Cyclohexa-1,5-Diene-1-Carbonyl-Coenzyme A (CoA) Hydratases of
Geobacter metallireducens and Syntrophus aciditrophicus: Evidence
for a Common Benzoyl-CoA Degradation Pathway in
Facultative and Strict Anaerobes

Franziska Peters,1 Yoshifumi Shinoda,1 Michael J. McInerney,2 and Matthias Boll3*

Institute for Biology II, Microbiology, University of Freiburg, 79104 Freiburg, Germany1; Department of Botany and
Microbiology, University of Oklahoma, Norman, Oklahoma 730192; and Institute of
Biochemistry, University of Leipzig, 04103 Leipzig, Germany3

Received 17 September 2006/ Accepted 13 November 2006

In the denitrifying bacterium Thauera aromatica, the central intermediate of anaerobic aromatic metabolism, benzoyl-Coenzyme A (CoA), is dearomatized by the ATP-dependent benzoyl-CoA reductase to cyclohexa-1,5-
diene-1-carboxyl-CoA (dienyl-CoA). The dienyl-CoA is further metabolized by a series of β-oxidation-like reactions of the so-called benzoyl-CoA degradation pathway resulting in ring cleavage. Recently, evidence was obtained that obligately anaerobic bacteria that use aromatic growth substrates do not contain an ATP-
dependent benzoyl-CoA reductase. In these bacteria, the reactions involved in dearomatization and cleavage of the aromatic ring have not been shown, so far. In this work, a characteristic enzymatic step of the benzoyl-CoA pathway in obligate anaerobes was demonstrated and characterized. Dienyl-CoA hydratase activities were determined in extracts of Geobacter metallireducens (iron reducing), Syntrophus aciditrophicus (fermenting), and Desulfococcus multivorans (sulfate reducing) cells grown with benzoate. The benzoate-induced genes putatively coding for the dienyl-CoA hydratases in the benzoate degraders G. metallireducens and S. aciditrophicus were heterologously expressed and characterized. Both gene products specifically catalyzed the reversible hydration of dienyl-CoA to 6-hydroxycyclohexenyl-CoA (K_m 80 and 35 μM; V_max, 350 and 550 nmol min^{-1} mg^{-1}, respectively). Neither enzyme had significant activity with cyclohex-1-ene-1-carbonyl-CoA or crotonyl-CoA. The results suggest that benzoyl-CoA degradation proceeds via dienyl-CoA and 6-hydroxycyclohexanoyl-CoA in strictly anaerobic bacteria. The steps involved in dienyl-CoA metabolism appear identical in all nonphoto-
synthetic anaerobic bacteria, although totally different benzene ring-dearomatizing enzymes are present in facultative and obligate anaerobes.
The corresponding gene, from *Geobacter metallireducens* (28). However, the further steps of aromatic metabolism, including ring dearomatization and cleavage, have not been demonstrated so far in any obligate anaerobe.

In a recent proteomic approach, 44 benzoate-induced genes were identified in *G. metallireducens*; several of their products were identified by mass spectrometric analysis (11, 28). The genes are organized in two clusters comprising those putatively involved in the benzoyl-CoA degradation pathway (bam genes [benzoic acid metabolism]) and in β-oxidation reactions yielding acetyl-CoA and carbon dioxide. The *bam* genes include the structural gene of benzoate-CoA ligase (*bamY*). Amino acid sequence similarities of products from other genes (bamA, *bamQ*, and *bamR*) with enzymes from *Thauera aromatica* suggested that similar steps may be involved in the conversion of the dearomatized product to an aliphatic C7 compound (reactions II to IV in Fig. 1, pathway A). The gene products BamB to -I were considered to be involved in benzoyl-CoA dearomatization (reaction II in Fig. 1, pathway A). However, attempts to determine a benzoyl-CoA reductase activity in strict anaerobes have failed so far (23, 28); as a consequence, information is lacking about the product of benzene ring dearomatization and the further metabolism of the dearomatized product.

In the genome of *G. metallireducens*, the product of the *bamR* gene (gi 7822357) was annotated as an enoyl-CoA hydratase. However, BamR showed exceptionally high amino acid sequence identities (68 to 72%) to dienoyl-CoA hydratases from *T. aromatica* and *Magnetospirillum* species (28). In contrast, no gene with such high similarities to dienoyl-CoA hydratase of *T. aromatica* is present in the genome of the fermenting, benzoate-degrading *Syntrophus aciditrophicus* (NC_007759). In this organism, a product of a gene coding for a putative enoyl-CoA hydratase (gi 85860872) showed amino acid sequence identities (47%) to enoyl-CoA hydratases from aromatic compounds degrading *Azotobacter* species (20, 24). Notably, thermodynamic considerations argue that the amount of energy available to fermentative, iron-reducing and sulfate-reducing anaerobes is not sufficient to support an ATP-dependent, two-electron reduction of benzoyl-CoA to a dienoyl-CoA intermediate (26). For this reason, it has been proposed that energetically more favorable four-electron reduction reactions occur, forming cyclohex-1-enyl-1-carboxylic-CoA from benzoyl-CoA (corresponding to pathway B in Fig. 1).

In order to investigate the benzoyl-CoA pathway in strict anaerobes for the first time, *bamR* from *G. metallireducens* (referred to *bamR*<sup>Ge</sup>) and the corresponding gene of *S. aciditrophicus* (referred to *bamR*<sup>S</sup>) were heterologously expressed with a His tag, purified, and characterized. The results clearly identified the gene products as highly specific, cyclohexa-1,5-diene-1-carboxylic-CoA hydratases. Dienoyl-CoA hydratase activities were also determined in extracts from cells of *G. metallireducens*, *S. aciditrophicus*, and *D. multivorans* grown with an aromatic substrate. These results strongly suggest that—except for *R. palustris* (Fig. 1B)—the benzoyl-CoA pathways are indeed identical in strictly and facultatively anaerobic bacteria metabolizing aromatic growth substrates, independent of the overall energy metabolism and the mode of benzoyl-CoA dearomatization.

**MATERIALS AND METHODS**

**Growth of bacterial cells and preparation of cell extracts.** *G. metallireducens* (DSMZ-Nr. 7210) and *D. multivorans* (DSMZ-Nr. 2059) were obtained from Deutsche Sammlung von Mikroorganismen. *S. aciditrophicus* was from the culture collection of M. McNerney. *G. metallireducens* (21), *S. aciditrophicus* (13), and *D. multivorans* (25) were cultured anaerobically in a mineral salt medium as described previously. The cells were harvested in the exponential growth phase by centrifugation (10,000 × g) and were stored in liquid nitrogen. For the preparation of crude extracts, frozen cells were suspended in 20 mM triethanolamine hydrochloride–KOH buffer, pH 7.3 (1 g cells in 1.5 ml buffer), 10 mM MgCl<sub>2</sub>, 10% glycerol, and 0.1 mg of DNase I. Cell lysates were obtained by passage through a French pressure cell at 137 MPa. After centrifugation at 100,000 × g (1 h at 4°C), the supernatant was used for further studies.

**Synthesis of CoA esters.** Crotonyl-CoA was purchased from Fluka (Ulm, Germany). Benzoyl-CoA and cyclohexenoyl-CoA were enzymatically synthesized from the corresponding carboxylic acids and CoA by using purified His-tagged benzoate-CoA ligase from *G. metallireducens* (specific activity with benzoate was 16 μmol min<sup>−1</sup> mg<sup>−1</sup>) (28). This enzyme catalyzes the following reaction: car-

**FIG. 1.** Initial steps of benzoyl-CoA pathway in *Thauera aromatica* (A) and *Rhodopseudomonas palustris* (B). In *T. aromatica*, reaction I is catalyzed by ATP-dependent benzoyl-CoA reductase (A). Further reactions are catalyzed by (d)ienoyl-CoA hydratase (reaction II), alcohol dehydrogenase (reaction III), and a ring-opening hydrolase (reaction IV). The following compounds are involved: compound 1, benzoyl-CoA; compound 2, dienoyl-CoA; compound 3, 3-hydroxypimelyl-CoA; compound 4, 6-oxocyclohex-1-ene-1-carbonyl-CoA; compound 5, 3-hydroxypimelyl-CoA; compound 6, cyclohex-1-ene-1-carbonyl-CoA; compound 7, 6-hydroxycyclohexane-1-carbonyl-CoA; compound 8, 6-oxocyclohexane-1-carbonyl-CoA; and compound 9, pimelyl-CoA. These compound numbers are used in the text to refer to the individual structures shown here. Fd<sub>red</sub> and Fd<sub>ox</sub>, reduced and oxidized ferredoxin, respectively.
The enoyl-CoA hydratase activity was determined by either a continuous or a discontinuous assay at 30°C. The continuous spectrophotometric assay is based on the difference in absorbance between dienoyl-CoA and 6-OH-cyclohexenoyl-CoA at 320 nm with \(\Delta A_{320} = 1700 \text{ M}^{-1} \text{ cm}^{-1}\) (19); it was routinely used during enzyme purification and for the determination of native molecular mass. For this purpose, cell extracts of the individual organisms grown with benzoyl-CoA were incubated with dienoyl-CoA, 6-OH-cyclohexenoyl-CoA, cyclohexenoyl-CoA, and 6-hydroxycyclohexenoyl-CoA were present in equal concentrations. Isolation and tests for purity of the coenzyme A esters were performed by preparative high-performance-liquid chromatography as described previously (18).

Cloning and expression of genes. Standard protocols were used for DNA isolation and amplification (2).

The bamRgeo gene (gi 78223357) was amplified from G. metallireducens DNA by using the primer pair ATGGAGCAAGGCTCTCTGAA (forward primer) and CCGGATCTCTGAGGGGCCC (reverse primer); for the bamRsyn gene (gi 85805872), the primer pair ATGGATCCTACACATTTTCTT (forward) and TTGGTCTTGAACCGCCTTTT (reverse) was used. Primers were designed in a way that the native stop codon was removed. Cloning positioned the gene of interest in frame with the DNA encoding a C-terminal peptide containing six histidines. Primers were synthesized by Biomers (Ulm, Germany).

The following PCR program using Taq and Pfu polymerase (18:1) was applied for amplification: 30 cycles of 94°C for 30 s, 55°C for 45 s, and 1°C for 45 s (bamRgeo) and 1°C for 1 min, 59°C for 1 min, and 72°C for 70 s (bamRsyn). The program started with 2 min at 94°C after the 30-cycle samples were incubated for a further 5 min at 72°C. The amplified genes were transferred into the pCRT7/CT-TOPO expression vector according to the manufacturer’s instructions (Invitrogen Life Technologies, Karlsruhe, Germany). The constructs were transformed into One Shot TOP10F’ and One Shot BL21(DE3)pLysS cells for propagation, analysis, and maintenance, as well as for the expression of the protein, respectively, according to the manufacturer’s instructions. Expression of the gene was performed at 18°C (bamRgeo) or 25°C (bamRsyn) overnight; induction was carried out when the cells reached an optical density at 578 nm of 0.5 by addition of 0.8 mM IPTG (isopropyl-\(\beta\)-D-thiogalactopyranoside). The temperature was chosen in order to obtain the highest amount of soluble gene product. Cells were harvested at an OD578 of 0.5 by addition of 0.8 mM IPTG (isopropyl-\(\beta\)-D-thiogalactopyranoside). The enoyl-CoA hydratase activity was determined by either a continuous or a discontinuous assay at 30°C. The continuous spectrophotometric assay is based on the difference in absorbance between dienoyl-CoA and 6-OH-cyclohexenoyl-CoA at 320 nm with \(\Delta A_{320} = 1700 \text{ M}^{-1} \text{ cm}^{-1}\) (19); it was routinely used during enzyme purification and for the determination of native molecular mass. For this purpose, cell extracts of the individual organisms grown with benzoyl-CoA were incubated with dienoyl-CoA, 6-OH-cyclohexenoyl-CoA, cyclohexenoyl-CoA, and 6-hydroxycyclohexenoyl-CoA were present in equal concentrations. Isolation and tests for purity of the coenzyme A esters were performed by preparative high-performance-liquid chromatography as described previously (18).

RESULTS AND DISCUSSION

Cyclohexa-1,5-dienoyl-CoA hydratase activities in cell extracts of G. metallireducens, S. aciditrophicus, and D. multivorans. A recent proteomic study with G. metallireducens suggested that steps II to IV in Fig. 1 are similar or identical in facultative and strict anaerobes. However, no experimental evidence for any of the enzymatic steps of the benzoyl-CoA pathway in strict anaerobes has been given so far. For this reason, the presence of dienoyl-CoA hydratases putatively catalyzing the second step in benzoyl-CoA degradation pathway in obligate anaerobes was studied in G. metallireducens, S. aciditrophicus, and D. multivorans. For this purpose, cell extracts of the individual organisms grown with benzene were incubated with dienoyl-CoA (compound 2) and the time-dependent conversion to 6-OH-cyclohexenoyl-CoA (compound 3) was determined by HPLC analysis as described previously (16, 18, 19).

Cell extracts from G. metallireducens rapidly converted dienoyl-CoA to 6-OH-cyclohexenoyl-CoA (2.5 \(\mu\text{mol mg}^{-1} \text{ min}^{-1}\)) (Table 1) in a protein- and time-dependent manner. In a second phase, both compounds were then slowly converted, most probably to compounds 4 and 5 (reactions III and IV in Fig. 1) (data not shown). Upon addition of NAD\(^+\) (0.5 mM) to the assay, the conversion of 6-OH-cyclohexenoyl-CoA was sifold increased, suggesting that NAD\(^+\) is the cosubstrate of 6-OH-cyclohexenoyl-CoA dehydrogenase catalyzing reaction III (Fig. 1). In contrast, virtually no conversion of cyclohexenoyl-CoA-CoA to 6-OH-cyclohexenoyl-CoA was observed in extracts of cells grown with benzene (Table 1). In addition, no significant conversion of dienoyl-CoA to 6-OH-cyclohexenoyl-CoA was observed in extracts of G. metallireducens cells grown with acetate (Table 1).

The results are in accordance with the observed induction of bamR during growth on benzene (28).

Extracts from D. multivorans and S. aciditrophicus cells...
TABLE 1. Hydratase activities in extracts of cells from strict anaerobes grown on different substrates

<table>
<thead>
<tr>
<th>Organism and growth substrate</th>
<th>Hydratase activity (U mg⁻¹)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dienoyl-CoA</td>
</tr>
<tr>
<td>G. metallireducens</td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Acetate</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S. aciditrophicus; benzoate</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>D. multivorans</td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.1</td>
</tr>
</tbody>
</table>

¹ The substrates tested for hydratase activities were cyclohex-1,5-diene-1-carboxyl-CoA (dienoyl-CoA; compound 2) and cyclohex-1-ene-1-carboxyl-CoA (cyclohexenoyl-CoA; compound 6). Activities with dienoyl-CoA were tested using the direct spectrophotometric assay; activity with cyclohexenoyl-CoA was tested by HPLC analysis of the substrate consumed/product formed. The mean value ± standard deviation from at least three determinations are indicated. One unit refers to μmol min⁻¹. ND, not determined.

grown with benzoate also showed a high level of dienoyl-CoA hydratase activity (Table 1). As described above, 6-OH-cyclohexenoyl-CoA was further converted to more polar products. In D. multivorans, the dienoyl-CoA hydratase activity in extracts from cells grown on lactate was only 5% of that in extracts of cells grown on benzoate (Table 1). In contrast to G. metallireducens, cell extracts of D. multivorans also catalyzed the hydration of cyclohexenoyl-CoA. However, the cyclohexenoyl-CoA hydration activity was even higher in extracts from cells grown with lactate (Table 1). Thus, this activity does not appear to be specifically associated with anaerobic aromatic acid metabolism.

The presence of benzoate-induced dienoyl-CoA hydratases in three different obligately anaerobic bacteria strongly suggests that dienoyl-CoA is an intermediate of the aromatic acid metabolism in strictly anaerobic bacteria, making it unlikely that the reduction of benzoyl-CoA occurs by a four-electron reaction, the dehydration of 6-OH-cyclohexenoyl-CoA to a more polar product in a time- and enzyme-dependent fashion (19). Clearly indicated that the product formed was 6-OH-cyclohexenoyl-CoA by direct analysis of the substrate consumed or product formed. The putative substrates or products dienoyl-CoA (compound 2) and 6-OH-cyclohexenoyl-CoA (compound 3) were enzymatically synthesized by the combined action of enriched benzoate-CoA ligase, benzoyl-CoA reductase, and dienoyl-CoA hydratase from T. aromatica (19).

To test whether BamRGeo and BamRSyn code for dienoyl-CoA hydratases, the substrate preferences of the enzymes were investigated in view of their possible roles in benzoate metabolism. For this purpose, the putative hydratase activities of the produced proteins were monitored in a discontinuous assay followed by HPLC analysis of the substrate consumed or product formed. The putative substrates or products dienoyl-CoA (compound 2) and 6-OH-cyclohexenoyl-CoA (compound 3) were enzymatically synthesized by the combined action of enriched benzoate-CoA ligase, benzoyl-CoA reductase, and dienoyl-CoA hydratase from T. aromatica (19).

Expression of bamRGeo and bamRSyn and purification of the gene products. Results from the in vitro dienoyl-CoA assays indicated that cells from G. metallireducens, S. aciditrophicus, and D. multivorans grown with benzoate contain benzoate-induced dienoyl-CoA hydratases. In a recent study, the benzoate-induced bamR gene (gi 78223357), annotated as an enoyl-CoA hydratase, has been suggested to code for a putative dienoyl-CoA hydratase (28). In contrast, in the recently sequenced genome of the fermenting, benzoate-degrading S. aciditrophicus (NC_007759), no open reading frame with high similarities to dienoyl-CoA hydratase of T. aromatica is present. Instead, an open reading frame coding for a putative enoyl-CoA hydratase (gi 85860872) showed amino acid identities (47%) to benzoate-induced putative dienoyl-CoA hydratase from Azoarcus species (20, 24). Both genes (referred to as bamRGeo and bamRSyn) were amplified with a C-terminal His₆ tag by PCR, and the DNA obtained was cloned and overexpressed in Escherichia coli.

Expression of bamRGeo and bamRSyn in E. coli yielded soluble proteins of approximately 30 kDa (Fig. 2). The molecular mass corresponded to that predicted from the gene sequence (30.4 kDa for BamRGeo, and 29.9 kDa for BamRSyn [including His tags]). Each protein was purified from the soluble protein fraction of recombinant E. coli cell extracts by Ni-chelating chromatography in a single step. BamRGeo was eluted between 100 to 200 mM imidazole, and BamRSyn was eluted between 280 and 400 mM imidazole. Almost all of the soluble protein of E. coli eluted with the equilibration buffer (Fig. 2).

Properties of BamRGeo and BamRSyn. The native molecular masses were determined by gel filtration, giving 92 ± 15 kDa for BamRGeo and 115 ± 10 kDa for BamRSyn. The mass of BamRGeo suggested an unusual α₅ composition; for this reason, an additional Ferguson blot analysis (12) was carried out, giving a mass of 70 ± 10 kDa. The data suggest the composition of BamRSyn to be α₄, whereas the composition of BamRGeo is suggested to be either α₃ or α₅. The subunit architecture of BamRGeo corresponds to that of dienoyl-CoA hydratase of T. aromatica, which may result from the higher amino acid sequence similarities between BamRGeo and dienoyl-CoA hydratase from T. aromatica (19).

To test whether BamRGeo and BamRSyn code for dienoyl-CoA hydratases, the substrate preferences of the enzymes were investigated in view of their possible roles in benzoate metabolism. For this purpose, the putative hydratase activities of the produced proteins were monitored in a discontinuous assay followed by HPLC analysis of the substrate consumed or product formed. The putative substrates or products dienoyl-CoA (compound 2) and 6-OH-cyclohexenoyl-CoA (compound 3) were enzymatically synthesized by the combined action of enriched benzoate-CoA ligase, benzoyl-CoA reductase, and dienoyl-CoA hydratase from T. aromatica (19).
coelution with the conjugated 1,5-dienoyl-CoA compound and by the typical UV spectrum (19). With the spectrophotometric assay, following the hydration of dienoyl-CoA to 6-OH-cyclohexenoyl-CoA (0.5 mM each), rates of 550 \(\text{mol min}^{-1} \text{mg}^{-1}\) (BamRGeo) and 350 \(\text{mol min}^{-1} \text{mg}^{-1}\) (BamRSyn) were determined; the rate in the reverse reaction was 730 \(\text{mol min}^{-1} \text{mg}^{-1}\) for BamRGeo (Table 2). The apparent \(K_m\) values for dienoyl-CoA were 85 \(\mu\text{M}\) (BamRGeo) and 30 \(\mu\text{M}\) (BamRSyn); the apparent \(K_m\) for 6-OH-cyclohexenoyl-CoA for BamRGeo was 50 \(\mu\text{M}\). The equilibrium concentration ratio between the substrate and the corresponding product was nearly 1:1 (Fig. 3). The discontinuous HPLC assay was also used to monitor a possible hydration of cyclohexenoyl-CoA (compound 6) or crotonyl-CoA (each at 1 mM) by both BamR enzymes. Neither substrate was converted at a significant rate by BamRGeo and BamRSyn.

The results obtained clearly indicate that BamRGeo and BamRSyn code for highly specific dienoyl-CoA hydratases catalyzing the second step in the benzoyl-CoA pathway in \(G.\) \textit{metallireducens} and \(S.\) \textit{aciditrophicus}. This result is in accordance with the activity measurements in cell extracts.

**Phylogenetic analysis of dienoyl-CoA hydratases.** The unambiguous identification of the function of the two dienoyl-CoA hydratases allowed a phylogenetic analysis and comparison with other enoyl-CoA hydratases. Obviously dienoyl-CoA hydratases can be distinguished from other enoyl-CoA hydratases by their amino acid sequence (Fig. 4). The results obtained suggest that there are two phylogenetic clusters of dienoyl-CoA hydratases: one including the enzymes from \(T.\) \textit{aromatica}, \(G.\) \textit{metallireducens}, and deduced gene products from \(Magnetospirillum\) species and a second with the enzyme from \(Azoarcus\) species. The putative dienoyl-CoA hydratases from \(Magnetospirillum\) and \(Azoarcus\) species are deduced from the amino acid sequence. In panel B, only some representative enoyl-CoA hydratases are shown from bacteria that are not capable growing on aromatics along with enoyl-CoA hydratases of unknown function from the genomes of \(G.\) \textit{metallireducens} and \(S.\) \textit{aciditrophicus}.

**FIG. 3.** Conversion of 6-OH-cyclohexa-1,ene-1-carbonyl-CoA and cyclohexa-1,ene-1-carbonyl-CoA (0.5 mM each) by purified BamRGeo. The numbers of the peaks refer to the structures shown in Fig. 1. Shown is the time-dependent dehydration of 6-OH-cyclohexenoyl-CoA (peak 3) to dienoyl-CoA (peak 2) from HPLC analysis of samples taken at 0 s (A), 15 s (B), 45 s (C), and 180 s (D). The reaction leveled off after 3 min by reaching the 1:1 equilibrium between substrate and product. Detection of all compounds was carried out by a UV monitor at 260 nm.

**FIG. 4.** Phylogenetic tree of dienoyl-CoA hydratases (A) and other enoyl-CoA hydratases (B). Enzymes from organisms in boldface have been characterized as dienoyl-CoA hydratases.

**TABLE 2.** Properties of cyclohexa-1,5-diene-1-carbonyl-CoA hydratases from \(G.\) \textit{metallireducens} (BamRGeo) and \(S.\) \textit{aciditrophicus} (BamRSyn)

<table>
<thead>
<tr>
<th>Hydratase</th>
<th>Substrate used</th>
<th>Substrates not used</th>
<th>(K_m) ((\mu\text{M}))</th>
<th>Sp act ((\text{\textmu}\text{mol min}^{-1} \text{mg}^{-1}))</th>
<th>Catalytic no. for hydration ((\text{s}^{-1}))</th>
<th>Subunit composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamRGeo</td>
<td>Dienoyl-CoA</td>
<td>Cyclohexa-1,ene-1-carbonyl-CoA, crotonyl-CoA</td>
<td>85 ± 20 (dienoyl-CoA), 50 ± 20 (6-OH-cyclohexenoyl-CoA)</td>
<td>550</td>
<td>730</td>
<td>280</td>
</tr>
<tr>
<td>BamRSyn</td>
<td>Dienoyl-CoA</td>
<td>Cyclohexa-1,ene-1-carbonyl-CoA, crotonyl-CoA</td>
<td>30 ± 10 (dienoyl-CoA)</td>
<td>350</td>
<td>175</td>
<td>(\alpha_4)</td>
</tr>
</tbody>
</table>

* The subunit was 30 kDa.
from S. aciditrophicus and deduced gene products from Azoarcus species. It is very likely that the deduced putative dienoyl-CoA hydratases from Magnetospirillum magnetotacticum (ZP 100053821) and Magnetospirillum sp. strain TS-1 (BAD 42370), both showing 68% and 67% amino acid sequence identities to BamRGeo, and the enzymes from Azoarcus sp. (Azoarcus sp. strain EbN1, YM 160034; Azoarcus evansii, CAD 21363; and Azoarcus sp. strain CIB, AAQ08815) are also specific dienoyl-CoA hydratases catalyzing the second step in anaerobic benzoate metabolism in both facultative anaerobes.

It is interesting to note that the phylogenetic dienoyl-CoA hydratase clusters represent neither the phylogenetic relationship nor a common overall energy metabolism of the organisms (facultative versus obligate anaerobes) but rather suggest that distribution of dienoyl-CoA hydratase genes occurred by horizontal gene transfer. The high and distinguishing amino acid sequence similarities of dienoyl-CoA hydratases, especially of the Thauera/Geobacter type, may allow the construction of gene probes for conserved regions. With such gene probes, both facultative and obligately anaerobic bacteria with the capacity to degrade aromatic compounds could be detected in less defined samples (enrichment cultures, environmental samples, etc.). So far gene probes for the ATP-dependent deamortizing benzoyl-CoA reductases only detect facultatively anaerobic aromatic compounds degrading bacteria (27).

Conclusions. In this work, evidence was obtained that with exception of R. palustris, dienoyl-CoA hydratases are involved in the aromatic acid metabolism of all facultatively and strictly anaerobic bacteria, independent of the overall energy metabolism (nitrate-, iron-, and sulfate-reducing and -fermenting bacteria) and the nature of the benzoyl-CoA deamortizing enzyme. This result has an impact on the question how the aromatic ring is deamortized in strictly anaerobic bacteria. A hydroxylation of the ring or a reduction by more than two electrons would not require the expression of a highly specific dienoyl-CoA hydratase during growth on aromatic compounds and can now be ruled out. Despite the fact that for unknown reasons a benzoyl-CoA-reducing activity could not be determined so far in any strictly anaerobic bacterium, the results provide a first biochemical evidence that the central benzoyl-CoA pathway proceeds via identical intermediates in nearly all anaerobes.

ACKNOWLEDGMENTS

We are grateful to S. Wischgoll (Freiburg) for help in cultivation of G. metallireducens.

This work was funded by the Deutsche Forschungsgemeinschaft. The Yamada Science Foundation (Osaka, Japan) gave Y.S. financial support for this study.

REFERENCES