Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Electronic Supplementary Information

Delineating the earliest steps of gilvocarcin biosynthesis: role of GilP and GilQ in starter unit specificity

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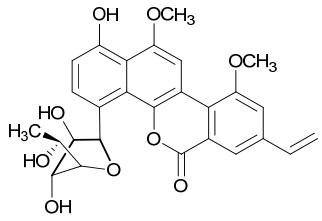

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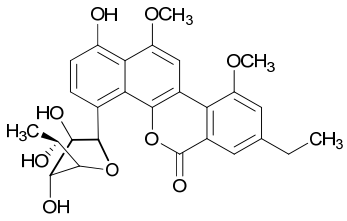

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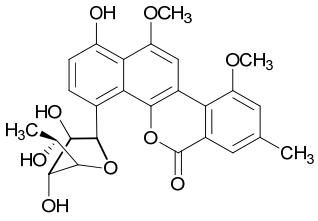


3
gilvocarcin M (GM)

Microorganisms and culture conditions: All *in vivo* experimentation was carried out in the heterologous host *S. lividans* TK24.[1] DNA introduction into *S. lividans* was facilitated by either conjugal transfer (cosG9B3-P^{-} and cosG9B3-Q^{-}) or via protoplast transformation (pGilQ and pGilQGilOIII) according to standard protocol.[1] Conjugation was carried out on MS agar[1] supplemented with 10 mM $MgCl_2$ and overlaid with nalidixic acid with appropriate antibiotics after 18 hours. Ex-conjugates were grown on solid M2 media (4 g/L glucose, 10 g/L malt extract, 4 g/L yeast extract, 1g/L $CaCO_3$, 15 g/L agar) supplemented with appropriate antibiotics. Protoplasts were regenerated on R2YE agar media[1] and overlaid after 18 hours with R3 soft agar (171 g/L sucrose, 10 g/L glucose, 4g/L peptone, 0.5 g/L K_2SO_4, 8.1g/L $MgCl_2 \cdot 6\ H_2O$, 2.2 g/L $CaCl_2$, 8.8 g/L agar) supplemented with appropriate antibiotics. For gilvocarcin production studies, all strains were grown in liquid SG media (20 g/L glucose, 10 g/L soy peptone, 2 g/L $CaCO_3$, 0.001 g/L cobalt-II-chloride, pH 7.2) with appropriate antibiotics. Each strain was grown in 3x100 mL liquid SG using 250 mL baffled Erlenmeyer flasks. After 4 days at 28 ^{o}C and 250 rpm, 50 mL of each culture flask was extracted with 50 mL ethyl acetate three times. Ethyl acetate was removed under vacuum and replaced with 3 mL of methanol. Of the 3 mL extraction, 50 μL was run on HPLC-MS, and "area under the curve" was calculated for GV (**1**), GE (**2**), and GM (**3**). These data were used to produce gilvocarcin congener ratios as well as determine overall gilvocarcin yields by comparing experimental data with a HPLC-MS "area

under the curve" standard curve of **1**. *Escherichia coli* (*E. coli*) strains were cultured in solid or liquid Lysogeny broth (LB), and supplemented with antibiotics when necessary. DNA manipulations such as plasmid isolation, ligation, and restriction digest analyses were carried out according to standard protocols.[1, 2] Bacterial strains used in this study can be found in **table S1**.

Antibiotics/antifungals used in this study: apramycin (apr; 50 μg/mL), chloramphenicol (cat; 50 μg/mL), ampicillin (amp; 100 μg/mL), thiostrepton (tsr; 25 μg/mL), kanamycin (kn; 50 μg/mL), nalidixic acid (na; 25 μg/mL).

Table S1. Bacterial strains used in this study.

Bacterial Strains	**Relevant characteristics**	**References**
E. coli XL1Blue	Cloning host	Stratagene
E. coli BL21 (DE3)	Host for protein expression	Stratagene
E. coli BW25113/pKD20 (CGSC#7637)	Host for homologous recombination, amp^r	[3]
E. coli ET12567/pUZ8002	Conjugation host with non transmissible plasmid	[4, 5]
S. lividans TK24	Heterologous expression host	[1]
S. lividans TK24(cosG9B3)	*S. lividans* TK24 transformed with cosG9B3	[6]
S. lividans TK24(cosG9B3-P$^-$)	*S. lividans* TK24 transformed with cosG9B3-P$^-$	This study
S. lividans TK24(cosG9B3-Q$^-$)	*S. lividans* TK24 transformed with cosG9B3-Q$^-$	This study
S. lividans TK24(cosG9B3-pGilQ)	*S. lividans* TK24-cosG9B3 transformed with pGilQ	This study
S. lividans TK24(cosG9B3-pGilQGilOIII)	*S. lividans* TK24-cosG9B3 transformed with pGilQGilOIII	This study

Generation of inactivation mutants: Inactivation experiments were carried out in cosG9B3, a pOJ446 derived cosmid containing the entire gilvocarcin gene cluster.[6] The deletion of *gilP* and *gilQ* from cosG9B3 was generated using a modified PCR-targeting REDIRECT protocol described previously.[7] Briefly, a chloramphenicol (cat^r) resistance gene flanked with FRT (flippase recognition target) and FLP (flippase) sites was used as a template to amplify inactivation cassettes. Inactivation cassettes for *gilP* and *gilQ* were amplified using primer pairs GilP-Inact-F and GilP-Inact-R and GilQ-Inact-F and GilQ-Inact-R, respectively (**Table S2**). The amplified cassettes were then introduced into *E. coli* BW25113/pKD20 (amp^r) harboring cosG9B3 (apr^r) by electroporation. With the loss of the temperature sensitive pKD20, chl and apr

resistant colonies were obtained indicating the replacement of *gilP* or *gilQ* with the inactivation cassette. Control primers flanking the start and stop codon of *gilP* and *gilQ* (GilP-Cntrl-F and GilP-Cntrl-R and GilQ-Cntrl-F and GilQ-Cntrl-R, respectively) were used to further confirm the replacement of the targeted genes with the chloramphenicol cassette (**Table S2**). Removal of the inactivation cassette via FLP-mediated excision was carried out as described previously.[7] The 82 bp in-frame scar left after FLP-mediated excision was confirmed in cosG9B3-P$^-$ and cosG9B3-Q$^-$ using control primers discussed previously. The resultant mutated cosmids were introduced into *E. coli* ET12567/pUZ8002 and conjugally transferred to *S. lividans* TK24 producing *S. lividans* TK24(cosG9B3-P$^-$) and *S. lividans* TK24(cosG9B3-Q$^-$) according to standard protocols (**Table S1**).[1]

Table S2. Primers used in this study separated by their use in either *in vivo* or *in vitro* studies.

Primer Name	Oligonucleotide sequence
For *in vivo* work	
GilQ-Inact-F	5'-CGTCCTTCCTGGCCGGCGTGACGGGCTGGTGAGCCGGTGATTCCGGGGATCCGTCGACC-3'
GilQ-Inact-R	5'-CATGGCCGGCCTTGCGCGTTGCACGTAGTGCAATGGTCATGTAGGCTGGAGCTGCTTC-3'
GilQ-Cntrl-F	5'-TCTAGAGCAAGGTCCTCTCGGGATTC-3'
GilQ-Cntrl-R	5'-ATGGGTGCCTCCCGGGAACTCG-3'
GilP-Inact-F	5'-GCGCCTCCACGACGACTTGGAACCCGAGGTGACGCGGTGATTCCGGGGATCCGTCGACC-3'
GilP-Inact-R	5'-ATGCGGCACTGCGTGCTTCACCGGCTCACCAGCCCGTCATGTAGGCTGGAGCTGCTTC-3'
GilP-Cntrl-F	5'-TCTAGATCCGTCCCGATCACCACTG-3'
GilP-Cntrl-R	5'-GACTCCCATGGCAGCGAACTG-3'
GilQ-F1	5'-GGC*CTGCAG*GGCTGGTGAGCCGGTGAAGCACGCA-3'
GilQ-R1	5'-GCGT*GCTAGC*T*TCTAGA*ATGGTCAACAGAATTCCTCGGCGACCT-3'
GilQ-F2	5'-TG*GCTAGC*CGGAGAAGCACGCAATGCCGCATCAGGCAACC-3'
GilQ-R2	5'-CGG*TCTAGA*GCGTTGCACGTAGTGCAATGGTCA-3'
GilOIII-F	5'-CC*TCTAGA*GGAAAGGATGAAGCGATGATCTCCACA-3'
GilOIII-R	5'-GTC*GAATTC*GACCACCGTCACGTCCTCGACG-3'
For *in vitro* work	
GilP-F	5'-AT*CATATG*AGAGCGTTCCTGTTCCCCGGT-3'
GilP-R	5'-AT*GAATTC*TCACCAGCCCGTCACGCC-3'
GilQ-F3	5'-AA*CATATG*GTGCCGCATCAGGCAACC-3'
GilQ-R3	5'-AA*GGATCC*TCAACAGAATTCCTCGGC-3'
RavC-F	5'-ATT*CATATG*AGCAGCTTCTCGATCGACGACCTC-3'

RavC-R	5'-ATT*GAATTC*TCAGCCGCGCGGCGCGGG-3'
RavC1-F	5'-ATT*CATATG*ACCACCGGCACGTTCACC-3'
RavC1-R	5'-ATT*GAATTC*TCACGCCGCGTTGACCAGCTC-3'
GilP-S90A-F	5'-CATCGCGGGCCACGCTCTGGGCGAGTAC-3'
GilP-S90A-R	5'-GTACTCGCCCAGAGCGTGGCCCGCGATG-3'
GilQ-S111A-F	5'-GCTGGTCGGTCACGCCGTGGGCGAGCTG-3'
GilQ-S111A-R	5'-CAGCTCGCCCACGGCGTGACCGACCAGC-3'
RavC-S39A-F	5'-CTGGGGTACGACGCCCTGGCGCTGC-3'
RavC-S39A-R	5'-GCAGCGCCAGGGCGTCGTACCCCAG-3'

Restriction site sequences are shown in italics.

Construction of overexpression constructs pGilQ and pGilQGilOIII: Primers GilQ-F1 and GilQ-R1 were used to amplify the entire *gilQ* nucleotide sequence including the natural putative ribosomal binding site (RBS) from cosG9B3. The PCR product was then cloned into the TOPO (Invitrogen) cloning vector, producing pGilQ-1-TOPO, and sequenced to ensure no errors were incorporated during amplification. *GilQ* was recovered from pGilQ-1-TOPO as a 1 kb *PstI*/*NheI* fragment and was then ligated downstream of the *ermE** promoter in pEM4 (*PstI/XbaI*),[8] producing pGilQ. Similarly, primer pairs GilQ-F2 and GilQ-R2 and GilOIII-F and GilOIII-R were used to amplify *gilQ* and *gilOIII* from cosG9B3. The amplified genes were then cloned into TOPO generating pGilQ-2-TOPO and pGilOIII-TOPO. *GilQ* was recovered from pGilQ-2-TOPO as a 1 kb *NheI*/*XbaI* fragment and was then ligated downstream of the *ermE** promoter in pEM_4 (*XbaI*), producing p1Q. Additional restriction digest analysis (*HindIII/XbaI*) and sequencing was required to confirm the correct orientation of *gilQ* in p1Q. GilOIII-TOPO was digested and *gilOIII* was recovered as a 1.2 kb *XbaI/EcoRI* fragment. This was then ligated into the same restriction sites of p1Q producing pGilQGilOIII. Both overexpression constructs, pGilQ and pGilQGilOII, were then transformed into *S. lividans* TK24 cosG9B3 via protoplast transformation creating *S. lividans* TK24/cosG9B3-pGilQ and *S. lividans* TK24/cosG9B3-pGilQGilOIII (**Table S1**). All plasmids used or created in this study can be found in **table S3**.

Table S3. Plasmids and cosmids used in this study.

Plasmids/Cosmids	Relevant characteristics	References
pIJ790	pKD20 derived plasmid, cat^r	[9]
PCR-Blunt II-TOPO	Vector for cloning PCR products, kn^r	Invitrogen
pET-28a(+)	Vector for expressing protein in *E. coli*, kn^r	Invitrogen
pEM4	*E. coli-Streptomyces* shuttle vector with *ermE**, amp^R, tsr^R	[8]
cosG9B3	pOJ446 derived cosmid containing all gilvocarcin genes, apr^r	[6]
cosRav32	pOJ446 derived cosmid containing all ravidomycin genes, apr^r	Not published
pGilQ-1-TOPO	*gilQ* (*PstI/NheI*) cloned into TOPO, kn^r	This study
pGilQ-2-TOPO	*gilQ* (*NheI/XbaI*) cloned into TOPO, kn^r	This study
pGilOIII-TOPO	*gilOIII* cloned into TOPO, kn^r	This study
pGilQ-3-TOPO	*gilQ* (*NdeI/BamHI*) cloned into TOPO, kn^r	This study
pRavC$_1$-TOPO	*ravC$_1$* cloned into TOPO, kn^r	This study
pET-GilP	*gilP* cloned into pET-28a(+),kn^r	Not published
pET-GilQ	*gilQ* cloned into pET-28a(+),kn^r	This study
pET-RavC	*ravC* cloned into pET-28a(+),kn^r	Not published
pET-RavC$_1$	*ravC$_1$* cloned into pET-28a(+),kn^r	This study
pGilQ	*ermE**promoter, gilQ cloned into pEM$_4$	This study
p1Q	*ermE**promoter, gilQ cloned into pEM$_4$	This study
pGilQGilOIII	*ermE**promoter, gilQ and gilOIII cloned into pEM$_4$	This study
cosG9B3-P$^-$	gilP deletion mutant of cosG9B3	This study
cosG9B3-Q$^-$	gilQ deletion mutant of cosG9B3	This study
pBS18	svp cloned into the pQE-70 expression vector, amp^r	[10]

cat^r, chloramphenical resistance; kn^r, kanamycin resistance; amp^r, ampicillin resistance; tsr^r, thiostrepton; apr^r apramycin resistance; *ermE*,* constitutive promoter found in erythromycin biosynthesis.

Construction of pET-GilQ and pET-RavC$_1$: Primer pairs GilQ-F3 and GilQ-R3 and RavC1-F and RavC1-R were used to amplify *gilQ* and *ravC$_1$* from cosG9B3 and cosRav32, respectively. As discussed above, amplified *gilQ* and *ravC$_1$* were cloned into TOPO producing GilQ-3-TOPO and RavC$_1$-TOPO. *GilQ* was then taken as a 1 kb *NdeI/BamHI* and cloned into the corresponding sites of pET-28a(+) (Invitrogen) generating pET-GilQ. Similarly, *ravC$_1$* was taken as a 250 bp *NdeI/EcoRI* fragment and cloned into pET-28a(+) producing pET-RavC$_1$. The expression constructs for the remaining proteins used in this study (GilP, RavC, and Svp) were reported elsewhere.[10, 11]

Expression and purification of GilP, GilQ, RavC, RavC$_1$, and Svp: The expression constructs for GilP, GilQ, RavC, RavC$_1$, and Svp were transformed into *E. coli* BL21 (DE3) according to

standard protocols.[2] Single colonies of each transformation was inoculated into 10 mL of LB containing appropriate antibiotics and grown for 5 hours at 37 ^{o}C to prepare seed cultures. One liter cultures (100 mL in 10 x 250 mL Erlenmeyer baffled flasks) were then inoculated with each seed culture and grown in the presence of appropriate antibiotics at 37 ^{o}C with shaking at 250 rpm. At an OD_{600} of 0.5, IPTG was added to a final concentration of 0.05 mM and placed at 18^{o}C with shaking at 250 rpm. After 16 hours the cell pellet was collected by centrifugation (4000 x g, 15 min) and washed twice with 25 mL lysis buffer (50 mM KH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.6). The final pellet was stored at -80 ^{o}C overnight and then taken for cell disruption. Cell lysis was achieved using a French Press (Thermo Electron Corporation), and crude protein was recovered by centrifugation (18000 x g, 30 min). All proteins were expressed with an *N*-terminal $(His)_6$-tag and were purified and desalted using a profinia protein purification system (Bio-Rad) utilizing immobilized metal affinity chromatography (IMAC) and desalting cartridges. Purified and desalted proteins were then concentrated using Amicon Ultra centrifugal filters (Millipore) and final concentrations were determined using the Bradford protein assay method.[12] The purity of the proteins was visualized by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses. The calculated sizes of the proteins (GilP, 33.7 kDa; GilQ, 38.9 kDa; RavC, 11.3 kDa; $RavC_1$, 10.8 kDa; Svp, 25.6 kDa) were close to the observed sizes seen upon SDS-PAGE analyses (**Figure S1**). Svp runs high on SDS-PAGE gels as reported previously.[10]

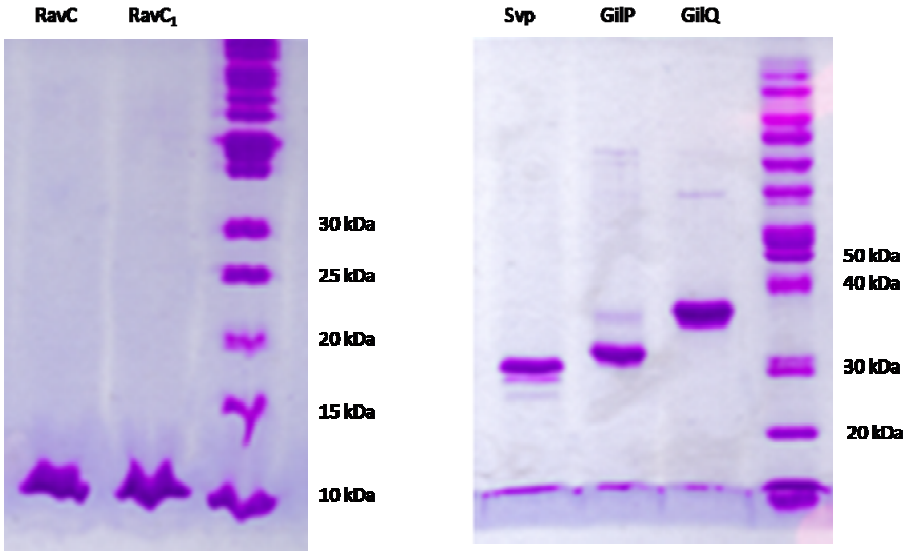
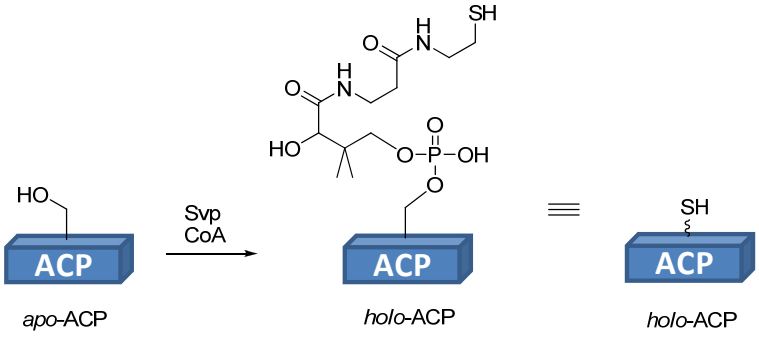


Figure S1. SDS-PAGE analysis of *N*-terminal $(His)_6$ – tagged proteins used in this study.

Production of *holo*-ACP using Svp: RavC and $RavC_1$ were expressed in *E. coli* almost exclusively (>95%) as the inactive *apo*-ACP. Svp, a promiscuous phosphopantetheinyl transferase (PPTase), was used to completely convert the purified inactive *apo*-ACP to the functional *holo*-ACP in the presence of Coenzyme A (CoA) (**Scheme S1**).[10]



Scheme S1. The functional *holo*-ACP is produced by the transfer of the phosphopantetheinyl prosthetic group from CoA to *apo*-ACP via a phosphopantetheinyl transferase, such as Svp.

The production of both *holo*-RavC and *holo*-RavC$_1$ was obtained through a modified protocol presented elsewhere.[13] The reaction conditions were as follows: 50 mM KH_2PO_4 (pH 7.5), 12.5 mM $MgCl_2$, and a 1:20:200 molar ratio of Svp:*apo*-ACP:CoA. The reaction was incubated at 30 ^oC and 10 μL samples were taken at 60 min time intervals and analyzed by HPLC with a Platinum C4-EPS-300 column (250 x 4.6 mm, 5 µm; Grace, Deerfield, IL). Samples were eluted with linear gradients from solvent A (0.1% trifluoroacetic acid in 10% acetonitrile) to solvent B (0.1% trifluoroacetic acid in 90% acetonitrile): 0 to 5 min, 5% B; 5 to 32 min, gradient from 5 to 95% B; 32-40 min, hold at 95% B; and 40 to 45 min, gradient from 95 to 5% B. Peaks were collected, dried under vacuum and analyzed by MALDI-TOF mass spectrometry. Under these conditions *apo*-RavC and *apo*-RavC$_1$ were converted to their active *holo*-forms in 60 min (**Figure S2**). Peak **1** had a mass of 11187 Da and was representative of *apo*-RavC (cal. 11197 Da), and peak **2** had a mass of 11524 Da indicating the presence of holo-RavC (cal. 11537 Da).

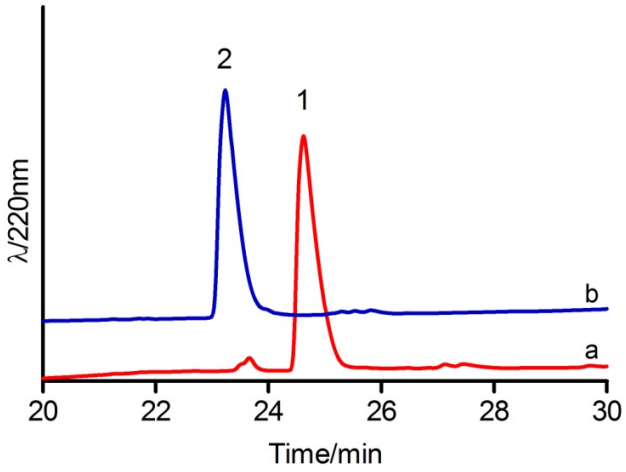


Figure S2. Conversion of *apo*-RavC (peak 1) to *holo*-RavC (peak 2) at time points 0 min (a) and 60 min (b)

Acyl transfer assay (GilP, GilQ and RavC): An acyl transfer assay was set up to determine the specific activity of GilP and GilQ to form radiolabeled acyl-RavC. The radiolabeled acyl-CoAs used in this study were malonyl-CoA (malonyl-2-^{14}C, Perkin Elmer), acetyl-CoA (acetyl-1-^{14}C, Perkin Elmer), methylmalonyl-CoA (methyl-^{14}C, American Radiolabeled Chemicals), and propionyl-CoA (propionyl-1-^{14}C, American Radiolabeled Chemicals). 100 μL reactions were set up consisting of 50 mM KH_2PO_4 (pH 7.5), 12.5 mM $MgCl_2$, 1 mM *tris*(2-carboxyethyl)phosphine (TCEP), 8 μM *holo*-RavC, and 0.8 μM purified GilP or GilQ (**Table S4**). This was allowed to equilibrate at 30 ^{o}C for 5 min and then the reaction was initiated by the addition of 8 μM radiolabeled acyl-CoA. The reaction was quenched with trichloroacetic acid (TCA) after 30 seconds at 30 ^{o}C to a final concentration of 7% TCA. 200 μg of bovine serum albumin (BSA) was added to the quenched reaction and was kept on ice for 15 min. Protein was collected by centrifugation (13000 x g, 10 min) and washed twice with cold 7% TCA. The pellet was then dissolved with 100 μL of a 1:1 solution of 2 M NaOH and 2 M Tris Base. The reconstituted pellet was then combined with 500 μL of scintillation cocktail (Research Products International) and analyzed on a liquid scintillation analyzer (2200CA TRI-CARB, Packard).

Table S4. List of reaction combinations for acyl transfer assays (1- 30) and initial rate studies (31-33). Final concentrations for 100 µL reactions are shown in parenthesis. All reactions contained 50 mM KH_2PO_4, 12.5 mM $MgCl_2$, and 1 mM TCEP.

Reaction #	*holo*-RavC (8 µM)	GilP (0.8 µM)	GilQ (0.8 µM)	M-CoA (8 µM)	A-CoA (8 µM)	MM-CoA (8 µM)	P-CoA (8 µM)
1,2,3	X	X	-	-	-	-	-
4,5,6	X	X	-	X	-	-	-
7,8,9	X	X	-	-	X	-	-
10,11,12	X	X	-	-	-	X	-
13,14,15	X	X	-	-	-	-	X
16,17,18	X	-	X	-	-	-	-
19,20,21	X	-	X	X	-	-	-
22,23,24	X	-	X	-	X	-	-
25,26,27	X	-	X	-	-	X	-
28,29,30	X	-	X	-	-	-	X
31	X	-	-	X	-	-	-
32	-	X	-	X	-	-	-
33	X	X	-	X	-	-	-

(X), added to reaction; (-), not added to reaction; M-CoA, ^{14}C-malonyl-CoA; A-CoA, ^{14}C-acetyl-CoA; MM-CoA, ^{14}C-methylmalonyl-CoA; P-CoA, ^{14}C-propionyl-CoA

The self loading properties of *holo*-ACPs have been well documented.[14] Non-selective self loading of *holo*-RavC was observed for all radiolabeled acyl-CoAs tested (data not shown). The initial rate of *holo*-RavC self loading was calculated to ensure the scintillation data was reflective of acyltransferase assisted acyl-CoA transfer and not due to the intrinsic self loading properties of *holo*-RavC. Reactions 31-33 (**Table S4**) were used to calculate the initial rate of *holo*-RavC and GilP self loading as well as the initial rate of GilP assisted transfer of malonyl-CoA to *holo*-RavC. Instead of single 30 second time points taken as discussed above, reactions 31-33 were quenched at 0 sec, 10 sec, 30 sec, 1 min, 3 min, 5 min, 10 min, 30 min, and 60 min. The ^{14}C-labeled proteins were collected and quantitated by liquid scintillation counting as described above. The initial rate of *holo*-RavC and GilP self loading of ^{14}C-malonyl-CoA under the aforementioned conditions were calculated to be 0.03 μM min^{-1} and 0.67 μM min^{-1},

respectively. The initial rate of ^{14}C-malonyl-CoA transfer by GilP to form ^{14}C-malonyl-CoA:RavC was calculated to be 3.81 μM min^{-1} (**Figure S3**). These experimental data show the initial rate of self loading by *holo*-RavC or GilP, and could not significantly contribute to the ^{14}C-radiolabeled proteins in the acyl transfer assay.

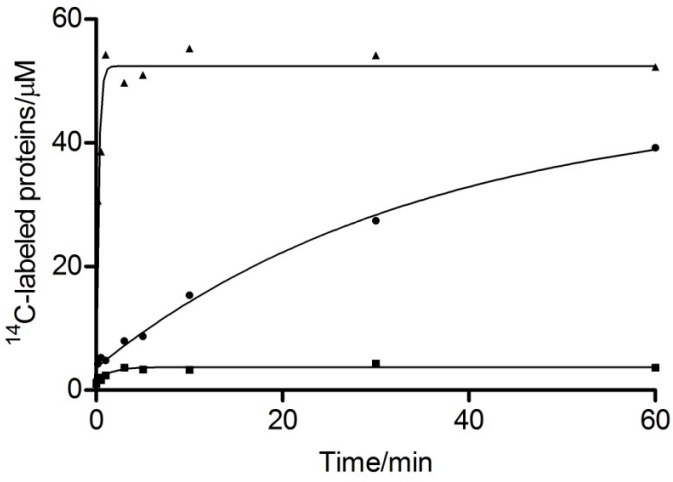


Figure S3. Non-linear regression curve of self loading properties of *holo*- RavC (●) and GilP (■) compared to *holo*-RavC + GilP (▲).

Preparation of GilPS$_{90}$A, GilQS$_{111}$A, and RavCS$_{39}$A: Bioinformatic analyses of GilP, GilQ, and RavC revealed the presence of their expected active site motif; xGHSxGE and LGxDSLxxVE for acyltransferases and acyl carrier proteins, respectively. The active site Ser residue was mutated to an Ala in all three proteins to determine the activity seen in the acyl transfer assays were due to the active site Serine. Primer sets GilP-S90A-F and GilP-S90A-R, GilQ-S111A-F and GilQ-S111A-R, and RavC-S39A-F and RavC-S39A-R were used with the QuickChange lightning site-directed mutagenesis kit (Stratagene) to produce GilPS$_{90}$A, GilQS$_{111}$A, and RavCS$_{39}$A. These proteins were expressed and purified as discussed previously.

An acyl transfer assay was then carried out using the mutated proteins under the same reaction conditions as described above (**Table S5**). Instead of using scintillation counting, however; 25 µL of the reaction (100 µL, 30 sec) was directly loaded to an SDS-PAGE gel. The gel was dried using cellophane paper (Idea Scientific Company), placed on storage phosphor screens (Amersham Biosciences) for 3 days and visualized using a Typhoon biomolecular imager (GE Healthcare). The inability of the mutated proteins to load or transfer ^{14}C-labelled CoA substrates clearly shows the active site Ser is in fact the catalytic residue of these proteins (**Figure S4**).

Table S 5. List of reaction combinations used to test the loading and transfer functionality of the mutated proteins GilPS$_{90}$A, GilQS$_{111}$A, and RavCS$_{39}$A. Final concentrations for 100 µL reactions are shown in parenthesis. All reactions contained 50 mM KH_2PO_4, 12.5 mM $MgCl_2$, and 1 mM TCEP.

Reaction #	***holo*-RavC (8 μM)**	**GilP (0.8 μM)**	**GilQ (0.8 μM)**	**RavCS$_{39}$A (8 μM)**	**GilPS$_{90}$A (0.8 μM)**	**GilQS$_{111}$A (0.8 μM)**	**M-CoA (8 μM)**	**P-CoA (8 μM)**
34	X	X	-	-	-	-	X	-
35	-	X	-	X	-	-	X	-
36	X	-	-	-	X	-	X	-
37	-	-	-	X	X	-	X	-
38	-	-	X	-	-	-	-	X
39	-	-	-	-	-	X	-	X

(X), added to reaction; (-), not added to reaction; M-CoA, ^{14}C-malonyl-CoA; P-CoA, ^{14}C-propionyl-CoA

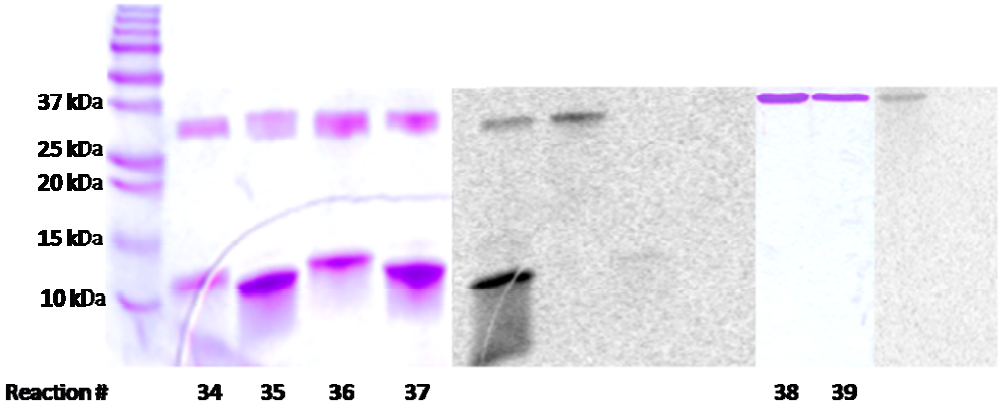


Figure S4. ^{14}C-acyl transfer assay using mutated GilPS$_{90}$A , GilQS$_{111}$A, and RavCS$_{39}$A. The slight radiolabeled band seen corresponding to lane 36 is due to the self loading properties of *holo*-RavC.

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