RedH and PigC Catalyze the Biosynthesis of Hybrubins via Phosphorylation of 4'-Methoxy-2,2'-Bipyrrole-5'-Carbaldehyde

Qingshan Long,^a Daniel E. Jeffries,^b Shuangjun Lin,^a Xuefei Chen,^a Weijun He,^a Yemin Wang,^a Zixin Deng,^a Craig W. Lindsley,^{b,c} Meifeng Tao^a

^aState Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

^bDepartment of Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, Tennessee, USA

^cDepartment of Pharmacology, Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, Tennessee, USA

ABSTRACT Hybrubins are “unnatural” alkaloids with the same 4'-methoxy-2,2'-bipyrrole-5'-methine moiety found in prodiginines and a different ring derived from tetramic acids. Here, we demonstrated that RedH, a homologue of prodigiosin synthetase PigC, was responsible for the biosynthesis of hybrubins A and B in *Streptomyces lividans*. *In vitro* reactions indicated that RedH and PigC catalyzed the intermolecular condensation between 4'-methoxy-2,2'-bipyrrole-5'-carbaldehyde (MBC) and (Z)-5-ethylidenetetramic acid (ETA) to produce hybrubin B. Moreover, we demonstrated that RedH and PigC activated MBC via phosphorylation of the aldehyde group to form an intermediate P_i-MBC and that the subsequent condensation between P_i-MBC and (Z)-5-ethylidenetetramic acid occurs in a nonenzymatic way.

IMPORTANCE Hybrubins are an emerging class of prodiginines possessing a new C ring derived from 5'-substituted tetramic acids and the methylene bridge connecting the C ring at a different position. We have supposed that condensation between 4'-methoxy-2,2'-bipyrrole-5'-carbaldehyde (MBC) and 5-ethylidenetetramic acid (ETA) yields the hybrid natural products hybrubins, which was proposed to be catalyzed by the undecylprodigiosin synthetase RedH. However, it is doubted whether RedH is able to catalyze another type of condensation between MBC and tetramic acids. In this study, we have demonstrated that the MBC-ETA condensation proceeds through RedH/PigC-catalyzed enzymatic activation of MBC via phosphorylation and a nonenzymatic condensation of P_i-MBC with ETA. Since MBC analogues have been shown to be accepted by PigC, more hybrubin analogues might be produced by using combinations of MBC analogues and other tetramic acids in future studies.

KEYWORDS MBC-ETA condensation, phosphorylated intermediate, hybrubins, RedH, PigC

Prodigiosin (compound 1) and prodigiosin-like compounds (prodiginines) are red-pigmented tripyrrolic alkaloids isolated from *Serratia marcescens* (prodigiosin), *Streptomyces* spp. (undecylprodigiosin [compound 2] and streptorubin [compound 3]), and many other bacteria (1). Prodiginines exhibit a wide range of bioactivities, including antimicrobial (2), antimalarial (3–5), immunosuppressive (6–8), and anticancer (9–14) properties. One synthetic prodigiosin analogue, obatoclax (GX15-070), was given orphan drug status by the U.S. Food and Drug Administration for treating chronic lymphocytic leukemia and was shown to be a pan-Bcl-2 inhibitor (15). Prodiginines possess three pyrrolic rings, of which the A and B rings are derived from 4'-methoxy-2,2'-bipyrrole-5'-carbaldehyde (MBC) and the C ring from monopyrroles, with a C-5'-carbaldehyde-derived methine bridge connecting the C ring at the C-5'' position (1) (Fig. 1).

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Address correspondence to Meifeng Tao, tao_meifeng@sjtu.edu.cn.

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FIG 1 (Undecyl)prodigiosin, streptorubin, and hyrubins. The A, B, and C rings are labeled. Note the difference in the C rings.

Hyrubins constitute an emerging class of prodigiosin analogues with the same 4'-methoxy-2,2'-bipyrrole-5'-methine moiety (A and B rings) of prodigiosin and a new C ring derived from 5-substituted 2,4-pyrrolidinone (also named 5-substituted tetramic acid). Moreover, in hyrubins, the MBC-derived methine bridge connects the C ring at the C-3'' position (16) (Fig. 1). Hyrubins A, B, and C are mixtures of two *cis-trans* isomers (16). The structure of hyrubin A₁ has been further confirmed by chemical synthesis (17). Although both of the pharmacophores prodiginine and tetramic acid are known to display significant cytotoxicity (18, 19), hyrubin A was noncytotoxic to tested cell lines (16). Additionally, Jeffries and Lindsley found that hyrubin A₁ selectively binds adenosine receptors and FMS-like tyrosine kinase 3 (FLT3), which is a target in the treatment of acute myeloid leukemia (AML) (17).

The distinguishing structure and properties of hyrubins motivated us to study their biosynthetic mechanisms. To produce hyrubins, the bacterial artificial chromosome clone pHZZL3, which bears a 13-kb genomic DNA fragment containing tetramic acid biosynthetic genes from *Streptomyces variabilis* Snt24, was heterologously expressed in *Streptomyces lividans* Δ redKL mutant strains such as SBT5 and GX28 (16, 20). Wild-type strains of *Streptomyces coelicolor* and *S. lividans* carry a *red* gene cluster for the biosynthesis of undecylprodigiosin and streptorubin, which proceeds via a convergent pathway dependent upon the two key intermediates MBC and 2-undecylpyrrole (2-UP) (21–23) (Fig. 1). Two sets of genes, i.e., *redMOWXNVIF* and *redQPRJLK*, are involved in the biosynthesis of MBC and 2-UP, respectively. RedH is responsible for the condensation of MBC and 2-UP to produce undecylprodigiosin (24). The assembly of prodigiosin in *S. marcescens* proceeds through an analogous bifurcated biosynthetic pathway, in which the same bipyrrolic intermediate MBC and another monopyrrole intermediate, i.e., 2-methyl-3-aminopyrrole (MAP), are synthesized and joined to generate the final product. The prodigiosin synthetase PigC, which is a homologue of RedH, catalyzes the MBC-MAP condensation in *S. marcescens* (25) (Fig. 1). In the hyrubin-producing strains *S. lividans* GX28/pHZZL3 and SBT5/pHZZL3, MBC may accumulate by the hosts since the biosynthesis of 2-UP is blocked due to deletion of the type I polyketide synthase gene *redL* and the oxidoreductase gene *redK* (16), both of which are essential for the biosynthesis of 2-UP (21); as pHZZL3 carries an *hbn* gene cluster for the biosynthesis of 5-substituted tetramic acids, both of the necessary compounds, i.e., MBC and 5-substituted tetramic acid, would be available for hyrubin synthesis in these strains (16). Therefore, we hypothesized that, in *Streptomyces*, condensation between MBC and 5-substituted tetramic acid yields the hybrid natural product hyrubin, with the condensation reaction proposed to be catalyzed by RedH (16).

However, it is questionable whether the undecylprodigiosin synthetase RedH is able to catalyze another type of condensation between MBC and (Z)-5-ethylidenepyrrolidine-2,4-dione (ETA). Moreover, it has been shown that tetramic acids reacted spontaneously with electrophiles such as aldehydes (26). Herein, we describe *in vitro* and *in vivo* functional analyses of RedH in the biosynthesis of hyrubins.

FIG 2 Verifying the function of *redH* in the biosynthesis of hybrubins via gene deletion and genetic complementation. (A) Construction of the *redH* gene deletion mutant LQS18 via homologous recombination. Two 5-kb chromosomal DNA segments next to both ends of the open reading frame (ORF) of *redH* were amplified to construct the gene deletion plasmid pJLQ10107. Homologous recombination between GX28 chromosome and pJLQ10107 gives rise to LQS18, i.e., the $\Delta redH$ mutant. ΔKL is the fused truncated nonfunctional ORF generated by previous *redK* and *redL* gene deletions (16). Double-headed arrows indicate the PCR-amplified products of the *redH* loci using the primer pair $\Delta redH$ -ve-F/ $\Delta redH$ -ve-R and genomic DNA of GX28 or LQS18 as the template. (B) Verification of the gene deletion by PCR amplification of the *redH* loci. GX28, *S. lividans* parental strain; SC, single-crossover strain carrying both wild-type and deleted loci; LQS18, four mutants carrying only the deleted locus. M1 and M2, molecular weight markers. (C) Reduction in pigment production with deletion of *redH*. GX28/pHZL3, parental strain; LQS18/pHZL3, mutant lacking *redH*. (D) Analysis of the production of hybrubins by the parental strain GX28/pHZL3, the $\Delta redH$ mutant (LQS18/pHZL3), and the gene complementation strain (LQS18/pHZL3+*redH*) by HPLC (at 500 nm). Vector, the empty vector control pJTU1278. Hybrubin A (compound 4), hybrubin B (compound 5), hybrubin C (compound 6): standard compounds purified from a culture of GX28/pHZL3.

RESULTS

Determination the role of *redH* in the hybrubin biosynthesis in *S. lividans*. We deleted *redH* from *S. lividans* GX28 by homologous recombination between the chromosome and the gene replacement construct to yield *S. lividans* LQS18 (Fig. 2; see also Fig. S1 in the supplemental material). When grown on R3 agar medium, the red pigment decreased sharply in *S. lividans* LQS18/pHZL3 compared with that in *S. lividans* GX28/pHZL3 (Fig. 2C). High-performance liquid chromatography (HPLC) analysis of the ethyl acetate extract of the LQS18/pHZL3 culture revealed that production of hybrubin A and B was abolished (Fig. 2D). However, the production of hybrubin C was not abolished in the $\Delta redH$ mutant but rather was increased to around 2-fold of the level in the parental culture. This result suggested that the production of hybrubins A and B, but not of hybrubin C, required the *redH* gene. To rule out the potential polar effect and confirm that the loss of hybrubins A and B was caused by the *redH* gene deletion, we cloned and introduced the wild-type *redH* gene into *S. lividans* LQS18/pHZL3 using the high-copy vector pJTU1278 under the *ermE** promoter (27). Fermentation and subsequent metabolite analysis of the complementation strain demonstrated the restoration of hybrubin production, validating the indispensability of RedH activity for the biosynthesis of hybrubins A and B (Fig. 2D).

Characterization of the RedH-catalyzed MBC-ETA condensation *in vitro*. We purified the soluble recombinant RedH protein (see Fig. S2) and ran reactions with chemically synthesized MBC (compound 7) and (Z)-5-ethylidenepyrrolidine-2,4-dione (ETA; compound 8) as the substrates, under reaction conditions adapted from Chawrai et al. (28). A significant quantity of hybrubin B was detected in the reaction mixture. In contrast, only trace amounts of hybrubin A and B were observed in the absence of

FIG 3 Condensation between MBC (compound 7) and ETA (compound 8) catalyzed by RedH or PigC. (A) HPLC analysis (at 500 nm) of the reaction mixtures. Each reaction mixture contained 10 mM MgCl_2 , 100 μM EDTA, 25 mM NH_4Cl , 1.25 mM ATP-Na_2 , 100 μM MBC, 100 μM ETA, 10% DMSO, and 10 μM enzyme in 100 mM HEPES (pH 7.0), and each reaction was conducted at 30°C for 24 h. (B) Enzymatic kinetic analysis of the reaction. Each of the ten data points in the initial velocity/MBC concentration curve was collected from three parallel replicates.

RedH or ATP (Fig. 3A). The hybrubin B standard is a mixture of two isomers according to previous nuclear magnetic resonance (NMR) data, one of which adopting an (*E*)-configuration at the C-5'' to C-6'' double bond of the C ring and supported by nuclear Overhauser effect spectroscopy (NOESY) data has been designated hybrubin B₁ (16). The product of the RedH-catalyzed MBC-ETA condensation, retaining a (*Z*)-configuration at the C-5'' to C-6'' double bond from the (*Z*)-form substrate and being eluted at the same retention time as the hybrubin B standard in HPLC analysis, should be the other isomer of hybrubin B and was therefore designated hybrubin B₂ (compound 5, structure shown in Fig. 3B). We determined kinetic parameters of the RedH-catalyzed MBC-ETA condensation: $K_m = 48.55 \pm 4.25$ (μM), $k_{\text{cat}} = 0.13 \pm 0.01$ (min^{-1}), $k_{\text{cat}}/K_m = 2.68$ ($\text{mM}^{-1} \text{min}^{-1}$) (Fig. 3B). We also expressed and purified soluble recombinant PigC protein, derived from *S. marcescens* SM02 (33), and repeated the reactions using this protein instead of RedH. The accumulation of hybrubin B was also observed in the reaction mixture (Fig. 3A). These results suggested that both RedH and PigC can catalyze the ATP-dependent MBC-ETA condensation to yield hybrubin B *in vitro*.

Identification of key amino acids involved in MBC-ETA condensation. Both RedH and PigC contain three protein domains: the N-terminal ATP-binding domain and the C-terminal phosphoryl-transfer domain, which are homologous to the corresponding domains of phosphoenolpyruvate synthase (PEPS) and pyruvate phosphate dikinase (PPDK) (1, 21), and the central domain, which shows no homology with any protein in the databases and has been presumed to bind substrates (22). The key conserved residues in the ATP binding domain and in the phosphoryl-transfer domain had been predicted based on the crystal structure of PPDK (PDB code 1JDE) (29) and were shown to play pivotal roles in the enzymatic activity of the prodigiosin synthetases PigC and HapC (28). Sequence alignment of the *Streptomyces* RedH proteins with PEPS, PPDK, and prodigiosin synthetases (PigC and HapC) identified the conserved residues Glu-307 and Arg-321 in the ATP-binding domain of RedH and His-869 in the phosphoryl-transfer domain (see Fig. S3). We mutated these residues to alanine to give the RedH variants E307A, R321A, and H869A, respectively. *In vitro* biochemical reactions using these RedH variants showed that the E307A and H869A mutants completely lost enzymatic activity in the MBC-ETA condensation, whereas the activity of the R321A mutant sharply decreased to approximately 10% of that of the wild-type protein (Fig. 4). These results suggested that both the ATP-binding and phosphoryl-transferring functions of the

FIG 4 HPLC analysis of the reaction products of MBC-ETA condensation catalyzed by RedH proteins with mutations in conserved amino acids. RedH was mutated by site-directed mutagenesis. HPLC was monitored at 500 nm.

enzyme are essential for the MBC-ETA condensation, implying that the RedH-catalyzed MBC-ETA condensation proceeds through a putative phosphorylated MBC intermediate, analogous to the previously proposed mechanism for prodigiosin biosynthesis (28).

Characterization of the phosphorylated MBC intermediate. To detect the putative phosphate intermediate and verify our hypothesis regarding the condensation pathway, we separated the MBC-ETA condensation into two half-reactions. In the first half-reaction, the first substrate MBC and the cosubstrate ATP were incubated with either RedH or PigC. The reaction mixture was boiled and extracted with methanol before liquid chromatography-mass spectrometry (LC-MS) analysis. The MS analysis displayed a definite peak for the molecular ion of phosphorylated MBC (P_i -MBC) (Fig. 5A and B), demonstrating the presence of the predicted phosphorylated intermediate in the RedH- and PigC-catalyzed reactions. The tandem mass spectrometry (MS/MS) analysis of P_i -MBC displayed 6 major collision fragments (Fig. 5C) whose m/z values are consistent with the calculated values (Fig. 5D). These results also suggested that P_i -MBC is fairly stable during the treatment.

Next, for the second half-reaction, the protein-free extract of the incubated mixture of the first half-reaction was dried, dissolved in buffer, and supplemented with ETA (compound 8). Surprisingly, after incubation, hybrubin B was detected in the reaction mixture by HPLC analysis; moreover, the yield of hybrubin B from the step-by-step reactions was comparable with the yield from the one-pot reactions (Fig. 5E). These results suggested that the RedH- or PigC-catalyzed condensation between MBC and ETA actually proceeds in a two-step fashion: first, the enzyme catalyzes the formation of a P_i -MBC intermediate, and then P_i -MBC condenses with ETA spontaneously to give the final product hybrubin B.

Determination of the effect of RedH on the second half-reaction. RedH was added to the reaction mixture and the production was monitored within 12 h (see Fig. S4). Both reaction groups with and without RedH showed the same rate of hybrubin production in the second half-reaction, suggesting that RedH does not promote the condensation reaction between P_i -MBC and ETA.

DISCUSSION

Chawrai et al. had proposed that natural MBC-MAP condensation proceeds through a phosphorylated intermediate, and they demonstrated that the ATP-binding domain and

FIG 5 Analysis of the intermediate and step-by-step reactions of RedH/PigC-catalyzed MBC-ETA condensation. (A) Extract ion chromatography (EIC) analysis of the reaction substrate (MBC) and intermediate in negative-ion mode. MBC was incubated with ATP and RedH or PigC for 24 h, and then the mixture was analyzed by high-resolution mass spectrometry to observe the intermediate. (B) The observed molecular anion and adduct ion of the intermediate P_i-MBC. (C) Tandem mass spectrometry of P_i-MBC. (D) Schematic elucidation of the MS/MS fragments. (E) HPLC analysis of the step-by-step reaction of RedH- or PigC-catalyzed MBC-ETA condensation. [Enzyme] = 10 μ M. Step 1, MBC was incubated with enzyme and ATP for 24 h; step 2, enzyme was removed thoroughly by boiling and extraction; then, compound 8 was added to the protein-free extract and incubated for 12 h. One-pot reaction mixtures were incubated for 24 h. HPLC was monitored at 500 nm.

the phosphoryl-transfer domain of PigC/HapC are responsible for activating MBC (28). Here, we determined the structure of the key phosphorylated intermediate, P_i-MBC, providing direct evidence that RedH and PigC activate the aldehyde MBC by phosphorylation (Fig. 5). On the other hand, in their pioneering searches for new prodiginines, the Challis and Leeper groups obtained derivatives of prodiginosin and undecylprodiginosin, in which the native pyrrole A ring was replaced by phenyl, indolyl, furyl, and thienyl groups, by feeding chemically synthesized MBC analogues into MBC nonproducing mutants (24, 25). We believe that RedH or PigC also activated these MBC analogues bearing different A rings, plausibly via phosphorylation on the formaldehyde group as well.

The observed nonenzymatic condensation between P_i-MBC and ETA explains the substrate promiscuity of the one-pot MBC-ETA condensation. The nonenzymatic

FIG 6 Proposed mechanism for the biosynthesis of hybrubin A/B. RedH- or PigC-catalyzed MBC-ETA condensation generating hybrubin A/B (compound 4/5). MBC, 4'-methoxy-2,2'-bipyrrole-5'-carbaldehyde. P_i-MBC, phosphorylated MBC.

P_i-MBC-ETA condensation may happen via nucleophilic attack of P_i-MBC by the enol form of ETA, followed by elimination of the phosphate group and accompanied deprotonation to produce hybrubin products (Fig. 6). In other words, the observed substrate “promiscuity” of RedH and PigC toward ETA is mainly due to the nonenzymatic property of the last half of the MBC-ETA condensation. Since the $\Delta redH$ mutant still produced hybrubin C (Fig. 2), we propose a revised hybrubin C biosynthetic pathway as shown in Fig. S5 in the supplemental material. After HbnAB catalyzed the formation of ETA (16), an unknown enzyme(s) in the cell catalyzed the oxidation of ETA to give pyrrolidine-2,3,5-trione (compound 9). Subsequently, nonenzymatic MBC-compound 9 condensation yielded the final product hybrubin C.

Interestingly, the natural products prodigiosin and undecylprodigiosin have distinct alkyl chain lengths, and additionally, PigC has been shown to display remarkable substrate promiscuity for the monopyrrole species (30). For example, 13 different new prodiginines were generated in which the C ring of prodigiosin was replaced with a series of monopyrroles of different alkyl chain lengths in the C-3 and C-2 positions, utilizing *Pseudomonas putida* pig-r2 $\Delta pigD$ and the condensing enzyme PigC (30). To reveal the mechanism of the substrate promiscuity of RedH and PigC toward different monopyrroles, it will be important in future studies to test whether the second half of MBC-monopyrrole condensation takes place in a nonenzymatic fashion similar to MBC-ETA condensation.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *Escherichia coli* DH10B was used as the host for plasmid construction, and *E. coli* BL21(DE3) was used as the host for protein expression. *E. coli* ET12567/pUB307, containing the *oriT_{RC}*-plasmid, was used as the donor strain in *E. coli*-*Streptomyces* intergeneric conjugation (31, 32). *Serratia marcescens* SM02 was used as the source of the DNA template for amplification of *pigC* (33). *Streptomyces lividans* GX28, derived from *S. lividans* SBT5, was used for the production of hybrubins in high yield (20). pHZZL3 is an integrative bacmid carrying the *hbn* biosynthetic gene cluster used for the production of hybrubin in *S. lividans* GX28 (16). pMD18-T (TaKaRa) was used for TA cloning and sequencing of DNA fragments. pOJ260, containing an origin of transfer (*oriT_{RC2}*), was used as a suicide vector for the construction of a gene disruption plasmid (34). pET-28a and pET-30a (Novagen) were used as protein expression vectors. pJTU1278, an *E. coli*-*Streptomyces* shuttle vector (27), was used to clone *redH* to yield the *redH* gene complementation plasmid.

E. coli strains were cultured in Luria-Bertani (LB) broth at 37°C. *S. marcescens* was cultured in LB broth containing 0.2% KH₂PO₄ at 30°C. *Streptomyces* strains were grown on solid mannitol soya flour (M5) medium at 30°C. *Streptomyces* was fermented for the production of hybrubin on R3 agar medium at 30°C for 6 to 7 days. *E. coli*-*Streptomyces* intergeneric conjugation was carried out according to the literature (35). Spores of

Streptomyces were suspended in 2× yeast extract-tryptone (YT) medium, heat shocked for 10 min at 50°C, cooled to room temperature, and mixed with the *E. coli* donor. The *E. coli*-*Streptomyces* conjugation mixture was spread on MS agar plates and incubated at 30°C for 3 to 5 days.

Molecular cloning and DNA manipulations in *E. coli*, *Serratia*, and *Streptomyces* were performed according to standard procedures. All primers used in this study were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and are listed in Table 1. In addition, unless stated otherwise, all biochemicals and chemicals were purchased from commercial suppliers and employed without further purification.

Construction of the *S. lividans* Δ redH mutant LQ518. The *redH* gene was deleted by homologous recombination between the chromosome of the parental strain *S. lividans* GX28 and the gene disruption plasmid pJLQ10107, which carries homologous arms amplified from the genomic DNA (gDNA) of GX28. pJLQ10107 was constructed as follows: a 5-kb DNA segment downstream of *redH* (armL) was amplified using the primers Δ redH_armL-F and Δ redH_armL-R, and a 5.1-kb segment upstream of *redH* (armR) was amplified using the primers Δ redH_armR-F and Δ redH_armR-R. The PCR-amplified fragments were then ligated into the pMD18-T vector, verified by DNA sequencing, and subcloned into pOJ260 sequentially to yield pJLQ10107. pJLQ10107 was then introduced into *S. lividans* GX28 by intergeneric conjugation. The double-crossover recombinants, named *S. lividans* LQ518, were finally acquired by two rounds of cultivation without antibiotics and screening for double-crossover mutants by plate stamping (for loss of the vector), followed by PCR amplification to verify the loss of *redH*.

Construction of the *redH* gene complementation mutant. A 2.8-kb DNA fragment containing the intact *redH* open reading frame was amplified from the gDNA of *S. lividans* GX28 using the primers *redH*-cp-F and *redH*-cp-R, followed by digestion of the fragment with NotI and BamHI and insertion into the plasmid pLB139 (possessing an *ermE** promoter) (36) to produce pLB139-*redH*. Then, the DNA fragment containing the intact *redH* and the *ermE** promoter (3,059 bp) was digested with HindIII and EcoRI and inserted into the *E. coli*-*Streptomyces* shuttle vector pJTU1278 to produce the complementation plasmid pJLQ12781. pJLQ12781 was then introduced into *S. lividans* LQ518/pHZL3 by conjugation to yield the complemented strain LQ518/pHZL3+*redH*.

Site-directed mutagenesis of RedH. Overlap-extension PCR was used to generate the amino acid substitutions in the *redH*_E307A, *redH*_R321A, and *redH*_H869A mutants. For the construction of the *redH*_E307A mutant, two pairs of primers, *redH*_E307A-F/*redH*-ex-R and *redH*-ex-F/*redH*_E307A-R, were used to amplify two DNA fragments from *S. lividans* GX28 gDNA. The two DNA fragments were then mixed as the templates and amplified by overlap-extension PCR with primers *redH*-ex-F and *redH*-ex-R. The PCR product, carrying the *redH*_E307A gene, was then subcloned into shuttle vector pET-28a to yield pET28-*redH*_E307A. pET28-*redH*_R321A and pET28-*redH*_H869A were similarly constructed. All clones were verified by restricted digestion and DNA sequencing.

Analysis of hybrubins. *S. lividans* strains GX28/pHZL3, LQ518/pHZL3, LQ518/pHZL3+vector, and LQ518/pHZL3+*redH* were fermented on R3 agar medium for 7 days at 30°C. Solid ferments were diced and extracted twice with ethyl acetate (EtOAc) and concentrated under reduced pressure rotary evaporation at 38°C. The crude extracts were dissolved in 1 ml methanol (MeOH) and subsequently analyzed by HPLC (Agilent 1260) coupled with a diode array detector. Hybrubins and analogues were analyzed on a reversed-phase C₁₈ column (Agilent Zorbax SB-C₁₈, 5 μ m, 4.6 mm by 250 mm) and eluted with H₂O (0.1% formic acid; phase A) and MeOH (phase B) under the following gradient: 0 min with 60% A-40% B, 5 min with 35% A-65% B, 15 min with 20% A-80% B, 20 to 25 min with 100% B, and 26 to 35 min with 60% A-40% B at a flow rate of 0.6 ml/min (16). The UV-visible (UV-Vis) spectra were monitored at 500 nm.

Protein expression and purification. Gene *redH* was amplified from the gDNA of *S. lividans* GX28 using primer pair *redH*-ex-F/*redH*-ex-R and inserted into pET-28a at XhoI-NcoI sites to generate the *redH* expression plasmid pET28-*redH*. Gene *pigC* was amplified from the gDNA of *S. marcescens* SM02 using primer pair *pigC*-ex-F/*pigC*-ex-R and cloned into pET-30a at XhoI-NdeI sites to yield the *pigC* expression plasmid pET30-*pigC*. Both RedH and PigC recombinant proteins carry a C-terminal His6 tag.

E. coli BL21(DE3) carrying the expression plasmids was cultured at 37°C on a shaker at 220 rpm in LB broth supplemented with kanamycin (final concentration, 50 μ g/ml) to an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.6. Cultures were then induced with 0.4 mM isopropyl- β -D-thio-galactopyranoside (IPTG) at 16°C for 20 to 24 h. As for induction of the recombinant strain expressing PigC, the IPTG concentration was 0.1 mM. Samples were collected before and after the induction with IPTG to detect the expression of protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells were harvested by centrifuging at 4,000 rpm for 15 min at 4°C after inducing with IPTG for 20 to 24 h. The resultant cell pellets were resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and lysed by sonication for 30 min. Cellular debris was removed by centrifuging at 10,000 rpm for 1 h at 4°C, and the supernatant was used to purify the protein by nickel-affinity chromatography with standard protocols. The protein was eluted with an increasing gradient of buffer B (500 mM imidazole in buffer A). Purified protein was concentrated and exchanged into buffer C (50 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol, pH 7.5) with centrifugal filters (Millipore Amicon). The protein was stored in buffer C at -80°C. Protein concentration was determined with the Bradford assay using bovine serum albumin (BSA) as a standard.

Chemical synthesis or source. Published procedures were used for the synthesis of 4'-methoxy-2,2'-bipyrrrole-5'-carboxaldehyde (MBC; compound 7) and 5-ethylidenetetramic acid (ETA; compound 8) (17, 37).

Biochemical assays and product analyses. The reaction mixture (200 μ l), adapted from Chawrai et al. (28), for RedH and PigC contained 10 mM MgCl₂, 100 μ M EDTA, 25 mM NH₄Cl, 1.25 mM ATP-Na₂, 100 μ M MBC, 100 μ M tetramic acid (TA) or analogue, 10% dimethyl sulfoxide (DMSO), and 1 to 10 μ M enzyme in 100 mM HEPES (pH 7.0), and the reaction was conducted at 30°C for 12 to 24 h. Reaction mixtures were quenched, extracted twice with ethyl acetate, and dried under vacuum,

TABLE 1 Primers used in this study

Primer name	Primer sequence (5'→3') ^a	Product size ^b (bp)	Purpose
<i>ΔredH_armL-F</i>	CCCAAGCTTGACCAACCGAGTGAACGCGTGAG	5,018	Generate the left homologous arm to knock out <i>redH</i>
<i>ΔredH_armL-R</i>	GCTCTAGAGCCGCCACCGCCGCAACACC	5,120	Generate the right homologous arm to knock out <i>redH</i>
<i>ΔredH_armR-F</i>	GCTCTAGAGCGGGTGTCTCTCGGCGG		
<i>ΔredH_armR-R</i>	GACTAGTAGTGAGATCTCGACGGCGC		
<i>ΔredH-ve-F</i>	TGCCGTGATGTCGCGCCAGAGCAC	WT, 3,239; SC, 3,239 and 442; DC, 442	<i>redH</i> knockout confirmation
<i>ΔredH-ve-R</i>	CAGGCTGCTCTACCACTCTCTGC		<i>redH</i> complementation
<i>redH-cp-F</i>	AAGGAAAAAGCGCCGCAAAAGGAAATCAGTCGGTGGCTCCGGCC	2,856	
<i>redH-cp-R</i>	CGCGGATCCAGCGAAGGAGATATCATATGGACACGACGTCAGCGACACGA		
<i>redH-ex-F</i>	CCGCTCGAGTCTGGTGGTCTCGGCGG	2,814	<i>redH</i> expression with C-terminal His tag
<i>redH-ex-R</i>	CATGCCATGGACACGACGTCAGCGACACGAC		
<i>redH_E307A-F</i>	CGCCGTCGCGCACGCCCAACCGGATGCTCTGCTGCTGCCG		Contains single point mutation for generating <i>redH</i> E307A mutant
<i>redH_E307A-R</i>	CGGCAGCAGCAGGACATCGCGTGGGGCGTGGCGACGGCG		
<i>redH_R321A-F</i>	CGGGCCGGTGGTGTATCGGGGCGGCTTGAAACAGGTGGAG		Contains single point mutation for generating <i>redH</i> R321A mutant
<i>redH_R321A-R</i>	CTTCCACTGTTCAGACCCGCCCGGATCACACCCGGCCCG		
<i>redH_H869A-F</i>	CGCGGGCCACGATGGAACCTGGCCGAGAGCATCGACCCGATG		Contains single point mutation for generating <i>redH</i> H869A mutant
<i>redH_H869A-R</i>	CATCGGTGATGCTCTCGGCCAGTTCCATCTGTCGCCCCGCG		
<i>pigC-ex-F</i>	CCGCTCGAGGCCATCGGCACGTTCTCCGCGT	2,683	<i>pigC</i> expression with C-terminal His tag
<i>pigC-ex-R</i>	GGAAATTCATATGAATCCTACCCCTGGTGTG		

^aRestriction sites are underlined; mutated codons are indicated by underlining and boldface font.

^bWT, wild type; SC, single crossover; DC, double crossover.

followed by suspension in 20 μ l methanol and centrifugation at 13,000 rpm for 10 min to separate the insoluble matter. Soluble portions were injected into an HPLC system (Agilent 1260) with column Agilent Zorbax SB-C₁₈ (5 μ m, 4.6 mm by 250 mm) at a flow rate of 0.6 ml/min. The mobile phase and the gradient were the same as for the above-mentioned analysis of hybrubin with detection at 500 nm.

Ultrahigh-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) was performed on an Agilent 1200 Series with a 6230 Accurate-Mass TOF LC-MS system in negative-ion mode (for P_i-MBC and hybrubins) with an Agilent Zorbax SB-C₁₈ column (5 μ m, 4.6 mm by 250 mm). The mobile phase was composed of H₂O (0.1% formic acid; phase A) and MeOH (phase B) under the following gradient: 0 min with 60% A–40% B, 5 min with 35% A–65% B, 15 min with 20% A–80% B, 20 to 25 min with 100% B, and 26 to 35 min with 60% A–40% B for hybrubin analogues; 0 min with 95% A–5% B, 5 min with 60% A–40% B, 15 min with 20% A–80% B, 20 to 25 min with 100% B, and 26 to 35 min with 95% A–5% B for P_i-MBC. The flow rate for both analyses was 0.4 ml/min.

Step-by-step reaction. The biochemical assay was split into two half-reactions, both of which were conducted in the same buffer as described in “Biochemical assays and product analyses,” above. In the first half-reaction, substrate MBC was incubated with ATP and RedH or PigC for 24 h at 30°C. To remove protein, the reaction product (as a mixture) was boiled at 100°C for 10 min and centrifuged at 13,000 rpm for 10 min. The supernatant was then placed in a –20°C freezer for 30 to 60 min and immediately transferred to a lyophilizer for overnight drying. The dried sample was dissolved in methanol and centrifuged at 13,000 rpm for 10 min to remove insoluble substances such as protein and salts. The supernatant was used to detect the reaction intermediate or transferred to a new microcentrifuge tube, dried under vacuum thoroughly, and used as the starting material for the second half-reaction. In the second half-reaction, buffer (without ATP) and the second substrate 5-ethylidenetetramic acid were added to the microcentrifuge tube and incubated for 12 h at 30°C. A double volume of ethyl acetate was added to terminate the reaction, and the sample was extracted twice and dried under vacuum. The resultant sample was dissolved in methanol, centrifuged to remove insoluble matter, and analyzed by HPLC or UPLC-QTOF-MS.

Effect of RedH on the second half-reaction (i.e., the condensation reaction of P_i-MBC with ETA).

The experimental group (added RedH) and the control group (no RedH) were set using equal amounts of starting material for the second half-reaction, which contained intermediate P_i-MBC. Three parallel experiments were set for each group. ATP-free buffer and ETA were added to both groups and mixed. The production was monitored continuously at 500 nm within 12 h by microplate reader.

LC-MS/MS analysis of the intermediate P_i-MBC. The substrate MBC, cosubstrate ATP, and RedH were incubated together at 30°C for 24 h. To remove water, the reaction mixture was placed at –20°C for 30 to 60 min and immediately transferred to a lyophilizer overnight. To denature and remove the protein, a 10-fold volume of methanol was added to the dried mixture and centrifuged at 13,000 rpm for 10 min. Then, the supernatant was transferred to a new tube and the solvent was air dried. Finally, a small amount of methanol was added to the tube and centrifuged at 13,000 rpm for 10 min to remove impurities. The supernatant was uploaded to an ultraperformance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) system, which model was Agilent 1290 Infinity II equipped with 6545 QTOF LC-MS. The column, mobile phase, elution method, and ion mode were the same as described in “Biochemical assays and product analyses,” above.

Determination of the kinetic constant of RedH. The second substrate ETA, given a saturation concentration, was employed here to couple the RedH-catalyzed reaction to generate red product hybrubin B, which can be measured by a microplate reader. First, a standard curve of the relationship between the OD₅₀₀ value and the concentration of the product was established. Then, the amount of RedH and the reaction time were determined at 5 μ M and 2 h, respectively. Finally, the production was monitored by a microplate reader at different MBC concentrations, and the data were obtained using software GraphPad Prism 7 for analyzing kinetic constants, including the Michaelis constant K_m , the maximum reaction rate V_{max} , and the catalytic constant k_{cat} of the enzyme.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.8 MB.

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