Published in final edited form as:

J Am Chem Soc. 2012 December 5; 134(48): 19552–19555. doi:10.1021/ja3093828.

Tetarimycin A, an MRSA active antibiotic identified through induced expression of environmental DNA gene clusters

Dimitris Kallifidas, Hahk-Soo Kang, and Sean F. Brady

Laboratory of Genetically Encoded Small Molecules, The Rockefeller University, Howard Hughes Medical Institute, 1230 York Avenue, New York, NY 10065

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. Gene silencing however, often hampers functional characterization of gene clusters captured on environmental DNA clones. Here we show that the over-expression 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 co-transformed with an environmentally-derived type II polyketide biosynthetic gene cluster and its pathway-specific SARP (Streptomyces antibiotic regulatory protein) regulator protein 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 possible to easily 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 is 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 over-expression 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 (Fig. 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 a methicillin resistant *Staphylococcus aureus* (MRSA).

A structurally diverse collection of aromatic metabolites, including many antimicrobial and anticancer agents, arises from Type II (iterative) polyketide synthases. While the gene clusters that encode for the biosyntheses of these molecules are very different in their details, they all contain a conserved minimal polyketide synthase (PKS) that is composed of two ketosynthases (KS $_{\alpha}$ and KS $_{\beta}$) and an acyl carrier protein (ACP).^{10,11} Using degenerate primers designed to recognize conserved regions in the minimal PKS, we have recovered a

Correspondence to: Sean F. Brady.

Supporting Information Available: NMR spectra, additional methods, RT-PCR data and gene tables are available free of charge at <http://pubs.acs.org>.

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 (Fig. 2a). Each recombinant expression plasmid was introduced into *Streptomyces albus* harboring its corresponding minimal PKS containing clone, and culture broth extracts from the resulting co-transformants were screened for activity against prokaryotic and eukaryotic cell lines.

A culture broth extract exhibiting activity against methicillin resistant *Staphylococcus aureus* (MRSA) was selected for further analysis (Table 1). The eDNA cosmid clone used in this culture, AZ60 (GenBank Accession 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 metabolites whose production was dependent on the presence of AZ60 as well as the constitutive expression of a SARP (*Streptomyces* antibiotic regulatory protein)-like transcription factor gene, *tamI*, found on this clone (Fig. 2b–i to iv). TamI belongs to the ATPases subfamily of SARPs that have a nucleotide binding domain in addition to helix-turn-helix domain and a transcriptional activation (BTAD) domain (Fig. 4a and S2).^{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* (Fig. 4b). RT-PCR (reverse transcription polymerase chain reaction) based analyses using PCR primers designed to recognize *tamKLM* cDNAs confirmed a dramatic *tamI* expression dependent increase in Tam gene expression (Fig. S3).

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 (sup mat).¹⁷ When the bacterial artificial chromosome containing this larger fragment was conjugated into *S. albus* it conferred the production of the same metabolites to the host (Fig. 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 the AZ60 starting at ORF10 also confers the production of the tetarimycins to *S. albus* (Fig. 2b–v) indicating that the Tam 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 predicted phenazine biosynthetic gene cluster (Table S2), while the 3' 18 kb contains a collection of Type II PKS related biosynthesis genes (Fig. 3a), which we have called the *tam* gene cluster.

Bio-assay 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** as well as a major inactive metabolite tetarimycin B (**2**) were elucidated using a combination of HRMS and NMR data (Fig. 5). The structure of **1** was also subsequently confirmed by single crystal X-ray diffraction analysis (Cambridge Crystallographic Data

Centre under accession number CCDC 902189) (Fig. 5). Both compounds are novel tetracyclic natural products.

The biosynthesis of the tetarimycins can be rationalized based on the predicted gene functions of the *tam* genes (Fig. 3b). In our proposed biosynthetic scheme the minimal PKS (TamKLM) generates a decaketide that undergoes an initial TamG dependent 9,14 cyclization. The TamG cyclase/aromatase shows high sequence identity to ElmNI and FdmI cyclases from elloromycin and fredericamycin biosynthesis respectively, both of which carry out similar initial cyclizations of non-reduced polyketide precursors.^{18,19} TamH is a predicted second/third ring cyclase based on high sequence similarity to the analogous cyclases from the biosynthesis 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 either one or both of the predicted oxidoreductases, TamF/TamB, yields a quinone that upon methylation by TamO gives **1**. Reduction of the tetracyclic intermediate by the aldo/keto-reductase TamC prior aromatization followed by methylation by TamO would give **2** instead.

TamO is a 37 KDa putative 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 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 as that predicted to give rise to the tetarimycins (Fig. 3b and 6); however, as a result of the geminal-dimethyl functionality seen on the tetarimycin “B ring” 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 encoding 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 KS α sequence that is 89% identical to *tamL* (Fig. S1) suggesting 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, like the one outlined here are amenable to high throughput formatting which should permit the screening of culture broth extracts derived from large collections of novel activated eDNA derived gene clusters in diverse biomedically relevant assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH GM077516. SFB is an HHMI Early Career Scientist. We thank Dr. Jason Sello (Brown University, USA) for the kind gift of pIJ10257.

References

1. Torsvik V, Ovreas L, Thingstad TF. *Science*. 2002; 296:1064–6. [PubMed: 12004116]
2. Ekkers DM, Cretoiu MS, Kielak AM, Elsas JD. *Appl Microbiol Biotechnol*. 2012; 93:1005–20. [PubMed: 22189864]
3. Pham VH, Kim J. *Trends Biotechnol*. 2012; 30:475–84. [PubMed: 22770837]
4. Simon C, Daniel R. *Appl Environ Microbiol*. 2011; 77:1153–61. [PubMed: 21169428]
5. Stewart EJ. *J Bacteriol*. 2012; 194:4151–60. [PubMed: 22661685]
6. Vartoukian SR, Palmer RM, Wade WG. *FEMS Microbiol Lett*. 2010; 309:1–7. [PubMed: 20487025]
7. Brady SF, Simmons L, Kim JH, Schmidt EW. *Nat Prod Rep*. 2009; 26:1488–503. [PubMed: 19844642]
8. Lewis K, Epstein S, D'Onofrio A, Ling LL. *J Antibiot (Tokyo)*. 2010; 63:468–76. [PubMed: 20648021]
9. Challis GL. *Microbiology*. 2008; 154:1555–69. [PubMed: 18524911]
10. Hertweck C. *Angew Chem Int Ed Engl*. 2009; 48:4688–716. [PubMed: 19514004]
11. Rawlings BJ. *Nat Prod Rep*. 1999; 16:425–84. [PubMed: 10467738]
12. Feng Z, Kallifidas D, Brady SF. *Proc Natl Acad Sci U S A*. 2011; 108:12629–34. [PubMed: 21768346]
13. Bibb MJ. *Curr Opin Microbiol*. 2005; 8:208–15. [PubMed: 15802254]
14. Osbourn A. *Trends Genet*. 2010; 26:449–57. [PubMed: 20739089]
15. Wietzorrek A, Bibb M. *Mol Microbiol*. 1997; 25:1181–4. [PubMed: 9350875]
16. Tanaka A, Takano Y, Ohnishi Y, Horinouchi S. *J Mol Biol*. 2007; 369:322–33. [PubMed: 17434533]
17. Kim JH, Feng Z, Bauer JD, Kallifidas D, Calle PY, Brady SF. *Biopolymers*. 2010; 93:833–44. [PubMed: 20577994]
18. Rafanan ER Jr, Le L, Zhao L, Decker H, Shen B. *J Nat Prod*. 2001; 64:444–9. [PubMed: 11325225]
19. Wendt-Pienkowski E, Huang Y, Zhang J, Li B, Jiang H, Kwon H, Hutchinson CR, Shen B. *J Am Chem Soc*. 2005; 127:16442–52. [PubMed: 16305230]
20. Gullon S, Olano C, Abdelfattah MS, Brana AF, Rohr J, Mendez C, Salas JA. *Appl Environ Microbiol*. 2006; 72:4172–83. [PubMed: 16751529]
21. Lomovskaya N, Doi-Katayama Y, Filippini S, Nastro C, Fonstein L, Gallo M, Colombo AL, Hutchinson CR. *J Bacteriol*. 1998; 180:2379–86. [PubMed: 9573189]
22. Schenk A, Xu Z, Pfeiffer C, Steinbeck C, Hertweck C. *Angew Chem Int Ed Engl*. 2007; 46:7035–8. [PubMed: 17691088]
23. Feng Z, Chakraborty D, Dewell SB, Reddy BV, Brady SF. *J Am Chem Soc*. 2012; 134:2981–7. [PubMed: 22224500]
24. Ames BD, Korman TP, Zhang W, Smith P, Vu T, Tang Y, Tsai SC. *Proc Natl Acad Sci U S A*. 2008; 105:5349–54. [PubMed: 18388203]
25. Reddy BV, Kallifidas D, Kim JH, Charlop-Powers Z, Feng Z, Brady SF. *Appl Environ Microbiol*. 2012; 78:3744–52. [PubMed: 22427492]

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

Figure 2.

(a) Induced expression screening. Positively 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* is 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, (vi) a 79 kb BAC and the TamI expression construct. The utilization of compatible ϕ C31 (cosmid vector) and ϕ BT1 (*ermE** expression vector) -based integrative cloning systems, allows for the co-integration 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.

Figure 3.

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

Figure 4.

(a) Phylogenetic tree of SARP HTH-BTAD di-domain sequences. (b) Conserved SARP binding sequence found in AZ60 compared to SARP recognition sequences from known biosynthetic gene clusters. *fdm*, fredericamycin; *dnr*, daunorubicin; *act*, actinorhodin.

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

Table 1

Minimum inhibitory concentrations ($\mu\text{g/ml}$) for the tetarimycins against a panel of bacterial pathogens and yeast.

	<i>E. coli</i>	<i>S. aureus</i> 6538P	<i>S. aureus</i> USA 300 -MRSA	<i>E. faecalis</i> 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

N/A = not assayed. VRE: vancomycin resistant enterococci, Yeast: *S. cerevisiae* W303. Crude = AZ60/TamI co-expression crude extract