

Genetic Characterization of the Resorcinol Catabolic Pathway in *Corynebacterium glutamicum*[∇]

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Corynebacterium glutamicum grew on resorcinol as a sole source of carbon and energy. By genome-wide data mining, two gene clusters, designated NCgl1110-NCgl1113 and NCgl2950-NCgl2953, were proposed to encode putative proteins involved in resorcinol catabolism. Deletion of the NCgl2950-NCgl2953 gene cluster did not result in any observable phenotype changes. Disruption and complementation of each gene at NCgl1110-NCgl1113, NCgl2951, and NCgl2952 indicated that these genes were involved in resorcinol degradation. Expression of NCgl1112, NCgl1113, and NCgl2951 in *Escherichia coli* revealed that NCgl1113 and NCgl2951 both coded for hydroxyquinol 1,2-dioxygenases and NCgl1112 coded for maleylacetate reductases. NCgl1111 encoded a putative monooxygenase, but this putative hydroxylase was very different from previously functionally identified hydroxylases. Cloning and expression of NCgl1111 in *E. coli* revealed that NCgl1111 encoded a resorcinol hydroxylase that needs NADPH as a cofactor. *E. coli* cells containing Ncgl1111 and Ncgl1113 sequentially converted resorcinol into maleylacetate. NCgl1110 and NCgl2950 both encoded putative TetR family repressors, but only NCgl1110 was transcribed and functional. NCgl2953 encoded a putative transporter, but disruption of this gene did not affect resorcinol degradation by *C. glutamicum*. The function of NCgl2953 remains unclear.

Various resorcinol compounds are produced in nature as secondary plant products (4). Early studies indicated that resorcinol was degraded via three different pathways in bacteria: In *Azotobacter vinelandii*, resorcinol was converted into pyrogallol, and subsequently the aromatic ring was cleaved by a pyrogallol 1,2-dioxygenase (9). *Pseudomonas putida* apparently adopted two different pathways: Resorcinol was converted into hydroxyquinol, and hydroxyquinol was subsequently degraded by (i) 2,3,5-trihydroxytoluene 1,2-dioxygenase (*meta* cleavage) (3) and (ii) hydroxyquinol 1,2-dioxygenase (*ortho* cleavage) (4). For all three degradative pathways, degradation of resorcinol was initiated by hydroxylation, although the hydroxylation happened at different positions of the aromatic ring: C-2 for *A. vinelandii* and C-4 or C-6 for *P. putida*. Two resorcinol hydroxylases from *P. putida* were purified, but they were not characterized at the genetic level (16, 17). Evidence supporting the conversion of resorcinol into pyrogallol by resorcinol-induced cells of *A. vinelandii* was obtained, but attempts to detect resorcinol 2-hydroxylase activity failed (9). To the best of our knowledge, no amino acid sequence of any resorcinol hydroxylase has been reported.

Not only is hydroxyquinol involved in resorcinol degradation; it is also the key intermediate during microbial degradation of a range of aromatic compounds, such as chlorophenol (15), 2,4,6-trichlorophenol (14), dibenzo-*p*-dioxin (1), 4-aminophenol (27), and 2-aminobenzoate (24). Consequently, the degradation of hydroxyquinol and its derivatives is of importance for

the understanding of microbial processes that govern the metabolism of aromatic compounds and for the understanding of the geobiochemical cycling of aromatic compounds. Recently, advances have been made in the understanding of aromatic compound degradation by *Corynebacterium glutamicum* (7, 25, 26). Here we describe the genetic characterization of the resorcinol catabolic pathway in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown aerobically on a rotary shaker (150 rpm) at 37°C in Luria-Bertani (LB) broth or on an LB plate with 1.5% (wt/vol) agar. *C. glutamicum* was routinely grown at 30°C in LB or in mineral salts medium (MM) (31), which was adjusted to pH 8.4 and supplemented with 0.05 g of yeast extract liter⁻¹ on a rotary shaker (150 rpm). Aromatic compounds were added at final concentrations of 2 mM (sterilized by filtration through 0.2- μ m-pore-size filters) when they served as carbon and energy sources. Cellular growth was monitored by measuring the turbidity at 600 nm. For generation of mutants and maintenance of *C. glutamicum*, BHIS (brain heart broth with 0.5 M sorbitol) medium was used. When needed, antibiotics were used at the following concentrations: kanamycin, 50 μ g ml⁻¹ for *E. coli* and 25 μ g ml⁻¹ for *C. glutamicum*; chloramphenicol, 20 μ g ml⁻¹ for *E. coli* and 10 μ g ml⁻¹ for *C. glutamicum*; ampicillin, 100 μ g ml⁻¹ for *E. coli*.

Analysis of sequence data. The genome sequence of *C. glutamicum* ATCC 13032 (accession no. NC 003450) was retrieved from GenBank. Sequence comparisons and database searches were carried out using BLAST programs at the BLAST server of the NCBI website.

DNA extraction and manipulation. The total genomic DNA of *C. glutamicum* was isolated according to the procedure of Tauch et al. (28). Plasmid isolation, DNA manipulation, and agarose gel electrophoresis were carried out as described by Sambrook et al. (21). DNA restriction enzymes, ligase, and DNA polymerase were used as recommended by the manufacturer. Restricted DNA fragments were separated by agarose gel electrophoresis and were purified by using the agarose gel DNA fragment recovery kit (TIANGEN, Beijing, China).

Amplification of DNA fragments and construction of plasmids. Target DNA fragments were PCR amplified by using *Pyrobest* DNA polymerase (TaKaRa) or *Taq* DNA polymerase (Promega, Madison, Wis.). PCR products were purified

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics ^a	Source, reference, or note
Strains		
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i> [F' <i>traD36 proAB lacZΔM15</i>]	Stratagene (catalog no. 200235)
BL21(DE3)	<i>hsdS gal (λclt857 ind-1 Sam7 nin-5 lacUV5-T7 gene 1)</i>	Novagen (catalog no. 69387-3)
<i>C. glutamicum</i>		
RES167	Restriction-deficient mutant of ATCC 13032; $\Delta(cglIM-cglIR-cglIIR)$	University of Bielefeld
RES167 Δ NCgl1110	A fragment of DNA coding for amino acids 105 to 215 of NCgl1110 was deleted	This study
RES167 Δ NCgl1111	A fragment of DNA coding for amino acids 123 to 375 of NCgl1111 was deleted	This study
RES167 Δ NCgl1112	A fragment of DNA coding for amino acids 114 to 222 of NCgl1112 was deleted	This study
RES167 Δ NCgl1113	A fragment of DNA coding for amino acids 122 to 180 of NCgl1113 was deleted	This study
RES167 Δ NCgl(2950-2953)	A fragment of DNA coding for amino acids 170 to 1288 of NCgl(2950-2953) was deleted	This study
RES167 Δ NCgl(2950-2953) Δ NCgl1112	Fragments of DNA coding for amino acids 114 to 222 of NCgl1112 and 170 to 1288 of NCgl(2950-2953) were deleted	This study
RES167 Δ NCgl(2950-2953) Δ NCgl1113	Fragments of DNA coding for amino acids 122 to 180 of NCgl1113 and 170 to 1288 of NCgl(2950-2953) were deleted	This study
Plasmids		
pK18 <i>mobsacB</i>	Mobilizable vector, allows for selection of double crossover in <i>C. glutamicum</i>	Schäfer et al. (22)
pK18 <i>mobsacB</i> - Δ NCgl1110	Carrying NCgl1110 deletion (see RES167 Δ NCgl1110)	This study
pK18 <i>mobsacB</i> - Δ NCgl1111	Carrying NCgl1111 deletion (see RES167 Δ NCgl1111)	This study
pK18 <i>mobsacB</i> - Δ NCgl1112	Carrying NCgl1112 deletion (see RES167 Δ NCgl1112)	This study
pK18 <i>mobsacB</i> - Δ NCgl1113	Carrying NCgl1113 deletion (see RES167 Δ NCgl1113)	This study
pK18 <i>mobsacB</i> - Δ NCgl(2950-2953)	Carrying NCgl(2950-2953) deletion [see RES167 Δ NCgl(2950-2953)]	This study
pXMJ19	Shuttle vector (Cam ^r <i>Ptac lacP³ pBL1 oriV_{C. glutamicum} pK18 oriV_{E. coli}</i>)	Jakoby et al. (11)
pXMJ19-NCgl1110	Carrying NCgl1110 (to generate complementation for NCgl1110)	This study
pXMJ19-NCgl1111	Carrying NCgl1111 (to generate complementation for NCgl1111)	This study
pXMJ19-NCgl1112	Carrying NCgl1112 (to generate complementation for NCgl1112)	This study
pXMJ19-NCgl1113	Carrying NCgl1113 (to generate complementation for NCgl1113)	This study
pXMJ19-NCgl2951	Carrying NCgl2951 (to generate complementation for NCgl2951)	This study
pXMJ19-NCgl2952	Carrying NCgl2952 (to generate complementation for NCgl2952)	This study
pET28a	Expression vector with N-terminal hexahistidine affinity tag	Novagen
pET28a-NCgl1111	pET28a derivative for expression of NCgl1111	
pET28a-NCgl1112	pET28a derivative for expression of NCgl1112	This work
pGEM-T Easy	Cloning of PCR products	Promega
Primers		
1110F(a)	<u>GAT</u> <u>TCTAGAAAAGGAGGAC</u> ACATATGCCACGCCTTCG (XbaI)	To generate pK18 <i>mobsacB</i> - Δ NCgl1110 and pXMJ19-NCgl1110
1110R	AGCAAGCTT <u>GAGCTCCCTGAGTCTGGTGCTT</u> (HindIII, SacI)	
1111F(a)	<u>GAT</u> <u>TCTAGAAAAGGAGGACC</u> ATATGTCACCAATAACTTCG (XbaI, NdeI)	To generate pK18 <i>mobsacB</i> - Δ NCgl1111, pXMJ19-NCgl1111, and pET28a-NCgl1111
1111R	AGCAAGCTT <u>CCCGGGCCTCAC</u> TGAACCGAAC (HindIII, SmaI)	
1112F(a)	<u>GAT</u> <u>TCTAGAAAAGGAGGACA</u> ACCATGTCTTTACAGTTCGAT (XbaI)	To construct pK18 <i>mobsacB</i> - Δ NCgl1112 and pXMJ19-NCgl1112
1112R	ACAGAATTCACACGAGCGTTTATACC (HindIII, SacI)	
1112EF	<u>GTCGCTAGCATGTCTTTACAGTTCGATC</u> (NheI)	
1112ER	AGCAAGCTT <u>GAAATCCGTAGTTC</u> ACTTCTTCT (HindIII, SacI)	
1113a	<u>GAT</u> <u>TCTAGAAAAGGAGGACA</u> ACCATGACTATTTACAGCACAA (XbaI)	To construct pK18 <i>mobsacB</i> - Δ NCgl1113, pXMJ19-NCgl1113, and RT-PCR
1113b	<u>GGTATTCGCCGTTTCTGT</u> CATCTCCGTTTTG	
1113c	GAAAACGGCGAATACC	
1113d	AGCAAGCTT <u>GAGCTCTCTCAA</u> ATTGCCAAAA (HindIII, SacI)	
2950-2953a	<u>GAT</u> <u>TCTAGAAAATGCCCTCGTTG</u> AAAA (XbaI)	To generate pK18 <i>mobsacB</i> - Δ NCgl(2950-2953)
2950-2953b	AGCAAGCTT <u>GTCGCACCCCTGTC</u> TAAA (HindIII)	
2950-2953c	<u>TTAGGACAGGGGTGCGGAC</u> AAGCTTATCCTGGTCCGTAGAACTGC (HindIII)	
2950-2953d	AGCGAATTCACAGGGCTAAATGCTCCAA (EcoRI)	
1110b	AATCCGCCCAAATCC	To perform RT-PCR on the fragments of the related genes
1111b	AATGATGGCGGAGTTTT	
1112b	AACCATTTGAGACCAGA	
2950a	<u>GAT</u> <u>TCTAGAAAAGGAGGAC</u> ACATATGAATCTGAAAGATCTC (XbaI, NdeI)	
2950b	TCATTGAAAAGCCACTC	
2951a	<u>GAT</u> <u>TCTAGAAAAGGAGGAC</u> ACATATGACAACCACCACCG (XbaI, NdeI)	
2951b	TTCCTTCAATCCACGC	
2952a	<u>GAT</u> <u>TCTAGAAAAGGAGGAC</u> ACATATGAACAACTACTCGCATT (XbaI, NdeI)	
2952b	GAGCACCTTCAGATCAACA	

^a Restriction enzyme sites are underlined. Ribosome binding sites are boldfaced. Nucleotides that are complementary to primer 2950-2953b are italicized.

using an agarose gel DNA fragment recovery kit. PCR fragments were cloned using the pGEM-T Easy vector system (Promega, Madison, Wis.).

Various plasmids (Table 1) were constructed with pK18*mobsacB* (22), pXMJ19 (11), or pET28a. The primers used are listed in Table 1. Genes on plasmids were deleted either by gene splicing by overlap extension (10)

[NCgl1113 and NCgl(2950–2953)] or by deletions of regions of each gene (NCgl1110, NCgl1111, or NCgl1112) through restriction enzyme digestion (see Fig. 1B). The disrupted genes were fused into pK18*mobsacB* to generate plasmids pK18*mobsacB* Δ NCgl1110, pK18*mobsacB* Δ NCgl1111, pK18*mobsacB* Δ NCgl1112, pK18*mobsacB* Δ NCgl1113, and pK18*mobsacB* Δ NCgl(2950–2953). For complemen-

tation, plasmids pXMJ19-NCgl1110, pXMJ19-NCgl1111, pXMJ19-NCgl1112, pXMJ19-NCgl1113, pXMJ19-NCgl2951, and pXMJ19-NCgl2952 were created by insertion of each PCR-amplified intact gene into pXMJ19 (11). Plasmids for expression of the target genes in *E. coli* were constructed from each PCR-amplified gene and pET28a.

Genetic disruption and complementation in *C. glutamicum*. The pK18*mobsacB* derivatives for gene disruption and the pXMJ19 derivatives for complementation described above were transformed into *C. glutamicum* RES167 and its mutants by electroporation (29). Screening for the first and second recombination events, as well as confirmation of the chromosomal deletion, was performed as described by Schäfer et al. (22). The resulting strains were designated *C. glutamicum* RES167 Δ NCgl1110 to RES167 Δ NCgl1113 and RES167 Δ NCgl(2950–2953) (Table 1). The deletions of the target genes in pK18*mobsacB* derivatives and in *C. glutamicum* mutants were verified by PCR and DNA sequencing. The expression of each gene in *C. glutamicum* was induced by addition of 0.6 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to the culture broth.

Heterologous expression of genes in *E. coli*, preparation of cellular lysate, and enzymatic activity assays. Plasmids (pET28a-NCgl1111 and pET28a-NCgl1112, described above) were electroporated into *E. coli* BL21(DE3). Synthesis of recombinant proteins was initiated by addition of 0.6 mM IPTG, and cultivation was continued for an additional 3 h. Cells were harvested by centrifugation at 10,000 \times g, washed twice with ice-cold 50 mM Tris-HCl buffer (pH 8.0), resuspended in the same buffer, and disrupted by sonification in an ice-water bath. After centrifugation at 20,000 \times g for 30 min at 4°C, the clear supernatant was used for enzymatic assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. All enzymatic assays were performed in 50 mM Tris-HCl buffer (pH 8.0). Resorcinol hydroxylase activity was measured spectrophotometrically by measuring the decrease in absorption at 340 nm due to oxidation of NADPH and was calculated with an extinction coefficient of 6,220 M⁻¹ · cm⁻¹ (17). Maleylacetate reductase activities were determined by monitoring NADH/NADPH oxidation as described by Schlömann et al. (23). Hydroxyquinol 1,2-dioxygenase activity was measured by determination of the increase in absorbance at 243 nm that resulted from the formation of maleylacetate, according to the method of Travkin et al. (30). An extinction coefficient of 4,440 M⁻¹ · cm⁻¹ was used for the calculation of enzymatic activity.

Conversion of resorcinol into maleylacetate via hydroxyquinol. *E. coli* BL21(DE3)/pET28-NCgl1111 and *E. coli* BL21(DE3)/pET28-NCgl1113 were cultivated and induced as described above. Cells were harvested by centrifugation, washed twice with Tris-HCl (50 mM; pH 8.0), and suspended in the same buffer at a final optical density at 600 nm of 2.5. Resorcinol at a final concentration of 2 mM was added to the cell suspensions of *E. coli* BL21(DE3)/pET28-NCgl1111 and *E. coli* BL21(DE3)/pET28-NCgl1113, and the mixtures were incubated at 30°C. After 0.5, 1, 2, and 3 h, samples (500 μ l each time) were collected, acidified to pH 2.0, extracted with ethyl acetate, and finally dried by N₂ blowing. In parallel, cell suspensions of *E. coli* BL21(DE3)/pET28a and *E. coli* BL21(DE3)/pET28a-NCgl1113 were used for this conversion (as negative controls).

Operation of MS. The extracted samples described above were subjected to mass spectrometry (MS) analysis. Mass spectrometry was performed on an electrospray ionization ion trap mass spectrometer (LCQ Deca XPPlus; ThermoFinnigan, San Jose, Calif.) operated in negative-ion mode. Typical parameters were as follows: temperature for ion transfer tube, 160°C; spray voltage, 3.3 kV. The samples were dissolved in methanol. For elucidation of the structure of the product, tandem MS (MS/MS) was performed with 35% normalized collision energy.

SDS-PAGE. SDS-PAGE was conducted with a 5% stacking gel and a 12% resolving gel and was run in a Mini-PROTEIN II electrophoresis cell (Bio-Rad) according to the manufacturer's instructions. After electrophoresis, the protein bands were visualized by Coomassie brilliant blue staining. Protein concentrations were determined according to the method of Bradford (2), with bovine serum albumin as the standard.

Enzymatic preparation of maleylacetate. Maleylacetate, the substrate for maleylacetate reductase, was freshly prepared by cleavage of 2 ml of 10 mM hydroxyquinol with hydroxyquinol 1,2-dioxygenase (10 μ l) in 50 mM Tris-HCl buffer (pH 6.3) and was monitored by determination of UV absorption at 243 nm. After the conversion was completed (~2 min), the pH of the buffer was adjusted to 8.0. This preparation was used as a substrate without further purification.

RT-PCR. *C. glutamicum* was cultivated either with MM broth containing resorcinol or with LB broth. Total RNA was isolated with a TRNzol extraction kit (TIANGEN, Beijing, China). The RNA preparation was first treated with DNase I and then used as a template for reverse transcription with Moloney murine leukemia virus reverse transcriptase (TIANGEN, Beijing, China). The

reverse transcription-PCR (RT-PCR) products (cDNAs) were used to amplify approximately 350- to 500-bp fragments of target genes. The primers used in RT-PCR are listed in Table 1. In order to exclude the possibility that residual DNA was amplified, negative controls were run in parallel, except that the Moloney murine leukemia virus reverse transcriptase was omitted from the reaction mixtures.

RESULTS AND DISCUSSION

Genome data-mining and bioinformatic analyses. By use of the hydroxyquinol 1,2-dioxygenase of *Arthrobacter chlorophenolicus* (15) and BLAST searches, the NCgl1113 and NCgl2951 genes, encoding putative hydroxyquinol 1,2-dioxygenases, were identified from the sequenced genome of *C. glutamicum* (Fig. 1). NCgl1113 and NCgl2951, encoding hydroxyquinol 1,2-dioxygenases, had been confirmed previously in recombinant *E. coli* (25), and their in vivo involvement in resorcinol assimilation was confirmed by gene disruption and complementation in this study (Fig. 2D). Analyses of their neighbor genes led to the discovery that two sets of genes at positions 1212.4 to 1216.8 (NCgl1110-NCgl1113) and 3255.7 to 3260.0 (NCgl2950-NCgl2953) were possibly involved in resorcinol degradation (Fig. 1).

The NCgl1111 gene encodes a resorcinol hydroxylase. The mutant RES167 Δ NCgl1111 lost the ability to grow on resorcinol (Fig. 2B), but its ability to grow on hydroxyquinol was the same as that of the wild-type strain (data not shown). A reasonable explanation for these phenotypes was that the NCgl1111 gene was involved in the conversion of resorcinol into hydroxyquinol through hydroxylation. When the mutant RES167 Δ NCgl1111 was complemented with the NCgl1111 gene, the ability to grow on resorcinol was partially restored (Fig. 2B). RT-PCR showed that NCgl1111 was transcribed in wild-type RES167 when resorcinol was provided in the culture medium (Fig. 2F, lane 2).

The translational product of the NCgl1111 gene showed only limited identity to the putative monooxygenase and hydroxylase (Table 2). In order to understand its biochemical properties, NCgl1111 was cloned into *E. coli* BL21(DE3)/pET28a. The recombinant *E. coli* cells actively synthesized a protein with a molecular mass of ca. 63 kDa. The cellular lysate of the recombinant *E. coli* cells showed resorcinol hydroxylase activity (Fig. 3), and this activity depended solely on the presence of NADPH. In addition to the enzyme activity, the cellular conversion of resorcinol also supported the hypothesis that NCgl1111 encodes a hydroxylase (Ncgl1111) that is involved in the conversion of resorcinol to hydroxyquinol. The recombinant cells of *E. coli* BL21(DE3)/pET28a-NCgl1111 converted resorcinol into hydroxyquinol. When Ncgl1111 was coupled with a hydroxyquinol 1,2-dioxygenase [Ncgl1113, encoded by NCgl1113 and expressed in *E. coli* BL21(DE3)/pET28a-NCgl1113] (25), resorcinol was converted to a product that was identified as maleylacetate by MS and MS/MS (data not shown).

Ncgl1111 is the first resorcinol hydroxylase that has been characterized at the genetic level. Although a similar resorcinol hydroxylase (68 kDa) had been purified from *P. putida* previously, neither the DNA sequence nor the amino acid sequence of this resorcinol hydroxylase was disclosed (17). Attempts to find an Ncgl1111 homolog in the *P. putida* genome failed, suggesting that the *P. putida* resorcinol hydroxy-

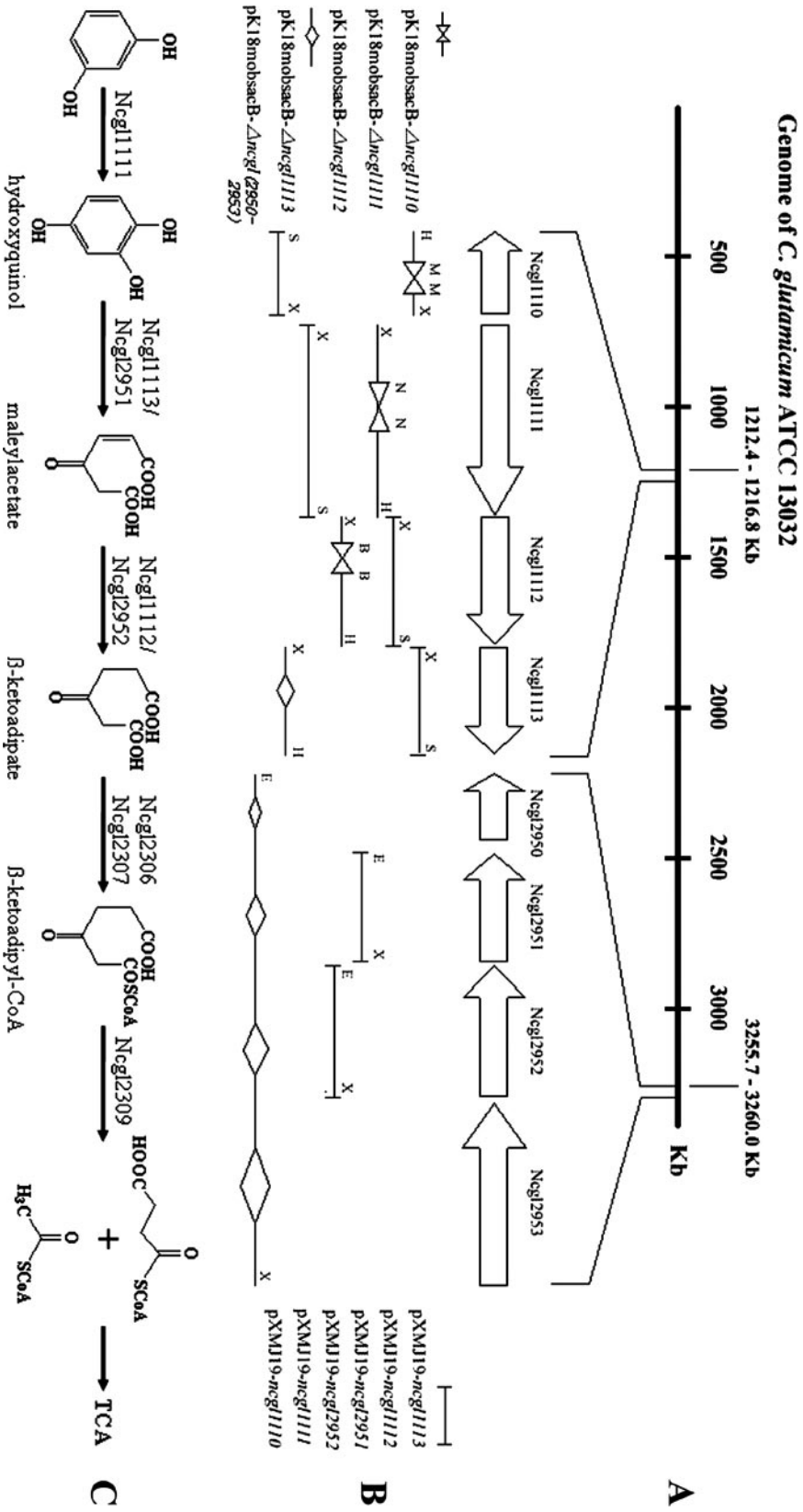


FIG. 1. Physical map and genetic organization (A) of the resorcinol pathway (○) in *C. glutamicum* and construction of plasmids for gene disruption and complementation (B). Restriction sites are abbreviated as follows: X, XbaI; H, HindIII; E, EcoRI; S, SacI; B, BamI. Right and left triangles with points touching represent deletion by restriction enzyme digestion; wide diamonds represent deletion by gene splicing by overlap extension.

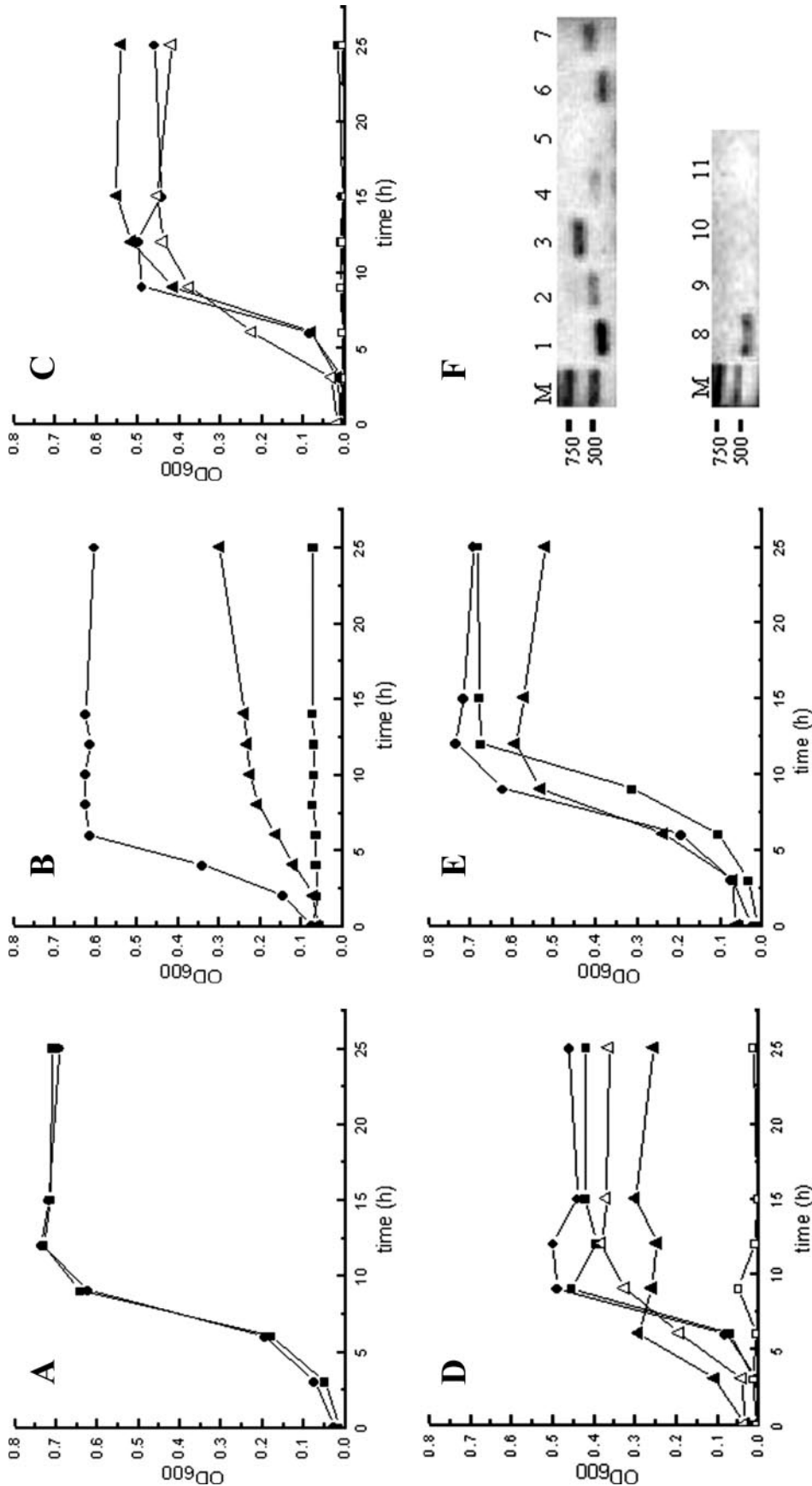


FIG. 2. (A through E) Growth curves of various *C. glutamicum* mutants cultivated with resorcinol; (F) transcriptional analysis by RT-PCR of gene expression during growth in MM broth with resorcinol or in LB broth. According to genome data analysis, NCgl1110 and NCgl2952 encode putative TetR family regulators; NCgl1111 encodes a putative hydroxylase; NCgl1112 and NCgl2952 encode putative maleylacetate reductases; NCgl1113 and NCgl2951 encode putative hydroxyquinol 1,2-dioxygenases; and NCgl2953 encodes a putative transporter. Symbols: ●, RES167; (A) ■, RES167ΔNCgl(2950–2953); (B) ■, RES167ΔNCgl1111; ▲, RES167ΔNCgl1111/pXMJ19-NCgl1111; (C) ■, RES167ΔNCgl1112; □, RES167ΔNCgl(2950–2953)/ΔNCgl1112; ▲, RES167ΔNCgl(2950–2953)/ΔNCgl1112/pXMJ19-NCgl1112; △, RES167ΔNCgl(2950–2953)/ΔNCgl1112/pXMJ19-NCgl1112/ΔNCgl1113; □, RES167ΔNCgl(2950–2953)/ΔNCgl1113; ▲, RES167ΔNCgl(2950–2953)/ΔNCgl1113/pXMJ19-NCgl1113; △, RES167ΔNCgl(2950–2953)/ΔNCgl1113/pXMJ19-NCgl1113; (E) ■, RES167ΔNCgl1110; ▲, RES167ΔNCgl1110/pXMJ19-NCgl1110; (F) Strain RES167 was grown in MM with resorcinol (lanes 1 to 7) or in LB broth (lanes 8 to 11). Total RNAs extracted were used as templates for RT-PCR to detect the transcription of each gene as follows: lane 1, NCgl1110 (420 bp); lane 2, NCgl1111 (464 bp); lane 3, NCgl1112 (550 bp); lane 4, NCgl1113 (410 bp); lane 5, NCgl2950 (no corresponding fragment); lane 6, NCgl2951 (430 bp); lane 7, NCgl2952 (464 bp); lane 8, NCgl1110; lane 9, NCgl1112; lane 10, NCgl1113; lane 11, NCgl2952.

TABLE 2. Blast search results and identified functions of genes involved in resorcinol catabolism with *Corynebacterium glutamicum*

Gene	Identified function of gene product	Sequence identity ^a	Related gene product (source, accession no.)
NCgl1110	Transcriptional regulator	70/218 (32) 75/232 (32)	Putative TetR family transcriptional regulator (<i>Streptomyces avermitilis</i> , BAC68276.1) Putative TetR family transcriptional regulator (<i>Streptomyces avermitilis</i> , BAC68295.1)
NCgl1111	Resorcinol hydroxylase	210/534 (39)	Putative FAD-dependent monooxygenase, geldanamycin biosynthesis gene cluster (<i>Streptomyces hygroscopicus</i> , AAO06914.1) (19)
NCgl1112	Maleylacetate reductase	163/548 (29) 257/355 (72) 189/351 (53)	3-(3-Hydroxyphenyl) propionate hydroxylases (<i>E. coli</i> , CAA70747.1) (8) Putative maleylacetate reductase (<i>Arthrobacter chlorophenolicus</i> , AAO46999.1) (15) Maleylacetate reductase (<i>Pimelobacter simplex</i> , ABA61848.1) (41)
NCgl1113	Hydroxyquinol 1,2-dioxygenase	216/290 (74) 204/290 (70)	Hydroxyquinol 1,2-dioxygenase (<i>Arthrobacter chlorophenolicus</i> , AAN08758.1) (15) Hydroxyquinol 1,2-dioxygenase (<i>Arthrobacter chlorophenolicus</i> , AAN08760.1) (15)
NCgl2950	Transcriptional regulator	125/185 (67) 52/180 (28)	Putative transcription regulator (<i>Corynebacterium efficiens</i> , BAC19720.1) Putative TetR family transcriptional regulator (<i>Streptomyces avermitilis</i> , BAC72413.1)
NCgl2951	Hydroxyquinol 1,2-dioxygenase	220/294 (74) 209/299 (69)	Hydroxyquinol 1,2-dioxygenase (<i>Arthrobacter chlorophenolicus</i> , AAN08760.1) (15) Hydroxyquinol 1,2-dioxygenase (<i>Arthrobacter chlorophenolicus</i> , AAN08758.1) (15)
NCgl2952	Maleylacetate reductase	251/354 (70) 197/351 (56)	Putative maleylacetate reductase (<i>Arthrobacter chlorophenolicus</i> , AAN08761.1) Maleylacetate reductase (<i>Pimelobacter simplex</i> , ABA61848.1) (30)
NCgl2953	Unknown	268/480 (55) 187/468 (39)	Metabolic transport protein (<i>Corynebacterium glutamicum</i> , CAF18749.1) Putative sugar permease gene (<i>Lactobacillus casei</i> , AAF74348.1)

^a Expressed as number of identical amino acids/number of amino acids in the related gene product (percent).

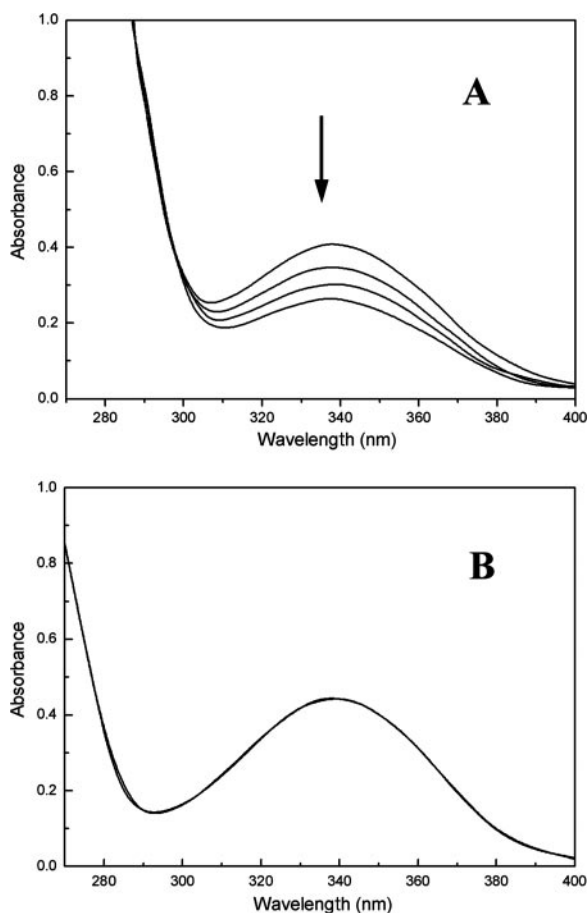


FIG. 3. (A) Resorcinol hydroxylase activity in recombinant *E. coli* pET28a-NCgl1111. (B) *E. coli*/pET28a was used as a negative control. The absorbances of the reaction mixture at wavelengths of 270 to 400 nm were recorded at time zero (top line) and at 60, 120, and 180 s (bottom line). Hydroxylase activity was also determined with 3-hydroxybenzoate, 3-aminophenol, 3,5-dihydroxytoluene, and 2,4-dihydroxybenzoate, but no activity was detected.

lase might not be homologous to the *C. glutamicum* resorcinol hydroxylase. Moreover, the *C. glutamicum* NCgl1111 did not show significant identities to previously identified 4-hydroxybenzoate 3-hydroxylases and 3-hydroxybenzoate 6-hydroxylases, which catalyze hydroxylation at the *ortho* and *para* positions, respectively. Since NCgl111 is the first functionally identified hydroxylase that takes resorcinol as a substrate, we propose that it represents a new type of hydroxylase that is involved in aromatic compound catabolism.

Both the NCgl1112 gene and the NCgl2952 gene code for maleylacetate reductases. Disruption of NCgl1112 resulted in the mutant RES167 Δ NCgl1112, which was not able to grow on resorcinol (Fig. 2C). The mutant RES167 Δ (NCgl2950-NCgl2953), in which the NCgl2952 gene was deleted, did not show disturbed assimilation of resorcinol. The double mutant RES167 Δ (NCgl2950-NCgl2953)/ Δ NCgl1112 was created and was used for complementation with NCgl1112 or NCgl2952. The results indicated that either NCgl1112 or NCgl2952 restored the ability to grow on resorcinol (Fig. 2C). Based on BLAST results showing high identities of NCgl1112 and NCgl2952 to maleylacetate reductases that had been functionally identified previously (Table 2), both NCgl1112 and NCgl2952 were assumed to code for maleylacetate reductases. When NCgl1112 was heterologously expressed in *E. coli*, the cellular lysate showed significant maleylacetate reductase activity. The results also indicated that the maleylacetate reductase preferred NADH (0.072 U/mg) to NADPH (0.047 U/mg) as a cofactor. It was also found that both NCgl1112 and NCgl2952 were transcribed in wild-type RES167 when resorcinol was provided in the culture broth (Fig. 2F, lanes 3 and 7).

NCgl1110, but not NCgl2950, codes for a negative transcriptional regulator. According to the BLAST search results, both NCgl1110 and NCgl2950 encode putative TetR family transcriptional regulators. To determine whether the two genes were functional, NCgl1110 and NCgl2950 were individually disrupted. The resulting mutants, RES Δ NCgl1110 and RES Δ NCgl(2950-2953), were not phenotypically affected when resorcinol was provided as a source of carbon and energy. However, when these mutants were cultivated in LB

broth, significant hydroxyquinol 1,2-dioxygenase activity (0.305 U/mg) was observed for the mutant RES Δ NCgl1110 but not for the mutant RES Δ NCgl(2950–2953) or wild type RES167. When the NCgl1110 gene was introduced (complementation) into the mutant RES167 Δ NCgl1110, hydroxyquinol 1,2-dioxygenase activities disappeared. RT-PCR indicated that only NCgl1110 was transcribed (Fig. 2F, lane 1), and NCgl2950 was not transcribed (Fig. 2F, lane 5), when RES167 was cultivated with resorcinol. Similarly, when RES167 was cultivated in LB broth, only NCgl1110 was transcribed (Fig. 2F, lane 8). These results clearly demonstrate that Ncgl1110 functioned as a repressor and that its repression was neutralized by resorcinol induction. The function of Ncgl2950 was not clear.

The TetR family regulators are usually transcriptional repressors for the biosynthesis of antibiotics, efflux pumps, and osmotic stress (18). There are 15 TetR family members on the genome of *C. glutamicum*, and 3 were functionally identified (12, 13, 20). But none of the three functioned in aromatic degradation. Our results confirmed that Ncgl1110 was a transcriptional repressor of resorcinol degradation in *C. glutamicum*. Previously, a TetR family regulator, CymR, was determined to be involved in the regulation of *p*-cymene (*cym*) and *p*-cumate (*cmt*) degradation in *P. putida* (6). As far as we know, Ncgl1110 is the second example of a TetR family regulator that is involved in the regulation of aromatic compound catabolism.

The NCgl2953 gene is not necessary for resorcinol assimilation. According to the results of sequence analysis and BLAST searches, the NCgl2953 gene encodes a protein that is possibly involved in membrane transportation of metabolic substrates or products, and we had deduced that it encoded a resorcinol or hydroxyquinol transporter. However, genetic disruption of the NCgl2953 gene or the whole NCgl2950-NCgl2953 genetic cluster did not affect aromatic compound assimilation. The mutants RES167 Δ NCgl2953 and RES167 Δ NCgl(2950–2953) grew as well as wild-type RES167 did, and this excluded the necessity of NCgl2953 for resorcinol assimilation. The function of this gene needs more investigation.

The two genetic clusters NCgl2950-NCgl2953 and NCgl1110-NCgl1113 function differently for resorcinol assimilation. As shown above, all the genes at the NCgl1110-NCgl1113 genetic cluster were involved in resorcinol assimilation, but the genes at the NCgl2950-NCgl2953 genetic cluster were different: the mutant RES167 Δ NCgl(2950–2953), in which in the NCgl2950-NCgl2953 genetic cluster was deleted, grew as well as the wild-type strain, RES167, when resorcinol was provided (Fig. 2A), indicating that this genetic cluster was not necessary for resorcinol assimilation. Interestingly, the RT-PCR results showed that NCgl1112 and NCgl1113 at the NCgl1110-NCgl1113 gene cluster, and NCgl2951 and NCgl2952 at the NCgl2950-NCgl2953 gene cluster, were all transcribed when *C. glutamicum* was cultivated with resorcinol. Apparently, the NCgl2951 and NCgl2952 genes, coding for hydroxyquinol 1,2-dioxygenase and maleylacetate reductase, respectively, were redundant to the NCgl1113 and NCgl1112 genes. Such catabolic redundancy has also been found for *A. chlorophenolicus* (15) and *Pimelobacter simplex* (30). One reasonable explanation for this redundancy is that some genes are expressed only under certain conditions: In *Burkholderia xenovorans*, there are three benzoate-catabolic pathways, and the benzoyl coenzyme

A pathways are preferentially active under reduced oxygen tension (5).

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