Roseobacticides: Small Molecule Modulators of an Algal-Bacterial Symbiosis

Mohammad R. Seyedsayamdost,† Gavin Carr,‡ Roberto Kolter,‡ and Jon Clardy*†

†Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, United States
‡Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, United States

Supporting Information

ABSTRACT: Marine bacteria and microalgae engage in dynamic symbioses mediated by small molecules. A recent study of Phaeobacter gallaeciensis, a member of the large roseobacter clade of α-proteobacteria, and Emiliania huxleyi, a prominent member of the microphytoplankton found in large algal blooms, revealed that an algal senescence signal produced by E. huxleyi elicits the production of novel algaeicides, the roseobacticides, from the bacterial symbiont. In this report, the generality of these findings are examined by expanding the number of potential elicitors. This expansion led to the identification of nine new members of the roseobacticide family, rare bacterial troponoids, which provide insights into both their biological roles and their biosynthesis. The qualitative and quantitative changes in the levels of roseobacticides induced by the additional elicitors and the elicitors’ varied efficiencies support the concept of host-targeted roseobacticide production. Structures of the new family members arise from variable substituents at the C3 and C7 positions of the roseobacticide core as the diversifying elements and suggest that the roseobacticides result from modifications and combinations of aromatic amino acids. Together these studies support a model in which algal senescence converts a mutualistic bacterial symbiont into an opportunistic parasite of its hosts.

INTRODUCTION

Investigating the chemistry underlying microbial symbioses provides opportunities to discover new small molecules in the context of the biological roles they have evolved to fulfill. In a recent example of this search strategy, we described roseobacticides A and B (Figure 1, 1, 2), which contain the previously unreported 1-oxazalan-2-one core, and their ability to affect marine phytoplankton with nM potency. The bacterial symbiosis partner, or symbiont, that produces these roseobacticides, Phaeobacter gallaeciensis BS107, belongs to the roseobacter clade, a large, phylogenetically related group of marine α-proteobacteria that account for up to 25% of all bacteria in typical coastal communities. P. gallaeciensis BS107 is easily cultured in the laboratory and under these conditions produces a number of secondary metabolites including the antibiotic tropodithietic acid (3), its precursor 4, and the plant growth promoter phenylacetic acid (5). P. gallaeciensis BS107 associates with Emiliania huxleyi, a globally distributed single-celled microalga covered with ornate CaCO3 disks. E. huxleyi is a major contributor (80–90%) to massive (103–105 km3) seasonal algal blooms that are easily visible in satellite images, and it, along with other microphytoplankton, produces nearly half of the Earth’s atmospheric oxygen. In addition to fixing CO2 through photosynthesis, E. huxleyi sequesters CO2 in the CaCO3 disks that surround each algal cell, and also plays a role in the global sulfur cycle by reducing dissolved sulfate to methionine, cysteine, and dimethylsulfoniopropionate (DMSP, 6). DMSP attracts roseobacter (and other) bacteria, which use it as a carbon and sulfur source. The bacteria can metabolize DMSP to volatile DMS, which in the atmosphere is converted to condensation nuclei for water droplets. Thus, roseobacter-microalgal symbioses play key roles in important biogeochemical processes.

Numerous studies had shown that the symbioses between bacteria, including those in the roseobacter clade like P. gallaeciensis BS107, and microphytoplankton, like E. huxleyi, were dynamic; that is, the partners were at times attracted to and at other times repelled by one another. It seemed likely that small molecule messages exchanged between the partners elicited these changes in their relationship status. Recent findings by the Harwood and Greenberg laboratories indicated that terrestrial plant-associated bacteria can respond to monomeric components of the heteropolymer lignin that are released into the surrounding soil when plants senesce. As lignin components have been identified in green, red and brown algae, a similar response could plausibly occur in marine plant-bacterial interactions. Examination of E. huxleyi, revealed production of significant quantities of p-coumaric acid (pCA, 7), making E. huxleyi

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Under these conditions, the healthy algal host provides DMSP (promoter 5) biosynthesized from E. huxleyi to pCA by producing the roseobacticides, potent algaecides that kill E. huxleyi and a parasitic phase, where the bacterial partner converts the algal host into the environment. The presence of pCA stimulates the interaction between P. gallaeciensis and E. huxleyi to transform molecules that facilitate algal growth to potent and selective phytotoxins. The model in Figure 1 was based on one member of the large roseobacter clade, one member of the microphytoplankton family, one elicitor, and a few bacterial metabolites, and this restricted basis set raises questions about the generality of our model and the small molecules involved. In this report we begin to address some of these questions by examining a larger panel of elicitors, compounds released by the algal host that stimulate production of bacteral secondary metabolites. These studies led to a dramatic expansion of the roseobacticide family through complex quantitative and qualitative changes in bacterial metabolism. We also expand the study to include other members of the roseobacter clade and their responses to the larger panel of elicitors.

**MATERIALS AND METHODS**

**Materials and Strains.** Candidate elicitors 7–11 (Figure 2) and sea salt used for preparation of culture media were obtained from Sigma-Aldrich. Other media components were from Becton-Dickinson. Roseobacter strains P. gallaeciensis BS107 and P. gallaeciensis 2.1015f were obtained from Prof. Rebecca Case (University of Alberta). Strains Phaeobacter inhibens (DSMZ 16374) and Marinorium algicola (DSMZ 10251) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

**General Procedures.** HPLC purifications were carried out on an Agilent 1200 Series analytical or preparative HPLC system equipped with a photodiode array detector. Low-resolution HPLC-MS analysis was performed on the same analytical system equipped with a 6130 Series ESI mass spectrometer using an analytical Phenomenex Luna C18 column (5 μm, 4.6 × 100 mm) operating at 0.7 mL/min with a gradient of 30% MeCN in H2O to 100% MeCN over 20 min. High resolution (HR)-HPLC-ESI-MS and HR tandem ESI-MS (HR-MS/MS) were carried out on an Agilent 1200 Series HPLC equipped with a photodiode array detector and a 6520 Series LC/Q-TOF using the same column and gradient as above. HR-MS and HR-MS/MS were calibrated to within 3 ppm and 12 ppm, respectively. 1H, 13C and 2D NMR spectra were recorded in the inverse-detection probe of a Varian Inova spectrometer (600 MHz for 1H, 150 MHz for 13C). Chemical shifts were referenced to the residual solvent peaks in acetone-d6 or methanol-d4.

**Cultivation of Roseobacter Strains.** Preparative-scale (2–8 L) cultivation of P. gallaeciensis BS107 (or other roseobacter strains) was carried out in half-strength yeast extract-triptone-sea salt (YTSS) medium, which consists of (per L): 20 g Sigma sea salt, 2 g yeast extract, and 1.25 g tryptone. P. gallaeciensis BS107 (or other roseobacter strains) were streaked out from frozen culture stocks and maintained on Marine Broth.
agar plates (Difco 2216) at 30 °C. Overnight cultures were initiated by inoculating 5 mL YTSS medium in 15 mL culture tubes and shaking these overnight at 250 rpm and 30 °C. A 0.5 L Erlenmeyer flask containing 50 mL YTSS medium was inoculated with 0.5 mL of the overnight culture and grown for 12–18 h at 30 °C and 160 rpm. Large 4 L Erlenmeyer flasks, each containing 0.4 L YTSS medium, were inoculated with 4 mL of the overnight culture and supplemented with 1 mM of each of the elicitors (7–11). The cultures were grown for 3 d at 30 °C and roseobacticides purified as described below.

**Elicitor Dose–Response Analysis.** Eight to ten 0.25 L Erlenmeyer flasks each containing 25 mL of YTSS medium and a range of elicitor concentrations (between 0 and 1.2 mM) were inoculated with 0.25 mL of an overnight *P. gallaeciensis* BS107 culture prepared as described above. These were grown at 30 °C and 160 rpm. After 3 d, each culture was extracted twice with 25 mL of EtOAc. The organic phase was combined, dried over Na2SO4, and subsequently dried in vacuo. The residue was resuspended in 0.3 mL MeOH and analyzed by HPLC-MS as described above. The amount of roseobacticide B (2) produced, quantified by mass-ion extraction ([M + H]+ = 269), was plotted against the concentrations of the elicitor. The maximal amount of 2 was normalized to 100%, and the EC50, the elicitor concentration where production of 2 was half-maximal, was obtained by fitting the data to eq 1, where Bmax and Bmin are the maximal (∼100%) and minimal (∼0%) amounts of 2 and p is a Hill slope parameter to account for variations in the slope.

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    \text{amount of } 2 = B_{\text{min}} + \frac{B_{\text{max}} - B_{\text{min}}}{1 + 10^{(EC_{50} - [\text{elicitor}]) \cdot p}}
\]  

**Purification of Roseobacticides.** After 3 days, the large-scale cultures were extracted twice with an equal volume of EtOAc. The organic phase was combined, dried over Na2SO4, and subsequently dried in vacuo. The residue was weighed, resuspended in a small volume of MeOH, mixed with a 3-fold excess of Celite (by weight), and dry-loaded onto a C18-functionalized silica gel column (~3 g, d = 15 mm, l = 40 mm), which had been equilibrated in 15% MeCN in H2O. The column was then washed with 10 column volumes (CV) of 15% MeCN, and roseobacticides eluted with a step gradient of 10 CV of 30% MeCN, 10 CV of 75% MeCN, which contained roseobacticides, and 10 CV of 100% MeCN. The 75% MeCN fraction was dried in vacuo and purified on a preparative Phenomenex Phenyl-Hexyl column (5 μm, 21.2 × 250 mm) operating at 12 mL/min with a gradient of 40% MeCN in H2O to 100% MeCN over 40 min. Fractions that contained roseobacticides, as judged by their UV-visible spectra and by analytical HPLC-MS, were further purified on a semipreparative Agilent Eclipse XDB-C8 column (5 μm, 9.4 × 250 mm) operating at 3 mL/min using a gradient of 35% MeCN in H2O to 80% MeCN over 40 min. Replication of the material onto the same column (or a Supelco Discovery C18 column (10 μm, 10 × 250 mm) or a Phenomenex Luna Phenyl-Hexyl column (5 μm, 10 × 250 mm), depending on the roseobacticide) using the same flow rate and gradient afforded pure material.

**Structural Elucidation.** Structures of roseobacticides were elucidated using standard 1D (1H and 13C) and 2D (gCOSY, gHSQC, gHMBC, NOESY) NMR spectra. In addition, HR-MS and HR-MS/MS data were utilized as described above. 1H NMR spectra, tables of 2D NMR data, HR-MS and HR-MS/MS results for each compound are shown in the Supporting Information. Degradation analysis for 13–15, 19, and 20 was carried out by incubating a small amount of each compound (~100 μL, ~5 μg) with 5 mM (~10 μL) of the disulfide reducing agent dithiothreitol in MeOH for 1–3 h at room temperature, followed by analysis of the reaction products (~50 μL) by HPLC-MS as described above.

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**RESULTS AND DISCUSSION**

**Additional Roseobacticide Elicitors.** In addition to indicating that algal cell wall components may act as elicitors of bacterial metabolite production, our previous results also suggested that *P. gallaeciensis* BS107 is an opportunistic symbiont that could interact with a wide range of hosts. Bioinformatic analyses showed that *E. huxleyi* appears to only contain a pathway for the biosynthesis of H-lignin, the polymer resulting from linkage of pCA units.2 However, as the nature of lignin components varies with algal hosts,20* P. gallaeciensis* BS107 could also encounter and respond to lignin monomers other than pCA. To test this hypothesis, *P. gallaeciensis* BS107 was incubated with various concentrations of pCA, sinapic acid (8) and ferulic acid (9), known components of cell wall lignin, as well as with cinnamic acid (10) and caffeic acid (11), intermediates in the biosynthesis of 7–9 (Figure 2), and the level of secondary metabolite production was assessed by HPLC-MS methods. Using 7, 8, and 9 as elicitors led to the production of a variety of new metabolites, 10 generated less dramatic results, and 11 produced no observable changes (Supporting Information Figure S1). These results indicate that, in addition to 7, the lignin precursors 8–10 also elicit roseobacticide production in *P. gallaeciensis* BS107 in support of the proposed mutualist-to-parasite switch in our dynamic symbiosis model (Figure 1). To find optimal conditions for roseobacticide production, a dose-response analysis was carried out with each of the main elicitors. *P. gallaeciensis* BS107 was incubated with varying concentrations of the elicitor, and roseobacticide B production was quantified using HPLC-ESI-MS. The analysis previously indicated a half-maximal effective concentration (EC50) of 0.79 ± 0.03 mM with pCA.2 With 8 and 9, we obtained EC50 values of 0.43 ± 0.03 and 0.16 ± 0.02 mM, respectively, indicating that these are more potent elicitors of roseobacticide B production in *P. gallaeciensis* BS107 (Figure 3). Each elicitor also shows...
quantitative changes in the levels of roseobacticide B produced, which may have implications for the interaction of *P. gallaeciensis* BS107 with its algal hosts (see below).

**Elucidation of New Roseobacticide Structures.** The compounds induced by pCA (7), sinapic acid (8) and ferulic acid (9) were purified from large-scale production cultures of *P. gallaeciensis* BS107 in the presence of each elicitor using standard solid-phase extraction and HPLC methods. The structures were subsequently solved by 1D and 2D NMR spectroscopy, HR-HPLC-ESI-MS and HR-MS/MS. All structures reported below have an H/C ratio < 1, and NMR analysis alone was usually not sufficient for structural elucidation necessitating HR-MS/MS and chemical degradation analyses. Using these techniques, we were able to elucidate the structures of nine new roseobacticides, which fall into four classes (Figure 4): (1) A phenol family with compounds 1, 13, and 17, which contain a thiomethyl, a methyl persulfide, or a p-hydroxybenzenethiol moiety at C7 and a phenol group at C3; (2) A phenyl family with compounds 2, 14, 16, and 18 containing a thiomethyl, a methyl persulfide, a sulfonate, or a p-hydroxybenzenethiol at C7 and a phenyl group at C3; (3) An indole family with roseobacticides C (12) and F (15), which contain a thiomethyl or a methyl persulfide at C7, and an indole at C3; and (4) A dimer family with roseobacticides J (19) and K (20), which consist of two roseobacticides joined through a disulfide linkage.

The structure of the first indole analog, roseobacticide C (Figure 4), was solved readily from 1D and 2D NMR spectra and HR-ESI-MS (Supporting Information Tables S1 and S2). The 'H NMR spectrum revealed a pattern diagnostic of the 1-oxazuln-2-one core with a different substituent at C3 (Supporting Information Figure S2). 'H NMR, COSY, HSQC, and HMBC spectra (Supporting Information Figure S2 and Table S3) indicated an indole group in agreement with a molecular formula of 
C\textsubscript{18}H\textsubscript{13}NO\textsubscript{2}S ([M + H]\textsuperscript{+} calcd 308.0745, exp 308.0738). HR-MS/MS analysis was consistent with this assignment (Supporting Information Table S2). As with 1 and 2, the NOESY spectrum of 12 revealed a cross peak between the methyl protons and the proton at C6 (Supporting Information Figure S2). The nature of the substituent at C3 in 1, 2, and 12, points to aromatic amino acids as precursors in roseobactide biosynthesis. In addition, the presence of indole at the C3 position implicates indoleacetic acid as an intermediate in the biosynthesis of 12.\textsuperscript{7} Because indoleacetic acid is a prominent plant and algal growth promoter,\textsuperscript{22} the presence of 12 further supports our model in which the mutualist-to-parasite switch results in a conversion of growth-promoting metabolites into phytotoxins. This finding further highlights the dynamic nature of the algal–bacterial symbiosis (Figure 1).

HR-ESI-MS analysis of roseobacticides D, E, and F indicated that they contain an additional sulfur atom relative to roseobacticides A, B, and C, respectively (Supporting Information Table S1). On the basis of the \(^{13}\text{C}\) chemical shifts of the methyl groups in 13–15, (22–23 ppm, Supporting Information Figures S3–S5, Tables S4–S6) compared to that of the methyl groups in 1, 2, and 12 (0–15 ppm, Supporting Information Table S3 and ref 2), we suspected that the former contained a methyl persulfide rather than a thiomethyl group at C7. Incubation of 14 with the reducing agent dithiothreitol (DTT) followed by low-resolution HPLC-MS analysis gave a fragment consistent with loss of methanethiol (Supporting Information Figure S6 and Scheme 1, 14, [M + H]\textsuperscript{+} calcd 255.1, exp 255.1) in agreement with a methyl persulfide functionality. In addition, HR-MS/MS analysis with 14 (Figure S) gave fragments resulting from the loss of a methyl group ([M + H]\textsuperscript{+} calcd 286.0117, exp 286.0163), loss of a thiomethyl group ([M + H]\textsuperscript{+} calcd 254.0396, exp 254.0436) and loss of a methyl persulfide ([M + H]\textsuperscript{+} calcd 222.0675, exp 222.0699) establishing the structure of 14 as shown in Figure 4. The corresponding fragments were also obtained with 13 and 15 (Figure S and Supporting Information Table S2). The NOESY spectra of 13–15 did not reveal a cross peak between the methyl protons and the C6-proton

![Figure 4. Structures of roseobacticides A–K, of which C–K have been determined in this work. See text for a description.](image-url)
The 1H NMR and HSQC spectra of 16 revealed a pattern similar to that of 2, but with major differences in 1H and 13C chemical shifts (Supporting Information Figure S8 and Table S7). The nature of these shifts and the broad peak of this compound during chromatography, even in the presence of 0.1% formic acid, suggested an acidic functionality. HR-MS yielded a formula of C15H10O5S in line with the presence of a sulfonic acid at C7. HR-MS/MS gave fragments consistent with the loss of SO2, which is diagnostic for aromatic sulfonates, as well as with the loss of SO3H and CO (Supporting Information Table S2). The 1H NMR and HSQC spectra of 17 and 18 were in line with the presence of the 1-oxaazulan-2-one core with the thiomethyl group at C7 replaced with a phenol-containing moiety at C7. HR-ESI-MS gave fragments consistent with the loss of SO2 and CO (Supporting Information Table S2, [M + H]+ calcd 254.0402, exp 254.0420). In HR-MS/MS, which originate from cleavage of the disulfide bond (Supporting Information Figure S10), in line with the molecular formula of C21H14O4S ([M + H]+) calcd 507.0725, exp 507.0736). During purification of 19, we also observed a faster-migrating fraction with a similar UV-visible spectrum. The molecular formula of C30H18O5S2 is consistent with 20, as are the two main fragments observed by HR-MS/MS, which originate from cleavage of the disulfide bond (Supporting Information Table S2, [M + H]+) calcd 270.0351, exp 270.0383 and [M + H]+) calcd 254.0402, exp 254.0398), in line with the molecular formula of C30H18O5S2 ([M + H]+) calcd 507.0725, exp 507.0736). During purification of 19, we also observed a faster-migrating fraction with a similar UV-visible spectrum. The molecular formula of C30H18O5S2 is consistent with 20, as are the two main fragments observed by HR-MS/MS, which originate from cleavage of the disulfide bond (Supporting Information Figure S10, [M + H]+) calcd 255.0480, exp 255.0483). HR-MS/MS analysis confirmed the structure of 19 revealing a major fragment arising from cleavage of the disulfide bond (Supporting Information Table S2, [M + H]+) calcd 254.0402, exp 254.0398), in line with the molecular formula of C30H18O5S2 ([M + H]+) calcd 507.0725, exp 507.0736). During purification of 19, we also observed a faster-migrating fraction with a similar UV-visible spectrum. The molecular formula of C30H18O5S2 is consistent with 20, as are the two main fragments observed by HR-MS/MS, which originate from cleavage of the disulfide bond (Supporting Information Table S2, [M + H]+) calcd 270.0351, exp 270.0383 and [M + H]+) calcd 254.0402, exp 254.0420). In addition, treatment of 20 with DTT led to its disappearance and formation of new peaks, one consistent with 21 (Supporting Information Figure S12, [M + H]+) calcd 255.0480, exp 255.0490), and another consistent with 22 ([M + H]+) calcd 271.0429, exp 271.0439, see Scheme 1). The structure of 20 has been assigned based on its migratory properties, UV−vis spectrum, HR-MS, and HR-MS/MS. This compound was produced in very small quantities insufficient for NMR analysis. Thus, the structure shown for 20 remains tentative.

Together, elucidation of the additional elicitors and roseobacticides considerably expands the diversity of small molecules that are likely exchanged in the dynamic roseobacter-algal interaction (Figure 1).

### Table 1. Amount of Roseobacticides (mg/L) Produced by P. gallaeciensis BS107 and 2.10 as a Function of Elicitor

| Roseobacticides | 7   | 8   | 9   | 10  |
|-----------------|-----|-----|-----|-----|
| A               | 0.29| 0.08| 0.2 | 0.04|
| B               | 0.11| 1.1 | 0.5 | 0.2 |
| C               | 0.29| 0.45| 0.25|
| D               | 0.1 | 0.09|
| E               | 0.18| 0.47| 0.2 |
| F               | 0.15| 0.19|
| G               | 0.6 | 0.13|
| H               | 0.2 | 0.12|
| I               | 0.06|
| J               | 0.06| 0.15| 0.1 |
| K               | 0.012|

*Values are averages from three (7, 8) or two (9, 10) independent isolations from large-scale cultures. Standard deviations ranged from 5–40%. **Denotes amounts below 0.04 mg/L. *An estimate from HPLC-MS comparisons with 19. Values are averages from two independent experiments from small-scale cultures and comparison with known amounts of roseobacticides. Note that, unlike the data for P. gallaeciensis BS107, these are not isolation yields. Standard deviations ranged from 10–35%.

HPLC-HR-MS monitoring of this reaction corroborated the assignment of the new peak as 21 ([M + H]+) calcd 255.0480, exp 255.0483). HR-MS/MS analysis confirmed the structure of 19 revealing a major fragment arising from cleavage of the disulfide bond (Supporting Information Table S2, [M + H]+) calcd 254.0402, exp 254.0398), in line with the molecular formula of C30H18O5S2 ([M + H]+) calcd 507.0725, exp 507.0736). During purification of 19, we also observed a faster-migrating fraction with a similar UV-visible spectrum. The molecular formula of C30H18O5S2 is consistent with 20, as are the two main fragments observed by HR-MS/MS, which originate from cleavage of the disulfide bond (Supporting Information Table S2, [M + H]+) calcd 270.0351, exp 270.0383 and [M + H]+) calcd 254.0402, exp 254.0420). In addition, treatment of 20 with DTT led to its disappearance and formation of new peaks, one consistent with 21 (Supporting Information Figure S12, [M + H]+) calcd 255.0480, exp 255.0490), and another consistent with 22 ([M + H]+) calcd 271.0429, exp 271.0439, see Scheme 1). The structure of 20 has been assigned based on its migratory properties, UV−vis spectrum, HR-MS, and HR-MS/MS. This compound was produced in very small quantities insufficient for NMR analysis. Thus, the structure shown for 20 remains tentative.

Together, elucidation of the additional elicitors and roseobacticides considerably expands the diversity of small molecules that are likely exchanged in the dynamic roseobacter-algal interaction (Figure 1).

### Host-Targeted Roseobacticide Production

Having characterized the structures of the new roseobacticides, we examined the elicitor-dependent differential production of each analog. Table 1 summarizes the amount of each roseobacticide obtained.
as a function of elicitors 7–10. While there were batch-to-batch variations, sinapic acid (8) was consistently the most effective elicitor with *P. gallaeciensis* BS107 both in the amount and diversity of roseobacticides stimulated followed by PCA (7), ferulic acid (9), and cinnamic acid (10). As lignin monomers vary depending on the algal host,\(^{180}\) the quantitative and qualitative changes observed in Table 1 may indicate host-specific production of roseobacticides. These results also indicate that *P. gallaeciensis* BS107 produces a library of roseobacticides, but each in relatively small quantities, perhaps because of the potency of roseobacticide activity, which has been observed with 1 and 2, and the broad range of hosts with which *P. gallaeciensis* BS107 likely interacts.\(^2\)

**Roseobacticide Production by *P. gallaeciensis* BS107.** Because of the ecological contributions of algal–bacterial symbioses, it is important to identify new roseobacticide producers as a measure of the potential environmental significance of this compound class. To assess how widespread roseobacticide production is, and whether the interaction in Figure 1 may be extended to other roseobacter, we examined three of the closest relatives to *P. gallaeciensis* BS107: *Phaeobacter gallaeciensis* 2.10,\(^{15f}\) isolated from the green macroalga, *Ulva lactuca*; *Phaeobacter inhibens*,\(^2,25\) isolated from the German Wadden Sea; and *Marinovum algicola*,\(^26\) isolated from the dinoflagellate *Proorocentrum lima*. Each strain was grown under identical conditions as *P. gallaeciensis* BS107 in the presence of 7–10. No roseobacticides were observed with *P. inhibens* or *M. algicola* under these conditions. In the case of *P. gallaeciensis* 2.10, various roseobacticides were produced as a function of the elicitor examined (Supporting Information Figure S13); the data are summarized in Table 1. Compound 7 induced the production of 1, 2, 17, and 18, while 9 stimulated production of large quantities of 2 and 18. Sinapic acid (8) resulted in production of only 2 at approximately similar levels as obtained with 10. In contrast to *P. gallaeciensis* BS107, 10 was a good elicitor in *P. gallaeciensis* 2.10. Overall, a different trend was observed with *P. gallaeciensis* 2.10 in that 9 was the strongest elicitor, followed by 10, 7, and 8. Production of roseobacticides by *P. gallaeciensis* 2.10 suggests they may be active against macroalgae, or that *P. gallaeciensis* 2.10 is, like its BS107 relative, also an opportunistic algal symbiont. We previously examined *Ruegeria pomeroyi* DSS-3 and *Ruegeria sp.* R11, both of which did not produce roseobacticides.\(^2\) Thus, within the still restricted number of roseobacter members investigated, roseobacticide production appears to be limited to *P. gallaeciensis* strains.

### CONCLUSIONS

In this study, we have identified three additional elicitors of roseobacticide production, nine new roseobacticides, and an additional member of the roseobacter clade that also produces these interesting and novel troponoids. The identification of other lignin monomers that lead to roseobacticide production provides support for a model in which algal senescence signals convert a mutualistic interaction into a parasitic one (Figure 1). They also argue that roseobacticide producers are opportunistic algal pathogens that interact with a variety of algal hosts. The structures of the new members of the roseobacticide family provide some insight into their biosynthesis: the substituents at C3 and C7 generate the family’s diversity. The nature of the substituents at C3 points to an aromatic amino acid origin in which the three monomeric families originate from phenylalanine, tyrosine, and tryptophan. The diversity and nature of the C7-substituents, coupled with the knowledge that *P. gallaeciensis* BS107 produces dozens of sulfur-, disulfide-, and thiol-containing compounds,\(^7,24\) suggest spontaneous addition of a variety of thiols. These two observations are consistent with a specific pathway for generation of the 1-oxazulan-2-one core derived from aromatic amino acids, followed by (possibly spontaneous) sulfur-dependent chemistry to provide substituents at the C7 position. Our results also show qualitative and quantitative changes in roseobacticide production depending on the nature of the elicitor in both *P. gallaeciensis* strains. Future biological assays with each roseobacticide variant against a panel of potential algal hosts will elucidate whether the elicitor-dependent changes result from host-targeted roseobacticide production.\(^27,28\) This study has established a large part of the diversity of roseobacticides produced in *P. gallaeciensis* setting the stage for examination of the molecular mechanisms that generate this diversity.

### ASSOCIATED CONTENT

**Supporting Information.** HPLC-ESI-MS analysis of the extracts of *P. gallaeciensis* BS107 and *P. gallaeciensis* 2.10 in the presence of 7–11, 1D/2D NMR spectra, HR-ESI-MS, and HR-MS/MS for 12–20, and degradation analysis of 14, 19, and 20. This material is available free of charge via the Internet at http://pubs.acs.org.

### AUTHOR INFORMATION

**Corresponding Author**

jon_clardy@hms.harvard.edu

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(28) Comprehensive activity assays of all roseobactidates described herein vs a panel of microalgae is currently in progress.