A Novel Alternate Anaplerotic Pathway to the Glyoxylate Cycle in Streptomyceses

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ccr encoding crotonyl coenzyme A (CoA) reductase (CCR), which catalyzes the conversion of crotonyl-CoA to butyryl-CoA in the presence of NADPH, was previously cloned from Streptomyces collinus. We now report that a complete open reading frame, designated meaA, is located downstream from ccr. The predicted gene product showed 35% identity with methylmalonyl-CoA mutases from various sources. In addition, the predicted amino acid sequences of S. collinus ccr and meaA exhibit strong similarity to that of adhA (43% identity), a putative alcohol dehydrogenase gene, and meaA (62% identity) of Methylobacterium extorquens, respectively. Both adhA and meaA are involved in the assimilation of C1 and C2 compounds in an unknown pathway in the isocitrate lyase (ICL) - negative Methylobacterium. We have demonstrated that S. collinus can grow with acetate as its sole carbon source even though there is no detectable ICL, suggesting that in this organism ccr and meaA may also be involved in a pathway for the assimilation of C2 compounds. Previous studies with streptomyceses provided a precedent for a pathway that initiates with the condensation of two acetyl-CoA molecules to form butyryl-CoA, which is then transformed to succinyl-CoA with two separate CoB12-mediated rearrangements and a series of oxidations. The biological functions of ccr and meaA in this process were investigated by gene disruption. A ccr-blocked mutant showed no detectable crotonyl-CoA reductase activity and, compared to the wild-type strain, exhibited dramatically reduced growth when acetate was the sole carbon source. An meaA-blocked mutant also exhibited reduced growth on acetate. However, both methylmalonyl-CoA mutase and isobutyryl-CoA mutase, which catalyze the two CoB12-dependent rearrangements in this proposed pathway, were shown to be present in the meaA-blocked mutant. These results suggested that both ccr and meaA are involved in a novel pathway for the growth of S. collinus when acetate is its sole carbon source.

Aerobic organisms growing with acetate as their sole carbon source require the glyoxylate cycle for the biosynthesis of cellular substances (21). This cycle catalyzes the net synthesis of succinate from two acetyl coenzyme A (CoA) molecules (Fig. 1a). The first two steps of the process involve the formation of citrate from a condensation of acetyl-CoA and oxaloacetate and a subsequent isomerization to form isocitrate and are catalyzed by two citric acid cycle enzymes. The resulting isocitrate is either oxidatively decarboxylated to form α-ketogluta- rate in the citric acid cycle or converted to succinyl-CoA and oxaloacetate in the glyoxylate cycle (22). The later part of the process is catalyzed by isocitrate lyase (ICL). Glyoxylate thus generated is condensed with the second molecule of acetyl-CoA in order to regenerate oxaloacetate. ICL therefore has a central role in metabolism and in regulating the fate of isocitrate.

Some bacteria do not appear to have an ICL but nonetheless grow with acetate as their sole carbon source (3). The pathway by which these organisms can assimilate acetate has presented a perplexing metabolic problem for several decades. Two pathways in which acetate is first converted to glyoxylate (which is further metabolized by a series of anaplerotic reactions) have been considered for the case of the ICL-negative methylotrophs, as exemplified by Methylobacterium extorquens (9, 31). There is no data, however, to suggest that either of these pathways actually functions in ICL-negative bacteria for their growth on acetate. Furthermore, results from the recent analysis of two genes, meaA and adhA in M. extorquens, which are required for growth on acetate, have been inconsistent with such a function for these pathways (9, 31). The role of meaA and adhA in the acetate assimilation process remains unknown, although analysis of meaA suggests that it may encode a CoB12-dependent mutase.

In this study, we report the cloning of two genes (meaA and ccr) from Streptomyces collinus whose amino acid sequences show similarity to those of the M. extorquens genes. A gene disruption approach was used to investigate the role of these genes in a novel anaplerotic pathway for synthesis of succinyl-CoA from acetyl-CoA that involves a butyryl-CoA intermediate (Fig. 1b). Consistent with this proposal are the observations that both the ccr and meaA mutants had dramatically impaired growth on acetate (compared to the wild-type strain) and that the ccr mutant was no longer able to convert crotonyl-CoA to butyryl-CoA. However, the levels of both isobutyryl-CoA mutase and methylmalonyl-CoA mutase (MCM), which catalyze the two separate CoB12-dependent rearrangements in the proposed pathway, were unchanged in the meaA mutant. These results provide evidence that meaA encodes a novel CoB12-dependent mutase involved in a pathway of acetate assimilation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacteria, plasmids, phages, and cosmids used in this study are listed in Table 1. The S. collinus cosmid library was kindly provided by Claudio Denoya at Pfizer Inc. Escherichia coli TG1 was grown in Luria-Bertani medium at 37°C supplemented with ampicillin (100 μg/ml) when necessary (28). E. coli DH10B was grown in Luria-Bertani medium supplemented with tetracycline (5 μg/ml) and kanamycin (5 μg/ml) when necessary. Cultures of S. collinus and Streptomyces lividans were normally grown in MYG medium (containing 10 g of malt extract, 4 g of yeast extract, and 4 g of glucose in 1 liter of distilled water [pH 7.0]) at 28°C. The following antibiotic concentrations were used for Streptomyces: thiostrepton (25

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Enzyme assays. The enzyme assay for crotonyl-CoA reductase activity has been described previously (37). For measuring ICL activity, S. collinus mycelia from a 48-h fermentation were harvested and washed twice with a buffer containing 50 mM morpholino propane sulfonic acid, 5 mM dithiothreitol, 15 mM MgCl₂, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride at pH 7.3. The cell pellet was resuspended in the same buffer, and the resulting cell suspension was passed through a French pressure cell (Aminco) at 80 to 100 MPa. The cell lysate was centrifuged (30,000 g for 1 h) and the supernatant containing 5 mM NADH was used for the ICL assay. ICL activity was assayed spectrophotometrically in a coupled enzyme assay with lactate dehydrogenase (11). The 1-ml crude extract, 18 U of lactate dehydrogenase, 0.2 mM NADH, and 5 mM of threo-(-)-isocitrate. Protein concentrations were determined by the Bradford (6) method standardized with bovine serum albumin and iminocin (200 μg/ml). R2YE medium was used for the regeneration of Streptomyces protoplasts (16). Minimal medium (16) was used for growth of Streptomyces mutants for phenotypic analysis and for fatty acid analysis. Perdeuterated precursors were added to minimal medium at the time of inoculation. For ICL activity, S. collinus was grown in minimal medium containing Tween at a final concentration of 1% (wt/vol) (17).

DNA isolation and manipulation. Streptomyces chromosomal DNA was prepared as described by Hopwood et al. (16). The alkaline lysis method of Kieser (20) was used to prepare plasmids from S. lividans. E. coli plasmid DNA was prepared with the Qiagen kit (Chatsworth, Calif.) or the alkaline lysis method of Sambrook et al. (28). The probes were radiolabeled with the random primers of PCR amplification of S. collinus ccr according to the method of Wallace et al. (36). The enzyme assay for crotonyl-CoA reductase activity has been described previously (37).

Hybridizations. For Southern hybridization, genomic DNA or cosmid DNA was completely digested with selected restriction endonucleases, and the fragments, separated by agarose gel electrophoresis, were transferred to nylon membranes (33). For colony hybridization, E. coli colonies were lifted onto nylon membranes which were subsequently denatured and neutralized according to the method of Sambrook et al. (28). The probes were radiolabeled with the random primers of PCR amplification of S. collinus ccr according to the method of Wallace et al. (36). The enzyme assay for crotonyl-CoA reductase activity has been described previously (37).

Transormations. Competent E. coli cells were prepared and transformed by standard methods (28). Protoplasts of S. collinus and S. lividans were prepared and transformed according to Hopwood et al. (16).

Enzyme assays. The enzyme assay for crotonyl-CoA reductase activity has been described previously (37). For measuring ICL activity, S. collinus mycelia from a 48-h fermentation were harvested and washed twice with a buffer containing 50 mM morpholino propane sulfonic acid, 5 mM dithiothreitol, 15 mM MgCl₂, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride at pH 7.3. The cell pellet was resuspended in the same buffer, and the resulting cell suspension was passed through a French pressure cell (Aminco) at 80 to 100 MPa. The cell lysate was centrifuged (30,000 g for 1 h) and the supernatant containing 5 mM NADH was used for the ICL assay. ICL activity was assayed spectrophotometrically in a coupled enzyme assay with lactate dehydrogenase (11). The 1-ml crude extract, 18 U of lactate dehydrogenase, 0.2 mM NADH, and 5 mM of threo-(-)-isocitrate. Protein concentrations were determined by the Bradford (6) method standardized with bovine serum albumin.

Nucleotide sequence analysis. DNA fragments were subcloned in pBluescript II KS(+) (+) and overlapping deletion clones were generated. Single-stranded DNA templates were isolated from cultures of E. coli TG1 phageinfected transformants (35). DNA sequencing was carried out by the dideoxy chain-termination method of Sanger et al. (29) with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and 32P-labeled dATP. Sequence ambiguities were resolved by automated DNA sequencing with MacVector software (version 4.0; Eastman Kodak Company). The BLAST family of programs (1, 2) was used to compare nucleotide and deduced amino acid sequences. For Southern hybridization, genomic DNA or cosmid DNA was completely digested with selected restriction endonucleases, and the fragments, separated by agarose gel electrophoresis, were transferred to nylon membranes (33). For colony hybridization, E. coli colonies were lifted onto nylon membranes which were subsequently denatured and neutralized according to the method of Sambrook et al. (28). The probes were radiolabeled with the random primers of PCR amplification of S. collinus ccr according to the method of Wallace et al. (36). The enzyme assay for crotonyl-CoA reductase activity has been described previously (37).

RESULTS

Comparison of the deduced amino acid sequence of S. collinus ccr with the database sequences. Crotonyl-CoA reductase, which catalyzes the conversion of crotonyl-CoA to butyryl-CoA in the presence of NADPH, has previously been shown to have 35% identity with members of the quinone oxidoreductase superfamily (37). Analysis of an updated database showed

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**TABLE 1. Strains, plasmids, cosmids, and phages used**

<table>
<thead>
<tr>
<th>Strain, plasmid, phage, or cosmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. collinus Tu1892</td>
<td>Wild type</td>
<td>36</td>
</tr>
<tr>
<td>H1</td>
<td>Mutant carrying ermE after ccr replacement</td>
<td>This work</td>
</tr>
<tr>
<td>H2</td>
<td>Mutant carrying ermE after meaA replacement</td>
<td>This work</td>
</tr>
<tr>
<td>S. lividans TK24</td>
<td>SLP + str</td>
<td>15</td>
</tr>
<tr>
<td>E. coli TG1</td>
<td>Δ(lac-pro)supE thi s hexD5F</td>
<td>8</td>
</tr>
<tr>
<td>DH10</td>
<td>Host strain of the cosmids library</td>
<td>14</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript II</td>
<td>Phagemid vector (amp lacZ')</td>
<td>Stratagene</td>
</tr>
<tr>
<td>KS(+)</td>
<td>Streptomyces-E. coli bidirectional vector (ser amp lacZ')</td>
<td>23</td>
</tr>
<tr>
<td>pHJL400</td>
<td>pUC18 carrying ermE</td>
<td>Pfizer Inc.</td>
</tr>
<tr>
<td>pZYB2</td>
<td>pUC18 with a 6.9-kb BamHI insert from S. collinus</td>
<td>37</td>
</tr>
<tr>
<td>pLH1</td>
<td>pHJL400 with a 6.9-kb insert from pZYB2</td>
<td>This work</td>
</tr>
<tr>
<td>pLH2</td>
<td>pHJL1 with a 2.5-kb-segment deletion</td>
<td>This work</td>
</tr>
<tr>
<td>pLH3</td>
<td>pHJ2 with ermE inserted in the ccr coding region</td>
<td>This work</td>
</tr>
<tr>
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<td>pKS(+) with a 3.2-kb BglII insert from Cos21B5</td>
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</tr>
<tr>
<td>pLH6</td>
<td>pLH5 with ermE inserted in the meaA coding region</td>
<td>This work</td>
</tr>
<tr>
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<td>VCSM13</td>
<td>Km' derivative of M13K07</td>
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<tr>
<td>Cosmid</td>
<td>Cos21B5</td>
<td>Cosmid clone isolated from the cosmids library of S. collinus</td>
</tr>
</tbody>
</table>

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**Comparative genomics.** A comparison of the deduced amino acid sequence of S. collinus ccr with the database sequences. Crotonyl-CoA reductase, which catalyzes the conversion of crotonyl-CoA to butyryl-CoA in the presence of NADPH, has previously been shown to have 35% identity with members of the quinone oxidoreductase superfamily (37). Analysis of an updated database showed
that the predicted ccr product has stronger similarity overall (43% identity, 54% similarity) to the predicted adhA product (ADH) of M. extorquens, which is involved in the pathway for the assimilation of C₁ and C₂ compounds (9). The catalytic function of ADH is unknown.

Cloning and sequence analysis of S. collinus meaA. Sequencing and analysis of a 1.0-kb DNA region on pZYB2 immediately downstream of ccr revealed a partial open reading frame (ORF) (Fig. 2a) with the established pattern of GC bias and preferred codon usage for streptomycetes (39). In order to clone the remaining gene, a 0.5-kb PstI-BamHI fragment of pZYB2 containing this ORF was used to screen the S. collinus cosmid library. Of 285 clones screened, 1 showed strong hybridization to the probe and was designated Cos21B5. Southern analysis of the Cos21B5 DNA with the same probe showed a 3.2-kb BglII hybridizing fragment which contains a 1.7-kb DNA region downstream of the partial ORF. The fragment was subcloned in both orientations in pBluescript II KS(+) and the 1.7-kb DNA region was sequenced (Fig. 3).

Codon preference analysis of the 1.7-kb DNA sequence together with the upstream 1.0-kb DNA sequence showed one complete ORF and one incomplete ORF, designated meaA and ORF1, respectively (Fig. 2a). The nucleotide sequences of the two ORFs indicated that they are transcribed in the same direction as the upstream ccr. The most likely start codon of meaA is the ATG at nucleotides (nt) 569 to 571, which is

FIG. 2. (a) Restriction map of the 8.5-kb fragment of S. collinus genomic DNA cloned as overlapping inserts in pZYB2 and Cos21B5. The location of ccr described by Wallace et al. (37) is shown. Arrows indicate ORFs and their orientations. The arrow with a dashed line shows the position and orientation of the incomplete ORF. (b) Diagram showing the construction of pLH3 and the disruption of S. collinus ccr by insertion of an erythromycin-resistance gene (ermE). (c) Diagram showing the construction of pLH6 and the disruption of S. collinus meaA. Abbreviations: B, BamHI; Bg, BglII; M, MluI; P, PstI; X, XbaI.
preceded by a putative ribosome-binding site (RBS; GAGG) 6 nt upstream. The putative translational start codon of meaA overlaps the stop codon of ccr. The meaA gene product is a polypeptide of 676 amino acids with a calculated molecular mass of 73,797 Da and an estimated isoelectric point of 5.22. The stop codon of meaA overlaps the start codon (ATG at nt 2590 to 2592) of ORF1. ORF1 is likely preceded by a putative RBS (GGAGG) 7 nt upstream of its start codon. No in-frame stop codon was present in the sequenced region downstream of the ORF1 start codon.

Deduced functions of meaA and ORF1. Comparison of the deduced amino acid sequence of meaA with database sequences showed 62% identity with the meaA product of M. extorquens (Fig. 4) (9, 31) and only 35% identity with the large subunits of MCMs from prokaryotic as well as eukaryotic sources (4, 13, 18, 38) and 35% identity with the large subunits of isobutyryl-CoA mutases from Streptomyces cinnamonensis and Streptomyces coelicolor (27). It has been reported that the meaA product of M. extorquens also has about 35% identity with subunits of MCMs (31). A putative CoB 12-binding motif of S. collinus meaA is located between residues 547 and 620 (Fig. 4). The M. extorquens meaA product appears not to be an MCM and has been shown to be involved in C1 and C2 compound assimilation, although the biochemical reaction which it catalyzes is unknown (9).

The deduced protein product of ORF1 showed 35% identity to the N-terminal sequences of citrate lyase of Klebsiella pneumoniae (5) and Haemophilus influenzae (12, 19).

Targeted disruption of the S. collinus ccr and meaA. Strong sequence similarity of S. collinus ccr and meaA with the M. extorquens adhA and meaA. In M. extorquens, adhA and meaA are divergently transcribed and are separated from an ORF whose deduced product shows strong similarity to catalases of various sources (9) (Fig. 5). Both adhA and meaA of M. extorquens are thought to be involved in a pathway which is responsible for growth on C1 and C2 compounds. The S. collinus ccr and meaA are transcribed in the same direction, and there is no intergenic noncoding region between the two ORFs (Fig. 5).

FIG. 3. Nucleotide and corresponding amino acid sequences of meaA and ORF1 of S. collinus. Putative RBSs and significant restriction enzyme recognition sequences are underlined. Translational start codons are identified as the beginning of the ORFs. Stop codons are identified by asterisks.
possibility was investigated with a gene replacement strategy. The vector used is a Streptomyces-E. coli shuttle vector, pHJL400, that lacks the partition function of plasmid SCP2 and is thus segregationally unstable in streptomycetes grown on media without thiostrepton selection (23). To construct pH3, the 6.9-kb BamHI fragment of pZYB2 was first subcloned in the BamHI site of pHJL400 to generate pLH1 (Fig. 2b). Probing of BgII digests of S. collinus wild-type genomic DNA with cer gave signals at 5.5 and 2.8 kb, while S. collinus wild-type genomic DNA BamHI digest gave one signal at 6.9 kb (data not shown). The signals seen in S. collinus H1 were consistent with the integration of emrE into the wild-type S. collinus cer chromosomal region by homologous recombination (Fig. 2b). Probing of BgII digests of S. collinus wild-type and H4 genomic DNA with the 0.5-kb PstI/BamHI DNA fragment of meaA gave signals at 3.2 and 4.8 kb, respectively (data not shown). The 1.6-kb increase in the size of the BgII fragment seen in H4 indicated that double crossover had indeed occurred (Fig. 2c).

Crotonyl-CoA reductase activity of the ccr-blocked mutant. Crotonyl-CoA reductase activity was assayed in wild-type S. collinus and in the ccr-blocked mutant. A similar level of crotonyl-CoA reductase activity was found in the wild type as reported previously (37). However, the ccr-blocked mutant showed no detectable crotonyl-CoA reductase activity under the same conditions.

Isobutyryl-CoA mutase activity of the meaA-blocked mutant. Since meaA has similarity in its predicted amino acid sequence to the genes encoding isobutyryl-CoA mutases, the presence of isobutyryl-CoA mutase activity was measured in the meaA-blocked mutant and in wild-type S. collinus. Both the wild type and the mutant were grown in minimal medium containing perdeuterated valine at a final concentration of 100 mM, and their fatty acids were extracted and analyzed. The fatty acid profile of the mutant strain was essentially identical to that of the wild type. In addition, in both strains, approximately 86% of the isopalmitate pool was labeled with seven deuteriums, consistent with the degradation of the labeled valine to isobutyryl-CoA and the utilization of this material as a starter unit consistent with the isomerization of perdeuterated valine to isobutyryl-CoA mutase activity was measured in the wild type as described above.

The 1.6-kb BglII fragment of pHJL6 was subcloned into the BamHI site within the meaA coding region to generate pH6. In both plH3 and pH6, emrE was oriented such that its direction of transcription was the same as that of the interrupted gene. Each construct was first introduced into S. lividans TK24, and plasmid DNA isolated from S. lividans transformants was used to transform S. collinus. Most colonies on the regeneration plates upon transformation of S. collinus were thiostrepton resistant (Ts') and lincomycin resistant (Lm'). Ts' Lm' colonies were isolated after the colonies resistant to both thiostrepton and lincomycin were propagated without thiostrepton selection. Three such colonies, designated S. collinus H1, H2, and H3, were isolated upon transformation of S. collinus with pH3 and propagation of the transformants; two colonies, S. collinus H4 and H5, were isolated from S. collinus pH6 transformants. The genotypes of the Thi' Lm' colonies were confirmed by Southern hybridization of chromosomal DNA restriction digests with either cer or meaA. Probing of BamHI digest of S. collinus H1 genomic DNA with cer gave signals at 5.5 and 2.8 kb, while S. collinus wild-type genomic DNA BamHI digest gave one signal at 6.9 kb (data not shown). The signals seen in S. collinus H1 were consistent with the integration of emrE into the wild-type S. collinus chromosomal region by homologous recombination (Fig. 2b). Probing of BgII digests of S. collinus wild-type and H4 genomic DNA with the 0.5-kb PstI/BamHI DNA fragment of meaA gave signals at 3.2 and 4.8 kb, respectively (data not shown). The 1.6-kb increase in the size of the BgII fragment seen in H4 indicated that double crossover had indeed occurred (Fig. 2c).

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Isobutyryl-CoA mutase activity of the meaA-blocked mutant. Since meaA has similarity in its predicted amino acid sequence to the genes encoding isobutyryl-CoA mutases, the presence of isobutyryl-CoA mutase activity was measured in the meaA-blocked mutant and in wild-type S. collinus. Both the wild type and the mutant were grown in minimal medium containing perdeuterated valine at a final concentration of 100 mM, and their fatty acids were extracted and analyzed. The fatty acid profile of the mutant strain was essentially identical to that of the wild type. In addition, in both strains, approximately 86% of the isopalmitate pool was labeled with seven deuteriums, consistent with the degradation of the labeled valine to isobutyryl-CoA and the utilization of this material as a starter unit for branched-chain fatty acid biosynthesis. Approximately 14% of the palmitate pool in both strains was labeled with either six or seven deuteriums, consistent with the isomerization of perdeuterated isobutyryl-CoA to n-butyryl-CoA and the utilization of this material as a starter unit for straight-chain fatty acid biosynthesis.
acid biosynthesis (36). This labeling of the palmitate in the mutant clearly indicated the presence of an active isobutyryl-CoA mutase, suggesting that S. collinus meaA does not encode an isobutyryl-CoA mutase.

**Phenotype analysis of both ccr- and meaA-blocked mutants.** The S. collinus wild type, the ccr-blocked mutant, and the meaA-blocked mutant were grown on minimal agar medium containing a single carbon source, including acetate, crotonic acid, and butyrate. Wild-type S. collinus barely grew on minimal medium containing crotonic acid, probably indicating poor uptake. Both the ccr- and meaA-blocked mutants exhibited poor growth compared to that of the wild type on minimal medium (Fig. 6). Surprisingly, both mutants also exhibited poor growth compared to that of the wild type in minimal medium with acetate as the sole carbon source (Fig. 6). In contrast, the two mutants showed essentially identical growth capabilities compared to that of the wild type in minimal medium containing glucose as well as in a variety of complex media (Fig. 6). Surprisingly, both mutants also exhibited poor growth compared to that of the wild type on minimal medium containing butyrate.

**Acetate uptake by the ccr-blocked mutant.** S. collinus H1 and the wild type were grown in minimal medium containing 1% glucose and 10 mM d3-acetate, and their fatty acids were extracted. The fatty acid profile of the mutant was essentially identical to that of the wild-type strain. Moreover, similar levels of labeled acetate incorporation into each of the malonate-derived positions of the fatty acids (8 ± 2%) were observed for S. collinus H1 and the wild type. This result indicated that the poor growth of the ccr-blocked mutant in minimal medium containing acetate as the sole source of carbon was not due to decreased uptake.

**ICL activity of S. collinus.** S. collinus ccr and meaA have strong similarities to adhA and meaA, respectively, of M. extorquens, whose products are involved in growth when acetate is the sole carbon source in this ICL-negative methylotroph. S. collinus was examined for ICL activity. When S. collinus was grown in minimal medium with acetate as the sole source of carbon, no detectable ICL activity was observed. When S. collinus was grown in minimal medium containing Tween as a carbon source, ICL activities of approximately 18 mU of protein per mg were observed (a unit of enzyme activity was defined as the oxidation of 1 µmol of NADH per min). The two cell-free extracts contained comparable protein concentrations. These experiments were repeated three times, with the same observations. Similar observations regarding ICL activity in S. coelicolor have been made previously (17).

**DISCUSSION**

When acetate is the sole source of carbon, most bacteria and plants are able to grow by means of the glyoxylate cycle, in which ICL plays a key role (Fig. 1a). Some bacteria, however, are able to grow when acetate is the sole carbon source even though they lack an apparent ICL (10). The pathway of C2 assimilation in these organisms is unknown (9).

The ICL-negative methylotrophs, exemplified by M. extorquens, are able to assimilate C1 compounds by using the well-characterized serine cycle. One of the critical steps in this process is the oxidation of acetyl-CoA to glyoxylate by a process that does not require ICL. The biochemical pathway involved in this transformation is unknown, although two pathways have been proposed. In the so-called 3-hydroxypropionate cycle, acetyl-CoA is first converted to propionyl-CoA via 3-hydroxypropionate (Fig. 7a) (34). Propionyl-CoA is then converted via succinyl-CoA to malyl-CoA, which is cleaved to form acetyl-CoA and glyoxylate. The second pathway involves the formation of α-ketoglutarate through the condensation between acetyl-CoA and oxaloacetate catalyzed by the tricarboxylic acid cycle enzymes (30). α-Ketoglutarate is then converted through multiple steps to β-methylmalyl-CoA, which is then cleaved to generate glyoxylate and propionyl-CoA. The latter is used to regenerate oxaloacetate (Fig. 7b).

Three genes, adhA, meaA, and pccA, specifically involved in the conversion of acetyl-CoA to glyoxylate have been identified in complementation experiments with a mutant of M. extorquens (9, 31). The pccA gene product has clearly been demonstrated to be a propionyl-CoA carboxylase, a catalytic activity required for both of the acetyl-CoA oxidative pathways described above (Fig. 7). However, neither the meaA nor the adhA products appear to have any function in these proposed pathways. No steps in either pathway are alcohol dehydrogenations (the proposed role of ADH), and meaA apparently encodes a novel CoB12-dependent mutase other than MCM or glutamate mutase, the only mutases involved in these pathways (9, 31). The roles of adhA and meaA and the pathway for the conversion of acetyl-CoA to glyoxylate in ICL-negative methylotrophs thus remain a mystery.

When grown in the presence of Tween, S. collinus is not an ICL-negative organism. However, when S. collinus was grown in a typical complex medium or only in the presence of acetate, no detectable ICL activity was observed. S. collinus was clearly shown to be able to grow with acetate as its sole carbon source,
CoA (24, 25). Finally, polyketide biosynthesis studies with \textit{S. cinnamonensis} have demonstrated that methylmalonyl-CoA generated from d$_3$-acetate retains all three deuteriums in the methyl positions (32). This result is entirely consistent with the notion of the butyryl-CoA pathway (Fig. 1b) and inconsistent with the glyoxylate cycle (Fig. 1a), the 3-hydroxypropionate cycle (Fig. 7a), and the \(\beta\)-methylmalyl pathway (Fig. 7b).

Crotonyl-CoA reductase is thought to play a key role in the catalysis of the last reductive step in the biosynthesis of butyryl-CoA from two acetyl-CoA molecules in \textit{M. extorquens} (36). The observation that the ability of the \textit{ccr} mutant of \textit{S. collinus} to grow on acetate was dramatically reduced is consistent with the proposed role of this enzyme in the butyryl-CoA pathway (Fig. 1a). The reason for the decreased growth of this mutant on butyrate compared to that of the wild type is not clear, however. It is reasonable to suggest based on the sequence similarity of \textit{S. collinus} CCR and \textit{M. extorquens} ADH and the comparable phenotypes of the two corresponding mutants that ADH may also catalyze the reduction of crotonyl-CoA.

We speculated that \textit{meaA} may encode isobutyryl-CoA mutase, which plays a central role in the proposed butyryl-CoA pathway. This possibility has not been investigated for the \textit{M. extorquens meaA}. Consistent with this suggestion were (i) the demonstration that in both \textit{S. collinus} and \textit{M. extorquens} the \textit{meaA} gene product is required for growth on acetate, (ii) the proximity of the \textit{ccr} (\textit{adhA}) and \textit{meaA} genes, and (iii) the similarity between the \textit{meaA} gene products and methylmalonyl-CoA mutases, which catalyze a reaction with many similarities to that catalyzed by isobutyryl-CoA mutase (4). Surprisingly, however, in vivo analysis of the \textit{S. collinus meaA} mutant clearly demonstrated no effect upon the isobutyryl-CoA mutase. Recently, genes encoding the large subunit of the isobutyryl-CoA mutases of \textit{S. cinnamonensis} and \textit{S. coelicolor} have been cloned and sequenced. The predicted gene products have approximately 92\% identity but only 37\% amino acid identity (58\% similarity) with the \textit{S. collinus meaA} gene product (27). These results are inconsistent with the hypothesis that \textit{meaA} encodes either the large or the small subunit of isobutyryl-CoA mutase. It is also unlikely that \textit{meaA} encodes a methylmalonyl-CoA mutase; MCM levels were unaffected by the disruption of this gene in either \textit{M. extorquens} (9, 31) or \textit{S. collinus} (data not shown).

In conclusion, \textit{S. collinus}, like \textit{M. extorquens}, uses a pathway other than the glyoxylate cycle for growth on acetate. This pathway involves \textit{ccr}, which encodes crotonyl-CoA reductase, and therefore likely also involves a butyryl-CoA intermediate and \textit{meaA}, which apparently encodes a mutase other than the MCM and isobutyryl-CoA mutases. In the case of \textit{M. extorquens}, this pathway also appears to require propionyl-CoA carboxylase. These results are inconsistent with any of the proposed alternate pathways for acetate assimilation (Fig. 1 and 7), indicating the presence of a novel pathway for acetate assimilation. The caveats for this conclusion are that the attribution of the mutase function to the \textit{meaA} gene product is correct and that the products of \textit{ccr} and \textit{pccA} do not have functions other than those already observed (9, 36).

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A large number of isotopic incorporation experiments probing fatty acid and polyketide biosynthesis in various streptomycetes have provided substantial evidence for the butyryl-CoA pathway. Labeled acetate molecules have been shown to be incorporated into the butyrate-derived positions of a number of polyketides (36, 37). The presence of a reversible CoB$_{12}$-dependent isobutyryl-CoA mutase in streptomycetes has been demonstrated in vivo and in vitro experiments (7, 25, 26, 36). The valine catabolite isobutyryl-CoA has been shown to be converted via methacryl-CoA intermediate to methylmalonyl-CoA (24, 25). Finally, polyketide biosynthesis studies with \textit{S. cinnamonensis} have demonstrated that methylmalonyl-CoA generated from d$_3$-acetate retains all three deuteriums in the methyl positions (32). This result is entirely consistent with the notion of the butyryl-CoA pathway (Fig. 1b) and inconsistent with the glyoxylate cycle (Fig. 1a), the 3-hydroxypropionate cycle (Fig. 7a), and the \(\beta\)-methylmalyl pathway (Fig. 7b).

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