

A Specialized Citric Acid Cycle Requiring Succinyl-Coenzyme A (CoA):Acetate CoA-Transferase (AarC) Confers Acetic Acid Resistance on the Acidophile *Acetobacter aceti*^{∇†}

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Microbes tailor macromolecules and metabolism to overcome specific environmental challenges. Acetic acid bacteria perform the aerobic oxidation of ethanol to acetic acid and are generally resistant to high levels of these two membrane-permeable poisons. The citric acid cycle (CAC) is linked to acetic acid resistance in *Acetobacter aceti* by several observations, among them the oxidation of acetate to CO₂ by highly resistant acetic acid bacteria and the previously unexplained role of *A. aceti* citrate synthase (AarA) in acetic acid resistance at a low pH. Here we assign specific biochemical roles to the other components of the *A. aceti* strain 1023 *aarABC* region. AarC is succinyl-coenzyme A (CoA):acetate CoA-transferase, which replaces succinyl-CoA synthetase in a variant CAC. This new bypass appears to reduce metabolic demand for free CoA, reliance upon nucleotide pools, and the likely effect of variable cytoplasmic pH upon CAC flux. The putative *aarB* gene is reassigned to SixA, a known activator of CAC flux. Carbon overflow pathways are triggered in many bacteria during metabolic limitation, which typically leads to the production and diffusive loss of acetate. Since acetate overflow is not feasible for *A. aceti*, a CO₂ loss strategy that allows acetic acid removal without substrate-level (de)phosphorylation may instead be employed. All three *aar* genes, therefore, support flux through a complete but unorthodox CAC that is needed to lower cytoplasmic acetate levels.

Acetic acid bacteria (AAB) are gram-negative, acidophilic α -proteobacteria that oxidize ethanol to acetic acid using membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase enzymes. AAB are remarkably resistant to the membrane-permeable toxic compounds ethanol and acetic acid at a low pH. Members of the AAB genus *Acetobacter* were historically differentiated from those of the genus *Gluconobacter* by a preference for ethanol and the ability to “overoxidize” acetate to CO₂, usually when ethanol is unavailable (5). Acetate oxidation implicates the oxidative decarboxylations performed by citric acid cycle (CAC) dehydrogenases and a complete CAC.

The molecular mechanisms of acetic acid resistance in *Acetobacter aceti* include adaptation of the cytoplasmic components (9, 14) to internal acidification (32), acetic acid efflux via the AatA acetic acid:proton antiporter (31, 39), and production of acid-inducible proteins identified by proteomic screens, many with undefined biochemical roles (28, 52).

A. aceti strain 1023, an especially acid-resistant and comparatively thermotolerant vinegar factory isolate (41, 42), requires acetic acid resistance genes *aarABC* for growth on >50 mM acetic acid at a low pH (16). AarA is a NADH-insensitive hexameric form of citrate synthase (15). AarB is predicted to be a basic protein of 154 amino acids with no homologues or known function (16). AarC is required for acetate oxidation

and resembles several acyl-coenzyme A (CoA):carboxylate CoA transferases but is not acetyl-CoA synthetase (17).

The apparent requirement of the *A. aceti* CAC for acetic acid resistance may indicate a direct role in depleting cytoplasmic acetate by way of acetyl-CoA oxidation to CO₂ or a general contribution to energy production. *A. aceti* requires vigorous oxygenation at high acetate levels and low pHs (37), which is consistent with either CAC role. However, a draft genome sequence of *A. aceti* strain 1023 (T. J. Kappock, S. W. Clifton, and R. K. Wilson, unpublished data) lacks malate dehydrogenase (Mdh) and succinyl-CoA synthetase (SCS) genes.

The goal of this study was to learn if the *A. aceti* CAC is interrupted. We found that *A. aceti* contains a complete but modified CAC, enabling direct acetate incorporation by an internal shunt. Mdh and SCS are functionally replaced by malate:quinone oxidoreductase (Mqo) and succinyl-CoA:acetate CoA-transferase (SCACT), respectively. SCACT is encoded by *aarC*. This biochemical function explains the genetic evidence for the essential roles of *aarC* in acetic acid resistance, assimilation, and oxidation (16, 17).

MATERIALS AND METHODS

Reagents and general analytical methods. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher (Houston, TX). Dethiaacetyl-CoA was synthesized as described previously (15). Oligodeoxynucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA) and used without further purification. *A. aceti* citrate synthase with a hexahistidine affinity tag (CSH6_{Aa}) was purified as described previously (15). Restriction enzymes, DNA-modifying enzymes, and DNA size standards were purchased from New England Biolabs (Beverly, MA). Absorbance measurements were recorded on a Cary Bio 100 UV-visible spectrophotometer (Varian, Palo Alto, CA) or a diode-array spectrophotometer (Agilent Technologies, Santa Clara, CA). Analytical gel filtration, electrospray ionization-mass spectrometry (ESI-MS), and protein quantitation by the method of Bradford were performed as described previously (14).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source
Strains		
<i>A. aceti</i> 1023	Acid-tolerant factory strain	Etsuko Entani ^b
<i>A. aceti</i> 2002	<i>A. aceti</i> type strain, DSM 2002	DSMZ
<i>E. coli</i> C41	F' <i>ompT hsdS_B(r_B m_B⁻) gal dcm</i> (DE3) with an uncharacterized mutation	Avidis ^c
Plasmids		
pET23a	T7 promoter expression vector, Ap ^r	Novagen
pET23d	T7 promoter expression vector, Ap ^r	Novagen
pJK286	<i>uctB</i> ligated into NdeI and HindIII sites in pET23a	This study
pJK357	<i>aarC</i> ligated into NdeI and XhoI sites in pET23a	This study
pJK358	<i>uctD</i> ligated into NdeI and EcoRI sites in pET23a	This study
pJK359	<i>uctA</i> ligated into NdeI and EcoRI sites in pET23a	This study
pJK360	<i>uctC</i> ligated into NcoI and EcoRI sites in pET23d ^d	This study
pJK385	Derivative of pJK357 that produces AarCH6 ^e	This study

^a Ap, ampicillin.

^b Described in reference 41.

^c Described in reference 35.

^d Encodes a Thr2→Ala mutation at the unique NcoI site.

^e QuikChange mutagenesis (Stratagene) of pJK357 was performed to introduce a Ter506→Ser mutation in *aarC*, adding SLEHHHHHH to the AarC C terminus.

Bacterial strains, media, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *A. aceti* strains were propagated in Difco yeast extract-peptone-dextrose medium (Becton Dickinson, Franklin Lakes, NJ) supplemented with 2.5% ethanol at 30°C as described previously (14). DNA was isolated and manipulated using standard protocols (2). PCRs were performed using *A. aceti* genomic DNA (isolated with DNazol; Molecular Research Center, Cincinnati, OH), appropriate oligodeoxynucleotide primers, and Vent DNA polymerase (New England Biolabs). All clones contained the expected DNA sequences.

HPLC-coupled SCACT assays. SCACT assays were performed at 25°C in 50 mM potassium phosphate (pH 8.0), 100 mM KCl, the indicated substrates, and AarC with a C-terminal hexahistidine-containing fusion peptide (AarCH6; 5 to 50 ng), which was used to initiate the reaction. Forward-direction reaction mixtures (LCF assays) contained 350 mM potassium acetate, 0.2 mM succinyl-CoA, or varied amounts of one substrate. Reverse-direction reaction mixtures (LCR assays) contained 20 mM succinic acid, 1 mM acetyl-CoA, or varied amounts of one substrate. After 5 min, an aliquot (0.1 ml) of the reaction mixture was transferred into 6.25% trichloroacetic acid (0.4 ml), vortexed briefly, and centrifuged at 16,100 × g for 3 min. The soluble portion was then transferred to an autosampler vial and analyzed by high-performance liquid chromatography (HPLC) using the method described in the supplemental material. No-enzyme controls were processed in the same manner.

Peak areas were referenced to an acetyl-CoA calibration curve ($\epsilon_{260\text{ nm}} = 16.4$

$\text{mM}^{-1}\text{ cm}^{-1}$) (12), and velocities were determined from the product CoA thioester peak area. Under the fixed-time assay conditions used, the rate of product formation was linear in each direction for at least 10 min. Velocities were averages of three or four determinations unless otherwise noted. Kinetic constants were determined using Prism (GraphPad) to fit equations for Michaelis-Menten kinetics, competitive inhibition, or substrate inhibition (8). A sum-of-squares *F* test was used to discriminate between alternative kinetic models.

Inactivation experiments. AarCH6 (0.5 μg) was incubated for 10 min at 25°C in a 0.5-ml mixture containing 50 mM potassium phosphate (pH 8.0), 100 mM KCl, and 100 μM acetyl-CoA. Sodium borohydride was added to 10 mM, followed by incubation for an additional 10 min. An aliquot (5 μl) of the enzyme solution was then immediately added to an otherwise complete LCR assay to measure the residual enzymatic activity. The control reactions lacked sodium borohydride, acetyl-CoA, or both. The remainder of the treated protein solution was flash frozen and analyzed later by ESI-MS.

Nucleotide sequence accession numbers. The sequences reported in this paper have the following GenBank accession numbers: *uctA*, DQ668371; *uctB-oxc-uctC-duf1275*, DQ668372; *uctD*, DQ668373; *aarA-sixA-tyrA-orf1-aarC*, DQ631551; and *mgo*, DQ674275.

RESULTS

Inferred metabolic fates of acetate. A draft of the 3-Mb *A. aceti* strain 1023 genome sequence, analyzed as described in the supplemental material, contains all genes needed for six of the eight enzymes of the canonical CAC. No genes for SCS, Mdh, or glyoxylate shunt enzymes were identified. Membrane-bound Mqo, a replacement for Mdh in diverse bacteria (18, 36), is present. The enzymes needed to convert acetate to acetyl-CoA, allowing acetate removal by the CAC, are also present: acetyl-CoA synthetase (*Acs*) or acetate kinase (*AckA*) and phosphotransacetylase (*Pta*).

The genome sequence contains five CoA-transferases, four with unassigned functions (*uctA* to *uctD*) and *aarC*. The new *aarC* sequence encodes a protein of 505 amino acids with a predicted isoelectric point of 6.16. The originally reported *aarC* gene (GenBank accession number D13291) has 496 amino acids and a different sequence in residues 1 to 40 (new AarC residues 1 to 54) (16). The only coding region sequence difference is an insertion of a G after nucleotide 178 in the original sequence report (G210 in the revised sequence).

Gene reannotation in the *aarB* region. Conditionally essential acetic acid resistance genes were identified in mutant *A. aceti* 1023 strains that could not survive exposure to 50 mM acetic acid at a low pH but that were able to grow at an equivalent pH generated by the membrane-impermeant mineral acid HCl (16). Insertional inactivation experiments were used to define the *aarABC* genes and an apparently essential gene, X (Fig. 1) (4).

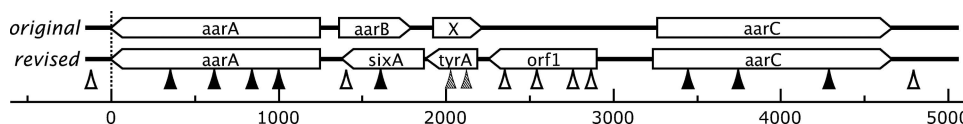


FIG. 1. Revised annotation of the *aar* region in *A. aceti* strain 1023. The ORFs from the original gene assignment (top) were assigned using a kanamycin cassette insertional inactivation at the restriction sites indicated with vertical triangles (16). Insertion points were characterized as resistant to 50 mM acetic acid (open triangles), sensitive (filled triangles), or apparently essential (hatched triangles). The original assigned functions were as follows: *aarA*, citrate synthase; *aarB*, unknown; X, unknown; and *aarC*, possible CoA-transferase or -hydrolase. As of this writing, neither *aarB* nor X has significant similarity to other inferred protein sequences. The new assigned functions are as follows: *orf1*, putative metallophosphoesterase; *tyrA*, chorismate mutase; and *sixA*, a phosphoprotein phosphatase that affects the ArcA/ArcB two-component regulator. The revised assignments include a single-base insertion in the *aarC* region that lengthens the ORF slightly. An insertion at the NsiI site, which does not disrupt acetic acid resistance, would disrupt the *aarB* gene product after Ala19 or would remove or alter seven or fewer C-terminal amino acids in SixA. The scale bar is in units of bp.

TABLE 2. Enzyme activities in *A. aceti* strains

Step no.	Enzyme	Activity (nmol product formed/min/mg protein) in <i>A. aceti</i> strain ^a :		Assay direction ^b	pH
		2002	1023		
1	Citrate synthase	1,050 ± 75	1,200 ± 60	F	8.0
2	Aconitase	5,460 ± 4	2,160 ± 150 3,170 ± 230 ^c	H	8.0
3	Isocitrate dehydrogenase	530 ± 80 (NAD ⁺) 520 ± 45 (NADP ⁺)	1,280 ± 12 (NAD ⁺) 590 ± 20 (NADP ⁺)	F	8.0
4	α-Ketoglutarate dehydrogenase	98 ± 2	28 ± 4	F	7.0
5	Succinyl-CoA synthetase	ND ^d	ND	R	7.2
5'	Succinyl-CoA:acetate CoA-transferase	114 ± 2 53 ± 5	170 ± 4 83 ± 18	F ^e R ^f	8.0
6	Succinate dehydrogenase	21 ± 2	22 ± 3	F	8.0
7	Fumarase	720 ± 115	570 ± 110	R	7.0
8	Malate dehydrogenase	ND	ND	R	8.0
8'	Malate:quinone oxidoreductase	69 ± 3	46 ± 2	F	7.5

^a Experimental procedures are described in the supplemental material. Values given are means ± SD of experiments performed in triplicate.

^b F, biosynthetic; R, nonbiosynthetic; H, citrate→aconitate (half-reaction).

^c Determined using slow-spin lysate.

^d ND, activity not detected.

^e Determined using the VisF assay.

^f Determined using the VisR assay. In preliminary experiments, 15 carboxylic acids were examined as potential substrates in a variant of the VisR assay (described in the supplemental material). Only succinate supported activity.

New gene annotations predicted three open reading frames (ORFs) on the opposite strand: *orf1*, encoding a putative metallophosphoesterase; *tyrA*, encoding chorismate mutase; and *sixA*, encoding a transcriptional regulator involved in a phospho-histidine relay system. Gene synteny in the four available AAB genomes supports the new assignments (19, 45; Kappock et al., unpublished; http://genome.jgi-psf.org/finished_microbes/acicr/acicr.home.html). AAB have a colinear *orf1-sixA-gltA* arrangement (*gltA*, citrate synthase, is the equivalent of *A. aceti aarA*), except in *Acidiphilium cryptum*, which has the putative phosphatase *orf1* located elsewhere. In *A. aceti* and *Gluconobacter oxydans*, *tyrA* is inserted between *orf1* and *sixA*, while *Granulibacter bethesdensis* has two genes inserted between *sixA* and *gltA*. In the revised assignment, *aarC* is divergently transcribed from all other genes in the *aar* gene region. Transcriptional analyses of the *aar* genes are in progress.

Enzyme activity assays. As anticipated from the genome sequence data, six of the eight canonical CAC enzyme activities were detected in *A. aceti* cell lysates (Table 2). No activity was detected for the glyoxylate shunt enzymes isocitrate lyase and malate synthase (data not shown), SCS, or Mdh, but SCAT and Mqo activities were found. SCAT activity was initially detected as the succinate-dependent decomposition of acetyl-CoA, using a variant of the VisR assay that is described in the supplemental material. Succinate was the only one of 15 carboxylic acids that supported activity.

Previously published “single-pot” CoA-transferase assays using citrate synthase to quantitate acetyl-CoA were unsuitable for the kinetic characterization. The discontinuous VisF/VisR methods described in the supplemental material were developed for use with complex cell lysates. HPLC-based LCF and LCR assays were used to quantitate individual (acyl)-CoAs, allowing discrimination between acyl-CoA hydrolase and transferase activities. Reaction-quenching conditions that minimized but did not eliminate acyl-CoA decomposition over a timescale of hours were identified. Replicate experiments were

performed such that the mean velocity at each concentration was determined with the same average delay. Velocity corrections based on the observed rates of acyl-CoA decomposition would increase the reported k_{cat} values by less than 16%.

Candidate SCAT gene expression and activity screening. Candidate SCAT genes *uctA* to *uctD* and *aarC* were overexpressed as soluble proteins in *Escherichia coli* (Fig. 2). Only cells producing UctD or AarC had any activity above that of a vector-only control (VisR assay). The specific activity of SCAT was much higher in the lysate of cells expressing *aarC*,

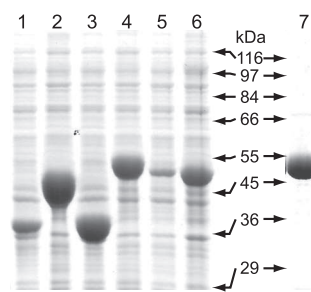


FIG. 2. Expression of candidate SCAT genes in *E. coli* strain C41(DE3). Left lanes, cleared cell lysates (centrifugation at 16,000 × *g* for 10 min) for strains producing an *A. aceti* CoA-transferase were assayed for the ability to consume acetyl-CoA (VisR assay) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The relative activity of each protein in the same culture volume is expressed as a percentage of the AarC activity (a vector-only control lysate has 0.008% relative activity): UctA (lane 1, 41.5 kDa expected; 0.007%), UctB (lane 2, 47.6 kDa expected; 0.01%), UctC (lane 3, 41.8 kDa expected; 0.01%), UctD (lane 4, 55.5 kDa expected; 0.3%), and AarC (lane 5, 54.8 kDa expected; 100%). Lane 6 shows a comparable amount of total cell protein for cells producing AarC, showing the limited solubility of this protein. A parallel experiment with cells producing AarCH6 (not shown) showed 74% relative activity but also a lower fraction of soluble protein than AarC-producing cells. Right lane, purified AarCH6 (lane 7; 5 μg protein). Size standard positions are indicated.

even though AarC was the least soluble of the five CoA-transferases. Thus, SCACT appears to be produced solely from the *aarC* gene. We plan to examine *aarC*-deficient strains to confirm that *A. aceti* contains only one SCACT gene.

AarC with a C-terminal hexahistidine-containing fusion peptide (AarCH6) was produced abundantly at 15°C but appeared to be slightly less soluble than the untagged protein (data not shown). The clarified lysates of *E. coli* cells producing AarCH6 had nearly the same activity as those producing AarC, indicating that the C-terminal fusion peptide does not significantly inhibit enzymatic activity.

Purification and properties of AarCH6. AarCH6 was purified using an ammonium sulfate fractionation procedure, previously developed for untagged AarC, and an immobilized-metal affinity step that are described in the supplemental material. An 8-liter pJK385/C41(DE3) culture yielded 9.5 mg of purified AarCH6 (Fig. 2).

ESI-MS analysis indicated AarCH6 is a 513-residue protein lacking Met1 (55,847 ± 2 Da observed; 55,847.2 Da expected). Analytical gel filtration gave a single peak with a molecular mass of 160 kDa, consistent with a trimeric or elongated dimeric solution state.

AarCH6 fluorescence emission showed a maximum at 343 nm, which was quenched 8% by the addition of dethiaacetyl-CoA, a nonhydrolyzable analogue of acetyl-CoA that has a methylene group replacing the sulfur atom (30). Fluorescence titrations, performed as described in the supplemental material, were used to determine an apparent K_d (dissociation constant) (Table 3). No fluorescence changes were noted upon the addition of 10 mM succinate to a solution containing 1 μM AarCH6 and 10 μM dethiaacetyl-CoA.

Mechanism of AarC. AarC is predicted to be a class I CoA-transferase from active site sequence alignments that identify Glu435 as a key active site residue (29). Class I CoA-transferases first bind the acyl-CoA substrate, forming a carboxylate product and an enzyme-CoA thioester intermediate (48, 50), which accounts for the observed ping-pong kinetics (22) and susceptibility to borohydride inactivation (23, 25, 50). Sodium borohydride treatment of AarCH6 in the presence of acetyl-CoA caused near-complete loss of enzyme activity (0.7% relative activity), consistent with the reduction of the glutamyl-CoA thioester intermediate to 5-hydroxynorvaline. ESI-MS analysis of the borohydride-treated enzyme complex showed the expected -14-Da change (55,832 ± 2 Da observed; 55,833.2 expected). AarCH6 treated with borohydride in the absence of an acyl-CoA substrate showed partial inactivation (71% activity) relative to a control lacking both acetyl-CoA and sodium borohydride (100% activity; 74 s⁻¹). Partial inactivation may indicate that some of the enzyme is isolated as an AarCH6-CoA adduct.

Steady-state experiments with duplicate LCR assays showed nonintersecting lines in double-reciprocal plots, consistent with ping-pong kinetics and a modified-enzyme mechanism (see Figure S2 in the supplemental material).

Kinetic characterization of AarCH6. The determination of accurate kinetic parameters for AarCH6 was complicated by the apparent acetate substrate inhibition (Fig. 3A) and the presence of CoA, a common contaminant in commercial acyl-CoAs (~5% in acetyl-CoA, ~11% in succinyl-CoA) that hinders substrate saturation. Accurate acid substrate kinetic con-

TABLE 3. Kinetic constants for AarCH6^a

Substrate	Parameter	Value
Acetate ^b	k_{cat} (s ⁻¹)	280 ± 37
	K_m (mM)	70 ± 20
	k_{cat}/K_m (M ⁻¹ s ⁻¹)	(4 ± 1) × 10 ³
Succinyl-CoA ^b	K_i , acetate (mM)	1,600 ± 780
	k_{cat} (s ⁻¹)	201 ± 8
	K_m (μM)	22.1 ± 3.6
Succinate ^c	k_{cat}/K_m (M ⁻¹ s ⁻¹)	(9 ± 2) × 10 ⁶
	k_{cat} (s ⁻¹)	70.9 ± 1.9
	K_m (mM)	0.90 ± 0.10
Acetyl-CoA ^c	k_{cat}/K_m (M ⁻¹ s ⁻¹)	(7.9 ± 0.9) × 10 ⁴
	k_{cat} (s ⁻¹)	75.2 ± 1.5
	K_m (μM)	22.3 ± 2.0
SCACT reaction	k_{cat}/K_m (M ⁻¹ s ⁻¹)	(3.4 ± 0.3) × 10 ⁶
	K_i , CoA (μM) ^d	15.9 ± 2.1
	K_{eq} ^e	0.137 ± 0.053
Acetoacetate ^f	k_{cat} (s ⁻¹)	36.5 ± 2.6
	K_m (mM)	130 ± 14
	k_{cat}/K_m (M ⁻¹ s ⁻¹)	(2.8 ± 0.4) × 10 ²
Dethiaacetyl-CoA	K_d (μM) ^g	0.75 ± 0.18
	K_i (μM) ^h	16.6 ± 3.0

^a Determined at 25°C and pH 8.0. Turnover numbers assume one active site per 55.8-kDa subunit, such that 1 s⁻¹ = 1.07 units/mg.

^b LCF assay. Determined using a fixed concentration of either succinyl-CoA (0.2 mM) or acetate (350 mM).

^c LCR assay. Determined using a fixed concentration of either acetyl-CoA (1 mM) or succinate (20 mM).

^d Modified LCR assay. Determined using a variable concentration of acetyl-CoA (5 to 1,200 μM) and fixed concentrations of succinate (20 mM) and CoA (unadjusted or adjusted to a final concentration of 40, 70, or 100 μM). Data were fit to a competitive inhibition model.

^e Determined using k_{cat}/K_m values as (succinyl-CoA × acetate)/(acetyl-CoA × succinate) (7).

^f Modified LCF assay. Determined using a fixed concentration of succinyl-CoA (0.2 mM) and a variable concentration of lithium acetoacetate.

^g Determined by fluorescence titration at 20°C and pH 8.0.

^h Modified LCR assay. Determined using a variable concentration of acetyl-CoA (10 to 300 μM) and fixed concentrations of succinate (20 mM) and dethiaacetyl-CoA (0, 5, 25, and 50 μM). Data were fit to a competitive inhibition model.

stants for class I CoA-transferases require the saturation of the enzyme with the acyl-CoA substrate. LCF/LCR assays allowed the simultaneous determination of CoA levels and enzyme activity. Similar kinetic parameters were obtained using both LCF/LCR and VisF/VisR assays (Fig. 3A).

Kinetic analysis was performed for each substrate at a single, saturating, but minimally inhibitory concentration of the second substrate. LCR assays spiked with CoA showed that CoA is an effective competitive inhibitor versus acetyl-CoA. A plot of velocity versus the concentration of acetyl-CoA is only satisfactorily fit if inhibition due to residual CoA is taken into account (Fig. 3B). The increased scatter in velocities determined with succinyl-CoA (LCF assay) gave fits that were not improved by correcting for CoA inhibition. If CoA is an equally potent competitor for each acyl-CoA substrate, the values given underestimate the succinyl-CoA K_m and k_{cat} values by ~15% but have a negligible effect on K_{eq} (equilibrium constant). The uninhibited kinetic constants obtained by individual substrate analysis (Table 3) are in reasonable agreement with the values obtained by the preliminary double-reciprocal analysis (data not shown).

Acetoacetate is a cosubstrate for several CoA-transferases that convert succinyl-CoA to succinate (10, 24). LCF assays containing acetoacetate instead of acetate showed the forma-

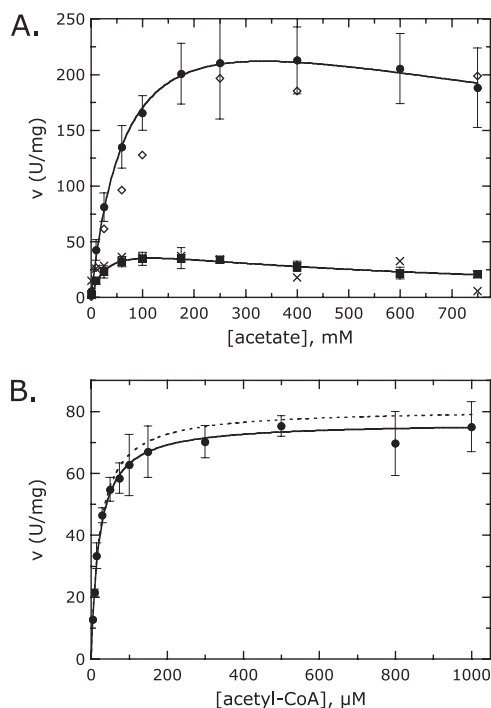


FIG. 3. Kinetic characterization of AarCH6. (A) Acetate saturation data at pH 8.0 (solid circles, LCF assay; open diamonds, VisF assay) and pH 5.0 (solid squares, LCF assay; crosses, VisF assay). The solid lines are fits to each LCF data set, assuming substrate inhibition. The concentration of succinyl-CoA was 0.2 mM. Fitted parameters are given in the text and Table 3. (B) Acetyl-CoA saturation data at pH 8.0 obtained with the LCR assay (solid circles) at 20 mM succinate. The solid line is a fit to the data accounting for competitive inhibition due to CoA (determined in assay mixtures spiked with CoA). The dotted line is a simulation using the fitted parameters at a CoA concentration of 0.

tion of acetoacetyl-CoA, identified by comparison to the HPLC retention time of an authentic standard. The k_{cat}/K_m for acetoacetate was 7% of that for acetate under the same conditions (Table 3).

pH dependence of acetate kinetic parameters. Acetate is anticipated to be the true substrate for SCACT. Its mole fraction should decrease as the pH drops below the pK_a of acetic acid, 4.76. Kinetic parameters were determined with LCF assays at pH 8.0 (Table 3), pH 6.0 ($k_{cat} = 147 \text{ s}^{-1}$; $K_m = 51 \pm 11 \text{ mM}$; $K_i = 1,100 \pm 310 \text{ mM}$), and pH 5.0 ($k_{cat} = 49 \text{ s}^{-1}$; $K_m = 29 \pm 8 \text{ mM}$; $K_i = 500 \pm 130 \text{ mM}$). The increased substrate inhibition at a lower pH may be due to the increased fraction of acetic acid (Fig. 3A). LCF and VisF velocities were comparable at all pH values tested.

DISCUSSION

Genetic screens have identified two categories of *Acetobacter* genes affecting acetic acid resistance: (i) those that improve the acetic acid resistance of *A. acetii* strains when introduced or overexpressed, including the *aarABC* gene cluster (16) and the aconitase gene (38), and (ii) those that improve the acetic acid resistance of *E. coli*, including the RecG (51) and AatA (39) genes. The current study was designed to

assess the potential role of the CAC and related pathways in acetic acid removal.

The *A. acetii* CAC converts acetate to CO_2 , a characteristic capability of *Acetobacter* strains. A prerequisite is the efficient conversion of acetate to acetyl-CoA, which can be performed by AckA/Pta or Acs. Pta and Acs activities increase in *A. acetii* during acetate oxidation (49). Sustained acetyl-CoA oxidation and acetate assimilation require a complete CAC. However, SCS was not found in a draft *A. acetii* genome sequence, and no activity was present in cell lysates.

When grown in the presence of glucose, *A. acetii* is able to assimilate carbon from acetate (39) or ethanol (46) by way of acetate. Isocitrate lyase and malate synthase activities have been reported in *A. acetii* cells grown on acetate as the sole carbon source (53), which would be consistent with acetate assimilation by the glyoxylate shunt. However, the genes for glyoxylate shunt enzymes have not been found in any AAB genome (19, 45). Thus, *A. acetii* must assimilate acetate by a different route. Alternative pathways are present in several other α -proteobacteria that lack isocitrate lyase (1).

Our findings show that *A. acetii* contains a complete but unorthodox CAC in which the acetic acid resistance protein AarC (SCACT) converts succinyl-CoA and acetate to succinate and acetyl-CoA. This bypass facilitates the metabolic activation and oxidation of acetate and constitutes part of a conditionally essential detoxification pathway that is required for survival at high acetate concentrations at a low pH but is not required for growth at low acetate concentrations, even at the same low pH.

AarC homologues. SCACT has not to our knowledge been identified in another aerobic α -proteobacterium. Of the four AAB with currently available genome sequences, only *G. oxydans* has an incomplete CAC (20, 45). The complete genome sequences of the AAB *G. betshdensis* CGDNIH1 (19) and *A. cryptum* contain SCS genes. *A. cryptum* also contains a close match to AarC (see Table S1 in the supplemental material). Genes resembling *aarC* are found in numerous proteobacteria, but sequences uniquely associated with SCACT activity have not been identified.

Anaerobic ATP synthesis in trichomonad hydrogenosomes and the distinctive mitochondria of trypanosomes and some helminths require both SCACT and SCS, which convert acetyl-CoA, ADP, and P_i to acetate, CoA, and ATP in the presence of succinate (55, 56). Hydrogenosomal SCACT from *Trichomonas vaginalis* (55) closely resembles AarC, but the sequence of mitochondrial SCACT from *Trypanosoma brucei* (47) is similar only in the active site region, (S/T)E(Q/N)GL.

SCACT activity is found in *Aspergillus nidulans* (6). The recently identified enzyme responsible for this activity (CoaT) has equal activity with acetate, propionate, and acetoacetate in the presence of succinyl-CoA (13). *A. nidulans* CoaT and similar inferred proteins in six related fungi, all of which contain putative mitochondrial targeting sequences, closely resemble AarC (see Table S1 in the supplemental material) and may function as mitochondrial SCACT enzymes. SCACT is one of a small number of enzyme activities found in both hydrogenosomes and some mitochondria (21). Each organelle is thought to be derived from the α -proteobacteria, a group that includes the AAB. The use of SCACT for a different metabolic purpose may explain the distinctive distribution of this gene.

Comparison with other CAC bypasses. *Helicobacter pylori* lacks several typical CAC components (27, 44), including Mdh, which is replaced by Mqo, and SCS, which is replaced by succinyl-CoA:acetoacetate CoA-transferase (10). AarC is also able to support succinyl-CoA:acetoacetate CoA-transferase activity, albeit with a substantially lower k_{cat}/K_m than that of the SCACT reaction (Table 3). Given the likelihood that the concentration of acetate is much greater than the concentration of acetoacetate in the *A. aceti* cytoplasm and that genetic experiments associate *aarC* with acetate metabolism (16, 17), AarC is likely to function principally as SCACT.

Mycobacterium tuberculosis replaces α -ketoglutarate dehydrogenase and SCS with α -ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase (54). (Activity for these enzymes was not detected in *A. aceti* lysates [data not shown].) Like the *A. aceti* SCACT bypass, the succinic semialdehyde bypass avoids a CoA- and nucleotide-dependent step and was detected by the analysis of inconsistent cofactor requirements.

All three bypasses preserve the typical cyclic course of the CAC and thereby differ from the "branched" CAC variants in facultative and obligate anaerobes (11).

Potential advantages of SCACT. The thioesterification reactions performed by CoA-transferases tend to have small $|\Delta G|$ values. For SCACT, the slightly unfavorable reaction ($K_{eq} = 0.14$; $\Delta G = +1.2$ kcal/mol) is mitigated at high acetate levels or when acetyl-CoA is rapidly consumed by the CAC, i.e., during vigorous aeration. The near-thermoneutrality of the SCACT reaction is likely to be maintained in *A. aceti* as the cytoplasmic pH drops (32), whereas the equilibrium of the substrate-level phosphorylation performed by SCS is likely to depend on pH.

The use of SCACT bypasses a nucleotide requirement in the CAC and the need for initial acetate phosphorylation or adenylation (Fig. 4), decreasing the sensitivity of flux through the CAC to the energy state of the cell. The metabolic demand for free CoA is similarly reduced. Thus, acetic acid detoxification by irreversible oxidation would require only the favorable regeneration of reduced pathway cofactors.

Acetate dissimilation. Reliance on a complete CAC for acetate removal might explain the vigorous oxygenation requirements of *A. aceti* cultures, which become critical at high acetic acid levels and low pHs (34, 37). Together with ADH-mediated ethanol oxidation, the CAC supplies energy for many cellular processes, including the maintenance of a proton gradient. Transient O_2 deprivation sharply decreases cell viability within minutes, which is not consistent with the energy loss due to a slow collapse of the transmembrane pH gradient. Instead, the evidence presented here indicates that a complete CAC including SCACT supports the eight-electron oxidation of acetate to two CO_2 molecules (Fig. 4). Cytoplasmic acetate would rapidly accumulate if cofactor reoxidation slows, which is consistent with the rapid loss of cell viability during inadequate culture aeration at high external acetic acid levels.

In many gram-negative bacteria, NADH limits CAC flux in anaerobic conditions. Citrate synthase (40) and Mdh (57) are susceptible to allosteric and product inhibition, respectively. However, both the acid-tolerant *H. pylori* and the acidophilic *A. aceti* contain citrate synthase forms that are insensitive to NADH (15, 44). Both organisms also replace Mdh with Mqo. Ubiquinone reduction by Mqo is more favorable than the Mdh

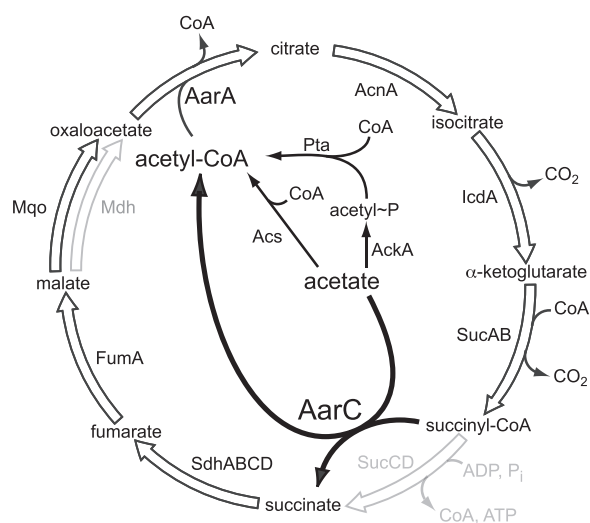


FIG. 4. An unorthodox *A. aceti* CAC oxidizes acetate. The gray arrows indicate CAC genes that are not found in a draft *A. aceti* 1023 genome sequence (Kappock et al., unpublished). Each has a functional replacement. Using periplasmic dehydrogenases, *A. aceti* produces large quantities of acetic acid from ethanol and must contend with a constant influx of the former. Together with a complete oxidative phosphorylation pathway, this variant CAC functions in the eight-electron oxidation of acetic acid: $CH_3COOH + 2 O_2 \rightarrow 2 CO_2 + 2 H_2O$. This pathway skips AckA, Acs, and SCS (*sucCD*), the only steps that would be directly influenced by cytoplasmic nucleotide pools, and reduces the number of enzymes that have free CoA substrates from two to one. The initial electron acceptors, two NAD^+ , one FAD, and one ubiquinone (presumed to be Q9 in *A. aceti*), would give a lower energy yield than a canonical CAC but have an additional irreversible step, the Mqo-mediated quinone reduction. Genes for the glyoxylate shunt enzymes isocitrate lyase and malate synthase are not found in *A. aceti* 1023.

reaction and is largely independent of the ratio of the concentration of NADH to the concentration of NAD^+ , both of which would tend to favor the production of oxaloacetate. In *A. aceti*, these adaptations may avoid the suppression of acetate dissimilation during periods of acetic acid stress.

Kinetic characterization. The kinetic and mechanistic parameters of SCACT delineate its potential effect(s) on metabolic flux. *A. aceti* survives in >0.5 M acetic acid at a low pH, but *aar*-disrupted strains are unable to survive in 50 mM external acetic acid (16). Since there is likely no difference in acetate permeation, cytoplasmic acetate removal by oxidation appears to become critical at $\sim 10^{-2}$ M. The observed acetate K_m values are in this range (Table 3), consistent with a conditionally essential detoxification role for *aarC*. Near the K_m , SCACT velocity depends linearly on the cytoplasmic concentration of acetate, diminishing as acetate is depleted. At a lower pH, the acetate K_m is smaller, which may increase the rate of acetate oxidation during intense acid stress.

An environmentally appropriate overflow pathway? Acetate secretion by many bacteria is due to the activation of the carbon overflow pathway during anaerobiosis or when the central metabolic pathways are overtaxed (58). NAD^+ and CoA are recovered as acetyl-CoA is converted to CoA and acetate, a diffusible compound that leaves the cytoplasm but can be recovered in the stationary phase. In AAB, vigorous periplasmic ethanol oxidation ensures that the acetate concentration

gradient is always directed inward, precluding the diffusive loss of excess cytoplasmic acetate. The oxidation of acetate to CO₂ by the SCACT-modified *A. acetii* CAC allows diffusive carbon loss, since CO₂ is favored over bicarbonate at a low pH and can traverse bacterial cell membranes (33).

A CAC regulator in the *aarA*-to-*aarC* intergenic region. New assignments in the *A. acetii* *aarA*-to-*aarC* intergenic region explain several previously confusing observations, including the singular gene X and *aarB* sequences that are apparently unique to *A. acetii*. Gene X is replaced by *tyrA* (chorismate mutase), which performs a key step in aromatic amino acid biosynthesis. Disruptants in the *X/tyrA* region were inviable (16), a finding that might now be attributed to a nutritional deficiency. Additionally, *aarB* is replaced by *sixA*. SixA regulates ArcA/ArcB, one of a small number of global regulators in *E. coli*, which represses the synthesis of aerobic metabolic enzymes during anaerobic conditions (3, 26). An ArcA knockout has increased CAC enzyme activities (43), suggesting that the SixA-dependent suppression of this inhibitory regulator would stimulate flux through the CAC. The reassignment of this conditionally essential sequence region to *sixA* is congruent with known AarA and AarC functions and suggests an important role for O₂ sensing.

Conclusion. The *A. acetii* strain 1023 *aarC* gene product is SCACT. This assignment reconciles previously confusing biochemical and genetic data regarding the important role(s) of *aarC* in acetic acid resistance and metabolism. SCACT bypasses SCS, which is missing from the genome; allows acetate incorporation without substrate-level phosphorylation; and enables the removal of diffusively trapped cytoplasmic acetate, by acetyl-CoA oxidation, as the readily diffusible CO₂.

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