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Tetarimycin A, an MRSA-Active Antibiotic Identified through Induced Expression of Environmental DNA Gene Clusters

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Supporting Information

ABSTRACT: The propagation of DNA extracted directly from environmental samples in laboratory-grown bacteria provides a means to study natural products encoded in the genomes of uncultured bacteria. However, gene silencing often hampers the functional characterization of gene clusters captured on environmental DNA clones. Here we show that the overexpression of transcription factors found in sequenced environmental DNA-derived biosynthetic gene clusters, in conjunction with traditional culture-broth extract screening, can be used to identify new bioactive secondary metabolites from otherwise-silent gene clusters. Tetarimycin A, a tetracyclic methicillin-resistant *Staphylococcus aureus* (MRSA)-active antibiotic, was isolated from the culture-broth extract of *Streptomyces albus* cultures cotransformed with an environmentally derived type-II polyketide biosynthetic gene cluster and its pathway-specific *Streptomyces* antibiotic regulatory protein (SARP) cloned under the control of the constitutive ermE* promoter.

Most bacteria present in the environment remain recalcitrant to culturing using methods that are easily compatible with natural product discovery programs.[1−6] The cloning of DNA extracted from environmental samples provides a means of studying biosynthetic gene clusters found in the genomes of these environmental bacteria.[7,8] Although it is easy to clone large numbers of novel biosynthetic gene clusters directly from the environment, these clusters often remain functionally silent in existing heterologous expression models. A similar phenomenon has been reported in culture-based studies, where large numbers of cryptic or silent biosynthetic gene clusters are often found in the genomes of even well-characterized model prokaryotes.[9] We have explored the possibility that the systematic overexpression of transcription factors found in sequenced environmental DNA (eDNA)-derived gene clusters, in conjunction with traditional culture-broth extract screening, could be used to identify novel bioactive secondary metabolites from otherwise-silent gene clusters. Here we describe the isolation and characterization of the tetarimycins (Figure 1) from an antibacterially active culture-broth extract identified in our initial effort to use this screening strategy to identify novel bioactive natural products. Tetarimycin A (**1**) is an antibiotic with activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

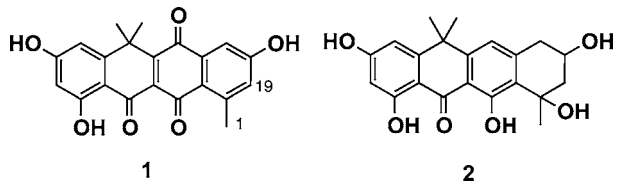

Figure 1. Tetarimycin A (**1**) and B (**2**).

A structurally diverse collection of aromatic metabolites, including many antimicrobial and anticancer agents, arises from type-II (iterative) polyketide synthases (PKSs). While the gene clusters that encode for the biosyntheses of these molecules are very different in their details, they all contain a conserved minimal PKS composed of two ketosynthases (KS_α and KS_β) and an acyl carrier protein (ACP).[10,11] Using degenerate primers designed to recognize conserved regions in the minimal PKS, we recovered a large collection of eDNA clones containing type-II minimal PKS systems. Our initial functional analyses of these clones led to the discovery of a number of metabolites with either new or rare carbon skeletons.[12] Full sequencing of the remaining clones in this collection revealed highly diverse biosynthetic systems that unfortunately remained silent in our initial heterologous expression studies.

Natural product biosynthetic gene clusters are often tightly regulated by both positive- and negative-acting transcription factors, resulting in the silencing of gene clusters in the laboratory setting.[13] In an effort to identify metabolites encoded by silent eDNA-derived gene clusters, sequenced minimal-PKS-containing clones were screened for genes predicted to encode transcription factors. These genes were then cloned into an integrative conjugative expression plasmid downstream of the strong, constitutive ermE* promoter (Figure 2a). Each recombinant expression plasmid was introduced into *Streptomyces albus* harboring its corresponding minimal-PKS-containing clone, and culture-broth extracts from the resulting cotransformants were screened for activity against prokaryotic and eukaryotic cell lines.

A culture-broth extract exhibiting activity against MRSA was selected for further analysis (Table 1). The eDNA cosmid clone used in this culture, AZ60 (GenBank accession code JX843821), was originally recovered from a cosmid library containing DNA isolated from Arizona desert soil. Reversed-phase HPLC analysis of the active extract identified a set of

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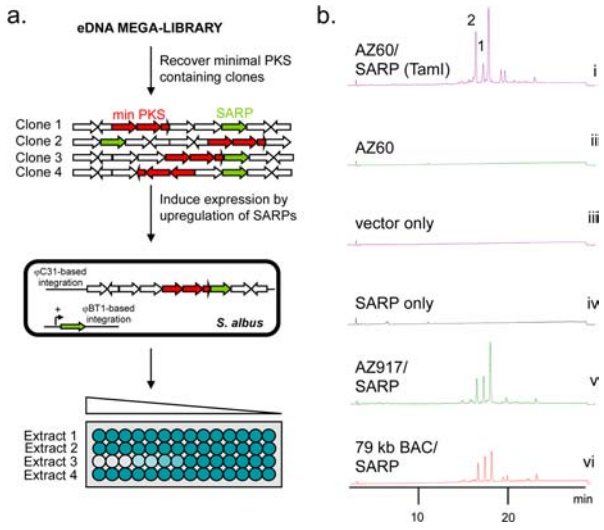

Figure 2. (a) Induced expression screening. Positive-acting SARP transcription factors found in minimal-PKS-containing eDNA clones were cloned under the control of the ermE* promoter. Extracts from *S. albus* cultures transformed with this construct and the corresponding minimal-PKS-containing clone were screened for bioactivities. (b) HPLC traces of *S. albus* culture-broth extracts. *S. albus* was transformed with (i) AZ60 and the TamI expression construct, (ii) AZ60 alone, (iii) an empty cosmid vector, (iv) the TamI expression construct alone, (v) AZ917 and the TamI expression construct, and (vi) a 79 kb BAC and the TamI expression construct. The utilization of compatible φC31 (cosmid vector)- and φBT1 (ermE* expression vector)-based integrative cloning systems allowed for the cointegration of both a biosynthetic gene cluster and a corresponding induced transcriptional activator into two distinct chromosomal sites in *S. albus*, resulting in the successful activation of previously silent eDNA gene clusters.

Table 1. Minimum Inhibitory Concentrations (μg/mL) for the Tetarimycins against a Panel of Bacterial Pathogens and Yeast[a]

	E. coli	*S. aureus* 6538P	*S. aureus* USA300 MRSA	*E. faecalis* EF16 VRE	yeast
crude	>25	1.5	6.25	25	>50
1	>25	0.39	0.78	3.125	>50
2	>25	25	>25	25	>50
apramycin	1.5	6.25	3.125	N/A	N/A
ampicillin	N/A	0.78	6.125	25	N/A

[a]Abbreviations: N/A = not assayed; VRE = vancomycin-resistant enterococci; yeast = *Saccharomyces cerevisiae* W303; crude = AZ60/TamI coexpression crude extract.

metabolites whose production was dependent on the presence of AZ60 as well as the constitutive expression of a *Streptomyces* antibiotic regulatory protein (SARP)-like transcription factor gene, *tamI*, found on this clone [Figure 2b(i–iv)]. TamI belongs to the ATPase subfamily of SARPs that have a nucleotide binding domain in addition to a helix–turn–helix (HTH) domain and a transcriptional activation (BTAD) domain [Figure 3a and Figure S2 in the Supporting Information (SI)].[14,15] Although the actual function of the ATP binding domain is unclear, its presence correlates with the modulation of DNA binding and transcriptional activation.[16] A predicted SARP binding site composed of an almost-perfect direct heptameric repeat is located just upstream of the −10 region within the promoter of the ABC transporter gene *tamA*

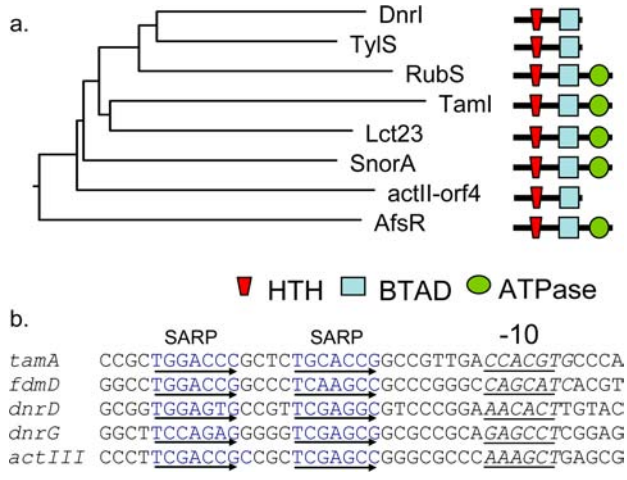

Figure 3. (a) Phylogenetic tree of SARP HTH–BTAD didomain sequences. (b) Comparsion of the conserved SARP binding sequence found in AZ60 with SARP recognition sequences from known biosynthetic gene clusters (*fdm*, fredericamycin; *dnr*, daunorubicin; *act*, actinorhodin).

(Figure 3b). Reverse-transcription polymerase chain reaction (RT-PCR)-based analyses using PCR primers designed to recognize *tamKLM* complementary DNAs confirmed a dramatic *tamI*-expression-dependent increase in Tam gene expression (Figure S3 in the SI).

Additional eDNA clones overlapping each end of AZ60 were recovered from the Arizona soil eDNA library in which AZ60 was originally found and then used to reconstruct a larger 79 kb continuous fragment of eDNA by transformation-assisted recombination in yeast (see the SI).[17] When the bacterial artificial chromosome (BAC) containing this larger fragment was conjugated into *S. albus*, it conferred the production of the same metabolites to the host [Figure 2b(vi)], indicating that the full tetarimycin, or *tam*, gene cluster is present in AZ60. A second clone recovered from the same Arizona library, clone AZ917, which overlaps the 5′ end of AZ60 starting at open reading frame (ORF) 10, also conferred the production of the tetarimycins to *S. albus* [Figure 2b(v)], indicating that the tam gene cluster is found in its entirety within the terminal 25 kb of clone AZ60. The 5′ end of this 25 kb region is predicted to encode a phenazine biosynthetic gene cluster (Table S2 in the SI), while the 3′ 18 kb contains a collection of type-II PKS-related biosynthesis genes (Figure 4a) that we have identified as the *tam* gene cluster.

Bioassay-guided fractionation of the *S. albus* AZ60/SARP culture-broth extract yielded a single *tamI*-expression-dependent, antibacterially active metabolite, tetarimycin A (**1**). Tetarimycin A is a Gram-positive-specific antibiotic with potent activity against MRSA. The structures of **1** and a major inactive metabolite, tetarimycin B (**2**), were elucidated using a combination of high-resolution mass spectrometry (HRMS) and NMR data (Figure 5). The structure of **1** was also subsequently confirmed by single-crystal X-ray diffraction analysis (data from which were deposited with the Cambridge Crystallographic Data Centre under accession number CCDC 902189) (Figure 5). Both compounds are novel tetracyclic natural products.

The biosynthesis of the tetarimycins can be rationalized on the basis of the predicted gene functions of the *tam* genes (Figure 4b). In our proposed biosynthetic scheme, the minimal

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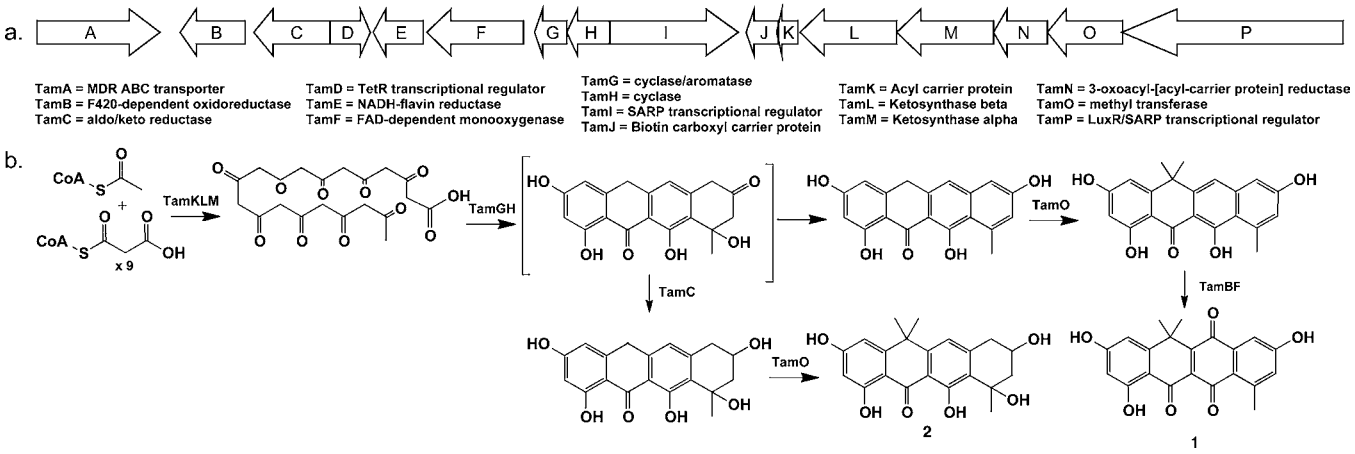

Figure 4. (a) Tam gene cluster and general function prediction for the *tam* genes. (b) Proposed biosynthetic scheme for the tetarimycins.

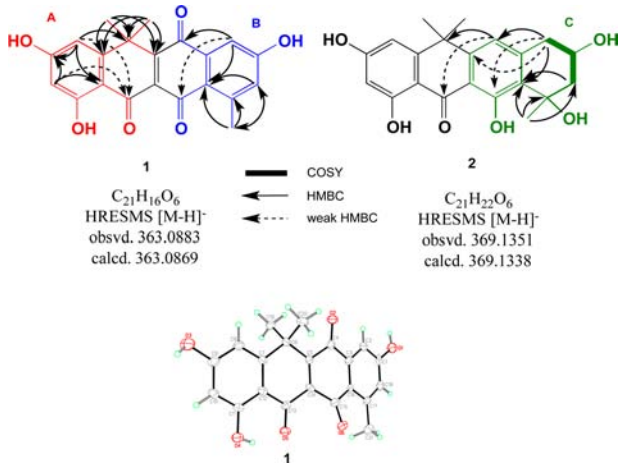

Figure 5. (top) HRMS data and key NMR correlations used to define the structures of **1** and **2**. (bottom) X-ray structure of **1** (gray = carbon, red = oxygen, green = hydrogen).

PKS (TamKLM) generates a decaketide that undergoes an initial TamG-dependent 9,14 cyclization. The TamG cyclase/aromatase shows high sequence identity to the ElmNI and FdmI cyclases from elloromycin and fredericamycin biosynthesis, respectively, both of which carry out similar initial cyclizations of nonreduced polyketide precursors.[18,19] TamH is predicted to be a second/third-ring cyclase on the basis of its high sequence similarity to the analogous cyclases from the biosyntheses of the anthracyclines steffimycin (StfY) and doxorubicin (DpsY).[20,21] Whether the fourth ring is also formed by TamH or formed spontaneously is not clear. In our proposal, the oxidation of the resulting aromatic tetracyclic intermediate by one or both of the predicted oxidoreductases TamF and TamB yields a quinone that upon methylation by TamO gives **1**. Reduction of the tetracyclic intermediate by the aldo/ketoreductase TamC followed by methylation by TamO would give **2** instead.

TamO is a 37 KDa putative *S*-adenosylmethionine (SAM)-dependent methyl transferase. In benastatin biosynthesis, a single SAM-dependent methyl transferase (BenF) has been shown to carry out the addition of two methyl groups on a similarly activated benzylic carbon to yield a *gem*-dimethyl functional group.[22] The *gem*-dimethyl moiety seen in the fasamycins is also thought to arise from the action of a single SAM-dependent methyl transferase.[23] TamO is therefore predicted to install both methyl groups of the *gem*-dimethyl functionality seen in the tetarimycins.

The anticancer antibiotics tetracenomycin and elloramycin arise from the same decaketide cyclization scheme that is predicted to give rise to the tetarimycins (Figures 4b and 6);

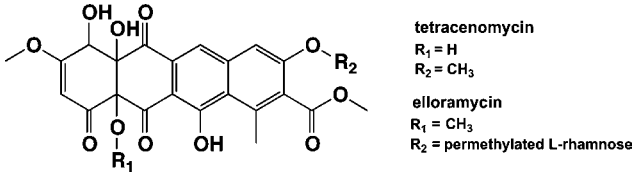

Figure 6. Decaketides with the same tetracyclic cyclization pattern as in the tetarimycins.

however, as a result of the *gem*-dimethyl functionality on the B ring of the tetarimycins and the absence of the C-19 ester, the tetarimycins represent a new carbon skeleton within the rare tetracenomycin family of aromatic polyketides.[24]

To assess whether there might be additional gene clusters that encode for tetarimycin relatives in the environment, we screened three archived soil eDNA libraries for sequences showing high identity to *tamL*. For aromatic polyketide biosynthesis, our lab and others have shown that gene clusters encoding metabolites within the same general family often contain closely related minimal PKS gene sequences (>85% identity).[25] In just this small sample of environmental biosynthetic diversity, we identified a KSα sequence that is 89% identical to *tamL* (Figure S1). This suggests that even though no members of the tetarimycin family have been identified in culture-based studies, there are likely to be numerous *tam*-like gene clusters in the environment that encode other members of this new class of antibiotics.

The cloning of natural product biosynthetic gene clusters from the environment is now routine; however, these gene clusters often remain silent under laboratory growth conditions, rendering them useless as sources of novel metabolites for high-throughput screening programs. With the extraordinary biosynthetic diversity known to be present in soil microbiomes and the continued rapid reduction in sequencing costs, directed gene cluster induction strategies such as the one outlined here are amenable to high-throughput formatting, which should permit the screening of culture-broth extracts derived from

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large collections of novel activated eDNA-derived gene clusters in diverse biomedically relevant assays.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra, additional methods, RT-PCR data, and gene tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Torsvik, V.; Ovreas, L.; Thingstad, T. F. *Science* **2002**, *296*, 1064−6.
(2) Ekkers, D. M.; Cretoiu, M. S.; Kielak, A. M.; Elsas, J. D. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 1005−20.
(3) Pham, V. H.; Kim, J. *Trends Biotechnol* **2012**, *30*, 475−84.
(4) Simon, C.; Daniel, R. *Appl. Environ. Microbiol.* **2011**, *77*, 1153−61.
(5) Stewart, E. J. *J. Bacteriol.* **2012**, *194*, 4151−60.
(6) Vartoukian, S. R.; Palmer, R. M.; Wade, W. G. *FEMS Microbiol. Lett.* **2010**, *309*, 1−7.
(7) Brady, S. F.; Simmons, L.; Kim, J. H.; Schmidt, E. W. *Nat. Prod. Rep.* **2009**, *26*, 1488−503.
(8) Lewis, K.; Epstein, S.; D'Onofrio, A.; Ling, L. L. *J. Antibiot.* **2010**, *63*, 468−76.
(9) Challis, G. L. *Microbiology* **2008**, *154*, 1555−69.
(10) Hertweck, C. *Angew. Chem., Int. Ed.* **2009**, *48*, 4688−716.
(11) Rawlings, B. J. *Nat. Prod. Rep.* **1999**, *16*, 425−84.
(12) Feng, Z.; Kallifidas, D.; Brady, S. F. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 12629−34.
(13) Bibb, M. J. *Curr. Opin. Microbiol.* **2005**, *8*, 208−15.
(14) Osbourn, A. *Trends Genet.* **2010**, *26*, 449−57.
(15) Wietzorrek, A.; Bibb, M. *Mol. Microbiol.* **1997**, *25*, 1181−4.
(16) Tanaka, A.; Takano, Y.; Ohnishi, Y.; Horinouchi, S. *J. Mol. Biol.* **2007**, *369*, 322−33.
(17) Kim, J. H.; Feng, Z.; Bauer, J. D.; Kallifidas, D.; Calle, P. Y.; Brady, S. F. *Biopolymers* **2010**, *93*, 833−44.
(18) Rafanan, E. R., Jr.; Le, L.; Zhao, L.; Decker, H.; Shen, B. *J. Nat. Prod.* **2001**, *64*, 444−9.
(19) Wendt-Pienkowski, E.; Huang, Y.; Zhang, J.; Li, B.; Jiang, H.; Kwon, H.; Hutchinson, C. R.; Shen, B. *J. Am. Chem. Soc.* **2005**, *127*, 16442−52.
(20) Gullon, S.; Olano, C.; Abdelfattah, M. S.; Brana, A. F.; Rohr, J.; Mendez, C.; Salas, J. A. *Appl. Environ. Microbiol.* **2006**, *72*, 4172−83.
(21) Lomovskaya, N.; Doi-Katayama, Y.; Filippini, S.; Nastro, C.; Fonstein, L.; Gallo, M.; Colombo, A. L.; Hutchinson, C. R. *J. Bacteriol.* **1998**, *180*, 2379−86.
(22) Schenk, A.; Xu, Z.; Pfeiffer, C.; Steinbeck, C.; Hertweck, C. *Angew. Chem., Int. Ed.* **2007**, *46*, 7035−8.
(23) Feng, Z.; Chakraborty, D.; Dewell, S. B.; Reddy, B. V.; Brady, S. F. *J. Am. Chem. Soc.* **2012**, *134*, 2981−7.
(24) Ames, B. D.; Korman, T. P.; Zhang, W.; Smith, P.; Vu, T.; Tang, Y.; Tsai, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5349−54.
(25) Reddy, B. V.; Kallifidas, D.; Kim, J. H.; Charlop-Powers, Z.; Feng, Z.; Brady, S. F. *Appl. Environ. Microbiol.* **2012**, *78*, 3744−52.