BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

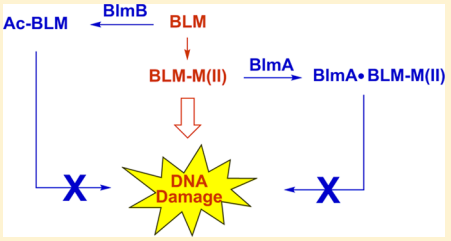
BlmB and TlmB Provide Resistance to the Bleomycin Family of Antitumor Antibiotics by *N*-Acetylating Metal-Free Bleomycin, Tallysomycin, Phleomycin, and Zorbamycin

Jane M. Coughlin,[†] Jeffrey D. Rudolf,[‡] Evelyn Wendt-Pienkowski,[†] Liyan Wang,[†] Claudia Unsin,[†] Ute Galm,[†] Dong Yang,[‡] Meifeng Tao,[†] and Ben Shen*[,†,‡,§,∥]

[†]Division of Pharmaceutical Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53705, United States
[‡]Department of Chemistry, The Scripps Research Institute, Jupiter, Florida 33458, United States
[§]Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, Florida 33458, United States
[∥]Natural Products Library Initiative, The Scripps Research Institute, Jupiter, Florida 33458, United States

Supporting Information

ABSTRACT: The bleomycin (BLM) family of glycopeptide-derived antitumor antibiotics consists of BLMs, tallysomycins (TLMs), phleomycins (PLMs), and zorbamycin (ZBM). The self-resistant elements BlmB and TlmB, discovered from the BLM- and TLM-producing organisms *Streptomyces verticillus* ATCC15003 and *Streptoalloteichus hindustanus* E465-94 ATCC31158, respectively, are *N*-acetyltransferases that provide resistance to the producers by disrupting the metal-binding domain of the antibiotics required for activity. Although each member of the BLM family of antibiotics possesses a conserved metal-binding domain, the structural differences between each member, namely, the bithiazole moiety and C-terminal amine of BLMs, have been suggested to instill substrate specificity within BlmB. Here we report that BlmB and TlmB readily accept and acetylate BLMs, TLMs, PLMs, and ZBM *in vitro* but only in the metal-free forms. Kinetic analysis of BlmB and TlmB reveals there is no strong preference or rate enhancement for specific substrates, indicating that the structural differences between each member of the BLM family play a negligible role in substrate recognition, binding, or catalysis. Intriguingly, the *zbm* gene cluster from *Streptomyces flavoviridis* ATCC21892 does not contain an *N*-acetyltransferase, yet ZBM is readily acetylated by BlmB and TlmB. We subsequently established that *S. flavoviridis* lacks the homologue of BlmB and TlmB, and ZbmA, the ZBM-binding protein, alone is sufficient to provide ZBM resistance. We further confirmed that BlmB can indeed confer resistance to ZBM *in vivo* in *S. flavoviridis*, introduction of which into wild-type *S. flavoviridis* further increases the level of resistance.



Bleomycins (BLMs), glycopeptide-derived antibiotics produced by *Streptomyces verticillus*, are clinically relevant natural products used in chemotherapy treatments against lymphomas, squamous cell carcinomas, and testicular cancer.[1−4] BLMs cause sequence-specific oxidative cleavage of double-stranded DNA through a metal-dependent mechanism.[5,6] BLMs contain four functional domains (Figure 1): (i) the metal-binding domain comprised of pyrimidoblamic acid and the β-hydroxyl histidine, (ii) the DNA-binding domain consisting of the bithiazole moiety and C-terminal amine tail in cooperation with the aminopyrimidine ring, (iii) a linker region connecting the metal-binding and DNA-binding domains, and (iv) the disaccharide moiety responsible for cell selectivity[3,7−10] and DNA cleavage activity.[11] Five nitrogen atoms within the metal-binding domain (Figure 1) coordinate iron and oxygen in the active form of the antibiotic. A crystal structure of BLM bound to BlmA, the BLM-binding protein found in the native producer, revealed that the primary amine of the β-aminoalanine moiety of BLM is the axial ligand to the metal ion in the active BLM−metal complex form.[12]

The tallysomycins (TLMs), phleomycins (PLMs), and zorbamycin (ZBM), members of the BLM family of antitumor antibiotics, are structurally related glycopeptides.[13−16] There are three major structural differences among these compounds. (i) The bithiazole moieties in the BLMs and TLMs are replaced with thiazolinyl-thiazole moieties in the PLMs and ZBM. (ii) ZBM contains a unique terminal amine compared with the BLMs, TLMs, and PLMs, which share many of the same C-terminal amines. (iii) The BLMs, TLMs, and PLMs share the same disaccharide moiety; ZBM features a unique disaccharide, and TLM possesses an additional sugar in the linker region. The metal-binding domain, however, is strictly conserved among all members of the family.

Antibiotic producers must contain elements within their genomes capable of providing self-resistance to the compounds they produce. Generally, there are four major mechanisms of resistance to antibiotics: antibiotic modification or destruction,

Received: September 5, 2014
Published: October 9, 2014

   dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901−6909

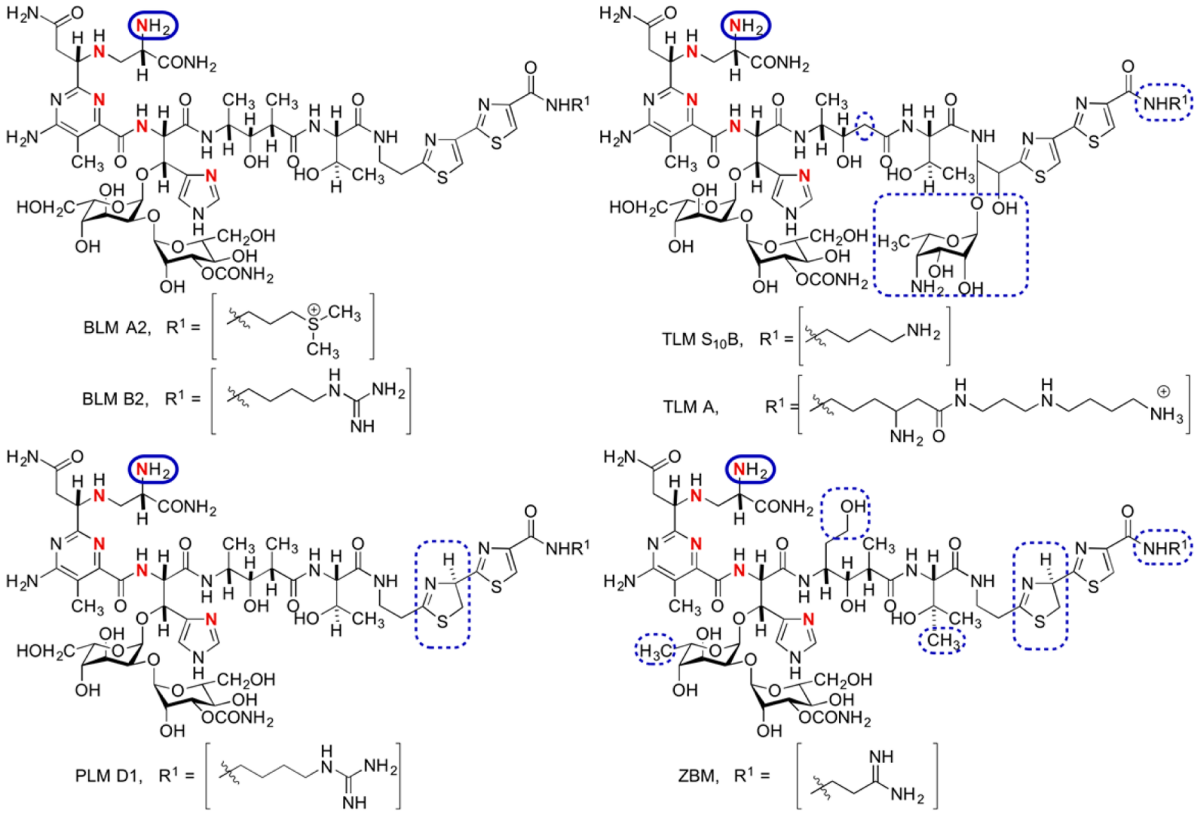


Figure 1. Structures of selected members of the bleomycin (BLM) family of antitumor antibiotics. Structural differences between the antibiotics are highlighted with dotted blue boxes; the five nitrogen atoms that coordinate the metal ion are shown in bold and colored red, and the nitrogens acetylated by BlmB and TlmB are highlighted with a solid blue box. The four members of the BLM family selected for this study are BLM B2, TLM A, PLM D1, and ZBM.

target modification, antibiotic efflux, or metabolic pathway circumvention.[17] Antibiotic sequestration, usually stoichiometric, is a fifth possible mechanism.[18−20] *Streptomyces verticillus* contains at least two of these mechanisms. BlmA, the BLM-binding protein, noncovalently binds metal-free and metal-bound BLMs, sequestering them and thereby preventing them from becoming active and cleaving DNA.[6,12,20] BlmB, an *N*-acetyltransferase that has been previously termed BAT,[21,22] transfers an acetyl group from acetyl-CoA to the primary amine of the β-aminoalanine moiety, the axial ligand of BLMs' metal-binding domain.[21,23] Although acetylation does not preclude metal from binding to BLMs, acetylation inhibits the metal-chelated complex from coordinating to and/or reducing molecular oxygen, thereby preventing the formation of activated BLMs.[23,24] The resistance provided by BlmB has been used as a selectable marker in bacteria, fungi, and mammalian cells.[25−28]

Comparison of the biosynthetic gene clusters of BLM from *S. verticillus* ATCC15003,[29] TLM from *Streptoalloteichus hindustanus* E465-94 ATCC31158,[30] and ZBM from *Streptomyces flavoviridis* ATCC21892[31] reveals all three clusters possess homologues of the BLM-binding protein (*blmA*, *tlmA*, and *zbmA*, respectively) (Figure S1 of the Supporting Information).[13] However, only the *blm* and *tlm* gene clusters contain genes encoding an *N*-acetyltransferase (*blmB* and *tlmB*, respectively), with *S. flavoviridis* lacking a *blmB* or *tlmB* homologue within the cloned *zbm* cluster. A protein sequence alignment showed BlmB is 57% identical (64% similar) to TlmB. As described above, ZBM is structurally distinct from BLMs and TLMs by the presence of a thiazolinyl-thiazole moiety and a unique disaccharide moiety (Figure 1). The lack of a *blmB*/*tlmB* homologue within the *zbm* gene cluster led to the speculation that (i) ZBM might be sufficiently different and is not accepted by an *N*-acetyltransferase as a substrate, (ii) a *blmB*/*tlmB* homologue may reside outside the cloned *zbm* gene cluster, or (iii) ZbmA alone is sufficient to confer resistance to ZBM in *S. flavoviridis*.

Given the structural similarity among the BLMs, TLMs, PLMs, and ZBM, specifically the conserved metal-binding domain, it is easy to imagine BlmB or TlmB would accept any member of the BLM family of antibiotics and catalyze acetylation. Surprisingly, BlmB was reported to be incapable of acetylating PLM or bleomycinic acid, BLM lacking the C-terminal amine.[21,32] Recently, the crystal structure of BlmB was determined, suggesting that a three-aromatic amino acid canyonlike groove is responsible for bithiazole specificity, while an electrostatic interaction between the negatively charged Glu188 and the positively charged terminal amine is responsible for the lack of activity with bleomycinic acid.[22]

Here, we report the *in vitro* characterization of the two known members of the BLM family of *N*-acetyltransferases, BlmB and TlmB, and an *in vivo* investigation of the resistance mechanisms of ZBM in *S. flavoviridis* SB9001, a ZBM overproducer derived from wild-type *S. flavoviridis* ATCC21892.[16] Our findings unveiled that (i) BlmB and TlmB acetylate all four members of the BLM family of antitumor antibiotics at the same primary amine of the β-aminoalanine moiety, (ii) BlmB and TlmB react only on the metal-free forms of the antibiotics, consistent with the crystal structure of BlmB,[22] (iii) there is no significant preference or

dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901−6909

rate enhancement by BlmB or TlmB for any of the four members of the BLM family, (iv) *S. flavoviridis* does not possess a *blmB*/*tlmB* homologue outside the cloned *zbm* gene cluster, indicating ZbmA is sufficient to confer resistance to ZBM in *S. flavoviridis*, and (v) BlmB can confer resistance to ZBM *in vivo* in *S. flavoviridis*, introduction of which into wild-type *S. flavoviridis* further increases ZBM resistance.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Biochemicals, and Chemicals. Strains, plasmids, and polymerase chain reaction (PCR) primers used in this study are summarized in Tables S1−S3, respectively, of the Supporting Information. *Escherichia coli* XL1 Blue MR was used for common subcloning and plasmid preparation.[33] *E. coli* BL21(DE3) was used for protein overproduction. Oligonucleotide primer synthesis and DNA sequencing were performed by the University of Wisconsin—Madison Biotechnology Center. The Expand High Fidelity PCR System (Roche) was used for PCR amplification. Commerical kits (Promega) were used for gel extraction and plasmid preparation. All restriction endonucleases and T4 DNA ligase were purchased from NEB and reactions conducted according to the manufacturer's protocols. Cu(II)-ZBM and metal-free TLM A were isolated from *S. flavoviridis* SB9001 and *Sa. hindustanus* E465-94 ATCC31154, respectively, as previously reported.[16,30] Cu(II)-BLM B2 (Nippon Kayaku, Tokyo, Japan), Cu(II)-PLM D1 (Cayla, Toulouse, France), metal-free BLM B2 (VWR International), [*acetyl*-1-^{14}C]acetyl-CoA (Moravek Biochemicals), and EcoLume scintillation cocktail (MP Biomedicals) were purchased from commercial sources. Other common biochemicals and chemicals were purchased from standard commercial sources and used directly.

Cloning of *blmB* and *tlmB*. The *blmB* gene from *S. verticillus* ATCC15003 was amplified from pBS9[29] using the Expand High Fidelity PCR System according to the manufacturer's instructions with primers blmB_for_pET and blmB_rev_pET (Table S3 of the Supporting Information). The resulting PCR products were cloned into pGEM-T Easy and verified by DNA sequencing. The *blmB* gene was subcloned into the *Nde*I and *Bam*HI sites of pET14b to yield pBS64. The *tlmB* gene from *Sa. hindustanus* E465-94 ATCC31158 was amplified from pBS8001[30] with primers tlmB_for_pET and tlmB_rev_pET (Table S3 of the Supporting Information) and cloned into pET14b as described for *blmB* to yield pBS8019.

Overproduction and Purification of BlmB and TlmB. The expression constructs pBS64 and pBS8019 were transformed into *E. coli* BL21(DE3), and the resultant recombinant strains were grown overnight in lysogeny broth (LB) medium containing 50 μg mL^{-1} ampicillin. A 1 mL aliquot of the overnight culture was used to inoculate 1 L of LB containing 50 μg mL^{-1} ampicillin and incubated at 37 °C while being shaken at 250 rpm until an OD_{600} of ∼0.6 was reached. The temperature was reduced to 18 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM. The cells were incubated for an additional 18 h and harvested by centrifugation at 4000*g* and 4 °C for 20 min. The harvested cells were resuspended in lysis buffer [100 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 2 mM β-mercaptoethanol] and lysed by sonication. Cell lysates were clarified by centrifugation at 10000*g* and 4 °C for 30 min, and the resulting supernatants were loaded onto 1 mL of Ni-NTA agarose (Qiagen). BlmB and TlmB were purified by nickel affinity chromatography and desalted using PD-10 desalting columns (GE Healthcare) using storage buffer [25 mM Tris-HCl (pH 8.0) containing 50 NaCl and 2 mM β-mercaptoethanol]. The proteins were assessed for purity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Figure S2 of the Supporting Information), and protein concentrations were determined by the Bradford method.

Preliminary BlmB Activity Study by High-Performance Liquid Chromatography (HPLC) Analysis. BlmB (485 nM) was incubated in Tris-HCl buffer (pH 7.5) containing 500 μM Cu(II)-BLM B2, 1 mM acetyl-CoA, and varying concentrations of EDTA (0, 0.125, 0.25, 0.5, 1, 3, 5, and 10 mM) for 30 min at 37 °C. The relative percent conversion of BLM B2 to acetylated BLM B2 was evaluated by HPLC analysis with UV detection at 300 nm.

HPLC Analysis and Characterization of the Acetylated Products. *N*-Acetyltransferase reactions were performed in 20 mM Tris-HCl buffer (pH 7.5) containing 250 μM Cu(II)-bleomycin analogue, 500 μM acetyl-CoA, 25 mM EDTA, and 485 nM BlmB or TlmB in a total volume of 60 μL. For metal-free TLM A, no EDTA was added to the reaction mixture. Incubations were conducted at 37 °C for 5, 10, 15, 30, or 60 min. The reactions were quenched by removing 25 μL, adding $CuSO_4$ to a final concentration of 250 μM, and boiling the mixture for 3 min. Negative controls were performed in parallel using boiled BlmB or TlmB. The resultant mixtures were analyzed by HPLC on a Varian Prostar System equipped with an Apollo C-18 column (250 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL min^{-1} and a Varian Prostar 330 PDA detector at 300 nm.

For TLM A, the reaction mixture was separated using a linear gradient of solvent A (0.2% acetic acid in water) and solvent B (0.2% acetic acid in methanol): 100% A for 5 min, 0 to 75% B for 25 min, and 75 to 100% B for 5 min. Acetylated Cu(II)-TLM A was collected, pooled, and subjected to high-resolution matrix-assisted laser desorption ionization mass spectrometry (HR-MALDI-MS) analysis, yielding the $[M]^+$ ion at m/z 1835.693 (calculated $[M]^+$ ion for $C_{70}H_{112}N_{22}O_{28}S_2Cu$ at m/z 1835.674).

For BLM B2, the reaction mixture was separated using 10% solvent B in solvent A for 5 min, 10 to 50% solvent B for 15 min, and 50 to 100% solvent B for 3 min. Acetylated Cu(II)-BLM B2 was collected, pooled, and subjected to HR-MALDI-MS analysis, affording the $[M]^+$ ion at m/z 1529.501 (calculated $[M]^+$ ion for $C_{57}H_{86}N_{20}O_{22}S_2Cu$ at m/z 1529.496).

For ZBM, the reaction mixture was separated using a solvent gradient of solvent C [1% ammonium acetate in water (pH 6.5)] and methanol: 25% methanol in solvent C for 5 min, 25 to 60% methanol for 17 min, and 60 to 100% methanol for 3 min. Acetylated Cu(II)-ZBM was collected, pooled, and subjected to HR-MALDI-MS analysis, affording the $[M]^+$ ion at m/z 1516.500 (calculated $[M]^+$ ion for $C_{57}H_{87}N_{19}O_{22}S_2Cu$ at m/z 1516.501).

For PLM D1, the reaction mixture was separated using a solvent gradient of solvent C and methanol: 20% methanol in solvent C for 5 min, 20 to 80% methanol for 17 min, and 80 to 100% methanol for 1 min. Acetylated Cu(II)-PLM D1 was collected, pooled, and subjected to HR-MALDI-MS analysis, yielding the $[M]^+$ ion at m/z 1531.511 (calculated $[M]^+$ ion for $C_{57}H_{88}N_{20}O_{22}S_2Cu$ at m/z 1531.512).

BlmB Activity Study Using ^{14}C-Labeled Acetyl-CoA as a Substrate. Alternatively, the acetyltransferase reactions were performed in 20 mM Tris-HCl buffer (pH 7.5) containing the

dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901−6909

selected member of the BLM family of antibiotics at 50 μM [i.e., metal-free BLM B2, Cu(II)-BLM B2, or Cu(II)-PLM D1], 500 μM [*acetyl*-1-^{14}C]acetyl-CoA (6.7 μCi μmol^{-1}), and 1.45 μM BlmB in a total volume of 150 μL. The reactions were performed in the absence or presence of 5 mM EDTA. Incubations were conducted at 30 °C, and 25 μL portions were removed and spotted on Whatman P81 paper after 20 or 40 min. The filter papers containing the dried reaction portions were washed with 3 × 300 mL of water followed by 200 mL of ethanol. After drying, the filter papers were vortexed in EcoLume scintillation cocktail and analyzed by liquid scintillation on a Packard Tri-Carb Liquid Scintillation Analyzer 1900 TR.

Kinetic Studies of BlmB and TlmB with Four Selected Members of the BLM Family. For kinetic analyses of BlmB and TlmB, the four selected members of the BLM family, all in their metal-free forms, were used. Metal-free BLM B2 was commercially available (VWR International); metal-free TLM A was isolated from *Sa. hindustanus* E465-94 ATCC31158 as previously reported,[30] and metal-free ZBM and PLM D1 were obtained by treating the Cu(II)-complexed forms with a solution of 0.5 M EDTA (pH 8.0) and purifying by HPLC as previously described.[30] All kinetic assays were performed in 10 mM Tris-HCl buffer (pH 7.5) in a total volume of 30 μL and incubated at 30 °C, and ^{14}C-labeled acetyl-CoA was used as a substrate to facilitate quantitative analysis. After incubation, the reaction mixtures were spotted on Whatman P81 paper, washed, dried, and scintillation counted as described above. Each reaction was limited to <15% completion. For acetyl-CoA, the assays contained 320 nM BlmB, 1 mM metal-free BLM B2, and [*acetyl*-1-^{14}C]acetyl CoA concentrations of 3, 6, 12, 24, 50, 100, and 253 μM and were incubated for 3 min. For BLM B2, TLM A, ZBM, and PLM D1, the assays contained 31−271 nM BlmB or 10−203 nM TlmB, 500 μM [*acetyl*-1-^{14}C]acetyl-CoA (33 μCi μmol^{-1}), and each member of the BLM family at concentrations of 10−150 μM and were incubated for 10 min. Rates (k_{cat}) and Michaelis constants (K_M) were obtained from nonlinear regression analysis by SigmaPlot12.5.

Construction of *S. flavoviridis* Recombinant Strain SB9031 with the *zbm* Cluster Deleted. The 5′-distal end of the *zbm* cluster was constructed by cloning the apramycin resistance gene [*aac(3)IV*] into the *Eco*RI and *Nsi*I sites of pBS9024,[31] yielding pBS9073. The 3′-distal end was constructed by first subcloning a 7 kb *Pst*I/*Eco*RI fragment from pBS9004[31] into the same sites of pGEM-3Zf and then inserting a 5 kb *Eco*RV/*Hin*dIII fragment into the *Hin*dIII and *Ecl*136I sites of pGEM-7Zf to yield pBS9075. The two flanking ends of the *zbm* cluster were combined by cloning the inset from pBS9073 into the *Xba*I and *Pst*I sites of pBS9075, followed by removal of *aac(3)IV* via *Eco*RI and *Cla*I digestion, treatment with Klenow, and religation to afford pBS9077. The insert was then subcloned into the *Xba*I and *Pst*I sites of pSET151[34] to yield pBS9078. pBS9078 was finally transformed into *E. coli* S17-1 and introduced into *S. flavoviridis* SB9001[16] by intergeneric conjugation following previously described methods.[31,35] Homologous recombination by a double-crossover event between *S. flavoviridis* SB9001 and pBS9078 afforded the mutant strain *S. flavoviridis* 9031, whose genotype of a *zbmA-orf38* deletion from the *zbm* cluster was confirmed by Southern analysis (Figure S7 of the Supporting Information).

Construction of *S. flavoviridis* Recombinant Strains SB9032, SB9033, SB9034, and SB9035 That Express *blmB*. The *blmB* gene, amplified via PCR from pBS9[29] using primers blmB_for_whm and blmB_rev_whm (Table S3 of the Supporting Information) and cloned into pGEM-T Easy, was subcloned as an *Xba*I/*Sph*I fragment into the same sites of two *Streptomyces* expression vectors, pWHM860[36] and pWHM1250,[37] to yield pBS65 and pBS66, respectively. While the expression of *blmB* in pBS65 is under the control of its putative native promoter, pBS66 differed from pBS65 by placing *blmB* under the control of the strong promoter *ErmE**, thereby ensuring its expression in *Streptomyces*.[35] Introduction of plasmids pBS65 and pBS66 into *S. flavoviridis* SB9001 and SB9031, respectively, was accomplished by protoplast transformation following standard procedures,[35] affording recombinant *S. flavoviridis* strains SB9032 (i.e., SB9001/pBS65), SB9033 (i.e., SB9001/pBS66), SB9034 (i.e., SB9031/pBS65), and SB9035 (i.e., SB9031/pBS66).

ZBM Resistance Determined by a Disk Diffusion Assay. A standard disk diffusion assay was used to determine the susceptibility of wild-type and recombinant *S. flavoviridis* strains to Cu(II)-ZBM. Briefly, quantities of Cu(II)-ZBM (0−500 μg) in methanol were applied to 7 mm paper disks (Whatman) and dried. The disks were placed on solid TSB agar plates applied with a liquid spore suspension containing 2 × 10^8 spores of the recombinant *S. flavoviridis* strains, SB9031, SB9032, SB9033, SB9034, and SB9035, with the ZBM overproducer *S. flavoviridis* SB9001[16] as a control. These plates were incubated at 30 °C for ∼48 h to determine the zones of inhibition as a measurement of ZBM resistance.

RESULTS

Cloning, Gene Expression, and Purification of BlmB and TlmB. The *blmB* gene from *S. verticillus* ATCC15003 and the *tlmB* gene from *Sa. hindustanus* E465-94 ATCC31158 were amplified by PCR, cloned into pET14b to afford pBS64 and pBS8019, respectively, and transformed into *E. coli* BL21(DE3) for overexpression. BlmB and TlmB were overproduced as N-terminal His$_6$-tagged fusion proteins and purified to homogeneity by Ni-NTA agarose affinity chromatography. The purified proteins showed single bands upon SDS−PAGE analysis consistent with the predicted molecular masses of BlmB (34388.8 Da) and TlmB (34422.2 Da) (Figure S2 of the Supporting Information).

BlmB and TlmB Acetylate the Metal-Free Forms of BLM B2, TLM A, PLM D1, and ZBM. As previously reported,[22] BlmB is unable to catalyze the acetylation of metal-bound BLMs. Incubation of 500 μM Cu(II)-BLM B2 with 1 mM acetyl-CoA and 485 nM BlmB or TlmB showed <4% turnover after 30 min. The addition of EDTA led to >80% turnover of BLM B2 under the same conditions (Figure S3 of the Supporting Information). The formation of acetylated BLM B2 was confirmed by HR-MALDI-MS.

An end point assay utilizing ^{14}C-labeled acetyl-CoA ([*acetyl*-1-^{14}C]acetyl-CoA), BLM B2 or PLM D1, and BlmB further confirmed the metal-free antibiotics as the preferred forms of the substrates. Metal-free BLM B2 and Cu(II)-bound BLM B2 or PLM D1 in the presence of EDTA showed significant turnover compared to that of the Cu(II)-bound antibiotic in the absence of EDTA (Figure S4 of the Supporting Information).

Although previous studies reported BlmB did not acetylate bleomycinic acid or PLM,[21,32] our previous work on isolating TLMs[30] and ZBM[16,31] afforded us the opportunity to examine the substrate flexibility of BlmB comparatively with each of the four members of the BLM family. Both BlmB and TlmB were

dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901−6909

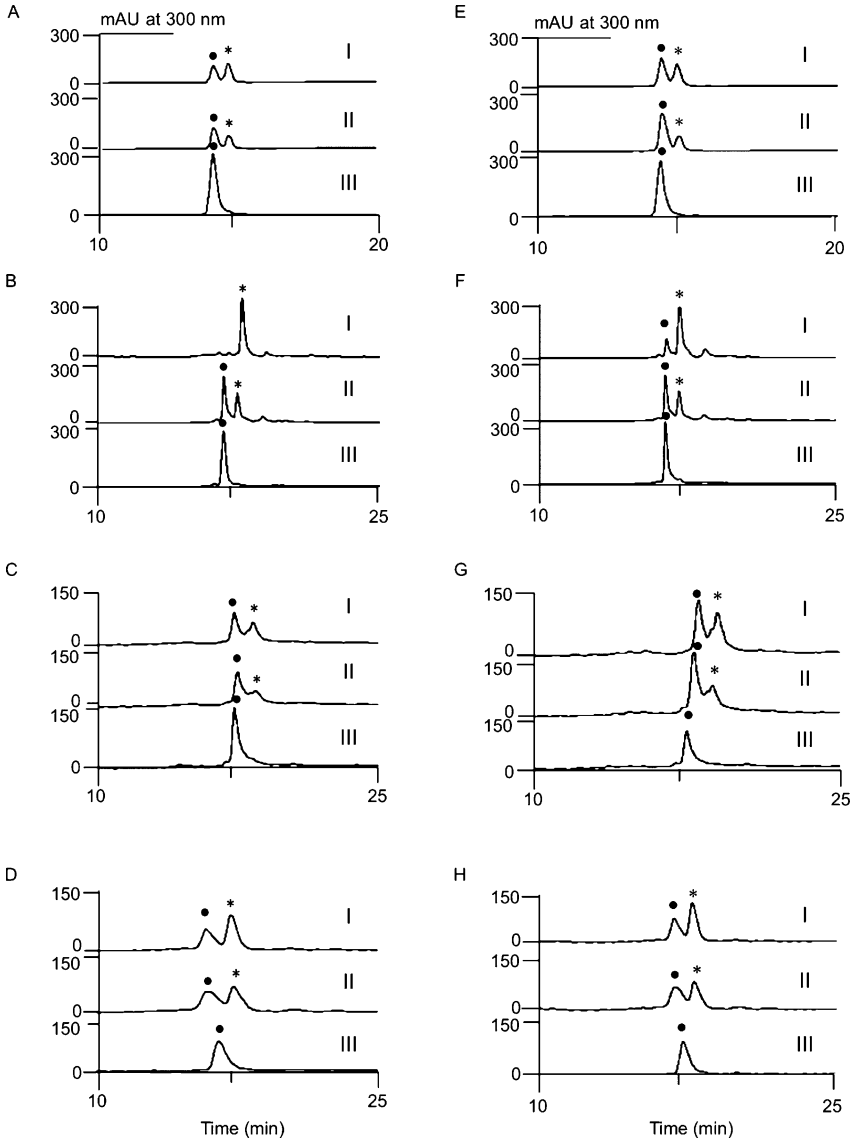


Figure 2. BlmB and TlmB acetylate four members of the BLM family of antitumor antibiotics, BLM B2, TLM A, PLM D1, and ZBM. HPLC analysis of *in vitro* enzyme reactions: (A) BLM B2 with BlmB, (B) TLM A with BlmB, (C) PLM D1 with BlmB, (D) ZBM with BlmB, (E) BLM B2 with TlmB, (F) TLM A with TlmB, (G) PLM D1 with TlmB, and (H) ZBM with TlmB. Panels A and E (BLM B2), B and F (TLM A), C and G (PLM D1), and D and H (ZBM) each show 30 min (I) and 15 min (II), 15 min (I) and 5 min (II), 60 min (I) and 30 min (II), and 10 min (I) and 5 min (II) reactions, respectively, and enzyme-boiled negative controls (III). Highlighted compounds are the antibiotic substrate (●) and acetylated product (*).

Table 1. Kinetic Parameters for BlmB and TlmB with Four Selected Members of the BLM Family of Antitumor Antibiotics[a]

	BlmB				TlmB			
	BLM B2	TLM A	PLM D1	ZBM	BLM B2	TLM A	PLM D1	ZBM
k_{cat} (min^{-1})	12 ± 2	8.2 ± 1.7	1.9 ± 0.5	29 ± 3	6.2 ± 0.6	4.4 ± 0.6	2.2 ± 0.4	21 ± 2
K_M (μM)	15 ± 5	45 ± 17	40 ± 25	21 ± 9	19 ± 5	42 ± 14	40 ± 16	25 ± 6
k_{cat}/K_M (s^{-1} M^{-1})	1.3×10^4	3.0×10^3	7.9×10^2	2.3×10^4	5.4×10^3	1.7×10^3	9.2×10^2	1.4×10^4

[a]The values are averages of at least two independent assays and are reported with standard errors.

assayed using Cu(II)-BLM B2, TLM A, Cu(II)-PLM D1, or Cu(II)-ZBM as a substrate with EDTA included in each reaction mixture. After 30 min, the reactions were quenched by boiling, and $CuSO_4$ was added to simplify HPLC analysis as the copper-bound and metal-free antibiotics elute at different retention times. HPLC analysis of each reaction mixture confirmed that both BlmB and TlmB are capable of acetylating BLM B2, TLM A, PLM D1, and ZBM (Figure 2). Each acetylated product was confirmed by HR-MALDI-MS.

Kinetic Characterization of BlmB and TlmB Reveals No Substantial Preference for Any Member of the BLM Family. Michaelis–Menten kinetic parameters for the four selected members of the BLM family, each in its metal-free form, are listed in Table 1. Rates (k_{cat}) and Michaelis constants (K_M) were determined from a nonlinear regression fit of initial

dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901–6909

velocities versus concentration (Figures S5 and S6 of the Supporting Information). For BlmB, the K_M for acetyl-CoA was determined to be 19 μM, which corresponded well with the previously reported value of 12 μM.[22] Previous kinetic studies with BlmB showed substrate inhibition at 12 μM BLM A2 at pH >6.0.[22] The reported K_M value for BLM A2 (at pH 6.0) was 11 μM,[22] correlating well with a previous K_M value for BLM A2 of 13 μM.[21] In our hands, BlmB and TlmB showed no substrate inhibition at pH 7.5 yet similar K_M values of 15 and 19 μM, respectively, for BLM B2. In fact, each member of the BLM family had comparable K_M values for both BlmB and TlmB. Rates (k_{cat}) for each substrate also had similar values, which were comparable with previously reported values using BLM A2,[21,22] with only ZBM showing a modest enhancement in rate versus those of the other members of the BLM family.

S. flavoviridis Does Not Possess a _blmB/tlmB_ Homologue Outside the Cloned _zbm_ Gene Cluster. Given the ability of BlmB and TlmB to efficiently acetylate ZBM *in vitro*, it is surprising that the cloned *zbm* gene cluster does not possess a *blmB/tlmB* homologue. To determine if a *blmB/tlmB* homologue resides outside the cloned *zbm* gene cluster, we deleted the *zbm* gene cluster from *S. flavoviridis* SB9001, a ZBM overproducer derived from the wild-type *S. flavoviridis* ATCC21892 strain,[16] to afford recombinant strain *S. flavoviridis* SB9031; the Δ*zbmA-orf38* genotype of *S. flavoviridis* SB9031 was confirmed by Southern analysis (Figure S7 of the Supporting Information). *S. flavoviridis* SB9031 is incapable of ZBM production and eliminates the only known form of self-resistance to ZBM, the ZBM-binding protein ZbmA. The susceptibility of *S. flavoviridis* SB9031 to Cu(II)-ZBM was determined using the disk diffusion assay. While *S. flavoviridis* SB9001 showed resistance up to 20 μg of ZBM per disk, the *S. flavoviridis* SB9031 mutant was sensitive at quantities as low as 0.5−1 μg of ZBM per disk, excluding the existence of other resistance elements on the *S. flavoviridis* SB9031 genome (Figure 3A).

Expression of _blmB_ Confers ZBM Resistance in _S. flavoviridis in Vivo_. To determine if BlmB is capable of acetylating ZBM *in vivo*, as demonstrated *in vitro*, two *blmB* expression plasmids, pBS65 and pBS66, placing *blmB* under its putative native promoter and the strong promoter *ErmE** to ensure its expression in *Streptomyces*, respectively, were constructed. Introduction of pBS65 and pBS66 into *S. flavoviridis* SB9001 and SB9031, respectively, afforded recombinant strains SB9032, SB9033, SB9034, and SB9035. These strains were assayed for Cu(II)-ZBM susceptibility using the disk diffusion assay with the parent *S. flavoviridis* SB9001 and SB9031 strains as controls. Expression of *blmB* in SB9031 indeed resulted in a significant increase in ZBM resistance. This was evident in *S. flavoviridis* SB9034, which showed resistance up to 10 μg of ZBM per disk, an increase of >10-fold versus that of the parent mutant SB9031. *S. flavoviridis* SB9035 showed a complete recovery of ZBM resistance as compared to the parent wild-type strain SB9001, presumably because of the enhanced expression of *blmB* under *ErmE** (Figure 3B). Similarly, expression of *blmB* in *S. flavoviridis* SB9001 also increased the level of ZBM resistance; this was more apparent in *S. flavoviridis* SB9033, where *blmB* expression was enhanced by *ErmE**, than in SB9032 (Figure S8 of the Supporting Information).

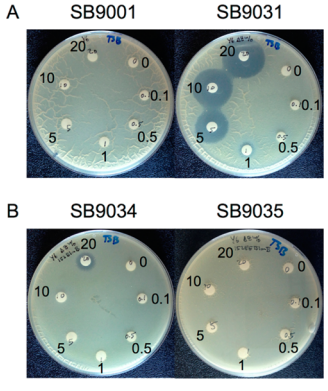


Figure 3. Disk diffusion assays for Cu(II)-ZBM susceptibility of selected *S. flavoviridis* recombinant strains in comparison with the wild-type strain. (A) SB9001 (wild-type) and SB9031 (Δ*zbmA-orf38*) challenged with 0, 0.1, 0.5, 1, 5, 10, and 20 μg of Cu(II)-ZBM (clockwise from the top right filter, respectively). (B) SB9034 (i.e., SB9031/pBS65 expressing *blmB* under its putative native promoter) and SB9035 (i.e., SB9031/pBS66 expressing *blmB* under the *ErmE** promoter) challenged with 0, 0.1, 0.5, 1, 5, 10, and 20 μg of Cu(II)-ZBM (clockwise from the top right filter, respectively).

DISCUSSION

The BLM *N*-acetyltransferase (BlmB) is a resistance element found within the *blm* gene cluster of *S. verticillus* ATCC15003 that bestows self-resistance to BLM by acetylating the primary amine of its β-aminoalanine moiety, the axial ligand of the BLM-metal ion-binding domain. As there are many examples of bacterial self-resistance elements conferring resistance to a family of antimicrobial agents,[19,38,39] it was surprising to discover BlmB was capable of acetylating only BLM and not other members of the BLM family of antitumor antibiotics, particularly PLM, a compound identical to BLM except for the presence of a thiazolinyl-thiazole moiety instead of the bithiazole moiety found in BLM.[21] A crystal structure of BlmB implied that a canyonlike groove of three aromatic side chains is too narrow to accommodate the nonplanar thiazoline ring of PLM.[22] Interestingly, the *zbm* gene cluster from *S. flavoviridis* ATCC21892 does not contain a *blmB/tlmB* homologue,[31] suggesting ZBM, a thiazolinyl-thiazole-containing analogue of BLM, would not be acetylated by a BlmB-like enzyme. In this study, we determined whether BlmB and TlmB can acetylate BLM B2, TLM A, PLM D1, and ZBM, four members of the BLM family, *in vitro* and investigated the resistance mechanisms of the ZBM producer *S. flavoviridis in vivo*.

Both BlmB and TlmB acetylate BLM B2, TLM A, PLM D1, and ZBM, but only the metal-free forms, *in vitro*. Although metal-free BLM as a requirement for BlmB activity was previously stated,[22] it appeared to be anecdotal as we found no experimental evidence in the literature. Comparisons of the BLM structures (Figure 4A),[12,22,40] free and enzyme-bound, reveal a probable cause for the lack of acetylation of the metal-bound forms of BLMs by BlmB and TlmB. When metal is coordinated to the five nitrogens of BLM, as in the case of the Cu(II)-BLM complex bound to BlmA or the Co(II)-BLM complex alone, the metal-binding domain forms a compact conformation. Conversely, BLM in BlmB must adopt an open conformation allowing the primary amine of BLM access to acetyl-CoA. The tight conformation of BLM when bound to metal likely precludes the correct binding orientation in BlmB necessary for catalysis and prevents BlmB from pulling apart the tightly chelated nitrogen atoms (Figure 4A).

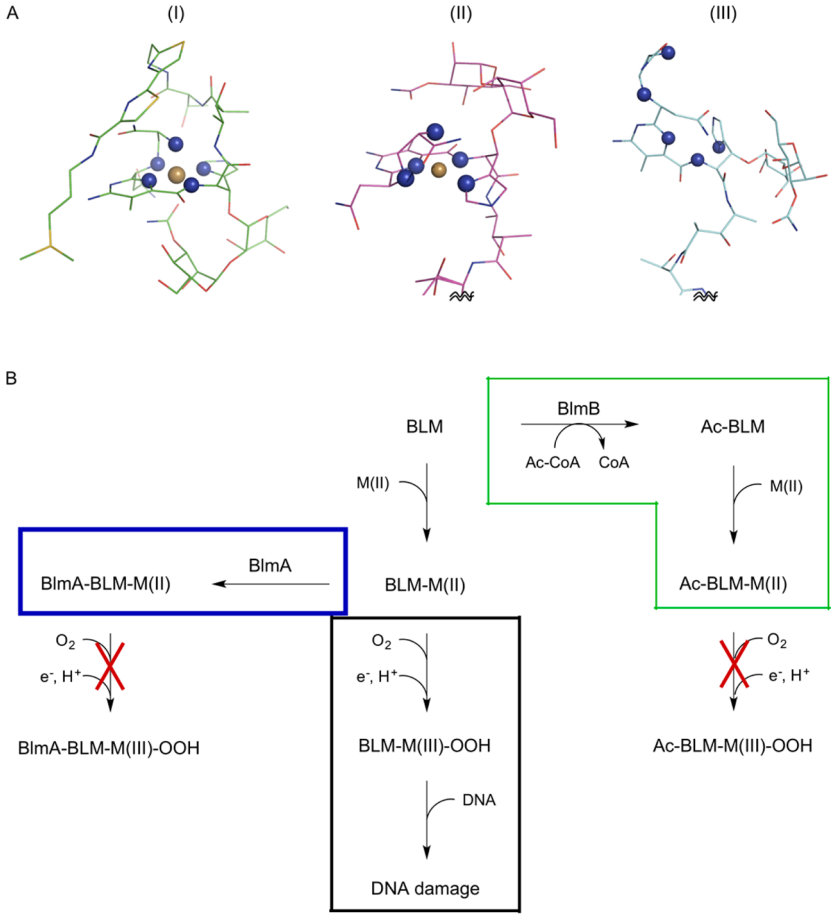


Figure 4. (A) Conformations of free and enzyme-bound BLM reveal its structural flexibility: (I) free Co(II)-BLM A2 (Protein Data Bank entry 1DEY), (II) Cu(II)-BLM A2 bound to BlmA (Protein Data Bank entry 1JIF), and (III) BLM A2 bound to BlmB (Protein Data Bank entry 2ZW7). Enzymes are not shown. Co(II) or Cu(II) atoms are shown as gold spheres. The five metal-coordinating nitrogens are shown as blue spheres. For II and III, the bithiazole moieties and C-terminal amines are cropped at the double wavy lines. The tight conformation of the metal-binding domain in metal-bound BLM likely prevents BLM from correctly binding to BlmB for acetylation. (B) Unified model for self-resistance within the producers of the BLM family of antitumor antibiotics as exemplified by the BLM producer. Activated BLM (or TLM or ZBM) is generated by binding to a metal ion [M(II)] and molecular oxygen (O_2), which is then reduced to form BLM-M(III)-OOH (black box). Resistance in the native producer is primarily achieved through sesquestration by BlmA (thick blue box), while antibiotic modification by BlmB (thin green box) provides an additional mechanism of resistance. BlmB acetylates the axial ligand of the BLM metal-binding domain, but only in the presence of metal-free BLM. Acetylated BLM (Ac-BLM) can bind metal, but O_2 binding or activation is inhibited. BlmA tightly binds metal-bound BLM, preventing O_2 binding and activation. For the sake of simplicity, the coordinated metal ion is represented as an M. Members of the BLM family are isolated as Cu(II) complexes; structures have been determined as Cu or Co complexes, and Fe(II) must bind before activated BLM is generated.

The BlmB and TlmB enzymes were kinetically characterized with four members of the BLM family, each in metal-free forms, and surprisingly, both the rates (k_{cat}) and Michaelis constants (K_M) for each member were essentially equivalent. Comparable Michaelis constants indicate each member, regardless of its differences in structure, binds favorably for the reaction to occur. Thus, the narrow bithiazole binding groove in BlmB does not prevent the correct binding orientation necessary for acetylation to occur in thiazolinyl-thiazole analogues. A sequence alignment of BlmB and TlmB reveals only two of the three aromatic side chains in the canyonlike groove are conserved (Figure S9 of the Supporting Information). One could imagine a Phe/Val substitution, as in the case of BlmB and TlmB, may provide the necessary space to better accommodate a nonplanar thiazoline ring. However, neither BlmB nor TlmB shows a preference for bithiazole as in BLM B2 and TLM A or thiazolinyl-thiazole as in PLM D1 and ZBM, suggesting the canyonlike groove plays little role in substrate recognition and specificity.

The BLM-binding proteins have long been known for their sequestration, and therefore inactivation, of members of the BLM family of antitumor antibiotics.[12,20,41,42] The structure of BlmA, the BLM-binding protein from *S. verticillus* ATCC15003, was determined with bound BLM B2 in both the metal-free and Cu(II)-complexed forms,[12] suggesting BlmA can bind either form of BLM and thus confer resistance in either situation. Given the significant structural similarity of both BlmA and BLM B2 in both the metal-free and Cu(II)-complexed forms and the fact that BLMs are isolated as Cu(II) complexes,[15,16,30] BlmA likely preferentially binds metal-bound BLMs *in vivo* (Figure 4B). As experimentally determined in this study, BlmB acetylates only the metal-free form of BLMs. As members of the metal-free forms of the BLM family are incapable of causing DNA damage,[3,43] BlmB and TlmB appear to inactivate an unharmful form of an antibiotic. Although counterintuitive, covalent modification of an inactive antibiotic by BlmB or TlmB immediately after its biosynthesis may provide a preemptive and complementary mechanism of resistance

dx.doi.org/10.1021/bi501121e | *Biochemistry* 2014, 53, 6901−6909

from self-imposed oxidative damage (Figure 4B) and/or a more stable, yet potentially reversible, inactivation. It is not clear, however, when and at what level the *blmB/tlmB* gene is expressed during biosynthesis. We suspect BlmB or TlmB acts on only the final biosynthetic product, a model that is consistent with our *in vitro* data, previously reported studies,[21] and the numerous contacts BlmB makes with the four functional domains of BLM.[22] This hypothesis would rule out BlmB acetylating a biosynthetic intermediate or precursor.

We previously reported that the cloned *zbm* cluster from *S. flavoviridis* ATCC21892 lacks the *blmB/tlmB* homologue, implying BlmA or TlmA performs the essential role in BLM resistance.[31] We now establish that *S. flavoviridis* does not possess a *blmB/tlmB* homologue in its genome. *S. flavoviridis* therefore appears to forego the BlmB resistance mechanism of antibiotic modification, and ZbmA alone must be sufficient to provide ZBM resistance. With these findings and the fact that Cu(II)-BLM complexes are isolated, we now propose that the mechanism of sequestration by BlmA, TlmA, and ZbmA is the primary mechanism of resistance. BlmB and TlmB may then be considered as a secondary mechanism of resistance. Indeed, we show that BlmB can confer resistance to ZBM *in vivo* in the ZBM-sensitive *S. flavoviridis* SB9031 strain and that introduction of BlmB into wild-type *S. flavoviridis* SB9001 further increases ZBM resistance as exemplified by the SB9033 strain. The reacquisition of ZBM resistance in sensitive strain *S. flavoviridis* SB9031 by expression of *blmB* confirms that ZBM can be inactivated by a BlmB homologue *in vivo* and proves BlmB can confer resistance as the sole resistance element.

This study highlights the biochemical functionality and antibiotic resistance mechanism of the BLM family of antitumor antibiotics by the BLM *N*-acetyltransferases. Comparison among the *blm*, *tlm*, and *zbm* gene clusters exposes the evolutionary relationship between the BlmB and TlmB *N*-acetyltransferases and the BlmA-, TlmA-, and ZbmA-binding proteins as mechanisms of resistance to the BLM family of drugs. On the basis of this study, we now propose a unified model for self-resistance within the producers of BLM, TLM, and ZBM, with BlmA, TlmA, and ZbmA providing the primary mechanism of resistance and BlmB and TlmB as a secondary additional mechanism of resistance.

■ ASSOCIATED CONTENT

Supporting Information

Strains, plasmids, and primers used in this study (Tables S1–S3), organization of the biosynthetic gene clusters of BLM, TLM, and ZBM (Figure S1), SDS–PAGE analysis of BlmB and TlmB (Figure S2), acetylation of Cu(II)-BLM by BlmB in the presence of EDTA (Figure S3), BlmB end point assay utilizing radiolabeled acetyl-CoA (Figure S4), Michaelis–Menten curves (Figures S5 and S6), Southern analysis of the Δ*zbmA-orf38* mutant *S. flavoviridis* SB9031 (Figure S7), disk diffusion assays for Cu(II)-ZBM susceptibility in *S. flavoviridis* SB9001, SB9032, and SB9033 (Figure S8), and a sequence alignment of BlmB and TlmB (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: shenb@scripps.edu. Telephone: (561) 228-2456. Fax: (561) 228-2472.

Author Contributions

J.C. and J.D.R. contributed equally to this work.

Funding

This work is supported in part by National Institutes of Health Grant CA094426 and the Natural Products Library Initiative at The Scripps Research Institute.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Bayer, R. A., Gaynor, E. R., and Fisher, R. I. (1992) Bleomycin in non-Hodgkin's lymphoma. *Semin. Oncol. 19*, 46–52.

(2) Einhorn, L. H. (2002) Curing metastatic testicular cancer. *Proc. Natl. Acad. Sci. U.S.A. 99*, 4592–4595.

(3) Chen, J., and Stubbe, J. (2005) Bleomycins: Towards better therapeutics. *Nat. Rev. Cancer 5*, 102–112.

(4) Galm, U., Hager, M. H., Van Lanen, S. G., Ju, J., Thorson, J. S., and Shen, B. (2005) Antitumor antibiotics: Bleomycin, enediynes, and mitomycin. *Chem. Rev. 105*, 739–758.

(5) Burger, R. M., Peisach, J., and Horwitz, S. B. (1981) Activated bleomycin. A transient complex of drug, iron, and oxygen that degrades DNA. *J. Biol. Chem. 256*, 11636–11644.

(6) Stubbe, J., and Kozarich, J. W. (1987) Mechanisms of bleomycin-induced DNA degradation. *Chem. Rev. 87*, 1107–1136.

(7) Yu, Z., Schmaltz, R. M., Bozeman, T. C., Paul, R., Rishel, M. J., Tsosie, K. S., and Hecht, S. M. (2013) Selective tumor cell targeting by the disaccharide moiety of bleomycin. *J. Am. Chem. Soc. 135*, 2883–2886.

(8) Chapuis, J.-C., Schmaltz, R. M., Tsosie, K. S., Belohlavek, M., and Hecht, S. M. (2009) Carbohydrate dependent targeting of cancer cells by bleomycin-microbubble conjugates. *J. Am. Chem. Soc. 131*, 2438–2439.

(9) Boger, D. L., and Cai, H. (1999) Review of bleomycin: Synthetic and mechanistic studies. *Angew. Chem., Int. Ed. 38*, 448–476.

(10) Hecht, S. M. (2000) Bleomycin: New perspectives on the mechanism of action. *J. Nat. Prod. 63*, 158–168.

(11) Huang, S.-X., Feng, Z., Wang, L., Galm, U., Wendt-Pienkowski, E., Yang, D., Tao, M., Coughlin, J. M., Duan, Y., and Shen, B. (2012) A designer bleomycin with significantly improved DNA cleavage activity. *J. Am. Chem. Soc. 134*, 13501–13509.

(12) Sugiyama, M., Kumagai, T., Hayashida, M., Maruyama, M., and Matoba, Y. (2002) The 1.6-Å crystal structure of the copper(II)-bound bleomycin complexed with the bleomycin-binding protein from bleomycin-producing *Streptomyces verticillus*. *J. Biol. Chem. 277*, 2311–2320.

(13) Galm, U., Wendt-Pienkowski, E., Wang, L., Huang, S.-X., Unsin, C., Tao, M., Coughlin, J. M., and Shen, B. (2011) Comparative analysis of the biosynthetic gene clusters and pathways for three structurally related antitumor antibiotics: Bleomycin, tallysomycin, and zorbamycin. *J. Nat. Prod. 74*, 526–536.

(14) Konishi, M., Saito, K., Numata, K., Tsuno, T., Asama, K., Tsukiura, H., Naito, T., and Kawaguchi, H. (1977) Tallysomycin, a new antitumor antibiotic complex related to bleomycin. II. Structure determination of tallysomycins A and B. *J. Antibiot. 30*, 789–805.

(15) Takita, T., Muraoka, Y., Nakatani, T., Fujii, A., Umezawa, Y., Naganawa, H., and Umezawa, H. (1978) Chemistry of bleomycin. XIX. Revised structures of bleomycin and phleomycin. *J. Antibiot. 31*, 801–804.

(16) 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) Glycopeptide antitumor antibiotic zorbamycin from *Streptomyces flavoviridis* ATCC 21892: Strain improvement and structure elucidation. *J. Nat. Prod. 70*, 402–406.

(17) Wright, G. D. (2011) Molecular mechanisms of antibiotic resistance. *Chem. Commun. 47*, 4055–4061.

(18) Reynolds, P. E. (1989) Structure, biochemistry, and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis. 8*, 943–950.

(19) Hayes, J. D., and Wolf, C. R. (1990) Molecular mechanisms of drug resistance. *Biochem. J. 272*, 281−295.
(20) Gatignol, A., Durand, H., and Tiraby, G. (1988) Bleomycin resistance conferred by a drug-binding protein. *FEBS Lett. 230*, 171−175.
(21) Matsuo, H., Mochizuki, H., Davies, J., and Sugiyama, M. (1997) Production of bleomycin N-acetyltransferase in *Escherichia coli* and *Streptomyces verticillus*. *FEMS Microbiol. Lett. 153*, 83−88.
(22) Oda, K., Matoba, Y., Noda, M., Kumagai, T., and Sugiyama, M. (2010) Catalytic mechanism of bleomycin N-acetyltransferase proposed on the basis of its crystal structure. *J. Biol. Chem. 285*, 1446−1456.
(23) Sugiyama, M., Kumagai, T., Shionoya, M., Kimura, E., and Davies, J. E. (1994) Inactivation of bleomycin by an N-acetyltransferase in the bleomycin-producing strain *Streptomyces verticillus*. *FEMS Microbiol. Lett. 121*, 81−86.
(24) Oppenheimer, N. J., Rodriguez, L. O., and Hecht, S. M. (1980) Metal binding to modified bleomycins. Zinc and ferrous complexes with an acetylated bleomycin. *Biochemistry 19*, 4096−4103.
(25) Brouns, S. J. J., Wu, H., Akerboom, J., Turnbull, A. P., de Vos, W. M., and van der Oost, J. (2005) Engineering a selectable marker for hyperthermophiles. *J. Biol. Chem. 280*, 11422−11431.
(26) Suzuki, S., Tada, S., Fukuoka, M., Taketani, H., Tsukakoshi, Y., Matsushita, M., Oda, K., Kusumoto, K.-I., Kashiwagi, Y., and Sugiyama, M. (2009) A novel transformation system using a bleomycin resistance marker with chemosensitizers for *Aspergillus oryzae*. *Biochem. Biophys. Res. Commun. 383*, 42−47.
(27) Sugiyama, M., Tanaka, M., and Kumagai, T. (1999) A mammalian vector carrying the bleomycin N-acetyltransferase gene from bleomycin-producing *Streptomyces verticillus* as a selective marker. *J. Antibiot. 52*, 895−899.
(28) Kumagai, T., and Sugiyama, M. (1998) Protection of mammalian cells from the toxicity of bleomycin by expression of a bleomycin-binding protein gene from *Streptomyces verticillus*. *J. Biochem. 124*, 835−841.
(29) Du, L., Sanchez, C., Chen, M., Edwards, D. J., and Shen, B. (2000) The biosynthetic gene cluster for the antitumor drug bleomycin from *Streptomyces verticillus* ATCC15003 supporting functional interactions between nonribosomal peptide synthetases and a polyketide synthase. *Chem. Biol. 7*, 623−642.
(30) Tao, M., Wang, L., Wendt-Pienkowski, E., George, N. P., Galm, U., Zhang, G., Coughlin, J. M., and Shen, B. (2007) The tallysomycin biosynthetic gene cluster from *Streptoalloteichus hindustanus* E465-94 ATCC 31158 unveiling new insights into the biosynthesis of the bleomycin family of antitumor antibiotics. *Mol. BioSyst. 3*, 60−74.
(31) Galm, U., Wendt-Pienkowski, E., Wang, L., George, N. P., Oh, T.-J., Yi, F., Tao, M., Coughlin, J. M., and Shen, B. (2009) The biosynthetic gene cluster of zorbamycin, a member of the bleomycin family of antitumor antibiotics, from *Streptomyces flavoviridis* ATCC 21892. *Mol. BioSyst. 5*, 77−90.
(32) Sugiyama, M., Thompson, C. J., Kumagai, T., Suzuki, K., Deblaere, R., Villarroel, R., and Davies, J. (1994) Characterization by molecular cloning of two genes from *Streptomyces verticillus* encoding resistance to bleomycin. *Gene 151*, 11−16.
(33) Sambrook, J., and Russel, D. (2001) *Molecular cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
(34) Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N., and Schoner, B. E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Stretpomyces* spp. *Gene 116*, 43−49.
(35) Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Stretpomyces genetics*, The John Innes Foundation, Norwich, U.K.
(36) Meurer, G., and Hutchinson, C. R. (1995) Functional analysis of putative β-ketoacyl:acyl carrier protein synthase and acyltransferase active site motifs in a type II polyketide synthase of *Streptomyces glaucescens*. *J. Bacteriol. 177*, 477−481.
(37) Madduri, K., Kennedy, J., Rivola, G., Inventi-Solari, A., Filippini, S., Zanuso, G., Colombo, A. L., Gewain, K. M., Occi, J. L., MacNeil, D. J., and Hutchinson, C. R. (1998) Production of the antitumor drug epirubicin (4′-epidoxorubicin) and its precursor by a genetically engineered strain of *Streptomyces peucetius*. *Nat. Biotechnol. 16*, 69−74.
(38) Davies, J., and Wright, G. D. (1997) Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol. 5*, 234−240.
(39) Rasmussen, B. A., and Bush, K. (1997) Carbapenem-hydrolyzing β-lactamases. *Antimicrob. Agents Chemother. 41*, 223−232.
(40) Lehmann, T. E., Serrano, M. L., and Que, L., Jr. (2000) Coordination chemistry of Co(II)-bleomycin: Its investigation through NMR and molecular dynamics. *Biochemistry 39*, 3886−3898.
(41) Dumas, P., Bergdoll, M., Cagnon, C., and Masson, J. M. (1994) Crystal structure and site-directed mutagenesis of a bleomycin resistance protein and their significance for drug sequestering. *EMBO J. 13*, 2483−2492.
(42) Kumagai, T., Nakano, T., Maruyama, M., Mochizuki, H., and Sugiyama, M. (1999) Characterization of the bleomycin resistance determinant encoded on the transposon Tn5. *FEBS Lett. 442*, 34−38.
(43) Sausville, E. A., Peisach, J., and Horwitz, S. B. (1978) Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. *Biochemistry 17*, 2740−2746.