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The Modified β-Ketoadipate Pathway in *Rhodococcus rhodochrous* N75: Enzymology of 3-Methylmuconolactone Metabolism

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Rhodococcus rhodochrous **N75 is able to metabolize 4-methylcatechol via a modified β-ketoadipate pathway. This organism has been shown to activate 3-methylmuconolactone by the addition of coenzyme A (CoA) prior to hydrolysis of the butenolide ring. A lactone-CoA synthetase is induced by growth of *R. rhodochrous* N75 on *p*-toluate as a sole source of carbon. The enzyme has been purified 221-fold by ammonium sulfate fractionation, hydrophobic chromatography, gel filtration, and anion-exchange chromatography. The enzyme, termed 3-methylmuconolactone-CoA synthetase, has a pH optimum of 8.0, a native M_r of 128,000, and a subunit M_r of 62,000, suggesting that the enzyme is homodimeric. The enzyme is very specific for its 3-methylmuconolactone substrate and displays little or no activity with other monoene and diene lactone analogues. Equimolar amounts of these lactone analogues brought about less than 30% (most brought about less than 15%) inhibition of the CoA synthetase reaction with its natural substrate.**

The degradation of methyl-substituted benzoates and phenols by *ortho* fission is precluded in many species of procaryotes due to the formation of dead-end lactone intermediates. For example, pseudomonads accumulate 4-methylmuconolactone (4-carboxymethyl-4-methylbut-2-en-1,4-olide) if 4-methylcatechol is directed by *ortho* fission through the β-ketoadipate pathway. 4-Methylmuconolactone cannot be degraded further in these organisms, because with a methyl substituent at C-4, there is no free proton to undergo the shift of the muconate isomerase reaction (5, 8, 17, 18, 20). While most methyl-substituted catechols are degraded by bacteria via *meta*-cleavage pathways, there have been some notable exceptions. Strains of rhodococci (5) and *Alcaligenes eutrophus* JMP134 (now *Ralstonia eutropha* JMP134) (20) have evolved a novel methyl lactone isomerase which transforms 4-methylmuconolactone into 3-methylmuconolactone (4-carboxymethyl-3-methylbut-2-en-1,4-olide), thereby overcoming the bacterial block. Purification and characterization of the methyl isomerases from *Rhodococcus rhodochrous* N75 (6) and *R. eutropha* JMP134 (21) showed significant differences and possibly different mechanisms for the isomerization reactions in the two organisms. Recent work by Prucha et al. (25) and Erb et al. (10) has provided evidence for an isomeric muconolactone isomerase in *R. eutropha* JMP134 which is induced during 4-methylmuconolactone metabolism and suggests that, in this organism, conversion of 3-methylmuconolactone to 4-methyl-3-ketoadipate occurs in a manner similar to that by which muconate is converted to 3-ketoadipate, though the appropriate enol-lactone hydrolase has still to be located. Eucaryotes, such as the fungus *Trichosporon cutaneum* (22) and *Aspergillus niger* (9), have overcome the procaryotic problem of the block at the lactonizing step by generating 3-methylmuconolactone directly from 3-methyl-*cis*,*cis*-muconate, which allows further degradation to 4-methyl-3-ketoadipate to occur by a mechanism analogous to that of the classical β-ketoadipate pathway (18, 22, 23). In *T. cutaneum* the 3-keto acid is further metabolized, via its coenzyme A (CoA) thioester, to the thioesters of 2-methylsuccinic, itaconic, and citramalic acids, but these steps have so far not been identified in *R. eutropha* JMP134 or in rhodococci. High concentrations of cell extract were shown to be required for the formation of 4-methyl-3-ketoadipate in *Rhodococcus rhodochrous* N75 (6), which implies that an additional cofactor may be necessary for delactonization to occur. In this paper we provide evidence that further degradation of 3-methylmuconolactone in *Rhodococcus rhodochrous* N75 occurs by a mechanism completely different from that observed in *T. cutaneum* or *R. eutropha* JMP134 and that an inducible lactone-CoA synthetase is involved in delactonization.

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MATERIALS AND METHODS

Chemicals. The chemicals used in this work were purchased from the following companies: Aldrich Chemical Company Ltd. (Gillingham, United Kingdom), Sigma Chemical Company Ltd. (Poole, United Kingdom), Fisons Scientific Equipment (Loughborough, United Kingdom), and BDH Ltd. (Poole, United Kingdom), unless otherwise stated. All chemicals were of analytical grade or above. Adenylate kinase, pyruvate kinase, and lactic dehydrogenase were obtained from Sigma. The biologically active forms of (+)-muconolactone and (*S*)-(+)-4-methylmuconolactone were prepared biologically (6), the racemic compounds were prepared by chemical synthesis (11), and the unnatural isomer (*R*)-(+)-3-methylmuconolactone was prepared by resolution of the ± isomer (11). Other lactones were obtained by feeding cultures with the appropriate precursors (6, 7). 3-Ketoadipic acid was synthesized by the method of Reigel and Lilienfeld (26), and 4-methyl-3-ketoadipic acid was synthesized by G. V. Rao, University of Glasgow, Glasgow, United Kingdom, by a modification of that method (details to be published elsewhere). 2-Methylmuconolactone was a gift from K.-H. Engesser, University of Stuttgart, Stuttgart, Germany.

Biological synthesis of 3-methylmuconolactone. 3-Methylmuconolactone was prepared biologically as described by Miller (17). The yield was 0.413 g (53%) of long white crystals. The ^{1}H nuclear magnetic resonance spectrum of the crystals coincided well with the reference data for 3-methylmuconolactone (8, 20). Mass spectra indicated a molecular ion with an *m*/*e* of 157, which corresponds to the formula $C_7H_8O_4$ for 3-methylmuconolactone. Purified 3-methylmuconolactone was found to be optically active; the specific rotation of sodium D line at 20°C in methanol was $-28.6 \pm 0.9°$.

Culture conditions. *Rhodococcus rhodochrous* N75 cultures were grown on minimal media supplemented with 10 mM *p*-toluate or 10 mM benzoate as a sole carbon source. The defined minimal medium, modified from the original recipe described by Miller (17), consisted of 4.0 g of K_2HPO_4 per liter, 0.4 g of KH_2PO_4 per liter, 1.0 g of $(NH_4)_2SO_4$ per liter, 0.1 g of $MgSO_4$ per liter, and 1 ml of the trace element solution described by Barnett and Ingram (2) per liter. Large

quantities of cell biomass were prepared with a 75-liter pilot-scale Chemap fermentor (Alfa-Laval Engineering, Brentford, United Kingdom), which was monitored with BioData Manager software. The dissolved-oxygen concentration was kept at ≥20% of saturation by a pO_2-agitation rate control loop, and air was supplied at a rate of 5 liters per min. The culture was grown for 18 to 20 h at 30°C, and cells were harvested by continuous-flow centrifugation at 15,000 rpm in a Sorvall RC-5C centrifuge fitted with a TZ-28 rotor. The pelleted cells were kept at −80°C until required. Recombinant strains of *Escherichia coli* containing the plasmids pVUC19 and pPAN30, which direct high expression of muconolactone isomerase and enol-lactone hydrolase, respectively, were kindly provided by L. N. Ornston (Yale University) and have been described elsewhere (13).

Preparation of cell extracts. Cells were resuspended at a concentration of 0.5 g (wet weight)/ml in 50 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.4) containing 1 mM dithiothreitol (DTT). The cell suspension was sonicated on ice in a Soniprep 150 MSE ultrasonic disintegrator (Fisons Instruments, FSA Ltd., Crawley, United Kingdom) at 12 to 18 μm 12 times for 15 s each time with 45 s of cooling between each cycle. The cell debris was removed by centrifugation in a Sorvall SS34 rotor at 20,000 rpm and 4°C for 1 h. The resulting cell extract was kept on ice until required.

Enzyme assays. Muconolactone isomerase was assayed according to the method of Meagher et al. (16).

3-Methylmuconolactone-CoA synthetase activity based on the detection of the CoA adduct was assayed by two methods. The hydroxamate method was used throughout the purification procedures, while the enzyme-coupled assay was used for the characterization of the enzyme when necessary. All assays were performed in duplicate. The method used for the acyl-CoA synthetase assay was based on an adaptation of the hydroxamate method of Overath et al. (19) as modified by Blakley (3). The reaction mixture contained the following in a total volume of 0.4 ml: 50 mM MOPS buffer (pH 7.8), 2.5 mM DTT, 10 mM ATP, 1 mM CoA, 10 mM $MgCl_2$, 3 mM 3-methylmuconolactone, and 0.5 M hydroxylamine-HCl freshly neutralized to pH 7.5. The reaction mixtures without enzyme were preincubated at 30°C for 5 min in 1.5-ml Eppendorf tubes. The reaction was initiated by the addition of enzyme and further incubated for up to 20 min. The reaction was then terminated by the addition of 0.3 ml of a 1:1 mixture of 10% (wt/vol) $FeCl_3$ in 0.1 M HCl and 12% (wt/vol) trichloroacetic acid in 3 M HCl. Precipitated proteins were removed by centrifugation in an MSE MicroCentaur microcentrifuge for 10 min. The remaining supernatants were then measured for absorbance at 540 nm. A standard curve was determined for the concentration of acyl hydroxamate on the basis of known concentrations of acetyl-CoA. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of acyl hydroxamate in 1 min at 30°C.

An enzyme-coupled assay was performed according to the method of Gibson et al. (12). The reaction mixture contained the following in a total volume of 1 ml: 50 mM Tris-HCl buffer (pH 8.0), 0.5 mM CoA (lithium salt), 1.0 mM ATP, 5 mM $MgCl_2$, 0.5 mM 3-methylmuconolactone, 10 mM KCl, 5 mM phosphoenolpyruvate (trisodium salt), 0.35 mM NADH, 2 U of adenylate kinase, 2 U of pyruvate kinase, and 2 U of lactic dehydrogenase. The activity was measured spectrophotometrically by the decrease in absorbance at 340 nm on the basis of the oxidation of NADH. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH in 1 min at 30°C.

Enzyme purification. Muconolactone isomerase was partially purified from cells (8 g [wet weight]) of *Rhodococcus rhodochrous* N75 grown at the expense of benzoate or *p*-toluate as the sole source of carbon. Cell extract was loaded onto a DEAE-Toyopearl 650C column (1.6 by 15 cm) that had been previously equilibrated with buffer A (50 mM MOPS [pH 7.4], 1 mM DTT). The column was then washed with the same buffer until no protein was evident in the eluate. Proteins were eluted with a linear gradient of 0 to 0.5 M KCl in a 240-ml volume at a flow rate of 1 ml/min. Active fractions were pooled, desalted, and then applied to a Mono Q column (HR 10/10). Proteins were eluted with a linear gradient of 20 to 40% buffer B (50 mM MOPS [pH 7.4] containing 1 mM DTT and 1 M NaCl) in a volume of 90 ml at a flow rate of 3 ml/min.

For the purification of 3-methylmuconolactone-CoA synthetase, cell extract was prepared with 20 g (wet weight) of *p*-toluate-grown cells. The extract was treated with ammonium sulfate to 40% saturation, and the protein precipitate was removed by centrifugation. The resulting supernatant was then raised to 55% saturation with additional finely powdered crystalline $(NH_4)_2SO_4$. After 40 min of equilibration, the protein precipitate was recovered by centrifugation and then redissolved in a small volume of buffer A. This solution was diluted with the same volume of buffer A containing 1 M $(NH_4)_2SO_4$ and then chromatographed on a phenyl agarose 6XL column (2.5 by 5 cm; Affinity Chromatography Ltd., Freeport, Ballasalla, Isle of Man, United Kingdom) that had been preequilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. Proteins were eluted with a decreasing gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in 200 ml of buffer A at a flow rate of 2 ml/min. Active fractions were pooled and then concentrated to a final volume of 2 ml by ultrafiltration with a YM 10 Diaflo membrane (10,000 M_r cutoff; Amicon). The concentrate was applied to a Sephacryl S-100 HR column (1.6 by 95 cm; Pharmacia Biotech, St. Albans, United Kingdom), and subsequent elution of the enzyme activities was carried out at 12 ml/h. Fractions with the highest enzyme activities were combined and applied to a Mono Q HR 5/5 (Pharmacia) column. The enzyme was eluted with a linear gradient of 20 to 50% buffer B in a volume of 30 ml at a flow rate of 0.5 ml/min. Fractions that contained the highest enzyme activities were combined and stored at 4°C until required.

Protein assays. Protein concentration was assayed by the method of Bradford (4) with a Coomassie blue dye-binding reagent (Pierce Ltd.) and bovine serum albumin as a standard.

Analytical methods. Thin-layer chromatography and high-pressure liquid chromatography (HPLC) were as described by Bruce and Cain (5). ^1H nuclear magnetic resonance spectroscopy was performed by the Department of Chemistry, University of Cambridge, on a Brucker 500-MHz spectrometer with trimethylsilane as an internal standard. Fast atom bombardment mass spectrometry was performed by the Department of Chemistry, University of Cambridge. Optical activity was measured with a Perkin-Elmer polarimeter with a 10-cm-long cell in the Department of Chemistry, University of Cambridge. Measurements were performed at 20°C and at a wavelength of 589 nm (sodium D line), with methanol as a solvent.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (15) with a Mini-Protean A apparatus (Bio-Rad Laboratories Ltd., Watford, United Kingdom). Native (nondenaturing) PAGE was performed at 4°C to retain enzyme activity by the same procedure but with the omission of SDS and sample denaturation.

For the determination of subunit compositions of enzymes, native PAGE of purified enzyme samples was performed at 4°C. Protein was detected by staining with Coomassie blue R-250. Enzyme activity was detected by assaying unstained gel slices from the same gel. Each gel slice was tested for enzyme activity by incubating with the appropriate substrate. A gel slice from the corresponding enzyme activity was then added to a small volume of SDS-PAGE sample buffer, boiled for 5 min, and then subjected to SDS-PAGE.

M_r determinations. The native M_r of purified 3-methylmuconolactone-CoA synthetase was determined by gel filtration chromatography by the method of Andrews (1). A prepacked column of Superose 6 was run at a flow rate of 0.2 ml/min. The following standards were used in different combinations for each enzyme: thyroglobulin (M_r, 669,000), ferritin (M_r, 440,000), catalase (M_r, 232,000), aldolase (M_r, 158,000), bovine serum albumin (M_r, 67,000), chymotrypsinogen (M_r, 25,000), and RNase A (M_r, 13,700).

RESULTS

Purification of muconolactone isomerase from cell extracts of *Rhodococcus rhodochrous*. Basal levels of delactonizing activity towards 3-methylmuconolactone had been previously seen with high levels of cell extracts of *Rhodococcus rhodochrous* (6). Partial purification of muconolactone isomerase (EC 5.3.3.4) from benzoate- or *p*-toluate-grown cells of *Rhodococcus rhodochrous* N75 was therefore performed in order to establish whether isozymes of muconolactone isomerase are induced for the further metabolism of 3-methylmuconolactone as seen in *R. eutropha* JMP134. Muconolactone isomerase eluted from extracts of *p*-toluate- and benzoate-grown cells in the DEAE-Toyopearl column at very similar concentrations of NaCl (0.15 to 0.22 and 0.18 to 0.22 M NaCl, respectively) and eluted in identical fractions when both protein mixtures were subjected to Mono Q chromatography. The specific activity of partially purified muconolactone isomerase was 513 U/mg from benzoate-grown cells, a yield of 38% at a purification factor of 55, and 11 U/mg from *p*-toluate-grown cells, a yield of 31% at a purification factor of 85. When active fractions from the Mono Q step for both sources of enzyme were mixed together, desalted, and reapplied to the Mono Q column, only a single peak of muconolactone isomerase activity was detected.

Neither preparation of muconolactone isomerase showed any activity against 3-methylmuconolactone when it was analyzed with the recombinant pseudomonad enol-lactone hydrolase, though it is possible that the (classical) enol-lactone hydrolase preparation (16, 24) used in the coupled assay is incapable of cleaving the putative enol form of 3-methylmuconolactone. *Rhodococcus rhodochrous* N75 therefore differs from *R. eutropha* JMP134, which, when induced with 4-methylcatechol, produces isomeric forms of muconolactone isomerase that can be separated by chromatographic techniques (10, 24, 25). Muconolactone isomerase activity is 50-fold greater in benzoate-grown cells of *Rhodococcus rhodochrous* N75 than in *p*-toluate-grown cells, even though both substrates support relatively similar growth rates (benzoate t_d = 3.2 h, *p*-toluate t_d =

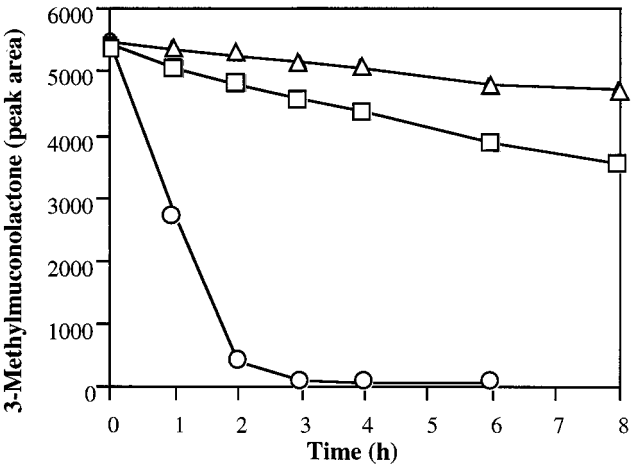

FIG. 1. Effect of CoA and ATP on the degradation of 3-methylmuconolactone. 3-Methylmuconolactone was incubated with dialyzed crude extract from benzoate-grown cells (□), dialyzed crude extract from *p*-toluate-grown cells in the absence of CoA and ATP (△), and dialyzed crude extract in the presence CoA and ATP (○). 3-Methylmuconolactone degradation was monitored by HPLC.

4.6 h), which further implies that muconolactone isomerase of the β-ketoadipate pathway does not mediate delactonization of 3-methylmuconolactone.

Evidence for the requirement of a cofactor in the degradation of 3-methylmuconolactone. Powlowski and Dagley (22) showed that favorable rates of conversion of 3-methylmuconolactone to 3-methyl-4-ketoadipate by extracts of the fungus *T. cutaneum* grown with *p*-cresol could be obtained only with high concentrations of cell extract protein. The corresponding rate of degradation of this lactone by comparably high concentrations of extract protein from cells of *Rhodococcus rhodochrous* N75 was still very much lower. The necessity for high levels of extract protein for activity may be due to the requirement of a dissociable cofactor in the cell extract. Dialysis of the cell extract from *p*-toluate-grown cells of *Rhodococcus rhodochrous* N75, against 2 liters of 50 mM potassium phosphate buffer (pH 7.3), clearly resulted in a further loss of degradative activity, implying that the delactonization reaction is cofactor dependent.

Desalted crude extracts from *p*-toluate-grown cells of *Rhodococcus rhodochrous* N75 rapidly transformed 3-methylmuconolactone (retention time = 6.1 min) to an unknown compound (retention time = 2.5 min) upon the addition of CoA, ATP, and Mg^{2+}, as monitored by HPLC. Controls which lacked one or more of the cofactors did not show CoA-dependent activity towards 3-methylmuconolactone (Fig. 1). The requirement for ATP, CoA, and Mg^{2+} for activity against 3-methylmuconolactone by cell extracts from *p*-toluate-grown cells of *Rhodococcus rhodochrous* N75 suggested the presence of an acyl-CoA synthetase active towards the carboxyl group of 3-methylmuconolactone. The formation of the hydroxamic acid derivative of the putative thioester further confirmed an acyl-CoA synthetase reaction. This assay system is known to be insensitive, and the specific activity of the acyl-CoA synthetase was typically low. As the responsible enzyme was presumed to catalyze the CoA esterification of 3-methylmuconolactone, it was termed 3-methylmuconolactone-CoA synthetase. Growth of *Rhodococcus rhodochrous* on *p*-toluate led to greatly enhanced CoA-dependent activity, some 11-fold greater than that found in benzoate-grown cells, and no significant activity was detected in succinate or yeast extract-grown cells. Interestingly, crude extract from benzoate-grown cells also exhibited a basal amount of degradation activity, which was faster than that observed with crude extract from *p*-toluate-grown cells in the absence of the cofactors. However, desalting cell extract from benzoate-grown cells did not cause a loss of this basal activity and the addition of cofactors to the extract did not further accelerate the rate. These results suggest that the limited 3-methylmuconolactone delactonization activity found in cell extract from benzoate-grown cells was probably due to muconolactone isomerase and enol-lactone hydrolase displaying very low rates of activity with 3-methylmuconolactone. These delactonizing enzymes are present in *Rhodococcus rhodochrous* N75 when it grows on *p*-toluate; however, the specific activity of muconolactone isomerase extract from benzoate-grown cells is some 50-fold greater than that observed in *p*-toluate-grown cells, which would account for the difference in basal rates of delactonization activity between benzoate- and *p*-toluate-grown cells.

Purification of 3-methylmuconolactone-CoA synthetase. To determine if 3-methylmuconolactone was the immediate substrate for the acyl-CoA synthetase, extract from *p*-toluate-grown cells was subjected to ammonium sulfate fractionation, hydrophobic interaction chromatography, gel filtration chromatography, and anion-exchange chromatography. Methylmuconolactone-CoA synthetase was purified 222-fold, resulting in a highly purified preparation of the acyl-CoA synthetase with a specific activity of 3.99 U/mg. The results are summarized in Table 1. SDS-PAGE revealed a few minor contaminating bands (data not shown); however, no muconolactone isomerase activity was present in the final preparation. Attempts to purify the acyl-CoA synthetase further to homogeneity were frustrated by recovery of low activities.

pH optimum. The pH optimum of the purified 3-methylmuconolactone-CoA synthetase activity was determined by the hydroxamate method with 150 mM MOPS or 150 mM Tris-HCl buffer at various pH levels. The maximum activity was observed at pH 8.0 in Tris-HCl buffer.

***M*$_r$.** The native M_r of 3-methylmuconolactone-CoA synthetase was estimated to be 128,000 by Superose 6 gel filtration chromatography. When a gel slice displaying 3-methylmuconolactone-CoA synthetase activity was excised from a nondenaturing polyacrylamide gel and subjected to SDS-PAGE, as described in Materials and Methods, only a single protein band was detected at an M_r of 62,000. This result suggests that the native enzyme is homodimeric, with a subunit M_r of 62,000.

Substrate specificity and inhibitors of the acyl-CoA synthetase. As both (+)-muconolactone and 4-methylmuconolactone chemically reacted with the components of the hydroxamate assay (not observed with 3-methylmuconolactone), 3-methylmuconolactone-CoA synthetase activities for a range of lactone compounds were measured by the enzyme-coupled assay. The ability of various compounds to act as substrates was investigated by replacing 3-methylmuconolactone in the reaction mixture with each compound at a concentration of 3 mM. Table 2 shows that the enzyme is highly specific for 3-methylmuconolactone, with very little activity displayed towards 4-methylmuconolactone or other lactones. No enzyme activity was observed with 2-methylmuconolactone, which also explains the limited catabolism of *m*-toluate by *Rhodococcus rhodochrous* N75. Although *m*-toluate can be metabolized to 2-methylmuconolactone, this lactone accumulates at this stage of the pathway. None of the other lactone analogues exhibited any significant activity; more significantly, 3-keto acids and 4-methyl-3-ketoadipate were not substrates for this enzyme, though these compounds are known to appear as CoA thioesters in the classical and modified β-ketoadipate pathways (14, 22). Phenylacetate, benzoate, and succinate, which are

TABLE 1. Purification of 3-methylmuconolactone-CoA synthetase[a]

Purification step	Vol of extract (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
None (crude extract)	42	810	14.62	0.018	1	100
$(NH_4)_2SO_4$ treatment	28	404	14.42	0.036	2.0	99
Phenyl agarose chromatography	90	39.6	8.77	0.222	12.3	60
Sephacryl S-100 chromatography	6	2.78	4.13	1.487	82.6	28
Mono Q chromatography	2	0.68	2.71 (11.186[b])	3.991 (16.450[b])	221.7	19

[a] Activity was measured by the hydroxyamate method.
[b] Value in parentheses indicates activity measured by the enzyme-coupled assay.

known as substrates for other types of acyl-CoA synthetases, did not show activity with 3-methylmuconolactone-CoA synthetase. The relative rate of activity with the racemic (±)-3-methylmuconolactone was approximately 88% of that seen with (−)-3-methylmuconolactone, which implies that the (+) isomer of 3-methylmuconolactone is less active, confirmed later by the very low activity observed with the resolved unnatural (+) isomer.

A number of lactone analogues and enzyme-inactivating agents were tested as inhibitors (Table 3). 2-Methylmuconolactone and 4-methylmuconolactone did not cause a significant inhibition, whereas (+)-muconolactone, 3-methylmuconolactone, 3-methyldienelactone, and 3,4-dimethylmuconolactone exhibited a slight inhibition. Divalent ions such as Cu^{2+} and Zn^{2+} significantly inhibited the enzyme activity. The substrate analogues did not inhibit the enzymes required for the coupled assay.

Further metabolism of the 3-methylmuconolactone-CoA ester. The mechanism for the delactonization of 3-methylmuconolactone-CoA is still unclear; however, hydrolytic cleavage via an enol-lactone would theoretically result in the formation of 4-methyl-3-ketoadipyl-CoA. Previous work on the metabolism of *p*-toluate by *Rhodococcus rhodochrous* N75 (5) demonstrated that cell extract from *p*-toluate-grown cells was able to transform 3-methylmuconolactone to 4-methyl-3-ketoadipate, albeit very slowly and at high concentrations of protein. CoA esters are extremely unstable, which may be the reason why 4-methyl-3-ketoadipate-CoA was not found to accumulate when the reaction product was examined by mass spectrometry. However, 3-ketoadipyl-CoA is known to absorb at 305 nm (14); therefore, in order to provide spectroscopic evidence that 4-methyl-3-ketoadipyl-CoA was formed from 3-methylmuconolactone-CoA, the following experiment was performed. 3-Methylmuconolactone (0.2 mM) was incubated in a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 0.5 mM CoA, 1 mM ATP, 1 mM $MgCl_2$, and 18 mU of purified 3-methylmuconolactone-CoA synthetase in a total volume of 1 ml. After a period of 30 min to permit formation of the lactone thioester, crude extract (0.26 mg of protein) from *p*-toluate-grown cells was added to the incubation mixture and the reaction was monitored spectrophotometrically at 305 nm. Figure 2 shows an increase in absorbance of light at 305 nm due to the formation of the magnesio adduct of a 3-ketoacyl-CoA. No increase in absorbance was observed when the crude extract was replaced with boiled extract or when the crude extract was omitted. Cell extract from benzoate-grown cells did not result in the production of a compound absorbing light at 305 nm, suggesting that the activity catalyzing the delactonization of the lactone-CoA ester is induced specifically by growth on *p*-toluate in cells of *Rhodococcus rhodochrous* N75. With or without the preincubation with purified 3-methylmuconolactone-CoA synthetase, extracts of *p*-toluate-grown or benzoate-grown cells showed no increase in absorbance at 305 nm when the 3-methylmuconolactone in the incubation mixture was replaced with 3-ketoadipate or 4-methyl-3-ketoadipate, indicating the absence of a CoA-dependent synthetase acting directly on these 3-keto acids.

TABLE 2. Substrate specificities of 3-methylmuconolactone-CoA synthetase[a]

Substrate	Activity (%)
(−)-3-Methylmuconolactone	100 (100[b])
(±)-3-Methylmuconolactone (racemic)	87.6 (80.8[b])
(+)-3-Methylmuconolactone (unnatural isomer)	5[c]
(+)-4-Methylmuconolactone	4.8
2-Methylmuconolactone	0 (1[b])
(+)-Muconolactone	7.6
1-Methylmuconodilactone	8.5[c]
(−)-3-Ethylmuconolactone	4.2 (2.7[b])
(−)-3-Isopropylmuconolactone	1.8[c]
3,4-Dimethylmuconolactone	<1 (0[b])
3-Methyl-*trans*-dienelactone	0
3-Ketoadipate	0
4-Methyl-3-ketoadipate	0
Phenylacetate	0
Benzoate	<1
Succinate	0

[a] 3-Methylmuconolactone-CoA synthetase activities were measured by the enzyme-coupled assay described in Materials and Methods. Approximately 0.8 μg of the purified enzyme was used per assay. Activities are quoted as percentages of the activity with 3-methylmuconolactone (10.2 U/mg) as the substrate.
[b] Measured by the hydroxamate method.
[c] Methylmuconolactone synthetase activity measured with cell extract from *p*-toluate-grown cells of *Rhodococcus rhodochrous*.

DISCUSSION

3-Methylmuconolactone, now fully recognized as an intermediate in the modified β-ketoadipate pathway of (methyl) aromatic compound catabolism in fungi (9, 22) and both gram-

TABLE 3. Inhibition of 3-methylmuconolactone-CoA synthetase by substrate analogues and enzyme-inhibiting agents[a]

Inhibitor (concn, mM)	Activity (%)
(+)-Muconolactone	74
2-Methylmuconolactone	94
(+)-4-Methylmuconolactone	96
3,4-Dimethylmuconolactone	68
(−)-3-Ethylmuconolactone	88
3-Methyl-*trans*-dienelactone	82
p-Chloromercuribenzoate (1)	0
$CuCl_2$ (1)	17
$ZnCl_2$ (1)	0

[a] The enzyme activity was measured by the enzyme-coupled assay described in Materials and Methods, with lactone analogues as inhibitors at a concentration of 0.5 mM. *p*-Chloromercurybenzoate, $CuCl_2$, and $ZnCl_2$ were incubated in the reaction mixture for 5 min prior to addition of the substrate. Activities are relative to those obtained in the absence of inhibitors.

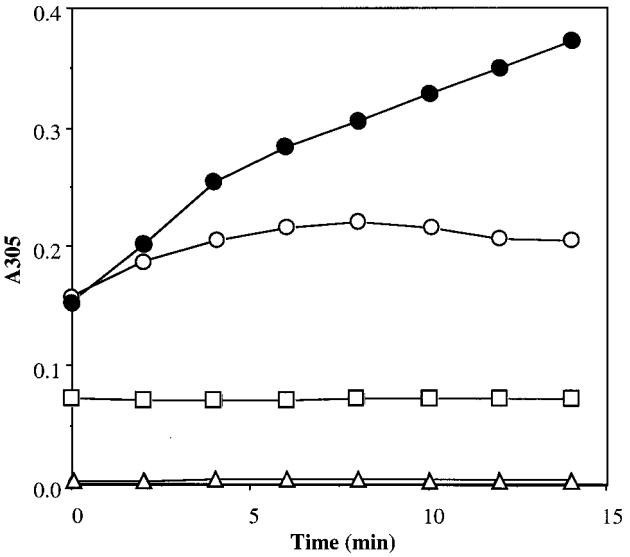

FIG. 2. Formation of a β-ketoacyl-CoA thioester. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 0.2 mM (○) or 0.5 mM (●) 3-methylmuconolactone, 0.5 mM CoA, 1 mM ATP, 1 mM $MgCl_2$, and 18 mU of 3-methylmuconolactone-CoA synthetase in a total volume of 1 ml. After a 30-min incubation, crude extract from *p*-toluate-grown cells (0.26 mg of protein) or benzoate-grown cells (0.21 mg of protein [□]) was added to the reaction mixture and the reaction was monitored by determining A_{305}. △, control with no crude extract.

negative (21) and gram-positive (5) bacteria, continues to offer novel features and to suggest that the modified 3-methylmuconolactone pathway may have evolved independently in these microbial groups. For instance, the muconate cycloisomerases of *Rhodococcus erythropolis* 1CP are recognizably distinct in both N-terminal sequence and the mechanisms of lactonization of chloromuconates from the corresponding enzymes of gram-negative genera (27, 28).

In *R. eutropha* (*Alcaligenes eutrophus*) JMP134, a (methyl) muconolactone isomerase (MMLI) encoded by the *mmlJ* gene (10) has been recognized as an isoenzyme of the muconolactone isomerase (EC 5.3.3.4) of the classical β-ketoadipate pathway (24, 25). This enzyme product of *mmlJ* was distinguished from classical muconolactone isomerase by the relative k_{cat} values with (+)-muconolactone, (4*R*,5*S*)-(+)-5-chloromuconolactone, and (+)-5-chloro-3-methylmuconolactones, for which the methyl-substituted analogue lowered the k_{cat} value of constant muconolactone isomerase significantly but had little effect on the MMLI isoenzyme form. Such activity is absent in *Rhodococcus rhodochrous*, which produces only one muconolactone isomerase enzyme on either benzoate or *p*-toluate, as described in this paper.

The nucleotide sequence of the *mmlI* gene of *R. eutropha* JMP134, which encodes its 4-methylmuconolactone methylisomerase, which generates the 3-methylmuconolactone substrate for the MMLI (21), has similarly been determined (10). It specifies a 113-amino-acid polypeptide (with two cysteine residues which are believed to be involved in the catalytic reaction) that dimerizes into the holoenzyme. Significantly, a 33-residue amino acid sequence from the tetrameric methylmuconolactone of *Rhodococcus rhodochrous* N75 shows no homology with any part of the *Ralstonia* sequence and, furthermore, the N-terminal residue of the rhodococcal enzyme is blocked (6a).

In addition to the distinctions between the rhodococcal and *Ralstonia* catabolism of methyl aromatics indicated above, this study produces compelling evidence that the further metabolism of 3-methylmuconolactone in *Rhodococcus rhodochrous* N75 may also differ substantially from that of *R. eutropha* JMP134 (9) and *T. cutaneum* (22, 23). The classical enol-lactone hydrolase (EC 3.1.1.24) of the β-ketoadipate pathway, which is responsible for the hydrolytic cleavage of the product of muconolactone isomerase or MMLI action on (+)-muconolactone, is inactive with the methyl-substituted product of muconolactone isomerase or MMLI action on 3-methylmuconolactone, and no novel isoenzymic enol-lactone hydrolase with this ability has yet been identified in *R. eutropha* or in rhodococci, the only two groups of bacteria known to process 4-methylmuconolactone to 4-methyl-3-ketoadipate. This study shows, in contrast, that the substrate specificity of the lactone-CoA synthetase and its appearance at high activity only in cells grown with *p*-cresol and *p*-toluate but not benzoate strongly imply that conversion of 3-methylmuconolactone to 4-methyl-3-ketoadipate occurs in the form of their CoA thioesters. Catabolism of similar furan compounds, e.g., furan 2-carboxylate, through their CoA thioesters has been known for some time (29, 30) and, significantly, also involves a double-bond shift, analogous to that observed in the muconolactone isomerase or MMLI reactions, to produce an enol-lactone structure that undergoes the hydrolytic step and keto-enol tautomerism to a keto acid, in this case 2-ketoglutarate. The 3-keto acid product of metabolism of 3-methylmuconolactone (Fig. 3) is seen to carry the CoA thioester on the same carboxylate group as that generated by the direct CoA esterification of 4-methyl-3-ketoadipate in *T. cutaneum* (22), which permits further metabolism in *Rhodococcus* by the route described for that fungus. *Rhodococcus rhodochrous* N75 did not, however, utilize 2-methylsuccinate, itaconate, or citramalate as a carbon source (28a). Extracts of *p*-toluate-grown cells of *Rhodococcus rhodochrous* N75 also showed no enzyme activities for the interconversion of methylsuccinyl-CoA, itaconyl-CoA, or citramalyl-CoA, though the assays, when checked with extracts of *p*-cresol-grown *T. cu-*

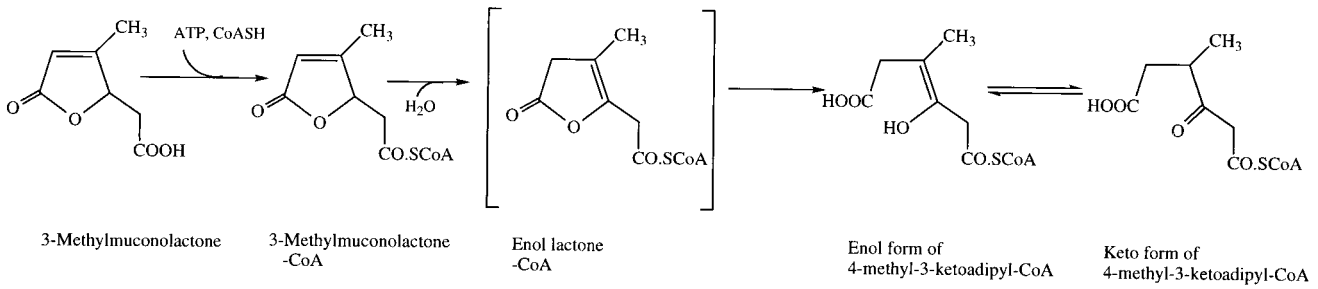

FIG. 3. Proposed pathway for the dissimilation of 3-methylmuconolactone in *Rhodococcus rhodochrous* N75.

taneum (22), were entirely satisfactory; only weak activity for the thiolysis of citramalyl-CoA was observed (26a).

While the distal steps of *p*-toluate catabolism in the rhodococci await further elucidation, it is clear that the genus continues to present novel features both in the biochemistry and enzymology of its utilization of alkylaromatic compounds.

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