

UC San Diego

UC San Diego Previously Published Works

Title

Identification of Thiotetronic Acid Antibiotic Biosynthetic Pathways by Target-directed Genome Mining

Permalink

<https://escholarship.org/uc/item/8jn8d1fh>

Journal

ACS Chemical Biology, 10(12)

ISSN

1554-8929

Authors

Tang, Xiaoyu
Li, Jie
Millán-Aguiñaga, Natalie
et al.

Publication Date

2015-12-18

DOI

10.1021/acscchembio.5b00658

Peer reviewed

Identification of Thiotetronic Acid Antibiotic Biosynthetic Pathways by Target-directed Genome Mining

Xiaoyu Tang,^{†,||} Jie Li,^{†,||} Natalie Millán-Aguíñaga,[†] Jia Jia Zhang,[†] Ellis C. O'Neill,[†] Juan A. Ugalde,[§] Paul R. Jensen,[†] Simone M. Mantovani,^{*,†} and Bradley S. Moore^{*,†,‡}

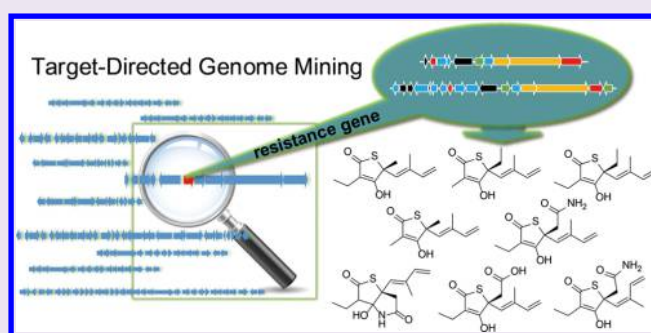
[†]Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California, United States

[‡]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California, United States

[§]Centro de Genómica y Bioinformática, Facultad de Ciencias, Universidad Mayor, Campus Huechuraba, Camino a la Pirámide 5750, Santiago, Chile

S Supporting Information

ABSTRACT: Recent genome sequencing efforts have led to the rapid accumulation of uncharacterized or “orphaned” secondary metabolic biosynthesis gene clusters (BGCs) in public databases. This increase in DNA-sequenced big data has given rise to significant challenges in the applied field of natural product genome mining, including (i) how to prioritize the characterization of orphan BGCs and (ii) how to rapidly connect genes to biosynthesized small molecules. Here, we show that by correlating putative antibiotic resistance genes that encode target-modified proteins with orphan BGCs, we predict the biological function of pathway specific small molecules before they have been revealed in a process we call target-directed genome mining. By querying the pan-genome of 86 *Salinispora* bacterial genomes for duplicated house-keeping genes colocalized with natural product BGCs, we prioritized an orphan polyketide synthase-nonribosomal peptide synthetase hybrid BGC (*tlm*) with a putative fatty acid synthase resistance gene. We employed a new synthetic double-stranded DNA-mediated cloning strategy based on transformation-associated recombination to efficiently capture *tlm* and the related *ttm* BGCs directly from genomic DNA and to heterologously express them in *Streptomyces* hosts. We show the production of a group of unusual thiotetronic acid natural products, including the well-known fatty acid synthase inhibitor thiolactomycin that was first described over 30 years ago, yet never at the genetic level in regards to biosynthesis and autoresistance. This finding not only validates the target-directed genome mining strategy for the discovery of antibiotic producing gene clusters without *a priori* knowledge of the molecule synthesized but also paves the way for the investigation of novel enzymology involved in thiotetronic acid natural product biosynthesis.



Microbial natural products are of paramount biomedical importance, serving as antibiotics against a variety of pathogenic bacteria.^{1,2} The profound emergence of bacterial resistance over the past several decades has marginalized many important antibiotics, thereby necessitating the need for new antibiotic discovery.³ Antibiotics from microbes are directly linked to clusters of genes that code for proteins associated with biosynthesis, resistance, regulation, and transport. The ability to connect natural antibiotics to gene clusters and vice versa, along with ever-increasing knowledge of biosynthetic machineries, has spawned a new field of natural product genome mining for the rational discovery of new chemical entities.^{4–6} At the same time, DNA sequence data from a great variety of microbial genomes and environmental metagenomes has rapidly accumulated in public databases through sophisticated sequencing technologies, with more than 4000 complete and 35 000 draft sequences of prokaryotic genomes in the NCBI database as of August 2015. Recently, an *in silico* bioinformatic analysis of 1154 prokaryotic genome sequences predicted a

total of 33 351 putative natural product biosynthetic gene clusters (BGCs),⁷ of which the vast majority could be considered “orphan” in that they could not be bioinformatically linked to the small molecules they produce. Thus, there is now considerable interest in prioritizing and developing new methods to study the products of these orphan BGCs, especially those with antibiotic activity.⁵

One of the significant challenges in the field of natural product genome mining is how to prioritize BGCs when mining for desired bioactivity, without prior knowledge of the biological targets of the compounds produced.⁸ Current genome mining strategies are often based on established biosynthetic information for known microbial metabolites,^{9–12} limiting the potential for linking chemistry with biology. Once

Received: August 14, 2015

Accepted: October 12, 2015

Published: October 12, 2015

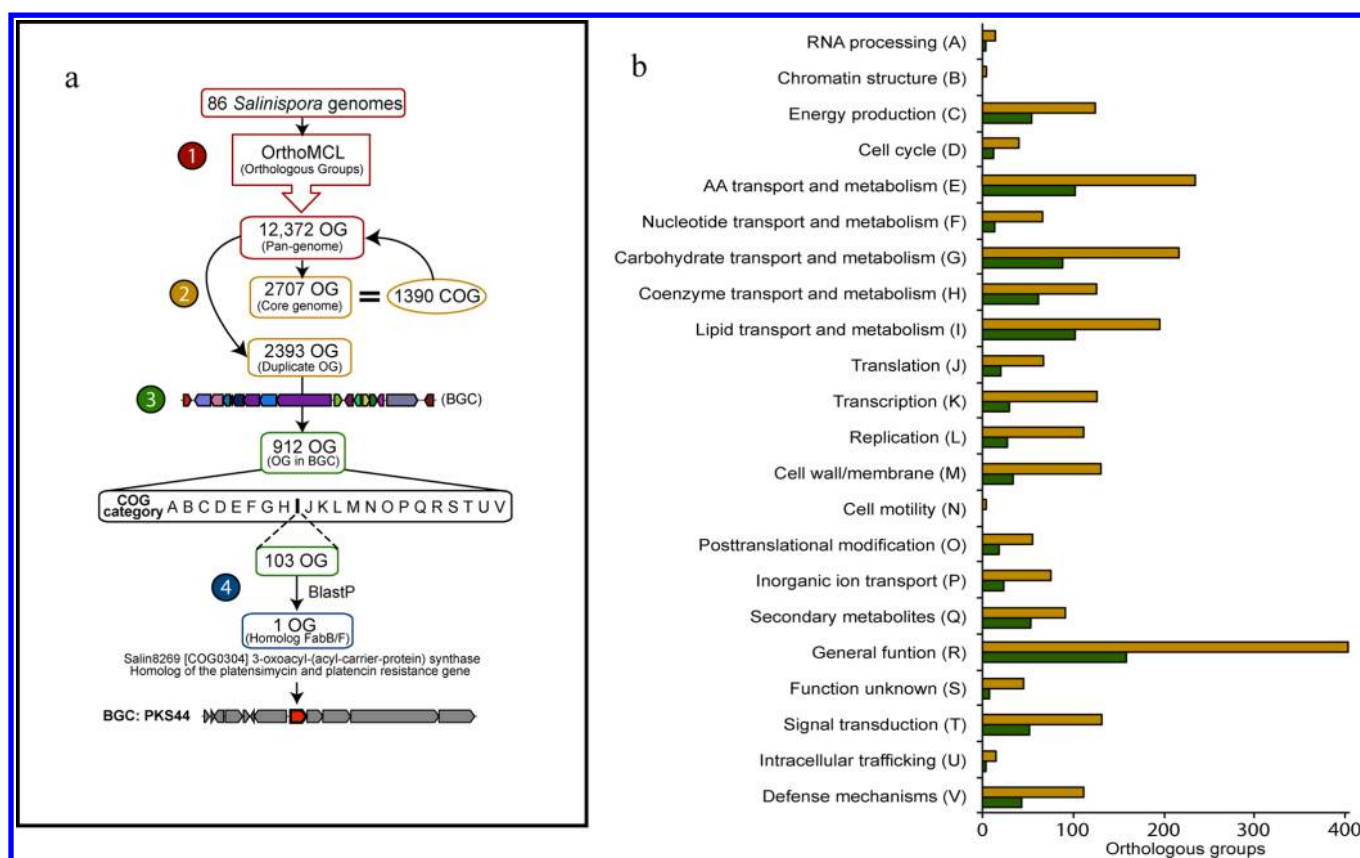


Figure 1. Bioinformatic identification of candidate resistance genes. (a) The workflow for identification of duplicated orthologous groups (OGs) associated with biosynthetic gene cluster (BGC) from 86 *Salinispora* strains: (1) Application of OrthoMCL to 86 *Salinispora* genomes resulted in the identification of 12 372 OGs, representing the pan-genome of the strains in the present study. (2) From the 12 372 OGs, application of custom python scripts identified 2707 OGs that are shared among all 86 strains (core genome). The core genome is comprised of 1390 unique COG numbers. Python scripts matched these unique COG numbers to identical COG numbers in the pan-genome, identifying a total of 2393 OGs (duplicate OGs). (3) Application of PHP scripts for identifying duplicated OGs associated with predicted BGCs resulted in the identification of 912 OGs from 20 COG categories being associated with BGCs. (4) 103 of the 912 OGs were affiliated with COG category “I” (lipid transport and metabolism). These OGs were annotated by BlastP searches, identifying one OG with homology to FabB/F, a known platensimycin and platencin resistance gene. This OG is located within the BGC PKS44 (*tlm*). (b) Distribution of the total number of duplicated OGs (yellow) and the OGs located within predicted BGC boundaries (green) among the COG categories.

potential orphan BGCs are prioritized for further investigation, rapidly connecting genes to molecules becomes another challenging task.⁸ Current strategies fall into two categories: (i) metabolic profiling coupled with mutagenesis of orphan genes^{13,14} and (ii) heterologous expression of whole biosynthetic pathways in a well-established host.^{15,16} The first approach relies heavily on the ability to genetically manipulate individual microbial genera and is often not applicable to genera without amenable genetic approaches or in microbes that presently cannot be cultured. In contrast, the heterologous expression of gene clusters, usually in a well-investigated and genetically amenable host, is often more facile and practical. However, this demands cloning of the entire BGC into suitable expression vectors by laborious traditional cloning methods.⁸

Genes encoding self-resistance mechanisms are a characteristic trait associated with antibiotic-producing bacteria.^{17,18} Self-resistance features are generally less favorable for host growth and survival and are thus only expressed concurrently with antibiotic biosynthesis.¹⁹ The most efficient way for bacteria to link these partners, and to ensure efficient cohorizontal gene transfer, is to include the resistance gene within or adjacent to the corresponding antibiotic BGC.¹⁷ Host bacteria have evolved several resistance strategies to avoid self-toxicity,

including product modification, binding and export, and target modification.²⁰ Of these mechanisms, target modification uniquely correlates an antibiotic to its mode of action. The antibiotics novobiocin (gyrase B),²¹ platensin (FabB/F),²² and griselimycin (DnaN),²³ for instance, represent a few examples in which target-duplicated resistance genes are coclustered with BGCs (Supporting Information Figure S1). With the notion in mind that antibiotic-producing bacteria often duplicate and mutate genes encoding targeted proteins to confer resistance, we reasoned that identifying putative resistance genes within BGCs would provide insight into the molecular targets of BGC chemical products prior to their isolation and structure elucidation.

The marine actinomycete genus *Salinispora* has proven to be a remarkably prolific source of structurally diverse and biologically active secondary metabolites.²⁴ These compounds span virtually all known biosynthetic classes, including the β -lactone proteasome inhibitor salinosporamide A²⁵ and the polyketide cytotoxin lomaiviticin A.²⁶ Recently, high quality draft genomes of 75 *Salinispora* strains revealed 124 discrete nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) BGCs that unmasked fundamental information about the evolution and distribution of secondary metabolite

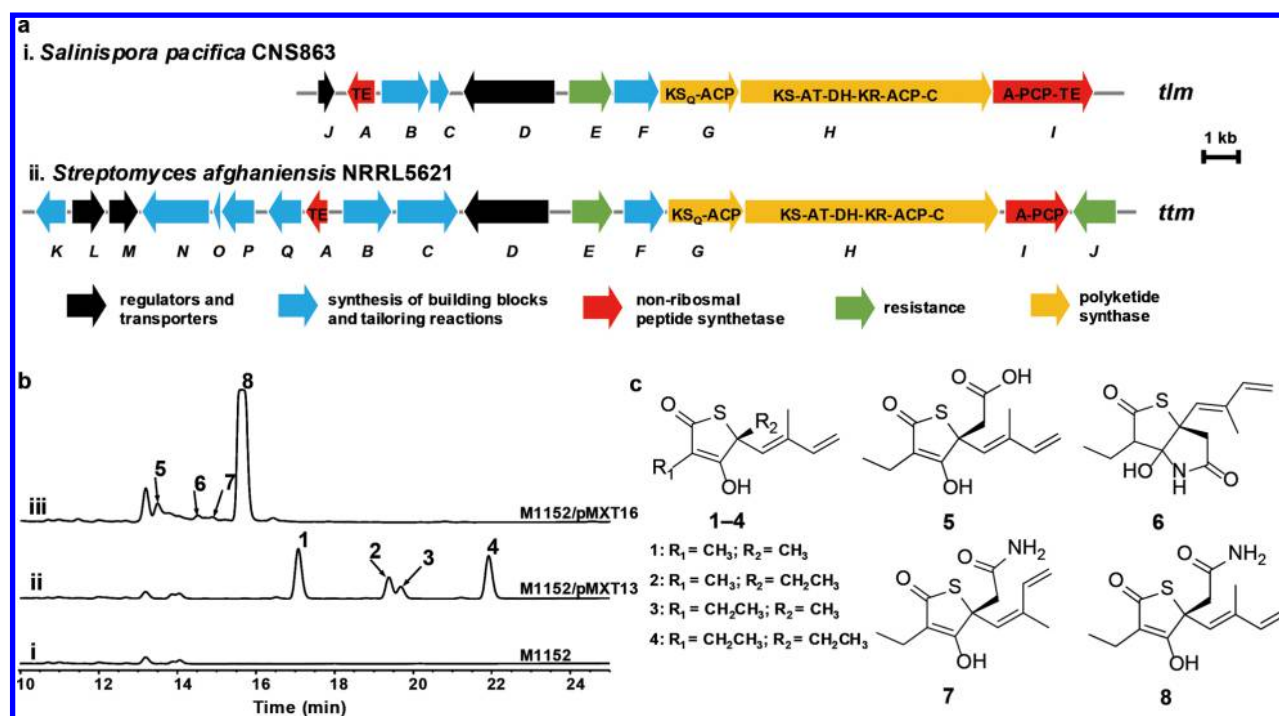


Figure 2. Identification of orphan BGCs with an extra copy of the *fabB/F* housekeeping gene and their metabolites. (a) Orphan gene cluster annotation. (b) HPLC profile of extracts from (i) *S. coelicolor* M1152, (ii) *S. coelicolor* M1152/pMXT13 (*tlm*), and (iii) *S. coelicolor* M1152/pMXT16 (*ttm*). Detection at 239 nm. (c) Structures of thiolactomycin (TLM, 1) and analogues identified in this work.

gene clusters in bacteria.²⁷ Here, we use *Salinispora* as a model organism to showcase target-directed genome mining for prioritizing orphan antibiotic BGCs, allowing us, for the first time, to systematically link two unusual PKS-NRPS hybrid BGCs to a series of structurally rare thiotetronic acid fatty acid synthase inhibitors.

RESULTS AND DISCUSSION

Mining *Salinispora* BGCs for Putative Antibiotic Resistance Genes. We queried the genomes of 86 *Salinispora* strains (10 *S. tropica*, 43 *S. arenicola*, and 33 *S. pacifica*) for putative target modifying resistance genes associated with natural product BGCs. First, we identified groups of related protein coding genes, or orthologous groups (OGs), using the program OrthoMCL,²⁸ which revealed a total of 12 372 OGs within the *Salinispora* pan-genome (Figure 1a). Of these, 2707 OGs were conserved across all *Salinispora* strains analyzed and thus comprise the *Salinispora* core genome. These core (housekeeping) genes could be further delineated into 1390 unique clusters of orthologous groups (COGs), each of which could be assigned a generalized function. We next searched the pan-genome for additional OGs with the same COG numbers as those found in the core genome, which suggests they have been duplicated or were acquired by horizontal gene transfer. We detected 2393 duplicate OGs, and among these, 912 (~38%) were associated with BGCs previously identified in the *Salinispora* strains.²⁷ These OGs were sorted based on COG categories to assess their potential role in resistance and to identify BGCs that may encode small molecules that act on specific targets of interest (Figure 1b and Supporting Information Methods). As proof of principle, this approach correctly identified the duplicated 20S proteasome β -subunit gene within the *sal* BGC, which confers resistance to the anticancer agent salinosporamide A in *S. tropica*.²⁹

Among the COG categories assigned to the duplicate OGs, we were particularly focused on identifying BGCs with the potential to produce compound(s) that inhibit bacterial fatty acid synthesis. The bacterial fatty acid synthase (FASII) is considered an attractive target for antibacterial drug discovery,^{30–32} as bacterial FASs are significantly distinct from the large, multifunctional type I synthases of mammals. Thus, we focused our attention on analyzing the BGCs linked to duplicate OGs assigned to COG category “I” of lipid transport and metabolism. BLAST analysis of the 103 “I” category OGs identified 44 related to core enzymes involved in bacterial fatty acid synthesis, including 18 similar to *fabG* (ketone reduction), 12 similar to *fabB/F* (elongation), six similar to *fabI* (enone reduction), five similar to *fabH* (chain initiation), and three similar to *fabD* (malonyl transacylation; Supporting Information Table S1). We reasoned that while many of these genes likely encode biosynthetic enzymes important for the construction of lipid components to natural products, others could be associated with antibiotic resistance. In particular, Salin8269 (from *S. pacifica* CNS-863), a homologue of *FabB/F*, notably showed very high amino acid sequence similarity to the characterized self-resistant proteins PtmP3 (65%/79%, identity/similarity-) and PtnP3 (65%/79%, identity/similarity) from BGCs associated with the hybrid diterpenoid FASII inhibitors platensimycin and platencin,²² respectively (Supporting Information Figure S2). Thus, we suspected that Salin8269 could serve a role in antibiotic resistance rather than natural product biosynthesis. The associated BGC, PKS44, is classified as a noncanonical hybrid PKS-NRPS and not linked to diterpenoid production, as is the case for platensimycin and platencin,³³ which prompted us to investigate this orphan BGC.

Discovery of the Thiolactomycin (*tlm*) Gene Cluster by Direct Cloning and Heterologous Expression. The PKS44 gene cluster was detected in four *S. pacifica* strains

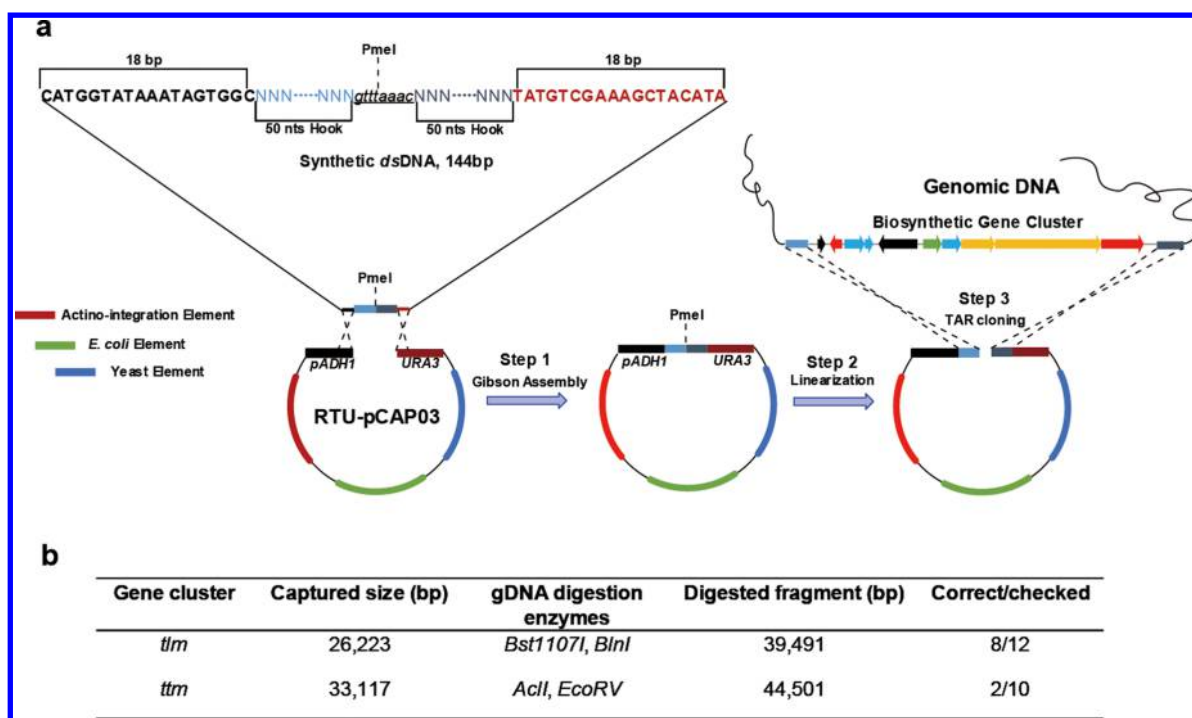


Figure 3. High-efficiency TAR based direct cloning strategy for capture of natural product gene clusters. (a) The high-efficiency TAR cloning involves three steps. In step 1, the capture vector is constructed in one step by Gibson Assembly using 144 bp synthetic *dsDNA*. In step 2, the capture vector is linearized by the restriction enzyme *Pme*I. In step 3, recombination in yeast. (b) Tabulation of direct cloning parameters of two orphan gene clusters.

isolated from Fiji (CNS-863, CNS-996, CNT-045, and CNS-860);²⁷ Supporting Information Figure S3a). Closer inspection of the gene cluster (renamed *tlm*) from *S. pacifica* CNS-863 revealed a ~22 kb PKS-NRPS hybrid gene cluster of 10 open reading frames (Figure 2a). In addition to the Salin8269 FabB/F homologue TlmE, the putative resistance gene, the core region of the *tlm* gene cluster codes for three PKS-NRPS proteins collectively containing 11 enzymatic domains (TlmG–TlmI) and a cytochrome P450 oxidoreductase (TlmF; Figure 2a and Supporting Information Table S2). We additionally annotated five genes upstream of the *tlmE–I* operon encoding a type II thioesterase (TE; TlmA), two enzymes for generating ethylmalonyl-CoA (TlmB and TlmC), and two transcriptional regulators (TlmD and TlmJ; Figure 2a and Supporting Information Table S2). Surprisingly, we also identified two additional *S. pacifica* strains (CNT-084 and CNT-609) in which *tlmF–I* were specifically lost from the *tlm* locus (Supporting Information Figure S3), suggesting these strains do not produce the *tlm* product but maintain the resistance phenotype putatively conferred by *tlmE*.

In order to identify the product(s) of the *tlm* gene cluster, we directly cloned an encompassing 26 kb genomic region using a synthetic double-stranded DNA-mediated (*dsDNA*-mediated) cloning strategy based on transformation-associated recombination (TAR) in the yeast *Saccharomyces cerevisiae*. Although we previously developed a TAR-based platform for the direct capture of natural product BGCs from genomic DNA for targeted expression,¹⁶ the original protocol demands construction of a cluster-specific capture vector by laborious traditional cloning methods. Furthermore, we observed high levels of plasmid recircularization due to nonhomologous end joining (NHEJ), repeatedly resulting in capture rates below 2%. This inefficiency necessitated extensive screening of hundreds

of colonies. To overcome these drawbacks, we revised the approach to eliminate the need for PCR for capture vector construction while introducing a counter-selectable marker to improve efficiency. We first introduced the *URA3* gene under the strong promoter of the *Schizosaccharomyces pombe* *ADH1* gene (*pADH1*) into pCAP01 as a counter selectable marker, to produce pCAP03 (Supporting Information Figure S4). *pADH1* can tolerate an insertion of up to 130 bp between the TATA box and the transcription initiation site.³⁴ This method allows the use of shorter capture arms and conveniently selects against NHEJ in the presence of 5-fluoroorotic acid (5-FOA).³⁵ We then designed a synthetic *dsDNA* fragment that could be directly inserted into the ready-to-use pCAP03 (RTU-pCAP03, Figure 3a and Supporting Information Figure S4) using Gibson Assembly (GA).³⁶ The 144 bp *dsDNA* contained two 18 bp overlapping fragments with the vector backbone for GA, two 50 bp capture hooks for recombination, and an 8 bp *Pme*I blunt end restriction site for linearization (Figure 3a). This approach not only greatly simplifies the procedure for constructing the capture vector but also significantly increased the percentage of positive yeast transformants. In the capture of the *tlm* gene cluster, we identified eight positive clones after the screening of 12 transformants by colony PCR, and four of them were transferred into *E. coli* and further confirmed by restriction digestion (Figure 3b, Supporting Information Figures 5 and 6).

We introduced the resulting plasmid pMXT13 into *S. coelicolor* M1152 by triparental intergeneric conjugation and selected three kanamycin resistant clones. The transformed strains were cultured and extracted for HPLC analyses. The results showed that all of the *S. coelicolor* M1152/pMXT13 strains produced at least four new UV absorbent peaks in comparison with the untransformed *S. coelicolor* M1152 host (Figure 2b). These molecules were purified via preparative

HPLC (Supporting Information Methods). Structural characterization by high-resolution mass spectrometry and extensive NMR spectroscopy identified the compounds as a group of previously described FASII inhibitors,^{37–40} including thiolactomycin (TLM, 1) and three analogues (2–4),^{41,42} with 3 being isolated as a new natural product (Figure 2c, Supporting Information Table S4 and Supporting Information Figures 7, 12, and 14–29). The production of 1–4 was additionally identified in the native producer *S. pacifica* CNS-863, which was aided with the results from the heterologous expression experiments (Supporting Information Figure S8).

TLM and its analogues share an unusual thiotetronic acid unit that is rare among natural products. The biosynthetic origin of TLM was previously reported to derive from a polyketide pathway involving acetate and three propionate building blocks and the sulfur atom from cysteine.⁴³ Inspection of the *tlm* locus suggests several unconventional biosynthetic features to account for these observed metabolic substrates. An AT-independent KS-ACP (TlmG) didomain appears to initiate polyketide chain priming with acetate, and a potential iterative PKS module TlmH (KS-AT-DH-KR-ACP-C) may elongate with three branched chain malonates to construct the carbon backbone of TLM. The incorporation of sulfur from cysteine appears to originate from the NRPS TlmI (A-PCP-TE) by an unknown mechanism. Recently, Shen and co-workers established cysteine as the precursor of the sulfur atom in leinamycin and characterized two new and unrelated PKS domains involved in the transformation.⁴⁴ We thus assume that the sulfur atom of the TlmI-activated cysteine residue is oxidatively removed and added to the TlmH polyketide intermediate to construct the thiotetronic acid pharmacophore.

Discovery of the Thiotetramide (*ttm*) Gene Cluster by PCR-independent Cloning and Heterologous Expression. Following the successful capture and heterologous expression of the *tlm* gene cluster, we identified a related gene cluster (*ttm*) from *Streptomyces afghaniensis* that contained two FabB/F homologues by BLAST search (Figure 2a and Supporting Information Table S3). Like the thiolactomycin TlmE, these two proteins were similarly related to PtmP3 (TtmE, 65%/78%, identity/similarity; TtmJ, 85%/91%, identity/similarity) and PtmP3 (TtmE, 66%/78%, identity/similarity; TtmJ, 83%/91%, identity/similarity).²² The detection of two copies of the putative resistance gene led to the hypothesis that the homologous *S. afghaniensis* pathway may produce a more potent series of FASII antibiotics. The *ttm* locus is similar to the *tlm* gene cluster in the core structure, except that the NRPS TtmI lacks the terminal thioesterase (TE) domain in comparison to TlmI (Figure 2a and Supporting Information Table S3). Furthermore, the 17 open reading frame *ttm* gene cluster contains five additional biosynthetic enzymes, including a short-chain dehydrogenase (TtmK), an aminotransferase (TtmN), a ferredoxin (TtmO), an additional cytochrome p450 oxidoreductase (TtmP), and a ketoacyl-acyl carrier protein synthase III (TtmQ; Figure 2a and Supporting Information Table S3).

In order to identify the product(s) of the homologous *ttm* gene cluster, we cloned a 33 kb genomic region containing the 29 kb gene cluster using the PCR-independent cloning approach, resulting in two positive colonies out of 10 transformants (Figure 3b). The resulting vector pMXT16 was verified (Supporting Information Figure S6) and heterologously expressed in *S. coelicolor* M1152. HPLC analysis of the heterologous expression system led to the isolation and

identification of a new series of TLM analogues (5–8; Figure 2b, Supporting Information Table S5 and Supporting Information Figures 7, 13, and 30–45). Among this series, 6 and 7 are new compounds, while 8 was previously reported as Tü 3010⁴⁵ and 5 was generated through alkaline hydrolysis of 8⁴⁵ (Figure 2c). Compounds 6–8 each contain a terminal amide functionality presumably derived from the pathway specific tailoring enzymes TtmK, TtmP, and TtmN and were accorded the trivial names thiotetramides (TTM) A–C. In contrast, 5 contains a carboxylic acid group and is proposed as an intermediate before terminal amination; hence it was assigned the name prethiotetramide C.

Confirmation of *ttmE* and *ttmJ* as Self-Resistance Elements. In order to test the hypothesis that the *tlm* and *ttm*-associated FabB/F homologues are associated with TLM/TTM resistance and not biosynthesis, we focused on the *ttm* gene cluster since it contained two FabB/F encoding genes—one shared with the *tlm* locus (*ttmE* and *tlmE*) and the second being unique (*ttmJ*). We first evaluated common *Streptomyces* heterologous host strains and found that *S. coelicolor* M1152 was susceptible to TTM C (8) with a minimum inhibitory concentration (MIC) of 12 µg/mL (Supporting Information Figure S9). *S. coelicolor* M1152 harboring the complete *ttm* BGC (*S. coelicolor* M1152/pMXT16), however, was resistant to 300 µg of TTM C on agar diffusion assays (Figure 4 and Supporting Information Figure S10).

We generated three mutants in which the FabB/F homologous genes were individually (*S. coelicolor* M1152/pMXT16Δ*ttmEscar* and *S. coelicolor* M1152/pMXT16Δ*ttmJ*) and collectively (*S. coelicolor* M1152/pMXT16Δ*ttmEscar*Δ*ttmJ*) inactivated. All three mutants retained the ability to heterologously produce the TTM compounds in the same

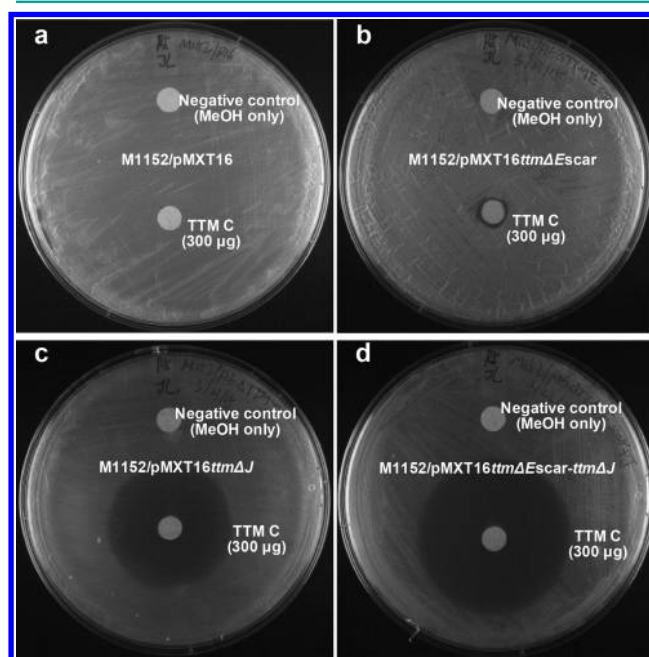


Figure 4. Representative disk diffusion assay plates for TTM C (300 µg) against *S. coelicolor* M1152 integrated with (a) the *ttm* gene cluster (designated as M1152/pMXT16) and (b–d) three mutants: (b) the *ttmE* deleted mutant *S. coelicolor* M1152/pMXT16Δ*tmeScar*, (c) the *ttmJ* deleted mutant *S. coelicolor* M1152/pMXT16Δ*ttmJ*, and (d) the *tmeE*–*ttmJ* double mutant *S. coelicolor* M1152/pMXT16Δ*tmeScar*Δ*ttmJ*.

distribution (Supporting Information Figure S11), thereby establishing that the FabB/F genes *ttnE* and *ttnJ* are not required for biosynthesis. We also observed that deletion of the resistance genes did not compromise the production of TTM. This may be attributed to the low yield of TTM C in the heterologous host (around 0.5 $\mu\text{g}/\text{mL}$), which is much lower than the MIC (12 $\mu\text{g}/\text{mL}$). The susceptibility of these recombinant *S. coelicolor* strains to TTM C was next determined using a disk diffusion assay. When a high concentration of TTM C (300 μg) was tested, the *ttnE* deletion strain *S. coelicolor* M1152/pMXT16 Δ *ttnE*Escar, the *ttnJ* knockout strain *S. coelicolor* M1152/pMXT16 Δ *ttnJ*, and the *ttnE* and *ttnJ* double-deletion strain *S. coelicolor* M1152/pMXT16 Δ *ttnE*Escar- Δ *ttnJ* showed inhibition zones of the sizes 0.9 ± 0.08 cm, 3.2 ± 0.3 cm, and 4.4 ± 0.2 cm (Figure 4), respectively. Smaller inhibition zones were observed when lower concentrations of TTM C (50–200 μg) were applied to the *ttnJ* deletion strains, while the *ttnE* deletion strain showed complete resistance at these concentrations (Supporting Information Figure S10). These data confirm that *ttnE* and *ttnJ* provide inherent resistance to TTM C, the major compound produced by the *ttn* BGC. Since deletion of *ttnE* did not compromise resistance of the strain at these concentrations, we suggest that TtmE plays a relatively minor role in resistance. Rather, the deletion experiments indicate that *ttnJ* is the major resistance element for TTM biosynthesis, as all *ttnJ* deletion mutants exhibited high susceptibility to TTM C. In line with our hypothesis, previously reported antibacterial activity tests showed that the MIC of TMM C is 16–32-fold lower than TLM and its analogues,³⁰ supporting the molecular logic that the *ttn* pathway requires a second resistance gene. Previous studies have shown various resistance mechanisms against TLM in pathogenic bacteria, including the *emrAB* encoded multidrug-resistant efflux pump in *E. coli*,⁴⁶ FabB point mutation in *E. coli*,⁴⁷ and FabH point mutations in *Staphylococcus aureus*.³⁷ Our findings reveal a distinct resistance mechanism associated with TLM/TTM producing strains.

Conclusion. We report here a new strategy for mining orphan biosynthetic pathways, which we refer to as target-directed genome mining. By using *Salinispora* as a model, the present study demonstrates a systematic approach to identify BGCs with duplicated housekeeping genes that may be candidates for conferring resistance against the product of the BGCs. Combined with the synthetic *ds*DNA-mediated cloning method, we showed that putative *fabB/F* resistance genes associated with orphan BGCs in *Salinispora* and *Streptomyces* bacteria are associated with FAS inhibitors of the thiotetronic acid family, antibiotics that were first described over 30 years ago yet never at the genetic level. Our work also paves the way for investigating the biosynthesis of the structurally unique thiotetronic acid natural products, a topic that we are actively pursuing and will report elsewhere. Significantly, this work shows the first demonstration of target-directed genome mining strategy for the discovery of antibiotic producing gene clusters, without *a priori* knowledge of the molecule synthesized. Our comparative genomic analysis provides an indication of the frequency that housekeeping genes are involved in secondary metabolic processes. Based on the rapid increase in genome sequence data in public databases, we envision that this strategy may be widely applicable to genomics-driven natural product discovery and innovate the manner in which antibiotics are discovered, offering an efficient, hypothesis-driven genome

mining platform for the development of new antibacterial drug candidates.

METHODS

Cultivation of the Strains. Seventy-five *Salinispora* genome sequences were obtained as previously described.²⁷ A highly transformable *S. cerevisiae* strain VL6–48N (MAT α , *his3-D200*, *trp1- Δ 1*, *ura3- Δ 1*, *lys2*, *ade2-101*, *met14*, *psi+ Δ ci ρ*)³⁵ was used as a host for gene cluster direct cloning experiments. The yeast cells were grown in liquid YPD medium (Yeast extract Peptone Dextrose medium; 2% D-glucose, 1% yeast extract, and 2% peptone (w/v)) supplemented with 100 mg/L adenine and used for spheroplasting prior to transformation-associated recombination (TAR). Yeast transformants were selected on synthetic tryptophan drop-out agar (SD-Trp agar) containing 5-fluoroorotic acid (5-FOA; SD-Trp-5-FOA agar) consisting of 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Sigma), 0.19% yeast synthetic drop-out medium supplements without tryptophan (Sigma), 1 M sorbitol, 2% D-glucose, 0.5% ammonium sulfate, 100 mg/L adenine, 2% agar, and 0.001% 5-FOA (w/v) (Zymo Research). *S. coelicolor* M1152 (SCP1–, SCP2–, Δ *act*, Δ *red*, Δ *cpk*, Δ *cda*, and *rpoB*[C1298T])⁴⁸ and their respective derivatives were maintained and grown on MS agar (2% soy flour, 2% mannitol, 2% agar (w/v for all); components purchased from BD Biosciences) or Tryptic Soy Broth (TSB) medium (BD Biosciences). *Salinispora pacifica* CNS-863 was maintained on A1 agar as previously reported,⁴⁹ and *Streptomyces afghaniensis* NRRL 5621 (accession no. NZ_AOPY01000000) was maintained on MS agar. *E. coli* strains were cultivated in LB medium (components purchased from BD Biosciences or Fisher Scientific) supplemented with the appropriate antibiotics. DNA isolation and manipulations were carried out according to standard methods for *E. coli* and *Streptomyces*.

Construction of the Counter-selectable Capture Vector pCAP03. *pADH* and *URA3* were amplified from pARS-VN³⁵ by using the primer pairs *ADH-fw/ADH-rev* and *URA3-fw/URA3-rev* (Table S6), respectively. The two PCR amplified fragments were then combined and assembled into a single piece by PCR with primers *ADH-fw* and *URA3-rev*. The assembled fragment was digested with *SpeI* and *KpnI* and introduced into the same sites of pCAP01¹⁶ to obtain pCAP03. In order to easily linearize pCAP03, the 1369 bp apramycin resistance cassette (*acc(3)IV*) was amplified from pJ773⁵⁰ by using the primers *acc(3)IV-pCAP03-fw* and *acc(3)IV-pCAP03-rev* (Supporting Information Table S6). The resulting PCR product was digested by *XhoI* and *NdeI* and cloned into the same restriction sites of pCAP03 to obtain pCAP03-*acc(3)IV*. The circular construct pCAP03-*acc(3)IV* was digested with *NdeI* and *XhoI* and agarose gel purified, as ready-to-use pCAP03 (RTU-pCAP03) for assembly of cluster-specific capture vectors.

Construction of Specific Capture Vectors. The 144 bp *ds*DNA fragments were designed as shown in Figure 3 and synthesized by Integrated DNA Technologies, Incorporation, La Jolla, US. The 100 ng *ttn*-hooks and *ttn*-hooks (Supporting Information Table S6) were assembled with 50 ng of the RTU-pCAP03 following the instructions for the Gibson Assembly Kit (New England BioLabs Inc.) to generate the capture vectors pMTZ01 and pMTZ02, respectively. Prior to direct TAR cloning, the capture vectors were digested by *PmeI*.

Preparation of Genomic DNA Fragments for TAR Capturing. Genomic DNA was isolated from mid log phase cells by standard procedures.⁵¹ Approximately 200 μg of genomic DNA was digested with appropriate restriction enzymes (Figure 3b), in an overnight reaction at 37 °C. The digested genomic DNA fragments were precipitated with isopropanol and washed with 70% ethanol. The resulting DNA pellet was dissolved in 100 μL of Tris buffer (10 mM Tris-HCl, pH 8.0).

Direct Capture of the *ttn* and *ttn* Gene Clusters. Direct TAR cloning of the *ttn* and *ttn* gene clusters from genomic DNA were carried out using a previously reported protocol with minor modifications.^{16,52} *S. cerevisiae* strain VL6–48N was grown in 50 mL of YPD medium supplemented with adenine (100 mg/L) at 30 °C with shaking until an OD₆₀₀ of 0.7–1.0 was reached. The cells were

harvested and washed with ice-cold water and osmotically stabilized in 1 M sorbitol at 4 °C overnight prior to spheroplasting. Preparation of spheroplast cells was carried out using a lytic enzyme (Zymolyase-20T, MP Biomedicals, US) at a final concentration of 0.1 mg mL⁻¹, with 30–40 min incubation. A total of 2.5 to 3 μg of genomic DNA fragments and 0.5 μg linearized specific capture vector were added to spheroplast cells, and the transformation was mediated by PEG8000 (Sigma, US). The transformed spheroplasts were mixed with 8 mL of synthetic tryptophan drop-out (SD-Trp) top agar (containing 3% agar) at 55 °C and overlaid on SD-Trp-5-FOA agar. The plates were incubated at 30 °C for 4–5 days. Normally, around 10–20 transformants appeared per plate and were picked and transferred onto new SD-Trp agar plates and incubated for 2 days at 30 °C. Cells were lysed using Zymolyase-20T at 37 °C for 2 h and subsequently boiled at 98 °C for 5 min. The captured *tlm* and *ttm* gene clusters were screened by the primer pairs of *tlm*-test-fw/*tlm*-test-rev and *ttm*-test-fw/*ttm*-test-rev, respectively (Supporting Information Table S6). Plasmids were extracted from PCR positive clones and then transferred into *E. coli* Top10 cells by electroporation. The plasmids were purified from kanamycin resistant *E. coli* clones, and the resulting constructs were confirmed by restriction analysis. The vector containing *tlm* gene cluster and *ttm* gene cluster were designated as pMXT13 and pMXT16, respectively.

Heterologous Expression of the *tlm* and *ttm* Gene Clusters. pMXT13 and pMXT16 were transferred into *E. coli* ET12567 and introduced into *S. coelicolor* M1152 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307.⁵³ Kanamycin resistant clones were selected, confirmed by PCR, and designated as *S. coelicolor* M1152/pMXT13 and *S. coelicolor* M1152/pMXT16. Three milliliters of TSB medium were inoculated with a spore suspension of *S. coelicolor* M1152 or a derivative thereof. The cultures were incubated for 2 days at 30 °C at 220 rpm. For the production of the compounds, 1 mL of preculture was inoculated into 50 mL of the production medium containing 1% soytone, 1% soluble starch, and 2% D-maltose (w/v) adjusted to pH 6.7 (components purchased from BD Biosciences, US). The cultures were incubated for 7 days at 30 °C with 220 rpm shaking. The culture supernatant was adjusted to pH 4 with acetic acid and subsequently extracted with an equal volume of ethyl acetate. The organic phase was evaporated, and extracts were dissolved in 1 mL of methanol. Each extract was monitored at 239 nm during separation by HPLC using a Luna 100A-C18 column (5 μm, 250 × 4.6 mm; Phenomenex, US) as follows: 0–23 min, 35%–70% B; 24–28 min, 70%–100% B; 29–33 min, 100% B; 34–35 min, 100%–35% B; 36–40 min, 35% B (solvent A, water/trifluoroacetic acid (999:1); solvent B, acetonitrile/trifluoroacetic acid (999:1)).

Other Methods. Other methods, including the identification of orthologous groups, BGC annotation, disk diffusion assays, isolation, and structural elucidation of thiolactomycins and thiotetraamides, are described in the Supporting Information Methods.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.5b00658.

Supporting materials and methods, NMR methods and general instrumentation for structural characterization, Tables S1–S6, and Figures S1–S45 (PDF)

Accession Codes

Eleven genome sequences reported in this paper have been deposited in the Joint Genome Institute's Integrated Microbial Genomes (IMG) database, <http://img.jgi.doe.gov/> (accession nos. 2540341193, 2524614530, 2540341192, 2528311034, 2524614561, 2524023246, 2526164509, 2528311033, 2524614529, 2524614515, 2521172655). The nucleotide sequences of the thiolactomycin gene cluster from *Salinispora pacifica* CNS-863 and the thiotetraamide gene cluster from

Streptomyces afghaniensis NRRL 5621 have been deposited in the GenBank database (accession nos. KT282100 and KT282101, respectively).

■ AUTHOR INFORMATION

Corresponding Authors

*Tel.: +1(858)822-6650. Fax: +1(858)534-1318. E-mail: smantovani@syntheticgenomics.com.

*Tel.: +1(858)822-6650. Fax: +1(858)534-1318. E-mail: bsmoore@ucsd.edu.

Author Contributions

These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank V. Larionov (National Cancer Institute, NIH) for providing *S. cerevisiae* strain VL6-48N and plasmid pARS-VN, Mervyn Bibb (John Innes Centre, UK) for providing *Streptomyces coelicolor* M1152, the Agricultural Research Service (ARS) culture collection for providing the *Streptomyces afghaniensis* NRRL 5621 strain, and N. Ziemert (Scripps Institution of Oceanography, UCSD) and M. Wietz (ICBM, University of Oldenburg) for valuable discussions. This work was supported by U.S. National Institutes of Health grants R01-GM085770 and U19-TW007401, the Roddenberry Foundation, Fondecyt Grant (11140666 to J.A.U.), and graduate fellowships from the NSF (to J.J.Z.) and Consejo Nacional de Ciencia y Tecnología (CONACYT-213497 to N.M.A.). Genome sequencing was conducted by the U.S. Department of Energy Joint Genome Institute and supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

■ ABBREVIATIONS:

PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; TAR, transformation-associated recombination; dsDNA, double-stranded DNA; NHEJ, nonhomologous end joining

■ REFERENCES

- (1) Clardy, J., Fischbach, M. A., and Walsh, C. T. (2006) New antibiotics from bacterial natural products. *Nat. Biotechnol.* 24, 1541–1550.
- (2) Demain, A. L. (2009) Antibiotics: natural products essential to human health. *Med. Res. Rev.* 29, 821–842.
- (3) Cooper, M. A., and Shlaes, D. (2011) Fix the antibiotics pipeline. *Nature* 472, 32.
- (4) Corre, C., and Challis, G. L. (2009) New natural product biosynthetic chemistry discovered by genome mining. *Nat. Prod. Rep.* 26, 977–986.
- (5) Genilloud, O. (2014) The re-emerging role of microbial natural products in antibiotic discovery. *Antonie van Leeuwenhoek* 106, 173–188.
- (6) Milshcheyn, A., Schneider, J. S., and Brady, S. F. (2014) Mining the metabiome: identifying novel natural products from microbial communities. *Chem. Biol.* 21, 1211–1223.
- (7) Cimermancic, P., Medema, M. H., Claesen, J., Kurita, K., Wieland Brown, L. C., Mavrommatis, K., Pati, A., Godfrey, P. A., Koehrsen, M., Clardy, J., Birren, B. W., Takano, E., Sali, A., Lington, R. G., and Fischbach, M. A. (2014) Insights into secondary metabolism from a global analysis of prokaryotic biosynthetic gene clusters. *Cell* 158, 412–421.

- (8) Rutledge, P. J., and Challis, G. L. (2015) Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat. Rev. Microbiol.* 13, 509.
- (9) Eustaquio, A. S., Nam, S. J., Penn, K., Lechner, A., Wilson, M. C., Fenical, W., Jensen, P. R., and Moore, B. S. (2011) The discovery of salinosporamide K from the marine bacterium "*Salinispora pacifica*" by genome mining gives insight into pathway evolution. *ChemBioChem* 12, 61–64.
- (10) Kaysser, L., Tang, X., Wemakor, E., Sedding, K., Hennig, S., Siebenberg, S., and Gust, B. (2011) Identification of a napsamycin biosynthesis gene cluster by genome mining. *ChemBioChem* 12, 477–487.
- (11) Kersten, R. D., Yang, Y. L., Xu, Y., Cimermancic, P., Nam, S. J., Fenical, W., Fischbach, M. A., Moore, B. S., and Dorrestein, P. C. (2011) A mass spectrometry-guided genome mining approach for natural product peptidogenomics. *Nat. Chem. Biol.* 7, 794–802.
- (12) Kersten, R. D., Ziemert, N., Gonzalez, D. J., Duggan, B. M., Nizet, V., Dorrestein, P. C., and Moore, B. S. (2013) Glycogenomics as a mass spectrometry-guided genome-mining method for microbial glycosylated molecules. *Proc. Natl. Acad. Sci. U. S. A.* 110, E4407–4416.
- (13) Lautru, S., Deeth, R. J., Bailey, L. M., and Challis, G. L. (2005) Discovery of a new peptide natural product by *Streptomyces coelicolor* genome mining. *Nat. Chem. Biol.* 1, 265–269.
- (14) Laureti, L., Song, L., Huang, S., Corre, C., Leblond, P., Challis, G. L., and Aigle, B. (2011) Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambifaciens*. *Proc. Natl. Acad. Sci. U. S. A.* 108, 6258–6263.
- (15) Fu, J., Bian, X., Hu, S., Wang, H., Huang, F., Seibert, P. M., Plaza, A., Xia, L., Muller, R., Stewart, A. F., and Zhang, Y. (2012) Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. *Nat. Biotechnol.* 30, 440–446.
- (16) Yamanaka, K., Reynolds, K. A., Kersten, R. D., Ryan, K. S., Gonzalez, D. J., Nizet, V., Dorrestein, P. C., and Moore, B. S. (2014) Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proc. Natl. Acad. Sci. U. S. A.* 111, 1957–1962.
- (17) D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006) Sampling the antibiotic resistome. *Science* 311, 374–377.
- (18) Thaker, M. N., Wang, W., Spanogiannopoulos, P., Waglechner, N., King, A. M., Medina, R., and Wright, G. D. (2013) Identifying producers of antibacterial compounds by screening for antibiotic resistance. *Nat. Biotechnol.* 31, 922–927.
- (19) Andersson, D. I., and Levin, B. R. (1999) The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* 2, 489–493.
- (20) Wright, G. D. (2011) Molecular mechanisms of antibiotic resistance. *Chem. Commun. (Cambridge, U. K.)* 47, 4055–4061.
- (21) Steffensky, M., Muhlenweg, A., Wang, Z. X., Li, S. M., and Heide, L. (2000) Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. *Antimicrob. Agents Chemother.* 44, 1214–1222.
- (22) Peterson, R. M., Huang, T., Rudolf, J. D., Smanski, M. J., and Shen, B. (2014) Mechanisms of self-resistance in the platensimycin- and platencin-producing *Streptomyces platensis* MA7327 and MA7339 strains. *Chem. Biol.* 21, 389–397.
- (23) Kling, A., Lukat, P., Almeida, D. V., Bauer, A., Fontaine, E., Sordello, S., Zaburanyi, N., Herrmann, J., Wenzel, S. C., Konig, C., Ammerman, N. C., Barrio, M. B., Borchers, K., Bordon-Pallier, F., Bronstrup, M., Courtemanche, G., Gerlitz, M., Geslin, M., Hammann, P., Heinz, D. W., Hoffmann, H., Klieber, S., Kohlmann, M., Kurz, M., Lair, C., Matter, H., Nuermberger, E., Tyagi, S., Fraisse, L., Grosset, J. H., Lagrange, S., and Muller, R. (2015) Antibiotics. Targeting DnaN for tuberculosis therapy using novel griselimycins. *Science* 348, 1106–1112.
- (24) Jensen, P. R., Moore, B. S., and Fenical, W. (2015) The marine actinomycete genus *Salinispora*: a model organism for secondary metabolite discovery. *Nat. Prod. Rep.* 32, 738–751.
- (25) Gulder, T. A., and Moore, B. S. (2010) Salinosporamide natural products: Potent 20 S proteasome inhibitors as promising cancer chemotherapeutics. *Angew. Chem., Int. Ed.* 49, 9346–9367.
- (26) Colis, L. C., Woo, C. M., Hegan, D. C., Li, Z., Glazer, P. M., and Herzon, S. B. (2014) The cytotoxicity of (–)-lomaiviticin A arises from induction of double-strand breaks in DNA. *Nat. Chem.* 6, 504–510.
- (27) Ziemert, N., Lechner, A., Wietz, M., Millan-Aguinaga, N., Chavarria, K. L., and Jensen, P. R. (2014) Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proc. Natl. Acad. Sci. U. S. A.* 111, E1130–1139.
- (28) Li, L., Stoeckert, C. J., Jr., and Roos, D. S. (2003) OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* 13, 2178–2189.
- (29) Kale, A. J., McGlinchey, R. P., Lechner, A., and Moore, B. S. (2011) Bacterial self-resistance to the natural proteasome inhibitor salinosporamide A. *ACS Chem. Biol.* 6, 1257–1264.
- (30) Young, K., Jayasuriya, H., Ondeyka, J. G., Herath, K., Zhang, C., Kodali, S., Galgoci, A., Painter, R., Brown-Driver, V., Yamamoto, R., Silver, L. L., Zheng, Y., Ventura, J. I., Sigmund, J., Ha, S., Basilio, A., Vicente, F., Tormo, J. R., Pelaez, F., Youngman, P., Cully, D., Barrett, J. F., Schmatz, D., Singh, S. B., and Wang, J. (2006) Discovery of FabH/FabF inhibitors from natural products. *Antimicrob. Agents Chemother.* 50, 519–526.
- (31) Balemans, W., Lounis, N., Gilissen, R., Guillemont, J., Simmen, K., Andries, K., and Koul, A. (2010) Essentiality of FASII pathway for *Staphylococcus aureus*. *Nature* 463, E3, discussion E4.
- (32) Yao, J., and Rock, C. O. (2015) How bacterial pathogens eat host lipids: implications for the development of fatty acid synthesis therapeutics. *J. Biol. Chem.* 290, 5940–5946.
- (33) Smanski, M. J., Yu, Z., Casper, J., Lin, S., Peterson, R. M., Chen, Y., Wendt-Pienkowski, E., Rajski, S. R., and Shen, B. (2011) Dedicated ent-kaurene and ent-atiserene synthases for platensimycin and platencin biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 108, 13498–13503.
- (34) Furter-Graves, E. M., and Hall, B. D. (1990) DNA sequence elements required for transcription initiation of the *Schizosaccharomyces pombe* ADH gene in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 223, 407–416.
- (35) Noskov, V. N., Kouprina, N., Leem, S. H., Ouspenski, I., Barrett, J. C., and Larionov, V. (2003) A general cloning system to selectively isolate any eukaryotic or prokaryotic genomic region in yeast. *BMC Genomics* 4, 16.
- (36) Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.
- (37) Parsons, J. B., Yao, J., Frank, M. W., and Rock, C. O. (2015) FabH mutations confer resistance to FabF-directed antibiotics in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 59, 849–858.
- (38) Jackowski, S., Murphy, C. M., Cronan, J. E., Jr., and Rock, C. O. (1989) Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. *J. Biol. Chem.* 264, 7624–7629.
- (39) Hayashi, T., Yamamoto, O., Sasaki, H., Kawaguchi, A., and Okazaki, H. (1983) Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 115, 1108–1113.
- (40) Price, A. C., Choi, K. H., Heath, R. J., Li, Z., White, S. W., and Rock, C. O. (2001) Inhibition of beta-ketoacyl-acyl carrier protein synthases by thiolactomycin and cerulenin. Structure and mechanism. *J. Biol. Chem.* 276, 6551–6559.
- (41) Sasaki, H., Oishi, H., Hayashi, T., Matsuura, I., Ando, K., and Sawada, M. (1982) Thiolactomycin, a new antibiotic. II. Structure elucidation. *J. Antibiot.* 35, 396–400.
- (42) Hayashi, T., Yamamoto, O., Sasaki, H., Okazaki, H., and Kawaguchi, A. (1984) Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. *J. Antibiot.* 37, 1456–1461.
- (43) Brown, M. S., Akopiants, K., Resceck, D. M., McArthur, H. A., McCormick, E., and Reynolds, K. A. (2003) Biosynthetic origins of the

natural product, thiolactomycin: a unique and selective inhibitor of type II dissociated fatty acid synthases. *J. Am. Chem. Soc.* **125**, 10166–10167.

(44) Ma, M., Lohman, J. R., Liu, T., and Shen, B. (2015) C-S bond cleavage by a polyketide synthase domain. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 10359–10364.

(45) Rapp, C., Jung, G., Isselhorst-Scharr, C., and Zähler, H. (1988) A new member of the class of antibiotics with thiotetronic acid structure isolated from *Streptomyces olivaceus* Tü 3010. *Liebigs Annalen der Chemie* **1988**, 1043–1047.

(46) Furukawa, H., Tsay, J. T., Jackowski, S., Takamura, Y., and Rock, C. O. (1993) Thiolactomycin resistance in *Escherichia coli* is associated with the multidrug resistance efflux pump encoded by *emrAB*. *J. Bacteriol.* **175**, 3723–3729.

(47) Jackowski, S., Zhang, Y. M., Price, A. C., White, S. W., and Rock, C. O. (2002) A missense mutation in the *fabB* (beta-ketoacyl-acyl carrier protein synthase I) gene confers thiolactomycin resistance to *Escherichia coli*. *Antimicrob. Agents Chemother.* **46**, 1246–1252.

(48) Gomez-Escribano, J. P., and Bibb, M. J. (2012) *Streptomyces coelicolor* as an expression host for heterologous gene clusters. *Methods Enzymol.* **517**, 279–300.

(49) Jensen, P. R., Gontang, E., Mafnas, C., Mincer, T. J., and Fenical, W. (2005) Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environ. Microbiol.* **7**, 1039–1048.

(50) Gust, B., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1541–1546.

(51) Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, UK.

(52) Kouprina, N., and Larionov, V. (2008) Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*. *Nat. Protoc.* **3**, 371–377.

(53) Flett, F., Mersinias, V., and Smith, C. P. (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. *FEMS Microbiol. Lett.* **155**, 223–229.