Transposon Mutagenesis in Streptomycetes

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SUMMARY

Recent whole genome sequencing programs have revealed that the biosynthetic potential of *Actinomycetales* has been even underexplored with traditional approaches. With the advent of next-generation DNA sequencing techniques, we can access the huge amount of genetic information, which awaits development into new chemical and biological entities. Therefore efficient methods for the genes characterization are of great importance. *In vivo* transposon-based strategy is a valuable tool to identify functions of a number of genes and to construct random mutant libraries for diverse applications. Despite the wide availability of transposon systems, few options exist for use in actinomycetes. The aim of this project is to establish a system for random transposon mutagenesis in streptomycetes.

According to this aim the nucleotide content of *Himar1* gene was adapted to the high GC-content of streptomycetes. Set of plasmids for transposon mutagenesis had been constructed and transposon mutant libraries of streptomycetes species had been obtained (*S. coelicolor* M145, *S. albus* J1074 and *S. lividans* 1326). The system was used for identification of novel regulatory genes of actinorhodin biosynthesis in *S. coelicolor* and for random integration of *gusA* reporter gene and antibiotic biosynthetic cluster into chromosome of *S. albus* J1074 with further investigation of position effect in this strain. Also the secondary *attB* site, discovered in the genome of *S. albus* J1074, was characterized.

ZUSAMMENFASSUNG

Die Methoden der Next-Generation DNA-Sequenzierung erlauben uns Zugang zu einer großen Menge an genetischen Informationen, die intensiv in den Bereichen der Biologie und Chemie genutzt werden sollten. Deswegen ist die Entwicklung von effektiven Methoden für eine funktionelle Gen-Charakterisierung heutzutage sehr wichtig. Die *in vivo*-Transposon-basierte Strategie ist ein wertvolles Instrument, das man für die Identifizierung der Funktionen zahlreicher Gene und die Konstruktion von Random-Mutanten-Bibliotheken für vielfältige Anwendungen nutzen kann. Trotz der breiten Verfügbarkeit von Transposon-Systemen sind nur wenige davon zuverlässig auf Streptomyceten anwendbar. Das Ziel dieser Arbeit ist es, ein System für Random-Transposon-Mutagenese in Streptomyceten zu entwickeln.

Hierfür wurde der Codongebrauch des Himar*1* Gens an den hohen GC-Gehalt der Streptomyceten angepasst. Basierend auf diesem Gen wurden die Plasmide für die Transposon-Mutagenese konstruiert und die Bibliotheken der Mutanten von verschiedenen Streptomyceten erzeugt. Das System wurde in *S. coelicolor* für die Identifizierung von neuen regulatorischen Genen in der Actinorhodin Biosynthese verwendet. Außerdem wurden mit Hilfe unseres Systems das Reporter-Gen *gusA* und biosynthetische Antibiotika-Cluster zufällig in das *S. albus*-Chromosom integriert. Damit wurde der Positions-Effekt in diesem Stamm erforscht. Es wurde auch eine sekundäre *attB*-Stelle von φC31-basierten Plasmiden entdeckt und charakterisiert.

1. INTRODUCTION

1.1. Streptomycetes, organisms with outstanding potential

1.1.1. Phylogeny of actinomycetes

Actinomycetes include a wide range of morphologically diverse prokaryotes from micrococci to pleomorphic roods and branched filamentous forms (Goodfellow, 1989). About one third of all bacteria belong to this group - they are the most common and widespread soil, freshwater, and marine bacteria (Hodgson, 2000; Kieser *et al.*, 2000). A common feature of actinomycetes is a positive reaction on Gram staining. It was believed that they have a high guanine and cytosine content (greater than 55 %) in the DNA, until some freshwater Actinobacteria with low GC content were identified (Ghai *et al.*, 2012). Classification and genus delimitation of actinomycetes based on morphology alone is difficult, but incorporation of molecular techniques like partial sequencing of the 16s ribosomal subunit DNA has a considerable impact on this process (Embley and Stackebrandt, 1994).

Members of the Actinobacteria phylum are well known as producers of a number of bioactive natural products responsible for non-life-essential functions, such as sexual hormones, ionophores, defence against other organisms, or communication signals (Demain and Adrio, 2008). Various species of the *Micromonospora* and *Saccharopolyspora* genera produce aminocyclitoles and macrolides; ansamycins are produced by some *Amycolatopsis* strains (Hopwood, 2007). However, the actinomycete genus that gained the most popularity due to its ability to produce a huge spectrum of different antibiotics is *Streptomyces* (Hodgson, 2000).

1.1.2. *Streptomyces*

Streptomyces is the type genus of the *Streptomycetaceae* family (Anderson and Welington, 2001). This genus currently includes Gram-positive aerobic bacteria with a complex life-cycle that is in many ways strikingly similar to that of filamentous fungi. The number of *Streptomyces* species keeps increasing every year (Labeda, 2010).

Streptomycetes have a complex life cycle. After a suitable germination trigger, a single spore grows into a colony: it forms germ tube that develops into branching hyphae, called vegetative mycelium (Hopwood, 1999). After the formation of a vegetative mycelium, as a response to some extracellular signals, e.g. nutrient depletion, process of specialized reproductive structures growth could be launched. These structures are called aerial mycelium; it is formed on the surface of the colony, grows mostly by tip growth and develops a chain of thick-wall spores (Flärdh and

Buttner, 2009), which represent semi-dormant stage of life cycle. In this stage organism can remain intact in soil for long periods of time (Mayfield *et al.*, 1972). Therefore, spores are a good adaptation of streptomycetes for dispersal in the environment. Thus, it is not surprising that streptomycetes adapted successfully to life in a wide range of different niches, like soil and water; some strains evolve into pathogens of plants and animals (Flärdh and Buttner, 2009). Interestingly, the vegetative mycelium serves as nutrients source during formation of aerial mycelium.

Genomes of streptomycetes are represented by single bacterial chromosomes – genophores, and may contain different plasmids, mostly self-transmissible fertility factors (Hopwood, 1999). Unlike other bacteria, chromosomes of streptomycetes are linear (Lin *et al.*, 1993). Both free 5' ends are covalently bound to proteins that probably act as primers for Okazaki fragment necessary for replication. Replication process proceeds in two directions after initiation at centrally located *oriC*. At chromosome ends there are long terminal repeats (LTRs). Their size varies in range 24-600 kb in different species. The first sequenced genome of streptomycete was the one of *S. coelicolor* M145, published in 2002 (Bentley *et al.*, 2002). With about 8 thousand genes it became the largest known bacterial genome. Nowadays the biggest sequenced streptomycete genome is the one of *S. scabies*, pathogenic streptomycete causing potato scab disease. It was sequenced by the Sanger Institute and contains 9107 genes with a total genome size of 10,1 mbp. The results of genomes sequencing have revealed that streptomycetes contain numerous "cryptic" clusters responsible for production of natural products, which are however not expressed under standard conditions (Medema *et al.*, 2011).

1.1.3. Exploiting the potential of streptomycetes as antibiotic producers.

As one of the most useful sources of antibiotics, streptomycetes produce more than 80% of all antibiotics identified in actinomycetes and more than half of all known antibiotics (Hodgson, 2000). When the costs for genome sequencing decreased, many putative natural products clusters had been identified in genomes of streptomycetes *in silico* (Medema *et al.*, 2010). Results of the first (Bentley *et al.*, 2002) and other sequenced streptomycetes genomes demonstrated that diversity of natural products that can be produced by these organisms was largely underestimated. *S. coelicolor* is known to produce five natural products but analysis of its genome unveiled 18 additional putative clusters encoding natural products (Bentley *et al.*, 2002). However, as it was mentioned above, large number of the clusters responsible for the production of natural products remain silent: they are not expressed under laboratory conditions and their products are therefore

unknown. Similar results were obtained after genome sequencing of the other streptomycetes. Industrial strain *S. avermitilis*, known for avermectins production, contains 30 putative clusters of natural products, while only three natural products have been isolated and characterized from this strain (Ikeda *et al.*, 2003). Streptomycin-producer *S. griseus* is known to produce six natural products, however its genome contains 34 putative gene clusters (Ohnishi *et al.*, 2008). Number of sequenced genomes of streptomycetes has been increased thus widening a collection of cryptic, potentially important biosynthetic gene clusters. In these circumstances combining new tools which simplify manipulations of streptomycetes (Siegl and Luzhetskyy, 2012) with efficient and reliable system for *in vivo* transposon mutagenesis will intensify exploration of streptomycetes genomes and give access to their enormous potential.

1.1.4. *Streptomyces coelicolor* M145

S. coelicolor M145 is a derivative of *S. coelicolor* A3(2), genetically the most studied representative of the genus. In contrast to the parental strain, it lacks two plasmids in the genome: 365 kb long linear plasmid SCP1 and 31 kb long circular plasmid SCP2 (Bentley *et al.*, 2002). As it was already mentioned, the genome of *S. coelicolor* was sequenced in 2002. It contains a single linear chromosome 8,667,507 bp long, with 7,825 predicted genes and centrally located origin of replication. By comparison, the genome of Gram-negative *E. coli* has 4,289 predicted genes and in the genome of lower eukaryote, *Saccharomyces cerevisiae*, 6,203 genes were identified. The essential genes, like those involved in cell division or DNA replication, are located near the centre of the chromosome, in the so called genome "core", and nonessential genes are more distant to *oriC*, located along the "arms" of the bacterial chromosome (Bentley *et al.*, 2002).

The genome of *S. coelicolor* encodes 18 gene clusters responsible for production of known or predicted natural products. To the known antibiotics belong methylenomycin, calcium-dependent antibiotic (CDA), undecilprodigiosin (Red), actinorhodin (Act), and γ-actinorhodin (Kieser *et al.*, 2000). Last three antibiotics are easily detectable due to the specific coloration and it makes *S. coelicolor* an attractive object for studying common mechanisms of antibiotic production (Coco *et al.*, 1991; Bystrykh *et al.*, 1996; Borodina *et al.*, 2008). Actinorhodin and undecilprodigiosin clusters include genes from *sco5071* to *sco5092* and from *sco5877* to *sco5898*, respectively (Bentley *et al.*, 2002). Production of these antibiotics is dependent on the phase of growth – it starts in liquid culture by entering stationary phase and on solid medium by start of morphological differentiation. Also, production may be influenced by physiological stresses (Hobbs *et al.*, 1992) and accumulation of γ-butyrolactone (Takano *et al.*, 2000).

1.1.5. *Streptomyces albus* J1074

Streptomyces albus J1074 strain used in this work is *S. albus* G-mutant isolated after ultraviolet irradiation (Chater and Wilde, 1976). In contrast to the parental strain it lacks *SalI*-restriction activity and is an isoleucine-plus-valine auxotroph. Like other streptomycetes, *S. albus* J1074 contains a single linear chromosome with centrally-located origin of replication (*oriC*). The genome of *S. albus* J1074 was sequenced in 2014 (Zaburannyi *et al.*, 2014) and, with the total size of 6,841,649 bp and 5832 predicted protein coding sequences (CDS), it is the shortest streptomycetes genome sequence published to date. For the comparison, the first sequenced streptomycetes genome of *S. coelicolor* A3(2), had 7825 predicted genes (Bentley *et al.*, 2002), one of the last sequenced *S. viridosporus* genome contains 7552 predicted genes (Davis *et al.*, 2013). The GC content (73.3%) of *S. albus* J1074 genome is also one of the highest among the streptomycetes (Zaburannyi *et al.*, 2014). The "core" region covers almost the whole chromosome (~90%), from approximately 0.3 Mb to 6.4 Mb. The "arms" are limited only to the regions from beginning of the chromosome to 0.3 Mb and from 6.4 Mb to the end of the chromosome (Zaburannyi *et al.*, 2014). These "arms", despite their minor role in the streptomycete life cycle, require additional time and resources from the cell for their replication and logistics, and are an additional source of genetic instability during genetic manipulations or expression of heterologous genes. It was shown (Zaburannyi *et al.*, 2014), that the difference in genome size of *S. albus* J1074 and other streptomycetes is caused mostly by reduction of these "arms", therefore it is not surprising that *S. albus* J1074 differs from other streptomycetes, e.g. *S. coelicolor* M145 or *S. lividans* 1326, by higher genetic stability and faster growth. Another interesting feature of the *S. albus* J1074 is deregulated γ-butyrolactone system (Zaburannyi *et. al.* 2014), the system which is involved in the regulation of secondary metabolism. All these factors made the *S. albus* J1074 strain an attractive heterologous host for expression of biosynthetic gene clusters (Winter *et al.*, 2007; Feng *et al.*, 2009; Kim *et al.*, 2009).

1.1.6. *Streptomyces lividans* 1326

Strain of *S. lividans* 1326 is closely related to *S. coelicolor* A3(2). For last half of century it became one of the most studied and used model organisms of the genus. This strain is known mainly because of its ability to accept methylated DNA and for low endogenous protease activity. These two factors made *S. lividans* 1326 a superior cloning and heterologous host (Anne *et al.*, 2012).

In contrast to *S. coelicolor*, *S. lividans* produces the same coloured products, actinorhodin and undecilprodigiosin, only under certain conditions, and is resistant to high concentrations of

mercury (Nakahara *et al.*, 1985), arsenic and zinc (Cruz-Morales *et al.*, 2013). Moreover, it was demonstrated that *S. lividans* requires copper for the mycelium development (Keiser *et al.*, 2000; Worrall and Vijgenboom, 2010).

Genome of *S. lividans* 1326 was sequenced in 2013 (Cruz-Morales *et al.*, 2013). Its chromosome is 8,496,762 bp long and encodes 8 083 proteins. 367 of *S. lividans* genes have no homologs in *S. coelicolor*. The genome also contains two plasmids, termed SLP2 and SLP3. The 50 kb long SLP2 was isolated (Chen *et al.*, 1993) and sequenced (Huang *et al.*, 2003) earlier. The second plasmid, SLP3, was characterized a decade later (Cruz-Morales *et al.*, 2013). The latter study also demonstrated that SLP3 contains two "cryptic" biosynthetic gene clusters involved in metal homeostasis.

Due to the active research efforts, this strain of *S. lividans* is also a parental strain for many transposons, which were identified in its genome and then developed into independent systems for transposon mutagenesis (Solenberg and Baltz, 1994; Baltz *et al.*, 1997). These systems, however, are not widely used due to their low efficiency.

1.2.   Transposon mutagenesis
1.2.1. Transposons in nature

Transposons, or transposable elements, are discrete segments of DNA that can relocate from one location to another (Hayes, 2003). They are present in both prokaryotes and eukaryotes (Craig, 1997) and form a significant part of their genomes: around 40% for human (Lander *et al.*, 2002), mouse (Waterson *et al.*, 2002), and rice (Goff *et al.*, 2002), and from 1% to 5% for lower eukaryotes and bacteria (Curcio and Derbyshire, 2003). Transposons can significantly influence host genome, causing activation or inactivation of genes or promoting inversions and deletions of chromosomal DNA. The bacterial transposons may encode antibiotic resistance markers and virulence factors (Curcio and Derbyshire, 2003).

Classical transposon contains two key parts: a gene of the transposone-specific transposase and two specific recognition sequences for this transposase. These sequences are inverted or highly homologous versions of each other and are called terminal repeats. The transposase binds to the terminal repeats, forms a transposase-DNA synaptic complex and catalyses translocation (Reznikoff and Winterberg, 2008). Usually, this reaction requires Mg^{2+} and may require some additional factors.

Due to its wide distribution among all living organisms, many different types of transposons were identified. The most informative and universal way to classify them is according to the type of a transposase protein that dictates a translocation mechanism (Curcio and Derbyshire, 2003). At the time, five types of transposases have been discovered: DDE-transposases, Y2-transposases, tyrosine-transposases, serine-transposases, and RT/En-transposases. All these proteins catalyze transposition by different mechanisms and some of these proteins "cut" their transposons out from the former location and "paste" it into a new one, whereas others do not cut the transposon out, but just replicate or "copy" it and "paste" this copy into a new location.

The best studied family of transposable elements is the DDE-type (*mariner*, *Ac/Ds*, Tn*5*). Transposases of this type have conservative aspartate(D)-aspartate(D)-glutamate(E) motif in the active centre and catalyse translocation through protein-DNA complex, transposome. The transposome contains donor and acceptor sites, transposase protein and, sometimes, host factors. The reaction occurs via of the "cut-and-paste" mechanism.

Tyrosine- (Tn916) and serine-transposases (IS607) also use the "cut-and-paste" type of translocation. The reaction includes excision, circularization and insertion of the transposon into new location.

The Y2-transposases use the "copy-and-paste" mechanism and require DNA-replication machinery of the host to replicate the transposon in the way, that each copy contains one old and one newly synthesized strand.

The RT/En-transposases (retrotransposases) are copying the transposon into acceptor DNA using an RNA-copy of the transposon, this RNA-copy is synthesised by reverse transcription. Not all transposons of this type contain terminal repeats.

1.2.2. Transposons as genetic tools

Main advantages of transposons making them useful tool in biotechnology are randomness of their transposition, self-sufficiency of a transposase and possibility to clone any desired sequence in between the terminal repeats. With the advent of genome projects, number of full genome sequences has increased, but these sequences consist mostly of genes with putative or unknown function. The ability to integrate stably into the host DNA made transposons a useful tool for identification of new genes of unknown function. With this aim native transposons are modified to fit the conditions of experiment in a desired organism. Usually, it means that the gene of transposase is cloned into easily curable vector outside from the sequence designated for

transposition. The sequence that is used for the transposition usually does not contain any DNA elements related to the native transposon except the sequences necessary for the recognition by the transposase. In addition, it contains an antibiotic resistance marker and other tools, available for a desired organism (Rubin *et al.*, 1999; Lyell *et al.*, 2008). Transposon and transposase can be delivered into the cell by electroporation (Beare *et al.*, 2008), transfection (Sohaskey *et al.*, 1992) or intergeneric conjugation (Petzke and Luzhetskyy, 2009), on a single plasmid (Rholl *et al.*, 2008) or on two separate plasmids (Beare *et al.*, 2008).

Diverse transposon-employing methods have been developed: transposons may be used to help with sequencing of problematic DNA regions. With this aim, the transposon has to be randomly inserted into a fragment of interest and using sequencing primers that anneal near the end of the transposon set of overlapping sequences can be generated and assembled into entire sequence of fragment (Griffin *et al.*, 1999). Transposons were also used to produce random transcriptional or translational fusions between gene of interest and reporter gene (Casadaban and Cohen, 1979). In actinomycetes, transposon based strategies most often were used for insertional inactivation and identification of regulatory genes involved in the regulation of natural products production (Solenberg and Baltz, 1991). Also, transposons found their wide application for the inactivation of one of competing pathways and thus enhancing outcome of the other, or for activation of silent clusters by cloning of highly active promoters into the transposon (Baltz, 2001). The *Himar1* based system had been successfully applied for identification of factors responsible for production, activity and secretion of listeriolysin O, toxin produced by human pathogen *Listeria mocytogenes*, (Zemanskyy *et al.*, 2009). Development of reliable method for *in vivo* transposon mutagenesis will make many of these methods available or more convenient for the application in streptomycetes.

1.2.3. Transposons in streptomycetes

The attempts to adapt the system of random transposon mutagenesis for streptomycetes can be divided in two categories. To the first category belong experiments with native transposons isolated from different streptomycetes, while the second includes an application of transposons from nonrelated species.

One of the first successful examples was Tn*4556*, isolated from *Streptomyces fradiae* (Chung, 1987). Its derivative, Tn*4560*, carrying viomycin resistance gene, was constructed and applied for mutagenesis of *S. lividans*, *S. coelicolor*, *S. lincolnensis* and *S. avermitilis* (Chung, 1987; Chung and Crose 1989; Ikeda *et al.*, 1993). Further development of Tn*4556*, Tn*5353*, adopted for the

transfection with φC31, was fused with the reporter gene of luciferase and employed for monitoring of transcription from the chromosomal promoters of streptomycetes (Sohaskey *et al.*, 1992). However, this transposon demonstrated low frequency of transposition and difficulties to cure the vector after the transposition occurred (Sohaskey *et al.*, 1992; Ikeda *et al.*, 1993). The first attempts to isolate the *S. coelicolor* mutants containing copy of transposon in the genome had failed (Chung, 1987; Sohaskey *et al.*, 1992), further experiments demonstrated, that introduction of IS*4560* into genome of *S. coelicolor* led to instability near the native insertion sequence IS*1649* (Widenbrant and Kao, 2007). Yagi and Ikeda reported that transposon insertions of Tn*4560* were not randomly distributed in the genome of *S. avermitilis* if transposition was performed at 30°C (Yagi, 1990; Ikeda *et al.,* 1993). Only increasing temperature to 37°C solved this problem, but obtained integrations were not stable and some of obtained auxotrophic mutants reversed to prototrophic, but remained resistant to viomycin (Ikeda *et al.*, 1993). Also, because of transposition immunity the Tn*4556* derivatives were presented by one copy in the genome (Chung, 1987).

Another attempt to adapt a native transposon was made when IS*493*, isolated from *S. lividans*, was used for transposon mutagenesis of *S. ambofaciens*, *S. cinnamonensis*, *S. coelicolor* and others (McHenney and Baltz, 1991). In further studies several transposons were developed from IS*493* (Solenberg and Baltz, 1994; Baltz *et al.*, 1997): Tn*5096*, Tn*5099* and several more, containing different resistance genes. These transposons were employed for physical mapping of genes involved in the daptomycin production in *S. roseosporus* and for cloning of the daptomycin biosynthetic genes. However, analysis of the insertions indicated that IS*493* and its derivatives have quite specific target site (Solenberg and Baltz, 1994) and demonstrate much lower frequency of transposition than Tn*4560* (Kieser *et al.*, 2000).

The second transposon isolated from *S. lividans* was IS*1373* (Volff and Altenbuchner, 1997). But the application of this transposon as a tool for the mutagenesis is limited because it has low transposition frequency, demonstrates some integration preferences and causes instability in *S. lividans* genome (Volff and Altenbuchner, 1997).

In parallel, also transposons from other actinomycetes were tested for streptomycetes. IS*6100* was isolated as a part of the transposon Tn*610* from *Mycobacterium fortuitum* (Martin *et al.*, 1999) and employed for transposon mutagenesis of *S. lividans* (Smith and Dyson, 1995) and *S. avermitilis* (Weaden and Dyson, 1998). In both cases transposition system included a temperature sensitive vector for deployment of transposon into the cell and the thiostrepton-inducible promoter to induce the transposase gene. In further studies its derivative Tn*1792*, with the gentamycin

resistance gene, was used for mutagenesis of *S. coelicolor* and *S. lividans* (Herron *et al.*, 1999). However, application of IS*6100* transposon in streptomycetes was remarked by difficulties – it demonstrated tendency to integrate the whole plasmid into the chromosome that caused instability of integrations. Also, it was impossible to induce the thiostrepton promoter in *S. avermitilis* (Weaden and Dyson, 1998). These difficulties in combination with the report that IS*6100* may cause instability in one shoulder of *S. lividans* chromosome (Günes *et al.*, 1999) limited the use of this transposon as a tool for streptomycetes.

Another transposon isolated from *Nocardia asteroides* YP21 was tested in streptomycetes was IS*204* (Yao *et al.*, 1994). Suicidal plasmid containing this transposon was used for transposon mutagenesis of *S. coelicolor* M145 (Zhang *et al.*, 2012). Analysis of the obtained mutants revealed, that not only the transposon, but the whole plasmid was integrated into the genome and this may cause the instability of the chromosome. Also, the authors suggest, that genome of *S. coelicolor* M145 may lack some host factors required for the efficient transposition.

The first transposon from an unrelated organism applied for mutagenesis in streptomycetes was the Tn*5* derivative, Tn*5493*, (Volff and Altenbuchner, 1997). It was used for transposon mutagenesis of *S. lividans* TK64. However, such drawbacks as low transposition efficiency (3% for *S. lividans* TK24), use of native *tn5*-transposase gene (AT-rich), and an absence of possibility to quickly identify the insertion locus restricted the application of this system for other streptomycetes strains.

Further attempts were concentrated on the application of Tn*5* as tool for *in vitro* transposon mutagenesis of streptomycetes (Sprusansky *et al.*, 2003). With this aim several Tn*5* derivatives were constructed. Using one of them, Tn*5062* (Bishop *et al.*, 2004), 311 cosmids of *S. coelicolor* were mutagenized and library with 6482 disrupted genes (83% of genome) was obtained. Derivatives of Tn*5062*, carrying different antibiotic resistant markers (hygromycin and spectinomycin resistance) and other genetic features expanding application of these transposons (*loxP* sites for recombination, *luxAB* genes, *e.t.c.*) were constructed. However, application of this system is complicated by necessity first to mutagenize the cosmid library and only then to introduce the mutagenezed cosmid into the genome.

Successful attempt to adapt the Tn*5* for streptomycetes *in vivo* was made few years later (Petzke and Luzhetskyy, 2009). The developed system is based on synthetic gene of Tn*5*-transposase and demonstrates high integration frequency together with the stability of insertion (Petzke and Luzhetskyy, 2009). This system was already applied for the identification of the regulatory genes involved in the landomycin E biosynthesis in *S. globisporus* 1912 (Horbal *et al.*, 2013).

1.2.4. *Himar1*.

The *Himar1*-transposon belongs to Tc1/*mariner* family of transposons. These transposons are probably the most widespread transposons in nature. First representative of Tc1/*mariner* was discovered in 1983 in *Caenorhabditis elegans* (Emmons *et al.*, 1983). Later these transposons were found in fungi, plants and animals (Plasterk *et al.*, 1999). These transposons may be divergent in their nucleotide sequences (up to 15% homology) but they share similar structural features and mechanism of transposition. Size of the transposon varies from around 1,3 kb (*Himar1*) to 2,4 kb (Pogo-transposon). It includes single transposase- encoding gene framed by two inverted terminal repeats (ITR), containing binding sites (BS) for transposase (Fig. 1.1). Sizes of ITR and BS also vary from 31 bp and 28 bp, respectively, for *Himar1* and up to 462 bp and 33 bp, respectively, for Tc3.
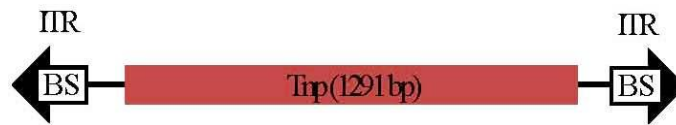
Figure 1.1. Structure of *Himar1* transposon. Central transposase gene (Tnp; red block) is flanked by two inverted terminal repeats (ITR; black arrows), containing binding sites for transposase (BS; white blocks) (Plasterk *et al.*, 1999).

Himar1-transposase belongs to DDE-family of transposases and its activity is sufficient to provide full excision and integration of the transposon *in vitro* (Lampe *et al.*, 1996). By choosing target for new integration, *Himar1*-trasposase shows a preference for regions with AT-duplications and integrates the transposons in between the TA sequence (Craig, 1997), leaving 2bp footprint on the donor DNA (Plasterk *et al.*, 1999). Such selectivity of choosing the integration site might seem problematic, but even in organisms with high GC content at least one TA dinucleotide is present in each gene.

Detailed mechanism of *Himar1* transposition (Fig. 1.2), based on biochemical analysis of early transposition events was proposed by Butler and colleagues (Butler *et al.*, 2006). According to this model, the first active molecules of transposase monomer have to be synthesized. Two such molecules bind separately to two ITRs at transposons' poles. During the second step, the transposase dimers form by recruiting the second monomer through protein-protein interactions. If at this stage concentration of transposase monomers is optimal, two poles of transposon drift to each other and form catalytically active complex, containing transposase tetramer and two ITRs (Lipkow *et al.*, 2004; Auge-Gouillou *et al.*, 2005). This complex is cleaved out from the old

site and reinserted at a new TA position elsewhere in the genome, thereby completing the transposition reaction (Lampe *et al.*, 1996; Tosi and Beverley, 2000).
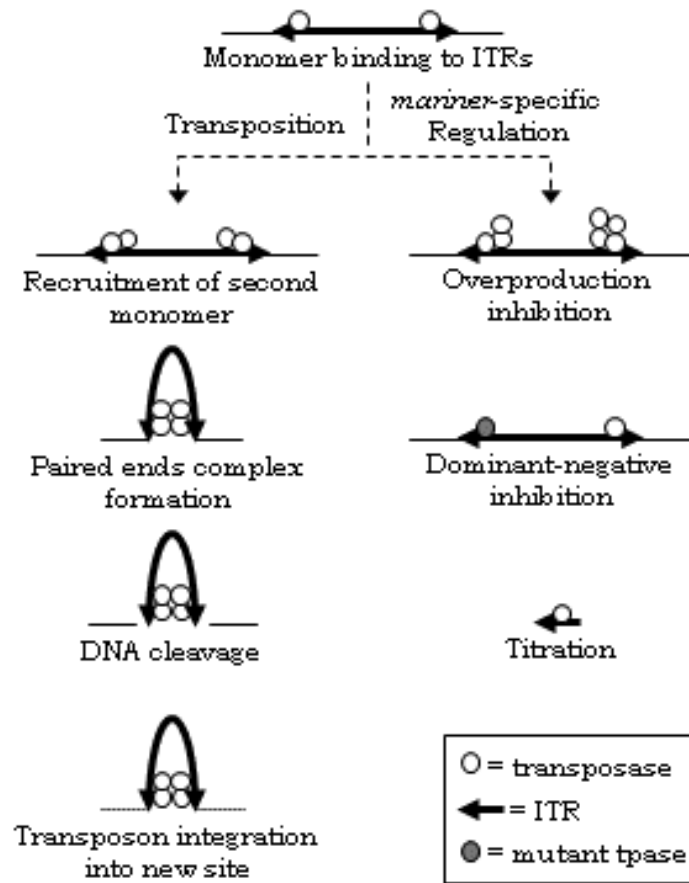
Figure 1.2. Model for *Himar1* mariner transposase transposition and regulation (Butler *et al.*, 2006).

Transposase activity can be regulated in several different ways (Butler *et al.*, 2006). If concentration of transposase is too high, multimers of transposase can be formed at each of two ITRs leading to inhibition of the transposition reaction (Lohe and Hartl, 1996; Hartl *et al.*, 1997; Lampe *et al.*, 1998). Also, the reaction is inhibited, when mutated transposase subunit, that cannot correctly catalyse transposition, or mutated ITR, that cannot be cleaved, participate in the reaction (Butler *et al.*, 2006; Hartl *et al.*, 1997). Then all other counterparts of reaction find themselves blocked by mutated reaction compound (subunit or ITR) and unable to accomplish the reaction. Three described types of inhibition are called overproduction inhibition, dominant-negative inhibition and inhibition by titration, respectively (Butler *et al.*, 2006).

While most transposons are limited to their own host range, the *Himar1* remains active in different organisms and was already adopted for *E. coli* and *Mycobacterium smegmtis* (Rubin *et al.*, 1999), *Methanosarcina acetivorans* (Zhang *et al.*, 2000), *Leptospira biflexa* (Louvel *et al.*, 2005), cell

cultures of mice and rabbits (Keravala *et al.*, 2006), *Frascinella tularensis* (Maier *et al.*, 2006), *Coxiella burnetii* (Beare *et al.*, 2008), *Burkhoderia pseudomallei* (Rholl *et al.*, 2008) and *Geobacter sulfurreducens* (Rollefson *et al.*, 2009) demonstrating satisfactory randomness and stability of integrations (Rubin *et al.*, 1998; Maier *et al.*, 2005; Louvel *et al.*, 2005) and high transposase activity (Lampe *et al.*, 1999; Rholl *et al.*, 2008). These advantages of *Himar1* over other existing transposons made it the most promising candidate for development of *in vivo* transposon mutagenesis system for streptomycetes.

1.3.   Position effect

Position effect is a term describing differences in genes expression caused by the location of genes on the chromosome. Such differences include variations in a phenotype, transcription level, recombination frequency, or replication timing (Gottschling *et al.,* 1990). Position effect can affect not only expression of native genes after spontaneous translocations but, also, transegenes inserted into different regions of a genome.

Well known example of position effect was described for *Drosophila melanogaster* (Weiler and Wakimoto, 1995). In the wild type strain, the gene responsible for red eye pigmentation is located in the euchromatin region and thus is easily accessible for transcription by the RNA- polymerase. If this gene is translocated closer to the heterochromatin region, the gene is no more accessible for transcription and eyes of such mutants are characterized by mottled appearance of white and red sectors, as the gene is expressed in some cells in the eyes and not in others. Such variegation caused by the gene inactivation in some cells through its abnormal translocation next to the heterochromatin region is called position-effect variegation.

In eukaryotic microorganisms position effect was demonstrated by Gottschling *et al.* 1990 for yeast *Saccharomyces cerevisiae*. Its ADE2-gene codes for one of adenine biosynthesis enzymes and at its normal chromosomal location it is expressed in all cells. In this "classical position-effect experiment" (Chen *et. al.* 2012), this gene was moved to heterochromatin region at the end of the yeast chromosome and was no longer expressed in most cells of the population thus leading to accumulation of a red pigment in the yeast cells. So, wild type colonies remained white, while mutant colonies, where the expression of ADE2 gene was altered, became red.

As mentioned above, also the position of heterologous gene in the host genome can influence its expression level. In experiment with 18 *S. cerevisiae lacZ*-integrants 14-fold variation in expression level was demonstrated (Thompson and Gasson, 2001).

Studies of the position effect in prokaryotes are limited at the moment to three organisms, *Escherichia coli* (Beckwith *et al.* 1966; Sousa *et al.* 1997), *Salmonella typhimurium* (Schmid and Roth, 1987) and *Lactococcus lactis* (Thompson and Gasson, 2001). Sousa (Sousa *et al.* 1997) demonstrated threefold variation in β-galactosidase activity in response to translocation of its gene in the chromosome. This is similar to results obtained after Schmid and Roth analysed 16 *Salmonella typhimurium* mutants containing randomly inserted *his* operon cluster (Schmid and Roth, 1987), where threefold variation in expression level was observed. Also, *L. lactis* mutants showed threefold difference in *gusA* expression level (Thompson and Gasson, 2001).

Main factors causing such a variability in the gene expression are (i) level of DNA compactiation (Gottschling *et al.*, 1990), (ii) variation in a promoter strength, and (iii) distance to the origin of replication (Thompson and Gasson, 2001). Impact of the first two factors is more critical for eukaryotic organisms possessing more perfect mechanism of DNA compactization and greater variation in promoter strengths, which could influence a downstream heterologous gene. The last factor, distance to an origin of replication is the major factor of variability in the gene expression in prokaryotic cells, as they contain single origin of replication per genophore. It means that gene placed closer to origin of replication is replicated before the one located near the terminus and therefore has an operative increase in gene dosage (Paavitt and Higgins, 1993).

At this time phenomenon of position effect was not investigated in streptomycetes despite the fact, that these organisms are important natural products producers and are commonly used as heterologous hosts. In a forecast we can expect that genes expression in streptomycetes is also influenced by the position effect, as all factors causing it are also present in cells of streptomycetes.

1.4. Attachment sites of streptomycetes bacteriophages

After infection of host cell, virulent bacteriophage starts to replicate and destroy infected cell. This provides release of new phage particles into surrounding medium and infection of new host cells. In contrast, 'temperate' phages may choose a lysogenic lifestyle of hiding in host genome. It allows them to be passed on in many generations of bacterial host (Stark, 2011).

To establish such lysogenic life style genomes of many bacteriophages contain integrase, an enzyme necessary for integration of the phage genome into the host chromosome (Campbell, 2006; Landy, 1989). The integrase catalyses site specific recombination between the phage and the host attachment sites, *attP* and *attB*, and forms two hybrid sites, *attL* and *attR*, with prophage genome in between. To enter the lytic lifestyle, prophage DNA should be excised by similar

integrase-mediated reaction between hybrid sites, *attL* and *attR*. In most cases direction of reaction catalysed by integrase is determined by the presence or the absence of a viral encoded protein, the recombination directionality factor (RDF) (Stark, 2011).

1.4.1. ΦC31-phage

One of the typical temperate phages, φC31, was isolated from *S. coelicolor* A3(2) (Lomovskaya *et. al.*, 1971). Its genome encodes an integrase that belongs to the large serine recombinases and uses serine residues to break target DNA strands (Thorpe and Smith, 2002). Integration reaction does not require any cofactors (Thorpe and Smith, 1998) is stable in the absence of RDF (Stark, 2011), and recognizes relatively short DNA-sequences: minimal size for *attB* and *attP* is only 34 bp and 39 bp, respectively (Groth *et al.*, 2000). These two factors are the main advantage of φC31-integrase over other recombinases, such as *Cre*, *Dre* or *Flp* and made it a widely applied tool in biotechnology of streptomycetes for construction of versatile, low-copy-number, and convenient vectors (Bierman *et al.* 1992; Kieser *et al.*, 2000). Also, φC31-integrase remains active in a wide range of other species: *Schizosaccharomyces pombe*, *Xenopus laevis* embryos, cultured silkworm cells, *Drosophila*, plants, mice, rabbit and human cells (Li *et al.*, 2011; Groth *et al.,* 2000).

An important feature of φC31-integrase is a control over direction of the recombination (Thorpe *et al.*, 2000). It means that during the integration, the right shoulder of *attP* becomes joined to the left shoulder of *attB*, and *vice versa*, giving *attL* and *attR*, respectively (Fig. 1.3). Mechanism of such polarity was investigated by Smith *et al.* 2004. It was shown that the polarity is ensured only by the so called core sequence consisting of two base pairs (TT) where crossover occurs. This dinucleotide forms sticky ends necessary for subsequent religation of recombinant products. Moreover, polarity could be manipulated if the core sequences of both sites are replaced by different combinations of the complementary dinucleotides. Seemingly, the integrase is able even to synapse and activate strand exchange even when due to mismatches in the core sequences, the process cannot be completed (Smith *et al.*, 2004).

Despite the fact that whole process of recombination is under strict control of integrase, scrupulosity of this enzyme by choosing the attachment site for integration is not always so precise: numerous secondary or pseudo-*attB* sites in *S. coelicor* were identified (Combes *et al.*, 2002). However, conjugation frequency for pSET152 reduces 300-fold when only these sites are present in the genome of recipient strain. Interestingly, one of the pseudosites, *pseB*2, has noncanonical core sequence TC, but no clear explanations were found, how the recombination could occur when the mismatches in core dinucleotides are present. So, despite of its broad

application as a tool for gene transfer, the mechanism of recombination catalysed by φC31-integrase may hide unexpected surprises.
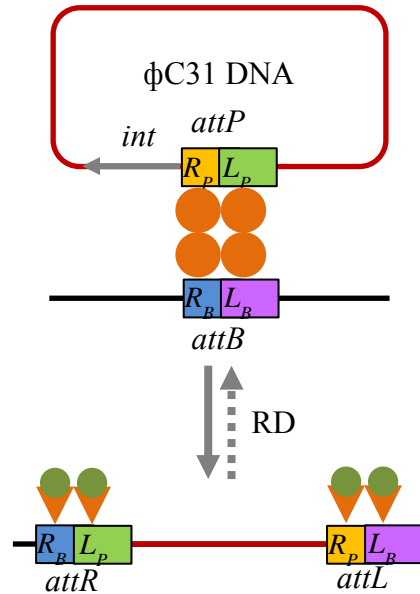
Figure 1.3. φC31 integration and excision mechanism (Stark, 2011). Integrase is marked by orange circles and triangles to indicate possible conformational changes; RDF is marked by green circles.

1.4.2. VWB-phage

The VWB-phage is a temperate phage of streptomycetes and was first isolated from soil using *S. venezuelae* ETH14630 as indicator strain (Anne *et al.*, 1984). It has a narrow host range, but could be introduced into several other streptomycetes, e.g. *Streptomyces lividans* TK24, by transfection. Its genome is 47,3 kb large with GC content of 63,9% (Anne *et al.*, 1985) and remains stable by carrying up to 4 kb of additional DNA (Van Mellaert *et al.*, 1998). Integration into the host chromosome occurs *via* the site-specific recombination between VWB *attP* and chromosomal *attB* site. As a result the host-phage junctions *attL* and *attR* are formed. Analysis of *attB*, *attP*, *attL* and *attR* sequences revealed presence of 45 bp of common core sequence. In the chromosome this sequence is presented by 3'-end of tRNAArg(AGG)-gene. The *attP* site contains 3' end of the tRNA gene so that the integration does not disrupt this gene.

In further studies (Van Mellaert *et al.*, 1998) functional integrative vector, based on VWB-encoded site specific recombination system, was constructed and tested in *S. venezuelae* ETH14630 and *S. lividans* TK24. As the tRNA genes are conservative within the genus, VWB-based integration system became a popular tool for genetic manipulations in streptomycetes. It was found to be active in other model strains, such as *S. coelicolor* M145 (Herrmann *et al.*, 2012)

and *S. albus* J1074 (this work). However, the fact that even for a thoroughly studied φC31-integrative system previously unknown attachment site in *S. albus* was detected (this work) promotes the idea that more attention to other phages-attachment sites in this strain should be paid.

1.5.   Aims of this work

The goal of this work is to establish an *in vivo Himar1*-based system for transposon mutagenesis for streptomycetes. Cornerstone of this project is a synthetic gene of transposase, *himar1(a)*, optimised for actinomycetes codon usage. This gene was tested for ability to provide expression of functional transposase that catalyses transposition of synthetic transposons, containing inverted terminal repeats (ITR), antibiotic resistance genes and different previously developed genetic tools, from replicative or suicidal plasmids into genomes of streptomycetes.

This work demonstrates new opportunities in exploring streptomycetes genetics that became available by adaptation of *Himar1* transposon mutagenesis system. First, this system was used for identification of novel regulatory genes of *S. coelicolor* involved in actinorhodin biosynthesis. Second, the transposon mutagenesis system and combination of this system with φC31 recombination system were used for random integration of *gusA* reporter gene and antibiotic biosynthetic cluster into chromosome of *S. albus* J1074 with further investigation of position effect in this strain.

During construction of *S. albus* recipient strain for random integration of antibiotic biosynthetic cluster, previously unknown predominant secondary attachment site for φC31-based plasmids was identified and characterized.

2. MATERIALS AND METHODS

2.1. LIST OF CHEMICALS

2.1.1. Components of media and buffers

Table 2.1. Components of media and buffers

Chemicals	Manufacturers
Agar	Roth
$CaCl_2$ x $2H_2O$	Roth
Casamino Acids	Sigma-Aldrich, Seelze
D-Mannitol	Roth
EDTA (disodium salt)	Sigma-Aldrich, Seelze
Glucose	Roth
Glycerol	Roth
HCl	Roth
K_2HPO_4	Merck
K_2SO_4	Roth
K_3PO_4 x $3H_2O$	Merck
KH_2PO_4	Merck
LiCl	Roth
Maleic Acid	Roth
Malt extract	Becton Dickinson, Heidelberg
$MgCl_2$ x $6H_2O$	Roth
$MgSO_4$ x $7H_2O$	Merck
NaCl	Roth
$(NH_4)_2SO_4$	Merck
NaOH	Roth
Polyethylene Glycol (PEG) 1000	Merck
Potassium Acetate (KAc)	Roth

Proline	Roth
SDS	Roth
Sodium Citrate	Roth
Sodium N-Lauroyl Sarcosine	Fluka, Taufkirchen
Soy flour	W. Schoenenberger GmbH & Co KG, Magstadt
Sucrose	Fluka, Taufkirchen
TES	Roth
Trace Elements	Sigma-Aldrich, Seelze
TRIS (base)	Roth
TRIS-HCl	Roth
Tryptone / Peptone from Casein	Roth
Tween® 20	Roth
Yeast extract	Roth

2.2.   ENZYMES AND KITS

Table 2.2. Enzymes and kits used in this work

Enzyme or kit	Manufacturer
DNA Restriction Endonuclease	Promega, Fermentas, NEB
Lyzozyme	Roth
Polymerases (*Pfu*, *Taq*, Phusion)	Fermentas
T4-DNA-Ligase	Fermentas
FastAP	Fermentas
Wizard® Plus SV Minipreps DNA Purification System	Promega
Wizard® SV Gel and PCR Clean-Up System	Promega
PureYield™ Plasmid Midiprep System	Promega

BACMAX™ DNA Purification Kit	Epicentre

2.3.   BUFFERS AND SOLUTIONS

Table 2.3. Buffers for chromosomal DNA isolation from streptomycetes

Buffer	Component	Volume/Concentration
TE Buffer	1M Tris HCl, pH 8,0	25 ml/l
	0,5M EDTA	50 ml/l

Table 2.4. Buffers and solutions for agarose gel electrophoresis

Buffer	Component	Volume/Concentration
50xTAE	Tris (Base)	242,2 g/l
	EDTA	18,62 g/l
		á pH 7,6 with glacial acetic acid
1xTAE	50xTAE	20 ml/l
Agarose Gel Solution	Agarose	0,35 g
		á H_2O 500 ml
Stainbath for Agarose Gel	Ethidium Bromide/Roti-Safe	1 µg/ml

Table 2.5. Buffers and solutions for hybridization

Buffer/Solution	Component	Volume/Concentration
Depurination Solution	25% HCl	36,2 ml/l
Denaturation Solution	NaOH	20 g/l
	NaCl	87,6 g/l
20xSSC Buffer	NaCl	175,3 g/l
	Sodium Citrate	88,3 g/l
		á pH7,0
Buffer for Hybridization	20x SSC Buffer	12,5 ml
	1% Sodium N-Laurylsarcosine	5 ml

	10% SDS	100 µl
		á H_2O 50 ml
Prehibridization Buffer	Buffer for Hybridization	50 ml
	Skim-milk Powder	1,5 g
Hybridization Buffer	Buffer for Hybridization	50 ml
	Skim-milk Powder	0,75 g
Blot Buffer 1	Maleic Acid	11,6 g/l
	NaCl	8,77 g/l
		á pH 7,0
Blot Buffer 3	100 mM Tris HCl, pH9,5	34,75 ml
	5M NaCl	0,75 ml
Antibody Solution	Anti-Dioxigenin-AP	5 µl
	Blot Buffer 1	50 ml
	Skim-milk Powder	0,75 g
Blocking Solution	Blot Buffer 1	50 ml
	Skim-milk Powder	1,5 g
0,5x Wash	20xSSC Buffer	1,25 ml
	10% SDS	0,5 ml
		á H_2O 50 ml
2x Wash	20xSSC Buffer	5 ml
	10% SDS	0,5 ml
		á H_2O 50 ml

Table 2.6. Buffers for measurement of glucuronidase activity

Buffer/Solution	Component	Volume/Concentration
Buffer 1	KH2PO4xH2O	3 g/l
	K2HPO4	4,1 g/l
	Triton X-100	1 g/l
	DTT (before usage)	0,771 g/l
Buffer 2	Buffer 1	50 ml
	Lysozyme	200 mg
Buffer 3	Buffer 1	50 ml
	p-nitrophenyl-β-D-glucuronide solution	500 µl

Note: p-nitrophenyl-β-D-glucuronide solution: 0,063 g/ml DMSO

2.4. CULTIVATION MEDIAS

A final concentration of agar for all solid media was 14 g/l unless otherwise stated. Antibiotics and other components were added after sterilization and under sterile conditions.

Table 2.7. *E. coli* cultivation media

LB (Manniatis *et al.*, 1989)	
NaCl	5 g/l
Yeast extract	5 g/l
Tryptone	10 g/l
á pH 7,3	
SOC	
Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0,58 g/l
KCl	0,185 g/l

Table 2.8. Streptomycetes cultivation media

HA-Medium (Hopwood *et al.*, 2000)	
Glucose	4 g/l
Yeast extract	4 g/l
Malt extract	10 g/l
á pH 7,4	
MS-Medium (Hopwood *et al.*, 2000)	
Soy Fluor	20 g/l
Mannitol	20 g/l
$MgCl_2$	10 mM
á pH 7,2	

R2YE (Hopwood *et al.*, 2000)	
Solution A	
K_2SO_4	0,25 g/l
$MgCl_2 \cdot 6H_2O$	10,12 g/l
$CaCl_2 \cdot 2H_2O$	2,95 g/l
Glucose	10 g/l
L-Proline	3 g/l
Casaminoacids	0,1 g/l
Solution B	
Sucrose	103 g/l
TES	5,73 g/l
Yeast extract	5 g/l
á pH 7,4	

Note: all components were added based on a final 1l volume; after autoclaving solution 1 ml of trace elements solution and 5 ml of 0,5% KH_2PO_4 were added to Solution B and then mixed with Solution A.

Minimal Medium (Hopwood *et al.*, 2000)	
L-Asparagine	0,5 g/l
K_2HPO_4	0,5 g/l
$MgSO_4\cdot 7H_2O$	0,2 g/l
$FeSO_4\cdot 7H_2O$	0,01 g/l
Glucose	10 g/l
á pH 7,4	
NL5	
NaCl	1g
K_2HPO_4	1g
$MgSO_4x7H2O$	0,5g
Trace Elements Solution	2ml
Glycerol	24ml
L-Glutamine	5,8g
à pH 7,3	

Note: for aranciamycin production experiments 1 g/l of yeast extract was added

2.5.   ANTIBIOTIC SOLUTIONS

Table 2.9. Antibiotic solutions (All antibiotic stock solutions were stored at -20°C).

Antibiotic	Solvent	Concentration	Target
Ampicillin	H_2O	100 µg/l	*E. coli*
Apramycin	H_2O	50 µg/l	*E. coli/Streptomyces*
Chloramphenicol	95% Ethanol	50 µg/l	*E. coli*
Hygromycin	H_2O	100 µg/l	*E. coli/Streptomyces*
Kanamycin	H_2O	30 µg/l	*E. coli*
Nalidixic Acid	0,4N NaOH	25 µg/l	*E. coli*
Phosphomycin	H_2O	200 µg/l	*E. coli*
Spectinomycin	H_2O	100 µg/l	*E. coli/Streptomyces*
Thiostrepton	DMSO	50 µg/l	*Streptomyces*

2.6. BACTERIAL STRAINS

Table 2.10. *E. coli* strains

ET12567	F⁻, *dam*-13::Tn9, *dcm*-6, *hsd*M, *hsd*R, *zij*-202 ::Tn10, *rec*F143, *gal*K2, *Gal*T22, *ara*-14, *lac*Y1, *xyl*-5, *leu*B6, *thi*-1, *ton*A31, *rps*L136, *His*G4, *tsx*-78, *mtl*-1, *gln*V44
TransforMax™ EC100D™ pir-116 electrocompetent cells	F⁻, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ☐80d*lacZ*ΔM15, Δ*lac*X74, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ⁻, *rps*L, *nup*G, *pir*-116(DHFR) (Epicentre®)
XL1-Blue	F´::Tn10 proA⁺B⁺ lacI�q Δ(lacZ)M15/recA1 endA1 gyrA96(Nalʳ) thi hsdR17 (rₖ⁻mₖ⁺) glnV44 relA1 lac
GB05red	Derived from GB2005 by integration of genes necessary for λ-mediated recombination

Table 2.11. Streptomycetes strains

S. coelicolor M145	Prototrophic derivative of *S. coelicolor* A3(2) missing SCP1, SCP2 plasmids (Kieser *et al.*, 2000)
S. albus J1074	Isoleucine-plus-valine auxotrophic derivative of *S. albus* G (Chater and Wilde, 1976), lacking *Sal*I-restriction activity
S. lividans 1326	Wild type of *S. lividans*, SLP2+, SLP3+ (Kieser *et al.*, 2000)

2.7. VECTORS

Table 2.12. Existing plasmid constructs

Name	Description	Reference or Source
pSET152	Integrative vector for actinomycetes containing *oriT, int*, and *attP* (*phiC31*), *aac(3)IV*	Bierman *et al.*, 1992
pAL1, pNL1	Replicative vectors for actinomycetes containing pSG5-rep, *ori*T, and *tipA* promoter	Fedoryshyn *et al.*, 2008
pNLTn5	Replicative vector for actinomycetes containing pSG5-rep, *ori*T, and *tn5* gene under *tipA* promoter	Dissertation of Dr. Lutz Petzke
p31Him	Suicide vector for actinomycetes containing *himar1(a)* gene under *phi*C31-integrase promoter	Bilyk *et al.*, 2012
pALTEAm	replicative plasmid derived from pTNM (Petzke and Luzhetskyy, 2009), containing two oriented outward	Horbal *et al.*, 2013

	constitutive promoters in minitransposons	
pUWL-Dre	Plasmid for inducing *Dre/rox* recombination containing synthetic *dre(a)* gene under the *tipA* promoter, pSG5rep, and *ori*T	Fedoryshyn *et al.*, 2008
pUWL-Cre	Plasmid for inducing *Cre/loxP* recombination containing synthetic *cre(a)* gene under the *tipA* promoter, pSG5rep, and *ori*T	Fedoryshyn *et al.*, 2008
pUWL-FLP	Plasmid for inducing *FLP/FRT* recombination containing synthetic *flp(a)* gene under the *tipA* promoter, pSG5rep, and *ori*T	Fedoryshyn *et al.*, 2008
pHP45Ω	Plasmid containing two antibiotic resistance genes with convenient restriction sites	Prentki and Kirsch 1984
pIJ773	pBluescript II SK-derivative, containing *aac(3)IV* and *ori*T flanked by two *loxP*-sites	Gust *et al.*, 2003
pIJ774	pBluescript II SK-derivative, containing *aac(3)IV* and *ori*T flanked by two *FRT*-sites	Gust *et al.*, 2003
p412C06	pOJ436 derivative, containing aranciamycin biosynthetic cluster	Luzhetskyy *et al.*, 2007
pTOS	Integrative vector for actinomycetes containing *oriT, aac(3)IV, int* and *attP* (VWB)	Herrmann *et al.*, 2012

Table 2.13. Cosmids and new plasmid constructs

Name	Vector	Size, kb	Marker	Description
p*Himar1*	n/a	3,4	*bla*	Plasmid with synthetic *himar1(a)* gene (GeneScript, NJ, USA)
pALHim	pAL1	9,6	*hph*	Plasmid derived from pAL1, containing *himar1(a)* gene
pNLHim	pNLTn5	9,2	*aac(3)IV*	Plasmid derived from pNL1, containing *himar1(a)* gene
pNLPr21	pNLHim	9,2	*aac(3)IV*	Plasmid derived from pNLHim, containing *himar1(a)* gene under strong synthetic promoter Pr21
pAHS	p31Him	4,2	*aac(3)IV*	Plasmid derived from p31Him with *hph* replaced by *aac(3)IV*
pPGP1,	pTOS	7,4	*aac(3)IV*	Plasmids derived from pTOS, both containing

pPGP2				gusA gene flanked by *att*P and *pse*B4 of *phi*C31; *pse*B4 is in direct (pPGP1) and inverted (pPGP2) orientations
p1F17, p1O6	pSMART®		*cam*	Cosmids from *S. albus* J1074 cosmid library with genome regions containing *att*B of *φC31*
p1E6, p2M18	pSMART®		*cam*	Cosmids from *S. albus* J1074 cosmid library with genome regions containing *pse*B4 of *φC31*

Table 2.14. New plasmid constructs carrying minitransposons

Name	Vector	Size, kb	Tns	Marker	Description
pITR	n/a	3,2	*himar1*	*bla*	Synthetic plasmid containing R6Kγ origin, two *lox*P and two *rox* sites framed by two ITRs
pTn5Oks	n/a	3,6	*himar1, tn5*	*bla*	PCR-derivative containing R6Kγ origin flanked by two MEs and two ITRs (Shine Gene, PRC)
pNheIaac	pITR	3,6	*himar1*	*bla*	pITR-derivative with one *lox*P-site
pHTM	pALHim	11,3	*himar1*	*aac(3)IV*	Minitransposon with *aac(3)IV*, R6Kγ origin, flanked by ITRs
pHSM	pALHim	12,9	*himar1*	*aadA(1)*	Minitransposon with *aadA(1)*, R6Kγ origin, flanked by ITRs
pHAH	pNLHim	11,1	*himar1*	*hph*	Minitransposon with *hph*, R6Kγ origin, flanked by ITRs
pHAH(II)9	pNLHim	11,1	*himar1*	*hph*	Minitransposon with *hph*, *phi*C31-*att*B, R6Kγ origin, flanked by ITRs
pHAT(II)3	pNLTn5	11,5	*tn5*	*hph*	Minitransposon with *hph*, *phi*C31-*att*B, R6Kγ origin, flanked by MEs
pNPT6	pNLPr21	11,1	*himar1*	*hph*	Minitransposon with *hph*, *phi*C31-*att*B, R6Kγ origin, flanked by ITRs
pAHT	pAHS	6,0	*himar1*	*hph*	Minitransposon with *hph*, *phi*C31-*att*B, R6Kγ origin, flanked by ITRs
pALG	pALHim	13,2	*himar1*	*aac(3)IV*	Minitransposon with *aac(3)IV*, *gusA* framed by two *fd* terminators, R6Kγ origin, flanked by ITRs

Table 2.15. Cosmids used for gene inactivation in *S. coelicolor* M145

Cosmid	Inactivated gene	Encoded protein
SCE9.1.A07_040722223J	*sco3390*	Putative two component sensor kinase
SCGD3.1.F04_04040113T4	*sco3812*	Putative gntR-family transcriptional regulator
SCQ11.2.B04_04111017BE	*sco3919*	Putative lysR-family transcriptional regulator
SCD66.1.C06_02021922EY	*sco4192*	Hypothetical protein
2StD46.2.C11	*sco4197*	Putative MarR-family regulator
SCD66.1.E01_02022009V9	*sco4198*	Putative DNA-binding protein
8F4.1.D01	*sco5222*	Putative lyase

2.8. METHODS IN MICROBIOLOGY

2.8.1. Cultivation conditions

2.8.1.1. Cultivation of *E. coli* strains

Cultivation of *E. coli* was carried out in 100 ml of the LB liquid medium in 300 ml Erlenmeyer shake flasks for the main cultures and in 20 ml LB medium in 100 ml Erlenmeyer shake flask for the pre-cultures at 37°C and 200 rpm overnight. For cultivation on a solid medium, LB agar was used, The culture was grown at 37°C overnight.

2.8.1.2. Cultivation of streptomycetes

The pre-culture was prepared as follow: 100 µl of a frozen stock mycelia were grown 48 h in 20 ml of TSB medium in a 100 ml Erlenmeyer flask containing three agitators. Afterwards 1 ml of the pre-culture was added to 100 ml of TSB medium in a 300 ml Erlenmeyer shake flask with four agitators. The main culture was grown for 24 h or until stationary phase of growth was reached, but not exceeding 48 h if further DNA isolations should be carried out. Alternatively, spores from the entire agar plate were inoculated in 20 ml TSB as above and grown for 48 h. The flasks were incubated at 28 °C and 200 rpm. For sporulation on solid medium, 1 ml of a culture was plated onto MS agar medium to harvest spores.

2.8.1.3. Sucrose cultures preparation

The desired culture of streptomycetes was grown in 20 ml of liquid TSB until stationary phase was reached. 10 ml of this culture was transferred into 15 ml falcon tube and centrifuged. The pellet was resuspended in 5 ml of 25% sucrose and placed on -80°C.

2.8.2. Transformation of DNA into *E. coli* (Maniatis *et al.*, 1989)

2.8.2.1. Electroporation

Preparation of electrocompetent E. coli cells

The glycerol stock of electrocompetent cells (50 μl) of an appropriate strain was inoculated into 100 ml shake flask with 20 ml of LB and cultivated overnight at 37 °C, 180 rpm. 2 ml of pre-culture was transferred into 4 shake flasks with 100 ml LB and incubated at 37 °C, 180 rpm until value of OD_{600} reached 0,5. Then the cells were centrifuged in 8x50 ml falcon tubes for 10 min at 4000 rpm, 4 °C. Pellet was resuspended in 5 ml of 10 % glycerol, combined into one tube, and washed with a volume up to 40 ml. Cells were pelleted again and washed with an additional 40 ml of 10 % glycerol. The pellet was resuspended in 1 ml of 10 % glycerol and distributed in 50 ml aliquots. Aliquots were stored at -80 °C.

Electroporation of electrocompetent E. coli cells

Electrocompetent cells were retrieved from the stock and placed on ice, where they thawed for 5 min., while electroporation cuvettes were cooled at -20°C. Then, the cells were carefully transferred into electroporation cuvettes and 5 μl of plasmid DNA was added and resuspended. Before electroporation the cuvette was wiped down on metal contacts to prevent short circuit and inserted into the cuvette-nest of "*E.coli* pulser" electroporator (BIORAD). Shock pulse of 1,8 KV was administered, 1ml of LB-medium was added to the electroporated cuvette, mixed, and the obtained suspension was transferred into new tube. The cells were placed on 37°C and incubated for 30 min to recover. Then, the cells were plated on the appropriate antibiotic medium. Plates were incubated at 37°C for up to 24 h.

2.8.2.2. Chemical transformation

Preparation of chemically competent E.coli cells

A glycerol stock of chemically competent cells (50 μl) of an appropriate strain was inoculated into 100 ml shake flask with 20 ml of LB and cultivated overnight at 37 °C, 180 rpm. 2 ml of this pre-

culture was transferred into 4 shake flasks with 100 ml LB and incubated at 37 °C, 180 rpm until OD_{600} reached 0,5 value. The cells were centrifuged in 8x50 ml falcon tubes for 10 min at 4000 rpm, 4 °C. In the following steps, the cell suspension was kept on ice as long as necessary. The pellet was resuspended in 30 ml of ice cold 0.1 M $MgCl_2$, centrifugated and incubated for 20 min in 0.1 M $CaCl_2$. After incubation the cells were centrifuged again and resuspended in 3 – 5 ml ice cold 0.1 M $CaCl_2$ – 15% glycerol solution and aliqouted. Aliquots were stored at -80 °C.

Transformation of chemically competent cells by heat shock

Frozen chemically competent cells were placed on ice and to thaw for 4 minutes. 5 µl of a plasmid were added into the thawed cell suspension and mixed. The mixture was incubated on ice for 15 min. A heat shock was administered for 1 min in a 42 °C water bath and cells were placed back onto ice and 1ml of LB was added. The cells were placed on 37°C and incubated for 30 min to recover. Afterwards, the cells were plated on the appropriate selective medium. Plates were incubated at 37°C for up to 24 h.

2.8.3. Intergeneric conjugation of *E. coli* with streptomycetes

To introduce a desired plasmid into the genome of streptomycetes method of intergeneric conjugation was used. This method is based on the RP4 conjugation system with a relaxed recipient specificity and imply transfer of DNA from non-methylating *E. coli* ET12567 donor strain containing the RP4 derivative plasmid pUZ8002 (Blaesing *et al.*, 2005) to the recipient streptomycetes strain.

2.8.3.1. Preparation of strains

A desired plasmid was introduced into the chemically competent *E. coli* ET12567/pUZ8002 cells by the heat shock transformation. Transformed cells were selected with two antibiotics, kanamycin (30 µg/ml) for pUZ8002 selection and appropriate antibiotic necessary for the selection of a corresponding plasmid. One of the obtained double resistant colonies was picked up and spread on the entire surface of the fresh selective medium and grown for 12 h at 37°C.

A frozen mycelia stock, cell culture or spores of the streptomycetes strain were spread on the entire surface of the fresh MS agar medium and grown for 48 h or until sporulation was obtained at 28°C.

2.8.3.2. Conjugation

Spores of arecipient streptomycetes strain were washed from the of surface of MS agar plate with 9 ml of TSB and transferred into 2 ml tubes, 1 ml aliquot each and heat-shocked for 15 min at 42°C. The cells of the donor strain were collected and mixed with heat-shocked spores of the recipient strain. The mixture was vortexed and plated onto MS agar. The conjugation plates were incubated at 28°C. After 12-18 h of the incubation all plates except the positive control were overlayed with phosphomycin, 200 μg/ml, or nalidixic acid, 25 μg/ml, to get inhibit the growth of the donor strain and with appropriate antibiotic to select the transconjugants of the recipient strain with a desired plasmid.

2.8.4. Transposon mutagenesis in streptomycetes

Spores of streptomycetes exconjugants transformed with an appropriate plasmid were scraped from the MS agar plate, transferred in 100 ml Erlenmeyer shake flask containing 20 ml TSB and 200 μg/ml of phosphomycin or 25 μg/ml of nalidixic acid and an appropriate antibiotic for a plasmid selection. If the induction of *himar1(a) via* thiostrepton inducible promoter was required, 2 μg/ml of thiostrepton after 24 h of cultivation were added. After a stationary phase was reached, 1 ml of culture was transferred into 100 ml Erlenmeyer flask with 20 ml of the TSB medium and phosphomycin and incubated at 37°C, 180 rpm until the stationary phase was reached. This step was repeated twice. It was necessary to get rid of replicating plasmid using the inability of pSG5 replicon to replicate at temperatures higher than 34°C. After three passages, 1 ml of the transposon mutant's culture was plated on MS agar plate with phosphomycin and an appropriate antibiotic. Spores were collected, and serial dilutions were made. 1 ml of each dilution starting from 10^{-6} was plated on R2YE agar plate. After 3 to 5 days of growth at 28°C single colonies appeared. The obtained colonies were picked to TSB medium and tested for a loss of the plasmid.

2.8.5. Rescue cloning

To generate rescue plasmids, genomic DNAs of desired strains was isolated (see 2.9.1) and digested with *Bam*HI, *Kpn*I, *Nco*I, *Not*I, *Pst*I or *Sac*II for 4 h at 37°C, then the chromosomal DNA was precipitated with ethanol, dissolved in 16 μl of water and, after 2 μl of ligation buffer and 2 μl of T4-DNA ligase were added, selfligated overnight at 4°C. In the morning additional 1 μl of T4-DNA ligase was added to the ligation mixture and reaction was proceeded for 2h at 18°C. 5

µl of selfligated DNA was transformed into the *E. coli* TransforMax™ EC100D™ pir-116 electrocompetent cells or into the *E. coli* XL1 by electroporation (E. coli Pulser Bio-Rad™) and plated on selective LB medium. The rescue plasmids were isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega™) and sequenced by 4Base-Lab or by GATC-Biotech.

2.8.6. Expression of *Dre, Cre* and *FLP* recombinases

Spores of streptomycetes transconjugants with a desired plasmid containing a recombinase gene were collected from an MS agar plate and transferred into 100 ml shake flask with 20 ml of TSB, 50 µg/ml of hygromycin and 400 µg/ml of phosphomycin. After a stationary growth phase was reached (2-3 days), 10 µg/ml of thiostrepton for the induction of the *tipA* promoter were added. After 12 h, 1 ml of the culture was plated onto 50 µg/ml hygromycin MS agar and grown for 3 days until the culture started to sporulate. The spores were harvested, reinoculeted into fresh TSB medium and the induction step was repeated. 1 ml of induced culture was transferred onto MS agar plates and incubated at 28°C for 2-3 days. The spores were harvested and serial spore dilutions were made. 1 ml of each dilution starting from 10^{-6} was plated on MS agar plates. After 3 to 5 days of growth at 28°C single colonies appeared. These colonies were patched on the non-selective MS-medium and on the MS-medium containing corresponding antibiotic (resistance gene to which should have been excised). The patches that did not grow on the selective medium were inoculated into liquid TSB, their chromosomal DNA was isolated and tested by PCR to prove the loss of the resistance marker.

2.9. METHODS IN MOLECULAR BIOLOGY
2.9.1. Genomic DNA isolation of streptomycetes

A fragment of a desired colony was braked off with the sterile pipette tip and transferred to 20 ml of TSB. After a stationary phase of growth was reached, 200-300 mg of pelleted mycelia were collected in 2 ml eppi and resuspended in 500 µl of TE buffer and centrifugated at 5000 rpm for 8 min. Supernatant was discarded and 500 µl of fresh TE buffer with 4 mg/ml lysozyme was added to the samples. Samples were then incubated for 30-45 min at 37°C and inverted each 5 min. 120 µl of 10% SDS and 50 µl of 5M NaCl were added to the sample and sample was vortexed for 20 sec. Sample was incubated at 65°C for 30 min. Afterwards, samples were cooled to RT and 240 µl of 3M CH_3COOK were added. Samples were incubated for 10m at 4°C, centrifugated at 14000 rpm for 12 min, 4°C. A supernatant was transferred to a new tube and 600

µl of pure isopropanol were added. Samples were gently mixed several times and incubated at -20°C for 10 min, then centrifugated at 14000 rpm, 4°C for 12 min. A supernatant was discarded and the pellet was washed once with 70% ethanol (14000 rpm, 4°C, 5 min). Supernatant was discarded and the pellet was allowed to dry for 30 min at 37°C. 200-400 µl of water was added to the pellet. The chromosomal DNA solution was stored at -20°C.

2.9.2. Measurement of DNA concentration

DNA concentration was measured according to Beer-Lambert Law. Due to this the nucleic acids reach their characteristic absorption maximum at 260 nm. Extinction in one unit corresponds to 50 µg/ml of DNA concentration. DNA purity was measured at 280 nm corresponding to absorption maximum of aromatic amino acids. The ratio between 260nm and 280 nm absorptions may not exceed 1,8. The measurements were carried out by spectrophotometer.

2.9.3. DNA agarose gel electrophoresis

For gel preparation 0,7% agarose melted in 1x TAE was used. DNA was separated at 80 V in 1xTAE buffer at RT. For fragment size identification 1kb DNA ladder (Fermentas) was used. The gel was stained for 15 min in a water bath with ethidium bromide or Roti-safe concentration 2 µg/ml. DNA fragments were visualized with UV-transilluminator.

2.9.4. Purification of DNA from agarose gels

The gel was stained for 20 min and the fragment of a desired size was excised under UV light and transferred to the eppi. DNA from this fragment was isolated using the Promega™ Wizard® GEL and PCR Clean-Up System according to manufacturer instructions and eluted in 55 µl of nuclease free water and stored at -20°C.

2.9.5. DNA-digestion

DNA was digested by restriction endonucleases according to manufacturer protocols in enzyme specific buffer and at appropriate temperature for a time of 1 h for the analytical digestion and for 2,5 h for the preparative digestion. For analytical purposes 20 µl of total volume were used and preparative digestions were carried out in a total volume of 70 µl. 10 units of a desired

enzyme were used for the analytical purposes and 30 units of enzyme for the preparative digestion.

2.9.6. DNA-ligation

T4-DNA-ligase (Fermentas) was used for ligation of DNA fragments. in the reaction volume of 20 µl contained 10% of ligase buffer, 2 µl of ligase and insert and vector in ratio from 1:1 to 3:1. Rescue plasmids generation was carried out in 20 µl of reaction volume, containing 10% of ligase buffer, 2 µl of ligase and 16 µl of previously digested chromosomal DNA. Reactions were incubated at +4°C overnight, afterwards additional 1µl of T4-DNA ligase was added to the mixture and the reaction mixture was incubated at 18°C for another 1-2 h.

2.9.7. DNA-precipitation with ethanol

The isolated DNA was dissolved in 200 µl of nuclease free water and mixed with 20 µl of 3M solution of sodium acetate and 600 µl of 99% ethanol. Samples were incubated for 10 min at -20°C and DNA-precipitate was collected by centrifugation for 12 min at 14000 rpm, 4°C. After centrifugation the pellet was washed with 70% ethanol, air dried and dissolved in a desired volume (50 – 100 µl) of nuclease free water.

2.9.8. DNA-dephosphorylation

Dephosphorylation of DNA was carried out using FastAP Thermosensitive Alkaline Phosphatase (Fermentas). With this aim 1 µl of FastAP was added to a restriction reaction, the mixture was incubated at 37°C for 10 min, and then the reaction was stopped by heating at 65°C for 15 min.

2.9.9. Southern hybridization
2.9.9.1. Preparation

The nylon membrane was touched only with nitrile glowes. Buffers and solutions were prepared using distilled water. The probes for labeling were amplified by PCR, purified and dissolved in 16 µl H_2O. Genomic DNA of samples was digested overnight. Reaction was carried out in 200 µl of reaction volume containing 10% of enzyme specific buffer, 40 units of desired enzyme and from 100 to 150 µl of genomic DNA. After digestion, DNA was precipitated with 99% ethanol and

resuspended in 35 µl of H$_2$O. To estimate digestion quality control gel was done: 4 µl of the DNA sample were mixed with 1 µl of bromphenol blue loading dye and loaded onto the 0,7% agarose gel.

2.9.9.2. Labeled probe preparation

16 µl of the purified probe was incubated at 95°C for 10 min. The probe was placed on ice and allowed to cool. The probe was first incubated for 18 h at 37°C with 4 µl of Dig-High Prime (Roche) and then frozen at -20°C to stop the reaction. The labeled probe was mixed with 50 ml of hybridization solution (blotting buffer 1 + 1,5% milk powder).

2.9.9.3. Separation of DNA

A standard 0,7% agarose gel was used for the samples separation. 30 µl of each sample were mixed with 4 µl of bromphenol blue loading dye and loaded onto the gel. Identification of fragments size was guided by a DIG labeled ladder III (Roche) and by 1 kb DNA ladder (Fermentas). Wild type DNA and amplified plasmid were used as negative and positive controls, respectively. The gel was run at 50 V for 2 h. After this, the gel was placed with pockets faced down into plastic container of appropriate size and washed on the table-top shaker in 250 mM HCl for 10 min. The gel was shortly washed in water and then two times for 15 min in the denaturation buffer.

2.9.9.4. DNA transfer to nylon membrane

DNA-transfer was carried out in alkaline conditions. With this aim a plastic container was filled with 500 ml of 0,4M NaOH. A plastic box, 10 cm high, was placed in the middle of the container. Two strips of blotting paper were placed on the top of the container and their edges were immersed into NaOH. The gel was removed from denaturation buffer and placed with the pockets faced down on the top of this "bridge". The nylon membrane was placed onto the gel and the construction was soaked with 0,4M NaOH. Two sheets of blotting paper were placed onto the top of the membrane and soaked with NaOH. Stack of paper towels was placed on the top of the construction. The apparatus was weighted down by a metal block (app. 500g) to ensure good and even contact between gel and membrane. DNA transfer was left overnight for 20 h. Afterwards, the blot apparatus was disassembled. To bind the transferred DNA to

membrane covalently, the membrane was exposed to ultraviolet radiation (2 min top side and 1 min back side). The membrane was washed with water and air dried.

2.9.9.5. Prehybridization and hybridization

The membrane was placed into a hybridization cylinder and incubated with 50 ml of prehibridization buffer in a hybridization oven for 4 h, at 65°C. Meanwhile the labeled probe, dissolved in 50 ml of hybridization buffer was denaturated by the heat at 95°C for 10 min. Then the prehybridization solution was discarded and the activated probe was added to the membrane. Incubation in hybridization oven was done overnight at 65°C.

2.9.9.6. Membrane treatment and visualization

The labeled probe solution was discarded and the membrane was washed twice with 2x wash buffer for 10 min at RT and twice with 0,5x wash buffer for 15 min at 56°C. Then the membrane was washed with the blocking buffer for 30 min, with the antibody solution for 1 h and with the blot buffer 1 containing 0,3% Tween for 15 min. After all washing steps were completed the membrane was placed in 35 ml of the blot buffer 3. Hybridization bands were visualized by incubation for 3 h in the water solution of NBT/BCIP Tablets (Roche Diagnostics GmbH).

2.9.10. Polymerase chain reaction (PCR)

To amplify a desired DNA fragment the polymerase chain reaction was used. The *Pfu* polymerase (Fermentas) was used to amplify the fragments for clonning, while *Taq* polymerase (Fermentas) was used to amplify the fragments for analytical needs. Extension time (T_E) was determined specifically for each fragment: for a regular reaction it was calculated with ratio 20 sec. for Phusion polymerase, 1 min for *Taq* polymerase and 2 min for *Pfu* polymerase per 1 kb of the fragment length. The standard reaction mixtures and protocols used for fragments amplification are shown in table below (Tabs.3.16-3.19).

Table 2.16. Standard PCR reaction for *Pfu* and *Taq* polymerases

Component	Volume	Final Concentration
DNA-template	0,5 µl	
Pfu/Taq, 10x Buffer	5 µl	
dNTPs Mix, 10 mM	1 µl	0,2 mM
Primers, 10 µM	1 µl of each	0,2 µM
DMSO	5 µl	
H_2O	á 49 µl	
Pfu-/Taq-polymerase	1 µl	2,5 u

Table 2.17. Standard PCR reaction for Phusion polymerase

Component	Volume	Final Concentration
DNA-template	0,2 µl	
HF/GC, 5x Buffer	2 µl	
dNTPs, 10 mM	0,2 µl of each	0,1 mM
Primers, 10 µM	0,2 µl of each	0,1 µM
DMSO	0,3 µl	
H_2O	á 20 µl	
Pfu-/Taq-polymerase	0,2 µl	0,4 u

Table 2.18. Standard PCR protocol for *Pfu* and *Taq* polymerases

Reaction Step	Temperature, °C	Duration, min:sec	Number of Cycles
Initial Denaturation	95	2:00	1
Denaturation	95	1:00	
Annealing	T_A	0:30	30
Extension	72	T_E	
Final Extension	72	5:00	1

Table 2.19. Standard PCR protocol for Phusion polymerase

Reaction Step	Temperature, °C	Duration, min:sec	Number of Cycles
Initial Denaturation	98	1:00	1
Denaturation	98	0:20	
Annealing	T_A	0:20	30
Extension	72	T_E	
Final Extension	72	4:00	1

2.9.10.1. Primers and PCR modifications

Primers used for fragments amplification were synthesized by Eurofins MWG Operon. The annealing temperature (T_A) for PCR-reaction was calculated as temperature 5°C lower than the melting temperature of primer with lower melting temperature if the *Pfu* or *Taq* polymerases were used and as identical to melting temperature of primer with lower melting temperature if Phusion was used. If such approach was unfruitful, then the gradient-PCR was carried out and different annealing temperatures were tested. If the primers carried any additional features needed to be brought into the amplified fragment (*e.g.* restriction sites, promoters, terminators) **two-step-PCR** with two annealing temperatures was carried out: first annealing temperature was calculated for the primer fragment homologous to the template and the second annealing temperature was calculated for the whole primer length including additional sequences. In case when concentration of unspecific fragments amplified by PCR was too high, the **nested PCR** was

carried out: whole volume of PCR-mixture was separated by agarose gel electrophoresis; then the fragment of the desired size was cut out, eluted and used as a template for the next PCR reaction. Primers sequences with annealing temperatures and PCR features are listed in the tables below (Tabs. 2.20-2.23).

Table 2.20. Primers used for plasmid construction

#	Name	Primer sequence (in 5'->3')	Features
1.	Fr-*M*I-*hph*	ccccc*caattg*gggtcgcagggcgtgcccttgggctccccgggcg cgtaccgtatttgcagtaccagcgt	*Mun*I (in italics)
2.	aac-f	acgtacc*gaattc*ggttcatgtgcagctccatcagc	*Eco*RI (in italics)
3.	aac-r	acgtac*gaattc*atgagctcagccaatcgactgg	*Eco*RI (in italics)
4.	Fr-*M*I-*attB*-*hph*	ccccc*caattg*CGGGTGCCAGGGCGTGCCCT TGGGCTCCCCGGGCGCGTAC*ccgtatttgca gtaccagcgt*-3	*Mun*I (in italics), *attB* (in capitals)
5.	Rs-*X*I-*hph*	ccccc*tctaga*gaataggaacttcggaatagg	*Xba*I (in italics)
6.	Fr-*H*3-*S*I-Pr21-*bla*	ccccc*aagctt*GCTTGGTACCTTGCTCATCCT ACCATACTAGGACGTGTTAGAGCCCG CACA*atttaaat*gttaa ctacgtcaggtggcac	*Hind*III and *Swa*I (in italics), Pr21 (in capitals)
7.	Rs-*H*3-*S*I-*bla*	ccccc*aagcttatttaaat*ttaccaatgcttaatcagtgagg	*Hind*III and *Swa*I (in italics)
8.	Fr-*X*I-*ep*1-*gusA*	ccccc*tctaga*GTTGTGGGCTGGACAATCGT GCCGGTTGGTAG GATCCAGCGatgctgcggcccgtcga aaccc	*Xba*I (in italics); *erm*Ep1 (in capitals)
9.	Rs-*M*I-t*fd*-*gusA*	ggggg*caattg*AAAAAAAAAGGCTCCAAAAG GAGCCTTTTAAtcactgcttcccgccctgctg	*Mun*I (in italics); *fd* terminator (in capitals)
10.	Fr-*E*RI-t*fd*-*aac*	ccccc*gaattc*AAAAAAAAAGGCTCCAAAAG GAGCCTTTTAAcacggtaactgatgccgtatt	*Eco*RI (in italics); *fd* terminator (in capitals)
11.	Rs-*E*RI-*aac*	ccccc*gaattc*ggaataggaacttatgagctc	*Eco*RI (in italics);

Table 2.21. Primers used for Redirect

#	Name	Primer sequence (in 5'->3')
1.	Fr-pIJ774 -*attB*-del	ctggtggatgaaggtcccgtcgatgatgtacgtgaccgtATTCCGGGGATCCGTCGACC
2.	Rs-pIJ774 -*attB*-del	caactacaagcacctggatccgttcatcatgatggaccaTGTAGGCTGGAGCTGCTTC
3.	Fr-pIJ773-*attB*2-del(II)	ctggtggcggccggggcctggctggtcttccgcgtccgcATTCCGGGGATCCGTCGACC
4.	Rs-pIJ773-*attB*2-del(II)	ctccgcgagaggggcgctgctgtcgttgctcatgccgggTGTAGGCTGGAGCTGCTTC
5.	Fr-*hph/aac(3)IV*	gaataggaacttcggaataggaacttcaggcgccggggGGGAATAGGAACTTATGAGCT
6.	Rs-hph/aac(3)IV	ccgtatttgcagtaccagcgtacggcccacagaatgatgGGTTCATGTGCAGCTCCAT

Table 2.22. Primers used for plasmids sequencing

#	Name	Primer sequence (5'->3')	Sequenced plasmids
1.	P1-*himar1(a)*-ch	gggggaactcgttgtccagcca	pALHim, pNLHim
2.	P2-*himar1(a)*-ch	ccgaaggagtactaccagaac	
3.	P1-*tn5(a)*-ch	cggcgcgggtcgcccagcgcc	pNLTn5
4.	P2- *tn5(a)*-ch	aggcgctccagtcgaagctgg	
5.	P1-*attB*40-ch	cgttagccatgagggtttagt	pTn5Oks*hph*, pTn5Oks*attBhph*
6.	P1-*hph*-ch	tgggggccgccggacaccgcc	
7.	P1-*aac(3)IV*-ch	ccgccagaggcgggatgcgaa	pALG
8.	P2-*aac(3)IV*-ch	aggccggcatttttcagcgtga	
9.	P1-*ermE*p1-ch	gtgagccgccgcgtgccgtcg	
10.	Fr-*hph/aac(3)IV*-ch	tcatgtgcagctccatcagca	pAHS
11.	Rs-*hph/aac(3)IV*-ch	gaaagacaatccccgatccgc	

12.	pMOD-for	ccaacgactacgcactagccaac	Rescue plasmids pHTM- and pHSM-based
13.	pTn5Oks-for	attcaggctgcgcaactg	Rescue plasmids pHAH-based
14.	Fr-pSET152-Rp	tacacgacgcccctctatggcccg	Rescue plasmids pSET152- and pOJ436-based
15.	Rs-pSET152-Rp	ggcgctacgctgtgtcgctgggct	
16.	Fr-*attB*-del-ch	cgggtccttcatcttgtccttggc	p1F17- and p1O6-based vectors
17.	Fr-*attB*-del-ch	atgcccgcagtgaccgttgagaat	
18.	Fr-*attB*2-del-ch(II)	tcgacatcgcgcccgggcaggacg	p1E6- and p2M18-based vectors
19.	Rs-*attB*2-del-ch(II)	gtgaagtaccagacggcggactgg	

Table 2.23. Specific PCR-features

#	Fragment	Primers	T_A, °C	t_E,	Polymerase	Template	PCR-type
1.	*hph*	Fr-*MI-hph*, Rs-*XI-hph*	55, 62	0:45	Phusion	pAL1	two step
2.	*aac(3)IV*	aac-f, aac-r	50	2:00	*Pfu*	pIJ773	one step
3.	*attB*40*hph*	Fr-*MI-attB-hph*, Rs-*XI-hph*	55, 62	0:45	Phusion	pAL1	nested
4.	*bla*-Pr21	Fr-*H3-SI-Pr21-bla*, Rs-*H3-SI-bla*	55, 63	0:30	Phusion	pLitmus38	nested
5.	*hph/aac(3)IV*	Fr-*hph/aac(3)IV*, Rs-hph/aac(3)IV	50, 55	0:40	Phusion	pIJ773	two step
6.	*aac(3)IV*+t*fd*	Fr-*ERI-tfd-aac*, Rs-*ERI-aac*	50, 60	0:40	Phusion	pIJ773	two step
7.	*ermEp1+gusA*+t*fd*	Fr-*XI-ep1-gusA*, Rs-*MI-tfd-gusA*	56, 64	1:00	Phusion	pSET*gusA*	two step
8.	*attB/aac(3)IV+oriT*	Fr-pIJ-*attB*-del, Rs-pIJ-*attB*-del	50, 55	0:40	Phusion	pIJ774	two step
9.	*pseB4/aac(3)IV+oriT*	Fr-pIJ-*attB*2-del(II), Rs-pIJ-*attB*2-del(II)	50, 55	0:40	Phusion	pIJ773	two step

2.9.11. Red/ET-recombination

2.9.11.1. Fragment preparation for cosmid targeting

For a fragment deletion following primers were designed (Tab. 2.21). Each of these primers contains on 5'-end 39 pb matching the sequence on the chromosome flanking the region to be deleted and on the 3'-end 19 or 20 pb fragment matching right and left shoulder of a disruption cassette.

To amplify the disruption cassette, the plasmid containing this cassette was digested by restriction enzymes with sites flanking the cassette (*e.g.*, *Eco*RI and *Hind*III for pIJ773 and pIJ774). Then the fragments were separated by the agarose gel electrophoresis with subsequent elution of the fragment of a desired size. The disruption cassette was amplified by PCR using previously designed primers and purified once again. To prevent occurrence of false-positive transformants after λ-red mediated recombination, highly competent *E. coli* XL1 cells were chemically transformed with 1 μl of the purified fragment. An absence of colonies proves the purity of the eluted fragment.

2.9.11.2. Λ-red mediated recombination in *E. coli* GB05red

A vector containing the region to be disrupted or substituted was purified using BAC-MAX or Promega Midiprep Kit. Thereafter 5 μl of the purified vector were transformed by electroporation into electrocompetent cells of *E. coli* GB05red, the transformants were recovered for 1 h at 37°C and grown overnight on selective medium at 37°C.

On the next day one isolated colony was inoculated into 5 ml of the selective LB medium. After 24 h of growth 0,5 ml of the culture were transferred into fresh 5 ml of the LB medium containing 10 mM of L-arabinose (for induction of genes responsible for λ-red mediated recombination). Culture was grown for 2 h at 37°C till OD_{600} of 0,6 was reached. Then the cells were pelleted, washed twice with ice cold water and resuspended in 50 μl of ice-cold water. 2 μl of PCR amplified disruption cassette were added and this mixture was transferred into 1mm-electroporation-cuvette and electroporated (200 Ω, 25μF, 1,8 kV). The transformants were recovered in SOC medium for 1,5 h at 37°C, then plated on the selective LB medium (selection was carried out, if possible, against both, the disruption cassette and the vector) and grown overnight at 37°C.

After 24 h single colonies of transformants were detected, one colony was isolated and inoculated into 200 ml of the selective LB-medium. Cosmid or plasmid DNA of this colony was isolated

using BAC-MAX or Promega MidiPrep Purification Kits, respectively, and the recombination was verified by the amplification of the disrupted fragment by PCR.

2.9.11.3. Transfer of recombined cosmid into *S. albus* J1074

Cosmid containing the disruption cassette was introduced into *E. coli* ET12567/pUZ8002 by chemical transformation and thereby transferred into *S. albus* J1074 by intergeneric conjugation. Transformants were inoculated into selective TSB medium, incubated for 48 h at 28°C and plated on selective MS agar plates. After sporulation dilutions to single colonies were made. To verify loss of the cosmid vector pSMART the plates with transconjugants were overlayed with X-Gluc. Colonies that did not turn blue were patched onto a new MS-agar plate and tested for the GusA-activity. One patch that did not demonstrate GUS-activity was inoculated into selective TSB, cultivated and plated onto MS agar plate. Serial dilutions to single spores of this mutant were made. One colony was isolated, multiplied and its chromosomal DNA was isolated and tested by PCR.

After the mutant was obtained, the disruption cassette was excised by expression of recombinase gene (see 2.8.6).

2.10. METHODS IN BIOCHEMISTRY
2.10.1. Measurment of glucuronidase activity
2.10.1.1. Spectrophotometric measurment of glucuronidase activity

To measure GUS activity instreptomycetes mutants, their mycelium from frozen, stock was inoculated into 20 ml of liquid TSB and incubated for 48 h at 28°C, 220 rpm. After the stationary phase was reached, 0,5 ml of each culture was transferred to fresh 20 ml of the TSB medium and incubated for another 48 h at 28°C, 220 rpm. After cultivation, 2 ml of mycelia were harvested by centrifugation, washed twice with distilled water and resuspended in 0,5 ml of buffer 2 and incubated for 30 min at 37°C. After incubation the samples were centrifugated at 14000 rpm, 10 min, 4°C and 100 µl of supernatant were transferred into 96-well plate. 100 µl of buffer 3 were added to each sample. The extinction values were measured at 415 nm, at 37°C, for 35 min each minute. Values between 10th and 30th minute were used to calculate average for one minute. The mixture of lysate mixed with buffer 1 was used as a reference.

2.10.1.2. Dry weight calculation

After stationary phase of growth was reached, 10 ml of the main culture were transferred into 15 ml-falcon tubes. Mycelium was pelleted by centrifugation, the supernatant was discarded and the samples were dried for 24 h at 65°C. After 24 h the falcon tubes with dry mycelium were weighted.

2.10.1.3. Calculation of glucuronidase activity.

Glucuronidase activity of each sample was calculated using following formula:

$$A = l \times C \times e,$$

where "A" is absorption, l – optical path (in our case 1 cm), C – molar concentration, e – coefficient of molar extinction (14000 for p-nitrophenol).

Molar concentration could be calculated as follows:

$$C = n/V, \; A/l \times e = n/V,$$

Where n is quantity of moles (in our case moles of p-nitrophenol), and V – volume (100 µl of lysate correspond 400 µl of culture).

Therefore quantity of moles can by calculated as follows:

$$n = (A \times V)/(l \times e).$$

Activity of an enzyme is calculated in units, where one unit is the amount of micromoles of substance generated in one minute. It leaded us to:

$$\text{Units} = n/(t \times 10^{-6}), \; \text{Units} = A/(1000 \times e \times t \times 10^{-6}) = (1000 \times A)/(14000 \times 20) =$$

$$= A/280$$

The obtained activity corresponded to activity in 400 µl of main culture. To calculate activity for 1 mg of dry mycelium total activity of culture was calculated and then divided on dry weight of mycelium calculated for the volume of culture (in mg).

2.10.2. Strains cultivation and extracts preparation for HPLC

2.10.2.1. Cultivation conditions

The streptomycetes strains were grown in 20 ml of TSB medium containing apramycin and nalidixic acid for 48 h at 30°C. Then 200 µl of preculture were transferred into 15 ml of NL5+YE medium and cultivated for another 120 h at 30°C. After cultivation, 5 ml of mycelia were harvested by centrifugation for 30 min at 4000 rpm.

2.10.2.2. Extraction from the liquid culture

Supernatant was transferred into new falcon tube and mixed with 5 ml of ethylacetate and rotated on a rotator for 30 min. Then the samples were centrifugated at 4000 rpm for 10 min and upper phase of samples was transferred to glass vials and dried under N_2. Dry pellet was dissolved in acetonitrile and centrifugated at 14 000 rpm for 10 min to remove the cell debries. 80 µl of extracts were transferred into vials and analyzed by HPLC.

2.10.2.3. Extraction from the solid culture

The solid culture extraction was performed in the same way as the extraction from the liquid culture but instead of ethylacetate acetone-methanol mixture (1:1) was used.

2.10.3. HPLC data analysis

The HPLC-ESI-MS-UV-Vis analysis was done on a Dionex Ultimate 3000 HPLC system (Thermo Fisher Sientific) connected to ESI-MS amazon (Bruker). The HPLC-system was equipped with column BEH C18, 100 x 2.1mm, 1.7 µm (Waters), column BEH C18, 50 x 2.1mm, 1.7 µm (Waters) or Luna C18, 100 x 2 mm, 2.5 µm (Phenomenex) depending on the method. For unkown extracts a 18 min gradient mainly on the long column and for prepurified or known extracts a 9 min gradient was choosen. All gradient methods start with 5% B and increase during 9 or 18 min to 95% of B. Solvent A was H_2O + 0.1% FA, solvent B contained ACN + 0.1% FA. The flow for BEH C18 columns was 0.6 ml/min, for Luna C18 0.4 ml/min, UV-Vis detection from 210–600 nm) Most of the time the ESI-MS was used in alternating mode (positive and negative). Depending on the method only a Full scan MS was recorded or additional with MS^2 data. Both systems HPLC and MS were combined through the program Hystar (Bruker). Due to this standard LC-MS experiments were selectable as supermethods. Those describe the selected

column (Luna = Luna C18, 100 = BEH C18 100 mm, 50 = BEH C18 50 mm), the gradient (9 min, 18 min) and the MS mode (MS only, ms2 posneg).

High resolution ESI-MS were measured on a Maxis Q-Tof 4G (Bruker) or an Orbitrap LTQ (Thermo Fisher Scientific) connected to the same HPLC-system used for standard LC-MS.

3. RESULTS

3.1.   Development of random transposon mutagenesis system for streptomycetes

3.1.1.   Construction of pNLHim and ALHim

The synthetic *himar1(a)* gene framed by convenient restriction sites was provided on a p*Himar1* plasmid (GeneScript, NJ, USA). The plasmid was digested with *Hin*dIII and *Xba*I and the fragment containing *himar1(a)* gene was cloned into pNL1 and pAL1 plasmids downstream of thiostrepton inducible promoter, giving pNLHim (Fig. 3.1.A) and pALHim (Fig. 3.2.A), respectively.

Main features of both plasmids are: *oriT*, an origin of transfer of broad specificity conjugative plasmid RP4 for intergeneric conjugation between *E. coli* and streptomycetes, the synthetic *himar1(a)* gene under control of thiostrepton inducible promoter, the *tipA*p inducible promoter, that allows to control transposition, and pSC5rep, the temperature sensitive replicon for actinomycetes unable to replicate at temperatures higher than 34°C, that facilitate loss of vector after transposition.

Obtained plasmid constructs were verified by analytical restrictions. Digestion of pNLHim (Fig. 3.1.B) and pALHim (Fig. 3.2.B) with *Xba*I and *Hin*dIII gave one 1 kb fragment, corresponding to *himar1(a)* gene. Digestion with *Eco*RV demonstrated that this restriction site is unique for both plasmids and can be used for further cloning of minitransposons.

A   B
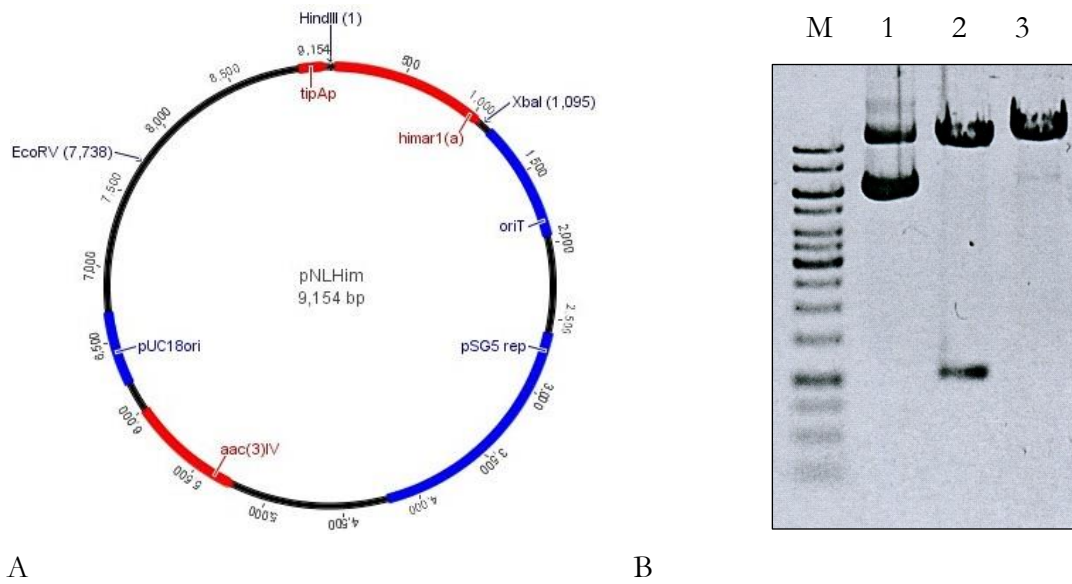
Figure 3.1. The map (A) and analytical restriction (B) of pNLHim. (A) Plasmid contains following features: *oriT* **– origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon ;** *himar1(a)* **– synthetic transposase gene, under control of** *tipA*p **– thiostrepton inducible promoter;** *aac(3)IV* **– apramycin resistance marker. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with** *Xba*I **and** *Hin*dIII**; 3 – plasmid digested with** *Eco*RV**. The** *himar1(a)* **fragment is visible as 1000 bp and the vector backbone as 10 kbp.**

A   B
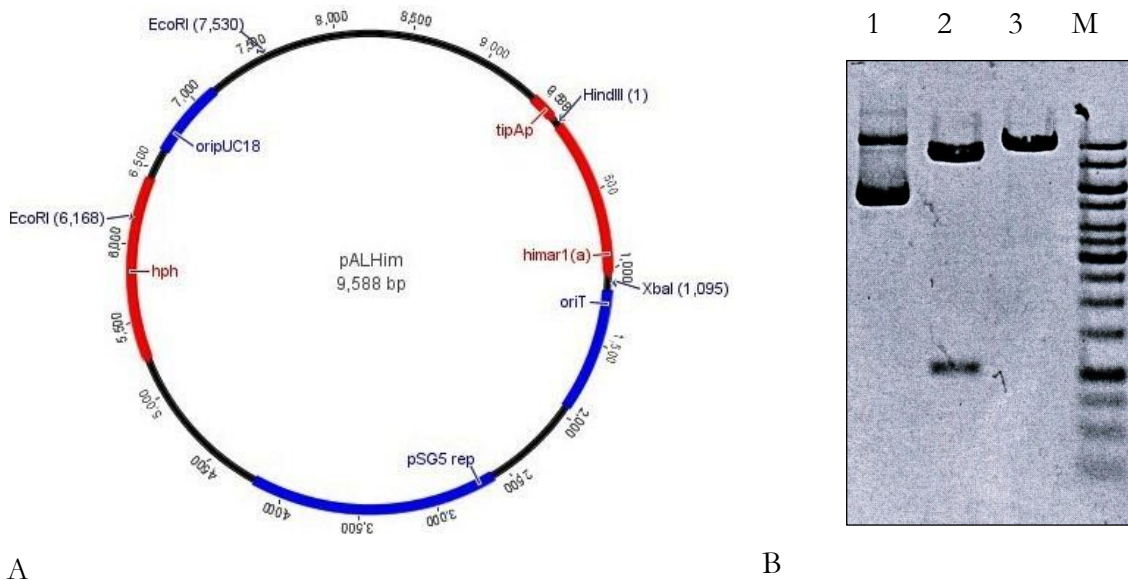
Figure 3.2. The map (A) and analytical restriction (B) of pALHim. (A) Plasmid contains following features: *oriT* **– origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon;** *himar1(a)* **– synthetic transposase gene, under control of** *tipA*p **– thiostrepton inducible promoter;** *hph* **– hygromycin resistance marker. (B) 1 – undigested plasmid; 2 – plasmid digested with** *Xba*I **and** *Hin*dIII**; 3 – plasmid digested with** *Eco*RV**; M - 1kb DNA Ladder. The** *himar1(a)* **fragment is visible at 1000 bp and the backbone at 9 kbp.**

3.1.2. Construction of pHAH, pHTM and pHSM

3.1.2.1. Construction of pHAH

The gene for hygromycin resistance, *hph*, was amplified using pAL1 as a template and Fr-*M*I-*hph* with *Xba*I site as a forward primer and Rs-*X*I-*hph* with *Mun*I site as a reverse primer (Tab. 2.20). The amplified fragment was cloned into pTn5Oks (ShineGene Molecular Biotech, Inc.) via the *Mun*I and *Xba*I restriction sites leading to pTn5Oks*hph*. The *Eco*RV fragment, containing the *hph* gene and the R6Kγ origin, both flanked by ITR sites, was cut out from pTn5Oks*hph* and cloned into pNLHim digested with *Eco*RV, yielding pHAH (Fig. 3.3.A).

The constructed plasmid was verified by analytical digestion with *Eco*RV. The obtained 1,9 kb fragment corresponds to minitransposon. The orientation of minitransposon was determined by an additional analysis with *Xba*I (data not shown).

A   B
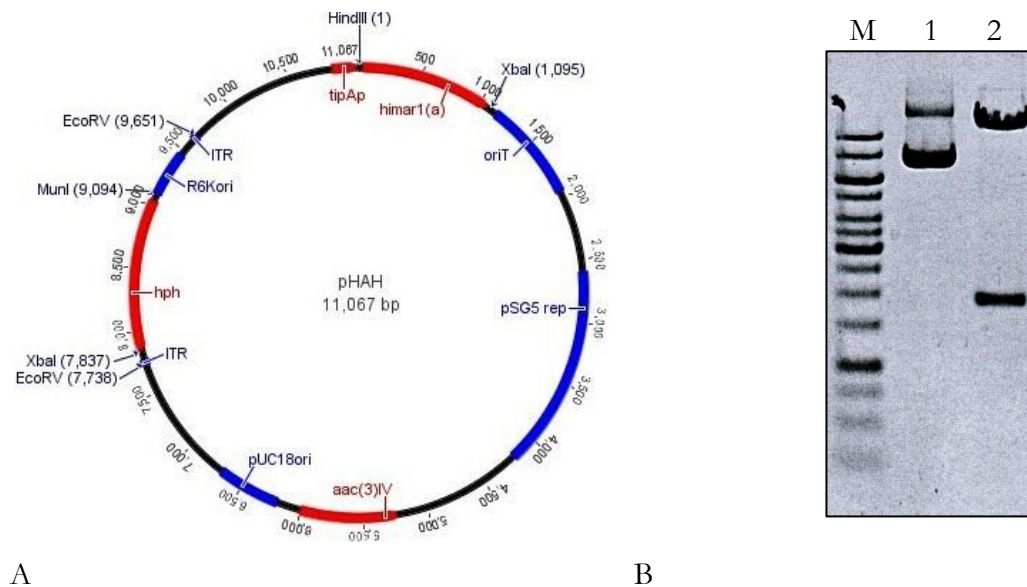
Figure 3.3. The map (A) and analytical restriction (B) of pHAH. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; *himar1(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6K◻-ori* – origin of replication for rescue cloning. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Eco*RV. The minitransposon fragment is visible as 1,9 kbp and the backbone as 10 kbp.

3.1.2.2.   Construction of pHTM

The apramycin resistance gene, *aac(3)IV*, was amplified from pIJ773 with the aac-f forward primer and the aac-r reverse primer both containing *Eco*RI restriction sites (Tab. 2.20). The obtained PCR product was cloned into the plasmid pITRΔNheI (derived from pITR, GenScript, New Jersey, USA; Weber, 2010) into the *Eco*RI site, leading to pITRΔNheIaac. *Pvu*II was used to excise a fragment from pITRΔNheIaac containing the *aac(3)IV* gene and the R6Kγ origin, all flanked by the ITR sites. This fragment was cloned into pALHim cleaved with *Eco*RV, yielding pHTM, (Fig. 3.4.A).

Analysis of pHTM plasmid by digestion with *Eco*RI demonstrated, that the *aac(3)IV* gene of minitransposon is present in the construct (Fig. 3.4.B). Other fragments present in Fig. 3.4.B are resulted from the vector backbone.

A   B
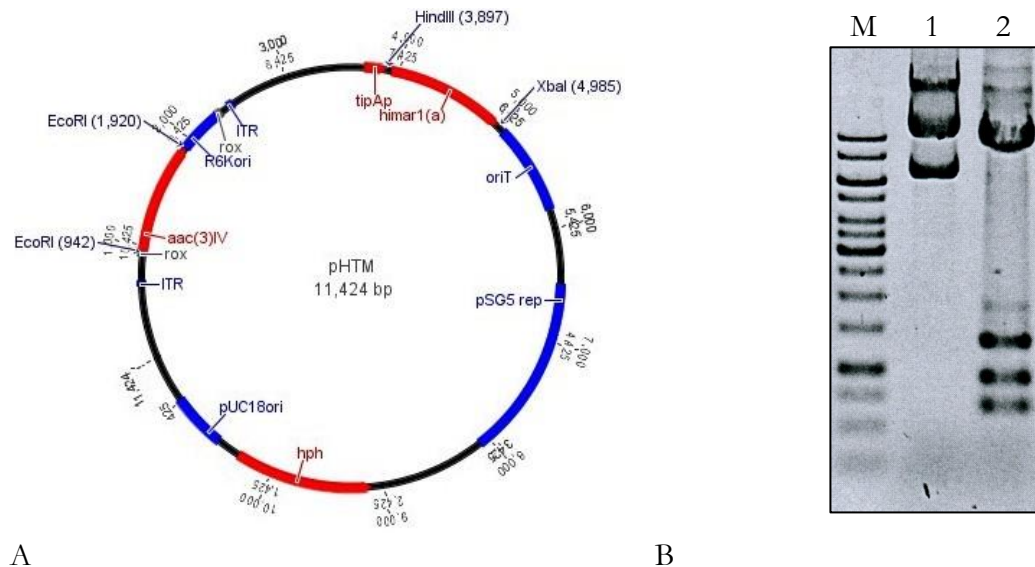
Figure 3.4. The map (A) and analytical restriction (B) of pHTM. (A) The plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; *himar1(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6Kᴑ-ori* – origin for rescue cloning; *rox* – site for *Dre/rox* recombination. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Eco*RV. The *aac(3)IV* fragment is visible as 900 bp.

3.1.2.3. Construction of pHSM

In a similar procedure, the spectinomycin resistance gene, *aadA(1)*, from pHP45Ω (Tab. 2.12) was excised by *Eco*RI and cloned into pITRΔNheI, giving pITRΔNheIaad. pITRΔNheIaad was then digested with *Pvu*II for blunt end cloning into the *Eco*RV site of pALHim, giving pHSM (Fig. 3.5.A).

Analytical digestion of the pHSM plasmid with *Eco*RI demonstrated, that the *aadA(1)* gene of minitransposon is present in the construct (Fig. 3.5.B). Other two fragments in Fig. 3.5.B are from the plasmid backbone digestion.

A   B
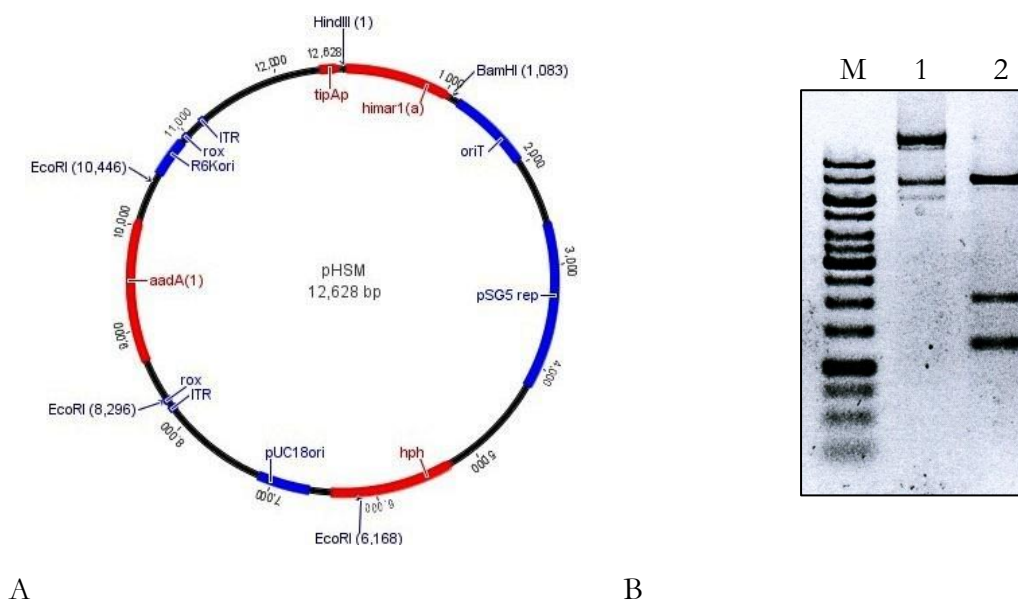
Figure 3.5. The map (A) and analytical restriction (B) of pHSM. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; *himar1(a)* – synthetic transposase gene, under control of tipAp – thiostrepton inducible promoter; *aadA(1)* – spectinomycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6Kγ-ori* – origin for rescue cloning; *rox* – site for *Dre/rox* recombination. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Eco*RV. The *aadA(1)* fragment is visible as 2,1 kbp.

3.1.3. Transposon mutagenesis of *Streptomyces coelicolor* M145

The plasmids pHTM and pHSM were introduced into *S. coelicolor* M145 by intergeneric conjugation. The exconjugants were cultivated through several passages as described above (see 2.8.4). Aliquots of the cultures were plated on the HA agar plates, the spores were recovered, diluted to 10^{-7} and plated out again. Single colonies were obtained and transferred to the HA agar

plates containing apramycin or spectinomycin. To verify the elimination of the pALHim backbone carrying hygromycin resistance gene, transposon mutants of *S. coelicolor* M145 were also plated on the HA agar plates containing hygromycin and cultivated at 28°C for 7 days. Of the 264 tested mutants *S. coelicolor* M145::pHTM carrying an apramycin resistance marker only one colony retained hygromycin resistance. All of 88 tested *S. coelicolor* M145::pHSM mutants were hygromycin sensitive. To demonstrate the stable inheritance of *Himar1* insertions, 10 randomly selected apramycin resistant and 2 spectinomycin resistant colonies were inoculated into liquid TSB and cultivated for approximately 100 generations in the absence of antibiotic selection. After the cultivation, cultures were plated onto the R2YE agar plates with the respective antibiotic and were all found to be antibiotic resistant. These results demonstrated effectiveness of synthetic hyperactive *Himar1*-based system for the generation of stable transposon libraries in actinomycetes.

3.1.4. Transposon mutagenesis of *Streptomyces albus* J1074

The same protocol for the random transposon mutagenesis was applied in *S. albus* J1074. The plasmid pHAH was introduced into this strain by intergeneric conjugation. The exconjugants were carried through all cultivation stages necessary for transposon library generation (see 2.8.4) and, as a result, transposon mutant library of *S. albus* J1074 was obtained. Due to the high sensitivity of *S. albus* J1074 to thiostrepton its concentration had to be reduced by ten times down to 200 pg/ml. Mutants were also tested for loss of the pNLHim plasmid backbone and for the stable inheritance of transposon.

3.1.5. Rescue plasmids isolation and identification of the insertion loci

All transposons constructed for this experiment contained R6Kγ ori that allows rescue cloning of the transposons and thereby identifying the insertion locus. With this aim chromosomal DNA of selected mutants from the transposon libraries of *S. albus* J1074 and *S. coelicolor* M145 was isolated according to the protocol, digested with *Sac*II or with *Not*I and self-ligated. These two enzymes produces relatively small DNA fragments (≤1kb) resulting in the efficient generation of rescue plasmids. The ligation mixtures were transformed into *E. coli* recipient strain and then plated on selective LB-agar. The obtained colonies were inoculated into selective LB and incubated overnight with the subsequent isolation of rescue plasmids.

Table 3.1. Loci of transposon insertion in *S. albus* J1074 identified by rescue plasmid sequencing. IGR – intergeneric region; CHP – conserved hypothetical protein

Ins. locus (XNR)	Ins. locus (SSHG)	Coded protein	First 10 b
0178	05739	ATP-dependent helicase	taggcgtgga
0617	05308	cytochrome c oxidase subunit I	tacaacgtct
1114	04808	two-component system response regulator	tatatgaaga
1498	04425	conserved hypothetical protein	tacatgacgg
1854/5	04075/6	IGR btw. lysR-fam. transcript. reg. and predicted	tacttctgat
1859	04070	cell division protein	tacaagcact
2294	03625	tetR family transcriptional regulatory protein	tagctgttga
2653	03282	dimethylmenaquinone methyltransferase	tactcggtgc
2675	03259	secreted protein	tacaggtccc
2914/5	03030/1	IGR btw. two conserved hypothetical proteins	tatcggtatg
3318	02619	conserved hypothetical protein	tacgtcgccc
3461	02475	goadsporin biosynthetic protein	tacgtggtgc
3556/7	02371/2	IGR btw. secreted protein and phosphoglycerate	tacaacgccg
4037	01897	cystathionine gamma-synthase	tacgtggaca
4136	01802	pknB-group protein kinase	tagggggggcg
4378/9	01556/7	IGR btw. CHP and coproporphyrinogen III	tagaagtccg
4449	01486	pyruvate phosphate dikinase	tactcgacga
5042	00906	acetyltransferase	taggtggcgt
5168	00779	glycerol kinase 1	tacgccctgg
5198/9	00748/9	IGR btw. RarA and cytochrome P450	tattgccctc
5453	00496	retrotransposon protein	tatcacggag

Table 3.2. Loci of transposon insertion in *S. coelicolor* M145 identified by rescue plasmid sequencing. Bolded entries indicate two mutants with the spectinomycin resistance marker. The 42 other entries contain the apramycin resistance marker

Ins. locus (SCO)	Coded protein	First 10 b
1097	putative hydrolase	tacgactccg
1531	threonine-tRNA synthetase	tagatgtgcg
2189	putative acetyltransferase	tacgagtcgg
2706	putative transferase	taggcgttgt
2730	putative regulator	tagacggtgg
2741	putative secreted protein.	taggtcgcct
2758	beta-N-acetylglucosaminidase (putative secreted protein)	tacgggttgc
2773	acyl CoA thioesterase II	tacgtctcck
2792	*araC*-family transcriptional regulator	tatgagccac
2992	hypothetical protein SCE50.21	tacgacctgt
3053	**putative secreted esterase**	**tacggccagg**
3057	putative dipeptidase	tacgacgcgg
3382	L-aspartate oxidase	tagtgggcgg
3390	putative two-component sensor kinase	tacgaggagg
3391	hypothetical protein	taggggtaca
3638	putative two-component system sensor kinase	tacgccgaca
3650	putative uridine 5'-monophosphate synthase	tagtagtcgg
3811	putative D-alanyl-D-alanine carboxypeptidase	tacagcggag
3826	putative ion channel membrane protein	tacaaggtga

3861	putative membrane protein	tacgtctccg
3871	putative decarboxylase	tacgtcgatc
3919	putative *lysR*-family transcriptional regulator	tatttatagt
4022	putative glycosyl transferase	taggggaacg
4048	conserved hypothetical protein	tacggctcag
4068	phosphoribosylamine-glycine ligase (EC 6.3.4.13)	tacgtcgtca
4119	**putative NADH dehydrogenase**	**taggcgtcgg**
4128	putative membrane protein	tacatggcgt
4138	phosphate transport protein	tacgtgaaga
4192	hypothetical protein	tagttgacgt
4199	hypothetical protein	tacttggygg
4211	putative integrase	tacgcctcgg
4508	putative cell division-related protein	tacttgtcct
4576	hypothetical protein	tacttcaggg
4581	conserved hypothetical protein	tatgccgttg
4776	putative serine/threonine protein kinase	tagacgacct
4787	putative aldolase	tacgaggacc
5165	putative hydrolase	tacgagtcgg
5487	conserved hypothetical protein SC2A11.21c	tacgggctgc
5495	secreted chitinase	tacgtctatc
5540	putative membrane protein SC1C2.21c	tactcagcagt
5673	secreted chitinase	tacgtctatc
5719	hypothetical protein SC3C3.06c	taggtgcgca
5905|5906	intergenomic region	taacacgccc
7329	putative long-chain-fatty-acid-CoA ligase	taggccacca

The isolated rescue plasmids were sequenced using a primer oriented outward from the transposon into genomic DNA. Thereby approximately 800 bp of flanking chromosomal DNA region could be identified. Sequence data were used for the BLAST analysis against the genomes of *S. albus* J1074 and *S. coelicolor* A(3)2 (Tabs. 4.1, 4.2). Analysis showed that transposon insertions were randomly distributed within the genomes of *S. albus* J1074 and *S. coelicolor* A(3)2 (Fig. 3.6).
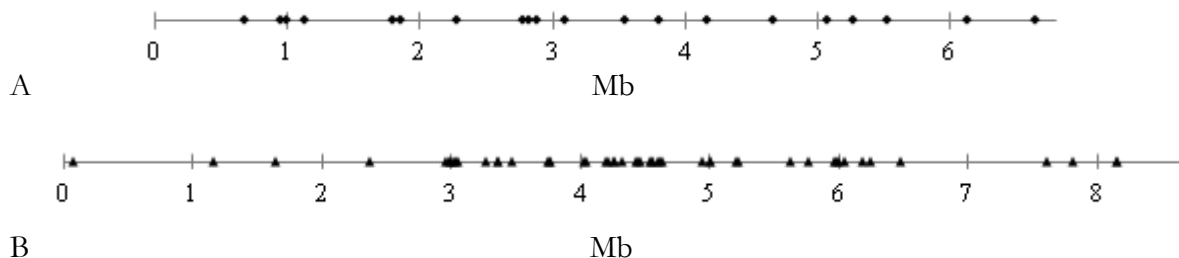
Figure 3.6. Distribution of insertion loci for *Himar1* transposons in *S. albus* J1074 (A, rhombs, insertions oriented according to SSHG genes location) and *S. coelicolor* M145 (B, triangles) chromosomes.

3.1.6. Analysis of integration frequency

To determine if a given transposon insertion was unique Southern blot analysis of mutant genomic DNAs was carried out. For this aim the genomic DNA from two-days-old TSB culture of streptomycetes transposon mutants were isolated. The samples were digested with *Nco*I for the *S. albus* J1074 strains and with *Bam*HI for the *S. coelicolor* M145 derivatives, separated by an agarose gel electrophoresis and transferred to a positively charged nylon membrane. A 0,4-kb R6Kγ origin from pTn5Oks (for *S. albus* J1074 mutants) and a 0,8-kb *aac(3)IV* gene from pHTM (for *S. coelicolor* M145 mutants) were prepared and used as the templates for probes labeling with digoxigenin-dUTP by the random priming method. The hybridization, washing, and signal detection were carried out as described by Roche Diagnostics. Genomic DNAs of *S. albus* J1074 and *S. coelicolor* M145 wild types were used as negative controls. The linearised pTn5Oks and pHTM were used as positive controls.
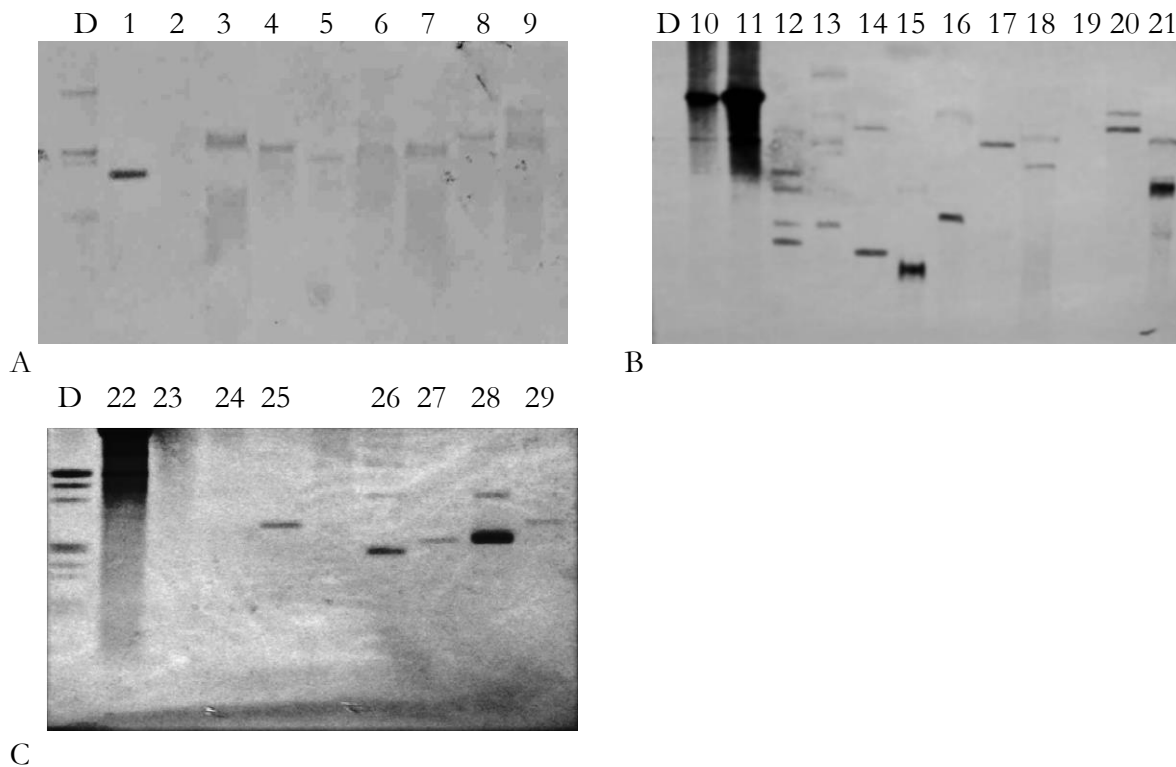
Figure 3.7. The hybridization membranes after Southern blot hybridization of *Himar1*-mutants. D - DIG labeled ladder III (Roche); 1 – positive control (pTn5Oks/*Hin*dIII); 10, 11, 22 – positive control (pHTM/*Nde*I); 2 – negative control (wild type *S. albus* J1074 DNA); 19, 23 – negative control (wild type *S. coelicolor* M145 DNA); 3-9 – *S. albus* J1074 transposon mutants (*tipAp* induced); 12-18, 20, 21 – *S. coelicolor* M145 transposon mutants (*tipAp* induced); 24-29 – *S. coelicolor* M145 transposon mutants (*tipAp* not induced).

For each of seven analyzed *S. albus* J1074 mutants only unique transposon integration sites were detected (Fig. 3.7.A). Conversely, all but one of analyzed *S. coelicolor* M145 mutants showed

multiple transposon integration sites (Fig. 3.7.B). This difference could be attributed to the high sensitivity of *S. albus* J1074 strain to thiostrepton and thus inability to induce well the thiostrepton inducible promoter during the exponential phase of growth.

Meanwhile tendency to tolerate multiple transposon integrations in the genome demonstrated by *S. coelicolor* M145 will make hard the interpretation of observed phenotypes. This problem was solved by omitting the induction step with the thiostrepton after the conjugation with the transposon-containing plasmid: exconjugants were washed from MS into liquid medium and immediately placed on 37°C to cure the plasmid. Subsequent analysis of obtained in this way transposon mutants with Southern blot demonstrated that residual activity of the *tipA* promoter was sufficient to cause the transposition and at the same time avoiding inductor increased number of mutants with single insertion (Fig. 3.7.C).

3.1.7. Transposon mutagenesis of *S. albus* J1074 using suicide plasmid

Most replicative transposon delivery vectors contain the temperature sensitive pSG5 replicon, which is not supported in some *Streptomyces* strains. In such cases suicide vectors can substitute replicative vectors. To establish such a system, the transposase gene expression should start immediately after vector introduction into the recipient cell. To accomplish this, the promoter of the *φC31* integrase from pSET152 had been used, since this integrative plasmid does not replicate, and without rapid expression of the integrase gene, it would be lost like a suicidal vector. Based on the suicide vector pKCLP2the suicide vector for transposon mutagenesis in *Streptomyces*, pHAM, was constructed (Dr. Maksym Myronovskyi). This vector contains a *Himar1* transposase encoding gene under the control of the *φC31* integrase promoter, the *Himar1* transposon and origin from the *oriT*. After introduction of pHAM in *S. albus*, transposon mutants were obtained with a frequency of between 10^{-3}- 10^{-4} (based on input recipient spores). This means that the transposase gene under the *φC31* integrase promoter expresses early enough to permit the transposition from the backbone of non replicative plasmid.

3.1.8. Expression of *Dre*-recombinase

To assess *Dre*-mediated marker excision, pUWL-Dre plasmid was transferred into ten *S. coelicolor* M145::pHTM strains. This plasmid contains synthetic gene encoding *Dre*-recombinase and marker of thiostrepton resistance. Exconjugants were collected and inoculated into 100 mL of TSB containing 50 μg/mL thiostrepton and 200 μg/mL phosphomycin. Cultures were grown for 3 days. Aliquots were plated onto 50 μg/mL thiostrepton MS agar and grown for 3 days until

sporulation. Spores were collected and grown in TSB with thiostrepton for 2 days at 28°C. Serial dilutions of obtained cultures were plated on MS agar. Single colonies were tested for apramycin resistance. Apramycin-sensitive colonies were readily obtained with marker excision efficiencies reaching 100%. All of the marker-free mutants were verified by PCR analysis (data not shown).

3.1.9. Identification of new regulatory genes of *S. coelicolor* M145 involved in secondary metabolite production

After the transposon mutagenesis, a library of mutants with a variety of phenotypes was obtained. Several of selected transposon mutants were tested for dynamic of actinorhodin and prodigiosin production (Fig. 3.8). With this aim ca. 250 mg of mycelium was inoculated into 12 well plates with R2YE and cultivated for 96 h at 28°C. The plates were photographed after 16h, 40, 50, 64 and 96 hours of cultivation. Most of the strains started to produce actinorhodin after 50 h of growth and after this time point no significant changes were observed.

Four transposon mutants with disrupted *sco3812* (putative GntR-family transcriptional regulator), *sco4197* (putative MarR family regulator), *sco4198* (putative DNA binding protein), *sco4192* (hypothetical protein) demonstrated impaired actinorhodin production (Fig. 3.8, red squares).
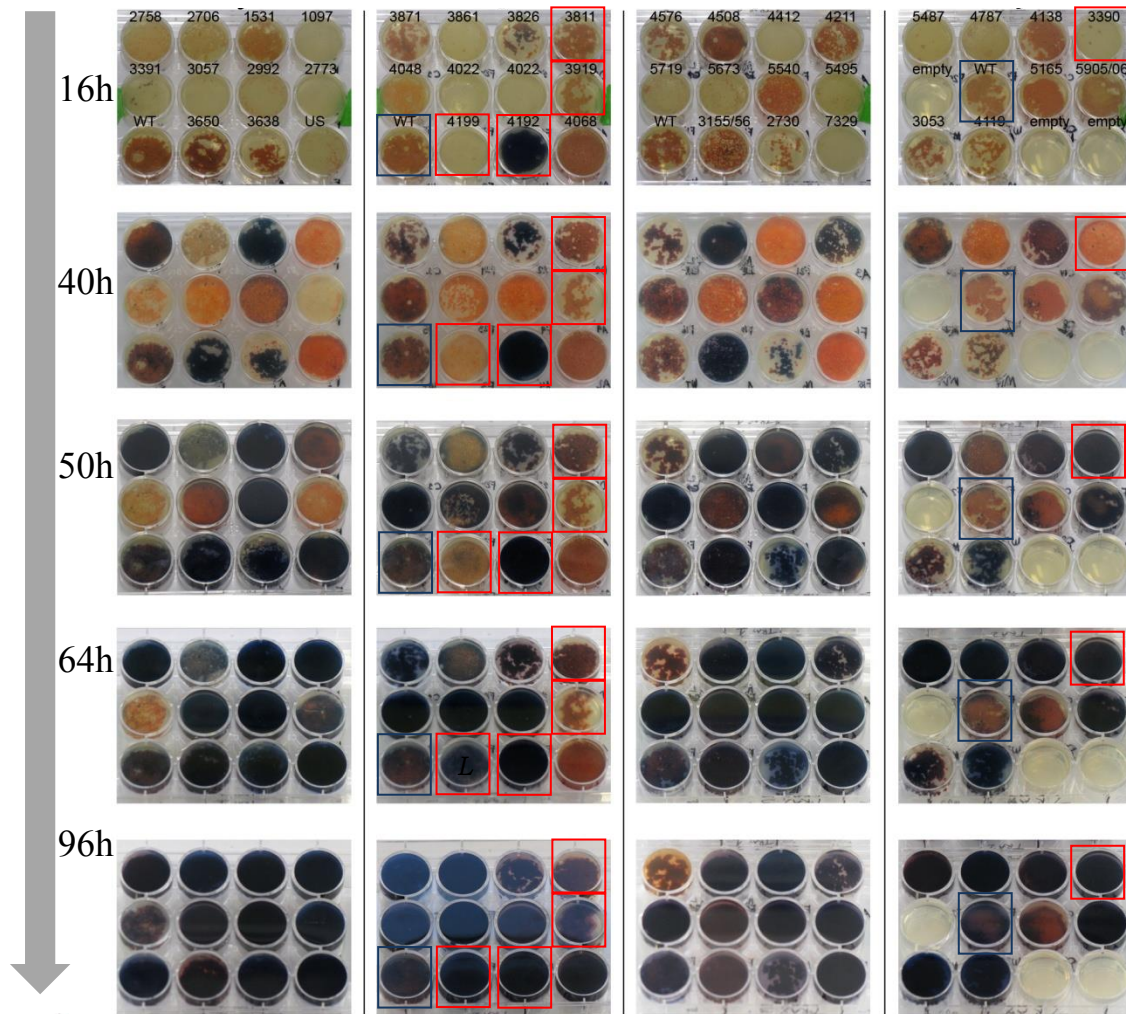
Figure 3.8. Comparison of antibiotic production by different *S. coelicolor* M145 transposon mutants on R2YE medium. Numbers represent the identification number of CDS where transposon insertion identified by sequencing of rescue plasmids; US, unsequenced; WT, *S. coelicolor* M145.

To ensure that the observed phenotype resulted from the inactivation of identified ORFs (*sco3812*, *sco4197*, *sco4198* and *sco4192*) these genes were disrupted via homologous recombination in a clean genetic background of *S. coelicolor* M145. The obtained mutants of *S. coelicolor* M145 did not produce actinorhodin on the R2YE agar and overproduced this antibiotic on the minimal medium (MM) and NL5 agar plates, in contrast to the wild type strain (Fig. 3.9, 3.10). Actinorhodin production was blocked upon substitution of glucose in MM with sucrose or glycerol. Addition of glycerol to MM induced production of yellow pigment, coelimycin P1 (Gomez-Escribano *et al.*, 2012) by all mutants (Fig. 3.9). In contrast to the wild type, all four mutants showed actinorhodin production on NL5 medium where glutamine was used as a

carbon source. The supplementation of the NL5 medium with glycerol, glucose, or sucrose blocked actinorhodin production by all mutants (Fig. 3.10).
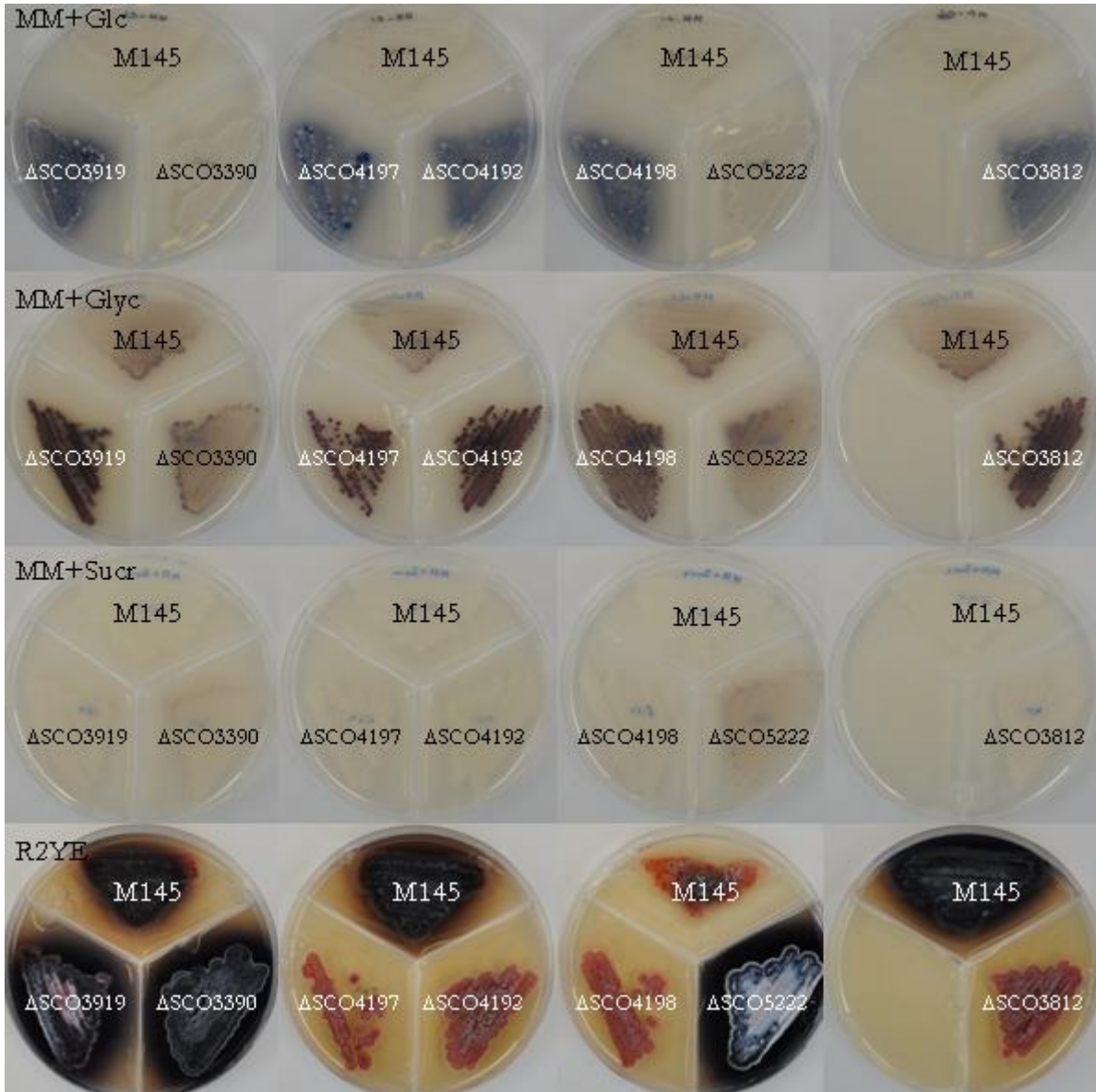
Figure 3.9. The comparative growth of *S. coelicolor* M145 wild type strain and deletion mutants on minimal medium with different carbon sources and on R2YE. Strains were grown at 30°C for 5 days. Glc – glucose; Glyc – glycerol; Sucr – sucrose.

Another two transposon mutants with affected secondary metabolism had insertions in *sco3390* and *sco3919* genes, encoding a putative two component system sensor kinase and putative LysR-family transcriptional regulator, respectively. Deletions of these two genes were also made via homologous recombination yielding two strains: *S. coelicolor* M145 B04 (with disrupted *sco3919*) and *S. coelicolor* M145 A07 (with disrupted *sco3390*). Both mutants showed slight actinorhodin overproduction on RY2E agar, while *S. coelicolor* M145 B04 in contrast to the wild type and *S. coelicolor* M145 A07 produced actinorhodin on MM (Fig. 3.9). Also, it has to be emphasised, that

actinorhodin production capabilities of *S. coelicolor* M145 B04 are very similar to the four *S. coelicolor* M145 mutants with inactivated *sco3812*, *sco4197*, *sco4198* and *sco4192* genes.
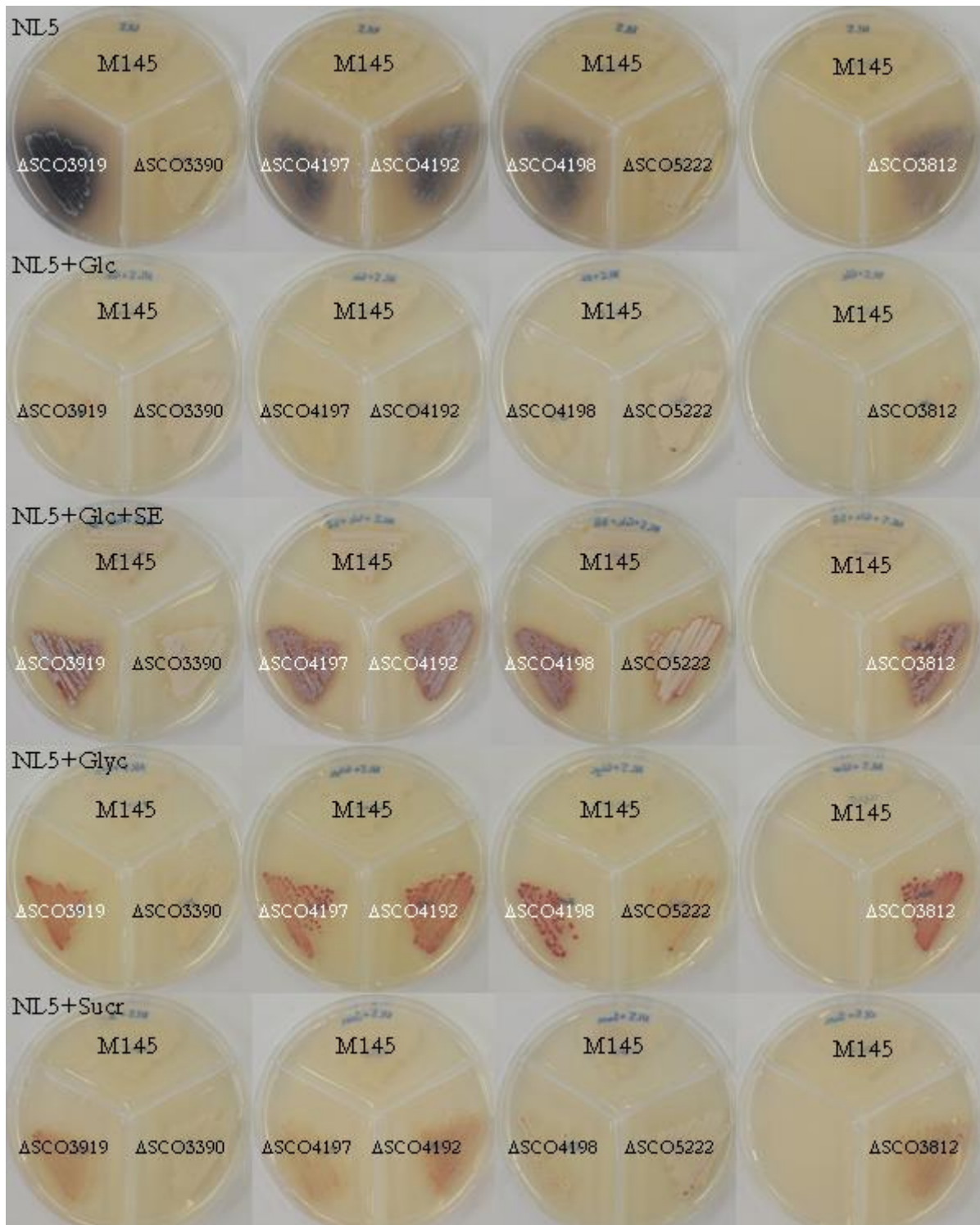
Figure 3.10. The comparative growth of *S. coelicolor* M145 wild type strain and its deletion mutants on NL5 medium with different carbon sources. Strains were grown at 30°C for 5 days. Glc – glucose; SE – trace elements; Glyc – glycerol; Sucr – sucrose.

Another transposon mutant showing impaired actinorhodin production contained insertion in the *sco5222* gene encoding a putative lyase. To prove this phenotype inactivation of the respective gene has been performed. However, obtained mutant did not show any differences in antibiotic production when compared to the wild type strain (Fig. 3.9, 3.10). Obviously, the effect on actinorhodin production in this strain was caused by some additional insertions that were not identified during rescue plasmids cloning.

3.1.10. Transcriptional fusion of *gusA* gene with *act*II-ORF4 promoter

In order to investigate in more details role of *sco3812, sco3919, sco4192, sco4197* and *sco4198* in regulation of actinorhodin biosynthesis the integrative plasmid containing *act*II-ORF4 promoter fused with *gusA* reporter gene (plasmid provided by Dr. Lilia Horbal) was introduced into *S. coelicolor* ΔSCO3812, ΔSCO3919, ΔSCO4192, ΔSCO4197 and ΔSCO4198 strains. Obtained exconjugants were patched on rich R2YE and on minimal medium with or without X-Gluc (Fig. 3.11).
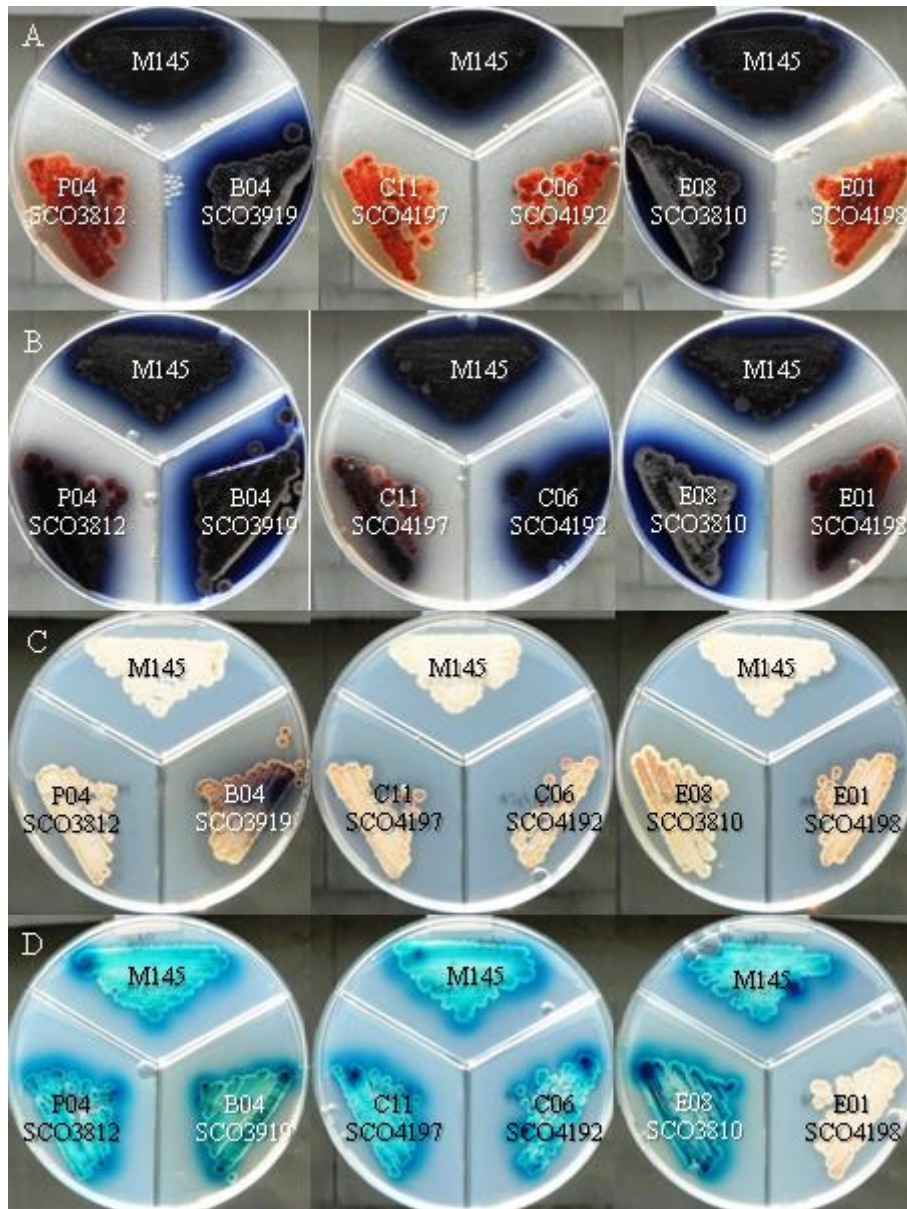
Figure 3.11. The comparative growth of *S. coelicolor* M145 wild strain and its deletion mutants, containing pGUSactII. Strains were grown for 3 days on R2YE (A), R2YE with X-Gluc (B), minimal medium (C) and minimal medium with X-Gluc (D).

The *actII-ORF4* promoter did not show a detectable activity in the strain with deletion of *sco4198* on MM and R2YE media, although the very weak actinorhodin production has been observed on MM (Fig. 3.11). Only very weak GusA-activity has been detected in the ΔSCO4197 and ΔSCO3812 mutants on R2YE agar which perfectly correlates with the level of actinorhodin production by these strains (Fig. 3.11.B). In contrast, strain ΔSCO4192 failed to produce actinorhodin, but still shows a high level of *gusA* expression driven by *actII-ORF4* promoter on all media (Fig. 3.11).

3.1.11. Transposon mutagenesis of *Streptomyces lividans* 1326

Since phenotypes of mutants obtained by transposon mutagenesis is predominantly caused by gene inactivation, selection of strains with genes expression hyper-activation remains neglected. In order to identify genes which overexpression influence secondary metabolism or/and morphological differentiation, plasmid, pALTEAm (Horbal *et al.*, 2013) was introduced into *S. lividans* 1326. This plasmid contains Tn*5*-based minitransposon flanked by two outward-oriented promoters, *erm*Ep1 (promoter of erythromycin resistance gene) and tcp830 (tetracycline inducible promoter). Such minitransposon structure provides high-level transcription of adjacent genes in loci of insertion.
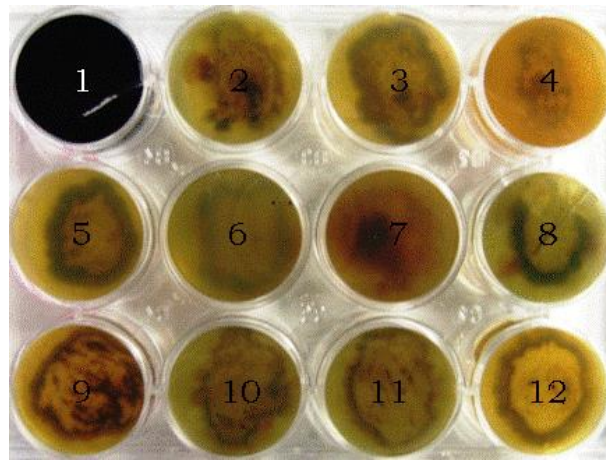
Figure 3.12. The plate with transposon mutants of *S. lividans* 1326::pALTEAm after 72h of growth at 28°C on R2YE medium. Actinorhodin (1) and undecilprodigiosin (7) producers can be easily recognized by abnormal blue and red coloration, respectively. 1-11 – transposon mutants of *S. lividans* 1326::pALTEAm; 12 – *S. lividans* 1326.

The *S. lividans* 1326::pALTEAm exconjugants were treated according to protocol for transposon mutagenesis described above. When the spores of transposon mutants were recovered, around four thousands of single colonies were screened for abnormal coloration. Mutants, seemingly producing actinorhodin, were plated on R2YE agar media in 12-well plates and allowed to grow for 72h.

Two of isolated mutants demonstrated intriguing phenotypes. One of them, *S. lividans* 1326::pALTEAm-7 (Fig. 3.12, 7) demonstrated low intensity of sporulation. Rescue plasmids, that were isolated, revealed, that the insertion is located in *sli4360* (putative two-component system response regulator). Another mutant, *S. lividans* 1326::pALTEAm-1, produced actinorhodin in significant amounts (Fig. 3.12, 1). Sequencing results showed that transposon is located in *sli1416* (TctA citrate transporter).

3.2. Investigation of position effect in *S. albus* J1074

3.2.1. Investigation of position effect using *gusA*-reporter system

3.2.1.1. Construction of plasmid containing *gusA* gene in transposon

The *gusA* gene was amplified using pSET*gusA* plasmid as a template, the forward primer, Fr-*XI*-*e*p1-*gusA*, carrying the *Xba*I site and *ermE*p1, a *Saccharopolyspora erythraea ermE* gene promoter (Bibb *et al.* 1994), and the reverse primer, Rs-*MI*-t*fd*-*gusA*, carrying the *fd*-phage terminator and the *Mun*I site (Tab. 2.20). The PCR fragment was ligated into pITRΔ*Nhe*I*aac* linearised by *Xba*I and *Mun*I leading to p11-8. The *aac(3)IV* gene was amplified using pIJ773 as a template, the forward primer, Fr-ERI-t*fd*-*aac*, carrying the *fd* terminator and the reverse primer, Rs-ERI-*aac*; both primers carry the *Eco*RI site (Tab. 2.20). The fragment was cloned into the *Eco*RI site of p11-8 replacing an existing *aac(3)IV*, resulting in p11-8*aac*t*fd*. The *Pvu*II fragment of p11-8*aac*t*fd*, containing transposon, was ligated to pALHim linearised with *Eco*RV, to yield pALG (Fig. 3.13.A).
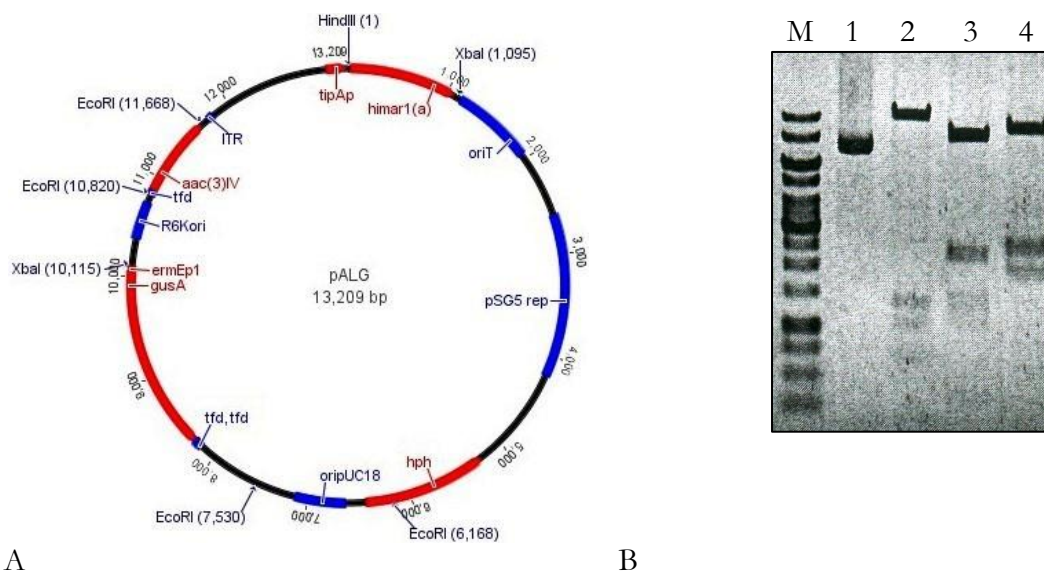
Figure 3.13. The map (A) and analytical digestion (B) of pALG. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon ; *himar1(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6K◻-ori* – origin for rescue cloning. *gusA* – glucuronidase gene; *ermE*p1 - promoter 1 of erythromycin resistance gene; t*fd* – terminator of *fd*-phage. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid restricted with *Eco*RI; 3 - plasmid restricted with *Hin*dIII and *Xba*I; 4 - plasmid restricted with *Mun*I and *Xba*I.

The correct size of cloned fragments was verified by analytical restriction endonuclease mapping (Fig. 3.13.B). The correct incorporation of the apramycin resistance gene was confirmed by digestion with *Eco*RI. The presence of *himar1(a)*-gene was confirmed by a double digestion with

*Hin*dIII and *Xba*I. In addition, the 2,0 kb fragment after double digestion with *Mun*I and *Xba*I corresponds to *gusA* gene framed by *erm*Ep1 and t*fd* was observed.

3.2.1.2. Generation of *S. albus* J1074::pALG transposon mutants library and measuring expression level of reporter gene

The pALG plasmid was introduced into *S. albus* J1074 by intergeneric conjugation with *E. coli* ET12567/pUZ8002 and a mutant library was generated as described above (see 3.1). Serial dilutions to single colonies were made and one hundred colonies, each representing a unique transposon mutant, were patched on the selective MS agar plate and overlaid with X-Gluc (Fig. 3.14). All mutants exhibited GusA-activity. Twenty four of them were inoculated into 20 ml of TSB for further analysis.
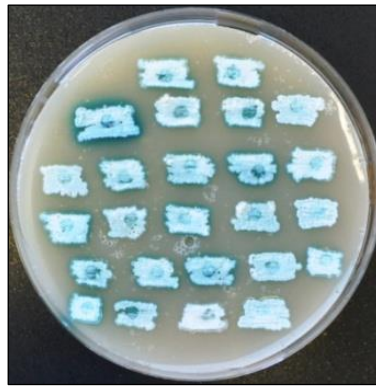
Figure 3.14. The *S. albus* J1074::pALG-mutants patched on selective MS medium, exhibiting GusA-activity. The mutants were grown for 48 h at 28°C and then 5 µl of X-Gluc solution were added in the middle of each patch and the plate was incubated for another 6 h at 28°C.

The inoculated cultures were grown for 48 h at 28°C. Then 2 ml of each pre-culture were transferred into three flasks with 20 ml of fresh TSB medium and cultivated for another 48 h at same conditions. Afterwards, 2 ml of culture were used for preparation of lysates to measure the GusA-activity and 1 ml was used for the isolation of chromosomal DNA.

The analyzed mutants demonstrated a six fold variations in GusA-activity (Fig. 3.15). The lowest value observed was 2,7 U/mg and the highest was 15,4 U/mg. Nine of 24 strains (38%) exhibited activity between 7,5 and 10 U/mg; six of them (25%) showed activity level higher than 11 U/mg. Such six fold variation in the expression level of heterologous gene stands out if to compare these results with similar experiments performed with other prokaryotes, where only 2-3 fold variations in activity were observed (Schmid and Roth, 1987; Sousa *et al.*, 1997; Thompson and Gasson, 2001).

Isolation of rescue plasmids was performed as described previously. To facilitate rescue cloning, chromosomal DNA was digested with *Sac*II, as this enzyme cuts off part of *gusA* gene, decreasing the size of fragments for rescue cloning and thus increases efficiency of their re-ligation. The digested DNA was precipitated, ligated and transformed into *E. coli* Transformax. Isolated rescue plasmids were sequenced. Our previous finding showed that usually S. *albus* J1074 transposon mutants contain one copy of minitransposon, so multiple integrations events were not checked by Southern blot in this particular experiment. However, to exclude the possibility of multiple insertions, two separate rescue plasmids for each mutant strain were isolated. When obtained plasmids were of the same size, only one of them was sequenced, if rescued plasmids had different sizes, both of them were sequenced. However, in 18 cases sizes of rescue plasmid were identical. In other cases, one of two isolated plasmids failed to be sequenced indicating to be a cloning artifacts.

GusA-activuty, U/mg

S. albus **J1074::pALG-mutants**
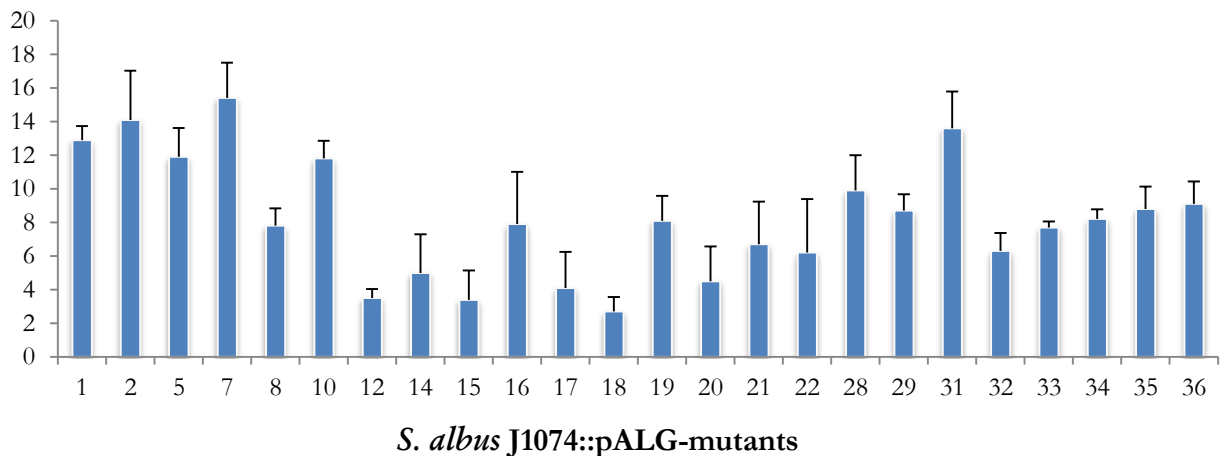
Figure 3.15. β-Glucuronidase activity of different *S. albus* J1074::pALG-mutants. 1-3, 5-22, 28-36 – *S. albus* J1074::pALG-mutants. Strains were grown for 48 h at 28°C.

Analysis of the obtained sequencing results showed, that M01, M05, M29 and M32 mutants are clones of the same mutant with insertion in intergenic region between *sshg01734* and *sshg01735*. As well as M16 and M34 have same insertion loci inside of *sshg04625*; M17 and M20 are carrying the transposon within *sshg02810* ORF. M18 and M19 also are identical and contain the transposon in *sshg02638* gene.

Table 3.3. Loci of transposon insertion in *S. albus* J1074 identified by rescue plasmid sequencing. M. – mutant; IGR – intergenic region; CHP – conserved hypothetical protein

M.	Ins. locus (XNR)	Ins. locus (SSHG)	Gene function	first 10 bps
M01	4204/5	01734/5	IGR btw. two predicted proteins	atccggtcat
M02	2599	03334	predicted protein/adenylate cyclase	tacgaggtcc
M05	4204/5	01734/5	IGR btw. two predicted proteins	atccggtcat
M07	1880	04048	phosphatase	tagaagcgcg
M08	5168	00779	glycerol kinase 1/2	taccgggtca
M10	2358	03563	reg. prot./MerR-family transcriptional reg.	tagccgacct
M12	2981	02962	integral membrane protein	tctggatgta
M14	2883	03064	TetR-family transcriptional regulator	tacgtctacg
M15	2683	03253	beta-lactamase	tacgcggcct
M16	1302	04625	urease subunit α 1	taggcgtgcg
M17	3133	02810	peptidase C14 caspase catalytic subunit	taccttcggg
M18	3300	02638	conserved hypothetical protein	tagcgcacgg
M19	3300	02638	conserved hypothetical protein	tagcgcacgg
M20	3133	02810	peptidase C14 caspase catalytic subunit	taccttcggg
M21	2089	03841	integral membrane protein	tacatcctgg
M22	5663	00286	succinate dehydrogenase flavoprotein subunit	tacgcgccct
M28	3826	02105	conserved hypothetical protein/DNA-binding prot.	agccgatgcg
M29	4204/5	01734/5	IGR btw. two predicted proteins	atccggtcat
M31	4675/6	01262/3	IGR btw. cons. hyp. protein and phospholipase	tacatcctta
M32	4204/5	01734/5	IGR btw. two predicted proteins	atccggtcat
M33	1853	04077	bi-functional transferase/deacetylase	tacgtgcaca
M34	1302	0462	urease subunit α 1	taggcgtgcg
M35	5073/4	00877/8	IGR btw. transmembr. transporter and integral membr. prot.	tacctcccag
M36	2035	03896	cons. hyp. prot./biosynthesis docking scaffold prot., SagD fam.	tactaccacg

As a result, 16 unique randomly distributed insertions were identified (Tab. 3.3) and mapped on the chromosome (Fig. 3.16). All identified insertions were situated in core region of chromosome.
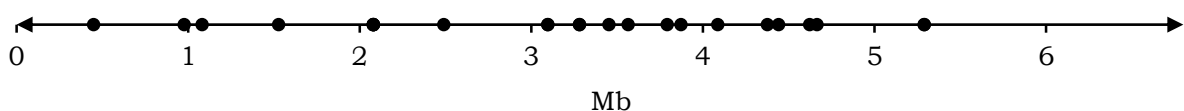
Figure 3.16. Distribution of insertion loci for pALG derived transposons in *S. albus* J1074 chromosome (insertions oriented according to SSHG genes location).

3.2.1.3.   Analysis of chromosome factors impact on heterologous gene expression

To identify specific chromosomal aspects that regulate expression of heterologous genes, the correlation analysis of GusA-activities with several other parameters was performed.
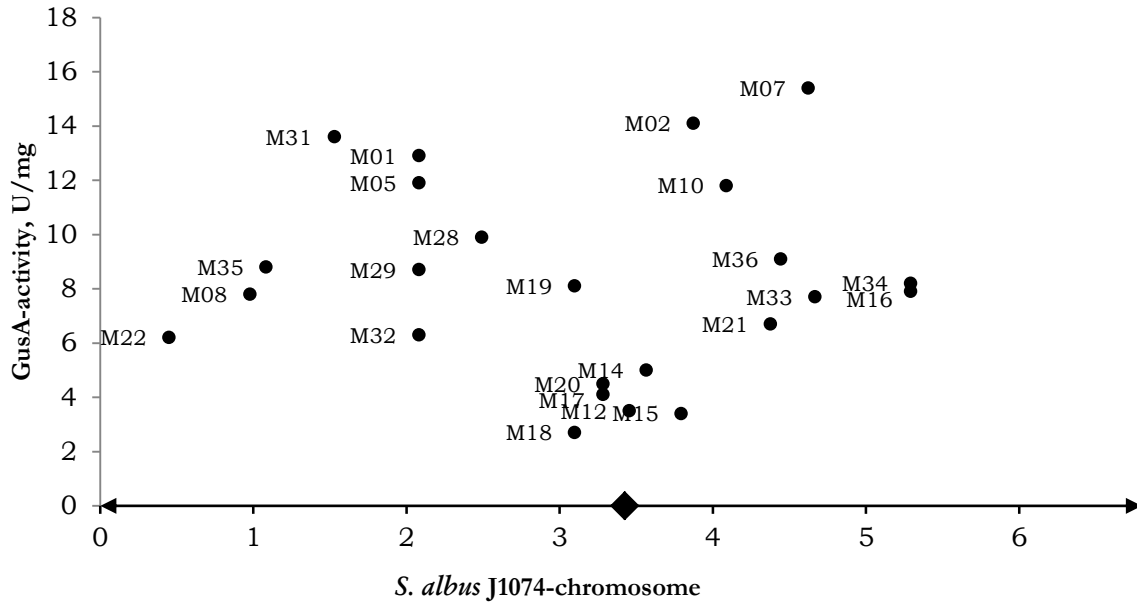
Figure 3.17. Activity of gusA (Y) in transposon mutant strains according to chromosome location (X). Mutants were grown at 28°C in TSB for 48 h. Dots correspond to transposon mutants; rhomb on X-axis corresponds to *oriC* located between M17/20 and M21 (insertions oriented according to SSHG genes location).

At first, we examined assertion that gene expression decreases with distance from *oriC* due to decreasing gene dosage. Therefore measured GusA-activities of mutants were plotted against the locations of a respective transposon insertion in *S. albus* J1074 chromosome (Fig. 3.17). The obtained results didn't show any correlation between these two parameters: levels of activity varied along the whole chromosome and the mutants with insertions adjacent to *oriC* (e.g. M14, M15, M17/M20 and M21) demonstrated same or even lower levels of enzyme activity than the mutants where insertions were located close to "arms" of the chromosome (e.g. M16/34 and M22). The Pearson correlation coefficient (PCC) was only +0,16.

The next aim was to study the correlation between the GusA-activity and the overall transcriptional activity of the transposon insertion site. For this analysis we have chosen ten mutants with lowest deviation values: M02, M07, M08, M10, M12, M31, M31, M33, M34, M35 and M36.

Table 3.4. Estimation of gene expression for genes with promoters that can modulate *gusA* expression by read-through effect

Mutant	Gene	RPKM, 36h	RPKM, 60h
M08	*sshg0783*	53	31,3
M35	*sshg0876*	21,9	17,7
M31	*sshg1268*	2,9	0,6
M12	*sshg2963*	70,1	52,8
M02	*sshg3335*	1,8	1,8
M10	*sshg3563*	24,2	16,1
M36	*sshg3896*	0,3	2,2
M07	*sshg4050*	36,5	21,5
M33	*sshg4077*	12,3	14,7
M34	*sshg4630*	12,2	3,9

Comparison of GusA-activity and RNA-seq results
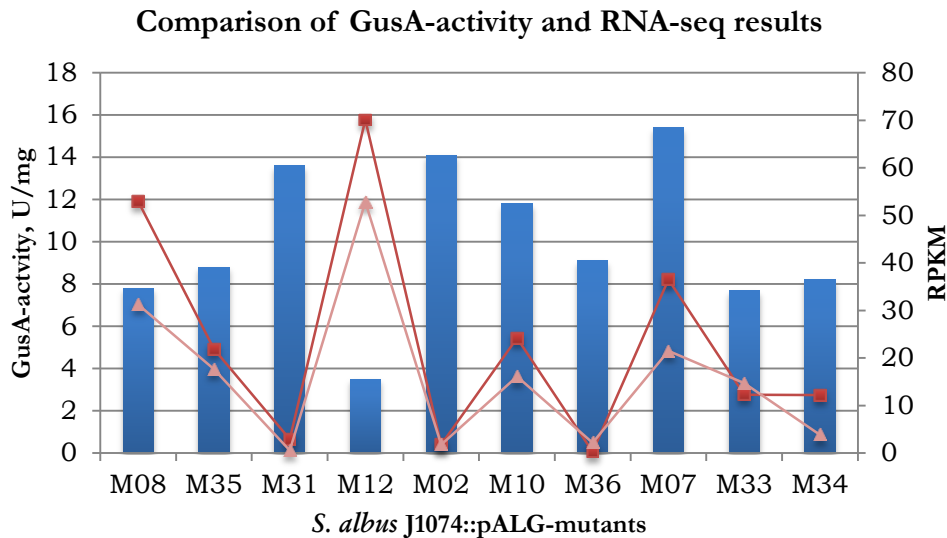
Figure 3.18. The comparison of GusA-activity levels with expression level of adjacent genes. Blue columns correspond to values of GusA-activity; red squares correspond to RPKM after 36h of cultivation; pink triangles correspond to RPKM after 60h of cultivation. Mutants are placed according to location of their transposons on the chromosome.

As the *gusA*-gene was framed by two *fd*-terminators, activity of local promoters should not have any impact on its expression. To provide assessment of this, GusA-activities of 14 mutants were compared to *S. albus* J1074 RNA-seq data after 36h and 60h of cultivation in TSB-medium.

For this aim we took values of reads per kilobase per million reads (RPKM) for the genes, promoters of whose are located in genome of *S. albus* J1074 upstream and in the same orientation as *gusA*, so that they can modulate *gusA* expression by read-through effect (Tab. 3.4).

The results demonstrated that in the obtained transposon mutants local promoters had minor effect on level of *gusA* expression: mutant M12 with the highest number of RPKM for neighbor

gene in the same time exhibited the lowest level of GusA-activity (Fig. 3.18). In the same time, mutants with relatively low RPKM of flanking genes (M31, M02 and M36) were characterized by high level of GusA-activity (Fig. 3.18). PCCs of GusA-activity with 36h and 60h RPKM were -0,51 and -0,60, respectively.

Table 3.5. Average reads coverage for TA-dinucleotide of transposon integration point	
Mutant	Reads coverage
M08	34
M35	28
M31	29
M12	30
M02	22
M10	28
M36	38
M07	27
M33	33
M34	9

Another parameter we tested was correlation of GusA-activity with the number of reads per nucleotide obtained after sequencing of *S. albus* J1074-chromosome. It was considered, that the DNA-regions less accessible for DNA-polymerase would have less number of reads per nucleotide. In the same time expression of heterologous genes located in such regions may be lower as they will be less accessible for the transcription initiation complex.

To examine this speculation we calculated average number of reads for each TA-dinucleotide, where integration of transposon occurred (reads coverage data provided by Nestor Zaburannyi). Obtained results showed that for 5 of 10 mutants this value lies between 27 and 30, for three TA-dinucleotides this parameter is higher than 30: M08, M33 and M36 and for two – less than 27 times: M02 and M34 (Tab. 3.5).

These results were plotted against GusA-activity of the mutant strains (Fig. 3.19). However, no correlation between these two parameters was observed. Calculated PCC was -0,15.

In summary, any of analyzed parameters did not show some significant correlation with deviations of GusA-activity.

Comparison of GusA-activity and reads coverage
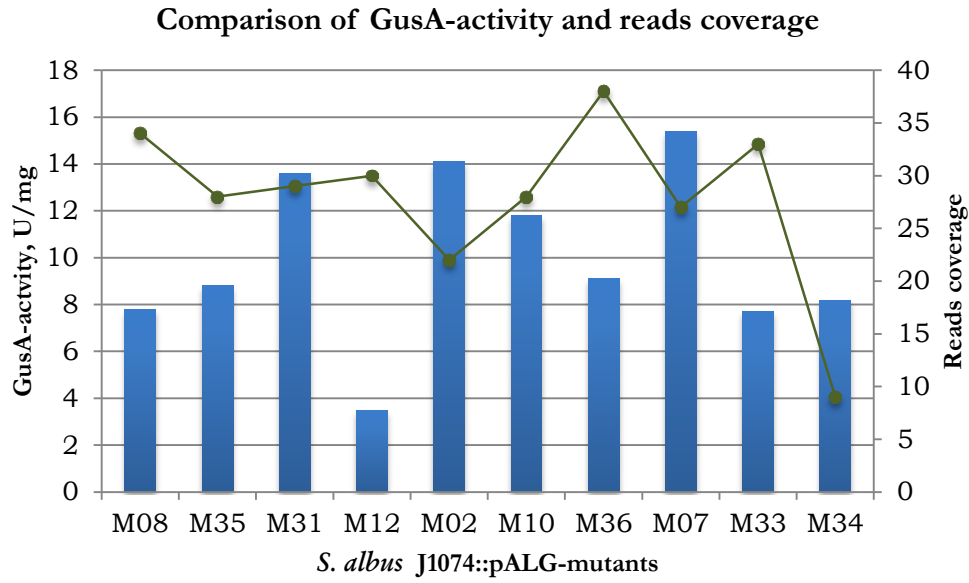
Figure 3.19. The comparison of GusA-activity levels with number of reads of TA-dinucleotides at transposon integration point. Blue columns correspond to values of GusA-activity; green dots correspond to average number of reads for TA-dinucleotide. Mutants are placed according to location of the transposon on the chromosome.

3.2.2. Investigation of position effect by integration of antibiotic biosynthesis gene cluster

3.2.2.1. Generation of plasmids containing minitransposon with φC31 attachment site

3.2.2.1.1. Construction of pHAH(II).

The *hph* gene was amplified using pAL1 as a template, Fr-*Mu*I-*attB*-*hph* as a forward primer, carrying *attB* and the *Mun*I restriction site, and Rs-*Xb*I-*hph* as a reverse primer carrying the *Xba*I site (Tab. 2.20). The amplified fragment was cloned into the *Mun*I and *Xba*I sites of pTn5Oks resulting in pTn5Oks*attBhph*(II). The *Eco*RV fragment from pTn5Oks*attBhph*(II), containing the transposon, was ligated to linearised by *Eco*RV pNLHim, to give pHAH(II) (Fig. 3.20.A).

To verify the obtained construct, analytical restriction mapping with *Eco*RV was performed. The obtained 1,9 kb fragment (Fig. 3.20.B) corresponds to minitransposon construct cloned from pTn5Oks*attBhph*(II).

A B
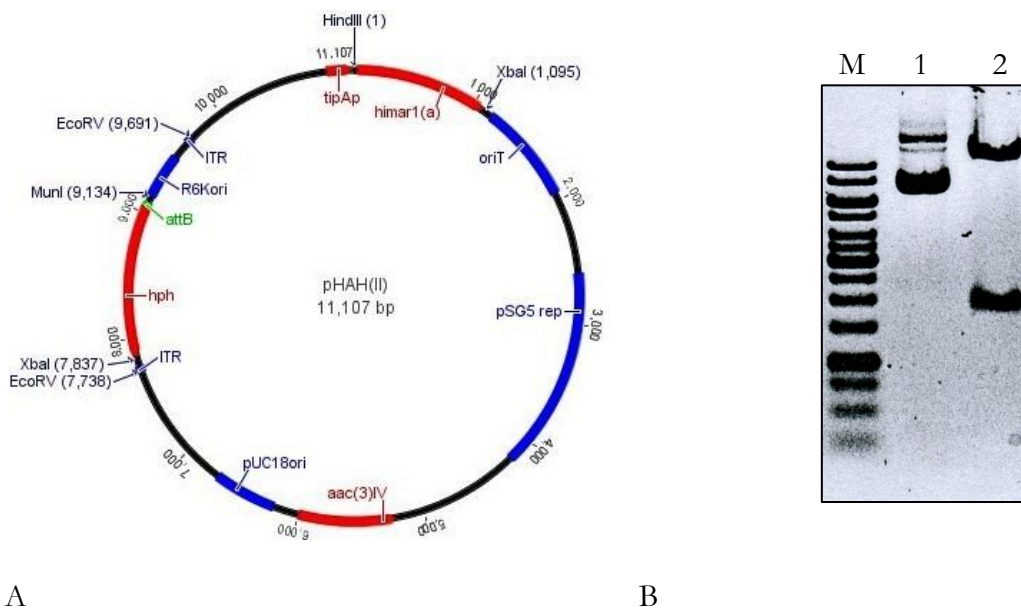
Figure 3.20. The map (A) and analytical restriction (B) of pHAH(II). (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – temperature-sensitive replicon in actinomycetes; *himar1(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6K-ori* – origin for rescue cloning; *attB* – φC31 phage attachment site. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Eco*RV. The minitransposon fragment is visible as 1,9 kbp and the backbone as 10 kbp.

3.2.2.1.2.   Construction of pHAT and pNPT

As it was described above, the majority of transposon mutants of *S. albus* contain single unique insertion. Possible reason of this fact could be insufficient activity of *Himar1* transposase in *S. albus* genome caused by the natural properties of this protein or by low expression of its gene predetermined by inability to induce effectively the *tipA*p with thiostrepton due to high sensitivity of *S. albus* to this antibiotic. To overcome the problem two additional plasmids for transposon mutagenesis were constructed. In one of them, pHAT, the *himar1(a)* gene was replaced by the *tn5(a)* transposase gene, while in the second delivery plasmid, pNPT, the *himar1(a)* gene was cloned under the control of the strong constitutive synthetic promoter Pr21 (sequence provided by Dr. Theresa Siegl).

A useful feature of pTn5Oks plasmid is that the transposon is flanked by recognition sequences for both transposases, *Himar1* and Tn5. This feature provides a possibility to facilitate a transposition of the construct using both systems.

A   B

Figure 3.21. The map (A) and analytical restriction (B) of pHAT. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; *tn5(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ME – mosaic end recognition sequence for transposase; *R6K◻-ori* – origin for rescue cloning; *attB* – φC31 phage attachment site. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Hin*dIII and *Xba*I. The transposase fragment is visible as 1,5 kbp and the backbone as 10 kbp.

Construction of pHAT. To yield pHAT the *Pvu*II fragment of pTn5Oks*attBhph*(II), containing transposon, was blunt-end ligated to pNLTn5 linearised by *Eco*RV (Fig. 3.21.A).

The obtained construct was digested with *Xba*I and *Hin*dIII to verify presence of the *tn5(a)* transposase gene. The obtained 1,5 kb fragment (Fig. 3.21.B) corresponds to the expected size.

Construction of pNPT. To yield pNPT, the ampicillin resistance gene, *bla*, was amplified using pLitmus38 as a template, the forward primer, Fr-*H*3-*S*I-Pr21-*bla*, containing Pr21 with *Hin*dIII and the reverse primer, Rs-*H*3-*S*I-*bla* with the *Swa*I site (Tab. 2.20). The fragment was ligated into *Hin*dIII digested pNLHim leading to pNLPr21*bla*. In this plasmid the *himar1(a)* gene is transcribed from the strong promoter Pr21. pNLPr21*bla* was digested with *Swa*I to remove the *bla* gene and self-ligated yielding pNLPr21. Then *Eco*RV-fragment from pTn5Oks*attBhph*(II), containing the transposon, was ligated to *Eco*RV of linearised pNLPr21, to give pNPT (Fig. 3.22.A).

To verify the obtained construct, analytical mapping with *Eco*RV was performed. Obtained 1,9 kb fragment (Fig. 3.22.B) corresponds to minitransposon cloned from pTn5Oks*attBhph*(II).

A   B
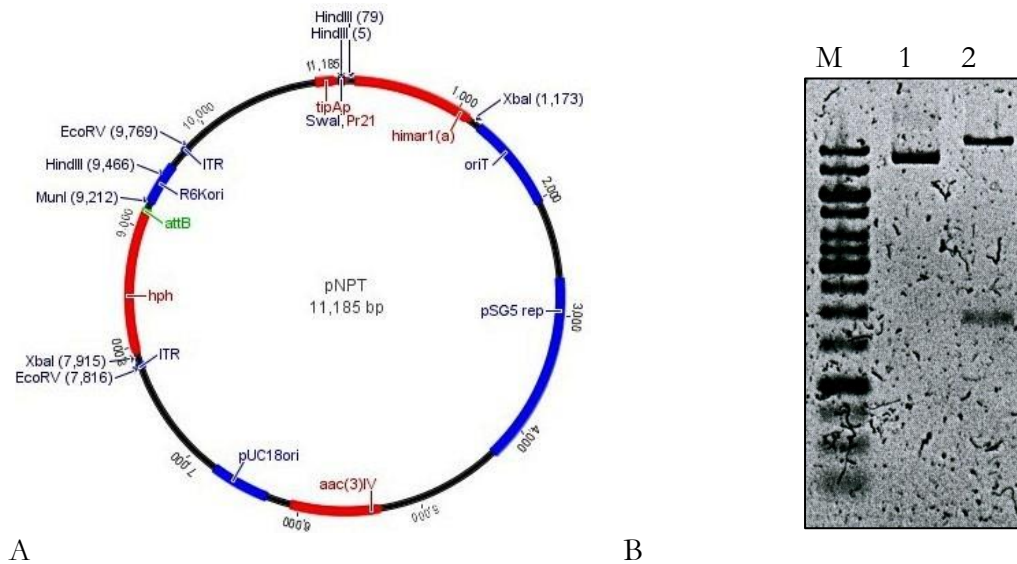
Figure 3.22. The map (A) and analytical restriction (B) of pNPT. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; pSG5rep – actinomycetes temperature-sensitive replicon; *himar1(a)* – synthetic transposase gene, under control of *tipA*p – thiostrepton inducible promoter and Pr21 – strong synthetic promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6K▫-ori* – origin for rescue cloning; *attB* – φC31 phage attachment site. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Eco*RV. The minitransposon fragment is visible as 1,9 kbp and the backbone as 10 kbp.

3.2.2.1.3. Construction of pAHT

Usually, a mutagenesis of streptomycetes with a transposon carried on a replicative plasmid is a time consuming process. This approach is comfortable if the obtaining and screening of large number, of mutants is required. In case when only few dozens of transposants are needed to be analyzed the replicative vectors could be replaced by suicide.

To construct such suicide delivery system applicable for our experiment, the hygromycin resistance gene in p31Him was replaced by the apramycin resistance gene using λ-red mediated recombination. The primers Fr-*hph/aac(3)IV* and Rs-*hph/aac(3)IV* (Tab. 2.21) were used to amplify the fragment for recombination. The obtained plasmid, pAHS, was linearised by *Eco*RV and ligated with the transposon from the pTn5Oks*attBhph* plasmid, giving pAHT (Fig. 3.23.A).

The obtained plasmid was verified by analytical restriction mapping. The digestion with *Xba*I and *Hin*dIII indicated a presence of 1,0 kbp *himar1(a)* gene while the digestion with *Eco*RV demonstrated a presence of 1,9 kbp fragment corresponding to the minitransposon cloned from pTn5Oks*attBhph* (Fig. 3.23.B).
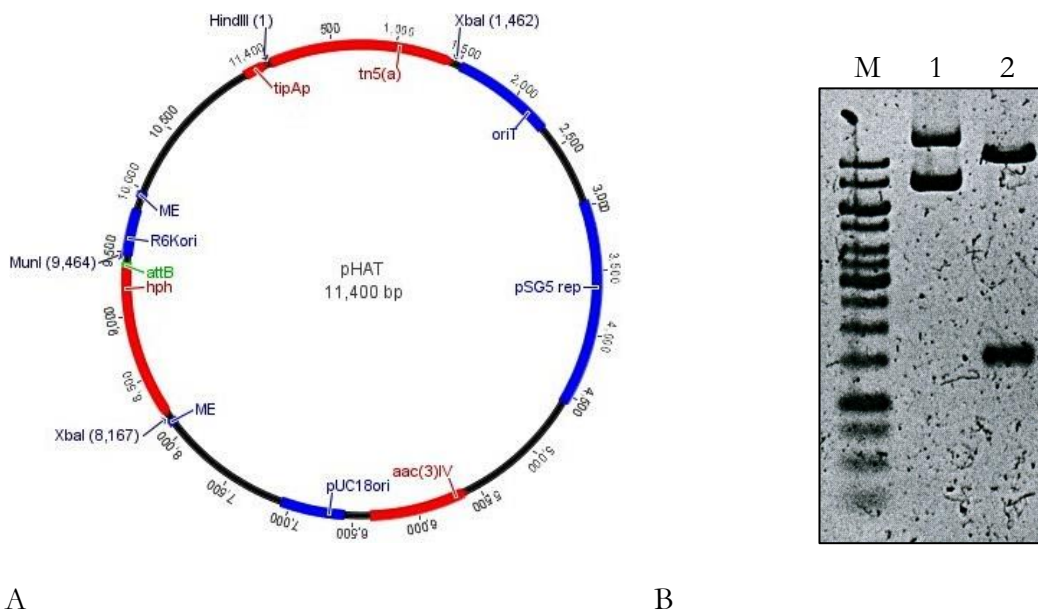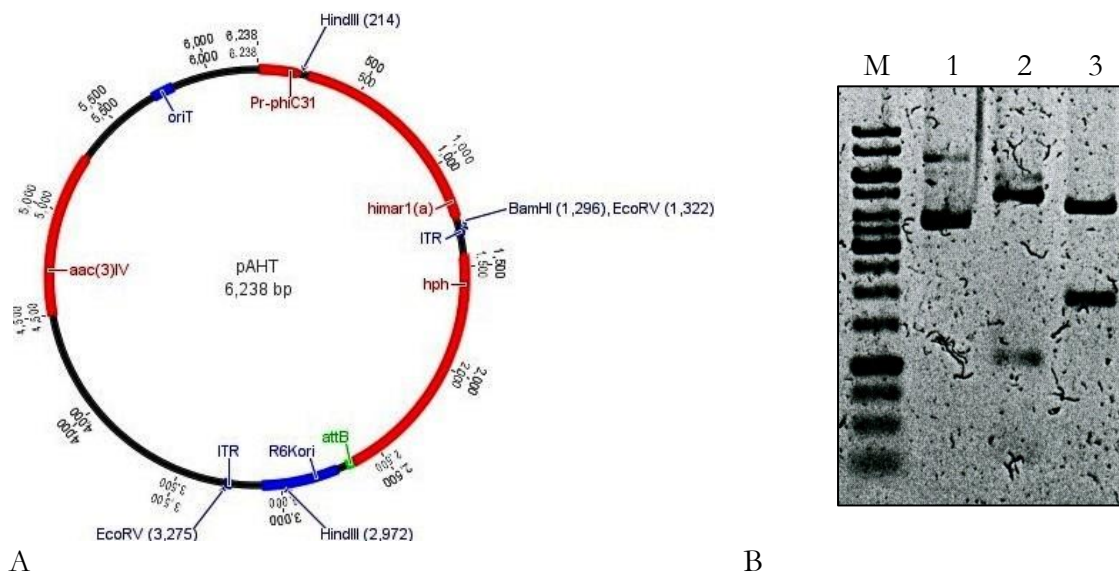

Figure 3.23. The map (A) and analytical restriction (B) of pAHT. (A) Plasmid contains following features: *oriT* – origin of plasmid transfer; *himar1(a)* – synthetic transposase gene, under control of Pr-φC31 – □C31-int promoter; *aac(3)IV* – apramycin resistance marker; *hph* – hygromycin resistance marker; ITR – inverted terminal repeats; *R6K□-ori* – origin for rescue cloning; *attB* – φC31 phage attachment site. (B) M - 1kb DNA Ladder; 1 – undigested plasmid; 2 – plasmid digested with *Bam*HI and *Hin*dIII; 3 – plasmid digested with *Eco*RV. Transposase fragment is visible as 1 kbp in lane 2; minitransposon fragment is visible as 1,9 kbp in lane 3.

3.2.2.2. Designing of *S. albus* recipient strain

3.2.2.2.1. Deletion of *attB* site in *S. albus* J1074

In order to carry the experiments in a defined genetic background, it was necessary to delete the native φC31-phage attachment site from the host strain chromosome. In the genomes of *S. coelicolor* M145 and *S. lividans* 1326 the attachment site of φC31 is located in the highly conserved gene encoding chromosome condensation protein. However it was demonstrated, that the disruption of the gene containing *attB* in genomes of these two strains is not lethal (Combes *et al.*, 2002). According to the BLAST analysis of *S. albus* J1074 genome sequence, we have determined, that *attB* is located in locus *sshg02858*, also encoding chromosome condensation protein.

The *attB* site was deleted from *S. albus* J1074 chromosome by λ-red mediated recombination. With this aim, two BACs p1F17 and p106, which contained the gene *sshg02858*, were isolated from *S. albus* J1074 BAC library and transformed into *E. coli* GB05red. These BACs are based on pSMART vector with the *gusA* reporter gene in a backbone. The 1,5 kb-disruption cassette containing the apramycin resistance gene, *aac(3)IV*, and the origin of conjugation, *oriT*, all framed by two *loxP* sites, was amplified using the Fr-pIJ774-*attB*-del and Rs-pIJ774-*attB*-del primers (Tab. 2.21) and pIJ774 as a template.

A   B
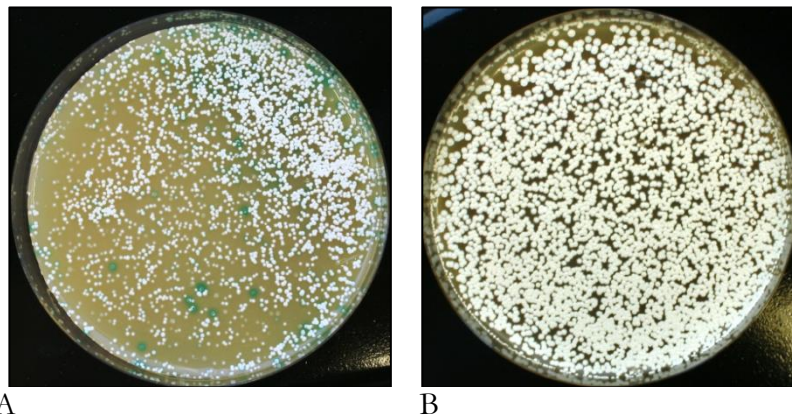
Figure 3.24. (A) The *S. albus* J1074::p1F17::*aac*74-exconjugants after 72 h of growth overlaid with X-Gluc. Blue colonies correspond to single crossover mutants, white colonies correspond to double crossover mutants; (B) The *S. albus* SAM1(Δ*attB*):: pSET152-exconjugants after 72 h of growth. Both plates were overlaid with apramycin, 50 μg/ml, and with phosphomycin, 200 μg/ml, 14 hours after conjugation.

The PCR generated fragment was transformed into *E. coli* GB05red::p1F17 and *E. coli* GB05red::p1O6 with induced expression of λ-red recombinase encoding genes. BACs with replaced *attB* site, p1F17::aac74 and p106::aac74, were introduced into *S. albus* J1074 by conjugation (Fig. 3.24). To select for double crossover-mutants, exconjugants were passed through several rounds of sporulation on a selective medium containing X-Gluc. The mutants

lacking GusA-activity and the resistance to apramycin were inoculated into liquid TSB and their chromosomal DNA was isolated and tested by PCR to prove the replacement of the *attB* site with the disruption cassette BACs with the replaced *attB* site were also proved by PCR (Fig. 3.25).
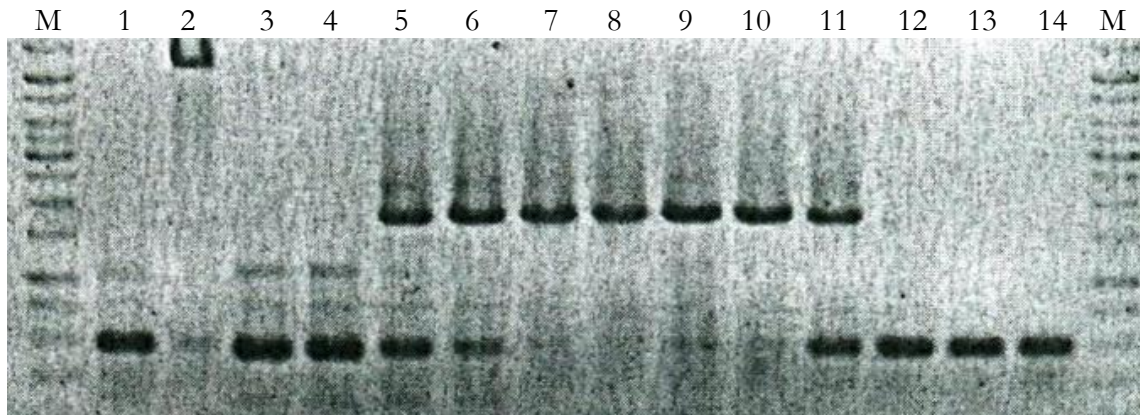
Figure 3.25. PCR confirming of correct *attB* deletion. M – 1kb DNA Ladder; 1 – *S. albus* J1074; 2 – negative control; 3 – p1O6; 4 – p1F17; 5 – p1O6::*aac*74; 6 – p1F17::*aac*74; 7, 8 – *S. albus* J1074::(p1O6::*aac*74) double crossover mutants; 9, 10 – *S. albus* J1074::(p1F17::*aac*74) double crossover mutants; 11 - *S. albus* J1074::(p1O6::*aac*74) single crossover mutant; 12 - [*S. albus* J1074::(p1O6::*aac*74)]::pUWL-Cre; 13 - [*S. albus* J1074::(p1F17::*aac*74)]::pUWL-Cre; 14 - *S. albus* SAM1(Δ*attB*)::pSET152. Primers for control PCR were homologous to chromosome app. 250 bp upstream and downstream to *attB*.

Sequencing of the PCR generated *attB*-containing fragment revealed, that *attB* of φC31 is replaced by the disruption cassette. Two analyzed exconjugants *S. albus* J1074::(1F17::*aac*74) and *S. albus* J1074::(p1O6::*aac*74) were selected for further work. To remove the resistance marker introduced by the replacement cassette, pUWL-Cre was introduced into obtained strains and *Cre*-recombinase mediated loss of the apramycin resistance phenotype in 70% of analyzed colonies. The genomic DNA of several apramycin sensitive colonies was tested with PCR and the obtained 0,5 kb fragment was sequenced to confirm removing of the disruption cassette. One of these strains named *S. albus* SAM1(Δ*attB*) obtained from *S. albus* J1074::(1F17::*aac*74), in which regarding sequencing data, the φC31*attB* site was replaced with 81 p.b. scar remaining after the marker excision, was chosen for further studies.

A

B a

NruI(1)   *attL*(10,2)   *attR*(15,9) NruI(16,5)

B b

NruI(1)   *pseR*4(4,8)   *pseL*4(10,5)NruI(13,5)
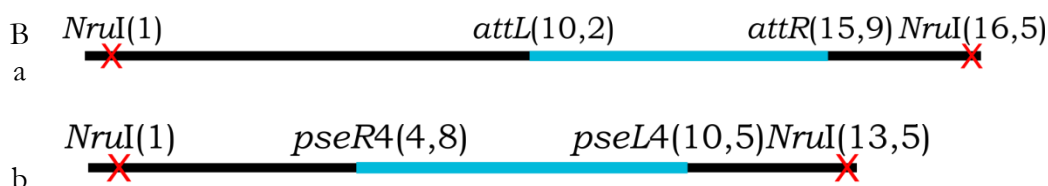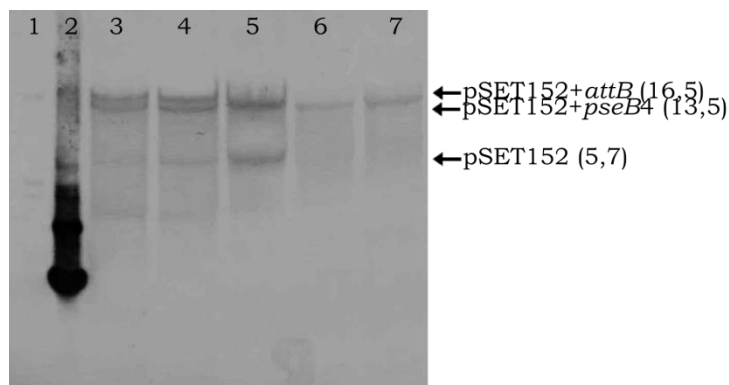
Figure 3.26. Analysis of pSET152 integration into *S. albus* J1074 and Δ*attB* strain genomes. (A) Hybridization membrane with genomic DNA probed with fragment containing the *aac(3)IV* gene. 1 – DIG marker; 2 – Positive control; 3, 4 – *S. albus* J1074::pSET152 transconjugants; 5, 6, 7 – *S. albus* SAM1(Δ*attB*)::pSET152 trans-conjugants. The genomic DNA was digested with *Nru*I, separated in 0,7% agarose gel, transferred on nylon membrane in denaturing conditions and hybridized with 1,2 kb fragment containing *aac(3)IV* gene of pIJ773. Size of fragments is shown in brackets. (B) Scheme of pSET152 integration into *attB* (a) and *pseB*4 (b).

3.2.2.2.2. Introduction of pSET152 into the *S. albus* SAM1(Δ*attB*) strain

To functionally verify deletion of *attB*, the pSET152 vector, based on the φC31 recombination system, was introduced into *S. albus* SAM1(Δ*attB*) by conjugation. The wild type *S. albus* J1074 was used as a positive control. The number of exconjugants obtained after the conjugation of pSET152 into *S. albus* SAM1(Δ*attB*) was the same or even higher than number of exconjugants obtained in experiment with *S. albus* J1074. This surprising result could be explained by the presence of previously unknown second *attB* site in the genome of *S. albus*.

To facilitate the identification of this insertion site, chromosomal DNA of *S. albus* J1074::pSET152 and *S. albus* SAM1(Δ*attB*)::pSET152 was isolated, digested with *Nru*I and hybridized with the *aac(3)IV* probe. This experiment should revile a number of pSET152 copies integrated into genome of each strain. If pSET152 was integrated into the native *attB*, band of 16,5 kb should be observed, 5,7 kb-bands of covalently closed pSET152 may also be seen (Fig. 2.26). These bands are clearly present in the samples of two analyzed *S. albus* J1074 exconjugants; 5,7 kb-bands are also present in one sample of three analyzed *S. albus* SAM1(Δ*attB*) exconjugants (Fig. 3.26). However, all exconjugants of both J1074 and Δ*attB* strains contained one additional

band approximately 10-15kb in size. This band was accounted for the fragment created by the insertion of pSET152 into pseudo-*attB* site. The obtained results demonstrated that pSET152 can integrate into the pseudo-*attB* site even if native *attB* is uninjured. The new pseudo-*attB* site was called *pseB*4, according to the nomenclature established previously (Combes *et al.*, 2002).

3.2.2.2.3. Identification of pseudo-*attB* site

In order to identify the *pseB*4 site, the rescue cloning of plasmids from chromosomal DNA of two *S. albus* SAM1(Δ*attB*)::pSET152 mutants was performed. For this aim, the isolated DNA was digested with *Kpn*I and self-ligated. The ligation mixture was then transformed into *E. coli* DH5α and the transformants resistant to apramycin were obtained. The plasmids rescued in this way should contain whole pSET152 and two fragments of the chromosome flanking the plasmid. Two plasmids were isolated from two independent *S. albus* SAM1(Δ*attB*)::pSET152 strains. Three of four isolated plasmids had size of native pSET152 and only one was of size larger than 5,7 kbp. It means that pSET152 is aberrantly presented in the most samples as it was also reported previously (Combes *et al.*, 2002).

Figure 3.27. Sequences of native *attB*, secondary *attB* of *S. albus* J1074 and secondary sites of *S. coelicolor* M145.

The plasmid containing fragments of chromosome was sequenced from the primers Fr-pSET152-Rp and Rs-pSEt152-Rp (Tab. 2.22) and the location of *pseB*4 was identified. The locus is situated in *sshg03147* ORF encoding conserved hypothetical protein. This location of *pseB*4 was also confirmed by Southern hybridization, as the size of the band corresponding to pSET152 integrated into *pseB*4 is predicted to be 13,5 kb when chromosomal DNA was digested with *Nru*I. The shoulders of the *pseB*4 site, are named, R_{pseB4} and L_{pseB4}, respectively, and the hybrid sites formed by recombining of L_P of pSET152 *attP* sequence with R_{pseB4} and R_P with L_{pseB4} – *pseR*4 and

pseL4, respectively. Remarkably, that identified *pseB*4 does not contain conserved TT- but TA dinucleotide in the core sequence (Fig. 3.27).

3.2.2.2.4. Deletion of *pseB*4 in *S. albus* J1074 and *S. albus* SAM1(Δ*attB*) strains

In order to study the position effect in *S. albus* J1074, the *pseB*4 had also to be deleted from the genomes of *S. albus* J1074 and *S. albus* SAM1(Δ*attB*). The same like by the deletion of *attB*, first *pseB*4 was replaced with the disruption cassette amplified from pIJ773 on a BAC, then modified BAC was introduced into the recipient strains and the apramycin resistance marker was excised by FLP-recombinase. The deletions were confirmed by PCR and sequencing analysis. The obtained mutants, named *S. albus* SAM2(Δ*pseB*4) and *S. albus* SAM3(Δ*attB*·Δ*pseB*4), were used in further experiments.

3.2.2.3. Establishing of transposon mutant library and analysis of mutants

The plasmids carrying transposon with the φC31 attachment site (pHAH(II), pHAT, pNPT and pAHT) were introduced into *S. albus* SAM3(Δ*attB*·Δ*pseB*4) by intergeneric conjugation. Different workflows for the generation of transposon mutant libraries were applied because of the different vectors features (Fig. 3.28). In the case of plasmids pHAH(II) and pHAT containing *himar1(a)* and *tn5(a)* transposase genes under control of *tipA*p, an additional thiostrepton induction step was included. In contrast, pNPT has the transposase gene under the constitutive promoter Pr21 making induction step redundant. All three plasmids are replicative and after the transposition step, the delivery vector has to be cured. The transposase gene in the suicide pAHT plasmid is cloned under φC31 *int* gene promoter and therefore do not require any additional efforts for the induction of the transposase or curing the plasmid backbone. The obtained exconjugants already contain copy of transposon inserted in the genome. However, the time gap, between the moment when the plasmid is introduced into the *S. albus* cell and when it is eliminated is not sufficient enough to produce significant number of mutants. Only dozen of colonies per plate are usually obtained after conjugation with pAHT.
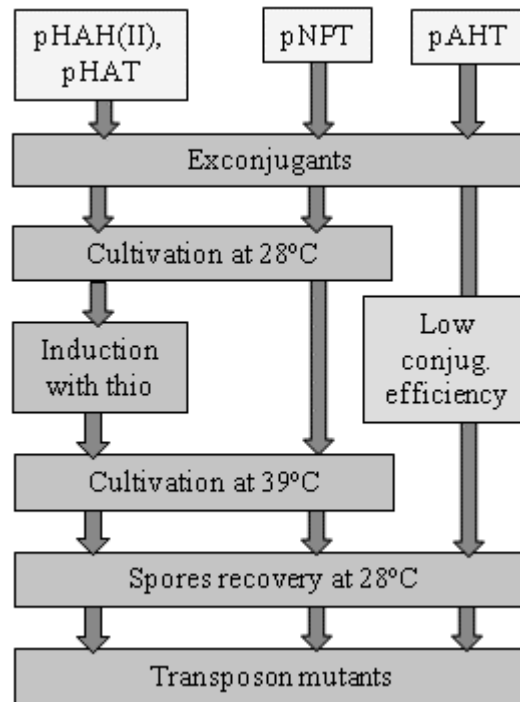
Figure 3.28. Variations of workflow for generation of transposon mutant library of *Streptomyces albus* **SAM3(Δ*attB*·Δ*pseB*4) with pHAH(II), pHAT, pNPT and pAHT. Replicative plasmids with inducible promoter require several additional steps, while transconjugants obtained with suicide plasmid already contain unique insertion of transposon. However, the efficiency of this approach is not sufficient to perform experiments where large number of mutants is required.**

Southern blot analysis of randomly chosen mutants demonstrated that eight selected strains obtained after the transposon mutagenesis with the suicide plasmid contain a unique transposon insertion (Fig. 3.29). The same results were observed in the case of six pNPT-transposon mutants. In case of Tn*5*-based replicative plasmid one of six analysed mutants contained two copies of transposon inserted in the chromosome (Fig. 3.29; L4).

These results demonstrated once again that the most of *S. albus* J1074 transposon mutants unlike *S. coelicolor* and *S. lividans* contain one copy of transposon in the chromosome. However, few colonies with the multiple insertions of a minitransposon are also present in the population.
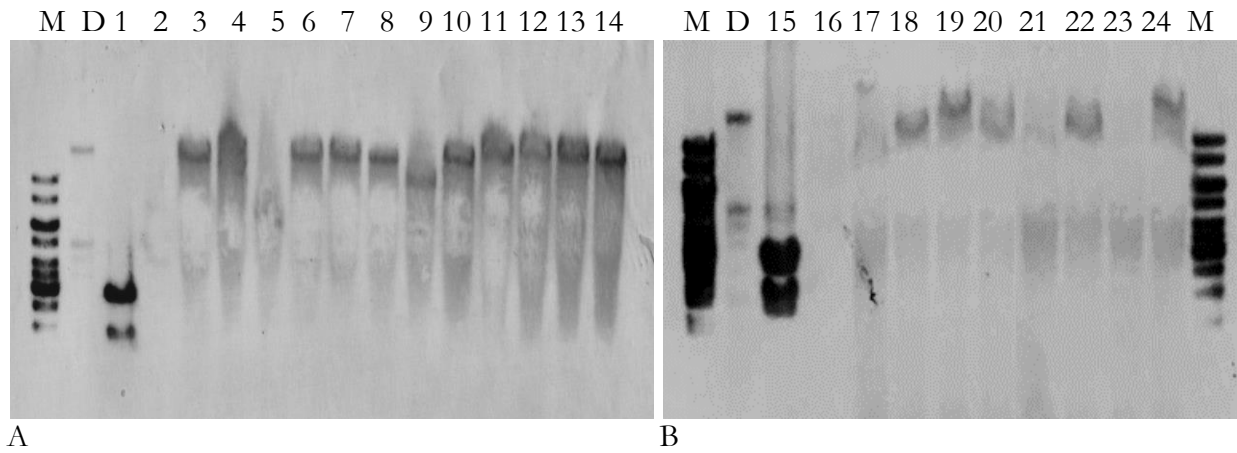
Figure 3.29. Hybridization membranes after Southern blot hybridization of transposon-mutants. M – 1kb DNA Ladders; D – DIG Markers; 1, 15 – positive controls; 2, 16 – *S. albus* SAM1(Δ*attB*), 3-8 – *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pHAT-mutants; 9-14 – *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pNPT-mutants; 17-24 – *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pAHT-mutants.

3.2.2.4. Integration of aranciamycin biosynthetic cluster and measuring of aranciamycin production level

To investigate the chromosomal position effect on the expression of heterologous natural products gene clusters, 30 *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pAHT-mutants were selected. These mutants contain minitransposons with *attB* inserted in different sites of chromosome. As a model cluster, the aranciamycin biosynthetic gene cluster was used. The *ara* gene cluster was present on the pOJ436 based cosmid p412C06, that contains 35,9 kb fragment of *S. echiatus* Tü303 chromosome with 24 ORFs responsible for aranciamycin biosynthesis. The pOJ436 cosmid vector contains the *attP* site and the *int* gene of φC31 allowing to be used in this experiment. Previously p412C06 was successfully expressed in *S. lividans*, *S. fradiae* A0 and *S. diastatochromogenes* Tü6028 (Luzhetskyy *et al.*, 2007).

To test the chromosomal position effect, the p412C06 was introduced into the selected pAHT mutants and in this way strains carrying aranciamycin biosynthetic cluster at random chromosomal positions were obtained.

Interestingly, that during cultivation in liquid medium, transconjugants that contained p412C06 started to produce red pigment (Fig. 3.30). This can be a result of modification of aranciamycin molecule by *S. albus* strain.
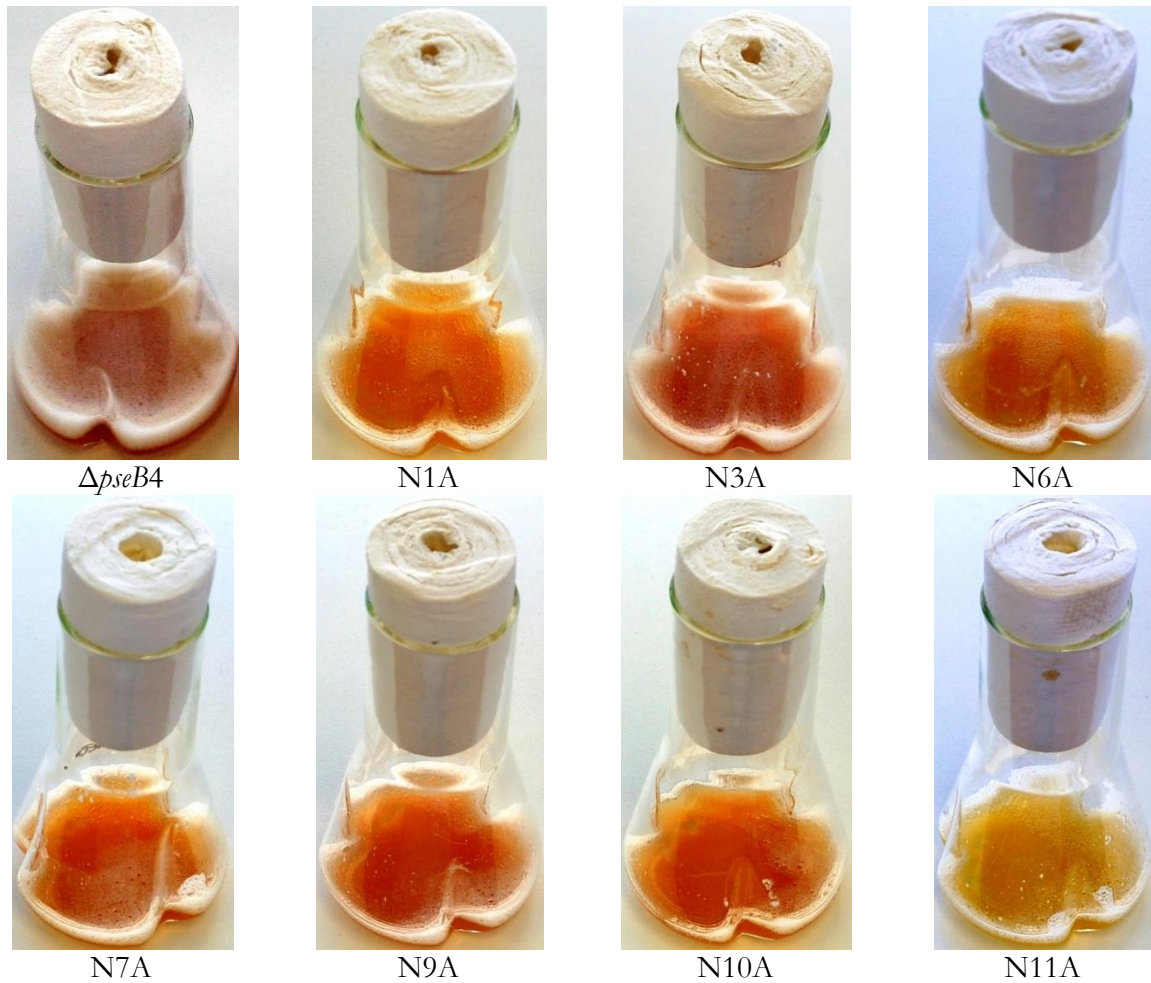
Figure 3.30. Transconjugants of *S. albus* SAM2(Δ*pseB*4)::pAHT::p412C06 and *S. albus* SAM3(Δ*attB·*Δ*pseB*4)::pAHT::p412C06 producing red pigment, apparently, aranciamycin derivative. Mutants were grown in TSB for 48 h at standard conditions.

To further estimate the aranciamycin production, a mycelium of the obtained mutants was cultivated in the NL5 liquid media supplemented with 0,1% of yeast extract. After five days of cultivation crude extracts from the cultural medium were obtained and the production profiles of the mutant strains were analyzed by HPLC and compared with that of the wild type. The production of aranciamycin by different mutants was estimated by the comparison of peaks areas of this antibiotic on the chromatograms (Fig. 3.31). Measurements were triplicated. The analyzed cultures demonstrated an eight fold variation in aranciamycin accumulation (Fig. 3.32; compare N16A and N03A).
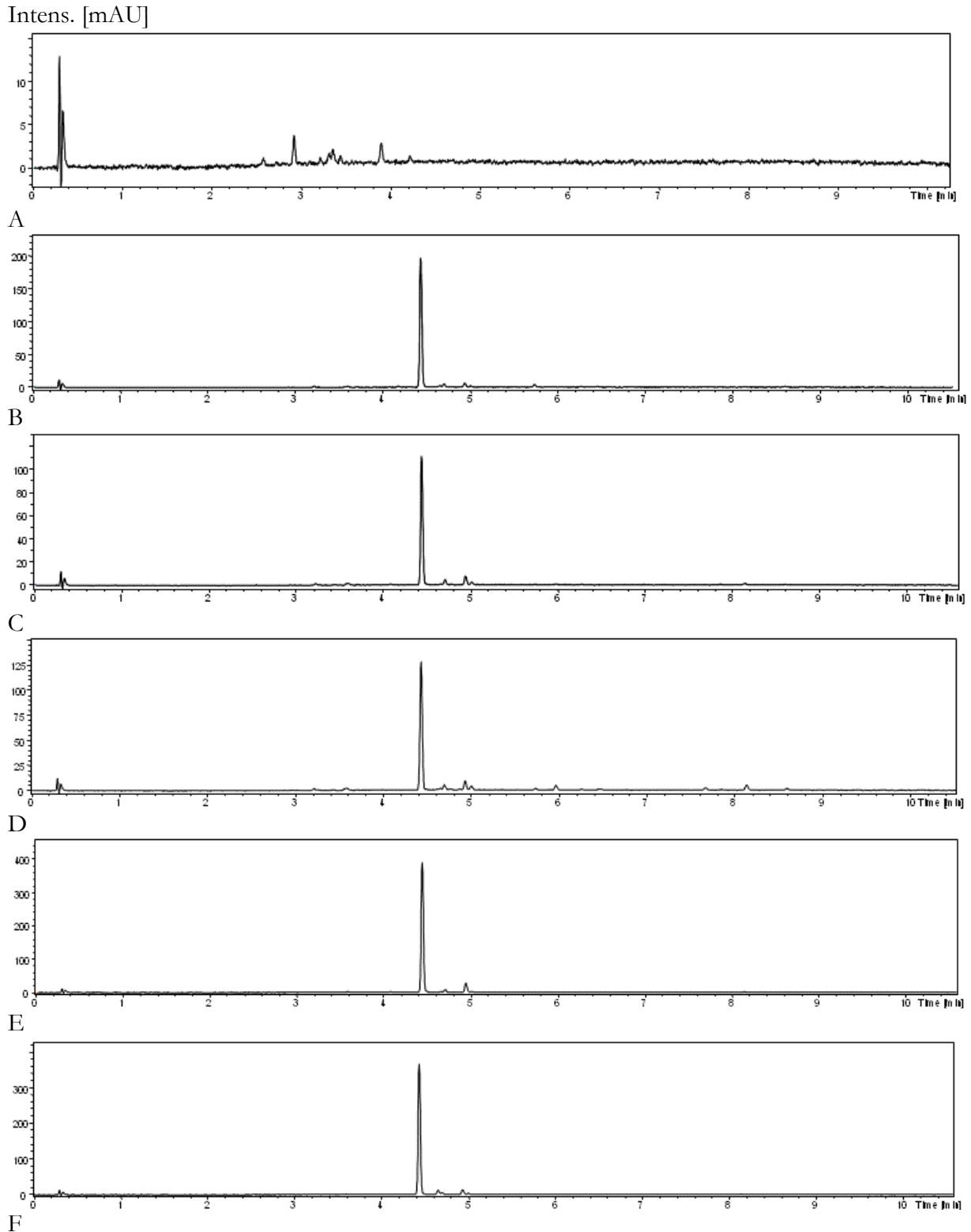
Figure 3.31. HPLC/ESI-MS analysis of crude extracts of *S. albus* mutants (428 nm). A. – *S. albus* J1074; B – *S. albus* J1074::p412C06; C – *S. albus* SAM1(Δ*attB*)::p412C06; D – *S. albus* SAM2(Δ*pseB*4)::p412C06; E, D – *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pAHT::p412C06-mutants (N03A and N05A, respectively)

The highest antibiotic concentration was observed by wild type *S. albus* J1074::p412C06 (11,1) as it carries two copies of cluster. Level of production of aranciamycin by Δ*attB* and Δ*pseB*4 strains

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was almost the same (5,08 and 5,47, respectively) and about two times lower than in the case of wild type strain. Five of 25 Tn strains 20%) produced (more compound than Δ*attB* and Δ*pseB*4 strains: N01A (6,39), N03A (8,24), N05A (6,41), N30A (6,70) and N31A (5,91). The lowest activity demonstrated N16A-mutant (1,04). Such eight fold variation in expression level of heterologous cluster correlates with results obtained for *gusA* reporter gene expression as described above (see 3.2.1).

Relative concentration of aranciamycin, 1/g
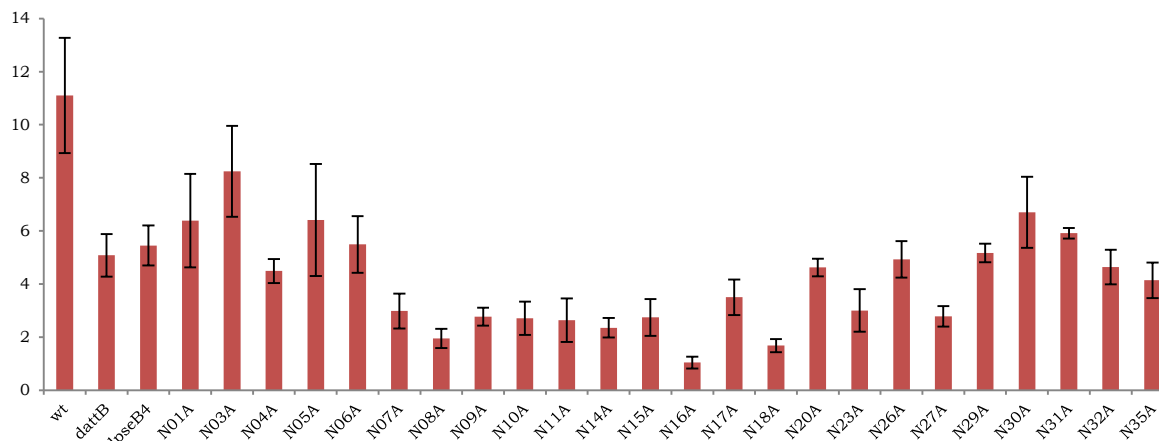
Figure 3.32. Production of aranciamycin by different mutants per 1 g of dry biomass. Strains were grown for 120 h at 30°C, 5 ml of culture was extracted with EtAc, concentrated up to 80 μl in acetonitrile and analyzed by HPLC. Relative values of aranciamycin concentrations were obtained after recalculation of peaks areas corresponding to this compound. Data was normalized for 1 g of dry biomass.

3.2.3. Introduction of additional *attB*-sites into *S. albus*-genome

Mutants with random integration of aranciamycin cluster didn't show any increase in aranciamycin production. So the efforts were switched to introduction of additional *attB* sites for cluster integration. To deliver these attachment sites the plasmid pHAT(II)3 had been conjugated into *S. albus* J1074. This plasmid was chosen, as Tn*5*-transposase had shown itself more reliable for multicopy transposon integration in *S. albus* (Fig. 3.28.A). After Southern blot analisis of obtained mutants (data not shown) two mutants, *S. albus* T1 and *S. albus* T11, with additional *attB*, were obtained and taken for further researches. Analysis production facilities of these strains will be carried out in next weeks.

3.3. Investigation of bacteriophages integration sites

3.3.1. Investigation of φC31 pseudo-attachment site

3.3.1.1. Introduction of pOJ436-based cosmid into the *S. albus* SAM1(Δ*attB*) strain

In order to investigate the aranciamycin production, the cosmid p412C06 was introduced into the genomes of *S. albus* J1074 and *S. albus* SAM1(Δ*attB*) by conjugation. Interesting feature was that all exconjugants of the Δ*attB*-strain were formed spores, while the most except dozen transconjugants of wild type strain remain bald (Fig. 3.33). To test if all exconjugants contain two copies of cosmid, two sporulating and two bald transconjugants of *S. albus* J1074::p412C06 and two transconjugants of *S. albus* SAM1(Δ*attB*)::p412C06 were grown in TSB, their chromosomal DNA was isolated, digested with *Pst*I and hybridized with the *aac(3)IV* probe.
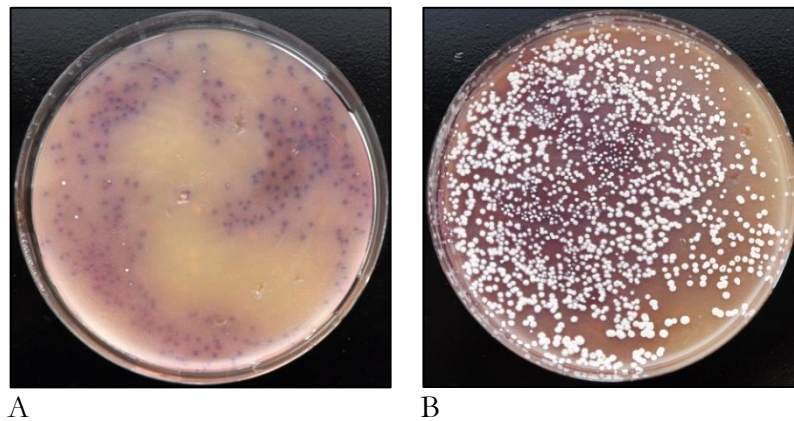
Figure 3.33. *S. albus* J1074::p412C06 (A) and *S. albus* SAM1(Δ*attB*)::p412C06 exconjugants (B) after 72 h of growth. Plates were overlaid with apramycin, 50 µg/ml, and with phosphomycin, 200 µg/ml, 14 hours after conjugation.

This hybridization should detect number of copies of p412C06 integrated into the genome of each strain. *Pst*I cuts inside of p412C06, so that the band visualized by Southern blot should contain 2,6 kb-fragment of the plasmid, including *aac(3)IV* and the left shoulder of *attP*, the right shoulder of *attB* or *pseB*4 and 3,1 kb (*attB*) or 12,9 kb (*pseB*4) genomic region adjacent to integration point. So, if p412C06 was integrated into native *attB*, the band of 5,7 kb should have been detected (Fig. 3.33.B). In case of integration of the cosmid into the *pseB*4 size, the corresponding band on the membrane should be visible as a 15,5 kb fragment (Fig. 3.33.B). The band of 5,7 kb is presented in all wild type probes (Fig. 3.33A). The band of 15,5 kb is presented in three of four analyzed probes of wild type and in one of two analyzed probe of Δ*attB*-strain (Fig. 3.33A). Probes of two other strains do not contain the band corresponding to *pseB*4-integration, but contain smaller band, of the approximate size of 9-10 kb (Fig. 3.33A). To explain

the appearance of this band the genomic region adjacent to *pseB*4 was analyzed. It was found that the size of this unexpected signal corresponds to the size of the DNA fragment that would be formed by the 2,6 kb plasmid fragment with left shoulder of *attP* and 6,4 kb genomic region containing left shoulder of *pseB*4 (Fig. 3.33B). It would be possible only if these two shoulders could recombine with each other and thus no control over polarity of integration is provided by integration of p412C06 into *pseB*4.
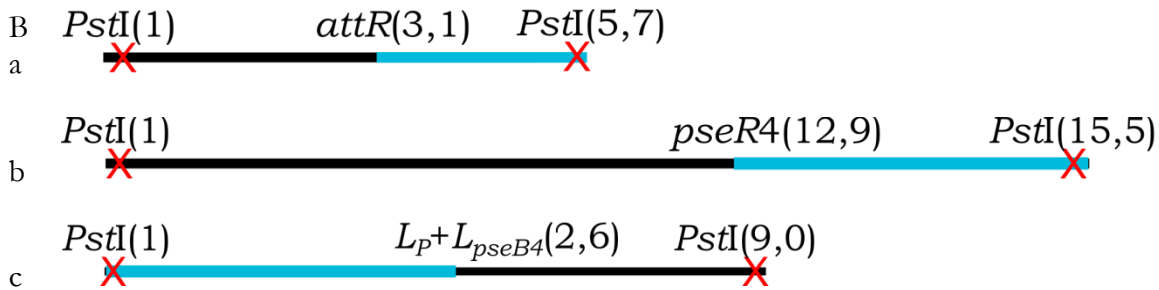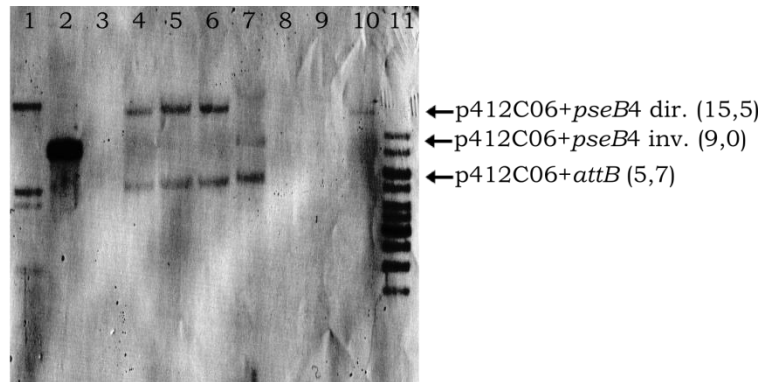
Figure 3.34. Analysis of p421C06 integration into *S. albus* J1074 and Δ*attB* strains chromosome. (A) Hybridization membrane with genomic DNA probed with fragment containing the *aac(3)IV* gene. 1 – DIG marker; 2 – positive control; 3 - *S. albus* J1074; 4-7 – *S. albus* J1074::p412C06 transconjugants; 8 – *S. albus* SAM1(Δ*attB*); 9, 10 – *S. albus* SAM1(Δ*attB*)::p412C06 transconjugants; 11 – 1kb DNA ladder. The genomic DNA was digested with *Pst*I, separated in 0,7% agarose gel, transferred on nylon membrane in denaturing conditions and labeled with 1,2 kb fragment containing *aac(3)IV* gene of pIJ773. Size of fragments is shown in brackets. (B) Scheme of p412C06 integration into *attB* (a), *pseB*4 in direct orientation (b) and *pseB*4 in inverted orientation (c).

3.3.1.2. Investigation of integration specificity into *pseB*4

To verify our assumption that the integration of φC31 based plasmid might be bidirectional into the *pseB*4 site in contrast to the native *attB* site, we have cloned and sequenced the rescue plasmids from the genome of *S. albus* SAM1(Δ*attB*)::p412C06. Obtained sequencing results supported the bidirectional integration of p412C06 into the *pseB*4 site (Fig. 3.35). In the sequences of the *attB* and *attP* sites a core sequence, TT, where cleavage, forming of staggered

break and joining of chains occurs, is situated in the centre of each of these sites. After the analysis of the hybrid sites, created after the recombination between L_{attP} and L_{pseB4} in one case and between L_{attP} and R_{pseB4} in another (Fig. 3.35) any such kind of core sequence could be observed: the fragment homologous to the phage attachment site is followed by the fragment homologous to the bacterial attachment site with no sequence that overlaps both sites. Only possible explanation can be, that by catalysis of the integration reaction into $pseB4$ the φC31-integrase does not form staggered but blunt break in the strand. To examine if this is particular event or legitimate case, the chromosomal DNA of six *S. albus* SAM1($\Delta attB$)::pSET152 transconjugants obtained previously was isolated and their regions that include hybrid *L* and *R* sites were amplified by PCR and sequenced (Fig. 3.35.B and C). Obtained result indicated, that after the integration of pSET152 also no staggered break of chains occurs. Interestingly, after the integration of pSET152 in the direct orientation, the breakpoint of the *pseB4* chain occurs few nucleotides to the left or to the right from core A-nucleotide (Fig. 3.35.B and D).

Figure 3.35. Sequences of left and right endpoints after integration of pSET152 and p412C06 in *S. albus* J1074. (A) – sequences of *pseB4* and *attP*; (B) – integration in direct orientation; (C) – integration in inverted orientation; (D) – integration in direct orientation with overlap; bases in dotted boxes correspond to bases analyzed in (B), (C) and (D); bases in yellow boxes correspond to bases that overlap between *attP* and *pseB4*.

3.3.1.3. Verification of integration features of *pseB*4

Construction of pIGP2 and pDGP1. To demonstrate the ability of *pseB*4 to recombine with *attP* in any orientation, two plasmids containing *gusA* gene flanked by *attP* and *pseB*4 once in direct (pIGP2) and once in inverted (pDGP1) orientations were constructed.
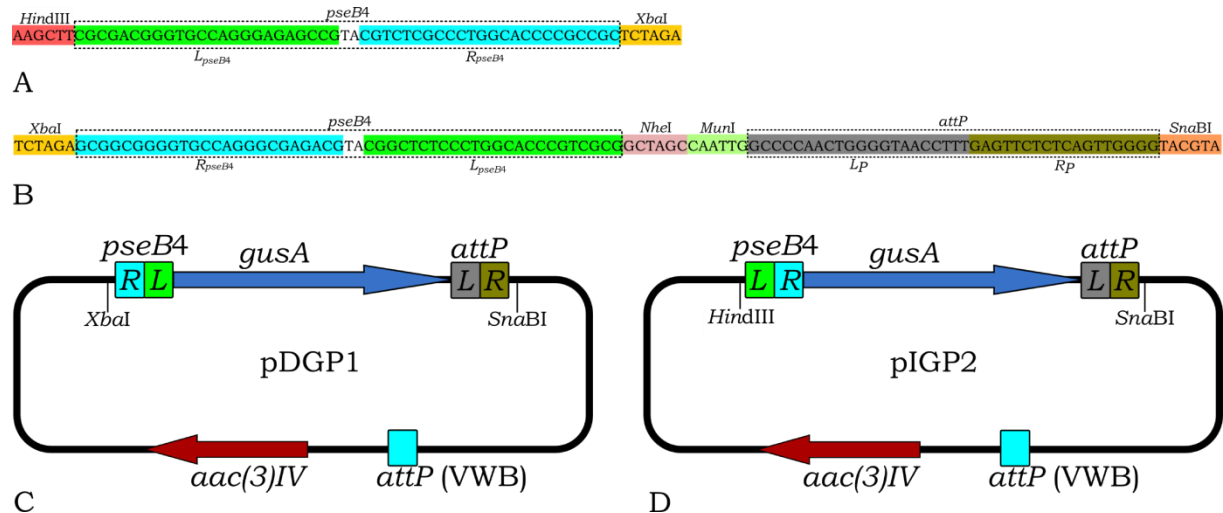
Figure 3.36. Construction of pIGP2 and pDGP1. (A) and (B) syntetic fragments of pBB1 and pBB2 were used for construction of pTOS based pIGP2 (C) and pDGP1 (D), respectively. *aac(3)IV* – apramycin resistence gene; *attP* (VWB) – phage attachment site of VWB for integration; *attP* – phage attachment site of φC31 for integration; *gusA* – gene of glucuronidase; *pseB*4 – bacterial secondary attachment site of *S. albus* J1074.

With this aim two synthetic constructs, one carrying *pseB*4, flanked by *Hin*dIII and *Xba*I, second carrying *pseB*4 and *attP* with *Nhe*I and *Mun*I in between, all flanked by *Xba*I and *Sna*BI, were synthesized by GenScript (NJ, USA) and provided on plasmids pBB1 and pBB2, respectively (Fig. 3.36.A and B).

To generate pDGP1, the fragment containing the *gusA* gene was amplified using the Fr-*X*I-*e*p1-*gusA* and Rs-*M*I-t*fd*-*gusA* primers (Tab. 2.20) and pSET152*gusA* as a template. The amplified fragment was digested with *Mun*I and *Xba*I and cloned into pBB2, linearised with *Mun*I and *Nhe*I, yielding pBB2*gusA*. The *gusA*-containing *Xba*I-*Sna*BI-fragment from this plasmid was cloned into respective sites of pTOS, giving pDGP1 (Fig. 3.36.C).
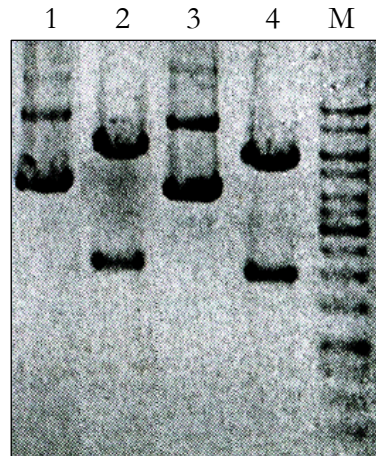
Figure 3.37. Analytical restrictions of pDGP1 and pIGP2 plasmids. 1 – undigested pDGP1; 2 – pDGP1 digested with *Xba*I and *Sna*BI; 3 – undigested pIGP2; 4 – pIGP2 digested with *Hin*dIII and *Sna*BI. Fragment containing *gusA* framed by *pseB*4 and *attB* is visible as 2 kb.

To generate pIGP2, the fragment containing *gusA* gene and *attP* was amplified by PCR using the Fr-*X*I-*e*p1 and Rs-*X*I-*S*BI-*P*38 primers (Tab. 2.20) and pBB2*gusA* as a template. The amplified fragment was digested with *Xba*I and cloned into linearised by *Xba*I pBB1. The obtained plasmid with the appropriate orientation of insertion was named pBB1*gusA*. The *gusA*-containing *Hin*dIII-*Sna*BI fragment from this plasmid was cloned into respective sites of pTOS, giving pIGP2 (Fig. 3.36.D).

Both plasmids, pDGP1 and pIGP2, were verified by digestion with *Xba*I and *Sna*BI or with *Hin*dIII and *Sna*BI, respectively. Observed 2 kb bands corresponded to the fragment containing *gusA* flanked by two attachment sites (Fig. 3.37).

***Introduction of pIGP2 and pDGP1 into S. albus* SAM3(Δ*attB*·Δ*pseB*4).** To demonstrate ability of *pseB*4 to recombine with *attP* in any orientation, the plasmids pIGP2, containing the *gusA* reporter gene flanked by *attP* and *pseB*4 in the direct orientation, and pDGP1, containing the *gusA* gene flanked by *attP* and *pseB*4 in the inverted orientation, were introduced separately into *S. albus* SAM3(Δ*attB*·Δ*pseB*4). To detect GusA-activity directly on plate, solution of the chromogenic substrate X-Gluc was used (Myronovskyi and Luzhetskyy, 2012). Strong GusA-activity was observed in all exconjugants. In order to test the ability of φC31 integrase to catalyze the recombination between *attP* and two orientations of *pseB*4, two exconjugants, one *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pDGP1, second *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pIGP2, were conjugated with pKHInt31, a plasmid containing the φC31 integrase gene. After one passage of *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pDGP1 and *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pIGP2 exconjugants with pKHInt31 in a liquid medium, serial dilutions of mycelium were made and 25 colonies of each

type were analyzed for the GusA-activity. Twenty three clones of *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pIGP2 and 24 clones of *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pDGP1 turned white, while parental strains continued to demonstrate GusA-activity. This fact indicates that integrase of φC31 phage could provide recombination between *attP* and *pseB*4 in direct orientation in 92% of clones and between *attP* and *pseB*4 in inverted orientation in 96% of clones (Fig. 3.38A and B).
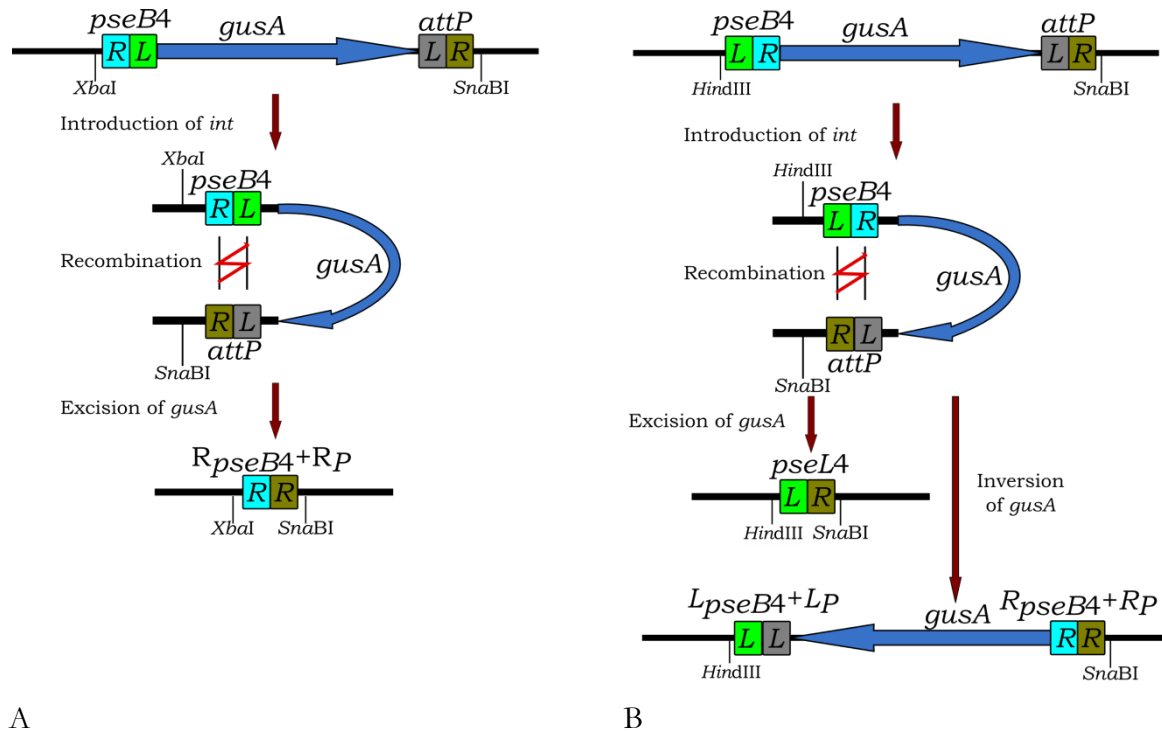
Figure 3.38. Scheme of *gusA* excision from *S. albus* SAM3(Δ*attB*·Δ*pseB*4):: pDGP1::pKHInt31 (A) and *S. albus* SAM3(Δ*attB*·Δ*pseB*4)::pIGP2::pKHInt31 (B). In case of pIGP2 two rescue plasmids were sequenced, one where *gusA* was excised, second where *gusA* was inverted.

3.3.1.4. Mutual inhibition of *attB* and *pseB*4

In order to compare the mutual and particular activity of *attB* and *pseB*4 and activity of remained secondary sites, the pSET152 was conjugated into strains *S. albus* J1074, *S. albus* SAM1(Δ*attB*), *S. albus* SAM2(Δ*pseB*4) and *S. albus* SAM3(Δ*attB*·Δ*pseB*4) (Fig. 3.39). The frequency of the pSET152 conjugation into these strains was $1{,}2\cdot10^{-6}$, $2{,}5\cdot10^{-6}$, $7\cdot10^{-5}$ and $8{,}3\cdot10^{-9}$ per spore for *S. albus* J1074, *S. albus* SAM1(Δ*attB*), *S. albus* SAM2(Δ*pseB*4) and *S. albus* SAM3(Δ*attB*·Δ*pseB*4), respectively. This indicates increasing in the frequency of conjugation into Δ*attB* and Δ*pseB*4 strains of approximately 2-fold and 58-fold, respectively, compared to wild type. Some residual integration activity demonstrated by pSET152 in Δ*attB*·Δ*pseB*4-strain can be accounted to the integration into other secondary *attB* sites of the genome.

Possible reason of increased conjugation frequency of pSET152 in the $\Delta attB$ and $\Delta pseB4$ strains could be, that when this two attachment sites are present in the genome, some part of catalytic activity of φC31 integrase is directed on interaction or even recombination of these two sites with each other. It may cause some lethal rearrangements or deletions in genome and so number of transconjugants is lower. Following this hypothesis, even expression of φC31-integrase gene in genome of *S. albus* J1074 should cause the same lethal effect. To test this assumption, plasmid containing *φC31-int* gene, pUWLInt31, was introduced into genomes of desired strains. However, the conjugation frequency of this plasmid was $1{,}25 \cdot 10^{-8}$, $2 \cdot 10^{-8}$, $1{,}1 \cdot 10^{-8}$ and $0{,}6 \cdot 10^{-8}$ per spore for wild type, $\Delta attB$, $\Delta pseB4$ and $\Delta attB \cdot \Delta pseB4$ strains, respectively. It demonstrates that possible interaction between *attB* and *pseB*4 do not play any significant role in increasing of conjugation frequency of pSET152 into genomes of *S. albus* SAM1($\Delta attB$) and *S. albus* SAM2($\Delta pseB4$) strains.
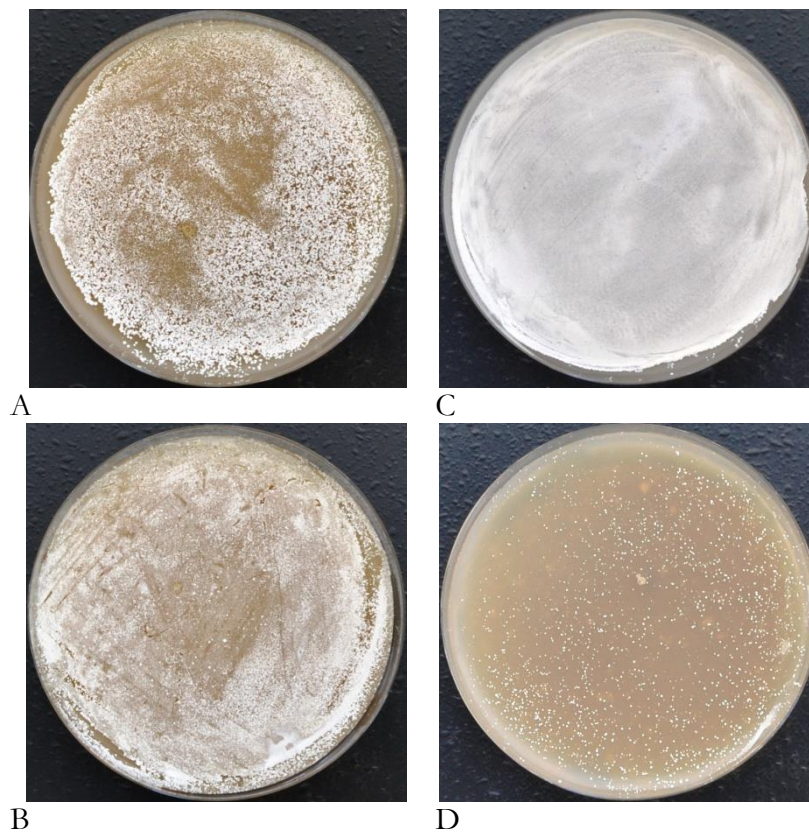
Figure 3.39. *S. albus* **J1074::pSET152-exconjugants (A),** *S. albus* **SAM2($\Delta pseB4$):: pSET152-exconjugants (B),** *S. albus* **SAM1($\Delta attB$)::pSET152-exconjugants (C) and** *S. albus* **SAM3($\Delta attB \cdot \Delta pseB4$)::pSET152-exconjugants (D) after 72 h of growth. Plates were overlaid with apramycin, 50 μg/ml, and with phosphomycin, 200 μg/ml, 14 hours after conjugation.**

3.3.2. Investigation of VWB attachment site

The identification of previously unknown site of φC31 phage integration in *S. albus* J1074 motivates us to test this issue for other bacteriophages in chromosome of this strain. To verify this idea, the attachment site of VWB phage, streptomycetes bacteriophage widely used in biotechnology, was analyzed. The VWB-based plasmid, pTOS, was introduced into the genome of *S. albus* J1074 by conjugation. The obtained exconjugants were grown on the MS-agar medium with apramycin, and then transferred into liquid TSB supplemented apramycin for stable plasmid maintenance. After the stationary phase was reached, serial dilutions of culture were plated on selective MS media, single colonies were obtained and chromosomal DNA of 8 independent clones were isolated and digested with *Nco*I. These samples were separated by agarose gel electrophoresis and transferred to the nylon membrane. The membrane then was blotted with the probe containing *aac(3)IV*.

Figure 3.40. Fragment of *S. albus* J1074 chromosome with VWB-phage attachment site and *Nco*I restriction sites.

Sequence of *S. venezuelae* 84 b. p. VWB *attB* was taken from Van Mellaert *et al.* 1998. As expected, BLAST analysis of *S. albus* J1074 genome showed that VWB *attB* site is located in tRNA^{Arg} gene (Fig. 3.40). Regarding this the expected size of hybridizing fragments generated by the integration of pTOS into the VWB *attB* site of *S. albus* J1074 were predicted. *Nco*I cuts inside of pTOS, so that the band visualized by Southern blot should contain 3,6 kb-fragment of the plasmid, including *aac(3)IV,* the right shoulder of *attP* and 1,4 kb genomic region to the left of integration point (Fig. 3.41.A).

A   B
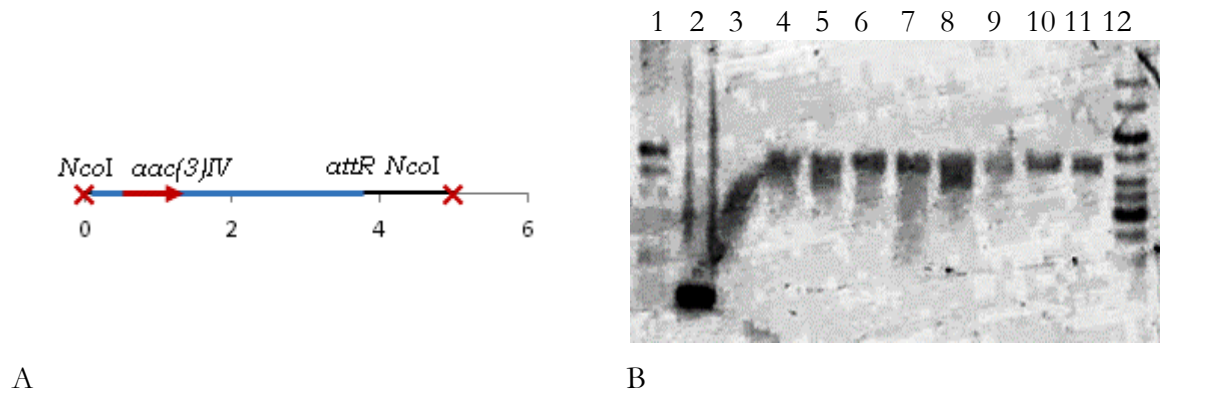
Figure 3.41. Analysis of pTOS integration into *S. albus* J1074. (A) Scheme of pTOS integration into *attB*. (B) Hybridization membrane with genomic DNA probed with fragment containing the *aac(3)IV* gene. Samples were loaded as follows: 1 – DIG marker, 2 – positive control, 3 – *S. albus* J1074, 4-11 – *S. albus* J1074::pTOS transconjugants, 12 – 1kb DNA ladder.

So, in the case of pTOS integration into the native *attB*, band of around 5,0 kb should be observed. In the case if the secondary *attB* site is present within the chromosome of *S. albus*, additional signals should be detected by hybridization experiment. After the hybridization, band of slightly smaller size then 5,0 kb was present in all probes except parental strain (used as negative control) and any other additional bands were observed (Fig. 3.41.B). According to these results, observed bands are corresponds to the fragment produced after integration of pTOS into *attB* of VWB. Any other additional attachment sites of this phage were detected in genome of *S. albus* J1074. Additionally, rescue plasmids from two *S. albus* J1074::pTOS-mutants were isolated and sequenced. According to sequencing results, pTOS integrated into predicted *attB* of VWB-phage.

4. DISCUSSION

Transposons became versatile tools for genetic manipulations and analysis of bacteria (Berg *et al.*, 1989; Weaden and Dyson, 1998; Petzke and Luzhetskyy, 2009, Damasceno, 2010; Bilyk *et al.*, 2012). They are used or potentially may find application for (i) creation of random knockout mutations; (ii) generation of gene/operon fusions to reporter functions; (iii) activation of cryptic genes or clusters by promoter insertions; (iv) locating primer binding sites for DNA sequence analysis; (v) up-regulation of genes involved in the biosynthesis of precursors or cofactors in natural products production by promoter insertions (Baltz 1992, 1993). Transposon's application is also intensively penetrating into such new fields as genomics and transcriptomics because the transposition can be used for establishing mutant libraries with random insertions and for elucidation of gene functions.

This work describes establishing of a random transposon mutagenesis system for streptomycetes. The system is based on the synthetic *Himar1* transposase gene and designed for *in vivo* application. The *Himar1* transposon does not require any host-specific factors for transposition (Lampe, 1996) and has low site specificity (Rubin, 1999; Maier, 2006). Despite it originating from an organism with the relatively high AT content, *Himar1* was able to transpose with almost 99% efficiency *in vivo* into the GC-rich chromosomes of *S. albus* J1074 and *S. coelicolor* M145.

Several novel regulatory genes of actinorhodin biosynthesis were identified by using the *Himar1*-based transpson system. The *Himar1* transposon mutagenesis was applied for a random insertion of the *gusA* gene and the aranciamycin biosynthetic cluster into the *S. albus* J1074 chromosome and, thus, helps to investigate the position effect of the expression of heterologous genes and clusters in this strain. Therefore, adaptation of *Himar1* for use in streptomycetes contributes a new tool for efficient investigation of these organisms.

While deleting *attB* site of φC31 from *S. albus* J1074 chromosome, intriguing pseudo-attachment site of this phage was discovered. Further investigations had shown that integration of φC31-based plasmids in this site is unpolar and unprecise.

4.1.   Current transposon mutagenesis systems available for streptomycetes

Several attempts were made previously to develop transposon mutagenesis systems for streptomycetes. These systems were based both on native streptomycetes transposons, and on transposons isolated from other species (Baltz *et al.*, 1997).

The native Tn*4556*transposon isolated from *S. fradiae* was used for transposon mutagenesis of its host strain, *S. lividans* and *S. coelicolor*, but the integration stability of this transposon remains unclear (Chung, 1987), and integrations of its derivative, Tn*4560* (Ikeda *et al.*, 1993), were not completely random. Also, introduction of Tn*4560* into the genome of *S. coelicolor* provokes instability near the native insertion sequence IS*1649* (Windenbrant and Kao, 2007). Isolated from *S. lividans* 66 IS*493* showed tendency to integrate into DNA regions with relatively low GC-content (Solenberg and Baltz, 1991). Several other transposons were developed from IS*493* (Baltz *et al.*, 1997), but their transposition frequency was 10^6-10^3 times lower than transposition frequency of Tn*4560* (Kieser *et al.*, 2000).

Among the transposons isolated from other organisms and adapted for use in streptomycetes is IS*6100*. It was isolated from *M. fortuitum* and used for mutagenesis in *S. avermitilis*. However, application of this transposon is limited due to its tendency to cointegrate whole plasmid into the genome. Furthermore, integration of IS*6100* into the *S. lividans* chromosome leads to genetic instability of the genome (Günes *et al.*, 1999). The derivative of Tn*5*, transposon Tn*5493* was employed for mutagenesis of *S. lividans* TK64 (Volff and Altenbuchner, 1997b). However, application of this system in other organisms was limited due to the use of the native AT-rich gene of the Tn*5*transposase, and therefore it cannot be expressed effectively in streptomycetes.

Recently developed transposon based on the synthetic gene of Tn*5* hypertransposase had been shown as an efficient tool for generation of transposon mutants in streptomycetes (Petzke and Luzhetskyy, 2009). It exhibits a high frequency of transposon mutagenesis and fast detection of integration loci. However, Tn*5* transposons have a slight tendency to integrate into GC rich sequences (Fernandez-Martinez, 2011). Derivative of Tn*5*, Tn*5062*, was used for *in vitro* mutagenesis of *S. coelicolor* cosmid library resulting in integration into approximately 6,5 thousands genes and a total of more than 35 thousands of insertions (Fernandez-Martinez, 2011).

4.2.   Advantages of *Himar1* transposon mutagenesis system
4.2.1.   Synthetic transposase gene

Genomes of different Actinomycetes representatives are well known for their high GC-content that can reach up to 70% (Sanli *et al.*, 2001). Furthermore, there is some evidence that codons containing G and C residues in the wobble position are more preferable for translation (Leswik *et al.*, 1991). It was shown that in *Streptomyces coelicolor* only one tRNA for the leucine-encoding codone TTA is present and it is encoded by *bldA*-gene (Kwak *et al.*, 1996). This gene controls the expression of nonessential genes, expressed in late growth phases, and thus is not critical for

viability. Other genes bearing TTA codons are poorly expressed and developmentally regulated (Craney *et al.*, 2006). Such preferences in codon usage can be limiting also for applicability of different heterologous genes, e.g. are the luciferase encoding reporter genes (Craney *et al.*, 2006) and the Flp recombinase encoding gene (Fedoryshyn *et al.*, 2008).

The most convenient way to express AT-rich heterologous gene in the high GC-content host is to design a synthetic gene, where all rare codons are replaced by synonymous ones with higher GC-content. Using this approach, synthetic *luxCDABE* gene cluster was successfully expressed in *S. coelicolor* after its GC-content was increased from 31% to 69% (Craney*et al.*, 2006).

The native *himar1*-gene has 56% AT-content and several AT-rich codons with A or T in a wobble position. To overcome this obstacle, the sequence of synthetic *himar1(a)*-gene was designed *in silico*, substituting codons enriched in GC residues and known to be frequently found in *S. coelicolor* genes. In addition, the codon usage was balanced in order not to overload particular tRNAs pool. The resulting gene had a GC content of 63%, which is satisfactory for *S. coelicolor*, as the designed *Himar1* transposon system showed >99% efficiency.

4.2.2.   Plasmids for transposon delivery

A wide range of vectors can be used for delivery of transposons into cells offers researchers flexibility of using different strategies and approaches for transposon mutagenesis.

The replicative vectors pALHim and pNLPr21 are derived from pNLHim, which in its turn is a derivative of pKC1139. This vector harbours temperature sensitive replicon, pSG5 (Muth*et al.*, 1995). So the plasmid could be maintained in the culture until required and then easily eliminated by simple increase of cultivation temperature up to more than 34°C. Such relatively long presence of the whole transposon mutagenesis machinery in the cell may cause instability of its genome, so the gene of transposase was placed under control of inducible promoter *tipA*p (Murakami *et al.*, 1989), whichprovides easy controllable gene expression. On the other hand, to ensure the high intensity of transposition we constructed pNLPr21, where the transposase gene was placed under the strong constitutive promoter Pr21 (Siegl *et al.*, 2012). The pSG5 replicon limits the application of pNLHim-based plasmids to strains where this replicon is maintained or strains which cannot grow at temperatures higher than 34°C. To circumvent these limitations the backbones of the vectors could be modified to accommodate other conditionally maintained replicons.

An additional option in such cases is the employment of suicide vectors. To establish such a system, expression of transposase gene should start immediately after entering of the vector into

the cell. This requires placing the transposase gene undercontrol of a promoter that ensures its rapid expression. We have used the promoter of the *φC31*integrase gene, which ensure the rapid expression of the *int*-gene for the efficient integration of the corresponding phage into the genome of actinomycetes. The first suicidal vector for transposon mutagenesis, p31Him, was constructed by Dr. Maksym Myronovkyi (Bilyk *et. al.*, 2013). It is based on the suicide pKCLP2 vector and contained *himar1(a)*-gene under control of the *φC31*integrase promoter and the hygromycin resistance gene, *hph*. The suicide vector pAHS is a derivative of p31Him where the hygromycin resistance was replaced with apramycin resistance cassette.

A minitransposon derived from pTn5Oks could be easily used with both *Himar1* and Tn*5* systems, as the region of minitransposon in this vector is flanked with both ME-sites and ITR-sites. Moreover, each vector for the transposon mutagenesis (pNLHim, pALHim, pNLTn5, pNLpr21 and pAHS) contains a unique blunt-end restriction site (*Eco*RV or *Pvu*II) for the insertion of the desired minitransposons. This design enables users to insert the variety of transposons with any tailored feature into any of above mentioned vectors.

The main method used for plasmids delivery into the streptomycetes cell is intergeneric conjugation from *E. coli* ET12567/pUZ8002. This process requires the presence of *oriT (origin of transfer)* within the vector to be transferred. Therefore, all our transposon delivery plasmids carry the *oriT*-site. The selection of the exconjugants carrying the vector is the next important issue. To provide this selection, for each plasmid two markers are required, expressed in both *E. coli* and streptomycetes. Different combinations of hygromycin, spectinomycin and apramycin resistant genes within the constructed plasmids are available giving the wide opportunity for application to various actinomycetes. As the number of efficient resistance markers for actinobacteria is limited, resistance tagging of mutants can significantly impair other genetic applications, such as complementation, double mutant isolation, or heterologous gene expression. This obstacle was overcome by placing the resistance genes between two *rox*-sites that allows removing of unnecessary resistance.

4.2.3. Mutagenesis workflow

All plasmids constructed for transposon mutagenesis were introduced into streptomycete by an intergeneric conjugation from *E. coli* ET12567::pUZ8002. This method of introduction was chosen because of its simplicity and no need to prepare and regenerate protoplasts. The non-methylating strain of *E. coli* was used to avoid the digestion of methylated plasmid by restriction enzymes of *S. coelicolor* M145 (Kieser *et al.*, 2000). A conjugation of replicative plasmids into

streptomycetes was around 10^{-6} — few thousands of exconjugants per dish. Such large starting number of exconjugants is ideal for the construction of a comprehensive transposon mutant library. In case of suicidal plasmid, frequency of the exconjugants was thousand fold lower, only 10^{-9}. Nevertheless, suicide plasmids are useful when small number of mutants for further analysis is required. Main advantage of conjugation with suicide plasmids is a simplified workflow (Fig. 3.19). While the isolation of single clones after the conjugation with a replicative plasmid requires additional cultivation and dilution steps, every clone obtained after the conjugation with suicidal plasmid is an independent transposon mutant and could be immediately analyzed or used in further experiments.

4.3. Integration of minitransposons into *S. albus* J1074 and *S. coelicolor* M145 chromosomes

4.3.1. Analysis of integration frequency

In their natural environment transposons could be characterized as genetic parasites. During ages of mutual development with their hosts transposons distributed their DNA among host's one and thus now are forming significant part of hosts genetic material. Interestingly, organisms did not develop any kind of more or less specific anti-transposon-protection system, as it was the case with other parasites. Apparently, inventing and supporting such a system would enhance evolutionary pressure affecting the species and would require more resources than carrying and supporting additional genetic luggage generated by transposon activity.

Main property which allowed transposons to successfully colonize genomes of their hosts was a specific selection of transposase proteins. Transposons containing highly-active transposase had no chance to survive, as high transposase activity led to instability of the host genome and, consequently, to death. This is why transposases in their natural environment are not highly active enzymes: their activity is sufficient to ensure some level of transposition activity, but insufficient to cause disintegration of host organism.

As transposons became an important genetic tool, the low transposition activity became a limiting factor of their application for genetic manipulations. Several successful attempts to increase activity of these elements were made (Lampe *et al.*, 1999; Baus *et al.*, 2005).

Combining of hyperactive transposase forms with the regulated promoters would give an ability to regulate expression of transposase gene and thus transposition frequency. It could be useful, as multiple insertions caused by the high frequency of transposition provides an advantage for phenotype screening. At the same time, the presence of two or more insertions complicates

identification of the locus responsible for a mutant phenotype. On the other hand, when screening for a specific mutant phenotype the high integration frequency would decrease the number of mutants to be screened.

A Southern blot analysis of *S. coelicolor* M145 mutants demonstrated that the basal level of gene expression from the *tipA* promoter is sufficient to cause single insertions into the genome, while induction of the promoter caused rise of mutants with multiple insertions. During transposon mutagenesis of *S. albus* J1074 the main problem was high sensitivity of this strain to thiostrepton which made impossible the promoter induction during an exponential phase of growth, when the majority of transposition events occur. To solve this problem, the *himar1(a)* gene was expressed froma strong constitutive promoter Pr21. It was expected that it should provide higher transposition frequency, but no rise of number of mutants with multicopy insertions was observed. After applying of the Tn*5*-transposase, mutants with two transposon insertions were isolated. One of the explanations is that, apparently, Tn*5*-transposase has less transposon specific regulation mechanisms that reduce its activity than *mariner* transposases (Reznikoff*et al.*, 1993; Hartl*et al.*, 1997).

4.3.2. Determination of integration loci

Equipping all minitransposons with RK6γ origin enable us to find the insertion loci of the transposon within a chromosome by cloning rescue plasmids. This method was previously applied in streptomycetes (Ou *et al.*, 2009) as well as in other prokaryotes (Lyell *et al.*, 2008; Sandmann *et al.*, 2009) and proved to be a convenient and quick way to map the insertion loci. *E. coli* TransforMax™ EC100D™ *pir*-116 electrocompetent cells express the π protein, necessary for activation of the RK6γ origin of replication. The chromosomal DNA of transposon mutants was digested with a restriction enzyme that cuts the chromosome with high frequency and has no sites within the transposon. The rescue plasmids were cloned by self-ligation of this mixture of DNA fragments, so one of these fragments contained the transposon and a part of a gene downstream of the sequencing primer binding site. This rescue plasmid could be easily isolated on the selection medium after transformation into *E. coli* Transformax™. After sequencing, the integration loci could be identified by BLAST-analysis.

The important conclusion from mapping of integration loci is that all mutated genes are not essential for growth or viability of *S. albus* J1074 and *S. coelicolor* M145 under the laboratory conditions.

4.3.3. Distribution of *Himar1* insertions

Accordingto the specific organisation of streptomycetes genomes, they contain essential genes in the central part of the linear chromosome and nonessential genes — in arms of the chromosome. Thus, it was expected that insertions might show the tendency to concentrate in the arms, as the mutants with insertions in the core would not survive. However, analysis of 38 transposon mutants of *S. albus* J1074 did not confirm this expectation - the insertions were distributed uniformly along the chromosome, and analysis of 48 *S. coelicolor* M145 transposon mutants demonstrates that most inserts occurred in the core region. Only 1 of 38 mutants of *S. albus* and 12 of 48 mutants of *S. coelicolor* M145 had insertions in arms regions. Furthermore, deeper analysis demonstrated preference of *Himar1* to insert into central part of chromosome in both analysed strains, *S. coelicolor* M145 and *S. albus* J1074 (Fig. 4.1). However, it can be explained by the fact that most transpositions occur when the chromosome is replicated and relative gene dosage of genes located closer to the origin of replication is higher than of those located in the arms, therefore probability of transposition into the first ones is higher.
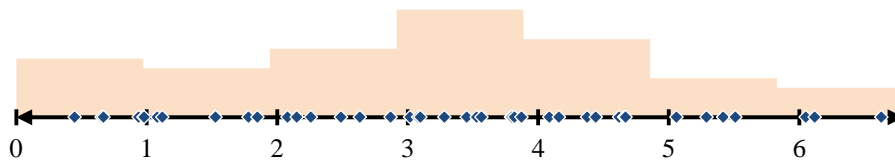
Figure 4.1. The final diagram of all transposon *Himar1* insertions identified for *S. albus* J1074. Blue rhombs represent transposon insertions; orange columns represent distribution of insertions (insertions oriented according to SSHG genes location).

Another interesting observation was made when the culture of *S. albus* J1074 transposon mutants was repeatedly cultivated at 28°C for 200 h, each 48 h 1 ml of old culture was transferred into new flask with TSB. Ten mutants after each such passage were isolated and insertion loci were identified. Mutants obtained after first passage all contained insertions in different loci, while in later passages we observed the higher number of mutants with the certain mutations. Possible explanation could be that the mutants that forced out all other mutants from the culture after continuous incubation had insertions that caused more intensive growth then other mutants. Deeper investigation of this phenomenon can help find mutants with the better fitness and determine genes responsible for this.

The half of analyzed mutants of second passage contained insertion in *sshg04808*.This gene encodes a regulator of two-component regulatory system. BLAST analysis showed that *sshg04808* is conserved among streptomycetes. Its locus tag in *S. coelicolor* M145 is *sco5749* and had been

already well characterized. This gene is called *osaB* (Bishop *et al.*, 2004) and is essential for morphological development when the organism is grown under the continual hyperosmotic growth conditions (Martinez *et al.*, 2009). The mutants of *S. coelicolor* with deleted *osaB* exhibit a bald phenotype and three to fivefold overproduction of actinorhodin and prodigiosin. However, previously any link between the response to osmotic stress and improved fitness was not detected: any deviations in growth intensity of *osaB*‾-mutants were not observed and their growth curves do not differ from those of wild type (Bishop *et al.*, 2004).

4.4. Determination of novel regulatory genes

The *S. coelicolor* M145 strain produces two antibiotics that are easily detectable due to their specific coloration: this is blue pigmented actinorhodin and red pigmented prodigiosin. Mutants obtained after transposon mutagenesis of *S. coelicolor* M145 differed by variety of phenotypes. To demonstrate the utility of the *Himar1* mutagenesis system, we have identified novel regulatory proteins involved in actinorhodin or prodigiosin production. Several mutants with abolished production of actinorhodin were identified (with insertions into *sco3390*, *sco3811*, *sco3919*, *sco4192* and *sco4199*).

Three of these mutants (*sco3390*, *sco4192* and *sco4199*) are overproducers of actinorhodin (Fig. 3.8). The *sco3390* encodes signal transducer of two-component signal-transducing system. Such systems allow organism to respond to changes in many different environmental conditions. (Mascher *et al.*, 2006). The genes *sco4192* and *sco4199* encode the hypothetical proteins so no suggestion about their functions could be made. Upstream of *sco4199* there are two potentially interesting genes, as they might be influenced by the polar effect caused by transposon. The first gene *sco4197*, encodes MarR-family regulator (multiple antibiotic resistance regulator). The regulators of this type are critical for control of response to antibiotic and oxidative stresses and catabolism of environmental aromatic compounds (Wilkinson and Grove, 2006). The second gene, *sco4198*, encoding DNA-binding protein, has been characterized previously (Hesketh *et al.*, 2007). It was demonstrated, that this gene may be significantly induced by ppGpp and suggested, that it plays a role in mediating the ppGpp-dependent rise in transcription of the *act*II-ORF4 regulator. The mutants with disrupted *sco4198* had reduced production of actinorhodin on certain media (Hesketh *et al.*, 2007).

Two other mutants carrying the transposons in *sco3811* and *sco3919* were reduced in their ability to produce actinorhodin. Downstream to *sco3811* is located a gene encoding gntR-family transcriptional regulator, *sco3812*. The regulators of this type act as environmental sensors and

thus control genes involved in responding to external stimuli. To gntR-regulators belong pleiotropic transcriptional repressor DasR and *agl3R* involved in the regulation of antibiotic production.The mutant with deleted *agl3R* fails to form spores and to produce blue pigment (Hillerich and Westpheling., 2006). Deletion of *dasR* gene also results in a bald phenotype and abolished antibiotic production (Rigali *et al.*, 2008). So it may be that disability of ΔSCO3811-mutant to produce actinorhodin is caused by the polar effect of the transposon on the *sco3812* gene. The gene *sco3919* encodes lysR-family transcription regulator. This gene is highly conserved among all streptomycetes and is called *abaB* (Scheu *et al.*, 1997). The first chromosomal fragment containing *abaB* was isolated from *S. antibioticus* ATCC11891, as it was able to stimulate actinorhodin and prodigiosin production in *S. lividans* TK21. When the promoter region of *abaB* gene was cloned in the high copy number into *S. lividans* TK21 it led to overproduction of both antibiotics (Scheu *et al.*, 1997).

Inactivation of the *sco3812*, *sco4192*, *sco4197* and *sco4198* genes by gene knock-outs in a clean genomic background led to the complete abolishment of actinorhodin production on the complex R2YE medium and to the activation of its production on the minimal medium. Adding of sucrose or glycerol into the minimal medium led to the abolishment of actinorhodin production. Thus, all four corresponding proteins act as activators of actinorhodin production if the strain grows on a rich media and as repressors if the strain grows on the minimal medium.

Inactivation of *abaB* did not cause abolishment of actinorhodin production on R2YE but led to activation of the production of this antibiotic on the minimal medium. Similarly, adding of sucrose or glycerol into the minimal medium impaired actinorhodin production. Thus, this gene acts as a repressor of actinorhodin production when the strain grows on the minimal medium. Also the increased biomass production in liquid medium was observed when compared to the wild type.

The obtained results also show that the genes *sco3812*, *sco3919*, *sco4192*, *sco4197* and *sco4198* are involved in a regulatory cascade sensing glycerol and glucose.

In contrast, the deletion of *sco3390* did not cause any influence on actinorhodin production, so probably this gene is not involved in any regulatory cascade responsible for actinorhodin production.

4.4.1. Actinorhodin biosynthesis and activity of *act*II-ORF4 promoter

Gene *act*II-ORF4 encodes a pathway-specific activator for actinorhodin production (Hesketh *et al.*, 2001). Fusion of its promoter with the *gusA* gene allows monitoring of the expression of *act*II-ORF4 in different mutant backgrounds. Interestingly, results of this monitoring demonstrated that GUS activity does not always correlate with actinorhodin production. For example, it was suggested, that *sco4198* may be involved in the signal transduction pathway involving *act*II-ORF4 (Hesketh *et al.*, 2007). In the mutant with inactivated *sco4198* no activity of the *act*II-ORF4 promoter on MM was observed while the blue pigment was produced. It may indicate that in this case the actinorhodin gene cluster was activated by another regulatory mechanism. In the ΔSCO4192 mutant the promoter of *act*II-ORF4 was very active on R2YE, but no actinorhodin production on this medium could be observed. It might indicate that actinorhodin biosynthesis is repressed on the level of mRNA translation.

In other mutants (with inactivated *sco3812*, *sco3919* and *sco4197*) activity of GUS correlated with actinorhodin production so in this case regulation mechanism involves *act*II-ORF4 gene.

4.4.2. Analysis of *S. lividans* 1326 transposon mutants

Unlike *S. coelicolor,* production of actinorhodin by *S. lividans* 1326 in the laboratory conditionsis blocked despite the corresponding gene cluster of this metabolite is present in its genome. Previously it was described that inactivation of polyphosphate kinase gene in *S. lividans* TK24 lead to accumulation of polyphosphates and activation of actinorhodin production (Ghorbel *et al.,* 2006a, b).

To demonstrate the utility of transposon mutagenesis for discovering novel genes in *S. lividans* which can launch production of actinorhodin, plasmid pALTEAm was introduced into *S. lividans* 1326. The advantage of pALTEAm over plasmids used previously for the identification of regulatory genes in *S. coelicolor* is a presence of two outward oriented promoters. Therefore, the transposon integration can result not only in the inactivation of a gene hitting it with transposon, but also in the overexpression of genes adjacent to the transposons' promoters.
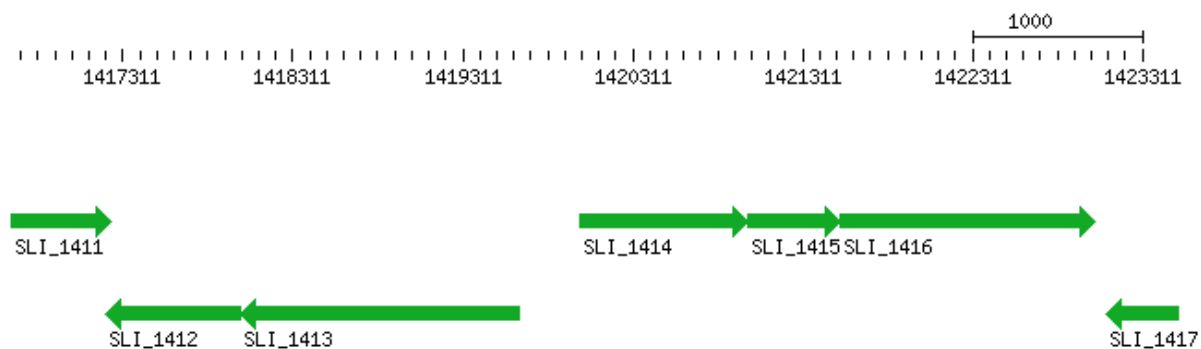
Figure 4.2. Location of genes involved in citrate metabolism in the genome of *S. lividans*. *sli_1412* encodes responseregulator CitB of citrate metabolism; *sli_1413* encodes signaltransduction histidine kinase CitA regulating citrate metabolism; *sli_1414* encodes TctCcitrate transporter; *sli_1415* encodes TctBcitrate transporter; *sli_1416* encodes TctAcitrate transporter.

Indeed the actinorhodin producing mutants were obtained after the mutagenesis with pALTEAm. The analysis of the transposon integration locus revealed insertion into *sli_1416* of *S. lividans* 1326. This gene belongs to the group of five genes compactly located on the chromosome and involved in citrate metabolism: *sli_1412* is a gene of response regulator CitB of citrate metabolism while *sli_1413* encodes a signal transduction histidine kinase CitA regulating citrate metabolism. Seemingly their products are the components of two-component regulatory system. Next three genes, *sli_1414*, *sli_1415* and *sli_1416* encode citrate transporter proteins, TctC, TctB and TctA, respectively (Fig. 4.2). According to the position of pALTEAm-transposon, activation of actinorhodin biosynthesis can be caused by the disruption of *sli_1416* or by the overexpression of two regulatory genes, *sli_1412* and *sli_1413*.

The link between citrate and actinorhodin production can be explained by the involvement of the first in the tricarboxylic acid cycle (Fig. 4.3). The first reaction of TCA cycle converts acetyl-CoA and oxaloacetate into citrate and coenzyme A. Acetyl-CoA is also a main precursor source for biosynthesis of actinorhodin (Baltz, 1998). In the previous experiments deletion of important genes of the TCA cycle enzymes also affected natural products production in *S. coelicolor* (Viollier *et al.*, 2006 a, b). Therefore, the inactivation of the citrate transporter gene or overexpression of the citrate metabolism regulators may cause shifts in TCA cycle and lead to the accumulation of intermediates common for TCA cycle and Act biosynthesis, and thus trigger actinorhodin production.
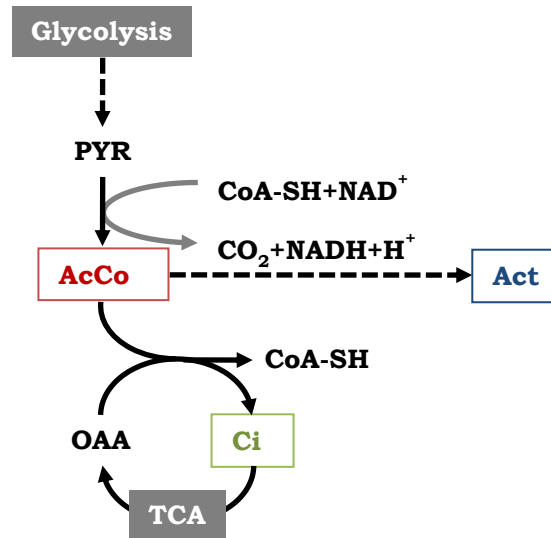
Figure 4.3. Fragment of primary carbon metabolism and relations between citric acid and production of actinorhodin. PYR – piruvate; AcCoA – acetyl-CoA; Cit – citrate; OAA – oxaloacetate; CoA-SH – coenzymeA; TCA - tricarboxylic acid cycle; Act – actinorhodin.

4.5.   Chromosomal position effect in *S. albus*-chromosome

In order to demonstrate the utility of the developed system, the depemdence of chromosomal location on the heterologous genes expression in streptomycetes was investigated.

The random integration of *gusA* and *lacZ* was published and this was achieved by asymmetrical ligation of random chromosomal fragments into a suicide vector (Thompson and Gasson, 2001) or by transposon mutagenesis (Sousa *et al.*, 1997). As the model organism used in this study, *S. albus* J1074, is widely used as host for heterologous expression of antibiotic biosynthetic clusters, we studied the impact of the position effect not only on a single reporter gene, but also on the entire antibiotic biosynthetic cluster. Examination of position effect using *gusA* expression level was simple, as only a slight modification of available plasmids was needed; it could be performed on wild type strain and in one conjugation-step.

A random integration of an entire antibiotic cluster would allow us to investigate the chromosome location effect not only on single gene but on a more complex transcription unit. However, integration of such a large construct is more laborious. Because of a large size of the chosen cluster (aranciamycin biosynthetic cluster, 35,9 kb) its introduction into the *S. albus* chromosome directly on the transposon could result in very low or even zero conjugation frequency. To circumvent this problem we combined a random transposon mutagenesis with the φC31-encoded site-specific recombination system.

4.5.1. Random introduction of *gusA* into *S. albus*-chromosome and analysis of integrations

The data described in the literature show that the major factors which may influence the *gusA* expression are: (a) gene dosage associated with distance to *oriC* (*gusA* integrated closer to *oriC* has higher relative gene dosage and therefore is expressed stronger); (b) strengths of local promoters (strong promoter located upstream to *gusA* may significantly increase its expression); (c) level of DNA compactization (DNA accessibility for RNA polymerases depends on its compactization). In order to study some of these factors in S. albus, we have modified available minitransposon. The reporter gene, *gusA*, was placed between two terminators of fd-phage. This terminator was shown to terminate transcription in both orientations (Gentz*et al.*, 1981) and therefore should prevent transcription of *gusA* from upstream and promoters (Gross and Hollatz, 1988). The gene of apramycin resistance, *aac(3)IV*, was also cloned in the orientation inverted to *gusA*, to avoid any read-through products from *aac(3)IV* promoter.

The *gusA*-gene was cloned under control of *ermE*p1, one of two promoters that form promoter region *ermE*p of *Saccharopolyspora erythraea* erythromycin resistance gene (Bibb *et al.*, 1994).

To be able to identify an insertion locus, the minitransposon was equipped with the R6Kγ-origin of replication. The plasmid pALHim was chosen as transposon-delivery vector, because of its simple workflow. In this case multicopy integrations should not be an obstacle, as in experiments with pNLHim and *S. albus* only mutants with single-copy insertions were obtained and it was expected that pALHim, as a derivative of pNLHim, will demonstrate the same behaviour. Standard procedures were used to generate the mutant library with random distribution of *gusA* and to identify insertion loci.

After measuring GusA-activity of 25 different mutants, 6-fold variation of activity had been observed. It demonstrates a presence of the chromosomal position effect in the *S. albus* chromosome. However, we could not find any correlations between the GusA-activity and different chromosomal factors (distance to *oriC*, local promoters strength). Interestingly, after isolating rescue plasmids we once again observed that mutants with certain insertions are more frequent in mutant pool. The mutants with insertions into intergenic region between genes of two predicted proteins, *sshg01734* and *sshg01735*, was isolated four times, mutants with insertion into *sshg02638* (conserved hypothetical protein), *sshg02810* (peptidase C14 caspase catalytic subunit) and *sshg04625* (urease subunit α1) were isolated two times each. In the previous chapter this phenomenon was explained from several perspectives: a) these mutants, as a consequence of gene inactivation, might have acquired benefits in growth over other mutants; or b) after

numerous cultivation steps, when only 1% of the culture is transferred to the next step, mutants pool is very limited. However, as two mutants with the same integration loci have been isolated in two independent experiments, we believe that it may be caused by its improved fitness. The mutant M08 contains the transposon insertion in the same locus (*sshg00779*). The *sshg00779* gene encodes glycerol kinase 1, an enzyme catalysing the formation of glycerol 3-phosphate from glycerol. It provides a way for glycerol to enter the glycolytic pathway. BLAST-analysis revealed that this gene is homologous to *sco1660* of *S. coelicolor*. In both strains these genes are part of glycerol-inducible, glucose-repressible *gylCABX* operon that consists of two genes encoding glycerol catabolic enzymes, glycerol kinase (*sco1660* and *sshg00779*), sn-glycerol-3-phosphate dehydrogenase (*sco1661* and *sshg00780*; Seno and Chater, 1983), and the membrane facilitator protein (*sco1659* and *sshg00778*; Fig. 4.4).
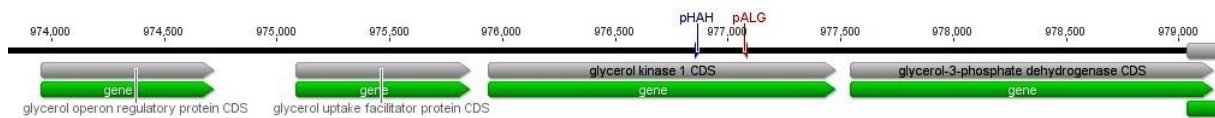
Figure 4.4. Organisation of glycerol-inducible glucose-repressible operon in genome of *S. albus*J1074 and integration points of minitransposons from pHAH (blue) and pALG (red).

4.5.2. Introduction of aranciamycin biosynthetic cluster into *S. albus*-chromosome at random locations

As mentioned above, because of the size of chosen antibiotic biosynthetic cluster, it was decided to introduce it into *S. albus* in two stages. Firstly, the mutant library with randomly distributed *attB*-containing transposons was generated and afterwards the cosmid, containing aranciamycin biosynthetic cluster, has been integrated via *attB*x*attP* recombination. The disadvantage of this strategy was the necessity to use two resistance markers, since we are limited in effective antibiotic selective markers for streptomycetes. On the other hand such two-steps strategy offered an advantage: mutants that would demonstrate high aranciamycin production could be reused for expression of any other cluster located on *attP*-containing vector.

The strategy also implied a construction of *S. albus*-*attB*-free recipient strain, as presence of two copies of cluster in a genome would complicate interpretation of obtained results. During deletion of the *attB* a secondary attachment site for φC31-based constructs was identified. These sites were stepwise replaced by the resistance cassettes, which were subsequently removed from the genome by site-specific recombinases.

During the first stage, which included transposon mutagenesis, it was decided to explore different variants of transposon delivery vectors for conjugation into streptomycetes. As mentioned above, we failed to isolate any *S. albus* transposon mutant with more than one transposon copy. One of the possible reasons of this observation might be insufficient activity of transposase-enzyme. To overcome the problem, the thiostrepton-inducible promoter was replaced by the strong constitutive promoter P21. The second variant implied replacing *himar1(a)*-gene with *tn5(a)*-gene, as the activity of the *Himar1*-transposase is suppressed by the *Himar1*-specific regulatory mechanisms in contrast to Tn*5* transposase. To simplify and speed up obtaining of transposon mutants a suicide vector for transposon delivery was constructed. The replicative plasmids can be used for the generation of comprehensive representative mutant libraries, while suicidal plasmids are preferable when only small number of mutants (up to 100) is required.

After integration of the cosmid, containing aranciamycin biosynthetic cluster, into genomes of obtained mutants, we were able to compare production levels of this antibiotic in different strains. As a result, after analysis of 26 mutants, any strain that produces aranciamycin in higher amounts than the wild type strain had been isolated (Fig. 3.31). Relocation of aranciamycin biosynthetic cluster through over the chromosome led mostly to significant decrease of aranciamycin production. Meanwhile, presence of two biosynthetic clusters in wild type strain gave 2-fold increase in production if to compare with Δ*attB* and Δ*pseB4* strains. Thus, the attempts to obtain aranciamycin were switched to introduction of additional aranciamycin clusters into genome of *S. albus* J1074.

4.6. Investigation of predominant secondary φC31 attachment site

In this work, a new secondary attachment site, *pseB4*, for integration of φC31-derived plasmids and cosmids was identified in the genome of *S. albus*J1074. BLAST analysis revealed that genomes of other streptomycetes do not contain sequences with the significant similarity to *pseB4* or to the gene where this site is located (*sshg03147*). Additionally, we identified two secondary attachment sites in the genome of *S. albus*J 1074, *pseB1* and *pseB3*, in the *sshg02502* (dihydropteroate synthase CDS) and *sshg02228* (aspartate aminotransferase CDS) genes, respectively.

The difference in conjugation frequency among the wild type, Δ*attB*, Δ*pseB4* and Δ*attB*·Δ*pseB4* strains demonstrates that, even though the insertion into *pseB4* was 29-fold less efficient than that into *attB*, it was still 300-fold more efficient than integration into the remaining pseudo-*attB* site in the *S. albus* J1074 genome. Comparison of *pseB4* with the secondary attachment sites of *S.*

coelicolor (Combes *et al.*, 2002) showed that higher activity of *pseB*4 could result from the significantly higher homology of this site than the other pseudo-sites to *attB*, e.g., 63% against 34%, 52% and 50% for *pseB*1, *pseB*2 and *pseB*3, respectively (38 bp of each site were analysed). This homology increases if only positions critical for synapse formation and DNA cleavage (Gupta *et al.*, 2007) are taken into account: 71% in *pseB*4 against 35%, 64% and 57% in *pseB*1, *pseB*2 and *pseB*3, respectively.

As mentioned above, φC31 integrase is able to control the direction of recombination (Thorpe *et al.*, 2000) and this control is ensured by the TT-core sequence in the centre of the *attB* and *attP* sites. Apparently, integrase is even able to synapse and activate strand exchange, even when the process cannot be completed because of mismatches in the core sequences (Smith *et al.*, 2004).

Previous studies of the integrase-*attB* interaction showed that the left shoulder of *attB* is the leading shoulder and it plays a greater role in *attB* activity than the right shoulder (Gupta *et al.*, 2007). Sequence analyses of *pseB*1, *pseB*2 and *pseB*4 indicated that their homology to *attB* in their left shoulders was slightly higher than that in their right shoulders (42% against 26%, 57% against 47% and 79% against 47% for *pseB*1, *pseB*2 and *pseB*4, respectively). Therefore, ability of the φC31-derived vectors to integrate into *pseB*4 in the inverted orientation can be explained by the finding that the right shoulder of *pseB*4 also has 71% homology to the left shoulder of *attB*. High homology of both shoulders can result in bidirectional integration.

Preliminary analysis of the *pseB*4 sequence revealed that the core region, where crossover should occur, consists of the TA dinucleotide (Fig. 3.35). Further analysis of the integration sequences demonstrated that, during the integration into *pseB*4, crossover indeed occurs at this position, but the point of cleavage can shift by one or two nucleotides. Breaks formed by the integrase at the *pseB*4 and *attP* sites are blunt; consequently, the sequences of *pseB*4 and *attP* do not overlap during rejoining of the DNA strands and formation of the hybrid sites. It was previously described (Qu *et al.*, 2012) that recombination between *attB* and pseudo *attP* in the bovine genome can be imprecise. However, in this case, the preciseness of the φC31 integrase can be influenced by the context of the mammalian genome. In case of *S. albus*, impreciseness of this enzyme, in terms of integration orientation and breakpoint formation, was demonstrated within the streptomycetes genome.

In summary, we identified a new *attB* site within streptomycetes at which integration occurs with a high frequency. The loss of integration polarity is very surprising for the native *attB* sites and reveals the previously unknown feature of the φC31 integrase to cleave the *att* sites at different positions. The availability of the two highly active *attB* sites in the *S. albus* genome at least partially

explains the ability of the strains to heterologously produce significant quantities of natural products, as demonstrated using the aranciamycin gene cluster.

4.7. Conclusions

At present the described above *Himar1*-based system for *in vivo* random mutagenesis is very efficient for streptomycetes and offers numerous genome enginerring possibilities.

Using this system, (a) transposon-mutant libraries for *S. albus* J1074 and *S. coelicolor* M145 were generated and locations of 44 insertions for each strain was identified; (b) after deeper analysis of *S. coelicolor* mutants with abolished actinorhodin production novel regulatory genes involved in the regulation of actinorhodin production were identified; (c) using randomness of transposon insertions the impact of chromosomal position on expression of heterologous genes had been explored and factors that may cause this effect were analyzed, it was shown, that any of analyzed factors (distance to *oriC* and others) had predominant influence on expression of heterologous genes; (d) using transposon mutagenesis additional attachment sites for φC31-based recombination system were introduced randomly into the *S. albus* genome and aranciamycin biosynthetic clusters were inserted using *attB×attP* recombination, analysis of aranciamycin production demonstrated that copy number of antibiotic biosynthetic clusters is important for increase of antibiotic production; (e) predominant pseudo-attachment site for φC31-based vectors was identified and explored.

Main advantages of this system over other transposon-based systems for streptomycetes are:

-stable translation of *himar1(a)* gene in genome of streptomycetes. It was insured by a modification of its sequence according to streptomycetes codon usage;

- usage of suicidal and pSG5rep-replicative vectors for transposon delivery ensures effective elimination of vector after mutagenesis and expands range of strains where the system can be applied;

- integration of R6Kγ origin of replication into transposon enabled rescue cloning and thus facilitated identification of insertion loci;

- integration of recognition sequences for site-specific recombination allows excision and reuse of antibiotic resistance markers;

- combination of the system with other genetic tools (reporter genes, phage-based recombination systems etc.) enriches arsenal for exploration of streptomycetes genomics and construction of antibiotic overproducers.

4.8.   Outlook for random transposon mutagenesis in streptomycetes

This dissertation represents development and application of system for random transposon mutagenesis in streptomycetes. Different minitransposons, based on *Himar1* and Tn*5*, were used to obtain mutant libraries with random insertions in *S. albus* J1074, *S. coelicolor* M145 and *S. lividans* 1326, to identify new regulatory genes, chromosomal position effect and construct host strains for antibiotic production.

Previous realization of these experiments in proposed manner in streptomycetes was impossible due to the lack of effective system for random transposon mutagenesis *in vivo*. The stable expression of developed system in streptomycetes is ensured by synthetic transposase gene that is modified according to streptomycetes codon usage. This method represents a convenient way to adopt genetic tools from other organisms in hosts with high GC-content. Requirement for new and highly effective system for transposon mutagenesis in streptomycetes is confirmed by the fact that after publication of our results (Bilyk *et al.*, 2012) around 30 work groups all over the world requested our plasmids with minitransposons.

5. APPENDIX

5.1. Sequences of *Himar1* transposase

5.1.1. Amino-acid sequence of *Himar1* transposase

```
  1  mekkefrvli kycflkgknt veaktwldne fpdsapgkst iidwyakfkr gemstedger sgrpkevvtd
 71  enikkihkmi lndrkmklie iaealkiske rvghiihqyl dmrklcakwv preltfdqkq qrvddsercl
141  qlltrntpef frryvtmdet wlhhytpesn rqsaewtatg epspkrgktq ksagkvmasv fwdahgiifi
211  dylekgktin sdyymaller lkveiaakrp hmkkkkvlfh qdnapchksl rtmakihelg fellphppys
281  pdlapsdffl fsdlkrmlag kkfgcneevi aeteayfeak pkeyyqngik klegrynrci alegnyve
```

5.1.2. Nucleotide sequence of *Himar1* transposase

```
  1  atggagaaga aggagttccg ggtcctgatc aagtactgct tcctcaaggg caagaacacc gtcgaggcca
 71  agacctggct ggacaacgag ttccccgact ccgccccggg caagtccacc atcatcgact ggtacgccaa
141  gttcaagcgg ggcgagatga gcaccgagga cggcgagcgc tccggccgcc cgaaggaggt cgtcaccgac
211  gagaacatca agaagatcca caagatgatc ctcaacgacc ggaagatgaa gctcatcgag atcgccgagg
281  ccctgaagat cagcaaggag cgggtcggcc acatcatcca ccagtacctg gacatgcgca agctgtgcgc
351  gaagtgggtc ccccgggagc tcaccttcga ccagaagcag cagcgggtcg acgactccga gcggtgcctc
421  cagctcctca cccggaacac cccggagttc ttccgccgct acgtcacgat ggacgagacg tggctccacc
491  actacacgcc cgagtccaac cggcagtccg ccgagtggac cgccaccggc gagcccagcc cgaagcgggg
561  caagacccag aagagcgccg gcaaggtcat ggcgagcgtg ttctgggacg cccacggcat catcttcatc
631  gactacctgg agaagggcaa gaccatcaac tcggactact acatggccct gctcgagcgc ctcaaggtcg
701  agatcgccgc caagcgcccc cacatgaaga agaagaaggt cctgttccac caggacaacg cccccctgcca
771  caagtccctc  cggacgatgg cgaagatcca cgagctgggc ttcgagctgc tgccccaccc gccgtactcc
841  ccggacctgg cccccctccga cttcttcctg ttctccgacc tgaagcggat gctggcgggc aagaagttcg
911  gctgcaacga ggaggtgatc gccgagaccg aggcctactt cgaggccaag ccgaaggagt actaccagaa
981  cggcatcaag aagctcgagg ccgctacaa ccgctgcatc gccctggagg gcaactacgt cgagt
```

5.2. Abreviations

A	adenine
aac(3)IV	apramycin resistance-imparting aminoglycoside acetyltransferase gene
aadA	spectinomycin resistance-imparting aminoglycoside adenylyltransferase gene
aphII	kanamycin resistance-imparting aminoglycoside phosphotransferase gene
bid.	bidistillled
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin

C	cytosine
Cre	cyclization recombination
DM	Davis minimal medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside-5´-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eppi	eppendorf tube
G	guanine
hph	hygromycin phosphotransferase
ITR	inverted terminal repeat
kb	kilo base
LB	Lennox broth
loxP	locus of X-over P1
M	Molar
Mb	mega base
ME	mosaic end
MS	mannitol-soy
OD_{600}	optical density at 600 nm wavelength of light
ori	origin of replication
oriT	origin of plasmid transfer
PCR	polymerase chain reaction
pSG5 rep	pSG5 temperature sensitive replicon
R6Kγ *ori*	origin of replication in *E. coli*
RNA	ribonucleic acid

RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
S. albus	*Streptomyces albus* J1074
S. coelicolor	*Streptomyces coelicolor* A3(2) M145
S. lividans	*Streptomyces lividans* 1326
SDS	sodium dodecyl sulfate
T	thymine
tcr	tetracycline resistance gene
tipA	thiostrepton inducible promoter gene
TRIS	tris(hydroxymethyl)aminomethane
TSB	tryptic soy broth
tsr	thiostrepton resistance gene
U	Units
WT	wild type

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