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Protein Assembly Line Components in Prodigiosin Biosynthesis: Characterization of PigA,G,H,I,J

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The tripyrrolic products of the prodiginine class of natural products are signature antibiotics of *Serratia* and *Streptomyces* bacterial strains and have a plethora of activities in multicellular eukaryotes.[1a−d] Prodigiosin, the red pigment produced by *Serratia marcescens*, has a prototypic scaffold with the A and B pyrrole rings connected directly as a bipyrrole unit, while the B and C rings have the methylene bridging connectivity typically found in heme-type pigments of life. Prodiginine biosynthetic gene clusters from *Streptomyces coelicolor*[2] and from *Serratia marcescens*[3] have been sequenced and bioinformatics-based pathways of tripyrrole assembly proposed. Each of the three pyrrole rings is proposed to arise from a different amino acid, ring A from L-proline, ring B in part from L-serine, and ring C in part from glycine.[4]

In this study, we have overproduced five of the *Serratia* sp. ATCC 39006 enzymes in *E. coli*, purified them to heterogeneity, and assigned carrier protein or catalytic function (Figure S1). These five proteins, PigA,G,H,I, and J, comprise the assembly line portion of the pathway and include a nonribosomal peptide synthetase (NRPS)−polyketide synthase (PKS) interface that the growing acyl chain must traverse.

The first three steps in the assembly of the tripyrrole scaffold involve shunting a fraction of the L-proline pool in the *Serratia* sp. cell to the covalently tethered pyrrolyl-2-carboxyl moiety in the thioester linkage to the phosphopantetheinyl arm of a peptidyl carrier protein (PCP) (PigG) (Scheme 1) similar to the biosynthesis of the pyrrole in undecylprodigiosin, pyoluteorin, clorobiocin, and coumermycin A_1.[5a−c] Indeed, we could show that the 54 kDa adenylation (A) domain PigI converts L-proline to L-prolyl-AMP and then tethers it to the phosphopantetheinylated form of PigG, a free-standing 10.4 kDa PCP. The third enzyme, the 42 kDa flavoprotein desaturase PigA, then effects the four-electron, two-step tandem desaturation of the L-prolyl-*S*-PigG to the pyrrolyl-2-carboxyl-*S*-PigG as shown by nanospray-Fourier Transform Mass Spectrometry (nFTMS) and radio-HPLC (Scheme 1, Figure S2) that serves as a precursor to pyrrole ring A of the tripyrrolic scaffold. Alternatively, pyrrolyl-*S*-PigG was generated via the incubation of synthetic pyrrolyl-2-carboxyl-CoA[6] with PigG and Sfp, a promiscuous phosphopantetheinyl transferase from *Bacillus subtilis*.[7]

The heterocycle on this acyl-*S*-carrier protein is the scaffold for the subsequent construction of the connected pyrrole ring B. Inspection of the *pig* gene cluster[4] suggested three candidate orfs, PigH,J,L, as catalysts for subsequent elaboration of the growing chain. PigJ is predicted to be a polyketide synthase (PKS) subunit containing an active ketosynthase (KS) domain and a chain length factor (CLF) partner domain that potentially decarboxylates the malonyl of PigH or is catalytically silent. PigH is predicted to have three domains, an unusual tandem pair of ACP domains (ACP_1 and ACP_2) and a pyridoxal phosphate (PLP)-containing domain, designated SerT as a putative seryltransferase.

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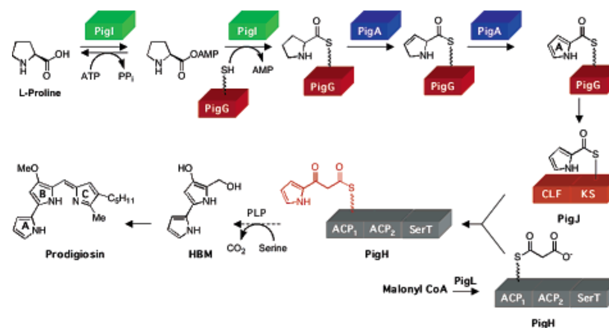
Scheme 1. Dipyrrole Formation during Prodigiosin Biosynthesis



Overproduction and purification of the three-domain PigH (ACP_1-ACP_2SerT) from *E. coli* led to evaluation of its capacity to undergo post-translational modification at each of the three domains. The conversion of each of the ACP domains from the inactive apo form to the active holo and malonyl-*S*-PigH forms by action of the phosphopantetheinyl transferase Sfp could be assessed both via incorporation of a radiolabel from [^{14}C]-acetyl-CoA and [^{14}C]-malonyl-CoA and/or by the gain of 340 and 426 mass unit increases observed by nFTMS, for the holo and malonylated forms of the ACP_1 domain of the peptide. Following a trypsin digestion of PigH, ACP_1 could be readily identified by nFTMS, but ACP_2 could not. The active-site Ser to Ala mutants in each of the ACP_1 and ACP_2 domains of PigH were generated to resolve any ambiguity that both domains could be activated by Sfp.

The third domain of PigH is predicted to bind PLP as cofactor for generating a C_2 fragment from L-serine for pyrrole ring B formation.[4] PigH as isolated from heterologous *E. coli* overexpression is faintly yellow due to substoichiometric PLP loading. Additional binding of PLP to the SerT domain of PigH could be shown by addition of a solution of pyridoxal phosphate to PigH. The absorption maximum of 388 nm, characteristic of the free aldehyde form of PLP, was shifted to 414 nm, typical of an aldimine linkage of PLP to an active-site lysine[8] (Figure S3) in a 1:1 ratio. Thus, all three domains of PigH undergo post-translational modifications[9] in preparation for their assembly-line functions.

At this juncture, incubation of the three proteins pyrrolyl-*S*-PigG, PigJ, malonyl-*S*-PigH led to PigH-mediated decarboxylation of C_3 of the malonyl group, a typical reaction for a KS−CLF protein with elongation function in type II PKS assembly lines.[10a,b] The decarboxylation catalyzed by PigJ generates a C_2 carbanion of acetyl-*S*-PigH as carbon nucleophile and is required for pyrrolyl group transfer. As shown in Scheme 1, the heterocyclic pyrrole group moves from the PCP domain of PigG to the active-site Cys

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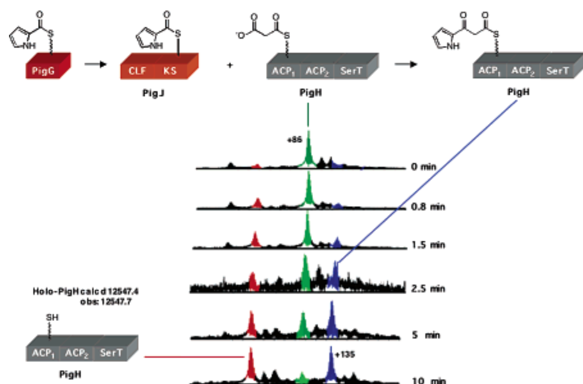

Figure 1. Identification of malonyl-*S*-PigH formation and pyrrolyl transfer from PigJ by ESI−FTMS (charges of the ions shown are 7+).

residue of the KS domain of PigJ, and then gets captured by the C_2 carbanion, creating the pyrrolyl-β-ketoacyl-*S*-PigH. This chain elongation by two-carbon extension to a β-ketoacyl-*S*-PigH product is a typical PKS-type of Claisen condensation, although the pyrrolyl-2-carbonyl starter unit is most unusual. We could observe this key carrier protein-bound pyrrolyl-β-ketoacyl-*S*-PigH intermediate on ACP_1 grow over time by high-resolution mass spectrometry (Figure 1, increase of 49 Da compared to the malonyl-*S*-ACP_1).

In the absence of PigJ, little to no pyrrolyl-β-ketoacyl-*S*-ACP_1 is formed (Figures S6 and S7). This is in agreement with the KS domain of PigJ, generating a transient pyrrolyl-*S*-cysteinyl PigJ intermediate that serves as the acyl donor to malonyl-*S*-PigH. The PigG/PigJ/PigH interaction constitutes an interface between the NRPS portion and the start of the PKS portion of the Pig assembly line.

Because we failed to observe the second ACP of PigH, a PigH construct lacking the SerT (PigHACP_1ACP_2) was used to decipher if the di-ACP would generate the dipyrrolyl-β-ketoacyl-*S*-PigHACP_1-ACP_2. By nFTMS, we show, using the PigHACP_1ACP_2 construct, that both ACPs are loaded with a pyrrolyl-β-ketoacyl as both a 49 and a 98 Da increase was observed compared to the dimalonyl-*S*-PigHACP_1ACP_2 (Figure S8). To verify that both active sites were capable of forming the pyrrolyl-β-ketoacyl functionality or if the dipyrrolyl-β-ketoacyl-*S*-PigHACP_1ACP_2 was a result of transthiolation (e.g., transfer from ACP_1 to ACP_2), an HPLC assay that detected the hydrolyzed pyrrolyl-β-ketoacyl group was developed so that both PigH mutants could be analyzed. With both PigH S45A and S139A, pyrrolyl-β-ketoacyl was observed (Figure S4, right panel), suggesting there is not a different role for each of the ACP domains. This finding is consistent with other tandem carrier domains containing proteins, such as PksL[11] and Mup,[12] that do not have any apparent different functions but may be responsible for increased metabolite production.[12]

As observed by reversed-phase HPLC and mass spectrometry (Figures S4, left panel, S6, and S7), the pyrrolyl-β-ketoacyl moiety is bound to PigH unless L-serine is added in the presence of PLP, which leads to a discharge of the covalent acyl chain. It remains to be seen if this Ser-mediated release is concomitant with the proposed Ser decarboxylation by the PLP cofactor in the SerT domain active site and intramolecular cyclization and release of the anticipated bipyrrolic (ring A−ring B) alcohol HBM. HBM could not be detected by LC-MS and HPLC. Enzymatic oxidation of the primary alcohol in HBM would create the aldehyde and provide the one-carbon bridge to the pyrrole ring C fashioned separately from long chain fatty acid and glycine by an analogous PLP-mediated decarboxylative condensation.[4]

At this point, functions for the first five proteins of the *Serratia* prodigiosin biosynthetic pathway are established. Detection of four covalently tethered acyl thioester protein intermediates that undergo redox tailoring and chain extension have been measured and identified in single turnover fashion. The nFTMS analysis, while the growing acyl chains are still attached to proteins via thermodynamically activated thioester linkages, provides insight into the logic and machinery of an assembly line to build a 2,2-bipyrrole unit.

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Supporting Information Available: Experimental details for protein preparation, activity assays by UV, HPLC, and FTMS studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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