Supporting Information

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

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Experimental:

Preparation of pPigI-pET28a, pPigG-pET22b, pPigA-pET22b pPigJ-pET22b, pPigH-pET22b, pPigHACP$_1$ACP$_2$-pET28a, and pPigHACP$_2$SerT-pET28a overexpression constructs. The genes encoding PigI, PigG, PigA, PigJ and PigH were PCR amplified genomic DNA isolated from *Serratia* sp. ATCC 39006 using Qiagen DNeasy® Tissue kit. The primers used for the amplification of each gene are listed in Table 1. PCRs were carried out using *Pfu* Turbo DNA polymerase as described by Stratagene. The amplified genes were inserted into the linearized pET28a (*pigI*, *pPigHACP$_1$ACP$_2$*, and *pPigHACP$_2$SerT*) and pET22b (*pigG*, *pigA*, *pigJ*, and *pigH*) vectors via the corresponding *Nde*I/*Xho*I restriction sites. Expression of pPigI-pET28a, pPigG-pET22b, pPigA-pET22b pPigJ-pET22b, pPigH-pET22b, pPigHACP$_1$ACP$_2$-pET28a, and pPigHACP$_2$SerT-pET28a was done following transformation into *E. coli* TOP10 competent cells. All expression clones were characterized by DNA sequencing (Dana Farber Cancer Institute) and compared to their corresponding gene sequence from *Serratia* sp. (GenBank entrie AJ833001).

Table 1. Primers used for the PCR amplification of the prodigiosin and prodigiosin mutant genes

Gene	5' Primer	3' Primer
pigI	GCTTAACATATGACAATATCCACTCCCGTG	GTTTATCTCGAGTCATACCGCAACCACCCCGG
pigG	GAGTTCCATATGTTAGAAAGTAAATTG	TTACATCTCGAGTGCCGCACCTCCCTG
pigA	GAGTGTCATATGGATTTTAACCTGTCAAATAG	GTTGAACTCGAGTCATGATGATGTTCCCCTT
pigJ	GTCATACATATGAGTAATGATAAACACATCG	CAGTGCCTCGAGAGACGATTGTCTGATGACAAGG
pigH	GTCATACATATGAACGATGTAACGACCGAG	CAGTGCCTCGAGAGCCTCCGCGACACTCGCG
pigHS45A mutant	GTGGAT**GCG**ATCGCGTTGGCAGAGATT	CGCGAT**CGC**ATCCACCCCCAGATCATTTTC
pigHS139A mutant	ATCGAC**GCG**GTGTCGGTGGCGTCCGCTCAG	CGACAC**CGC**GTCGATCCCCAGATCACTTTC
pigHACP$_1$ACP$_2$	GTCATACATATGAACGATGTAACGACCGAG	GCCCTCCTCGAGTTAGTTCAAACCAATCCTGACCTTT
pigHACP$_2$SerT	ATTGCTCATATGGGGGACGCTGACCTTTGGCTTGG	CAGTGCCTCGAGAGCCTCCGCGACACTCGCG

The introduced restriction sites are underlined for each primer. All the 5' primers introduced an *Nde*I restriction site. All the 3' primers included an *Xho*I restriction site. The Serine to Alanine mutation point is in bold.

Preparation of pPigHS45A-pET22b and pPigHS139A-pET22b mutants overexpression constructs. Genomic DNA isolated from *Serratia* sp. ATCC 39006 was used for PCR amplification for the construction of the PigH mutants. The primers used for the amplification of each gene are listed in Table 1. PCRs were carried out using *Pfu* Turbo DNA polymerase as described by Stratagene. Mutants were constructed using the SOE method.[1] In the first round of PCR, the sequence downstream and upstream of the mutation were separately amplified using genomic DNA as a template in conjunction with the 5'primer for the *mutant* with the 3' primer for *pigH* and the 5' primer for *pigH* with the 3' primer for the *mutant*, respectively (Table 1). The resulting amplified PCR fragments were gel-purified and subjected to a second round of PCR using the forward and reverse primers for *pigH* (Table 1). The newly amplified fragments were then digested with *Nde*I and *Xho*I and subcloned into the linearized pET22b vector via the

corresponding *Nde*I/*Xho*I restriction sites. Expression of pPigHS45A-pET22b and pPigHS139A-pET22b was done following transformation into *E. coli* TOP10 competent cells. All expression clones were characterized by DNA sequencing (Dana Farber Cancer Institute).

Overproduction and purification of PigI, PigG, PigA, PigJ, PigH, PigHACP$_1$ACP$_2$, PigHACP$_2$SerT, PigHS45A mutant, and PigHS139A mutant. Purified pPigI-pET28a, pPigG-pET22b, pPigA-pET22b pPigJ-pET22b, pPigH-pET22b, pPigHACP$_1$ACP$_2$-pET28a, pPigHACP$_2$SerT-pET28a, pPigHS45A-pET22b, and pPigHS139A-pET22b plasmids were transformed into *E. coli* BL21(DE3) competent cells for production and purification. Transformants harboring the pPigI-pET28a, pPigHACP$_1$ACP$_2$-pET28a, and pPigHACP$_2$SerT-pET28a constructs were grown in Luria-Bertani (LB) medium (6 X 1L batches) supplemented with kanamycin (50 μgmL^{-1}), whereas LB broth supplemented with ampicillin (100 μgmL^{-1}) was used for the growth of transformants harboring the pPigG-pET22b, pPigA-pET22b pPigJ-pET22b, and pPigH-pET22b constructs. All cells were grown at 25 °C to an OD_{600} of around 0.5. They were subsequently induced by addition of IPTG (final concentration, 100 μM), and shaken for an additional 14 h at 25 °C. Cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C, Sorvall RC5B centrifuge, SLA-3000 rotor) and resuspended in buffer A [25 mM Tris-HCl (pH 8.0), 400 mM NaCl, and 10% (v/v) glycerol]. Resuspended cells were lysed (2 passes at 10000-15000 psi, Avestin EmulsiFlex®-C5 high-pressure homogenizer), and the cell debris was removed by centrifugation (35000 rpm, 30 min, 4 °C, Beckman L7 Ultracentrifuge, 70Ti rotor). Imidazole (final concentration, 2 mM) was added to the supernatant, which was then incubated with 2 mL of Ni-NTA Agarose resin (Qiagen) at 4 °C for 2 h with gentle rocking. The resin was loaded onto a column and washed with 10 mL of buffer A containing 2 mM imidazole and with 10 mL of buffer A containing 5 mM imidazole. The desired proteins were eluted from the column in a stepwise imidazole gradient (5 mL fractions of 20, 40, 60, 200, and 500 mM imidazole). Fractions containing the pure target proteins (as determined by SDS-PAGE) (Figure S1) were combined and dialyzed overnight at 4 °C against 1 L of buffer B [50 mM Tris-HCl (or HEPES for all PigH constructs) (pH 8.0 adjusted at rt), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol]. A second dialysis was carried out for 4 h at 4 °C in 1 L of buffer C [50 mM Tris-HCl (or HEPES for all PigH constructs) (pH 8.0 adjusted at rt), 100 mM NaCl, 1 mM DTT and 10% (v/v) glycerol]. Proteins were concentrated using either Amicon Ultra PL-10 or Amicon Ultra PL-5 for either PigI, PigA, PigJ, PigH, and PigH constructs and mutants or PigG, respectively. Proteins were flash-frozen in liquid nitrogen and stored at –80 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad). PigA was obtained in its pure form as a yellow protein. The yellow color as determined by HPLC is due to FAD binding.

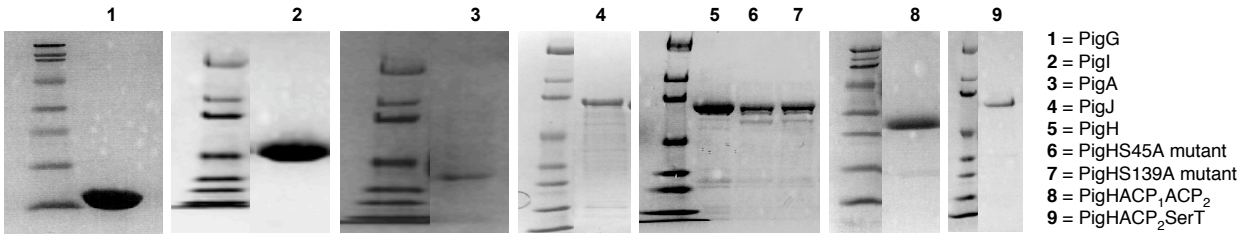

Figure S1. 4-15% Tris(hydroxymethyl)aminomethane (Tris)/HCl SDS-PAGE of purified PigI [lane 2], PigA [lane 3], PigJ [lane 4], PigH [lane 5], PigHS45A mutant [lane 6], PigHS139A mutant [lane 7], and PigHACP$_2$SerT [lane 9] detected by coomassie blue staining. 15% Tris(hydroxymethyl)aminomethane (Tris)/HCl SDS-PAGE of purified PigG [lane 1] and PigHACP$_1$ACP$_2$ [lane 8] detected by coomassie blue staining.

ATP-[^{32}P]PP_i exchange assays for L-prolyl-AMP formation by PigI. To determine PigI activity, ATP-[^{32}P]PP_i reactions (100 μL) containing Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (5 mM), ATP (5 mM), L-proline (5 mM), and 1 mM [^{32}P]PP_i (55 Ci/mM) were performed at 25 °C. The reactions were started by addition of PigI at a final concentration of 1 μM. Reactions were incubated for 2 h before quenching with charcoal suspensions (500 μL) [1.6% (w/v) activated charcoal, 4.5% (w/v) tetrasodium pyrophosphate, and 3.5% (v/v) perchloric acid in H_2O]. Centrifugation was applied to provide the charcoal pellet. The pellet was then washed twice with the wash solution (500 μL) [4.5% (w/v) tetrasodium pyrophosphate and 3.5% (v/v) perchloric acid in H_2O], resuspended in H_2O (500 μL), and submitted for liquid scintillation counting (Beckman LS6500, Beckman Coulter, Fullerton, CA). Using this assay, it was determined that pyrrolyl-2-carboxylic acid is not activated by PigI.

HPLC analysis of purified apo, holo, and L-prolyl-PigG and pyrrolyl-*S*-PigG. Apo-, holo-, and acylated-PigG were separated by HPLC (Beckman System Gold) with a Vydac protein and peptide C18 column (250 mm x 4.6 mm) at a flow rate of 1 $mLmin^{-1}$ (λ = 220 nm). The HPLC solvents were A: H_2O (0.1% TFA) and B: acetonitrile. The elution gradient was 20-60%B over 20 min followed by 60-100%B over 10 min. The conversion from apo to holo- and pyrrolyl-*S*-PigG was achieved using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), ATP (5 mM), PigG (15 μM), Sfp (1 μM), and CoA or pyrrolyl-CoA (100 μM). The formation of the L-prolyl-PCP was performed using Tris-HCl (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (5 mM), ATP (5 mM), holo-PigG (8 μM), PigI (1 μM), and L-proline (0.25 mM).

Pyrrolyl-*S*-PigG formation by action of PigI and PigA. PigG (45 μM) was pantetheinylated in HEPES buffer (pH 8.0) (75 mM) using Sfp (1.5 μM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (5 mM), and CoA (200 μM). After overnight incubation at rt, ATP (5 mM), L-proline (5 mM), and PigI (8 μM) were added. After 6 h, FAD (150 μM) and PigA (10 μM) were added and the reaction was left at rt for an additional 12 h before quenching with 10% TCA (800 μL). The protein was pelleted by centrifugation (13000 rpm, rt, 6 min) and washed with 10% TCA. The pyrrolyl moiety was cleaved by KOH (0.1 M, 100 μL). The reaction mixture was acidified with 50% TFA (10 μL) before analysis by radiolabeled reversed-phase HPLC with a Vydac C18 small pore column (250 mm x 4.6 mm) at a flow rate of 1 $mLmin^{-1}$ (Figure S2). The HPLC solvents were A: H_2O (0.1% TFA) and B: MeCN. The elution gradient was a 0-80%B over 40 min. Elution of the products resulting from KOH hydrolysis was monitored at 270 nm. Control experiments omitting PigG in the reaction mixture showed that L-proline can't be converted to pyrrolyl-2-carboxylic acid by action of PigI and PigA. This confirmed that L-proline has to be covalently attached to PigG for its conversion to pyrrolyl. Attempts to form pyrrolyl-*S*-PigG using the dehydrogenase CloN3 from the clorobiocin system (shown to perform oxidation on a prolyl moiety attach to the PCP of pyoluteorin, clorobiocin, and coumermycin A_1) were unsuccessful indicating that mixing and matching of domain does not work in the prodigiosin system (Figure S2). By ESI-FTMS we were also able to confirm that PigA is responsible for the conversion of L-prolyl-*S*-PigG to pyrrolyl-*S*-PigG. A mass of 11028.62 Da corresponding to pyrrolyl-*S*-PigG, which is ~433 Da larger than 10595.56, the mass of apo-PigG, was observed when PigA was present in the reaction mixture and was not when PigA was omitted. In addition, we observed by ESI-FTMS that in the absence of PigA in the initial reaction, only malonyl-*S*-$PigHACP_1$ is detected indicating that without PigA the L-prolyl-*S*-PigG is not converted to pyrrolyl-*S*-PigG and can therefore not be transferred to PigH.

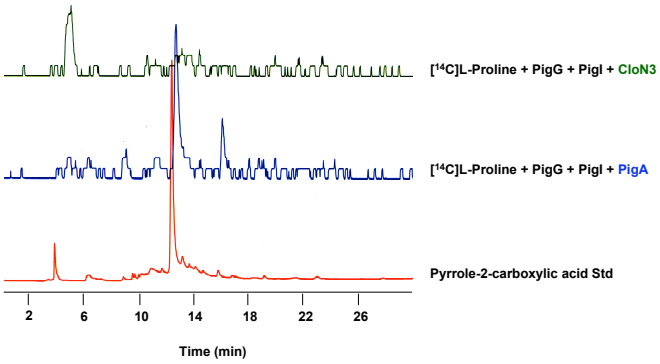

Figure S2. Reversed-phase HPLC traces for the formation of pyrrolyl-*S*-PigG. The red UV trace is the standard pyrrolyl-2-carboxylic acid. The blue trace shows the co-elution of pyrrolyl-2-carboxylic acid formed by action of PigA on L-prolyl-*S*-PigG. The green trace indicates the absence of oxidation of pyrrolyl-*S*-PigG in the presence of the dehydrogenase CloN3.

Determination of PigH activity. PigH contains a SerT domain that is proposed to be PLP dependent. Upon purification PigH is pale yellow, which indicates the presence of PLP bound to the enzyme. By titration of PLP into PigH by increments of 0.22, 0.45, 0.67, 0.9 and 1 eq of PLP we could determine that PLP binds to PigH in a 1:1 ratio (Figure S3). The formation of malonyl-*S*-PigH was determined by trichloroacetic acid (TCA) precipitation assays using [^{14}C]-malonyl-CoA in the presence of Sfp. The reaction mixture (100 µL) contained HEPES (pH 8.0) (100 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), [^{14}C]-malonyl-CoA (50 µM), PigH (15 µM), and Sfp (1 µM). Samples of 25 µL were removed at 10 and 60 min and added to 10% TCA (100 µL). Protein was pelleted by centrifugation, washed with 10% TCA (100 µL), washed twice with H_2O (1 mL), and resuspended in 88% formic acid. The radiolabeled product was counted by liquid scintillation counting.

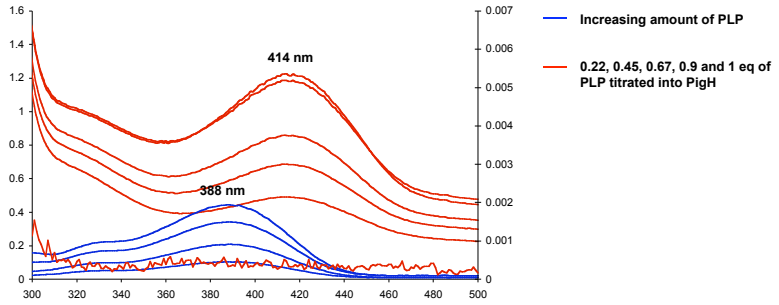

Figure S3. UV traces of PLP titration into PigH. The absorption maximum of 388 nM is characteristic of the free aldehyde form of PLP. The absorption maximum of 414 nM is typical of an aldimine linkage of PLP to an active site lysine.

Reconstitution of pyrrolyl-β-ketoacyl-*S*-PigH by reversed-phase HPLC. To confirm the pyrrolyl-β-ketoacyl-*S*-PigH formation, HPLC assays of base hydrolyzed enzyme-bound intermediates were utilized (Figure S4, left panel). Formation of pyrrolyl-*S*-PigG and malonyl-*S*-PigH were first achieved separately in 1 h. The reaction mixture (30 µL) for the pyrrolyl-*S*-PigG formation contained HEPES (pH 7.5) (75 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM),

pyrrolyl-2-carboxyl-CoA (50 μM), PigG (15 μM), and Sfp (5 μM). The reaction mixture (30 μL) for the malonyl-*S*-PigH formation contained HEPES (pH 8.0) (100 mM), $MgCl_2$ (10 mM), TCEP (pH 7.0) (1 mM), malonyl-CoA (200 μM), PigH (50 μM), and Sfp (5 μM). After 1 h (for each of the separate formation of loaded PigG and PigH), the 2 reaction mixtures were combined and 1 eq. of PigJ was added. After 15 min the reaction mixture was either quenched with cold MeOH (200 μL) or L-serine (1 mM) and PLP (0.5 μM) were added and reacted for an extra 15 min (to release the pyrrolyl-β-ketoacyl moiety potentially as the dipyrrole HBM) prior to quenching with cold MeOH (200 μL). The quenched reactions were kept a –20 °C for at least 30 min before centrifugation (13000 rpm, 10 min, 4 °C) to precipitate the proteins. The precipitated protein pellets were treated with 0.1 M KOH (100 μL) for 10 min at 65 °C and acidified with 50% TFA (10 μL). After centrifugation (13000 rpm, 10 min, 4 °C) the supernatant was analyzed by reversed-phase HPLC (Beckman System Gold) using a Vydac small pore C18 column (250 mm x 4.6 mm) at a flow rate of 1.0 $mLmin^{-1}$. The HPLC solvents were A: H_2O (0.1% TFA) and B: MeCN. The elution gradient was a 0-100%B over 30 min. Product elution was monitored at 270 nm.

To verify on which ACP of PigH the transfer of the pyrrolyl moiety takes place, the mutants PigHS45A and PigHS139A were utilized in HPLC assays using the reaction conditions described above (Figure S4, right panel). The finding that the pyrrolyl-β-ketoacyl moiety is formed on both mutants indicates that both ACP_1 and ACP_2 can achieve the same function. We did not observe different functions eventhough we looked for it. The presence of pyrrolyl-2-carboxylic acid in the two top traces of Figure S4 right panel is believed to be the result of a side reaction that is PigJ dependent given that the pyrrolyl-*S*-ACP only appears at a later time point in the ACP_1 construct. Eventhough the rate for the reaction on ACP_1 (Figures 1 and S8) appears to be slower than for the reaction on ACP_2, careful kinetic experiments (many factors to be considered: amount of CoA in the commercial samples of CoA that vary over time due to hydrolysis, and amount of PigJ added to the reaction that influences the rate of the reaction) beyond the scope of this communication would be require to confirm this observation.

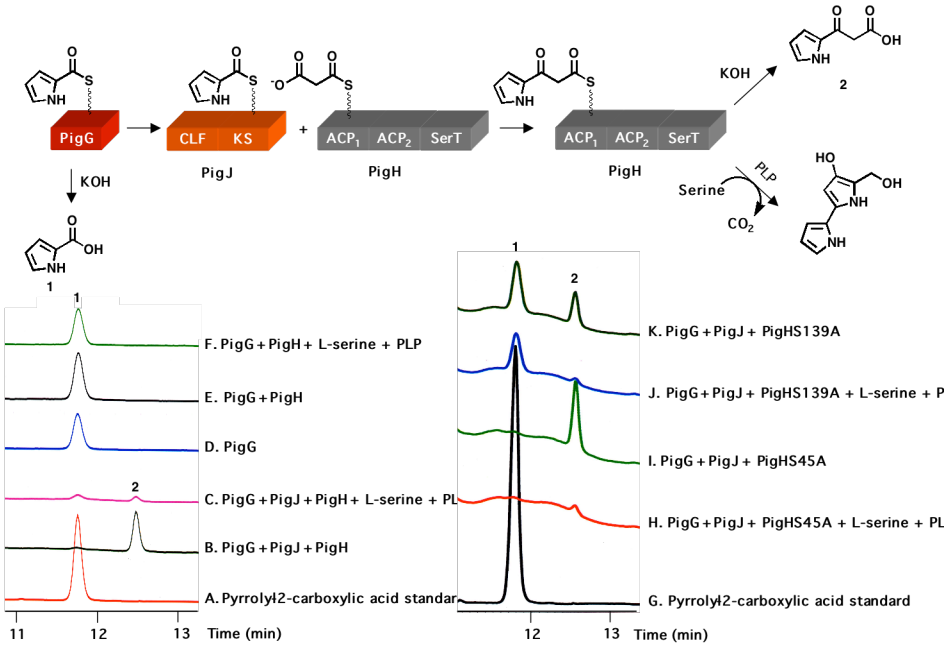

Figure S4. *Left panel:* HPLC traces for the reconstitution of pyrrolyl-β-ketoacyl-*S*-PigH using WT PigH. Traces: (A) Standard of pyrrolyl-2-carboxylic acid; (B) Pyrrolyl-β-ketoacid from KOH hydrolysis of PigG,J,H reaction; (C) Addition of L-serine and PLP to PigG,J,H reaction releases the acyl moiety from PigH resulting in disappearance of pyrrolyl-β-ketoacid; (D) Pyrrolyl-2-carboxylic acid from KOH hydrolysis of PigG reaction; (E and F) Pyrrolyl-2-carboxylic acid from KOH hydrolysis of reaction mixtures where PigJ was omitted. *Right panel:* HPLC traces for the reconstitution of pyrrolyl-β-ketoacyl-*S*-PigH using PigH mutants (S45A and S139A). Traces: (G) Standard of pyrrolyl-2-carboxylic acid; (H and J) Addition of L-serine and PLP to PigG,J,HS45A and PigG,J,HS139A reactions releases the acyl moiety from PigH resulting in disappearance of pyrrolyl-β-ketoacid; (I and K) Pyrrolyl-β-ketoacid from KOH hydrolysis of PigG,J,H mutants reactions.

Reconstitution for pyrrolyl-β-ketoacyl-*S*-PigH(ACP_1) active site for detection by mass spectrometry. A typical reconstitution assay was performed as follows. 200 µL PigG (~300 µM), 2 µL $MgCl_2$ (800 mM), 30 µL CoA (3.2 mM), 4 µL Sfp (1.2 $mgmL^{-1}$) was incubated for 60 min. After these 60 min, 10 µL ATP (100 mM), 5 µL L-proline (500 mM), and 5 µL of PigI (2 $mgmL^{-1}$) were added and the mixture was incubated for an additional 20 min. This was followed by the addition of 12 µL of PigA (3 $mgmL^{-1}$), 4 µL FAD (6 mM) and incubated for a total of 120 additional min to generate reaction mixture A. At 90 min of incubation into the formation of reaction mixture A the following 300 µL PigH (68 µM), 6 µL Sfp (1.2 $mgmL^{-1}$), 12 µL malonyl-CoA (14 mM), 3 µL $MgCl_2$ (800 mM) were added and incubated for 30 min to generate reaction B. Then 66 µL of reaction mixture A and 75 µL of reaction mixture B were combined, and 5 µL PigJ (1.2 $mgmL^{-1}$) was added. To quench the reaction, 5 µg of sequence grade trypsin (17000 U/mg) in 20 µL of 1 mM formic acid and 20 µL of 1 M Tris (pH 8.0) were added, which were allowed to incubate for 5 min at rt before 50 µL of 10% formic acid was used to stop the trypsin digestion. The digest was then separated by HPLC over a 60 min acetonitrile gradient (Table 2). The HPLC column used for all desalting steps and separations was a Jupiter 5µ C4 300 Å column from Phenomenex. The protein domain that corresponded to the ACP_1 active site eluted between 47 and 50 min. The active site was verified by tandem mass spectrometry (Figure S4). For unknown reasons, the ACP_2 active site could not be found from the

intact PigH construct even when different digestion methods and HPLC gradients were employed.

Time (min)	0.0	10.0	15.0	55.0	60.0	60.1	60.2	62.6	63.0	65.0	66.0
%A	90	90	70	30	10	10	95	95	5	5	90
%B	10	10	30	70	90	90	5	5	95	95	10

Table 2. HPLC gradient for active site purification, solvent A is water (0.1% TFA) and solvent B is acetonitrile (0.1% TFA). The reason for the unusual gradient above 60 min is to wash the column prior to the next injection to avoid contamination from a previous run.

Pyrrolyl-β-ketoacyl-*S*-PigHACP$_1$ACP$_2$ reconstitution and preparation for mass spectrometry. 120 μL of PigG (300 μM), 1.5 μL of $MgCl_2$ (800 mM), 25 μL of pyrrolyl-2-carboxyl-CoA and 5 μL of Sfp (1.2 mgmL^{-1}) was incubated for 60 min to generate reaction mixture A. At the same time, 320 μL PigHACP$_1$ACP$_2$ (3 mgmL^{-1}), 3 μL of $MgCl_2$, 11 μL of malonyl-CoA (14 mM) and 8 μL of Sfp (1.2 mgmL^{-1}) were allowed to incubate for 60 min to generate reaction mixture B. At the 60 min time point, 151.5 μL of reaction mixture A, 285 μL of reaction mixture B (the remainder of this reaction mixture was used as time = 0 min time point) and the addition of 15 μL of PigJ (1.2 mgmL^{-1}). At time points 1, 2, 4, 8 and 16 min, 90 μL of this reaction mixture was added 60 μL of 10% formic acid to stop the reaction. The loaded di-ACP domain was then desalted by HPLC using a 30 min acetonitrile gradient (Table 3). The HPLC column used for all desalting steps and separations was a Jupiter 5μ C4 300 Å column (150 X 4.6 m) from Phenomenex. And the fractions containing the PigHACP$_1$ACP$_2$ protein were analyzed by mass spectrometry.

Time (min)	0.0	10.0	12.5	25.0	30.0	30.1	30.2	32.6	33.0	35.0	36.0
%A	90	90	70	30	10	10	95	95	5	5	90
%B	10	10	30	70	90	90	5	5	95	95	10

Table 3. HPLC Gradient for active site purification, solvent A is water (0.1% TFA) and solvent B is acetonitrile (0.1% TFA). The reason for the unusual gradient above 30 min is to wash the column prior to the next injection to avoid contamination from a previous run.

Effect of the addition of L-serine by mass spectrometry. To demonstrate the effect of L-serine, the pyrrolyl-β-ketoacyl-*S*-PigH was prepared as described above in the section "Pyrrolyl-β-ketoacyl-*S*-PigHACP$_1$ reconstitution and preparation for mass spectrometry". Once the pyrrolyl-β-ketoacyl-*S*-PigHACP$_1$ was prepared the reaction was incubated with 100 μM PLP and 1mM L-serine for 7 min before ACP$_1$ was digested trypsin, quenched with 10% formic acid and purified by HPLC as described above.

Fourier-Transform Mass Spectrometry (FTMS). HPLC fractions containing the active sites prepared as described above were redissolved in 100-400 μL of 78% acetonitrile, 0.1% acetic acid or 49% methanol, 1% formic acid and analyzed by nFTMS. For mass spectrometric analysis, a custom 8.5 Tesla ESI-FTMS mass spectrometer equipped with a front-end quadrupole was used.[2] The samples were introduced into the FTMS using a NanoMate 100 for automated nanospray (Advion Biosciences, Ithaca, NY). Typically 500 ms ion accumulation per scan was

used and 30-200 scans were acquired per spectrum. The instrument was externally calibrated using ubiquitin, 8560.65 Da monoisotopic M_r value (Sigma). For the calculation of the masses of the proteins, the MIDAS analysis data-station was used.[3]

BODIPY loading of PigH and PigH mutants. Using Sfp, both mutants could be loaded with BODIPY-*S*-CoA (Figure S9). BODIPY-CoA was prepared by Shaun McLoughlin as described.[4,5] PigH (or mutants) (100 µL of 68 µM) was incubated with 1 µL $MgCl_2$ (800 mM) and 2 µL of Sfp (1.2 $mgmL^{-1}$) and 200 µM of BODIPY-CoA were incubated for 30 min. At this point 20 µL was diluted with 5 µL SDS-loading buffer (Laemmli sample buffer by Bio-Rad). The gel for the separation was a 4-20% Tris-HCl gel from Bio-Rad. To visualize the fluorescence, the gel was illuminated with a 366 nm UV light and the image captured with a digital camera (Canon). This image is shown in Figure S9.

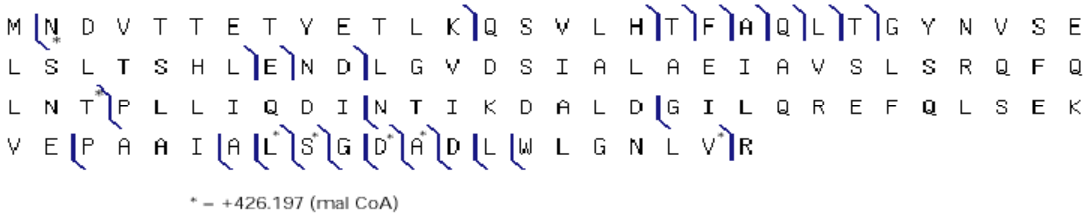

Figure S5. Active site verification of malonyl-S-PigH(ACP_1) by tandem mass spectrometry (collisionally activated dissociation).

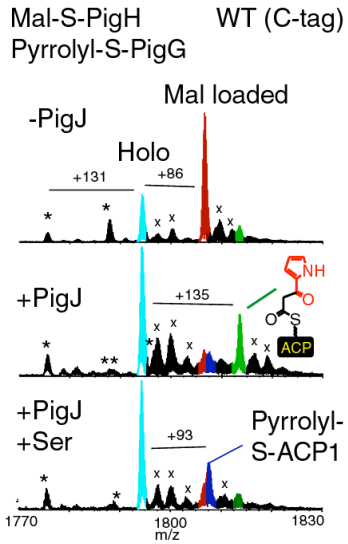

Figure S6. Pyrrolyl-β-ketoacyl-*S*-PigH(ACP_1) from WT PigH protein active site detection by mass spectrometry. Top panel malonyl-S-PigH, pyrrolyl-S-PigG. Middle panel same reaction conditions as the top panel but incubated with PigJ. Bottom panel same as the top panel but incubated with PigJ, PLP and L-serine. * indicates the 131 Da loss due to partial N-terminal loss of methionine. x = non-covalent sodium adduction generated during the nanospray-process.

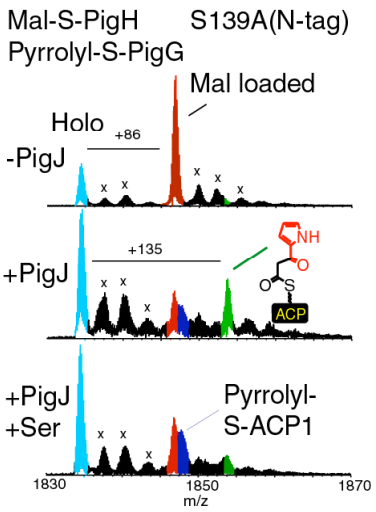

Figure S7. pyrrolyl-β-ketoacyl-*S*-PigH(ACP$_1$) from S139A PigH protein active site detection by mass spectrometry. Top panel malonyl-S-PigH (S139A), Pyrrolyl-S-PigG. Middle panel same reaction conditions as the top panel but incubated with PigJ. Bottom panel same as the top panel but incubated with PigJ, PLP and L-serine. x = non-covalent sodium adduction generated during the nanospray-process.

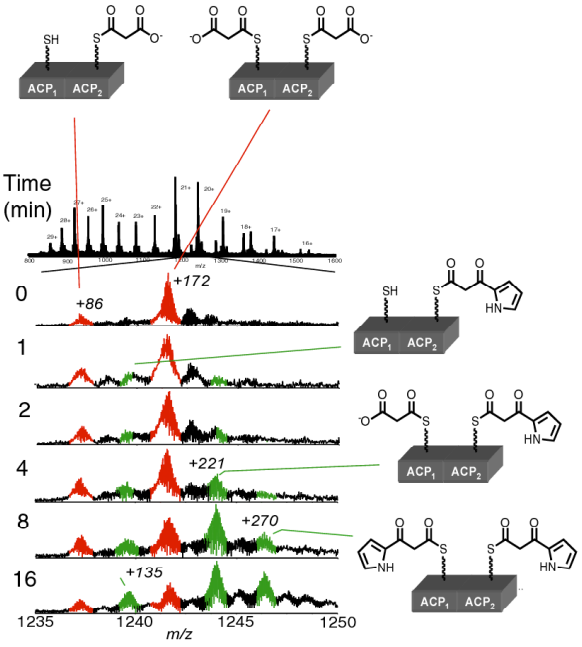

Figure S8. A time course for the formation of dipyrrolyl-β-ketoacyl-*S*-PigHACP$_1$ACP$_2$ from dimalonyl-S-PigHACP$_1$ACP$_2$ upon incubation with PigJ and pyrrolyl-*S*-PigG. The masses indicated are the mass increases compared to holo-PigHACP$_1$ACP$_2$. Even if only one regioisomer is shown for each mass, both regioisomers are possible (*e.g.* top left structure: ACP$_1$ is shown as holo and ACP$_2$ as loaded with malonyl, however for the same mass it could be holo ACP$_2$ and malonyl-*S*-ACP$_1$).

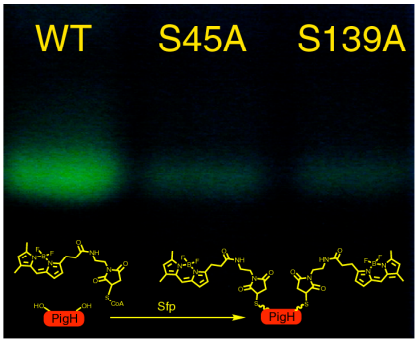

Figure S9. Loading of PigH WT, PigHS45A, and PigHS139A with BODIPY-S-CoA to verify loading of the mutants by Sfp.

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