

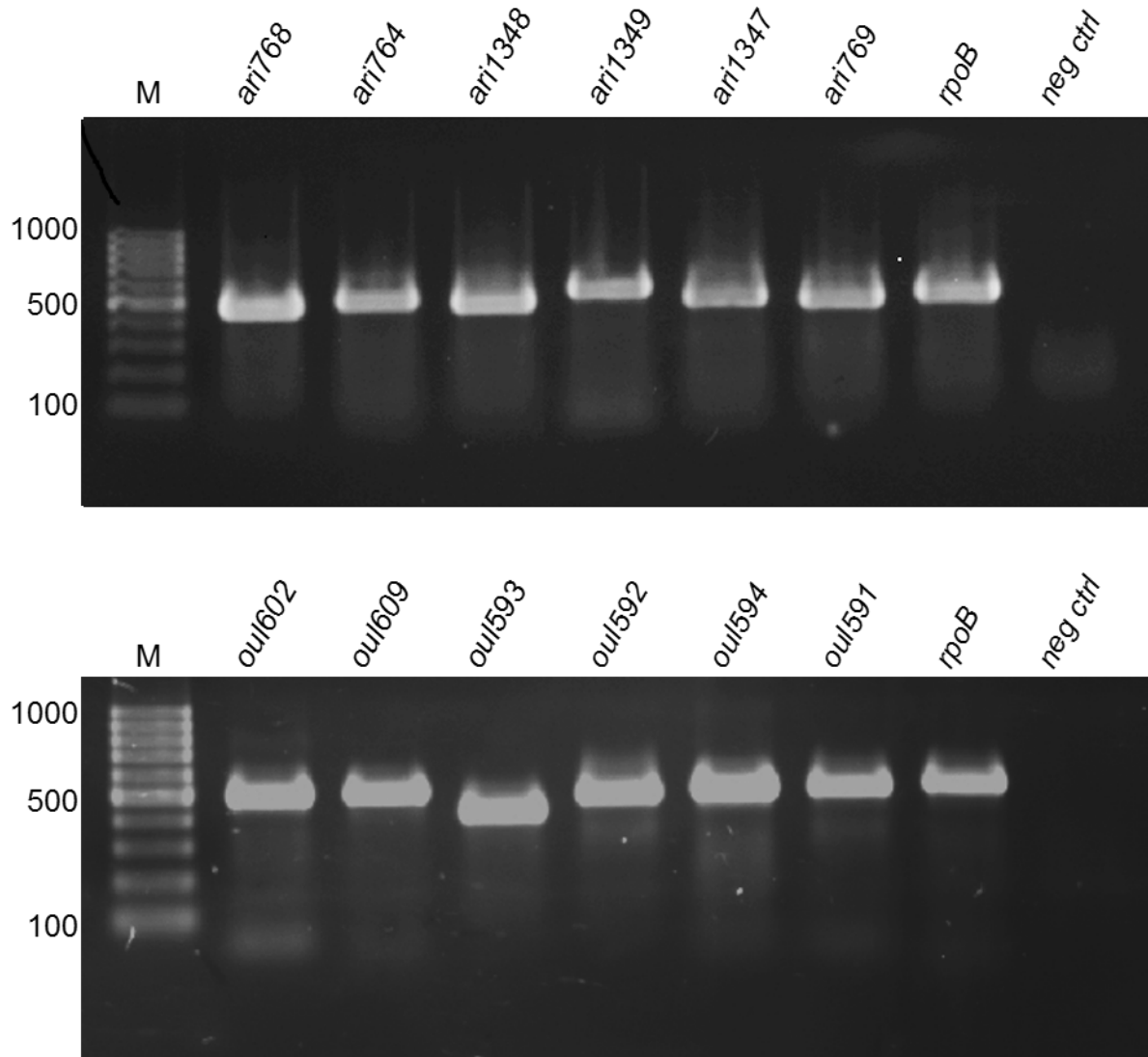
Supplementary Information

The predominance of nucleotidyl activation in phosphonate biosynthesis

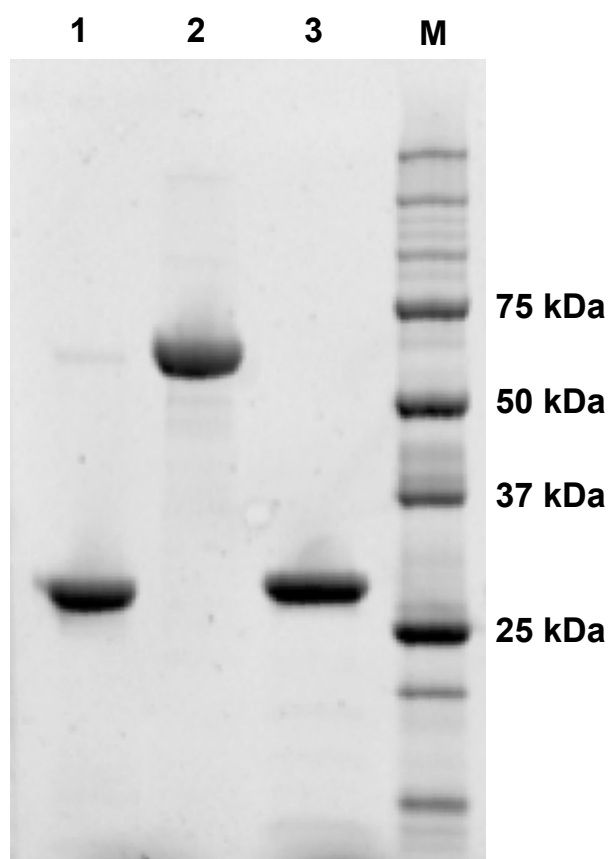
K. Rice *et al.*

Supporting Information

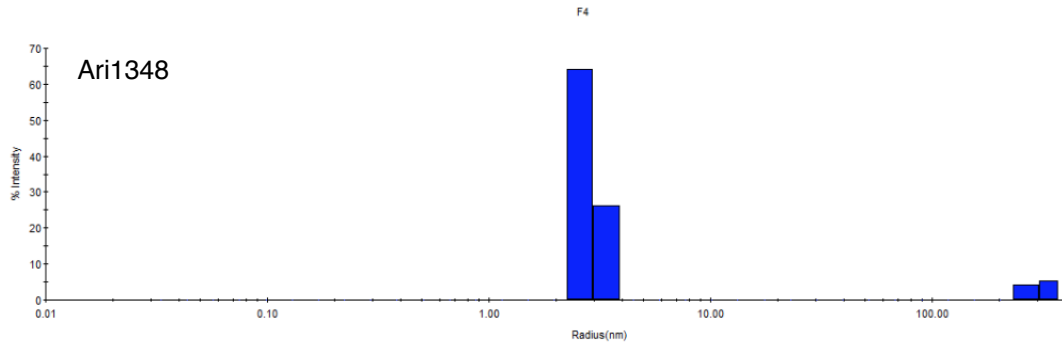
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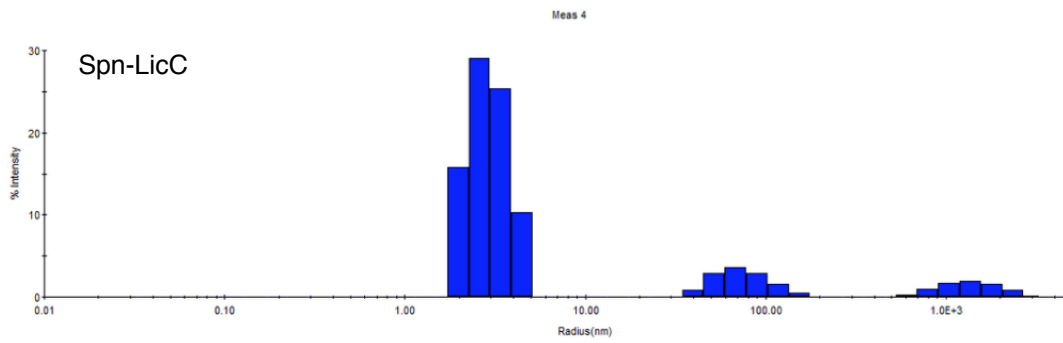
Supplementary Figure 1. RT-PCR analysis demonstrates expression of genes from clusters shown in Figure 3 for *A. rimae* (top) and *O. uli* (bottom). The *rpoB* gene was used as a positive control.¹ Primers used are listed in Supplementary Table 4.



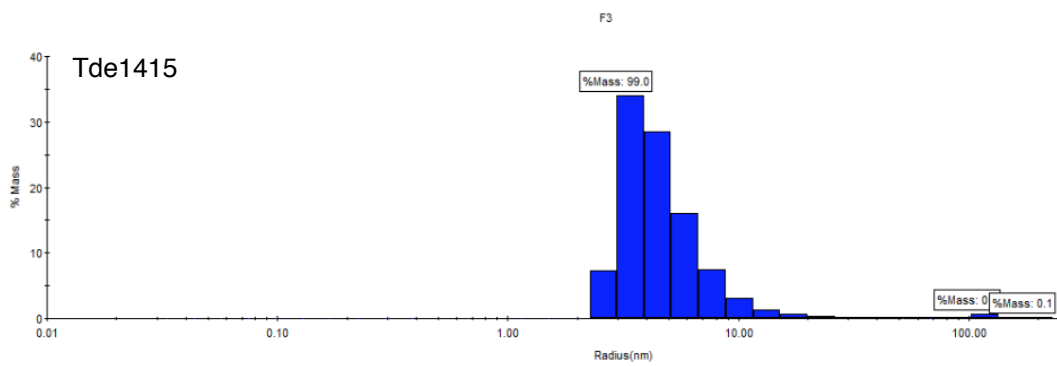
Supplementary Figure 2. SDS-PAGE of Spn-LicC (lane 1), Tde1415 (lane 2), Ari1348 (lane 3), and Bio-Rad Precision Plus Protein Standard (lane M).



Intensity Distribution	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
<input checked="" type="checkbox"/>	Peak 1	2.830	38	90.4	100.0
<input checked="" type="checkbox"/>	Peak 2	313.323	2329710	9.6	0.0

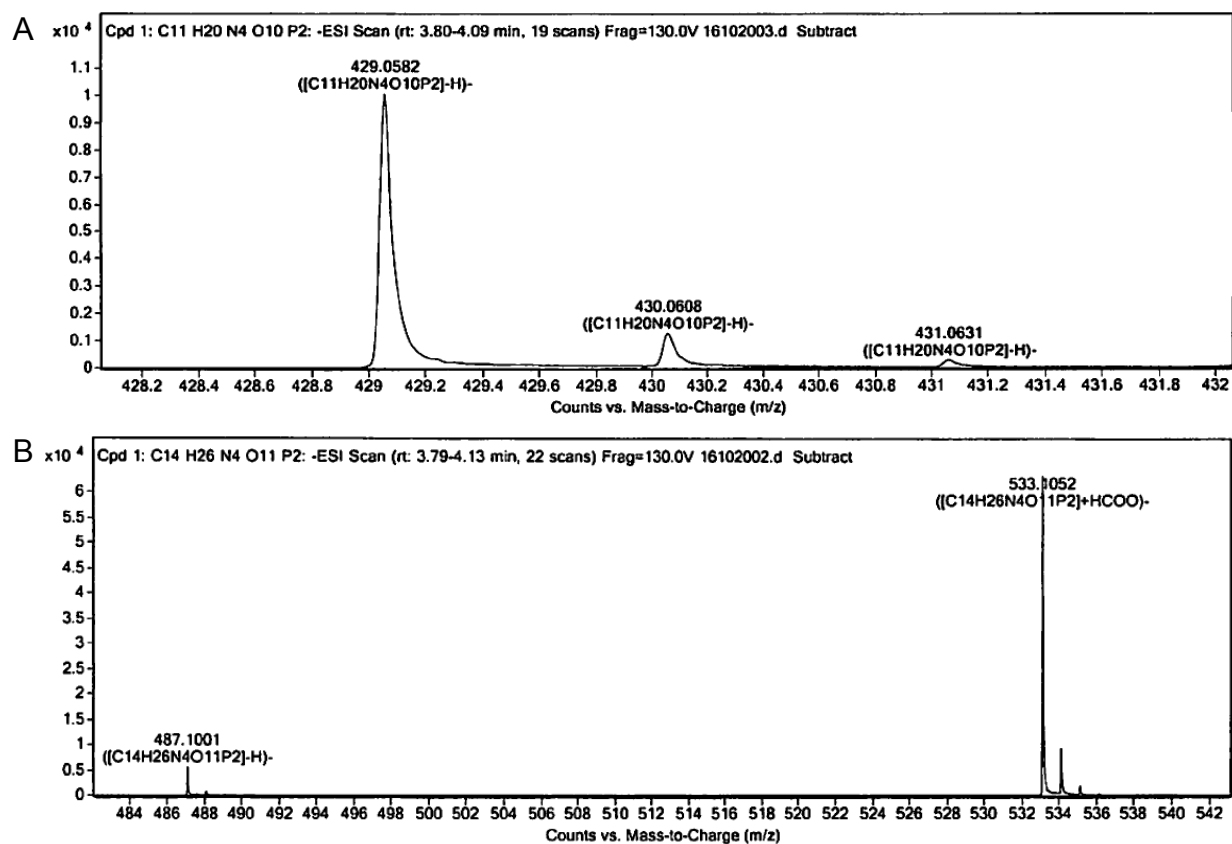


Intensity Distribution	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
<input checked="" type="checkbox"/>	Peak 1	2.967	43	80.4	99.9
<input checked="" type="checkbox"/>	Peak 2	77.675	89136	12.3	0.0
<input checked="" type="checkbox"/>	Peak 3	1425.910	80746700	7.3	0.1

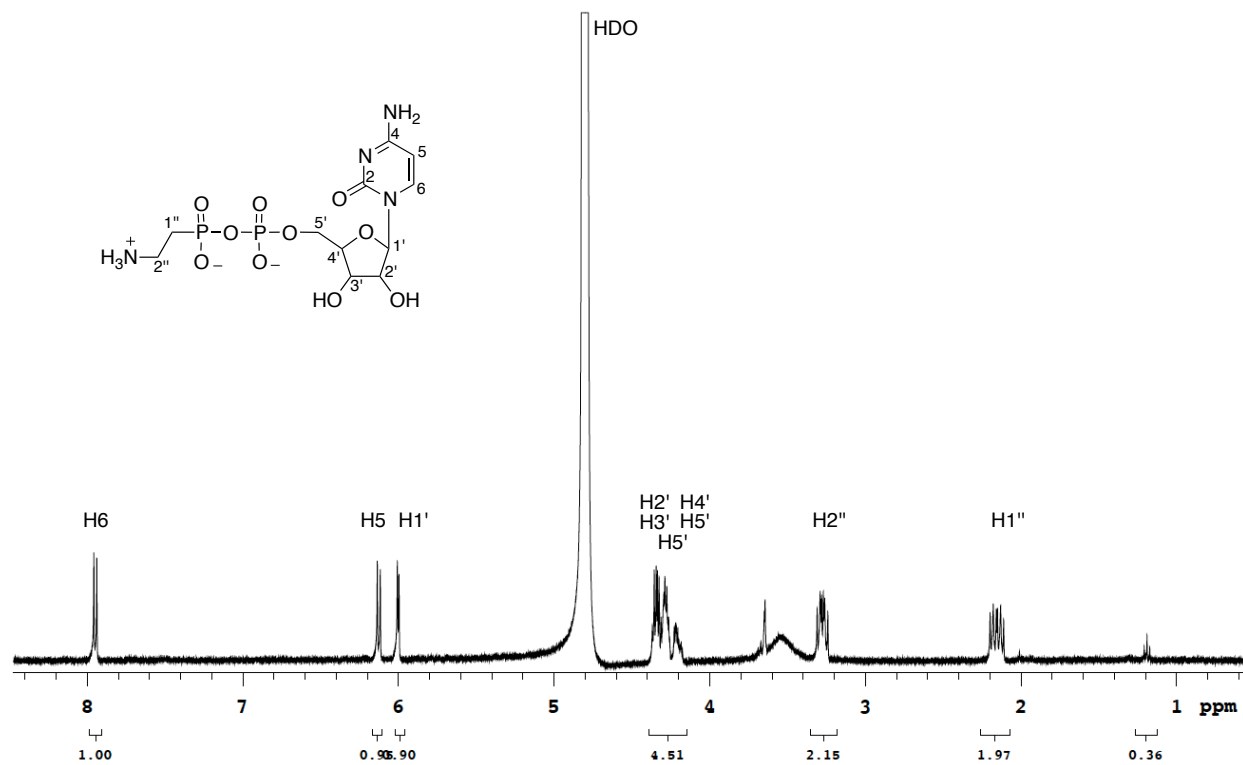


Mass Distribution	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
<input checked="" type="checkbox"/>	Peak 1	5.011	146	80.2	99.0
<input checked="" type="checkbox"/>	Peak 2	118.041	237311	17.9	0.9
<input checked="" type="checkbox"/>	Peak 3	205.078	864189	1.8	0.1

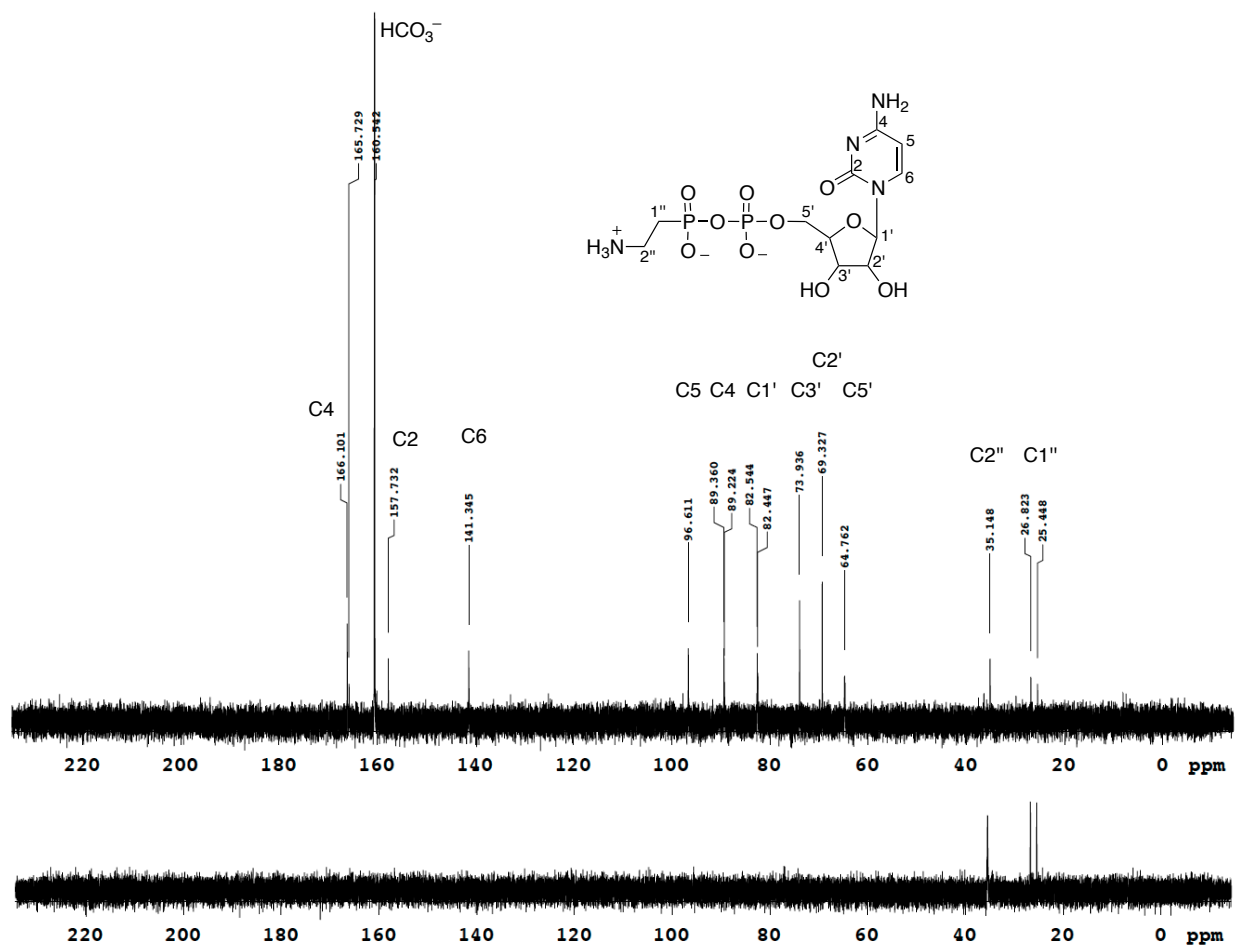
Supplementary Figure 3. Dynamic light scattering data showing molecular weights of 38 kDa for Ari1348, 43 kDa for Spn-LicC, and 146 kDa for Tde1415.



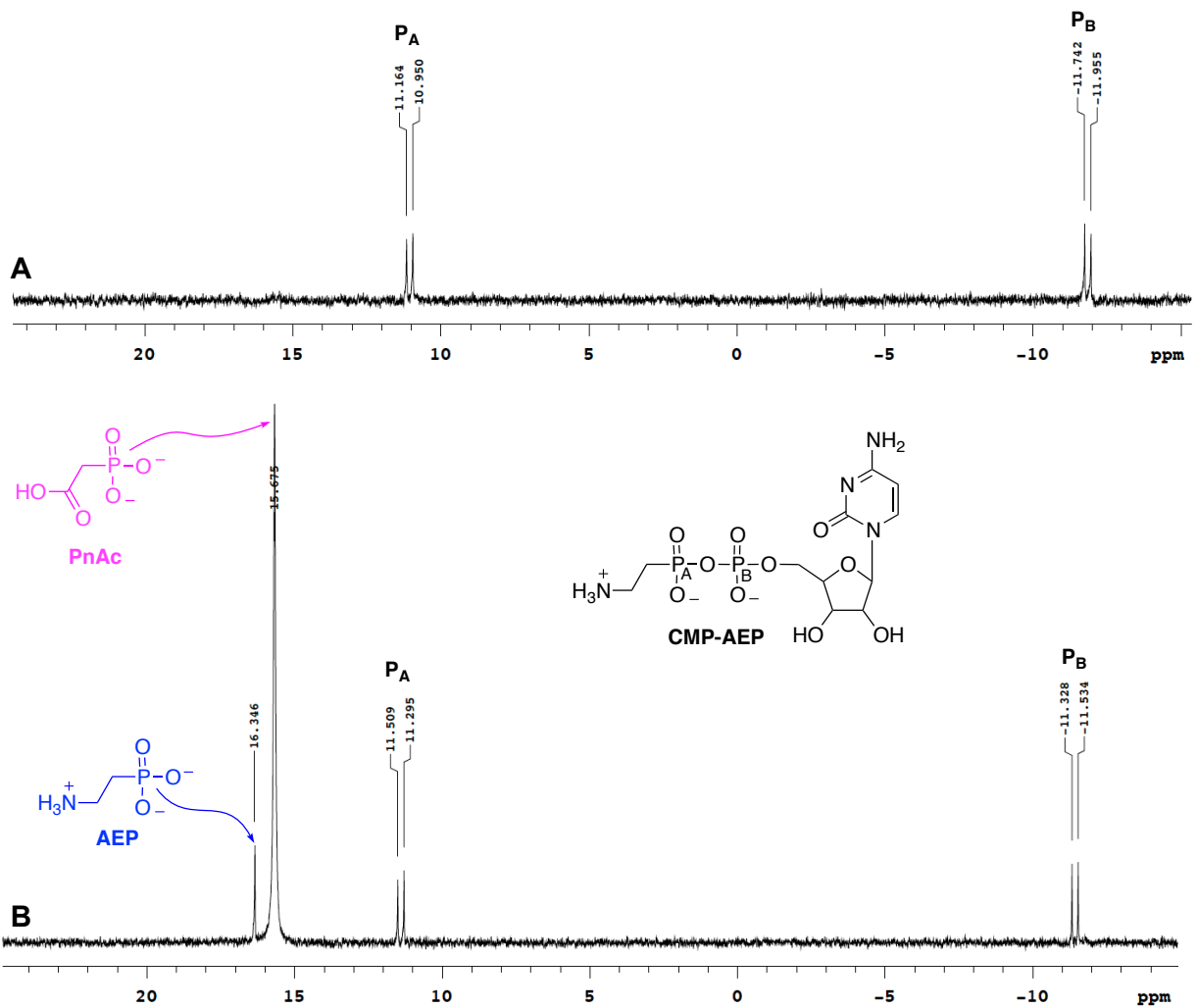
Supplementary Figure 4. HR-ESI-MS of (A) CMP-AEP revealing the $[M-H]^-$ ion at m/z 429.0582 consistent with a molecular mass of 430.0655 for molecular formula C₁₁H₂₀N₄O₁₀P₂ (calculated 430.0655), and (B) CDP-Cho revealing the $[M-H]^-$ ion at m/z 487.1001 consistent with a molecular mass of 488.1071 (calculated 488.1073).



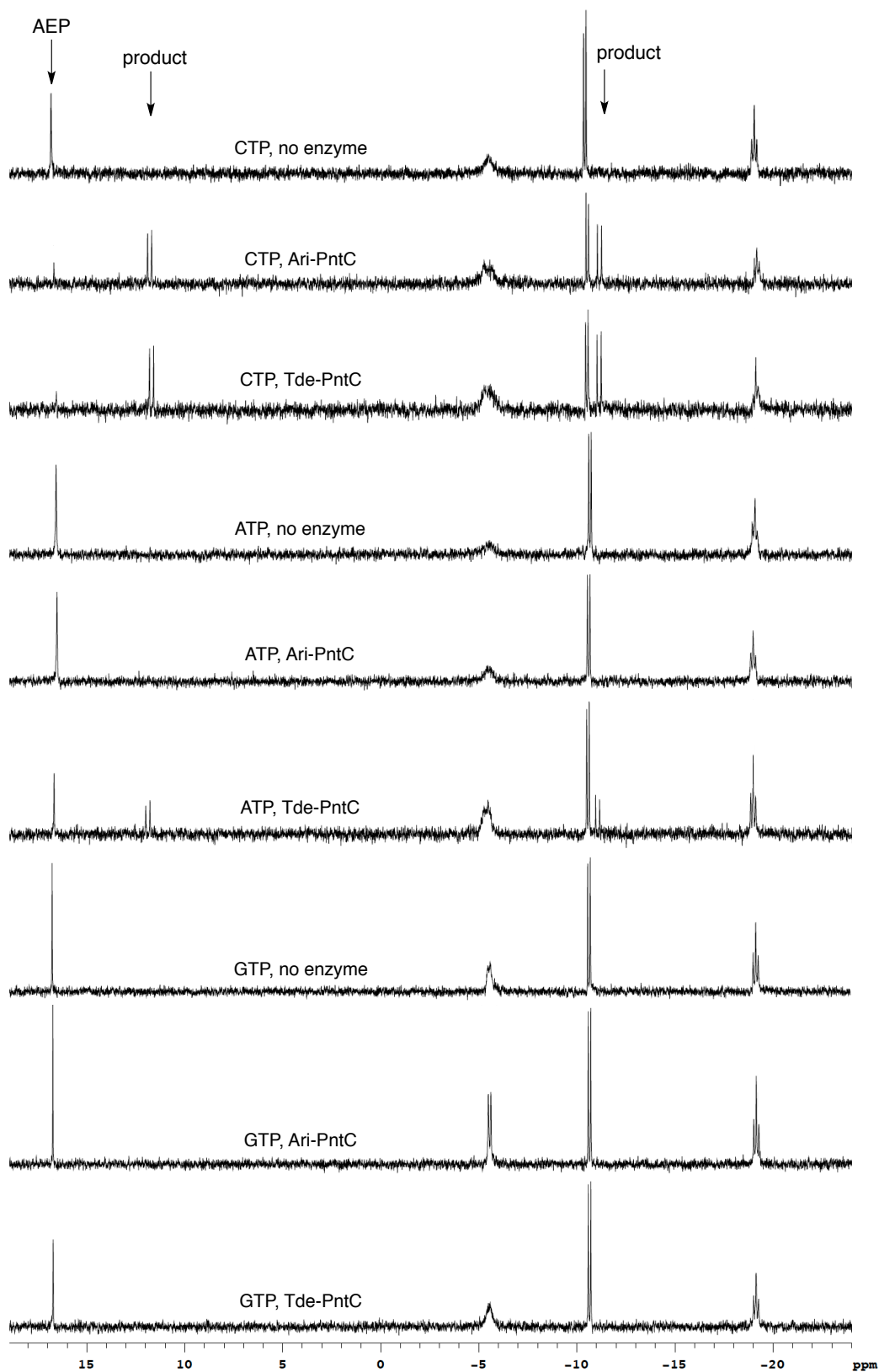
Supplementary Figure 5. ¹H NMR (D₂O, 400 MHz) spectra of CMP-AEP: δ 7.95 (d, 1H, H6, J = 7.6 Hz), 6.12 (d, 1H, H5, J = 7.6 Hz), 6.00 (d, 1H, H1', J = 4.0 Hz), 4.82-4.18 (m, 5H, H2', H3', H4', H5'), 3.29 (m, 2H, H2''), 2.15 (m, 2H, H1'')



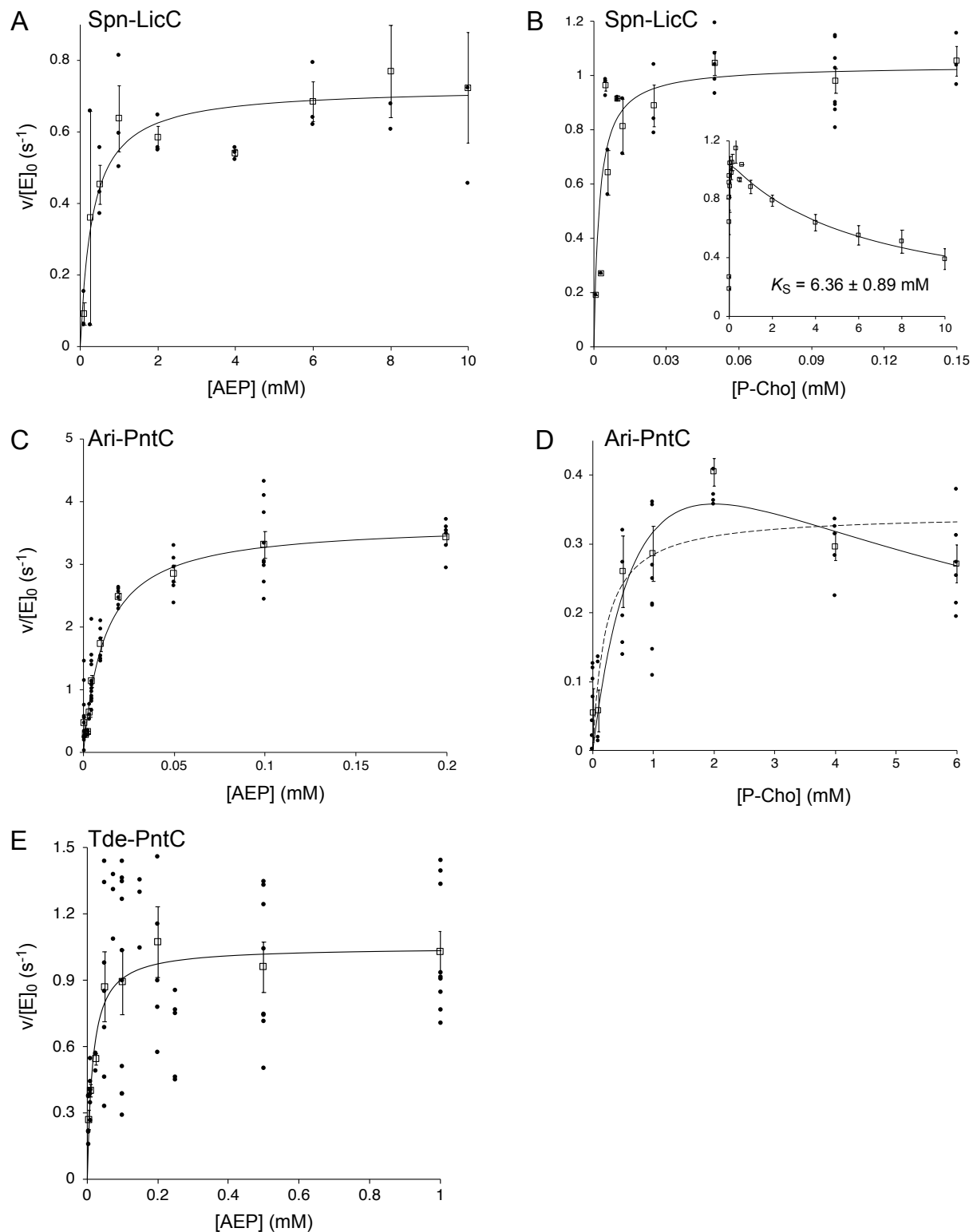
Supplementary Figure 6. ¹³C NMR (D₂O, 100 MHz) spectra of CMP-AEP: δ 166.1 (C4), 157.7 (C2), 141.3 (C6), 96.6 (C5), 89.3 (C4'), 82.5 (C1'), 73.9 (C3'), 69.3 (C2'), 64.8 (C5'), 35.1 (C2''), 26.1 (C1''); $^1J_{CP} = 130$ Hz). Bottom spectrum represents commercial AEP alone.



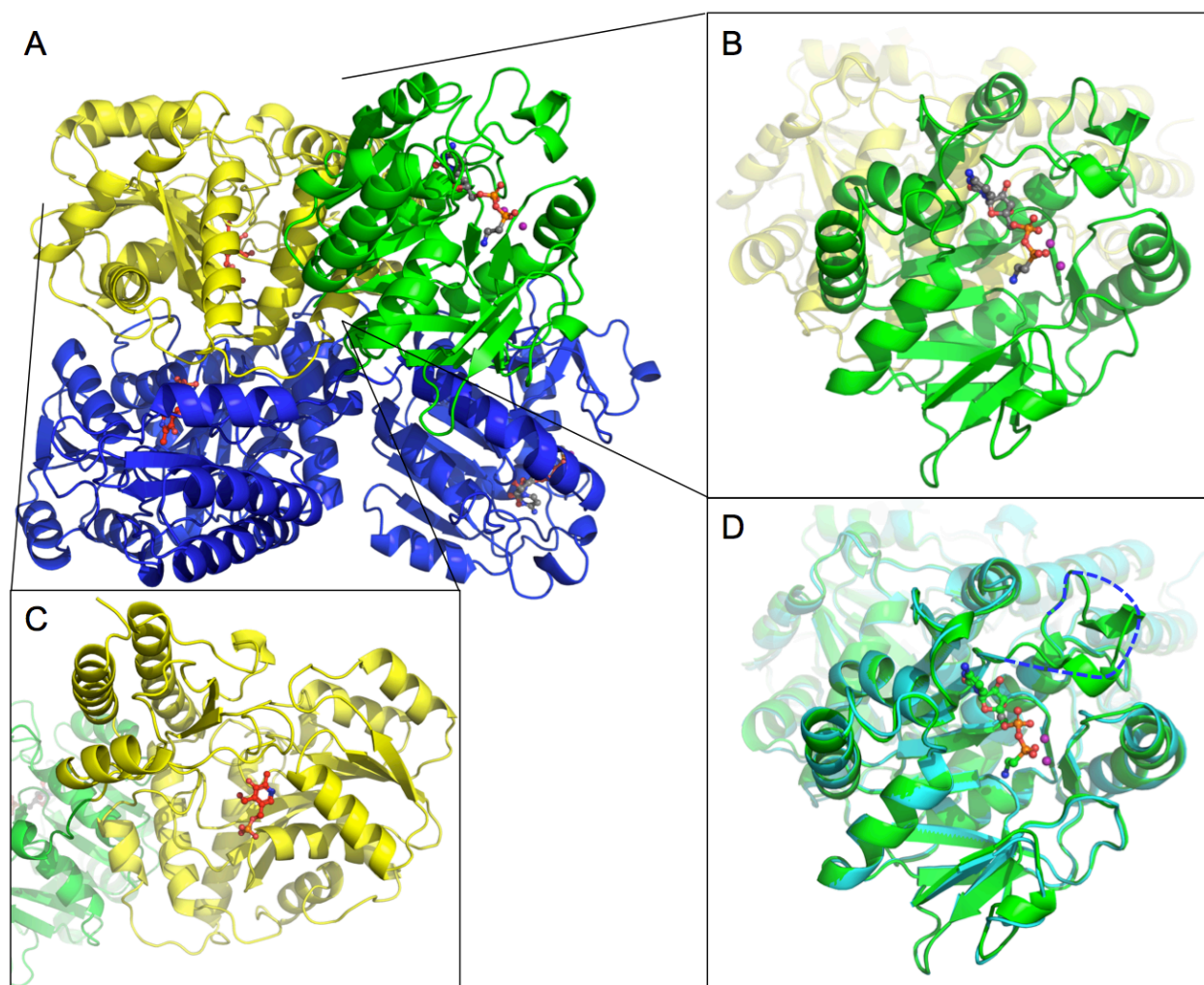
Supplementary Figure 7. ^{31}P NMR (D_2O , 121 MHz) spectra of (A) CMP-AEP alone and (B) with phosphonoacetic acid (PnAc) and 2-ethylaminophosphonate (AEP). Using PnAc as the internal standard with a reported chemical shift of 15.7,² our measured chemical shifts of CMP-AEP: δ 11.4 (P_A), -11.4 (P_B), $^3J = 25.6$ Hz.



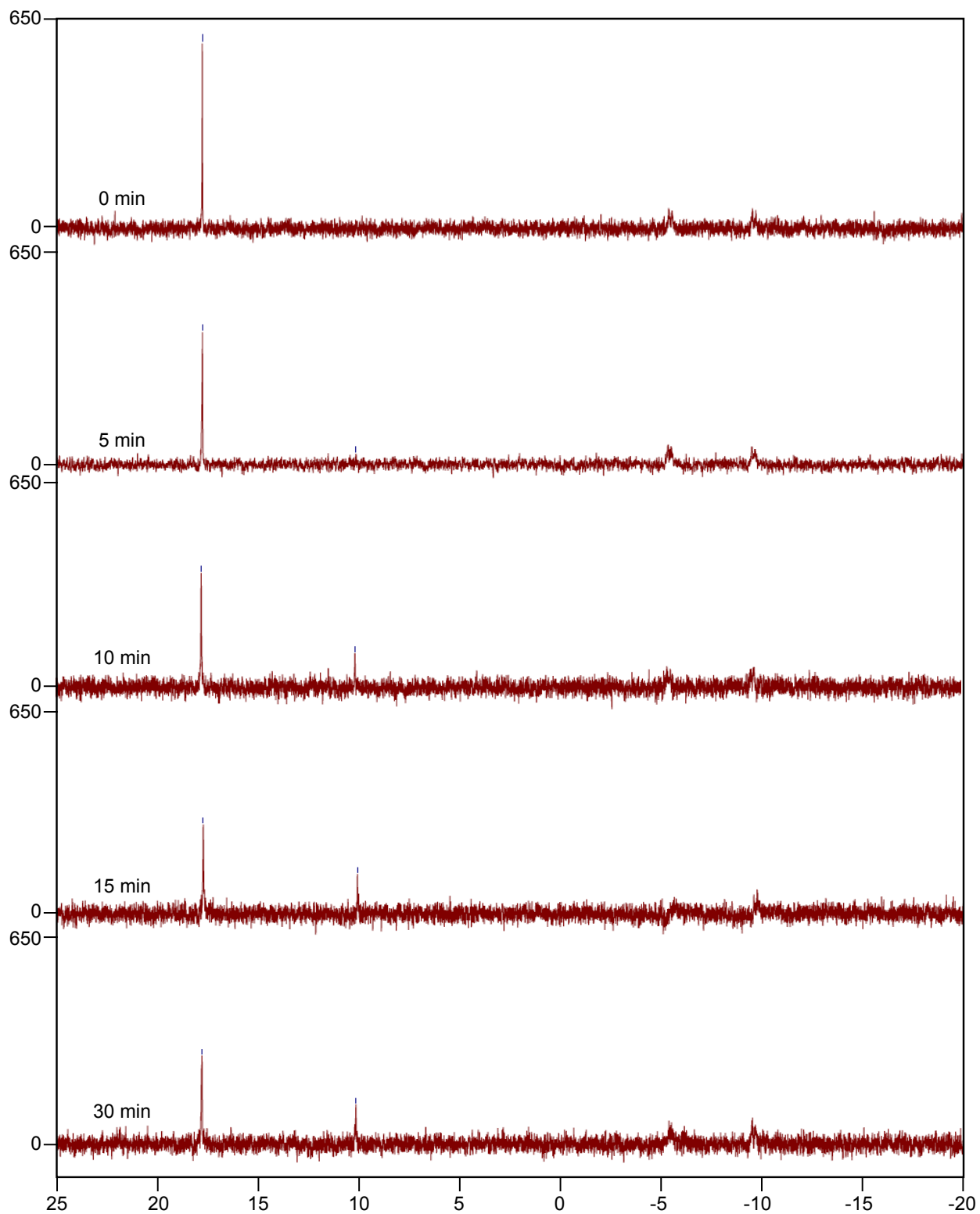
Supplementary Figure 8. ^{31}P NMR analysis of 1-hour reactions of 10 μM PntC enzyme with 1 mM AEP and 2 mM of CTP, ATP, or GTP in 50 mM Tris-Cl and 7 mM MgCl_2 , pH 8.0.



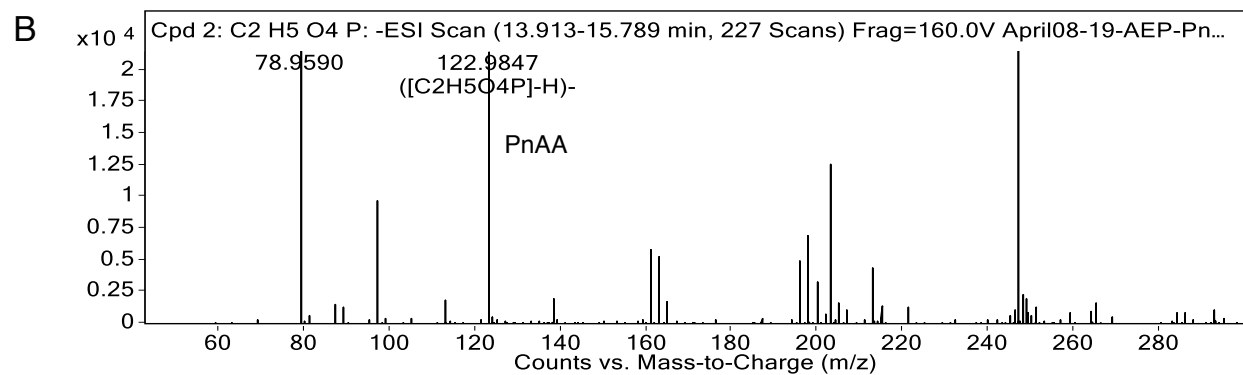
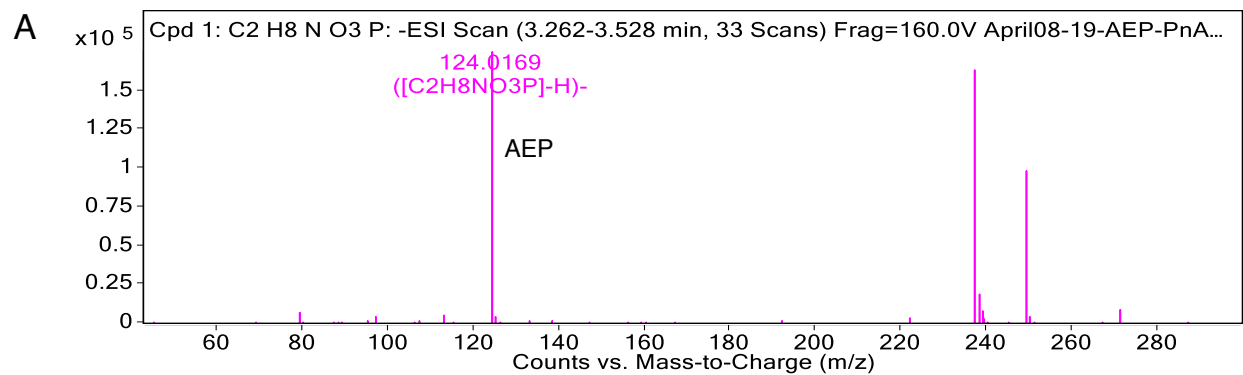
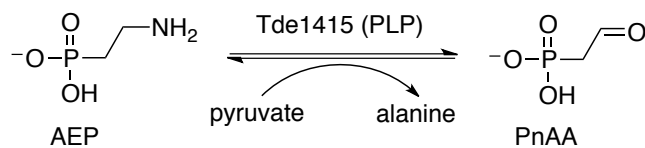
Supplementary Figure 9. Steady-state kinetic analysis of cytidyltransferase activities of (A) Spn-LicC with AEP, (B) Spn-LicC with P-Cho, (C) Ari-PntC with AEP, (D) Ari-PntC with P-Cho, and (E) Tde-PntC with AEP. Activity not detected for Tde-PntC and P-Cho. Error bars show standard errors of the mean (open square), with all data points shown as filled circles.



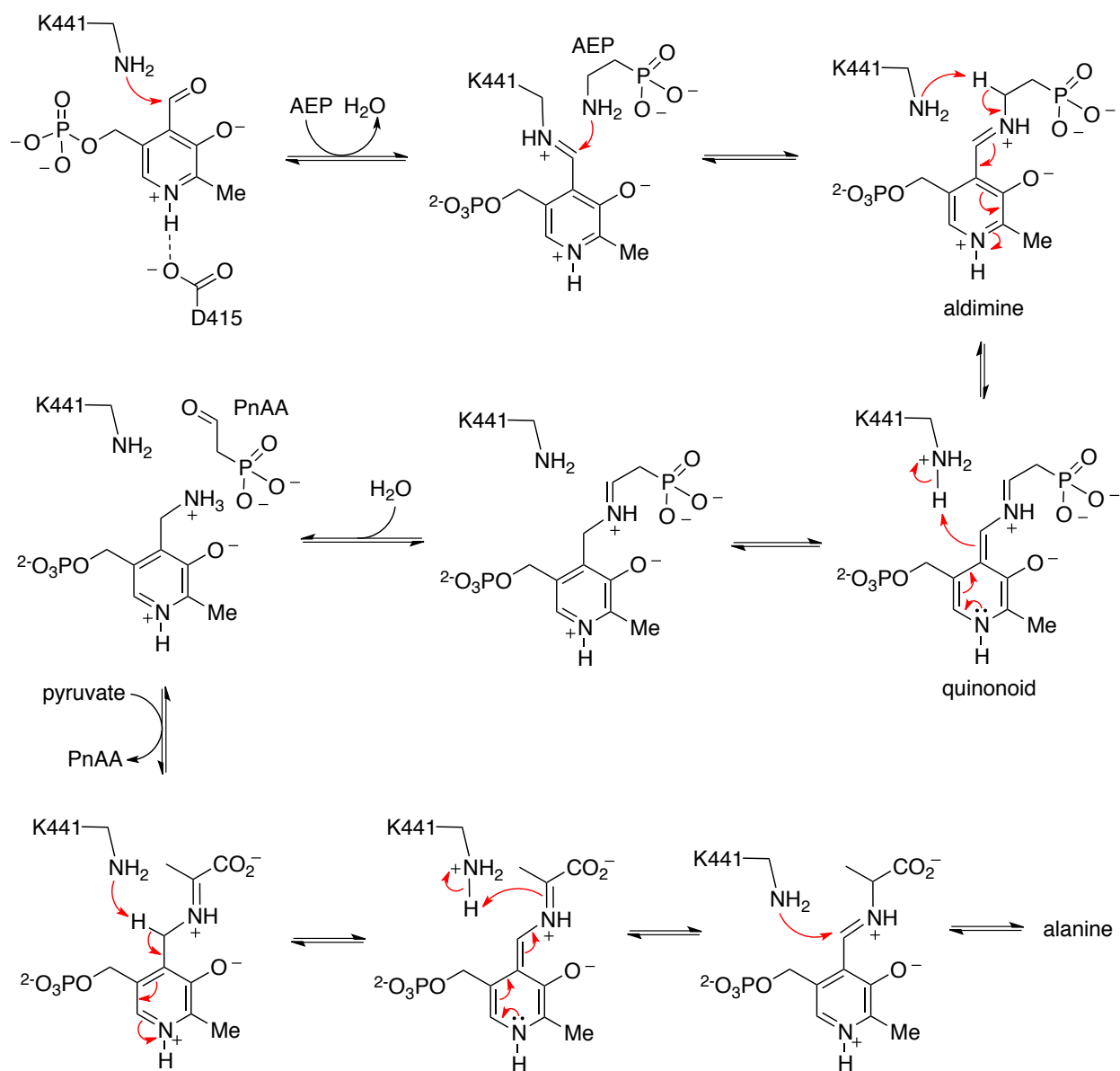
Supplementary Figure 10. Cartoon representations of Tde1415 crystal structures. (A) Overall dimeric structure showing molecule A in blue and molecule B is bi-colored yellow (residues 1-249) and green (residues 250-615). (B) Tde-PntC cytidyltransferase domain (green) in complex with CMP-AEP. (C) AEPT domain (yellow) complexed with PLP. (D) Superposition of Tde-PntC-apo (cyan) and Tde-PntC:CMp-AEP (green) reveals low RMSD ranging from 0.53 to 0.86 Å. The exception is the disordered residues 14-20 of Tde-PntC-apo (dotted blue line).



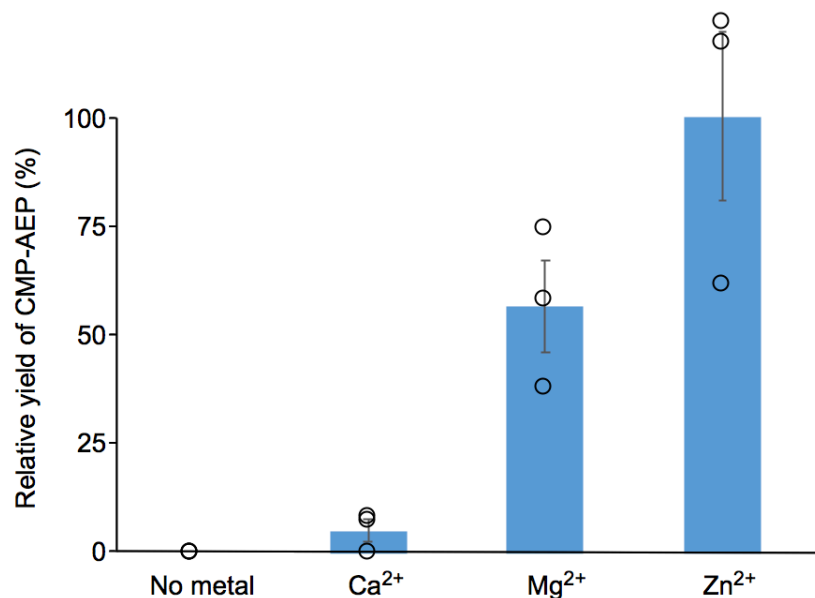
Supplementary Figure 11. Time-dependent conversion of 1.5 mM AEP to PnAA catalyzed by 50 nM Tde1415 in the presence of 6 mM pyruvate, 30 μ M PLP, and 300 mM NaCl in Tris-Cl, pH 8. Reactions were run at 20 $^{\circ}$ C and quenched with an equal volume of methanol at each time point.



Supplementary Figure 12. Mass spectrometry revealing the aminotransferase activity of Tde1415 as shown. A) Detection of AEP (calculated mass = 124.0169) in the presence of Tde1415 (also observed in no enzyme control) and B) detection of PnAA (calculated mass = 122.9853).

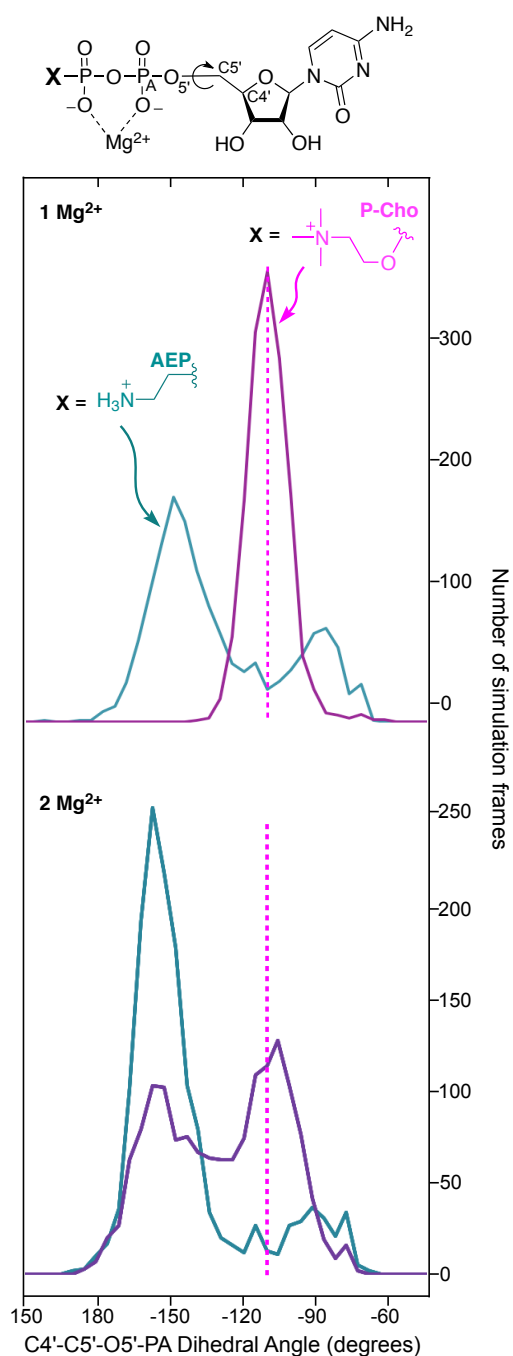


Supplementary Figure 13. Proposed mechanism for aminotransferase activity of Tde1415 catalyzing the interconversion of AEP and PnAA. The first structure represents the crystallographically observed state, which unusually does not have an imine linkage between K441 and PLP.

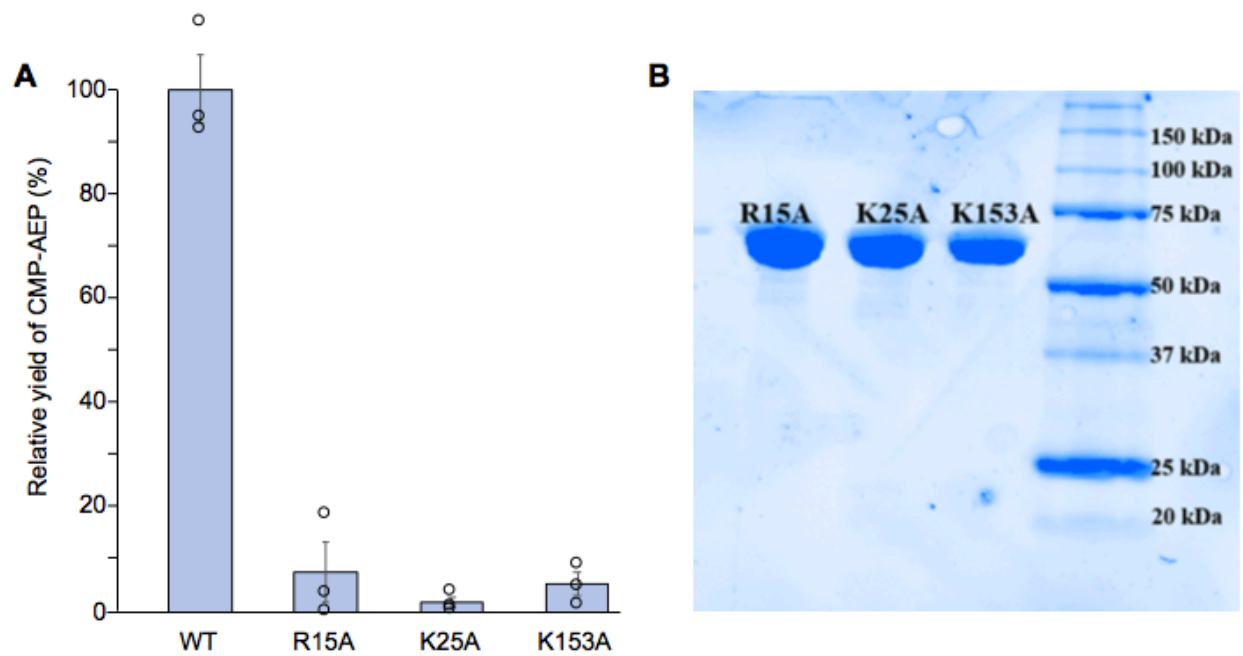


Supplementary Figure 14. Comparison of PntC activity in the presence of different metals. The activity of EDTA-treated enzyme (“No metal”) was compared against the same enzyme supplemented with Ca²⁺, Mg²⁺, or Zn²⁺. All four treatments were repeated in triplicate with and are reported with standard errors. Assays were carried out in 50 mM Tris-Cl pH 8.0 with 7.0 mM metal, 7.0 mM CTP, 3.0 mM AEP, and 5.5 μM enzyme. Reactions were allowed to proceed at 20 °C for 2 h, then an internal standard (6.0 mM phosphonoacetic acid) was added immediately prior to acquiring ³¹P NMR spectra. Peak integrations for CMP-AEP were calculated relative to the internal standard. Bars represent mean values with standard errors shown. Individual data points (three for each metal) are shown as open circles. The yield of CMP-AEP product is assigned as 100% for the most active condition (Zn²⁺).

Supplementary Figure 15. (A) Clustal Omega multiple sequence alignment of selected PF12804 cytidyltransferases. Amino acid sequences were retrieved from NCBI with the following accession numbers: Bfr-PntC from *Bacteroides fragilis* 638R (CBW22390); Tde-PntC from *Treponema denticola* ATCC 35405 (NP_992021); Cj1416 from *Campylobacter jejuni* (CAI38904); Oul-PntC from *Olsenella uli* DSM 7084 (ADK67708); Ari-PntC from *Atopobium rimae* ATCC 49626 (ZP_03568201); Spn-LicC from *Streptococcus pneumoniae* R36A (AAK94072); Hin-LicC from *Haemophilus influenzae* C486 (AJO89865); FrbH from *Streptomyces rubellomurinus* (ABB90397); YgbP (or IspD, CDP-ME synthase) from *Escherichia coli* K-12 (CQR82192). Box denotes the GXG(T/S)RX₈PK consensus sequence. The dotted line represents a salt bridge between Glu216 and Arg129 observed in the apo Spn-LicC crystal structure (PDB 1JYK).³ (B) Cladogram of cytidyltransferases from the alignment. YgbP is a MEP cytidyltransferase belonging to the IspD Pfam (PF01128), which possesses significant overlap with PF12804.



Supplementary Figure 16. The effects of a second Mg²⁺ ion on Spn-LicC MD simulations. The top panel reproduces the Spn-LicC data from Figure 5b in the main text, which resulted from simulations based on the crystallographic observation of a single Mg²⁺ ion in the active site. The bottom panel shows data resulting from the inclusion of a second Mg²⁺ ion in the simulations.



Supplementary Figure 17. Analysis of Tde1415 active site variants R15A, K25A, and K153A. (A) Activity of each variant based on integration of ^{31}P NMR peaks relative to a phosphonoacetic acid internal standard. Averages and standard errors of three measurements are shown as bars, with individual data points included as open circles. (B) SDS-PAGE analysis of purified variant enzymes.

Supplementary Table 1. X-ray data collection and refinement statistics (molecular replacement).*

	Tde-PntC-apo	Tde-PntC-CMP-AEP
Data collection		
Space group	P2 ₁	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	89.05, 129.01, 135.80	76.45, 154.05, 134.58
α , β , γ (°)	90, 92.99, 90	90, 90.09, 90
Resolution (Å)	38.00-2.72 (2.77-2.72)**	48.00-1.95 (2.06-1.95)
<i>R</i> _{merge}	0.097 (0.700)	0.188 (0.965)
CC _{1/2}	0.996 (0.728)	0.989 (0.767)
<i>I</i> / σ <i>I</i>	13.0 (2.1)	9.8 (2.1)
Completeness (%)	99.9 (99.7)	98.2 (97.1)
Redundancy	4.2 (4.3)	6.8 (7.0)
Refinement		
Resolution (Å)	2.72	1.95
No. reflections	342,389	1,510,620
<i>R</i> _{work} / <i>R</i> _{free}	0.199/0.233	0.220/0.254
No. atoms		
Protein (chains A, B, C, D)	19241	19277
Magnesium	4	8
PLP	-	64
CMP-AEP	-	108
gamma-PO ₄	-	20
Water	257	1614
<i>B</i> -factors (Å ²)		
Protein (chains A, B, C, D)	48.7	26.4
Magnesium	55.9	28.9
PLP	-	22.1
CMP-AEP	-	31.4
gamma-PO ₄	-	36.5
Water	48.6	28.7
R.m.s. deviations		
Bond lengths (Å)	0.005	0.011
Bond angles (°)	0.948	1.14

*One crystal was used for data collection and refinement. **Values in parentheses are for highest-resolution shell.

Supplementary Table 2. Strains used in this study.

Strain	Genotype/Description	Source (Reference)
<i>E. coli</i> DH5 α	<i>E. coli</i> host for general cloning	Life Technologies
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> host for protein production	Life Technologies
<i>E. coli</i> HG1000	pGH1000 in BL21(DE3)	This study
<i>E. coli</i> HG2000	pGH2000 in BL21(DE3)	This study
<i>E. coli</i> HG3000	pGH3000 in BL21(DE3)	This study
<i>A. rimae</i> ATCC 49626	VPI D140H-11A [NCFB 2896]	ATCC ⁴
<i>O. uli</i> ATCC 49627	VPI D76D-27C ^T = DSM 7084	ATCC ⁵

Supplementary Table 3. Plasmids used in this study.

Plasmid	Description	Source (Reference)
pGH1000	pET29 plasmid containing <i>Spn-licC</i> as an <i>NdeI/XhoI</i> fragment	This study
pGH2000	pET29 plasmid containing <i>ari1348</i> as an <i>NdeI/NotI</i> fragment	This study
pGH3000	pET21 plasmid containing <i>tde1415</i> as an <i>NdeI/NotI</i> fragment	This study

Supplementary Table 4. Primers used in this study.

Target gene	Primer Name	Sequence (5'→3')
<i>oul594</i>	ouPEPmut-F	cgagaggatacggctctacg
	ouPEPmut-R	ctccatgatgtcgtctatcgtgc
<i>oul592</i>	ouPnPy decarb-F	atcacatcatcgccgcaaac
	ouPnPy decarb-R	tggtggagacgatggggtc
<i>oul_rpoB</i>	ourpoB-F	tcgacgtgcgcttcgtc
	ourpoB-R	gacacgcgcaaggtcg
<i>oul602</i>	ouLicC(602)-F	cgaggaggcgggtctg
	ouLicC(602)-R	gagtgatcgaccgcc
<i>oul609</i>	ouLicD(609)-F	ctgcgtagggagggcg
	ouLicD(609)-R	gaaggtagggcatgccg
<i>oul593</i>	ouLicC(593)-F	ccggtcaagctcatcgaaaac
	ouLicC(593)-R	ggacagggcggattctg
<i>oul591</i>	ouLicD(591)-F	ctgggatgatgatcgcacatcgg
	ouLicD(591)-R	tgtctgggtgaagcggttc
<i>ari1347</i>	arPEPmut-F	ctatcttacgacgacgtgattgc
	arPEPmut-R	tatccccgtcaagaatgatgggttg
<i>ari1349</i>	arPnPy decarb-F	attatcaatcctgtggcttctctctg
	arPnPy decarb-R	cgtcaccgtcaatgcacc
<i>ari_rpoB</i>	arrpoB-F	catgaccgagcgcgg
	arrpoB-R	gctatctcaagaccaagcttctg
<i>ari1348</i>	arLicC-F	ggcgttgagaggatctctgtg
	arLicC-R	taattcggcaatatggtattcaccacatc
<i>ari764</i>	arLicD(764)-F	taccatctccatcggaatctttccg
	arLicD(764)-R	atctttcacttgccaaaatcaagtctg
<i>ari768</i>	arLicAC-F	ggactttctctcgtttgctc
	arLicAC-R	cacggtcaaaatacacgtgtcc
<i>ari769</i>	arLicD(769)-F	cttgggacgatgatattgacgtcg
	arLicD(769)-R	tggtcttcacgtgtgcatagc
<i>tde1415</i>	Fwd-NdeI	catatgattaagcaagcgggtgattctggc
	Rvs-XhoI	ctcgagaacgccaacgccgatgc
	Fwd-R15A	ctgggtagcgactgaaggataagaccaagacc
	Rvs-R15A	ggtcttggtcttatccttcagtgcgctaccag
	Fwd-K25A	gaccaagaccatgccggcgggtttctggagatc
	Rvs-K25A	gatctccagaaaacccgccgcatggtcttggtc
	Fwd-K153A	ctgaccggcctgagcgcgcaaccgtg
Rvs-K153A	cacggttgctcagcggcgtcag	

Supplementary References

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2. Weber, M. *et al.* Method development in quantitative NMR towards metrologically traceable organic certified reference materials used as (31)P qNMR standards. *Anal. Bioanal. Chem.* **407**, 3115–3123 (2015).
3. Kwak, B.-Y. *et al.* Structure and mechanism of CTP:phosphocholine cytidyltransferase (LicC) from *Streptococcus pneumoniae*. *J. Biol. Chem.* **277**, 4343–4350 (2002).
4. Olsen, I., Johnson, J. L., Moore, L. V. & Moore, W. E. *Lactobacillus uli* sp. nov. and *Lactobacillus rimae* sp. nov. from the human gingival crevice and emended descriptions of *Lactobacillus minutus* and *Streptococcus parvulus*. *Int. J. Syst. Bacteriol.* **41**, 261–266 (1991).
5. Göker, M. *et al.* Complete genome sequence of *Olsenella uli* type strain (VPI D76D-27C). *Stand. Genomic Sci.* **3**, 76–84 (2010).