

## Biochemical and Genetic Analysis of the $\gamma$ -Resorcylate (2,6-Dihydroxybenzoate) Catabolic Pathway in *Rhizobium* sp. Strain MTP-10005: Identification and Functional Analysis of Its Gene Cluster<sup>∇</sup>

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**We identified a gene cluster that is involved in the  $\gamma$ -resorcylate (2,6-dihydroxybenzoate) catabolism of the aerobic bacterium *Rhizobium* sp. strain MTP-10005. The cluster consists of the *graDAFCBEK* genes, and *graA*, *graB*, *graC*, and *graD* were heterologously expressed in *Escherichia coli*. Enzymological studies showed that *graD*, *graA*, *graC*, and *graB* encode the reductase (GraD) and oxygenase (GraA) components of a resorcinol hydroxylase (EC 1.14.13.x), a maleylacetate reductase (GraC) (EC 1.3.1.32), and a hydroxyquinol 1,2-dioxygenase (GraB) (EC 1.13.11.37). Bioinformatic analyses suggested that *graE*, *graR*, and *graK* encode a protein with an unknown function (GraE), a MarR-type transcriptional regulator (GraR), and a benzoate transporter (GraK). Quantitative reverse transcription-PCR of *graF*, which encodes  $\gamma$ -resorcylate decarboxylase, revealed that the maximum relative mRNA expression level ( $[5.93 \pm 0.82] \times 10^{-4}$ ) of *graF* was detected in the total RNA of the cells after one hour of cultivation when  $\gamma$ -resorcylate was used as the sole carbon source. Reverse transcription-PCR of *graDAFCBE* showed that these genes are transcribed as a single mRNA and that the transcription of the gene cluster is induced by  $\gamma$ -resorcylate. These results suggested that the *graDAFCBE* genes are responsible as an operon for the growth of *Rhizobium* sp. strain MTP-10005 on  $\gamma$ -resorcylate and are probably regulated by GraR at the transcriptional level. This is the first report of the  $\gamma$ -resorcylate catabolic pathway in an aerobic bacterium.**

$\gamma$ -Resorcylate decarboxylase (EC 4.1.1.x) reversibly catalyzes the decarboxylation of  $\gamma$ -resorcylate (2,6-dihydroxybenzoate) to resorcinol (18, 21, 25, 38, 39). The enzyme is expected to be useful for the industrial production of  $\gamma$ -resorcylate, which is an important intermediate in the synthesis of pharmaceuticals and agricultural chemicals (29, 30). Our research group, as well as a few other researchers, have reported the occurrence and biochemical properties of the enzyme (18, 25, 38, 39). We isolated a microorganism, identified as *Rhizobium* sp. strain MTP-10005, which shows a high level of  $\gamma$ -resorcylate decarboxylase activity (38). We then purified the enzyme and expressed its gene in *Escherichia coli* for the first time (38). Moreover, we determined the three-dimensional structure of the enzyme and found that one Zn<sup>2+</sup> binds to Glu8, His10, His164, and Asp287 in the active center of the enzyme and that the enzyme is a novel Zn<sup>2+</sup>-dependent decarboxylase (15). During the course of cloning of the  $\gamma$ -resorcylate decarboxylase gene, *graF*, we found that the homologous genes encoding a hypothetical resorcinol hydroxylase (*graA*), a hydroxyquinol 1,2-dioxygenase (*graB*), and a maleylacetate reductase (*graC*) were localized immediately upstream and downstream of it

(38). Recently, we found four additional genes homologous to an NAD(P)H-flavin oxidoreductase (*graD*), a MarR-type transcriptional regulator (*graR*), the hypothetical protein Atu2526 (*graE*), and a benzoate transport protein (*graK*). Genetic analysis of this  $\gamma$ -resorcylate catabolic gene cluster from aerobic bacteria has not been done up to now (21), but it deserves much attention, especially because of the first  $\gamma$ -resorcylate catabolic pathway in aerobic bacteria and the importance for the regulation of microbial production of  $\gamma$ -resorcylate and development of effective  $\gamma$ -resorcylate production systems in industry.

We here describe the genetic analysis of the  $\gamma$ -resorcylate catabolic pathway in the aerobic bacterium *Rhizobium* sp. strain MTP-10005, as well as the identification and functional analysis of its gene cluster.

### MATERIALS AND METHODS

**Materials.** *E. coli* BL21(DE3), *E. coli* NovaBlue, a pET3b vector, a pET14b vector, and a pT7Blue T-Vector were purchased from EMD Biosciences, Inc. (San Diego, CA). Ex Taq DNA polymerase, a DNA ligation kit (Mighty Mix), the LA PCR in vitro cloning kit, the chaperone plasmid pG-TF2, containing *groES*, *groEL*, and *tig*, and the restriction enzymes BamHI, EcoRI, NdeI, PstI, and SalI were purchased from TaKaRa Bio, Inc. (Shiga, Japan). BglII was purchased from Roche Diagnostics (Basel, Switzerland). *E. coli* BL21 Star (DE3), DNase I (amplification grade), and the SuperScript III Platinum SYBR green one-step quantitative reverse transcription-PCR (qRT-PCR) kit were purchased from Invitrogen Corp. (Carlsbad, CA). The UltraClean15 DNA purification kit was purchased from Mo Bio Laboratories, Inc. (West Carlsbad, CA). Ni-nitritotriacetic acid (NTA) agarose, the OneStep RT-PCR kit (OneStep RT-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<b>Strains</b>		
<i>Rhizobium</i> sp. strain MTP-10005	$\gamma$ -Resorcyolate decarboxylase-producing bacterium	38
<i>E. coli</i> BL21 (DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal dcm</i> (DE3)	EMD Biosciences
<i>E. coli</i> BL21 Star (DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal dcm me131</i> (DE3)	Invitrogen
<i>E. coli</i> NovaBlue	Tc <sup>r</sup> ; <i>endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> F'[ <i>proA</i> <sup>+</sup> <i>B</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> <i>Z</i> $\Delta$ M15::Tn10 (Tc <sup>r</sup> )]	EMD Biosciences
<b>Plasmids</b>		
Vector plasmids		
pET3b	Ap <sup>r</sup> ; expression vector	EMD Biosciences
pET14b	Ap <sup>r</sup> ; expression vector	EMD Biosciences
pT7Blue T-Vector	Ap <sup>r</sup> ; TA cloning vector	EMD Biosciences
Plasmids used for cloning of $\gamma$ -resorcyolate degradation genes		
pGWU1	Ap <sup>r</sup> ; pT7Blue T-Vector, a 1.2-kb SalI fragment	This study
pGWU2	Ap <sup>r</sup> ; pT7Blue T-Vector, a 0.75-kb PstI fragment	This study
pGWU3	Ap <sup>r</sup> ; pT7Blue T-Vector, a 1.0-kb SalI fragment	This study
pGWU4	Ap <sup>r</sup> ; pT7Blue T-Vector, a 0.75-kb BamHI fragment	This study
pGWD1	Ap <sup>r</sup> ; pT7Blue T-Vector, a 3.0-kb EcoRI fragment	This study
pGWD2	Ap <sup>r</sup> ; pT7Blue T-Vector, a 1.5-kb EcoRI fragment	This study
pGWD3	Ap <sup>r</sup> ; pT7Blue T-Vector, a 2.0-kb PstI fragment	This study
Plasmids used for expression of $\gamma$ -resorcyolate catabolic genes		
pGRA	Ap <sup>r</sup> ; pET14b- <i>graA</i>	This study
pGRA3	Ap <sup>r</sup> ; pET3b- <i>graA</i>	This study
pGRB	Ap <sup>r</sup> ; pET14b- <i>graB</i>	This study
pGRC	Ap <sup>r</sup> ; pET14b- <i>graC</i>	This study
pGRD	Ap <sup>r</sup> ; pET14b- <i>graD</i>	This study
pGRDA	Ap <sup>r</sup> ; pET14b- <i>graDA</i>	This study
Plasmids used for qRT-PCR		
pTGF	Ap <sup>r</sup> ; pT7Blue T-Vector- <i>graF</i>	38
pTR16	Ap <sup>r</sup> ; pT7Blue T-Vector-16S rRNA	38

PCR buffer and OneStep RT-PCR enzyme mix), and the RNeasy mini kit were purchased from QIAGEN (Hilden, Germany). A DNA ladder was purchased from New England Biolabs, Inc. (Ipswich, MA). Precision Plus Protein all-blue standards (Bio-Rad Laboratories, Inc., Hercules, CA) and protein molecular weight markers (Fermentas International, Inc., Burlington, ON, Canada) were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All other reagents were the best grade commercially available and were purchased from Kanto Kagaku Co. (Tokyo, Japan), Kishida Chemical Co. (Osaka, Japan), Sigma-Aldrich Co. (St. Louis, MO), Tokyo Kasei Kogyo Co. (Tokyo, Japan), or Wako Chemical Co. (Osaka, Japan) unless otherwise stated.

**Bacterial strains, plasmids, and primers.** The bacterial strains and plasmids used in this study are summarized in Table 1. The primer sequences used for genome-walking PCR, the construction of expression vectors, qRT-PCR, and RT-PCR are summarized in Table 2.

**Cloning and sequence analysis of *graA*, *graB*, *graC*, *graD*, *graE*, *graK*, and *graR*.** Genome-walking PCR was done with the LA PCR in vitro cloning kit to analyze the genes upstream and downstream of *graF*. The chromosomal DNA extracted from *Rhizobium* sp. strain MTP-10005 cells was digested with BamHI, which produces the same type of sticky end as Sau3AI, EcoRI, PstI, or SalI and is ligated to the Sau3AI, EcoRI, PstI, or SalI cassette, respectively. The DNA fragments obtained were used as the template for PCR amplifications. PCR amplification was carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Piscataway, NJ). The PCR fragments obtained were purified from the agarose gel with the UltraClean15 DNA purification kit and ligated into a pT7Blue T-Vector with the DNA ligation kit (Mighty Mix). The plasmids constructed (pGWU1, pGWU2, pGWU3, pGWU4, pGWD1, pGWD2, and pGWD3) were transformed into *E. coli* NovaBlue. The white ampicillin-resistant colonies were selected and grown in 5 ml of LB medium containing ampicillin (100  $\mu$ g/ml). The plasmid extracted from the cells was purified and sequenced by means of a DNA sequencer SQ5500E (Hitachi High-Technologies Corp., Tokyo, Japan) with a Thermo Sequenase primer cycle sequencing kit (GE Healthcare

Bio-Sciences Corp., Piscataway, NJ). A homology search was done with a BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

**Construction of expression vectors of *graA*, *graB*, *graC*, and *graD*.** *graA*, *graB*, *graC*, and *graD* were amplified from *Rhizobium* sp. strain MTP-10005 genomic DNA (253 ng) with the primers (10 pmol) by PCR. PCR amplification was carried out with Ex Taq DNA polymerase in a Gene Amp PCR system 9700. The PCR fragments obtained were purified from the agarose gel with an UltraClean15 DNA purification kit and subcloned into a pT7Blue T-Vector. After the DNA sequence was confirmed, the subcloned gene was digested with NdeI/BamHI and cloned into a pET14b vector digested with the same restriction enzymes to form pGRA, pGRB, pGRC, or pGRD. The *graDA* coexpression vector, pGRDA, was constructed as follows: *graA* was ligated into the NdeI/BamHI sites of pET3b vector; the plasmid obtained, pGRA3, was digested with BglII/EcoRI; and the fragment containing *graA* was ligated into pGRD digested with BamHI/EcoRI to form pGRDA.

**Expression of *graA*, *graB*, *graC*, and *graD* in *E. coli* BL21 Star (DE3).** pGRA, pGRB, pGRC, pGRD, or pGRDA was transformed into *E. coli* BL21 Star (DE3). The clones were grown in a Sakaguchi flask (500 ml) containing 200 ml of LB medium supplemented with ampicillin (100  $\mu$ g/ml). After cultivation at 37°C with shaking (130 rpm) until the optical density at 600 nm (OD<sub>600</sub>) reached approximately 0.6, the cultures were placed at 15°C for at least 30 min and cultivated again at 15°C for 24 h. pGRD was transformed into *E. coli* BL21(DE3) harboring a chaperone plasmid, pG-TE2, containing *groES*, *groEL*, and *tig*. The cells were grown in a Sakaguchi flask (500 ml) containing 200 ml of LB medium supplemented with ampicillin (50  $\mu$ g/ml) and chloramphenicol (20  $\mu$ g/ml). The cells were grown at 37°C with shaking (130 rpm), and tetracycline was added to the medium (final concentration, 10 ng/ml) when the OD<sub>600</sub> reached approximately 0.3. The cultures were then placed at 15°C for at least 30 min when the OD<sub>600</sub> reached approximately 0.6 and cultivated again at 15°C for 24 h.

**Preparation of cell extracts.** After cultivation, the cells were collected by centrifugation (6,500  $\times$  g, 15 min) and washed in a 20 mM Tris-HCl buffer (pH

TABLE 2. Primer sequences used in this study

Primer	Sequence <sup>a</sup>
Primers used for genome-walking PCR	
Sense primers used for PCR and nested PCR	
pGWU1 PCR sense primer.....	5'-GCA TTC AGC GAC AGG ATC ATG GT-3'
Nested PCR sense primer.....	5'-TCC ATC AGC TTC AGG CGC GTA TC-3'
pGWU2 PCR sense primer.....	5'-CGG TCA GAA GCG GCA TGT TG-3'
Nested PCR sense primer.....	5'-TGG GAA CAA AGA CAT CAT CGA GAA CCA-3'
pGWU3 PCR sense primer.....	5'-GGC GCT GGA CCG GAA AAG AT-3'
Nested PCR sense primer.....	5'-TGT GGC GCA TGG CTC ATA TCG-3'
pGWU4 PCR sense primer.....	5'-GAT CAC CCG TGG CAC AGG C-3'
Nested PCR sense primer.....	5'-ATC GTC GTG CGG AAA TTC TGG C-3'
pGWD1 PCR sense primer.....	5'-CTC GAA GAG CAT TTC GCA ATC CC-3'
Nested PCR sense primer.....	5'-GTG CCC GGT GAT TAC TGG AAG GAA CT-3'
pGWD2 PCR sense primer.....	5'-CAG AGG CGC TTT ATG CTC GGG A-3'
Nested PCR sense primer.....	5'-GCA CTT TAC GGC GCG TGG C-3'
pGWD3 PCR sense primer.....	5'-CTT CAC CAC GCT CGT CAC GC-3'
Nested PCR sense primer.....	5'-TGC GGT TTT CGG TGT GAA GGA AAG-3'
Antisense primers used for PCR and nested PCR with pGWU1, pGWU2, pGWU3, pGWU4, pGWD1, pGWD2, and pGWD3	
PCR antisense primer.....	5'-GTA CAT ATT GTC GTT AGA ACG CGT AAT ACG ACT CA-3'
Nested PCR antisense primer.....	5'-CGT TAG AAC GCG TAA TAC GAC TCA CTA TAG GGA GA-3'
Primers used for construction of expression vectors	
<i>graA</i> sense primer.....	5'-TCA TAT GAA CGA TAT GAG CCA TGC G-3'
Antisense primer.....	5'-AGG ATC CTC AAT ATT GGC CCT TGG-3'
<i>graB</i> sense primer.....	5'-CAT ATG GAC ATG AAA ACA ACC GGT GAC GAC G-3'
Antisense primer.....	5'-GGA TCC TCA TCG GGC GAG CAC G-3'
<i>graC</i> sense primer.....	5'-CGC ATA TGC AGC CGT TCG TCT ATA C-3'
Antisense primer.....	5'-GGA TCC TCA CTC CGG CCT TG-3'
<i>graD</i> sense primer.....	5'-CCA TAT GAC ATC AGC ACT GTT TGG CCT-3'
Antisense primer.....	5'-GGA TCC TCA GGC GGA CAG GC-3'
Primers used for qRT-PCR	
<i>graF</i> sense primer.....	5'-GGT GGA AAA GCT TGA TGT GC-3'
Antisense primer.....	5'-TGG CCA AGA ATG ATG TTG AG-3'
16S rRNA sense primer.....	5'-GAT CCT GGC TCA GAA CGA AC-3'
Antisense primer.....	5'-GGC TCA TCA TAC CCC GAT AA-3'
Primer sequences used for RT-PCR	
Sense primer for all amplifications.....	5'-CTG TTT GGC CTG AAC AAT CTT GC-3'
Antisense primer for <i>graDA</i> region.....	5'-CTT CGA CAA GCT CGG CAA ATT C-3'
Antisense primer for <i>graDAF</i> region.....	5'-TGG CCA AGA ATG ATG TTG AG-3'
Antisense primer for <i>graDAFC</i> region.....	5'-CCA TAG CGT CGA GAT CTT CC-3'
Antisense primer for <i>graDAFCB</i> region.....	5'-TGC CGA AAC GAT GTA GTG C-3'
Antisense primer for <i>graDAFCBE</i> region.....	5'-AGC CAT GAC GTC CTC GAT AC-3'

<sup>a</sup> Sequences shown in boldface type are as follows: CATATG, NdeI site; GGATCC, BamHI site.

8.0). The washed cells were suspended in a 20 mM Tris-HCl buffer (pH 8.0) and disrupted with an ultrasonic disintegrator, model UD-201 (Tomy Seiko Co., Tokyo, Japan). The disruption was done in an ice bath for 3 min and repeated twice after the output and duty cycle were adjusted to 6 and 30, respectively. The cell debris was removed by centrifugation (16,000 × g, 15 min), and the supernatant solution was used as a cell extract.

**Enzyme assays.** In all enzyme assays described below, a blank assay against the enzyme reaction was taken with a cell extract of the host cells harboring the corresponding empty vector, and the value obtained in the blank assay was subtracted as the background activity. We also subtracted the blank activities of the oxidation of NADH. One unit of enzyme was defined as the amount of enzyme that transforms 1 μmol of NADH, resorcinol, hydroxyquinol, or  $\gamma$ -resorcyate per min at 30°C. The molar extinction coefficients (M<sup>-1</sup>cm<sup>-1</sup>) were as follows: 6,220 for NADH at 340 nm and 42,000 for maleylacetate at 243 nm.

The flavin reductase (EC 1.5.1.30) activity was assayed by monitoring the oxidation rate of NADH spectrophotometrically at 340 nm. The reaction mixture (final volume, 3 ml) contained 0.1 mM FAD, 0.1 mM NADH, a 0.1 M potassium phosphate buffer (pH 7.0), and the cell extract. The reaction was started by the addition of NADH and was carried out at 30°C.

The resorcinol hydroxylase (EC 1.14.13.x) activity was assayed by measuring the remaining resorcinol by high-performance liquid chromatography (HPLC), according to the modified method of Otto et al. (26). The reaction mixture (final volume, 1 ml) contained 2 mM resorcinol, 50 mM NADH, a 50 mM Tris-HCl buffer (pH 8.0), 150 mM potassium formate, 0.5 U/ml formate dehydrogenase (Roche Diagnostics), 650 U/ml catalase (Wako Chemical Co.), and the cell extract. The reaction was started by the addition of NADH and was carried out at 30°C with shaking (150 rpm). After 0, 30, 60, 120, 180, 240, or 300 min, the reactions were stopped with 1 ml of acetonitrile containing 10% (vol/vol) acetic acid. The mixture (10 μl) was then subjected to HPLC analysis.

The hydroxyquinol 1,2-dioxygenase (EC 1.13.11.37) activity was assayed by measuring the produced maleylacetate spectrophotometrically at 243 nm (9, 12, 35). The reaction mixture (final volume, 3 ml) contained 0.5 mM hydroxyquinol, a 50 mM 2-morpholinoethanesulfonate-NaOH buffer (pH 6.0), and the cell extract. The reaction was started by the addition of hydroxyquinol and was carried out at 30°C.

The maleylacetate reductase (EC 1.3.1.32) activity was assayed by measurement of the oxidation rate of NADH spectrophotometrically at 340 nm. The reaction mixture (final volume, 3 ml) contained 0.5 mM hydroxyquinol, 0.2 mM

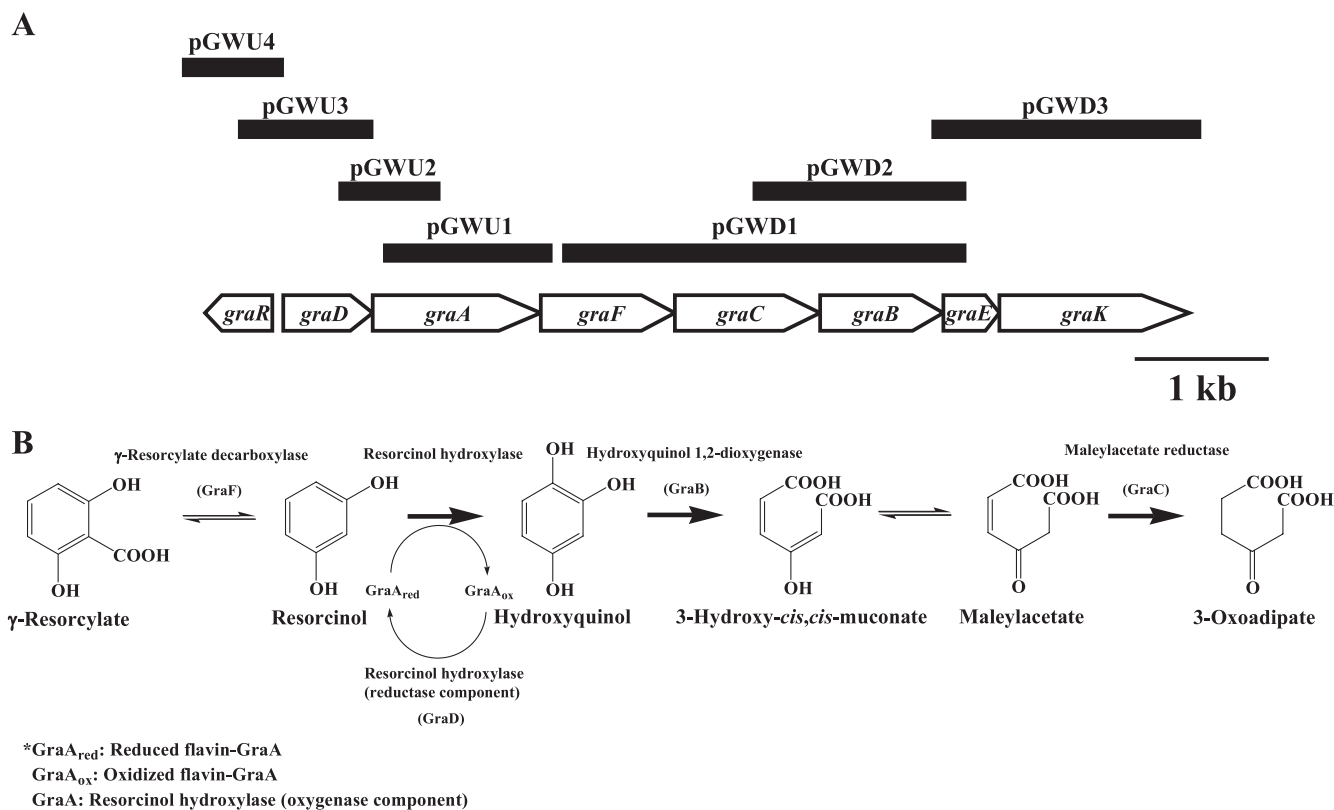


FIG. 1. Organization of the genes related to  $\gamma$ -resorcylate catabolism (A) and the  $\gamma$ -resorcylate catabolic pathway in *Rhizobium* sp. strain MTP-10005 (B).

NADH, a 50 mM 2-morpholinoethanesulfonate–NaOH buffer (pH 6.5), and the cell extract. The reaction was started by the addition of hydroxyquinol and was carried out at 30°C.

The  $\gamma$ -resorcylate decarboxylase (EC 4.1.1.x) activity was assayed according to a previously described method (38), except that the reaction time was changed to 10 min.

**qRT-PCR of *graF*.** *Rhizobium* sp. strain MTP-10005 was cultivated in an Erlenmeyer flask (100 ml) containing 20 ml of LB medium on a rotary shaker (150 rpm) at 30°C. After 21 h, 2 ml of the culture was transferred into a Sakaguchi flask (500 ml) containing 200 ml of LB medium and cultivated on a reciprocal shaker (130 rpm) at 30°C for 7 h. The cells were harvested by centrifugation (7,500  $\times$  g, 20 min), suspended in a Sakaguchi flask (500 ml) containing 200 ml of a minimal medium supplemented with 0.3% (wt/vol)  $\gamma$ -resorcylate or glycerol, and cultivated on a reciprocal shaker (130 rpm) at 30°C. After 0, 0.5, 1, 3, 6, or 9 h, 15 ml of the culture was taken from the flask and the cells were collected by centrifugation (7,500  $\times$  g, 20 min) and washed twice with a 10 mM potassium phosphate buffer (pH 7.0) containing 0.75% (wt/vol) NaCl. The washed cells were suspended in 750  $\mu$ l of a 10 mM potassium phosphate buffer (pH 7.0) containing 0.02% (wt/vol) 2-mercaptoethanol, and 500  $\mu$ l of the cell suspension obtained was subjected to an ultrasonic disintegrator, model UD-201 (Tomy Seiko Co.), at about 4°C (two times for 9 s; output, 6; duty cycle, 30). The cell debris was removed by centrifugation (16,000  $\times$  g, 15 min), and the supernatant solution was used as a cell extract. The total RNA was extracted from the remaining 250  $\mu$ l of cell suspension obtained above with the RNeasy mini kit according to the manufacturer's instructions and was treated with DNase I. qRT-PCR was performed with an Mx3000P Real-Time PCR system (Stratagene Corp., La Jolla, CA). The reaction mixture for qRT-PCR consisted of SuperScript III RT/Platinum *Taq* mix, 1 $\times$  SYBR green reaction mix, 1 mM MgSO<sub>4</sub>, 0.5  $\mu$ M ROX reference dye, 0.2  $\mu$ M sense and antisense primers, and 100 ng of total RNA. The housekeeping gene used as a control was 16S rRNA, and the mRNA expression level was normalized in such a way that the 16S rRNA expression level was equal to 1 and was averaged with three independent measurements.

**RT-PCR of the *graDAFCBE* genes.** Total RNA was extracted from *Rhizobium* sp. strain MTP-10005 cells that were cultivated as described above in a minimal

medium supplemented with  $\gamma$ -resorcylate or glycerol at 30°C for 1 h. RT-PCR was performed with an iCycler thermal cycler (Bio-Rad Laboratories, Inc.). The reaction mixture for RT-PCR consisted of a 1 $\times$  OneStep RT-PCR buffer, a 400  $\mu$ M deoxynucleoside triphosphate mix, 0.6  $\mu$ M sense and antisense primers, the OneStep RT-PCR enzyme mix, and 100 ng of total RNA.

**Analytical methods.** Spectrophotometric measurements were done with a UV/visual spectrophotometer U-3210 (Hitachi High-Technologies Corp.). The HPLC analysis was performed with HPLC system LC-10A (Shimadzu Corp., Kyoto, Japan). The column used was an Inertsil ODS-2 column (0.46 by 25 cm; GL Sciences, Inc., Tokyo, Japan), and resorcinol was detected at 280 nm. The mobile phase used was 15% (vol/vol) acetonitrile in water containing 0.1% (vol/vol) trifluoroacetic acid, and the flow rate was 0.6 ml/min. SDS-PAGE was performed by the method of Laemmli (22). The proteins in the gels were stained with Coomassie brilliant blue R-250. The protein concentration was measured by the method of Bradford (7), with bovine serum albumin (Wako Chemical Co.) used as the standard.

**Nucleotide sequence accession numbers.** The DNA sequences of *graA*, *graB*, *graC*, *graD*, *graE*, *graK*, and *graR* of *Rhizobium* sp. strain MTP-10005 were submitted to the GenBank database under accession numbers AB266210, AB266211, AB266212, AB266213, AB266214, AB266215, and AB266216, respectively.

## RESULTS AND DISCUSSION

**Cloning and sequencing of the  $\gamma$ -resorcylate catabolic gene cluster and analysis of its gene organization.** The organization of the  $\gamma$ -resorcylate catabolic gene cluster, based on genome-walking PCR, is summarized in Fig. 1A. The typical  $-35/-10$  promoter sequences were identified only upstream of the genes *graD*, *graR*, and *graK*. The distances between *graD* and *graA*, *graA* and *graF*, *graF* and *graC*, and *graC* and *graB* are 36,

TABLE 3. Characteristics of the  $\gamma$ -resorcyate catabolic gene cluster

ORF <sup>a</sup>	Gene	Length (aa)	Molecular wt	Identical protein(s)	Identity (%)	Accession no.
1	<i>graR</i>	183	20,868	Transcriptional regulator (MarR family) from <i>Azoarcus</i> sp. EbN1	26	CR555306
				Putative transcriptional regulator (MarR family) from <i>Agrobacterium tumefaciens</i> strain C58	91	AE009200
2	<i>graD</i>	179	19,429	NAD(P)H-flavin oxidoreductase from <i>Pseudomonas syringae</i> pv. tomato strain DC3000	38	AE016853
				Putative actinorhodin polyketide dimerase from <i>Agrobacterium tumefaciens</i> strain C58	94	AE009199
3	<i>graA</i>	409	43,305	<i>p</i> -Hydroxyphenylacetate hydroxylase C2, the oxygenase component from <i>Acinetobacter baumannii</i>	27	AY566612
4	<i>graF</i>	327	37,422	Putative oxidoreductase from <i>Agrobacterium tumefaciens</i> strain C58	92	AE009199
				2,3-Dihydroxybenzoate decarboxylase from <i>Aspergillus oryzae</i>	43	AP007151
5	<i>graC</i>	351	36,405	Hypothetical protein Atu2529 from <i>Agrobacterium tumefaciens</i> strain C58	96	AE009199
				Maleylacetate reductase from <i>Ralstonia eutropha</i> JMP134	46	U16782
6	<i>graB</i>	295	33,349	Putative maleylacetate reductase from <i>Agrobacterium tumefaciens</i> strain C58	90	AE009199
				Hydroxyquinol 1,2-dioxygenase from <i>Nocardioides simplex</i> 3E	46	AY822041
7	<i>graE</i>	112	12,195	Putative dioxygenase from <i>Agrobacterium tumefaciens</i> strain C58	94	AE009199
				Hypothetical protein Atu2526 from <i>Agrobacterium tumefaciens</i> strain C58	75	AE009199
8	<i>graK</i>	428	45,002	Benzoate transport protein from <i>Acinetobacter baylyi</i> ADP1	35	AF009224
				Putative MFS permease from <i>Agrobacterium tumefaciens</i> strain C58	94	AE009199

<sup>a</sup> ORF, open reading frame.

47, 3, and 39 bp, respectively. These results suggest that *graDAFCBE* forms an operon and that *graR* and *graK* may be independently transcribed. The putative Shine-Dalgarno sequences precede the start codons of *graR*, *graD*, *graA*, *graF*, *graB*, and *graK*, but such sequences do not exist in front of the start codons of *graC* and *graE*. Additionally, the start codon of *graE* overlaps the stop codon of *graB* by 4 bp, indicating that *graF* and *graC*, as well as *graB* and *graE*, are translationally coupled (19). The characteristics of the  $\gamma$ -resorcyate catabolic gene cluster are summarized in Table 3. The bioinformatics analysis suggests that *graR*, *graC*, and *graB* encode a MarR-type transcriptional regulator, a maleylacetate reductase, and a hydroxyquinol 1,2-dioxygenase, respectively. *GraK* shows similarity to a benzoate transport protein and is probably involved in the transport of  $\gamma$ -resorcyate. *GraA* shows similarity to the oxygenase component of *p*-hydroxyphenylacetate hydroxylase, suggesting that the hydroxylation of resorcinol may be catalyzed by the coupled reaction of *GraD* and *GraA*. The function of *GraE* cannot be predicted, because there are no functionally characterized homologues in the data bank.

**Expression of *graA*, *graB*, *graC*, and *graD* in *E. coli* BL21 Star (DE3).** The plasmids pGRD, pGRA, pGRC, pGRB, and pGRDA were constructed with a high-expression vector (pET14b) and *graD*, *graA*, *graC*, *graB*, and both *graD* and *graA*, respectively (Table 1), and each plasmid was introduced into *E. coli* BL21 Star (DE3) by transformation. The cell extracts of these clones were subjected to SDS-PAGE analysis (Fig. 2). Overexpressed proteins with molecular weights of approximately 22,100 (22.1K), 45.2K, 38.2K, 35.2K, and 21.2K or 43.2K were observed in *E. coli* BL21 Star (DE3) harboring pGRD, pGRA, pGRC, pGRB, and pGRDA, respectively (Fig. 2). The molecular weights of these overexpressed proteins agreed well with the expected values of the N-terminal His-tagged gene products of *graD* (21.6K), *graA* (45.5K), *graC* (38.6K), and *graB* (35.5K) and the gene product of *graA*

(43.3K). *GraD* expressed in *E. coli* BL21 Star (DE3) harboring pGRD was present mostly in the insoluble fractions. However, *GraD* was overexpressed in the soluble fractions of *E. coli* BL21(DE3) harboring pGRD when the chaperone plasmid pG-Tf2 was coexpressed (Fig. 2B). The chaperone probably helps in the correct folding of *GraD*.

**Identification of *GraA*, *GraB*, *GraC*, and *GraD*.** The enzyme activities of the expressed proteins were measured as described in Materials and Methods to clarify their functions. The experimental data of the enzyme activities are summarized in Table 4. The enzyme activities detected for *GraA*, *GraB*, *GraC*, and *GraD* agreed well with the expected functions based on the bioinformatic analysis.

Flavin reductase activity was detected when the cell extract of *E. coli* BL21 Star (DE3) harboring pGRD or *E. coli* BL21(DE3) harboring pGRD and the chaperone plasmid, pG-Tf2, were used. The specific activity for FAD was determined to be  $3.94 \pm 0.30$  U/mg for *E. coli* BL21 Star (DE3) harboring pGRD. About six-times-higher specific activity ( $24.4 \pm 1.3$  U/mg) was observed when the chaperone plasmid pG-Tf2 was coexpressed. However, the cell extract of *E. coli* BL21 Star (DE3) harboring pGRA did not show resorcinol hydroxylase activity by itself. The resorcinol hydroxylase activity was detected only when the cell extract of *E. coli* BL21(DE3) harboring pGRD and pG-Tf2 was mixed with that of *E. coli* BL21 Star (DE3) harboring pGRA. The specific activity was calculated to be  $(1.41 \pm 0.14) \times 10^{-3}$  U/mg. In addition, when *GraD* and *GraA* were coexpressed in *E. coli* BL21 Star (DE3) harboring pGRDA, resorcinol hydroxylase activity was also detected, and about 1.5-times-higher specific activity was observed ( $[2.11 \pm 0.15] \times 10^{-3}$  U/mg). Accordingly, the chaperone plasmid pG-Tf2 was effective for the expression of *GraD* in the soluble fractions of *E. coli* BL21(DE3). *GraD* and *GraA* were eluted separately when the cell extract of *E. coli* BL21 Star (DE3) harboring pGRDA was applied to a column of Ni-NTA aga-

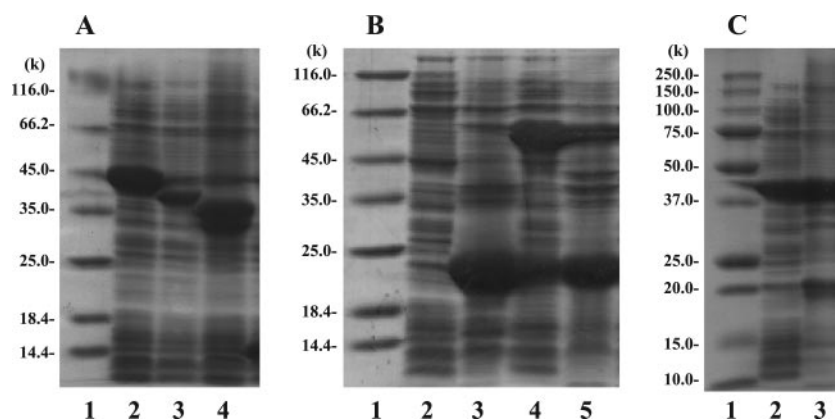


FIG. 2. SDS-PAGE of cell extracts from *E. coli* harboring  $\gamma$ -resorcyolate catabolic genes. (A) Lane 1, molecular weight marker (Fermentas International, Inc.); lane 2, cell extract of *E. coli* BL21 Star (DE3) harboring pGRA (38.1  $\mu$ g); lane 3, cell extract of *E. coli* BL21 Star (DE3) harboring pGRC (21.7  $\mu$ g); lane 4, cell extract of *E. coli* BL21 Star (DE3) harboring pGRB (39.2  $\mu$ g). (B) Lane 1, molecular weight marker (Fermentas International, Inc.); lane 2, cell extract of *E. coli* BL21 Star (DE3) harboring pGRD (16.6  $\mu$ g); lane 3, insoluble fraction of *E. coli* BL21 Star (DE3) harboring pGRD; lane 4, cell extract of *E. coli* BL21 (DE3) harboring pGRD and the chaperone plasmid pG-Tf2 (32.8  $\mu$ g); lane 5, insoluble fraction of *E. coli* BL21(DE3) harboring pGRD and the chaperone plasmid pG-Tf2. (C) Lane 1, molecular weight marker (Bio-Rad Laboratories, Inc.); lane 2, cell extract of *E. coli* BL21 Star (DE3) harboring pGRDA (24.6  $\mu$ g); lane 3, insoluble fraction of *E. coli* BL21 Star (DE3) harboring pGRDA.

rose (data not shown). These results suggest that GraD and GraA are not subunits but rather two essential components of resorcinol hydroxylase. By considering the sequence similarities (Table 3), GraA and GraD are identified as the oxygenase and reductase components of resorcinol hydroxylase, respectively. Two types of flavin-dependent aromatic hydroxylases, i.e., single-component and two-component enzymes, have been reported previously. The single-component enzyme contains one subunit with oxygenase and reductase functions, whereas the two-component enzyme has two components that catalyze the oxygenation of the substrate and transfer a reduced form of flavin to the oxygenase component separately. The single-component NADPH-dependent resorcinol hydroxylase from *Corynebacterium glutamicum* has recently been described (17), and the two-component *p*-hydroxyphenylacetate hydroxylases from *Acinetobacter baumannii* (6), *Pseudomonas aeruginosa* (6), and *E. coli* W ATCC 11105 (23) are well characterized.

The recombinant resorcinol hydroxylase from *Rhizobium* sp. strain MTP-10005 showed the enzyme activity only when both GraA (the oxygenase component) and GraD (the reductase component) existed in the assay mixture and therefore is classified as a two-component flavin-dependent aromatic hydroxylase. This is the first example of a two-component resorcinol hydroxylase. In contrast to the resorcinol hydroxylase from *C. glutamicum*, the reductase component of the resorcinol hydroxylase from *Rhizobium* sp. strain MTP-10005 requires NADH as a coenzyme. The sequence similarities between *C. glutamicum* resorcinol hydroxylase and GraA or GraD are 2.9 and 4.5%, respectively.

Hydroxyquinol 1,2-dioxygenase activity was detected in the cell extract of *E. coli* BL21 Star (DE3) harboring pGRB (specific activity,  $0.602 \pm 0.016$  U/mg). Accordingly, GraB is identified as a hydroxyquinol 1,2-dioxygenase. In the hydroxyquinol 1,2-dioxygenase reaction, 3-hydroxy-*cis,cis*-muconate produced

TABLE 4. Experimental data for measurement of enzyme activities<sup>a</sup>

Enzyme <sup>b</sup>	Change in value <sup>c</sup> /min	Total activity (U)	Total protein (mg)	Sp act (U/mg)
Flavin reductase				
GraD*	$0.271 \pm 0.021$	$65.4 \pm 5.0$	$16.6 \pm 0.1$	$3.94 \pm 0.30$
(GraD + chaperone)†	$0.332 \pm 0.018$	$801 \pm 44$	$32.8 \pm 0.2$	$24.4 \pm 1.3$
Resorcinol hydroxylase				
GraA*	0	0	$68.3 \pm 0.4$	0
(GraD + chaperone)† + GraA*	$1,390 \pm 140$	$(9.63 \pm 0.94) \times 10^{-2}$	$(58.6 \pm 0.3) + (68.3 \pm 0.4)$	$(1.41 \pm 0.14) \times 10^{-3}$
GraDA*	$2,960 \pm 220$	$0.103 \pm 0.008$	$48.9 \pm 0.1$	$(2.11 \pm 0.15) \times 10^{-3}$
Hydroxyquinol 1,2-dioxygenase				
GraB*	$0.165 \pm 0.004$	$23.6 \pm 0.6$	$39.2 \pm 0.6$	$0.602 \pm 0.016$
Maleylacetate reductase				
GraC*	$0.304 \pm 0.005$	$293 \pm 5$	$21.7 \pm 0.5$	$13.5 \pm 0.2$

<sup>a</sup> Cell extract was used for all enzyme assays. Values are means  $\pm$  standard deviations.

<sup>b</sup> Symbols: \*, expressed in *E. coli* BL21 Star (DE3); †, GroES + GroEL + Tig, expressed in *E. coli* BL21 (DE3).

<sup>c</sup> Units: flavin reductase,  $\Delta A_{340}$ /min; resorcinol hydroxylase, change in peak area of resorcinol per min; hydroxyquinol 1,2-dioxygenase,  $\Delta A_{243}$ /min; maleylacetate reductase,  $\Delta A_{340}$ /min. Hydroxyquinol and resorcinol were eluted at retention times of 5.06 and 10.1 min, respectively.

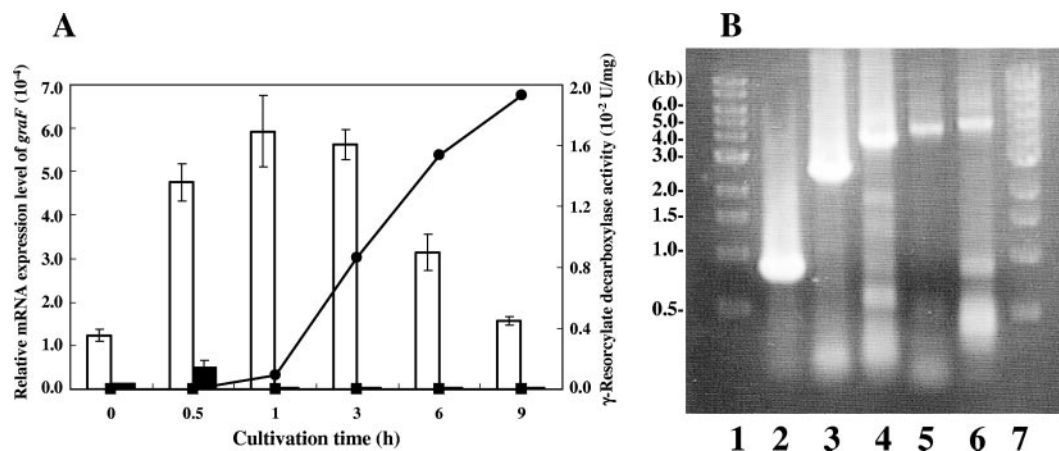


FIG. 3. qRT-PCR and RT-PCR analyses. (A) Relative mRNA expression levels of *graF* and  $\gamma$ -resorcylate decarboxylase activity versus cultivation time. White bars show the relative mRNA expression levels of *graF* in total RNA prepared from *Rhizobium* sp. strain MTP-10005 cells cultivated in a minimal medium supplemented with 0.3% (wt/vol)  $\gamma$ -resorcylate. Black bars show the relative mRNA expression levels of *graF* in the total RNA prepared from *Rhizobium* sp. strain MTP-10005 cells cultivated in a minimal medium supplemented with 0.3% (wt/vol) glycerol. ●,  $\gamma$ -resorcylate decarboxylase activity of cell extract prepared from *Rhizobium* sp. strain MTP-10005 cells cultivated in a minimal medium supplemented with 0.3% (wt/vol)  $\gamma$ -resorcylate; ■,  $\gamma$ -resorcylate decarboxylase activity of cell extract prepared from *Rhizobium* sp. strain MTP-10005 cells cultivated in a minimal medium supplemented with 0.3% (wt/vol) glycerol. The temperature profile used for qRT-PCR was as follows: RT step (one cycle, 50°C, 30 min), initial PCR activation step (one cycle, 95°C, 15 min), PCR step (50 cycles, 94°C, 15 s; 55°C for 30 s; 72°C for 1 min), final annealing step (one cycle, 55°C, 30 s), and final denaturation step (one cycle, 95°C, 30 s). The sequence-specific standard curves were described with 1 ng, 10 pg, 100 fg, and 1 fg of pTGF or pTR16. (B) RT-PCR analysis of the *graDAFCBE* operon. Lanes 1 and 7, 1-kb DNA ladder; lane 2, internal 796 bp of *graDA* region; lane 3, internal 2,494 bp of *graDAF* region; lane 4, internal 3,780 bp of *graDAFC* region; lane 5, internal 4,616 bp of *graDAFCB* region; lane 6, internal 5,041 bp of *graDAFCBE* region. The temperature profile used for RT-PCR was as follows: RT step (one cycle, 45°C, 30 min), initial PCR activation step (one cycle, 95°C for 15 min), PCR step (forty cycles, 94°C, 10 s; 55°C, 30 s; 68°C, 5 min), and final extension step (one cycle, 68°C, 10 min).

from hydroxyquinol is spontaneously and reversibly converted to its tautomer, maleylacetate. The three-dimensional structure of the hydroxyquinol 1,2-dioxygenase from *Nocardioideis simplex* 3E has been solved, and the essential amino acid residues for the enzyme activity have been determined (13). Tyr164, Tyr197, His221, and His223 form a polyhedron structure with Fe(III) that is essential for the catalytic activity, and a similar structure has been observed for the catechol 1,2-dioxygenase from *Acinetobacter baylyi* ADP1 (37). These amino acid residues are well conserved as Tyr165, Tyr200, His224, and His226 in the primary structure of the hydroxyquinol 1,2-dioxygenase from *Rhizobium* sp. strain MTP-10005 and could be the probable iron ligands.

Maleylacetate reductase activity was detected in the cell extract of *E. coli* BL21 Star (DE3) harboring pGRC in the presence of the cell extract of *E. coli* BL21 Star (DE3) harboring pGRB and hydroxyquinol to supply maleylacetate as a substrate. The specific activity was determined to be  $13.5 \pm 0.2$  U/mg. Accordingly, GraC is identified as a maleylacetate reductase. We found that Cys242 in the primary structure of the maleylacetate reductase from *Rhizobium* sp. strain MTP-10005 is conserved in the enzyme from *Ralstonia eutropha* JMP134. The *R. eutropha* JMP134 enzyme was inhibited by *p*-chloromercuribenzoate and stabilized by dithiothreitol (31). Accordingly, Cys242 is probably necessary for the catalytic activity of the maleylacetate reductase from *Rhizobium* sp. strain MTP-10005, too. Although the maleylacetate reductase gene from *Ralstonia eutropha* 335T was expressed in *E. coli* (32) and the enzymological characteristics of the *R. eutropha* JMP134 enzyme were well characterized (31, 36), the three-dimensional structures of the enzymes have not been determined.

Based on these enzymological studies, the  $\gamma$ -resorcylate catabolic pathway of *Rhizobium* sp. strain MTP-10005 is proposed in Fig. 1B.

**Regulation of the *graDAFCBE* operon.** The MarR-type transcriptional regulator homolog gene *graR* (Table 3) exists upstream of the *graDAFCBE* genes (Fig. 1A). The MarR transcriptional regulator from *E. coli* negatively regulates the expression of the antibiotic resistance genes, *marAB* (1, 4, 10, 24, 33). Inducers of these genes, such as 2-hydroxybenzoate and 2,4-dinitrophenol, have been shown to bind to the *marAB* operator region and allow transcription of the *marAB* operon. The relative mRNA expression level of *graF* was measured by qRT-PCR in the presence and absence of  $\gamma$ -resorcylate (Fig. 3A). The total RNA from cells grown on  $\gamma$ -resorcylate or glycerol was extracted and used. The relative mRNA expression level of *graF* increased significantly in the total RNA of the cells when  $\gamma$ -resorcylate was used as the sole carbon growth source, and the maximum relative value ( $[5.93 \pm 0.82] \times 10^{-4}$ ) was detected after 1 h of cultivation. Moreover,  $\gamma$ -resorcylate decarboxylase activity was detected after 1 h of cultivation ( $[8.97 \pm 0.08] \times 10^{-4}$  U/mg) and increased with the cultivation time up to 9 h (Fig. 3A). The results of RT-PCR against *graDAFCBE* genes showed that the amplified fragments with molecular sizes of approximately 796, 2,494, 3,780, 4,616, and 5,041 bp agree well with the expected values of those for *graDA* (790 bp), *graDAF* (2,453 bp), *graDAFC* (3,677 bp), *graDAFCB* (4,687 bp), and *graDAFCBE* (5,082 bp) (Fig. 3B), respectively, and that the *graDAFCBE* genes are transcribed as a single mRNA and the transcription of the gene cluster is induced in the presence of  $\gamma$ -resorcylate. These results suggest that the *graDAFCBE* genes are expressed as an operon for the growth

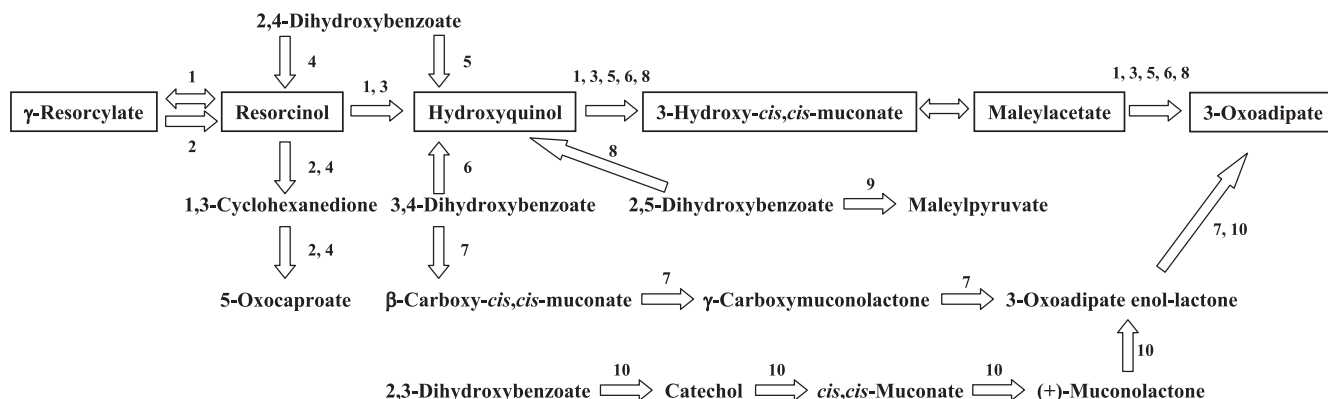


FIG. 4. Various dihydroxybenzoate catabolic pathways in microorganisms. 1,  $\gamma$ -resorcylylate pathway of *Rhizobium* sp. strain MTP-10005; 2,  $\gamma$ -resorcylylate pathway of *Clostridium* sp. KN 245 (21); 3, resorcinol pathway of *Corynebacterium glutamicum* (17), *Pseudomonas putida* (9), and *Trichosporon cutaneum* (14); 4, 2,4-dihydroxybenzoate pathway of *Clostridium* sp. KN 245 (21); 5, 2,4-dihydroxybenzoate pathway of *Pseudomonas* sp. BN9 (34) and *Sphingomonas* sp. strain RW1 (5); 6, 3,4-dihydroxybenzoate pathway of *Trichosporon cutaneum* (2); 7, 3,4-dihydroxybenzoate 3,4-cleavage pathway of *Agrobacterium* sp. (27, 28), *Bradyrhizobium* sp. (27), *Rhizobium* sp. (27), *Roseobacter* sp. (8), and *Streptomyces* sp. strain 2065 (19); 8, 2,5-dihydroxybenzoate pathway of *Trichosporon cutaneum* (2); 9, 2,5-dihydroxybenzoate 1,2-cleavage pathway of *Pseudomonas acidovorans* (16) and *Pseudomonas testosteroni* (16); 10, 2,3-dihydroxybenzoate pathway of *Aspergillus niger* (20) and *Trichosporon cutaneum* (2, 3).

of *Rhizobium* sp. strain MTP-10005 on  $\gamma$ -resorcylylate and are probably regulated by GraR at the transcriptional level. The homologous benzoate transport gene of GraK (Table 3) existed downstream of the *graDAFCBE* operon (Fig. 1A). BenK, the benzoate transport protein that is involved in the benzoate degradation of *Acinetobacter baylyi* ADP1, is induced by a benzoate metabolite, *cis,cis*-muconate, and transports benzoate and benzaldehyde into the cytoplasm (11). Judging from the sequence similarity, GraK is probably involved in transporting  $\gamma$ -resorcylylate.

**A novel pathway for  $\gamma$ -resorcylylate catabolism in *Rhizobium* sp. strain MTP-10005.** The biodegradation of various dihydroxybenzoates has been reported only in microorganisms, and their pathways are summarized in Fig. 4. The  $\gamma$ -resorcylylate pathway has been reported previously only for an anaerobic bacterium, *Clostridium* sp. KN 245 (21), in which  $\gamma$ -resorcylylate is converted into 5-oxocaproate via resorcinol and 1,3-cyclohexanedione (Fig. 4, pathway 2), and is quite different from the  $\gamma$ -resorcylylate pathway of the aerobic bacterium *Rhizobium* sp. strain MTP-10005 (Fig. 4, pathway 1). The present study is the first report of the  $\gamma$ -resorcylylate catabolic pathway in an aerobic bacterium. In the  $\gamma$ -resorcylylate pathway of *Rhizobium* sp. strain MTP-10005 (Fig. 4, pathway 1), the oxygenase component of the resorcinol hydroxylase requires an oxygen molecule to produce hydroxyquinol, whereas in that of *Clostridium* sp. KN 245 (Fig. 4, pathway 2), resorcinol produced from  $\gamma$ -resorcylylate is converted to 1,3-cyclohexanedione by the resorcinol reductase without the participation of oxygen. Accordingly, we can assume that these  $\gamma$ -resorcylylate catabolic pathways have evolved independently in bacteria adapted to oxic and anoxic environments. We also investigated whether the  $\gamma$ -resorcylylate catabolic pathway exists in microorganisms whose genomes have been sequenced. The results of BLAST analyses indicated that homologous genes for the  $\gamma$ -resorcylylate catabolic pathway of *Rhizobium* sp. strain MTP-10005 are present only in the genome of *Agrobacterium tumefaciens* strain C58 (Table 3). The genes are clustered in a manner similar to that of the *graDAFCBEK* genes and are likely to be functional, since

$\gamma$ -resorcylylate decarboxylase was purified from the other strain of *A. tumefaciens* (39).

We are currently studying the enzymological properties of GraD, GraA, GraC, and GraB in detail and trying to clarify the function of GraE.

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