Testing the utility of site-specific recombinases for manipulations of genome of moenomycin producer *Streptomyces ghanaensis* ATCC14672

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35 **Abstract**
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37 *Streptomyces ghanaensis* ATCC14672 is the producer of phosphoglycolipid antibiotics
38 moenomycins that for almost forty years were used worldwide as an animal feed additive. As the
39 use of moenomycins narrows down (due to bans in EU and some other countries), it opens the
40 opportunity to develop much-needed antibiotics against Gram-positive human pathogens, such as
41 cocci. It is desirable to develop ATCC14672 strains accumulating only certain members of
42 moenomycin family which would facilitate their purification, analysis and/or chemical
43 modification. Here we tested site-specific recombinases (SSRs) as a tool to manipulate the genome
44 of ATCC14672 and to achieve aforementioned goals. We show that of three SSRs tested –Cre, Dre
45 and Flp – the first two efficiently catalyzed recombination reactions, while Flp showed no activity
46 in ATCC14672 cells. Cre recombinase can be reused at least three times to modify ATCC14672
47 genome without detrimental effects, such as large-scale inversions or deletions. Properties of the
48 generated strains and SSRs are discussed.
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51 ***Keywords***: *Streptomyces ghanaensis*; SSRs; genome engineering; moenomycin.
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53 Actinomycete *Streptomyces ghanaensis* ATCC14672 produces a mixture of structurally related
54 phosphoglycolipid secondary metabolites collectively known as moenomycin (or flavomycin)
55 complex (Ostash and Walker 2010). Moenomycin was used as a feedstuff additive in many
56 countries for decades without any significant rise of resistance in animal microflora (Pfaller 2006).
57 This antibiotic possesses unique mechanism of antibacterial action not shared with any other
58 clinically useful drug (Welzel 2005; Gampe et al. 2011); it displays extremely high activity against
59 many Gram-positive pathogens including vancomycin- and methicillin-resistant cocci; and low
60 incidence of moenomycin resistance was reported to date (Ostash and Walker 2010; Rebets 2014).
61 For these reasons moenomycin is also considered a blueprint for new class of antibiotics much
62 needed to counter the rise of multidrug-resistant infections.

63 Because moenomycin complex of ATCC14672 consists of 10-15 very similar compounds and
64 they all are produced at low level (Ostash and Walker 2010), it is challenging to use the strain as a
65 source of a single moenomycin for biological testing and/or chemical manipulations. Nevertheless,
66 the immense chemical complexity of the moenomycins guarantees that their fermentation-based
67 production is the only viable route of supply. ATCC14672 genome sequence is available (Ostash et
68 al. 2007), which opens the door to genetic engineering of moenomycin pathway. Through judicious
69 deletions of certain *moe* genes it should be possible to simplify the naturally produced moenomycin
70 mixture down to two-three compounds of interest. However, generation of multiple mutations in
71 ATCC14672 genome is limited by a small number of selectable markers. Excision of a marker gene
72 after knockout and its subsequent re-use is one of the ways around this problem, and such a kind of
73 genome engineering can be achieved with the help of site-specific recombinases (SSRs). Indeed,
74 technology of markerless deletions is well established for many groups of organisms (Schweizer
75 2003), and recently it was introduced to actinomycetes (Siegl and Luzhetskyy 2012). Moreover, we
76 have shown that SSR Cre efficiently promotes one round of marker eviction in *S. ghanaensis*
77 (Makitrinskyy et al. 2013). It remained unknown as to whether SSRs other than Cre can function in
78 ATCC14672. In general, the efficiency of different SSRs varies dramatically from species to
79 species, necessitating direct trials for a given strain (Siegl and Luzhetskyy 2012). Also, there is no
80 information on whether the same SSR can be used several times to perform recombination in
81 different chromosomal loci without detrimental effects. Little information on this issue is available
82 for any actinomycete, given that SSRs were introduced recently to this field. Here we tested three
83 SSRs as a tool for ATCC14672 genome engineering – Flp, Cre and Dre, all of which are encoded
84 by synthetic genes codon-optimized to match codon usage of actinomycete chromosomes (Siegl
85 and Luzhetskyy 2012). Actinophage φC31-based plasmids carrying apramycin resistance cassette
86 (*aac(3)IV-oriT*) flanked with *loxP* (pINT13; see Electronic Supplementary Materials (ESM) for full
87 procedures and plasmid description), *FRT* (pINT3) or *rox* (pINTROX) recombination sites were

88 transferred conjugally into ATCC14672 cells. It is known that ATCC14672 genome contains single
89 *attB*$^{\varphi C31}$ site (Ostash et al. 2009). The apramycin-resistant (Am^r) transconjugants thus contained
90 single copy of the cassette flanked with homotypic sites for one of the three SSRs. Then SSR gene
91 was introduced into the transconjugants on replicative plasmids, either pAL-based (moderate copy
92 number *ts*-replicon of pSG5; SSR gene is driven by thiostrepton-inducible promoter *tipAp*) or
93 pUWL-based (high copy number replicon of pIJ101, SSR gene is controlled by strong constitutive
94 promoter *ermEp**; see also Table S1). The resultant transconjugants (carrying both constructs) were
95 selected for thiostrepton resistance. 200 thiostrepton resistant clones of three types (*loxP*+Cre,
96 *rox*+Dre and *FRT*+Flp) were directly checked for apramycin susceptibility (Am^s; loss of cassette
97 because of SSR activity). The Cre and Dre expression led to immediate eviction of the cassette in
98 85-95 % (mean values of three independent experiments; standard deviations did not exceed 15% of
99 the mean) of all initial transconjugants being tested. 100% eviction of the cassette from Am^r clone
100 carrying either *loxP* or *rox* cassette was achieved after applying the marker eviction procedure
101 described in ESM (one passage in TSB in the absence of antibiotics, plating onto oatmeal agar and
102 resistance check via replica plating; 200 colonies were tested). In contrast, no one of the 200 tested
103 colonies carrying pINT3+pALFLP plasmids was Am^s. We passaged three independent Am^r
104 colonies under nonselective conditions, yet this did not yield Am^s clones as well. It is possible that
105 *flp(a)* gene is for some reasons poorly expressed from thiostrepton-inducible promoter (pALFlp) in
106 ATCC14672. We therefore tested high copy number Flp expression plasmid pUWLFlp (see above).
107 No Am^s clones were isolated in this case as well. We further speculated that inability to excise
108 FRT-flanked apramycin cassette can be caused by its position, namely *attB*$^{\varphi C31}$ site or its
109 surroundings somehow interfere with recombination reaction. Therefore, an attempt has been made
110 to evict FRT-flanked *aac(3)IV-oriT* cassette from *S. ghanaensis* dH5 genome. In dH5 strain the
111 cassette is located within *moe* cluster 2, in place of amidotransferase gene *moeH5* (Ostash et al.
112 2013). However, in spite of repeated attempts we failed to isolate even one colony exhibiting Am^s
113 phenotype. We scaled up the experiment and screened 2000 pINT3$^+$ clones for marker loss, yet no
114 one Am^s clone was found. The obtained results imply that efficiency of Flp-mediated marker
115 excision is below 0.05% (less than 1 clone out of 2000 tested). This is in contrast to literature data
116 (Fedoryshyn et al. 2008), where efficiency of Flp was within 10-40% range. Finally, we showed via
117 semiquantitative RT-PCR analysis that *flp(a)* gene is transcribed relatively well from pALFLP and
118 pUWLFLP constructs, the latter giving more transcript (ESM Fig. S1). Hence, failure to detect Flp
119 recombinase activity is not related to poor or absent gene transcritption.

120 It was shown by us previously that in ATCC14672 cell φC31-based integrative plasmids can
121 exist in extrachromosomal state, most likely because of excision of tandemly duplicated copies of
122 the plasmid (Ostash et al. 2009). We took advantage of this fact to retrieve and sequence pINT3,

123 pINT13 and pINTROX derivatives from *S. ghanaensis* after expression of SSRs. For this purpose
124 *E. coli* DH5α was transformed to ampicillin resistance (marker gene in the vector scaffold of the
125 aforementioned plasmids) with total DNA isolated from the transconjugants. Indeed, in all three
126 cases we were able to obtain ampicillin-resistant *E. coli* clones carrying expected plasmids.
127 Sequencing of pINT13 and pINTROX derivatives revealed scar sequences instead of *aac(3)IV-oriT*
128 cassette, while pINT3 remained intact.

129 Next we focused on Cre recombinase and set out to study its potential for multiple rounds of
130 marker eviction. *S. ghanaensis* ΔabsB, carrying scar sequence in place of $absB_{gh}$ gene
131 (SSFG_02129, see Fig. 1) after Cre-mediated marker eviction (Makitrinskyy et al. 2013), has been
132 chosen as a host to generate unmarked *moeH5* deletion. Helper plasmid pALCre was introduced
133 into *moeH5* knockout strain B38 (see ESM Methods and Fig. S1), and Am^s clones were readily
134 isolated from the primary transconjugants (out of 200 clones checked 80% displayed the desired
135 phenotype). One clone, labeled as B38.3 (Fig. S2), was chosen for further studies. PCR analysis
136 confirmed marker eviction from B38.3 (Fig. S3). Also, mass-spectrometry analysis showed that
137 B38.3 produced expected phosphoglycolipid compound, nosokomycin A (ESM, Fig. S4). We
138 detected no clones with impaired growth and/or sporulation upon pALCre expression in B38 strain,
139 which would be an indicative of large-scale inversion between *loxP* sites at *absB* and *moe* cluster
140 loci (Fig. 1). Although our experimental procedures cannot completely rule the inversions, we think
141 this kind of genome rearrangement is rare, since the frequency of Am^s clones in this experiment
142 was at the level observed in model (pINT13-based) studies.

143 Next we decided to generate markerless deletion of *moeGT3moeR5moeS5* genes in B38.3
144 genome (already containing two *loxP* scars) using Cre-*loxP* system. First we generated strain
145 B38.3/8 (as described in ESM) carrying *loxP*-flanked *aac(3)IV-oriT* cassette in place of moeGT3-
146 S5 (Fig. 1 and S2). Here *loxP* scar in place of *moeH5* is located 11 kb away from *loxP* sites flanking
147 the cassette. Also, the latter were in orientation opposite to the *loxP*-scar in place of *moeH5*. Upon
148 expression of Cre recombinase in B38.3/8 we observed 20% of Am^s clones. It is likely that Cre is as
149 efficient in B38.3/8 as it was in B38, but in the former case part of recombinase activity resulted in
150 rearrangements that are lethal (large deletions) or did not lead to marker loss (such as inversions).
151 PCR analysis of one clone, labeled as B38.3/80, confirmed marker eviction from *moeGT3-R5-S5*
152 knockout site (Fig. S2, S3). Among 100 tested Am^s clones we found one that did not produce
153 moenomycin A. Analysis of this clone, referred to as B38.3/21, revealed the inversion of *moe*
154 cluster segment between two *loxP*-scars in the cluster (Fig. S2, S5). We wondered as to whether the
155 loss of moenomycin production by B38.3/21 is indeed a consequence of inversion, or it is a result of
156 the other, unanticipated mutations/rearrangements in genome of this strain. To address this issue,
157 Cre-expressing construct pALCre was re-introduced into B38.3/21 cells, and the resultant

158 transconjugants were checked for moenomycin production. The rationale behind this experiment
159 was as follows. Expression of Cre SSR in B38.3/21 cells would lead (presumably, at low
160 frequency) to restoration of initial order of *moe* genes within the cluster and moenomycin
161 biosynthesis, provided that inversion was sole reason for blocked antibiotic production. Indeed, of
162 the 150 clones checked, three produced moenomycin at the level of initial (B38.3) strain, lending
163 support for the above mentioned suggestion.

164 In conclusion, we show here that SSR Dre and Cre are efficient tools for manipulation of
165 ATCC14672 genome. The latter can be reused several times to generate markerless deletions of
166 distantly located genome loci. As a proof of principle, unmarked deletion of amidotransferase gene
167 *moeH5* has been generated, yielding the strain B38.3 with moenomycin production pattern
168 narrowed down to nosokomycin A. We also show that, at low frequency, Cre recombinase can
169 cause additional rearrangements between proximal *loxP* scars, such as inversion in case of B38.3/21
170 clone. Although undesired from the moenomycin production engineering point of view, the latter
171 result demonstrates the significance of *moe* gene order for their expression and/or regulation, and
172 subsequent moenomycin production.

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226 Legends to figures

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228 **Fig. 1.** Manipulating ATCC14672 genome with SSRs. **A.** Genomic loci of ATCC14672 that were
229 subject to manipulations with SSRs (shaded in grey). Approximate distances from the “left” end of
230 the chromosome and between *moeH5* and *moeGT3* genes are indicated. The distances were
231 obtained from available whole-genome sequencing data for ATCC14672 genome (available at
232 www.broadinstitute.org). Only relevant *moe* genes are shown. Cosmids moeno38 and moeno40
233 carrying overlapping fragment of *moe* cluster are shown at the bottom. **B.** Schematic representation
234 of relative localization of *loxP* sites (black triangles) in genomes of the strains mentioned in the
235 text. Double slash line on the schematic represents distant location (approx. 600 kb) of *loxP* scar in
236 place of *absB* gene relative to *moe* genes. More details are given in ESM Fig. S1, S2, S4.
237 Abbreviations: M – marker gene; H5, KO, GRS – genes *moeH5*, *moeK5-moeX5* segment,
238 *moeGT3moeR5moeS5*, respectively.

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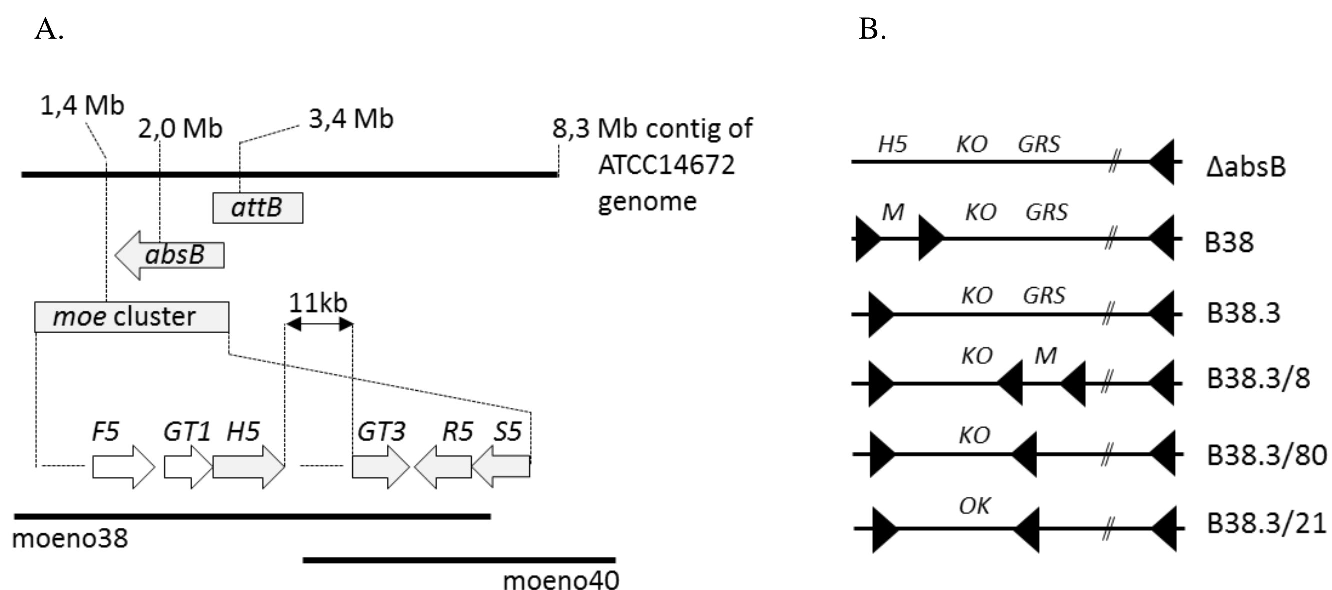

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