In Vivo Manipulation of the Bleomycin Biosynthetic Gene Cluster in *Streptomyces verticillus* ATCC15003 Revealing New Insights into its Biosynthetic Pathway

Ute Galm,[†] Liyan Wang,[†] Evelyn Wendt-Pienkowski,[†] Runying Yang,[¥] Wen Liu,[†] Meifeng Tao,[†] Jane M. Coughlin,[†] and Ben Shen[†,‡,§,¥*]

From the [†]Division of Pharmaceutical Sciences, [‡]University of Wisconsin National Cooperative Drug Discovery Group, and [§]Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, 53705, and [¥]Department of Chemistry, University of California, Davis, California 95616

Running title: *In Vivo* Manipulation of the Bleomycin Biosynthetic Gene Cluster.

Address correspondence to: Ben Shen, Division of Pharmaceutical Sciences, School of Pharmacy, University of Wisconsin-Madison, 777 Highland Ave., Madison, WI 53705, Tel: (608) 263-2673; Fax: (608) 262-5245; Email: bshen@pharmacy.wisc.edu

SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Sequencing and computer assisted sequence analysis—Sequencing of an additional 5385 bp beyond the downstream end of the published sequences was performed by primer walking using the dideoxynucleotide chain termination method. Sequencing reactions were run using Big Dye Terminator mix (Applied Biosystems, Foster City, CA), cleaned using CleanSeq magnetic beads (Agencourt Biosciences, Beverly, MA), and sequenced by the University of Wisconsin Biotechnology Center. Samples were electrophoresed on an Applied Biosystems 3700 automated DNA sequencing instrument, and data were analyzed using PE-Biosystems version 3.7 for Sequence Analyses. ORF assignments were made with the assistance of Codon Preference (GCG package, Madison, WI) and BLAST analyses. The DNA sequence reported in this work was determined from pBS40 and has been deposited at GenBank under the accession no. EU805703.

Isolation and analysis of BLM and decarbamoyl-BLM—To analyze the production of BLM and decarbamoyl-BLM produced by *S. verticillus* wild-type and the respective recombinant strains, a typical fermentation broth (50 mL) was adjusted to pH 7.0 with 1.0 N HCl and loaded onto an Amberlite IRC50 column (H^+ type, 7-10 mL). After washing the column with 10 bed volumes of H_2O, BLM and intermediates were eluted with 40 mL of 0.2 N HCl. The resulting eluent was neutralized with 1.0 N NaOH and then evaporated *in vacuo*. The dry material was dissolved in 1 mL of H_2O. Analytical HPLC was carried out on an Apollo C18 column (5 μm, 250 × 4.6 mm, Alltech Associates, Inc., Deerfield, IL). The column was equilibrated with 100% solvent A (1% HOAc) and 0% solvent B (1% HOAc, 99% methanol) and developed with a linear gradient (0 to 5 min, from 100% A to 90% A/10% B; 5 to 10 min, from 90% A/10% B to 16% A/84% B; 10 to 30 min, from 16% A/84% B to 100% B) at a flow rate of 0.7 mL/min and with UV detection at 300 nm using a Varian Prostar 330 PDA detector (Varian, Palo Alto, CA). The isolated BLM or intermediate at this step was about 90% pure. The identity of BLM was confirmed by LC-ESI mass spectrometry on an Agilent 1100 HPLC-MSD SL quadrupole mass spectrometer.

Large-scale isolation of decarbamoyl-BLM—The fermentation culture (10.0 L) was centrifuged at 3000 rpm for 30 min, and the supernatant (8.5 L) was collected, adjusted to pH 7.0 with 1.0 N HCl, and loaded onto an HP-20 column (1.8 L). After washing the column with 10 bed volumes of H_2O, 3-MOP-decarbamoyl-BLM was eluted with 6.0 L of 50% MeOH. The resulting eluate was concentrated to 10 mL *in vacuo*, loaded onto a Sephadex C-25 column, and eluted with 100 mL of H_2O, 0.05 M NH_4OAc, and 0.15 M NH_4OAc, successively. The eluates were collected and the 3-MOP-decarbamoyl-BLM was found in the 0.15 M NH_4OAc eluate. The 0.15 M NH_4OAc eluate was mixed with 20 mL of Amberlite XAD-2 for 30 min. The resin was then washed with excessive water to desalt, and the 3-MOP-decarbamoyl-BLM was eluted with 80% MeOH. Final purification of decarbamoyl-BLM was achieved by semipreparative HPLC on an Altima C18 column (5 μm, 250 × 10 mm, Alltech Associates, Inc.). HPLC isolation was carried out as follows using the same instrument and detector as stated above. The column was equilibrated with 100% solvent A (0.1% TFA) and 0% solvent B (0.1% TFA, 99.9% methanol) and developed with a linear gradient (0 to 20 min, from 90% A/10% B to 40% A/60% B) at a flow rate of 3 mL/min and with UV detection at 300 nm. Under these conditions, decarbamoyl-BLM was eluted with a retention time of 15.2 min. The methanol was removed from the eluate by evaporation at 40°C, and the final pure decarbamoyl-BLM-Cu complex (10.0 mg) was obtained by freeze drying. Cu-free decarbamoyl-BLM was obtained by treating the decarbamoyl-BLM-Cu complex with 0.5 M EDTA-Na (pH 7.3) solution. After the final HPLC purification, Cu-free decarbamoyl-BLM was obtained as a pale white power (8.0 mg).

TABLE S1. Plasmids used in this study.

Plasmids	Description	Source/reference
SuperCos1		Stratagene
pBluescript II SK (+)		Stratagene
pKC1139	Temperature sensitive pSG5 derived replicative vector	(1)
pKC1218	SCP2* derived replicative vector	(1)
pHZ1358	pIJ101 derived replicative vector	(2)
pSET152	ΦC31 derived integrative vector	(1)
pRT801	phiBT1 derived integrative vector	(3)
pSOK804	VWB derived integrative vector	(4)
pIJ773	*oriT-aac(3)IV* cassette for λRED-mediated PCR-targeting	(5)
pSET151	Non-replicative vector in streptomycetes	(1)
pBS37	Cosmid containing upstream part of the BLM cluster	this study
pBS38	Cosmid containing central part of the BLM cluster	this study
pBS39	Cosmid containing downstream part of the BLM cluster	this study
pBS40	Cosmid containing downstream part of the BLM cluster	this study
pBS41	*blmIV* inactivation construct in pSET151 (4.1-kb and 2.3-kb flanking regions)	this study
pBS42	*blmIV* inactivation construct in pSET151 (9.8-kb and 7.8-kb flanking regions)	this study
pBS43	pBS38 derived *blmIV* gene replacement construct (18.9-kb and 12.9-kb flanking regions)	this study
pBS44	pBS37 derived *blmVIII* gene replacement construct (23.5-kb and 12.2-kb flanking regions)	this study
pBS45	pBS37 derived *blm-orf31/32* gene replacement construct (6.0-kb and 33.1-kb flanking regions)	this study
pBS46	pBS40 derived *blm-orf(-1)* gene replacement construct (17.6-kb and ~20-kb flanking regions)	this study
pBS47	pBS39 derived *blmD* gene replacement construct (21.0-kb and ~15-kb flanking regions)	this study
pBS48	pBS47 derived *blmD* in-frame deletion construct (*bla* gene on SuperCos1 backbone)	this study
pBS49	pBS48 derived *blmD* in-frame deletion construct (*aac(3)IV-oriT* cassette on SuperCos1 backbone)	this study

TABLE S2. Bacterial strains used in this study

Strains	Description	Source/reference
Escherichia coli XL1 Blue MR		Stratagene
Escherichia coli DH5α		(6)
Escherichia coli S17-1		(7)
Escherichia coli ET12567(pUZ8002)		(8)
Escherichia coli BW25113/pIJ790		(5)
Streptomyces verticillus ATCC15003	Wild type BLM producer	American Type Culture Collection, Rockville, MD, USA
SB1	*S. verticillus* ATCC 15003*ΔblmIV::aac(3)IV* mutant	this study
SB2	*S. verticillus* ATCC 15003*ΔblmVIII::aac(3)IV* mutant	this study
SB3	*S. verticillus* ATCC 15003*Δblm-orf31/32::aac(3)IV* mutant	this study
SB4	*S. verticillus* ATCC 15003*Δblm-orf(-1)::aac(3)IV* mutant	this study
SB5	*S. verticillus* ATCC 15003*ΔblmD* mutant	this study

TABLE S3. Oligonucleotides used in this study

Oligonucleotides	Sequence	Source/reference
- for λRED-mediated PCR-targeting		
blmIV-1f	5'-gccctgtcccccgaacgccgggcggcgttcgagaagatg ATTCCGGGGATCCGTCGACC-3' [a)]	this study
blmIV-1r	5'-cggggagcagtgtcggcgtgggccgggcgctcgtcactc TGTAGGCTGGAGCTGCTTC-3' [a)]	this study
blmVIII-1f	5'-ggtggccgcgcgccgcaggaaaggcggcggacgatgagc ATTCCGGGGATCCGTCGACC-3' [a)]	this study
blmVIII-1r	5'-gctcggtgagcaggtcggtgatgcggggcgtggtcacag TGTAGGCTGGAGCTGCTTC-3' [a)]	this study
NRPS10-1f	5'-acgccgtccgcagcgtctcgtgccgtcccaccagcatcc ATTCCGGGGATCCGTCGACC-3' [a)]	this study
PHNA-1r	5'-agtgctctcgtgaccgagaaccttccgtcgtgccccgaa TGTAGGCTGGAGCTGCTTC-3' [a)]	this study
treh-1f	5'-gccgccccctgggacacgacggaagcgaggccggtgatg ATTCCGGGGATCCGTCGACC-3' [a)]	this study
treh-1r	5'-ccatccgcacagggtgaatggccgggggccgacgcctca TGTAGGCTGGAGCTGCTTC-3' [a)]	this study
blmD-1f	5'-gagcccccaccccgtgacgatcaggagtaccccatgagc ATTCCGGGGATCCGTCGACC-3' [a)]	this study
blmD-1r	5'-gccgctgacgatgaggcccgggagcaccaggtggtcgag TGTAGGCTGGAGCTGCTTC-3' [a)]	this study
f-pMT3	5'-CTCACGTTAAGGGATTTTGGTCATG-3'	(9)
r-pMT3	5'-GGAAGAGTATGAGTATTCAACATTTCCGTG TCGCCCTTATGCATGCCTGCAGGTCGACGG-3'	(9)
- for PCR and Southern blot verification of mutant strains		
blmIV-2f	5'-GCTGACCTCGATCCCGGCAC-3'	this study
blmIV-2r	5'-CGGGTGCGGTGCGGTCGTTC-3'	this study
blmVIII-2f	5'-AGGAGGACGCGCGCGAGACG-3'	this study
blmVIII-2r	5'-CCGTCGGCCGTGAGGGTCAC-3'	this study
C-PHNA-f1	5'-GCAGCGTCATGAACAGGGTG-3'	this study
C-PHNA-r1	5'-CCGGACCATCATGTAGCGAC-3'	this study
C-treh-f1	5'-GGTCGACATCGTCGGTCACTCG-3'	this study
C-treh-r1	5'-CACGCACACGATCGAAGACCTG-3'	this study
C-blmD-f2	5'-CCGCACTGGAACAGGAGATG-3'	this study
C-blmD-r	5'-GTCTAGATGTCGCGTTCGAG-3' [b)]	this study
blmIII/IVprobe-f	5'-TCGCACGAGGTGCGCGACGC-3'	this study
blmIII/IVprobe-r	5'-GTACCGGCGGCCCGGGTTCG-3'	this study
blmVIIIprobe-f	5'-ACGAGCCCACGGTGGCGGCG-3'	this study
blmVIIIprobe-r	5'-GCGCGACCGGCTCGGCGATC-3'	this study
Treh-p-f1	5'-GTGTCCAGGACACGGCAGTC-3'	this study
Treh-p-r1	5'-GCGGCATCATGCCACCTTGC-3'	this study
C-blmE-f2	5'-CCTACCACGTGGTGGTCAAC-3'	this study
C-blmE-r	5'-AGTCTAGACGGAGAGGATCG-3' [b)]	this study

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

[b)] underlined sequence represents an introduced *Xba*I restriction site

FIGURE S1. Determination of the *blm* gene cluster boundaries by gene replacements of *blm-orf31/32* and *blm-orf (-1)*. Construction of the *blm-orf31/32* (A) and *blm-orf(-1)* (C) gene replacement mutants and restriction map of *S. verticillus* wild-type (A and C), SB3 (A) and SB4 (C) mutant strains showing fragment sizes upon *Bam*HI (A) and *Sst*I (C) digestion, respectively. (B) Southern analysis of *S. verticillus* wild-type (B, lane 2; D, lane 3), SB3 (B, lane 1), and SB4 (D, lane 2) genomic DNA digested with *Bam*HI (B) and *Sst*I (D), respectively, using a 1.446-kb *Bam*HI fragment of a 2.788-kb PCR amplified product (B) and a 1.110-kb PCR-amplified fragment (D) as probes. Molecular weight marker, lane 3 (B) and lane 1 (D). B, *Bam*HI; S, *Sst*I; Apra^R, apramycin resistant; Apra^S, apramycin sensitive.

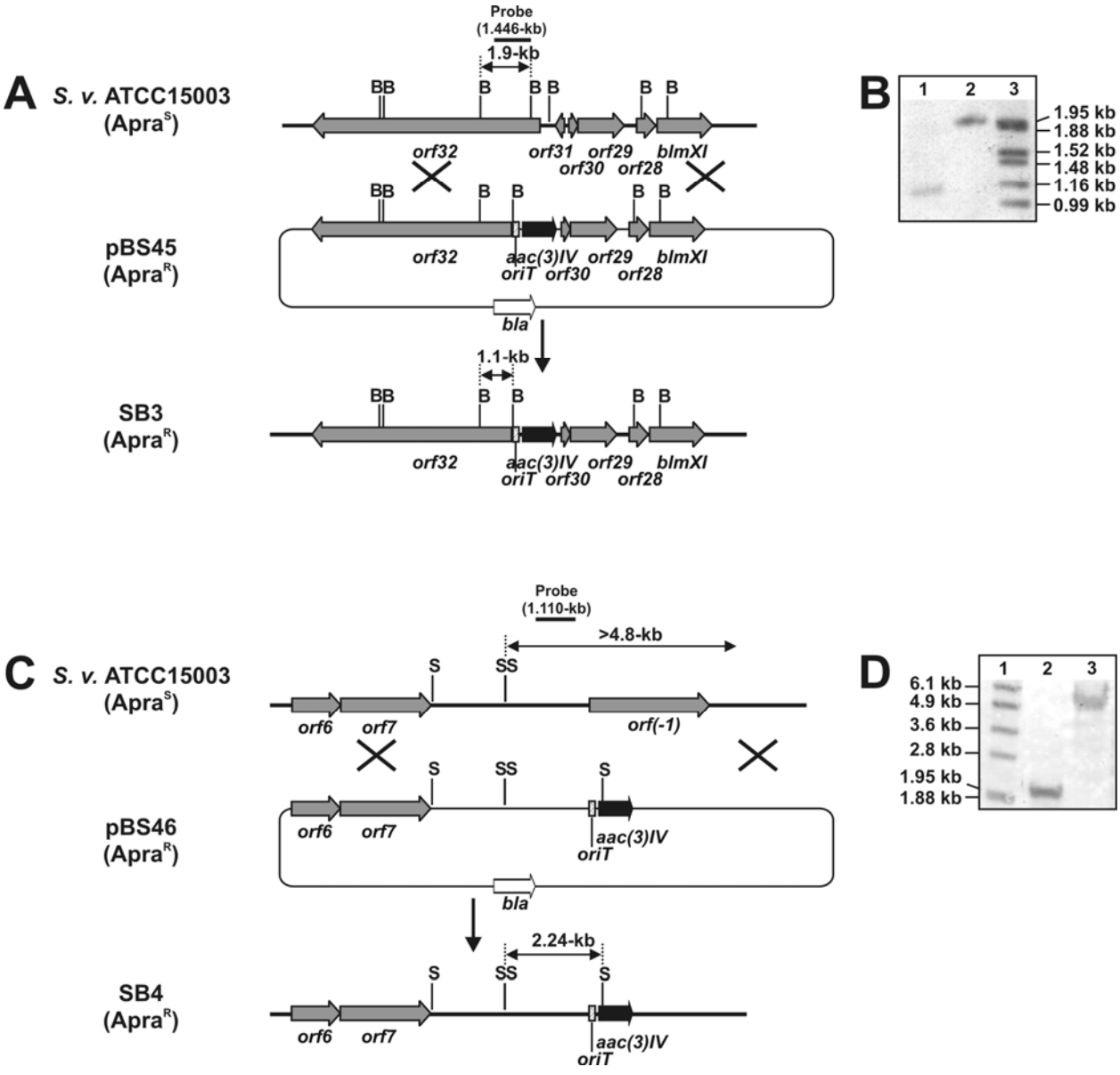
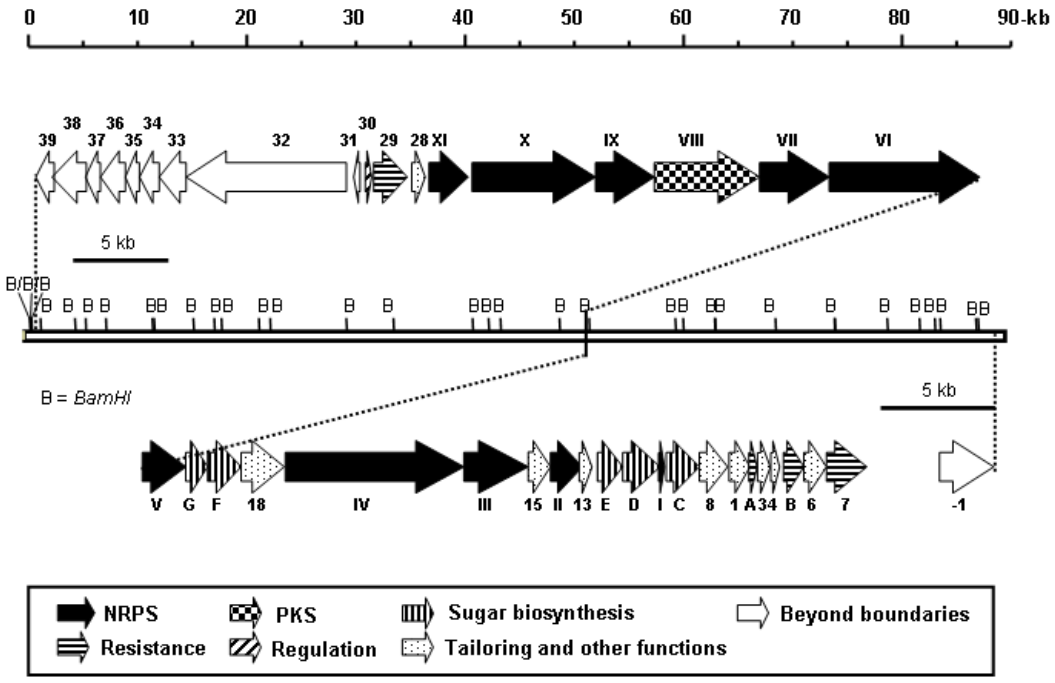


FIGURE S2. Restriction map of the sequenced 90-kb DNA region from *S. verticillus* ATCC 15003 and genetic organization of the *blm* biosynthetic gene cluster. The cluster boundaries have been determined experimentally in this study to be between *orf31* and *orf30* (upstream) and between *orf7* and *orf(-1)* (downstream), respectively.



REFERENCES

1. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992) *Gene* **116**, 43-49.
2. Sun, Y., Zhou, X., Liu, J., Bao, K., Zhang, G., Tu, G., Kieser, T., and Deng, Z. (2002) *Microbiology* **148**, 361-371.
3. Gregory, M.A., Till, R., and Smith, M.C. (2003) *J. Bacteriol.* **185**, 5320-5323.
4. 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.
5. Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1541-1546.
6. Sambrook, J., Russell, D.W. (2001). *Molecular cloning: a laboratory manua,.* 3rd Ed., Cold Spring Harbor Laboratory Press, New York.
7. 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.
8. Paget, M.S., Chamberlin, L., Atrih, A., Foster, S.J., and Buttner, M.J. (1999) *J. Bacteriol*. **181**, 204-211.
9. 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.