Convergent evolution of a modified, acetate-driven TCA cycle in bacteria

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Abstract

The tricarboxylic acid (TCA) cycle is central to energy production and biosynthetic precursor synthesis in aerobic organisms. There exist few known variations of a complete TCA cycle, with the common notion being that the enzymes involved have already evolved towards optimal performance. Here, we present evidence that an alternative TCA cycle, in which acetate:succinate CoA-transferase (ASCT) replaces the enzymatic step typically performed by succinyl-CoA synthetase (SCS), has arisen in diverse bacterial groups, including microbial symbionts of animals such as humans and insects.

Keywords

citric acid cycle; Krebs cycle; acetate; gut microbiota; *Snodgrassella alvi*

Main text

The tricarboxylic acid (TCA) cycle arose during early evolution and represents a core process in aerobic respiration and production of carbon-based precursor molecules needed for the biosynthesis of amino acids, nucleotides and cofactors. The enzymes that complete the cycle show homology across Bacteria, Archaea, and Eukaryota, and are highly efficient[1,2]; consequently, there are relatively few known variations of a complete TCA cycle. Many bacteria have incomplete cycles, while some autotrophs use a reverse (reductive) cycle to fix carbon from CO_2[3]. Alternative oxidative cycles have been proposed to operate in Cyanobacteria[4], *Mycobacterium*[5], and *Helicobacter*[6]. Another variant cycle was identified in *Acetobacter aceti*, whereby succinyl-CoA is converted to succinate by an acetate:succinate CoA-transferase (ASCT) instead of the typical succinyl-CoA synthetase

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(SCS)[7,8]. The variant ASCT pathway is thought to enhance tolerance to the high levels of acetate produced by *Acetobacter* during fermentation[7,8].

In the course of an investigation into the genome of *Snodgrassella alvi*, a resident gut bacterium of honey bees, we noticed the absence of the two genes encoding SCS subunits (*sucC* and *sucD*) from an otherwise complete TCA cycle[9]. This obligately aerobic heterotroph also lacks pathways for glycolysis and the glyoxylate shunt, implying reliance on the TCA cycle for energy and biosynthesis. We located a gene (hereafter referred to as *asct*) encoding a protein with sequence similarity to CoA-transferases, which we hypothesized might fulfill the missing role of interconverting succinate and succinyl-CoA (Fig. 1A). This gene has been shown to confer a strong fitness benefit. Based on a recent genome-wide transposon mutagenesis screen[10], *S. alvi* suffered a 38.4-fold fitness reduction in the bee gut when *asct* was disrupted ($p_{adj} = 5.5 \times 10^{-10}$), which was similar to the reductions caused by disruption of other TCA cycle genes (32.2-fold average, $p_{adj} < 10^{-5}$).

S. alvi's *asct* shows sequence homology (55% amino acid identity) to *aarC*, which encodes the previously characterized acetate:succinate CoA-transferase that completes the TCA cycle in *Acetobacter aceti*[7,8]. This homology suggests that the ASCT family of transferases may have a more widespread and substantive role in central carbon metabolism than previously reported. We searched the prokaryotic genome database on NCBI for genomes harbouring ASCT. Of the 5,014 complete genomes queried, 1,067 had positive hits; however, many of these matches may represent CoA-transferases specific for other substrates. Focusing only on genomes that also lacked *sucC* and/or *sucD* narrowed these candidates to 180 strains. These represent species from at least 3 phyla, and include several prominent bacteria such as those used in industrial vinegar fermentation (*Acetobacter* spp.), human commensals (*Kingella* spp. and *Neisseria* spp.), and opportunistic pathogens (*Moraxella* spp. and *Corynebacterium diphtheriae* – the causative agent of diphtheria).

To determine if the putative *asct* homologs in these diverse bacteria could functionally replace SCS and complete the oxidative TCA cycle as predicted from their genomes, we cloned and expressed several of these genes in *E. coli* strains lacking *sucC* or *sucD* (Table S1). Upon cultivation in minimal media with acetate as the sole carbon source, only strains complemented with *asct* were able to grow (Fig. 1B), indicating successful rescue of TCA cycle function. Enzymatic assays confirmed the production of acetyl-CoA in *asct*-bearing *E. coli*, which verified that the TCA cycle was being completed via the modified route (Table 1). Interestingly, *asct*-complemented *sucC* knockouts (*sucC*) grew at a faster rate than *asct*-complemented *sucD* strains. Given the upstream location of *sucC*, *sucC* knockouts are expected to more fully disrupt expression of *sucCD* and abolish SCS function (Fig. 1B). The faster growth of *sucC* mutants, and the lower growth of stains co-expressing *sucCD* and *asct* (Supplementary Fig. 1) suggests potential competitive inhibition between SCS and ASCT, at least within the *E. coli* metabolic and gene regulatory framework. Although not tested here, further experiments with double-mutants and tuning of enzyme expression may help uncover these interactions.

The choice of using SCS or ASCT to fulfill TCA cycle function involves a trade-off between the ATP gain with SCS from substrate-level phosphorylation, and the more efficient

catabolism of acetate via ASCT[7] (Fig. 1A). In acetate-rich environments, such as some animal guts, growth may be enhanced by silencing or deleting *sucCD* and relying exclusively on ASCT to complete the TCA cycle. However, their presence is not mutually exclusive – in fact, most of the putative *asct* homologs we detected are found in genomes with complete *sucCD* genes. A phylogeny of *asct* shows widespread co-occurrence of ASCT with SCS, as well as multiple evolutionarily independent lineages with dedicated, ASCT-dependent TCA cycles (Fig. 1C, Supplementary Fig. 2). The *asct* sequences grouped largely according to bacterial clades, suggesting a deep origin and subsequent diversification of this gene. Horizontal gene transfers between distantly related bacteria are also evident, such as in the acquisition of *asct* by the gammaproteobacterium *Moraxella*, which has a copy clustering with sequences from Betaproteobacteria. Additionally, some members of the Alpha-, Beta-, and Gammaproteobacteria possess *asct* from a cluster that tends to co-occur with *sucCD*.

Taking a closer look at the *Neisseriaceae*, an obligately aerobic family of Betaproteobacteria, using whole-genome phylogenetics, we find evidence of repeated transitions between SCS- and ASCT-dependent TCA function (Supplementary Fig. 3). At least 14 species in the clade containing *Neisseria* spp. likely have ASCT-dependent TCA cycles, with the switch from SCS to ASCT occurring at least once and from ASCT to SCS occurring twice. Evolutionary transitions between exclusively ASCT or SCS pathways require both the gain of one enzyme and the loss of the other, a feat that is improbable unless motivated by redundancy and, perhaps, an adaptive advantage in only retaining one pathway.

Surprisingly, we also detected putative *asct* homologs in a number of obligate anaerobes, such as *Bacteroides* species, which are abundant in the gut communities of mammals, including humans. Here, these genes may operate in a reductive manner, converting succinate to succinyl-CoA for synthesis of 2-oxoglutarate or further degradation[11]. It is notable that, as with TCA-oxidizing bacteria, some of these reverse-TCA utilizers have lost SCS and instead appear to rely on ASCT (Fig. 1C). We tested the function of the *asct* homologs present in *Bacteroides fragilis*, an obligate anaerobe, and *Acinetobacter baumannii*, an obligate aerobe, both of which also carry SCS, and discovered that both enzymes can successfully rescue TCA cycle function (Fig. 1B). These findings support our conclusion that ASCT can replace the function of SCS, but also that the retention of both ASCT and SCS in some organisms may be beneficial.

Further analyses will be required to better characterize ASCT and its interactions with other components of cellular metabolism. The fulfillment of critical metabolic duties by ASCT is not unprecedented: in the hydrogenosomes of some protozoans, ATP production is mediated by an ASCT in a coupled cycle with SCS[12]. In bacteria, the repeated evolution of ASCT-dependent TCA cycles may be a consequence of niche-specialization, reflecting adaptation to life in acetate-rich environments[7,9,13]. Our results demonstrate the inherent diversity and flexibility of the TCA cycle[14], and should prompt a re-evaluation of the role of unorthodox enzymes in central metabolic processes.

Methods

Heterologous expression of ASCT

The genes coding for ASCT in *S. alvi* wkB2, *Acetobacter cerevisiae* ATCC 23765, *C. diphtheriae* NCTC 13129, *Moraxella catarrhalis* ATCC 25240, *Acinetobacter baumannii* ATCC 19606, and *Bacteroides fragilis* NCTC 9343 were amplified by PCR from genomic DNA and cloned into the plasmid pBad-EBFP2[15] (Table S1). The existing blue fluorescent protein gene, EBFP2, was excised by digesting with NdeI and EcoRI (New England BioLabs) prior to cloning in the ASCT genes via blunt-end ligation with T4 DNA ligase (Promega). The plasmids were transformed into the Keio collection *E. coli* strains CGSC 8788 (*sucC*) and CGSC 11810 (*sucD*)[16]; the parent strain, CGSC 7636, was used as a control. To test for rescue of TCA cycle function, strains were grown in M9 minimal media (1% w/v M9 salts, 1mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.3% w/v sodium acetate. Growth curves of cultures, grown at 37°C aerobically, were quantified by measuring absorbance at 600 nm on a Tecan Spark 10M. Growth assays were conducted in triplicate, with average values plotted in Fig. 1B. pBad-EBFP2 and pWK31 (Supplementary Table 1) were used as vector controls.

Phylogenetic analysis

Prokaryotic genomes were obtained from the NCBI Genomes database. To identify bacteria with putative ASCT, genomes were searched by BLASTP using the ASCT of *Acetobacter aceti* 1023 (AarC) as the query. To determine the presence/absence of SCS, genomes were searched by BLASTP using the SucC and SucD sequences of *E. coli* MG1655 as queries. For select strains of interest, searches for other genes of the TCA cycle were also conducted to verify cycle completeness. BLASTP hits with over 75% query coverage and 40% similarity were retained.

The phylogeny of *asct* genes (Fig. 1C) was built with codon-aligned nucleotide sequences in RAxML version 8 using the maximum-likelihood algorithm and a GTRGAMMAI substitution model with 1,000 bootstraps[17]. For the whole-genome *Neisseriaceae* phylogeny, single copy orthologs were identified from representative genomes for all available *Neisseriaceae* species using OrthoFinder[18]. Their amino-acid sequences were then extracted and aligned using MUSCLE[19], concatenated, and degapped. The tree was built using the maximum-likelihood algorithm in RAxML version 8 with the PROTCATLG model and 1,000 bootstraps.

Enzyme activity assays

E. coli strains were were grown at 37°C in 2-liter flasks containing 1 liter of LB medium with ampicillin for 2 hours, and induced with 0.02% arabinose at OD_{600nm} of 0.2–0.4. Cells were grown continuously until OD_{600nm} = 1.0, and harvested by centrifugation. The harvested cells were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5; 100 mM NaCl) and sonicated 20 times with bursts of 10 s followed by 10 s for cooling. Cell debris was removed by centrifugation at 4°C for 20 min at 20,000 *g* (fast spin lysate) or 10 min at 2,700 *g* (slow spin lysate). The protein in the supernatant was quantified with the Quick Start^TM Bradford Protein Assay kit using the Bovine Serum Albumin Standard Set (Bio-Rad Inc.).

The ASCT activity assay was performed as described by Mullins *et al.*[7]. In brief, the reaction was performed in 50 mM potassium phosphate buffer (pH 8.0) with 100 mM KCl, 350 mM potassium acetate, and 0.5 mM succinyl-CoA. The reaction was initiated by adding 150–200 μg of the fast spin cell lysate. After 5 min, 20 μl of the reaction mixture was used to measure the amount of produced acetyl-CoA using the Acetyl-Coenzyme A Assay Kit (Sigma-Aldrich Inc.). Acetyl-CoA Quencher supplied in the kit was used to correct for background created by free Coenzyme A or succinyl-CoA in the samples. Succinyl-CoA synthetase activity was assayed using the Succinyl-CoA Synthetase Activity Colorimetric Assay Kit (BioVision Inc.) with the slow spin lysate following the kit protocol. Detection limits for ASCT and SCS assays are 0.2 and 20 nmol product/min/mg protein, respectively.

Data availability

Sequence data used are available from GenBank and accession numbers are referenced in the supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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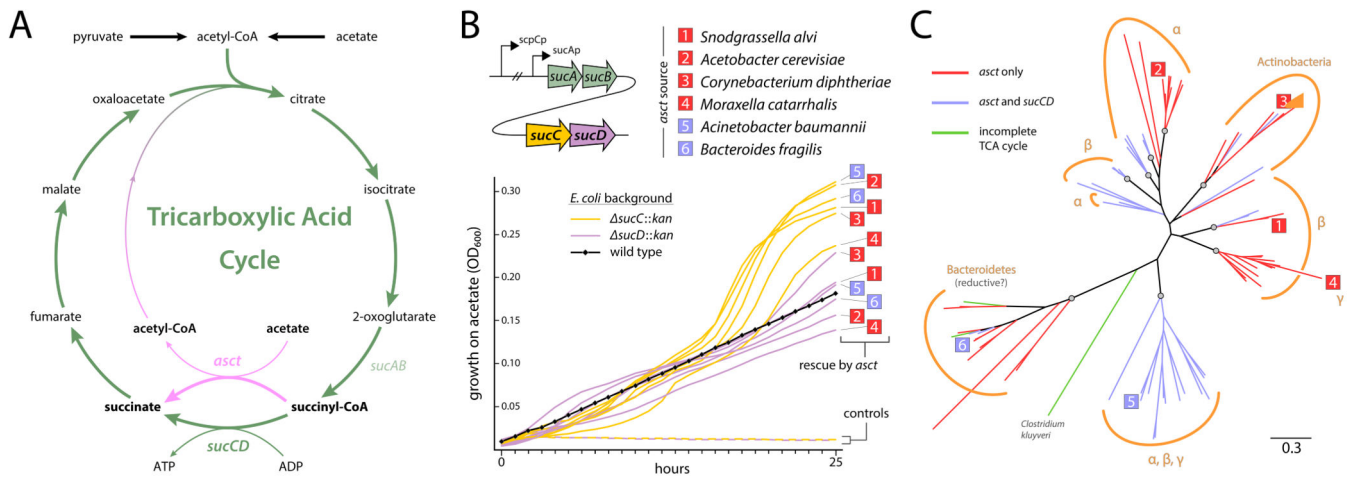
Figure 1.

An acetate-driven TCA cycle in diverse bacteria. (A) The TCA cycle (green) and the *asct* modification (pink). (B) Restoration of TCA cycle function by *asct* from diverse bacteria (labeled 1–6) in *E. coli* succinyl-CoA synthetase (SCS) knockouts. Operon structure of *sucCD* in *E. coli* is shown at upper left (not to scale); black arrows indicate promoters. Δ, deletion; *::kan*, kanamycin resistance gene insertion. Control strains, carrying a plasmid without *asct* (pBad-EBFP2), are denoted by dashed lines. Colour of lines indicate *E. coli* background of *asct*-complemented or control strains; strains are listed in Supplementary Table 1. Growth curves represent average of three replicates; see Supplementary Table 2 for means and standard deviations at each time point. (C) Phylogeny of *asct* from representative species of phyla Bacteroidetes, Actinobacteria, and Proteobacteria (α, β, γ; classes Alpha-, Beta-, and Gamma-proteobacteria, respectively). Circles indicate nodes with > 95% bootstrap support from maximum-likelihood analysis. Taxa used to build this tree are shown in Supplementary Fig. 2. Numbered labels indicate positions of taxa in (B), with color scheme reflecting presence (blue) or absence (red) of co-occurring *sucD*.

Table 1

Enzyme activities of ASCT and SCS.

E. coli strain	Activity (nmol product formed/min/mg protein)	
	ASCT[a]	SCS[b]
CGSC 8788 (sucC) + pWK1 (asct from S. alvi)	75 ± 8	—
CGSC 8788 (sucC) + pBad-EBFP2 (control)	—	—
CGSC 7636 (wild type) + pBad-EBFP2 (control)	—	234 ± 15

Means ± s.d. (n = 3) shown. —, activity not detected.

[a]Succinyl-CoA + Acetate → Succinate + Acetyl-CoA

[b]Succinyl-CoA + Pi + ADP → Succinate + CoA + ATP