Electronic Supplementary Materials for the paper of M. Lopatniuk *et al*.: Testing the utility of site-specific recombinases for manipulations of genome of moenomycin producer *Streptomyces ghanaensis* ATCC14672

Materials and methods

Bacterial strains and culture conditions. Strains and DNA constructs used in this work are listed in Table S1. *E. coli* DH5α was used for routine subcloning. *E. coli* ET12567 (pUZ8002) was used to perform intergeneric conjugation from *E. coli* to *Streptomyces ghanaensis*. *E. coli* BW25113 with pIJ790 was used to carry out RedET-mediated gene replacement (Gust et al. 2004). Solid oatmeal (g/L: oat flour (tolokno) – 34, agar – 18) and SM media (Kieser et al. 2000) were used to harvest *Streptomyces* spores suspension, maintain strains and to plate *E. coli – Streptomyces* matings. TSB medium was used to grow ATCC14672 strains for biomass and moenomycin assays. *E. coli* strains were grown as described elsewhere.

Marker eviction. The same procedure was used throughout this work; it is detailed below for pINT13^{+} transconjugants of ATCC14672 (*loxP*-flanked *aac(3)IV-oriT* cassette in *attB*$^{\varphi C31}$ site). The eviction was carried out using helper plasmid pALCre (Siegl and Luzhetskyy 2012). The helper plasmid was conjugally transferred into pINT13^{+} ATCC14672 and the transconjugants were selected for thiostrepton resistance (to maintain pALCre). The thiostrepton-resistant colonies were directly checked for loss of apramycin resistance (an indicative of the cassette loss) by replica plating. Usually the majority of the colonies have already lost the cassette. Transconjugant colonies exhibiting apramycin resistance were pre-cultured in liquid medium TSB for 48 h at 37°C. 0.3 ml of the preculture was transferred into fresh TSB medium (30 ml in 300-ml flask) and grown for 48 h. The passage was repeated one more time, and the resultant culture was diluted and plated to obtain 50-200 colonies per plate. Their antibiotic resistance phenotype was checked by replica plating. The helper plasmid was lost after two subsequent passages of selected apramycin-sensitive clone in the absence of thiostrepton. As a control, pINT13^{+} transconjugants in the absence of pALCre were tested in parallel, and no apramycin-sensitive colonies were observed (data not shown).

DNA manipulations and gene knockouts. Genomic DNA from *Streptomyces* and plasmid DNA from *E. coli* were isolated using standard protocols (Kieser et al. 2000; Sambrook and Russell 2001). RedET-mediated gene replacements (Datsenko and Wanner 2000) in cosmids moeno38 and moeno40 (Fig. 1) were carried out with the help of REDIRECT system (Gust et al. 2003). *E. coli* transformation and intergenetic *E. coli–Streptomyces* matings were performed as described by Kieser et al. (2000). DNA sequencing was performed at GATC-Biotech (Konstanz, Germany).

Gene knockouts in *S. ghanaensis* were verified via PCR analysis. *moeH5 markerless deletion*. The *aac(3)IV-oriT* cassette from pIJ774 was amplified with primers moeH5up and moeH5down. The resulting *loxP*-flanked amplicon was used to replace the *moeH5* gene in cosmid moeno38 with *aac(3)IV-oriT*, yielding moeno38-dH5loxP. *S. ghanaensis* transconjugants carrying moeno38-dH5loxP were selected on plates overlaid with apramycin and kanamycin (single cross-over between regions of homology on the cosmid and in the genome). One such Am^rKm^r colony was then subjected to three rounds of propagation in the absence of selection to allow for the second cross-over. One Am^rKm^s colony obtained, designated B38, was confirmed to be the desired deletion mutant by PCR. The marker was evicted as descrived above. moeGT3-moeS5 markerless deletion. The *aac(3)IV-oriT* cassette from pIJ774 was amplified with primers moeGT3up and moeS5down. The resulting *loxP*-flanked amplicon was used to replace the fragment with *moeGT3-moeR5-moeS5* genes in cosmid moeno40 with *aac(3)IV-oriT*, yielding moeno40-dGTRSloxP. The generation of marked and markeless deletion of *moeGT3-R5-S5* genes in ATCC14672 genome were carried out and PCR-verified as described for *moeH5* deletion.

The presence and stability of inheritance of φC31-based constructs in *S. ghanaensis* were checked as described earlier (Ostash *et al*., 2009).

Analysis of moenomycin production. Growth of the strains, moenomycin purification, LC-MS conditions and analysis of the data are described in Makitrinskyy *et al*. (2010). LC-MS and MS^2 data were acquired on Bruker Esquire 3000 ESI-MS spectrometers.

Table S1. Strains and plasmids used in the work

Strain or plasmid/cosmid	Description	Source or reference
Streptomyces ghanaensis ATCC14672	Moenomycin producer, wild type (WT)	ATCC
S. ghanaensis ΔabsB	WT derivative, $absB_{gh}$ deletion	Makitrinskyy et al. (2013)
S. ghanaensis dH5	WT derivative, *moeH5* replacement with *aac(3)IV-oriT* cassette, *FRT*-flanked	Ostash et al. 2013
S. ghanaensis B38	ΔabsB derivative, *moeH5* replacement with *aac(3)IV-oriT* cassette, *loxP*-flanked	This work
S. ghanaensis B38.3	B38 with evicted *aac(3)IV-oriT* cassette	This work
S. ghanaensis B38.3/8	B38.3 derivative, *moeGT3-S5* replacement with *aac(3)IV-oriT* cassette, *loxP*-flanked	This work
S. ghanaensis B38.3/80	B38.3/8 derivative with evicted *aac(3)IV-oriT*	This work
S. ghanaensis B38.3/21	B38.3/8 derivative with inversion *loxP* scars	This work
Escherichia coli DH5α	Routine cloning host	Life Technologies
E. coli ET12567 (pUZ8002)	Host for conjugative DNA transfer	Kieser et al. (2000)
E. coli BW25113 (pIJ790)	Host for recombineering experiments	Gust et al. (2004)
Bacillus cereus	Moenomycin production test culture	ATCC
pIJ773	pBluescriptKS(+) carrying *aac(3)IV-oriT* cassette flanked with *FRT* sites	Gust et al. (2004)
pIJ774	pBluescriptKS(+) carrying *aac(3)IV-oriT* cassette flanked with *loxP* sites	Gust et al. (2004)
pINT3	pIJ773 carrying *int-attP* fragment from actinophage phiC31	Fedoryshyn et al. (2008)
pINT13	pIJ774 carrying *int-attP* fragment from actinophage phiC31	Fedoryshyn et al. (2008a)
pINTROX	Integrative plasmid carrying *aac(3)IV-oriT* cassette flanked with *rox* sites	Herrmann et al. (2012)
pALFlp	Replicative plasmid (*ts*-replicon of pSG5) carrying *flp(a)* gene under *tipA* promoter	Fedoryshyn et al. (2008)
pUWLFlp	Replicative plasmid (replicon of pIJ101) carrying *flp(a)* gene under *ermE** promoter	Fedoryshyn et al. (2008)
pALCre	Replicative plasmid (*ts*-replicon of pSG5) carrying *cre(a)* gene under *tipA* promoter	Fedoryshyn et al. (2008a)
pALDre	Replicative plasmid (*ts*-replicon of pSG5) carrying *dre(a)* gene under *tipA* promoter	Herrmann et al. (2012)
moeno38	SuperCos1-based cosmid carrying *moe* cluster 2 except *moeR5moeS5* genes	Ostash et al. (2007)
moeno40	SuperCos1-based cosmid carrying *moe* cluster 2 fragment, from *moeD5* to *moeS5*	Ostash et al. (2007)
moeno38-dH5loxP	moeno38 derivative, *moeH5* replacement with *aac(3)IV-oriT* cassette, *loxP*-flanked	This work
moeno40-dGTRSloxP	moeno40 derivative, *moeGT3-R5-S5* replacement with *loxP*- cassette, *loxP*-flanked	This work

Table S2. Primers used in this study

Primer name	Sequence	Purpose
moeH5up	AGGCCGCCCTCCAGCCCCTGCTGGACGCCCGATGACGGTATTCCGGGGATCCGTCGACC	Knock-out of *moeH5* gene with *aac(3)IV-oriT* cassette, *loxP*-flanked (upstream primer)
moeH5down	TCTCGTGAAGTGGGGGTCTGCGGCGGTCCGGCCCCGCTATGTAGGCTGGAGCTGCTTC	Knock-out of moeH5 gene with *aac(3)IV-oriT* cassette, *loxP*-flanked (downstream primer)
moeGT3up	GTGGCCGTCCTCCGCGGTGACGACGAGGCGCTCCCCCACTGTGTAGGCTGGAGCTGCTTCG	Knock-out of moeGT3-R5-S5 genes with *aac(3)IV-oriT* cassette, *loxP*-flanked (upstream primer)
moeS5down	GTGAGAGTTCTTGTCGTCGGCGGGAGCGGCTTCCTCGGGATTCCGGGGATCCGTCGACCC	Knock-out of moeGT3-R5-S5 genes with *aac(3)IV-oriT* cassette, *loxP*-flanked (downstream primer)
moeH5for	CATGGAGCGGTGGGTGCCG	To check *moeH5* knock-out (upstream primer)
moeH5rev	GTCGTGATGGCGCACCAGG	To check moeH5 knock-out (downstream primer)
moeGT3for	TGGTGGTGGTCGATCCGGACG	To check moeGT3-R5-S5 knock-out (upstream primer)
moeS5rev	AGAACCCGGCTCCCTGTCGTA	To check moeGT3-R5-S5 knock-out (downstream primer)
flp_rt_up	CCACATCGGTCGCCACCTG	RT-PCR analysis of
flp_rt_rp	GTTGATGTAGCTGCTCAGG	flp(a) expression (350-bp 3'-terminal fragment)

* Restriction sites are underlined

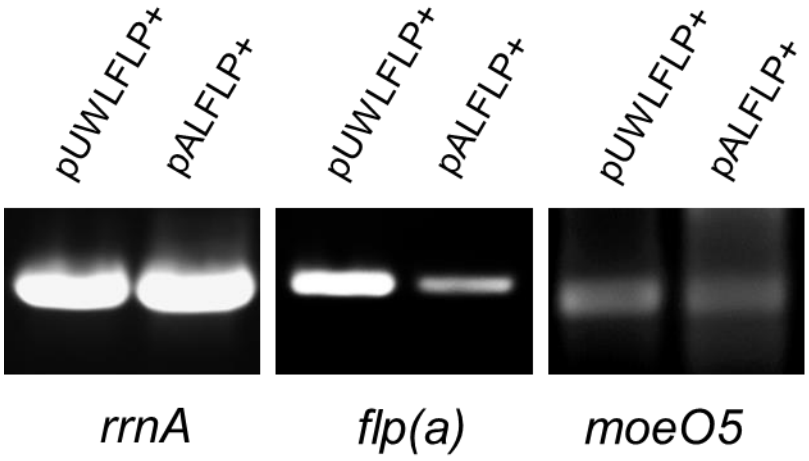

Fig. S1. Semiquantitative RT-PCR analysis of expression of *flp(a)* gene in *S. ghanaensis* strains carrying either pUWLFLP (pUWLFLP+) or pALFLP (pALFLP+). As controls, *rrnA* and *moeO5* genes were amplified. Equal amounts of RNA (prepared as described in Makitrinskyy et al. 2010) were used for reverse transcription (RT) steps. Amplification of *rrnA* from RNA samples in the absence of RT step yielded no signal, confirming the quality of RNA preparations (data not shown). In all cases approximately 350-bp fragments were amplified, *rrnA* and *moeO5* primers are described in Makitrinskyy et al. 2010.

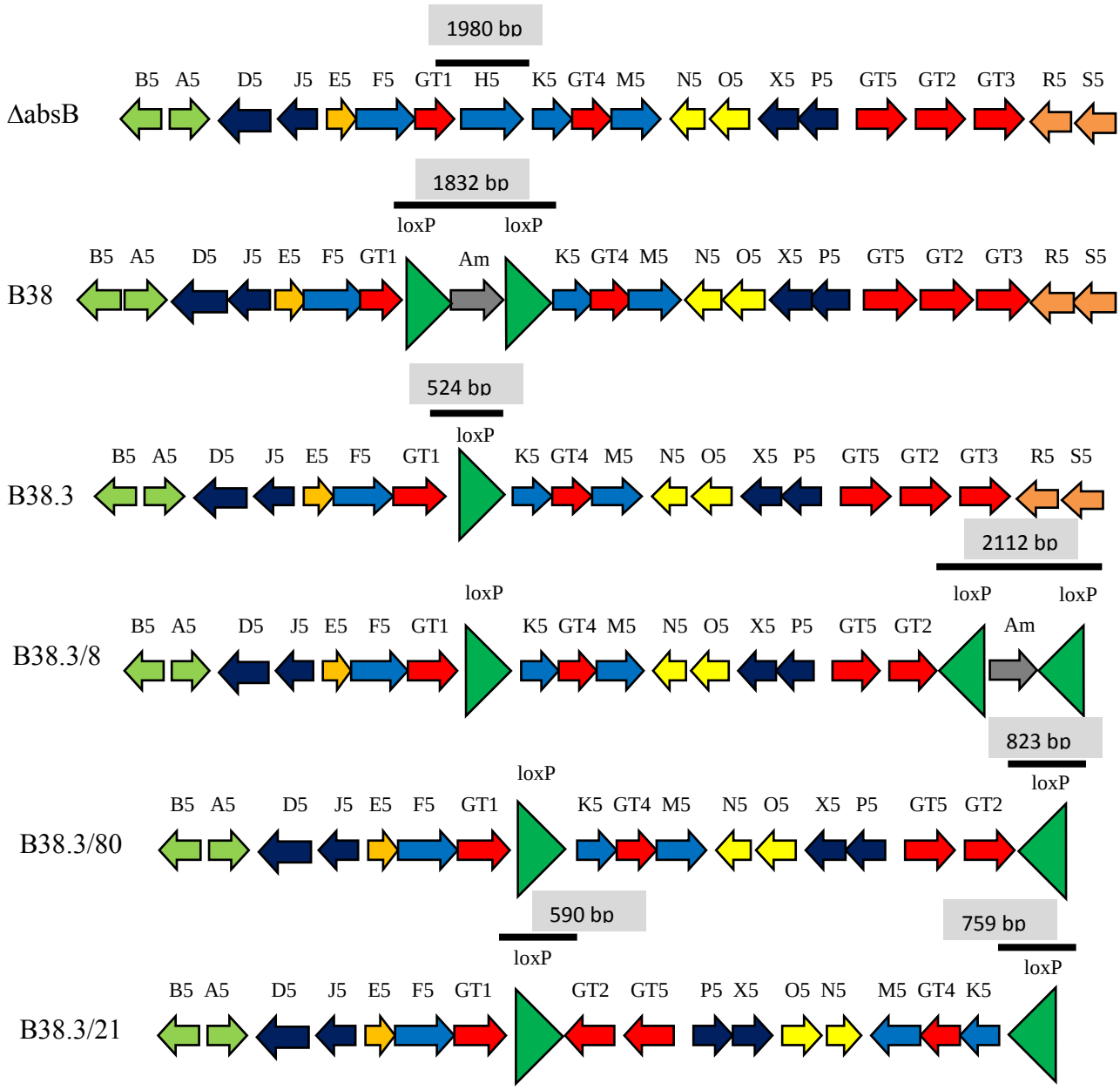

Fig. S2. Location of *loxP* sites (green triangles) within the *moe* cluster of the strains mentioned in the main text. The *loxP* scar sequence in place of $absB_{gh}$ gene in the genome ΔabsB and all its derivative strains is located approximately 600 kb away from *moe* cluster (see Fig. 1, main text). Black lines indicate regions of moe cluster analyzed via diagnostic PCR (see Fig. S3, S5). Expected sizes of the amplicons are shown above the lines (grey rectangles).

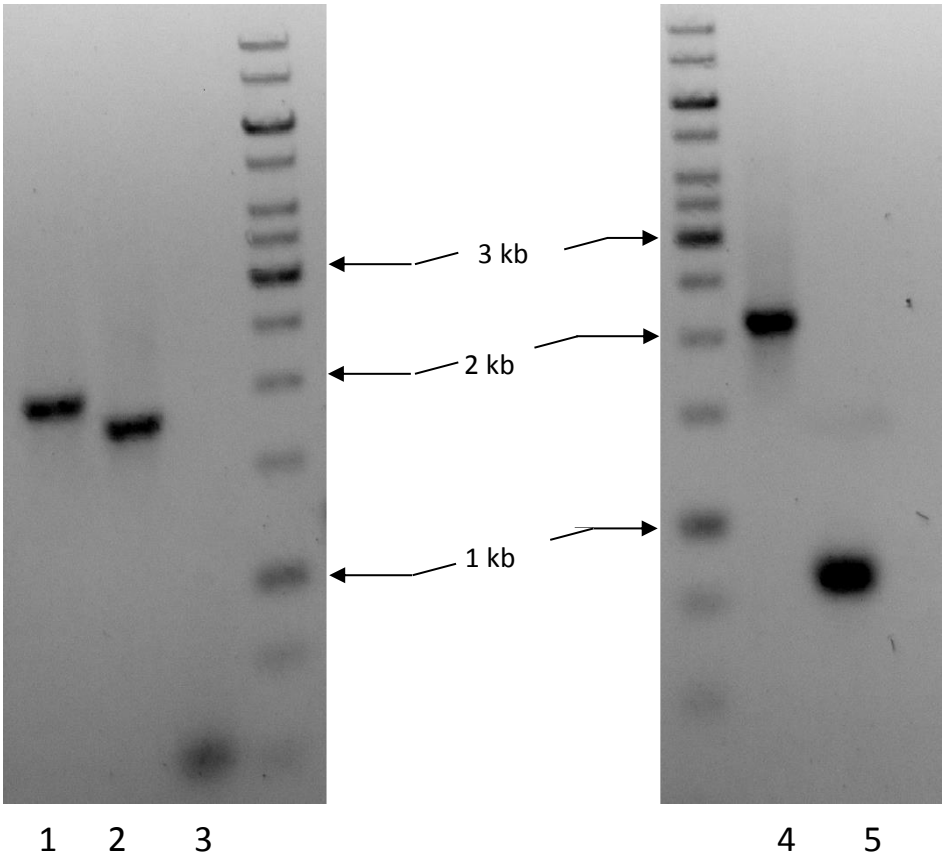

Fig. S3. PCR analysis of SSR-manipulated sites of genomes of *S. ghanaensis* strains: ΔabsB, primers to flanking regions of *moeH5* gene (1980 bp; lane **1**); B38, primers moeH5for and moeH5rev (Table S2) to flanking regions of *moeH5* gene (**2**; amplification of 1832 bp *aac(3)IV-oriT* cassette); B38.3, primers to flanking regions of *moeH5* gene (**3**; amplification of 524 bp *loxP* scar sequence + surrounding regions); B38.3/8, forward primers to *moeGT3*, reverse – to *moeS5* flanking regions (**4**, 2112 bp *aac(3)IV-oriT* cassette+ flanking regions); B38.3/80 – forward primers to *moeGT3*, reverse – to *moeS5* flanking regions (**5**; amplification of 832 bp *loxP* scar sequence + surrounding regions). Molecular marker – 1 kb DNA ladder (Thermo). See Fig. S2 for a scheme of location of SSR sites.

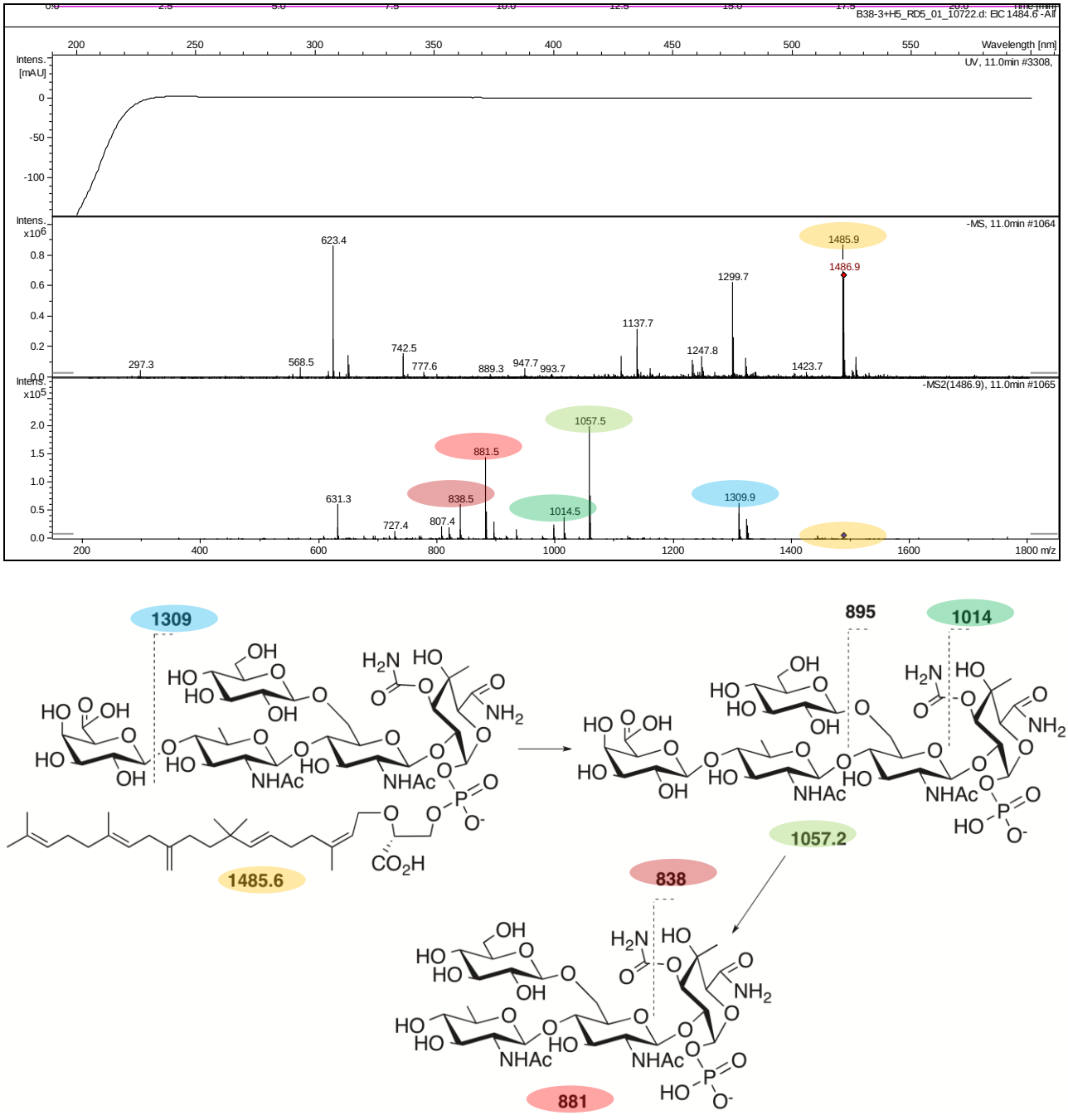

Fig. S4. MS-MS analysis of 1485.6 Da compound (nosokomycin A) produced by *S. ghanaensis* B38.3 strain. The observed pattern of fragmentation of 1485.6 Da compound is fully consistent with the fragmentation pathway (shown below the MS-MS chromatogram) for moenomycins, as it is proposed on the basis of published data by Eichhorn *et al*. (2005) and Uchida *et al*. (2010). Please note the formation of major diagnostic 1057 Da peak (carbohydrate portion after the loss of the lipid) and its descarbamoylated analog (minus 43 Da – 1014 Da).

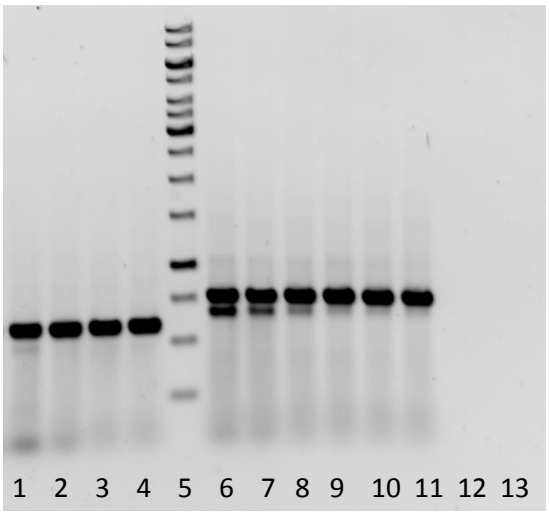

Fig. S5. PCR analysis of inversion of *moe* cluster segment in B38.3/21 strain (see Fig. S1). Lanes 1-4: gradient PCR analysis of left junction (GT1-GT2) with primers moeH5for and moeGT3for (590 bp amplicon); lanes 6-11: gradient PCR analysis of right junction (K5-GT3) with primers moeH5rev and moeS5rev (759 bp amplicon). Lane 12, 13 – negative control (moeH5for-moeGT3for and moeH5rev-moeS5rev primer pairs used against B38.3 genome as a template). Lane 5 – 1 kb DNA ladder. See Fig. S2 for a scheme of location of SSR sites in B38.3/21 genome.

Supplementary references

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