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Synthetic Biology of Antibiotic Production

Assembly and Re-factoring of Secondary Metabolite Biosynthesis Gene Clusters for Heterologous Expression in Genetically Engineered Bacterial Host

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Preface

This project is a part of ‘Activation and heterologous expression of secondary metabolite gene clusters’ that is led by Professor Sergey Zotchev. The lab works were conducted from Nov 2012 to May 2014 in a continuous way in the department of biotechnology, NTNU, and the report was written in spring 2014.

My principal supervisor is Professor Sergey Zotchev. He has given sound instructions and suggestions in the project ideas and experiments data analysis as well as insightful advice in thesis writing. The project provided by him is right what I am interested. Therefore, I would honestly thank him for excellent supervision in the project. Researcher Olga Sekurova is my lab supervisor. She has provided outstanding guidance on analysis of lab work results and useful advice on writing thesis, besides, her teaching in lab work execution is both patient and friendly. I, hereby, express my sincere acknowledgement for her student-oriented supervision.

In addition, I would like to thank Professor Atle M. Bones for lending cell plates, researcher Ertesvåg Helga and postdoctor Bin Liu for their instruction in using spectrophotometer, and researcher YiQian Sun for assistance in Bradford assay. Last but not least, I would express my acknowledgement to all people for creating a sound study environment, from laboratory colleagues to administrative staff, etc.

Summary

The issues in new antibiotic discovery are pressing, because the frequent re-discovery of antibiotic scaffolds leads to few novel antibiotics discovered, besides, with the widespread use of antibacterial agents, multi-resistant pathogens are emerging, which poses more huge challenge in antibiotic discovery. However, next generation sequencing technology and bioinformatics have revealed that many secondary metabolite biosynthesis gene clusters possess the potential of producing new bioactive secondary metabolites (BSMs), which were ignored previously. Among the microorganisms, *actinomycetes* species are the best sources for those gene clusters. Synthetic biology is the enabling technology to activate those silent gene clusters, which aims to engineer organisms for expected applications with combination of various biotechnologies.

The project employs the reciprocal regulation system between jadomycin (Jd) and chloramphenicol (Cm) in *S. venezuelae*: JadR1 activates Jd synthesis while represses Cm synthesis with ethanol shock. This system can be used to rationally engineer *S. venezuelaee* for heterologous production of BSMs with re-factored gene clusters containing appropriate control elements: deletion of the *jadR1* gene shall lead to down-regulation of Jd production, simultaneously induce overproduction of Cm due to the relieved repression of the Cm structural genes' promoters. Besides, the *cml* gene cluster should be completely deleted to avoid interfering with the introduced gene cluster. The appropriate control element is an inducible promoter screened out with GUS assay among *cmlFp, cmlIp, cmlXp*, *jadJp*. The inducible promoter would be used to construct an inducible system for industrial scale production of BSMs, because constitutive heterologous expression of BSMs is harmful for producing hosts.

The *jadR1*$^{-}$ *cml*$^{-}$ mutants were successfully generated with Gibson Assembly, transconjugation, double crossover and replica plating. The gene cluster MP112-09-Lac was cloned with native promoter and *ermE** respectively and transconjugated to *jadR1*$^{-}$ *cml*$^{-}$ mutant, however, cloing of MPS05-B41-Lin was hindered by wrong PCR amplification. The four promoters were tested with GUS assay, based on MYM medium and *cmlF* is speculated to be the most desirable inducible promoter.

Abbreviations

sd H2O	distilled sterile water
EHF	Expand high fidelity
EtOH	ethanol
ES+/-	ethanol shock/no ethanol shock
Double check	restriction enzyme digestion and PCR verification for assembled plasmid
GUS	β-Glucuronidase
Cm	Chloramphenicol
Jd	Jadomycin
Kan	Kanamycin
Nal	Nalidixic acid
BSM	bioactive secondary metabolite
OD	Optical Density
oriT	origin of transfer
oriR	origin of replication
pDNA	plasmid Deoxyribonucleic Acid (DNA)
PNDG	p-nitrophenyl-β-D-glucuronide
SD	standard deviation
WT	wild type
DD	double deletion (*jadR1*$^-$ *cml*$^-$) mutant

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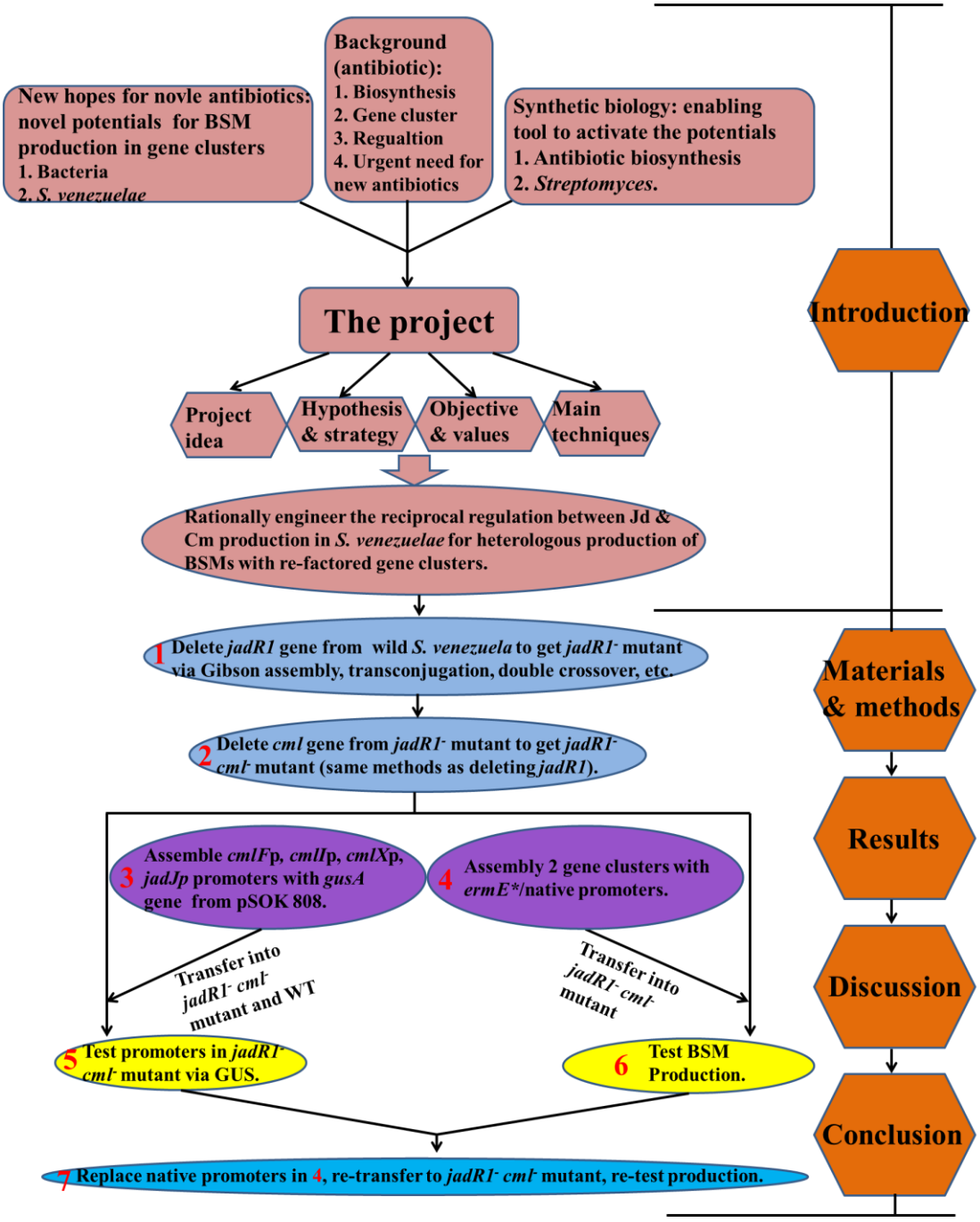
Graphic Representation of the Content



1. Introduction

1.1 Background

1.1.1 What is Antibiotic?

Antibiotics are secondary metabolites synthesized by bacteria, fungi, plants, and some animals. Most antibiotics belong to one of the three categories: (i) natural products produced by microorganisms, (ii) semi-synthetical products derived from natural products, (iii) chemically synthesized products based on the structure of the natural products. (Nikodinovic, Barrow et al. 2003) They are not essential for the host organisms, but can inhibit or even kill other competing species so as to utilise more surrounding nutrition. The mechanism of antibiotic action is complex. Briefly, the antibiotic molecules act on various cellular targets such as DNA replication, RNA synthesis, cell wall synthesis, protein synthesis via physical interaction that involves biochemical, molecular, and structural changes. (de Lima Procópio, da Silva et al. 2012)

In 1928, A. Fleming noticed that a Petri dish culture of *Staphylococcus aureus* was contaminated by a mould and the bacteria colonies surrounding the mould were dissolved. Later he figured out that it was a substance produced by the mould that killed the bacteria. The mould was identified as *Penicillium notatum*, and the substance was named penicillin that became the first antibiotic in human history. Penicilin was found to be a potent antibacterial compound and thus well used in medical treatment in 1940s. Enlightened by Fleming, researchers discovered thousands of more antibiotics in the natural products such as chloramphenicol and streptomycin. Among all the microbiological antibiotics, the most important ones include penicillins, cephalosporins, tetracyclines, aminoglycosides, chloramphenicol, macrolides, and some glycopeptides. (Demain 2009) Since they exhibit novel properties of antimicrobial, antitumor, and insecticidal activities, they have been playing significant role in medical treatment of infections, cancers, agriculture, etc. (Zotchev 2008; Demain and Sanchez 2009)

1.1.2 Antibiotic Biosynthesis

In general, the antibiotic biosynthesis process can be divided into three steps. First, Building blocks (precursors) are obtained, which are typically from primary metabolites such as malonyl-CoA, amino acids, S-adenosyl methionine, formate, carbamoyl phosphate, nucleotides, however, some natural products often serve as building blocks as well. If not already activated, the building blocks are activated before assembly in ways of adenylation, phosphorylation, attachment of nucleotide moieties, etc. Second, antibiotic scaffolds are assembled from those active building blocks, which are catalyzed by specific enzymes. Those enzymes are modular polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS). They bind different reaction sites of individual building blocks such as to make condensation and modification reactions, working in an assembly-line manner. (Hertweck 2009; Condurso and Bruner 2012) In the last step, the scaffolds are widely modified by tailoring enzymes, the process of which can be significantly different for different antibiotics. Those tailoring enzymes span from oxidoreductases, methyltransferases, halogenases, carbamoyltransferases, acyltransferases, glycosyltransferases. (Zotchev 2008; Weber 2014)

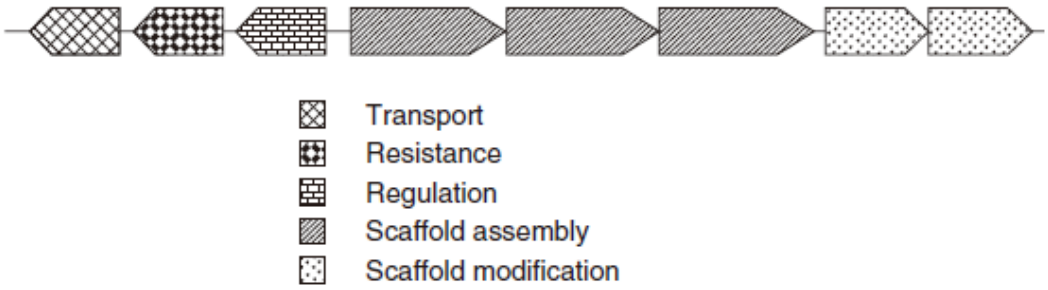
1.1.3 Antibiotic Biosynthesis Gene Clusters

1.1.3.1 Typical Antibiotic Biosynthesis Gene Cluster

Biosynthesis of antibiotics is a complex metabolism and is strictly controlled so as not to put heavy burden on the host organisms. To produce antibiotics in a benign way to the synthesizing organisms, biosynthetic genes are clustered in the genomes of synthesizing organisms such that the corresponding enzymes are expressed in an orderly manner, which ensures coordinated enzymatic reactions in scaffold assembly and modification. (Zotchev 2008)

With the significant advances in bioinformatics, analysis on the entire antibiotic biosynthetic pathways and genes is possible. Besides, genetic approaches such as mutational analysis, enzyme assays enable researchers to identify the function of those biosynthetic genes. Therefore, more and more antibiotic biosynthesis gene clusters are identified such as bioactive polyketides, antifungal polyene macrolides. (Aparicio, Caffrey et al. 2003) **Figure 1.1A** shows the typical organisation of an

antibiotic biosynthesis gene cluster. In the cluster, the genes responsible for the synthesis of scaffolds (PKS, NRPS, glycosyltransferase, etc.) are the nuclei. Close to the nuclei are the genes that encode modification enzymes, e.g. hydroxylases, methyltransferases, acyltransferases, halogenases, glycosyltransferases. Typically, pathway-specific regulatory genes exist in the cluster, regulating the enzyme expressions in a harmonious way. In addition, the cluster provides resistance to its own antibiotic, which is accomplished in two strategies: first, inactivate the intracellularly accumulated antibiotics or mask the antibiotic target. Particularly, antibiotic inactivation is performed mainly by three enzymes: β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases; second, transport the antibiotic outside with efflux pumps encoded by the cluster, which can inhibit or kill the competing organisms by releasing antibiotics in outer surroundings. (Zotchev 2008; Giedraitienė, Vitkauskienė et al. 2011)



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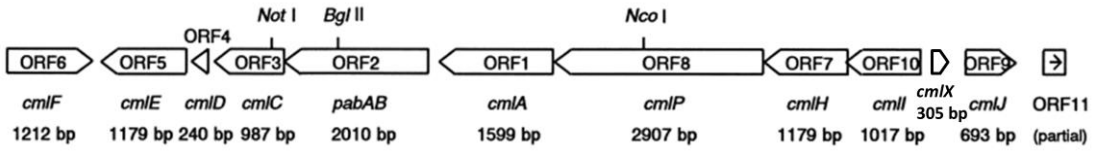

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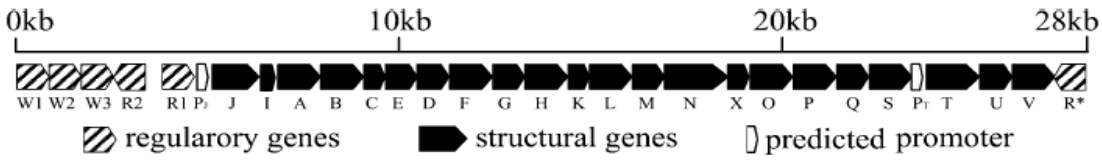


C

Figure 1.1 Antibiotic biosynthesis gene clusters. A: Typical organisation of an antibiotic biosynthesis gene cluster. Five primary functions are conferred by the cluster: transport, resistance (to avoid suicide), regulation, scaffold assembly, scaffold

modification. See text for details. (Zotchev 2008) **B: Chloramphenicol (Cm) biosynthesis gene cluster.** The Cm biosynthesis cluster is separated by *pabAB.* Disruption of *cmlB/pabAB, cmlC, cmlH, cmlP or cmlJ* blocks Cm production. *cmlF* is postulated to be responsible for Cm efflux. (He, Magarvey et al. 2001) **C: Jadomycin gene cluster.** Regulatory genes, structural genes, and two predicted promoters are presented. (Zheng, Wang et al. 2007)

1.1.3.2 Chloramphenicol and Jadomycin Biosynthesis Gene Clusters

Cm biosynthesis genes in *S. venezuelae* ISP5230 are clustered in separate regions flanking ADC (4-amino-4-deoxychorismate) synthase gene *pabAB* (*cmlB*) (**Figure 1.1B**). Cm biosynthesis begins in a secondary metabolic branch of the shikimate pathway that produces PAPA (p-amino phenylalanine). Initially, the chorismic acid generated from shikimate pathway is catalyzed by ADC synthase encoded by *pabAB* (*cmlB*). Then the produced ADC is converted into amino deoxyprephenic acid, which is catalyzed by a monofunctional ADC mutase encoded by the gene *cmlD*. *cmlE* encodes DAHP (deoxy-arabino-heptulosonate-7-phosphate) synthase that initiates the shikimate pathway, leading to chorismic acid formation and subsequent chorismate branching reaction that forwards to Cm synthesis. The main function of *cmlE* is perhaps to regulate the shikimate pathway. The inactivation of genes *pabAB* and *cmlC* can cause significant decrease in Cm production, since the chorismic acid precursor from shikimate pathway cannot be utilized by the Cm biosynthesis. In comparison, the disruption of *cmlF* only slightly affects Cm production, thus *cmlF* is supposed to responsible for Cm efflux that transports Cm into extracellular environment and protects normal intracellular biosynthesis activities from inhibition. However, researchers discovered a discrete set of *cml* efflux genes located at least 30 kb from *pabAB*, far from Cm biosynthesis cluster. Similar to *pabAB /cmlB*, *cmlC*, disruption of

cmlH, *cmlP*, *cmlJ* can inhibit Cm biosynthesis as well. (Brown, Aidoo et al. 1996; He, Magarvey et al. 2001)

Jadomycin (Jd) biosynthesis gene cluster (**Figure 1.1C**) stringently regulates the Jd production. The regulating genes are located at the left end of the cluster: *jadW1*, *W2*, *W3*, *R2* and *R1*. JadR1 is a necessary positive regulator for Jd production, because disruption or deletion of *jadR1*disables *S. venezuelaee* to synthesis Jd. In contrast, *jadR2* is negative regulator, which represses Jd production, since deletion of this genes leads to Jd production in the absence of environmental stress, and applied stress increases Jd yield. (Yang, Han et al. 1995; Yang, Han et al. 2001) Next to the left of *jadR2* are *jadW1*, *W2*, and *W3*, though their definite regulating roles are not understood, but their involvement in the regulation of Jd synthesis is undoubted. *jad W1* has sequence similarity with *afsA*, and is speculated to encode an enzyme that synthesizing γ-butyrolactone. Disruption of this gene makes *S. venezuelaee* unable to produce Jd and Cm as well as affecting its morphology.(Wang and Vining 2003) The regulating roles of *jadW2* and *W3* can be indicated by the corresponding mutants, but not clearly figured out. To the right of *jadR1*, are the structural genes that are clustered in the same transcription direction with short intergenic spaces. This organization means the same promoter can control all the structural genes. Two promoters are identified: P_J and P_T, upstream of *jadJ* and *jadT* respectively. P_J is speculated to control structural genes *jadJ-S* while P_T to control *jadT-V*. (Zheng, Wang et al. 2007)

1.1.4 Regulation of Antibiotic Biosynthesis

Antibiotic biosynthesis is tightly controlled, as it competes for metabolites (building

blocks) with primary metabolism, posing a burden for the synthesizing organism itself. The regulating mechanism can be divided into local and global types. Antibiotic gene clusters contain some regulatory genes clustered with genes for biosynthesis, secretion and resistance to the antibiotics. These regulating genes are local and usually pathway-specific. In contrast, other regulatory genes are global pleiotropic regulators that control different metabolic pathways, not linked to specific biosynthetic gene clusters. Some researchers proposed that pleiotropic regulators activate the pathway-specific regulators located within gene clusters. (Horbal, Rebets et al. 2010) The global regulators are sensitive to many nutritional or environmental factors, e.g. emergence of competing organisms, nutrition shortage, the presence of chitin, cell wall damage, phage infection, pH stress, etc. In these cases, the antibiotic production is induced and is beneficial to producing species. (Martín and Liras 2010) But in other circumstances, excessive level of inorganic phosphate inhibits antibiotic production. (Bibb 2005)

Some organisms are able to synthesis more than one antibiotics harbouring quite different properties, which means the competition for antibiotic precursors can occur among different antibiotic synthesizing pathways. In this case, the priority is given to the antibiotic that benefits the host organism most in the current environment. (Zotchev 2008)

Besides, it is worthy of mentioning the regulation of antibiotic resistance by the producing organism. The primary mechanism is antibiotic efflux that relies on ATP energy. In addition, other mechanisms also confer producing organisms resistance, such as target modification, degradation/modification of endogenously accumulated

antibiotics. (Zotchev 2008)

1.1.4.1 Regulation of Secondary Metabolite Biosynthesis in *Stryptomyces*

In *Stryptomyces* species, there are two regulatory mechanisms that are best investigated. The first one is the pyramidal system responsible for the biosynthesis of streptomycin, tylosin and cephamycic/clavulanic acid. In this system, a butyrolactone receptor protein (Brp) exists in the top position of the regulatory cascade. External signals can activate the butyrolactone-Brp system that activates regulatory genes encoding SARP (*Streptomyces* antibiotic regulatory proteins). SARP serve as the final checkpoint in regulation of certain antibiotic biosynthesis, and most of them are positive regulators. (Horbal, Rebets et al. 2010) The second mechanism is the so-called two-component systems (TCSs) or global regulators responding to external stresses. The best-known one in *Streptomyces* species is the PhoR–PhoP TCS, where PhoR is a standard membrane sensor kinase while PhoP is a DNA-binding response regulator. (Martín and Liras 2010)

1.1.4.2 Cross-regulation of Jd and Cm Biosynthesis

Streptomyces venezuelaee ISP5230 produces two antibiotics, chloramphenicol and jadomycin B, in response to disparate conditions: the former is produced in situation of moderate nutrient limitation, by contrast, the latter is synthesized only in response to highly limited sources of carbon, nitrogen, etc. and additional stress of shock, phage infection or toxic concentrations of ethanol. (Yang, Han et al. 1995) The regulation of jadomycin and chloramphenicol is a cross-circuit involving a key component JadR2. JadR2 is a pseudo GBL (γ-butyrolactone) receptor, since unlike

the genuine GBL receptor binding specific ligand, JadR2 binds to both ligands of Jd and Cm that are chemically different. Specifically, the pseudo GBL receptor JadR2 directly represses the transcription of JadR1 and subsequently repressed Jd synthesis, since JadR1 is a cluster-situated regulator initiating the Jd production by activating the expression of structural genes. Disruption of *jadR2* leads JdB production without ethanol stress. Besides, JadR1 represses Cm production by direct binding to the *cmlI-cmlJ* intergenic region (**Figure 1.1B**), which is necessary for Cm biosynthesis. JadR2 also positively regulates Cm production at least in the absence of ethanol, which is supported by the unusual fact that the Cm biosynthetic gene cluster has no cluster-situated regulators, meaning outside-cluster regulators are required. Jd and Cm could directly bind to JadR2, which dissociates JadR2 from the *jadR1* promoter. Overall, Jd and Cm production is reciprocally regulated by JadR1, with Jd synthesis activated while Cm repressed (**Figure 1.2**). (Xu, Wang et al. 2010)

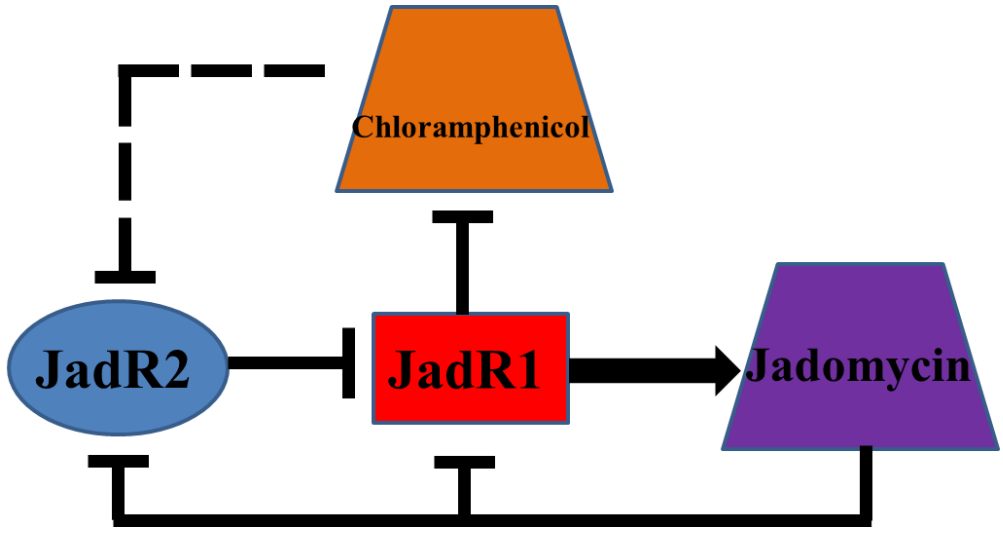


Figure 1.2 Regulation between jadomycin and chloramphenicol. JadR1 activates Jd production while represses Cm production. JadR2 represses Jd production by inactivating JadR1 and activates Cm production at least in the absence of ethanol. Both Cm and Jd can dissociate JadR2 from *jadR1* promoter. JadR1 is the low level regulator directly controlling Cm and Jd biosynthesis, whereas JadR2 is the higher

level signal coordinator that senses the metabolites signal and functions by regulating *jadR1* expression. (Xu, Wang et al. 2010)

1.1.5 Benefits and Issues from Antibiotics

1.1.5.1 Benefits Generated by Antibiotics

During the 20th century, antibiotics benefited human well-being dramatically, because of the versatile function in combating infections. They doubled our life span, released sufferings, and brought a revolution in medicine. In US, for example, the average life span was increased from 47 years to 74 and 80 years for men and women respectively during 1900 and 2000. (Lederberg 2000; Demain 2009) Moreover, antibiotics boosted worldwide economy significantly: antibiotics shared a worldwide market of $26 billion, $32 billion and $55 billion (including antiviral agents) in 1996, 2001 and 2000 respectively. (Erdmann 1999; Projan and Youngman 2002) And amazingly, the market of *Streptomyces* antibiotics alone accounted for $25 billion in 2001. (Hranueli, Cullum et al. 2005)

1.1.5.2 The Urgent Need for New Antibiotics

Despite the enormous benefits to human societies, some urgent issues have emerged from antibiotics. Initially, the "golden age" of antibiotic production began with penicillin in late 1940s and progressed till 1970s, after which it slowed down due to the increasing difficulty in discovering novel antibiotics. The primary reason is each antibiotics class share a common scaffold. The majority of current antibiotics were originated from scaffolds discovered between the mid-1930s and the early 1960s (**Figure 1.3**). For instance, during 1960s and 2000, all approved antibiotics were

derivatives of known scaffolds, with exemption of the introduction of carbapenems in 1985. Besides, among all the new antibiotics filed between 1981 and 2005, only the scaffolds of cephalosporins, penicillins, quinolones, and macrolides—were responsible for 73%. (Newman and Cragg 2007)

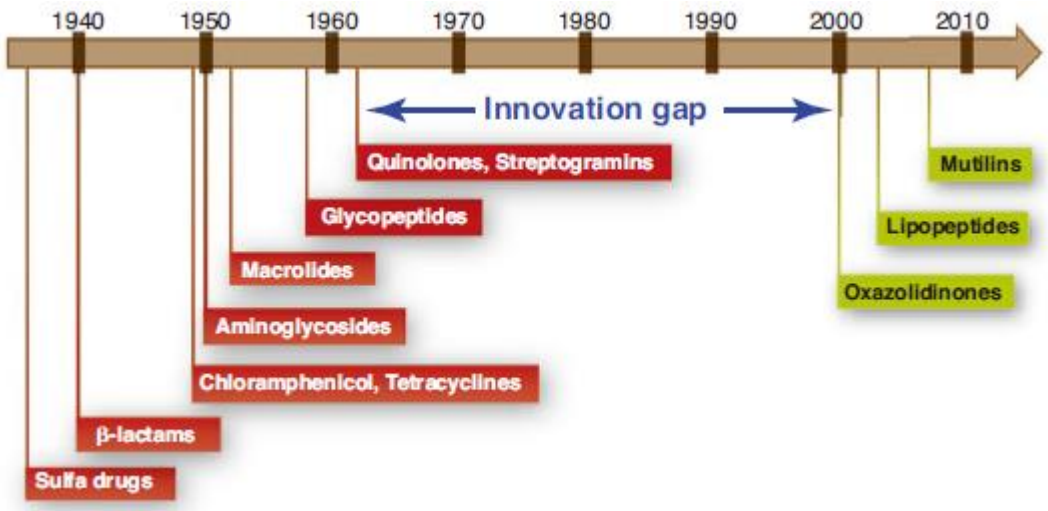


Figure 1.3 Discovery pace of new antibiotics. Antibiotic discovery experienced a "golden age" between 1940s and 1960s-1970s, since 1970s it has been difficult to identify new antibiotics, especially between 1962 and 2000, there was an innovation gap. (Fischbach and Walsh 2009)

Furthermore, due to the widely used antibacterial agents, pathogenic bacteria with antibiotic-resistance are becoming increasingly prevalent in both hospitals and the community. The emergence of multidrug resistant pathogens makes matters worse. (Fischbach and Walsh 2009; Giedraitienė, Vitkauskienė et al. 2011) There are mainly three types of antibiotic resistant pathogens posing threat to public health: (i) *Staphylococcus aureus* (MRSA); (ii) *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*; (iii) *Mycobacterium Tuberculosis*. (Fischbach and Walsh 2009) The antibiotic resistance of pathogens can be innate or

acquired. The latter is caused by either mutation in genes or gene transfer among different organisms by plasmids, transposon, integrons and bacteriophages. (Giedraitienė, Vitkauskienė et al. 2011)

Besides these problems, the antibiotic field is being ignored by many large pharmaceutical companies due to profit concerns, hence small companies, and the research institutions are taking much responsibility in novel antibiotic discovery. (Demain 2009)

Overall, discovery of new antibiotics is facing crisis, thus new sources for antibiotic discovery and novel enabling technologies are urgently needed to address the crisis.

1.2 New Hope for Novel Antibiotic Discovery

1.2.1 Bacteria Provide Rich Resources for Novel Antibiotic Discovery

In the crisis of novel antibiotic discovery (**seen in 1.1.5.2**), bacteria provide a promising solution, because the microbial natural products and their derivatives provide rich sources for antibacterial discovery, despite its decline for some time, with more than 60% of the used drugs originating from natural products. (Molinari 2009) The discovery of platensimycin demonstrates that novel scaffolds are still available in microbial products if proper tools and technologies are applied.(Genilloud, Gonzalez et al. 2011) Among those natural products obtained so far, 45% are synthesized by *actinomycetes*, 38% by fungi, and 17% by eubacteria, which indicates that *actinomycetes* play the pivotal role in producing antibacterials. Besides, *actinomycetes* products are the sustained sources for novel antibiotics that combat the pathogens without harming the host tissues. (Mahajan and Balachandran 2012) Moreover, the advanced technology in next generation sequencing and bioinformatics unrevealed that microbes have novel potentials, previously unrecognized, to produce new bioactive products with antibiotic property, which is in the form of gene clusters. (Zotchev, Sekurova et al. 2012) These potentials could be fulfilled by an enabling tool—synthetic biology (**seen in 1.3**).

In this project, the gene clusters analysed are MP112-09-cluster 6-Lactococcin972-like (MP112-09-Lac) and MPS05-B41-cluster 28-Lincocin-M18-like (MPS05-B41-Lin) (**Figure 1.4).** MP112-09-Lac from *Nocardiopsis.sp* MP112-09, is predicted by informatics technology to be able to synthesize lactococcin-972-like bacteriocin that might have similar antimicrobial activities with lactococcin 972: active on all

lactococci tested to date. (Martinez, Suárez et al. 1995) MPS05-B41-Lin from *Streptomyces sp.* MPS05-B41, is predicted to be able to synthesize bacteriocin Linocin M18 that is typically produced by *Brevibacterium linens* M18. This bacteriocin inhibits the growth of *Listeria* spp., several coryneform as well as other gram-positive bacteria. (Valdés-Stauber and Scherer 1994)

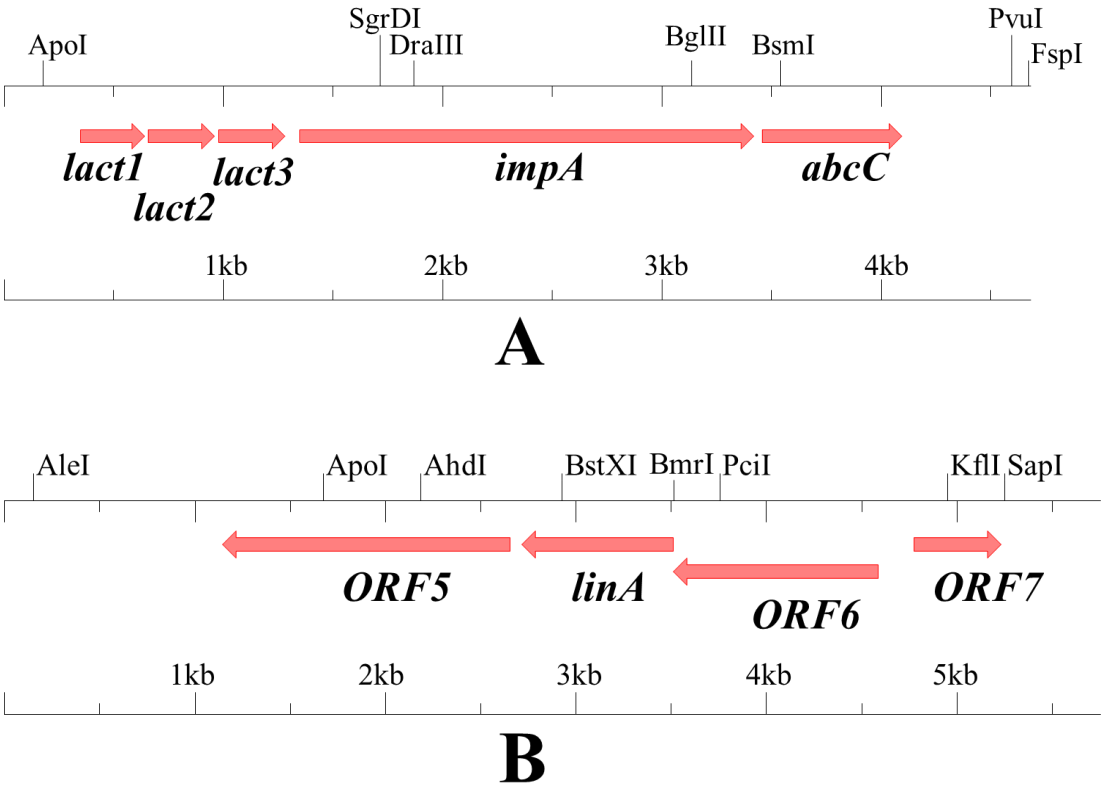


Figure 1.4 MP112-09-Lac and MPS05-B41-Lin maps (made with Clone Manager version 6, selected genes are shown). A: MP112-09-Lac. The genes *lact1-3* are responsible for lactococcin-972-like bacteriocin chain A-C respectively, *impA* encodes bacteriocin-associated integral membrane (putative immunity) protein, whereas *abcC* can synthesise putative bacteriocin exporter. The five genes seem associated with baceriocin biosynthesis and export, thus are perhaps controlled by one common promoter. The simplicity of one common promoter is the reason why this gene cluster was analysed with native promoter and *ermE**/inducible promoter

replacing its naïve promoter in this project. **B: MPS05-B41-Lin.** The gene *linA* is expected to encode Linocin-M18 bacteriocin protein while ORF5-7 is responsible for diaminopimelate decarboxylase, Dyp-type peroxidase family, putative beta-lactamase or peptidase respectively. The four genes seem encode different kinds of proteins, therefore are perhaps controlled by different promoters. This gene cluster was designed to be analysed only with its native promoter due to the presumed complexity of different promoters.

1.2.2 Actinomycetes and Streptomyces

Actinomycetes are filamentous, Gram-positive bacteria. The most important genera include *Streptomyces; Micromonospora actinoplanes; Streptosporangium, Dermatophilus*, *Thermoactinomyces*, *Microbispora*, *nocardia. (madurae* type*); Nocardia (*type *farcinica asteroides); Actinomyces (israeli* type*); Actinomyces (bovis*-type*).* They possess both fungal and bacterial properties. Specifically, *actinomycetes* form hyphae that have true branching, therefore their morphology is similar to filamentous fungi that have a vegetative thallus during part of their life cycle. Like fungi, *actinomycetes* can generate multi-hyphal strands forming upright coremia-like structures or linking adjacent colonies. Besides, *actinomycetes* have many bacterial features. Their hyphae have the diameter of 1 μ, which is the same with the bacterial order of magnitude. The cell walls of *actinomycetes* are chemically similar to those of Gram-positive bacteria, since their cell walls do not contain chitin or cellulose. (Avery and Blank 1954; Cummins and Harris 1956) *Actinomycetes* are susceptible to antibiutics that are effective against Gram-positive bacteria while are resistant to the strictly antifungal antibiotics (e.g. polyenes). This is probably due to the lack of sterols in *actinomycetes*/bacteria. (Lechevalier and Lechevalier 1967)

Actiomycetes are capable of producing many secondary metabolites in response to some changes, such as slowing down of the primary metabolism at the end of growth, unusual growth phase caused by some reasons. Due to the useful products such as antibiotics and vitamins they produce, *Actinomycetes* have become extremely important, both commercially and scientifically. (Lechevalier and Lechevalier 1967)

Streptomyces is a genus of *Actinomycetes*, Gram-positive bacteria. It grows in diverse environments, with a filamentous morphology similar to fungi. A unique feature of this organism among Gram-positives bacteria is the morphological differentiation forming a layer of hyphae, which can differentiate into a chain of spores.

Streptomyces have a complex multicellular development. When conditions of temperature, nutrients, and moisture are advantageous, spores would form germ tube, and the hyphae would develop, including the aerial hyphae. Then the continuous growth starts. Esporogenic cells may contain more than 50 copies of the chromosome in linear order, forming multinuclear aerial mycelium with septa at regular intervals. The septa divide an apical cell into a chain of uninucleated spores that would disperse when conditions are favorable (**Figure 1.5**). The most important characteristic of *Streptomyces* is the ability to synthesize bioactive secondary metabolites (BSMs) such as antifungals, antivirals, antitumoral, anti-hypertensives, immunosuppressives, and especially antibiotics. Most antibiotics are species specific, which are important for the producing *streptomyces* to compete with other organisms, even the same genre. (de Lima Procópio, da Silva et al. 2012)

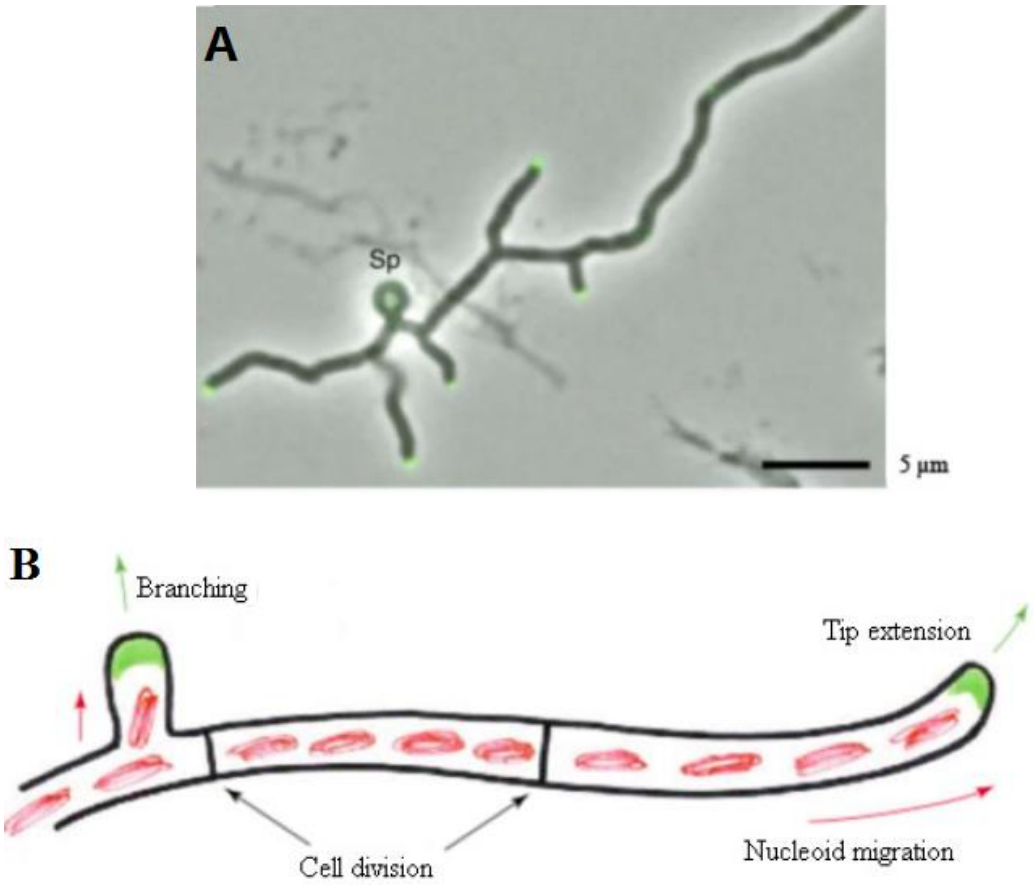
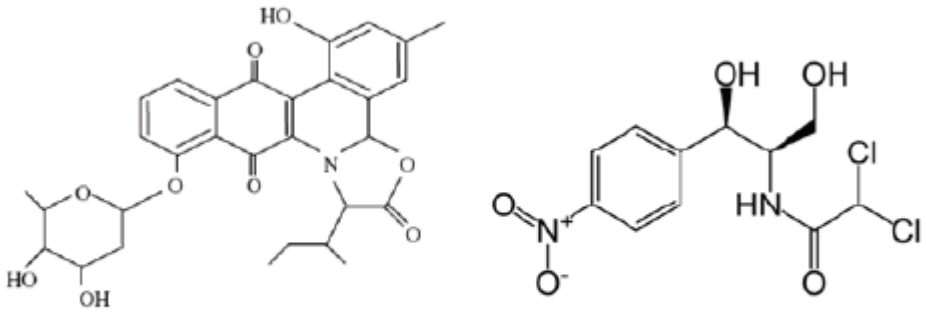


Figure 1.5 Growth of *Streptomyces*. A: the phase-contrast image of *Streptomyces* mycelium growing out of a spore (Sp). Green colour indicates an apical protein complex for tip extension. **B: Illustration of polarised growth in *Streptomyces* hyphae**. The apical cell extends its cell wall only at the tip (green), and its division by a new hyphal-cross wall makes the subapical daughter cell unable to grow. But the subapical daughter cell eventually generates a lateral branch with a new extending tip. (Flärdh 2003)

1.2.3 Jadomycin and Chloramphenicol

Jadomycin (Jd) and chloramphenicol (Cm) **(Figure 1.6)** are synthesized by *Streptomyces venezuelaee*. The jadomycins are a unique family of angucycline-derived antibiotics because of their pentacyclic 8H-benz[b]oxazolo[3,2-f]-phenanthridine backbone that includes a dihydropyridine and an oxazolone ring.(Rix, Zheng et al. 2004) Jadomycins are considered to be a promising cancer treatment drugs and favorable antibiotic, because it has versatile properties such as antibacterial, antitumor, antifungal, enzyme inhibitory and cytotoxic properties to cancer cells. The jadomycin production requires three conditions: (i) exhaustion of carbon, nitrogen or phosphate in the growth medium, (ii) addition of amino acids, (iii) ethanol/heat shock or phage infection. (Doull, Singh et al. 1994) Under the three conditions, if the addition of amino acids is isoleucine, jadomycin B is the principal products. The replacement of isoleucine by other amino acids can lead to generation of novel analogues of jadomycin B. (Rix, Zheng et al. 2004) The cyclized product of jadomycin is synthesized by the presence of amino acids in the medium which has a biosynthetic aldehyde precursor that generates a reactive aldimine to form jadomycin. The mass of vegetative cells of *Streptomyces venezuelaee* inoculated into production media can affect the jadomycin yield.



(a) Jadomycin B (b) Chloramphenicol

Figure 1.6 Chemical structures of jadomycin B and chloramphenicol. (Marianne 2012**)**

Chloramphenicol is the only antibiotic produced by *Streptomyces venezuelaee* under normal growth conditions. (Rix, Zheng et al. 2004) This antibiotic is the first natural product that contains a nitro group and also the first natural product of a derivative of dichloroacetic acid, which makes it unique. Due to the relatively simple structure and great clinical applications, Cm was modified to generate a large number of analogues. But no analogue has proved superior to the natural antibiotic in terms of antibacterial activity. Primarily, Cm is a bacteriostatic agent that completely inhibits all the true bacteria and organisms quite closely related (rickettsias, blue green algae, spirochetes) at low concentrations from 1 to 10 ug per ml. Besides, some protozoa and animal cell lines appear to be also sensitive while fungi and plants are quite resistant to this antibiotic. This selectivity serves as the basis of chloramphenicol acting as a chemotherapeutic agent. (Brock 1961)

1.3 Synthetic Biology: the Novel Enabling Technology for Novel Antibiotic Discovery

1.3.1 What is Synthetic Biology?

The generation of synthetic biology was driven by studies on mathematical logic in gene regulation in the 1960s (e.g. the lac operon) and achievements in genetic engineering in the 1970s (e.g. recombinant DNA technology). This subject aims to engineer organisms for expected applications by modifying their behaviors. (Andrianantoandro, Basu et al. 2006) There are four important levels underlying synthetic biology: (i) parts-promoters, translation start sites, ORFs, terminators, regulatory elements, etc. These individual parts are assembled into a gene of specific functions in an ordered manner. (ii) devices-combination of parts designed for particular functions. A device stands for sets of biochemical reactions such as transcription, translation, protein phosphorylation, allosteric regulation, enzymatic reactions, etc. (iii) modules-compartments of functionally connected devices. A module can be specific pathways such as metabolic pathways or signal transduction pathways. (iv)chassis-engineered hosts where the introduced parts/devices are able to form expected pathways or give predictable responses. The chassis can be obtained with synthetic genomes generated from combining devices, modules or by deleting non-essential genes in the genome of natural organisms (e.g. *E. coli*). The ideal chassis are expected to only have designed pathways and are able to accommodate any desired devices for their optimal function. (Andrianantoandro, Basu et al. 2006; Ellis, Adie et al. 2011)

The application of synthetic biology is promising in many areas. For example, in fermentation, it is required to monitor batch cultures and add inducers to induce gene expression. But engineered bacteria can avoid this time-consuming and expensive procedure because of designed coordination. (Farmer and Liao 2000; Chen and Weiss 2005) Synthetic biology would revolutionise the engineering of biosystems, and its development could influence many other scientific and engineering fields, eventually our daily life. (Andrianantoandro, Basu et al. 2006)

1.3.2 Synthetic Biology Applied to Antibiotic Biosynthesis

The advances in genome-sequencing have revealed thousands of uncharacterized secondary metabolite biosynthetic pathways, organized in the form of gene clusters, many of which are expected to produce novel antibiotics. The enabling tools of synthetic biology can be used to tune these valuable pathways, and the tuned clusters can be easily inserted into pre-engineered microbial hosts (chassis) and used in a plug-and-play fashion, in which the cellular machinery is optimized for overproduction of novel compounds such as antibiotics (**Figure 1.7**). Moreover, further engineering the biosynthetic pathways through intrinsic levels of cluster modularity can lead to the optimal production. (Medema, Breitling et al. 2011)

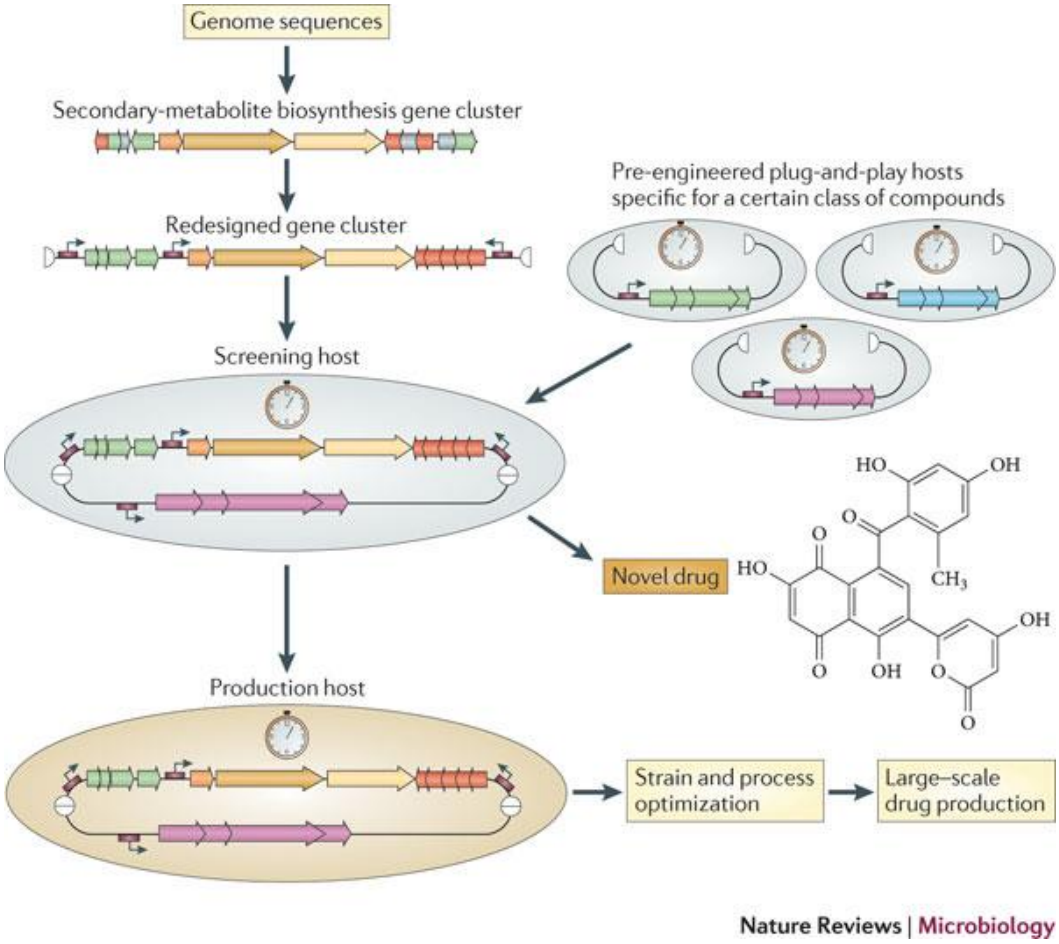


Figure 1.7 Overview of plug-and-play expression of unknown secondary metabolite biosynthetic pathways. Selected gene clusters are redesigned for streamlined expression in pre-engineered screening hosts. These hosts are specifically optimized for overproduction of a certain class of secondary metabolites via timing of gene expression (signified by the clocks) and the spatial control that guides the metabolic fluxes towards the end compound. Each screening host has a counterpart of

a complementary production host on which further synthetic tuning would be applied for efficient production of new compounds. (Medema, Breitling et al. 2011)

Initially, the silent gene clusters for new antibiotic biosynthesis can be activated for high titer production in some ways. One is based on regulatory genes that control the expression of structural biosynthetic genes. Manipulation on the pathway-specific regulators such as inactivation of a repressor, overexpression of a positive regulator can increase the production of cluster-specified antibiotics. Another strategy is the tandem amplification of a biosynthesis gene cluster, which was demonstrated in *S. coelicolor* that showed 20-fold increase in the actinorhodin yield via tandem amplification. (Murakami, Burian et al. 2011; Zotchev, Sekurova et al. 2012)

Furthermore, synthetic biology enables us to engineering the antibiotic production in both temporal and spatial scales. In temporal scale, timing the expression of pathway components contributes to the optimal antibiotic biosynthesis. During the intense constitutive production, the continuous supply for production can collide with the need for cellular survival, thus dynamic regulation of antibiotic biosynthesis pathway in time usually benefits the efficient production. One example is timing enzyme expression. The synthesis of enzymes involved in antibiotic production is one of the most expensive processes, since it costs much energy and resources, especially when those enzymes become available before meeting their substrates, their production is kind of wasteful. (Wagner 2007) Hence, fine-tuning enzyme expression is important for the producing hosts to optimally channel flux towards to antibiotic precursors, which could be achieved through engineering the transcriptional units, promoters and ribosome-binding sites. Other methods of temporal engineering include allosteric control, population synchronization of metabolic programmes, etc. In spatial scale, the spatial engineering of metabolism in producing strains is critical for optimal cellular machinery redesign, because the rate of antibiotic production in a specific pathway is dominated by the local metabolite concentrations. (Conrado, Varner et al. 2008) One strategy is scaffolding of enzyme complexes. The synthetic protein scaffolds are employed to combine proteins into complexes such that local enzyme concentrations are increased and subsequently the metabolic flux towards a specific pathway. (Dueber, Wu et al. 2009) Other strategies include cellular compartmentalization, designing microbial consortia. (Medema, Breitling et al. 2011)

However, there are also many challenges in antibiotic biosynthesis, such as challenges in efficient cloning of the gene clusters, the unpredictable outcomes originating from transplanting gene clusters to new producing hosts, etc. But with advanced techniques

in synthetic biology such as assembling small parts into genes, pathways and even the complete genomes and the synthetic genomes, etc. It is possible to build microbial systems specifically designed for novel antibiotic production, which can subsequently be upgraded for the large-scale production. (Medema, Breitling et al. 2011; Zotchev, Sekurova et al. 2012)

1.3.3 Synthetic Biology in *Streptomyces*

Streptomyces are producers of most clinically used antibiotics and several widely used drugs against common diseases, including cancer, being the main sources of bioactive compounds. Besides, genome sequencing has suggested that *Streptomyces* possess larger potentials for the production of novel secondary metabolites than previously perceived, which are organized in gene clusters. Synthetic biology is a desirable tool to utilize the treasure resources, which is promoted by the innate module of secondary metabolite biosynthetic pathways (e.g. modular polyketide synthases) as well as gene cassettes/operons and biosynthetic gene clusters. The molecular biology in *Streptomyces* has already engendered specific tools for exploiting the silent potentials in *Streptomyces*, ranging from cloning vectors to inducible promoters and translational control elements. (Medema, Breitling et al. 2011)

There are some important engineering strategies and tool boxes for synthetic biology application in *Streptomyces*. First, iterative engineering of the secondary metabolite gene clusters is a promising strategy, because our knowledge about gene regulation in *Streptomyces* is not yet enough, thus insertion of a whole re-factored gene cluster at once can lead to problems that cannot easily be attributed to a particular gene. Therefore, it is reasonable to individually optimize the divided units of a whole gene cluster in an iterative way. Second, transcriptional engineering can be applied to avoid suicide in *Streptomyces*. For example, the removal of repression on actinorhodin transporter expression avoids suicide by reducing cellular toxicity. *Streptomyces* have different transcriptional control of gene expression from most organisms as well as the high genomic GC content, as a result, regarding transcriptional engineering, common tools and kits are not ready to use in *Streptomyces*. But some inducible/constitutive promoters and terminators have been available, such as *tipA/ermE**and *Fd* respectively. (Bibb, Janssen et al. 1985; Ward, Janssen et al. 1986; Takano, White et al. 1995) Third, in the translational engineering, since RBS function is context-dependent, which means translational efficiency can be different form the wild-type if the wild-type RBSs are used, therefore, new synthetic RBSs are required

to restore the wild-type stoichiometry of the proteins. (Salis, Mirsky et al. 2009) The tool boxes in the translational level include RBSs from ribosomal proteins, antisense RNA, etc. (Takano, White et al. 1995) In terms of vectors, self-replicating plasmids are most commonly used. Besides, integrating vectors are particularly suitable for synthetic biology applications, since they are favorable for their stability, especially in terms of large inserts. (Medema, Breitling et al. 2011)

In addition, there are also many difficulties in the synthetic application in *Streptomyces*. For instance, standardized parts for *E.coli* may not function optimally in *Streptomyces*; *Streptomyces* have diverse genus members, thus much metabolic difference exists among different species such as sigma factors and 16S rRNA sequences. This means optimal expression in one host may not succeed in another. However, *Streptomyces* with genetic manipulation are popular hosts for heterologous expression of secondary metabolites from foreign sources, and many strains have showed successful heterologous expression with improved titer of antibiotics, such as *Streptomyces parvulus, S. coelicolor M145, Streptomyces avermitilis*, etc. (Zotchev, Sekurova et al. 2012) Therefore, synthetic biology application in *Streptomyces* for novel antibiotic production is still optimistic.

1.4 The Project

1.4.1 How the Project Idea is Originated?

In the crisis of novel antibiotic discovery (**seen in 1.1.5.2**), bacteria provide a promising solution (**seen in 1.2**), because the microbial natural products account for more than 60% of the used drugs. (Molinari 2009) Among those natural products obtained so far, *Actinomycetes* are the main antibacterial producer. *Streptomyces*, a genus of *Actinomycetes*, are favored in new antibiotic discovery due to three primary reasons: (1) the advanced technology in next generation sequencing and bioinformatics unrevealed that *Streptymyces* have novel potentials, previously unrecognized, to produce new bioactive products with antibiotic property, which is in the form of gene clusters. (Zotchev, Sekurova et al. 2012) (**seen in 1.2.2**) (2) Synthetic biology, an enabling tool to fulfill such potentials, is available (seen in **1.3.3**). (3) they are popular hosts for heterologous expression of secondary metabolites from foreign sources. For example, *Streptomyces parvulus*, *S. coelicolor* M145, *Streptomyces avermitilis*, etc., already showed some successful heterologous expression with improved titer of antibiotics. (Zotchev, Sekurova et al. 2012)

Streptomyces venezuelaee ATCC 10712 (ISP5230) possess a distinctive property: the reciprocal regulation between jadomycin (Jd) and chloramphenicol (Cm) production with the condition of ethanol shock (**seen in 1.1.4.2**). The project is fundamentally based on the reciprocal regulation mechanism: take advantage of this regulation system to rationally engineer *S. venezuelaee* for heterologous production of BSMs by introducing re-factored secondary metabolite biosynthesis gene clusters containing appropriate control elements. The detailed hypothesis is presented below.

1.4.2 Hypothesis and Strategies

In *Streptomyces venezuelae* ATCC 10712 (ISP5230), Ja and Cm syntheses are reversely regulated by JadR1 that activates Jd synthesis while represses Cm synthesis

with ethanol shock (**seen in 1.1.4.2**). This system can be adopted to rationally engineer *S. venezuelaee* for heterologous production of BSMs by using re-factored gene clusters containing appropriate control elements: deletion of the *jadR1* gene from the *S. venezuelaee* chromosome shall lead to down-regulation of Jd production, simultaneously induce overproduction of Cm due to the relieved repression of the Cm structural genes' promoter. This and other promoters from the *cml* gene cluster could be tested for their efficiencies with *gusA* reporter, then the efficient promoters would be introduced upstream of an exogenous BSM gene cluster, which generates a re-factored gene cluster that shall efficiently express in *jadR1*$^-$ host. Besides, the *cml* gene cluster should be completely deleted in order avoid interfering with the expression of the exogenous gene cluster.

Based on the above hypothesis, the project shall be conducted in the following procedures (simplified):

1. Delete the *jadR1* gene from the *S. venezuelae* chromosome using suicide vector and double-crossover to get *jadR1*$^-$ mutant.

2. Delete the entire *cml* gene cluster from the *S. venezuelae* genome using suicide vector and double-crossover to get *jadR1*$^-$*cml*$^-$ mutant.

3. Assemble two BSM gene clusters with native/*ermE*p* in a specially designed vector, and transfer them to *jadR1*$^-$*cml*$^-$ mutant, then evaluate the BSM production.

4. Test the expression of the reporter gene *gusA* under control of *cmlFp, cmlIp, cmlXp, jadJp* promoters in *jadR1*$^-$*cml*$^-$ mutant and find the best inducible promoter. This step can be conducted in parallel with step 3.

5. Replace native/*ermE*p* in step 3 with the best inducible promoter in step 4, transfer the two BSM gene clusters with the new promoter to *jadR1*$^-$*cml*$^-$ mutant and re-check production of novel compounds.

1.4.3 Objective and Value

Based on the hypothesis above, this project aims to establish an inducible system where novel BSMs are synthesized from re-factored gene clusters on industrial scale. The inducible promoter confirmed by GUS assay would be the switch of the inducible system. For industrial production of novel BSMs, the inducible system is necessary, since if the production is constitutive, the accumulation of BSMs from heterologous gene expression could kill hosts, as a result, no sufficient biomass is achieved and industrial scale production cannot be guaranteed. By comparison, the inducible system allows biomass accumulation at the early stage, then after the inducer (ethanol shock) is applied, the BSM production is initiated rapidly, leading to large scale production.

Provided this objective is achieved, the silent BSM gene clusters would be exploited to produce novel BSMs at large scale, and hopefully, it would overcome the pressing issues of novel antibiotic discovery and benefit the wellbeing of society.

1.4.4 Main Techniques

1.4.4.1 Gibson Assembly

The discovery of DNA ligase and restriction endonucleases engendered the recombinant DNA technology. Various methods for assembling DNA molecules through the use of restriction enzymes and PCR have been developed. (Yount, Denison et al. 2002; Shetty, Endy et al. 2008) Gibson assembly is novel in that it is capable of assembling and repairing overlapping DNA molecules in a single isothermal step. Besides, the joined DNA molecules can be as large as 583 kb and the resulted products as large as 300 kb can be clone in *E. coli*. The required reagents and enzymes are commercially available, which can be made into assembly mixture aliquots stored at –20 °C for more than one year. This method is significantly simple, since the assembly is completed by mixing the overlapping DNA molecules with one assembly aliquot and incubating the mixture at 50 °C for only 15min. **Figure 1.8** specifies the detailed process. Besides, it has other advantages: (1) exonucleases chewing back at 5’ end of double-stranded DNA do not compete with polymerase

activity, which means all enzymes necessary for DNA assembly would be simultaneously active in the single isothermal reaction. (2) circular products are not processed by the three enzymes (T5 exonuclease, Phusion DNA polymerase, Taq DNA ligase) in the mixture. (Gibson, Young et al. 2009)

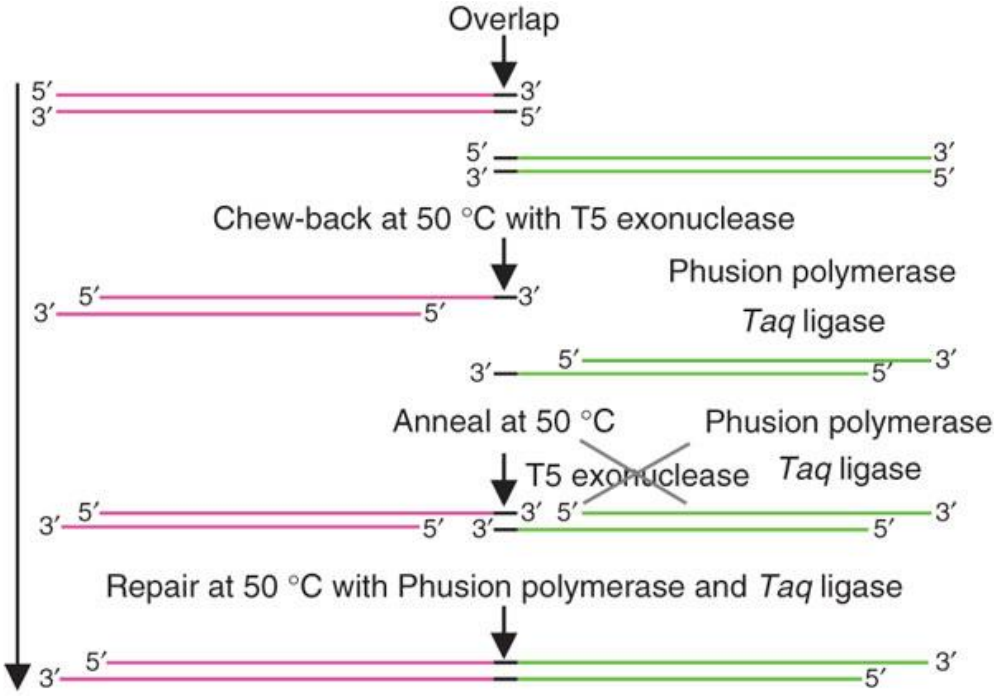


Figure 1.8 One-step isothermal *in vitro* assembly. The T5 exonuclease at 50 °C chews back at the 5' ends of double stranded DNA, exposing complementary single-stranded DNA overhangs (black). After the DNA overhangs are annealed, phusion DNA polymerase fills the gaps and Taq DNA ligase seals the nicks. T5 exonuclease is sensitive to heat, inactivated in the 50 °C incubation. As a result, DNA molecules (magenta and green) sharing terminal overlaps (black) are combined into a covalently sealed molecule. (Gibson, Young et al. 2009)

1.4.4.2 Transconjugation to *Streptomyces venezuelae*

Streptomyces are desirable candidates for new antibiotic discovery. To exploit their new antibiotic potential, genetic manipulation is necessary and therefore it is imperative that DNA can be transferred into the host strain such as to inactivate or replace specific genes. Generally, methods (e.g. polyethylene glycol (PEG)-dependent

electroporation) for introducing DNA into *Streptomyces* species are inhibited by various factors, such as breakdown of foreign DNA recognized by methylation patterns, lack of efficient gene transfer systems, and unavailability of suitable cloning vectors. (Nikodinovic, Barrow et al. 2003) However, a method, circumventing these barriers, of intergeneric conjugation was developed (Mazodier, Petter et al. 1989), and subsequently was improved in many strains. This approach relies on introducing recombinant plasmids into a donor of methylation-deficient *E.coli* and subsequently transferring them to a recipient. One of such *E.coli* is *E. coli* ET12567 that carries pUZ8002 helper plasmid assisting transfer. (MacNeil, Occi et al. 1992) Most of the transferred plasmids carry the *attP* site, integrase gene of ϕC31 phage, *RP4 oriT* (origin of ssDNA transfer), *oriR* (replication origin) and a selectable marker. (Bierman, Logan et al. 1992) ϕC31 integrase mediates the site-specific recombination between the chromosomal *attB* site (34 pb) and plasmid *attP* site (39 pb) while *RP4 oriT* is the site where helper plasmid functions in *trans*. The DNA sequences of *attB* are proved to be highly conserved in various *Streptomyces* strains. (Sioud, Aigle et al. 2009) Plasmids without the above features are either in autonomous replication or integrated into host genome through homologous recombination. (Bierman, Logan et al. 1992)

In *Streptomyces*, transconjugation takes place during in the early growth phase when *Streptomyces* form mycelia on solid media containing $MgCl_2$. The transconjugation mechanism in mycelial *streptomycetes* is different from that in unicellular Gram-positive and Gram-negative bacteria in the way that the unprocessed double-stranded DNA molecule is translocated into the host by a plasmid-encoded septal DNA translocator TraB that non-covalently interacts with the DNA. (Reuther, Gekeler et al. 2006)

In the project, *E. coli* ET12567 (pUZ8002) was used to transfer recombinant plasmids to *Streptomyces venezuelae.* The recombinant plasmids p-*jad*-D/p-*cml*-D (pSOK 201 derived) were integrated to chromosome via homologous recombination, due to lack of *attP* and *int* in pSOK201, while pLacNat/pLacNP (pSOK 804/pSOK 806 derived) and *gusA* plasmids (pSOK 808 derived) were integrated by site-specific recombination, due to the presence of *attP* and *int* (refer to **2.1.4**).

1.4.4.3 Double-crossover for Deletion Mutants

Traditionally, gene-deleted mutants are generated based on the homologous recombination carried out by double-crossover at one step. If the two flanking fragments are large enough, this method can be efficient, however, this method as well as the others often introduces a selectable marker into the genome that remains in the chromosome permanently. (Kieser 2000) This is not favourable due to some disadvantages: the resistance marker remained in the chromosome can execute polar effects on the expression of nearby genes; the expression of selectable markers may influence bacterial metabolism as well; in terms of *actinomycete* genomes, the resistance markers are limited, which means multiple gene deletions in the same genetic background are also restricted. (Siegl and Luzhetskyy 2012)

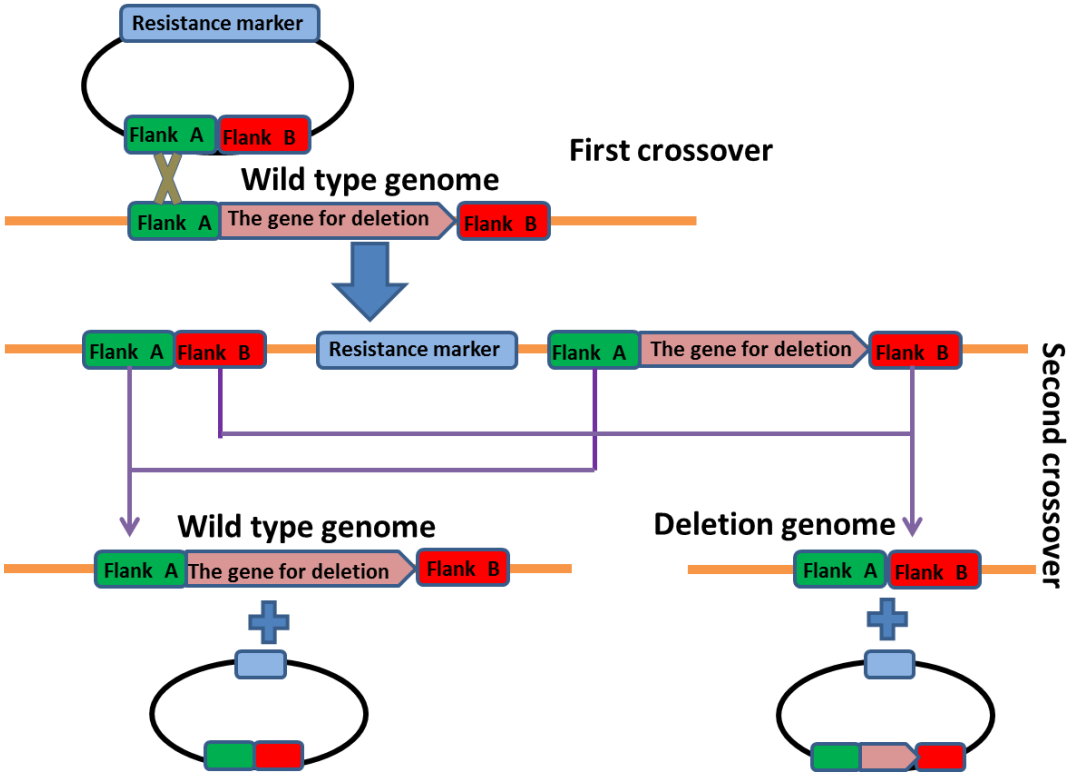


Figure 1.9 Double crossover processes for deletion mutants. Two flanking fragments A (green) and B (red) of the gene for deletion were assembled in a plasmid containing a resistance marker. The first crossover resulted in the integration of the assembled plasmid into the genome DNA. The resulted genome was selected through resistance marker and verified by PCR. The second crossover resulted in looped out

plasmids and simultaneously generated wild type genome and deletion genome. The deletion genome was identified by PCR.

To circumvent such demerits, in this project, the double-crossover was carried out in two steps, leaving no selectable marker in the deletion mutant. The plasmid with apramycin (Am) resistance was integrated into genomic DNA through the first homologous recombination (**first-crossover**), and the first cross-over mutants would be selected against Am, subsequently verified by PCR. Then among the genuine first-crossover mutants, those losing Am resistance were assumed to be the deletion mutants or wild type, generated through the second homologous recombination (**second-crossover**), and the deletion mutants could be identified by PCR. **Figure 1.9** represents the detailed process.

1.4.4.4 Replica Plating

In microbiology, a frequent chore is the transferring isolates from one substrate to other selective or indicator agar media. In place of an inoculating needle, Lederberg & Lederberg (1952) devised the replica plating technique that employed a velveteen pad to replicate randomly distributed colonies rapidly from initial plates and print the replica onto other media. This technique is useful for routine tests requiring repetitive inoculations of many isolates on different media. (Lederberg and Lederberg 1952) There are mainly two applications of replica plating. The first one is about gene recombination that exploits nutritional requirements as genetic characters, whereas the second one is the isolation of mutants. It has been successfully applied to bacteria, *actinomycetes* and unicellular algae. (Roberts 1959)

Replica plating consists of three steps: (1) spreading a suspension of organisms on a solid medium so that the organisms on the initial plate can form single colonies after incubation. (2) using a pad of sterile velveteen or filter-paper to make replica inoculations from the initial plate onto a series of plates with different media. (3) comparing the responses of individual colonies to different media (the series of replica plates) or comparing the replica plates with the initial plates to find mutants. One important factor affecting the efficiency of the technique is the colony density, therefore, when preparing initial plates it is reasonable to strike a balance between the

number of colonies on each plate and the fact that more dense the colonies, more mutants that will not be detected because of mixed growth. In order to well control the colony density, initial plates are prepared by spreading a dilute spore suspension to yield 100-200 discrete colonies on each plate that is incubated until good sporulation appears. (Roberts 1959)

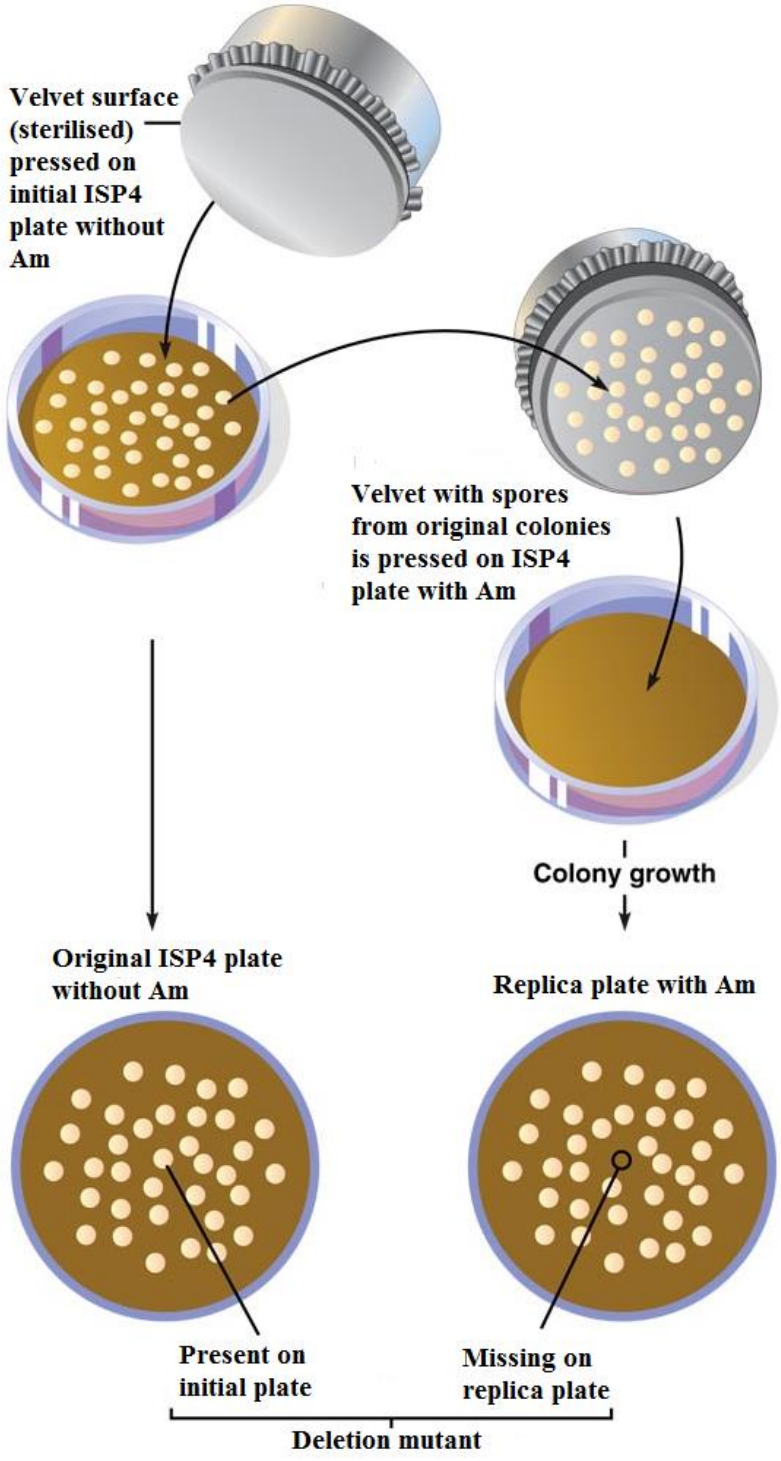


Figure 1.10 Replica plating process (Pearson Benjamin Cummings, 2006) Am: apramycin. First, discrete spores formed on initial ISP4 plate. Then, a piece of sterilised velvet was pressed on initial plate such that spores from each colony were replicated on the velvet surface. The velvet with spores was pressed on ISP4 plate with Am 50 μl/ml, incubated at 30°C overnight. Colonies present on initial plate while missing on replica plate were candidate mutants and subjected to PCR verification. (StudyBlue 2014)

In this project, velvet was used and the spores were diluted from 10^{-1} to 10^{-6} (or 10^{-7}) to ensure 100-200 discrete colonies per plate. **Figure 1.10** illustrates the process.

1.4.4.5 β-Glucuronidase (GUS): A Sensitive and Versatile Reporter

The clinically important secondary metabolites produced by *Actinomycetes* is tightly controlled by various regulatory proteins, therefore, understanding the cryptic regulatory cascades should benefit the generation of antibiotic overproducers and clarification the roles of the natural products in the producing bacteria. (Baltz 2001; Bibb 2005) Reporter genes are important for the investigation on the gene expression regulation, as after fused with regulatory elements and introduced into the biological system, they enable the quantification of biochemical process by providing visual signals.(Casadaban, Chou et al. 1980; Shuman and Silhavy 2003)

Among the reporter genes used in *actinomycete* species, the most popular ones are GFP and luciferase. GFP from jellyfish is a common reporter used to monitor the trafficking and subcellular localization of proteins living organisms, because of its ability to tolerate N- and C-terminal translational fusions. (Kain, Adams et al. 1995) In gene expression research, however, GFP is inferior due to some reasons. First, GFP is less sensitive owing to much background fluorescence and no enzymatic signal amplification. Second, UV-induced toxicity in GFP assay limits the period of observation and analysis. Moreover, *actinomycetes* often exhibit high levels of auto-

fluorescence, making the analysis more complicated. (Kieser, Bibb et al.) Luciferase assays are advantageous in monitoring the dynamics of gene transcription due to the rapid turnover of luciferases. Nonetheless, the dependence on multiple reagents ($FMNH_2$, O_2, ATP, Mg_2O_2) in enzymatic reactions make luciferase inferior. Besides, in *actinomycetes*, the lack of auto-luminescence causes a high signal-to-noise ratio in luciferase assays. (Jefferson, Kavanagh et al. 1987; Craney, Hohenauer et al. 2007)

By comparison, β-glucuronidase (GUS) encoded by gene *gusA* is a sensitive and versatile reporter in *actinomycetes*. Initially, GUS can hydrolyze various β-glucuronides, allowing assays in various types in cheap and simple ways (e.g. spectrophotometric: p-nitrophenyl-β-D-glucuronide and phenolphthalein-β-D-glucuronide, chemiluminescent: 1, 2-dioxetane-β-D-glucuronide). (Jefferson, Kavanagh et al. 1987) In addition, GUS exhibits high specific enzymatic activity as well as stability, thus offers an unparalleled sensitivity. Moreover, this enzyme tolerates the most commonly used chemicals and assay conditions (e.g., pH and temperature) and large N- and C-terminal translational fusions. (Jefferson 1989; Hull and Devic 1996) In terms of *streptomycetes*, most species do not possess any endogenous GUS activity. The GUS system has been successfully employed in gene expression studies at the levels of transcription and translation, and would have more application areas. (Jefferson, Kavanagh et al. 1987)

The promoter of the erythromycin resistance gene (*ermE*) is known to be constitutive and partially inducible in its native context. Its variant *ermE*p* with one base pair mutation acquires enhanced promoter activity and thus has been frequently utilised in in *Streptomyces* and related bacteria as a strong constitutive promoter for both native and heterologous gene expression. (Wilkinson, Hughes-Thomas et al. 2002; Medema,

Breitling et al. 2011) Thus, *gusA* under control of *ermE*p* can be used as positive control.

In this project, GUS/p-nitrophenyl-β-D-glucuronide system was used. Specifically, the *gusA* was fused with *ermE*p* or *cml/jad* promoters and integrated into *jadR1*⁻*cml*⁻ mutant, respectively. The promoter efficiencies were expressed in Miller units of glucuronidase per mg of total protein and compared to find the best promoter. The total protein concentrations were measured with Bradford assay.

1.4.4.6 Bradford Assay

A fast and accurate measurement of protein concentration is necessary in many biological researches. Many methods for estimating protein concentrations have been developed, some of which are not protein specific, such as Kjeldahl or elemental analysis. By contrast, other methods are available with greater specificity for protein, which are based on reduction, such as Lowry method of copper reduction, bicinchoninic acid, silver binding, ultraviolet, biuret. The main drawback of these approaches is the interference of compounds (e.g. phenolics) causing oxidoreduction. Besides, methods relying on protein precipitation (TCA, amido black) or hydrolysis (ninhydfin, OPA) have been developed as well, but they are inferior due to the time-consuming steps and some other limitations. (Jones, Hare et al. 1989)

A method described by Bradford has been favored in quantifying protein in many research areas, because this approach is faster, simpler, more sensitive and cheaper than most other methods. For example, compared with Lowry method, Bradford is more sensitive and less susceptible to the interference of common reagent and non-protein molecules. (Kruger 1994) This technique exploits Coomassie brilliant blue G-250 dye that exists in three forms: cationic, neutral, and anionic. Only the anion can bind to protein to produce a complex with absorbance at 595 nm. Anion is not available at the dye reagent pH while available in the presence of proteins. The dye has specific binding requirements for macromolecules, which primarily are arginine

and basic and aromatic amino acid residues. Moreover, the dye does not react with free amino acids, low-molecular-weight polypeptides, or many other nitrogen-containing or protein-like molecules. (Compton and Jones 1985; Jones, Hare et al. 1989) However, some chemical-protein and chemical-dye interaction can interfere with the assay. For instance, non-protein compounds can interfere by shifting the equilibrium of the dye among the three species; some detergents, flavonoids, and basic protein buffers, can stabilise the neutral dye species by direct binding. (Bio-Rad 2014)

The Bradford method generates relative measurements with spectrophotometer, because it is common to use a purified protein standard. With the absence of purified protein being assayed as ideal standard, BSA serves as the most common protein standard for protein assays. In this project, the standard curve was made from BAS 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, and the samples were 20× diluted. By doing this, the samples were positioned in the linear range (for most spectrophotometers is 0.2 - 0.8 O.D. units) of the standard curve, which made the measurement more accurate than otherwise.

2. Materials and Methods

In this section, chemicals, strains and instruments are presented first, then the protocols are exhibited. The all plasmids maps and media recipes are given in **Appendix B** (Page 116-118) and **Appendix C** (Page 119-122), respectively.

2.1 Chemicals, Strains and Instruments

2.1.1 Chemicals

The main chemicals/reagents used in this project are presented below in **table 2.1**.

Table 2.1 Chemicals/reagents/enzymes used in the project

Chemicals/Reagents	**Manufacturer**
Agar bacteriological	OXOID LTD
BSA	New England Biolabs Inc.
Difco ISP Medium 4	Becton, Dickinson and Company
DMSO	Sigma-Aldrich
dNTP's	Promega
EDTA (0.5 M)	Merck
Ethanol (96 %)	VWR
Expand High Fidelity (EHF) DNA polymerase	Roche
GelGreen Nucleic Acid Stain (10 000x) (Cat: 41005)	Biotium
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific Inc.
Glycerol bidistillied (99.5 %)	AnalaR NORMAPUR, VWR Prolab

MasterAmp™ Extra-Long DNA Polymerase Mix (2.5 U/µl)	Epicentre
High Fidelity 2x Long PCR premixes (1-9)	Epicentre
Phusion High-Fidelity DNA Polymerase (2 U/µL)	Thermo Fisher Scientific Inc.
Isopropanol	Arcus
Kanamycin	AppliChem
Nalidixic acid sodium salt	Sigma-Aldrich
Apramycin	Sigma-Aldrich
Chloramphenicol	AppliChem
Ampicillin sodium salt	BioChemica, AppliChem
Lysozyme (> 30 000 FIP U/mg)	Merck
Malt extract	Sigma-Aldrich
Maltose (Lot 109H1049)	Sigma-Aldrich
MgCl2 (25mM)	Roche
MOPS sodium salt (99 %)	AppliChem
NaCl	VWR
Na_2HPO_4-$2H_2O$ (99.5 %)	Merck
$NaH2PO_4$-H2O (99.0 %)	Merck
D-galactose	SIGMA
L-isoleucine	SIGMA
KH_2PO_4	ACROS ORGANICS

K_2HPO_4	Merck
NaOH (99 %)	Merck
$MgSO_4$-$7H_2O$	VWR International AS
$CaCl_2$-$2H_2O$	VWR International AS
$FeSO_4$-$7H_2O$	Merck
NEB buffers	New England Biolabs Inc.
p-nitrophenyl-β-D-glucuronide (PNDG) (99.4 %)	CalbioChem
Primers (attachment)	SIGMA-ALORICH
*Pst*I, *Sac*I, *Bmr*I, *Age*I, *Not*I, *Bam*HI, *Kas*I, *Nar*I, *Apo*I, *Dpn*I	New England Biolabs Inc.
CutSmart 10×	New England Biolabs Inc.
T5 exonuclease (10 U/µl)	New England Biolabs Inc.
Taq DNA ligase (40 000 U/ml)	New England Biolabs Inc.
Triton X-100 SigmaUltra	Sigma-Aldrich
Tryptone	OXOID LTD
Tryptone soya broth (TSB)	OXOID LTD
Yeast extract	OXOID LTD
Wizard Genomic DNA Purification Kit	Promega
QIAquick PCR Purification Kit	QIAGEN
QIAquick® Gel Extraction Kit	QIAGEN
QIAGEN DNeasy® Blood & Tissue kit	QIAGEN

2.1.2 Instruments

The main instruments used this project are listed in **Table 2.2**.

Table 2.2 Instruments used in the project.

Equipment	**Specification**	**Manufacturer**
Autoclave	SX-500E	Tomy
DNA gel electrophoresis power source	Power PAC	Bio-Rad
Laminar hood	S-2020 1.2	Heto-Holten
DNA gel electrophoresis systems	Owl Easycast B1A Mini	Thermo scientific
Freezer (- 20 °C)		Electrolux
Freezer (- 80 °C)	C66085	New Brunswick Scientific
Eppendorf tubes	1.5 ml	Sarstedt
ChemiDoc™ XRS+ Imaging System with Image Lab™ Software	Universal HoodII	Bio-Rad
Microcentrifuge	5415 R	Eppendorf AG
PCR machine	5331 46612	Eppendorf

Petri plates	90×16.2mm	Gosselin
pH-meter	PHM92	Unigen
Pipettes	10 µl, 200 µl, 1000 µl	Eppendorf
Pipette tips	200 µl, 1 ml	Fisher Scientific
Pipette tips	10 µl	Molecular BioProducts
Pyrex^{R} baffled Erlenmeyer flask	125 ml	Sigma-Aldrich
NanoDrop	ND-1000	Saveen Werner
Incubators (30 °C, 37 °C)	474	ASSAB
Shaking incubators (30 °C, 37 °C)	28573	Infors HT multitron
Spectrophotometer	TECAN INFINITE TE 200 PRO	Noax Lab AS
24-well Cell Culture Plate	1.9cm^2 /well	Fisher Scientific
96-well Cell Culture Plate	0.32cm^2/well	Fisher Scientific

2.1.3 Strains/Plasmids

The strains/plasmids used in this project are listed in **Table 2.3**. **Plasmid maps** are presented in **Appendix B** (Page 116-118).

Table 2.3 Strains and plasmids used in the project.

For visual convenience, they are marked with different colours by different categories.

Bacterial strains	**Genotype/ Phenotype**	**Source/Reference**
Escherichia coli DH5α	Competent cloning strain, heat-shock transformed. Genotype: *LuxS supE44 ΔlacU169, (ϕ80 lacZΔM15) hsdR17, recA1, endA1, gyrA96, thi-1, relA1*	(Wood, Barrios et al. 2006)
Escherichia coli ET125671 (pUZ8002)	Methylation deficient (*dam*$^{-}$, *dcm*$^{-}$, *hsdM*$^{-}$). Mediates conjugative DNA transfer from RP4 oriT with helper plasmid pUZ8002 (Kan^{R}, Cm^{R}).	(MacNeil, Occi et al. 1992)
Streptomyces venezuelaee ATCC10712 (ISP5230)	Wild type, GC-rich, linear chromosomes, chloramphenicol and jadomycin B producer (in response to disparate conditions) (refer to **1.2.2**).	Sergey B. Zotchev /Olga N. Sekurova (Yang, Han et al. 1995)
Nocardiopsis.sp MP112-09	High GC. Colonies are slow growing, aerobic, Gram-positive, nonacid-fast, coarsely wrinkled to folded, and white or pink to red in color.	(Meyer 1976)
Streptomyces sp. MPS05-B41	High GC, Gram+, aerobic. The threadlike mycelia bear chains of spores at maturity (refer to **1.2.2**).	(Britannica 2014)
S. venezuelae- p201-*jad*-D	Wild type genome integrated with p201-*jad*-D (first crossover).	**The project**
jadR1^{-} mutant of *S. venezuelae*	*jadR1* deleted from wild type genome	**The project**

	(second crossover).	
jadR1^{-} mutant of *S. venezuelae*-p201-*cml*-D	*jadR1*$^{-}$ mutant genome integrated with p201-*cml*-D (first crossover)	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*	*cml* deleted from *jadR1*$^{-}$ mutant genome (second crossover).	**The project**
Wild type *S. venezuelae*-pSOK808-*cmlFp*	Wild type genome integrated with pSOK808-*cmlF*.	**The project**
Wild type *S. venezuelae*-pSOK808-*cmlIp*	Wild type genome integrated with pSOK808-*cmlI*.	**The project**
Wild type *S. venezuelae*-pSOK808-*cmlXp*	Wild type genome integrated with pSOK808-*cmlX*.	**The project**
Wild type *S. venezuelae*-pSOK808- *jadJp*	Wild type genome integrated with pSOK808- *jadJp*.	**The project**
Wild type *S. venezuelae*-pSOK808	Wild type genome integrated with pSOK808.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK808-*cmlFp*	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK808-*cmlFp*.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK808-*cmlIp*	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK808-*cmlIp*.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK808-*cmlXp*	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK808-*cmlXp*.	**The project**

jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK808- *jadJp*	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK808- *jadJp*.	**The project**
Wild *S. venezuelae*-pSOK808	Wild *S. venezuelae* genome integrated with pSOK808.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK808	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK808.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK804-lacNat	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK804-lacNat.	**The project**
jadR1^{-}*cml*$^{-}$ mutant of *S. venezuelae*-pSOK804-NP	*jadR1*$^{-}$*cml*$^{-}$ mutant genome integrated with pSOK804-NP.	**The project**
Plasmid	**Genotype**	**Source**
pSOK 201	Replication initiator protein gene, Am^{R}, *RP4 oriT*, *ColEI* replication origin.	Sergey B. Zotchev /Olga N. Sekurova
pSOK 804	*ColEI* replication origin, Am^{R}, *RP4 oriT*, *attP*, *int*.	Sergey B. Zotchev /Olga N. Sekurova
pSOK 806	*ColEI* replication origin, Am^{R}, *RP4 oriT*, *attP*, *int*, *ermE*	Sergey B. Zotchev /Olga N. Sekurova
pSOK 808	*ColEI* replication origin, Am^{R}, *RP4 oriT*, *attP*, *int*, *ermE*, ***gusA*.**	Sergey B. Zotchev /Olga N. Sekurova
pSOK201-*jad*-D	Two *jadR1* flanks, *RP4 oriT*, Am^{R}	**This project**

	gene.	
pSOK201-*cml*-D	Two *cml* flanks, *RP4 oriT*, Am^R gene, *hbs'* (truncate globin protein).	**This project**
pSOK808-*cmlFp*	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, *gusA*, *cmlFp*.	**This project**
pSOK808-*cmlIp*	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, *gusA*, *cmlIp*.	**This project**
pSOK808-*cmlXp*	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, *gusA*, *cmlXp*.	**This project**
pSOK808-*jadJp*	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, *gusA*, *jadJp*.	**This project**
pSOK804-lacNat	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, a gene cluster of *MP112-09*-cluster 6-Lactococcin972-like.	**This project**
pSOK806-NP	*ColEI* replication origin, Am^R, *RP4 oriT*, *attP*, *int*, a gene cluster of *MPS05-B41*-cluster 28-Lincocin-M18-like.	**This project**

2.2 Protocols

2.2.1 PCR Reaction

The generalized PCR reaction recipes and programmes are presented in **Table 2.3and 2.4, Table 2.6** respectively. **Table 2.5** shows the fragments and templates for each assembled plasmid, which are used to specify a particular PCR reaction. Primers are listed in **Appendix A** (Page 114-115).

Table 2.3 PCR reaction mixture I (based on Expand High Fidelity PCR system)

Components	**Amount [µl]**
Buffer (vial 2, 10 × $MgCl_2$)	5
DMSO	3.5
BSA (1 mg/ml)	4
dNTPs (2.5 mM)	1
Forward and reverse primer (each 10 µM, diluted with Buffer EB)	1 (each)
Template (100ng)	1
DNA polymerase (Expand High Fidelity)	0.8
sd H_2O	32.7
Total: 50 µl. Prepare on ice.	

Table 2.4 PCR reaction mixture I I (based on MasterAmp™ Extra-Long PCR Kit)

Premix 9: pDNA template. Premix 8: linear DNA template. DNA polymerase Expand High Fidelity or Phusion High-Fidelity was used for amplifying DNA size less than 7kb. MasterAmp™ Extra-Long DNA Polymerase was used for amplifying DNA size longer than 7kb. The mixture was prepared on ice.

Components	**Amount [µl]**
sd H_2O	16
MasterAmp™ Extra-Long PCR 2× Premix	20

Template	1
Forward and reverse primer (each 10 μM, diluted with Buffer EB)	1+1
DNA polymerase	1
Total	40

Table 2.5 Fragments (with size) and templates for assembled pDNA.

The pSOK201 and pSOK808 were initially provided by researcher Olga Sekurova and then were cloned in *E.coli* DH5α.

Assembled pDNA in the project	**Assembled fragment**	**Template**
pSOK201-*jad*-D	p201 vector fragment (Am^R) (3.1kb)	pSOK 201
	jadR1-FlankA (2.1kb) and *jadR1*-FlankB (2.2kb)	gDNA of wild type *Streptomyces venezuelaee* ATCC 10712
pSOK201-*cml*-D	p201 vector fragment (Am^R) (3.1kb)	pSOK 201
	cml-FlankA (2.2kb) and *cml*-FlankB (2.3)	gDNA of wild type *Streptomyces venezuelaee* ATCC 10712
pSOK808-*cmlFp*, pSOK808-*cmlIp*, pSOK808-*cmlXp*, pSOK808-*jadJp*	*cmlFp*(314bp), *cmlIp*(233bp), *cmlXp*(233bp), *jadJp*(331bp).	gDNA of wild type *Streptomyces venezuelaee* ATCC 10712
	P808 vector fragment (Am^R) (7.6kb (*cmlFp*), 7.5kb (*cmlIp*), 7.5kb (*cmlXp*), 7.6kb (*jadJp*))	pSOK808
pSOK804-lacNat	MP112-09-Lac fragment (4.7kb)	gDNA of *Nocardiopsis.sp* MP112-09

	P804 vector fragment (Am^R) (5.4kb)	pSOK804
pSOK806-NP	MP112-09-Lac fragment (4.7kb)	gDNA of *Nocardiopsis.sp* MP112-09
	P804 vector fragment (Am^R) (5.0kb)	pSOK804
pSOK804-Lin plasmid.	MPS05-B41-Lin (5.8kb)	gDNA of *Streptomyces sp.* MPS05-B41
	P804 vector fragment (Am^R) (5.4kb)	pSOK804

Table 2.6 PCR programmes (apply to PCR reaction mixture I and II).

The elongation time was set 2 min longer than theoretical time that was calculated based on the approximate amplification speed 1kb/min. For example: 6min elongation for amplifying 4kb DNA (theoretical elongation is 4min). The fragment sizes are available in **table 2.6**.

Step	**Temperature (°C)**	**Time (min)**
1. First denaturation	95	5
2. Denaturation	95	1
3. Annealing	56	1
4. Elongation	68	2 min + theoretical time
5.Continued elongation	68	7
6. Repeat 2-4 for 25 cycles		
7. Hold	4	∞

PCR verification of assembled pDNA after restriction enzyme check: the candidates passing enzyme digestion were used as templates to amplify the fragments used for assembling the same pDNA, then the products were compared with their purified counterparts (**2.3.1.1**) for assembly on gel. This measurement was taken to avoid the wrong plasmids resulted from byproducts in assembled fragments, since though the fragments were checked before ligation on gel (to decide volume ratio in ligation), the misprimed fragments, if not fully eliminated in purification, might be too less to observe, consequently, wrong assembled plasmids could be engendered (seen in **3.1.1.1**). Plasmids generated from p-*cml*-D onward were subjected to both restriction

enzyme check and PCR verification (**double check**), because a wrong p-*cml*-D passing digestion check led to no double mutant generated, and finally was identified by PCR verification.

PCR verification of first crossover and double crossover: the primer pair of FlankA forward and FlankB reverse were used, since this primer pair could amplify the fragment FlankA+FlankB that characterises the real mutants. Right mutants were determined by the right band on gel (*jadR1* deletion: 4.3kb; *cml* deletion: 4.5kb), with WT and p-*jad*-D/p-*cml*-D as negative and positive control respectively.

2.2.2 Gel Electrophoresis

Gel electrophoresis is used to separate charged biological macromolecules with different sizes, such as nucleic acids and proteins (SDS-PAGE). Negatively charged DNAs are separated in gel with electrical field, with smaller fragments moving faster. (Slater and Noolandi 1986) In this project, this technique was used to check PCR products and restriction enzyme digestion patterns.

Materials:

0.8 % Agarose gel. 1 × TAE-buffer. Casting tray. Gel Doc 2000. Loading dye. DNA ruler. Power supply.

Procedures:

1. The 0.8 % agarose solution was poured into a casting tray (without bubbles) with a comb placed to make wells, cooled down for 20 min.
2. Samples were mixed with loading dye (1 volume of loading dye to 5 volumes of purified DNA). Samples and DNA ladder (0.8 μl) were applied to separate

wells. Usually, 2-5 μl sample of PCR products was used, depending on the DNA concentration. If isolating DNA of a specific size, or analyze digested pDNA, all samples were applied.

3. The power supply was turned on to run gel. For large tray (12×14 cm): 75V, 270 min (get results on the same day) or 20V, overnight. For small tray (8×8 cm), 60V, 120min.
4. DNA bands were visualised with imaging device. Bands with a particular size could be cut off under UV light in the imaging device (need to wear goggles). (Aaij and Borst 1972)

2.2.3 PCR Product Purification

The PCR product needs to be purified for assembly or digestion. The purification was conducted according to instruction from QIAquick PCR Purification Kit.

Materials:

QIAquick PCR Purification Kit. Microcentrifuge.

Notes before starting:

Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of $\leqslant$7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

Procedures:

1. Five volumes Buffer PBI was added to 1 volume of the PCR reaction and mixed. If the color of the mixture was orange or violet, 10 µl 3 M sodium acetate, pH 5.0 would be added, and mixed. The color of the mixture would turn yellow.
2. A QIAquick column was placed in a provided 2 ml collection tube or into a vacuum manifold (Details on how to set up a vacuum manifold seen in the QIAquick Spin Handbook).
3. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30–60 s or a vacuum was applied to the manifold until all the samples had passed through the column. The flow-through was discarded and the QIAquick column was placed back in the same tube.
4. To wash, 0.75 ml Buffer PE was added to the QIAquick column, centrifuged for 30–60s or vacuum applied. The flow-through was discarded and the QIAquick column was placed back in the same tube.
5. The QIAquick column was centrifuged once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Each QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) was added to the center of the QIAquick membrane, centrifuged for 1 min. For increased DNA concentration, 30 µl elution buffer was added to the center of the QIAquick membrane, after standing for 1 min, the column was centrifuged.
8. If the purified DNA was to be analyzed on a gel, 1 volume of Loading Dye was added to 5 volumes of purified DNA. The solution was mixed by

pipetting up and down before loaded on the gel.

2.2.4 Purify PCR Products Exercised from Gel

If byproducts are amplified, the total PCR products should be subjected to gel electrophoresis so as to isolate the right products by excising the right band. If the template in PCR reaction is pDNA, the product should be isolated from contaminating template in the same way. The excised PCR product from gel was purified according to instruction from QIAquick® Gel Extraction Kit.

Materials:

QIAquick® Gel Extraction Kit. Microcentrifuge.

Notes before starting:

The yellow color of Buffer QG indicates a pH ≤7.5.

Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume). Isopropanol (100%) and a heating block or water bath at 50°C are required.

All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge.

Procedures:

1. The DNA fragment was excised from gel with a clean, sharp scalpel.
2. After weighed the gel slice in a colorless tube, 3 volumes Buffer QG was added to 1 volume gel (100 mg ~ 100 μl). For >2% agarose gels, 6 volumes Buffer QG was added.
3. The tube was incubated at 50°C for 10 min (or until the gel slice was completely dissolved), vortexed every 2–3 min to help dissolve gel.

4. After the gel slice was dissolved completely, check that the color of the mixture should be checked whether it was yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture was orange or violet, 10 µl 3 M sodium acetate, pH 5.0 was added and mixed. The color of the mixture would turn yellow.
5. One gel volume of isopropanol was added to the sample, mixed.
6. A QIAquick spin column was placed in a provided 2 ml collection tube or into a vacuum manifold.
7. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min or the manifold was applied with vacuum until all the samples had passed through the column. The flow-through was discarded and the QIAquick column was placed back into the same tube. For sample volumes of >800 µl, the steps were repeated.
8. If the DNA would subsequently be used for sequencing, in vitro transcription, or microinjection, 0.5 ml Buffer QG was added to the QIAquick column and centrifuged for 1 min or vacuum applied. The flow-through was discarded and the QIAquick column was placed back into the same tube.
9. To wash, 0.75 ml Buffer PE was added to QIAquick column and centrifuged for 1 min or vacuum applied. The flow-through was discarded and the QIAquick column was placed back into the same tube.
10. The QIAquick column was centrifuged once more in the provided 2 ml collection tube for 1 min at 13,000 rpm to remove residual wash buffer.
11. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.
12. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water was added to the center of the QIAquick membrane and centrifuged for 1 min. For

increased DNA concentration, 30 µl Buffer EB was added to the center of the QIAquick membrane, after standing for 1 min, the column was centrifuged for 1 min. After the addition of Buffer EB to the QIAquick membrane, the yield of purified DNA could be increased by increasing the incubation time to up to 4 min.

13. If the purified DNA was to be analyzed on a gel, 1 volume of Loading Dye would be added to 5 volumes of purified DNA. The solution was mixed by pipetting up and down before loaded on the gel.

2.2.5 *Dpn*I digestion

Vector DNAs amplified from pDNAs that are purified from a dam^+ strain shall be subjected to DpnI digestion, because the template pDNAs are contamination in Gibson Assembly mixture. *Dpn*I cleaves only when its recognition site is methylated, thus it only cleaves pDNA from a dam+ strain, leaving the vector DNAs intact. (BioLabs 2014) Specifically, the total vector DNA product was applied on gel and excised from contaminating template band, after purified, it was subjected to *Dpn*I digestion (37 °C, 3 h) to eliminate the remaining template.

Table 2.7 *Dpn*I digestion.

The mixture was incubated at 37 °C, 3h. *Dpn*I was inactivated at 80 °C, 20 min, then the mixture was held at 4 °C.

Components	**Amount**
Vector DNA fragment	29.5 µl
CutSmart (or NE Bufer 4)	3.5 µl
*Dpn*I	2 µl

2.2.6 Gibson Assembly

Gibson Assembly was used to assemble plasmids in this project, due to its novelty in assembling DNA fragments in a single isothermal step (seen in **1.4.4.1**). (Gibson, Young et al. 2009) The recipes used in this project are listed below.

Table 2.8 Gibson 5× isothermal reaction buffer

Ingredients	**Amount**
Tris-HCl (1 M)	3 ml
MgCl2 (2 M)	150 µl
dGTP, dATP, dCTP, dTTP (each100 mM)	Each 60 µl
DTT (1 M)	300 µl
Polyethyleneglycol (PEG-8000)	1.5 g
NAD (100 mM)	300 µl

Table 2.9 Gibson Assembly Master Mix.

The 0.64 µl of T5 exonuclease was 10 × diluted (9 µl 1×T5 exonuclease buffer + 1 µl T5 exonuclease) so as to avoid variance from adding 0.064 µl (too small volume) 1×T5 exonuclease. The 1×T5 exonuclease buffer was made by adding 1 µl 10×T5 exonuclease buffer to 9 µl sd H_2O.

Ingredients	**Amount**
5× isothermal reaction buffer	32 µl
Phusion DNA polymerase	2 µl
Taq DNA ligase	16 µl
T5 exonuclease	0.64 µl
sd H2O	69.36 µl
Total: 120 µl, divided into aliquots of 15 µl, stored at -20 °C.	

Table 2.10 Gibson Assembly Reaction Mix

p-*jad*-D/p-*cml*-D	***gusA* plasmid**
One Master Mix aliquot (15 µl) + 5 µl purified DNA fragments based amount	One Master Mix aliquot (15 µl) + 5 µl purified DNA fragments based amount

ratio of p201 vector fragment: flankA: flankB = 1: 2: 2, estimated from bands density on gel image (e.g. if three bands densities are same, 1µl vector, 2µl flank A, 2µl flank B).	ratio of p808 vector fragment: promoter fragment = 2: 3, estimated from bands density on gel image (e.g. if three bands densities are same, 1µl vector, 2µl flank A, 2µl flank B).
Control: One aliquot (15 µl) + 1µl vector + 4 µl sd H_2O.	Control: One aliquot (15 µl) + 2µl vector + 3 µl sd H_2O.
Total: 20 µl. 50 °C, 1h.	

2.2.7 Prepare Competent *E. coli* DH5α and *E. coli* ET12567

E. coli DH5α is competent for intracellular plasmid cloning, and *E. coli* ET12567 is competent for conjugative DNA transfer into *S. venezuelae* (seen in **1.4.4.2**). Both competent cells are highly capable of accepting plasmids. Their stocks need to be prepared for frequent use.

Materials:

Glycerol stock of *E. coli* cells from the -80 °C freezer: *E. coli* DH5α, *E. coli* ET12567. LB medium. TSS-buffer. Ice. Cold centrifuge

Procedures:

1. *E. coli* DH5α or *E. coli* ET12567 cells from a glycerol stock were inoculated in LB 2 ml medium. The *E. coli* ET12567 cells were incubated with Cm (30 mg/ml) and Kan (40 mg/ml) to select the helper plasmid. Cultures were incubated at 37 °C, 225 rpm overnight.
2. Overnight culture 0.4 ml was inoculated in 40 ml LB medium, incubated for approximately 2 h at 37 °C, 225 rpm. For *E. coli* ET12567, Cm (30 mg/ml)

and Kan (40 mg/ml) were added. Before 2h, sample was taken to measure OD_{600} till OD_{600} reached between 0.4– 0.6.

3. Cell suspension was spun down at 4500 rpm, 4 °C, 5 min, supernatant discarded, and the pellet was placed on ice. Cells were re-suspended in 4 ml TSS- buffer (pre-cooled to 4°C).
4. The cell suspension was kept on ice for 1 hour, then aliquots of 100 µl/sterile Eppendorf tube were made (conducted on ice). Cells were ready for transformation in 1 hour. Aliquots were stored in -80 °C. (Inoue, Nojima et al. 1990)

2.2.8 Transformation of *E.coli*

In this project, *E.coli* DH5α and *E. coli* ET12567 (pUZ8002) were transformed with heat shock for purpose of cloning assembled pDNA and transconjugation respectively.

Materials:

Competent *E.coli* cells. Microcentrifuge. LB medium. LA plate with antibiotic(s). Inoculation loop.

Note: Before transformation, cells were always carried on ice.

Procedures:

1. Competent *E.coli* cells (stocks) were taken from –80 °C freezer, and melt on ice (5 min). For transforming a DNA construct, 50 µl of competent cells were used. For transforming a ligation, 100 µl of competent cells were used. The cell amount was decided by how competent they were.
2. Five µl ligation mixture was added into *E.coli* cells, mixed and incubated on ice for 15 min. For transforming a DNA construct, 1-2 µl (50 ng) circularised DNA was used.

3. Heat shock: ligation mixture/circularized DNA and *E.coli* (in tubes) were put into water bath at 42°C for 45 seconds.
4. Tubes were kept on ice to reduce damage to the *E.coli* cells, 5-10 min.
5. The LB medium 800 µl was added.
6. Tubes were incubated for 1 hour at 37°C.
7. The resulting culture 100 µl was spread on LA plates (with appropriate antibiotic added – in this project Am 100 µg/ml). The rest of the culture was spun down at 2 min 13,000 rpm. The supernatant was discarded and cells were re-suspended in residual liquid. All of the suspension was spread on a second LA plate (same as the first one). Both plates were incubated at 37°C overnight. Same procedure was applied with controls, in which ligation mixture/circularized DNA was replaced with the same volume of sd water.
8. Surviving colonies were picked up on the following day. (Inoue, Nojima et al. 1990)

2.2.9 Isolate pDNA from *E. coli*

After cloned in *E.coli*, the pDNA needs to be isolated. In this project, the pDNA isolation from *E.coli* was performed according to instruction of Wizard® *Plus* SV Minipreps DNA Purification System of Promega.

Materials: Wizard® *Plus* SV Minipreps DNA Purification kit from Promega. Microcentrifuge. Sterile Eppendorf tubes 1.5 ml.

Note: All centrifugation steps were carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge.

Procedures

Production of Cleared Lysate

1. Overnight culture was transferred into sterile Eppendorf tubes, 1.5 ml/tube, pelleted for 5 minutes.
2. Pellet was thoroughly resuspended with 250µl of Cell Resuspension Solution. In order to protect the pDNA integrity, the suspension should not be vortexed after step 2.
3. Cell Lysis Solution 250µl was added to each sample, inverted 4 times to mix, and incubated 5 minutes at room temperature.
4. Neutralization Solution 350µl was added, inverted 4 times to mix.
5. The lysate solution was centrifuged at top speed for 10 minutes at room temperature.

Binding of Plasmid DNA

6. Spin Column was inserted into Collection Tube.
7. Cleared lysate was decanted into Spin Column.
8. Spin Column was centrifuged at top speed for 1 minute at room temperature. Flowthrough was discarded, and Column was reinserted into Collection Tube.

Washing

9. Wash Solution (ethanol added) 750µl was added, centrifuged at top speed for 1 minute. Flowthrough was discarded, and Column was reinserted into Collection Tube.
10. Step 10 was repeated with 250µl of Wash Solution.

11. Collection Tube with Column was centrifuged at top speed for 2 minutes at room temperature.

Elution

12. Spin Column was transferred to a sterile 1.5ml microcentrifuge tube, without any of the Column Wash Solution in the Spin Column. If some Column Wash Solution was left in Spin Column, the Column would be centrifuged again for 1 minute at top speed, then transferred to a new, sterile 1.5ml microcentrifuge tube.
13. Nuclease-Free Water 100µl was added to the Spin Column, centrifuged at top speed for 1 minute at room temperature.
14. Column was discarded, and plasmid was stored at –20°C or below.

2.2.10 Digest pDNA with Restriction Enzymes

After isolated from *E. coli*, the pDNAs were subjected to restriction enzyme digestion for checking the rightness. The digestion mixture was separated by gel electrophoresis. The right pDNA was determined by comparing the resulted band pattern with DNA ladder. The digestion recipe is illustrated below.

Table 2.11 Digestion recipe.

The mixture was incubated 2 h. Incubation temperature was enzyme-specific and could be found in 'Performance Chart for NEB Restriction Enzymes (BioLabs®). If inactivation was required, the mixture could be incubated 20 min at the given temperature from manufacturer. CutSmart could be replaced with NEBuffers specified by the manufacturer. (BioLabs 2014)

Components	Amount
sd H2O	13.5 µl

CutSmart (NEBuffer)	2 µl
BSA	0.5 µl
pDNA	3 µl
restriction enzyme	1 µl
Total	20 µl

Table 2.12 Digestion enzyme, temperature and time for each plasmid.

Plasmid	**Restriction enzyme**	**Incubation temperature [°C]**	**Incubation time [h]**
pSOK201-*jad*-D	*Sac*I	37	2
pSOK201-*cml*-D	*Age*I	37	2
pSOK808-*cmlFp*	*Bam*HI	37	2
pSOK808-*cmlIp*	*Bam*HI	37	2
pSOK808-*cmlXp*	*Bam*HI	37	2
pSOK808-*jadJp*	*Kas*I	37	3
pSOK804-lacNat	*Age*I	37	2
pSOK806-NP	*Not*I	37	2
pSOK804-Lin	*Not*I	37	2

2.2.11 Transfer Recombinant pDNA to *S. venezuelae*

The *E. coli* ET12567 (pUZ8002) (**ET**) strain is competent in transferring pDNA to *S. venezuelae* (seen in **1.4.4.2**). It is a non-methylating host (*dam*$^-$, *dcm*$^-$) carrying

pUZ8002 helper plasmid that provides transfer functions from *RP4 oriT*. The strain is resistant to both Cm (30 g/ml) and Kan (40 g/ml). (Flett, Mersinias et al. 1997)

ET cells was transformed with the verified recombinant pDNA (seen in **2.2.8**), and spread on **LA plate 1** (100 µg/ml Am), incubated at 37 °C, overnight (in this project all the assembled plasmids possessed the Am^R marker). The well-growing colonies were inoculated on to **LA plate 2** (100 µg/ml Am, 25 µg/ml Cm, 20 µg/ml Kan), incubated at 37 °C, overnight. The difference between transconjugation of p-*jad*-d/p-*cml*-D and *gusA* plasmids was that the former plasmids were integrated to chromosome via homologous recombination (finally verified by PCR) while the latter via site specific integration (finally verified by Am^R) (refer to **1.4.4.2, 1.4.4.3** and **Appendix B**, Page 116-118).

Materials:

E. coli ET12567 (pUZ8002) cells transformed with specific constructs on a fresh LA plate with Am, Cm and Kan. ISP4 plate with *S. venezuelae* ATCC 10712 spores/glycerol stock of *S. venezuelae* ATCC 10712 spores. ISP4 plates + $MgCl_2$ (0.01M, added after autoclave). LB medium. 2×YT medium. Sterile syringes (5ml) with cotton wool filter. Inoculation loop.

Procedures:

1. Spore suspension of *S. venezuelae* was collected in sd water by washing off spores from ISP4 plates with 5.0 ml sd water and filtered through the sterile cotton wool in syringe to remove mycelia.
2. Filtered spore suspension/frozen spore suspension 50 µl was added to 500 µl 2×YT medium, mixed and incubated for 5 min at 50 °C (germination

induced). The heat-shocked spore suspension was cooled down at room temperature (15-20 min).

3. ET cell suspension was prepared by sampling cells from **LA plate 2** and suspending them in 500 µl 2×YT medium.
4. Heat-shocked spore suspension (550 µl) was mixed with 100 µl ET cell suspension by pipetting.
5. The mixture was spun down at a table centrifuge for 1 min, and 550 µl of the supernatant was removed. The pellet was re-suspended in the residual medium (100 µl) and spread on ISP4 + $MgCl_2$ (MgCl2 was used to increase the conjugation efficiency). The pletes were incubated at room temperature on the laboratory bench for 14-23 h until a thin mycelium layer was present (less time for fresh spores than glycerol stock).
6. Antibiotic solution was made for selecting transconjugants by mixing nalidixic acid (Nal) and apramycin (Am) in sterile distilled water (add nalidixic acid first to avoid precipitation). The medium volume per plate was assumed to be 30 ml, thus for each plate 30 µl Nal and 15 µl Am were added in 1ml sterile distilled water to create final concentration of Nal 30 µg/ml and Am 50 µg/ml in the medium. The total antibiotic solution volume was calculated according to this assumption. Nal was used to select against contaminating *E. coli*, since *Streptomycetes* were naturally resistant to Nal, while *E. coli* was sensitive to it. The Am was used to select transconjugants, since the transferred plasmid was Am^R.
7. Antibiotic mixture was spread by 1 ml per ISP4 + $MgCl_2$ plate with conjugation mix (**first selection**). After drying out, the plates were incubated

at 30°C for further growth. The control was performed with untransformed ET.

8. After 3-4 days incubation, the surviving colonies were inoculated onto ISP4 plates with Nal (30 µg/ml) and Am (50 µg/ml) (**second selection**), and incubated at 30 °C. Eventually, the PCR verification was exploited to determine the genuine transconjugants (**third selection**). (Flett, Mersinias et al. 1997)

2.2.12 Isolate gDNA from *S. venezuelae*

The gDNA of wild *S. venezuelae* were required for amplify *jad/cml* flanking fragments and *jad/cml* promoters while gDNAs of *S. venezuelae* first and second crossover candidates were required to verify the real mutants. The gDNA isolation of *S. venezuelae* was performed according to instruction of QIAGEN DNeasy® Blood & Tissue kit. The following protocol was optimized for *actinomtcetes*.

Materials:

QIAGEN DNeasy® Blood & Tissue kit. Table-top microcentrifuge. Water bath. Heating block. Enzymatic lysis buffer. TSB medium.

Procedures:

1. Overnight culture 1 ml plus 500 µl sdH2O (8,000 rpm, 3 min) was spun down in one 1.5 ml Eppendorf tube.
2. Pellet was re-suspended in 180 µl enzymatic lysis buffer (prepared immediately before use) containing 20 mg/ml lysozyme, incubated at 37 °C for 15 min and vortexed every 5 min.

3. Proteinase K 25 µl (kit) was added, mixed by pipetting, then 200 µl buffer AL (kit) was added, incubated at 55 °C for 30 min. (proteinase K should not be added directly to Buffer AL.)
4. EtOH 96% 200 µl was added and mixed by pipetting. Solution was applied on the DNeasy Mini spin column (kit) and spun at 13,000 rpm for 5 min. (It was important that the sample and the ethanol were mixed thoroughly to yield a homogeneous solution. A white precipitate was formed on addition of ethanol. It was essential to apply all of the precipitate to the DNeasy Mini spin column. The procedure was not interfered with by the precipitate.)
5. The column was washed with 500 µl buffer AW1 (8,000 rpm, 1 min).
6. The column was washed with 500 µl buffer AW2 (13,000 rpm, 3 min). (It was important to dry the membrane of the column, since subsequent reactions might be interfered with by residual ethanol. The centrifugation step was taken to ensure that no residual ethanol would be carried over during the following elution)
7. Column was placed in a sterile 1.5ml Eppendorf tube. DNA was eluted with 125 µl buffer AE (preheated to 50 °C), incubated at room temperature, 15 min.
8. DNA was spun down at max speed (14,000 rpm) for 1 min.
9. The gDNA concentration was measured with NanoDrop. Concentration usually ranged from 10-100 ng/µl.

2.2.13 Make Glycerol Stock

Bacterial strains that might be used in later works should be stored at -80 °C in a glycerol solution. The *E. coli* ET12567 (pUZ8002) transformed with assembled plasmids and the first and second crossover mutants of *S. venezuelae* were made into glycerol stocks at -80 °C.

Materials:

Overnight culture of *E. coli*/ISP4 plate with *S. venezuelae*. Sterile 20 % Glycerol solution. Sterile cryo vials. Sterile syringes (5ml) with cotton wool filter. Microcentrifuge.

Protocol for *E. coli* glycerol stock:

1. Overnight culture 1.5 ml was transferred to an sterile Eppendorf tube.
2. The culture was spun down at 13 000 rpm, 4 min, then the supernatant was discarded.
3. The pellets were re-suspended in 20 % Glycerol (1.5 ml) and cell suspension

was transferred to a cryo vial, stored at -80 °C. (Lab of Charles R. Sanders 2011)

Protocol for *S. venezuelae* glycerol stock:

1. The sterile glycerol solution (20 %, 5 ml) was applied onto an ISP4 plate with fresh *S. venezuelae* spores and the spores were detached with a pipette tip.
2. The spore suspension was collected and filtered through a sterile cotton wool filter, which was used to remove the mycelia. The filtrate was transferred to a cryo vial, stored at -80 °C. (Lab of Charles R. Sanders 2011)

2.2.14 Replica Plating

In this project, replica plating was used for selecting *jadR1*$^-$ and *jadR1*$^-$ *cml*$^-$ mutants, since this technique is a favorable technique for selecting mutants (seen in **1.4.4.4**).

Materials:

Velvets. Wood block. Sterile distilled water. Microcentrifuge tubes. Pure ISP4 plates. ISP4 plates with Am (50 µl/ml).

Procedures:

1. Spore suspension was made by collecting spores from ISP4 plates in sd H_2O.

2. The 10-fold series dilution from 10^{-1} to 10^{-6} (or 10^{-7}) was prepared: 200 µl spore suspension was added to 1800 µl sd H_2O (total 2ml) and mixed well. (The dilution was used to ensure 100-200 discrete colonies per plate.)

3. The 10^{-6} (or 10^{-7}) dilution 100 µl was evenly distributed on each of 20 pure ISP4 plates, incubated at 30 °C for 2-3 days.

4. When small colonies were present (not connected with one another), sterile velvets and wood block were used to print colonies from the 20 pure ISP4 plates onto another 20 ISP4 plates with Am 50 µl/ml, respectively, incubated at 30°C for one day. (One piece of velvet was used for one plate. The plate should not be rotated when printing and markers were left on each plate for later comparison.)

5. Colonies on each pure ISP4 plate were compared with ISP4 plates with Am according to the markers. Colonies present on the former plate but absent on the later plate were candidate mutants, then would be subjected to PCR verification. (Lederberg and Lederberg 1952)

2.2.15 GUS Assay

In this project, GUS assay was used to compare promoter efficiencies through *gusA*, because this method is relatively cheap, simple and sensitive compared with other methods such as GFP, luciferase, etc. (seen in **1.4.4.5**). The raw data in this project are listed in **Appendix D** (Page 122-129).

Materials:

24-well plates (Corning® Costar®). β-Glucuronidase (GUS). Lysozyme. Heating block. Spectrophotometer. Stopwatch.

Procedures:

Prepare Strain Cultures

Twelve strains were cultured: ten transconjugants from **3.1.4.2**, one wild *S. venezuelae* and one double deletion as negative controls.

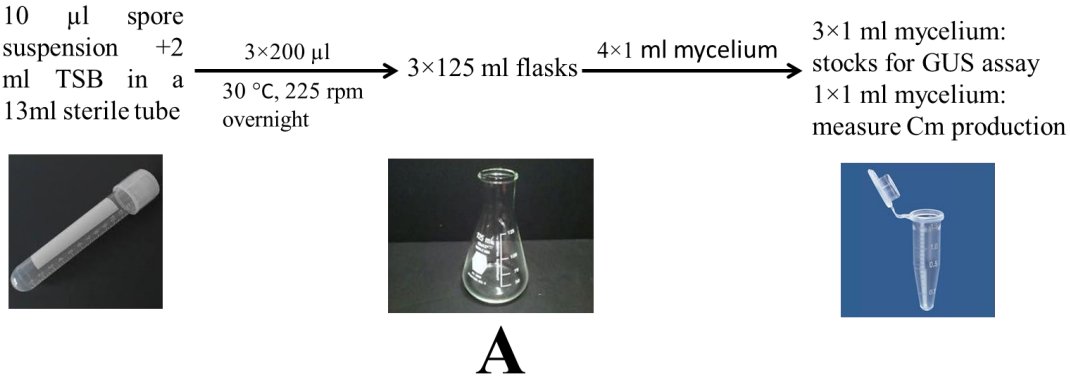
1. For each strain, 10 µl spore suspension was added to 2 ml liquid TSB in a 13 ml sterile plastic tube, incubated at 30 °C, 225 rpm overnight.

2. For each strain, three biological replicates were prepared by inoculating 3×200 µl overnight culture into 3×125 ml flasks (10 ml liquid MYM (or GI) /flask) respectively, incubated at 30 °C for 24 hours without ethanol shock. The steps were repeated with 600 µl ethanol added to each flask after 10 h incubation.

3. For each strain (three flasks), 4×1 ml mycelia were harvested in 4 microcentrifuge tubes per flask (12×1 ml mycelia/strain). Three tubes could be used in three independent tests if the test needed to be repeated while the fourth tube (not centrifuged as bellow) was used for measuring Cm production. Total harvest: 6 (strains in WT or DD) × 3 (replicates/strain) × 4 (harvest/replicate) × 2 (ethanol-/+) × 2 (WT and DD) = 288 tubes. (**Figure 2.1A**)

4. The mycelia were centrifuged at 13000 rpm, 3 min, supernatant removed, and cells re-suspended in 1 ml distilled water by vortexing.

5. The suspension was re-centrifuged at 13000 rpm, 3 min and supernatant was removed. Pellets could be stored at -80 °C or used in GUS assays immediately.



A

plate 1					
WT 1	WT 2	WT 3	WT-F 1	WT-F 2	WT-F 3
WT-I 1	WT-I 2	WT-I 3	WT-X 1	WT-X 2	WT-X 3
WT-J 1	WT-J 2	WT-J 3	WT-8 1	WT-8 2	WT-8 3
plate 2					
DD 1	DD 2	DD 3	DD-F 1	DD-F 2	DD-F 3
DD-I 1	DD-I 2	DD-I 3	DD-X 1	DD-X 2	DD-X 3
DD-J 1	DD-X 2	DD-X 3	DD-8 1	DD-8 2	DD-8 3

B

Figure 2.1 A: Culture preparation for each strain. Three biological replicates were prepared by inoculating 3×200 µl overnight culture into 3×125 ml flasks (10 ml liquid MYM (or GI) /flask) respectively, incubated at 30 °C for 24 hours without ethanol shock. Mycelia 4×1 ml were harvested in 4 microcentrifuge tubes per flask. The steps were repeated with 600 µl ethanol added to each flask after 10 h incubation. **B: Layout of GUS assay in 24-well plate.** WT: wild type *S. venezuelae*. DD: *jadR1*$^{-}$*cml*$^{-}$ double deletion mutant. F, I, X, J, 8 represent WT/DD integrated with pSOK808-*cmlF*, pSOK808-*cmlIp*, pSOK808-*cmlXp*, pSOK808-*jadJp*, pSOK 808 respectively. Each strain had three replicates.

GUS Test

6. Three replicates (pellet from 1 ml culture) without ethanol shock and three replicates with ethanol shock were used for each strain. Pellet from 1 ml culture was resuspended in 1 ml lysis buffer containing 4 mg/ml lysozyme (prepared just before the experiment).

7. The suspension was incubated for 30 min at 37 °C, gently flipped every 10 min, then centrifuged for 5 min at 13000 rpm.

8. Lysate 0.5 ml was mixed with 0.5 ml Z-buffer in 24-well plates (**Figure 2.1B**). But if reaction proceeded too quickly, i.e. if promoter was particularly strong, 0.1 ml lysate would be used with 0.9 ml Z-buffer.

9. The 24-well plate with mixtures was incubated at 37 °C for 5 min.

10. The reaction was initiated by adding 0.2 ml 4 mg/ml p-nitrophenyl-β-D-glucuronide (PNDG) (in Z-buffer), incubated at 37°C, and stopwatch was started (PNDG is unstable so make fresh solution before use).

11. When the first yellow colour was observed in a certain reaction mixture, the optical density (OD) was measured at both 420nm and 550nm. Ideally the reading should be 0.6-0.9. The OD_{550} was used to correct light scattering, but this reading should be low, as the lysate was spun to remove cell debris.

12. The measuring was repeated at different times, with the time recorded, according to the yellow colour development. This was critical to avoid measuring data after enzymatic reaction plateau, since the Miller units would be smaller than real value if data were measured after plateau according formula in step 14. After the intense yellow colour was developed, the reaction could be stopped by adding 0.5 ml of a 1M Na_2CO_3 solution (The reaction could take 1-4 hours, but if yellow colour was not developed during this time, samples could be wrapped in aluminium foil and left overnight. In this case, a control of 1ml Z-buffer with 5ul 0.2M p-nitrophenyl-β-D-glucuronide but no cell extract was required).

13. The remaining lysate was used to calculate amount of total protein released from mycelium in a Bradford assay.

14. Miller units of glucuronidase was calculated: $[1000 \times (OD_{420} - 1.75 \times OD_{550})]$ /[time of reaction x volume of culture assayed] and expressed as Miller units per mg of total protein. (Prof Mervyn Bibb, John Innes Centre, UK)

2.2.16 Bradford assay

The Bradford method has been favored in quantifying protein in many research areas, because this approach is faster, simpler, more sensitive and cheaper than most other methods (seen in **1.4.4.6**). This approach generates relative measurements with

spectrophotometer. In this project, the most commonly used standard BSA was used. The raw data are listed in **Appendix D** (Page 122-129).

Materials:

96-well plate (Corning® Costar®). BSA 1 mg/ml. Spectrophotometer. Bradford Reagent (BioRad). sd water.

Procedures:

1. Bradford Reagent (BioRad) was diluted by 5x with sd water.
2. The spectrophotometer machine was turned on to allow bulb warm up (approx. 10 min before use).
3. Standards were prepared by diluting BSA 1 mg/ml into BSA 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml.
4. The samples (remaining lysate) was diluted (e.g. by 20×) with sd water such that sample OD values would be positioned in linear range (for most spectrophotometers is 0.2 - 0.8 O.D. units) of standards. (If the concentration of protein could not be estimated, make several dilutions.)
5. Standards were placed in triplicate (10 ul per well) from in column 2 in 96-well plate. Column 1 was left as blank.
6. Samples were placed in triplicate (10 ul per well).
7. Diluted Bradford reagent 200 µl was added to each well, left standing 5 min.
8. OD was measured at 595 nm.
9. The results were used to graph the standard curve (Axes are commonly labeled as y=A, 600nm and x=mg/mL). The curve and data from Bradford were used to calculate lysate protein concentrations with Microsoft Excel. (lab 2014)

3. Results

In this chapter, the overall strategies are displayed first so as to create understanding convenience for results in the following section: generate *jadR1*$^-$ mutant, generate *jadR1*$^-$*cml*$^-$ mutant, cloning gene clusters in *jadR1*$^-$*cml*$^-$ mutant, test promoters with GUS, replace the native promoter in gene clusters, which are presented in order of the procedures.

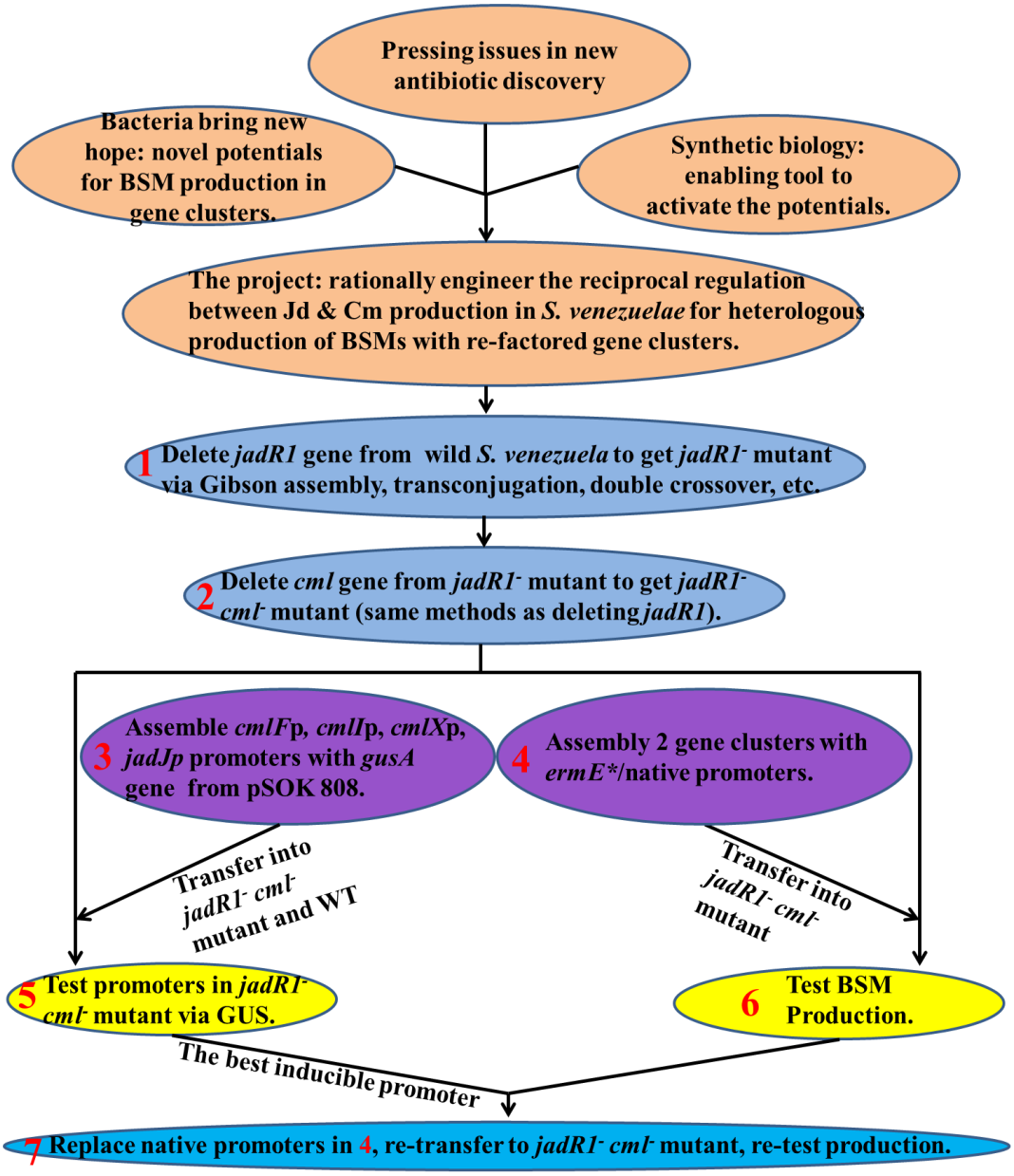


Figure 3.1 Schematic overview of the procedures. The project relies on the reciprocal regulation of Jd and Cm biosynthesis (seen in **1.4**) and can be divided into

five steps. Part 3 and 5, 4 and 6 are presented as one part respectively below, and they can be conducted in parallel.

3.1 Overall strategies

The project consists of five steps. **Figure 3.1** is the sketch representation of the general strategies.

3.1.1 Delete *jadR1* from the *S. venezuelaee* Chromosome Using Suicide Vector and Double-crossover

Traditionally, gene deletion could be carried out by double-crossover (homologous recombination) at one step. However, this and other methods often introduce a selectable marker into the genome, (Kieser 2000) which engenders some disadvantages (**seen in 1.4.4.3**). (Siegl and Luzhetskyy 2012) In this project, to avoid the drawbacks, the double-crossover was carried out in two steps, no selectable marker introduced. The deletion plasmid p-*jad*-D was made and integrated to *S. venezuelae* chromosome thorough first crossover, then the *jadR1*$^{-}$ mutant was generated by second crossover (double crossover).

3.1.1.1 Assemble *jadR1* Deletion Plasmid

PCR amplification

The web-based software tool j5 (Hillson, Rosengarten et al. 2011) was used to design primers (seen in **Appendix A**, Page 116-118). Three DNA fragments for constructing *jadR1* deletion plasmid (**p-*jad*-D**) were amplified by PCR (seen in **2.2.1):** the p201 vector fragment (Am^{R}), *jadR1*-FlankA and *jadR1*-FlankB.

Digestion and purification

The PCR products were visualized by gel electrophoresis to check byproducts (seen in **2.2.2**), if byproducts of a certain fragment were detected, then the total amount of this fragment would be applied on gel, excised from byproducts and purified (seen in

2.2.4). The p201 vector product was subjected to DpnI digestion (37 °C, 3 h) to eliminate the contaminating template, because DpnI only cleaves pDNA from a dam+ strain, leaving the vector fragment intact (seen in **2.2.5**). (BioLabs 2014) The digestion mixture and the two flanking fragments were purified (seen in **2.2.3**).

Gibson assembly

The three purified fragments were assembled through Gibson Assembly (seen in **2.2.6**).

3.1.1.2 Clone and Verify p201-*jad*-D

Transform *E.coli* DH5α with p201-*jad*-D

The *E.coli* DH5α competent cells were prepared as **2.2.7**. The *E.coli* DH5α (stock) was transformed with Gibson Assembly mixture, including control (transformation with control assembly mixture) and spread on LA plate (100 μg/ml Am) to select right transformants (seen in **2.2.8**).

Verify p201-*jad*-D

The survival colonies were picked up individually into 2 ml LB (100 μg/ml Am), incubated at 37 °C, overnight. pDNAs were individually isolated from the overnight cultures and digested with *Sac*I, 37 °C 2h (seen in **2.2.10**). The digestion patterns were visualised through gel electrophoresis to find the right p-*jad*-D displaying right pattern (seen in **2.2.2**).

3.1.1.3 Transfer p-*jad*-D from *E. coli* ET12567 (pUZ8002) to *S. venezuelae*

The transconjugation of *S. venezuelae* through *E. coli* ET12567 (pUZ8002) is proved as an efficient way, due to the *int, attp, RP4 oriT, attB* system between helper plasmid and host strain. (Bierman, Logan et al. 1992) Thereofore, it was exploited in this project.

Transform *E. coli* ET12567 (pUZ8002) (ET) with p-*jad*-D

The ET competent cells were prepared as **2.2.7**. ET cells was transformed with the right p-*jad*-D, and spread on **LA plate 1** (100 μg/ml Am), incubated at 37 °C, overnight (seen in **2.2.8**). The well-rowing colonies were inoculated on to **LA plate 2** (100 μg/ml Am, 25 μg/ml Cm, 20 μg/ml Kan), incubated at 37 °C, overnight. The glycerol stock was made for ET on **LA plate 2 (**seen in **2.2.13**).

Transfer p-*jad*-D to *S. venezuelae*

The transconjugation was made between ET from **LA plate 2** and *S. venezuelae* (seen in **2.2.11**). After 3-4 days incubation, p-*jad*-D was expected to be integrated into chromosome through homologous recombination (**first crossover**) due to lack of *int, attP* (seen in **Appendix B**, Page 113-115). The surviving colonies were inoculated onto ISP4 plate with Nal (30 μl/ml) and Am (50 μl/ml), incubated at 30°C for two days. The surviving colonies were inoculate into 2 ml TSB (Am 50 μl/ml), with WT as control, incubated at 30°C overnight. Genome DNAs of all overnight cultures were isolated and DNA concentrations were measured with NanoDrop so as to add the right amount of templates in PCR reaction later. PCR verification was made to identify the right transconjugants (with 4.3kb band) (seen in **2.2.1**). The glycerol stock of right transconjugants was made **(**seen in **2.2.13**).

3.1.1.4 Generate *jadR1*$^{-}$ Mutant

Three Rounds of Sporulation

The right transconjugant was inoculated onto an ISP4 plate, incubated at 30°C for four days, which was repeated for another two times by inoculating spores from the former plate. The first two rounds could be replaced in the following way: 0.5 ml overnight TSB culture of right transconjugants was inoculated into 10 ml TSB in a 125ml flask, incubated at 30°C overnight, which was repeated once by inoculating 0.5 ml overnight culture from the former flask. Then 100 μl liquid culture was inoculated onto an ISP4 plate for making glycerol stock **(**seen in **2.2.13**).

After the three-round sporulation, the second crossover was expected to have occurred in the transconjugants chromosome through homologous recombination, yielding double mutant or wild *S. venezuelae* (refer to **1.4.4.3**)

Replica Plating

This technique was employed to select the candidates of double crossover mutant (*jadR1*$^{-}$) as described in **2.2.14**.

Verify *jadR1*$^{-}$ Mutant

The gDNAs of *jadR1*$^{-}$ candidates was isolated, and PCR verification was made to identify the right mutants (seen in **2.2.1**). The controls were wild gDNA of *S. venezuelae* (negaive) and p-*jad*-D (positive).

3.1.2 Delete the Entire *cml* Gene Cluster from the *jadR1*$^{-}$ Mutant Genome Using Suicide Vector and Double-crossover to Get the *jadR1*$^{-}$*cml*$^{-}$ Mutant

The *cml* gene deletion was conducted with the same procedures as *jadR1* deletion with the difference specified as below: two *cml* flanks were amplified in the beginning; p-*cml*-D candidates were treated with *Age*I digestion and PCR verification (seen in **2.2.1**); *jadR1*$^{-}$ mutant was used as recipient in transconjugation.

3.1.3 Transfer Gene Clusters to the *jadR1*$^{-}$*cml*$^{-}$ Mutant and Check Novel Compounds Production

The heterologous expression of BSMs might be harmful for producing host, especially for *S. venezuelae*. Thus it is reasonable to know how the expression of introduced gene clusters affects *jadR1*$^{-}$*cml*$^{-}$ mutant. To answer this question, the re-factored gene clusters under control of native/*ermE** promoters were introduced to *jadR1*$^{-}$*cml*$^{-}$ mutant and the results was being evaluated, besides, the results could also be used as control at later stage when the inducible promoter (from **3.1.4**) would replace native/*ermE** promoters. The stages of **3.1.3** and **3.1.4** were conducted in parallel. The brief steps in **3.1.3** are shown below.

Two gene clusters were chosen to analyse, due to their simplicity and predicted potential for novel BSMs by bioinformatics methods. The gene cluster MP112-09-cluster 6-Lactococcin972-like (MP112-09-Lac) was assembled with two vectors to

generate two plasmids: (1) pSOK804-lacNat: MP112-09-Lac with its native promoter with a pSOK804 fragment. (2) pSOK804-NP: MP112-09-Lac with a pSOK806 fragment with *ermE*p* replacing its native promoter. Because the bacteriocin genes are perhaps controlled by the same promoter, which is easy to manipulate (**Figure 1.4A**). The gene cluster MPS05-B41-cluster 28-Lincocin-M18-like (MPS05-B41-Lin) was assembled with its native promoter with a pSOK804 fragment to get pSOK804-Lin plasmid. Since the genes in this cluster are probably controlled by different promoters, not easy to manipulate (**Figure 1.4B**). The assembly was carried out through Gibson Assembly (seen in **2.2.6**).

The three recombinant plasmids (maps seen in **Appendix B**, Page 113-115) above would be transferred to *S. venezuelaee jadR1⁻cml⁻* mutant (seen in **2.2.11**), followed by effects evaluation.

3.1.4 Test the Expression of the Reporter Gene *gusA* under Control of Selected Promoters in the *jadR1⁻cml⁻* Mutant and Wilde Type

In this stage, four promoters *cmlFp, cmlIp, cmlXp, jadJp* were chosen to test, because *cmlIp, cmlXp* were known to control the essential genes in Cm biosynthesis, besides, according to the project hypothesis, overproduction of Cm was expected in *jadR1⁻* mutant, which meant *cmlIp, cmlXp* shall be active promoters in *jadR1⁻* mutant and thus could be used to drive expression of exogenous gene clusters. Before assembling with exogenous gene clusters, it was necessary to test whether *cmlIp, cmlXp* were genuine efficient promoters. The *cmlFp* is responsible for Cm transport to extracellular environment, which means it might be active under stress conditions (ethanol shock), hence it was chosen to test as well. The *jadJp* is speculated to be necessary for Jd production, thus was also chosen to test (seen in **1.1.3.2**). (He, Magarvey et al. 2001) (Zheng, Wang et al. 2007)

GUS (encoded by *gusA*) is shown to be a versatile reporter in *actinomycetes* compared to other methods, such as eGFP, luciferase, because this method is relatively sensitive, simple and cheap (seen in **1.4.4.5**). Therefore, *gusA* reporter was employed in this project.

3.1.4.1 Assembly *gusA* Plasmids

Four promoters of *cmlFp, cmlIp, cmlXp, jadJp* were independently amplified from wild *S. venezuelae*, with corresponding vector fragments amplified from pSOK 808. Each vector (Am^R) contained *gusA* gene without a promoter, thus each of the four promoters was placed upstream the *gusA* gene via Gibson Assembly (seen in **2.2.6**). Four *gusA* plasmids were generated: pSOK808-*cmlFp*, pSOK808-*cmlIp*, pSOK808-*cmlXp*, pSOK808-*jadJp*.

3.1.4.2 Transfer *gusA* Plasmids to *jadR1⁻cml⁻* Mutant and Wild *S. venezuelae*

The verified *gusA* plasmids were transferred to *jadR1⁻cml⁻* mutant and WT (controls) (seen in **2.2.11**), and integrated to chromosome through site-specific integration due to *int, attP* (maps seen in **Appendix B**, Page 113-115). Ten transcojugants were generated: four wild *S. venezuelaes* integrated with one of the four *gusA* plasmids respectively; four *jadR1⁻cml⁻* mutants integrated with one of the four *gusA* plasmids respectively; one wild *S. venezuelae* and one *jadR1⁻cml⁻* mutant integrated with pSOK808 respectively (used as positive controls). In pSOK808, *gusA* was under control of *ermE*p*, hence it could be used as positive control.

3.1.4.3 Compare Promoter Efficiencies through GUS Test

This test was first conducted based on the growth medium of MYM, if results would not be desirable, the producing medium of GI would be exploited later. Because in the industrial scale production, the inoculation from growth medium to producing medium is both time consuming and susceptible to contamination, if the growth medium shows good results, then large scale production could be focused on MYM rather than GI.

Prepare Strain Cultures

Twelve strains were cultured and harvested (seen in **2.2.15**): ten transconjugants from **3.1.4.2**, one wild *S. venezuelae* and one double deletion as negative controls. Total harvest: 288 tubes.

GUS Test

Three replicates (pellet from 1 ml culture) without ethanol shock and three replicates with ethanol shock were used for each strain. Miller units of glucuronidase were calculated as: [1000 x (OD420 – 1.75 x OD550)] / [time of reaction x volume of culture assayed] (seen in **2.2.15**). The remaining lysate was used to calculate amount of total protein released from mycelium in a Bradford assay (seen in **2.2.16**). Miller units were expressed as per mg of total protein, which was used to decide the best inducible promoter.

3.1.5 Clone Gene Clusters with the Inducible Promoter

Provided no significant negative effects was detected in **3.1.3**, the native promoters would be replaced with the best inducible promoter from **3.1.4.3** through Gibson Assembly, and subsequently the plasmids with new promoters would be transferred to the *S. venezuelaee jadR1*$^{-}$*cml*$^{-}$ mutant, followed by re-checking BSM production.

3.2 Experiment Results

The experiment results below are presented with the order in the overall strategies.

3.2.1 Generate *jadR1*$^{-}$ Mutant

The *jadR1*$^{-}$ mutant was obtained through suicide vector and double crossover. Results in this step are presented in three parts: **assemble p-*jad*-Del, select first crossover mutant, select double crossover mutant**.

3.2.1.1 Assemble p-*jad*-Del

As stated in section **3.1.1,** to avoid the drawbacks of introduced selectable marker in traditional method (seen), the double-crossover was carried out in two steps, no selectable marker introduced.

At the beginning, the suicide vector p201-*jad*-Del (**p-*jad*-D**) was assembled. Unexpectedly, when checking byproducts in two flanking fragments, two bands were present in flank A, with sizes right above 2 kb and right below 1.5kb respectively (estimated from ladder). Since the real size of flankA is 2.1kb, hence the band with larger size was isolated and purified. The two fragment and *Dpn*I-treated pSOK 201 fragment were visualised on gel to compare their relative density based on band intensities (**Figure 3.2 A**), which was used to set amounts ratio of pSOK 201 fragment: *jadR1*-FlankA: *jadR1*-FlankB =1: 2: 2 in Gibson Assembly. According to **Figure 3.2 A**, 1 µl 201 fragment, 3 µl *jadR1*-FlankA, and 1µl *jadR1*-FlankB were added in Gibson Assembly. Two colonies survived on the LA plate (Am 100µg/ml) and their pDNAs were treated with SacI digestion after isolation. The digestion pattern of candidate 2 was right (**Figure 3.2 B**), then p-*jad*-D2 was used in following procedures. But candidate 1 was perhaps right as well, because its 2 kb band was much wider than more intense than its counterpart in candidate 2. This could be explained by incomplete digestion.

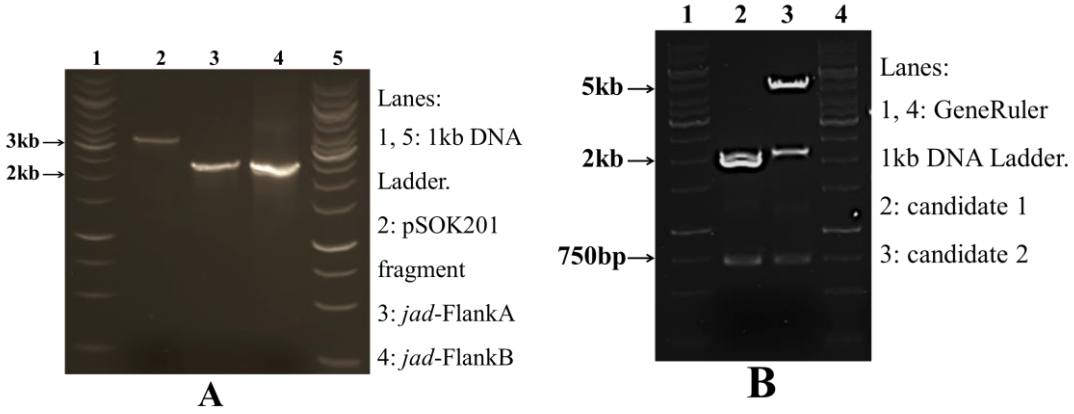


Figure 3.2 A: Relative densities of overlapping fragments of p-*jad*-D. Based the intensities, 1 µl 201 fragment, 3 µl *jadR1*-FlankA, and 1µl *jadR1*-FlankB were added in Gibson Assembly. **B: Digestion patterns of p-*jad*-D candidates.** Two p-*jad*-D candidates were treated with *Sac*I digestion. Candidate 2 (lane 3) was right, because

of its expected digestion pattern: 4.6+2.1+0.75kb, whereas candidate 1 was perhaps right as well, because its 2 kb band was much wider than more intense than its counterpart in candidate 2, which was perhaps caused by incomplete digestion.

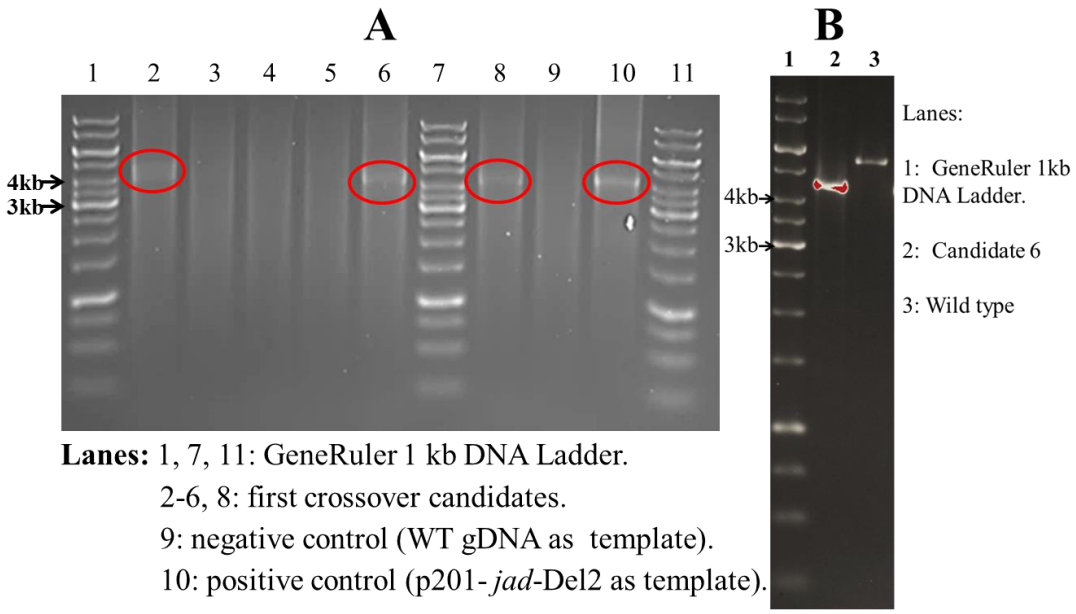
3.2.1.2 Select First Crossover Mutant

In this stage, *E. coli* ET12567 (pUZ8002) (**ET**) transconjugating-system was exploited, because this system is proved to be efficient in *Streptomyces* (seen in **1.4.4.2**). First, p-*jad*-D was transconjugated from ET (Am^R, Cm^R, Kan^R) to *Streptomyces venezuelae* ATCC 10712 (ISP5230) (WT), and shall be integrated into *S. venezuela* chromosome via homologous recombination (first crossover, seen in **1.4.4.3**) Next, transconjugants were selected against Am, which were subsequently verified by PCR.

In PCR verification, the primer pair *jadR1*-FlankA forward and *jadR1*-FlankB reverse were used. Theoretically, three fragments would be amplified: *jadR1*-FlankA+*jadR1*-FlankB (4.3kb), *jadR1*-FlankA+ *jadR1*+*jadR1*-FlankB (5.1kb), p201-*jad*-Del 2+*jadR1*+*jadR1*-FlankB (10.4kb) (refer to **1.4.4.3**), but only the 4.3kb was observed (**Figure 3.3A).** The absence of the other two fragments could be ascribed to the inefficiency of EHD DNA polymerase and non-optimal condition for 5.1 kb and 10.4 kb amplification. However, as long as a certain candidate was Am^R and the 4.3 kb product was obtained, it was confident to conclude it was the first crossover mutant. This reasoning was backed up by the fact that the 4.3kb fragment was absent in negative control using WT gDNA as template and present in positive control using p-*jad*-D2 as template. Therefore, three candidates (lane 2, 6 and 8) were confirmed as genuine first crossover mutants. The only problem was that the 5.1kb fragment was absent in WT (lane 9). Then PCR reaction was repeated with WT and one genuine mutant-**candidate 6** (lane 8). **Figure 3.3B** indicates the 5.1kb and 4.3kb fragment was

achieved in WT and candidate 6 respectively. Eventually, **candidate 6** (lane 8) was adopted in the following steps for double crossover.

The absence of 4.3kb fragment in the rest candidates (lane 3-5) could be explained by a deletion eliminating part or all 4.3kb fragment because of the unstability in genome. Alternatively, it was probably caused by errors in PCR reaction, which could be enlightened by the fact that the 5.1kb band in WT was absent in the same batch of PCR reaction and present in new amplification (**Figure 3.3B**).



Lanes: 1, 7, 11: GeneRuler 1 kb DNA Ladder.
2-6, 8: first crossover candidates.
9: negative control (WT gDNA as template).
10: positive control (p201-*jad*-Del2 as template).

Figure 3.3 PCR verification of the first crossover mutant for *jadR1* deletion. A: Six candidates (lane 2-6 and 8) were analysed. Lane 9 and 10 was negative control (WT gDNA as template) and positive control (p-*jad*-D2 as template) respectively. The primer pair *jadR1*-FlankA forward and *jadR1*-FlankB reverse were used in PCR reaction, but only 4.3kb rather than 5.1kb and 10.4 kb was observed in three candidates (lane 2, 6 and 8, red circles). The three candidates were identified as genuine first crossover mutants, because they were Am^R, and the 4.3kb fragment was absent in negative control, present in positive control (red circle in lane 10). The low

intensity of bands was caused by insufficient samples applied. **B:** the 5.1kb (lane 3) and 4.3kb (lane 2) fragment was achieved in WT and candidate 6 respectively.

3.2.1.3 Select *jadR1*$^{-}$ Mutant

The candidate 6 of genuine first crossover mutant for *jadR1* deletion (**C6GFJD**) was treated with three-round sporulation (ISP4 plate based) to create condition for occurrence of double crossover. After replica plating selection and Am^{S} test in TSB (overnight culture), three candidates were screened out. In order to identify the *jadR1*$^{-}$ mutant and exclude reverted wild type *S. venezuelae*. PCR verification was performed.

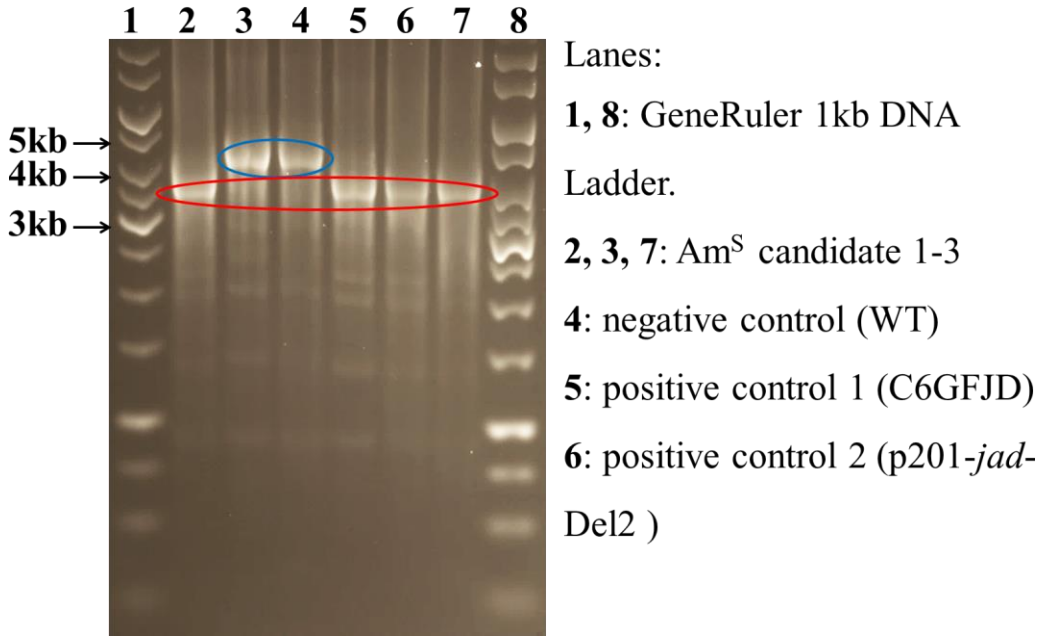


Figure 3.4 PCR verification of *jadR1*$^{-}$ mutant. The candidate 2 (lane 3) had the same band (5.1kb, blue circle) with negative control (lane 4, WT gNDA as template), whereas candidate 1 (lane 2) and 3 (lane 7) had the same band (4.3kb, red circle) with positive control 1 (lane 5, C6GFJD gDNA as template) and 2 (lane 6, p201-*jad*-Del2 as template). Candidate 2 (lane 3) shall be reverted WT while candidate 1 (lane 2) and 3 (lane 7) shall be real *jadR1*$^{-}$ mutant. The bands in candidate 2 (lane 3) and negative control (lane 4) should be 5.1kb and bands in lane 2, 5-7 should be 4.3kb.

Figure 3.4 shows that candidate 1 (lane 2) and 3 (lane 7) were real *jadR1*$^{-}$ mutants while candidate 2 (lane 3) was reverted wild type, because: first, their Am^{S} property suggested they lost Am^{R} gene by double crossover; second, candidate 2 (lane 3) had

the same band (5.1kb) with negative control (lane 4, WT gNDA as template), whereas candidate 1 (lane 2) and 3 (lane 7) had the same band (4.3kb) with positive control 1 (lane 5, C6GFJD gDNA as template) and 2 (lane 6, p-*jad*-D2 as template). Bands 5.1kb and 4.3 kb did not align well with the corresponding bands in ladder lane, but this variance is acceptable, since gel electrophoresis is a rough method to identify DNA sizes. In this project the well aligned bands between candidate 1 (lane 2), 3 (lane 7) and positive control 1, 2 was sound proof of genuine *jadR1*$^-$ mutant. Therefore, candidates 1 and 3 are real *jadR1*$^-$ mutant, and candidate 3 was used in the following steps.

3.2.2 Generate *jadR1*$^-$*cml*$^-$ Mutant

The *jadR1*$^-$*cml*$^-$ mutant was generated with the same methods as *jadR1*$^-$ mutant. Similarly, three bands should be amplified in theory: *cml*-FlankA+*cml*-FlankB (4.5kb), *cml*-FlankA+*cml*+*cml*-FlankB (19.1kb), p201-*cml*-Del+*cml*+*cml*-FlankB (24.5kb), but only the 4.5kb was obtained in practice. The real double mutant was obtained in the third attempt. In the first two attempts, only reverted WTs were generated, but the undesirable results gave valuable lessons for improving the method: executing **double check** on deletion plasmids and limiting PCR cycles, which is presented below.

3.2.2.1 Reverted Wild Type

In the first attempt, the first crossover mutant for *cml* deletion was confirmed by both Am^R and PCR verification, however, all the double crossover mutants were reverted WT, judged from Am^S property and PCR verification. Then the problems were checked from analysing pSOK201-*cml*-Del (**p-*cml*-D**) by amplifying two flanks of *cml* (*cml*-FlankA *cml*-FlankB**)** and pSOK201 fragment with p-*cml*-D as template. **Figure 3.5A** presents that *cml*-FlankB was wrong because its size 0.5kb in gel was

much smaller than the real size 2.3kb, whereas *cml*-FlankA (2.2kb) and 201 fragment (3.1kb) were right. **Figure 3.5B** further proves p-*cml*-D was wrong, as the digestion pattern in gel was 4kb+2.5kb, which was different from expectation: 3.8+2.8+1.0 kb. This could be the reason why no *jadR1*$^{-}$*cml*$^{-}$ mutant was obtained, because the deletion mutant and reverted WT is achieved when homologous recombination happens in FlankB and FlankA respectively (refer to **2.2.3**). The wrong FlankB might be caused by **mispriming** (primer annealing to the wrong DNA sequence).

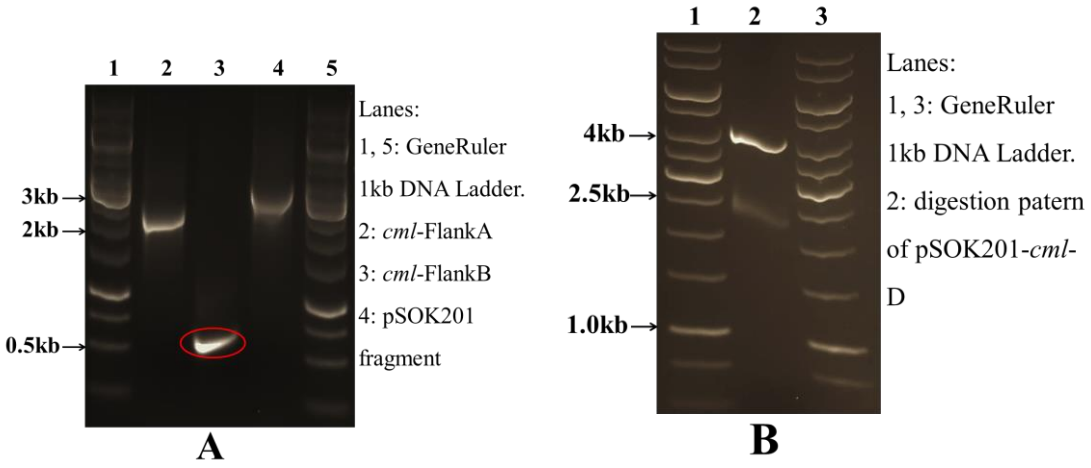


Figure 3.5 Analysis on p-*cml*-D. **A**: *cml*-FlankA**,** *cml*-FlankB and the pSOK201 fragment amplified from p-*cml*-D. *cml*-FlankB (lane 3, red circle) was wrong because its size 0.5kb in gel was much smaller than the real size 2.3kb. **B**: *Bmr*I digestion pattern of p-*cml*-D. The digestion pattern in gel was 4kb+2.5kb, different from right pattern: 3.8+2.8+1.0 kb.

The error in p-*cml*-D suggests that only restriction enzyme digestion is not enough to check the rightness of plasmids, therefore, **in the following procedures, recombinant plasmids were double checked by both enzyme digestion and PCR verification.**

In the second attempt, p-*cml*-D was re-assembled with the same purified fragments as the first attempt. The adopted p-*cml*-D was screened out by **double check** (enzyme digestion and PCR verification). The first crossover mutant was confirmed as well. After three-round sporulation, the *jadR1*$^{-}$*cml*$^{-}$ mutant still could not be obtained, since the replica plating was performed three times, and generated totally 14 candidates (first time: 7, second time: 2, third time: 5). Five of the 14 candidates were Am^{S}, but could not past PCR verification, no 4.5 kb band of *cml*-FlankA+*cml*-FlankB observed, hence they should be reverted WT. For comparison convenience, the five candidates were collected on the same gel in **Figure 3.6**, where the 4.5kb band (red circles) was present in positive control while absent in the negative controls and five Am^{S} candidates. Since the occurrence probability of *jadR1*$^{-}$*cml*$^{-}$ mutant and reverted WT shall be 50% (refer to **1.4.4.3**) respectively, meaning at least one should be the double mutant among the five Am^{S} candidates, thus no double deletion mutant suggests the possible DNA sequence mutation in *cml*-FlankB, which hindered homologous recombination. This mutation could be caused by excessive cycles (35) in PCR reaction of *cml*-FlankB at the beginning. Then in the third attempt, PCR cycle was reduced from 35 to 25.

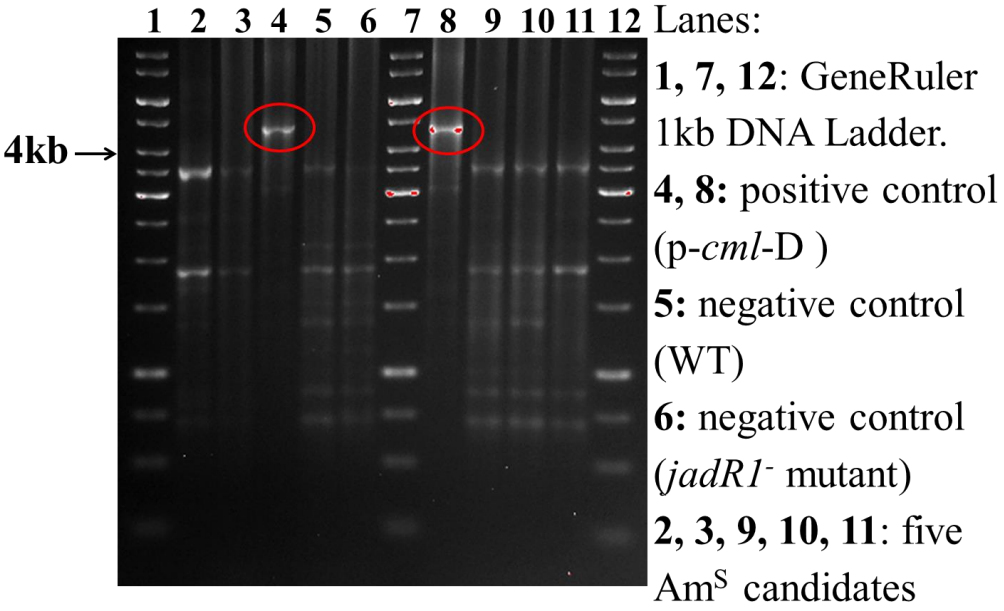


Figure 3.6 Reverted wild type in the second attempt. The positive control used p-*cml*-D that passed double check as template (lane 4 and 8 were same). In the figure it was clear that 4.5kb band (red circles) was present in positive control while absent in the negative controls and five Am^S candidates. Thus the five candidates were speculated to be reverted wild type. The other bands were possibly resulted from mispriming.

3.2.2.2 Genuine *jadR1*$^-$*cml*$^-$ Mutant

In the third attempt, three p-*cml*-D candidates were obtained, and their *Age*I-digestion patterns (5.8kb+1.6kb+0.2kb) seemed right (**Figure 3.7A**). Since the 0.2kb bands were shaded by heavy dye, then the digestion mixture of candidate 1 (lane 2 in **Figure 3.6A**) was re-analysed on gel (**Figure 3.7B**), where the 0.2kb band was visible. After that, PCR verification was conducted, in which *cml*-FlankA, *cml*-FlankB and p201 fragment were amplified from candidate 1 and compared with their purified

counterparts for Gibson assembly (**Figure 3.7C**). Candidate 1 (**p-*cml*-D1**) passed double check, and was adopted in the following procedures.

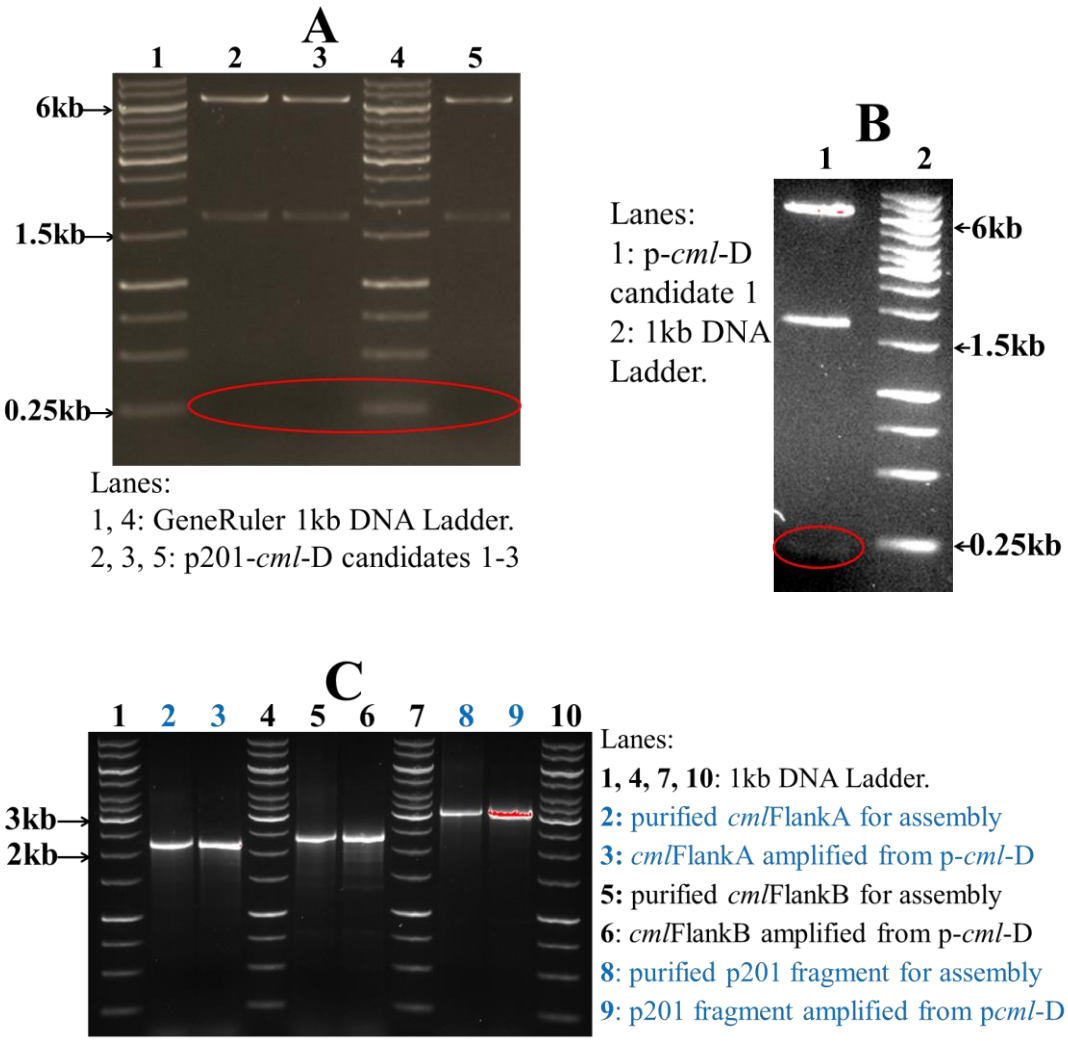


Figure 3.7 pSOK201-*cml*-D verification. A: three p-*cml*-D candidates were subjected to *Age*I-digestion that should give pattern of 5.8kb+1.6kb+0.2kb. Their patterns were right except that the 0.2kb bands were shaded by heavy dye (red circle). **B**: digestion mixture of candidate 1 (lane 2 in **Figure 3.7A**) were re-analysed on gel, where the 0.2kb was visible (red circle). **C:** the PCR verification was performed on candidate 1, in which *cml*-FlankA, *cml*-FlankB and p201 fragment were amplified from candidate 1 and compared with their purified counterparts for Gibson assembly.

The three pairs of fragments aligned well. Therefore, candidate 1 was confirmed as real p-*cml*-D.

The p-*cml*-D1 was transconjugated to *jadR1*$^{-}$ mutant and five Am^{R} candidates of first crossover mutant for *cml* deletion (**CFCD**) were chosen. **Figure 3.8A** shows the PCR verification, in which all candidates had the 4.5kb bands, same as positive control (lane 5), and no 4.5kb bands were detected in negative controls (lane 7, 8). Candidate 1 (lane 2 in **Figure 3.8A**) was treated with three round sporulation (TSB-based, time-saving). Four Am^{S} *jadR1*$^{-}$ *cml*$^{-}$ candidates were chosen after replica plating, and their PCR verification is shown in **Figure 3.8B,** where candidates 3 (land 4) and 4 (lane 5) had the 4.5kb bands, same as positive control (lane 7), and no 4.5kb bands appeared in negative controls (lane 8 and 9). Therefore, candidates 3 and 4 were right *jadR1*$^{-}$ *cml*$^{-}$ mutant, and candidate 4 was used in the following stages.

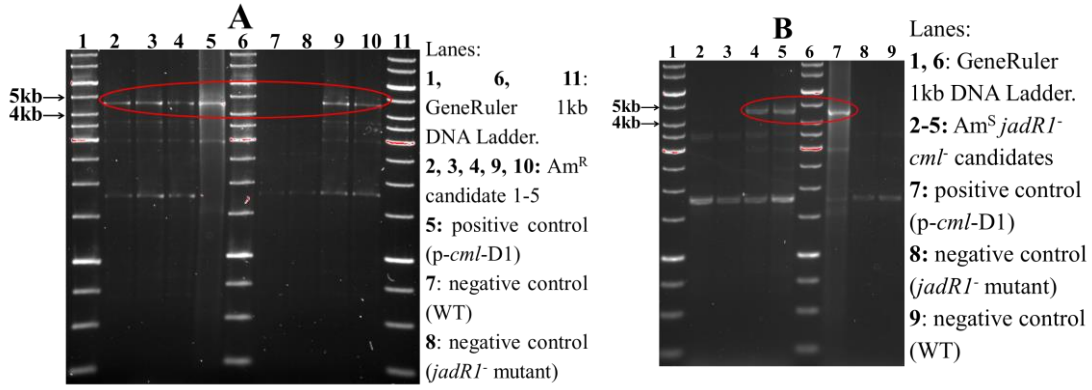


Figure 3.8 PCR verification of first and second crossover mutants for *cml* deletion. A: five Am^{R} candidates (lane 2, 3, 4, 9, 10) had the 4.5kb bands (red circle), which were same as positive control (lane 5), and no 4.5kb bands was observed in negative controls (lane 7, 8). **B**: two Am^{S} candidates (lane 4, 5) had the 4.5kb bands (red circle), which were same as positive control (lane 7), and no 4.5kb bands was present in negative controls (lane 8 and 9), therefore candidates in lane 4, 5 were

genuine *jadR1*$^{-}$ *cml*$^{-}$ mutant. The other two Am^{S} candidates (lane 2, 3) had no 4.5kb bands, and thus were considered as reverted *jadR1*$^{-}$ mutant. The other bands outside red cycles were probably caused by mispriming.

3.2.3 Test Gene Clusters in the *jadR1*$^{-}$*cml*$^{-}$ Mutant

Two gene clusters, MPS05-B41-Lin and MP112-09-Lac, that were predicted to have potentials to produce BSMs were cloned to generate three plasmids: pSOK804-lacNat (pLacNat), pSOK806-NP (pLacNP), pSOK804-Lin (pLin). In pLacNat, a pSOK804 fragment was assembled with MP112-09-Lac, with native promoter included, by contrast, due to the possible presence of single promoter (**Figure 1.4A**), in pLacNP a pSOK806 fragment was assembled with MP112-09-Lac, with the native promoter replaced by a strong constitutive promoter *ermE** (seen in **1.4.4.5**). Because of the possible presence of different promoters (**Figure 1.4B**), in pLin, a pSOK804 fragment was assembled with MPS05-B41-Lin, only with native promoter (refer to **3.1.3**). Gibson Assembly method was employed to construct the three plasmids. For pLacNat, one out of 15 candidates was proved to be genuine by double check (**Figure 3.9A, C**), by contrast, for pLacNP, four out of ten candidates were proved to be genuine (**Figure 3.9B, C**). The pLacNat (lane 3 in **Figure 3.9 A**) and pLacNP (lane 2 in **Figure 3.9 B**) were, in later stage, tranconjugated to *jadR1*$^{-}$ *cml*$^{-}$ mutant via site-specific recombination (refer to **1.4.4.2**), and the results evaluation is in progress. pLacNat pLacNP

However, the pLin could not be obtained. At first, the product of MPS05-B41-Lin did not show right size on gel, after increasing the annealing temperature from 56 °C to 65 °C, the product with right size was obtained. Afterwards, though 30 colonies were checked successively, no right pLin was revealed. Then the error-finding action was carried out with restriction enzyme digestion (EcoRV, StuI) of PCR product and no right digestion pattern was detected. The reason could be mispriming of MPS05-B41-Lin in PCR, or the special DNA structure at MPS05-B41-Lin, which inhibited primer annealing at the right place.

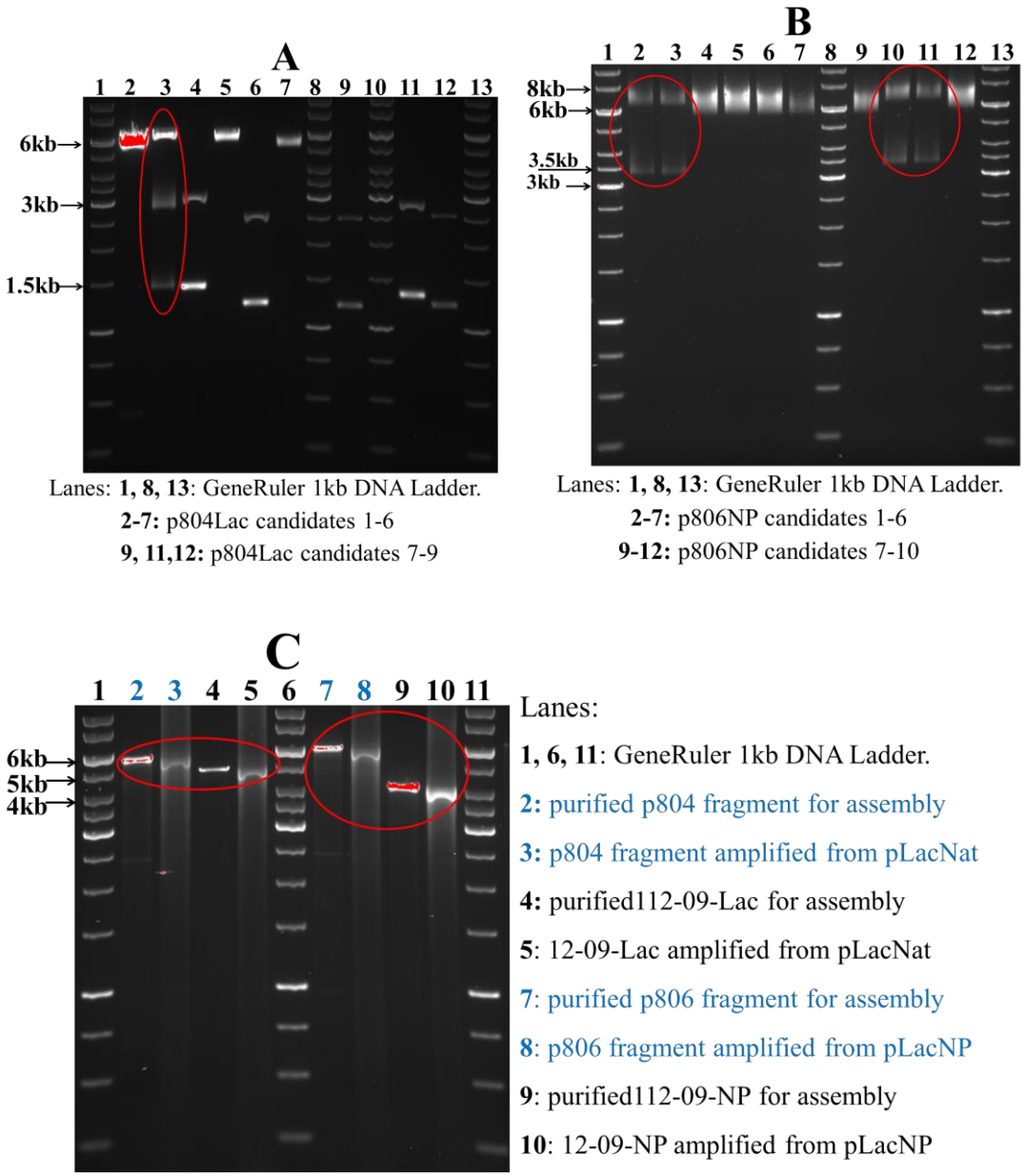


Figure 3.9 Double check of pLacNat and pLacNP. A: AgeI digestion of pLacNat. Totally, 15 colonies were analysed in three times. In the first two times, three colonies showed partially right pattern could be attributed to incomplete digestion, thus they were re-digested (lane 9, 11, 12) with another six colonies (lane 2-7) in the third time. Only one candidate had the right pattern: 6.0+2.7+1.4kb (lane 3, red circle). **B: *Not*I digestion of pLacNP.** Totally, 10 colonies were analysed. Lane 2, 3, 10, 11 showed right digestion pattern: 6.5+3.3kb (red circles). Lane 2, 5, 7 in A and lane 4-7, 9, 12 in B showed one band, and thus could be contaminating pSOK804 and pSOK806, respectively, according to clone manager. The contamination might be introduced by insufficient *Dpn*I digestion. Lane 4 and 11 in A might be right pLacNat, because the

small bands around 1.5kb were much intense and perhaps were caused by incomplete digestion, whereas lane 6, 12 in A could be unknown contamination, because the patterns could not be explained by known plasmids. **C: PCR confirmation of pLacNat and pLacNP.** The gene cluster fragments and vector fragments were amplified from pLacNat (lane 3 in A) and pLacNP (lane 2 in B) respectively, and compared with their counterparts for Gibson Assembly. Good size alignment was observed (red circles), therefore pLacNat (lane 3 in A) and pLacNP (lane 2 in B) passed double check and were used in the following steps.

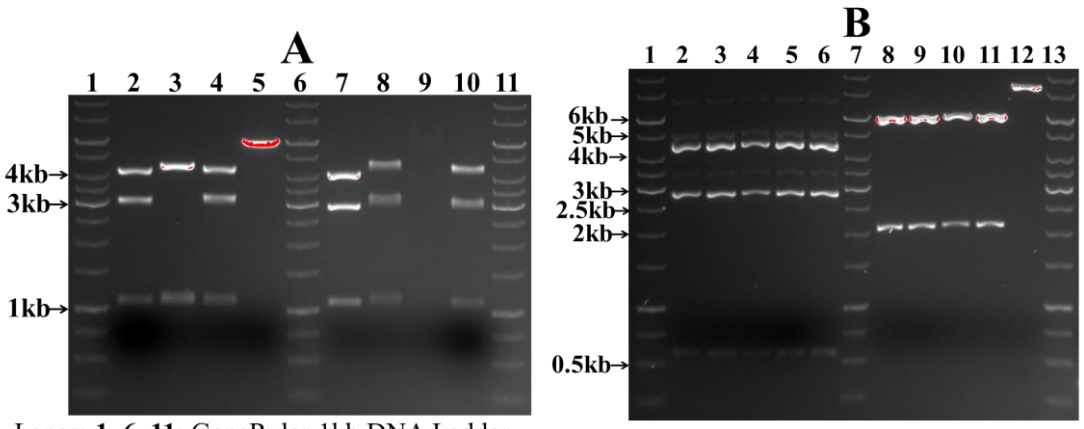
3.2.4 Test reporter *gusA* with *cmlFp, cmlIp, cmlXp, jadJp* in the *jadR1*$^{-}$*cml*$^{-}$ mutant and Wild Type

In this stage, four promoters *cmlFp, cmlIp, cmlXp, jadJp* were chosen to test, because *cmlIp, cmlXp* control the essential genes in Cm biosynthesis, thus they might be active in *jadR1*$^{-}$ mutant where Cm production could be initiated. The *cmlFp* is responsible for Cm transport to extracellular environment, thus it might be activated under stress conditions (ethanol shock). The *jadJp* is speculated to be necessary for Jd production, thus was also chosen to test (seen in **3.1.4**). (He, Magarvey et al. 2001) (Zheng, Wang et al. 2007) GUS assay was employed in this project, because it is shown to be a versatile reporter in *actinomycetes* compared to other methods, such as eGFP, luciferase (seen in **1.4.4.5**).

3.2.4.1 Assemble *gusA* plasmids

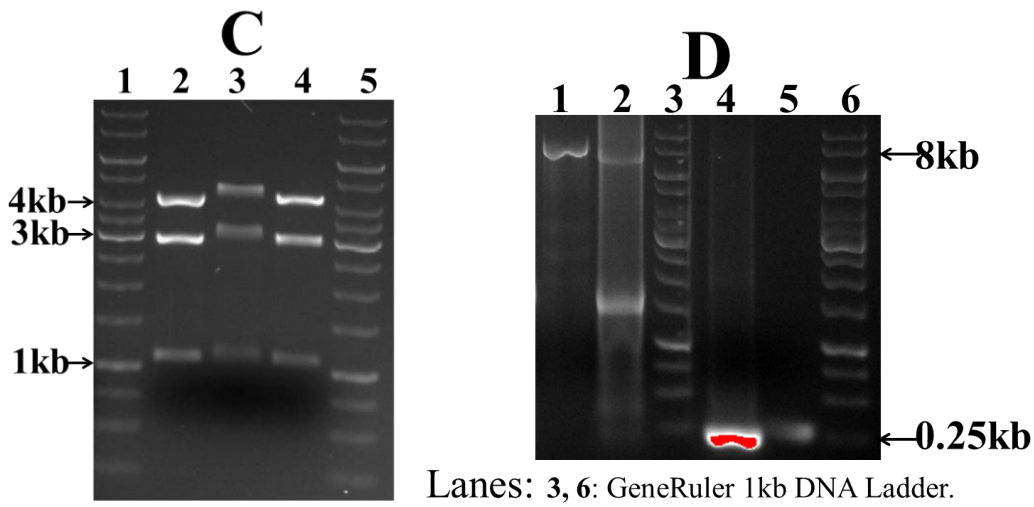
The *gusA* plasmids were made by assembling each of *cmlFp, cmlIp, cmlXp, jadJp* with *gusA* gene (in a pSOK 808 fragment) (seen in **3.1.4.1**). The resulted plasmids were: pSOK808-*cmlFp* (**pF*gusA***), pSOK808-*cmlIp* (**pI*gusA***), pSOK808-*cmlXp* (**pX*gusA***), pSOK808-*jadJp* (**pJ*gusA***). In the first attempt, the mutated *gusA* from pSOK805 was introduced in the resulted plasmids, then the assembly was repeated with the repaired *gusA* in pSOK808. Four pF*gusA* and four pX*gusA* candidates were checked by *Bam*HI digestion, and two of each showed right digestion pattern (**Figure 3.10A**). Five pJ*gusA* candidates were checked by *Kas*I digestion first, but the incomplete digestion was obvious, then *Apo*I and *Nar*I digestions were applied later, which confirmed four candidates (**Figure 3.10B**). Two out of three pI*gusA* candidates

were confirmed by *Bam*HI digestion (**Figure 3.10C**). Then PCR verification was carried out on candidate 1 of each *gusA* plasmids (**Figure 3.10D-E**).



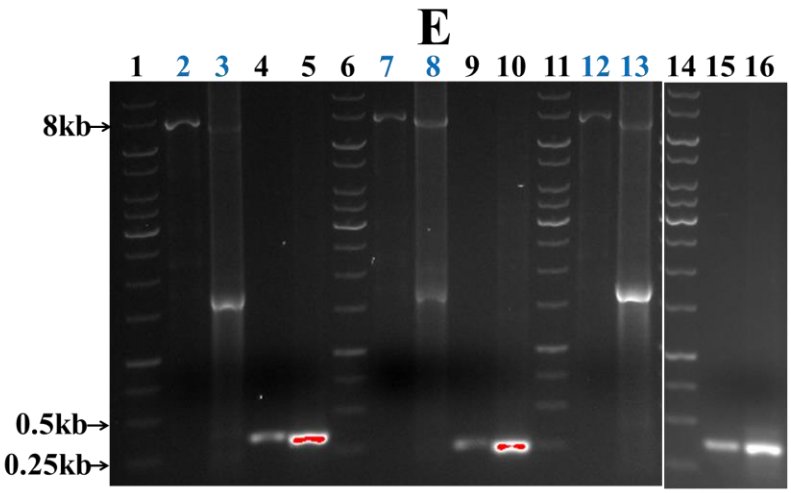
Lanes: **1, 6, 11**: GeneRuler 1kb DNA Ladder.
2-5: pF*gusA*candidates
7-10: pX*gusA*candidates

Lanes: **1, 7, 13**: GeneRuler 1kb DNA Ladder.
2-6: *Nar*I digestion of pJ*gusA* candidates
8-12:*Apo*I digestion of pJ*gusA* candidates



Lanes:
1, 5: 1kb DNA Ladder.
2-4: pI*gusA*candidates

Lanes: **3, 6**: GeneRuler 1kb DNA Ladder.
1: purified p808X vector for assembly
2: p808X fragment amplified from pX*gusA*
4: purified *cmlXp* for assembly
5: *cmlXp* amplified from pXgusA



Lanes:

1, 6, 11, 14: GeneRuler 1kb DNA Ladder.
2: purified p808F vector for assembly
3: p808F fragment amplified from pF*gusA*
4: purified *cmlFp* for assembly
5: *cmlFp* amplified from pF*gusA*
7: purified p808I vector for assembly
8: p808I fragment amplified from pI*gusA*
9: purified *cmlIp* for assembly
10: *cmlIp* amplified from pI*gusA*
12: purified p808J vector for assembly
13: p808J fragment amplified from pJ*gusA*
15: purified *cmlJp* for assembly
16: *cmlJp* amplified from pJ*gusA*

Figure 3.10 Double check of four *gusA* plasmids. A: restriction digestion of pF*gusA* and pX*gusA*. Both were treated with *Bam*HI digestion, and two candidates of each *gusA* plasmids showed right pattern-pF*gusA*: 3.8+2.8+1.0kb (lane 2, 4), pX*gusA*: 3.8+2.7+1.0kb (lane 7, 10). **B: restriction digestion of pJ*gusA*.** The *Kas*I digestion was applied on five pJ*gusA* candidates firstly, but the right candidates could not be picked up, due to the incomplete digestion (not shown), then *Nar*I and *Apo*I digestions were applied simultaneously, which implied right pattern 4.3+2.7+0.6kb and 5.6+2.0kb respectively. Four candidates (lane 2-5/8-11) were right (**Figure 3.9B**). **C: restriction digestion of pI*gusA*.** Two out of three candidates showed right *Bam*HI-digestion pettern: 3.8+2.7+1.0kb (lane 2, 4). The candidate in lane 3 might be genuine as well, because the variance of pattern could be accounted for less sample applied (judged by less band intensity compared with lane 2 and 4). **D: PCR confirmation of pX*gusA*.** The vector p808X (7.3kb) and *cmlXp* (233bp) were amplified from candidate 1 (lane 7 in **A**) and compared with their counterparts for Gibson Assembly. Good size-alignment was shown in **D**. **E: PCR confirmation of pF*gusA*, pI*gusA* and pJ*gusA*.** The vector p808F (7.3kb) and *cmlFp* (314bp) were amplified from candidate 1 (lane 2 in **A**) and compared with their counterparts for Gibson Assembly (lane 2-5). The same comparison was carried out on pI*gusA* (lane 7-

10, p808I: 7.3kb, *cmlIp*: 233bp) and pJ*gusA* (lane 12-13, 15-16, p808J: 7.3kb, *cmlJp*: 331bp), where pI*gusA* and pJ*gusA* candidate 1 was lane 2 in **C** and lane 2/8 in **B**. All the three comparisons showed good alignment, therefore, genuine *gusA* plasmids were confirmed.

3.2.4.2 GUS Test

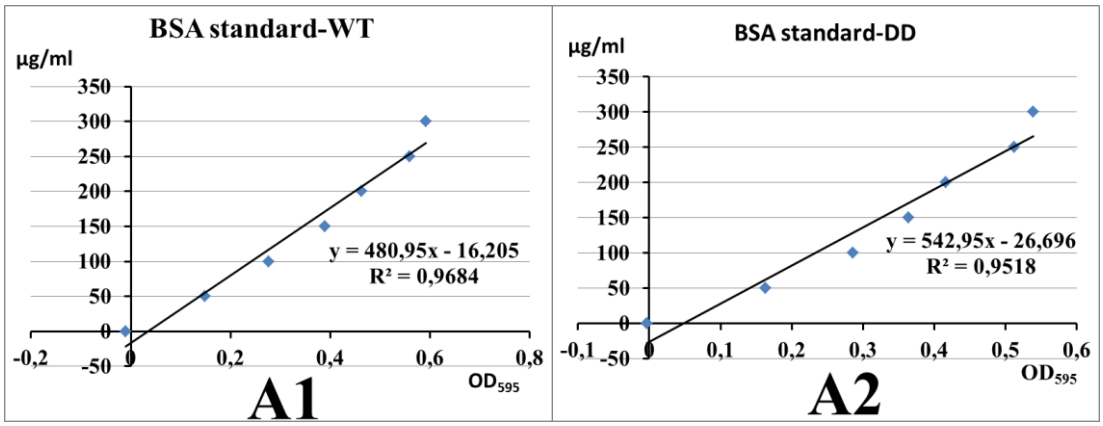
As plan, the promoters shall be first tested with culture from growth medium MYM, if results are not desirable then turn to the production medium GI, because if the culture medium is good to use, much industrial costs and procedures could be saved without inoculation to production medium. By now the GUS test has been conducted with MYM medium. The candidate 1 of each *gusA* plasmids that passed double check was transconjugated to *jadR1*$^{-}$ *cml*$^{-}$ mutant (**double deletion/DD**) (refer to **1.4.4.2**), then the promoter efficiencies were tested by GUS and Bradford assay (seen in **2.2.15; 2.4.16**), with the results shown in **Figure 3.11**. The Miller unites of GUS were measured at different times (30 min and 67 min) depending on the yellow color development to ensure appropriate data were taken, since data measured at reaction reaching beyond plateau point would generate biased smaller results (refer to formula in **2.2.15**).

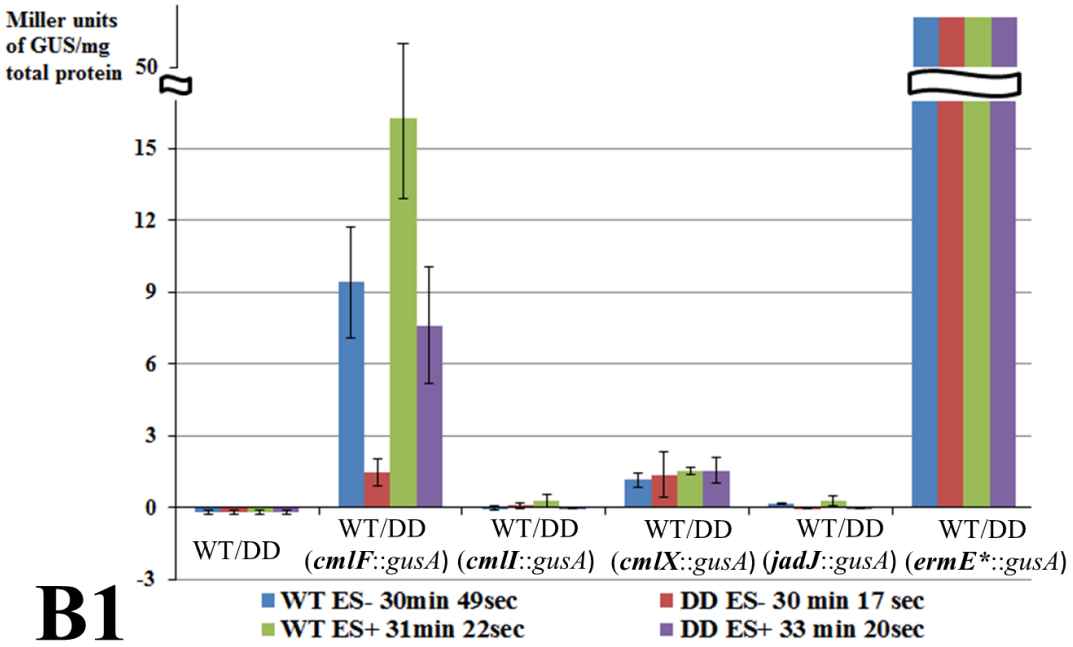
No GUS was detected in WT/DD (negative control) and GUS level was too high to measure in WT/DD(*ermE**p::*gusA*) (positive control). In WT/DD(*cmlFp*::*gusA*) , the inducing effect of ES+ in DD was interesting: the measured GUS level in DD ES+ was 5.1 and 4.4 times as high as DD ES- at 30min and 67min respectively. This effect was obvious compared with WT: the measured GUS level of WT ES+ was 1.7 times as high as WT ES- at 30min. Moreover, in DD(*cmlFp*::*gusA*) the Miller units of GUS/mg total protein from 30min (DDES-: 1.5, DDES+:7.6) to 67min (DDES-: 1.7, DDES+:7.4) was almost constant. Besides, GUS level in WT(*cmlFp*::*gusA*) ES- was 6.3 and 4.6 times as high as in DD(*cmlFp*::*gusA*) ES- at 30 min and 67 min respectively. The decreased values in WT(*cmlFp*::*gusA*) ES- and ES+ at 67 min might be explained by reaction reached plateau before 67 min. It is important that the error bars in DD(*cmlFp*::*gusA*) both at 30 min and 67 min are not overlapping, thus the 5.1/4.4 folds inducing effect of ES+ is meaningful.

In WT/DD(*cmlIp*::*gusA*) and WT/DD(*cmlXp*::*gusA*) at 30 min and 67min, the measured GUS level in DD ES- was higher than in WT ES-, most probably because deletion of *jadR1* in DD leads to *cmlI* and *X* unrepressed (seen in **1.1.4.2**).

In WT/DD(*jadJ*::*gusA*) , no GUS expression was detected in DD ES- and ES+ while it was detected in WT ES- and ES+ though at low levels, and WT ES+ was higher than WT ES-, this might be explained by that *jadJ* initiation requires JadR1, which can be enhanced by ethanol shock (seen in **1.1.4.2** and **1.2.3**).

Therefore, the *cmlF* was the best inducible promoter for designing the inducible system (refer to **1.4.3**), according to the current data.





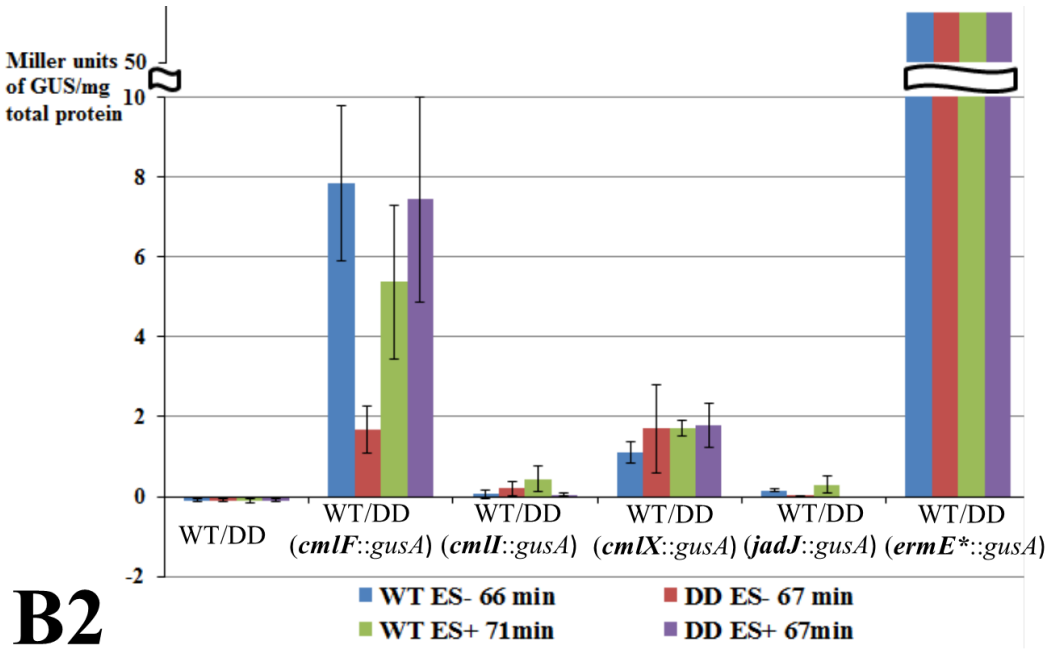


Figure 3.11 GUS test of promoter efficiencies. The GUS expression level was measured as OD first, then for comparison convenience, it was calculated as unified format-Miller units of GUS/mg total protein that is shown in B1 (at reaction time about 30 min) and B2 (at reaction time about 67 min). The values of WT/DD(*ermE**p::*gusA*) were out of measurement, due to the strong promoter *ermE**. Error bards in B1 and B2: means±1 S.D. generated from three independent replicates. WT: wild type, DD: double deletion, ES-: no ethanol shock, ES+: ethanol shock. **A1: BSA standard curve for WT strains.** The standard curve was made from BSA and was used to measure protein concentrations of WT and WT strains introduced pF*gusA*, pI*gusA*, pX*gusA*, pJ*gusA*, pSOK808 respectively. Each sample (BSA and strain lysate) had three replicates in measurement. **A2: BSA standard curve for DD strains.** The standard curve was made in the same way as A1, and was used to measure protein concentrations of DD and DD strains introduced pF*gusA*, pI*gusA*, pX*gusA*, pJ*gusA*, pSOK808 respectively. **B1: Miller units of GUS per mg total protein at '30min'.** The small plus values in WT/DD(*cmlIp*::*gusA*) and

WT/DD(jadJ::*gusA*) suggested low expression. The values in WT/DD(*cmlIp*::*gusA*) were almost same (about 1.6). In WT/DD(*cmlFp*::*gusA*) , the measured GUS level of ES+ was 1.7 and 5.1 times as high as WT ES- and DD ES- respectively. **B2: Miller units of GUS per mg total protein at '67min'.** Compared to 30min, the measured GUS levels were generally higher in WT/DD + (*cmlIp*::*gusA*)/(*cmlXp*::*gusA*)/ (jadJ::*gusA*), lower in WT(*cmlFp*::*gusA*) of both ES- and ES+, and higher and stable in DD(*cmlFp*::*gusA*) of ES- and ES+ respectively, but the measured GUS level in DD(*cmlFp*::*gusA*) ES+ was equal to 4.4 times DD(*cmlFp*::*gusA*) ES-. At both 30 min and 67 min, the small minus values in WT/DD indicated no GUS expression while the values from positive control WT/DD(*ermE**p::*gusA*) were too high to measure.

3.2.5 Replace Native Promoters with the Inducible Promoter

This step has not been initiated.

4. Discussion

4.1 Generate Double Mutant

4.1.1 Recombinant Plasmids

The Gibson Assembly technique is a favorable method for recombinant plasmids, because it combines the digestion of overlapping ends and ligation in one step, which is time saving and simple. (Gibson, Young et al. 2009) However, problems arising from **mispriming** (primer annealing to the wrong DNA sequence) of PRC products and mutation in PCR reaction weaken this technique. Specifically, as shown in **Figure 3.5,** the wrong p-*cml*-D might be caused by the introduced wrong *cml*-FlankB that resulted from mispriming in PCR (similar to wrong MPS05-B41-Lin in **3.2.3**), therefore, the primer quality shall be emphasized so as to avoid such problems. Though the fragments are checked before ligation on gel (to decide volume ratio in ligation), the misprimed fragments, if not fully eliminated in purification, might be too less to observe on gel, consequently, they could engender wrong assembled plasmids. Therefore, **double check** (restriction enzyme digestion and PCR verification) on recombinant plasmids is recommended to discover such problems. Besides, the PCR cycles should be limited when amplifying DNA for ligation, because, as shown in **3.2.2.1**, excessive cycles can give rise to mutation in DNA products, which could lead to failed homologous recombination (failed double crossover in *cml* deletion in this project) or wrong gene products in other cases. In this project, after the 'PCR mutation' problem was inferred, the PCR cycles were reduced from 35 to 25 to reduce mutation probability, and right double mutant was generated.

In addition, *Dpn*I digestion is indispensable for vector DNA amplified from pDNAs originating from dam^+ strains, because it cleaves only at the methylated recognition site, thus the contaminating pDNAs are eliminated, with vectors intact. (BioLabs 2014) Also, enzyme digestion in checking pDNAs should be sufficient, otherwise, the incomplete digestion pattern on gel makes it hard to identify the right candidates, leading to additional jobs.

4.1.2 Double Crossover Mutants

In the transconjugation of *S. venezuelae* through *E. coli* ET12567 (pUZ8002) (seen in **1.4.4.2**), no problems occurred, thus this technique was further proved as efficient. It is important to pick up the surviving colonies at appropriate time after overlying Nal and Am, because early time can reduce the occurrence of right transconjugants while later time can give rise to more false positive candidates.

The double crossover approach to delete genes in *S. venezuelae* is advantaged in that no selectable marker is introduced. However, besides the drawback from 'mutation in flank' in **4.1.1**, it is necessary to distinguish between the reverted WT and genuine deletion mutant.

The replica plating technique has merits in isolation of mutants and has been successfully applied to bacteria, *actinomycetes* and unicellular algae. (Roberts 1959) When perform this experiment, it is critical to make sure the density of 100-200 discrete colonies per plate, otherwise co-growth between colonies makes it difficult to identify real mutants. In this project, the three-round sporulation before replica plating can be performed on plates or liquid medium. The former is time-consuming (three-four days/each round) but is convenient to discover contamination by checking the colony morphology, whereas the latter is time saving (overnight/each round) but is difficult to discover contamination if it happens.

In the PCR verification of first crossover mutants, the primer pairs of FlankA forward and FlankB reverse were used, and three fragment should be obtained theoretically: FlankA+FlankB, FlankA+ gene for deletion + FlankB, p201-Del + gene for deletion + FlankB (refer to **1.4.4.3**), but only the smallest fragment was detected. This could be explained by inefficient DNA polymerase or non-optimal PCR reaction condition for large fragments, because the EHD DNA polymerase can only amplify DNA fragment smaller than 8kb. In order to get the other two larger fragments, more efficient polymerase and exploration of optimal reaction conditions could be exploited. But the genuine first crossover mutants could be identified as long as they are Am^R and the smallest fragments are detected. In terms of the double crossover mutants, as long as they are Am^S and the smallest fragments are detected, they are genuine (refer to **1.4.4.3**), because second crossover deletes Am^R gene and leaves FlankA and FlankB

connected in the real double crossover mutants, which makes them distinctive from reverted WT.

Bands on gel may not align well with the corresponding bands in ladder lane, but the small variance is acceptable, since gel electrophoresis is a rough method to identify DNA sizes, which can be affected by sample density, GC content in DNA, buffer, gel quality (e.g. bubbles, homogeneity), etc. Therefore, it is reasonable to include positive controls with which right bands can be compared if variance with ladder is too much.

4.2 Clone Gene Clusters

The gene cluster MP112-09-Lac was successfully cloned in pLacNat and pLacNP, but the ratio of right plasmids to total candidates (**Figure 3.9**) was smaller than *gusA* plasmids (**Figure 3.10**), which might be concluded as the smaller fragments the easier to assembly in Gibson Assembly.

The gene cluster MPS05-B41-Lin could not be amplified, because though the product size on gel was right, it did not show right digestion pattern. The reason could be mispriming of MPS05-B41-Lin in PCR, improper PCR condition, or the inhibition of primer annealing at the right place by special DNA structure at gene cluster MPS05-B41-Lin.

The product with right size on gel was obtained by increasing the annealing temperature from 56 °C to 65 °C after failures with 56 °C. Though this product was proved pseudo by enzyme digestion, it revealed that the annealing temperature is critical for amplifying specific products.

4.3 Test Promoters with GUS Assay

4.3.1 GUS Assay

GUS is a sensitive and versatile reporter because of its wide substrates, high specific enzymatic activity and stability, tolerance to the most commonly used chemicals and assay conditions. In *streptomycetes*, most species do not possess any endogenous

GUS activity (refer to **1.4.4.5**). The project benefits from such merits, but the problem came from the strong promoters that made the yellow colour development too rapid to measure. In other words, expression of *gusA* gene under strong promoters provided large amounts of GUS, which converted substrate to a yellow product too fast to ensure accurate measurements. Therefore, pre-experiments are required to decide the optimal lysate amount used in GUS reaction. Besides, it is also critical to measure data at different times such that data before reaction plateau point are taken, since the Miller unites of GUS is calculated according to [1000 x (OD_{420} − 1.75 x OD_{550})] / [time of reaction x volume of culture assayed], if measurement is after the enzymatic reaction plateau, the Miller unites would be smaller than real value. The first measurement should be executed on first appearance of yellow colour, then subsequent measurements shall be carried out depending how rapid the colour develops.

4.3.2 The Inducible Promoter

As planned, the promoters are first tested with culture from growth medium MYM, if results are not desirable then turn to the production medium GI (seen in **1.4.3**). According to MYM data (**Figure 3.11**), in WT/DD no GUS was detected and in WT/DD(*ermE*p*::*gusA*) GUS level was too high to measure, which contributed to the reliability of results in other samples by serving as desirable negative or positive controls.

In WT/DD(*cmlFp*::*gusA*) , ES+ caused 5.1 and 4.4 times inducing effect in DD at 30min and 67min respectively, whereas this effect was 1.7 times in WT at 30min. Thus, *cmlF* could be recognized as the inducible promoter. Besides, GUS level in WT(*cmlFp*::*gusA*) ES- was five to six times as high as in DD(*cmlFp*::*gusA*) ES-. The decreased values in WT(*cmlFp*::*gusA*) ES- and ES+ at 67 min might be explained by reaction had reached plateau before 67 min. Based on those data and the existing knowledge (refer to **1.1.4.2**), the relationship between ES, JadR2, JadR1, *cmlF* is proposed in **Figure 4.1**. Specifically, JadR1 induces Jd but represses Cm biosynthesis, simultaneously repressing *cmlF* that is responsible for Cm transport to extracellular environment. JadR1 and JadR2 mutually repress and the inhibition of JadR2 on *cmlF* is stronger than JadR1 on *cmlF*, because deletion of *jadR1* led to down regulation of

cmlF (**Figure 3.11**). The ES inhibits JadR2 in an unknown way, because ES+ engendered 5.1 and 4.4 folds inducing effect in DD(*cmlFp*::*gusA*) at 30min and 67min respectively (**Figure 3.11**).

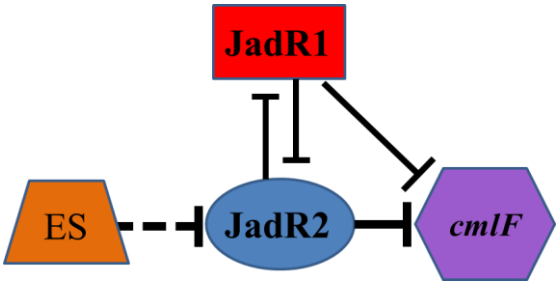


Figure 4.1 Speculated relationship between ES, JadR2, JadR1, *cmlF*. JadR1 and JadR2 are encoded by *jadR1* and *jadR2* respectively. Ethanol shock inhibits JadR2 in an unknown way. JadR2 and JadR1 inhibit each other, and both can repress *cmlF*, but JadR2 represses *cmlF* more intensively than JadR1.

Additionally, in DD(*cmlFp*::*gusA*) the Miller units of GUS/mg total protein from 30min to 67min were almost stable (**Figure 3.11**). This at least means the result at 30min was before reaction plateau, because the productivity-time relationship in enzymatic reaction is relative linear before plateau (Chaplin 2012), if plateau was at 30min or before, the result at 67min would be half or less than half of 30min, according to the formula in **4.3.1**. Therefore, the 5.1 time ES+ inducing effect in DD(*cmlFp*::*gusA*) should be reliable.

In WT/DD(*cmlIp*::*gusA*) and WT/DD(*cmlXp*::*gusA*) at 30 and 67min, the measured GUS level in DD ES- was higher than in WT ES- (**Figure 3.11**), which could be explained by deletion of *jadR1*, thus this results was consistent with current knowledge-JadR1 represses *cmlI* and *X* (seen in **1.1.4.2**) and the project hypothesis-deletion of *jadR1* shall cause up-regulation of Cm production that relies on *cmlI* and *cmlX* (seen in **1.4.2**). In WT/DD(*jadJ*::*gusA*), GUS expression was absent and present (though at low levels) in DD (ES-, ES+) and WT (ES-, ES+) respectively, and it was higher in WT ES+ than WT ES- (**Figure 3.11**), this was in accordance with existing knowledge as well: *jadJ* initiation requires JadR1, which can be enhanced by ethanol shock (seen in **1.1.4.2** and **1.2.3**).

In summary, *cmlFp*, as the best inducible promoter, is possible to meet the project objective for designing the inducible system (refer to **1.4.3**), according to the current

data. The relatively long error bars in **Figure 3.11** mar the results, but it is worth mentioning that the error bars in DD(*cmlFp*::*gusA*) both at 30 min and 67 min are far from overlapping, thus the 5.1/4.4 folds inducing effect of ES+ is meaningful.

4.4 Future Works

In the following jobs, MYM based GUS assay would be repeated so as to reduce the error bars and further corroborate the conclusion. Besides, the GI-based GUS assay might also be performed, since though 5.1/4.4 folds inducing effects in *cmlF* is valuable, more significant inducing effects might be exposed in *cmlF* itself or other promoters in GI-based results. Besides, the PCR condition should be optimized for MPS05-B41-Lin amplification so as to get this fragment. Alternatively, choose other gene clusters to test. Then after decided the most desirable inducible promoter, the gene clusters MPS05-B41-Lin and MP112-09-Lac would be assembled under control of this promoter respectively and transconjugated to *jadR1*$^{-}$*cml*$^{-}$ mutant, followed by BSM measurement.

5. Conclusion

The *jadR1*$^{-}$ and *jadR1*$^{-}$ *cml*$^{-}$ mutants were successfully generated with Gibson Assembly, transconjugation, double crossover and replica plating. The gene cluster MP112-09-Lac was cloned with the native promoter and *ermE** respectively and successfully transconjugated to *jadR1*$^{-}$ *cml*$^{-}$ mutant, however, cloning of MPS05-B41-Lin was hindered by wrong PCR amplification. The four *gusA* plasmids were successfully obtained by assembling each of *cmlFp, cmlIp, cmlXp, jadJp* with *gusA* gene (in a pSOK 808 fragment) and transconjugated to *jadR1*$^{-}$ *cml*$^{-}$ mutant. The four promoters were tested with GUS assay, based on MYM medium. The *cmlF* promoter is speculated to be the most desirable inducible promoter and hopefully it could meet the project objective.

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Appendix

A: Primers

All the following primers were designed by J5. (Hillson, Rosengarten et al. 2011)

Assembly of deletion plasmids

p-*jad*-D

SOK201jr1_F CGACATCAAGGACTGCGTAATCATGTCATAGCTGTTTCC
SOK201jr1_reverse GAGTCCGTGGTACAGGTCGACGGATCTTTTCC
JR1del1_forward GATCCGTCGACCTGTACCACGGACTCCTCGACC
JR1del1_reverse GGATGGTGATCACCACTTCTACGGACGTCAGGC
JR1del2_forward GTCCGTAGAAGTGGTGATCACCATCCGAGGC
JR1del2_R CTATGACATGATTACGCAGTCCTTGATGTCGCAGATGG

p-*cml*-D

p201*cml*D-F CGACGAACTGCTGCGTAATCATGTCATAGCTGTTTCC

p201*cml*D-R CCTCTCAACATAGCAACAGGTCGACGGATCTTTTCCGCTGC

*cml*D1-F GATCCGTCGACCTGTTGCTATGTTGAGAGGTATGTCGAGTCC

*cml*D1-R GTACTGCTCGTAGCACGAGTTGCTGCTGCTGCCG

*cml*D2-F GCAGCAACTCGTGCTACGAGCAGTACCACCAGTTCCTCG

*cml*D2-R GCTATGACATGATTACGCAGCAGTTCGTCGTCCGGC

Assembly of *gusA* plasmids

pSOK808::*cmlFp*

*cmlFp*_forward CTTCGCACTTCGTGGTCGTCATGAACACTCCTTCTCCGCG
*cmlFp*_reverse CCTTCGATCGATGGCTCCAACTACATCGCAGAAAGGGG
808NP-F ATGTAGTTGGAGCCATCGATCGAAGGAGAGTTCACCATGCTGA
808NP_R TGTTCATGACGACCACGAAGTGCGAAGTTCACCGAAGAGC

pSOK808::*cmlIp*

*cmlIp*_forward CTTCGCACTTCGTGCTCCGTCACCTTAAGGCCTCCG
*cmlIp*_reverse CCTTCGATCGATGGTCTCGAAGTCTGTGGATATCGGTCGCG
808NP_F CCACAGACTTCGAGACCATCGATCGAAGGAGAGTTCACCAT
GCTGA
808NP_R CCTTAAGGTGACGGAGCACGAAGTGCGAAGTTCACCGAAGAGC

pSOK808::*cmlXp*

*cmlXp*_F GGTGAACTTCGCACTTCGTGGTCTCGAAGTCTGTGGATATCGG

TCGCG
*cmlXp*_R CCTTCGATCGATGCTCCGTCACCTTAAGGCCTCCG
808NP_F TAAGGTGACGGAGCATCGATCGAAGGAGAGTTCACCATGCTGA
808*gus*NP_R ACAGACTTCGAGACCACGAAGTGCGAAGTTCACCGAAGAGC

pSOK808::*jadJp*
*jadJp*_forward CTTCGCACTTCGTGGACGCTGTCCGAGCGGACACC
*jadJp*_reverse CCTTCGATCGATGGACTCGCCTTCTCCGTACCCG
808NP_F GGAGAAGGCGAGTCCATCGATCGAAGGAGAGTTCACCATG
CTGA
808NP_R GCTCGGACAGCGTCCACGAAGTGCGAAGTTCACCGAAGAGC

Assembly of gene cluster plasmids

pSOK804_05-41_lin
SOK804_forward ATCTCGGCCGCTTCTGCAGGTCGACTCTAGAGGATCCGC
SOK804_reverse ACCATGATGAAGGCGAAGTTCACCGAAGAGCGCATTTTCG
S05-41_lin_forward TCGGTGAACTTCGCCTTCATCATGGTGGCCTTCAGGTGC
S05-41_lin_reverse AGTCGACCTGCAGAAGCGGCCGAGATGGACATCG

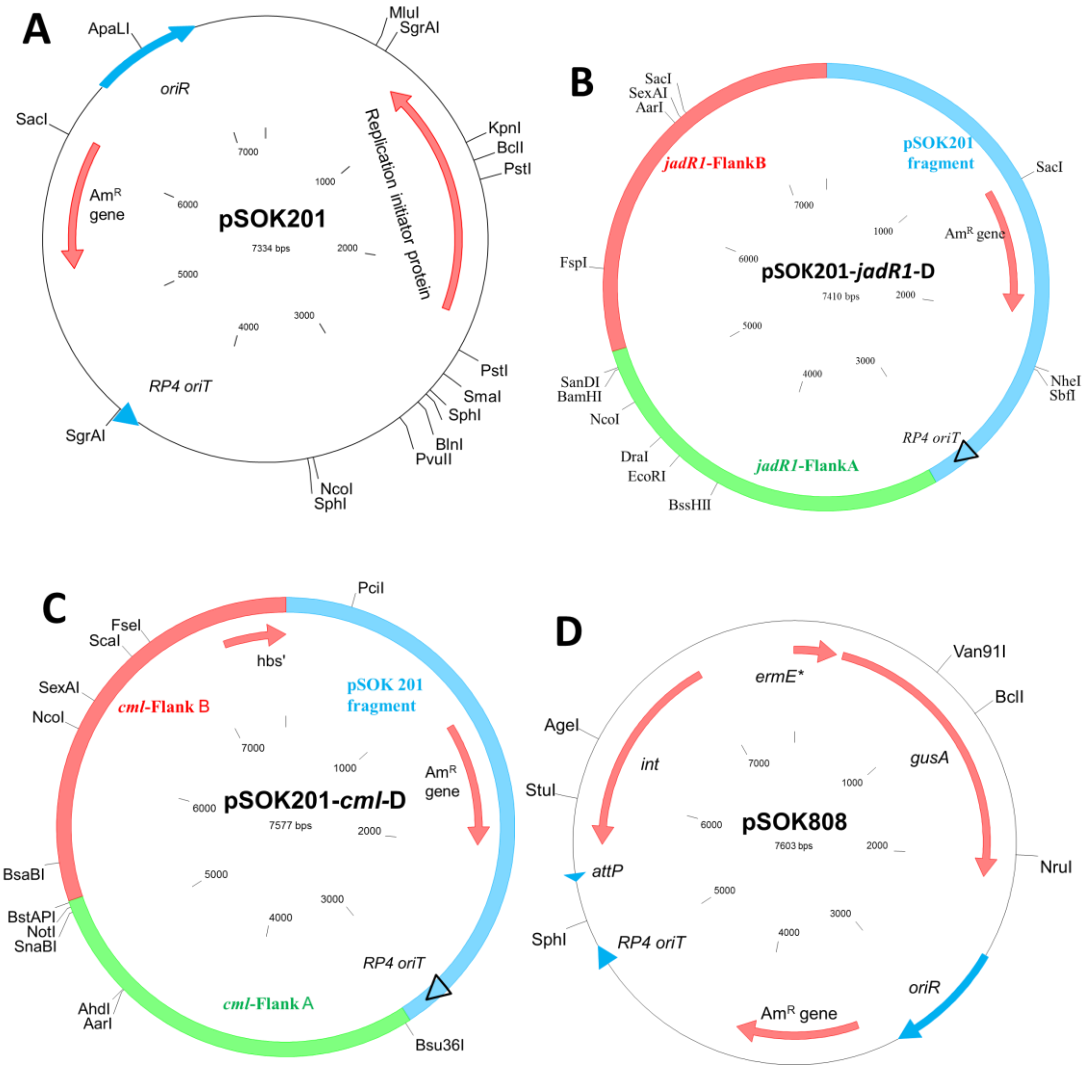
pSOK804_112-09_lac
SOK804_forward TCTGCGCAACATCCTGCAGGTCGACTCTAGAGGATCCGC
SOK804_reverse GCTGCGGATGCGAAGTTCACCGAAGAGCGCATTTTCG
112-09_Lact_F AATGCGCTCTTCGGTGAACTTCGCATCCGCAGCATCTTC
GGCAAGC
112-09_Lact_R AGTCGACCTGCAGGATGTTGCGCAGATCCAGGTCC

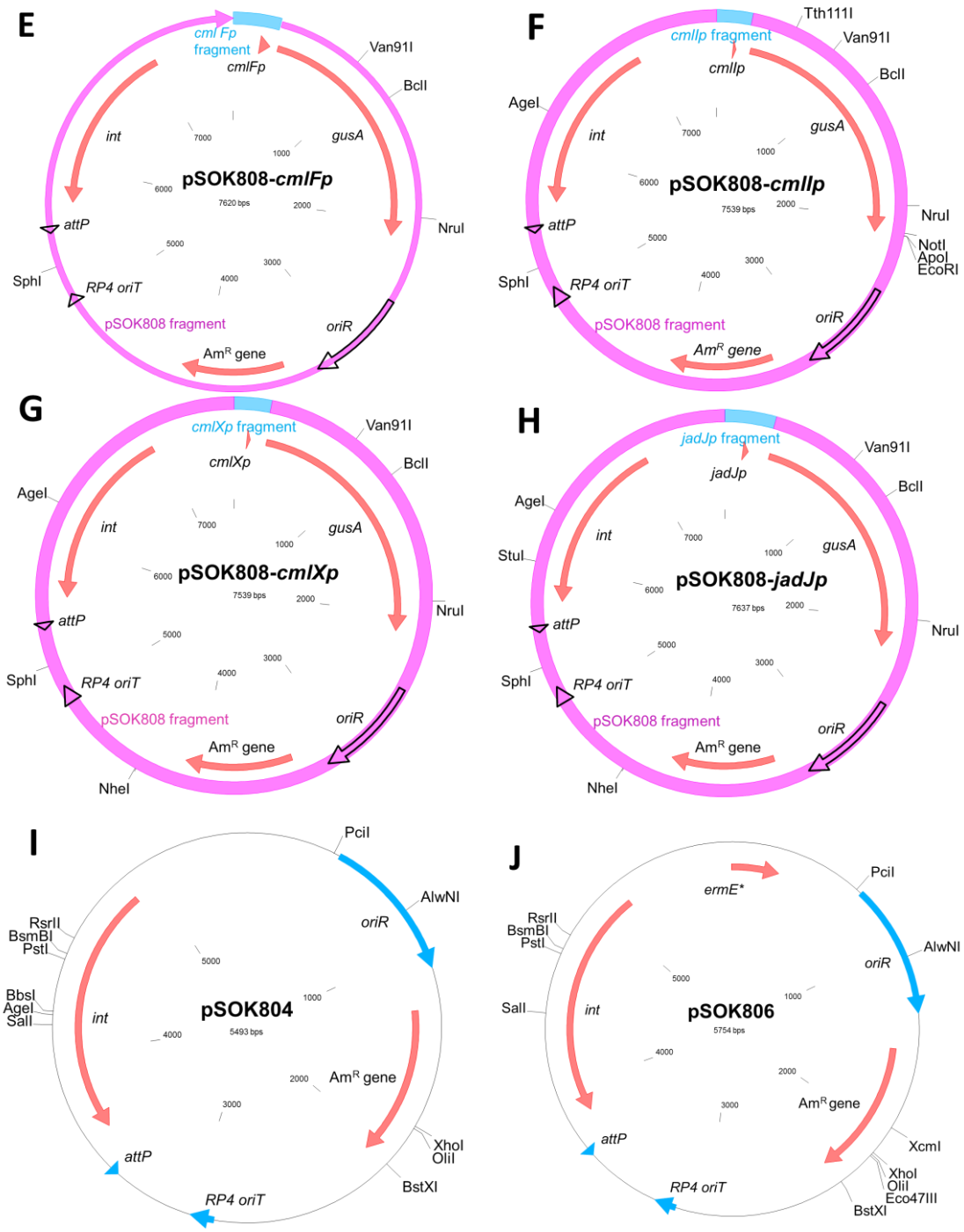
pSOK806_112-09_lacNP
SOK806_forward TGTAGCATCTGAGCTGCAGGTCGACTCTAGAGGATCCG
SOK806_reverse CGAGGTGCGCTTCGATCCTACCAACCGGCACGATTGTCC
112-09_lacNP_forward GGTTGGTAGGATCGAAGCGCACCTCGCGCCAGC
112-09_lacNP_reverseCCTCTAGAGTCGACCTGCAGCTCAGATGCTACAG
CTCTGACGCGG

B: Plasmid Maps

In this project, eight plasmids were successfully generated from pSOK201, pSOK808, pSOK804, and pSOK806. pSOK201-*jad*-D and pSOK201-*cml*-D were assembled with two flanks of *jadR1* or *cml* and a pSOK201 fragment. pSOK808-*cmlFp*, pSOK808-*cmlIp*, pSOK808-*cmlXp*, pSOK808-*jadJp* were assembled with one

promoter of *cmlFp, cmlI, cmlXp, jadJp* and a corresponding pSOK808 fragment. pSOK804-*MPS05*-Lin was designed to be assembled with the gene cluster of *MPS05-B41*-cluster 28-Lincocin-M18-like (including native promoter) and a pSOK804 fragment. pSOK804-*MP112*-Lac was assembled with the gene cluster of *MP112-09*-cluster 6-Lactococcin972-like (including native promoter) and a pSOK804 fragment. pSOK806-MP112-LacNP was assembled with the gene cluster of *MP112-09*-cluster 6-Lactococcin972-like with a pSOK806 fragment, in which the *ermE** promoter replaced the native promoter. **Figure B1** represents the details.




E
cmlFp fragment
cmlFp
Van91I
BclI
NruI
int
gusA
pSOK808-cmlFp
7620 bps
attP
SphI
RP4 oriT
pSOK808 fragment
oriR
AmR gene
F
cmllp fragment
cmllp
Tth111I
Van91I
BclI
AgeI
int
gusA
pSOK808-cmllp
7539 bps
NruI
NotI
ApoI
EcoRI
attP
SphI
RP4 oriT
pSOK808 fragment
oriR
AmR gene
G
cmlXp fragment
cmlXp
Van91I
BclI
AgeI
int
gusA
pSOK808-cmlXp
7539 bps
NruI
attP
SphI
RP4 oriT
pSOK808 fragment
oriR
AmR gene
NheI
H
jadJp fragment
jadJp
Van91I
BclI
AgeI
StuI
int
gusA
pSOK808-jadJp
7637 bps
NruI
attP
SphI
RP4 oriT
pSOK808 fragment
oriR
AmR gene
NheI
I
PciI
AlwNI
oriR
RsrII
BsmBI
PstI
BbsI
AgeI
SalI
int
pSOK804
5493 bps
AmR gene
XhoI
OliI
BstXI
attP
RP4 oriT
J
ermE*
PciI
RsrII
BsmBI
PstI
AlwNI
oriR
SalI
int
pSOK806
5754 bps
AmR gene
XcmI
XhoI
OliI
Eco47III
BstXI
attP
RP4 oriT

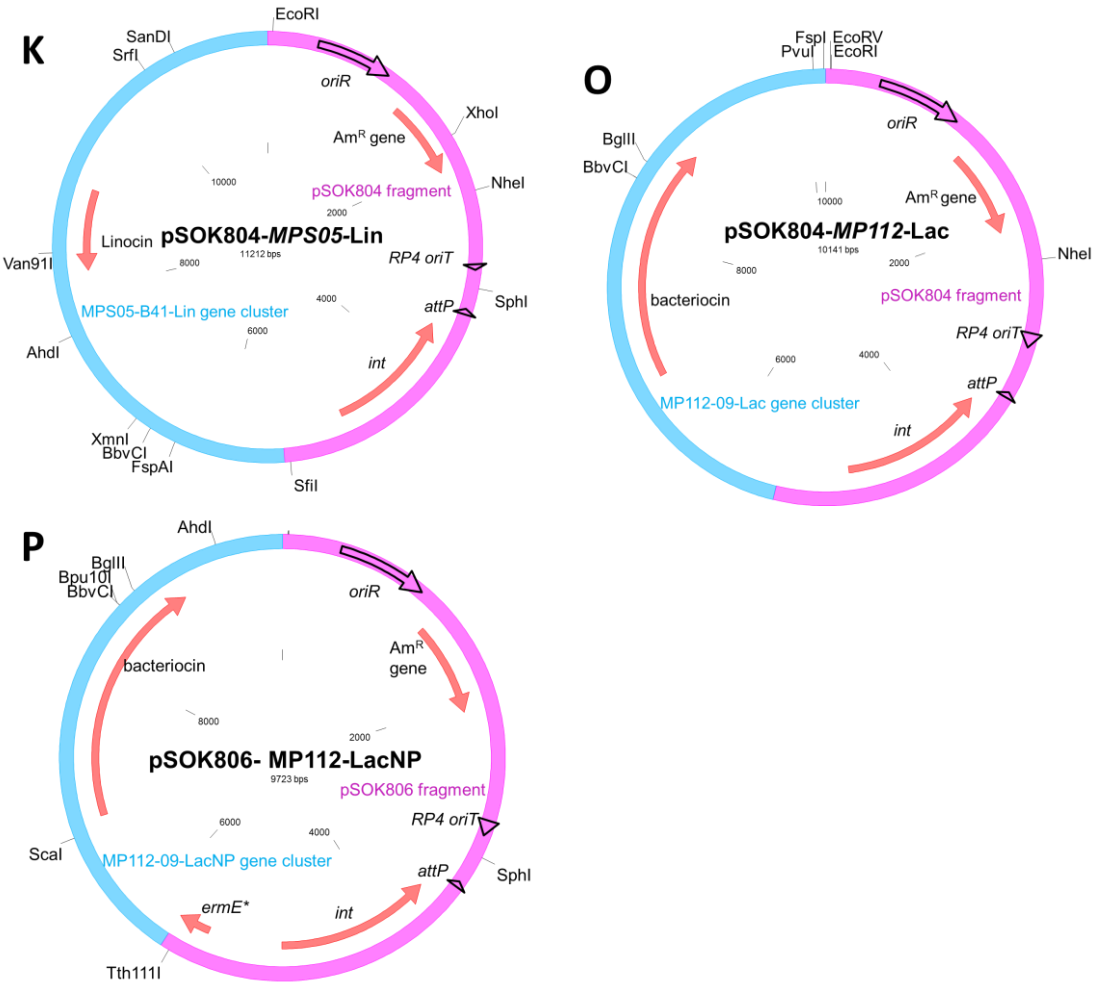


Figure B1 Schematic representation (made with Clone Manager version 6) of plasmids involved in the project. A: pSOK201. The *oriR* is replication origin while *RP4 oriT* is the origin of ssDNA transfer, where helper plasmid functions in *trans.* Besides, there is also an Am^R gene (selection marker) and a gene encoding replication initiator protein. **B**: pSOK201-*jadR1*-D. The green and red regions are two flanking fragments of *jadR1*and the blue is amplified from pSOK201. This plasmid contains a *RP4 oriT* but no *oriR*, because it was designed to be transferred to *S. venezuelae* and integrated to chromosome through homologous recombination without autonomous replication (suicide vector). **C**: pSOK201-*cml*-D. It is the same with pSOK201-*jad*-D except that the red and green are two *cml* flanks. **D**: pSOK808. The *gusA* is under control of *ermE**, and the *int* encodes an integrase that mediates site specific recombination between *attP* and *attB* in bacterial chromosome (seen in **1.4.4.2**). Besides this plasmid harbours *oriR, RP4 oriT,* and an Am^R gene as well. **E-H:** plasmids constructed by replacing *ermE** with one of *cmlFp, cmlI, cmlXp, jadJp*, respectively. The four plasmids were integrated to host chromosome through site-

specific instead of homologous recombination and used in GUS assays. **I**: pSOK804 (a variant of pSOK808). This plasmid lacks *ermE** and *gusA* compared to pSOK808. **J:** pSOK804 (a variant of pSOK808). This plasmid lacks *gusA* compared to pSOK808. **K**: pSOK804-*MPS05*-Lin. The blue part is the gene cluster of *MPS05-B41*-cluster 28-Lincocin-M18-like that includes the native promoter while the red part is a pSOK804 fragment that includes the *oriR*, Am^R gene, *RP4 oriT*, *attP*, and *int* without *ermE**. Thus this plasmid could be integrated to host chromosome thorough site-specific recombination and the gene cluster was under control of its native promoter. **O**: pSOK804-*MP112*-Lac. This plasmid is same with **K** except that the gene cluster is *MP112-09*-cluster 6-Lactococcin972-like under control of its native promoter. **P**: pSOK806- MP112-LacNP. This plasmid is the same with **O** except that the native promoter was replaced with *ermE**.

C: Media Recipes

Table C1 TAE buffer (50 ×).

Diluted to 1× TAE buffer for DNA gel electrophoresis. Prepared by technicians. (BioinformationWeb 2013)

Ingredients	**Amount/L sd H_2O**
Tris-base	242.0 g
Acetic acid (100 %)	57.1 ml
EDTA (0.5 M) pH 8	100 ml

Table C2 Agarose (0.8 %)

The ingredients were melted in a microwave machine. The solution was stored at 60 °C. Prepared by technician. (BioinformationWeb 2013)

Ingredients	**Amount**
SeaKem LE Agarose	2.4 g
1×TAE buffer	300 ml
GelGreen Nucleic Acid Stain (10 000×)	30 µl

Table C3 TSS-buffer.

The pH was adjusted to 6.5, autoclaved (121 °C, 20min), then 5 % DMSO (volume/volume) was added. (LabLife 2011)

Ingredients	**Concentration**
LB-media	85 % (volume/volume)

PEG6000	10 % (weight/volume)
$MgCl_2$	50 mM

Table C4 LB/LA medium.

Ingredients were dissolved in distilled water, and autoclaved at 121 °C for 20 min. For LA medium, different antibiotic(s) could be added when it had cooled down to 50-60 °C. (Inoue, Nojima et al. 1990)

Ingredients	**Concentration**
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L
Agar (**only for LA medium**)	15 g/L

Table C5 2×YT medium.

The ingredients were dissolved in distilled water. The pH was adjusted to 7.0, autoclaved at 121 °C for 20 min. (theLabRat 2005)

Ingredients	**Concentration[g/L]**
Bacto Tryptone	16
Bacto Yeast extract	10
NaCl	5

Table C6 Antibiotics. All were stored at - 20 °C after sterile filtered.

Name	**Concentration of stock solution (mg/ml)**	**Concentration in use (μg/ml)**
Ampicillin	100 in sdH2O	100
Chloramphenicol	25 in 50%-100% EtOH	25
Kanamycin	40 in sdH_2O	20
Nalidixic	30 in 0.1 M NaOH	30
Apramycin	100 in sdH20	100 for *E.coli*. 50 for *S. venezuelae.*

Table C7 Enzymatic lysis buffer.

This buffer was used for isolating gDNA from *S. venezuelae*, made according to instruction of QIAGEN DNeasy® Blood & Tissue kit.

Components	**Concentration**
Tris•HCl, pH 8.0	20 mM
sodium EDTA	2 mM
Triton® X-100 (volume/volume)	1.2%

Table C8 MYM medium.

Ingredients were dissolved in distilled water before autoclaved at 121 °C for 20 min. (Doull, Singh et al. 1994)

Ingredients	**Concentration [g/L]**
Maltose	4
Yeast extract	4
Malt extract	10

Table C9 Lysis buffer.

This buffer was vacuum filtered and kept in room temperature, used for GUS assay. (Prof Mervyn Bibb, John Innes Centre, UK)

Components	**Concentration**
$Na_2HPO_4\,7H_2O$	8 g/L
$NaH_2PO_4\,H_2O$	2.35 g/L
triton X-100	0.1% (volume/volume)

Table C10 Z-buffer.

The pH was adjusted to 7, vacuum filtered and kept in room temperature, used for GUS assay. (Prof Mervyn Bibb, John Innes Centre, UK)

Components	**Concentration [**g/L**]**
$Na_2HPO_4\,7H_2O$	16.1
$NaH_2PO_4\,H_2O$	5.5
KCl	0.75
$MgSO_4\,7H_2O$	0.246

Table C11 D-galactose-L-isoleucine (GI) medium.

This medium was used for jadomycin production. The ingredients were dissolved in sd H_2O, with pH adjusted to 7.5, then autoclaved at 121 °C, 20 min. (Doull, Singh et al. 1994)

Components	**Concentration [g/L]**
D-galactose	18.2
L-isoleucine	3.94
KH_2PO_4	0.95
K_2HPO_4	0.52
$MgSO_4$-$7H_2O$	0.2
$CaCl_2$-$2H_2O$	0.09
NaCl	0.09
$FeSO_4$-$7H_2O$	0.009
Trace mineral solution	4.5 ml

TSB (Tryptone Soya Broth) medium

30 g powder TSB/L distilled water, autoclaved at 121 °C for 20 min.

ISP4 medium

37 g powder media/L distilled water.

Autoclaved at 121 °C for 20 min. In this project three kinds ISP4 media plates were prepared: pure ISP4; ISP4 + $MgCl_2$ (0.01M, added after autoclave); ISP4 + antibiotic(s) (added to the medium when it had cooled down to 50-60 °C).

D: Raw Data in GUS Assay

Table D1 BSA standard for WT and DD.

	WT				DD			
BSA µg/ml	Triplicate			Blank	Triplicate			Blank
0	0,326	0,328	0,328	0,327	0,325	0,327	0,331	0,323
50	0,483	0,482	0,494	0,347	0,496	0,496	0,491	0,328
100	0,596	0,636	0,610	0,339	0,628	0,593	0,629	0,339

150	0,711	0,734	0,736	0,340	0,673	0,715	0,696	0,345
200	0,774	0,814	0,815	0,334	0,727	0,767	0,747	0,331
250	0,866	0,879	0,947	0,346	0,836	0,840	0,854	0,332
300	0,929	0,920	0,942	0,332	0,975	0,924	0,711	0,317

Table D2 OD_{595} of WT ES- lysate (20×) in Bradford assay.

Each biological replicate from flask culture had one triplicate.

	Triplicate of biological replicate 1, ES-			Triplicate of biological replicate 2, ES-			Triplicate of biological replicate 3, ES-		
WT	0,516	0,502	0,530	0,573	0,536	0,528	0,463	0,500	0,495
WT + pF*gusA*	0,463	0,477	0,468	0,518	0,544	0,539	0,446	0,455	0,466
WT + pI*gusA*	0,489	0,511	0,496	0,521	0,533	0,543	0,456	0,466	0,471
WT + pX*gusA*	0,534	0,548	0,540	0,456	0,478	0,468	0,537	0,524	0,506
WT + pJ*gusA*	0,576	0,597	0,588	0,527	0,548	0,557	0,625	0,628	0,641
WT + p8*gusA*	0,504	0,516	0,510	0,585	0,571	0,570	0,484	0,516	0,516

Table D3 OD_{595} of WT ES+ lysate (20×) in Bradford assay.

Each biological replicate from flask culture had one triplicate.

	Triplicate of biological replicate 1, ES+			Triplicate of biological replicate 2, ES+			Triplicate of biological replicate 3, ES+		
WT	0,527	0,541	0,536	0,471	0,461	0,465	0,501	0,508	0,501

WT + pF*gusA*	0,500	0,495	0,482	0,451	0,451	0,440	0,512	0,531	0,521
WT + pI*gusA*	0,426	0,432	0,425	0,535	0,546	0,528	0,468	0,503	0,503
WT + pX*gus A*	0,435	0,424	0,434	0,489	0,505	0,502	0,476	0,450	0,492
WT + pJ*gusA*	0,557	0,572	0,578	0,517	0,546	0,558	0,561	0,607	0,576
WT + p8*gusA*	0,508	0,502	0,512	0,477	0,484	0,481	0,451	0,456	0,463

Table D4 OD_{595} of DD ES- lysate (20×) in Bradford assay.

Each biological replicate from flask culture had one triplicate.

	Triplicate of biological replicate 1, ES-			Triplicate of biological replicate 2, ES-			Triplicate of biological replicate 3, ES-		
DD	0,519	0,516	0,492	0,517	0,552	0,516	0,498	0,484	0,479
DD + pF*gusA*	0,507	0,540	0,517	0,446	0,456	0,446	0,530	0,531	0,525
DD + pI*gusA*	0,530	0,502	0,524	0,520	0,526	0,583	0,518	0,554	0,546
DD + pX*gus A*	0,526	0,562	0,553	0,493	0,493	0,488	0,470	0,451	0,464
DD + pJ*gusA*	0,558	0,564	0,544	0,484	0,509	0,485	0,541	0,524	0,539
DD +	0,445	0,449	0,453	0,459	0,473	0,461	0,510	0,502	0,525

p8*gusA*									

Table D5 OD_{595} of DD ES+ lysate (20×) in Bradford assay.

Each biological replicate from flask culture (each member in triplicate of GUA assay) had one triplicate.

	Triplicate of biological replicate 1, ES+			Triplicate of biological replicate 2, ES+			Triplicate of biological replicate 3, ES+		
DD	0,453	0,471	0,466	0,431	0,426	0,427	0,428	0,437	0,432
DD + pF*gusA*	0,558	0,549	0,560	0,462	0,469	0,470	0,454	0,470	0,456
DD + pI*gusA*	0,492	0,481	0,492	0,486	0,479	0,492	0,389	0,407	0,384
DD + pX*gusA*	0,523	0,541	0,558	0,463	0,498	0,494	0,419	0,426	0,425
DD + pJ*gusA*	0,510	0,516	0,534	0,558	0,581	0,585	0,538	0,631	0,647
DD + p8*gusA*	0,515	0,562	0,542	0,525	0,547	0,524	0,545	0,563	0,545

Table D6 OD_{420} and OD_{550} of WT ES-/+ in GUS assay at '30min'.

'OVER' means the OD value exceeded the measurement limitation, and was not calculated for the final results.

		OD_{420}			OD_{550}		
0.1 ml lysate	WT	0,0477	0,0612	0,0898	0,0378	0,0677	0,0864
	WT + pF*gusA*	1,9674	1,9286	1,8742	0,0804	0,0802	0,0805

	WT + p*IgusA*	0,1351	0,1142	0,0443	0,0633	0,0612	0,0385
	WT + p*XgusA*	0,3713	0,3711	0,3690	0,0697	0,0603	0,0381
	WT + p8*gusA*	OVER	OVER	OVER	0,0868	0,0869	0,0884
0.5 ml lysate	WT + p*JgusA*	0,3426	0,4509	0,5831	0,0413	0,0778	0,1066
0.1 ml lysate	WT	0,0565	0,0661	0,0998	0,0437	0,0733	0,0927
	WT + p*FgusA*	3,0930	3,6657	3,2081	0,0838	0,0845	0,0822
	WT + p*IgusA*	0,1480	0,1685	0,2503	0,0711	0,0728	0,0768
	WT + p*XgusA*	0,5979	0,4150	0,4406	0,0736	0,0629	0,0406
	WT + p8*gusA*	OVER	OVER	OVER	0,0889	0,0862	0,0909
0.5 ml lysate	WT + p*JgusA*	0,3008	1,1078	0,5469	0,0435	0,0635	0,1093

Table D7 OD_{420} and OD_{550} of DD ES-/+ in GUS assay at '30min'.

'OVER' means the OD value exceeded the measurement limitation, and was not calculated for the final results.

			OD_{420}			OD_{550}		
30min, 17sec, ES-	0.1 ml lysate	DD	0,0551	0,0633	0,0952	0,0442	0,0733	0,0909

		DD + pF*gusA*	0,6094	OVER	0,9617	0,086	0,0838	0,0869
31min, 49 sec, ES-	0.1 ml lysate	DD + pI*gusA*	0,0784	0,1185	0,2592	0,0398	0,0651	0,0965
		DD + pX*gusA*	0,2475	0,4435	0,5983	0,0864	0,0877	0,0908
		DD + p8*gusA*	OVER	OVER	OVER	0,0752	0,0675	0,0395
	0.5 ml lysate	DD + pJ*gusA*	0,1511	0,1658	0,1315	0,0896	0,1004	0,0923
33min, 20sec, ES+	0.1 ml lysate	DD	0,0479	0,0623	0,0929	0,0382	0,0683	0,0914
		DD + pF*gusA*	1,9175	1,814	2,114	0,0879	0,0862	0,0871
		DD + pI*gusA*	0,1049	0,1056	0,1279	0,0705	0,0688	0,0719
		DD + pX*gusA*	0,4442	0,5327	0,4346	0,0753	0,0617	0,0383
		DD + p8*gusA*	0,0667	0,1061	0,1411	0,0425	0,0797	0,1172
	0.5 ml lysate	DD + pJ*gusA*	OVER	OVER	OVER	0,0899	0,0924	0,0998

Table D8 OD_{420} and OD_{550} of WT ES-/+ in GUS assay at '67min'.

'OVER' means the OD value exceeded the measurement limitation, and was not calculated for the final results.

			OD_{420}			OD_{550}		
66min, ES-	0.1 ml lysate	WT	0,0473	0,0617	0,0889	0,0382	0,072	0,0862

		WT + pF*gusA*	3,3824	3,3006	3,243	0,0774	0,0765	0,0787
		WT + pI*gusA*	0,1929	0,1565	0,0446	0,064	0,0627	0,0386
		WT + pX*gusA*	0,6324	0,6352	0,6611	0,0669	0,0607	0,0378
		WT + p8*gusA*	OVER	OVER	OVER	0,0885	0,0878	0,0899
	0.5 ml lysate	WT + pJ*gusA*	0,5974	0,7722	0,9875	0,0414	0,0809	0,1069
66min, ES+	0.1 ml lysate	WT	0,0574	0,0721	0,1004	0,0435	0,0812	0,0928
		WT + pF*gusA*	3,867	3,9389	OVER	0,0821	0,0849	0,0823
		WT + pI*gusA*	0,232	0,2734	0,4508	0,0744	0,0759	0,0756
		WT + pX*gusA*	1,2053	0,8251	0,9134	0,0714	0,0653	0,0404
71min, ES+	0.1 ml lysate	WT + p8*gusA*	OVER	OVER	OVER	0,0867	0,0889	0,0907
	0.5 ml lysate	WT + pJ*gusA*	0,6054	2,2926	1,0494	0,0416	0,0763	0,1078

Table D9 OD_{420} and OD_{550} of DD ES-/+ in GUS assay at '67min'.

'OVER' means the OD value exceeded the measurement limitation, and was not calculated for the final results.

			OD_{420}			OD_{550}		
67min, ES-	0.1 ml lysate	DD	0,0582	0,066	0,096	0,0486	0,075	0,0912

		DD + pF*gusA*	1,3566	OVER	2,1639	0,0875	0,0868	0,0888
68min, ES-	0.1 ml lysate	DD + pI*gusA*	0,1157	0,1936	0,4813	0,0405	0,0701	0,0967
		DD + pX*gusA*	0,4708	0,9364	1,2869	0,0867	0,0918	0,0914
		DD + p8*gusA*	OVER	OVER	OVER	0,0864	0,0969	0,0907
	0.5 ml lysate	DD + pJ*gusA*	0,2129	0,23	0,1669	0,0894	0,0732	0,0393
67min, ES+	0.1 ml lysate	DD	0,048	0,0613	0,0965	0,0383	0,0682	0,0928
		DD + pF*gusA*	3,5688	3,4866	3,8616	0,0864	0,0846	0,085
		DD + pI*gusA*	0,1417	0,144	0,1921	0,0716	0,0712	0,0746
		DD + pX*gusA*	0,8761	1,0849	0,9031	0,0782	0,0618	0,04
		DD + p8*gusA*	OVER	OVER	3,904	0,0876	0,0904	0,097
	0.5 ml lysate	DD + pJ*gusA*	0,0854	0,1287	0,1571	0,0442	0,0792	0,1131