Characterization of Catechol Catabolic Genes from *Rhodococcus erythropolis* 1CP

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The biochemical characterization of the muconate and the chloromuconate cycloisomerases of the chlorophenol-utilizing *Rhodococcus erythropolis* strain 1CP previously indicated that efficient chloromuconate conversion among the gram-positive bacteria might have evolved independently of that among gram-negative bacteria. Based on sequences of the N terminus and of tryptic peptides of the muconate cycloisomerase, a fragment of the corresponding gene has now been amplified and used as a probe for the cloning of catechol catabolic genes from *R. erythropolis*. The clone thus obtained expressed catechol 1,2-dioxygenase, muconate cycloisomerase, and muconolactone isomerase activities. Sequencing of the insert on the recombinant plasmid pRER1 revealed that the genes are transcribed in the order *catA catB catC*. Open reading frames downstream of *catC* may have a function in carbohydrate metabolism. The predicted protein sequence of the catechol 1,2-dioxygenase was identical to the one from *Arthrobacter* sp. strain mA3 in 59% of the positions. The chlorocatechol 1,2-dioxygenases and the chloromuconate cycloisomerases of gram-negative bacteria appear to be more closely related to the catechol 1,2-dioxygenases and muconate cycloisomerases of the gram-positive strains than to the corresponding enzymes of gram-negative bacteria.

In aerobic degradation of aromatic compounds by bacteria, catechol plays a key role, since it is a ring cleavage substrate in which an exceptionally large number of peripheral pathways converge (57). It can be further catabolized to intermediates of the central metabolism either following *meta* cleavage adjacent to the hydroxyl groups or following *ortho* cleavage between them. On the latter metabolic route, catechol is initially converted to 3-oxoadipate by the action of four enzymes (Fig. 1): catechol 1,2-dioxygenase (EC 1.13.11.1), muconate cycloisomerase (EC 5.5.1.1), muconolactone isomerase (EC 5.3.3.4), and 3-oxoadipate enol-lactone hydrolase (EC 3.1.1.24).

In the degradation of chlorinated aromatic compounds, chlorocatechols occupy a position corresponding to that of catechol in the catabolism of nonhalogenated compounds. The chlorocatechols are converted to 3-oxoadipate also by the action of four enzymes (Fig. 1). Three of them, i.e., chlorocatechol 1,2-dioxygenase (EC 1.13.11.-), chloromuconate cycloisomerase (EC 5.5.1.7), and dienelactone hydrolase (EC 3.1.1.45), catalyze reactions which have a counterpart in catechol catabolism but which require (at least) an altered substrate specificity (7, 53). The fourth enzyme, maleylacetate reductase (EC 1.3.1.32), compensates for the different oxidation states and yields 3-oxoadipate by simple reduction of maleylacetate or by two subsequent reductive reactions when acting on 2-halomaleylacetates (30, 64).

The metabolic pathways for catechol and chlorocatechol degradation outlined above on the one hand are relatively complex, suggesting that quite a number of genetic events were necessary for their evolution. On the other hand, the existence of chemical necessities and the limited availability of recruitable biochemical modules constrained the choices for evolution to generate these pathways. This raises the question (57) whether they are the result of divergent evolution from a single

origin or whether the constraints on the evolution led to several independent occurrences of these pathways by convergent evolution.

For catechol catabolism, sequencing of the corresponding *cat* genes from several gram-negative strains has shown that isofunctional genes in these bacteria are clearly homologous (1, 10, 26, 32, 43, 44, 55). Similarly, the catechol 1,2-dioxygenase of *Arthrobacter* sp. strain mA3, the only gram-positive strain for which sequence information for *cat* genes is available thus far, was shown to be identical to the corresponding enzymes from gram-negative strains in 27 to 31% of the residues (9). Thus, the catechol branch of the 3-oxoadipate pathway seems to have evolved from a common origin in all bacteria in which it has been genetically analyzed until now.

The chlorocatechol catabolic enzymes encoded by plasmids of gram-negative strains, such as pJP4, pAC27, or pP51, likewise appear to have a common origin (51). Sequencing of the corresponding gene clusters from these plasmids showed that (i) the chlorocatechol dioxygenases and chloromuconate cycloisomerases are homologous to the respective catechol pathway enzymes and (ii) the chlorocatechol catabolic enzymes show more sequence similarity with each other than with the respective catechol degradative enzymes (15, 48, 62). The catabolic genes of many other strains were shown by hybridization to be related to those of pJP4, pAC27, or pP51 (e.g., see references 2, 16, 29, and 60). In other cases, no hybridization of the respective chlorocatechol genes was observed under the hybridization conditions employed (2, 16, 25, 29), possibly indicating, but not yet convincingly proving, the existence of a separate line of chlorocatechol pathways in the respective bacteria.

During recent investigations of the muconate and the chloromuconate cycloisomerases of the chlorophenol-degrading strain *Rhodococcus erythropolis* 1CP (18), biochemical evidence which suggests that the chlorocatechol pathway present in this strain diverged from catechol catabolism independently of chlorocatechol pathways of gram-negative bacteria was obtained (56). Both cycloisomerases, in contrast to their coun-

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FIG. 1. Degradative pathways for catechol, 4-chlorocatechol, and 3,5-dichlorocatechol. Enzyme names as well as their designations as gene products as previously assigned for gram-negative or gram-positive bacteria are given. Enzymes with a specific function in chlorocatechol catabolism are indicated by heavy arrows, and those involved in catechol degradation are indicated by light arrows.

terparts in gram-negative strains, strongly discriminated between the two possible directions of 2-chloro-*cis,cis*-muconate cycloisomerization by forming only (+)-5-chloromuconolactone, the product of 3,6-cycloisomerization. Also in contrast to their counterparts in gram-negative strains, both *R. erythropolis* cycloisomerases were unable to convert (+)-2-chloromuconolactone, which can be formed by muconate cycloisomerases of gram-negative bacteria from 2-chloro-*cis,cis*-muconate by 1,4-cycloisomerization (65). If the differences in the ability to discriminate between cycloisomerization directions and to convert 2-chloromuconolactone indicate a basic divergence of cycloisomerases, then the altered substrate specificity allowing efficient turnover of 3-chloro- and 2,4-dichloro-*cis,cis*-muconate should have evolved at least twice independently, at least once among the gram-negative bacteria and at least once among the gram-positive bacteria. Such a functionally convergent evolution could provide a basis for new insight into the adaptations necessary for productive chlorocatechol conversion, and it would have to be taken into consideration in ecological investigations concerning the occurrence and diversity of chlorocatechol pathways in nature. A proof for the hypothesis that chlorocatechol catabolism in *R. erythropolis* 1CP diverged from catechol catabolism independently of chlorocatechol pathways of gram-negative strains can be provided only by sequence information on both the catechol and the chlorocatechol gene clusters of this strain or its relatives. In the present paper, we report on the cloning and characterization of part of the catechol gene cluster from *R. erythropolis* 1CP.

(Some of the results presented here have recently been reported in preliminary communications [13, 14].)

MATERIALS AND METHODS

Strains, plasmids, and cultivation conditions. *R. erythropolis* 1CP has previously been isolated with 2,4-dichlorophenol as a carbon source (18). Among other substrates, it also utilizes 4-chlorophenol, phenol, and benzoate. *Escherichia coli* DH5 α was obtained from GIBCO BRL. The plasmids used in this study are summarized in Table 1.

R. erythropolis 1CP cells for the purification of catechol 1,2-dioxygenase were grown in a 10-liter fermentor at 30°C in a mineral medium (8) with 50 mM phosphate buffer (pH 7) and with five portions of 5 mM sodium benzoate. Cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at -20°C. Biomass for the purification of muconate cycloisomerase and muconolactone isomerase was available from a previous cultivation (56).

Usually, *E. coli* was grown aerobically with constant shaking at 37°C in Luria-Bertani (LB) medium or TB medium (59), if appropriate, with 100 μ g of ampicillin per ml. For induction of *E. coli* DH5 α harboring pRER1, 0.4 ml of overnight LB precultures was used to inoculate 200 ml of LB medium (incubation at 37°C). After induction with 0.4 mM IPTG (isopropylthio- β -D-galactoside) at an optical density at 600 nm of 1, incubation was further continued for 3 h at 30°C.

Preparation of cell extracts. Frozen cells of *R. erythropolis* 1CP were thawed on ice. MnSO₄ was added to a final concentration of 2 mM. The cells were disrupted by being ground with glass beads in a Bachofen Dyno-Mill type KDL homogenizer (80 ml of cell suspension with 120 ml of 0.3-mm-diameter glass beads, 6 min at 0 to 8°C). The extracts were washed from the glass beads twice with 50 mM Tris-HCl (pH 7.5)-2 mM MnSO₄. Cell debris and unbroken cells were removed by centrifugation (30 min, 130,000 $\times$ g, 4°C). The supernatant solutions were passed through 0.22- μ m-pore-size filters and were used as crude cell extracts for the enzyme purifications. *E. coli* cells were harvested by centrifugation and resuspended in 0.01 volume of 50 mM Tris-HCl (pH 7.5)-1 mM MnSO₄. Disruption was performed by passage through a precooled Aminco French pressure cell (115 MPa), and cell debris was removed by centrifugation as described above.

Enzyme assays and estimation of protein concentration. The activities of catechol 1,2-dioxygenase and muconate cycloisomerase were measured spectrophotometrically at 260 nm, and those of muconolactone isomerase and 3-oxoadipate enol-lactone hydrolase were measured spectrophotometrically at 230 nm

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics ^a	Reference or source
pBluescript II SK (+)	Phagemid derived from pUC19; P _{lac} <i>lacZ'</i> Ap ^r ; f1(+) origin; ColE1 origin	Stratagene
pBluescript II KS (+)	As pBluescript II SK (+), but multiple cloning site in opposite orientation	Stratagene
pRERT1	Ap ^r ; 1-kb PCR product of primers N80.50 and N-NT in <i>EcoRV</i> site of pBluescript II KS (+)	This study
pRERT2	Ap ^r ; 0.85-kb PCR product of primers N50.19 and N-NT in <i>EcoRV</i> site of pBluescript II SK (+)	This study
pRER1	Ap ^r ; 4-kb <i>Bam</i> HI fragment of <i>R. erythropolis</i> 1CP DNA in pBluescript II KS (+)	This study
pRER11	Ap ^r ; 0.9 kb from pRER17 on <i>Pst</i> I- <i>Sac</i> II fragment in pBluescript II KS (+)	This study
pRER12	Ap ^r ; 0.45 kb from pRER17 on <i>Pst</i> I- <i>Sac</i> II fragment in pBluescript II KS (+)	This study
pRER13	Ap ^r ; 0.28 kb from pRER1 on <i>Pst</i> I fragment in pBluescript II KS (+)	This study
pRER14	Ap ^r ; 0.18 kb from pRER1 on <i>Pst</i> I fragment in pBluescript II KS (+)	This study
pRER17	Ap ^r ; 1.4 kb from pRER1 on <i>Pst</i> I fragment in pBluescript II KS (+)	This study

^a Ap^r, plasmid carries information for ampicillin resistance. Fragment sizes are given as determined by agarose gel electrophoresis.

as described by Schlömann et al. (52). The assay mixtures for muconolactone isomerase and 3-oxoadipate enol-lactone hydrolase contained 0.67 mM racemic muconolactone (prepared by the method of Elvidge et al. [11]) and in addition partially purified preparations of 3-oxoadipate enol-lactone hydrolase or muconolactone isomerase, respectively (45, 46). *cis,cis*-Muconic acid for the assays was kindly provided by J. E. Adamus (Celgene Corp., Warren, N.J.). The following extinction coefficients were used: for the formation or consumption of *cis,cis*-muconate, $16,800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (7); for the conversion of muconolactone to 3-oxoadipate, usually $1,430 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $2,050 \text{ M}^{-1} \cdot \text{cm}^{-1}$, if preparations of auxiliary enzymes contained muconate cycloisomerase (45, 46).

Protein concentrations during purification and expression experiments were determined by the method of Bradford (5) with bovine serum albumin as the standard. The determination of the protein concentration for a tryptic digest of muconate cycloisomerase was carried out by the more accurate Scopes (54) procedure.

Protein purifications. Muconate cycloisomerase was purified as described by Solyanikova et al. (56). Cell extract (volume, 320 ml; protein, 592 mg; total activity, 182 U; specific activity, 0.31 U/mg) was incubated at 60°C for 20 min, centrifuged, and then chromatographed first on a Pharmacia Q Sepharose Fast Flow XK 50/20 column (bed volume, 200 ml) and subsequently on a Pharmacia Phenyl Superose HR 10/10 column. This resulted in a preparation (volume, 14 ml; protein, 2.31 mg; total activity, 27.9 U) with a specific activity of 12.1 U/mg (39-fold purification; 15% yield). The fraction with the highest activity was used for tryptic digestion (2.25 U/ml; 197 µg of protein/ml).

For the purification of muconolactone isomerase, cell extract (volume, 190 ml; protein, 160 mg; total activity, 195 U; specific activity, 1.2 U/mg) was chromatographed on a Q Sepharose HP column (HR 16/10; bed volume, 20 ml) with 50 mM Tris-HCl (pH 7.5) and a linear NaCl gradient (0 to 0.5 M over 300 ml) for elution. The most active fractions (eluting at ca. 0.3 M NaCl) were pooled (volume, 15 ml; protein, 12.7 mg; total activity, 207 U). Proteins were precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 70% saturation, and the preparation was stored at 4°C for 3 days. After centrifugation and dissolution of the resulting pellet in 1.5 ml of 50 mM Tris-HCl (pH 7.5), $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of ca. 2 M and the preparation was centrifuged again. The supernatant was loaded onto a Phenyl Superose HR 10/10 column from which proteins were eluted by a decreasing gradient of 2 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in 50 mM Tris-HCl (pH 7.5) over 100 ml [activity peak at ca. 0.67 M $(\text{NH}_4)_2\text{SO}_4$]. The combined fractions (volume, 3 ml; protein, 0.63 mg; total activity, 64 U; specific activity, 102 U/mg) were desalted by using Pharmacia PD-10 columns and were subjected to chromatography on Pharmacia Mono Q (HR 5/5) by using 50 mM Tris-HCl (pH 7.5) and an NaCl gradient (0 to 0.25 M over 10 ml, 0.25 to 0.45 M over 60 ml, and 0.45

to 1 M over 14 ml) for elution. The fractions with the highest level of activity were pooled (volume, 2 ml; protein, 0.48 mg; total activity, 56 U), desalted, and rechromatographed on Mono Q (NaCl gradient: 0 to 0.16 M over 10 ml, 0.16 to 0.35 M over 70 ml, and 0.35 to 0.5 M over 10 ml). The most active fractions (activity peak at ca. 0.24 M NaCl) resulted in a preparation (volume, 3 ml; protein, 0.26 mg; total activity, 40 U) with a specific activity of 154 U/mg, equivalent to a 128-fold purification.

Purification of catechol 1,2-dioxygenase was achieved by following a scheme different from that previously published by Maltseva et al. (38) for this enzyme. Cell extract (volume, 170 ml; protein, 612 mg; total activity, 192 U; specific activity, 0.31 U/mg) was chromatographed on a Q Sepharose HP column (HR 16/10; bed volume, 20 ml) with 50 mM Tris-HCl (pH 7.5) and an increasing NaCl gradient (0 to 0.15 M over 60 ml, 0.15 to 0.5 M over 350 ml, and 0.5 to 1 M over 60 ml) for elution. Dioxygenase-containing fractions (eluting at about 0.27 to 0.3 M NaCl) were pooled (volume, 20 ml; protein, 38.6 mg; total activity, 77.4 U; specific activity, 2.0 U/mg). After addition of $(\text{NH}_4)_2\text{SO}_4$ to a 1.4 M final concentration, the preparation was filtered and subjected to Phenyl Superose HR 10/10 chromatography. Elution was achieved with a decreasing gradient of 1.4 to 0.56 M $(\text{NH}_4)_2\text{SO}_4$ over 85 ml and 0.56 to 0 M over 15 ml. The fraction with the highest activity, eluting at 0.80 to 0.85 M $(\text{NH}_4)_2\text{SO}_4$ (volume, 2 ml; protein, 1.19 mg; total activity, 28.2 U; specific activity, 23.7 U/mg), was then loaded onto a Pharmacia Superdex 200 Prep-Grade gel filtration column (HiLoad 16/60; bed volume, 120 ml). Proteins were eluted with 50 mM Tris-HCl (pH 7.5) plus 0.15 M NaCl, which resulted in a preparation (volume, 1 ml; protein, 0.20 mg; total activity, 5.64 U) with a specific activity of 28.2 U/mg, equivalent to a 91-fold purification.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out to determine the purity of enzyme preparations. It was performed by a modified Laemmli (34) procedure on self-casted, 0.5-mm-thick, 7-cm-long vertical slab gels with 10% (wt/vol) acrylamide in the separation gel. The gels were stained by a modification of the method of Merrill et al. (41) with a Bio-Rad silver-staining kit.

Protein cleavage, isolation of peptides, and sequencing of peptides and N termini. Trypsin digestion of muconate cycloisomerase and subsequent separation of tryptic peptides by reversed-phase high-pressure liquid chromatography (HPLC) were performed as described by Stone et al. (58). The amount of muconate cycloisomerase after precipitation, washing, and drying was 61 µg. The protein was digested for 24 h at 37°C with 2.4 µg of HPLC-pure trypsin from Sigma (dissolved in 0.01% trifluoroacetic acid). Peptides were separated by reversed-phase HPLC using a Nucleosil (Grom, Herrenberg, Germany) C₁₈ reversed-phase column (particle size, 5 µm; length, 25 cm; internal diameter, 4

TABLE 2. Isolated tryptic peptides and the N terminus of muconate cycloisomerase with deduced oligonucleotides for PCR

Peptide (elution time in min) or N terminus	Amino acid sequence ^a	Sequence of deduced oligonucleotide	Name of primer
40.14	YVP		
45.37	TWTR		
50.19	<u>EVVAIAK</u>	TTBGCATBGCSACSACYTC	N50.19
70.69	AAVDVALHDAW		
80.50	<u>YADGQVFLPE</u> GPG	GAAVACYTGVCCTCBGCGTA	N80.50
82.84	LVEGGVELIEQPTPGEQLEVLAEINR	CCBGGSGTBGGYTGYTCGAT	N82.84
N terminus ^b	TDLSIVSVETITLDVPLVRP	ATCGTBGARACSACSATCYT	N-NT

^a Segments used for construction of oligonucleotides are underlined.

^b Information from Solyanikova et al. (56).

FIG. 2. Restriction map of the insert of pRER1 carrying 3,945 bp of *R. erythropolis* 1CP DNA (white bar) in the multiple cloning site of pBluescript II KS (+) (grey bar). The arrangement of some of the smaller subclones was evident only after DNA sequencing. The inserts of subclones hybridizing to the insert of pRERT1 (black bars) and the location and orientation of open reading frames (bottom) are indicated. ORF, open reading frame.

mm), an aqueous solution of 0.06% (vol/vol) trifluoroacetic acid as solvent A, 0.052% (vol/vol) trifluoroacetic acid and 80% acetonitrile in water (vol/vol) as solvent B, and the following gradient: 0 to 60 min, 2 to 37% solvent B; 60 to 90 min, 37 to 75% solvent B; and 90 to 120 min, 75 to 98% solvent B (flow rate, 0.5 ml/min; detection at 210 nm). The column effluent during detection of distinct peaks was collected (100 to 300 μ l, containing about 1 nmol of the respective peptide).

Cyanogen bromide cleavage of purified catechol 1,2-dioxygenase was performed as described by Matsudaira (40). After concentration of the preparation to 0.6 mg/ml, an aliquot of 100 μ l was desalted by repeated dilution with water and subsequent ultrafiltration. The protein was dried in a vacuum centrifuge and then solubilized in 100 μ l of 70% formic acid. Two crystals of CNBr were added and dissolved by gentle vortexing. The tube was flushed with nitrogen and capped. The reaction mixture was incubated in the dark at room temperature for 18 h. The digest was quenched by evaporating the formic acid under a vacuum in the presence of NaOH pellets for 15 min. It was then dried completely in a vacuum centrifuge, solubilized in 6 M guanidine-HCl–0.1 M Tris-HCl (pH 8.5)–0.1 M dithiothreitol, and injected onto a Pharmacia ProRPC HR 5/10 reversed-phase column. The peptides were eluted by a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile over 18 ml (flow rate, 0.3 ml/min). The column effluent during detection of distinct peaks was collected (50 to 300 μ l, containing about 1 nmol of the respective peptide).

Selected peptide-containing fractions from reversed-phase HPLC were subjected to automated sequencing (Applied Biosystems model 473A). Purified muconolactone isomerase from *R. erythropolis* 1CP prior to sequencing was partially desalted by changing the buffer to 5 mM sodium phosphate buffer (pH 7.0) by ultrafiltration.

Purification of genomic and plasmid DNAs. Genomic DNA from *R. erythropolis* 1CP was prepared from 50-ml cultures grown in the same mineral medium as described above (30°C, 125 rpm), but with 2 g of glucose and 50 mg of 4-chlorophenol per liter as carbon and energy sources in the presence of 20 g of glycine per liter. The cells were centrifuged, washed with 50 mM Tris-HCl (pH 8), and centrifuged again. They were then resuspended in 3 ml of sucrose (100 g/liter)–10 mg of lysozyme per ml in 50 mM Tris-HCl (pH 8) and incubated at 37°C for 1.5 h. The cells were then pelleted by centrifugation and resuspended in 2.8 ml of TE buffer (10 mM Tris-HCl [pH 8], 1 mM sodium EDTA [pH 8]). Pronase E (Fluka)

and SDS were added to final concentrations of 100 μ g/ml and 5 g/liter, respectively, in a total volume of 3 ml. The mixture was incubated at 37°C for 2 h. Purification of DNA was achieved by two extractions with 3 ml of phenol and one extraction with 3 ml of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]). The aqueous phase was then dialyzed overnight against TE buffer at 4°C.

Plasmid DNA from *E. coli* DH5 α on a small scale was isolated with a Pharmacia Flexiprep kit. The method of Lee and Rasheed (35) was scaled up and used for plasmid isolation from 25-ml *E. coli* cultures.

General in vitro manipulations of DNA. Vector DNA digested with only one enzyme was dephosphorylated prior to ligation. For cloning of PCR products, a T-vector was prepared as described by Marchuk et al. (39). Insert DNA for ligation was isolated from gels by use of a GeneClean II kit (Bio 101, Vista, Calif.). Transformation of *E. coli* DH5 α was achieved by the method of Chung et al. (6) or that of Inoue et al. (28). Loss of LacZ complementation by insertion of DNA into vectors was detected by using LB plates with 0.13 mM IPTG and 32 μ g of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) per ml.

PCR. Oligonucleotides were custom synthesized according to the sequences of the previously determined N terminus (56) and the sequences of three internal peptides (Table 2). Degeneracy of the oligonucleotides was reduced by using codon usage tables previously published for *Rhodococcus* genes (20, 27). The reaction mixtures (50 μ l) contained 50 pmol of each primer, 0.5 μ g of chromosomal template DNA, 100 μ M deoxynucleoside triphosphates, 1 $\times$ optimized PCR buffer (Biometra), 0.2 to 1.0 U of DNA polymerase from *Thermus brockianus* F500 (Biometra), and various concentrations of denaturing agents, such as dimethyl sulfoxide (2 to 10%) or formamide (5 to 10%) (63). The PCR was performed with 30 cycles of denaturing (95°C, 30 s), annealing (50°C, 1 min), and polymerization (72°C, 1 min), with an additional 4.5 min of denaturing during the first cycle and an additional 14 min of polymerization during the last cycle.

Cloning strategy and hybridization procedures. The PCR products were initially ligated into the T-tailed EcoRV site of pBluescript II SK (+) and in one case were subcloned after HindIII/XbaI digestion into pBluescript II KS (+), thus yielding pRERT1 and pRERT2, respectively. The insert of pRERT1 was labeled with digoxigenin by using a DIG DNA Labeling and Detection Kit Nonradioactive (Boehringer). All labeling and hybridization procedures were performed as described in the Boehringer manual. The probe was then used to detect the corresponding fragment on a Southern blot of 5 μ g of BamHI-digested *R. erythropolis* DNA run on a 1% agarose gel with TAE (4.84 g of Tris base, 1.14 ml of glacial acetic acid, 2 ml of 0.5 M EDTA [pH 8] per liter). Prior to blotting of the DNA onto a Pall Biodyne B transfer membrane (0.45 μ m), the gel had been incubated in 0.25 M HCl twice for 15 min and subsequently in 1.5 M NaCl–0.5 M NaOH for denaturation. From a second gel, an area which corresponded in size to the hybridization signal (ca. 3 to 5 kb) was excised. DNA was eluted from the gel slice and ligated into pBluescript II KS (+). After transformation of the ligation mixture into *E. coli* DH5 α , the labeled insert of pRERT1 was used to identify the correct clones by colony hybridization. For this, cell material was carried over to Pall membranes which were subsequently incubated in 1.5 M NaCl–0.5 M NaOH twice for 5 min each and neutralized in 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl twice for 5 min each. Cell debris and nonlysed cells were removed from the membranes with Whatman 3MM paper. The membranes were washed in 50 mM NaCl and air dried. Fixation of DNA on the membranes was achieved by incubation at 70°C for 2 to 12 h.

TABLE 3. Enzyme activities expressed from pRER1

Enzyme	Sp act (U/mg) ^a of <i>E. coli</i> DH5 α carrying:	
	pRER1	pBluescript II KS (+)
Catechol 1,2-dioxygenase	0.015	<0.001
Muconate cycloisomerase	0.127	<0.001
Muconolactone isomerase	1.65	<0.02
3-Oxoadipate enol-lactone hydrolase	<0.1	<0.1

^a Details on induction and activity assays are given in Materials and Methods.


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3001 CCAGCCCGACATCATGCGGGAGTTTCAGGCTGCACCCGCGTTCCGGTGTGCGACGGTCCCGGGGAGSGTGATCCCGATCCCGACCGTCCGTCCCGGGTCTCGCCAGACTCTTCGCGACGCG
GGTCGGGCTGTAGTACCCCTCAAGTCCGACGTGGGCGCAAGCCACAGCTCCAGGGCCCCCTCCGACTAGGGCTAGGGCTGGCAGGCGAGGGCGCCAGAGCGGGCTCTGAGAGCGCGCTCGCG
W G S M L P S C G R E T D V T G P L S I G I G V T R G A T E G L S E R L A

3121 GATGAGGCGTTTGCGCGATGACGGGCGATCAGTTTCGGCCGGCTGGTGGCGATCTCTGCTCCAGCTCCTCGGCGACGATGACGTCGCGCTGCGAGGTGCGAGATGGCCAGCTGGGTGCGGCT
CTACTCCGCAACGCGCTACTGCCCCGTAGTCAAGCCGGCCGACACCGCTAGAGGAGGAGGTGCAGGAGCCGCTGCTACTGCGCGGACGCTCCAGCGTCTACCGGTGACCCACGCGA
I L R Q A I V P M L E A P S T G I E Q E V D E A V I V D G A L D C I A L Q T R S

3241 CCGACCGATGGCGCCGACACGACGACACCGGCTGAACGAGAGCTTGACCGGGCGGCGACCGCGGTGGAGACGCCCTCTCGCGTTTCGACGACGAGCCCGAGGCGCTGCAG
GGCTGGCTACCGGCGGCTGTGCTGCTGTGGCCGACTTGCACTTTGCTCTCGAACTGGCCGCGGCTGGCGGCGACCTCTGCGGGAGGAGCGCAAGCTGCTGCTCGGGCTTCGGGACGTC
R G I A A S L V V G A Q V H F S L K V P P R G G T S V G E E R E V V L G S A Q L

3361 CGAAGTCACCCCGGCGCGGACAGCGGTGCGGGAGAGGCGGTGAGGGTTCCGATCTCGGTGCGCGCTGCTTGCCTTGGCGGAATGAGGGCGAAGACGTCGCCACCCGTCGCGGGGA
GCTTCAGTGGGCGCGGCGTGTGCGCCAGGCTCTCGGCCACTCCCAAGGCTAGAGGACGCGCGAGCAACGAGGACGCGCTTACTCCCGCTTTCGACGCGTGGGCGAGGCGGCTCT
S T V R A A V A T R S L G T L T G I E T R T T A Q G A R I L A F V D G G T A P S

3481 GGTCTGCGAGCGAGGCGTCCGCGATGCGAGGCGATTCAACCAACCGGACACGCGTCTGACAACGTAAGGCGGTGACCTTTGTTCGCGAATACTTAAGTCTGTATTGATCAAAAGCCAAAG
CCAGCAGCTCGCTCCGAGGCGTA
T T S R P T R M
< ORF4

3601 GTCCGCTACTGTGCTGTGACGCGCCACACATCCGGCTTTCAGAGGAGATGACATGTACCAGCGACACCGTGGAACCGTGCGATTTCGTGCTGTTCGGCGGAACCGGCGACCTGGG
M S F A T T V E P C D F V V F G G T G D L A
ORF5 →

3721 GGTTCTGTAAGCTGCTTCCCGCGCTTACCTGCGCGATCGGGACGCGCAGCTGCCCCCGAAACCCGCGTGATCGGTGCTTCGCGAGCCGCTTCGACACCGACGCGCTACCGCGACAAGGT
V R K L L P A L Y L R D R D G Q L P A E T R V I G A S R A G L D T D G Y R D K V

3841 CGCGGGAGAACTCGCGCGGTTCTGCTTCGAGCGATCAGCTGGATTTCGAGACCTGTGAGCGATTTCTCAATCGCCTCGAATACGCTCCGTCGACGCGCGGCGATCC
R G E L A R F V S S D Q L D S D T C E R F L N R L R Y V S V D A A D

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FIG. 3.—Continued.

DNA sequence analysis. For generation of nested deletions in pRER1, a double-stranded nested deletion kit from Pharmacia was used. The nucleotide sequence of pRER1 was determined partly by the dideoxy chain termination method of Sanger et al. (50), using a Pharmacia ¹⁷Sequencing kit with Deaza G/A ¹⁷Sequencing mixes in order to eliminate band compressions in sequencing gels, and partly by cycle sequencing using an Applied Biosystems Prizm kit with subsequent electrophoresis and analysis in an Applied Biosystems A373 sequencer. Computer-based sequence analyses were performed mainly with the PC/GENE program package (Intelligenetics Inc., Mountain View, Calif.). FASTA (47), as implemented in PC/GENE, was used under default conditions to screen databases, while multiple sequence alignments were performed by the PC/GENE version of CLUSTAL (23) with open and unit gap costs set to 10. Dendrograms based on the Fitch-Margoliash algorithm were obtained by using the programs PROTDIST, FITCH, and DRAWTREE of the PHYLIP program package (14a) with default settings unless otherwise specified.

Nucleotide sequence accession number. The 3,945-bp sequence of the insert on pRER1 shown in Fig. 3 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. X99622.

RESULTS

Cloning of the muconate cycloisomerase-encoding region from *R. erythropolis* 1CP. In order to obtain sufficient amounts of protein for the isolation of tryptic fragments, the muconate cycloisomerase was purified from an extract of benzoate-grown cells of *R. erythropolis* 1CP basically by scaling up a previously developed procedure (56). An aliquot of the purified protein was subjected to trypsin digestion, and the resulting peptide mixture was separated by reversed-phase HPLC. The sequence information obtained from some of the isolated peptides (Table 2) was used for the design of oligonucleotides which then served as primers for PCR experiments. With primers N-NT and N50.19, a 0.85-kb fragment was produced, and with N-NT and N80.50 a 1-kb fragment was produced. Neither fragment was detectable when a PCR was performed under identical conditions but with only one of the two primers. The 0.85-kb fragment and the 1.0-kb fragment were cloned into pBluescript vectors. DNA sequencing into the cloned inserts demonstrated that they contained the respective primers and between them a DNA fragment with sequence similarities to known cycloisomerases (see below). The larger insert (in pRER1) was digoxigenin labeled and then used for the identification of corresponding bands on a Southern blot as well as for the identification of colonies of the desired clones. A clone carry-

ing a 4-kb *Bam*HI insert in pBluescript II KS (+) was isolated and designated pRER1 (Fig. 2).

Expression of *cat* genes from pRER1. *E. coli* DH5 α strains harboring pRER1 in addition to muconate cycloisomerase activity expressed catechol 1,2-dioxygenase and muconolactone isomerase activities (Table 3). None of the three enzymes was expressed in *E. coli* DH5 α harboring pBluescript II KS (+) without an insert. A 3-oxoadipate enol-lactone-hydrolyzing enzyme could not be detected in the extracts of *E. coli* DH5 α (pRER1).

Sequence analysis of pRER1. DNA sequencing of the insert of pRER1 revealed five open reading frames (Fig. 3). The first one, positions 1 to 813, was incomplete and lacked the N terminus. It showed high sequence similarities to known catechol and chlorocatechol 1,2-dioxygenases (Fig. 4; Table 4), especially to the catechol 1,2-dioxygenase of *Arthrobacter* sp. strain mA3 (9), with which it shares 58.9% identical positions in the alignment of Fig. 4. Both cyanogen bromide fragments of purified catechol 1,2-dioxygenase which were subjected to amino acid sequencing were found to occur in the predicted sequence (Fig. 3). Thus, the first open reading frame clearly is part of the *catA* gene.

The second open reading frame, positions 843 to 1964, was similar to muconate and chloromuconate cycloisomerases (Fig. 5; Table 4) and was therefore designated *catB*. All determined sequences of internal peptides of the muconate cycloisomerase of *R. erythropolis* 1CP (Table 2) were found to be contained in the open reading frame (Fig. 3). The predicted subunit molecular mass of the protein expressed from this open reading frame is 39,608 Da.

The third open reading frame, positions 1980 to 2261, showed high sequence similarities to the muconolactone isomerases of *Pseudomonas putida* strains (47.3 and 49.5% identical positions, respectively, in the alignment of Fig. 6) and to the enzyme of *Acinetobacter calcoaceticus* (61.3% identical positions). The predicted amino acid sequence of the N terminus of the third open reading frame was found to be identical to the sequence obtained for the muconolactone isomerase purified from benzoate-grown cells of *R. erythropolis* 1CP

FIG. 4. Sequence alignment of catechol 1,2-dioxygenases (CatA and PheB) and chlorocatechol 1,2-dioxygenases (TfdC, ClcA, and TcbC) as calculated by the CLUSTAL program. At positions 175 to 178, gaps have been aligned manually. Numbers above the sequences refer to positions in the alignment, not in single sequences. Amino acids in positions in which at least eight of the nine sequences are identical (highlighted) and histidine and tyrosine residues previously reported to be involved in iron binding (21, 37) (arrows) are indicated. In some regions (positions 1 to 32, 99 to 104, 124 to 137, 175 to 184, and 269 to 320) the positioning of gaps is questionable, as indicated by the fact that in these regions the alignment given here differs from a previous one of six of the sequences (9). The N-terminal 10 amino acids of the *R. erythropolis* CatA sequence have been predicted from the nucleotide sequence of an overlapping clone (12). Accession numbers and references for the published sequences (order as in the alignment) are as follows: U12557 (26), M57500 (32), M76991 (44), Z36909 (10), M35097 (48), M16964 (15), M57629 (62), and M94318 (9). Two more catechol 1,2-dioxygenase sequences (D37782 and D37783 [43]) which are very similar to the one from *P. putida* PRS2000 shown here have been omitted for clarity. *P. put.*, *P. putida* PRS2000; *P. EST*, *Pseudomonas* sp. strain EST1001; *A. cal.*, *A. calcoaceticus* ADP1 (catechol catabolism); *A. cal. Ph.*, *A. calcoaceticus* NCIB8250 (phenol catabolism); *Arthr.*, *Arthrobacter* sp. strain mA3; *R. ery.*, *R. erythropolis* 1CP.

TABLE 4. Sequence similarities between different groups of catechol and chlorocatechol 1,2-dioxygenases as well as muconate and chloromuconate cycloisomerases

Groups compared	Avg ratio of identical positions in pairwise comparisons (range) ^a	
	1,2-Dioxygenases	Cycloisomerases
Catechol enzymes of gram-negative bacteria versus chlorocatechol enzymes of gram-negative bacteria	28.1 (25.5–30.6)	41.5 (39.5–44.1)
Catechol enzymes of gram-negative bacteria versus catechol enzymes of gram-positive bacteria	28.8 (27.3–30.9)	39.0 (38.1–39.9)
Catechol enzymes of gram-positive bacteria versus chlorocatechol enzymes of gram-negative bacteria	34.7 (33.8–36.2)	38.0 (37.0–39.7)

^a The calculations (expressed as percentages) are based on the sequence alignments shown in Fig. 4 and 5.

(Fig. 3). The predicted subunit molecular mass of the protein expressed from this open reading frame is 10,911 Da.

A fourth open reading frame (bases 3504 to 2311), probably transcribed in the direction opposite to that of the *cat* genes, was detected downstream from *catC*. It showed significant sequence similarity to regulatory proteins of carbohydrate catabolism, e.g., to XylR, a repressor of xylose utilization by *Bacillus subtilis* (accession number M27248), and to NagC, a repressor of *N*-acetylglucosamine utilization by *E. coli* (accession number X14135).

The fifth open reading frame extended from base 3656 to the end of the cloned fragment. The predicted amino acid sequence in a FASTA run yielded the highest scores with the sequences of several glucose-6-phosphate dehydrogenases, e.g., from *Zymomonas mobilis* (accession number M37982) and *E. coli* (accession number M55005).

DISCUSSION

The cloned *R. erythropolis* 1CP DNA which was characterized in the present investigation does not contain the complete *catA* gene for catechol 1,2-dioxygenase. The comparison of the

FIG. 5. Sequence alignment of muconate cycloisomerases (CatB) and chloromuconate cycloisomerases (TfdD, ClcB, and TcbD) as calculated by the CLUSTAL program. At positions 23 and 97, gaps have been aligned manually. Numbers above the sequences refer to positions in the alignment, not in single sequences. Amino acids in those positions in which at least six of the seven sequences are identical (highlighted), aspartate and glutamate residues involved in manganese coordination (arrows pointing upward), as well as lysine and glutamate residues directly involved in the enzyme mechanism (arrows pointing downward) (17, 22, 24) are indicated. Accession numbers and references for the published sequences (order as in the alignment) are as follows: U12557 (26), M19460 (1), M76991 (55), M35097 (48), M16964 (15), and M57629 (62). The N-terminal amino acid of TfdD has been determined as methionine (19), although the respective codon is GTG (48). *P. put.*, *P. putida*; *A. cal.*, *A. calcoaceticus* ADP1; *R. ery.*, *R. erythropolis* 1CP.

predicted amino acid sequence to that of the corresponding enzyme from *Arthrobacter* sp. strain mA3 (9) suggests that 30 bp encoding the 10 N-terminal amino acids are missing. Moreover, the clone comprises only one dioxygenase gene, while the catechol 1,2-dioxygenase of *R. erythropolis* 1CP has previously been reported to consist of two subunits of 33 and 35 kDa (38). Nevertheless, the protein expressed from pRER1 clearly had catechol 1,2-dioxygenase activity (Table 3), implying that one subunit is sufficient for a functional enzyme. In addition, SDS gel electrophoresis of catechol 1,2-dioxygenase purified from benzoate-grown wild-type cells and sequencing of cyanogen bromide fragments of the enzyme did not give an indication for the presence of two different subunits. Correspondingly, the catechol 1,2-dioxygenase from *R. erythropolis* AN-13 has also previously been reported to consist of one or more copies of a single subunit (3). However, our data also do not exclude the possibility that the enzyme of strain 1CP may, under certain

conditions, occur in an $\alpha\beta$ form which has been described for the catechol 1,2-dioxygenase of *P. putida* C-1 (42).

In the cluster of the *R. erythropolis* *cat* genes, *catA* is directly followed by *catB* and *catC* (Fig. 2 and 7). This arrangement as a whole is unique among the known clusters of catechol and chlorocatechol catabolic genes. However, *catC* has been observed to follow *catB* in all *cat* gene clusters examined so far, and therefore this might be an ancient property. (There is no *catC* equivalent in chlorocatechol degradation.) That *catB* directly follows *catA* as in *R. erythropolis* 1CP has previously not been observed for other *cat* genes, but, interestingly, a corresponding arrangement has been described for all chlorocatechol gene clusters of plasmids from gram-negative bacteria (Fig. 7).

Previously, the characterization of the catalytic properties of various muconate and chloromuconate cycloisomerases, especially with respect to 2-chloro-*cis,cis*-muconate conversion, had

FIG. 6. Sequence alignment of muconolactone isomerases as calculated by the CLUSTAL program. The gap at position 49 was aligned manually. Numbering above the sequences refers to positions in the alignment, not in single sequences. Amino acids in positions in which all sequences are identical (highlighted) and amino acid residues previously reported to contribute to the active site (31) (arrows) are indicated. Accession numbers and references for the published sequences (order as in the alignment) are as follows: U12557 (26), M19460 (1), and M76991 (55). *P. put.*, *P. putida*; *A. cal.*, *A. calcoaceticus* ADP1; *R. ery.*, *R. erythropolis* 1CP.

FIG. 7. Structures of catechol and chlorocatechol catabolic gene clusters. Homologous genes are presented in the same shade of grey. Information was taken from Fig. 3, references given in an earlier review (51), and other publications (26, 33, 36, 49). The gene cluster shown for *P. putida* is that of strain PRS2000 (26). With respect to the distances between genes, it differs from that of strain RB1 (1). Since sequences for *P. aeruginosa* are not available, the direction of transcription of the *catA* gene is not given and the genes are assumed to be of lengths similar to those in *P. putida*. The insertion sequence-like element (IS), reported to occur in the *tfdT* gene (36), is not necessarily drawn in the correct position. *A. calcoac.*, *A. calcoaceticus*; *P. aerugin.*, *P. aeruginosa*; *R. erythr.*, *R. erythropolis*; ORF, open reading frame.

shown that muconate and chloromuconate cycloisomerases of *R. erythropolis* 1CP share properties not shared by the corresponding enzymes from gram-negative strains and vice versa (56). These findings suggested that the muconate and chloromuconate cycloisomerases of gram-positive strains are more closely related to each other than to muconate and chloromuconate cycloisomerases of proteobacterial origin. This would imply that the adaptation to chlorosubstituted substrates occurred independently among gram-negative and gram-positive bacteria. Simple comparisons of cycloisomerase sequences set the muconate cycloisomerase of *R. erythropolis* 1CP apart from the enzymes of gram-negative strains, quite in line with the hypothesis outlined above. Thus, the *Rhodococcus* enzyme shares ca. 39% identical residues with proteobacterial muconate cycloisomerases and ca. 38% identical residues with corresponding chloromuconate cycloisomerases, while the average degree of identical positions shared by the latter groups is 41.5% (Table 4).

However, the same analyses of the catechol and chlorocatechol 1,2-dioxygenases revealed a different pattern of sequence similarities. Here, the catechol 1,2-dioxygenases of the gram-positive *Arthrobacter* and *Rhodococcus* strains turned out to be relatively similar to the chlorocatechol dioxygenases of gram-negative strains (ca. 35% identical positions), while the catechol dioxygenases of *Pseudomonas* and *Acinetobacter* strains showed less sequence similarity to the other two groups (Table 4). The different similarity patterns are also obvious in dendrograms based on the unweighted pair-group method with arithmetic averages (UPGMA) (Fig. 8A). There are examples of gene clusters for phenol degradation which comprise catechol 1,2-dioxygenase genes but apparently no muconate cycloisomerase genes (10, 32), indicating that the dioxygenases and cycloisomerases do not necessarily coevolve. However, in most cases, dioxygenase and cycloisomerase genes have been found in the same operons or superoperonic clusters (Fig. 7). Thus, their dendrograms could be expected to have similar

branching patterns, implying that one of those in Fig. 8A might not reflect the evolutionary relationships correctly.

Sequence analyses using the Fitch-Margoliash algorithm for the dioxygenases resulted basically in the same picture as the UPGMA (Fig. 8), while for the cycloisomerases the two methods gave dendrograms with different branching patterns. In correspondence to the dioxygenase tree, the dendrogram of Fig. 8B shows the muconate cycloisomerase of *R. erythropolis* on the same branch as the chloromuconate cycloisomerases of plasmids from gram-negative bacteria. It is also obvious that the branch leading to the *Rhodococcus* enzyme is considerably longer than those to the other cycloisomerases. Thus, differences in the evolutionary rates of the cycloisomerases are likely to have resulted in a misleading dendrogram when the UPGMA was used, which assumes an evenly running evolutionary clock. It might be pointed out, however, that the branch leading to the muconate cycloisomerase of *R. erythropolis* 1CP is not significantly longer than those to the catechol 1,2-dioxygenases of the gram-positive strains (drawn to the same scale in Fig. 8B). In contrast, the branches to the muconate and chloromuconate cycloisomerases of gram-negative bacteria are not only shorter than that to the *Rhodococcus* enzyme, but also considerably shorter than the branches to the corresponding dioxygenases (Fig. 8B).

The proximity of the branching points of the major clusters to each other as well as bootstrap analyses (data not shown) suggests that the topology of the dendrograms in Fig. 8B may have to be changed upon incorporation of new sequences or use of other analytical methods. However, even the finding that the chlorocatechol enzymes encoded by plasmids of gram-negative bacteria appear to have originated from the branches to enzymes of gram-positive strains is still compatible with the hypothesis, derived from biochemistry of the cycloisomerases, that chlorocatechol conversion among the gram-positive bacteria evolved in functional convergence to that in proteobacteria. Two assumptions are necessary. (i) The common precur-

FIG. 8. Dendrograms showing the relatedness of catechol and chlorocatechol 1,2-dioxygenases (left) as well as muconate and chloromuconate cycloisomerases (right). Clustering was performed by UPGMA of the program CLUSTAL (A) and by the Fitch-Margoliash procedure of the PHYLIP program package (B). The dioxygenase and the cycloisomerase dendrograms in panel B are drawn to the same scale (in centimeters per unit branch length). To indicate the position of the roots in the dendrograms of the Fitch-Margoliash procedure, the following sequences served as out-groups: PcaH, the β -subunit of protocatechuate 3,4-dioxygenases (M33798, M30791, and L14836), known to be homologous to catechol and chlorocatechol 1,2-dioxygenases (21); and MdlA, the mandelate racemase of *P. putida* (J05293), and GalD (DgoD), the galactonate dehydratase of *E. coli* (L10328 and U19577), which are homologous to muconate and chloromuconate cycloisomerases (4, 61). The Fitch-Margoliash dendrograms shown were calculated from the complete alignments including regions in which the alignment was not certain. Basically the same tree topologies were obtained also when only regions in which the alignments were considered correct were used, as judged, for example, by proper alignment of iron-coordinating amino acid residues (Fig. 4) or manganese- or magnesium-coordinating and other active-site residues (Fig. 5).

sor of the cycloisomerases would be assumed to have the catalytic properties shared by the muconate and chloromuconate cycloisomerases of the gram-negative bacteria. (ii) The chlorocatechol pathway of the gram-positive bacteria diverged from the branch to the catechol enzymes of these bacteria later than the chlorocatechol enzymes encoded by plasmids of gram-negative strains. Work to test the second hypothesis is in progress.

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