Epimerization of an L-Cysteinyl to a D-Cysteinyl Residue during Thiazoline Ring Formation in Siderophore Chain Elongation by Pyochelin Synthetase from *Pseudomonas aeruginosa*†

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ABSTRACT: The thiazoline-containing siderophores pyochelin, yersiniabactin, and Micacocidin A all have D-thiazoline rings, participating in high-affinity chelation of ferric iron. However, studies with pyochelin (Pch) synthetase and yersiniabactin (Ybt) synthetase reconstituted from pure protein components have shown that only L-cysteine is activated and tethered as a covalent aminoacyl−S−enzyme intermediate. Nor are any of the canonical epimerase domains of nonribosomal peptide synthetase (NRPS) assembly lines found in the Ybt or Pch synthetase modules. Here, we report that the PchE subunit of the Pch synthetase exchanges solvent deuterium into the C_2 center of the thiazoline moieties during siderophore chain elongation. Both PchE and HMWP2, from Ybt synthetase, subunits have a 310−360-residue insert in their amino acid activation domains that look like defective methyltransferase (MT) domains. We suggest these inserts are noncanonical epimerase domains, reversibly deprotonating and reprotonating acyl−S−enzyme intermediates at the C_2 locus. The PchE subunit does not epimerize the Cys−S−enzyme intermediate, but once amide bond formation from a benzoyl−S−PchE donor is catalyzed by the cyclization (Cy) domain of PchE, the *N*-benzoyl-Cys−S−PchE intermediate is present as a D,L-mixture. The subsequent phenylthiazolinyl−S−PchE intermediate, arising from cyclodehydration of the *N*-benzoyl-Cys−S−PchE intermediate, is likewise a D,L-mixture on hydrolytic release and enantiomer analysis. These results suggest a default role for MT domains of NRPS assembly lines in generating α-carbanionic species from thioester intermediates during siderophore chain elongation.

Under iron-limiting conditions, many bacteria produce and secrete low-molecular mass iron chelators. These chelators many times have been found to be virulence factors associated with cholera (*1*), tuberculosis (*2*, *3*), cystic fibrosis (*4*, *5*), and other diseases. These low-molecular mass siderophores generally fall into three types with hydroxamates, catechols, or thiazolines as iron-coordinating functional groups, but all three types can be assembled by nonribosomal peptide synthetases (NRPS)[1] (*6*). NRPS are multimodular enzymes responsible for the biosynthesis of a diverse group of peptide-based secondary metabolites ranging from siderophores [yersiniabactin (*7*, *8*) and pyochelin (*9*)] to compounds used clinically as antibiotics [chloroeremomycin (*10*)], antitumor compounds [epothilone (*11*, *12*)], and immunosuppressants [cyclosporin (*13*)]. The thiazoline-containing siderophores [pyochelin (*9*), yersiniabactin (*7*, *8*), and micacocidin (*14*, *15*)] contain cysteine-derived heterocycles with a range of additional modifications, such as heterocycle reduction, N-methylation, and C-methylation (Figure 1). The thiazolines arise from cyclodehydration (*16*−*19*) of the *N*-acylcysteinyl precursors and dramatically alter the peptide bond connectivity, proteolytic susceptibility, and architecture of these NRP products. The cysteine-derived heterocycle in the NRPS/PKS antibiotic epothilone is a thiazole ring that arises from the NRPS oxidation of a thiazoline (*20*). We examine the epimerization of the C_2 configuration of the first thiazoline ring of pyochelin and demonstrate that it occurs during siderophore assembly.

Epimerization (E) domains are found as 50 kDa domains in NRPS modules where L-amino acids are selected and activated by the NRPS assembly line but downstream chain extension proceeds via the D-isomer. The presence of E domains in an NRPS module is highly predictive that a D-amino acid residue will be found in the elongating chain. A few cases are known where D-amino acids are incorporated but E domains are absent from those NRPS modules (*21*,

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[1] Abbreviations: T, thiolation; MT, methyltransferase; TE, thioesterase; C, condensation; Cy, cyclization; A, adenylation; CoA, coenzyme A; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SOE, splicing by overlap extension; TCEP, tris(2-carboxyethyl)phosphine; TIPS, triisopropylsilane; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; LSC, liquid scintillation counting; HPT, hydroxyphenylthiazoline; HPT−COOH, hydroxyphenyl-thiazolinyl-carboxylic acid; PT, phenylthiazoline; HBC, hydroxybenzoylcysteine; HPTT−COOH, hydroxyphenyl-bisthiazolinyl-carboxylic acid; HPT−Cys, hydroxyphenyl-thiazolinyl-cysteine; NRPS, nonribosomal peptide synthetases; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; SAM, *S*-adenosylmethionine; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form.

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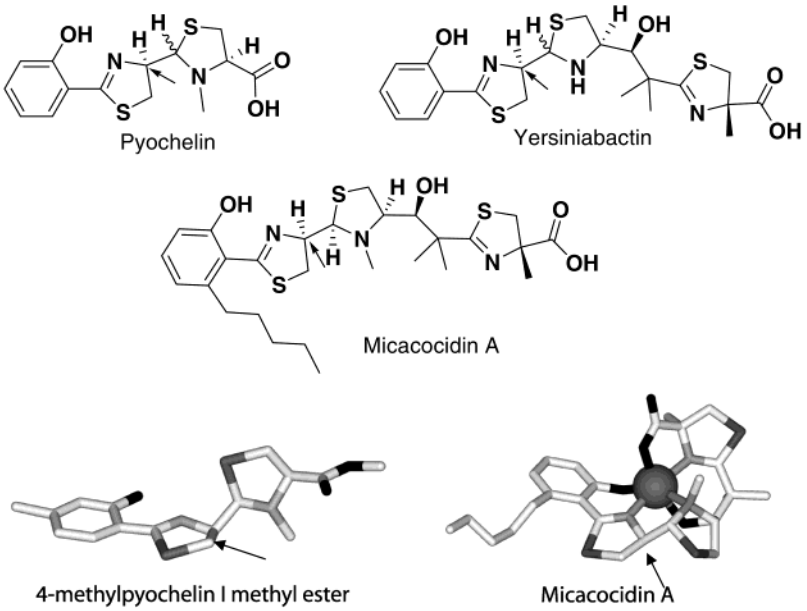


FIGURE 1: Structures of thiazoline-containing siderophores. The crystal structures of 4-methylpyochelin I methyl ester (*23*) and Micacocidin A (complexed with zinc) (*15*) are shown. Elements are depicted as follows: carbon in white, oxygen in black, nitrogen in light gray, sulfur in dark gray, and zinc as a large ball. The arrows indicate the stereocenter of interest.

22). Stereochemical assignment or three-dimensional structure determination of pyochelin (*23*), yersiniabactin (*24*), and micacocidin (*15*) (Figure 1) has shown that the first thiazoline ring in each has the C_2 stereochemistry derived from D-cysteine. Yet, previous studies with the reconstituted pyochelin and yersiniabactin synthetases from this group have shown that PchE (*18*) and HMWP2 (*25*) activate and load L-cysteine only, and sequence analysis of the Pch and Ybt gene clusters does not show any traces of canonical epimerase domains.

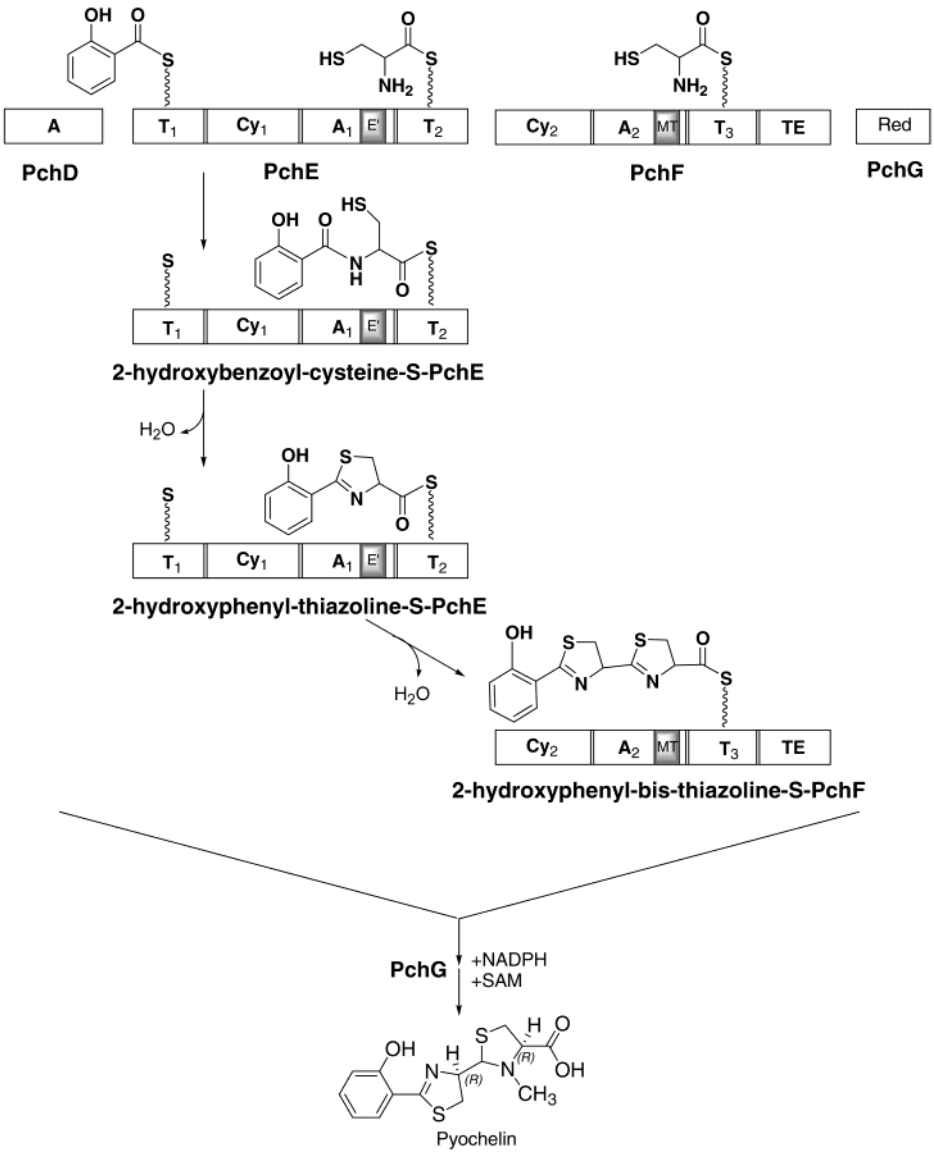
Prior studies from our group have led to overexpression of the four proteins Pch D−G in *Escherichia coli*, purification, and functional characterization (*18*, *26*, *27*). Sequence alignment analysis had previously indicated an ~320-amino acid insert within the adenylation (A) domain of PchE that had homology elements reminiscent of a methyltransferase domain (*28*). There is N-methylation of the thiazolidine ring during pyochelin assembly (Scheme 1), but it was *a priori* assumed that methylation would occur while the growing pyochelin chain was transiting through the PchF subunit. Indeed, our biochemical characterization of the reconstituted pyochelin synthetase indicated that the comparably placed ~400-residue insert within the adenylation domain of PchF contained the *N*-methyltransferase responsible for the methylation of the second thiazoline/thiazolidine ring (*27*). In this study, we have addressed the possibility that the MT-like insert in the PchE adenylation domain could function as a novel epimerase domain.

To probe this possibility, we took advantage of several partial reactions carried out by the isolated PchE subunit. The A domain of PchE activates L-cysteine as Cys-AMP and transfers it to the *in cis* T_2 in its posttranslationally phosphopantetheinylated holo form (*18*). The Cy domain catalyzes salicyl transfer from the salicyl−S−PchE to the cysteinyl−S−PchE intermediate in an amide bond-forming step, followed by cyclodehydration of the proposed *N*-salicylcysteinyl−S−PchE intermediate to the heterocyclized hydroxyphenylthiazolinyl (HPT)−S−PchE intermediate (Scheme 1). This can be slowly hydrolyzed *in vitro* (*18*) and *in vivo* (*28*, *29*) to release the free HPT−COOH, also known as dihydroaeruginoic acid. In this study, we focus on the stereochemical identity of the cysteinyl−, *N*-acylcysteinyl−, and arylthiazolinyl−S−PchE covalent intermediates (Scheme 1).

EXPERIMENTAL PROCEDURES

Materials and Recombinant DNA Techniques. Luria-Bertani (LB) medium was prepared and used for culturing *E. coli* and *Pseudomonas aeruginosa* strains (*30*). GGP medium with 100 μM phenanthroline (*29*) was also used for culturing *P. aeruginosa* strains (*31*). Pyochelin production in *P. aeruginosa* strains in iron-limited GGP medium was confirmed by HPLC and thin-layer chromatography as reported previously (*9*, *32*, *33*), and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Pyochelin produced by *P. aeruginosa* strains was purified as reported with modifications (*32*). Competent cells of *E. coli* strains DH5α and Top10 were purchased from GibcoBRL and Invitrogen, respectively. Plasmid pET27b-(+) was obtained from Novagen. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs and Stratagene. All organic solvents that were used were anhydrous and were bought from Aldrich with highest purity. All commercially available reagents were purchased from Sigma and were used without further purification unless otherwise noted. [^{14}C]-L-Cystine and [^{35}S]-L-cysteine were purchased from Perkin-Elmer LifeSciences (formerly New England Nuclear). SrfA-D (*34*) was provided by R. Kohli. VibB, -E, and -F were provided by G. Marshall (*35*). YbtE and HMWP2 were provided by D. Miller (*25*). Plasmid DNA preparation was performed using the Qiaprep kit (Qiagen). Gel extraction of DNA fragments and purification of DNA amplified by polymerase chain reaction (PCR) were performed using the Qiaquick kit (Qiagen) followed by dialysis with water to remove residual salt. PCRs were carried out using *Pfu* DNA polymerase as described by the enzyme

Scheme 1: Representation of Pyochelin Synthetase[a]



[a] The conversion of the covalently loaded monomers (salicylate and cysteine) to 2-hydroxybenzoylcysteine−S−PchE and 2-hydroxyphenylthiazoline−S−PchE intermediates is shown. Subsequently, PchF condenses and cyclodehydrates with the HPT−S−PchE donor and creates the bis-thiazoline intermediate, which is then competent for reduction and methylation to produce the mature pyochelin. The absolute stereochemistry is indicated in italics.

supplier (Stratagene), except for the addition of DMSO (10%) and glycerol (3%) to the reaction mixtures. The fidelity of PCR-amplified DNA fragments was established by nucleotide sequencing after subcloning into the corresponding expression vector. DNA sequencing was performed on double-stranded template DNA by the Molecular Biology Core Facility of the Dana Farber Cancer Institute (Boston, MA). Oligonculeotides were obtained from Integrated DNA Technologies Inc. Protein concentrations were calculated from the protein's predicted molar extinction coefficient and absorbance at 280 nm.

Cloning pch Genes and Plasmid Construction. Cloning of the *pch* genes *pchD*−*pchG* and variant gene *pchF-TE* and construction of plasmids pPchD, pPchE, pPchF, pPchG, and pPchF-TE were described previously (*18*, *26*, *27*).

The multicloning site of pET27b(+) was subcloned into the pUCPSK shuttle vector (*36*) to create a shuttle vector for transferring *pchE* from pPchE into a vector suitable for cloning in *P. aeruginosa*. The pET27b(+) vector was digested with *Xba*I and *Bsp*EI and the cloning region ligated to pUCPSK digested with *Xba*I and *Xho*I. The resulting linear fragment was purified, blunt ended using the Klenow fragment of DNA polymerase I, and ligated to create a circular vector. Restriction analysis showed the desired construct. This vector was named pSK27. A variant, pKS27, was also constructed by digesting pSK27 with *Apa*I and *Sac*II and ligating to pUCPKS (*36*) digested with the same enzymes.

The *pchE* gene was transferred from pPchE to pSK27 by digesting pPchE with *Xba*I and *Hin*dIII and ligating to pSK27 digested with the same enzymes. This vector, pSK27PE, was used for further expression, mutagenesis, and complementation studies.

Construction of PchE Epimerase Mutants. The genes for the PchE variant proteins, PchE(DE1057−8AA), PchE-(R1129A), PchE(EE1154−9AA), PchE(D1195A), and PchE-

Table 1: Oligonucleotides

PchE variant	extension direction relative to ORF	primer name	primer[a]
all	forward	HP12	GTTATCGCAACGATCCCGAACTCAG
all	reverse	HP28	TAACGTACGCCGAAACGCTCACGTAT
DE1057−8AA	reverse	HP14	GCAACGC**TG**C**TG**CGAGCGGC
DE1057−8AA	forward	HP15	CGCCGCTCG**CA**G**CA**GCGTTG
R1129A	reverse	HP46	AACGCCTG**GC**GGTGGCGGTG
R1129A	forward	HP45	CACCGCCACC**GC**CAGGCGTT
EE1154−9AA	reverse	HP16	GCTGCGT**G**CGAAGAGGGTCAGT**G**CCAACC
EE1154−9AA	forward	HP17	GGTTGG**C**ACTGACCCTCTTCG**C**ACGCAGC
D1195A	reverse	HP18	GCTGATCACCCG**TG**CGTAGC
D1195A	forward	HP19	GGTCGCTACG**CA**CGGGTGAT
H1204A	reverse	HP48	CGCGCTG**GC**CGCCTACGCGG
H1204A	forward	HP47	GCGTAGGCG**GC**CAGCGCGGCGA

[a] The bold letters denote the nucleotides that have been changed.

(H1204A), were constructed via the splicing by overlap extension (SOE) method (*37*) using pSK27PE as a template and the primers shown in Table 1. To make each variant, the 5′ and 3′ fragments of a mutant insert were amplified in the first round of PCR using primer HP12 with the "reverse" primers in Table 1 (HP14, HP46, HP16, HP18, and HP48) to create the 5′ fragment and HP28 with the "forward" pimers in the table (HP15, HP45, HP17, HP19, and HP47) to generate the 3′ fragment. The fragments were gel-purified and the respective combinations mixed and used as a template for the second round of PCR with primers HP12 and HP28. The resulting products were digested with *Sgf*I and *Bsi*WI and ligated to the corresponding sites in pSK27PE, creating pSK27PE1057−8, pSK27PE1129, pSK27PE1154−9, pSK27PE1195, and pSK27PE1204. These plasmids were introduced into *E. coli* Top10 cells, and subsequently into *P. aeruginosa* strain PAO6332 (*pvdB*Δ*pch*) (*26*) for overproduction of the variant PchE protein and *P. aeruginosa* strain PAO6310 (*pchE*Δ*EcoRI*) (*28*) for complementation studies. *P. aeruginosa* cells were transformed by electroporation (*38*).

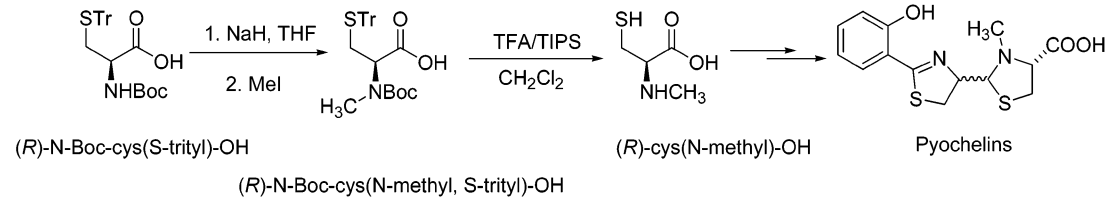
Overproduction and Purification of Pch Proteins in E. coli or P. aeruginosa. Overproduction and purification of PchF, PchD, and PchG were carried out as previously described (*18*, *26*, *27*). Purfied PchF was incubated in dialysis buffer with 10 mM $MgCl_2$, 2 μM Sfp, and 5 mM CoA for 3 h. Following incubation, phosphopantetheinylated proteins were purfied on a Source15Q anion exchange column (Pharmacia), also previously described (*39*). To minimize copurification of a contaminant and to minimize variations between complementation studies and biochemical analysis, *P. aeruginosa* PAO6332 strains containing the pSK27PE (or *pchE* variants) plasmid were cultivated (1 L) with shaking (200 rpm) at 20 °C in LB medium containing 250 μg/mL carbenicillin. Incubation was continued for 48 h, and cells were harvested. As previously described (*18*), cells were lysed and the protein was purified. Purified proteins were concentrated prior to dialysis by ultrafiltration on Centricon-50 membranes.

Production and Detection of Incorporation of Deuterium into Enzymatically Produced HPT and Pyochelin. Enzymatic reaction mixtures for detecting incorporation of deuterium into enzymatically produced HPT contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 1 mM salicylate, 5 mM cysteine, 10 μM PchD, 10 μM PchE (or variant), and 1 μM SrfA-D. Enzymatic reaction mixtures (100 μL) for detecting incorporation of deuterium into enzymatically produced pyochelin contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 1 mM salicylate, 5 mM cysteine, 1.0 mM NADPH, 1.0 mM SAM, 10 μM PchD, 10 μM PchE (or variant), 2 μM PchF, and 10 μM PchG. For deuterium exhange reactions, enzymes were dialyzed into D_2O buffered with 20 mM Tris (pH 7.5) containing 5% glycerol for 8 h, and substrates were dissolved in D_2O or MeOD prior to addition (the final H_2O content was assumed to be less than 10%). After addition of ATP, incubation was continued for 2 h followed by acidification with 10 μL of 1 N HCl. Products were extracted with ethyl acetate (1 mL) as reported previously (*25*). The organic fraction (900 μL) was dried under reduced pressure; the residue was dissolved in a minimal volume of acetonitrile and subjected to MALDI-TOF mass spectroscopy on a PerSeptive Biosystems Voyager-DE STR mass spectrometer. The samples were also analyzed by HPLC using a Vydac C18 reverse phase column (4.6 mm × 25 cm) on a Beckman HPLC system with monitoring at 254 nm. Mobile phases were as follows: mobile phase A being 0.1% TFA in 1 L of water and mobile phase B being acetonitrile. Samples were eluted at 1 mL/min using a linear gradient of mobile phase B from 0 to 100% over the course of 30 min.

Detection and Enantiomeric Separation of Covalently Bound Cysteine. Unless otherwise specified, enzymatic reaction mixtures for incorporation of [^{35}S]cysteine and subsequent separation contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 1 mM [^{35}S]-L-cysteine (1052 Ci/mol), and 10 μM PchE or 10 μM PchF. After addition of ATP, samples (100 μL) were incubated for 60 min at 25 °C and reactions quenched with addition of 1 mL of ice-cold acetone. The precipitate was pelleted by centrifugation at 4 °C, washed four times with acetone (1 mL), and dried prior to addition of 50 μL of 20 mM NaOH. After a 60 min incubation at 50 °C, 2 μL of 1 N HCl was added. A sample of this mixture was added to a racemic mixture of 20 mM cysteine in 98% methanol and centrifuged, and the supernatant was resolved by HPLC (Beckman) on a Chirobiotic T column (Advanced Separation Technologies, Inc., 250 mm × 5 mm) with a detector wavelength of 220 nm and simultaneously by an on-line radioactivity detector tuned for ^{35}S. The mobile phase consisted of 100% methanol at a flow rate of 1 mL/min.

Production of Benzoylcysteine and Phenylthiazoline. Unless otherwise specified, enzymatic reaction mixtures for

Scheme 2: Representation of the Reaction Scheme for Synthesizing *N*-Methylcysteine and Subsequently Pyochelin I and II and Neopyochelin I and II



production of [^{14}C]cysteine-containing benzoylcysteine and phenylthiazoline compounds contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 5 mM benzoate, 13 μM [^{14}C]-L-cysteine (150 Ci/mol), 10 μM PchD, and 10 μM PchE or PchE variant. [^{14}C]-L-Cystine was treated with 1 mM TCEP at 50 °C for 30 min prior to addition to produce L-cysteine. A multiple-turnover system was created by addition of 1 μM SrfA-D during incubation of the reaction mixtures. These reactions were allowed to continue for 1 h at 25 °C, and reactions (100 μL samples) were quenched with addition of 10 μL of 1 N HCl. Acidified reaction mixtures from either system were extracted with 1 mL of ethyl acetate, and 900 μL of organic fraction was dried under reduced pressure and solubilized in a minimal volume of methanol. Chiral separation of benzoylcysteine was achieved by HPLC on a Chirobiotic T HPLC column with a detector wavelength of 254 nm and simultaneously by an on-line radioactivity detector tuned for ^{14}C. The mobile phase consisted of 40% methanol in 1% acetic acid at a flow rate of 1 mL/min. Separation of phenylthiazoline isomers was achieved by drying a representative reaction mixture under reduced pressure, solubilizing in methanol, resolving on chiral TLC (Aldrich) with an acetonitrile/water/methanol (4:1:1) mobile phase, and visualizing using BAS-TR2040 image plates read by a BAS1000 Bio-Imaging Analyzer (Fuji).

Covalent Incorporation of [^{14}C]Salicylate into PchE and [^{14}C]Salicylate Transfer from PchE to PchF. To visualize the incorporation of [^{14}C]salicylate into PchE and PchF, reaction mixtures (20 μL), unless otherwise noted, contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 5 mM cysteine, 4 μM PchD, 0.1 μM Sfp, 1 μM PchE or 1 μM PchE(H1204A), 5 μM PchF-TE, and 90 μM [^{14}C]-salicylate (55 Ci/mol). Reaction mixtures were incubated for 30 min at 25 °C prior to addition of PchF-TE to allow loading of salicylate to PchE or PchE(H1204A). After addition of PchF-TE, samples were allowed to incubate for varying times, reactions quenched by addition of 10 μL of 3× SDS−PAGE sample buffer without a reducing agent, and mixtures heated at 80 °C for 2 min. The samples were resolved using 7.5% gels (Bio-Rad), stained with Coomassie Blue, dried at 80 °C under vacuum, and visualized using a BAS1000 Bio-Imaging Analyzer (Fuji).

Synthesis of Standard Compounds. All reactions were performed under a dry Ar atmosphere unless otherwise indicated. All reactions were monitored by analytical thin-layer chromatography performed using the indicated solvent on E. Merck silica gel 60 F_{254} plates (0.25 mm), unless otherwise indicated. Compounds were visualized by either UV light (λ_{254}) or fluorescence. Flash column chromatography was performed using the indicated solvent on E. Merck silica gel 60 (40−63 m). NMR (^{1}H and ^{13}C) spectra were recorded on a Bruker spectrometer (300 MHz). Chemical shifts (δ) are quoted in parts per million and referenced to $CDCl_3$ (^{1}H NMR, 7.26; ^{13}C NMR, 77.0, center line). LC−MS condition: Shimadzu module, Vydac C18 column (5 μm, 250 mm × 1.0 mm), 0.1% formic acid in water (buffer A) and 0.1% formic acid in CH_3CN (buffer B) as the mobile phase, 0 to 100% buffer B gradient over the course of 35 min.

The L- and D-isomers of benzoylcysteine (*40*) and phenylthiazoline (*41*) were prepared according to published procedures.

The four diastereomers of pyochelin were synthesized by published procedures (*23*, *24*) except that an alternate route to *N*-methylcysteine was employed as shown in Scheme 2.

(1) (R)-N-Boc-Cys(N-methyl,S-trityl)-OH. NaH (60% in mineral oil, 1.03 g, 0.026 mol) was suspended in anhydrous THF (30 mL), followed by slow addition of (*R*)-*N*-Boc-*S*-tritylcysteine (5 g, 0.0108 mol) in anhydrous THF (10 mL) via a syringe and then iodomethane (5.38 mL, 12.27 g, 0.086 mol). The mixture was stirred in the same ice bath and naturally warmed to room temperature. The reaction was monitored by TLC (2:1 hexane/ethyl acetate mixture with 1% acetic acid) and LC−MS (a t_R of 22.6 min for product vs a t_R of 22.0 min for the starting material and t_R of 24.8 min for the bisalkylated byproduct). After 20 h, the reaction was quenched by the addition of phosphate buffer (pH 7, 50 mL) followed by removal of THF upon rotary evaporation. The resulting aqueous mixture was washed with hexanes (50 mL) and extracted with ether (3 × 50 mL). The combined ether layer was washed with saturated NH_4Cl (50 mL) and brine (50 mL) and dried over Na_2SO_4. After solvent evaporation under vacuum, *N*-Boc-Cys(*N*-methyl,*S*-trityl)-OH was obtained as a white foam (4.32 g, 84%). The crude product was carried over to the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) of a mixture of two rotamers: δ 9.1 (br, 1H), 7.18−7.33 (m, 15H, aromatic), 3.57−3.90 [two sets of dd, 3.88 (minor), dd, J = 3.0, 4.5 Hz; 3.60 (major), dd, J = 3.0, 7.5 Hz, total 1H], 2.56−2.76 [two sets of dd, 2.74 (major), J = 3.0, 7.5 Hz; 2.56−2.60 (minor), total 2H], 2.56−2.60 [two sets of s, 2.60 (major), 2.56 (minor), total 3H], 1.28−1.34 [two sets of s, 1.34 (major), 1.28 (minor), total 9H]. ^{13}C NMR (75 MHz, $CDCl_3$) of the major rotamer: δ 175.6, 155.3, 144.9, 129.9, 128.4, 127.2, 81.5, 67.4, 60.8, 34.4, 32.3, 28.7. ESI *m*/*z* calcd for $[M + Na]^+$: 500.6. Found: 500.6. LC−MS: t_R = 22.6 min.

(2) (R)-N-Methylcysteine-OH. (*R*)-*N*-Boc-Cys(*N*-methyl,*S*-trityl)-OH (2.38 g, 4.99 mmol) was dissolved in CH_2Cl_2 (8 mL) followed by the addition of TIPS (5 mL, 24.4 mmol) and TFA (10 mL, 129.8 mmol) under Ar. The reaction was monitored by TLC (1:1 hexane/ethyl acetate mixture with 1% acetic acid) and LC−MS (t_R = 22.6 min) following the disappearance of the starting material. After 2 h, the volatile components were removed with a rotary evaporator. The

resulting oily mixture was washed with ether (3 × 20 mL) to afford Cys(*N*-methyl)-OH after high vacuum as an oily compound (970 mg, 78%). The crude product was carried over to the next step without further purification. ^{1}H NMR (300 MHz, D_2O): δ 4.02 (dd, J = 4.5, 6.8 Hz, 1H), 3.10 (dd, J = 6.8, 15.9 Hz, 1H), 2.98 (dd, J = 4.5, 15.9 Hz, 1H), 2.65 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 62.8, 31.9, 23.0.

The remaining syntheses of the four pyochelin diastereomers follow by a two-step literature protocol starting with methyl 2-(2-hydroxyphenyl)-2-thiazoline-4-carboxylate (*23*, *24*). The reactions were monitored by TLC and LC−MS. The final four pyochelin diastereomers could be separated by TLC (2:2:1:1 BuOH/H_2O/MeOH/hexanes mixture, t_R = 0.42, 0.35, 0.26, and 0.16) with the first two fluorescent compounds being natural products (the first and last spots have stronger fluorescence), and LC−MS (t_R = 15.06, 16.01, 17.03, and 17.65 with a ratio of approximately 4:2:1:2) with the middle two being natural products by coelution. ESI *m*/*z* calcd for $[M]^+$: 324.0. Found: 324.0.

RESULTS

Cloning and Sequencing of pch Genes and Variants. Cloning of the genes encoding PchD−PchG was described previously (*18*, *26*). To evaluate the potential role of the A domain 320-residue insert in PchE, mutations were designed in the conserved residues of the putative epimerase domain. For construction of the PchE variants, a fragment of the 4317 bp *pchE* gene was amplified from the pSK27PE vector by mutagenic primers and cloned into a vector containing the remaining, unmutated, portions of *pchE*, to produce plasmids pSK27PE1057−8, pSK27PE1129, pSK27PE1154−9, pSK27PE1195, and pSK27PE1204. Five sets of primers introduced seven mutations (Table 1) in the proposed epimerase domain. Upon alignment of the proposed epimerase domain of PchE with homologous sequences in PchF, HMWP1, and HMWP2, these aspartate, glutamate, arginine, and histidine residues were shown to be conserved (Figure 2) and hypothesized potentially to be involved in the catalysis of the deprotonation or reprotonation steps of an epimerase reaction. Though Asp1057 and Glu1154 were not conserved across all four proteins, they were mutated with the nearby residues because of the ease of including these on the same primers. Also, His1204 was not conserved in PchF's *N*-methyltransferase, but we have hypothesized that a basic residue may not be required for an *N*-methyltransferase mechanism, but required for both C-methylation and epimerization (see the Discussion). The conserved arginine at PchE position 1171 was not altered.

Sequencing of all mutants showed that all were free of PCR errors except for PchE(H1204A), which contained two PCR errors resulting in two additional amino acid changes (A1168T and L1272P).

Purification of PchD−PchG. The heterologous expression and purification from *E. coli* of the four proteins PchD−PchG that comprise pyochelin synthetase were as previously described (*18*, *26*, *27*), with modification to overproduce PchE in *P. aeruginosa* to eliminate the copurification of a difficult to remove 100 kDa contaminating protein and to minimize differences between complementation studies and biochemical analysis. Growing *P. aeruginosa* PAO6332 transformed with pSK27PE and purifying PchE by previously described methods (*18*) completely eliminated the copurifying 100 kDa *E. coli* contaminant protein and produced comparable protein yields (10 mg/L). For unknown reasons, none of the PchE variants are expressed as well as the wild-type protein and they gave lower yields and purity (1 mg/L); however, all mutants were active in cysteine activation and loading assays (data not shown). Expression of *pchE* in *P. aeruginosa* eliminated the need for *in vitro* phosphopantetheinylation (data not shown) of apo-PchE with a phosphopantetheinyl transferase as the endogenous PPTase (*42*) which sufficed for creation of the holo forms of the T_1 and T_2 domains of PchE.

Evidence that Pyochelin Synthetase Indeed Contains an Epimerase Domain. To determine if either PchD or PchE contained any epimerization activity, we examined the proteins' ability to incorporate deuterium from D_2O into the growing pyochelin peptide. Although not all mechanisms of enzyme-mediated epimerization would require exchange of a proton with solvent, we assumed that any observed deuterium incorporation into product would provide a marker for epimerization activity (*21*). The hydroxyphenylthiazoline−COOH intermediate (HPT−COOH) and the final product pyochelin were isolated from reaction mixtures containing the enzymes PchDE and PchD−PchG, respectively. To isolate sufficient quantities of HPT−COOH for MS analysis, we used the external thioesterase enzyme from the surfactin biosynthesis system, SrfA-D (*34*), to cleave the HPT−S−PchE covalent linkage and release HPT−COOH (Scheme 3). MALDI-TOF analysis of the enzymatically produced molecules showed that the HPT−COOH ($[M + H]^+$ = 224.1) intermediate was isolated with a +1 Da shift when H_2O was replaced with D_2O (Figure 3A,B) and that pyochelin ($[M + H]^+$ = 325.1) produced in this fashion in the PchD−PchG incubations also contained a +1 Da shift (Figure 3C,D). As a control for the solvent deuterium incorporation into the thiazolinyl ring, we turned to the reconstituted vibriobactin synthetase (*35*). It was previously shown that VibBEF proteins were able to generate the dihydroxyphenylthiazoline−cysteine intermediate (DHPT−Cys) (*43*), and sequence analysis did not indicate either a classical 50 kDa epimerase domain or the 35 kDa MT-like inserts in the A domain of the Vib module. Isolation of DHPT−Cys from VibBEF and HPT−Cys from PchDE under similar conditions showed that a +1 Da shift was observed for the product of PchDE but not in the case with VibBEF (data not shown). These deuterium wash in experiments indicated that PchE, or formally PchD, contained a domain actively catalyzing solvent deuterium exchange, a hallmark of a cysteinyl− or thiazolinyl−S−epimerase intermediate.

Cysteine Covalently Bound to PchE and PchF Does Not Undergo Epimerization. To identify the step in the synthesis of HPT during which the epimerization reaction equilibrating the C_2 configuration of the cysteine-derived moiety occurred, we released the covalent intermediates in the multistep process and subjected them to chiral chromatography to resolve enantiomers. Previous experiments showed that PchE would only activate and load L-cysteine, not D-cysteine (*18*). We determined that the stereochemistry of the L-cysteine covalently loaded on PchE and PchF retained the L-configuration in the absence of the upstream acyl donor and PchD. Direct separation of L- and D-cysteine was possible

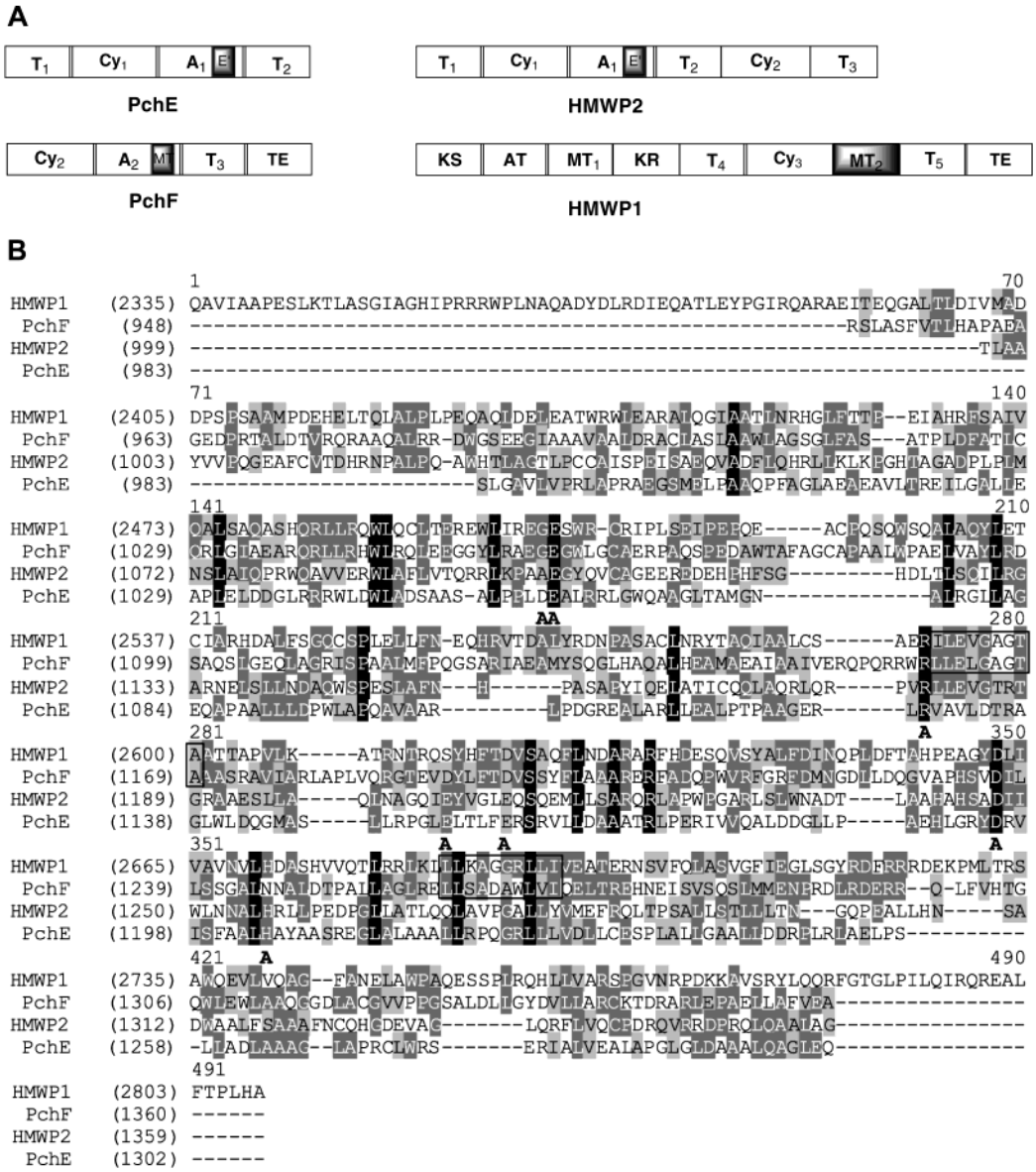


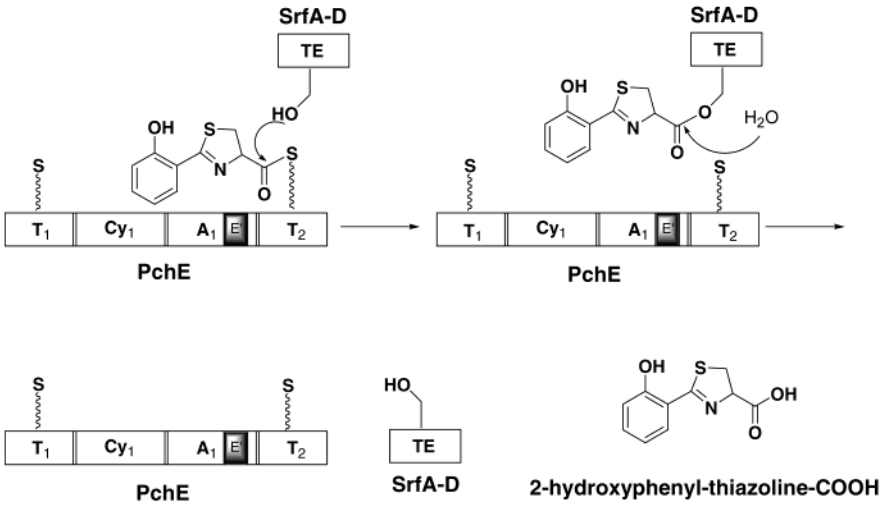
FIGURE 2: (A) Domain organization of PchE, PchF, HMWP2, and HMWP1. The aligned portions are indicated by the shaded regions. (B) Sequence alignment of methyltransferase and methyltransferase-like domains of HMWP1, PchF, HMWP2, and PchE. The alignment was done using AlignX (InforMax, Inc.) with a BLOSUM62 similarity matrix (white on black for identitical, white on gray for conservative, and black on gray for a block of similar). Pairwise similarity values were as follows: 22% for HMWP1 and PchF, 16% for HMWP1 and HMWP2, 13% for HMWP1 and PchE, 31% for PchF and HMWP2, 28% for PchF and PchE, and 40% for HMWP2 and PchE. The PchE residues replaced with alanines are indicated with an A underneath the PchE sequence. The boxed sequences correspond to motif I and III of methyltransferases found in PchF and HMWP1.

using a Chirobiotic T HPLC column. Therefore, [^{35}S]-L-cysteine was self-loaded onto holo-PchE and holo-PchF and the protein isolated. The [^{35}S]cysteinyl−S−PchE intermediate was cleaved by KOH hydrolysis, and the released [^{35}S]-cysteine was chromatographed on chiral HPLC. The cysteine covalently attached in a thioester linkage to the carrier protein domains on either PchE or PchF was shown to coelute with L-cysteine (Figure 4).

Epimerization of the Covalently Tethered Cysteine in PchE Catalysis Occurs after Condensation with the Upstream Donor. The next proposed intermediate on the reaction pathway to HPT is the 2-hydroxybenzoylcysteinyl−S−PchE-(HBC) intermediate (Scheme 1). HBC is the result of the condensation reaction (amide bond formation) of the PchE Cy domain prior to dehydration and cyclization. We could not release and isolate sufficient quantities of the HBC intermediate from D_2O reaction conditions for MALDI-TOF analysis due to its cyclodehydration to the HPT acyl moiety by the PchE Cy domain or selectivity of SrfA-D. Chiral separation of HBC proved to be troublesome using Cu-ligand exchange TLC. However, replacing the aryl acid starter unit salicylate (2-hydroxybenzoate) with benzoate in the PchDE system produced the benzoylcysteinyl−S−PchE intermediate in sufficient yield for chiral analysis. [^{14}C]Benzoylcysteine isolated from PchE by the SrfA-D-mediated thioester cleavage procedure was chromatographed on a Chirobiotic T column and coeluted with the L- and D-enantiomers (Figure 5).

We concluded that epimerization had occurred on the acyclic amide-condensed product. We then expected that the subsequent cyclodehydration catalysis by the PchE Cy domain should also yield a D,L-mixture of the phenylthia-

Scheme 3: Mechanism of External Thioesterase-Mediated Thioester Cleavage at Physiologic pH[a]



[a] HPT loaded on T_2 of PchE is transferred from its phosphopantetheine tether (−SH) to an active site serine (−OH) of the TE, forming the acyl−enzyme intermediate and regenerating active PchE. The intermediate on the TE breaks down by the attack of water to give the carboxylic acid product and regeneration of the TE for further catalysis. This method of thioester cleavage allows for multiple turnovers of the PchDE system.

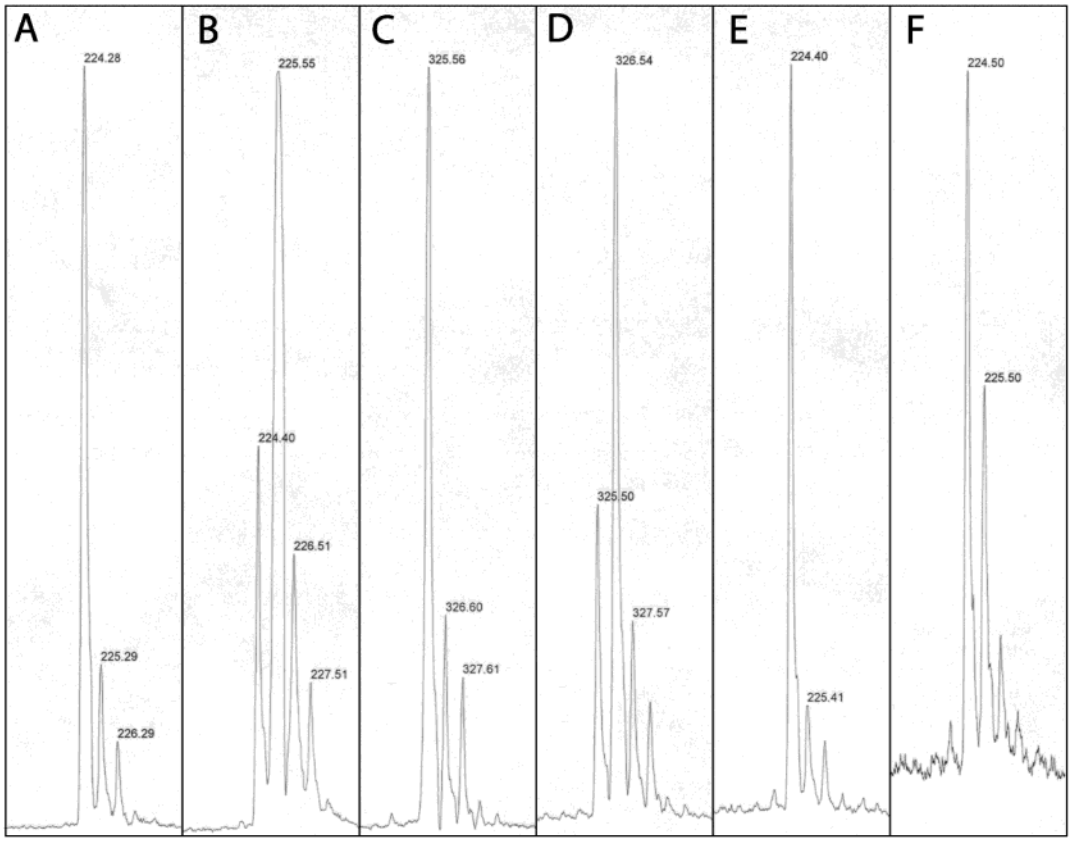


FIGURE 3: MALDI-TOF analysis of enzymatic products performed in H_2O (A, C, and E) or D_2O (B, D, and F). All reaction mixtures contained PchD, cysteine, ATP, and salicylate; reaction mixtures used for panels C and D also contained PchF, NADPH, and SAM. Additionally, mixtures for panels A−D contained PchE, and mixtures for panels E and F contained PchE(H1204A). See Experimental Procedures for reaction conditions.

zolinyl−S−PchE intermediate, starting from benzoate, and the D,L-hydroxyphenylthiazolinyl (HPT)−S−PchE intermediate, starting from salicylate. In incubations of salicylate with PchDE, HPT−COOH could be released from the carrier protein domain. Chiral separation of HPT also proved to be troublesome using Cu-ligand exchange TLC or Chirobiotic T HPLC. Similarly, replacing the starter unit salicylate with benzoate in the PchDE system produced the phenylthiazoline−COOH intermediate (PT−COOH) in sufficient yield for analysis. When PchDE was incubated with benzoate and [^{14}C]-L-cysteine and the mixture allowed to incubate at 25 °C, the phenylthiazoline that was released enzymatically with the thioesterase SrfA-D (Figure 6) or by KOH hydrolysis (data not shown) showed that an approximately 1:1 mixture of L- and D-isomers existed by comparison to synthetic standards.

Mutational Analysis. We had determined that the PchDE system could release an epimerized benzoylcysteine and

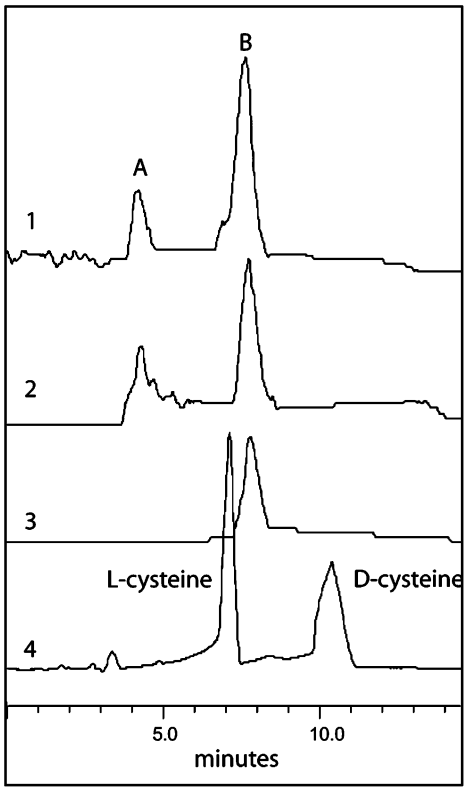


FIGURE 4: HPLC traces showing the stereochemical identity of the cysteinyl−S−enzyme intermediates. (1) Radio-HPLC trace of [^{35}S]-cysteine isolated from PchF. (2) Radio-HPLC trace of [^{35}S]cysteine isolated from PchE. (3) Radio-HPLC trace of pure [^{35}S]-L-cysteine. (4) HPLC trace of the authentic cysteine standards added to radioactive products as cold carriers, which were monitored by an UV detector at 220 nm. Peak A represents an unknown radioactive contaminant. Peak B represents [^{35}S]-L-cysteine. The delay between the UV signal and the radioisotope signal is attributed to the fact that each detector is aligned in series following the HPLC column. See Experimental Procedures for details.

phenylthiazoline, presumably from the D,L-benzoyl-Cys−S−PchE and D,L-phenylthiazolinyl−S−PchE intermediates. To determine if the ~320-residue insert in the adenylation domain was indeed the source of the epimerase activity, we generated mutations in the region. The residues were chosen by aligning the inserts in the adenylation domains of PchE, PchF, and HMWP2 as well as the *C*-methyltransferase of HMWP1 (Figure 2) and locating aligned acidic or basic residues that could participate in the proposed deprotonation/reprotonation epimerase reaction mechanism. Five variant *pchE* genes were created: *pchE*(DE1057−8AA), *pchE*(R1129A), *pchE*(ED1154−9AA), *pchE*(D1195A), and *pchE*(H1204A). These five variant genes and the wild-type gene were transformed into a Δ*pchE* strain of *P. aeruginosa*, and the secreted amount of pyochelin was quantified by HPLC analysis. The strain carrying *pchE*(ED1154−9AA) produced approximately 5% of the amount of pyochelin as the wild type, and no pyochelin was detected in the supernatants of strains carrying *pchE*(H1204A). The five mutants were purified and their activities assayed by deuterium wash-in and phenylthiazoline (PT) synthesis. Only the PchE(H1204A) mutant was defective in its ability to create the D-PT enantiomer (Figure 6) and was unable to wash in deuterium into HPT (Figure 3EF), nor was it able to produce enough pyochelin to be detected by HPLC (Figure 7) or MALDI-TOF analysis (data not shown). Though the PchE1154−9 enzyme was not able to wash deuterium into the final product pyochelin as efficiently as the wild type, it was capable of washing in deuterium into HPT as well as making D-PT (data not shown) and was not further characterized. The PchE(H1204A) variant was also analyzed for its ability to produce benzoylcysteine. As expected, PchE(H1204A) was not able to epimerize benzoylcysteine. These results indicated that the PchE(H1204A) was completely deficient in the ability to catalyze the epimerization of either the benzoylcysteine−S−PchE or phenylthiazoline−S−PchE intermediate, and it was concluded that a mutation in this region of PchE has an effect on the stereochemistry of the hydroxyphenylthiazoline generated by the mutant PchE acting in combination with wild-type PchD.

The PchF Cy Domain Is Selective for D-HPT. To address the question of whether the PchF Cy domain discriminated between the L-HPT and D-HPT enantiomers for its donor substrate, experiments were conducted with the PchF-TE domain mutant to block any acyl chain release and with [^{14}C]-salicylate as a substrate in the presence of PchD and PchE or PchE(H1204A) to generate the acyl donor substrate HPT−S−T_2 (Scheme 1). At the end of incubations, proteins were separated by SDS−PAGE and subjected to autoradiography to detect radiolabeled protein. As seen in Figure 8, incorporation of [^{14}C]salicylate into protein was ATP-dependent (lane 1). Lanes 2−4 show transfer of salicylate from PchE to PchF over time, while lanes 5−7 show that the PchE(H1204A) mutant was incapable of presenting donor substrate to PchF on the time scale of the incubations. The presence of only the natural isomers of pyochelin in reconstitution reactions with wild-type PchE (Figure 7), in combination with these results, indicates that the PchF Cy domain provides the proofreading step required to discriminate between the enantiomeric mixture of donor substrates on T_2 of PchE.

DISCUSSION

The siderophore pyochelin, elaborated by *P. aeruginosa* in times and locales of iron depletion, serves as a virulence factor for infection in vertebrate hosts (*4*, *44*). This iron chelator is produced from salicylate and two molecules of L-cysteine by a three-module NRPS-type siderophore synthetase assembly line, spread over four proteins, PchD−PchG (*18*, *26*, *27*) (Scheme 1). While PchD (A domain) and PchG (reductase domain) contribute only one domain each, to the first and third modules, respectively, PchE and PchF constitute the second and third modules, respectively, and each has the triad of core domains (condensation, adenylation, and thiolation) found in all NRPS modules involved in chain elongation and/or chain termination (*45*). Each A domain activates L-cysteine and tethers it *in cis* to the posttranslationally primed phosphopantetheine-containing T domain. The condensation domains of both PchE and PchF fall into the subset of NRPS domains that have the hallmarks of heterocyclization (Cy) domains (*28*), and this informatics-driven assignment is validated by the observation that the reconstituted PchE and PchF subunits do convert the salicylate and two cysteines into a hydroxyphenyl-thiazolinyl-thiazolinyl−S−PchF covalent enzyme intermediate (Scheme 1) (*18*).

Inspection of the pyochelin structure and stereochemistry indicates three other tailoring reactions have occurred during

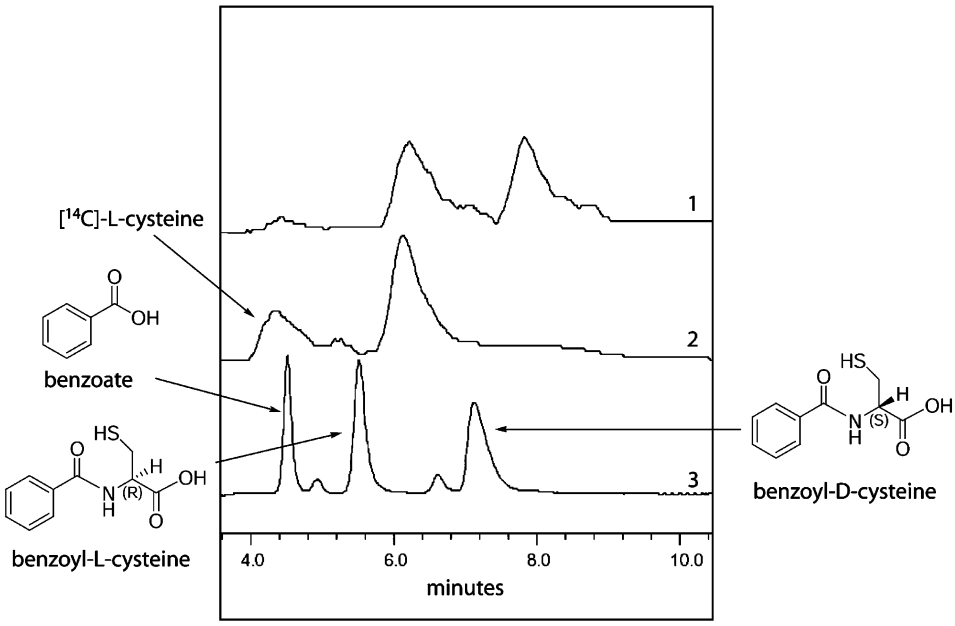


FIGURE 5: HPLC traces showing the stereochemical identity of the benzoylcysteinyl−S−enzyme intermediates. (1) Radio-HPLC trace of benzoyl[^{14}C]cysteine isolated from reaction mixtures containing PchD and PchE. (2) Radio-HPLC trace of benzoyl[^{14}C]cysteine isolated from reaction mixtures containing PchD and PchE(H1204A). (3) HPLC trace of the authentic benzoylcysteine standards added to radioactive products as cold carriers, which were monitored by an UV detector at 254 nm. The delay between the UV signal and the radioisotope signal is attributed to the fact that each detector is aligned in series following the HPLC column. See Experimental Procedures for details.

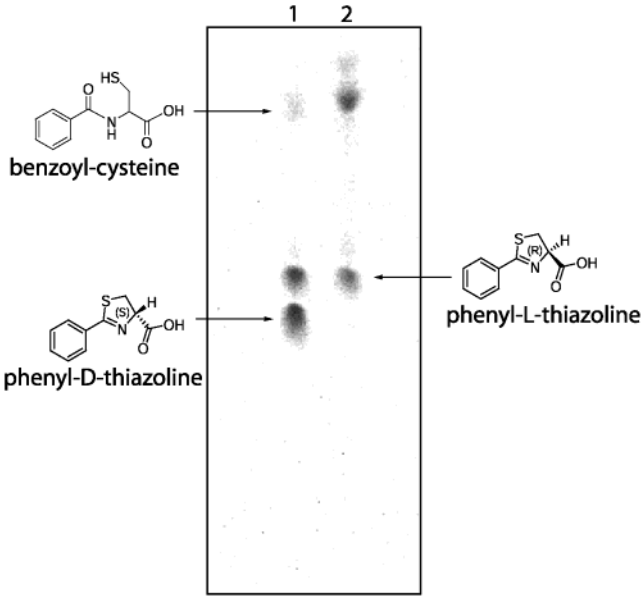


FIGURE 6: Phosphorimage of the chiral TLC plate analysis of the PchDE SrfA-D released products starting with benzoic acid and [^{14}C]-L-cysteine. Lane 1 shows the product when wild-type PchE is included in the reaction mixture. Lane 2 shows the product when PchE(H1204A) is included in the reaction mixture, in place of wild-type PchE. The spotted radioactivity was normalized to show the identity of peaks, and spot sizes do not reflect the relative amounts of the amount produced by each reaction. See Experimental Procedures for details.

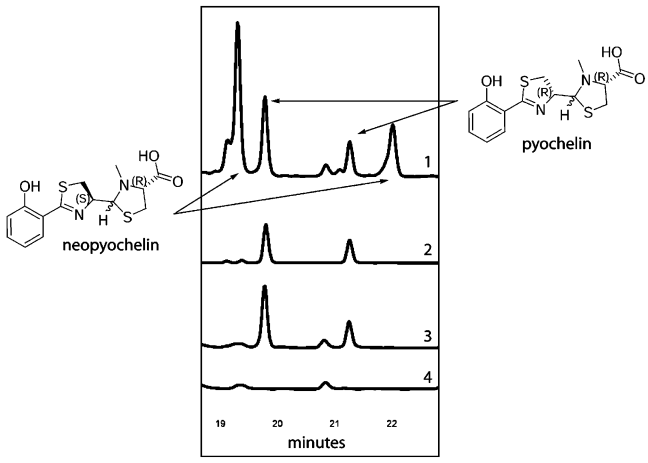


FIGURE 7: HPLC traces showing the pyochelin isolated from reconstitution reactions. (1) HPLC trace of the synthetic pyochelin standards, in which all four diastereomers are present. (2) HPLC trace of the naturally occurring pyochelin standards isolated from culture supernatants of *P. aeruginosa*, in which only the naturally occurring diastereomers are present. Reaction mixtures contained PchD, PchF, PchG, ATP, salicylate, L-cysteine, NADPH, SAM, and (3) wild-type PchE or (4) PchE(H1204A) (a full reconstitution reaction). The HPLC trace of the pyochelins was monitored by an UV detector at 254 nm. See Experimental Procedures for details.

chain elongation down the three-module NRPS assembly line. One is reduction of the second thiazoline ring, from the dihydro oxidation state to the tetrahydro oxidation state of the thiazolidine ring. The second is the subsequent N-methylation of the now basic nitrogen of the thiazolidine nucleus (Scheme 1). The third is the conversion at some stage of the first L-cysteinyl moiety to a D-thiazoline ring, a net inversion of stereochemistry at the original C_2 of the first cysteinyl moiety, to produce pyochelin with *R,R*-stereochemistry as indicated in Scheme 1. One expects three additional domains, one for each of these transformations, in the assembly line, as discussed below.

We have previously shown that the reduction of the imine C=N linkage, the thiazoline to thiazolidine ring conversion, occurs via NADPH by the catalytic action of PchG while the HPTT−S−enzyme intermediate is lodged on the T_3 domain of PchF (*26*, *27*). Then the thiazolidine ring nitrogen becomes a competent amine nucleophile, and N-methylation from SAM ensues via the PchF MT domain (Scheme 4B) (*27*). This two-stage reduction and N-methylation completes

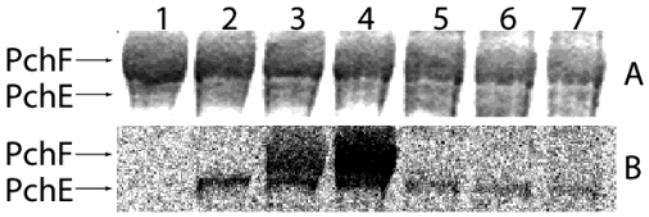


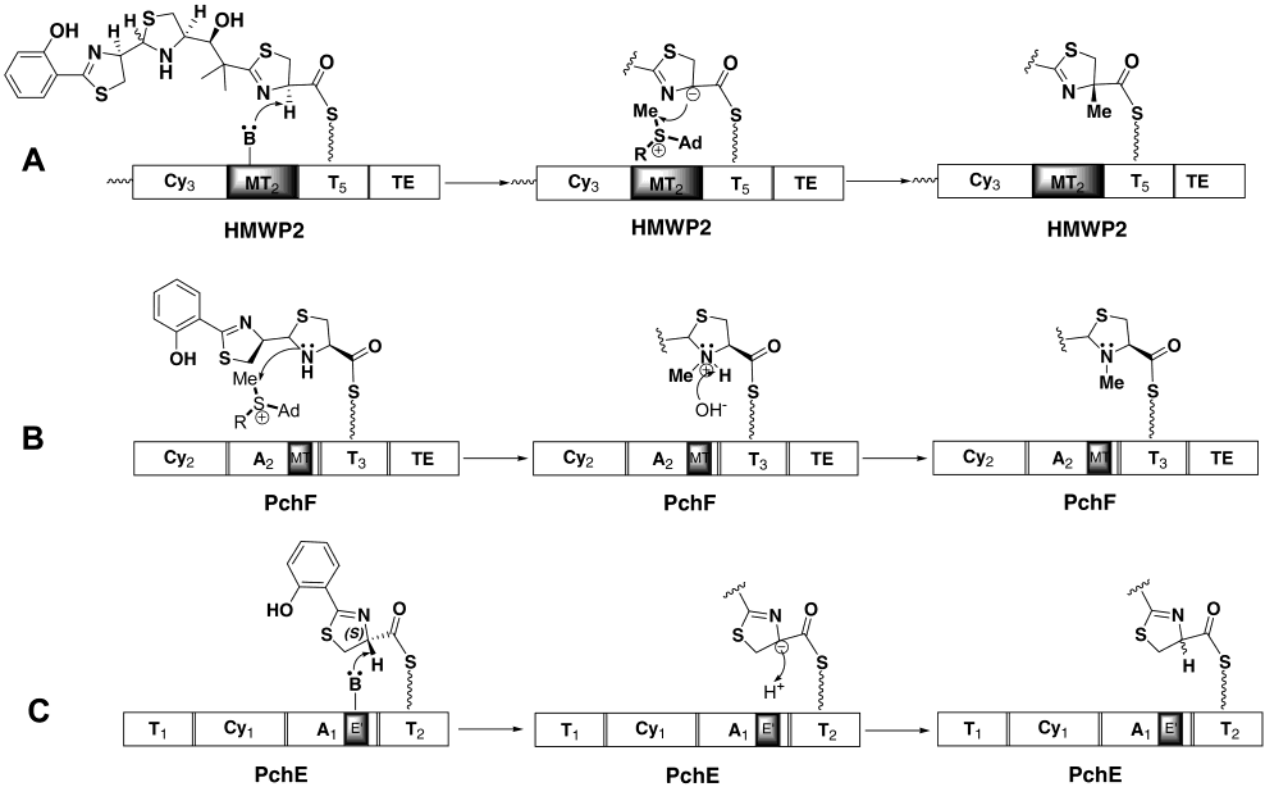
FIGURE 8: Transfer of a salicylate-containing intermediate from PchE to PchF. Panel A shows Coomassie blue-stained bands of Pch proteins, and panel B shows autoradiographs of the corresponding proteins. Reaction mixtures contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM L-cysteine, 90 μM [^{14}C]salicylate (55 Ci/mol), 2 μM PchD, 5 μM PchF-TE, 1 μM PchE (lanes 1−4), 1 μM PchE(H1204A) (lanes 5−7), and 5 mM ATP (lanes 2−7). After addition of PchF-TE, reaction mixtures were incubated for 0 s (lanes 2 and 5), 60 s (lanes 3 and 6), or 15 min (lanes 4 and 7) prior to quenching. No ATP was added to the reaction mixture in lane 1. See Experimental Procedures for details.

the maturation of the pyochelinyl chain, and its hydrolytic release is then accelerated by the TE domain of PchF. By this analysis, PchF is a five-domain NRPS module [Cy_2-A_2-(MT)-T_3-TE], with a sixth domain, the reductase domain of PchG, acting *in trans*. We noted that the 35 kDa MT domain in PchF is actually inserted within the A domain, between motifs A8 and A9 (*46*), a placement that is also seen in the seven *N*-methyltransferase domains in the 11-module fungal NRPS catalyst, cyclosporin synthetase (*13*).

We have also noted that such a similar MT-like insert is found, in the same location, in the A domain of PchE [T_1-Cy_1-A_1("E′")-T_2] and also in the A domain of the HMWP2 subunit of yersiniabactin synthetase from *Yersinia pestis* (*25*, *47*). This is so despite the lack of any obvious methylation tailoring of the growing chain of pyochelin or yersiniabactin while lodged in thioester linkage to the T_2 domain of PchE or HMWP2. On the other hand, the first thiazoline ring has C_2 D-stereochemistry in these two siderophores. We have conclusively shown with purified PchE on its own, and in full reconstitution of the NRPS assembly line, that the A domains in this module activate only L-cysteine, not D-cysteine (*17*, *18*).

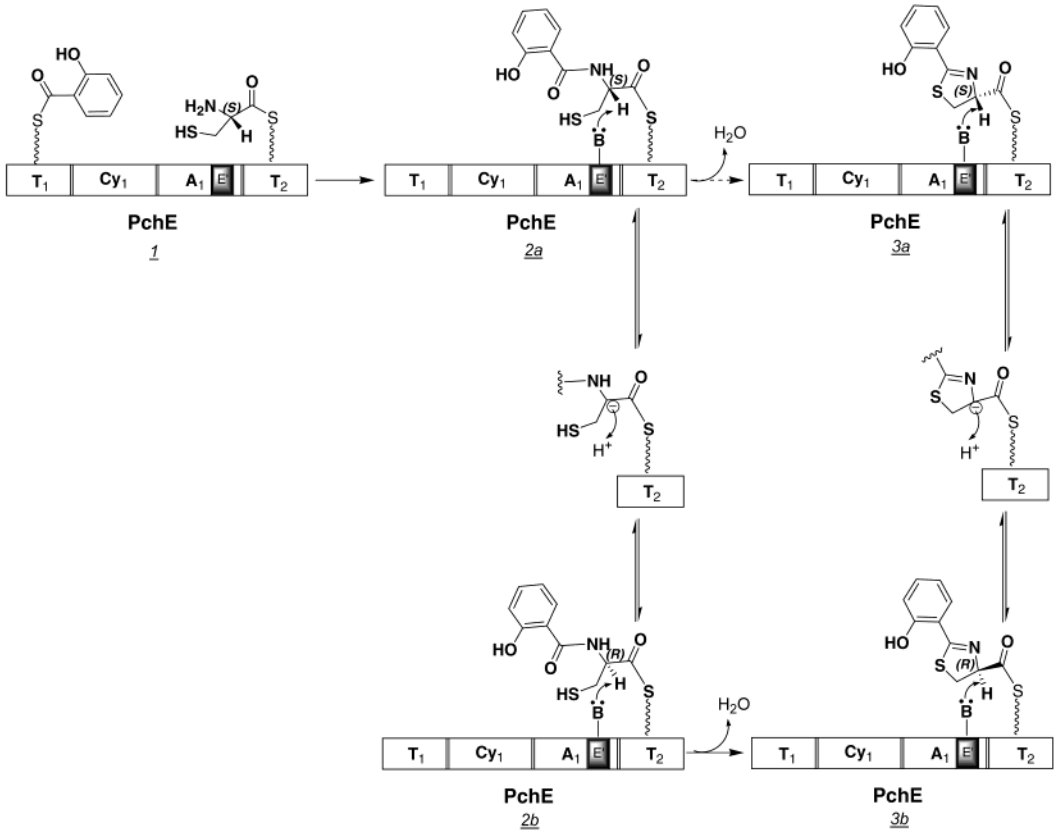
The net inversion of C_2 stereochemistry from L-cysteine to D-thiazoline in the HPT moiety most likely reflects an epimerization process during the action of PchE, with subsequent utilization of only the D-isomer by the Cy_2 domain of the PchF module. Such epimerization and chiral chain selection by the C-domains of the next modules is typical in several NRPS assembly lines (*48*, *49*), where 50 kDa epimerization (E) domains are embedded just downstream of T domains. Thus, a recognizable C-A-T-E four-domain signature is characteristic and predictive of NRPS modules that will select and activate the L-isomer of an amino acid, tether it in a thioester linkage to the T domain, and then epimerize it. The epimerization may happen to the aminoacyl−S−enzyme (before condensation) or to the peptidyl−S−enzyme (after condensation but before translocation to the next downstream module) intermediate, depending on the placement of the E domain in an initiation (A-T-E) or elongation (C-A-T-E) module (*49*−*51*). The epimerization happens on the tethered aminoacyl or peptidyl thioesters because the C_2−H cleavage step is energetically favored since the C_2 carbanion is stabilized by the adjacent thioester linkage (*49*, *52*).

Scheme 4: Proposed Mechanism of Thiazoline C-Methylation (A), N-Methylation (B), and Epimerization (C)[a]



[a] Mechanism A shows base-mediated deprotonation producing a carbon nucleophile required to attack the electropositive SAM methyl group. Mechanism B depicts the nucleophilic amine directly attacking the SAM methyl group followed by deprotonation by solvent. In the epimerization mechanism (C), base deprotonation results in the formation of a C_2 carbanion which leads to sterically random reprotonation from solvent.

Scheme 5: Epimerization of the Hydroxybenzoylcysteine−S−PchE and Hydroxyphenylthiazoline−S−PchE Intermediates[a]



[a] **1** depicts PchE loaded with monomers, salicylate, and L-cysteine. **2a** and **2b** represent the acyl-L-cysteine−S−PchE and acyl-D-cysteine−S−PchE intermediates, respectively. **3a** and **3b** represent the aryl-L-thiazoline−S−PchE and aryl-D-thiazoline−S−PchE intermediates, respectively. The conversion of **2a** and **2b** to **3a** and **3b**, respectively, may be reversible steps.

There is no such 500-residue E domain that can be detected in either PchE or HMWP2, ruling out the participation of a canonical epimerase domain. However, the 35 kDa MT-like inserts (E′) in the A domain of PchE and HMWP2 raised a mechanistic prospect that these could initiate chemistry that would result in the generation of the D-configuration of one or more of the cysteinyl-derived acyl−S−enzyme forms of PchE. In particular, consideration of a likely catalytic cycle for the SAM-dependent *C*-methyltransferase domain found later, in the HMWP1 subunit, of the yersiniabaction synthetase assembly line (*53*, *54*), would be the generation of the stabilized C_2 carbanion of a thiazolinyl−S−T_5 intermediate to generate the carbon nucleophile required to attack the electropositive CH_3 moiety in SAM (Scheme 4A). Thus, one predicts that the MT domain will be able to deprotonate the C_2−H bond of a thiazolinyl−S−enzyme intermediate. If the E′ insert in HMWP2 and in PchE similarly labilize the equivalently acidic C_2−H bond of the HPT−S−enzyme intermediate, then sterically random reprotonation would equilibrate the C_2 configuration (Scheme 4C).

The experiments reported here validate that PchE, on its own or in full assembly line reconstitution, does catalyze complete exchange of the thiazoline C_2−H bond with deuterium from D_2O on covalently tethered substrates and/or intermediates. This is not merely due to the chemical lability of the C_2−H bond of the thiazoline rings since the reconstituted vibriobactin synthetase system, making a hydroxyphenylthiazolinyl−S−enzyme intermediate, does not exchange in solvent deuterium. The A domain of the VibF subunit lacks such an MT-like 30−35 kDa insert.

To follow up these indirect indicators of reversible C_2 hydroxyphenyl-thiazolinyl−S−PchE carbanion formation, we examined the chirality of the covalent intermediates accumulating on the T_2 domain of PchE. L-Cysteine was covalently loaded by PchE on its T_2 domain. On base-mediated cleavage of the Cys−S−enzyme linkage and analysis of the released cysteine, only L-cysteine was detected, proving that the C_2 chirality of the aminoacyl−S−PchE intermediate was retained and that this first acyl−enzyme intermediate was not the substrate for epimerization (Figure 4). On addition of salicylate (or benzoate), ATP, and PchD, salicyl-AMP (benzoyl-AMP) is generated, transferred to the T_1 domain of PchE, and then used by the Cy_1 domain for amide bond formation (to the *N*-salicoyl-cysteinyl−S−PchE intermediate transiently) and subsequent cyclodehydration to yield the hydroxyphenylthiazolinyl (HPT)−S−PchE intermediate. In the absence of PchF, this acyl−enzyme intermediate accumulates. It can leak off hydrolytically to form HPT−COOH [also seen *in vivo* in *P. aeruginosa* as dihydroaeruginoate (*28*, *55*)], or it can be removed catalytically by an external thioesterase enzyme as noted in this study. The dihydroaeruginoate isolated from culture supernatants of *Pseudomonas fluorecens* has also been shown to be the D-isomer (*29*), consistent with PchE-mediated epimerization occurring some time after amide bond formation (Scheme 5). When salicylate was replaced with benzoate and the [^{14}C]acyl−S−PchE intermediate continuously hydrolyzed by addition of exogenous thioesterase, multiple turnovers ensue. [^{14}C]PT−COOH is released as the major species, but small amounts of [^{14}C]-*N*-benzoylcysteine, most importantly

both the L- and D-isomers, are also released, reflecting accumulation of the *N*-benzoylcysteinyl−S−PchE stage, after amide bond condensation (step one of Cy domain catalysis) but before cyclization and dehydration to the arylthiazolinyl−S−PchE intermediate (Figure 5). The results show two things. First, the Cy_1 domain of PchE makes the acyclic amide (*N*-benzoyl-Cys−S−T_2) linkage before the cyclodehydration step (Scheme 5). Second, the E′ domain has equilibrated the C_2 configuration at the *N*-acyl-Cys−S−PchE intermediate stage. We do not yet know if **3a** and **3b** can be equilibrated back to **2a** and **2b**, respectively, and therefore do not know what is the favored route from **1** to **3b** (Scheme 5). The altered ratio of benzoylcysteine to phenylthiazoline released from the PchE(H1204A) mutant (Figure 6) suggests to us that the preferred path is from **2a** to **2b** to **3b** (which then equilibrates to **3a**); however, fast kinetic studies will be required to deconvolute this pathway.

To further tie the PchE-associated epimerase activity toward *N*-aryl-Cys−S−enzyme and arylthiazolinyl−S−enzyme intermediates to the MT-like insert (E′) in the A domain, we turned to mutagenesis. Alignment of the bona fide N-MT and C-MT domains of NRPS enzymes and the MT-like inserts in PchE and HMWP2 (Figure 2) suggested some conserved acidic/basic side chains. Not much is known about the architecture and substrate-binding sites of these subfamilies of protein MT domains other than some SAM recognition sites, and at least one of those is missing in the PchE insert, consistent with it being defective for SAM binding (Figure 2). Three of the five E′ domain mutant genes proved to behave like the wild type upon transformation into the Δ*pchE* strain. Of the two mutants which failed to complement the chromosomal deletion, PchE(H1204A) was substantially reduced in the rate of solvent wash-in to the HPT−S−PchE intermediate, inactive for epimerization of the *N*-benzoyl-Cys−S−PchE or phenylthiazoline−S−PchE intermediate, and unable to support pyochelin formation in an *in vitro* reconstitution reaction. These results strongly support the ∼320-residue insert as the deprotonation/reprotonationation catalyst on the *N*-acyl-Cys−S−enzyme and HPT−S−enzyme intermediates docked in the thioester linkage at the T_2 domain of PchE. Interestingly, histidine 1204 was not conserved in PchF's *N*-methyltransferase, reflecting the need for a basic residue in a *C*-methyltransferase and epimerase mechanism, but perhaps not in an *N*-methyltransferase mechanism (Scheme 4).

Thus, we define the MT-like inserts in PchE and the HMWP2 subunit of yersiniabactin synthetase as noncanonical epimerase (E′) domains. They may only be catalyzing the initial deprotonation step, and stereorandom reprotonation from solvent protons and/or deuterons may be uncatalyzed (Scheme 5). The mutant insert data suggest that the H1204A mutation has reduced the catalytic efficiency of the deprotonation step such that the *N*-acyl-Cys−S−enzyme or the HPT−S−enzyme intermediate does not exchange its C_2−H bond on the time scale of the incubations. Therefore, as shown in Scheme 5, the E′ domain of PchE can act on the second and/or third of the three acyl enzyme forms that are tethered in the thioester linkage at the T_2 domain.

The C_2−H bond of the HPT−S−enzyme intermediate is expected to be substantially more acidic than the uncyclized *N*-acyl-Cys−S−enzyme precursor acyl enzyme, borne out by a 2 h half-life of phenyl-thiazolinyl-SNAC in uncatalyzed D_2O exchange (*56*). It will be of interest to see if the MT-like domain inserts into A domains of other NRPS have the general capability to deprotonate and so lead to racemization of aminoacyl− and peptidyl−S−enzyme intermediates. The observation that the *N*-acyl-Cys−S−enzyme (and the cyclized HPT−S−enzyme intermediate) but not the Cys−S−PchE intermediate with the free amino group is epimerized (Scheme 5) is also in accord with kinetic preferences our laboratory has recently reported for a canonical E domain in module 4 of tyrocidine synthetase. There, the peptidyl−S−enzyme (*N*-acyl-Phe_4−S−enzyme) but not the H_2N-Phe_4−S−enzyme intermediate is epimerized, perhaps reflecting an electronic barrier to cleavage of the C_2−H bond from the free base form of the aminoacyl−S−enzyme intermediate (*49*) and the consequent destabilization of the resultant C_2 carbanion of the thioester by the amine lone pair.

Given that both the N-acylated and cyclodehydrated forms of the Cys−S−PchE intermediate are subject to epimerization at C_2, subsequent fast kinetic studies will be required to evaluate if C_2 configurational equilibration is complete (an approximately 1:1 D/L mixture) before intersubunit chain transfer to the PchF module. We have shown that the PchE(H1204A) mutant is defective in its ability to produce pyochelin and that wild-type PchE only produces the naturally occurring diastereomers of pyochelin (Figure 7); we have also demonstrated that the PchE(H1204A) mutant, which can only create the L-HPT−S−PchE intermediate, is unable to transfer its covalent intermediate to PchF through the action of the PchF Cy domain (Figure 8). Thus, we have indications that the PchF Cy domain is D-specific/selective for the thiazolinyl ring of the HPT-acyl donor on T_2 of PchE. The enantiomeric discrimination of the downstream Cy domain is in agreement with the observations of studies of canonical epimerase domains, where the downstream C domain preferentially condenses with only one stereoisomer present on the preceding T domain (*49*).

In conclusion, we noted that D-amino acid-derived residues are one of the hallmarks of peptide-based natural products of nonribosomal origin. Occasionally, a D-residue comes from a separate amino acid racemase encoded in the biosynthetic gene cluster as in an alanine racemase dedicated for providing D-Ala_1 incorporated into cyclosporin (*22*). Most often, canonical E domains act *in cis* in elongation modules of NRPS assembly lines to equilibrate the configuration of peptidyl−S−T_2 domain intermediates (*19*, *21*, *49*). In this work, we describe a third route, in siderophore synthetases activating and tailoring cysteine residues, where methyltransferase-like domains, acting in default E′ modes, make C_2 carbanionic species on *N*-acyl-Cys−S−enzyme or arylthiazoline−S−enzyme intermediates to create D-centers that are selectively processed by the enzymatic assembly lines. The four-protein, three-module pyochelin synthetase (PchD−PchG) uses 12 domains and a cascade of covalent acyl−S−pantetheinyl-enzyme intermediates to transform an acyl−dipeptide scaffold into a potent heterocyclic scavenger for ferric iron.

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