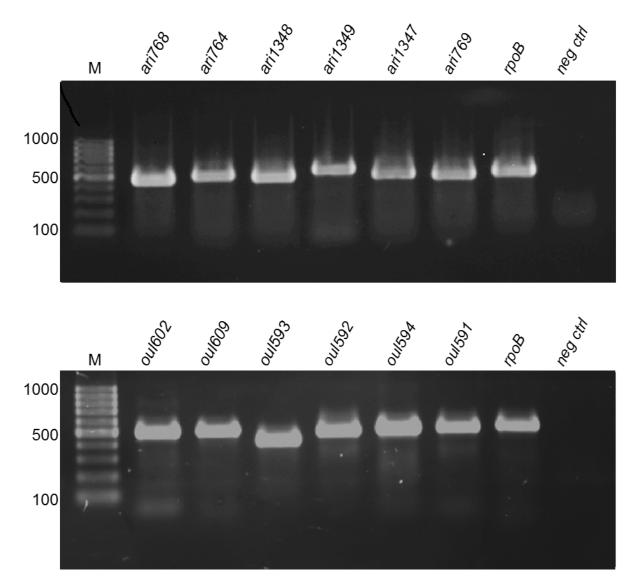
# **Supplementary Information**

The predominance of nucleotidyl activation in phosphonate biosynthesis

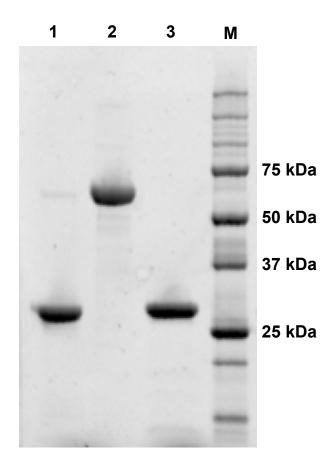
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# **Supporting Information**

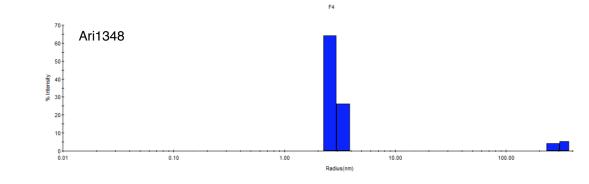
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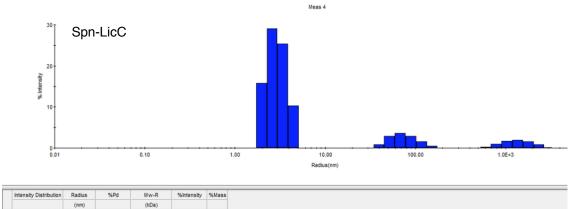
**Supplementary Figure 1.** RT-PCR analysis demonstrates expression of genes from clusters shown in Figure 3 for *A. rimae* (top) and *O. uli* (bottom). The *rpoB* gene was used as a positive control.<sup>1</sup> Primers used are listed in Supplementary Table 4.



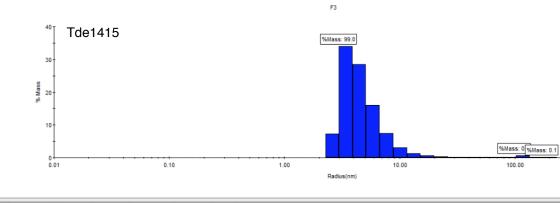
**Supplementary Figure 2**. SDS-PAGE of Spn-LicC (lane 1), Tde1415 (lane 2), Ari1348 (lane 3), and Bio-Rad Precision Plus Protein Standard (lane M).



Intensity Distribution	Radius	%Pd	Mw-R	%Intensity	%Mas
	(nm)		(kDa)		
Peak 1	2.830	13.0	38	90.4	100.0
Peak 2	313.323	13.2	2329710	9.6	0.0

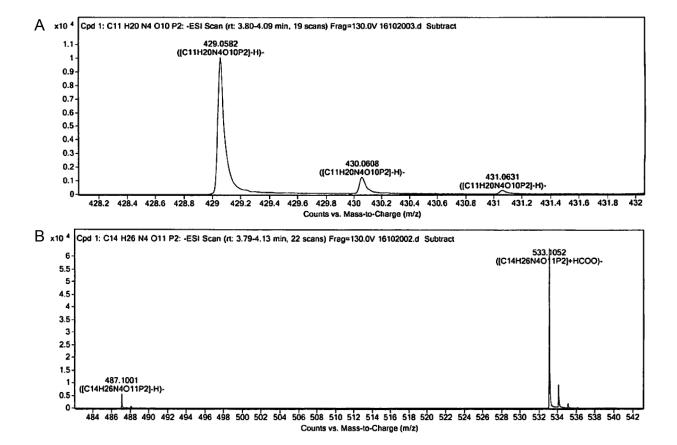


	1	Peak 3	1425.910	38.3	80746700	7.3	0.1
	$\checkmark$	Peak 2	77.675	36.5	89138	12.3	0.0
1	V	Peak 1	2.967	26.0	43	80.4	99.9
			(nm)		(kDa)		
	intens	ity Distribution	Radius	%Pd	Mw-R	%Intensity	%Mass

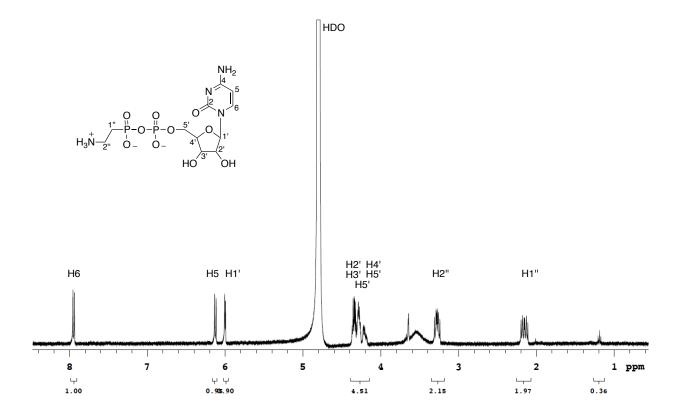


Ma	ss Distribution	Radius	%Pd	Mw-R	%Intensity	%Mass
		(nm)		(kDa)		
	Peak 1	5.011	68.5	146	80.2	99.0
V	Peak 2	118.041	13.5	237311	17.9	0.9
	Peak 3	205.078	5.4	864189	1.8	0.1

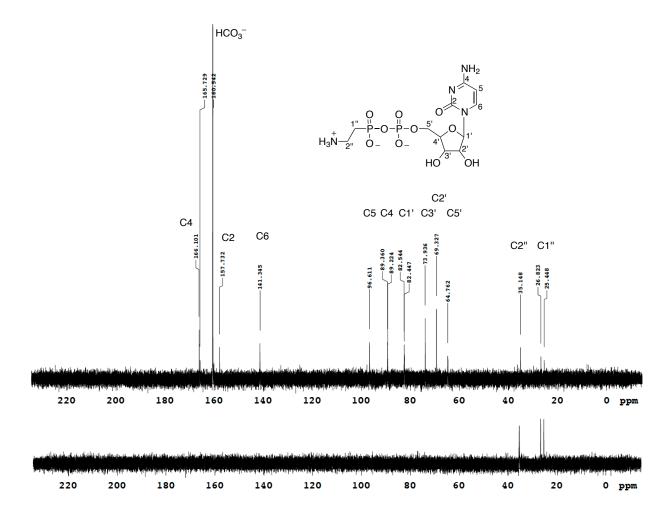
**Supplementary Figure 3.** Dynamic light scattering data showing molecular weights of 38 kDa for Ari1348, 43 kDa for Spn-LicC, and 146 kDa for Tde1415.



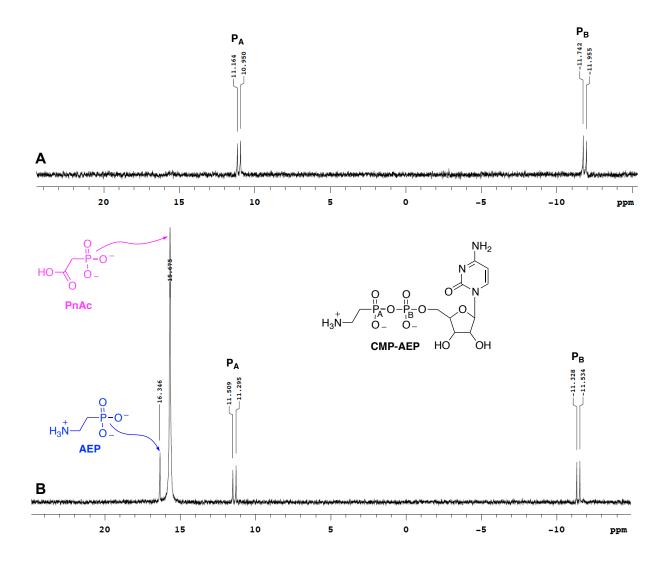
**Supplementary Figure 4.** HR-ESI-MS of (A) CMP-AEP revealing the  $[M-H]^-$  ion at m/z 429.0582 consistent with a molecular mass of 430.0655 for molecular formula  $C_{11}H_{20}N_4O_{10}P_2$  (calculated 430.0655), and (B) CDP-Cho revealing the  $[M-H]^-$  ion at m/z 487.1001 consistent with a molecular mass of 488.1071 (calculated 488.1073).



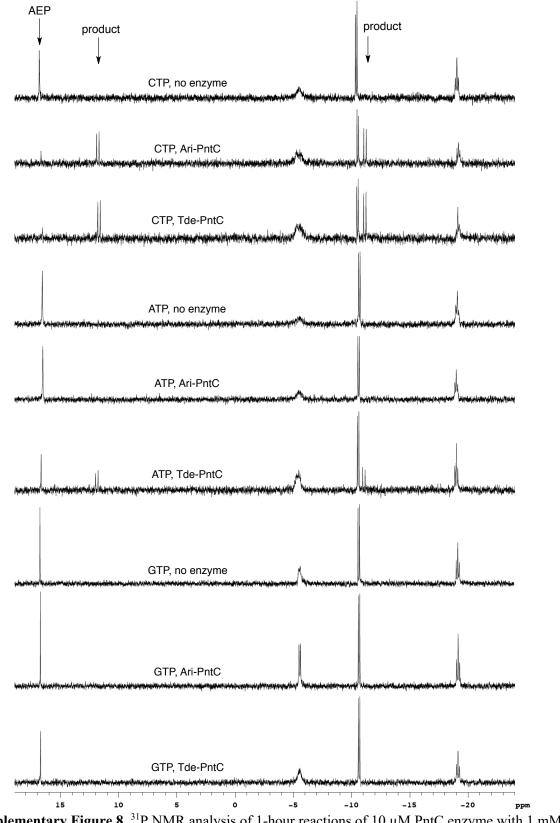
**Supplementary Figure 5.** <sup>1</sup>H NMR (D<sub>2</sub>O, 400 MHz) spectra of CMP-AEP: δ7.95 (d, 1H, H6, *J* = 7.6 Hz), 6.12 (d, 1H, H5, *J* = 7.6 Hz), 6.00 (d, 1H, H1', *J* = 4.0 Hz), 4.82-4.18 (m, 5H, H2', H3', H4', H5'), 3.29 (m, 2H, H2"), 2.15 (m, 2H, H1")



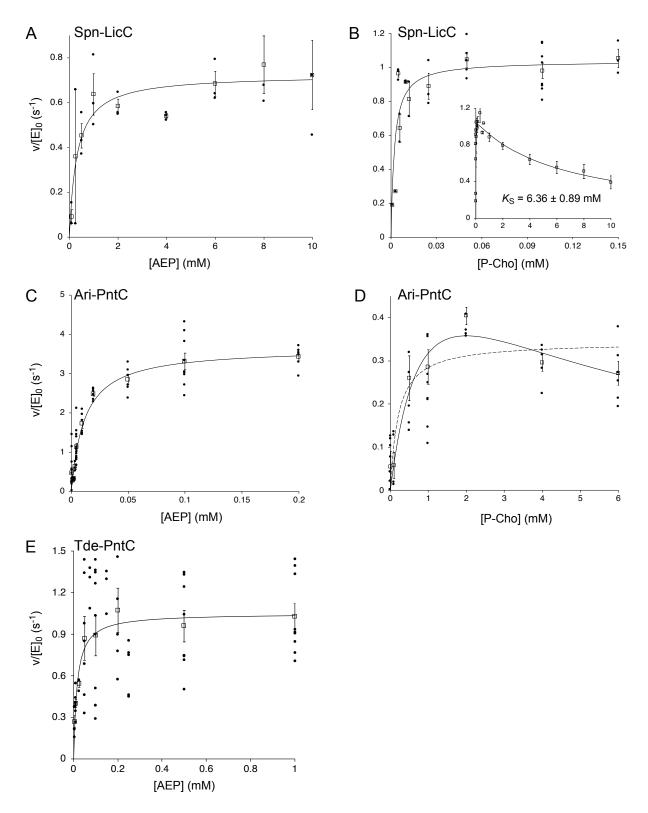
**Supplementary Figure 6.** <sup>13</sup>C NMR (D<sub>2</sub>O, 100 MHz) spectra of CMP-AEP:  $\delta$  166.1 (C4), 157.7 (C2), 141.3 (C6), 96.6 (C5), 89.3 (C4'), 82.5 (C1'), 73.9 (C3'), 69.3 (C2'), 64.8 (C5'), 35.1 (C2''), 26.1 (C1'';  ${}^{1}J_{CP} = 130$  Hz). Bottom spectrum represents commercial AEP alone.



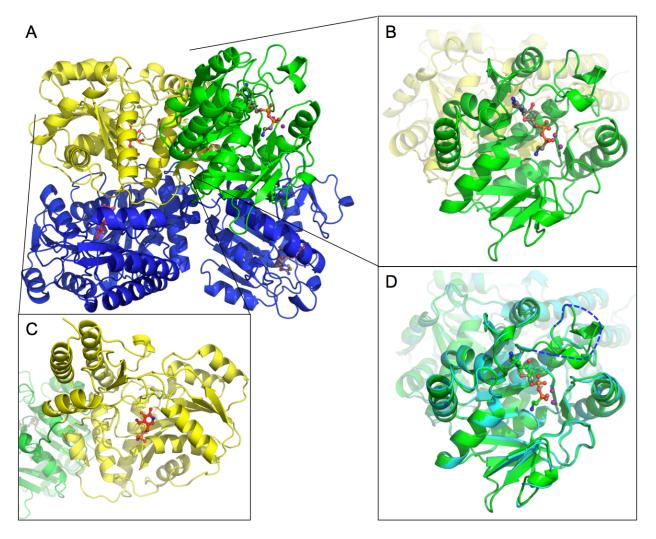
**Supplementary Figure 7.** <sup>31</sup>P NMR (D<sub>2</sub>O, 121 MHz) spectra of (A) CMP-AEP alone and (B) with phosphonoacetic acid (PnAc) and 2-ethylaminophosphonate (AEP). Using PnAc as the internal standard with a reported chemical shift of 15.7,<sup>2</sup> our measured chemical shifts of CMP-AEP:  $\delta$  11.4 (P<sub>A</sub>), -11.4 (P<sub>B</sub>), <sup>3</sup>J = 25.6 Hz.



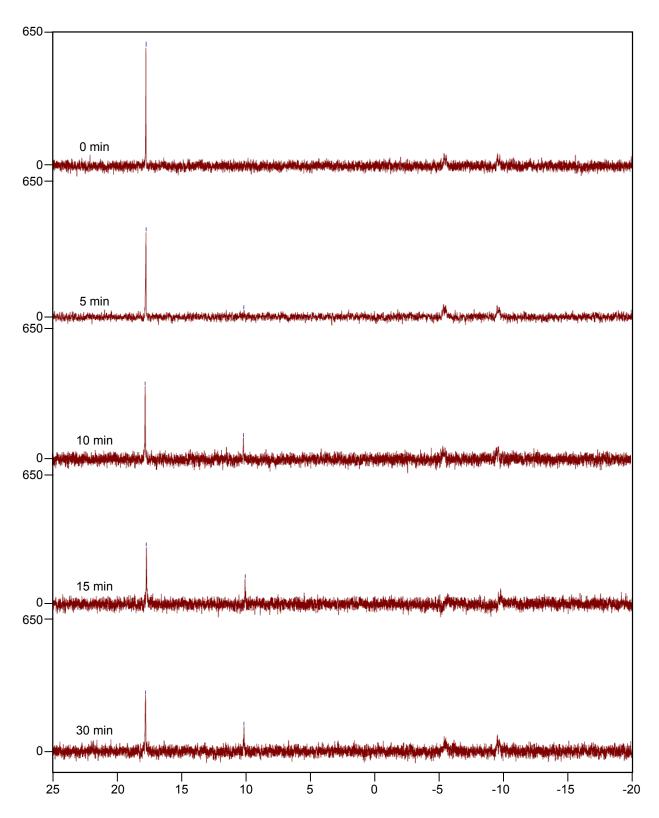
Supplementary Figure 8. <sup>31</sup>P NMR analysis of 1-hour reactions of 10  $\mu$ M PntC enzyme with 1 mM AEP and 2 mM of CTP, ATP, or GTP in 50 mM Tris-Cl and 7 mM MgCl<sub>2</sub>, pH 8.0.



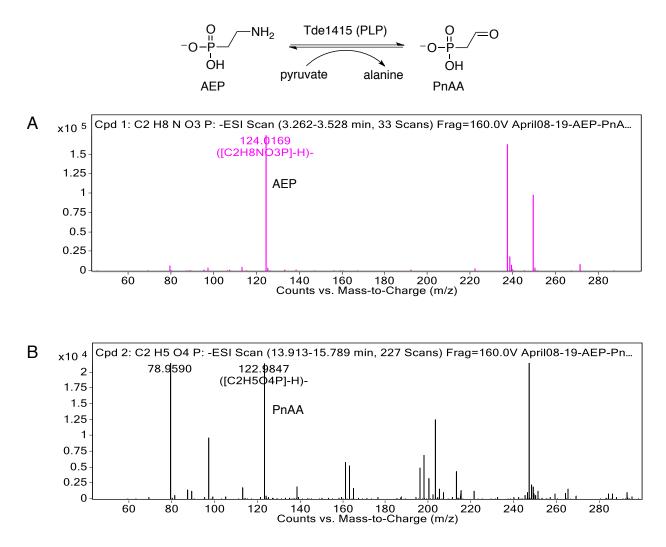
**Supplementary Figure 9.** Steady-state kinetic analysis of cytidylyltransferase activities of (A) Spn-LicC with AEP, (B) Spn-LicC with P-Cho, (C) Ari-PntC with AEP, (D) Ari-PntC with P-Cho, and (E) Tde-PntC with AEP. Activity not detected for Tde-PntC and P-Cho. Error bars show standard errors of the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



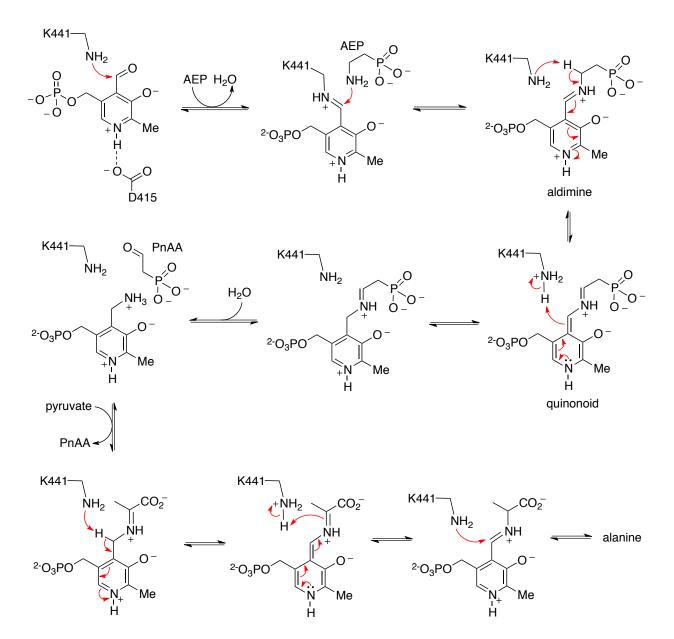
**Supplementary Figure 10.** Cartoon representations of Tde1415 crystal structures. (A) Overall dimeric stucture showing molecule A in blue and molecule B is bi-colored yellow (residues 1-249) and green (residues 250-615). (B) Tde-PntC cytidylyltransferase domain (green) in complex with CMP-AEP. (C) 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).



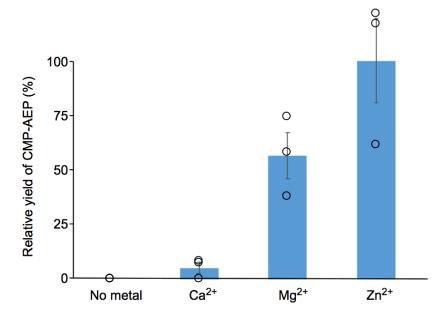
**Supplementary Figure 11.** Time-dependent conversion of 1.5 mM AEP to PnAA catalyzed by 50 nM Tde1415 in the presence of 6 mM pyruvate, 30  $\mu$ M PLP, and 300 mM NaCl in Tris-Cl, pH 8. Reactions were run at 20 °C and quenched with an equal volume of methanol at each time point.



**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).



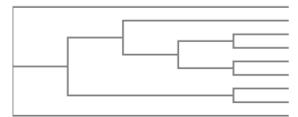
**Supplementary Figure 13.** Proposed mechanism for aminotransferase activity of Tde1415 catalyzing the interconversion of AEP and PnAA. The first structure represents the crystallographically observed state, which unusually does not have an imine linkage between K441 and PLP.



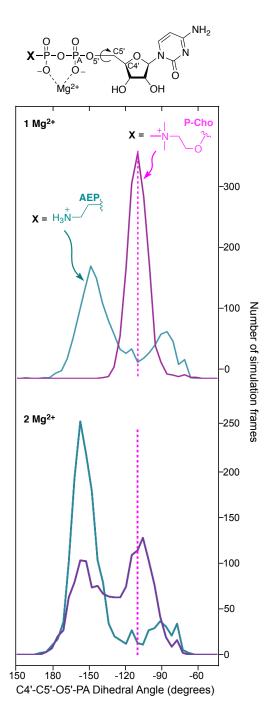
**Supplementary Figure 14.** Comparison of PntC activity in the presence of different metals. The activity of EDTA-treated enzyme ("No metal") was compared against the same enzyme supplemented with  $Ca^{2+}$ ,  $Mg^{2+}$ , or  $Zn^{2+}$ . All four treatments were repeated in triplicate with and are 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  $\mu$ M 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 <sup>31</sup>P 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 (Zn<sup>2+</sup>).

А		
A YqbP	MATTHLDVCAVVPAAGFGRRMQTECPKDYLSIGNOTILEHSVHALLAHPRVKR	53
Cj1416	MAITHEDVCAVVFAAGFGAAAQTECFACTESIGNQIILEHSVNALLAAFKVKK MNAIILAAGFGSRLMPLTKDOPKCMVEYKNKKIIDYEIEALKSAGI-NE	48
Oul-PntC	MARITIAN CSALL BIRDER AND TRACKTIDISTAL AND TRACKTIDIST	43
Ari-PntC	MACVKGSNAEKTNAIIMAAGLGTRMAPLTKTTPKPLISVNGTPMIETVINALVTAGV-ER	59
Spn-LicC	MKAIILANGLGTRLRPMTENTPKALVQVNQKPLIEYQIEFLKEKGI-ND	48
Hin-LicC	MNAIILAAGLGSRFKDITOSTHKSLLDIHGTPNLERTLTFLRQANI-DN	48
FrbH	AVVLAAGLGSRLGEPSSRRPKPLTPVAGRPILAHTLGHLAGVGV-QE	46
Bfr-PntC	MIKTAMIMAAGLGTRFGHYTELVPKGFVEVGGKPMIIRSIETLLSCGI-ER	50
Tde PntC	MIKQAVILAGCLGSRLKDKTKTMPKGFLEIGGTAIVEQSVQKLLAHGI-EK	50
	*.*:* * : *	
YgbP	VVIAISPGDSRFAQLPLANHPQITVVDGGDERADSVLAGLKAAGDAQWVLVHD	106
Cj1416	IAVVGGYLNDVLKNYLNKY-DIEHFFINSKYDKTNMVHTFFCAKDFMLKCIKEKOD	103
Oul-PntC	IYVVVGYLKDQFRYLEERY-GPVKLIENPEYLSKNNISSIYAAIEVLEQGDAFICEAD	100
Ari-PntC	ISVVVGILKEQFCYLEERY-PAVVLVENTEYLEKNNISSIYAAVDVLEQGATFICEAD	116
Spn-LicC	IIIIVGYLKEQFDYLKEKY-GV-RLVFNDKYADYNNFYSLYLVKEELANSYVIDAD	102
Hin-LicC	IVIVTGYLHEQFEYLKKKY-DC-TLIYNEKYREYNSIYSFSLAQDFFSDCYVIDAD	102
FrbH	VVLVVGHLREAVRELAGGEYAGMKIHYVVNPDPSTTNNLRSVRLAR-EFLDQDVFLLEGD	105
Bfr-PntC	IILGTGYKKEAYEALQADF-PQIETCFSPRYADTNSMYTLYNTRDVIGDDSFLLLESD	107
Tde PntC	IVIGTGHCNEYYDNLAKKY-PAIITVKNENYANTGSMGTLEVCA-SFVNESFLLLESD	106
Vala		152
YgbP	AARPCLHQDDLARLLALSETSRTGGILAAPVRDTMKRAEPGKNAIA	163
Cj1416 Oul-PntC	LIVSYADIVYFQDCVQKLINIKEELAIVVDKSWRKLWSKRFANPLEDAETLKMTNGYIIE LVVSESGIFSDLPSKSCYFGRMTEGYTDDWAFDLDTTGRITR	142
Ari-PntC	LVVSESGIFSDLFSRSCIFGRKFEGITDDWAFDLDDIGRITR LVISDEHIFOPRPSRSCYFGRKFSGHTGDWVFDLDDSGKIVR	158
Spn-LicC	NYL-FKNMFRNDLTRSTYFSVYREDCTNEWFLVYGDDYKVQD	143
Hin-LicC	VVL-NRNIFLTKPSHSKYFTVIRSKTHNEWLPILNSNGQVIR	143
FrbH	VVEPAVLERLAAAPEASAVAASRPLRPLAGTVVRADADGVLTD	149
Bfr-PntC	LVFERKAILSLLDDEFPDVMLVSSLTKFODOYYVEYDRNHILTS	151
Tde PntC	LIYDSAGLFSLINDERKNLILASGATKSGDEVYLEADEKNCLTG	150
_	:	
YgbP	HTVDRNGLWHALTPQFFPRELLH-DCLTRALNEGATITDEASAL-	195
Cj1416	LGKKANAYDEIEAQYIGLFKFSYQFLSEVIAFYEMLDRDILYDNKNFENM-	213
Oul-PntC	VGKGAVNSYAMVGISFFKGGDAAHLARCIRSAYTQEGHERL-	183
Ari-PntC	IGKGGSDTYAMVGLSYFSAPDAKRLARFMHDAYKETGHEQL-	199
Spn-LicC	IIVDSKAGRILSGVSFWDAPTAEKIVSFIDKAYVSGEFVDL-	184
Hin-LicC	IEIGSLNQSSLSGVSYWTTRDCDIILTLLKEYTSEVRLKNPKL-	186
FrbH	YVDDRRQAG-AFDH-PGALKTANLYLLREAFLRERFLPALEELDRRLAGQG	198
Bfr-PntC	CSVDKDALE-AKGELVGIHKLSNTFYRRMCADYATILESQPKL- LSKNRDALKNIFGELVGITKLTKSTLDKMCAYAKIHHSDLPKM-	193 193
Tde_PntC	LSKARDALRAIFGELVGITKLIRSTLDRACAIARIHHSDLPRA-	195
YgbP	EYCGFHPQLVEGRADNIKVTRPEDLALAEFYLTRTIHQENT 236	AEP/ChoP binding
Cj1416	YMTSFLQALIEKYNNAKAVEIDGNWCEIDFMSDLEVQIDK 253	
Oul-PntC	FWDDIVNQHIDEFDLRIRPVLEGQVVELDTIEELAAFDPSYSDQLRSNIDEG 235	Metal coordination
Ari-PntC	FWDDVVNNHIAELDLSIHPVEAQQIAELDSVAELAAFDHGYVYLLRS 246	Cytosine binding
Spn-LicC	YWDNMVKDNIKELDVYVEELEGNSIYEIDSVQDYRKLEEILKNEN 229	Phosphate binding
Hin-LicC	YWDTIPMEYIEKLNIYTEQLNSDDIFEMDNLDDYHHILQKLTPNKEK- 233	
FrbH	YYDYAVSDGLAAGGHAWRVADISDLAWYEVDDPGDQRQAD238	Ribose binding
Bfr-PntC	GYEYELLR-MSRSVSPVRVLRVEGLKWYEIDDEADLSYAEEHIIRYC 239	···· Apo salt bridge
Tde_PntC	EYEHALLE-AAK-TIPVAIKRIEYFVWREIDNEDHLEMA 230	

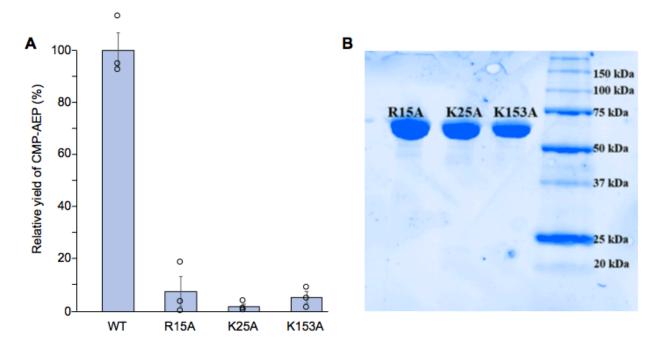
В



YgbP 0.41142 Cj1416 0.38747 Oul-PntC 0.18227 Ari-PntC 0.15686 Spn-LicC 0.30922 Hin-LicC 0.3196 Bfr-PntC 0.28599 Tde\_PntC 0.2948 FrbH 0.36747 **Supplementary Figure 15.** (A) Clustal Omega multiple sequence alignment of selected PF12804 cytidylyltransferases. 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)RX<sub>8</sub>PK consensus sequence. The dotted line represents a salt bridge between Glu216 and Arg129 observed in the apo Spn-LicC crystal structure (PDB 1JYK).<sup>3</sup> (B) Cladogram of cytidylyltransferases from the alignment. YgbP is a MEP cytidylyltransferase belonging to the IspD Pfam (PF01128), which possesses significant overlap with PF12804.



**Supplementary Figure 16.** The effects of a second  $Mg^{2+}$  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  $Mg^{2+}$  ion in the active site. The bottom panel shows data resulting from the inclusion of a second  $Mg^{2+}$  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 <sup>31</sup>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.

	Tde-PntC-apo	Tde-PntC-CMP-AEP
Data collection	-	
Space group	P21	P21
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	89.05, 129.01, 135.80	76.45, 154.05, 134.58
$\alpha, \beta, \gamma$ (°)	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)
R <sub>merge</sub>	0.097 (0.700)	0.188 (0.965)
CC <sub>1/2</sub>	0.996 (0.728)	0.989 (0.767)
Ι/σΙ	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
Rwork / Rfree	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 <sub>4</sub>	-	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 <sub>4</sub>	-	36.5
Water	48.6	28.7
R.m.s. deviations		
Bond lengths (Å)	0.005	0.011
Bond angles (°)	0.948	1.14

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

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

Supplementary Table 2. Suams	used in this study.	
Strain	Genotype/Description	Source (Reference)
<i>E. coli</i> DH5α	E. coli host for general cloning	Life Technologies
E. coli BL21(DE3)	E. coli 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
A. rimae ATCC 49626	VPI D140H-11A [NCFB 2896]	$ATCC^4$
<i>O. uli</i> ATCC 49627	$VPI D76D-27C^{T} = DSM 7084$	ATCC <sup>5</sup>

### Supplementary Table 2. Strains used in this study.

### Supplementary Table 3. Plasmids used in this study.

Supprementary rank 5. Trasmus used in this study.						
Plasmid	Description	Source (Reference)				
pGH1000	pET29 plasmid containing Spn-licC as an	This study				
	NdeI/XhoI fragment					
pGH2000	pET29 plasmid containing ari1348 as an	This study				
	NdeI/NotI fragment					
pGH3000	pET21 plasmid containing <i>tde1415</i> as an	This study				
	NdeI/NotI fragment					

Target gene	Primer Name	Sequence $(5' \rightarrow 3')$
oul594	ouPEPmut-F	cgagaggatacggctctacg
	ouPEPmut-R	ctccatgatgtcgtctatcgtgc
oul592	ouPnPy decarb-F	atcacatcatcgccgcaaac
	ouPnPy decarb-R	tggtggagacgatggggtc
oul_rpoB	ourpoB-F	tcgacgtgcgcttcgtc
	ourpoB-R	gacacgcgcaaggtcg
oul602	ouLicC(602)-F	cgaggaggcgggtctg
	ouLicC(602)-R	gagtgatgcgaccgcc
oul609	ouLicD(609)-F	ctgcgtagggagggcg
	ouLicD(609)-R	gaaggtagggcatgcccg
oul593	ouLicC(593)-F	ccggtcaagctcatcgaaaac
	ouLicC(593)-R	ggacagggcggattctg
oul591	ouLicD(591)-F	ctgggatgatgatatcgacatcgg
	ouLicD(591)-R	tgtctgggtgaagcggttc
ari1347	arPEPmut-F	ctatctttacgacgacgtgattgc
	arPEPmut-R	tatccccgtcaagaatgatgggtttg
ari1349	arPnPy decarb-F	attatcaatcctgtggcttctcttctg
	arPnPy decarb-R	cgtcaccgtcaatgcacc
ari_rpoB	arrpoB-F	catgaccgagcgcgg
	arrpoB-R	gctatctcaagaccaagcttcttg
ari1348	arLicC-F	ggcgttgagaggatctctgtg
	arLicC-R	taattcggcaatatggttattcaccacatc
ari764	arLicD(764)-F	taccatctccatcggaatctttccg
	arLicD(764)-R	atetttecaettgecaaaateaagtetg
ari768	arLicAC-F	ggactttcttctcgctttgctc
	arLicAC-R	cacggtcaaaatacacgtgtcc
ari769	arLicD(769)-F	cttgggacgatgatattgacgtcg
	arLicD(769)-R	tggtcttcacgtgtgcatagc
tde1415	Fwd-NdeI	catatgattaagcaagcggtgattctggc
	Rvs-XhoI	ctcgagaacgccaacgccgatgc
	Fwd-R15A	ctgggtagcgcactgaaggataagaccaagacc
	Rvs-R15A	ggtcttggtcttatccttcagtgcgctacccag
	Fwd-K25A	gaccaagaccatgccggcgggttttctggagatc
	Rvs-K25A	gatetecagaaaaaccegecaggeatggtettggte
	Fwd-K153A	ctgaccggcctgagcgcaaaccgtg
	Rvs-K153A	cacggtttgcgctcaggccggtcag

# Supplementary Table 4. Primers used in this study.

#### **Supplementary References**

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