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Supplemental Data

In Vivo and In Vitro *Trans*-Acylation by BryP,

the Putative Bryostatin Pathway Acyltransferase

Derived from an Uncultured Marine Symbiont

Nicole B. Lopanik,[1,7] Jennifer A. Shields,[2] Tonia J. Buchholz,[1] Christopher M. Rath,[1,3] Joanne Hothersall,[2] Margo G. Haygood,[4] Kristina Håkansson,[3] Christopher M. Thomas,[2] and David H. Sherman[1,3,5,6,*]

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning of *bryP*

The AT domains of *bryP* were cloned intact, and separately, using a subcloned fragment of the fosmid MM5 as the PCR template. BryP AT_1 was cloned into pET28b (Novagen) by engineering *EcoRI* and *XhoI* sites into PCR primers (Table S2), and amplifying the product from the fosmid subclone using a high fidelity *Taq* polymerase (ExTaq, Takara). After double digestion of the PCR product and vector with *EcoRI* and *XhoI* (New England Biolabs) and purification, the insert and vector were ligated at 14°C overnight using T4 DNA ligase (Invitrogen). The ligation reaction was transformed into *Escherichia coli* XL1-Blue (Stratagene). Colonies were screened by colony PCR using the T7 and T7 terminator primers. The plasmid was purified from positive transformants and sequenced to verify the insert. Because many of the genes in the bryostatin biosynthetic cluster are AT-rich, this plasmid (pNL020) was transformed into *E. coli* BL21 (DE3) (Stratagene) bearing a coexpression plasmid that overexpresses rare codons (pRARE_CDF). In general, for all overexpression experiments, starter cultures were grown overnight in LB broth with the appropriate antibiotics. Overexpression cultures (TB media) were inoculated with the starter cultures at a 1:100 ratio. These cultures were grown to OD_{600} of approximately 1 at 30 °C and the temperature was reduced to 16 °C. After approximately 1 hr, IPTG was added to a concentration of 0.1 mM. The cultures grew overnight, and were harvested by centrifugation the next day.

Repeated attempts at cloning and overexpression of BryP AT_2 and the didomain, BryP AT_1AT_2 in pET28b resulted in insoluble protein. Therefore, we redirected our efforts into overexpressing the proteins with both a maltose-binding fusion protein and a 6X histidine tag. Ligation-independent cloning (LIC) (Aslanidis and de Jong, 1990) was used to clone the mono- and didomain into pMCSG9 (Donnelly et al., 2006). Multiple constructs of BryP AT_2 and BryP AT_1AT_2 with different start and stop sites were generated based on secondary structure and disorder predictions of the amino acid sequences in order to generate a construct that produced soluble protein (Supp. Fig. 3). The plasmids were transformed into *E. coli* BL21 (DE3) with the pRARE_CDF coexpression plasmid and overexpressed on a small scale (0.5 mL) as described above. The cells were lysed using CellLytic (Sigma-Aldrich), purified by Ni-NTA resin affinity purification, and the elutions were separated and visualized on the Caliper system.

Each AT domain in the didomain construct of BryP AT_1AT_2 was inactivated by site-directed mutagenesis of the active site serine residues (S88A and S410A). In a similar manner, the double mutant (S88A/S410A) was generated. The QuikChange Primer Design Program (Stratagene) was used to design the primers (Supp. Table 2), and the QuikChange protocol (Stratagene) was followed to perform the mutagenesis. The reaction was transformed into *E. coli* XL1-Blue. To prepare the double mutant, the BryP AT_1AT_2° mutant plasmid was used as a template for the AT_1 mutation reaction. All site-direct mutagenesis was confirmed by DNA sequencing.

Cloning of Genes for Carrier Proteins and Modules

Genes for several carrier proteins (CPs) were cloned and overexpressed in order to probe the specificity of the BryP proteins. Cloning and overexpression of CloN5, a PCP from the clorobiocin gene cluster was prepared as described previously (Garneau et al., 2005). BryB M7 ACP, an integrated ACP from the bryostatin gene cluster, was cloned into pET28b by PCR amplifying the insert (Table S2) from a fosmid subclone, and ligating the *EcoRI*- and *XhoI*-digested insert with the digested vector at 14°C overnight. A second integrated ACP from the bryostatin gene cluster (BryA M3 ACP) was cloned into pMCSG7 (Stols et al., 2002) via LIC cloning (Table S2). An ACP from the pikromycin gene cluster, PikAIII M5 ACP (Xue et al., 1998), was cloned into pET28b. PikAIII M5 ACP was amplified from cosmid DNA containing a portion of the pikromycin gene cluster [pLZ71 (Xue et al., 1998)], and cloned into the *NdeI* and *HindIII* sites of pET28b (Table S1).

Three modules from PKS gene clusters were targeted for cloning and overexpression, including the terminal modules of the erythromycin [EryAIII module 6: KS, AT, KR, ACP, TE (Donadio et al., 1991)] and pikromycin gene clusters [PikAIV module 6: KS, AT, ACP, TE (Xue et al., 1998)], and one module from the bryostatin gene cluster, BryB M4 [Fig. 1A; unknown domain, KS, MT, ACP (Sudek et al., 2007)]. The wild type EryAIII M6 was cloned into pET21c as previously described (Gokhale et al., 1999). PikAIV cloning and overexpression is described in (Beck et al., 2003). AT mutants were made for both of these modules by site-directed mutagenesis (EryAIII M6: S2107A, and PikAIV M6: S652A). For BryB M4, several constructs of were made based on amino acid secondary structure and disorder predictions. BryB M4 was PCR amplified from a fosmid subclone with Phusion DNA polymerase (Finnizymes) and cloned into pMCSG9 via LIC (Table S1).

Protein Overexpression and Purification

The plasmids were transformed into *E. coli* BL21 (DE3) strains carrying various coexpression plasmids. Because many of the genes in the bryostatin biosynthetic cluster are AT-rich, the AT, ACP, and module plasmids (pNL020, pNL091, pNL106, pNL101-104, and pNL109) were transformed into *E. coli* BL21 (DE3) bearing a coexpression plasmid that overexpresses rare codons (pRARE_CDF, W. Clay Brown HTP). In addition, some constructs were overexpressed in *E. coli* BL21 (DE3) with a plasmid that expresses *Bacillus subtilis sfp* (pSG701), a protein that adds a phosphopantetheine arm to the active-site serine in the carrier proteins (Lambalot et al., 1996). This modification is necessary to generate *holo* carrier proteins. Some carrier proteins were expressed with and without *sfp* to produce *apo* carrier protein for use as a negative control and for *in vitro* phosphopantetheinylation reactions. Overexpression was conducted as described above for BryP.

For protein purification, cells were resuspended in lysis buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, and 1 mM EDTA, 10 mM imidazole, pH 8) for those constructs with only a 6X Histidine tag (BryP AT_1, BryA M3 ACP, BryB M7 ACP, PikAIII M5 ACP, and EryAIII M6 wt and AT°) or lysis buffer 2 (100 mM sodium phosphate buffer, 300 mM NaCl, 25% glycerol, 1 mM EDTA, pH 7.2) for those constructs with a 6X His-tag and maltose binding protein (BryP AT_1AT_2 wt and mutants, three BryP AT_2 constructs, and BryB M4). The cells were lysed by sonication and the cell debris pelleted by centrifugation at 30,000 *x g* for 10 min at 4°C. For purification of the 6X His-tag proteins, the soluble fraction was incubated with Ni-NTA resin (Qiagen) for >45 min, and the proteins were purified following the manufacturer's protocol. Briefly, the resin was rinsed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, and 1 mM EDTA, 30 mM imidazole, pH 8) until assays with Bradford reagent (BioRad) demonstrated that the rinse was free of protein. The target proteins were then eluted from the column (elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, 1 mM EDTA, 250 mM imidazole, pH 8). For the MBP affinity chromatography, amylose resin (New England Biolabs) was used. This resin was rinsed with lysis buffer 2, and the target proteins were eluted with 100 mM sodium phosphate buffer, 300 mM NaCl, 25% glycerol, 1 mM EDTA, 10 mM maltose, pH 7.2. After elution, the proteins were desalted on a PD10 column (GE Healthcare), and concentrated in an Amicon Ultra Centrifugation Unit (Millipore). The AT proteins were stored in 50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, and 1 mM EDTA, pH 8. The carrier proteins (including modules containing ACPs) were stored in 100 mM sodium phosphate buffer, pH 7.2, 300 mM NaCl, 1 mM EDTA, 2 mM DTE, and 20% glycerol. Protein concentrations were quantified by the Bradford assay using BSA as the standard.

FT-ICR MS Analysis of BryB M4

Because the bryostatin native module BryB M4 was only partially soluble and unable to be purified by conventional methods, we used MS analysis to confirm the identity of it in a SDS-PAGE gel. Preparations of BryB M4 were separated by SDS-PAGE, and visualized by Coomassie blue staining. The putative BryB M4+MBP band at 200 kDa was excised from the gel, reduced, alykylated with iodoacetic acid and digested with trypsin for 16 hours at 37°C. Samples were desalted as for BryP AT_1 using centrifugal spin columns, but using C18 instead of C4. Samples were directly infused into the FT-ICR MS and analyzed as above, with a loop value of 4, and ion accumulation time of 1s. Protein Prospector MS digest was utilized to predict digestion fragments with methionine oxidation and missed cleavages considered. Protein prospector MS product was also utilized for initial assignment of CID MS/MS spectra. Spectrum were processed in Midas analysis using the Thrash algorithm (Horn et al., 2000). Peptide mass-mass fingerprinting data was then manually assigned.

SUPPLEMENTAL RESULTS

Plasmid Construction and Protein Purification

To investigate BryP function in vitro, *bryP* AT_1 was cloned into pET28b, and overexpressed as an N-terminal 6x His tag fusion protein that was purified to homogeneity via nickel-NTA resin (Supp. Fig. 4). Size exclusion chromatography indicated that BryP AT_1 exists as a monomer. Producing soluble full length BryP and BryP AT_2 was more challenging. Ligation-independent cloning (LIC) (Aslanidis and de Jong, 1990) was used to efficiently clone inserts with a variety of start and stop sites into vectors encoding one of three purification/solubility enhancing tags [6X Histidine alone, 6X His + Maltose binding protein [MBP], or 6X His + Mocr (DelProposto et al., 2008)]. After small scale overexpression and purification via Ni-NTA resin, protein eluents were separated on a Caliper LabChip 90 and quantified to identify the constructs yielding the most soluble BryP or BryP AT_2. Four constructs (BryP AT_1AT_2: residues 1-574; BryP AT_2(construct #32): 288-633, BryP AT_2(37): 294-574; and BryP AT_2(47): 320-588; Supp. Fig. 3) were chosen for large-scale expression as N-term 6X His + MBP fusion proteins. However, these proteins could only be partially purified (Supp. Fig. 3).

Several ACP domains and one peptidyl carrier protein (PCP) domain were chosen to assay the substrate flexibility of BryP, including two ACPs excised from the bryostatin gene cluster (BryA M3 ACP and BryB M7 ACP, Fig. 1A), one ACP from the pikromycin gene cluster (PikAIII M5 ACP), and a PCP domain from the clorobiocin gene cluster (CloN5). These carrier proteins were overexpressed in *E. coli* as 6X His fusion proteins, and purified to homogeneity using Ni-NTA agarose. In addition, three complete PKS modules were targeted for overexpression, including EryAIII M6, PikAIV M6, and BryB M4. EryAIII M6 and PikAIV M6, and their site-directed AT° mutants were overexpressed as 6X His fusion proteins and purified to homogeneity using Ni-NTA agarose. BryB M4 was overexpressed as a MBP fusion protein and was isolated using amylose resin.

FT-ICR MS Analysis of BryB M4

As BryB M4 (cloned as an excised monomodule from the BryB PKS) was only partially purified, we sought to more rigorously identify the protein band at ~ 220 kDa by FT-ICR analysis. Putative BryB M4 protein eluent was separated by SDS-PAGE electrophoresis and the gel was stained with Coomassie Brilliant Blue. The presumed BryB M4-MBP fusion protein band was excised, digested, and peptides were analyzed for protein identification by FT-ICR MS (Supp. Fig. 5). One-hundred twenty out of 253 peptides observed could be assigned to BryB M4 with a mass accuracy of <15 ppm, representing approximately 75% sequence coverage. Fourteen of these peptides were subjected to collision induced dissociation MS/MS and assignments were consistent with those obtained by accurate mass.

Table S1. Plasmids Used in this Study

Name	Plasmid Description	Source/ref
pAKE604	5.9 kb, pMB1 replicon, Ap^r, *oriT*, *lacZα, sacB*	(El-Sayed et al., 2001)
pJH10	14.5 kb, IncQ replicon, Tc^r, Sm^r, *oriT*, *tac*p, *lacI*^{q}	(El-Sayed et al., 2003)
pJHAT101	pAKE604 + 1102 bp PCR fragment with deletion of MmpC AT_1	This study
pJS164	BryP AT_1 cloned *EcoRI-SalI* into pGEM-T-Easy; start-stop 1-285	This study
pJS261	BryP AT_1 cloned *EcoRI-SacI* into pJH10	This study
pJS262	BryP AT_1AT_2 PCR product cloned *EcoRI-SacI* into pJH10; start-stop 1-603	This study
pNL020	BryP AT_1; 0.8 kb PCR product from fosmid subclone (MM5_2_B07) cloned into pET28b; start-stop 1-285	This study
pNL101	BryP AT_1AT_2; 1.7 kb PCR product from MM5_2_B07 cloned into pMCSG9; start-stop 1-574 (89)	This study
pNL102	BryP $AT_1°AT_2$; site-directed mutant of pNL101	This study
pNL103	BryP $AT_1AT_2°$; site-directed mutant of pNL101	This study
pNL104	BryP $AT_1°AT_2°$; double mutant of pNL101	This study
pNL105	BryP AT_2; 1.0 kb PCR product from MM5_2_B07 cloned into pMCSG9; start – stop 288-633 (32)	This study
pNL106	BryP AT_2; 1.0 kb PCR product from MM5_2_B07 cloned into pMCSG9; start – stop 294-574 (37)	This study
pNL107	BryP AT_2; 1.0 kb PCR product from MM5_2_B07 cloned into pMCSG9; start – stop 320-588 (47)	This study
pNL053	BryB M7 ACP; 0.3 kb PCR product from MM5_4_C05 cloned into pET28b	This study
pNL091	BryA M3 ACP; 0.4 kb PCR product from MM20_1_D02 cloned into pMCSG7	This study
pCloN5-pET16b	CloN5; PCR product from cosmid DNA cloned into pET16b	(Garneau et al., 2005)
pMPA404	PikM5 ACP; 0.5 kb PCR product from cosmid DNA (pLZ71) cloned into pET28b	This study
pDHS4188	PikAIV M6 wt; 4.0 kb PCR product from cosmid DNA (pLZ71) cloned into pET24b	(Beck et al., 2003)
pDHS4198	PikAIV M6 AT° (S652A); site-directed mutant of pDHS4188	This study
pJK025	EryAIII M6; PCR product from cosmid DNA into pET21c	Based on
pNL111	EryAIII M6 AT°; site-directed mutant of pJK025 (S622A)	This study
pNL109	BryB M4; 4.7 kb PCR product from subclone MM5_2_B12 cloned into pMCSG9	This study

Table S2. Primers Used to Generate Constructs used in this Study

Primers for *P. fluorescens* MmpC mutant complementation plasmids

BryP_AT1_F	cagaattcatgaaaactatttatcttttcc
BryP_AT2_R	tggagctctcaaaacccatgttttaaagag
Primer_AT11F	ggatccgatacaccgctctgcgcac
Primer_AT11R	aagcttcatgtgacggtattgcgac
Primer_AT12F	aagcttcggagatcatggctatgaaac
Primer_AT12R	gaattccaatctgcgtacgcacgct

Overexpression constructs

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
BryP AT_1	gaggtgaattccatgaaaactattt	gatgatctcgagtttcaaatatgtaaag
BryP AT_1AT_2	fLIC-gtgaagactatttatcttttccctgg	rLIC-atattaaattttcggaattaattgtgtttcttat
BryP AT_2(32)	fLIC-aacttatcatcccatcaaaataaatc	rLIC-aatacagttgaaattatttattaaatgattgattgattc
BryP AT_2(37)	fLIC-aacaaatctaaagaaaattaccaag	rLIC-atattaaattttcggaattaattgtgtttcttat
BryP AT_2(47)	fLIC-atgtattctggacaggg	rLIC-atcaattaaaatattattgcttgtgttct
BryA M3 ACP	fLIC-gaagattccgacaggcttg	rLIC-tatagatggttgatgct
BryB M7 ACP	gagatgaattctgacatcagtc	ctgtatactcgagtttcattctatt
PikAIII M5 ACP	ggcagcatatggcgctggccgcg	ccgcgaagctttcaggtgttacg
BryB M4	fLIC-gtgagttatcgagatatt	rLIC-cgttgctaaccactgcgc

Site-directed mutagenesis

Construct	Forward primer (5'→3')
BryP AT_1°	ccatcatatgttgcaggccatgctttaggtgaatacaatgcact
BryP AT_2°	ccagacgcatttttaggacatgccttaggagaatatattgctgc
EryAIII M6 AT°	ccgtcatcggccatgcgcagggcg
PikAIV M6 AT°	gtcatcggccacgcccagggcgaga

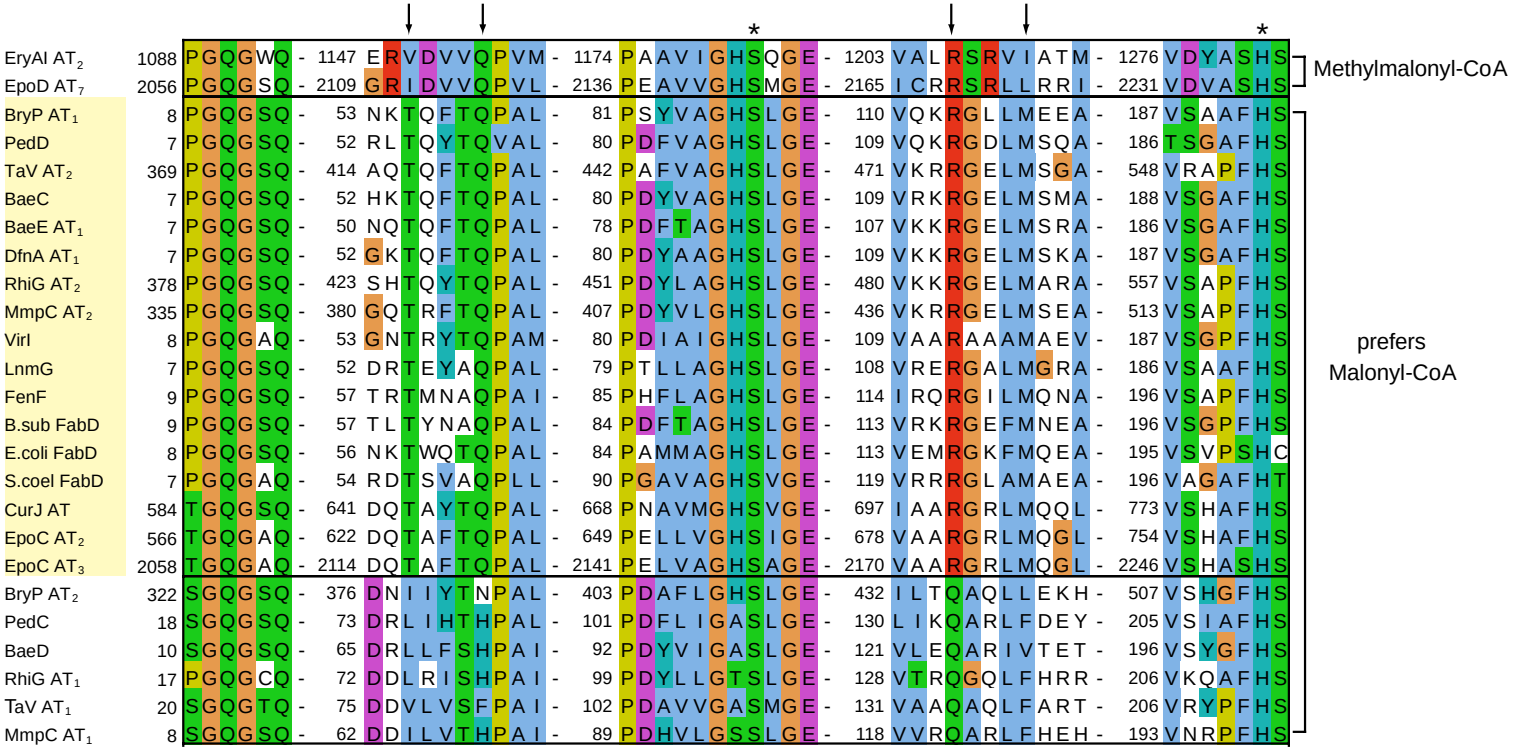


Figure S1. Amino Acid Alignment of Discrete AT Domains from Various PKS and FAS Gene Clusters
Also included are two methylmalonyl-CoA embedded AT domains (EryAI AT_2 and EpoD AT_7) and three malonyl-CoA embedded AT domains (CurJ AT, EpoC AT_2 and AT_3). * indicate active site residues, arrows indicate residues thought to play a role in substrate selection.

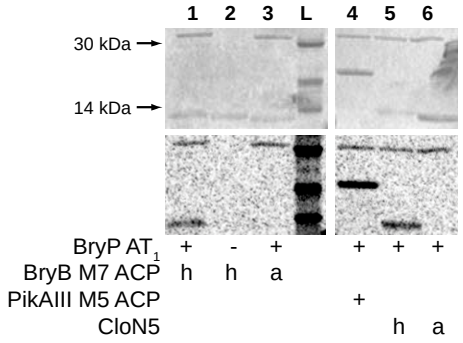
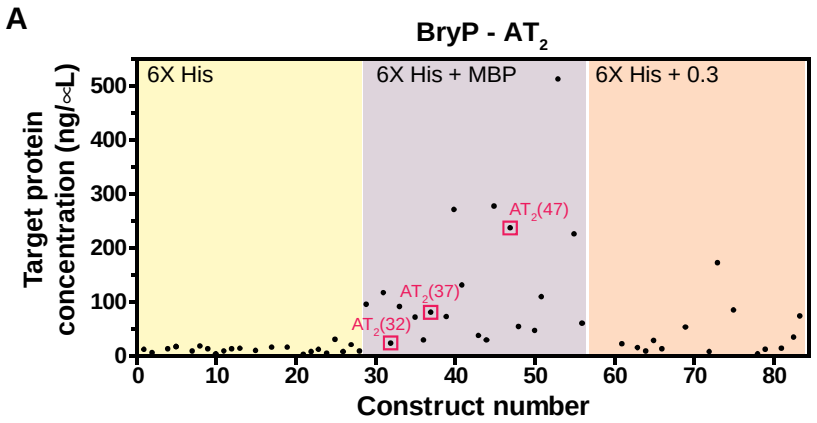
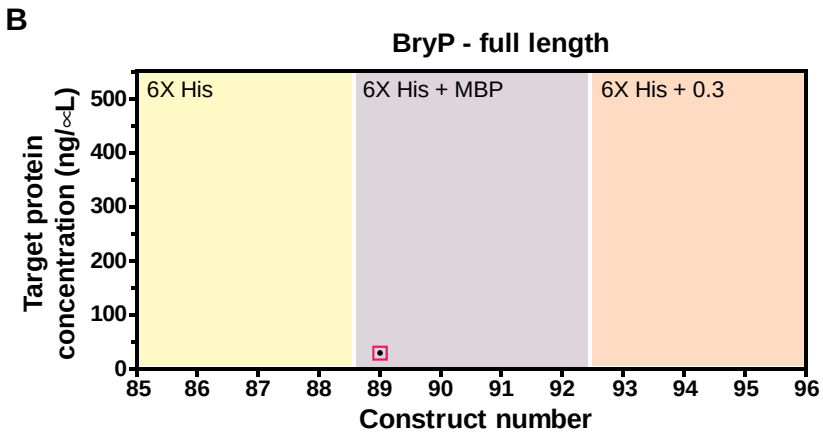
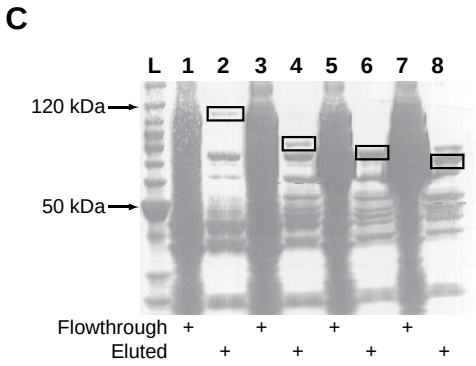


Figure S2. Autoradiography of SDS-PAGE of BryP AT_1 Loading of [^{14}C]-malonyl-CoA onto Various Carrier Proteins

Lane 1 = BryB M7 ACP + BryP AT_1, lane 2 = BryB M7 ACP + no BryPAT_1, lane 3 = *apo* BryB M7 ACP + BryP AT_1, lane L = ladder, lane 4 = PikAIII M5 ACP + BryP AT_1, lane 5 = CloN5 (Garneau et al., 2005) + BryP AT_1, and lane 6 = *apo* CloN5 + BryP AT_1. a = *apo*, h = *holo*.







Construct #			Start-Stop
1	29	57	288-574
2	30	58	288-582
3	31	59	288-588
4	32	60	288-633
5	33	61	291-574
6	34	62	291-582
7	35	63	291-588
8	36	64	291-633
9	37	65	294-574
10	38	66	294-582
11	39	67	294-588
12	40	68	294-633
13	41	69	299-574
14	42	70	299-582
15	43	71	299-588
16	44	72	299-633
17	45	73	320-574
18	46	74	320-582
19	47	75	320-588
20	48	76	320-633
21	49	77	339-574
22	50	78	339-582
23	51	79	339-588
24	52	80	339-633
25	53	81	346-574
26	54	82	346-582
27	55	83	346-588
28	56	84	346-633

85	89	93	1-574
86	90	94	1-582
87	91	95	1-588
88	92	96	1-633

Figure S3. Yield of Protein of Expected Size from Various Constructs of BryP AT_2 and BryP AT_1AT_2

(A–C) SDS-PAGE of His-tag purified overexpressed BryP AT constructs BryP AT_1AT_2 (B) and BryP AT_2 (A). Lanes 1 & 2 = BryP AT_1AT_2, lanes 3 & 4 = BryP AT_2(32), lanes 5 & 6 = BryP AT_2(37), lanes 7 & 8 = BryP AT_2(47). Lanes 1, 3, 5, 7 = flow-through. Lanes 2, 4, 6, 8 = eluted protein.

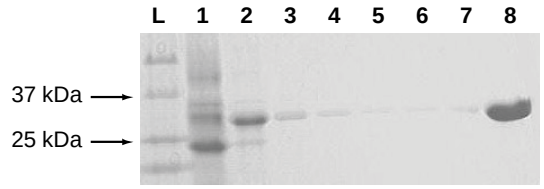


Figure S4. SDS-PAGE of Overexpressed BryP AT Constructs BryP AT_1
Lane L = ladder. Lane 1 = flow-through, lanes 2-7 = rinses, and lane 8 = purified BryP AT_1.

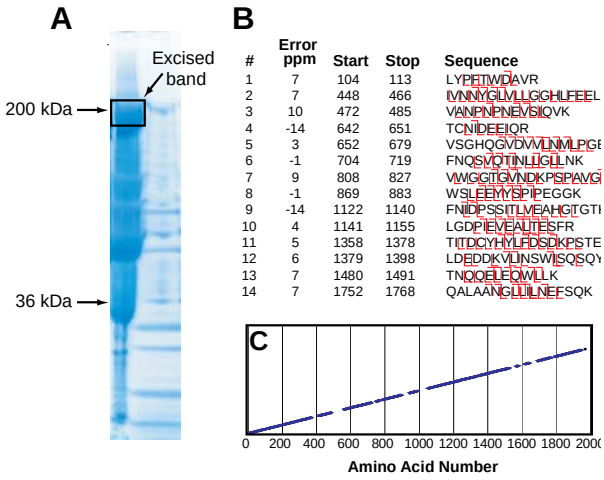


Figure S5.
(A) Putative BryB M4 SDS-PAGE band excised for in-gel digest and FT-ICR MS. Note that gel is over-loaded to increase signal.
(B) Peptides selected for MS/MS confirmation following accurate mass identification. Fragments assigned as a, b, and y-ions displayed.
(C) Sequence coverage, 75%, from accurate mass assignment of tryptic peptides, <15ppm.

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