**Citrate synthase and 2-methylcitrate synthase: structural, functional and evolutionary relationships**

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Following the complete sequencing of the *Escherichia coli* genome, it has been shown that the proposed second citrate synthase of this organism, recently described by the authors, is in fact a 2-methylcitrate synthase that possesses citrate synthase activity as a minor component. Whereas the hexameric citrate synthase is constitutively produced, the 2-methylcitrate synthase is induced during growth on propionate, and the catabolism of propionate to succinate and pyruvate via 2-methylcitrate is proposed. The citrate synthases of the psychrotolerant eubacterium DS2-3R, and of the thermophilic archaea *Thermoplasma acidophilum* and *Pyrococcus furiosus*, are approximately 40% identical in sequence to the *Escherichia coli* 2-methylcitrate synthase and also possess 2-methylcitrate synthase activity. The data are discussed with respect to the structure, function and evolution of citrate synthase and 2-methylcitrate synthase.

**Keywords:** citrate synthase, 2-methylcitrate synthase, propionate, metabolism

**INTRODUCTION**

Citrate synthase (EC 4.1.3.7) catalyses the entry of carbon into the citric acid cycle:

\[ \text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \rightarrow \text{Citrate} + \text{CoA} \]

Eukarya, Gram-positive eubacteria and archaea possess a homo-dimeric form of the enzyme, whereas in the majority of Gram-negative eubacteria the citrate synthase is a homo-hexamer. Irrespective of their oligomeric nature, all citrate synthases have polypeptide subunits of M, 42000–49000, and their sequences are homologous (Muir et al., 1995).

Recently, we have reported that *Escherichia coli* may possess a second citrate synthase (Patton et al., 1993). In outline, a citrate-synthase-negative strain of *E. coli* (K114) was mutated and a citrate-synthase-positive revertant (K11r4) was isolated. It was shown that both K114 and K11r4 produce an inactive hexameric citrate synthase in which a catalytically essential Asp has been replaced by an Asn. However, K11r4 additionally produces an active dimeric citrate synthase whose N-terminal sequence demonstrates that it must be encoded by a gene distinct from that producing the hexameric enzyme.

The sequencing of the *E. coli* genome has now been completed (Kroeger & Wahl, 1997). In the present paper we identify from the genome sequence the gene encoding the ‘second’ citrate synthase and show that the expressed protein is in fact a 2-methylcitrate synthase (EC 4.1.3.31) with partial citrate synthase activity. 2-Methylcitrate synthase catalyses the reaction

\[ \text{Propionyl-CoA} + \text{Oxaloacetate} + H_2O \rightarrow 2-\text{Methylcitrate} + \text{CoA} \]

and the role of this enzyme in propionate metabolism in *E. coli* is characterized. Furthermore, we report the relative activities with acetyl-CoA and propionyl-CoA of citrate synthases from other organisms and comment on the evolutionary and structural relationships between 2-methylcitrate synthase and citrate synthase.

**METHODS**

**Strains and plasmids.** Bacterial strain DS2-3R is a psychrotolerant isolate from Antarctica (Gerike et al., 1997). *E. coli* strain JM109 (Sambrook et al., 1989) and the citrate-synthase-deficient strain *E. coli* W620 (gltA6 relA1 supE44 thi-1 pyrD36 galK30 rpsL129 Smr) (Donald & Duckworth, 1986) were used for cloning and expression studies, respectively. Expression vector pREC7/NdeI was kindly provided by Dr L. C. Kurz.
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Molecular biology methods. Established protocols (Sambrook et al., 1989) were used for routine work with recombinant DNA. The gene encoding the putative dimeric citrate synthase (Patton et al., 1993) was subcloned by the following procedure. E. coli chromosomal DNA was isolated according to Ausubel et al. (1990) and the gene encoding the dimeric citrate synthase was PCR-amplified using primer 1 (5'-GGAATTCATATGGCGACACACGATCCCTG-3') and primer 2 (5'-GGCCGATTCTTCCGATCAATTCCGG-3'). The restriction sites for NdeI (primer 1) and BamHI (primer 2), used for cloning into the expression vector pREC7/Ndel, are underlined. Amplification was performed in a 50 µl incubation mixture containing 1 µg chromosomal DNA, 100 pmol of each primer, 0.2 mM each of dATP, dTTP, and primer 2 fragments were purified from agarose gels with the Quiapex and 2 U Vent DNA polymerase (New England Biolabs). DNA cut with NdeI and BamHI. The resulting plasmid (pRES1) contains the start codon of the dimeric citrate synthase gene at an optimal distance of 6 bp from the Shine-Dalgarno sequence.

Growth and induction. A 15 ml volume of an overnight culture of E. coli W620 transformed with pRES1 was inoculated into 300 ml Luria broth (containing 150 µg ampicillin ml-1 and 50 µg streptomycin ml-1) (Sambrook et al., 1989) and incubated at 37 °C. When the culture reached exponential phase (OD600 0.2), expression was induced with 15 ml of an overnight culture of E. coli (35 °C), and gene-derived translational start of the protein sequence.

For the determination of Kₘ and Vₘₐₓ values, kinetics analysis was performed using the Software package from the Genetics Computer Group of the University of Wisconsin (GCC, version 8). The E. coli prp genes were retrieved from the E. coli database collection [ECDC; Justus-Liebig-University Giessen, Germany] (Wahl & Kroeger, 1995; Kroeger & Wahl, 1997). The prp operon of Salmonella typhimurium is accessible in GenBank with the accession number US1879.

RESULTS

Identification of the gene encoding the second citrate synthase of E. coli

We have previously proposed the presence of a second, dimeric, citrate synthase in E. coli and determined the N-terminal amino acid sequence of the purified protein (Patton et al., 1993). Using this peptide sequence, the E. coli database collection was searched and a perfect match for the second citrate synthase gene was revealed. However, the N-terminal sequence obtained from the purified enzyme starts 17 amino acids later than the gene-derived translational start of the protein sequence. This might be due to non-specific proteolytic degradation of the N-terminus of the purified protein, or the result of an in vivo processing system.

This second citrate synthase showed highest homology (96% identity at the amino acid sequence level) to a putative citrate synthase of Salmonella typhimurium (prpC) but was only 30% identical to the hexameric citrate synthase of E. coli (gltA). The prpC gene is located within the prp operon of the S. typhimurium genome. This operon consists of four genes and has been implicated in propionate metabolism (Horswill & Escalante-Semerena, 1997). Interestingly, the same number and order of genes is found on the E. coli chromosome. The order of the prp genes of S. typhimurium and E. coli, and the percentage amino acid identity of their gene products, are: prpB (90%), prpC (96%), prpD (94%) and prpE (88%).

Cloning the gene for the second citrate synthase of E. coli

As shown by Horswill & Escalante-Semerena (1997), the prp operon of S. typhimurium is involved in propionate metabolism. In addition to other pathways, propionate can be metabolized by the 2-methylcitric acid pathway in the yeasts Saccharomyces cerevisiae (Prong et al., 1994) and Yarrowia lipolytica (formerly Candida lipolytica) (Uchiyama & Tabuchi, 1976). This pathway involves the formation of 2-methylcitrate from the condensation of propionyl-CoA and oxaloacetate, via a 2-methylcitrate synthase, resembling the formation...
of citrate from acetyl-CoA and oxaloacetate catalysed by citrate synthases.

To investigate the function of the dimeric citrate synthase in *E. coli*, the prpC gene of *E. coli* was cloned into the expression vector pREC7/Ndel to give plasmid pRES1. After transformation of pRES1 into the *E. coli* mutant strain W620, which does not produce an active hexameric citrate synthase, both citrate synthase and 2-methylcitrate synthase activities were detected in cell extracts. \( K_m \) values were determined for acetyl-CoA (101 ± 37 \( \mu \)M; mean ± SEM), and propionyl-CoA (37 ± 6 \( \mu \)M); the \( K_m \) value for oxaloacetate (5 ± 6 \( \mu \)M) was the same with both acyl-CoAs. At saturating concentrations of substrates, specific activities in the cell extracts were 0.11 (±0.01) nmol min\(^{-1}\) (mg protein\(^{-1}\)) for citrate synthase and 0.33 (±0.01) nmol min\(^{-1}\) (mg protein\(^{-1}\)) for 2-methylcitrate synthase.

Given that cell extracts of the host strain, *E. coli* W620, possess neither citrate synthase nor 2-methylcitrate synthase activities when grown on Luria broth, the presence of these two enzymes in cells transformed with pRES1 must arise from the same recombinant protein. The relative \( k_{cat}/K_m \) values with acetyl-CoA and propionyl-CoA in the cell extracts are 1:8, suggesting that the second citrate synthase of *E. coli* is primarily a 2-methylcitrate synthase. This contrasts with data for the *E. coli* hexameric citrate synthase, which has virtually no activity with propionyl-CoA (Man *et al.*, 1995).

### Growth on glucose and propionate

To investigate the role of the *E. coli* citrate synthase and proposed 2-methylcitrate synthase further, *E. coli* wild-type strain JM109 and *E. coli* mutant strain W620 (citrate synthase negative) were grown on glucose and propionate minimal media. Cells growing exponentially in glucose minimal medium were washed and added to the respective minimal media. As expected, both strains immediately entered an exponential growth phase on transfer to the glucose medium, but on transfer to propionate a significant lag phase was observed (Fig. 1), presumably reflecting the adaptation of the cells to this new single carbon and energy source. The absence of the hexameric citrate synthase seems to influence the growth rate on propionate, as strain W620 grows significantly more slowly than strain JM109 (Fig. 1).

Cells from both *E. coli* strains grown on glucose or propionate were used to measure citrate synthase and 2-methylcitrate synthase activities in cell extracts (Table 1). When grown on glucose, *E. coli* W620, which we have shown does not produce an active citrate synthase due to a mutation in the gene encoding the hexameric enzyme (Patton *et al.*, 1993), produced no detectable 2-methylcitrate synthase activity. However, both 2-methylcitrate synthase and citrate synthase activities were found in cells grown on propionate, indicating that the 2-methylcitrate synthase gene is inducible by propionate and that the enzyme has citrate synthase activity as a minor (10%) component.

A similar induction of 2-methylcitrate synthase by propionate was observed with the *E. coli* wild-type strain JM109 (Table 1). However, this carbon source also increased the levels of citrate synthase activity by an amount greater than can be accounted for by the

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**Table 1.** Citrate synthase and 2-methylcitrate synthase activities determined in cell extracts of *E. coli* JM109 (wild-type), *E. coli* W620 and DS2-3R grown on glucose or propionate

<table>
<thead>
<tr>
<th>Carbon source in growth medium</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>E. coli</strong> JM109</td>
<td><strong>E. coli</strong> W620</td>
</tr>
<tr>
<td><strong>Enzyme activity [nmol min(^{-1}) (mg protein(^{-1})]</strong></td>
<td><strong>Citrate synthase</strong></td>
<td><strong>2-Methylcitrate synthase</strong></td>
</tr>
<tr>
<td>10 mM glucose</td>
<td>608</td>
<td>6</td>
</tr>
<tr>
<td>20 mM propionate</td>
<td>1160</td>
<td>85</td>
</tr>
</tbody>
</table>
Table 2. Substrate specificity for acetyl-CoA and propionyl-CoA of citrate synthases from different organisms

Purified enzymes were used in all cases and the assay contained 0.2 mM oxaloacetate and either 0.2 mM acetyl-CoA or 0.17 mM propionyl-CoA.

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Growth temp. of organism (°C)</th>
<th>Assay temp. (°C)</th>
<th>Catalytic activity (%) with propionyl-CoA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS2-3R</td>
<td>15</td>
<td>23</td>
<td>75.8</td>
</tr>
<tr>
<td>Pig</td>
<td>37</td>
<td>37</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>T. acidophilum</td>
<td>55</td>
<td>50</td>
<td>18.3</td>
</tr>
<tr>
<td>P. furiosus</td>
<td>100</td>
<td>68</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*Expressed relative to the activity with acetyl-CoA for the respective enzyme, taken as 100%.

Table 3. Kinetic parameters of citrate synthase purified from the psychrotolerant Antarctic bacterium DS2-3R

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase activity</td>
<td></td>
</tr>
<tr>
<td>$K_m$ acetyl-CoA</td>
<td>229 ($\pm$ 133) µM</td>
</tr>
<tr>
<td>$K_m$ oxaloacetate</td>
<td>7 ($\pm$ 2) µM</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>30 ($\pm$ 6) µmol min$^{-1}$ (mg protein)$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
<td>21 ($\pm$ 4) s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ acetyl-CoA</td>
<td>9 ($\pm$ 5) x 10$^4$ M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>2-Methylcitrate synthase activity</td>
<td></td>
</tr>
<tr>
<td>$K_m$ propionyl-CoA</td>
<td>16 ($\pm$ 2) µM</td>
</tr>
<tr>
<td>$K_m$ oxaloacetate</td>
<td>3 ($\pm$ 1) µM</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>12 ($\pm$ 1) µmol min$^{-1}$ (mg protein)$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
<td>8 ($\pm$ 1) s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_m$ propionyl-CoA</td>
<td>52 ($\pm$ 9) x 10$^4$ M$^{-1}$ s$^{-1}$</td>
</tr>
</tbody>
</table>

*Means ($\pm$ SEM).

Increased levels of 2-methylcitrate synthase. This suggests that propionate is an inducer of the hexameric citrate synthase gene, which would be consistent with some of the propionate being metabolized via pyruvate and acetyl-CoA as shown by Evans et al. (1993).

Do citrate synthases from other organisms have 2-methylcitrate synthase activity?

It has already been reported that the hexameric citrate synthase of E. coli has a very low activity with propionyl-CoA as substrate (Man et al., 1995) and our data in Table 1 would indicate that it is approximately 1% of that with acetyl-CoA. It was therefore interesting to examine the substrate specificity of citrate synthases from other organisms. The relative activities for propionyl-CoA in comparison to acetyl-CoA determined with purified enzymes from the psychrotolerant bacterium DS2-3R, pig, T. acidophilum and P. furiosus are shown in Table 2. Propionyl-CoA serves as substrate for the P. furiosus, T. acidophilum and DS2-3R enzymes, although the pig citrate synthase exhibited virtually no activity.

Given that the cold-active enzyme from DS2-3R has almost equal citrate and 2-methylcitrate synthase activities using the standard assay concentrations of substrates, it was decided to characterize this enzyme further. The kinetic parameters with acetyl-CoA and propionyl-CoA as substrates are summarized in Table 3; both citrate synthase and 2-methylcitrate synthase activities followed Michaelis–Menten kinetics. From these data, the calculated $k_{\text{cat}}/K_m$ values, which determine the specificity for competing substrates, are in the ratio of 6:1 in favour of propionyl-CoA.

In the light of the situation in E. coli, DS2-3R was also grown on glucose and propionate minimal media and the activities of citrate and 2-methylcitrate synthases were determined (Table 1). No significant increase of 2-methylcitrate synthase activity was found in cells grown on propionate, and the ratio of 2-methylcitrate and citrate synthase activities, 1:2 on glucose as growth substrate and 1:3 on propionate as growth substrate, coincided with the ratio of true $V_{\text{max}}$ values for propionyl-CoA and acetyl-CoA (Table 3) determined for the purified enzyme of DS2-3R (1:2.5).

These data are consistent with there being either a single enzyme with dual specificity for acetyl-CoA and propionyl-CoA or two separate enzymes that are constitutively produced. To distinguish these two possibilities, extracts of DS2-3R cells grown on Nutrient Broth (0.3% beef extract powder, 0.5% peptone, pH 6.8; Difco) were assayed at various concentrations of acetyl-CoA and propionyl-CoA, both individually and together. The data given in Table 4 indicate that DS2-3R possesses a single enzyme with both citrate synthase and 2-methylcitrate synthase activities.

DISCUSSION

In this paper, we have provided evidence that E. coli possesses both a citrate synthase and a 2-methylcitrate synthase, and that the two genes encoding these activities
Table 4. Substrate competition between acetyl-CoA and propionyl-CoA in cell extracts of DS2-3R grown on Nutrient Broth

The theoretical enzyme activity for one enzyme utilizing both acetyl-CoA (A) and propionyl-CoA (B) is given by Segel (1975):

\[ V_{\text{total}} = \frac{V_A \left(1 + \frac{[A]}{K_A}ight) + V_B \left(1 + \frac{[B]}{K_B}\right)}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B}} \]

where \( V_A \) and \( V_B \) are enzyme velocities with acetyl-CoA or propionyl-CoA alone and the \( K_v \) values are those given in Table 3. The reactions with acetyl-CoA and propionyl-CoA as substrates (citrate synthase and 2-methylcitrate synthase activities, respectively) are given in the Introduction, and the assay methods are described in Methods.

<table>
<thead>
<tr>
<th>Substrate(s) in assay</th>
<th>Observed enzyme activity [nmol min(^{-1}) (mg protein)(^{-1})]*</th>
<th>Theoretical enzyme activity [nmol min(^{-1}) (mg protein)(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One enzyme</td>
<td>Two separate enzymes</td>
</tr>
<tr>
<td>600 ( \mu )M acetyl-CoA</td>
<td>1.75</td>
<td>1.32</td>
</tr>
<tr>
<td>320 ( \mu )M propionyl-CoA</td>
<td>1.18</td>
<td>2.93</td>
</tr>
<tr>
<td>Both substrates together</td>
<td>1.69</td>
<td></td>
</tr>
<tr>
<td>90 ( \mu )M acetyl-CoA</td>
<td>0.59</td>
<td>1.14</td>
</tr>
<tr>
<td>12 ( \mu )M propionyl-CoA</td>
<td>1.14</td>
<td>1.30</td>
</tr>
<tr>
<td>Both substrates together</td>
<td>1.30</td>
<td>1.18</td>
</tr>
<tr>
<td>300 ( \mu )M acetyl-CoA</td>
<td>1.30</td>
<td>1.73</td>
</tr>
<tr>
<td>16 ( \mu )M propionyl-CoA</td>
<td>0.78</td>
<td>1.38</td>
</tr>
<tr>
<td>Both substrates together</td>
<td>1.57</td>
<td>2.08</td>
</tr>
</tbody>
</table>

*The results are from representative experiments. Each of the three experiments was repeated with essentially identical results.

are transcribed and regulated in an independent manner.
The citrate synthase is a hexameric enzyme produced constitutively, whereas the 2-methylcitrate synthase is a dimeric protein induced during growth on propionate. However, while citrate synthase does not possess 2-methylcitrate synthase activity, the 2-methylcitrate synthase accepts both acetyl-CoA and propionyl-CoA as substrate.

These data are consistent with our previous observations on citrate synthase in *E. coli* and associated mutational studies. That is, it is possible to generate a citrate synthase-negative strain of *E. coli* by mutation within the *gltA* gene, as 2-methylcitrate synthase (which also exhibits citrate synthase activity) is not produced unless propionate is present in the growth medium. Furthermore, when we mutated the citrate-synthase-negative strain to create a revertant that had regained the citrate synthase activity (Danson *et al.*, 1979), we presumably had caused the constitutive expression of the 2-methylcitrate synthase gene. Consequently, this revertant still produced an inactive hexameric citrate synthase, the gene sequence of which was identical to that of the original citrate-synthase-negative parent (Patton *et al.*, 1993), plus the 2-methylcitrate synthase with its associated citrate synthase activity.

The presence of a 2-methylcitrate synthase that is within a putative *prp* operon and which is induced during growth on propionate suggests that *E. coli* metabolizes propionate via 2-methylcitrate, in addition to its catalysis via the acrylate (Evans *et al.*, 1993) and the methylmalonyl-CoA pathways (Wegener *et al.*, 1968) (Fig. 2). By analogy with the metabolism of propionate in *Yarrowia lipolytica* (Tabuchi & Uchiyama, 1975), we suggest that 2-methylcitrate would be further catalyzed in *E. coli* via 2-methylaconitate and 2-methylisocitrate to succinate and pyruvate (Fig. 2). Interestingly, the 2-methylcitrate synthase from *Y. lipolytica* also possesses citrate synthase activity (Uchiyama & Tabuchi, 1976).

Hexameric and dimeric 'citrate synthases' have also been found in mutants of *Acinetobacter lwoffi* (Weitzman *et al.*, 1978) and of *Pseudomonas aeruginosa* (Mitchell *et al.*, 1995). In both cases evidence was provided for these being different gene products, raising the possibility that the dimeric enzyme might be principally a 2-methylcitrate synthase. This suggestion is strengthened by the recent determination of the N-terminal amino acid sequence of the dimeric 'citrate synthase' from *P. aeruginosa* (Mitchell & Anderson, 1996), and its similarity with the N-terminus of what we have now shown to be the *E. coli* 2-methylcitrate synthase.
Fig. 2. Schematic diagram of three pathways of propionate degradation: (1) the acrylate pathway, (2) the 2-methylcitrate pathway, and (3) the methylmalonyl-CoA mutase pathway. The enzymes for the 2-methylcitrate synthase pathway are (a) 2-methylcitrate synthase, (b) and (c) 2-methylaconitase, and (d) 2-methylisocitrate lyase.

The identity of the E. coli 2-methylcitrate synthase with citrate synthases from the psychrotolerant bacterium DS2-3R (41%), P. furiosus (37%), and T. acidophilum (35%) led us to demonstrate that these enzymes also possess 2-methylcitrate synthase activity. Both P. furiosus and T. acidophilum are members of the Archaea which, on the basis of rRNA sequence comparisons, are thought to be closest to the root of the universal phylogenetic tree (Doolittle & Brown, 1994). Thus a logical scenario might be that citrate synthase and 2-methylcitrate synthase were originally activities of the same enzyme and that in some organisms the gene has undergone duplication and divergence to give separate enzymes. In Y. lipolytica, for example, the two enzymes are produced constitutively whereas in others, such as E. coli, the 2-methylcitrate synthase is inducible during growth on propionate. At this point, it should be noted that genetically distinct enzymes catalysing the same or similar metabolic reactions are seen in other citric acid cycle enzymes, such as aconitase and fumarase (Gruer et al., 1997).

Where the citrate synthase and 2-methylcitrate synthase activities arise from the one protein, a common catalytic mechanism is presumed for both acetyl-CoA and propionyl-CoA. In the case of P. furiosus citrate synthase, the critical involvement of two histidines and an aspartic acid residue in the condensation reaction between acetyl-CoA and oxaloacetate is suggested from the crystal structure (Russell et al., 1997) and from comparison with extensive data for the pig citrate synthase (reviewed by Remington, 1992). These residues are conserved in both the E. coli citrate synthase and 2-methylcitrate synthases, again supportive of a common evolutionary origin. The explanation of their differing specificities will have to await structural data on both the citrate synthase and 2-methylcitrate synthase from this organism.

In some organisms, gene duplication to give more than one citrate synthase has also been documented, examples including Bacillus subtilis (Jin & Sonenshein, 1994) and the mitochondrial and glyoxysomal isoenzymes of yeast (Rosenkrantz et al., 1986). Consequently, it may now be time to revisit the many citrate synthases that are currently in the sequence databases to see how many are partially or predominantly 2-methylcitrate synthases. Furthermore, now that the genome sequences of many organisms are being determined, our findings emphasize the need to characterize expressed gene products rather than to rely on sequence homologies for their identification.

NOTE ADDED IN PROOF


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Citrate synthase and 2-methylcitrate synthase


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