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Genetic engineering in *Actinoplanes* sp. SE50/110 – development of an intergeneric conjugation system for the introduction of actinophage-based integrative vectors

Tetiana Gren[a], Vera Ortseifen[b], Daniel Wibberg[b], Susanne Schneiker-Bekel[b], Hanna Bednarz[c], Karsten Niehaus[c], Till Zemke[d], Marcus Persicke[a], Alfred Pühler[b], Jörn Kalinowski[a,*]

[a] *Microbial Genomics and Biotechnology, Center for Biotechnology, Bielefeld University, Universitätsstraße 27, 33615 Bielefeld, Germany*
[b] *Senior Research Group in Genome Research of Industrial Microorganisms, Center for Biotechnology, Bielefeld University, Universitätsstraße 27, 33615 Bielefeld, Germany*
[c] *Department of Proteome and Metabolome Research, Faculty of Biology, Bielefeld University, Postfach 100131, D-33501 Bielefeld, Germany*
[d] *Product Supply, Bayer Pharma AG, Friedrich Ebert Str. 217-475, 42117 Wuppertal, Germany*

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ABSTRACT

The α-glucosidase inhibitor acarbose is used for treatment of diabetes mellitus type II, and is manufactured industrially with overproducing derivatives of *Actinoplanes* sp. SE50/110, reportedly obtained by conventional mutagenesis.

Despite of high industrial significance, only limited information exists regarding acarbose metabolism, function and regulation of these processes, due to the absence of proper genetic engineering methods and tools developed for this strain. Here, a basic toolkit for genetic engineering of *Actinoplanes* sp. SE50/110 was developed, comprising a standardized protocol for a DNA transfer through *Escherichia coli-Actinoplanes* intergeneric conjugation and applied for the transfer of ϕC31, ϕBT1 and VWB actinophage-based integrative vectors. Integration sites, occurring once per genome for all vectors, were sequenced and characterized for the first time in *Actinoplanes* sp. SE50/110. Notably, in case of ϕC31 based vector pSET152, the integration site is highly conserved, while for ϕBT1 and the VWB based vectors pRT801 and pSOK804, respectively, no sequence similarities to those in other bacteria were detected.

The studied plasmids were proven to be stable and neutral with respect to strain morphology and acarbose production, enabling future use for genetic manipulations of *Actinoplanes* sp. SE50/110. To further broaden the spectrum of available tools, a GUS reporter system, based on the pSET152 derived vector, was also established in *Actinoplanes* sp. SE50/110.

1. Introduction

Actinobacteria are Gram-positive filamentous soil bacteria that have a complex life cycle, embodying several stages of morphological differentiation. They produce many bioactive compounds as secondary metabolites, such as antibiotics, antitumor agents, immunomodulators, anthelmintic and insect control agents (Kieser et al., 2000).

Actinoplanes sp. SE50/110 belongs to so called, "rare actinomycetes" and is known for its ability to produce acarbose, which is one of its prominent secondary metabolites. Acarbose is mainly used for treatment of type II diabetes mellitus, but also can be regarded as a prevention drug. It helps patients with starch- and sucrose-containing diets delaying the digestion of ingested carbohydrates in the human intestine, thereby resulting in a smaller rise in blood glucose concentration (Puls and Keup, 1973; Truscheit et al., 1981). Structurally, acarbose can be characterized as a pseudotetrasaccharide, which consists of an unsaturateded cyclitol, 4-amino-4,6-dideoxyglucose and maltose (Wehmeier, 2003).

The extensive body of data on *Actinoplanes* sp. SE50/110 genomics, proteomics and transcriptomics was obtained in recent years. In 2011 the complete genome of *Actinoplanes* sp. SE50/110 was sequenced, analyzed and annotated. It was revealed that the genome with a high G + C content (71.32%) consists of one circular chromosome with a size of 9,239,851 bp hosting 8270 predicted protein coding sequences (Schwientek et al., 2012; Schwientek et al., 2013; Schwientek et al., 2014).

* Corresponding author.
E-mail address: Joern.Kalinowski@Cebitec.Uni-Bielefeld.DE (J. Kalinowski).

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In 2012, the first whole transcriptome analysis of *Actinoplanes* sp. SE50/110 using RNA sequencing technology for comparative gene expression studies between cells grown in minimal and complex growth media was conducted (Schwientek et al., 2013).

The *acb* gene cluster has been sequenced, cloned and found to consist of 22 genes, some of which were expressed and studied in heterologous conditions (Zhang, 2002; Wehmeier 2003; Hemker et al., 2001).

Also, studies on extra and intracellular proteome of the strain *Actinoplanes* sp. SE50/110 (Wendler et al., 2013, 2015, 2016) as well as metabolomics studies (Wendler and Ortseifen et al., 2014) were conducted. Based on these analyses and other studies, several models of acarbose metabolism were proposed (Zhang, 2002; Wehmeier 2003; Wendler et al., 2013; Wendler and Ortseifen et al., 2014).

Various *Actinoplanes* sp. SE50/110 strain derivatives, which are being used for industrial acarbose production, were reported to be exclusively obtained via mutagenesis and stepwise selection of overproducers. Although this strategy was proven to be effective in the past, it is certainly limited. Genetic engineering of the producer strain can be utilized as an alternative approach in this case. However, up to date there is no information regarding any genetic manipulations of *Actinoplanes* sp. SE50/110 and the genus *Actinoplanes* is described as a challenging object for any genetic manipulation (Vobis 2006).

Regarding the DNA uptake system, a broad choice of methods, available for Actinobacteria, exists. Among them, protoplast transformation is the route usually followed for *Streptomyces* spp. However, conditions for protoplast formation and regeneration have to be empirically determined in a set of laborious experiments and are known to be species- or even strain-specific (Marcone et al., 2010a,b). This approach was applied with limited success only for few non-*Streptomyces* strains (Marcone et al., 2010a,b). Intergeneric transfer of plasmid DNA from *E. coli* to *Streptomyces* spp. was reported first by Mazodier et al., 1989. Effective conjugation systems have since been developed for many actinomycetes, in which the incoming plasmid can be maintained episomally or integrated into the host chromosome by site-specific or homologous recombination (Stinchi et al., 2003).

Up to now, there are only two representatives of *Actinoplanaceae*, *Actinoplanes friuliensis* and *Actinoplanes teichomyceticus*, for which gene cloning systems were successfully developed and implemented being based on intergeneric conjugation strategies (Heinzelmann et al., 2003; Ha et al., 2008). Particularly prominent progress in establishing the genetic system was made for *A. teichomyceticus*, producer of lipoglycopeptide antibiotic teicoplanin. The procedure of intergeneric conjugation was used to transfer a set of commonly used integrative and replicative vectors to *A. teichomyceticus* cells. Later on, the above mentioned vectors were used for overexpression of two regulatory genes in a wild type strain, which led to the increase in teicoplanin production (Horbal et al., 2012; Ha et al., 2008).

Actinomycete bacteriophages or actinophages are frequently being used as a source to develop integrative vectors. Among the most well studied and used temperate actinophages are ϕC31 (Lomovskaya et al., 1972) and ϕBT1 (Gregory et al., 2003). Both of them use large serine recombinases for site specific integration without requiring any additional host or phage functions. Recombination occurs between specific *attP* and *attB* regions, which frequently have sequence similarity, resulting in a formation of hybrid *attL* and *attR* regions (Baltz, 2012). Importantly, these bacteriophages use unrelated chromosomal genes for their integration: *attB* sites for ϕC31 are located in homologs of genes encoding a pirin-like protein and ϕBT1 *attB* sites are contained in the coding regions of integral membrane proteins (Baltz, 2012; Combes et al., 2002).

The temperate bacteriophage VWB was originally isolated from *Streptomyces venezuelae* and characterized as having a narrow host range. It integrates into the host chromosomes by recombination with *attB* locus, similar to ϕC31 and ϕBT1. Till nowadays, *attB* sites of VWB were characterized only for two strains: *Streptomyces ghanaensis* and *S. venezuelae* (Ostash et al., 2009; Van Mellaert et al., 1998; Van Dessel et al., 2005). However, VWB-based integrative vectors were proven to be effective also in one representative of *Actinoplanes*, namely *A. teichomyceticus* (Horbal et al., 2012).

Use of reporter genes gained more attention in the last decades as a promising approach to study gene regulation and for the visualization of multiple biological processes directly *in vivo*. These genes are fused to various regulatory sequences, and introduced into a strain of interest. The signal detected from the expression of reporters can be quantified, which gives the possibility to study the effect of various physiological conditions, stresses and other factors (Myronovskyi et al., 2011). The β-glucuronidase enzyme (GUS) hydrolyzes several β-glucuronides. The GUS reporter system appeared to be highly sensitive due to the stability and high specific activity of the GUS enzyme (Myronovskyi et al., 2011). It was successfully applied also in one representative of the *Actinoplanes* genus, *A. teichomyceticus* (Horbal et al., 2013).

In this paper, we present the development of a gene cloning system for *Actinoplanes* sp. SE50/110. Namely, the selection of appropriate solid media, the determination of antibiotic resistance spectra, the development of the *Actinoplanes* – *E. coli* conjugation procedure, and its application for transfer of three different integrative vectors are described. Finally, the GUS reporter system was applied in *Actinoplanes* sp. SE50/110 using a pSET152 derivative as vehicle for genetic engineering.

2. Methods

2.1. Strains, media, growth conditions and reagents

To maintain and isolate plasmid DNA, *Escherichia coli* DH5αMCR strain was used as a host. *E. coli* ET12567 pUZ8002 (*dam*-13:*Tn*9, *dcm*-6, *hsdM*, *hsdS*) (Kieser et al., 2000) was used for intrageneric conjugation with *Actinoplanes* sp. SE50/110 (ATCC 31044). The site-specific integration vectors, pIJ6902 (7.4 kb) and pSET152 (5.7 kb), pSOK804 (5.5 kb), pRT801 (5.2 kb), contain ϕC31, VWB and ϕBT1 *int* and *attP* genetic regions respectively. All of the vectors contain and *oriT* of RK2, as well as an apramycin resistance gene for selection in actinomycetes and *E. coli* (Bierman et al., 1992). These plasmids do not contain the replicative functions and can be maintained in recipient strains only in the chromosomally integrated state.

Plasmids pSET152, pSOK804, pRT801 and pIJ6902 were received from B. Ostash (Ivan Franko National University of Lviv, Ukraine).

pSETGUS (7.7 kb) vector is based on pSET152, contains an apramycin resistance gene for selection in actinomycetes and *E. coli*, ϕC31 *int* and *attP* genetic regions and *gusA* gene, cloned under *tipA* promoter. pSETGUS was received from A. Luzhetskyy (Saarbrücken, Germany) (Myronovskyi et al., 2011).

All cultivations of *E.coli* strains were done in LB-media, prepared as described in Kieser et al., 2000. The growth conditions were 37 °C and 180 rpm in a GFL shaking incubator 3032 (GFL, Burgwedel, Germany). All *Actinoplanes* strains were grown on soy flour medium (SFM; 20 g/L soy flour, 20 g/L mannitol, 20 g/L agar, tapped water to 1 L; pH adjusted to 8.0 prior to autoclaving; autoclaved twice), oatmeal medium (OM; oatmeal flour 38 g/L, agar 10 g/L; tapped water to 1 L; pH adjusted to 8.0 prior to autoclaving; autoclaved twice). SFM plates, used for setting *Actinoplanes-E.coli* conjugations, were additionally dried at 37 °C before the experiment to assure absence of water on a plate surface. Minimal, complete and R2 medium were prepared as described in Kieser