Investigation of the Genetics and Biochemistry of Roseobacticide Production in the Roseobacter Clade Bacterium Phaeobacter inhibens

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ABSTRACT  Roseobacter clade bacteria are abundant in surface waters and are among the most metabolically diverse and ecologically significant species. This group includes opportunistic symbionts that associate with micro- and macroalgae. We have proposed that one representative member, Phaeobacter inhibens, engages in a dynamic symbiosis with the microalga Emiliania huxleyi. In one phase, mutually beneficial molecules are exchanged, including the Roseobacter-produced antibiotic tropolactone acid (TDA), which is thought to protect the symbiotic interaction. In an alternative parasitic phase, triggered by algal senescence, the bacteria produce potent algaecides, the roseobacticides, which kill the algal host. Here, we employed genetic and biochemical screens to identify the roseobacticide biosynthetic gene cluster. By using a transposon mutagenesis approach, we found that genes required for TDA synthesis—the tda operon and paa catabolon—are also necessary for roseobacticide production. Thus, in contrast to the one-cluster–one-compound paradigm, the tda gene cluster can generate two sets of molecules with distinct structures and bioactivities. We further showed that roseobacticide production is quorum sensing regulated via an N-acyl homoserine lactone signal (3-OH–C10-HSL). To ensure tight regulation of algaecide production, and thus of a lifestyle switch from mutualism to parasitism, roseobacticide biosynthesis necessitates the presence of both an algal senescence molecule and a quorum sensing signal.

IMPORTANCE  Marine Roseobacter species are abundant in the oceans and engage in symbiotic interactions with microscopic algae. One member, P. inhibens, produces the antibiotic TDA and a growth hormone thought to protect and promote algal growth. However, in the presence of molecules released by senescing algae, the bacteria produce potent algaecides, the roseobacticides, which kill the host. We examined the regulatory networks and biosynthetic genes required for roseobacticide production. We found that P. inhibens uses largely the same set of genes for production of both TDA and roseobacticides, thus providing a rare case in which one gene cluster synthesizes two structurally and functionally distinct molecules. Moreover, we found roseobacticide production to be regulated by quorum sensing. Thus, two small molecules, the algal metabolite and the quorum-sensing signal, ensure tight control in the production of roseobacticides. These results highlight the role of small molecules in regulating microbial symbioses.

The wealth of bacterial genome sequences has deeply impacted our ability to connect an isolated secondary metabolite to its biosynthetic gene cluster. With recent advances in bioinformatics, the first step in this process typically involves a computational approach (1). However, in the case of small molecules that belong to a new chemotype and do not represent the typical structural classes, such as nonribosomal peptides (NRP) or polyketides (PK), this process is much more challenging. Identifying the biosynthetic gene clusters of these types of molecules requires bacterial genetic methods with screens tailored toward the biological or chemical properties of the molecule of interest. Roseobacticides, a family of tropolactone natural products, represent an example of this category of molecules (2). They are not derived from NRP/PK biosynthetic pathways, contain a unique chemical scaffold, and are synthesized by an as-yet-unknown biosynthetic pathway and gene cluster.

Roseobacticides are produced by Phaeobacter inhibens, a member of the Roseobacter clade of alphaproteobacteria, which are abundant in the oceans, especially in coastal regions during algal blooms (3, 4). Aside from their abundance, Roseobacter are known for their ability to colonize biotic and abiotic surfaces and interact with microalgae, macroalgae, and other eukaryotes (5–7). Numerous studies have demonstrated beneficial roles for bacterial colonization of algal surfaces, suggesting a mutualistic association (8–10). On the basis of recent results, we have proposed that P. inhibens engages in a biphasic symbiosis with microalgae, such as Emiliania huxleyi, with each phase of the symbiosis characterized by a set of small molecules (Fig. 1) (2, 11). In the mutualistic phase, the algae provide dimethylsulfoniopropionate (DMSP), which the bacteria move toward via chemotaxis and use as a source of carbon and sulfur (12–14). In return, the bacteria generate phenylacetic acid, which has been shown to serve as a growth...

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Wang et al.

FIG 1 Model for algal-bacterial symbiosis involving *P. inhibens* and *E. huxleyi*. The symbiosis comprises two modes, a mutualistic phase (green arrows) and a parasitic phase (red arrows) (2). In the mutualistic phase, phenylacetic acid (PAA) provides a precursor for TDA, and both metabolites serve as beneficial molecules to the algae, which provide the bacteria with food in the form of DMSP. In the parasitic phase, the senescing algal host releases pCA. The bacteria respond by combining fragments of DMSP, PAA, and pCA to synthesize the algaecide roseobacticide A (23).

The biochemical pathway for TDA production has been investigated in fungi (27), TDA biosynthesis occurs by a different strategy (15, 28, 29). Much like roseobacticides, the related tropone-bearing TDA is polycyclic and contains a tropone motif (Fig. 1). The troponoid inducers, such as pCA or sinapic acid (2, 11), are cryptic metabolites: their production is tightly regulated, that is, they are not observed under a number of conditions lacking pCA and have only been observed in the presence of phenylpropanoid inducers, such as pCA or sinapic acid (2, 11).

Aside from their important biological activity, roseobacticides constitute a new structural class of natural products and as such their biosynthesis is of great interest, as it promises to uncover new enzymatic transformations. Our recent examination of the biosynthetic pathway of roseobacticides by isolate feeding experiments revealed that phenylacetic acid, phenylglyoxylic acid, and Cys—which is derived from DMSP in a natural setting—are utilized as roseobacticide precursors. Not only did these studies provide a biosynthetic model for the roseobacticides, they also demonstrated a remarkable metabolic economy in the conversion of molecules involved in the mutualistic phase (DMSP and phenylacetic acid) into toxins in the parasitic phase (Fig. 1).

Much like roseobacticides, the related tropone-bearing TDA is also structurally unusual (Fig. 1) (24–26). While tropone-containing metabolites have been shown to be produced in a polyketide synthase-dependent manner in fungi (27), TDA biosynthesis in *P. inhibens* occurs by a different strategy (15, 28, 29). The biochemical pathway for TDA production has been investigated for over 20 years, but a consensus pathway has yet to be determined. The regulatory and biosynthetic genes have been identified through a comprehensive transposon mutagenesis screen, which utilized the potent antibacterial activity of TDA against *Vibrio anguillarum* to search for mutants that no longer exhibited growth-inhibitory effects (28). This approach identified the *tda* operon, as well as a number of other biosynthetic loci that in a concerted fashion give rise to TDA. These genes code for small biosynthetic enzymes, most without recognizable signature motifs, and as such, identifying them by bioinformatic means would be exceedingly difficult. Using a similar approach, we hoped to answer questions regarding the regulation and biosynthesis of roseobacticides. Here, we have employed random transposon mutagenesis along with a high-throughput fluorescence assay to identify genes involved in roseobacticide synthesis. We report that roseobacticide production is regulated via quorum sensing (QS) and that it shares a late common intermediate with the TDA biosynthetic pathway, as these two processes share a number of enzymes. Thus, the *tda* locus gives rise not only to TDA but also to roseobacticides, providing an unusual case in which one gene cluster is responsible for the synthesis of two structurally and functionally distinct secondary metabolites.

**RESULTS AND DISCUSSION**

**Choice of random mutagenesis and assay systems.** To identify the biosynthetic genes required for roseobacticide production, we initiated a transposon mutagenesis study, in which we planned to generate a library of mutants and identify those that do not produce roseobacticides in the presence of pCA. Roseobacticides do not harbor antibacterial activity that can be carried out in a high-throughput fashion. While they kill *E. huxleyi* (with a half-maximal inhibitory concentration of 0.1 μM in the case of roseobacticide B), carrying out this assay with thousands of mutants proved impractical.

Given that the bioactivity of roseobacticides is difficult to test in a high-throughput format, we turned our attention to their chemical properties. Roseobacticides display absorption features with a λ_{max} of 430 nm and extinction coefficient (ε) of 6,000 M⁻¹ cm⁻¹ (see Fig. S1 in the supplemental material). These properties, however, were not sufficient to discern the presence of roseobacticides in cultures, due to interference from the medium of choice, which gave background absorption at 430 nm. Fluorescence spectroscopy, however, provided a viable alternative (Fig. 2). The fluorescence emission spectrum of *P. inhibens* cultures showed an emission wavelength (λ_{em}) of 510 nm (excitation wavelength [λ_{ex}] of 430 nm) only in the presence of the inducer sinapic acid. This was not due to the yeast extract-tryptone-sea salt (YTSS) medium or sinapic acid and correlated with roseobacticide production (Fig. 2). Thus, fluorescence emission coupled with the EZ-Tn5 transposome system offered an efficient mutagenesis and assay system. We optimized a workflow in which a library of Tn5 mutants was generated on agar and subsequently arrayed into 96-well plates. Assay plates containing the inducer sinapic acid were then inoculated with the arrayed library plate, grown for 3 days, and then analyzed by fluorescence spectroscopy. The use of black-walled, clear-bottom 96-well plates allowed us to determine the optical density at 600 nm (OD_{600}) and fluorescence emission spectra for each mutant, thus removing from our analysis mutants that failed to grow while also enabling normalization for cell density. Mutants were defined as those that displayed <5% of the λ_{em} intensity of wild-type (wt) cells with sinapic acid or yielded entirely different emission spectra.

**Transposon screen and validation.** *P. inhibens* contains 3,960 open reading frames. We generated an array library of ~8,000
the cluster (involved in aerobic degradation of phenylacetic acid), and DNA repair, substrate/product transport, phage-related genes, several physiological pathways, including primary metabolism, resulted in 48 unique genes, whose disruption appeared to abolish transposon insertion was determined using arbitrary PCR and (~1%) exhibited a roseobacticide-deficient phenotype. The site of then screened as described above. From our library, 85 mutants, giving a ~2-fold genome coverage. These mutants were

mutants, giving a ~2-fold genome coverage. These mutants were then screened as described above. From our library, 85 mutants (~1%) exhibited a roseobacticide-deficient phenotype. The site of transposon insertion was determined using arbitrary PCR and resulted in 48 unique genes, whose disruption appeared to abolish roseobacticide production (Fig. 3A). These 48 genes grouped into several physiological pathways, including primary metabolism, DNA repair, substrate/product transport, phage-related genes, transcriptional regulators, sulfur insertion genes, the paa gene cluster (involved in aerobic degradation of phenylacetic acid), and the tda gene cluster (responsible for TDA biosynthesis). The possible biosynthetic genes are shown in Table 1, and all other genes are summarized in Table S1 in the supplemental material. To corroborate these results, a high-performance liquid chromatography-mass spectrometry (HPLC-MS) assay was conducted for each of the 85 Tn mutants, in which the presence of roseobacticide, if any, was quantitated. The results showed that 28 out of the 48 mutants were completely deficient in roseobacticide synthesis, while 18 mutants generated less than 5% of the roseobacticide generated by wt cultures. The remaining 2 mutants exhibited more than 5% roseobacticide compared with wt P. inhibens (Table 1; see also Table S1).

Of the category of genes detected, the tda, paa, and sulfur insertion genes represented the only potential biosynthetic genes, along with a number of genes of unknown function (Fig. 3A; Table 1) (28–32). The involvement of the paa cluster may have been surprising. The screen revealed that Tn insertion into numerous tda genes results in roseobacticide-deficient mutants, indicating that roseobacticide and TDA production share a number of biosynthetic enzymes. A number of genes of unknown function (Fig. 3A), notably a cluster of three such genes adjacent to the tda cluster, were uncovered in our screen as well. These did not display sequence similarities to any characterized genes in the known database and may be involved in the final stages of roseobacticide biosynthesis. The results above also indicate that enzymes encoded from at least three different genetic loci are necessary for roseobacticide production. As with TDA, a distinct coregulated biosynthetic gene cluster bearing all the necessary biosynthesis, transport, and resistance genes does not exist (28). Instead, the roseobacticide genes appear to be spread in several genetic loci.

An efficient method for creating targeted knockout mutants in P. inhibens. To further corroborate the transposon screening results described above and to test the idea that roseobacticide production requires biosynthetic genes from a number of genetic loci, we sought to generate site-directed gene inactivation mutants. Current strategies for mutagenizing Roseobacter utilize insertion of an antibiotic selection marker and growth on the requisite antibiotic (33). In our hands, this method frequently resulted in formation of merodiploid mutants. We addressed this shortcoming by creating a reliable mutagenesis procedure involving antibiotic selection followed by counterselection using the pheS toxic gene, which has previously been used for genetic manipulation of Burkholderia strains (34, 35). The product of pheS, an engineered tRNA synthetase, selectively incorporates p-chlorophenylalanine (p-Cl-Phe) into proteins. Cells that harbor this gene and are cultured in the presence of p-Cl-Phe incorporate the unnatural amino acid into the proteome, resulting in cell death.
TABLE 1 Putative biosynthetic genes required for roseobacticide production by *P. inhibens*.

| Metabolic pathway and locus tag | Gene | % relative production | Predicted function |
|--------------------------------|------|-----------------------|-------------------|
| Phenylacetae catabolism        |      |                       |                   |
| PGA1_c04090                    | paaA | 0                     | LysP homolog      |
| PGA1_c04080                    | paaB | 0                     | LysP homolog      |
| PGA1_c04060                    | paaC | 0                     | LysP homolog      |
| PGA1_c04040                    | paaE | 0                     | LysP homolog      |
| Sulfur metabolism              |      |                       |                   |
| PGA1_c0760                     | cysA | 4                     | Sulfite reductase  |
| PGA1_c00860                    | patB | 0                     | Cystathionine β-lyase |
| TDA biosynthesis               |      |                       |                   |
| PGA1_262p00980                 | tdaA | 0                     | LysR transcriptional regulator |
| PGA1_262p0970                  | tdaB | 0                     | β-Etherase        |
| PGA1_262p0960                  | tdaC | 0                     | Prephenate dehydratase |
| PGA1_262p0950                  | tdaD | 0                     | Thioesterase      |
| PGA1_262p0940                  | tdaE | 0                     | Acyl-CoA dehydrogenase |
| PGA1_262p0800                  | paaZ2| 0                     | Enoyl-CoA hydratase |
| PGA1_262p0840                  | PUF | 0                     |                   |

*See Table S1 in the supplemental material for a list of all other genes identified using the Tn mutagenesis screen.*

*Production with each Tn mutant, relative to that of wt, determined using an HPLC-MS assay for direct detection of roseobacticide B.*

*Predicted functions are based on protein homology to proteins in the IMG database.*

*See reference 31.*

*See reference 28.*

*PUF, protein of unknown function.

The successful application of *pheS* as well as combination of an efficient selection and counterselection scheme enabled rapid and reliable production of marked and unmarked site-directed mutants in *P. inhibens*.

**Implications for roseobacticide biosynthesis: the sulfur source.** With a robust mutagenesis method in hand, we began to test the pathway for roseobacticide production. The genes implicated in sulfur insertion from our transposon data are PGA1_c20760, a putative sulfite reductase, and PGA1_c00860, a putative cysI (compound 3). The experimental details of this model need to be investigated, it does provide a direct pathway for incorporation of the DMSP-sulfur into roseobacticides via PatB. There are only a few biosynthetic pathways for insertion of thiols into small molecules (39). The experimental evidence thus far is consistent with a new biosynthetic mechanism for thiol group insertion in the production of roseobacticides. A similar conclusion was recently reached by Brock et al. for the biosynthesis of TDA (29).

**Origins of the tropone.** In *P. inhibens*, two pathways have been proposed for production of tropone: one of these involves the ring-expanding PaaN enzyme, while the second implicates the *paa* catabolon (Fig. 5A, paaN pathway versus *paa* catabolon pathway) (15, 30–32, 40). Our mutagenesis data implicated the *paa* catabolon in the production of roseobacticides (Table 1). These results were verified with a targeted *paaE* knockout mutant, which gave a roseobacticide-deficient phenotype (Fig. 3B). We propose that the *paa* catabolon provides the tropone precursor for roseobacticide synthesis.

Surprisingly, the *tda* locus was also implicated in our screen (Table 1). To verify this finding, we generated targeted knockout mutants for *tdaA*, a LysR-type transcriptional regulator that positively regulates TDA production in response to 3-OH-decanoyl-homoserine lactone (3-OH–C10-HSL) (41, 42), *tdaB*, a putative acyl-CoA dehydratase, *tdaC*, a putative acyl-CoA thioesterase, and *tdaD*, a putative acyl-CoA dehydrogenase. With the exception of *tdaC*, we found all mutants to be deficient in roseobacticide biosynthesis, confirming the importance of these genes (Fig. 3B). The involvement of multiple *tda* genes in roseobacticide production suggests that the TDA and roseobacticide biosynthetic pathways share an intermediate, from which each pathway could diverge. Thus, elucidation of one pathway will necessitate understanding of the other.

The involvement of TdA, TdB, TdC, and TdE provides clues regarding roseobacticide synthesis. Brock et al. have proposed the most comprehensive model for TDA production (29). These enzymes are common to both pathways, whereas TdC is
not required for either TDA or roseobacticide synthesis (28). In our current working model (Fig. 5A), which is based on the proposed pathway by Brock et al. (29), PaaABCDE generate epoxide 8 from phenylacetyl-CoA (compound 7), which is synthesized from phenylacetic acid by PaaK (30–32). Epoxide 8 is converted to oxepin-CoA (compound 9) by PaaG, which undergoes ring opening by PaaZ to give a linear product (compound 10). Two gene copies of a PaaZ derivative, which encode PaaZ1 and PaaZ2 with important E256Q and C295R mutations relative to PaaZ, then may catalyze formation of compound 11 (29). This compound may undergo dehydration, possibly spontaneously, to give intermediate compound 12, which could serve as a substrate for TdaB and TdaF. These enzymes could insert a thiol group by using S-thio-Cys, as previously proposed (29). The product of this reaction, compound 13, may be the last common intermediate in the production of TDA and roseobacticides. Insertion of a second thiol, followed by oxidation and CoA-thioester hydrolysis would furnish TDA, while reaction with the glyoxylyl-CoA (compound 14), as previously proposed (23), would lead to roseobacticides.

Consistent with the idea that the tda operon can synthesize two distinct compounds is the common substructure in TDA and roseobacticides involving the 1H9252-thiotropone moiety (Fig. 5B). That TDA and roseobacticide production share common intermediates and biosynthetic enzymes is an unexpected finding, one that has implications for the algal-bacterial symbiosis (see below).

Source of the aromatic side chain. Collectively, the data above provide insights into thiol insertion and the origin of the seven-membered ring in roseobacticides. A cluster of three hypothetical genes adjacent to the tda cluster were also implicated by our mutagenesis data (Fig. 3A; Table 1). HPLC-MS assays showed that Tn insertion into one of these genes abolished roseobacticide synthesis. Biochemical studies are necessary to investigate their possible role in incorporation of the amino acid portion of roseobacticides, as indicated by isotope feeding studies (23). Alternatively, it is
possible that our screen did not capture the genes responsible for this aspect of roseobacticide production and additional studies may be necessary to uncover all the genes required for roseobacticide synthesis.

**Regulation of roseobacticide biosynthesis.** The transposon screen provided two clues regarding the regulation of roseobacticide synthesis. First, the involvement of the *tda* cluster, which is regulated by the autoinducer 3-OH-C_{10}-HSL (42), suggests that roseobacticide biosynthesis may also be QS regulated. Second, two transcriptional regulators were found in the transposon screen that resulted in a roseobacticide-deficient phenotype. We first sought to corroborate these results and eliminate the possibility of polar effects resulting from transposon insertion. Both transcriptional regulator mutants retained the ability to generate roseobacticides, inconsistent with a role in its synthesis. To examine possible QS regulation, we generated a homoserine lactone synthase mutant strain, strain ΔpgaI, which lacks the ability to produce 3-OH-C_{10}-HSL, the signal that induces the *ida* cluster (42). HPLC-MS assays showed that this mutant also lost the ability to synthesize roseobacticides (Fig. 6). However, when purified 3-OH-C_{10}-HSL was added to the ΔpgaI strain cultures, or when the mutant was cocultured with a ΔtdaA strain, roseobacticide synthesis was restored (Fig. 6). Accordingly, one strain, the ΔtdaA strain, secretes the 3-OH-C_{10}-HSL signal, while the ΔpgaI strain can generate roseobacticides. Further, examination of the time course of roseobacticide production showed that it is not produced at low cell densities, but rather in late stationary phase. Addition of the QS signal 3-OH-C_{10}-HSL led to an earlier induction of roseobacticide biosynthesis (see Fig. S4 in the supplemental material). Col-

**FIG 6** Roseobacticide biosynthesis is regulated by quorum sensing via 3-OH-C_{10}-HSL. HPLC-MS profiles are shown for extracts of wt *P. inhibens* and selected mutant strains in the presence of sinapic acid (SA). The peak in the wt trace corresponds to roseobacticide B. The ΔtdaA and ΔpgaI strains individually failed to produce roseobacticides, while a coculture of these mutant strains, or a ΔpgaI strain culture supplemented with 3-OH-C_{10}-HSL, generated roseobacticides. The traces have been vertically offset for clarity.
for some roseobacticides, such as roseobacticide A (see Fig. 1) (23), while for other analogs, such as roseobacticide B or C, it acts only as an inducer.

A preliminary regulatory framework for roseobacticide biosynthesis may be conceived. Under normal, mutualistic conditions, production and detection of 3-OH-C_{10}-HSL at high bacterial cell densities leads to synthesis of TDA, which has been proposed to protect the algal host from pathogenic bacteria (Fig. 7). In this mode of interaction, roseobacticide synthesis is turned off. When the host senescences, it releases pCA, which is presumably detected at the cell surface by \textit{P. inhibens}. The combination of pCA and 3-OH-C_{10}-HSL then leads to production of TDA and roseobacticides (Fig. 7). To ensure tight regulation of algaecide production and firm control over a proposed lifestyle switch from mutualism to parasitism, roseobacticide biosynthesis necessitates the presence of two signals, algal pCA and bacterial 3-OH-C_{10}-HSL.

**Conclusions.** In response to algal phenylpropanoids, \textit{P. inhibens} synthesizes roseobacticides, which has been proposed to initiate a mutualist-to-parasite switch in its symbiosis with an algal host. Much like other critical cellular processes, such as sporulation or virulence factor production, this lifestyle switch is tightly regulated (43–47). Herein, we begin to delineate the components of this regulatory process. Not only is the algal metabolite pCA or a similar inducer, such as sinapic acid, necessary for roseobacticide production, but this process is also under QS control and requires the autoinducer 3-OH-C_{10}-HSL. Roseobacticides join a long list of other toxins that are activated by QS, but in this case, QS is necessary but not sufficient. While our findings have identified a key feature in the regulatory cascade of roseobacticide synthesis, this picture is far from complete and will receive further attention in the future.

We further defined the molecular underpinnings of the rapid switch-like behavior from mutualism to parasitism by \textit{P. inhibens}. TDA and roseobacticides are largely generated by the same biosynthetic enzymes. This enables \textit{P. inhibens} to merely divert an already active biosynthetic pathway toward the synthesis of roseobacticides, rather than inducing a new pathway. By intertwining roseobacticide and TDA biosyntheses, the bacterium preserves precious nutrients and precursors, allowing it a rapid lifestyle switch in response to algal senescence. In addition, the production of two distinct metabolites from largely one set of genes amplifies the secondary metabolic potential of \textit{P. inhibens}.

Our studies set the stage for the characterization of a number of enzymes involved in production of both rare troponoid compounds. Initial forays in this regard have begun to delineate a pathway for sulfur and phenylacetic acid insertion into the roseobacticides. While the biochemical steps still need to be elucidated, the evidence thus far suggests that new enzymatic transformations remain to be discovered in the production of these troponoids, especially with respect to tropolactone formation and the insertion of thiol groups.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** \textit{P. inhibens} DSM 17395 was used throughout this study (48). It was routinely cultured in marine broth (MB; Difco) or in half-strength YTSS medium (referred to as \(1/2\)YTSS, containing, per liter, 20 g Sigma sea salt, 2 g yeast extract, 1.25 g tryptone) (2).

**DNA manipulations.** Genomic DNA was isolated using the Wizard gDNA isolation kit (Promega). Transposon mutagenesis was carried out with the EZ-Tn5 transposome kit (Epigenetec) (49). PCRs were performed with \textit{Taq} DNA polymerase (NEB) for arbitrary PCR or with QS DNA polymerase (NEB) for cloning and genetic manipulations. T4 DNA ligase (NEB) was used for ligation reactions. \textit{E. coli} DH5α cells (NEB) and
BL21(DE3) cells (NEB) were used for cloning and expression, respectively.

Transposon mutagenesis. Competent P. inhibens cells were created via the procedure previously described, with minor modifications (42). Briefly, a 5-ml overnight culture of P. inhibens in marine broth was grown in a 14-ml sterile culture tube at 30°C and 250 rpm. It was then diluted 50-fold into 175 ml of marine broth in a 1-liter Erlenmeyer flask. After 7 to 8 h, an OD$_{600}$ of ~0.5 was reached, at which point the flask was incubated in ice-water for 15 min and gently swirled several times during that period. Cells were then pelleted by centrifugation (4°C, 2,500 × g, 8 min) and resuspended in 120 ml of ice-cold sterile 10% (vol/vol) glycerol. This process of centrifugation and resuspension in 10% glycerol was repeated four more times. After the last spin cycle, the cells were resuspended in 0.7 ml of 10% glycerol, distributed in 40-μl aliquots, and flash-frozen in liquid N$_2$. To create P. inhibens mutants strains, an aliquot was thawed on ice, 1 μl of the EZ-Tn5 transposome mix was added, and the mixture was transferred to a 1-mm electroporation cuvette. The mixture was electro-porated at 1,375 V for 5 ms, then immediately returned to ice, supplemented at 1,375 V for 5 ms, then immediately returned to ice, supplemented with 1 mM ATP, 0.1 mM ATP, 5 mM MgCl$_2$, and 10 μM NusA-PatB. The mixture was then incubated at 37°C for 30 min, and 1 μl of the library plate using a CyBi-well automated liquid transfer system (CyBio). The plates were sealed with a membrane and grown at 37°C for 3 days. After 3 days, expression spectra were recorded with a H1MF plate reader (BioTek) by excitation at 430 nm and emission between 450 and 700 nm.

Creating targeted knockout mutants in P. inhibens. Gene deletion mutants were created with a new procedure using gentamicin positive selection and a pheS counterselection cycle (see Table S2 in the supplemental material). The pheS gene was cloned from vector pEX18Km-phaS (35) using the primers 5'-AGCAATTTCAGCTCCCAGCGTAAGCGGA-3' and 5'-AGCAATTCAGCACGGCCTAACATGCTACTGAGGCC-3'. The reaction mixture contained, in a final volume of 100 μl of 10 mM Tris-HCl (pH 8.0), 1 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM NADH, and 10 μM purified NusA-PatB. The increase in absorbance at 412 nm was measured at an interval of 0.02 min for 5 min at room temperature. A molar extinction coefficient of 13,400 M$^\text{−1}$ cm$^\text{−1}$ was used to determine NusA-PatB concentrations.

Cloning, expression, and purification of PatB. patB was amplified from P. inhibens gDNA by using primers 5'-GGCGCCGAATTCCTTGGAGGATTG822 and 5'-CGCTCGGAGATCC-3' (Table S1, DOCX file, 0.02 MB). The amplified fragment was cloned into pBluescript KS(−) and transformed to E. coli BL21(DE3) cells in LB. Large-scale cultures (four 1-liter LB cultures in 4-liter Erlenmeyer flasks) were grown at 37°C to an OD$_{600}$ of ~0.6 and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside. The temperature was lowered to 18°C, and expression was carried out for 18 h. At that point, the cells were pelleted by centrifugation, flash-frozen in liquid N$_2$, and stored at −80°C. Protein purification was carried out at 4°C. Each gram of cell pellet was resuspended in 5 ml of buffer A (20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM imidazole, 5% glycerol, and 0.1% Triton) and supplied with a protease inhibitor cocktail (Sigma). The homogenized cells were lysed by sonication using a Branson ultrasonic cell disrupter operating at 65% power with 15 iterations of 10-s on/20-s off cycles. Cell debris was removed by centrifugation, and the crude extract was passed over an Ni$^2$+-nitriotriacetic acid column (5 ml, 4-cm length, 1.25-cm diameter; Clontech). NusA-PatB was eluted with a step gradient of buffer A containing 50 mM, 100 mM, 200 mM, and 500 mM imidazole. NusA-PatB was detected in the last two fractions by SDS-PAGE. The protein was exchanged into storage buffer (50 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.2 mM EDTA, and 5% glycerol) on a Sephadex G-25 column (30 ml, 25-cm length, 1.25-cm diameter) and stored at a concentration of 36.3 mg/ml at −80°C. An extinction coefficient of 98,945 M$^\text{−1}$ cm$^\text{−1}$ was used to determine NusA-PatB concentrations.

Characterization of PatB. PatB activity was assayed in a continuous spectrophotometric assay using the thiol-reactive agent DTNB. The reaction mixture contained (in a final volume of 300 μl) 100 mM Tris-HCl (pH 9.0), 0.2 mM DTNB, 0.1 mM PLP, 2 mM substrate (L-threonine, L-cystathionine, or L-cystine), and 10 μM purified NusA-PatB. The increase in absorbance at 412 nm was measured at an interval of 0.02 min for 5 min at room temperature. A molar extinction coefficient of 13,400 M$^\text{−1}$ cm$^\text{−1}$ for the aryl mercaptide was used to calculate the amount of product formed. To verify formation of pyruvate as a product in the NusA-PatB reaction, a coupled lactate dehydrogenase (LDH) assay was carried out. The reaction mixture contained, in a final volume of 300 μl, 100 mM Tris-HCl (pH 9.0), 0.1 mM PLP, 2 mM L-cystine, 0.2 mM NADH, 10 μM purified NusA-PatB, and 6 U of LDH. The consumption of NADH was measured at 340 nm at an interval of 0.02 min for 5 min at room temperature.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02118-15/-/DCSupplemental.

Figure S1, EPS file, 1.5 MB.
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