Benzoate Metabolism Intermediate Benzoyl Coenzyme A Affects Gentisate Pathway Regulation in *Comamonas testosteroni*

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A previous study showed that benzoate was catabolized via a coenzyme A (CoA)-dependent epoxide pathway in *Azoarcus evansii* (R. Niemetz, U. Altenschmidt, S. Brucker, and G. Fuchs, Eur. J. Biochem. 227:161-168, 1995), but gentisate 1,2-dioxygenase was induced. Similarly, we found that the *Comamonas testosteroni* strain CNB-1 degraded benzoate via a CoA-dependent epoxide pathway and that gentisate 1,2-dioxygenase (GenA) was also induced when benzoate or 3-hydroxybenzoate served as a carbon source for growth. Genes encoding the CoA-dependent epoxide (*box* genes) and gentisate (*gen* genes) pathways were identified. Genetic disruption revealed that the *gen* genes were not involved in benzoate and 3-hydroxybenzoate degradation. Hence, we investigated *gen* gene regulation in the CNB-1 strain. The P_{genA} promoter, a MarR-type regulator (GenR), and the GenR binding site were identified. We found that GenR took gentisate, 3-hydroxybenzoate, and benzoyl-CoA as effectors and that binding of GenR to its target DNA sequence was prohibited when these effectors were present. *In vivo* studies showed that the CNB-1 mutant that lost benzoyl-CoA synthesis was not able to activate P_{genA} promoter, while transcription of *genA* was upregulated in another CNB-1 mutant that lost the ability to degrade benzoyl-CoA. The finding that benzoyl-CoA (a metabolic intermediate of benzoate degradation) and 3-hydroxybenzoate function as GenR effectors explains why GenA was induced when CNB-1 grew on benzoate or 3-hydroxybenzoate. Regulation of gentisate pathways by MarR-, LysR-, and IclR-type regulators in diverse bacterial groups is discussed in detail.

Bacteria adopt three different strategies for benzoate degradation. (i) Under anaerobic conditions, benzoate is first converted to benzoyl coenzyme A (benzoyl-CoA), which is subsequently reduced to cyclohex-1,5-diene-1-carbonyl-CoA; the latter compound is finally cleaved into acetyl-CoA (1). (ii) Under aerobic conditions, benzoate is initially oxidized by mono-oxygenases into dihydroxylated intermediates such as catechol (2, 3), protocatechuate (3, 4), or gentisate (3, 5); the dihydroxylated intermediates are cleaved at the 1,2 or 3,4 position and linearized by various dioxygenases, such as catechol 1,2-dioxygenase (6, 7), protocatechuate 3,4-dioxygenase (8, 9), and gentisate 1,2-dioxygenase (7, 9). (iii) The CoA-dependent epoxide pathway (10, 11) (Fig. 1A) is used. The CoA-dependent epoxide pathway begins at activation of benzoate to benzoyl-CoA by benzoate-CoA ligase. Benzoyl-CoA is subsequently converted into an epoxide (2,3-epoxybenzoyl-CoA) (12), which is catalyzed by benzoyl-CoA oxygenase (BoxB) and reductase (BoxA) (11). 2,3-Epoxybenzoyl-CoA is hydrolyzed into formic acid and 3,4-dehydroadipyl-CoA semialdehyde by benzoyl-CoA hydratase (BoxC) (13). The latter intermediate is subsequently oxidized by 3,4-dehydroadipyl-CoA semialdehyde dehydrogenase (BoxD) to 3,4-dehydroadipyl-CoA (14). Further metabolism of 3,4-dehydroadipyl-CoA proceeds via reactions similar to β -oxidation and the β -ketoadipate pathway (3, 15), with succinyl-CoA and acetyl-CoA as products (11). Thus far, the CoA-dependent epoxide pathway has been found in *Pseudomonas* species (16), *Escherichia coli* (17-19), *Azoarcus evansii*, and *Bacillus* species (10, 11, 20).

Multiple metabolic pathways for benzoate degradation may occur simultaneously in bacteria, making benzoate degradation a complex process. A catechol *ortho*-cleavage pathway and two benzoyl-CoA pathways are involved in benzoate degradation in *Burk-*

holderia xenovorans LB400 (21), and transcription and expression of genes in these pathways are dependent on the growth phase of this bacterium (22). Recently, benzoate degradation in *A. evansii* (formerly *Pseudomonas* strain KB 740) was shown to proceed through a CoA-dependent epoxide pathway (11). However, gentisate 1,2-dioxygenase activity was induced in this particular strain (23). Niemetz et al. deduced that benzoate was degraded via 3-hydroxybenzoyl-CoA and gentisate (24), but subsequent studies did not support this hypothesis (25). To date, an explanation for induction of gentisate 1,2-dioxygenase activity in KB 740 is lacking.

The *Comamonas testosteroni* strain CNB-1 was isolated for degradation of 4-chloronitrobenzene (4-CNB) (26). In addition to 4-CNB, CNB-1 also uses benzoate and many other aromatic compounds as carbon sources for growth (26, 27) and has been applied for rhizoremediation of CNB-polluted soil (28). Our previous investigations revealed that the CNB-1 strain metabolizes 4-CNB via a partial reductive pathway and metabolizes 3-hydroxybenzoate, 4-hydroxybenzoate, protocatechuate, and vanillate via the proto-

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FIG 1 The CoA-dependent epoxide pathway (A) and its genetic cluster (B) and the gentisate 1,2-dioxygenase pathway (C) and its genetic cluster (D) in *C. testosteroni*. Abbreviations: BCL, benzoate-CoA ligase; BoxA, benzoyl-CoA reductase; BoxB, benzoyl-CoA oxygenase (2,3-epoxidase); BoxC, 2,3-epoxybenzoyl-CoA dihydrolase; BoxD, 3,4-dehydroadipyl-CoA semialdehyde dehydrogenase; GenA, gentisate 1,2-dioxygenase; GenC, maleylpyruvate isomerase; GenB, fumarylpyruvate hydrolase. In panel A, gene tags for these strain CNB-1 enzymes are beneath the arrows.

catechuate 4,5-cleavage pathway. CNB-1 grows on benzoate, but how it degrades benzoate has not been investigated. Previous studies reported that gentisate 1,2-dioxygenase activity was induced when CNB-1 grew on benzoate (27). In the present study, we showed that CNB-1 degrades benzoate via a CoA-dependent epoxide pathway (Fig. 1A) but not a gentisate pathway (Fig. 1C). We identified the promoter (P_{genA}) of the *gen* cluster, a MarR-type transcriptional regulator (GenR), and gentisate, benzoyl-CoA, and 3-hydroxybenzoate as effectors of GenR. We further demonstrated that benzoyl-CoA induced transcription of *genA*, and thus, benzoyl-CoA was responsible for the induction of gentisate 1,2-dioxygenase activities during benzoate degradation in CNB-1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown aerobically on a rotary shaker (200 rpm) at 37°C in Luria-Bertani (LB) broth or on LB plates with 1.5% (wt/vol) agar. *C. testosteroni* was cultivated and maintained in LB medium or in minimal salt broth (MSB) (29) containing 1 g/liter of ammonium chloride as the nitrogen source at 30°C. Benzoate, gentisate, pyruvate, or succinate was added at a final concentration of 2 mM when used as a carbon and energy source. Cellular growth was monitored by measuring the optical density of the culture at a wavelength of 600 nm (OD_{600}). If necessary, antibiotics were used at the following concentrations: kanamycin, 50 μ g/ml (*E. coli*) and 150 μ g/ml (*C. testosteroni*); tetracycline, 20 μ g/ml (for both *E. coli* and *C. testosteroni*).

Comparative proteomic analysis. Comparative proteomic studies were conducted as previously described (30). Briefly, cells were harvested at the late exponential phase of growth and sonicated on ice. Supernatants of cellular lysates (ca. 300 μ g proteins) were analyzed by 2-dimensional electrophoresis (2-DE). Isoelectric focusing and sodium dodecyl sulfate-

polyacrylamide gel electrophoresis were conducted as previously described (30). Proteins were digested with trypsin, and the resultant peptides were detected by mass spectrometry. Protein identification was carried out according to peptide fingerprint analysis. A two-tailed Student's *t* test was adopted to evaluate the spot differences between the control and experimental gels ($P < 0.05$). Significant change in protein abundance was defined as a 2-fold increase above the normalized volume in the two sets of 2-DE spots. Three biological tests were run in parallel.

DNA extraction, plasmid isolation, PCR amplification, and DNA sequence analysis. CNB-1 genomic DNA was isolated and purified using the SiMax genomic-DNA extraction kit (SBS Genetech, Beijing, China). DNA manipulation, plasmid preparation, agarose gel electrophoresis, ligation, and transformation were performed using standard methods (31). Plasmids were extracted and purified with the plasmid minispin HP kit (Vigorous, Beijing, China). Restriction endonucleases, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturer's instructions (New England BioLabs, Beijing, China).

Primers used for DNA amplification in this work are listed in Table S1 in the supplemental material. To facilitate cloning, forward and reverse primers were flanked with restriction endonuclease sites (see Table S1). PCRs were carried out in a Biomtra thermocycler (Analytik Jena, Germany) using *Pfu* or *Taq* DNA polymerases (TransGen Tech, Beijing, China). PCR products were analyzed by electrophoresis using 0.8% agarose gels and purified from gels with the Tiagel midi purification kit (Tiangen, Beijing, China). Fragments were digested using the corresponding endonucleases and ligated into the pEASY-T1 vector.

The chromosome sequence of *C. testosteroni* CNB-2 (accession no. NC_013446) (32), a plasmid-curing derivative of the *C. testosteroni* strain CNB-1 (26, 29), was retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). Sequence comparisons and database searches were performed using BLAST programs at the BLAST server of the NCBI website.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>Comamonas testosteroni</i>		
CNB-1	Wild type	26
CNB-1 Δ 0097 <i>bcl</i>	A fragment of 0097 <i>bcl</i> encoding amino acids 152 to 436 was deleted	This study
CNB-1 Δ 0097 <i>bcl</i> /pBBR1MCS3- <i>bcl</i>	Complementation for CNB-1 Δ 0097 <i>bcl</i>	This study
CNB-1 Δ 0394 <i>bcl</i>	A fragment of 0394 <i>bcl</i> DNA was deleted	This study
CNB-1 Δ 0097_0394 <i>bcl</i>	Carries mutations of 0097 <i>bcl</i> and 0394 <i>bcl</i>	This study
CNB-1 Δ boxAB	DNA fragment encoding BoxAB was deleted	This study
CNB-1 Δ boxAB/pBBR1MCS3- <i>boxBA</i>	Complementation for CNB-1 Δ boxAB	This study
CNB-1 Δ boxC	A fragment of <i>boxC</i> encoding amino acids 15 to 472 was deleted	This study
CNB-1 Δ boxC/pBBR1MCS3- <i>boxC</i>	Complementation for CNB-1 Δ boxC	This study
CNB-1 Δ genA	A fragment of <i>genA</i> encoding amino acids 1 to 332 was deleted	This study
CNB-1 Δ genA_ <i>boxC</i>	Carries mutations of <i>genA</i> and <i>boxC</i>	This study
<i>E. coli</i>		
DH5 α	ϕ 80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44</i> Δ <i>lacU169</i>	31
BL21(DE3)	F [−] <i>ompT hsdSB</i> ($r_B^- m_B^-$) <i>gal dcm</i> λ DE3 (harboring gene 1 of the RNA polymerase from the phage T7 under the control of the <i>P_{lacUV5}</i> promoter)	31
Rosetta(DE3)	F [−] <i>ompT hsdSB</i> ($r_B^- m_B^-$) <i>gal dcm</i> (DE3) pRARE (Cam ^r)	Novagen
Plasmids		
pK18 <i>mobsacB</i>	Mobilizable vector; allows selection of double crossover in CNB-1	34
pK18 <i>mobsacB</i> - Δ 0097 <i>bcl</i>	Construction of CNB-1 Δ 0097 <i>bcl</i> and CNB-1 Δ 0097_0394 <i>bcl</i>	This study
pK18 <i>mobsacB</i> - Δ 0394 <i>bcl</i>	Construction of CNB-1 Δ 0394 <i>bcl</i>	This study
pK18 <i>mobsacB</i> - Δ boxAB	Construction of CNB-1 Δ boxAB	This study
pK18 <i>mobsacB</i> - Δ boxC	Construction of CNB-1 Δ boxC and CNB-1 Δ genA_ <i>boxC</i>	This study
pK18 <i>mobsacB</i> - Δ genA	Construction of CNB-1 Δ genA	This study
pBBR1MCS3	Tc ^r , <i>lacPOZ'</i> broad-host-range vector with R-type conjugative origin	54
pBBR1MCS3- <i>bcl</i>	Carries 0097 <i>bcl</i> to generate complementation for 0097 <i>bcl</i>	This study
pBBR1MCS3- <i>boxBA</i>	Carries <i>boxBA</i> to generate complementation for <i>boxAB</i>	This study
pBBR1MCS3- <i>boxC</i>	Carries <i>boxC</i> to generate complementation for <i>boxC</i>	This study
pBBR1MCS2- <i>P_{genA}::eGFP</i>	pBBR1MCS2 derivative for fusion of <i>P_{genA}</i> and eGFP	This study
pET28a	Expression vector	Novagen
pET28a- <i>bcl</i>	pET28a derivative for expression of 0097 <i>bcl</i>	This study
pET28a- <i>boxA</i>	pET28a derivative for expression of <i>boxA</i>	This study
pET28a- <i>boxB</i>	pET28a derivative for expression of <i>boxB</i>	This study
pET28a- <i>boxC</i>	pET28a derivative for expression of <i>boxC</i>	This study
pET28a- <i>boxD</i>	pET28a derivative for expression of <i>boxD</i>	This study
pET28a- <i>genA</i>	pET28a derivative for expression of <i>genA</i>	This study
pET28a- <i>genB</i>	pET28a derivative for expression of <i>genB</i>	This study
pET28a- <i>genC</i>	pET28a derivative for expression of <i>genC</i>	This study
pET28a- <i>genR</i>	pET28a derivative for expression of <i>genR</i>	This study

Construction of mutants and genetic complementation. Truncated target genes were obtained by gene splicing and overlap extension (33). For genetic disruption and complementation, pK18*mobsacB* and pBBR1MCS3 vectors were used. Various plasmids derived from these vectors were constructed, and their relevant characteristics are listed in Table 1. Plasmids were electroporated into CNB-1, and the mutants obtained were screened according to the method of Schäfer et al. (34), except that LB medium was supplemented with sucrose at a final concentration of 20%. Deletion of the target genes in pK18*mobsacB* derivatives and in the CNB-1 mutants was verified by PCR amplification. Complementation of the target genes was conducted by introducing pBBR1MCS3 derivatives into the mutants.

Overexpression and purification of enzymes. His₆-tagged fusion proteins of benzoate-CoA ligase, BoxAB, BoxC, or BoxD were produced in *E. coli* BL21(DE3). His₆-tagged *genR* was produced in *E. coli* Rosetta(DE3). Syntheses of the His₆-tagged fusion proteins were induced by addition of isopropyl- β -D-thiogalactopyranoside when the OD₆₀₀ of the cultures reached 0.4. Cells were continuously incubated at a decreased

temperature of 16°C for 4 h before being harvested. Cells were pelleted by centrifugation at 8,000 $\times$ g for 10 min, and the cell pellet was suspended in binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole [pH 7.9]) and then lysed by sonication for 10 min. The cellular lysate was centrifuged at 12,000 $\times$ g for 15 min, and the supernatant was filtered through a 0.22- μ m Millipore Express membrane (Millipore, Carrigtwohill, Ireland) and then applied to a Ni²⁺-charged His $\cdot$ Bind column (Novagen, Frankfurt, Germany) that was pre-equilibrated with binding buffer. Proteins were eluted with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole [pH 7.9]), and the separated protein fractions were analyzed by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel. The fractions containing a single target protein were pooled, desalted with the PD MiniTrap G-25 kit (GE Healthcare, Shanghai, China), and concentrated with Amicon Ultra-15 centrifugal filter units (Millipore, Beijing, China). The proteins obtained were stored in 10% (wt/vol) glycerol at -80°C until use. Protein concentration was determined according to the Bradford method (35).

Enzyme activity assays. Benzoate-CoA ligase activity was determined at 30°C using the indirect assay method of Schühle et al. (36), which was originally described by Ziegler et al. (37). Briefly, benzoate-CoA ligase activity was coupled to myokinase, pyruvate kinase, and lactate dehydrogenase. The reaction mixture (1 ml) contained 100 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 5 mM MgCl₂, 1 mM ATP, 0.4 mM CoA, 0.4 mM NADH, 1 mM phosphoenolpyruvate, 0.5 mM benzoic acid, myokinase (1 U), pyruvate kinase (1 U), lactate dehydrogenase (1.5 U), and 10 µl protein fraction (0.2 to 0.4 mg). Reactions were initiated by the addition of benzoic acid, and the decrease of adsorption at 365 nm, which reflects NADH oxidation, was recorded. Benzoyl-CoA oxygenase/reductase activity was measured spectrophotometrically at 340 nm at 30°C for NADPH oxidation (12). The assay mixture (1 ml) contained 100 mM Tris-HCl (pH 8.0), 0.2 mM FAD, 0.6 mM NADPH, 0.2 mM benzoyl-CoA, and 0.04 mg purified BoxA, and the reaction was started by the addition of 0.8 mg purified BoxB. A control test without addition of BoxB was run in parallel.

2,3-Epoxybenzoyl-CoA dihydrolase (BoxC) activity was analyzed by following the decrease of 2,3-epoxybenzoyl-CoA at a wavelength of 310 nm (13). The catalytic product of BoxAB (2,3-epoxybenzoyl-CoA) was used, and 0.08 mg BoxC was added to the assay mixture.

Photometric assays of 3,4-dehydrodipyl-CoA semialdehyde dehydrogenase (BoxD) were carried out by following NADP⁺ reduction at 340 nm (14). The catalytic product of BoxC (3,4-dehydrodipyl-CoA semialdehyde) was used, and 0.04 mg BoxD was added to the assay mixture.

Gentisate 1,2-dioxygenase activity was assayed spectrophotometrically by measuring the increase of absorption at 330 nm derived from maleylpyruvate. The mixture (total volume, 2 ml) contained 0.1 mM gentisate, 10 µl of recombinant gentisate 1,2-dioxygenase from *E. coli*, and 50 mM Tris-HCl buffer (pH 8.0) (38). Maleylpyruvate isomerase was measured by following the disappearance of maleylpyruvate at 330 nm. Crude maleylpyruvate was prepared from gentisate by gentisate 1,2-dioxygenase digestion at room temperature for 5 min as described above; 0.5 µM glutathione (GSH) was added, and the reaction was initiated by adding 10 µl of maleylpyruvate isomerase (39). Fumarylpyruvate hydrolase activity was followed by measuring the disappearance of fumarylpyruvate absorption at 340 nm. The assay mixture consisted of 0.1 mM gentisate, 0.5 µM GSH, 10 µl of recombinant gentisate 1,2-dioxygenase, and maleylpyruvate isomerase in 50 mM Tris-HCl buffer (pH 8.0); 10 µl of fumarylpyruvate hydrolase was added 15 min later at room temperature to initiate the reaction (40).

All spectrophotometric assays were performed on a SPECORD 205 UV/Vis spectrophotometer (Analytik Jena AG, Jena, Germany).

Preparation of benzoyl-CoA. Benzoyl-CoA was chemically synthesized from CoA and benzoic acid anhydride according to previously published procedures (41). To purify benzoyl-CoA, the reaction mixture was applied to a solid-phase extraction column (Supelclean LC-18 SPE tube; bed weight, 500 mg; volume, 3 ml; Supelco, Sigma-Aldrich, Bellefonte, PA) equilibrated with 20 mM ammonium formate (pH 3.5) containing 2% (vol/vol) methanol (as the equilibration buffer). The column was washed with 6 ml of the equilibration buffer, and purified benzoyl-CoA was eluted with 80% (vol/vol) methanol. The eluate fraction was evaporated under reduced pressure and lyophilized.

Extraction of total RNA and quantitative RT-PCR (qRT-PCR). CNB-1 and mutant cells growing on benzoate, gentisate, or pyruvate were harvested at mid-exponential growth phase (OD₆₀₀, ~0.15). For determination of benzoyl-CoA as a GenR effector *in vivo*, CNB-1Δ0097bcl and CNB-1ΔboxAB cells were cultivated on pyruvate, followed by incubation with 2 mM benzoate for 2 h. Total RNA was extracted using TRIzol reagent (Life Technologies, Shanghai, China) following the manufacturer's instructions. Reverse transcription was conducted according to the instructions for the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). qRT-PCR was performed using a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Boston, MA) according to the manufacturer's protocol with a LightCycler 480 real-time PCR system (Roche Applied Sci-

ence, Basel, Switzerland). The specific primer pairs used for qRT-PCR analyses are listed in Table S1 in the supplemental material. The housekeeping gene *rpoB* of *C. testosteroni* CNB-1 was used as an internal standard for normalization. Each sample was run in triplicate. The relative levels of target gene expression in wild-type CNB-1 and its mutants under defined culture conditions were evaluated using LightCycler relative quantification software (LightCycler 480; Roche Applied Science).

Construction of a transcriptional fusion reporter plasmid and microscopy analysis of recombinant cells. The pBBR1MCS2-eGFP (enhanced green fluorescent protein) vector was used to construct the plasmid for transcriptional fusions. The *genA* promoter was PCR amplified using the primers P_{genA}F and P_{genA}R (see Table S1 in the supplemental material), digested with KpnI and HindIII, and ligated upstream of the eGFP gene in the digested pBBR1MCS2-eGFP vector. The resulting pBBR1MCS2-P_{genA}::eGFP plasmid was verified by DNA sequencing and subsequently electroporated into CNB-1, CNB-1Δ0097bcl, or CNB-1Δ0097bcl/pBBR1MCS3-bcl cells (Table 1). Transformants were selected with kanamycin (150 µg/ml) and cultivated in MSB medium with benzoate or succinate as the carbon source. Cells were obtained from cultures at mid-exponential growth phase (OD₆₀₀, ~0.15). Microscopy analyses were performed with a confocal laser scanning microscope (TCS SP8 microscope; Leica, Bensheim, Germany) with a 63× objective (Plan-Neofluar; numerical aperture, 1.4) and using LAS AF software (Leica Microsystems).

Electrophoretic mobility shift assay (EMSA). A 219-bp DNA fragment containing the promoter region of *genA* was obtained from CNB-1 genomic DNA by PCR using the primer pairs listed in Table S1 as a probe. A 215-bp DNA fragment upstream of *boxD* was used as a nonspecific control probe. Binding experiments were performed as previously described (42). The DNA probe (10 ng) was incubated with various concentrations of purified GenR at 25°C for 30 min in binding buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol (DTT), 5 mM MgCl₂, 0.5 mg/ml calf bovine serum albumin (BSA), and 5% (vol/vol) glycerol in a total volume of 20 µl. Mixtures were then loaded onto native 4% (wt/vol) polyacrylamide gels (mono/bis, 80:1) in 0.5× TBE (90 mM Tris-boric acid and 2 mM EDTA). Gels were stained with SYBR gold nucleic acid gel stain (Invitrogen) for 30 min in TBE buffer and photographed under UV transillumination.

To analyze disassociation of the DNA probe and GenR, effector molecules of benzoate, 3-hydroxybenzoate, gentisate, protocatechuate, benzoyl-CoA, acetyl-CoA, and HS-CoA were added to the reaction mixture at various concentrations.

DNase I footprinting. To determine the GenR binding sites in the *genA* promoter region, a DNase I footprinting assay was performed using a fluorescent-labeling procedure (43). Briefly, the DNA fragment was prepared by PCR using the P_{genA}F and 5'-labeled hexachlorofluorescein phosphoramidite (HEX)-P_{genA}R primers listed in Table S1. The labeled DNA fragment (100 to 150 ng) was purified from an agarose gel, and various concentrations of His₆-GenR were added to the footprinting reaction mixture containing 20 mM HEPES (pH 7.4), 2 mM DTT, 100 mM KCl, 5 mM MgCl₂, 0.5 mg/ml calf BSA, and 5% (vol/vol) glycerol in a total volume of 50 µl. After incubation at 25°C for 30 min, DNase I digestion was conducted for a further 1 min at 25°C and then terminated by addition of stop buffer (20 mM EGTA, pH 8.0) (Promega, Madison, WI, USA). The purified samples were loaded into an Applied Biosystems 3730xl DNA genetic analyzer along with an internal lane size standard (GeneScan 500 LIZ; Applied Biosystems, Beijing, China). The electropherograms generated were then analyzed with GeneMarker v2.2 (SoftGenetics, State College, PA).

Fluorescent primer extension. To analyze the transcriptional start site of the *genA* gene, we performed fluorescent primer extension following the method of Lloyd et al. (44). CNB-1 cells that were growing on benzoate were harvested at mid-exponential growth phase (OD₆₀₀, ~0.15). Total RNA was prepared from *C. testosteroni* strain CNB-1 as described above. Total RNA (30 µg) was treated with 30 U of RNase-free

TABLE 2 Proteins related to aromatic compound degradation^a

No.	ORF	Protein name; putative function	Score ^b	Match ^c	Coverage (%) ^d	pI/MW ^f	
						Theoretical ^e	Experimental
B6	CtCNB1_2779	GenB; fumarylpyruvate hydroxylase	77	12	58	5.31/26	5.47/27
B13	CtCNB1_0066	BoxB; benzoyl-CoA oxygenase component B	133	18	43	5.63/54	5.83/55
B14	CtCNB1_0065	BoxA; benzoyl-CoA oxygenase; reductase	120	29	45	5.66/47	5.94/55
B15	CtCNB1_0097	BCL; benzoate-CoA ligase family	69	15	31	5.90/57	6.10/59
B17	CtCNB1_0097	BCL; benzoate-CoA ligase family	61	15	31	5.90/57	6.30/59
B19	CtCNB1_2778	GenA; gentisate 1,2-dioxygenase	54	11	34	6.10/42	6.40/47
B22	CtCNB1_0093	LivG; ABC-type branched-chain amino acid transport systems, ATPase component	52	13	48	5.99/28	6.51/27

^a Proteins related to aromatic compound degradation were induced in cells grown with benzoate as the sole carbon source. The proteomes of CNB-1 cells grown on succinate were used for reference. The 2-DE gel images of proteomes from CNB-1 cells grown on benzoate and succinate are provided in Fig. S1 in the supplemental material. For all comparisons, *P* was <0.05; i.e., the spot was found only on gels where CNB-1 was grown on benzoate as opposed to succinate. Induction was observed for each protein.

^b Mascot protein score among the fractions where the protein was identified.

^c Number of spectra matched to the protein.

^d Protein sequence coverage for the fraction.

^e From a Mascot search.

^f MW, molecular weight (in thousands).

DNase I (Promega) for 30 min at 37°C and then hybridized to HEX-*P_{genA}*R. The final volume was adjusted to 20 µl using DEPC-treated water in a sterile DEPC-treated microcentrifuge tube. The tube was heated to 70°C for 5 min and then cooled on ice for 10 to 30 min. Then, 400 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 40 U of RNasin RNase inhibitor, and 2 mM concentrations of deoxynucleoside triphosphates (dNTPs) in 1× M-MLV reaction buffer (Promega) were added to the annealed primer-RNA mixture. The final volume was adjusted to 40 µl with DEPC-treated water. The primer extension reaction mixture was incubated at 42°C for 60 min. After addition of 1 µl RNase A (10 mg/ml; Sigma-Aldrich), the sample was incubated for 30 min at 37°C. HEX-labeled cDNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with DNAmate (TaKaRa, Dalian, China) according to the manufacturer's instructions. Capillary electrophoresis was performed on an Applied Biosystems 3730xl DNA genetic analyzer with GeneScan 500 LIZ (Applied Biosystems) as the internal lane size standard. The length of the HEX-labeled cDNA product was calculated by GeneMarker v 2.2 software (SoftGenetics).

Surface plasmon resonance (SPR). SPR experiments were performed on a Biacore 3000 apparatus (GE Healthcare, Buckinghamshire, United Kingdom) with a running buffer composed of 20 mM Tris-HCl, 150 mM NaCl, and 0.005% Tween 20 (pH 8). To determine the association and dissociation of the operator *P_{genA}* and GenR after the addition of effector molecules, a 5'-end-biotinylated double-stranded *P_{genA}* DNA fragment was immobilized on a streptavidin-coated SA sensor chip (GE Healthcare), and His₆-GenR (32 nM, 30 µl) with benzoate, 4-hydroxybenzoate, protocatechuate, succinate, or acetyl-CoA (1 mM, 30 µl) or running buffer was injected with a COINJECT pattern and with various concentrations of 3-hydroxybenzoate, gentisate, and benzoyl-CoA with a KINJECT pattern at a flow rate of 30 µl/min. At the end of each cycle, the sensor chip was regenerated by injecting 5 µl of running buffer plus 0.02% sodium dodecyl sulfate.

RESULTS

Two putative metabolic pathways for benzoate degradation in *C. testosteroni* CNB-1. CNB-1 grows on benzoate as the sole carbon source. The only detectable aromatic ring cleavage dioxygenase activity from benzoate-grown cells was gentisate 1,2-dioxygenase activity (27), indicating that benzoate was metabolized via the gentisate 1,2-cleavage pathway (Fig. 1C). Indeed, two representative enzymes of the gentisate pathway, gentisate 1,2-dioxygenase (B19, GenA) and fumarylpyruvate hydrolase (B6, GenB), were detected in benzoate-grown

CNB-1 cells (Table 2; also, see Fig. S1 in the supplemental material). A genetic cluster (CtCNB1_2776 to CtCNB1_2780, designated the *genABC* cluster) that encodes a complete gentisate 1,2-cleavage pathway was identified in this study in the CNB-2 genome (Fig. 1D; Table 3). GenA, GenC, and GenB were confirmed as gentisate 1,2-dioxygenase, maleylpyruvate isomerase, and fumarylpyruvate hydrolase, respectively (see Fig. S2 in the supplemental material).

In addition to GenA and GenB, the comparative proteomic studies on benzoate- and succinate-grown cells also revealed proteins that were induced in CNB-1 when benzoate was used the carbon source. Benzoate-CoA ligase (B15, B17, BCL), benzoyl-CoA reductase (B14, BoxA), and benzoyl-CoA oxygenase (B13, BoxB) were induced (Table 2; also, see Fig. S1 in the supplemental material). This observation prompted us to consider a different pathway alternative to the above-mentioned gentisate 1,2-cleavage pathway (Fig. 1A), i.e., the recently identified CoA-dependent epoxide pathway in *A. Evansii* (11). Data mining of the CNB-2 genome revealed that two genetic clusters (CtCNB1_0065 to CtCNB1_0067 and CtCNB1_0097 to CtCNB1_0098, designated *box* clusters) (Table 3) possibly encode the conversion of benzoate to 3,4-dehydroadipyl-CoA (Fig. 1B).

CNB-1 metabolism of benzoate via the CoA-dependent epoxide pathway and identification of genes involved in the CoA-dependent epoxide pathway in CNB-1. To determine whether benzoate was metabolized via gentisate 1,2-cleavage or the CoA-dependent epoxide pathway, the genes putatively encoding gentisate 1,2-dioxygenase (*genA*, CtCNB1_2778) and 2,3-epoxybenzoyl-CoA dihydrolase (*boxC*, CtCNB1_0067) were disrupted. Like CNB-1, the CNB-1Δ*genA* mutant also grew on benzoate (Fig. 2A). This result indicated that benzoate degradation was independent of the gentisate pathway. Furthermore, our results (Fig. 2) indicated that disruption of *box* genes resulted in CNB-1 mutants that were not able to grow on benzoate. These results clearly demonstrated that benzoate was metabolized via the CoA-dependent epoxide pathway in CNB-1.

The CoA-dependent epoxide pathway starts by the activation of benzoate, which is catalyzed by benzoate-CoA ligase. Two candidate benzoate-CoA ligase genes (*bcl*) were identified in the genome, which were tagged as CtCNB1_0097 and CtCNB1_0394.

TABLE 3 Genomic analysis of benzoate- and gentisate-degradative pathways in *C. testosteroni* strain CNB-1^a

Gene (ORF)	Gene product (no. of aa)	Related gene product			% identity; no. of aa	Accession no.
		Name	Function	Organism		
CtCNB1_0065	BoxA (433)	BoxA	Benzoyl-CoA oxygenase component A	<i>Azoarcus evansii</i>	59; 414	Q9AIX6
CtCNB1_0066	BoxB (474)	BoxB	Benzoyl-CoA oxygenase component B	<i>Azoarcus evansii</i>	72; 473	Q9AIX7
CtCNB1_0067	BoxC (552)	BoxC	Benzoyl-CoA-dihydrodiol lyase	<i>Azoarcus evansii</i>	67; 555	Q84HH6
CtCNB1_0091	TE (157)	PaaI	Thioesterase	<i>Haemophilus influenzae</i>	47; 138	1SC0_A
CtCNB1_0092	LivF (233)	LivF	ABC transporter, ATPase component	<i>Azoarcus</i> sp. strain CIB	57; 257	CCD33112
CtCNB1_0093	LivG (256)	LivG	ABC transporter, ATPase component	<i>Azoarcus</i> sp. strain CIB	57; 252	CCD33113
CtCNB1_0094	LivM (364)	LivM	ABC transporter, permease component	<i>Azoarcus</i> sp. strain CIB	47; 329	CCH23034
CtCNB1_0095	LivH (289)	LivH	ABC transporter, permease components	<i>Azoarcus evansii</i>	62; 288	AAN39369
CtCNB1_0096	LivK (387)	LivK	ABC transporter, periplasmic component	<i>Azoarcus evansii</i>	70; 391	AAL02078
CtCNB1_0097	BCL (521)	BCL	Benzoate-CoA ligase	<i>Rhodopseudomonas palustris</i>	64; 524	4EAT_A
CtCNB1_0098	BoxD (531)	BoxD	Aldehyde dehydrogenase	<i>Burkholderia xenovorans</i> LB400	64; 532	2VRO_A
CtCNB1_0099	BoxR (300)	BoxR	Transcriptional regulator	<i>Azoarcus</i> sp. strain CIB	51; 300	CCD33120
CtCNB1_2776	LysR (300)	LysR	Transcriptional regulator, LysR family	<i>Pseudomonas</i> sp. strain 19-rlim	55; 298	AEO27403
CtCNB1_2777	GenR (200)	GenR	Transcriptional regulatory protein	<i>Comamonas testosteroni</i>	55; 172	BAF34929
CtCNB1_2778	GenA (375)	GenA	Gentisate 1,2-dioxygenase	<i>Rhodococcus</i> sp. strain NCIMB 12038	61; 359	ADT78164
CtCNB1_2779	GenB (239)	GenB	Fumarylpyruvate hydroxylase	<i>Ralstonia</i> sp.	78; 192	O86042
CtCNB1_2780	GenC (214)	GenC	Maleylpyruvate isomerase	<i>Ralstonia</i> sp.	70; 212	O86043

^a aa, amino acids.

The theoretical translational products of CtCNB1_0097 and CtCNB1_0394 showed sequence identities of 53% and 36%, respectively, to the benzoate-CoA ligase from *A. evansii*. When the two candidate *bcl* genes were individually disrupted, only the CNB-1Δ0097*bcl* mutant was unable to grow on benzoate. Furthermore, the growth phenotype on benzoate was restored by expression of the CtCNB1_0097 (*bcl*) gene in the CNB-1Δ0097*bcl* mutant (Fig. 2B). This indicates that CtCNB1_0097 is the authentic *bcl* gene that has an *in vivo* benzoate degradation function in the CNB-1 strain. Indeed, benzoate-CoA ligase activity was detected when CtCNB1_0097 was expressed and purified from *E. coli* cells (see Fig. S3A in the supplemental material).

BoxAB catalyzes the conversion of benzoyl-CoA to 2,3-epoxybenzoyl-CoA in the CoA-dependent epoxide pathway (12). We showed that CtCNB1_0065 and CtCNB1_0066 encode BoxAB in CNB-1. The CNB-1Δ*boxAB* mutant was not able to grow aerobically on benzoate (Fig. 2C). The CtCNB1_0065 and CtCNB1_0066 genes were cloned and expressed in *E. coli*, and their translational products (BoxAB) showed benzoyl-CoA oxygenase/reductase activities (see Fig. S3B in the supplemental material). We cloned and expressed the BoxC gene (CtCNB1_0067), and enzymatic assays showed that the purified BoxC exhibited 2,3-epoxybenzoyl-CoA dihydrolase activity (see Fig. S3 in the supplemental material). Oxidation of 3,4-dehydroadipyl-CoA semi-aldehyde to the corresponding 3,4-dehydroadipyl-CoA acid is catalyzed by BoxD (14). When the CtCNB1_0098 gene, encoding aldehyde dehydrogenase in CNB-1, was heterologously expressed in *E. coli*, its translation product showed aldehyde dehydrogenase activity (BoxD) (see Fig. S3D in the supplemental material).

GenR negatively regulates transcription of the *gen* cluster. The above results clearly show that benzoate was metabolized via the CoA-dependent epoxide pathway, but not the gentisate pathway, in the CNB-1 strain. To explain why gentisate 1,2-dioxygenase was induced when CNB-1 grew on benzoate, we investigated the regulation of the *gen* cluster for the gentisate pathway. The *gen* cluster is organized into two transcriptional units, *lysR-genR* and

genABC (see Fig. S4 in the supplemental material). Two putative regulators are encoded by CtCNB1_2776 (LysR type, named *lysR*) and CtCNB1_2777 (MarR type, named *genR*), and they are oriented divergently to other genes of the *gen* cluster. To clarify which regulator governed regulation of the *gen* genes, quantitative RT-PCR was performed with CNB-1 and the mutants CNB-1Δ*lysR* and CNB-1Δ*genR*. Results showed that the transcription of *lysR*, *genA*, *genB*, and *genC* increased in CNB-1Δ*genR* compared to that of CNB-1 (Fig. 3). Transcription of *gen* cluster genes was not significantly influenced when the *lysR* gene was deleted (Fig. 3). Therefore, these results indicate that GenR negatively regulated transcription of the *gen* cluster genes in CNB-1.

GenR specifically binds to a 41-bp sequence in the intergenic region of *genR* and *genA*. To identify the *gen* cluster promoter and characterize the interaction between GenR and its binding sequence, *genR* was expressed and purified from *E. coli* Rosetta(DE3) cells. Native His₆-GenR had an apparent molecular mass of 51.6 kDa (the monomer has a calculated molecular mass of 23.8 kDa), suggesting that it was a homodimer. The interaction between the GenR regulator and the *genR-genA* intergenic region was next examined. GenR bound to the *genR-genA* intergenic region in a concentration-dependent manner (Fig. 4A). To pinpoint the specific GenR-binding sequences, DNase I footprinting was performed using a capillary sequencer. The results showed that GenR bound to a 41-nt sequence (ACGCATATCAACATTATGCTAATCATCAGTGTGCTGTTTAT) (Fig. 5A). The transcription start site for *genA* was determined (Fig. 5B), and the detailed structure of its promoter region (*P_{genA}*) is shown in Fig. 5C.

3-Hydroxybenzoate, gentisate, and benzoyl-CoA affect GenR binding to *P_{genA}*. To identify effectors that affect the binding of GenR to *P_{genA}*, a range of molecules, including succinate, benzoyl-CoA, acetyl-CoA, HS-CoA, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, gentisate, and protocatechuate, were tested. EMSA results showed that 3-hydroxybenzoate, gentisate, and benzoyl-CoA dissociated the GenR-*P_{genA}* complex, while the other compounds showed no observable effects (Fig. 4B). The

FIG 2 Genetic disruption and complementation of *genA* and *boxC* (A), *bcl* (B), and *boxAB* (C) in *C. testosteroni*.

effect of 3-hydroxybenzoate, gentisate, and benzoyl-CoA on dissociation of the GenR- P_{genA} complex was further observed with SPR assays (Fig. 4C to F).

Benzoyl-CoA functions *in vivo* as an effector for the regulator GenR. As demonstrated above, we found that benzoyl-CoA antagonized the binding of GenR to the *genA* promoter *in vitro*. To

FIG 3 GenR negatively regulates the transcription of genes encoding the gentisate pathway. The housekeeping gene *rpoB* of *C. testosteroni* was used as an internal control. Relative transcription levels are defined as the transcription ratios of the target genes in mutants to their counterparts in wild-type CNB-1.

establish whether benzoyl-CoA is an effector for GenR *in vivo* and regulates the transcription of *gen* cluster, we analyzed the transcription of *genA* and *genR* in CNB-1, CNB-1Δ0097bcl, and CNB-1ΔboxAB. As shown in Fig. 6A, the transcription of *genA* was mostly unchanged in CNB-1Δ0097bcl (benzoyl-CoA synthesis was disabled), but it increased significantly in CNB-1ΔboxAB (benzoyl-CoA degradation was disabled). We also observed that the transcription of *genR* slightly decreased in both CNB-1Δ0097bcl and CNB-1ΔboxAB. These data indicated that benzoyl-CoA affected *in vivo* the transcription of the *gen* genetic cluster.

To demonstrate *in vivo* the effect of benzoyl-CoA on the interaction between promoter P_{genA} and GenR, we constructed a promoter-reporter to visualize the effect of benzoyl-CoA. The promoter-reporter plasmid carried the pBBR1MCS2- P_{genA} ::eGFP fusion, and the promoter activity was dependent on the presence of benzoyl-CoA. The pBBR1MCS2- P_{genA} ::eGFP reporter plasmid was electroporated into CNB-1, CNB-1Δ0097bcl, and CNB-1Δ0097bcl/pBBR1MCS3-bcl cells. No GFP fluorescence was observed in CNB-1Δ0097bcl, while its complementary strain (CNB-1Δ0097bcl/pBBR1MCS3-bcl) and wild-type CNB-1 showed strong GFP fluorescence (Fig. 6B). These data clearly indicated that benzoyl-CoA affected *in vivo* the interaction between promoter P_{genA} and GenR.

DISCUSSION

The present study demonstrated that CNB-1 degrades benzoate via a CoA-dependent epoxide pathway, although a previous investigation (27) and this study both observed that gentisate 1,2-dioxygenase and other enzymes of the gentisate pathway were induced when the CNB-1 strain was cultivated with benzoate as the carbon source. Rather et al. (11) reported that 4 to 5% of the sequenced microbial genomes carry genes putatively encoding this CoA-dependent epoxide pathway. We further explored the updated NCBI databank using BoxB and BoxC sequences as queries and found that the CoA-dependent epoxide pathway is identifiable in *Actinobacteria* (3.1% of a total 160 genomes), *Firmicutes* (0.8% of a total 240 genomes), and *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* (10.3%, 36.7%, 0.4%, and 6.1% of a total of 150, 98, 230, and 49 genomes, respectively). Thus, the CoA-dependent epoxide pathway seems to be more widely distributed among *Betaproteobacteria* (36.7%) than in the other bacteria mentioned above. Several bacterial species

FIG 4 Electrophoretic mobility shift assays (EMSA) for GenR binding to the *genR-genA* intergenic region (A) and determination of effector molecules by EMSA (B) and by surface plasmon resonance (SPR) analysis (C to F). (A and B) Each lane contained 10 ng of DNA probe. (A) Lane 1, P_{genA} probe alone; lane 2, 1.5 μ M BSA as a control protein; lanes 3 to 6, retardation assays using 2 nM, 20 nM, 100 nM, 2 μ M, and 4 μ M His₆-GenR proteins, respectively. DNA upstream of *boxD* was used as a nonbinding control. (B) Lane 1, P_{genA} probe alone; lane 2, the P_{genA} probe and 4 μ M His₆-GenR proteins. Lanes 3 to 15 contain, in addition to the P_{genA} probe and 4 μ M His₆-GenR proteins, 2 mM benzoate (Ben; lane 3), protocatechuate (PCA; lane 10), acetyl-CoA (AcA; lane 14) and HS-CoA (CoA; lane 15), and various concentrations of 3-hydroxybenzoate (3HB; lanes 4 to 6, 10 μ M, 20 μ M, and 30 μ M), gentisate (Gen; lanes 7 to 9, 3 μ M, 10 μ M, and 30 μ M), or benzoyl-CoA (BzA; lanes 11 to 13, 10 μ M, 20 μ M, and 50 μ M). (C to F) Biotinylated P_{genA} was immobilized on a streptavidin-coated SA sensor chip. Detailed conditions are described in Materials and Methods. 4HB, 4-hydroxybenzoate; PCA, protocatechuate; 3HB, 3-hydroxybenzoate.

FIG 5 DNase I footprinting of the coding strand of the *genA* promoter region. (A) Fluorograms correspond to the control (DNA plus 10 μ M BSA) and to the protection reactions (with concentrations of 0.9, 2.7, and 9 μ M His₆-GenR protein). (B) Transcription start site analysis of *genA* as determined by fluorescent primer extension. (C) Nucleotide sequence of the *genR*-*genA* intergenic region. The numbers on the left indicate the nucleotide positions. The *genA* transcription start site (TSS) is indicated by a bent arrow and an asterisk. Presumptive -10 and -35 regions of the *genA* promoter are underlined. The sequence protected from DNase I digestion is indicated by a shaded box. The GenR and GenA translational start codons are boxed, and the translated amino acids are shown below the nucleotide sequence.

carrying *box* genetic clusters encoding the CoA-dependent epoxide pathway were confirmed as being able to grow on benzoate (11, 21). Nonetheless, the functionalities of the CoA-dependent epoxide pathway have so far been identified only in *A. Evansii* (11), *B. xenovorans* (21), and *C. testosteroni* (this study). The genes involved in the CoA-dependent epoxide pathway in CNB-1 are interrupted by putative ABC transporter genes, which differ from

the *box* genes of *A. Evansii*, which are continuously organized in one genetic cluster (45).

The discovery that the MarR-type regulator GenR regulates the gene encoding the gentisate pathway and that it accepts benzoyl-CoA as an effector explains the induction of gentisate 1,2-dioxygenase and other enzymes of the gentisate pathway when was CNB-1 grown on benzoate: benzoate was converted into benzoyl-

FIG 6 *In vivo* determination of benzoyl-CoA regulation of transcription of *genA* by qRT-PCR (A) and a GFP promoter-reporter system (B).

CoA, which then released the GenR repression of *genA* in CNB-1. The *genA* gene was subsequently transcribed, and gentisate 1,2-dioxygenase was synthesized. The induction of gentisate 1,2-dioxygenase activity with benzoate was observed previously in the *A. evansii* strain KB 740 (23), which harbors the same CoA-dependent epoxide pathway (24) for benzoate degradation as that found in CNB-1. To date, there are no genome data or published studies on the genetics of gentisate degradation in *A. evansii*. Hence, we

explored the available genomic data for other *Azoarcus* members, i.e., *Aromatoleum aromaticum* EbN1 (synonym *Azoarcus* sp. strain EbN1) (46) and *Azoarcus* sp. strain BH72 (47). As shown in Fig. 7, the *gen* clusters in the EbN1 and BH72 strains are very different from that of CNB-1. There are no MarR-type regulators in EbN1 and BH72. In fact, only putative LysR-type regulators are associated with the *gen* clusters in EbN1 and BH72. A putative LysR-type regulator was also observed in the CNB-1 strain, which is adjacent

FIG 7 Genome data-mining for the gentisate catabolism pathway and its regulator proteins. Boldface type indicates that the species' regulator proteins belong to the MarR family.

to GenR in the cluster. Our results demonstrated that this putative LysR regulator in CNB-1 did not regulate the *gen* cluster. Therefore, we deduced that gentisate 1,2-dioxygenase activity is regulated differently in *Azoarcus* species than in *C. testosteroni*, although benzoyl-CoA might be the common molecule involved in such regulation. Previous literature showed that gentisate 1,2-dioxygenase was coinduced in *Pseudomonas testosteroni* when 3-hydroxybenzoate was fed as the carbon source, although *P. testosteroni* metabolizes 3-hydroxybenzoate exclusively through a protocatechuate pathway (48, 49).

The mechanism by which GenR regulates the *gen* cluster in CNB-1 has been illustrated by the present study: GenR binds to its target DNA sequence (ACGCATATCAACATTATGCTAATCATCAGTGTGCTGTTTAT) in the absence of effectors and represses gene transcription of the *genABC* genes. When effectors such as gentisate, 3-hydroxybenzoate, or benzoyl-CoA are present, GenR protein is released from its DNA binding site and the repression of transcription is abolished. According to our study (Fig. 6A), GenR also exerts positive self-regulation of its own transcript unit, i.e., the *lysR-genR* unit. GenR is a MarR-type regulator, and several MarR-type regulators have been found to be involved in regulation of aromatic compound degradation. For examples, BadR activates benzoyl-CoA reductase (50), CouR governs *p*-coumarate degradation in *Rhodopseudomonas palustris* (51), CbaR controls the *cbaABC* operon for 3-chlorobenzoate degradation in *C. testosteroni* BR60 (52), and FerC regulates the ferulate catabolic operon in *Sphingobium* sp. strain SYK-6 (53). *p*-Coumaroyl-CoA and feruloyl-CoA have been identified as effectors for CouR and FerC, respectively. This study corroborated that benzoyl-CoA is an effector of GenR. These aromatic CoA thioesters represent a new category of effectors for MarR-type regulators. More putative MarR-type proteins, as well as LysR- and IclR-type proteins that potentially regulate gentisate degradation, have been identified in the genomes of diverse bacterial species (Fig. 7). These represent new candidate regulators for gentisate degradation regulation that merit further investigation.

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