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METHODS AND PROTOCOLS

In vivo random mutagenesis of streptomycetes using mariner-based transposon *Himar1*

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Abstract We report here the in vivo expression of the synthetic transposase gene *himar1*(*a*) in *Streptomyces coelicolor* M145 and *Streptomyces albus*. Using the synthetic *himar1*(*a*) gene adapted for *Streptomyces* codon usage, we showed random insertion of the transposon into the streptomycetes genome. The insertion frequency for the *Himar1*-derived minitransposons is nearly 100 % of transformed *Streptomyces* cells, and insertions are stably inherited in the absence of an antibiotic selection. The minitransposons contain different antibiotic resistance selection markers (apramycin, hygromycin, and spectinomycin), site-specific recombinase target sites (*rox* and/or *loxP*), I-*Sce*I meganuclease target sites, and an R6Kγ origin of replication for transposon rescue. We identified transposon insertion loci by random sequencing of more than 100 rescue plasmids. The majority of insertions were mapped to putative open-reading frames on the *S. coelicolor* M145 and *S. albus* chromosomes. These insertions included several new regulatory genes affecting *S. coelicolor* M145 growth and actinorhodin biosynthesis.

Keywords *Himar1* · *Actinobacteria* · Transposon mutagenesis · Recombinases · Rescue plasmids

Introduction

Members of the order *Actinomycetales* are the most productive bacteria with respect to the synthesis of bioactive metabolites. These bacteria have been in the focus of industrial application for many decades, e.g., in the production of tetracycline, erythromycin, and vancomycin. Recent whole genome sequencing programs have revealed that the biosynthetic potential of *Actinomycetales* has been greatly underexplored with traditional approaches. A common feature of all actinobacterial genomes is a length of about 4–11 Mb and the presence of approximately 20–30 gene clusters encoding the synthesis of secondary metabolites. 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 functional genes characterization are of great importance.

Transposition became a powerful tool in genetic analysis, with applications in creating insertional knockout mutations, generating gene–operon fusions to reporter functions, providing physical or genetic landmarks for the cloning of adjacent DNAs, and locating primer binding sites for DNA sequence analysis (Damasceno 2010; Petzke and Luzhetskyy 2009; Weaden and Dyson 1998). The number and uses of transposons continue to expand into new fields such as genomics and transcriptomics. 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.

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Several in vivo transposon mutagenesis systems have been applied for actinobacteria, such as the native actinobacteria transposons IS*6100* (Weaden and Dyson 1998), Tn*4560* (Ikeda 1993), Tn*1792* (Herron 1999), or the foreign transposon Tn*5*. A recently developed transposon based on Tn*5* hypertransposase synthetic encoding gene has been shown as an efficient tool for the generation of random mutants in streptomycetes (Petzke and Luzhetskyy 2009). It combines very high transposon mutagenesis frequency with fast detection of insertion loci. Tn*5* transposon insertions are however slightly biased to GC-rich sequences (Fernandez-Martinez 2011). Therefore, using different transposon systems in addition to Tn*5* could minimize gaps in chromosome coverage attributed to insertion-site biases. The transposon we established for random in vivo mutagenesis in streptomycetes is a derivative of the transposon *Himar1*, a *mariner* family element isolated from the horn fly *Haematobia irritans*. The *mariner* transposon has a remarkable lack of host specificity as it does not require any host-specific factors for transposition (Lampe 1996). The *Himar1* transposon has previously been shown to have little site specificity in vitro and in vivo (Rubin 1999; Maier 2006). Apart from showing a preference for AT-rich DNA, *Himar1* only requires the presence of a TA dinucleotide for insertion (Craig 1997). Even in members of the actinobacteria with the highest GC contents, it is most likely that at least one TA dinucleotide is present in each gene. *Himar1* has been shown to transpose in distantly related insects, even more distantly related protozoa, vertebrate cells, and many Gram-positive and Gram-negative bacteria, while most transposons are limited to their own host range (Rubin 1999).

As of yet, in vivo application of the *Himar1* transposon in *Mycobacteria* showed randomness without recognizable sequence determinants (Rubin 1999). Highly efficient stable integrations were also reported in *Francisella tularensis*, suggesting that comprehensive transposon libraries could be generated with the procedure (Maier 2004). In vivo mutagenesis of *Burkholderia pseudomallei* was reported to be highly efficient with up to 44 % integration success (Rholl 2008). Use of a mutant *Himar1* transposase in *Escherichia coli* was also shown to be efficient by increasing the activity up to 50 times over the native transposase (Lampe 1999). For phenotypic analysis, a *Himar1* mutagenesis approach in *Leptospira biflexa* produced randomly distributed mutations which could be screened for phenotypes affecting several aspects of metabolism and physiology (Louvel 2005). It was also shown that transgenes flanked by inverted terminal repeats could be integrated into the *Myxococcus xanthus* genome in a *MycoMar* transposon, suggesting that *mariner* family elements could be used to deliver large gene clusters (Fu 2008). Nonetheless, there are no reports to date of successful application of *mariner* transposons in streptomycetes.

In this paper, we introduce a transposon mutagenesis system based on the synthetic *Himar1* encoding transposase gene under *Streptomyces* specific promoter. Several aspects make this system superior for generating mutant libraries among other applications: *Himar1* offers an unparalleled efficiency and randomness of the transposon insertions; the minitransposon is equipped with *loxP* and *rox* sites allowing excision of transposon resistance markers by *Cre* and/or *Dre* recombinase and their further reutilization in the same genetic background; pSG5 replicon allows to eliminate the replicative plasmid after mutagenesis; the presence of the R6Kγ origin of replication allows fast and easy insertion loci identification; possibility to generate transposon mutant libraries for single or multiple insertion mutants with a slight method modification.

Materials and methods

Bacterial strains and media

Streptomyces coelicolor M145 and *Streptomyces albus* J1074 were used to express the synthetic *himar1* gene. *E. coli* DH5α (Hanahan 1983) was used for cloning, and the non-methylating *E. coli* ET12567/pUZ8002 was used to drive conjugative transfer of non-methylated DNA to *Actinobacteria* as described previously (Luzhetskyy 2006). *E. coli* TransforMax™ EC100D™ pir-116 (Epicentre, Madison) was used for transfer and recovery of rescue plasmids derived from chromosomal DNA. Cultivation of all *E. coli* strains was performed as described previously (Sambrook 1989). For conjugation, *Streptomyces* strains were grown on mannitol soy flour agar plates (MS agar); for standard cultivation, *S. coelicolor* M145 and *S. albus* J1074 were either grown on HA agar plates or on R2YE agar plates. For liquid cultivation purposes, tryptone soy broth (TSB) was used. For antibiotic phenotyping R2YE, NL5 and minimal medium (MM) were used (Kieser 2000). All seed cultures were started from frozen mycelia stock and cultured in flasks with a 10-cm coil to promote dispersed growth. Apramycin, spectinomycin, and hygromycin were added to the media to final concentrations of 50, 100, and 100 μg/mL, respectively.

Plasmid construction

The plasmids used in this work are listed in Table 1. DNA manipulation and cloning were carried out according to standard protocols (Sambrook 1989). Plasmid constructs were confirmed by DNA sequencing. The synthetic gene *himar1*, flanked by *Hind*III and *Xba*I restriction sites, was synthesized by GenScript (New Jersey, USA) and provided on the plasmid p*Himar1*. This plasmid was digested with

*Hind*III and *Xba*I. The fragment containing the synthetic *himar1* gene was cloned into pAL1 and pNL1 (Fedoryshyn 2008a) to yield pALHim and pNLHim.

Four plasmids with different minitransposons were constructed, two containing the apramycin resistance gene *aac(3)IV*, one containing the spectinomycin resistance gene *aadA(1)*, and one containing the hygromycin resistance gene *hph* (pHTM, pHAM, pHSM, and pHAH, respectively). All plasmids contained the R6Kγ origin, and inserts were flanked on each side by ITR sites.

Construction of pHTM

The gene *aac(3)IV* was amplified from pIJ773 with forward primer 5′-acgtacc*gaattc*ggttcatgtgcagctccatcagc-3′ and the reverse primer 5′-acgtac*gaattc*atgagctcagccaatcgactgg-3′ containing *Eco*RI restriction sites. The PCR product was cloned into the plasmid pITRΔNheI (derivated from pITR, GenScript, New Jersey, USA) by *Eco*RI restriction sites, 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 ITR sites. This fragment was cloned into pALHim restricted with *Eco*RV, yielding pHTM (Fig. 1a).

Construction of pHSM

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

Construction of pHAH

The gene *hph* was amplified by PCR using pAL1 as a template, the forward primer 5′-ccccc*tctaga*gaataggaacttcg gaatagg-3′ with a *Xba*I site (in italics) and the reverse primer 5′-ccccc*caattg*g-ggtcgcagggcgtgcccttgggctccccgggcgcg taccgtatttgcagtaccagcgt-3′ with a *Mun*I site. The amplified fragment was cloned into pTn5Oks (ShineGene Molecular Biotech, Inc.) via *Mun*I and *Xba*I restriction sites leading to pTn5Oks*hph*. An *Eco*RV fragment was cut from pTn5Oks*hph* containing the *hph* gene and the R6Kγ origin, all flanked by ITR sites, and cloned into pNLHim restricted with *Eco*RV, yielding pHAH (Fig. 1c).

Construction of pHAM

The promoter of φ*C31*integrase was amplified from pSET152 (Bierman 1992) using the forward primer5′-aaaa gatctccccgtgccggagcaatcgc-3′ and the reverse primer 5′-aaaaagcttatgtcggcgaccctacgccc-3′. The PCR product was digested with *Bgl*II and *Hind*III enzymes and cloned into the respective sites of pKCLP2, yielding pKCLP2phiCpr. The promoterless transposase of the *Himar1* transposon was recovered as *Hind*III *BamH*I fragment from pHTM and cloned into the respective sites of pKCLP2phiCpr, yielding p31Him. The Himar transposon was excised from pITRΔNheIaac as an *Eco*RV fragment and cloned into the *Eco*RV site of p31Him to make pHAM (Fig. 1d).

Rescue plasmid generation and recovery

To generate rescue plasmids, genomic DNA was isolated from *S. coelicolor* M145 and *S. albus* J1074 wild-type strains and corresponding transposon mutants grown for 2 days at 28 °C in 30 mL TSB liquid cultures (Kieser 2000). The DNA was either digested with *Bam*HI, *Nco*I, *Not*I, *Pst*I, or *Sac*II and self-ligated with T4-DNA ligase. Where required, the DNA was transformed into *E. coli* TransforMax™ EC100D™ pir-116 electrocompetent cells by electroporation (*E. coli* pulser Bio-Rad™). Plasmids were recovered with the Wizard® Plus SV Minipreps DNA Purification System (Promega™). Chromosome–*Himar1* junction sequences were determined using the sequencing primer pMODfor (5′-ccaacgactacgcactagccaac-3′) for *S. coelicolor* M145 mutants and pTn5Oksfor (5′-attcaggctgcgcaactg-3′) for *S. albus* mutants.

Expression of Dre recombinase

Spores of *S. coelicolor* M145 transposon mutants were conjugated with the pUWL-Dre plasmid, containing the *Dre*-recombinase gene. Exconjugants were collected and inoculated to 100 mL TSB with 50 μg/mL thiostrepton and 200 μg/mL phosphomycin. Cultures were grown for 3 days. Aliquots were plated onto 50 μg/mL thiostrepton HA agar and grown for 3 days to sporulation. Spores were collected and inoculated to TSB with thiostrepton 2 days at 28 °C. Aliquots were plated onto MS agar with an appropriate antibiotic and grown for 3 days at 28 °C.

Southern hybridization of genomic DNA

Southern blot analysis was carried out on genomic DNA of *S. coelicolor* M145 and *S. albus* J1074 *Himar*1-transposon mutants. Genomic DNAs were digested with *Bam*HI for *S. coelicolor* M145 and with *Nco*I for *S. albus* J1074 mutants, separated by agarose gel electrophoresis, and transferred to a positively charged nylon membrane. A 0.8-kb *aac(3)IV* gene from pHTM (for *S. coelicolor* M145 mutants) and 0.4-kb R6Kγ origin from pTn5Oks (for *S. albus* mutants) were prepared and used as template for probe labeling with digoxigenin-dUTP by the random priming method. Hybridization, washing, and signal detection were carried out as described by Roche Diagnostics.

Table 1 Plasmids used in this work

Plasmid	Description	Reference or source
pSET152	Integrative vector for actinomycetes containing *oriT*, *int*, and *attP* (*phiC31*), *aac*(*3*)*IV*	(Bierman 1992)
pAL1, pNL1	Replicative vectors for actinomycetes containing pSG5-rep, *ori*T, and *tipA* promoter	(Fedoryshyn 2008a)
pUWL-Dre	Plasmid for inducing *Dre* recombination containing synthetic *dre*(*a*) gene under the *tipA* promoter, pSG5rep, and *ori*T	(Fedoryshyn 2008b)
pHP45Ω	Plasmid containing two antibiotic resistance genes with convenient restriction sites	(Prentki and Kirsch 1984)
pIJ773	pBluescript II SK-derivative containing *aac(3)IV* flanked with *FRT* sites	(Gust 2003)
pUZ8002	Non-transmissible mediator of intergeneric conjugation through *ori*T	(Blaesing 2005)
p*Himar1*	Synthetic plasmid with synthetic *Himar1* transposase gene	GenScript (NJ, USA)
pTn5Oks	pPCR derivative containing R6Kγ origin flanked by two ITR sites	ShineGene (PRC)
pALHim, pNLHim	Plasmids derived from pAL1 and pNL1, both containing *Himar1* transposase gene	This work
pITR	Synthetic plasmid with *Himar1* minitransposon with two *rox* and two *lox*P sites flanked by inverted terminal repeats	This work
pITRΔNheI	pITR derivative, with one *lox*P site	This work
pHTM	*Himar1* minitransposon with *aac*(*3*)*IV*, R6Kγ origin, flanked by inverted terminal repeats recognized by *Himar1* transposase	This work
pHSM	*Himar1* minitransposon with *aadA*(*1*), R6Kγ origin, flanked by inverted terminal repeats recognized by *Himar1* transposase	This work
pHAH	*Himar1* minitransposon with *hph*, R6Kγ origin, flanked by inverted terminal repeats recognized by *Himar1* transposase	This work
pHAM	*Himar1* suicide plasmide with *aac*(*3*)*IV* , R6Kγ origin, flanked by inverted terminal repeats recognized by *Himar1* transposase	This work

Results

Construction of the transposon vectors containing a synthetic Himar1 transposase gene

Actinobacteria usually have a GC content more than 70 %; therefore, their codon usage differs from that of most other bacteria. To circumvent the problem of inefficient translation of codons for which the corresponding amino-acyl-tRNA molecules are in limited supply or absent in, the genetic code of the transposase gene was modified in silico to make a synthetic *Himar1* transposase gene (GenScript, New Jersey, USA). Three vectors for its expression were constructed. The synthetic gene was cloned to pAL1 and pNL1, giving pALHim and pNLHim, respectively. pHSM and pHTM were obtained by cloning the apramycin and spectinomycin resistance genes linked to the R6Kγ origin flanked by ITR (transposase recognition sequence) sites to pALHim (Fig. 1a, b). pHAH was obtained by cloning the hygromycin resistance gene linked to the R6Kγ origin flanked by ITR sites to pNLHim (Fig. 1c). All plasmids carry the *oriT*, the thiostrepton-inducible promoter, *tipA* and the temperature-sensitive replicon, pSG5rep. It allows to transfer the plasmids from an *E. coli* host to different *Streptomyces* species by conjugation, to induce an expression of the transposase gene, and to select against the plasmids when the cultivation temperature is raised to 39 °C.

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, we used the promoter of the φ*C31* integrase from pSET152 since this integrative plasmid does not replicate, and without rapid expression of the integrase gene, it would be lost like a suicidal vector. We constructed pHAM based on the suicide vector pKCLP2. This vector contains a *Himar1* transposase encoding gene under the control of the φ*C31* 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 $10^{-3}$and 10^{-4} (based on input recipient spores). This means that the transposase gene under the φ*C31* integrase promoter expresses early enough to permit the transposition from the backbone of nonreplicative plasmid.

Himar1 mutagenesis of *S. coelicolor* M145 and *S. albus* J1074

We applied our system in *S. coelicolor* M145 with pHTM and pHSM. The plasmids were introduced into *S. coelicolor* M145 by intergeneric conjugation. The exconjugants were carried through several cultivation stages (Fig. 2). Aliquots of cultures were plated on HA agar plates, and spores were recovered and diluted to 10^{-7} and plated out again. Single colonies were obtained and transferred to HA agar plates

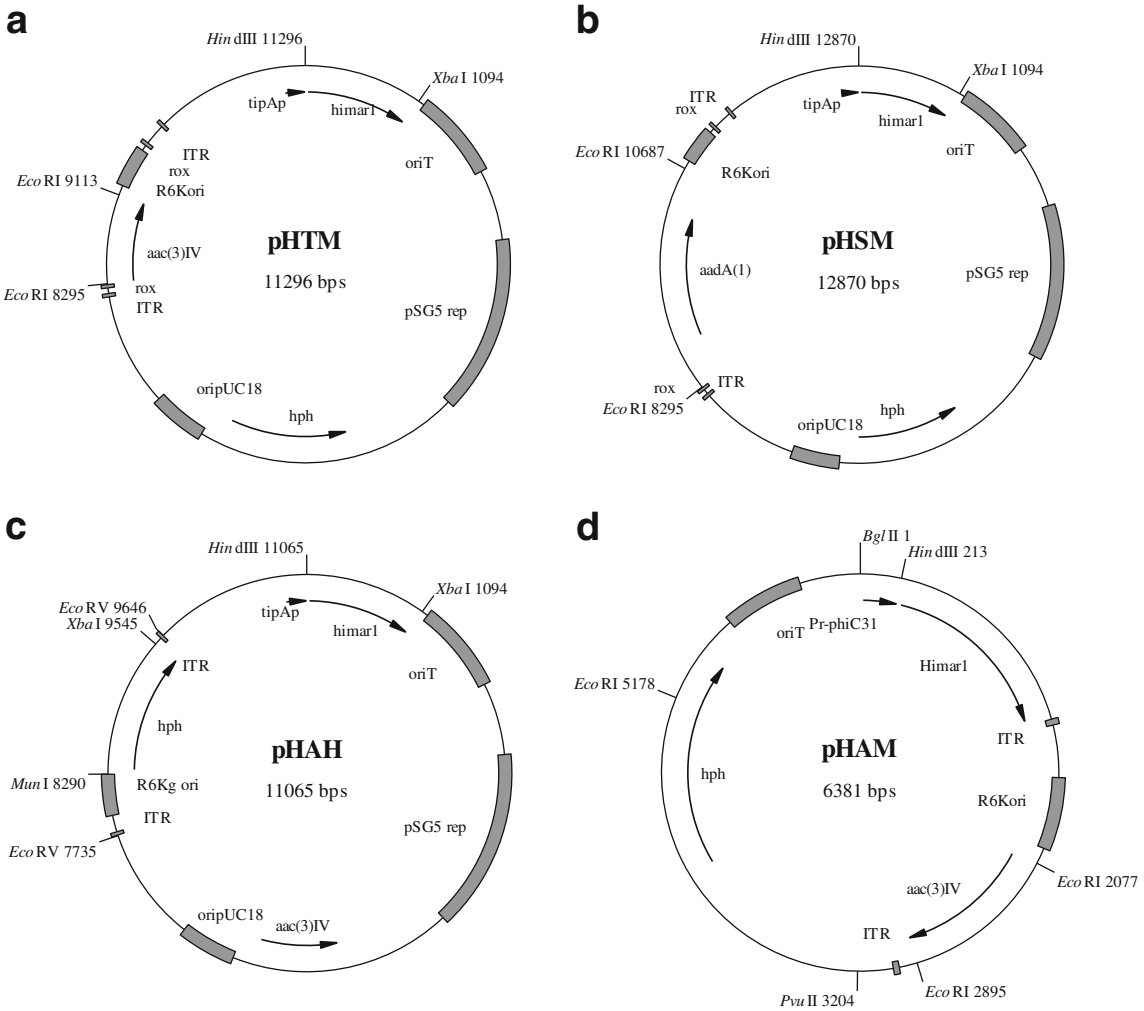

Fig. 1 Maps of four *Himar*1-containing plasmids. The plasmids contain the following shared features: ITR, inverted terminal repeat for *Himar1*; *oriT*, origin of plasmid transfer; *R6Kori*, origin of replication in *E. coli* for rescue plamids; *hph*, hygromycin resistance marker. Plasmids pHTM, pHAH, and pHAM contain *aac*(*3*)*IV*, apramycin resistance marker. pHSM contains *aadA*(*1*),spectinomycin resistance marker. Plasmids pHTM, pHSM, and pHAH contain *pSG5rep*, temperature-sensitive replicon in *Streptomyces*, and *tipAp*, thiostreptone-inducible promoter. Plasmid pHAM contains *Pr-φC31*, *φC31*integrase promoter. Plasmids pHTM and pHSM contain *rox*, recognition sites for *Dre* recombinase. Plasmids are not drawn to scale

containing apramycin and spectinomycin. Transposon mutants of *S. coelicolor* M145 were also plated to HA agar plates containing hygromycin and cultivated at 28 °C for 7 days. Of the 264 tested mutants with the apramycin resistance marker, a single colony was found to be hygromycin resistant. Eighty-eight mutants with the spectinomycin resistance marker were tested. All were hygromycin sensitive. To assess the stability of *Himar1* insertions, ten randomly selected apramycin-resistant mutants and two spectinomycin-resistant mutants were grown in liquid TSB cultures for approximately 100 generations in the absence of antibiotic selection. The cultures were plated onto their respective antibiotic R2YE agar and were all found to be antibiotic resistant. These results demonstrate the effectiveness of the synthetic hyperactive *Himar1*-based system for transposon mutagenesis. The same system of random transposon mutagenesis was applied in the commonly used heterologous host strain *S. albus* J1074.

Identification of the insertion loci and analysis of integration frequency

Rescue plasmids for *S. coelicolor* M145 and *S. albus* mutants were generated and sequenced to identify the loci of transposon insertion. Sequencing started from the transposon outwards into the genomic DNA. Thereby, approximately 800 bp of the connecting chromosomal DNA could be identified. Sequence data were used for BLAST analysis against the genomic DNA sequence of *S. coelicolor* A(3)2 and *S. albus* J1074 [Electronic supplementary material (ESM) Tables S1 and S2]. The transposon insertions have

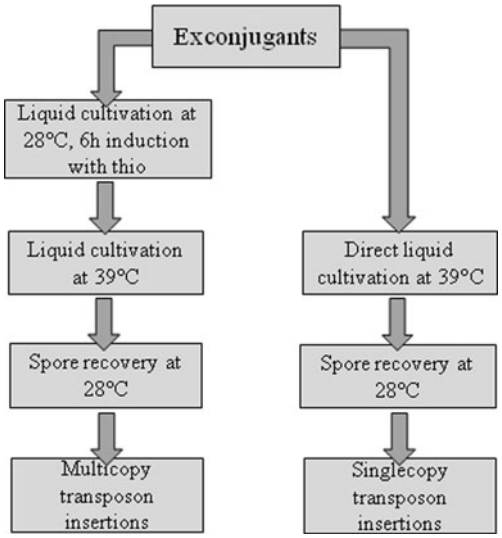

Fig. 2 Schematic diagram depicting the steps for obtaining a transposon mutant library of *S. coelicolor* M145 with pHTM/pHSM and *S. albus* J1074 with pHAH

been randomly distributed throughout the genomes of *S. coelicolor* M145 or *S. albus*.

Southern blot analysis was carried out to determine if a given transposon insertion was unique. All but one mutant of *S. coelicolor* M145 showed multiple transposon integration sites (Fig. 3a). Conversely, only one unique transposon integration site was detected for each of the *S. albus* J1074 mutants (Fig. 3c). This difference could be attributed to the inability to induce the thiostrepton-inducible promoter during the exponential growth phase of *S. albus*.

While multiple gene disruptions caused by multiple transposon insertions could give rise to new and interesting phenotypes or reduce the number of colonies, one would need to screen for a specific phenotype, the presence of two or more insertions in the genome of *S. coelicolor* M145 transposon mutants complicates relating an observed phenotype to the disrupted gene. We therefore developed a method to increase the number of mutants with unique transposon cassette in the genome (Fig. 2). Cultivation conditions optimized to cause loss of the plasmid in the early stage of cultivation increased the frequency of *S. coelicolor* M145 mutants with single insertion (Fig. 3b).

Excision of the apramycin resistance gene using *dre* recombinase

To assess *Dre*-mediated marker excision, selected apramycin resistance mutants were conjugated with pUWL-Dre containing a thiostrepton resistance marker, and *Dre* excision was performed as previously described (Herrmann 2012). As expected, apramycin-sensitive colonies were readily obtained with marker excision efficiencies reaching

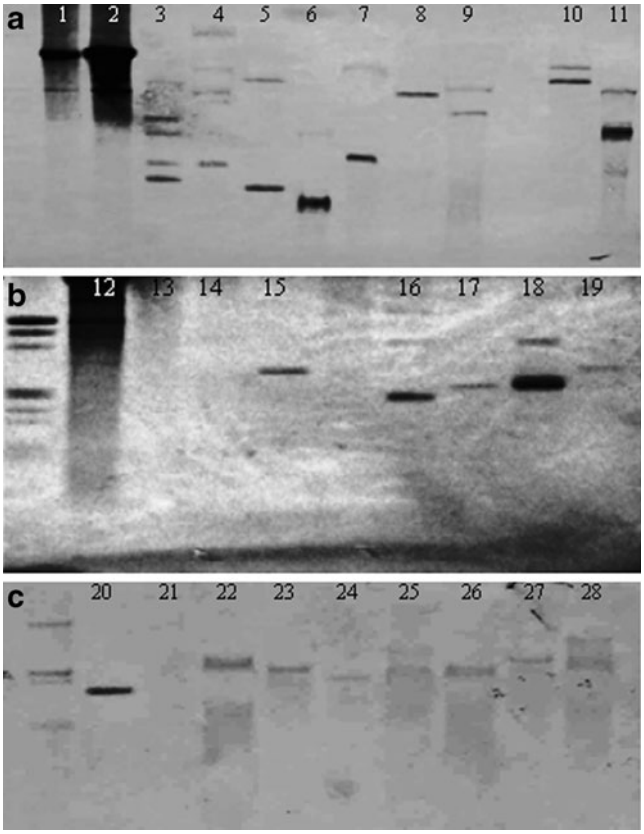

Fig. 3 Hybridization membrane after Southern blot hybridization of *Himar1*-mutants; *1*, *2*, *12* positive control (pHTM/*Nde*I); *20*, *29* positive control (pTn5Oks/*Hind*III); *13* negative control (wild-type *S. coelicolor* M145 DNA); *14*, *21* negative control (wild-type *S. albus* J1074 DNA); *3–11 S. coelicolor* M145 transposon mutants (*tipAp* induced); *15–19 S. coelicolor* M145 transposon mutants (*tipAp* uninduced); *22–28 S. albus* J1074 transposon mutants (*tipAp* induced)

100 %. All of the marker-free mutants were verified by PCR analysis.

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

After transposon mutagenesis, a mutant library with a variety of mutant phenotypes was obtained. Four transposon mutants of *S. coelicolor* M145 showing impaired actinorhodin production were selected for further analysis. Rescue plasmids were recovered from the mutants and sequenced. The BLAST analysis of four *S. coelicolor* M145 mutants revealed sequences identical to genes SCO3812 encoding a putative gntR-family transcriptional regulator, SCO4197 encoding a putative MarR family regulator, SCO4198 encoding a putative DNA binding protein, and to SCO4192 encoding a hypothetical protein (http://streptomyces.org.uk; Bentley 2002).The corresponding mutants showed a different phenotype on R2YE, MM, and NL5 agar plates compared to the wild type. To ensure that the observed phenotype was due to the

identified orfs (SCO3812, SCO4197, SCO4198, and SCO4192), we inactivated these genes via homologous recombination in a clean genetic background of *S. coelicolor* M145 (ESM Table S3). The obtained mutants of *S. coelicolor* M145 did not produce actinorhodin on the R2YE agar and overproduced this antibiotic on the MM and NL5 agar plates, in contrast to the wild-type strain (ESM Figs. S1 and S2). Actinorhodin production was blocked upon substitution of glucose in MM with sucrose or glycerol. Addition of glycerol to MM induced yellow pigment production by all mutants (ESM Fig. S1). In contrast to the wild type, all four mutants showed actinorhodin production on NL5 medium where glutamine was used as a carbon source. Addition of glycerol, glucose, or sucrose to the NL5 medium blocked actinorhodin production by all mutants (ESM Fig. S2).

Another two transposon mutants which were analyzed contained insertions in the SCO3390 and SC03919 genes, encoding for a putative two-component sensor kinase and putative LysR-family transcriptional regulator. We generated both knockouts via homologous recombination (ESM Table S3) yielding the strain *S. coelicolor* M145 B04 (with disrupted SC03919) and the strain *S. coelicolor* M145 A07 (with disrupted SCO3390). Both mutants showed slight actinorhodin overproduction on the RY2E agar, while *S. coelicolor* M145 B04 in contrast to the wild type and *S. coelicolor* M145 A07 produces actinorhodin on the MM (ESM Fig. S1). The 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.

The last transposon mutant showing impaired actinorhodin production contained insertion in the SCO5222 gene encoding a putative lyase. We have performed the respective gene inactivation experiments to prove the phenotypes (ESM Table S3). However, the mutant did not show any differences in antibiotic production (ESM Figs. S1 and S2). Obviously, actinorhodin production impairment was caused by some additional insertions.

Discussion

Implementing a transposon mutagenesis system generally requires optimization of several parameters. To obtain an efficient transposon system, we combined an optimized synthetic gene with high GC content encoding the *Himar1* transposase and several resistance markers as minitransposons. The availability of hygromycin, spectinomycin, and apramycin resistant genes within minitransposons might be important when working with different actinobacteria being resistant to some of those antibiotics. There is a limited choice of efficient resistance markers which can be used for genetic manipulation of actinobacteria. Therefore, apramycin, hygromycin, and/or spectinomycin resistance tagging of mutants can significantly impair downstream genetic applications such as complementation, double-mutant isolation, or heterologous gene overexpression in the same genetic background. This obstacle was overcome by flanking the minitransposons with a *Dre* recombinase-excisable resistance marker.

The development of the *Himar1* random mutagenesis system with the R6Kγ origin has a distinct advantage over other transposon systems that do not offer the possibility to replicate rescue plasmids in a foreign host. Rescue plasmid sequencing proved to be a quick and convenient way to map several insertion loci. TransforMax™ EC100D™ *pir*-116 electrocompetent *E. coli* cells constitutively express the π protein (*pir* gene product) for replication of plasmids containing the R6Kγ origin of replication. Upon digestion of *Streptomyces Himar1* mutant chromosomal DNA, several fragments of various sizes were formed. The enzyme used was confirmed to not cut within the antibiotic resistance marker or any other part of the transposon that would disrupt rescue plasmid replication or sequencing. Given the frequency of *Sac*II sites in the chromosome (on average every 570 bp), ligated rescue plasmids were statistically small and efficient to transform. The fragment containing the transposon and a piece of gene downstream of the sequencing primer binding site was simply ligated end to end to give a rescue plasmid that could be efficiently transformed to *E. coli* TransforMax™. Naturally, plasmids of several different sizes and religation possibilities are present, but only those with an R6Kγ origin are replicated providing an opportunity to identify a transposon insertion locus. The *Himar1* minitransposon system is not only an efficient tool for identifiable random mutagenesis (Fig. 4) but also a means of delivering useful markers or recognition sites into the chromosome.

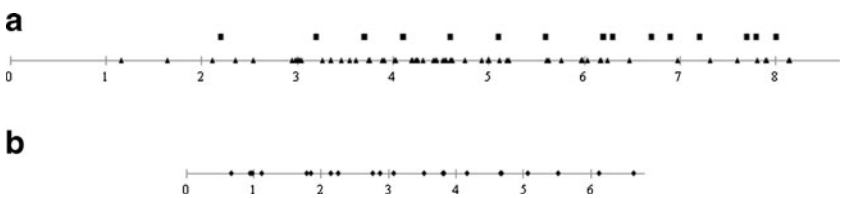

Fig. 4 Distribution of loci of insertion for *Himar1* (*triangles*) and Tn*5* (*squares*; Petzke and Luzhetskyy 2009) transposons in the *S. coelicolor* chromosome (**a**) and for *Himar1* (*rhombs*) transposons in the *S. albus* chromosome (**b**)

The *Himar1* minitransposons were designed with unique integrated *loxP* and I-*Sce*I sites. Introducing *loxP* sites into the chromosome generates several possibilities to experiment with multigene recombinations using the Cre recombinase (Fedoryshyn 2008b), while the presence of I-*Sce*I sites provides the possibility to facilitate homologous recombination in a certain locus (Siegl 2010). Transposon delivery vectors are very important during transposon mutagenesis. So far, only replicative vectors were used for introduction of transposons in *Streptomyces* cells. This has a few serious drawbacks, which reduce efficacy of mutagenesis dramatically. One of such problems is an early transposition effect. This problem arises due to the absence of a tight regulation of transposase gene expression and a low frequency of transposition vector delivery in *Strepomyces* cells. After the vector was introduced into the cell, the transposons jump into the chromosome even without induction of the transposase gene expression, as even a low level of its expression is sufficient for transposition. In the worst case, the number of individual mutants after mutagenesis will correspond to the number of transposon vector containing clones used in mutagenesis, or all the clones will have several transpositions among which one will be the same in most clones. Another problem arises when two subsequent transposon mutagenesis have to be applied. After the first round of mutagenesis, the vector should be cured in order to introduce the new vector with a new transposon. To avoid this unnecessary step, we have delivered the *Himar1* transposon into the cells on the suicide vector pKCLP2. In this case, every clone arising after conjugation is an independent transposon mutant. Using a suicidal vector solves the problem with early transposition events, multiple insertions of transposons, and vector curing.

By Southern blot analysis, the general frequency of insertion into the genome of *S. coelicolor* M145 for the *Himar1* transposon was determined. We observed both multiple and single transposon insertions, depending on the conditions during *Himar1* library preparation. Each method has its distinct advantages. Multiple insertions caused by high-frequency transposition present advantages for phenotype screening and discovery. A combination of more than one insertion could give rise to phenotypes like antibiotic overproduction that would otherwise not be seen with a single insertion. On the other hand, the presence of two or more insertions complicates the identification of a targeted DNA locus responsible for the mutant phenotype. Alternatively, where a specific phenotype is desired in order to discover the gene mutation responsible for it, multiple insertions would make it more likely that the applicable gene mutation occurred, reducing the number of colonies that would have to otherwise be screened. Having a single insertion is important for linking a mutant phenotype with the certain targeted gene.

To demonstrate the usefulness of our system, we identified several regulatory proteins involved in actinorhodin and prodigiosin biosynthesis. The inactivation of the first group of genes (SCO3812, SCO4197, SCO498, and SCO4192) leads to the complete abolishment of the actinorhodin production on rich R2YE medium and to activation of its production on minimal medium. Thus, all four corresponding proteins act as activators of antibiotic production if the strain grows on rich media, and as repressors if the strain is grown on minimal medium. Addition of sucrose and glycerol diminished the stimulatory effect of the mutations on actinorhodin production. This shows that the proteins encoded by SCO3812, SCO4197, SCO4198, and SCO4192 are part of the regulatory cascade sensing these two carbon sources.

In summary, we have demonstrated that the modified *Himar1* transposon generates random mutants in *Streptomycetes* with a high efficiency in vivo. The presence of the R6Kγ origin of replication in the transposon enables rapid identification and cloning of transposon insertion sites in rescue plasmids. Due to the *rox* sites flanking the resistance marker of the transposon, it is possible to remove the marker efficiently from the chromosome and reutilize it in further experiments. To demonstrate the usefulness of our transposon system, we identified four novel regulators responsible for actinorhodin production in *S. coelicolor* M145. In addition, the mutants were shown to be genetically stable. It is very probable that this transposon will be active in many bacterial strains containing GC-rich DNA (other *Actinobacteria*, myxobacteria) and will serve as an efficient system for different forward genetic approaches.

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