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Resolving the TCA cycle and pentose-phosphate pathway of *Clostridium acetobutylicum* ATCC 824 using isotopomer analysis, in vitro *Re*-citrate synthase activities and expression analysis

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Abstract

Solventogenic clostridia are an important class of microorganisms that can produce various biofuels. One of the bottlenecks in engineering clostridia stems from the fact that central metabolic pathways remain poorly understood. Here, we utilized the power of ^{13}C-based isotopomer analysis to re-examine central metabolic pathways of *C. acetobutylicum* ATCC 824. We demonstrate using [1,2-^{13}C]glucose, mass spectrometry analysis of intracellular metabolites, and enzymatic assays that *C. acetobutylicum* has a split TCA cycle where only *Re*-citrate synthase contributes to the production of α-ketoglutarate via citrate. Furthermore, we show that there is no carbon exchange between α-ketoglutarate and fumarate and that the oxidative pentose-phosphate pathway is inactive. Dynamic gene expression analysis of the putative *Re*-citrate synthase gene (CAC0970), its operon, and all glycolysis, pentose-phosphate pathway and TCA cycle genes identify genes and their degree of involvement in these core pathways that support the powerful primary metabolism of this industrial organism.

Keywords

Citrate synthase; TCA cycle; pentose-phosphate pathway; metabolic flux analysis; gene expression analysis

Solventogenic clostridia are an important class of microorganisms that can utilize simple and complex carbohydrates to produce various solvents, acids and other products by anaerobic fermentation [1, 2]. The biochemistry of solventogenesis has been extensively reviewed [1–4]; however, central metabolic pathways remain only partially resolved. Recently, two

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Conflict of interest statement

The authors have declared no conflict of interest.

Note by the Authors. An earlier version of this manuscript was submitted for publication on June 29, 2010. On July 9, the manuscript "Systems-level metabolic flux profiling elucidates a complete, bifurcated TCA cycle in Clostridium acetobutylicum" (doi:10.1128/JB.00490-10) by Amador-Noguez *et al.*, appeared in *Journal of Bacteriology* under *Manuscripts published ahead of print.* The Amador-Noguez *et al.* manuscript presents similar results and conclusions to our analysis.

genome-scale metabolic network reconstructions for *C. acetobutylicum* ATCC 824 were reported [5, 6]. Based on its genome sequence [4], it is accepted that in *C. acetobutylicum* the TCA cycle is degenerate (incomplete) and the oxidative branch of the pentose-phosphate pathway (oxPPP) is missing. Notably, given the inability to identify enzymes needed for the conversion of succinate to fumarate, succinyl-CoA to succinate, and oxaloacetate to citrate (a citrate synthase, CS), the biosynthesis of α-ketoglutarate, a precursor for glutamate, glutamine and proline, remains unknown. The two genome-scale models propose two different pathways for α-ketoglutarate biosynthesis. Senger and Papoutsakis hypothesized a previously unresolved pathway involving the urea cycle and arginine biosynthesis pathway operating in the reverse direction [5], and Lee et al. hypothesized that *C. acetobutylicum* has a reductive TCA cycle operating in the direction from oxaloacetate to fumarate and to α-ketoglutarate [6]. However, there is limited experimental evidence to support either hypothesis. Given that the missing proteins may be coded by previously unidentifiable proteins raises the possibility that the accepted hypotheses regarding these key steps of central metabolism require a careful re-examination. We undertook this re-examination driven by the recent discovery of a novel *Re*-CS in *C. kluyveri*, which has an identifiable ortholog (CAC0970) in *C. acetobutylicum*, and the power of ^{13}C-based isotopomer analysis for in vivo flux analysis [7–9]. Here, we show that *C. acetobutylicum* indeed utilizes *Re*-CS to produce α-ketoglutarate via citrate, that oxPPP is inactive, and that there is no carbon exchange between α-ketoglutarate and fumarate in the TCA cycle.

We used Metran software [8, 10] to design a tracer experiment to elucidate the metabolic pathways in the putative network model (Fig. 1). The optimal tracer was determined to be [1,2-^{13}C]glucose. Figure 2 illustrates the strategy for pathway elucidation from mass isotopomer distributions (MID) of intracellular metabolites that can be measured by GC-MS (see Supporting Information). *C. acetobutylicum* was grown anaerobically to mid-exponential phase in batch culture on defined clostridial growth medium CGM [11] with 20 g/L [1,2-^{13}C]glucose (99%) as the only carbon source. After 14 hrs, the cells produced 35±3 mM acetate and 21±1 mM butyrate. Only low levels of solvents were detected (6.8±1.4 mM ethanol, 1.1±0.2 mM butanol, and 1.5±0.2 mM acetone), indicating that the cells were in the acetogenic phase and had just initiated solvent production. The labeling of glucose in the medium and intracellular metabolites was measured by GC-MS (Waters Quattro-Micro) after *tert*-butyldimethylsilyl derivatization [10, 12] (Fig. 3A). MIDs of pyruvate, aspartate and fumarate were almost identical with M+2 (~40%) as the only enriched mass isotopomer. Loss of C1 of glucose in oxPPP would have resulted in M+1 enriched pyruvate. Thus, the absence of M+1 mass isotopomer indicated that oxPPP was inactive, as had been originally hypothesized [4].

MIDs of citrate, α-ketoglutarate, and glutamate had labeling patterns with M+2 (~37–48%) and M+4 (~16%) as the main enriched mass isotopomers. This labeling pattern suggested that the metabolites were formed by condensation of two ~40% M+2 labeled species, presumably acetyl-CoA and oxaloacetate by CS. The expected M+2 enrichment was 2×0.4×(1–0.4) = 0.48, and the expected M+4 enrichment was 0.4×0.4 = 0.16 (Fig. 2). The labeling pattern of glutamate fragment C2-C5 was characterized by M+1, M+2 and M+3 mass isotopomers, which was consistent with citrate being formed by *Re*-CS (Fig. 2), as *Si*-CS would have resulted in formation of M+2 and M+4 mass isotopomers. To examine the

completeness of the TCA cycle, fumarate labeling was analyzed. Fumarate labeling was identical to that of aspartate indicating that there was no carbon flow from α-ketoglutarate to fumarate, as that would have produced M+1 and M+3 mass isotopomers of fumarate. Moreover, we concluded that there was no carbon flow from fumarate to α-ketoglutarate, as that would have produced higher abundance of M+2 mass isotopomer of glutamate.

Cell-free protein extracts were assayed for CS activity using a commercial kit (Sigma-Aldrich). The specific CS activity in the presence of $MnCl_2$ (presumably then the *Re*-CS activity [13]), was 0.55±0.08 U/mg of total-cell-protein over its corresponding control without oxaloacetate. The specific enzyme activity was reduced to 0.20 U/mg of protein when 30 μl of cell-free protein extracts were treated with a final concentration of 0.3 mM EDTA. This observation suggested the possibility of small levels of non-metal dependent, *Si*-CS activity, although we could not ascertain that EDTA would remove the metal cofactor from the presumed *Re*-CS protein with 100% effectiveness. *Si*-CS activity would not be consistent with the ^{13}C-tracer data (Figs. 2 and 3A). Metal dependent CS activity was restored to levels similar to those observed without EDTA treatment upon adding 0.5 mM of $MnCl_2$ to the reaction mixture that was treated with EDTA. This further supported the assumption that most of the CS activity was derived from the *Re*-CS enzyme.

The gene coding for this activity has been tentatively identified as CAC0970 and is the first gene of a putative tricistronic operon (CAC0970–0971-0972) [13, 14], with the last two genes coding for aconitase and isocitrate dehydrogenase (Fig. 3B). These three genes code for the proteins catalyzing three sequential reactions of the TCA cycle (Fig. 1). The expression of these genes based on microarray studies [15] appears to be similar (Fig. 3C-D), thus supporting the prediction that the genes belong to the same operon. This operon is expressed well, and apparently at higher levels in the stationary phase of culture, which is consistent with the predicted σ^E promoter for this operon [14]. We also examined the gene expression patterns (Fig. S1) of all the genes putatively identified (Fig. 1) to support the reactions of glycolysis, pentose-phosphate pathway and TCA cycle. The goal was to examine the extent to which these expression patterns and levels were consistent with each other within each of the three pathways, identify which among possible multiple genes are likely involved in a reaction, and if a reaction is likely to operate in a particular stage of the fermentation. While there is no perfect correlation between gene expression and fluxes, previous analysis has shown [16] that gene expression analysis is consistent with results from flux analysis. Overall, most of the pentose-phosphate pathway genes are expressed low, while all identifiable core TCA genes appear to be expressed at high levels and apparently in a synchronized fashion.

The results from this work are significant because they provide a solid basis for future studies of *C. acetobutylicum* using ^{13}C-metabolic flux analysis [17–20]. In addition, our work identifies new targets for metabolic engineering of energy metabolism in *C. acetobutylicum* with implications for biofuel production. In particular, metabolic engineering of the TCA cycle is a potential strategy for improving biofuel yield now that it is possible to achieve limited growth of *C. acetobutylicum* under microaerobic conditions by manipulating the expression of the peroxide repressor-like *perR* gene [2, 21]. Growth under microaeronic conditions may not only simplify bioprocessing, but significantly, cells may be

able to derive biosynthetic energy (ATP) via oxygen utilization. This will have benefits for solvent production and especially the butanol yield by decreasing the need to produce high amounts of acetate for ATP production [2, 22–24].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

CS	citrate synthase
MID	mass isotopomer distribution
oxPPP	oxidative pentose-phosphate pathway

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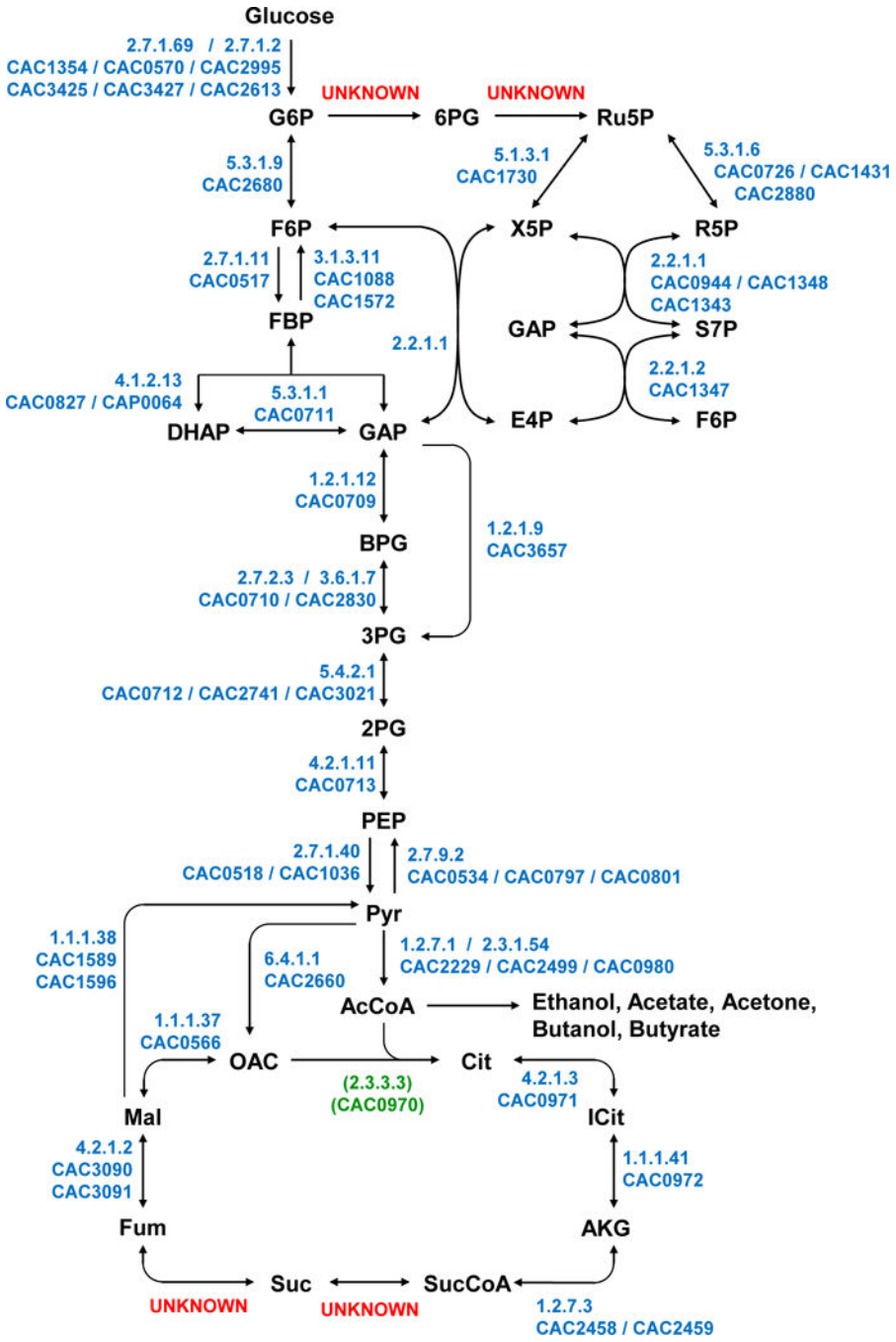

Figure 1. Central metabolic pathways of *C. acetobutylicum* based on the original genome annotation [4] and two recently constructed genome-scale models of *C. acetobutylicum* ATCC 824 [5, 6].

Unresolved or hypothesized pathways/reactions are indicated as UNKNOWN. The proposed/putative gene (CAC0970) coding for *Re*-citrate synthase is shown.

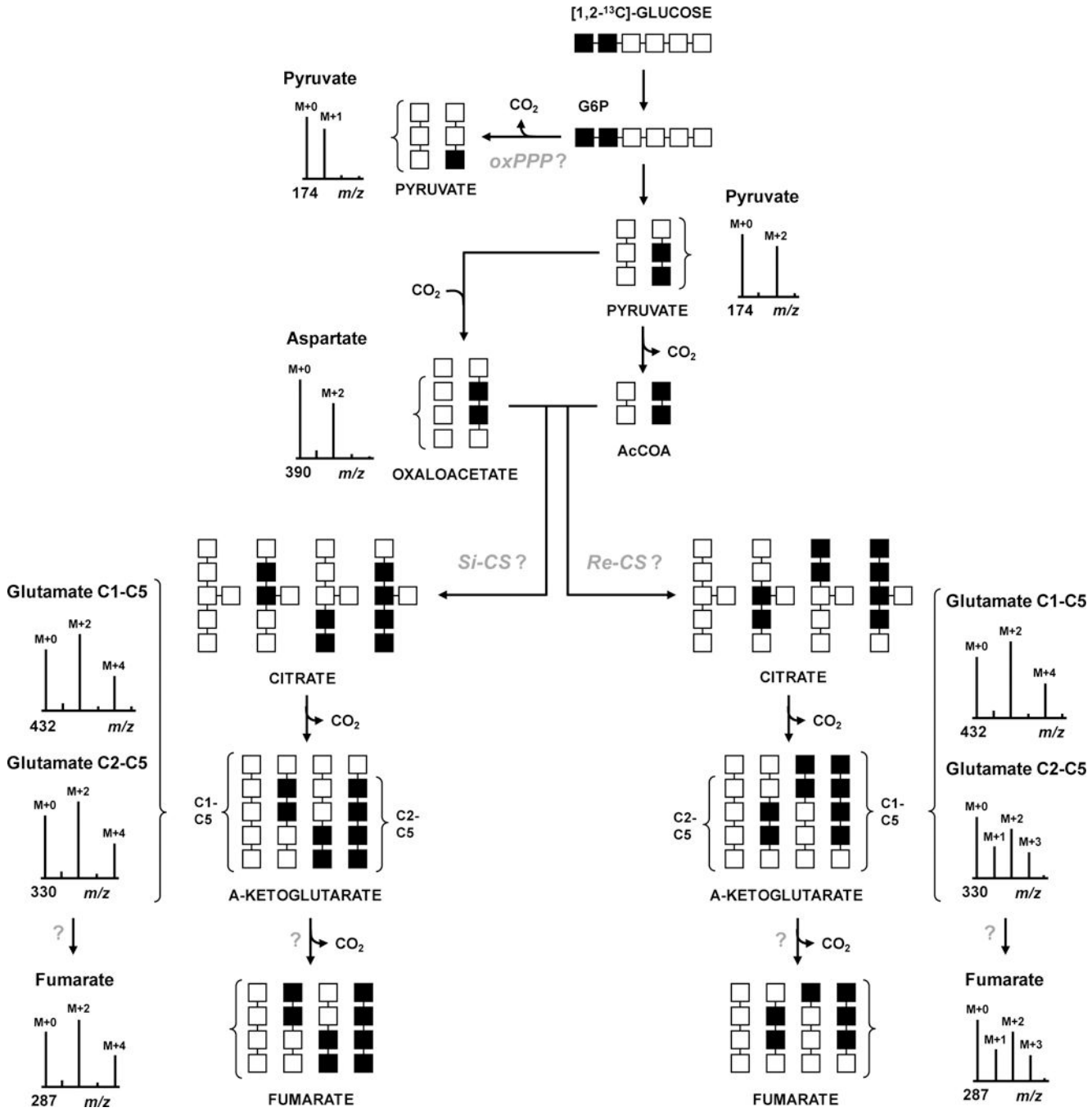

Figure 2. ^{13}C-Labeling experiment designed to elucidate central metabolic pathways in *C. acetobutylicum.*

C. acetobutylicum is grown on [1,2-$^{13}C_2$]glucose as the sole carbon source (■=^{13}C, □=^{12}C). The expected labeling profiles and MIDs of intracellular metabolites at key points in metabolism are shown. Presence or absence of oxPPP is determined from the labeling of pyruvate. Condensation of M+2 labeled oxaloacetate and acetyl-CoA via CS will result in a characteristic pattern of M+2 and M+4 mass isotopomers of glutamate (m/z 432 fragment). To determine the stereochemistry of CS, MID of glutamate fragment m/z 330 is measured, which retains carbon atoms C2-C5. For *Si*-CS, MID of m/z 330 fragment of glutamate will

be identical to that of m/z 432 fragment of glutamate; for *Re*-CS characteristic M+1 and M +3 isotopomers will be formed. To determine if the TCA cycle is complete, MID of fumarate is compared to that of glutamate fragment m/z 330 and aspartate.

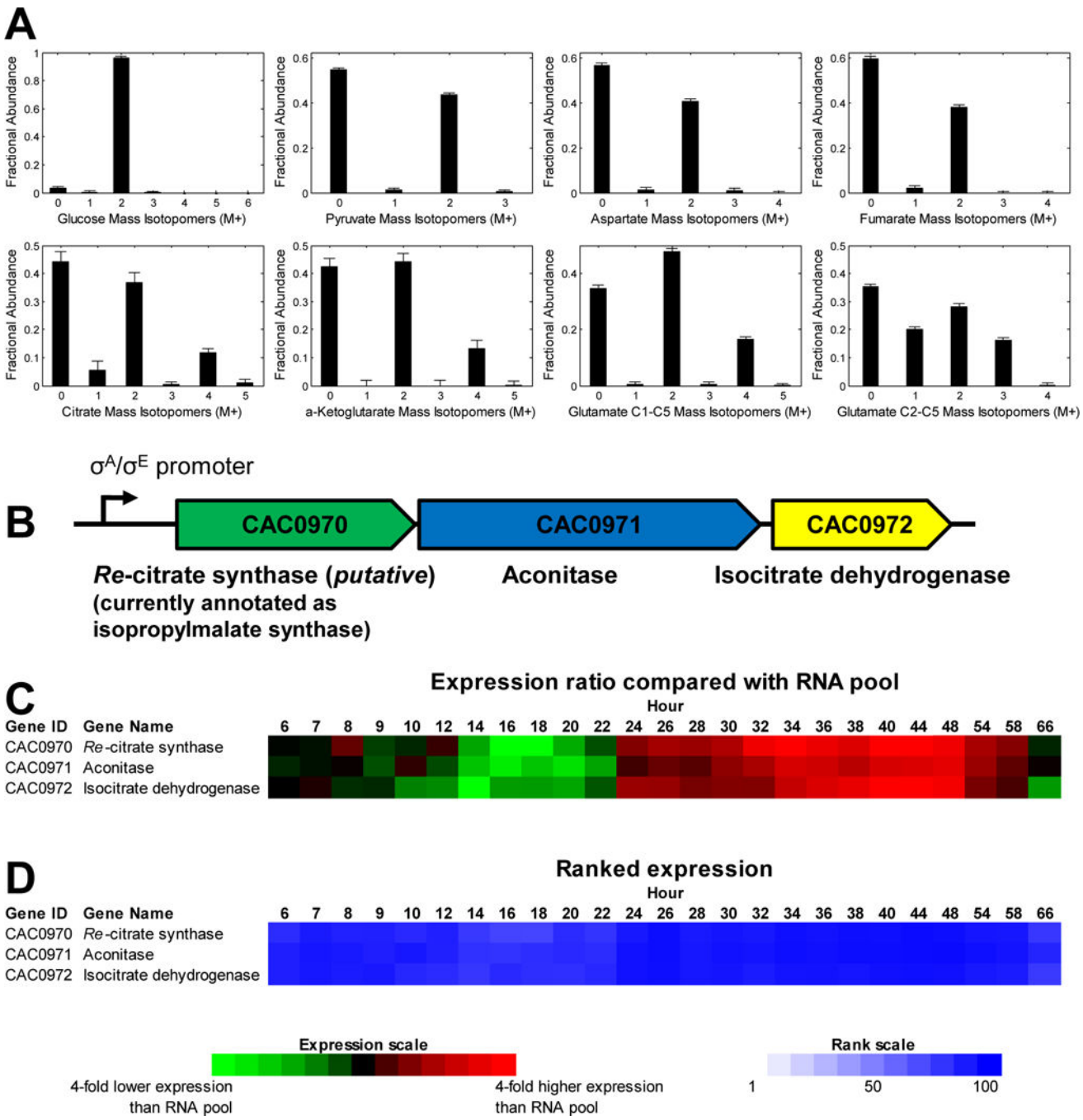

Figure 3. Mass isotopomer distributions of glucose and intracellular metabolites from *C. acetobutylicum* grown on [1,2-$^{13}C_2$]glucose, and putative *Re*-citrate synthase in *C. acetobutylicum* ATCC 824.

(A) MIDs of glucose, pyruvate, aspartate, fumarate, citrate, α-ketoglutarate, and two fragments of glutamate, m/z 432 fragment (C1-C5) and m/z 330 fragment (C2-C5). Data shown were corrected for natural isotope enrichments. (B) *Re*-citrate synthase is putatively coded by CAC0970 which is part of the tricistronic operon CAC0970–0971-0972. (C) Expression values are presented as ratios compared to a reference mRNA pool [15]. (D)

Level of expression as measured by the ranked expression intensity values for each gene. Ranks run from 100 to 1 based on all the expressed genes of the genome at each time point.