

In Vivo Manipulation of the Bleomycin Biosynthetic Gene Cluster in *Streptomyces verticillus* ATCC15003 Revealing New Insights into Its Biosynthetic Pathway^{*[5]}

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Bleomycin (BLM), an important clinically used antitumor compound, and its analogs are challenging to prepare by chemical synthesis. Genetic engineering of the biosynthetic pathway in the producer strain would provide an efficient and convenient method of generating new derivatives of this complex molecule *in vivo*. However, the BLM producing *Streptomyces verticillus* ATCC15003 has been refractory to all means of introducing plasmid DNA into its cells for nearly two decades. Several years after cloning and identification of the bleomycin biosynthetic gene cluster, this study demonstrates, for the first time, genetic accessibility of this pharmaceutically relevant producer strain by intergeneric *Escherichia coli*-*Streptomyces* conjugation. Gene replacement and in-frame deletion mutants were created by λ RED-mediated PCR targeting mutagenesis, and the secondary metabolite profile of the resultant mutants confirmed the identity of the BLM biosynthetic gene cluster and established its boundaries. Ultimately, the in-frame *blmD* deletion mutant strain *S. verticillus* SB5 resulted in the production of a bleomycin intermediate. The structure of this compound, decarbamoyl-BLM, was elucidated, and its DNA cleavage activity was compared with the parent compounds.

The bleomycins (BLMs)³ (1, 2) are important clinically used hybrid peptide-polyketide antitumor compounds. Combined with other agents, the BLMs are used clinically for the treatment of several types of tumors and marketed under the trade name Blenoxane[®], with BLM A2 and B2 as the principle constituents (3). Early development of drug resistance and cumu-

lative pulmonary toxicity are the major limitations of BLMs in chemotherapy (3, 4). Therefore, it is an important research goal to develop strategies to produce novel BLM analogs by microbial fermentation, particularly those unavailable or extremely difficult to prepare by chemical synthesis.

The BLMs are thought to exert their biological effects through a sequence-selective, metal-dependent oxidative cleavage of DNA and RNA in the presence of oxygen (5, 6). They can be dissected into four functional domains: (i) the metal-binding domain, which consists of the pyrimidoblastic acid subunit along with the adjacent β -hydroxyl histidine; (ii) the bithiazole and C-terminal amine, which confers the majority of BLM-DNA affinity; (iii) the (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid linker subunit, which plays an important role in the efficiency of DNA cleavage by BLMs; and (iv) the carbamoylated disaccharide moiety (4). The exact functional role of the sugars and the attached carbamoyl group remains controversial. They possibly contribute to cell recognition, cellular uptake of BLMs, metal ion coordination, and/or DNA affinity (4, 6).

The investigation of biosynthetic pathways and generation of new natural product analogs by genetic engineering critically depends on the availability of tools for recombinant DNA work in the producing organism. In general, this can be achieved by the development of an expedient genetic system for the native producer of the respective compound. Alternative solutions to this most commonly used approach are represented by the heterologous expression of the entire biosynthetic gene cluster in a suitable host strain or by the identification of genetically accessible producers of structurally closely related compounds. Although mobilization of entire biosynthetic pathways is often challenging because of their size or host-specific limitations (7, 8), producer strains of similar compounds can be found only in very few cases.

We have been studying the biosynthesis of BLM in *Streptomyces verticillus* ATCC15003 (9–16) and the closely related compounds tallysomycin in *Streptoalloteichus hindustanus* E465–94 ATCC31158 (17) and zorbamycin in *Streptomyces flavoviridis* ATCC21892 (18). Feeding experiments with isotope-labeled precursors and isolation of biosynthetic intermediates suggested that the BLM aglycone is derived from nine amino acids and one acetate, with methionine serving as two methyl donors (16). Cloning and analysis of the BLM, and more recently tallysomycin, and zorbamycin biosynthetic gene clus-

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains experimental procedures, Tables S1–S3, and Figs. S1 and S2.

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³ The abbreviations used are: BLM, bleomycin; NRPS, nonribosomal peptide synthetase; PKS, polyketide synthase; 3-MOP, 3-morpholinopropylamine; HPLC, high performance liquid chromatography; MS, mass spectrometry; cfu, colony-forming unit; TSB, tryptic soy broth; HMBL, heteronuclear multiple bond correlation.

ters suggested that this family of compounds is formed *via* a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS)-catalyzed enzymology from amino acid and carboxylic acid precursors (10, 12, 14, 17, 18).

Despite exhaustive efforts, *S. verticillus* remained refractory to all means of introducing plasmid DNA into its cells, a fact that has so far prevented us from carrying out genetic analysis of the BLM biosynthetic locus *in vivo*. Protoplast-mediated transformation was inhibited by high lysozyme resistance of the *S. verticillus* mycelia as well as low frequencies of protoplast formation and regeneration. *Escherichia coli*-*Streptomyces* conjugation was complicated by poor sporulation characteristics of *S. verticillus*. All attempts to carry out transformation by electroporation proved to be unsuccessful. Only in one case was successful plasmid DNA transfer into *S. verticillus* reported by phage transduction. The plasmid, however, could not be recovered from these mutants (19). Here we present (i) the development of a genetic system for the BLM producer *S. verticillus*, (ii) *in vivo* confirmation and boundary determination for the BLM biosynthetic gene cluster by gene inactivation in *S. verticillus*, and (iii) isolation of a BLM pathway intermediate, decarbamoyl-BLM, followed by its chemical and biochemical characterization.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—*S. verticillus* ATCC15003 (American Type Culture Collection, Manassas, VA) and recombinant strains generated in this study were routinely grown at 28 °C in tryptic soy broth (TSB) liquid medium (20). ISP4 medium supplemented with 0.05% yeast extract, 0.1% tryptone, and MgCl₂ to a final concentration of 20 mM was used to prepare *S. verticillus* spores and to plate out conjugation mixtures. For BLM production, *S. verticillus* and recombinant strains were cultured in 250-ml baffled flasks containing 50 ml of the previously described seed medium (10) for 2 days at 28 °C and 250 rpm. Then, 5 ml of the seed cultures were inoculated into 250-ml baffled flasks containing 50 ml of the production medium (10) supplemented with 5 μ l of 3-morpholino-propylamine (3-MOP) and fermented at 28 °C and 250 rpm for 6–8 days. *E. coli* XL1 Blue MR (Stratagene, La Jolla, CA) and *E. coli* DH5 α (21) were used for routine subcloning, plasmid preparations, and cosmid library preparation. *E. coli* S17-1 (20) or *E. coli* ET12567 (pUZ8002) (22) were the donor strains in *E. coli*-*S. verticillus* conjugation, and *E. coli* BW25113/pIJ790 was the host for λ RED-mediated PCR targeting mutagenesis (23).

Plasmids and Biochemicals—The plasmid vectors pKC1139 (24), pKC1218 (24), pHZ1358 (25), pSET152 (24), pRT801 (26), pSOK804 (27), pIJ773 (23), pSET151 (24), and the *aac(3)IV* apramycin resistance cassette (20) were described previously. SuperCos1 (Stratagene) was used for *S. verticillus* cosmid library preparation, and pBluescript II SK(+) (Stratagene) was employed for DNA cleavage assays. Common biochemicals, chemicals, media, restriction enzymes, and other molecular biology reagents were from standard commercial sources. Ampicillin (150 μ g/ml), apramycin (50 μ g/ml), and chloramphenicol (25 μ g/ml) were used for selection in *E. coli*. Apramycin (50–100 μ g/ml) and thiostrepton (25–60 μ g/ml) were used

for selection of *S. verticillus* recombinants. Nalidixic acid (50 μ g/ml) was used to select against the *E. coli* conjugal donor after conjugation.

Genetic Manipulation of *S. verticillus* ATCC15003—*E. coli*-*S. verticillus* conjugation was carried out using a protocol based on standard literature conditions (20). In brief, $\sim 10^9$ – 10^{10} *S. verticillus* spores were heat-shocked in 500 μ l of TSB medium supplemented with 0.4% glycine and 10.0% sucrose at 50 °C for 10 min, followed by incubation at 30 °C for 3–4 h as *S. verticillus* recipients. *E. coli* S17-1 containing the respective plasmid was grown in LB medium to an A₆₀₀ of 0.4–0.6. Cells from 10 ml of culture were pelleted, washed twice with LB medium, and resuspended in 500 μ l of LB medium as the *E. coli* donors. For conjugation, the donors (500 μ l) and recipients (500 μ l, 10^9 – 10^{10} spores) were mixed and distributed onto ISP4 plates supplemented with 0.05% yeast extract, 0.1% tryptone, and MgCl₂ to a final concentration of 20 mM. The plates were incubated at 30 °C for 16–20 h and overlaid with 1 ml of water containing final concentrations of 50 μ g/ml nalidixic acid to select against *E. coli* and 100 μ g/ml apramycin to select for *S. verticillus* exconjugants. Incubation was continued at 30 °C until exconjugants appeared.

DNA Isolation and Manipulation—DNA isolations and manipulations in *E. coli* (21) and *Streptomyces* (20) were carried out according to standard procedures. For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Roche Applied Science). A cosmid library of *S. verticillus* chromosomal DNA was constructed in SuperCos1 according to the manufacturer protocol (Stratagene, La Jolla, CA). This library was screened with digoxigenin-labeled fragments derived from the BLM biosynthetic gene cluster (10) as probes. A set of four cosmids, pBS37, pBS38, pBS39, and pBS40, covering the entire BLM biosynthetic gene cluster, was isolated and used for gene inactivation experiments.

Sequencing and Computer-assisted Sequence Analysis—Sequencing of an additional 5385 bp beyond the downstream end of the published sequences (accession numbers AF210249 (10) and L26955 (28)) was performed by primer walking using the dideoxynucleotide chain termination method. Detailed procedures are provided in the supplemental data. The DNA sequence reported in this work was determined from pBS40 and has been deposited at GenBankTM under accession number EU805703.

Cloned Inactivation Constructs for Gene Replacement of *blmIV* (NRPS)—A 6.4-kb DNA fragment containing part of *blmIV* was cloned into pSET151 and separated into a 4.1-kb left and 2.3-right arm by the inserted *aac(3)IV* gene to afford the first inactivation construct for *blmIV*, pBS41.

To generate the second *blmIV* inactivation construct, a 22.5-kb DNA fragment containing *blmIV* was cloned into pSET151, and a 5.9-kb internal fragment was then replaced with the *aac(3)IV* gene to afford pBS42. Regions of 9.8- and 7.8-kb in size flanking the *aac(3)IV* gene at the upstream and downstream end, respectively, were available for homologous recombination.

Inactivation of *blmIV* (NRPS), *blmVIII* (PKS), *blm-orf31/32* (PHNA/NRPS), *blm-orf(-1)* (Trehalose Hydrolase), and *blmD*

(Carbamoyl Transferase) via λ RED-mediated PCR Targeting—The *blmIV*, *blmVIII*, *blm-orf31/32*, *blm-orf(-1)*, and *blmD* genes located on pBS38, pBS37, pBS37, pBS40, and pBS39, respectively, were replaced by the *aac(3)IV-oriT* cassette from pIJ773 via λ RED-mediated PCR targeting (23) using oligonucleotide pairs *blmIV*-1f and *blmIV*-1r, *blmVIII*-1f and *blmVIII*-1r, *NRPS10*-1f and *PHNA*-1r, *treh*-1f and *treh*-1r, and *blmD*-1f and *blmD*-1r, respectively, to yield pBS43, pBS44, pBS45, pBS46, and pBS47, respectively. The regions flanking the *aac(3)IV* gene upstream and downstream were 18.9- and 12.9-kb for pBS43, 23.5- and 12.2-kb for pBS44, 6.0- and 33.1-kb for pBS45, 17.6- and ~20-kb for pBS46, and 21.0- and ~15-kb for pBS47.

To generate an in-frame deletion of *blmD*, the *aac(3)IV-oriT* cassette was then removed by FLP recombination from pBS47, resulting in pBS48. The *bla* gene on the SuperCos1 backbone of pBS48 was finally replaced by the *aac(3)IV-oriT* cassette from pSET152 via λ RED-mediated PCR targeting (23) using the previously described oligonucleotides f-pMT3 and r-pMT3 (17) to yield pBS49.

Generation of the Mutant Strains and Analysis of Their Secondary Metabolite Profile—The inactivation constructs pBS43, pBS44, pBS45, pBS46, and pBS49 were introduced into the wild-type *S. verticillus* strain by intergeneric conjugation. Exconjugants were selected by a combination of the apramycin-resistant phenotype and PCR screening using oligonucleotide pairs *blmIV*-2f and *blmIV*-2r for pBS43, *blmVIII*-2f and *blmVIII*-2r for pBS44, C-PHNA-f1 and C-PHNA-r1 for pBS45, C-treh-f1 and C-treh-r1 for pBS46, as well as C-*blmD*-f2 and C-*blmD*-r for pBS49, respectively, affording the mutant strains *S. verticillus* SB1, SB2, SB3, SB4, and SB5 (Fig. 1 and supplemental Fig. S1). Suitable probes were PCR-amplified using oligonucleotide pairs *blmIII/IV*probe-f and *blmIII/IV*probe-r for SB1, *blmVIII*probe-f and *blmVIII*probe-r for SB2, C-PHNA-f1 and C-PHNA-r1 for SB3 (only a 1.446-kb BamHI fragment of the 2.788-kb PCR product was used as probe), Treh-p-f1 and Treh-p-r1 for SB4, and C-*blmE*-f2 and C-*blmE*-r for SB5. The genotypes of all apparent double cross-over mutant strains were further confirmed by Southern blot analyses (Fig. 1 and supplemental Fig. S1). The mutants were cultured and investigated for BLM production with the wild-type *S. verticillus* strain as a control (Fig. 2, traces I, IV, III, II, VI, and V). The production levels of BLM from the SB3 and SB4 mutant strains and decarbamoyl-BLM from the SB5 mutant strain were 8–10 mg/liter (~95% of the wild-type strain), 8–10 mg/liter (~95% of the wild-type strain), and 6–8 mg/liter, respectively, as estimated by HPLC analysis. The 3-MOP-BLM-Cu and 3-MOP-decarbomoyl-BLM-Cu complexes were eluted with retention times of 17.1 and 16.0 min, respectively.

Isolation and Analysis of BLM and Decarbamoyl-BLM—BLM and decarbamoyl-BLM were first isolated as copper complexes. Copper-free BLM and decarbamoyl-BLM were obtained by treating the copper complexes with 0.5 M EDTA-Na, pH 7.3, solution. The identity of BLM was confirmed by liquid chromatography-electrospray ionization-mass spectrometry on an Agilent 1100 HPLC-MSD SL quadrupole mass spectrometer. The structure of decarbamoyl-BLM was established by spectroscopic analyses. Decarbamoyl-BLM-Cu complex: light blue powder; electro-

spray ionization-MS m/z 729.4 [3-MOP-decarbomoyl-BLM-Cu] $^{2+}$, calculated for 3-MOP-decarbomoyl-BLM-Cu, 1458. copper-free decarbamoyl-BLM: pale white powder; $[\alpha]_D^{25} +48.8$ (c 0.1, H₂O); UV (H₂O) λ_{max} 229 (shoulder), 288 (ϵ 17850) nm; IR (neat) ν_{max} 3400–3200, 1671, 1550, 1201, 1184, 1131 cm $^{-1}$; 1H and ^{13}C NMR data (see Table 1); high resolution matrix-assisted laser desorption-ionization fourier transform-MS m/z 1396.5664 $[M + H]^+$, C₅₆H₈₅N₁₇O₂₁S₂ + H $^+$ (calculated, 1396.5661). Detailed procedures are provided in the supplemental data.

DNA Cleavage Assay—The DNA cleavage assays were performed in 10 μ l (total volume) of 25 mM Tris-HCl buffer, pH 7.5, containing ~20 ng of pBluescript SK II(+) plasmid DNA, 10 μ M Fe(NH₄)₂(SO₄)₂·6 H₂O (freshly prepared solution in 1 mM H₂SO₄) and the appropriate concentrations of BLM A2, BLM B2, or 3-MOP-decarbomoyl-BLM. The reactions were incubated at 35 °C for 30 min and stopped by the addition of 5 mM EDTA and 5 μ l of loading dye (30% glycerol containing 0.25% (w/v) bromophenol blue). The samples were applied to a 0.8% agarose gel containing 1 μ g/ml ethidium bromide, and gel electrophoresis was carried out in 40 mM Tris acetate buffer, pH 8.0, containing 1 mM disodium EDTA at 90 V for 1 h. The DNA bands were evaluated under UV light.

Nucleotide Sequence Accession Number—The nucleotide sequences reported in this study are available in the GenBankTM data base under accession numbers L26955, AF210249, and EU805703.

RESULTS

Development of a Genetic System for *S. verticillus* ATCC15003—Aiming at the establishment of a genetic system for the BLM producer, the growth and sporulation conditions were evaluated and optimized. *S. verticillus* grows optimally between 28 and 30 °C. Common media such as TSB, ISP2, and R2YE (20) are suitable for vegetative growth of *S. verticillus*. However, for genomic DNA isolation, it is necessary to supplement TSB with 10% (w/v) sucrose and 0.4% (w/v) glycine to facilitate lysozyme digestion. SGGP medium (29) supplemented with 10% or 20% (w/v) sucrose and YEME medium (20) supplemented with 34% (w/v) sucrose proved most suitable for protoplast preparation. *S. verticillus* sporulates very poorly on common growth media such as TSB, ISP2, R2YE, and CM medium. Slightly green spore formation to an acceptable extent (~10⁷ spores/plate) was observed after 10 days of growth at 30 °C on ISP4 medium or ISP4 medium supplemented with 0.05% yeast extract, 0.1% tryptone, and MgCl₂ to a final concentration of 20 mM. The BLM producer is fairly sensitive to apramycin, thiostrepton, and kanamycin but fully resistant to even high concentrations (up to 200 μ g/ml) of hygromycin; therefore, 50–150 μ g/ml of apramycin and 25–60 μ g/ml of thiostrepton were used throughout this study for selection.

Intergeneric conjugation allowed the introduction of two of six plasmid vectors, pSET152 and pRT801, into *S. verticillus*. Although *S. verticillus* mycelium of the early exponential growth phase can be protoplasted in a respectable yield (20–30%), the protoplasts cannot be regenerated efficiently under all conditions tested (\leq 10%). All attempts to introduce plasmid DNA into *S. verticillus* by protoplast-mediated transformation failed to produce any true transformants, and this technique

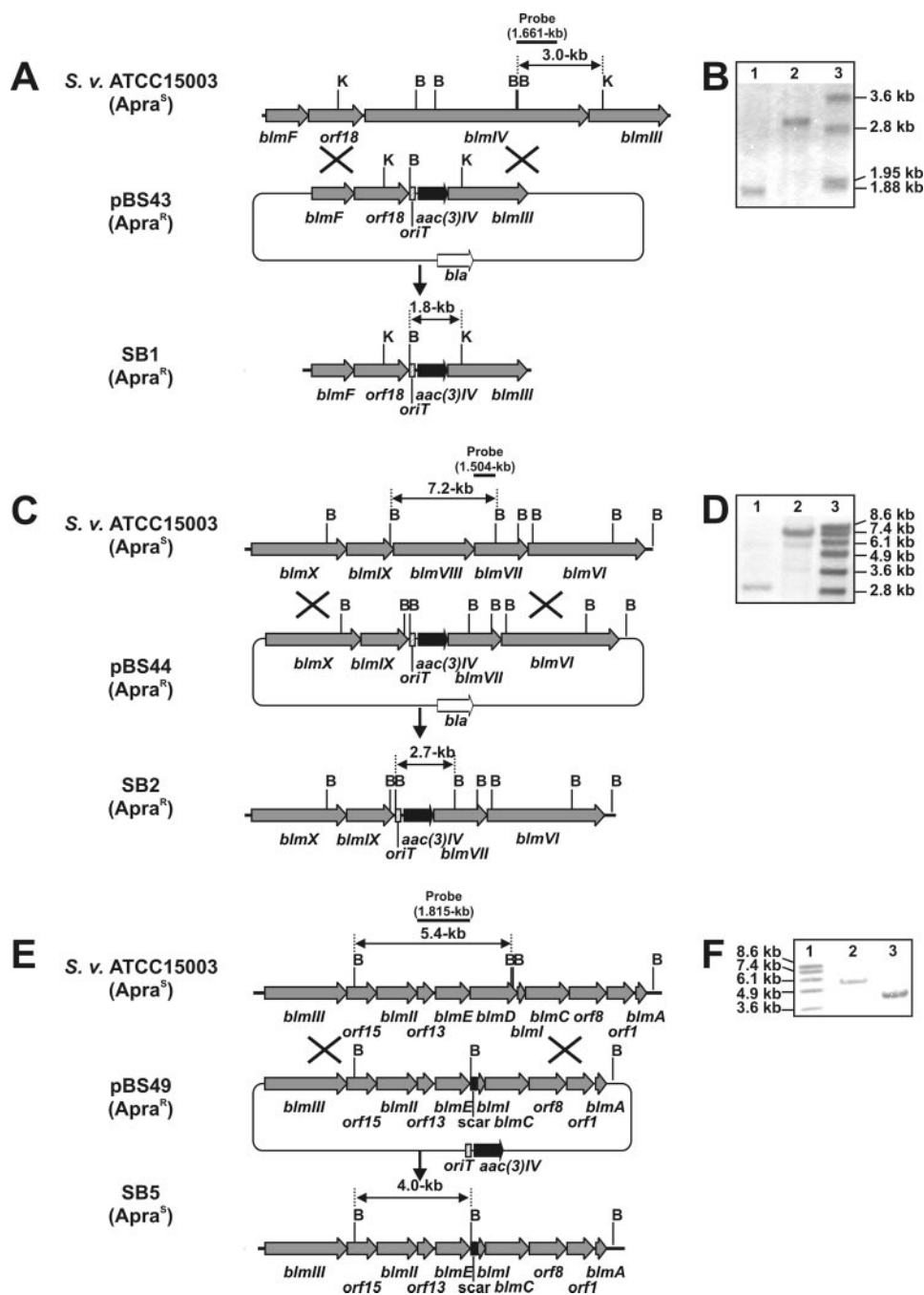


FIGURE 1. Inactivation of *blmIV*, *blmVIII*, and *blmD*. Shown are constructions of *blmIV* (A) and *blmVIII* (C) gene replacement as well as *blmD* (E) in-frame deletion mutants and restriction map of *S. verticillus* wild-type (A, C, and E), SB1 (A), SB2 (C), and SB5 (E) mutant strains showing fragment sizes upon BamHI-KpnI (A) and BamHI (C and E) digestion, respectively. B, Southern analysis of *S. verticillus* wild-type (B, D, and F, lanes 2) and SB1 (B, lane 1), SB2 (D, lane 1), and SB5 (F, lane 3) genomic DNA digested with BamHI and KpnI (B) or BamHI (D and F), respectively, using a 1.661-kb (B), a 1.504-kb (D), and a 1.815-kb (F) PCR-amplified fragment, respectively, as probes. Molecular weight marker, lane 3 (B and D) and lane 1 (F). B, BamHI; K, KpnI; *Apra^r*, apramycin-resistant; *Apra^s*, apramycin-sensitive.

was subsequently abandoned. In contrast, we were successful in developing a protocol for conjugation between *E. coli* S17-1 and *S. verticillus*. Following a literature procedure (20) with modifications, we found that conjugation between *E. coli* S17-1 and *S. verticillus* occurred with the highest frequency on modified ISP-4 agar freshly supplemented with 20 mM MgCl₂ when 10⁹–10¹⁰ *S. verticillus* recipient cells and *E. coli* S17-1 donor cells harvested from 10 ml of culture grown to an A₆₀₀ of 0.4–0.6

were used. Utilization of the methylation deficient *E. coli* ET12567/pUZ8002 as donor strain failed to result in higher exconjugant frequencies than the methylation proficient *E. coli* S17-1. The true nature of exconjugants was verified by PCR and Southern blot hybridization.

Self-replicating plasmids derived from three different replicons (the temperature-sensitive pSG5 derivative pKC1139 (24), the SCP2* derivative pKC1218 (24), and the pIJ101 derivative pHZ1358 (25)) and integrative vectors derived from three different bacteriophages (the ϕ C31 derived pSET152 (24), the phiBT1-derived pRT801 (26), and the VWB-derived pSOK804 (27)) were tested in a series of *E. coli*-*S. verticillus* intergeneric conjugation experiments. All replicative vectors and one of the integrative vectors, pSOK804, failed to yield any true exconjugants. The highest conjugation frequencies were obtained with pSET152 and pRT801 at 10⁻⁷–10⁻⁸ exconjugants/cfu.

Homologous Recombination in *S. verticillus* ATCC15003—Gene replacement for *blmIV* and *blmVIII* via double cross-over homologous recombination was carried out to confirm, for the first time, the identity of the BLM biosynthetic gene cluster by gene inactivation. Construct pBS41, which contains a 6.4-kb fragment of *blmIV* separated into a 4.1-kb upstream and a 2.3-kb downstream region by the insertion of the *aac(3)IV* apramycin resistance gene, was first chosen for this experiment. Construct pBS42, which harbors a 9.8-kb upstream fragment and a 7.8-kb downstream fragment, was also generated for homologous recombination. No true exconjugants were obtained after conjugal

transfer of these plasmids into *S. verticillus*.

A newly prepared cosmid library of *S. verticillus* genomic DNA was then employed for gene inactivation by λ RED-mediated PCR targeting mutagenesis (23). Replacement of the NRPS encoding *blmIV* gene by the *aac(3)IV* gene on pBS38 generated 18.9-kb upstream and a 12.9-kb downstream flanking regions accessible for homologous recombination in pBS43 (Fig. 1A). Similarly, inactivation of the PKS

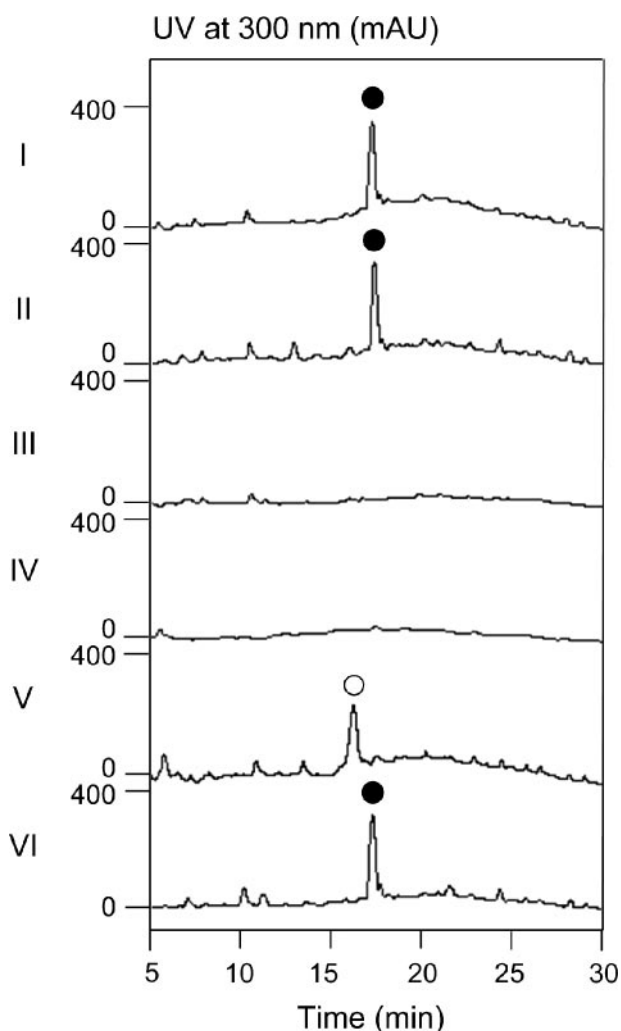


FIGURE 2. HPLC analysis of 3-MOP-BLM (●) production in *S. verticillus* wild-type (I) and mutant strains SB3 (II), SB2 (III), SB1 (IV), and SB4 (VI) and of 3-MOP-decarbamoyl-BLM (○) production in mutant strain SB5 (V).

encoding *blmVIII* gene on pBS37 yielded 23.5-kb upstream and 12.2-kb downstream regions in pBS44 (Fig. 1C). After introduction of pBS43 and pBS44 into *S. verticillus* by conjugation, exconjugants resistant to apramycin were isolated, and the double cross-over mutants SB1 and SB2, respectively, were selected (Fig. 1, B and D). Both mutant strains completely lost their ability to produce BLM (Fig. 2, traces IV and III), confirming that the NRPS encoding *blmIV* and the PKS encoding *blmVIII* are indeed required for BLM biosynthesis. The conjugation frequencies observed for both inactivation constructs were in the same range as for the integrative model plasmid pSET152 (10^{-7} – 10^{-8} exconjugants/cfu).

Determination of the Boundaries for the BLM Biosynthetic Locus by Gene Inactivation—Through inactivation of selected genes (*blm-orf31/32* and *blm-orf(-1)*) residing at distal ends of the BLM biosynthetic gene cluster, the upstream and downstream *blm* cluster boundaries were assigned to reside between *blm-orf31/32* and *blm-orf30* and between *blm-orf7* and *blm-orf(-1)*, respectively (supplemental Fig. S2). At the upstream end of the cluster, *blm-orf30* and *blm-orf29* had previously been proposed to play a role in the regulatory and self-resistance

mechanisms in BLM production, respectively (10). The predicted gene products of *blm-orf31* (a protein with similarity to phosphonoacetate hydrolases) and *blm-orf32* (an NRPS protein) were thought to be dispensable for BLM biosynthesis. Gene replacement of *blm-orf31* together with part of *blm-orf32* yielded the mutant strain SB3 that exhibited BLM production levels similar to the wild-type *S. verticillus* strain (~95% of the wild-type strain; Fig. 2, traces II and I).

At the downstream boundary, *blm-orf7* encoding a transport protein with a possible function in BLM resistance mechanisms of the producer strain represented the last gene of the sequenced region (accession number L26955) (28). Sequencing and analysis of ~5.4-kb beyond the published nucleotide sequence revealed a ~3.2-kb gap without any apparent open reading frames downstream of *blm-orf7* followed by *blm-orf(-1)*. The *blm-orf(-1)* gene product (802 amino acids) exhibits high similarity to hydrolases such as a putative glycosyl hydrolase from *Streptomyces ambofaciens* (accession number CAJ88378; 67% identity and 76% similarity) and a hypothetical protein from *Streptomyces coelicolor* (accession number NP_624928; 68% identity and 76% similarity). Inactivation of *blm-orf(-1)* yielded the mutant strain SB4 that had little effect on BLM production (~95% of the wild-type strain; Fig. 2, traces VI and I), confirming that its product is not required for BLM biosynthesis.

In-frame Deletion of *blmD* Resulting in the Accumulation of a Pathway Intermediate—Inactivation of the *blmD* gene resulted in the accumulation of a pathway intermediate, decarbamoyl-BLM, which was characterized by MS and NMR spectroscopic analyses. In-frame deletion was chosen for the inactivation of the carbamoyl transferase encoding *blmD* gene. This strategy ensures the generation of nonpolar mutants, in contrast to gene replacement mutants bearing an antibiotic resistance cassette. The selected double cross-over mutant, SB5, completely lost its ability to produce BLM and instead accumulated a new compound, decarbamoyl-BLM, with a slightly different retention time (Fig. 2, traces V and I). This new compound (6–8 mg/liter) was isolated, and the pure intermediate (10.0 mg) was obtained as a copper complex. The decarbamoyl-BLM-Cu complex was further treated with EDTA to remove copper and subsequently subjected to HPLC on a C18 column to afford the copper-free molecule (8.0 mg) as a pale white powder.

Structure elucidation of the intact molecule of 3-MOP-decarbamoyl-BLM was carried out by a combination of MS along with ^1H , ^{13}C , and two-dimensional NMR spectroscopic analyses, as well as by comparing the resultant NMR data with those reported in the literature, particularly of BLM A2 (30) (Fig. 3). Upon electrospray ionization-MS analysis, the 3-MOP-decarbamoyl-BLM-Cu complex yielded a molecular ion peak (m/z) at 729.4, consistent with the $[\text{M} + \text{Cu}]^{2+}$ ion for the 3-MOP-decarbamoyl-BLM-Cu complex (calculated, 1458.5). Upon high resolution matrix-assisted laser desorption-ionization fourier transform MS analysis, copper-free 3-MOP-decarbamoyl-BLM showed a molecular ion peak at m/z 1396.5664 $[\text{M} + \text{H}]^+$, which agreed well with the copper-free 3-MOP-decarbamoyl-BLM molecular formula of $\text{C}_{56}\text{H}_{85}\text{N}_{17}\text{O}_{21}\text{S}_2 + \text{H}^+$ (calculated, 1396.5661). Table 1 summarizes the ^1H and ^{13}C NMR data of copper-free 3-MOP-de-

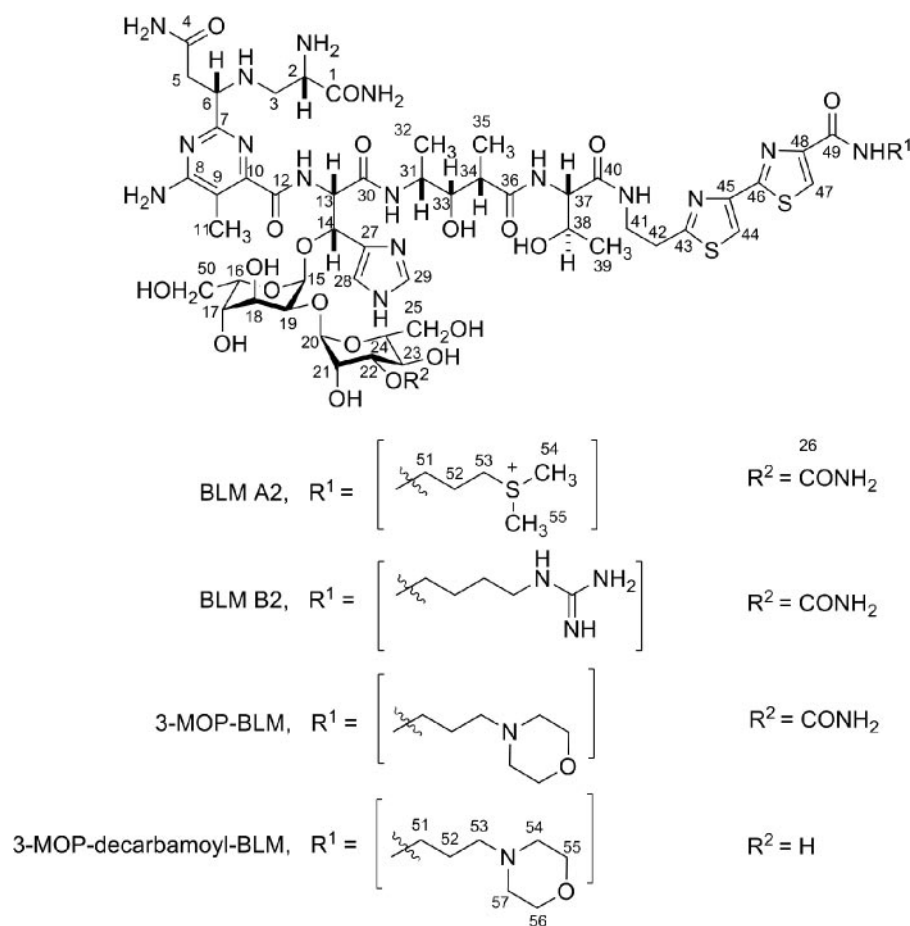


FIGURE 3. Structures of the antitumor antibiotics BLM A2 and B2, 3-MOP-BLM, and the newly characterized pathway intermediate 3-MOP-decarbamoyle-BLM.

carbamoyl-BLM in D_2O . The ^{13}C NMR spectrum combined with HSQC spectrum showed a total of 54 carbons including 4 methyl, 11 methylene, 23 methine, and 16 quaternary carbons. The ^{13}C NMR spectrum appeared to lack two signals (C_{54}) relative to the total carbon number proposed for the molecular formula of 3-MOP-decarbamoyle-BLM by high resolution MS ($\text{C}_{56}\text{H}_{85}\text{N}_{17}\text{O}_{21}\text{S}_2$) because of the symmetric structure of the morpholino ring. The signals at δ_{C} 55.0 and δ_{C} 67.7, however, were approximately twice as high as the ordinary carbon signals, suggesting that these two signals represent the symmetric carbons of the morpholino ring. Analyses of ^1H - ^1H COSY and TOCSY spectra lead to the partial structures of C2-C3, C5-C6, C13-C14, C32-C31-C33-C34-C35, C39-C38-C37, C41-C42, C51-C52-C53, C54,57-C55,56, and two sugar moieties, which were subsequently connected using heteronuclear multiple bond correlation (HMBC) (Fig. 4). The ^1H and ^{13}C NMR spectra of 3-MOP-decarbamoyle-BLM are almost identical to the ones of BLM except that (i) they lack the signals from the carbamoyl carbonyl group (C26) attached to the disaccharide moiety of BLM and (ii) the signals attributed to the 3-MOP (δ_{H} 3.50, δ_{C} 39.8 (C51), δ_{H} 2.02, δ_{C} 27.1 (C52), δ_{H} 2.96, δ_{C} 58.0 (C53), δ_{H} 3.03, δ_{C} 55.0 (C54,57), δ_{H} 3.89, δ_{C} 67.7 (C55,56)) differed from the signals obtained with the BLM A2 amine (30) (Fig. 3). The C-21, 22, and 23 of the mannosyl moiety of 3-MOP-decarbamoyle-BLM clearly showed a des-acylation shift compared with those signals of BLM ($\Delta\delta_{\text{H}22} = -0.92$,

$\Delta\delta_{\text{C}21} = +1.5$, $\Delta\delta_{\text{C}22} = -4.2$, and $\Delta\delta_{\text{C}23} = +2.0$ with $\Delta\delta$ defined as $\Delta\delta = \delta_{3\text{-MOP-decarbamoyle-BLM}} - \delta_{\text{BLM}}$). These data suggested that the isolated intermediate represents 3-MOP-decarbamoyle-BLM and confirmed BLM as the carbamoyl transferase.

DNA Cleavage Activity of Decarbamoyle-BLM—Loss of the carbamoyl group attached to the BLM-disaccharide resulted in a decrease in DNA cleavage activity by a factor of 10. Decarbamoyle-BLM was compared with BLM A2 and B2 for its ability to cleave pBluescript II SK(+) supercoiled plasmid DNA in the presence of Fe^{2+} . BLM-mediated single-strand cleavage first results in the conversion of supercoiled plasmid DNA (form A) to open circular plasmid DNA (form B), and double-strand cleavage subsequently generates linearized plasmid DNA (form C). In this assay, BLM A2 and B2 showed nearly 100% plasmid relaxation and ~50% plasmid linearization at concentrations of $1\text{ }\mu\text{M}$ each, whereas decarbamoyle-BLM exhibited ~10-fold reduced DNA cleavage activity, resulting in a mixture of super-

coiled, relaxed, and linearized plasmid DNA at a concentration of $5\text{ }\mu\text{M}$ (Fig. 5). The observed DNA cleavage activity was concentration-dependent for all of the compounds tested.

DISCUSSION

More than four decades after the discovery of the clinically important anticancer compound BLM (1), its native producer *S. verticillus* unambiguously proved genetically accessible for the first time. Although some improvement of protoplast formation and regeneration was achieved during these studies, DNA transfer by protoplast-mediated transformation failed to yield any true transformants. Intergeneric *E. coli*-*Streptomyces* conjugation under optimized conditions, however, facilitated the development of a genetic system for this pharmaceutically important microorganism.

From an entire set of six different replicative and integrative vectors employed as test plasmids, two of three integrative vectors, pSET152 and pRT801, yielded true exconjugants at frequencies of 10^{-7} – 10^{-8} exconjugants/cfu, which is 10^5 – 10^6 -fold lower than reported for *Streptomyces lividans* ($\sim 10^{-2}$ exconjugants/cfu) under similar conditions (31). Failure to introduce the self-replicating vectors originating from three different *Streptomyces* replicons indicated that this host strain is unable to propagate any of the well known *E. coli*-*Streptomyces* shuttle vectors, thereby significantly complicating expression and complementation experiments in this strain.

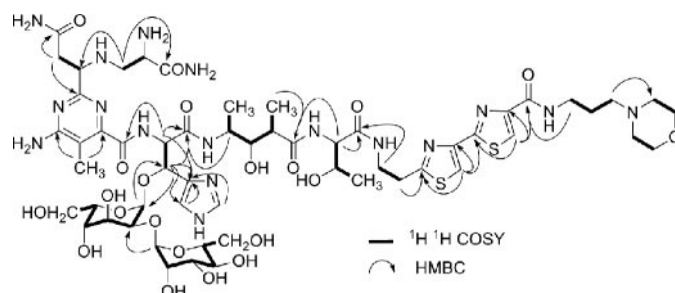
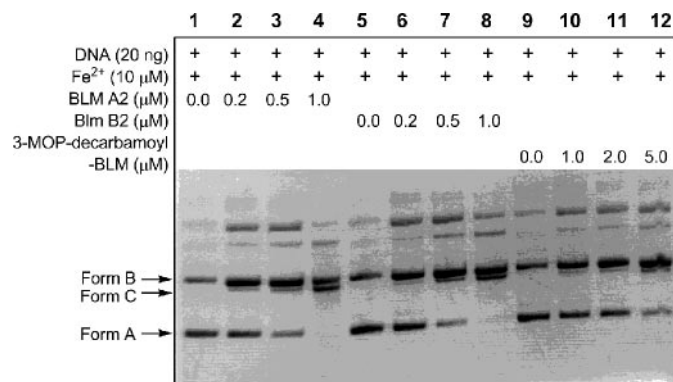
TABLE 1
¹H and ¹³C NMR data of 3-MOP-decarbamoyle-*BLM* (D₂O, TSP, δ (ppm) (*J* = Hz))

 Assignments were confirmed by DEPT, ¹H-¹H COSY, TOCSY, HSQC, HMBC, spectra obtained at 500 and 125 MHz, pH adjusted to 7.0 by NaOD.

No.	δ _C	δ _H
1	175.6	
2	55.6	3.94 m ^a
3	50.6	2.97 dd (13.5, 7.5), 2.95 m ^a
4	179.0	
5	43.0	2.64 dd (14.5, 8.5), 2.71 dd (14.5, 6.0)
6	62.5	3.97 dd (8.5, 6.0)
7	168.4	
8	167.2	
9	114.4	
10	156.0	
11	13.6	1.95 s
12	170.6	
13	59.5	5.11 d (7.0)
14	76.5	5.18 d (7.0)
15	101.07	5.26 d (3.5)
16	69.6	4.02 m ^a
17	71.9	3.89 m ^a
18	70.6	4.11 m ^a
19	72.3	4.03 m ^a
20	100.97	5.02 brs
21	73.0	3.93 m ^a
22	73.3	3.76 m ^a
23	69.9 ^a	3.63 m ^a
24	76.3	3.69 m ^a
25	63.9	3.79 m, 3.91 m ^a
27	137.9	
28	120.7	7.29 s
29	139.8	7.82 s
30	172.0	
31	50.3	3.92 m ^a
32	17.7	1.20 d (7.0)
33	77.2	3.76 m ^a
34	45.4	2.50 m
35	14.8	1.15 d (7.0)
36	180.4	
37	61.9	4.27 d (4.5)
38	69.9 ^a	4.13 m ^a
39	21.7	1.12 d (7.0)
40	174.8	
41	41.9	3.64 m ^a
42	34.8	3.28 t (6.0)
43	173.5	
44	121.8	8.02 s
45	149.8	
46	165.5	
47	127.8	8.18 s
48	151.7	
49	166.1	
50	62.8	3.49 m, 3.33 dd (11.0, 5.0)
51	39.8	3.50 m ^a
52	27.1	2.02 m
53	58.0	2.96 m ^a
54, 57	55.0	3.03 m
55, 56	67.7	3.89 m ^a

^a Overlapped signals.

Typically, the frequency of site-specific integration into the host chromosome is much higher than that of homologous recombination for a specific *Streptomyces* strain. Based on the observation of site-specific integration frequencies as low as 10⁻⁷–10⁻⁸ exconjugants/cfu for *S. verticillus*, failure to obtain gene inactivation mutants by homologous recombination was not surprising. Because the frequency for homologous recombination is also proportional to the size of the DNA regions available for the recombination event (20), a recently developed technology (λ RED-mediated PCR targeting mutagenesis) (23) provided a promising new strategy to genetically modify this strain allowing the facile increase of inactivation constructs. As opposed to 2.3- and 4.1-kb homologous arms and 9.8- and 7.8-kb homologous arms in the earlier constructs pBS41 and


FIGURE 4. Key two-dimensional NMR correlations of 3-MOP-decarbamoyle-*BLM*.

FIGURE 5. DNA cleavage activities of BLM A2, B2, and 3-MOP-decarbamoyle-*BLM* as observed in plasmid relaxation assays with pBluescript II SK(+). Form A, supercoiled plasmid DNA; form B, open circular plasmid DNA; form C, linearized plasmid DNA.

pBS42, respectively, flanking regions of >12-kb on at least one side of the targeted gene in constructs pBS43, pBS44, pBS45, pBS46, and pBS49 enabled the generation of the desired mutant strains at surprisingly high frequencies, very comparable with site-specific integration of pSET152 and pRT801. From these data we conclude that for *S. verticillus*, the minimal size range of DNA fragments allowing for homologous recombination events to occur lies between 10 and 12 kb, which is uncommonly large compared with other *Streptomyces* strains (20).

To date, many other microbial producers of important natural products have been reported to be genetically inaccessible or extremely hard to manipulate (32–34). However, the availability of an efficient genetic system is one of the key prerequisites for successful engineering of natural product biosynthetic pathways to generate new biologically active derivatives using combinatorial biosynthesis. The results from our studies indicate a potential solution for this widely experienced obstacle in microbiology, especially in cases where the alternative approach of heterologously expressing the desired biosynthetic pathway has failed or is not feasible.

Multiple gene knock-out experiments by gene replacement and in-frame deletion confirmed the identity of the BLM biosynthetic locus *in vivo*, experimentally defined the BLM cluster boundaries, and revealed new insights into the timing of BLM-carbamoylation by BlmD. Five gene inactivation experiments proved the involvement of *blmIV*, *blmVIII*, and *blmD* in BLM biosynthesis and confirmed the predicted cluster boundaries to be located downstream of *blm-orf31/32* and upstream of *blm-orf(-1)*. All gene replacement mutants showed either the expected BLM producing phenotype for targeted genes located

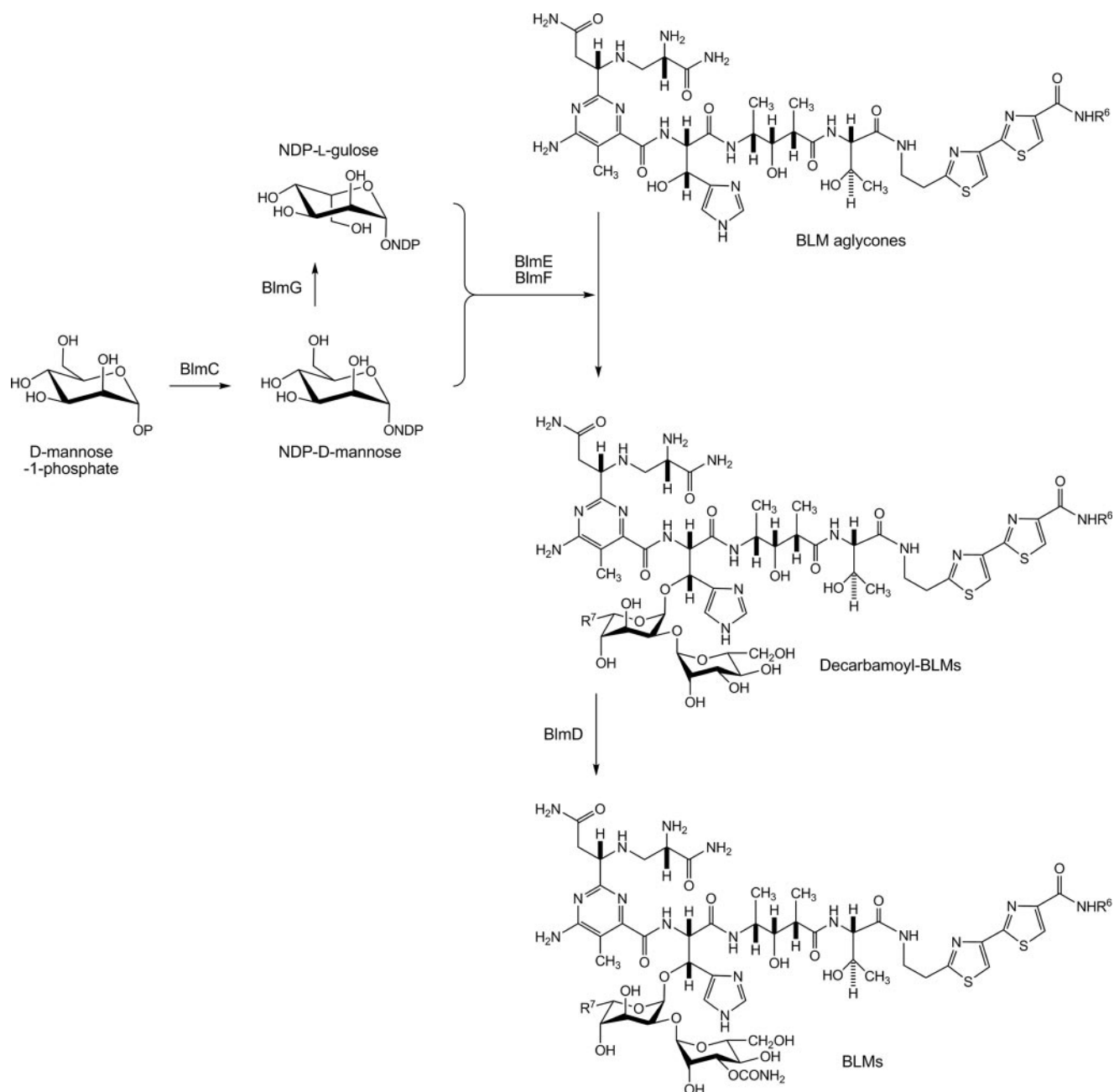


FIGURE 6. Revised biosynthetic pathway for formation of the disaccharide moiety of BLM from D-mannose, its attachment, and carbamoylation.

outside the cluster boundaries (*blm-orf31/32* and *blm-orf(-1)*) or complete abolishment of BLM production for genes proposed to be involved in BLM biosynthesis (*blmIV* and *blmVIII*). Only the in-frame deletion of *blmD*, however, yielded the anticipated pathway intermediate, decarbamoyl-BLM. It may be speculated that early pathway intermediates stay tethered to their respective NRPS or PKS enzymes and therefore could not be isolated from the respective mutant fermentations. Moreover, intermediates lacking the sugar moiety may not be recognized by the respective transport proteins for their export out of the cells and hence escape detection in the culture broth. Isolation of 3-MOP-decarbamoyl-BLM from the cell free supernatant of the respective fermentations, in contrast, indicates efficient transport of late biosynthetic intermediates into the

production medium. A one-step purification procedure using Amberlite IRC-50 resin (H^+ type) allowed us to estimate the titer of 3-MOP-decarbamoyl BLM to be 6–8 mg/liter representing ~70% of the 3-MOP-BLM production observed in wild-type *S. verticillus* (8–10 mg/liter) under equivalent fermentation conditions. The 3-MOP-decarbamoyl BLM was then purified from preparative scale fermentation.

Extensive analysis of MS, 1H , ^{13}C , 1H - 1H COSY, TOCSY, HSQC, and HMBC data for this compound resulted in the full 1H and ^{13}C NMR spectroscopic assignments (Fig. 4 and Table 1), and their comparison with the BLM A2 spectroscopic data reported in the literature (30) confirmed that the isolated intermediate indeed represents 3-MOP-decarbamoyl-BLM. Decarbamoyl-BLM has previously been reported as a partial hydroly-

ysis product of BLM under basic aqueous conditions after prolonged storage but has only been accessible in very small quantities (35, 36). Additionally, decarbamoyl-BLM demethyl-A2, lacking one methyl group in the A2 terminal amine, was isolated as a byproduct during the tedious multi-step total chemical synthesis of BLM demethyl-A2 (37). From a structural point of view, the carbamoyl group has been speculated to represent one of the ligands involved in formation of the active metal complexed BLM. Detailed mechanistic investigations, however, have so far been hampered by the limited availability of decarbamoyl-BLM. The accessibility of this particularly important compound in large quantities by simple fermentation and compound isolation procedures from the mutant of a highly appreciated, pharmaceutically relevant *Streptomyces* strain now facilitates such studies.

Our data presented here support the hypothesis that carbamoylation is the final step of BLM biosynthesis, revising the previous assumptions for disaccharide formation and attachment (Fig. 6) (10). This is in agreement with the novobiocin biosynthetic pathway for which carbamoylation of the noviose sugar was also reported to be the final catalytic step (38). The previously discussed alternative of the mannose moiety first being carbamoylated by BlmD and then transferred onto the BLM-monosaccharide by the respective glycosyltransferase (BlmE or BlmF) seems less likely, because this pathway would require broad substrate flexibility of the glycosyltransferase to catalyze decarbamoyl-mannose transfer to form 3-MOP-decarbamoyl-BLM in the *blmD* defective mutant strain.

Investigation of 3-MOP-decarbamoyl-BLM for DNA relaxation and linearization in comparison with BLM A2 and B2 indicates an important role of the carbamoyl group for efficient cleavage activity. Although several *in vitro* studies have shown that deglyco-BLMs exhibit reduced DNA cleavage activities compared with BLM A2 or B2 (36, 39, 40), the role of the carbamoyl group remained controversial. Decarbamoyl-BLM was reported to show significantly reduced malondialdehyde forming activity, but similar oligonucleotide cleavage activity compared with BLM A2 (36), and similar DNA cleavage activity as BLM in the presence of Fe^{2+} , but reduced activity in the presence of Cu^{2+} and dithiothreitol (39), and oligonucleotide cleavage activities much closer to deglyco-BLM A2 rather than BLM A2 (41). Our results obtained with 3-MOP-decarbamoyl-BLM, however, indicate a significant impact of the carbamoyl group on DNA cleavage activity resulting in ~10-fold reduced plasmid relaxation efficiency compared with BLM A2 and B2. This observation may originate from the carbamoyl group directly contributing to the coordination of the metal ion for DNA cleavage or from being involved in DNA binding. Although there has been speculation about the former hypothesis (41), the latter is supported by the recently published crystal structure of DNA-bound Co(III)-BLM B2; the carbamoyl NH_2 forms a hydrogen bond with the minor groove, whereas the function of the disaccharide seems to be to correctly position and stabilize the entire complex (42). In addition to the likely reduced DNA affinity of 3-MOP-decarbamoyl-BLM caused by the lack of the carbamoyl group, the different terminal amines of 3-MOP-decarbamoyl-BLM, BLM A2, and B2 may also contribute to the variation in DNA cleavage

activity. In depth spectroscopic and mechanistic studies of 3-MOP-decarbamoyl-BLM in comparison with BLM will now allow elucidation of the exact contribution of the carbamoyl group to DNA binding, cleavage activity, cytotoxicity, and bioavailability of the BLM family of compounds.

In summary, since the identification and sequence analysis of the BLM biosynthetic gene cluster several years ago (10, 12, 14), the inaccessibility of *S. verticillus* to genetic manipulation has prevented us from in depth *in vivo* investigations of its biosynthetic pathway. Moreover, all attempts to express the entire ~65-kb BLM biosynthetic gene cluster in a heterologous host and to engineer the respective DNA sequence therein have been unsuccessful as well. Although progress in genetic manipulation of the related tallsomycin and zorbamycin producers has been made recently (17, 18), this study represents the first report of a new BLM derivative generated *in vivo* by engineering of the respective producer strain and demonstrates the feasibility of BLM analog production in suitable mutants on a large scale in the future. Moreover, the availability of an *S. verticillus* mutant strain producing 3-MOP-decarbamoyl-BLM in respectable yields will now greatly facilitate spectroscopic and mechanistic studies, providing insight into the exact mode of action of the BLM family of antibiotics.

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REFERENCES

1. Umezawa, H., Maeda, K., Takeuchi, T., and Okami, Y. (1966) *J. Antibiot.* **19**, 200–209
2. Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Umezawa, Y., Naganawa, H., and Umezawa, H. (1978) *J. Antibiot.* **31**, 801–804
3. Sikic, B. I., Rosenzweig, M., and Chater, S. K. (1985) *Bleomycin chemotherapy*, Academic Press, New York
4. Galm, U., Hager, M. H., Van Lanen, S. G., Ju, J., Thorson, J. S., and Shen, B. (2005) *Chem. Rev.* **105**, 739–758
5. Hecht, S. M. (2000) *J. Nat. Prod.* **63**, 158–168
6. Chen, J., and Stubbe, J. (2005) *Nat. Rev. Cancer* **5**, 102–112
7. Galm, U., and Shen, B. (2006) *Expert Opin. Drug Discov.* **1**, 409–437
8. Wenzel, S. C., and Muller, R. (2005) *Curr. Opin. Biotechnol.* **16**, 594–606
9. Du, L., and Shen, B. (1999) *Chem. Biol.* **6**, 507–517
10. Du, L., Sanchez, C., Chen, M., Edwards, D. J., and Shen, B. (2000) *Chem. Biol.* **7**, 623–642
11. Du, L., Chen, M., Sanchez, C., and Shen, B. (2000) *FEMS Microbiol. Lett.* **189**, 171–175
12. Shen, B., Du, L., Sanchez, C., Edwards, D. J., Chen, M., and Murrell, J. M. (2001) *J. Ind. Microbiol. Biotechnol.* **27**, 378–385
13. Sanchez, C., Du, L., Edwards, D. J., Toney, M. D., and Shen, B. (2001) *Chem. Biol.* **8**, 725–738
14. Shen, B., Du, L., Sanchez, C., Edwards, D. J., Chen, M., and Murrell, J. M. (2002) *J. Nat. Prod.* **65**, 422–431
15. Du, L., Chen, M., Zhang, Y., and Shen, B. (2003) *Biochemistry* **42**, 9731–9740
16. Shen, B., Du, L. C., Sanchez, C., Chen, M., and Edwards, D. J. (1999) *Bioorg. Chem.* **27**, 155–171
17. Tao, M., Wang, L., Wendt-Pienkowski, E., George, N. P., Galm, U., Zhang, G., Coughlin, J. M., and Shen, B. (2007) *Mol. Biosyst.* **3**, 60–74
18. Wang, L., Yun, B.-S., George, N. P., Wendt-Pienkowski, E., Galm, U., Oh, T. J., Coughlin, J. M., Zhang, G., Tao, M., and Shen, B. (2007) *J. Nat. Prod.*

- 70, 402–406
19. Burke, J., Schneider, D., and Westpheling, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6289–6294
20. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, 2nd Ed., John Innes Foundation, Norwich, UK
21. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Paget, M. S., Chamberlin, L., Atrih, A., Foster, S. J., and Buttner, M. J. (1999) *J. Bacteriol.* **181**, 204–211
23. Gust, B., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1541–1546
24. Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) *Gene (Amst.)* **116**, 43–49
25. Sun, Y., Zhou, X., Liu, J., Bao, K., Zhang, G., Tu, G., Kieser, T., and Deng, Z. (2002) *Microbiology* **148**, 361–371
26. Gregory, M. A., Till, R., and Smith, M. C. (2003) *J. Bacteriol.* **185**, 5320–5323
27. Sekurova, O. N., Brautaset, T., Sletta, H., Borgos, S. E., Jakobsen, M. O., Ellingsen, T. E., Strom, A. R., Valla, S., and Zotchev, S. B. (2004) *J. Bacteriol.* **186**, 1345–1354
28. Calcutt, M. J., and Schmidt, F. J. (1994) *Gene (Amst.)* **151**, 17–21
29. Yamamoto, H., Maurer, K. H., and Hutchinson, C. R. (1986) *J. Antibiot.* **39**, 1304–1313
30. Calafat, A. M., Won, H., and Marzilli, L. G. (1997) *J. Am. Chem. Soc.* **119**, 3656–3664
31. Flett, F., Mersinias, V., and Smith, C. P. (1997) *FEMS Microbiol. Lett.* **155**, 223–229
32. Eustaquio, A. S., Gust, B., Galm, U., Li, S. M., Chater, K. F., and Heide, L. (2005) *Appl. Environ. Microbiol.* **71**, 2452–2459
33. Mutka, S. C., Carney, J. R., Liu, Y., and Kennedy, J. (2006) *Biochemistry* **45**, 1321–1330
34. Wenzel, S. C., Gross, F., Zhang, Y., Fu, J., Stewart, A. F., and Müller, R. (2005) *Chem. Biol.* **12**, 349–356
35. Naganawa, H., Muraoka, Y., Takita, T., and Umezawa, H. (1977) *J. Antibiot.* **30**, 388–396
36. Sugiyama, H., Ehrenfeld, G. M., Shipley, J. B., Killuskie, R. E., Chang, L. H., and Hecht, S. M. (1985) *J. Nat. Prod.* **48**, 869–877
37. Katano, K., An, H. Y., Aoyagi, Y., Overhand, M., Sucheck, S. J., Stevens, W. C., Hess, C. D., Zhou, X., and Hecht, S. M. (1998) *J. Am. Chem. Soc.* **120**, 11285–11296
38. Freil Meyers, C. L., Oberthur, M., Xu, H., Heide, L., Kahne, D., and Walsh, C. T. (2004) *Angew. Chem. Int. Ed. Engl.* **43**, 67–70
39. Ehrenfeld, G. M., Shipley, J. B., Heimbrook, D. C., Sugiyama, H., Long, E. C., van Boom, J. H., van der Marel, G. A., Oppenheimer, N. J., and Hecht, S. M. (1987) *Biochemistry* **26**, 931–942
40. Boger, D. L., Teramoto, S., and Zhou, J. C. (1995) *J. Am. Chem. Soc.* **117**, 7344–7356
41. Sugiyama, H., Killuskie, R. E., Chang, L. H., Ma, L. T., Hecht, S. M., Vandermaarel, G. A., and Vanboom, J. H. (1986) *J. Am. Chem. Soc.* **108**, 3852–3854
42. Goodwin, K. D., Lewis, M. A., Long, E. C., and Georgiadis, M. M. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 5052–5056