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MoeH5: a natural glycorandomizer from the moenomycin biosynthetic pathway

Bohdan Ostash^{1,2,*}, Jennifer Campbell^{2,†}, Andriy Luzhetskyy³, and Suzanne Walker^{2,*}

¹Department of Genetics and Biotechnology, Ivan Franko National University of Lviv, 4 Hrushevskoho st., Lviv 79005, Ukraine

²Department of Microbiology and Immunobiology, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115, USA

³Helmholtz Institute for Pharmaceutical Research, Saarland Campus, Building C2.3, 66123 Saarbrücken, Germany

Summary

The biosynthesis of the phosphoglycolipid antibiotic moenomycin A attracts the attention of researchers hoping to develop new moenomycin-based antibiotics against multidrug resistant Gram-positive infections. There is detailed understanding of most steps of this biosynthetic pathway in *Streptomyces ghanaensis* (ATCC14672), except for the ultimate stage, where a single pentasaccharide intermediate is converted into a set of unusually modified final products. Here we report that only one gene, *moeH5*, encoding a homologue of the glutamine amidotransferase (GAT) enzyme superfamily, is responsible for the observed diversity of terminally decorated moenomycins. Genetic and biochemical evidence support the idea that MoeH5 is a novel member of the GAT superfamily, whose homologues are involved in the synthesis of various secondary metabolites as well as K and O antigens of bacterial lipopolysaccharide. Our results provide insights into the mechanism of MoeH5 and its counterparts, and give us a new tool for the diversification of phosphoglycolipid antibiotics.

Introduction

Moenomycins are a small group of phosphoglycolipid secondary metabolites of actinomycete origin that have potent antibacterial activity (Ostash and Walker, 2010). They act through direct inhibition of peptidoglycan glycosyltransferases (PGTs) involved in bacterial cell wall formation (Welzel 2005; Lovering *et al.*, 2007; Gampe *et al.*, 2011). Moenomycins are the only known natural products that directly target PGTs and they are active against vancomycin- and methicillin-resistant Gram-positive pathogens (Ostash and Walker, 2005; Halliday *et al.*, 2006). In fact, the average minimal inhibitory concentration (MIC) of moenomycins against Gram-positive bacteria is among the lowest for all known antibiotics, highlighting the exquisite adaptation of these compounds to PGT inhibition (Chen *et al.*, 2003; Yuan *et al.*, 2008). Furthermore, a mixture of naturally occurring moenomycins has been successfully used in animal nutrition under the trademark Flavomycin® or Flavophospholipol® for more than 40 years, and no moenomycin resistance has been observed (Pfaller, 2006).

* Address for correspondence: 4 Blackfan Circle, HIM Room 1013 Boston, MA 02115 Tel/fax: 617-432-5488/617-738-7664 suzanne_walker@hms.harvard.edu. Address for correspondence: 4 Hrushevskoho st. Rm 102 Lviv 79005 Tel/fax: 380-032-2394407/032-3294475 bohdanostash@gmail.com.

† Present address: Fraunhofer CMI, 15 Saint Mary's Street, Brookline, MA 02446, USA

Moenomycin A (MmA, Fig. 1), a prototypical member of the family, was first isolated from *Streptomyces ghanaensis* (ATCC14672) almost 50 years ago (Wallhauser *et al.*, 1965). MmA is considered a blueprint for the development of a new class of antibiotics to counter the growing threat of antibiotic resistance in hospitals and in the community (Ostash *et al.*, 2010). Despite numerous positive traits, the use of MmA in medicine is complicated by low oral bioavailability and an extremely long half-life in the bloodstream (Ostash and Walker, 2010). It is therefore important to explore the chemical space around the moenomycin scaffold to understand how various changes in structure influence the activity and pharmacokinetics, and, subsequently, to generate improved analogs.

Structural variations around the carbohydrate B ring are the source of the greatest natural diversity in the moenomycin antibiotic family. The B ring is present in its simplest form, D-galacturonic acid, in nosokomycin A (NoA; Fig. 1). Nosokomycin B (NoB) carries a carboxamide instead of the free carboxyl group of the B ring (Uchida *et al.*, 2010), while in MmA, the B ring is extended with the chromophore, 2-amino-3-hydroxy-2-cyclopenten-1-one (A ring). Although no rigorous structural exploration has been carried out, early studies of *Streptomyces bambergiensis* and *S. prasinus* suggested that glycine can also be attached to the B ring of moenomycins produced by these strains, (Weisenborn *et al.*, 1967; Schacht and Huber, 1969).

To our knowledge, all of the above mentioned carbohydrate modifications are extremely rare or entirely unprecedented in natural products. The carboxy-amidated sugar, 2-acetamido-2-deoxy-D-galacturonamide (D-GalNAcAN), has only been revealed as a part of lipopolysaccharide (LPS) in the outer membrane of *Escherichia coli* serotype O121 (*Shigella dysenteriae* D7) and *Pseudomonas aeruginosa* O4a (Knirel *et al.*, 1988). K and O antigens of the LPS of certain *Escherichia*, *Proteus*, *Providencia* and *Vibrio* strains were shown to contain amides of D-galacturonate/glucuronate with L-lysine, L-alanine, L-serine or L-threonine (Linnerborg *et al.*, 1997; Kondakova *et al.*, 2003; Ovchinnikova *et al.*, 2005; Whitfield, 2006). No uronate amides with glycine have been reported so far. Although gene clusters directing the biosynthesis of some of the respective polysaccharides have been identified, the biochemistry of these aforementioned modifications and (in the case of amino acid-decorated sugars of O antigens) even their genetic determinants remain elusive (Belanger *et al.*, 1999; Feng *et al.*, 2004; Liu *et al.*, 2008; Wang *et al.*, 2010).

Our previous work on moenomycin biosynthesis suggested that the *moeH5* gene, encoding a putative glutamine amidotransferase (GAT) superfamily enzyme, controls the carboxyamidation of the B ring of NoA, thus leading to NoB (Ostash *et al.*, 2007; Ostash *et al.*, 2009). However, it is not known whether *moeH5* controls the conversion of NoA into NoB alone, or whether it acts together with another GAT-encoding gene *moeF5*, located within the *moe* cluster. Moreover, the attachment of the A ring remained speculative, and the presence of glycine-containing moenomycins was completely unexplored. Given the unique chemical nature of these carbohydrate modifications and the significant contribution of the A ring to bioactivity, we undertook a detailed investigation to gain a complete understanding of the genetics of B ring tailoring reactions during moenomycin biosynthesis. Here, we report that the amidotransferase MoeH5 alone controls the conversion of NoA into a diverse set of more advanced metabolites that includes NoB, MmA and several amino acid-containing moenomycins. Our findings broaden the understanding of the functional diversity of GAT superfamily enzymes, point to their possible roles in LPS production, and offer new prospects for the combinatorial biosynthesis of phosphoglycolipid antibiotics.

Results

Streptomyces ghanaensis produces glycine-decorated moenomycin

It is important to identify all types of terminally decorated moenomycins produced by ATCC14672 if one wants to dissect the biosynthetic basis of B ring modification. Although the presence of the glycine-bearing moenomycins in the extracts from certain strains was suggested decades ago (Schacht and Huber 1969), they were not observed during the recent and very detailed mass spectrometry (MS)-based studies of the industrially-used moenomycin (Flavomycin) complex (Eichhorn and Aga, 2005; Zehl *et al.*, 2006; Gallo *et al.*, 2010). There may be several reasons for that, such as the nature of the strain used for industrial production, peculiarities of the manufacturing process, or the innate lability of amino acid-decorated moenomycins. We therefore explored the possibility of glycine-containing moenomycin production by ATCC14672. The strain was grown in tryptic soy broth (TSB), which supports MmA production. Methanol extracts of the cells were promptly and minimally processed in order not to lose any moenomycin variants (see Methods). Careful analysis of the extract led to identification of a novel compound, referred to as moenomycin G (MmG; Fig. 1), that clearly carried a glycine residue instead of the A ring. The structure of MmG is supported by the results of accurate mass determination, its retention time during liquid chromatography (which is similar to other known moenomycins; Table 1), and its fragmentation pattern in MS² experiments (see Supplemental Information (SI), Fig. S1).

Due to the low productivity of ATCC14672 and difficulties in separation from the other moenomycins, MmG could not be purified in sufficient quantity to determine its antibacterial properties nor its quantitative contribution to the moenomycin mixture. Nevertheless, we (Makitrinsky *et al.*, 2010) and others (Gallo *et al.*, 2010; Zehl *et al.*, 2006) observed that major moenomycins ionize with equal efficiency during routine MS calibration experiments (see also *Methods*). It is reasonable to assume that this is the case for MmG as well. Indeed, the phosphate group of moenomycins is completely deprotonated over a wide pH range irrespective of the structure of the carbohydrate portion (Lantzsche *et al.*, 1998). Hence, it can be inferred from MS data that MmA and NoB were accumulated in roughly equal amounts, making up no less than 90% of the mixture, while MmG accounted for no more than 10% of it. Other known moenomycins, such as A₁₂, C₁, C₄, etc (Ostash and Walker, 2010), were observed in trace amounts and their accumulation did not follow a regular pattern.

Several additional experiments were carried out in an attempt to study the influence of nutritional conditions on MmG production. Particularly, *S. ghanaensis* was grown in several complex liquid media recommended for Flavomycin production (Endler *et al.*, 1998; Subramaniam-Niehaus *et al.*, 1997) as well as in TSB supplemented with glycine to a final concentration of 0.5, 1 or 2% (w/v). Under all conditions tested, MmG remained a minor fraction, quantitatively the same as it is in the absence of exogenously added glycine. Using LC-MS, we further tested for the presence of moenomycins carrying all 20 possible proteinogenic amino acids instead of the A ring, in TSB and TSB supplemented with the respective amino acid (1%). No production of amino acid-decorated moenomycins other than MmG were observed (data not shown).

Genetic evidence that moeH5, and not moeB4, is responsible for incorporation of the A ring, glycine and other amino acids into moenomycins

An initial hypothesis about the genetic control of the B ring modification assumed that MoeB4 is necessary for transfer of the A ring to NoA, and that MoeH5 somehow assists MoeB4 in this reaction (Ostash *et al.*, 2009). The importance of MoeB4 for A ring transfer

was supported mainly by reports of MoeB4 homologues involved in the biosynthesis of A ring-bearing polyketide antibiotics (Zhang *et al.*, 2010; Rui *et al.*, 2010). To verify this hypothesis, we generated two *S. ghanaensis* ATCC14672 mutants, referred to as dH5 and dB4. In the first one, the GAT gene *moeH5* is replaced with the apramycin resistance gene *aac(3)IV*, while the second one carries the *aac(3)IV* in place of amide synthase *moeB4* (see Fig. 2 for location of the *moe* genes). The dB4 mutant showed neither qualitative nor quantitative change in moenomycin production as compared to wild type, whereas dH5 produced NoA exclusively, a moenomycin with an unmodified B ring. (We note that possible changes in production of other secondary metabolites by dB4 were not monitored). Introduction of *moeH5* under control of the *ermEp** promoter (plasmid pOOB47h, Fig. 2) into dH5 restored the production of all moenomycins typical for the wild type strain (Fig. 3). Overexpression in dH5 of another GAT gene, *moeF5* (plasmid pOOB48a), did not complement the *moeH5* deletion.

The dispensability of *moeB4* for production of B ring-decorated moenomycins was also confirmed under conditions of heterologous expression, as detailed in SI Fig. S2–S3. For this purpose we used *S. lividans* TK24 and *S. albus* J1074, which lack the capacity to produce moenomycins (Makitrinsky *et al.*, 2010). Cosmid moeno38-5 in TK24 and J1074 directed the production of nosokomycin B₂ and moenomycin G₂, carrying carboxamide and glycine on their B rings, respectively. Coexpression of moeno38-5 with plasmid pOOB64bd (*moe* cluster 2 lacking *moeB4*, Fig. 2) resulted in production of A ring-containing moenomycin A₇. (Fig. 1 and Table 1). Deletion of *moeH5* (cosmid moeno38-6, Fig. 2) abrogated the production of all B ring-decorated moenomycins, irrespective of the presence or absence of full or truncated *moe* cluster 2 (plasmids pOOB64b and pOOB64bd). We had to conclude that *moe* cluster 2 only serves to produce the A ring, whereas its attachment to nosokomycin A is controlled by the *moe* cluster1-situated gene *moeH5*, either alone or with assistance from other as-yet-unknown proteins. Our results are in stark contrast to those where the role of MoeB4 homologues in the transfer of the A ring to polyketide acceptor substrates was firmly established (Zhang *et al.*, 2010; Rui *et al.*, 2010).

A wider set of amino acids is incorporated into moenomycins under conditions of amino acid feeding and *moeH5* overexpression

The involvement of *moeH5* in the transfer of moieties as diverse as ring A, amine and glycine prompted us to carefully re-investigate the degree of substrate ambiguity of this enzyme. Here, we resorted to our aforementioned biotransformation approach, with the exception that *S. lividans* moeno38-5⁺ and *S. ghanaensis* strains overexpressing *moeH5* (plasmid pOOB47a) were used. All biogenic amino acids as well as some D-forms were fed to the strains and extracts were analyzed via high-resolution mass spectrometry. We detected very low but reproducible accumulation in the biomass of serine-, cysteine- and alanine-containing moenomycins, referred to as I, K, and L, respectively (Fig. 1, Table 1) upon addition of the respective amino acids to TSB (Fig. S4). Production of these compounds was observed in the presence of either L- or D-forms of these amino acids, and the nature of the isomeric form of the amino acids did not influence the yield of the novel compounds (data not shown). Adding other amino acids and cyclopentylamine to the fermentation medium (see Fig. 1, substituent z) did not result in the production of novel moenomycins.

Insights into a potential MoeH5 mechanism through analysis in silico

It is informative to compare MoeH5 with another GAT protein, MoeF5, involved in the carboxyamidation of the F ring of moenomycins (Fig. 1). Function of the latter has been established in a series of genetic (Ostash *et al.*, 2009) and biochemical experiments (S. Walker and D. Perlstein, unpublished data). Results of the domain analysis of MoeF5 and MoeH5, which share 22% identical and 28% similar amino acids, are summarized in SI Fig.

S5–S6. MoeF5 is a typical member of the GAT superfamily with an Ntn-type asparaginase domain for glutamine hydrolysis and an AsnB-like asparagine synthetase domain. An absolutely conserved cysteine Cys₁, a hallmark of the Ntn domain, is present in MoeF5 together with other conserved amino acids (Zalkin 1993). The conserved amino acid motif of the synthetase domain is less recognizable, although most of the conserved residues (along with essential E₂₈₆) and nucleotide-binding motifs can be located (Zalkin 1985). In comparison to MoeF5, the Ntn domain of MoeH5 is significantly truncated, while the synthetase domain is well preserved (Fig. S6). Our findings suggest that MoeH5 is incapable of hydrolyzing glutamine to produce free amines. To convert NoA into NoB, MoeH5 would need to use ammonium ions that passively diffuse into the enzyme, as described for some GATs (Zalkin and Smith, 1998). It is also possible that MoeF5 could cooperate with MoeH5 to provide the latter with ammonia. From this analysis, MoeH5 appears to have evolved from a GAT into a ligase-type enzyme capable of transferring complex amide donor molecules onto NoA.

Database and literature searches demonstrated that GAT-encoding genes are rare in carbohydrate metabolism, and they are confined to moenomycin and LPS biosynthesis (see Introduction). Yet, the GAT superfamily is relatively well represented in gene clusters for biosynthesis of various actinomycete natural products, mostly of polyketide origin. Here, GATs are involved in a number of tailoring reactions, where carboxyamidation is the most common (see Fig. S7). Our attention was attracted to GATs PdmN and Orf3-K40. According to genetic evidence, PdmN transfers an alanine residue to the carboxyl group of the pradimicin A precursor (Zhan *et al.*, 2009), while Orf3-K40 produces an amide from glucuronic acid and L-serine during biosynthesis of the K40 antigen of *E. coli* (Amor *et al.*, 1999). Like MoeH5, PdmN and Orf3-K40 lack the critical Cys₁ residue within the Ntn domain that abolishes its ability to hydrolyze glutamine to amine and glutamate (Zalkin and Smith, 1998). Closer inspection of amino acid sequences of different GATs from secondary metabolic pathways revealed more proteins lacking Cys₁ (Table S1). In all of these cases, the respective natural products carry nitrogen substituents other than carboxamide, or contain no nitrogen moiety at all (e.g., rubromycin, Fig. S7). A phylogenetic tree of GAT proteins involved in secondary metabolism and LPS biosynthesis was built using several different tree-building approaches (e.g., neighbor joining, maximum likelihood, and maximum parsimony). Irrespective of the algorithm being used, the trees had essentially the same topology featuring two clearly distinct clades. One (minor) clade was represented by MoeH5 proteins from phosphoglycolipid biosynthetic pathways of different streptomycete species (Svir, MoeH5-cl etc). These included Orf3-R40 along with its homologues and a GAT of unknown function from exopolysaccharide biosynthetic pathway (Fig. 4). All of the proteins listed above are involved in the modification of carbohydrate moiety, whereas representatives of the major clade have more diverse functions in secondary metabolism and LPS production. The common ancestry of MoeH5 and MoeF5 could not be inferred from this dataset.

MoeH5 alone controls all B ring modifications

All of the results presented above attest to the importance of *moeH5*, although they do not show that *moeH5* alone is responsible for the transfer of three quite different moieties to the carboxylic group of NoA. It is safe to suppose that no genes outside of the *moe* clusters assist *moeH5* in these reactions, because it was possible to recreate production of moenomycins in two heterologous hosts through coexpression of *moe* cluster 1 and *moeC4moeA4*. However, the participation of *moe* cluster 1 genes other than *moeH5* in this process is impossible to rule out based on *in vivo* studies. As mentioned above, MoeF5 may be a MoeH5 partner. We therefore set out to re-create *in vitro* the reactions of conversion of NoA into MmA, NoB and MmG. Purification of active His-tagged MoeF5 (rMoeF5) has

been carried out according to established protocols (D. Perlstein, unpublished data; see also Experimental Procedures section). After a period of unsuccessful experimentation with different *E. coli* expression systems, we were able to produce soluble C-terminally hexahistidine tagged MoeH5 (rMoeH5; plasmid pOOB83e, Fig. 2) in *Streptomyces lividans* TK24 (Fig. 5). Although His-tags decreased the *in vivo* activity of rMoeH5 (Fig. S8), it was active enough for initial characterization of this enzyme.

We incubated rMoeH5 or rMoeH5+rMoeF5 in suitable buffer and in the presence of ATP, acceptor substrate (NoA) and various amide donor substrates – D,L-glutamine, NH₄Cl, glycine, L-serine A ring, and cyclopentylamine. Results of these experiments are summarized in Fig. 6 and Fig. S9–S12. Glutamine did not support the conversion of NoA into NoB, while NH₄Cl did. MmG and MmI were produced in the presence of glycine and serine, respectively, and MmA was produced in the presence of A ring. Production of novel moenomycin J (Fig. 1) was observed in the presence cyclopentylamine. All reaction products were readily observed in the presence of MoeH5 only, and their yield was not changed by the presence of MoeF5. Omission of ATP from the reaction mixtures completely abolished the production of the aforementioned products.

B ring modifications render moenomycins more antibacterially active

We compared the antibacterial activities of MmA, NoB and NoA against several *Staphylococcus aureus* strains, including two methicillin-resistant ones. These results are summarized in Table 2 and clearly show that substituents on the B ring carboxyl group increase the potency of moenomycins. Namely, NoA is approximately 5 times less active than NoB, and the latter is more than 10 times less active than MmA.

Discussion

The biosynthesis of MmA has been fertile ground for the discovery of highly unusual biotransformations (Schuricht *et al.*, 2001; Ostash *et al.*, 2009; Doud *et al.*, 2011; Ren *et al.*, 2012). To date, these were limited to the initial steps of the biosynthesis, namely the assembly of the lipid-disaccharide portion of MmA (units E-F-G-H, see Fig. 1). Here, we elucidate the biosynthetic logic behind the ultimate step of phosphoglycolipid antibiotic biosynthesis – conversion of NoA into a diverse set of B ring-decorated moenomycin congeners. Results of *in vivo* and *in vitro* experiments strongly suggest that the single GAT-type enzyme MoeH5 transfers onto NoA different amine-bearing groups, as diverse as ammonia, glycine, serine, A ring, and cyclopentylamine. To the best of our knowledge (Whitfield, 2006; Liu *et al.*, 2008; Thibodeaux *et al.*, 2008), this is the first description of a carbohydrate modification enzyme that leads to formation of both carboxamide- and amino acid-tailored oligosaccharides. Our findings have a number of implications for a better understanding of carbohydrate metabolism and the chemical diversity controlled by GAT-like enzymes, particularly with respect to moenomycin biosynthesis.

Characterization of MoeH5 also sheds new light onto the biosynthesis of other natural compounds. As mentioned in the Introduction, structures of carbohydrate units within a few K- and O-polysaccharide moieties of LPS offer the only structural analogs to the B ring-decorated moenomycins. Biosynthesis of D-GalNAcAN in *E. coli* O121 and *P. aeruginosa* O4 is believed to result from the action of the GAT-like enzyme WbpS (WbqG; Belanger *et al.*, 1999; Feng *et al.*, 2004). It was suggested that carboxyamidation occurs prior to the sugar transfer, at the stage of UDP-activated D-GalNAcAN precursor (Belanger *et al.*, 1999; Feng *et al.*, 2004). Our data are in agreement with an alternative scenario, where carboxyamidation of galacturonic acid takes place after its attachment to the oligosaccharide. It is likely that the same is true for LPS biosynthesis. Our phylogenetic analysis (see Fig. 4) showed that MoeF5 groups together with WbpS/WbqG and MoeH5 is

on one clade with Ste10. The latter is encoded within a gene cluster for the biosynthesis of an exopolysaccharide of unknown structure (Wang *et al.*, 2003). We can now suggest that Ste10 controls the formation of an amide-bearing carbohydrate in its respective biosynthetic pathway.

Whereas the genetics and biochemistry of galacturonamide units found in O antigens and moenomycins appear to be similar, the same cannot be suggested for amino acid-decorated counterparts. Only the *E. coli* O8-K40 K antigen biosynthesis gene cluster contains a MoeH5 homologue, Orf3-K40, responsible for L-serine transfer to a glucuronate residue (Amor *et al.*, 1999). The *Vibrio vulnificus* (ATCC27562) gene cluster responsible for production of L-serine-decorated capsule polysaccharide does not contain an Orf3-K40 homologue. In ATCC27562, the function of serine transfer was assigned to protein CppA (Nakhamchik *et al.*, 2010), which, according to our BLAST-assisted analysis, is similar to a number of glycosyltransferases. Likewise, there is plenty of genomic data on enterobacterial strains producing alanine-, lysine- and serine-tailored O antigens, and they clearly show the absence of WbpS/MoeH5 homologues within O antigen biosynthetic loci or elsewhere in the genomes (Liu *et al.*, 2008; Pearson *et al.*, 2008; Wang *et al.*, 2010). However, using the structural homology search program HHpred (Soding *et al.*, 2005), we revealed within K/O antigen biosynthesis a group of genes for ATP-grasp amino acid ligases, originally annotated as glycosyltransferases. The group includes proteins WemR, WemB, WfdG and WcvD encoded within gene clusters for production of amino acid-decorated K/O antigens; several other proteins are from strains whose O antigen structures remain unknown (Fig. S13). Interestingly, these proteins strongly resemble a number of amidoligases involved in cell wall biosynthesis and glycopeptide resistance, and they are also moderately similar to the larger subunit of carbamoyl-phosphate synthetase (CPS). The latter is known to form a complex with class I GAT enzymes, in order to be provided with ammonia (Thoden *et al.*, 2002).

Significant mechanistic differences exist between GAT and ATP-grasp amidoligases. Whereas GAT proteins activate carboxylate substrates through formation of acyl-AMP intermediates, the amidoligases proceed through acylphosphate intermediates (Fawaz *et al.*, 2011). The biosyntheses of teichurono peptide, D-Ala-D-Ala, mycothiol and purines provide well-studied examples of amidoligase-mediated transfer of peptides or amino acids onto the carbohydrate unit, which demonstrate similarities to and differences from the GAT-like mechanism of MoeH5/WbpS (Iyer *et al.*, 2009; Newton *et al.*, 2008). Thus, whereas GAT superfamily enzymes can govern carboxyamidation of galacturonate in moenomycin and LPS biosyntheses, two different strategies may lead to uronate amides with amino acids. MoeH5 produces the latter in the moenomycin biosynthetic pathway, while related ATP-grasp amidoligases are likely candidates for that purpose in antigen pathways. Although MoeH5/WbpS are phylogenetically different from WemR-type proteins, the loss of the functional glutaminase domain by MoeH5 (and, most likely, by its homologues – Orf3-K40, PdmN, RubR, GrhP, TblS, PieD) is indicative of its evolution into an amidoligase enzyme. In this regard, we note that ATP-grasp ligases (such as D-Ala-D-Ala ligase) and the aforementioned GAT enzyme PdmN use D-amino acids. In our work, D- and L-forms of amino acids were used in biotransformation and *in vitro* experiments, and it remains to be studied whether both forms or just one of them were used by MoeH5. Nevertheless, it is reasonable to expect that MoeH5, like its homologue PdmN, possesses certain potential to accept D-amino acids. MoeH5 therefore represents an interesting expansion of functional diversity of the GAT enzyme superfamily, which will surely be broadened in due course by investigations into MoeH5 homologues.

The two-cluster organization of the *moe* genes was one of the distinctive features of genetics of moenomycin biosynthesis (Fig. 2). Two genes within *moe* cluster 2, *moeA4* and *moeC4*,

are involved in A ring production (Ostash *et al.*, 2007). The role of amide synthetase gene *moeB4* in A ring attachment to MmA precursor was indirectly supported by the biosynthetic studies of A ring decorated polyketides (Rui *et al.*, 2010; Zhang *et al.*, 2010). Nevertheless, our results show that *moeB4* is not a part of the moenomycin biosynthetic pathway. We note that the main *moe* cluster carries homologs of *moeA4* and *moeC4* – *moeB5* and *moeA5*, respectively (Fig. 2), both of which appear to be nonfunctional (Ostash *et al.*, 2007). Based on our results, we suggest that the two-clustered organization of the MmA biosynthetic pathway in *S. ghanaensis* is not a consequence of the rearrangement of a single *moe* cluster. Rather it could be a result of fortuitous co-occurrence of a fully functional *moe* cluster for MmA biosynthesis and that for A ring tailored polyketide biosynthesis. In the absence of selective pressure, initially functional *moeB5* and *moeA5* accumulated mutations, rendering these genes obsolete and making MmA biosynthesis dependent on *moe* cluster 2. Although the order of acquisition of the *moe* and polyketide gene clusters by *S. ghanaensis* (probably, via lateral gene transfer) is not known, the above hypothesis offers the most parsimonious explanation for the organizational and functional peculiarities of MmA production. Two observations lend support to our conjecture. First, the gene cluster for moenomycin biosynthesis has been recently discovered as a part of the *S. clavuligerus* megaplasmid (Medema *et al.*, 2010). According to our analysis, this cluster contains functional *moeA4* and *moeC4* homologues (and no *moeB4*) together with the rest of the *moe* genes (data not shown). Second, *moe* cluster 2 for A ring production by *S. ghanaensis* is indeed located near the PKS type I gene cluster, suggesting a functional link of *moeB4* to polyketide biosynthesis. The identification of moenomycin biosynthetic pathways from the other actinomycetes will provide better understanding of the evolutionary trajectories of this exceptional secondary metabolic pathway.

Via LC-MS analysis of moenomycin extracts and the elucidation of MoeH5 function, we show that moenomycin biosynthesis culminates with a mixture of final products, of which MmA and NoB are equidominant, and MmG is a minor product. All of these products are a result of the activity of GAT enzyme MoeH5 transferring different amide donors onto NoA, as summarized on Fig. 7. Being deprived of the status of the only final product, MmA is still the most potent moenomycin and PGT inhibitor known to date, as our comparison of MmA, NoB and NoA activities has shown. This underscores significant contribution of the B ring modification to antibacterial activity and justifies further efforts aimed at diversification of moenomycins around the B ring. The undesired properties of moenomycins (such as extremely low oral bioavailability and long half-life in the bloodstream) are thought to be caused by the long C₂₅ isoprene moiety; however, its truncation severely reduces the antibacterial activity (Ostash *et al.*, 2009; Fuse *et al.*, 2010). It will therefore be desirable to compensate the reduced activity of moenomycins with shorter lipids with modification to other parts of the molecule. Recent structural studies show that there is plenty of room in the PGT active site cleft around the chromophore (Ostash and Walker, 2010). More reactive or bulkier functionalities may be introduced in place of the chromophore to create additional contacts to the target, thereby increasing potency. The glycine-bearing moenomycins (presumably, corresponding to MmG described in this work) were reported to exhibit somewhat increased activity against certain bacteria (Schacht *et al.*, 1969). However, past attempts to improve moenomycins via A/B ring modification were unsuccessful due to the low or inadequate structural diversity of the analogs being tested (Ruhl *et al.*, 2003; Ostash and Walker, 2010). In this regard, MoeH5 can be a useful tool for the chemoenzymatic production of novel moenomycins. We note that glycine, alanine, serine and cysteine resemble each other and part of the A ring, and these are the only amino acids shown to be recognized by MoeH5 *in vivo*. However, only glycine and D,L-serine were tested *in vitro*, making it difficult to define the true limit of donor substrate specificity of MoeH5. More extensive research and protein engineering would therefore be needed to fully embrace the potential of MoeH5, both for *in vivo* and *in vitro* approaches towards novel moenomycins.

Antigen biosynthetic pathways might be another source of tools for moenomycin diversification. In fact, our search for *moeH5* counterparts within these gene clusters led to the identification of a group of genes for putative amidoligases, which might transfer amino acids to galacturonate or glucuronate. The possibility of using the discovered K/O antigen biosynthetic genes to produce novel moenomycins is currently being investigated in our laboratories.

Experimental procedures

Chemicals and antibiotics

Pure MmA and freshly synthesized 2-amino-3-hydroxy-2-cyclopenten-1-one hydrochloride, or A ring (Ebenezer 1991; Taylor *et al.*, 2006) were kindly provided by Prof. D. Kahne (Department of Chemistry and Chemical Biology, Harvard University). NoA was purified from *S. ghanaensis* *moeH5* mutant dH5 according to described procedures (Yuan *et al.*, 2008). Cyclopentylamine and amino acids of the highest available purity were purchased from Sigma. For recombinant strain selection, commercially available antibiotics were used ($\mu\text{g/ml}$): ampicillin (100), chloramphenicol (25), kanamycin (50), apramycin (50), and hygromycin (100).

Bacterial strains and culture conditions

Strains, cosmids and plasmids used in this study are listed in Table 3. *E. coli* was grown in liquid Luria-Bertani (LB), or on LB-agar (Sambrook *et al.*, 1989). TSB (Difco) was used to grow *S. ghanaensis* and *S. lividans* strains for moenomycin production and analysis. Modified R5A medium (Makitrinskyy *et al.*, 2010) was used to grow *S. albus* strains for moenomycin production. Unless otherwise stated, *S. ghanaensis* was grown at 37°C, while other streptomycetes were grown at 30°C, all with shaking at 200 rpm. All constructs were transferred into *Streptomyces* conjugally. The presence and stability of inheritance of ϕC31 -based constructs in streptomycetes were checked as described earlier (Ostash *et al.*, 2009a; Ostash *et al.*, 2012).

Procedures for DNA manipulation

Oligonucleotides used in this work are listed in Table 4. Standard procedures were used for plasmid/chromosomal DNA isolation, subcloning and analysis (Sambrook *et al.*, 1989). Polymerase chain reactions (PCR) were performed using recombinant KOD Hot Start DNA polymerase (EMD) and all PCR products were sequenced. RedET-mediated gene replacements in cosmids and plasmids were carried out with the help of the REDIRECT system (Gust 2009).

Moenomycin production analysis

Growth of the strains, moenomycin purification, LC-MS conditions and quantitative analysis of the data are described in Ostash *et al.* (2009), Makitrinskyy *et al.* (2010) and Doud *et al.* (2011). The levels of moenomycin production were normalized to equal amounts of dry biomass (10 mg) in different strains. The cells were exhaustively extracted three times with 8 mL of methanol; the fourth extraction did not contain any measurable amounts of moenomycins, confirming that all moenomycin had already been recovered (data not shown). Under the described LC-MS and MS² conditions pure equimolar samples of MmA, NoB and NoA yield essentially equal ion counts, enabling the use of MS for their quantification. The compounds monitored via LC-MS in the extracts are shown on Fig. 1. LC-MS and MS² data were acquired on Agilent 6520 Q-TOF, Agilent 1110 LC/MSD and Bruker Esquire 3000 ESI-MS spectrometers.

Biotransformation experiments

This experiment was carried out with *S. ghanaensis* and *S. lividans* TK24 moeno38-5⁺ strains carrying *moeH5* expression plasmid pOOB47a. The latter was constructed as follows. A fragment of *moe* cluster 1 encompassing the entire *moeH5* coding region as well as the 3'-end of *moeGT1* was amplified with primers *moeGT1*XbaIup and con73start. The amplicon was digested with restriction endonucleases XbaI and EcoRI and cloned into their respective sites of pKC1139E. The resulting plasmid, pOOB47a, contained *moeH5* under the control of the strong constitutive promoter *ermEp*. This plasmid was conjugally transferred into ATCC14672 and TK24 strains, and transconjugants were verified by PCR. Spore suspensions of pOOB47a⁺ strains of *S. ghanaensis* ATCC14672 and *S. lividans* TK24 moeno38-5⁺ (200 µl; 3–4×10⁸ cfu ml⁻¹) were inoculated into 0.3-L flasks containing 35 ml of TSB and grown for two days at 30 °C. Three milliliters of this preculture were added to a 0.3-L flask containing 35 ml of TSB+ 1% (v/w) amino acid and grown for 72 h. The biomass was extracted with methanol for 4 h. The extracts were dried, dissolved in 100 µl of deionized water, filtered (Sartorius, 0.2 µm) and immediately subjected to LC-MS analysis.

Knockout of the *moeB4* gene in *S. ghanaensis*

The cosmid, moeno5-dB4, for *moeB4* gene knockout was generated as follows. The *aac(3)IV-oriT* cassette from pIJ773 was amplified with primers *moeB4*-red-up and *moeB4*-red-rp. The resulting amplicon was used to replace the *moeB4* gene in cosmid moeno5 with *aac(3)IV-oriT*, yielding moeno5-dB4. *S. ghanaensis* transconjugants carrying moeno5-dB4 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 dB4, was confirmed to be the desired deletion mutant by PCR (Fig. S14). LC-MS revealed no changes in moenomycin production by dB4 compared to its parent strain (see Fig. 3).

Knockout of the *moeH5* gene in *S. ghanaensis*

The *aac(3)IV-oriT* cassette from pIJ773 was amplified with primers *moeH5*-up-aac and *moeH5*-rp-aac. The resulting amplicon was used to replace the *moeH5* gene in cosmid moeno38 with *aac(3)IV-oriT*, yielding moeno38-dH5. Generation of the *moeH5*-deficient mutant dH5 was carried out similarly to that of the dB4 strain. PCR with *aac(3)IV*- and *moeGT1*-specific primers confirmed the replacement of *moeH5* with the *aac(3)IV-oriT* cassette. NoA was the exclusive product of the *moe* pathway in the dH5 strain, (Fig. 3 and Fig. S15); the yield of NoA was approximately 2 mg/L. Production of NoB, MmG and MmA was restored to dH5 upon introduction of plasmid pOOB47h (Fig. 3), confirming that *moeH5* knockout caused no polar effects on the expression of surrounding *moe* genes. Plasmid pOOB47h is a derivative of pOOB47a (see *Biotransformation experiments* section), in which the apramycin resistance marker gene (*aac(3)IV*) was replaced with the hygromycin resistance marker gene (*hyg*) via recombineering (primers p1Am-Hyg-up and p2Am-Hyg-rp).

Generation of heterologous strains coexpressing *moe* cluster 1 and genes *moeA4moeC4*

Plasmid pOOB64bd, a derivative of pOOB64b that carries *hyg* in place of *moeB4*, was generated via recombineering (primers *moeB4*HY-red-up and *moeB4*HY-red-rp). The pOOB64bd⁺ transconjugants of *S. lividans* TK24 moeno38-5⁺ and *S. albus* J1074 moeno38-5⁺ strains were obtained and analyzed as described above. Co-expression of *moe* cluster 1 with the entire *moe* cluster 2 (pOOB64b) was reported earlier to produce the same compounds as observed in this study (Ostash *et al.*, 2009).

Protein expression and purification

MoeF5 with a C-terminal (His)₆ tag was expressed from plasmid pCXL5 in BL21(DE3) pLysS cells. An overnight culture was grown at 37°C in LB. When OD₆₀₀ = 0.5, the culture was cooled to 18°C for 30 min, IPTG was added (1 mM) and cells were harvested ~16 h after IPTG addition. Cell paste (4 g from 1 L culture) was frozen overnight at -80 °C, then thawed and resuspended in 20 mM Tris pH 7.5 (at room temperature), 200 mM NaCl, 0.5% CHAPS, 5 mM imidazole, 1 mM PMSF (added from a 100 mM stock solution in isopropanol) or protease inhibitor mix, and 5% (v/v) glycerol to a final volume of 90 mL. Benzonase (0.1 kU) and rLysozyme (20 kU) were added and the mixture was stirred at room temperature for 45 min. Cell debris was removed by centrifugation (12,000 × g, 45 min, 4 °C) and the soluble fraction was incubated with 2 mL His-bind resin equilibrated in wash buffer (TBS, 30 mM imidazole, 5% glycerol) for 30–60 min at 4°C. Resin was collected and washed with 20 mL wash buffer and eluted with 25 mM Tris pH 7.5 (at room temperature), 150 mM NaCl, 200 mM imidazole, and 5% glycerol. The protein was concentrated by Amicon Ultra with a 50 kDa MWCO membrane to ~0.3 mL and then dialyzed overnight against 1 L of 20 mM Tris pH 8 (at room temp), 1 mM DTT, and 10% (v/v) glycerol.

MoeH5 with a C-terminal (His)₆ tag was expressed from plasmid pOOB83e. The *moeH5* coding sequence and its RBS were amplified from the *S. ghanaensis* genome with primers *moeGT1XbaIup-int* and *h5exp6hisEcoRI* (six His and stop codons were introduced into *moeH5* with the latter), digested with XbaI and EcoRI, and cloned into the respective sites of pKC1139E. The plasmid was introduced into *S. lividans* TK24. A spore suspension of the pOOB83e⁺ strain was inoculated into 35 ml of TSB and grown at 30°C (250 rpm) for two days. Five milliliters of this preculture were added to a 1-L flask containing 150 ml of TSB and grown for 72 h at 30°C. The biomass was centrifuged (6000 rpm, 10 min, 4°C), washed once in ice cold 20% glycerol, and then with ice cold HEPM buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, from 100 mM isopropanol stock, 1 mM 2-mercaptoethanol, and 5% glycerol). Typically, 4 g of biomass (wet weight) was harvested from the 150 ml culture. The biomass pellet was stored at -80°C. The biomass (4 g) was thawed on ice for 30 min, then resuspended in 40 ml of HEPM buffer supplemented with rLysozyme (100 kU), and 35 µl benzonase (1 kU). The mixture was incubated at room temperature on a rocking platform for 30 min, then sonicated. The biomass was then French-pressed twice and centrifuged (12,000 × g, 45 min, 4°C). Imidazole was added to the supernatant (to a final concentration 5 mM) and it was subjected to an IMAC column (2.5 mL of the resin). Wash buffer: 20 mM HEPES pH 8.0, 150 mM NaCl, 30 mM imidazole, and 5% glycerol). Elution buffer: 20 mM HEPES pH 8.0, 150 mM NaCl, 200 mM imidazole, and 5% glycerol). The fractions of the eluted MoeH5 were pooled together, concentrated on MWCO 50 kDa Amicon columns and dialyzed against HEPM buffer. Both rMoeH5 and rMoeF5 were kept on ice and used for *in vitro* reactions within 12 h of purification, since we have not determined suitable conditions for long-term storage of these proteins.

MoeH5 activity assay

Reactions (50 µL) in HEPES buffer (50 mM, pH 8) contained NoA (~25 µM), DTT (4 mM), MgCl₂ (10 mM), ATP (10 mM), and a donor substrate – either NH₄Cl, glycine, D,L-glutamine, D,L-serine, A ring, or cyclopentylamine (all at 10 mM, except for A ring, which was 5 mM). MoeH5, alone or in combination with MoeF5, was added (40 nM total) and reactions were incubated at 30°C for 4 h. Reactions were terminated by boiling (5 min) and precipitated protein was removed by centrifugation. The protein pellet was washed with H₂O+0.1% NH₄OH and supernatants were combined, concentrated and analyzed by LC-MS. The percent conversion of NoA into reaction products was calculated through integration and normalization of mass-peak areas corresponding to NoA and reaction

products. It was the lowest for serine and cyclopentylamine (2–3%), while 10–20% conversion was observed for the other donor substrates. MS-MS analysis of the more abundant MoeH5 reaction products (MmA, NoB, MmG) confirmed their identities (Fig. S10–S12, SI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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	R¹	R²	[M-H]⁻
moenomycin A	a	H	1580.6
moenomycin A ₇	a	OH	1596.6
nosokomycin A	OH	H	1485.6
nosokomycin B	NH ₂	H	1484.6
nosokomycin A ₂	OH	OH	1501.6
nosokomycin B ₂	NH ₂	OH	1500.6
moenomycin G	x	H	1542.6
moenomycin G ₂	x	OH	1558.6
moenomycin I	y	H	1572.6
moenomycin I ₂	y	OH	1588.6
moenomycin K	u	H	1588.6
moenomycin K ₂	u	OH	1604.6
moenomycin L	t	H	1556.6
moenomycin L ₂	t	OH	1572.6
moenomycinJ	z	H	1552.6

Fig. 1.
Structures of the moenomycins mentioned in this work.

Fig. 2.

Genes and constructs described in this work. **A.** Genetic organization of *moe* clusters 1 and 2. The fragment of *moe* cluster 1 between *moeK5* and *moeR5*, not relevant to this study, is not shown on the figure. Amide synthase gene *moeB4* and GAT superfamily genes *moeF5* and *moeH5* are shown as grey arrows. Cosmids and plasmids used to express segments of the *moe* clusters are shown below. Genes *moeH5* and *moeB4* were replaced in moeno38-6 and pOOB64bd, respectively, with the kanamycin resistance cassette *neo* (marked as grey rectangle). **B.** Relative localization of *moe* clusters 1 and 2 in the *S. ghanaensis* chromosome (according to available WGS data at www.broadinstitute.org).

Fig. 3. Moenomycin production profiles of *moeB4*- and *moeH5*-deficient *S. ghanaensis* mutants. Note the four distinct x-axes. Extracted ion chromatograms showing the presence of moenomycins in the methanol extracts from *S. ghanaensis* strains dB4 (ΔmoeB4), dH5 (ΔmoeH5) and complemented dH5 ($\Delta\text{moeH5+pOOB47h}$). The *moeGT1*-deficient moenomycin nonproducer mutant (ΔmoeGT1 ; Ostash *et al.*, 2007) was used as a negative control. Compounds are shown as graphical symbols, where the rectangle indicates NoA; masses (in Da) are given to the right. Red arrows indicate peaks corresponding to NoA (1485 Da), that overlap with the 1485.6 isotope of NoB.

Fig. 4.

Unrooted maximum-likelihood tree showing the relationships within a set of selected MoeH5 homologues found in primary and secondary metabolic pathways. Complete amino acid (aa) sequences of the proteins were used for analysis. Double black triangles mark the proteins having truncated Ntn domains (see Fig. S6, ESM), while single triangles mark those with complete Ntn domains that lack the invariable Cys₁ residue. The tree was built with the help of phylogeny.fr v.2 server (Dereeper *et al.*, 2008) using default parameters. Confidence indices aLRT (Anisimova and Gascuel 2006) are shown on the nodes; the branches having aLRT values below stringent threshold level (0.75) were collapsed. The scale bar represents 2.0 aa substitution per aa position. Source data are given in supplementary table S1.

Fig. 5. Purification of proteins MoeH5 and MoeF5. **A.** SDS-PAGE of protein fraction obtained after IMAC of lysate from *S. lividans* strain TK24 pOOB83e⁺. Lanes: 1: soluble fraction; 2–4: fractions eluted with 200 mM imidazole; 5–6: 250 mM imidazole. The 56 kDa band corresponds to rMoeH5 as judged by Western blot analysis (not shown). The lower band is a MoeH5 degradation product. Lane 7: marker (Precision Plus protein ladder, BioRad) **B.** SDS-PAGE of concentrated MoeH5 (lane 1) and MoeF5 (lane 2). Lane 3: Long Range protein ladder (Sigma).

Fig. 6.

In vitro assay of rMoeH5 activity. NoA, ATP and rMoeH5 were present in each reaction mixture. Conversion of NoA into respective products was measured using 10 mM ATP, 10 mM amide donor substrate, 25 μ M NoA and ~40 nM enzyme. Conversion was between 2 and 20% for different donor substrates. Reactions were run for 4 h, quenched and immediately analyzed by MS and MS-MS. Extracted ion chromatograms (representative result of at least four independent experiments) showing production of moenomycins by MoeH5 in the presence of D,L-glutamine (A), D,L-glutamine and MoeF5 (B), NH₄Cl (C), NH₄Cl and MoeF5 (D), glycine (E), D,L-serine (F), A ring (G) and cyclopentylamine CPA (H).

(H). Masses of the compounds are given in Table 1. Relevant control reactions are given in SI, Fig. S9; MS-MS data are given in Figs. S10–S12.

Fig. 7.
Summary of the final stage of moenomycin biosynthesis in *S. ghanaensis*.

Table 1

LC-MS data for moenomycins described in this work

Compound name	-COOH of B ring modified with:	Min ¹	Accurate mass, m/z [M-H] ⁻	
			Calculated	Observed ²
Moenomycin A	A ring	12.6	1580.6541	1580.6532
Moenomycin A ₇	A ring	12.6	1596.6490	1596.6492
Nosokomycin A	No decoration	12.5	1485.6169	1485.6195
Nosokomycin B	-NH ₂	12.8	1484.6329	1484.6326
Nosokomycin A ₂	No decoration	12.4	1501.6118	1501.6122
Nosokomycin B ₂	-NH ₂	12.8	1500.6278	1500.6273
Moenomycin G	Glycine	12.6	1542.6384	1542.6385
Moenomycin G ₂	Glycine	12.5	1558.6333	1558.6331
Moenomycin I	Serine	12.6	1572.6489	1572.6479
Moenomycin I ₂	Serine	12.5	1588.6438	1588.6435
Moenomycin K	Cysteine	12.6	1588.6261	1588.6258
Moenomycin K ₂	Cysteine	12.5	1604.6210	1604.6201
Moenomycin L	Alanine	12.6	1556.6540	1556.6532
Moenomycin L ₂	Alanine	12.5	1572.6489	1572.6455
Moenomycin J	Cyclopentylamine	13.0	1552.6955	1552.6949

¹ Agilent C₁₈ column (5 μm, 250×4.6 mm) at 30 °C; solvent system: acetonitrile–water with 0.05% ammonium formate as a solvent modifier. LC conditions are as previously reported (Ostash *et al.*, 2007); ² mass error tolerance of 5 ppm was used

Table 2MIC values for MmA, NoB and NoA against three *S. aureus* strains

Compound	MIC ¹ , µg ml ⁻¹		
	Newman ²	MW2 ³	COL ⁴
MmA	0.08	0.04	0.08
NoB	1.25	0.63	0.63
NoA	2.5	5.0	2.5

¹ average of 10 measurements, carried out according to Campbell *et al.* (2011);

² sensitive to methicillin;

³ intermediate resistance to methicillin;

⁴ highly resistant to methicillin

Table 3

Strains and plasmids used

Strain/plasmid	Description/ function	Source
Strains		
<i>S. ghanaensis</i> ATCC14672	Wild type (WT) moenomycin producer	ATCC
<i>S. ghanaensis</i> GT1	WT, <i>moeGT1</i> inactivation; Am ^r Km ^r	Ostash <i>et al.</i> (2007)
<i>S. ghanaensis</i> dB4	WT, Δ <i>moeB4</i> deletion mutant; Am ^r	This study
<i>S. ghanaensis</i> dH5	WT, <i>AmoeH5</i> deletion mutant; Am ^r	This study
<i>S. lividans</i> TK24	Host for moenomycin production and rMoeH5 protein expression; Sm ^r	Kieser <i>et al.</i> (2000)
<i>S. lividans</i> TK24 moeno38-5 ⁺	TK24 carrying cosmid moeno38-5	Ostash <i>et al.</i> (2009)
<i>S. lividans</i> TK24 moeno38-6 ⁺	TK24 carrying cosmid moeno38-6	Ostash <i>et al.</i> (2009)
<i>S. albus</i> J1074	Host for moenomycin production	Makitrinsky <i>et al.</i> (2010)
<i>S. albus</i> J1074 moeno38-6 ⁺	J1074 carrying cosmid moeno38-6	This study
<i>E. coli</i> XL1 Blue MR	Host strain for DNA cloning	Stratagene
<i>E. coli</i> BL21(DE3) pLysS	Host for MoeF5 protein expression	Novagen
<i>E. coli</i> ET12567	Host for DNA conjugation	Kieser <i>et al.</i> (2000)
<i>E. coli</i> BW25113	Host for DNA recombineering	Gust (2009)
Cosmids		
moeno38	SuperCos1-based, carries entire <i>moe</i> cluster 1 except <i>moeR5moeS5</i> , Km ^r Ap ^r	Ostash <i>et al.</i> (2007)
moeno38-1	moeno38, replacement of <i>neo</i> with <i>int⁰C31-oriT^{RK2}-hyg</i> cassette, Hy ^r	Ostash <i>et al.</i> (2007)
moeno38-5	moeno38-1 Δ <i>moeA4</i> Δ <i>moeB4</i> , Hy ^r Km ^r	Ostash <i>et al.</i> (2009)
moeno38-6	moeno38-1 Δ <i>moeH5</i>	Ostash <i>et al.</i> (2009)
moeno38-dH5	moeno38 Δ <i>moeH5</i>	This study
moeno5	SuperCos1-based, carries entire <i>moe</i> cluster 2	Ostash <i>et al.</i> (2007)
moeno5-dB4	moeno5, Δ <i>moeB4</i>	This study
Plasmids		
pSOK804	VWB-based integrative vector, Am ^r	Sekurova <i>et al.</i> (2004)
pOOB64b	pSOK804 carrying entire <i>moe</i> cluster 2	Ostash <i>et al.</i> (2009)
pOOB64bd	pOOB64b, Δ <i>moeB4</i> deletion, Am ^r Hy ^r	This study
pKC1139E	pKC1139 carrying <i>ermEp</i> , Am ^r	Ostash <i>et al.</i> (2007)
pOOB47a	pKC1139E, <i>ermEp-moeH5</i> fusion, Am ^r	This study
pOOB47h	pKC1139E, <i>ermEp-moeH5</i> fusion, Hy ^r	This study
pOOB48a	pKC1139E, <i>ermEp-moeF5</i> fusion, Am ^r	Ostash <i>et al.</i> (2009)
pOOB83e	pKC1139E, <i>ermEp-moeH5-6His</i> , Am ^r	This study
pCXL5	pET24a, <i>moeF5</i> for production of C-terminally 6His-tagged MoeF5, Km ^r	D. Perlstein
pHYGI	pUC19 carrying <i>hyg</i> gene, Ap ^r Hy ^r	J. Salas
pIJ773	Source of <i>aac(3)IV-oriT</i> cassette	Gust (2009)
pUZ8002	Expression of proteins for plasmid conjugation <i>in trans</i> , Km ^r	Kieser <i>et al.</i> (2000)
pIJ790	Expression of proteins Bet, Exo and Gam for recombineering, Cm ^r	Gust (2009)

Am^r, apramycin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Ap^r, ampicillin resistant; Hy^r, hygromycin resistant; Cm^r, chloramphenicol resistant.

Table 4

Oligonucleotides used

Name	Sequence, 5'→3'	Purpose
plAm-Hyg-up	GTGCAATACGAATGGCGAAAAGCCGAGCTCATCGGTCAGCCCGTAGAGATTGGCGATCCC	Replacement of <i>aac(3)IV</i> in
p2Am-Hyg-rp	TCATGAGCTCAGCCAATCGACTGGCGAGCGGCATCGCATCAGGCGCCGGGGCGGTGTC	pOOB47a with <i>hyg</i>
aac-up	GGGCCACAGGCAGAGCAGA	Diagnostic primers to amplify
aac-rev	GAGCCACCTGTCCGCAAG	
apra_i	AGTTGTCTCTGACACATTCT	<i>aac(3)IV</i> and <i>moeB4</i> flank
moeB4up	CGCGCGATCATGTAAGTCTGCAC	
moeB4-red-up	GAAGGCAAGGCCCCGGTTGTGGCGGTGCTGACCGTCACCATTCCGGGGATCCGTCGACC	Replacement of <i>moeB4</i> within
moeB4-red-rp	GATCGCGGCGGTCAGCTCCGGTTCGCCCCAGTAGCCGAGTGTTAGGCTGGAGCTGCTTC	moeno5 with <i>aac(3)IV-oriT</i>
moeB4HY-red-up	GAAGGCAAGGCCCCGGTTGTGGCGGTGCTGACCGTCACCCCGTAGAGATTGGCGATCCC	Construction of pOOB64bd
moeB4HY-red-rp	GATCGCGGCGGTCAGCTCCGGTTCGCCCCAGTAGCCGAGCCAGGCGCCGGGGCGGTGTC	
moeH5-up-aac	AGGCCGCCCTCCAGCCCCTGCTGGACGCCCGATGACGGTATTCCGGGGATCCGTCGACC	Replacement of <i>moeH5</i> within
moeH5-rp-aac	TCTCGTGAAGTGGGGGTCTGCGGCGGTCCGGCCCCGCTATGTAGGCTGGAGCTGCTTC	moeno38 with <i>aac(3)IV-oriT</i>
moeGT1XbaIup-int	AAATCTAGAACCAGCGGTACATGGCCCTCATG	Cloning of <i>moeH5</i>
h5exp6hisEcoRI	AAAGAATTCAGTGGTGGTGGTGGTGGTGGCGGCCGGCGGGCCCTTCG	+ 6 His tag
moeGT1XbaIup con73start	AAATCTAGATGCTCCTGGACACCCTCTCAAAAAGCTTGCTGATGTCTCGTGAAGTGG	Cloning of <i>moeH5</i>