Supporting Information

Characterization of a Unique Pathway for 4-Cresol Catabolism Initiated by Phosphorylation in *Corynebacterium glutamicum*

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Running title: *The 4-Cresol Catabolic Pathway Initiated by Phosphorylation*

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Keywords: 4-cresol, biodegradation, *Corynebacterium glutamicum*, cytochrome P450 monooxygenase, phosphorylation

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Supporting Information Text

Synthesis of diethyl (4-formylphenyl) phosphate. To a solution of 4-hydroxybenzyl aldehyde (2.3 g, 19 mmol) dissolved in carbon tetrachloride (20 mL) was added diethyl phosphite (2.9 mL, 23 mmol) at 0 ºC under argon atmosphere. Triethylamine (3.2 mL, 23 mmol) was carefully added drop-wise to the mixture by using a dropping funnel. The mixture was stirred overnight at room temperature. Water (50 mL) was added and the organic layer was separated. The organic layer was washed twice with dilute hydrochloric acid (2 × 25 mL), four times with dilute sodium hydroxide solution (4 × 25 mL), and twice with brine (2 × 25 mL) before being dried over anhydrous $MgSO_4$. Removal of solvent on rotary evaporator gave a crude, which was further purified by silica gel column chromatography using a petroleum ether/ethyl acetate (4/1: v/v) mixture to afford diethyl (4-formylphenyl) phosphate (3.5 g). Yield: 71%.

Synthesis of diethyl (4-(hydroxymethyl)phenyl) phosphate. To a solution of diethyl (4-formylphenyl) phosphate (1.5 g, 5.8 mmol) dissolved in absolute ethyl alcohol (20 mL) at 0 ºC was added sodium borohydride (0.29 g, 7.5 mmol) portion wise. The reaction mixture was then warmed to room temperature and stirred for 30 min. The reaction was quenched with water (10 mL). Ethanol was evaporated and the resulting mixture was extracted with CH_2Cl_2 (2 × 30 mL). The extract was washed with brine (30 mL), and dried over anhydrous $MgSO_4$. Removal of solvent on rotary evaporator gave a crude, which was further purified by silica gel column chromatography using a petroleum ether/ethyl acetate (1/2: v/v) mixture to afford diethyl (4-(hydroxymethyl)phenyl) phosphate (1.4 g). Yield: 95%.

Synthesis of compound benzylalcohol-4-phosphate. Diethyl (4-(hydroxymethyl)phenyl) phosphate (0.74 g, 2.9 mmol) was dissolved in dry CH_2Cl_2 (25 mL). Then bromotrimethylsilane (3.8 mL, 10 equiv. to phosphate ester) was added to the solution and the mixture was heated to reflux for 18 h under argon atmosphere. The solvent was evaporated and the residue was dissolved in anhydrous methanol (25 mL). The solution was stirred for 30 min at 40 ºC. Then the resulting solution was concentrated to give benzylalcohol-4-phosphate (0.53 g). Yield: 90%.

Synthesis of compound benzylaldehyde-4-phosphate. The detailed procedures were the same as synthesis of benzylalcohol-4-phosphate with using diethyl (4-formylphenyl) phosphate as the reaction substrate. Yield: 91%.

Synthesis of diethyl 4-tolyl phosphate. The detailed procedures were the same as synthesis of diethyl (4-formylphenyl) phosphate with using 4-cresol as the reaction substrate. Yield: 80%.

Synthesis of compound 4-methylbenzyl phosphate. The detailed procedures were the same as synthesis of benzylalcohol-4-phosphate with using diethyl 4-tolyl phosphate as the reaction substrate. Yield: 92%.

Table S1: Strains and plasmids used in this study

Strain, plasmid	genotype/phenotype	Source
E. coli BL21(DE3)		Novagen
E. coli DH5α		Invitrogen
C. glutamicum RES167	restriction-deficient mutant of ATCC13032, Δ(*cglIM-cglIR-cglIIR*)	Li T, *et al.*, 2014
pET28b	expression vector	Novagen
pET28b-*creC*	pET28b derivative for expression of *creC*	Present study
pET28a-*creD*	pET28a derivative for expression of *creD*	Li T, *et al.*, 2014
pET28a-*creE*	pET28a derivative for expression of *creE*	Li T, *et al.*, 2014
pET28a-*creF*	pET28a derivative for expression of *creF*	Li T, *et al.*, 2014
pET28a-*creG*	pET28a derivative for expression of *creG*	Li T, *et al.*, 2014
pET28b-*creH*	pET28b derivative for expression of *creH*	Present study
pET28b-*creI*	pET28b derivative for expression of *creI*	Present study
pET28a-*creJ*	pET28a derivative for expression of *creJ*	Li T, *et al.*, 2014

Table S2: Primers used in this study

Primers	Sequences (5'-3')	Description
creC-F	CCGCCATATGCCGATGAATGCTGCAACCA	To construct pET28b-*creC*
creC-R	GCGCGGATCCGTGTTTAGCTGAGAACTTTCAG	To construct pET28b-*creC*
creD-F	GACACATATGACTCGCAGTAATTTACCCGC	To construct pET28b-*creD*
creD-R	CGGAATTCGAGAAGCACGCCTGGTTG	To construct pET28b-*creD*
creE-F	GACACATATGAATACTTCAGCTGAAACTGGA	To construct pET28b-*creE*
creE-R	GGAGCTCTCCCAAGCGGGTAAAT	To construct pET28b-*creE*
creF-F	GCGCCATATG AAGATCATGTCTACTATTCATT	To construct pET28b-*creF*
creF-R	TCATAAGCTT TCACACTTGCGTTTCTGGCG	To construct pET28b-*creF*
creG-F	GACACATATGCCTAGTCCACGCACTGTTC	To construct pET28b-*creG*
creG-R	CGGAATTCAGTAGACATGATCTTCTCCTTAG	To construct pET28b-*creG*
creH-F	GCGCCATATGGCTAATAAATCTTTCCCCAAGCCC	To construct pET28b-*creH*
creH-R	ACGCGGATCCGTGGACTAGGCATGTGTATC	To construct pET28b-*creH*
creI-F	TCGCCATATGGGAGACACCATGACCAACAGT	To construct pET28b-*creI*
creI-R	GCCCAAGCTTAACGAGGTAGTACGGGTACA	To construct pET28b-*creI*
creJ-R	CGTGCCATGGGCACAATGACTTCCCAGACT	To construct pET28b-*creJ*
creJ-R	CGGGAAGCTTAGCGTTCCAAGTCACGGGAA	To construct pET28b-*creJ*
CB-F	TCTCGGTGGCTTGGTATT	RT-PCR. To amplify region a in Figure S11.
CB-R	AAGATTCGGCGATGACTG	RT-PCR. To amplify region a in Figure S11.
DC-F	GGCTTCTAGCTCATGGTTCG	RT-PCR. To amplify region b in Figure S11.
DC-R	AATGGGCGGATGTCTTCG	RT-PCR. To amplify region b in Figure S11.
DE-F	CTCAAACTACAAATTGCAGGAC	RT-PCR. To amplify region c in Figure S11.
DE-R	CGGAGATGATTCGGCTCT	RT-PCR. To amplify region c in Figure S11.
FE-F	GGCGGTTCCTTATCGTGT	RT-PCR. To amplify region d in Figure S11.
FE-R	GATCCGTCGTCGTTCTTTT	RT-PCR. To amplify region d in Figure S11.
GF-F	ACCAGGGTGCTTGCTAAA	RT-PCR. To amplify region e in Figure S11.
GF-R	GGACTGCGGTCTCCATTA	RT-PCR. To amplify region e in Figure S11.
HG-F	AAAACCGGCGATTACCTC	RT-PCR. To amplify region f in Figure S11.
HG-R	GCTCTTTAGCAAGCACCCT	RT-PCR. To amplify region f in Figure S11.
IH-F	AAGCCCGTTGATGAAGAA	RT-PCR. To amplify region g in Figure S11.
IH-R	TGAATCGCAGAACCAGAAT	RT-PCR. To amplify region g in Figure S11.
JI-F	TGGTTTCGGCATCCACTA	RT-PCR. To amplify region h in Figure S11.
JI-R	GAAGCCCAGCATTTACGG	RT-PCR. To amplify region h in Figure S11.
RJ-F	CGCTATCTCCCGATCCTT	RT-PCR. To amplify region i in Figure S11.
RJ-R	AACATCACTGGCTCTTCCT	RT-PCR. To amplify region i in Figure S11.
R-F	CGATGCGGTATAGAACGA	RT-PCR. To amplify region j in Figure S11.
R-R	GAAACGAGACGGTGGGTG	RT-PCR. To amplify region j in Figure S11.

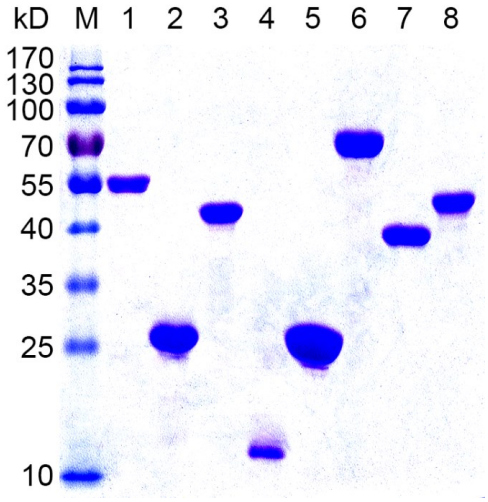

Figure S1. SDS-PAGE analysis of purified proteins. M, marker; 1, CreC; 2, CreD; 3, CreE; 4, CreF; 5, CreG; 6, CreH; 7, CreI; 8, CreJ.

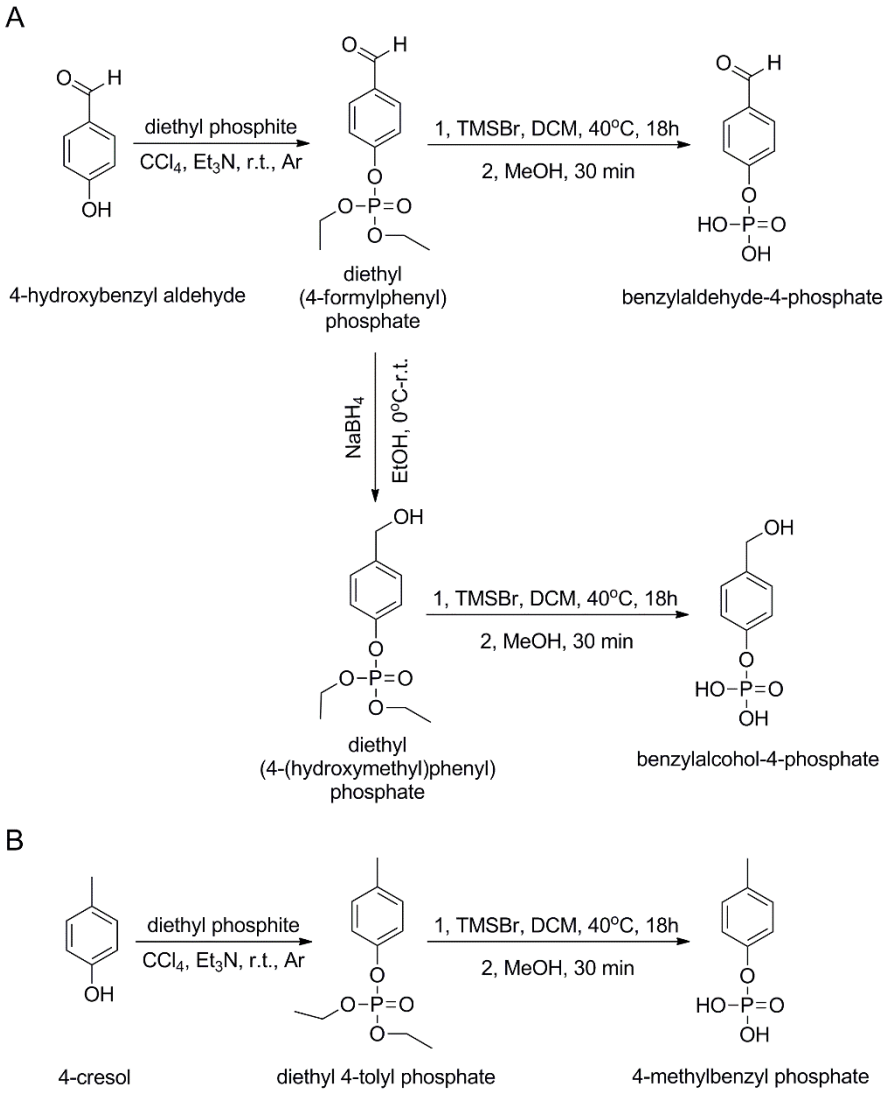

Figure S2. Chemical synthesis routes of 4-methylbenzyl phosphate, benzylalcohol-4-phosphate and benzylaldehyde-4-phosphate.

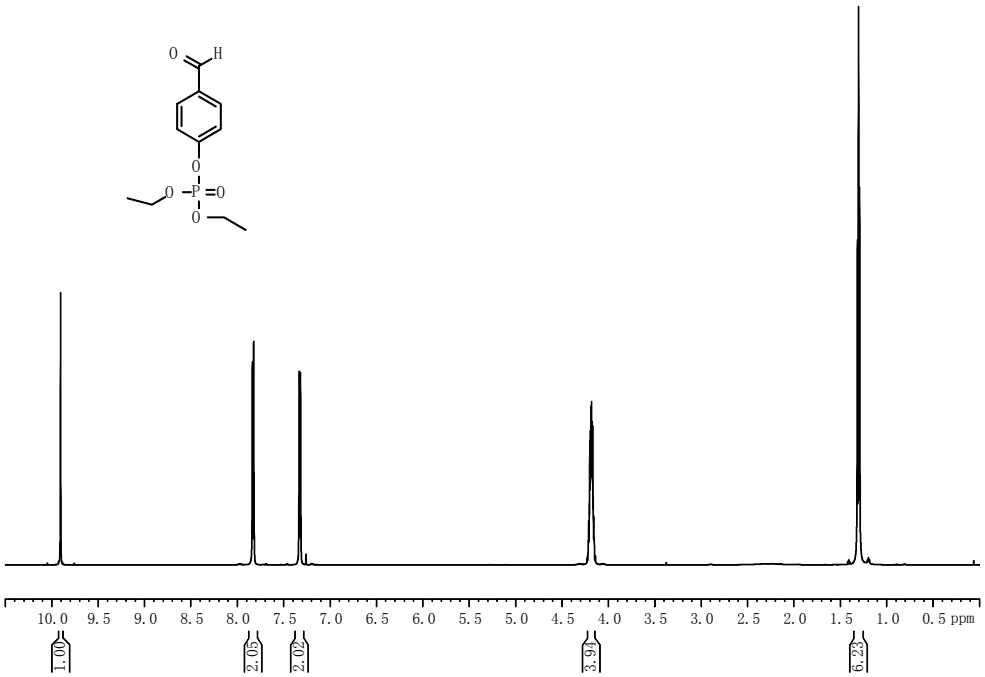

Figure S3. ^{1}H NMR spectrum of diethyl (4-formylphenyl) phosphate in $CDCl_3$.

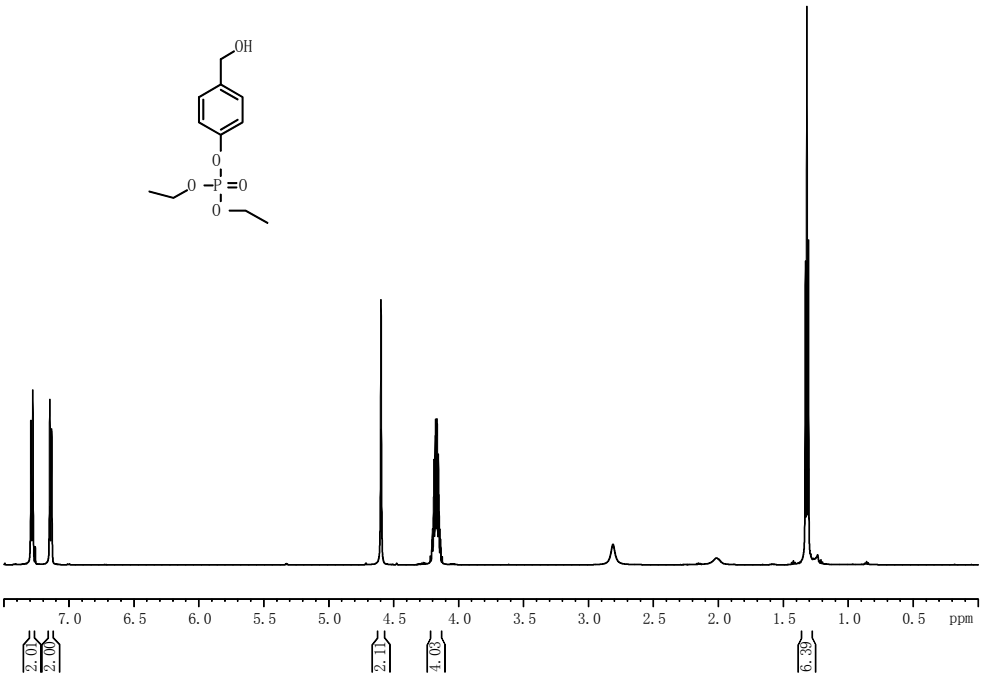

Figure S4. ^{1}H NMR spectrum of diethyl (4-(hydroxymethyl)phenyl) phosphate in $CDCl_3$.

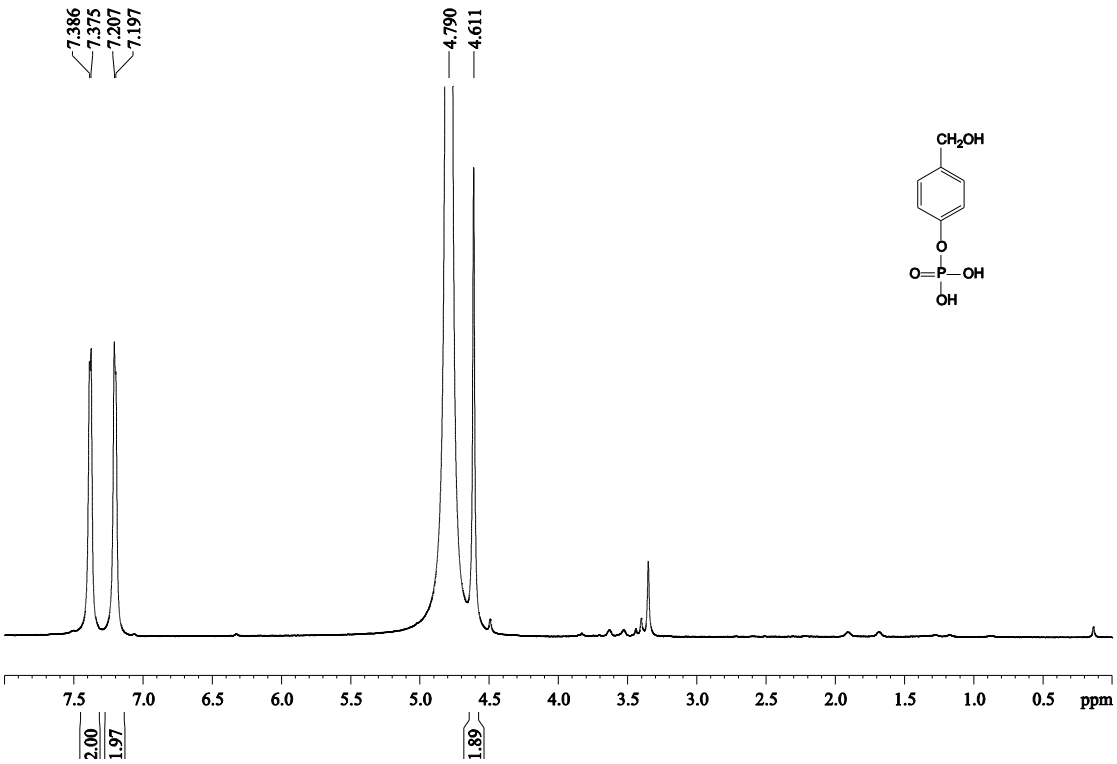

Figure S5. ^{1}H NMR spectrum of benzylalcohol-4-phosphate in D_2O.

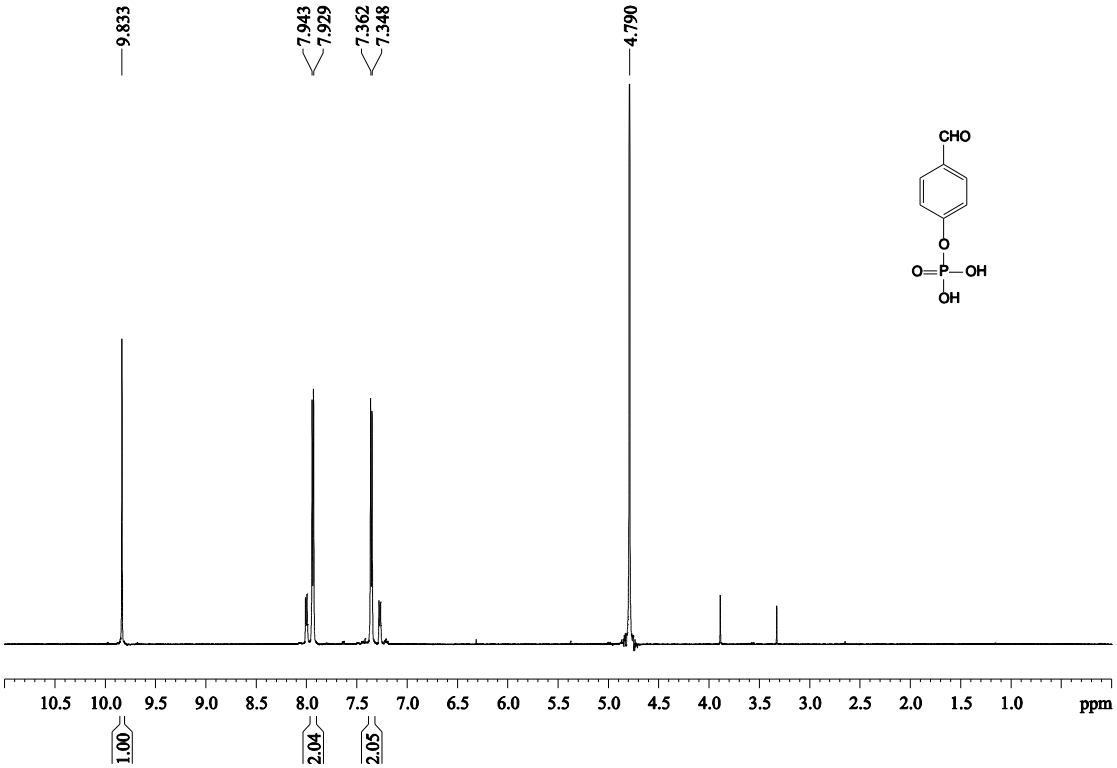

Figure S6. ^{1}H NMR spectrum of benzylaldehyde-4-phosphate in D_2O.

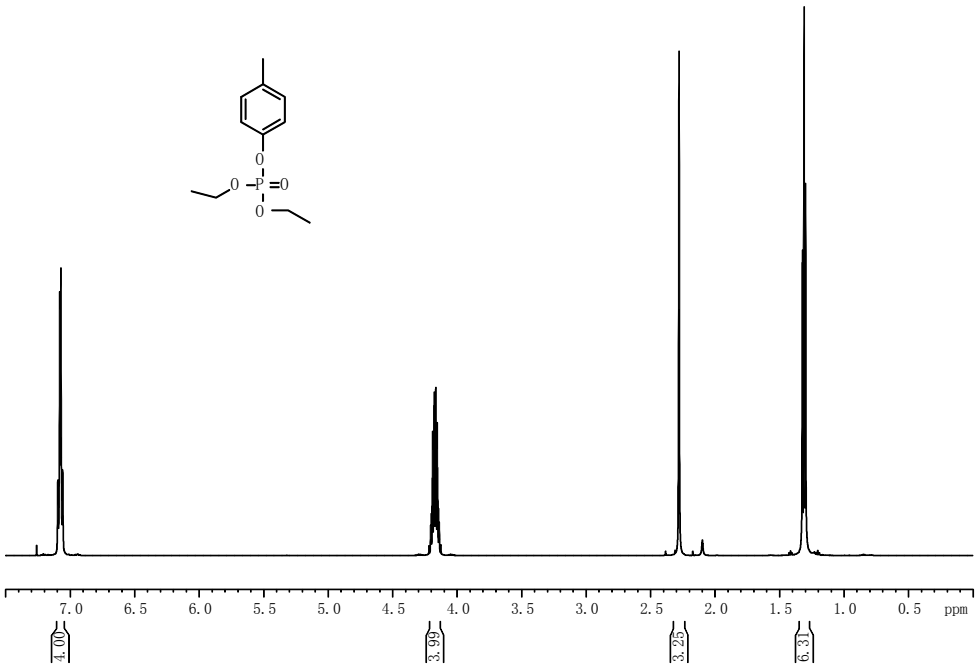

Figure S7. ^{1}H NMR spectrum of diethyl 4-tolyl phosphate in $CDCl_3$.

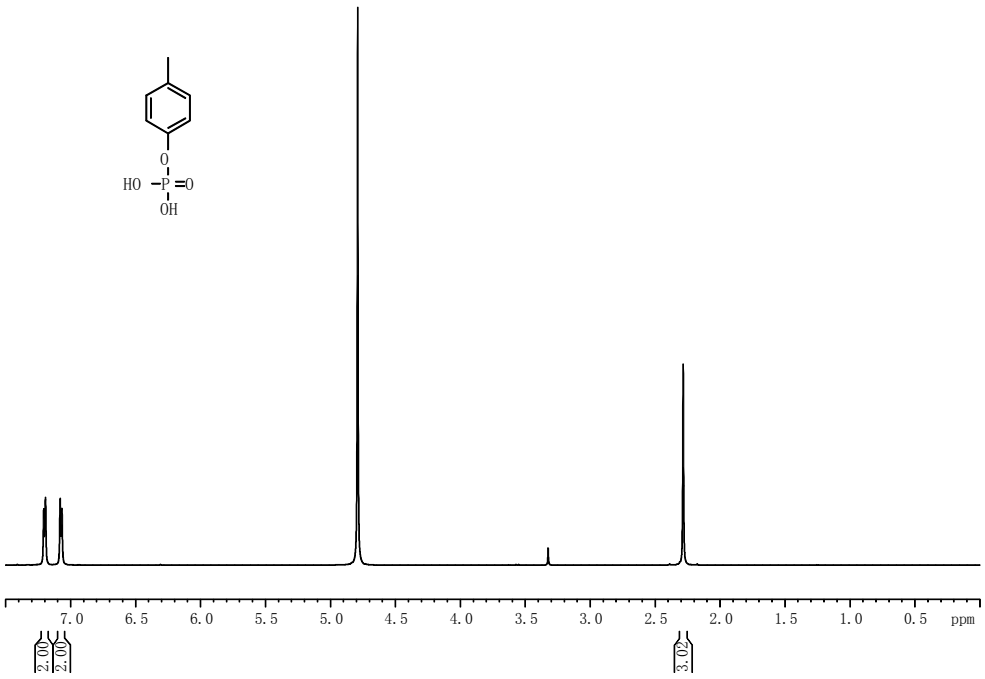

Figure S8. ^{1}H NMR spectrum of 4-methylbenzyl phosphate in D_2O.

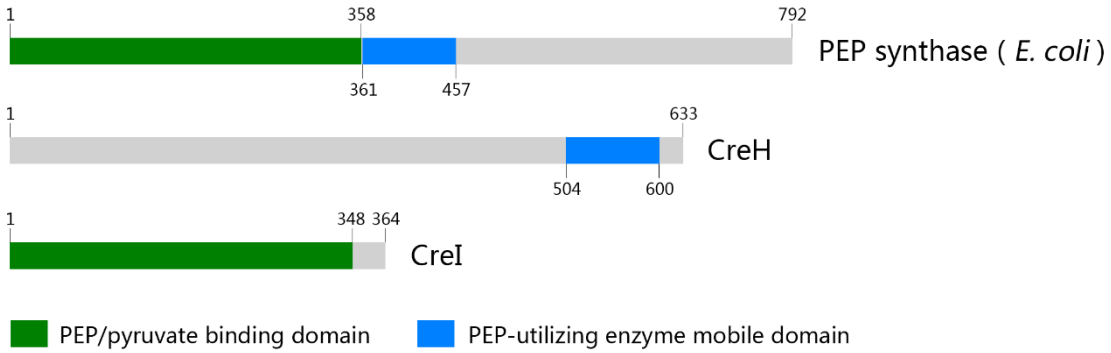

Figure S9. Conserved domain analysis of the PEP synthase of *E. coli*, CreH and CreI.

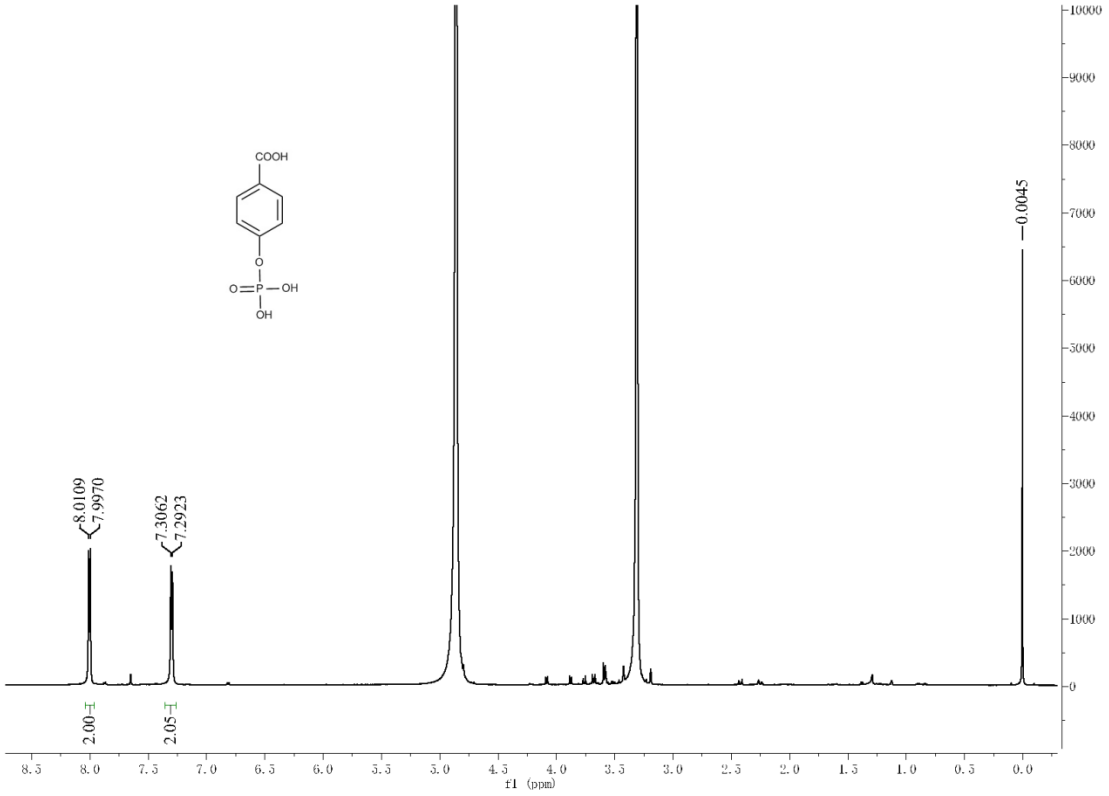

Figure S10. ^{1}H NMR spectrum of benzoate-4-phosphate in D_2O.

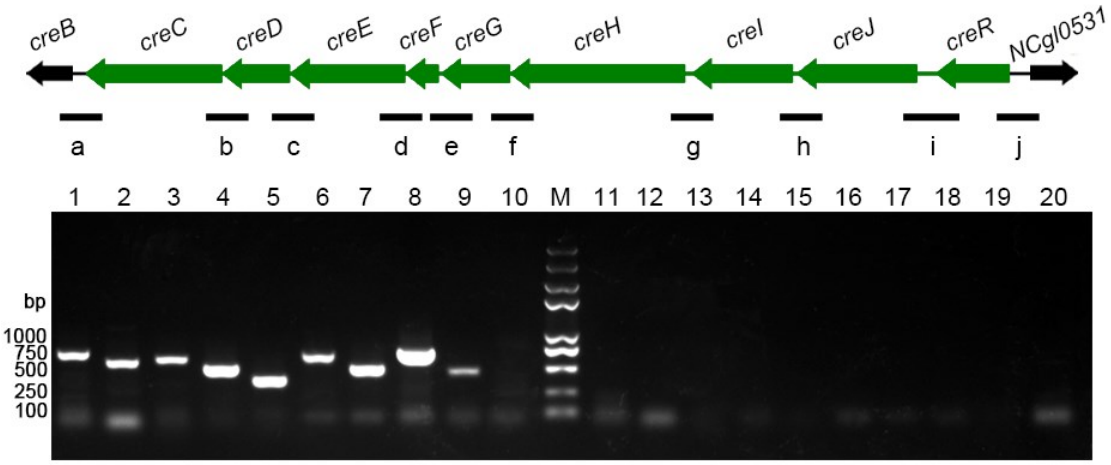

Figure S11. Results of RT-PCR assays. The cDNA prepared from *Corynebacterium glutamicum* RES167 grown in LB broth containing 2 mM 4-cresol for 48 h was used as template. Black bars below the gene cluster (a to j) represent the locations of fragments amplified from RT-PCR assays. Lanes 1-10 show the a-j RT-PCR products. Lanes 11-20 were negative controls with mRNA as PCR template. M: Molecular size standard.

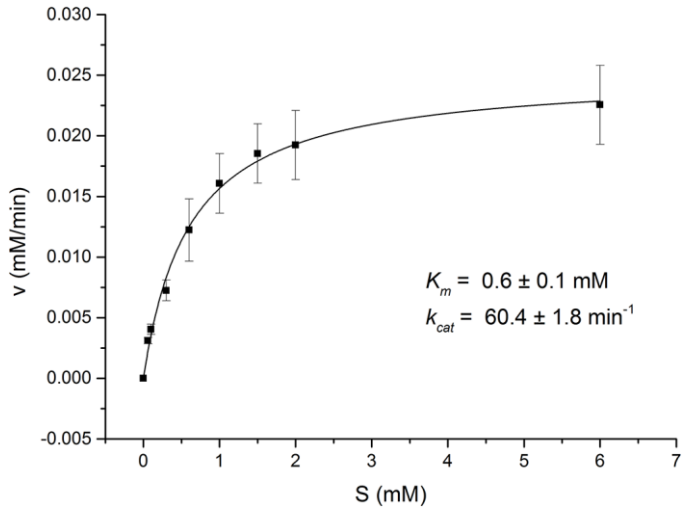

Figure S12. The kinetic curve of CreHI with 4-cresol as substrate. ([CreH] = [CreI] = 1.7 μM)

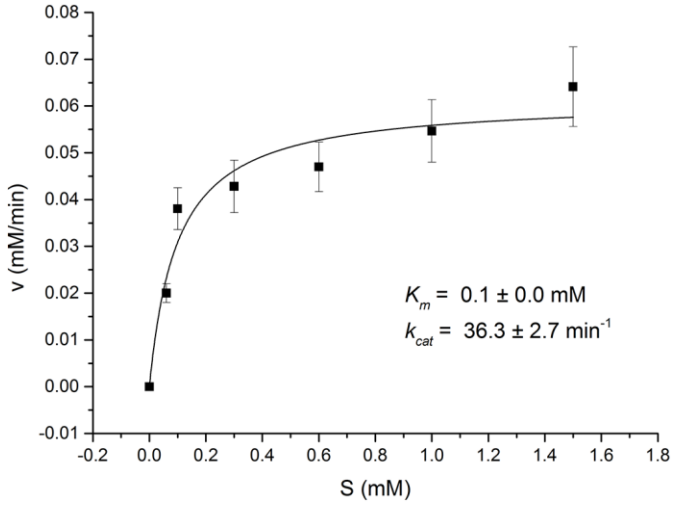

Figure S13. The kinetic curve of CreHI with 4-hydroxybenzyl alcohol as substrate. ([CreH] = [CreI] = 1.7 μM)

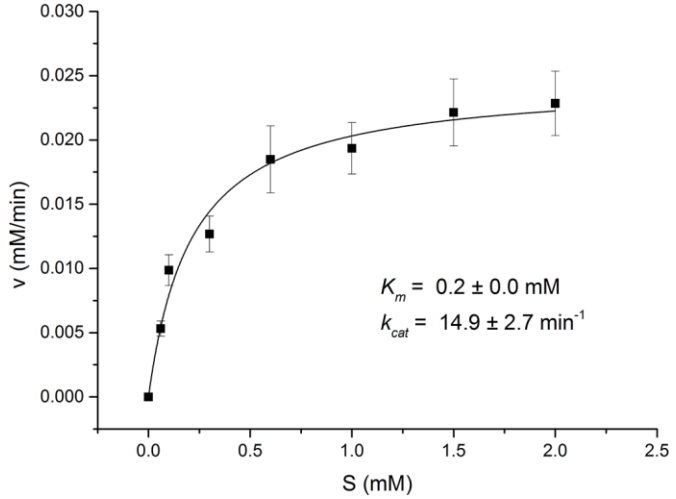

Figure S14. The kinetic curve of CreHI with 4-hydroxybenzyl aldehyde as substrate. ([CreH] = [CreI] = 1.7 μM)

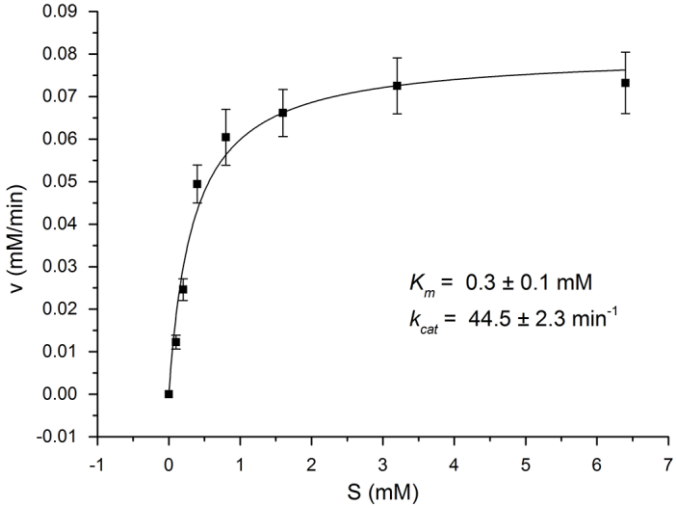

Figure S15. The kinetic curve of CreJEF with 4-methylbenzyl phosphate as substrate. ([CreJ] = [CreE] = [CreF] = 0.8 μM)

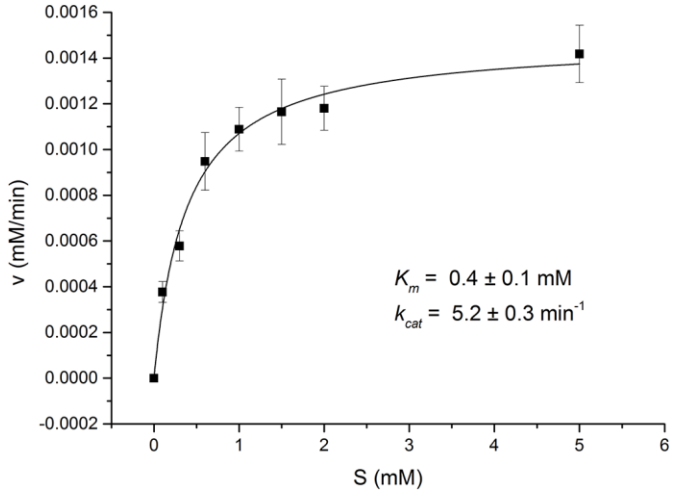

Figure S16. The kinetic curve of CreJEF with benzylalcohol-4-phosphate as substrate. ([CreJ] = [CreE] = [CreF] = 0.8 μM)

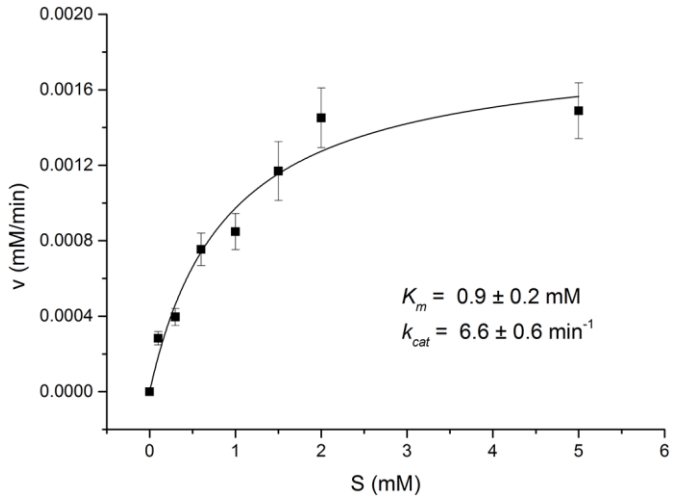

Figure S17. The kinetic curve of CreJEF with benzylaldehyde-4-phosphate as substrate. ([CreJ] = [CreE] = [CreF] = 0.8 μM)

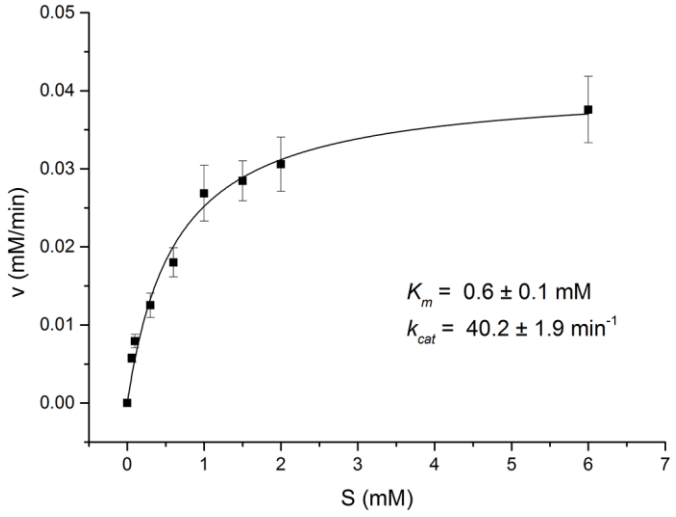

Figure S18. The kinetic curve of CreD with 4-methylbenzyl phosphate as substrate. ([CreD] = 1.0 μM)

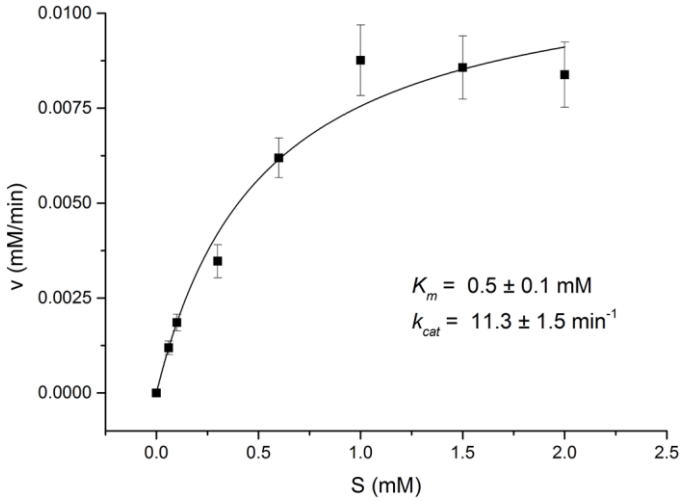

Figure S19. The kinetic curve of CreD with benzylalcohol-4-phosphate as substrate. ([CreD] = 1.0 μM)

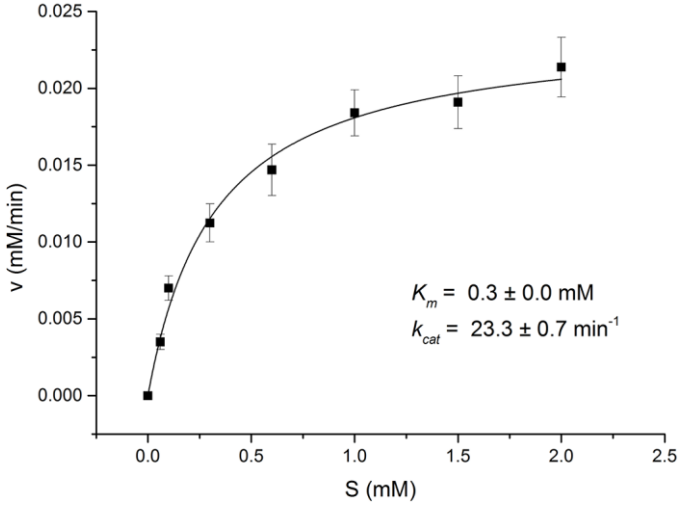

Figure S20. The kinetic curve of CreD with benzylaldehyde-4-phosphate as substrate. ([CreD] = 1.0 μM)

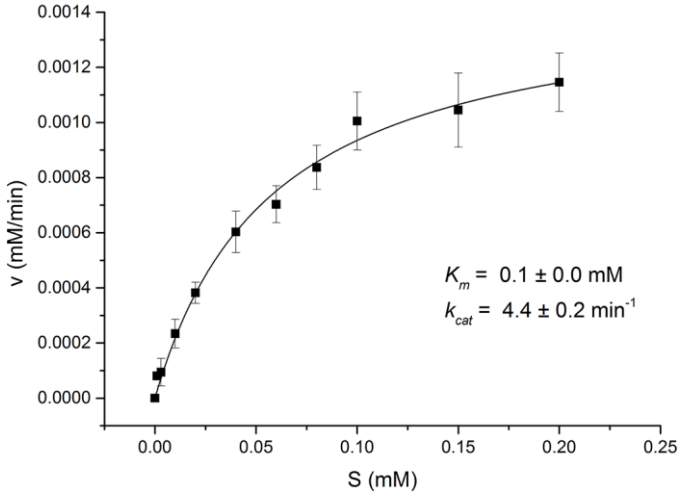

Figure S21. The kinetic curve of CreC with 4-hydroxybenzyl aldehyde as substrate. ([CreC] = 0.3 μM)

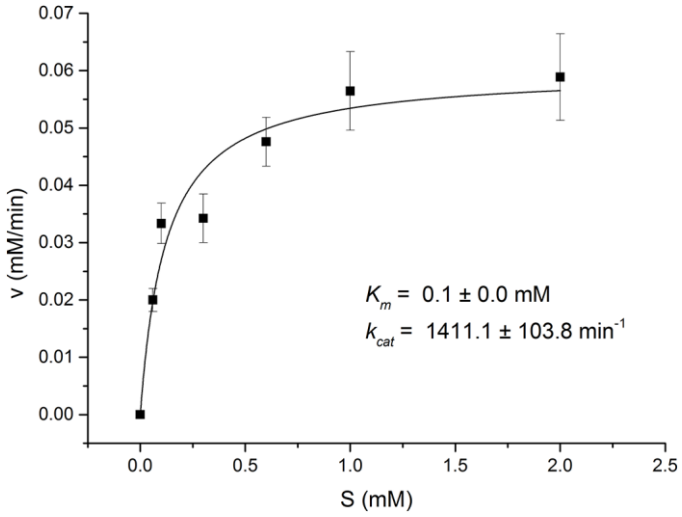

Figure S22. The kinetic curve of CreC with benzylaldehyde-4-phosphate as substrate. ([CreC] = 0.04 μM)

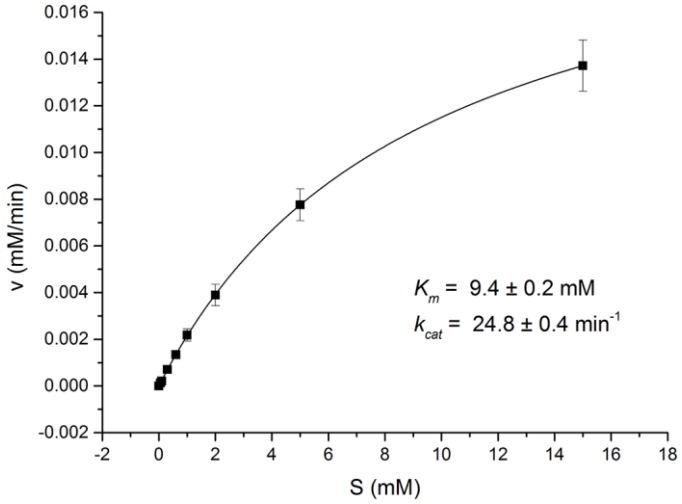

Figure S23. The kinetic curve of CreG with 4-hydroxybenzyl alcohol as substrate. ([CreG] = 0.9 μM)

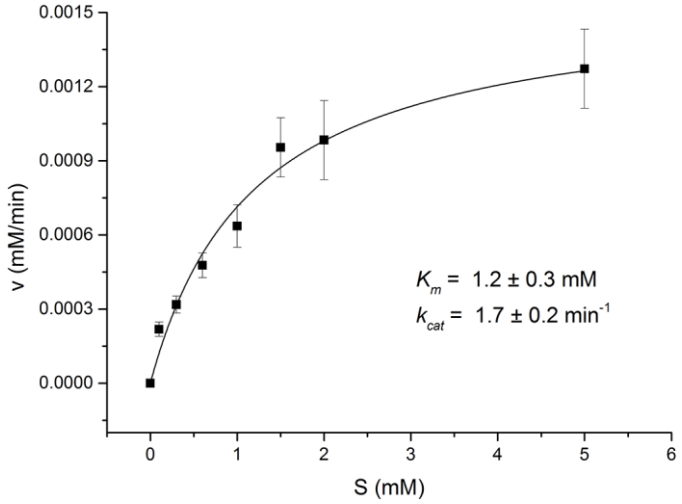

Figure S24. The kinetic curve of CreG with benzylalcohol-4-phosphate as substrate. ([CreG] = 0.9 μM)

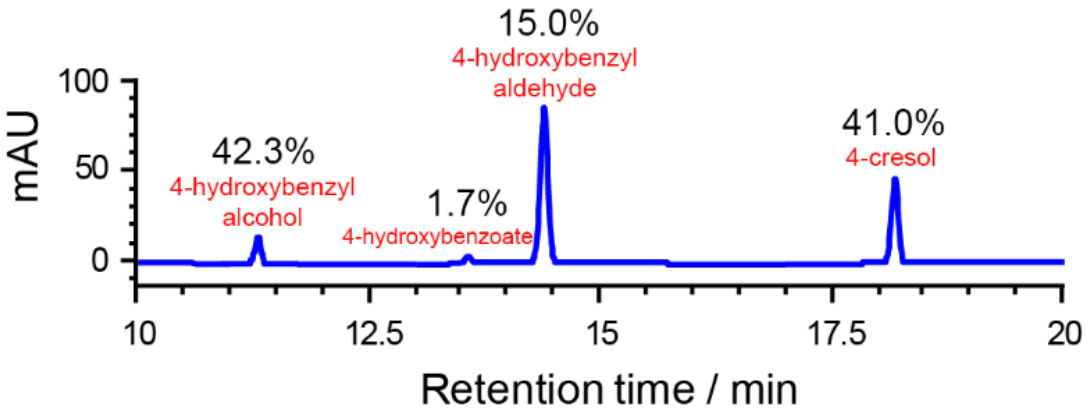

Figure S25. HPLC analysis of the competition assay between CreJEF and CreD. In this assay, 1 mM 4-methylbenzyl phosphate was used as substrate, and all involved enzymes were normalized to the same concentration of 12.0 μM. Assays were carried out in 100 mM Tris-HCl buffer (pH 8.0) at 30°C for 120 min. Each compound was quantified based on area under curve of corresponding HPLC peak relative to that of authentic standard compound with known concentration. Due to distinct extinction coefficients, the peak intensity of compounds do not necessarily reflect relative amounts shown in percentage.