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

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4-Cresol is not only a significant synthetic intermediate for production of many aromatic chemicals, but also a priority environmental pollutant because of its toxicity to higher organisms. In our previous studies, a gene cluster implicated to be involved in 4-cresol catabolism, creCDEFGHIR, was identified in Corynebacterium glutamicum and partially characterized in vivo. In this work, we report on the discovery of a novel 4-cresol biodegradation pathway that employs phosphorylated intermediates. This unique pathway initiates with the phosphorylation of the hydroxyl group of 4-cresol, which is catalyzed by a novel 4-methylbenzyl phosphate synthase, CreHI. Next, a unique class I P450 system, CreJEF, specifically recognizes phosphorylated intermediates and successively oxidizes the aromatic methyl group into carboxylic acid functionality via alcohol and aldehyde intermediates. Moreover, CreD (phosphohydrolase), CreC (alcohol dehydrogenase), and CreG (aldehyde dehydrogenase) were also found to be required for efficient oxidative transformations in this pathway. Steady-state kinetic parameters (Km and kcat) for each catabolic step were determined, and these results suggest that kinetic controls serve a key role in directing the metabolic flux to the most energy effective route.

The aromatic compound 4-cresol (i.e. p-cresol or p-methylphenol) is an important synthetic precursor for manufacturing a great variety of chemical products including synthetic resins, disinfectants, antioxidants, preservatives, fumigants, explosives, and others (1). However, it is also a priority environmental pollutant because of its high toxicity to many higher organisms including humans (2, 3). This compound is mainly derived from diverse industrial processes such as coal gasification and fractionation of coal tar. In nature, 4-cresol is generated by anaerobic bacteria as a byproduct during the metabolism of phenylalanine and tyrosine (4–6).

Like other aromatic compounds, 4-cresol in polluted environments is degraded by various aerobic and anaerobic microorganisms. Thus, studies on biodegradation mechanisms of 4-cresol hold significant potential for industrial application in environmental protection. In the past decades, a growing number of microorganisms capable of degrading 4-cresol have been discovered, and great efforts have been made to elucidate their biodegradation pathways (7–16).

Currently known 4-cresol catabolic pathways can be classified into one of three categories based on the initial step (Fig. 1). The microorganisms falling into the first category (Fig. 1A) begin their degradation of 4-cresol from a methyl hydroxylating step. For instance, in Gram-negative Pseudomonas species, 4-cresol is initially oxidized to 4-hydroxybenzyl alcohol, and then to 4-hydroxybenzaldehyde by the same enzyme 4-cresol methylhydroxylase (PCMH) (17–20). Subsequently, 4-hydroxybenzaldehyde undergoes variant modifications to form protocatechuate acid (i.e. 3,4-dihydroxybenzoate) or benzoyl-CoA, both of which can be diverted into the central metabolism (i.e. TCA) (9, 13, 15, 19, 21).

In the second category, the obligate anaerobe Desulfbacterium cetonicum (12) initiates the degradation of 4-cresol by linking a fumarate moiety to the methyl group to generate 4-hydroxybenzyl succinate, which is further degraded to 4-hydroxybenzyl-CoA via β-oxidation (Fig. 1B). Subsequent reduction to benzoyl-CoA leads the metabolic flux to the central metabolism.

The fungus Aspergillus fumigates likely adopts the third type of degradation pathway to assimilate 4-cresol (14). To form protocatechuate acid, the hydroxylation of 4-cresol by an NADPH-dependent hydroxylase first occurs on aromatic ring to produce 4-methylcatecol, followed by a series of methyl oxidations (Fig. 1C). The intermediates and the enzyme activities for the proposed steps have been identified using cell free extracts (14). In yeast Trichosporon cutaneum (16), an ortho-fission enzyme directly transforms 4-methyl-
catecol into 3-methyl-\textit{cis},\textit{cis}-muconic acid, which can readily enter the central metabolism via the $\beta$-ketoadipate pathway (8) (Fig. 1C).

More recently, focus has shifted toward understanding the capacity of the Gram-positive bacterium \textit{Corynebacterium glutamicum} to metabolize diverse aromatic compounds including 4-cresol (22–31). This high GC content bacterium is not only an important industrial microorganism for production of amino acids and vitamins (32–37), but is also a useful model system to understand the genetics, biochemistry, and mechanisms for biodegradation, especially for assimilation of aromatic compounds (23).

FIGURE 1. \textit{Known pathways for 4-cresol catabolism by variant microorganisms}. Degradation of 4-cresol begins from methyl hydroxylation (A), linking fumarate to the methyl group (B), and direct aromatic ring hydroxylation (C).
In our previous studies, we employed proteomic analysis and genome mining to identify a gene cluster in *C. glutamicum*, creABCDEFGHJIR, which is involved in 4-cresol catabolism (22, 26). Specifically, six (creD, creE, creG, creH, creL, and creF) of eleven cre genes were successfully knocked out from the genome of *C. glutamicum* RES167. Each knock-out strain lost the ability of the wild-type strain to grow in the minimal medium in which 4-cresol was the sole carbon source. For each mutant, genetic complementation with the corresponding gene restored the wild-type phenotype (22). These results clearly demonstrated that at least these six genes were required for 4-cresol biodegradation in *C. glutamicum*. Furthermore, subjecting the six mutants to medium containing diverse aromatic compounds as the sole carbon source, such as 4-hydroxybenzyl alcohol, 4-hydroxybenzyl aldehyde, and 4-hydroxybenzoate, suggested a catabolic pathway responsible for the degradation of 4-cresol (22). However, details of this putative catabolic pathway could not be rationalized from our previous experiments.

Although the cre pathway was genetically identified in part, the catalytic functions of the majority of enzymes were not determined. Of the remaining five genes in the pathway, creR is a putative regulatory gene and is therefore not predicted to function as a catabolic catalyst. Based on bioinformatics analysis, creA and creB are believed to reside outside of the cre gene cluster boundary. Interestingly, both *Corynebacterium efficiens* YS-314 and *Arthrobacter* sp. FB24 possess the identical cre genetic organization to that of *C. glutamicum* with the noted absence of homologues to creA and creB. Previously, we generated the creA and creB deletion mutant in *C. glutamicum* and observed that each resulting strain grew similarly to wild type on the medium supplemented with 4-cresol (22). Thus, neither creA nor creB are predicted to be involved in 4-cresol biodegradation. Finally, numerous attempts to generate creC and creF deletion mutants were unsuccessful. Therefore, the role of these genes products in 4-cresol degradation could not be anticipated.

In the current work, all biodegradation enzymes encoded by the cre gene cluster of *C. glutamicum* were cloned, expressed, and functionally characterized using in vitro enzymatic assays. Through these efforts, an unprecedented 4-cresol catabolic pathway was unveiled that features a novel activating mechanism and a group of enzymes possessing remarkable substrate flexibility. Of particular interest, a unique phosphorylation reaction mediated by CreHI was identified as a novel initiating step of 4-cresol biodegradation. Further, a class I cytochrome P450 (P450) system (38) consisting of CreJ (P450 enzyme), CreF (ferredoxin), and CreE (ferredoxin reductase) was elucidated during this investigation. In vitro characterization of this system demonstrated that it accepts multiple phosphorylated aromatic compounds as substrates and plays a central role in assimilation of 4-cresol.

**Experimental Procedures**

**Strains, Plasmids, and Culture Conditions**—The bacterial strains and plasmids used in this study are listed in supplementary Table S1. *C. glutamicum* was routinely cultivated in Luria-Bertani (LB) broth with a rotary shaker (150 rpm) at 30 °C. *Escherichia coli* strains were grown at 37 °C in LB broth with a rotary shaker (220 rpm) or on LB agar (2% w/v) plates. For protein expression and purification, *E. coli* strains were cultured in Terrific Broth medium (39). When required, kanamycin was added to a final concentration of 50 μg/ml. *E. coli* DH5α was used for plasmid construction, storage, and isolation. *E. coli* BL21 (DE3) was used for recombinant protein expression.

**DNA Isolation and Manipulation**—The genomic DNA of *C. glutamicum* was isolated by following the Kirby mix procedure (40). Plasmid DNA was isolated using an E.Z.N.A.™ plasmid miniprep kit (Omega Biotek, Norcross, GA). DNA fragments were purified using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Ligation, transformation, agarose gel electrophoresis, and other standard techniques for *E. coli* were performed as previously described (41).

**Construction of Expression Vectors for cre Genes**—The complete open reading frames of creC, creD, creE, creF, creG, creH, creL, and creF were PCR-amplified (35 cycles of 95 °C for 20s, 62 °C for 20s, and 72 °C for 60s) from genomic DNA of *C. glutamicum* using *Pfu* DNA polymerase (Transgen, Beijing, China). Primers used to amplify the DNA fragments of target genes are listed in supplemental Table S2. Appropriate restriction sites were introduced into the PCR primers for cloning purposes. Resulting PCR amplicons were doubly digested with a specific pair of restriction enzymes and ligated into similarly digested pET-28b (Novagen). All plasmid constructs were confirmed by DNA sequencing (Sangon Biotech, Shanghai, China). Upon sequence verification, plasmids were then transformed into *E. coli* BL21 (DE3) for protein expression.

**Protein Expression and Purification**—The *E. coli* BL21 (DE3) cells harboring the expression plasmids were grown at 37 °C overnight in LB broth containing 50 μg/ml kanamycin. The overnight culture was used as the seed culture to inoculate (1:100 dilution) 1 liter of Terrific Broth medium containing 5% (w/v) glycerol, and 20 mM imidazole. The cells were grown at 37 °C with shaking until the A600 reached ~0.6. Protein expression was initiated by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.2 mM. Cultivation continued at 18 °C for an additional 20 h. Purification of His-tagged proteins was carried out as described elsewhere (42) with minor modifications. All protein purification steps were performed at 4 °C. Briefly, the cultures were harvested by centrifugation (5000 × g for 5 min). The cell pellet was resuspended in 40 ml of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10% (w/v) glycerol, and 10 mM imidazole, pH 8.0), and the resuspended cells were lysed by sonication. The cell lysate was clarified by centrifugation at 12,000 × g for 30 min to remove cellular debris. Nickel-nitrilotriacetic acid resin (1 ml) (Qiagen) was added to each cell lysate with subsequent incubation with gentle agitation for 30 min. This slurry was loaded into an empty column and washed with ~200 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 10% (w/v) glycerol, and 20 mM imidazole, pH 8.0) until no protein was detected in flow-through. Next, the nickel-bound protein was eluted from the resin with 10 ml of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 10% (w/v) glycerol, and 250 mM imidazole, pH 8.0) using a PD-10 desalting column (GE Healthcare). The resulting
purified proteins were flash frozen by liquid nitrogen and stored at −80 °C for future use.

Purity Qualification and Concentration Determination of Purified Proteins—The purity of all purified proteins was evaluated by SDS-PAGE (supplemental Fig. S1). The concentration of P450 enzyme CreJ was determined according to the method described by Omura and Sato (43). Briefly, the CO-bound reduced difference spectrum of CreJ was recorded by a UV-visible spectrophotometer DU 800 (Beckman Coulter, Fullerton, CA). The concentration of functional P450 was subsequently calculated using the extinction coefficient (ε450−490) of 91,000 M−1 cm−1. Concentrations of non-P450 enzymes were determined by the Bradford method using BSA as the standard (44).

Enzymatic Assays—The assay employed to evaluate the activity of CreHI was developed based on the previous report by Schmeling et al. (45). The reaction mixture contained 1.0 mM of 4-cresol, 4-hydroxybenzyl alcohol, 4-hydroxybenzyl aldehyde, or 4-hydroxybenzoate as substrate, 20 mM MgCl2, 1 mM MnCl2, 2 mM ATP, and 40 μM purified CreH and CreI.

For the assay of CreD, the reaction mixture contained 1.0 mM of 4-methylbenzyl phosphate, benzylalcohol-4-phosphate, benzylaldehyde-4-phosphate, or benzoate-4-phosphate as substrate; 20 mM MgCl2; and 40 μM purified CreD.

For the assay of P450 monoxygenase activity (CreJEF), the reaction mixture contained 1.0 mM of 4-methylbenzyl phosphate, benzylalcohol-4-phosphate, or benzylaldehyde-4-phosphate as substrate, 2.0 mM NADPH, and 40 μM purified CreE, CreF, and CreJ (42).

The assays of CreG and CreC were performed following the previous method (22) with minor modifications. In brief, the reaction system was comprised of 1.0 mM alcohol substrate (for CreG) or aldehyde substrate (for CreC), 2.0 mM electron acceptor (NAD+ for CreG, NADP+ for CreC), and 40 μM purified CreG or CreC.

For the one-pot reaction, 200 μM 4-cresol was added, and all involved enzymes were normalized to the same concentration of 12 μM. All the above described assays were carried out in 100 mM Tris–HCl buffer (pH 8.0) at 30 °C for 120 min. Reactions were quenched by the 1:1 addition of methanol (MeOH) containing 0.2% (v/v) TFA.

Enzymatic Kinetics—The Km value of each enzyme was determined under the identical condition for its enzymatic activity assay (see above). The concentration of substrates varied from 0.1 to 15 mM, and any required cofactors were added to excess. Each enzyme was diluted to a suitable concentration to ensure that the consumption of substrate was within the linear range during the reaction. Samples from each reaction were taken at 0, 2, and 5 min. The concentrations of products or substrates were determined based on the integrations of chromatographic peak areas observed during HPLC analysis. The Km and Vmax values were calculated by nonlinear regression fitting to the Michaelis-Menten equation. All reactions were carried out in triplicate, and the data were reported as the means ± S.D.

Analytical Methods—Reaction mixtures in MeOH/H2O (1:1) with protein removed by centrifugation (20,000 × g for 10 min) were analyzed on an Agilent 1260 infinity HPLC system (Agilent Technologies) equipped with an ultraviolet detector. Specifically, compounds were separated on a ZORBAX SB-C18 column (Agilent Technologies, Wilmington, DE) using a linear mobile phase gradient that ranged from 2% (v/v) MeOH in 0.1% (v/v) TFA aqueous solution to 70% (v/v) MeOH in 0.1% (v/v) TFA aqueous solution over 20 min at a flow rate of 1 ml/min. The wavelength of detection was 275 nm. Structural identification was performed by comparison of retention times of detected compounds with corresponding synthesized authentic standards. Further, eluents were collected and subjected to high resolution mass spectrometry (HRMS) examination. Spectra of electrospray ionization–MS were recorded in the negative ionization mode on an LCQ Deca XP ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA).

Chemical Synthesis of Phosphorylated Compounds—The phosphorylated compounds were synthesized through chemical methods with reference to previous reports by McKenna et al. (46) and Kenner and Williams (47) (supplemental Fig. S2). The specific procedures are detailed in the supplemental materials. The structures of all the synthetic phosphorylated compounds were confirmed through NMR spectroscopy (supplemental Figs. S3–S8). Benzoate-4-phosphate was produced from benzylaldehyde-4-phosphate through enzymatic reaction catalyzed by the cytochrome P450 system comprised of CreJ, CreE, and CreF. The proteins of this enzymatic system in crude benzoate-4-phosphate solution were removed through heat denaturation and centrifugation.

Results

4-Cresol Catabolic Pathway Initiated by Phosphorylation

The phosphocontaining 4-cresol was activated via phosphorylation by a new 4-Methylbenzyl Phosphate Synthase CreHI—Based on our previous genetic knock-out and complementation studies of cre genes and also on the identification of the metabolic intermediate 4-hydroxybenzoate (22), it was deduced that the class I P450 inactivation of 4-cresol. Similar hydroxylation of the methyl group by 4-cresol methylhydroxylase (a flavocytochrome) has been identified in Pseudomonas putida (49) and Geobacter metallireducens (50). However, we failed to detect hydroxylation of 4-cresol in reactions employing the CreJEF P450 system. Moreover, when we tested the in vitro ability of individual enzymes (CreC, CreD, CreG, CreH, or CreI), in the presence of required cofactors, to hydroxylate 4-cresol, no conversion was observed (data not shown). Taken together, these results suggest that an enzyme complex might be required for initiating the modification of 4-cresol.

Subsequent bioinformatics analysis revealed that CreH contains a phosphoenolpyruvate (PEP)-utilizing enzyme mobile domain (supplemental Fig. S9), whereas CreI contains a PEP/pyruvate binding domain (supplemental Fig. S9) (51). Their homologues Orf1 and Orf2 subunits identified in Thauera aromatica, which have 62%/45% and 63%/46% amino acid similarity/identity to CreH and CreI, respectively, were reported to be responsible for converting phenol into phenylphosphate when acting synergistically (45). In view of this report, as well as the structural similarity between 4-cresol and phenol, we hypothesized that CreH and CreI might be required in tandem to phosphorylate 4-cresol.
To test this hypothesis, recombinant CreH and CreI were produced and purified from *E. coli* (supplemental Fig. S1), and their combined *in vitro* catalytic activity against 4-cresol was evaluated. In the reaction mixture containing both CreH and CreI, as well as ATP, Mg²⁺/H₁₁₀₀₁ and Mn²⁺/H₁₁₀₀₁ (all required), 4-cresol was converted to a new product (Fig. 2A). The identity of this product was determined to be 4-methylbenzyl phosphate by HRMS (Fig. 2B) and was confirmed by comparison with the chemically synthesized authentic standard (Fig. 2A). CreHI was also able to catalyze the phosphorylation of 4-hydroxybenzyl alcohol/aldehyde (but not 4-hydroxybenzoate), into their corresponding phosphorylated products benzylalcohol-4-phosphate and benzylaldehyde-4-phosphate, respectively (Fig. 2, A, C, and D). A similar ion-dependent phenylphosphate synthase has been discovered in an anaerobic phenol degrading *T. aromaticum* strain (45); however, the newly identified CreHI 4-methylbenzyl phosphate synthase differentiates from the *T. aromaticum* phenylphosphate synthase by featuring a distinct substrate profile.

**CreJEF Represents a Unique Class I Cytochrome P450 System That Successively Oxidizes 4-Methylbenzyl Phosphate, Benzyllcohol-4-phosphate, and Benzylaldehyde-4-phosphate**—Based on DNA sequence analysis, creJ putatively encodes a cytochrome P450 enzyme. The coexistence of the ferredoxin gene creF and the ferredoxin reductase gene creE in the same gene cluster strongly suggests that CreJEF should form a three-component class I cytochrome P450 system (52). CreJEF cannot directly oxidize 4-cresol; however, the discovery that CreHI produces phosphorylated products naturally led to the hypothesis that CreJEF might require phosphorylated substrate(s). As expected, CreJEF recognized 4-methylbenzyl phosphate as substrate. Interestingly, three products were observed (Fig. 3A), two of which were identified as benzyllcohol-4-phosphate and benzylaldehyde-4-phosphate (see above). The third product was determined to be benzoate-4-phosphate based on HRMS (Fig. 3B) and the proton NMR of the enzymatically prepared product (supplemental Fig. S10). Not surprisingly, CreJEF also catalyzed the oxidation of benzylalcohol-4-phosphate into benzylaldehyde-4-phosphate (Fig. 3A). Taken together, our results demonstrate that CreJEF is a class I P450 system that catalyzes the sequential oxidations from 4-methylbenzyl phosphate to benzoate-4-phosphate.

**CreG Is a 4-Hydroxybenzyl Alcohol Dehydrogenase**—It was previously reported that creG encodes an NAD⁺/H₁₁₀₀₁-dependent 4-hydroxybenzyl alcohol dehydrogenase that oxidizes 4-hydroxybenzyl alcohol into 4-hydroxybenzyl aldehyde (22). In the present work, we further demonstrated that CreG was also able to oxidize benzylalcohol-4-phosphate into benzylaldehyde-4-phosphate (Fig. 4A). Steady-state kinetic analysis indicated that CreG had higher affinity to benzylalcohol-4-phosphate (Kₘ =
**4-Cresol Catabolic Pathway Initiated by Phosphorylation**

**FIGURE 3. CreJEF catalyzed reactions. A, upper panel, CreJEF reactions scheme. Lower panel, HPLC analysis (275 nm) of CreJEF catalyzed reactions. s1 and s2, authentic standards; i, 4-methylbenzyl phosphate as substrate; ii, benzaldehyde-4-phosphate as substrate; iii, benzaldehyde-4-phosphate as substrate. Traces in blue represent corresponding control reactions without addition of enzymes. B, HRMS of benzoxo-4-phosphate. Note that because of distinct extinction coefficients, the peak intensity of compounds do not necessarily reflect relative amounts.**

1.2 ± 0.3 mM) than to 4-hydroxybenzyl alcohol (\(K_m = 9.4 \pm 0.2\) mm) (Table 1). Notably, CreG used NAD\(^+\) as a preferred cofactor. The dehydrogenase activity of CreG supported by NADP\(^+\) was observed to be 1.3 \(\times\) 10\(^3\) times lower than that by NAD\(^+\), when 4-hydroxybenzyl alcohol was employed as substrate.

**CreC Is a Benzaldehyde-4-phosphate Dehydrogenase**—Conserved domain searches suggested that CreC belongs to the aldehyde dehydrogenase superfamily containing an aldehyde dehydrogenase domain and an NAD(P)\(^+\) cofactor-binding domain. Based on protein sequence alignment, CreC displays 34% amino acid identity to PcuC, a known 4-hydroxybenzyl aldehyde dehydrogenase from *Pseudomonas* species (7, 8). Catalytically, CreC was able to oxidize both 4-hydroxybenzyl aldehyde and benzaldehyde-4-phosphate to corresponding carboxylic acids (Fig. 4B), respectively. Evaluation of the catalytic efficiency with both substrates revealed that CreC was much more efficient in its ability to oxidize benzaldehyde-4-phosphate (\(k_{cat}/K_m = 1.2 \times 10^8\) mm\(^{-1}\) min\(^{-1}\)) than in oxidizing 4-hydroxybenzyl aldehyde (\(k_{cat}/K_m = 77\) mm\(^{-1}\) min\(^{-1}\)) (Table 1). Unlike CreG, CreC can only employ NADP\(^+\) as a cofactor, because no catalytic activity was detected in the presence of NAD\(^+\). This feature is distinct to other benzaldehyde dehydrogenases, which have been reported to have the capacity to utilize both NAD\(^+\) and NADP\(^+\) as cofactors (53–55).

**CreD Is a Universal Phosphohydrolase**—Our previous study demonstrated that CreD displayed phosphohydrolase activity against the unnatural substrate 4-nitrophenylphosphate (22). However, its physiological function remained elusive because there were no substrate candidates available for testing at that time. In this work, the discovery that CreHI catalysis produced phosphorylated products inspired us to examine the activity of CreD against these phosphorylated compounds. As expected, CreD hydrolyzed 4-methylbenzyl phosphate, benzalcohol-4-phosphate, benzaldehyde-4-phosphate, and benzoyl-4-phosphate into 4-cresol, 4-hydroxybenzyl alcohol, 4-hydroxybenzyl aldehyde, and 4-hydroxybenzoate, respectively (Fig. 5).

**In Vitro Assembly of the 4-Cresol Catabolic Pathway**—Elucidation of the catalytic activities of CreHI, CreJEF, CreG, CreC, and CreD has led to the proposal of a unique 4-cresol biodegradation pathway in *C. glutamicum* (Fig. 6). This pathway has two unusual features. First, all involved biocatalysts can accept multiple metabolic intermediates as substrates, which results in two alternative but interactive routes. The first route of 4-cresol degradation proceeds through phosphorylated intermediates (steps 1 \(\rightarrow\) 3 \(\rightarrow\) 4 \(\rightarrow\) 10 \(\rightarrow\) 14), whereas the second route proceeds through nonphosphorylated compounds (steps 1 \(\rightarrow\) 3 \(\rightarrow\) 7 \(\rightarrow\) 8 \(\rightarrow\) 13). Second, CreHI and CreD catalyze reverse reactions. This unique paradigm appears to be energy wasteful because the net outcome of ATP consuming phosphorylation reaction catalyzed by CreHI plus phosphohydrolysis by CreD is equivalent to the uneconomical consumption of ATP.

To understand this seemingly unreasonable network with enzyme functional redundancies (i.e. a common reaction catalyzed by different enzymes) and unfavorable energy economy, we carried out a one-pot enzymatic reaction to evaluate the efficiency of ATP utilization. In this one-pot pathway reconstitution experiment, all enzymes were normalized to the same concentrations (12.0 \(\mu\)M) in the reaction mixture containing 200 \(\mu\)M of 4-cresol and all required cofactors. This experimental design is based on the assumption that all involved enzymes are physiologically expressed simultaneously. This assumption was supported by a series of RT-PCR experiments. To determine whether all *cre* genes were expressed coordinately, we attempted to amplify all intergenic regions between two adjacent genes within the *cre* cluster using a common cDNA sample as template. The RT-PCR results (supplemental Fig. S11) indicate that *cre-C-R* were transcribed as a single operon and likely expressed coordinately. However, the cellular enzyme levels could vary dynamically because of differential affinity of ribosome binding sites across the polycistronic mRNA or diverse epigenetic effects. Thus, the one-pot reaction containing the same levels of enzymes represented a simplified model system for understanding the kinetic behavior of mixed 4-cresol degrading enzymes.

To initiate the multienzymatic catalysis, varying ATP doses (200, 400, and 600 \(\mu\)M, corresponding to molar ratios of 1:1, 1:2, and 1:3 for -cresol:ATP, respectively) were added into the reaction mixtures. Results showed that 40.8% and 78.8% of 4-cresol
were consumed when the substrate:ATP ratios were 1:1 and 1:2, respectively. 4-Cresol was completely consumed when the 4-cresol:ATP ratio was 1:3 (Fig. 7A). These quantitative results indicate that 4-cresol can be efficiently converted into downstream metabolic intermediates; meanwhile the futile ATP consumption indeed occurred to some extent.

Enzyme Kinetics Regulates the Metabolic Flux in the 4-Cresol Biodegradation Pathway—To further understand the metabolic flux in the complex 4-cresol biodegradation pathway, the steady-state enzymatic kinetic parameters of each step were determined (Table 1 and supplemental Figs. S12–S24). The phosphorylation of 4-cresol by CreHI had an apparent $K_m$ value of 0.61 ± 0.06 mM and a $k_{cat}$ value of 60.4 ± 1.8 min⁻¹, resulting in a calculated catalytic efficiency ($k_{cat}/K_m$) of 99 min⁻¹·mM⁻¹ for this initial step. The catalytic efficiency of CreHI was slightly higher than the phosphohydrolysis of 4-methylbenzyl phosphate ($k_{cat}/K_m = 65$ min⁻¹·mM⁻¹) by CreD. Interestingly, the oxidation of 4-methylbenzyl phosphate by CreJEF was more efficient than the phosphohydrolysis by CreD, because CreJEF had lower $K_m$ and higher $k_{cat}$ (~2-fold more efficient than CreD for 4-methylbenzyl phosphate). The competition experiment between CreJEF and CreD showed that the final ratio between oxidation and hydrolysis products was 59:41 (supplemental Fig. S25), which is consistent with the kinetic data.

Next, additional one-pot reactions were performed to evaluate the contribution of CreC and CreG in this catabolic pathway (Fig. 7B). Again, 4-cresol was completely converted to 4-hydroxybenzoate in the one-pot reaction containing all enzymes. When both CreC and CreG were absent, the main products turned out to be 4-hydroxybenzyl alcohol (64.2%) and 4-hydroxybenzoate (33.5%), whereas the terminal-product 4-hydroxybenzoate in the one-pot reaction containing all enzymes. This result is consistent with the enzyme kinetic studies, where the $k_{cat}/K_m$ values of step 4 (14 mm⁻¹·min⁻¹) and step 9 (7.4 mm⁻¹·min⁻¹) were lower than those of step 7 (22 mm⁻¹·min⁻¹) and step 12 (72 mm⁻¹·min⁻¹), respectively. When only CreC was omitted, 4-hydroxybenzyl alcohol was the predominant product, while 4-hydroxybenzoate was produced in trace amounts.

FIGURE 4. Reactions catalyzed by CreG and CreC. A, upper panel, CreG reactions scheme. Lower panel, HPLC analysis (275 nm) of CreG catalyzed reactions. s1 and s2, authentic standards; i, 4-hydroxybenzyl alcohol as substrate; ii, benzylalcohol-4-phosphate as substrate. B, upper panel, CreC reactions scheme. Lower panel, HPLC analysis of CreC catalyzed reactions. s1 and s2, authentic standards; i, 4-hydroxybenzyl aldehyde as substrate; ii, benzylaldehyde-4-phosphate as substrate. Traces in blue represent corresponding control reactions without addition of enzymes. Note that because of distinct extinction coefficients, the peak intensity of compounds do not necessarily reflect relative amounts.

TABLE 1

Steady-state kinetics of enzymes encoded by the cre cluster

| Enzyme | Substrate | $K_m$ [mM] | $k_{cat}$ [min⁻¹] | $k_{cat}/K_m$ [min⁻¹·mM⁻¹] |
|--------|-----------|-----------|-----------------|--------------------------|
| CreHI  | 4-Cresol  | 0.61 ± 0.06 | 60.4 ± 1.8     | 99                       |
| CreHI  | 4-Hydroxybenzyl alcohol | 0.10 ± 0.03 | 36.3 ± 2.7 | 1050                     |
| CreHI  | 4-Hydroxybenzyl aldehyde | 0.21 ± 0.02 | 14.9 ± 2.7 | 71                       |
| CreJEF | 4-Methylbenzyl phosphate | 0.34 ± 0.06 | 44.5 ± 2.3 | 1300                     |
| CreJEF | Benzylalcohol-4-phosphate | 0.38 ± 0.09 | 5.21 ± 0.33 | 14                      |
| CreJEF | Benzylaldehyde-4-phosphate | 0.90 ± 0.22 | 6.61 ± 0.59 | 74                       |
| CreD   | 4-Methylbenzyl phosphate | 0.62 ± 0.09 | 40.2 ± 1.9 | 65                       |
| CreD   | Benzylalcohol-4-phosphate | 0.52 ± 0.06 | 11.3 ± 1.5 | 22                       |
| CreD   | Benzylaldehyde-4-phosphate | 0.32 ± 0.02 | 23.3 ± 0.7 | 72                       |
| CreC   | 4-Hydroxybenzyl aldehyde | 0.058 ± 0.006 | 4.83 ± 0.20 | 77                       |
| CreC   | Benzylaldehyde-4-phosphate | 0.12 ± 0.04 | (1.41 ± 0.10) × 10³ | 1.2 × 10³ |
| CreG   | 4-Hydroxybenzyl alcohol | 9.4 ± 0.2 | 24.8 ± 0.4 | 26                       |
| CreG   | Benzylalcohol-4-phosphate | 1.2 ± 0.3 | 1.74 ± 0.20 | 1.5                       |

The values were calculated by nonlinear regression fitting to the Michaelis-Menten equation. All reactions were carried out in triplicate, and the data are reported as means ± S.D.
product (92.4%), 4-hydroxybenzoate was the minor product (7.6%), whereas 4-hydroxybenzyl alcohol was not detected. If only CreG was omitted, 4-hydroxybenzyl alcohol and 4-hydroxybenzoate accounted for 61.0 and 39.0% of products, respectively. In sum, the involvement of CreC and CreG in the reaction pathway serves to make these oxidative conversions more efficient and energy-effective.

**Discussion**

Two unusual enzymatic systems represent the most important discoveries of the current study: 1) CreHI initiates the pathway by phosphorylating 4-cresol to give 4-methylbenzyl phosphate, and 2) CreJEF catalyzes the three-step sequential oxidation of 4-methylbenzyl phosphate to benzylalcohol-4-phosphate, benzylaldehyde-4-phosphate, and benzoate-4-phosphate.

Conserved domain searches revealed that CreH has a PEP-utilizing enzyme mobile domain, whereas CreI has a PEP/pyruvate binding domain (51). Interestingly, both CreH and CreI possess respective conserved domains with strong similarity to different parts of the PEP synthase of *E. coli* (supplemental Fig. S9), suggesting a possible evolutionary relationship between CreH, CreI, and PEP synthase. The Orf1 and Orf2 subunits from the anaerobic *T. aromatica*, which show 45 and 46% amino acid identity to CreH and CreI, respectively, are responsible for converting phenol into phenylphosphate (45, 56, 57). The *creHI* homologous genes are also found in many other microbial genomes such as *C. efficiens*, Cladosporium halotolerans, Brevibacterium flavum, Arthrobacter species, Kocuria palustris, Bradyrhizobium species, and Runella slithyformis. Taken together, these gene products may form a new enzyme family capable of phosphorylating phenolic compounds.
compounds. Moreover, the genomes of *C. efficiens*, *C. halotolerans*, *B. flavum*, *Arthrobacter* species, and *K. palustris* contain gene clusters with strong homology to the *cre* gene cluster of *C. glutamicum*, suggesting that these bacteria are likely able to degrade 4-cresol using the same mechanism employed by *C. glutamicum*.

CreJEF is able to catalyze the successive oxidations of phosphorylated intermediates. It is possible that the phosphate group of substrates could act as an anchoring group that delivers the substrate to the correct position within the active site of CreJ, as done by the deoxysugar that is attached to substrates of the PikC P450 enzyme (58, 59). To the best of our knowledge, this is the first reported example of a P450 enzyme that recognizes a spectrum of substrates via a phosphate group. This discovery may have great biotechnological potential for selective oxidation of unactivated C–H bonds.

Some known P450 enzymes are capable of catalyzing the complete oxidation of a C–H bond into a carboxylic acid. One important example is the multifunctional *ent*-kaurene oxidase encoded by the P450–4 gene in the gibberellin biosynthetic pathway of *Gibberella fujikuroi*. This P450 enzyme catalyzes three successive oxidation steps between *ent*-kaurene and *ent*-kaurenioic acid (60). Another example is CYP71AV1 from *Artemisia annua*, which performs a three-step oxidation of amorpha-4,11-diene to form artemisinic acid (61). To complete this challenging six-electron oxidation, CYP71AV1 recruits an artemisinic aldehyde dehydrogenase and an alcohol dehydrogenase to achieve the optimal transformation (62). This is similar to what we have observed with CreJEF, CreC, and CreG. Likewise, in the tirandamycin biosynthetic pathway, Tam1 P450 enzyme employs the FAD-containing Tam1 oxidase to overcome the conversion from an alcohol to a keto group (63). These examples suggest that the successive oxidations are likely a heavy burden for P450 enzymes to accomplish on their own; therefore, assistance is required from other types of oxidases.

It is obvious that the initial oxidation of 4-cresol requiring a prephosphorylation step in *C. glutamicum* is more complicated than the direct oxidation catalyzed by a 4-cresol methylhydroxylase (PCMH). Kinetically, the latter flavocytochrome is much more efficient. For example, the PCMH from *Achromobacter* sp. exhibited *K*ₘ and *k*ₐ₅ values for 4-cresol of 21 μM and 112 s⁻¹, respectively (15). The *k*ₐ₅/*K*ₘ value of another PCMH from *P. putida* NCIMB 9869 was calculated to be 2.9 × 10⁶ M⁻¹ s⁻¹ (17). Both enzymes are >2000 times more efficient than CreJEF using 4-methylbenzyl phosphate as substrate (*K*ₘ = 0.34 mM, *k*ₐ₅ = 44.5 min⁻¹; Table 1). This is not surprising because the *k*ₐ₅ values of many P450 enzymes are within 1–300 min⁻¹, which can mainly be attributed to the slow electron transfer step (64). Considering that the additional enzymes in the pathway (except for CreC against benzylaldehyde-4-phosphate) also displayed relatively high *K*ₘ and low *k*ₐ₅ values (Table 1), *C. glutamicum* appears not to be a good 4-cresol assimilating microorganism. However, without a PCMH gene on its genome (data not shown), it is intriguing to speculate that *C. glutamicum* was forced to evolve a novel, albeit less efficient, pathway for 4-cresol degradation.

Based on the kinetic analyses and competition experiments, we propose that the primary degradation pathway for 4-cresol in *C. glutamicum* is the phosphorylated route (Fig. 6): 4-cresol is first activated by CreHI via conversion into 4-methylbenzyl phosphate, and subsequent oxidations of phosphorylated intermediates are co-mediated by CreJEF, CreG, and CreC. This proposed pathway sequence is supported by the fact that the conversion from benzoate-4-phosphate to 4-hydroxybenzoate is a unidirectional reaction (Fig. 6), because CreHI does not take 4-hydroxybenzoate as substrate.
4-Cresol Catabolic Pathway Initiated by Phosphorylation

However, it remains yet premature to suggest that the primary route consisting of steps 1 → 3 → 4 → 10 → 14 represents the physiological pathway for 4-cresol degradation in _C. glutamicum_ because of details lacking with respect to the cellular concentration and localization of enzymes, availability of cofactors, and other intracellular environmental factors. It is noteworthy that there is both agreement as well as inconsistency between the proposed pathway in this study and the previous _in vivo_ results (22). For example, according to the pathways shown in Fig. 6, the inactivation of CreJEF, CreHI, or CreD would block the major catabolic pathway, thus abolishing the ability of knock-out strains to grow on 4-cresol as the sole carbon source. This observation is consistent with the previous observations (22). In contrast, based on the results obtained from the one-pot reactions (Fig. 7B), CreG and CreC appear to be unnecessary in the major route from 4-cresol to 4-hydroxybenzoate. This observation, however, contradicts our earlier finding that the _creG_ knock-out strain was unable to grow using 4-cresol as the sole carbon source. We reason that this inconsistency might be derived from a yet unknown function of CreG or the result of the complexity within the _in vivo_ environment such as balances of energy and cofactors or potential toxicity of produced intermediates that could adversely affect cell growth.

Another interesting aspect of the 4-cresol catabolic pathway is the differential usage of cofactors by CreJEF (NADP^+), CreG (NAD^+), and CreC (NADP^+). This may be of physiological importance, because these enzymes may rely on a cofactor balancing system during degradation of 4-cresol. For instance, CreJEF and CreC may form an NADPH/NADP^+ recycling system _in vivo_ to avoid too much loss of reducing power during multiple P450 oxidations.

It is intriguing to speculate that the substrate flexibility of the catabolic enzymes involved in 4-cresol degradation may have physiological significance because this pathway could also assimilate other phenolic compounds. To further assess this possibility, we are currently pursuing more detailed biochemical studies in our laboratories. Enzyme function redundancy may be useful when microorganisms face harsh polluted environments because multiple enzymes with a common functionality could maximally release the metabolic potential by better taking advantage of substrates, cofactors, and energy stored in different forms.

**Author Contributions**—L. D., X. W., S.-J. L., and S. L. conceived this study, analyzed the results, and wrote the manuscript. L. D., L. M., F. Q., X. Z., C. J., and A. L. conducted experiments. All authors read and approved the manuscript.

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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 °C 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 MgSO₄. 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 °C 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 CH₂Cl₂ (2 × 30 mL). The extract was washed with brine (30 mL), and dried over anhydrous MgSO₄. 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 CH₂Cl₂ (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 °C. 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%.
| 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            |
| pET28a-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            |
| Primers | Sequences (5'→3') | Description |
|---------|-------------------|-------------|
| creC-F  | CCGCCATATGCGGATGAATGCTGCAACCA | To construct pET28b-creC |
| creC-R  | GGCAGGCTCGGTGTAGAGGCTGAAACTTTCAG | To construct pET28b-creC |
| creD-F  | GACACATAGACTCGCAGTAATTTACCCGC | To construct pET28b-creD |
| creD-R  | CGGAATTCCGAGAACGCACCGCTGGTTG | To construct pET28b-creD |
| creE-F  | GACACATAGATACTCGGACGCTGAAACTTTCAG | To construct pET28b-creE |
| creE-R  | GGAGCTCTCCACGCGGCTATAATT | To construct pET28b-creE |
| creF-F  | GCGCCATATGCCGATGAATGCTGCAACCA | To construct pET28b-creF |
| creF-R  | TCATAAGCTTTCACTATGCTGGTTTCTGGCG | To construct pET28b-creF |
| creG-F  | GACACATATGACTCGCAGTAATTTACCCGC | To construct pET28b-creG |
| creG-R  | GGAGCTCTCCACGCGGCTATAATT | To construct pET28b-creG |
| creH-F  | GACACATATGACTCGCAGTAATTTACCCGC | To construct pET28b-creH |
| creH-R  | GGAGCTCTCCACGCGGCTATAATT | To construct pET28b-creH |
| creJ-F  | GACACATATGACTCGCAGTAATTTACCCGC | To construct pET28b-creJ |
| creJ-R  | GGAGCTCTCCACGCGGCTATAATT | To construct pET28b-creJ |
| creF-R  | GACACATATGACTCGCAGTAATTTACCCGC | To construct pET28b-creF |
| creJ-R  | GGAGCTCTCCACGCGGCTATAATT | To construct pET28b-creJ |
| CB-F    | TCTCGGTGCGTTTGGATT | RT-PCR. To amplify region a in Figure S11. |
| CB-R    | AAGATTCGCGATGACTG | RT-PCR. To amplify region a in Figure S11. |
| DC-F    | GCTCCATTAGCTCATGCTGGTTCG | RT-PCR. To amplify region b in Figure S11. |
| DC-R    | AATGGGCGGATGCTGTCTTCG | RT-PCR. To amplify region b in Figure S11. |
| DE-F    | CTCAAAATACAAATGCAAGGAC | RT-PCR. To amplify region c in Figure S11. |
| DE-R    | CGGAGATGATCGCTGGTTCG | RT-PCR. To amplify region c in Figure S11. |
| FE-F    | GGCGGTTTCTTATGCTGT | RT-PCR. To amplify region d in Figure S11. |
| FE-R    | GATCGGTCGTTCTTATGCTGT | RT-PCR. To amplify region d in Figure S11. |
| GF-F    | ACCACGGTGCGTTTGGATT | RT-PCR. To amplify region e in Figure S11. |
| GF-R    | GACACATAGATACTCGGACGCTGAAACTTTCAG | RT-PCR. To amplify region e in Figure S11. |
| HG-F    | AAAACCGGGCGGCGTTCGTTTATGCTGT | RT-PCR. To amplify region f in Figure S11. |
| HG-R    | GCTCTTCTAGGCAAGACCACTTCTATTAG | RT-PCR. To amplify region f in Figure S11. |
| IH-F    | AAGGCCCCGTGCGGTTGATGAAAGA | RT-PCR. To amplify region g in Figure S11. |
| IH-R    | TGAATCCGCAACGAGAAT | RT-PCR. To amplify region g in Figure S11. |
| JI-F    | TGGTTTCTGGCATCCTACTTA | RT-PCR. To amplify region h in Figure S11. |
| JI-R    | GAAGCCCCGACATTACGG | RT-PCR. To amplify region h in Figure S11. |
| RJ-F    | CGCATGTGTCCTCGTGCTGCT | RT-PCR. To amplify region i in Figure S11. |
| RJ-R    | AACACATGCGGATGCTGTCTTCTATTAG | RT-PCR. To amplify region i in Figure S11. |
| R-F     | CGATCGGTTATAAGAAGAAT | RT-PCR. To amplify region j in Figure S11. |
| R-R     | GAAGCCCCGACGAGGCTGGTG | 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$_2$O.

Figure S6. $^1$H NMR spectrum of benzylaldehyde-4-phosphate in D$_2$O.
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$_2$O.
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$_2$O.
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 benzaldehyde-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.
Characterization of a Unique Pathway for 4-Cresol Catabolism Initiated by Phosphorylation in Corynebacterium glutamicum

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