence due to high selectivity of $KS_S$ for the α,β-functionalization of the incoming substrates shown by phylogenetic (Irschik *et al*., 2010; Nguyen *et al*., 2008; Teta *et al*., 2010) and their other unpublished data. Therefore, they interpret the biosynthetic blocks as a result of faulty in β-processing, which in turn prevents further elongation of incorrectly processed intermediates.

In conclusion, we have presented new data indicating that TmlU, the amide synthetase in thiomarinol cluster, is not able to work instead of MupU (the Acyl CoA synthase) in mupirocin system, but rather it interferes with the biosynthesis of the 9HN part of mupirocin, which MmpB is predicted to be responsible for (El-Sayed *et al*., 2003). TmlU blocks the $2^{nd}$ and the $3^{rd}$ round of condensation in the synthetic stages of 9HN, but did not block the $1^{st}$ round of condensation, without affecting the conversion from PA-B to PA-A. By using NCIMB10586 strains with different forms of mutation in the $ACP_S$ of MmpB, we have been able to show that TmlU is not targeting the active site serine of the $ACP_S$ for the interaction with mupirocin cluster. In addition, we have obtained some data that indicates that TmlU may interacts with all the $ACP_S$ (ACP5, ACP6, and ACP7) in the tandem ACP of MmpB, while

deletion of ACP6 reduces the sensitivity to the negative effects of *tmlU* expression. To our knowledge, these observations are very significant since it provides the first direct evidence that MmpB is involved in 9HN production, and in addition, it provides evidence that the 2$^{nd}$ ($C_5$-$C_7$) and the 3$^{rd}$ ($C_7$-$C_9$) round of condensation might happen on MmpB, while the 1$^{st}$ ($C_3$-$C_5$) condensation might happen outside MmpB since this last condensation was not affected by TmlU.

# CHAPTER 4

## INVESTIGATING THE ROLE OF THE SECOND MODULE WITHIN TmpB OF THE THIOMARINOL BIOSYNTHETIC PATHWAY IN *Pseudoalteromonas* spp SANK 73390

## 4.1 Introduction

Modular (type I) polyketide synthases ($PKS_S$) (Donadio *et al*., 1991), are multifunctional enzymes that are involved in the biosynthesis of structurally diverse natural products of medicinally important (Brautaset *et al*., 2003). In a similar fashion to fatty-acid synthases, $PKS_s$ catalyse the decarboxylative condensation of basic carboxylic acids into the growing polyketide chain. However, as $PKS_S$ can use of different primer and extender units, they are more diverse in their catalytic reactions. Several different domains with distinct catalytic functions during the biosynthetic pathway are brought together in a single module which is responsible for a single condensation step during $PKS_S$ catalysis. Ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) are the minimal domains essential for a single condensation. Further additional domains which are responsible for various degrees of the reduction state of each $\beta$-carbon within the polyketide chain represented by ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) may be present in various combinations (Brautaset *et al*., 2003). The order of functional domains in modular $PKS_S$ reflect the series of reactions being carried out on the growing polyketide chain so that the most important part of the programming of this type of polyketide biosynthesis is essentially organisational (Tsukamoto *et al*., 1996).

Based on the structure of $PKS_S$, Staunton and Weissman, (2001) suggested that modules prime with a KS domain and end in an ACP, and in order for the polyketide chain to transfer and progress between modules, the ACP of one module requires partner KS in the next module. Only the linker region located in front of the next module but not its entire KS domain makes the modular unit. Also they reported the necessity of downstream linker for the first module and the high degree of tolerance exhibited by the second module to the protein structure that precedes it. Deletion of one or more specific modules could change the

chain length, while swapping one module with a heterologous one derived from another PKS (differ by a combination of enzymatic domains) could promote diversity, for example by changing the extent of processing of the polyketide chain by that module (Lal *et al*., 2000).

MmpB in the mupirocin biosynthesis cluster is proposed to be responsible for synthesis of 9-HN (Hothersall *et al*., 2007; Martin *et al*., 1989; El-Sayed *et al*., 2003). MupU is proposed to be responsible for the transfer of an intermediate (PA-B with C8-OH) to mAcpE which is then further processed by MupO, V, C and F for the production of PA-A (mupirocin) (Cooper *et al*., 2005a; Hothersall *et al*., 2007). Therefore, it is proposed that PA-B is acting either like a precursor or a side product of PA-A (Cooper *et al*., 2005a). Since there is no C8-OH in the structure of thiomarinol, we were interested to know how thiomarinol system get ride of C8-OH. In contrast to mupirocin system, the absence of mAcpE in the thiomarinol system and the presence of an extra KS and ACP (Figure 1.20) in TmpB (that is absent from MmpB in mupirocin system) suggest the possibility that this step in processing the intermediate for thiomarinol production might happen on TmpB prior to its release by the TE of TmpB (Fukuda *et al*., 2011). This raises the possibility that such an arrangement might lower the potential for the release of incomplete products (Fukuda *et al*., 2011) and could be the reason for not observing detectable amounts of thiomarinols, the equivalent to pseudomonic acid B which is with C8-OH (Murphy *et al*, 2011). Therefore, this work aimed to test that hypothesis by deleting the KS-B2/ACP-B2 represented as a second module of *tmpB* to see whether we could get an accumulation of molecules with an extra OH at C8.

## 4.2 Materials and methods

### 4.2.1 Bacterial strains and plasmids

*Escherichia coli* DH5α was used for plasmid transformation and propagation. *Pseudoalteromonas* spp SANK 73390 was used as the wild-type thiomarinol–producing bacterium. *E. coli* S17-1 was used to mobilise the suicide vector into *Pseudoalteromonas* spp by bacterial conjugation. *Bacillus subtilis* 1064 (thiomarinol-sensitive organism), was used in disc bioassays to detect the antibacterial activity of thiomarinol (Materials and Methods 2.2.6). DNA isolation and manipulations were described in Chapter 3. Samples preparation and HPLC analysis of the extracted thiomarinol was described in Chapter 2. Bacterial strains and plasmids used in this study are listed in Table 4.1 and 4.3, respectively.

### 4.2.2 Growth media and culture conditions

*Pseudoalteromonas* spp SANK 73390 was grown at 23$^{o}$C in marine broth and marine agar described in Chapter 2. *E. coli* strains DH5α and S17-1 were grown at 37$^{o}$C in L-broth and L-agar described in Chapter 3 and supplemented with 50 µg/ml kanamycin, while *Bacillus subtilis* 1604 was grown at 37$^{o}$C in L-broth only without any supplement. Antibiotics used during this study are listed in Table 4.2. All medium was sterilised by autoclaving (121$^{o}$C at a pressure of 15 pounds per square inch for 15 min) before adding antibiotics or sucrose.

**Table 4.1 Bacterial strains used or constructed during this study.**

| Bacterial strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli* DH5α | F⁻ *Φ80dlacZΔM15, recA1, endA1, gyrA86, thi-1, HsdR17($r_{k^-}$, $m_{k^+}$), supE44, relA1, deoR, Δ(lacZYA-ArgF)U169* | Hanahan, 1983 and 1985 |
| *Pseudoalteromonas* spp SANK 73390 | Wild type (WT) strain which produces thiomarinol | Fukuda *et al.*, 2011 |
| *Escherichia coli* S17-1 | *RecA pro hsdR RP4-2-Tc::Mu-km::Tn7* | Simon *et al.*, 1983 |
| *Bacillus subtilis* 1604 | *Trp*C2 | Moir *et al.*, 1979 |
| *Pseudoalteromonas* spp SANK 73390 Δ*tmpB* 2nd module | *Pseudoalteromonas* spp SANK 73390 with deletion of 2nd module (KS-ACP) in *tmpB* gene | This study |

**Table 4.2 Antibiotics used in this study.**

| Antibiotic | Stock solution (mg/ml) | Working concentration (µg/ml) |
|---|---|---|
| Ampicillin | 100 | 100 |
| Kanamycin sulphate | 50 | 50 |

**Table 4.3 Plasmids used and constructed in this study.**

| plasmid | Size (Kb) | Genotype Description | Reference |
|---|---|---|---|
| pAKE604 | 7.2 | $Ap^R$, $Km^R$, *oriT*, *lacZα*, *sacB* | El-Sayed *et al*., 2001 |
| pGEM-T Easy | 3.0 | $Ap^R$, *lacZα* (PCR cloning vector) | Promega |
| pAMG1 | 3.672 | 0.672 kb *Eco*RI-*Bam*HI fragment containing N-terminal fragment of 2nd module of *tmpB* cloned into pGEM-T Easy | This study |
| pAMG2 | 3.521 | 0.521 kb *Bam*HI-*Xba*I fragment containing C-terminal fragment of 2nd module of *tmpB* cloned into pGEM-T Easy | This study |
| pAMKE | 8.393 | 1.193 kb (arm1 672 bp *Eco*RI-*Bam*HI and arm 2 521 bp *Bam*HI-*Xba*I) PCR products for deletion of 2nd module of *tmpB* cloned into *Eco*RI-*Xba*I of pAKE604. | This study |

**Table 4.4 Primers used in this study.**

| Gene | Primer | Length (bp) | Primer sequence, 5'→3' | Purpose | Restriction sites |
|---|---|---|---|---|---|
| *tmpB* | FmutB1 | 30 | CACGAATTCATTGAGATCTCAGCCACGCTG | In frame Deletion of the 2nd module (KS-B2-ACP-B2) of *tmpB* | *EcoRI* |
| | RmutB1 | 29 | GCCGGATCCCAGATAATCTACCAGCGCCG | | *BamH1* |
| | FmutB2 | 31 | GCCGGATCCATTACCGAACGGTTAATAGAGG | | *BamH1* |
| | RmutB2 | 30 | CGCTCTAGAGCTTGCGTTAATGGCTCTATC | | *XbaI* |

### 4.2.3 Polymerase chain reaction

The PCR procedure (Saiki *et al*., 1985; Saiki *et al*., 1988) allows the *in vitro* amplification of specific DNA regions by multimillion fold replication using nanogram amounts of a specific segment of DNA as a template, and two specific primers complementary to the ends of the amplified DNA sequence.

Either plasmid or chromosomal DNA was used as a template for PCR prepared using the boil preparation technique. Colonies from young bacterial cultures were picked up by a sterile 200 µl micropipette tip and placed into a sterilised microfuge tube containing 50 µl sterilised distilled water and vortex-mixed to make a homogenous suspension. The re-suspended bacterial colonies were boiled for 10 min with the microfuge lid closed with a special plastic lock to prevent contamination with the boiled water. The cells were pelleted by centrifugation at 14,000 *g* for 5 min and the supernatant (DNA) transferred to another sterile microfuge tube and kept in the ice. The dried oligonucleotide primers were purchased from Alta Biosciences, University of Birmingham and resuspended using the recommended amount of sterile distilled water to make up 100 pmol/ µl concentration (100 µM) with gentle mixing. From this, 10 µM stocks were prepared and the rest stored at -20$^{o}$C. The 10 µM was stock used whenever PCR reactions were performed using the Bioline Velocity kit for the amplification of DNA fragments for cloning since it is provided with proofreading activity. Taq DNA polymerase (Invitrogen) was used whenever proofreading was not essential, for example when checking for mutations introduced into the DNA. The annealing temperature was set to 5$^{o}$C less than the melting temperature of the primers, and the extension times were calculated based on 15-30 second per each kb of the amplified DNA, which is equal to 0.06s per each base pair.

### 4.2.3.1 PCR using Velocity™ DNA polymerase (Bioline)

Amplification of the DNA fragments for cloning carried out using Velocity kit (Bioline), which includes a highly thermostable proofreading DNA polymerase that provides 50-fold higher fidelity than Taq DNA polymerase and results in higher yields of the PCR products. It includes 5'-3' DNA polymerase and 3'-5' proofreading exonuclease activities as well. In addition, it is desirable for amplification of difficult templates and for mutagenesis. A standard reaction contained 48 µl of the below mixture:

| Reagent | Volume per Reaction (µl) |
|---|---|
| 5x Hi-Fi Reaction Buffer (contains 10 mM $Mg^{2+}$) | 10 |
| dNTPs (2.5 mM) | 5 |
| Forward primer (10 µM) | 2 |
| Reverse primer (10 µM) | 2 |
| DMSO | 1.5 |
| Water ($ddH_2O$) | 27.0 |
| Velocity DNA polymerase | 0.5 |

Due to the inherent 3'-5' exonuclease activity of the Velocity DNA polymerase's, the enzyme was added as last ingredient or just before dispensing and adding DNA so as to prevent the primers from the degradation. Amplification of each DNA fragment was carried out using four reactions, therefore, the above reaction mixture was prepared according to the number of the reactions. 48 µl of the reaction mixture was added to each of the four reactions. To three of them 2 µl of prepared DNA was added, while 2 µl of SDW was added to the fourth one as a control. PCR reactions carried out using SensoQuest Lab-cycler and the programme with the following parameters:

| | | | | |
|---|---|---|---|---|
| **Step 1** | **Initial denaturation** | **98°C** | **2 min** | **1 cycle** |
| **Step 2a** | **Denaturation** | **98°C** | **30 s** | **25-35 cycles** |
| **Step 2b** | **Annealing** | **(Tm-5)°C** | **30 s** | |
| **Step 2c** | **Extension** | **72°C** | **15-30s /kb** | |
| **Step 3** | **Extension** | **72°C** | **7 min** | **1 cycle** |

Since Velocity generates blunt-ended amplicons which are not suitable for effective integration into TA cloning vectors such as pGEM-T Easy vector (Promega), therefore an A' overhang was added to each PCR product (section 4.2.4) to facilitate cloning into sequencing vectors.

### 4.2.3.2 PCR using Taq DNA polymerase (Invitrogen)

For checking bacterial isolates for any mutation introduced into the DNA, Taq DNA polymerase obtained from *Thermus aquaticus* was used for performing PCR reactions. The following 45 µl ingredients were included in the mixture of PCR reactions:

| Reagent | Volume per Reaction (µl) |
|---|---|
| 10X PCR Buffer minus Mg | 5 |
| dNTPs (2.5 Mm) | 4 |
| Taq DNA polymerase | 0.6 |
| 50% Glycerol | 10 |
| Forward primer (100 µM) | 0.3 |
| Reverse primer (100 µM) | 0.3 |
| 50 mM Magnesium Chloride | 1.0 |
| SDW | 23.8 |

According to the number of PCR reactions, enough of the above mixture was prepared, and then 45 µl was dispensed into each PCR reaction tube. 5 µl of the DNA boil prep from the wild type strain (control negative), and from the bacterial isolates to investigate the mutation was added to separate reaction tubes, respectively. 1 µl of plasmid DNA, the suicide vector that has been used for mutagenesis plus 4 µl of sterile distilled water were added to another reaction tube as positive control, while only 5 µl of SDW was added to the last reaction tube as a control negative.

The SensoQuest Lab-cycler was adjusted to the desired PCR programme suitable for Taq DNA polymerase performance was consist of the following parameters:

| | | | | |
|---|---|---|---|---|
| **Step 1** | **Initial denaturation** | **94$^{o}$C** | **2 min** | **1 cycle** |
| **Step 2a** | **Denaturation** | **94$^{o}$C** | **15 s** | **29 cycles** |
| **Step 2b** | **Annealing** | **(Tm-5)$^{o}$C** | **30 s** | |
| **Step 2c** | **Extension** | **72$^{o}$C** | *** s** | |
| **Step 3** | **Extension** | **72$^{o}$C** | **7 min** | **1 cycle** |

* Length of the sequence in base pair was taken as an estimate to calculate the extension times per seconds

## 4.2.4 Addition of ‘A’-overhangs to purified PCR products

PCR products were run in agarose gel electrophoresis and purified from the gel as described in Chapter 3. Since the Velocity DNA polymerase generate PCR products with blunt-ended which makes them not ready for ligation into pGEM-T Easy vector which has ‘T’-overhangs on both 3´-ends. Therefore, an ‘A’-tail must be added to the 3’-ends of the gel

purified and eluted PCR products in order to make them suitable for ligation into pGEM-T Easy vector. 6 µl of the eluted PCR product was added to a sterile microfuge tube. To this, 1 µl of 10X buffer without magnesium chloride, 1 µl of 2 mM dATP (which was prepared by mixing 2 µl of 100 mM dATP with 98 µl SDW), 1 µl magnesium chloride, and 1 µl Taq DNA polymerase were added. The whole contents were mixed gently and then heated at 70$^{o}$C for 30 minutes using hot block.

### 4.2.5 Cloning into the pGEM-T Easy vector

The prepared PCR products with 'A'-overhangs added to the 3´-ends were used for cloning into pGEM-T Easy vector (Promega). A linearized pGEM-T Easy vector contains a single 3´-thymidine at both ends, which makes it compatible to the 'A'- tailed PCR products. In addition, the presence of the '3'-T overhangs at the insertion site of the vector improves the efficiency of the ligation into this vector and prevents it from re-circularization as well. The following ligation mixture in a total 12 µl worked efficiently:

| **Reagent** | **Volume per Reaction (µl)** |
|---|---|
| 2x Rapid Ligation Buffer | 6 |
| pGEM-T Easy vector (50 ng/ µl) | 1 |
| 'A'-overhangs insert DNA | 4 |
| T4 DNA Ligase (3 units/ µl) | 1 |

The ligation mixture was incubated at 4$^{o}$C overnight. Half of the ligation reaction (5-6 µl) was used for the transformation of competent DH5α, and the rest kept at 4$^{o}$C. 50 µl and 200 µl of the transformation mixture was plated out onto two separate LB-agar plates

supplemented with X-gal (80 µg/ml), IPTG (0.5 mM) and ampicillin (100 µg/ml) and incubated overnight at 37$^{o}$C for blue/white colonies selection.

### 4.2.6 DNA sequencing

For sequencing the genes or the PCR products cloned into pGEM-T Easy vector, plasmids were isolated from 5 ml overnight cultures as described in Chapter 3 using the *AccuPrep* plasmid extraction kit (Bioneer). 4 µl of eluted plasmid was then mixed with 3 µl of 1.1 µM universal forward primer once and 3 µl of 1.1 µM universal reverse primer in the second tube, and make up to a final volume of 10 µl with SDW. The whole 10 µl was loaded into micro-titration plate in the Functional Genomic Laboratory, University of Birmingham, and sequencing performed using the Sanger dideoxy method (Sanger *et al*., 1977) by using the ABI 3700 analyzer.

### 4.2.7 Sequencing analysis

The sequence of the cloned DNA compared to the original (wild type) sequence using the programmes provided by the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov), which were also used for translation of ORFs. The New England Biolabs (www.tools.neb.com/NEBcutter2/) was used for mapping the restriction sites. The Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html) was used for obtaining the reverse complement strand (5´ to 3´) for designing the reverse primers.

### 4.2.8 Construction of deletion mutant using suicide vector strategy

The strategy used by Rahman *et al*., (2005) and illustrated in Figure 4.1 was used for the construction of mutants with in-frame deletions. The protocol started by designing two different sets of primers for the PCR amplification of 672bp and 521bp-flanking regions on either side of the gene to be deleted and ligating them. Then the 1.163 kb insert was introduced into the suicide vector pAKE604 containing the $Amp^R$, $Kan^R$,*lacZα*, *sacB* as selectable markers, *E.coli* S17-1 was then transformed to mobilize the plasmid into *Pseudoalteromonas* spp SANK 73390. This suicide plasmid pAKE604 constructed in this way was transferred from *E.coli* S17-1 into wild type *Pseudoalteromonas* spp SANK 73390. Later on these two strains were mixed and then filtered via 0.45 μm sterile Millipore filter, followed by placing the filter on marine-agar plates without antibiotics, followed by overnight incubation at 23$^o$C in order for the conjugation to take place. The filter was then removed from the plate, and transferred into 1 ml of marine broth and vortex mixed to re-suspend the cells. Serial dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ of bacterial suspension were prepared using marine broth. 100 μl from each dilution was then spread on marine agar with kanamycin for the selection of *Pseudoalteromonas* spp SANK 73390 with pTML1 integrated suicide plasmid, which incubated at 23$^o$C for 2-3 days. Typical colonies of SANK 73390 were then selected and used for the inoculation of marine broth without antibiotic for the excision of the suicide vector, incubated overnight at 23$^o$C. Then serial dilutions prepared from the broth culture, and 100 μl of the dilutions was spread on marine agar containing 5.5% (w/v) sucrose for the selection of the strains in which the suicide plasmid had been excised from pTML1, followed by overnight incubation at 23$^o$C for 2-3 days. Selected colonies were picked up, and further screening for the sensitivity of those strains to kanamycin confirmed that excision of the suicide plasmid was successful. PCR of the wild type and the new strains gave quite reliable evidence that mutation occurred.

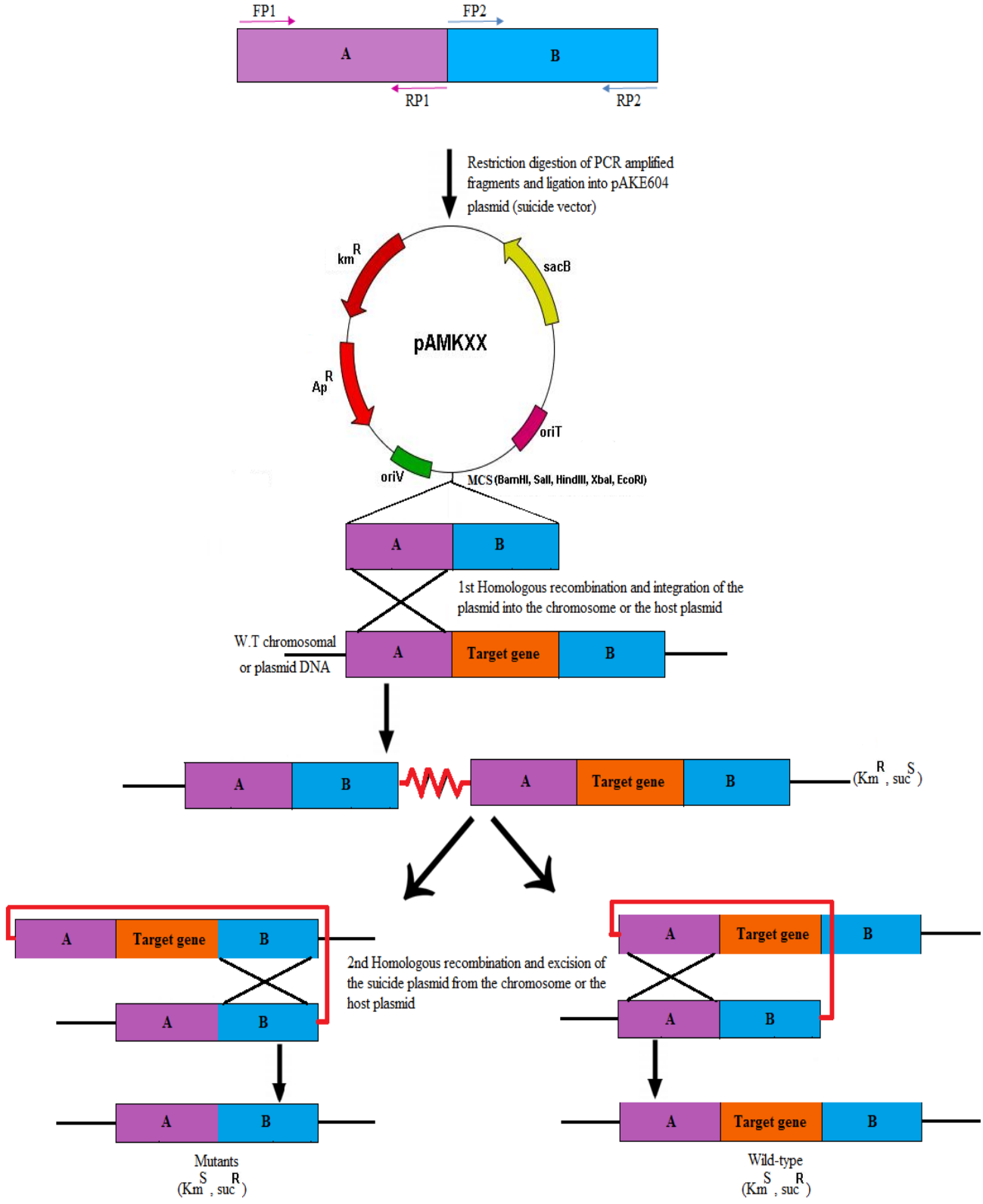



**Figure 4.1 Strategy for the suicide vector integration and excision.** pAMKXX is the suicide vector pAKE604, which XX refers to the specificity.

## 4.3 Results

### 4.3.1 Deletion of KS-B2 and ACP-B2 from TmpB does not block antibiotic production

Using the standard suicide vector strategy, in-frame deletion of the second module of *tmpB* in the thiomarinol system was achieved, removing KSB2/ACPB2 which are proposed to be non-elongating. Two mutants (different isolates) were confirmed as lacking the 2$^{nd}$ module. Since it was proposed that this extra KS and ACP might have a role in the removal of the $C_8$-OH group from marinolic acid (PKS part) of thiomarinol, strains lacking them either might switch on the system totally to compounds retaining $C_8$-OH group (compounds like PA-B in mupirocin) or at least might raise the level of such compounds. Disc bioassays were performed to check the biological activity of thiomarinol compounds extracted from mutants with KS-B2/ACP-B2 deletion using the WT *Pseudoalteromonas* spp SANK 73390 as a control. The area of the zone of inhibition was estimated and was taken as an indicator of the potency of the compounds produced by the mutants in comparison to those of the wild type. Production of thiomarinol by the mutants decreased compare to the WT *Pseudoalteromonas* spp SANK 73390 (Figure 4.2), and a reduction was about 30% less using the WT as 100% (Figure 4.3). Since the major compound produced by the WT strain is thiomarinol A (TH-A), the most potent thiomarinol compound, this strain should give 100% productivity and the biggest zone of inhibition. A smaller zone of inhibition with any mutants might be an indicator of the production of less TH-A, but could also indicate that the thiomarinol system could have shifted to some extent to a pathway specific for minor compounds with lower bacteriocidal activity. The latter effect was what we were proposing for the extra KSB2/ACPB2 in the biosynthesis of thiomarinol.

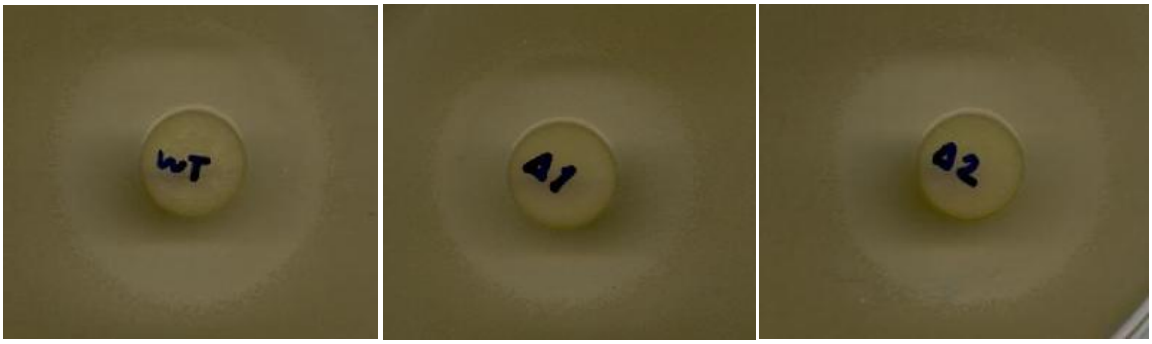



**Figure 4.2 Disc bioassay of thiomarinol produced by the KS-B2/ACP-B2 mutant using thiomarinol produced by the WT *Pseudoalteromonas* spp SANK 73390 as control.** From left to right WT: wild type strain, Δ1: ΔKS-B2/ACP-B2 isolate1, and Δ2: ΔKS-B2/ACP-B2 isolate2.

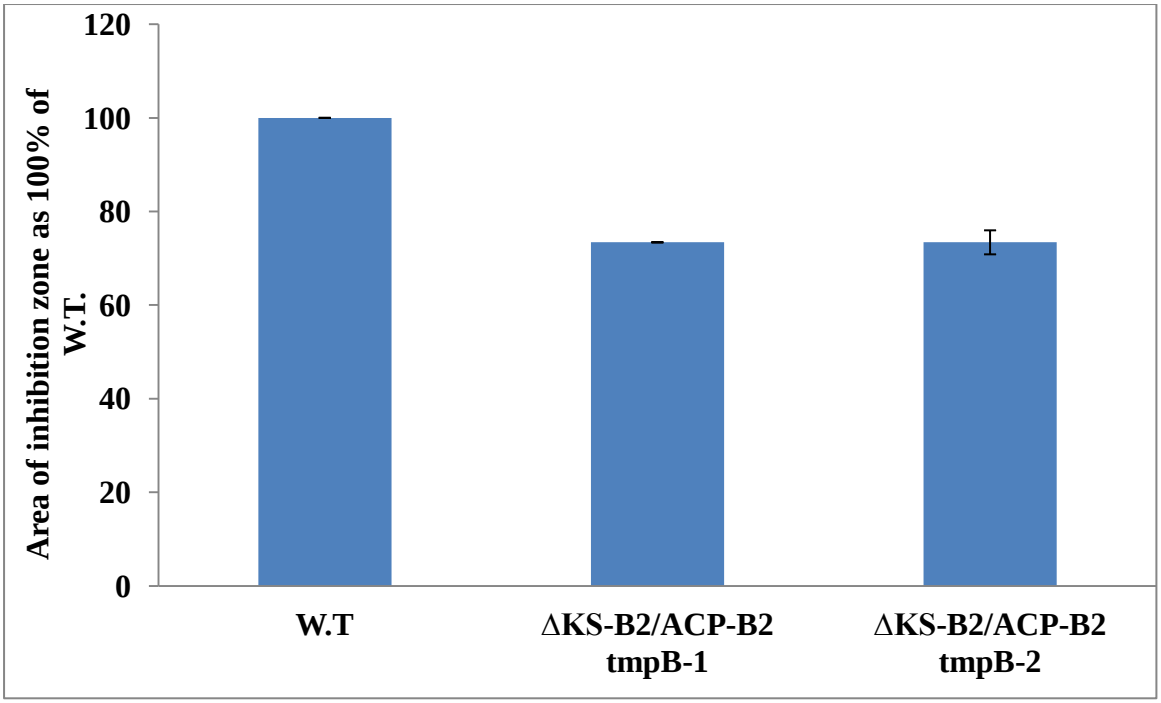



**Figure 4.3 Quantitative bioassay of *Pseudoalteromonas* spp SANK 73390 with ΔKS-B2/ACP-B2 of *tmpB* in comparison to the WT strain.** Triplicate samples were used.

### 4.3.2 HPLC analysis for thiomarinol production

Thiomarinol extracted with the standard technique using acetone and ethyl acetate, was subjected to HPLC analysis to investigate the effect of the in frame deletion of the KS-B2/ACP-B2 of *tmpB* on thiomarinol production. HPLC chromatograms obtained from the WT SANK 73390 and the two isolates (mutant) showed a detectable peak of thiomarinol (Figure 4.4) indicating that the mutants still able to produce thiomarinol but with a much lower yield.

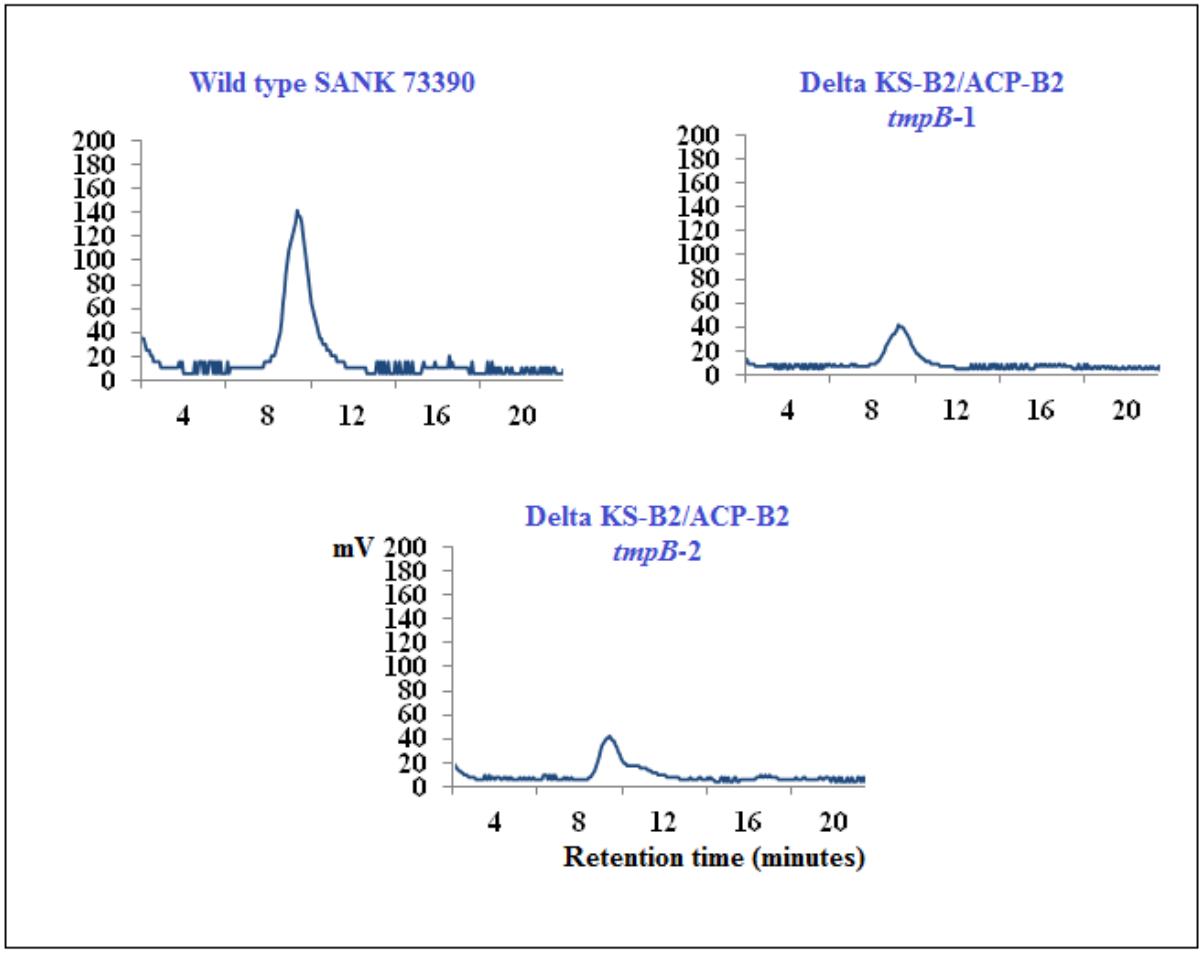



**Figure 4.4 HPLC chromatograms of thiomarinol produced by *Pseudoalteromonas* spp SANK 73390 with ΔKS-B2/ACP-B2 of *tmpB* using WT strain as control.**

Quantitative HPLC analysis showed a strong reduction in the production of thiomarinol by the mutant (isolates) to about 30% of the amount produced by the WT strain (Figure 4.5 and

4.6). This observation clearly indicated the negative effect of KS-B2/ACP-B2 deletion of *tmpB* on the strain productivity. However, the deletion did not block thiomarinol production, as effect which might be due to the reduction in the production of thiomarinol A in favour of other thiomarinol compounds.

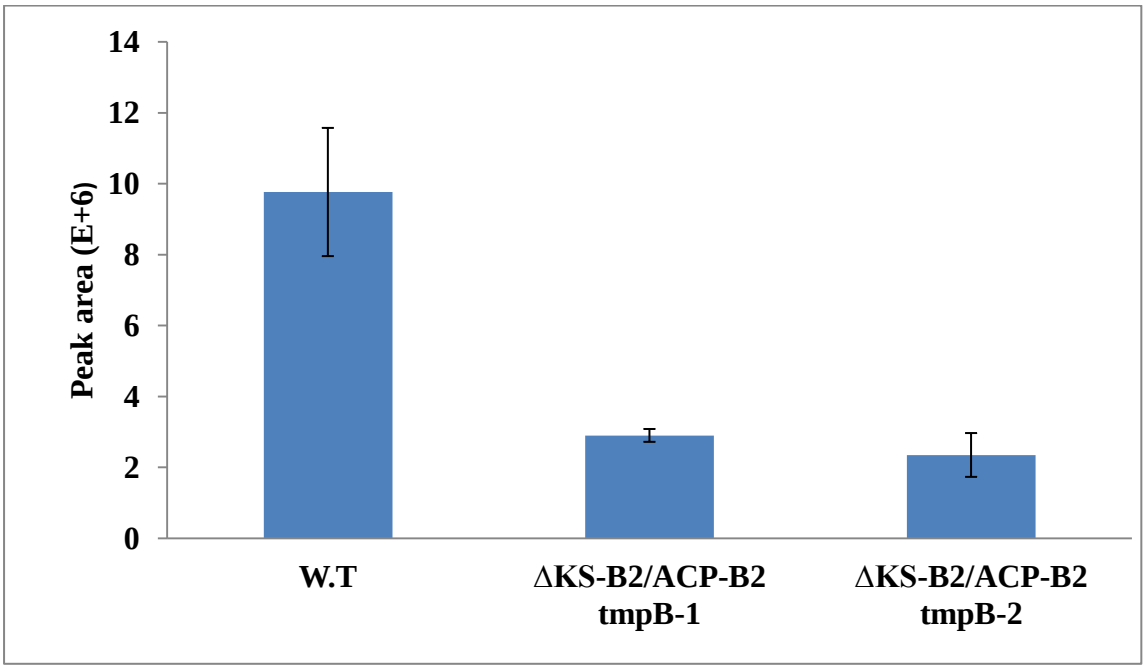



**Figure 4.5 HPLC analysis of production of thiomarinol by the WT *Pseudoalteromonas* spp SANK 73390 and strains with ΔKS-B2/ACP-B2 of *tmpB*, and using triplicates.**

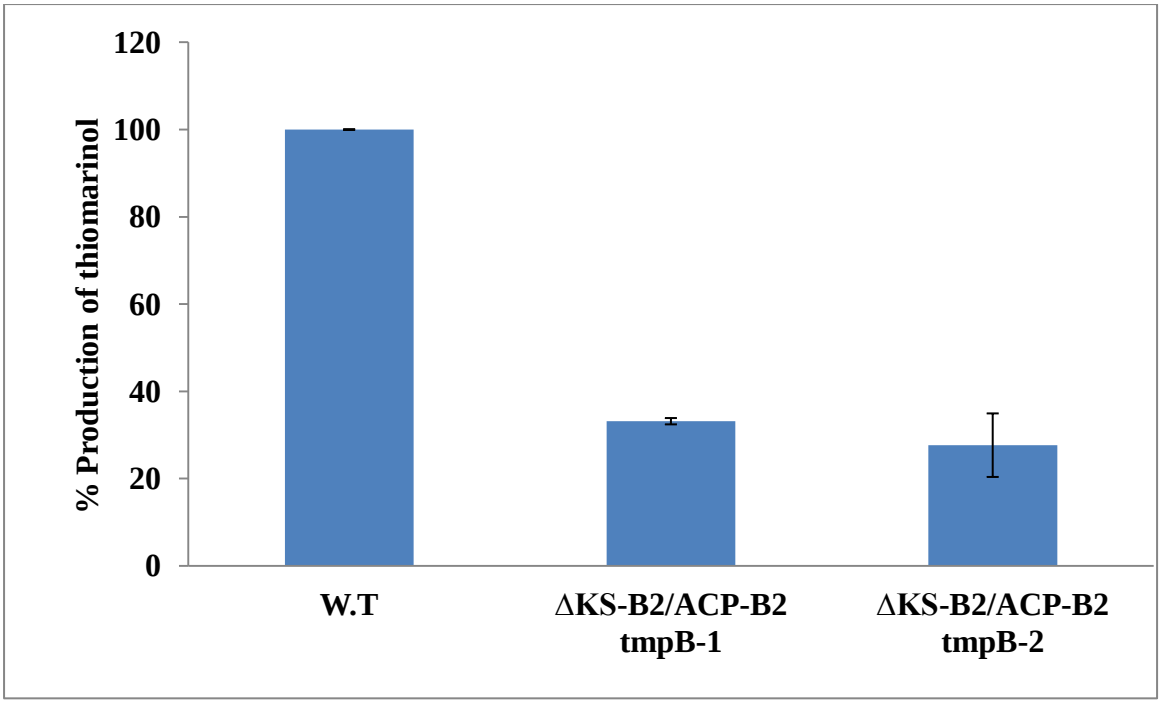



**Figure 4.6 Quantitative HPLC analysis of production of thiomarinol by the WT *Pseudoalteromonas* spp SANK 73390 and strains with ΔKS-B2/ACP-B2 of *tmpB*.**

## 4.3.3 Detection of PA-B analogue (TH-H) by LC-MS analysis

To analyse the change in the product profile in more detail the extracts were subject to LC/MS analysis by our colleagues at the University of Bristol. The results presented in Figure 4.7 shows various thiomarinol compounds produced by the WT *Pseudoalteromonas* spp SANK 73390 and the mutants with KS-B2/ACP-B2 deletion from *tmpB*, and the percent productivity of each compound by the mutants relative to the WT ones in Figure 4.8. Thiomarinol A, the major compound of the WT is still produced by the mutants but with a much lower yield (approx. 35.6%). However, production of other compounds such as thiomarinol C was increased significantly in the mutants by more than two-fold. Both the wild type and the mutants produced marinolic acid as expected and with a similar yield. However, while we predicted a decrease in the production of thiomarinol B by the mutants, a

small peak with the same MW as TH-B identified although it has not been confirmed as TH-B or even related. Nevertheless, the most striking result was a profound increase (about 3-fold compare to the WT) in the production of a compound with MW 656. This compound has been confirmed structurally as a hybrid of TH-A and TH-G (with C8-OH), which is an analogue of pseudomonic acid B in the mupirocin system and we call it TH-H. Since we get an accumulation of this compound which has an extra OH at C8, therefore, this strongly indicates that our hypothesis was correct and that this extra KS-B2/ACP-B2 ($2^{nd}$ module) of *tmpB* is non elongating module where the removal of the C-8 OH occurs.

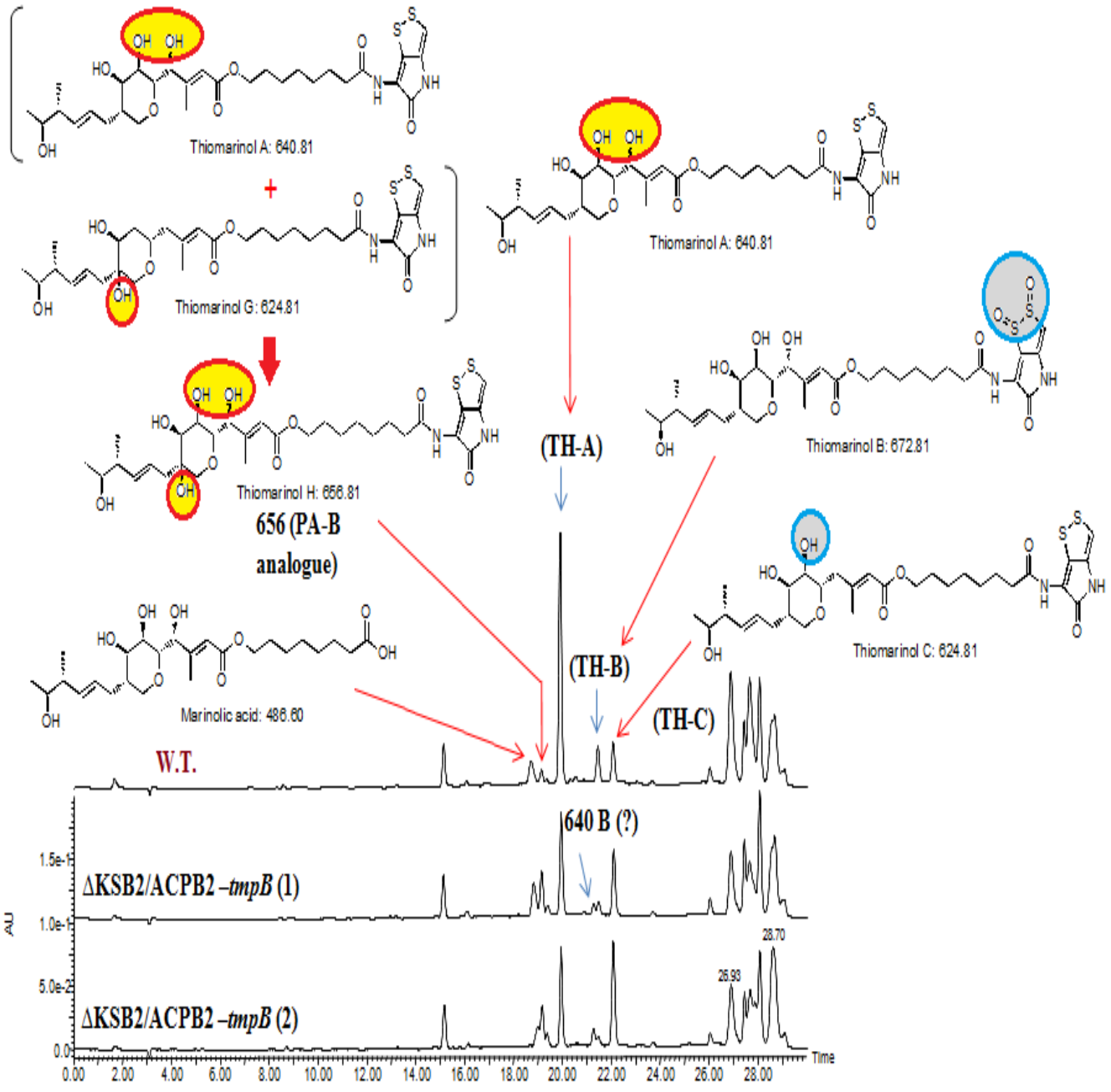



**Figure 4.7 Comparison of various thiomarinol compounds produced by the WT strain *Pseudoalteromonas* spp SANK 73390 and isolates with ΔKS-B2/ACP-B2 of *tmpB*.** Compounds were analysed using diode array, LC-MS and ELS detection. Compounds from left to right; marinolic acid with m/e 486.60; hybrid of thiomarinol A and G (compound with C8-OH named as TH-H), the pseudomonic acid B analogue with m/e 656.81; thiomarinol A with m/e 640.81; unknown compound (TH-B?) with m/e 640; thiomarinol B with m/e 672.81 and thiomarinol C with m/e 624.81 (data from Dr. Song in Bristol).

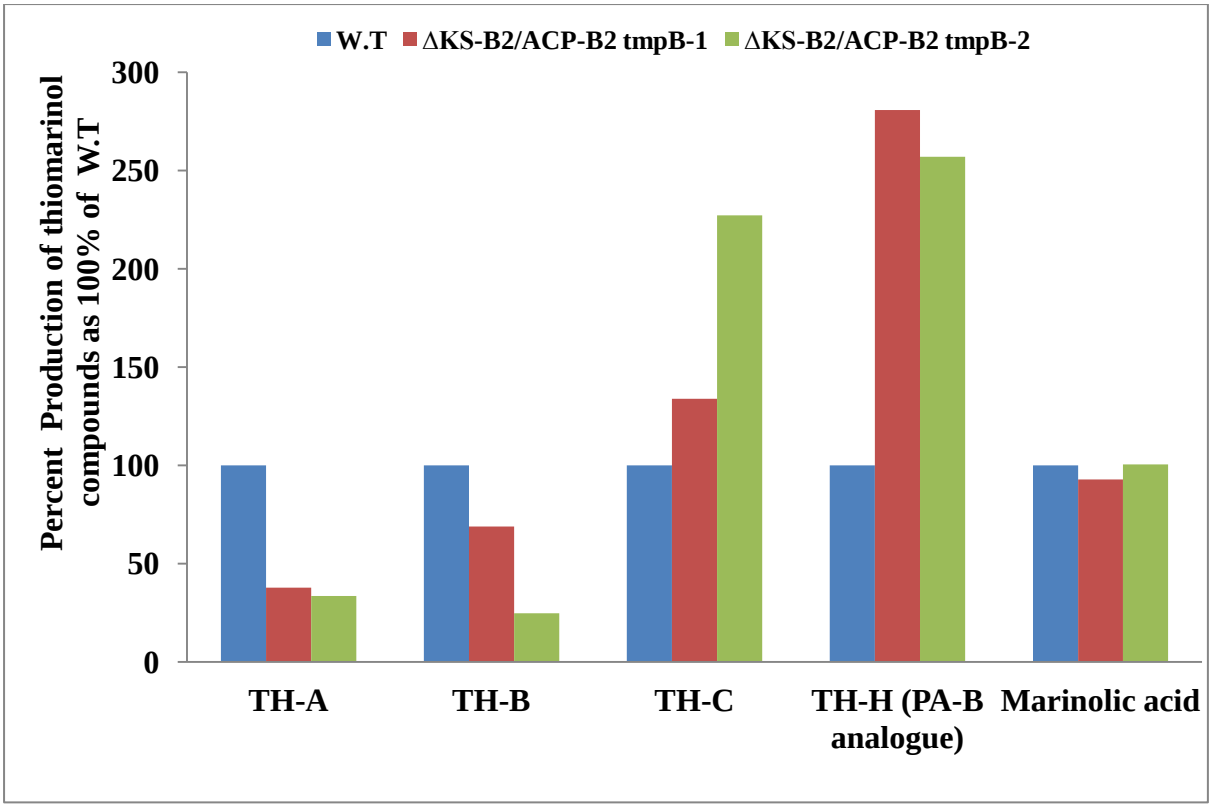



**Figure 4.8 Quantitative analysis of various thiomarinol compounds produced by isolates of SANK 73390 with ΔKS-B2/ACP-B2 of *tmpB* relative to thiomarinol A and using WT ones as 100%.** Compounds were analysed using diode array, LC-MS and ELS detection. Significant decrease in the yield of both TH-A and TH-B were observed, while profound increase in the yield of TH-H (compound with extra OH at C8) and a valuable increase in TH-C could be seen by the mutant in comparison to the WT ones (data from Dr. Song in Bristol).

## 4.4 Discussion

Progressive research on modular polyketides, clearly provides the possibility of modifying the structure of complex polyketides by introducing direct changes within PKSs (Katz, 1997). More specifically, Tsukamoto *et al*., (1996) confidentially support the idea of producing new polyketide products by dealing with the enzymatic domains using diverse ways like addition, deletion, swapping, or rearrangements. However, keeping the balance between substrate recognition and permissiveness characteristic of any of the included enzymatic roles or activities is required for the success of such a strategy. Retaining the most essential functions including within the TE as a termination domain, provides evidence that the steps of releasing, cyclising and post-PKS modifications of the products does not seem to be a major consequence of deleting modules from this PKS (Kao *et al*., 1994 and 1995; Cortes *et al*., 1995). TmpB is one of the multifunctional megaproteins in the PKS part of the thiomarinol pathway, and is constituted with a $2^{nd}$ module extra KS-B2/ACP-B2 designated as a putative non-elongating KS. The absence of mupirocin mAcpE's analogue from thiomarinol system led to the proposal that it is involved in the last few tailoring steps of pseudomonic acid production; namely of conversion of PA-B with OH at C8 to PA-A. This implies that the extra module of TmpB could be involved in the equivalent process. Therefore, the present study aimed to test the hypothesis of accumulation of thiomarinol molecules with extra OH at C8 as a result of deletion of the extra KS-B2/ACP-B2 from *tmpB.*

By suicide mutagenesis, mutants of *Pseudoalteromonas* spp SANK 73390 with KS-B2/ACP-B2 deletion from *tmpB* was obtained and thiomarinol extracted from mutants as well as the WT using optimal condition of extraction. Disc bioassay showed inhibition of the sensitive bacteria tested by thiomarinol extracted from mutants but with less effectiveness than compounds extracted from the WT. HPLC analysis carried out to confirm the amount of

thiomarinol produced by the deletion mutants in comparison to the WT ones, showed a significant reduction in the production of biologically active thiomarinol (mostly TH-A) by the mutant to about 1/3 the amount produced by the WT. This might explain that the module, or at least what has been deleted, is important for the biosynthetic pathway since the profile is clearly changed.

To investigate in more detail the various compounds that had been produced, we analyzed the metabolite profile of the ΔKS-B2/ACP-B2 of *tmpB* mutant by LCMS and found that both thiomarinol A and thiomarinol B decreased significantly compare to the WT ones, while marinolic acid was produced in the same range. Strikingly, the product retaining an extra OH at C8 with MW= 656 dalton has been detected in the mutant with a ratio more than the WT by about 3 folds. According to LCMS and fragmentation pattern the structure of this compound was elucidated to be a hybrid of TH-A and TH-G (with OH at C8) as an analogue of pseudomonic acid B in mupirocin system and we designated it TH-H (Figure 4.9). This result provides good evidence that our hypothesis about the possible implication of the extra KS and ACP of *tmpB* in the removal of the extra OH at C8 from thiomarinol structure is correct. Moreover, it might indicate that this extra module of *tmpB* is a non-elongating module and that the KS-B2 is working as a transferase rather that to function in a Claisen condensation since the deletion mutant did not change the carbon backbone of thiomarinol. In that case the ACP-B2 in the 2$^{nd}$ module might function like a tailoring enzyme in thiomarinol system. Another exciting result with this mutant is the significant increase in the production of thiomarinol C, the compound lacking OH at C4 by approximately two fold than the WT. This observation might indicate that somehow ACP-B2 is involved in the removal of OH group at C8 while retaining it at C4 (C4-hydroxlyation). The prediction which is consistent with Murphy's suggestion that C4-hydroxylation does not have to happen before or even

during the elongation of the polyketide backbone (Murphy *et al*., 2011), since this deletion did not change the polyketide chain (acts in tailoring steps only).

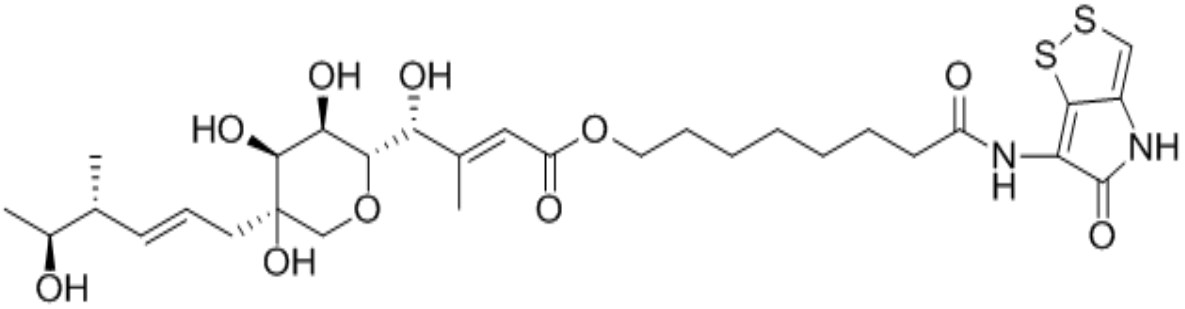



**Figure 4.9 Predicted chemical structure of thiomarinol compound with molecular weight 656.81 with an extra OH group at C8 produced by the mutant ΔKS-B2/ACP-B2 of *tmpB* by about three fold more than the WT.**

In summary, it is obvious that deletion of the KS-B2/ACP-B2 of *tmpB* has a profound effect on the biosynthesis pathway of thiomarinol by reducing but not eliminating thiomarinol A and thiomarinol B production. It also increased the production of thiomarinol C, the compound lacking OH at C4. We have also been able to demonstrate the accumulation of thiomarinol molecule with extra OH at C8 in the mutant of ΔKS-B2/ACP-B2 of *tmpB*. Presumably, this might be due to the role of either, both the KS-B2 and ACP-B2, or just the ACP-B2 that works like a tailoring enzyme, since we observed that this module is a non elongating module and that the KS-B2 function may be as a transferase instead of working in chain extension by a Claisen condensation.

# CHAPTER 5
# MANIPULATION OF *mupU* AND *macpE* GENES FOR INCREASED PA-A PRODUCTION

## 5.1 Introduction

During the past two decades, tailoring enzymes in modular PKSs have been shown to play a striking role in the conversion of the core products of PKSs into the active form through further modifications. In mupirocin they are represented by mono-functional enzymes. For example, epoxidation of C-8,-16 double bond, as an essential step in the formation of the tetrahydropyran ring, has been proposed to possibly be due to the catalytic role of both MupW and MupT. This process apparently makes C-16 more receptive to assault by C-5 hydroxyl group. Two further steps that may occur either before or after this, including esterification with 9-HN and C-10,-11 epoxidation, result in pseudomonic acid B. Knocking out *mupW* and *mupT* produced a new metabolite, mupirocin W, that lacks the tetrahydropyran ring but still has the fatty acid side chain (9-HN) attached, a fact that confirms the critical role of MupW/MupT in the biosynthesis of mupirocin. However, other genes like *mupO*, *mupU*, *mupV*, and *macpE* (Figure 5.1) are necessary for PA-A production but not PA-B (Cooper *et al.*, 2005a), and the latest evidence indicates that PA-B is a precursor of PA-A (Gao *et al*., unpublished data). Therefore, it is hypothesised that MupU is responsible for loading mAcpE with an intermediate, which in turn is processed by MupO, aided by MupV/C/F for the production of PA-A. Also, since mupirocin is produced as a mixture of pseudomonic acids, and it is likely that different tailoring enzymes are responsible for the different forms (compounds), then manipulation of the level of those tailoring enzymes might change the balance of the different forms.

The commercial importance of PA-A as the most potent product of the mupirocin cluster led Macioszek in 2009 to demonstrate that over-expression of *macpE* and *mupO*/*U*/*V*/ *C*/*F* genes could increase the production of both PA-A and PA-B more than two fold. Other experiments demonstrated increase in the production of mupirocin and novel metabolites by

about 17-fold resulting from *in trans* expression of *mupR*, the transcriptional regulator, both in the wild type and mutants (Hothersall *et al*., 2011). Furthermore, tandem acyl carrier proteins both in mupirocin and curacin were shown to play a crucial role in promoting the enzymatic reactions consecutively, and thus increasing the polyketide production (Rahman *et al*., 2005; Liangcia *et al*., 2011).

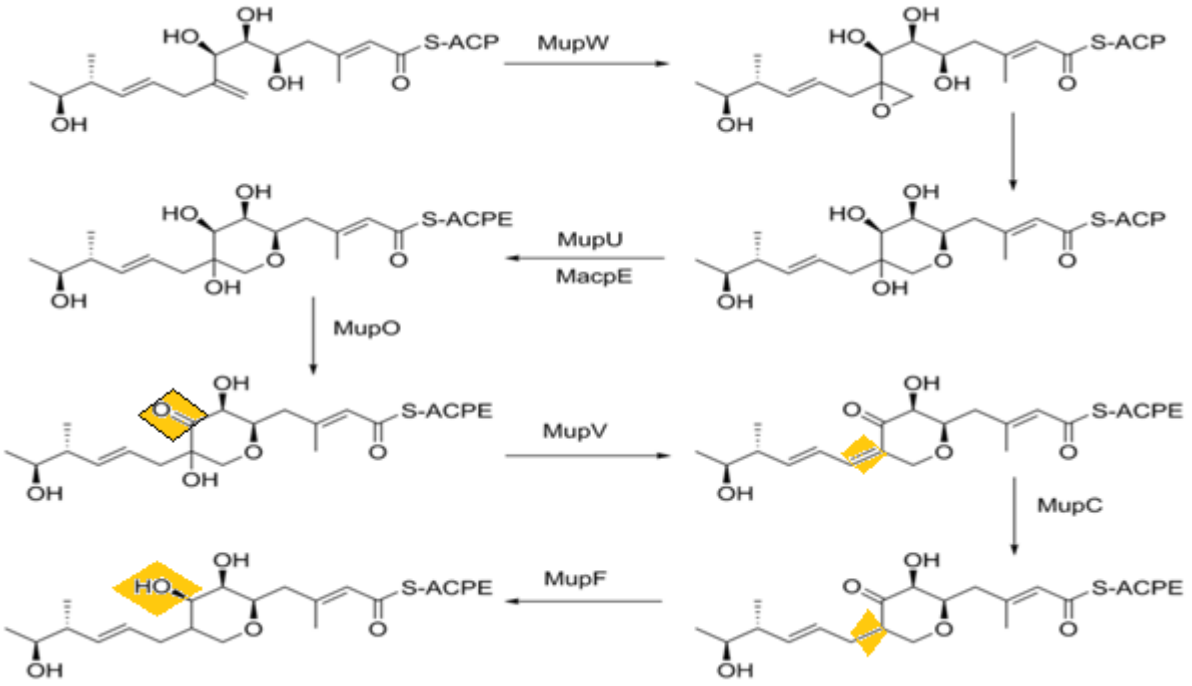



Figure 5.1 Multistep reactions for mupirocin production proposed to explain why in-frame deletion of open reading frames *mupO*, *mupU*, *mupV* and *macpE* abrogate PA-A production but not PA-B production, demonstrating that PA-B is either a precursor or, a side product to PA-A (Cooper *et al*., 2005a).

It is predicted that one of the reasonable explanations for why over expression of *macpE* and *mupO, U, V, C, F* (essential genes for PA-A production) was not a complete strategy to shut down PA-B production, is that the thioesterase associated with MmpB (Figure 5.2a) could possibly release PA-B. This means that there may be a need for more flux onward in

the mupirocin pathway to transfer an ACP bound intermediate to mAcpE by MupU in order to convert it to PA-A (Macioszek *et al*., unpublished data).

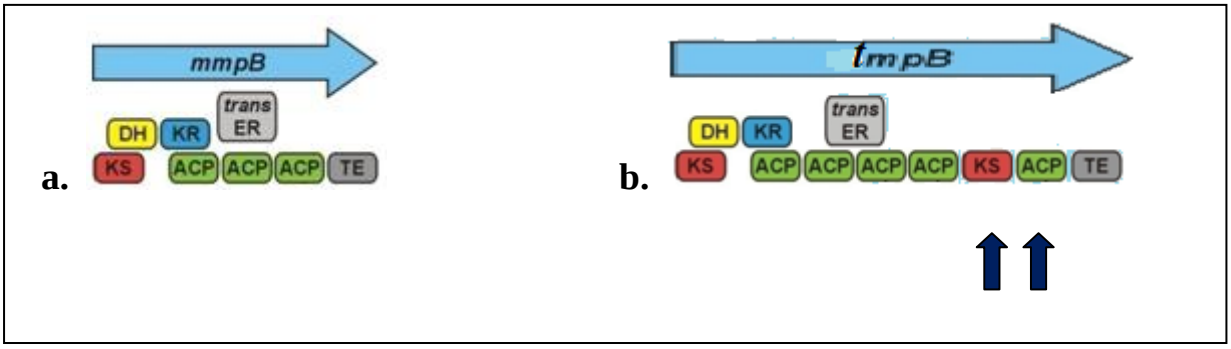



**Figure 5.2 Differences in the proposed domain structure responsible for fatty acid synthesis in mupirocin and thiomarinol production.** A tandem ACP in mupirocin is followed by TE, while in thiomarinol there is an extra non elongating module designated by the arrows that separate tandem ACP from TE.

Three valuable strategies were used for the purpose of this study. The first was to construct a strain of *P. fluorescens* NCIMB 10586 with a new promoter upstream *mupU* (proposed to be responsible for the transfer of intermediates to mAcpE), in order to increase the flux of intermediates and finally more production of PA-A. The second was to examine whether it is possible to increase the flow from PA-B to PA-A by increasing the expression of fused *mupU* (-STC)-*macpE*, based on the concept that to increase flux we need more ACPs, and the enzyme essential to transfer the intermediate onto the ACP. The third was based on the fact that in TmpB (Figure 5.2b) of the thiomarinol cluster there is an extra module separating the last ACP from the TE domain, which is demonstrated in Chapter 4 of this thesis to have a probable role in the removal of $C_8$-OH group, and the release of thiomarinol PA-B analogue (TH-G or C and TH-H). This led to an attempt by atomic level surgery to insert fused *mupU* (-STC)–*macpE*(-STC) into *mmpB* before TE in order to reduce PA-B, which in turn should increase PA-A to the highest level.

## 5.2 Materials and Methods

### 5.2.1 Bacterial strains and plasmids

Bacterial strains used or constructed in this study are listed in Table 5.1. The growth conditions and media used with their supplements are described in Chapter 3, and only kanamycin (50 μg/ml) as an extra antibiotic was used whenever strains carried pAKE604 plasmid. DNA isolation and manipulations was described in Chapter 3. Bacterial conjugation and DNA transfer via bi-parental mating was described in Chapter 3. Suicide vector strategy for in-frame deletion of open reading frames was described in Chapter 4, except that saline solution and L-agar were used instead of marine broth and agar, respectively, and the growth at 30$^{o}$C, during the excision of the suicide vector. Plasmids used and constructed during the present study are listed in Table. 5.2.

**Table 5.1 Bacterial strains used and constructed during this study.**

| Bacterial strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli* DH5α | F⁻ *Φ80dlacZΔM15, recA1, endA1, gyrA86, thi-1, HsdR17(r$_{k}^{-}$, m$_{k}^{+}$), supE44, relA1, deoR, Δ(lacZYA-ArgF)U169* | Hanahan, 1983 and 1985 |
| *Pseudomonas fluorescens* NCIMB10586 | Wild type (WT) strain which produces mupirocin | Whatling *et al.*, 1995 |
| *Escherichia coli* S17-1 | *recA pro hsdR RP4-2-Tc::Mu-km::Tn7* | Simon *et al.*, 1983 |
| *Bacillus subtilis* 1604 | *trp*C2 | Moir *et al.*, 1979 |
| NCIMB10586 Δ*macpE* | *P. fluorescens* with deletion of *macpE* gene | Cooper *et al.*, 2005a |

| | | |
|---|---|---|
| NCIMB10586 Δ*mupU*/Δ*macpE* | *P. fluorescens* with deletion of *mupU* and *macpE* genes | This study |
| NCIMB10586Δ*UE* (*UE* in *mmpB*) | NCIMB10586Δ*mupU*/Δ*macpE* with *mupU* (-stop codon)-Linker (20 $aa_s$)-*macpE* (-stop codon) inserted into *mmpB* (between ACP7 and TE) | This study |
| NCIMB10586Δ*UE* (*UE* after *mmpA*) | NCIMB10586Δ*mupU*/Δ*macpE* with *mupU* (-stop codon)-Linker (20 $aa_s$)-*macpE* (-stop codon) inserted after ACP4 of *mmpA* | This study |
| NCIMB10586 *mupUp* | *P. fluorescens* with a new promoter upstream *mupU* | This study |

**Table 5.2 Plasmids used and constructed in this study.**

| plasmid | Size (kb) | Genotype Description | Reference |
|---|---|---|---|
| pGEM-T Easy | 3.0 | $Ap^R$, *lacZα* (PCR cloning vector) | Promega |
| pAKE604 | 7.2 | $Ap^R$, $Km^R$, *oriT, lacZα, sacB*, multiple cloning sites (*Bam*HI-*Eco*RI) | EL-Sayed *et al*., 2001 |
| pJH10 | 13.76 | $T_C^R$; *lacI*$^q$, *tacp*, IncQ replicon, multiple cloning site (*Eco*RI-*Sac*I) | EL-Sayed *et al*., 2003 |
| pJH2 | 14.482 | *mupR* cloned into pJH10 | Hothersall *et al*., 2011 |
| $pAMGU_{PA1}$ | 3.574 | 574 bp *Bam*HI-*Sac*II PCR product containing arm 1 for putting promoter upstream *mupU*, cloned into pGEM-T Easy vector | This study |

| | | | |
|---|---|---|---|
| $pAMGU_{PA2}$ | 3.585 | 585 bp *Sac*II-*Eco*RI PCR product containing arm 2 for putting promoter upstream *mupU*, cloned into pGEM-T Easy vector | This study |
| pAMK1 | 8.3 | 1.15 kb {arm1 (574 bp *Bam*HI-*Sac*II) PCR and arm 2 (585 bp *Sac*II-*Eco*RI)} PCR products for putting promoter upstream *mupU*, cloned into *Bam*HI-*Eco*RI of pAKE604. | This study |
| $pAMGU_{A1(deletion)}$ | 3.513 | 513 bp *Bam*HI-*Xba*I PCR product containing arm 1 for *mupU* deletion, cloned into pGEM-T Easy vector | This study |
| $pAMGU_{A2(deletion)}$ | 3.618 | 618 bp *Xba*I-*Eco*RI PCR product containing arm 2 for *mupU* deletion, cloned into pGEM-T Easy vector | This study |
| pAMK2 | 8.3 | 1.13 kb {arm1 (513 bp *Bam*HI-*Xba*I) PCR and arm 2 (618 bp *Xba*I-*Eco*RI)} PCR products for deletion of *mupU*, cloned into *Xba*I-*Eco*RI of pAKE604. | This study |
| pAMGU | 4.6 | 1.6 kb *Kpn*I-*Mfe*I PCR product containing *mupU* (-stop codon) cloned into pGEM-T Easy vector | This study |
| pAMGE1 | 3.26 | 260 bp *Cla*I-*Xba*I PCR product containing *macpE* cloned into pGEM-T Easy vector | This study |
| pAMGE2 | 3.26 | 260 bp *Cla*I-*Xba*I PCR product containing *macpE* (-stop codon) cloned into pGEM-T Easy vector | This study |
| pAMG(ACP7-*mmpB*) | 3.516 | 516 bp *Bam*HI-*Eco*RI PCR product containing ACP7 of *mmpB*, cloned into pGEM-T Easy vector. | This study |
| pAMG(TE-*mmpB*) | 3.549 | 549 bp *Xba*I-*Eco*RI PCR product containing TE of *mmpB*, cloned into pGEM-T Easy vector. | This study |
| pAMG(ACP4-*mmpA*) | 3.495 | 495 bp *Bam*HI-*Xba*I PCR product containing ACP4 of *mmpA*, cloned into pGEM-T Easy vector. | This study |

| | | | |
|---|---|---|---|
| pAMG(*mupB*) | 3.490 | 490 bp *Xba*I-*Eco*RI PCR product containing *mupB*, cloned into pGEM-T Easy vector. | This study |
| pAMH1 | 13.86 | 96 bp *Kpn*I-*Nhe*I-*Mfe*I---20aa---*Cla*I-*Nhe*I-*Xba*I, cloned into pJH10 vector | This study |
| pAMH2-a | 15.3 | 1.6 kb *Eco*RI, *Kpn*I-*Mfe*I PCR product containing *mupU* (-stop codon), cloned into pAMH1 vector with multiple cloning sites *Kpn*I-*Xba*I and 20aa linker | This study |
| pAMH2-b | 15.3 | 1.6 kb *Eco*RI, *Kpn*I-*Mfe*I PCR product containing *mupU* (-stop codon), cloned into pAMH1 vector with multiple cloning sites *Eco*RI, *Kpn*I-*Xba*I and 20aa | This study |
| pAMH3 | 15.6 | 260 bp *Cla*I-*Xba*I PCR product containing *macpE*, cloned into pAMH2-a {with *mupU* (-stop codon) and 20aa} vector | This study |
| pAMH4 | 15.6 | 260 bp *Cla*I-*Xba*I PCR product containing *macpE* (-stop codon), cloned into pAMH2-b {with *mupU* (-stop codon) and 20aa} vector | This study |
| pAMH5 | 16.1 | 516 bp *Bam*HI-*Eco*RI PCR product containing ACP7 of *mmpB* cloned into pAMH4{with *mupU* (-stop codon)--20aa--*macpE* (-stop codon)}vector | This study |
| pAMK3 | 7.75 | 549 bp *Xba*I-*Eco*RI PCR product containing TE of *mmpB* cloned into pAKE604 vector | This study |
| pAMK4 | 10.2 | 2.5 kb *Bam*HI-*Xba*I fragment containing {ACP7 of *mmpB*, *mupU* (-stop codon)--20aa-*macpE* (-stop codon)} in pAMH5 cloned into pAMK3 vector. | This study |
| pAMH6 | 16.1 | 495 bp *Bam*HI-*Eco*RI PCR product containing ACP4 of *mmpA* cloned into pAMH4{with *mupU* (-stop codon)--20aa--*macpE* (-stop codon)} vector | This study |

| | | | |
|---|---|---|---|
| pAMK5 | 7.75 | 490 bp *Xba*I-*Eco*RI PCR product containing *mupB*, cloned into pAKE604 vector. | This study |
| pAMK6 | 10.2 | 2.5 kb *Bam*HI-*Xba*I fragment containing {ACP4 of *mmpA*, *mupU* (-stop codon)--20aa-*macpE* (-stop codon)} in pAMH6, cloned into pAMK5 vector. | This study |

**Table 5.3 Primers used during this study.**

| Gene | Primer | Primer Length (bp) | Primer sequence, 5'⟶3' | Purpose |
|---|---|---|---|---|
| *mupU* | FP1*mupU*$_{PR}$ | 27 | CGCGGATCCGAGCCACATGTGCCACTG | Putting *mupU* under the control of new promoter |
| | RP1*mupU*$_{PR}$ | 26 | CGCCCGCGGTGTCAAGCGAAGCAGGC | |
| | FP2*mupU*$_{PR}$ | 40 | GCCCGCGGCAATGTGCGTCTATCATTTTTGGTATGGCAAG | |
| | RP2*mupU*$_{PR}$ | 41 | GCGAATTCCGTATAGAGGATCAGCGCTGGATGATCGCCTGC | |
| *mupU* | FP1*mupU*$_{Del}$ | 24 | GGATCCCGTGAGCAGTGAGGTCGT | Deletion of *mupU* |
| | RP1mupU$_{Del}$ | 24 | TCTAGAGCACACCTGCGCTAATGC | |
| | FP2*mupU*$_{Del}$ | 24 | GCTCTAGACTTCAAGCGGCCTCGC | |
| | RP2*mupU*$_{Del}$ | 24 | GCGAATTCCTCCACCTCACCCGAA | |
| *mupU* | FP*mupU*$_{Construction}$ | 26 | CGGGTACCGGTATGGCAAGGGAATGT | Construction of *mupU* |
| | RP*mupU*$_{Construction}$ | 26 | CGCAATTGGGCCGGTTCATGGGCCTG | |
| | FP*mupU*$_{Internal}$ | 36 | GGATCCGTGCTCCGTTTTTCCTGGTATCGATGAGCT | Internal primers for checking of |

| | | | | |
|---|---|---|---|---|
| | RP*mupU*$_{\text{Internal}}$ | 36 | GAATTCATGTACCAACCGTCCTCTGTGAAGGCGTCG | *mupU* sequence |
| *macpE* | FP*macpE* Construction | 26 | CGATCGATCATCCACAGGAGGCGTAG | Construction of *macpE* |
| | RP*macpE* Construction | 26 | CGTCTAGATCACTGCTCACGTTGGGC | |
| *macpE* (-stop codon) | FP*macpE* Construction | 29 | CGATCGATCGGCATCCACAGGAGGCGTAG | Construction of *macpE* (-stop codon) |
| | RP*macpE* Construction | 29 | CGTCTAGACTGCTCACGTTGGGCGAACCA | |
| Fused (*mupU-macpE*) -stop codon | FP$_{\text{ACP7-}mmpB}$ | 27 | GGATCCCTGCTCGATGAGTTGGTGGCA | Insertion of fused *mupU-macpE* into *mmpB* |
| | RP$_{\text{ACP7-}mmpB}$ | 27 | GAATTCCAATGCCTCCAGGCTGACCAC | |
| | FP$_{\text{TE-}mmpB}$ | 27 | TCTAGAGCCGAGCAGTTGCGCGCCTAT | |
| | RP$_{\text{TE-}mmpB}$ | 27 | GAATTCCTCGAAAGCCACGCTGGCCCC | |
| Fused (*mupU-macpE*) -stop codon | FP$_{\text{ACP4-}mmpA}$ | 27 | GGATCCCCCGCCGACGGCATTTATACC | Insertion of fused *mupU-macpE* after ACP4 of *mmpA* |
| | RP$_{\text{ACP4-}mmpA}$ | 27 | GAATTCCTACCGCTGCGCCAACACGTC | |
| | FP$_{\text{ACP4-}mupB}$ | 27 | TCTAGACAACCATGACTTTCAATGTCG | |
| | RP$_{\text{ACP4-}mupB}$ | 27 | GAATTCGAACCGGAAAGCTTCGTCTTC | |
| Any Cloned Gene(s) | pJH10 pre *Eco*RI primer* | 22 | CCGACATCATAACGGTTCTGGC | Sequencing of pJH10 for any gene cloned into MCS *Eco*RI-*Sac*I |

♦ The restriction sites are shown by red.

* Designed by Prof. C.M. Thomas

## 5.2.2 Oligonucleotide annealing

Purchased oligonuclotides from Alta Biosciences were dissolved in sterile distilled water to make up a concentration of 100 pmol/µl. A special PCR tube was used for mixing equal volumes (20µl or 25µl) of the two complementary oligonucleotides. The reaction was carried out in PCR machine, which was programmed for 10 min denaturation step at 98$^{o}$C followed by ramp cooling to 25$^{o}$C.

**Table 5.4 Oligonucleotides used in this study.**

| Purpose | Primer sequence, 5'⟶3' | Restriction sites |
|---|---|---|
| To add 20aa Between fused *mupU-macpE* | CGCTAGCCAATTGGGCGGCGGCAGCGGCGGCGGCAGCGGTAC<br>CGGCGGCGGCAGCGGCGGCGGCAGCGGCATCGATGCTAGCT | *Kpn*I<br>*Nhe*I |
| | CTAGAGCTAGCATCGATGCCGCTGCCGCCGCCGCTGCCGCCGC<br>CGGTACCGCTGCCGCCGCCGCTGCCGCCGCCCAATTGGCTAGC<br>GGTAC | *Mfe*I<br>*Cla*I<br>*Xba*I |

## 5.2.3 Construction of insertion mutant using suicide vector strategy

The strategy presented in Figure 5.3 illustrate the manner which was followed for the insertion of the fused *mupU*(-STC)-*macpE*(-STC) into the *mup* cluster in NCIMB10586Δ*mupU*/Δ*macpE*. The procedure started by designing two different sets of primers (Table 5.3) for the PCR amplification of two adjacent regions from the *mup* cluster

where the fused *mupU/macpE* planned to be inserted. These two regions were then ligated on either side of the fused *mupU*(-STC)-*macpE*(-STC) for the construction of the suicide vector pAKE604 (pAMK4 and pAMK6, respectively). *E. coli* S17-1 was then transformed with the suicide vector and the procedure described in Chapter 3 (Materials and Methods section 3.2.7) was followed to mobilise the plasmid into NCIMB10586Δ*mupU*/Δ*macpE* by conjugation. Then the steps described in Chapter 4 (Materials and Methods section 4.2.8) were followed for the excision of the suicide vector, with the exception of using saline solution and L-agar instead of marine broth and agar, whenever required, in addition to the incubation of the plates at 30$^{o}$C.

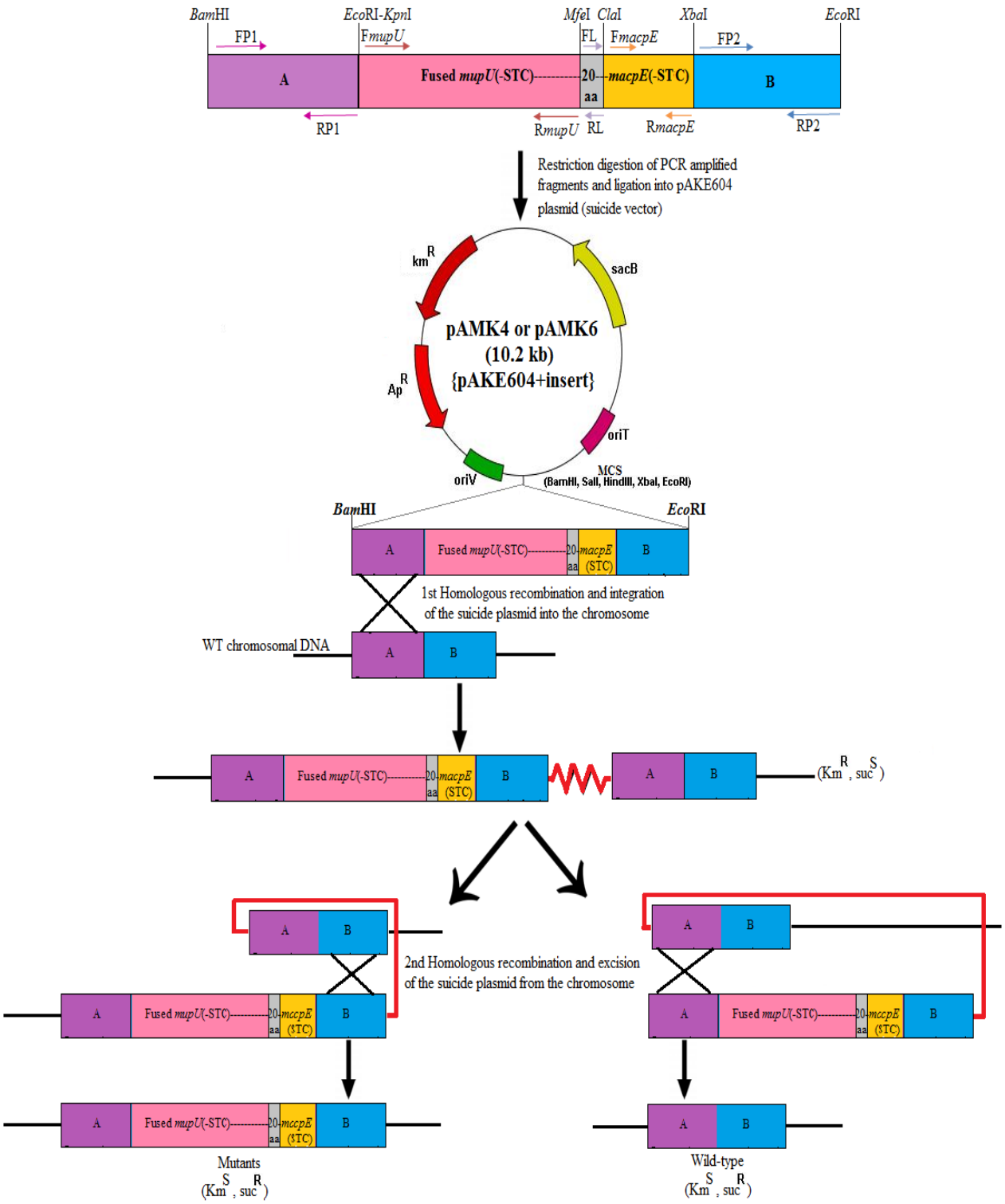



**Figure 5.3 Strategy for the insertion of the fused *mupU*(-STC)---*macpE*(-STC) into the chromosome of *P. fluorescens*.**

## 5.3 Results

### 5.3.1 Construction of *P. fluorescens* strains with promoter upstream of *mupU* encoding the acyl CoA synthase involved in PA-A production

MupU is proposed to load PA-B or related substrate onto mAcpE as the first step in conversion to PA-A. It may therefore represent a rate limiting step in the production of PA-A so increasing the level of MupU relative to the other enzymes of the factory may help to reduce PA-B relative to PA-A. Since *mupU* appears to be far away from the second promoter in the *mup* operon a strategy was devised to create a promoter immediately upstream of *mupU* in order to increase the transcription of *mupU* and therefore level of MupU. This was proposed to reduce PA-B production and increase the production of PA-A, the major component and the most active mupirocin compound.

An existing *Sac*II site that is flanked by two regions that were looking similar to -35 and -10 regions respectively, has been used for joining them together after they had been improved and the distance between them reduced as well (Figure 5.4). The forward primer for the upstream region contained a *Bam*HI restriction site, while the reverse primer of the downstream region contained an *Eco*RI restriction site. The PCR amplified products of the two regions (Material and Methods, Section 4.2.5) were each ligated into vector pGEM-T Easy (Table 5.2), separately, and the required changes within the two regions confirmed by DNA sequencing. These two regions inserted successfully in a single ligation reaction into the *Bam*HI and *Eco*RI site of pAKE604 vector to create pAMK1 plasmid. Standard suicide vector strategy was followed for the construction of *P. fluorescens* with the new promoter upstream of *mupU*. PCR checking using external primers for the strains with the new promoter upstream *mupU* was not helpful as long as the changes were very specific and different from checking in-frame deletion. Therefore, PCR products out of the expected

isolates were gel purified and ligated into pGEM-T Easy vector for sequencing. Results out of sequencing confirmed two isolates as having the correct new promoter upstream of *mupU* perfectly.

Bioassay did show a reasonable increase in the production of mupirocin up to more than two fold by the strains with the new promoter (Figure 5.5 and 5.6, respectively) using *B. subtilis* 1604 as a sensitive organism and WT *P. fluorescens* as a control. This result confirms the significance of MupU in increasing the production of total mupirocin, however, it did not show whether there was a change in the ratio of PA-A to PA-B, which needed to be determined by HPLC analysis.

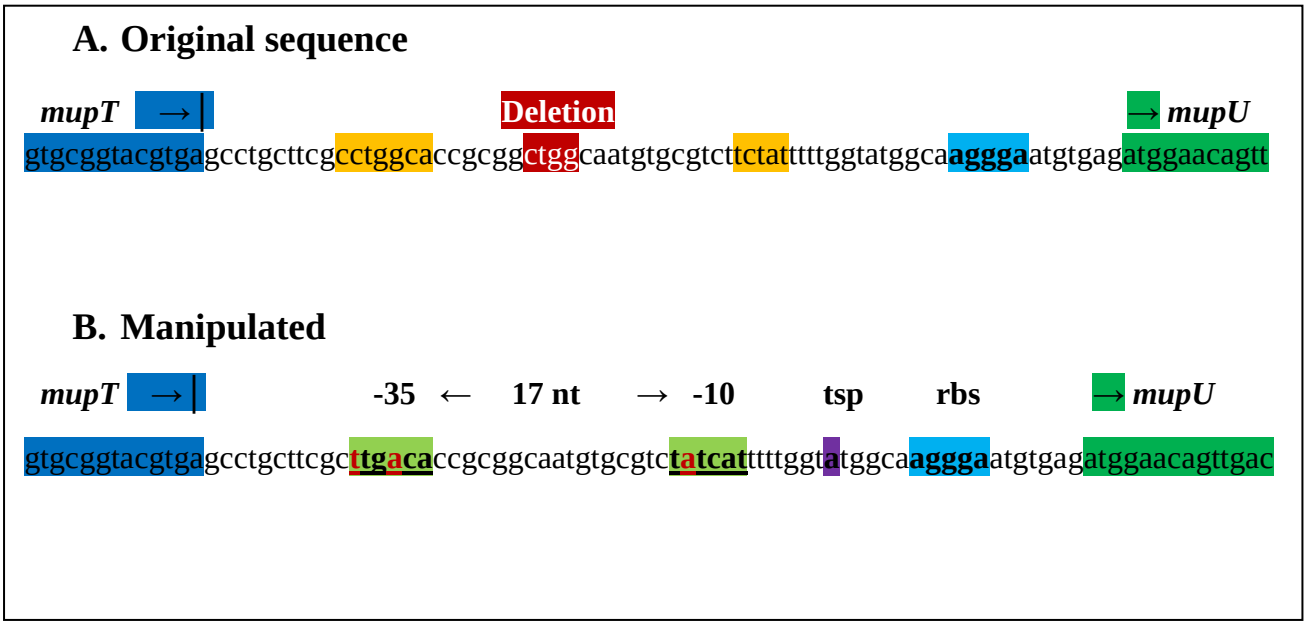



**Figure 5.4 Scheme showing the strategy for putting *mupU* under the control of a new promoter.** The original DNA targeted sequence is shown in A, while the new promoter based on genetic manipulation is shown in B.

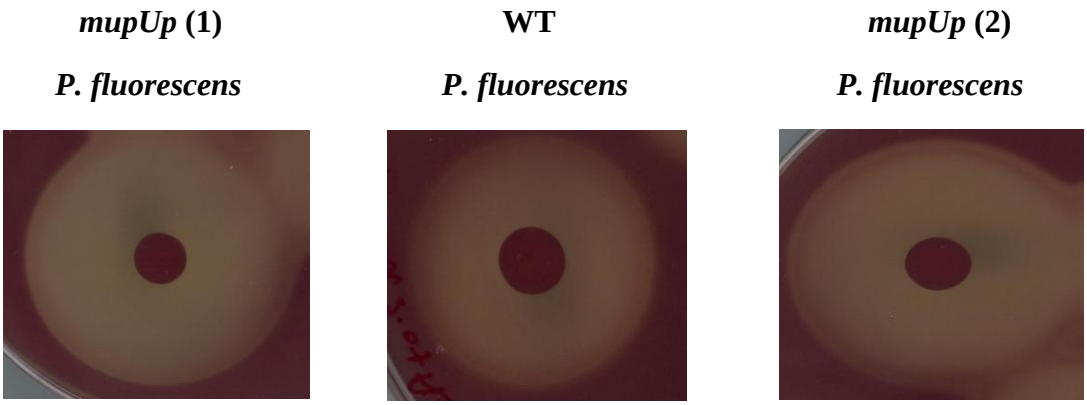



**Figure 5.5 Bioassay to determine the productivity of the isolates with a new promoter upstream of *mupU* relative to the WT.**

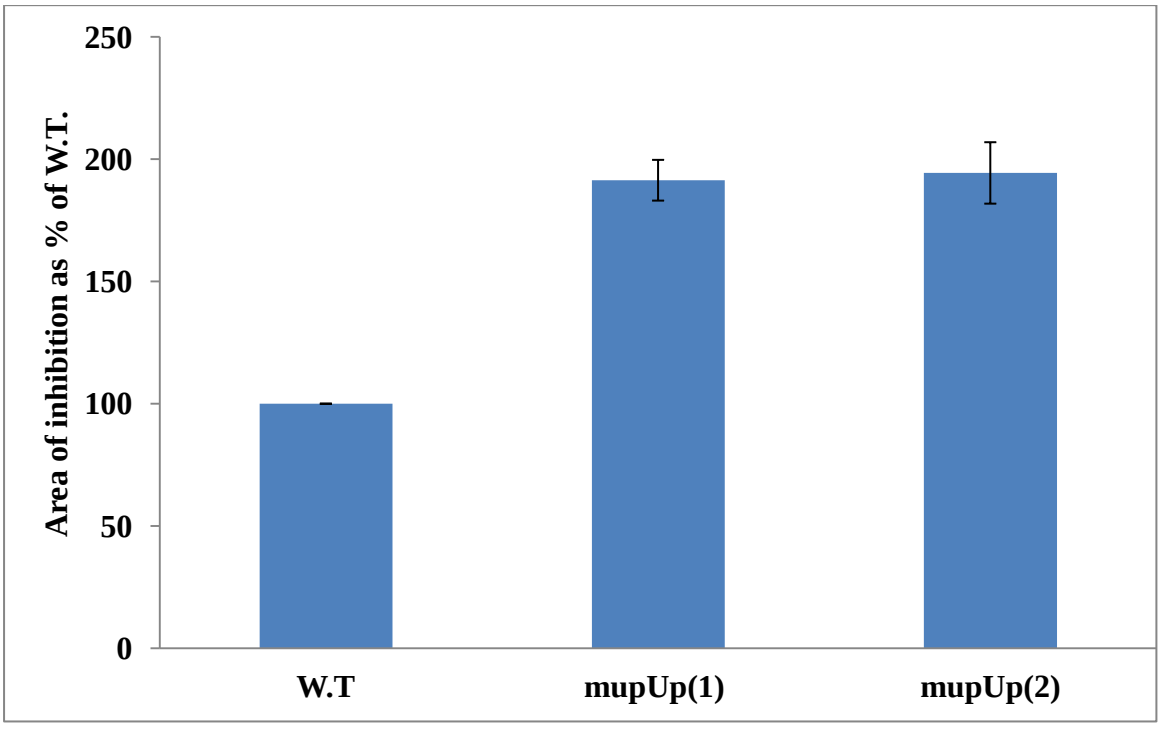



**Figure 5.6 Quantitative bioassay of *P. fluorescens* isolates with a new promoter upstream of *mupU* in comparison to the WT using triplicate for each.**

### 5.3.2 Quantitative analysis of PA-A and PA-B produced by strains with promoter upstream of *mupU*

HPLC analysis was performed to check whether over expression of *mupU* by isolates with a new promoter affects the yield of the different pseudomonic acids. According to our proposal for increasing the production of PA-A relative to PA-B we need more ACPs as well as more of the enzyme that transfers the intermediate to ACP. Therefore, as a first option to test this hypothesis, *mupU* was over-expressed via a new promoter inserted just upstream. WT *P.fluorescens* was used as control, also as 100% expression for both PA-A and PA-B. HPLC analysis (Figure 5.7) showed that over-expression of *mupU* did not abolish PA-B production. However, the production of PA-A (Figure 5.8) was increased by 3.1-fold in comparison to the wild type.

Quantitative HPLC analysis of isolates with the new promoter upstream of *mupU* showed an increase in the production of PA-A (Figure 5.9) by more than three fold in comparison to the wild type. Although of an increase in the production of PA-A by the new isolates, production of PA-B was not abolished, and even it increased by not less than two fold compare to the wild type PA-B. Barely, this might indicate that production of PA-B and PA-A is concomitant. In addition, mass spectrometry analysis done in Bristol by Dr. Song, confirmed the production of more antibiotic (mupirocin) by isolates with the new promoter (Figure 5.10), in comparison to the WT, and about the same productivity of WT with *in trans* expression of *mupR.* Also, it did show an increase in the production of both PA-A and PA-B (Figure 5.11) to about 4 to 5 fold with the new isolates, but without a significant difference compare to those in the WT.

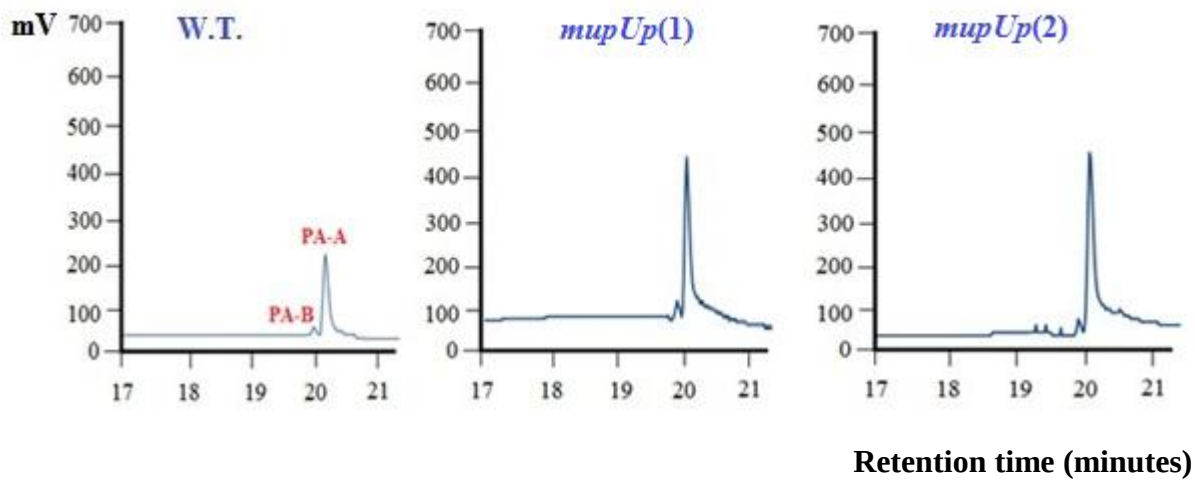



**Figure 5.7 HPLC chromatograms of pseudomonic acids produced by *P. fluorescens* isolates with new promoter upstream of *mupU*, using WT strain as control.**

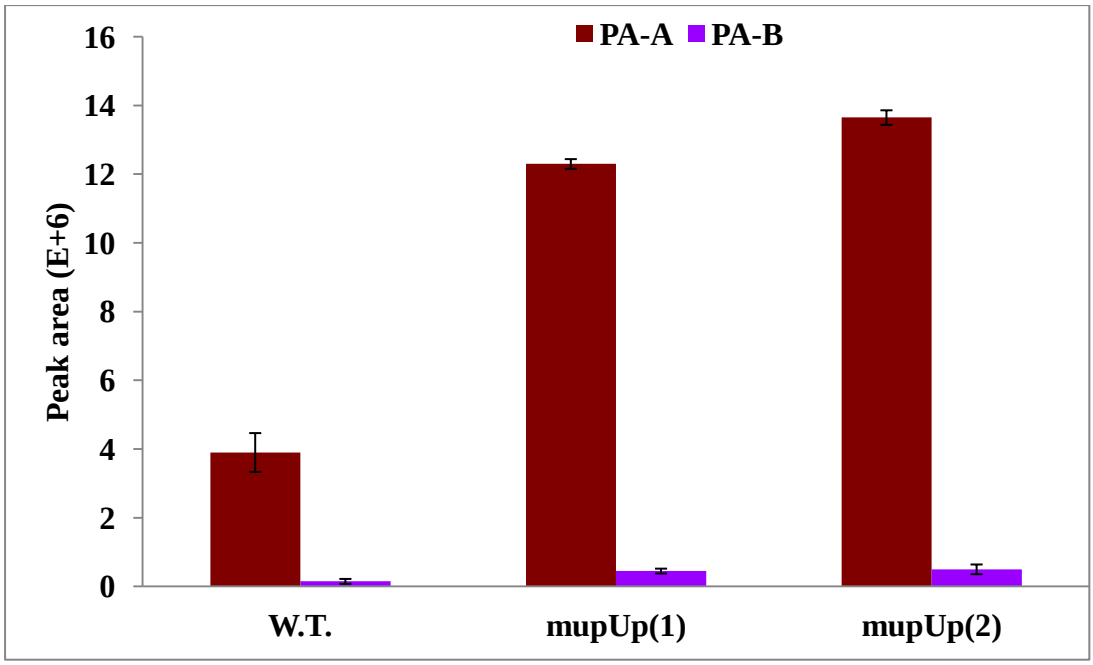



**Figure 5.8 HPLC analysis of production of PA-A and PA-B by the WT *P. fluorescens* and strains with new promoter upstream of *mupU*. Samples were collected from cultures grown under similar conditions, and triplicates were used for each isolate.**

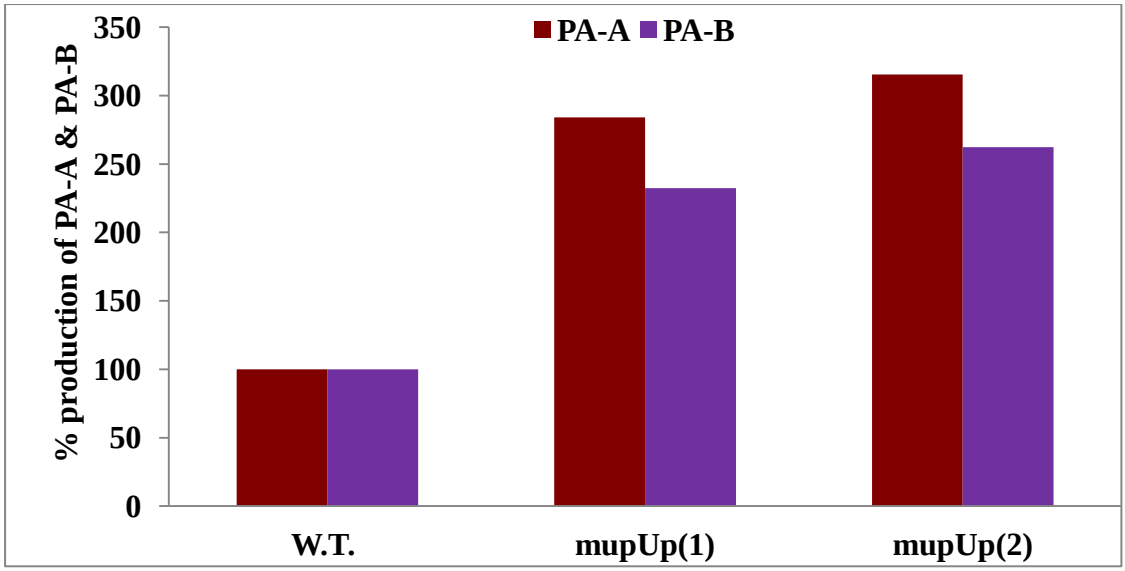



**Figure 5.9 Quantitative HPLC of PA-A and PA-B production by isolates with the new promoter upstream of *mupU*, in comparison to the WT strain of *P.fluorescens*.**

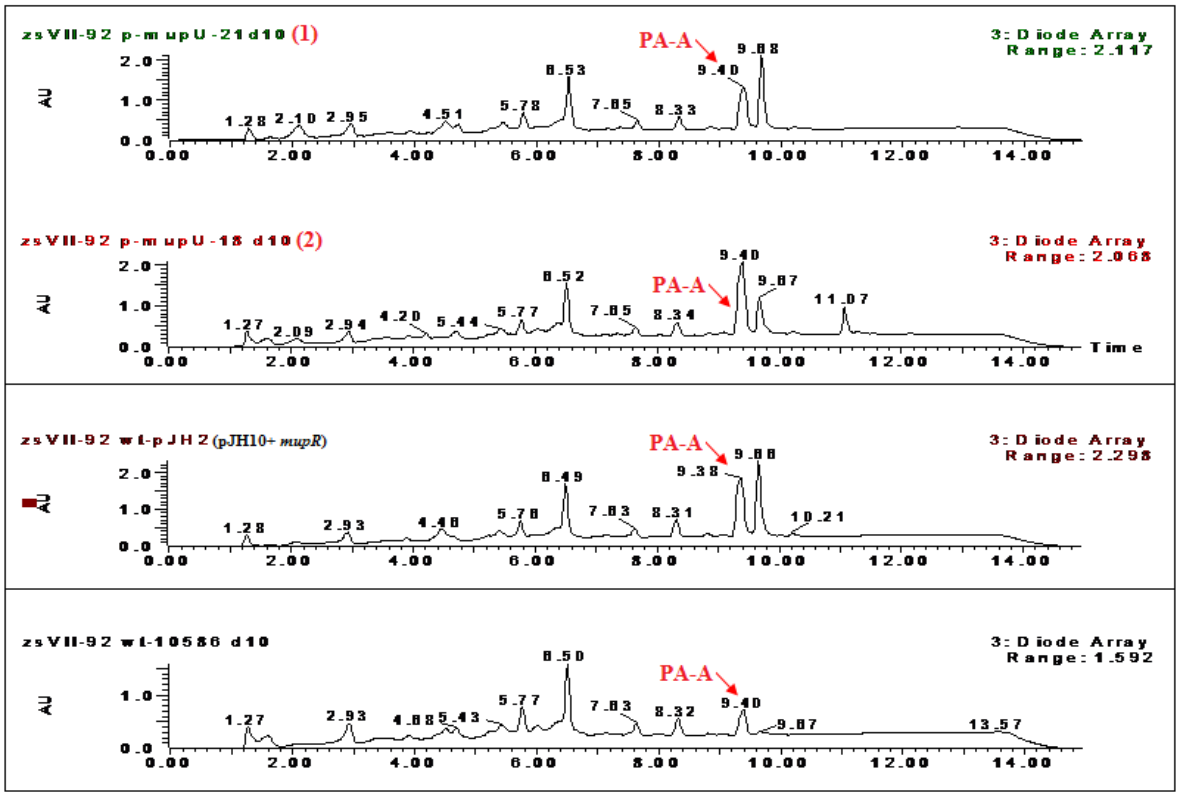



**Figure 5.10 Diode array and LC-MS of mupirocin production by isolates of *P. fluorescens* with new promoter upstream of *mupU* (at the top), in comparison to the WT (bottom one) and WT with *mupR* (the transcriptional activator) expression *in trans* from pJH2 (pJH10) plasmid (in the middle). Analysis done by Dr. Song in Bristol.**

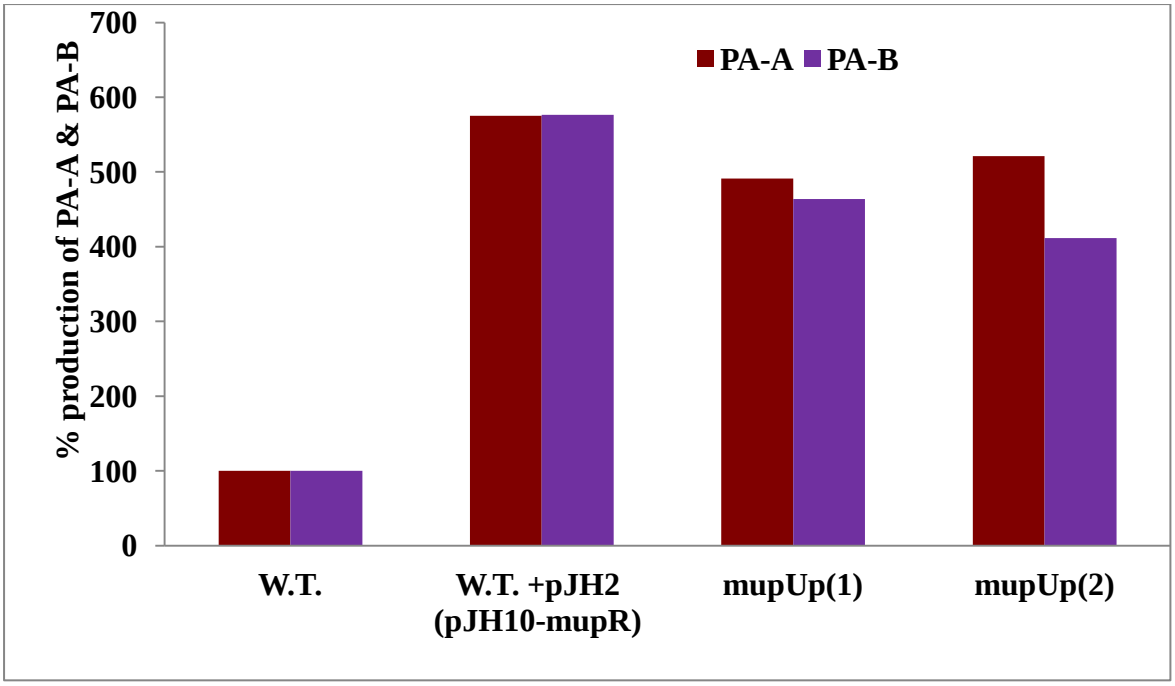



**Figure 5.11 Quantitative MS analysis of PA-A and PA-B production by isolates with the new promoter upstream of *mupU*, in comparison to the WT strain of *P. fluorescens* and WT with *mupR* provided *in trans* by pJH2 (pJH-*mupR*).**

### 5.3.3 Construction of *P. fluorescens* strain with Δ*mupU* and Δ*macpE*

Going back to our proposal that in order to increase the production of PA-A relative to PA-B, we need more ACPs as well as more of the enzyme that transfers the intermediate to ACP. Since the first attempt through increasing transcription of *mupU* (the acyl CoA synthase) gene did not have the desired effect of reducing PA-B production, it was decided to fuse *mupU*, and *macpE* to increase the efficiency of transfer. To test the function of such a hybrid, a double deletion strain was needed. NCIMB10586Δ*macpE* was used for adding an in-frame deletion of *mupU* as well. To do so, two regions, upstream and downstream of *mupU* were PCR amplified using specific primers designed for that purpose. The forward

primer of the upstream arm and the reverse primer of the downstream arm contained *Bam*HI, and *Eco*RI restriction endonuclease sites, respectively. However, both the reverse primer of the upstream arm, and the forward primer of the downstream arm contained an *Xba*I restriction endonuclease site. Both of those two arms were ligated into pGEM-T Easy vector (Table 5.2), separately, and the arms that did not show any mismatches from the DNA sequencing, were selected and inserted into the *Bam*HI-*Eco*RI site of the suicide (pAKE604) vector to create pAMK2 vector. This new pAMK2 plasmid was used in the standard way to create strains with both Δ*macpE* and the additional in-frame deletion of *mupU* which was confirmed as successful by PCR, and designated as NCIMB10586Δ*mupU*/Δ*macpE*.

This new strain was used for both *in trans* expression of fused *mupU*(-STC)--*macpE*(+STC) while looking for complementation study and atomic level surgery to insert fused *mupU*(-STC)--*macpE*(-STC) into different locations within the mupirocin gene cluster, described subsequently in this Chapter.

### 5.3.4 Construction of a *mupU-macpE* expression plasmid

For expression of fused *mupU-macpE in trans* in NCIMB10586Δ*mupU*/Δ*macpE*, pAMH3 plasmid was constructed (Figure 5.12). To create this plasmid, the oligonucleotide linker with 20 amino acids shown in Table 5.4 was first inserted into *Kpn*I-*Xba*I sites of pJH10 vector under the control of *tac* promoter, and the insertion confirmed by different digestion like, *Kpn*I-*Mfe*I, *Kpn*I-*Xba*I, and *Cla*I-*Xba*I.

In order to create a fusion of *mupU* and *macpE*, the stop codon was removed from the *mupU* gene, and both of *mupU* and *macpE* were amplified by PCR using specific primers, which were then ligated into pGEM-T Easy vector for DNA sequencing (Table 5.2).

However, it should be mentioned that internal primers (Table 5.3) were used for further DNA sequencing of *mupU* because it has a long sequence (1.6 kb). PCR amplified *mupU* and *macpE* genes which were confirmed as not having any mismatches were cloned into *Eco*RI-*Mfe*I and *Cla*I-*Xba*I sites of the pJH10 vector with the oligonucleotide linker, respectively, creating pAMH3 plasmid, so that the orfs would be joined by the short orf encoded in the linker. Moreover, the reason for using this linker was to leave enough space to achieve the proper folding of fused proteins and to get translated into a functional polypeptide.

### 5.3.5 Complementation analysis of *mupU* and *macpE* expressed from pAMH3 plasmid

Plate bioassay was used to check the expression and function of the fused *mupU-macpE* downstream from *tac* promoter. Plasmid pAMH3 carrying the fused *mupU-macpE* genes, was transferred to NCIMB10586Δ*mupU*/Δ*macpE* by bacterial conjugation. The inhibitory (clearance) zone around the spot of bacterial culture under investigation was used as an estimate of antibacterial activity against sensitive bacteria. Plates of L-agar, without induction as well as with 0.5 mM IPTG induction were used for performing the bioassay. The bioassay result showed that the fused genes were expressed from pAMH3 plasmid and were functional, and antibiotic production was restored in NCIMB10586Δ*mupU*/Δ*macpE* even without IPTG (Figure 5.13). Induction with 0.5 mM IPTG was found to improve antibiotic production significantly by about 15 %. However, induction with 0.5 mM IPTG decreased the antibiotic production (Figure 5.14) in NCIMB10586Δ*mupU*/Δ*macpE*, in the presence of empty pJH10 vector.

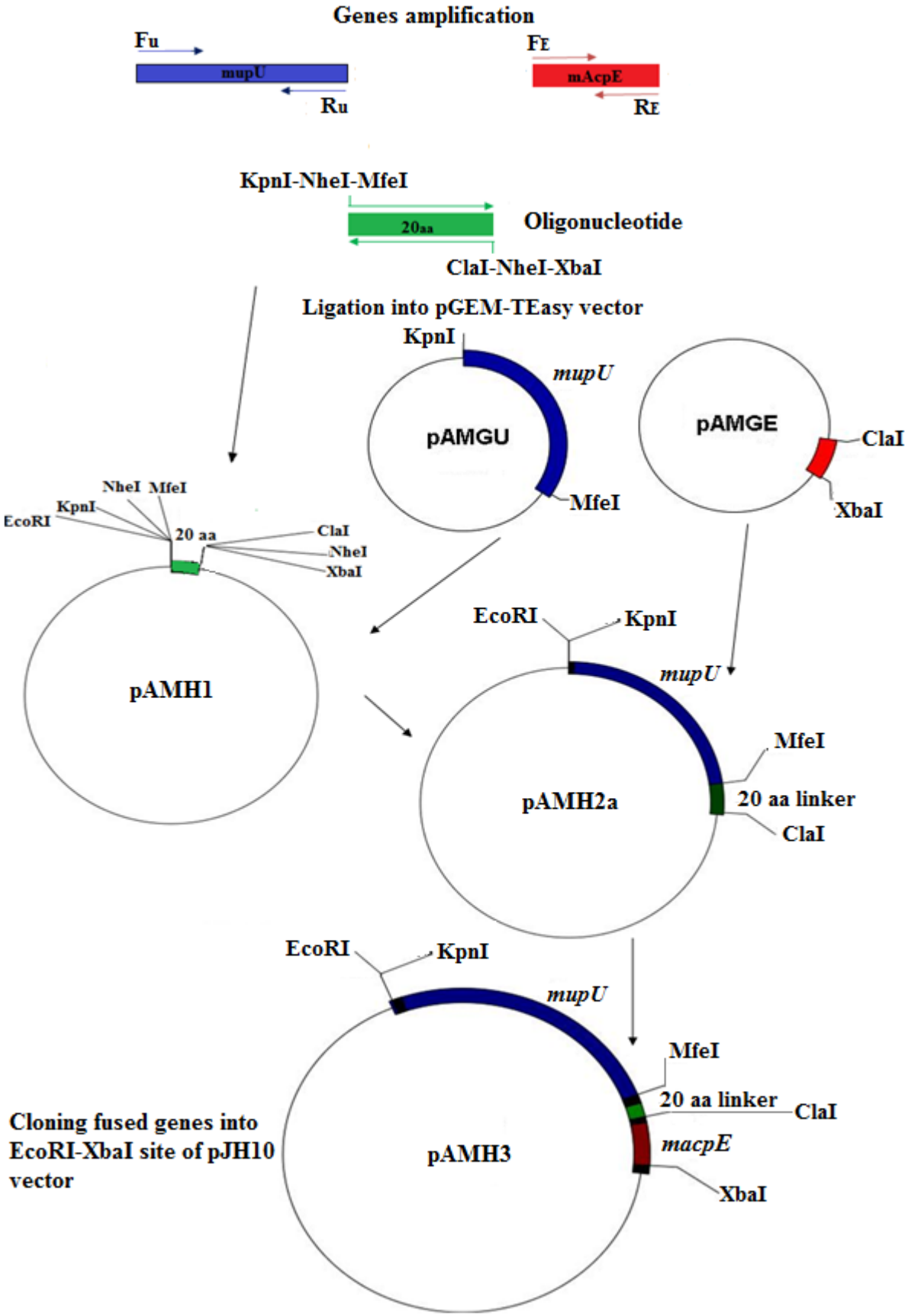



**Figure 5.12 Strategy for the construction of pAMH3 plasmid.**

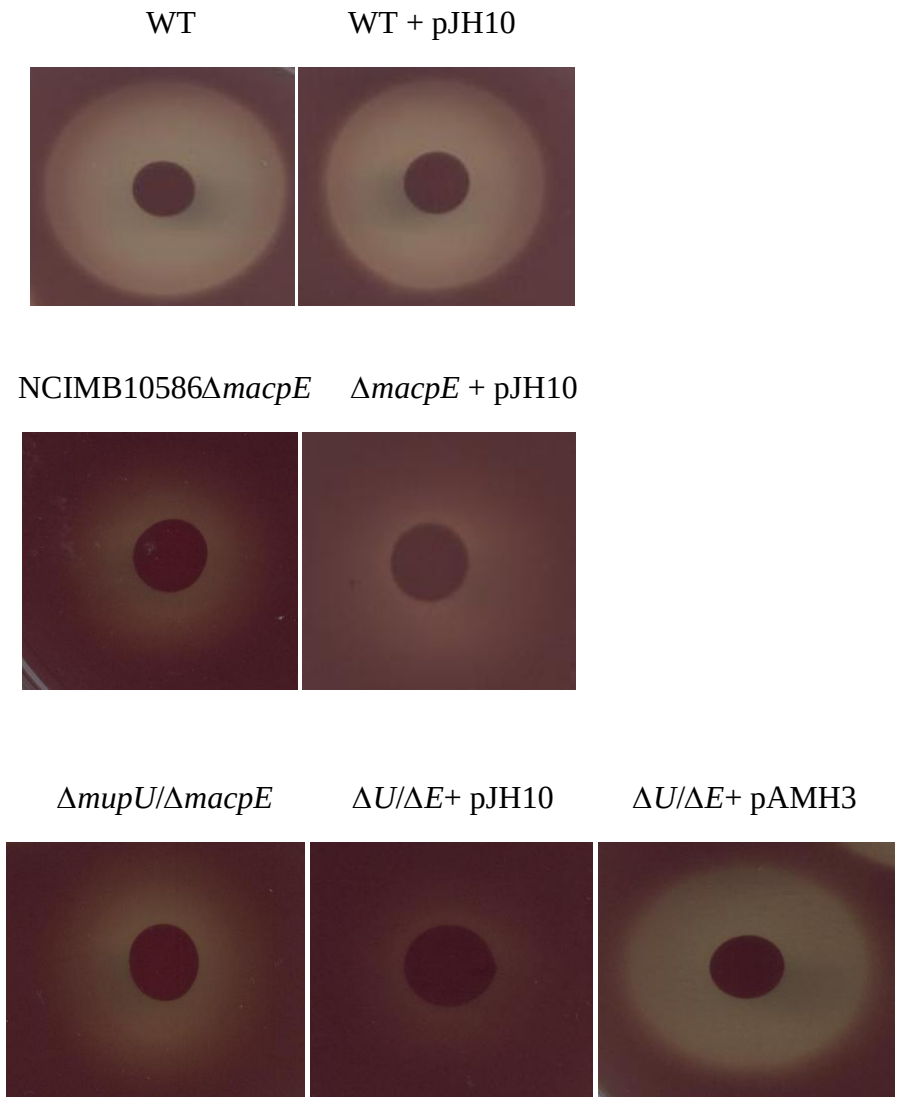



**Figure 5.13 Bioassay to determine complementation of NCIMB10586Δ*mupU*/Δ*macpE* by the *in trans* expression of fused *mupU*/*macpE* genes from pAMH3 plasmid without IPTG.**

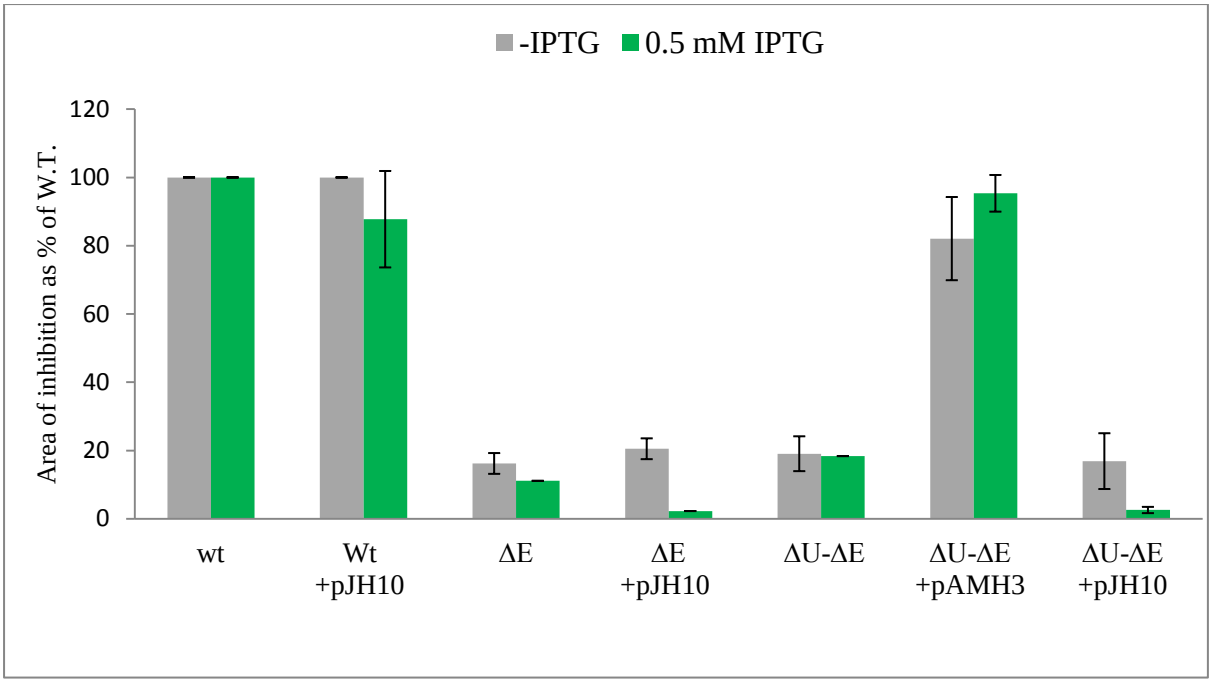



**Figure 5.14 Quantitative bioassay of NCIMB10586Δ*mupU*/Δ*macpE* strain expressing fused *mupU/macpE in trans* from pAMH3 plasmid in comparison to the wild type (WT) performed with and without 0.5 mM IPTG and with triplicates.**

## 5.3.6 Quantitative analysis of PA-A and PA-B produced by NCIMB10586Δ*mupU*/Δ*macpE* expressing fused *mupU*/*macpE*

More precise data about complementation of NCIMB10586Δ*mupU*/Δ*macpE* strain expressing fused *mupU*(-STC)-*macpE* from pAMH3 plasmid obtained via HPLC analysis. According to our hypothesis that MupU and mAcpE are working in succession, therefore, expression of fused *mupU*(-STC)-*macpE in trans* from pAMH3 plasmid should complement the strain with deletion in those two genes. HPLC chromatograms showed that without IPTG induction either there was about 50% PA-A, or much less compare to PA-B. However, full complementation and even production of a little more of both PA-A and PA-B was observed

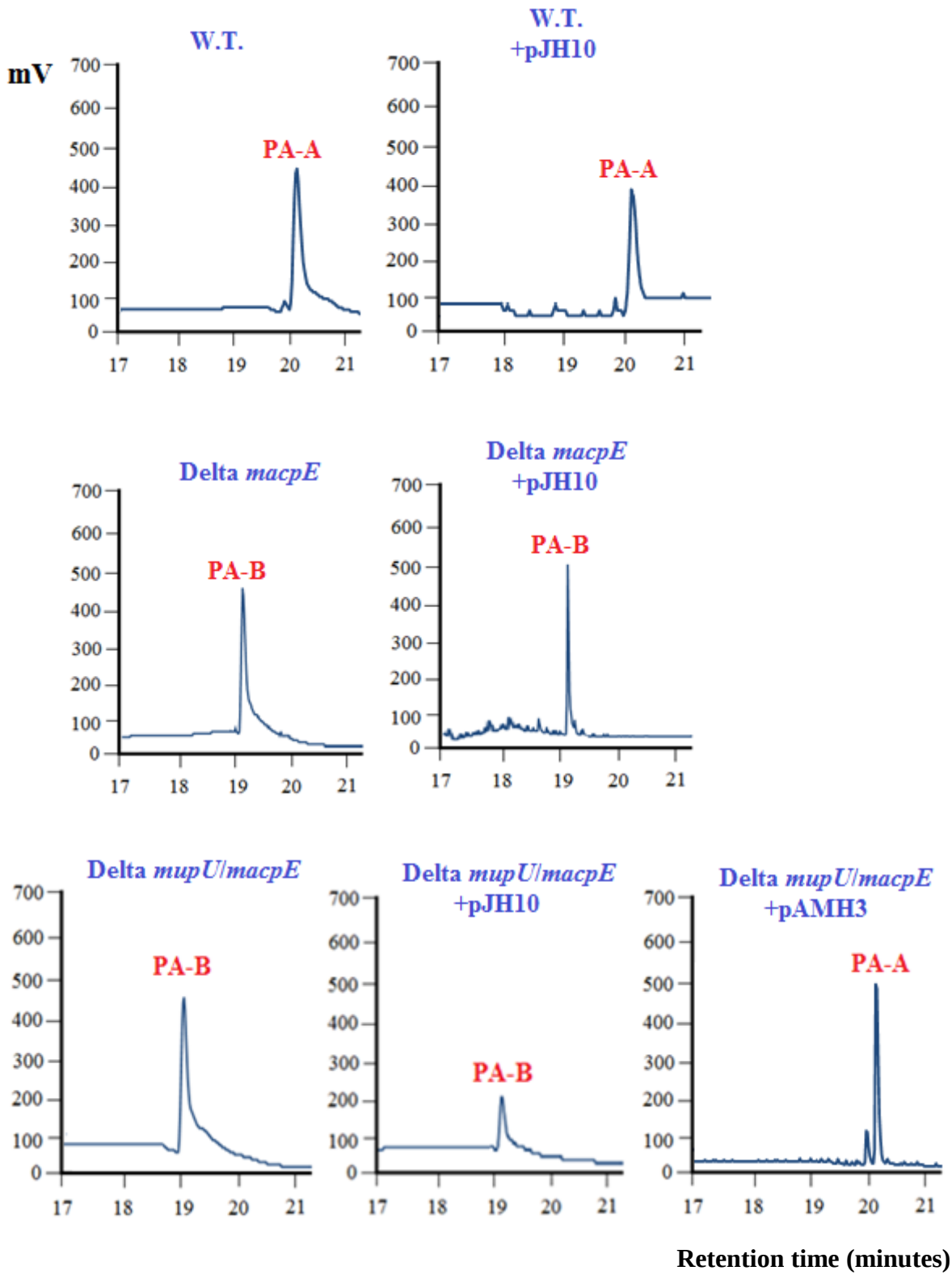



**Figure 5.15 HPLC chromatograms of NCIMB10586Δ*mupU*/Δ*macpE* strain with pAMH3 plasmid expressed *in trans*. NCIMB10586 WT, NCIMB10586Δ*macpE*, and NCIMB10586Δ*mupU*/Δ*macpE* strains with and without pJH10 are taken as controls.**

compared to the wild type when the *tac* promoter induced with 0.5 mM IPTG (Figures 5.15, 5.16, and 5.17). This might indicate that MupU and mAcpE are playing their role successively, and are able to work together when they get fused and translated into a single polypeptide.

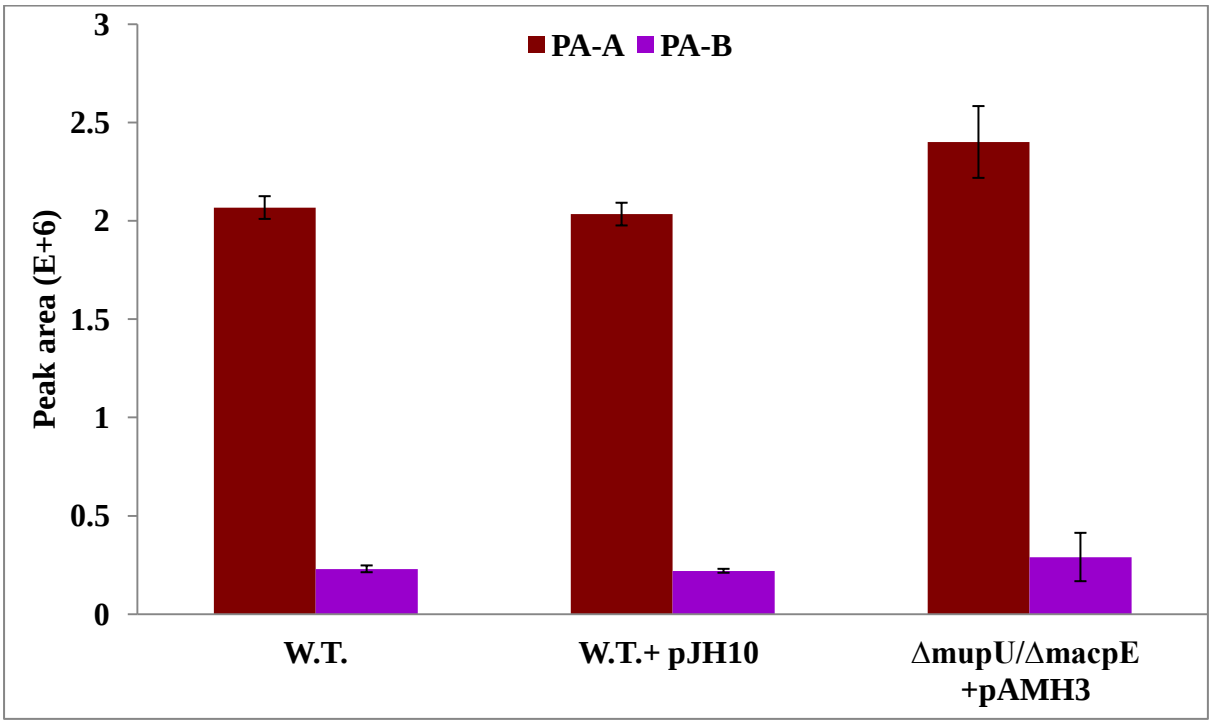



**Figure 5.16 HPLC analysis of PA-A and PA-B production by the wild type strain of *P. fluorescens*, and strain of NCIMB10586Δ*mupU*/Δ*macpE* complemented through *in trans* expression of fused *mupU*/*macpE* by pAMH3. Triplicate samples were collected from induced cultures grown under similar conditions.**

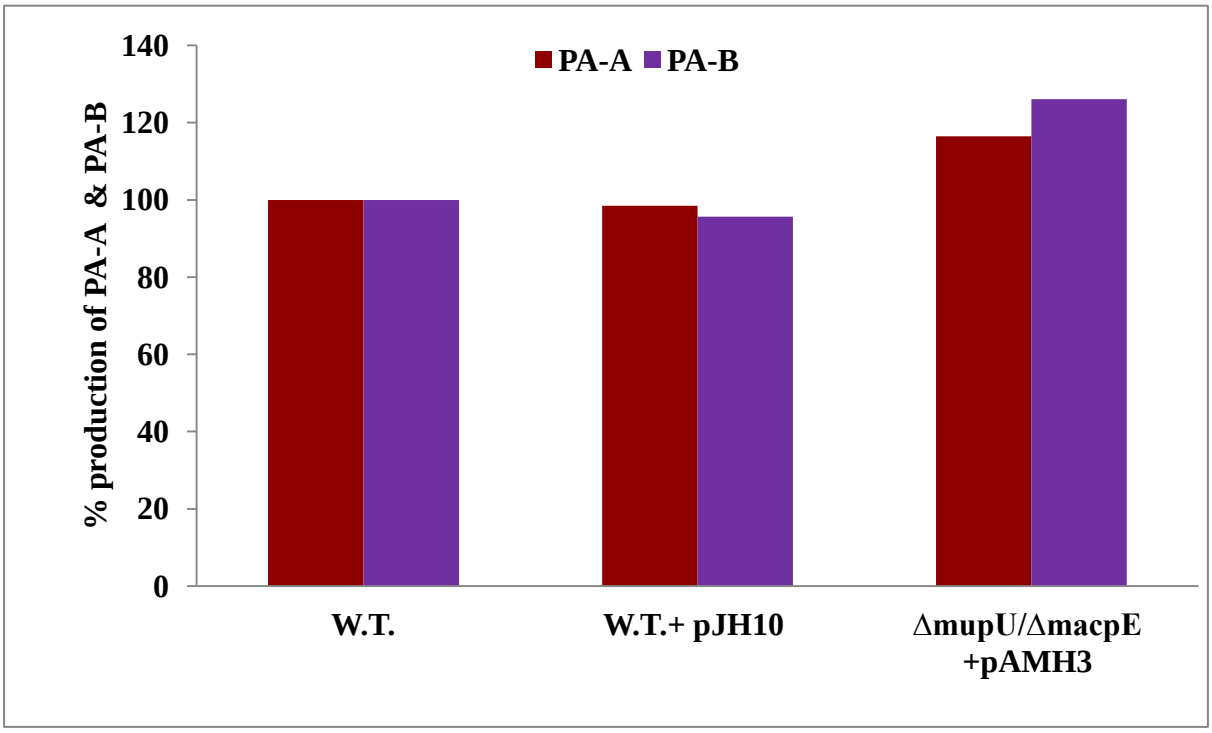



**Figure 5.17 Quantitative HPLC of PA-A and PA-B production by NCIMB10586Δ*mupU*/Δ*macpE* strain, complemented through *in trans* expression of fused *mupU*/*macpE* from pAMH3, in comparison to the wild type strain with IPTG induction.**

## 5.3.7 Bioassay and HPLC analysis of NCIMB10586Δ*mupU*/Δ*macpE* strain with fused *mupU*/*macpE* insertion either after ACP7(Bc) of *mmpB* or after ACP4(A3b) of *mmpA*

Because we had been able to demonstrate that a fusion protein of MupU-mAcpE can complement NCIMB10586Δ*mupU*/Δ*macpE* strain through *in trans* expression of the fused *mupU*(-STC)-*macpE*, this added extra supportive evidence for the proposed role of MupU and mAcpE in the conversion of PA-B to PA-A. Also, the absence of an *macpE* homologue

in thiomarinol gene cluster and the presence of extra KS and ACP in TmpB (Figure 5.2b) before the TE domain, suggested that this extra non-elongating module could be responsible for all the late tailoring steps (Fukuda *et al*., 2011) in thiomarinol, just as mAcpE is implicated in during the production of mupirocin (Cooper *et al*., 2005a). In addition, results in Chapter 4 of this thesis demonstrated the probable role of this extra module in the removal of $C_8$-OH group, and the release of thiomarinol PA-B analogue (HT-G or C and TH-H). Therefore, and as a third attempt to reduce PA-B, and to increase PA-A the highest level, we have planned to insert the fused *mupU*(-STC)-*macpE*(-STC) into *mmpB* in the linker region between ACP7 and the TE domain. This insertion might fit with the suggestion that both the $C_9$ and $C_8$ fatty acid side chains in mupirocin and thiomarinol respectively, seems to be built up on a PKS derived (monic acid and marinolic acid) part by a successive elongations, rather than by the ligation of the fully assembled hydroxy acids (Murphy *et al*., 2011). However, the second insertion of the fused *mupU*(-STC)-*macpE*(-STC) was planned to be inserted before the stop codon of ACP4 of *mmpA*, which fits with the idea that monic acid in mupirocin might get loaded onto mAcpE first.

To create the suicide vectors for both of the two insertions, and to be certain that the fused *mupU*/*macpE* is going to be included and translated as a single functional polypeptide in each insertion, new primers were used for the PCR amplification of *macpE* without the stop codon as long as we had *mupU*(-STC). Those two genes were inserted into *Cla*I-*Xba*I and *Eco*RI-*Mfe*I of the pAMH1 (pJH10 plasmid with a 20 aa linker) plasmid, respectively, creating pAMH2-b and pAMH4 plasmids (Table 5.2). Upstream and downstream arms for both of the two insertions were amplified by PCR using specific primers designed for that purpose. The upstream arms (ACP7-linker, and ACP4, respectively) both had *Bam*HI-*Eco*RI restriction sites and were cloned into pAMH4 plasmid separately using same restriction site to create pAMH5 and pAMH6 plasmids, respectively. However, downstream arms (linker-TE, and

*mupB*, respectively) contained *Xba*I-*Eco*RI restriction sites and were inserted into same restriction site in pAKE604 vector (Figure 5.18) separately to create pAMK3 and pAMK5, respectively. Then both pAMH5 and pAMH6 plasmids were digested with *Bam*HI-*Xba*I restriction endonuclease to release the upstream arm, and fused with *mupU*(-STC)-*macpE*(-STC) and ligated into pAMK3 and pAMK5 plasmids to create the final suicide vectors pAMK4 and pAMK6, respectively. However, it should be mentioned that all the PCR amplified genes were sequenced using pGEM-T Easy vector, labelled pAMG, which followed by a letter as a symbol for the gene (Table 5.2).

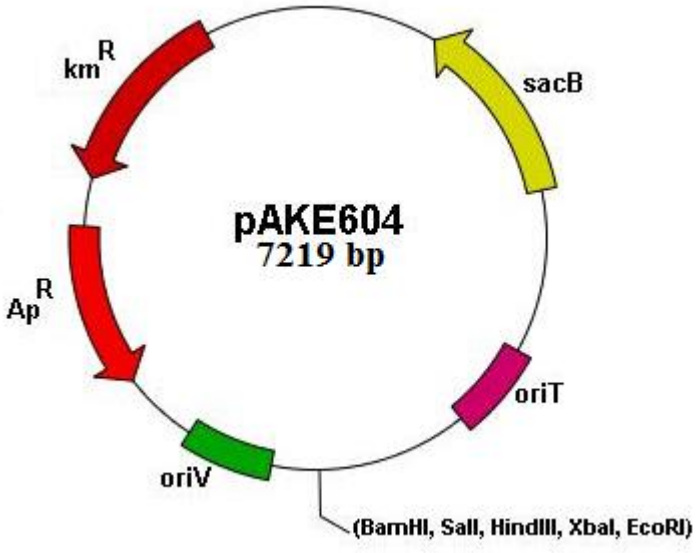



**Figure 5.18 Plasmid pAKE604 that was used to create the suicide vectors pAMK4, and pAMK6, that were used for insertion mutation in the mupirocin cluster (El-Sayed *et al*., 2001).**

Plasmid pAMK4 was used as a suicide vector for the insertion of the fused *mupU*(-STC) – *macpE*(-STC) into *mmpB* of NCIMB10586Δ*mupU*Δ*macpE*, and pAMK6 was used similarly

for the insertion after ACP4 of *mmpA* of the same NCIMB10586Δ*mupU*/Δ*macpE* strain. Selected bacterial isolates were tested by PCR to confirm the insertion of the fused *mupU*(-STC)-*macpE*(-STC) into mupirocin genes cluster. Two mutants were obtained for each type insertion. The insertion into *mmpB* was in the linker region between ACP7 and TE. The *mupU* and *macpE* orfs had been joined by the short orf of linker and were kept in frame with the orf in *mmpB* so that they would get translated into a single functional polypeptide. However, it was discovered that the other insertion was after the stop codon of ACP4 of *mmpA*, which means it was not included into *mmpA*, and this was a mistake which would need to be rectified if there was more time.

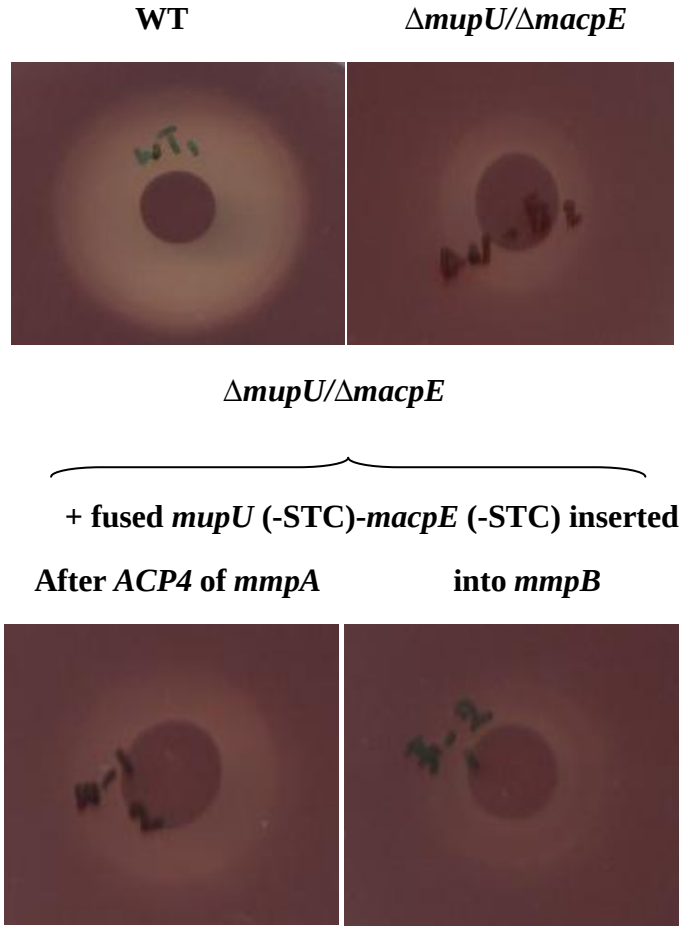



**Figure 5.19 Bioassay to determine the effect of fused *mupU*(-STC)-*macpE*(-STC) insertion into either *mmpB* or after ACP4 of *mmpA* of NCIMB10586Δ*mupU*/Δ*macpE* using WT NCIMB10586 and NCIMB10586Δ*mupU*/Δ*macpE* strains as controls.**

Bioassay results did not show a significant difference between the NCIMB10586Δ*mupU*/Δ*macpE* strain and derivatives with fused *mupU*(-STC)-*macpE*(-STC) inserted into either *mmpB* or after *mmpA* with regard to the production of biologically active product (Figure 5.19 and 5.20). Moreover, the percent production was about 1/3 of the WT NCIMB10586. Therefore, this means that we did not achieve complementation, and this might be due to improper folding of the fused proteins in MmpB. However, the case is different with the second insertion as long as the insertion was not included into *mmpA*.

HPLC analysis (Figure 5.21) showed the production of PA-B by mutant strains with fused *mupU*(-STC)-*macpE*(-STC) inserted after ACP4 of *mmpA*, which was similar to the original NCIMB10586Δ*mupU*/Δ*macpE*. However, there was no clear cut evidence of a peak related to any biological compounds (PA-A or PA-B) with the insertion of the same fused genes into *mmpB*. Further mass spectrometry analysis confirmed HPLC results.

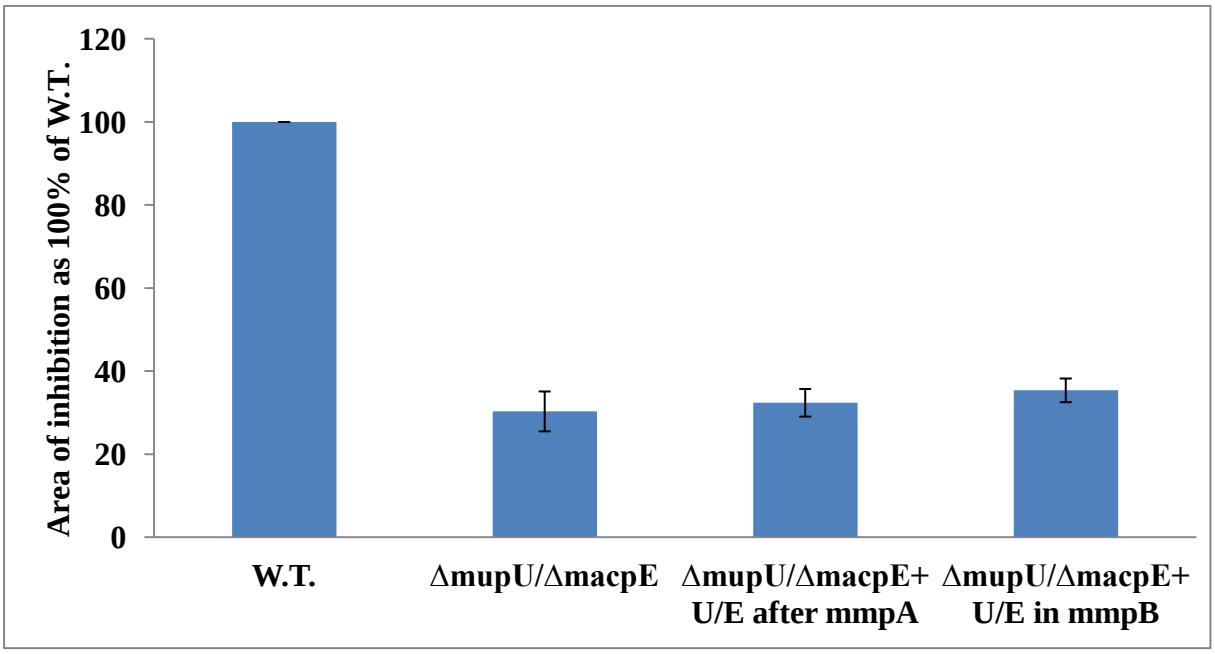



**Figure 5.20 Quantitative bioassay of NCIMB10586Δ*mupU*/Δ*macpE* with fused *mupU*(-STC)-*macpE*(-STC) inserted into either *mmpB* or after ACP4 of *mmpA* using WT NCIMB10586 and NCIMB10586Δ*mupU*/Δ*macpE* strains as controls. Data was collected from more than triplicate samples.**

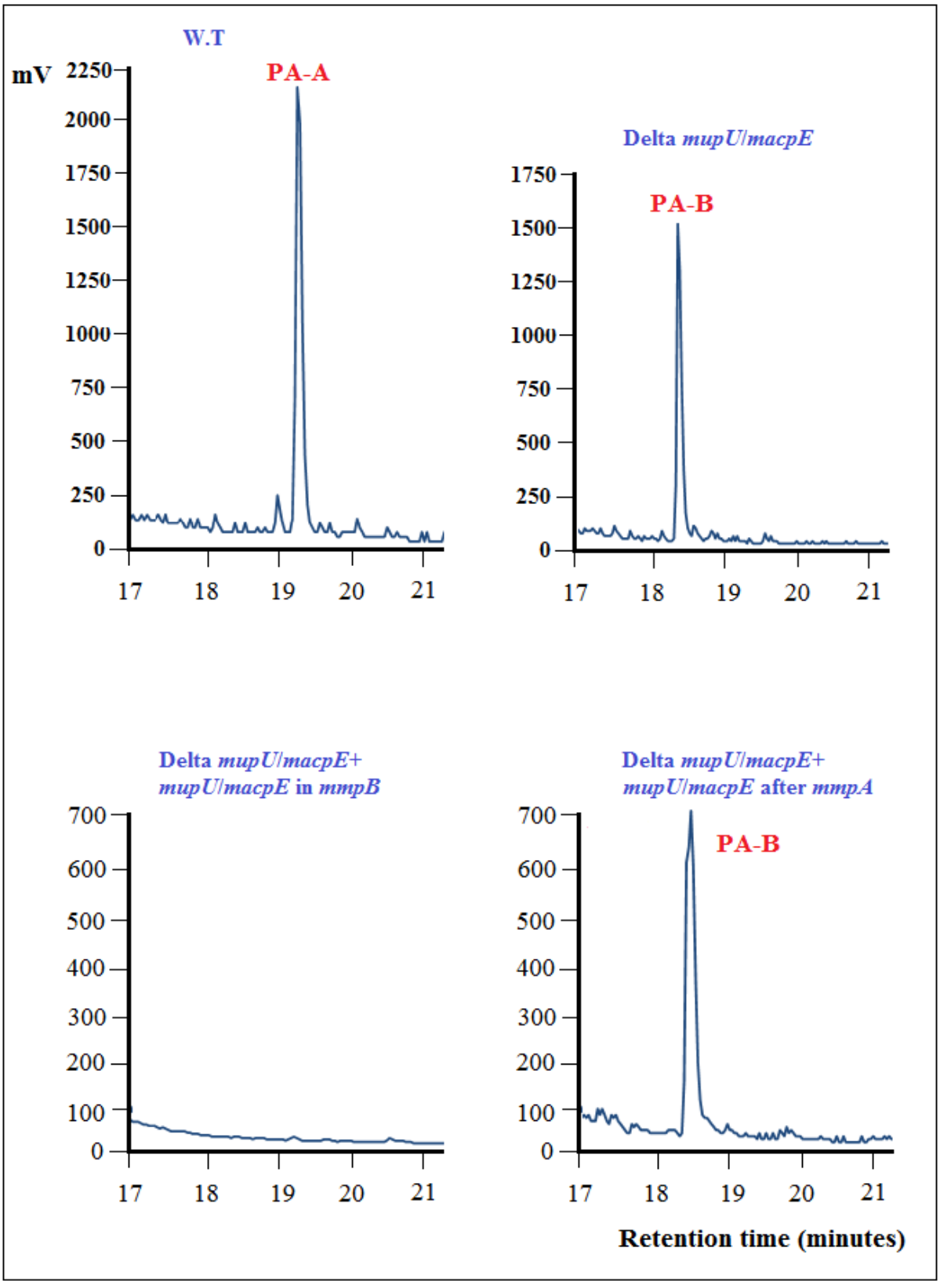



**Figure 5.21 HPLC chromatograms of NCIMB10586Δ*mupU*/Δ*macpE* with fused *mupU*(-STC)-*macpE*(-STC) inserted into either *mmpB* or after *mmpA* using WT NCIMB10586 and NCIMB10586Δ*mupU*/Δ*macpE* strains as controls.**

## 5.4 Discussion

The idea of attempting to decrease the production of PA-B, as a minor product of mupirocin started when Cooper *et al*., (2005b) showed that in-frame deletion of *mupO, mupU, mupV* and *macpE* abolished PA-A production, which means they are essential for PA-A. Also that two additional genes, *mupC* and *mupF* were proposed to be implicated in the conversion of PAB to PA-A (Hothersall *et al*., 2007). In this study we used three strategies for reaching that purpose. The first was to investigate the effect of increasing the expression of *mupU*, the gene that is proposed to be involved in the transfer of the intermediate to mAcpE, on the production of PA-A, through putting a new promoter upstream *mupU* . The second was to test the capability of over-expression of fused *mupU*(-STC)-*macpE in trans* in NCIMB10586Δ*mupU*/Δ*macpE* strain to complement the double deletion, and to divert the production away from PA-B in favour of PA-A. The third was to construct something like in TmpB (Figure 2b) of the thiomarinol cluster by inserting fused *mupU*(-STC)-*macpE*(-STC) into *mmpB* of NCIMB10586Δ*mupU*/Δ*macpE* strain once, and after ACP4 of *mmpA* as a second to reduce PA-B, which in turn should increase PA-A to the highest level.

Genetically manipulated strains of *P. fluorescens* with a new promoter upstream *mupU* were constructed successfully, and the effect on the production of pseudomonic acids was determined using HPLC analysis. While constructing these strains, all the positions within the consensus sequence (-35 region, spacer 17 nucleotide, and -10 region) were conserved. In addition, the transcriptional start point was kept as typical as possible within 7 bases downstream the -10 region of the promoter, and the natural RBS of *mupU* was conserved before the start codon in order to create the optimum conditions for translation. Results of bioassay showed a significant increase in the production of mupirocin up to more than two fold compare to the wild type strain. This increase in the production of mupirocin might be

due to increased expression of all the other genes downstream *mupU* as well. Therefore, this might be due to not only the role of MupU, but also it might be due to the role of other proteins including MupV, and MupR (the transcriptional activator). This is consistent with both Macioszek's observation while over-expressing *mupV in trans* ((Macioszek, PhD thesis, University of Birmingham, 2009), and over-expression of *mupR in trans* (Hothersall *et al*., 2011) in the WT NCIMB10586.

HPLC and LC-MS analysis showed that genetically improved strains did not abolish PA-B production as predicted by our hypothesis. Even though, both PA-A and PA-B was increasing up to more than three fold in comparison to the WT NCIMB10586, and approximately the same productivity of WT strain expressing *mupR in trans*. Therefore, although this result represents a new approach to increase the production of total antibiotic, it does not provide a practical way to shutt down PA-B pathway. This result is consistent with the result of over expression of *mupO*/*macpE*/*U*/*V*/*C*/*F in trans* in the wild type of mupirocin producer (Macioszek, PhD thesis, University of Birmingham, 2009). One possible explanation for the inability to shut down the PA-B pathway by this approach might be due to the level of other rate-limiting proteins implicated in PA-A production. For example mAcpE, which receives the intermediates from MupU, that needs to be processed further by MupO/V/C/F for the production of PA-A. Therefore, in the availability of more MupU, there might be less mAcpE, which means less processing of the intermediate, and ultimately less conversion of PA-B to PA-A. This is consistent with the concept that to increase the flux from PA-B to PA-A, we need more ACP (represented by mAcpE) and the enzyme (MupU) essential to transfer the intermediates onto the ACP.

To over express the fused *mupU*(-STC)-*macpE in trans* and to determine the influence on the level of pseudomonic acids by HPLC, both *mupU*(-STC) and *macpE* were fused together by a short orf encoded by a 20 aa linker while keeping their RBS intact with a successful

cloning into pAMH3 plasmid. Data from the bioassay of over-expression of fused *mupU*(-STC)-*macpE in trans* from the *tac* promoter of pAMH3 plasmid showed that NCIMB10586Δ*mupU*/Δ*macpE* strain restored antibiotic production even without induction. However, full complementation and production of antibiotic to the wild type level were observed when 0.5 mM IPTG was used for induction. This results indicate that MupU, and mAcpE were able to complement NCIMB10586Δ*mupU*/Δ*macpE* strain when they were translated as a single functional polypeptide.

More precisely, HPLC analysis of *in trans* expression of fused *mupU*(-STC)-*macpE* from pAMH3 plasmid in NCIMB10586Δ*mupU*/Δ*macpE* strain showed production of both PA-A and PA-B either in similar quantities or very less PA-A without induction. Surprisingly, a smaller increase in the production of both PA-A and PA-B than the wild type level was observed while 0.5 mM IPTG used for the induction of *tac* promoter. The inability to achieve full complementation of NCIMB10586Δ*mupU*/Δ*macpE* strain and the production of PA-A as a major product without induction, might have been due to insufficient fused MupU, and mAcpE. However, although full complementation of the NCIMB10586Δ*mupU*/Δ*macpE* strain was achieved while over-expressing fused *mupU*(-STC)-*macpE in trans*, it does not provide a way to abolish PA-B. Presumably, this might have been due to the *in trans* expression of fused *mupU*(-STC)-*macpE*, which makes some difference if they are expressed *in cis* with the whole biosynthetic gene cluster. Therefore, it might be that there is inevitable release of a small amount of PA-B until the fused MupU/mAcpE starts playing its role in the biosynthetic pathway.

Atomic level surgery followed through inserting the fused *mupU*(-STC)-*macpE*(-STC) into *mmpB* of NCIMB10586Δ*mupU*/Δ*macpE* strain and exactly in the linker region between ACP7 and the thioesterase (TE). This is to test the expected position where MupU and mAcpE are proposed to work in the mupirocin system during the biosynthesis pathway, and

makes something similar to the KS°-ACP° (2nd TmpB module) in the thiomarinol system. Also it was proposed that this might shut down PA-B and divert the pathway to the major compound PA-A, as in the case of thiomarinol normally there is a release of a very tiny amount of PA-B analogue (TH-G). Concomitantly, fused *mupU*(-STC)-*macpE*(-STC) proposed to be inserted before the stop codon of ACP4 in *mmpA* of NCIMB10586Δ*mupU*/Δ*macpE,* as it was expected that the MA (PKS part) of mupirocin might be loaded onto mAcpE by MupU before the esterification with 9-HN, the fatty acid side chain of mupirocin. Unfortunately, the fused genes were inserted after the stop codon of ACP4, which means it was not included in *mmpA*. Bioassay result of mutants with the insertions did not show either complementation of NCIMB10586Δ*mupU*/Δ*macpE* strain by production of PA-A, or a remarkable increase in the biological activity compare to NCIMB10586Δ*mupU*/Δ*macpE* strain in either case against the sensitive bacteria tested. HPLC analysis confirmed production of PA-B, quite similar to the NCIMB10586Δ*mupU*/Δ*macpE* strain for the insertion after *mmpA*, indicate that the inserted genes are not interfering with the biosynthetic pathway in a major way. However, and surprisingly, no detectable compound was observed in mutants with the insertion of the fused genes in *mmpB*. This may indicate that MupU and mAcpE are normally not acting in the biosynthesis of mupirocin in this position, therefore, the whole biosynthesis blocked, or it might be simply be due to the disruption of the region between ACP7 and TE, that affects the action MmpB. However, we wanted to prevent disrupting the linker that join ACP7 to TE in *mmpB*, which consists of 160 amino acids by inserting the fused *mupU*(-STC)-*macpE*(-STC) in the middle of that linker while keeping the genetic codes conserved, and to keep both ACP7 and TE apart from the inserted genes. This result is consistent with the observation made by Tran et al., (2008), that there is no sequence conservation of the linker between ACP6 and TE in the DEBS system. However, it appears as if the amino acid sequence of the

linkers keeps the domains apart yet permits them moving about relative to each other, as they confer a degree of stiffness (Weissman, 2004).

Kira Weissman (Nancy University), Peter Leadlay (Cambridge University) and their colleagues suggested that the TE interacts more significantly with the ACP's attached acyl moiety than the ACP itself, rather than the idea that phosphopantetheinylation and/or acylation makes some structural changes to the ACP that promotes its binding to TE (Tran et al., 2008). They also reported that DEBS TE interacts with the ACP in the erythromycin system through covalent linkage, and that both the transfer and the release of the chain will occur ideally if the proper architectural relationship is maintained and the substrate on the ACP contains suitable recognition features for the TE. Therefore, it might be that the mAcpE attached acyl group was facing the TE in case of mutants with fused *mupU*(-STC)-*macpE*(-STC) insertion into *mmpB* before its loading onto MupV/C/F for the production of PA-A, and that the TE was not able to recognise the mAcpE's attached acyl group in order to release it. Moreover, it is principally agreed that engineering experiments including altering domains (deletions, additions, and swaps) that alter the enzyme organization might translate into a predictable change in the structure of the polyketide. However, many of these experiments are not successful in giving the expected polyketides, and the yields are often too low even if they can be made, that are not useful for large scale production (drug synthesis), and trials to trace the problems are usually difficult (Weissman, 2004).

We have demonstrated that genetically manipulated strains of *P. fluorescens* with a new promoter upstream of *mupU* could have a very strong effect on increasing the production of total antibiotic, but not diverting one compound (PA-B) in favour of the other (PA-A). This might be due to the imbalance of the level of different proteins essential for a specific pathway (PA-A). Over-expression of fused *mupU*/*macpE*, the two important factors in PA-A production were able to restore antibiotic production in the mutant

NCIMB10586Δ*mupU*/Δ*macpE* when they were translated into a single polypeptide but they were unable to abolish the PA-B pathway by increasing the flux of the intermediates. This also might be due to low level of other rate limiting enzymes essential for PA-A production as long as it appears like normally Mup proteins are expressed at low levels. Genetically engineered NCIMB10586Δ*mupU*/Δ*macpE* with *mupU*/*macpE* fused into *mmpB* did not complement the deleted genes, which indicates that they are unable to act in that position. However, it might be a good idea of splicing the KS°-ACP° and the TE from TmpB in thiomarinol with the tandem ACP of MmpB instead of the TE in mupirocin, which might be helpful to switch on the whole mupirocin biosynthesis into PA-A.

# CHAPTER 6

# ATTEMPTS TO TRANSFER THIOMARINOL PRODUCTION PLASMID pTML1 TO INVESTIGATE EXPRESSION AND REGULATION OF THE BIOSYNTHETIC GENES

## 6.1 Introduction

Understanding the mechanisms or the aspects of the genetic regulation of antibiotic production by the bacteria that produce them will open a strong and rapid way of improving strains with higher productivity. In addition, it will facilitate both combinatorial biosynthesis and synthesis of novel compounds that are needed to overcome the critical problem of antibiotic resistance. Thiomarinol, the naturally hybrid antibiotic that we are working on, is encoded by a novel 97 kb circular plasmid designated pTML1, and is made up of two parts, the pyrrothine (holomycin) part of a dithiolopyrrolone class of antibiotic attached to 8-hydroxyoctanoic acid side-chain, the mupirocin like component termed marinolic acid, via an amide linkage (Fukuda *et al*., 2011). Although it is known that the biosynthesis of mupirocin is regulated via a quorum-sensing dependent system it is still not known how thiomarinol biosynthesis is regulated. Nevertheless, data in Chapter two of this thesis showed that the thiomarinol gene cluster appears to be regulated so as to give expression in late exponential or early stationary phase, and it would not make sense for genes to be on a plasmid and able to move between bacteria if they could not carry their regulatory system with them. Therefore, the hypothesis is to transfer pTML1 to a new host to see if the genes are expressed and can be regulated in the same way, or whether it needs one or more chromosomal genes from *Pseudoalteromonas* spp. SANK 73390 to allow activation and expression.

Different approaches have been used to determine the mechanism by which the regulatory system exerts global control over antibiotic gene expression. For example, AbrB protein, the repressor that is produced by *Bacillus subtilis*, was confirmed by gel mobility shift experiments to bind to the regulatory region for tyrocidine synthetase I of the tryrocidine biosynthetic pathway in *Bacillus brevis* (Robertson *et al*., 1989). Using quantitative real-time PCR for the number of transcripts, both in-frame deletion and insertion of a terminator

cassette into the biosynthetic gene cluster of novobiocin confirmed transcriptional regulation to be governed by two regulatory genes, *novE* and *novG*, which act in a cascade-like mechanism (Dangel *et al*., 2009). Although Geistlich *et al*., (1992) identified *srmR* (*srm22*) as a regulatory gene for the biosynthesis of spiramycin in *Streptomyces ambofaciens*, Karray *et al*., (2010) identified *srm40* as being a pathway-specific activator that depends on *srm22* for its expression using reverse transcription-PCR (RT-PCR) analysis for genes in the wild type and deletion mutants. Moreover, the *aur1P* gene upstream of the biosynthetic gene cluster of the polyketide auricin was identified as an essential gene that produces Aur1P regulator that activates expression of all the genes included in the auricin biosynthetic gene cluster (Novakova *et al*., 2005). In *Streptomyces coelicolor*, the producer of four genetically and structurally different antibiotics, AbsB protein (an RNase III homologue) was identified as having a role in stabilizing the pathway-specific transcription regulator (Price *et al*., 1999). However, the pathway specific regulator within each antibiotic cluster that is designated as a cluster situated regulator ($CSR_S$) is found to have pleiotropic actions (cross-regulation) via controlling other antibiotic biosynthetic gene clusters in *Streptomyces coelicolor*, and the cross regulation activity is demonstrated to be modulated globally and differentially during the growth cycle by the RNase III homologue AbsB (Huang *et al*., 2005).

Heterologous expression of a cosmid carrying the entire gene cluster of medermycin from *Streptomyces* sp. AM-7161 in *Streptomyces coelicolor* CH999 was successful in proving the completeness of the clone (Ichinose *et al*., 2003). Two overlapping cosmids of *Streptomyces clavuligerus*, producer of a dithiolopyrrolone antibiotic, holomycin, was constructed using an integrative *Streptomyces-E.coli* shuttle vector, induced the production of holomycin when heterologously expressed in *Streptomyces albus* (Huang *et al*., 2011).

El-sayed *et al*., (2001) identified MupR, the transcriptional activator, and MupI the AHL producer in the soil gram-negative bacteria *Pseudomonas fluorescens* NCIMB 10856, as two

regulatory proteins regulating mupirocin production via quorum-sensing regulation. Surprisingly, production of AHLs signals, the quorum-sensing communication in marine snow bacteria such as *Roseobacter* spp. was reported for the first time by Gram *et al*., (2002) using well diffusion and thin-layer chromatography (TLC) assays, and it was found that AHLs are likely to govern a variety of phenotypic traits including antibiotic production.

Production of lankacidin and lankamycin, antibiotics encoded on a giant linear plasmid pSLA2-L, and morphological differentiation in *Streptomyces rochei* were found to be under the control of complex regulatory (*γ*-butyrolactone receptor) system including four different genes (Arakawa *et al*., 2007). Therefore, the purpose of this study is to identify how the thiomarinol system is regulated via capturing and mobilising pTML1 out of *Pseudoalteromonas* spp SANK 73390 to heterologous host. Three different strategies have been followed using different suicide vectors constructed according their ability to recombine with pTML1, and based on carrying a piece of DNA from pTML1 that is outside the thiomarinol biosynthetic gene cluster and not essential for replication or stability. One such strategy provided evidence of successful integration into pTML1 although we were not successful in recovering the hybrid in *E.coli*.

We have found that it is essential to give a brief description about the original plasmids that have been used for the construction of the suicide vectors during different strategies of this study. RK2 plasmid (Figure 6.1), a 60 kb broad-host-range replicon of the IncPα incompatibility group (Chikami *et al*., 1985), is not distinguishable from other isolates R18, R68, RP1 and RP4 of the same plasmid (Pansegrau *et al*., 1994). This plasmid was identified

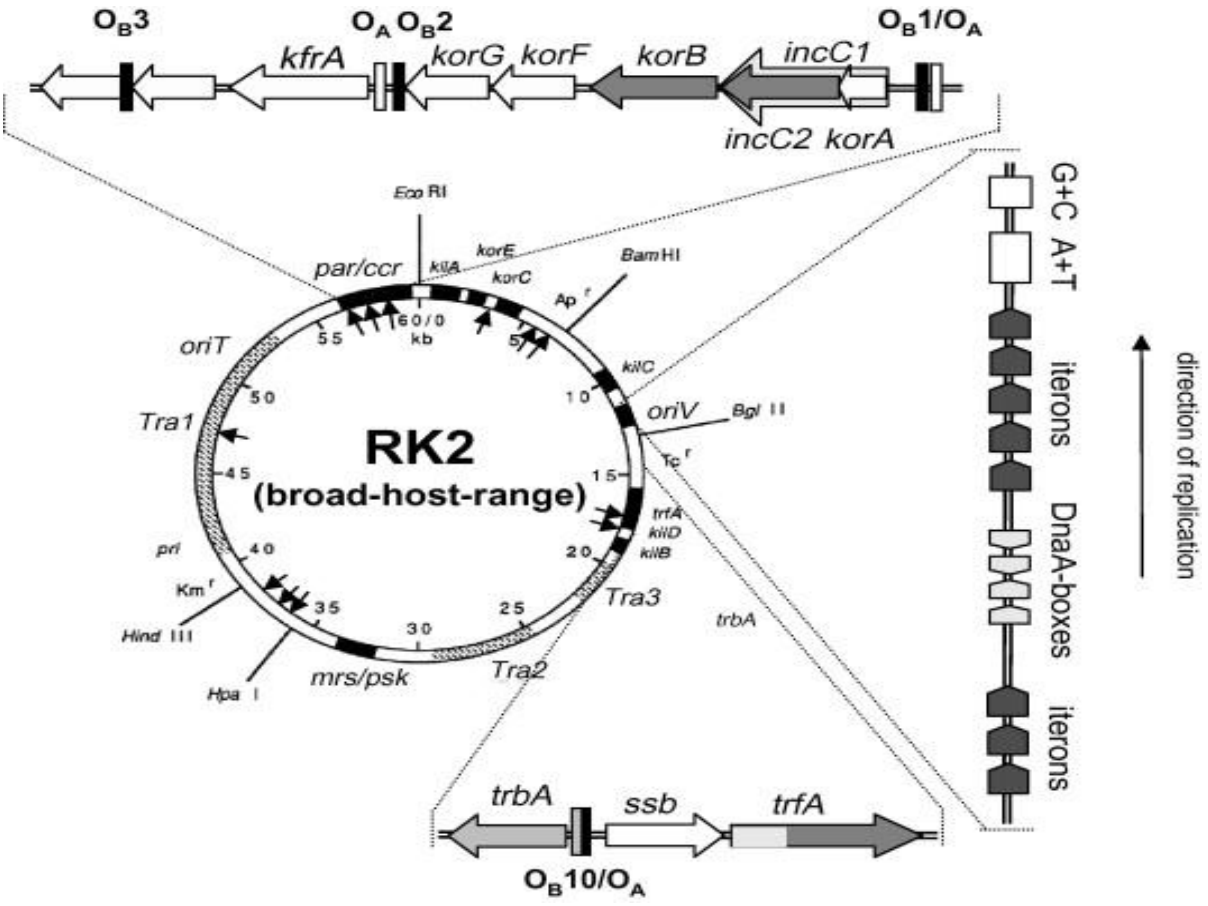



**Figure 6.1 Map of broad-host-range plasmid RK2 showing regions responsible for replication, partitioning and stable maintenance of the plasmid.** (Top) Central control region of RK2. Genes *korA* and *korB* are encode repressor genes marked with arrows; *incC1* and *kfrA* are regulatory genes. (Right) Structure of RK2's origin (*oriV*) containing motifs required for replication: four DnaA boxes (depicted in light grey) where host DnaA protein binds during replication initiation; eight repeated iteron sequences (dark grey), binding sites for RK2 initiator protein TrfA. (Bottom) Genes encoding replication proteins (*trfA*, *ssb*) and the gene for the global regulatory protein *(trbA*) (from Kolatka *et al*., 2010).

originally as an agent conferring resistance to ampicillin, kanamycin and tetracycline (Meyer and Helinski, 1977) to the clinical isolates like *Klebsiella sp*. and *Pseudomonas sp*. isolated from burn patients in Birmingham Accident Hospital (Lowbury *et al*., 1969). RK2 plasmid replicates in *Escherichia coli* at a low number of four to eight copies per chromosome (Kues and StahI, 1989; Thomas and Helinski, 1989). Owing to the ability of RK2 to transfer by

conjugation, and its stable maintenance in almost all gram-negative bacteria (Thomas and Smith, 1987), with the exception of *Bacterioides* and *Myxococcus* (Thomas and Helinski, 1989), it becomes an ideal vector for genetic manipulations in diverse bacterial species (Thomas and Smith, 1987). Different regions have been identified within the RK2 genome in addition to the antibiotic resistance genes, including *oriV* as a region for vegetative replication, the *trfA* gene encoding a replication initiation protein, (*oriT*, *tra*) as two regions for conjugal transfer, (mrs/psk) as an encoding region for both multimer resolution system and post-segregational killing function, respectively, and ccr (central control region), consisting of a region combining partitioning function with a region encoding for its regulation as well (from Kolatka, *et al*., 2010).

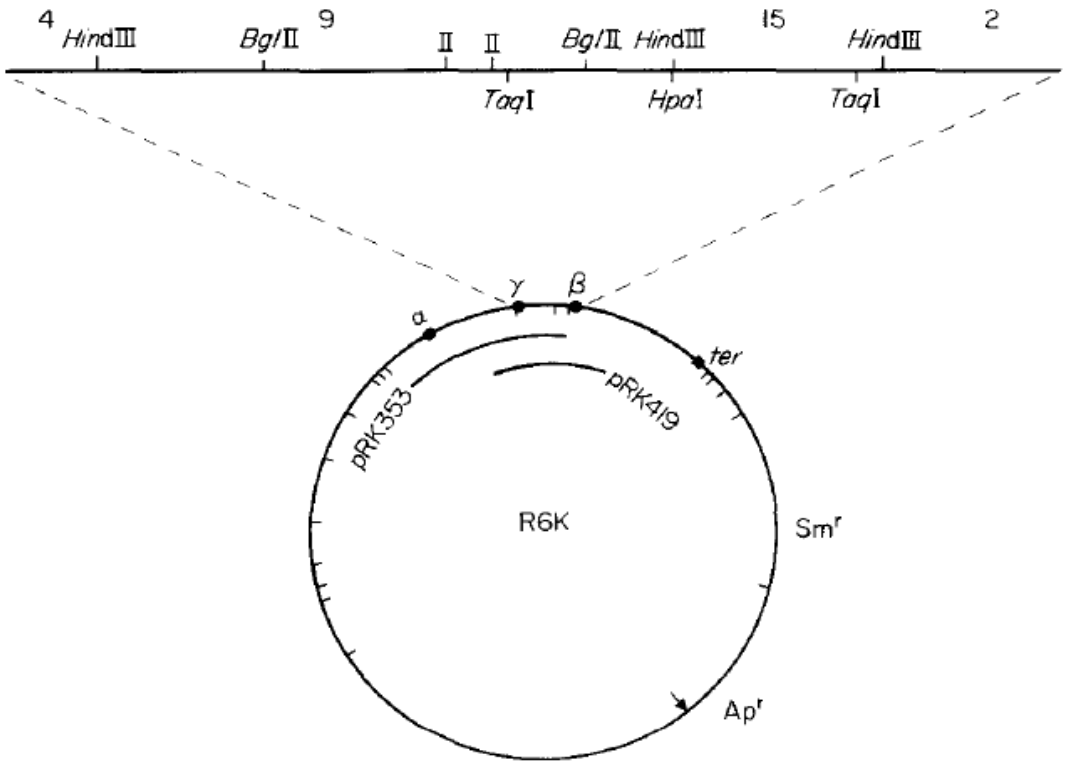



**Figure 6.2 Map of the antibiotic resistance plasmid R6K illustrating the 3 origins of DNA replication α, β, and γ, respectively.** The asymmetric terminus of replication defined as *ter*. Essential restriction sites are indicated. 11.05 kb pRK353, is a self-replicating derivative of R6K. (from Stalker *et al*., 1982).

R6K, a 39.9 kb (Figure 6.2) self-transmissible plasmid (Rakowski and Filutowicz, 2013) that occurs naturally in *E.coli* has a high copy number and specifies resistance to both ampicillin and streptomycin (Kolter and Helinski, 1978b) with a relaxed mode of replication (Kontomichalou *et al*., 1970). Three distinct origins, designated α, β, and γ, have been identified *in vitro* as regions from which initiation of DNA replication starts (Kolter and Helinski, 1978a; Crosa, 1980). However, smaller derivatives of R6K have been constructed, including pRK353, which identified a 2.5 kb segment of the plasmid DNA that provides sufficient information for autonomous replication in *E.coli* (Kolter and Helinski, 1978b). Further investigation confirmed this segment as a piece of DNA that contains both the γ-origin of DNA replication and a structural gene (*pir*) that codes for the π- protein, that is essential for the initiation of DNA replication of R6K *in vitro* (Inuzuka and Helinski, 1978).

P15A plasmid is one of the smallest known naturally occurring replicons, which is able to replicate autonomously derived from *E. coli* 15 (Cozzarelli *et al*., 1968). Since P15A replication system was found to be a reasonable source for the construction of a variety of special-purpose plasmid cloning vehicles, a series of cloning vehicles derived from P15A have been constructed (Chang and Cohen, 1978). Construction of those plasmids including pACYC184 (Figure 6.3) carried out through attaching chloramphenicol and tetracycline as selectable "marker" genes to P15A, and those assayable genes contained cleavage sites for a number of commonly employed site-specific endonucleases. The ability of pACYC184 as an amplifiable, multicopy DNA cloning vehicle to co-exist compatibly with plasmids derived from ColE1 in the same bacterial cells, in addition to several other properties, makes it useful as a general and special-purpose cloning vehicle. The complete nucleotide sequence of pACYC184 has been reported by Rose, (1988) as 4245 bp nucleotide in length. However, Mok *et al*., (1991) discovered that the recognition site of the restriction endonuclease *Bsi*YI

that recognizes 5' CCNNNNNNNGG 3' was wrongly sequenced in P15A, pACYC177, and pACYC184, respectively.

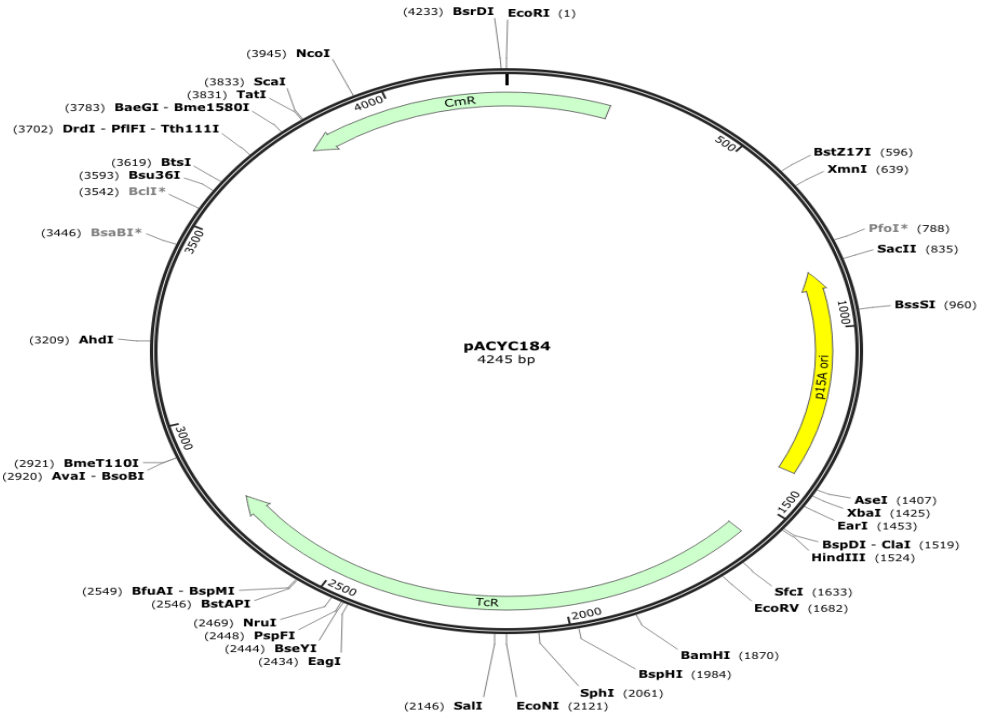



**Figure 6.3 Map of pACYC184 cloning vehicle.** pACYC184 is an *E.coli* 15 plasmid cloning vector with a low copy number at about 15 copies per cell. Restriction enzymes are designated. pACYC184 includes a tetracycline resistance gene ($Tc^R$), a chloramphenicol resistance gene ($Cm^R$) and a P15A origion of replication (ori). (Figure from NEB; snapgene, and Chang and Cohen, 1978).

In this study, we used three different strategies for the purpose that has been mentioned earlier, and pACYC184 was really successful as a cloning vehicle for cloning DNA fragments whenever required. A suicide RK2 vector that depends on $oriV_{RK2}$ and consisting of a piece of DNA from pTML1 outside the thiomarinol biosynthetic gene cluster has been used in the first strategy. The $oriV_{RK2}$ in the first suicide vector has been replaced by the R6K replicon using R6K derivative pRK353 as a source of R6K replicon, and the constructed suicide vectors was used in the second strategy. In the third strategy, a suicide RK2 plasmid only with R6K γ-*ori* which was incapable of autonomous replication and required

chromosomal expression of π-protein was constructed in *E. coli* strains ECF529 and ECF530, respectively, and was successfully integrating into pTML1, but not successful in moving it outside *Pseudoalteromonas* spp SANK 73390.

## 6.2 Materials and Methods

### 6.2.1 Bacterial strains

In addition to *E.coli* DH5α that has been described in Chapter 3, all the other strains used during this study are listed in Table 6.1.

### 6.2.2 Growth media and culture conditions

Media used were L-broth (LB) and L-agar for the growth of *E.coli* strains, M-agar and broth described in Chapter 4 used for the growth of *Pseudoalteromonas* spp. SANK 73390. These media were supplemented with antibiotics as listed in Table 6.2 whenever required. In addition, media were supplemented with 0.05% and 0.2% L-arabinose to induce π protein production under the control of $P_{BAD}$ promoter in ECF529 and ECF530 strains with the *pir* gene in the chromosome. However, when those two strains were transformed with ligation reactions, transformants were incubated in LB with 0.005% arabinose for 1 hour before plating (Bowers et al., 2004). M9 Histidine medium was used for the growth of C2110 ($PolAI^-$), and M9 Casamino acids medium (Kahn *et al*., 1979) was used for the growth of MV10 whenever carrying plasmids with $trpE^+$. The procedure described in section 4.2.8 (the conjugation part) of Chapter 4 was used for conjugation with *Pseudoalteromonas* spp. SANK

73390 whenever required. *E.coli* strains were grown at 37$^{o}$C, while strains of *Pseudoalteromonas* spp. SANK 73390 were grown at 23$^{o}$C.

**Table 6.1 Bacterial strains used during this study.**

| Bacterial strain | Genotype | Reference |
|---|---|---|
| *Escherichia coli C600* | *F⁻ tonA21 thi-1 thr-1 leuB6 lacY1 glnV44 rfbC1 fhuA1 λ⁻* | Bachmann, 1987 |
| *Escherichia coli* K12 (MV10) | C600 Δ*trpE5* | Hershfield *et al*., 1974 |
| *Escherichia coli* C2110 | K-12 *pol*A1 *his rha* | Stachel *et al*., 1985 |
| WM2949 | *lacI$^{Q}$*, Δ*araBAD* Δ*rhaBAD* Δ*araFGH* Δ*araE* | J. Cronan: (Datsenko and Wanner, 2000) |
| ECF529 with wt *pir* gene | WM2949:attB_::$P_{BAD}$-*pir rrnB*PI(CTC-AGA) -*lacY*A177C | M. Filutowicz; Sheryl. A. Rakowski, University of Wisconsin-Madison (Bowers *et al*., 2004) |
| ECF530 With hyperactive variant of *pir* gene | WM2949:attB_::$P_{BAD}$-*pir*P106L^F107S *rrnB*PI(CTC-AGA)-*lacY*A177C | |
| *Pseudoalteromonas sp* SANK 73390 | Wild type (W.T) strain which produces thiomarinol (yellow strain) | Fukuda *et al*., 2011 |
| *Pseudoalteromonas sp* SANK 73390 | pTML1 (thiomarinol gene cluster removed) cured (white strain) | Fukuda *et al*., 2011 |

**Table 6.2 Antibiotics used in this study.**

| Antibiotic | Stock solution (mg/ml) | Working solution (µg/ml) |
|---|---|---|
| Ampicillin | 100 | 100 |
| Kanamycin sulphate | 50 | 50 |
| Chloramphenicol | 34 | 34 |
| Ampicillin | 25 | 25 |

**Table 6.3 Primers used in this study.**

| Gene | Primer | Length (bp) | Primer sequence, 5'→3' | Purpose | Rerstriction sites |
|---|---|---|---|---|---|
| pTML1-Junk DNA | $FP_{Junk\ DNA}$ | 17 | AGGGATCCGCTGTGTTT | Junk DNA Construc-tion | *Bam*HI |
| | $RP_{Junk\ DNA}$ | 21 | AGAAGCTTTTTTACTTGGTCA | | *Hin*dIII |
| RK6 gamma (γ) region | $FP_{R6K\text{-}\gamma\text{-}origin}$ | 24 | GAATTCTGTCAGCCGTTAAGTGTT | R6K Gamma origin construc-tion | *Eco*RI |
| | $RP_{R6K\text{-}\gamma\text{-}origin}$ | 24 | TCTAGAGTCCATCATGACCTTGAG | | *Xba*I |
| *klaC*-RK2 | $FP_{klaC\text{-}RK2}$ | 42 | AAGCTTCGAGATCTCGTGTACACGG GTTCGTGCGATCCGTCT | klaC con-struction | *Hin*dIII, *Bgl*II *Bsr*GI |
| | $RP_{klaC\text{-}RK2}$ | 26 | TCTAGACCAGCCCAACAAAGCCGG TT | | *Xba*I |
| pTML1 capture | $FP_{(A)\text{-}pTML1\text{-}Junk}$ | 27 | GGTACCCATTAACAACGCAGCAAT ATA | For check-ing the integration of suicide vector without *pir* gene into pTML | *Bam*HI |
| | $RP_{(B)\text{-}pTML1\text{-}Junk}$ | 27 | CTGCAGGTTGGGCAGAATCTAATTA TT | | *Pst*I |
| | $FP_{(D)\text{-}R6K\text{-}Junk}$ | 27 | CTGCAGGAGGCTATTTAAGTTGCTG AT | | *Pst*I |
| | $RP_{(C)\text{-}tet^{R}\text{-}Junk}$ | 27 | GGTACCCAGAGCCATGTAGACAAC ATC | | *Kpn*I |

**Table 6.4 Plasmids used or constructed during this study.**

| Plasmid | Size (kb) | Relevant properties | Reference or source |
|---|---|---|---|
| pACYC184 | 4.245 | $Mob^{-}$, *cat*($Cm^{R}$), $Tc^{R}$ , P15A_replicon | Chang and Cohen, 1978 and NEB |
| RK2 | 60.0 | $Tc^{R}$ , $Ap^{R}$, $Km^{R}$, oriV replication | Jobanputra and Datta, 1974 |

| | | | |
|---|---|---|---|
| pRK353 | 11.05 | *TrpE* $^{+}$, R6K replicon | Kolter and Helinski, 1978b |
| pGEM-T Easy | 3.0 | $Ap^{R}$, *lacZα* (PCR cloning vector) | Promega |
| pGEM-T Easy -Junk | 3.5 | 0.5 kb *Bam*HI-*Hin*dIII PCR fragment of pTML1-Junk DNA cloned into pGEM-T Easy vector | Anthony. S. Haines |
| pACYC184 -Junk | 4.745 | 0.5 kb *Bam*HI-*Hin*dIII PCR fragment of pTML1-Junk DNA cloned into pACYC184 | Anthony. S. Haines |
| pAM01 | 6.045 | 1.3 kb *Bam*HI-*Sal*I $Tc^{R}$ gene from RK2 cloned into *Bgl*II-*Sal*I sites of pACYC184-Junk | This study (Fig. 6.4) |
| pAM02 | 66.04 | 60.0 kb RK2 with pAM01 (RK2- pACYC184 derivative or hybrid) | This study (Fig. 6.6) |
| pAM03 | 49.7 | *Eco*RI deletion of pAM02 plasmid (RK2-pACYC184 derivative or hybrid) | This study (Fig. 6.6) |
| pAM04 | 60.75 | pAM03 plasmid with R6K replicon-pRK353 (RK2-pACYC184-R6K derivative) orientation I | This study (Fig. 6.8) |
| pAM05 | 60.75 | pAM03 plasmid with R6K replicon-pRK353 (RK2-pACYC184-R6K derivative) orientation II | This study (Fig. 6.8) |
| pAM06 | 6.55 | 0.51 kb *Hin*dIII,*Bgl*II,*Bsr*GI-*Xba*I PCR fragment of *klaC*-RK2 cloned into pAM01 (pACYC184 derivative) | This study (Fig. 6.11) |
| pAM07 | 7.02 | 0.47 kb *Bgl*II-*Bsr*GI fragment of *γ*-region digested from R6K replicon and cloned into pAM06 (pACYC184 derivative) | This study (Fig. 6.11) |
| pAM08 | 67.02 | 60.0 kb RK2 with pAM07 (RK2-R6K-pACYC184 derivative). HR via *klaC* | This study (Fig. 6.11) |
| pAM09 | 67.02 | 60.0 kb RK2 with pAM07 (RK2-R6K-pACYC184 derivative). HR via $Tc^{R}$ gene | This study (Fig. 6.11 and 6.13) |
| pAM10 | 62.75 | *Eco*RI deletion of pAM08; ( plasmid with $oriV_{RK2}$ & P15A replicon ) (- Junk DNA) | This study (Fig. 6.11) |
| pAM11 | 50.72 | *EcoR*I deletion of pAM09; (non self-replicating plasmid (depends on chromosomally expressed *pir* gene) ) (suicide vector) | This study (Fig. 6.13) |

## 6.3 Results

### 6.3.1 Construction of a suicide RK2 derived vector depending on the pACYC184 plasmid

For mobilising pTML1 plasmid carrying the biosynthetic genes of thiomarinol, and as a 1st attempt, a suicide vector was designed that would have lost $oriV_{RK2}$ constructed through a series planned steps to capture pTML1 plasmid and move it out of the original host *Pseudoalteromonas* spp. SANK 73390 to strains like *E.coli* and *Pseudomonas putida*.

#### 6.3.1.1 Cloning a non-essential DNA segment from pTML1 and RK2 tetR gene into pACYC184 plasmid

A piece of DNA (0.5 kb) designated "junk DNA" because it does not appear to encode an essential part of the plasmid pTML1 (that carries the thiomarinol biosynthetic genes) was amplified and sequenced using vector pGEM-T Easy. The junk DNA was then cloned into *Bam*HI-*Hin*dIII sites of pACYC184 using DH5α as a host and selecting $Cm^R$. The new plasmid was designated pACYC184-Junk (Figures 6.4 and 6.5A). This part of the project was completed by Dr. Anthony Haines and Prof. C. M. Thomas. However, I used the primers designed for the junk DNA later on to confirm junk DNA in the wild type *Pseudoalteromonas* spp. SANK73390, and its absence in pTML1 cured mutant strain.

DNA of broad host range vector RK2 plasmid was prepared by Maxi- prep as described in part 3.2.3.1 of Chapter 3 of this thesis. Then DNA of this plasmid was digested with *Bgl*II-*Sal*I to release 1.3 kb fragment of tetR from $Tc^R$ (tetracycline resistance) gene (Figure 6.5B). This DNA was ligated with DNA of pACYC184-Junk that was digested with *Bam*HI-*Sal*I and transformed into DH5α selecting $Cm^R$. A number of $Cm^R$ plasmids were isolated by this method. One such plasmid, pAM01 plasmid (Figure 6.4), had the tetR fragment inserted

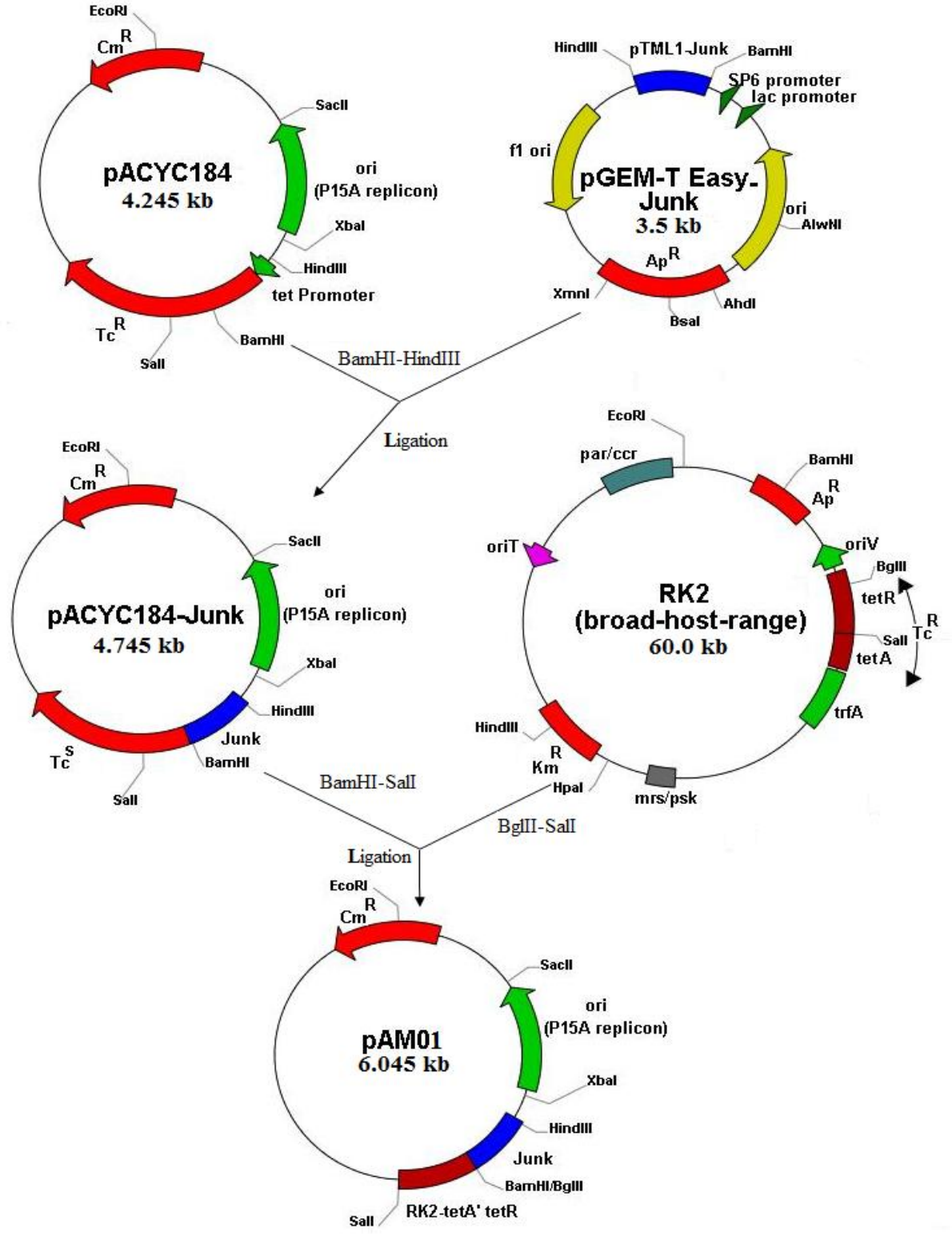



**Figure 6.4 Construction of pACYC184-Junk, and pAM01.** pGEM-T Easy-Junk and pACYC184 were digested with *Bam*HI-*Hin*dIII to clone the junk fragment into pACYC184 and then ligated. BHR RK2 vector was digested with *Bgl*II-*Sal*I to clone tetR into *Bam*HI-*Sal*I of pACYC184-Junk and then ligated.

downstream of the Junk region, while others possible clones did not. This result was confirmed by *Sal*I-*Hin*dIII digestion which released a DNA fragment of 1.8-2.0 kb (Figure 6.5C).

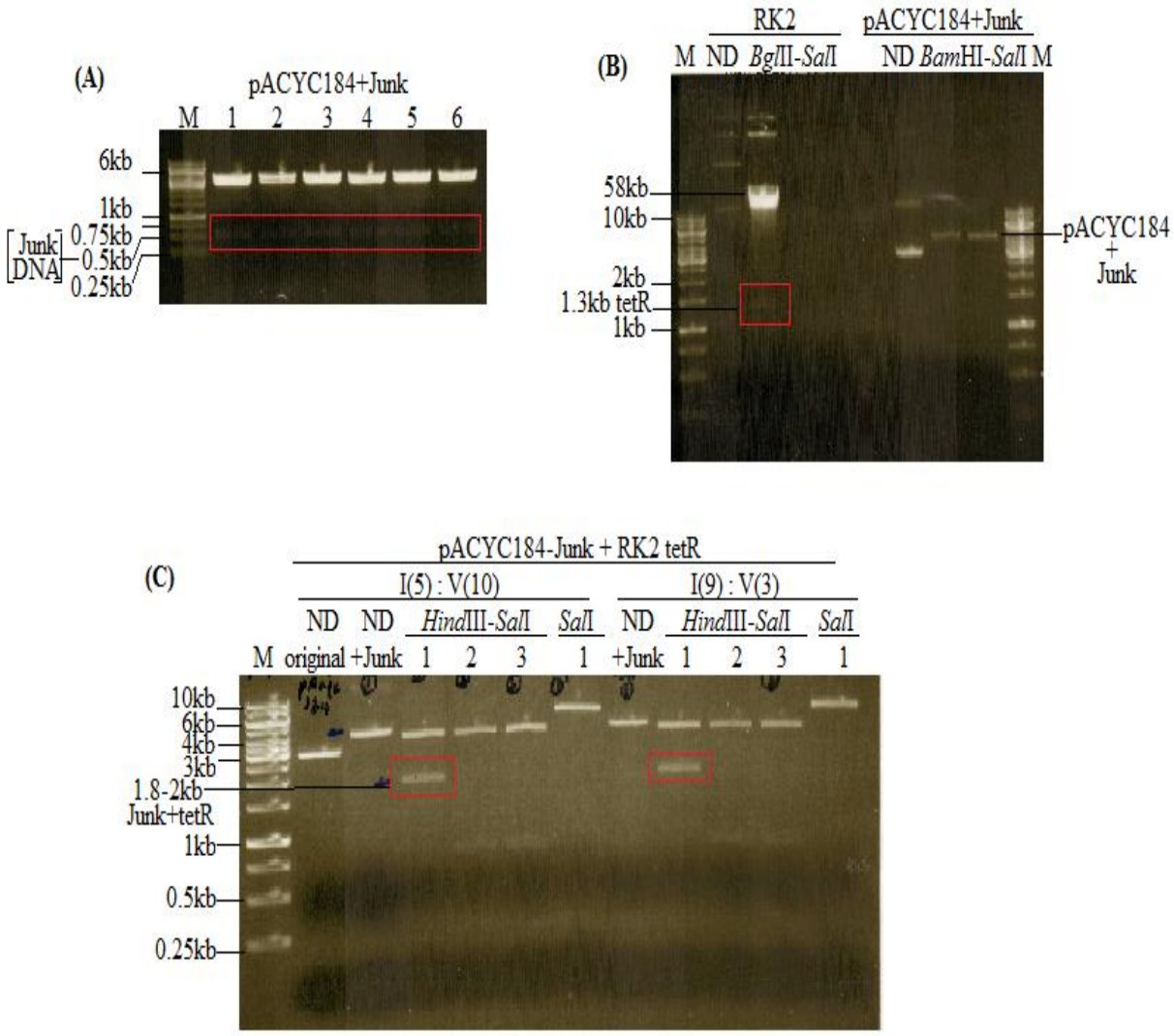



**Figure 6.5 Construction of pACYC184 (pAM01) plasmid carrying the junk fragment and RK2-tetR.** Junk fragment cloned into *Hin*dIII-*Bam*HI of pACYC184 in A**,** the release of 1.3kb tetR from RK2 by *Bgl*II-*Sal*I digestion and *Bam*HI-*Sal*I digested pACYC184-Junk in B, and *Hin*dIII- *Sal*I digestion confirmed pACYC184 carrying both junk DNA and RK-tetR in C.

### 6.3.1.2 Recombination of pAM01 plasmid with RK2 in C600 and construction of pAM02 in C2110

Transformation of pAM01 plasmid (with RK2-tetR) into *E.coli* C600 harbouring RK2 selecting $Cm^R$ and $Km^R$ to allow low frequency homologous recombination to take place to create pAM02 (Figure 6.6) was performed. However, pAM01 plasmid is able to replicate in this strain because C600 is *polA1*$^+$. This strain was used for conjugation with C2110 (*polA1*$^-$) selecting $Cm^R$ and $Km^R$ in order to isolate pure pAM02 plasmid and to exclude the possibility of having pAM01 plasmid as well (plasmids without HR), in which the later one is (*polA1*$^-$) and unable to replicate in C2110.

To confirm the right organisation, pAM02 plasmid was digested using single *Eco*RI digestion and different combinations of restriction endonucleases, such as *Eco*RI-*Bam*HI, *Eco*RI-*Bam*HI-*Xba*I, *Eco*RI-*Bgl*II, *Eco*RI-*Bgl*II-*Xba*I, *Bam*HI-*Bgl*II and *Eco*RI-*Hin*dIII (Figure 6.7). In order to remove $oriV_{RK2}$, pAM02 plasmid digested with *Eco*RI, was heat treated at 80$^o$C for 20 minutes to inactivate the enzyme, and then ligated back to construct pAM03 (under the control of P15A replicon) plasmid (Figure 6.6). This trial was repeated several times, but they were not successful to construct pAM03 plasmid at this stage since *Eco*RI digestion did not give the plasmid. However, this plasmid (pAM03) was constructed later on and confirmed as $Km^R$, $Ap^S$, $Cm^S$ in section 6.3.3 of this Chapter when combined with pRK353 (with R6K replicon) for the construction of pAM04 and pAM05 plasmids.

## 6.3.2 Attempt to capture and mobilise pTML1 using pAM02 plasmid under the control of $oriV_{RK2}$

As a 1$^{st}$ attempt to mobilise pTML1, C2110 carrying the confirmed pAM02 plasmid, the co-integrant with the Junk DNA was used for conjugation with *Pseudoalteromonas* spp.

SANK 73390 carrying pTML1 plasmid. Selecting $Km^R$ and $Cm^R$. C600 with RK2 was used for conjugation as a control selecting $Km^R$. Since both of the two plasmids, pAM02 and RK2, transferred to *Pseudoalteromonas* spp. SANK 73390 similarly with a high frequency, the fact which indicates that there is no need for pAM02 plasmid to integrate into pTML1 plasmid in the new host in order to be replicated. Plasmids were isolated out of *Pseudoalteromonas* spp. SANK 73390 for both of the two cases and checked by comparing with pAM02 plasmid. Although the isolated plasmids looked different in size, there was no clear evidence for the integration of the suicide pAM02 plasmid into pTML1, as most of the plasmids were faint and smeary. Also it was difficult to conclude that the larger sized plasmids are the suicide vector with pTML1. Therefore, to determine whether pTML1 plasmid was captured or not, the same *Pseudoalteromonas* spp. SANK 73390 isolates which had been used previously for plasmid isolation were re-used for conjugation with DH5α and C2110 selecting $Km^R$, and $Km^R$, $Cm^R$, respectively, moving out the suicide vector possibly with pTML1 captured. The same hosts with $Km^R$ selection had been used for moving the control RK2 plasmid out of *Pseudoalteromonas* spp. SANK 73390. In order to determine whether pTML1 was captured and moved out of the original host (*Pseudoalteromonas* spp. SANK 73390), plasmids isolated from DH5α and C2110 were compared with control RK2 vector isolated from same strains as well. Although plasmids with possible pTML1 captured should look quite different from RK2 vector, there was no indication of pTML1 with the suicide vector that was used for mobilising it. Therefore, this result indicates that our suicide vector was unable to mobilise the plasmid (pTML1) carrying thiomarinol biosynthetic genes cluster, and this is mostly due to the fact that $oriV_{RK2}$ and *trfA* must still be functional in this host and makes it able to replicate in *Pseudoalteromonas* spp. SANK 73390, so that there is no need for it to integrate into pTML1 in order to replicate.

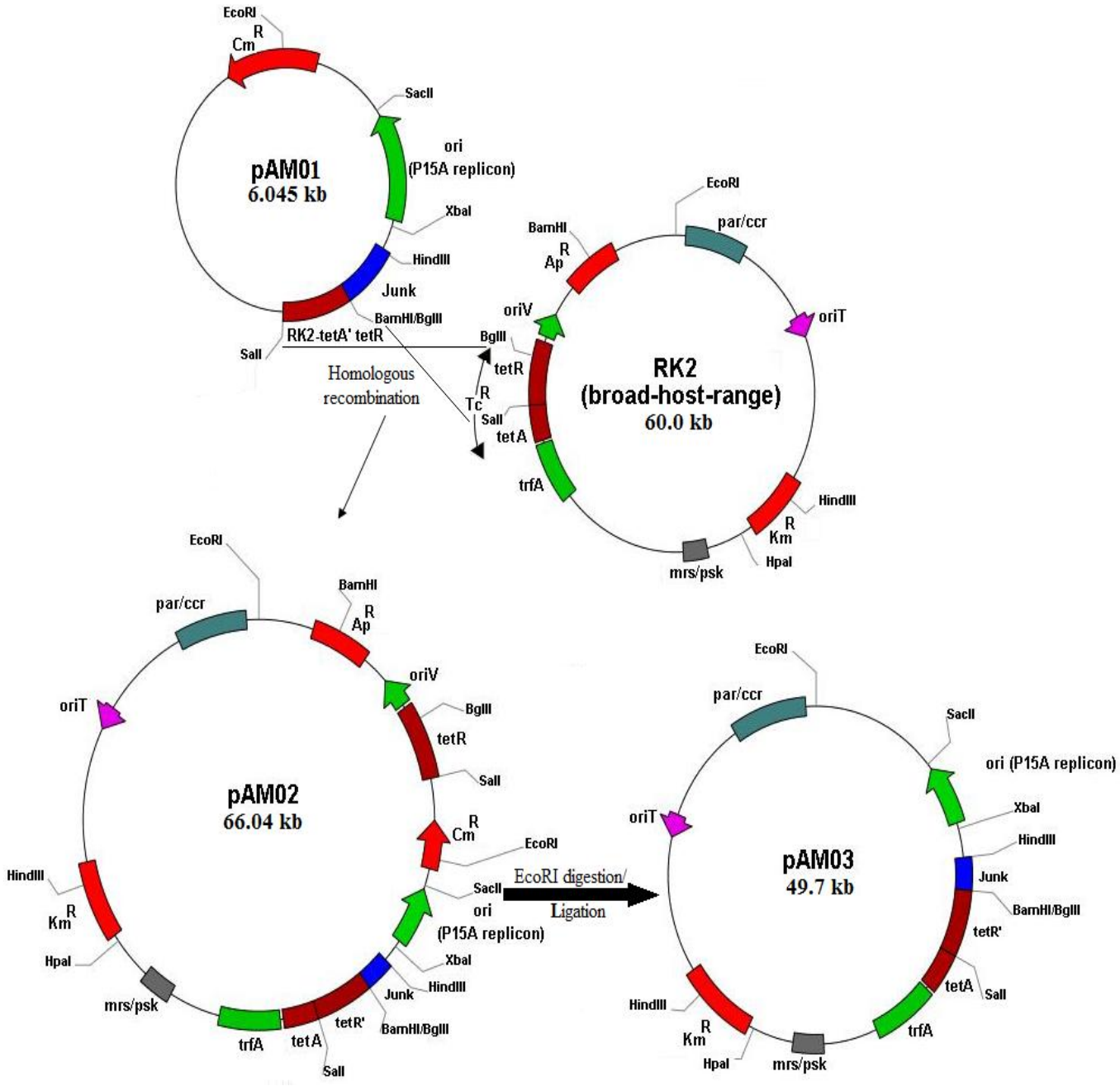



**Figure 6.6 Construction of pAM03 (-*oriV*$_{RK2}$) vector.** Plasmid pAM01 with RK2-tetA′ tetR was used for HR with RK2 in C600. Plasmid pAM02 isolated from C2110 was digested with *Eco*RI to remove *oriV*$_{RK2}$ for the construction of pAM03.

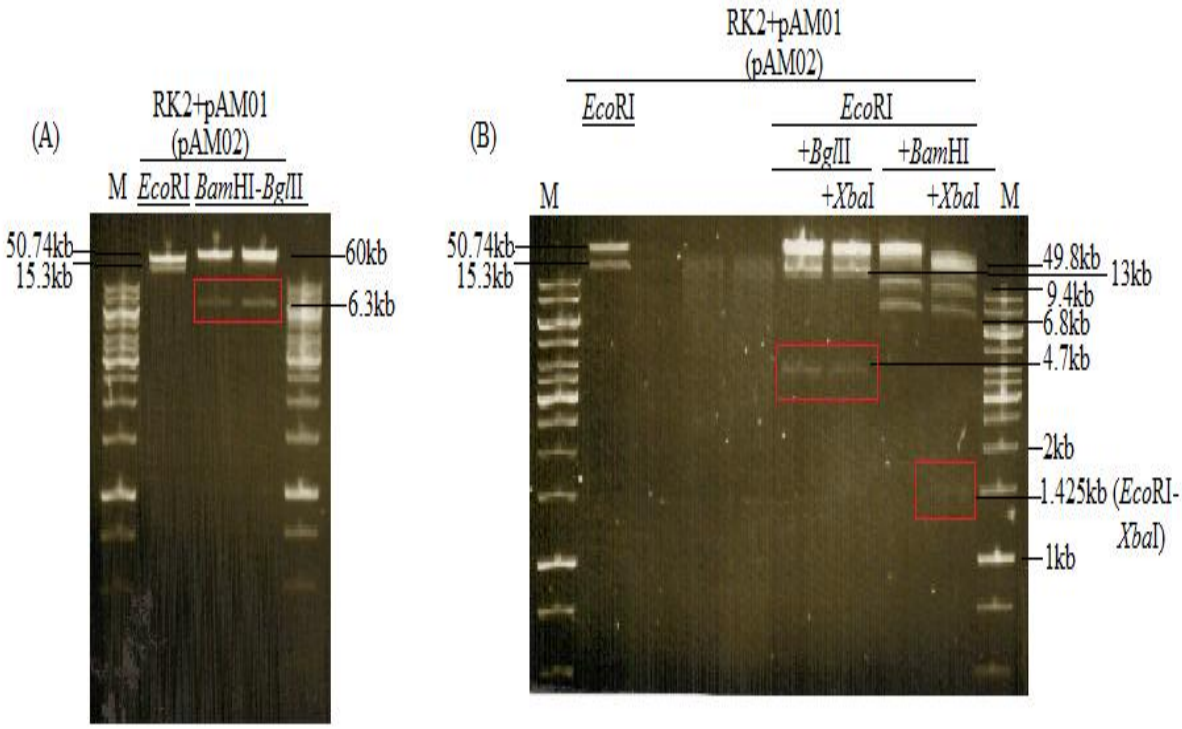



**Figure 6.7 Confirmation of the right orientation (I) of pAM02 plasmid recombined with RK2 (HR).** Orientation I would give plasmid with only *oriV*$_{RK2}$ located between the two *Eco*RI sites, while both *oriV*$_{RK2}$ and P15A replicon will be located between the two *Eco*RI sites with orientation II and *Eco*RI digestion will remove *oriT*$_{RK2}$. *Eco*RI and *Bam*HI-*Bgl*II digestion in A, and digestion with different combinations of *Eco*RI-*Bgl*II/*Xba*I and *Eco*RI-*Bam*HI/*Xba*I in B. These digestions would release fragments smaller than these in case of orientation II and that is based on the position of the junk DNA within the plasmids (orientation I and II).

### 6.3.3 Construction of a suicide vector depending on R6K replicon

An alternative strategy adopted to demonstrate whether it could be feasible to capture pTML1 and mobilise it out of *Pseudoalteromonas* spp. SANK 73390, is to put the suicide pAM03 plasmid under the control of another replicon. This strategy was achieved by replacing the $oriV_{RK2}$ by the R6K (*PolA*-independent) replicon because it was thought that the R6K replicon had a narrower host range and therefore may not function in *Pseudoalteromonas* spp.

The 11.05 kb pRK353 (*TrpE* $^{+}$) plasmid (Figure 6.8) with R6K replicon isolated from MV10 was precisely confirmed by digestion with *Hin*dIII in addition to *Eco*RI, and *Eco*RI-*Bam*HI digestion (Figure 6.9B). DNA of this molecule was digested with *Eco*RI to produce a single cut per plasmid DNA molecule. This DNA was ligated with plasmid DNA of pAM02 that had been digested with *Eco*RI (deleting $oriV_{RK2}$ and $Ap^R$ gene to produce pAM03 plasmid) and transformed into MV10 selecting $Km^R$ (Figure 6.9C). Several $Km^R$ plasmids were isolated by this method. Although the orientation is not a problem with these plasmids, *Hin*dIII digestion of those plasmids confirmed that there were plasmids with both orientations (Figures 6.8 and 6.9D) designated pAM04 and pAM05, respectively. Those two new plasmids with the R6K replicon were confirmed as $Km^R$, $Ap^S$, $Cm^S$.

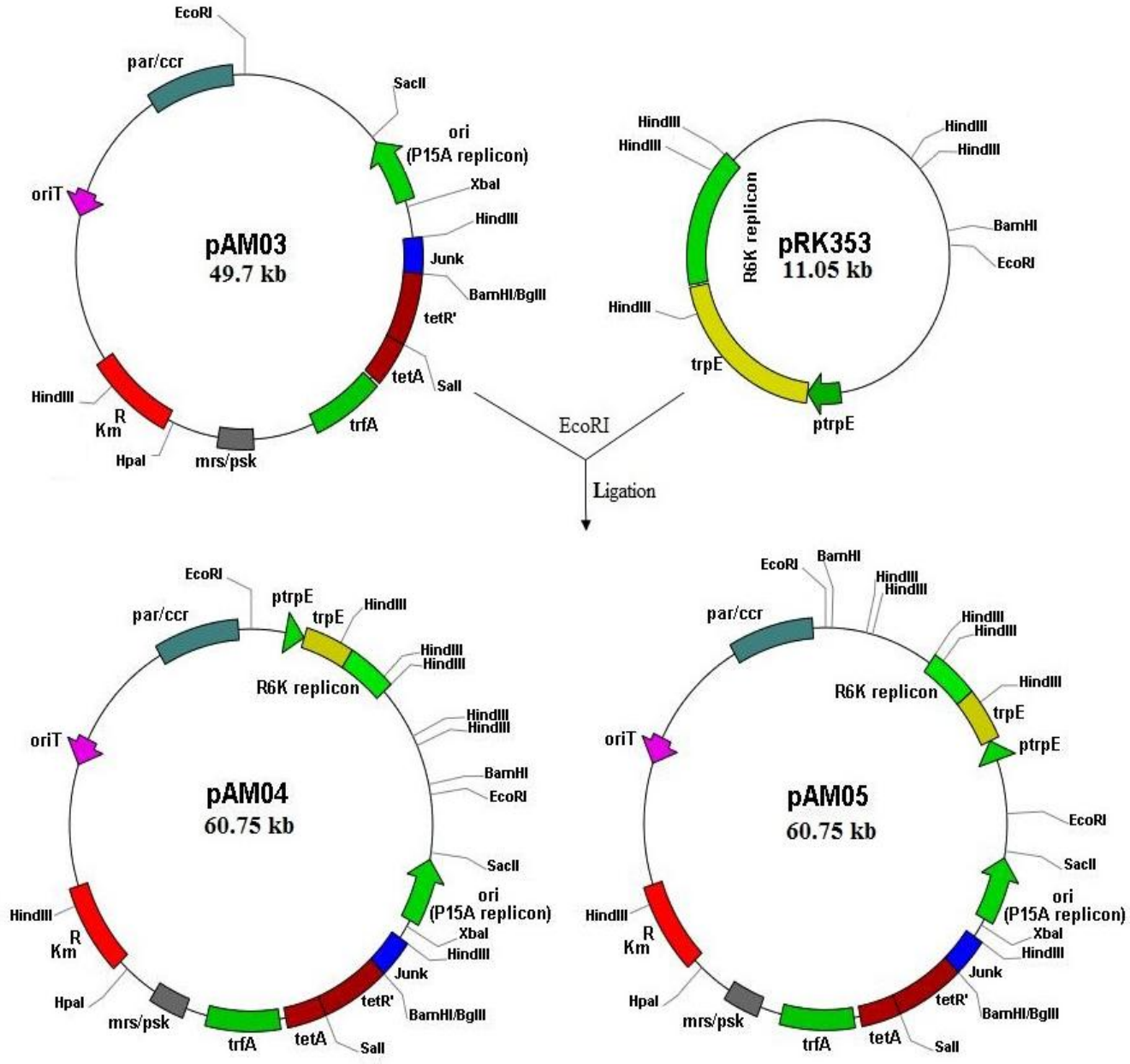



**Figure 6.8 Construction of hybrid RK2-R6K vector.** pAM03 (-*oriV*$_{RK2}$) and pRK353 (self-replicating R6K derivative) were digested with *Eco*RI and then ligated. Km$^R$ colonies obtained by transformation of MV10 with this DNA were screened for Ap$^S$ and Cm$^S$, while colonies with Km$^R$, Ap$^S$ and Cm$^S$ were examined for plasmid DNA. Two sorts of plasmids differing by orientation of pRK353 with R6K replicon were constructed. These plasmids designated as pAM04 and pAM05, respectively.

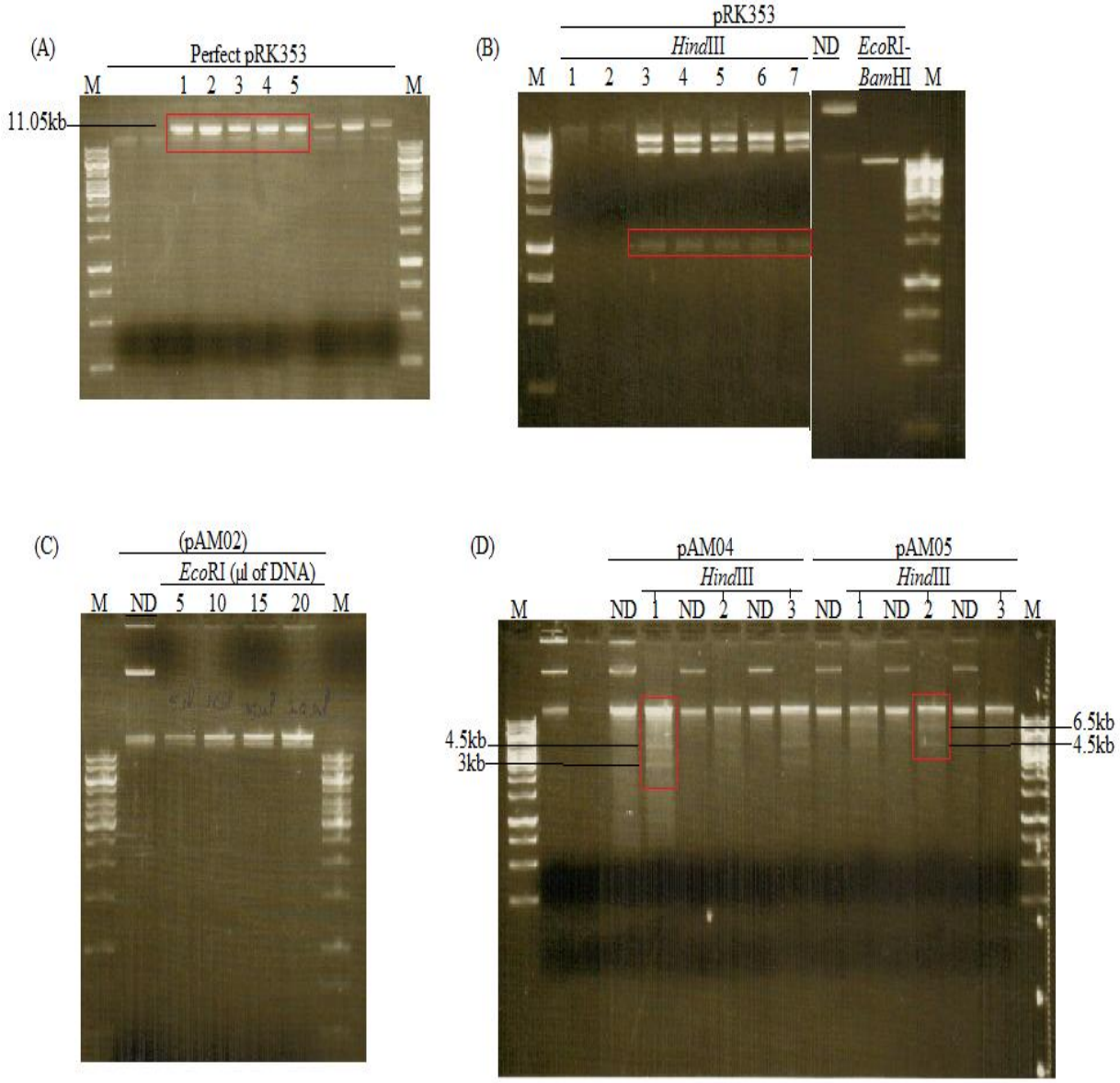



**Figure 6.9 pRK353, a derivative of R6K plasmid and the construction of pAM04 and pAM05 plasmids.** A, intact pRK353 presented and confirmed by *Hin*dIII and *Eco*RI-*Bam*HI digestion in B. *Eco*RI digestion of pAM02 plasmid (C), and pAM04 and pAM05 plasmids with R6K replicon confirmed by *Hin*dIII digestion (D).

### 6.3.4 Attempt to capture and mobilise pTML1 using suicide pAM04 and pAM05 vector with R6K replicon

The new suicide vector (pAM04 and pAM05) with the R6K replicon, rather than *oriV*$_{RK2}$ from the (BHR) RK2 plasmid was used for the attempted integration into pTML1. MV10 with either of those two vectors separately was used for bacterial conjugation with *Pseudoalteromonas* spp. SANK 73390 carrying pTML1 and SANK 73390 cured of pTML1, respectively, selecting Km$^{R}$. Surprisingly, growth was detected for both strains, which indicate that the suicide vectors with the R6K replicon are able to replicate in the SANK strains. Most of the plasmid DNA preparations isolated out of bacteria resulting from the conjugation were smeary. However, a few of them were better, but still did not provide any evidence of integration with pTML1. Moreover, plasmid DNA isolated from the pTML1 cured strain were also smeary and even heat treatment of plasmid DNA did not help to clarify them. This might indicate that the suicide vector was able to replicate autonomously, and replication in bacteria cured of pTML1 could be taken as good evidence for that. Furthermore, it should be noted that the DNA molecules isolated from the pTML1 cured bacteria were not looking like chromosomal DNA, but rather were plasmid DNA. Additionally, the presence of the "junk DNA" in the wild type *Pseudoaltermonas* spp. SANK 73390 carrying pTML1 plasmid, suicide vector (with R6K replicon), and the trans-conjugant of the wild type SANK 73390 with suicide vector, and its absence in SANK 73390 strain that is cured of pTML1 was confirmed by PCR (Figure 6.10). It therefore appears that the R6K replicon can function in *Pseudoalteromonas* spp and therefore this plasmid is not suitable to be used as a suicide plasmid.

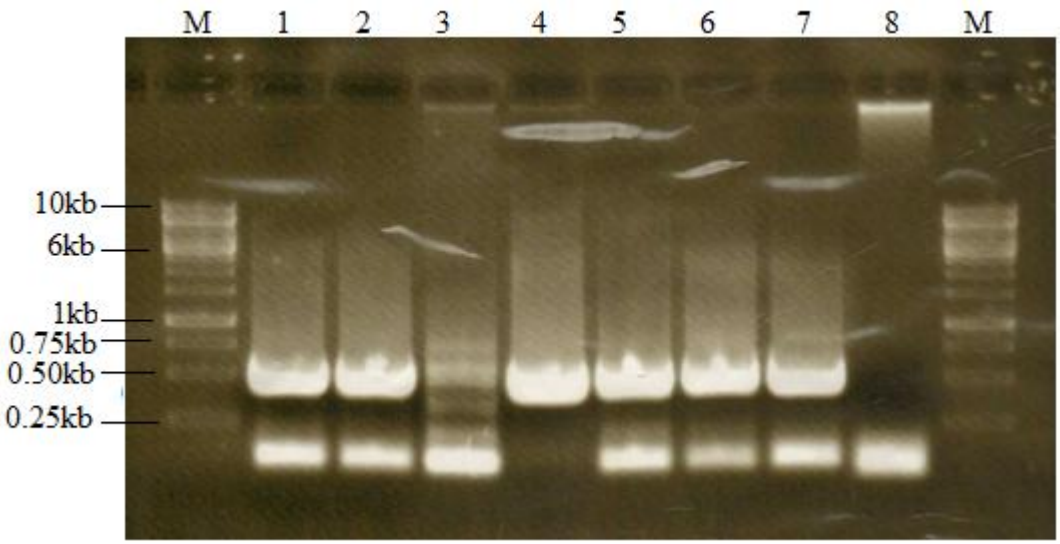



**Figure 6.10 PCR for presence of 0.5 kb "junk DNA".** Lanes from left to right; M: 1 kb marker ladder, Lane 1 and 2: *Pseudoalteromonas* spp. SANK 73390 with pTML1, Lane 3: SANK 73390 cured of pTML1, Lane 4: suicide RK2-R6K hybrid (PAM05) plasmid, Lane 5, 6 and 7: conjugant of WT SANK 73390 with the suicide vector, and Lane 8: control negative.

### 6.3.5 Construction of RK2 suicide vector with junk DNA depending on chromosomal expression of π-protein for replication and maintenance

Because neither of the previous strategies to capture pTML1, based on replacement of the *ori*$V_{RK2}$ with the P15A replicon or the R6K replicon, had worked for different reasons an alternative approach was devised. This involved constructing a suicide vector that depends on induction of chromosomally expressed π-protein which is essential for replication from the R6K replication origin using strains from Marcin Filutowicz as described in Table 6.1. These strains have the *pir* (encoding the R6K π-protein) gene inserted in the chromosome. In order to construct that vector, two different routes were followed.

### 6.3.5.1 Substituting R6K and P15A replicons in pAM05 vector with R6K γ-origin

The key requirement for this new strategy was to have the R6K replication origin separate from the gene, *pir*, encoding the essential replication protein. According to the map of R6K plasmid, the location of the *pir* gene spans the region between two *Hin*dIII fragments designated as 9 and 15, while that of the γ-origin was specified to the junction of *Hin*dIII fragments 4 and 9 (Stalker *et al*., 1982). It has also been demonstrated that *in trans* expression of *pir* gene (encoding π- protein) and γ-origin supports the replication of R6K derivatives that only have γ-origin (Kolter *et al*., 1978), in contrast to derivatives with either α or β origins, respectively (Shafferman *et al*., 1981). Therefore, the γ-origin was PCR amplified using the R6K sequence available from Sanger Institute and using pRK353 (R6K derivative) plasmid as a template without including *pir* gene. The forward primer contained an *Eco*RI site while the reverse primer contained an *Xba*I. To construct a plasmid with γ-origin only (depending on chromosomal expression of π- protein), pAM05 plasmid DNA was digested with *Eco*RI-*Xba*I to remove both R6K and P15A replicons. This DNA was ligated with the amplified DNA of the γ-origin that has the same sticky ends and transformed into ECF529 and ECF530 (with *pir* gene in the chromosome) strains, using wild type WM2949 as control, selecting $Km^R$ with arabinose. Plasmid DNA was isolated from ECF529 and ECF530 (with hyperactive *pir* gene), but these plasmid bands were faint and could not be digested with *Eco*RI-*Xba*I. This step was carried out so as to clone RK2-*klaC* subsequently into *Xba*I-*Hin*dIII sites of pAM05 plasmid downstream the R6K γ-origin. However, since cloning of R6K γ-origin apparently was not successful, and we found that *Hin*dIII is cutting within the sequence of the R6K γ-origin if we try to clone RK2-*klaC* into *Xba*I-*Hin*dIII sites, therefore, we moved to an alternative plan.

### 6.3.5.2 Construction and characteristics of the γ-*ori* expression vector

The first attempt to replace the R6K and P15A replicons in pAM05 plasmid with γ-origin was unsuccessful as mentioned in the previous section. The alternative plan was to use a linker to clone RK2-*klaC* first into pAM01 plasmid to avoid *Hin*dIII cutting within γ- origin, then cloning the γ-*ori* of R6K replicon (Figure 6.11). A 0.51 kb segment of *klaC* of RK2, one of the genes in the *kilA* operon was amplified and sequenced (Figure 6.12A) and then cloned into the *Hin*dIII-*Xba*I sites of pAM01 (Figure 6.12B) using a small linker *Hin*dIII, *Bgl*II, *Bsr*GI- *Xba*I selecting $Cm^R$. Isolated plasmids were confirmed as having *klaC*, and designated as pAM06. DNA of this plasmid was digested with *Bgl*II-*Bsr*GI and ligated with *Bgl*II-*Bsr*GI fragment (γ-origin) of pRK353 with the R6K replicon (Figure 6.12C) and transformed into DH5α selecting $Cm^R$. Transformants were confirmed as carrying plasmid pAM07 (Figures 6.11 and 6.12D) having both *klaC* and R6K γ-origin cloned accurately. As pAM01 plasmid had the *tetR* gene from RK2, therefore, pAM07 plasmid is deliberately carrying two DNA fragments from RK2, *tetR* and *klaC* genes, allowing sequential double recombination which should give the plasmid that we wished to construct.

For recombination with RK2 via one of the RK2 fragments, C600 was transformed with pAM07 selecting $Km^R$ and $Cm^R$. *Xba*I–*Sal*I digestion of plasmids isolated from transformants of C600 confirmed *klaC* and R6K γ-origin in pAM07 vector with preliminary evidence of recombination, using RK2 as a control (Figure 6.12E). The transformants were used for conjugation with the strains ECF529 and ECF530 that provide the R6K replication protein and would therefore allow the product of resolution to survive if they carry the R6K replication origin. $Km^R$ and $Cm^R$ were selected and 0.05% arabinose was added to induce expression of the *pir* gene. *Eco*RI digestion of isolated plasmids showed that both possible recombinations of the component segments had been obtained and these were designated

pAM08 and pAM09 (Figure 6.11). In theory, *Eco*RI digestion and re-ligation of these two plasmid should give pAM10 (Figure 6.11) and pAM11 (Figure 6.13) plasmids, respectively. The recombinant releasing a 4.4-4.8 kb *Eco*RI fragment (pAM08) is not the required plasmid because deletion of the small *Eco*RI fragment would generate a plasmid without either the junk DNA or the R6K γ-origin (pAM10) (Figure 6.12F). By contrast, the plasmid that released an *Eco*RI fragment of 16.0-16.3 kb (pAM09) was the preferred recombinant because *Eco*RI deletion would generate a plasmid (pAM11) that would depend on the π- protein (Figure 6.14A). Therefore, plasmids that release a fragment of about 16.0-16.3 kb were digested with *Eco*RI, the enzyme heat-inactivated and ligation then performed followed by transformation of ECF530 selecting $Km^R$ and $Cm^R$; $Km^R$; and $Cm^R$, respectively. Transformants resistant to kanamycin were confirmed as chloramphenicol sensitive. Unfortunately, the isolated plasmids out of ECF530 were still able to replicate in WM2949 (-*pir*), and even in C2110 which excluded the possibility of replicating via the P15A replicon. Further *Eco*RI, and *EcoRI-Sal*I digestions showed the 16.0 kb *Eco*RI fragment re-ligated back to the vector.

The gel purified and ligated plasmid resulting from the *Eco*RI digestion of the plasmid pAM09 was another trial to construct plasmid pAM11, which it did not replicate in the WT WM2949, but it did replicate in ECF530 as required. However, this was also not helpful, because *Eco*RI digestion of the isolated plasmids did not give an intact plasmid DNA. Therefore, 10 of the plasmids (pAM09) isolated out of ECF529 and ECF530 described previously, that were confirmed having the 16.0 kb *Eco*RI fragment, were digested with *Eco*RI, the enzyme heat-inactivated and ligation performed in a larger volume (100 µl), then followed by transformation of ECF529 and ECF530 selecting $Km^R$ with 0.05% arabinose only, since these plasmids were confirmed as chloramphenicol sensitive. From the isolated plasmids digested with *Eco*RI, two of them had lost the 16.0 kb fragment, which means they

should have no *oriV*$_{RK2\text{-}Tn1}$ region (Figures 6.14C). Further digestion using *Eco*RI-*Bsr*GI, *Xba*I-*Bsr*GI, *Bsr*GI-*Bgl*II, confirmed both of these two plasmids as having the γ-origin (Figure 6.14B), and *Eco*RI-*Bam*HI showed that they have lost the Amp$^{R}$ gene. Moreover, digestion with *Eco*RI, *Eco*RV, *Eco*RI-*Eco*RV (Figure 6.14C, D and E), more accurately confirmed that they are the right suicide vector (pAM11) using RK2 plasmid, and plasmids from both C600 and C2110 as controls. Faint growth of ECF530 (with $P_{BAD}$ promoter and *pir* gene) strain with these plasmids in broth culture without arabinose was observed, while better growth was observed with 0.05% arabinose. This is consistent with Bowers *et al*., (2004) observation that $P_{BAD}$ promoter is leaky enough to give support of maintenance of γ-*ori* plasmids even in the absence of arabinose by supplying sufficient levels of π-protein.

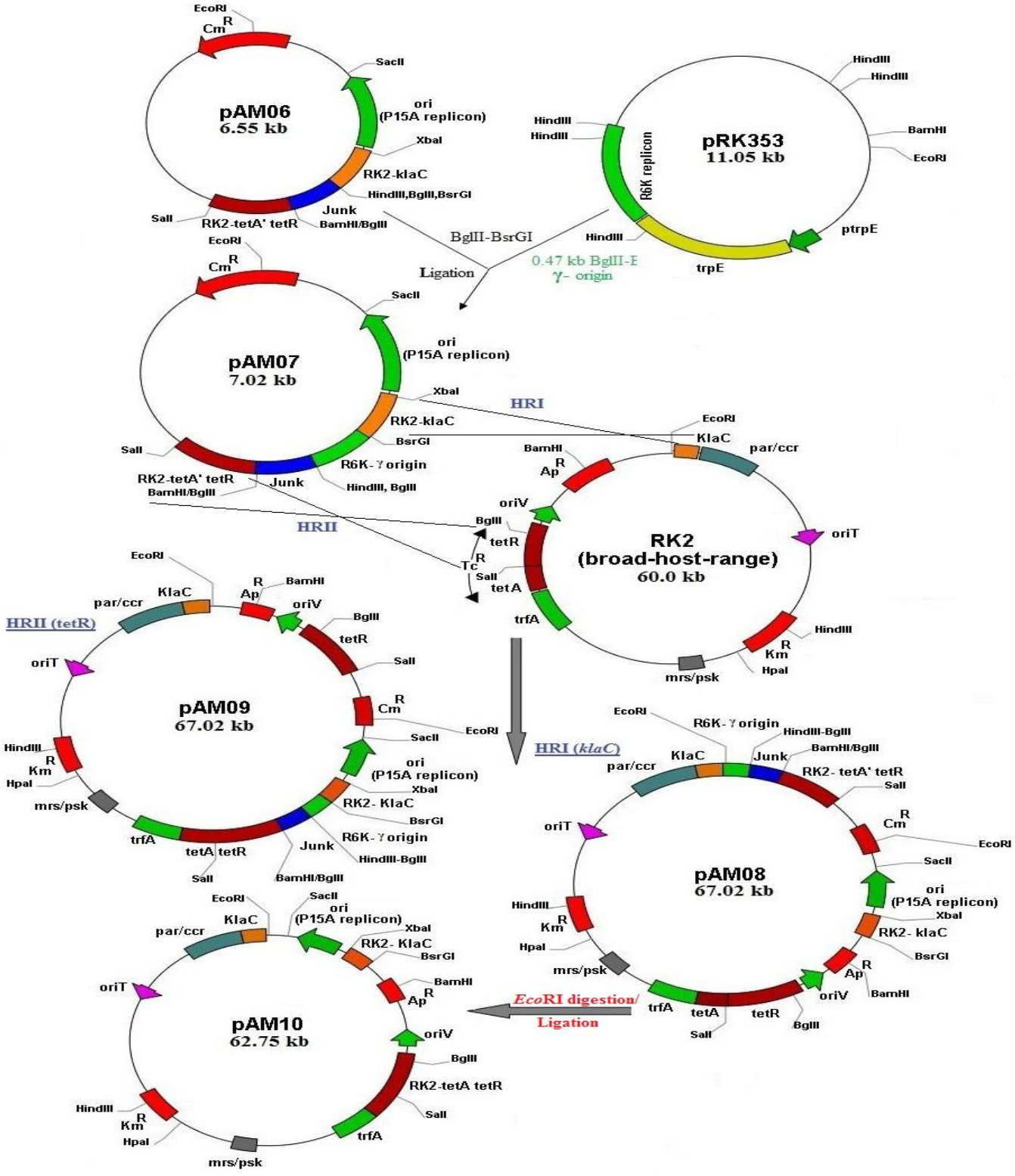



**Figure 6.11 Scheme for the construction of the suicide vector (hybrid of RK2-R6K γ-*ori*) but showing the undesired plasmids.** Plasmid pRK353 digested with *Bgl*II-*Bsr*GI released a 0.47 kb γ-*ori* fragment which was ligated between the *Bgl*II-*Bsr*GI sites of pAM06 to give pAM07 plasmid. *Eco*RI digestion of pAM08 plasmid out of recombination of pAM07 plasmid with RK2 via *klaC* region (HRI) would give plasmid pAM10 which does not have the desired properties and was not constructed.

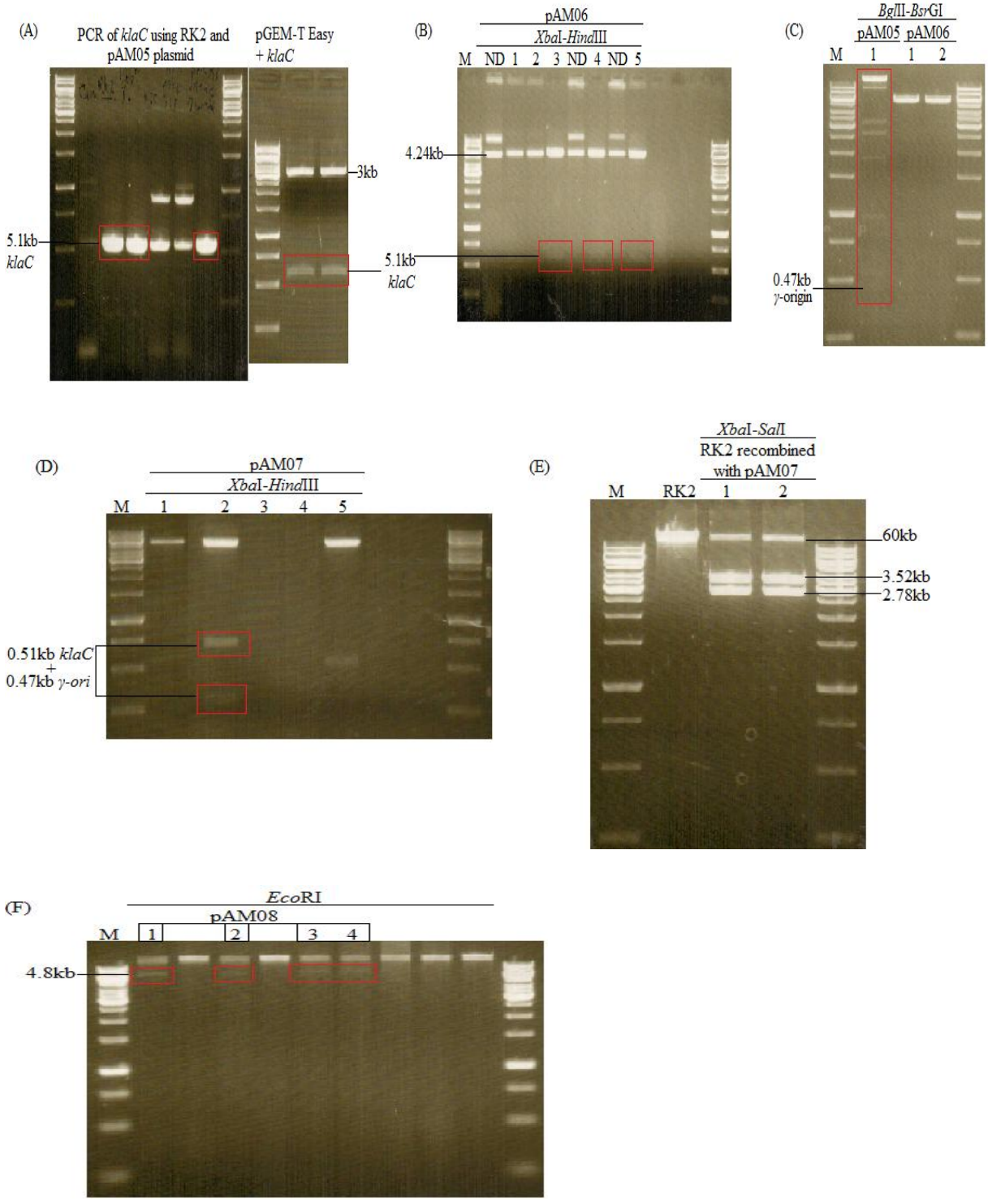



**Figure 6.12 Cloning *klaC* and R6K γ-*ori* into pAM01 plasmid for recombination with RK2.** Using RK2 and pAM05 plasmids 0.51 kb *klaC* amplified (A) and cloned into pAM01 for the construction of pAM06 (B) which was digested with *Bgl*II-*Bsr*GI to clone 0.47 kb R6K γ-*ori* out of pAM05 plasmid (C). Construction was confirmed by *Xba*I-*Hin*dIII (D) and *Xba*I-*Sal*I (E) digestions. *Eco*RI digestion of pAM08 plasmid out of recombination of pAM07 with RK2 via RK2-*klaC* (HRI) released 4.8 kb designated as pAM10.

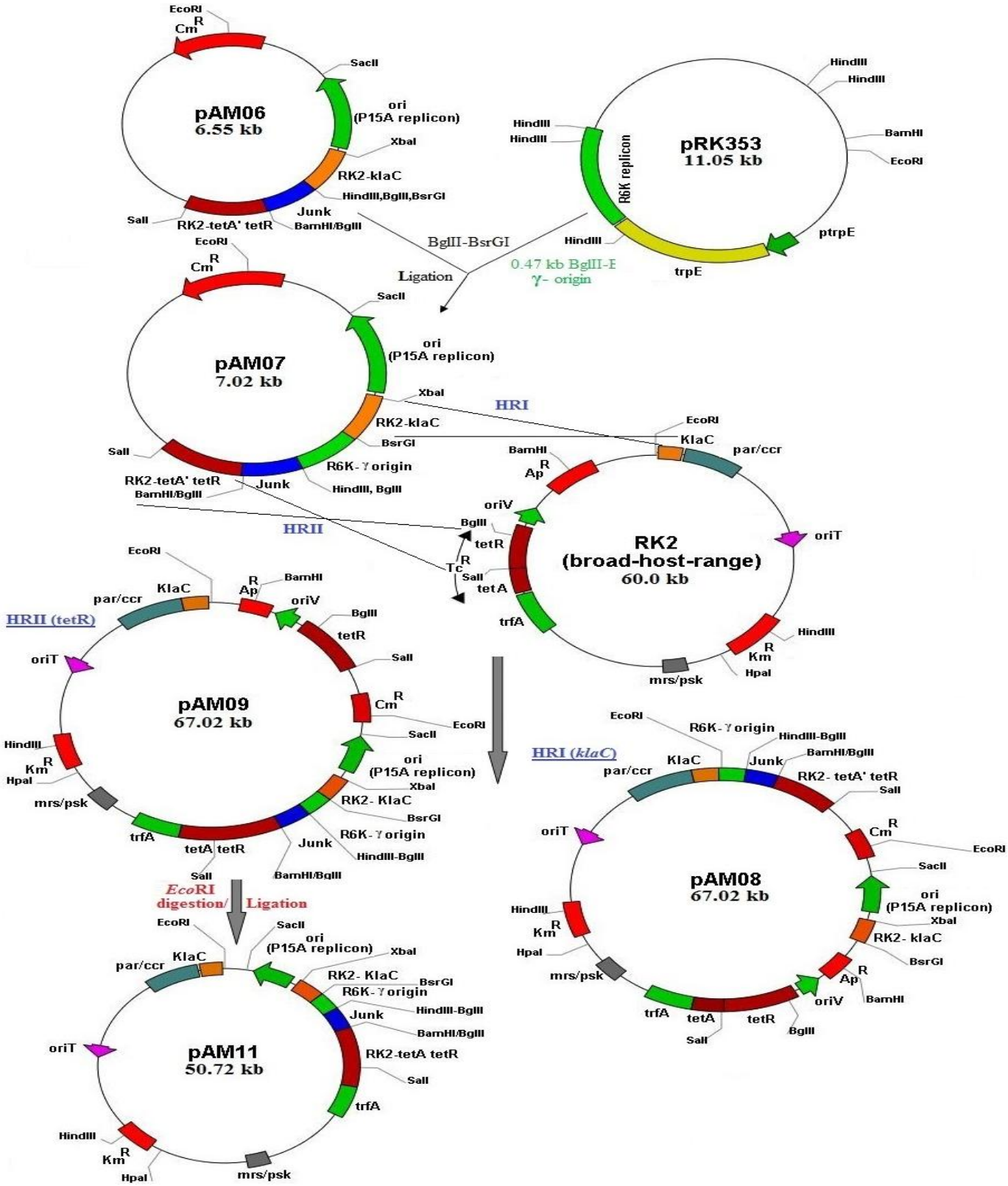



**Figure 6.13 Construction of suicide vector (hybrid of RK2-R6K γ-*ori*).** Plasmid pRK353 digested with *Bgl*II-*Bsr*GI released a 0.47 kb γ-*ori* fragment which was ligated between the *Bgl*II-*Bsr*GI sites of pAM06 to give pAM07 plasmid. *Eco*RI digestion of pAM09 plasmid out of recombination of pAM07 plasmid with RK2 via tetR region (HRII) of tetracycline resistance gene gave the suicide vector pAM11 with R6K γ-*ori*.

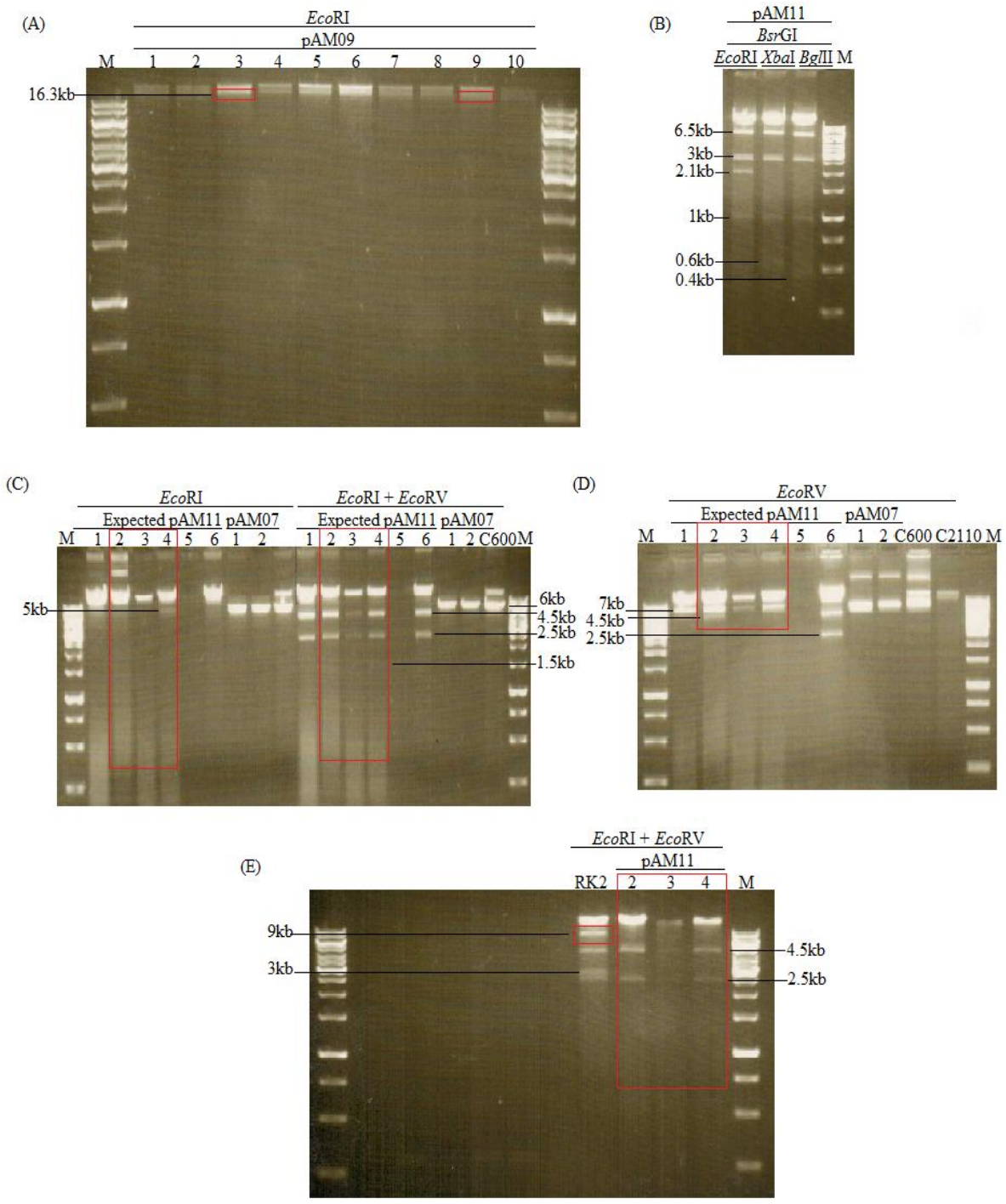



**Figure 6.14 Confirmation of the construction of the pAM11 suicide vector (RK2-R6K γ-*ori* hybrid) with the junk DNA.** *Eco*RI digestion of pAM09 plasmid out of recombination of pAM07 with RK2 via RK2-tetR (HRII) released 16.3 kb designated as pAM11 (A) which had been digested with *Bsr*GI/*Eco*RI, *Bsr*GI/*Xba*I and *Bsr*GI/*Bgl*II, respectively (B), and confirmed by *Eco*RI, *Eco*RV, and *Eco*RI/*Eco*RV digestions (C, D and E) using RK2 and plasmids out of C600 and C2110 as controls.

### 6.3.6 Successful integration but not mobilisation of pTML1 by suicide vector RK2 with R6K γ-*ori* depending on chromosomal expression of π-protein

The suicide vector based on RK2 with R6K γ-*ori* and without *oriV*$_{RK2\text{-}Tn1}$ region constructed in section 6.3.5.2 in ECF530 with *pir* gene in the chromosome was next used for conjugation with *Pseudoalteromonas* spp SANK 73390 selecting Km$^R$, using pTML1 cured SANK 73390 as a control. The experiment was performed more than in triplicate, and the result showed transconjugants on plates with both the wild type SANK 73390 and the pTML1 cured strain as well. However, growth of typical colonies of SANK 73390 with optimum numbers was seen only on plates from the conjugation with the wt carrying pTML1. In contrast, mostly small to tiny colonies were seen on plates from the conjugation with pTML1 cured strain, and those might have been just the donor *E. coli* ECF530 (Table 6.5).

| **Transconjugation replicates** | **WT SANK 73390** | **pTML1 cured SANK 73390** |
|---|---|---|
| | **Numbers and description of colonies on plates with different dilutions** | |
| 1 | Typical colonies up to $10^{-5}$ (a few colonies on $10^{-4}$ and $10^{-5}$) | Mixed of S and M colonies up to $10^{-4}$ (3-4 colonies on $10^{-4}$) |
| 2 | Typical colonies up to $10^{-5}$ (3-4 colonies on $10^{-4}$ and $10^{-5}$) | S and M colonies up to $10^{-3}$ (no colonies on $10^{-4}$ and $10^{-5}$) |
| 3 | Typical and small colonies up to $10^{-5}$ | 90% tiny colonies up $10^{-4}$ |
| 4 | Typical and small colonies up to $10^{-5}$ | Tiny colonies on some of the plates |
| 5 | Typical colonies on $10^{-2}$ and $10^{-3}$ only | 90% tiny colonies up to $10^{-2}$ only |
| 6 | Typical colonies up to $10^{-5}$ | S and M colonies up to $10^{-4}$ |

**Table 6.5 Showing number of the colonies on plates of different dilutions out of transconjugation of the suicide (RK2-R6K-γ-*ori* hybrid) vector with the WT and the pTML1 cured strains of SANK 73390.**

To check for suicide vector integration into pTML1 in the WT SANK 73390, PCR was performed using primers labelled A to D described in Table 6.3 and designed to give a 1.0 kb product. Suicide vector (RK2 with R6K γ-*ori*), WT SANK73390, SANK 73390 (-pTML1 cured) without and with suicide vector (trans-conjugant) were used as controls. Ideally, primers A and B should amplify a fragment running through the junk DNA in the WT carrying pTML1, and should give 1.0 kb product in case of the WT strain but not in the pTML1 cured strain. Primers C and D should amplify a fragment of RK2-tetR and a fragment of R6K γ-*ori* while running across the junk DNA in the suicide vector pAM11 (a hybrid of RK2-R6K γ-*ori*) to give 1.0 kb product (Figure 6.15). PCR using A and B primers gave a 1.0 kb product in case of WT with pTML1, but not with pTML1 cured strain and the negative control. In addition, PCR using C and D primers gave a 1.0 kb product with suicide vector (RK2 -R6K *γ-ori* hybrid), but not with the negative control (Figure 6.16). These results are exactly what we were expecting for two reasons, first it confirmed the presence of pTML1 in the WT SANK 73390, and its absence in pTML1 cured strain; second it indicates the presence of the junk DNA and R6K γ-*ori* in the suicide vector. Therefore, and based on recombination between the suicide vector and pTML1 in the WT SANK 73390, PCR was performed using combinations of primers, A with D and B with C to check for suicide integration into pTML1 of WT strain (Figure 6.15). Trans-conjugants with the pTML1 cured strain which cannot undergo recombination with pTML1 were used as a negative control. The 1.0 kb PCR product (Figure 6.16) observed with both combinations of primers in case of trans-conjugant with WT (carrying pTML1), indicated the correct integration of the suicide vector into pTML1. The first combination of primers did not give PCR product in case of trans-conjugant with the pTML1 cured strain, which is expected, however, and surprisingly, a faint 1.0 kb product observed with the second combination of primers (B and C). This is rele-

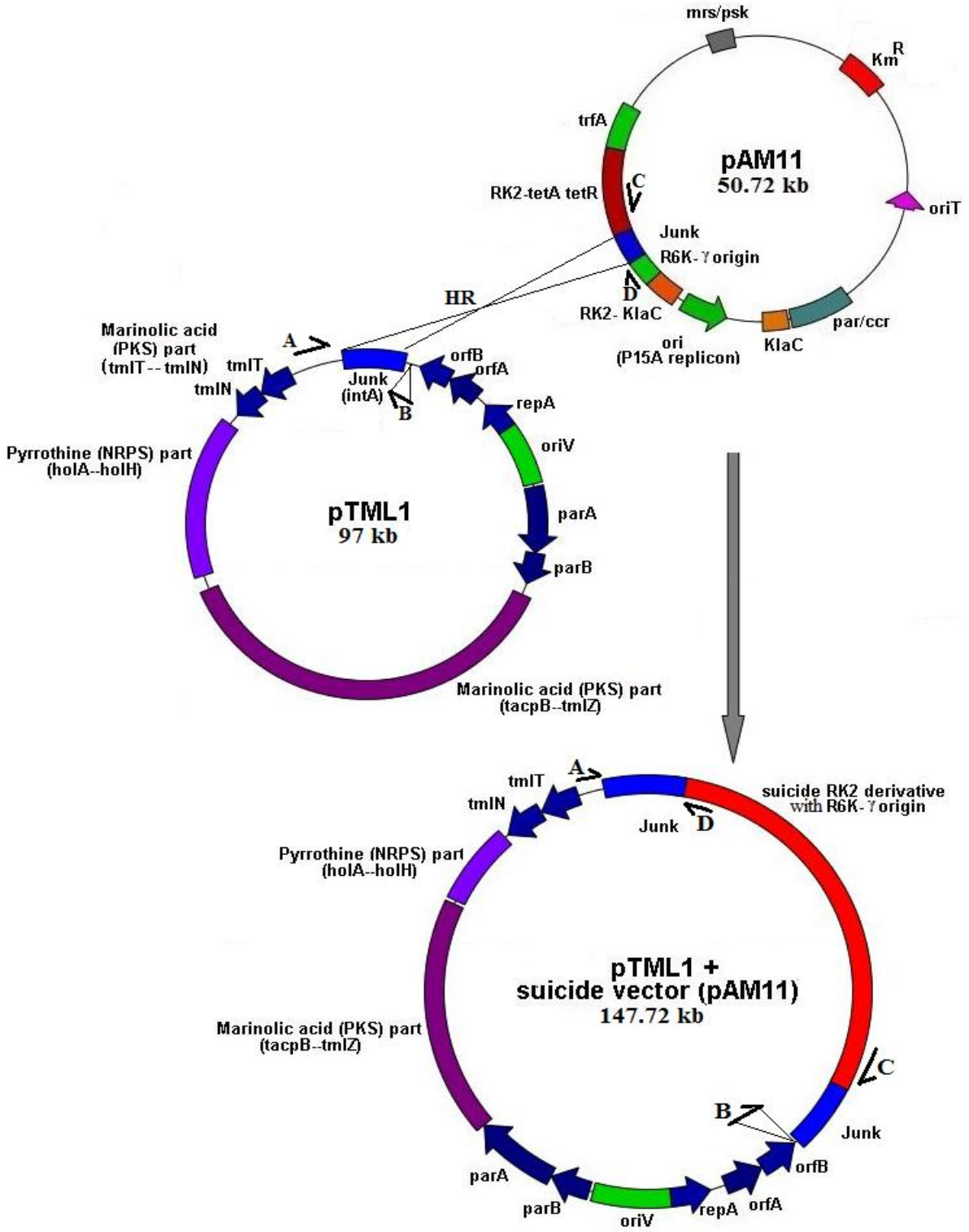



**Figure 6.15 Investigation of the suicide (RK2-R6K γ-*ori* hybrid) vector integration into pTML1 in the WT SANK 73390.** Primers A and B designed to amplify a 1.0 kb fragment running across the junk DNA in the WT carrying pTML1, and primers C and D designed to amplify a 1.0 kb fragment in the suicide vector running through the junk DNA including part of RK2-tetR in one side and part of R6K γ-*ori* in the other side.

vant with primer C since it normally amplifies part of RK2-tetR before running through the junk DNA in the suicide vector, which it might be able to replicate in the pTML1 cured strain. However, it is peculiar with primer B, since we precisely confirmed that SANK pTML1 cured strain is lacking pTML1, but it might be due to annealing of primer B somewhere else within the vector as the PCR product looks different in size compare to the 1.0 kb product of trans-conjugant with WT.

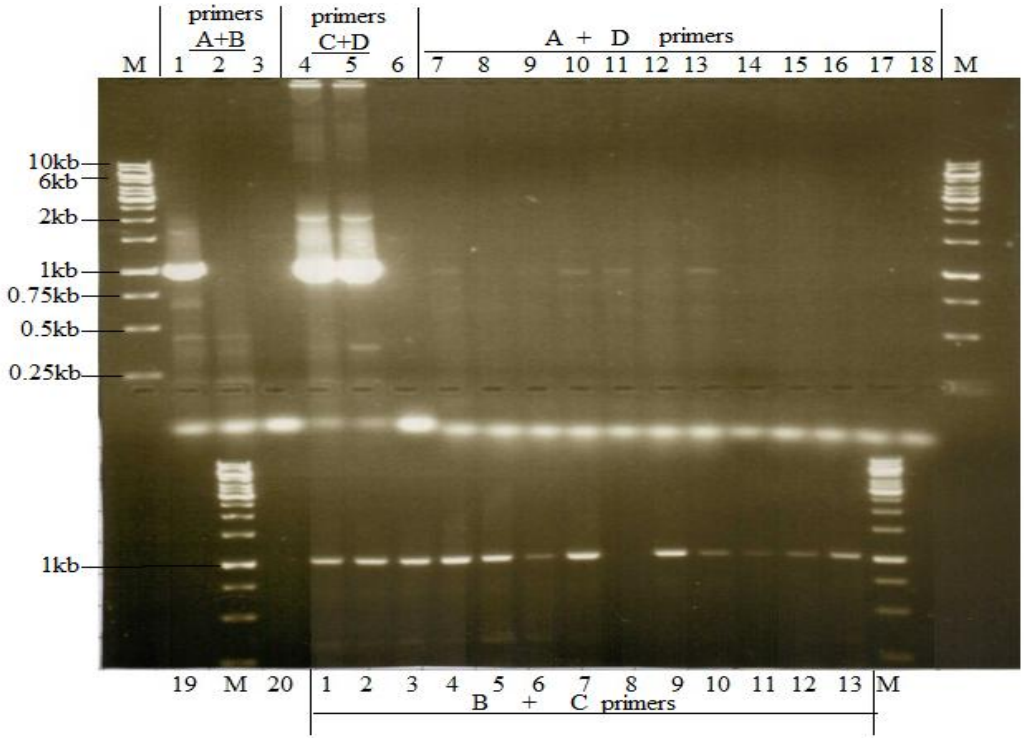



**Figure 6.16 Confirmation of the suicide vector integration into pTML1 plasmid.** Top lanes from left: M, 1 kb marker ladder; lane1, WT SANK 73390; lane 2, SANK 73390 pTML1-cured; lane 3 and 6, negative controls; lane 4 and 5, suicide vector with R6K γ-*ori*; lanes 7 to 13, suicide integration into pTML1 of WT SANK; lanes 14 to 18 at the top, 19 and 20 at the bottom left, SANK pTML1-cured. Bottom lanes from left: 1 to 7, suicide integration into pTML1 of WT SANK; unused lane 8; lanes 9 to 13, SANK pTML1-cured.

In order to be certain about the correct direction of the primers, for example primers A in the WT SANK with pTML1 and primer C in the suicide vector that are running through the

*Bam*HI site of the junk DNA, the fact which provides good evidence to the previous result of the suicide integration into pTML1. PCR was performed using normal combination of primers (A+B and C+D) to check the trans-conjugant. Results showed a 1.0 kb product with both combination of primers in case of trans-conjugant with WT carrying pTML1, and only with the combination of C and D primers in the case of trans-conjugants with pTML1 cured, while no product was observed when A and B primers were used for trans-conjugant with pTML1 cured (Figure 6.17). The presence of products with C and D might indicate the possibility of having different copies of the plasmid in the trans-conjugant with WT, such as pTML1, suicide vector (RK2 with R6K γ-*ori*), and suicide vector integrated into pTML1, but only the presence of the suicide vector in the trans-conjugant with pTML1 cured strain. Since these results were encouraging, therefore, conjugation with *E. coli* strains ECF529 and ECF530 were performed to move the hybrid plasmid (RK2 with R6K γ-*ori*) possibly with pTML1 captured out of trans-conjugant of WT SANK 73390 selecting $Km^R$ with 0.05% arabinose. Unfortunately, plasmids isolated out of ECF strains looked similar to the suicide vector (RK2 with R6K *γ*-*ori*).

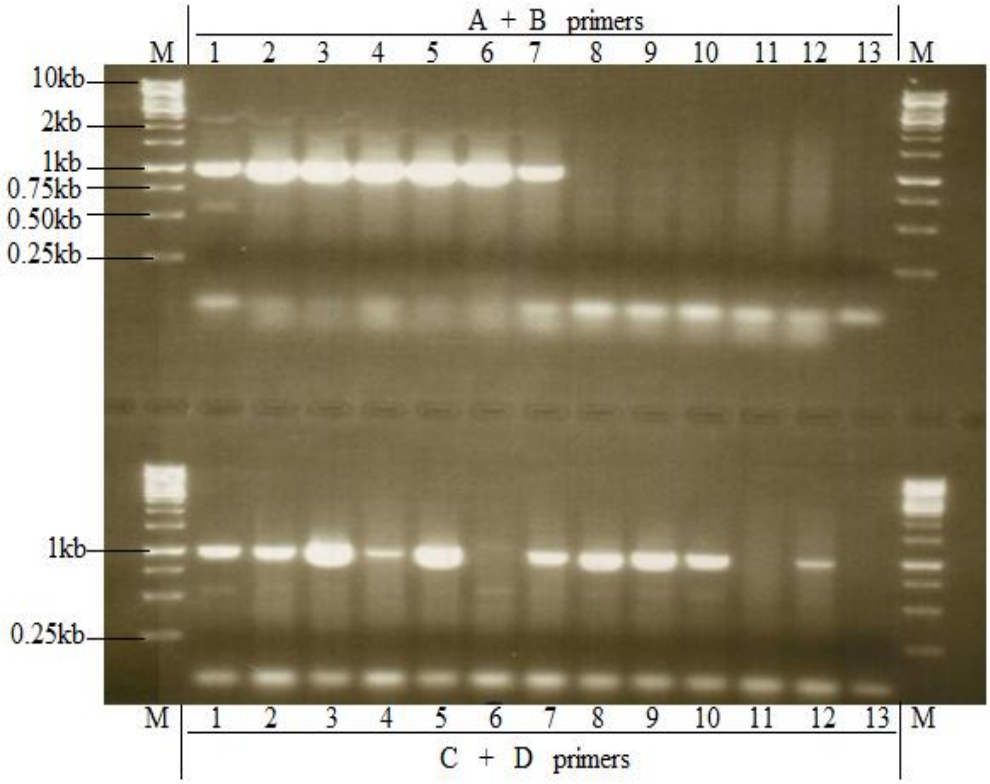



**Figure 6.17 PCR checking of vector integration into pTML1 plasmid.** Top and bottom lanes from left: M, 1 kb marker ladder; lanes 1 to 7, WT SANK 73390; lanes 8 to 12, SANK 73390 pTML1-cured; lane 13, control negative.

## 6.4 Discussion

Regulation of thiomarinol biosynthetic genes carried on pTML1 plasmid, are predicted to be encoded by gene(s) located on the plasmid itself. This prediction was based on preliminary data in Chapter 2 of this thesis showing that thiomarinol production appears to be regulated so as to give expression in late exponential or early stationary phase. Moreover, if the plasmid is able to replicate autonomously in the cytoplasm of the host cell, and able to transfer to a recipient cell through the mode of conjugation, as two fundamental properties of transmissible plasmids (Willetts, 1972), then it should carry its own regulator. However, although the majority of essential requirements for a typical mobile DNA including replication, partitioning, transposition and integration were identified on five orfs, occupying only 7.6 kb of pTML1 DNA molecule, there was no evidence of conjugation transfer function (Fukuda *et al*., 2011). In addition, plasmids that are not self-transmissible can often be mobilised by other replicons, allowing them to adapt and colonize new bacterial species (del Solar and Espinosa, 2000). More specifically, and at the level of gene, Thomas and Nielsen (2005) showed the probability and usefulness of broad-host-range plasmids for the spread of a gene between bacteria in case the gene moves onto the vector. Therefore, the purpose of the study described in this Chapter was different attempts to mobilise pTML1 plasmid out of *Pseudoalteromonas* spp. SANK 73390 to investigate expression and regulation of thiomarinol biosynthetic genes.

The first attempt to capture and mobilise pTML1 plasmid was by utilising RK2 vector. For this plasmid to be able to integrate into pTML1 through homologous recombination (HR) it needs to have some homology with its target. Therefore, a suicide RK2 (pAM02) vector under the control of $oriV_{RK2}$ with a piece of DNA, designated as 'junk', out of the biosynthetic gene cluster of thiomarinol has been constructed using plasmid pACYC184 as

an intermediate vector. Data from using this vector to capture and mobilise pTML1 showed the inefficiency of this vector to do so while using RK2 vector as a control. This might be due to the ability of pAM02 plasmid to replicate in *Pseudoalteromonas* spp. SANK 73390, which minimised the possibility of integration into pTML1 plasmid via the junk DNA.

To overcome the problem of the suicide vector replication in SANK73390, pRK353 plasmid (Figure 6.18), a derivative of R6K replicon was used to replace the $oriV_{RK2}$ in pAM02 plasmid when pAM03 plasmid constructed. pRK353 plasmid with R6K replicon had been selected according to three characteristics that made it preferable vehicle in molecular cloning (Kolter and Helinski, 1978b). The most relevant one is the absence of selectable

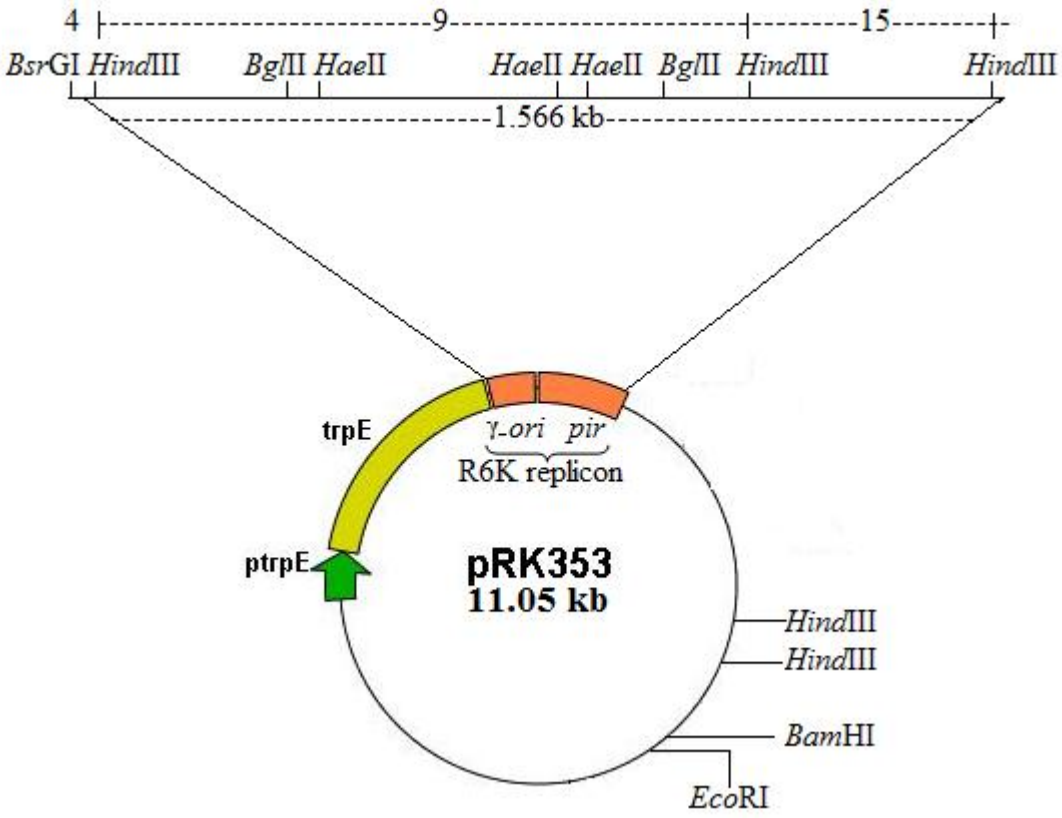



**Figure 6.18 Map of autonomously replicating pRK353.** Restriction sites for *Eco*RI, *Bam*HI, *Hind*III, *Bsr*GI, *Bgl*II, and *Hae*II are shown. Sufficient R6K replicon present in this plasmid represented by γ-origin and *pri* gene and the size indicated. p*trpE*, and *trpE* refers to tryptophan E gene and the promoter region.

markers (antibiotic resistance) in pRK353 makes it a reasonable plasmid for cloning DNA fragments with *Bam*HI, *Eco*RI, or *Bam*HI-*Eco*RI. Therefore, suicide RK2 vector with R6K replicon with two orientations (pAM04 and pAM05) was constructed, and both were used for conjugation with *Pseudoalteromonas* spp. SANK 73390, using SANK pTML1 cured strain as a control. Apparently, these new suicide vectors with R6K replicon were also able to replicate both in the wild type carrying pTML1 and pTML1 cured strain as well, which excluded the possibility of capturing and mobilising pTML1.

Because the initially constructed suicide vectors were unable to mobilise pTML1, another suicide vector with R6K γ-*ori* (pAM11) was constructed so that it could not replicate without an exogenous source of π protein. This type of vector has been confirmed as a valuable tool in molecular applications (Bowers *et al*., 2004), including recombination studies (Alexeyev and Shokolenko, 1995; Metcalf *et al*., 1996). *E.coli* strains with chromosomally inserted *pir* gene, ECF529 (with wt *pir* gene) and ECF530 (with hyperactive *pir* gene) has been used for providing enough π protein for the initiation of replication from γ-*ori* of the suicide vector, and maintaining the plasmid copy number at an accurate levels in the cell. This is in agreement with Bowers *et al*., (2004) since a supply of π protein from the chromosome is preferable over provision from another helper plasmid encoding π protein. One of the approaches used to identify whether this new suicide vector with R6K γ-*ori* was able to integrate into pTML1 and mobilise it, was specific PCR primers designed to identify the suicide integration into pTML1. Results of using different combinations of PCR primers (A+D and C+B) indicated the suicide integration into pTML1 in case of transconjugants of WT *Pseudoalteromonas* spp. SANK 73390. Suicide (RK2-R6K- γ-*ori*) vector, WT SANK 73390, and pTML1 cured strain were used as controls. This result was encouraging and provides a preliminary evidence of possible mobilisation of pTML1 out of the WT SANK 73390. Using the same combination of primers for the transconjugants with the SANK

pTML1 cured, primers A with D did not produce any product which was as expected, however, primers B with C gave a faint product which looks different compare to the 1.0 kb product of transconjugants with WT SANK with pTML1. Presumably, this might be due to the annealing of primer B somewhere else within the suicide vector since there is no pTML1 to anneal with. When the usual combination of primers (A+B) was used, only transconjugants with the WT SANK 73390 showed a 1.0 kb product since the primers run across the junk DNA, which was as expected. However, and unexpectedly, both of the transconjugants with the WT SANK and pTML1 cured showed a 1.0 kb product while using primers C and D, which usually runs across the junk DNA in the suicide (RK2-R6K γ-*ori*) vector. The presence of different copies of plasmids including the suicide (RK2-R6K γ-*ori* hybrid) vector and the suicide vector integrated into pTML1 in the transconjugant of WT SANK may be the possible explanation. However, the presence of suicide (RK2-R6K γ-*ori* hybrid) vector in the transconjugants of SANK pTML1 cured strain can be the only possible explanation for the 1.0 kb product. Since we confirmed accurately the absence of $oriV_{RK2}$ in this suicide vector, but dependence on $oriT_{RK2}$ for conjugal transfer, survival of the plasmid in these strains might be due to the presence of P15A replicon when there is no obvious exogenous source of π protein. Also it might be due to the host-encoded integration host factor (IHF), if there is any, which has been demonstrated to permit the replication from γ-*ori* through the binding to one IHF site (*ihf*1) only, but not the second IHF site (*ihf*2) within the γ-*ori* when the level of π protein is inhibitory (Dellis *et al*., 1996).

Subsequently, and as the other side of the previous approach, plasmids isolated from ECF529 and ECF530 when suicide RK2 derivative (RK2-R6K γ-*ori* hybrid) possibly with pTML1 moved out of SANK strains to *E. coli* strains by conjugation. Unfortunately, the plasmids seem to be similar to the suicide vector. Therefore, although previous data in SANK 73390 with pTML1 and pTML1 cured strains did show the suicide integration into pTML1

was which was really encouraging, however, the reason for not been able to mobilise pTML1 might be due to the size of both pTML1 (97 kb) and the suicide vector pAM11 (50.72 KB), respectively.

Overall, this study demonstrated the ability to integrate with pTML1 plasmid carrying the biosynthetic gene cluster of thiomarinol using suicide RK2-R6K (*γ-ori* only) hybrid vector. However, the reason why attempts to mobilise pTML1 were unsuccessful is unclear. Further experiments need to be performed to identify possible problems and explore associated solutions. One possibility is that the plasmid does transfer to *E. coli* but then rapidly breaks down by recombination. In this case a recombination deficient recipient might overcome the problem. It might also be worth trying to make this suicide vector smaller if it is possible, then using it for the mobilisation.

# CHAPTER 7

# GENERAL DISCUSSION AND FUTURE WORK

## 7.1 General comments

Over the last fifty years, a huge number of diverse polyketides (PKs) was found, some of which are given as examples in Chapter 1. Among them, a good number have entered clinical use, serving medicine via different ways, acting as anti-bacterial (antibiotics), anti-fungal, anti-parasitic and anti-cancer agents as well as various immunological purposes and lowering cholesterol levels. In addition, various other compounds including non-ribosomal peptides (NRPSs) and of PKS-NRPS hybrids were discovered, which added extra power and interest to this field, although some are still underway to be used medicinally. Understanding the assembly lines of modular polyketides and the synthesis pattern of type II and type III PKSs, has already provided a critical path to genetically engineering pathways of various specific PKs, to produce compounds with the required alterations (Weissman and Leadlay, 2005). However, all the way through, combinatorial biosynthesis is still under development, aiming to improve the process of manipulation by overcoming some key steps, which will facilitate different strategies. These include attempts to develop a set of universal 'interdomain' and 'intermodular' linkers that could be used successfully for joining any pieces of PKS together, even if they are different based on their sequences, so as to make (reprogramming) a functional assembly line (Weissman, 2004). It also includes attempts to develop a set of orthogonal pairs of docking domains (normally located at the ends of PKS proteins which resist forming incorrect associations) that can simply be attached to the ends of the newly assembled (engineered) PKS proteins from different pathways to make sure that the reprogramming of the assembly line joined together in a functional and correct order (Weissman, 2004). The outcome of engineering the PKS and NRPS is hoped to give compounds with stronger activity, less toxicity, high production and various other desirable properties. Erythromycin is given as a model example for understanding the biosynthetic pathway, partly because it was the first studied in detail but also because the order of the PKS

genes in the cluster is colinear with its structure. However, in the mupirocin system, there are several interesting features which made it of a significant value. These features includes the order of the genes in *mup* cluster which is not colinear with mupirocin structure, a *tran*-AT domains, tandem ACP and a system which includes a set of reductive (tailoring) enzymes. In addition to further other interesting specificities, this clearly indicates that the system is really valuable and very important to understand biosynthesis and adds further realistic and valuable engineering mechanisms to combinatorial biology. These critical tools may provide a way to develop mupirocin so that it could be used systemically. Thiomarinol, the hybrid of PKS (pseudomonic acid) and NRPS (pyrrothine) is another more interesting antibiotic, with a high potency (broad spectrum activity) against a wide range of bacterial groups. However, it still has not yet been used clinically, since more things need to be solved or discovered, including the way that the system is regulated. All these might be helpful in removing the toxic effect of this antibiotic to eukaryotic cells, if there is any, and further important aspects which needs to be taken into consideration, when it comes to be used in the clinic. In addition, creation of novel compounds through successful mutasynthesis is one of the most interesting features of thiomarinol, which suggests a great future with diverse and strong novel compounds that could be used not only for the treatment of MRSA as the case with mupirocin, or thiomarinol, that can overcome mupirocin resistance, but expansion to more than that.

## 7.2 Discussion of key conclusions

### *7.2.1 Thiomarinol production is regulated, its extraction improved by adjusting the pH to acidic conditions and using acetone in combination with ethyl acetate*

Production of thiomarinol by *Pseudoalteromonas* spp SANK 73390 after 9 h of growth, which is late logarithmic phase, is an indicator that the production is under regulation.

Logarithmic phase was confirmed as the period from 3 to 9 h after inoculation followed by stationary phase where the production of thiomarinol fluctuated to give a higher level at 18 h (the first 24 h) and 36 h (the second 24 h). Removing thiomarinol that was already produced in the seed culture before carrying on the growth and production in fresh medium was essential for the accurate designation of the initial point of thiomarinol production. In agreement with Shiozawa *et al*., (1993), a combination of acetone with ethyl acetate was found to give efficient extraction of the majority of the thiomarinol. Also acidic conditions gave improved extraction, possibly through stabilising the compound, which is consistent with Takahashi *et al*., (1995) that thiomarinol compounds can be extracted by organic solvents under neutral or acidic conditions. In addition, since thiomarinol is hydrophobic (Takahashi *et al*., 1995), 35% acetonitrile and 65% water were typical for the detection and discrimination of thiomarinol by HPLC analysis.

### *7.2.2 TmlU, the thiomarinol amide ligase, interferes with mupirocin biosynthetic pathway through making pseudomonic acids with shorter fatty acid side chain (C5 PA-B and PA-A)*

TmlU, the amide ligase in the thiomarinol system acts by linking marinolic acid (mupirocin analogue) and pyrrothine via an amide linkage. It was also proposed to play an additional role equivalent to the role of MupU in the mupirocin system, in transferring the growing PK chain (intermediate) to mAcpE, based on the weak similarity between them (Fukuda *et al*., 2011). However, the inability of *tmlU* to cross-complement *mupU* indicates that TmlU is responsible for a quite distinct and specific function. Comparison of amino acid sequence of TmlU with its homologues, SimL from simocyclinone (Luft *et al*., 2005; Pacholec *et al*., 2005) and NovL in novobiocin biosynthesis (Steffensky *et al*., 2000), suggested that it creates the amide linkage during the biosynthesis, which has been confirmed

tentatively and mentioned earlier by Fukuda *et al*., (2011). Not only did *tmlU* not complement *mupU* but it also interferes with the biosynthesis of mupirocin to cause the accumulation of a C5-pseudomonic acid A and B depending on the level of induction of *tmlU*. It therefore appears to block the second ($C_5$-$C_7$) and the third ($C_7$-$C_9$) round of elongation for the synthesis of 9HN (which is predicted to be the product of MmpB with tandem ACPs/-Ba, -Bb and -Bc (El-Sayed *et al*., 2003), without affecting the first condensation step ($C_3$-$C_5$). This was also the case with the wild type *P. fluorescens* NCIMB 10586 and 10586Δ*mupX*, since it was expected that MupX (amidase) in the WT counteracts TmlU function, which in turn caused the truncation of mupirocin. Further analysis confirmed that TmlU is not working like an abnormal acyl hydrolase to release the PK chain when it was expressed in NCIMB10586ΔTE. Expression of *tmlU* in 10586 strains with the active site serine of the ACPs of MmpB mutated to alanine showed that TmlU is not targeting the active site. Additional data indicates that TmlU may interact with all the ACPs of MmpB, when *tmlU* was expressed in NCIMB10586 strains with single or double deletion of these ACPs. However, the deletion of ACP6 (Bb) significantly reduced the sensitivity of mupirocin biosynthesis to the negative effect of TmlU. These observations are important, since they provide the first evidence that MmpB is involved in the production of 9HN. But they also indicate that while the second and the third elongation steps happen on MmpB, it is possible that the first round of condensation might happen on a protein other than MmpB. It also adds another indication to the idea that the saturated acyl side chain in the case of mupirocin and thiomarinol are built up through successive elongations of the PKS derived product, rather than via the ligation of fully assembled fatty acids ($C_9$ or $C_8$, respectively) (Murphy *et al*., 2011) with the PK part.

*7.2.3 The extra KS-B2/ACP-B2 (2nd module) of TmpB represents a non-elongating module in thiomarinol biosynthesis pathway, and the ACP-B2 has a role in the removal of C8-OH.*

In the mupirocin cluster, *macpE*, which has an essential role in the last steps in the production of pseudomonic acid A but not pseudomonic acid B (Cooper *et al*., 2005a), is one of the genes (along with *mupU* and *mupV*) that has no equivalent in the thiomarinol biosynthetic cluster. However, the presence of the extra KS-B2/ACP-B2 in TmpB of the thiomarinol biosynthesis pathway, and its absence in MmpB of mupirocin, suggests that all the last tailoring steps in the production of thiomarinol might happen on TmpB (Fukuda *et al*., 2011). In-frame deletion of this extra module in TmpB minimised the production of thiomarinol A and B but did not eliminate thiomarinol production. This is a good indication that this extra KS-B2/ACP-B2 works as a non-elongating module, and that the KS-B2 functions as a transferase, since it is not implicated in a Claisen condensation. The deletion also revealed the accumulation of thiomarinol molecules with an extra OH at C8, indicating that the ACP-B2 might be implicated in this tailoring step. Accumulation of thiomarinol C, which lacks OH at C4 is also interesting which might indicate a link between the removal of OH at C4 and hydroxylation at C8 as a result of KS-B2/ACP-B2 deletion.

*7.2.4 Genetic manipulation increased mupirocin production but does not reduce PA-B in favour of PA-A. Fused MupU-mAcpE expressed in trans as a single functional protein is able to convert PA-B to PA-A, does not function when inserted into mmpB*

The proposed pathway which includes the transfer of the intermediate by MupU to mAcpE, following by further processing via MupO, V, C and MupF, is predicted to be sufficient for the conversion of PA-B to PA-A (Hothersall *et al*., 2007). In trans expression of those genes in the WT NCIMB 10586 was successful in increasing total mupirocin (PA-A

and PA-B) by about two fold, but did not achieve its primary goal of shutting down PA-B in favour of PA-A (Macioszek, PhD thesis, University of Birmingham, 2009). This was predicted to be as a result of the release of the ACP bound acyl intermediate (PA-B) in the pathway, catalysed by the thioesterase (TE) at the end of MmpB and because of a limit in the onward flux of the intermediate. Over-expression of *mupU* through genetic manipulation to increase the onward flux of the intermediate in the PA-A pathway, was demonstrated to increase the production of mupirocin by about three to four fold, but without reducing the level of PA-B. The increase in production might be due to an increase in the expression of the other genes downstream of *mupU*, including *mupR* (transcription activator), which was shown by Hothersall *et al*., (2011) to have a profound role in increasing the yield of mupirocin. Fusion of *mupU* and *macpE* via a small linker was performed, so that the two functions would be translated into a single functional polypeptide. Complementation achieved while expressing in trans the fused *mupU-macpE* in NCIMB10586Δ*mupU*Δ*macpE*, confirmed that they were translated into a polypeptide that retained both functions. In contrast, most of the hybrid *pik*AI complementation plasmids were not detectably functional to restore pikromycin production in a *pik*AI deletion mutant (Kim *et al*., 2004). However, complementation was not achieved with the insertion of the fused *mupU-macpE* into *mmpB* of NCIMB10586Δ*mupU*Δ*macpE* to make something like the extra *KS-ACP* of *tmpB* in thiomarinol. Indeed, the insertion blocked the biosynthetic pathway. This might indicate that MupU and mAcpE are not working normally at this point in the pathway, i.e after full elongation of the 9HN moiety. It could also indicate that the whole segment between ACP-Bc and TE needs to be kept intact so that TE could hydrolase and release the compound. However, when various reductive domains (KR, DH and ER), either singly or in combinations, were inserted into the second module of DEBS 1-TE version between the AT and the ACP by either of two linkers, production of a triketide by most of the hybrids

indicates that the reductive loop was treated as a single functional unit (Kellenberger *et al*., 2008). Also, since the second insertion of the fused *mupU-macpE* was not included in *mmpA* of NCIMB10586Δ*mupU*Δ*macpE*, PA-B was the sole product, indicating that no alteration in the enzyme organisation of the biosynthesis pathway had been achieved.

### 7.2.5 *Creation of a hybrid of RK2-R6K γ-ori suicide vector depending on chromosomal expression of the pir gene integrates into pTML1 but did not succeed in recovering it to E. coli*

The demonstration that the thiomarinol biosynthetic genes cluster is located on plasmid pTML1 of *Pseudoalteromonas* spp SANK 73390, was really interesting, but, the ambiguity of the regulatory gene and the absence of the conjugative transfer functions (Fukuda *et al*., 2011) makes the search for the regulation complicated. Attempts to mobilise pTML1 plasmid to another host using different suicide vectors carrying a non essential DNA segment of pTML1 were implemented. The first suicide vector constructed depends on $oriV_{RK2}$ has been constructed and used for capturing pTML1, but since the vector was able to replicate independently in SANK 73390, it ruled out the possibility of the integration into pTML1 and the mobilisation. In addition, when the $oriV_{RK2}$ of the suicide vector was replaced with the R6K replicon for the construction of the second vector, no changes could be detected, due once again to the ability of the vector to replicate again. However, when a new vector constructed devoid of both $oriV_{RK2}$ and R6K replicon, but only with the γ-origin of R6K, which depends on chromosomal expression of π-protein (*E. coli* strains ECF529 and ECF530) for replication, a great improvement was made. It was demonstrated that this new vector was successfully integrated into pTML1 plasmid but unfortunately it was not possible to recover the suicide vector with pTML1 in the *E. coli* strains ECF529 and ECF530 with

chromosomally integrated *pir* gene. The reason for why pTML1 mobilisation was unsuccessful is unclear. However, one possibility is that the plasmid does transfer to *E. coli* strain but it rapidly breaks down by recombination. Non-mobilised (non-conjugative) plasmids were transmitted at high frequencies between strains of *Lacococcus lactis*, when a specific fragment, minimal mobilisation (mob) locus of pIP501 cloned into the target plasmids and the transfer (tra) functions were provided *in trans* by a pIP501 derivative (Langella *et al*., 1993). pHel4, and pHel12, are examples of plasmids identified in many strains of *Helicobacter pylori* (*Hp*), which contain a mobilisation region, but are deficient in cognate type IV secretion system (T4SS), essential for conjugative transfer. However, certain Hp strains, e.g. strain P12 carrying plasmid pHel12, can harbour up to four types of T4SSs in their genome, and *comB* T4SS identified as the major mediator of plasmid DNA transfer between strains of Hp, in addition to providing evidence which indicates the existence of a T4SS-independent mechanism of DNA transfer (Rohrer *et al*., 2012) .

## 7.3 Future work

One of the biggest issue that still needs more input, is switching on mupirocin biosynthesis pathway totally into pseudomonic acid A (PA-A) since the enzymes essential for this specific pathway have been elucidated, and the specific role determined. Manipulating the ribosome binding site of *mupU* could be useful in this regard, since it will be translated from the same amount of mRNA. Fused *mupU-macpE* has been cloned into an expression vector and can be utilised in future work via different strategies. Over-expression of fused *mupU-macpE* in the WT NCIMB 10586 could be really useful, since over-expression in NCIMB10586Δ*mupU*Δ*macpE* was complemented. Insertion of the fused *mupU-macpE* into *mmpB* by avoiding the sequence of the linker which connects ACP7 (Bc) with thioesterase

(TE) using two different options; the first insertion before the linker and the second after, could be successful. It might also be worth trying to insert the fused *mupU-macpE* after *ACP-A3b* (before the stop codon) of *mmpA.* Over-expression of Malgorzata Macioszek's plasmid (pMMH6), which consists of all the six genes found to be sufficient to convert PA-B to PA-A, in NCIMB10586Δ*mupU*Δ*macpE* may give what is being sought.

The effect of TmlU on the biosynthesis of mupirocin, and the clue that has been given about the biosynthesis of the 9HN part should also be continued. Inactivation of TmlU by point mutation, then over-expression in WT NCIMB 10586 would be very useful in the process of investigating the negative effect of TmlU, to see whether it is still able to interact with mupirocin biosynthesis. Since it appears as if TmlU is targeting MmpB, and NCIMB10586Δ*ACP6 (Bb)* showed less sensitivity, it could be useful to develop NCIMB10586 strains with all the possible options of swapping the ACPs (Ba, -Bb and –Bc) of MmpB and then testing the effect of TmlU. Substituting the tandem ACPs of MmpB in the WT NCIMB 10586 with the last three ACPs of TmpB, followed by the expression of TmlU in-trans would be another opportunity. An attempt to co-purify TmlU once with TmpB (from thiomarinol), and second with MmpB, would also serve as a proof that TmlU is targeting MmpB in mupirocin system. This could be achieved by expression and purification of cloned His-tagged TmlU, TmpB and MmpB, respectively, removing the His-tag from TmlU possibly then trying to elute TmlU with TmpB and MmpB, separately. While the first step of elongation (unaffected by TmlU) in the biosynthesis of 9HN is expected to happen outside MmpB, and based on the prediction that MupS, MupQ and mAcpD are implicated in the biosynthesis of 3HP, the starter unit of 9HN, it might be worth trying to express TmlU in NCIMB10586Δ*mupS*, NCIMB10586Δ*mupQ* and NCIMB10586Δ*macpD*, respectively.

The ability of suicide (hybrid of RK2 and R6K γ-ori) vector to integrate into pTML1 plasmid, but still allow recovery in *E. coli* strains ECF529 and ECF530 with chromosomally

integrated *pir* gene, needs further work to be performed to identify the possible problems and to explore the associated solutions. Since it has been proposed that the plasmid does transfer to *E. coli* but rapidly undergoes degradation as a result of recombination, therefore, using another recipient with a recombination deficiency might be useful to overcome the problem. Also dealing with the size of the suicide vector to make it smaller might be helpful for the mobilisation. Since the main goal is looking for the regulation of the thiomarinol biosynthesis pathway, if it is possible, it could be reasonable to construct a cosmid library of biosynthetic genes cluster of thiomarinol from pTML1 using an integrative shuttle vector. Introducing the cosmid into a strain like *Streptomyces albus*, fermentation and then checking the antibacterial activity to explore thiomarinol production. The demonstration of the initial point of thiomarinol production relative to growth could be useful for studying the gene expression via quantifying the level of RNA using the available techniques. In addition, identifying promoters within the thiomarinol cluster and studying the inverted repeats located in the space between the two divergent transcriptional units, which are predicted to be the operator sequences through which the genes are regulated, could be another meaningful path.

Regardless of all the future projects which have been mentioned earlier, leading from this project there are still some additional logistic steps about mupirocin which need to be understood, and more than that about thiomarinol needs to be discovered. In case of mupirocin, resolving all the critical points related to the biosynthesis of the 9HN moiety and the esterification with the monic acid moiety can solve the problem of mupirocin dissociation in the human blood circulation, which then could be used systemically. This might also improve combinatorial biosynthesis in mupirocin. Since there are still quite a few genes in the thiomarinol cluster that need to be investigated, it might be helpful to find additional gene (s) that confers resistance to the pyrrothine element, if there is any. It is hoped that NCIMB10586ΔAT1 could be complemented with the type II TEs from other PKSs including

those encoded by the NRPS gene clusters, since they apparently are not playing an essential role in the biosynthesis. Complementation with the editing hydrolases (AHs) described by Jensen *et al*., (2012) might also be useful, or even insertion of these acyl hydrolases into the biosynthetic pathway of mupirocin might be useful to release the ACPs bound stalled intermediates from the blocked modules.

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