NTNU – Trondheim
Norwegian University of
Science and Technology

Re-wiring of antibiotic biosynthesis regulation in Streptomyces venezuelae for inducible heterologous production of secondary metabolites

Joanna Pawlikowska

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Supervisor: Sergey Zotchev, IBT
Co-supervisor: Olga Sekurova, IBT

Norwegian University of Science and Technology
Department of Biotechnology

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Table of contents

Abbreviations

Am	apramycin
antiSMASH	Antibiotics & Secondary Metabolite Analysis SHell
bp	base pair
Cml	chloramphenicol
CPEC	Circular Polymerase Extension Cloning
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dsH2O	distilled sterile water
DTT	dithiothreitol
dTTP	thymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
GAS	Group A *Streptococcus*
gDNA	Genomic DNA
Glu	glutamic acid
GUS	β-Glucuronidase
jad	jadomycin
Kan	kanamycin
kb	kilo base pairs
LA	Luria Agar medium
LB	Luria Broth medium
LiAc	lithium acetate
LL-DAP	LL isomer of diaminopimelic acid
MAGIC	Mating-Assisted Genetically Integrated Cloning

Mb	Megabase
MRSA	Methicillin-resistant Staphylococcus aureus
NAD	nicotinamide adenine dinucleotide
Nal	nalidixic acid
OD	Optical Density
OmpR-type ARR	OmpR-type "atypical" response regulator
oriT	origin of transfer
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
PIPE	Polymerase Incomplete Primer Extension Cloning
PKSII	type II polyketides synthase
reg	Regulator
SBSPKS	Structure Based Sequence Analysis of Polyketide Synthases
Ser	Serine
SLIC	Sequence and Ligase Independent Cloning
SLiCE	Seamless Ligation Cloning Extract
ss-carrier	single-stranded carrier DNA
SSRTA	Site-Specific Recombination-based Tandem Assembly
TMS1	trace elements for PM4-1 medium
UPLC	Ultra high Performance Liquid Chromatography
USER	Uracil-Specific Excision Reagent cloning
UTI	urinary tract infection
VRE	Vancomycin-Resistant *Enterococcus*
WT	wild type
YAC	Yeast Artificial Chromosome

Abstract

Streptomyces venezuelae ATCC10712 which is an object of research investigation during this master thesis belongs to *Actinomycetales* which are widely recognized as a producer of antibiotics. Secondary metabolites synthesis in *S. venezuelae* varies depending from the conditions of incubation. If stress factors like ethanol are present in the medium, microorganism switches to production of jadomycin. In normal conditions however, nutrient depletion leads to subsequent synthesis of chloramphenicol. Regulation of aforementioned compounds is precisely, internally regulated by the set of adjacent genes. *jadR1* and *jadR2* were identified as genes having major influence in these pathways.

The idea of this project was to re-engineer the antibiotic biosynthesis regulatory circuit of chloramphenicol and jadomycin in *S. venezuelae*, in order to make recombinant strain's chloramphenicol production dependent on ethanol stress. Following steps took place to achieve this goal.

Firstly, construction of recombinant strain with deletion of *jadJ-E* structural genes from *S. venezuelae* chromosome and their replacement with a reporter gene *gusA*. Another assembly was constructed with the aim of replacing *cmlI-X* promoter in deletion mutant with *jadR1-jadJ* promoter region in the chloramphenicol biosynthetic gene cluster. Planning was based on the fact that since those genes are under negative regulation of JadR1 regulator, exchange of the promoter will lead to its activation and subsequent chloramphenicol biosynthesis. Due to the difficulties which arose from the mutations in *gusA* gene, additional assembly with the deletion of of *jadJ-E* genes in *S. venezuelae* with another version of *gusA* region had to be constructed.

Chloramphenicol production was assessed in *S. venezuelae* mutant carrying substituted promoter by UPLC analysis. Results revealed 5 fold increase of chloramphenicol expression under ethanol shock in recombinant strain, in comparison with wild-type *S. venezuelae* in the same conditions. When uninduced, mutant produced no chloramphenicol. This experiment was the main objective of this master thesis.

In addition, construct for heterologous expression of chloramphenicol antibiotic gene cluster in *Streptomyces albus* was assembled. Strain was assessed for the production of this antibiotic by UPLC analysis.

Introduction

I. The discovery, biology and classification of Actinomycetes

Actinomycetes were firstly described in 1877 as a causative agent of "lumpy jaw" disease which was found in cattle. Symptoms were manifesting in the form of severe swellings on the side of the face caused by proliferation and unnatural deviation of the bone. After certain period of time this resulted in an animal's difficulty to eat. Carl Otto Harz (1842-1906) was the first one to elucidate the morphological structure of the causative organism. Mistakenly, due to similar appearance, he thought it was a fungus. For this reason he called it *Actinomyces* which means "ray fungus". In 1875 Ferdinand Cohn (1828-1898) published his observations of a range of microbes. He included one called *Streptothrix foesteri* where streptothrix means "twisted hair". Second part of the name originates from his friend, R. Foerster who provided material for his research. In 1916, R.E. Buchanan recognized officially group *Actinomycetales*. Due to multiplicity of the names, classification of this group was very problematic at the beginning. In 1943 another attempt to classify Actinomycetes has been done by S. Waksman and Henrici. The degree of branching was used as a main criterion to divide organisms into three groups. Two of them were subdivided afterwards, what resulted in five genera altogether. Moreover, *Streptomyces* ("twisted fungus") name emerged and replaced previous one – *Streptothrix*. Results of their work are presented in Figure 1.

Established classification was based on morphology of the microorganism. Relationships between species however still remained unresolved. Until later, when Waksman decided to research soil microorganism, Actinomycetes seemed not to be considered a group of interest. The key observation which led to further findings was that disease-causing bacteria survive in a soil only for a short period of time. Further on, he assumes that this has to happen due to soil microbes which are actively destroying them.

Term antibiotic was invented by P. Vuillemin in 1889. At this time, it was described as a destruction of one organism by the other. Waksman proposed to modify it with the respect to the origin of the compound – synthetic or natural. In this way, following description emerged: "chemical substances that are produced by microorganism and that have the capacity, in dilute solution, to selectively inhibit the growth or even to destroy other microorganism".

Sometime after the discovery of Penicillin it became known that it is influencing only gram-positive bacteria. For this reason, Waksman decided to focus on the other group of microorganisms – gram-negative ones. As he committed his life to finding new antibiotics, he started screening different soil microorganisms. First test group consisted of three of them: bacteria, fungus and actinomycetes. Last one turned out to be amazingly productive, with 106 strains out of 244 which exhibited antibiotic properties. Year 1943 happened to be a breakthrough in Waksman's research. One of his graduate students, Albert Schatz discovered an antibiotic produced by a strain of *Streptomyces griseus* which was effective both against gram-negative pathogens and also tubercule bacillus. This led Waksman to be awarded with Nobel Prize for Physiology or Medicine in 1952 (Hopwood, 2007).

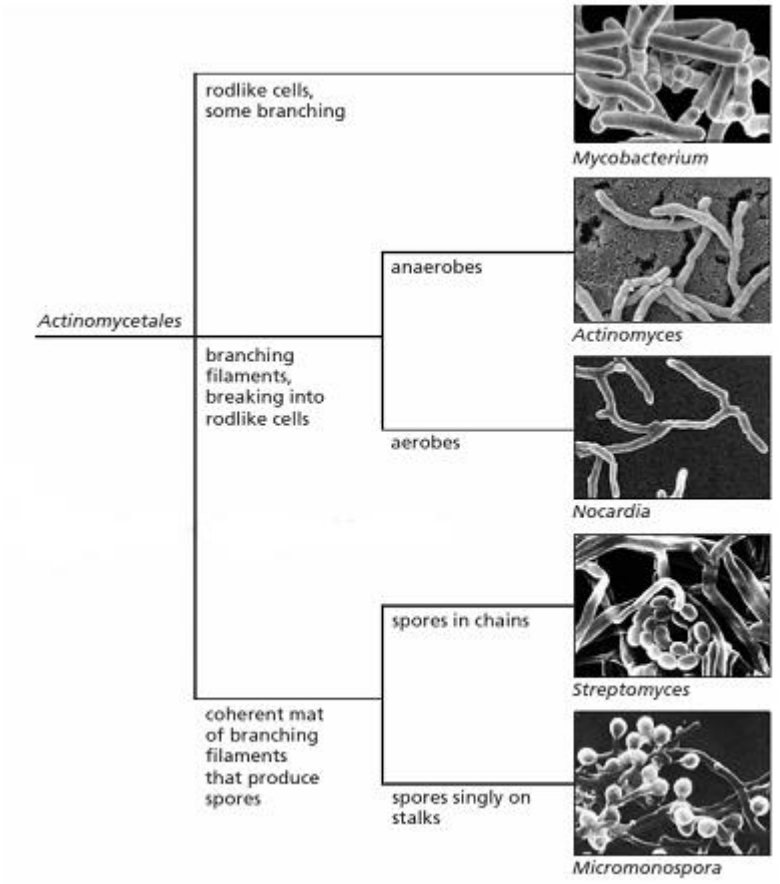


Figure 1. Classification of the actinomycetes by Waksman and Henrici. Scanning electron micrographs (Hopwood, 2007).

1. Actinomycetes

As it was mentioned before, actinomycetes belong to prokaryotes. Their genomes are approximately 8-10 Mb in size, and they contain 20-30 gene clusters encoding secondary metabolites (Siegl and Luzhetskyy, 2012). Actinomycetes were isolated from many natural habitats such as soil, marine and lake water, insects, sand, pollen grain (Mahajan and Balachandran, 2012).

Gram-positive bacteria can be subdivided into following groups: low G+C organisms – which include for instance: *Bacillus, Clostridium, Staphylococcus* and *Streptococcus* and high G+C organisms – actinomycetes. Latter ones tend to develop mycelia which is considered to be a hallmark of actinomycetes. Formation of branched threads and rods comprise characteristics of their growth. They frequently give a rise to unicellular mycelium. Mycelium may be of two types: vegetative – growing in the substrate or aerial – which means, that it is produced above the vegetative growth. Hyphae, which are generally not-separate may dissociate under special conditions. Strains of *Streptomyces* are characterized by the presence of aerial mycelium. Reproduction of actinomycetes involves either special sporulating bodies or parts of vegetative mycelium (Mahajan and Balachandran, 2012). It is important to note however, that some actinomycetes do not form aerial mycelium, and do not sporulate. Thus, the most important taxonomic tool in classification of actinomycetes is a partial sequence analysis of 16S ribosomal RNA (Kieser et al., 2000).

2. *Streptomyces*

Microorganism which was an object of this master thesis is *Streptomyces venezuelae* ATCC10712.

Streptomyces contain large linear chromosome and were identified by presence of LL isomer of diaminopimelic acid (LL-DAP) as the diamino acid in the peptidoglycan. This feature combined with characteristic substrate and aerial mycelium was a diagnostic feature of *Streptomyces* (Dyson, 2009; Kieser et al., 2000).

Morphological development of *Streptomyces* is a quite complex process which includes four phases: mycelial growth, multicellular behaviour, intercellular communication and morphological differentiation. Life cycle ends with production of spores, as presented in Figure 2.

When the spore is placed in an optimal environment, it starts to germinate. This includes swelling of the spore, establishment of the call polarity and apical growth. In addition, it leads to the outgrowth of one or more hyphae. The hyphae grow into mycelia networks by tip extension and branching. Due to this networks *Streptomyces* reminiscence more filamentous fungi than other bacteria (McCormick and Flärdh, 2012).

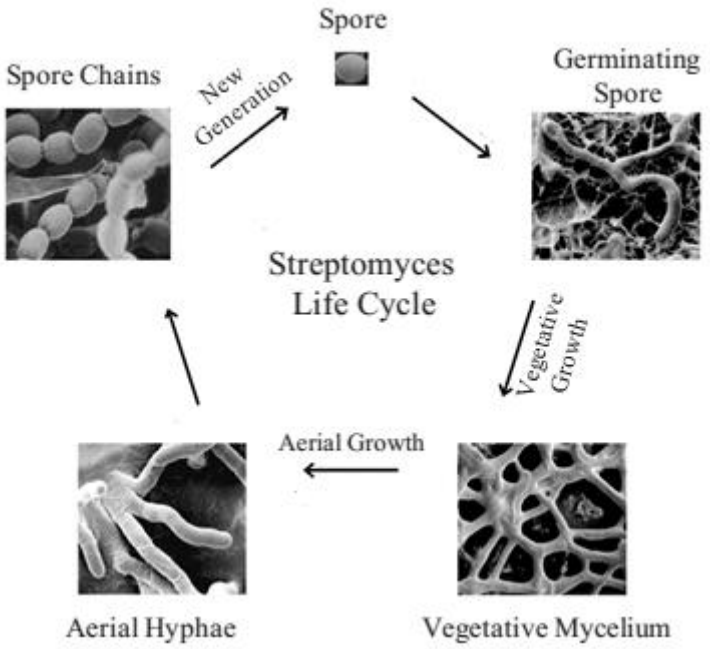


Figure 2. Development of Streptomyces (Brooks et al., 2012).

Streptomyces produce antibiotics in the soil which makes them potential antagonists for soil-borne pathogens. Production of antibiotics appears to be growth phase-dependent. In liquid culture, biosynthesis starts as culture enters stationary phase. In solid agar plates, it starts with the stage of morphological differentiation. Antibiotics are produced as a result of expression of specific gene clusters (Kieser et al., 2000).

II. Antibiotics

Up to date definition of antibiotics runs as follows: substances produced as a secondary metabolites by many bacterial, fungal and some animal species. They are not required for a normal growth of the organism. They possess antibiotic activity that is one which inhibits the growth or kill other living organism (Behal, 2000).

The function of these compounds is undergoing an extensive debate. Some say, they inhibit the growth of competing organisms in order to allow others take an advantage from surrounding environment (Zotchev, 2012). On the other hand, many researchers think that this is rather form of a defence of a colony biomass against overgrowth by other organism during the autolysis at the certain stage of development (Chater et al., 2010). Concentration of antibiotics is however very low in natural conditions, which may imply the other alternatives. Antibiotics are known to modulate gene expression in bacteria in subinhibitory concentrations (Zotchev, 2012). This may imply role as a signalling molecules which provide ways of communication between different species. In addition, microorganisms might control the development of themselves by producing signals promoting aerial growth and sporulation or inhibitors of spore germination. In symbiotic interactions, antibiotics protect a symbiotic partner against infection by other microorganism (Chater et al., 2010).

Production of antibiotics is species specific. *Actinomycetales* produce over 60% of all compounds with antibiotic activity (Zotchev, 2012).

1. Discovery of Penicillin

Development of antibiotics started with discovery of penicillin. In summer 1928 Alexander Fleming who was a physician and bacteriologist at Inoculation Department at St. Mary's Hospital in London came back from long holidays and to his surprise noticed large splotch of mould on the plate which he previously inoculated with staphylococcus bacteria. The fact wouldn't be anything weird except that there was significant growth inhibition zone of growing staphylococci around the mould. Due to his previous work on disintegration effect of lysosyme on bacteria, Fleming recognized growth inhibition phenomenon. After closer investigation he discovered that the mould belongs to the type of *Penicillium* and besides staphylococci, affects whole range of other bacteria. This research led to publication about substance called "penicillin" in 1929. Discovery of penicillin was connected with whole series

of lucky coincidents. Firstly, it turned out that the spore of mould didn't come from the random spore happened to drift around in the air. It appears it came from the laboratory of mycologist – C. J. La Touche which was located just on the floor below. Also, since penicillin exerts the effect only on growing bacteria, arrival of the spore, timing of Fleming's vacation and local weather at this time put a mark on this remarkable discovery. Due to the difficulties on the way connected with lack of knowledge and problems with repetition of the experiment, penicillin research was dropped until the decade after. In late 1930s Ernst Chain and Howard Florey stumbled upon penicillin while researching the effects of lysozyme on disintegration of cell wall in bacteria. Due to his biochemistry background Chain managed to produce a small amount of concentrated penicillin which started miraculously curing people without evoking any toxic effect. With the time and other fortunate incidents production was upscaled and in 1944 there was enough penicillin to treat masses of people (Queijo J., 2010).

2. Classification of antibiotics

Since Fleming times, several classes of antibiotics have been developed. Representatives of each along with their bioactivity and mechanism of action are summarized in the table below.

Table 1. Classification of antibiotics (Coates et al., 2011; Moore, 2013).

Class	Representatives	Biological activity	Mechanism of action
β-lactamase inhibitors	clavulanate	*Staphylococcus aureus; Staphylococcus epidermis; Escherichia coli; Klebsiella;*	Cell wall synthesis inhibition
β-lactams (Penicillins)	penicillin G	Gram-positive bacteria	Cell wall synthesis inhibition
Aminoglycosides	gentamycin	Aerobic Gram-negatives; *Enterobacteriaceae; Pseudomonas;*	Protein synthesis inhibitors (inhibit 30S subunit)
Angucyclines	jadomycin	Gram-positive; Gram-negative bacteria; Yeast; Antifungal; Antitumor;	Exact mechanism not elucidated yet
Bacitracin	bacitracin	Topical Gram-positive	Cell wall

		infections	synthesis inhibition
Carbapenems	imipenem	Very broad activity (except MRSA, *Mycoplasma*)	Cell wall synthesis inhibition
Cephalosporins	*First generation*: cephalothin	*S. aureus;* *S. epidermidis;* Some Gram-negatives: *E. coli;* *Klebsiella;*	Cell wall synthesis inhibition
	Second generation: cefprozil	Above + ↑ Gram-negative	
	Third generation: cefotaxime	Above + ↑ Gram-negative *Pseudomonas*	
	Fourth generation: cefpirome	Above + ↑ Gram-negative *Bacteroides spp.;* *Haemophilus influenzae* *Moraxella catarrhalis;* *Morganella morganii;* *Providencia rettgeri;* *Serratia spp.;* *Streptococcus pneumonia;* *Streptococcus pyogenes;*	
	Fifth generation: ceftaroline	Above + ↑ Gram-negative + resistant phenotypes	
Chloramphenicol	chloramphenicol	*H. influenza;* Bacterial meningitis; Brain abscess;	Protein synthesis inhibitors (inhibit 50S subunit)
Glycopeptides	vancomycin	MRSA; Penicillin/Cephalosporin allergies *S. aureus;* *S. epidermidis;*	Cell wall synthesis inhibition
Ketolides	telithromycin	Gram-positive bacteria; some intercellular pathogens;	Protein synthesis inhibitors (inhibit 50S subunit)
Lincosamides	clindamycin	*Bacteroides fragilis;*	Protein synthesis

		S. aureus; Coagulase-negative *Staphylococcus & Streptococcus;* Protozoa;	inhibitors (inhibit 50S subunit)
Lipopeptides	daptomycin	Gram-positives	Disruption of the cell membrane
Macrolides	erythromycin	*Streptococcus; H. influenza; Mycoplasma pneumoniae;*	Protein synthesis inhibitors (inhibit 50S subunit)
Monobactams	aztreonam	Aerobic Gram-negatives	Cell wall synthesis inhibition
Oxazolidinones	linezolid	Resistant Gram-positives	Protein synthesis inhibitors (inhibit 50S subunit)
Polimyxins	polimyxin B	Topical Gram-negative infections	Disruption of the outer membrane
Quinolones	nalidixic acid	*Steptococcus; Mycoplasma;* Aerobic Gram-positive;	DNA Synthesis Inhibitors
Rifamycins	rifampicin (also called rifampin)	*Staphylococcus; Mycobacterium tuberculosis;*	RNA synthesis Inhibitors
Streptogramins	quinupristin	VRE; GAS; *S. aureus* skin infections;	Protein synthesis inhibitors (inhibit 50S subunit)
Sulphonamides	sulfamethoxazole	UTI organisms; *Proteus; Enterobacter;*	Folic Acid synthesis inhibitors
Tetracyclines	tetracycline	*Rickettsia; Mycoplasma; Spirochetes;*	Protein synthesis inhibitors (inhibit 30S subunit)

3. Chloramphenicol

Chloramphenicol is a compound which was isolated from *Streptomyces* species from a soil sample obtained from a field near to Caracas. Due to the place where it has been found, bacterium got the name *Streptomyces venezuelae*.

Chloramphenicol is a small molecule, poorly soluble in water. It exists in four possible stereoisomers but only one of them possesses antibacterial activity.

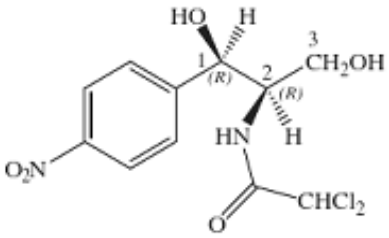


Figure 3. Chloramphenicol structure (Anderson et al., 2012).

Experiments have shown that chloramphenicol has very broad spectrum of action. It exhibits both bacteriostatic and bactericidal activity against various strains of gram-positive and gram-negative bacteria, spirochetes and obligatory intracellular pathogens like mycoplasma or chlamydiae. In the treatment of typhoid it is considered as a drug of a choice (Mahajan and Balachandran, 2012).

Mechanism of action involves binding to the large ribosomal subunit – 50S at the peptidyl transferase centre A site. In this way it prevents binding next charged tRNA and overall inhibits protein synthesis. It appears that chloramphenicol binds very selectively. The reason for that may be difference in conformation between bacterial and eukaryotic peptidyl transferase. Experiments have shown that it binds only to bacterial ribosome (Anderson et al., 2012).

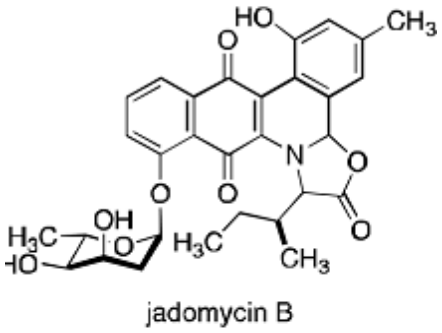
It was firstly released on the market as Chloromycetin. Nowadays it is manufactured completely synthetically. It is sold as a chloramphenicol palmitate ester which is its inactive form. Hydrolysis in the gastrointestinal tract allows it to be absorbed as a free molecule (Mahajan and Balachandran, 2012).

Due to the risk of developing serious haematological effects, systemic use of chloramphenicol is highly restricted. It is used only in life-threatening infections caused particularly by

Haemophilus influenza. Topical use of chloramphenicol is quite common, especially in the superficial eye infections (Anderson et al., 2012).

4. Jadomycin

Jadomycins are a class of angular tetracyclines which is called angucyclines. They are also considered as members of type II polyketides (PKSII) produced by *S. venezuelae* (Cossy and Arseniyadis, 2012). On the contrary to chloramphenicol, jadomycin is produced only under specific conditions i.e. induction by heat shock, ethanol stress or phage infection.



jadomycin B

Figure 4. Jadomycin B structure (Sharif and O'Doherty, 2012).

They consist of 8H-benzo-[b]-phenanthridine backbone with fused nitrogen and oxygen in various combinations (Sharif and O'Doherty, 2012). PKSII-derived natural product contains five-membered oxazolone ring. Formation of oxazolone ring happens via reaction of amino acid from the culture medium with biosynthetic aldehyde precursor. This results in reactive aldimine which undergoes several latter reactions (Jakeman et al., 2009). Jadomycin B which is a focus in this thesis is glycosylated with a L-digitoxose sugar unit. Glycosylation exerts its role in the bioactivity of the compound formed (Sharif and O'Doherty, 2012).

Jadomycin biosynthesis happens in nutrient-deprived amino acid rich medium. The way to achieve this condition is exhaustion of carbon, nitrogen or phosphate from the medium together with supplying it with amino acids. Production is induced by the factors mentioned before like ethanol and heat shock (Brooks et al., 2012).

Depending on the amino acid provided in the medium, jadomycin has several analogues B, L, F, G, Y, N. Different types arise from aromatic, polar, aliphatic α-L-amino acids and α-D-

amino acids. Incorporation of such a novel non-natural amino acids broadens the potential of the secondary metabolites (Jakeman et al., 2005).

Jadomycins are broad spectrum antimicrobials, and were found to be active against gram-positive, gram-negative bacteria and yeast. However, they tend to give better effect against gram-negative microbes. Additionally, it was proven that they demonstrate antitumor, antifungal, enzyme inhibitory and cytotoxic activity to cancer cells (Brooks et al., 2012; Rix et al., 2004).

III. Regulation of antibiotic gene clusters

1. Antibiotic biosynthesis gene clusters and its regulation

Microorganisms are very rich reservoir of antibiotics and other biologically active secondary metabolites. Function of many of them is still unknown. Before sequencing and analysis of *Streptomyces coelicolor* genome in 2002, many thought natural sources for discovery of new compounds might become depleted and finding new ones may be excessively difficult. . This was mainly induced by the high rediscovery rate of compounds which were already known. With the development of sequencing technology i.e. in 2002, researchers came across amazingly high numbers of chemical diversity encoded in antibiotic clusters which were not detected before. It turned out that each strain has a potential to produce 20-40 distinct secondary metabolites. Reason for not noticing them before could be unstable, no or very low expression level, undetectable with up-to-date methods. Need for understanding rich actinomycetes genomes promoted development of new approaches and tools. This was a beginning of a new era for bioprospecting. Efforts of the researchers started to be redirected from environmental screening and rather focused on studies on regulation mechanisms. It was discovered that stress responses like heat or ethanol shock may induce expression of silent gene clusters. An example of this type of regulation is production of jadomycin B from *S. venezuelae* (Zotchev et al., 2012; Rebets et al., 2014; Yoon and Nodwell, 2014). In addition, Brian Bachmann and John McLean, biochemists at Vanderbilt University discovered while investigating development of bacterial drug resistance that many bacteria in response to antibiotic assault express hundreds of compounds which are not produced by their wild types. Some of them have a potential to be a novel secondary metabolites (Derewacz et al., 2013). Cryptic antibiotic clusters are detected with the help of several technologically-advanced bioinformatic tools like antiSMASH or SBSPKS software which allows localizing them in the organisms like bacteria and fungi. In addition, it provides information about cluster abundance and potential produced substance, which may facilitate estimation of probability of discovery a novel compound. Finding a cluster of interest is followed by wet-lab approaches aiming its activation by inactivation of a repressor, overexpression of positive regulator or heterologous expression. Modern synthetic biology approaches helping in achieving this goal will be described in the section Synthetic biology and its possible applications in *Streptomyces* bacteria (Zotchev et al., 2012; Rebets et al., 2014; Yoon and Nodwell, 2014).

2. Molecular regulation of antibiotic biosynthesis in *Streptomyces venezuelae*

As it was mentioned before, *S. venezuelae* produces two antibiotics – chloramphenicol and jadomycin. While first one is produced in standard conditions, jadomycin biosynthesis is induced through environmental stress factor like for example 6% ethanol addition in culture medium. In order to control this mechanism in an efficient way, *S. venezuelae* developed very efficient regulatory system of cross-regulation in an antagonistic mode. Jadomycin cluster is consisted of five regulatory genes: *jadW1,-W2,-W3,-R2,* and *-R1* (Wang et al., 2009). Main molecules which govern its regulation are JadR1 and JadR2. *jadR1* is located upstream of *jadJ* which comprises first structural gene from jadomycin biosynthesis cluster. It contains winged-like helix-turn-helix motif at C-terminal output domain and two aspartic acid residues substituted by Glu49 and Ser50 in N-terminal receiver domain (Liu et al., 2013). In addition to that, JadR1 is an OmpR-type ARR ("atypical" response regulator). This means that it is a part of two-component transduction system, typically comprised from sensor histidine kinase and cognate response regulator. It is described as "atypical" due to decreased number of conserved residues in N-terminal receiver domain. Interestingly, determinants of such systems are located in a considerable distance from their histidine kinase gene (Wang et al., 2009). Regulatory genes *jadW1, jadW2* and *jadW3* appear to exert very strong amino acid sequence similarity when compared to gene products connected with regulation of morphological differentiation as well as secondary metabolism through γ-butyrolactones in many *Streptomyces* species (Kharel et al., 2011).

In order to start biosynthesis from the cluster, JadR1 has to sense signalling molecules. It occurs due to the fact that JadR1 can't be phosphorylated. When concentration of jadomycin B is low in the environment, JadR1 activates the expression of the antibiotic from the cluster. On the contrary however, high concentration causes JadR1 to dissociate from target promoter. Concluding, activation of the jadomycin cluster is autoregulated in dose-dependent manner. In addition to that, it appears that JadR1 is involved at the same time in repression of chloramphenicol by binding to an extended sequence in this antibiotic cluster. Place of binding is located downstream to the transcription start site of *cmlJ*.

jadR2 which is a gene adjacent to *jadR1* inhibits its transcription. As a result, during the repression of JadR1 by JadR2, chloramphenicol biosynthesis is turned on. In order to release

JadR1, jadomycin or chloramphenicol has to bind to JadR2. This makes JadR2 to have a role of biosensor. Same effect can be obtained by ethanol shock mentioned in the previous paragraph. Unfortunately mechanism behind this phenomenon was not elucidated yet. Finally, accumulation of JadR1 leads to repression of chloramphenicol and activation of jadomycin synthesis. In the case of deletion mutant with jadR2, jadomycin B expression is switched on in the absence of normally, compulsory stress factors. On the other hand, production of chloramphenicol is repressed at the same time (Liu et al., 2013).

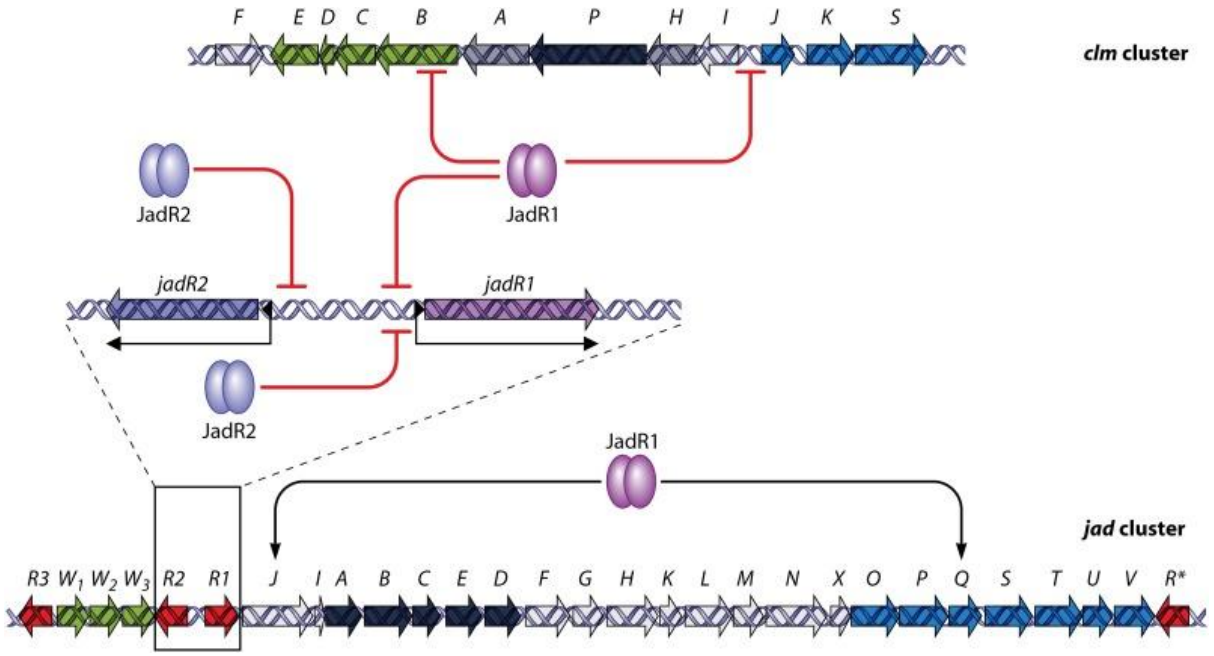


Figure 5. Scheme presenting cross-coordination of different antibiotic biosynthetic pathways in *S. venezuelae* (Liu et al., 2013).

Table 2. Jadomycin B biosynthetic genes and proposed functions (Kharel et al., 2011).

Gene	Function	Gene	Function
jadW1	Regulator	jadK	Hydrolase
jadW2	Regulator	jadL	Efflux pump
jadW3	Regulator	jadM [2]	Phosphopantetheinyl transferase

jadR2	Transcriptional reg.	jadN	Malonyl-coenzyme A decarboxylase
jadR1	Transcriptional reg.	jadX	Unknown
jadJ [2]	Acetyl-coenzyme A carboxylase	jadO	NDP-hexose-2,3-dehydratase
jadI	Cyclase	jadP	NDP-hexose-3-ketoreductase
jadA[1]	Ketoacyl synthase	jadQ	NDP-glucose synthase
jadB[1]	Chain length factor	jadS	Glycosyltransferase
jadC[1]	Acyl carrier protein	jadT	NDP-hexose-4,6-dehydratase
jadE[1]	Ketoreductase	jadU	NDP-hexose-5-epimerase
jadD[1]	Cyclase/Dehydratase	jadV	NDP-hexose-4-ketoreductase
jadF	Oxygenase/Dehydratase	jadR*	tetR homologue
jadG	Anthrone oxygenase	jadY	Unknown
jadH	Oxygenase/Dehydratase	jadZ	Unknown

[1]PKS genes

[2]genes required for biosynthesis of jadomycin B

3. γ-butyrolactones

γ-butyrolactones are signalling molecules in *Streptomyces* regulating secondary metabolism and morphological differentiation. They belong to a family of TetR regulators. They are synthesized in big amounts just before the growth of the organism is stopped (Wang et al., 2003). They exist in a form of ligand and cognate to it receptor. Most receptors belong to repressor proteins family. Mechanism of inhibition involves binding to receptor protein which resides in cytoplasm. Since binding of one target molecule is retarded, expression of the other is turned on (Takano, 2006). By this means they can have a significant role in cross-regulation between various antibiotic pathways (Wang et al., 2003). γ-butyrolactones molecules are

common for *Streptomyces* but they vary significantly between species. Some researchers refer to them as "pseudo" γ-butyrolactones due to the fact that on the contrary to genuine receptors, which bind only to specific γ-butyrolactones molecules, they act as signal coordinating antibiotic biosynthesis by binding and responding to different antibiotics. In this way, antibiotics facilitate intracellular signalling and induce downstream responses. In several publications they are said to resemble quorum sensing molecules. By definition, quorum sensing is a way for bacteria to translate their population density into cellular responses (Xu et al., 2010). As it was mentioned before *jadR1* and *jadR2* are involved in regulation of jadomycin synthesis. JadR1 has a role of cluster-situated regulator, while JadR2 is a pseudo γ-butyrolactone receptor. Research has proved involvement of few more genes from the same cluster. These are *jadW1, jadW2* and *jadW3*. Especially *jadW1* turned out to be one of a major importance (Wang et al., 2009). It is said to resemble by sequence *afsA* which encodes a key element for widely characterized A-factor synthesis found in many *Streptomyces* species (Bibb, 2005). Moreover, inactivation of *jadW1* has the influence on both antibiotic production and morphology. If *jadW1* is lost, *S.venezuelae* is no longer able to produce neither jadomycin nor chloramphenicol. However, when the gene is restored, production of jadomycin in mutant strain is increased 2.5 fold. The reason for that might be presence of several copies of *jadW1* gene due to multiple chromosomal integrations by shuttle vector. It is appears that *jadR2* codes for pseudo γ-butyrolactone which represses the expression of *jadW1* (Zheng et al., 2007; Kharel et al., 2011; Liu et al., 2013). *jadR2* through binding to the promoter region of *jadW1* which is coding for putative γ-butyrolactone synthase, directly shuts down its transcription. Summing up, JadR2 has two binding sites: one in the promoter region of *jadR1* and the other in *jadW1* (Wang and Vining, 2003).

IV. Synthetic biology and its possible applications in *Streptomyces* bacteria

In 1974, Polish geneticist Wacław Szybalski presented for the first time novel term of synthetic biology. Until the year 2004, when the international meeting on synthetic biology was held at MIT (USA), it did not gained much of popularity. Just after this event, synthetic biology slowly found its place in mainstream science and gained the belief of tremendous positive impact on future biotechnology prospects. By the definition, it is a multidisciplinary science joining together fields like genetics, nanotechnology, chemical engineering, robotics, system biology. Main characteristics comprise connecting basic elements of the cell which regulate the operational functionality of the microorganism to design, engineer and build new synthetic entities as well as re-engineer already existing ones. It aims to give the cells new properties and characteristics which may turn out to be beneficial for industrial application. Current synthetic biology focus evolves mainly around therapeutics, biofuel production and bioremediation (Jain et al., 2012). This chapter is committed for the review of some of the synthetic biotechnology approaches available up-to-date, with the highlights of the methods employed for the purposes of this thesis.

1. Modern methods of cloning

One of the important tools in synthetic biology are methods for DNA assembly which like the field itself are still relatively new. They aim to reconstruct natural pathways but also make new ones. Methods may be classified into those based on the homology and ligation.

Approaches based on the homology require that the neighbouring DNA fragments possess an identical sequences which allows for splicing to happen by homologous recombination *in vivo* or extension of the homologous ends *in vitro*.

Gibson assembly which was used in this master thesis is a method where DNA fragments containing homologous ends are joined together by three enzymatic reactions. Benefits of this approach are scarless, mostly sequence-independent and multi-part method allowing for one-tube isothermal reaction with big assemblies up to 300 kb. Limitations are quite expensive enzyme mix, parts smaller than 250 bp seems not suitable and secondary structures especially in the case of strains with high G+C content of the genome may impede DNA assembly. Details can be found in the Materials and Methods section.

Sequence and ligase independent cloning also referred as SLIC similarly to Gibson exploits 3'exonuclease and T4 DNA polymerase ability to chew-back 3' ends and fill the gaps. It is also a scarless and mostly sequence and ligase-independent reaction but allows only for assembly constructs up to 20 kb. Same like in the case of Gibson, secondary structures may inhibit the acquisition of final product.

PIPE stands for polymerase incomplete primer extension cloning. Its main principle is based on keeping single-stranded 3' ends by incomplete primer elongation during PCR cycle.

Uracil-specific excision reagent cloning (USER) creates single-stranded 3' ends by involvement of both uracil-containing primers and uracil-specific glycosylase and endonuclease.

With the exception of Gibson, none of the above approaches use ligase. Its function is replaced by nick-sealing *in vivo* which happens after the construct in transformed in the host of interest. In addition to that, SLIC and PIPE undergo *in vivo* also gap-filling process.

CPEC stand for circular polymerase extension cloning. Parts of assembly with homologous ends undergo cycles of denaturation, annealing and extension in order to form duplexes which after many cycles are joined together *in vivo*.

Site-specific recombination-based tandem assembly (SSRTA) uses *Streptomyces* phage φBT1 for joining the fragments together. Reaction takes place in vitro and flanking of the fragments by a set of orthogonal recombination sites are necessary. For that reason, it leaves scars which may present significant constrain. Despite of this disadvantage, method offers high specificity of φBT1 integrase and removal of polymerase extension step which avoids introducing mutations.

DNA Assembler is a technique which involves *S. cerevisiae* as a host for the assembly reaction of DNA fragments with overlapping, homologous, terminal sequences. Reaction allows for assembly and transformation in one reaction as well as selection of the homologous recombinant by incorporation of a specific marker.

Interestingly, another *in vivo* assembly example is Red recombination system, where amplification of homologous recombination in *E. coli* is caused by expression of Redαβ proteins from lambda prophage or RecET proteins from Rac prophage.

Mating-assisted genetically integrated cloning which is referred as MAGIC takes advantage of bacterial conjugation in order to transfer donor plasmid to the hosts' strain receiver plasmid. In order to generate linear fragments and enhance homologous recombination endonuclease and lambda recombinases are supplemented.

SLiCE stands for seamless ligation cloning extract which is a technique providing benefits of cheap, scarless, one-tube reaction which is in most of the cases sequence-independent and ensures high fidelity. Unfortunately, it has not been tested neither for big (more than 4kb), multi-part nor high G+C DNA assembly content.

In order to standardize cloning techniques which is one of the goals of synthetic biology, several 'genetic devices' were designed.

BioBrick assembly is one of the examples. Underlying principle is based on the restriction digest and ligation reaction to be conducted at the same time. This leads to removal of recognition sites in between the fragments but retaining those at their ends. Assembly has wide range of varieties, is cheap and simple and it is performed with the help of commercial kits. Drawbacks of the method are scars in the sequence and time consuming assembling process due to the possibility of joining only two pieces at the time.

Another approach based on the use of restriction enzymes is Golden Gate assembly and its variants. It employs type IIS restriction endonucleases which are able to bind specific recognition sites but cut in the significant distance from it. Method is quasi-scarless, offers multi-part assembly where homology of the parts is not a requirement. It is important to mark however that internal sites for the restriction enzymes should be eliminated (Zotchev et al., 2012; Cobb et al., 2014).

Assembly of bigger constructs turns out to be successful in yeast which was proved by the results in this thesis. Biosynthetic gene cluster assembly in yeast followed by transfer to *Streptomyces* host and subsequent heterologous expression was one of objects of investigation.

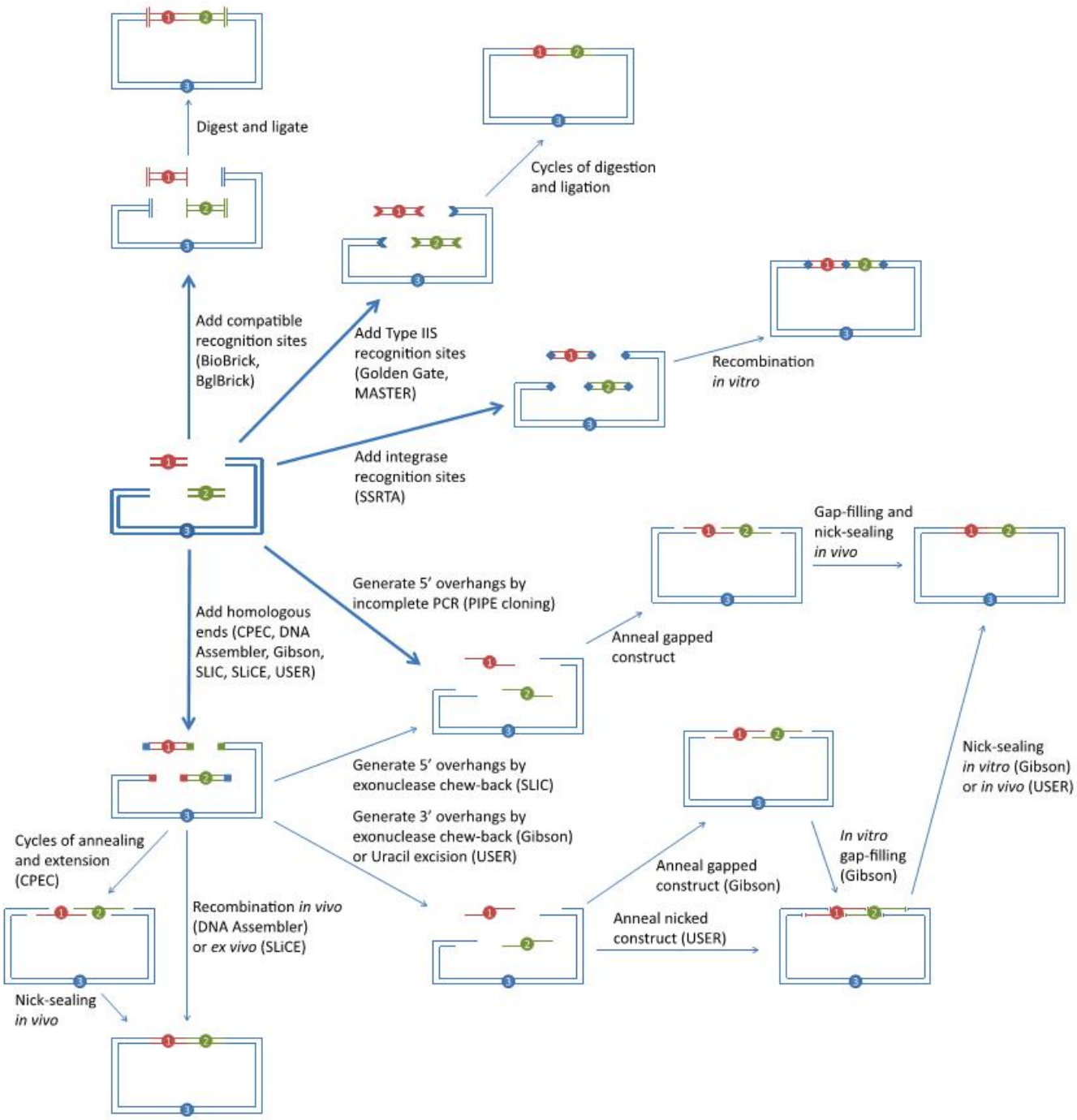


Figure 6. Modern techniques for multi-part DNA assembly (Cobb et al., 2014).

2. Reporter genes

Reporter markers are used in a bacterial genetics to study processes like gene regulation, gene expression activity, translation and transcription of operons, gene improvement and more. Their function is to visualize various biological processes in living cells. Reporter genes available in *Streptomyces* are: *neo* (encoding neomycin phosphotransferase), *cat* (encoding

chloramphenicol acetyltransferase), *xylE* (encoding catechol 2,3-dioxygenase), green fluorescent protein, luciferase, *gusA* (Myronovskyi et al., 2011; Siegl and Luzhetskyy, 2012). Marker genes facilitate production and subsequent identification of transgenic organisms. Two groups of marker genes exist: selectable marker genes and nonselectable – also called scorable marker or reporter genes. Among selectable markers, researchers distinguish two groups: positive – which include giving a selective advantage to a cell and negative which means selective killing (Stewart, 2008). Since *gusA* was used during the development of this master thesis, it will be described below.

gusA gene encodes for β-glucuronidase enzyme in *E. coli*. When fused to regulatory sequences or introduced into biological system, it generates a signal which may be quantified. Detected signal is directly correlated with the activity of promoter (Ramakrishna, 2009). In this way, it is possible to assess influence of various factors affecting the cell like for instance stress or trans-acting factors. These factors modulate expression at transcriptional or translational level. These changes are possible to detect thanks to advantage of reporter markers.

There are several advantages of using GUS (*gusA*) reporter system. Benefits arise from the stability of the enzyme which does not require any cofactors and also high specific activity which makes it very sensitive. In addition, β-glucuronidase is unusually tolerant to most used chemicals and conditions in the assay like pH and temperature. Conveniently, most of the *Streptomyces* strains have been found to lack β-glucuronidase endogenous activity. Summing up, assay is relatively simple, inexpensive and sensitive. It also exists in a wide range of commercially available substrates for various assay types and formats like: spectrophotometric, fluorimetric, chemiluminescent, chromogenic (Myronovskyi et al., 2011).

3. Heterologous expression of natural product biosynthetic gene clusters

Heterologous expression is one of the approaches of genome mining technique. As it is described by Gomez-Escribano and Bibb (2013), genome mining is "the use of bioinformatics, molecular genetics, and natural product analytical chemistry to access the metabolic product of a gene cluster found in the genome of an organism". Genome mining may be employed in two ways. Firstly, by activating the silent gene cluster either by manipulation involved with deletion or over-expression of negative or positive transcriptional regulators, respectively. An alternative method consists of cloning the fragment of DNA of

producing microorganism along with gene cluster of interest and attempt to express it in suitable host. Heterologous expression applications are connected with confirmation of complete transfer of genes from the cluster of interest, attempt of acquisition metabolic product from cryptic gene cluster or difficult to culture or not genetically amenable organism, obtaining unnatural metabolites or expressing mutated gene clusters. Hosts like *E. coli*, *S. cerevisiae* or several *Streptomyces* species are used for these purposes. Advantage of *E. coli* host is a wide range of possible vectors and promoters and opportunity to express genes with G+C sequence as high as 73%. Drawbacks include inability of recognition of promoters originating from *Streptomyces*, lack of production of some precursors which are necessary for biosynthesis of compounds and frequent incorrect folding of type I polyketides synthase proteins. Successful attempts of initial cloning of selected genes in *E. coli* vectors which bear yeast promoters and then subsequent assembly and expression in YAC system resulted in production of several flavonoids. However, in order to achieve that expression, in most of the cases genetic engineering modifications are required. The odds for sustainable expression increase if the source genes and the host strain are closely related to each other. The ideal *Streptomyces* host for synthetic biology purposes should posses following, desirable characteristics. Firstly, it should offer a wide range of vectors and markers. In addition, it was proved that removal or limited production of all endogenous, active secondary metabolite gene clusters is an advantage. In this way, chances of interfering with the activity of target gene cluster are significantly decreased. Pathways for biosynthesis should be well understood. Catabolic pathways should utilize cheap nutrients in order to ensure financial sustainability. Strain should easily accumulate biomass without losses in productivity. More than that efficiency of cluster cloning is another difficult step. Heterologous expression may turn out to be useful, when natural producer fails to provide sufficient titers of metabolite allowing for researching its biosynthetic pathway (Zotchev et al., 2012; Gomes-Escribano and Bibb, 2014)

V. The aims of this study

The aim of this study was to re-engineer the antibiotic biosynthesis regulatory circuit in *S. venezuelae* in order to evoke inducible heterologous production of secondary metabolites, using jadomycin and chloramphenicol as models. This was planned to be achieved by exchange of the promoter which drives expression of the *cml* structural genes. Since these genes are under repression of JadR1 regulator, it was thought to be sustainable to substitute the *cml* promoter with a promoter which is activated by JadR1. In this way, upon ethanol shock, instead of jadomycin production, chloramphenicol biosynthesis would take place. The specific goals to reach overall aim were:

1. Deletion of *jadJ-E* structural genes from *S. venezuelae* chromosome and replacement with a reporter gene *gusA*
2. Testing *gusA* expression upon ethanol shock
3. In case the expression of a reporter is shown to be dependent on the ethanol, replacing *cmlI-X* promoter in deletion mutant with *jadR1-jadJ* promoter region in the chloramphenicol biosynthetic gene cluster
4. Testing the recombinant strain for dependence of chloramphenicol production on ethanol stress

Materials and Methods

Four constructs were planned to be constructed throughout the span of this master thesis.

1. **pJP1** – construct for deletion of of *jadJ-E* genes in *S. venezuelae*
2. **pJP1gusA** – construct for deletion of of *jadJ-E* genes in *S. venezuelae* with another version of gusA region
3. **pJP2** – construct for replacement of the *cmlI* promoter in *S. venezuelae* with *jadJ* promoter
4. **pCML3** – construct for heterologous expression of chloramphenicol antibiotic gene cluster

Labwork was performed according to the flowcharts and detailed description presented below. Boxes marked orange are common for all the charts; blue ones are distinct for the particular workflow.

Need for exchange of *gusA* fragment from the initial construct i.e. pJP1 arose from the fact of discovery a mutation preventing *gusA* from correct functioning. For that reason pJP1gusA construct appeared.

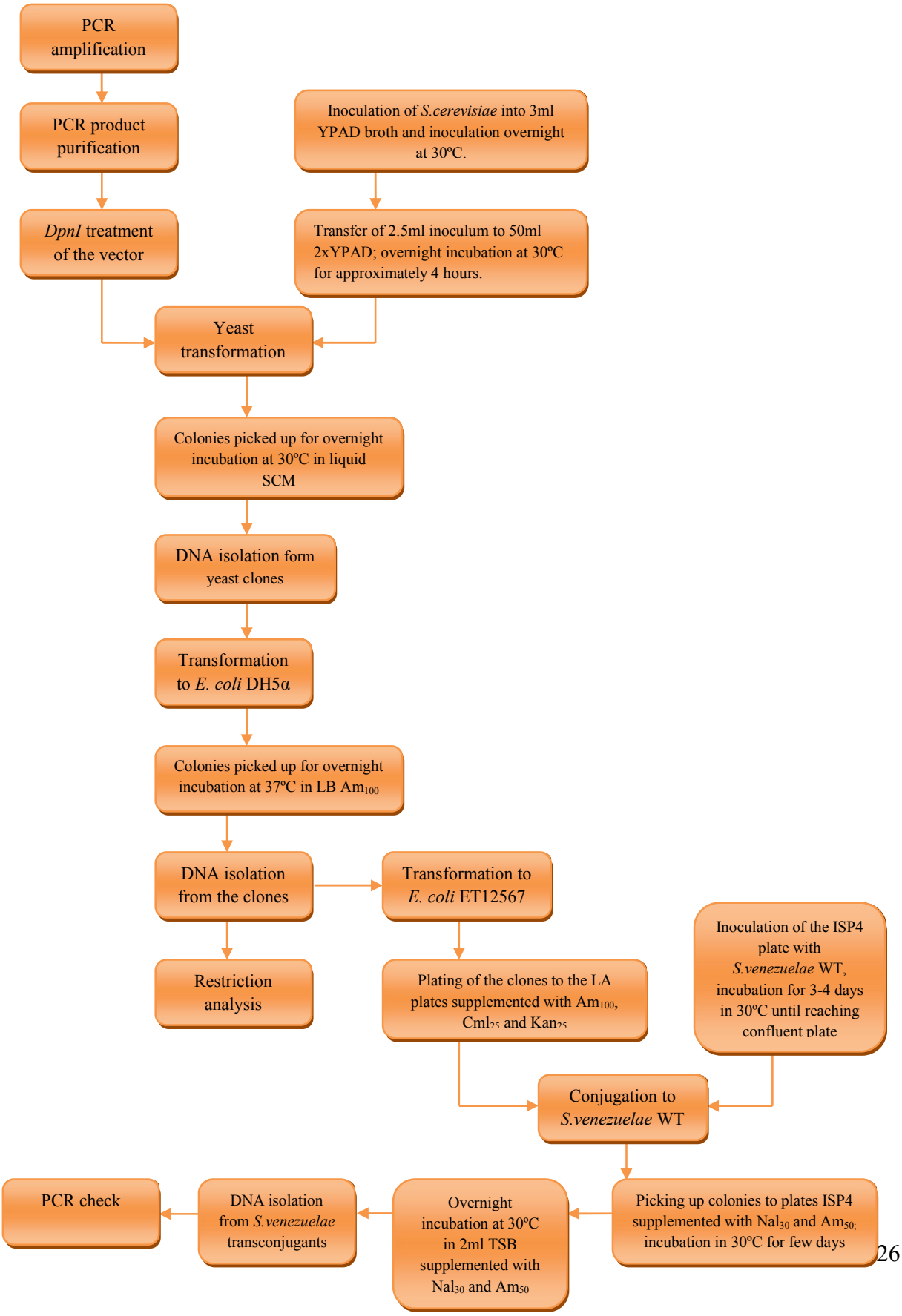
All the procedures described in the following sections can be found in Lab protocols chapter of this master thesis.

pJP1 construct fragments were initially amplified by PCR (description: Lab protocols, PCR amplification, PCR I). After this step 2µl of the product was used to perform gel electrophoresis and the rest was subsequently purified. Afterwards, 2µl purified product was once more checked on the gel. Conditions: 55min, 100V. Vector was treated with *DpnI* in order to get rid of the template residues.

Yeast transformation using protocol for 'Yeast Transformation by LiAc/SS Carrier DNA/PEG Method' was subsequently employed. Fragments were mixed in the following volumes: vector - 3µl, left flank – 3µl, right flank – 3µl, gusA – 4µl, water – 21µl. Resulting colonies were picked up for overnight incubation, and next day DNA was isolated. DNA from the yeast clones was electroporated to *E. coli* DH5α. DNA from colonies was isolated and analysed by restriction cutting with *BamHI* in order to determine whether the correct assembly was constructed.

After successful confirmation, DNA was transformed to *E. coli* ET12567 cells and plated on LA plates supplemented with Am_{100}, Cml_{25} and Kan_{25}. ET12567 clones were afterwards conjugated to *S. venezuelae* WT. Obtained colonies were preliminarily screened on ISP4 medium with Nal_{30} and Am_{50} and incubated at 30ºC until visible growth appeared. TSB supplemented with Nal_{30} and Am_{50} was inoculated for overnight incubation at the same temperature, DNA isolated and checked by PCR.

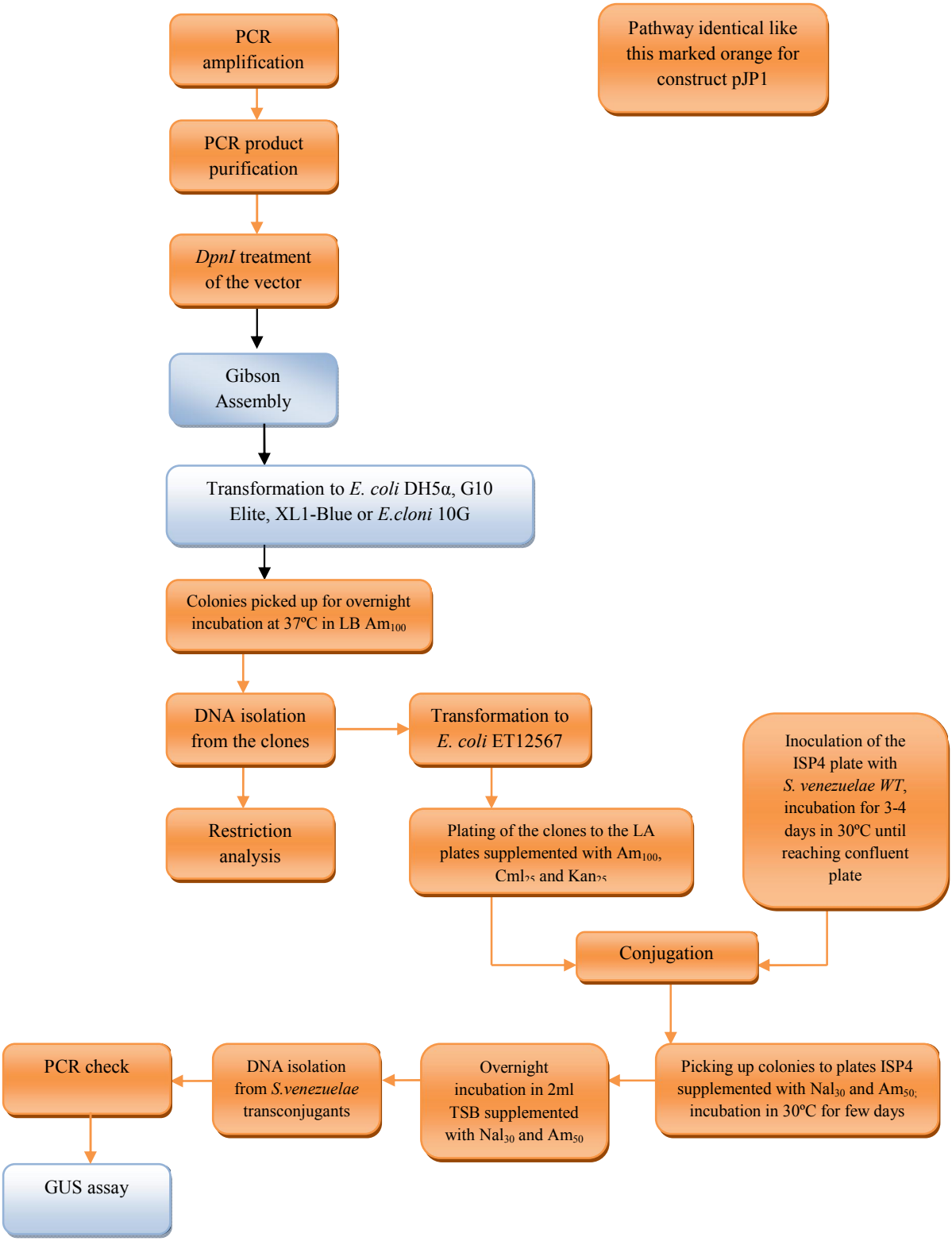
I. Flowchart of labwork for the construct pJP1.



As it was mentioned previously, **pJP1gusA** construct was assembled in order to exchange mutated *gusA* gene in pJP1. Fragments were amplified by PCR (description: Lab protocols, PCR amplification, PCR IV), purified and checked on the agarose gel following each of those two steps. *gusA* fragment was amplified using pSOK808 template. Subsequently, vector was treated with *DpnI*.

Fragments were mixed and assembled using Gibson assembly method in following ratio: 4.5µl GusA, 0.5µl vector. Gibson mix was later on transformed to couple of potential hosts like *E. coli* DH5α, G10 Elite, XL1-Blue and *E. cloni* 10G. DNA from the transformants was isolated and restriction analysis with several enzymes like *BamHI, PstI, AseI, MluI* was performed. After several attempts, no right construct was obtained and due to the time constrains this part of the project was not continued.

II. Flowchart of labwork for the construct pJP1gusA.



Fragments for **pJP2** assembly were amplified by PCR (description: Lab protocols, PCR amplification, PCR II), checked on the gel, purified and checked on the gel for the second time. Vector was *DpnI* treated. Assembly using 'Yeast Transformation by LiAc/SS Carrier DNA/PEG Method' was performed. Fragments were mixed in following ratio: vector - 1µl, left flank - 3µl, right flank - 3µl, promoter region - 8µl, water 18µl. Colonies resulting from this transformation were inoculated overnight at 30ºC to liquid SCM and DNA was isolated the following day. Plasmid DNA isolated from yeast cells, have been transformed into *E.coli* strain. After isolation of the DNA from the transformants, it was analysed by restriction digest and checked on the gel.

Correct clone was introduced to *E. coli* ET12567 cells., and selected on LA with Am. 3 clones were plated onto LA Am_{100}, Cml_{25} and Kan_{25} plates, and one of them subsequently subjected to intergeneric conjugation with *S. venezuelae* WT.

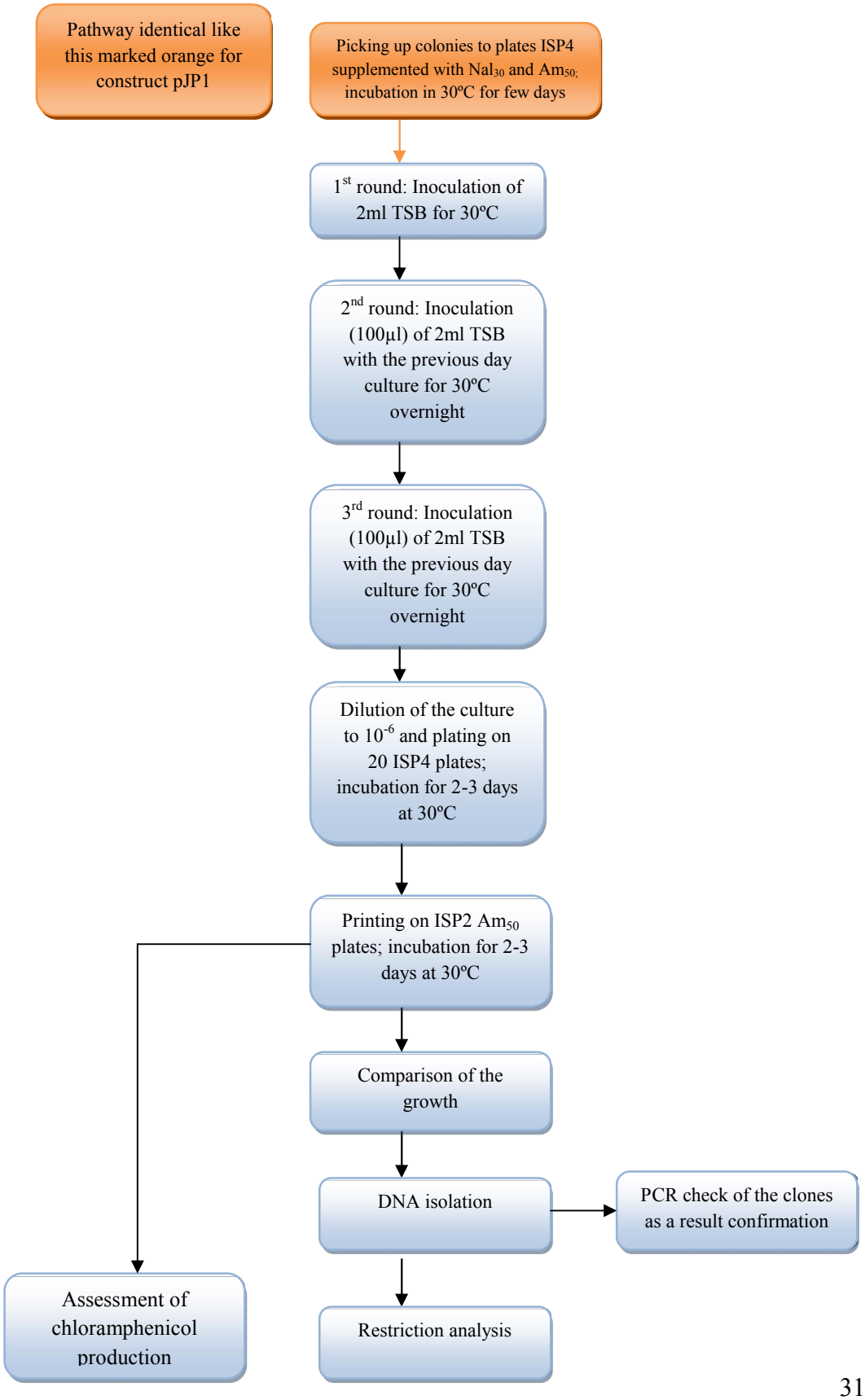
Resulting transconjugants were checked using PCR method and after this confirmation test, selection for the second crossover took place. It was performed by three-day series of overnight incubations at 30ºC in 2 ml TSB without antibiotics. Dilution 10^{-6} from the culture was plated in 100µl aliquotes over 20 ISP4 plates in order to get single colonies. Plates were incubated at 30ºC for 2-3 days until visible growth appeared.

Following this, replica plating technique was employed. Sterile velvet pads were placed over the wooden stamp and pressed to ISP4 plates, and then gently pressed in the same position to ISP2 plates supplemented with Am_{50}. After few days results of selection were visible. Plates were compared with each other. Successful potential second-crossover colonies were able to growth on ISP4 but lost the ability to grow in the presence of apramycin. Genomic DNA from the candidates for second crossover was isolated and checked using PCR and restriction cutting with *HindIII*.

Assessment of chloramphenicol production from the recombinant strain was performed. 1 ml out of 3 ml overnight TSB culture at 30ºC of pJP2 in *S. venezuelae* was used to inoculate 30 ml of MYM medium and incubate it for 9h at 30ºC. After this time, culture was divided in two flasks 15 ml each. One of them was supplemented with 1 ml 96% ethanol. At the same time 1ml of purified water was added to the other flask which comprised the control. Cultures were incubated for further 48h in the same conditions. Subsequently chloramphenicol was

extracted using ethyl acetate method. Results were assessed by UPLC analysis. Control with *S. venezuelae* WT was conducted alongside to the recombinant strain. Results from the UPLC analysis of both strains were compared to each other.

III. Flowchart of labwork for the construct pJP2.



Similarly to other constructs, fragments for **pCML3** were amplified by PCR (description: Lab protocols, PCR amplification, PCR III), 2µl of DNA were checked on the agarose gel, and product was purified and once more checked by electrophoresis. *DnpI* treatment of the vector was performed.

As it was also mentioned in the other descriptions, this construct was also assembled by yeast transformation with LiAc/SS Carrier DNA/PEG. DNA fragments were mixed by following: vector - 3µl, Cml3H1 - 3µ, Cml3H2 - 6µl, water - 22µl. Plasmid DNA was isolated from yeast clones and transformed into *E. coli* strain to check the assembly. Plasmid DNA from *E. coli* was cut by *EcoRI and PvuII* to find the correct clones.

Transformation to *E. coli* ET12567 and plating of the resulting colonies on the plates with the addition of Am_{100}, Cml_{25} and Kan_{25} was a pre-step for intergeneric conjugation to *S. albus*. Similarly to *S. venezuelae*, transconjugants of *S. albus* were checked both by plating on the SFM plate with Nal_{30} and Am_{50} and also with PCR amplification and subsequent agarose gel.

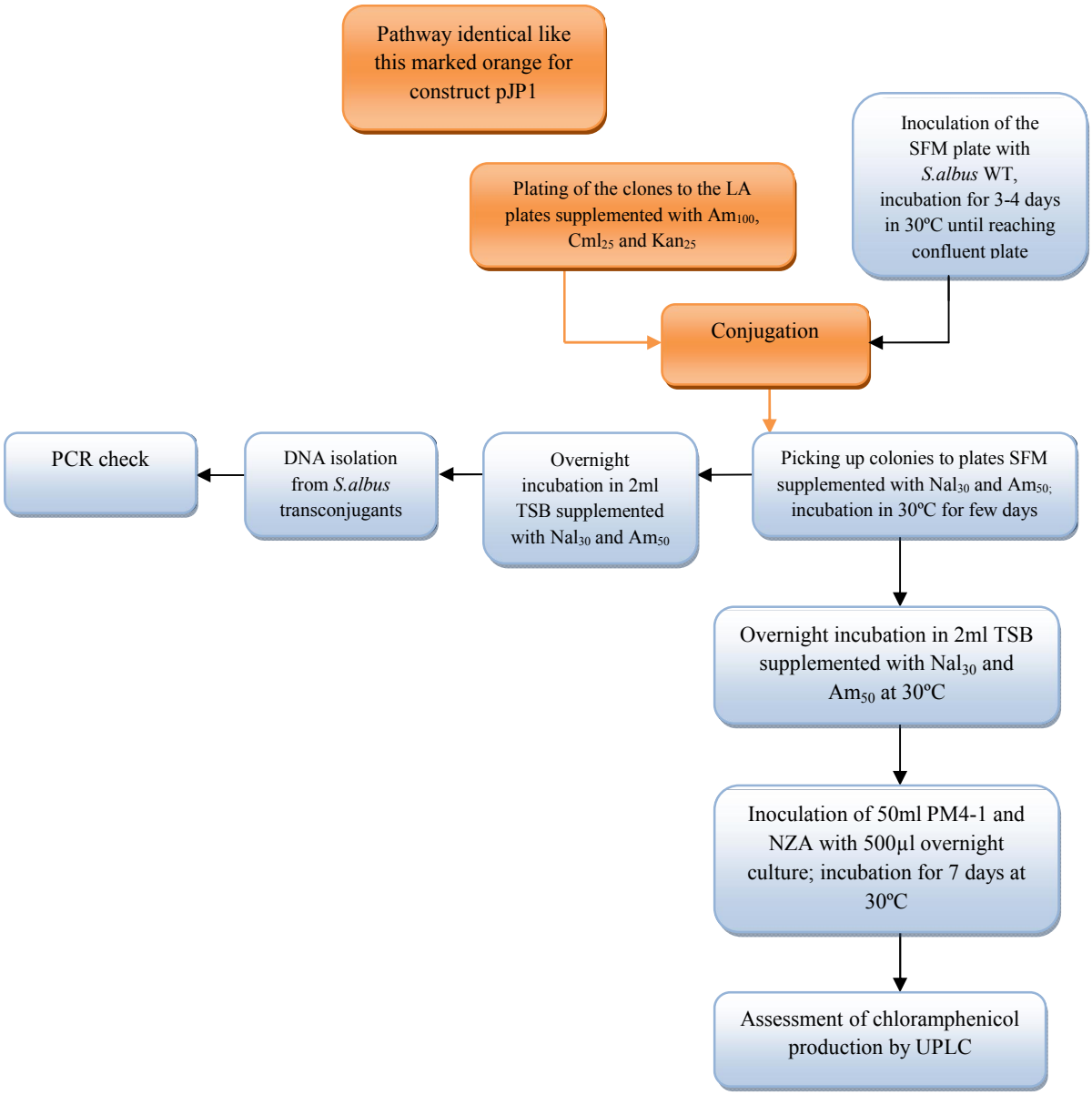
After confirmation of the correct constructs, *S. albus* transconjugants were incubated overnight in 2ml TSB at 30ºC. On the very next day, 50ml PM4-1 and NZA media were inoculated with 500µl of the overnight culture and incubated in the same conditions at the shaker for next 7 days. After this time, chloramphenicol was extracted using method involving ethylacetate. Results were analysed externally by UPLC analysis.

- **Complementation experiment**

It was performed in order to check if the introduction of construct pCML3, containing the whole cml cluster, is able to restore the production of Cml in *JadRI/CmlΔ* mutant, non-producer of Cml. Double deletion *S. venezuelae* mutant was provided - *JadRI/CmlΔ*. It was conjugated with pCML3. Transconjugants were selected by plating them on ISP4 medium with Nal_{30} and Am_{50} and then confirmed PCR amplification and electrophoresis analysis.

Similarly to the previous assembly, 2ml TSB was inoculated by spores from the complementation strain for overnight incubation at 30ºC. 500µl of overnight culture was used to start the 7 days fermentation in 50 ml PM4-1 in shaking incubator at 30ºC. After 7 days, chloramphenicol was extracted using method with ethylacetate. In order to assess the amount of the antibiotic in the cultures, samples were sent for UPLC analysis.

IV. Flowchart of labwork for the construct pCML3.



1. Flowchart of the complementation experiment

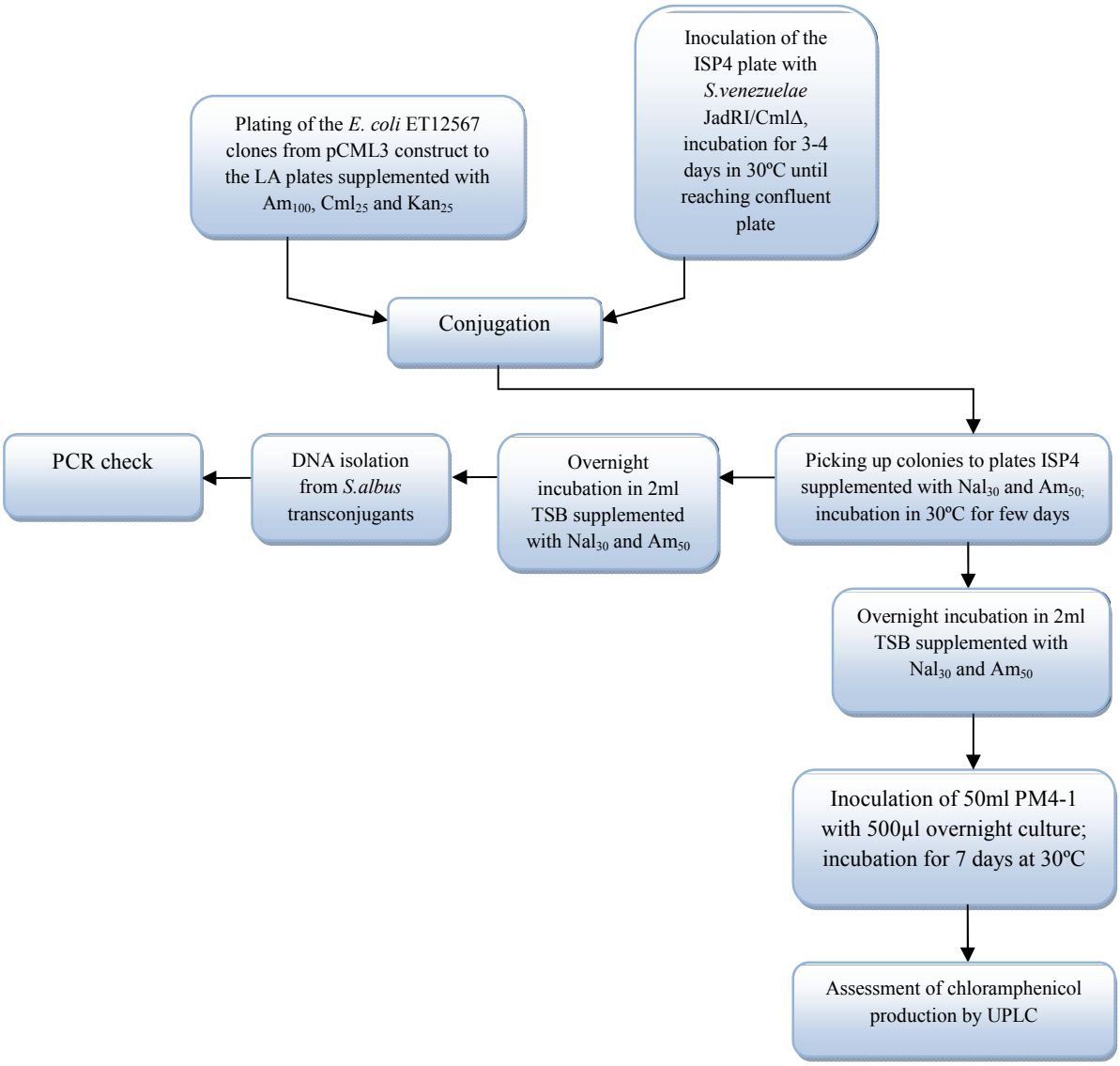


Table 3. Bacterial strains and plasmids used in this thesis

Strain or plasmid	Characteristics/genotype	Reference or source
Streptomyces strains		
S. venezuelae ATCC 10712	wild-type strain, producer of jadomycin and chloramphenicol	Doull et al., 1993
S. venezuelae JadRI/CmlΔ	chloramphenicol deleted from jadR1Δ mutant genome (second crossover)	Jianhai Zhang, unpublished
S. albus J1074	*S. albus* G1 (DSM 41398) derivative with the defective *Sal*GI restriction modification system heterologous host	Myronovskyi et al., 2014
E.coli strains		
DH5α	*supE44 ΔlacU169 recA1 endA1 gyrA96 thi-1 relA1*	Reisner et al., 2003
ET12567 (pUZ8002)	Methylation deficient strain - *dam⁻, dcm⁻, hsdM⁻* which contains helper plasmid pUZ8002 facilitating conjugative DNA transfer from RP4 oriT	MacNeil et al., 1992
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]*	Stratagene Technical Services, 2004
E. cloni 10G	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 φ80dlacZΔM15 ΔlacX74 araD139 Δ (ara,leu) 7697 galU galK rpsL (StrR) nupGλ tonA*	Lucigen
Yeast strains		
S. cerevisiae BY4742	Host for TAR-assisted DNA assembly; *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Risler et al., 2012
Plasmids		
pCLY10	ori15A, VWBint, attP, RP4oriT, *aac3(IV),* LEU2, CEN6-ARS4	Sekurova, unpublished
pSOK808	oriColEI, VWBint, attP, RP4oriT, *aac3(IV), ermE*p*::*gusA*	Zotchev, unpublished

pUC57 –gusA	oriColEI, *bla, gusA*	Zotchev, unpublished
pJP1	ori15A, RP4oriT, *aac3(IV),* LEU2, CEN6-ARS4, *jadJ*p::*gusA*	This work
pJP1gusA	ori15A, RP4oriT, *aac3(IV),* LEU2, CEN6-ARS4, *jadJ*p::*gusA* (fixed)	This work
pJP2	ori15A, RP4oriT, *aac3(IV)*, *cmlI*p->*jadJ*p	This work
pCML3	pCLY10::cml	This work

V. Lab protocols

1. DNA isolation

A. DNA isolation from bacterial cultures

Procedure was performed according to the instruction for use of products from technical bulletin of Wizard® *Plus* SV Minipreps DNA Purification System.

1) 2ml of the overnight bacterial culture was harvested by centrifugation for 5 minutes at 10,000 x g in a tabletop centrifuge. Subsequently supernatant was discarded and tubes blotted on a paper towel to remove excess media.
2) 250µl of Cell Resuspension Solution was added to the samples. Pellet was completely resuspended by pipetting in a 1,5ml microcentrifuge tubes.
3) 250µl of Cell Lysis Solution was added to the samples and mixed by pipetting until partial clearing of the lysate was achieved (no more than 5 minutes).
4) 10µl of Alkaline Protease Solution was added. Tubes were mixed by inverting 4 times and afterwards incubated for 5 min in room temperature. Due to the risk of nicking the plasmid DNA due to the protease activity 5 min was never exceeded.
5) 350µl of Wizard® Neutralization Solution was added and tubes were immediately mixed by inverting 4 times.
6) Bacterial lysate was centrifuged at maximum speed (13,000 x g) in a microcentrifuge for 10 min at room temperature.

7) Cleared lysate was transferred to prepared beforehand Spin Columns. In case white precipitate was still present in the lysate, samples were centrifuged additionally for another 5 to 10 minutes.
8) After applying supernatant to the column, tubes were centrifuged at maximum speed for 1 min at room temperature. Flowthrough was discarded and columns were reinserted to Collection Tubes.
9) 750µl of Column Wash Solution was applied on the column. Solution was diluted with 95% ethanol prior to use according to producer description on the package.
10) Tubes were centrifuged for another 1 min at maximum speed at room temperature. Flowthrough was discarded and columns were reinserted into Collection Tubes.
11) Procedure has been repeated with 250µl of Column Wash Solution.
12) Subsequently probes were centrifuged at maximum speed for 2 minutes at room temperature.
13) Spin Columns were transferred to new 1,5ml microcentrifuge tube avoiding accidental transfer of any residual Column Wash Solution.
14) Plasmid DNA was eluted with 30 or 50µl of Elution Buffer applied to the centre of membrane depending from expected DNA yield.
15) Samples were centrifuged at maximum speed for 1 min at room temperature.
16) Spin Columns were discarded and purified samples later on used for another tests or stored frozen in -20ºC.

B. DNA isolation from yeast cultures

This protocol is based on the previous one i.e. DNA isolation from bacterial cultures. Due to the nature of the organism which DNA is isolated from, certain modifications have been applied.

1) 3ml of the overnight yeast culture in SCM medium was harvested by centrifugation for 5 minutes at 10,000 x g in a tabletop centrifuge. Subsequently supernatant was discarded and tubes blotted on a paper towel to remove excess media.
2) 250µl of Cell Resuspension Solution mixed with 3µl of Zymolyase were added to the samples. Pellet was completely resuspended by pipetting in a 1,5ml microcentrifuge tubes.

3) Samples were incubated for 1hour in 37ºC. Probes were tipped every 20 min throughout the whole time of incubation in order to ensure even distribution of the enzyme.
4) Rest of the steps starting from the step number 3 were followed according to the protocol number A i.e. **DNA isolation from bacterial cultures.**

C. DNA isolation from *S.venezuelae*

DNA from *Streptomyces* was isolated using Dneasy tissue kit from QIAGEN. Protocol was supplemented by the manufacturer, and was modified in our group for isolation of genomic DNA specifically from streptomycetes.

1) Inoculated spores from well-sporulated colonies incubated overnight in 2 ml TSB medium were prepared.
2) 1 ml of the culture was transferred into another eppendorf tube. In addition 0.5 ml of sterile water was supplemented. Samples were mixed thoughrouly and spinned down for 3 min at 13,000 rpm. Supernatant was discarded, probes were centrifuged shortly again, and later on the rest of the cultivation media was removed.
3) Pellet was resuspended by adding180µl of Lysis Buffer and pipetting the solution up and down several times. Lysis Buffer contains 20mM Tris HCl, pH 8.0; 10mM EDTA pH 8.0; 1.2% Triton X100; 20 mg/ml lysosyme. Lysosyme was added to earlier prepare pre-lysis mix just before the experiment.
4) Mixed was incubated for 15 min at 37ºC. During this time, samples were tipped every 5 min.
5) After the incubation 25µl of Proteinase K was added and mixed by pipetting. Later on, 200µl of buffer AL was supplemented and mixed by pipetting several times. Suspension was incubated for 30 min at 55ºC.
6) 200µl of 96% ethanol was added and mixed thoroughly by pipetting. Mixture was applied on the column inserted into 2 ml collection tube provided by the producer. Solution was centrifuged at 13,000 rpm for 2 min.
7) Columns were transferred to another 2 ml collection tube and 500µl of AW1 buffer was applied. Samples were spinned for 1 min at 13,000 rpm.
8) Columns were transferred to new 2ml collection tubes and 500µl of AW2 buffer was applied. Samples were spinned for 3 min at 13,000 rpm.

9) Columns were transferred to new eppendorf tubes and 125µl of previously preheated to 50ºC buffer AE was applied onto them. Probes were incubated 15 min at room temperature.
10) Samples were centrifuged for another 1 min at 13,000 rpm in order to elute DNA, columns were discarded, and DNA was stored in -20ºC.

2. PCR amplification

PCR is an abbreviation from Polymerase Chain Reaction and was invented by Kary Mullis. It in an in vitro amplification reaction with a set of enzymes and different temperature steps throughout. They comprise three essential for PCR steps: denaturation, annealing and extension. During the first one, strands of the DNA template are separated in high temperature reaching 94-95ºC. Later in annealing step, by lowering the temperature primers are able to attach to their complementary sequences on the template strands. Here, temperature of the reaction varies with the respect of the used primers in order to ensure highest possible specificity. The higher annealing temperature, highest specificity. In the last step, temperature is adjusted to the DNA polymerase activity which facilitates elongation process throughout several cycles (McPherson and Moller, 2006). In this master thesis PCR amplification was used in following setup:

PCR Mix	13µl
Template	1µl
Primers (forward and reverse)	1µl + 1µl
Polymerase	1µl
H_2O	8µl
Total 25µl	

Two types of polymerases were used: Expand High Fidelity Polymerase PCR System 3.5 U/µl from Roche Diagnostics GmbH and MasterAmp™ Extra Long Polymerase Mix 2.5 U/µl from Epicentre. PCR mixes were a part of MasterAmp™ Extra-Long PCR Kit.

PCR I

1) For *jad* deletion construct (pJP1)
    a. Setup for vector:

PCR Mix	Number 1
Template	pCLY10
Primers (forward and reverse)	Y10_SVF1 + Y10_SVR1
Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

b. Setup for left and right flank:

PCR Mix	Number 7
Template	*S. venezuelae* gDNA
Primers (forward and reverse)	JJEdel-F + R2; JJEdelR-F + R
Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

c. Setup for *gusA* part:

PCR Mix	Number 7
Template	pUC57
Primers (forward and reverse)	gusAF2 + R2
Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

PCRII

2) For promoter replacement construct (pJP2)

a. Setup for vector

PCR Mix	Number 9
Template	pCLY10
Primers (forward and reverse)	Y10_SVcml-F + R

Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

b. Setup for left and right flank

PCR Mix	Number 7
Template	*S. venezuelae* gDNA
Primers (forward and reverse)	CmlI-F + R; CmlX-F + R
Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

c. Setup for promoter region amplification

PCR Mix	Number 7
Template	*S. venezuelae* gDNA
Primers (forward and reverse)	jadJp-F + R
Polymerase	Expand High Fidelity
H_2O	H_2O
Program: SZLG	

PCR III

3) Fragments for chloramphenicol assembly with long overlaps (pCML3)

a. Setup for vector

PCR Mix	Number 9
Template	pCLY10
Primers (forward and reverse)	Y10_cml3-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG2	

b. Setup for remaining fragments

PCR Mix	Number 7
Template	*S. venezuelae* gDNA
Primers (forward and reverse)	Cml3H1-F + R; Cml3H2-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG2	

PCR IV

4) For gusA exchange (pJP1gusA)

a. Setup for amplification of the whole assembly except *gusA* region

PCR Mix	Number 9
Template	pJP1 (assembly no 1)
Primers (forward and reverse)	Y10_cml3-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG	

b. Setup for gusA part

PCR Mix	Number 9
Template	pSOK808
Primers (forward and reverse)	gusA_JP-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG	

Checking of mutant strains by PCR

Setup for pJP1

PCR Mix	Number 7
Template	DNA from transconjugant
Primers (forward and reverse)	JJEdel-F + JJEdelR-R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG	

Setup for pJP2

PCR Mix	Number 7
Template	DNA from transconjugant
Primers (forward and reverse)	Y10_SVcml-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG	

PCR Mix	Number 7
Template	DNA from transconjugant
Primers (forward and reverse)	CmlI-F + CmlX-R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG	

Setup for pCML3 and complementation experiment

PCR Mix	Number 7
Template	DNA from transconjugant
Primers (forward and reverse)	Cml3H1-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O

Program: SZLG	
PCR Mix	Number 7
Template	DNA from transconjugant
Primers (forward and reverse)	Cml3H2-F + R
Polymerase	Master Amp. Polymerase
H_2O	H_2O
Program: SZLG2	

As a control DNA from *S. venezuelae* WT as a template was used.

All PCR product sizes were simulated in Clone Manager.

PCR programs

PCR programs used in this master thesis are specified in the tables below:

SZLG

Step	Temperature	Time [h:m:s]
Denaturation	95.0ºC	00:03:00
Continued denaturation	94.0ºC	00:00:45
Annealing	56.0ºC	00:00:45
Elongation	68.0ºC	00:10:00
-	Go to 2 rep 25	-
Continued elongation	68.0ºC	00:10:00
Hold	4.0ºC	∞

SZLG2

Step	Temperature	Time [h:m:s]
Denaturation	95.0ºC	00:03:00
Continued denaturation	94.0ºC	00:00:45
Annealing	56.0ºC	00:00:45

Elongation	68.0ºC	00:22:00
-	Go to 2 rep 25	-
Continued elongation	68.0ºC	00:25:00
Hold	4.0ºC	∞

OSGIB

Step	Temperature	Time [h:m:s]
Incubation	50.0ºC	01:00:00
Hold	4.0ºC	∞

DPNJP

Step	Temperature	Time [h:m:s]
Incubation	37.0ºC	03:00:00
Inactivation	80.0ºC	00:20:00
Hold	4.0ºC	∞

3. PCR product purification

PCR purification was performed according to the protocol QIAquick PCR Purification Kit Protocol using a microcentrifuge from QIAquick® Spin Handbook for QIAquick PCR Purification Kit from QIAGEN. Protocol was designed in order to purify single- or double-stranded DNA fragments from PCR. Procedure allows for removal of primers, nucleotides and buffer component as well as nontarget amplification products.

Note: All centrifugation steps were performed at maximum speed i.e. 13,000 rpm at room temperature in a tabletop microcentrifuge.

1) 5 volumes of Buffer PB previously supplemented with pH indicator were added to 1 volume of the PCR sample and mixed by pipetting.
    a. Indicator was added to the Buffer PB in ratio 1:250 prior to the procedure.
    b. The color of the mixture should be yellow.

c. If the color of the mixture turned orange or violet, 10µl of 3M sodium acetate, pH5.0 was added.

2) QIAquick spin column was placed in 2 ml collection tube.
3) Samples were applied to the columns and spinned for 30-60s.
4) Flowthrough have been discarded and subsequently 750µl of Buffer PE was added to wash the columns. This step was followed by centrifugation for 30-60s.
5) Flowthrough have been discarded and samples were spinned for additional 1 min in order to remove all residual ethanol coming from Buffer PE.
6) Probes were placed into new 1.5ml microcentrifuge tubes.
7) DNA was eluted by overlaying the column membrane with 50µ of Buffer EB.
8) Columns were standing for 1 min and then spinned down for another 1 min in order to remove DNA from the membrane.
9) Samples were stored in -20ºC.

A. Purification of DNA by excision from agarose gel

It was performed in accordance to the protocol QIAquick Gel Extraction Kit Protocol using a microcentrifuge found in QIAquick Spin Handbook supplied by the manufacturer. This approach was designed to extract and purify DNA fragments ranging from 70bp to 10kbs from standard or low-melt agarose gels in TAE or TBE buffer.

Note: All centrifugation steps were performed at maximum speed i.e. 13,000 rpm at room temperature in a tabletop microcentrifuge.

1) DNA bands were visualized on the gel using UV light and afterwards excised using sharp scalpel.
2) 3 volumes of Buffer QG were added to 1 volume of the gel piece.
3) Tubes were incubated at 50ºC for 10 min or until the pieces were completely dissolved. Samples were mixed by tapping the tube every 2-3 min.
4) Yellow is an expected color of the solution after the gel is dissolved. If the mixture turned orange or violet, 10µl of 3 M sodium acetate, pH 5.0 was added.
5) 1 gel volume of isopropanol were added to the sample and mixed.
6) QIAquick spin columns were placed in a provided 2 ml collection tubes.

7) In order to bind DNA, volume of the sample was applied on the column and subsequently spinned down for 1 min.
8) Flowthrough was discarded and QIAquick columns were placed back in the same collection tubes.
9) In order to wash, 750µ of Buffer PE was added on the top of the column and then centrifuged for 1 min.
10) Flowthrough was discarded and column was spinned down for additional 1 min.
11) Collection tubes were discarded and columns were placed into clean 1.5 microcentrifuge tubes.
12) To elute DNA, 30-50µl of the Buffer EB was applied to the centre of the membrane.
13) Columns were let to stand for 1 min and then spinned for additional 1 min in order to elute DNA suspension.
14) Samples were stored at -20ºC.

B. Purification of DNA by QIAEX II silica particles suspension

Principles of purification DNA by QIAEX II system involve solubilization of agarose and subsequent selective adsorption of nucleic acids. They bound reversibly to QIAEX II silica-gel particle in the surrounding of chaotropic salt. This approach allows for separation DNA from salts, agarose, polyacrylamide, dyes, proteins, and nucleotides.

1) DNA bands were visualized on the gel using UV light and afterwards excised using sharp scalpel.
2) 400µl of QG solubilization and binding buffer was added along with 10µl f bead suspension.
3) Samples were mixed and incubated for 10 min at 50ºC. Tubes were gently tipped during the incubation every 2 min.
4) Tubes were spinned down for 1 min at 13,000 rpm.
5) Supernatant was removed.
6) After adding 500µl of QG buffer steps 4 and 5 were repeated.
7) After adding 500µl of PE buffer steps 4 and 5 were repeated.
8) After adding 500µl of PE buffer steps 4 and 5 were repeated once more.
9) Supernatant was completely removed and tubes were dried for 15 min at the table.

10) After overlaying the pellet with 20µl of Elution Buffer (EB), samples were incubated for 10 min at 50ºC.
11) When the incubation was finished, tubes were spinned down for 1 min at 13,000 rpm and the pellet was transferred to new tubes.
12) Samples were stored at -20ºC.

4. Gel electrophoresis

Electrophoresis is a separation technique which is based on the ability of charged molecules to move in an electric field. Its main application is analysis and purification of large molecules i.e. proteins or nucleic acids. Procedure is carried out by loading a sample into a well moulded in a porous matrix. Afterwards voltage is applied to the entire system. Molecules which are sized, shaped or charged differently will move though the matrix at various velocities finally creating visible bands. Matrix components can be versatile, like gels made of polyacrylamide, agarose, or starch. Concentration of the gel determines the pores size. This affects molecules retardation in a matrix, since small molecules will move faster than the big ones. DNA is negatively charged due to its phosphate group and it will migrate from the katode (-) to the anode (+) (Western Blotting Principles and Methods; GE Healthcare handbook). During this master thesis horizontal system with 0.8% agarose gel was used. It was applied in order to determine the size and the purity of the amplified PCR product as well as check the DNA digestion pattern during the restriction analysis.

Agarose 0.8%

Per 300 ml of 1 x TAE buffer:

SeaKem LE Agarose	2.4g
Gel Green Nucleic Acid Stain (10000x)	20µl

Agarose solution was prepared by dissolving agarose powder in 300 ml of 1 x TAE buffer simultaneously warming it up in a microwave until it is completely dissolved. 20µl of Gel Green Nucleic Acid Stain (10 000x) manufactured by Biotium was added subsequently and the solution was mixed thoroughly. Ready-to- use mix was stored at 60ºC.

Protocol:

1) 0.8% agarose gel with 20µl of Gel Green was prepared according to the description in a section above.
2) Liquid, hot gel was poured into the gel-form placed into the electrophoresis chamber, comb with the appropriate number of wells was applied and solution was left to stiffen for approximately 30 min.
3) After this time, electrophoresis chamber was filled with 1xTAE buffer.
4) DNA ladder was applied to the first well (1 - 2µl).
5) Samples supplemented with loading dye were loaded into the wells.
   - For PCR product check: 2µl DNA + 7 µl water + 1 µl loading dye
   - For purified PCR product check: 2µl DNA + 7 µl water + 1 µl loading dye
   - For check of restriction cut isolated DNA: 10µl of reaction mix + 1µl loading dye
   - For excision of the DNA from the gel: Entire DNA volume + 2µl loading dye
6) After assembling the chamber, 100V voltage was applied. Gel was run depending on the level of required separation i.e. from 55 min to 120 min.
7) Resulting bands were visualized with UV-light using Gel Doc 2000. Readout was accomplished by comparison of the sample bands to the sizes on the ladder.

5. Restriction analysis

Restriction analysis of plasmid DNA was performed in order to find the clones containing correct plasmids. It is based on the possibility to cut DNA with the enzymes in its adequate restriction sites. Reaction was first designed in Clone Manager and later on executed in the lab. Results were visualized on the agarose gel by electrophoresis and sizes of the bands were determined according to the ladder. Predicted and actual results of the digestion were compared and allowed to conclude if the construct is an expected result or not.

Restriction analysis procedure was set up according to the following ratio:

DNA……2µl

Enzyme……...….0.5µl

Buffer……..2µl

H_2O…….15.5µl Total: 20µl

All the components were mixed together and then incubated in a water bath for 1.5 hour for standard enzyme or 30 min for High Fidelity enzyme at the optimal for enzymes temperatures.

Results were recorded in a way described in section 4 of this chapter.

6. *DpnI* treatment

In order to get rid of the original template for the PCR amplification, and digest all methylated pDNA, fragments were treated with *DpnI*.

Reaction was set up according to the following:

Purified vector DNA……17µl

DpnI …….....….1µl

NEB Buffer 4…….....…..2µl Total: 20µl

Components were mixed together in a PCR tube and placed in a cycler set to the program DPNJP. Details of the program can be found in a table below.

Step	Temperature	Time
Enzyme digestion	37ºC	3h
Enzyme inactivation	80ºC	20 min
Final hold	4ºC	∞

7. Preparation of chemically competent cells

Chemically competent cells are used for heat-shock transformation. In other words, it is an introduction of a plasmid into the cell. During this project, several strains of competent cells were prepared like for instance: DH5α or ET12567. Cell strains used in this thesis are listed in the Table 3.

Protocol:

1) Competent cells from frozen stock or single colony from agar plate were incubated overnight at 37ºC in 2 ml of LB medium.
   Note: In case of ET12567, cells were incubated with addition of 2µl of chloramphenicol (25mg/l) and 1µl kanamycin (50 mg/l) in order to select for the helper plasmid.
2) 0.4ml of the overnight culture was transferred to 40 ml of LB medium and incubated for approximately 2h at 37ºC in a shaking incubator (225rpm). Incubation was continued until the OD_{600} reached 0.4 - 0.6.
   Note: As it was noted previously, antibiotics solutions have to be added to ET12567 cells at this stage as well – chloramphenicol 40µl and kanamycin 20µl.
3) The cell suspension was spinned down at 4500 rpm for 5 min at 4ºC after reaching desired concentration.
4) Supernatant was removed from the tube and replaced by 4 ml cold TSS-buffer.
5) Cells were incubated on ice for 1 hour and then aliquoted to several tubes 100µl each.
6) Cells were stored at -80ºC.

8. Preparation of electrocompetent cells

Electrocompetent cells are used during the transformation using the electroporation process. Its basis consists of employing electrical pulses to create pores which allow genetic material to permeate though the bacterial membrane. DH5α cells were prepared for the need of this master thesis.

Note: All the washing steps were performed on ice.

1) 300 ml of LB was inoculated with 3 ml of the overnight bacterial culture and incubated at 37ºC in a shaker until the OD_{600} reached 0.35 – 0.4.
2) When the right concentration of the cells was reached, flask was placed on ice for 20-30 minutes and occasionally mixed to ensure even cooling of the culture.
3) In the meantime, overall volume of the culture was divided among 6 cold cubic tubes, resulting in 50 ml of the culture per tube.
4) The cells were harvested by centrifugation at 2400 rpm for 20 min at 4ºC.
5) Supernatant was removed and cells were resuspended in 40 ml of ice cold dsH_2O.
6) Step 4 and 5 were repeated with 20 ml of ice cold dsH_2O.
7) Suspensions of 2 bottles were combined resulting in 3, 40ml flasks.

8) Cells were spinned down at 2400 rpm for 20 min at 4ºC.
9) Supernatant was removed and each pellet was resuspended in 20 ml of ice cold, 10% glycerol.
10) Tubes were centrifuged at 2400 rpm for 20 min at 4ºC.
11) Supernatant was aspirated not disturbing loosely adherent pellet. Cells were gently resuspended in 0.5ml ice cold 10% glycerol.
12) Suspension was aliquoted in a volume of 100µl to 1.5ml microcentrifuge tubes and immediately frozen and stored in -80ºC.

9. Transformation

Transformation is a vital technique used in molecular biology in order to research the genomics and proteomics of the microorganisms. It was firstly mentioned by Griffith in 1928 as a way of uptake of DNA by both prokaryotes and eukaryotes. In his project, he was focusing on *Pneumococcus* and studying acquisition of virulence by the non-virulent strain which lack the polysaccharide capsule. He discovered that this feature may be acquired by injection of heat-killed virulent bacteria with non-virulent inoculum to the peritoneal cavity of the mouse. He called this process "transformation". Further studies performed by Avery, McLeod, and McCarty identified the "transformation factor" which was DNA.

It is a process aiming to introduce extracellular DNA into the competent cells. During the heat shock or electropulsing step cell wall destabilizes which allows for plasmid to enter the cell. Introduced DNA often confers antibiotic resistance genes which make it possible to select transformed colonies on the plate. Only colonies bearing the resistance gene will survive on a plate with appropriate antibiotic.

A. by heat shock

1) Probe with frozen competent cell was slowly melted on ice.
2) 100µl of competent cells were mixed together with isolated DNA or Gibson ligation mix (1-3µl).
3) Solution was incubated on ice for 30 min.
4) After this time, cells were heat shocked for 30 sec at 42ºC. Immediately after, cells were put back on ice for 2 to 5 min.

5) 450-500µ of LB media was added to the samples, mixed and placed for 1 hour incubation at 37ºC.
6) 100µl of transformation mix was spreaded on the plate supplemented with antibiotics.
7) Plates were incubated overnight at 37ºC.

B. by electroporation

1) Probe with frozen electrocompetent cell was slowly melted on ice.
2) 100µl of competent cells were mixed together with isolated DNA or Gibson ligation mix (1-3µl).
3) Solution was incubated on ice for 30 min.
4) Mix was transferred to an already pre-chilled electroporation cuvette.
5) Cells were pulsed according to the following setup:
    - Voltage: 2500V
    - Capacitance: 25C
    - Resistance: 100Ω
    - Cuvette: 1mm
6) Electroporated solution was mixed with 450-500µ of LB media in a 1.5ml microcentrifuge tube and placed for 1 hour incubation at 37ºC.
7) 100µl of transformation mix was spreaded on the plate supplemented with antibiotics.
8) Plates were incubated overnight at 37ºC.

10. Yeast transformation

Yeast transformation using protocol for 'Yeast Transformation by LiAc/SS Carrier DNA/PEG Method' was employed.

First successful transformation to *Saccharomyces cerevisiae* was performed by Oppenoorth in 1960 but unfortunately his results turned out to be impossible to replicate (Gietz and Woods, 2001). Several methods were developed up-to-date. To mention some of them: lithium acetate/single-stranded carrier DNA/polyethylene glycol method, electroporation, agitation with glass beads, bombardment with DNA-coated microprojectiles and conversion of spheroplasts by treatment with Zymolyase.

Technique of yeast transformation which was used during this project is LiAc/SS Carrier DNA/PEG method. In order to obtain large number of transformants high-efficiency protocol was used. Gietz and Woods work managed to make significant advancements in the available protocols resulting in development of four protocols tailored to meet up the needs of several projects i.e. the rapid transformation protocol for the introduction of a plasmid into a yeast strain; the high-efficiency transformation protocol for recovering large numbers of transformants; the large-scale transformation protocol for screening complex plasmid libraries such as those required for a two-hybrid screen and the microtiter plate transformation protocols 3 for the simultaneous transformation of multiple strains or multiple samples of a single strain Gietz and Woods, 2002).

First reports regarding yeast transformation after treatment with alkali cations appeared in 1983. It was proved that yeast cells can be stimulated to uptake DNA plasmids by addition of monovalent alkali cations like Na^+, K^+, Rb^+, Cs^+, and Li^+ along with PEG (Gietz and Woods, 2001). After several modifications, efficiency of this method was increased from 400 to more than 1 x 10^6 transformants/µg plasmid DNA. The key to such an improvement was an addition of single – stranded carrier DNA which is a component of transformation mix. Since this breakthrough, technique acquired today's name. Even though the main principles of the method were established, it was still possible to better it. As a result of tremendous work, technique got simplified and optimized to a present shape. Some of the modifications which took place: reduction of exposure to LiAc and removal of TE buffer from the transformation mix.

Main advantage of this protocol which was utilized in this master thesis is an introduction of specific plasmid into particular strain of yeast with the focus to recover and analyze number of transformants (Gietz and Woods, 2006). Thanks to incubation in double – strength YPAD, growth and transformation efficiency significantly increased. After this step cells were harvested and washed with water. Further on, they were resuspended in the transformation mix which is composed from LiAc, ss-carrier DNA, PEG and plasmid DNA. After incubation at 42 ° for 40 minutes, cells were centrifuged, mix was removed and after addition of water they were ready for plating. Dilutions were plated onto Synthetic Complete (SC) selection medium (Gietz and Woods, 2002). Protocol has many other applications like screening multiple yeast genomes for plasmids that are able to complement particular mutation, transformation of yeast strain with an integrating plasmid or oligonucleotide, simultaneous

transformation of a yeast strain to different plasmids and also transformation of a plasmid library into two-hybrid yeast strain. Technique can be employed as a way to generate large number of transformants which is needed to screen eukaryotic cDNA libraries (Gietz and Woods, 2006). Advantages of this technique in comparison to other transformation methods are high efficiency of the process, relatively simple and easily optimized protocol which allows for using frozen cells, low cost and finally no demand for any special equipment. Drawbacks on the other hand are: requirement of growing cells in overnight culture and variability of efficiency in different strains (Gietz and Woods, 2001).

Transformation mix:

PEG3500 50% [w/v]……..…240µl

LiAc 1.0M…….....….36µl

SS carrier DNA (0.2 mg/…...……..50µl

Plasmid DNA…….……..34µl

Total: 360µl

Protocol:

Day 1

1) 3ml of YPAD broth was inoculated with the loop of yeast *S. cerevisiae* BY4742 or 10µl of overnight culture and incubated overnight in shaker at 30ºC.

Day 2

1) 2.5ml of the culture inoculated the previous day was transferred to 50ml 2xYPAD and incubated overnight in a shaker at 30ºC for approximately 4 hours.
2) In the meantime 1ml of carrier DNA was denatured in a boiling water bath for 5 min and afterwards chilled immediately on ice.
3) Cells after the incubation were harvested by centrifugation at 3000g for 5 min.
4) Pellet was washed twice in 25ml dsH_2O and the cells were finally resuspended in 1ml of dsH_2O.
5) Cell suspension was transferred to 1.5ml microcentrifuge tube and spinned down for 30s. Supernatant was discarded.

6) Cells were resuspended in 1ml of sterile water and aliquoted, 100µl per each transformation reaction.
7) Tubes were centrifuged at 13,000 rpm for 30s and supernatant was removed.
8) Transformation mix was prepared according to the description in a section above.
9) 360µ of mix was added to each transformation tube and resuspended vigorously.
10) Tubes were placed on a floating rack in a water bath for incubation at 42ºC for 40 min.
11) Samples were centrifuged for 1 min and Transformation mix was removed.
12) 500µl of dsH_2O was added to each transformation tube. Pellet was resuspended by pipetting or vortexing.
13) Cell suspension was divided into 5 and plated 100µl per plate on SCM medium.
14) Plates were incubated for 3-4 days at 30ºC.

11. Conjugative transfer of recombinant plasmid from *E. coli* ET12567 to *S. venezuelae*

First reports of intergeneric conjugation between *E. coli* and *Streptomyces* are dated 1989. From this time, process has been demonstrated also for many *Streptomyces* species. Intergeneric conjugation is used as a way of plasmid transfer where *E. coli* serves as a donor. This technique makes possible to construct and manipulate recombinant plasmids placed in *E. coli* which are later on transferred to *Streptomyces* (Mazodier et al., 1989).

E. coli ET12567 containing pUZ8002 which is a RK2 derivative was used in this protocol. RK2 represent that the organism is able to replicate in a wide-range of single-cellular hosts. pUZ8002 is capable of transferring functions to *oriT*-carrying plasmids, but due to the mutation in its own *oriT*, its mobility is inhibited (Paget et al., 1999).

Preparation

- Spores of *S. venezuelae* were inoculated on ISP4 plate and incubated at 30ºC for approximately 4 days until achievement of dense, confluent lawn.
- Recombinant plasmid has been transformed to *E. coli* ET12567 selecting only for one vector marker i.e. apramycin.

3 transformants were picked from the plate and spread in three sector-divided plate onto LA supplemented antibiotics in order to select for the original pUZ8002 and assembly-encoded plasmid i.e. with apramycin 100µl/l, chloramphenicol 25µl/l and kanamycin 25µl/l. Plates were incubated overnight at 37ºC.

Protocol

1) Spore suspension of *S. venezuelae* was prepared by pouring 5ml of sterile water onto the plate and scrapping off the mycelium with the pipette, resuspending it up and down and finally filtering the suspension through the sterile cotton pad placed in 5ml syringe.
2) 50µl of spore suspension was transferred to 350µl of 2xYT medium in eppendorf tubes. Suspension was thoroughly mixed and incubated for 5min at 50ºC. Samples were cooled down at room temperature for the next 5 min.
3) All the *E. coli* cells were scrapped off from the corresponding sectors and resuspended in 500µl of 2xYT medium in eppendorf tubes.
4) 100µl of *E. coli* suspension was added to heat-shocked spores of *S.venezuelae*, mixed and spinned down at 13,000 rpm for 2 min.
5) Supernatant was removed leaving only 100µl of the total volume of the sample. Pellet was resuspended and mixture was spreaded onto the ISP4 plate supplemented with $MgCl_2$.
   Note: Plates were dried for 15 minutes at the sterile hood prior to plating.
6) Conjugation plates were incubated in room temperature for approximately 20 hours loosely covered with aluminium foil.
7) After this time each conjugation plate was overlayed with 1 ml of antibiotic solution containing nalidixic acid and apramycin in final concentrations 30µl/ml and 15µl/ml respectively. Solution was distributed gently over the entire surface using sterile spreader.
8) Plates were incubated for 3-4 days at 30ºC until visible colonies appeared.

Modifications for *S. albus* conjugation:

- Spores solution was heat-shocked for 10 min at 50ºC.
- Instead of incubation of conjugation plates in room temperature for 20 hours, plates were placed for 20 hours at 30ºC.

- Instead of ISP4 with $MgCl_2$, samples were plated on SFM medium supplemented with. $MgCl_2$.

12. Second crossover selection

Second crossover selection was performed in pJP2 *S.venezuelae* construct. After obtaining first crossover strain by conjugation, microorganism was cultivated in three rounds of growth, one day each.

- Experiment was conducted in 2ml TSB medium without antibiotics, incubated at 30ºC overnight.
- 100µl culture from the previous day was used as the inoculum for the next one.
- On the third day 10^{-6} dilution of the culture suspension was prepared and plated on 20 ISP4 plates, incubated for 2-3 days until growth was visible at 30ºC.
- Plates were printed on ISP2 plates supplemented with Am_{50} using replica plating method and incubated for 2-3 days at 30ºC.
- Results were compared. If the second crossover was successful, clones should lose Am^R. In this way, clones should grow on ISP4 plate but not on ISP2 Am_{50}. Comparison was conducted by putting plates on one another and observing growth.

13. Replica plating

First general description of replica plating technique was provided by Lederberg & Lederberg in 1952. In this master thesis it was used to select the mutants which underwent second crossover. Selective medium was ISP2 supplemented with Am_{50}. Mutants susceptible for apramycin did not grow on ISP2 Am_{50} plates.

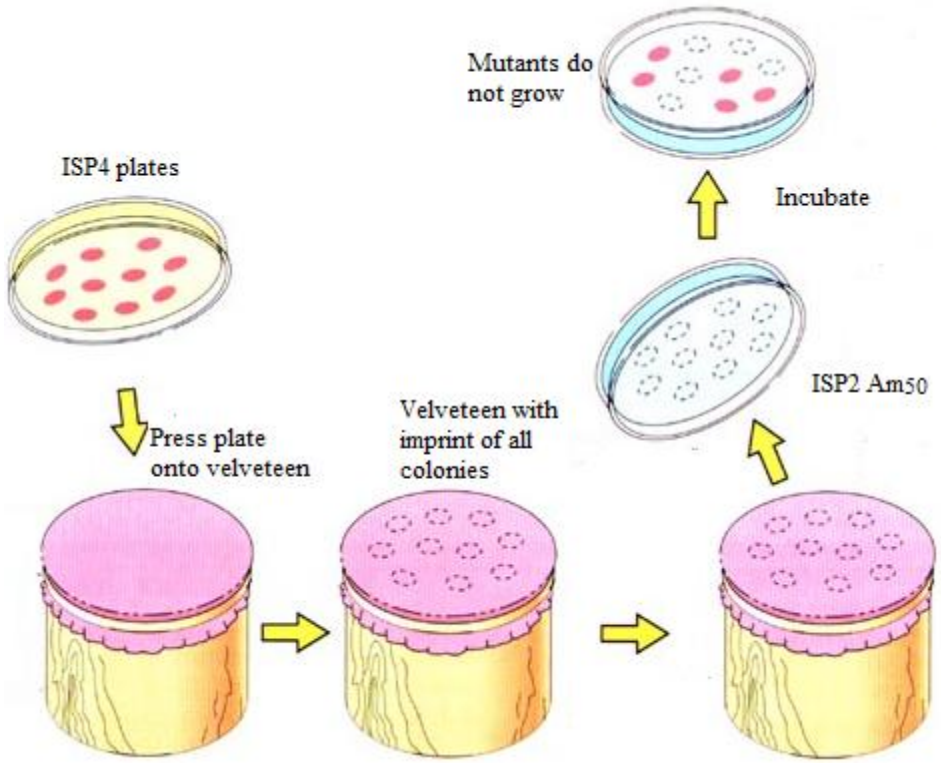


Figure 7. Scheme of replica plating method (modified from the webpage http://utminers.utep.edu/rwebb/html/isolation_of_spontaneous_mutat.html)

14. Gibson Assembly

Gibson assembly is a cloning method which is referred to as "seamless cloning". It was developed by the Daniel G. Gibson from J. Craig Venter Institute (JCVI) along with collaboration with Synthetic Genomics Inc. (SGI). Method combines attributes from several other solutions in order to create simple, one-step isothermal *in vitro* recombination technology.

Enzymes used in Gibson Assembly are: 5′ T5 exonuclease, Phusion DNA polymerase and *Taq* DNA ligase. They exploit following enzyme specificities: exonuclease activity – which chews back the ends of DNA fragments exposing single stranded DNA overhangs which can anneal to their single stranded complement DNA, DNA polymerase activity which fills the gaps in the annealed product and DNA ligase activity which covalently seals nicks appeared as a result of the assembly (New England Biolabs website). It is possible to use *Taq* DNA polymerase in the place of Phusion DNA polymerase, but since latter possess proofreading

activity which avoids presence of noncomplementary sequences, it is highly preferred (Gibson et al., 2009). Gibson Assembly gained its popularity due to the simplicity and robustness of the method. In order to acquire the successful product, DNA fragments with appropriate overlaps and mix of three enzymes with aforementioned specificity are needed. Reagents are mixed and incubated at 50ºC for 1h. Resulting product without any further modifications is suitable for downstream processing (New England Biolabs website). Gibson Assembly can be used for joining fragments as large as 583 kb and to clone joined products in Escherichia coli the size of 300 kb (Gibson et al., 2009).

There are several advantages of the Gibson Assembly. Firstly, it allows for insertion of one or more DNA fragments into theoretically any possible position of the linearized vector. Moreover, it is not dependent on the presence of restriction sites in the target sequence. Fragments are designed in a way to contain 20 – 40 bps overlaps. Thanks to this method, unwanted insertions may be avoided. In addition, significantly larger amount of DNA fragments can be joined together. Lastly, reaction is much faster than any other this type of solution available so far (New England Biolabs website). The reason for that is possibility of mixing all compounds in one isothermal reaction. Since enzymes are not competing with each other they can act simultaneously (Gibson et al., 2009).

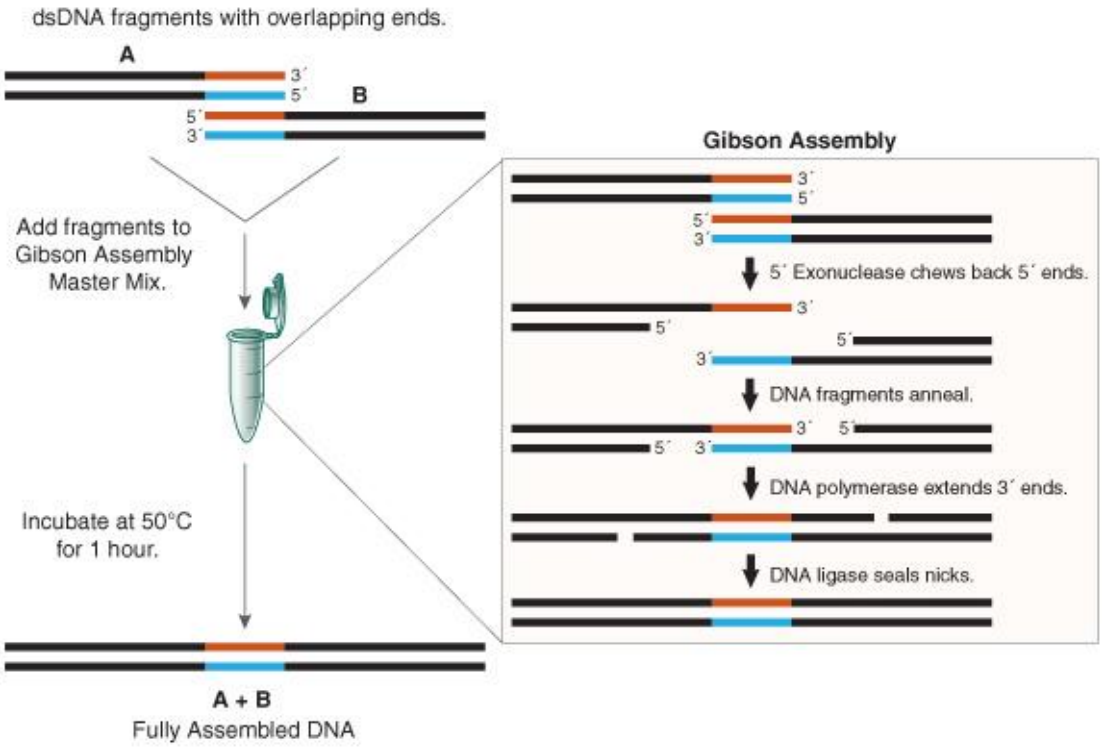


Figure 8. Overview of the Gibson Assembly Cloning Method (New England Biolabs website).

In order to prepare Gibson Mix following components were mixed together:

5 x isothermal reaction buffer*……320µl

10U T5 exonuclease…….....….0.64µl

2U Phusion DNA polymerase……..20µl

40U Taq DNA ligase……..160µl

H_2O……699.36µl

Total: 1.2ml

Gibson Mix was aliquoted to PCR tubes 15µl each and stored in -20ºC.

*components of 5 x isothermal reaction buffer:

Tris-HCl (1M)……..…3ml

$MgCl_2$ (2M).....150µl

dGTP (100mM).....60µl

dATP (100mM)..... 60µl

dTTP (100mM)..... 60µl

dCTP (100mM)……...……...60µl

DTT (1M)…….300µl

PEG_{8000}……..…….1.5g

NAD (100mM)……..…300µl

Gibson reaction protocol:

1) 15µl of Gibson reaction mix was slowly thawed on ice.
2) 5µl of DNA was added to the tube with the mix.

3) Sample was placed in PCR cycler set up for the program OSGIB.
4) 1-3µl of the reaction mix was used subsequently for transformation, either by heat-shock or electroporation. Protocol for transformation can be found in 9th section of Lab Protocols chapter of this master thesis.

15. Extraction of chloramphenicol from cultures of *S. albus*

Protocol

1) 1ml of culture was centrifuged at 13,000 rpm for 5 min, then 0.5ml of supernatant was transferred to sterile microcentrifuge tube.
2) 0.5ml of supernatant was overlayed with 0.2ml ethyl acetate and extracted by vortexing for 1 min and subsequently centrifuged at 13,000 rpm for 2 min. Arising upper phase was transferred to a clean tube.
3) Ethyl acetate extraction was repeated 2 more times with same volume of ethylacetate.
4) All the extracts were combined in one tube, making 0.6ml of the liquid.
5) Samples were open under the fume hood and let stand until all the ethylacetate was evaporated. This may last even overnight. For faster evaporation, vacuum drying was recommended.
6) Dry matter was dissolved in 100µl of water and methanol in the ratio 50:50 and stored in -20ºC.

16. Glycerol stocks

Glycerol stocks were used for long storage of bacterial suspensions at -80ºC.

Protocol for *E. coli* glycerol stocks:

1) 2 ml of overnight culture was transferred to 1.5 microcentrifuge tube, spinned down for 2 min at 13,000 rpm. Supernatant was removed.
2) Pellet was resuspended in 1.5 ml 20% glycerol solution, transferred to Cryo tube and stored at -80ºC.

Protocol for *S. venezuelae* glycerol stocks:

1) 5 ml of sterile water was poured onto the confluent plate using the pipette. Surface of the plate was gently rubbed, resulting in a spore suspension which was transferred to a syringe supplemented with cotton pad sitting in a 5 ml tube.

2) Filtered suspension was centrifuged at 5,000 rpm for 5 min.
3) Supernatant was removed and pellet was resuspended in 1.5 ml 20% glycerol solution, transferred to Cryo tube and stored at -80ºC.

17. Assessment of chloramphenicol production for pJP2 construct

1) 2 tubes of 3 ml TSB inoculated one with *S. venezuelae* wild-type and second with final pJP2 construct in *S. venezuelae* were incubated overnight at 30ºC.
2) 1 ml of each starting culture was transferred to 30 MYM medium and incubated further 9 hours.
3) After this time, each culture was divided into two; one part was supplemented with 1 ml of 98% ethanol, the other with 1 ml of purified water. Culture was incubated for 48h at 30ºC in shaking incubator.
4) Chloramphenicol was extracted using ethyl acetate method.
5) Samples were sent over for UPLC analysis.

Results and discussion

I. pJP1 and pJP1gusA construct assembly

pJP1 construct (see Appendix D for plasmid map) was assembled via yeast transformation from PCR amplified and purified fragments (according to the setup PCR I which can be found in section V. Lab protocols; 2. PCR amplification). It consists of suicide vector amplified from pCLY10 template, *gusA* gene amplified from pUC57 template and its two flanking regions amplified from *S. venezuelae* genomic DNA containing regulatory gene *jadR1* and structural genes *jadF* and *jadG'*. The purpose of creating this plasmid was to remove *jadJ-E* structural genes of *S. venezuelae* and replace them with reporter gene which when expressed, produces β-glucuronidase allowing for detection of quantifiable signal. Since JadR1 acts as a positive activator of deleted structural jadomycin gene cluster, functionality of the GUS system was planned to be assessed under ethanol shock induction.

Assembly in *S. cerevisiae* BY4742 was subsequently transformed to *E. coli* DH5α. The results of restriction analysis with *BamHI* revealed expected pattern of bands during gel electrophoresis i.e. 5.9 kb, 2.3 kb, 1.1 kb, 0.95 kb, 0.46 kb. Due to the slower movement of the high G+C content DNA through the gel, bands are positioned slightly higher.

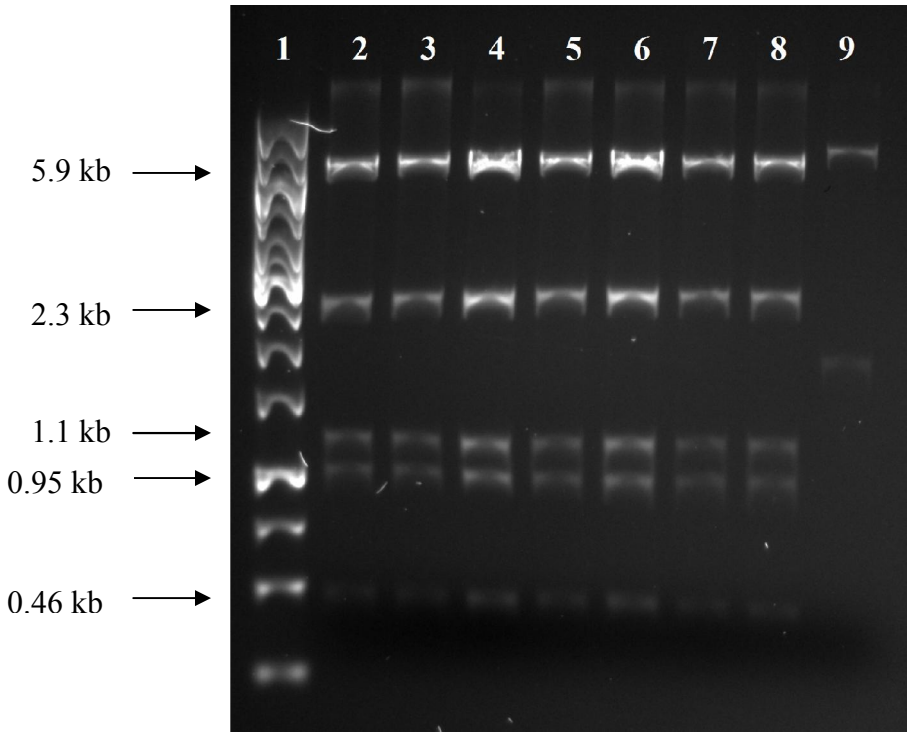


Figure 9. Results of restriction analysis of pJP1 construct in *E. coli* DH5α. Wells from the left: DNA ladder; *BamHI* digested clone no. 1-7, pCLY10

pJP1 construct was subsequently transformed to *E. coli* ET12567 and then conjugated to wild-type strain of *S. venezuelae*. However, due to the discovered mutation in the *gusA* gene this fragment had to be replaced.

pJP1gusA assembly - construct for deletion of *jadJ-E* genes in *S. venezuelae* with another version of *gusA* region was performed by firstly amplifying *gusA* gene from pSOK808 and rest of the assembly using as a template pJP1. Gibson Assembly with subsequent transformation to strain like *E. coli* DH5α, G10 Elite, XL1-Blue or *E. cloni* 10G was attempted. Restriction cutting revealed that none of the clones resulted in acquisition of the right construct. This was the reason why GUS assay could not be executed as it was initially planned. Gibson Assembly difficulties in obtaining the right construct were expected to be connected with high G+C content of *Streptomyces* genome which facilitates secondary structures formation. These may significantly impede formation of the correct construct. Also, polymerase mistakes introduced during the PCR could remove restriction sites which resulted in wrong pattern of the restriction cutting.

II. pJP2 construct assembly

pJP2 (see Appendix D for plasmid map) assembly fragments were firstly amplified by PCR (according to the setup PCR II which can be found in section V. Lab protocols; 2. PCR amplification) and subsequently purified on the column. pJP2 is a construct for replacement of the *cmlI* promoter in *S. venezuelae* with *jadJ* promoter. It consists of suicide vector amplified from pCLY10 template, *jadJ* promoter and its two flanking regions composed of structural genes of chloramphenicol antibiotic cluster amplified from *S. venezuelae* genomic DNA. The idea was to replace *cmlI-X* promoter with *jadJ* promoter which is positively activated by *jadR1* upon ethanol shock and in this way regulate chloramphenicol production. Construct was firstly assembled in yeast and then transformed to *E. coli* DH5α cells. DNA was isolated and subjected to restriction analysis. Restriction cutting with *EcoRI, EcoRV* and *PstI* and subsequent gel electrophoresis resulted in expected pattern of bands. For *EcoRI* – 3.8 kb, 3.7 kb, 1.9 kb; *EcoRV* – 5.2 kb, 3.5 kb, 0.6 kb; *PstI* – 5.6 kb, 3.8 kb. Pattern for control with pCLY10: *EcoRI* – 4.3 kb, 3.7 kb; *EcoRV* – 4.4 kb, 3.5 kb; *PstI* – 5.6 kb, 1.1 kb, 0.7 kb, 0.6 kb. Digestion with *PstI* resulted in something which may be suspected as star activity of the enzyme. Due to that reason, band on the agarose gel are significantly smeared.

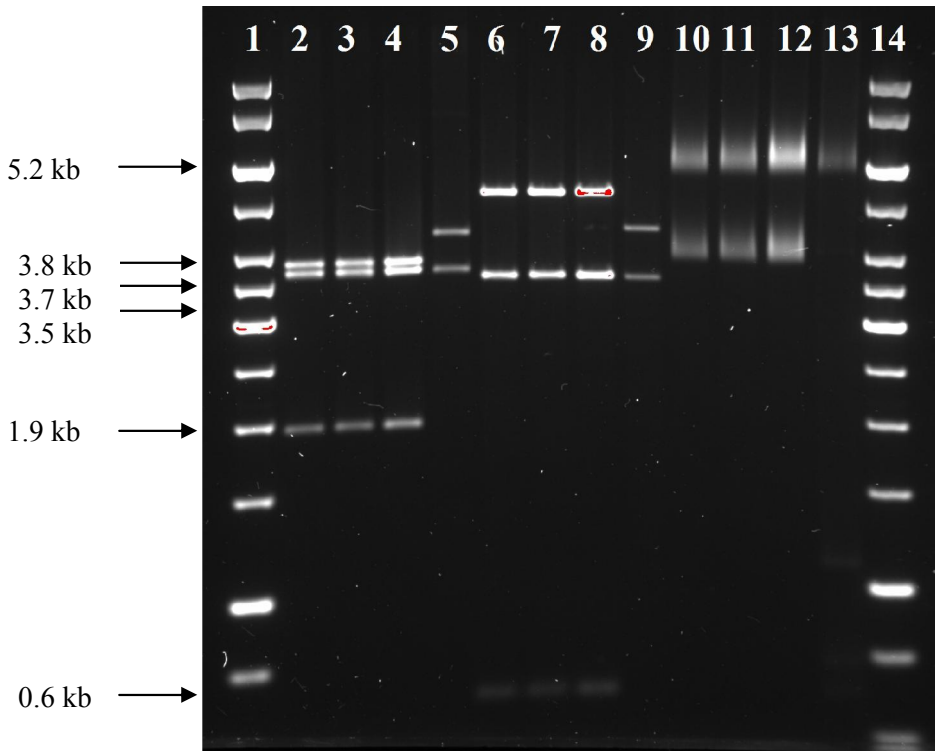


Figure 10. Results of restriction analysis of pJP2 construct in *E. coli* DH5α. Wells from the left: DNA ladder; *EcoRI* digested clone no. 1, 2, 3, pCLY10; *EcoRV* digested clone no. 1, 2, 3, pCLY10; *PstI* digested clone no. 1, 2, 3, pCLY10; ladder.

pJP2 construct in *E. co*li DH5α was subsequently transformed to *E. coli* ET12567 and conjugated to wild-type strain of *S. venezuelae*. PCR products from confirmation test ran on the agarose gel resulted in desired band pattern i.e. 5.7 kb for the pair of primers Y10_SVcml-F + R, 3.8 kb for the pair CmlI-F + CmlX-R. Control with *S. venezuelae* wild-type was included which revealed the following band pattern: no product for the Y10_SVcml-F + R pair; 3.5 kb for CmlI-F + CmlX-R. Size of the bands was predicted in Clone Manager software. Setup for all PCR programs can be found in Materials and Methods section.

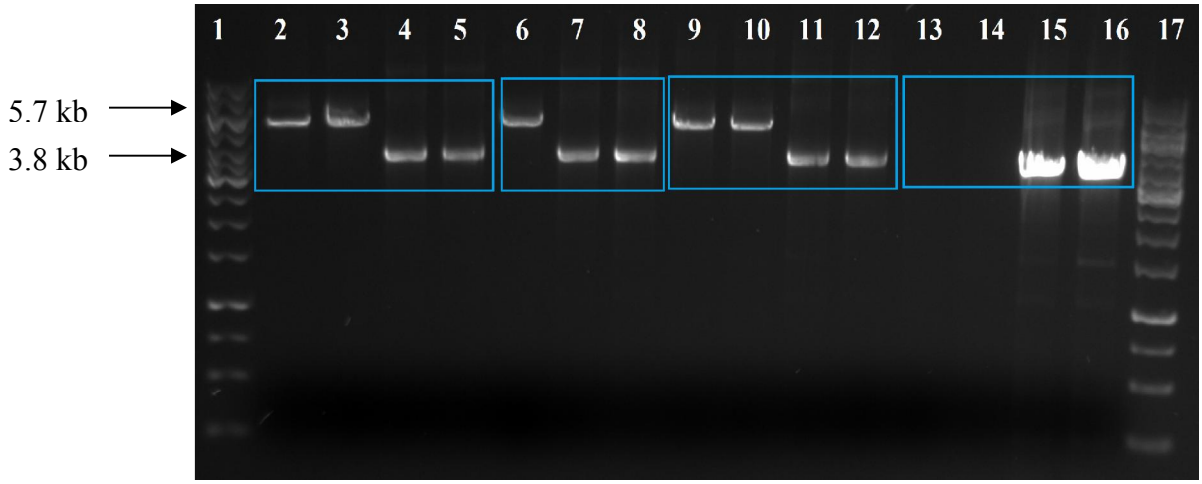


Figure 11. Results of PCR confirmation test. Well order: 1, 17 - ladder; 2-3 clone no.1, 6 clone no.2, 9-10 clone no.3, 13-14 control - Y10_SVcml-F + R primers; 4-5 clone no.1, 7-8 clone no.2, 11-12 clone no.3, 15-16 control - CmlI-F + CmlX-R primers.

After the positive confirmation of *S. venezuelae* transconjugants, second crossover selection was performed. Protocol can be found in section V. Lab protocols; 12. Second crossover selection. Second crossover selection was based on the hypothesis that due to the homologous recombination, construct will loose resistance to apramycin. Using replica plating technique, colonies grown on the ISP4 plates were stamped onto $ISP2_{Am50}$. After 2-3 day incubation, results were compared. Clones which kept Am^R formed colony, those who lost it however, did not. In this way, based on antibiotic resistance selection, screening for second-crossover mutants was performed. After total DNA isolation, PCR check was conducted. Control with

wild-type of *S. venezuelae* strain was added. As in the previous gel, no band with Y10_SVcml-F + R primers and 3.8 kb band with CmlI-F + CmlX-R primers was expected. Since the clones were sensitive to apramycin, they must have lost the vector, and thus seemed to be correct.

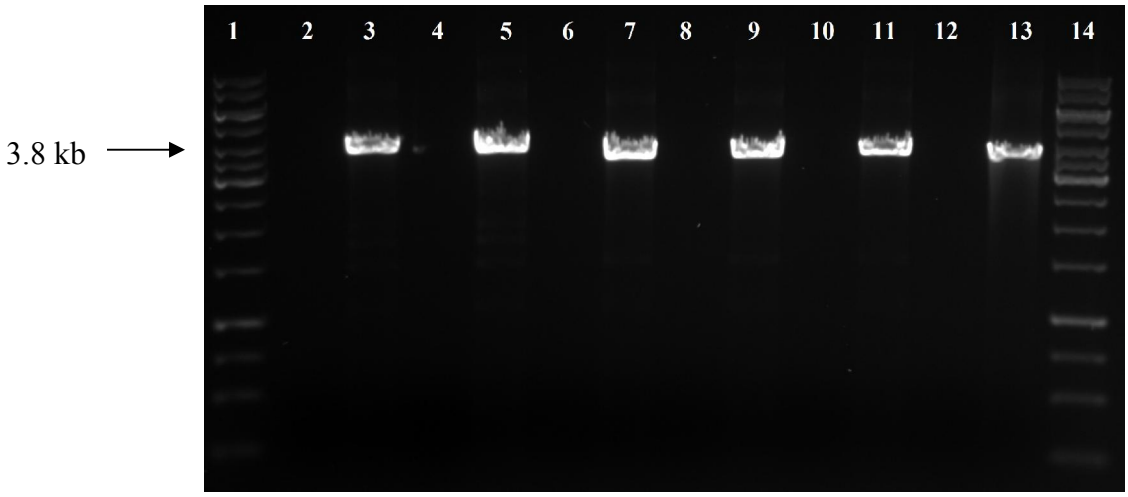


Figure 12. Second crossover selection PCR check. Well order: 1, 14 - Ladder, 2, 4, 6, 8, 10, 12 – clones no. 1, 2, 3, 4, 5, control respectively with Y10_SVcml-F + R primers; 3, 5, 7, 9, 11, 13 – clones 1, 2, 3, 4, 5, control respectively with CmlI-F + CmlX-R primers.

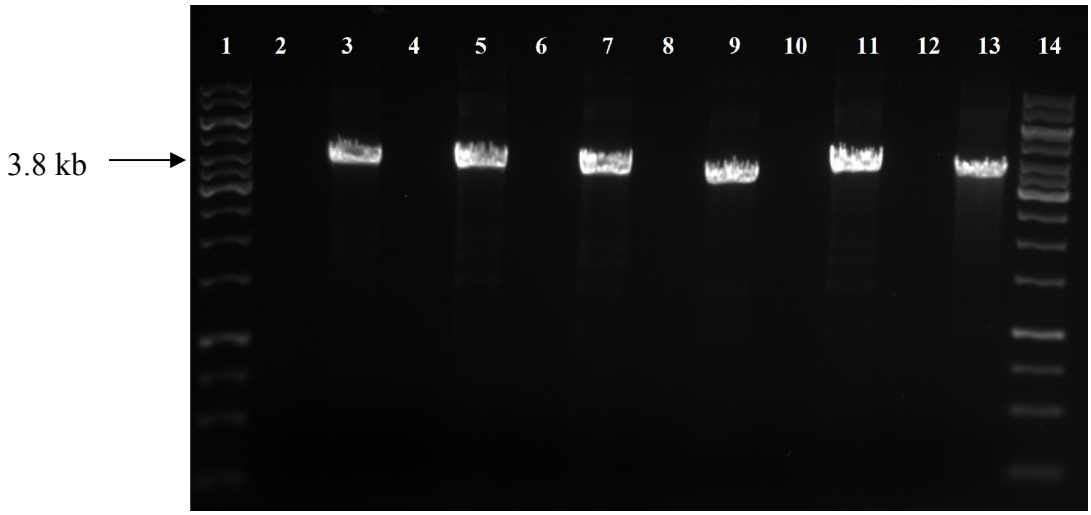


Figure 13. Second crossover selection PCR check. Well order: 1, 14 - Ladder, 2, 4, 6, 8, 10, 12 – clones no. 6, 7, 8, 9, 10, control respectively with Y10_SVcml-F + R primers; 3, 5, 7, 9, 11, 13 – clones 6, 7, 8, 9, 10, control respectively with CmlI-F + CmlX-R primers.

Since all the clones looked potentially as expected, restriction analysis with *HindIII* was performed as a supplementary test. Simulation in Clone Manager and subsequent electrophoresis revealed following band sizes for the right clones: for the pair of primers CmlI-F + CmlX-R – 1.9 kb and 1.8 kb. Clones marked red were identified as successful double crossovers.

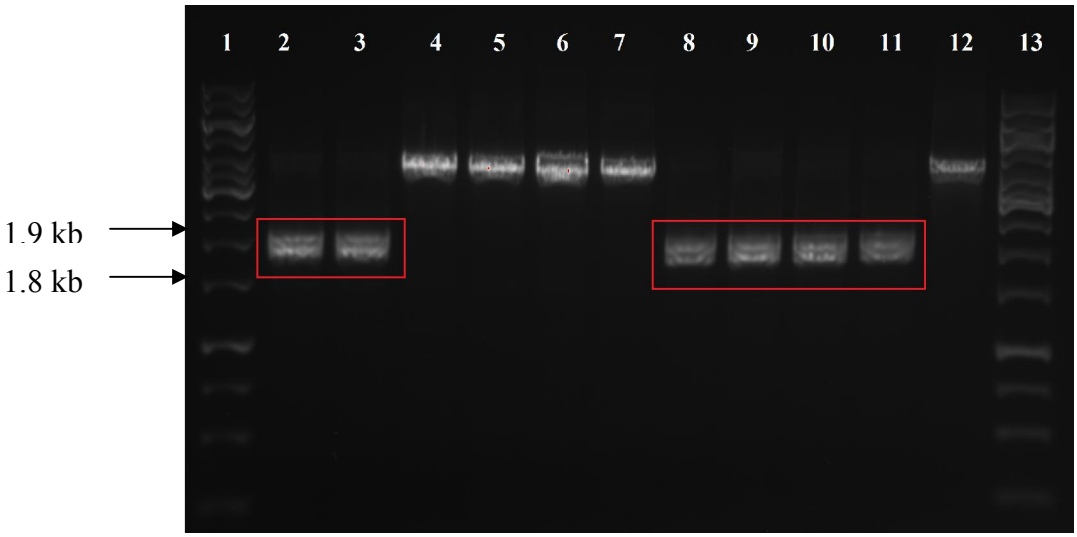


Figure 14. Second crossover transconjugants after restriction cutting with *HindIII*. PCR products from the previous check were subjected for analysis. Wells: 1, 13 – ladder; 2-12 clones.

Digestion with *HindIII* revealed also some false-positive clones.

Testing of the recombinant *S. venezuelae* JP2 strain for dependence of chloramphenicol biosynthesis on ethanol shock was performed. Experiment was executed according to the protocol found in section V. Lab protocols; 17. Assessment of chloramphenicol production for pJP2 construct. Chloramphenicol was extracted using ethyl acetate method from four samples: **1**. *S.venezuelae* wild-type; control with water, **2**. *S. venezuelae* wild-type; ethanol added, **3**. *S.venezuelae* JP2 construct; control with water, **4**. *S.venezuelae* JP2 construct; ethanol added. Samples were sent over for UPLC analysis. Raw results from UPLC are presented in the Appendix F. Chloramphenicol concentration was calculated according to the following equation, taking under consideration its molar mass 323.13 g/mol.

C_{Cml}[µg/l] = µM in the sample x molar mass, converted to [µg/ml]

Table 4. Results from UPLC analysis.

No.	Strain of *S. venezuelae*	Ethanol	Chloramphenicol concentration [µM]	C_{Cml}[µg/ml]
1.	Wild-type	-	0.3	0,10
2.	Wild-type	+	14	4,52
3.	JP2 mutant	-	0.0	0,00
4.	JP2 mutant	+	67.6	21,84

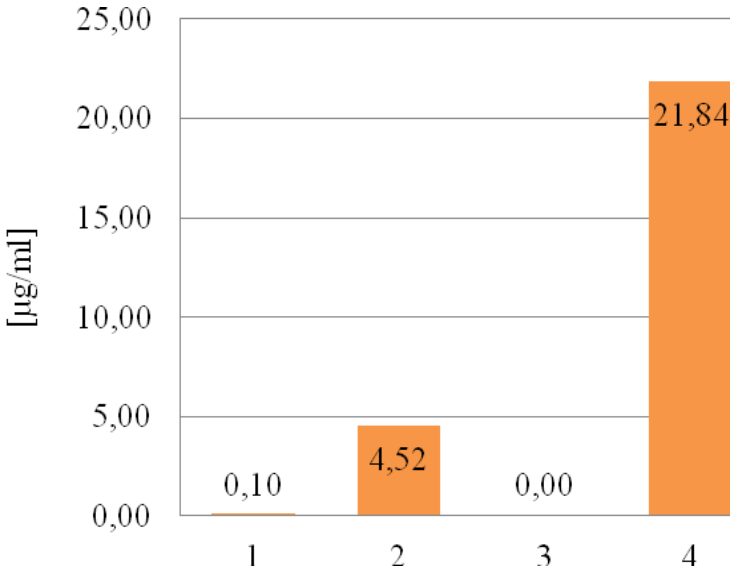


Figure 15. Chart representing chloramphenicol concentration in the samples from UPLC analysis. Numbers on the horizontal axis correspond with number of the sample in the Table 3.

Results from the UPLC revealed surprisingly low chloramphenicol production in *S. venezuelae* wild-type which was not induced by ethanol shock in comparison to the treated one. It is unusual due to the fact, that chloramphenicol is a metabolite which is expressed in standard conditions. JP2 mutant on the other hand, when not induced, produces absolutely no chloramphenicol. When exposed to ethanol shock however, it produces five times more antibiotic than ethanol-shocked wild-type strain.

This data support the main objective of this master thesis which was re-wiring of the antibiotic biosynthesis regulation in *S. venezuelae*. Exchange of the promoter which drives expression of chloramphenicol structural genes caused its production completely dependent on the ethanol shock.

III. pCML3 construct assembly

pCML3 is a construct assembled for heterologous expression of antibiotic gene cluster from *S. venezuelae* in *S. albus*. *S. albus* was chosen as a suitable organism for this experiment because it doesn't produce chloramphenicol. *S. venezuelae* on the other hand is a natural producer of chloramphenicol. Approach consisted of cloning the fragment of DNA of producing microorganism along with gene cluster of interest and attempting to express it in suitable host.

PCR of the fragments was performed according to the setup described in section PCR III which can be found in V. Lab protocols; 2. PCR amplification. Assembly consists from vector amplified from pCLY10 template and two additional fragments using genomic DNA from *S. venezuelae* as a template. They include structural genes of chloramphenicol biosynthetic gene cluster. After purification of the fragments they were transformed to yeast and subsequently introduced to *E. coli* DH5α. Digestion with *EcoRI* and *PvuII* resulted in desired band localization on the agarose gel during electrophoresis. For *EcoRI*: 15.9 kb, 7.5 kb 3.7 kb, 3.0 kb*; PvuII*: 12.0 kb, 6.9 kb, 6.0 kb, 2.1 kb, 1.8 kb, 1.2 kb. Control with pCLY10 cut with *EcoRI*: 4.3 kb, 3.7 kb; *PvuII*: 8 kb. As a supplementary ladder due to the big size of construct, isolated DNA from phage lambda cut with *HindIII* was used. Fragments are 23.1 kb, 9.4 kb, 6.6 kb, 4.3 kb, 2.3 kb, 2.0 kb, 0.5 kb.

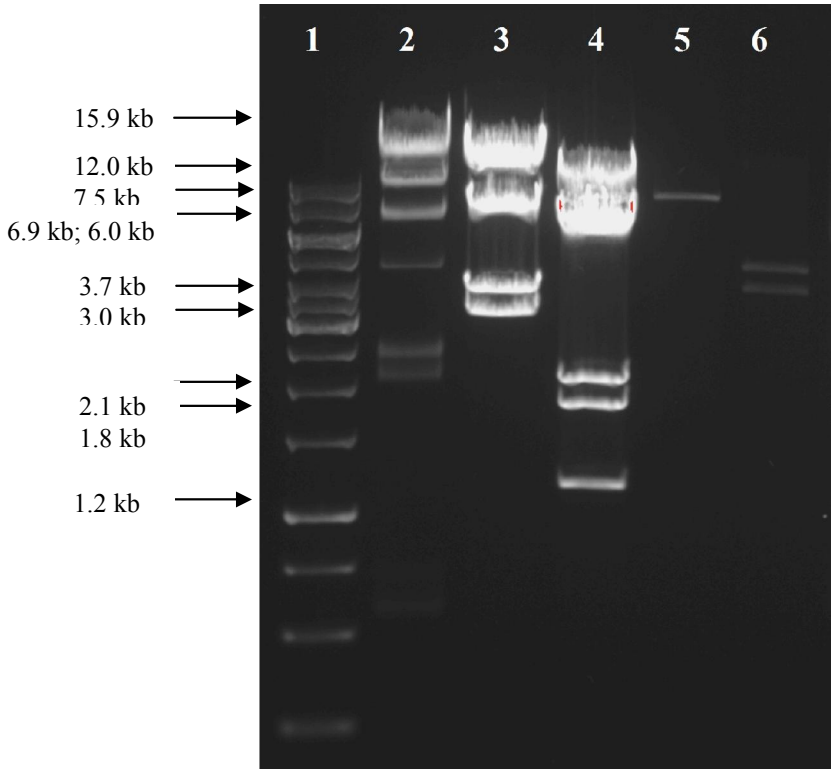


Figure 16. Restriction analysis of DNA isolated from *E. coli* DH5α clone. Wells from the left: 1. 1 kb Ladder, 2. Lambda *HindIII* Ladder, clone no. 1 cut with *EcoRI*, clone no. 1 cut with *PvuII*, clone, pCLY10 cut with *PvuII*, pCLY10 cut with *EcoRI*.

Subsequent transformation to *E. coli* ET12567 cells and conjugation to *S. albus* J1074 resulted in one transconjugant from which DNA was isolated and checked by PCR. Control with *S. venezuelae* wild-type carrying chloramphenicol cluster was added and samples were run on the agarose gel. Expected band pattern was obtained and it allowed proceeding to the further part of the experiment. Simulation of PCR product with primers Cml3H1-F + R resulted in fragment of 11.69 kb; with Cml3H2-F + R – 11.77 kb. Control had same fragment sizes.

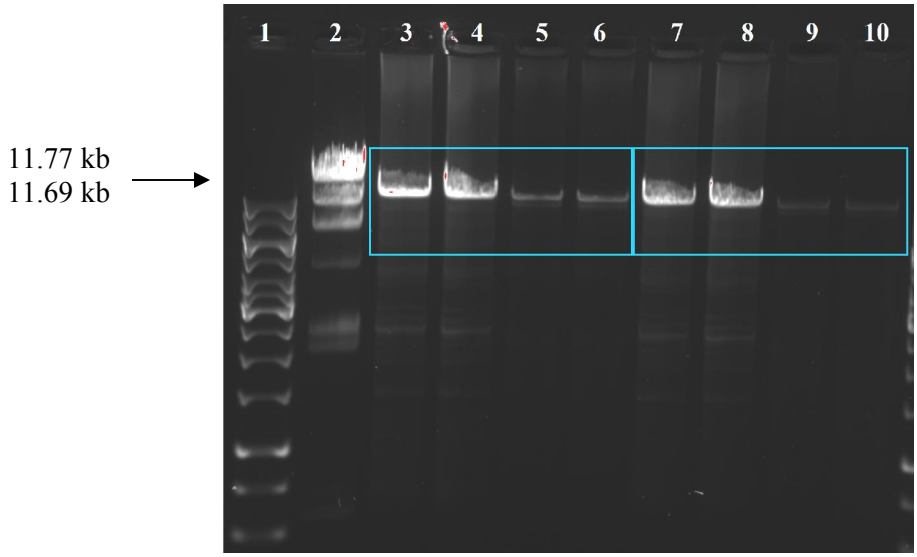


Figure 17. PCR confirmation check. Wells: 1. 1 kb Ladder; 2. Lambda *HindIII* Ladder; 3, 4 – clone, Cml3H1-F + R primers; 5, 6 – clone, Cml3H2-F + R primers; 7, 8 – *S. venezuelae* control, Cml3H1-F + R primers; 9, 10 - *S. venezuelae* control, Cml3H2-F + R primers.

Recombinant *S. albus* carrying pCML3 was then subjected to incubation, first in 2 ml TSB with Nal_{30} and Am_{50} overnight at 30ºC. Consequently, 500µl of the starter culture was transferred to 50 ml PM4-1 and NZA media, and incubated for 7 days in shaking incubator at 30ºC. Negative control with pCLY10 in *S. albus* was added. After that time, cultures were extracted with ethyl acetate for assessment of chloramphenicol production. Tubes were handed out for assessment of chloramphenicol content by UPLC analysis to external lab. Unfortunately, no chloramphenicol production was detected for the tested strains.

1. Complementation experiment

In addition to the attempt of heterologous expression of chloramphenicol cluster from pCML3, complementation experiment was performed. Double deletion *S. venezuelae* mutant - *JadRI/CmlΔ* was provided by the colleague from the lab Jianhai Zhang. The idea was to introduce chloramphenicol structural genes and complement the deletion. In order to ensure that and avoid interference, chloramphenicol gene cluster in deletion mutant had to be completely removed. In addition, due to the deleted *jadR1* gene, introduction of chloramphenicol genes will lead to its overproduction. It is caused by the lack of repression normally enforced by *jadR1*. *JadRI/CmlΔ S. venezuelae* was conjugated with pCML3 *E. coli* ET12567. Transconjugants were checked by PCR amplification and subsequent

electrophoresis analysis. Bands were simulated in Clone Manager and expected as follow: for the clones – primers Cml3H1-F + R 11.69 kb and primers Cml3H2-F + R 11.77 kb. Control with *S. venezuelae* WT was added. Size of the bands is the same like for the transconjugants. Correct results are marked red.

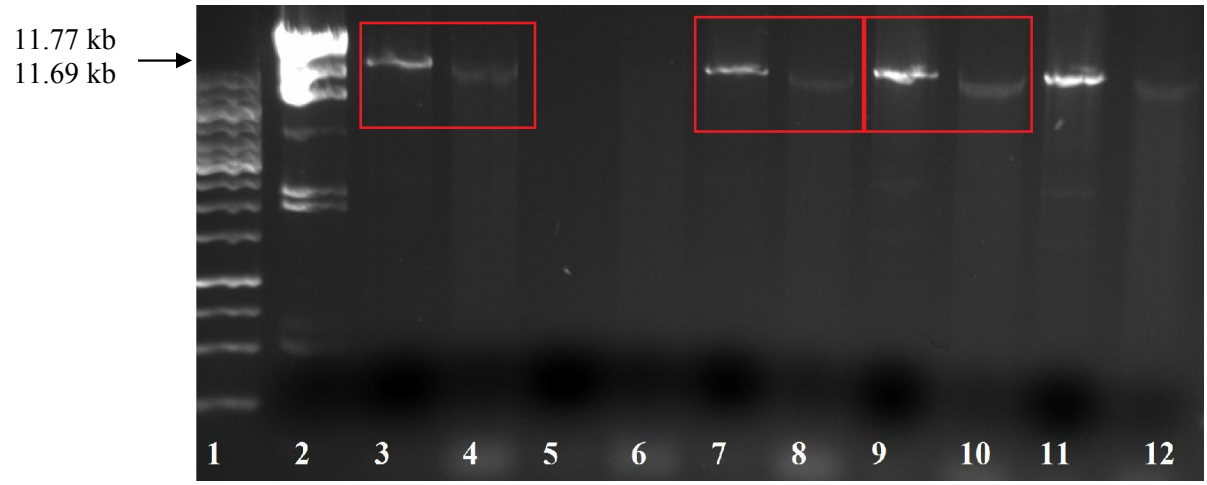


Figure 18. PCR confirmation check. Wells: 1. 1 kb Ladder; 2. Lambda *HindIII* Ladder; 3, 5, 7, 9, 11 – clones no. 1, 2, 3, 4, control - Cml3H1-F + R primers; 4, 6, 8, 10, 12 – clones no, 1, 2, 3, 4, control - Cml3H2-F + R primers.

Similarly to the previous experiment, 2ml TSB was inoculated with complemented *S. venezuelae* mutant for overnight incubation at 30ºC. 500µl of next day culture served as inoculum for 7 days incubation in 50 ml PM4-1 in shaking incubator at 30ºC. Along with that construct, positive control with *S. venezuelae* wild-type and negative with *S. venezuelae JadRI/CmlΔ* mutant were conducted. After incubation, chloramphenicol was extracted with ethyl acetate, and in order to assess the amount of the antibiotic in the cultures, samples were sent for UPLC analysis. No chloramphenicol production was observed in the complemented mutant. Negative control – non-complemented mutant, resulted in no chloramphenicol production. Positive control - wild-type *S. venezuelae* produced significant amount of chloramphenicol.

pCML3 construct was successfully assembled during the span of this master thesis, demonstrating usefulness of both pCLY10 vector and yeast-based DNA assembly for rapid cloning of gene clusters. PCR confirmation tests revealed appearance of the correct clones. When checking the functionality of the construct by assessment of chloramphenicol production by UPLC, it was revealed that the cluster is not working properly, as no

chloramphenicol was detected in the samples. It might be expected that the reason for non-functional construct are mistakes introduced during PCR by polymerase. Due to the high G+C content of the *Streptomyces* DNA, polymerase is more likely introducing mutations. Nothing happen when the place of mutation resides in not essential part of the genome. However if it disrupts one of the vital genes, it may impede the functionality of the whole cluster. Most likely this was the case here.

Future recommendations

Many of the significant obstacles in this master thesis most likely arose from the mutations introduced by DNA polymerase during PCR. If those could be avoided, results of the experiments wouldn't be based in such a great extent on chance. Artificial gene synthesis offers solution to these problems but it is too expensive at the moment to include it in everyday lab routines. With the time, like any other developed technology so far, it will become certainly cheaper. Affordable price will make it possible to increase its use.

Conclusions

During the time span of this master thesis four constructs were attempted to be assembled. **pJP1** – construct for deletion of of *jadJ-E* genes in *S. venezuelae;* **pJP1gusA** – construct for deletion of the *jadJ-E* genes in *S. venezuelae* with another version of *gusA* region; **pJP2** – construct for replacement of the *cmlI* promoter in *S. venezuelae* with *jadJ* promoter and **pCML3** – construct for heterologous expression of chloramphenicol antibiotic gene cluster. pJP1 assembly due to the discovery of mutations in *gusA* gene had to be discontinued, and a pJP1gusA version carrying verified functional reporter should have been constructed instead. Experiment was designed in a way, that functional version of *gusA* will be joined together with remaining part of the plasmid via Gibson Assembly. Due to several possible reasons, assembly did not work out and GUS assay for testing *gusA* gene expression upon ethanol shock could not be carried out. As mentioned before, most of the difficulties arose due to the mutations acquired during PCR amplification. In the end, however, the main goal of the master thesis was achieved by assembling pJP2 construct and replacing cmlIp with jadJp promoter. The results from this experiment, confirmed possibility of re-engineering antibiotic biosynthetic regulation by exchanging the promoter governing the expression of structural genes. Analysis of chloramphenicol production in the engineered strain revealed that it produced no chloramphenicol without ethanol shock, while in the case of ethanol addition, production was increased by 5 fold in comparison to the ethanol-shocked wild-type strain. Another important observation is that upon continuous cultivation in the MYM medium ethanol shock induces chloramphenicol biosynthesis, which has not been reported previously.
Additional experiment regarding heterologous expression of antibiotic gene clusters was performed. Results confirm positive construction of the assembly in heterologous host i.e.

S. albus as well as complementation of the double mutant strain in *S. venezuelae*, but due to mutations introduced by PCR, both constructs turned out to be non-functional. For that reason, it is difficult to drag any final conclusion regarding these experiments, while high-fidelity DNA polymerase capable of amplifying DNA with high GC content (>70%) may solve the problem.

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Appendix

I. Appendix A: Media recipes

1. ISP2 medium

ISP2 and ISP4 media were developed for characterization of *Streptomyces* species according to International Streptomyces Project (ISP).

ISP Medium 2 is also referred in literature to as Yeast Extract-Malt Extract Agar.

Content: (Per 1L of water)

Yeast Extract	4.0 g
Malt Extract	10.0 g
Dextrose	4.0 g
Agar	20. g

38g of powder Difco™ ISP Medium 2 from dehydrated product was suspended in 1L of purified water. Subsequently medium was autoclaved at 121ºC for 20 min.

For conjugation purposes, plates with ISP4 were supplemented with 1M $MgCl_2$ in concentration 1 ml per 100 ml media.

2. ISP4 medium

ISP Medium 2 is also referred in literature to as Inorganic Salts-Starch Agar.

Content: (Per 1L of water)

Soluble Starch	10.0 g
Dipotassium Phosphate	1.0 g
Magnesium Sulfate USP	1.0 g
Sodium Chloride	1.0 g
Ammonium Sulfate	2.0 g
Calcium Carbonate	2.0 g
Ferrous Sulfate	1.0 mg

Manganous Chloride	1.0 mg
Zinc Sulfate	1.0 mg
Agar	20.0 g

37g of powder Difco™ ISP Medium 4 from dehydrated product was suspended in 1L of purified water. Medium was autoclaved at 121ºC for 20 min. Prior to dispensing it to Petri dishes, medium was thoroughly mixed.

3. ISP4 medium with $MgCl_2$

It was used as conjugation plate with the procedure of conjugative transfer of recombinant plasmid from *E. coli* to *S. venezuelae*.

Medium was prepared according to the procedure described above. After autoclaving 1M $MgCl_2$ was added in amount 1ml per 100 ml medium.

4. LA medium

Luria Agar (LA) medium is nutritionally rich medium widely used in cultivation and maintenance of various strains of *E. coli*.

Per 1L of purified water:

Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

Ingredients were suspended in water and then autoclaved at 121ºC for 20 min.

5. LB medium

Luria Broth (LB) medium has the same content as previously described LA medium, with the omission of agar in its components. Thus, its properties are same as well.

6. MYM medium

MYM medium was used for cultivation of *S. venezuelae*.

Per 1L of purified water:

Maltose	4.0 g
Malt extract	10.0 g
Yeast extract	4.0 g
MOPS Na-salt	1.9 g

Ingredients were suspended in water and then autoclaved at 121ºC for 20 min.

7. NZA medium

It is used for cultivation and maintenance of several *Streptomyces* species.

Per 1L of purified water:

Starch soluble	20.0 g
Glucose	10.0 g
Yeast extract	5.0 g
N-Z Amine	5.0 g
Calcium Carbonate	1.0 g
Agar	15.0 g

All the components were mixed in 1L of water and then autoclaved at 121ºC for 20 min.

8. PM4-1 medium

Per 1L of purified water:

Glucose	15.0 g
Soy meal	15.0 g
Corn Steep solids	5.0 g
$CaCO_3$	2.0 g

TMS 1 (trace elements)*	6.0 ml

Composition of TMS 1 stock solution (filter-sterilized) per 1L water:

$FeSO_4 \cdot 7H_2O$	5000 mg
$CuSO_4 \cdot 5H_2O$	390 mg
$ZnSO_4 \cdot 7H_2O$	440 mg
$MnSO_4 \cdot H_2O$	150 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
$CoCl_2 \cdot 6H_2O$	20 mg
HCl	50 ml

Prior to preparation the medium 150 g/l sterile glucose solution was prepared. 5g of corn steep solids was dissolved in 890 ml distilled water. After addition of magnetic stirrer to the bottle, 15 g of soy meal and 2 g of $CaCO_3$ was added to the solution. At this stage, medium was sterilized for 20 min at 121ºC. 100 ml glucose solution and 6 ml TMS1 was supplemented subsequently.

9. SCM medium

SCM is an abbreviation from Synthetic Complete Selection Medium. It is used for selection of transformants with specific genetic marker carried by plasmid by omission of specific components from Amino Acid Mix.

Per 800 ml purified water:

Yeast Nitrogen Base w/o amino acids	5.4 g
Amino acid mix	1.6 g
Glucose	16.0 g
Agar	12.0 g

Amino Acid Mix contains:

Adenine SO_4	**0.5 g**

Arginine	2.0 g
Aspartic Acid	2.0 g
Glutamic Acid	2.0 g
Histidine HCl	**2.0 g**
Inositol	2.0 g
Isoleucine	2.0 g
Leucine	**4.0 g**
Lysine HCl	2.0 g
Methionine	2.0 g
Phenylalanine	2.0 g
Serine	2.0 g
Threonine	2.0 g
Tryptophan	**2.0 g**
Tyrosine	2.0 g
Uracil	**2.0 g**
Valine	2.0 g
p-Aminobenzoic acid	0.2 g

Components marked with bold type are omitted in SC selection medium.

Medium was prepared by mixing all the ingredients together but glucose which was supplemented after autoclaving. It was sterilized for 20 min at 121ºC. Medium has to be stored in 4ºC and the darkness due to its light-sensitivity.

10. SFM medium

Often referred in literature as to Mannitol Soy Agar medium.

Per 1L of purified water:

Mannitol	20.0 g
Soya flour	20.0 g
Agar	20.0 g

Medium was sterilized for 20 min at 121ºC. Prior to pouring onto the plates, it was mixed vigorously in order to rise up the pellet and ensure equal distribution of the components on each plate.

For conjugation purposes, SFM plates were supplemented with 1M $MgCl_2$ in concentration 1ml per 100ml media.

11. TSB medium

Name is an abbreviation from Tryptic Soy Broth, called also Soybean-Casein Digest Medium. It is used as general purpose medium, being successfully implemented as a medium used for cultivation a wide range of microorganisms. It is consisted of following compounds per liter of purified water:

Enzymatic Digest of Casein	17.0 g
Enzymatic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dipotassium Phosphate	2.5 g
Dextrose	2.5 g

It was prepared from the ready-to-make powder mix from a supplier according to the instructions on the package. Subsequently, medium was autoclaved for 20 min at 121ºC.

12. YPAD Broth medium; 2xYPAD Broth medium

Abbreviated name from Yeast Extract-Peptone-Adenine-Dextrose Medium. Commonly, it is used in research as a general medium for cultivation of yeast strains. Double strength alternative of the solution allows for re-growing culture suspension back to log-phase.

Medium can be prepared in three variants: YPAD Agar, YPAD Broth and 2xYPAD Broth. Recipe to obtain 800 ml is presented below.

	YPAD Agar	YPAD Broth	2xYPAD Broth
Bacto YPD Agar	50.0 g	-	-
Bacto YPD Broth	-	40.0 g	80.0 g
Distilled/deionized water	800 ml	800 ml	800 ml

Medium was sterilized for 20 min at 121ºC.

13. 2xYT medium

It is very rich medium, facilitating and excellent growth of many recombinant strains of *E. coli*. It has been also exploited in the propagation of M13 bacteriophage.

Per 1L of purified water:

Tryptone	16.0 g
Yeast Extract	10.0 g
NaCl	5.0 g

Medium was autoclaved for 20 min at 121ºC.

II. Appendix B: Buffers and Solutions

1. Antibiotic solutions

Antibiotic stock solutions were prepared by dissolving antibiotic salt in an adequate amount of solvent. Solutions were stored in -20ºC.

Apramycin (100mg/ml)

Apramycin	0.5g
dsH_2O	5 ml

Chloramphenicol (25mg/ml)

Chloramphenicol	125 mg
Absolute ethanol	5 ml

Kanamycin (25mg/ml)

Kanamycin	125 mg
dsH_2O	5 ml

Nalidixic acid (30mg/ml)

Nalidixic acid sodium salt	150 mg
NaOH (1M)	5 ml

2. Buffers

50 x TAE buffer

Used for gel electrophoresis.

Recipe per 1L of purified water:

Tris base	242g
EDTA 0.5M pH 8	100 ml

Acetic Acid (100%)	57.1g

Buffer was autoclaved for 20 min at 121ºC.

TSS buffer

Used for preparation of competent cells.

For 50 ml of the ready-to-use buffer following components were mixed:

PEG8000	5g
MgCl2 (1M)	1.5ml
DMSO	2.5ml
LB medium	42.5ml

Solution omitting DMSO was autoclaved for 20 min at 121ºC. DMSO was added subsequently and buffer was stored at 4ºC.

III. Appendix C: List of primers

Primer	Sequence
Y10_SVF1	TGGACGTTCGAGGAGAACCTGCAGGTCGACTCTAGAGG
Y10_SVR1	TAATGGCGGCGTCACTTCCACACGCGTCAGACGGAC
JJEdel-F	GTCCGTCTGACGCGTGTGGAAGTGACGCCGCCATTAAC
JJEdelL-R2	CTCGACCGGCCTCAGCATGACGGTTTCAACTCCTGATCG
JJEdelR-F	TCCGACCCGGCGAAGCTTCCTGGCAGAGAAGAGACGA
JJEdelR-R	TCTAGAGTCGACCTGCAGGTTCTCCTCGAACGTCCAGT
gusAF2	TCAGGAGTTGAAACCGTCATGCTGAGGCCGGTCGAG
gusAR2	CGTCTCTTCTCTGCCAGGAAGCTTCGCCGGGTCGGATAC
Y10_SVcml-F	GCTCAGCAGCGGCAGCACCTGCAGGTCGACTCTAGAGG
Y10_SVcml-R	CTTCGACGAACTGCTCAGCACACGCGTCAGACGGAC
CmlI-F	GTCCGTCTGACGCGTGTGCTGAGCAGTTCGTCGAAGG
CmlI-R	TCAGGAGTTGAAACCGTCATGCGTGACCACACGGACG
jadJp-F	GTCCGTGTGGTCACGCATGACGGTTTCAACTCCTGATCG
jadJp-R	CGGTTCCTTCAGTTGCACCACATTCCCGTCCTCGTGATCC
CmlX-F	CACGAGGACGGGAATGTGGTGCAACTGAAGGAACCGGAC
CmlX-R	TCTAGAGTCGACCTGCAGGTGCTGCCGCTGCTGAGC
Y10cml3-F	AACTGCCGGACGACGAACTGCTGCAGGTCGACTCTAGAGGATCCG
Y10cml3-R	TATCCGCAGCGCCGCCTGTTCGAAGTTCACCGAAGAGCGCATTTTC
Cml3H1-F	GCGCTCTTCGGTGAACTTCGAACAGGCGGCGCTGCGGATATTACG
Cml3H1-R	TCCACTGCGGCTACCAGGTCAACCAC
Cml3H2-F	GTCGTAGTACTCGGAGGCGTACATCAG
Cml3H2-R	CCTCTAGAGTCGACCTGCAGCAGTTCGTCGTCCGGCAGTTCCTTC
JP1-F	CGACTCTAGAGGATCGATCACAGACCCTGACCACACTC
JP1-R	CGATCGATGCGATCGACTCGCCTTCTCCGTACCC
gusA_JP-F	GGAGAAGGCGAGTCGATCGCATCGATCGAAGGAGAGTTCACCA
gusA_JP-R	GGTCAGGGTCTGTGATCGATCCTCTAGAGTCGACCTGCAGT

IV. Appendix D: Plasmid maps

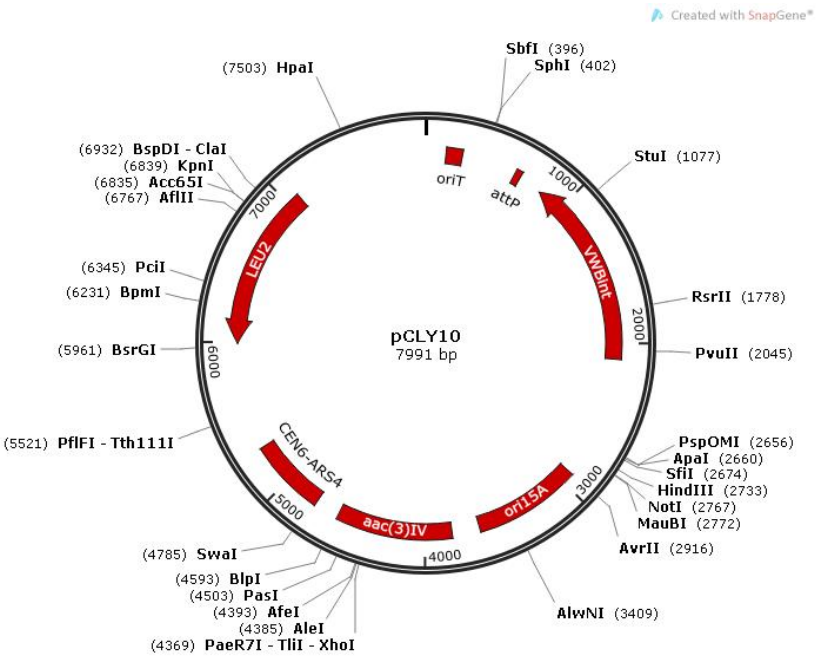

Figure 19. Template vector for construction of assemblies.

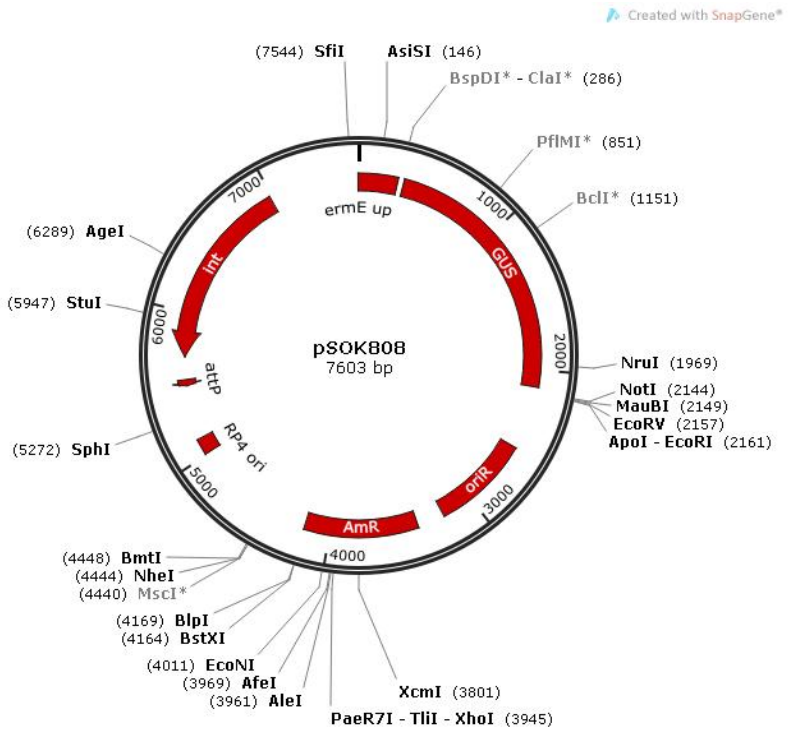

Figure 20. Template vector for *gusA* gene.

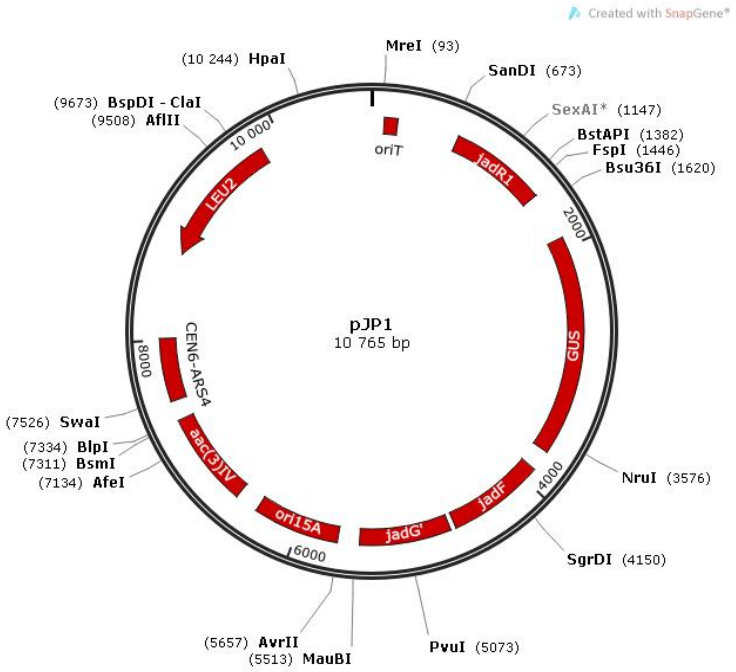

Figure 21. Construct for deletion of *jadJ-E* genes in *S. venezuelae*.

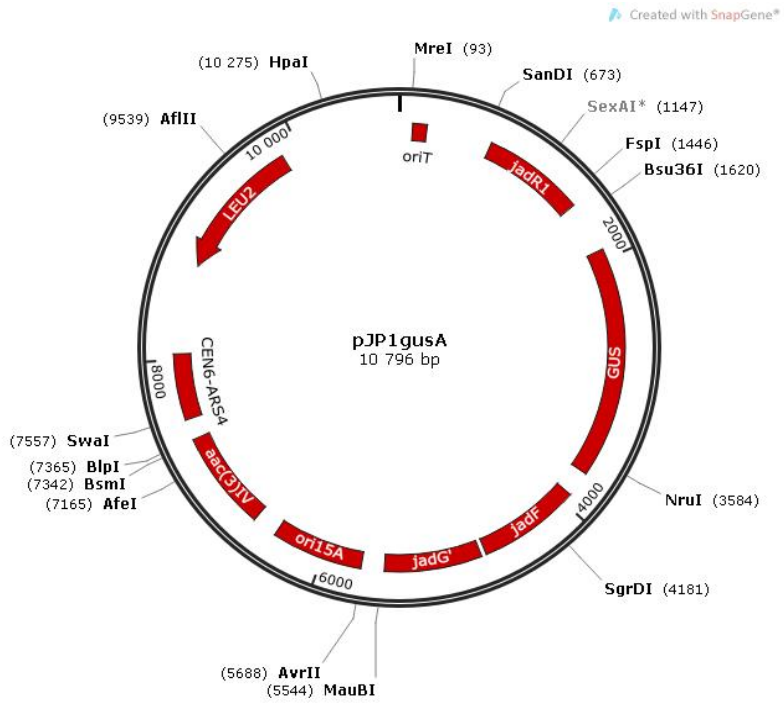

Figure 22. Construct for deletion of *jadJ-E* genes in *S. venezuelae* with the exchanged *gusA* region.

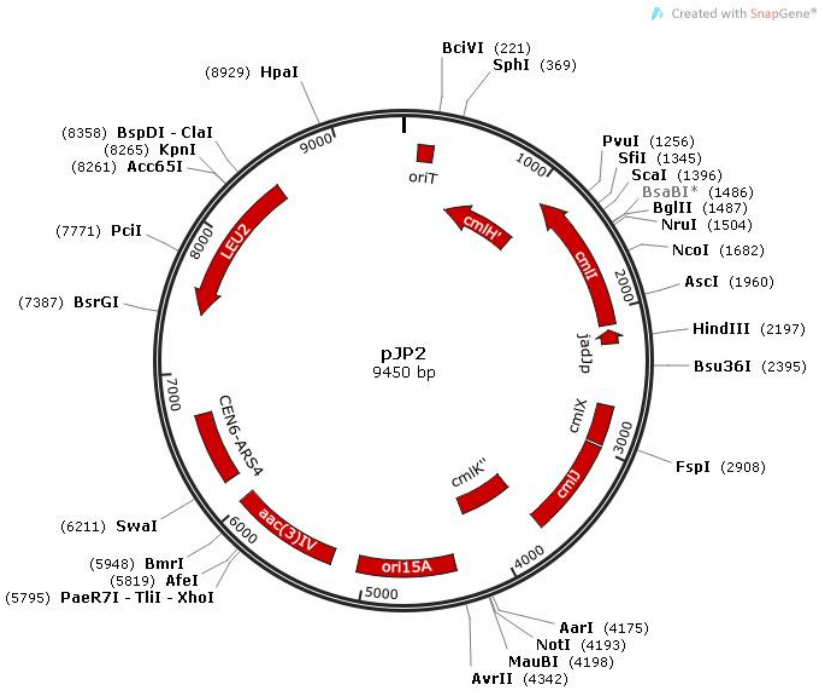

Figure 23. Vector for replacement of the *cmlI* promoter in *S. venezuelae* with *jadJ* promoter.

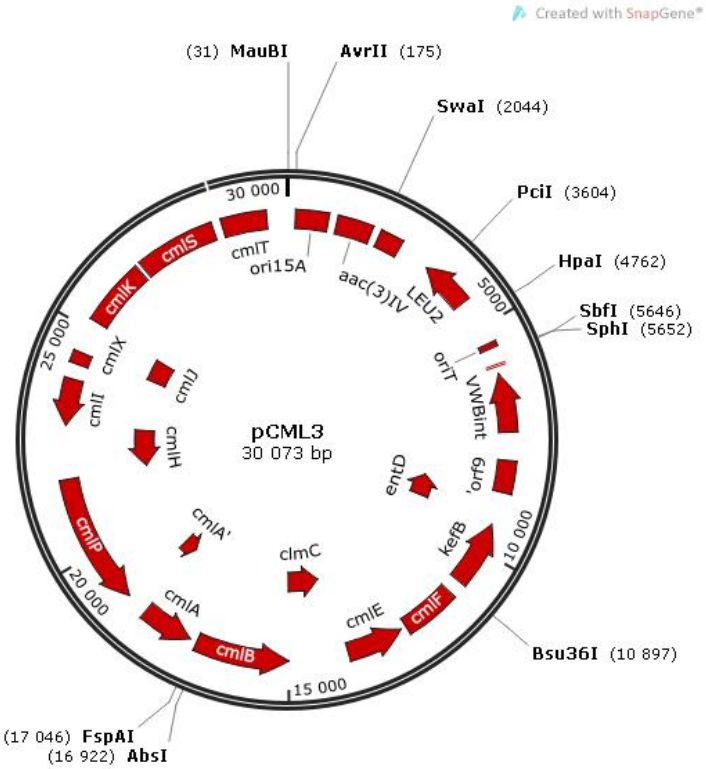

Figure 24. Construct for testing possible heterologous expression from the antibiotic cluster.

V. Appendix E: Gel electrophoresis ladder

The ladder used in electrophoresis is presented in a figure below.

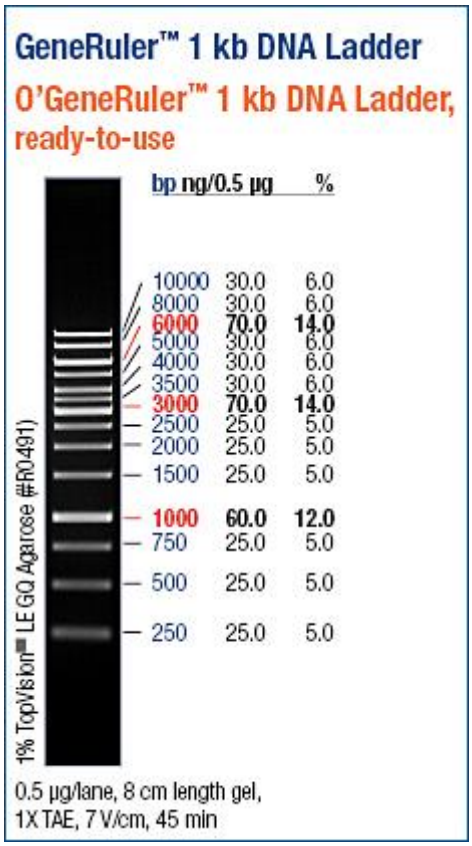

Figure 25. The sized of the bands using Thermo Scientific O'Gene Ruler 1kb DNA ladder ready-to-use 0.1µg/µl.

VI. Appendix F: Results from UPLC analysis

Raw data

Strain of *S. venezuelae*	Ethanol	Chloramphenicol concentration [μM]
Wild-type	-	0.3
Wild-type	+	14
JP2 mutant	-	0.0
JP2 mutant	+	67.6