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

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The tricarboxylic acid (TCA) cycle is central to energy production and biosynthetic precursor synthesis in aerobic organisms. There are 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.

The tricarboxylic acid (TCA) cycle arose during early evolution and represents a core process in aerobic respiration and production of the 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 efficient3,4. 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 CO2 (ref. 3). Alternative oxidative cycles have been proposed to operate in Cyanobacteria5, Mycobacterium6 and Helicobacter7. Another variant cycle was identified in Acetobacter aceti, in which succinyl-CoA is converted to succinate by an acetate:succinate CoA-transferase (ASCT) instead of the typical succinyl-CoA synthetase (SCS)5,7. The variant ASCT pathway is thought to enhance tolerance to the high levels of acetate produced by Acetobacter during fermentation5,7.

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 cycle8. This obligately aerobic heterotroph also lacks pathways for glycolysis and the glyoxylate shunt, implying a 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 hypothesize 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 screen9, S. alvi suffered a 38.4-fold fitness reduction in the bee gut when asct was disrupted (Padj = 5.5 × 10−10), which is similar to the reductions caused by disruption of other TCA cycle genes (32.2-fold average, Padj < 1 × 10−5).

The S. alvi asct gene shows sequence homology (55% amino acid identity) to aarC, which encodes the previously characterized ASCT that completes the TCA cycle in A. aceti7,8. 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 three 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 whether the putative asct homologues in these diverse bacterial groups could functionally replace SCS and complete the oxidative TCA cycle as predicted from their genomes, we cloned and expressed several of these genes in Escherichia coli strains lacking sucC or sucD (Supplementary Table 1). 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 (ΔsucC) grew at a faster rate than asct-complemented ΔsucD 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 ΔsucC mutants and the lower growth of strains co-expressing sucCD and asct (Supplementary Fig. 1) suggest 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 to uncover these interactions.

The choice of using SCS or ASCT to fulfill TCA cycle function involves a trade-off between the adenosine triphosphate (ATP) gain with SCS from substrate-level phosphorylation and the more efficient catabolism of acetate via ASCT10 (Fig. 1a). In acetate-rich environments, such as the guts of some animals, 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 homologues that 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 and Supplementary Fig. 2). The asct sequences group 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. probably 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 homologues 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. 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.

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 (labelled 1–6) in E. coli succinyl-CoA synthetase (SCS) knockouts. The operon structure of sucCD in E. coli is shown (top 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. Colours of lines indicate E. coli background of asct-complemented or control strains; strains are listed in Supplementary Table 1. Growth curves represent the average of three replicates. Supplementary Table 2 shows means and standard deviations at each time point. c, Phylogeny of asct from representative species of phyla Bacteroidetes, Actinobacteria and Proteobacteria (α, β and 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 colour scheme reflecting presence (blue) or absence (red) of co-occurring sucCD.
on ASCT (Fig. 1c). We tested the function of the \textit{asc} homologues in \textit{Bacteroides fragilis}, an obligate anaerobe and \textit{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\(^1\). 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\(^2,3\). Our results demonstrate the inherent diversity and flexibility of the TCA cycle\(^4\) and should prompt a reevaluation of the role of unorthodox enzymes in central metabolic processes.

### Methods

**Heterologous expression of ASCT.** The genes coding for ASCT in \textit{S. ali} wk82, \textit{Acetobacter cerevisiae} ATCC 23765, \textit{C. diphteriae} NCTC 13129, \textit{Moraxella catarrhalis} ATCC 25240, \textit{A. baumannii} ATCC 19606 and \textit{B. fragilis} NCTC 9343 were amplified by PCR from genomic DNA and cloned into the plasmid pBAD-EBFP2 (Supplementary Table 1)\(^5\). The existing blue fluorescent protein gene, EBFP2, was excised by digesting with NdeI and EcoRI (New England BioLabs) before cloning in the ASCT genes via blunt-end ligation with T4 DNA ligase (Promega). The plasmids were transformed into the Keio collection \textit{E. coli} strains CGSC 8788 (\textit{ΔsucC}) and CGSC 11810 (\textit{ΔsucC} and \textit{ΔCSCT})\(^6\); the parent strain, CGSC 7636, was used as control. To test for rescue of TCA cycle function, strains were grown in M9 minimal medium (1% wt/vol M9 salts, 1 mM MgSO\(_4\), 0.1 mM CaCl\(_2\)) supplemented with 0.3% wt/vol 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 \textit{ASCT} of \textit{A. acet} 1023 (AarC) as the query. To determine the presence/absence of SCS, genomes were searched by BLASTP using the SucC and SucD sequences of \textit{E. coli} MG1655 as queries. For select strains of interest, searches were performed using an Acetyl-CoA Assay Kit (Sigma-Aldrich). Acetyl-CoA Quencher, succinyl-CoA synthetase activity was assayed using a.

**Table 1** Enzyme activities of ASCT and SCS.

| E. coli strain                      | Activity (nmol product formed per min per mg protein) |
|------------------------------------|-----------------------------------------------------|
| \textit{CGSC} 8788 (\textit{ΔsucC} + pWK1) (asc from \textit{S. ali}) | \textit{ASCT*} 75 ± 8                                 |
| \textit{CGSC} 8788 (\textit{ΔsucC} + pBAD-EBFP2 (control)) | 234 ± 15                                           |

Data presented as mean ± s.d. (\(n = 3\)). *Sucinyl-CoA + acetate → succinate + acetyl-CoA. + Sucinyl-CoA + P + ADP → succinate + CoA + ATP. ADP, adenosine diphosphate; P\(_i\), inorganic phosphate.

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**Acknowledgements**

This work was supported by the Canadian Natural Sciences and Engineering Research Council through Postgraduate Scholarship award PGSD-3-420434-2012 (to W.K.K.), the US National Science Foundation Dimensions of Biodiversity awards 1046153 and 1415604 and the US National Institutes of Health award 1R01GM108477-01 (to N.A.M.).

**Author contributions**

Conceptualization, formal analysis, visualization and writing of the original draft were carried out by W.K.K. Methodology and investigation were performed by W.K.K. and H.Z. Writing, reviewing and editing of the manuscript were carried out by W.K.K., H.Z. and N.A.M. Resources and supervision were provided by N.A.M.

**Additional information**

Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to W.K.K.

How to cite this article: Kwong, W. K., Zheng, H. \& Moran, N. A. Convergent evolution of a patented metabolic cycle in bacteria. \textit{Nat. Microbiol.} 2, 17967 (2017).

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**Competing interests**

The authors declare no competing financial interests.