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Supplemental Data

Cloning, Expression, and Biochemical Characterization of *Streptomyces rubellomurinus* Genes Required for Biosynthesis of Antimalarial Compound FR900098

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Supplemental Experimental Procedures

Bacterial Strains and Plasmids. *S. rubellomurinus* strain 5818 (ATCC 31215) was provided by the Fujisawa Pharmaceutical Co. (Osaka, Japan). *S. lividans* 66 was obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL). WM3118 (*mcrA*, Δ(*mrr*, *hsdRMS*, *mcrBC*), ϕ80(Δ*lacM15*), Δ*lacX74*, *endA1*, *recA1*, *deoR*, Δ(*ara*, *leu*)*7697*, *araD139*, *galU*, *galK*, *nupG*, *rpsL*, *λattB*::pAMG27(P*rhaB*::*trfA33*)) is an *E. coli* DH10B derivative that contains the *trfA33* gene under the control of a rhamnose-inducible promoter, which, when induced by addition of 20 mM rhamnose, allows replication of pJK050 as a high-copy plasmid. WM4489 ((*mcrA*, Δ(*mrr*, *hsdRMS*, *mcrBC*), ϕ80(Δ*lacM15*), Δ*lacX74*, *endA1*, *recA1*, *deoR*, Δ(*ara*, *leu*)*7697*, *araD139*, *galU*, *galK*, *nupG*, *rpsL*, *λattB*::pAE12(P*rhaB*::*trfA33*, Δ*oriR6K-cat::frt5*)) is similar to WM3118, except that the antibiotic resistance marker has been deleted. *E. coli* WM3608(Blodgett *et al.*, 2005) was used as a donor strain for conjugal transfer of plasmids into *S. lividans*. Plasmids used for cloning in the study are shown in Figure S2.

The biosassay strain *E. coli* WM6242 *(lacIq, rrnB3, del(lacZ4787), hsdR514, attP22(EcoB), del(araBAD)567, del(rhaBAD)568, rph-1, del(phnC-P), del(phoA), HK-attB::pJK077(del aadA-oriR6K), lambda-attB::pJK074(delcat-oriR6K)* expresses the phosphonate uptake system encoded by *phnCDE* under the control of a P_{tac} promoter. Induced expression of *phnCDE* by addition 0.5 mM IPTG increases sensitivity to many phosphonate antibiotics. WM6242 is a derivative of BW26678(Haldimann and Wanner, 2001). The plasmids containing *phnCDE*, pJK074 and pJK077, are derivatives of pAE12. The *phoA* and *phn* alleles of BW26678 were deleted by red-recombination, and then the *phnCDE* plasmids were introduced to make WM6186 (*lacIq, rrnB3, del(lacZ4787), hsdR514, attP22(EcoB), del(araBAD)567, del(rhaBAD)568, rph-1, del(phnC-P), del(phoA), HK-attB::pJK077, lambda-attB::pJK074*). The drug markers and plasmid origins were then removed from pJK074 and pJK077 by Flp-mediated recombination to create WM6242.

Identification of PEP Mutase Gene in *S. rubellomurinus*. A single colony of *S. rubellomurinus* 5818 grown on ISP medium 2 agar (Difco) was mixed into 100 µL DMSO, and 1 µL of the mixture was used as a template for PCR in a total volume of 35 µL. PCR was carried out in Failsafe buffer G (Epicentre, Madison, WI) using Taq polymerase and the primers CHIpepmutF1 (5'-CGCCGGCGTCTGCNTNGARGAYAA-3') and CHIpepmutR2 (5'-GGCGCGCATCATGTGRTTNGCVYA-3'), which were designed using CODEHOP(Rose *et al.*, 1998) based on known PEP mutase sequences available in Genbank. The 406 bp product was cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) and sequenced at the University of Illinois Urbana-Champaign Biotechnology Center. The specific primers rubellFor (5'-GACCAGGAGACGATCGAGAG-3') and rubellRev (5'-CTTGGAGTGGATCAGGATGG-3') were designed based on the resulting sequence using the Primer3 software(Rozen and Skaletsky, 2000).

***S. rubellomurinus* Genomic Library Construction.** Genomic DNA was prepared from *S. rubellomurinus* using a modification of the method described by Kieser *et al.*(2000). Briefly, a 20 mL culture of *S. rubellomurinus* 5818 was grown in MYG broth (1 % malt extract, 0.4 % yeast extract, 0.4 % dextrose, pH 7.2) for 48 h, and 3 mL of this culture were used to inoculate 150 mL of S medium(Okanishi *et al.*, 1974) + 0.75% glycine in a 1 L flask baffled with a coiled steel spring. After a 50 h incubation at 30 °C, 25 mL were removed and homogenized using a sterile glass tissue homogenizer. The homogenized cells were pelleted by centrifugation (2000×*g* for 10 min), washed with 10 mL TE25S buffer (25 mM Tris-HCl, 25 mM EDTA, 0.3 M sucrose, pH 8), and resuspended in 10 mL TE25S with 2 mg/mL lysozyme (Sigma-Aldrich, St. Louis, MO). After incubation at 37 °C for 20 min, the now protoplasted cells were pelleted by centrifugation, washed once with 10 mL TE25S, resuspended in 10 mL TE25S and Proteinase K (Invitrogen Corp., Carlsbad, CA) was added to a concentration of 0.15 mg/mL. After a 30 min incubation at 50 °C, sodium dodecyl sulfate (SDS) was then added to a final concentration of 0.5 %. Followed by an additional 5 min incubation at room temperature. The resulting lysate was sequentially extracted with buffer saturated phenol, chloroform, and isoamyl alcohol (25:24:1) and chloroform and isoamyl alcohol (24:1). Genomic DNA was precipitated from the aqueous layer by addition of 0.1 vol of 5 M NaCl and 0.7 vol isopropanol, spooled onto a glass rod, washed three times with 70 % EtOH and once with 100 % EtOH, dried, and resuspended in 2 mL TE buffer, pH 8.0.

Approximately 5 µg of this genomic DNA was partially digested with Sau3AI (New England Biolabs, Beverly, MA) to yield fragments of ~ 20-60 kb, which were then treated with shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) and ligated into BamHI- and NheI-digested pJK050. This vector was treated with shrimp alkaline phosphatase between the NheI and BamHI digests to prevent formation of vector concatamers. The ligated DNA was purified by EtOH precipitation and packaged into lambda phage using the MaxPlax packaging extract (Epicentre, Madison, WI) according to the manufacturer's instructions. *E. coli* WM3118 cells were transfected with the packaged library and plated on LB agar + 12 µg/mL chloramphenicol (Cm).

Screening of Library for PEP Mutase-Encoding Clones. The *E. coli* library was screened by PCR for clones containing the PEP mutase sequence. Each reaction consisted of 1 µL culture broth, 500 nM of each primer (rubellFor and rubellRev), and Taq polymerase in Failsafe buffer G (Epicentre, Madison, WI), and the annealing temperature

was 52 °C. Fosmid DNA was isolated from positive clones grown overnight in 5 mL LB + 12 μg/mL Cm + 20 mM rhamnose using a Qiagen Miniprep kit (Qiagen Inc., Valencia, CA). To add the functions necessary for transfer and integration into *S. lividans*, the purified fosmids were individually recombined *in vitro* with pAE4 using BP clonase (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Fosmid:pAE4 cointegrants were isolated after transformation of *E. coli* DH5α with selection on LB + 12 μg/ml Cm + 50 μg/ml apramycin (Apr) and subsequently moved into the conjugal donor strain *E. coli* WM3608(Blodgett *et al.*, 2005) for transfer to *S. lividans.* Conjugal transfer to *S. lividans* 66 was performed as described by Martinez *et al.*(Martinez *et al.*, 2004), with the exception that the entire *E. coli*/*S. lividans* mixture was spotted onto R2 (minus sucrose) agar in 2 μL aliquots. After 16-20 h at 30 °C, plates were flooded with 2 ml of a mixture of 1 mg/mL each nalidixic acid (Nal) and Apr and incubated at 30 °C for an additional 5-7 days, at which point exconjugants were picked and restreaked on LB + Nal (50 μg/mL) + Apr (50 μg/mL) + Cm (12 μg/mL) and grown for 3-4 days.

Production of FR900098 by *S. lividans* 4G7. *S. lividans* 4G7 was plated on Hickey-Tresner agar(Kieser *et al.*, 2000) or ISP4 and incubated at 30 °C. After 4 days, the agar-solidified medium was liquefied by freezing and subsequent thawing, and the liquid portion (containing the produced antibiotic) was decanted from the remaining agar residue.

Purification of *E. coli* DXP Reductoisomerase. The *E. coli dxr* gene was amplified from genomic DNA using PCR with colidxrF (5'-GCTCTAGAGCATATGAAGCAACTCACCATTCTGG- 3') and colidxrR (5'-GCGCAGATCTCAGCTTGCGAGACGC-3') primers (35 cycles, annealing temp 55 °C). The resulting product was digested with NdeI and BglII (New England Biolabs, Beverly, MA) and cloned into the same sites in pET15b (Novagen, San Diego, CA). The resulting plasmid was transformed into *E. coli* Rosetta (DE3) (Novagen, San Diego, CA), and expression of the *dxr* gene was induced by addition of IPTG to a final concentration of 0.5 mM in a 500 mL culture grown in LB broth + 12 μg/mL Cm + 50 μg/mL ampicillin at 30 °C to an OD_{600} of 0.8. The culture was incubated at 30 °C for an additional 4 h, at which point the cells were harvested and lysed by addition of lysozyme. The lysate was cleared by centrifugation, and the enzyme was purified by metal affinity chromatography using Ni-NTA agarose (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The purified enzyme was concentrated and stored in 20 mM Tris, 0.2 mM DTT, 10 % glycerol, pH 8.0.

Enzyme-Inhibition Assay for FR900098 Detection. Activity of the *E. coli* DXR enzyme was measured spectrophotometrically by observing the decrease of absorbance at 340 nm resulting from oxidation of NADPH during the reaction. Each reaction contained 100 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 1 mg/ml BSA, 100 μM 1-deoxy-D-xylulose 5-phosphate (DXP; Echelon Biosciences Inc., Salt Lake City, UT), 150 μM NADPH, and ~ 15 nM DXR in a total volume of 120 μL. Spectra were recorded every 5 s for 5 min on a Hewlett-Packard 8453 diode-array spectrophotometer. The background rate of NADPH oxidation was recorded before addition of enzyme to each reaction. To determine the presence or absence of FR900098 in culture supernatant, 5 μL of unconcentrated supernatant was included in the enzyme reaction, and the reaction rate was compared to a control with 5 μL of supernatant from a non-producing strain.

Phosphonate Specific Bioassay. WM6242 was grown to an OD_{600} of 0.8 in LB at 37 °C. Three mL of LB top agar, with and without 1 mM IPTG, was inoculated with 50 µl of the culture before addition to corresponding LB plates with and without 1 mM IPTG. Filter discs (6 mm, Becton Dickinson and Company, Sparks, MD) were impregnated with 9 µl of the test solutions and placed on the surface of the solidified plates. The bioassay plates were incubated at 37 °C and the size of the growth inhibition zone scored after 12 hours. To confirm the heterologous production of FR900098, inhibition zones from unconcentrated *S. lividans* 4G7 culture supernatant, were compared to supernatant from the *S. lividans* parent strain and a solution of authentic FR900098 (50µg/ml).

^{31}P-NMR Detection of FR900098. Culture supernatant was concentrated approximately 10-fold by evaporation, and D_2O was added to 15 % as a lock solvent. ^{31}P-NMR spectra were recorded on a Varian Unity 500 spectrophotometer with a 5-mm QUAD probe (Nalorac Cryogenics, Martinez, CA) at the Varian Oxford Instruments Center for Excellence in the Nuclear Magnetic Resonance Laboratory at the University of Illinois at Urbana–Champaign. Spectra were externally referenced to an 85 % phosphoric acid standard set at 0 ppm. To confirm that the observed peak corresponded to FR900098, an authentic sample of FR900098 (Invitrogen Corp., Carlsbad, CA) was added to a concentration of 100 µg/mL, and the spectrum of the sample was recorded again under identical conditions.

Mass Spectrometric Detection of FR900098, N-acetyl-3-Aminophosphonate, and 2-Phosphonomethylmalate. Culture supernatant was concentrated approximately 10-fold by evaporation, and methanol was added to a final concentration of 90%. Precipitated material was removed by centrifugation, and the samples were again concentrated 10-fold by evaporation. Precipitated material was removed by centrifugation, and the samples were brought up to 75% acetonitrile for HILIC chromatography. The samples were injected onto a 2.1 × 50 mm Atlantis HILIC silica column (Waters) flowing at 200 µL / minute and eluted over 70 minutes in a gradient from 5% 100mM ammonium acetate (pH 4) in 95% acetonitrile + 0.1% acetic acid to 60% 100mM ammonium acetate (pH 4) in 40% acetonitrile + 0.1% acetic acid. The eluent was infused into a LTQ-FT mass spectrometer (ThermoFisher Scientific) for negative ion FTMS detection and the identities of FR900098 and *N*-acetyl-3-aminophosphonate were confirmed by both accurate mass analysis (< 2 ppm) and tandem MS fragmentation.

2-phosphonomethylmalate was analyzed by injection of 20 µL of the *in vitro* reaction mixture (*vide infra*) onto a 2.1 × 100 mm C18 Synergi Fusion column (Phenomenex). Compounds were eluted with a 20 minute gradient of 5% acetonitrile + 0.1% formic acid in 95% water + 0.1% formic acid to 50% water + 0.1% formic acid in 50% acetonitrile + 0.1% formic acid into a custom 12T LTQ-FT Ultra mass spectrometer (ThermoFisher Scientific) for negative ion FTMS detection. Transfer of an acetyl group to phosphonopyruvate to generate 2-phosphonomethylmalate was confirmed by both accurate mass analysis (< 1 ppm) and high-resolution tandem MS fragmentation.

Sequencing of Fosmid 4G7. A library of transposon insertions was generated using the mini-Mu transposon encoded in pAE5. Transposition reactions of BglII-digested pAE5 (mini-MuAE5 transposon) and the pAE4-retrofitted fosmid 4G7 (target DNA) were conducted *in vitro* using MuA transposase (MJ Research, Waltham, MA) according to the manufacturer's instructions. *E. coli* WM4489 was transformed with the reaction products, and successful insertions were selected on LB + 25 µg/mL kanamycin (Km). Fosmid

DNA was isolated from approximately 200 colonies that were individually picked into 2 mL LB + 20 mM rhamnose + 12 μg/mL Cm + 25 μg/mL Km and incubated overnight at 37 ºC. The fosmid DNA was sequenced using the primers seqAETnR (5'-TAGGAACTTCGGGATCCGTT-3') and SEQAETnL (5'-TCGCCTTCTTGACGAGTTCT-3') at the University of Illinois Urbana-Champaign Biotechnology Center. The sequence was assembled using Sequencher (Gene Codes Corp., Ann Arbor, MI) and the remaining gaps were filled in by obtaining further sequence using specifically designed primers. Potential open-reading frames were identified using BLAST analysis(Altschul *et al.*, 1990), GeneMark(Borodovsky and McIninch, 1993), and visual inspection.

Deletion Analysis of the Biosynthetic Cluster. To determine the portion of the cosmid required for antibiotic production, various deletions were made by site-specific recombination between frt or loxP sites present adjacent to the cloning site of the fosmid vector and the matching site on selected mini-Mu transposon insertions. These deletions remove all DNA between the site of the transposon insertion (chosen based on the DNA sequencing results) and the cloning junction in fosmid 4G7. To do this, cells of *E. coli* WM4489 containing the selected 4G7::mini-MuAE5 plasmids were transformed with either pCP20 (expressing Flp recombinase under control of a temperature sensitive promoter(Cherepanov and Wackernagel, 1995)) or pAE19 (expressing Cre recombinase under control of the same promoter), and transformants were selected on LB + 100 μg/mL ampicillin (Amp) at 30 ºC. After single colony isolation, the transformed cells were plated on LB + 12 μg/mL Cm and incubated at 42 ºC. Individual colonies were then screened for sensitivity to Km and Amp. The resulting recombinant plasmids were integrated into *S. lividans* 66 as described above, and exconjugants were assayed for the ability to produce FR900098 using the *E. coli* DXR inhibition assay. Production was further verified by mass spectrometry as described above. The complete sequence of this 14.4 kb region has been deposited in GenBank with accession number DQ267750.

Cloning, Expression and Purification of FrbC and FrbD. The *frbC* and *frbD* genes were obtained by PCR amplification from the fosmid 4G7. Primers for *frbC* (5'-GCTATTAAT*CATATG*CGCAACGACTTAGTGCTCGAGG-3' and 5'-CGAAATAT*AAGCTT*TCAGGCGGCGGCCTTGTTGTAGATC-3') and *frbD* (5'-GCTATTAAT*CATATG*ACCAAGCGAACCATGTTACGCAG-3' and 5'-CGAAATAT*AAGCTT*TCAGCGCTGGAGCTCGAAGACCTCG -3') contained *Nde*I and *Hind*III sites (restriction sites underlined and in italics), and the amplified genes were cloned into the corresponding sites of pET28a. *E. coli* BL21(DE3) (Novagen, San Diego, CA) was transformed with the resulting plasmids. The correct sequences of the cloned expression vectors were confirmed by DNA sequencing.

E. coli BL21(DE3) cells overexpressing *frbC* or *frbD* were grown in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg mL^{-1}). Cells were grown at 37 °C to OD_{600}=0.6-0.8 and then induced by the addition of IPTG to a final concentration of 0.2 mM. The culture was incubated at 30 °C for an additional 6-8 h, after which the cells were harvested by centrifugation (15 min at 10,000×g) and resuspended in 20 mM Tris-HCl (pH 7.65 at 4 °C), 15% glycerol, and 0.5 M NaCl (buffer A). Lysozyme (1 mg/mL) was added to the resuspended cells and the sample was freeze-thawed. The cells were further lysed by sonication (Fisher Scientific Model 500 Ultrasonic

Dismembrator) with the amplitude set at 30% and with a pulse sequence of 5s on and 10s off, for 5 min. The cell debris was removed by centrifugation (20 min at 10,000×g).

The N-His_6-tagged-proteins were purified using immobilized metal affinity chromatography (IMAC) coupled with fast performance liquid chromatography (FPLC). TALON™ Superflow Co^{2+} resin (Clontech, Mountain View, CA) was charged and equilibrated according to the manufacturer's instructions. Proteins were concentrated and desalted using a Millipore Amicon Ultra-4 Centrifugal Filter Devices (MWCD 10 kDa) at 4000×g at 4 °C, with 50 mM MOPS buffer (pH 7.2). Concentrated protein was stored at -80 °C. Protein concentration was determined using the calculated molar extinction coefficient at 280 nm (FrbC, ε_{280}= 30500 $M^{-1}cm^{-1}$; FrbD, ε_{280}=16560 $M^{-1}cm^{-1}$).

Phosphonomethylmalate Formation. Phosphonomethylmalate synthase activity was assayed by tracking CoASH formation in a reaction similar to that used for phosphinomethylmalate synthase(Shimotohno *et al.*, 1988). The assay mixture (1 ml) contained 50 mM HEPES-K^+ (pH 7.2), 5 mM $MgCl_2$, 0.3 mM acetyl-CoA, 5 mM phosphoenolpyruvate or 1 mM phosphonopyruvate, 0.2 mM 5,5'-dithiobis(2-nitro-benzois acid) (DTNB), and enzyme (1.6 µM FrbC and/or 2.5 µM FrbD). The reaction was incubated at 30 °C and was initiated by addition of PEP or PnPy. The reaction was followed for 300 s by the increase in absorbance at 412 nm (extinction coefficient 13.6 mM^{-1} cm^{-1}). Phosphonomethylmalate product formation was confirmed by LCMS as described above.

Supplemental References

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Table S1. Homologs of Putative Proteins Encoded by the FR900098 Biosynthetic Gene Cluster

Predicted ORF	Top BLASTp Hit in Genebank[1]	Identities[2]	Expect[3]
FrbA (886 aa)	ZP_01724555.1 *Bacillus sp.* B14905 Aconitate hydratase (901 aa)	444/885 (55%)	0.0
FrbB (352 aa)	NP_926034.1 *Gloeobacter violaceus* PCC 7421 Isocitrate dehydrogenase (359 aa)	68/262 (25%)	7e-09
FrbC (373 aa)	AAW57042.1 cyanobacterium endosymbiont of *Rhopalodia Gibba* Homocitrate synthase (377 aa)	160/361 (44%)	1e-83
FrbD (276 aa)	ZP_02023307.1 *Nitrosopumilus maritimus* SCM1 Putative phosphoenolpyruvate phosphomutase (299 aa)	126/257 (49%)	2e-64
FrbE (365 aa)	NP_926034.1 *Gloeobacter violaceus* PCC 7421 Isocitrate dehydrogenase (359 aa)	181/363 (49%)	1e-82
FrbF (286 aa)	YP_001375271.1 *Bacillus cereus* subsp. cytotoxis NVH 391-98 Aminoglycoside N3'-acetyltransferase (269 aa)	115/252 (45%)	8e-63
FrbG (437 aa)	NP_793346.1 *Pseudomonas syringae* pv. tomato str. DC3000 hypothetical protein (466 aa)	134/433 (30%)	6e-38
FrbH (628 aa)	NP_928710.1 *Photorhabdus luminescens* subsp. laumondii TTO1 hypothetical protein: putative aminotransferase (618 aa)	193/601 (30%)	8e-82
FrbI (206 aa)	YP_001539547.1 *Salinispora arenicola* NUDIX hydrolase CNS-205 (188 aa)	83/171 (48%)	2e-39
FrbJ (339 aa)	ZP_01742840.1 *Rhodobacterales* bacterium HTCC2150 hypothetical protein: putative aKG-dependent oxygenase (328 aa)	106/325 (32%)	7e-43
DxrB (417 aa)	NP_823739.1 *Streptomyces avermitilis* MA-4680 1-deoxy-D-xylulose 5-phosphate reductoisomerase (394 aa)	253/389 (65%)	7e-128

[1]Results are from a BLAST search of the GenBank protein database on November, 2007.
[2]Identities in the aligned region.
[3]Expectation value.

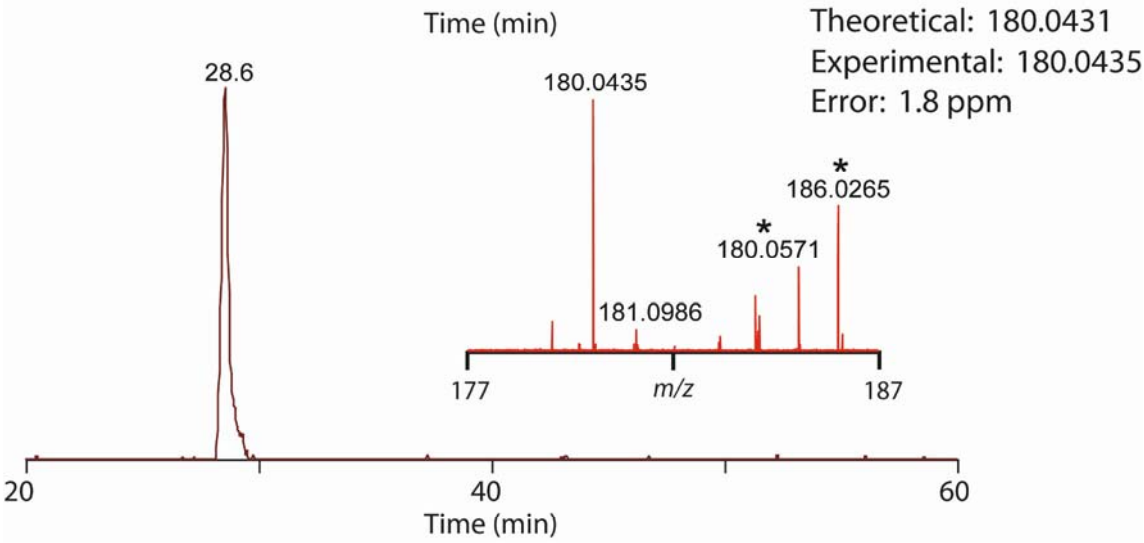

Figure S1. Selected Ion Chromatogram for N-Acetyl-3-Aminophosphonate +/- 2 ppm From an Injection of Culture Supernatant With Summed Mass Spectrum

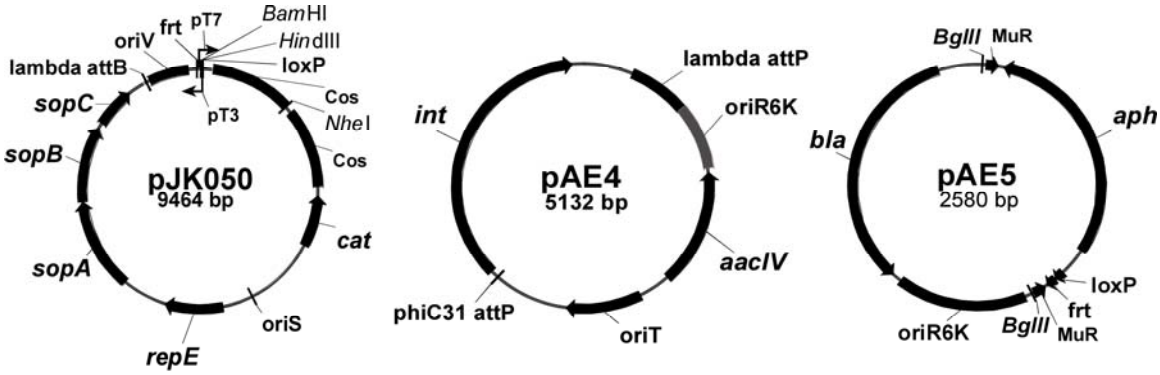

Figure S2. Graphic Maps of pJK050, pAE4 and pAE5
pJK050 is a double-cos(Bates, 1987) fosmid vector that has *oriV* (from plasmid RP4) to allow copy control in hosts that allow regulated expression of the gene for the replication protein TrfA. Either BamHI or HindIII can be used for cloning sites. Note that the insert can be transcribed via flanking T7 and T3 promoters. Note also that the cloning sites are flanked with loxP and frt sites (the recognition sites for the Flp and Cre recombinases, respectively) to allow deletion of DNA from the cloning junction up to the sites of insertion of the mini-MuAE5 transposon. These are very useful to delimit the DNA within the insert needed for expression of a trait encoded by the insert (e.g., antibiotic production). The plasmid has lambda *attB* to allow efficient retro-fitting with helper plasmids such as pAE4. pAE4 encodes *oriT* to allow mobilization of the plasmid into

other bacteria via the RP4 *tra* system, along with the phiC31 *int* gene and *attP* to allow insertion into the chromosome of most actinomycetes via site specific recombination(Bierman et al., 1992). It encodes the *aacIV* gene, which confers resistance to apramycin in both *E. coli* and *Streptomyces* spp. (Kieser et al., 2000). It can be efficiently inserted into pJK050-derived clones using BP-clonase (Invitrogen, Carlsbad, CA) in an *in vitro* reaction. Retrofitting is mediated by site-specific recombination between the lambda *attB* on pJK050 and lambda *attP* on pAE4. pAE5 carries a mini-Mu transposon (designated mini-MuAE5) that can be simultaneously used for DNA sequencing and for functional analysis of plasmid clones. In addition to serving as a mobile priming site for DNA sequencing reactions, the transposon also has loxP and frt sites at one end. These can recombine with the frt or loxP sites flanking the cloning site on pJK050, thus deleting all DNA between the transposon insertion and the cloning junction with pJK050. Complete plasmid sequences and details of construction are available upon request from the authors.