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Article

A Streamlined Metabolic Pathway for the Biosynthesis of Moenomycin A

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SUMMARY

Moenomycin A (MmA) is a member of the phosphoglycolipid family of antibiotics, which are the only natural products known to directly target the extracellular peptidoglycan glycosyltransferases involved in bacterial cell wall biosynthesis. The structural and biological uniqueness of MmA make it an attractive starting point for the development of new antibacterial drugs. In order both to elucidate the biosynthesis of this unusual compound and to develop tools to manipulate its structure, we have identified the MmA biosynthetic genes in *Streptomyces ghanaensis* (ATCC14672). We show via heterologous expression of a subset of *moe* genes that the economy of the MmA pathway is enabled through the use of sugar-nucleotide and isoprenoid building blocks derived from primary metabolism. The work reported lays the foundation for genetic engineering of MmA biosynthesis to produce novel derivatives.

INTRODUCTION

The emergence of resistance to existing antibiotics represents a significant threat to public health. The increasing frequency of resistance to the cell wall inhibitor vancomycin is of particular concern because this antibiotic is the last line of defense against methicillin-resistant Gram-positive infections. New antibiotics with activity against resistant bacterial strains are desperately needed. Antibiotics that inhibit validated bacterial targets but are structurally and mechanistically unrelated to compounds in clinical use may be especially attractive candidates for further development. One example of such a compound is moenomycin A (MmA) (Figure 1), a phosphoglycolipid antibiotic that is several orders of magnitude more potent than vancomycin against many Gram-positive pathogens, including the vancomycin-resistant pathogens [1]. MmA is a representative member of a family of compounds that inhibit the peptidoglycan glycosyltransferases (also known as transglycosylases) involved in bacterial cell wall formation [2]. Vancomycin also inhibits these enzymes, but the mechanisms of vancomycin and MmA are completely unrelated. Whereas vancomycin binds to peptidoglycan intermediates, which are substrates for the peptidoglycan glycosyltransferases [3], MmA binds to the peptidoglycan glycosyltransferases themselves. The moenomycins are the only natural products known to have such a mechanism of action.

Paralleling the unusual mechanism of action of the moenomycins is an unusual structure that contains an unprecedented phosphoglycerate-ether linkage to an irregular isoprenoid chain. This structural feature, which is of considerable interest from a biosynthetic standpoint, is also believed to be the primary cause of the moenomycins' poor physicochemical properties, which have hampered their development as therapeutic agents for human use. In the hope of learning how the phospholipid of MmA is assembled and attached, and of acquiring tools to enable the production of MmA analogs with better properties, we sought to identify the genes for MmA production. Access to these genes could enable the preparation of a range of MmA derivatives to probe the mechanism of peptidoglycan glycosyltransferase inhibition and the structural requirements for activity, work that may lead eventually to the discovery of peptidoglycan glycosyltransferase inhibitors for therapeutic use. Here, we report the identification of the MmA biosynthetic genes from *Streptomyces ghanaensis* (ATCC14672) by using a whole-genome scanning approach combined with gene knockouts and complementation experiments. Analysis of the gene cluster suggests that the MmA biosynthetic pathway differs from previously characterized carbohydrate-containing antibiotic gene clusters in that the sugar-nucleotide building blocks, as well as most of the other building blocks of the molecule, are derived exclusively from primary metabolism. We have confirmed this hypothesis by demonstrating that heterologous expression of a subset of the MmA biosynthetic genes in *S. lividans* TK24 leads to the production of a moenomycin pentasaccharide derivative. Thus, the MmA cluster provides an alternative paradigm for the biosynthesis of glycosylated secondary metabolites that may influence genome-scanning approaches to identify other carbohydrate-rich natural products. The availability of a heterologous expression system for moenomycin production will facilitate analysis of gene function, and it may yield intermediates that can be chemically modified to produce novel antibiotics or small-molecule probes for bacterial peptidoglycan glycosyltransferases.

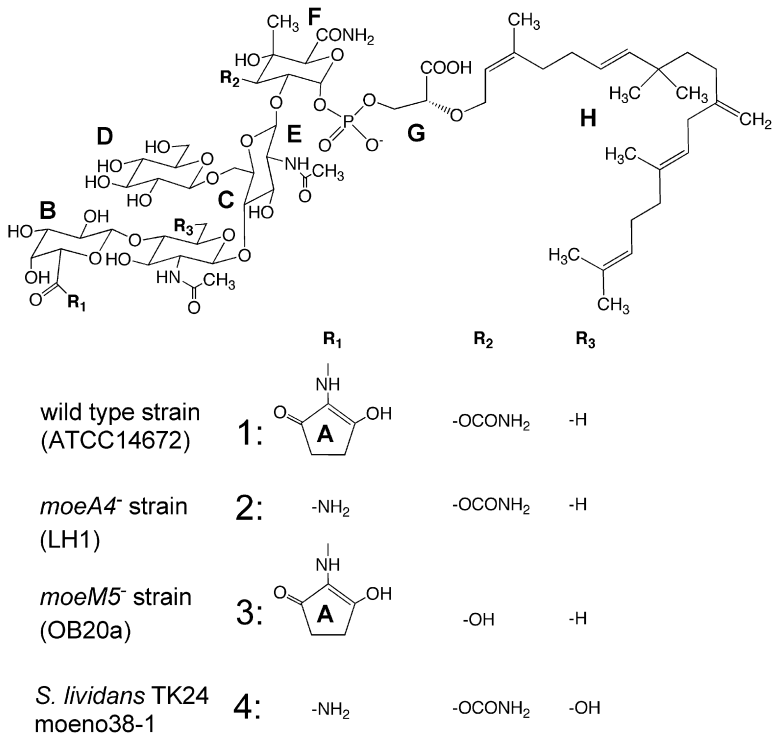

Figure 1. Structures of MmA and Proposed Structures of Moenomycins Produced by Recombinant *S. ghanaensis* and *S. lividans* Strains Described in This Work

Capital letters A–H indicate the eight "building blocks" of MmA.

RESULTS

Identification of the Genes for C5N Subunit Biosynthesis

Many gene clusters for antibiotic production have been readily identified by degenerate primer-based PCR amplification of highly conserved genes [4]. Although structurally unique, MmA does contain some features found in other secondary metabolites. The most recognizable one is perhaps the C_5N subunit (A ring) (Figure 1). The biosynthesis of this subunit involves an aminolevulinate synthase (ALS) [5], and we were able to amplify an internal fragment of a putative ALS gene, *moeC4*, from the *S. ghanaensis* genome by using degenerate primers based on sequences of ALS genes proposed to govern the production of asukamycin and the polyene ECO-02301 [5, 6]. Using this amplicon as a probe for screening a *S. ghanaensis* SuperCos1-based gene library, we retrieved a cosmid, moeno5, carrying the three-gene operon *moeA4 moeB4 moeC4* (referred to here as *moe* cluster 2) (Figure 2). Analysis of an *S. ghanaensis moeA4*-deficient strain, LH1, prepared as described in Experimental Procedures, showed that it did not produce MmA. Instead, it accumulated an intermediate lacking the C_5N chromophore that is proposed to have the structure **2** (Figures 1 and 3B). Although interruption of unit A attachment to the pentasaccharide was expected to exclusively yield the galactopyranuronic acid form of MmA (which was observed in the mutant as a minor intermediate; data not shown), the exact mass of the major product supports the proposed structure (calculated mass of the negative ion: 1484.6329, found: 1484.6371; MS^2 spectra provided in Supplemental Data available with this article online).

Compound **2** showed antibiotic activity, consistent with experiments suggesting that the C_5N subunit is not critical for biological activity [7]. The introduction of plasmid pKC11395-8 (which carries the *moeC4-moeB4* intergenic region and the entire *moeB4 moeA4* genes) into the LH1 strain restored MmA biosynthesis, as evident from LC-MS analysis. Expression of the *moeB4* gene alone (plasmid pOOB12) in the LH1 strain did not lead to MmA production, implying that only *moeA4* is affected in the mutant. Based on sequence analysis, MoeA4 is proposed to be an acyl-CoA ligase that cyclizes aminolevulinate, while MoeB4 is proposed to be an amide synthetase that couples the C_5N unit to the C6 acid of the MmA B ring sugar (Figure 4). Accumulation of the galactopyranouronamide derivative **2** in MmA producers has been reported by other laboratories [8] and may be due to amidation of unit B by the same enzyme(s) that operates on unit F (Figure 1). Although the preceding results establish a role for *moeA4 moeB4 moeC4* genes in MmA production, none of the genes flanking these three genes proved to be involved in MmA production. We concluded that the primary *moe* cluster must be elsewhere in the chromosome.

Identification of the Primary MmA Biosynthetic Gene Cluster, *moe* Cluster 1

We attempted to clone the main MmA gene cluster by targeting genes proposed to be involved in the biosynthesis of the isoprenoid chain and some of the sugar subunits. The isoprenoid chain in moenomycin was shown, by using

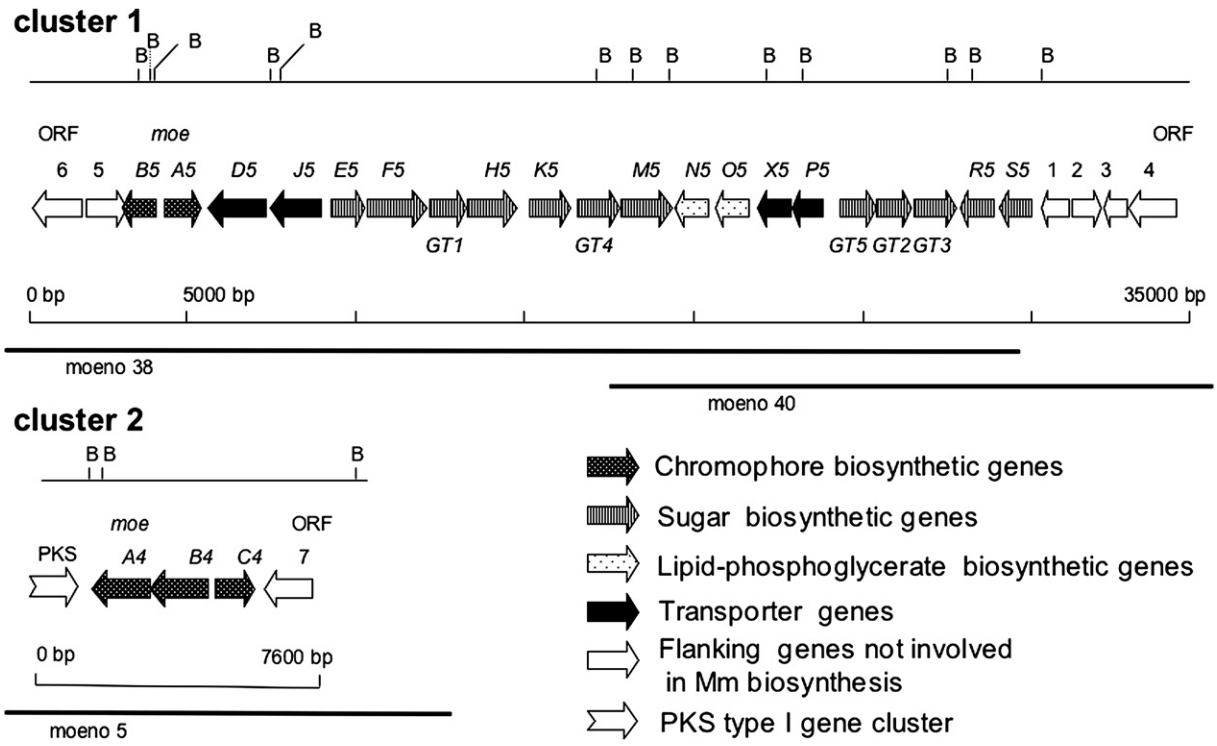

Figure 2. Genetic Organization of MmA Biosynthetic Gene Clusters 1 and 2
The *moe* genes are located within cosmids moeno5, 38, and 40. B, BamHI restriction sites.

tracer experiments, to be of nonmevalonate origin [9–11]. Nonmevalonate (also called DOXP)-pathway enzymes are involved in the biosynthesis of all primary as well as some secondary isoprene-derived metabolites in streptomycetes. In one case, genes encoding DOXP-pathway enzymes have been found to be clustered with a natural product pathway [12]. Efforts to identify the primary *moe* cluster by targeting DOXP-pathway enzymes were unsuccessful, however. Furthermore, all other attempts to clone *moe* genes via a degenerate primer-based PCR approach also failed: although a number of putative hexose 4,6-dehydratase, carbamoyltransferase, and prenyltransferase genes were cloned, none of them proved to be involved in MmA production.

We considered several other strategies to identify the primary *moe* cluster, including in vivo approaches (transposon mutagenesis followed by screening for altered MmA production/sensitivity; complementation of MmA nonproducing mutants) and in silico whole-genome scanning. After some experimental work aimed at testing various in vivo approaches, we concluded that genome scanning would be the most direct and reliable way to identify the *moe* gene cluster. Advances in sequencing and software technologies have reduced both the cost and the time of the process to the point where whole-genome sequencing is competitive with other approaches as a tool for the discovery of novel metabolic pathways.

The genome of *S. ghanaensis* ATCC14672 (~8.6–9.0 Mb) was shotgun sequenced to 6.6× coverage and was partially assembled at the Broad Institute (Cambridge, MA); this process yielded 1018 contigs ranging from 1 to 95 kb in size. The contigs cover 7.4 Mb of the strain's chromosome. They were analyzed for the presence of candidate *moe* genes by using the BLASTX and FRAMEPLOT [13] online programs. Contig 908 contained the previously identified *moe* cluster 2. Four other neighboring contigs (70–73), located more than 1 Mb away from contig 908 in the assembled genome, contained groups of genes that appeared functionally capable of governing the biosynthesis of MmA. Two cosmids (moeno38 and moeno40) covering contigs 70–73 were retrieved from an *S. ghanaensis* genomic library and fully sequenced to identify a cluster of 20 *moe* genes (*moe* cluster 1) (Figure 2). The boundaries of cluster 1 are defined by regions of extensive synteny to the terminal region of the *S. coelicolor* chromosome [14]. The predicted functions of all *moe* genes are presented in Table 1.

We confirmed the involvement of *moe* cluster 1 in MmA biosynthesis through disruptions of genes *moeGT1* and *moeM5*, which encode a putative glycosyltransferase (Gtf) and a carbamoyltransferase, respectively. Cell extracts from the mutants were analyzed for both antibiotic activity and the presence of MmA analogs. The *moeGT1*-deficient *S. ghanaensis* strain OB21e showed no antibiotic activity, and no peaks for MmA or its anticipated intermediates could be detected upon mass analysis of OB21e semipurified extracts (Figure 3C) or culture broths. Complementation of the OB21e mutant with plasmid

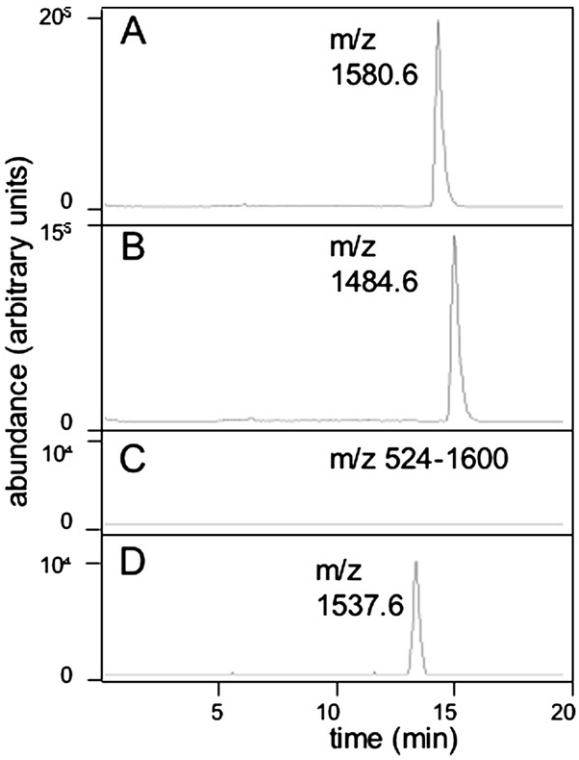

Figure 3. HPLC-MS Analysis of Moenomycin Production by Wild-Type and Recombinant *S. ghanaensis* Strains

(A–D) HPLC-MS analysis (ESI, negative mode) showing the moenomycins found in cell extracts of *S. ghanaensis* (A) wild-type, (B) *moeA4* mutant LH1, (C) *moeGT1* mutant OB21e, and (D) *moeM5* mutant OB20a.

pOOB41, which carries a P_{ermE}-*moeGT1* fusion, restored MmA production, as judged from bioassays and LC-MS analysis. The MmA trisaccharide degradation product (units C, E, F, G, and H, Figure 1) is reported to have substantial antibacterial activity [15], suggesting that the *moeGT1* knockout blocks transfer of the C, E, or F rings. The *moeM5* null strain, OB20a, produced a compound with greatly reduced antibiotic activity and a mass of 1537.6 Da ($[M\text{-}H]^-$) (Figure 3D), consistent with the loss of the NH_2CO group from the C′-3 position of ring F of MmA (Figure 1). Complementation of OB20a with pOOB43, which carries a P_{ermE}-*moeM5* fusion, restored production of MmA, as judged by the reappearance of a mass peak at 1580.6 Da. We suggest that MoeM5 acts as a late-stage tailoring enzyme to install the carbamoyl group, which is an important element in the MmA pharmacophore [1].

Description of *moe* Cluster 1

Cluster 1 contains genes encoding two prenyltransferases, five Gtfs, a 4,6-dehydratase/ketoreductase pair putatively involved in C ring biosynthesis, a possible epimerase, and several sugar-tailoring genes (Figure 2 and Table 1) that are presumed to be involved in functionalization of the F ring after pentasaccharide assembly. It also contains a second ALS gene and a gene for ALA cyclization that contains an in-frame deletion and is presumed to be nonfunctional; however, there is no gene for attachment of the cyclized C_5N unit. The biosynthetic *moe* cluster 2 most likely arose from duplication of a chromophore biosynthetic operon from *moe* cluster 1, whereupon the redundant chromophore operon in cluster 1 subsequently underwent deletion events. In addition to the MmA biosynthetic genes, four genes encoding components of two ATP-binding cassette (ABC) transport systems have been identified in *moe* cluster 1. ABC-type transporters are found in many antibiotic gene clusters and are typically thought to be involved in the transport of natural products or precursors across bacterial cell membranes [16]. Full descriptions of the *moe* genes are provided in Supplemental Data.

The structures of the individual sugars in MmA and the paucity of sugar biosynthetic genes in the cluster led us to surmise that all of the NDP sugars in MmA must be derived from building blocks produced by primary metabolism. For example, the D ring donor is predicted to be UDP-glucose; the E and C rings are predicted to share a common precursor, UDP-GlcNAc, which is used without further modification as the E ring donor and is converted to UDP-chinovosamine by MoeR5 and MoeS5 to make the C ring donor (Figure 4). The B and F rings in MmA are both derived from C6 acid precursors, epimeric with respect to the C4 hydroxyls. The B and F rings are predicted to share a common precursor, however, because *S. ghanaensis* also produces a moenomycin variant in which the methyl substituent on the F ring is lacking [2], and in this compound the C4 hydroxyl has the same stereochemistry as the B ring. The common precursor is speculated to be either UDP-glucuronic acid or UDP-galacturonic acid, both of which are used in exopolysaccharide biosynthesis in streptomycetes. Uncertainty in the precise nature of the donor arises because *moe* cluster 1 contains a putative epimerase, MoeE5, that could modify UDP-glucuronic acid to UDP-galacturonic acid. Other sugar-tailoring genes include *moeM5* for the carbamoyltransferase described above, as well as *moeF5*/*moeH5* and *moeK5*, which encode the putative amidotransferase subunits and a putative methylcobalamin-dependent radical SAM methyltransferase [17, 18], respectively, that modify the F ring (Figure 4).

The hypothesis that the sugars in MmA are derived from primary metabolism is supported by sequence analysis of the *moe* Gtfs themselves. The vast majority of Gtfs involved in secondary metabolite biosynthesis are predicted to have a characteristic two-domain fold, known as the GT-B superfamily fold [19]. These enzymes share an easily recognizable sequence motif. Only one of the Gtfs in *moe* cluster 1 is predicted to resemble Gtfs typically found in antibiotic production. This Gtf, MoeGT1, shows strongest homology to MurG, an UDP-N-acetylglucosaminyl transferase involved in transferring GlcNAc to a glycolipid during peptidoglycan biosynthesis [20, 21]. Therefore, we suggest that MoeGT1 carries out a similar reaction during MmA biosynthesis, namely, the transfer of N-acetylglucosamine to a lipid-linked F ring precursor

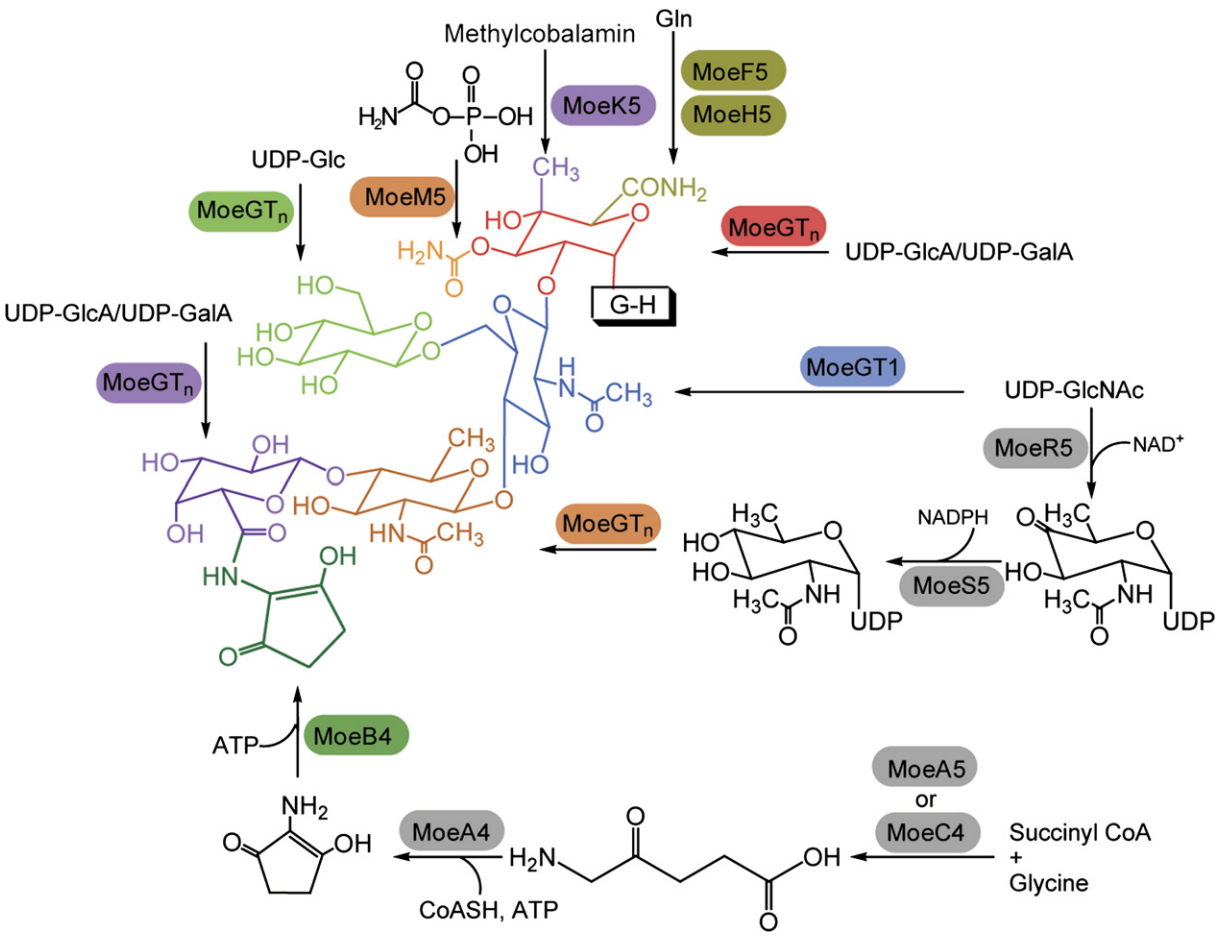

Figure 4. Proposed Biosynthetic Scheme for the Chromophore and Pentasaccharide Portions of MmA

Letters G–H in the shaded rectangle mark the lipid-phosphoglycerate moiety of MmA. MoeGTn represents any of four Moe Gtfs (MoeGT2–5). UDP-GlcNAc, UDP-N-acetyl glucosamine; UDP-Glc, UDP-glucose; UDP-GlcA/UDP-GalA, UDP-glucuronic acid/UDP-galacturonic acid.

(Figure 4). The other Gtfs show homology to Gtfs proposed to be involved in exopolysaccharide biosynthesis in various organisms. Thus, the Gtfs in *moe* cluster 1 are more similar to enzymes known or thought to be involved in primary metabolic processes than to enzymes typically involved in natural product biosynthesis.

The use of building blocks from primary metabolism apparently extends to the biosynthesis of the lipid-phosphoglycerate chain of MmA. There are only two putative prenyltransferases in *moe* cluster 1, one of which, MoeN5, shows homology to other prenyltransferases involved in isoprenoid biosynthesis and is thus predicted to be involved in the synthesis of the C_{25} lipid chain. Schuricht et al. have proposed a mechanism for the formation of the irregular isoprenoid chain of MmA that involves the coupling of C_{15} and C_{10} subunits, both of which could be derived from precursors produced in primary metabolic pathways [1, 9–11]. Thus, a relatively complex isoprenoid structure is apparently governed by a single gene operating on available cellular metabolites (Figure 5).

The second prenyltransferase, MoeO5, is predicted to catalyze formation of the ether linkage between 3-R-phosphoglycerate, an abundant metabolite produced during glycolysis, and the isoprenoid chain (Figure 5). MoeO5 shows sequence homology to the archaeal enzymes that form the first ether linkage between isoprenoid precursors and glycerol phosphate in the biosynthesis of the unusual membrane lipids that distinguish Archaea from the other kingdoms of life [22]. These archaeal prenyltransferases have a TIM barrel fold, one of the most common folds in nature, but unknown in other prenyltransferases [23]. MoeO5 is also predicted to have a TIM barrel fold, making it the first, to our knowledge, bacterial homolog of the archaeal prenyltransferases to be putatively assigned a function. We note that MoeO5 homologs exist in a range of bacteria and may catalyze similar prenyltransfer reactions, which would suggest that bacteria produce other metabolites that resemble archaeal membrane lipids. Indeed, there is recent evidence that this is the case [24].

Heterologous Expression of a Subset of *moe* Cluster 1 in *Streptomyces lividans* TK24

We have proposed that *moe* cluster 1 encodes all of the genes required to assemble a bioactive MmA derivative

Table 1. Deduced Functions for Genes in Moenomycin A Gene Clusters 1 and 2

Protein	AA	Homolog	ID%/SI%	Accession Number	Proposed Function
MoeA4	516	Putative acyl CoA ligase (*Streptomyces aizunensis*)	63/73	AAX98210.1	Acyl CoA ligase
MoeB4	521	SimL (*Streptomyces antibioticus*)	45/62	AAG34163.1	Amide synthetase
MoeC4	412	HemA-AsuA (*Streptomyces asukaensis*)	70/83	AY240962	Aminolevulinate synthase
MoeB5	301	Putative acyl CoA ligase (*S. aizunensis*)	58/76	AAX98210.1	Nonfunctional acyl CoA ligase[a]
MoeA5	394	HemA-AsuA (*S. asukaensis*)	64/78	AY240962	Aminolevulinate synthase
MoeD5	591	Putative ABC transporter (*Symbiobacterium thermophilum*)	41/55	YP075256.1	ABC transporter
MoeJ5	564	Same as above	45/61	YP075255.1	ABC transporter
MoeE5	340	Putative UDP-glucose 4-epimerase (*Symbiobacterium thermophilum*)	46/58	YP074610.1	NDP-hexose 4-epimerase
MoeF5	645	WbpS (*Pseudomonas aeruginosa*)	29/43	AAF24002.1	Aminotransferase subunit 1
MoeGT1	402	Putative Gtf (*Polaromonas sp*)	27/40	EAM38951.1	Glycosyltransferase
MoeH5	513	AsnB-like amidotransferase (*Azoarcus sp*)	32/48	CAI08539.1	Aminotransferase subunit 2
MoeK5	407	Putative methyltransferase (*Pyrococcus horikoshii*)	34/52	NP_142754.1	Methyltransferase
MoeGT4	427	Putative Gtf (*Mycobacterium vahbaalenii*)	27/38	EAS23724.1	Glycosyltransferase
MoeM5	530	GdmN (*Streptomyces hygroscopicus*)	29/44	AAO06921.1	Carbamoyltransferase
MoeN5	260	Putative prenyltransferase (*Loktanella vestfoldensis*)	29/43	EAQ07619.1	Prenyltransferase
MoeO5	281	GGGPS (*Thermoplasma acidophilum*)	27/43	JC7965	Prenyl 3-phosphoglycerate synthase
MoeX5	226	Putative membrane protein (*Mycobacterium sp*)	26/40	EAS99725.1	ABC transporter membrane protein
MoeP5	223	ABC transporter ATPase (*Mycobacterium flavescens*)	43/58	EAS11435.1	ABC transporter ATP-binding protein
MoeGT5	312	Gtf MoeGT4 (*S. ghanaensis*; see above)	45/59		Glycosyltransferase
MoeGT2	286	Putative Gtf (*Methylococcus capsulatus*)	35/51	AAU93096.1	Glycosyltransferase
MoeGT3	414	Putative Gtf (*Kineococcus radiotolerans*)	44/56	ZP_00616987.1	Glycosyltransferase
MoeR5	374	CapD (*Nocardioides sp*)	53/68	EAO07657.1	Hexose-4,6-dehydratase
MoeS5	282	SCO7194 (*Streptomyces coelicolor*)	62/75	CAC01594.1	Hexose-4-ketoreductase

AA, amino acids. For references citing *moe* gene homologs, see Supplemental Data.
[a] See Supplemental Data.

lacking the chromophore unit (compound **2**, Figure 1) from cellular pools of available metabolites. To test this hypothesis, we constructed cosmid moeno38-1 from cosmid moeno38, in which the *neo* gene marker has been replaced with the 5.3 kb *hyg-oriT*^{RK2}-*int*$^{\phi C31}$ PCR fragment of vector pOOB40 by using the λ-RED recombination approach [25, 26]. In a similar manner, the *neo* gene was replaced in the SuperCos1 vector to produce SuperCos1Hy. Cosmid moeno38-1 contains all of the *moe* cluster 1 genes except *moeR5 moeS5*, which are proposed to convert UDP-GlcNAc to UDP-chinovosamine. This cosmid was transferred conjugally into *S. lividans* TK24, and the presence of moeno38-1 in the TK24 strain was confirmed by PCR with primers specific to *moeGT1*, *moeGT3*, and *attR*$^{\phi C31}$ [27]. Biochromatography showed that the cell extract and culture broth from the moeno38-1^{+} TK24 transconjugant, but not from a strain harboring the empty vector SuperCos1Hy, contain a biologically active compound (Figure 6). HPLC analysis showed that the compound has the same mobility and UV spectrum as the MmA precursor **2** lacking the C_5N chromophore unit. High-resolution MS analysis showed that the new compound has a mass of 1500.6278 Da ([M-H]$^{-}$). MS/MS analysis of the compound revealed several characteristic mass peaks commonly observed during fragmentation of moenomycins (Supplemental Data). Based on HPLC mobility, exact mass,

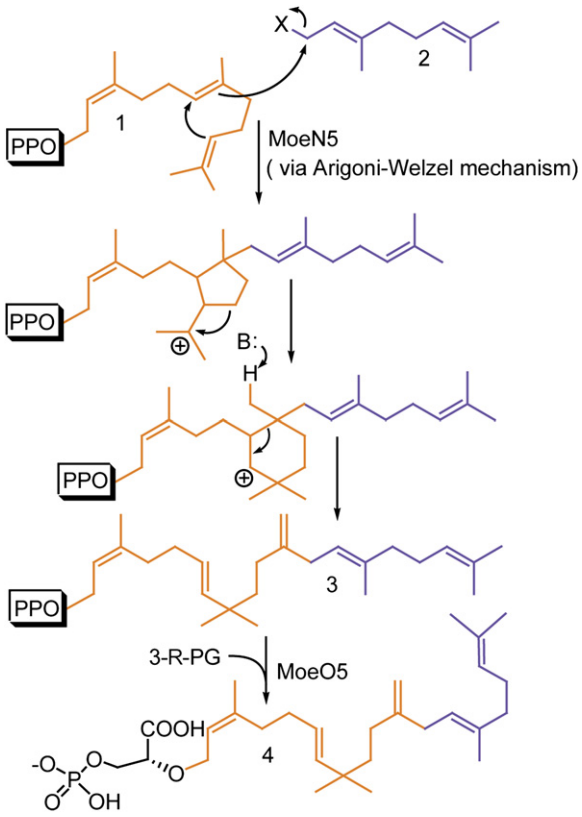

Figure 5. Proposed Scheme for the Biosynthesis of Lipid-Phosphoglycerate Moiety 4 of MmA

The letters "PPO" in the shaded rectangle represent pyrophosphate. **1**, farnesyl pyrophosphate; **2**, C_{10} isoprenoid precursor. Intermediates on the pathway to moenocinol pyrophosphate **3** were postulated by Schuricht et al. [10]. 3-R-PG, 3-phosphoglycerate. The formation of **1** with the *cis* configuration could be governed by a primary metabolism enzyme similar to *M. tuberculosis* Z-farnesyl diphosphate synthase [43].

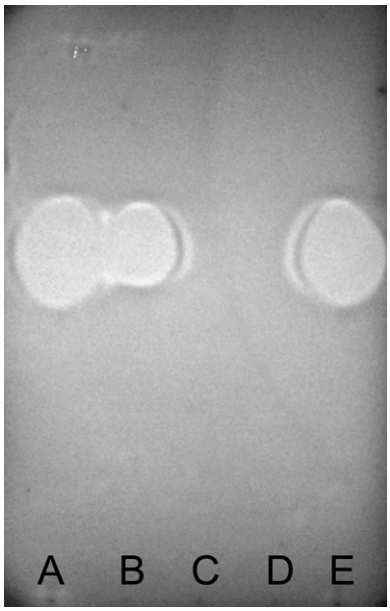

Figure 6. Biochromatography of Methanol Extracts

Biochromatography of *S. lividans* (lane A) TK24 moeno38-1$^+$ cell extracts and (lane B) culture broth and *S. lividans* (lane C) TK24 SuperCos1Hy$^+$ cell extracts and (lane D) culture broth. The MmA standard (10 mcg) is in lane E.

and biological data, the compound is proposed to be a pholipomycin derivative lacking the chromophore unit (compound **4**, Figure 1; calculated mass of the negative ion: 1500.6278 Da). Pholipomycin is a known member of the moenomycin family in which the C ring is N-acetylglucosamine rather than chinovosamine [2]. Exclusive production of a pholipomycin precursor by the recombinant *S. lividans* strain is consistent with the proposed roles of the *moeR5 moeS5* genes in the biosynthesis of UDP-chinovosamine from UDP-GlcNAc. These data also indicate that the Gtf responsible for unit C attachment can accept not only UDP-chinovosamine, but also UDP-GlcNAc. This finding is consistent with recent studies showing that the moenomycin complex of antibiotics purified from *S. ghanaensis* also contains pholipomycin (or 6C-hydroxy-MmA) as a component [8]. Most important, the results show that cosmid moeno38-1 carries all of the genes necessary for formation of the moenomycin pentasaccharide phospholipid scaffold, confirming that the building blocks of the molecule are supplied from cellular metabolite pools. Based on sequence analysis and the results of heterologous *moe* gene expression, we suggest that production of a fully active 1.4 kDa MmA precursor will take only nine dedicated genes (~12 kb DNA fragment), six of which govern the assembly of the phosphoglycolipid scaffold and three of which direct two biologically important sugar-tailoring reactions (carbamoylation and carboxyamidation of unit F).

DISCUSSION

To our knowledge, the cloning and analysis of the genes governing MmA production provide the first insight into the genetics of phosphoglycolipid antibiotic biosynthesis. Certain features of the MmA biosynthetic pathway merit further comment. First, the involvement of groups of genes at two widely separated loci in natural product biosynthesis is rare, but not completely unprecedented [28, 29]. More unusual is the size of the primary *moe* cluster 1, which is remarkably small (<30 Kb) given the size of the molecule produced (1583 Da). The antibiotic calicheamicin, which has a mass similar to that of MmA (1364 Da) and contains four sugar subunits, is produced by a gene cluster that is more than three times larger and contains many more ORFs than *moe* cluster 1 [30]. A comparison of the clusters for the biosynthesis of MmA, calicheamicin, and other antibiotics containing multiple sugar subunits reveals a striking difference: the *moe* cluster contains very few genes involved in the biosynthesis of the individual sugar subunits. With few exceptions [31, 32], gene

clusters for glycosylated metabolites contain nucleotidyltransferases. However, there is not a single nucleotidyltransferase gene in the *moe* cluster, and other sugar biosynthetic genes are sparse as well.

We have demonstrated via heterologous expression of a subset of genes from *moe* cluster 1 that all of the sugar building blocks (as well as the building blocks of the moenocinol-phosphoglycerate moiety) are derived from primary metabolism and are used either directly or with minimal modification to produce the pentasaccharide scaffold of MmA, which is subsequently tailored on the B and F rings. Although many other types of antibiotics derive building blocks from cellular metabolite pools, carbohydrate-containing antibiotic gene clusters typically contain a great many genes devoted to sugar biosynthesis. In fact, it was recently suggested that the formation of NDP sugar precursors by dedicated nucleotidyltransferases represents a crucial branching point of secondary carbohydrate metabolic pathways from primary metabolism [33]. The discovery of *moe* cluster 1 shows that there is another paradigm for the synthesis of carbohydrate-rich secondary metabolites.

MmA is produced late in the streptomycetes' life cycle [34], consistent with its role as a secondary metabolite, but the building blocks used to assemble the molecule are expected to be present throughout cell growth. Apparently, they are used to construct MmA only when the *moe* genes are transcribed. The regulation of *moe* cluster gene expression is unclear because it appears to lack dedicated regulatory genes. However, the *moeA5*, *moeO5*, *moeR5*, and *moeE5* genes all contain rare TTA leucine codons. In *S. coelicolor,* 5′-processed $_{Leu}$tRNA^{UUA} is accumulated in significant quantities only in late stationary phase [35], thus setting an important regulatory switch for antibiotic production [36]. Fewer than 0.2% of genes in the *S. coelicolor* genome contain TTA codons [36]. Therefore, the appearance of this codon in 20% of the genes within *moe* cluster 1 seems significant and suggests that, in *S. ghanaensis*, MmA production is coincident with the increased accumulation of the mature tRNA for TTA codon expression late in growth.

It is worth asking whether the MmA biosynthetic pathway, with its heavy reliance on available primary metabolite pools, represents a special case or a common paradigm for the synthesis of glycosylated secondary metabolites. We favor the latter possibility, and we suggest that an appropriate search of bacterial genomes will turn up previously unrecognized natural product gene clusters having a genetic architecture similar to that members of *moe* cluster 1. For example, the genome of the enterobacterium *Photorhabdus luminescens* contains a cluster of genes (*plu3353–plu3368*) encoding five putative Gtfs (one shows homology to both MoeGT4 and MoeGT5), one MoeO5 homolog, a prenyltransferase, as well as a set of hypothetical proteins [37]. The genetic capacity of this cluster is enough to direct the formation of a phosphoglycolipid molecule. This and other clusters may have been overlooked as potential determinants of antibiotics or other bioactive compounds both because of their economy and because many of the carbohydrate-processing genes, like those in the MmA cluster, bear a stronger resemblance to genes involved in primary metabolism than to those involved in secondary metabolism. Thus, besides providing us with genetic tools for manipulating MmA structure, the discovery of the MmA cluster provides new, to our knowledge, insights into the biosynthesis of carbohydrate-containing natural products that may be useful in the annotation of bacterial genomes.

SIGNIFICANCE

MmA is a chemically and biologically unique phosphoglycolipid antibiotic with potent activity against Gram-positive bacteria, including vancomycin- and methicillin-resistant pathogens. Development of novel antibacterial drugs based on the MmA pharmacophore is hampered by our poor understanding of the mechanisms of MmA inhibition as well as limited chemical tools for manipulating MmA structure. The cloning of the MmA biosynthetic genes paves the way for the combinatorial biosynthesis and chemoenzymatic synthesis of moenomycin derivatives for use as antibiotics and as chemical probes to understand better the mechanism of action of bacterial peptidoglycan glycosyltransferases involved in peptidoglycan biosynthesis and remodeling. The genetic organization of MmA pathway itself is remarkable for the apparent absence of genes for the formation of activated sugars, isoprenoid metabolism, and regulation, as well as for the presence of such novel genes as *moeO5*, which is homologous to prenyltransferases involved in archaeal membrane biosynthesis. Thus, impressive streamlining of the *moe* cluster and the presence of genes for unusual biotransformations are two salient features of the MmA biosynthetic pathway. To our knowledge, the MmA biosynthetic pathway provides new insights into secondary metabolism that may lead to the discovery of previously unrecognized natural product clusters in other bacterial genomes.

EXPERIMENTAL PROCEDURES

Antibiotics

Pure MmA was provided by M. Adachi and S. Fuse (Department of Chemistry and Chemical Biology, Harvard University). For recombinant strain selection, commercially available antibiotics were used (mcg/ml): ampicillin (100), chloramphenicol (35), kanamycin (50), apramycin (50), hygromycin (100), spectinomycin (200), and streptomycin (100).

Strains and Vector DNAs

Streptomyces ghanaensis ATCC14672 and *Bacillus cereus* ATCC19637 were obtained from ATCC. *S. lividans* TK24 was kindly provided by M. Kobayashi (University of Tsukuba, Japan). *E. coli* XL1 Blue MR and cosmid SuperCos1 were purchased from Stratagene. The methylation-deficient conjugative strain *E. coli* ET12567 (pUB307) [38] was obtained from Prof. C.P. Smith (Manchester University, UK). *E. coli* BW25113 (pIJ790) was obtained from the John Innes Center (Norwich, UK). *S. ghanaensis* strains LH1, OB20a, and OB21e with disrupted *moeA4*, *moeM5*, and *moeGT1* genes, respectively, were constructed as described in this work. The vector pKC1139

with a temperature-sensitive pSG5 replicon and integrative vector pSET152 [39] were obtained from Prof. C.P. Smith. Vector pMKl9 was constructed by the cloning of a 0.2 kb HindIII-XbaI fragment carrying the *ermE* promoter into respective sites of the pKC1139 polylinker (provided by I. Ostash, L'viv University, Ukraine). To generate vector pOOB40, the central portion of the apramycin-resistance gene *aac(3)IV* in vector pSET152 had been excised with SacI endonuclease, and the rest of the vector had been treated with Klenow fragment and ligated to the EcoRV-digested hygromycin-resistance cassette *hyg* [39]. Vector pOOB5, which carries the spectinomycin-resistance marker *aadA* instead of *aac(3)IV*, was generated in the same way.

DNA Manipulations and Analysis

Standard molecular biology procedures were used throughout this work [39, 40]. The plasmid and fosmid libraries for *S. ghanaensis* ATCC14672 genome sequencing were created at the Broad Institute. Sequencing of cosmids moeno5, 38, 40 and their subclones was done at the Biopolymers Facility of Harvard Medical School by using standard (M13, T7, T3) and custom-designed primers. The generation, assembly, and analysis of *S. ghanaensis* genomic sequences will be described separately. BLAST search tools (on the server of the National Center for Biotechnology Information, Bethesda, MD), FramePlot2.3.2 [13], CUPplot1.0 (at www.nih.go.jp/-jun/cgi-bin/frameplot.pl), and the Lasergene software package were used for *S. ghanaensis* sequence assembly, analysis, and annotation. The CDD search engine (BLAST server) and a set of programs (HHPred, Pfam, TMHHM) on the ExPaSy proteomics server were utilized for identification of topology and conserved domains of Moe proteins.

The *S. ghanaensis* genomic library has been constructed by using SuperCos1. DIG-labeled internal fragments of genes *moeC4*, *moeGT1*, and *moeGT3* amplified from the chromosome (primers are described in Supplemental Data) were used as hybridization probes for library screening. The 5.3 kb fragment of pOOB40 (*hyg-oriT-int* cassette) was engineered into cosmid moeno38 by utilizing the primers and the strategy reported by Yanai et al. [41].

Generation of *S. ghanaensis* Disruption Mutants and Their Analysis

S. ghanaensis transconjugants (obtained according to described procedures; [39]) carrying pKC1139-based disruption plasmids in replicative form were grown for 3 days in tryptic soy broth (TSB; Difco) at 30°C. The biomass was washed twice with water to remove apramycin used for plasmid selection, and ~10^5 cfu were inoculated into fresh TSB without antibiotic. The culture was incubated for 6 days at 40°C (to eliminate free plasmid), plated onto LB agar supplemented with apramycin, and grown for 4–5 days at 37°C. Colonies with disruption plasmids integrated into the gene of interest were obtained with a frequency of 3×10^{-5} to 4×10^{-5}. No apramycin-resistant colonies were detected in a control strain carrying the empty vector, implying that the frequency of nonspecific integration of pKC1139 is below the level of detection (we usually plated 3×10^8 cfu), and that the appearance of AmR colonies in the experimental strains is due to homologous integration. Moreover, ten independent colonies of each disruption mutant were assayed for MmA production, and in no case could we detect the MmA^+ phenotype due to possible nonspecific integration of the plasmid into the *S. ghanaensis* genome. We also did not detect reversions to the AmS and MmA^+ phenotypes when the strains were grown in the presence of apramycin at 37°C, indicating that, under the stated conditions, the pKC1139-based plasmids are stably integrated into the homology region of generated mutants (3000–4000 colonies have been assayed). Passage of wild-type strains under the cultivation conditions used to generate *moe* mutants did not affect MmA production. The knockouts were verified by Southern analysis by using the internal fragments of disrupted genes as probes. For complementation experiments, matings of *S. ghanaensis* mutants with donor *E. coli* strains were incubated at 37°C and were overlaid after 8 hr of growth. Control conjugations with empty vectors (either pOOB5 [Sp^r] or pOOB40 [Hy^r]) were performed, and the obtained transconjugants were analyzed in parallel with experimental strains to ensure that workup conditions did not affect the stability of MmA^- mutants.

Moenomycin Production Analysis

For all moenomycin extraction procedures, equal amounts of biomass (2 g, wet weight) and fermentation medium (30 ml) were used. For MmA production, *S. ghanaensis* mutants with disrupted *moe* genes and *S. lividans* strains were grown at 37°C for 4–5 days in mTSB (TSB supplemented with trace elements solution [39], 0.8 ml per 1 liter). For antibiotic disc diffusion assays, fermentation medium and concentrated methanol extracts of MmA from mycelium of streptomycete strains were applied to antibiotic assay discs (10 mm, Sigma) and stacked onto LA plates overlaid with soft agar containing MmA-sensitive *B. cereus*. Zones of *B. cereus* growth inhibition were observed after 12–14 hr of incubation at 30°C. Moenomycins were partially purified (60% purity) from the cell extracts by using a 100 mg C_{18} SPE cartridge (Waters) and were analyzed by LC-MS as described in [42] on an Agilent 1100 series LC/MSD machine. For biochromatography, moenomycin extracts were separated on silica gel aluminum TLC plates (mobile phase: methanol:acetonitrile:water at 35:40:25), which were dried and overlaid with soft agar containing *B. cereus*. After overnight incubation at 30°C, the plates were visualized with visible and UV light (254 nm). Accurate mass spectra of purified moenomycins from recombinant *S. ghanaensis* LH1 and *S. lividans* TK24 (moeno38-1) strains were acquired by using an Agilent LC/MSD TOF machine. For the LC analysis, a Gemini C18 column (5 μm, 4.6 mm × 100 mm) from Phenomenex was used (mobile phase A: H_2O and mobile phase B: acetonitrile, with 0.1% ammonium hydroxide as a solvent modifier). Samples were run in negative ionization mode; the capillary voltage was set to 4.0 kV, and the fragmentor voltage was set to 150 V. The drying gas temperature was 300°C, the drying gas flow rate was 7 l/min, and the nebulizer pressure was 15 psi. (–)ESI-MS^2 spectra of compounds **2** and **4** were acquired with a Micromass qTOF2 machine. Cone and capillary voltages were set to 35 V and 3 kV, respectively; collision energy was 30 V.

Supplemental Data

Supplemental Data, including a detailed description of *moe* genes, primer sequences, plasmid construction, mutant strain verification, and MS/MS analysis are available at http://www.chembiol.com/cgi/content/full/14/3/257/DC1/.

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Accession Numbers

The sequences reported in this paper have been deposited in the GenBank database with accession codes DQ988993 and DQ988994 for *moe* clusters 2 and 1, respectively.