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ABC Transporter Genes from *Streptomyces ghanaensis* Moenomycin Biosynthetic Gene Cluster: Roles in Antibiotic Production and Export

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Abstract

Streptomyces ghanaensis ATCC14672 produces antibiotic moenomycin A (MmA), which possesses strong antibacterial activity. The genetic control of MmA biosynthesis has been recently elucidated; nevertheless, little is known about the roles of two pairs of genes, *moeX5-moeP5* and *moeD5-moeJ5*, coding for ATP-dependent transporter systems. Here we report that both gene pairs form transcriptional units actively expressed during MmA production phase. *S. ghanaensis* mutants deficient in either (one) or both transporter systems are characterized by a decreased ability to produce moenomycins, and the Δ *moeP5moeX5* mutant exported less moenomycins. However, even the quadruple *S. ghanaensis* mutant (Δ *moeD5moeJ5*+ Δ *moeX5moeP5*) remains able to extrude significant amounts of moenomycin. Similar results were observed under conditions of heterologous expression of *moe* cluster. Transporter genes other than those located in *moe* cluster are likely to participate in moenomycin efflux.

Keywords

Streptomyces ghanaensis; moenomycin; ABC transporters

Introduction

Transport of antibiotics across the cytoplasmic membrane is an important and yet poorly understood step of secondary metabolism in actinomycetes. On one hand, antibiotics are believed to lack an essential role in the intracellular metabolism of the producing cells, and are therefore destined for extrusion to fulfill defensive or other functions. Indeed, almost all known antibiotic gene clusters contain genes for proton- or ATP-dependent transporters, most likely responsible for export of the corresponding secondary metabolites (Mendez and Salas 1998, Mendez and Salas 2001, Martin et al. 2005; Alkhatib et al. 2012). In some cases the formation of a secondary metabolite is coupled to its export (Olano et al. 1995, Bystrykh et al. 1996), showing the essentiality of transporters for antibiotic biosynthesis and resistance. On the other hand, there are numerous precedents of successful disruptions of transporter genes within antibiotic gene clusters that have no discernible phenotype or influence on antibiotic efflux/resistance (Sanchez et al. 2006, Ostash et al. 2007 and 2008). It is likely that the immense structural and biological diversity of secondary metabolites

dictated different pathways of evolution of their efflux systems, which can rely on genes located outside the antibiotic gene clusters (Blanc et al. 1995, Sheldon et al. 1999, Sletta et al. 2005).

The biosynthesis of cell wall active antibiotics is an interesting case from the “export” point of view. In actinomycetes, gene clusters for beta-lactams and glycopeptides all contain genes for transport-related proteins (Liras and Martin 2006, Menges et al. 2007). As the targets for these very potent antibiotics are situated on the cell surface, their export is not a resistance mechanism. Rather, it reflects the general need for secretion of the compounds for them to reach their targets on other bacteria, as evident from recent work on ABC transporter gene *tba* from the balhimycin gene cluster (Menges et al. 2007). Nevertheless, much remains to be learned about actual roles of transporter proteins in the biosynthesis of glycopeptides and other families of cell wall active antibiotics.

We recently identified a gene cluster for biosynthesis of moenomycin A (MmA, Fig. 1), a structurally unique antibiotic that inhibits peptidoglycan biosynthesis (Ostash et al. 2007). Whereas beta-lactams target the transpeptidase domain of penicillin binding proteins (PBPs), MmA inhibits the glycosyltransferase domain of PBPs (Ostash et al. 2010). MmA is considered a blueprint for the development of a new class of antibiotics against Gram-positive pathogens, necessitating further exploration of biosynthetic approaches to generation and/or overproduction of MmA analogs (Yuan et al. 2008, Ostash et al. 2009, Makitrinsky et al. 2010). To date, almost all MmA biosynthetic genes (*moe*) have been studied experimentally, with the exception of two pairs of transporter genes *moeD5moeJ5* and *moeX5moeP5* encoding different types of ABC transporters (Ostash and Walker 2010; Fig. 2). In this study, we characterize both gene pairs through gene disruption experiments. Our results show that these genes are important for efficient production of MmA. They also modestly influence the efflux of moenomycins and have no effects on the resistance of the producing organisms to MmA.

Materials and Methods

Microorganisms, vectors and culture conditions

S. ghanaensis ATCC14672 was used as a host for *moe* gene disruption and overexpression. *S. lividans* TK24 and *S. albus* J1074 (Makitrinsky et al. 2010) were used to express different cosmids harboring *moe* genes. *Escherichia coli* DH5 α was used for routine subcloning. *E. coli* ET12567 (pUB307) was used to perform intergeneric conjugation from *E. coli* to *Streptomyces ghanaensis*. *Bacillus cereus* ATCC19637 was used as a moenomycin-sensitive test-culture. RedET-mediated gene replacements (Datsenko et al. 2000) in cosmids moeno38 and moeno38–55 (Fig. 2) were carried out with the help of REDIRECT system (Gust et al. 2003). *Streptomyces* expression vectors pKC1139, pKC1139E and *moe* cluster-harboring cosmids moeno38, moeno38–55 were described previously (Kieser et al. 2000; Ostash et al. 2007; Makitrinsky et al. 2010). Plasmids pHP45 Ω and pHYG2 (Kieser et al. 2000) were used as a source of spectinomycin and hygromycin resistance genes *aadA* and *hyg*, respectively. For sporulation and intergeneric matings, *S. ghanaensis* strains were grown on OM medium at 30 °C (Luzhetskyy et al. 2001). For moenomycin production *S. ghanaensis* and heterologous strains were grown in TSB medium or TSB agar (TSA) at 37 and 28 °C, respectively. *E. coli* strains were grown under standard conditions (Sambrook et al. 2001).

Plasmids and cosmids construction

Plasmid pOOB38a for *moeD5moeJ5* expression was generated as follows. Both genes along with 200 bp upstream region were amplified from cosmid moeno38 with primers con71end

and con72start (see Table 1). The resulting 3.8 kb PCR product was digested with XbaI and EcoRI and cloned into respective sites of pMKI9 (Ostash et al. 2007). Plasmid pOOB62 for *moeX5moeP5* expression was constructed through amplification of these genes with primers moeP5XbaIup and moeX5EcoRIrp and cloning of the resultant 1.5kb PCR product into XbaI and EcoRI sites of pMKI9. The *ermEp-moeX5moeP5* fragment was retrieved as a HindIII-EcoRI fragment from pOOB62, treated with Klenow fragment and ligated to EcoRV-digested pOOB5 (Ostash et al. 2007) to give pOOB62f. Plasmid pOOB53 carrying *moeX5::aadA* allele was generated as follows. The gene *moeX5* was amplified with primers X5HindIIIup and moeX5EcoRIrp, digested with HindIII and EcoRI and cloned into respective sites of pKC1139 to give pOOB30. There is unique *Bam*HI site within pOOB30 located at the 3' end of *moeX5*. BamHI-digested pOOB30 was treated with Klenow fragment and ligated to *aadA* (retrieved as 1.6 kb DraI fragment from pHP45Ω), yielding pOOB53. Expression cosmid moeno38–311 (*moeD5moeJ5* replacement with *kanR*). Kanamycin resistance gene (*kanR*) from plasmid pKD4 (Datsenko et al. 2000) was amplified with primers 38start-KD4 and moeJ5-P1. The resulting amplicon was used to replace *moeJ5moeD5* gene pair as well as the entire nonessential “left arm” of moeno38–55 (Fig.1). We did not evict *kanR* gene region from moeno38–10 because it did not exert any negative effects on MmA production. Throughout this work, the replacement of *moe* genes was confirmed via diagnostic PCR (e.g., primers P2-KD4 and con72start for *moeD5moeJ5* knockout). Expression cosmid moeno38–201 (*moeD5moeJ5* replacement with *kanR* plus *moeX5::aadA* disruption). Gene *moeX5* was replaced in moeno38–311 with *moeX5::aadA* allele retrieved as 1.5kb HindIII-EcoRI fragment from pOOB53. Cosmids moeno38dDJ5 and moeno38dDJ5Hy for *moeD5moeJ5* knockouts in *S. ghanaensis*. The *aac(3)IV-oriT* cassette from pIJ773 was amplified with primers moeJ5-aac-up and moeD5-aac-rev. The resulting amplicon was used to replace *moeJ5moeD5* genes in cosmid moeno38 with *aac(3)IV-oriT*, yielding moeno38dD5J5. with Gene *hyg* was amplified with primers P1Am-Hyg-up and P2A-Hyg-rp and used to replace *aac(3)IV* in moeno38dD5J5, giving moeno38dDJ5Hy. Cosmid moeno38dXP5 for *moeX5moeP5* knockout in *S. ghanaensis*. The *aac(3)IV-oriT* cassette from pIJ773 was amplified with primers P5-RED-up-aac and X5-RED-rp-aac. The resulting amplicon was used to replace *moeX5moeP5* genes in cosmid moeno38 with *aac(3)IV-oriT*, yielding moeno38dXP5.

Generation and verification of the *S. ghanaensis* mutants and heterologous strains containing derivatives of cosmid moeno38–55

All constructs were transferred into *Streptomyces* conjugally. Gene replacements in *S. ghanaensis* were generated as described in (Gust et al. 2003). Kanamycin- and hygromycin-resistant transconjugants carrying cosmid moeno38–55 and *S. ghanaensis* mutants deficient in certain transporter *moe* genes were checked for the stability of inheritance of antibiotic resistance phenotype. 200 clones of each transconjugant were checked after three passages under nonselective conditions and all were found to retain the expected phenotype. PCR was employed to confirm the presence of the cosmids and expected gene replacements in the chromosomes of streptomycetes. For this purpose relevant primers were used as described in Table 1. For example, to check *moeD5moeJ5* replacement with *aac-oriT* cassette, *aac*-specific primer aac-rev and *moeJ5p*-specific primer (con72-start) were used in PCR assay. In all cases the expected pattern of amplification was observed, confirming the correct genetic rearrangements

MmA resistance determination

Survival of spore suspensions of various *S. ghanaensis* strains was determined after plating on TSB agar supplemented with increasing concentrations of MmA (0-20-50-100 mcg/ml). Pure MmA was provided by Prof. D. Kahne (Harvard University). Each experiment was duplicated and data have been averaged.

Determination of moenomycins production

This was carried out essentially as described in (Makitrinsky et al. 2010). Growth time was reduced to 72 h and quantity of moenomycins in biomass and spent medium was determined separately. The cells were exhaustively extracted three times; fourth extraction did not contain any measurable amounts of moenomycins, confirming that all antibiotic has already been recovered (data not shown). Following compounds (shown on Fig. 1) were monitored via LC/MS in the extracts: MmA ($[M-H]^- = 1580.6$ Da), nosokomycin B (NoB; $[M-H]^- = 1484.6$ Da; MmA precursor) and nosokomycin B₁ (NoB₁; $[M-H]^- = 1500.6$ Da; principal moenomycin accumulated by *S. lividans* and *S. albus* strains expressing moeno38–55 and its derivatives). All LC/MS data were acquired on an Agilent 6520 Q-TOF spectrophotometer. The levels of moenomycin production were referred back to equal amounts of dry biomass (10 mg) in different strains.

RT-PCR analysis

Qualitative analysis of transporter genes expression in *S. ghanaensis* was carried out as described (Makitrinsky et al. 2010). qRT-PCR of *moeO5* and *moeE5* expression in *S. ghanaensis* followed the procedure outlined in (Ostash et al. 2011).

Results

Characterization moenomycin production and *moe* transporter gene expression in the parent *S. ghanaensis* strain (ATCC14672)

Several previous studies centered on the fermentation of various moenomycin producers have suggested the amounts of moenomycin complex in spent medium (Sattler et al. 1975, Huber 1979). However, both different culture conditions and strains were employed in these experiments. Furthermore, since moenomycin production was analyzed during the late stages of fermentation, there is a possibility that part of the extracellular moenomycin came from lysed cells, confounding the contribution of transport processes. Since the precise nature and distribution of the moenomycin complex produced by ATCC14672 is crucial for our study, we revisited this question prior to undertaking experiments involving the exporter genes. Under our fermentation conditions, *S. ghanaensis* reached the peak of accumulation of viable mycelia after 70 h of growth (approximately $(3.7 \pm 0.6) \times 10^7$ cfu); after 80 h cfu number dropped 3-fold. Therefore, unless otherwise stated, for all measurements of moenomycin production/export we used 3-day old liquid cultures of *S. ghanaensis* and heterologous strains. At this timepoint, ATCC14672 accumulated roughly equal amounts of MmA (carboxyl group of terminal sugar decorated with chromophore; Fig. 1) and NoB (carboxyl group converted into carboxamide). Quantitatively, these two compounds (hereafter referred to as total moenomycin) constitute 95% of all moenomycins produced by ATCC14672; the rest are moenomycins A₁₂, C₁ (Ostash and Walker 2010) and other unidentified phosphoglycolipids whose production did not follow a regular pattern. The production profile in which MmA and NoB are equidominant species was not changed during late stages of fermentation (6 days), although the absolute amount of the total moenomycin increased 1.5–2 fold as compared to 3 days of growth (data not shown). We revealed that 25–30% of total moenomycin is located in the media (Fig. 3), a significantly higher value than previously suggested (around 10%), which may be accounted for by our more precise method measurements. We surmised that ATCC14672 is engaged in active export of moenomycins throughout the fermentation process.

Genes *moeD5-moeJ5* and *moeX5-moeP5* with high similarity to other ABC transporters were considered the primary candidates for moenomycin efflux since they are in the moenomycin biosynthetic cluster (Ostash et al. 2007). Also, homologs of the aforementioned genes are located in a hypothesized *moe* cluster in *S. clavuligerus* (Medema

et al. 2010, Ostash and Walker 2010). According to the established classification (Mendez and Salas 1998), *moeD5* and *moeI5* are typical type III ABC transporters with both transmembrane (TMD) and nucleotide binding (NBD) domains fused into one polypeptide. Genes *moeX5* and *moeP5* encode individual TMD and NBD, respectively, the two components of a type I ABC transporter. Both genes pairs are actively transcribed on the 3rd day of fermentation and they form transcriptional units, as evident from generation of amplicons S2 and S6 during RT-PCR (Fig. 4). We also checked the expression of these genes after 24 h and 48 h of growth, although ambiguous results (faint signals or their absence) did not allow thorough analysis (data not shown). Although transcriptional coupling of *moeX5* and *moeP5* was anticipated from physical overlap of these genes, the dicistronic *moeD5moeI5* transcript is rather unusual given the apparent 177 bp spacer between these genes.

Overexpression and disruption of *moe* transporter genes in *S. ghanaensis*

Introduction of pSG5-based replicative plasmids pOOB38a (*ermEp-moeD5moeI5*) and pOOB62 (*ermEp-moeX5moeP5*) for the overexpression of the respective *moe* transporter genes into *S. ghanaensis* did not influence moenomycin resistance or production (data not shown). Therefore, we resorted to a gene knockout approach to better understand the functions of the studied genes. Using REDIRECT technology, three *S. ghanaensis* mutants were generated: dDJ5 (Δ *moeD5moeI5*), dXP5 (Δ *moeX5moeP5*) and dDJXP (deletion of all 4 *moe* transporter genes). These mutants did not differ from the parental strain in resistance to MmA and other antibiotics, growth rate or sporulation (data not shown). In comparison to ATCC14672, all mutants produced less moenomycins (Fig. 3). This was particularly obvious for dXP5, whose average total moenomycin level was reduced twofold, while for dDJ5 we observed 1.5-fold decrease. When looking at the levels of extracellular and intracellular moenomycin separately, it appears that the disruption of *moeD5moeI5* did not influence moenomycin export while the *moeX5moeP5* knockout did. The dispensability of *moeD5moeI5* for moenomycin export was highlighted by the fact that *moeD5moeI5* disruption in context of dXP5 mutant (strain dDJXP) did not enhance its moenomycin transport deficiency. The quadruple mutant still excreted moenomycins (Fig. 3). Using pSET152-based plasmid pOOB62f, we reintroduced genes *moeX5moeP5* into *attB⁹C31* site of *S. ghanaensis* dXP5; the resulting transconjugant had moenomycin production and distribution similar to that of wild type strain (data not shown). This ruled out the contribution of unexpected genetic rearrangements or polar effects to dXP5 phenotype.

To evaluate whether the transporter deletion strains affected the transcription levels of any other biosynthetic genes, we examined the expression of key structural genes *moeE5* and *moeO5* in wild type and dXP5 strain. No decrease in *moeE5* and *moeO5* expression was revealed in dXP5 compared to wild type (Fig. 5). In fact, *moeO5* expression was slightly higher in dXP5, possibly because of the read-through from the *aac(3)IV* promoter of the resistance gene cassette used to replace *moeX5moeP5* genes.

Expression of cosmid moeno38–201 in *S. lividans* TK24 and *S. albus* J1074

The ability of the dDJXP strain to export moenomycins in the absence of MoeD5MoeI5 and MoeX5MoeP5 points to the existence of transporters encoded by genes outside *moe* clusters capable of excreting moenomycins. We wondered whether such transporter(s) are unique to *S. ghanaensis* or they are present in other streptomycetes. To address this issue, we expressed cosmids moeno38–55 (full *moe* cluster; Fig. 2) and moeno38–201 (*moe* cluster with deletion of 4 *moe* transporter genes) in *S. lividans* TK24 and *S. albus* J1074, two known heterologous hosts for moenomycin production (Makitrinsky et al. 2010). The cosmids direct the production of nosokomycin B₁ (NoB₁; Fig. 1). This is derivative of NoB, carrying N-acetylglucosamine instead of chinovosamine as a second carbohydrate ring

(counting from terminal carboxamide group of NoB) because genes *moeR5moeS5* are absent in moeno38-5 and its derivatives (Ostash et al. 2007). Results of these experiments, summarized in Fig. 6 agree with data from the analysis of the *S. ghanaensis* mutants, namely that moenomycin export continues in the absence of the *moe* transporter genes, although its production is clearly reduced.

Discussion

In this work we have carried out a set of experiments aimed to provide insights into the export of moenomycins from producing cells. Two transporter systems encoded by *moeD5-moeJ5* and *moeX5-moeP5* genes within *moe* cluster 1 were studied through gene disruptions. These studies showed that both transport systems are involved in modulation of MmA production level, but they do not contribute at all to MmA resistance. As to their role in MmA export, we revealed that *moeX5-moeP5* participate in this process, while *moeD5-moeJ5* genes apparently do not. The latter are actively transcribed during MmA production, suggesting their relation to its biosynthesis, although the exact mechanism still remains elusive. Further investigations are needed to fully understand how the disruptions of transporter genes decrease the level of moenomycin production. Analysis of *moeE5* and *moeO5* expression in wild type and dXP5 strains suggests that the deletions of transporter genes did not affect the transcription of structural *moe* genes. Hence, posttranscriptional regulatory mechanisms are the most likely explanation for the observed decrease in moenomycin production. For instance, MoeX5-MoeP5 and/or MoeD5-MoeJ5 could serve as the membrane “anchor” necessary for efficient formation of the complex of enzymes involved in MmA biosynthesis. Failure to form such a complex could greatly diminish the moenomycin production. This kind of regulatory mechanism has been described for virulence polyketide production by *Mycobacterium tuberculosis* (Jain and Cox 2005).

Although MoeX5-MoeP5 system is implicated in moenomycin export, clearly it is not the only player in this process. The ability of heterologous strains to extrude moenomycins in the absence of MoeD5-MoeJ5 and MoeX5-MoeP5 underscores the existence of MmA exporting genes outside the *moe* cluster. Actinomycetes are famous for carrying multidrug exporter genes in their genomes, (Lee et al. 2007). Alternatively, MmA extrusion could be mediated by exporters from primary metabolism, particularly those involved in cell wall biogenesis.

Secretion of antibiotics is a poorly understood aspect of the biology of their producers with relevant biotechnological value. For example, weak export of the antitumor polyketide doxorubicin from *S. peucetius* cells has been recently shown to decrease the overall productivity of the strain (Malla et al. 2010, Song et al. 2011). A similar situation probably applies to many recombinant pathways (Ostash et al. 2010a), effectively limiting the potential of combinatorial biosynthesis in drug development. This study shows that expression of *moe* transporter genes as well as the overall efficiency of moenomycin export must be taken into account during development of moenomycin overproducers.

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	R ₁	R ₂
moenomycin A		H
nosokomycin B	NH ₂	H
nosokomycin B ₁	NH ₂	OH

Fig. 1.
Structures of moenomycins (Mms) mentioned in this work

Fig. 2. Genetic organization of the MmA biosynthetic (*moe*) gene cluster 1. Seven black rectangles beneath the *moe* genes indicate fragments amplified during RT-PCR analysis of transporter *moe* genes. Cosmids *moeno38* and *moeno38-55* and transporter gene expression plasmids pOOB38, pOOB62, pOOB62f are shown schematically.

Fig. 3. Total moenomycin distribution between the biomass (grey rectangle) and spent medium (white) of parental strain (wt) and transporter mutants (dDJ, dXP, dDJXP) of *S. ghanaensis* (72 h of fermentation). Above the bars, a ratio of extracellular moenomycin to intracellular moenomycin is shown. Error bars indicate the standard deviations

Fig. 4. RT-PCR analysis of expression of *moe* transporter genes. **A.** Scheme showing the genetic organization of transporter genes and the location of seven 340–345 bp fragments (S1–S7) amplified during RT-PCR. **B.** Results of RT-PCR. C+, positive control (*rna* transcript); C–, negative control (*rna* amplification from RNA sample in absence of RT)

Fig. 5.

Expression of *moeE5* and *moeO5* genes in dXP5 mutant. Signals were standardized to *rrnA* levels, and fold changes in expression were calculated as the [value of *moe* expression in dXP5] / [value of *moe* expression in wt] ratio. Data represent the averages of two independent experiments and error bars indicate the standard deviations.

Fig. 6. Nosokomycin B₁ (NoB₁) distribution between the biomass (grey rectangle) and spent medium (white) of *S. lividans* TK24 and *S. albus* J1074 strains expressing either moeno38–55 cosmid (liv-55 and alb-55, respectively) or moeno38–201 cosmid (liv-201 and alb-201, respectively). Above the bars is a ratio of extracellular NoB₁ to intracellular NoB₁.

Table 1

Primers used in this work

Primer name	Sequence, 5'-3'	Purpose
moeD5_P1	CTGCGCGGCTCGGCCCGCACCTACTGG ACCCTCACCGGTGTAGGCTGGAGCTGCTTC	To replace <i>moeD5J5</i> in moeno38-1 with <i>kanR</i> gene
moeJ5_P1	CTGCGCGGTGAACGGACCGCCGTGGCGC TGCTCGCCCTCGTGTAGGCTGGAGCTGCTTC	
moeP5XbaIup	AAATCTAGACCGATACGCGCTATGTG	To clone <i>moeX5moeP5</i>
moeX5EcoRIrp	AAAGAATTCTGTGGGTAGGGAGACTTTG	
X5HindIIIup	AAAAGGCCCTGCCACCTGCGGATGGCG	To clone <i>moeX5</i> to clone <i>moeD5J5</i> genes
con71end	AAAGAATTCTCACCCGCGGGAGGGCT	
con72start	AAAGAATTCTAGAAATCCCCCTTGTTTCGCTC	
X5-RED-rp-aac	TCACGCGAATTGCAGATGGCGCCGGCGCGCAATCGGCCTGTA GGCTGGAGCTGCTTC	To replace <i>moeX5P5</i> in moeno38 with <i>aac-oriT</i>
P5-RED-up-aac	ATGGGCCATTCCGTCGGTGCCCGAGAGGGGTACCACGGCATTC CGGGATCCGTCGACC	
moeJ5-aac-up	CTGCGCGGTGAACGGACCGCCGTGGCGCTGCTCGCCCTCATTC GGGGATCCGTCGACC	To replace <i>moeD5J5</i> in moeno38 with <i>aac-oriT</i>
moeD5-aac-rev	TCACCCCGCGGGAGGGCTCTGCGGAGCGGGCCCGCGTCGTGA GGCTGGAGCTGCTTC	
P1Am-Hyg-up	GTGCAATACGAATGGCGAAAAGCCGAGCTCATCGGTCAGCCCG TAGAGATTGGCGATCCC	To replace <i>aac</i> gene in moenod5J5 with <i>hyg</i>
P2A-Hyg-rp	TCATGAGCTCAGCCAATCGACTGGCGAGCGGCATCGCATCAGG CGCCGGGGCGGTGTC	
moeD5rt-up	TGTTCCAGCGGCTGTTCGA	RT-PCR of <i>moeD5</i>
moeD5rt-rp	CGTCGATCCGCATCATCAC	
moeJ5rt-up	ACCTGTGCGCGGATTCAT	RT-PCR of <i>moeJ5</i>
moeJ5rt-rp	ACGAGGATGCCGACGATCA	
moeJ5rt-gap	GATCCTGGTGCTGGAGGAG	RT-PCR of <i>J5-D5</i> intergenic region
moeD5rt-gap	GGTGAGGGTCCAGTAGGTG	
moeX5rt-up	ATGACCTTCCGCGAACTGC	RT-PCR of <i>moeX5</i>
moeX5rt-rp	AGTGTGGTCAGCGCCAGTT	
moeP5rt-up	GGCGTCGTGAAACGCTACA	RT-PCR of <i>moeP5</i>
moeP5rt-rp	ACAGGTCCTTGCAAGGAC	
moeP5-gap	GTCTTGCCAAGGACCTGT	RT-PCR of <i>X5-P5</i> intergenic region
moeX5-gap	GCAGTTCGCGGAAGGTCAT	
aac-up	GGGCCACAGGCAGAGCAGA	Primers to check <i>aac(3)IV</i> gene
aac-rev	GAGCCACCTGTCCGCAAG	
RtE5R2	GGAAGAGCTTCCTCGAGAC	RT-PCR analysis of <i>moeE5</i> expression
RtE5F	CACACGGAACGGACTTAGC	
RtO5F	CTGTCGAGGTACTCGGTGA	RT-PCR analysis of <i>moeO5</i> expression
RtO5R2	GGAAGAGCTTCCTCGAGAC	