LETTER

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Natural Products from Environmental DNA Hosted in *Ralstonia metallidurans*

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ABSTRACT Metagenomic studies designed to access new small molecules from the heterologous expression of environmental DNA have focused on the use of two model systems, *Escherichia coli* and *Streptomyces* spp., as heterologous hosts. Accessing the biosynthetic potential of DNA extracted from the bacteria present in environmental samples will require the development of a more diverse collection of model bacterial hosts that can be used for screening environmental DNA libraries. In this study the bacterium *Ralstonia metallidurans* was explored as a heterologous host. Here we report the isolation and characterization of both novel and known metabolites from pigmented and antibacterially active clones found in *R. metallidurans* based environmental DNA libraries. The clones found in this study do not confer the production of clone-specific metabolites to *E. coli*, validating *R. metallidurans* as an orthogonal expression host that can be used to expand the number of metabolites found in future metagenomic discovery efforts.

It is now well-established that the vast majority of bacteria present in environmental samples are not easily grown in the laboratory (*1, 2*). Culture-dependent techniques for the discovery of natural products therefore fail to access the full chemical and metabolic diversity present within environmental samples. One means to access the biosynthetic potential of uncultured bacteria that circumvents the challenges of culturing environmental bacteria is to extract DNA directly from environmental samples (environmental DNA, eDNA) and introduce this DNA into bacteria that are amenable to culturing in the laboratory (*3*). All potential cultured bacterial hosts are, however, restricted by their intrinsic abilities to recognize and utilize the foreign DNA captured within eDNA libraries. To date, only eDNA libraries based in *Escherichia coli* and *Streptomyces* spp., the traditional model systems used to study natural product biosynthesis, have yielded novel metabolites (*4–7*). In this study, the β-Proteobacteria *Ralstonia metallidurans* was explored as a potential new host for eDNA libraries. Here we report the isolation and characterization of both novel and known metabolites produced by clones found from screening eDNA libraries hosted by *R. metallidurans*. The development of a phylogenetically diverse collection of model bacterial systems that can serve as heterologous hosts for eDNA libraries should expand the repertoire of natural products that can be accessed from uncultured bacteria.

A recent meta-analysis of soil metagenomic libraries found that the vast majority of bacteria present in environmental samples reside in five major divisions of bacteria: α-, β-, γ-Proteobacteria, Acidobacteria, and Actinobacteria (*8*). Although molecules have been found from screening eDNA libraries hosted in representative γ-Proteobacteria (*Escherichia coli*) and Actinobacteria (*Streptomyces* spp.), the remaining three major divisions of bacteria have largely been overlooked (*4–7*). We examined these three divisions for potential hosts that would be genetically tractable, easily screenable, and metabolically versatile. The β-Proteobacterium, *R. metallidurans* was found to meet all of these criteria. *Ralstonia* species are easily transformable, rapidly form small colonies that can be easily examined for interesting phenotypes, and harbor a large number of genes for the metabolism of aromatic compounds (*9–12*). *R. metallidurans* also contains a variety of cytochrome P450 monooxygenases that might be useful for transforming heterologously produced metabolites into novel entities (*13, 14*).

E. coli remains the most efficient bacterium for creating large genomic DNA libraries. Therefore the eDNA libraries used in this study were initially constructed in *E. coli* and then transferred to *R. metallidurans* by electroporation (*9, 15*). The resulting *R. metallidurans* based libraries were screened for the production of color and antibiosis. Similar assays have served as successful

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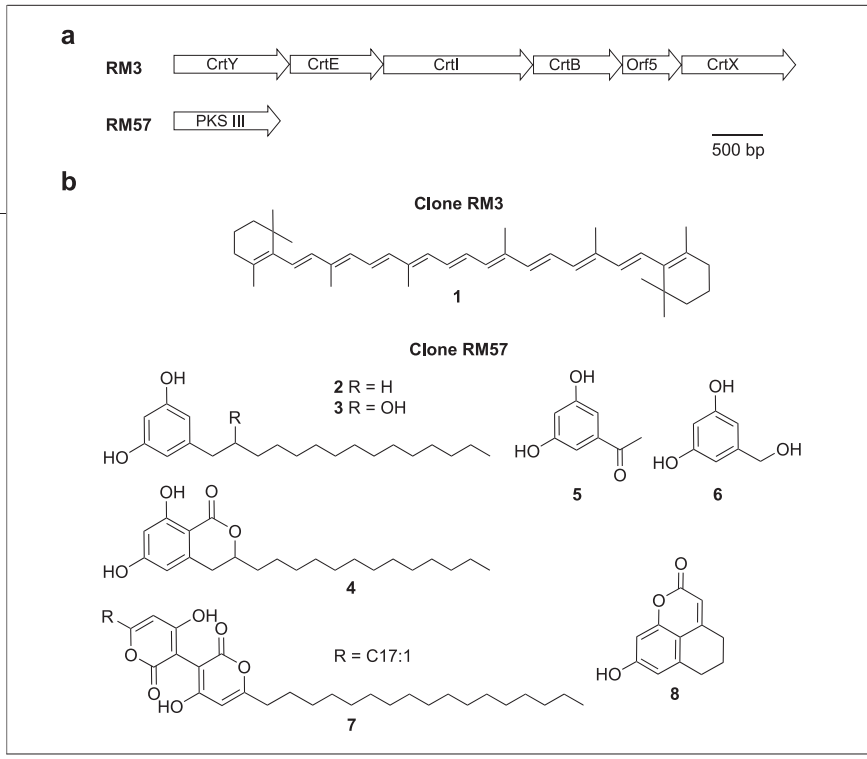

Figure 1. Environmental clones RM3 and RM57. a) The open reading frame(s) (ORF) responsible for the color and antibacterial phenotypes produced by RM3 and RM57, respectively. The ORF from RM57 is a predicted type III polyketide synthase (PKS) gene, and the ORFs from RM3 are related to genes for carotenoid (Crt) biosynthetic enzymes. b) Metabolites characterized from clones hosted in *R. metallidurans*. For compounds 2 and 3 only the major derivative present in the organic extract is shown. In addition to the C_{15} side chains shown, derivatives with $C_{15:1}$ and $C_{17:1}$ side chains were also detected in the organic extract (see Supporting Information).

high-throughput primary screens for identifying clones that produce small molecules in *E. coli* based eDNA libraries (*15*). Two representative clones, one colored (RM3) and one antibacterially active (RM57), that produce organic extractable small molecules were selected for further study. Ethyl acetate extracts of the culture broth from the colored clone (RM3) contained a yellow pigment, and extracts from the antibacterially active clone (RM57) contained seven clone-specific metabolites. Neither the cosmid from RM3 nor the cosmid from RM57 conferred the production of any detectable clone-specific small molecules to *E. coli*, the host used most frequently for eDNA studies.

In an attempt to identify the genes responsible for encoding the biosynthesis of the metabolites produced by these two clones, both cosmids were sequenced (GenBank accession nos. FJ151553 (RM3) and FJ151552 (RM57)). RM3 was found to contain a six-gene operon resembling a hybrid of the carotenoid gene clusters from *Bdellovibrio bacteriovorus* and *Xanthobacter autotrophicus* (Figure 1, panel a and Supplementary Table 1). 1D and 2D NMR and HRMS analysis of the yellow compound purified from cultures of RM3 showed that it was spectroscopically identical to the isoprenoid β-carotene (**1**).

Sequence gazing of the cosmid from RM57 did not identify any obvious candidate genes as the source of the clone-specific metabolites produced by RM57 (Supplementary Table 2). To identify the gene(s) responsible for the biosynthesis of these metabolites, the cosmid from RM57 was transposon mutagenized (<KAN-2> EZ-Tn5, Epicenter) and retransformed into *R. metallidurans*. Extracts from 97 transposon mutants were examined by TLC. Ninety-three mutant clones produced wild-type levels of the seven clone-specific metabolites isolated from cultures of RM57, one showed reduced production of all seven metabolites, and the remaining three produced no detectable clone-specific metabolites. The three knockout clones all contained transposon insertions in a putative type III polyketide synthase gene (RM57 protein, Rmp57). The transposon insertion in the mutant with reduced compound production is located just upstream of this ORF in a non-coding region that is likely the promoter for *Rmp57* (Supplementary Figure 1). *Rmp57* along with its promoter were subcloned from the RM57 cosmid as a 1680-bp PCR fragment and retransformed into *R. metallidurans*. Cultures of *R. metallidurans* transformed with *Rmp57* alone produced the same mixture of metabolites seen with the entire RM57 cosmid, indicating that Rmp57 is necessary and sufficient for the production of all seven clone-specific metabolites.

Rmp57 is most closely related (70% similar/53% identical) to a putative type III polyketide synthase found in the genome of *Acidobacteria bacterium Ellin345*. With few exceptions, members of the phylum Acidobacteria remain recalcitrant to culturing (*16, 17*). The metabolites produced by RM57 were isolated from culture broth extracts using a combination of normal phase chromatography and reversed phase HPLC, and the structure of each metabolite was determined by 1D and 2D NMR and HRMS (see Supporting Information). The family of clone-specific metabolites produced by RM57 is shown in Figure 1, panel b. The 1D and 2D NMR spectra for five of the seven metabolites (**2**−**6**) contained characteristic resorcinol-like aromatic signals that allowed us to easily assign their structures as long- and short-chain substituted resorcinol derivatives (*18, 19*). The NMR spectra for compounds **7** and **8** did not show the same characteristic signals and could not be reconciled with any known metabolites.

The ^{1}H spectrum of compound **7** contains a large methylene envelope similar to that seen in spectra from the long-chain resorcinol derivatives identified in this study. However, unlike the other metabolites derived from RM57 that each contain at least six deshielded carbon chemical shifts, the ^{13}C NMR spectrum of compound **7** contains only five deshielded carbons. The presence of only five deshielded carbons indicated

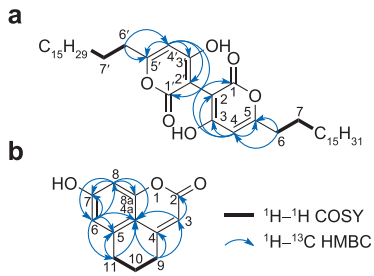

Figure 2. ^{1}H−^{1}H COSY and key ^{1}H−^{13}C HMBC correlations used for chemical structure determination. a) Compound 7. b) Compound 8.

that **7** could not contain the six-membered resorcinol headgroup seen in other RM57 metabolites. HMBC correlations from the lone olefinic proton singlet to the four remaining deshielded carbons along with HMBC correlations from the terminal methylene protons of the alkyl side chain to two of the deshielded carbons confirmed the presence of a tri-substituted six-membered pyrone headgroup in compound **7** (Figure 2). The molecular formula (HR-ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{44}H_{73}O_6$ 697.5407; found 697.5369) for compound **7** can only be reconciled with this NMR data if **7** is a dimer covalently linked through the C2 position of the pyrone headgroup. The most abundant pyrone dimer isolated from RM57 ethyl acetate extracts is a heterodimer that contains one saturated and one monounsaturated C_{17} side chain.

The ^{1}H spectrum of compound **8** contains neither the large methylene envelope nor the characteristic pair of resorcinol-derived doublets that are seen in other RM57 metabolites; ^{13}C chemical shift data together with HMBC correlations associated with H6 and H8 (Figure 2) did, however, suggest the presence of a tetra-substituted resorcinol-like system. This substituted resorcinol can be directly connected to a three-carbon methylene spin system (C9-C10-C11) by HMBC correlations from H11 to C4a, C5 and H6 to C11 (Figure 2). The other end of this spin system (C9) is linked by additional HMBC correlations to both the resorcinol-like ring and the C4-C3 olefin (Figure 2). The only way to place the final two atoms (CO) predicted by HRMS (HR-ESI-MS (*m*/*z*): $[M + H]^+$ calcd for $C_{12}H_{11}O_3$ 203.0708; found 203.0693) is to close the third ring and form the tricyclic isocoumarin (**8**).

To the best of our knowledge both compounds **7** and **8** are novel metabolites that have not been previously reported from studying cultured bacteria. The tricyclic isocoumarin-based carbon skeleton seen in compound **8** has not been previously reported as a natural metabolite nor as a synthetic compound. Its closest naturally occurring relatives are plant metabolites containing additional methyl and hydroxyl substituents (*20, 21*). While compound **7** has not been reported as a natural product, short-chain derivatives of this metabolite have been produced in studies designed to synthesize unnatural variants of type III polyketide synthase derived natural products (*22*). Alkylresorcinol (**2**), the major known product isolated from RM57 culture extracts, and the two novel metabolites (**7** and **8**) were assayed for antibacterial activity against *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. At the highest concentration tested (100 μg mL^{-1}) compound **2** showed no activity against any of these bacteria. The pyrone dimer (**7**) exhibited an MIC of 25 μg mL^{-1} against both *B. subtilis* and *S. aureus*, and the tricyclic isocoumarin (**8**) exhibited an MIC of 100 μg mL^{-1} against *B. subtilis*. At 100 μg mL^{-1}, compound **8** showed no activity against *S. aureus*, and neither compound **7** nor **8** exhibited activity against *E. coli*.

Although a bacterial type III polyketide synthase that produces both resorcylic metabolites as well as six-membered pyrone ring containing metabolites was recently reported (*23*), the possibility that a single type III polyketide synthase could generate all of the molecules produced by RM57 seemed unlikely. To explore the origin of these metabolites, Rmp57 was cloned under an inducible promoter in *E. coli*. In this system we found only pentadecylresorcinol (**2**) in the culture broth extracts. To investigate the possibility that many of the compounds characterized from *R. metallidurans* RM57 cultures might arise from a mixed biosynthetic pathway involving both the eDNA derived type III polyketide synthase and endogenous *R. metallidurans* enzymes, compound **2** was fed (0.2 mg mL^{-1}) to cultures of *R. metallidurans* harboring the empty cosmid vector. After 5−6 days of incubation at 30 °C both compounds **6** and **8** accumulated in the culture broth of fed cultures. Neither compound appeared in control cultures that were not fed with compound **2**.

A proposed biosynthetic scheme that explains the biogenesis of the metabolites isolated from RM57 is shown in Figure 3 (*23, 24*). In this scheme the formation of a pyrone or a resorcinol head group depends on the length of the starter unit used by Rmp57. The incorporation of a C_{16} starter unit results in the enzyme-catalyzed condensation of three malonyl-CoA extender units, and upon cyclization and decarboxylation, a resorcinol-based compound. The incorporation of a longer C_{18} starter unit largely results in the enzyme-catalyzed condensation of only two malonyl-CoA extender units, which upon cyclization yields a pyrone-based compound. Endogenous *R. metallidurans* oxidases could then convert these long-chain resorcinol-based metabolites into compounds **6** and **8** and pyrone monomers into dimers. The biosynthesis of the various metabolites extracted from cultures of RM57 can thus be rationalized by the capacity of Rmp57 to produce both resorcinol- and pyrone-based compounds depending on the pool of available starter units and conversion of these compounds to novel metabolites by endogenous *R. metallidurans* oxidases.

Compounds **7** and **8** are the first new metabolites to be identified from the heterologous expression of eDNA in a host other

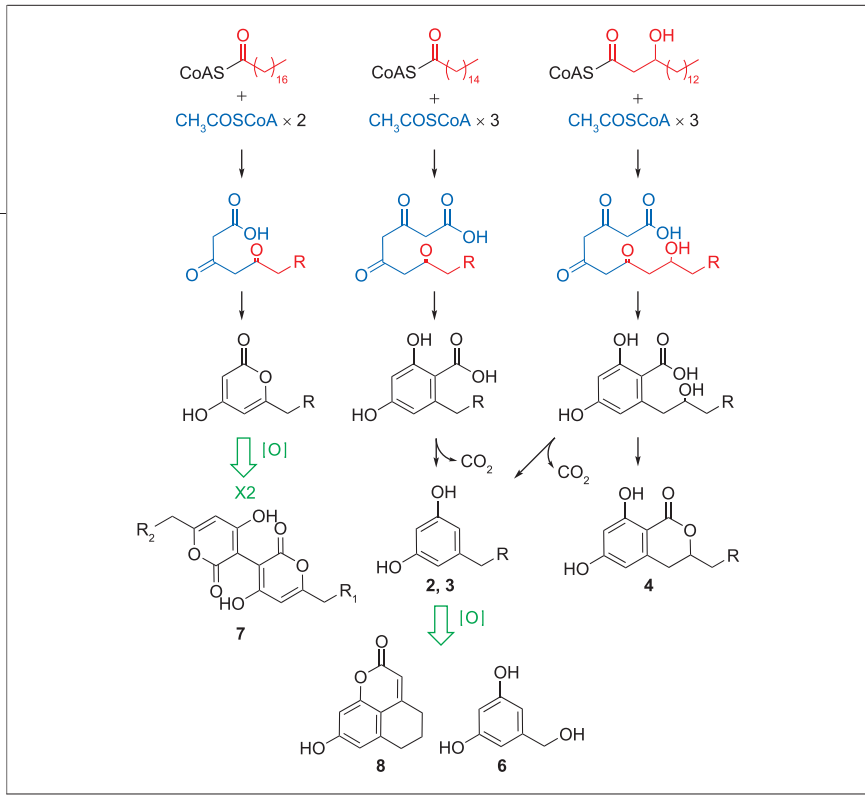

Figure 3. A biosynthetic scheme that includes both eDNA derived enzymatic steps (black arrows) and host enzymatic or spontaneous oxidation steps (green arrows) can be used to explain the biosynthesis of the clone-specific metabolites produced by RM57. Compound 5 was present in such low quantities in RM57 culture extracts (25 μg L^{-1}) that it was not possible to determine its biosynthetic origin.

than *E. coli* or *Streptomyces* spp. The novel metabolites characterized in this study appear to arise from the interaction of host enzymes with the metabolites produced from the expression of cloned eDNA. Both a host's native biosynthetic capacity as well as its propensity to recognize foreign promoters should be taken into consideration when choosing additional bacterial hosts for future eDNA studies. Accessing the biosynthetic potential encoded by DNA extracted from the mixtures of bacteria present in environmental samples will require the development of a diverse collection of model bacterial hosts that can be used to screen eDNA libraries. *R. metallidurans* represents a new model system that can now be used to expand the collection of small molecules found in future metagenomic studies.

METHODS

Broad-Host-Range Cosmid Vector Design. The 8.1-kb RK2-derivative plasmid pTR101 was used as the foundation for the construction of a broad-host-range cosmid vector (*25*). A 756-bp λ-cos containing ApoI fragment from pWEB was ligated into *Eco*RI-digested pTR101. pTR101-cos was then digested again with *Eco*RI and ligated to an 1828-bp *Eco*RI fragment containing a SacB gene with an internal ScaI restriction site. pTR101-cos-SacB was then digested with *Kpn*I and ligated to a 3507-bp fragment of pLAFR3 containing the KorB/IncC/KorA stability region of RK2, obtained by PCR amplification (primer pair: *Kpn*I-*Bam*HI-Kor-F: 5′-GGTACCGGATCCTTTAGCTGAACAGTTCTCGACTTA-3′, Kor-PmlI-*Bam*HI-R: 5′-CACGTGGGATCCCACGTGTTTAGCGGCTAAAGGTGTTGACGT-3′). This final cosmid vector, pJWC1, was twice digested with ScaI, CIP-treated, and agarose gel purified in preparation for the blunt-end cloning of environmental DNA.

Environmental DNA Isolation and Library Construction. Crude eDNA samples were prepared from soil using standard eDNA isolation methodology (*15*). In brief, a 1:1 mixture of soil and lysis buffer (100 mM Tris-HCl, 100 mM Na EDTA, 1.5 M NaCl, 1% (w/v) CTAB, 2% (w/v) SDS, pH 8.0) was heated for 2 h at 70 °C, followed by removal of soil particulate matter by centrifugation (4,000 × *g*, 30 min). Crude environmental DNA was precipitated from the supernatant with the addition of 0.7 vol of isopropanol, collected by centrifugation (4,000 × *g*, 30 min), washed with 70% (v/v) ethanol, and then resuspended in TE buffer (10 mM Tris-HCl, 1 mM Na EDTA, pH 8.0). The remaining soil particulate matter and humic substances were removed by large-scale gel purification on a 1% agarose gel (16 h at 20 V). Purified high molecular weight eDNA was then recovered by electroelution (2 h at 100 V) and concentrated by isopropanol precipitation. Environmental DNA was blunt-ended (Epicenter, End-It), ligated into a ScaI-digested and CIP-treated broad-host-range cosmid vector, packaged into lambda phage (Epicenter, MaxPlax Packaging Extracts), and transfected into EC100 *E. coli*. After overnight selection on LB (20 μg mL^{-1} tetracycline) agar plates, the resulting cosmid library was resuspended from the selection plates, and aliquots of the resuspended library were then used to inoculate liquid cultures. DNA miniprepped from these liquid cultures was used to electroporate *Ralstonia metallidurans*. For this study 125,000 cosmid clones were constructed from Oregon soil eDNA, and 450,000 cosmid clones were constructed from Pennsylvania soil eDNA.

Transformation of *Ralstonia metallidurans* CH34 with eDNA Cosmid Libraries and Phenotypic Screening for Antibacterial Activity. Electrocompetent cells were prepared according to established protocols (*9*). Electroporation reactions (1.0 mm cuvette, 1.8 kV pulse for 6 ms) for library production contained 2.5 μg of cosmid DNA and 80-μL aliquots of electrocompetent *Ralstonia metallidurans* CH34. After the electorporation pulse and the addition of 1 mL of LB supplemented with 10 mM $MgSO_4$, the cells were incubated with shaking at 37 °C for 1.5−2.5 h and then plated onto 150 mm diameter LB-tetracycline (20 μg mL^{-1}) selection plates at a titer of 1,500−2,500 colonies per plate. After 3−6 days at 30 °C the plates were screened by visual inspection for colored colonies, and a thin layer of LB-top-agar (15 mL per plate) containing *Bacillus subtilis* 1E9 (1:200 dilution of a culture at OD_{600} = 1.0) was poured onto each plate. After 1−2 days at 30 °C the plates were screened for colonies that produced zones of growth inhibition. Active colonies were picked with a sterile toothpick and struck onto triple-selection media (tetracycline 20 μg mL^{-1}, kanamycin 3 μg mL^{-1}, chloramphenicol 1.5 μg mL^{-1}) to remove contaminating *B. subtilis*.

Organic Extraction and Compound Isolation. For RM3 carotenoid extraction and isolation, the cell pellet from 1-L liquid cultures of *R. metallidurans* CH34 (LB with tetracycline 20 μg mL^{-1} and kanamycin 3 μg mL^{-1}) grown at 30 °C (200 rpm) for 5 days was extracted with acetone. Acetone extracts were then subjected to normal-phase flash chromatography with 100% hexanes. Fractions containing pure yellow pigment by analytical TLC were pooled, yielding compound **1**. For RM57 metabolite extraction and isolation, 1-L liquid cultures of *R. metallidurans* CH34 (LB with tetracycline 20 μg mL^{-1} and kanamycin 3 μg mL^{-1}) grown at 30 °C (200 rpm) for 3−6 days were extracted with ethyl acetate, and the dried extract was partitioned by normal-phase flash chromatography (step gradient = 100% hexanes, 95:5 hexanes/ethyl acetate, 90:10 hexanes/ethyl acetate (containing compound **4**), 75:25 hexanes/ethyl acetate (containing compound **2**), 50:50 hexanes/ethyl acetate (containing compounds **2** and **3**), 25:75 hexanes/ethyl acetate (containing compounds **3**, **5**, and **8**), 100% ethyl acetate, 90:10 ethyl acetate/methanol (containing compounds **6** and **7**). Fractions containing compound **4** were subjected to a second round of normal phase flash chromatography (step gradient = 100% chloroform, 99:1 chloroform/methanol (containing compound **4**), 95:5 chloroform/methanol). Pooled fractions containing compound **4** were then subjected to preparative TLC (95:5 acetone/dichloromethane) to give purified **4** (50 μg L^{-1}). Pooled fractions containing compound **3**, **5**, or **6** from the ini-

tial flash chromatography step were separately subjected to a second round of normal phase flash chromatography (step gradient = 100% chloroform, 98:2 chloroform/methanol, 95:5 chloroform/methanol (containing either compound **3** or compound **5**), 90:10 chloroform/methanol (containing compound **6**), 75:25 chloroform/methanol). Pooled fractions containing either compound **5** or **6** were subjected to preparative TLC: compound **5**, 90:10 dichloromethane/methanol, yield 25 μg L^{-1} or compound **6**, 80:20 dichloromethane/methanol, yield 100 μg L^{-1}. Pooled fractions containing compound **3** were subjected to preparative TLC (50:50 methanol/acetone) to yield purified **3** (150 μg L^{-1}). Pooled fractions containing either compound **2** or compound **8** from the initial round of flash chromatography were separately subjected to a second round of normal-phase flash chromatography (four step gradient = 100% chloroform, 99:1 chloroform/methanol, 97.5:2.5 chloroform/methanol (containing compound **8**), 90:10 chloroform/methanol (containing compound **2**, 2 mg L^{-1}). Pooled fractions containing compound **8** were subjected to preparative TLC (100% methanol) resulting in a final yield of 200 μg L^{-1}. Pooled fractions containing compound **7** from the initial round of flash chromatography were subjected to a second round of normal phase flash chromatography (four step gradient = 100% chloroform, 99:1 chloroform/methanol, 95:5 chloroform/methanol (containing compound **7**), 90:10 chloroform/methanol. Pooled fractions containing compound **7** were then subjected to preparative HPLC (Waters XBridge C18 column (10 × 150 mm), 7 mL min^{-1}, 90:10 H_2O/MeOH with 0.1% formic acid to 100% MeOH with 0.1% formic acid over 7 min, then holding at 100% MeOH with 0.1% formic acid for 8 min, resulting in a final yield of 200 μg L^{-1}. Compound **7**: ^1H NMR (methanol-d_4) δ 5.94 (2H, s, H4/H4′), 2.49 (4H, t, 7.6, H6/H6′), 1.67 (4H, p, 7.3, H7/H7′), 1.37−1.29 (56H, m, H8−H21/H8′−H21′), 0.92 (6H, t, 7.0, H22/H22′); $^1H-^{13}C$ HMBC derived ^{13}C (methanol-d_4) (C1−C6, C7−C21, C22) δ 161.4, 87.7, 172.5, 100.3, 166.4, 31.3, 28.9−21.9, 13.0. Compound **8**: ^1H NMR (methanol-d_4) δ 6.62 (1H, t, 1.1, H6), 6.57 (1H, d, 2.2, H8), 5.98 (1H, s, H3), 2.92 (2H, t, 6.1, H11), 2.84 (2H, td, 6.1, 1.0, H9), 1.98 (2H, p, 6.2, H10); $^1H-^{13}C$ HMBC derived ^{13}C (methanol-d_4) (C2−C11) δ 162.9, 105.9, 156.0, 109.4, 140.3, 111.3, 161.2, 99.9, 155.3, 29.4, 22.0, 28.7.

Cloning and Expression of Rmp57 in *E. coli*. The 1077-bp *Rmp57* gene was amplified (30 cycles of 97 °C for 15 s, 55 °C for 20 s, and 72 °C for 60 s, NEB Taq and Thermopol buffer) from environmental clone RM57 using the following primers: AE57PKS3*Eco*RIF: 5′-ATCCCCGGGAATTCAATGCGAATTGCATCTGTAGCAACAGCC-3′, AE57PKS3*Eco*RIR: 5′-GATAACGGGAATTCATCATGTACTTGAACCACGGATCAGTCC-3′, which introduce *Eco*RI restriction sites upstream and downstream from *Rmp57*. The PCR amplicon was digested with *Eco*RI, ligated into *Eco*RI-digested/CIP-treated pGEX-3X, and then transformed into S17.1 *E. coli*. Clones testing positive for the presence of the *Rmp57* gene in the correct orientation were transferred to liquid cultures (LB-ampicillin 50 μg mL^{-1}) and incubated at 20, 30, and 37 °C with shaking, in both the presence and absence of 1.0 mM IPTG. After 3−5 days, cultures were extracted with an equal volume of ethyl acetate, dried under vacuum, and analyzed by normal-phase TLC and reversed-phase HPLC.

Subcloning of Rmp57 in *R. metallidurans*. The *Rmp57* gene and its upstream promoter region were amplified (30 cycles of 97 °C for 20 s, 50 °C for 20 s, and 68 °C for 120 s, NEB Taq and Thermopol buffer) from environmental clone RM57 using the following primers: XbaI500Rmp57Ups: 5′-TACGTAAATCTAGAAAGACGCCGCGACGAGCCAATCAGCAGATG-3′, AE57PKS3*Eco*RIR: 5′-GATAACGGGAATTCATCATGTACTTGAACCACGGATCAGTCC-3′. The PCR amplicon was blunt-ended (Epicenter, End-It) and ligated into the ScaI-digested and CIP-treated broad-host-range cosmid vector, then transformed into EC300 *E. coli*. DNA miniprepped from successful ligation transformants was then electroporated into *R. metallidurans*. Liquid cultures of *R. metallidurans* containing *Rmp57* and its endogenous promoter were incubated at 30 °C with shaking for 4 days, after which they were extracted with an equal volume of ethyl acetate, dried under vacuum, and analyzed by normal-phase TLC and reversed-phase HPLC.

Antibacterial and Antifungal Activity Assays. Activity assays were carried out against *S. aureus* (ATCC 6538), *B. subtilis* 1E9, and *E. coli* DRC39 ΔAcrAB. Overnight cultures grown in LB were diluted 10^6-fold, 200 μL of cell suspension was aliquoted into each well of the first row of a 96-well microtiter plate, and 100 μL of cell suspension was then aliquoted into all remaining wells. Compounds were resuspended in DMSO, added to the first well within a row (to a final concentration of 100 μg mL^{-1}), and then serially diluted in two-fold steps down each respective row. Plates were incubated overnight at 30 °C with shaking, and the minimum inhibitory concentrations were recorded the following morning.

Accession Codes: RM3 carotenoid gene cluster: GenBank accession no. FJ151553. RM57 environmental DNA insert: GenBank accession no. FJ151552.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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