Investigating the role of MmyB-like pathway-specific transcriptional activators in antibiotic production

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List of Abbreviations

AmpR	ampicillin resistance
antiSMASH	antibiotics and secondary metabolite analysis shell
ATP	Adenosine tri-phosphate
B2	Binding buffer
B. subtilis	*Bacillus subtilis*
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CDA	Calcium dependent antibiotic
CLS	Cell lysis solution
CRS	Cell re-suspension solution
DEPC	diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dH2O	distilled water
dNTPs	Deoxyribonucleotide triphosphates
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
LB	Luria broth
LAL	Large ATP binding of LuxR
MIC	minimum inhibitory concentration
Mm	Methylenomycin
MMF	Methylenomycin furan
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NRP	Non-ribosomal peptide

NRPS	Non-ribosomal peptide synthetase
NSB	Neutralisation solution
OD	Optical density
PCR	Polymerase chain reaction
PK	Polyketide
PKS	Polyketide synthase
RBS	Ribosome binding site
Red	Prodiginine
RNA	Ribonucleic acid
RNase	ribonuclease
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer RNA
Rpm	Round per minute
RT-PCR	reverse transcriptase PCR
RT	Room temperature
SARP	*Streptomyces antibiotic regulatory* protein
S. coelicolor	*Streptomyces coelicolor*
SCP1	*Streptomyces coelicolor plasmid 1*
SFM	Soya flour mannitol
SMMS	supplemented minimal medium solid
TAE	Tris- Acetate electrophoresis buffer
TBS	Tris buffer saline
TFR	TetR family regulator
W1	Washing buffer

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Thanks to fabrizio Alberti and Ian Lidbury for helping me greatly and supportively in my PhD. I never forget my colleagues; Vincent Poon for his support toward my method. I would thank Kathryn Styles specifically for her major effort, support and clarification on many key and sensitive points. And the rest of the Core Group for their assistance, I would like to thanks everyone because they are always helpful and supportive.

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Abstract

Streptomyces bacteria are known to produce a large number of bioactive secondary metabolites. The genome sequence of the model organism *Streptomyces coelicolor* A3(2) revealed the presence of multiple previously unknown antibiotic-like gene clusters (Bentley *et al*., 2002). A transcriptional activator named MmyB was proposed to specifically trigger the production of the methylenomycin antibiotics in *S. coelicolor* A3(2). *S. coelicolor* A3(2) itself, which encodes 16 proteins highly similar to MmyB (Xu *et al*., 2012), each of them proposed to control the expression of a distinct biosynthetic gene cluster or to control the expression of other uncharacterised genes was our main target micro-organism. (O'Rourke *et al*., 2009).

The aim of this project was to clone a series of *mmyB*-like putative pathway-specific transcriptional activators under the control of a *Streptomyces* constitutive promoter in integrative vectors. These vectors were then introduced in *Streptomyces* bacteria, using intergenic conjugation. The effect of *mmy*B-like gene overexpression was then investigated by: i) analysing the expression of specific sets of antibiotic biosynthetic genes using RT-PCR, ii) assaying the antibiotic activity of metabolic extracts where *mmyB*-like genes were overexpressed versus negative controls and iii) To identify, isolate and elucidate the structure of antimicrobial using analytical chemistry (LC-MS, HPLC, NMR) in collaboration with analytical chemists. *S. coelicolor* A3(2) itself which encodes 16 proteins highly similar to MmyB (Xu *et al*., 2012), each of them proposed to control the expression of a distinct biosynthetic gene cluster or to control the expression of other uncharacterised genes was our main target micro-organism.

This project successfully revealed that overexpression of transcriptional activators was a powerful strategy to discover new natural products by switching on silent biosynthetic gene clusters. Our understanding of the molecular mechanisms by which particular families of transcriptional activators function was essential in order to determine which specific genes within a cluster would be overexpressed and would result in overproduction of cryptic metabolites.

The antibiotic activity of culture extracts and purified metabolites from engineered *S. coelicolor* strains overexpressing *mmyB*-like transcriptional activators was then assessed against *Bacillus subtilis*. In addition to confirming methylenomycin

overproduction, these essays also revealed the production of new natural products (lantibiotic-derived and poly unsaturated fatty acid-derived).

Chapter 1: Introduction

1.1 Antibiotic history

The term “antimicrobial agent” refers to the group of drugs including antibiotics, antivirals, antifungals and anti-protozoal agents. Antibiotics are often produced by microorganisms such as bacteria and fungi (Scholar and Pratt, 2000). The term antibiotic refers to a drug that cures infections caused by bacteria. An antibiotic has the ability to either kill other bacteria (bactericidal) or inhibit the growth of other bacteria (bacteriostatic activity) (Walsh, 2003). Antibiotics are very important and widely used in the prevention and treatment of infectious diseases (Bentley, 2000).

Sir Alexander Fleming discovered the first natural antibiotic used in medicine in 1928 in London and used during the Second World War, when he observed that the mould *Penicillium chrysogenum* produced a substance able to kill *Staphylococcus sp*. This is now known of as penicillin (Houbraken *et al.*, 2011). Following the discovery of penicillin, many more antibiotics were discovered and developed rapidly. This resulted in an ability to cure many infectious diseases (Fleming, 1929).

Once antibiotics started being used regularly, resistant microbes quickly appeared due to incorrect and over-usage. Over-prescription and the non-exclusive usage of antibiotics for only bacterial infections remains a global problem in modern health care, as well as in agriculture (Davies *et al*., 2010, Fleming, 1944, Frearson and Wyatt, 2010, Harbarth *et al*., 2015).

Many scientists are still constantly searching for novel antibiotics (Wain *et al.*, 1997). A potential source of novel antibiotics is *Streptomyces* bacteria (Procopio *et al*., 2012, Shlaes *et al.*, 2004, Wright, 2010).

1.2 *Streptomyces*

Streptomyces bacteria are found naturally in the soil (Zhou *et al*., 2011). They belong to the Actinomycete class (Aigle and Corre, 2012, Anné *et al.*, 2012, Hindra *et al.*, 2014, Watve *et al.*, 2001). They are Gram- positive and characterized by the high G+C content in their DNA and the erection of aerial hyphae around 0.5–2.0 µm long (Bhave *et al*., 2013).

S*treptomyces* has a complex life cycle which starts with single spores that germinate with the right nutrients present, then grow and branch to form a vegetative mycelium. Thereafter, they will grow upright forming aerial hyphae caused by poor environmental conditions such as reduction in the quantity of nutrients (Flardh and Buttner, 2009). Later, dependence on low nutrients (such as high mineral composition, neutral pH) within a given growth area leads to the formation of spores enabling survival , the mature spores are released to start the life cycle again (Flardh and Buttner, 2009, Hodgson, 2000, Hopwood, 1999, Zhou *et al*., 2011) **(Figure 1)**.

It has been well-known that sporulation coincides with biosynthesis of antibiotics such as actinorhodin in *Streptomyces coelicolor* A3(2) (Slama *et al.*, 2014, Watve *et al*., 2001). These bacteria have the ability to produce a large number of unique bioactive secondary metabolites that are not essential for growth but have a wide range of pharmacological and agricultural applications. Examples of these antibiotics include streptomycin (Distler *et al*., 1987), chloramphenicol (Akagawa *et al.*, 1975), tetracycline (Procopio *et al.*, 2012) and neomycin (Dulmage, 1953), as well as antifungal agents for example nystatin (Brautaset *et al*., 2008), as well as anti-parasitic such as avermectins (Fisher and Mrozik, 1992), antitumor drug, antiviral, anti-hypertensive and immunosuppressant agents (Demain and Vaishnav, 2011).

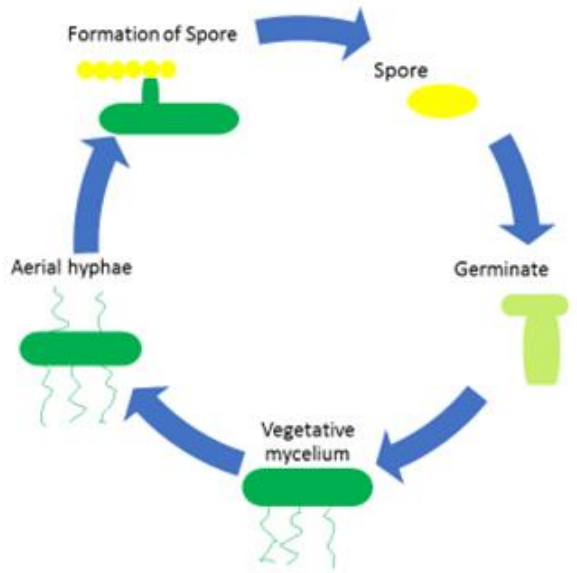


Figure 1: The life cycle of Streptomyces , picture adapted from (Flardh and Buttner, 2009).

The first antibiotic isolated from *Streptomyces* species suitable for human medicine with a broad spectrum of activity was the aminoglycoside streptomycin. It was discovered by Waksman's group. Isolated from *S. griseus* in 1944 and used in the treatment of tuberculosis. It targets and inhibits protein synthesis in *Mycobacterium tuberculosis* (Finken *et al.*, 1993, Kincade *et al.*, 1948, Singh and Mitchison, 1954, Waksman *et al*., 1946).

After the discovery of streptomycin, there was a large number of newly discovered important antibiotics suitable for human medicine found between 1944 and 1960, also referred to as the "the golden age of antibiotics" (Procopio *et al.*, 2012). *Streptomyces* species are able to produce many diverse bioactive secondary metabolites (Nett *et al*., 2009). They are natural products not typically essential for growth. Some of these molecules provide a defence mechanism to fight other bacteria that are competing for resources and many can therefore be used as antibiotics **(Figure 2).** Approximately two thirds of clinically useful antibiotics originate from *Streptomyces* species (Kieser, 2000, Procopio *et al.*, 2012, Ventura *et al.*, 2007).

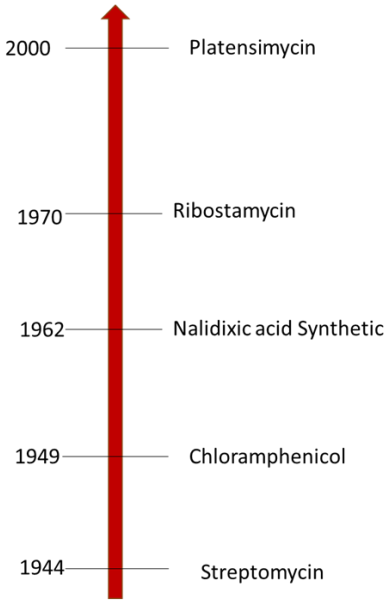


Figure 2: An example antibiotics produced by *Streptomyces*, the Image adapted from (Procopio *et al*., 2012).

Streptomyces coelicolor A3(2) has been a model organism, easily recognisable due to the colour of the pigments of some compounds produced by this organism such as actinorhodin (blue) and prodiginines (red).

There are five compounds that were found to be produced before its genome was sequenced: calcium-dependent antibiotic (CDA), grey spore pigment, prodiginines (Red), actinorhodin (Act), and methylenomycin (Mm) compounds (Bentley *et al*., 2002, Liu *et al.*, 2013).

S. coelicolor A3(2) genome was one of the first sequenced bacteria published. This bacterium has a single linear chromosome around 8 667 507 base pairs (bps) which contain 72 % GC. The genome also contains two extra-chromosomal plasmids; the linear SCP1 around 365,000 bps which contain 69 % GC (Bentley *et al*., 2002, Bentley *et al*., 2004) and the circular SCP2 around 31,000 bps and contain 72 % GC (Bentley *et al*., 2002, Haug *et al*., 2003). The genome contains around 25 cryptic biosynthetic gene clusters predicted to be involved in secondary metabolite biosynthesis, many of the gene clusters were under further investigation but they could not be expressed in laboratory growth conditions and sometimes needed either an inducer molecule or

very specific environmental conditions to induce biosynthesis of the natural product by switching on the silent cryptic gene cluster (Bentley *et al.*, 2002).

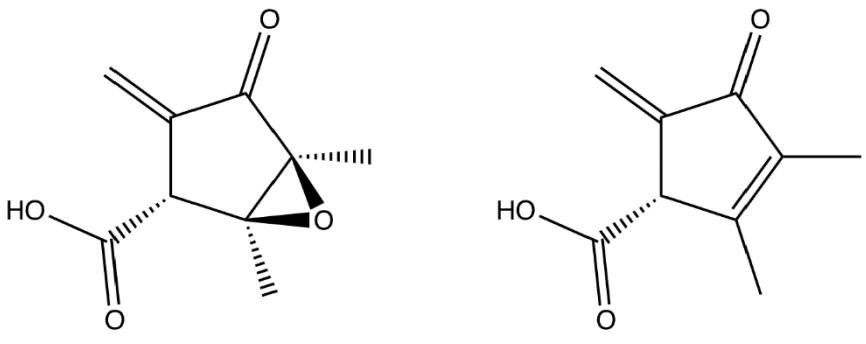
Over the last decade genome mining approaches have been developed to discover new natural products and it has helped identify gene clusters by specific domain analysis and annotation (Aigle & Corre, 2012) more information about the genome mining can be found in the section (1.5 Genome mining, page 22).

1.2.1 Methylenomycin

Both *Streptomyces coelicolor* A3(2) and *Streptomyces violaceoruber* SANK 95570 can produce methylenomycin (Chater and Bruton, 1985) but *S. violaceoruber* was not used in this study.

1.2.1.1 Types of methylenomycins

S. coelicolor A3(2) can produce methylenomycins A and C. The structures of these methylenomycins are illustrated in **(Figure 3)**. These two methylenomycin antibiotics are natural product. Methylenomycin C is the precursor for the cyclopentanoid antibiotic. Methylenomycin A. Acidic shock and limiting alanine conditions were shown to trigger the production of methylenomycin A in *S. coelicolor* A3(2) but it is still poorly understood in terms of the mechanisms involved in responses to pH and heat shock (Hayes *et al*., 1997, Yoon and Nodwell, 2014). Methylenomycin B another cyclopentanoid antibiotic (not shown here) appears to derive from the decarboxylation of methylenomycin A(HANEISHI *et al*., 1974).



Methlenomycin A Methlenomycin C

Figure 3: Structure of antibiotics methylenomycin A and methylenomycin C, picture taken from thesis (Styles, 2016)

1.2.1.2 Methylenomycin Biosynthetic Gene Cluster

The 19 kb *mmy* methylenomycin gene cluster is located on the linear plasmid SCP1 and contains 21 genes. The genes responsible for producing the biosynthetic machinery needed for methylenomycin A and C synthesis form several operons; *mmyBQEDXCAPK*, *mmyTOC*, and *mmmYF*. The *mmfLHP* operon is responsible for the production of enzymes required to synthesise the five methylenomycin furan (MMF) signalling molecules. Also in the methylenomycin gene clusters are *mmfR* and *mmyR*, coding for DNA-binding transcriptional repressors, *mmyJ* and *mmr*, which are involved in methylenomycin resistance, and mmyB, a transcriptional activator methylenomycin antibiotics.(Corre *et al*., 2008),

The structures of these methylenomycin gene clusters are illustrated in **(Figure 4)** (O'Rourke *et al*., 2009). The production of methylenomycin is dependent on the expression of *mmyB.* The MmyB activator binds to the "B-box" sequence to activate expression of methylenomycin biosynthetic genes. However, deletion of *mmyB* renders the bacterium incapable of producing the methylenomycin antibiotics (O'Rourke *et al*., 2009).

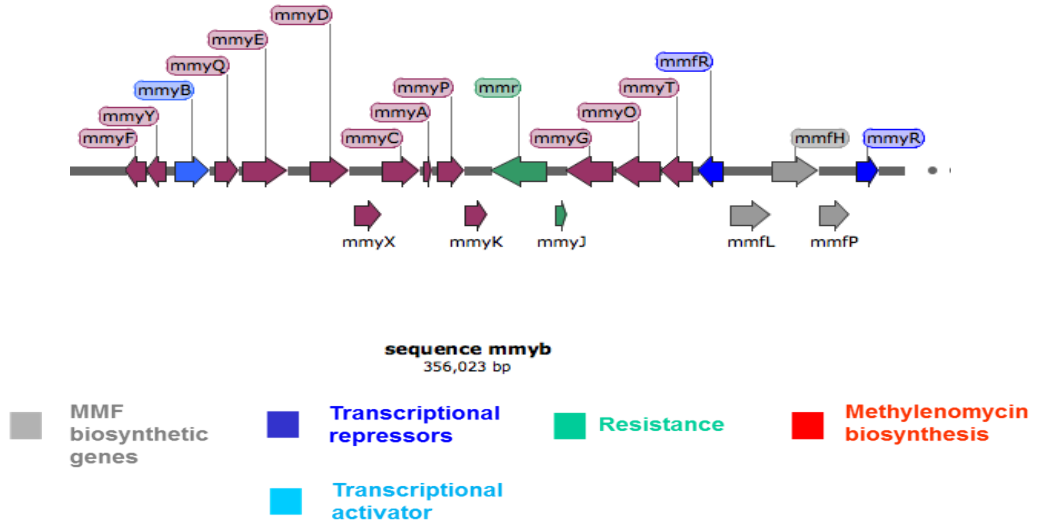


Figure 4: The gene for antibiotic biosynthesis methylenomycin are clustered in *S, coelicolor* A3(2). These set of genes typically include biosynthetic genes, resistance mechanism gene and pathway-specific transcription regulators. Picture adapted from (O'Rourke et al., 2009).

1.3 Transcriptional regulation

Transcriptional regulation is very important step for controlling gene expression. *Streptomyces* regulatory systems often include both transcriptional activators and repressors, signal molecules and sigma factors this can control biosynthesis gene cluster to final production of any natural products (Aigle and Corre, 2012, Jacob and Monod, 1961, O'Rourke *et al.*, 2009).

1.3.1 Transcriptional Repression

Transcriptional repressors are proteins which can bind to specific nucleotide sequences in operator regions located near a promoter and block RNA polymerase. They can often inhibit their own expression (Payankaulam *et al*., 2010). The TetR family of transcriptional regulators mainly act as repressors (Cuthbertson and Nodwell, 2013). It was discovered in *Escherichia coli* regulator of resistance to the antibiotic tetracycline. The TetR repressor protein functions as a homodimer with each subunit consisting of a ligand-binding domain and an N-terminal DNA-binding domain (Cuthbertson and Nodwell, 2013, Ramos *et al*., 2005). Another example for TetR family member transcription repressor is MmfR and MmyR (O'Rourke *et al*., 2009).

1.3.1.1 Transcriptional Regulation by MmfR

MmfR is a member of the TetR family of transcriptional repressors as is its paralogue MmyR. It form a homodimer with a N-terminal DNA binding region and a C-terminal ligand-binding domain (Chater and Bruton, 1983). Both MmyR and MmfR are similar in sequence to the *Streptomyces griseus* DNA-binding protein, ArpA the first studied ArpA repressor in *Streptomyces* (O'Rourke et al., 2009).

MmfR shows 32% identity and 58% similarity to Streptomyces griseus DNA-binding protein ArpA, the first studied ArpA repressor in Streptomyces. ArpA binds to the promoter region of adpA, encoding the pathway-specific activator for overproduction streptomycin biosynthesis (Chater and Horinouchi, 2003).

MmfR is very important repressor of the production of methylenomycin antibiotic in *S. coelicolor* and binds to specific DNA sequence regions known as methylenomycin auto-regulatory response elements (MARE) in the operator region upstream of the *mmy*B gene ; in the presence of MMF, MmfR is released from the MARE sequence (O'Rourke *et al*., 2009).

MmfR is proposed to bind to three different intergenic locations within the methylenomycin cluster, thereby blocking the promoters for mmfR, mmfL, mmyY, mmyB and mmyR cluster biosynthesis and block promoters binding to *mmfR - mmfL* and *mmyY - mmyB* intergenic region (O'Rourke *et al.*, 2009). The binding of MmfR to the three MAREs is illustrated in **(Figure 5).**

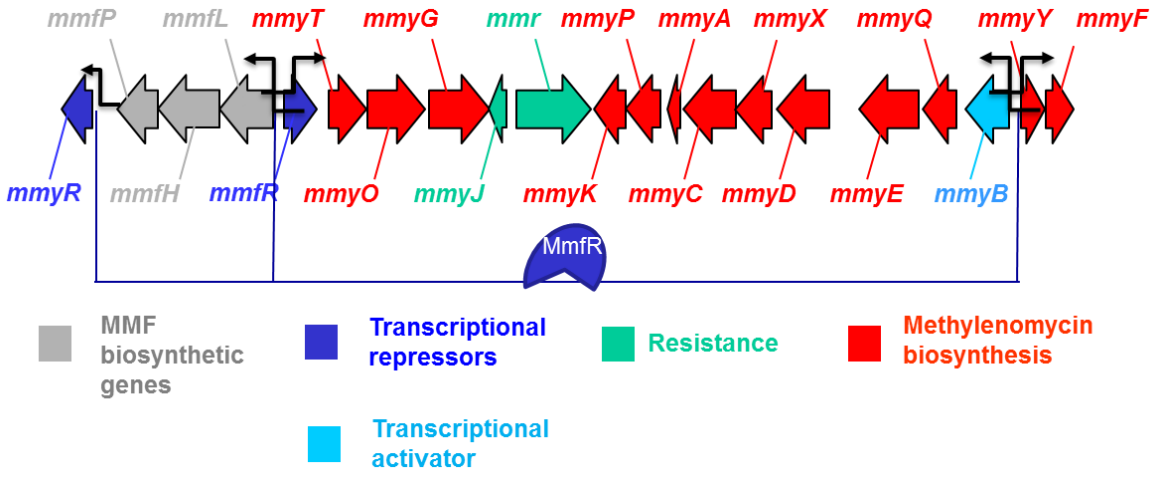


Figure 5: Methylenomycin gene cluster from *S. coelicolor* regulated by transcriptional repressors MmfR and focus on the areas surrounding the MAREs the image adapted from (Corre *et al*., 2008).

1.3.1.2 Transcriptional Regulation by MmR

MmR is also a TetR family member of transcriptional repressors. It has 43% identity and 65% similarity to MmfR (O'Rourke *et al*., 2009).). In the absence of MMF signalling molecules, these repressors prevent expression of the transcriptional activator MmyB via binding to the *mmyB* operator, in presence of MMF, MmfR is released from the MARE sequence and leads to methylenomycin production. For example, the *S. coelicolor* methylenomycin overproducing strain W89 was generated by replacing mmyR with an apr gene (for Apramycin resistance), this confirmed that

MmyR represses Mm biosynthesis. Both MmyR and MmfR have been shown to bind to the promoter region of *mmyB* (Corre *et al*., 2010).

1.3.2 Transcriptional Activators

Transcriptional activators are proteins which can bind to specific sequences in a DNA site located near a promoter then recruit RNA polymerase that will cause stimulation and transcription of nearby genes thus activating the expression of biosynthetic genes cluster then help to produce natural product (Barnard *et al*., 2004). *S. coelicolor* gene cluster often contain transcriptional activators to control the production of metabolite for example *actII-orf4* (actinorhodin), *redD* (streptorubin), and *mmyB* (methylenomycin) (Aigle and Corre, 2012, O'Rourke *et al.*, 2009).

There are two main families of pathway-specific positive regulators that have been described in *Streptomyces*; the numbers of these regulatory genes present vary between BGC's. The first is the SARP (Streptomyces Antibiotic Regulatory Proteins) family; these have been found in a wide variety of BGC's, such as type I and II PKS clusters and non ribosomal peptide synthetize (NRPS) clusters but only in actinomycetes. The second family of regulators is the LAL (Large ATP-binding regulators of the LuxR family) family, found in both proteobacteria and widely in actinomycetes. (Aigle and Corre, 2012).

1.3.2.1 Transcription regulator MmyB

The MmyB protein is found in *Streptomyces coelicolor* A3(2). It is a proposed pathway specific transcriptional activator for the methylenomycin biosynthetic gene cluster.

By knocking *mmyB* out it is hypothesised that this will prevent the expression of the methylenomycin biosynthetic gene cluster and subsequent production of the methylenomycin antibiotic (O'Rourke *et al.*, 2009).

A MmyB-like transcription regulator protein has been characterized and the crystal structure generated for MltR in *Chloroflexus aurantiacus*. It has an N-terminal DNA-

binding domain and a C-terminal ligand-binding module (Xu *et al*., 2012) (more detail in page 12).

Expression of *mmyB* is repressed by the transcriptional repressor MmfR but upon the addition of a methylenomycin furan (MMF), which is a hormonal regulatory system widely found in *Streptomyces* species (belonging to the group of 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs) that are structurally distinct from the GBL family (Corre *et al.,* 2008). MMF is able to make a conformational change of MmfR and MmR then these repressors are released from the DNA from the *mmyB* promoter site. The *mmyB* is able to express after the removal of repression at the MARE (O'Rourke *et al.*, 2009), the resulting MmyB protein then binds to a pseudo-palindromic sequence called the B-box (16 bps sequence which is located near *mmyB* - *mmyY* intergenic region and promoter of *mmyT*) in the methylenomycin cluster and help to recruit RNA polymerase then activates expression of the methylenomycin biosynthetic genes cluster. These are illustrated in (**Figure 6)** (O'Rourke *et al*., 2009).

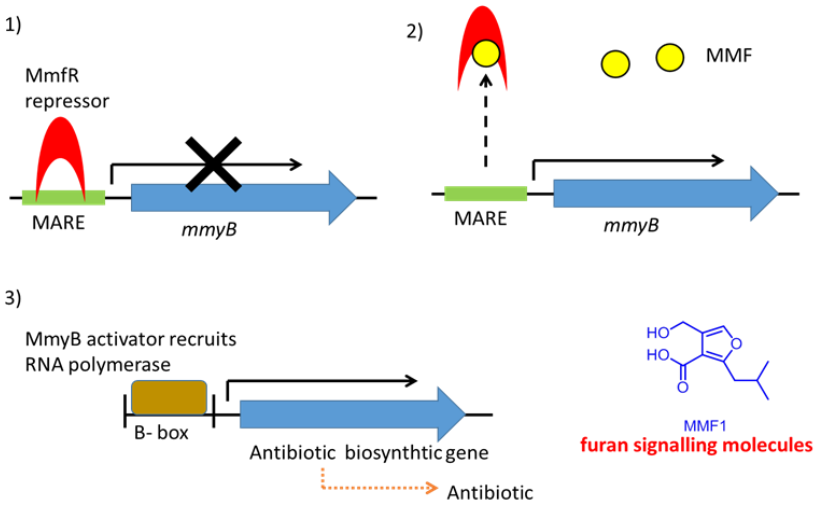


Figure 6: Proposed mechanism by which the pathway-specific transcription activator MmyB controls production of the methylenomycin (Mm) antibiotics in *S. coelicolor*. In absence of methylenomycin furan inducers, the MmfR repressor binds to a specific DNA sequence Methylenomycin Auto-regulatory Response Element (MRAE) in the operator region upstream of the *mmy*B gene (1). In presence of MMFs the MmfR is released from the MARE sequence and *mmy*B is expressed (2). The DNA-binding protein MmyB can then bind to B-boxes and recruit RNA polymerase thus activating the expression the antibiotic biosynthetic genes (3) the Image adapted from (O'Rourke *et al*., 2009).

1.3.2.1.1 MmyB homologues in *S. coelicolor* A3(2)

S. coelicolor has 16 proteins highly similar to MmyB each of them responsible for controlling different pathways, these protein may be involved in promoting biosynthesis of other natural product **(Table 1)** (Xu *et al*., 2012). The MmyB alignment is reported below in **Figure 7.**

Table 1: A Protein Blast was carried out and revealed the presence of conserved MmyB-like in *S. coelicolor* species (Xu *et al*., 2012).

Protein names	Percentage identify/ similarity and over number of amino acids (compared to MmyB)	Total number of amino acids
SCO2537	43/58 over 255	294
SCO0110	37/51 over 263	297
SCO4944	37/54 over 275	291
SCO7706	37/50 over 257	284
SCO0307	37/53 over 246	295
SCO0891	36/52 over 255	279
SCO0236	33/46 over 262	290
SCO2501	37/54 over 234	298
SCO7817	34/50 over 214	280
SCO7767	32/34 over 252	275
SCO4680	37/49 over 216	296
SCO0233	34/44 over 252	279
SCO7140	36/49 over 152	276
SCO6926	29/43 over 183	274

```
SCO6926    -------mcgaikdgggegestgigallrvfraaag-ttlg-------------rp-ilq   38
SCO0891    ----------------mtatefgralrlrrdrvspeaag----lpaggh-rraaglrr   37
SCO4680    ----------------mkyaelgsflrsrrerirpadvg----lpagpr-rrvpglrr   37
SCO7817    ----------------------madflrhrrealrpedvg----lspgar-rrarglrr   32
SCO0233    ----------------midrsglaqflrhrrealqpedvg----lprgrr-rrtsglrr   38
SCO7140    ----------------madraalaaflrarrealqpedvg----lprgrr-rrtgglrr   38
SCO7767    ----------------mssqeladflrrrredlrpedvqaettlppsrrarrtpglrr   42
SCO0236    --------------mkdesgnrlgsylrarrelitpaqag----ippggn-rrvpglrr   40
SCO7706    ----------------mddnhlgeflrarraglrpqdvn----mashgl-rrvaglrr   37
SCO4944    -------------dgqldrraelseflrtrrarlkpedvg----lesygrqrrvpglrr   42
SCO0307    ----------------msselgdflkarrrelspptag----lpadgr-rrvpglrr   36
SCO0110    -------------mptntnraelrdflrsrrarvspedvg----ipatv-rrrtpglrr   41
SCO2501    mtgretavqspprdtgseirrhelaaflrsrreriapeqvg----lprgr-rrrtpglrr   55
MmyB       -masvdgvakdstvcspkrqrealrhflrsrrarlspddvg----llatg-rrhtpglrr   54
SCO2537    -------mitragtagrdrrrselreflmsrrarvspaevg----lpdggarrrtpglrr   49
                         :  *  *                  : :

SCO6926    revadalhrserwyrdleggvitrpltrheldtigtllgldrvqrralflvsnggglssp   98
SCO0891    eelallagisvdyvtrleqgraanps-aqvvealvralrlpaeerahlfrlaglapp-gp   95
SCO4680    eevahlagasvdyyneleргagsqps-eqmlaalaralrlsaderdylyrladrpvp-vp   95
SCO7817    eevaalalmstdyytrleqrrgpqps-ermldslaralrltraerdylyrvaghnap-as   90
SCO0233    eevaalchmstdyyarlerergpqps-qqmivsiaqglhlslderdhlfrlaghtpp-pr   96
SCO7140    eevaalcdmsvdyysrleqprgphps-eqmltsmarglrlsleerdllfqlaghalp-rr   96
SCO7767    eevaalaqvsvsyyerleqaraprps-pqvlsalatalqltdaerdhlarlagqvlp-ae   100
SCO0236    eevallagispdyylrlergrdknps-pqvleslarvlqlddiertyllglaaarpr-ap   98
SCO7706    eevavlagvnadyytrleqgrerhps-pqvldalgralrldpearahlhrlagvspa-gr   95
SCO4944    eelaqlagvsvayytrleqgngqnvs-aevldaiaralrltdaeqahlthlarpkqq-rr   100
SCO0307    eevavlasispghytrleqgrrr-as-epvldalaralrlsaderaylfelsgkdag-rp   93
SCO0110    eevaqlagvgitwytwleqgrpinvs-sqvldavartlildaaerdhlyrlaevpqp-pv   99
SCO2501    eevaqlsavgvtwytwleqardiqvs-vqvldalartllldpterahlfqlagsvdp-tp   113
MmyB       eevaviagvsaswytwleqgrdikvs-dgvlnaisqalrlddterahlyrlagvnpp-qs   112
SCO2537    eevavlagvgaswyqwleqgrdisvs-pqvldavgrvlrlsnterrhlyvlaglnpp-aa   107
           .*:*    .     **          : ::   * *    :  *  ::

SCO6926    e---tqeppriside-lrlldqqpfpayvidatwnvlavntsmaalfpwst---apganl   151
SCO0891    e----avpayippsvhrlldrlagtpvavydamwtlllanppyaalmgdpsewhgperng   151
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SCO7767    n---dgtpehvpedaqqllgrldgipayivndrqdivawnaaaaalitdfsrltpdernl   157
SCO0236    r---rkrpehvparvhell-ahlpipafvegrafdvlasnpmavalsprlrp----gqnr   150
SCO7706    d--slhatervgpalrqlmdgyahtpafvmsrtldvlaanaladalyapftp----adnl   149
SCO4944    ragaargkqpvrvallqlldsidtvpayvsgrrseilawnrmaaalfgdwgklpaaernw   160
SCO0307    l---grpgrqvrpqlrrlddlthtpalvlgrctdilawnppaaalftdfallsrdernf   150
SCO0110    i--sdppt-elpghldtvlealdplpamlvdartdvlrwnrayaalhpalvsappgkrnt   156
SCO2501    a--tdcpa-itp-avralleqfepypacvqnsrydilahnrtygllcdldavppedrnc   169
MmyB       v--patagqtetsrlqlivdgwlpapafvvdrywntlaanqaars---algvgag-dqny   166
SCO2537    e--vepskrdmceglrrlidtwmpypahimdryyncvlyndaaat---vlgmrpettwnc   162
                     ::     *. :     : .             .
```

Figure 7: Amino acid sequence alignment of MmyB-like proteins from *S. coelicolor*

1.3.2.1.2 Structure of MmyB-like transcription regulator (MltR)

The structure of the MmyB-like transcription regulator (MltR) has been discovered in *Chloroflexus aurantiacus* which is a Gram-negative bactrium. It is homolog of the methylenomycin activator MmyB. The crystal structure of transcriptional regulator MltR has been determined by X-ray crystallography and has been shown to form a complex with fatty acid (meristic acid). MltR consists of (Xenobiotic Response Element) Xre-type N-terminal DNA-binding domain and a C-terminal ligand-binding module that is related to the Per-Arnt-Sim (PAS) domain **(Figure 8)** (Xu *et al*., 2012).

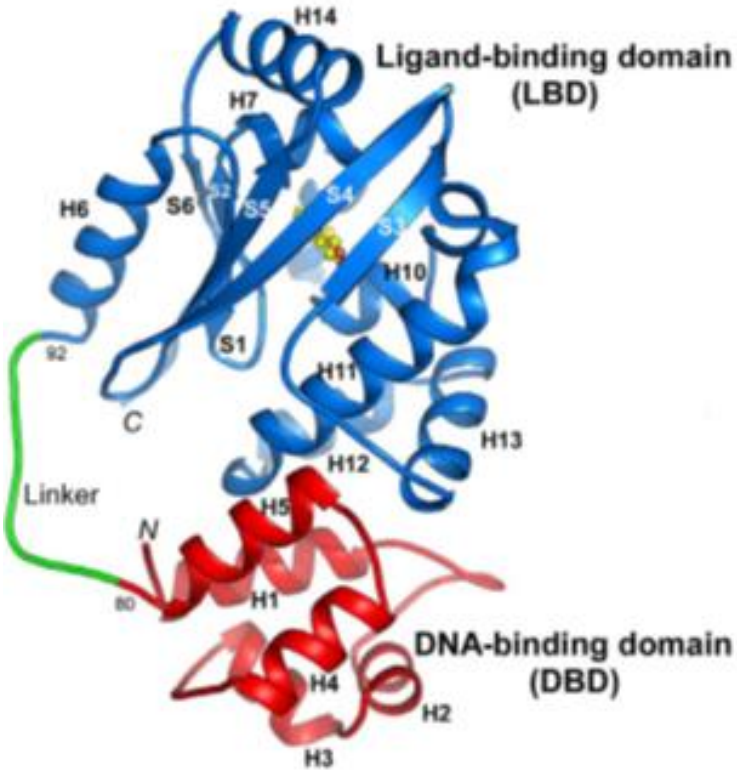


Figure 8: Structure of the MmyB-like transcription regulator (MltR) from *Chloroflexus aurantiacus*. (Xu *et al*., 2012) (A)

1.4 Natural products

The story of bioactive natural products started more than 80 years ago. Natural products refer to chemical compounds or is any substance that derived from the living organisms such as plants, animals and microorganisms which able to inhibit the growth bacteria and even to destroy other microorganisms (Berdy, 2005).

Natural products, which are also called bioactive secondary metabolites, are known to include a wide range of antibiotic and they are not essential for bacteria growth (Procopio *et al*., 2012).

It is important to note that not all organisms produce their full range of clinically useful natural products and secondary metabolites under lab conditions. (Bibb, 2005, van Wezel and McDowall, 2011). Only a few organism produce substances that are useful in research, these are known as producers. A number of them have been used to develop new drugs to combat infection diseases and cancer. Apart from their application in medicine, the natural products have also been used in the agricultural sector (Clark *et al.,* 2013).

Natural products can be developed synthetically to produce more useful product with less toxic effects. Normally some bacteria can switch on the biosynthesis of secondary metabolites that they need in response to specific extracellular stimuli normally not present in laboratory conditions such as particular environmental conditions or inducer molecules for natural product biosynthesis to be switched on (van Wezel and McDowall, 2011, Zhu *et al.*, 2014).

So it is very important to understand the regulation of a gene cluster order to carry out its genetically manipulation or introduction it into a heterologous expression host (Gomez-Escribano *et al*., 2014) Some of the best sources of antimicrobial agents are secondary metabolites produced by *Streptomyces.* The new strategies for the isolation of new natural products are described in the (**Figure 9)** (Aigle and Corre, 2012).

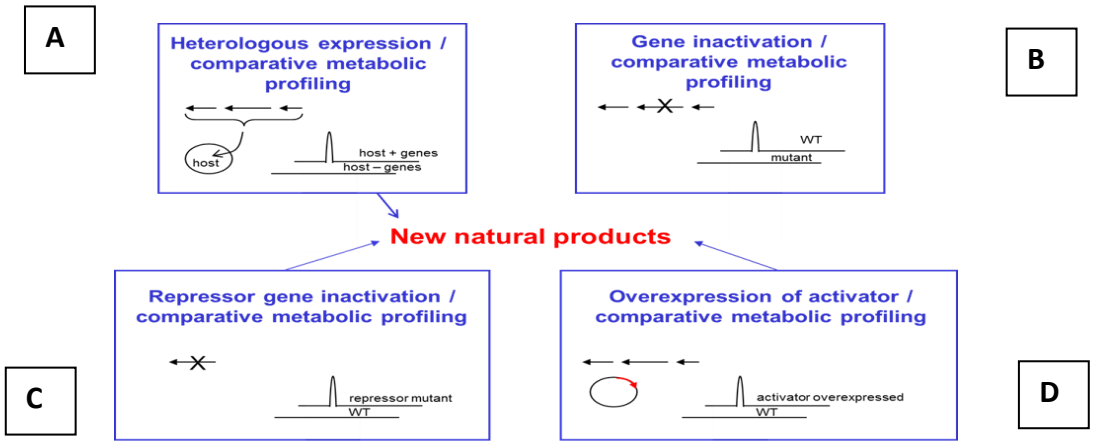


Figure 9: New natural product discovery relies on, heterologous expression of putative biosynthetic gene cluster in heterologous hosts (A). The biosynthetic gene cluster responsible for not producing the novel natural compound can be confirmed by inactivation the essential gene within the biosynthetic pathway (B). Inactivation of repressor gene enables the switching on silent natural products gene clusters (C), in addition, adding transcriptional activators can activate silent natural product gene clusters (D) Image adapted from (Aigle and Corre, 2012).

1.5 Genome mining

Genome mining is important to study because there are a lot more useful natural products yet to be discovered (Lerner *et al.*, 2007). Genome mining refers to searching an organisms genome for genes that encode enzymes thought to be involved in the biosynthesis of natural products, often via the comparison to known motifs from homologous proteins in other organisms (Challis, 2008).

Computational software methods are rapidly advancing as an approach for annotation of genomic data (Weber *et al.*, 2016) Examples of online resources includes the widely used the Antibiotics and Secondary Metabolite Analysis SHell (antiSMASH) (Blin *et al.*, 2017, Weber *et al.*, 2015). These resources are also useful for novel natural product discovery. Bioinformatic tools such as National Centre for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) are used to compare known motifs from homologous proteins from different organisms. Genome sequences can also be analysed by using bioinformatics tools to identify the biosynthetic gene clusters for the natural product of interest (Challis, 2008, Lerner *et al.*, 2007).

Nowadays, tools like antiSMASH and BLAST could be used to technically identify whole biosynthetic gene clusters the for analysis of secondary metabolites (Blin *et al.*, 2013, Medema *et al.*, 2013).

Bacterial genome mining has revealed a vast untapped reservoir of novel bioactive products (Bentley *et al.*, 2002). Numerous cryptic gene clusters predicted to direct the biosynthesis of novel natural products, in particular antibiotic, have indeed be identified in the genome *Streptomyces* bacteria (Corre *et al*., 2008). However under laboratory conditions, many of these cryptic gene clusters are not expressed, it needs very specific environmental conditions necessary to activate the production of the compounds (Corre and Challis, 2007). Hence a new approach for antibiotic discovery that aims to overcome these challenges can be developed by exploiting pathway-specific transcriptional activators (Laureti *et al.*, 2011). Genome sequence analysis has facilitated the identification of additional BGC's that were previously unknown; these include those encoding non-ribosomal peptide synthetises (NRPSs), polyketide synthases (PKSs) and terpene synthases (Rutledge and Challis, 2015).

1.6 Hypothesis statement

The main hypothesis of this project as follow:

- Could we turn on silent biosynthetic pathways by overexpressing *mmyB* and *mmyB*-like genes?
- Would this novel strategy result in the overproduction and discovery of new natural products?

1.7 Aims

- To clone *mmyB* and a series of *S. coelicolor mmyB*-like pathway-specific transcriptional activators under the control of a *Streptomyces* constitutive promoter.

- To introduce these vectors, using intergenic conjugation in *Streptomyces coelicolor*.

- To analyse the expression profile of *mmyB*-like genes and that of putative biosynthetic genes located next to the *mmyB*-like regulatory genes using RT-PCR.

- To test the antibiotic activity of metabolic extracts where *mmyB*-like genes have been overexpressed versus negative controls.

- In collaboration with colleague to compare the metabolic profiles of the engineered *Streptomyces* strain using LC-MS analyses and possibly isolate and elucidate the structure of novel antimicrobials using additional analytical chemistry techniques (mass spectrometry and NMR spectroscopy).

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Microbial strains

Table 2: list of microbial strain used in this study

Strain	Uses/ Genotype	Source
Escherichia coli DH5α	General cloning host. *fhuA2 lac(del)U169 phoA glnV44 80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
Escherichia coli Top10	Strain used in antimicrobial assay General cloning host. *F- mcrA Δ(mrr- hsdRMS-mcrBC) 80dlacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara, leu)7697 galE15 galK16 rpsL(StrR) endA1*	Invitrogen
Escherichia coli ET12567/pUZ8002	Non- methylating host for the transfer of DNA into *Streptomyces* species. ET12567: *dam- dcm- hsdS cat tet* pUZ8002 plasmid: *tra neo RP4*	JIC, Norwich, UK
S. coelicolor W81	SCP1 was deleted *mmyLPH*	C. Corre group
S. coelicolor+pJH2	*mmyB* ligated in pOSV556 integrated in *S. coelicolor* W81	Generated in this project
S. albus+pJH2	*mmyB* ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pJH2	*mmyB ligated in pOSV556 integrated in S. xanthocidicus*	Generated in this project
S. coelicolor M145	*S. coelicolor* SCP1-, SCP2 -	JIC, Norwich, UK
S. coelicolor M145+pJT1	*sco*6926 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus+pJT1	*sco*6926 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pJT1	*sco*6926 *ligated in pOSV556 integrated in S. xanthocidicus*	Generated in this project

S. coelicolor M145+pVN3	*sco*0110 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus M145+pVN3	*sco*0110 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. coelicolor M145+pHAM3	*sco*7140 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus M145+pHAM3	*sco*7140 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pHAM3	*sco*7140 ligated in pOSV556 integrated in *S. xanthocidicus*	Generated in this project
S. coelicolor M145+pNBH3	*sco*7706 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus +pNBH3	*sco*7706 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pNBH3	*sco*7706 ligated in pOSV556 integrated in *S. xanthocidicus*	Generated in this project
S. coelicolor M145+pWAL3	*sco*0236 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus +pWAL3	*sco*0236 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pWAL3	*sco*0236 ligated in pOSV556 integrated in *S. xanthocidicus*	Generated in this project
S. coelicolor M145+pAMR3	*sco*7817 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus M145+pAMR3	*sco*7817 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pAMR3	*sco*7817 ligated in pOSV556 integrated in *S. xanthocidicus*	Generated in this project
S. coelicolor M145+pAML3	*sco*4680 ligated in pOSV556 integrated in *S. coelicolor* M145	Generated in this project
S. albus M145+pAML3	*sco*4680 ligated in pOSV556 integrated in *S. albus*	Generated in this project
S. xanthocidicus+pAML3	*sco*4680 ligated in pOSV556 integrated in *S. xanthocidicus*	Generated in this project
Bacillus subtilis 168	Strain used in antimicrobial assay	Hodgson Group, University of Warwick, Coventry, UK
Staphylococcus aureus	Strain used in antimicrobial assay	Wellington Group, University of Warwick, Coventry.

2.1.2 Plasmids

Table 3: plasmids used in this study

Plasmids	Properties	Source
pOSV556	*ampR, hygR, ermE*, oriT , int pSAM2.*	Pernodet Group, University of Paris-sud, France
pJH2 (pC-0015)	*mmy*B gene ligated in pOSV556,cloned into the *Hind*III and *Pst*I sites.	This study
pJT1 (pC-0016)	*sco*6926 gene ligated in pOSV556, cloned into the *Hind*III and *Pst*I sites.	This study
pVN3 (pC-0017)	*sco0*110 gene ligated in pOSV556, cloned into the *HindIII* and *PstI* sites.	This study
pWAL3 (pC-0018)	*sco*0236 gene ligated in pOSV556, cloned into the *HindIII* and *PstI* sites.	This study
pNBH3 (pC-0019)	sco7706 gene ligated in pOSV556, cloned into the *HindIII* and *PstI* sites.	This study
pHAM3 (pC-0020)	*sco*7140 gene ligated in pOSV556, cloned into the *HindIII* and *Pst1* sites.	This study
pAMR3 (pC-0021)	*sco*7817 gene ligated in pOSV556, cloned into the *HindIII* and *stu1* sites.	This study
pAML3 (pC-0022)	*sco*4680 gene ligated in pOSV556, cloned into the *HindIII* and *Pst1* sites.	This study

2.1.3 Primers

The primers were provided at stock concentration of 100 pmol/µl. Primers were then diluted to a final concentration of 100 mM with sterile dH_2O and stored at -20 ºc.

Table 4: Primers used in PCR to amplify of interest.

Primers number	Primers	Sequences
T01	***mmy*B and mmyB-like genes used in PCR**	Restrictions enzyme *Hind*III and *Pst*1: Red colour and *Stu1* green colour. Extra nucleotides: under line. Ribosome binding side (RBS): orange colour. *mmy*B, *sco*0110 *and sco*6926 genes and other *mmyB*-like: blue colour.
T01-F	*mmyB* Forward	ACTAAGCTTAAAGGAGGGGCGCCATGGCTAGCGTC
T01-R	*mmyB* Reversed	TGTTGCTGCAGCCGCGCAG
T02	***sco*0110 gene used in PCR**	
T02-F	*sco*0110 Forward	ACTAAGCTTAAAGGAGGGTGCCGACAAACACGAAC
T03-R	*sco*0110 Reversed	TGTTGCTGCAG TCACAGGTGCGTCGCCTG
T03-F	*sco*6926 Forward	ACTAAGCTTAAAGGAGG ATGTGCGGGGCCATAAAG
T03-R	*sco*6926 Reversed	TGTTGCTGCAGTCACGTTGCTTGGACAGG
T04	*sco*7706 Forward and reversed	F: ACTAAGCTTAAAGGAGGATGGACGACAACCACCTC R: TGTTGCTGCAGTCAATTCCCGGCCGAGTC
T05	*sco*0236 Forward *and reversed*	F: ACTAAGCTTAAAGGAGGATGAAGGACGAATCCGGC R: TGTTGACTGCAGGCTTCCGGAGCGCGGCG
T06	*sco*7140 Forward *and reversed*	F: ACTAAGCTTAAAGGAGGATGGCAGACCGCGCGGCG R:TGTTGCTGCAGTCACGAGACCGGCAGCAAG
T07	*sco*4680 *Forward and reversed*	F: ACTAAGCTTAAAGGAGGGTGAAGTACGCCGAGCTG R*:* TGTTGCTGCAGTCACCCCTGCCGCCTGG
T08	*sco*7767 *Forward and reversed*	F: ACTAAGCTTAAAGGAGGATGGACGACAACCACCTC R: TGTTGCTGCAGTCAATTCCCGGCCGAGTC
T09	*sco*7817	F: ACTAAGCTTAAAGGAGGATGGCCGACTTCCTGCGC

	Forward and reversed	R*:* TGTTGAGGCCTTCACCGCACCTGGTCCTC
T10	*sco*0891 *Forward and reversed*	F: ACTAAGCTTAAAGGAGGATGACGGCTACGGAGTTC R*:* TGTTGCTGCAGCTACTCCACCAGCTCCTG

Table 5: primers for methylenomycin genes used in RT-PCR

Primers number	Gene name	Primers sequences 5' to 3'
H01	*mmy*B	F CAAAGATTCGACGGTGTGCA R GATGACTGCGACTTCCTCAC
H02	*mmy*O	F GATTCGGTGCTTTCCTCTCC R CAGGGTGATACGACGGGTC
H03	*mmy*J	F GATCACGACAGAGCGCAT R CGAGGTGCCGATGTAGTACT
H04	*mmy*D	F CTGTTCCATCGCTCTGTCGA R GATCACCAGGACGTCGACC
H05	*mmy*E	RT-F ACTGTTCGGGCCCTTCAC RT-R CTGTGGATCCGGGGAACTG
H06	*mmy*F	F CCGGAGTGGTCGTGATCA R GGGATGCCGGTGAATTTGTC
H07	*mmy*G	F CACCGAACCGATACGCATC R CATGGTCCGGCACTTTCAC
H08	*mmy*Q	F CATCATCTCCACCCTCGACC R AGATCGACGGGGCTGAATC
H09	*mmy*T	F CTCGGATGGCAGAAGAGACT R TGTGCCCGTAGAACAGGAAG
H10	*mmy*r	F CTGAAGAGTTCGCCCTGCA R TGTCGTGCTTCATTTGACGG
H11	*mmy*P	F CACCACCATGTTCGACTTCC R CGGTCGAGGAACCATCGT
H12	*mmy*A	F GCGATCATCTGCGAACACTT R CTTCGCCGATGGTCCTGC
H13	*mmy*X	F AGGCCTCGTACAACTCCTTC R GACATCTGCGGTGTGAAGTC
H14	*mmy*Y	F GATCATTGAGCTGTGCACCC R AAGTGCTGTGTGGCTTGC
H15	*mmy*C	F AGGACCTGATCGTGATACCG R CGACGGCATGCAGGATCC
H16	*mmy*K	F TCCTGCACGAGTGGATCTAC R CAGGTCGTACGTGTCGAAAG

Table 6: primers for polyunsaturated fatty acid genes used in RT-PCR

Primers number	Gene name	Primers sequences 5' to 3'
R01	*sco*0108	F: ATCAAGGTCCAGTGGCTCG R: GCAGAGCTTTCAAGGACACC
R02	*sco*0110	F: GTGCCGACAAACACGAACC R: GCATCCAGAATCAGCGTACG
R03	*sco*0117	F: ATGGAACTGAAGAACGCGGT R: CCGCGTTGTTGATCAGAAGT
R04	*sco*0124	F: CTGTTACGTCGTCTGCTCG R: GTCTCCACCACCCTCAGC
R05	*sco*0127	F: GGACGGGTTCTGGGAGAA R:GAATCCCTCGACGTACCCC

Table 7: primers for lantibiotic genes used in RT-PCR

Primers number	Gene name	Primers sequences 5' to 3'
B01	*sco*6926	F: CCAGGA CCCAGACCTTCAG R: TGGGTCAGGATCGTCATGC
B02	*sco*6927	F: ATGGACGTCGGCCAGATAG R: CTTGTTCAGGGCGACGGT
B03	*sco*6932	F: ATGCAGAACGACGAATTCCG R: TCAGGCCACGTTGGTG
B04	*sco*6933	F: ATCCTGGGAGTCACGGGAAT R: TGACGGCATATCTCGGTTCA

2.1.4 Molecular microbiology material used

Table 8: Materials used

supplier	Material
Fermentas	Spectra™ Multicolor Low Range Protein Ladder
GE Healthcare	Ni Sepharose™ 6 Fast Flow
Invitrogen	Quick Gel Extraction and PCR purification combo kit.
	T4 DNA ligase
New England Biolabs (UK)	NEB 5-alpha Competent *Escherichia coli*
	*Hind*III and *Pst*I
Roche Diagnostics	Expand High Fidelity PCR System
Sigma Aldrich	Primers
	Antibiotics; apramycin, ampicillin, kanamycin and chloramphenicol
Thermo Fisher Scientific - Fermentas	FastRuler™ DNA ladders
	GeneJET™ Plasmid Miniprep kit
	Phusion High Fidelity DNA polymerase
	Restriction enzymes and buffers
	Routine PCR Taq DNA polymerase kit

2.1.5 Equipment used

Table 9: Equipment used

Supplier	Equipment	
	Name	**Type**
Beckman Coulter	Centrifuge	Avanti j-25
Bio-Rad Laboratories Ltd	Vertical electrophoresis system	Mini-PROTEAN® Tetra Cell, 1.0 mm gel
	Horizontal electrophoresis system	Wide Mini-Sub cell GT cell
Eppendorf	Microcentrifuge	5424 R
	PCR machine	Mastercycler Nexus
	PCR machine	Mastercycler ep*gradient*
Fisher Scientific	pH meter	Acument basic AB15
	NanoDrop	NanoDrop 2000 Spectrophotometer
Grant	Water bath	JB1
Greiner Bio-One Ltd	White tissue culture 96-well plates	Polystyrene, sterile
Hettich	Centrifuge	Rotina 46R
INFORS HT	Incubator shaker	Multitron
New Brunswick Scientific	Incubator shaker	C24

2.1.6 Buffer recipes

Buffers for preparation of competent cells:

TfbI solution	30 mM K-acetate
	50 mM $MnCl_2$
	100 mM KCl
	10 mM $CaCl_2$
	15% glycerol
	pH 7.4
TfbII solution	10 mM Na-MOPS
	75 mM $CaCl_2$
	10 mM KCl
	15% glycerol
	pH 7.4

2.1.7 Culture media recipes

Media

Table 10: Media used

LB media	**LB agar**
10 g tryptone	10 g tryptone
5 g yeast extract	5 g yeast extract
10 g NaCl	10 g NaCl
Make up to 1 L with distilled water	15 g agar
pH 7.0	Make up to 1 L with distilled
	pH 7.0
Mix together and then autoclave	Mix together and then autoclave

SFM (soya flour mannitol)

400 mL tap water

8 g bacto-agar

8 g soya flour

8 g mannitol

Mix together and then autoclave

2XYT broth

16 g tryptone

10 g yeast extract

5 g NaCl

1 L water

Mix and autoclave

SMM agar (Supplemented minimal media)

2 g Casaminoacids

5.73 g TS Buffer

15 g Bacto- agar

1000 mL distilled water

pH=7.2

SMM broth (Supplemented minimal media)

2 g Casaminoacids

5.73 g TS Buffer

1000 mL distilled water

pH=7.2

Add the following after autoclaving per litter

10 mL $NaH_2 PO_4 + K_2HPO_4$

5 mL $Mg SO_4$

18 mL glucose

1 mL trace element solution

trace element solution

0.1 g $ZnSO_4$
0.1 g $FeSO_4$
0.1 g $MnCl_2$

0.05g $CaCl_2$

0.1 g NaCl

1000 mL distilled water

2.1.8 Antibiotics solution

All antibiotics has been filtered before using then stored in 4 °C

Table 11: List of antibiotics solution used in this project

Antibiotics	Stock Solution (mg/mL)	*E. coli* (µg/mL)	*Streptomyces* (µg/mL)
Ampicillin	100	100	-
Chloramphenicol	25	25	-
Hygromycin	50	-	100
Kanamycin	50	50	-
Nalidixic Acid	25	25	-

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Isolation and purification of plasmids

E. coli DH5α glycerol stocks were plated on Lysogeny Broth (LB) plates with ampicillin selection (100 µg/mL) and incubated overnight at 37 °C. Single colonies were selected and incubated in 10 mL LB with ampicillin (100 µg/mL) as overnight cultures. The vector pOSV556 was isolated using a Gene JET plasmid mini-prep kit (Thermo Scientific United Kingdom) according to the supplier sinstructions with some modification, as described below. The overnight LB cultures were centrifuged for 10 minutes at 4000 rpm at 4 °C. The supernatant was discarded and the pellet re-suspended in 250 µL cell re-suspension solution (CRS) and vortex mixed until completely re-suspended. 250 µL of cell lysis solution (CLS) was added thereafter and mixed by inverting the tube 4 – 6 times (not vortex mixing), before leaving the solution to incubate at room temperature for 2 - 3 minutes. 350 µL of neutralisation solution (NSB) was added and mixed with inverting the tube 4 – 6 times followed by centrifugation at 15,000 rpm for 5 minutes at 4 °C. The supernatant was transferred onto the spin column (provided in the kit) and centrifuged for 60 second at 8,000 rpm at 4 °C. The flow through was discarded and column washed with 550 µL of washing buffer (include ethanol), by centrifugation at 8,000 rpm for 60 second at 4 °C thereafter discarding the flow through. This washing was repeated twice and the column placed

in a flow cabinet to evaporate off any remaining ethanol. The plasmid was eluted from the column into a new sterile micro-tube with 50 - 55 µL of 65 ^{o}C filtered sterile distilled water then centrifuging the column at 15,000 rpm for 120 second. A NanoDrop ND-2000 spectrophotometer (Thermo Scientific United Kingdom) was used to measure the concentration of the purified plasmid.

2.2.1.2 Agarose gel electrophoresis

DNA sample were analysed on 1% agarose gels (AGTC Bioproducts) prepared in 1x Tris- Acetate electrophoresis buffer (TAE) with 5 µL of red gel solution per 100 mL gel. 5 µl DNA sample was mixed with 1 µL from 6x MassRuler DNA loading Dye (Thermo Scientific United Kingdom) before loading in the gel. The gel was then analysed bynelectrophoresis at 100 V for 40 minutes. The DNA size was compared relative to a FastRuler high or middle range DNA ladders (Thermo Scientific United Kingdom). The gels were then visualised with ultraviolet light.

2.2.1.3 Polymerase Chain Reaction (PCR)

PCR was used to amplify the DNA target sequence of gene using two primers:

1. Forward primers '*mmyB, sco*6926 *and sco*0110 were designed with the restriction enzyme *Hind*III sequence,
2. Revers primers '*mmyB, sco*6926 *and sco*0110 were designed with the restriction enzyme *Pst*I sequence. Also other *mmyB*-like genes see **(Table 4).**

The master mix for PCR reaction consisted of a NEB Phusion High Fidelity Polymerase and a fixed molar concentration of $MgCl_2$ in Phusion High Fidelity buffer 5x (include 15mM $MgCl_2$) and 10 mM dNTPs are required for DNA template synthesis **(Table 12).** The reaction was 30 cycles, to gain the amplification of DNA target sequence, run into an automated thermal cycler following the steps reported in **Table 13.**

Table 12: Components and volumes used for PCR reaction to amplify the DNA target.

Component	Volume
High fidelity phusion buffer (5x)	10 µL
dNTP mix. (10 mM)	1 µL
Primer forward-*Hind*III	1 µL
Primer reverse –*Pst*I or using *Stu*1	1 µL
Templet (100 ng/µL)	0.5 mL
Dimethyl sulfoxide (DMSO) (100 %)	2.5 µL
Phusion High Fidelity-DNA Polymerase (5U/µL)	1 µL
Sterile distilled water up to 50 µL	34 µL
Total	50 µL

Table 13: PCR cycling programme used.

Cycle	Temperature	Time	Total cycles
Initial denaturation	98 ^{o}C	2 minutes	
Denaturation	98 ^{o}C	45 seconds	30 cycles
Annealing	55 ^{o}C	45 seconds	
Extending	72 ^{o}C	1 minute	
Final extending	72 ^{o}C	15 minutes	
Cooling down	4 ^{o}C	∞	

2.2.1.4 Purification of amplification products from the agarose gel

The PCR amplified band on an agarose gel was purified by using the GeneJET gel extraction kit (Thermo Scientific United Kingdom). After cutting the DNA band of

interest under UV-light with a clean and sharp scalpel, the sliced gel is weighed. The protocol was then followed as outlined in the manufacturer`s protocol.

2.2.1.5 Restriction enzymes used to digest both vector and gene of interest

Both purified gene products and plasmids were digested separately using the restriction enzymes *Hind*III and *Pst*I in two steps, (Thermo Scientific United Kingdom) as follows using supplier instructions with some modification

First step: The construct were digested by incubating 1 µL restriction enzyme *Hind*III with 1 µg of substrate DNA and 5 µL of the relevant 10 x buffers. Sterile distilled water was added up to 50 µL before incubation of digestion mix for 2 hours at 37 °C singly digested plasmid and gene were then purified by using Quick Gel Extraction and PCR purification combo kit (Invitrogen) protocol as outline below:

Add 4 volumes of binding buffer (B2) with isopropanol to 1 volume of the samples. Mix well then transferred into a spin column which has the filter called PureLink spin column filter (provided by the kit) and centrifuged for 60 second for 4000 rpm at 4 ^{o}C discard the flow through and place the column in the same wash tube and wash with 650 µL of washing buffer (W1) (containing ethanol) before centrifuged again at 4000 rpm for 60 second at 4 ^{O}C, discard the flow through and place the column in the same wash tube, then centrifuged again for 10000 rpm for 60 second at 4 ^{O}C to remove any remaining ethanol, discard the flow through then leave the filter spin column fume hood for 5 - 10 minutes to dry all the ethanol , the plasmid and gene was eluted in clean micro tube by added 30 - 35 µL of 60 ^{o}C filtered sterile distilled water then centrifuged at maximum speed (15000 rpm) for 120 second. NanoDrop ND-2000 spectrophotometer (Thermos Scientific United Kingdom) was used to measure the concentration of the cloning and purified plasmid and gene.

Second step: Double digestion was achieved by incubating 1 µL restriction enzyme *Pst*I with 1 µg of singly digested and purified DNA and 5 µL of the relevant 10x buffers (New England Biolabs). Sterile distilled water was again added up to 50 µL before incubation of digests for 2 hours at 37 °C. Plasmid and gene were purified by using Quick Gel Extraction and PCR purification combo kit (As previously described).

NanoDrop ND-2000 spectrophotometer (Thermo Scientific United Kingdom) was used to measure the concentration of the plasmid and gene.

2.2.1.6 Ligation of plasmid and gene by using T4 DNA ligase

After restriction digestion of plasmid and gene the ends were ligated using a molar ratio of 1:3 vector to insert for the indicated DNA sizes. The ligation calculator was used web site NEBio (https://nebiocalculator.neb.com/#!/ligation).

Expanded Formula (required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector lengths).

This reaction was performed by using T4 DNA Ligase (400 u/µL Invitrogen) **(Table 14).** Keep the micro tube incubation overnight at 16 ^{o}C. Followed by the transformation of plasmid in *E. coli* TOP 10.The resultant vector harbouring the gene of interest was named as pJH2, pJT1 and pVN3.

Table 14: Components and volumes were used for ligation between pOSV556 and gene

Components	Volumes
pOSV556	6 µL
Gene interest	2 µL
5x T4 ligase buffer	2 µL
T4	1.5 µL
Distilled water up to 20 µL	8.5 µL

2.2.1.7 RNA extraction and purification

Total RNA was extracted from both *Streptomyces coelicolor and Streptomyces coelicolor* with integrated genes (pHJ2, pJT1 and pVN3) grown in MMS until the optical density at 600 nm (OD_{600}) reached at 1.0 (Van Dessel *et al.*, 2004) using the hot acid-phenol method with some modification (Gilbert et al., 2000). For RNA work, all plastic ware, tips, micro-tube, PCR tubes, pipette tips etc were used RNase-free. The cultures were harvested in RNase free Falcon tubes, centrifuged for 5 minutes at 3500 rpm at 4 ^{O}C. The supernatant was kept for antibiotic assay and the pellet re-suspend with 250 µL solution A which contains both (0.3 M sucrose and 0.01 M

sodium acetate pH=4.5) and vortex mixed until it completely re-suspended. 250 µL of solution B which contains (2% sodium dodecyle sulfate and 0.01 M sodium acetate pH=4.5) was added and then transfer to ribolyser tube then add 500 µL before leaving the solution to incubate at room temperature for 2 - 3 minutes. Cell were lysed by using Habaid Ribolyser (speed 6 for 20 to 40s) each time and the sample keep the on the ice for 1 min, it was then centrifuged for 5 minutes at 15000 rpm at 4 ^{O}C and aqueous phase was transferred to new tube 1.5 ml Eppendorf. 500 µL of phenol was added and incubated 4 min at 64 ^{o}C then direct frozen in dry ice ethanol for 10s and mixed with inverting the tube 4 – 6 times followed by centrifugation at 15,000 rpm for 5 minutes at 4 ^{O}C, transfer Aquose phase to new tube 1.5 ml Eppendorf. 500 µL of phenol- chloroform were added and mixed by vortex for 45s and centrifuged for 5 min at 15,000 rpm at 4 ^{o}C. The nucleic acid was predicated with 60 µL 0.01 M sodium acetate pH=4.5 and 940 µL of 100 % ethanol and keep the tube in – 20 ^{o}C overnight then centrifugation at 15,000 rpm for 30 min at 4 ^{O}C thereafter discarding the supernatant and the pellet should be washed by 70 % ethanol. To remove all ethanol, leave the tube in room temperature to dry for 10 – 15 min. RNA was dissolved in of diethylpyrocarbonate (DEPC) treated water add 50 µL. The quantity and concentration of RNA was determined by spectrophotometrically using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific United Kingdom) by measuring the absorbance ratio between A_{260} and A_{280}. In addition, the RNA was checked by electrophoresis on a 1% (w/v) TAE agarose gel.

2.2.1.8 Genomic DNA degradation

The genomic DNA was removed from the total RNA extract by using RNase-free, DNase I (New-England Biolab), following the manufacturer's instructions.

2.2.1.9 Purification of RNA

To removal of all traces of DNA, RNA was cleaned and concentrated according to the manufacturer's instructions (Zymo Research). Finally RNA cleaned was dissolved in 40 µl of diethylpyrocarbonate (DEPC) treated water.

2.2.1.10 First strand complementary DNA (cDNA) synthesis

RNA was reverse transcribed to first strand complementary DNA (cDNA) using cDNA Superscript II RT. First-Strand Synthesis kit (Invitrogen, UK), according to the manufacturer's instructions. Between 100 and 200 ng of total RNA was used as a template for cDNA synthesis. cDNA formed was mixed with 50 µl of diethylpyrocarbonate (DEPC) treated water, control PCRs were similarly performed with RNA untreated by reverse transcriptase to confirm the absence of contaminating DNA in the RNA preparations cDNA, store it at -20ºC for future use.

2.2.1.11 Reverse transcription- polymerase chain reaction (RT-PCR)

RT-PCR was carried out using a cDNA template to investigate the expression genes form selected antibiotic biosynthesis gene clusters (methylenomycin, lantibiotic, poly unsaturated fatty acid) in *Streptomyces coelicolor* W81and M145. These set of genes typically include biosynthetic genes, resistance genes and pathway-specific transcriptional activators for methylenomycin (O'Rourke et al., 2009) and lantibiotic, poly unsaturated fatty acid. PCR was used to amplify the cDNA target sequence of gene using two designated primers (see previous method for PCR **Table 12 and 13**). Primer sequences are summarized in **Table 5, 6 and 7**. PCR product samples were analysed on 1% agarose gels (AGTC Bioproducts) prepared in 1x Tris- Acetate electrophoresis buffer (TAE).

2.2.1.12 *Streptomyces* genomic DNA extraction

Genomic DNA extraction from *Streptomyces* was done using FastDNA ^{TM} spin kit for soil (Thermo scientific) as outlined in the manufacturer`s protocol with some modification. 20 µL of the bacteria sample was added to Lysing Matrix E tube along with 978 µL sodium phosphate buffers and 122 µL MT buffer before overtaxing for 2 minute. The sample was then centrifuged for 5 minutes at 4000 rpm at 4 ^{O}C and the supernatant was transferred to a clean 2.0 mL micro centrifuge tube and the pellet discarded. This was followed by adding 250 µL of protein precipitation solution (PPS) and mix by using inverting the tube 6 – 10 times (do not use vortex). Tube was then spun down at 15000 rpm for 5 minutes at 4 ^{O}C and the supernatant transferred to a

clean 15 mL tube. Add 1000 µL re-suspend binding matrix to this 15 mL tube. The tube should be inverted for 2 minutes that will help the binding of the DNA then place the tube in a rack for 2 – 3 minutes to allow setting of the silica matrix. Remove and discard 500 µL of supernatant. The re-suspended binding matrix was then transferred into a spin column with a filter (provided by the kit) and centrifuged for 60 seconds at 15000 rpm at 4 ^{o}C and the flow through discarded. The remaining mixture was then added to the spin filter and centrifuge as before, then washed with 500 µL SEWS-M, gently suspending the pellet using the force of the liquid from the pipette tip, then centrifuged for 15000 rpm for 60 second at 4 ^{O}C, discard the flow through, then centrifuged again for 15000 rpm for 2 minute at 4 ^{O}C to remove ethanol, discard the flow through then leave the filter spin column in safety cabinet for 5 - 10 minutes to dry all the ethanol , the plasmid was eluted in clean micro tube by adding 50 - 55 µL of filtered sterile distilled water (was kept at 60 ^{o}C) then centrifugation at maximum speed 15000 rpm for 2 minute. NanoDrop ND-2000 spectrophotometer (Thermo Scientific United Kingdom) was used to measure the concentration of the cloned and purified plasmid.

2.2.2 Transformation

Two different protocols were employed for transformation of *E.coli* with plasmid DNA cells.

2.2.2.1 Preparation of chemically competent *E. coli*

The *E. coli* strain TOP10 is widely used as a host cell for gene cloning. A sterile loop was used to streak *E. coli* TOP10 from frozen stock on the surface of a Luria Broth agar (LB agar) plate (without using antibiotic) and then the plate was incubated for around 17 - 19 hours at 37 ^{o}C. After incubation a single colony was transferred into 10 mL of a Luria Broth (LB). The cells were grown for around 17 - 19 hours at 37 ^{O}C. The following day 1000mL of LB broth was inoculated with 10 mL of the overnight culture and cells were incubated for 2.5 to 3 hours at 37 °C or until the optical density at 600 nm (OD_{600}) reached around 0.4 – 0.5. Thereafter, cells were harvested by centrifugation at 4000 rpm for 10 minutes and then the cell pellet was re-suspended in 30 mL sterile ice - cold Tfbl buffer which was composed of 30 mM K-acetate, 50 mM $MgCl_2$, 100 mM KCl, 10 mM $CaCl_2$ and 15% glycerol, pH 7.4 and incubated on ice

for 30 minutes. The cell pellet was collected by centrifuge at 4000 rpm for 10 minute in 4 ^{o}C and after that the pellet re-suspended in 4 mL TfbII buffer, which was composed of 10 mM Na-MOPS, 75 mM $CaCl_2$, 10 mM KCl and 15% glycerol, pH 7.4, 50 µL of the competent cells were then aliquoted into micro-centrifuge tubes and transferred immediately to -80 ^{O}C or was kept on ice for immediate transformation (Hanahan, 1983).

2.2.2.2 Transformation of chemically competent *E. coli*

3 µl of the ligation product (pJH2, pJT1 and pVN3) were transferred into 50 µl of chemical competent *E. coli* TOP10 strain then mixed gently and incubated on ice for 15 - 20 minutes after which the cells were heat shocked in water bath at 37 ^{O}C for 2 minutes before being immediately transferred onto ice again where they were incubated for 5 minutes. 50 µL of this mixture was then transferred into falcon tube containing 500 µL of LB broth and incubated at 37 ^{O}C for 1 – 1.5 hours with shaking. After that two different volumes were plated on LB media has ampicillin (100 µg/mL) for example 10 µL on one and 460 µL on the other plate. These were then incubated for overnight at 37 ^{O}C. After this, a single colony was picked up using a sterile tip and used to inoculate 10 mL of LB broth medium containing 100 µg/mL of ampicillin before incubation overnight at 37 ^{O}C with shaking 200 RPM. The plasmid DNA was then isolated and purified by using Gene JET plasmid mini-prep (Thermos Scientific United Kingdom). The solution should be run on 1% agarose gel electrophoresis in order to check the size of the expected band.

2.2.2.3 Preparation of electro-competent cells

A sterile loop was used to streak *E.coli* (strain ET12567/ pUZ8002) from frozen stock on the surface of a Luria Broth agar (LB agar) plate used contain kanamycin 50 (µg/mL) and chloramphenicol 35 µg/ml then the plate was incubated for an around 17 - 19 hours at 37 ^{o}C. After incubation, a single colony was transferred into 10 mL of a Lysogeny Broth (LB) with the same selection antibiotics. The cells were grown for around 17 - 19 hours at 37 ^{O}C. The *E.coli* electro-competent cells were prepared by adding 100 µL of an overnight cell culture to 10 ml of LB and incubating until the culture reached an $OD_{600} \approx 0.4$ - 0.5 nm. The culture was subjected to centrifugation

and then the supernatant was removed. The cell pellet was washed two times with 10 mL of sterile ice-old 10% glycerol. Finally, the pellet was re-suspended in 70 µL of 10% glycerol. It then was ready for the electroporation technique.

2.2.2.4 Electroporation

For electroporation, a 70 µL aliquot of electro-competent cells were mixed with 3 µL of plasmid DNA in an electro-cuvette. Cuvettes were pulsed once with a time constant of ~ 23 ms and the following settings: 2.5 kV (voltage), 2.5 µF (capacitance). After electroporation the mixture 1 ml of ice-cold LB was immediately added and the mixture incubated for 1 hour at 37°C with gentle shacking at 210 rpm. The bacterial cells were then grown on LB plates containing kanamycin 50 µg/mL, ampicillin 50 µg/mL and chloramphenicol 35 µg/mL overnight at 37 ^{o}C.

2.2.3 Assessment of the DNA fragments size.

The purified DNA construct ligated into vector was digested (Thermo Scientific United Kingdom) as follows using supplier instructions with some modification using the following protocol:

1. Linearized:

Restriction enzyme *Hind*III	1µL
DNA plasmid	1.5 µg
10X buffer	5µL
Sterile distilled water up to	50µL

The reaction mixture was incubated either in water bath at 37 ^{o}C for 2 hours or overnight at 4 ^{O}C. After incubation period, this reaction solution was run on 1% agarose gel electrophoresis in order to check the size of the expected band.

2. Excised:

Restriction enzyme *Hind*III and *Pst*I	1 µL
DNA plasmid	1.5 µg
10X buffer	5 µL
Sterile distilled water up to	50 µL

The reaction mixture was incubated either in water bath at 37 ^{o}C for 2 hours or overnight at 4^{o}C. After incubation period, this reaction solution was run on 1% agarose gel electrophoresis in order to check the size of the expected band.

2.2.3.1 **Screening the plasmid constructs by PCR**

PCR screening was used to amplify the DNA target sequence gene using two designated primers, the forward primer was designed using a restriction enzyme *Hind*III site and the reverse primer included the site for the restriction enzyme *Pst*I (**Table 6**).

The PCR master mix consisted of a taq DNA polymerase and buffer 10x, 25mM $MgCl_2$ and dNTPs (**Table 15**). The reaction mixture went through 30 PCR cycles, to gain the amplification of DNA target sequence, in automated thermal cycler machine (**Table 16**).

Table 15: Components and volumes were used for PCR reaction to amplify the DNA target.

Component	Volume
Taq DNA buffer (10 x)	5 µL
dNTP mix. (10 mM)	1 µL
forward primer *mmy*B forward-*Hind*III	1 µL
revers primer *mmy*B reverse -*Pst*I	1 µL
Plasmid sample	0.5 µL
Dimethyl sulfoxide (DMSO)	2.5 µL
Taq DNA polymerase	1 µL
$MgCl_2$ (25 mM)	3 µL
Distilled water up to 50 µL	35 µL
Total	50 µL

Table 16: Cycling programme was used for PCR screen DNA gene ligated with pOSV556.

Cycle	Temperature	Time	Total cycles
Initial denaturation	98 ^{o}C	2 Minute	
Denaturation	98 ^{o}C	45 second	30 cycles
Annealing	55 ^{o}C	45 second	
Extending	72 ^{o}C	1 minute	
Final extending	72 ^{o}C	15 minute	
Cooling down	4 ^{o}C	∞	

2.2.3.2 Assessment of the DNA by doing DNA sequencing

Sequencing of specific DNA regions were done by GATC Biotech AC (European Custom Sequencing Center, Gottfried-Hagen-Strobe 20, 51105 Cologne, Germany). The total reaction volume was 10 µL composed of 5 µL of plasmid DNA, 2.5 µL of both primer and 2.5 µL sterile distilled water.

2.2.4 Growth and storage the bacteria

2.2.4.1 Growth *E. coli* bacteria

E. coli cultures were grown at 37 °C using LB for overnight in solid or liquid cultures, shaking at 200 rpm in flasks for liquid cultures.

2.2.4.2 *E. coli* glycerol stocks preparation

E. coli cultures in broth LB media overnight at 37^{o}C and diluted with equal volume with sterile 50% (v/v) glycerol to a final concentration of 25% before being stored at -80 °C.

2.2.5 Growth of *Streptomyces* bacteria

Streptomyces cultures were grown at 30 °C using SFM agar for 6-7 days in liquid or solid cultures, shaking at 200 rpm in flasks containing stainless steel spring for liquid cultures.

2.2.5.1 *Streptomyces* glycerol stocks preparation

Streptomyces glycerol spore stocks were prepared (Shepherd *et al.*, 2010) with some modification. After incubating at 30 °C for six days spores were grown on SFM plates with appropriate antibiotics. Around 3 mL sterile water was added to each plate. A sterile loop was used to free the spores and do suspension. The suspension was filtered through a sterile syringe with non-absorbent cotton wool to remove any agar that had been picked up and pelleted by centrifugation (3,500 rpm for 5 minutes). The supernatant was removed. The spore pellet was re-suspended in the residual liquid exactly 1 ml then add equal volume with sterile 50% (v/v) glycerol to a final concentration of 25% before being stored at -80 °C.

2.2.5.2 Transfer of the plasmid with a gene cassette into *Streptomyces*

The plasmid (pJH2, pJT1 and pVN3) were introduced by transformation into the non-methylation *E. coli* ET12567/pUZ8002. The plasmid was then transferred to *Streptomyces coelicolor* by intergenic conjugation according to standard methods (Kieser, 2000) with some modification as described below.

After incubation a single colony which carried a plasmid was transferred into 10 mL of a Luria Broth agar (LB agar) which contained of ampicillin (100 µg/mL), kanamycin (50 µg/mL) and chloramphenicol (23 µg/mL). The cells were grown for 17 - 19 hours at 37 ^{O}C. The culture was subjected to centrifugation and then the supernatant was removed. The cell pellet was washed two times with 10 mL of sterile LB media to remove antibiotics that might inhibit *Streptomyces* then re-suspend in 500 µL of LB. Add 10 µL of *Streptomyces coelicolor* spores to 500 mL 2XYT, then heat shock at 50°C for 10 min, then allowed cooling. Mix 500µL of *E. coli* ET12567/pUZ8002 cell suspension and 500 µL heat-shocked spores and spin briefly at 5000 rpm for 30 second, re-suspension the pellet in the residual liquid. Serial dilution should be done from 10^{-1} to 10^{-4}. Of each dilution 100 µL was used to inoculate plates on SFM which containing of 10 mM $MgCl_2$ (to help the colonies sporulation) these were then incubated for 18- 20 hours at 30 ^{O}C. The next day, overlay the plate with 1 mL sterile distilled water containing 20 µL naldixic acid (from 25 mg/mL stock) to kill *E. coli*, 25 µL of 100 mg/mL stock ampicillin and 25 µL of 50 mg/mL stock hygromycin to select the *Streptomyces* exconjugants. A spreader was

not used for this, instead the plate was tilted until evenly covered with the antibiotic solution, then wait until the plate have dried and then keep it again in incubation at 30 ^{o}C for 6 days to allow growth of the exconjugants.

2.2.6 Analytical chemistry

2.2.6.1 Organic extraction

Two different protocols were employed for organic solid-liquid and liquid-liquid extraction.

2.2.6.1.1 Organic extraction Liquid-Liquid

Organic Liquid-Liquid extraction means that two liquids are used in the extraction procedure using a separator funnel. The culture was centrifuged around 4,000 rpm for 15 - 20 minutes to remove the bacteria. The liquids must be immiscible: this means that they will form two layers when added together. First of all, using pJH2 integrated into the *S. coelicolor* W81 chromosome, 10 µL were transferred into 50 µL of SMM broth then add equal volume of Ethyl acetate the sample should be acidified to pH 3 with 37% Hcl. The solution should be mixed then a separator funnel used to separate the two liquid layers separate. The organic solvent and water are not miscible with each other. Add the liquid to separator funnel. Shake the funnel vigorously for a few seconds. Put the funnel back into the ring until the layers are clearly separated. While waiting, remove the stopper and place a beaker or flask under the separator funnel. Carefully open the stopcock and allow the lower layer to drain into the flask. The upper layer should separate from the funnel in clean flask, this was labeled and magnesium sulfate added to remove all water before clean filter paper was used to remove all magnesium sulfate. The remaining solvent should be removed by using a rotary evaporator. Add 150 µL DMSO to dissolve the entire component in the flask. The solution was eluted into clean micro tube which is ready to test in antibiotic assay or re-dissolved with HPLC grade methanol and water re-dissolved in 500 µL 50:50 to run in LCMS by using the Agilent 1200 HPLC coupled to the Bruker High Capacity Trap (HCT) Ultra mass spectrometer.

2.2.6.1.2 Organic extraction Liquid-Solid

Organic extraction liquid-solid used agar media, add 30 mL Ethyl acetate. It should be acidified to pH 3 with 37% Hcl, same technique used in Liquid-Liquid but without using magnesium sulfate.

2.2.6.1.3 Liquid chromatography – mass spectrometry (LC - MS)

The LC-MS analysis of any crude extract results to compare of two samples. Organic extracts were filtered on a spin column around 5000 rpm for 5 minutes then injected 20 µL through Agilent ZORBAX Eclipse Plus column C18, 46 x 150 mm, particle size 5 µm connected to Agilent 1200 HPLC coupled to the Bruker High Capacity Trap (HCT) Ultra mass spectrometer, operating in positive ion mode, The solvent and linear gradient profile with a flow rate of 1 mL/minute were used in LC-MS analysis see **Table 17**.

Table 17: Gradient profile for LC-MS analysis

Time (minutes)	**Water + 0.1% formic acid (%)**	**Methanol + 0.1% formic acid (%)**
0	95	5
5	95	5
30	0	100
35	0	100
40	95	5
55	95	5

2.2.7 Antibiotic assay

All media were prepared according to the manufacturer's instructions, and sterilised by autoclaving at 121°C for 15 min. The antibiotic sensitivity assay shows the ability of drug in inhibiting the growth of microorganisms. *Bacillus subtilis, Staphylococcus aureus and E. coli* stocks were plated on LB agar and incubated for around 17 - 19 hours at 37 ^{o}C after incubation a single colony was transferred into 10 mL of a Luria Broth (LB). The cells were grown an around 17 - 19 hours at 37 ^{O}C in a shaking incubator at 200 rpm. The bacteria cells were then prepared for the antibiotic assay by adding 200 µl of this overnight cell culture to 10 ml of LB and incubating until the culture reached an $OD_{600} \approx 0.4$ - 0.5 nm. 50 µL of this bacteria strain culture then added to 1 mL LB broth and used to overlay LB agar plate not containing antibiotic, this was done without using spreader and tilting the plate instead and wait until all the solution has evaporated. The sterile filter paper to 6 mm discs were then placed onto of the bacteria plated before adding 21 µL either direct from culture or from organic extraction liquid-liquid as described previous then incubated overlay LB agar around 18 - 20 hours at 37 ^{O}C.

Chapter 3: Overexpression of the transcriptional activator gene *mmyB* in *S. coelicolor*

The MmyB protein is found in *Streptomyces coelicolor* A3(2). It is a proposed to be a transcriptional activator for the methylenomycin biosynthetic gene cluster. The methylenomycin biosynthetic gene cluster is tightly regulated at the transcriptional level, and is usually not produced and if it is produced, then it is produced in a very small quantity in laboratory growth conditions (O'Rourke *et al*., 2009).

Overexpression of transcriptional activators under the control of *ermEp** (*ermEp** is promoter which originated from erythromycin resistant gene) is expected to unlock the production of compounds from neighbouring cryptic biosynthetic gene clusters. To investigate this, an experiment was performed determine if overexpression of this activator will switched on the methylenomycin biosynthetic genes cluster and express other *mmy* gene to produce the methylenomycin antibiotics illustrated in **Figure 4**. To this end, it is very important to clone the *mmyB* gene in the pOSV556 vector (which has the ermEp* promotor) and then introduce it into *S. coelicolor* W81.

In order to do this, PCR was used to amplify specific the *mmyB* gene. Two primers were designed (*mmyB* forward and reverse) (Primers number **T01 in Table 4**). PCR products were analysed by electrophoresis on a 1% agarose gel in order to verify the size of the construct which yields a band at the expected 994 bps (**Figure 10)**. A NanoDrop ND-1000 spectrophotometer was used to measure the concentration of the *mmyB* construct (273.4 ng/ µl).

The pOSV556 plasmid was isolated from *Escherichia coli* DH5α and purified using a Gene JET plasmid mini-prep kit yielding purified pOSV556 plasmid. A NanoDrop ND-1000 spectrophotometer was used to measure the concentration (963 ng/µl). The plasmid produced a band at the expected 9009 bps on a 1% agarose gel. The plasmid contains ampicillin and hygromycin resistant genes which were used as selection markers for successful transformations when these vectors were introduced into S. coelicolor strains using intergenic conjugation.

This process was facilitated with various genes included in the vector such as:

1. *attP* – which allows integration into *S. coelicolor,*

2. *OriT* – Origin of transfer (allows conjugation and recognition by *Streptomyces* and helps with transfer of genes),
3. Ter – transcriptional terminator (where the mRNA finishes) (**Figures 11A &B).**

The plasmid created by this ligation was termed pJH2 (containing *mmyB* under the control of *ermEp**). Plasmid pJH2 was grown in *E.coli* Top10 and purified using the mini-prep procedure; the agarose gel run following the mini-prep of the construct is shown in **Figure 12**. This construct was then ready to transform into non-methylation *E. coli* ET12567/pUZ8002 host cell (by electroporation) to introduce into *S. coelicolor* W81 because the *S. coelicolor* W81 contains a methyl-sensing restriction system (MacNeil *et al*., 1992).

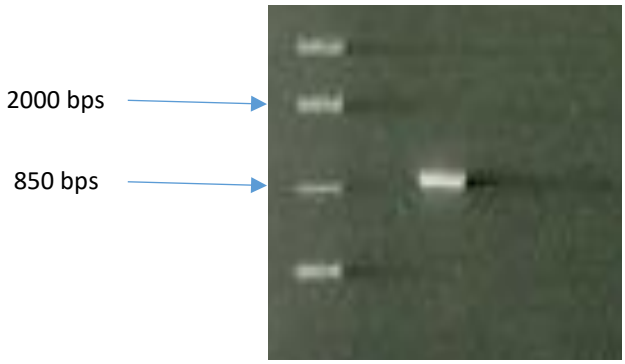


Figure 10: PCR was used to amplify *mmy*B. This reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 994 bps (Lane 1) compared to a FastRuler middle range DNA ladder (Track L).

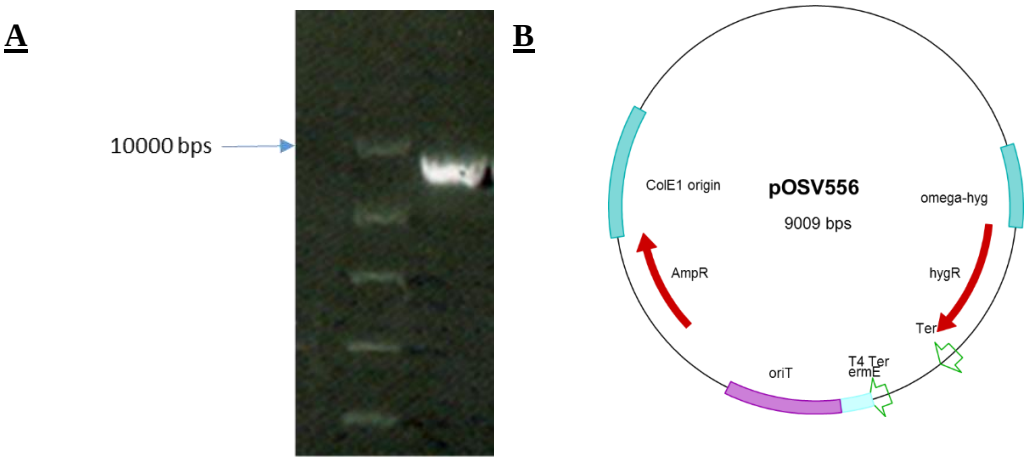


Figure 11: **A)** Purification of plasmid pOSV556 to verify the size of the expected band 9009 bp (Track 1 and 2) by comparing it to a FastRuler high range DNA ladder (Track L). **B)** Schematic map diagram for vector pOSV556, which has a size of 9009 bps.

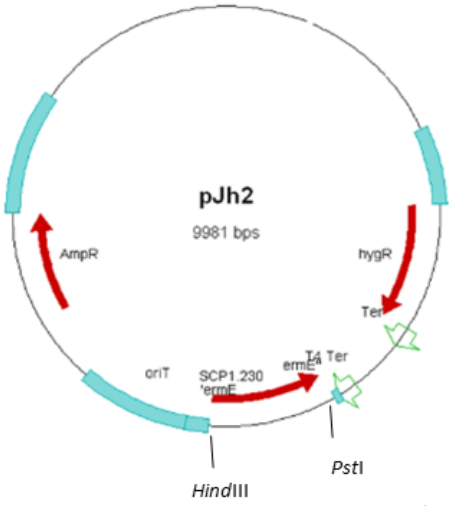


Figure 12: Schematic map for the *mmyB* ligated into pOSV556 which has been named pJh2 and is 9981 bps in size.

Screen of the vector for correct insert

The pOSV556 vector containing the cloned *mmyB* gene (pJh2) was linearized with *Hind*III giving a band of 9981 bps. The cloned *mmy*B gene was recovered from the plasmid using restriction enzymes *Hind*III and *Pst*I giving the expected band size of 9009 bps for the vector and a band at 994 bp for *mmy*B (**Figure 13**).

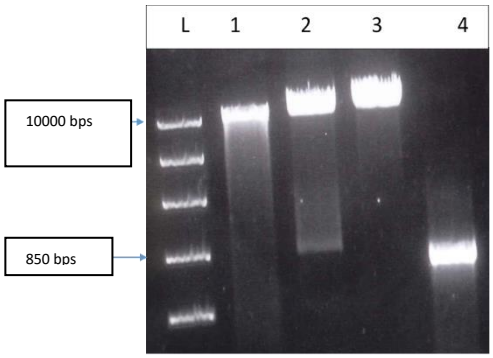


Figure 13: Assessment of the pJH2 fragment size. The purified *mmyB* construct was ligated into pOSV556 vector and was digested using the restriction enzyme *Hind*III and *Pst*I. This was then run on a 1% agarose gel in order to check the size of the expected bands. FastRuler high range DNA ladder (Track L), the size of the linearized plasmid was 9981 bps (Track 3), the size of the excised the *mmyB* insert 994 bps and vector is 9009 bps (Track 2). The pOSV556 band 9009 bps as positive control for the vector (Track 1). Positive control for the *mmyB* gene produced from the PCR reaction (Track 4).

3.1 Screen of the vector via PCR amplification

PCR screening was used to amplify the *mmyB* target sequence of gene using two primers **(T01 in Table 4)**. PCR products were run on a 1% agarose gel in order to verify the size of the expected band which was 994 bps **(Figure 14).** At this stage, the *mmyB* construct after cloning was sent for sequencing analysis to verify that no mutations had taken place during the cloning steps and this was confirmed in the returned sequencing data.

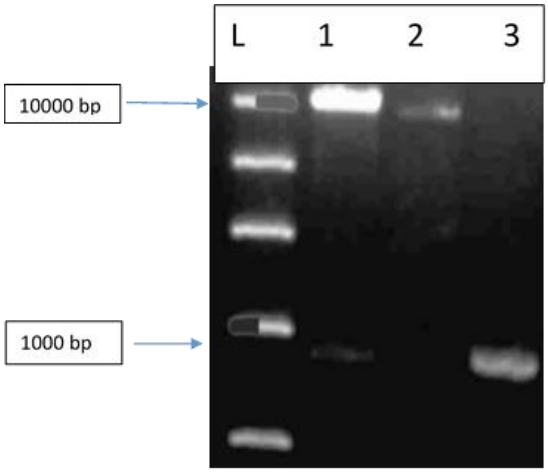


Figure 14: Assessment of the *mmyB* by doing PCR amplification used two primers. This was then run on a 1% agarose gel in order to check the size of the expected band. FastRuler high range DNA ladder (Track L). The *mmyB* insert around 994 bps and vector is 9009 bps (Track 1). The pOSV556 band around 9009 bps as positive control (Track 2). Positive control was for the *mmy*B gene (Track 3).

3.2 Assessment of pJh2 in *S. coelicolor* W81 using genomic DNA extraction and PCR screening

To introduce the pJh2 plasmid into *S. coelicolor* W81, non-methylating *E. coli* ET12567/pUZ8002 host cells (by electroporation) were required to transfer the plasmid using intergenic conjugation, this is because *S. coelicolor* W81. The construct was successfully introduced through conjugation in *S. coelicolor* W81. After genomic DNA extraction of pJh2, which was integrated into *S. coelicolor* W81, a concentration of 317 ng/µl for the purified plasmid was obtained. PCR screening was used to amplify the DNA target sequence of the gene using two designed primers. The PCR products were run on 1% agarose gel and observed a single band around the expected size of 994 bps **(Figure 15).**

Using intergenic conjugation, we were hence able to successfully integrate the *mmyB* gene into *S. coelicolor* W81 yielding the desired pJh2 plasmid.

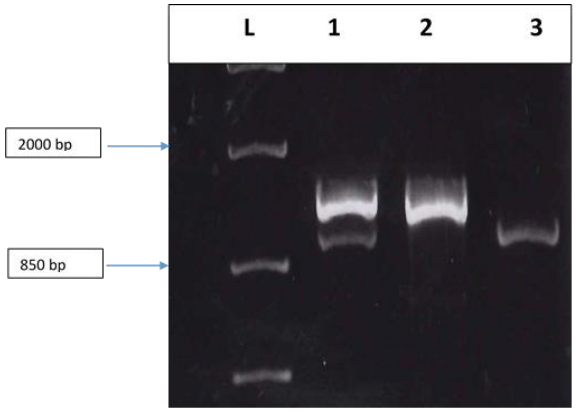


Figure 15: Assessment of the genomic *mmyB* by using PCR amplification for pJh2 integrated in *S. coelicolor* W81 after genomic DNA extraction technique (Track 1). FastRuler High range DNA ladder (Track L). Positive control used *mmyB* ligated pOSV556 (Track 2). *S. coelicolor* W81 only was used as negative control (Track 3).

3.3 Transcription analysis of the methylenomycin biosynthetic genes through RT-PCR

To find the expression-specific pathway after overproduction of pJH2 integrated in S. coelicolor, reverse transcription-PCR was performed on the extracted total RNA from pJH2 integrated into *S. coelicolor* W81 and wild type *S. coelicolor* W81.

Total RNA was extracted from *S. coelicolor* W81 strain in which pJh2 was integrated into. Three expected bands representing intact ribosomal RNA were observed with the total RNA concentration of 779 ng/ µl. RNA products were run on 1% agarose gel (in order to verify the size of the expected bands 5s, 16s and 23s) **(Figure 16).**

The RNA was converted into single stranded complementary DNA (cDNA) by using cDNA Superscript II RT. cDNA products were analysed by electrophoresis on 1% agarose gel as shown in **Figure 17**. As a control, a sample with no reverse transcriptase used to ensure that the results observed are that of the amplification of cDNA and not DNA contamination.

The cDNA synthesised were used as a template in thermal cycler reactions to determine if *mmy*B gene was transcribed. PCR products were analysed by electrophoresis on 1% agarose gel (**Figure 18 A & B)**.

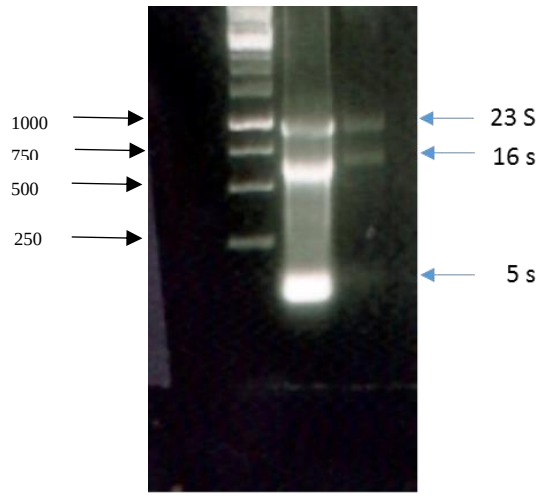


Figure 16: Total RNA was extracted from *S. coelicolor* W81/pJH2 grown in MMS broth. This reaction solution was run on 1% agarose gel and ribosomal RNA were clearly observed in three bands (Track 1) "S" stands for Svedberg's unit. A 1 kb ladder was used (Track L).

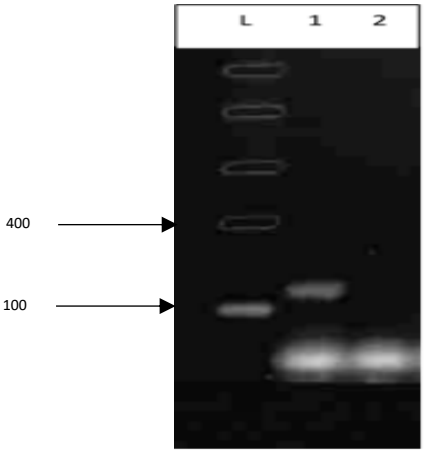


Figure 17: mRNA isolated from pJH2 integrated in *S. coelicolor* W81 was reverse transcribed to complementary DNA (cDNA) using cDNA Superscript II RT (Track 1). The negative control was performed using without reverse transcriptase to cDNA to confirm no DNA contamination (Track 2). FastRuler middle range DNA ladder (Track L).

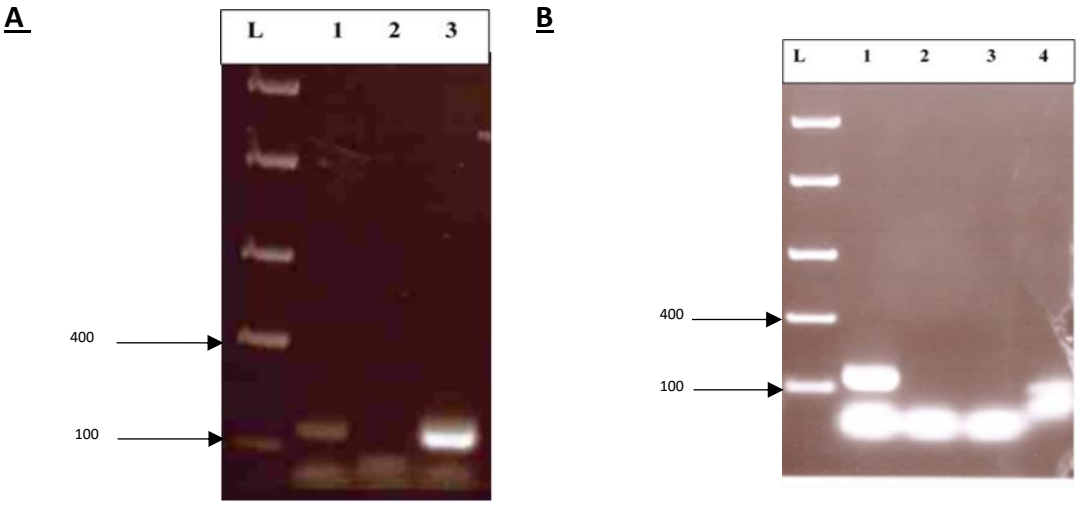


Figure 18: A) pJH2 integrated in *S. coelicolor* W81 expression *mmyB* using RT-PCR (Track 1). The negative control was performed using *S. coelicolor* W81 without pJH2 (Track 2) and the positive control is construct used to amplify the DNA target sequence of gene using same two designated primers by using template genomic DNA extracted from *S. coelicolor* A3 (2) (Track 3). FastRuler middle range DNA ladder (Track L). B) The second negative control was performed using minus RT-PCR using same two designated primers (Track 3). Track 4 shows control primers designed to amplify the *hrdB* gene which was considered to be constitutively expressed.

3.3.1 Transcription analysis by RT-PCR for *mmyO, mmyJ, mmyD, mmyF, mmyG, mmyQ* and *mmyB*

RT-PCR was used to test expression of selected gene from methylenomycin biosynthetic gene cluster. For this purpose sixteen different primer-pairs **(Table 7)** representing eight members of *mmy* genes (*mmyO, J, D, E, F, G, Q,* and *B)* were designed and their RT-PCR products were run on an agarose gel 1% and are shown in **Figure 19.**

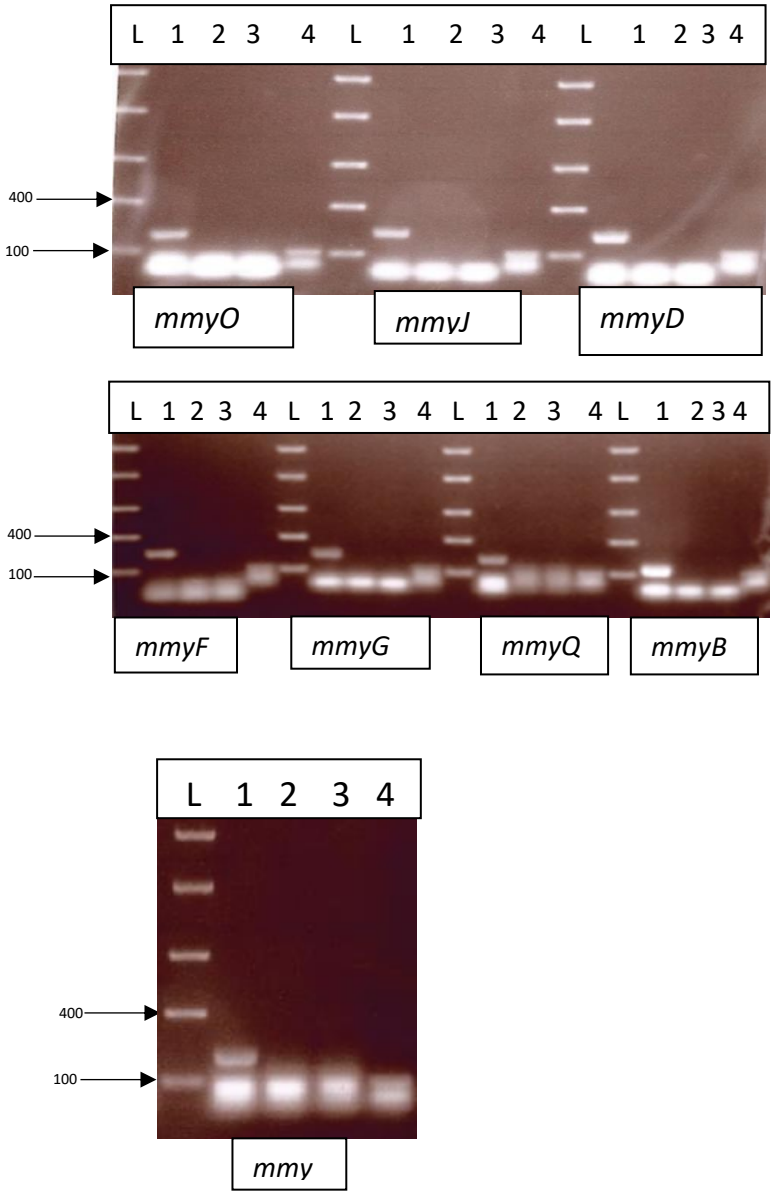


Figure 19: Analysis of the expression of a selection *mmy* genes *mmyO, J, D,E,F,G,*and *Q* in *S. coelicolor* W81 pJH2 using RT-PCR (Track 1). The negative control was performed using *S. coelicolor* W81 (Track 2) and also negative control was performed using minus RT-PCR using same two designated primers (Track 3). In Track 4 using as a control primers designed to amplify the *hrdB* gene considered to be constitutively expressed. FastRuler middle range DNA ladder (Track L).

3.4 Methylenomycin production by overexpression of *mmyB*

3.4.1 *S. coelicolor* W81 with and without pJH2 growth conditions

In order to confirm that the construct has been incorporated in *S. coelicolor*, the pOSV556 vector containing the cloned *mmy*B gene (pJh2) was transferred to *Streptomyces coelicolor* by intergenic conjugation then kept in incubation at 30 ^{o}C for 6 days to allow growth of the exconjugants in SFM agar. It was observed that the plate with integrated overproduction with pJH2 shows growth and produced dark blue pigments (plate 1 Figure 29). However the wild type shows red pigment. From this it can be concluded that after integration of the pJh2 plasmid, it was able to switch on the silent pathway and produced methylenomycins antibiotics. However, in the wild type, this is not observed **Figure 20**.

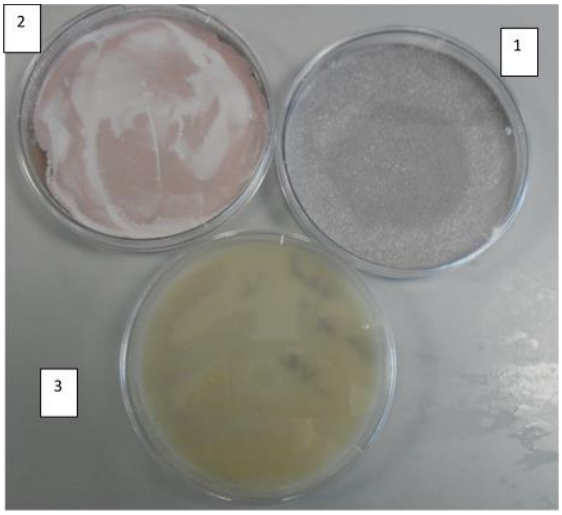


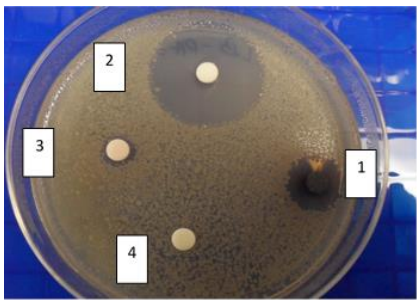
Figure 20: Culture plates of *streptomyces coelicolor* W81 after growth for 6 days at 30 ^{o}C which contain the plasmid pJh2 that was integrated into *S. coelicolor* W81 (plate 1), *S. coelicolor* W81 growth without integrated pJH2 as wild type (plate 2) and using SFM media without inoculation of any bacteria as negative control (plate 3).

3.4.2 Antibiotic assay

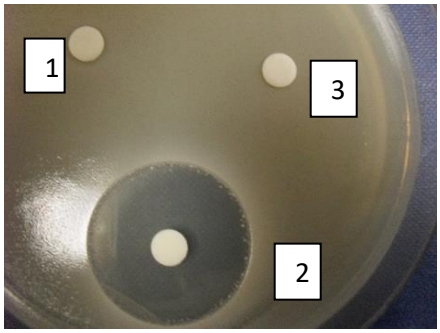
In order to confirm that after integration of the pJh2 plasmid, it was able to switch on the silent pathway and produced methylenomycins antibiotics. All the eight *mmy* genes (*mmyB, O, J, D, F, G, Q* and *mmyE*) were expressed when the *mmyB* was integrated in *S. coelicolor* W81. Antimicrobial activity of *S. coelicolor* W81/ pJh2 was tested using antibiotic sensitivity assay. The antibiotic sensitivity assay shows the ability of a drug in inhibition zone for the growth of the bacteria such as *Bacillus subtilis, E. coli* and *S. aureus* around the *S. coelicolor* W81/ pJh2 (**Figure 21).** The positive control was Apramycin Track 2, the antibiotic drug of the plasmid pJh2 was integrated into S. coelicolor W81 and metabolites were tested after organic extraction showing zone of inhibition (Track 1).

Hence it can be deduced that methylenomycin antibiotic are produced.

Bacillus subtilis



E. coli



S. aureus

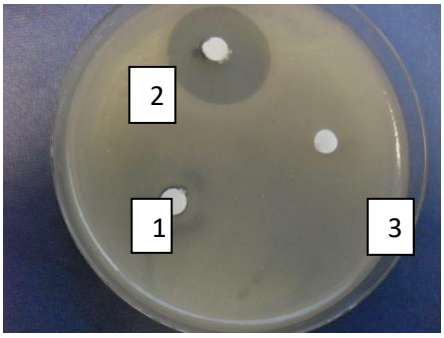


Figure 21: Antibiotic assay used *Bacillus subtilis* on LB agar pH 5. The plasmid pJH2 was integrated into *S. coelicolor* W81 and metabolites were tested after organic extraction showing zone of inhibition (Track 1). Apramycin used as positive control (Track 2), *S. coelicolor* W81 only without the pJH2 was used as negative control (Track 3), Track 4 for negative controls containing DMSO only.

Chapter 4: Lantibiotic gene clusters regulated by SCO6926

4.1 Lantibiotics

Lantibotics act as antibiotics and are produced by a different Gram-positive bacteria (Jung *et al.*, 1991) and can kill other Gram-positive bacteria by inhibiting cell wall synthesis (VÉRIEST et al., 1999). Lantibiotics are ribosomally synthesised peptides, in which some amino acids are post-transitionally modified to non-natural amino acids (Sahl *et al.*, 1995). Some examples of the non-natural amino acid that are created in lantibiotics are didehydroalanine, lanthionine, didehydrobutyrine and β-methyllanthionine (Asaduzzaman *et al.*, 2009). Some of the well-known members of lantibiotics are shown in **(Figure22)** (Molloy *et al.*, 2012).

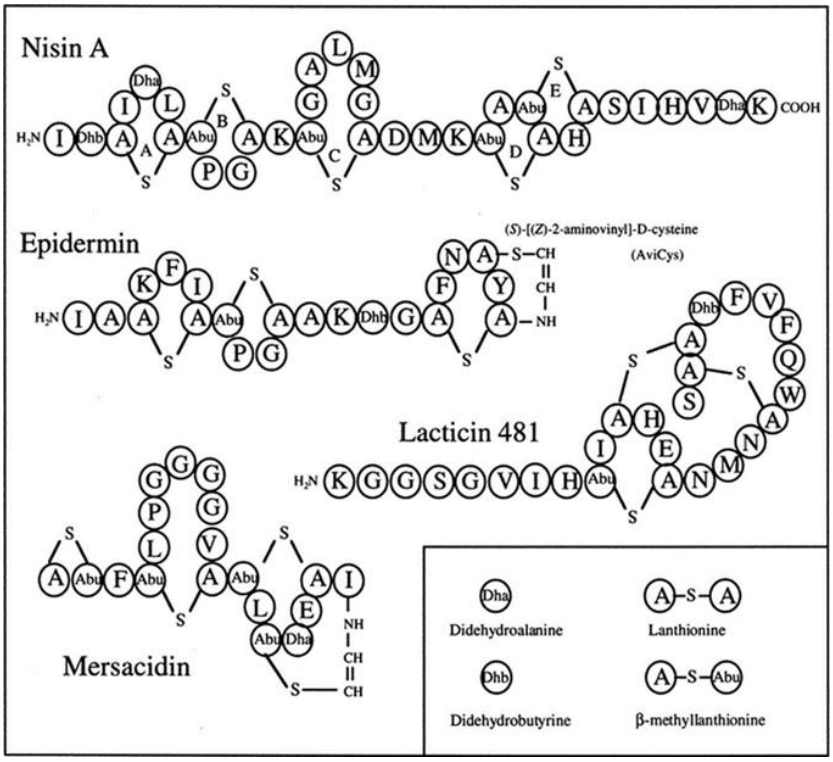


Figure 22: The various commonly found lantibiotics include nisin A, The various commonly found lantibiotics include nisin A, Epidermin, Lacticin and Mersacidin. The encircled letters represent the amino acid (single letter code) (-S-) represents the thioether bound: A=Alanine, D=Aspartic acid, F=Phenylalanine, G=Glycine, H= Histidine, I= Isoleucine, K=Lysine, N= Asparagine, M= Methionine, P= Proline, S=Serine, V=Valine and Y=Tyrosine this picture taken from (McAuliffe *et al.*, 2001).

The lantibiotic is created by the dehydration of certain serine (Ser) and threonine (Thr) residues with the addition of intramolecular of Cys thiols to the resulting in an unsaturated amino acids with the polypeptide chain (**Figure 23**) (McAuliffe *et al.*, 2001).

Lantibiotics are considered as a member of bacteriocins that are classified according to their extent of post-translational modification. The lantibiotics can be categorised as extensively modified Class I bacteriocins. Bacteriocins for which disulphide bonds are the only modification to the peptide are Class II bacteriocins (McAuliffe *et al.*, 2001)

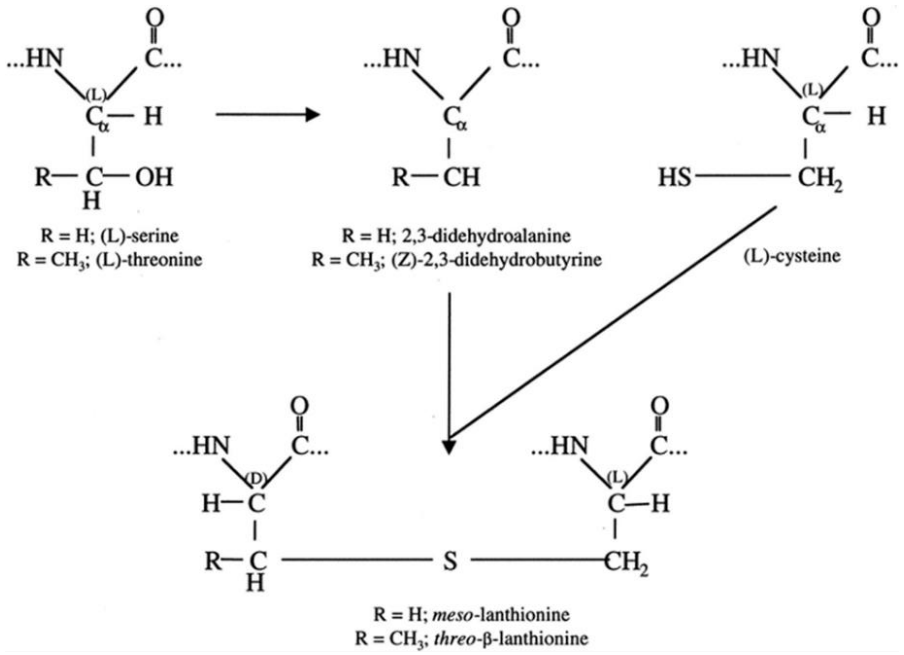


Figure 23: Lantibiotics synthesis, the picture taken from (McAuliffe *et al.*, 2001).

Another classification of lantibiotics divides them into type A and type B based on their structural and functional features **Table 18** (Jung *et al*., 1991).

Table 18: Different type of lantibiotics (Jung *et al*., 1991).

Lantibiotics tybes	Structure	Length	Functions	An example
Type A	Elongated	Cationic peptides up to 34 residues	Disrupting the membrane integrity of target organisms	nisin, subtilin, and epidermin
Type B	Globular	Cationic peptides up to 19 residues	Inhibition of cell wall biosynthesis	Mersacidin, actagardine, and cinnamycin.

4.1.1 Mechanism of action

Lantibiotics show high specificity for some components of the bacterial cell membrane especially of Gram-positive bacteria. Small quantities of lantibiotics type A can kill Gram-positive bacteria rapidly because they can form pores in the cell wall. However, type B lantibiotics can inhibit the cell wall peptidoglycan biosynthesis (Brötz and Sahl, 2000).

Lantibiotics contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, in addition to the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid. Lanthionine is composed of two alanine residues that are cross-linked on their β-carbon atoms by a thioether (monosulfide) linkage. Nisin and epidermin are well-studied lantibiotics that bind to lipid II, and disrupt cell wall production. On the other hand, the duramycin family of lantibiotics binds phosphoethanolamine in the membranes of its target cells leading to the disruption essential physiological functions. Interestingly, some lanthionine containing peptides have been discovered that do not have any antibiotic activity (Goto *et al*., 2010). Some lantibiotic produced from different show in **Table 19.**

Table 19: Lantibiotic produced from different strains of Gram-positive bacteria, the table taken from (McAuliffe *et al*., 2001).

Lantibiotics	Producing Strain
Nisin A	*Lactococcus lactis* NIZOR5, 6F3, NCFB894, ATCC11454
Nisin Z	*Lactococcus lactis* N8, NIZO22186
Subtilin	*Bacillus subtilis* ATCC6633
Epidermin	*Staphylococcus epidermidis* Tu3298
Gallidermin	*Staphylococcus gallinarum* Tu3928
Mutacin B-Ny266	*Streptococcus mutans*
Mutacin 1140	*Streptococcus mutans* JH1000
Pep5	*Staphylococcus epidermidis 5*
Epicidin 280	*Staphylococcus epidermidis* BN280
Epilancin K7	*Staphylococcus epidermidis* K7
Lacticin 481	*Lactococcus lactis* CNRZ481, ADRIA85LO30
Cytolysin	*Enterococcus faecalis* DS16
Lacticin 3147	*Lactococcus lactis* DPC3147
Staphylococcin C55	*Staphylococcus aureus* C55
Salvaricin A	*Streptococcus salvarius* 20P3
Lactocin S	*Lactobacillus sake* L45
Streptococcin A-FF2	*Streptococcus pyogenes* FF22
Sublancin 168	*Bacillus subtilis* 168
Carnocin U149	*Chryseobacterium piscicola*
Variacin 8	*Micrococcus varians* MCV8
Cypemycin	*Streptomyces ssp*
Cinnamycin	*Streptomyces cinnamoneus*
Duramycin B	*Streptoverticillium ssp*
Duramycin C	*Streptomyces griseoluteus*
Ancovenin	*Streptomyces ssp*
Mersacidin	*Bacillus subtilis* HIL Y-85, 54728
Actagardine	*Actinoplanes*

4.2 Bioinformatics analysis for lantibiotic gene clusters regulated by *sco6926*

Discovery of new natural products can be achieved via the genome mining approach; the genome sequence of a microorganism of interest can be obtained and analysed using bioinformatics tools to identify potential cryptic biosynthetic gene clusters (BGC's). *S. coelicolor* has 16 proteins highly similar to MmyB shown in **(Table 1)** each of them responsible for controlling different pathways (Xu *et al*., 2012), SCO6926 may be involved in promoting biosynthesis of lantibiotic. Bioinformatics helps to identify the lantibiotics biosynthesis secondary metabolite gene clusters in *S. coelicolor*. Tools to do this include antiSMASH (http://antismash.secondarymetabolites.org/) and a lantibiotic biosynthetic gene cluster in *S. coelicolor* was successfully identified using AntiSMASH (**Figure 24 A & B)** and **(Figure 25)**.

In addition, it is useful for novel natural products discovery and bioinformatics tools such as the National Centre for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and useful to identify proposed function and proteins for all the genes of lantibiotic metabolite gene clusters in **Table 20.** The SCO6926 protein is found in *Streptomyces coelicolor* A3(2). It is a proposed pathway specific transcriptional activator for the lantibiotic biosynthetic gene cluster.

A)

Identified secondary metabolite clusters

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID
The following clusters are from record NC_003888.3:					
Cluster 1	T1pks-Otherks	86637	139654	Leinamycin_biosynthetic_gene_cluster (2% of genes show similarity)	BGC0001101_c1
Cluster 2	Terpene	166501	192038	Isorenieratene_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000664_c1
Cluster 3	Lantipeptide	235986	271084	Sanglifehrin_A_biosynthetic_gene_cluster (4% of genes show similarity)	BGC0001042_c1
Cluster 4	Nrps	493989	544920	Coelichelin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000325_c1
Cluster 5	Bacteriocin	791584	801799	Informatipeptin_biosynthetic_gene_cluster (42% of genes show similarity)	BGC0000518_c1
Cluster 6	T3pks	1257625	1298749	Herboxidiene_biosynthetic_gene_cluster (8% of genes show similarity)	BGC0001065_c1
Cluster 7	Ectoine	1995500	2005898	Ectoine_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000853_c1
Cluster 8	Melanin	2939306	2949875	Lactonamycin_biosynthetic_gene_cluster (3% of genes show similarity)	BGC0000238_c1
Cluster 9	Siderophore	3033895	3045682	Desferrioxamine_B_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000940_c1
Cluster 10	Nrps	3523335	3603988	Calcium-dependent_antibiotic_biosynthetic_gene_cluster (90% of genes show similarity)	BGC0000315_c1
Cluster 11	T2pks	5509801	5552424	Actinorhodin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000194_c1
Cluster 12	Terpene	5671016	5692101	Albaflavenone_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000660_c1
Cluster 13	T2pks	5766945	5809487	Spore_pigment_biosynthetic_gene_cluster (66% of genes show similarity)	BGC0000271_c1
Cluster 14	Siderophore	6335587	6347533	-	-
Cluster 15	T1pks	6429549	6476442	Undecylprodigiosin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0001063_c1
Cluster 16	Bacteriocin	6632343	6643659	-	-
Cluster 17	Terpene	6656219	6678399	-	-
Cluster 18	Siderophore	6842315	6855522	Enduracidin_biosynthetic_gene_cluster (8% of genes show similarity)	BGC0000341_c1
Cluster 19	T1pks-Butyrolactone	6871293	6951959	Coelimycin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000038_c1
Cluster 20	Nrps	7088264	7136089	Nogalamycin_biosynthetic_gene_cluster (40% of genes show similarity)	BGC0000249_c1
Cluster 21	Lantipeptide	7409664	7432456	SAL-2242_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000546_c1
Cluster 22	Terpene	7506017	7532758	Hopene_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000663_c1
Cluster 23	T1pks-Otherks	7570412	7618555	Arsenopolyketides_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0001283_c1
Cluster 24	Lantipeptide	7685016	7709795	-	-

B)

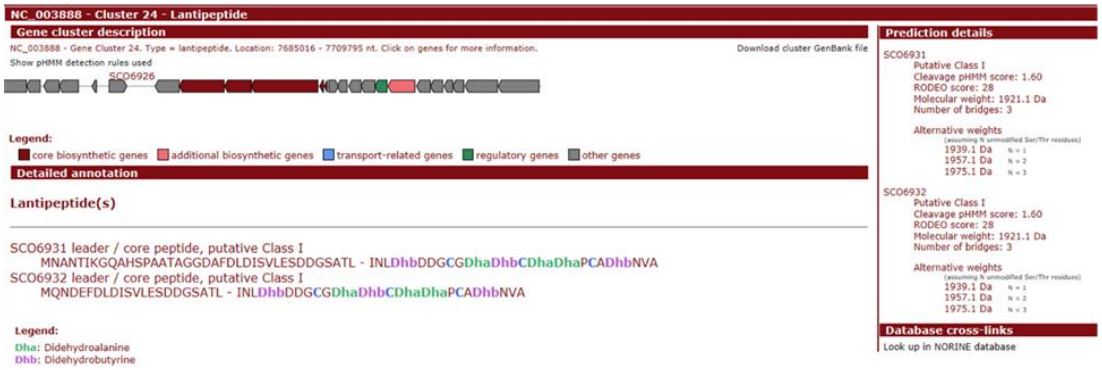


Figure 24: Identify lantibiotic secondary metabolite genes cluster in *S. coelicolor* by using antiSMAH

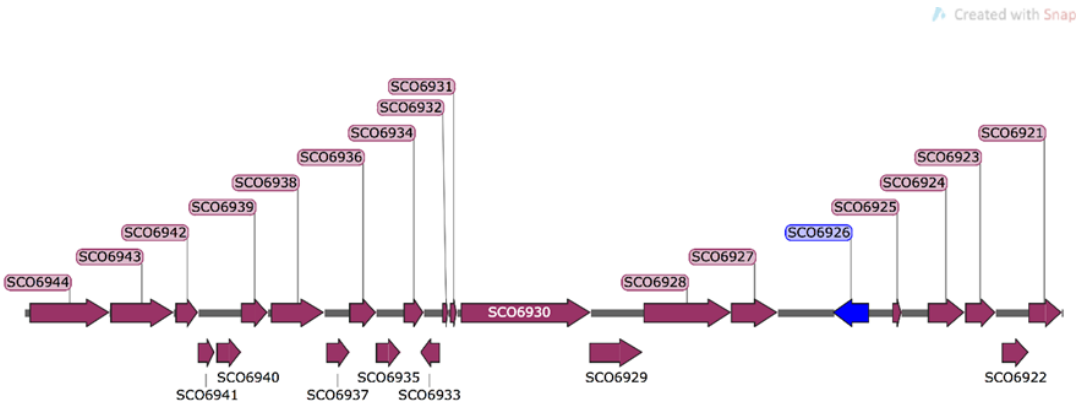


Figure 25: Proposed the lantibiotic genes cluster contain 24 genes

Table 20: Proposed function of the biosynthetic genes from the *sco6926* cluster.

Genes	Protein (number of aa) GenBank	Homologue (% identity / % similarity) Organism GenBank	Putative function
*sco*6921	WP_011031303.1 576 aa	Hypothetical protein (86%/89%) *Streptomyces aureofaciens WP_052837004.1*	Membrane protein
*sco*6922	WP_011031304.1 213 aa	WhiB family transcriptional regulator (92%/94%) *Streptomyces aureofaciens* WP_052837005.1	WhiB family transcriptional regulator
*sco*6923	WP_011031305.1 238 aa	Hypothetical protein (96%/98%) *Streptomyces* sp. NBRC 110030 WP_055469087.1	Hypothetical protein
*sco*6924	WP_011031306.1 298 aa	DNA-binding protein (91%/93%) *Streptomyces* sp. NRRL S-37 WP_030861947.1	DNA-binding protein
*sco*6925	WP_011031307.1 70 aa		Hypothetical protein
*sco*6926	WP_011031308.1 274 aa	Transcriptional regulator (96%/98%) *Streptomyces olivaceus* WP_031048052.1	Transcriptional regulator
*sco*6927	WP_011031309.1 374 aa	Hypothetical protein (84%/98%) *Streptomyces zinciresistens WP_007491153.1*	Hypothetical protein
*sco*6928	WP_011031310.1 696 aa	methyltransferase, FxLD system (94%/95%) *Streptomyces zinciresistens WP_007491154.1*	O-methyltransferase
*sco*6929	WP_011031311.1 419 aa	Lanthionine biosynthesis cyclase LanC (90%/93%)	Hypothetical protein

		Streptomyces silaceus WP_076683631.1	
*sco*6930	WP_011031312.1 1032 aa	Hypothetical protein (97%/98%) *Streptomyces zinciresistens* WP_007491156.1	Hypothetical protein
*sco*6931	WP_011031313.1 59 aa	FxLD family lantipeptide (85%/88%) *Streptomyces sp.* CCM_MD2014 WP_054100455.1	FxLD family lantipeptide
*sco*6932	WP_011031314.1 43 aa	FxLD family lantipeptide (98%/ 97%) *Streptomyces silaceus* WP_076683627.1	MULTISPECIES: FxLD family lantipeptide
*sco*6933	WP_011031315.1 149 aa	LLM class flavin-dependent oxidoreductase (34%/45%) *Mycetocola saprophilus* WP_051701837.1	Hypothetical protein
*sco*6934	WP_011031316.1 155 aa	Hypothetical protein (95%/96%) *Streptomyces sp.* CCM_MD2014 WP_054100456.1	Hypothetical protein
*sco*6935	WP_011031317.1 191 aa	Hypothetical protein (96%/96%) *Streptomyces zinciresistens* WP_007491163.1	Hypothetical protein
*sco*6936	WP_011031318.1 217 aa	Hypothetical protein (94%/94%) *Streptomyces zinciresistens* WP_086021316.1	Hypothetical protein
*sco*6937	WP_011031319.1 186 aa	DNA-binding protein (92%/95%) *Streptomyces zinciresistens WP_086021317.1*	DNA-binding protein
*sco*6938	WP_011031320.1 417 aa	Acyl-CoA dehydrogenase (91%/95%) *Streptomyces olivaceus* WP_031045354.1	Acyl-CoA dehydrogenase

*sco*6939	WP_011031321.1 213 aa	histidine kinase (91%/94%) *Streptomyces aureofaciens* WP_052837026.1	GAF domain-containing protein
*sco*6940	WP_011031322.1 192 aa	ATP-binding protein (95%/96%) *Streptomyces zinciresistens* WP_086021320.1	ATP-binding protein
*sco*6941	WP_011031323.1 135 aa	DUF742 domain-containing protein (94%/97%) *Streptomyces zinciresistens* WP_007491176.1	DUF742 domain-containing protein
*sco*6942	WP_011031324.1 171 aa	Hypothetical protein (94%/95%) *Streptomyces longwoodensis* WP_067230177.1	Hypothetical protein
*sco*6943	WP_011031325.1 505 aa	Histidine kinase (91%/92%) *Streptomyces zinciresistens* WP_007491180.1	Histidine kinase
*sco*6944	WP_011031326.1 633 aa	Glucoamylase (92%/95%) *Streptomyces aureofaciens* *WP_052837030.1*	Glucoamylase

4.3 Overexpression *sco6926* gene from *S. coelicolor* M145

The SCO6926 protein is found in *Streptomyces coelicolor* A3(2). It is proposed to be a transcriptional activator for the lantibiotic biosynthetic gene cluster. The lantibiotic biosynthetic gene cluster is tightly regulated at the transcriptional level, and is usually not produced or produced in a very small quantity in laboratory growth conditions.

Overexpression of transcriptional activators under the control of *ermEp** is expected to unlock the production of compounds from expression of the neighbouring cryptic biosynthetic gene clusters. To investigate this, an experiment was performed determine if overexpression of this activator switched on the lantibioitc biosynthetic genes cluster will expressed the own genes to produce the lantibiotic antibiotics. To this end, cloned *sco*6926 in to the pOSV556 vector under the control of the *ermEp** promotor and then introduced it into *S. coelicolor* M145.

PCR was used to amplify specific *sco*6926 sequences. Two primers were designed (forward and reverse) **(T03 in Table 4).** High-Fidelity PCR amplification was used to amplify a target region of *sco*6926 for cloning purposes using PCR reaction. PCR products were run on 1% agarose gel in order to verify the size of the expected band which was 825 bps (**Figure 26 A)**. A NanoDrop ND-1000 spectrophotometer was used to measure the concentration of the *sco6962* (304 ng/ µl).

Digestion and ligation of plasmid pOSV556 followed by insertion of *sco*6926 were performed as part of cloning and plasmid analysis. The plasmid created by this ligation is known as pJT1 (containing *sco*6926 under the control of ermEp*). Plasmid *sco6926* was amplified in *E.coli* Top10 strain and purified using the mini-prep procedure; the agarose gel run following the mini-prep of the construct is shown in **Figure 26 B**. This construct was then ready to transform into non-methylating *E. coli* ET12567/pUZ8002 host cell (by electroporation) to introduce into *S. coelicolor* M145.

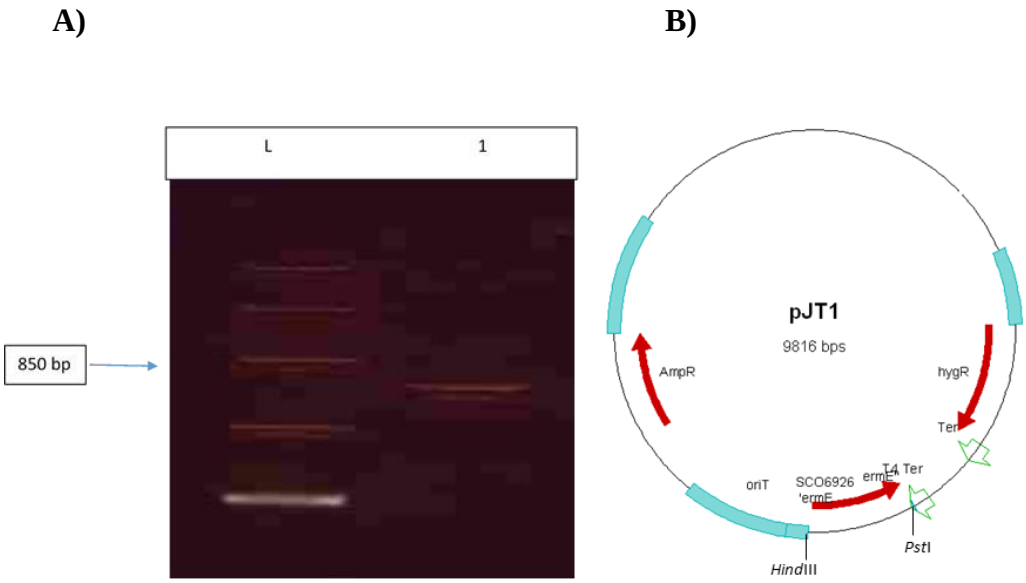


Figure 26: A) PCR was used to amplify *sco*6926. This reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 825 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). **B)** Schematic map for the sco6926 ligated into pOSV556which has been named pJT1 and is 9816 bps in size.

4.3.1 Screen of the vector via PCR amplification

PCR screening was used to amplify the *sco*6926 target sequence of gene using two primers **(T03 in Table 4)**. PCR products were run on 1% agarose gel in order to verify the size of the expected band which was 825 bps **(Figure 27).** At this stage, the DNA construct after cloning was sent for sequencing analysis to verify that no mutations had taken place during the cloning steps and this was confirmed in the returned sequencing data.

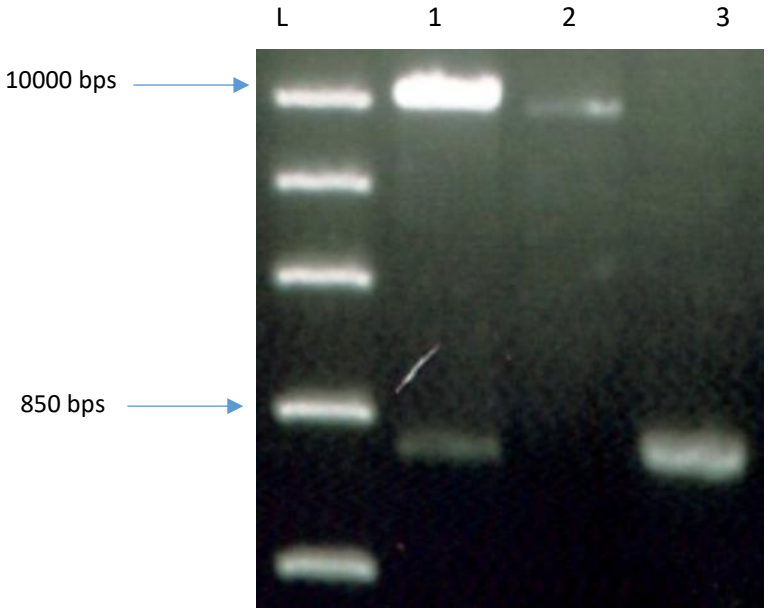


Figure 27*:* Assessment of the *sco6926* by doing PCR amplification used two primers. This was then run on a 1% agarose gel in order to check the size of the expected band. FastRuler high range DNA ladder (Track L). The *sco*6926 insert around 825 bps and vector is 9009 bps (Track 1). The pOSV556 band around 9009 bps as positive control (Track 2). Positive control was for the *sco6926* gene (Track 3).

4.3.2 Assessment of the pJT1 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

To introduce the pJT1 plasmid into *S. coelicolor* M145, non-methylating *E. coli* ET12567/pUZ8002 host cells (by electroporation) were required to transfer the plasmid using intergenic conjugation, this is because *S. coelicolor* M145 contains a methyl-sensing restriction system. The construct was successfully introduced through conjugation in *S. coelicolor* N145. After genomic DNA extraction of pJT1, which was integrated into *S. coelicolor* M145, a concentration of 201 ng/µl for the purified plasmid was obtained. PCR screening was used to amplify the DNA target sequence of the gene using two designed primers. The PCR products were run on 1% agarose gel and observed a single band around the expected size of 825 bps **(Figure 28).** Once screened, this plasmid was transferred into *S. coelicolor* M145 via conjugation.

Using intergenic conjugation, we were hence able to successfully integrate the *sco*6926 gene into *S. coelicolor* M145 yielding the desired pJT1 plasmid.

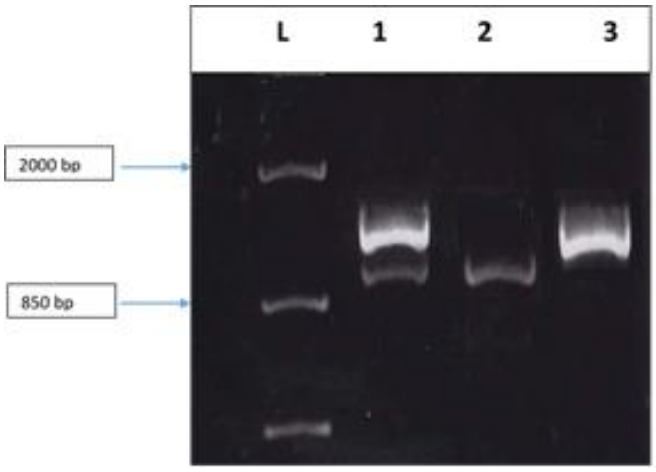


Figure 28*:* Assessment of the genomic *sco6926* by using PCR screen for pJT1 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). *). S. coelicolor* M145 only was used as negative control (Track 2). Positive control used construct of *sco*6269 ligated pOSV556 (Track 3). FastRuler high range DNA ladder (Track L).

4.4 Transcription analysis of the lantibiotic biosynthetic gene through RT-PCR

To find the expression-specific pathway after overproduction of pJT1 integrated in S. coelicolor, reverse transcription-PCR was performed on the extracted total RNA from pJT1 integrated into *S. coelicolor* M145 and wild type *S. coelicolor* M145.

Total RNA was extracted from *S. coelicolor* M145 strain in which pJT1 was integrated into. Three expected bands representing intact ribosomal RNA were observed with the total RNA concentration of 187 ng/ µl. RNA products were run on 1% agarose gel (in order to verify the size of the expected bands 5s, 16s and 23s) **(Figure 29).**

The RNA was converted into single stranded complementary DNA (cDNA) by using cDNA Superscript II RT. cDNA products were analysed by electrophoresis on 1% agarose gel as shown in **Figure 30**. As a control, a sample with no reverse transcriptase used to ensure that the results observed are that of the amplification of cDNA and not DNA contamination.

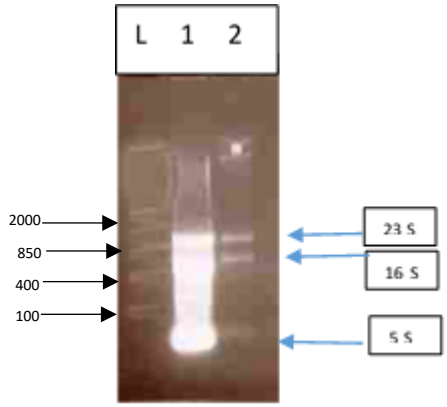


Figure 29: Total RNA was extracted from *Streptomyces coelicolor* M145 / pJT1 grow in MMS broth. This reaction solution was run on 1% agarose gel and ribosomal RNA were clearly observed (Track 1,2). 1 kb ladder was used (Track L).

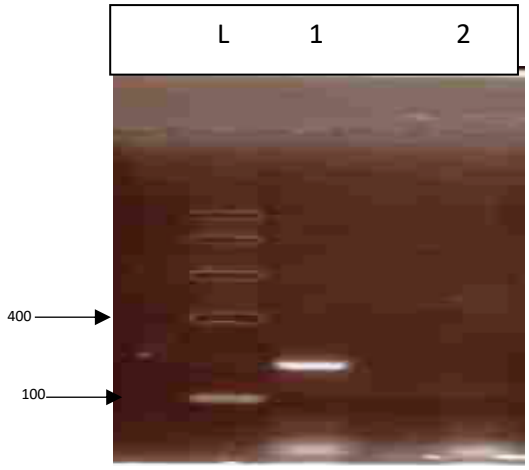


Figure 30: mRNA isolated from pJT1 integrated in *S. coelicolor* M145 was reverse transcribed to complementary DNA (cDNA) using cDNA Superscript II RT (Track 1). The negative control was performed using minus reverse transcriptase to cDNA to confirm no DNA contamination (Track 2).

4.4.1 Transcription analysis by RT-PCR for *Sco6926, sco6927, sco6932* and *sco6933*

RT-PCR was used as test expression of selected gene from lantibiotic biosynthetic gene cluster. For this purpose eight different primer-pairs **(Table 7)** representing four members of *sco* genes for examples *sco*6926, *sco*6927, *sco*6932 and *sco*6933 were designed and their RT-PCR products were run on an agarose gel 1% and are shown in **(Figure 31).** Additional experimental show in the appendix**.**

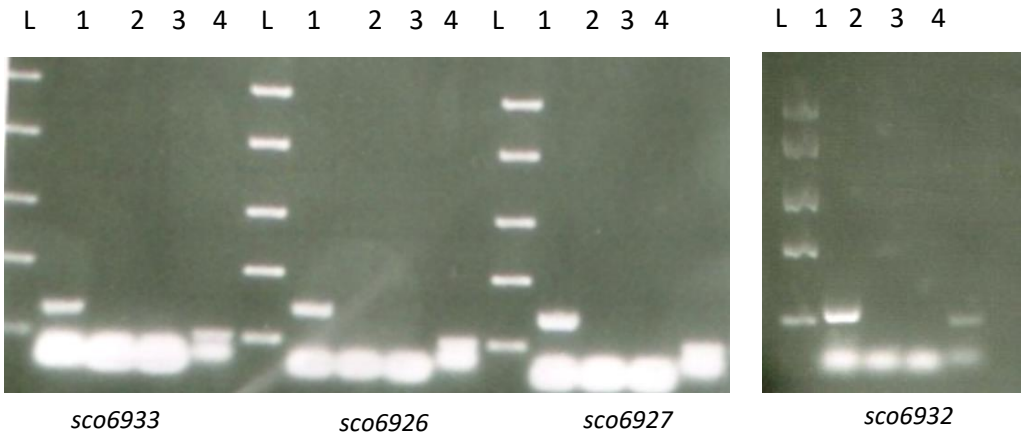


Figure 31*:* Analysis of the expression of a selection *sco* genes *sco*6926, 27, 32 *and sco*6933 in *S. coelicolor* M145/pJT1 using RT-PCR (Track 1). The negative control was performed using *S. coelicolor* M145 (Track 2) and also negative control was performed using minus RT-PCR using same two designated primers (Track 1). In Track 4 using as a control primers designed to amplify the hrdB gene considered to be constitutively expressed.

4.5 Bacterial growth conditions and antibiotic assay

4.5.1 *S. coelicolor* M145 with and without pJT1 growth conditions

In order to confirm that the construct has been incorporated in *S. coelicolor*, the pOSV556 vector containing the cloned *sco*6926 gene (pJT1) was transferred to *Streptomyces coelicolor* M145 by intergenic conjugation according to standard methods by (Kieser, 2000), then keep it in incubation at 30 ºC for 6 days to allow growth of the exconjugants in SFM agar **(Figure 32**). It was observed that the plate with integrated overproduction with pJT1 shows growth and produced bale red pigments (plate 1 Figure 42). However the wild type shows white pigment. From this it can be concluded that after integration of the pJT1 plasmid, it was proposed to switch on the silent pathway and produced lantibiotics antibiotics. However, in the wild type, this is not observed.

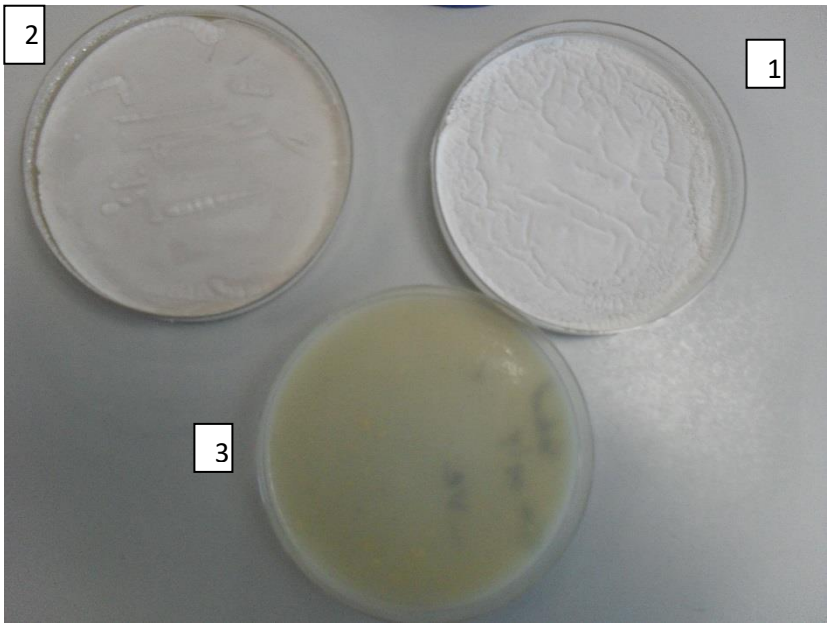
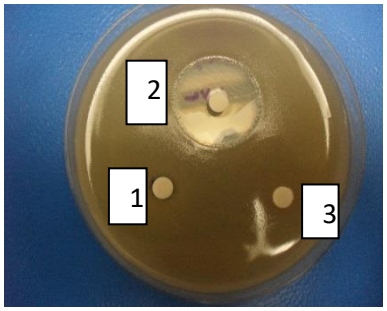


Figure 32: culture plates *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pJT1 was integrated into *S. coelicolor* M145 (plate 2), *S. coelicolor* M145 growth without integrated pJT1 as wild type (plate 1) and use SFM media without inoculation any bacteria as negative control (plate 3).

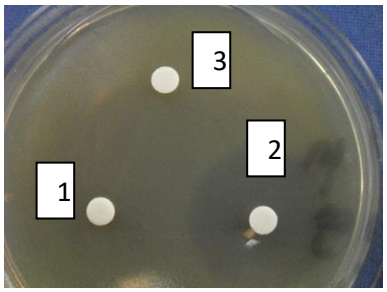
4.5.2 Antibiotic assay

In order to confirm that after integration of the pJh2 plasmid, it was able to switch on the silent pathway and produced methylenomycins antibiotics. All the four *sco*6926 genes (*sco*6926, *sco*6927, *sco*6932, *sco*6933) were expressed when the *sco*6926 was integrated in *S. coelicolor* M145. Antimicrobial activity of *S. coelicolor* M145/ pJT1 was test using antibiotic sensitivity assay. The antibiotic sensitivity assay shows the ability of drug in inhibition zone for the growth of the bacteria such as *B. subtilis S. aureus* but not active around the *E. coli* around the *S. coelicolor* M145/pJT1 **(Figure 33).**

Bacillus subtilis



E. coli



S. aureus

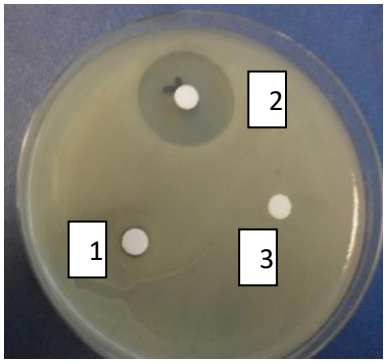
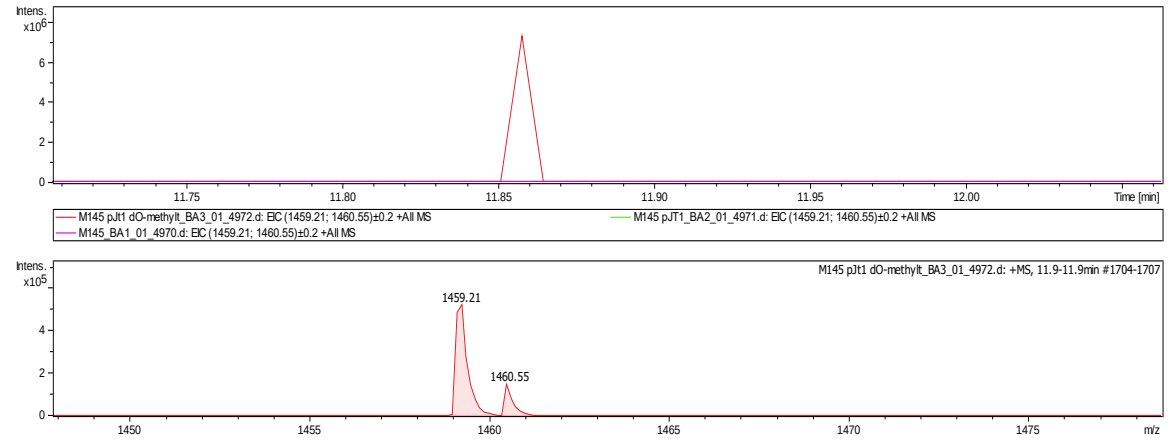


Figure 33: Antibiotic assay used *Bacillus subtilis*, *S. aureus* and *E. coli* on LB agar pH 5. The plasmid PJT1 was integrated into *S. coelicolor* M145 and metabolites were tested and showing zone inhibition aureus but not active around the *E. coli* (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the PJT1 was used as negative control (Track 3).

4.6 Identification of predicted lantibiotic compounds in *S. coelicolor* M145 heterologous hosts

In order to identify the predicted lantibiotics LC-MS of the SMM medium in which the cells are grown is performed. This is because any novel lantibiotics are synthesized by the cell after the incorporation of the new construct will be directed to the extracellular environment. Metabolites between the *S. coelicolor* with pJT1 homologous host strains and *S. coelicolor* M145 wild type were compared by LC-MS and two new compounds were identified. The m/z= 1459.21 and 1474.93 were only present in *S. coelicolor* strains containing pJT1 and were absent in *S. coelicolor* M145 control **(Figure 34 A & B).** Hence, we are able to identify two novel compounds which could potentially be implicated in antibiotic activity.

A



B

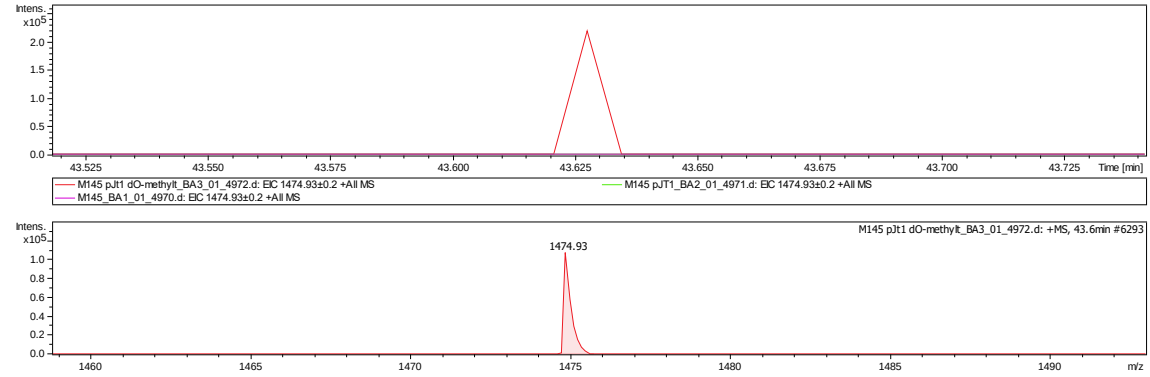


Figure 34: **A)** LC-MS trace of the predicted novel lantibiotic compound. Extracted ion chromatograms m/z = 1459.21. **B)** Extracted ion chromatograms m/z = 1474.93 which is not present when LC-MS of wild type is performed.

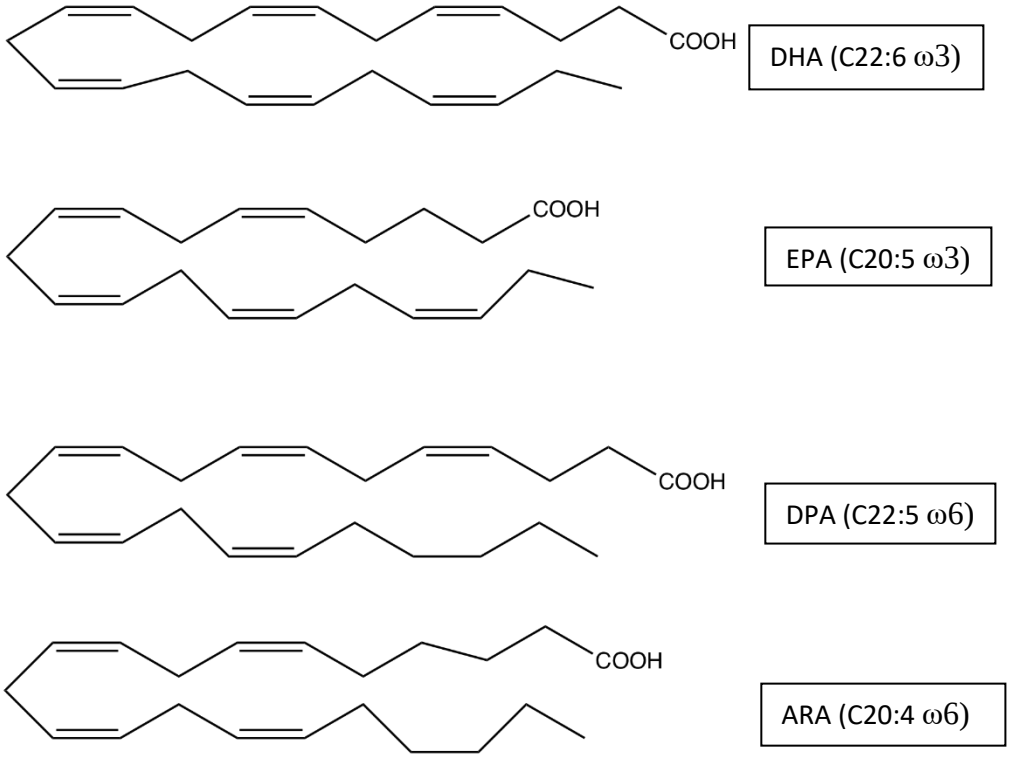
Chapter 5: Polyunsaturated fatty acid gene clusters regulated by *sco0110*

5.1 Polyunsaturated fatty acid gene

Lipids are fatty acid normally obtained from nutritional sources from foods. Fatty acids are carboxylic acids containing different lengths of carbon atoms that form a hydrocarbon chain. This chain is completed by carboxyl and methyl group. It is soluble in organic non-polar solvents. It can be classified like chains without double bonds between two adjoining carbon atoms are known as saturated fatty acids. Monounsaturated fatty acids have a single double bond, whereas polyunsaturated fatty acids two or more double bonds. the number of double bonds and the position of the initial double bond in the chain from the methyl functional group at the end of the chain, which is referred to as the 'n' or 'ω' carbon (Russell and Nichols, 1999, Yates *et al*., 2014). Polyunsaturated fatty acids (PUFAs) are important biomolecules in maintaining the health of living organisms. Therefore, they are known as essential fatty acids. However, animals cannot produce PUFAs because they lack some of the enzymes required for their biosynthesis. As a result, there is an increased interest in PUFA production in microorganisms (Elrazak *et al.*, 2013).

PUFAs can be referred to according to their organic structure. For example, arachidonic acid (ARA), which is presented as 20:4n−6, where 20 stands for the total number of carbon atoms, 4 indicates the number of double bonds and 6 shows that the position of the first double bond is on the 6th carbon from the terminal methyl (Abedi and Sahari, 2014, Yates *et al.*, 2014). It is possible to distinguish between various families of PUFAs using this naming system. Also, some common examples of PUFAs found in microorganisms include docosahexaenoic acid (DHA) is presented as 22:6n−3, where 22 stands for the total number of carbon atoms, 6 indicates the number of double bonds, and 3 shows that the position of the first double bond is on the 3rd carbon from the methyl group (Abedi & Sahari 2014). For eicosapentaenoic acid (EPA), the structure is indicated as 20:5n−3. For decosapentaenoic acid (DPA), the structure is C22:5ω6, and for arachidonic acid (ARA), it is 20:4ω6 **(Figure 35 A)** (Hayashi *et al*., 2016). For dihomo-γ-linolenic acid (DGLA), it is C20:3n-6, and for γ-linolenic acid (GLA), it is C18:3n-6 **(Figures 35 B)** (Desbois and Lawlor, 2013).

A)



B)

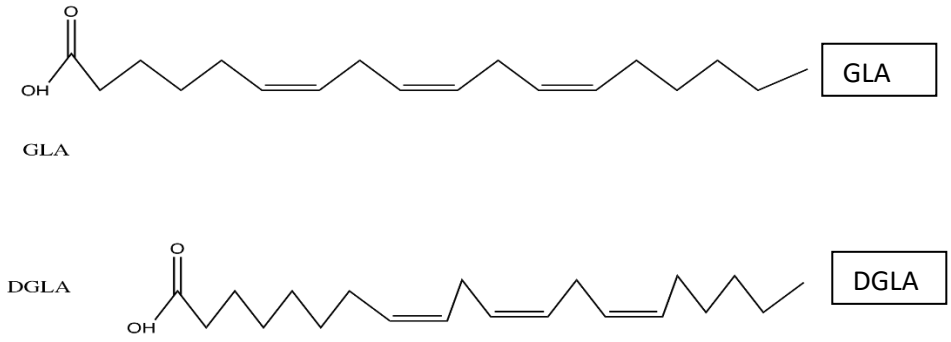


Figure 35:A) Structure of common PUFAs in bacteria the Image adapted from (Hayashi *et al*., 2016). **B)** Structure of GLA and DGLA this picture taken from (Desbois and Lawlor, 2013).

5.1.1 Biosynthesis of polyunsaturated fatty acids in bacteria

There are two biosynthetic pathways are used in the production of PUFAs:

The first pathway is the aerobic desaturase or elongase pathway that is prevalent in fungi, plants, bacteria and microalgae. It involves desaturase and elongase enzymes that catalyse reactions in oleic acid (C18:1 ω9) (Hayashi *et al.* 2016). PUFAs as a result of several desaturation cycles of oleic acid. DHA, EPA, and ARA are also known as long-chain PUFAs (LC-PUFAs) that are synthesised in eukaryotes and prokaryotes. Thus, the aerobic pathway for synthesising LC-PUFAs is based on desaturation and elongation (Hayashi *et al*. 2016).

The second pathway is the anaerobic PUFA synthase pathway is found in bacteria and eukaryotic microalgae. This pathway comprises PUFA synthases that use acetyl CoA and malonyl-CoA as the beginning and extender entities in the biosynthesis of PUFAs as shown in **Figure 36** (Hayashi *et al*., 2016).

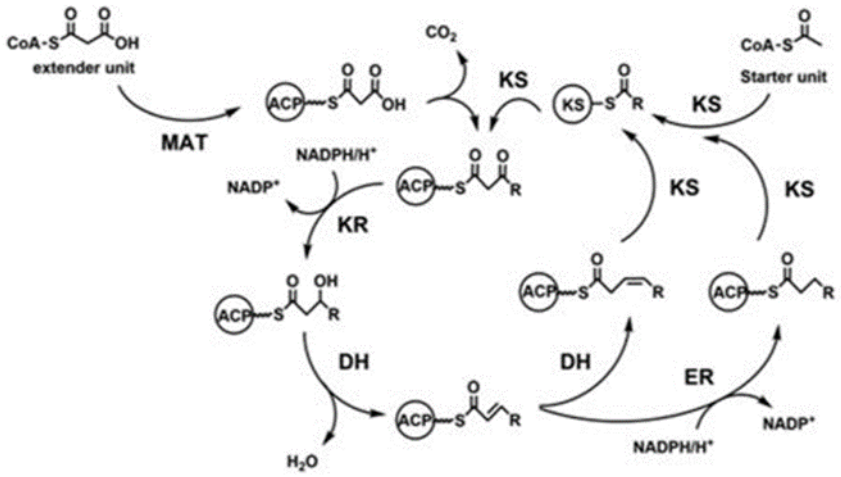


Figure 36: Biosynthesis of PUFA in bacteria, the picture taken from (Hayashi *et al*. 2016).

Long-chain PUFAs (LC-PUFAs) encompasses all PUFAs with 18 to 20 or more carbon atoms in their chains (Abedi and Sahari, 2014). The biosynthesis of LC-PUFAs in bacteria involves 3 main stages of initiation, extension and chain termination **(Figure 37).** At the initiation stage of biosynthesis of LC-PUFAs, the activation of the acyl-carrier proteins (ACPs) from their dormant forms is observed.

Phosphopantetheinase (PPTase) is a catalyst in this process. The next step is an extension process that consists of the elongation, formation of double bonds, and the generation of the structure of the final product. The activated ACP accepts acetyl and malonyl groups from acetyl-CoA and malonyl-CoA in reactions catalysed by acyltransferase (AT) and malonyl-CoA acyltransferase (MAT) (**Figure 37**) (Yoshida *et al*. 2016). The final stage of the biosynthesis of LC-PUFA is the incorporation of compound into lipids (Yoshida *et al*. 2016).

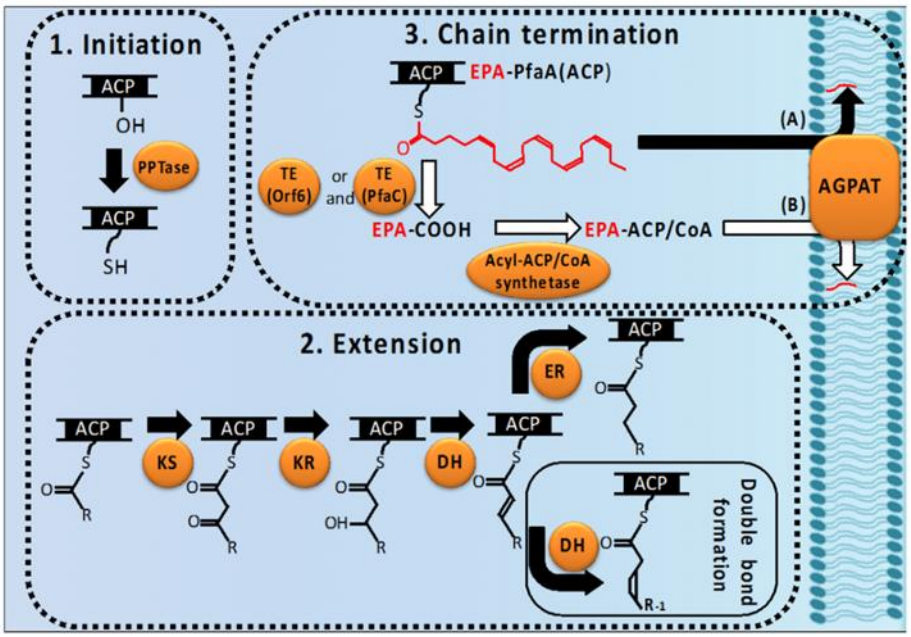


Figure 37: The Biosynthesis of LC-PUFAs in bacteria, the picture taken from (Yoshida *et al*. 2016).

5.1.2 Biological role of polyunsaturated fatty acids in bacteria

Normally, PUFAs are implicated in the adaptation of bacterial cells to osmotic stress. PUFAs in the membranes of bacterial cells play the same roles as in other organisms with the focus on guaranteeing the survival through energy storage (Yoshida *et al.* 2016).

According to (Elrazak and Glassey 2013) PUFAs are important for "modulating the architecture, dynamics, phase transition, and permeability of membranes and the cohesion of membrane-associated processes." PUFAs guarantee the selective permeability of membranes in cells in prokaryotes, as well as their significant

flexibility. Moreover, membrane-bound proteins are also regulated with the help of PUFAs.

The function of LC-PUFAs such as EPA, ARA and DHA is that they play a part in the resilience of bacteria against oxidising agents such as hydrogen peroxide (Fu *et al*., 2015, Yoshida *et al*., 2016). It has also been reported that EPA plays a role in the development of bacteria in conditions of low temperatures and high pressures, particularly in *Shewanella* species and EPA ensures the structural integrity of cells by influencing the extent of hydration and promotes cell division at elevated pressures. In addition (Yoshida *et al.* 2016, p. 10).

5.1.3 Antibiotic activity of polyunsaturated fatty acids in bacteria

LC-PUFAs have well-known antimicrobial and anti-inflammatory properties associated with preventing the growth of pathogenic microorganisms. As a result, LC-PUFAs are used in topical therapies for Gram-positive contagions infection. The antimicrobial properties of EPA, DHA, GLA, DGLA and 15-hydroxyeicosatrienoic were tested against *Staphylococcus aureus* and *Propionibacterium acnes*. The LC-PUFAs were shown to prevent the growth of the bacteria at minimum inhibitory concentrations ranging from 32 to 1024 mg/L (Desbois & Lawlor 2013). The LC-PUFAs inhibited the growth of *P. acnes* and demonstrated bactericidal properties on *S. aureus* only. *S. aureus* cells were killed within 15 to 30 minutes following exposure to the LC-PUFAs (Desbois & Lawlor 2013).

5.2 Bioinformatics analysis for polyunsaturated fatty acid gene clusters regulated by *sco*0110

S. coelicolor has 16 proteins highly similar to *sco*0110 shown in **(Table 1)** each of them responsible for controlling different pathways (Xu *et al*., 2012), the SCO0110 may be involved in promoting biosynthesis of polyunsaturated fatty acid. Bioinformatic analysis helped to identify the polyunsaturated fatty acids which are due to secondary metabolite gene cluster in *S. coelicolor*.

As explained in section 1.5, antiSMASH and BLAST can be used to proposed function of gene clusters. This was done for the *sco0110* gene as well.

Tools to do this antiSMASH (http://antismash.secondarymetabolites.org/). (**Figure 38 A & B)**. **).** AntiSMASH successfully identified a polyunsaturated fatty acid biosynthetic gene cluster in *S. coelicolor* **(Figure 39)**. In addition, it is useful for novel natural products discovery and bioinformatics tools such as the National Centre for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and useful to identify Proposed function for all this genes of polyunsaturated fatty acid metabolite gene clusters in (**Table 21).** The SCO0110 protein is found in *Streptomyces coelicolor* A3(2). It is a proposed pathway specific transcriptional activator for the polyunsaturated fatty acid biosynthetic gene cluster.

A)

Identified secondary metabolite clusters

Cluster	Type	From	To
The following clusters are from record AL939104.1:			
Cluster 1	T1pks-Otherks	86637	139654
Cluster 2	Terpene	166501	192038
Cluster 3	Lantipeptide	235986	271084

B)

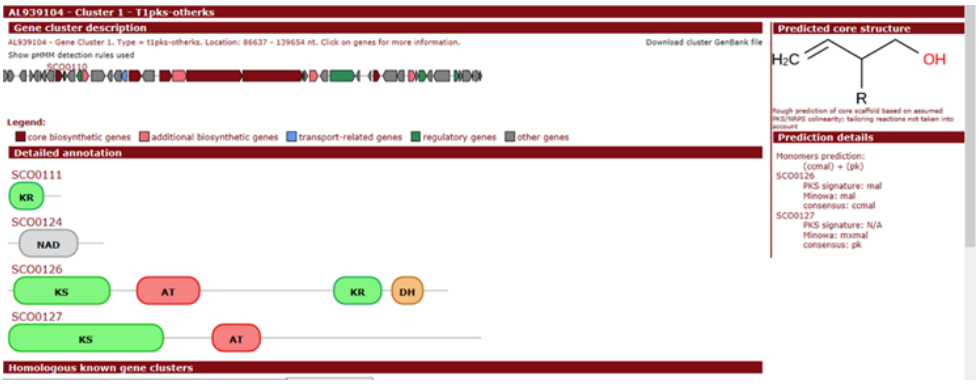


Figure 38: Identify polyunsaturated fatty acid secondary metabolite genes cluster in *S. coelicolor* by using antiSMASH

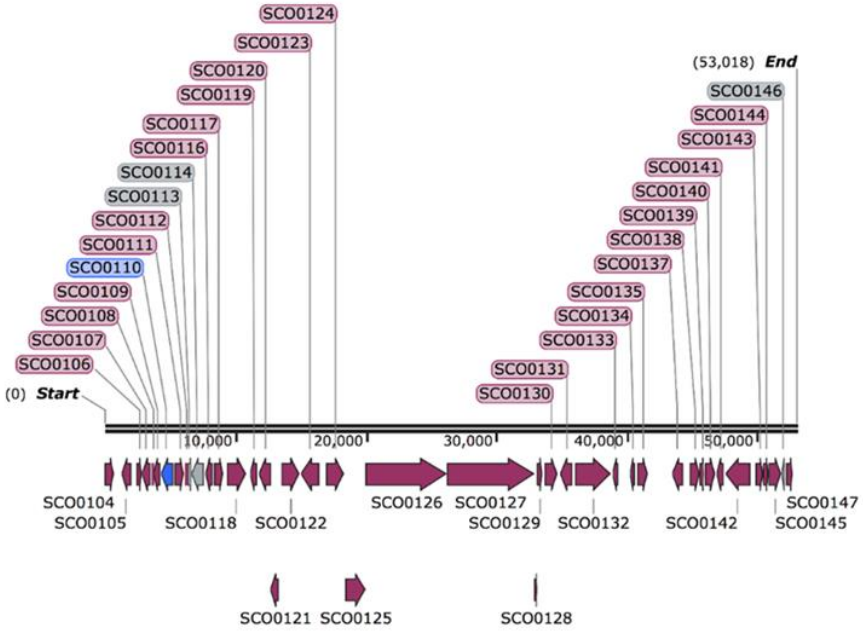


Figure 39: Proposed the polyunsaturated fatty acid genes cluster contain 42 genes.

Table 21: Proposed function of the biosynthetic genes from the sco0110 cluster.

Genes	**Protein (number of aa) GenBank**	**Homologue (% identity / % similarity) Organism GenBank**	**Putative function**
*sco*0108	WP_011026852.1 49 aa	LysR family transcriptional regulator (43% /60%) *Pseudomonas syringae* group WP_054085804.1	Hypothetical protein
*sco*0109	WP_011026853.1 156 aa	Hypothetical protein (89%/88%) *Streptomyces lividans* TK24 EFD71859.1	Hypothetical protein
*sco*0110	WP_011026854.1 279 aa	DNA-binding protein (37% /51%) *mmyB Streptomyces coelicolor* WP_030871026.1	DNA-binding protein
*sco*0111	WP_011026855.1 250 aa	Oxidoreductase (96%/97%) *Streptomyces coelicoflavus* WP_007387339.1	Oxidoreductase
*sco*0112	WP_011026856.1 51 aa	Hypothetical protein (96%/ 96%) *Streptomyces violaceoruber* WP_037897918.1	Hypothetical protein
*sco*0113	AL939104.1 No protein		pseudo
*sco*0114	AL939104.1 No protein		pseudo
*sco*0116	WP_011026857.1 188 aa	Transcriptional regulator, TetR family (99%/100%) *Streptomyces lividans* WP_003978762.1	TetR family transcriptional regulator
*sco*0117	WP_011026858.1 230 aa	Short-chain dehydrogenase (89%/93%) *Streptomyces griseofuscus WP_037664029.1*	Short chain dehydrogenase
*sco*0118	WP_011026859.1 509 aa	Glycoside hydrolase 43 family protein (99%/99%)	Xylosidase/arabinosidase

		Streptomyces violaceoruber WP_078653367.1	
*sco*0119	WP_011026860.1 173 aa	Hypothetical protein (38%/61%) *Streptomyces violaceoruber WP_074756121.1*	Hypothetical protein
*sco*0120	WP_011026861.1 291 aa	LysR family transcriptional regulator (99%/99%) *Pseudomonas abietaniphila WP_030871018.1*	Hypothetical protein
*sco*0121	WP_011026862.1 204 aa	ABC transporter permease (99%/99%) *Streptomyces violaceoruber]* WP_030871018.1	ABC transporter ATP-binding protein
*sco*0122	WP_011026863.1 458 aa	Monooxygenase (99%/98%) *Streptomyces violaceoruber WP_030871014.1*	Flavin-containing monooxygenase
*sco*0123	WP_011026864.1 448 aa	Hypothetical protein (99%/99%) *Streptomyces lividans* TK24 AIJ18330.1	Hypothetical protein
*sco*0124	WP_011026865.1 453 aa	Hypothetical protein (98%/98%) *Streptomyces violaceoruber WP_030871009.1*	Hypothetical protein
*sco*0125	WP_011026866.1 527 aa	*2-nitropropane dioxygenase (99%/100%) Streptomyces violaceoruber WP_030871006.1*	Oxidoreductase
*sco*0126	WP_011026867.1 2082 aa	Beta keto-acyl synthase (99%/99%) *Streptomyces violaceoruber* WP_078653366.1	Putative multi-domain beta keto-acyl synthase
*sco*0127	WP_011026868.1 2240 aa	MULTISPECIES: type I polyketide synthase (99%/99%) *Streptomyces sp* WP_038535457.1	Type I polyketide synthase

*sco*0128	WP_011026869.1 60 aa	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 96%/ 67% *Streptosporangium canum* SFI08982.1	Hypothetical protein
*sco*0129	WP_011026870.1 124 aa	Hypothetical protein 99%/ 99% *Streptomyces lividans* WP_078653365.1	Hypothetical protein
*sco*0130	WP_003978746.1 304 aa	MBL fold metallo-hydrolase *Streptomyces sp* 99%/100% WP_087804028.1	Beta-lactamase
*sco*0131	WP_011026871.1 279 aa	MULTISPECIES: secreted protein *Streptomyces sp* 99%/100% WP_016324965.1	Hypothetical protein
*sco*0132	WP_011026872.1 919 aa	helix-turn-helix transcriptional regulator *Streptomyces sp* 99%/100% WP_093455274.1	Transcriptional regulator
*sco*0133	WP_011026873.1 126 aa	MULTISPECIES: hypothetical protein *Streptomyces sp* 98%/100% WP_003978743.1	Hypothetical protein
*sco*0134	WP_003978742.1 101 aa	hypothetical protein 98%/100% Streptomyces sp. WP_106517060.1	Hypothetical protein
*sco*0135	WP_011026874.1 239 aa	MULTISPECIES: alpha/beta hydrolase *Streptomyces sp* 99%/100% WP_030870993.1	Hypothetical protein
*sco*0136	WP_014058145.1 517 aa	PTS ascorbate transporter subunit IIC 99%/100% *Streptomyces violaceusniger* WP_059148306.1	PTS system ascorbate-specific transporter subunit IIC

*sco*0137	WP_011026876.1 266 aa	MULTISPECIES: phosphotransferase system protein 99%/100% *Streptomyces sp* WP_016324970.1	Sugar-transport protein
*sco*0139	WP_011026877.1 257 aa	MULTISPECIES: SDR family NAD(P)-dependent oxidoreductase 99%/100% *Streptomyces sp* WP_030870984.1	Short chain dehydrogenase
*sco*0140	WP_003978737.1 106 aa	Nuclear transport factor 2 family protein 99%/100% *Streptomyces violaceoruber* WP_030870981.1	Hypothetical protein
*sco*0141	WP_011026878.1 275 aa	MerR family DNA-binding transcriptional regulator 99%/100% *Streptomyces sp*. WP_093455278.1	MerR family transcriptional regulator
*sco*0142	WP_011026879.1 183 aa	MULTISPECIES: EF-hand domain-containing protein 99%/100% *Streptomyces violaceorubidus* WP_030182965.1	Calcium-binding protein
*sco*0143	WP_011026881.1 178 aa	MULTISPECIES: TetR/AcrR family transcriptional regulator 99%/100% *Streptomyces sp* WP_093455281.1	Hypothetical protein
*sco*0144	WP_003978731.1 119 aa	WP_093455282.1 Hypothetical protein 99%/100% *Streptomyces sp* WP_093455282.1	Hypothetical protein
*sco*0145	WP_011026882.1 342 aa	Alpha/beta hydrolase 99%/100% *Streptomyces sp* WP_106517057.1	Hypothetical protein
sco0146	Unknown		Pseudo

*sco*0147	WP_011026883.1 163 aa	DUF2975 domain-containing protein 99%/100% *Streptomyces sp*. WP_106517056.1	Transmembrane transport protein

5.3 Overexpression *sco*0110 gene from *S. coelicolor* M145

SCO0110 protein is found in *Streptomyces coelicolor* A3(2). It is a proposed to be a transcriptional activator for the polyunsaturated fatty acid biosynthetic gene cluster. The polyunsaturated fatty acid biosynthetic gene cluster is tightly regulated at the transcriptional level, and is usually not produced and if it is produced, then it is produced in a very small quantity in laboratory growth conditions

Overexpression of transcriptional activators under the control of *ermEp** is expected to unlock the production of compounds from neighbouring cryptic biosynthetic gene clusters. To investigate this, an experiment is performed in which if overexpression of this activator will switch on the polyunsaturated fatty acid biosynthetic genes cluster and express other *sco* gene to produce the polyunsaturated fatty acid illustrated in **Figure 39**. To this end, it is very important to clone the *sco*0110 gene in the pOSV556 vector (which has the ermEp* promotor) and then introduce it into *S. coelicolor* m145.

In order to do this, PCR was used to amplify specific the *sco*0110 gene. Two primers were designed (*sco*0110 forward and reverse) (T02 in **Table 4**). PCR products were run on 1% agarose gel in order to verify the size of the construct which yields a band at the expected 840 bps (**Figure 40 A)**. A NanoDrop ND-1000 spectrophotometer was used to measure the concentration of the *mmyB* construct (109.4 ng/ μl).

Digestion and ligation of plasmid pOSV556 followed by insertion of *sco0110* were performed as part of cloning and plasmid analysis. The plasmid created by this ligation is known as pVN3 (containing *sco*0110 under the control of ermEp*). Plasmid DNA was amplified in *E.coli* Top10 strain and purified using the mini-prep procedure; the agarose gel run following the mini-prep of the construct is shown in **Figure 40 B**. This construct was then ready to transform into non-methylation *E. coli* ET12567/pUZ8002 host cell (by electroporation) to introduce into *S. coelicolor* M145.

The *S. coelicolor* M145 already contains a methyl-sensing restriction system (MacNeil *et al*., 1992).

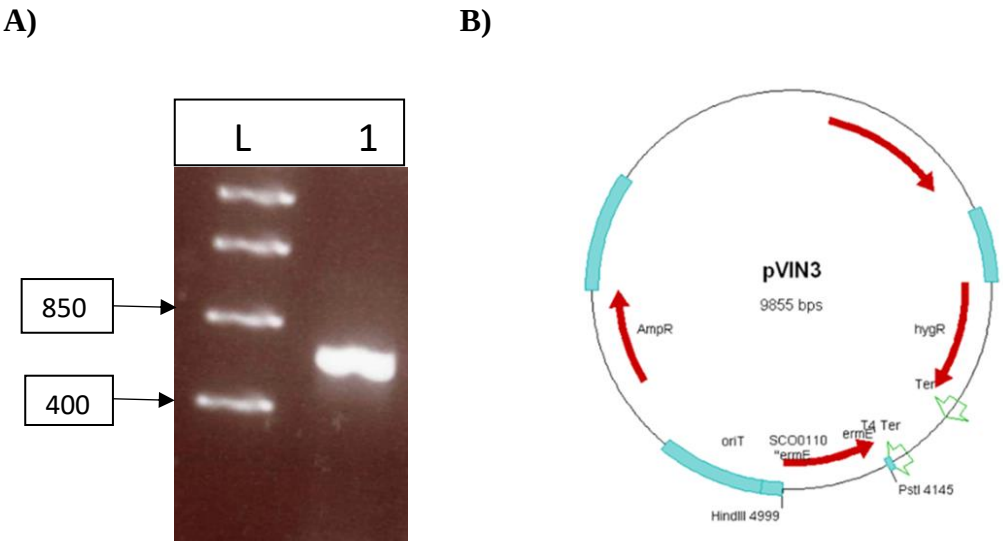


Figure 40: **A)** PCR was used to amplify *sco0110*. This reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 840 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). **B)** Schematic map for the *sco0110* ligated into pOSV556which has been named pVIN3 and is 9855 bps in size.

5.4 Screen of the vector via a PCR amplification

PCR screening was used to amplify the *sco0110* target sequence of gene using two primers **(primers number T02 in Table 4).** PCR products were run on 1% agarose gel in order to verify the size of the expected band which was 840 bps **(Figure 41).** At this stage, the DNA construct after cloning was sent for sequencing analysis to verify that no mutations had taken place during the cloning steps and this was confirmed in the returned sequencing data.

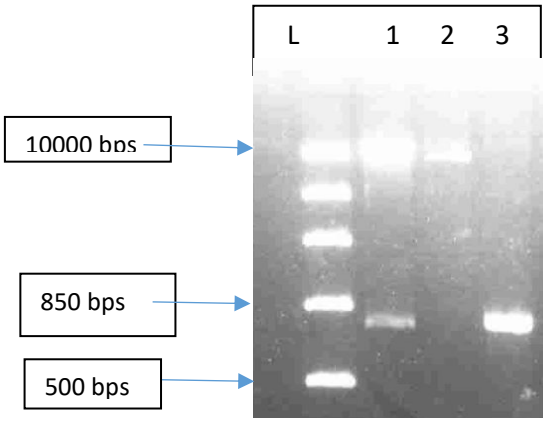


Figure 41: Assessment of the pVN3 by doing PCR amplification used tow primers. This was then run on a 1% agarose gel in order to check the size of the expected band. FastRuler high range DNA ladder (Track L). The *sco*0110 insert around 840 bps and vector is 9009 bps (Track 1). The pOSV556 band around 9009 bps as positive control (Track 2). Positive control was used *sco*0110 gene (Track 3).

5.5 Assessment of the pVN3 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

To introduce the pVN3 plasmid into *S. coelicolor* M145, non-methylation *E. coli* ET12567/pUZ8002 host cells (by electroporation) were required to transfer the plasmid using intergenic conjugation, this is because *S. coelicolor* M145 contains a methyl-sensing restriction system. The construct was successfully introduced through conjugation in *S. coelicolor* M145. After genomic DNA extraction of pVN3, which was integrated into *S. coelicolor* M145, a concentration of 211.9 ng/µl for the purified plasmid was obtained. PCR screening was used to amplify the DNA target sequence of the gene using two designed primers. The PCR products were run on 1% agarose gel and observed a single band around the expected size of 840 bps **(Figure 42).** Once screened, this plasmid was transferred into *S. coelicolor* M145 via conjugation.

Using intergenic conjugation, we were hence able to successfully integrate the *sco0110* gene into *S. coelicolor* M145 yielding the desired pVN3 plasmid.

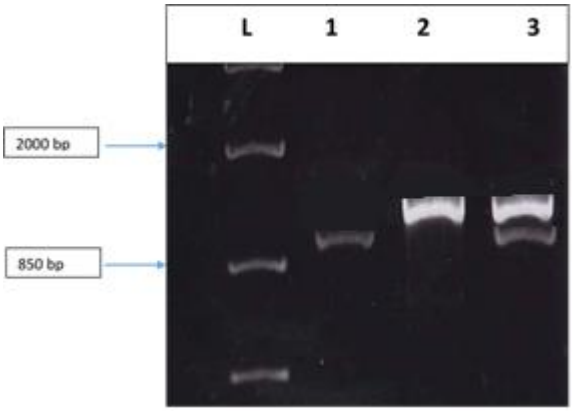


Figure 42: Assessment of the genomic DNA by using PCR amplification for pVN3 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). FastRuler high range DNA ladder (Track L). Positive control used *sco*0110 ligated pOSV556 (Track 2). *S. coelicolor* M145 only was used as negative control (Track 3).

5.6 Transcription analysis of the poly unsaturated fatty acid biosynthetic gene through RT-PCR

To find the expression-specific pathway after overproduction of pVN3 integrated in *S. coelicolor*, reverse transcription-PCR was performed on the extracted total RNA from pVN3 integrated into *S. coelicolor* M145 and wild type *S. coelicolor* M145.

Total RNA was extracted from *S. coelicolor* M145 strain in which pJT1 was integrated into. Three expected bands representing intact ribosomal RNA were observed with the total RNA concentration of 129 ng/ µl. RNA products were run on 1% agarose gel (in order to verify the size of the expected bands 5s, 16s and 23s) **(Figure 43).**

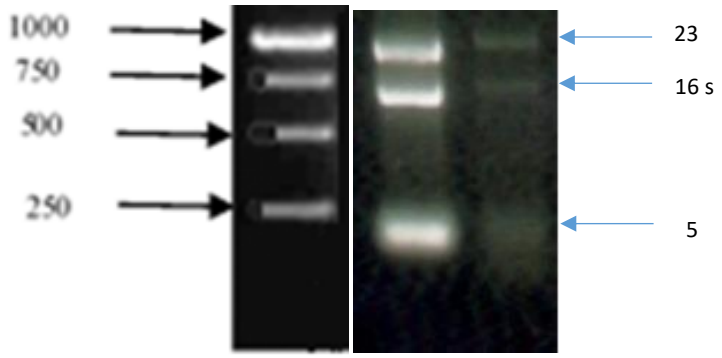


Figure 43: Total RNA was extracted from *S. coelicolor* M145 grow in MMS. This reaction solution was run on 1% agarose gel and ribosomal RNA were clearly observed (Track 1.2) 1kb ladder was used (Track L).

5.6.1 Transcription analysis by RT-PCR for *sco0108, 0110, 0117, 0124, and sco0127*

The RNA was converted into single stranded complementary DNA (cDNA) by using cDNA Superscript II RT. As a control, a sample with no reverse transcriptase used to ensure that the results observed are that of the amplification of cDNA and not DNA contamination. The cDNA synthesised were used as a template in thermal cycler reactions to determine if *sco*0110 gene was transcribed. PCR products were run on 1% agarose gel as shown in **Figure 44**.

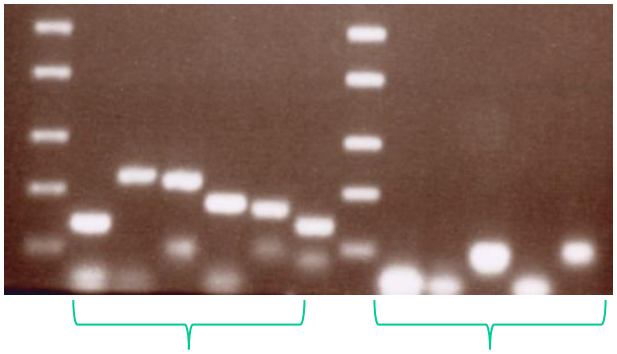


Figure 44: Analysis of the expression of a selection *sco* genes *sco0108, 0110.0117, 0124* and *sco0127*in *S. coelicolor* M145/ pVN3 using RT-PCR using as a control primers designed to amplify the *hrdB* gene considered to be constitutively expressed (Track 1). The negative control was performed using *S. coelicolor* M145 (Track 2).

5.7 Bacterial growth conditions and antibiotic assay

5.7.1 *S. coelicolor* M145 with and without pVN3 growth conditions

In order to confirm that the construct has been incorporated in *S. coelicolor*, the pOSV556 vector containing the cloned *sco*0110 gene (pVN3) was transferred to *Streptomyces coelicolor* M145 by intergenic conjugation according to standard methods by (Kieser, 2000), then keep it in incubation at 30 ºC for 6 days to allow growth of the exconjugants in SFM agar **(Figure 45).** It was observed that the plate with integrated overproduction with pJT1 shows growth and produced dark blue pigments (plate 2 Figure 56). However the wild type shows white colony. From this it can be concluded that after integration of the pJT1 plasmid, it was able to switch on the silent pathway and produced lantibiotic antibiotics. However, in the wild type, this is not observed.

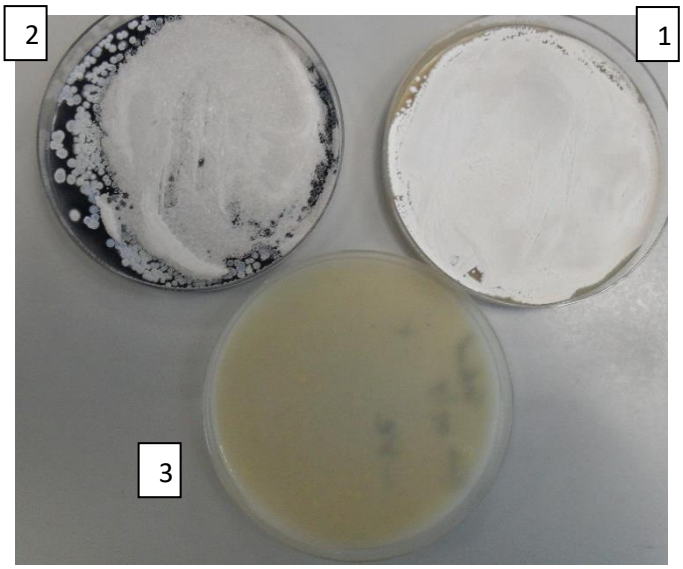
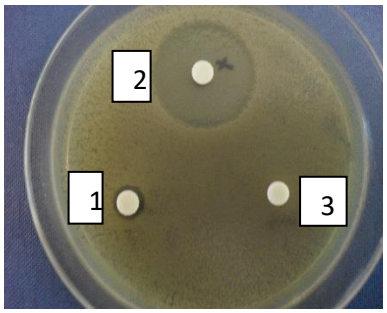


Figure 45: Culture *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pVN3 was integrated into *S. coelicolor* M145 (plate 2), *S. coelicolor* M145 growth without integrated pVN3 as wild type (plate 1) and use SFM media without inoculation any bacteria as negative control (plate 3).

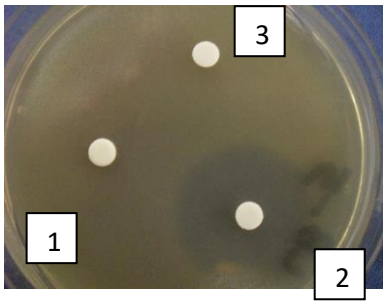
5.7.2 Antibiotic assay

In order to confirm that after integration of the pVN3 plasmid, it was able to switch on the silent pathway and produced polyunsaturated fatty acids antibiotics. All the five genes *sco*0108, *sco*0110, *sco*0117, *sco*0124, *sco*0127) were expressed when the *sco0110* was integrated in *S. coelicolor* M145. Antimicrobial activity of *S. coelicolor* M145/ pVN3 was test using antibiotic sensitivity assay. The antibiotic sensitivity assay shows the ability of drug in inhibition zone for the growth of the bacteria *Bacillus subtilis S. aureus* but not active around the *E. coli* **(Figure 46).** The positive control was Apramycin Track 2, the antibiotic drug of the plasmid pJh2 was integrated into S. coelicolor W81 and metabolites were tested after organic extraction showing zone of inhibition (Track 1). Hence it can be deduced that methylenomycin antibiotic are produced.

Bacillus subtilis



E. coli



S. aureus

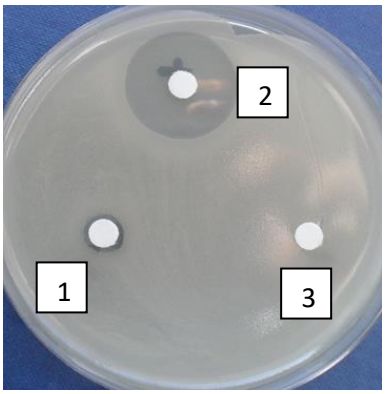


Figure 46: Antibiotic assay used *Bacillus subtilis*, *E. coli* and *S. aureus* on LB agar pH 5. The plasmid PVN3 was integrated into *S. coelicolor* M145 and metabolites were tested without organic extraction showing zone inhibition but not active around the *E. coli* (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the PVN3 was used as negative control (Track 3).

5.7.3 Identification of predicted lantibiotic compounds in *S. coelicolor* M145 heterologous hosts

Metabolites between the *S. coelicolor* with pVN3 heterologous host strains and *S. coelicolor* M145 wild type were compared by LC-MS and one new compound was identified. The m/z= 1475 was only present in *S. coelicolor* strains containing pVN3 and was absent in *S. coelicolor* M145 control (**Figure 47**).

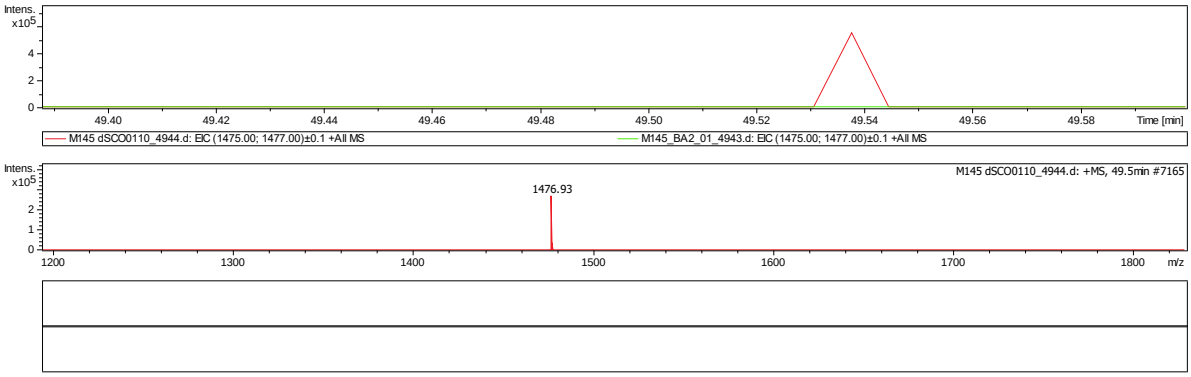


Figure 47: UV chromatogram the predicted antibiotic compound. Extracted ion chromatograms m/z = 1476.93 which is not present when LC-MS of wild type is performed.

5.8 Combined antibiotic activity

This section explores the combined antibiotic activity of novel *S. coelicolor* compounds against *B. subtilis* and *S. aureus.* Antimicrobial activity of *S. coelicolor* M145/pVN3, pJT1 and *S. coelicolor* W81/pJH2 was cultured in Smm broth **Figure 50** then tested using antibiotic sensitivity assay. The new compounds produced by *S. coelicolor* engineered strains was assayed against *B. subtilis* and *S. aureus*. The antibiotic sensitivity assay shows the ability of the antibiotics produced from a combination of these strains shows that there is a large drug inhibition zone which does not allow *B. subtilis* and *S. aureus* to grow. The overexpression of *mmyB* and *mmyB*-like genes together can turn on silent biosynthetic pathways, this novel strategy results in the overproduction and discovery of new natural products can be observed (**Figure 48 and 49).** LC-MS need to be performed on the metabolites to find the new compounds which may be suitable for human and agriculture.

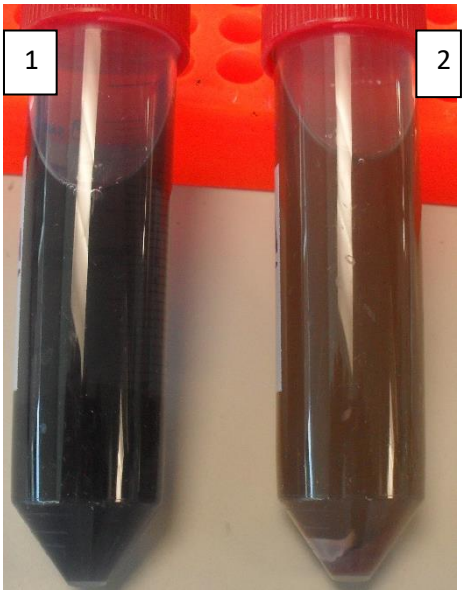
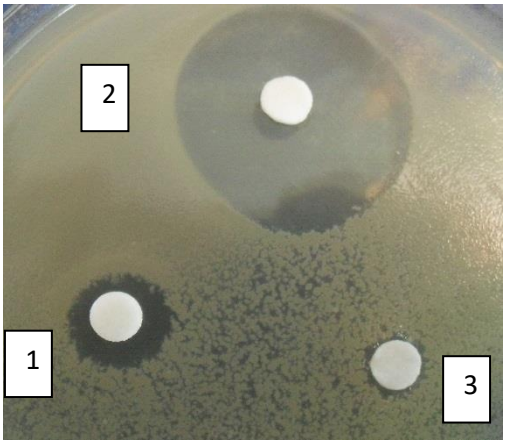


Figure 48: Culture *S. coelicolor* engineered strained (pJH2, pJT1 and PNV3) shows in MMS media dark blue Track 1. However the colour for wild type which was include both *S. coelicolor* W81/M145 has brown colour Track 2.

Bacillus subtilis



S. aureus

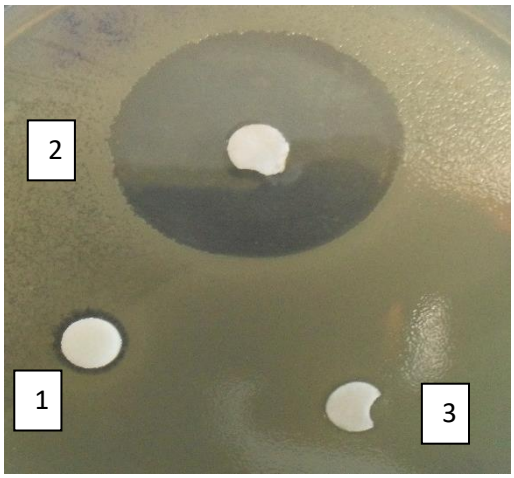


Figure 49: Antibiotic assay used *Bacillus subtilis* and *Staph aureus* on LB agar pH5. *S. coelicolor* engineered strained (pJH2, pJT1 and PNV3) metabolites were tested without organic extraction showing zone inhibition (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145/W81 without the engineered strained was used as negative control (Track 3).

By culturing the following:

1- *S. coelicolor* M145/ pVN3
2- *S. coelicolor* M145/pJT1
3- *S. coelicolor* W81/pJH2

S. coelicolor engineered strains were tested individually using antibiotic sensitivity assay. The new compounds produced by *S. coelicolor* engineered strains have been assayed against *B. subtilis*. The results show each compound that is produced from *S. coelicolor* engineered strains kills *B. subtilis* **(Figure 50 A).**

On the other hand, while mixing an equal amount 15 µL of *S. coelicolor* M145/ pVN3 with 15ml of *S. coelicolor* M145/pJT1, shows that the combination of the two *S. coelicolor* engineered strains was not active against *B. subtilis* and *S. aureus*. Also mixing another equal amount of either *S. coelicolor* M145/ pVN3 with *S. coelicolor* W81/pJH2 or mixing an equal amount of both *S. coelicolor* W81/pJH2 with *S. coelicolor* M145/pJT1 will still shows no activity against *B. subtilis* and *S. aureus.* Furthermore mixing all three *S. coelicolor* engineered strains, M145/ pVN3, M145/pJT1 and W81/pJH2 will still show no zones inhibition on the agar overlay plate **(Figure 50 B).**

A) **B)**

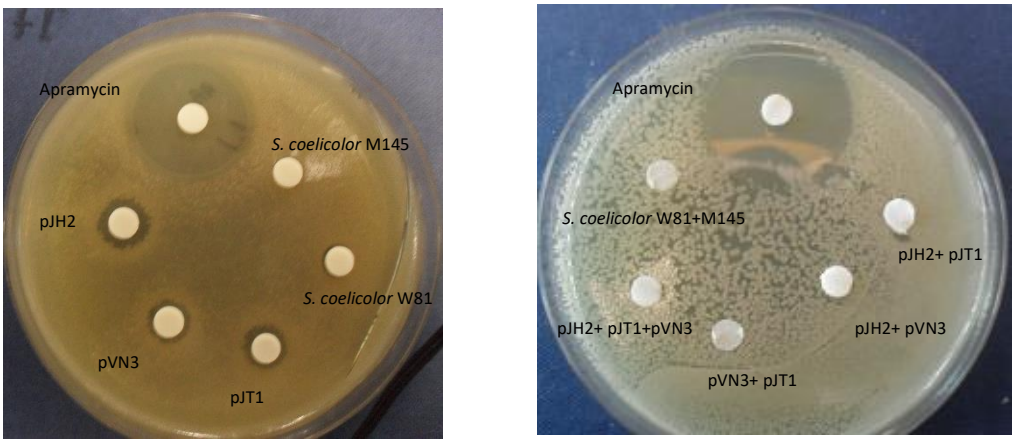


Figure 50: Antibiotic assay used *Bacillus subtilis* on LB agar pH5. *S. coelicolor* engineered strained (pJH2, pJT1 and PNV3) individually metabolites were tested without organic extraction individual was active zone inhibition *against B. subtilis*. However, while mixing an equal amount each of them 15 µL showed not active.

Chapter 6 Other mmyB-like protein

S. coelicolor has 16 proteins high similar to MmyB shown in (Table 1) . While 16 *mmyB*-like homologues are encoded within *S. coelicolor* each of them responsible for controlling different pathways some of the divergent proposed gene regulated show in the **Table 22.**

Table 22: *sco* all are putative DNA-binding protein

Divergent proposed gene regulated	Predicted function of gene cluster	comments
mmyf-mmyfR	Methylenomycin biosynthesis	In this project. Overexpression of transcriptional activators *mmyB* switching on the production of compounds from neighbouring cryptic biosynthetic gene clusters. was proved by RT-PCR
*sco*0104-*sco*0149	Polyunstaturated fatty acid biosynthesis	In this project. Overexpression of transcriptional activators *sco*0110 switching on the production of compounds from neighbouring cryptic biosynthetic gene clusters. was proved by RT-PCR
*sco*0237	short-chain dehydrogenase/reductase	
*sco*0890	short-chain dehydrogenase/reductase	
*sco*4681- *sco*4682	short-chain dehydrogenase/reductase, downstream gene encodes 4oxalocrotonate tautomeras	

*sco*6922-*sco*6944	lantibiotic biosynthesis	In this project. Overexpression of transcriptional activators *sco*6962 switching on the production of compounds from neighbouring cryptic biosynthetic gene clusters. was proved by RT-PCR
*sco*7141	short-chain dehydrogenase/reductase	
*sco*7707	Atu4866 family protein	SCO7705 similar to tetracyclin resistance oxidoreductase
*sco*7768 – *sco*7769	Nuclear transport factor 2-like protein, similar to RANGDPbinding; fused to gene for GDPsugar epimerase	Next to SCO7766 homolog of actVA4
*sco*7818	short-chain dehydrogenase/reductase (2,3-dihydro-2,3dihydroxybenzoate dehydrogenase)	Highly conserved in Streptomyces

The proteins high similar to MmyB Some of them as shown in **Table 23** successfully constructed after that introduce the *mmyB*-like gene in similar vectors into *S. coelicolor* using inter-generic conjugation then to assess the antibiotic assay after integrated *mmyB*-like in *S, coelicolor* which show after overexpression transctrapital activoter able to switch on gene clusters to produce natural products with mutant strain which inhipit growth *Bacillus subtilis. E. coli* and *S. aureus* except *sco*0236 and *sco*0891 were find this not active antibiotic activity see more details in appendix section.

All this test need more investigation to find the gene cluster by using AntiSmash for each *mmyB*-like. In additional, RT-PCR used to detection express gene cluster and by using chemical analysis for more identifications process.

Table23: Plasmids *mmyB*-like have been successfully constructed and test antibiotics activity.

Cloning genes in pOSV556	Conjugation in *S. coelicolor*	Antibiotic Activity	RT-PCR
sco0236	Done	Non active	Not done
sco0891	Done	Not done	Not done
sco7140	Done	Active	Not done
sco4680	Done	Active	Not done
sco7767	Done	Active	Not done
sco7817	Done	Active	Not done
sco7706	Done	active	Not done

6.1 Other mmyB-like transcriptional activators cloned under the control of a Streptomyces constitutive promoter

The same approach was used and the result as follow:

6.1.1 SCO0236

6.1.1.1 Cloning of *sco*0236

A) **B)**

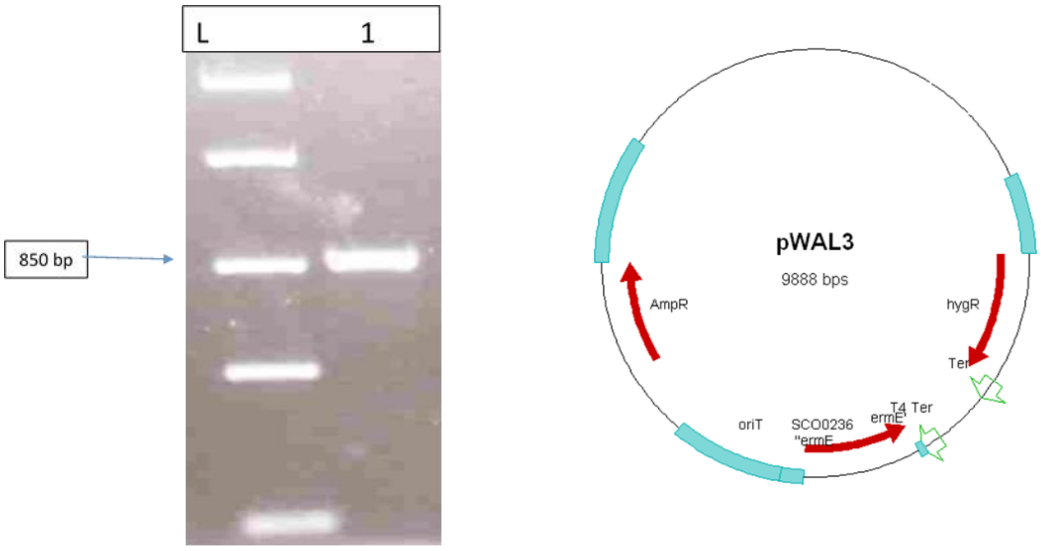


Figure 51:A) PCR was used to amplify *sco*0236 this reaction solution was run on 1% agarose gel in order to verify the size of the expected band 872 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). **B)** Schematic map for the sco0236 ligated into pOSCV556 which has been named pWA3 and it is 9888 bps in size.

6.1.1.2 Assessment of the pWAL3 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

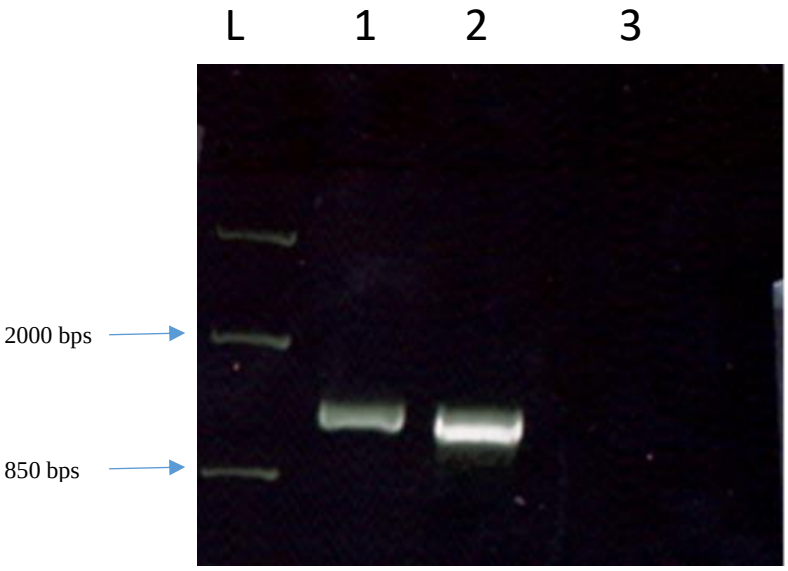


Figure 52: Assessment of the genomic DNA by using PCR screen for pWA3 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). FastRuler middle range DNA ladder (Track L). Positive control used cos0236 ligated pOSV556 (Track 2). *S. coelicolor* M145 only was used as negative control (Track 3).

6.1.1.3 Bacterial growth conditions and antibiotic

6.1.1.3.1 *S. coelicolor* M145 without pWAL3 growth conditions

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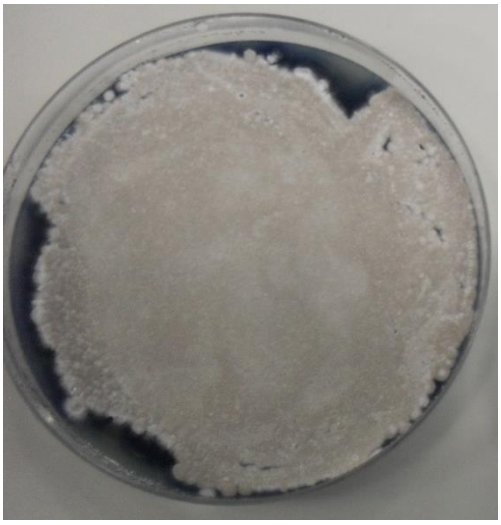
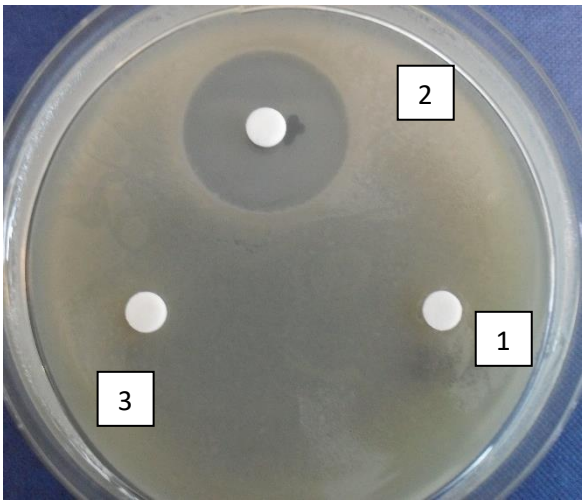


Figure 53: Culture *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pWAL3 was integrated into *S. coelicolor* M145.

6.1.1.3.2 Antibiotic assay

Bacillus subtilis



E. coli

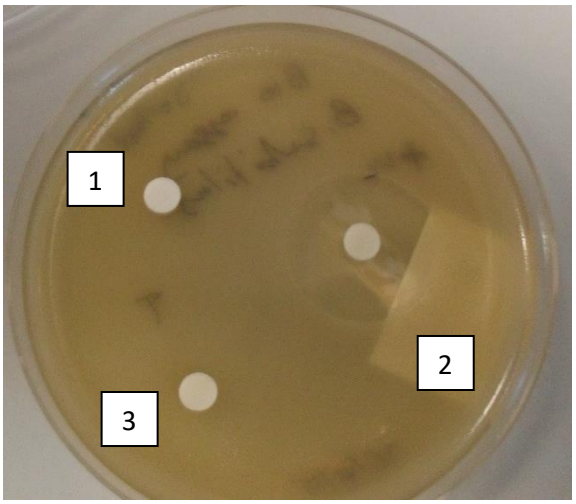


Figure 54: Antibiotic assay used *Bacillus subtilis and E. coli* on LB agar pH 5. The plasmid PWAL3 was integrated into *S. coelicolor* M145 and metabolites were tested and showing not inhibit growth of bacteria (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the PWAL3 was used as negative control (Track 3).

6.1.2 SCO7140

The same approach was used and the result as follow:

6.1.2.1 Cloning of *sco*7140

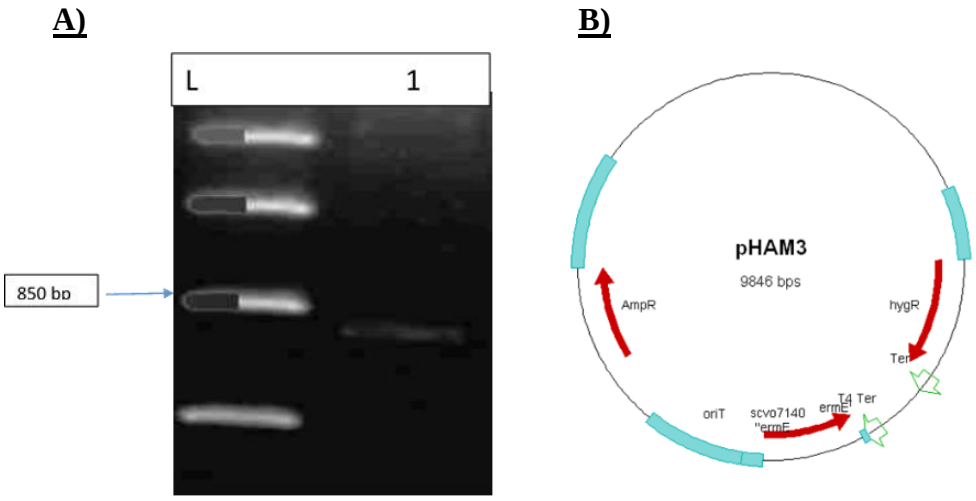


Figure 55: **A)** PCR was used to amplify *sco*7140. This reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 831 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). **B)** Schematic map for the sco7140 ligated into pOSV556 which has been named pHAM3 and is 9846 bps in size

6.1.2.2 Screen of the vector for correct insert

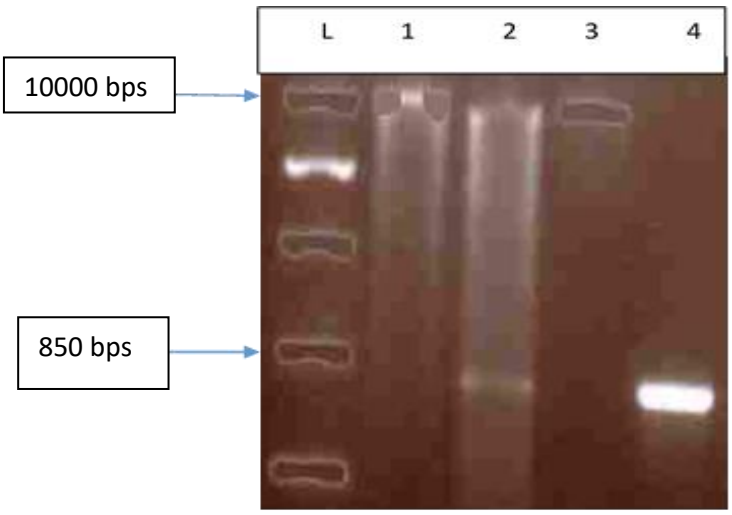


Figure 56: Assessment of the DNA fragment size. The purified DNA construct was ligated into pOSV556 vector and was digested using the restriction enzyme *Hind*III and *Pst*I. This was then run on a 1% agarose gel in order to chick the size of the expected bands. FastRuler high range DNA ladder (Track L), the size of the excised the *sco*7140 insert around 831 bps and vector is 9009 bps (Track 2). A positive control was used pOSV556 vector and was digested using the restriction enzyme *Hind*III (Track3) and the *sco*7140 gene produced from the PCR reaction (Track 4).

6.1.2.3 Assessment of the pHAM3 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

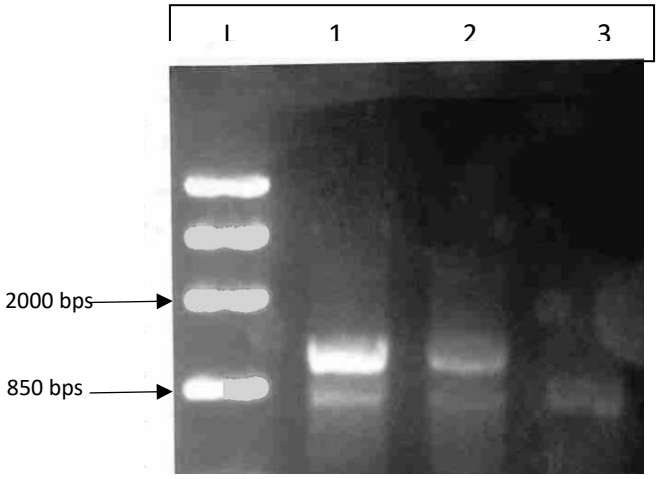


Figure 57: Assessment of the genomic DNA by using PCR screen for pHAM3 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). FastRuler high range DNA ladder (Track L). Positive control used *sco*7140 ligated pOSV556 (Track 2). *S. coelicolor* M145 only was used as negative control (Track 3).

6.1.2.4 Bacterial growth conditions and antibiotic

6.1.2.4.1 *S. coelicolor* M145 with and without pHAM3 growth conditions

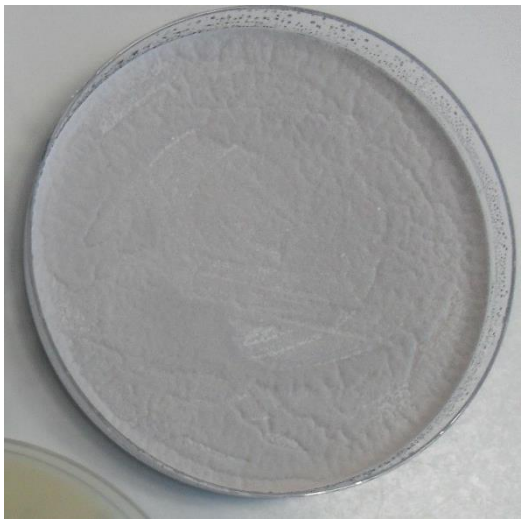
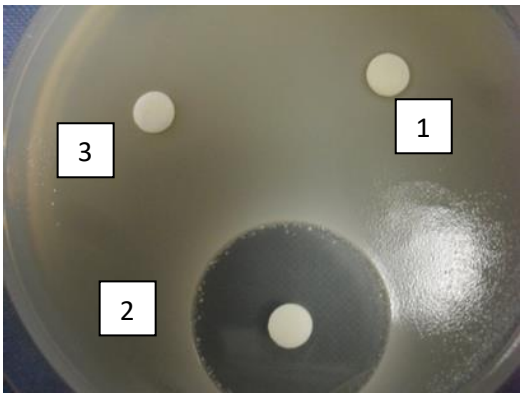


Figure 58: culture *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pHAM3 was integrated into *S. coelicolor* M145.

6.1.2.4.2 Antibiotic assay

Bacillus subtilis



E. coli

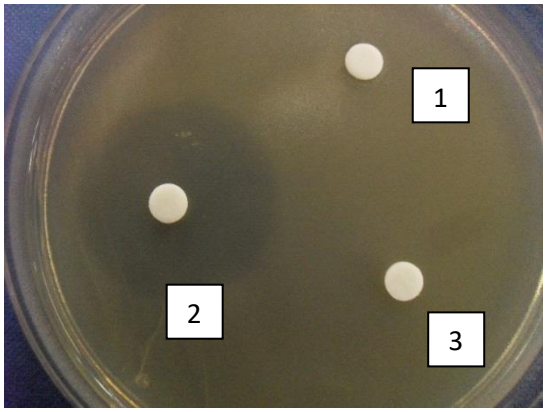


Figure 59: Antibiotic assay used *Bacillus subtilis* on LB agar pH 5. The plasmid pHAM3 was integrated into *S. coelicolor* M145 and metabolites were tested and showing zone inhibition (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the pHAM3 was used as negative control (Track 3).

6.1.3 SCO7706

The same approach was used and the result as follow:

6.1.3.1 Cloning of *sco*7706

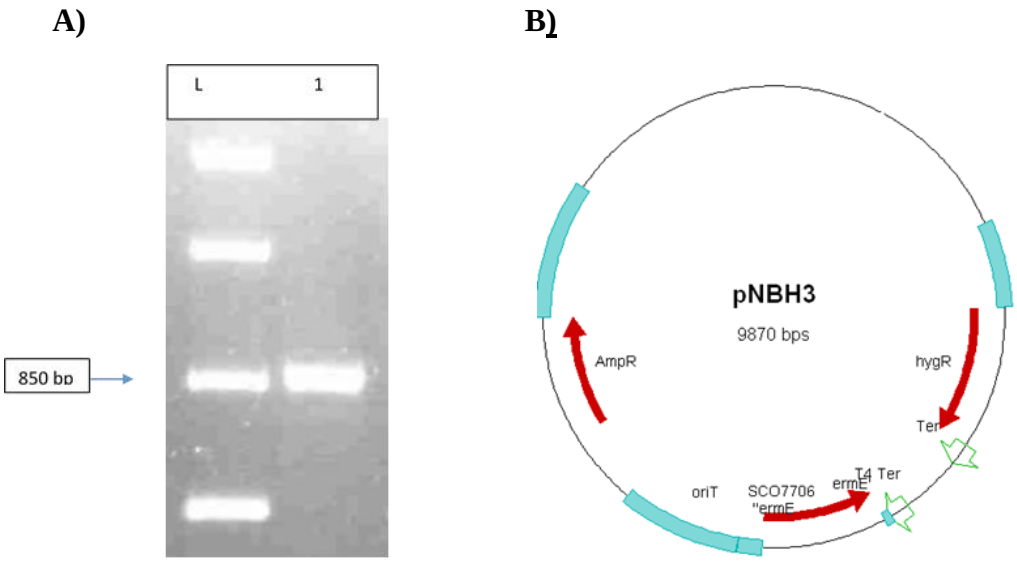


Figure 60: **A)** PCR was used to amplify *sco*7706 this reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 855 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). **B)** Schematic map for the sco7706 ligated into pOSV556 which has been named pNBH3 and is 9870 bps in size.

6.1.3.2 Screen of the vector via a PCR amplification

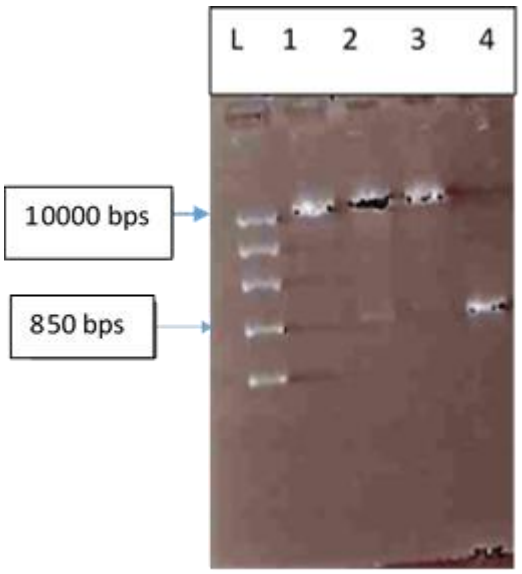


Figure 61: Assessment of the *sco7706* frament size by doing PCRAssessment of the sco0236 fragment size by doing PCR amplification used tow primers. This was then run on a 1% agarose gel in order to check the size of the expected band. FastRuler high range DNA ladder (Track L). The *sco0236* insert around 840 bps and vector is 9009 bps (Track 2&3). The pOSV556 band around 9009 bps as positive control (Track 1). Positive control was used *sco0236* gene (Track 3).

6.1.3.3 Assessment of the pNBH3 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

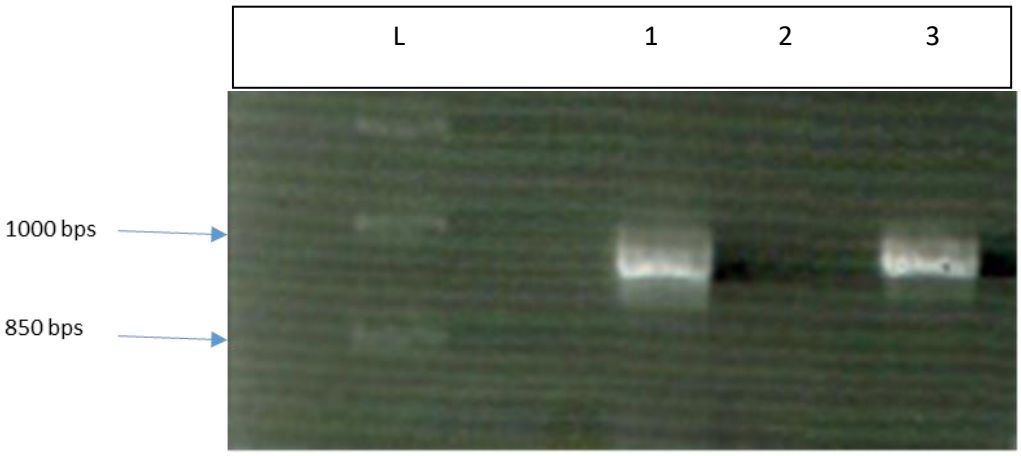


Figure 62: Assessment of the genomic DNA by using PCR screen for pNBH3 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). FastRuler high range DNA ladder (Track L). Positive control used *sco*7706 ligated pOSV556 (Track 2). *S. coelicolor* M145 only was used as negative control (Track 3).

6.1.3.4 Bacterial growth conditions and antibiotic

6.1.3.4.1 *S. coelicolor* M145 with and without pNB3 growth conditions

Figure 63: culture *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pNBH3 was integrated into *S. coelicolor* M145.

6.1.3.4.2 Antibiotic assay

Antimicrobial activity of *S. coelicolor* M145/ pNBH3 was test using antibiotic sensitivity assay. The antibiotic sensitivity assay shows the ability of drug in inhibiting the growth of microorganisms such as *Bacillus subtilis*, *E. coli* and *S. aureus* **Figure 82.**

Bacillus subtilis

E. coli

S. aureus

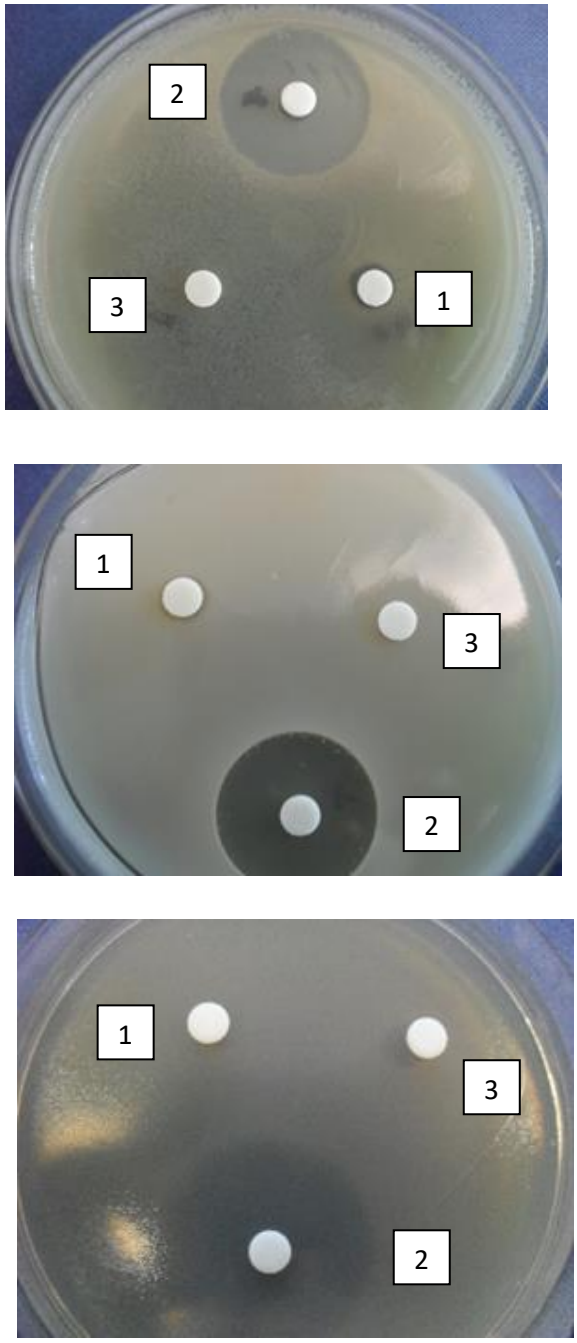


Figure 64: Antibiotic assay used *Bacillus subtilis E. coli* and *S. aureus* on LB agar pH 5. The plasmid pNBH3 was integrated into *S. coelicolor* M145 and metabolites were tested and showing zone inhibition both *Bacillus subtilis* and *S. aureus* (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the PNBH3 was used as negative control (Track 3).

6.1.4 *SCO*7817

The same approach was used and the result as follow:

6.1.4.1 Cloning of *sco*7817

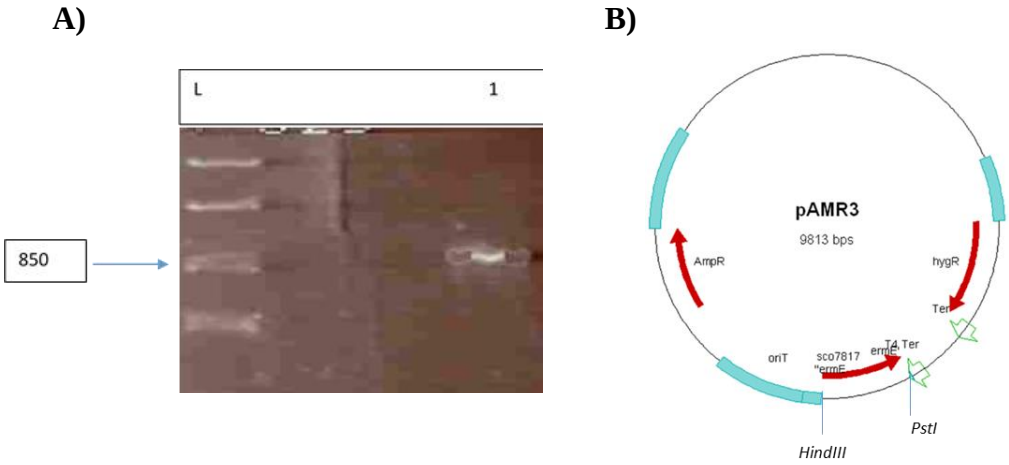


Figure 65: PCR was used to amplify *sco8717***.** This reaction solution was run on 1% agarose gel in order to verify the size of the expected band, at around 843 bps (Track 1) compared to a FastRuler middle range DNA ladder (Track L). B) Schematic map for the sco7817 ligated into pOSV556 which has been named pAMR3 and is 9813 bps in size.

6.1.4.2 Screen of the vector for correct insert

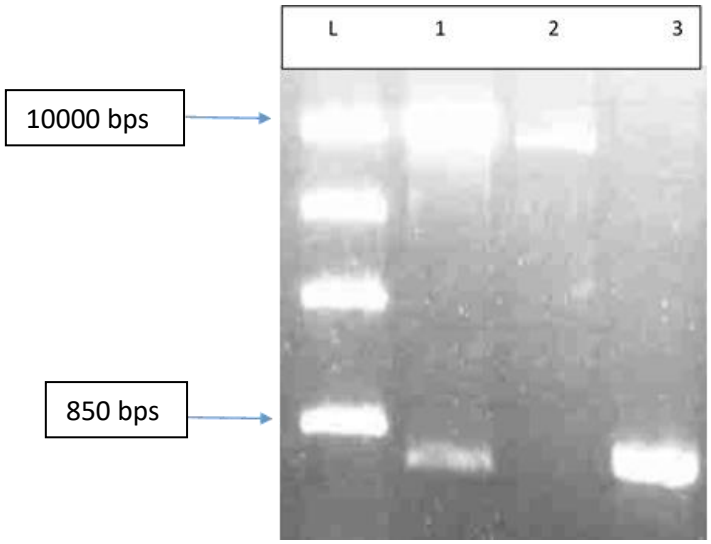


Figure 66: Assessment of the *sco7817* fragment size by doing PCR amplification used tow primers. This was then run on a 1% agarose gel in order to check the size of the expected band. FastRuler high range DNA ladder (Track L). The sco7817 insert around 840 bps and vector is 9009 bps (Track 1). The pOSV556 band around 9009 bps as positive control (Track 2). Positive control was used sco7817 gene (Track 3).

6.1.4.3 Assessment of the pAMR3 in *S. coelicolor* M145 using genomic DNA extraction and PCR screening

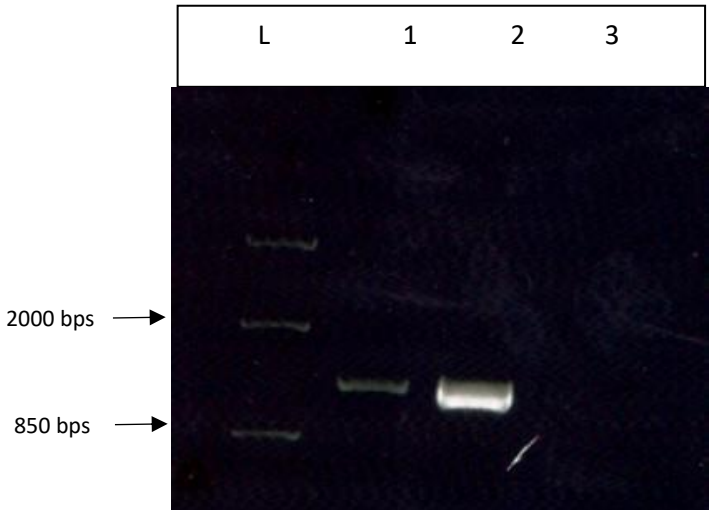


Figure 67: Assessment of the genomic DNA by using PCR screen for pAMR3 integrated in *S. coelicolor* M145 after genomic DNA extraction technique (Track 1). FastRuler middle range DNA ladder (Track L). Positive control used *sco*7817 ligated pOSV556 (Track 2). *S. coelicolor* M145 only was used as negative control (Track 3).

6.1.4.4 Bacterial growth conditions and antibiotic

6.1.4.4.1 *S. coelicolor* M145 with and without pNB3 growth conditions

The pOSV556 vector containing the cloned *sco*7817 gene (pAMR3) was transferred to *S. coelicolor* M145 by intergenic conjugation according to standard methods by (Kieser, 2000) with some modification. Overlay the plate with 1 mL sterile distilled water containing 20 µL nalidixic acid (from 25 mg/mL stock) to kill *E. coli*, 25 µL of 100 mg/mL stock ampicillin and 25 µL of 50 mg/mL stock hygromycin to select the *Streptomyces* exconjugants. Then keep it in incubation at 30 ºC for 6 days to allow growth of the exconjugants **Figure 88.**

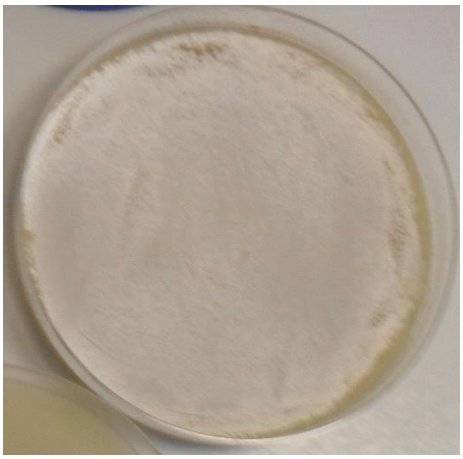
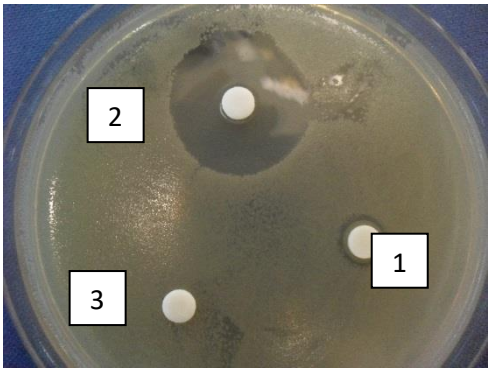


Figure 68: culture *S. coelicolor* M145 after growth 6 days at 30 ºC which contain the plasmid pAMR3 was integrated into *S. coelicolor* M145.

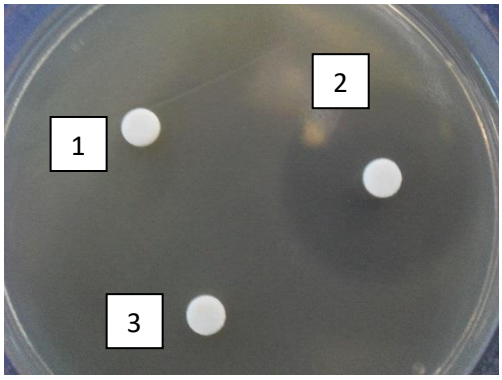
6.1.4.4.2 Antibiotic assay

Antimicrobial activity of *S. coelicolor* M145/ pAMR3 was test using antibiotic sensitivity assay. The antibiotic sensitivity assay shows the ability of drug in inhibiting the growth of microorganisms such as *Bacillus subtilis*, *E. coli* and *S. aureus* **Figure 89.**

Bacillus subtilis



E. coli



S. aureus

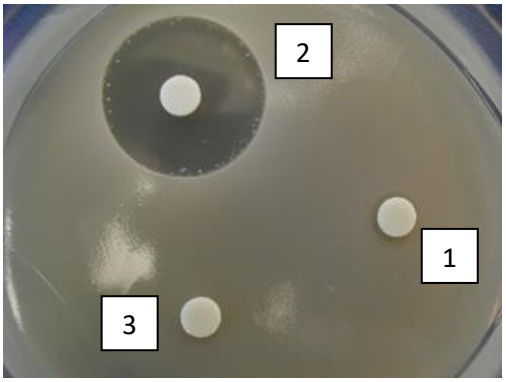


Figure 69: Antibiotic assay used *Bacillus subtilis, E. coli and S. aureus* on LB agar pH 5. The plasmid pAMR3 was integrated into *S. coelicolor* M145 and metabolites were tested and showing zone inhibition (Track 1). Apramycin used as positive control (Trach 2), *S. coelicolor* M145 only without the pAMR3 was used as negative control (Track 3).

Chapter 7: Final discussion and future work

Despite the success and discovery of approaches for natural product synthesis, there is still a need for novel antimicrobial agents because of the rise in multi-drug resistance microorganisms. In addition, the increase and spread of multidrug-resistant pathogens have become a growing threat to global common health.

The soil bacteria of the genus *Streptomyces* are a rich source of bioactive secondary metabolites. It contains dozens of gene clusters involved in the direct biosynthesis of specialised metabolites. Many gene clusters for natural product are cryptic and silent, they are produced in very small quantities or not activated under laboratory culture conditions (Bentley et al., 2002, Zerikly and Challis, 2009).

Streptomyces coelicolor A3(2) are the largest genus of Actinobacteria and are a rich source of antibiotics and bioactive secondary metabolites which may become suitable for human, veterinary medicine and agricultural applications (Genilloud, 2017). *Streptomyces* species are a good source for commercially viable antibiotics; (Kieser, 2000, Procopio *et al*., 2012, Ventura *et al*., 2007).

Streptomyces coelicolor A3(2) is widely used as a model organism to study the bacterial genome. When the genome was first sequenced, it identified many pathways with unknown natural products that might not be expressed in laboratory conditions and may need to be further investigated (Bentley *et al*., 2002). The metabolites are synthesised from biosynthetic gene clusters (BGCs): genes which typically remain silent unless stimulated. These clusters need very specific environmental conditions (such as pH, temperature and/or a specific nutrient) or physiological stimuli like inducer molecules to switch on so as to be able to produce natural products (Harrison and Studholme, 2014). Gene expression in *S. coelicolor* is tightly regulated and it has the genetic capability to produce natural products in a laboratory environment.(Bibb, 2005, Gottelt *et al*., 2010, van Wezel and McDowall, 2011).

It is very important for bacteria to regulate the production of antibiotics to prevent toxic stress on the producer strain which could subsequently lead to cell death (Ramos *et al*., 2005). *Streptomyces* regulatory systems often include both transcriptional activators and repressors to control the production of the secondary metabolites (Aigle and Corre, 2012). However, this is not always the case. For example, *S. venezuelae*

has a gaburedin biosynthetic gene cluster which does not have any transcriptional activators. Instead, it can de-repressed by the inactivation of the gbnR repressor (sven_4187) which leads to the upregulations of gaburedins (Sidda *et al*., 2014). However, many *Streptomyces* genomes contain one or more gene coding for a putative TetR repressor (Cuthbertson and Nodwell, 2013).

Recent research in the field has focussed on the genetic manipulation of *Streptomyces* in order to awaken silent cryptic clusters. BGCs can be unsilenced by genetic engineering via overexpression of pathway specific activators or disruption of pathway specific repressors (Aigle and Corre, 2012).

A method for awakening silent cryptic BGCs involved in activation of transcriptional repressors that bind to specific nucleotide sequences in operator region. These repressors inhibit expression of their own gene as well as indirectly repressing other gene as a result (Aigle and Corre, 2012).

Previous studies have primarily focussed on the deletion or inactivation of TetR-like transcriptional repressors to switch on the silent biosynthetic gene clusters; for example,

1. Deleting the gene *arpA* repressor in the *S. griseus* genome leads to the overproduction of streptomycin which can also develop aerial hyphae earlier than the wild type strain (Kato *et al.*, 2004).
2. Deleting the gene *mmR* repressor into the *S. coelicolor* leads to the overproduction of methylenomycin. (Aigle and Corre, 2012).
3. Deletion of the *scbR2* repressor gene into the *S. coelicolor* leads to the overproduction of the yellow pigment coelimycin (Gomez-Escribano *et al*., 2012).
4. Deletion of the *gbnR* repressor gene into the *S. venezuelae* leads to the overproduction of the gaburedins (Sidda *et al*., 2014).

The aforementioned examples confirm that the deletion of transcriptional repressors is a powerful strategy for the discovery of natural products and the identification of silent cryptic biosynthetic gene clusters.

In contrast, the genes in the biosynthetic pathway of interest may be targeted to switch on the 'silent' pathways. This allows the determination of cryptic natural products by overexpression of transcriptional activators.

In *Streptomyces coelicolor*, the bioactive secondary metabolites gene clusters often include regulatory genes controlling the production of that natural product. For example, genes *actII-orf*4, red and *mmyB* all encode for transcriptional activators which are found within the gene clusters for the biosynthesis of actinorhodin, streptorubin B and methylenomycin, respectively in *S. coelicolor*. Therefore, specific regulatory elements in the biosynthetic pathway of interest may be targeted in order to turn on potentially silent pathways and to determine the nature of the corresponding cryptic natural product.

The first method for awakening a silent cryptic BGCs to discover novel metabolites in *Streptomyces* involves overexpression of the ultimate activator to induce natural production. An example of this is the silent type I PKS gene cluster ATCC23877 in *S. ambofaciens,* which when activated results in the synthesis of macrolide compounds named the Stambomycins. Stambomycins are biosynthesised by a silent PKS gene cluster. The regulatory gene samR0484, encoding a positive LAL regulator was selected for overexpression and cloned into a conjugative and integrative plasmid (pIB139) which is under the control of the strong constitutive promoter *ermE**p (derivative named pOE-0484). This was then transformed into *E. coli* ET12567/pUZ8002 followed by transfer into a *Streptomyces* host through intergenic conjugation: a wild type strain and a mutant strain with presence of pOE-0484 led to unlocked the production of the stambomycins which were silent in the wild type strain (Laureti *et al*., 2011).

Methylenomycin is one of very few known antibiotics in which the whole biosynthetic cluster is entirely plasmid based. Methylenomycin gene cluster is found on a 19 kb region in the *S. coelicolor* SCP1 plasmid and has 21 biosynthetic genes clusters (Chater and Bruton, 1985).

Methylenomycin biosynthesis is strongly regulated by different mechanisms including

1. those that are through transcriptional activators,
2. those mechanisms which rely on the influence of the presence (and absence) of the methylenomycin furans and transcriptional repressors (O'Rourke *et al.*, 2009).

The original aims of this study were:

A. To clone *mmyB* and a series of *S. coelicolor mmyB*-like pathway-specific transcriptional activators under the control of a *Streptomyces* constitutive promoter.
B. To introduce these vectors, using intergenic conjugation in *Streptomyces coelicolor strain.*
C. To analyse the expression profile of *mmyB*-like genes and that of putative biosynthetic genes located next to the *mmyB*-like regulatory genes using RT-PCR.
D. To test the antibiotic activity of metabolic extracts where *mmyB*-like genes have been overexpressed versus negative controls.
E. Collaboration with a colleague to compare the metabolic profiles of the engineered *Streptomyces* strain using LC-MS analyses and compounds of interest (metabolites produced) that are normally detected by using High-Pressure Liquid Chromatography (HPLC) and possibly isolate and elucidate the structure of novel antimicrobials using additional analytical chemistry techniques (mass spectrometry and NMR spectroscopy). However, time constraints did not allow for HPLC and NMR experiments to be performed.

The objective of this study was to clone *mmyB* and a series of *mmyB*-like pathway-specific transcriptional activators under the control of *Streptomyces* constitutive promoter (*ErmE**p promoter) in *E.coli* / *Streptomyces* shuttle vector. This novel approach will allow the organism to express silent biosynthetic gene clusters. Resultantly, more of the natural products can be extracted from bacteria which would otherwise have not been possible.

We were able to achieve aims A,B,C,D, but E could not be completed. The first step taken was to clone the *mmyB* or *mmyB*-like cluster-associated transcriptional vector

into the integrative vector pOSV556. This was done using intergenic conjugation by designing a specific vector for introduction into the *Streptomyces* genome.

Overexpression of transcriptional activators such as *mmy*B and *mmy*B-like were found to be products of cryptic biosynthetic gene clusters which are activated by switching on the production of compounds from neighbouring cryptic biosynthetic gene clusters. This was done homologously in *S. coelicolor*. There were a series of activators that belong to pathway-specific regulators which are MmyB-like. There are 16 *mmy*B-like homologues which are encoded within *S. coelicolor* (Xu *et al.*, 2012). Overexpression of some of these activators resulted in the production of novel lantibiotic and polyunsaturated fatty acids (unpublished).

Methylenomycin gene cluster contains 21 genes (19 kb). The *mmyB* gene is found in *S. coelicolor* A3 (2) gene cluster located in the linear chromosome called SCP1 (365 000 bps). Based on the genetic organization, the *mmyB* gene is proposed to be a transcriptional activator for the methylenomycin biosynthetic gene cluster. Expression of *mmy*B is normally repressed by TetR-like transcriptional repressor MmfR and MmyR, bound upstream of its promoter at the Methylenomycin Auto-Regulatory Element (MARE). Both MmyR and MmfR have been shown to bind to the promoter region of *mmyB*. Furan ligands (MMFs) can bind and make conformational change to MmfR. MmfR by releasing them from the MARE sequence and hence allowing the expression of *mmyB*. The DNA-binding protein MmyB can then bind to B-boxes and recruit RNA polymerase thus activating the expression of antibiotic-like biosynthetic genes (O'Rourke *et al.*, 2009).

In this project, the cloning sequence for i) *mmy*B, and mmyB-like such as ii) *sco*6926, and iii) *sco*0110 were successfully amplified and ligated with vector pOSV556 (under the regulation of ermEp*). The new plasmids were named pJH2, pJT1 and pVN3 respectively. All the ligations were assessed through PCR and verified by DNA sequencing: forward and reverse sequencing confirms the cloning of *mmyB, sco*6926 and *sco*0110 with vector pOSV556. These were all successful with no errors.

The expression plasmids for pJH2, pJT1 and pVN3 were transformed into the non-methylated *E. coli* ET12567/pUZ8002 which can be used for conjugation with *Streptomyces*. There is evidence that DNA methylated by *E.coli* can prevent conjugation in *S. coelicolor* as the DNA is seen as foreign and removed from the

Streptomyces (Kieser et al., 2000, MacNeil *et al*., 1992). The constructs were integrated into the genome of *Streptomyces* via intergeneric conjugation, as described in sections 2.2.5.2. The methylenomycin antibiotic pathway has been silenced in the *S. coelicolor* strain W81. Therefore, this strain has lost the ability to make MMF because *mmyLHP* was deleted. The *mmy*B plasmid was transferred to *S. coelicolor* W81 and constitutive expression of the methylenomycin pathway-specific transcriptional activator MmyB was expected to stop the expression of methylenomycin. (Corre *et al.*, 2008). Additionally, *sco*6926 and *sco*0110 were transferred to *S. coelicolor* M145 by intergenic conjugation. This integration happens using the phage attP site in the pOSV556 vector matches with attB site in the target host system which comes from an 11 kb integrative element in *Streptomyces ambofaciens* (Zhang *et al.*, 2013)

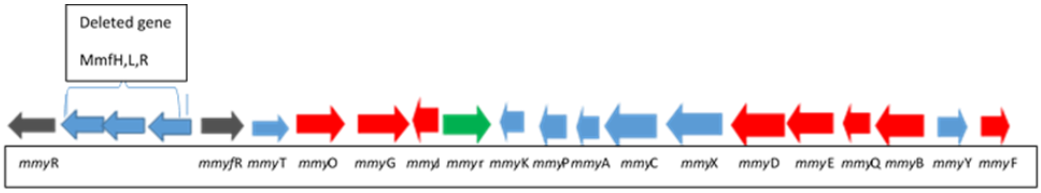


Figure 70*: S. coelicolor* W81 was deleted *mmyLPH* (not produce MMF), this strain was used to integrated pJH2 and expressed other *mmy* genes in some red and blue colours.

In order to confirm the expression of mmy gene clusters in the *S. coelicolor* after overexpression, each strain with the plasmid encoding for the transcriptional activators (and a negative control) was grown on supplemented minimal medium (SMM) which is a simple production medium for secondary metabolites. Each of these strains were grown for 6 days and they were analysed by RT-PCR, antibiotic assay and liquid chromatography-mass spectrometry (LCMS) analyses to confirm that the metabolites are overproduced in the strains containing pJH2, pJT1 and pVN3 relative to the wild type. Each of these will now be discussed further.

The metabolic profile using RT-PCR of *Streptomyces* strains constitutively expressing the transcriptional activator(s) was obtained by extracting the total RNA from *S. coelicolor* W81 wild-type and from *S. coelicolor* W81 with integrated pJH2. These were grown in MMS to evaluate the expression of a range of *mmy*B and other genes within the methylenomycin gene cluster. RT-PCR was performed to give single stranded complementary DNA (cDNA) synthesised from the DNA of *S. coelicolor*

W81 wild-type (negative control) and from the strain with integrated pJH2. They were both used as a template in PCR reactions to amplify the transcribed *mmyB* and other genes within the methylenomycin genes cluster. After integration of the plasmid (i.e. pJH2), all eight *mmy* genes were expressed in the *S. coelicolor* W81 but not in the wild-type strain, thus suggesting that the regulator is a pathway-specific activator of the gene cluster. Expression of nearby biosynthetic has been tested and it has been demonstrated that the activators turn on the expression of silent gene clusters to produce methylenomycin antibiotics (**Figure 70**). Complementary DNA from wild-type *S. coelicolor* W81 was used as a negative control in the PCR and same primers as with template (-) RT-PCR were used. The *hrdB* gene was used here as a positive control.

In addition, RT-PCR technique was used for the detection of lantibiotic and polyunsaturated fatty acids after integration of the plasmid into *S. coelicolor* M145 pJT1 and pVN3 respectively,

A. All the four *sco* genes (*sco*6926, 27, 32 and *sco*6933 were expressed when the *sco*6926 was integrated in *S. coelicolor* M145.
B. All the five *sco* genes (*sco*0108, 10, 17, 24 and *sco*0127) were expressed when the *sco*0110 was integrated in *S. coelicolor* M145.

All *sco* genes were expressed in the *S. coelicolor* M145 but not in the wild-type strain, thus suggesting that the regulator is a pathway-specific activator of the gene cluster and expression of nearby biosynthetic has been tested and demonstrated that the activators turned on the expression of silent gene clusters to produce lantibiotic and polyunsaturated fatty acids.

The noteworthy consequence of this is that after integration of the transcriptional activator, and cloning it with a strong promoter, the silent biosynthetic gene cluster for methylenomycin, lantibiotic and polyunsaturated fatty acids can be switched on.

The antibiotic sensitivity assay shows the ability of drug in inhibiting the growth of *Bacillus subtilis, S. auras* and *E. coli*. When overexpressing transcriptional activator genes from *S. coelicolor,* the antibacterial susceptibility was tested of the crude extracts (or organically extracted compound in the case of pJh2) by using a volume of 21 µl. The antibiotic activity was monitored using *S. coelicolor* W81/ pJh2 by using antibiotic sensitivity assay which involves loading a sample onto a sterile antibiotic

disc (6mm). The antibiotic sensitivity assay shows the ability of methylenomycin in inhibiting the growth of used *Bacillus subtilis, S. auras* and *E. coli.*

Lantibotics are antibiotics which can kill other Gram-positive bacteria. On the other hand, the antimicrobial activity of polyunsaturated fatty acid is also well-known. By comparing the metabolites produced between the *S. coelicolor* M145 engineered strains (pJT1 for lantibiotic and pVN3 for polyunsaturated fatty acids) and *S. coelicolor* M145 wild type, it was found that the former produced more active compounds against *Bacillus subtilis, S. auras*. However, in the case of *E. coli*, none of the strains showed any activity.

The active strains produced by the engineered strains required further characterisation. In the first instance, this was done by liquid chromatography mass spectrometry (LC-MS).

Various combinations of the metabolites were also tested. Surprisingly, when mixing an equal amount 15 µL of *S. coelicolor* M145/pVN3 with 15 µl of *S. coelicolor* M145/pJT1, it was observed that the combination of the two *S. coelicolor* engineered strains was not active against *B. subtilis*. Similarly, mixing equal amounts of either *S. coelicolor* M145/pVN3 with *S. coelicolor* W81/pJH2 or mixing equal amounts of both *S. coelicolor* W81/pJH2 with *S. coelicolor* M145/pJT1 will still show inactive results against *B. subtilis.* This probably shows an antagonism reaction has been occurred. (Antagonism reaction, is when two or more antibiotic join together and lose activity).

Furthermore mixing all three *S. coelicolor* engineered strains, M145/pVN3, M145/pJT1 and W81/pJH2 will show zones of inhibition on the agar overlay plate against *B. subtilis*. Interestingly, in this case, the zone of inhibition is much larger than that observed for the individual sets of metabolites. The exact reason for this rise in activity requires further investigation.

The LC-MS analysis of crude extracts results allowed comparison of two samples: metabolites produced from the engineered strains versus the wild type. After overexpression of the transcription activators, peaks can be observed in the LC-MS spectra of pJT1 and PVN3 which can be attributed to lantibiotics and polyunsaturated acids respectively. For the wild type, no peak was observed.

The metabolites produced after overexpression of the regulatory gene pJT1 in *S. coelicolor* M145 and wild type were compared by LC-MS. Two prospective novel compounds were identified with m/z = 1479.21 and 1474.93. These compound have proposed lantibiotic activity and were only found in the engineered *S. coelicolor* strain containing pJT1 and were absent in *S. coelicolor* M145 control.

Additionally, the *S. coelicolor* strain with overexpression of the regulatory pVN3 gene in the host strain and *S. coelicolor* M145 wild type were compared by LC-MS with another prospective novel compound being identified. A peak with m/z= 1476.93 was only present in *S. coelicolor* strains containing pVN3 and was absent in *S. coelicolor* M145 control.

On the other hand, after overexpression of the regulatory gene pJH2 in *S. coelicolor* W81 and wild type the mass of the methylenomycin was not detected or identified using LC-MS. However, after overexpression the regulatory gene pJH2 in *S. coelicolor* W81, the RT-PCR and antibiotic activity identify methylenomycin antibiotic production in *S. coelicolor* strains containing pJH2.

In conclusion:

Recently, there has been an increase in the number of natural novel products discovered via genomics-guided approaches. This project was focused on a specific secondary metabolite which is produced after a biosynthetic gene cluster is activated by the overexpression of a transcription activator. For example, constitutive overexpression of *mmyB* using a strong promotor (*ermEp**) resulted in the production of compounds from neighbouring cryptic biosynthetic gene clusters which in turn switched on the methylenomycin biosynthetic genes cluster to produce the methylenomycin antibiotics. This was confirmed by successfully performing RT-PCR and antibiotics assays.

Similarly overexpression *sco*6962 of lantibiotic and *sco*0110 of polyunsaturated fatty acid by using a strong *ermEp** promoter resulted in the production of compounds from neighbouring cryptic biosynthetic gene clusters which subsequently switched on the lantibiotic and polyunsaturated fatty acid biosynthetic genes clusters to produce the lantibiotic and polyunsaturated fatty acid respectively. This was confirmed by successfully performing RT-PCR, antibiotics assays and LC-MS.

Future work

Initially, attempts should be made to clone other *mmyB*-like pathway-specific transcription activators such as *sco0233, sco*0307, *sco*2501, *sco*2537 and *sco*4944 under the control of *Streptomyces* constitutive promoter into integrative vectors such as *S. coelicolor* M145. It would then be helpful to find the gene cluster by using AntiSmash for each mmyB-like transcription activator. Furthermore, it would be useful to analyse the expression profile of *mmyB*-like genes and that of putative biosynthetic genes located next to the *mmyB*-like regulatory genes using RT-PCR.

Next, the goal would be to introduce these plasmids using intergenic conjugation in other *Streptomyces* bacteria, such as *Streptomyces albus* (often used as a heterologous host for BGC expression) so as to activate silent pathways. One of the reasons to use this strain is that *S. albus* in particular has a wide and diverse geological distribution; it has been isolated from environments including sponges, sea sediments and insects. Strains of this *streptomyces* have been found to harbour 48 unique BGCs, 18 of these BGCs have been identified which give rise to its core secondary metabolome. The metabolic products of 29 out of the 48 gene clusters analysed are still unidentified; this shows promise on the continuing search for novel compounds with desired biological activities, such as antibacterial and anticancer treatments (Seipke, 2015).

The relative antimicrobial properties of the culture supernatants obtained from these bacterial strains should be assessed. LC-UHRMS analyses of the samples showing significantly improved antimicrobial properties compared to the parent strain can then be carried out. Differences in the metabolic profiles should be investigated and the molecular formulae of metabolites only appearing in the presence of the constitutively expressed activators can then be generated. Database searches (i.e. Reaxys) can then be used to reveal which newly produced compounds exhibit molecular formulae not previously known for a natural product. This important step aims at preventing antibiotic “rediscovery”.

Compounds of interest (metabolites produced) are normally detected by using High-Pressure Liquid Chromatography (HPLC) which compares metabolite profiles via ultraviolet (UV) absorption changes at specific wavelengths. Mass spectrometry allows deduction of a metabolite molecular formula from its m/z value and pattern of

fragment ions. Structure elucidation of pure samples and novel metabolites produced can then be purified and characterised via high-resolution mass spectrometry, NMR spectroscopy, and X-ray crystallography.

It is important to note that turning on the production of several specialised metabolic pathways at once in the parent strain might lead to synergistic antimicrobial properties. Depending on time and resources, the supernatants resulting from Streptomyces strains engineered with each of the 3 different transcriptional activators could also be combined and synergistic antimicrobial activities assessed. The mode of action and combination of targets hit by these "natural" mixtures of antimicrobial compounds could then be investigated.

This would allow for the assessment of the antibiotic activity of the metabolites produced by the engineered strains against pathogenic microbes.

The antibiotic activity of new metabolites will be assessed in liquid culture against a panel of dominant susceptible and resistant strains of key hospital and community-acquired pathogens.

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Appendix

SCO0110:

Reverse transcription-PCR was performed on the extracted total RNA from pN3 integrated into S. coelicolor M145 and wild type S. coelicolor M145. The RNA was subject into single stranded complementary DNA (cDNA) by using cDNA Superscript II RT. cDNA products were run on 1% agarose gel in Figure below. It can use the control with no reverse transcriptase added to ensure that direct result of the amplification of cDNA and not DNA contamination.

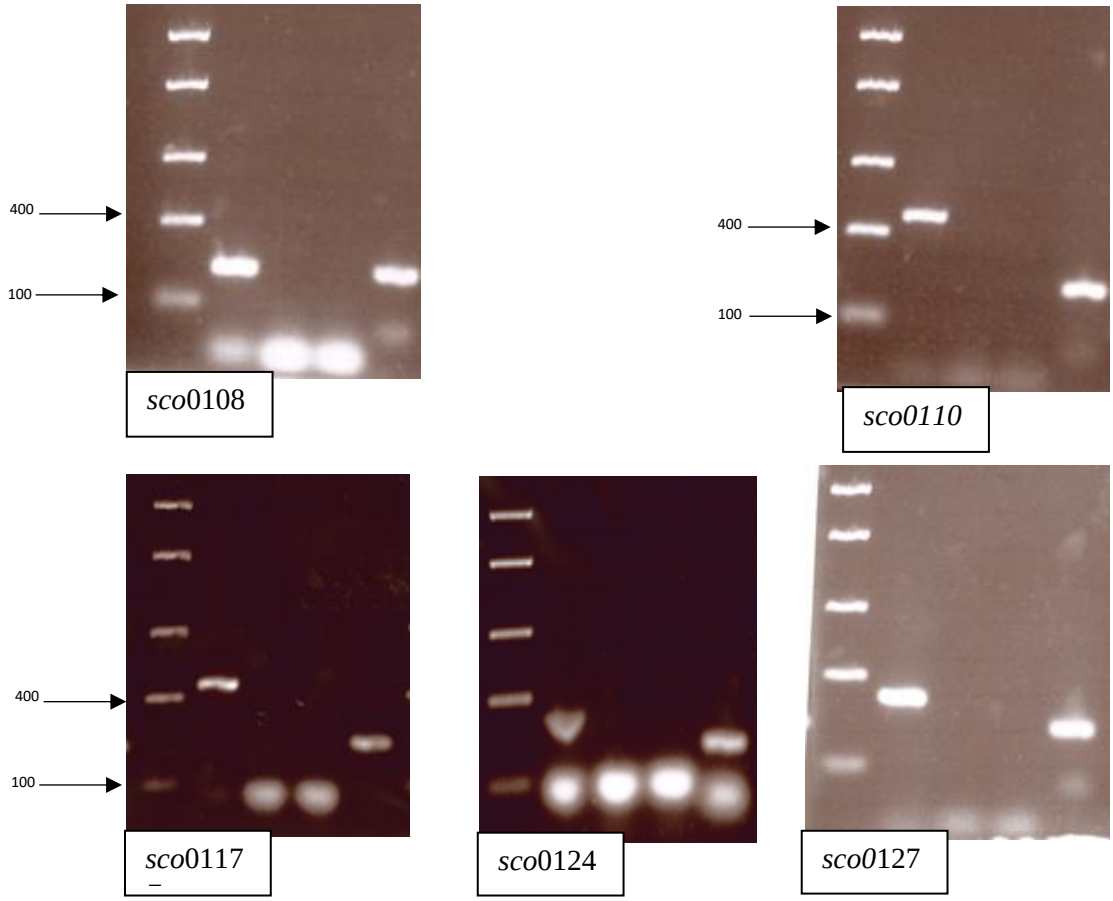

Figure: Detection of pVN3 integrated in S. coelicolor M145 *expression sco0108, sco0110, sco0117, sco0124* and *sco0127* genes using RT-PCR (Track 1). The negative control was performed using wild type of S. coelicolor M145 (Track 2) and also negative control was performed using minus RT-PCR using same two designated primers (Track 3). In Track 4 using as a control primers designed to amplify the *hrdB* gene considered to be constitutively expressed.