DOI: 10.1002/cbic.200800029

Reassembly of Anthramycin Biosynthetic Gene Cluster by Using Recombinogenic Cassettes

Yunfeng Hu,[a] Vanessa V. Phelan,[a] Chris M. Farnet,[b] Emmanuel Zazopoulos,[b] and Brian O. Bachmann*[a]

The reassembly and heterologous expression of complete gene clusters in shuttle vectors has enabled investigations of several large biosynthetic pathways in recent years. With a gene cluster in a mobile construct, the interrogation of gene functions from both culturable and nonculturable organisms is greatly accelerated and large pathway engineering efforts can be executed to produce "new" natural products. However, the genetic manipulation of complete natural product biosynthetic gene clusters is often complicated by their sheer size (10–200 kbp), which makes standard restriction/ligation-based methods impracticable. To circumvent these problems, alternative recombinogenic methods, which depend on engineered homology-based recombination have recently arisen as a powerful alternative. Here, we describe a new general technique that can be used to reconstruct large biosynthetic pathways from overlapping cosmids by retrofitting each cosmid with a "recombinogenic cassette" that contains a shared homologous element and orthogonal antibiotic markers. We employed this technique to reconstruct the anthramycin biosynthetic gene cluster of the thermotolerant actinomycete Streptomyces refuineus*, from two >30 kbp cosmids into a single cosmid and integrate it into the genome of* Streptomyces lividans*. Anthramycin production in the heterologous* Streptomyces *host confirmed the integrity of the reconstructed pathway and validated the proposed boundaries of the gene cluster. Notably, anthramycin production by recombinant* S. lividans *was seen only during growth at high temperature—a property also shown by the natural host. This work provides tools to engineer the anthramycin biosynthetic pathway and to explore the connection between anthramycin production and growth at elevated temperatures.*

Introduction

More often than not, natural product biosynthetic pathways are far from concise. They frequently entail dozens and sometimes over a hundred individual biosynthetic steps.[1,2] In bacteria and some fungi, the corresponding genes that comprise such pathways are generally clustered into 10–200 kbp loci, a convenient property that has allowed rapid cloning and sequencing of many complete natural product gene clusters in microorganisms.[3,4] However, the majority of natural-product-producing microorganisms are intransigent to standard gene manipulation techniques, and some are not cultivatable outside their ecological context.[5,6] For these reasons, the study and modification of whole pathways has greatly benefited from the ability to manipulate them in vectors, such as cosmids, fosmids, and bacterial artificial chromosomes (BACs).[7,8]

The practical difficulty of assembling and modifying large gene clusters by using restriction/ligation enzyme-based tools arises from the problem of identifying multiple unique restriction sites in large stretches of DNA, and also from the entropic challenge of circularizing large vectors. Additionally, many gene clusters are derived from microorganisms with GC-rich genomes, a factor that can complicate PCR-based cloning and expression.[9] Recently, the characterization of the phage λ homologous recombination system has enabled an alternative means of restriction/ligation-free gene manipulation.[10,11] It was discovered that in engineered *E. coli* that contain the Redα (exo), Redβ (bet), and Redγ (gam) proteins of the phage λ, allelic exchange can take place if a given DNA fragment is flanked at both ends by extensions of more than 35 nucleotides that are homologous to a target DNA region.[12,13] This observation was quickly applied by microbial geneticists for recombinogenic targeting of bacterial genomes as well as for manipulating small and large vectors, including cosmids[14] and BACs.[15] In the area of natural products biosynthetic genetics, this system, which is alternatively referred to the "λ-red PCR-targeting system" or "Red/ET cloning system" has proven to be a revolutionary method for rapid gene replacement and/or fusion in cosmids and BACs. These methods use PCR to amplify a selectable marker with flanking >35 nucleotide extensions followed by transformation of the linear PCR product into the appropriately modified *E. coli* strain that contains a target cosmid.[16]

The application of the λ-phage recombinogenic system for the functional reconstruction of large natural product biosyn-

[a] *Y. Hu, V. V. Phelan, Prof. B. O. Bachmann*
Chemistry Department, Vanderbilt University
7921 Stevenson Center, Nashville, TN 37235 (USA)
Fax: (+1) 615-341-1234
E-mail: brian.bachmann@vanderbilt.edu

[b] *C. M. Farnet, E. Zazopoulos*
c/o Thallion Pharmaceuticals Inc.
7150 Alexander-Fleming, Montreal
Quebec H4S 2C8 (Canada)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

thetic gene clusters from fragments on separate vectors[8,14] was pioneered for myxobacterial metabolites. Heterologous expression of the myxothiazole (57 kbp) and myxochromid (43 kbp) gene clusters, which were isolated from genetically refractory myxobacterial hosts has permitted significant new insights into the biosynthesis of these natural products. Very recently, these techniques have been extended to the actinobacterial metabolite phenalinolactone.[17] In this study we describe a concurrently developed variation of these techniques: a general method for recombining cosmids with overlapping sequence by retrofitting each cosmid with a "recombinogenic cassette" that contains orthogonal selectable markers and a shared homologous element. This method does not require PCR of cosmid DNA or enzymatic ligation, it does not introduce additional DNA into the gene cluster, and it requires only the identification of a single unique restriction enzyme in the overlap region of one of the donor cosmids.

As a case study to demonstrate the utility of the method, we targeted the anthramycin biosynthetic gene cluster from the thermotolerant microorganism *Streptomyces refuineus*,[18] which we have recently demonstrated to be present on a 35 kb region on two cosmids.[19] By retrofitting each cosmid with a recombinogenic cassette, we were able to efficiently reconstruct the anthramycin gene cluster on a single construct. Furthermore, subsequent to the addition of plasmid transfer and integration elements, we were able to demonstrate expression of the anthramycin gene cluster in the heterologous host *Streptomyces lividans*.

Results and Discussion

Anthramycin biosynthetic gene cluster

The 32.5 kb anthramycin gene cluster was previously identified through whole-genome scanning of *S. refuineus* and its role in anthramycin biosynthesis has recently been validated by a series of site-directed mutagenesis and chemical complementation studies.[19] Two cosmids, 024CA and 024CO, containing unequal portions of the anthramycin biosynthetic gene cluster with a 7 kb overlap were obtained from a genomic library in a SuperCos1 (Stratagene) modified cosmid vector that contains kanamycin (*neo*) and ampicillin (*bla*) resistance genes.

Design and introduction of recombinogenic cassettes

We observed that two of the disruption cassettes typically used in PCR-targeted gene replacements, which are derived from plasmids pIJ773 and pIJ778,[15] each contain a 341 bp region of identical sequence between the origin of transfer (*oriT*) and antibiotic-resistance genes (*aac(3)IV* and *aadA*, respectively). This property was exploited to create "recombinogenic cassettes" that could, once inserted into appropriate locations in donor cosmids, provide recombination sites to assemble gene clusters into a single cosmid (Figure 1). Correspondingly, an apramycin-resistance recombinogenic cassette (hereafter termed UPAcc) was generated from plasmid pIJ773 by PCR by using primers with added XbaI and SpeI recognition sites between the apramycin-resistance gene (*aac(3)IV*) and the 39 bp flanking sequences for PCR-targeted replacement. These sites were chosen because they are not present in the anthramycin biosynthetic gene cluster. In the same manner, a streptomycin resistance recombinogenic cassette (termed DNaad) was generated from plasmid pIJ778 by using primers with a XbaI and SpeI recognition site between the streptomycin-resistance marker (*aadA*) and the 39 bp flanking sequence for

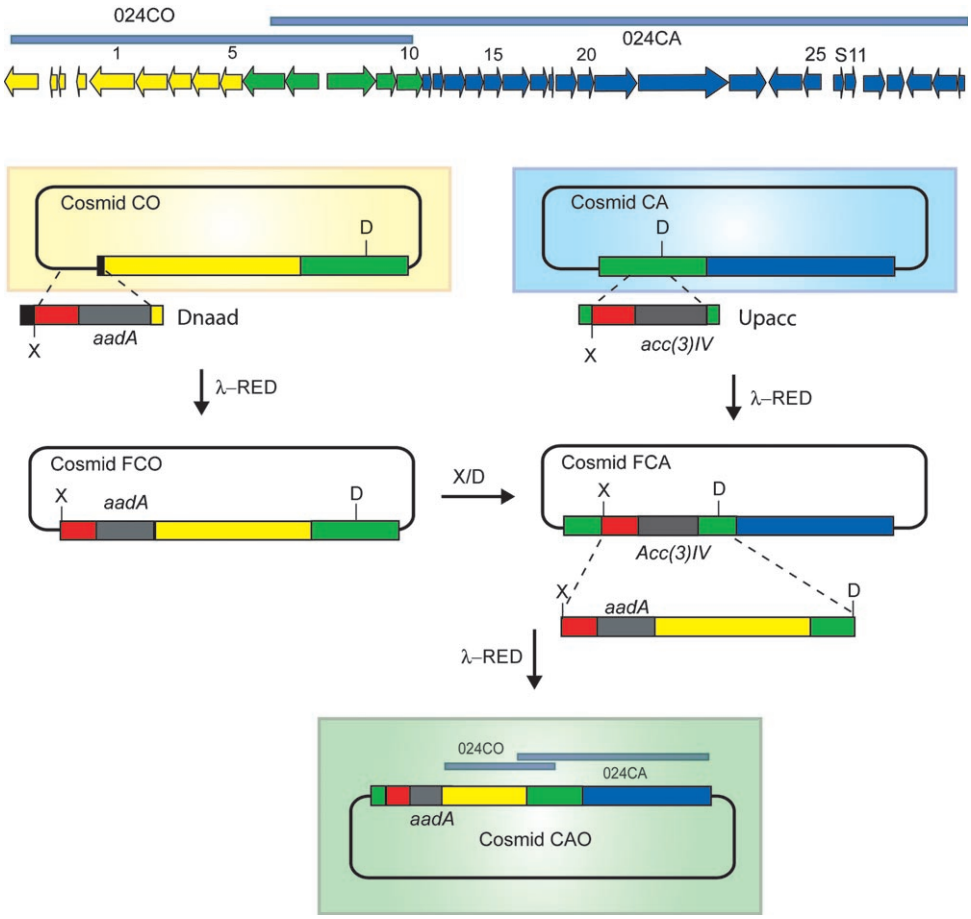


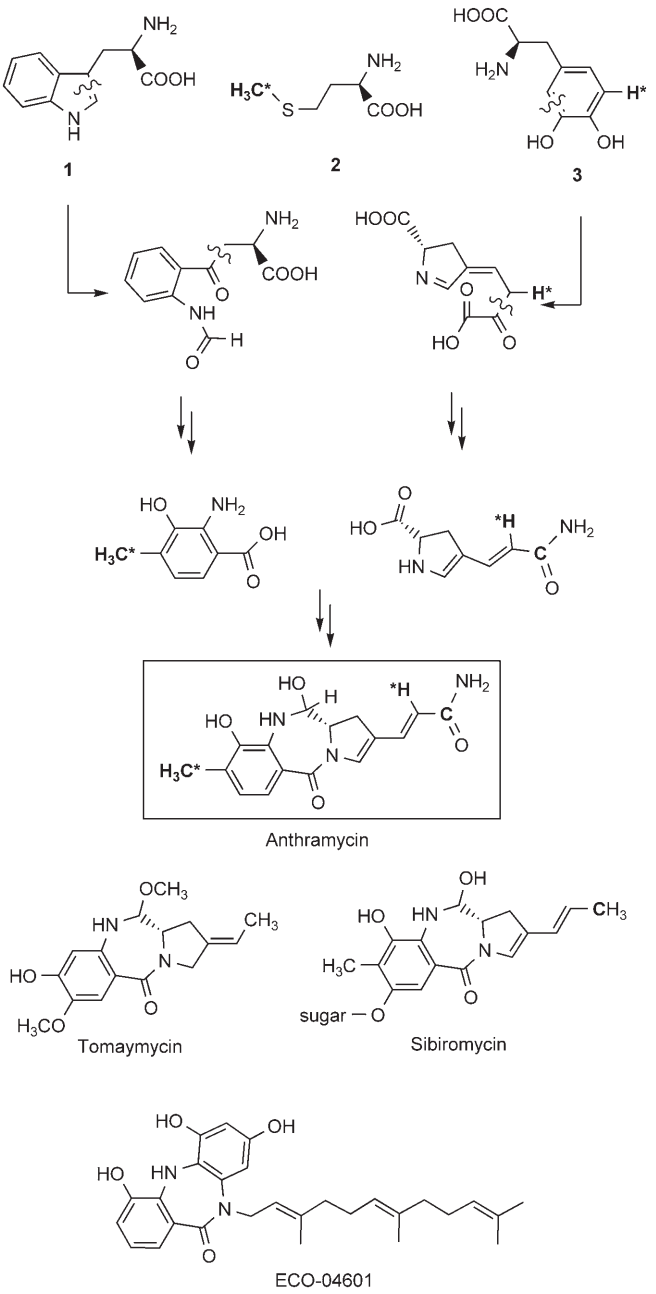
Figure 1. Fusion cosmid CAO was constructed from two donor cosmids by retrofitting each with a recombinogenic linker Dnaad and Upacc, which were generated by PCR. The red region in these linkers indicates the advantageous identical sequence found upstream of each antibiotic marker *aadA* (streptomycin) and *acc(3)IV* (apramycin). The green region in the donor cosmids represents the 7 kbp overlap between the upstream half of the cluster (yellow) and the downstream half (blue).

PCR targeting replacement. Flanking sequences were selected that targeted regions that would permit recombinant regeneration of the complete anthramycin gene cluster. In cosmid 024CA a 240 bp fragment within *orf7* of the gene cluster was targeted for replacement by the UPAcc recombinogenic cassette to yield cosmid FCA. In cosmid 024CO, a 200 bp fragment located 4425 bp upstream of *orf1* of the gene cluster was replaced by the DNaad recombinogenic cassette to yield cosmid FCO (Scheme 1).

Recombination of anthramycin biosynthetic gene cluster on a single cosmid

The linear recombinogenic cassettes UPacc and DNadd were transformed into separate *E. coli* BW25113/pIJ790 strains that contain the cosmids 024CA and 024CO, respectively. As previously stated, the sequences immediately upstream of the selectable markers are identical. Since there is only one DraI restriction site in the anthramycin gene cluster and it exists in the overlap region of the two partial anthramycin biosynthetic gene clusters, cosmid FCO was cut with XbaI and DraI to yield a 15.9 kb DNA fragment with sequences homologous to cosmid FCA, which flanked both ends. The 15.9 kb DNA fragment was gel-purified and transformed into *E. coli* BW25113/pIJ790, which contained cosmid FCA, and recombinants that contained cosmid CAO, which comprised the complete anthramycin biosynthetic gene cluster, were selected by isolating streptomycin-resistant colonies (Figure 1). PCR was used to verify the correct orientation of the inserted sequence (Scheme S1 and Table S1 in the Supporting Information). A small amount of the upstream region of the gene cluster is duplicated upstream of the introduced antibiotic-resistance marker in cosmid CAO. The sequence upstream of the first open-reading frame (*orf1*) was not known to us at the time of the primer design; this necessitated the duplication of a small amount of the gene upstream sequence. However, this duplication is a consequence of the specific design used in this case study, not the method in general.

It should be noted that this method requires the presence of a restriction site in the region of overlapping homology that is not repeated elsewhere in the donor fragment that is to be used for the final recombination step. In the 7 kbp of sequence homology shared between cosmids FCO and FCA, we identified at least six suitable restriction sites; this indicates that this requirement should not limit the usefulness of the approach.



Scheme 1. Benzodiazepine-containing natural products from bacteria can be grouped into two classes: the pyrrolo-benzodiazepines, including anthramycin, tomaymycin, and sibiromycin, which are derived from tyrosine and tryptophan, and the benzodiazepenones, such as ECO-4601, which remain uncharacterized.

Integration of the anthramycin biosynthetic gene cluster in *S. lividans* TK24

To integrate cosmid CAO into the genome of *S. lividans* TK24, it was necessary to retrofit this construct with an integrase gene (*int*), attachment site of phage c31 (*attP*), and origin of intergeneric transfer (*oriT*; Scheme S1). Correspondingly, the ampicillin-resistance gene (*bla*) in cosmid CAO was replaced by a DraI/BsaI fragment from plasmid pIJ787 with the *int* gene, *attP*, and the tetracycline-resistance gene as previously described[15] to yield cosmid CAO-2. A cassette that contained *oriT* and the apramycin-resistance gene (*aac(3)IV*) was obtained from pIJ773 by PCR and was used to replace the kanamycin-resistance gene (*neo*) of cosmid CAO-2 to yield cosmid CAO-3. Cosmid CAO-3 was transformed into *E. coli* ET12567/pUZ8002 and transferred into *S. lividans* TK24 and *S. coelicolor* M595 by intergeneric conjugation. Integration of cosmid CAO-3 was confirmed by PCR amplification of diagnostic DNA fragments from the interface of the cosmid–*aac(3)IV*/*oriT* sequence,[20,21] as well as from different sites of the anthramycin gene cluster, which

included the cluster-bracketing regions *orf1* and *orfS11*, and the internal gene *orf10* (Table S1).

Heterologous expression of the reconstructed anthramycin biosynthetic gene cluster

Anthramycin production was measured by analysis of whole-culture butanol extracts through TLC bioautography (anti-*Bacillus* activity) and HPLC–MS in triplicate fermentations. In initial experiments we assayed for the production of anthramycin by CAO-3 integrants of *S. lividans* at 30 °C but failed to observe production under these conditions. However, when *S. lividans*/COA-3 was cultivated at 47 °C, we observed levels of anthramycin production that were comparable to those seen in the natural host, *S. refuinius*, when it was grown under similar conditions (Figure 2). The optimal temperature range for anthramycin production in the heterologous host was determined to be

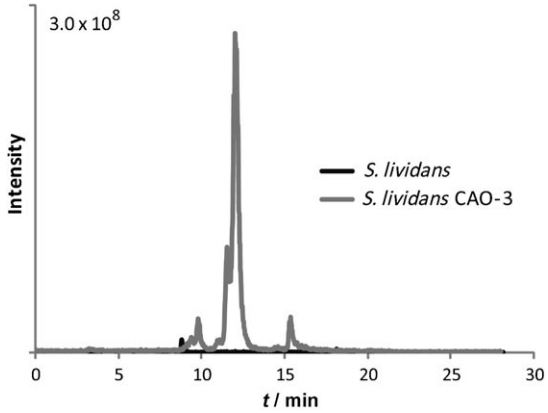


Figure 2. HPLC–MS chromatogram of anthramycin production (*m/z* 318 $[M+H]^+$) in *S. lividans*.

quite narrow; anthramycin was not detected during growth at 30, 37, or 52 °C despite robust cell growth at all temperatures, as indicated by culture biomass measurements (data not shown). These observations demonstrate that expression of the anthramycin gene cluster occurs only at elevated temperature even when transferred into a mesophilic host.

S. refuineus was isolated from an exothermically decaying compost heap and described as a "thermophilic" actinomycete based on its ability to grow at elevated temperatures (up to 55 °C[22,23]). While *S. refuineus* grows robustly at 30 °C, anthramycin production by this organism is strictly temperature dependent and occurs only at temperatures between 47–50 °C. *S. lividans* is commonly classified as a mesophilic actinomycete because it reportedly grows optimally at temperatures between 26–30 °C.[24,25] Our observation that cultures of *S. lividans* grow well at 47 °C suggests that actinomycetes might be more broadly thermotolerant than previously believed. Furthermore, the strict dependence of anthramycin production on elevated temperature in the heterologous *S. lividans* host suggests that higher growth temperatures might be required for the functional expression of the anthramycin biosynthetic pathway. Further work will be required to determine the factors responsible for the temperature-dependent expression of this pathway.

Conclusions

Large natural product gene clusters generally need to be reassembled from multiple clones that are isolated from a genomic library. Here, we demonstrate a recombination-based method to reassemble gene clusters that has advantages over prior methods. The recently reported method of Binz et al. employs a similar, though methodologically distinct strategy. The primary difference is that the method described here retrofits one of the donor cosmids in the overlap region with a cassette that is removed during the joining step. We used this method to reconstruct the anthramycin gene cluster and demonstrated that its expression remains strictly dependent on high temperatures, even when transferred into a mesophilic host. This work provides tools that should be generally useful in reconstructing large gene clusters, and it also provides an alternative system to engineer the anthramycin pathway and investigate the connection between anthramycin production and growth at elevated temperatures.

Experimental Section

DNA isolation/manipulation, cosmid preparation and gel electrophoresis were conducted according to standard methods.[26] Cosmid DNA was isolated and purified from *E. coli* strains by using plasmid miniprep and gel extraction kits (Qiagen). Genomic DNA from *Streptomyces* strains was isolated by using the Wizard™ genomic DNA purification kit (Promega). Primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA). All transformations in *E. coli* were performed by electroporation by using a GenePulser electroporator (Biorad) with a 100 V, 30 ms pulse. Intergeneric conjugation was performed according to Kieser et al.[6] with modifications as described below. The anthramycin gene cluster was deposited in GenBank under accession number EU195114.

Bacterial strains and media: Targeted gene replacement experiments were performed in *E. coli* strain BW25113 that contained the plasmid pIJ790.[16] *E. coli* ET12567 that contained the RP4 derivative pUZ8002 was used for intergeneric conjugation between *E. coli* and *Streptomyces*.[27] The heterologous expression host was *Streptomyces lividans* strain TK24.[28] *Bacillus* sp. TA (NRRL B-3167) was used as an indicator strain to test the activity of anthramycin.

E. coli strains were maintained in LB medium[26] that contained the appropriate antibiotics for selection. *S. refuineus* was grown in SRS medium (0.5% N-Z Amine B, 0.2% yeast extract, 0.2% soytone, 1.0% potato starch, 0.5% mannitol, 0.015% $FeSO_4$, 2% agar, pH 7.0) and anthramycin production was performed in production medium AP1 (1% corn starch, 2% peptonized milk, 0.3% yeast extract, pH 7.0).[29] AS1 medium (0.1% yeast extract, 0.02% L-alanine, 0.02% L-arginine, 0.05% L-asparagine, 0.5% starch, 0.25% NaCl, 1% Na_2SO_4, 2% agar) and MS medium (2% mannitol, 2% soya, 2% flour agar)[6] were used for conjugation.

Retrofitting donor cosmids with recombination cassettes: The apramycin-resistance gene (*aac(3)IV*) cassette was amplified by PCR from plasmid pIJ773 by using primers UPaacF/UPaacR (Table 1) and transformed into *E. coli* BW25113/pIJ790/CA. Transformants were

Table 1. PCR-targeting primers.

Primer	Sequence[a]
UPaccF	5′-ACCGAGCCGATCGGGTAGAGCACCGCACCGTAGCGGTCGC**TCTAGA**GCTGACGCCGTTGGATAC-3′
UPaccR	5′-CCCTGGTACGACGTGTGGCTCCCCGGGTCCGCCGTGGAGG**ACTAGT**GGAATAGGAACTTATGAGC-3′
DNaddF	5′-CACGTGCGGCCGCACGAGGGCGAGTCGGCCGCCGCGGAGG**TCTAGA**GCTGACGCCGTTGGATAC-3′
DNaddR	5′-GCGCCACCGTCACGCTCTCCACCCGCAGCCCCGGCACCAT**ACTAGT**GCGGCATCTTATTTGCCGAC-3′
ATF	5′-TCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCGGAATAGGAACTTATGAGC-3′
ATR	5′-GCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCGCAAGTTCCCGCCAGCCTC-3′

[a] Restriction sites are in bold; the homologous targeting sequence is underlined.

selected for resistance to apramycin at 30 μg mL^{-1}. The resulting cosmid FCA, in which a 240 bp DNA fragment in the anthramycin biosynthetic gene ORF7 was exchanged with PCR-targeted recombination, was purified by gel electrophoresis. A 200 bp DNA fragment that was 4425 bp upstream of the anthramycin biosynthetic cluster on cosmid CO was exchanged in a similar fashion. In this case, the streptomycin-resistance gene (*aadA*) cassette was amplified from plasmid pIJ778 with primers DNaadF/DNaadR and transformed into *E. coli* BW25113/pIJ790/CO. Transformants that were resistant to streptomycin (30 μg mL^{-1}) contained cosmid FCO, which was subsequently purified by gel electrophoresis. The insertion of recombinogenic cassettes was verified by PCR (Scheme S1 and Table S1).

Reconstruction of the complete anthramycin pathway: Cosmid FCO was restricted with XbaI and DraI and the resulting gel-purified 15.9 kb fragment was transformed into *E. coli* BW25113/pIJ790/024FCA by electroporation. Transformants that contained the resulting cosmid CAO, which contained a 15.9 kb fragment that was exchanged into cosmid FCA were selected by resistance to streptomycin (30 μg mL^{-1}). Cosmid CAO was purified by gel electrophoresis and confirmed by PCR with primers TFCA-F1/DNaddR (Table S1).

Retrofitting fusion cosmid with integrase and origin of transfer: Insertion of origin of transfer (*oriT*) and integrase (*int*) genes into the reconstructed cosmid was performed according to established protocols[15] with minor modifications. Plasmid pIJ787 was digested with DraI and BsaI, and the resulting 5 kb DNA fragment, which contained the integrase cassette flanked by approximately 100 bp of *bla* sequence upstream and 300 bp of *bla* sequence downstream of the integrase cassette, was gel purified and transformed into *E. coli* BW25113/pIJ790/CAO. Selection for ampicillin resistant (50 μg mL^{-1}) clones resulted in strains that contained cosmid CAO-2. The apramycin-resistance/origin of transfer gene (*aac(3)IV/oriT*) cassette was amplified from plasmid pIJ773 with primers ATF/ATR (Table 1). The resulting gel-purified PCR product was transformed into *E. coli* BW25113/pIJ790/CAO-2, so that apramycin resistance could replace the kanamycin gene in cosmid CAO-2 with *aac(3)IV/oriT*. The resulting cosmid 024CAO-3 was purified and transformed into *E. coli* ET12567/pUZ8002.

Heterologous expression of the anthramycin cluster: Cosmid CAO-3 was transformed into *Streptomyces lividans* TK24 by conjugation with *E. coli* ET12567/pUZ8002/024CAO-3. Transconjugants were selected on MS or AS-1 medium (50 μg mL^{-1} apramycin) and exconjugates were verified by amplifying isolated genomic DNA with four sets of primers on bracketing ends of the gene cluster and in the cosmid sequence (Table S1). A *Streptomyces lividans* TK24 integrant that harbored cosmid CAO-3 was cultured in AP1 seed medium (50 mL) at 47 °C for 24 h. A 5% inoculum was then added to production medium AP1 (50 mL) and incubated at 47 °C for 24 h. Anthramycin was extracted from the production medium with butanol (50 mL). Butanol fractions were dried over anhydrous $MgSO_4$, concentrated in vacuo, and redissolved in MeOH directly prior to analysis.

Assays for anthramycin: Antibacterial activity of anthramycin was detected by thin-layer chromatography bioautography and HPLC–MS. Anthramycin (dissolved in MeOH) was chromatographed on 25DC-Alufolien kieselgel plates (Merck) that were eluted with MeOH/$CHCl_3$ (1:9) and then visualized by bioautography. LB agar (~55 °C) that was inoculated with indicator strain *Bacillus* sp. TA was poured on TLC plates and cultured at 37 °C for 20 h to detect anti-*Bacillus* activity of anthramycin. Anthramycin production was further confirmed by HPLC–MS analysis by using a Jupiter™ minibore C-18 column (2.0 mm × 15 cm; Phenomenex, Torrance, CA, USA) with a linear water/acetonitrile gradient (H_2O/CH_3CN, 95:5 to 5:95) that contained NH_4OAc (10 mM) at 0.2 mL min^{-1} flow rate. Mass spectrometry was performed by using ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source that was outfitted with a deactivated fused Si capillary (100 μm i.d.). The injection volume was 10 μL. Mass spectrometery was performed in the positive and negative-ion mode, and the electrospray needle was maintained at 4200 V. The ion transfer tube was operated at 35 V and 342 °C (−35 V and 300 °C for negative). The tube lens voltage was set to 85 V (−220 V for negative). Source CID (offset voltage between skimmer and the first ion guide, Q00) was used at 15 V.

Acknowledgements

This work was financially supported by the Vanderbilt Institute for Chemical Biology. The cosmids that contained the biosynthetic gene cluster were isolated, sequenced, and initially annotated by Ecopia Biosciences Inc. (now Thallion Pharmaceuticals Inc.). All gene manipulations, expression, and analysis were envisioned and executed by the Vanderbilt team.

Keywords: anthramycin · benzodiazepine · gene expression · natural products · *Streptomyces*

[1] F. Karray, E. Darbon, N. Oestreicher, H. Dominguez, K. Tuphile, J. Gagnat, M. H. Blondelet-Rouault, C. Gerbaud, J. L. Pernodet, *Microbiology* **2007**, *153*, 4111–4122.
[2] D. G. Fujimori, S. Hrvatin, C. S. Neumann, M. Strieker, M. A. Marahiel, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16498–16503.
[3] S. G. Van Lanen, T. J. Oh, W. Liu, E. Wendt-Pienkowski, B. Shen, *J. Am. Chem. Soc.* **2007**, *129*, 13082–13094.
[4] J. Ahlert, E. Shepard, N. Lomovskaya, E. Zazopoulos, A. Staffa, B. O. Bachmann, K. Huang, L. Fonstein, A. Czisny, R. E. Whitwam, C. M. Farnet, J. S. Thorson, *Science* **2002**, *297*, 1173–1176.
[5] J. Handelsman, *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 669–685.
[6] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, **2000**.
[7] K. Narayanan, R. Williamson, Y. Zhang, A. F. Stewart, P. A. Ioannou, *Gene Ther.* **1999**, *6*, 442–447.

[8] S. C. Wenzel, F. Gross, Y. Zhang, J. Fu, A. F. Stewart, R. Muller, *Chem. Biol.* **2005**, *12*, 349–356.
[9] F. Hill, V. Benes, D. Thomasova, A. F. Stewart, F. C. Kafatos, W. Ansorge, *Genomics* **2000**, *64*, 111–113.
[10] Y. Zhang, F. Buchholz, J. P. P. Muyrers, A. F. Stewart, *Nat. Genet.* **1998**, *20*, 123–128.
[11] Y. Zhang, J. P. Muyrers, G. Testa, A. F. Stewart, *Nat. Biotechnol.* **2000**, *18*, 1314–1317.
[12] B. Gust, G. Chandra, D. Jakimowicz, T. Yuqing, C. J. Bruton, K. F. Chater, *Adv. Appl. Microbiol.* **2004**, *54*, 107–128.
[13] A. Raynal, F. Karray, K. Tuphile, E. Darbon-Rongere, J. L. Pernodet, *Appl. Environ. Microbiol.* **2006**, *72*, 4839–4844.
[14] O. Perlova, J. Fu, S. Kuhlmann, D. Krug, A. F. Stewart, Y. Zhang, R. Muller, *Appl. Environ. Microbiol.* **2006**, *72*, 7485–7494.
[15] A. S. Eustaquio, B. Gust, U. Galm, S. M. Li, K. F. Chater, L. Heide, *Appl. Environ. Microbiol.* **2005**, *71*, 2452–2459.
[16] B. Gust, G. L. Challis, K. Fowler, T. Kieser, K. F. Chater, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1541–1546.
[17] T. M. Binz, S. C. Wenzel, H.-J. Schnell, A. Bechthold, R. Müller, *ChemBioChem* **2008**, *9*, 447–454.
[18] W. Leimgruber, V. Stefanovic, F. Schenker, A. Karr, J. Berger, *J. Am. Chem. Soc.* **1965**, *87*, 5791–5793.
[19] Y. Hu, V. Phelan, I. Ntai, C. M. Farnet, E. Zazopoulos, B. O. Bachmann, *Chem. Biol.* **2007**, *14*, 691–701.
[20] K. Ichinose, M. Ozawa, K. Itou, K. Kunieda, Y. Ebizuka, *Microbiology* **2003**, *149*, 1633–1645.
[21] R. Zirkle, J. M. Ligon, I. Molnar, *Microbiology* **2004**, *150*, 2761–2774.
[22] C. M. Farnet, A. Staffa, *Vol. US2003/0077767*, United States, **2003**.
[23] C. Gairola, L. Hurley, *Eur. J. Appl. Microbiol.* **1976**, *2*, 95–101.
[24] D. A. Hopwood, H. M. Wright, *Mol. Gen. Genet.* **1978**, *162*, 307–317.
[25] M. J. Bibb, S. N. Cohen, *Mol. Gen. Genet.* **1982**, *187*, 265–277.
[26] J. Sambrook, T. M. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, **1989**.
[27] D. J. MacNeil, K. M. Gewain, C. L. Ruby, G. Dezeny, P. H. Gibbons, T. MacNeil, *Gene* **1992**, *111*, 61–68.
[28] Z. M. Wang, B. H. Bleakley, D. L. Crawford, G. Hertel, F. Rafii, *J. Biotechnol.* **1990**, *13*, 131–144.
[29] L. H. Hurley, M. Zmijewski, C. J. Chang, *J. Am. Chem. Soc.* **1975**, *97*, 4372–4378.

Received: January 17, 2008
Published online on June 2, 2008

