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Functional Identification of Putrescine *C*- and *N*-Hydroxylases

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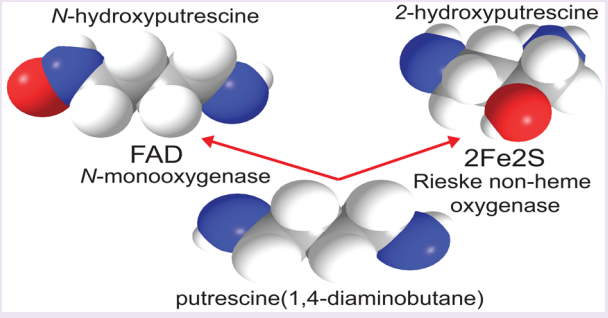
Supporting Information

ABSTRACT: The small polyamine putrescine (1,4-diaminobutane) is ubiquitously and abundantly found in all three domains of life. It is a precursor, through *N*-aminopropylation or *N*-aminobutylation, for biosynthesis of the longer polyamines spermidine, *sym*-homospermidine, spermine, and thermospermine and longer and branched chain polyamines. Putrescine is also biochemically modified for purposes of metabolic regulation and catabolism, *e.g.* *N*-acetylation and *N*-glutamylation, and for incorporation into specialized metabolites, *e.g.* *N*-methylation, *N*-citrylation, *N*-palmitoylation, *N*-hydroxylation, and *N*-hydroxycinnamoylation. Only one example is known where putrescine is modified on a methylene carbon: the formation of 2-hydroxyputrescine by an unknown *C*-hydroxylase. Here, we report the functional identification of a previously undescribed putrescine 2-hydroxylase, a Rieske-type nonheme iron sulfur protein from the *β*-proteobacteria *Bordetella bronchiseptica* and *Ralstonia solanacearum*. Identification of the putrescine 2-hydroxylase will facilitate investigation of the physiological functions of 2-hydroxyputrescine. One known role of 2-hydroxyputrescine has direct biomedical relevance: its role in the biosynthesis of the cyclic hydroxamate siderophore alcaligin, a potential virulence factor of the causative agent of whooping cough, *Bordetella pertussis*. We also report the functional identification of a putrescine *N*-hydroxylase from the *γ*-proteobacterium *Shewanella oneidensis*, which is homologous to FAD- and NADPH-dependent ornithine and lysine *N*-monooxygenases involved in siderophore biosynthesis. Heterologous expression of the putrescine *N*-hydroxylase in *E. coli* produced free *N*-hydroxyputrescine, never detected previously in a biological system. Furthermore, the putrescine *C*- and *N*-hydroxylases identified here could contribute new functionality to polyamine structural scaffolds, including C−H bond functionalization in synthetic biology strategies.



If it can be said that there is a core metabolome of life, then polyamines are an ancient part of it. Polyamines are small aliphatic polycations ultimately derived from amino acids and are found in most cells in all three domains of life.[1] Simple diamine putrescine (1,4-diaminobutane; Figure 1A) is the precursor for synthesis of triamines spermidine and *sym*-homospermidine. Spermidine is essential for growth and cell proliferation in all eukaryotes, in part due to the role of spermidine in the formation of the hypusine modification of translation elongation factor eIF5A.[2] The aminobutyl group of spermidine is transferred to a unique lysine residue in eIF5A by deoxyhypusine synthase to form the deoxyhypusine modification,[3] which is essential in cellular translation due to its role in enabling translation of mRNA encoding proteins containing polyproline tracts.[4] Bacteria do not possess eIF5A and therefore are not constrained by an essential requirement for spermidine in hypusine formation. This may explain why bacterial polyamine structures and metabolism are diverse relative to those of eukaryotes.[5]

Putrescine is fully protonated at physiological pH and is usually found as the unmodified form, although modified forms are found in many species, including *N*-acetylputrescine in *Escherichia coli*.[6] Other putrescine modifications include *N*-methylation as part of tropane alkaloid biosynthesis in plants, mediated by putrescine *N*-methyltransferase.[7] In addition, long chain polyamines found in diatoms are usually *N*-methylated.[8] Plants *N*-acylate putrescine with hydroxycinnamic acids to form, *e.g.*, caffeoylputrescine.[9] A gene identified from a metagenomic library encodes an acyltransferase that *N*-palmitoylates putrescine when expressed in *E. coli*.[10] Putrescine undergoes *N*-glutamylation in bacteria as part of a putrescine degradation pathway.[11] In some fungi and bacteria, putrescine is *N*-citrylated on each amine to form the siderophore rhizoferrin.[12,13] Putrescine is *N*-hydroxylated during the biosynthesis of the cyclic hydroxamate siderophore putrebactin (Figure 1B)[14] in the *γ*-proteobacterium *Shewanella putrefaciens*, probably by an as yet uncharacterized *N*-monooxygenase,[15] exhibiting homology to the lysine *N*6-hydroxylase of aerobactin biosynthesis.[16]

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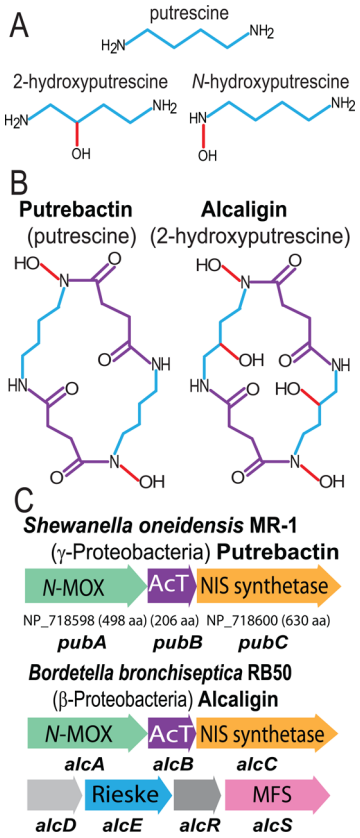

Figure 1. Identification of putrescine hydroxylase candidates. (A) Structures of putrescine (1,4-diaminobutane), 2-hydroxyputrescine, and *N*-hydroxyputrescine. (B) Structures of cyclic hydroxamate siderophores putrebactin and alcaligin. Putrescine backbone in blue, succinyl moiety in purple, hydroxyl groups in red. (C) Gene clusters potentially encoding putrebactin and alcaligin biosynthesis. *N*-MOX, *N*-monooxygenase; AcT, acyl(succinyl)-CoA transferase; NIS synthetase, nonribosomal peptide synthetase-independent siderophore synthetase; MFS, probable siderophore efflux protein.

In contrast to the diverse *N*-modifications of polyamines, there is only one known modification of a methylene carbon in the polyamine backbone, the 2-hydroxylation of putrescine, which has been found almost exclusively in the *β*-proteobacteria,[17,18] and the marine *γ*-proteobacterium *Alteromonas macleodii*.[19] The level of 2-hydroxyputrescine in any species is variable but can approach the level of putrescine. Unlike free 2-hydroxyputrescine, which is abundant in *β*-proteobacteria, free unmodified 1-hydroxyputrescine and *N*-hydroxyputrescine have never been detected in a biological system. A close structural analogue of putrebactin, known as alcaligin, is almost identical to putrebactin except for the presence of 2-hydroxyputrescine (Figure 1B). Alcaligin is synthesized in some *Bordetella* species including the causative agent of whooping cough, *B. pertussis*,[20] and may be critical for successful host colonization and infection establishment.[21]

The biosynthesis of 2-hydroxyputrescine is of biochemical interest because it is the only known modification of a polyamine methylene carbon and could be of use in synthetic biology to confer additional functionality to polyamine structural scaffolds. It is the defining polyamine of *β*-proteobacteria including pathogenic *Bordetella* and *Burkholderia* species, and furthermore, the presence of 2-hydroxyputrescine in a clinically relevant siderophore of the whooping cough agent is biomedically relevant as a potential novel target for chemotherapeutic intervention. It has been 46 years since 2-hydroxyputrescine was first detected in bacteria,[22] and until now the responsible enzyme had not been discovered. We sought to identify the gene encoding the putrescine 2-hydroxylase and functionally identified a previously undescribed Rieske-type nonheme iron sulfur protein from the small mammal pathogen *Bordetella bronchiseptica* and the globally distributed plant pathogen *Ralstonia solanacearum*[23,24] that hydroxylates putrescine on the *C*2 position. A putrescine *N*-hydroxylase from environmental *γ*-proteobacterium *Shewanella oneidensis*, exhibiting similarity to ornithine and lysine *N*-monooxygenases involved in siderophore biosynthesis, was also functionally identified. The proteins hydroxylating the *C*2 and *N* atoms of putrescine exhibit no detectable similarity to one another.

RESULTS AND DISCUSSION

Identification of a Candidate Putrescine 2-Hydroxylase. Due to difficulty in detecting 2-hydroxyputrescine by HPLC, traditional approaches to biochemically purifying a putrescine 2-hydroxylase activity from a *β*-proteobacterium were not feasible. We noticed that the cyclic hydroxamate siderophores putrebactin and alcaligin differ only in that putrebactin contains putrescine and alcaligin contains 2-hydroxyputrescine (Figure 1B). The gene clusters (Figure 1C) encoding putrebactin biosynthesis from *S. oneidensis* MR-1 and alcaligin biosynthesis from *B. bronchiseptica* RB50[15,25] each encode an *N*-monooxygenase with homology to the IucD lysine *N*-monooxygenase of *E. coli* aerobactin biosynthesis,[26] an *N*-acyltransferase and a nonribosomal peptide synthetase (NRPS)-independent siderophore (NIS) synthetase, with homology to the proteins encoded by the *iucB*, and the *iucA* and *iucC* genes of the *E. coli* aerobactin biosynthesis, respectively.[27] In *S. oneidensis* MR-1, these genes are *pubABC*, and in *B. bronchiseptica* RB50, they are *alcABC* (Figure 1C). The likely biosynthetic sequence is that PubA/AlcA *N*-hydroxylates putrescine/2-hydroxyputrescine, PubB/AlcB transfers a succinyl group from succinyl-CoA to *N*-hydroxyputrescine or *N*1-hydroxy 3-hydroxyputrescine, and PubC/AlcC is responsible for condensing two molecules of *N*1-succinyl *N*1-hydroxyputrescine (or its 2-hydroxyputrescine version) together followed by cyclization[15] to form putrebactin or alcaligin, respectively.

It is unknown whether the starting substrate in alcaligin biosynthesis is putrescine or 2-hydroxyputrescine, and it is formally possible that putrebactin is produced and subsequently the putrescine moiety is 2-hydroxylated. However, putrebactin has never been detected in *Bordetella* species in which alcaligin is synthesized. Within the extended alcaligin biosynthetic gene cluster is a gene, *alcE* (CAE35871), that exhibits homology to Rieske-type nonheme iron sulfur protein ring-hydroxylating dioxygenases. We used the AlcE protein sequence in a BLASTP search, and it detected homologues only in the *β*-proteobacteria and a few *γ*-proteobacteria species including *Alteromonas macleodii* that, like the *β*-proteobacteria, is known to accumulate 2-hydroxyputrescine.[19] Furthermore, encoded within the *B. bronchiseptica* RB50 genome is a paralogue of AlcE (CAE32403) that exhibits 71% amino acid identity with AlcE. On the basis of the "guilt by association" principle, both AlcE and the AlcE paralogue appeared to be reasonable candidates for a putrescine 2-hydroxylase.

Functional Identification of Putrescine 2-Hydroxylase. Synthetic genes with *E. coli*-optimized codons were fabricated

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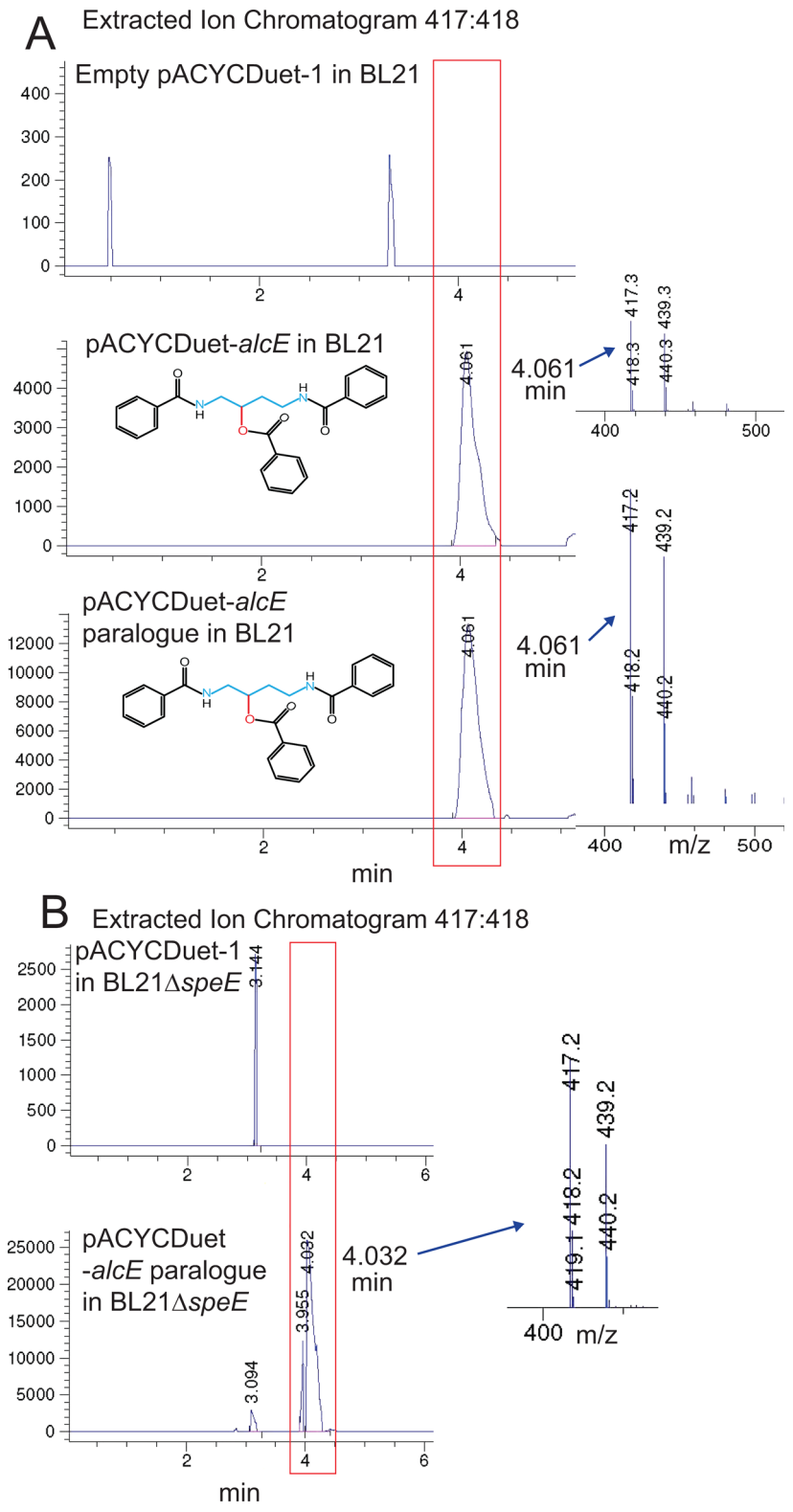

Figure 2. Heterologous production of 2-hydroxyputrescine. (A) Expression of the *B. bronchiseptica* RB50 *alcE* or *alcE* paralogue (*put2H*) ORFs from pACYCDuet-1 in *E. coli* BL21 (pACYCDuet-1 is the empty vector control). Extracted ion chromatograms (nonpolar solvent system) corresponding to the mass of tribenzoylated 2-hydroxyputrescine are shown on the left, and the corresponding mass spectra of the 4.0 min peaks are shown on the right. (B) Expression of the *B. bronchiseptica* RB50 *alcE* or *alcE* paralogue (*put2H*) ORFs in the *E. coli* BL21Δ*speE* (Δspermidine synthase) background.

to encode the *B. bronchiseptica* RB50 AlcE and AlcE paralogue proteins and were inducibly expressed in *E. coli* BL21 from the low copy number expression plasmid pACYCDuet-1. After overnight induction, polyamine contents of the *E. coli* cultures were extracted with trichloroacetic acid and then derivatized with benzoyl chloride to allow detection of the polyamines.

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Control *E. coli* BL21 cultures containing the empty pACYCDuet-1 expression vector, like most *E. coli* strains, accumulate the diamines putrescine and cadaverine and the triamine spermidine. Benzoylated extracts of the *E. coli* cells were subjected to LC-MS analysis using a nonpolar solvent system. The calculated mass of the tribenzoylated 2-hydroxyputrescine (Figure 2A) is 416.47 Da. The extracted ion chromatogram (EIC) for the masses 417:418 shows prominent peaks eluting at 4.06 min in *E. coli* BL21 expressing *alcE* or the *alcE* paralogue but not in control cells (Figure 2A). Mass spectra for the peak at 4.0 min show masses of 417 *m*/*z* and 418 *m*/*z*, representing protonated forms, with corresponding peaks of the sodium adducted forms at 439 *m*/*z* and 440 *m*/*z* (Figure 2A), confirming the presence of hydroxylated putrescine. The eluted peak detected in the EIC 417:418 was approximately 3 times bigger with the *alcE* paralogue than with *alcE*, which we have confirmed in multiple independent experiments, and therefore we named the *alcE* paralogue *put2H* for putrescine 2-hydroxylase. Although AlcE produces 2-hydroxyputrescine when expressed in *E. coli*, its location within the alcaligin biosynthetic cluster suggests that it is not involved in the constitutive production of 2-hydroxyputrescine, since it is cotranscribed under iron limitation with the other alcaligin biosynthetic genes.[28] We had previously noted that disruption of spermidine biosynthesis in *Salmonella enterica* serovar Typhimurium caused a large accumulation of putrescine.[29] To determine whether increasing putrescine levels in *E. coli* BL21 might increase the yield of 2-hydroxyputrescine, we deleted the *speE* (spermidine synthase) gene in *E. coli* BL21. When the *B. bronchiseptica* RB50 *put2H* gene was expressed in the BL21Δ*speE* background, the relative peak size for 2-hydroxyputrescine (eluting at 4.0 min) was approximately twice as high compared to expression in the normal BL21 background (Figure 2B). Depending on experiment, the relative peak size for tribenzoylated 2-hydroxyputrescine was approximately 4−12% of the peak size of the dibenzoylated putrescine.

Functional Identification of Putrescine *N*-Hydroxylase. Biosynthesis of putrebactin requires the *N*-hydroxylation of putrescine (Figure 1B), but it is not known whether alcaligin biosynthesis includes *N*-hydroxylation of putrescine or 2-hydroxyputrescine. We synthesized *E. coli* codon-optimized genes encoding the putative putrescine *N*-monooxygenases AlcA (CAE35867) and PubA (NP_718598; Figure 1C) from *B. bronchiseptica* RB50 and *S. oneidensis* MR-1, respectively, and expressed them from pACYCDuet-1 in *E. coli* BL21. No production of *N*-hydroxyputrescine, which has the same mass as 2-hydroxyputrescine, was detected by LC-MS after expression of either gene (results not shown). We then transferred the genes into pET28b, which has a higher copy number than pACYCDuet-1, and cotransformed the genes in pET28b, along with an empty pACYCDuet-1 plasmid into BL21 cells. After overnight induction of *S. oneidensis pubA* expression, the benzoylated cell extracts were analyzed by LC-MS with a nonpolar solvent system. From cells expressing *S. oneidensis pubA*, we detected a new peak, relative to the empty pET28b control, eluting at 4.88 min (Figure 3). The mass spectrum of this peak revealed the presence of tribenzoylated protonated forms of *N*-hydroxyputrescine (*m*/*z* 417, 418) and sodium adducted forms (*m*/*z* 439, 440) that are absent in the control strain (Figure 3). We did not detect any *N*-hydroxyputrescine after expression of *alcA* (data not shown), and this may have been due to a technical problem or it could

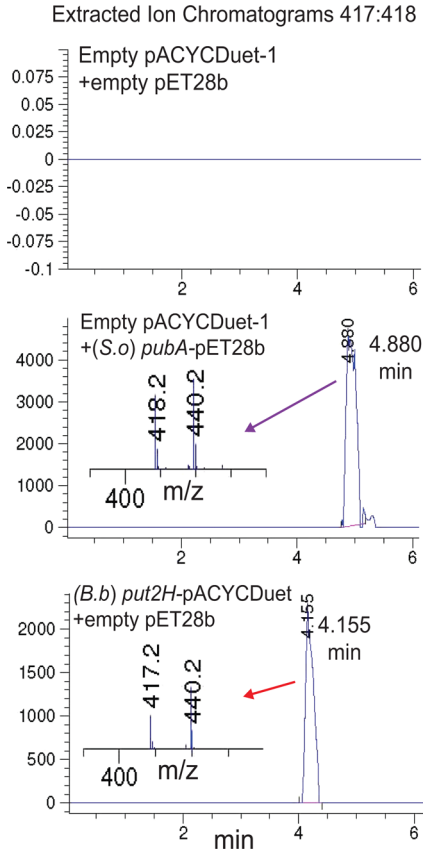

Figure 3. Heterologous production of *N*-hydroxyputrescine and 2-hydroxyputrescine. Extracted ion chromatograms (nonpolar solvent system) corresponding to the mass of tribenzoylated hydroxyputrescine. The control *E. coli* BL21 strain was cotransformed with two compatible plasmids (pACYCDuet-1 and pET28b). *S. oneidensis* MR-1 putrescine *N*-hydroxylase (*pubA*) was expressed from pET28b together with the empty pACYCDuet-1 plasmid. *B. bronchiseptica* RB50 putrescine 2-hydroxylase (*put2H*) was expressed from pACYCDuet-1 together with the empty pET28b plasmid.

be an indication that the substrate for the alcaligin biosynthetic enzyme AlcA is 2-hydroxyputrescine to form *N*1-hydroxy,3-hydroxyputrescine.

Although *N*-hydroxyputrescine and 2-hydroxyputrescine are structural isomers with the same mass, we wanted to determine whether they could be analytically distinguished from one another. The *B. bronchiseptica put2H* gene was transferred from pACYCDuet-1 into pET28b to determine if this would increase production of 2-hydroxyputrescine, but none was detected by LC-MS, probably due to formation of an insoluble protein. To be able to compare directly the chromatographic behavior of the structural isomers 2-hydroxyputrescine and *N*-hydroxyputrescine produced under equivalent physiological conditions, we cotransformed the *B. bronchiseptica* RB50 *put2H* in pACYCDuet-1 and the empty pET28b plasmid into BL21. In this way, the BL21 cells expressing *pubA* or *put2H* in different plasmids were subjected to the same antibiotic selection. The 2-hydroxyputrescine produced by the *put2H* gene (Figure 3) eluted sooner (4.15 min) than the *N*-hydroxyputrescine produced by the *pubA N*-monooxygenase (4.88 min), which is to be expected considering that the 2-hydroxylated putrescine has a shorter stretch of uninterrupted methylene groups that would reduce hydrophobic interactions with the LC column. Isomers 2-hydroxyputrescine and *N*-hydroxyputrescine were

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easily distinguished by the LC separation of the LC-MS analysis.

We were able to obtain a commercial custom synthesis of *N*-hydroxyputrescine through AKos GmbH. However, the synthesis and purification of 2-hydroxyputrescine failed several times due to its strong chelating behavior that led to the formation of stable complexes with metals and organic ligands used in the synthesis. Purity of the *N*-hydroxyputrescine authentic compound was reconfirmed by ^{1}H NMR (Supporting Information Figure 1). We then expressed the *S. oneidensis* MR-1 *pubA* from pET28b in *E. coli* BL21, and as a control we used the empty pET28b plasmid. After growth of the control strain, we added 100 μM of pure authentic *N*-hydroxyputrescine to the cells before polyamine extraction. Benzoylated cell extracts were analyzed by LC-MS using a nonpolar solvent system, and in this analysis, the peaks eluted later that those shown in Figure 3. The authentic *N*-hydroxyputrescine eluted at exactly the same time (5.7 min) as the *N*-hydroxyputrescine produced by the *S. oneidensis* MR-1 *pubA* *N*-monooxygenase (Figure 4).

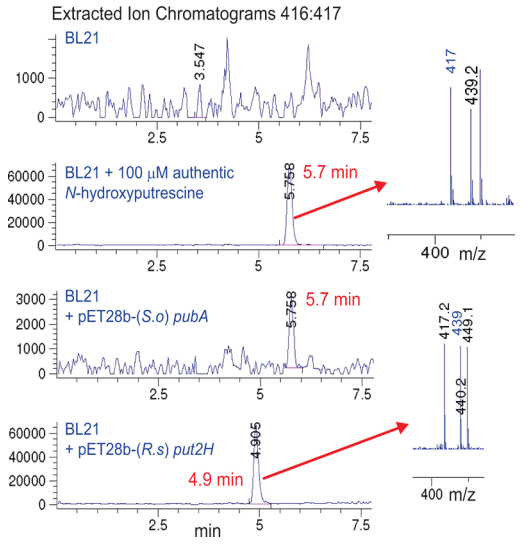

Figure 4. Confirmation of the identity of heterologously produced *N*-hydroxyputrescine. Extracted ion chromatograms (nonpolar solvent system) for the mass of tribenzoylated hydroxyputrescine (416:417) for extracts from *E. coli* BL21, BL21 + 100 μM pure authentic *N*-hydroxyputrescine, BL21 expressing the *S. oneidensis* putrescine *N*-hydroxylase (*S.o pubA*) from pET28b, and BL21 expressing the *R. solanacearum* putrescine 2-hydroxylase (*R.s put2H*) from pET28b. The peak eluting at 5.7 min has a mass of 417/418 (includes protonated form) with the sodium adducted form at 439/440 and corresponds to tribenzoylated *N*-hydroxyputrescine. The peak eluting at 4.9 min also has a mass of 417/418 and corresponds to tribenzoylated 2-hydroxyputrescine.

We also successfully expressed a codon-optimized *put2H* homologue (Rs11165) of the β-proteobacterium *Ralstonia solanacearum* GM1000 from pET28b in *E. coli* BL21 and detected 2-hydroxyputrescine eluting at 4.9 min (Figure 4). The peak height of the 2-hydroxyputrescine produced by the *R. solanacearum* GM1000 Put2H was the same as that of 100 μM pure *N*-hydroxyputrescine and was approximately 20-fold greater than the peak height of the *N*-hydroxyputrescine produced by the *S. oneidensis* MR-1 PubA *N*-monooxygenase.

Refining the Discrimination Between Structural Isomers 2-Hydroxyputrescine and *N*-Hydroxyputrescine. The more efficient production of 2-hydroxyputrescine by the *R. solanacearum* Put2H enabled us to further refine the analytical discrimination between 2-hydroxyputrescine and *N*-hydroxyputrescine by comparing the dibenzoylated pure authentic *N*-hydroxyputrescine (predicted mass 312.36 Da) with the dibenzoylated form of 2-hydroxyputrescine produced by the *R. solanacearum* Put2H, which has the same predicted mass. As a comparison, we detected the dibenzoylated form of cadaverine (1,5-diaminopentane) constitutively present in *E. coli* BL21 with a predicted mass of 310.39 Da. In BL21 cells expressing the empty pET28b plasmid, a peak corresponding to the expected mass of dibenzoylated cadaverine eluted at 3.8 min in the EIC 310:311 (Figure 5). The addition of 100 μM authentic *N*-hydroxyputrescine after growth of BL21 cells expressing the empty pET28b did not produce a detectable new peak in the EIC 310:311, but the peak for dibenzoylated cadaverine was present. Similarly, expression of the *R. solanacearum put2H* from pET28b in BL21 did not produce a new peak in the EIC 310:311, whereas dibenzoylated cadaverine was detected. In the EIC 312:313, which extracts the mass of dibenzoylated 2-hydroxyputrescine and *N*-hydroxyputrescine, a new peak was detected after the expression of *R. solanacearum put2H*, eluting at 3.39 min, and easily distinguishable from the peak corresponding to multiprotonated dibenzoylated cadaverine at 3.88 min, which is also present in the control BL21 with empty vector strain. The addition of 100 μM *N*-hydroxyputrescine did not produce any new peak in the EIC 312:313, suggesting that either the dibenzoylated form of *N*-hydroxyputrescine is not produced or it coelutes with the multiprotonated form of dibenzoylated cadaverine. (Figure 5). Together, these results show that the structural isomers 2-hydroxyputrescine and *N*-hydroxyputrescine can be readily distinguished chromatographically in both the triand dibenzoylated forms.

Depletion of 2-Hydroxyputrescine Biosynthesis in *Ralstonia solanacearum*. To further confirm the function of the *R. solanacearum* Put2H, the corresponding gene was disrupted in the *R. solanacearum* GM1000 strain, and the parental strain and Δ*put2H* gene mutant were analyzed by LC-MS. In the case of *R. solanacearum*, which is metabolically quite different from *E. coli*, we found that a polar rather than nonpolar solvent for the chromatographic separation provided easy detection of the dibenzoylated 2-hydroxyputrescine in the parental strain, eluting at 10.8 min in the EIC 312:313 (Figure 6). This peak disappeared in the Δ*put2H* gene mutant, providing robust support for the *in vivo* function of the *R. solanacearum* Put2H as a putrescine 2-hydroxylase. Depletion of 2-hydroxyputrescine has no discernible effect on growth of *R. solanacearum* GMI1000 Δ*put2H*.

Conclusion. The two putrescine hydroxylases described in this work are entirely unrelated proteins. Putrescine *N*-hydroxylase (*N*-monooxygenase) is homologous to well characterized lysine and ornithine *N*-monooxygenases involved in siderophore biosynthesis that are FAD-dependent and consist of two Rossmann-like nucleotide-binding domains for FAD and NADPH, and also a substrate-binding domain.[30−32] It is clear that the *S. oneidensis* putrescine *N*-hydroxylase involved in putrebactin biosynthesis must have some substrate flexibility because substrate feeding allows the incorporation of cadaverine, as well as putrescine, into the cyclic hydroxamate structure to form a mixed cadaverine/putrescine molecule (avaroferrin) and a fully cadaverine-based version (bisucaberin).[33] Putrescine 2-hydroxylase is a Rieske-type nonheme iron sulfur protein with homologues found throughout the β-

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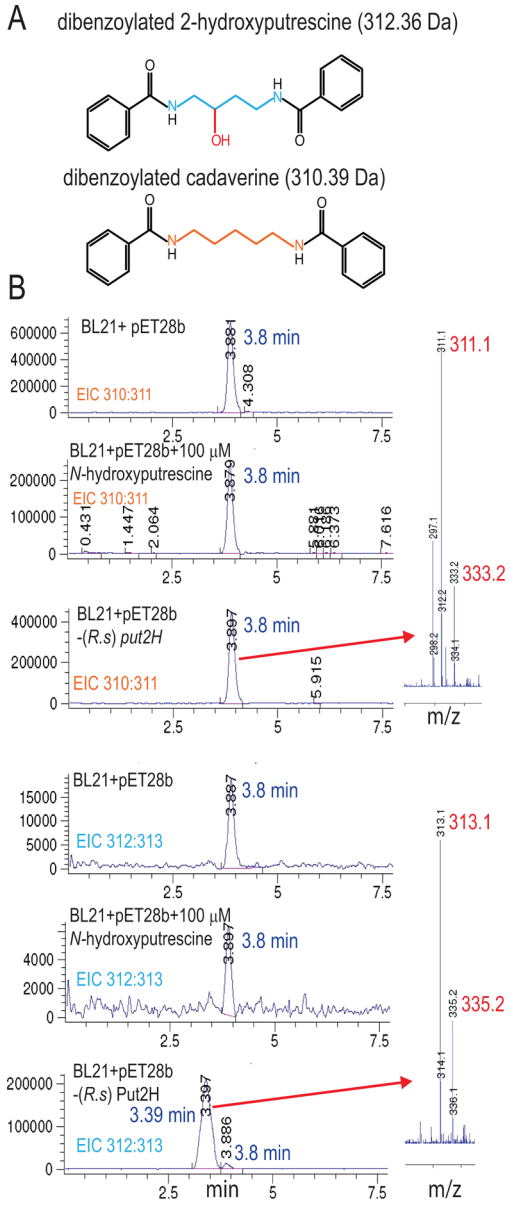

Figure 5. Distinguishing 2-hydroxyputrescine and cadaverine. (A) Structures of dibenzoylated 2-hydroxyputrescine and cadaverine (1,5-diaminopentane). (B) Extracted ion chromatograms (nonpolar solvent system) corresponding to the mass of dibenzoylated cadaverine (310:311) and hydroxyputrescine (312:313). Shown are the analyses of *E. coli* BL21, BL21 + 100 μM pure authentic *N*-hydroxyputrescine, and BL21 expressing the *R. solanacearum put2H* from pET28b, to detect cadaverine (31:311) and 2-hydroxyputrescine/*N*-hydroxyputrescine (312:313).

proteobacteria and a limited number in some γ-proteobacteria. The function of 2-hydroxyputrescine is an enigma. However, one possibility is suggested by the use of citrate as a siderophore in species such as the α-proteobacterium *Bradyrhizobium japonicum*.[34] Many siderophores include citrate in their structure; *e.g.*, rhizoferrin consists of two molecules of citrate attached to each amine group of putrescine.[12] Citrate alone was shown to act as an iron-binding molecule, and ferric citrate functioned as an iron source for growth of *B. japanonicum*.[34] In some β-proteobacterial species, 2-hydroxyputrescine is a component of the cyclic dihydroxamate siderophore alcaligin, the only difference with the siderophore putrebactin. It would be of interest to know whether alcaligin binds metals with greater avidity than putrebactin, and it would be interesting to determine whether 2-hydroxyputrescine itself could be a metal-binding molecule. The putrescine *C*- and *N*-hydroxylases described here have potential as new components in the natural product synthetic biology toolbox.

METHODS

Heterologous Expression in *E. coli*. All genes were synthesized by GenScript with *E. coli*-optimized codons. The *B. bronchiseptica* RB50 gene encoding AlcE (CAE35871:402 a.a.) was synthesized with a 5′ Nde1 site and a 3′ Xho1 site, and the *B. bronchiseptica* RB50 gene encoding Put2H (CAE32403:430 a.a.) was synthesized with a 5′ Nde1 and 3′ EcoRV site. They were ligated into separate plasmids in the corresponding sites in the expression vector pACYCDuet-1 (Novagen). The *S. oneidensis* MR-1 gene encoding putrescine *N*-hydroxylase (PubA, WP_014610805:505 a.a.) was synthesized with 5′ BamH1 and 3′ *Hin*dIII sites and ligated into the corresponding sites of pET28b-TEV, referred to throughout this paper as "pET28b" (Novagen). The *R. solanacearum* GMI1000 gene encoding Put2H was synthesized with 5′ BamH1 and 3′ *Hin*dIII sites and ligated into the corresponding sites of pACYCDuet-1 and pET28b-TEV. Plasmid DNA was prepared using the PureYield Plasmid Miniprep System (Promega) as described by the manufacturer. Recombinant plasmids were transformed into BL21(DE3) or BL21(DE3)Δ*speE* by electroporation. Induction of gene expression for production of hydroxylated putrescine is described below in the section covering polyamine extraction from *E. coli*.

Construction of Spermidine Synthase (*speE*) Deletion Mutant of *E. coli* BL21 (DE3). An *E. coli* gene deletion mutant where the spermidine synthase (*speE*) ORF is replaced by a kanamycin resistance cassette (Δ*speE*::kanFRT) was obtained from the KEIO collection (strain JW0117). The Δ*speE*::kanFRT sequence of JW0117 was transduced from JW0117 into BL21(DE3) (F^-*ompT gal dcm lon hsdSB* (r_B^- m_B^-) λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 sam7 nin5*]) using a cleared lysate of phage P1-infected JW0117 to infect BL21(DE3) followed by selection for kanamycin resistance. Replacement of the native BL21(DE3) spermidine synthase ORF by Δ*speE*::kanFRT was confirmed by PCR amplication of replicated colonies using the upstream primer 5′-atattgaccgtgcatccgcg-3′ and downstream primer 5′-gttttggcgtagcagatatcg-3′. The native *speE*$^+$ product produced a PCR product of 1.9 kbp, whereas the Δ*speE*::kanFRT replacement produced a product of 2.3 kbp. One of the positive colonies where spermidine synthase was replaced with Δ*speE*::kanFRT was named SK833. The strain SK833 (BL21 Δ*speE*) likely has a deficiency in *S*-adenosylmethionine decarboxylase (*speD*) expression due to a polar effect of the upstream *speE* disruption.

Construction of a *R. solanacearum* GMI1000 Putrescine 2-Hydroxylase Unmarked Deletion Mutant. An unmarked Δ*put2H* mutant was constructed using a pUFR80 suicide vector that is unable to replicate in *R. solanacearum*,[35] and which encodes kanamycin resistance and *sacB* (encoding levanosucrase conferring sucrose susceptibility). The genomic locus of the *R. solanacearum* GMI1000 *put2H* corresponds to RS11165 (also known as RSc2224). A 938 bp fragment upstream of *put2H* was PCR amplified using the primers (forward) 5′-cgacggccagtgccaCTTCATCATAGGCGCGATG-3′ and (reverse) 5′-caatagtccTATCCCCACTCCCAAGAAC-3′, and a 1082 bp fragment downstream of *put2H* was amplified using primers (forward) 5′-tggggataGGACTATTGATCCGTCCG-3′ and (reverse) 5′-acctgcaggcatgcaTCAGACTTCCGGGTGGAT-3′. Both fragments were assembled into the *Hin*dIII site of pUFR80. The assembled fragment was sequenced to confirm there were no nucleotide changes introduced. Plasmid pUFR80 containing the upstream and downstream fragments was introduced into *R. solanacearum* GMI1000 by electroporation, and cells were grown on kanamycin-containing plates to select for cells with the upstream and downstream fragments integrated into the host genome. A kanamycin-resistant clone was then counter-selected on 5% sucrose growth medium to select for loss of the pUFR80 vector backbone. The resulting sucrose-resistant colonies

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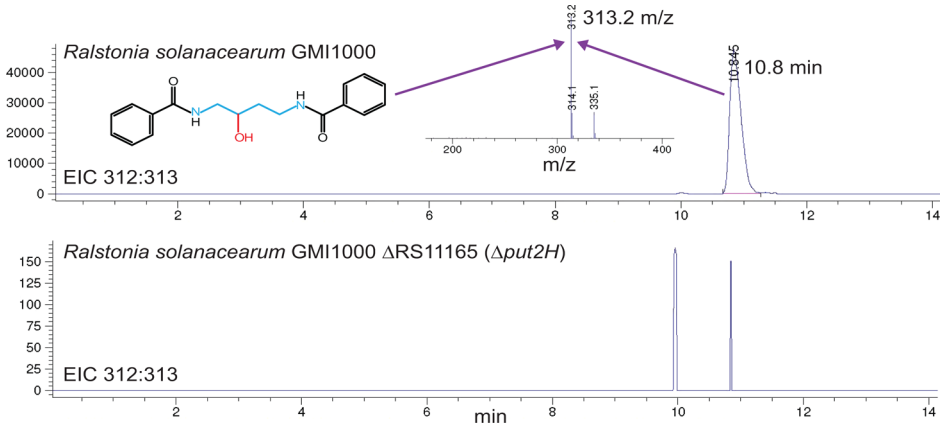

Figure 6. *R. solanacearum put2H* gene required for biosynthesis of 2-hydroxyputrescine. Wild-type and Δ*put2H* strain benzoylated extracts analyzed by LC-MS with a polar solvent system. Extracted ion chromatogram 312:313 for the detection of 2-hydroxyputrescine; the peak eluting at 10.8 min has a mass of 313.2 *m/z* with the sodium adducted form at 335.1 *m/z*.

were PCR screened to identify the *put2H* knockout mutants using the primers (forward) 5′-GAGGTGGGCGACTATCACAC-3′ and (reverse) 5′-CCGAATTCCCAGGTCAGGTC-3′. Mutants lacked the 485 bp PCR product produced by the screening primers. In a further confirmatory screen, the forward primer of the *put2H* upstream fragment and the reverse primer of the *put2H* downstream fragment described above were used to amplify a 3.1 kbp band in the wildtype strain and a 2.0 kbp band in the mutant.

Polyamine Extraction from *E. coli*. A single colony from plates containing solid LB medium was used to inoculate 2 mL of liquid LB medium with appropriate antibiotics and grown at 37 °C overnight. Then, 400 μL of the culture was used to inoculate 20 mL of liquid LB containing appropriate antibiotics and grown at 37 °C until an $O.D.600_{nm}$ of approximately 0.5 was met. At this point, the culture was adjusted to 0.5 mM isopropyl-β-D-thiogalactopyranodside (IPTG) to induce gene expression and induced at 16 °C overnight. Cells were then pelleted by centrifugation and washed three times by resuspension in PBS. Repelleted cells were resuspended in 200 μL of lysis buffer (100 mM MOPS pH 8.0, 50 mM NaCl, 20 mM $MgCl_2$), followed by freezing in liquid nitrogen and thawing at 37 °C, repeated three times. At this point, 100 μM authentic *N*-hydroxyputrescine was added where required. A total of al of 60 μL of 40% trichloroacetic acid was then added, mixed well, and kept on ice for 5 min. Cellular debris was pelleted by centrifugation at 4 °C, and the supernatant transferred to a new tube.

Polyamine Extraction from *R. solanacearum* GMI1000. Cells were grown overnight in 2 mL polyamine-free BMM glucose.[36] Approximately 1.7×10^9 cells were inoculated into 30 mL of BMM glucose in conical flasks and grown for 24 h. Cells were pelleted by centrifugation (6×10^9 to 1×10^{10} cells) and washed three times in water. Pellets were frozen and stored at −80 °C until processing. Frozen pellets were resuspended in 750 μL of lysis buffer (100 mM MOPS pH 8.0, 50 mM NaCl, 20 mM $MgCl_2$) and lysed by sonication in an ice−water bath. Cellular debris was precipitated with 225 μL of 40% trichloroacetic acid. Samples were mixed well, incubated on ice for 5 min, and centrifuged and supernatants transferred to a new tube.

Polyamine Benzoylation. To 200 μL of cell extract containing polyamines was added 1 mL of 2.0 M NaOH and 10 μL of benzoyl chloride, then vigorously mixed for 2 min and left at RT for 1 h. A total of 2 mL of saturated NaCl was added, followed by vortexing for 2 min, and then 2 mL of diethyl ether was added, vortexed for 2 min and left at RT for 30 min. The upper layer of diethyl ether containing the polyamines was transferred to a new tube and kept in a chemical hood until fully evaporated.

Liquid Chromatography−Mass Spectrometry. Benzoylated samples were dissolved in methanol with 0.1% formic acid (v/v) and run on an Agilent Infinity LC-MS or Agilent 1100 series LC-MS, with electrospray probes, using an Agilent Eclipse XDB-C18 column. The column was 4.6 × 150 mm (5 μm) Samples were injected using an autosampler. The solvent system used was: solvent A, water (HPLC grade, 0.1% v/v formic acid), and solvent B acetonitrile (HPLC grade, 0.1% v/v formic acid). Column flow, at RT, was 0.5 mL min^{-1}, the nonpolar solvent system was 30% solvent A/70% solvent B; the polar solvent system was 80% solvent A/20% solvent B. The analysis time was 20 min.

NMR Analysis of *N*-Hydroxyputrescine. ^1H NMR data were recorded at 600 MHz in DMSO-d_6 solution on a Varian System spectrometer, and chemical shifts were referenced to the corresponding residual solvent signal (δ_H 2.50 for DMSO-d_6.).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00629.

Supporting Figure 1 (PDF)

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Notes

The authors declare no competing financial interest.

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