

## Short Communication

# Identification and characterization of *Streptomyces ghanaensis* ATCC14672 integration sites for three actinophage-based plasmids

Bohdan Ostash<sup>a</sup>, Roman Makitrinsky<sup>a</sup>, Suzanne Walker<sup>b</sup>, Victor Fedorenko<sup>a,\*</sup>

<sup>a</sup> Department of Genetics and Biotechnology, Ivan Franko National University of L'viv, Grushevskogo st., 79005 L'viv, Ukraine

<sup>b</sup> Department of Microbiology and Molecular Genetics, Harvard Medical School, 02115 Boston, USA

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## ABSTRACT

*Streptomyces ghanaensis* produces the antibiotic moenomycin A, which is the only known direct inhibitor of bacterial peptidoglycan glycosyltransferases (transglycosylases). Recent progress in understanding moenomycin biosynthesis opens the door to the generation of novel moenomycins via biocombinatorial approaches. To realize the promise of such an approach, one needs better knowledge of the *S. ghanaensis* genome and diverse genetic tools for stable expression of recombinant constructs in this strain. In this respect, we report the intergeneric *Escherichia coli*-*S. ghanaensis* conjugal transfer of plasmids pRT801 and pSOK804 based on the actinophage BT1 and VVB integrase systems, respectively. The *attB* sites for these two plasmids and for pSET152 were characterized. In particular, sequencing revealed that a putative Arg-tRNA gene serves as an integration site for both phage VVB and pSAM2-like actinomycete integrative and conjugative element recently suggested to be widespread and functional in actinomycetes. The stability of the studied plasmids and their neutrality with respect to antibiotic production warrant their use for manipulations of *S. ghanaensis* genome.

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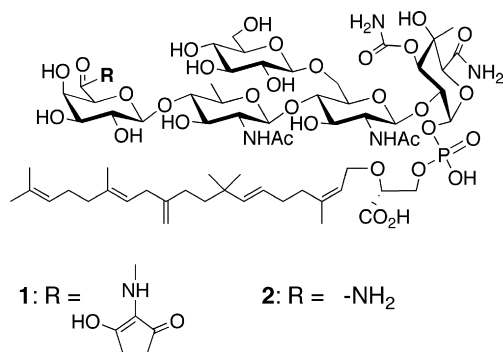
## 1. Introduction

*Streptomyces ghanaensis* produces the pentasaccharide antibiotic moenomycin A (MmA; Fig. 1), which is notable both for its unusual structure and for being the only known direct natural product inhibitor for an important class of peptidoglycan synthesizing enzymes, the bacterial peptidoglycan glycosyltransferases, also known as transglycosylases (Ostash and Walker, 2005). The worldwide rise of multidrug-resistant pathogenic bacteria has renewed interest in novel antibacterials and MmA is considered a promising lead for combating vancomycin- and methicillin-resistant pathogens (Goldman and Gange, 2000; Lovering et al., 2007; Yuan et al., 2008). However, MmA exhibits poor pharmacokinetics and is unsuitable for clinical use in humans, although it is used commercially in animal nutri-

tion. The ability to manipulate the structure of moenomycin could lead to the development of useful analogs; however, the chemical synthesis of this molecule is not trivial (Taylor et al., 2006) and production of novel MmA derivatives through fermentation and/or chemoenzymatic approaches would be highly desirable. The identification and initial characterization of the MmA biosynthetic (*moe*) genes (Ostash et al., 2007) has laid the groundwork for the diversification of the MmA scaffold via genetic engineering. Although expression of *moe* gene sets in *S. lividans* allowed rapid dissection of the logic of MmA biosynthesis, it is not clear at the moment whether heterologous expression will be a viable strategy for producing novel moenomycins on a large scale. Therefore, the ability to generate certain MmA derivatives in the native host, *S. ghanaensis*, via gene disruption and/or expression techniques is of great interest. In this respect, actinophage-based integrative vectors are especially valuable tools, since they allow stable expression of foreign DNA, usually with little or no perturbation of secondary metabolism. We previously

\* Corresponding author. Fax: +380 322 394475.

E-mail addresses: [suzanne\\_walker@hms.harvard.edu](mailto:suzanne_walker@hms.harvard.edu) (S. Walker), [v\\_fedorenko@franko.lviv.ua](mailto:v_fedorenko@franko.lviv.ua) (V. Fedorenko).



**Fig. 1.** Structures of moenomycin A (1) and its late precursor (2) lacking chromophore unit.

showed that the actinophage  $\phi$ C31-based vector pSET152 is useful for manipulation of *S. ghanaensis* (Ostash et al., 2007). Having only pSET152, our ability to modify the strain was limited to a single integration experiment. Here we demonstrate that two other similarly designed vectors derived from distinct actinophages can be efficiently transferred and stably maintained in *S. ghanaensis*, thus allowing the engineering of an MmA producer in a “multiplasmid” fashion. In the course of characterizing *S. ghanaensis* *attB* sites for all studied plasmids we have shown that a putative Arg-tRNA gene containing *attB*<sup>VWB</sup> also serves as an integration site for a pSAM2-like element widely distributed in actinomycete genomes (te Poele et al., 2008a,b).

## 2. Results

### 2.1. Identification of the *S. ghanaensis* *attB* <sup>$\phi$ C31</sup> site and properties of pSET152<sup>+</sup> transconjugants

There is one integration site for pSET152 (see Electronic Supplementary material 1 (ESM) for the map) within the *S. ghanaensis* chromosome (Ostash et al., 2007); however, its identity was unknown. We used a published PCR strategy to clone *attR* <sup>$\phi$ C31</sup> (Stinchi et al., 2003) and amplified a 0.6 kb chromosomal fragment of a pSET152<sup>+</sup> *S. ghanaensis* transconjugant. Sequencing of the resulting amplicon revealed the canonical “right arm” of the  $\phi$ C31 integration site, which is highly homologous to that of *Streptomyces coelicolor* A3(2). Since the genome of *S. ghanaensis* is being sequenced, we took advantage of the available whole genome shotgun sequence (WGS) database at the NCBI site ([www.ncbi.nlm.nih.gov/Traces](http://www.ncbi.nlm.nih.gov/Traces)) to identify the entire *attB* <sup>$\phi$ C31</sup> via a BLAST search and assembly of trace sequences. As was shown for *S. coelicolor* (Combes et al., 2002), *attB* <sup>$\phi$ C31</sup> of *S. ghanaensis* lies within a conserved gene for a putative chromosome condensation protein (Accession No. EU368673). The *attB* <sup>$\phi$ C31</sup> sites of *S. ghanaensis* and *S. coelicolor* are highly homologous; out of 51 bp, 47 are identical and mismatches lie beyond the core hexanucleotide recognition site.

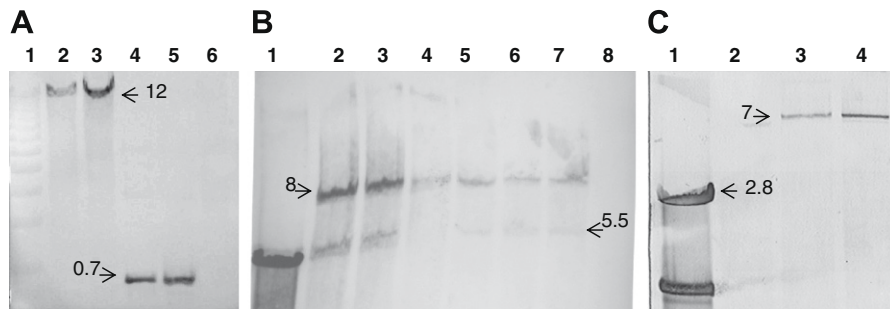
The presence of pSET152 in the extrachromosomal non-replicating form in mycelia of *S. ghanaensis* and other strains has been previously reported (Ostash et al., 2007;

Kieser et al., 2000). Probably, after either integration of tandemly duplicated molecules or successive integrations of pSET152 (1st—site-specific and 2nd—homologous), free circular plasmids are generated spontaneously through homologous recombination (they can be recovered by transformation of *Escherichia coli* with total DNA isolated from transconjugants). To test whether integration of pSET152 derivatives could be biased towards homologous recombination in the presence of long regions of homology, we constructed plasmid pSSG7, which is pSET152 carrying an anonymous 13 kb BamHI fragment of the *S. ghanaensis* genome (Fig. 2S (Supplementary material 1), ESM). The insert in pSSG7 was sequenced from one end. It displayed homology to a regulatory gene involved in the biosynthesis of the macrolide spiramycin (data not shown). The frequency of pSSG7<sup>+</sup> *S. ghanaensis* transconjugants was comparable to that of pSET152<sup>+</sup> ones (Ostash et al., 2007), implying that plasmid size did not affect the efficiency of conjugation. Subsequent Southern analysis showed that pSET152 and pSSG7 integration patterns are identical and that the amount of extrachromosomal plasmid DNA is not increased in the case of pSSG7<sup>+</sup> transconjugants (Fig. 2B). Thus pSET152 derivatives integrate site-specifically even when they carry long DNA fragments homologous to the *S. ghanaensis* genome.

### 2.2. Transfer of pSOK804 and identification of *attB*<sup>VWB</sup> and flanking sequences

Next we tested the utility of actinophage VWB-based vector pSOK804 (Sekurova et al., 2004; Fig. 1S (Supplementary material 1), ESM) for genetic manipulations of *S. ghanaensis*. The frequency of pSOK804<sup>+</sup> transconjugants selected for apramycin resistance (AmR) was  $1.4 \times 10^{-5}$  when the titer of recipient spores was  $2 \times 10^7$  and standard mating procedure was employed (Ostash et al., 2007). The transconjugants did not differ from the parental strain in their ability to grow, sporulate and produce moenomycin. The same was true for double transconjugants carrying both pSOK804 and pO0B5, a pSET152 derivative in which the apramycin resistance marker has been replaced with a spectinomycin one (Ostash et al., 2007). To study the stability of inheritance of pSOK804 in *S. ghanaensis*, 200 transconjugant colonies were tested after three passages under non-selective conditions. All the colonies were AmR. KpnI digests of total DNA of two pSOK804<sup>+</sup> transconjugants were probed with a DIG-labeled *ori*<sup>RK2</sup> fragment of pSET152 to determine the number of pSOK804 integration sites. We observed one hybridization signal, meaning that *S. ghanaensis* possesses 1 *attB*<sup>VWB</sup> site. (Fig. 2A). While free copies of pSET152 can be found in *S. ghanaensis* transconjugants using *E. coli* transformation approach (see previous chapter), no free copies of pSOK804 were found in pSOK804<sup>+</sup> cells.

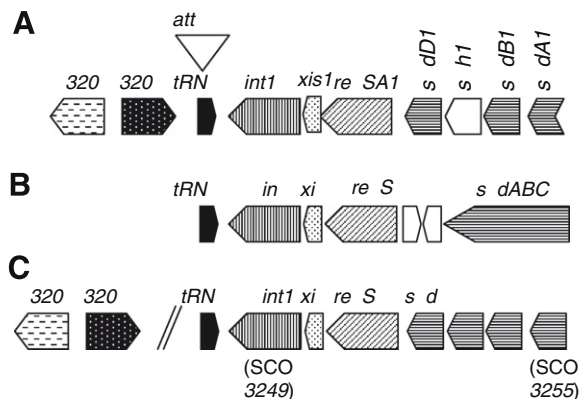
The only reported *attB*<sup>VWB</sup> site that has been characterized in a natural host for VWB phage is in *S. venezuelae* ETH14603 (Mellaert et al., 1998). Nothing is known about the genomic context and nature of *attB*<sup>VWB</sup> loci in other strains (Luzhetskyy et al., 2006). Such information would help understand whether *attB*<sup>VWB</sup> is conserved across species and what may cause the mobility of pSOK804 (e.g.



**Fig. 2.** Southern analyses of different *S. ghanensis* transconjugants. (A). KpnI-digests of genomic DNA isolated from two independent pSOK804<sup>+</sup> *S. ghanensis* transconjugants (lanes 2 and 3). Lane 1, 1 kb DNA ladder (NEB); lanes 4 and 5, 0.8 kb PstI fragment of pSET152 containing *oriT* (used as a DIG-labeled probe in hybridization); lane 6, KpnI digest of *S. ghanensis* ATCC14672 genomic DNA. (B). BamHI-digests of genomic DNA isolated from six independent pSSG7<sup>+</sup> *S. ghanensis* transconjugants (lanes 2–7). Lane 1, linearized pSET152; lane 8, BamHI digest of the wild type strain chromosome. (C). BamHI-digests of genomic DNA isolated from two independent pRT801<sup>+</sup> *S. ghanensis* transconjugants (lanes 3 and 4). Lane 1, HindIII–XhoI digests of pSET152 and pUCoriT; lane 2, BamHI digest of the wild type strain chromosome. Arrows indicates the sizes (in kb) of the hybridizing bands.

repetitive sequences, mobile genetic elements near *attB* etc). To clone the *attL<sup>VWB</sup>* from *S. ghanensis*, we took advantage of the fact that pSOK804 lacks KpnI sites (Fig. 2 and Fig. 3S (Supplementary material 1), ESM). Genomic DNA of a pSOK804<sup>+</sup> transconjugant was digested with KpnI, re-ligated and *E. coli* was transformed with ligation mixture. Plasmid DNA from resulting Am<sup>r</sup> *E. coli* clones was isolated and mapped with restriction endonucleases to confirm that it is pSOK804 plus the 6.7 kb KpnI fragment of *S. ghanensis* chromosome into which the plasmid had integrated. SphI fragments of this plasmid, pSSG12, carrying *attL<sup>VWB</sup>* and flanking DNA were subcloned into pUC19 and sequenced. This revealed that *attL<sup>VWB</sup>* of *S. ghanensis* is identical to that of *S. venezuelae* ETH14630. By inspection of the *S. ghanensis* sequence we identified a 6.5 kb segment of the *S. ghanensis* genome that contains the intact *attB<sup>VWB</sup>* site and several adjacent genes (Fig. 3; Accession No. EU368674). As in *S. venezuelae*, the *attB<sup>VWB</sup>* site of *S. ghanensis* is embedded into a putative tRNA<sup>Arg</sup> (AGG)

gene. The *S. ghanensis* tRNA<sup>Arg</sup> differs from the *S. venezuelae* one only in the absence of the terminal CCA triplet. The inverted repeats are present downstream of *S. venezuelae* tRNA<sup>Arg</sup> gene; however we did not observe ones downstream of the *S. ghanensis* tRNA<sup>Arg</sup> sequence. Upstream of the tRNA<sup>Arg</sup> gene is an open reading frame, SGH3208. The translation product of this ORF is highly homologous to a putative secreted protein SCO3208 from *S. coelicolor* (78% identity, 85% similarity). SGH3208 and four upstream genes form a region of synteny to the SCO3204–SCO3208 segment of the *S. coelicolor* chromosome (data not shown). Six open reading frames were detected downstream of the tRNA<sup>Arg</sup> gene. Their transcriptional direction is opposite to that of the tRNA<sup>Arg</sup> gene. The nucleotide sequence and overall genetic architecture of these six genes resemble that of the *attP*–*spdA* fragment of pSAM2 (Sezonov et al., 1998) and the SCO3249–SCO3256 actinomycete integrative and conjugative element of the *S. coelicolor* (Fig. 3; te Poele et al., 2008a; detailed description of the genes is given in ESM). We have not found other tRNA<sup>Arg</sup>-like sequences in the *S. ghanensis* WGS database, implying that the *attB<sup>VWB</sup>*-containing tRNA<sup>Arg</sup> gene might be essential.



**Fig. 3.** Scheme of genetic organization of the region of *S. ghanensis* genome containing *attB<sup>VWB</sup>* (A) in comparison to the syntenous segments of pSAM2 (Sezonov et al., 1998) (B) and *S. coelicolor* genome (C). Functionally similar genes are marked in the same way. White arrows indicate nonhomologous genes of unknown function. The *attB<sup>VWB</sup>* is embedded into putative tRNA gene (marked as a white triangle on top of the tRNA gene symbol). Gene names and functions—see the text.

### 2.3. Conjugal transfer of pRT801

The actinophage BT1-based vector pRT801 (Fig. 1S (Supplementary material 1), ESM) was tested in *E. coli*–*S. ghanensis* matings. The pRT801<sup>+</sup> transconjugants of *S. ghanensis* were obtained at a frequency of  $6.3 \times 10^{-7}$  (titer of recipient spores was  $2 \times 10^8$ ). As was the case for pSET152<sup>+</sup> and pSOK804<sup>+</sup> transconjugants, pRT801<sup>+</sup> ones were 100% stable and were not affected in primary metabolism, morphology or moenomycin production. Southern analysis revealed one pRT801 integration site within the *S. ghanensis* genome (Fig. 2C). Transformation of *E. coli* with total DNA isolated from 6 pRT801<sup>+</sup> transconjugants yielded no Am<sup>r</sup> clones, implying that hybridization signals do not correspond to the extrachromosomal plasmid. The *S. coelicolor* J1929 gene SCO4848 containing an integration site for  $\phi$ BT1 phage (Gregory et al., 2003) was used to retrieve the highly homologous ORF (Accession No. EU368672) from the *S. ghanensis* WGS database. The *S. ghanensis*

counterpart of SCO4848 contains a 73 bp region almost identical to *attB*<sup>φBT1</sup> from *S. coelicolor*. Taking into account the results of our *in vivo* and *in silico* studies, we propose that the *S. ghanaensis* *attB*<sup>φBT1</sup> site most likely resides within the identified SCO4848 homolog.

#### 2.4. Probing the utility of new tools: pSOK804-mediated duplication of *moe* genes as a test case

There is considerable interest in simplification of the complex mixture of related moenomycin antibiotics produced by streptomycetes, so that individual compounds can be easily purified. For instance, under conditions of submerged fermentation employed in our work *S. ghanaensis* accumulates both MmA and its late precursor **2** (lacks chromophore unit; see Fig. 1) as the major moenomycins (Table 1). A three-gene *moe* cluster 2 (*moeA4*, *moeB4*, *moeC4*) is responsible for biosynthesis and attachment of chromophore group (Ostash et al., 2007). Hence we supposed that an increase in *moeA4*, *moeB4* and *moeC4* genes dosage could enhance the conversion of **2** into MmA. To test this idea, we cloned *moe* cluster 2 into pSOK804 and introduced the resulting plasmid (pOOB64b; ESM) into *S. ghanaensis*. Compared to control strain, *S. ghanaensis* carrying pOOB64b produced roughly 1.8 times more MmA, while production of **2** decreased more than three times (Table 1). These results show that pSOK804 can be successfully used to manipulate the biosynthesis of moenomycins. The availability of several different integrative plasmids is particularly beneficial, since it allows further stable insertions to be made in the pOOB64b<sup>+</sup> strain. For example, major *moe* cluster 1 can be duplicated with the help of the pSET152-based cosmid moeno38-1 (Ostash et al., 2007) and then additional genes can potentially be expressed from pRT801.

### 3. Discussion

Our studies broaden the repertoire of actinophage-based vectors that may find use during the engineering of recombinant *S. ghanaensis* strains. Site-specific recombination is a dominant route for pSET152, pSOK804 and pRT801 integration into the *S. ghanaensis* chromosome. There is only one integration site for each plasmid, and the *attB* sites are located in different regions of the *S. ghanaensis* genome as judged from sequencing results. Integration sites for the studied plasmids in the *S. ghanaensis* genome are neutral in the context of moenomycin biosynthesis and sporulation. The data presented here indicate that the aforementioned vectors will be valuable tools for

**Table 1**

Quantitative analysis<sup>a</sup> of production of MmA **1** and its precursor **2** by *S. ghanaensis* strains (in arbitrary units per 10 mg of dry biomass).

Strain	Compound	
	MmA ( <b>1</b> )	MmA precursor ( <b>2</b> )
pSOK804 <sup>+</sup> (control)	(52 ± 10) × 10 <sup>3</sup>	(27 ± 4) × 10 <sup>3</sup>
pOOB64b <sup>+</sup>	(97 ± 7) × 10 <sup>3</sup>	(7 ± 3) × 10 <sup>3</sup>

<sup>a</sup> See ESM for description of the procedure.

studies on the genetics of secondary metabolism of this industrially important strain.

The genetic organization of the chromosomal region that comprises *attB*<sup>VWB</sup> has also been revealed with the help of the whole-genome shotgun sequence database for *S. ghanaensis*. We have found that the *S. ghanaensis* *attB*<sup>VWB</sup>-containing tRNA<sup>Arg</sup> gene is located near genes for certain integrative plasmid/element functions. The integration of *Streptomyces* plasmids into functional tRNA genes is well documented (Mellaert et al., 1998; te Poele et al., 2008a,b) and our data suggest that the tRNA<sup>Arg</sup> sequence served as an *attB* for a presumed pSAM2-like element in the evolutionary past of *S. ghanaensis*. Upon integration of such an element, the intact tRNA<sup>Arg</sup> gene was restored in the *attL* site, and now serves as an *attB*<sup>VWB</sup> site. Our work demonstrates that Arg-tRNA gene is a “hot spot” for integration of different genetic elements in *S. ghanaensis* and, probably, *S. coelicolor*. In the latter, a similar pSAM2-like genetic element was proposed to be functional (te Poele et al., 2008a); however this speculation awaits for experimental verification. It is not known whether the integrated element is stably maintained in the *S. ghanaensis* chromosome; even if this is not the case, it does not seem to affect the maintenance of pSOK804 (as judged from stability of AmR phenotype). Further sequencing of the discovered pSAM2-like element will help us elucidate its structure and function in more detail and may lead to construction of new vector molecules.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.plasmid.2008.12.002](https://doi.org/10.1016/j.plasmid.2008.12.002).

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