Comparative Analysis of the Biosynthetic Gene Clusters and Pathways for Three Structurally Related Antitumor Antibiotics Bleomycin, Tallysomycin and Zorbamycin[⊥]

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[†]Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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Supporting Information

Experimental Section

Bacterial strains and culture conditions. *S. verticillus* ATCC15003 (American Type Culture Collection, Rockville, MD, USA), *S. flavoviridis* SB9001,[1] and recombinant strains generated in this study were routinely grown at 28°C in TSB liquid medium (Table S2).[2] Spore preparation, *E. coli-Streptomyces* intergeneric conjugation, and *S. verticillus* as well as *S. flavoviridis* fermentations for BLM and ZBM production, respectively, were carried out as described previously.[1,3,4] *Escherichia coli* XL1 Blue MR (Stratagene, La Jolla, CA, USA) and *E. coli* DH5α[2] were used for routine subcloning and plasmid preparations. *E. coli* S17-1[2] was the donor in *E. coli-S. verticillus* and *E. coli-S. flavoviridis* SB9001 conjugations. *E. coli* BW25113/pIJ790 was the host for λRED-mediated mutagenesis (Table S2).[5]

Plasmids and biochemicals. The pGEM series of vectors (Promega, Madison, WI), Litmus 28 and 38 (New England Biolabs, Beverly, MA), and pSPORT1 (Invitrogen, Carlsbad, CA) were from commercial sources, pANT841,[6] pBS37,[4] pBS9005,[3] pBS9006,[3] pBS9010,[3] pBS9027,[3] pIJ773,[5] pOJ260,[7] pSET152,[7] pSET151,[7] pWHM1250,[8] and the *aac(3)IV* apramycin resistance cassette[2] were described previously (Table S1). Common biochemicals, chemicals, media, restriction enzymes, and other molecular biology reagents were from standard commercial sources. Ampicillin (150 μg ml^{-1}), apramycin (50 μg ml^{-1}), and chloramphenicol (25 μg ml^{-1}) were used for selection in *E. coli*. Apramycin (50-100 μg ml^{-1}) and thiostrepton (50-100 μg ml^{-1}) were used for selection of *S. verticillus* and *S. flavoviridis* recombinants. Nalidixic acid (50 μg ml^{-1}) was used to select against the *E. coli* conjugal donor after conjugation.

Isolation and analysis of BLM, ZBM and analogs. BLM, ZBM, and analogs produced by the respective recombinant strains were isolated and analyzed as described previously.[1,3,4] The identity of BLM, ZBM, and analogs was always confirmed by co-injection of the respective standards as well as by LC-ESI mass spectrometry on an Agilent 1100 HPLC-MSD SL quadrupole mass spectrometer or an Agilent 1100 HPLC-MSD SL ion trap mass spectrometer.

DNA isolation, manipulation, sequence analysis, and genetic manipulation of *S. verticillus* and *S. flavoviridis*. DNA isolations and manipulations in *E. coli*[9] and *Streptomyces*[2] 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 Diagnostics Corp., Indianapolis, IN, USA). Sequence alignments, identity and similarity calculations were carried out by AlignX in the Vector NTI Advance™ 10 program from Invitrogen. *E. coli-Streptomyces* conjugation was carried out as previously reported.[3,4]

Complementation of the Δ*zbm-orf2* mutant strain SB9008 with *zbm-orf1-3* and *zbm-orf2*. In order to create the *zbm-orf2* complementation construct spanning *zbm-orf1-3*, a *Hind*III-*Pst*I fragment from pBS9027 was cloned into pSPORT1, and then transferred into Litmus 38 containing the *ErmE** promoter on an *Eco*RI-*Bam*HI fragment as a 2350-bp *Eco*RV-*Hind*III fragment to create pBS9042. The entire insert was moved as an *Eco*RI-*Spe*I fragment from pBS9042 into the *Eco*RI-*Xba*I sites of pBS9010, generating the final *zbm-orf1-3* complementation construct pBS9043. For the *zbm-orf2* complementation construct covering *zbm-orf2* only, an *Ecl*136I-*Nco*I fragment from pBS9027 was cloned into Litmus 28 at *Eco*RV-*Nco*I, and then transferred into Litmus 38 containing the *ErmE** promoter on an *Eco*RI-*Bam*HI fragment as a *Pst*I-*Avr*II fragment into the *Pst*I-*Spe*I sites to create pBS9044. The entire insert of pBS9044 was moved as an *Eco*RI-*Xba*I fragment into the same sites of pBS9010 to create the *zbm-orf2* expression construct, pBS9045.

Inactivation and complementation of *zbmII*. A 9.5-kb *Xho*I fragment containing part of *zbmIV*, *zbmIII*, *zbm-orf19*, *zbmII*, *zbm-orf21*, *zbmE*, and part of *zbmD* was isolated from pBS9005 and cloned

into pGEM7Zf creating pBS9046. A ~5-kb fragment was then eliminated from pBS9046 by *Mlu*I restriction and religation, thereby deleting part of the *zbmII* gene and creating pBS9047. A *Pst*I-*Avr*I fragment was then removed from pBS9047 and replaced with a linker made by annealing oligonucleotides zbmII-lif and zbmII-lir (Table S3), thereby creating an in-frame deletion within *zbmII* and yielding pBS9048. The *Xba*I-*Nco*I fragment from pBS9048 and the *Nco*I-*Eco*RI fragment from pBS9046 were combined and ligated into pOJ260 at the *Eco*RI-*Xba*I sites to form pBS9049.

Inactivation of *blmXI* via λRED-mediated PCR-targeting. The *blmXI* gene in pBS37 was replaced by the *aac(3)IV-oriT* cassette from pIJ773 *via* λRED-mediated PCR-targeting method[4] using oligonucleotides blmXI-1f and blmXI-1r (Table S3), with lower case letters representing DNA sequence originating from *S. verticillus* and upper case letters representing DNA sequence flanking the *aac(3)IV-oriT* cassette from pIJ773, to yield pBS50.

Inactivation and complementation of *zbmXI*. A 9-kb *Sst*I fragment containing *zbmXI* was subcloned from pBS9005 into pSPORT1 such that the upstream end of the insert faced the *Pst*I end of the multiple cloning site, forming pBS9050. A 3-kb *Cla*I-*Bam*HI fragment was isolated from pBS9050 and cloned into pGEM7Zf to form pBS9051. A linker made by annealing oligonucleotides zbmXI-lif and zbmXI-lir (Table S3) was then used to replace a *San*DI-*Bgl*II fragment in pBS9051, thereby creating an in-frame deletion within the *zbmXI* gene and yielding pBS9052. The deletion-containing *Cla*I-*Bgl*II fragment of pBS9052 was then used to replace the wild-type *Cla*I-*Bgl*II fragment in pBS9050, creating pBS9053. The entire insert of pBS9053 was finally transferred to pSET151 as an *Eco*RI-*Xba*I fragment, forming pBS9054.

In order to create the *zbmXI* complementation construct, the *ErmE** promoter was cloned from pWHM1250 and moved first into pGEM7Zf and subsequently into pANT841 to create pBS9055. The *zbmXI* gene was then moved as a 3-kb *Cla*I-*Bam*HI fragment from pBS9051 into pBS9055 at the *Cla*I-*Bgl*II sites, thereby placing it under the control of the *ErmE** promoter and creating pBS9056. The entire insert of pBS9056 was then transferred to pSET152 as an *Eco*RI-*Xba*I fragment to create pBS9057.

Inactivation of *zbm-orf31*. An 11-kb *Bgl*II fragment containing *zbm-orf31* was isolated from pBS9005 and cloned into the *Bam*HI site of pGEM7Zf to form pBS9058 such that the downstream *Bgl*II faced the *Nsi*I restriction site of the multiple cloning site. A 6-kb *Nsi*I-*Bam*HI fragment of pBS9058 was subcloned into the *Pst*I-*Bam*HI sites of pGEM3Zf and then moved as a *Bam*HI-*Hind*III fragment into Litmus 28 to form pBS9059. A 5-kb *Eco*RI-*Kpn*I fragment containing the upstream portion of the pBS9058 insert was transferred to pGEM3Zf, and the 3.3-kb *Eco*RI-*Stu*I fragment was then subcloned into the *Eco*RI-*Eco*RV sites of pBS9059 to create pBS9060. The entire insert of pBS9060 was then transferred to pSET151 as an *Eco*RI-*Xba*I fragment to create the final in-frame deletion construct of pBS9061.

Inactivation and complementation of *zbmVIIb*. A 3.86-kb *Pst*I-*Bgl*II fragment containing *zbm-orf26*, *zbm-orf27*, and *zbmXI*, and a 2.35-kb *Bgl*II-*Pst*I fragment containing the *zbmVIIb* gene from pBS9005 were separately cloned into Litmus 28 creating pBS9062 and pBS9063, respectively. A 1640-bp fragment was then eliminated from pBS9063 by *Sph*I restriction and religation, thereby deleting most of the *zbmVIIb* gene in-frame to create pBS9064. The flanking regions on either side of the deletion were added a 2.7-kb *Pst*I-*Bam*HI fragment, containing parts of *zbm-orf30* and *zbm-orf31* from pBS9005, and a 3.9-kb *Bgl*II-*Avr*II fragment, containing parts of *zbm-orf26*, *zbm-orf27*, and *zbmXI* from pBS9062, into the *Pst*I-*Bam*HI and *Bgl*II-*Spe*I sites of pBS9064, respectively, to create pBS9065. The entire insert was then moved as an *Xba*I fragment into pSET151 to yield pBS9066.

In order to create the *zbmVIIb* complementation construct, the *zbmVIIb* gene was moved as an *Nco*I-*Bgl*II fragment from pBS9063 into pBS9055 at the same sites, thereby placing it under the control of the

*ErmE** promoter and creating pBS9067. The entire insert was then transferred to pSET152 as an *Eco*RI-*Xba*I fragment to create pBS9068.

Inactivation and complementation of ZbmVIIc. A 21-kb *Eco*RI fragment containing *zbmVIIc* from pBS9006 was ligated into Litmus 28 at the same site with the upstream end of the fragment facing the *Xho*I site to yield pBS9069. A 14.8-kb *Cla*I-*Hind*III fragment was removed from pBS9069 and after Klenow treatment, the vector-containing portion was religated upon itself to create pBS9070. An *Sfi*I-*Asc*I fragment was then removed from pBS9070 and replaced with a linker made by annealing oligonucleotides zbmVIIc-lif and zbmVIIc-lir (Table S3), thereby creating an in-frame deletion of zbmVIIc and yielding pBS9071. The resulting insert was then transferred as an *Xba*I-*Nsi*I fragment to pSET151 to create pBS9072.

Cross-Complementation of the *ΔzbmXI* mutant strain SB9015 with *blmXI* and *ΔzbmVIIb* mutant strain SB9017 with *blmVII*. In order to create a *zbmXI* cross-complementation construct, a 2.6-kb *Pst*I-*Xba*I fragment containing *blmXI* was cloned from pBS37 and ligated into pSET152 at the *Eco*RI-*Xba*I sites, along with an *Eco*RI-*Pst*I fragment containing the *ErmE** promoter[10] from pWHM1250, forming pBS51. For the *zbmVIIb* cross-complementation construct, a 6.7-kb *Pst*I-*Bgl*II fragment containing *blmVII* was cloned from pBS37 and ligated into pWHM1250 at *Bam*HI-*Pst*I behind the *ErmE** promoter to create pBS52. The entire insert of pBS52 was transferred as an *Eco*RI-*Pst*I fragment into Litmus 38 at the same sites, and then moved as an *Eco*RI-*Spe*I fragment into pSET152 at *Eco*RI-*Xba*I, yielding the final *blmVII* expression construct, pBS53.

Generation of the mutant and complementation strains and analysis of their secondary metabolite profile. The inactivation construct pBS50 (i.e., *ΔblmXI*) was introduced into the wild-type *S. verticillus* strain by intergeneric conjugation.[4] Exconjugants were selected by a combination of the apramycin-resistant phenotype and PCR screening using oligonucleotide pair C-blmXI-f2 and C-blmXI-r (Table S3) for pBS50, affording the mutant strain *S. verticillus* SB6 (Figure S1, C and D, Supplemental Data). A suitable probe was PCR amplified using oligonucleotide pair C-blmXI-f2 and C-blmXI-r (Table S3) for SB6 (only a 0.981-kb *Bam*HI fragment of the 2.688-kb PCR product was used as probe). The genotype of this apparent double crossover mutant strain was further confirmed by Southern blot analyses (Figure S1, D, Supplemental Data). The mutant was cultured and investigated for BLM production with the wild-type *S. verticillus* strain as a control (Figure 4, traces XV and XVI).[4]

The inactivation constructs pBS9049 (i.e., *ΔzbmII*), pBS9054 (i.e., *ΔzbmXI*), pBS9061 (i.e., *Δzbm-orf31*), pBS9066 (i.e., *ΔzbmVIIb*), and pBS9072 (i.e., *ΔzbmVIIc*) were introduced into the wild-type *S. flavoviridis* SB9001 strain by intergeneric conjugation.[3] Exconjugants were selected for single-crossover events by using the apramycin or thiostrepton resistant phenotype, and subsequently for double-crossover events by using the apramycin or thiostrepton sensitive phenotype, affording the mutant strains *S. flavoviridis* SB9014 (i.e., *ΔzbmII*), SB9015 (i.e., *ΔzbmXI*), SB9016 (i.e., *Δzbm-orf31*), SB9017 (i.e., *ΔzbmVIIb*), and SB9018 (i.e., *ΔzbmVIIc*) (Figure S1, Supplemental data). The genotypes of all apparent double crossover mutant strains were further confirmed by Southern blot analyses (Figure S1, Supplemental data). The mutants were cultured and investigated for ZBM production with the wild-type *S. flavoviridis* SB9001 strain as a control (Figure 4, traces I, V, VI, IX, X, XI, and XIV).[3] The isolated yields of ZBM from the SB9016 mutant strain was ~50% of the wild-type SB9001 strain.

The complementation and cross-complementation constructs pBS9043 (i.e., *zbm-orf1-3*), pBS9045 (i.e., *zbm-orf2*), pBS9057 (i.e., *zbmXI*), pBS9068 (i.e., *zbmVIIb*), pBS51 (i.e., *blmXI*), and pBS53 (i.e., *blmVII*) were introduced into the SB9008 (i.e., *Δzbm-orf2*), SB9015 (i.e., *ΔzbmXI*), and SB9017 (i.e., *ΔzbmVIIb*) mutant strains, respectively, by intergeneric conjugation. Maintenance of pBS9043, pBS9045, pBS9057, pBS9068, pBS51, and pBS53 in the resultant *S. flavoviridis* SB9019 (i.e., *Δzbm-*

orf2/zbm-orf1-3), SB9020 (i.e., *Δzbm-orf2/zbm-orf2*), SB9021 (i.e., *ΔzbmXI/zbmXI*), SB9022 (i.e., *ΔzbmVIIb/zbmVIIb*), SB9023 (i.e., *ΔzbmXI/blmXI*), and SB9024 (i.e., *ΔzbmVIIb/blmVII*) strains, respectively, was verified by PCR analysis. The complementation strains were cultured and investigated for ZBM production with the wild-type *S. flavoviridis* SB9001 and the parent SB9008, SB9015, and SB9017 strains as controls (Figure 4, traces I, II, III, IV, VI, VII, VIII, X, XI, XII, and XIII). The isolated yields of ZBM from the SB9019, SB9020, SB9021, and SB9022 complementation strains were ~50%, ~50%, ~100%, and ~50%, respectively, of the wild-type SB9001 strain.

Table S1. Plasmids used in this study

Plasmid	Description	Reference
pGEM vectors	Cloning vectors	Promega
Litmus 28 and 29	Cloning vectors	NE Biolabs
pSPORT1	Cloning vector	Invitrogen
pIJ773	*aac(3)-oriT* cassette from REDIRECT Technology kit	5
pANT841	Cloning vector	6
pWHM1250	Cloning vector containing the *ErmE** promoter	8
pSET151	Cloning vector	7
pSET152	Cloning vector	7
pOJ260	Cloning vector	7
pBS9005	Cosmid clone containing a part of the ZBM cluster	3
pBS9006	Cosmid clone containing a part of the ZBM cluster	3
pBS9010	pSET152-based cloning vector	3
pBS9027	pANT841-based clone containing a fragment of *zbm-orf(-1)*	3
pBS37	Cosmid clone containing a part of the BLM cluster	4
pBS50	pBS37-based construct for *blmXI* inactivation by λRED method	This study
pBS51	pSET152-based construct for expression of *blmXI* under *ErmE**	This study
pBS52	pWHM1250-based construct for expression of *blmVII* under *ErmE**	This study
pBS53	pSET152-based construct for expression of *blmVII* under *ErmE**	This study
pBS9042	Intermediate construct for *zbm-orf1-3* under *ErmE** in Litmus 38	This study
pBS9043	pBS9010-based construct for expression of *zbm-orf1-3* under *ErmE**	This study
pBS9044	Intermediate construct for *zbm-orf2* under *ErmE** in Litmus 38	This study
pBS9045	pBS9010-based construct for expression of *zbm-orf2* under *ErmE**	This study
pBS9046	Intermediate construct for *zbmII* inactivation in pGEM7Zf	This study
pBS9047	Intermediate construct for *zbmII* inactivation by deletion in pGEM7Zf	This study
pBS9048	Intermediate construct for in-frame deletion of *zbmII* in pGEM7Zf	This study
pBS9049	pOJ260-based construct for in-frame deletion of *zbmII*	This study
pBS9050	Intermediate construct for *zbmXI* inactivation in pSPORT1	This study
pBS9051	Intermediate construct for *zbmXI* inactivation in pGEM7Zf	This study
pBS9052	Intermediate construct for in-frame deletion of *zbmXI* in pGEM7Zf	This study
pBS9053	Intermediate construct for in-frame deletion of *zbmXI* in pSPORT1	This study
pBS9054	pSET151-based construct for in-frame deletion of *zbmXI*	This study
pBS9055	*ErmE** promoter in pANT841	This study
pBS9056	Intermediate construct for *zbmXI* under *ErmE** in pANT841	This study
pBS9057	pSET152-based construct for expression of *zbmXI* under *ErmE**	This study
pBS9058	Intermediate construct for *zbm-orf31* inactivation in pGEM7Zf	This study
pBS9059	Intermediate construct for *zbm-orf31* inactivation in Litmus 28	This study
pBS9060	Intermediate construct for in-frame deletion of *zbm-orf31* in Litmus 28	This study
pBS9061	pSET151-based construct for in-frame deletion of *zbm-orf31*	This study
pBS9062	Intermediate construct for *zbmVIIb* inactivation in Litmus 28	This study
pBS9063	Intermediate construct for *zbmVIIb* inactivation in Litmus 28	This study
pBS9064	Intermediate construct for in-frame deletion of *zbmVIIb* in Litmus 28	This study
pBS9065	Intermediate construct for in-frame deletion of *zbmVIIb* in Litmus 28	This study
pBS9066	pSET151-based construct for in-frame deletion of *zbmVIIb*	This study
pBS9067	Intermediate construct for *zbmXIIb* under *ErmE** in pANT841	This study
pBS9068	pSET152-based construct for expression of *zbmXIIb* under *ErmE**	This study
pBS9069	Intermediate construct for *zbmVIIc* inactivation in Litmus 28	This study
pBS9070	Intermediate construct for *zbmVIIc* inactivation in Litmus 28	This study
pBS9071	Intermediate construct for in-frame deletion of *zbmVIIc* in Litmus 28	This study
pBS9072	pSET151-based construct for in-frame deletion of *zbmVIIc*	This study

Table S2. Bacterial strains used in this study

Strains	Description	Reference
Escherichia coli		
XL1 Blue MR	Host for routine subcloning and plasmid preparation	Stratagene
DH5α	Host for routine subcloning and plasmid preparation	9
S17-1	Host for *E. coli-Streptomyces* conjugation	2
BW25113/pIJ790	Host for λRED-mediated, PCR-targeted gene inactivation	5
Streptomyces verticillus		
ATCC15003	Wild-type BLM producer	ATCC
SB6	*ΔblmXI* inactivation mutant by *ΔblmXI::aac(3)IV* replacement	This study
Streptomyces flavoviridis		
SB9001	Improved ZBM producer	1
SB9008	*Δzbm-orf2* inactivation mutant by *Δzbm-orf2::aac(3)IV* replacement	4
SB9014	*ΔzbmII* inactivation mutant by in-frame *ΔzbmII* deletion	This study
SB9015	*ΔzbmXI* inactivation mutant by in-frame *ΔzbmXI* deletion	This study
SB9016	*Δzbm-orf31* inactivation mutant by in-frame *Δzbm-orf31* deletion	This study
SB9017	*ΔzbmVIIb* inactivation mutant by in-frame *ΔzbmVIIb* deletion	This study
SB9018	*ΔzbmVIIc* inactivation mutant by in-frame *ΔzbmVIIc* deletion	This study
SB9019	SB9008/pBS9043 (*Δzbm-orf2* complemented with *zbm-orf1-3*)	This study
SB9020	SB9008/pBS9045 (*Δzbm-orf2* complemented with *zbm-orf2*)	This study
SB9021	SB9015/pBS9057 (*ΔzbmXI* complemented with *zbmXI*)	This study
SB9022	SB9017/pBS9068 (*ΔzbmVIIb* complemented with *zbmVIIb*)	This study
SB9023	SB9015/pBS51 (*ΔzbmXI* cross-complemented with *blmXI*)	This study
SB9024	SB9017/pBS53 (*ΔzbmVIIb* cross-complemented with *blmVII*)	This study

Table S3. Oligonucleotides used in this study

Oligonucleotides	Sequences[a]	Reference
blmXI-1f	5'-cgcgtacgaagcgatggcgaactggagggacacagcgtgATTCCGGGGATCCGTCGACC-3'	
blmXI-1r	5'-ggttcgattccgaccctaccactcactcattgcgcgttaTGTAGGCTGGAGCTGCTTC-3'	
C-blmXI-f2	5'-TAGTGGATCCCCAGGAGTC-3' (underlined sequence = introduced BamHI site)	
C-blmXI-r	5'-GATTCTAGACCCTACCACTC-3' (underlined sequence = introduced XbaI site)	
zbmII-lif	5'-GGCGGGAGATGCATGGAGC-3'	
zbmII-lir	5'-CTAGGCTCCATGCATCTCCCGCCTGCA-3'	
zbmXI-lif	5'-GGTCCCATGCATCAATTGA-3'	
zbmXI-lir	5'-GGATCTCAATTGATGCATG-3'	
zbmVIIc-lif	5'-CGGCCTCACCAGATCTGCGCATGGAAGTCGTCCCTCAGG-3'	
zbmVIIc-lir	5'-CGCGCCTGAGGGACGACTTCCATGCGCAGATCTGGTGAGGCCGTGG-3'	

[a]Lower case letters represent DNA sequence originating from the respective producer strain (*S. verticillus*) and upper case letters represent DNA sequence flanking the *aac(3)IV-oriT* cassette from pIJ773

Figure S1 Determination of functional involvement of various BLM and ZBM biosynthetic genes by gene replacement or in-frame deletion. A, *Avr*II; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; P, *Pst*I; S, *Sph*I; Sf, *Sfi*I; X, *Xho*I; ApraR, apramycin resistant; ApraS, apramycin sensitive.

(A) Construction of the *zbmII* in-frame deletion mutant and restriction map of *S. flavoviridis* SB9001 wild-type and SB9014 mutant strains showing fragment sizes upon *Bam*HI-*Kpn*I digestion.

(B) Southern analysis of *S. flavoviridis* SB9001 wild-type (lane 2) and SB9014 (lanes 3-7 are five individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *zbmII* (lane 8) locus as control digested with *Bam*HI-*Kpn*I using a 0.335-kb *Avr*II-*Bam*HI fragment as a probe. Lane 1, molecular weight marker.

(C) Construction of the *blmXI* gene replacement mutant and restriction map of *S. verticillus* ATCC15003 wild-type and SB6 mutant strains showing fragment sizes upon *Bam*HI digestion.

(D) Southern analysis of *S. verticillus* ATCC15003 wild-type (lane 6) and SB6 (lanes 2-5 are four individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *blmXI* (lane 1) locus as control digested with *Bam*HI using a 0.981-kb PCR-amplified fragment as a probe (primer pair C-blmXI-f2 and C-blmXI-r). Lane 1, molecular weight marker.

(E) Construction of the *zbmXI* in-frame deletion mutant and restriction map of *S. flavoviridis* SB9001 wild-type and SB9015 mutant strains showing fragment sizes upon *Pst*I-*Xho*I digestion.

(F) Southern analysis of *S. flavoviridis* SB9001 wild-type (lane 2) and SB9015 (lanes 3-4 are two individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *zbmXI* (lane 5) locus as control digested with *Pst*I-*Xho*I using a 1.650-kb *Pst*I-*Cla*I fragment as a probe. Lane 1, molecular weight marker.

(G) Construction of the *zbm-orf31* in-frame deletion mutant and restriction map of *S. flavoviridis* SB9001 wild-type and SB9016 mutant strains showing fragment sizes upon *Bam*HI digestion.

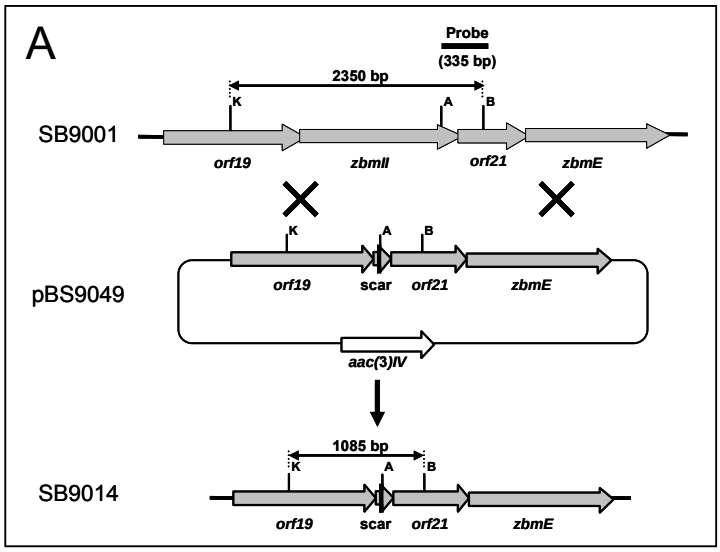
(H) Southern analysis of *S. flavoviridis* SB9001 wild-type (lane 2) and SB9016 (lanes 3-7 are five individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *zbm-orf31* (lane 8) locus as control digested with *Bam*HI using a 1.500-kb *Bam*HI-*Pst*I fragment as a probe. Lane 1, molecular weight marker.

(I) Construction of the *zbmVIIb* in-frame deletion mutant and restriction map of *S. flavoviridis* SB9001 wild-type and SB9017 mutant strains showing fragment sizes upon *Xho*I-*Pst*I digestion.

(J) Southern analysis of *S. flavoviridis* SB9001 wild-type (lane 2) and SB9017 (lanes 3-4 are two individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *zbmVIIb* (lane 5) locus as control digested with *Xho*I-*Pst*I using a 0.430-kb *Sph*I-*Pst*I fragment as a probe. Lane 1, molecular weight marker.

(K) Construction of the *zbmVIIc* in-frame deletion mutant and restriction map of *S. flavoviridis* SB9001 wild-type and SB9018 mutant strains showing fragment sizes upon *Bgl*II digestion.

(L) Southern analysis of *S. flavoviridis* SB9001 wild-type (lane 2) and SB9018 (lanes 3-4 are two individual isolates) genomic DNA and inactivation plasmid harbouring the mutated *zbmVIIc* (lane 5) locus as control digested with *Bgl*II using a 0.820-kb *Bgl*II-*Sfi*I fragment as a probe. Lane 1, molecular weight marker.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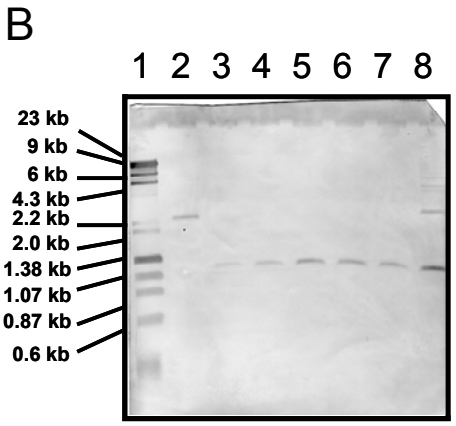
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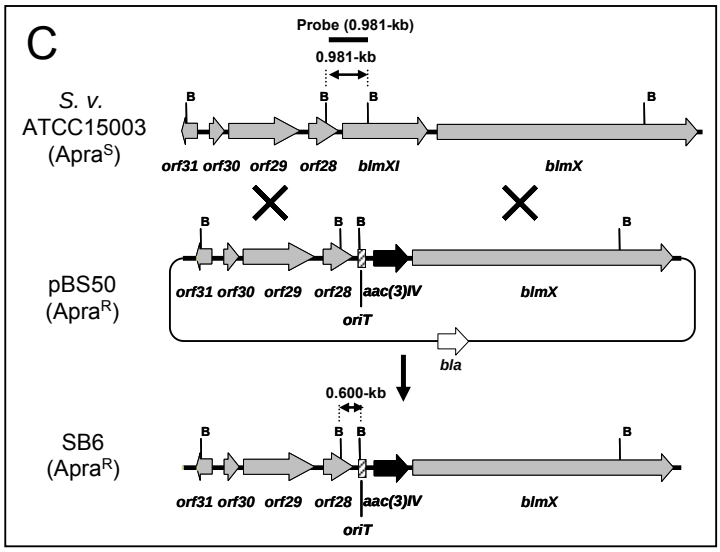
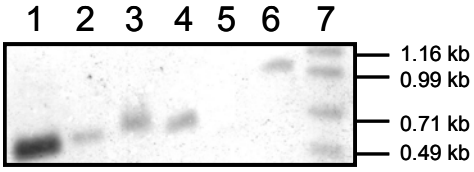

C
Probe (0.981-kb)
0.981-kb
S. v.
ATCC15003
(Apra^S)
orf31 orf30 orf29 orf28 blmXI
blmX
pBS50
(Apra^R)
aac(3)IV
oriT
bla
0.600-kb
SB6
(Apra^R)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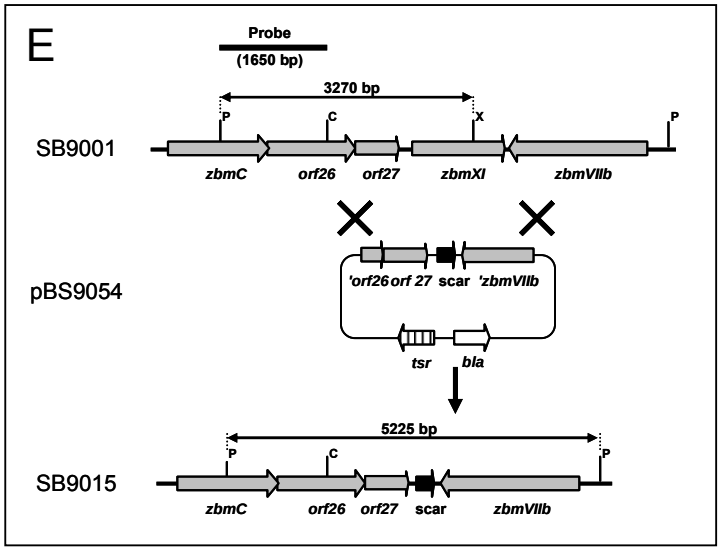
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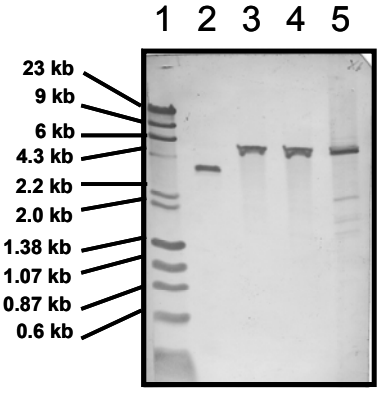
Probe
(1650 bp)
3270 bp
P
C
X
SB9001
zbmC
orf26
orf27
zbmXI
zbmVIIb
pBS9054
'orf26 orf 27
scar
'zbmVIIb
tsr
bla
5225 bp
SB9015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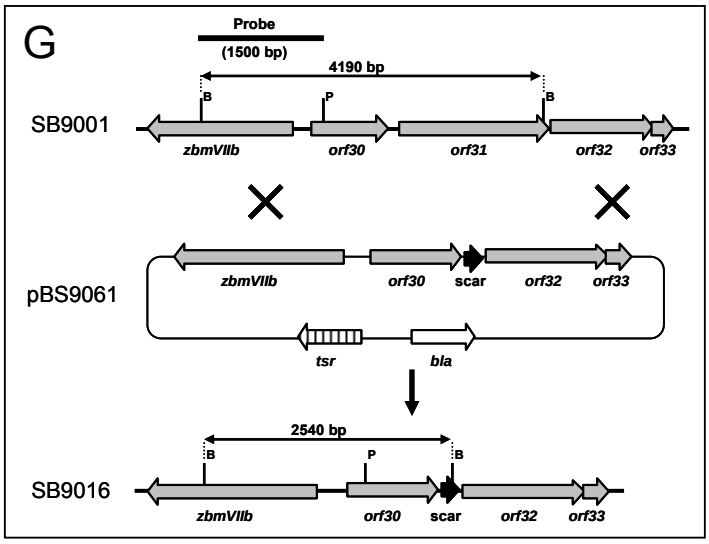
G
Probe
(1500 bp)
4190 bp
B
P
B
SB9001
zbmVIIb
orf30
orf31
orf32
orf33
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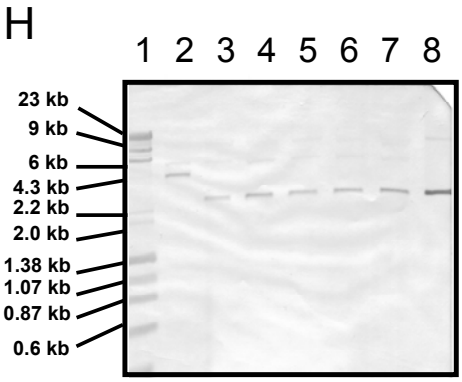
zbmVIIb
orf30
scar
orf32
orf33
tsr
bla
2540 bp
B
P
B
SB9016
zbmVIIb
orf30
scar
orf32
orf33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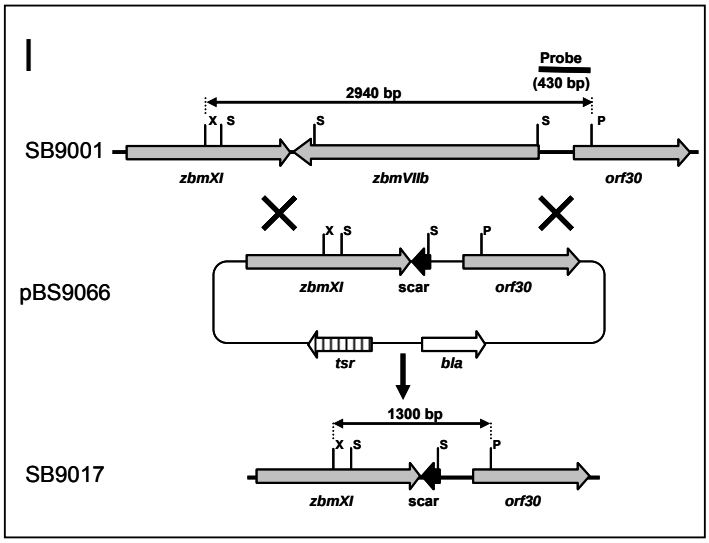
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S
S
S
P
SB9001
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orf30
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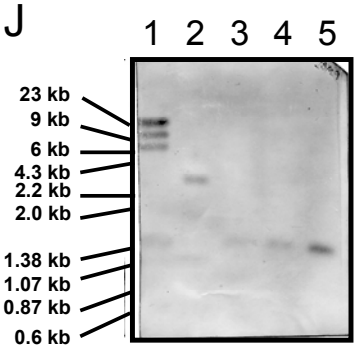
scar
orf30
tsr
bla
1300 bp
X
S
S
P
SB9017
zbmXI
scar
orf30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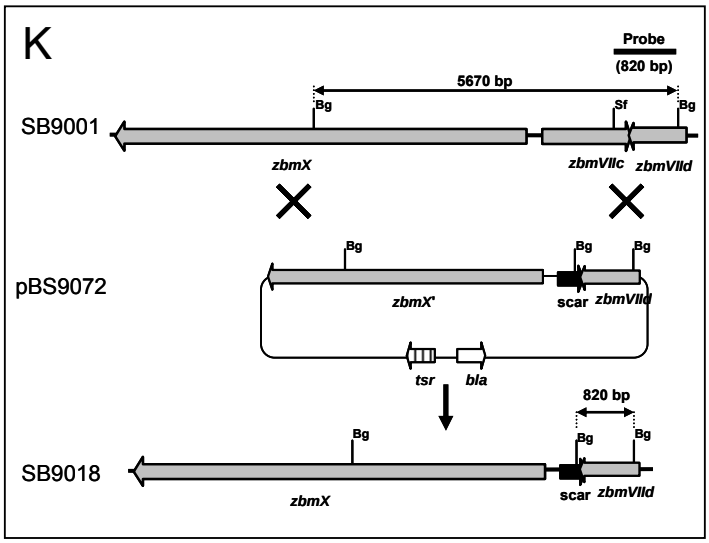
K
Probe
(820 bp)
5670 bp
Bg
Sf
Bg
SB9001
zbmX
zbmVIIc
zbmVIId
pBS9072
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Bg
Bg
Bg
SB9018
zbmX
scar
zbmVIId

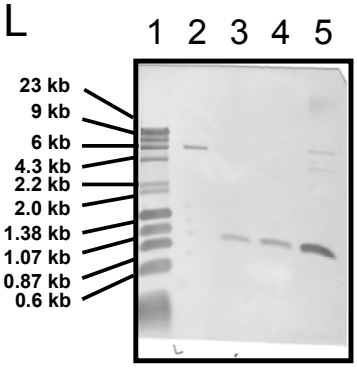
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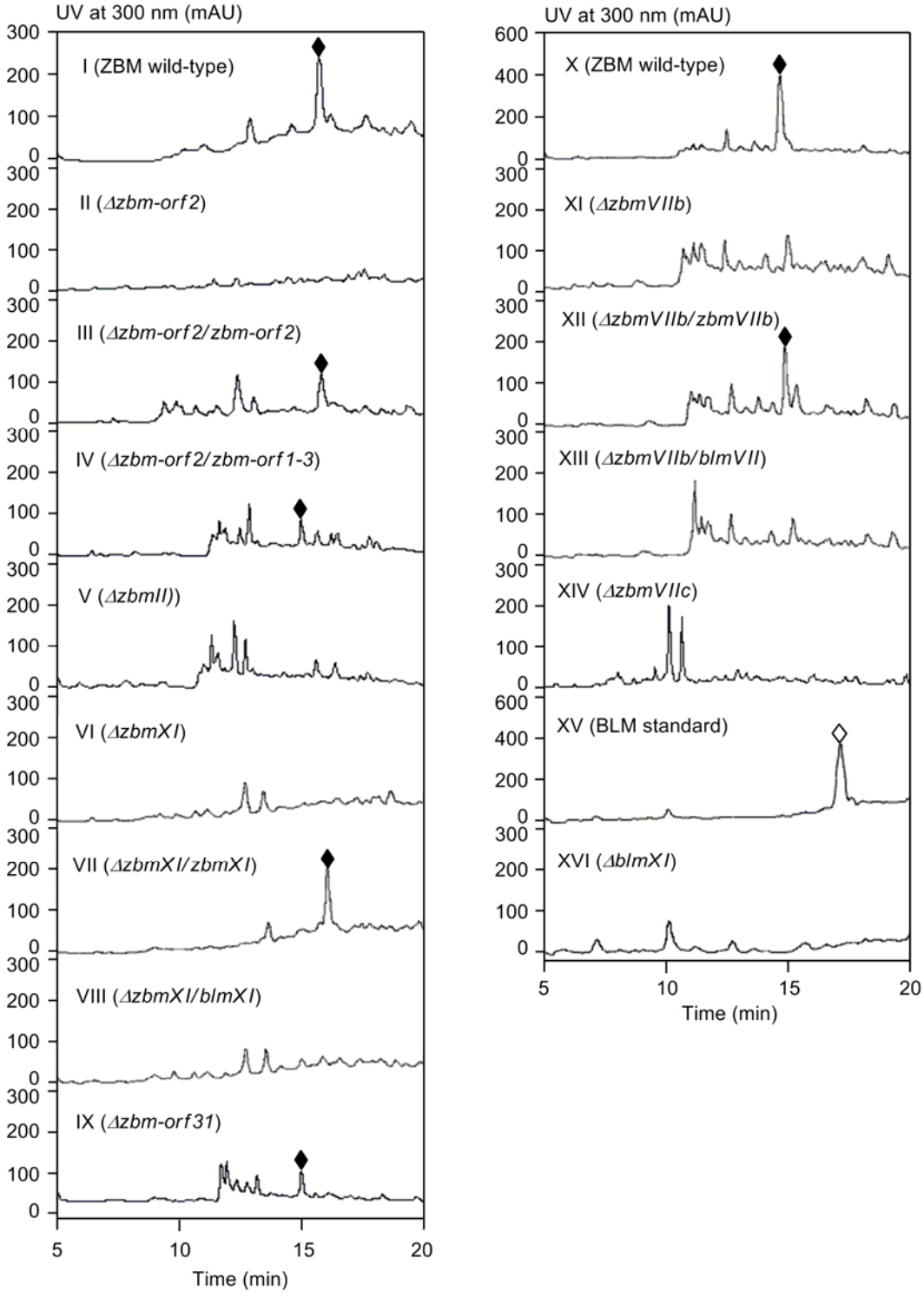
Figure S2 HPLC analysis of ZBM (♦) and BLM (◊) production in *S. flavoviridis* wild-type strain SB9001 (I and X), recombinant strains SB9008 (II), SB9019 (III), SB9020 (IV), SB9014 (V), SB9015 (VI), SB9021 (VII), SB9023 (VIII), SB9016 (IX), SB9017 (XI), SB9022 (XII), SB9024 (XIII), SB9018 (XIV), and *S. verticillus* recombinant strain SB6 (XVI), as well as authentic BLM standard (XV). Small discrepancies in retention time were always confirmed by co-injection of the respective standards as well as by MS analysis of the peaks.



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