In Vitro Reconstitution of the *Pseudomonas aeruginosa* Nonribosomal Peptide Synthesis of Pyochelin: Characterization of Backbone Tailoring Thiazoline Reductase and *N*-Methyltransferase Activities†

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ABSTRACT: During iron starvation the Gram-negative pathogenic bacterium *Pseudomonas aeruginosa* makes the nonribosomal peptide siderophore pyochelin by a four protein, 11 domain assembly line, involving a cascade of acyl-S-enzyme intermediates on the PchE and PchF subunits that are elongated, heterocyclized, reduced, and N-methylated before release. Purified PchG is shown to be an NADPH-dependent reductase for the hydroxyphenylbisthiazoline-S-PchF acyl enzyme, regiospecifically converting one of the dihydroheterocyclic thiazoline rings to a thiazolidine. The K_m for the PchG protein is 1 μM, and the k_{cat} for throughput to pyochelin is 2 min^{-1}. The nitrogen of the newly generated thiazolidine ring can be N-methylated upon addition of SAM, to yield the mature pyochelin chain still tethered as a pyochelinyl-S-PchF at the PCP domain. A presumed methyltransferase (MT) domain embedded in the PchF subunit catalyzes this N-methylation. Mutation of a conserved G to R in the MT core motif abolishes MT activity and subsequent chain release from PchF. The thioesterase (TE) domain of PchF catalyzes hydrolytic release of the fully mature pyochelinyl chain to produce the pyochelin siderophore at a rate of 2 min^{-1}, at least 30−40-fold faster than in the absence of hydroxyphenylbisthiazolinyl-COOH (HPTT-COOH) chain reduction and N-methylation. A mutation in the PchF TE domain does not catalyze autodeacylation and release of the pyochelinyl-S-enzyme. Thus, full reconstitution of the nonribosomal peptide synthetase assembly line by purified protein components has been obtained for production of this tandem bisheterocyclic siderophore.

The opportunistic Gram-negative bacterial pathogen *Pseudomonas aeruginosa,* like other bacteria in times of iron-limited growth, upregulates genes for siderophore biosynthesis, export, and import to scavenge ferric iron essential for continued growth. Both pyoverdin and pyochelin siderophores are nonribosomal peptides (*1*−*3*) and contribute to virulence of *P. aeruginosa* infections in animals (*3*−*6*). Genetic analysis has led to the identification of genes PchD−G (*7*−*9*) (Figure 1) required for pyochelin formation from salicylate, two cysteines, NADPH, and *S*-adenosylmethionine, while PchA,B (*10*) are required for salicylate biosynthesis. Prior studies from our group have led to overexpression of the three proteins PchD,E,F in *Escherichia coli*, purification, and functional characterization (*11*). The PchD subunit comprises an adenylation (A)[1] domain that activates salicylate as salicyl-AMP (*11*) for the start of pyochelin chain growth. PchE is the second subunit of the nonribosomal peptide synthetase assembly line and has four domains (ArCP-Cy1-A1-PCP1) of which two are catalytic (Cy1, A1) and two serve as carrier proteins (ArCP, PCP1). The A domain activates L-cysteine as Cys-AMP and transfers it to the *in cis* PCP1 in its posttranslationally phosphopantetheinylated holo form (*11*). The Cy domain of PchE catalyzes salicyl transfer from salicyl-S-PchE to cysteinyl-S-PchE in an amide bond-forming step, followed by cyclodehydration of the proposed *N*-salicylcysteinyl-S-PchE to the heterocyclized hydroxyphenylthiazolinyl (HPT)-S-PchE. This

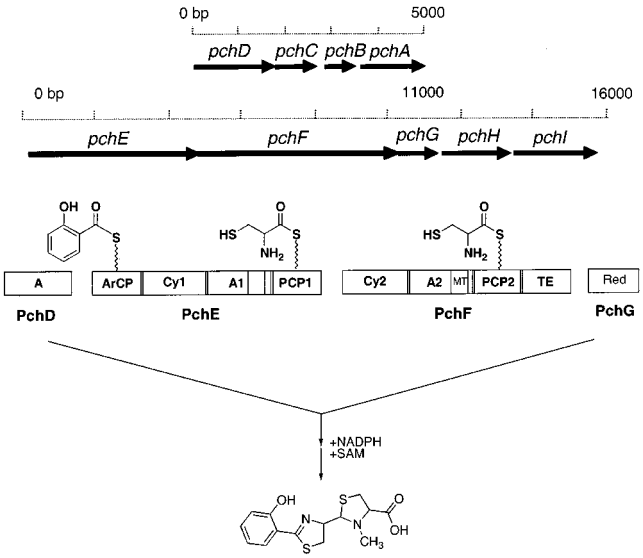

FIGURE 1: Organization of the *pch* genes and domain organization of the PchDEFG system. The adenylation (A) domains, cyclization (Cy) domains, aryl and peptidyl carrier protein (ArCP and PCP) domains, methyltransferase (MT) domain, thioesterase (TE) domain, and reductase (Red) domain are represented. The empty box within A2 of PchE represents an insert of ∼340 amino acids which has no known homology. The prediction of the domain organization for the PchDEFG system has been reported by Reimmann (*7*−*9*). The aminoacyl intermediates are shown attached to the ArCP and PCP domains as well as the final product, pyochelin.

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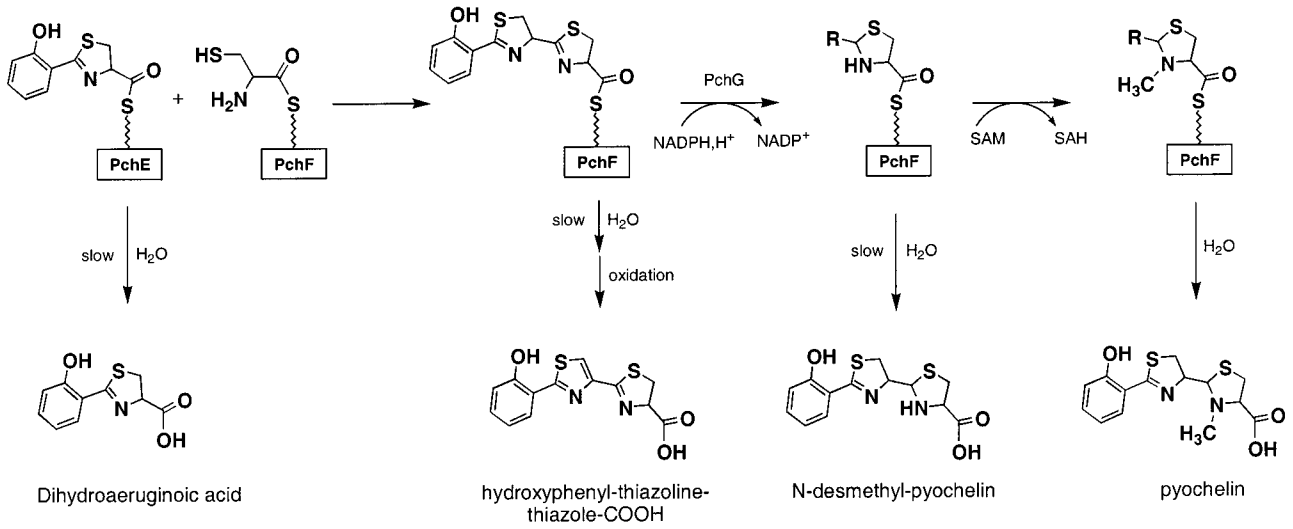
Scheme 1: Model for PchDEFG-Dependent Enzymatic Synthesis of Pyochelin[a]



[a] Intermediates, which have been isolated and characterized, are shown.

can be slowly hydrolyzed in vitro (*11*) and in vivo (*8*, *12*) to release the free HPT-COOH, also known as dihydroaeruginoic acid.

The PchF subunit provides domains 6 through 10 of the NRPS assembly line, (Cy2-A2-MT-PCP2-TE). The Cy2, A2, and PCP2 domains of PchF act analogously to those in PchE, activating a second cysteine, tethering it to holo-PCP2, and transferring the upstream HPT acyl moiety from PchE to Cys-S-PchF and heterocyclizing that to the tandem bis-heterocyclic HPTT-S-PchF (Scheme 1). At this point the chain stalls and is not released from its covalent tethering by the TE unless a fourth protein PchG is present (*9*). PchG acts as an NADPH-dependent thiazoline reductase on the acyl enzyme HPTT-S-PchF, thereby permitting N-methylation of the newly generated thiazolidine ring by SAM and the MT domain embedded in PchF, in turn allowing for chain transfer and release of soluble pyochelin by the TE domain of PchF. In this study, we focus on the MT, TE, and PchG activities, leading to complete in vitro reconstitution of the pyochelin synthetase from salicylate, L-cysteine, SAM, and NADPH.

EXPERIMENTAL PROCEDURES

Materials and Recombinant DNA Techniques. Luria−Bertani (LB) medium was prepared and used for culturing *E. coli* and *P. aeruginosa* strain PAO1 (*13*). Maximal-induction medium (MIM) was prepared and used for culturing *E. coli* (*14*). GGP medium (*12*) was used for culturing *P. aeruginosa* strain PAA4 (*15*). CAA medium was used for culturing *P. aeruginosa* strain PAO1 (*16*). Pyochelin production in *P. aeruginosa* strain PAO1 in iron-limited CAA medium was confirmed by HPLC and thin-layer chromatography as reported previously (*2*, *16*, *17*) and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Pyochelin produced by *P. aeruginosa* strain PAA4 was purified as reported with modifications (*16*). Competent cells of *E. coli* strains DH5α and BL12(DE3) were purchased from GibcoBRL and Novagen, respectively. Plasmid pET29b(+) was obtained from Novagen. Restriction endonucleases, T4 DNA ligase, and pUC19 were obtained from New England Biolabs. Coenzyme A (CoA), *S*-adenosylmethionine (SAM), and NADPH were purchased from Sigma. [^{14}C]Salicylate, adenosyl-L-methionine, *S*-[*methyl*-^{14}C]adenosyl-L-methionine ([^{14}C]SAM), and *S*-[*methyl*-^{3}H]adenosyl-L-methionine ([^{3}H]-SAM) were purchased from DuPont New England Nuclear. HPTT-COOH was synthesized and kindly provided by I. Mori. Sfp was purified as previously described (*18*). 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). PCRs were carried out using *Pfu* DNA polymerase as described by the enzyme 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 Techonologies Inc. The protein concentration was calculated from the protein's predicted molar extintion coefficient and absorbance at 280 nm.

Cloning pch Genes and Plasmid Construction. Cloning of the *pch* genes *pchD*, *pchE*, *pchF*, and *pchG* and construction of plasmids pPchD, pPchE, pPchF, and pPchG were described previously (*9*, *11*).

Construction of PchF Thioesterase and Methyl Transferase Mutants. Construction of the Cys1606Ala/Ser1607Ala double mutant of PchF was previously described (*11*). The Gly1167Arg mutant of PchF was constructed via the splicing

[1] Abbreviations: PCP, peptidyl carrier protein; ArCP, aryl carrier protein; MT, methyltransferase; TE, thioesterase; Cy, cyclization; A, adenylation; BSA, bovine serum albumin; CoA, coenzyme A; IPTG, isopropyl 1-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SOE, splicing by overlap extension; TCA, trichloroacetic acid; LSC, liquid scintillation counting; HPTT-COOH, hydroxyphenylbisthiazolinylcarboxylic acid; HPT-Cys, hydroxyphenylthiazolinylcysteine; NRPS, nonribosomal peptide synthetases; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; SAM, *S*-adenosylmethionine; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form.

by overlap extension (SOE) method (*19*). Using pPchF as a template, the 5′ and 3′ fragments of a mutant insert were amplified in the first round of PCR using the following primers: MT1 (5′-GCAGAGTCCGGAGGACGCCTGGACG-GCGTT-3′) and MT2 (5′-CGCGGCTGGCGGCGGCGGT-GCGGGCGCCAA-3′) (5′ fragment) and MT3 (5′-GCTG-GAGCTTGGCGCCCGCACCGCCGCCGCCA-3′) and MT4 (5′-GCCGTTGCCGGTGACCGGCAGGCGTT-3′) (3′ fragment). Fragments were gel-purified, combined, and used as a template for the second round of PCR with primers MT1 and MT4. The resulting product was digested with *Bsp*EI and *Bst*EII and ligated to the corresponding sites in pUC19-PchF(6458−10503) (*11*), creating pUC19-PchFMT. This plasmid was then digested with *Bsi*WI and *Hin*dIII, and the insert was recovered and ligated to the corresponding sites in digested pPchF, creating pPchFMT. This plasmid was introduced into *E. coli* DH5α and subsequently into *E. coli* BL21(DE3) for overproduction of PchF(Gly1167Arg)-H6 (PchFMT).

Overproduction and Purification of Pch Proteins in E. coli. Overproduction and purification of PchF, PchF variants (PchFTE and PchFMT), PchD, and PchG were done as previously described (*9*, *11*). To minimize copurification of a contaminant, *E. coli* BL21(DE3) strains containing the pPchE plasmid were cultivated (1 L) with shaking (300 rpm) at 25 °C in MIM medium containing 50 μg/mL kanamycin. Cultures were induced with 0.2 mM IPTG when they reached an OD_{600} of 1.8. After induction, incubation was continued, and cells were harvested prior to entrance into stationary phase (∼4 h). Cells were lysed, and protein was purified as previously described (*11*). When required, purified proteins were concentrated prior to dialysis by ammonium sulfate precipitation at 90% saturation.

Covalent Incorporation of [^{14}C]Methyl into PchF. For assessing the incorporation of the [*methyl*-^{14}C]SAM-derived methyl group into PchF, reaction mixtures (100 μL), unless otherwise noted, contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 5 mM cysteine, 0.1 mM CoA, 4 μM PchD, 0.1 μM Sfp, 1 μM PchE, 10 μM PchF, PchFMT, or PchFTE, 0.5 mM NADPH, and 35 μM [*methyl*-^{14}C]SAM (15 Ci/mol). Reactions were incubated at 25 °C for 30 min to allow phosphopantetheinylation prior to addition of ATP. After addition of ATP, samples were allowed to incubate for varying times and quenched with addition of 500 μL of 10% TCA and 100 μg of BSA. The precipitate was pelleted by centrifugation, washed two times with 10% TCA (1 mL), solubilized in 200 μL of formic acid, and added to 3.5 mL of liquid scintillation fluid. The incorporated ^{14}C label was quantified by LSC. To visualize the incorporation of [^{14}C]-SAM into PchF, reactions (50 μL), unless otherwise specified, were as described above. The reaction mixtures were incubated for 30 min to allow phosphopantetheinylation prior to addition of ATP. After reaction time (varied), reactions were quenched by addition of 50 μL of 2× SDS−PAGE sample buffer without reducing agent. The samples were resolved using 4−15% gels (Bio-Rad), stained with Coomassie blue, dried at 80 °C under vacuum, and visualized using a Fuji phosphorimager.

Production and Detection of Salicylate-Containing Compounds (Reconstitution Reactions). Unless otherwise specified, enzymatic reaction mixtures for production of salicylate-containing compounds (reconstitution reaction) contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM CoA, 1 mM salicylate, 5 mM cysteine, 1.0 mM NADPH, 1.0 mM SAM, 10 μM PchD, 10 μM PchE, 2 μM PchF, 10 μM PchG, and 0.1 μM Sfp. The reaction mixtures were incubated at 25 °C for 30 min to allow phosphopantetheinylation prior to addition of ATP. After addition of ATP, incubation was continued, and samples of 100 μL were taken at specific times, followed by acidification with 10 μL of 1 N HCl. Salicylate-containing compounds were extracted with ethyl acetate (1 mL) as reported previously (*20*). The organic fraction (900 μL) was dried under reduced pressure. The residue was dissolved in 150 μL of 10% acetonitrile/water and analyzed by HPLC as previously described (*9*, *11*). The previously reported HPLC conditions were modified: solvent A was 2.3 μM formic acid and 1.44 μM triethylamine in water, solvent B was 2.3 μM formic acid and 1.44 μM triethylamine in acetonitrile, and samples were eluted at a rate of 1 mL/min using a linear gradient of mobile phase B from 8% to 100% over 30 min. The amount of pyochelin present in reconstitution reaction mixtures was calculated from the peak's area by comparison to reference curves (area vs nanomoles) prepared using a sample of pyochelin purified from *P. aeruginosa* and its concentration calculated using published extinction coefficients ($\epsilon_{315} = 4200\ M^{-1}\ cm^{-1}$; $\epsilon_{248} = 9000\ M^{-1}\ cm^{-1}$) (*2*).

Production and Detection of [^{3}H]Methyl-Containing Compounds. Unless otherwise specified, enzymatic reaction mixtures for production of [^{3}H]methyl-containing compounds contained 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM CoA, 1 mM salicylate, 5 mM cysteine, 10 μM PchG, 10 μM PchE, 2 μM PchF, 10 μM PchD, 0.5 μM Sfp, 1.0 mM NADPH, and 1.0 mM [*methyl*-^{3}H]SAM (7 Ci/mol). The reaction mixtures were incubated at 25 °C for 30 min to allow phosphopantetheinylation prior to addition of ATP. After addition of ATP, incubation was continued, and samples of 100 μL were taken at specific times, followed by acidification with 10 μL of 1 N HCl. Reactions were extracted with 1 mL of ethyl acetate, and 900 μL of organic fraction was added to 3.5 mL of scintillation fluid. The amount of ^{3}H was quantified by LSC. The identity of the [^{3}H]methyl-containing compound was determined by drying a representative reaction under reduced pressure, solubilized in acetonitrile, resolving on a silica gel TLC with mobile phase choloroform:acetic acid:ethanol (90:5:2.5), and visualized using a Fuji phosphorimager.

RESULTS

Cloning and Sequencing of pch Genes and Variants. Cloning of the genes encoding PchDEFG and the PchFTE variant was described previously. For construction of the PchFMT mutant, a fragment of the 5430 bp gene *pchF* was amplified from the vector pPchF by mutagenic primers and cloned in two steps into a vector containing the remaining, unmutated, portions of *pchF* to produce plasmid pPchFMT. The primers introduced a mutation (Gly1167Arg) in the proposed methyltransferase domain. This conserved glycine was shown to be required for function of other methyltransferase enzymes in vivo and in vitro (*21*−*24*).

Purification of PchD,E,F,G. The heterologous expression and purification from *E. coli* of the four proteins PchD,E,F,G that comprise pyochelin synthetase were as previously described (*9*, *11*), with modification in the PchE expression

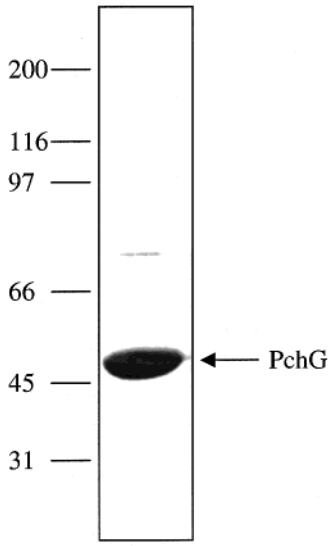


FIGURE 2: Purity of recombinant PchG-H6 overproduced in and purified from *E. coli*. Protein samples were resolved by SDS−PAGE (10%) and stained with Coomassie blue. The positions of molecular mass markers are denoted.

to minimize the coexpression of a difficult to remove 100 kDa contaminating protein. Maintaining cultures in exponential phase resulted in lower levels of the copurifying 100 kDa contaminant. The yield of PchE was 10 mg/L, PchF was 15−18 mg/L, and PchG was 25 mg/L (Figure 2), providing sufficient amounts of the enzymes for the kinetic characterizations reported below. In addition to the wild-type PchF, mutants in the methyltransferase (MT) module, termed PchFMT, and in the thioesterase module (*11*), PchFTE, were purified in comparable amounts.

Characterization of Reconstituted Pyochelin Synthetase Activity from Purified PchD,E,F,G. We have recently noted (*9*) that complete pyochelin synthetase activity can be detected when all four of the purified PchD,E,F,G proteins are incubated for 2 h. The released pyochelin was identified by HPLC and MALDI-TOF MS and compared with authentic pyochelin isolated from cultures of *P. aeruginosa.* In this work we explored the steady-state kinetics of siderophore production in this four-enzyme system. Figure 3a shows a time course for pyochelin production as described in Experimental Procedures with 10 μM PchD, PchE, and PchG and 2 μM PchF. Pyochelin formation, as determined in the HPLC assay, is linear out to 3 h, at which point some 300 catalytic turnovers (based on a limiting amount of PchF) were observed. The linear phase indicated a turnover rate of 1.8 min^{-1} under these incubation conditions.

Pyochelin contains an *N*-methylthiazolidine moiety, likely to arise from cysteine heterocyclization, thiazoline reduction, and then N-methylation with SAM (Scheme 1). Accordingly, catalytic turnover of pyochelin synthetase could be measured using tritium-labeled SAM, with ^3H in the transferring methyl group and the extracted pyochelin detected counted for radioactivity using LSC. This assay gave good sensitivity, with product detection in the first minute under the same assay conditions (Figure 3b). The data shown in Figure 3b give a net k_{cat} for [*methyl*-^3H]pyochelin of 1.6 min^{-1}, in good agreement with the 1.8 min^{-1} value determined by the HPLC assay.

These assays permitted evaluation of the apparent affinity of the various pyochelin synthetase subunits in catalytic turnover. One of the subunits, PchF, was set at a limiting fixed concentration of 2 μM, two other subunits were held at high (10 μM) fixed concentrations, and the fourth was varied to look for saturation behavior. As seen in Figure 4, saturation behavior was observed when PchD, PchE, and PchG were the varied proteins, respectively. For example, PchD shows a K_m of 200 nM and saturates at a velocity of 1.9 min^{-1}. Given that PchD saturates at a level 10-fold lower than PchF (200 vs 2000 nM), these results indicate that PchD acts catalytically and therefore associates and dissociates from PchF. Analogously, the PchE subunit showed a K_m of 200 nM and a k_{cat} of 2.3 min^{-1}, consistent also with its reversible binding to PchF in the turnover conditions. Since PchE and PchF both are NRPS modules, this suggests that any complex is dynamic. Finally, the PchG component also showed saturation, with a K_m of 1 μM and k_{cat} of 2.0 min^{-1}. The common k_{cat} values around the two per minute value are one index that all of the data are consistent.

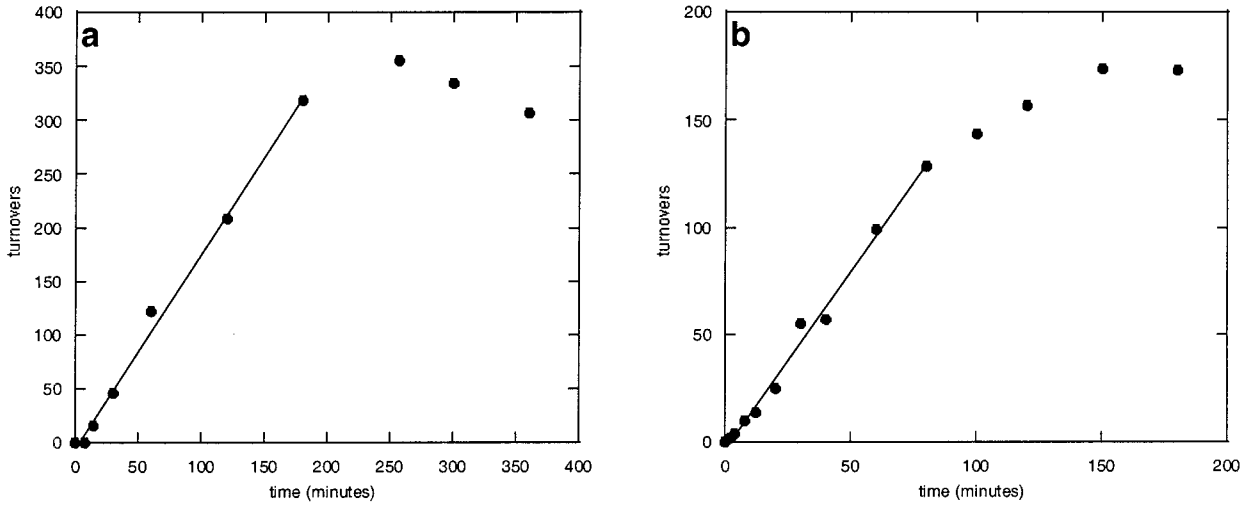


FIGURE 3: (a) Time-dependent accumulation of pyochelin (●) in reconstitution reaction mixtures containing PchD, PchE, PchF, and PchG. Pyochelin was detected by resolving on reverse-phase HPLC and quantification by comparison to a standard curve. (b) Time-dependent accumulation of a ^3H-containing product. Extracted material was resolved on TLC, and radioactive spots were lined up with natural pyochelin. See Experimental Procedures for details.

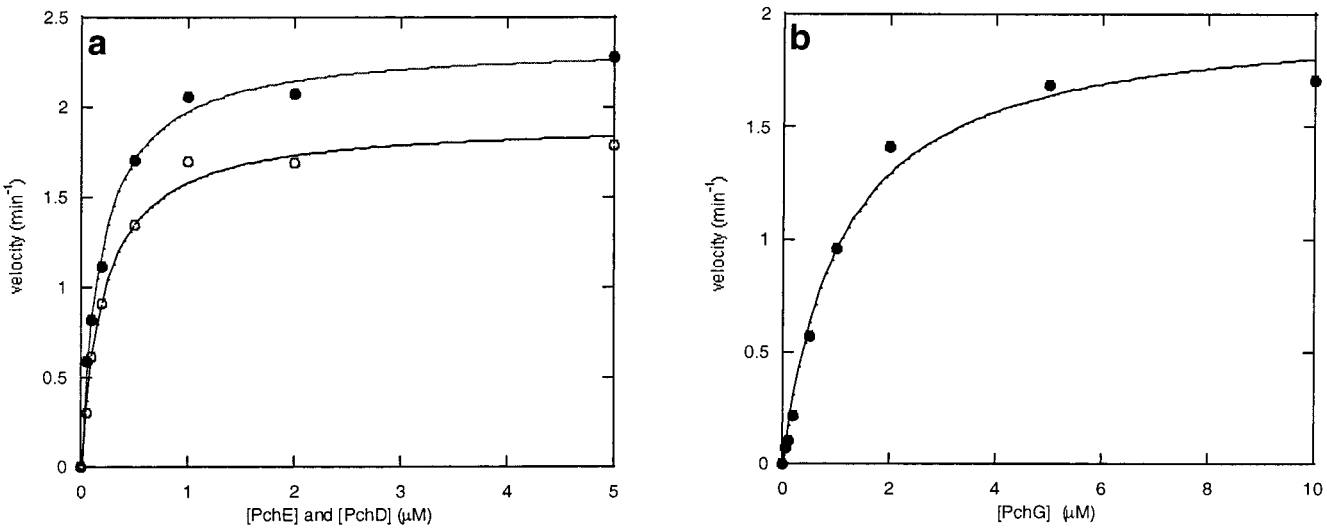


FIGURE 4: (a) Velocity of PchE (●) or PchD (○) dependent production of pyochelin. (b) Velocity of PchG (●) dependent production of pyochelin. Pyochelin was detected by resolving on reverse-phase HPLC and quantification by comparison to a standard curve. See Experimental Procedures for details.

In addition to the four proteins PchD,E,F,G there are four low molecular weight substrates in the pyochelin synthetase assays: salicylate, cysteine, NADPH, and SAM (Figure 1). We have previously determined the K_m of salicylate for PchD and cysteine for the adenylation domains in both PchE and PchF (*11*). When we sought to determine K_m values for NADPH and for SAM, the K_m values were too low to be detected reliably in the HPLC assay where sensitivity was an issue and the small amounts of NADPH and SAM were consumed before product detection could be monitored. We turned to the [^{3}H]SAM assay, but still the K_m values were each below 10 μM.

The TE Domain of PchF Is Required for Catalytic Turnover of Pyochelin Synthetase. In prior experiments performed in the absence of PchG (*11*), no pyochelin was detected. Rather, there was a very slow release ($k < 0.05$ min^{-1}) of an advanced intermediate, the *N*-desmethyl two-electron-oxidized form of the tandem bisheterocycle, hydroxyphenylthiazolinylthiazolinyl-COOH (HPTT-COOH), by slow hydrolysis of the HPTT-S-enzyme intermediate tethered on the PCP domain of the PchF subunit. HPTT-COOH release required a functional TE domain in PchF as determined by a double alanine mutant in the TE domain active site (Figure 5a) (*11*) which abolished HPTT-COOH detection. On addition of PchG the k_{cat} for product turnover was elevated at least 40-fold to 2 min^{-1}, as N-methylation and thiazoline ring reduction occur in the presence of PchD,E,F,G, and are totally dependent on the functional TE domain of PchF as shown in Figure 5b.

The MT Domain of PchF Is a Functional N-Methyl Transferase Acting on the Hydroxyphenylthiazolinylthiazolidinyl-S-PchF Acyl Enzyme Intermediate. Sequence analysis of the 200 kDa PchF subunit had suggested (*8*) that there might be five distinct domains in the NRPS module: cyclization (Cy), adenylation (A), methyltransferase (MT), peptidyl carrier protein (PCP), and thioesterase (TE). The putative MT domain appears to be inserted into the A domain between conserved sequences A8 and A9. To validate that the MT domain was functional, we mutated a conserved glycine, G1167, in the proposed MT core motif (Figure 6a) (*21*−*24*) to arginine (PchF G1167R = PchF-MT mutant),

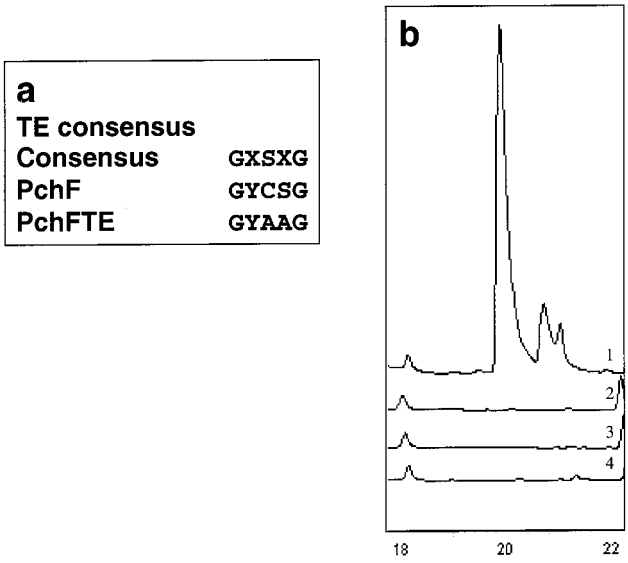


FIGURE 5: (a) Sequence alignment of the conserved TE domain with the homologous region in PchF and PchFTE. The proposed catalytic residues (cysteine or serine) have been mutated to alanine in PchFTE. (b) HPLC traces showing the production of pyochelin by reconstituted PchDEG with PchF or PchFTE. Trace 1 shows the pyochelin produced from reconstitution with 2 μM PchF. Trace 2 shows the extracted products of reconstitution with 2 μM PchFTE. Trace 3 shows the extracted products of reconstitution with 10 μM PchFTE. Trace 4 shows the extracted products of reconstitution with 2 μM PchF without ATP. See Experimental Procedures for details.

purified this form of the PchF enzyme, and tested it in the catalytic turnover assays. As shown in Figure 6b no detectable pyochelin was produced. At 5-fold higher levels of the PchF-MT mutant (10 μM), a peak corresponding to the *N*-desmethylpyochelin was detected on HPLC. SAM was tested at 100−500-fold over its estimated K_m limit with no effect. These results support the view that the ~380 amino acid insert in the PchF adenylation domain is indeed a functional *N*-methyltransferase, acting on the thiazolidinyl ring of the demethylpyochelinyl-S-PchF acyl enzyme.

To address the question of whether the MT domain indeed acted on the penultimate acyl-S-PchF covalent intermediate

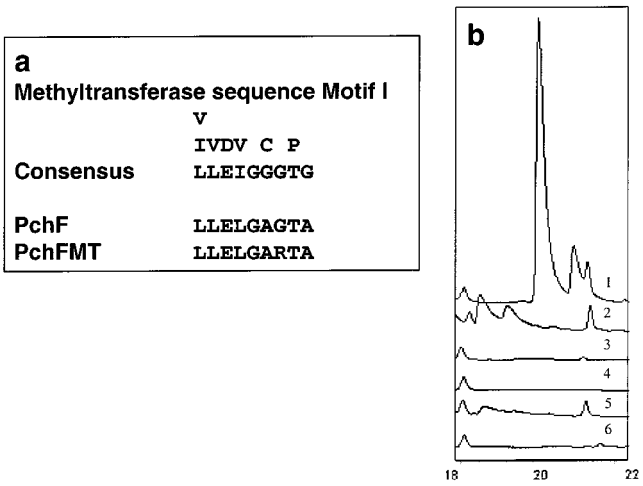


FIGURE 6: (a) Sequence alignment of conserved motif I of methyltransferases with the homologous region in PchF and PchFMT. The required glycine residue has been mutated to arginine in PchFMT. (b) HPLC traces showing the production of pyochelin by reconstituted PchDEG with PchF or PchFMT. Trace 1 shows the pyochelin produced from reconstitution with 2 μM PchF. Trace 2 shows the extracted products of reconstitution with 10 μM PchFMT. Trace 3 shows the extracted products of reconstitution with 2 μM PchFMT. Trace 4 shows the extracted products of reconstitution with 2 μM PchFMT with five times the SAM as used in the reaction shown in trace 3. Trace 5 shows the extracted products of reconstitution with 2 μM PchF without SAM. Trace 6 shows the extracted products of reconstitution with 2 μM PchF without ATP. See Experimental Procedures for details.

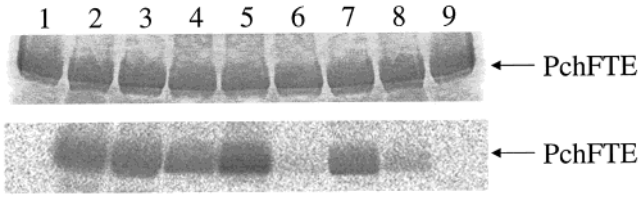


FIGURE 7: Incorporation of SAM-derived ^{14}C into PchFTE. The top panel shows Coomassie blue-stained bands of PchFTE (PchE also visible). The bottom panels show autoradiographs of the corresponding proteins. Lane 1 contained a −ATP control. Lanes 2 and 3 were included as a control for [^{14}C]salicylate incorporation into PchF, reactions without NADPH and SAM and with NADPH and SAM, respectively. Reactions for lanes 4 and 6 contained no NADPH and were allowed to incubate for 20 min and 30 s, respectively. Reactions for lanes 5 and 7 were full reconstitutions with all components and were allowed to incubate for 20 min and 30 s, respectively. Reactions for lanes 8 and 9 were full reconstitutions incubated for 30 min, though salicylate (lane 8) and PchG (lane 9) were not included. See Experimental Procedures for details.

or after TE-mediated release of desmethylpyochelin into solution, experiments were conducted with the PchF-TE domain mutant to block any acyl chain release and with [^{14}C]SAM as methylation cosubstrate in the presence of all soluble substrates and the PchD,E,G protein components. At the end of incubations, proteins were separated by SDS−PAGE and subjected to autoradiography to detect radiolabeled protein. As seen in Figure 7 incorporation of the [^{14}C]-methyl group covalently into protein was ATP-dependent (lane 1) as well as cysteine-dependent (data not shown). Lanes 2 and 3 were included as positive controls of [^{14}C]-salicylate incorporation and transfer (*11*). Lanes 5 and 7 contain reactions where all reaction components were included. These lanes indicate that near saturating levels of [*methyl*-^{14}C]SAM are incorporated within 30 s. However,

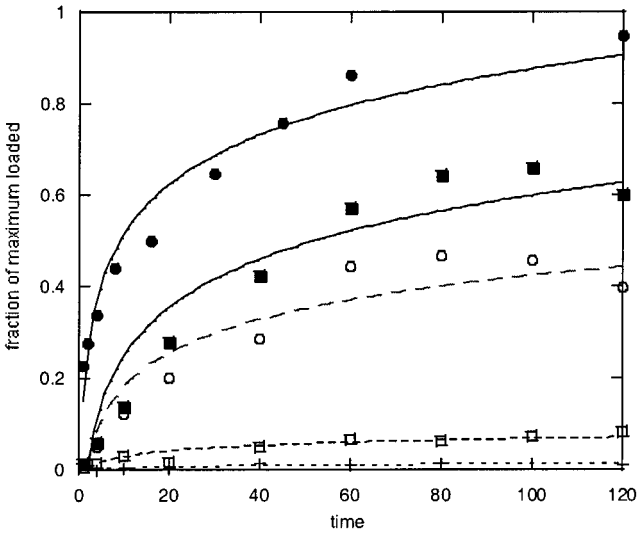


FIGURE 8: Time-dependent accumulation of SAM-derived ^{3}H into TCA insoluble material in reconstitution reaction mixtures containing PchD, PchE, PchF, PchG, salicylate, cysteine, ATP, and [^{3}H]-SAM. Reactions depicted contained all components (●), except NADPH (■), PchG (○), salicylate (□), or PchE (+).

in lanes 4 and 6, which did not include NADPH, we observed methylation in these reactions though it occurred at a slower rate than those depicted in lanes 5 and 7. Low levels of labeling were detected in lanes 8 and 9 (not visible in figure).

To characterize the rate of methylation, time courses were conducted, the reaction components were TCA precipitated, and protein-bound radioactivity was counted. These assays showed that the rate of label incorporation was 4-fold higher for the full reconstitution mixture when compared to reactions without reducing enzyme or NADPH. Also, the nonreduced reactions produced labeled proteins at only one-tenth the rate at early time points (5 min) compared to the level of the full reconstitution (Figure 8). The nature of this PchDEF-, salicylate-, and cysteine-dependent late methylation has not yet been deconvoluted.

DISCUSSION

The reconstitution of pyochelin synthetase activity, with a net turnover of 2 min^{-1}, from the four purified proteins PchD,E,F,G is in accord with prior genetic results that indicate that these four gene products were necessary and sufficient for biosynthesis of the nonribosomal peptide siderophore from salicylate and two molecules of L-cysteine. The four enzymes contain 11 identifiable domains that carry out at least 13 chemical steps in each turnover of the PchE/F NRPS assembly line: each of the three A domains (PchD,E,F) produces an acyl-AMP and then an acyl-S-enzyme on each of the three carrier protein domains. The two Cy domains (PchE,F) make the sal-Cys-S-PchE and HTP-Cys-S-PchF amide linkages, respectively, and then perform cyclodehydrations to make each thiazolinyl ring in the HPTT-S-PchF acyl enzyme. Then, as detailed in this paper there is the imine reduction in the C-terminal thiazoline by PchG, the N-methylation by the MT domain of PchF, and hydrolytic cleavage of the mature pyochelinyl-S-PchF. Notable among the tailoring reactions to the elongating N-capped Cys-Cys dipeptide chain as it is converted into pyochelin are the tandem bisheterocyclizations, regiospecific reduction of one of the thiazolines, and N-methylation.

The genetic clustering of PchG with the PchD,E,F genes was the first indication that it may be involved in the pyochelin pathway, and this has been corroborated by gene knockout (*9*). That PchG is an NADPH-oxidizing heterocyclic imine reductase acting on a thiazolinyl ring as an acyl constituent on the PCP domain of the PchF enzyme required an appropriate assay. The substrate for PchG, the HPTT-S-PchF, can be prepared in situ by action of PchD and PchE to build up the arylbisthiazolinyl acyl chain. The PchG reaction product is still covalently tethered in thioester linkage to the PCP domain of PchF and is most readily detected only after hydrolytic release. In the absence of SAM or when PchF has a nonfunctional MT domain, there is slow hydrolytic release of the *N*-desmethylpyochelin, which has been detected by HPLC and mass spectrometry (*9*). Given the difficulty of the acyl-S-protein substrate access, we have not attempted any probes of selectivity of PchG action yet, but the initial studies validate that PchG acts catalytically, enables multiple turnovers of the assembly line, and shows saturation behavior in pyochelin synthetase assays.

PchG acts only on the second thiazoline ring in the HPTT-S-PchF, and the imine reduction is most probably a hydride addition to the electropositive carbon and then protonation of the developing thiazolidine nitrogen. This conversion of a dihydro heteroaromatic five ring system to a tetrahydro five ring system has analogy to the well-precedented NADPH-dependent dihydro to tetrahydro six member ring reduction by dihydrofolate reductase (*25*). BLAST (*26*) search indicated homology of the N-terminal portion of PchG to biliverdin reductase, also an NADPH-dependent enzyme (*27*). Structural homology search using Swiss-Model (*28*) gave homology of the N-terminal portion of PchG to the C-terminal portion of mouse major urinary protein (MUPI) which was crystallized with 2-(*sec*-butyl)thiazoline (*29*).

Once the thiazoline ring in the maturing siderophore acyl chain has been reduced to the thiazolidine, the amine nitrogen will have a gain in basicity and also become a competent nucleophilic site for N-methylation. The 1809- residue PchF NRPS module is larger than the four domain PchE 1438-residue subunit, and much of that size increase can be attributed to an insert of ~380 some amino acids within the A domain of PchF. On the basis of the structure of the adenylation domain of the initiation module, GrsA, of the NRPS gramicidin synthetase (*30*), the ~380-residue insert would be at a surface loop between conserved motifs A8 and A9 of the adenylation domain and not interrupt the integrity of the cysteine-specific A domain of PchF (Figure 9).

Inspection of the putative MT insert reveals one of three (*23*) core sequence motifs (Figure 6a) that are found in other SAM-dependent methyltransferases. Our previous efforts to produce released pyochelin from HPTT-S-PchF (*11*) on addition of SAM and NADPH, *but in the absence of PchG*, had no effect. Now, once PchG has acted, the addition of SAM leads to an increase of >40-fold in k_{cat} for pyochelin turnover. Thus, the PchD,E,F,G contain a competent *N*-methyltransferase catalytic activity, and it is functional after adjustment of the last ring in the acyl chain to the thiazolidine, a tetrahydro oxidation state compared to the parent thiazoline ring.

To prove that the insert in PchF was indeed a functional N-MT domain, a mutation of the conserved Gly1167 in the core MT motif abolished the methylation and formation of pyochelin, with release of desmethylpyochelin. PchF is, therefore, a five domain NRPS module. It is worth noting that N-MT domains have been well-known in fungal nonribosomal peptide synthetases, such as cyclosporin synthetase (*31*), in those fungal NRPS assembly lines the N-MT domains are inserted into the A domain primary sequences. It does seem likely that the N-MT domain of PchF, embedded within the A domain, will act *in cis* on the desmethylpyochelinyl acyl chain tethered just downstream, via its 20 Å long pantetheinyl arm, on the adjacent PCP domain of PchF. This result derives from incorporation of the radioactive methyl group into the PchF enzyme when the acyl chain is stalled upstream of a mutant TE domain defective in release.

These studies, focusing on the five domain PchF NRPS subunit and the separate PchG as partner protein, have assigned catalytic or carrier functions to all 11 domains in the PchD,E,F,G components of pyochelin synthetase and have elucidated some new features of NRPS assembly line catalysis. In particular, from the point of view of tailoring reactions that modify the elongating peptidyl chain, three occur while the acyl chain is parked on PCP2 of PchF: dehydration of the HPT-Cys chain to the bisheterocyclic HPTT chain, reduction of the second thiazoline ring to the thiazolidine, and N-methylation. Two of these tailoring reactions are effected by PchF domains *in cis* (Cy and MT) and the third by the partner protein PchG acting *in trans*.

Unknown in any NRPS assembly line are what controls the rate of chain transfer from one domain or module to the next one downstream. The PchF module is intriguing because of the three maturation reactions noted above that occur while the HPT-Cys chain is heterocyclized, reduced, and N-methylated before the TE domain will process it for hydrolytic release. Since the mature pyochelinyl chain accumulates on the PCP domain in the inactive TE mutant of PchF, it may be that interdomain migration of the acyl chain, from the S-PCP to O-TE intermediate, is controlled by the reduction and methylation state of the siderophore. Certainly, the turnover number is affected at least 40-fold, and it will be of interest to see if that reflects slow transfer of the mature pyochelinyl chain out to the terminal TE domain and/or rate-limiting hydrolysis once it arrives there.

It is not yet clear why the dihydrothiazoline ring is reduced to the tetrahydrothiazolidine ring during siderophore maturation. This may have consequence for the chelation site or geometry as pyochelin coordinates ferric iron. A precedent for this role comes from the X-ray structure of the structurally related siderophore micacocidin A (Figure 10a) (*32*, *33*) in complex with zinc as a surrogate for iron. The nitrogen of the *N*-methylthiazolidine ring is indeed a direct ligand to the metal. Once the reduction has occurred though, the N-methylation may be crucial for stabilizing the tetrahydro ring against opening to the free thiol and the imine linkage which will be hydrolytically labile and induce fragmentation of the desmethylpyochelinyl chain (*2*, *34*).

Pyochelin (*2*, *35*), a siderophore of *P. aeruginosa*, has clear structural homology to the yersiniabactin (Ybt) siderophore from *Yersinia pestis* (*36*−*38*) and the micacocidin A from *Pseudomonas* sp. no. 57-250, each having the hydroxyphenylthiazolinylthiazolidine three ring moiety (Figure 10b). Ybt then deviates with a short polyketide-type linker before

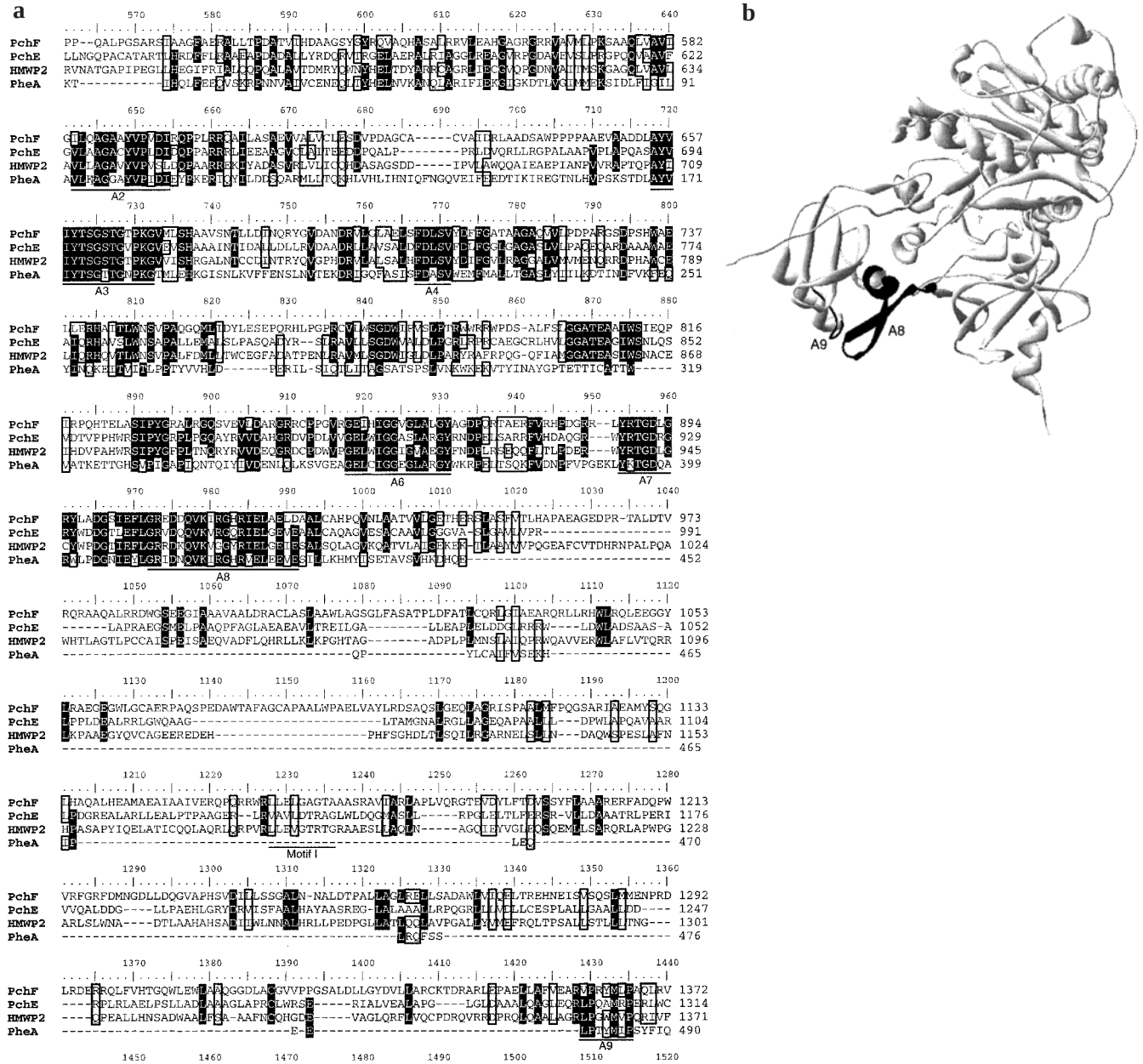


FIGURE 9: (a) Sequence alignment of PchF, PchE, HMWP2, and PheA. Identitical amino acids are highlighted in black whereas similar amino acids are highlighted in gray. The conserved sequences corresponding to adenylation domains and motif I of methyltransferases found in PchF are shown as labeled horizontal bars under the corresponding sequence. (b) Three-dimensional structure of PheA (*30*). The ~380 amino acid MT insert within PchF's A domain would lie within the highlighted (black) residues, corresponding to conserved sequences A8 (PheA residues 411−430) and A9 (PheA residues 479−485).

ending with a *C*-methylthiazolinyl-COOH moiety. The domain and module structure of the Ybt synthetase mirrors the homology, with the six domain high molecular weight protein 2 (HMWP2) subunit organized equivalently to the four domain PchE and the first two domains of PchF before divergence of Ybt synthetase to a PKS module (*20*, *39*−*41*). Ybt has three *C*-methyl groups derived from SAM and no *N*-methyl groups. There are three potential MT domains in the Ybt synthetase assembly line, one embedded in the HMWP2 A domain in the analogous position to the MT domain found in PchF and then two MT domains in the high molecular weight protein 1 (HMWP1) subunit. The MT-type insertion in the A domain of the Ybt HMWP2 subunit does not appear to be functional, by product analysis with SAM (Miller and Walsh, unpublished results) and by sequence comparison (Figure 9), where the conserved glycine in the MT core motif I is an arginine (similar to the Gly1167Arg mutation we employed to inactivate the PchF MT domain). Likewise, an analogously placed insert into the A domain of PchE (Figure 9) has the same G to R mutation. These may be vestiges of previously active MT domains, which may exist and still be active in the micacocidin synthetase subunits. Although the biosynthetic genes for micacocidin A have not yet been discovered, it is an even better hybrid of the design elements in the other two siderophores (Figure 10b) and has both the thiazolidine N-methylation of pyoch-

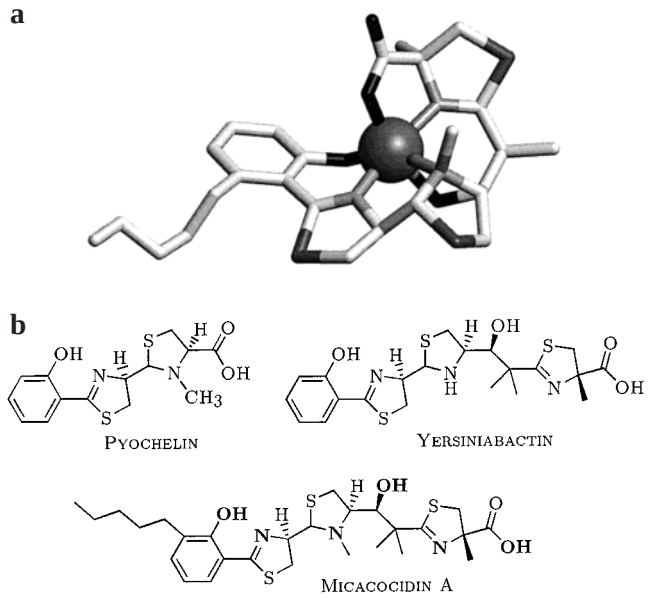


FIGURE 10: (a) Three-dimensional structure of micacocidin A (*33*). Elements depicted are as follows: carbon (white), oxygen (black), nitrogen (light gray), sulfur (dark gray), and zinc (large ball). (b) Three-dimensional structures of pyochelin, yersiniabactin, and micacocidin A. The boldface oxygens and nitrogens in the drawing of micacocidin A indicate chelating atoms seen in (a).

elin and the three C-methylations of Ybt. How specificity for C-methylation and against thiazolidine N-methylation is achieved by these MT domains is yet to be studied. The thiazoline to thiazolidine reductase in the Ybt synthetase assembly line is probably provided *in trans* by YbtU, shown genetically to be essential (*42*), and homologous to PchG. Whether domains can be mixed and matched between the Pch and Ybt and micacocidinA assembly lines or more generally in thiazoline- and thiazole-producing NRPS modules, e.g., for epothilone (*43*, *44*), myxothiazole (*45*), or bleomycin (*46*), will be of interest for combinatorial biosynthesis strategies.

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