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(71) Applicant (for all designated States except US): **UNIVERSITY OF MARYLAND BIOTECHNOLOGY INSTITUTE OFF. OF RESEARCH ADMIN/TECH.DEV.** [US/US]; 701 E. Pratt Street, Suite 200, Baltimore, MD 21202 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **BELAS, Robert** [US/US]; 110 Newburg Avenue, Catonsville, MD 21228 (US). **GENG, Haifeng** [US/US]; 120 Nunnery Lane, Apt.C, Baltimore, MD 21228 (US).(74) Agent: **HULTQUIST, Steven, J.**; Intellectual Property/Technology Law, P.o. Box 14329, Research Triangle Park, NC 27709 (US).

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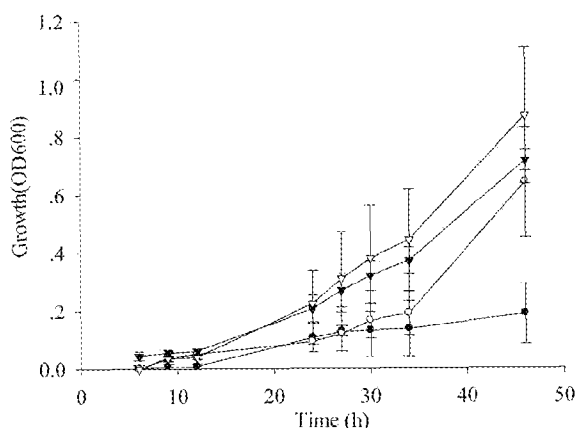
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(54) Title: BIOSYNTHETIC PATHWAY AND GENES REQUIRED FOR TROPODITHIETIC ACID BIOSYNTHESIS IN SILICIBACTER TM1040

(57) Abstract: The symbiotic association between the roseobacter *Silicibacter* sp. TM1040 and the dinoflagellate *Pfiesteria piscicida* involves bacterial chemotaxis to dinoflagellate-produced dimethylsulfoniopropionate (DMSP), DMSP demethylation, and ultimately a biofilm on the surface of the host. Biofilm formation is coincident with the production of an antibiotic and a yellow-brown pigment. The antibiotic is a sulfur-containing compound, tropodithietic acid (TDA). Using random transposon insertion mutagenesis, 12 genes were identified as critical for TDA biosynthesis by the bacteria, and mutation in any one of these results in loss of antibiotic activity (Tda) and pigment production. A biosynthetic and regulatory pathway for TDA biosynthesis in roseobacters is described.

strain	SO ₄	SO ₃	Met	Cys	DMSP
<i>cysI</i> growth	-	-	-	+	-
WT growth	+	+	+	+	+
<i>cysI</i> TDA	-	-	-	+	-
WT TDA	+	+	+	+	-



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**BIOSYNTHETIC PATHWAY AND GENES REQUIRED FOR TROPODITHIETIC
ACID BIOSYNTHESIS IN SILICIBACTER TM1040**

GOVERNMENT RIGHTS IN INVENTION

[0001] Work related to the invention was conducted in the performance of National Science Foundation Grant MCB0446001. The United States Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATION

[0002] This application claims the benefit of priority of U.S. Provisional Patent Application 60/861,117 filed November 27, 2006 in the names of Robert Belas, et al. for "BIOSYNTHETIC PATHWAY AND GENES REQUIRED FOR TROPODITHIETIC ACID BIOSYNTHESIS IN SILICIBACTER TM1040." The disclosure of U.S. Provisional Patent Application 60/861,117 is hereby incorporated herein by reference in its entirety, for all purposes.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] This invention relates to *Roseobacter* bacteria and to the production of antibiotics by use of such microbial species.

Description of the Related Art

[0004] Bacteria of the *Roseobacter* clade of marine alpha-Proteobacteria stand out as some of the most critical players in the oceanic sulfur cycle due to the ability of several genera to degrade dimethylsulfoniopropionate (DMSP). While roseobacters are wide-spread throughout the marine ecosystem, their abundance is significantly correlated with DMSP-producing algae, especially prymnesiophytes and dinoflagellates, such as *Prorocentrum*, *Alexandrium* and *Pfiesteria* species.

[0005] Roseobacters have abundant and diverse transporters, complex regulatory systems, multiple pathways for acquiring carbon and energy in seawater, with the potential to produce secondary, biologically active metabolites.

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SUMMARY OF INVENTION

[0006] The present invention relates to *Roseobacter* bacteria and to the production of antibiotic tropodithietic acid (TDA) by use of such microbial species.

[0007] In one aspect, the invention relates to an isolated nucleic acid encoding a megaplasmid (pSTM3) of *Silicibacter* sp. TM1040, wherein the nucleic acid comprises genes involved in tropodithietic acid biosynthesis of *Roseobacter* bacteria.

[0008] Another aspect of the invention relates to a protein encoded by a nucleic acid sequence represented by SEQ. ID. 1; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

[0009] Yet another aspect of the invention relates to a protein encoded by a nucleic acid sequence represented by SEQ. ID. 2; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

[0010] In another aspect, the invention relates to a protein encoded by a nucleic acid sequence represented by SEQ. ID. 3; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

[0011] An additional aspect of the invention relates to a protein encoded by a nucleic acid sequence represented by SEQ. ID. 4; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

[0012] A further aspect of the invention relates to an antibacterial composition comprising tropodithietic acid isolated from bacteria of the *Roseobacter* clade

[0013] Another aspect of the invention relates to a method for producing an antibacterial composition comprising tropodithietic acid, the method comprising:

a) culturing *Silicibacter* sp. TM1040 in a culture medium supporting growth of the bacterium and production of tropodithietic acid; and

- b) separating the tropodithietic acid from the culture medium; and
- c) purifying the tropodithietic acid by high performance liquid chromatography.

[0014] Still another aspect of the invention relates to a method for producing an antibacterial composition comprising tropodithietic acid, the method comprising:

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- a) culturing *Roseobacter* sp.27-4 in a culture medium supporting growth of the bacterium and production of tropodithietic acid; and
- b) separating the tropodithietic acid from the culture medium; and
- c) purifying the tropodithietic acid by high performance liquid chromatography.

10 [0015] A further aspect of the invention relates to a method of treating or preventing bacterial disease in a subject in need of such treatment or prevention, comprising administering to said subject an antibacterial composition comprising tropodithietic acid isolated from bacteria of the *Roseobacter* clade.

[0016] Yet another aspect of the invention relates to a plasmid pSTM3.

15 [0017] Another aspect of the invention relates to a compound selected from the group consisting of:

- 1, 2-dihydro-phenylacetyl-CoA;
- 2-hydroxy-7-oxo-cyclohepta-3,5-dienecarboxylic acid;
- 2,7-; dihydroxy-cyclohepta-1,3,5-trienecarboxylic acid;
- 20 2,7-dihydroxy-3-oxo-cyclohepta-1,4,6-trienecarboxylic acid;
- 2,7-dihydroxy-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid; and
- 7-hydroxy-2-mercapto-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid.

[0018] Other aspects, features and advantages of the invention will be more fully apparent from the ensuing disclosure and appended claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG. 1. When grown in static liquid media, *Silicibacter* sp. strain TM1040 produces a yellow-brown pigment and has a large amount of antibacterial activity, which was measured by a

well diffusion assay using *V. anguillarum* as the target organism (Materials and Methods). In contrast, pigment and antibacterial compound is very low under 30°C shaking conditions.

[0020] FIG. 2. Tropodithietic acid. C18 reverse phase HPLC chromatograms of ethyl acetate extracts from TM1040 and *Phaeobacter* 27-4. Insets show the UV spectra of the HPLC peak corresponding to the antibiotic activity. For 27-4, the peak is TDA.

[0021] FIG. 3. Genes required for synthesis of TDA in TM1040. The black boxes indicate the ORF interrupted by the transposon. Arrows indicate ORFs transcriptional orientations and hatch marks indicate a break in the region. (A) Sulfur assimilation genes, *tdaH*, *malY*, *cysI*, are located in the TM1040 chromosome. Phenylacetate catabolism genes are in the megaplasmid pSTM1.

(B) *tdaA~tdaF* genes reside on a cryptic plasmid, with their closest homologues found on the chromosome of *P. denitrificans* PD1222. *tdaH* encodes sulfite oxidase domain protein; *hik2* encodes two-component hybrid sensor and regulator; *malY* encodes β -C-S lyase (cystathionase); *asnC* encodes transcriptional regulator AsnC family; *cysG* encodes siroheme synthesis; *hypo* encodes hypothetical protein; *cysI* encodes sulfite reductase beta (siroheme- dependent); *cysH* encodes adenylylsulfate reductase; *gntR* encodes GntR family transcriptional regulator; *paaG*, *paaH*, *paaI*, *paaJ*, *paaK* encode respectively phenylacetic acid degradation protein complex protein 1,2,3,4,5; *tdaA* encodes LysR substrate binding domain protein; *tdaB* encodes β -etherase; glutathione-S-transferase; *tdaC* encodes prephenate dehydratase; *tdaD*, 4-hydroxybenzoyl-CoA thioesterase; *tdaE* encodes Acyl-CoA dehydrogenase; *tdaF* encodes phosphopantothienoylcysteine decarboxylase. *P. denitrificans* PD1222 genome contains two chromosomes and one plasmid, whereas *tdaAB*, *tdaCDE* and *tdaF* homologue genes located discretely in a 19 kb region of chromosome 1.

[0022] FIG. 4. Growth and TDA synthesis is affected by mutations in *cysI*. TM1040 (inverted triangles) and the *cysI* mutant (HG1220; circles) were grown in minimal medium containing either methionine (closed symbols) or methionine (open symbols), and growth was measured optically at 600 nm. Unlike the wild-type, the CysI mutant cannot grow methionine, but does utilize cysteine. Measurement of antibiotic activity indicates that the *cysI* defect also affects TDA synthesis, which is corrected by the addition of cysteine to the medium, but not methionine, DMSP, sulfite, or sulfate addition (table).

[0023] FIG. 5. TM1040 *tda* genes reside on a cryptic plasmid that undergoes a low frequency spontaneous loss. (A). Pigment synthesis. TM1040 (wt) produces a yellow-brown extracellular pigment that is correlated with TDA synthesis. In contrast, a *tdaE*:Tn mutant (strain HG1265) and a spontaneous mutant (sm; TM1040SM) are nonpigmented and have lost the ability to produce both TDA and pigment. (B). Spontaneous loss of pigment and antibiotic activity results from a loss of *tda* genes. PCR amplification of *tdaE* results in a band from wt and *tdaE*:Tn DNA, respectively, with the additional 2 kb in size of the *tdaE*:Tn product resulting from insertion of the transposon. No product was amplified from the spontaneous nonpigment mutant (sm). (C). PFGE separation of total DNA obtained from TM1040 (wt), the spontaneous nonpigmented mutant (sm), and the *tdaE*:Tn mutant. All three strains show a fuzzy band at ca. 130 kb, with a slight increases in the width of the wt and *tdaE* bands. (D). Southern blot hybridization of the PFGE gel to labeled *tdaE* DNA. The *tdaE* probe hybridized to the band migrating at ca. 130 kb in both wt and *tdaE*:Tn (first and third lanes), but failed to hybrid to the DNA obtained from the spontaneous nonpigmented mutant. (E). *Nco*I digestion of plasmid DNA isolated from TM1040 (wt), the spontaneous nonpigmented mutant (sm), and HG1265 (*tdaE*:Tn), respectively. The digested DNAs were separated by electrophoresis and the band patterns compared to each other and to an *in silico* *Nco*I digestion of pSTM2 (supplemental data). The pattern of fragments from sm DNA matched the predicted pSTM2 *Nco*I digestion, while both wt and *tdaE* DNA patterns showed evidence of additional restriction fragments. (F). Southern blot hybridization of *Nco*I-digested plasmid DNA to *tdaE*. A *tdaE* probe hybridizes to one fragment in wt and *tdaE*:Tn DNA cut with *Nco*I, but to any fragments produced from *Nco*I digestion of plasmid. The increase in the size of the fragment in *tdaE*:Tn results from the insertion of the 2 kb transposon.

[0024] FIG. 6. *Nco*I digestion patterns of pSTM3 transformed into *E. coli*. A mixture of plasmid pSTM3-1265 (pSTM3 harboring a transposon in *tdaE*) and pSTM2 was isolated from HG1265 and the DNAs used to transform *E. coli* (Materials and Methods). Each of the plasmids harbored in the resulting Kan^r transformants was purified, digested with *Nco*I, and the resulting DNA fragments separated by agarose gel electrophoresis. Of the total DNAs examined, four types of band patterns emerged and are shown in lanes 1-4, respectively.

[0025] FIG. 7. DNA from other roseobacter species hybridizes to *tda* DNA. Total DNA was extracted from 13 roseobacters, TM1040, and a non-roseobacter control species (*V. anguillarum*), and used in a slot blot hybridization with labeled *tda* DNA. Positive hybridization was strongly correlated with measurable antibiotic activity (indicated by an asterick). The strains used were: ISM: *Roseovarius* strain ISM; TM1038: *Roseobacter* sp. strain TM1038; TM1039, *Roseobacter* sp. strain TM1039; 33942, *Roseobacter denitrificans* ATCC 33942; SE62, *Sulfitobacter* strain SE62; 49566, *Roseobacter litoralis* ATCC 49566; DSS-3, *Silicibacter pomeroyi* DSS-3; EE36, *Sulfitobacter* strain EE36; 1921, *Sulfitobacter* strain 1921; TM1040, *Silicibacter* sp. TM1040; V. a, *Vibrio anguillarum*; 51442, *Roseobacter algicola* ATCC 51442; 27-4, *Phaeobacter* 27-4; TM1035, *Roseovarius* sp. strain TM1035; and, TM1042, *Roseovarius* sp. strain TM1042.

[0026] FIG. 8. The presence and relative abundance of each of the Tda proteins identified in TM1040 (rows) in the GOS metagenomic database (via the internet website at [hypertext transfer protocol address, camera.calit2.net/](http://hypertexttransferprotocoladdress.camera.calit2.net/)). The relative abundance is based on the total BLASTP matching sequences in the individual filters using a cutoff E value of 1E-20 (48). The distribution of Tda proteins harbored on pSTM3 (TdaA-F) in the sample is remarkably different from the distribution of Paa and sulfur metabolism proteins (CysI, MalY, and TdaH), which have a more even distribution throughout the series of samples. Relative abundance is indicated by the size of the circle. GOS sample numbers are indicated on the horizontal axis.

[0027] FIG. 9. A putative model of the TDA biosynthetic pathway based on the present genetic analysis. The suggested pathway involved phenylacetate derivation from shikimate-chorismate and degradation pathway providing precursors (step 1~6) and an core oxidative ring-expansion pathway forming the seven carbon tropolone skeleton (step7~10) followed by sulfur-oxygen exchange (step11~15), consistent with the proposed TDA synthesis based on chemical labeling studies in *Pseudomonas* CB-104 (14). The protein assignment was based on predicted functions.

DETAILED DESCRIPTION OF THE INVENTION

[0028] The present invention relates to *Roseobacter* bacteria and to the production of tropodithietic acid (TDA) by use of such microbial species.

[0029] The symbiotic association between the roseobacter *Silicibacter* sp. TM1040 and the dinoflagellate *Pfiesteria piscicida* involves bacterial chemotaxis to dinoflagellate-produced dimethylsulfoniopropionate (DMSP), DMSP demethylation, and ultimately a biofilm on the surface of the host. Biofilm formation is coincident with the production of an antibiotic and a yellow-brown pigment. The antibiotic is a sulfur-containing compound, tropodithietic acid (TDA). Using random transposon insertion mutagenesis, 12 genes were identified as critical for TDA biosynthesis by the bacteria, and mutation in any one of these results in loss of antibiotic activity (Tda⁻) and pigment production. Unexpectedly, six of the genes, referred to as *tdaA-F*, could not be found on the annotated TM1040 genome and were instead located on a previously unidentified cryptic megaplasmid (ca. 130 kB; pSTM3) that exhibited a low frequency of spontaneous loss. Homologs of *tdaA* and *tdaB* from *Silicibacter* sp. TM1040 were identified by mutagenesis in another TDA-producing roseobacter, *Phaeobacter* 27-4, which also possesses two large plasmids (ca. 60 and ca. 70 kb, respectively), and *tda* genes were found by DNA:DNA hybridization in 88 % of a diverse collection of 9 roseobacters with known antibiotic activity. These data suggest that roseobacters employ a common pathway for TDA biosynthesis that involves plasmid-encoded proteins. Using metagenomic library databases and a bioinformatics approach, a pronounced difference in the biogeographical distribution between the critical TDA synthesis genes was observed, implying substantial environmental preference differences among these genes.

[0030] The present invention in another specific aspect relates to the interaction of a roseobacter, *Silicibacter* sp. TM1040, and *Pfiesteria piscicida*. *Silicibacter* sp. TM1040 (hereafter referred to as TM1040) is isolatable from laboratory microcosm culture of heterotrophic DMSP-producing dinoflagellate *P. piscicida*. Marine algae are major producers of DMSP in the marine environment while members of the *Roseobacter* clade are capable of DMSP catabolism. TM1040 degrades DMSP via a demethylation pathway producing 3-methylmercaptopropionate (MMPA) as a major breakdown product. The bacteria respond via chemotaxis to dinoflagellate homogenates, and are specifically attracted to DMSP, methionine, and valine. TM1040 motility is important in the initial phases of the symbiosis. Once the bacteria are in close proximity to their host, TM1040 forms a biofilm on the surface of the dinoflagellate. The symbiosis includes

two parts: one that involves chemotaxis and motility, and a second step in which a biofilm predominates.

[0031] Specific phenotypes, e.g., the ability to produce antibacterial compounds and biofilm formation, may give members of the *Roseobacter* clade a selective advantage, and help to explain the dominance of members of this clade in association with marine algae. The production of an antibiotic activity is observed in roseobacters and is hypothesized to provide an advantage when colonizing phytoplanktonic hosts, such as dinoflagellates. The genome of TM1040 consists of a 3.2 Mb chromosome and two plasmids, pSTM1 (823 Kb) and pSTM2 (131 Kb) (41). A comparison between TM1040 and two other roseobacters (*Silicibacter pomeroyi* DSS-3 and *Jannaschia* sp. CSS-1) suggests that roseobacters have abundant and diverse transporters, complex regulatory systems, multiple pathways for acquiring carbon and energy in seawater, with the potential to produce secondary, biologically active metabolites.

[0032] Biologically active metabolites, including antibacterial compounds, are obtainable from roseobacters. A sulfur-containing antibiotic compound, tropodithietic acid (TDA), has been isolated and chemically characterized from *Phaeobacter* 27-4, hereafter called 27-4, and *Roseobacter* T5. The chemical backbone of TDA (shown in Fig. 2) is a seven member aromatic tropolone ring, which is highly significant as tropolone derivatives, notably hydroxylated forms, are widely seen as medically important sources of antibacterial, antifungal, antiviral, and antiparasitic agents. Thiotropocin, another trophione derivative closely related to TDA, can be synthesized from shikimate by an oxidative ring expansion of phenylacetic acid.

[0033] We have used both genomic and genetic techniques to identify the genes and proteins required for TDA synthesis in TM1040 and 27-4 as models for the *Roseobacter* clade. In the process of locating these genes, we discovered a megaplasmid critical for TDA biosynthesis that is part of the TM1040 genome, as hereinafter more fully described.

[0034] Materials and Methods

[0035] Bacteria and media

[0036] The strains used in our study are listed in (Table 1). *Silicibacter* sp. TM1040, *Phaeobacter* 27-4 and *Vibrio anguillarum* 90-11-287 were grown and maintained in 2216 marine broth or 2216 agar as recommended by the manufacturer (BD Biosciences, Franklin Lakes, NJ).

A marine basal minimal medium (MBM; per liter: 8.47g Tris HCl, 0.37 g of NH₄Cl, 0.0022 g of

K₂HPO₄, 11.6 g NaCl, 6 g MgSO₄, 0.75 g KCl, 1.47 g CaCl₂·2H₂O, 2.5 mg FeEDTA; pH 7.6, 1 ml of RPMI-1640 vitamins [Sigma R7256]) was used for determining carbon and sulfur requirements. Sole carbon sources were added at a final concentration of 1 g/l. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar containing 1.5% Bacto Agar (Becton Dickinson, Franklin Lakes, N.J.). As appropriate, kanamycin was used at 120 µg per ml for *Roseobacter* strains and 50 µg per ml for *E. coli*.

[0037] Characterization of antibiotic

[0038] Bacterial spent medium was either injected directly (up to 10 µL) or purified by mixed phase anion-exchange reversed phase mini column chromatography on Oasis MAX columns as previously described. Tropodithietic acid was analyzed by reverse phase liquid chromatography (LC) on an Agilent 1100 HPLC system equipped with a diode array detector (DAD). Separation was conducted using a Phenomenex (Torrance, CA) Curosil PFP 15 cm, 2 mm, 3 µm column using a water-acetonitrile (ACN) gradient system. Both solvents contained 200 µL/L trifluoroacetic acid, and started 35% ACN increasing this linear to 60% in 6 min. The wavelength 304 ± 4 nm was used for detection. LC-DAD with online high resolution mass spectrometry (HR-MS) using positive and negative electrospray was used for validation of tropodithietic acid detection as previous described.

[0039] Transposon mutagenesis and Tda⁻ screening

[0040] Electrocompetent roseobacter strains were prepared following the method described by Garg et al. as modified by Miller and Belas. Random transposon insertion libraries were constructed in TM1040 and 27-4 using the EZ-Tn5<R6K_{γori}/KAN-2>Tnp Transposome™ Kit (Epicentre, Madison, WI). Strains were spread onto 2216 plates containing kanamycin and incubated for 1 day at 30 °C. Individual Kan^r transposon insertion strains were transferred to 7x7- arrays on 2216 marine agar plus kanamycin to facilitate further screening. To screen for loss-of-function, antibiotic-negative (Tda⁻) mutants, a modification of the method described by Bruhn et al. was used. Bacteria were replicated, as a 7x7 array, to a lawn of *Vibrio anguillarum* strain 90-11-287, and incubated at 20°C for 24 h, after which a zone of clearing indicative of antibiotic production was measured and compared to the parental strain (TM1040 or 27-4). For purposes of this study, Tda⁻ is defined as a strain lacking a detectable zone of clearing on *V. anguillarum*. Strains determined to be Tda⁻ by the modified well-diffusion assay were further

tested by incubation at 30°C for 48 h in 2216 marine broth without shaking. Bacteria were removed by filtering through a 0.22 µm MCE membrane (Millex ,Millipore, Bedford, MA) and the antibacterial activity of the supernatant measured using the *V. anguillarum* well diffusion assay, as described by Bruhn et al.

5 **[0041] Sole carbon and sulfur source growth**

[0042] Bacterial utilization of sole carbon sources was determined by measuring growth in MBM broth that was modified by replacing glycerol with the carbon source to be tested. Carbon compounds tested included amino acids (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine); sugars (arabinose, fructose, galactose ,glucose, lactose, maltose, mannose, N-acetylglucosamine, ribose, sucrose, xylose); tricarboxylic acid cycle (TCA) intermediates (citrate, fumurate, succinate); as well as phenylacetic acid and sodium phenylpyruvate.

[0043] Sulfur utilization was tested by growth in MBM containing different sulfur sources:

15 DMSP, cysteine, methionine, sodium sulfate, and sodium sulfite.

[0044] Bioinformatics analysis

[0045] Approximately 1 µg of genomic DNA isolated from the candidate mutant was digested with *Nco* I, self-religated with T4 DNA ligase, and electroporated into DH5α (λpir). Following selection for kanamycin resistance, Kan^r colonies were picked and the plasmid isolated for bidirectional sequencing with transposon-specific primers as recommended by the supplier (Epicentre, Madison, WI). Nucleotide sequence thus obtained was analyzed by BLAST analyses using DNA-DNA homology searches again the *Silicibacter* sp. TM1040 genome (Accession numbers: NC_008044 , NC_008043 , NC_008042). The genes identified are listed in Table 2 for TM1040 and Table 3 for 27-4.

25 **Table 2. *Silicibacter* sp. TM1040 genes and encoded proteins required for the regulation and synthesis of tropodithietic acid.**

Gene Number	GenBank Assession Number	Gene Designation	Function	Best Hit Ortholog / E score
Ring Precursors, Oxidation, and Expansion				

TM1040_3728	CP000376	<i>paaK</i>	Phenylacetate oxidoreductase	<i>Roseobacter</i> sp. MED193 phenylacetic acid degradation oxidoreductase PaaK / 8e-161
TM1040_3726	CP000376	<i>paaI</i>	Phenylacetate oxygenase	<i>Roseobacter</i> sp. MED193 phenylacetic acid degradation protein PaaI / 4e-110
TM1040_3727	CP000376	<i>paaJ</i>	Phenylacetate oxygenase	<i>Roseobacter</i> sp. MED193 phenylacetic acid degradation protein PaaJ / 2e-69
EF139203	EF139203	<i>tdaD</i>	4-hydroxybenzoyl-CoA thioesterase	<i>Paracoccus</i> . <i>denitrificans</i> PD1222 conserved hypothetical protein / 2e-45
EF139204	EF139204	<i>tdaE</i>	Acyl-CoA dehydrogenase	<i>Paracoccus</i> <i>denitrificans</i> PD1222 acyl- CoA dehydrogenase / 9e-120
EF139201	EF139201	<i>tdaB</i>	β -etherase, glutathione S transferase	<i>Paracoccus</i> <i>denitrificans</i> PD1222 putative β -etherase (β -aryl ether cleaving enzyme) protein / 6e-56
EF130202	EF130202	<i>tdaC</i>	Prephenate dehydratase	<i>Paracoccus</i> <i>denitrificans</i> PD1222 hypothetical protein / 2e-45
Sulfur Metabolism and Addition				

TM1040_2581	CP000377	<i>malY</i>	β -C-S lyase (cystathionase); amino transferase	<i>Roseobacter</i> sp. MED193 aminotransferase, classes I and II / 0.0
TM1040_0961	CP000377	<i>tdaH</i>	Sulfite oxidase domain protein	<i>Sulfitobacter</i> sp. NAS-14.1 hypothetical protein / 7e-34
TM1040_1758	CP000377	<i>cysI</i>	Sulfite reductase	<i>Roseobacter</i> sp. MED193 sulfite reductase / 0.0
Co-enzyme A Metabolism				
EF139205	EF139205	<i>tdaF</i>	Phosphopantothienoylcysteine decarboxylase	<i>Paracoccus denitrificans</i> PD1222 flavoprotein / 2e-55
Regulatory Mechanism				
EF139200	EF139200	<i>tdaA</i>	LysR substrate binding domain protein	<i>Paracoccus denitrificans</i> PD1222 regulatory protein, LysR:LysR, substrate-binding / 1e-29

Table 3. Sole carbon source tested for TM1040 and mutants.

Gene	Cy s	Trp	Phe	Phenylaceti c acid	Sodium phenylpyruvat e	Sodium phenylbutyra te	2216	Other Amino acid
WT	+	+	+	+	+	+	+	+
<i>paal</i>	+	-	-	-	-	-	+	+
<i>paaJ</i>	+	-	-	-	-	-	+	+
<i>paaK</i>	+	-	-	-	-	-	+	+
<i>tdaA</i>	+	+	+	+	+	+	+	+
<i>tdaB</i>	+	+	+	+	+	+	+	+
<i>tdaC</i>	+	+	+	+	+	+	+	+
<i>tdaD</i>	+	+	+	+	+	+	+	+
<i>tdaE</i>	+	+	+	+	+	+	+	+
<i>tdaF</i>	+	+	+	+	+	+	+	+
<i>cysI</i>	+	-	-	-	-	-	+	-

<i>malY</i>	+	+	+	+	+	+	+	+
<i>tdaH</i>	+	+	+	+	+	+	+	+

[0046] Signature amino acid domains in the deduced amino acid sequence of the respective ORFs were identified using BLASTP, Pfam, SMART, and the Conserved Domains Database (CDD; <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). Homologs in roseobacters were identified using BLASTP analysis of Roseobase (<http://www.roseobase.org/>) and Gordon and Betty Moore Foundation Marine Microbial Genome databases (<https://research.venterinstitution.org/moore/>) with respective predicted protein sequence as the query sequence and a maximum E value of 1E-30. Homologs in the Global Ocean Sampling Expedition metagenomic libraries (<http://camera.calit2.net/index>) were identified by BLASTP analysis using a cutoff E value 1E-20.

[0047] DNA extraction and separation

[0048] Chromosomal DNA was extracted from bacterial cells by routine methods or by the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA). Plasmid DNA was prepared by the alkaline lysis method, digested with *NcoI* (New England Biolabs, Beverly, MA), and the resulting restriction fragments were separated by agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer.

[0049] Pulsed Field Gel Electrophoresis (PFGE) was performed using a CHEF DR-III clamped homogeneous electric field system (Bio-Rad, Richmond, Calif.) with a 1% agarose gel, a 3- to 15-s pulse ramp, an electrophoresis rate of 6.0 V/cm with an included angle of 120° at a constant temperature of 14°C, and a run time of 26 h. Gels were stained with ethidium bromide (EB) and visualized with a Typhoon 9410 (Amersham Biosciences, Piscataway, N.J.).

[0050] PCR amplification

[0051] Multiplex PCR amplification was used to screen for the presence of *tda* genes in Tda⁻ mutants. A 716-bp sequence internal to *tdaE* was amplified using primers 5'-CAGATGATGGTGCCAAAGGACTAT-3' and 5'-GGTCAGTTTCTTCTGCACATACTGG-3', while (in the same reaction), an internal 401-bp fragment of *flaA* (accession number: CP000377, locus tag: TM1040_2952) was also amplified using primers 5'-TTGCAGTATCCAATGGTCGTG-3' and 5'-TGAATTGCGTCAGAGTTTGCC-3' as a control. Standard PCR amplification conditions were 100 μM dNTP each, 0.2 μM of each primer, 1 U

Taq DNA polymerase (New England Biolabs, Beverly, MA) in 1× reaction buffer (New England BioLabs) with an initial denaturing step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min each, annealing at 55°C for 30 s, and an elongation at 72°C for 1 min.

[0052] To detect the *tdaA-E* locus, PCR amplification was conducted with a forward primer complementary to *tdaA* (5'-CGCTTTCCGGAAGTGGAGAT-3') and a reverse primer complementary to *tdaE* (5'-GGCTGCCGTATAGTTTCAGCA-3') using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) and the PCR program conditions and cycle parameters as described by the supplier.

[0053] DNA hybridization

[0054] DNA:DNA hybridization by Southern 'slot' blot (3) was used to detect the presence of *tda* genes in other roseobacters. The roseobacter strains used were: *Phaeobacter* strain 27-4, *Roseobacter algicola* ATCC 51442, *Roseobacter denitrificans* ATCC 33942, *Roseobacter litoralis* ATCC 49566, *Roseobacter* sp. strain TM1038, *Roseobacter* sp. strain TM1039, *Roseovarius* sp. strain TM1035, *Roseovarius* sp. strain TM1042, *Roseovarius* strain ISM, *Silicibacter pomeroyi* DSS-3, *Silicibacter* sp. strain TM1040, *Sulfitobacter* strain EE36, *Sulfitobacter* strain 1921, *Sulfitobacter* strain SE62, and *Vibrio anguillarum* 90-11-287.

[0055] Following extraction, 100 ng of total genomic DNA purified from each strain was spotted onto a positively charged nylon membrane (Roche). The DNA was cross-linked to the membrane with ultraviolet light using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA), followed by prehybridization of the membrane at 25°C for 30 min, using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) as described by the manufacturer. The membrane was incubated at 25°C overnight with a double-stranded DNA probe prepared by *Hind* III digestion of a plasmid bearing *tdaA* cloned from strain HG1310 that was labeled with digoxigenin-dUTP using random priming as recommended by the manufacturer (Roche). Unbound labeled DNA was removed from the membrane by 2 × 5 min in 2×SSC, 0.1% SDS followed by 2 × 15 min in 0.2×SSC, 0.1% SDS (3). In the southern blot, the membrane was prehybridized for 30 min in the same buffer to which was added a *tdaD* gene probe, and the probe allowed to hybridize overnight at 42°C. The blots were washed under high stringency conditions following the manufacturer's protocol (Roche applied science) and exposed to Lumi-

film chemiluminescent detection film (Roche) for subsequent detection of the hybridization signal.

[0056] RESULTS

[0057] TM1040 produces the sulfur-containing antibiotic tropodithietic acid

5 [0058] TM1040 produces an extracellular broad spectrum antibacterial compound capable of inhibiting or killing many bacteria. We found that greater antibacterial activity occurred when the bacteria were grown in a nutrient broth culture under static conditions, i.e., no shaking, compared to shaking conditions (11 mm; Fig. 1). Under static conditions, TM1040 cells attached to one another forming rosettes and produced a very distinct yellow-brown pigment (Fig. 1).
10 These phenotypes are consistent with *Phaeobacter* 27-4 and other roseobacters. During the course of this investigation, non-pigmented colonies were sometimes seen after TM1040 was incubated on nutrient agar, and subsequent analysis revealed that these 'white spontaneous mutants' also had lost antibacterial activity as well.

[0059] TM1040 produces an antibiotic and shares common phenotypic traits with other
15 roseobacters, notably *Phaeobacter* 27-4 whose antibiotic is tropodithietic acid (TDA). We therefore hypothesized that the antibacterial compound produced by TM1040 may also be tropodithietic acid. Cell-free supernatants were collected independently from both TM1040 and 27-4, ethyl acetate extraction of the supernatants was used to separate TDA from other compounds, and the concentrated extract was analyzed by HPLC. The resulting elution
20 chromatograms and subsequent UV spectra of the putative peak of TDA from TM1040 and 27-4 are shown in Fig. 2. Both chromatograms and UV spectra are nearly identical, indicating chemically similar metabolites are produced by both strains. A compound with a retention time of 4.2 min (indicated in Fig. 2) is observed in both chromatograms and has been positively identified as TDA in 27-4. The equivalent 'TDA peak' from TM1040 has a UV spectrum that
25 overlaps with that of published spectrum of TDA obtained from 27-4, with four major absorptions at 210 nm, 304 nm, 355 nm and 452 nm. Mass spectroscopy of the TM1040 'TDA peak' was used to confirm the efficacy of the compound as TDA. Taken together, the data corroborate TDA as the antibacterial metabolite produced by TM1040.

[0060] Identification of genes involved in the synthesis of TDA

[0061] With the exception of genes involved in shikimate and phenylacetate metabolism, an analysis of the genome of TM1040 does not provide much insight into genes likely to participate in the biosynthesis and regulation of TDA. To determine the genes required for TDA synthesis, a genome-wide random-insertion transposon bank of 11,284 Kan^r colonies was generated in
5 TM1040 and screened for antibiotic loss-of-function mutants (Tda⁻ phenotype). Approximately 0.7% of the transposon insertions (81 out of 11,284) were Tda⁻ mutants, all of which were defective in TDA synthesis as well as in pigment formation.

[0062] The location of the transposon insertion site in each of the 81 Tda⁻ mutants was determined by sequencing TM1040 DNA adjacent to the transposon. The pair of sequences
10 (both sides of the transposon insertion point) obtained from each mutant was used to search the annotated TM1040 genome to identify the mutated gene. Surprisingly, we were unable to find homologs in the genome for 32 or nearly 40% of the Tda⁻ mutants, yet these DNAs overlapped permitting assembly into one large contiguous DNA fragment of 4.5 kb harboring at least 6 ORFs that we have called *tdaA-F* (Table 2 and Fig. 3A). It is clear that *tdaA-F* represent DNA
15 that is not part of the original annotation of the genome, suggesting that this DNA may have been lost from the sequenced variant of TM1040. A thorough analysis of these 'orphan' genes is presented below (TDA biosynthesis genes resided on a 130 kb cryptic plasmid).

[0063] Forty nine Tda⁻ mutants had transposon insertions in genes found in one of the three DNAs that make up the genome. Due to the observation of a low frequency spontaneous loss of
20 TDA synthesis and knowledge of the existence of *tdaA-F*, we analyzed each of the 49 genomic Tda⁻ strains for the presence of *tdaA-F*. Nearly 90% (43 out of 49) did not harbor *tdaA-F*, as determined by PCR amplification with primers to *tdaE*, and had lost this DNA presumably resulting in their Tda⁻ phenotype. The transposon insertion in these strains may contribute to the Tda⁻ phenotype.

25 [0064] The sequences obtained from the remaining 6 Tda⁻ mutants were highly informative (Table 2).

[0065] An analysis of the genes identified from the 6 'genomic' TDA⁻ mutants revealed that the phenylacetate catabolism (*paa*) pathway is required for TDA synthesis (Fig. 3A). Transposon insertions were identified in homologs of *paaI*, *paaJ*, and *paaK*. The deduced amino acid
30 sequence from each of these ORFs had strong homology to similar proteins encoded by other

roseobacters. For example, TM1040 *paaI* is 79% similar to *paaI* of *Silicibacter pomeroyi* DSS-3, TM1040 *paaJ* is 74% similar to an ORF of *Roseobacter* sp. MED193, and *paaK* is 77% to *Roseobacter* sp. MED193 *paaK* (Table 2). In other bacteria, *paaGHIJK* encodes a ring-hydroxylating complex of proteins that is responsible for the first step in the aerobic catabolism of phenylacetate involving Coenzyme A (CoA) activation, producing 1, 2-dihydro-phenylacetate-CoA. The finding that mutations in *paa* genes affects TDA synthesis is consistent with the biochemical evidence of phenylacetate metabolism in thiotropocin synthesis.

[0066] Mutants with defects in phenylacetate metabolism were also unable to grow on phenylalanine, phenylacetic acid, tryptophan, sodium phenylpyruvate or phenylbutyrate as a sole carbon source (Table 3).

[0067] TDA is a disulfide-modified tropolone compound, indicating that sulfur metabolism must be involved in TDA synthesis. This hypothesis is supported by the identification of 3 Tda⁻ mutants (Table 2) each with a transposon inserted in a gene whose product is involved in sulfur metabolism: *cysI*, *malY*, and an ORF (*tdaH*) with homology to sulfite oxidase (Table 2). The identification of these genes suggests that sulfur from reductive sulfur pathways is used and incorporated into TDA, which was tested by observing growth of the sulfur-metabolism mutants on a minimal medium containing a sole sulfur source (Materials and Methods). The results are shown in Fig. 4. The *cysI* mutant grew when provided complex sulfur sources or cysteine and was unable to utilize DMSP, SO₃²⁻, SO₄²⁻, or methionine. The addition of cysteine to the medium resulted in enhanced growth of the *cysI* mutant as well as increased synthesis of TDA (Fig. 4).

[0068] TDA biosynthesis genes resided on a 130 kb cryptic plasmid

[0069] As previously described, *tdaA-F* genes were not part of the annotated TM1040 genome and were absent in spontaneous Tda⁻ mutants. We conducted a series of bioinformatic analyses to elucidate the potential function of these genes (Table 2) and their proteins. Interestingly, these genes share their strongest homology with a similar set of genes in *Paracoccus denitrificans* PD1222 chromosome 1 (Accession number: NC_008686), a non-motile alphaproteobacterium first isolated from soil by Beijerinck. As shown in Fig. 3B, the orientation and spacing between *tdaA* and *tdaB* suggests that these genes form a bicistronic message while *tdaC-E* are likely to compose an operon separate from *tdaAB*. *tdaF* is in a different operon (Fig. 3).

[0070] Amino acid domain identification was useful in assigning potential functions to the encoded proteins. For example, TdaA (Table 2) has homology with LysR regulatory proteins, possessing a helix-turn-helix and a LysR substrate-binding domain (57). TdaA is the only regulatory protein uncovered in this study, perhaps indicating that it is the sole regulator of TDA synthesis. The remaining ORFs encode putative enzymes. TdaB contains a glutathione S-transferase (GST) domain and belongs to the bacterial GST protein family (Table 1). TdaC has an amino acid domain with homology to prephenate dehydratase (PheA), an enzyme involved in the conversion of chorismate to prephenate, a step in the pathway leading to phenylacetate synthesis.

10 **Table 1. Bacterial strains and plasmids used.**

Strain/plasmid	Genotype/phenotype	Source of reference
<i>Escherichia coli</i>		
DH5 α	<i>F' endA1 hsdR17 (r_K⁻ m_K⁻) supE44 thi-1 recA1 gyrA96 relA1 Φ80dlacZΔM15</i>	(1)
DH5 α (λ pir)	DH5 α transduced with λ pir	(2, 3)
EC100D <i>pir</i> +	<i>F' mcrA D(mrr-hsdRMS-mcrBC) f80dlacZDM15 DlacX74 recA1 endA1 araD139 D(ara, leu)7697 galU galK l- rpsL nupG pir+(DHFR).</i>	Epicentre TM
Roseobacters		
<i>Silicibacter</i> sp. TM1040	Wild type, antibacterial activity	(4)
Mutants derived from TM1040		
<i>Silicibacter</i> sp.	None pigment and <i>tda</i> spontaneous strain	current study

¹ Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual., 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

² Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.

³ Kolter, R., M. Inuzuka, and D. R. Helinski. 1978. Transcomplementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell **15**:1199-1208.

⁴ Miller, T. R., and R. Belas. 2004. Dimethylsulfoniopropionate metabolism by *Pfiesteria*-associated *Roseobacter* spp. Appl. Environ. Microbiol. **70**:3383-3391

TM1040 SM		
HG1005	<i>paaK</i> ::EZ-Tn5,Kan	“
HG1015	<i>tdaB</i> ::EZ-Tn5,Kan	“
HG1050	<i>tdaF</i> ::EZ-Tn5,Kan	“
HG1056	<i>paaJ</i> ::EZ-Tn5,Kan	“
HG1080	<i>tdaC</i> ::EZ-Tn5,Kan	“
HG1110	<i>tdaD</i> ::EZ-Tn5,Kan	“
HG1213	<i>malY</i> ::EZ-Tn5,Kan	“
HG1220	<i>cysI</i> ::EZ-Tn5,Kan	“
HG1244	<i>tdaH</i> ::EZ-Tn5,Kan	“
HG1265	<i>tdaE</i> ::EZ-Tn5,Kan	“
HG1299	<i>paaI</i> ::EZ-Tn5,Kan	“
HG1310	<i>tdaA</i> ::EZ-Tn5,Kan	“
<i>Phaeobacter</i> sp. 27-4	Wild type, antibacterial activity	(5, 6)
Mutants derived form 27-4		
JBB1001	<i>tdaB</i> ::EZ-Tn5,Kan	current study
JBB1003	<i>tdbC</i> ::EZ-Tn5,Kan	“
JBB1005	<i>traI</i> ::EZ-Tn5,Kan	“
JBB1006	<i>clpX</i> ::EZ-Tn5,Kan	“
JBB1007	<i>tdbF</i> ::EZ-Tn5,Kan	“
JBB1009	<i>tdbA</i> ::EZ-Tn5,Kan	“
JBB1011	<i>tdbD</i> ::EZ-Tn5,Kan	“
JBB1029	<i>tdbE</i> ::EZ-Tn5,Kan	“
JBB1030	<i>tdaA</i> ::EZ-Tn5,Kan	“
JBB1044	<i>metF</i> ::EZ-Tn5,Kan	“

⁵ Bruhn, J. B., L. Gram, and R. Belas. 2007. Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. Appl. Environ. Microbiol. **73**:442-450.

⁶ Hjelm, M., O. Bergh, A. Riaza, J. Nielsen, J. Melchiorson, S. Jensen, H. Duncan, P. Ahrens, H. Birkbeck, and L. Gram. 2004. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst. Appl. Microbiol. **27**:360-371.

JBB1045	<i>tdbB::EZ-Tn5,Kan</i>	“
Other <i>Roseobacters</i>		
<i>Roseobacter algalicola</i> 51442	Wild type, none antibacterial activity	(5, 7)
<i>Roseobacter denitrificans</i> 33942	Wild type, none antibacterial activity	(5, 8)
<i>Roseobacter litoralis</i> 49566	Wild type, none antibacterial activity	(5, 8)
<i>Roseobacter</i> sp. TM1038	Wild type, antibacterial activity	(5, 4)
<i>Roseobacter</i> sp. TM1039	Wild type, antibacterial activity	(5, 4)
<i>Roseovarius</i> sp. ISM	Wild type, antibacterial activity	
<i>Roseovarius</i> sp. TM1035	Wild type, antibacterial activity	(5, 4)
<i>Roseovarius</i> sp. TM1042	Wild type, antibacterial activity	(5, 4)
<i>Silicibacter pomeroi</i> DSS- 3	Wild type, antibacterial activity	(5, 9)
<i>Sulfitobacter</i>	Wild type, none antibacterial activity	(5)

⁷ Lafay, B., R. Ruimy, C. Rausch de Traubenberg, V. Breittmayer, M. J. Gauthier, and R. Christen. 1995. *Roseobacter algalicola* sp. nov., a new marine bacterium isolated from the phycosphere of the toxin-producing dinoflagellate *Prorocentrum lima*. Int. J. Syst. Bacteriol. **45**:290-296.

⁸ Shiba, T. 1991. *Roseobacter litoralis* gen. nov., sp. nov., and *Roseobacter denitrificans* sp. nov., aerobic pink-pigmented bacteria which contain bacteriochlorophyll a. Syst. Appl. Microbiol. **14**:140-145.

⁹ Gonzalez, J. M., J. S. Covert, W. B. Whitman, J. R. Henriksen, F. Mayer, B. Scharf, R. Schmitt, A. Buchan, J. A. Fuhrman, R. P. Kiene, and M. A. Moran. 2003. *Silicibacter pomeroi* sp. nov. and *Roseovarius nubinihibens* sp. nov., dimethylsulfoniopropionate-demethylating bacteria from marine environments. Int. J. Syst. Evol. Microbiol. **53**:1261-1269.

sp.1921		
<i>Sulfitobacter</i> sp. EE36	Wild type, antibacterial activity	(5, 10)
<i>Sulfitobacter</i> sp. SE62	Wild type, none antibacterial activity	(5, 11)
<i>Vibrio</i> <i>anguillarum</i> 90- 11-287	Wild type, serotype O1, susceptible to tropodithietic acid	(5, 12)
<i>Plasmid</i>		
<i>pSTM3</i>	Harboring tda genes	current study
<i>pSTM3-1265</i>	pSTM3 carrying a Tn5 insertion in tdaE, derived from HG1265	current study

[0071] The involvement of CoA metabolism, addition, or modification is evident from the functional domains on TdaD and TdaE. TdaD is anticipated to be a member of the thioesterase superfamily of acyl-CoA thioesterases (Table 2), TdaE encodes a putative acyl-CoA dehydrogenase (ACAD), and TdaF has homology to aldehyde dehydrogenase.

[0072] The secondary evidence suggests that *tdaA-F* resides on a cryptic plasmid that may be spontaneously lost. To develop a means to test the hypothesis, we used three strains, TM1040, a spontaneous Tda⁻ nonpigmented strain of TM1040 (TM1040SM), and HG1265 (*tdaE*::Tn) (Fig. 5A and Table 1), along with a PCR amplification using primers for *tdaA-E*, predicted to generate a 3.8 kb product from wild-type DNA. As shown in Fig. 5B, PCR amplification of wild-type

¹⁰ Buchan, A., L. S. Collier, E. L. Neidle, and M. A. Moran. 2000. Key aromatic-ring-cleaving enzyme, protocatechuate 3,4-dioxygenase, in the ecologically important marine roseobacter lineage. Appl. Environ. Microbiol. **66**:4662-4672.

¹¹ Buchan, A., E. L. Neidle, and M. A. Moran. 2001. Diversity of the ring-cleaving dioxygenase gene *pcaH* in a salt marsh bacterial community. Appl. Environ. Microbiol. **67**:5801-5809.

¹² Skov, M. N., K. Pedersen, and J. L. Larsen. 1995. Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar O1. Appl. Environ. Microbiol. **61**:1540-1545.

DNA gave the predicted 3.8 kb band, a 5.7 kb product when *tdaE*:Tn DNA was used as a template, and no product when the DNA from the SM strain was amplified indicating that the SM strain had lost the *tdaA-E* locus.

[0073] Total DNA from TM1040, TM1040SM, and HG1265 (*tdaE*:Tn) was separated by PFGE.

5 As observed in Fig. 5C, all three strains had high molecular weight DNA, presumably a mixture of chromosomal and pSTM1 and a band or bands at ca. 130 kb, corresponding to the size of pSTM2 (132 kb) (41). Close inspection of this region and comparison between the SM DNA lane (middle, Fig. 5C) and either the TM1040 or *tdaE*:Tn DNA (left and right lanes, respectively) shows that the SM band is thinner than either TM1040 or *tdaE*:Tn hinting that SM
10 DNA is missing a DNA species in this size range that overlaps with pSTM2. Repeated attempts to change PFGE conditions did not resolve this region. To overcome this limitation, a Southern blot (Fig. 5D) using a *tdaD* DNA probe was performed on the gel shown in Fig. 5C, and the results confirmed that the SM DNA, while possessing a 130 kb band, fails to hybridize to *tdaD*. In contrast, both wild-type DNA and *tdaE*:Tn DNA hybridize to the expected band (ca. 130 kb).
15 This confirms the loss of *tda* DNA in SM and adds evidence supporting the hypothesis that the missing *tda* DNA is on a plasmid. It does not rule out the unlikely possibility that *tda* genes reside on pSTM2 and are somehow deleted from that known molecule.

[0074] To resolve the issue, we isolated plasmids from each of the three strains (TM1040, TM1040SM, and HG1265) and subjected each mixture to *Nco*I digestion (Fig. 5E), chosen
20 because an *in silico* *Nco*I digestion of pSTM2 provided a recognizable pattern of DNA fragments. As shown in Fig. 5E, the TM1040SM DNA digest had much fewer bands than wild-type DNA or DNA from *tdaE*:Tn. This would be expected if the TM1040SM strain lost a large plasmid. Consistent with this hypothesis, Southern blotting showed that a *tdaD* probe hybridized to a 4.5 kb fragment in wild-type plasmid DNA and to a 6.4 kb fragment from plasmids isolated
25 from the *tdaE*:Tn strain (Fig. 5E).

[0075] We reasoned that it is possible to transform a cryptic *tda* plasmid bearing a selectable marker into a suitable host and thereby provide proof of the existence of this plasmid. The transposon that we used, EZ:Tn, contains a kanamycin-resistance gene as well as the *ori*R6K origin of replication permitting replication in permissive hosts carrying the *pir* gene. Thus, the
30 plasmid from *tdaE*:Tn was used to transform *E. coli* EC100D (Table 1) with a subsequent

selection for kanamycin resistance (Materials and Methods). This transformation was successful despite a very low transformation efficiency resulting in 7 Colony Forming Units (CFUs) per µg of mixed plasmid DNA, and provides strong evidence for the existence of a cryptic ca. 130 kb plasmid harboring *tda* genes. This new plasmid was called pSTM3.

5 [0076] Twelve random colonies were chosen from the transformation with pSTM3 and the *Nco*I-digestion pattern of each compared. Fig. 6 shows the four common patterns resulting from this analysis. Although each plasmid was PCR positive for the *tda* genes (data not shown) and the set of four shared many common bands, they had remarkably different patterns indicating deletion and/or rearrangements had occurred during or after transfer of pSTM3 to *E. coli*. The
10 reason and molecular mechanism underlying these band pattern differences is not known; however, the sum of the results indicates that TM1040 harbors a ca. 130 kb plasmid, pSTM3, which is essential for TDA and pigment biosynthesis and which may be spontaneously lost in laboratory culture.

[0077] Distribution of *tda* genes in other *Roseobacters*

15 [0078] The *Roseobacter* clade produce an antibacterial activity. In light of the current findings, we sought confirmation that other roseobacters had *tda* genes as well, and chose *Phaeobacter* 27-4 as a suitable candidate.

[0079] We used the same transposon to construct a 6,321-member library and screened these mutants for the Tda⁻ phenotype. 37 Tda⁻ mutants were found of which 12 were analyzed further.
20 Two of the 12 ORFs mutated were similar to TdaA (identity 38%) and TdaB (identity 55%) from TM1040 (Table 4), suggesting that these two roseobacters share a common TDA biosynthesis and regulation scheme. The remaining 9 genes were not identified as important to TDA synthesis in TM1040 and had varying degrees of homology to genes in the annotated TM1040 genome, but, unlike TM1040, were not part of the phenylacetate or reductive sulfur pathways. The one
25 exception is 27-4 *metF* (Table 4), which may possibly be involved in sulfur metabolism.

[0080] We also used DNA:DNA hybridization to measure hybridization of a *tdaA-F* gene probe to DNA from 14 *Roseobacter* clade species (Fig. 7). The *tda* probe hybridized to 8 of the 9 roseobacters that have been established as producing antibacterial activity (Fig. 7), with the ninth, *Silicibacter pomeroyi* DSS-3, showing a low amount of hybridization. Three of 6 non-
30 antibiotic-producing roseobacters also positively hybridized to the *tda* DNA. This false positive

may have resulted from a strain that has very low *tda* expression and antibiotic activity below the detection limits of the well diffusion assay, or from spurious hybridization to non-*tda* DNA. The *tda* probe did not hybridize with DNA from *V. anguillarum*, implying that the second possibility is the more likely scenario.

5 **Table 4. *Phaeobacter* 27-4 genes and encoded proteins required for the regulation and synthesis of tropodithietic acid.**

Mutant Number	GenBank Assession Number	Gene Designation	Function	Best Hit Ortholog / E score
Ring Precursors, Oxidation, and Expansion				
JBB1001 / JBB1030	EF139212	<i>tdaB</i>	β -etherase, glutathione S transferase	<i>Sinorhizobium meliloti</i> putative β -etherase (β -aryl ether cleaving enzyme / 4e-52
Sulfur Metabolism and Addition				
JBB1044	EF139218	<i>metF</i>	5-methyltetrahydrofolate-homocysteine S-methyltransferase	<i>Silicibacter</i> sp. TM1040 MetF protein / 2e-77
Co-enzyme A Metabolism				
JBB1009	EF139215	<i>tdbA</i>	D- β -hydroxybutyrate dehydrogenase	<i>Roseovarius</i> sp. 217 D- β -hydroxybutyrate dehydrogenase / 2e-32
JBB1045	EF139216	<i>tdbB</i>	Phosphate acetyltransferase	<i>Roseobacter</i> sp. MED193 phosphate acetyltransferase / 8e-81
Transport: Import and Export				
JBB1003	EF139213	<i>tdbC</i>	Lytic transglycosylase, peptidase C14	<i>Roseobacter</i> sp. MED193 hypothetical protein / 6e-85
JBB1005	EF139221	<i>tral</i>	TraI, Type IV (Vir-like) secretion	<i>Rhodobacter sphaeroides</i> 2.4.1 TraI / 5e-58
JBB1011	EF139222	<i>tdbD</i>	Type I secretion target repeat protein	<i>Roseobacter</i> sp. MED193 type I secretion target repeat protein / 8e-54
JBB1029	EF139216	<i>tdbE</i>	Oligopeptide/dipeptide ABC transporter	<i>Silicibacter</i> sp. TM1040 binding-protein-dependent transport systems inner membrane component / 6e-124

Regulatory Mechanism				
JBB1006	EF139220	<i>clpX</i>	ATP-dependent Clp protease	<i>Silicibacter</i> sp. TM1040 ATP-binding subunit ClpX / 1e-47
JBB1007	EF139214	<i>tdbF</i>	Ribonuclease D	<i>Roseobacter</i> sp. MED193 ribonuclease D / 6e-49
JBB1030	EF139217	<i>tdaA</i>	LysR substrate binding domain protein	<i>Paracoccus denitrificans</i> PD1222 regulatory protein, LysR:LysR, substrate-binding / 3e-51

[0081] Distribution of *tda* genes in the environment

[0082] Marine genome and metagenomic databases were searched for sequences with homology to one of the 12 genes (Table 2) required for TDA synthesis by TM1040. While
5 homologs to the proteins involved in phenylacetate and reductive sulfur metabolism were found within the 14 selected roseobacter genomes in Roseobase (<http://www.roseobase.org/>) and the Gordon and Betty Moore Foundation Marine Microbial Genome databases (<https://research.venterlinstitute.org/moore/>), close homologs of TdaA-F were absent (at a BLASTP E value cutoff of 1E-30). While the reason for the absence of homologs is not known,
10 it is possible, although unlikely, that all 14 roseobacters do not produce TDA, produce an antibacterial activity that involves another compound, or lost their *tda* plasmid. The last possibility is most likely to have resulted from laboratory culturing, therefore we searched for Tda homologs in environmental metagenomic libraries (<http://camera.calit2.net/>) that should contain abundant uncultivated roseobacter DNA.

[0083] The data gathered from searching the CAMERA marine metagenomic GOS dataset database are shown graphically in Fig. 8, where a circle and its relative size indicates the presence and abundance (respectively) of a given protein. As was observed with the roseobacter genomes, phenylacetate and reductive sulfur metabolism proteins were found at numerous sites, with the greatest abundance of PaaIJK and CysI at site GS00a, a Sargasso Sea sample (31 32'6"
20 N, 63 35' 42" W). Positive Tda protein 'hits' were also recorded in a hypersaline pond sample (GS033) and a sample obtained from Lake Gatun, Panama Canal (Fig. 8). In no sample did we find hits to all 12 proteins involved in TDA biosynthesis.

[0084] Various members of the *Roseobacter* clade, whose genomes reveal a great potential for the synthesis of bioactive molecules, produce TDA. Many marine bacteria produce an antibiotic activity, including antibacterial activity from roseobacters, e.g., a compound that produces a probiotic effect on scallop larvae and is antagonistic to γ -Proteobacteria strains, as well as a compound that is antagonistic against fish larval bacterial pathogens. From our data, it is likely that much of the antibiotic activity seen in roseobacters is due to plasmid-borne *tda* genes that can be difficult to maintain in laboratory conditions.

[0085] There is a direct link between the spontaneous appearance of non-pigmented Tda⁻ colonies and the loss of pSTM3 of TM1040. Over 40 of the mutants initially screened as Tda⁻ were ultimately found to have lost pSTM3. This suggests that loss of pSTM3 is a relatively frequent event during laboratory cultivation of TM1040. Instability of the Tda⁺ phenotype is not unique to TM1040. The appearance of spontaneous nonpigmented Tda⁻ mutants or variants is characteristic of other roseobacters, including *Phaeobacter* 27-4 and *Roseobacter gallaeciensis* sp. T5. One possible explanation for the cause of these spontaneous mutants is a loss of a plasmid carrying one or more critical genes required for TDA synthesis. Indeed, 27-4 possesses at least two plasmids of ca. 60 kb and 70 kb respectively. One or both of these plasmids may be involved in TDA biosynthesis of 27-4 and *tdaA* and *tdaB*, identified by transposon insertion mutagenesis in 27-4 Tda⁻ mutants, reside on one of these plasmids.

[0086] Instability of pSTM3 is also apparent when the plasmid is transformed into a nonroseobacter host, e.g., *E. coli*. As shown in Fig. 6, at least four unique *NcoI*-restriction fragment patterns were observed from pSTM3 that had been successfully transformed into a new host. As a cause of this instability, it seems improbable that TDA biosynthesis is to blame, because the pSTM3 used to transform *E. coli* does not confer a TDA⁺ phenotype due to the presence of a transposon in *tdaE*. It is possible that, despite absence of TdaE, some other protein(s) encoded by other *tda* genes (*tdaABCD* or *-F*) may be detrimental when expressed in *E. coli*. While there is no evidence to directly link instability of pSTM3 in *E. coli* with spontaneous loss of the plasmid in TM1040, these phenomena may share a common cause. We have initiated efforts to sequence and annotate pSTM3 and compare it to the pSTM3 species obtained from *E. coli*. Preliminary evidence indicates that pSTM3 harbors a *repC* homolog upstream of *tdaA*. RepC forms a complex along with RepAB and is required for plasmid

replication and maintenance. It will be determined if the pSTM3 plasmid species obtained from *E. coli* transformation have defects in *repABC*.

[0087] The ability of pSTM3 to replicate in *E. coli*, albeit with significant alteration in the plasmid, suggests that pSTM3 also may be transferred to other marine bacteria, perhaps other roseobacters, or even to higher organisms, e.g., dinoflagellates. TM1040 possesses varied capabilities to achieve horizontal gene transfer, including the presence of several prophage genomes in the bacterium's genome, one of which is homologous to the gene transfer agent of other alphaproteobacteria, and many of the genes on pSTM2 are homologs of the *vir* system of *Agrobacterium tumefaciens*. The *A. tumefaciens* Ti plasmid, transferred by Vir Type IV secretion, requires RepABC, suggesting that a similar mechanism may allow pSTM3 transfer to other organisms. Plasmids similar to pSTM3, such as pSymA of *Sinorhizobium meliloti* and the Ti plasmid, are important for the proper interaction of those bacteria and their respective hosts, and TM1040 pSTM3 and pSTM2 may correspondingly serve to enhance the TM1040-dinoflagellate symbiosis.

[0088] It is important to note that TDA activity and biosynthesis depend on culture conditions and the physiology of TM1040. TDA activity is significantly enhanced when TM1040 is cultured in a static nutrient broth, a condition that accentuates biofilm formation. The symbiosis includes two phases: the motile phase in which TM1040 cells actively respond to dinoflagellate-derived molecules by swimming towards the host, and sessile phase, whereupon having located the zoospore, the bacteria cease to be motile and form a biofilm on the surface of the dinoflagellate. Thus, there is a direct correlation between biofilm formation and TDA biosynthesis.

[0089] Biosynthesis of TDA has several potentially beneficial effects on the TM1040-dinoflagellate symbiosis. TDA is likely to benefit the dinoflagellate by acting as a probiotic with antibacterial activity whose action prevents the growth and colonization of bacteria on the surface of the dinoflagellate that could potentially harm the zoospore. In turn, the antibacterial activity of TDA may enhance the growth of TM1040 cells attached to the zoospore by warding off other biofilm-forming bacteria that compete with TM1040 for space on the surface of and nutrients from *P. piscicida*. Interestingly, DMSP appears not to be a primary source of the sulfur

atoms of TDA. One or more non-DMSP sulfur-containing metabolites produced by the dinoflagellate may be used by TM1040 in the biosynthesis of TDA.

[0090] One of the unexpected results from our study is the paucity of homologous Tda proteins in either the genomes of other sequenced roseobacters or in the CAMERA metagenomic library (Fig. 8). There are several reasons why Tda proteins were not found. For example, amino acid sequence divergence between Tda proteins of TM1040 and other roseobacters could result in BLASTP E values greater than our chosen cutoff (1E-20). This argument may also be applied to the metagenomics search. In focusing on just the search for Tda homologs in roseobacter genomes, it is possible that, in culturing these roseobacter species in preparation for isolation and purification of their genomic DNAs, the bacteria lost a pSTM3-like plasmid harboring *tda* genes. Equally feasible is the possibility that TDA is but one of many antibiotic compounds produced by roseobacters or that more than one biochemical pathway exists to produce TDA. Both arguments may help explain the lack of Tda protein homologs in roseobacter genomes.

[0091] The lack of Tda protein homologs in the marine metagenomics database presents a much more difficult problem to interpret, especially in the context of PaaIJK and CysI searches that frequently identified their respective homologs in numerous samples within the database (Fig. 8). While the data do not provide definitive answers to this question, our data show that stability and retention of pSTM3 by TM1040 is greatest when the bacteria are directly associated with the dinoflagellate, i.e., the plasmid may be lost when TM1040 is grown in laboratory culture, yet retained when cultivated as part of the *Pfiesteria piscicida* mesocosm from which the bacteria were isolated. Close association of TM1040 with *P. piscicida* provides a selection to maintain the pSTM3; that selective pressure is lost when the bacteria are taken away from their host (as happens under laboratory culture). The CAMERA metagenomic samples analyzed were prepared after filtration to remove 0.8 μ m particles, which may have removed the portion of the roseobacter population harboring a *tda* plasmid like pSTM3.

[0092] The two metagenomic samples that showed relatively good Tda homolog hits were from a site in the Sargasso Sea and a hypersaline pond, respectively. DMSP is potentially useful by algae as an osmolyte that protects the cells against changes in salinity. Our results suggest that DMSP is not used as a sole sulfur source in the biosynthesis of TDA, and show that there is a correlation between salinity, DMSP, and the presence of Tda homologs.

[0093] The genetic data from the current study, specifically the identification of *paaIJK* and *tdaC* (prephenate dehydratase), indicate that TDA biosynthesis originates from the shikimate pathway and proceeds through phenylacetate (Fig. 9). The results also show that phenylacetate-CoA and CoA metabolism is vital to TDA production and are consistent with TdaD-F involvement in a ring expansion reaction that converts PAA-CoA to a seven-member tropolone ring (step 8 in Fig. 9). TdaB, a homolog of glutathione S-transferase, is a potential agent in the addition of sulfur to the nascent TDA molecule.

[0094] The compounds shown in Fig. 9 include the following:

Table 5

Compound Produced from Reaction:	IUPAC ¹³ name
6	1, 2-dihydro-phenylacetyl-CoA
8	2-hydroxy-7-oxo-cyclohepta-3,5-dienecarboxylic acid
9	2,7-dihydroxy-cyclohepta-1,3,5-trienecarboxylic acid
10	2,7-dihydroxy-3-oxo-cyclohepta-1,4,6-trienecarboxylic acid
11	2,7-dihydroxy-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid
13	7-hydroxy-2-mercapto-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid

[0095] Identification of a LysR homolog in TdaA is consistent with the regulation of TDA biosynthesis involving a cofactor. In other bacteria, LysR cofactors can function as precursor molecules required to synthesize the final product, implicating molecules in the shikimate pathway, phenylacetate, or other TDA precursors as being required for maximal expression of the *tda* genes. Consistent therewith, modifications of the broth by addition of phenylalanine and histidine significantly increase production of TDA from *Phaeobacter* T5.

[0096] We therefore disclose the genes and proteins required for TDA synthesis by roseobacters, and the occurrence of *tda* genes on a previously unknown megaplasmid (pSTM3) of TM1040, as aspects of the present invention. The backbone of TDA is a seven member aromatic tropolone ring, which is highly significant as tropolone derivatives, notably hydroxylated forms, are medically important sources of antibacterial, antifungal, antiviral, and antiparasitic agents.

¹³ International Union of Pure and Applied Chemistry

Chemical synthesis of tropolone and derivatives can be difficult, making natural sources of tropolone precursors often the preferred choice as starting material for the synthesis of new tropolone antibiotics. The mutants obtained in this study may lead to the development of bacterial sources of medically important tropolone compounds and a suite of new antimicrobial agents based on TDA.

[0097] Sequencing of the 130 kb pSTM3 plasmid bearing genes required for tropodithietic acid biosynthesis has been carried out with determination of the following sequences.

pSTM3 partial sequence:

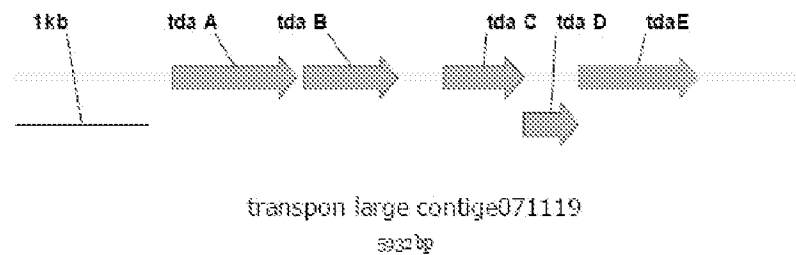
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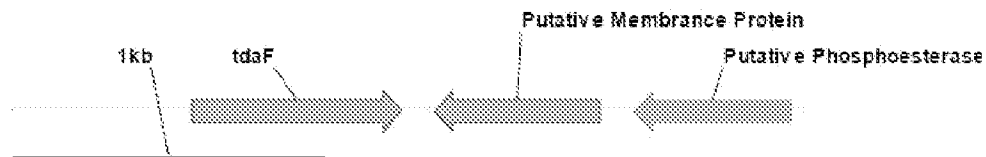
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tdaF and membrane protein gene:

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lipoprotein:

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 CTTTCTTAG

10 [0098] Another aspect of the invention relates to a methodology for purification of TDA and intermediate compounds, including the use of solid phase extraction techniques to obtain tropodithietic acid from *Silicibacter* sp. TM1040.

[0099] A still further aspect of the invention relates to a method of purification of TDA by HPLC techniques.

15 [00100] An illustrative purification technique is set out below.
 Purification of Compound.

1. *Roseobacter* 27-4 was grown in 500 ml MB in a 5 liter volumetric flask at 25°C for 4 days.
- 20 2. The cells were removed by centrifugation (10,000 x g for 10 min).
3. The pH of the supernatant was adjusted to 3.5
4. Extraction was carried out with 3 times 500 ml ethyl acetate acidified with 0.1% formic acid (FA)
5. The organic phase was transferred to a vessel and evaporated to dryness under nitrogen
 25 flow.
6. The dry ethyl acetate extract was redissolved in 3 x 3 ml acetonitrile (CAN)-water (1:19) containing 1% FA
7. The redissolved extract was sequentially applied to two 60 mg Oasis MAX columns (Waters, Milford, MA) which had previously been sequentially conditioned with 4 ml
 30 methanol (HPLC grade) and 3 ml CAN-water (1:19) containing 1% FA.

8. After loading the samples by gravity the columns were washed with 4 ml PBS buffer (pH 7).
9. 3.5 ml CAN-water (1:1) was passed through the column and collected (fraction 1)
10. 3.5 ml CAN-water (9:1) (fraction 2)
- 5 11. 3.5 ml CAN-water (1:1) with 2% FA (fraction 3)
12. 3.5 ml CAN-water (9:1) with 2% FA (fraction 4)
13. The solvents were then removed *in vacuo* on a SpeedVac (ThermoSavant, Holbrook, NY).

10

INDUSTRIAL APPLICABILITY

[00101] The invention provides an effective and useful biosynthetic capability for the production of tropodithietic acid (TDA) by use of *Roseobacter* bacteria. TDA is a useful sulfur-containing antibiotic compound. The biosynthetic route of the present invention enables
15 scalable production of TDA and TDA derivatives.

THE CLAIMS

What is claimed is:

- 5 1. An isolated nucleic acid encoding a megaplasmid (pSTM3) of *Silicibacter* sp. TM1040; wherein the nucleic acid comprises genes involved in tropodithietic acid biosynthesis of *Roseobacter* bacteria.
2. The isolated nucleic acid of claim 1, having a nucleic acid sequence comprising SEQ. ID. 1,
10 SEQ. ID. 2, SEQ. ID. 3, and SEQ. ID. 4.
3. The isolated nucleic acid of claim 1, having a nucleic acid sequence comprising SEQ. ID. 1.
4. The isolated nucleic acid of claim 1, having a nucleic acid sequence comprising SEQ. ID. 2.
15
5. The isolated nucleic acid of claim 1, having a nucleic acid sequence comprising SEQ. ID. 3.
6. The isolated nucleic acid of claim 1, having a nucleic acid sequence comprising SEQ. ID. 4.
- 20 7. A protein encoded by a nucleic acid sequence represented by SEQ. ID. 1; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.
8. The protein of claim 7, wherein the nucleic acid sequence represented by SEQ. ID. 1 is selected from the group consisting of *tdaA*, *tdaB*, *tdaC*, *tdaD*, and *tdaE*.
25
9. A protein encoded by a nucleic acid sequence represented by SEQ. ID. 2; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.
10. The protein of claim 9, wherein the nucleic acid sequence represented by SEQ. ID. 2 is *tdaF*.
30

11. A protein encoded by a nucleic acid sequence represented by SEQ. ID. 3; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

12. A protein encoded by a nucleic acid sequence represented by SEQ. ID. 4; wherein the protein is involved in the biosynthesis of tropodithietic acid by *Roseobacter* bacteria.

13. An antibacterial composition comprising tropodithietic acid isolated from bacteria of the *Roseobacter* clade.

14. The antibacterial composition of claim 13, isolated from *Silicibacter* sp. TM1040.

15. The antibacterial composition of claim 13, isolated from *Roseobacter* sp. 27-4.

16. A method for producing an antibacterial composition comprising tropodithietic acid, the method comprising:

- a) culturing *Silicibacter* sp. TM1040 in a culture medium supporting growth of the bacterium and production of tropodithietic acid; and
- b) separating the tropodithietic acid from the culture medium; and
- c) purifying the tropodithietic acid by high performance liquid chromatography.

17. A method for producing an antibacterial composition comprising tropodithietic acid, the method comprising:

- a) culturing *Roseobacter* sp.27-4 in a culture medium supporting growth of the bacterium and production of tropodithietic acid; and
- b) separating the tropodithietic acid from the culture medium; and
- c) purifying the tropodithietic acid by high performance liquid chromatography.

18. A method of treating or preventing bacterial disease in an subject in need of such treatment or prevention, comprising administering to said subject an antibacterial composition comprising tropodithietic acid isolated from bacteria of the *Roseobacter* clade.

5 19. The method of claim 18, wherein the tropodithietic acid is isolated from *Silicibacter* sp. TM1040.

20. The method of claim 18, wherein the tropodithietic acid is isolated from *Roseobacter* sp. 24-7.

10

21. The plasmid pSTM3.

22. A compound selected from the group consisting of:

1, 2-dihydro-phenylacetyl-CoA;

15 2-hydroxy-7-oxo-cyclohepta-3,5-dienecarboxylic acid;

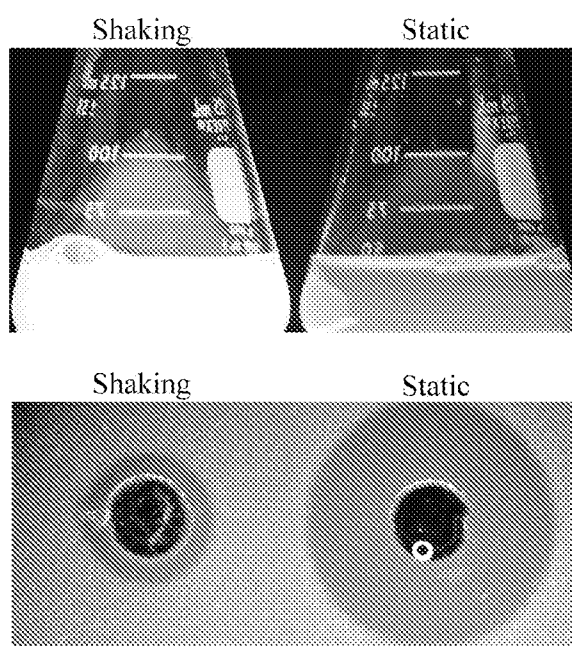
2,7-; dihydroxy-cyclohepta-1,3,5-trienecarboxylic acid;

2,7-dihydroxy-3-oxo-cyclohepta-1,4,6-trienecarboxylic acid;

2,7-dihydroxy-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid; and

7-hydroxy-2-mercapto-3-thioxo-cyclohepta-1,4,6-trienecarboxylic acid.

20

**FIG. 1**

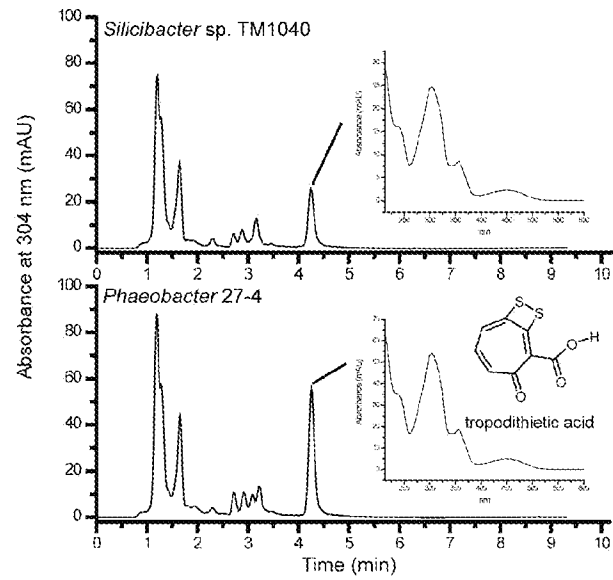


FIG. 2

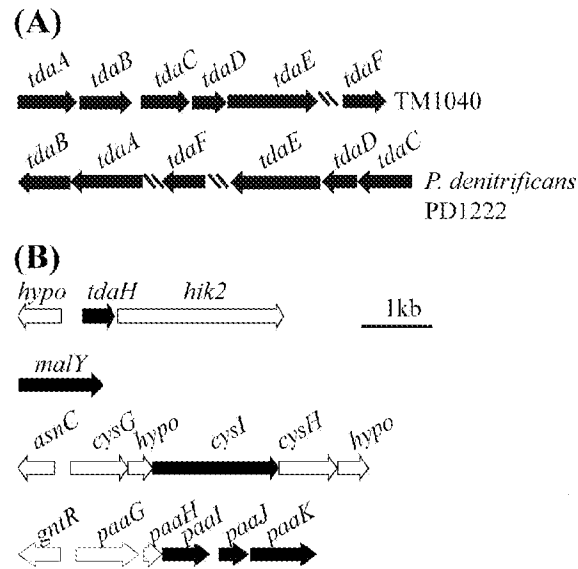
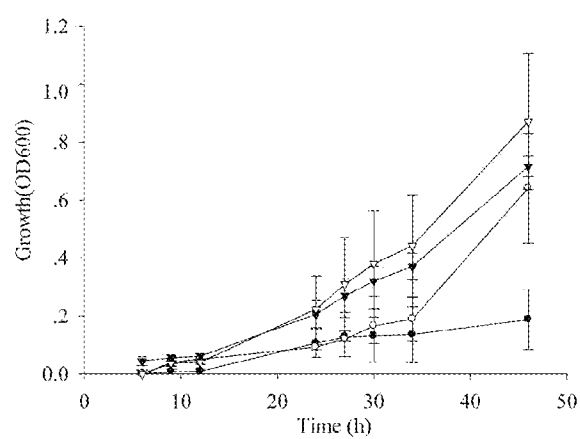


FIG. 3



strain	SO ₄	SO ₃	Met	Cys	DMSP
<i>cysI</i> growth	-	-	-	+	-
WT growth	+	+	+	+	+
<i>cysI</i> TDA	-	-	-	+	-
WT TDA	+	+	+	+	-

FIG. 4

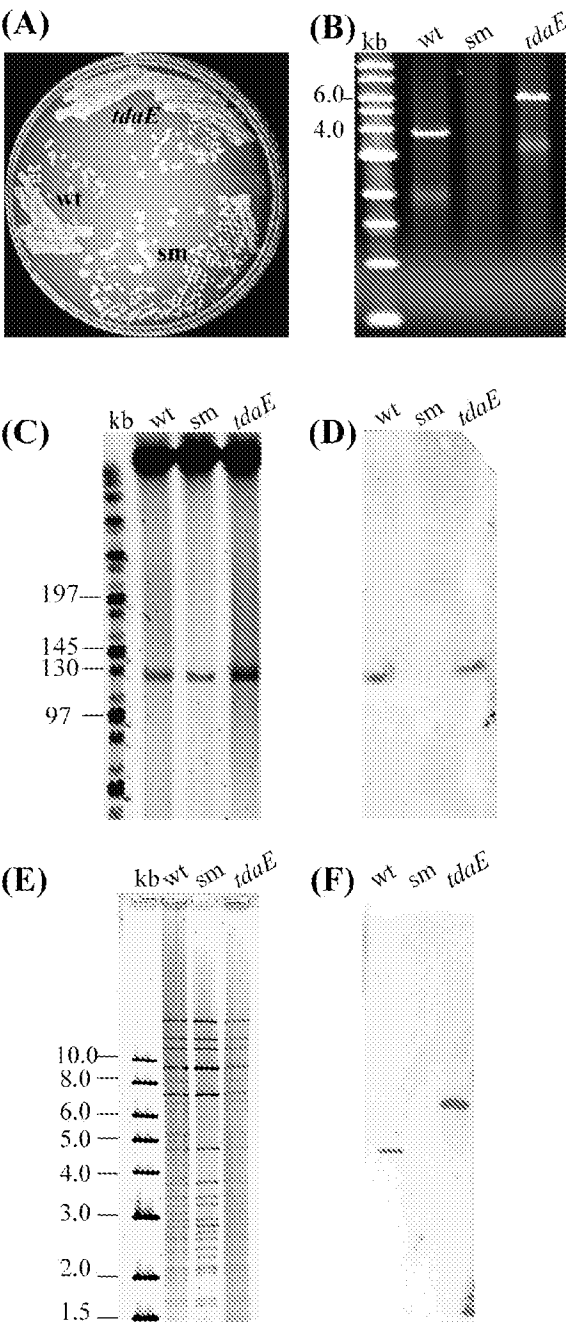


FIG. 5

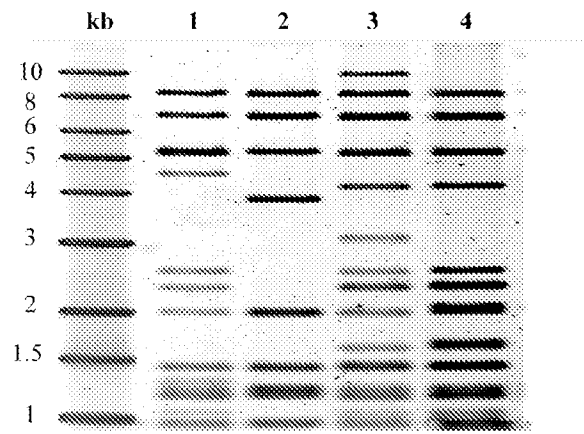


FIG. 6

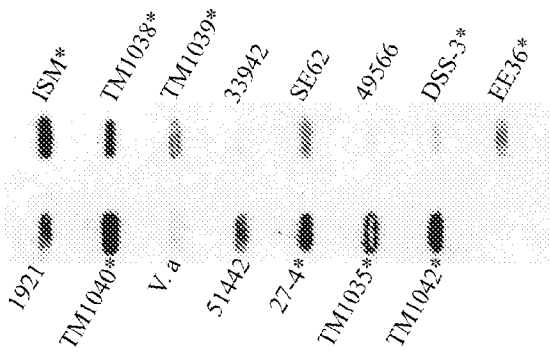


FIG. 7

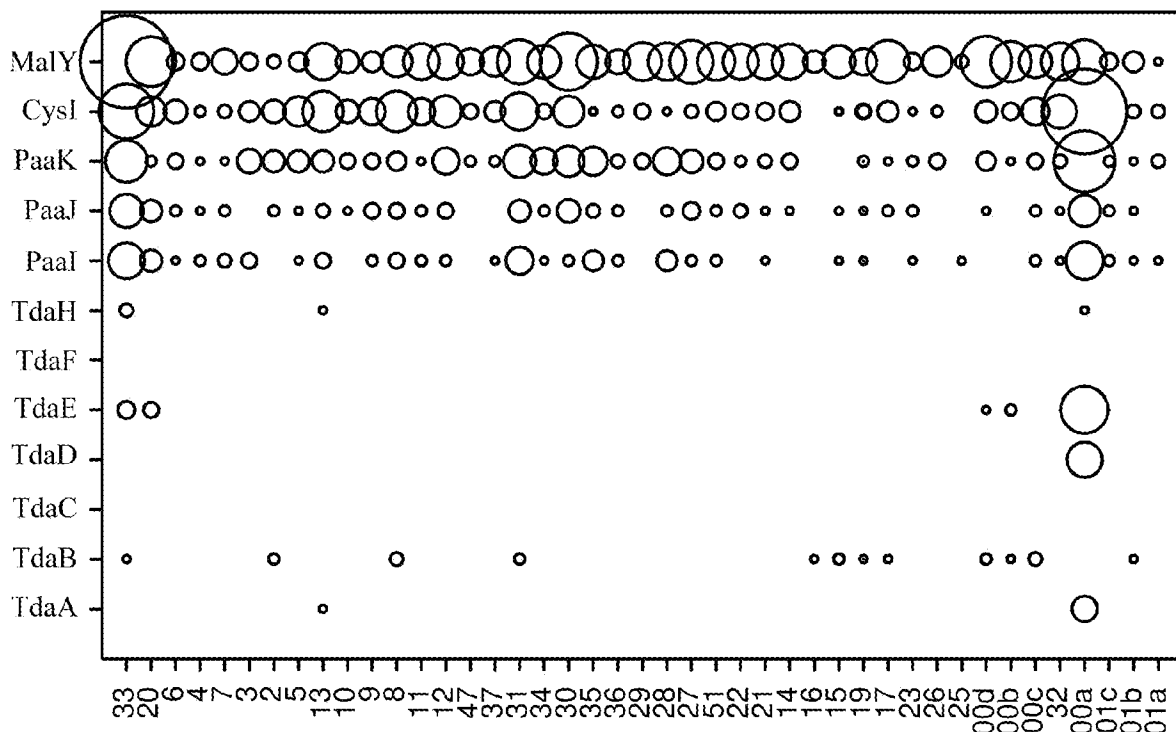


FIG. 8

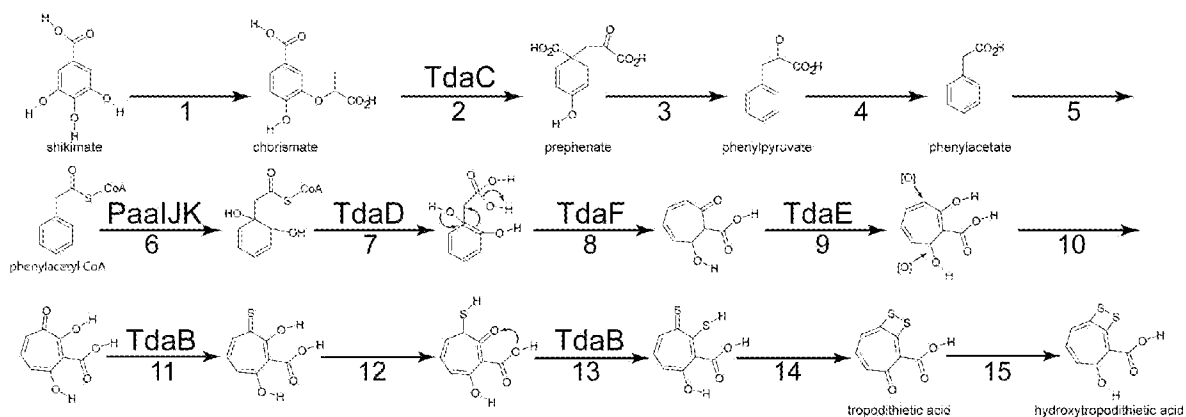


FIG. 9