Supplementary Information

Unusual transformations in the biosynthesis of the antibiotic phosphinothricin tripeptide

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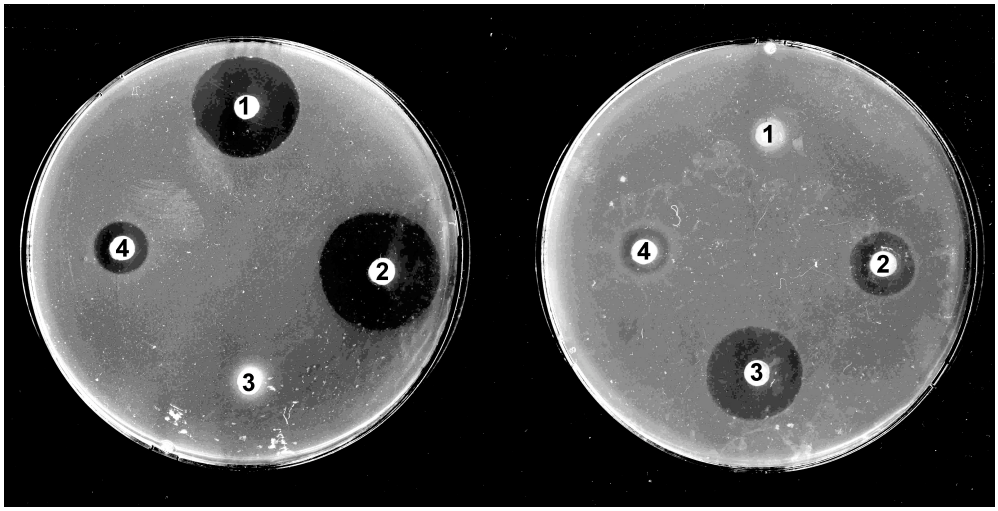
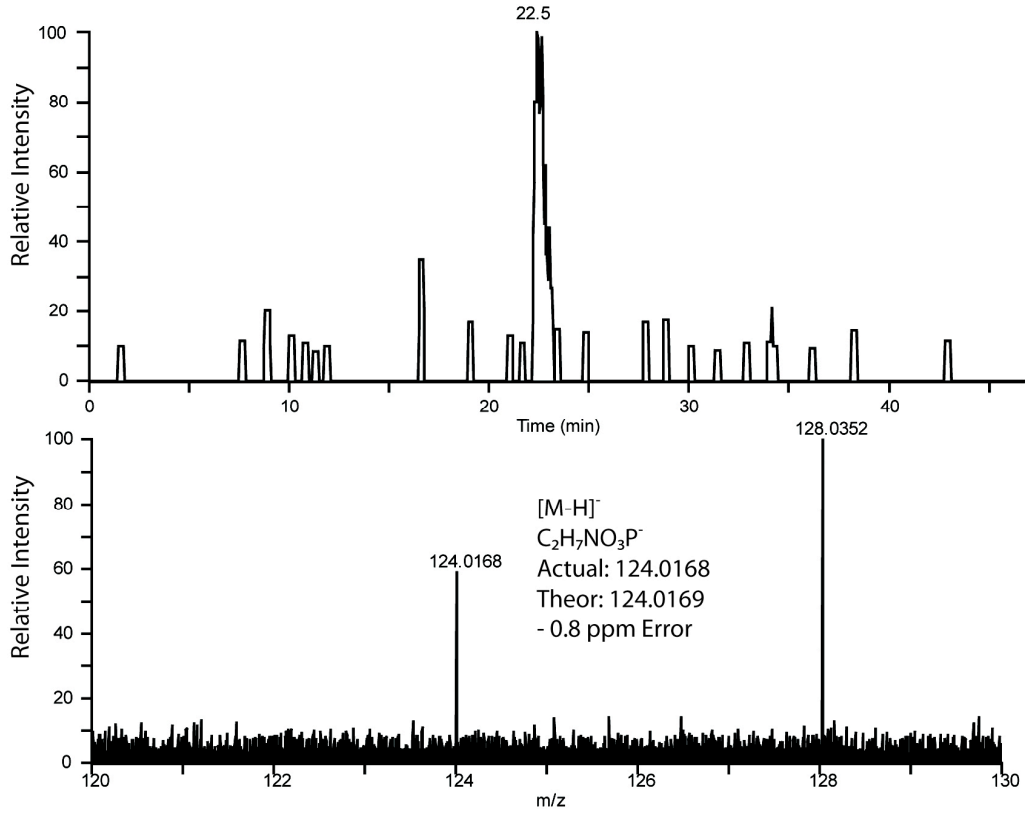
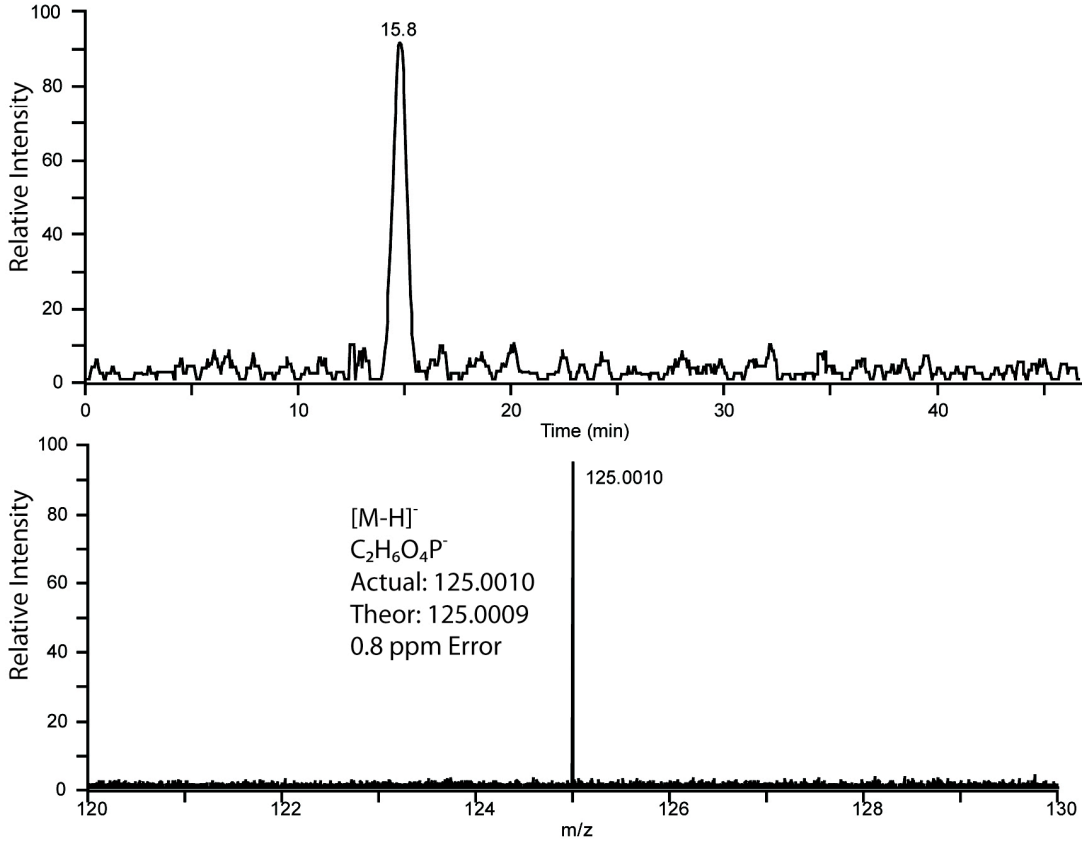
Supplementary Figures



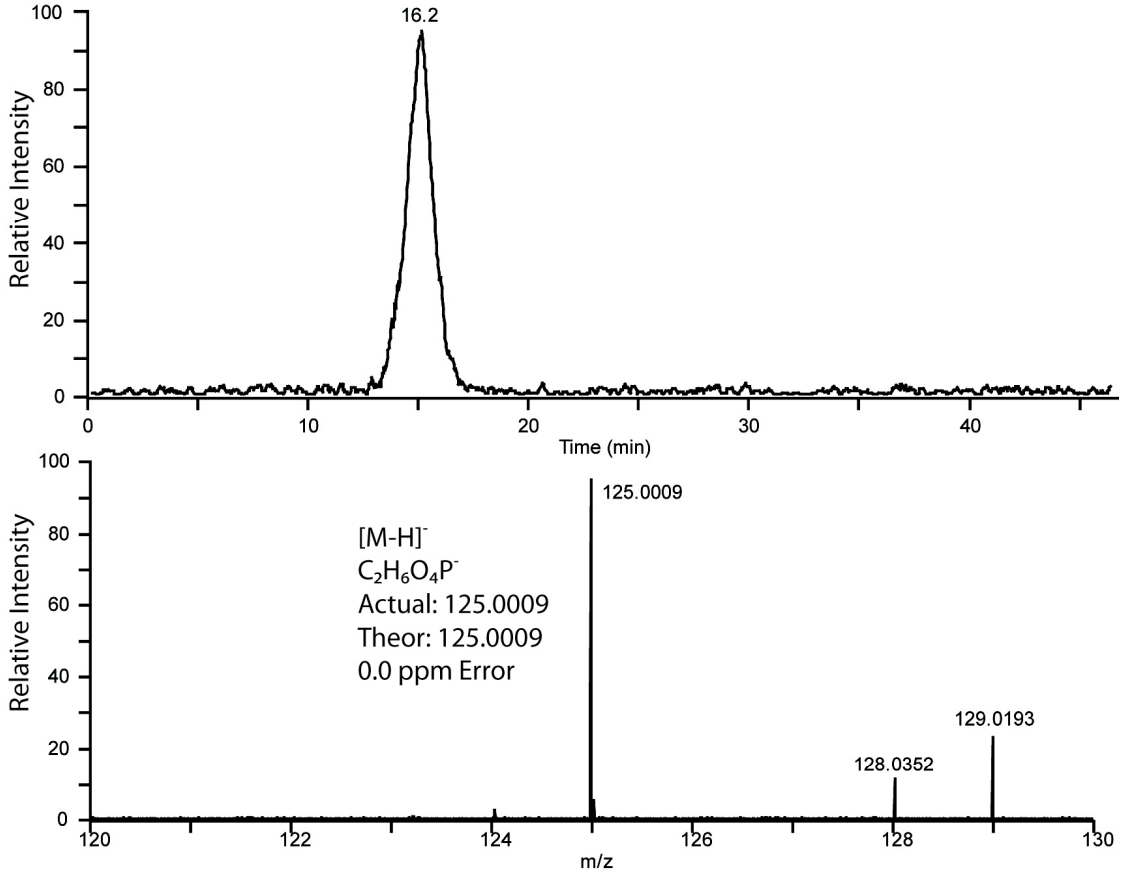
Supplementary Figure 1. Bioassays of agar cores cut from ISP2 plates inoculated with *Streptomyces viridochromogenes* Δ*php* mutants and plated against PTT-sensitive *Bacillus subtilis* indicator strain ATCC6633. Left bioassay plate, core 1 WM6548 (Δ*phpJ*), core 2 WM5302 (parent strain [+ control]), core 3 WM6601 (Δ*phpD*), core 4 WM6546 (Δ*phpF*). Right bioassay plate, core 1 WM6604 (Δ*phpH*), core 2 WM6549 (Δ*phpG*), core 3 WM6547 (Δ*phpC*), core 4 WM6602 (Δ*phpE*).



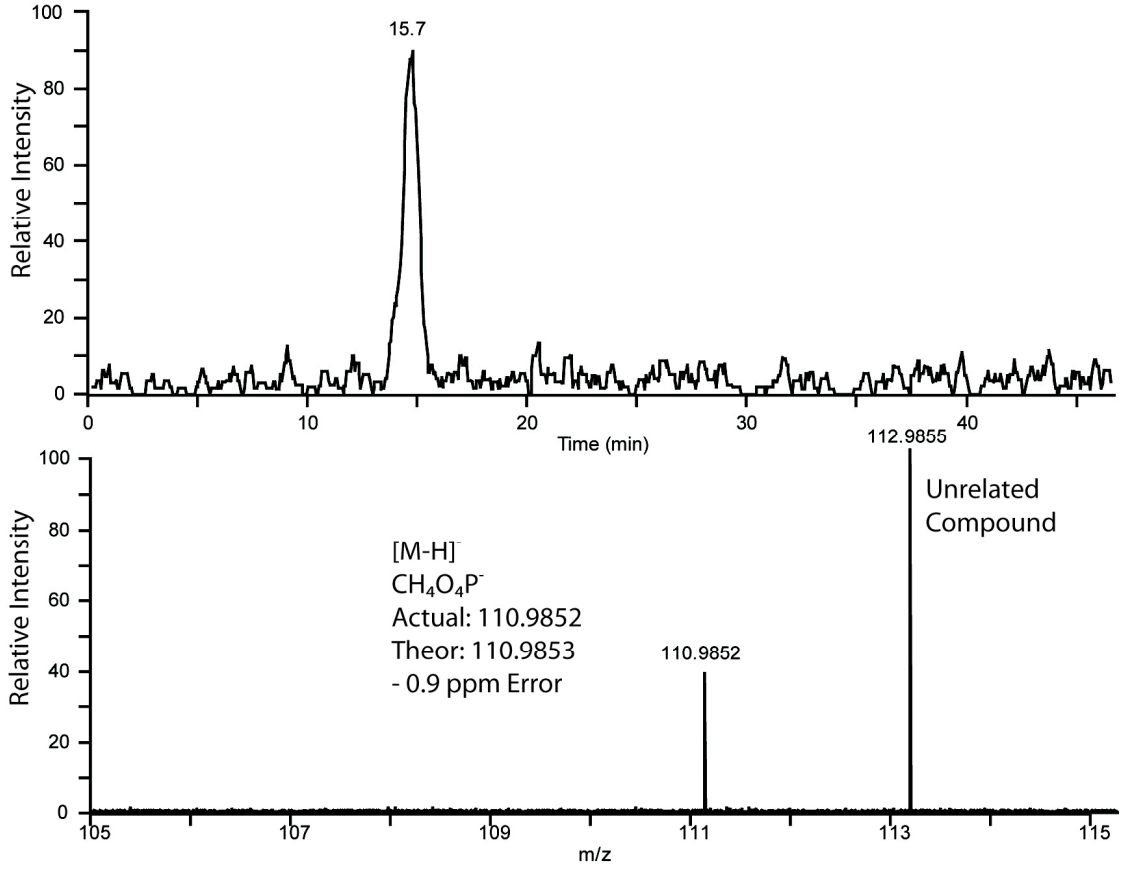
Supplementary Figure 2. LC/MS detection of 2-aminoethylphosphonate from culture supernatants of a *S. viridochromogenes* Δ*phpC* mutant. Upper panel, selected ion chromatogram for 2-aminoethylphosphonate +/- 0.01 Da from an injection of 50μL of concentrated WM6492 supernatant. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



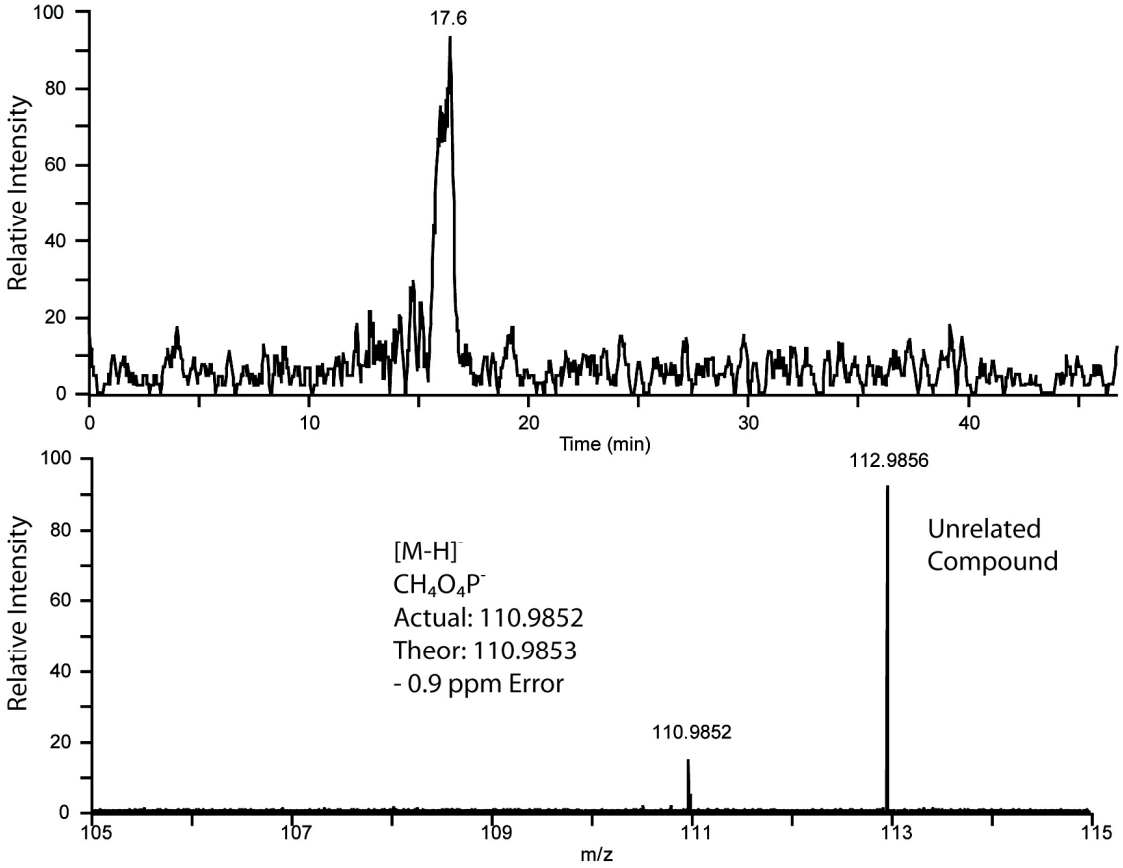
Supplementary Figure 3. LC/MS detection of hydroxyethylphosphonate from the *in vitro* reduction of phosphonoacetaldehyde by His-PhpC. Upper panel, selected ion chromatogram for 2-hydroxyethylphosphonate +/- 0.01 Da from an injection of 50μL reaction mixture. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



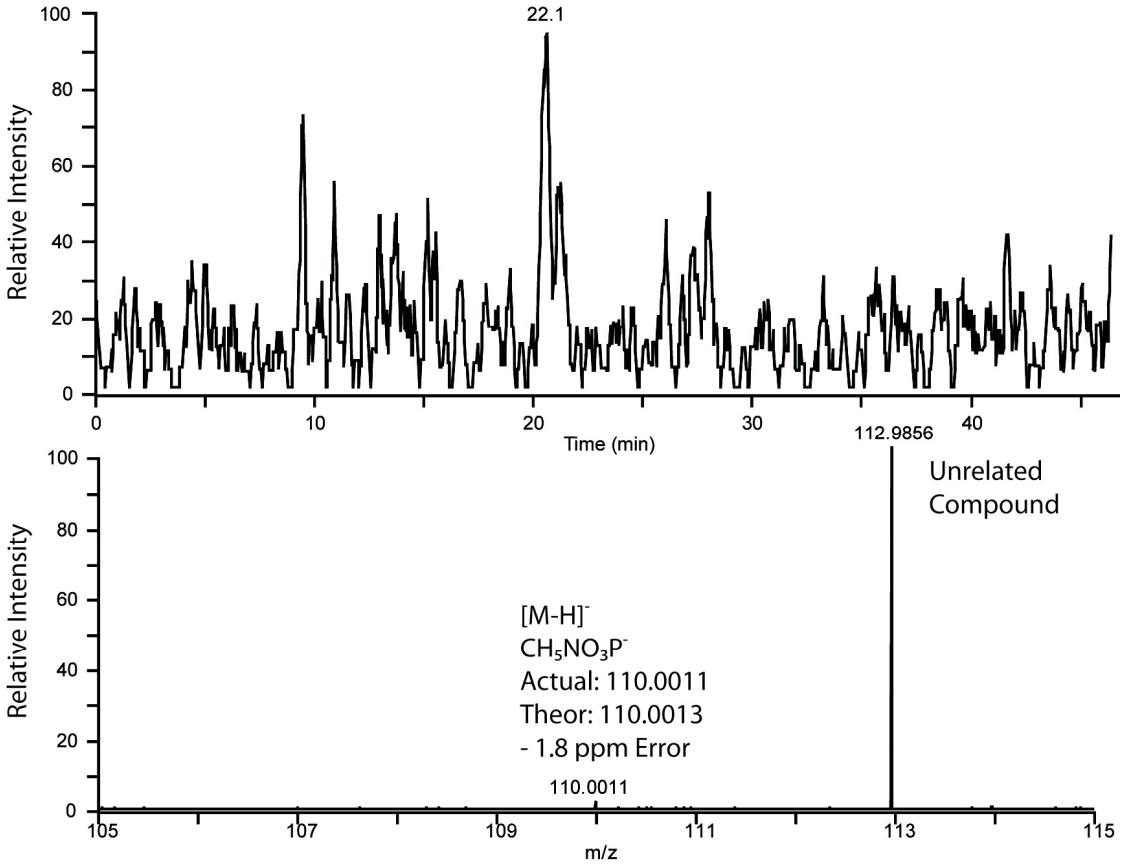
Supplementary Figure 4. LC/MS detection of 2-hydroxyethylphosphonate from culture supernatants of a *S. viridochromogenes* Δ*phpD* mutant. Upper panel, selected ion chromatogram for 2-hydroxyethylphosphonate +/- 0.01 Da from an injection of 50μL of concentrated WM6601 supernatant. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



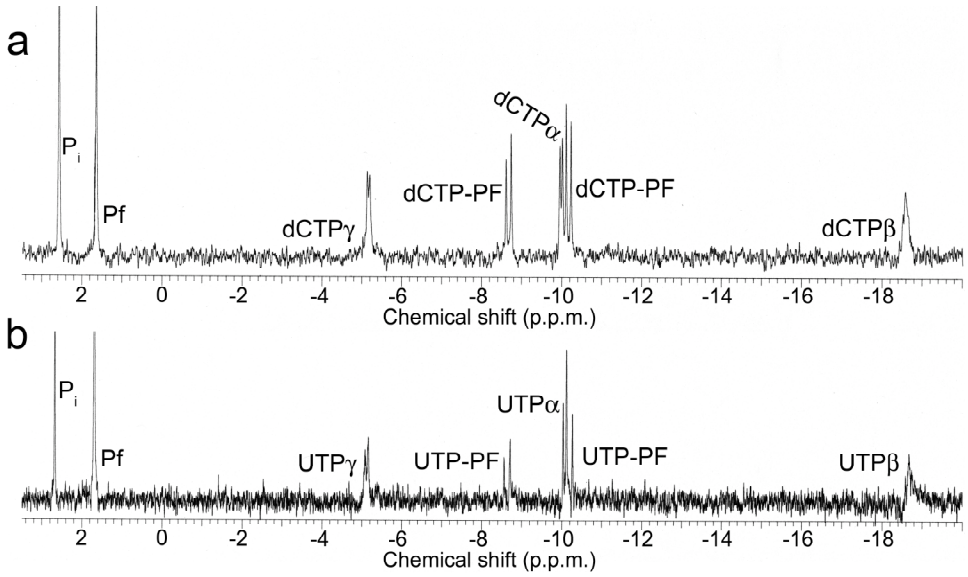
Supplementary Figure 5. LC/MS detection of hydroxymethylphosphonate from the *in vitro* cleavage of hydroxyethylphosphonate by PhpD. Upper panel, selected ion chromatogram for hydroxymethylphosphonate +/- 0.01 Da from an injection of 50μL of reaction mixture. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



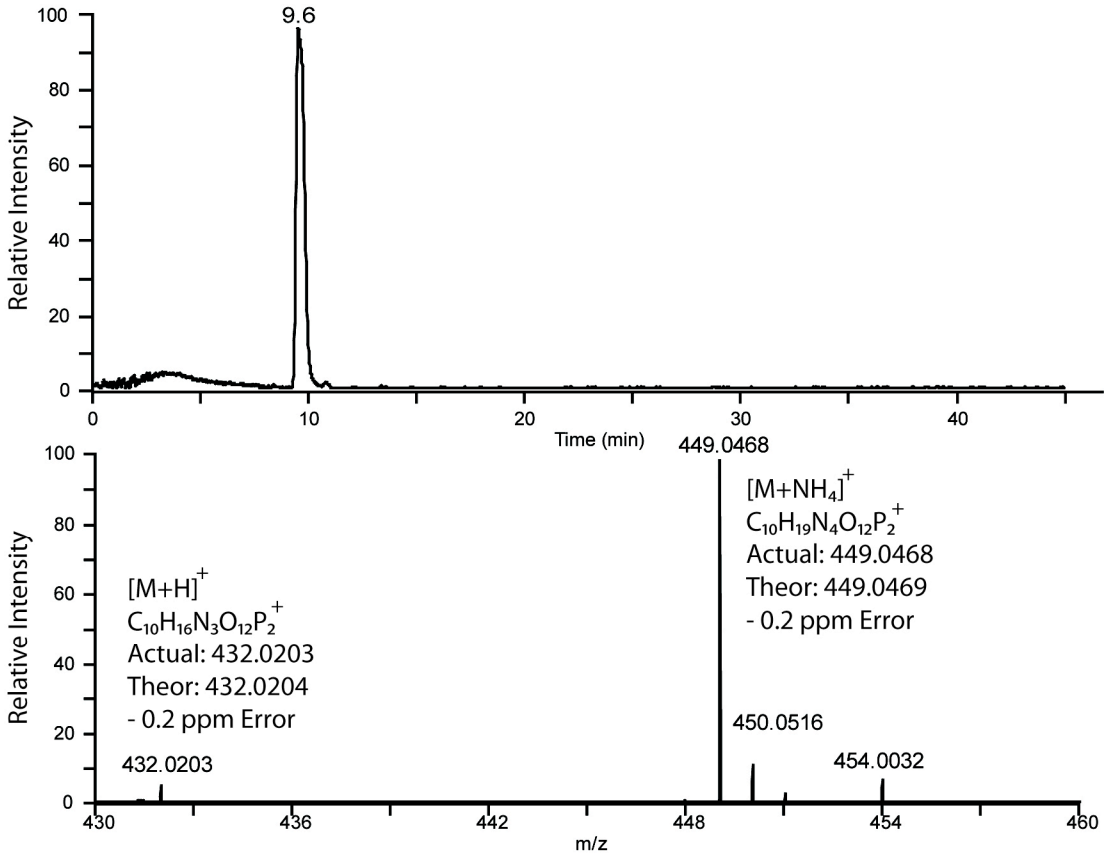
Supplementary Figure 6. LC/MS detection of hydroxymethylphosphonate from culture supernatants of a *S. viridochromogenes* Δ*phpE* mutant. Upper panel, selected Ion Chromatogram for hydroxymethylphosphonate +/- 0.01 Da from an injection of 50μL of concentrated WM6602 supernatant. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



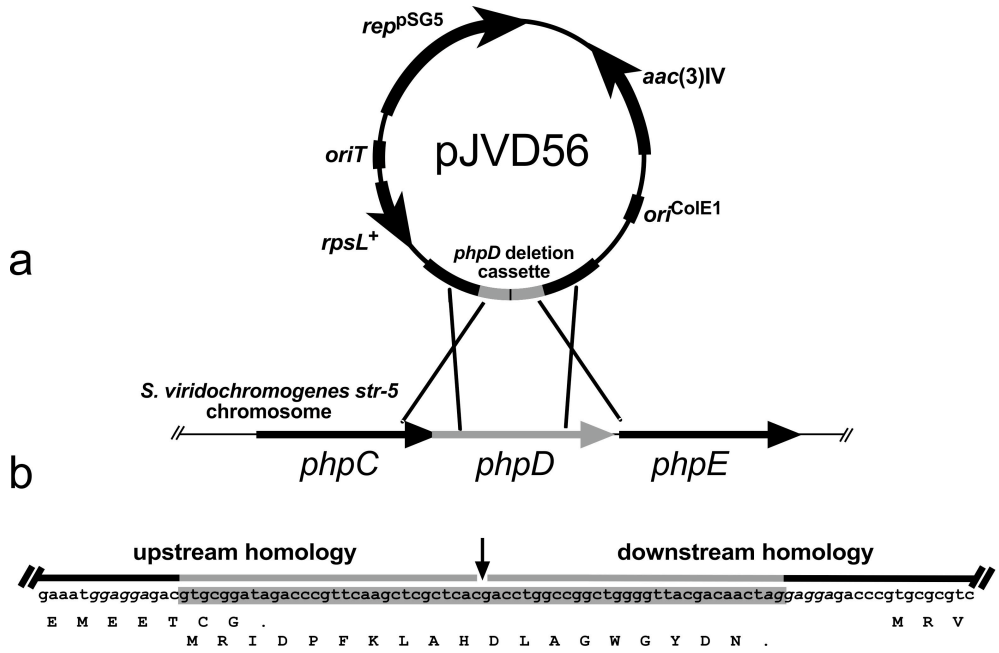
Supplementary Figure 7. LC/MS detection of aminomethylphosphonate from culture supernatants of a *S. viridochromogenes* Δ*phpJ* mutant. Upper panel, selected ion chromatogram for aminomethylphosphonate +/- 0.01 Da from an injection of 50μL of concentrated WM6488 supernatant. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors.



Supplementary Figure 8. ^{31}P NMR results of assays illustrating the incomplete conversion of nucleotides other than CTP to phosphonoformylated products with His-PhpF. (**a**) A reaction containing His-PhpF, inorganic pyrophosphatase, dCTP and phosphonoformate showing the partial conversion of phosphonoformate (Pf) and dCTP into dCMP-5'-phosphonoformate (dCMP-PF) and phosphate (P_i). (**b**) A reaction containing His-PhpF, inorganic pyrophosphatase, UTP and phosphonoformate showing the partial conversion of phosphonoformate and UTP into UMP-5'-phosphonoformate (UTP-PF) and phosphate. The putative α, β, and γ designations of the nucleotide phosphorus nuclei have been assigned by comparison to those analogously found in ATP [1]. The peaks associated with dCMP-PF and UMP-PF were assigned by comparison those analogously found for CMP-5'-phosphonoformate. In both spectra, the tops of the phosphate and phosphonoformate peaks are not shown to fit them to reasonably sized figures.



Supplementary Figure 9. LC/MS detection of CMP-5'-PF from the *in vitro* assay of His-PhpF. Upper panel, selected ion chromatogram for CMP-5'-PF +/- 0.01 Da from an injection of 50μL of reaction mixture. Lower panel, summed mass spectra from chromatographic peak showing HRMS errors for both the protonated species and the ammonium adduct.



Supplementary Figure 10. Scheme used for the deletion of phosphinothricin tripeptide biosynthetic genes. (**a**) The deletion of *phpD* has been chosen as a specific example, although all other deletions constructed for this work were similarly made. The gray areas within the 'deletion cassette' denote DNA sequence encoding portions of *phpD* (not to scale). The dark line separating the upstream and downstream regions of homology indicates the point of where the two fragments were joined by PCR fusion. Tandem recombination events between the chromosome and the deletion plasmid allows for the unmarked removal of most of the *phpD* coding sequence. (**b**) The chromosomal scar resulting from unmarked mutagenesis. The arrow indicates the junction between the upstream and downstream homology regions originally encoded by the deletion cassette. The nucleotides in gray encode the truncated PhpD protein, for which the complete translated sequence is presented directly below. Fragments of both the 3' terminus of *phpC* and the 5' terminus of *phpE* and the corresponding amino acid residues they encode have been included. They are shown clarity and illustrate the complicating issue of overlapping sequences in the PTT gene cluster. Stop codons have been designated by periods and putative ribosome-binding sites have been denoted in italics.

Supplementary Tables

Supplementary Table 1. Microorganisms and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference[a]
Escherichia coli		
DH5α/λ*pir*	λ*pir*/Φ80d*lac*ZΔM15, Δ(*lacZYA-argF*)U169, *rec*A*1*, *hsdR17, deoR, thi-1, supE44, gyrA96, relA1*	[2]
Rosetta(DE3)pLysS	F-, *ompT, hsdS*$_B$(r$_B$- m$_B$-), *gal, dcm* (DE3)/ pLysSRARE (Cam^R)	Novagen
WM3780	*dam*-3, *dcm*-9, *metB1*, *galK2*, *galT27*, *lacY1*, *tsx*-78, *supE44*, *thi-1*, *mel-1*, *tonA31 att*HK:: pJK202Δ(*ori*R6K-*aad*A)::Frt	[3]
WM6026	*lacI*^{q}, *rrnB3*, Δ*lacZ*4787, *hsdR514*, Δ*araBAD567*, Δ*rhaBAD568, rph-1, att*λ::pAE12(Δ*ori*R6K-*cat*::Frt5), Δ*endA*::Frt, *uidA*(ΔMluI)::*pir*, *att*HK::pJK1006Δ(*ori*R6K-*cat*::Frt5; *trfA*::Frt)	J.K. Zhang and W. Metcalf, unpublished
WM4491	Rosetta(DE3)pLysS/pJVD18	This study
WM6047	Rosetta(DE3)pLysS/pJVD26	This study
WM5392	Rosetta(DE3)pLysS/pJVD30	This study
Bacillus subtilis		
ATCC6633	PTT-sensitive bioassay strain	ATCC
WM4445	PTT-resistant derivative of ATCC 6633	[3]
Streptomyces coelicolor A3(2)	Wild type (NRRL B-16638)	ARS
Streptomyces lividans 66	Wild type (NRRL B-16148)	ARS
WM6409	*S. lividans*/pJVD63	This study
Streptomyces hygroscopicus	Wild type (ATCC 21705)	ATCC
Streptomyces viridochromogenes	Wild type (DSM 40736)	DSM
WM5302	*S. viridochromogenes str*-5	This study
WM6547	*S. viridochromogenes str*-5 Δ*phpC*	This study
WM6601	*S. viridochromogenes str*-5 Δ*phpD*	This study
WM6602	*S. viridochromogenes str*-5 Δ*phpE*	This study
WM6546	*S. viridochromogenes str*-5 Δ*phpF*	This study
WM6549	*S. viridochromogenes str*-5 Δ*phpG*	This study
WM6604	*S. viridochromogenes str*-5 Δ*phpH*	This study
WM6548	*S. viridochromogenes str*-5 Δ*phpJ*	This study
Plasmids		
pET11a	Amp^R *E. coli* protein expression vector	Novagen
pET15b	Amp^R *E. coli* histidine-tag fusion expression vector	Novagen
pET-DUET-1	Amp^R *E. coli* protein expression vector	Novagen
pAE4	Apr^R, *oriT*, *ori*R6K, ΦC31*att*P, ΦC31*int*, λ*attP* vector	[4]

pPB37	Amp^R *E. coli* cloning vector, *ori*R6K	[5]
pSH19	Thio^R *Streptomyces* protein over-expression vector	[6]
pKC1139	Apr^R , *oriT, rep*$^{pSG5(ts)}$, *rep*^{pUC} shuttle vector	[7]
pOJ260	Apr^R , *oriT, rep*^{pUC} *E. coli* cloning vector	[7]
fosmid 5-9G	Contains PTT biosynthetic gene cluster	[3]
pJVD17	pET11a digested with BamHI/XbaI and ligated to *ppd* PCR product amplified from *B.fragilis* chromosomal DNA with primers Fragilisppd FOR and Fragilisppd REV and digested with BglII/XbaI	This study
pJVD18	pJVD17 digested with BamHI/XbaI and ligated to *ppm* PCR product amplified from *B.fragilis* chromosomal DNA with primers Fragilisppm FOR and Fragilisppm REV and digested with BglII/XbaI	This study
pJVD25	pET-DUET digested with BglII/XbaI and ligated to *phpC* PCR product amplified from fosmid 5-9G with primers phpC FOR and phpC REV and digested with XbaI/BglII	This study
pJVD26	pET-DUET digested with BglII/XbaI and ligated to *phpD* PCR product amplified from fosmid 5-9G with primers phpD FOR and phpD REV and digested with XbaI/BglII	This study
pJVD29	pET-DUET digested with BglII/XbaI and ligated to *phpF* PCR product amplified from fosmid 5-9G with primers phpF FOR and phpF REV and digested with XbaI/BglII	This study
pJVD30	pJVD29 digested to release *phpF* with BglII/NdeI, ligated to pET15b digested with BamHI/NdeI	This study
pJVD39	pKC1139 digested with ClaI and ligated to *rpsLG* PCR product amplified from *S. coelicolor* genome with primers Sc rpsLG5' and Sc rpsLG3' and digested with ClaI	This study
pJVD40	pJVD39 digested with BamHI/EcoRI and ligated to fusion PCR generated *phpE* deletion cassette digested with BamHI/EcoRI	This study
pJVD41	pOJ260 digested with ClaI and ligated to *rpsLG* fragment from pJVD40 released by ClaI digest	This study
pJVD42	pJVD41 digested with EcoRV/XbaI and ligated to fusion PCR generated *phpC* deletion cassette digested with EcoRV/XbaI	This study
pJVD43	pJVD41 digested with EcoRI/XbaI and ligated to fusion PCR generated *phpD* deletion cassette digested with EcoRI/XbaI	This study
pJVD44	pJVD41 digested with EcoRI/XbaI and ligated to fusion PCR generated *phpF* deletion cassette digested with EcoRI/XbaI	This study

pJVD45	pJVD41 digested with EcoRI/XbaI and ligated to fusion PCR generated *phpG* deletion cassette digested with EcoRI/XbaI	This study
pJVD46	pJVD41 digested with EcoRI/XbaI and ligated to fusion PCR generated *phpH* deletion cassette digested with EcoRI/XbaI	This study
pJVD47	pJVD41 digested with EcoRI/XbaI and ligated to fusion PCR generated *phpJ* deletion cassette digested with EcoRI/XbaI	This study
pJVD48	pSH19 digested with SpeI and ligated to pPB37 digested with XbaI, yielding a fusion of both complete plasmids	This study
pJVD52.1	pKC1139 digested with BglII and ligated to *rpsL* PCR product amplified from *S.coelicolor* genome with primers Sc rpsL5' and Sc rpsL3' and digested with BglII	This study
pJVD53	pJVD48 digested with AscI and ligated to *oriT* fragment from pAE4 released by AscI digest	This study
pJVD56	pJVD52.1 digested with EcoRI/XbaI and ligated to the EcoRI/XbaI flanked *phpD* deletion cassette of pJVD43	This study
pJVD59	pJVD25 digested to release *phpC* with BglII/NdeI , ligated to pET15b digested with BamHI/NdeI	This study
pJVD63	pJVD59 digested to release *N-hisphpC* with EcoRI/XbaI, ligated to pJVD53 digested with EcoRI/XbaI	This study
pJVD73	pJVD52.1 digested with EcoRV/XbaI and ligated to the EcoRV/XbaI flanked *phpC* deletion cassette of pJVD42	This study
pJVD75	pJVD52.1 digested with EcoRI/XbaI and ligated to the EcoRI/XbaI flanked *phpF* deletion cassette of pJVD44	This study
pJVD76	pJVD52.1 digested with EcoRI/XbaI and ligated to the EcoRI/XbaI flanked *phpG* deletion cassette of pJVD45	This study
pJVD77	pJVD52.1 digested with EcoRI/XbaI and ligated to the EcoRI/XbaI flanked *phpH* deletion cassette of pJVD46	This study
pJVD78	pJVD52.1 digested with EcoRI/XbaI and ligated to the EcoRI/XbaI flanked *phpJ* deletion cassette of pJVD47	This study

[a]ATCC, American Type Culture Collection, Manassas, Va; ARS, U.S. Department of Agriculture Agricultural Resource Service, Peroria, Ill.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

Supplementary Table 2. Oligonucleotides used in this study

Oligonucleotide	DNA sequence
phpC KO F1	GGCGCGTCTAGAGCCCGACAGCCGGGAGATC
phpC KO F2	GTTTCCCGGTGAACTGCTGCGATCGGAAATGGAGGAG
phpC KO R1	GGCGCGCCGATATCGAGGCCATGGAGGCCGC
phpC KO R2	TCTCCTCCATTTCCGATCGCAGCAGTTCACCGGGAAACAC
phpD KO F1	GGCGCGCCTCTAGACCACACCGGACAGCGTCG
phpD KO F2	CCCGTTCAAGCTCGCTCACGACCTGGCCGGCTGGG
phpD KO R1	GGCGCGCCGAATTCAACGCCCTCGGCCACGC
phpD KO R2	CCCAGCCGGCCAGGTCGTGAGCGAGCTTGAACGGG
phpE KO F1	GGCGCGCCGAATTCCCTGAACAACGGCCACCTC
phpE KO F2	CTACACCGACCCCGCCTGGACCCCGCCGGCCGAGGG
phpE KO R1	GGCGCGCCGGATCCCCCTGCACCGGCGCGAG
phpE KO R2	CCCTCGGCCGGCGGGGTCCAGGCGGGGTCGGTGTAG
phpF KO F1	GGCGCGCCTCTAGACTGGGCAACATGACCAAC
phpF KO F2	GCAGATCGCCGGGACGGGCGGAATCGATGAGCGCATCC
phpF KO R1	GGCGCGCCGAATTCCGCGTCGGCCGGGTCCG
phpF KO R2	GGATGCGCTCATCGATTCCGCCCGTCCCGGCGATCTGC
phpG KO F1	GGCGCGCCTCTAGACGTCGGCGCCCACGCCC
phpG KO F2	CGGCGCCGACCGGCCGGTGCTCACCGGGTCGGACGGAG
phpG KO R1	GGCGCGCCGAATTCCTGACCGTGCCGGCCTG
phpG KO R2	CTCCGTCCGACCCGGTGAGCACCGGCCGGTCGGCGCCG
phpH KO F1	GGCGCGCCTCTAGAGGGACCCACAGACCCGGC
phpH KO F2	CTCCGTCAGGCTGCGCGGCTCCCTGGCGGGCCTCGTGA
phpH KO R1	GGCGCGCCGAATTCGGTGATCAGTCCTGTTCG
phpH KO R2	TCACGAGGCCCGCCAGGGAGCCGCGCAGCCTGACGGAG
phpJ KO F1	GGCGCGCCTCTAGAGACGCAAGGCACCTTCG
phpJ KO F2	CGGAGCACTGCAGCCGGCCACCCTTCCCGGCCCCTACG
phpJ KO R1	GGCGCGCCGAATTCGCGCATGGGGACGTTGAAG
phpJ KO R2	CGTAGGGGCCGGGAAGGGTGGCCGGCTGCAGTGCTCCG
phpC FOR	GGCGCGCCTCTAGAAATAATTGGATCCAACTTTAAGAAGGAGATATACATATGACCGCTGTGT TTCCCGG
phpC REV	GGCGCGCCAGATCTCTATCCGCACGTCTCC
phpD FOR	GGCGCGCCTCTAGAAATAATTGGATCCAACTTTAAGAAGGAGATATACATATGCGGATAGAC CCGTTC
phpD REV	GGCGCGCCAGATCTCTAGTTGTCGTAACCC
phpF FOR	GGCGCGCCTCTAGAAATAATTGGATCCAACTTTAAGAAGGAGATATACATATGAGCGCCGAG CAGATC
phpF REV	GGCGCGCCAGATCTTCAGGCCCGGATGCGC
Sc rpsL5’	GGCGCGCCAGATCTCTTCGTCCGCCACGACACG
Sc rpsL3’	GGCGCGCCAGATCTTTACTTCTCCTTCTTGGCGCC
Sc rpsLG5’	GGCGCGCCATCGATCTTCGTCCGCCACGACACG
Sc rpsLG3’	GGCGCGCCATCGATCGTCTTGCCCGCGTCGATG
ShygroPhpH F	GCCATCGAGGAGATCGAC
ShygroPhpH R	CGGAGAACACGTTGACCATC
ShygroPhpG F	AAGGTCGTGAAGGAGTGTGG
ShygroPhpG R	GGCACCCGAATCGGACT
FragilisPpm FOR	GGCGCGCCTCTAGAAATAATTGGATCCAACTTTAAGAAGGAGATATACATATGAACAAGAAA ATC
FragilisPpm REV	GGCGCGCCAGATCTTTATTTAGTGCCCGG
FragilisPpd FOR	GGCGCGCCTCTAGAAATAATTGGATCCAACTTTAAGAAGGAGATATACATATGGTAAGTGTA GAA
FragilisPpd REV	GGCGCGCCAGATCTTCATGAATGCGTATC

Supplementary Methods

DNA manipulation, PCR, cloning and sequencing. Plasmid constructions (Supplementary Table 1) were performed using standard techniques[8]. Restriction enzymes and T4 DNA ligase were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA). Plasmid DNA isolation was performed using Qiagen (Valencia, CA) miniprep or maxiprep kits. DNA fragments used in cloning and for sequencing were extracted from agarose gels using the Promega (Madison, WI) SV Wizard Gel and PCR Cleanup System. *Streptomyces* genomic DNA for hybridization analysis was isolated by the CTAB method[9]. DNA hybridization analyses were performed using Hybond-N nylon membrane (Amersham, Buckinghamshire, UK) and results were visualized using the DIG system (Roche Applied Science, Indianapolis, IN). PCR was routinely performed using FailSafe PCR PreMix buffers (Epicentre, Madison, WI) with KOD Hotstart DNA proofreading polymerase (Novagen, Madison, WI). *Streptomyces* colony PCR was performed using the method of Van Dessel *et al.*[10]. The oligonucleotides used in this study (Supplementary Table 2) were purchased from Integrated DNA Technologies (Coralville, IA). Portions of *Streptomyces hygroscopicus* PTT biosynthetic genes were amplified from chromosomal DNA by PCR and products were sequenced using the same primers used for amplification at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois.

Deletion of PTT biosynthetic genes in *S. viridochromogenes*. Deletion mutants of PTT genes in *S. viridochromogenes* were constructed using *rpsL* dominance in a

selection/counter-selection scheme[11] that results in unmarked deletions (Fig. S1). Mutagenesis was performed essentially as described elsewhere[12] except that the plasmids we used required Apr instead of Thio as the initial selective agent. To isolate a Str^R *S. viridochromogenes* mutant for deletion analysis, wild-type spores (~7.2×10^8) were applied to ISP2 Str^{20} plates. Resulting resistant colonies arose at a frequency of ~6.6×10^{-8} and were streaked to ISP2 Str^{100} to ensure stable streptomycin resistance. A subset of Str^R *S. viridochromogenes* isolates were examined for continued PTT production and sporulation. Based upon these criteria, one Str^R mutant, *S. viridochromogenes str-5*, was selected and subsequently used as parent for making all of the deletion mutants described here.

For our deletion experiments, we constructed two similar (*rpsL*$^+$) vectors, pJVD39 or pJVD52.1. Both are based upon the *Streptomyces- E. coli* shuttle vector pKC1139, which contains a *Streptomyces*-specific temperature-sensitive origin of replication and an apramycin resistance marker (*aac*(3)IV) for selection in both *Streptomyces* and *E. coli*. Plasmids pJVD39 and pJVD52.1 both contain the *rpsL*$^+$ gene of *Streptomyces coelicolor* (*rpsLG*$^+$ in pJVD39) that allows for a dominant streptomycin-sensitive phenotype in an *rpsL* (Str^R) background. The regions of homology necessary to delete genes by the unmarked method were constructed by two sequential sets of PCR reactions. First, the individual upstream and downstream regions of homology were PCR amplified individually. The upstream homology regions were PCR amplified by primer pairs designated KO F1 and KO R2 and the downstream homology regions were similarly amplified using primer pairs KO F2 and KO R1. The second round of PCR was done to fuse the upstream and downstream regions by PCR overlap extension[13, 14] to form a 'deletion cassette'. After cloning into a counter-selectable vector, the 'deletion cassettes'

are designed to delete the majority of a gene though they preserve the coding sequence of the first 10 amino acids fused in frame with sequence encoding the last 9 amino acids plus stop codon (Supplementary Figure 10). This results in a severely truncated gene product when translated, but preserves the coding sequences of flanking genes and minimizes the likelihood of polar effects. This was done because previous analysis of the PTT biosynthetic gene cluster indicated that many genes appeared to have start codons within the ends of upstream genes[3] and we did not want to delete bases common to other genes.

Deletion plasmids based upon the *Streptomyces- E. coli* shuttle vectors pJVD39 or pJVD52.1 were conjugated from the *E. coli* donor WM3780 to *S. viridochromogenes str-5*, selecting for Apr^R colonies at 30 °C. After streaking exconjugants on medium containing Apr^{30} and Nal^{25} (to select against the *E. coli* donor), individual colonies were picked to MYG liquid and grown non-selectively for at least 3 days at 39 °C to force integration of the deletion plasmids by homologous recombination. These cultures were spread to plates containing Apr at 39°C and individual colonies were streaked in parallel to medium containing Str at the same temperature. A subset of Apr^R Str^S colonies were cultured in MYG liquid without selection at 39°C to allow for the second recombination event. This culture was plated to ISP2 containing Str to select for segregants. Str^R colonies were screened for Apr^S, and Str^R Apr^S colonies were checked via colony PCR for the expected deletion events. Strains believed to be deletion mutants were subjected to two sequential rounds of sporulation before verification again by PCR and by DNA hybridization. In this manner, the following genes were deleted from *S. viridochromogenes str-5* (listed with the deletion vector used during mutagenesis): *phpC*

(pJVD73), *phpD* (pJVD56), *phpE* (pJVD40), *phpF* (pJVD75), *phpG* (pJVD76), *phpH* (pJVD77) and *phpJ* (pJVD78).

Overexpression, purification and activity assay of His-PhpF. WM5392 cells were grown in LB Cm^{12} Amp^{100} broth to mid-log phase at 37°C. IPTG was added to 1 mM final concentration and cells were harvested via centrifugation after 5 hours of induction. Cell pellets carrying overexpressed His-PhpF were resuspended in 50mM HEPES-Na^+ (pH 7.25) buffer containing 10% glycerol, 0.3 M NaCl, and 20 mM imidazole (buffer A). Cells were broken 2× in a French pressure cell (20,000 psi) and centrifuged at 13,000 ×*g* to pellet cell debris and the resultant supernatant was added to a Ni-NTA agarose (Invitrogen) gravity flow column pre-equilibrated with buffer A. Contaminating *E. coli* proteins were washed off the column in a stepwise fashion with buffers identical to buffer A, but with varying concentrations of imidazole (10, 50, 100 and 200 mM). His-PhpF was collected from the column after washing with buffers containing 250 or 500 mM imidazole. His-PhpF after affinity chromatography was purified to apparent homogeneity, as ascertained by SDS-PAGE followed by Coomassie Brilliant Blue staining. Although the protein appeared ~7 kDa larger than the expected based on SDS-PAGE, it had a mass of 22804.4 Daltons (based on FT-MS analysis), which is in close agreement with the theoretical mass of His-PhpF (minus the amino-teriminal methionine) of 22804.5 Da (2.5 ppm mass error). High-purity His-PhpF was dialyzed (Slide-A-Lyzer 10,000 MWCO, Pierce, Rockford, IL) overnight at 4 °C, with two buffer exchanges, against 50 mM HEPES-K^+ buffer (pH 7.25) containing 10% glycerol, 50mM sodium L-glutamate, 50 mM L-arginine monohydrochloride and 2 mM dithiothreitol (DTT). Glutamate and arginine were added to the dialysis buffer because they known to stabilize

proteins[15] and attempts to dialyze in the absence of these amino acids invariably led to precipitation of His-PhpF. Dialyzed protein was frozen at –70 °C until directly used for analysis.

Initial phosphonoformate-nucleotidyltransferase activity was assayed in 50 mM HEPES-K^+ 10 mM $MgCl_2$ buffer (pH 7.25) containing 1 mM CTP, 1 mM PF, 5U of yeast inorganic pyrophosphatase (Sigma) and 5 μg of His-PhpF. Reactions were incubated at 30 °C for 1 hour before analysis by ^{31}P NMR or LC/MS. Data from LC/MS experiments were acquired by sample separation on a Thermo Surveyor HPLC using a 2.1 mm x 50 mm Hypercarb column (ThermoFisher Scientific) operated at 500 μL/minute. A 30 minute gradient (100:0 A:B to 70:30 A:B where A was 25 mM ammonium acetate, pH 4 and B was acetonitrile + 0.1% acetic acid) was employed to separate the nucleotide from buffer salts. The eluent was directly infused into a LTQ-FT mass spectrometer where high-resolution Fourier-Transform mass spectrometry (FT-MS) data were acquired in positive ion mode.

Creation of a *Streptomyces* host for His-PhpC overexpression. His-PhpC was over-expressed in *Streptomyces lividans* using the "P_{nitA}-NitR" system recently developed by Herai et al[6]. We did not, however, directly use the published pSH19 vector for our studies because it does not have any *E. coli* functions to facilitate cloning and other common molecular methods. Instead, we modified pSH19 for use as a shuttle vector by the addition of an *E. coli* origin of replication (*ori*R6K) and resistance marker (*bla*). To facilitate *Streptomyces* strain construction, an origin of conjugal transfer (*oriT*) was also added, giving rise to the generally useful shuttle vector pJVD53. pJVD53 was used for

over-expression of HisPhpC after cloning *hisphpC* under control of P_{nitA} (pJVD63). The *S. lividans* over-expression strain, WM6409, was created by conjugation of pJVD63 from the *E. coli* donor WM6026 essentially as previously described[12]. The culture conditions for WM6026 differ from that of the other conjugal donor used in this study, WM3780, because it requires supplementation with 0.1mM diaminopimelic acid to overcome the *dap* auxotrophy (See Supplementary Table 1). This particular donor strain was used in this experiment for two reasons. First, pJVD63 requires the *pir* gene product supplied *in trans* for replication in *E. coli* hosts, an element not found in most available conjugal donors. Second, pJVD63 contains the *bla* gene for ampicillin selection, an antibiotic to which most conjugal donor strains are resistant.

Overexpression, purification and assay of His-PhpC. WM6409 mycelia grown on ISP2 Thio[50] plates were used to inoculate 15 ml MYG Thio[25] medium in a 125 ml Erlenmeyer flask equipped with 5 mm glass beads, giving rise to a starter culture. After 3 days, the starter culture was homogenized to break up mycelial aggregates and 100 µl was used to inoculate 1 L YEME (0.5% glycine) Thio[25] in a 3 L baffled flask. The culture was grown on a flatbed shaker for 72 hours at 30 °C prior to the addition of ε-caprolactam to 8.8 mM (final concentration) to induce His-PhpC over expression. After addition of the inducer, the culture was returned to the shaker for 24 hours before harvesting. Cells were harvested by centrifugation, re-suspended in 50 mM HEPES, 300 mM NaCl, 10% glycerol, 20 mM imidazole buffer (pH 7.25, Buffer A), and incubated on ice for 30 minutes after addition of lysozyme to 1 mg/mL final concentration. Cells were then broken 2× in a chilled French pressure cell (20,000 psi) and the lysate was cleared

of cell debris and insoluble protein *via* centrifugation for 30 minutes at 13,000× *g* at 4°C. Cleared supernatant was added to a column pre-equilibrated with lysis buffer containing 7 mL Ni-NTA affinity resin. Contaminating *S. lividans* proteins were washed off the column in a stepwise fashion with buffers identical to buffer A, but with either 50 or 100 mM imidazole. His-PhpC was eluted from the column after washing with buffer containing 200 or 250 mM imidazole. Protein was desalted using Zeba Desalt Spin Columns (7000 Dalton exclusion, Pierce) pre-equilibrated with storage buffer (50 mM HEPES, 10%glycerol, 100 mM NaCl, 2 mM DTT pH 7.25) and was frozen at -70 °C until use. His-PhpC was estimated to be at least 90+% pure by visual inspection of protein separated by SDS-PAGE and stained with Coomassie Brilliant Blue. His-PhpC activity assays were carried out at 30°C (1 hour) in 50 mM HEPES, 10% glycerol pH 7.25 buffer containing 91.5 μg purified protein, ~0.8 mM (estimated) enzymatically produced PnAA, and 1 mM reduced cofactor (NADH or NADPH). PnAA conversion to HEP was monitored both by ^{31}P NMR and LC/MS.

Overexpression and assay of PhpD. WM6047 cells were grown in LB Cm^{12} Amp^{100} broth to mid-exponential phase at 37°C. The culture was cold-shocked in an ice-water bath for 10 minutes, and IPTG was added to 1 mM final concentration. The culture was grown at 18 °C for an additional 21 hours before harvesting. Induced cells suspended in 50 mM HEPES, 10% glycerol buffer (pH 7.25) were broken 2× in a chilled French pressure cell (20,000 psi), and lysates were cleared of cell debris and insoluble protein via centrifugation for 30 minutes at 13,000× *g* at 4 °C. PhpD over expression was verified via SDS-PAGE. Assay of hydroxyethylphosphonate converting activity was performed at

30 °C in 50 mM HEPES, 10% glycerol buffer (pH 7.25) after addition of 1 mM HEP and 0.78 mg protein from WM6047 extract. HEP conversion to HMP was monitored both by ^{31}P NMR and LC/MS with conditions as mentioned above.

Phosphonic acids. Sodium phosphonoformate hexahydrate (95+% pure) was obtained from Alfa Aesar (Ward Hill, MA) and used without further purification. 2-aminoethylphosphonic acid and aminomethylphosphonic acid (99+% pure) were purchased from Sigma (St. Louis, MO). Sodium phosphite (98+% pure) was purchased from Riedel-de Haën (Seelze, Germany). The chemical syntheses of hydroxyethylphosphonate and hydroxymethylphosphonate are described below, as is the enzymatic synthesis of phosphonoacetaldehyde.

Enzymatic production of phosphonoacetaldehyde. Phosphonoacetaldehyde (PnAA) was enzymatically synthesized from phosphoenolpyruvate (PEP) by modification of the previously published method of Zhang *et al*[16]. Enzymatic activity for the production of the aldehyde was supplied from the extract of *E. coli* strain (WM4491) that carries genes for both phosphoenolpyruvate mutase and phosphonopyruvate decarboxylase (from *Bacteroides fragilis*) on plasmid pJVD18. The strain was grown in TYE Cm^{12} Amp^{100} broth at 30°C to mid-exponential phase and was induced for protein synthesis by addition of IPTG (1 mM final concentration). Cultures were induced for 6 hours before harvest. The cell pellet carrying the over expressed proteins was re-suspended in 50 mM HEPES-K^+ 10 mM $MgCl_2$ pH 7.25 buffer and lysed in a chilled French pressure cell. Lysates were centrifuged at 13,000 × *g* for 30 minutes at 4°C to remove cell debris and cell

extracts were used immediately without further processing to provide enzymatic activity. Protein expression was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

PnAA was routinely produced in reactions consisting of 10 mM PEP (PEP potassium salt; 99+% pure, Acros), 1.5 mM thiamine pyrophosphate (Sigma) and $1/10^{th}$ final assay vol. of WM4491 extract in 50 mM HEPES-K^+ 10mM $MgCl_2$ pH 7.25 buffer. Reactions were incubated for 1 hour at 30 °C and protein was removed from the reaction by filtration through a 3 kDa molecular cut off filter (Microcon, Amicon, Beverly MA). The resulting filtrate was analyzed for PEP conversion to PnAA by ^{31}P NMR analysis. After ^{31}P NMR verification, PnAA was directly used in assays.

Chemical synthesis of hydroxyethylphosphonate (HEP): To a solution of dibenzyl phosphite (0.524 g) in dimethylformamide (20 mL) was added cesium carbonate (1.96 g) and tetrabutylammonium iodide (2.42 g). The suspension was stirred at room temperature for 1 h, followed by addition of benzyl 2-bromoethyl ether (216 mg). The mixture was stirred at room temperature for two days. The resulting white suspension was diluted with water (30 mL) and extracted with ethyl acetate (3×30 mL). The organic layer was washed with water (3×30 mL) and brine (30 mL). The crude product was purified by silica gel chromatography eluting with hexane/EtOAc (1:2) to give a colorless liquid (326 mg, 83%). To this product (103 mg) was added palladium on carbon (20 mg) in methanol (5 mL). The suspension was stirred at room temperature under hydrogen atmosphere overnight. The resulting suspension was filtered, and the filtrate was concentrated to give

a colorless liquid (31 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.60 (dt, *J* = 12.4, 7.2 Hz, 2H),1.85 (dt, *J* = 18.0, 7.2 Hz, 2H). ^{31}P NMR (162 MHz, $CDCl_3$) δ 27.7.

Chemical synthesis of hydroxymethylphosphonate (HMP): Diethyl hydroxymethylphosphonate was purchased from Aldrich (Milwaukee, WI). The ethyl esters were hydrolyzed by adding 10 mL of 6 M HCl to 1.0 g of starting material. The mixture was heated at reflux for 12 h and then cooled to room temperature. Water and HCl were carefully removed under reduced pressure to produce a sticky oil, which solidified slowly at room temperature to give a white solid (0.66g, 99%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.60 (d, *J* = 7.5 Hz) ^{31}P NMR (162 MHz, $CDCl_3$) δ 22.8.

Supplementary Data

Additional structural data for the intermediate CMP-5'-PF.

Tandem mass spectrometry (MS^2) detected a fragment (HRMS (m/z): $[M+H]^+$ calcd for $C_9H_{16}N_3O_{10}P_2$, 388.0305; found 388.0314) that corresponds to the loss of CO_2 from CMP-5'-PF, likely from the terminal phosphonoformate moiety

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