# A Novel Alternate Anaplerotic Pathway to the Glyoxylate Cycle in Streptomycetes

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Received 14 March 1997/Accepted 13 June 1997

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  $C_1$  and  $C_2$  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 C<sub>2</sub> compounds. Previous studies with streptomycetes 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 CoB<sub>12</sub>-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 meaAblocked 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  $\alpha$ -ketoglutarate in the citric acid cycle or converted to succinyl-CoA and glyoxylate 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  $COB_{12}$ -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 CoB<sub>12</sub>-dependent rearrangements in the proposed pathway, were unchanged in the meaA mutant. These results provide evidence that meaA encodes a novel CoB<sub>12</sub>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  $\mu$ g/ml) when necessary (28). *E. coli* DH10B was grown in Luria-Bertani medium supplemented with tetracycline (5  $\mu$ g/ml) and kanamycin (5  $\mu$ g/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 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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FIG. 1. (a) The synthesis of succinyl-CoA from acetyl-CoA by the glyoxylate cycle. (b) The butyryl-CoA pathway. Abbreviations: CCR, crotonyl-CoA reductase; ICM, isobutyryl-CoA mutase.

 $\mu$ g/ml) and lincomycin (200  $\mu$ g/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 (28). Single-stranded DNA templates used for dideoxy DNA sequencing were prepared with helper phage VCSM13 (35).

**İybridizations.** 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-primer DNA labeling kit (GIBCO BRL) with  $[\alpha^{-32}P]dCTP$  (3,000 Ci/mmol; Amersham). Prehybridization was carried out for 2 h at 65°C in a solution containing 5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinyl pyrrolidone), 0.1% (wt/vol) sodium dodccyl sulfate (SDS), and 100 µg of denatured salmon sperm DNA per ml. Following prehybridization, the membranes were incubated with specific probes overnight under the same conditions. Membranes were washed once with 1× SSC containing 0.1% SDS and then twice with 0.1× SSC containing 0.1% SDS.

**Transformations.** 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 morpholinepropanesulfonic acid, 5 mM dithiothreitol, 15 mM MgCl<sub>2</sub>, 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 ysate was centrifuged (30,000 × g at 4°C for 60 min). The supernatant was collected and used for the ICL assay. ICL activity was assayed spectrophotometrically in a coupled enzyme assay with 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 phagemid transformants (35). DNA sequencing was carried out by the dideoxy chain-termination method

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

Strain, plasmid, phage, or cosmid	Description	Reference or source
S. collinus		
Tü 1892	Wild type	36
H1	Mutant carrying <i>ermE</i> after <i>ccr</i> replacement	This work
H2	Mutant carrying <i>ermE</i> after <i>meaA</i> replacement	This work
S. lividans TK24	SLP <sup>-</sup> str	15
E. coli		
TG1	$\Delta(lac-pro)supE \ thiS \ hsdD5/F'$	8
DH10	Host strain of the cosmid library	14
Plasmids		
pBluescript II KS(+)	Phagemid vector (amp $lacZ'$ )	Stratagene
pHJL400	<i>Streptomyces-E. coli</i> bifunctional vector ( <i>tsr amp lacZ</i> ')	23
nH4026	nUC18 carrying <i>ermE</i>	Pfizer Inc
nZYB2	pUC18 with a 6 9-kb BamHI	37
P2102	insert from S. collinus	57
pLH1	pHJL400 with a 6.9-kb insert from pZYB2	This work
pLH2	pLH1 with a 2.5-kb-segment deletion	This work
pLH3	pLH2 with <i>ermE</i> inserted in the <i>ccr</i> coding region	This work
pLH4	pKS(+) with a 3.2-kb <i>Bgl</i> II insert from Cos21B5	This work
pLH5	pHJL400 with a 3.2-kb BglII insert from Cos215B5	This work
pLH6	pLH5 with <i>ermE</i> inserted in the <i>meaA</i> coding region	This work
Phage		
VCSM13	Km <sup>r</sup> derivative of M13K07	Stratagene
Cosmid		
Cos21B5	Cosmid clone isolated from the cosmid library of <i>S. collinus</i>	This work

of Sanger et al. (29) with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and  $\alpha^{-35}$ C-labeled dATP. Sequence ambiguities were resolved by resequenced. Computer-assisted analysis of the DNA sequences was performed 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 with the public sequence databases. Sequence alignments were carried out with the GAP program of the Genetics Computer Group program package, version 9.1 (University of Wisconsin, Madison).

**Disruption of the** *S. collinus ccr* and *meaA* genes. The segregationally unstable shuttle vector, pHJL400 (23), was used to construct gene replacement vectors. The erythromycin-resistant gene (*ermE*) in pIJ4026 (Pfizer Inc.) was excised as a 1.6-kb fragment and inserted in the coding region of either *ccr* or *meaA*.

Fatty acid analysis. *Streptomyces* fatty acids were extracted and analyzed according to the method of Wallace et al. (36).

Nucleotide sequence accession number. The *S. collinus meaA* and ORF1 sequence reported here has been deposited in GenBank under accession no. AF008569.

## 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



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, *Bam*HI; Bg, *Bg*/II; M, *Mlu*I; P, *Pst*I; X, *Xba*I.

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  $C_1$  and  $C_2$  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-Bam*HI fragment of pZYB2 containing this ORF was used to screen the *S. collinus* cosmid library. Of 285 clones screened, 1 showed strong hy-

bridization to the probe and was designated Cos21B5. Southern analysis of the Cos21B5 DNA with the same probe showed a 3.2-kb *Bgl*II 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 BqlII

1 61

121

181 241

301

361

421

481

541

601

661

721

781

841

901

961

1021

1081

1141

1201

1261

1321

1381

1441

1501

1561

1621

1681

$ \sum_{k = k \le 0} (k \le k \le 0 \ k \le k$	BglII	1741	CGAGTCCGGGCAGGAGAAGATCGTCGGCGTCAACGTCTTCGAGGGCACCGAGCCGAACCC
TTTTSMAGAAGAGAGCAGCCCCAGAGCCCCGAGCCCCGAGCCGGAGCCCGGAGCCGGGAGCCCGGGAGCCGGGAGCCGGGGCGACCGCGGGGAGCCGGGGAGCCGGGGAGCCGGGGAGCCGGGGGAGCCGGGGGAGCCGGGGGAGCCGGGGGAGCGGGGGCGCCCCGGGGGG	<u>AGATCT</u> GCCGCTCGATGGGCGCCGAGGCGATCATCGACCGCAACGCCGAGGGCTACAAGT		E S G Q E K I V G V N V F E G T E P N P
$ \begin{aligned} & GCAGGCTGACCGGCGGCGCGCGCGCGCGCGCGCGCGCGCG$	TCTGGAAGGACGAGCACACCCAGGACCCCAAGGAGTGGAAGCGCTTCGGCAAGCGCATCC		
$ \begin{array}{c} TCGGGGGGCATCTCACCGCAAGGGGCAACCGCGTCACCGCCGCGGGGGGGG$	GCGAGCTGACCGGCGGCGAGGACATCGACATCGTCTTCGAGCACCCCGGCCGCGAGACCT	1801	GCTCACGGCCGACCTGGACACCGCGATCCAGACGGTGGACCCGGCGGTCGAGAACCGGGT
<pre>cesseric Ant PC ACCOUNT A</pre>	TCGGCGCCTCCGTCTACGTCACCCGCAAGGGCGGCACCATCACCACCTGCGCCTCGACCT		LTADLDTAIQTVDPAVENRV
$ \begin{array}{c} CTCOCCAPTCOCCAPTCOCCAPAGGGCCTAPTCOCCAPAGGCCTTATCOCCAPAGGCCTTATCOCCAPAGGCCTTATCOCCAPAGGCCTTATCOCCAPAGGCCTTATCOCCAPAGCCCTTATCOCCAPAGCCCTTATCOCCAPAGGCCTTATCOCCAPAGCCCCTTATCOCCAPAGCCCCTTATCOCCAPAGCCCCTTATCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCTTACCAPTCOCCAPAGCCCCCTTACCAPTCOCCAPAGCCCCCTTACCAPTCOCCAPAGCCCCTTCACCCCCCAPACCCCCTTCCCCCAPACCCCCTTCCCCCAPAGCCCCTTCCCCCAPACCCCCTTCCCCCAPACCCCCTTCCCCCAPACCCCCTTCCCCCAPACCCCCTTCCCCCAPACCCCCTTCCCCCAPACCCCCCTCCCCCCAPACCCCCCCC$	CGGGCTACATGCACGAGTACGACAACCGGTACCTGTGGATGTCCCTGAAGCGGATCATCG	1861	CGTCGAGTCCCTCAGGCACTGGCGGGACACCCCGTACCAGCCGCCCTTCAACCACCCGCG
<ul> <li>ALATCCACCGACGCTTCAAAGGCTAATGCGGGCAGGCGGGGCGGGGGGGG</li></ul>	GCTCGCACTTCGCCAACTACCGCGAGGCGTACGAGGCCAACCGCCTGATCGCCAAGGGCA		V E S L R H W R D T P Y Q P P F N H P R
$ \begin{array}{c} \label{eq:product} Correspondence Correspon$	AGATCCACCCGACGCTGTCGAAGACGTACTCCCTGGAGGAGACCGGCCAGGCGGCGTACG	1921	CCCGTGCAAGGCGCTGGAGCGGCTGAAGGAGGCCGCCAAGGGCACCGACAACCTGATGGA
<ul> <li>SCCTCGGGCGTGGGGGGGGGGGGGGGGGGGGGGGGGGGG</li></ul>	ACGTCCACCGCAACCTGCACCAGGGCAAGGTCGGCGTCCTGTGCCTCGCGCCGGAGGAAG		PCKALERLKEAAKGTDNIME
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	GCCTCGGCGTGCGCGACGCGGGGGGGGCGCCCAGCACATCGACGCCATCAACCGCTTCC	1981	GGCCACCCTGGAGTGCGCCCGCGCGCGGGGTCACCACCGGCGAGTGGGCGGGC
$ \begin{array}{c} GCARGGTCTGARGACCCCCGAGGAGGTCCCCCCCGAGGAGGTCCCCCCCCGAGGAGGTCCCCCCCC$	rhs meal>		ATLECARAGVTTGEWAGALR
$ \begin{array}{c} \text{CP} \text{C} \text{C} \text{C} \text{C} \text{C} \text{C} \text{C} C$		2041	CGAGGTGTTCGGCGAGTACCGGGCGCCCACCGGGGGTCTCCCCCGCGCGCG
$ \begin{array}{llllllllllllllllllllllllllllllllllll$			EVEGEVEAPTGVSSAPVAVA
$ \begin{array}{c} \text{A}  \text{E}  \text{T}  \text{Y}  \text{A}  \text{G}  \text{A}  \text{E}  \text{A}  \text{E}  \text{V}  \text{A}  \text{E}  \text{A}  \text{A}  \text{A}  \text{E}  \text{A}  \text{A}  \text{A}  \text{E}  \text{A}  \text$		2101	
<ul> <li>TUTGGCOAGGGCAACAGGGCATGTCTGCGGGAGGAGGGCGGGCTTGCGGGCGG</li></ul>	M D T V A C H C T A A V C N F I V D D N	5202	A E A G S A L A E V R A K V D A T A R E
$ \begin{array}{c} LA K K G Q T G S V A K P D L P T Q T Q T Q T Q T Q T Q T Q T Q T Q T$		2161	
LA K G V A K C V A V A V A V A V A V A V A V A V A V	I A K G O T G I G V A F D I D T O T G V	2101	L C V C K L P F L V C K D C L D C U C N
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$ \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c}$	CGACCCCGACCACCATCCTCGCGCGGGGGGGGGGGGGGG	2221	COGCOCCOAGAGAGAGAGGCOCOCOCOCGCOCGCOCGCOC
$ \begin{array}{c} \text{Celebrate} \begin{tabular}{lllllllllllllllllllllllllllllllllll$		2201	
$ \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c}$	COTOGGIGACATGIGCOGGOIGITCCAGGACATCCCCCOTGGAGCAGATGAACACCTCGAT	2201	
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		2241	
$ \begin{array}{c} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 $	GACGAICAACGCCACGGCCAIGIGGCIGCIGGCGCCICIACCAGGICGTCGCGGAGGAGCA	2371	
$ \begin{array}{c} \text{Green} Gale D \mid T \mid k \mid Q \mid G \mid T \mid Q \mid N \mid D \mid V \mid A \mid G \mid G \mid I \mid P \mid N \mid G \mid G \mid G \mid A \mid Q \mid V \mid A \mid G \mid G \mid I \mid P \mid N \mid G \mid G \mid G \mid A \mid A \mid A \mid A \mid A \mid A \mid A$		2401	
$ \begin{array}{c} G \ A \ D \ I \ V \ V \ V \ V \ V \ V \ V \ V \ V$	GGGCGCGGACATCACCAAGCTCCAGGGCACGACCCAGAACGACATCGTCAAGGAGTACCT	2401	IN A C A T D I D U I A C C I I D N C D A
$ \begin{array}{c} CGCGGARCACGGTCCCGGARGAGGACCCGARGAGACGCCGARGAGACCCCGARGAGACCCCGARGAGACCCCGARGAGACCCCCGARGAGACCCCCCCGARGAGACCCCCCCC$		2467	
$ \begin{array}{c} \text{S} \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \$	GTCCCGCGGGGACGCACGTCTTCCCGCCGGGGCCCTCGCTCG	2401	CGAGCAGCIGAAGGAAGCCGGAGIGGCCGCCGICIICACCCCGAAGGACTICGACATCAC
$ \begin{array}{c} CGGGAGGACGCGGACACCCGGAGACACCCGGAGAGACCCGGACACACCCGGAGAGACCCGGAGAGACCCGGAGAGAGCCGAGAGACCCCGGAAGAGCCGAACAGCCCCGGCGACCCGCGAAGAGCCGCGAAGAGCCGAAGAGCCGACCCCCGGCGACCACC$	S R G T H V F P P G P S L R L T T D M L		EQUREAGVAAVFIPRDFDII
$ \begin{array}{c} A & Y & T & C & S & H & I & P & K & W & N & P & I & N & I & C & S & Y & H & L \\ CACAGGAGGCCGGCGCCCCGGGGACCCCGGGACCCCGGGACCCCGGCGACCCCCC$	CGCGTACACGTGCTCCCCACATCCCGAAGTGGAACCCCGATCAACATCTGCAGCTACCACCT	0501	
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	AYTCSHIPKWNPINICSYHL	2521	CGGGATCATCGGCCGCATCGTCGACGAGATCCGGGAAAGCGAACAAGCTCGACCCCCT <u>GGA</u>
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	GCAGGAGGCCGGGGGCCACACCGGTGCAGGAGATCGCGTACGCGATGTCCACCGCGATCGC		GIIGRIVDEIRKANKLDPLE
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Q E A G A T P V Q E I A Y A M S T A I A	0501	
$ \begin{array}{c} V \ L \ D \ A \ V \ R \ D \ S \ G \ Q \ V \ P \ Q \ E \ R \ M \ G \ D \ V \ V \ P \ A \ M^{P} \ T \ P \ V \ K \ L \ P \ K \ K \ P \ K \ S \ C \ L \ A \ V \ C \ CCCCCCCCCCCCCCCCCCCCCCCCC$	CGTCCTCGACGCCGTCCGCGACAGCGGCCAGGTGCCGCAGGAGCGCATGGGGGACGTGGT	2581	GGTCCCCGCATGACCACCCCCGTCAACCGCCTTCGCCCGCGGCGTTCCTGCCTG
$ \begin{array}{c} Caccege Cacceler (Control (Cont$	V L D A V R D S G Q V P Q E R M G D V V	0.5.4.1	V P AM*T T P V N R L R P R R S C L A V
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	CGCCCGCATCTCCTTCGTGAACGCGGGCGTCCGCTTCATTGAGGAGATGTGCAAGAT	2641	CCGGGCAGCAACCCCCGCTTCCTGGAGAAGGCGCAGGGCCTCCCCCGCGGACCAGGTCTTC
$ \begin{array}{c} CCGGGCGCGCCGCCGCCGCCGCCGCCGCCGCGCGCGCG$	A R I S F F V N A G V R F I E E M C K M	0.001	P G S N P R F L E K A Q G L P A D Q V F
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	GCGGGCGTTCGGCCGCATCTGGGACAAGGTCACCCGTGAGCGGTACGGCATCGAGAACCC	2701	UTGGACCTGGAGGACGCGTGCGCCGCTCGCGAAGCCGGGAGGCCCGGCACACCATCGTC
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	RAFGRIWDKVTRERYGIENP	0.7.01	
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	CAAGCAGCGCCGCTTCCGCTACGGCGTCCAGGTCACCTCCCTC	2761	AAGTTCUTCAACGAGGGCGACTGGACGGGCAAGACCCGGGTGGTCCGCGTCAACGACTGG
$ \begin{array}{c} CCCGGAGAACACCTCCAGCGGATCGTCGCGGATCGTCGGCGGTGGACCCGGTCGTCGGCGGAGACCTCCTGGAGCGGCGGAGGCGCCGGGAGCGCCGGGAGCGTCGCCGGAGGCGCCGGGAGCGCCGGGAGCGCCGGGGCCGGGGCCGGGGCCGGGGCCGGGGCCGGGGCCGGGG$	K Q R R F R Y G V Q V T S L G L T E A Q		
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	GCCGGAGAACAACGTCCAGCGGATCGTGCTGGAGATGCTGGCGGTGACCCTGTCGAAGGA	282I	ACGACCGAGTGGACGTACCGCGACGTCGTCACGGTGGTCGAGGGCGCCGGCCAGAACCTC
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	PENNVQRIVLEMLAVTLSKD		TTEWTYRDVVTVVEGAGQNL
A R A R A V Q L P A W N E A L G L P R P CTGGGACCAGCAGGGGAGCTCCCAGCAGGGGCCCGCCCCCCCC	CGCACGCGCGCGTGCCGTGCAGCTGCCCGCCTGGAACGAGGCGCTGGGCCTGCCCCGGCC	788T	GACTGCATCATGCTGCCGAAGGTGCAGAACGCCGAGCAGGTCGTCGCCGCCTGGACCTGCTG
$\begin{array}{c} CTGGGACCAGAGATGGAGCCTGCGCATCGAGCAGCTGCCTACGAGAGCGACCTGCTGCCTACGAGAGGCCACCTGCTGCGCTAGAGAGCCTGAGAGGCCCTGCAGGAGGCCTGAGAGGCGCAGGCGCCTGAGGGCTCGAGGGCTGAGGGCGCGGGGCGCGGGAGGCCGCGGGGCCGCGGGCGCGGGGCGCGGGGCGCGGGGCGC$	ARARAVQLPAWNEALGLPRP		DCIMLPKVQNAEQVVALDLL
$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	CTGGGACCAGCAGTGGAGCCTGCGCATCCAGCAGGTGCTCGCCTACGAGAGCGACCTGCT	2941	CTGACGCAGATCGAGAAGACCATGGGCTTCGAGGTCGGTAAGATCGGCATCGAGGCGCAG
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	W D Q Q W S L R I Q Q V L A Y E S D L L		LTQIEKTMGFEVGKIGIEAQ
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	GGAGTACGAGGACATCTTCGAGGGCTCGAAGGTGATCGAGGCGAAGGTGGACCAGCTGGT	3001	ATCGAGAACGCGCAGGGCCTGAACAACGTCAACGCGATCGCCGAGGCCTCCCCGCGCCTG
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	EYEDIFEGSKVIEAKVDQLV		IENAQGLNNVNAIAEASPRL
$ \begin{array}{c} CACCGACGTCCTCCGGGAGATGGACGGATCGAGGAGATGGCGCGCGCGCGCGGCGAGGACGCCGCGCGGAGGACGCCGC$	BamHI	3061	GAGACGATCATCTTCGGCCCGGCCGACTTCATGGCCTCCATCAACATGAAGTCCCTCGTC
T D V L A E M D R I Q E M G G A M A A V GGAGTCCGGCTACCTGAAGTCGCAGCGCGCGCGCGCGCGC	CACCGACGTCCTCGCGGAGATGGACC <u>GGATCC</u> AGGAGATGGGCGGCGCGATGGCCGCCGT		ETIIFGPADFMASINMKSLV
GGAGTCCGGCTACCTGAAGTCGCAGCTGGTCGCCTCGCACGCGGAGCGCCGGGCCCGGAT E S G Y L K S Q L V A S H A E R R A R I BglII 3181 CTGATGGCCGCCGCGCCAACAACCTCCAGGCGATCGACGGCCCCTACCTCC <u>AGACTCT</u> L M A A R A N N L Q A I D G P Y L Q I	T D V L A E M D R I Q E M G G A M A A V	3121	GTGGGCGAGCAGCCGCCCGGCTACCCGGCGGACGCCTACCACTACATCCTGATGAAGATC
ESGYLKSQLVASHAERRARI Bglii 3181 CTGATGGCCGCCGCGCCAACAACCTCCAGGCGATCGACGGCCCCTACCTCCA <u>GGACGTC</u> LMAARANNLQAIDGPYLQI	GGAGTCCGGCTACCTGAAGTCGCAGCTGGTCGCCTCGCACGCGGAGCGCCCGGGCCCGGAT		V G E Q P P G Y P A D A Y H Y I L M K I
BglII 3181 CTGATGGCCGCCGCGCCAACAACCTCCAGGCGATCGACGGCCCTACCTCC <u>AGATCT</u> L M A A R A N N L Q A I D G P Y L Q I	ESGYLKSQLVASHAERRARI		
3181 CTGATGGCCGCCCGCGCCAACAACCTCCAGGCGATCGACGGCGCCCTACCTCC <u>AGATCT</u> L M A A R A N N L Q A I D G P Y L Q I			BglII
L M A A R A N N L Q A I D G P Y L Q I		3181	CTGATGGCCGCCCGCGCCAACAACCTCCAGGCGATCGACGGCCCCTACCTCC <u>AGATCT</u>
			LMAARANNLQAIDGPYLQI

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.

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<sub>12</sub>-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 C<sub>1</sub> and C<sub>2</sub> 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).

Comparison of the gene organization of S. collinus ccr and meaA with that of M. extorquens adhA and meaA. In M. extorquens, adhA and meaA are divergently transcribed and are separated by 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 C<sub>1</sub> and C<sub>2</sub> 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).

Targeted disruption of the S. collinus ccr and meaA. Strong sequence similarity of S. collinus ccr and meaA with the M. extorquens genes suggested that the S. collinus genes could also be involved in the assimilation of  $C_1$  and  $C_2$  compounds. This

М.	extorquens	7	VAEVKRDKPWIIRTYAGHSTAAESNKLYRGNLAKGQTGLSVAFDLPTQTG   : : : :  :	56			
S.	collinus	1	MTERQKDRPWLMRTYAGHSTAAVSNELYRRNLAKGQTGLSVAFDLPTQTG	50			
М.	extorquens	57	YDPDHELARGEVGKVGVSIAHLGDMRALFDQIPLAQMNTSMTINATAPWL	106			
5.	collinus	51	YDPDHILARGEVGRVGVPVAHLGDMCRLFQDIPLEQMNTSMTINATAMWL	100			
М.	extorquens	107	LSLYLAVAEEQGAPLAALQGTTQNDIIKEYLSRGTYVFPPAPSLRLTKDV	156			
s.	collinus	101	LALYQVVAEEQGADITKLQGTTQNDIVKEYLSRGTHVFPPGPSLRLTTDM	150			
М.	extorquens	157	ILFTTKNVPKWNPMNVCSYHLQEAGATPVQELSYALAIAIAVLDTVRDDP	206			
ς.	collinus	151	IAYTCSHIPKWNPINICSYHLQEAGATPVQEIAYAMSTAIAVLDAVRDSG	200			
М.	extorquens	207	DFDEASFSDVFSRISFFVAGMRFVTEICKMRAFAELWDEIAQERYGITD	256			
s.	collinus	201	QVPQERMGDVVARISFFVNAGVRFIEEMCKMRAFGRIWDKVTRERYGIEN	250			
М.	extorquens	257	AKKRIFRYGVQVNSLGLTEQQPENNVHRILIEMLAVTLSKRARARAVQLP	306			
<i>s</i> .	collinus	251	PKQRRFRYGVQVTSLGLTEAQPENNVQRIVLEMLAVTLSKDARARAVQLP	300			
М.	extorquens	307	AWNEALGLPRPWDQQWSMRMQQILAFETLLEYDDIFDGSTVIEARVEAL	356			
s.	collinus	301	AWNEALGLPRPWDQQWSLRIQQVLAYESDLLEYEDIFEGSKVIEAKVDQL	350			
М.	extorquens	357	KEQTRAELTRIAEIGGAVTAVEAGELKRALVESNARRISAIEKGEQIVVG	406			
<i>s</i> .	collinus	351	VTDVLAEMDRIQEMGGAMAAVESGYLKSQLVASHAERRARIESGQEKIVG	400			
М.	extorquens	407	VNKWQQGEPSPLTAG.DGAIFTVSETVEMEAETRIREWRSKRDERAVG.	453			
s.	collinus	401	VNVFEGTEPNPLTADLDTAIQTVDPAVENRVVESLRHWRDTPYQPPFNHP	450			
М.	extorquens	454	QALADLEQAARSGANIMPPSIAAAKAGVTTGEWGQRLREVFGEYRAP	500			
s.	collinus	451	RPCKALERLKEAAKGTDNLMEATLECARAGVTTGEWAGALREVFGEYRAP	500			
М.	extorquens	501	TGVTLQTVTSGAAEDARLLIADLGERLGETP.RLVVGKPGLDGHS	544			
S. cor	collinus msensus	501	TGVSSAPVAVAAEAGSALAEVRAKVDATARELGVGKLRFLVGKPGLDGHS D H	550			
М.	extorquens	545	NGAEQIALRARDVGFDVTYDGIRQTPTEIVAKAKERGAHVIGLSVLSGSH	594			
S. cor	collinus meensus	551	NGAEQIAVRARDAGFEVVYQGIRLTPEQIVDAALAEDV.TVGLSILSGSH G ${\bf S}$ L	599			
М.	extorquens	595	VPLVREVKAKLREAGLDHVPVVVGGIISTEDELVLKNMGVTAVYTPKDYE	644			
S. cor	<i>collinus</i> Isensus	600	ARLVPDVLQRLRVAGATDIPVIAGGIIPNGABQLKEAGVAAVFTPKDFD GG	649			
· · · · · · · · · · · · · · · · · · ·							
М.	extorquens	64	S LDKIMVGLAKVVERA 659 :   : ::				
s.	collinus	65	0 ITGIIGRIVDEIRKA 664				

FIG. 4. Alignment by GAP (Genetics Computer Group) of the amino acid sequence deduced from *meaA* of *S. collinus* and the predicted *meaA* gene product of *M. extorquens* (9, 31). Identical amino acids are indicated by bars, and similar amino acids are identified by double dots. Consensus sequence important for cobalamin binding is shown.

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 pLH3, the 6.9-kb BamHI fragment of pZYB2 was first subcloned in the BamHI site of pHJL400 to generate pLH1 (Fig. 2b). The 2.5-kb DNA region upstream of ccr was deleted by XbaI and MluI double digestion, followed by religation to generate pLH2. The 1.6-kb BglII fragment of pIJ4026, which carries the erythromycin-resistant gene (ermE) of Saccharopolyspora erythraea, was subcloned in the BglII site, which is in the coding region of ccr, to generate pLH3 (Fig. 2b). To construct pLH6, the 3.2-kb BglII fragment of Cos21B5 was subcloned in the BamHI site of pHJL400 to give pLH5 (Fig. 2c). The 1.6-kb BglII fragment of pIJ4026 was subcloned in the BamHI site within the meaA coding region to generate pLH6. In both pLH3 and pLH6, ermE 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<sup>r</sup>) and lincomycin resistant (Lin<sup>r</sup>). Ts<sup>s</sup> Lin<sup>r</sup> 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 pLH3 and propagation of the transformants; two colonies, S. collinus H4 and H5, were isolated from S. collinus pLH6 transformants. The genotypes of the This Linr colonies were confirmed by Southern hybridization of chromosomal DNA restriction digests with either ccr or meaA. Probing of BamHI digest of S. collinus H1 genomic DNA with ccr 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 ermE into the wild-type S. collinus ccr chromosomal region by homologous recombination (Fig. 2b). Probing of BglII 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 BglII 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 meaAblocked 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 deuteruims, 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



FIG. 5. Comparison of gene organization among genes encoding crotonyl-CoA reductase and alcohol dehydrogenase and novel mutases. (A) *S. collinus* gene cluster. (B) *M. extorquens* gene cluster (9). ORFs and their orientations are indicated by arrows. The arrow with a dashed line indicates an incomplete ORF. *ccr*, crotonyl-CoA reductase; *meaA*, a CoB<sub>12</sub>-dependent mutase; *adhA*, alcohol dehydrogenase; *katA*, catalase; *ilvC*, acetohydroxy acid isomeroreductase; *pccA*, propionyl-CoA carboxylase.



FIG. 6. Growth of S. collinus wild type, S. collinus H1, and S. collinus H4 on minimal medium containing different carbon sources. Abbreviations: MM, minimal medium; A, acetate; G, glucose.

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 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 d<sub>3</sub>-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  $\pm$  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  $\mu$ mol 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  $C_2$  assimilation in these organisms is unknown (9).

The ICL-negative methylotrophs, exemplified by M. extorquens, are able to assimilate  $C_1$  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  $\alpha$ -ketoglutarate through the condensation between acetyl-CoA and oxaloacetate catalyzed by the tricarboxylic acid cycle enzymes (30).  $\alpha$ -Ketoglutarate is then converted through multiple steps to  $\beta$ -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 CoB<sub>12</sub>-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,



FIG. 7. Two proposed pathways for the oxidation of acetyl-CoA to glyoxylate. (a) The 3-hydroxypropionate cycle (34). (b) The  $\beta$ -methylmalyl pathway (30). Abbreviations: PCC, propionyl-CoA carboxylase; GM, glutamate mutase.

suggesting the existence of an alternate pathway to the glyoxylate cycle for acetate assimilation. In addition to the two pathways described above, we have considered a third pathway in which two acetyl-CoA molecules are first condensed to form acetoacetyl-CoA, which is then converted to butyryl-CoA (Fig. 1b). The isomerization of this butyryl-CoA generates isobutyryl-CoA, which is oxidized via methacryl-CoA to methylmalonyl-CoA. This methylmalonyl-CoA is converted to succinyl-CoA by methylmalonyl-CoA mutase. Such a pathway, like the glyoxylate cycle, would provide one succinyl-CoA molecule from two acetyl-CoA molecules. The succinyl-CoA could be converted to malyl-CoA and cleaved to regenerate one acetyl-CoA molecule and glyoxylate.

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<sub>12</sub>dependent isobutyryl-CoA mutase in streptomycetes has been demonstrated in 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 methylmalonylCoA (24, 25). Finally, polyketide biosynthesis studies with *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 *S. collinus* (36). The observation that the ability of the *ccr* mutant of *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 *S. collinus* CCR and *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 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 M. extorquens meaA. Consistent with this suggestion were (i) the demonstration that in both S. collinus and M. extorquens the meaA gene product is required for growth on acetate, (ii) the proximity of the ccr (adhA) and meaA genes, and (iii) the similarity between the 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 S. collinus meaA mutant clearly demonstrated no effect upon the isobutyryl-CoA mutase. Recently, genes encoding the large subunit of the isobutvrvl-CoA mutases of S. cinnamonensis and 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 S. collinus meaA gene product (27). These results are inconsistent with the hypothesis that meaA encodes either the large or the small subunit of isobutyryl-CoA mutase. It is also unlikely that meaA encodes a methylmalonyl-CoA mutase; MCM levels were unaffected by the disruption of this gene in either M. extorquens (9, 31) or S. collinus (data not shown).

In conclusion, *S. collinus*, like *M. extorquens*, uses a pathway other than the glyoxylate cycle for growth on acetate. This pathway involves *ccr*, which encodes crotonyl-CoA reductase, and therefore likely also involves a butyryl-CoA intermediate and *meaA*, which apparently encodes a mutase other than the MCM and isobutyryl-CoA mutases. In the case of *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 *meaA* gene product is correct and that the products of *ccr* and *pccA* do not have functions other than those already observed (9, 36).

#### ACKNOWLEDGMENTS

We are grateful to I. S. Hunter for providing culture conditions for measuring ICL activity, to M. E. Lidstrom and P. Goodwin for helpful discussion and for providing the results of their research with *M. extorquens* prior to publication, to J. A. Robinson for apprising us of his progress with the *S. cinnamonensis* isobutyryl-CoA mutase and for running the comparison of this with the *S. collinus meaA* gene product, and to C. L. Hershberger of Eli Lilly & Company for the vector pHJL400. We thank Z. Bao for assistance in sequencing a portion of

*meaA* and Nilesh Banavali for conducting the MCM activity of the *meaA*-blocked mutant.

This work was supported by a grant from the National Institutes of Health (GM 50542).

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