

12-3-2013

Functional analysis of the acetic acid resistance (*aar*) gene cluster in *Acetobacter aceti* strain 1023

Elwood Mullins

Purdue University, elwood.mullins@Vanderbilt.Edu

T Joseph Kappock

Purdue University, tkappock@purdue.edu

Follow this and additional works at: <http://docs.lib.purdue.edu/biochempubs>

 Part of the [Biochemistry Commons](#)

Recommended Citation

Mullins, Elwood; Kappock, Thomas J. (2013). Functional analysis of the acetic acid resistance (*aar*) gene cluster in *Acetobacter aceti* strain 1023. *Acetic Acid Bacteria*. 2(s1):e3. doi: 10.4081/aab.2013.s1.e3.

This document has been made available through Purdue e-Pubs, a service of the Purdue University Libraries. Please contact epubs@purdue.edu for additional information.

Functional analysis of the acetic acid resistance (*aar*) gene cluster in *Acetobacter aceti* strain 1023

Elwood A. Mullins,^{1,2} T. Joseph Kappock¹

¹Department of Biochemistry, Purdue University, West Lafayette, Indiana;

²Department of Chemistry, Washington University in St. Louis, St. Louis, Missouri, USA

Abstract

Vinegar production requires acetic acid bacteria that produce, tolerate, and conserve high levels of acetic acid. When ethanol is depleted, aerobic acetate *overoxidation* to carbon dioxide ensues. The resulting diauxic growth pattern has two logarithmic growth phases, the first associated with ethanol oxidation and the second associated with acetate overoxidation. The vinegar factory isolate *Acetobacter aceti* strain 1023 has a long intermediate stationary phase that persists at elevated acetic acid levels. Strain 1023 conserves acetic acid despite possessing a complete set of citric acid cycle (CAC) enzymes, including succinyl-CoA:acetate CoA-transferase (SCACT), the product of the acetic acid resistance (*aar*) gene *aarC*. In this study, cell growth and acid production were correlated with the functional expression of *aar* genes using reverse transcription-polymerase chain reaction, Western blotting, and enzyme activity assays. Citrate synthase (AarA) and

SCACT (AarC) were abundant in *A. aceti* strain 1023 during both log phases, suggesting the transition to acetate overoxidation was not a simple consequence of CAC enzyme induction. A mutagenized derivative of strain 1023 lacking functional AarC readily oxidized ethanol but was unable to overoxidize acetate, indicating that the CAC is required for acetate overoxidation but not ethanol oxidation. The primary role of the *aar* genes in the metabolically streamlined industrial strain *A. aceti* 1023 appears to be to harvest energy via acetate overoxidation in otherwise depleted medium.

Introduction

The characteristic ability of acetic acid bacteria (AAB) to aerobically oxidize ethanol to acetic acid has been harnessed for millennia to produce vinegar.¹ Traditional acetification methods often involve diauxic growth.²⁻⁴ Cultures accumulate acetic acid in the first logarithmic growth phase (log phase) as ethanol is oxidized, maintain acetic acid during the first stationary phase, and then deplete acetic acid in the second log phase. Ideal strains for vinegar production exhibit little acetic acid consumption during the first log phase and an ability to tolerate high concentrations of acetic acid during a prolonged stationary phase.² Modern industrial acetification methods avoid the second log phase and concomitant loss of acetic acid.⁵

A genetic screen of the industrial vinegar production strain *Acetobacter aceti* 1023 identified a cluster comprised of five genes that was essential for acetic acid resistance on solid media.⁶ Three genes in the *aar* gene cluster are required for acetic acid resistance: *aarA* encodes citrate synthase (CS),^{6,7} *sixA* encodes a phosphoprotein phosphatase (SixA) proposed to modulate citric acid cycle (CAC) enzyme synthesis,⁸ and *aarC* encodes succinyl-CoA:acetate CoA-transferase (SCACT). AarA and AarC constitute part of a specialized, variant CAC that provides acetic acid resistance by catalyzing the *overoxidation* of cytoplasmic acetate to carbon dioxide (Figure 1). AarC facilitates this process by uncoupling catabolic activation of acetate from substrate-level phosphorylation and/or adenylation.

A. aceti strain 1023 lacks the glyoxylate shunt enzymes isocitrate lyase and malate synthase, which bypass the oxidative decarboxylations performed by CAC dehydrogenases, and succinyl-CoA synthetase,⁸ making flux through the CAC dependent upon AarC. In contrast, the type strain *A. aceti* NBRC 14818 possesses a complete CAC including succinyl-CoA synthetase, both glyoxylate shunt enzymes, and AarC (Figure 1).⁴

The feasibility of acetate assimilation as a means of rapidly reducing acetic acid levels in planktonic cultures has brought into question the relevance of the *aar* gene cluster for acetic acid resistance in liquid media.⁹ Acetate dissimilation, however, is compatible with an

Correspondence: T. Joseph Kappock, Department of Biochemistry, Purdue University, 175 South University Street, West Lafayette, Indiana 47907-2063, USA. Tel. +1.765.494.8383 - Fax: +1.765.494.7897.
E-mail: kappock@purdue.edu

Key words: AarA, SixA, AarC, citric acid cycle, acetate overoxidation.

Acknowledgments: the authors thank Dr. Koichi Kondo for providing the *A. aceti* strains used in this study, Kelly L. Sullivan for performing size-exclusion chromatography, and Kayleigh E. Nyffeler for performing PCR controls. This work was supported by the US National Science Foundation (MCB 0347250) and Purdue University Agricultural Research Programs.

Conference presentation: part of this paper was presented at the *3rd International Conference on Acetic Acid Bacteria. Vinegar and Other products*, 2012 Apr 17-20, Cordoba, Spain (<http://www.uco.es/aab2012/>).

Contributions: EAM acquired, analyzed, and interpreted data and drafted and revised the manuscript; TJK interpreted data and revised the manuscript.

Conflict of interests: the authors declare no potential conflict of interests.

Received for publication: 1 October 2012.

Revision received: 28 November 2012.

Accepted for publication: 3 December 2012

This work is licensed under a Creative Commons Attribution 3.0 License (by-nc 3.0).

©Copyright E.A. Mullins and T.J. Kappock, 2013

Licensee PAGEPress, Italy

Acetic Acid Bacteria 2013; 2(s1):e3

doi:10.4081/aab.2013.s1.e3

acetic acid resistance function.⁸ This study seeks to assess the importance of the *aar* products in liquid media and to correlate transcriptional and translational levels with acetic acid levels during diauxic growth.

Materials and Methods

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher (Houston, TX, USA) in the highest purity available. *A. aceti* strains were a generous gift from Dr. Koichi Kondo, Mizkan Group Corporation (Aichi, Handa, Japan) (Table 1)^{6,10-12}. Oligodeoxynucleotide (ODN) primers from Integrated DNA Technologies (Coralville, IA, USA) were used without further purification (Table 2). Restriction enzymes, DNA modifying enzymes, DNA polymerases, and DNA size standards were from New England Biolabs (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat α -rabbit IgG (H+L) and rabbit α -chicken IgY (H+L) antibodies were from Jackson ImmunoResearch (West Grove, PA, USA) and Thermo Scientific (Waltham, MA, USA), respectively. *A. aceti* CS (AarA), AarA with a C-terminal hexahistidine affinity tag (AarAH6), and *A. aceti* SCAC (AarC) with a C-terminal hexahistidine affinity tag (AarCH6) were isolated as described previously.^{7,8} Polyclonal antibodies were generated in rabbits (α -AarCH6 and α -SixAH6) or chickens (α -AarA) by Cocalico Biologicals (Reamstown, PA, USA) using pure recombinant proteins overproduced in *E. coli* C41(DE3).

General analytical methods

Absorbance measurements were recorded on an 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Protein quantitation by the method of Bradford¹³ was performed using bovine serum albumin as a standard. Chemiluminescence was recorded using a ChemImager 5500 imaging system (Alpha Innotech, San Leandro, CA, USA). DNA was sequenced by the staff of the Purdue University Genomics Core Facility. Protein mass was determined by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by the staff of the Purdue Proteomics Facility.

Isolation of genomic DNA and sequencing of the *aar* gene cluster

A. aceti genomic DNA (gDNA) was isolated using 20/G genomic tips (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) mixtures contained 1× Phusion HF buffer, 0.2 mM dNTPs, 125 ng gDNA, 0.2 μ M each of ODNs 2023/2053 (Table 2), and 1 unit Phusion DNA polymerase. Amplification was performed in three stages: stage 1 (1

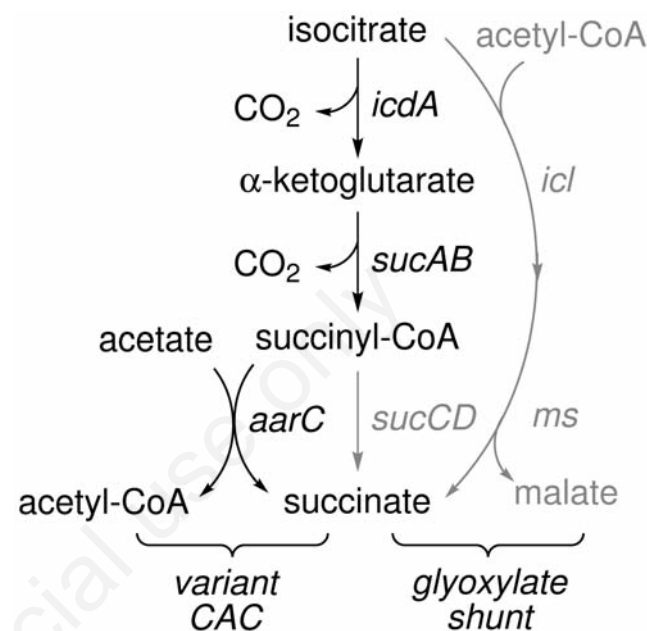


Figure 1. Citric acid cycle (CAC) variants and shunts found in diverse *A. aceti* strains. A portion of the canonical CAC is shown in the center. The AarC bypass and the glyoxylate shunt are shown to the left and right, respectively. Pathways absent in the vinegar production strain *A. aceti* 1023 are colored gray. All pathways are present in the type strain *A. aceti* NBRC 14818.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description*	Source
Strain		
<i>A. aceti</i>		
1023	Vinegar factory isolate, Ace ^R	Ref. 10
10-8 ^o	Derivative of strain 1023, pro ⁻ Ace ^R	Ref. 11
AS10 ^o	Derivative of strain 10-8, pro ⁻ Ace ^S aarC ⁻	Ref. 6
<i>E. coli</i>		
C41(DE3)	Derivative of strain BL21 [F' <i>ompT</i> ⁻ <i>hsdS</i> _B (r _B ⁻ m _B ⁻) <i>gal</i> ⁻ <i>dcm</i> ⁻ (DE3)] possessing additional mutation(s)	Avidis, Ref. 12
DH5 α	F' ϕ 80(<i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> ⁻ <i>recA1</i> ⁻ <i>endA1</i> ⁻ <i>hsdR17</i> (r _K ⁻ m _K ⁻) <i>phoA</i> ⁻ <i>supE44</i> ⁻ <i>thi-1</i> ⁻ <i>gyrA96</i> ⁻ <i>relA1</i> ⁻	Invitrogen
Plasmid		
pET23a	T7 promoter expression vector, Ap ^R	Novagen
pJK385	T7 promoter expression construct, encodes AarCH6, Ap ^R	Ref. 8
pJK502	T7 promoter expression construct, encodes SixAH6, Ap ^R	Present work
pJK504	T7 promoter expression construct, encodes AarCH6-C357Y, Ap ^R	Present work

**aarC* deficient (*aarC*⁻), acetic acid resistant (Ace^R), acetic acid sensitive (Ace^S), ampicillin resistant (Ap^R), and proline auxotrophic (*pro*⁻); ^omutant *A. aceti* strains were created by chemical mutagenesis using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine.^{6,11}

cycle), 2 min at 98°C; stage 2 (30 cycles), 10 s at 98°C, 0.5 min at 65°C, 2.5 min at 72°C; and stage 3 (1 cycle), 5 min at 72°C. PCR products were recovered using a kit (Qiagen) and sequenced using internal ODN primers. DNA sequences were assembled and edited using phred/phrap/consed.¹⁴⁻¹⁶ Terminator sequences were identified using the ARNold web server.¹⁷

Cloning and mutagenesis

AarCH6-C357Y production plasmid pJK504 was prepared using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), AarCH6 production plasmid pJK385, and ODNs 2028/2029 (Table 2). SixAH6 production plasmid pJK502 was prepared by cloning a *sixA* PCR product obtained using Vent DNA polymerase, *A. aceti* strain 1023 gDNA, and ODNs 2004/2005 (Table 2) into the *NdeI* and *XhoI* sites of plasmid pET23a (Novagen, Madison, WI, USA). ODN 2005 encodes a Ter175→Ser mutation, which adds SLEHHHHHH to the C-terminus of SixA.

Purification of SixAH6

E. coli C41(DE3) cells freshly transformed with plasmid pJK502 were propagated on Luria-Bertani medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, and 0.1 g/L ampicillin (LB/Amp) and supplemented with 0.2% (w/v) glucose. Production cultures (1 L) were inoculated with overnight cultures at a 1:100 dilution and grown at 37°C to an OD₆₀₀=0.6. Cells were then harvested by centrifugation and resuspended in 1 L LB/Amp supplemented with 0.4 mM isopropylthio-β-D-1-galactopyranoside (IPTG). Cultures were grown at 37°C an additional 3 h. Cells were then pelleted by centrifugation and stored at -80°C. All subsequent steps were performed at 4°C. Cells (typically 5 g/L culture) were resuspended in 5 mL/g 50 mM potassium phosphate, pH 6.0, and 100 mM potassium chloride and disrupted by three cycles of sonication. Lysate was cleared by centrifugation at 30,000 g for 30 min, addition of streptomycin to 1% (w/v) from a 10% (w/v) stock, incubation for 15 min, and additional centrifugation at 30,000g for 30 min. Solid ammonium sulfate was then added to the cleared lysate to 25% saturation (144 g/L) over 30 min. After equilibrating an additional 30 min, solids were removed by centrifugation at 30,000 g for 10 min. Solid ammonium sulfate was then added to the supernatant to 45% saturation (123 g/L) over 30 min. After equilibrating an additional 30 min, solids were collected by centrifugation at 30,000 g for 10 min, dissolved in a minimal volume of TK buffer (50 mM Tris•HCl, pH 8.0, and 100 mM potassium chloride), and applied to a Ni²⁺-charged nitrilotriacetic acid (NiNTA) agarose column (1.5×4.5 cm, 8.0 mL). The column was washed with 5 column volumes of TK buffer containing 20 mM imidazole and then developed in a linear gradient of imidazole (20→500 mM, 80×80 mL). Fractions containing SixAH6 were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and concentrated to >5 mg/mL by ultrafiltration (Amicon Ultra-15; 3000 MWCO). The concentration of imidazole was reduced by several cycles of dilution and reconcentration. Single-use aliquots were flash-frozen and stored at -80°C

Size-exclusion chromatography

Analytical size-exclusion chromatography was performed using an ÄKTA fast protein liquid chromatography system (Amersham Biosciences, Arlington Heights, IL, USA). SixAH6 (5 mg/mL in TK buffer adjusted to 5% glycerol) was filtered through a low-retention nylon membrane (0.22 μm, Fisher) and then centrifuged at 16,000g and 4°C for 10 min prior to injection (0.1 mL) onto a Superdex 200 column (1.6×60 cm, 120 mL). The column was developed at 4°C and 1 mL/min in TK buffer. Solution sizes were determined by reference to size standards (Sigma MW-GF-1000, Blue Dextran, and acetone) using $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the peak elution volume, V_0 is the void volume, V_t is the included volume of the gel bed, and K_{av} is the partition

coefficient (proportional to the logarithm of the solution molecular weight).

Heterologous overproduction of AarCH6-C357Y

E. coli C41(DE3) cells transformed with plasmid pJK504 were propagated on LB/Amp. Production cultures (1 L) were inoculated with overnight cultures at a 1:500 dilution and grown at 37°C to an OD₆₀₀=0.6. Production of recombinant AarCH6-C357Y was then induced by addition of IPTG to 0.4 mM. Cells were grown at 15°C an additional 16 h, harvested by centrifugation, and stored at -80°C. All subsequent steps were performed at 4°C. Cells (typically 5 g/L culture) were resuspended in 5 mL/g TK buffer and disrupted by three cycles of sonication. Lysate was cleared by centrifugation at 30,000 g for 30 min, addition of streptomycin to 1% (w/v) from a 10% (w/v) stock, incubation for 15 min, and additional centrifugation at 30,000g for 30 min.

Growth of *A. aceti* cultures

A. aceti strains 1023, 10-8, and AS10 (Table 1) were propagated on yeast extract-peptone-dextrose (YPD) medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose and supplemented with 2% (v/v) ethanol (YPDE). YPDE solutions (500 mL) in 2.8 L Fernbach flasks were inoculated from single colonies, and cultures were grown at 30°C with continuous agitation (200 rpm) in a C25 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) for 12 d. Large aliquots (10 mL) were periodically removed, and cells were harvested by centrifugation. Cell-free medium samples and cell pellets were stored separately at -80°C for subsequent analyses. Small aliquots (3 mL) were removed for RNA purification, mixed with 2 volumes of RNAProtect Bacteria reagent (Qiagen), and incubated at room temperature for 5 min. Cells were then harvested by centrifugation and stored at -80°C.

Titration of acetic acid in *A. aceti* cultures

Percent acidity in *A. aceti* cultures was determined by titration with 0.1 M sodium hydroxide (standardized with potassium hydrogen phthalate) as described previously.^{3,18} Uninoculated YPD medium was titrated to determine the background percent acidity.

Enzyme activity assays

Frozen cell pellets (large aliquots) were resuspended in 10 mL ice-cold water, repelleted by centrifugation, and then resuspended in 1 mL

Table 2. Oligodeoxynucleotides used in this study.

ODN	Sequence (5'→3')
413	GAGCTCGAATTCTTAGCGTTTGGCAAGCGGCACATAGTCAC
416	GATATACATATGAGCGCGTCGCAGAAAGAAGGTAAGCTATC
1221	CAGGAAGCCCTCATATGACAGAGCG
1280	GCAATACTCGAGTTATGATGGGAGC
2004	GCCGCCATATGCGCCGCTTGTCTG
2005*	GGCATCTCGAGTGGAGGTCGCGCGAATGT
2023	GCCGATACAACCACCAATAAAGCT
2028*	GTTTAGGCTACATTCATGCAACGGCATGA
2029*	TCATGCCGTTTATGCAATGTAGCCATAAC
2037	GCCGGCATATGACCGTGACAGACGACCA
2038	GCCGGCATATGCCAAACGGACTCCGCA
2053	GGTAGCGTTTATTTCGCTCTGCCG

*Changes in the coding region relative to the genomic sequence are underlined. ODN, oligodeoxynucleotide.

50 mM potassium phosphate, pH 8.0, and 100 mM potassium chloride. Cells were lysed by three cycles of sonication, and debris was removed by centrifugation (16,100 g and 4°C for 15 min). SCACT and CS activities in soluble lysates were determined using 5,5'-dithiobis-(2-nitrobenzoic acid) dependent VisR and CS assays as described previously.⁸

Isolation of RNA from *A. acetii* strain 1023

RNA was isolated from cell pellets (small aliquots) according to the RNeasy Mini kit enzymatic lysis and proteinase K digestion protocol 4 (Qiagen). Contaminating gDNA was removed by double on-column DNase treatment using the RNase-Free DNase set (Qiagen). RNA used for transcriptional mapping was also subjected to in-solution DNase treatment and then repurified using the RNeasy Mini kit.

Reverse transcription-polymerase chain reaction analysis

One-step reverse transcription-polymerase chain reaction (RT-PCR) mixtures contained 1× Green GoTaq Flexi buffer, 4 mM dithiothreitol, 1.25 mM magnesium chloride, 0.2 mM dNTPs, 1 µg DNase-treated total RNA, 0.3 µM of each DNA primer (Table 2: *aarA*, ODNs 413/416; *sixA*, ODNs 2004/2005; *aarC*, ODNs 1221/1280), 200 units Superscript II RNase H⁻ reverse transcriptase (RT, Invitrogen, Grand Island, NY, USA), and 2.5 units GoTaq DNA polymerase (Promega, Madison, WI, USA). RT was omitted from control reactions. Amplification was performed in three stages: stage 1 (1 cycle), 30 min at 50°C, 5 min at 94°C; stage 2 (20 cycles, *aarA*; 25 cycles, *sixA* and *aarC*), 0.5 min at 94°C, 1 min at 62.5°C, 1.5 min at 72°C; and stage 3 (1 cycle), 5 min at 72°C.

Transcript mapping was performed as described above except that the annealing temperature (stage 2) was reduced to 57.5°C and the number of cycles in stage 2 was increased to 30. RT was omitted from additional control reactions lacking RNA but containing 10 ng gDNA.

Western blot analysis

Soluble proteins present in cell-free lysates generated for *A. acetii* strain 1023 activity assays were separated by SDS-PAGE (12%), and proteins were transferred to a polyvinylidene difluoride membrane (0.2 µm, BioRad, Hercules, CA, USA) in Towbin buffer.¹⁹ [The AarC transfer buffer was supplemented with 0.1% (w/v) sodium dodecyl sulfate (SDS).] The membrane was blocked for 1 h with TTBS buffer¹⁹ and probed for 1 h with one of three custom primary polyclonal antibodies (diluted 1:50,000 in TTBS). After rinsing with TTBS, the membrane was probed with one of two secondary HRP-conjugate antibodies (α -rabbit, diluted 1:250,000 in TTBS; α -chicken, diluted 1:500,000 in TTBS). The membrane was rinsed with TTBS and soaked for 5 min in a 1:1 mixture of stable peroxide and luminol/enhancer solutions (Thermo Scientific). Chemiluminescence was promptly measured using 1-5 min exposures.

Data analysis and figure generation

ImageJ²⁰ was used to determine relative Western blot and RT-PCR band intensities. Molecular graphics images were created using

PyMOL.²¹ Models of mutant proteins were prepared using the PyMOL mutagenesis *wizard* and the lowest-energy side chain rotamer conformation.

Results

Overproduction, purification, and characterization of SixAH6

The *sixA* gene region was originally annotated as AarB,⁶ a protein with no known function and no known homologues. All attempts to heterologously overproduce AarB were plagued by apparent toxicity and failed to yield soluble protein (Susan C. Hockings, unpublished observations). Early attempts to clone *sixA* and overproduce SixAH6 were similarly hindered. However, soluble recombinant protein (~50 mg per L culture) was successfully produced using freshly transformed cells and growth medium supplemented with glucose to suppress leaky gene expression.²² SixAH6 was purified by ammonium sulfate fractionation and immobilized metal affinity chromatography. While SDS-PAGE and Western blots showed multiple bands that cross-reacted with a polyclonal α -SixAH6 antibody (Figure 2A,B), MALDI-TOF-MS indicated that SixAH6 was a homogeneous 183-residue protein retaining Met1 (Figure 2C). Size-exclusion chromatography suggested SixAH6 is primarily monomeric in solution (*data not shown*).

Diauxic growth of *A. acetii* cultures

A. acetii strains 1023, 10-8, and AS10 (Table 1) were propagated in YPD medium supplemented with 2% (v/v) ethanol. Both *A. acetii* strains 1023 and 10-8 exhibited biphasic growth (Figure 3A). The first log phase for all strains coincided with ethanol oxidation and acetic acid accumulation (Figure 3B). *A. acetii* strains 1023 and 10-8 maintained relatively constant levels of acetic acid throughout the first stationary phase but rapidly depleted levels of acetic acid as the second log phase began. In contrast, *A. acetii* strain AS10 remained in the first stationary phase and maintained a fixed level of acetic acid. Sequencing (≥ 4.3 -fold coverage) of the 5.1 kb PCR amplicon containing the complete *aar* gene cluster revealed three missense mutations, encoding both AarC (strain AS10) and AarA (strains AS10 and 10-8) mutants (Table 3).²³ Mutation of *aarC* may account for the Ace^S phenotype of *A. acetii* strain AS10. An Ace^R phenotype was conferred by plasmid pAR248, which contains the *orf1* – *aarC* intergenic region and all of *aarC* (GenBank™ accession number DQ631551, starting at nucleotide 3141; Table 3).⁶

Determination of citrate synthase and succinyl-CoA:acetate CoA-transferase activities

Cells were harvested from *A. acetii* cultures during the 12 d growth period, and CS and SCACT activities were assayed in soluble lysates (Figure 3C). *A. acetii* strain 1023 exhibited moderate CS and SCACT activities in the first log phase and early first stationary phase, as

Table 3. Sequence differences in the *aar* gene clusters of *A. acetii* strains 1023, 10-8, and AS10.

<i>A. acetii</i> strain	Mutation(s)	Mutant(s)	GenBank accession number	Contig size (bp)
1023	None	None	DQ631551*	5100
10-8	619G→A	AarA-A265V	JX475924	5041
AS10	1139C→T 4602G→A	AarA-E92K AarC-C357Y	JX475925	5061

*The sequence was updated with an A at nucleotide position 1263, not the G present in the original assembly.²³ This correction is a silent alteration of the AarA Phe50 codon.

ethanol was being converted to acetic acid and glucose was presumably being used as the primary carbon source. CS and SCACT activities dropped dramatically (~100-fold decrease) during the first stationary phase and then spiked (>200-fold increase) during the second log phase as acetic acid was depleted. In contrast, *A. aceti* strain 10-8 displayed very low CS and SCACT activities during both the first log and first stationary phases. Only upon onset of the second log phase did CS and SCACT activities rise to approximately those of *A. aceti* strain 1023. *A. aceti* strain AS10 exhibited CS and SCACT activities near the threshold of detection throughout the growth period.

Translational analysis of AarA, SixA, and AarC

Western blotting was performed to monitor AarA, SixA, and AarC levels in *A. aceti* strain 1023 (Figures 3E and 4). Congruent with activity assays, AarA and AarC levels were moderate in the first log phase, low in the mid first stationary phase, and highest in the second log phase

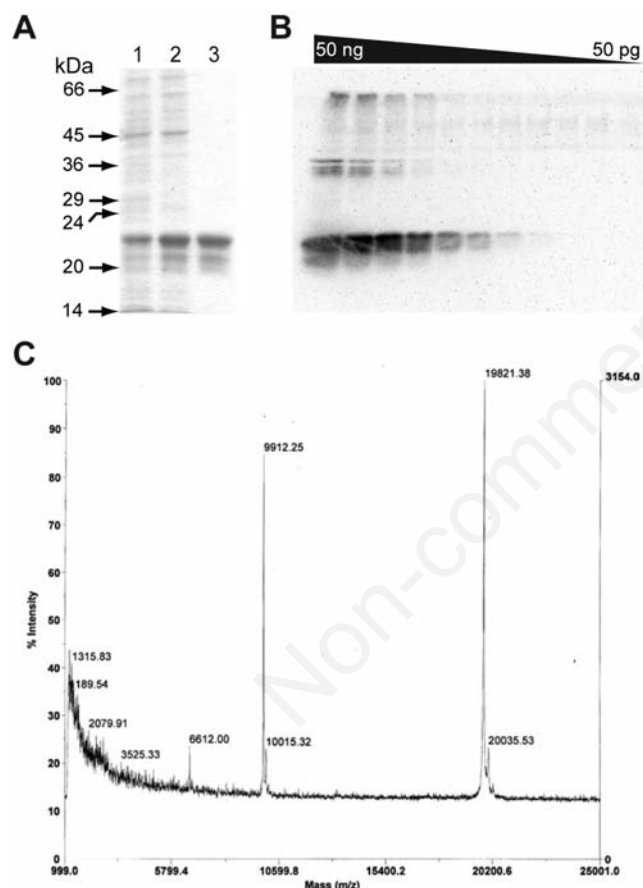


Figure 2. Isolation, analysis, and characterization of SixAH6. (A) Purification of SixAH6 monitored by SDS-polyacrylamide gel electrophoresis. Lane 1, clarified lysate; lane 2, 25–45% ammonium sulfate fraction; lane 3, pooled NiNTA fractions. Pure SixAH6 migrates more slowly than expected and smears, consistent with incomplete denaturation or partial refolding during electrophoresis. Each lane contains 5 μ g protein. Size standard positions are indicated. (B) Semi-quantitative Western blotting of SixAH6 using an α -SixAH6 antibody. Pure SixAH6 was added to *A. aceti* strain 1023 clarified lysate (112 h point in Figure 3; 5 μ g total protein per lane) to give a two-fold serial dilution series. The limit of detection was 0.4 ng SixAH6. (C) MALDI-TOF-MS analysis of SixAH6 (19,821 \pm 2 Da observed; 19,822 Da expected).

as acetic acid was depleted from the medium. SixA was not detected at any stage of the growth period. Spiking of *A. aceti* strain 1023 lysate with pure SixAH6 (Figure 2B) was used to rule out problems with protein transfer to the blotting membrane and to establish an upper limit for SixA levels in the cell (~500 molecules).

Transcriptional analysis of *aarA* and *aarC*

Qualitative RT-PCR experiments were performed to monitor expression of *aarA* and *aarC* in *A. aceti* strain 1023 during growth in YPDE medium (Figures 3D and 5). Transcription of *aarA* and *aarC* increased

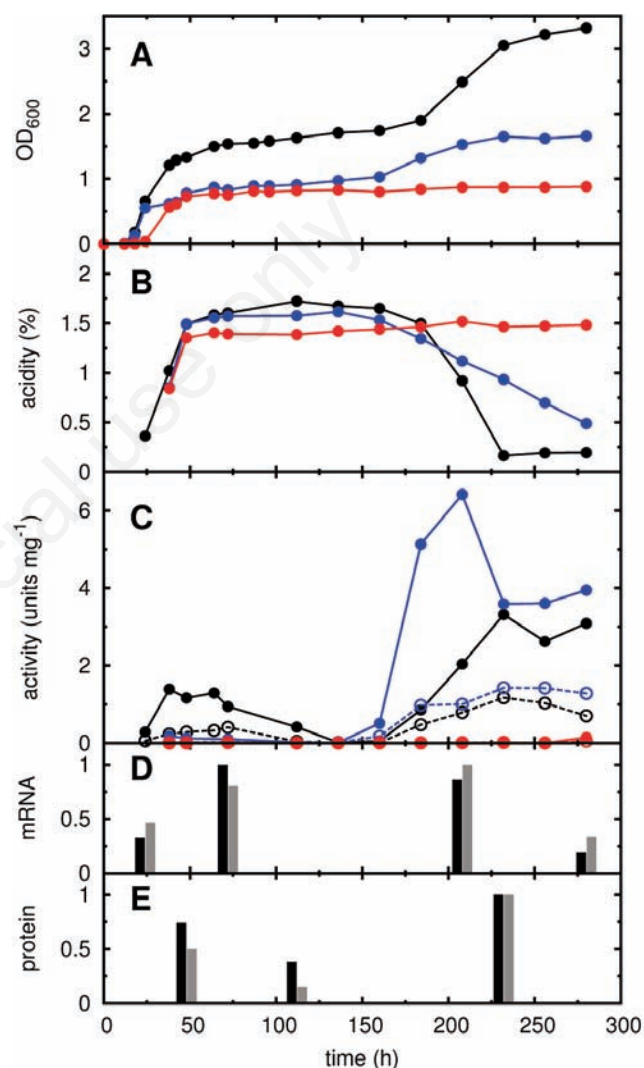


Figure 3. Propagation and analyses of *A. aceti* strains 1023 (*Ace*^R, black symbols), 10-8 (*pro*⁻ *Ace*^R, blue symbols), and AS10 (*pro*⁻ *Ace*^S *aarC*⁻, red symbols) in YPDE medium. (A) Cell density. (B) Titrable acidity. Complete conversion of 2% (v/v) ethanol (0.34 M) to acetic acid (0.34 M) would correspond to 2.1% acidity. (C) CS (*AarA*, solid lines) and SCACT (*AarC*, dashed lines) activities in soluble lysates. One unit is equivalent to one μ mol product formed per min. (D) Determination of *aarA* (black bars) and *aarC* (gray bars) levels in *A. aceti* strain 1023 by RT-PCR. Aliquots were taken at the time points indicated by the center of each pair of bars. Band intensities are given relative to the brightest band in Figure 5. (E) Determination of *AarA* (black bars) and *AarC* (gray bars) levels in *A. aceti* strain 1023 by semi-quantitative Western blotting. Aliquots were taken at the time points indicated by the center of each pair of bars. Band intensities are given relative to the darkest band (excluding lane C) in Figure 4.

as acetic acid was produced in the first log and early first stationary phases. Transcription of *aarC* further increased in the second log phase as acetic acid was consumed. The observed levels of expression correlate with AarA and AarC levels and CS and SCACT activities. In preliminary studies, transcription of *sixA* appeared to be greatest in the first log phase (*data not shown*).

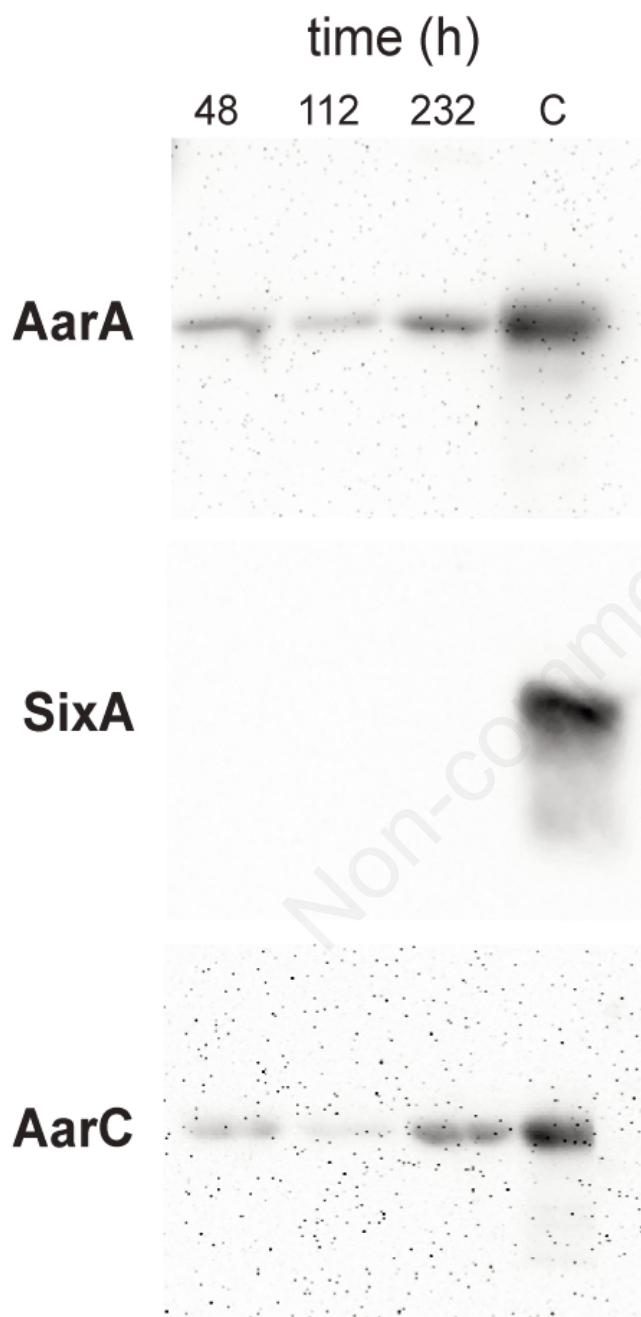


Figure 4. Analysis of AarA, SixA, and AarC by Western blotting. Lanes 48, 112, and 232 each contain 5 μ g total protein. Lane C contains 50 ng pure AarA, SixAH6, or AarCH6. SixA was not detected at any stage of the growth.

Mapping of transcripts produced from the *aar* gene cluster

The divergent orientation of *aarC* relative to the other four genes in the *aar* gene cluster implies that *aarC* is expressed as a separate transcript. The number of transcripts produced by the remainder of the gene cluster was established by RT-PCR, using primer pairs that spanned the intergenic regions. RT-PCR products were observed for *sixA* – *tyrA* and *sixA* – *orf1* but not for *aarA* – *sixA* (Figure 6A/B), suggesting that three *aar* transcripts are produced: a single polycistronic transcript including *sixA*, *tyrA*, and *orf1* (RNA-B) and two monocistronic transcripts including *aarA* (RNA-A) and *aarC* (RNA-C) (Figure 6C). Transcription of RNA-A and RNA-C likely ends at the two rho-independent terminators that demarcate the *aar* gene cluster.

In silico and *in vitro* characterization of AarA and AarC mutants

The crystal structures of AarA⁷ and AarCH6²⁴ show the positions and likely structural consequences of the mutations identified in *A. aceti* strains 10-8 and AS10. The AarA-A265V and AarA-E92K mutations are located at the subunit interface of the core dimeric unit in positions that are far-removed from the active site (Figure 7A/B). Ala265 and Ala265' (Ala265 from a partner subunit) are buried near each other and a pseudo-twofold symmetry axis (Figure 7D). A larger valine side chain at this position may wedge apart helices α M and α M'. Glu92 forms a solvent-accessible salt bridge with Arg436' (dotted line in Figure 7E) that would be disrupted by a positively charged lysine side chain. The AarCH6-C357Y mutation is located near but not in the active site (Figure 7C). Cys357 is buried within a cluster of highly ordered hydrophobic residues (Figure 7F). A considerably larger tyrosine side chain would disrupt hydrophobic packing interactions and likely hinder formation of the core β -sheet in the C-terminal domain. Nearby chloride binding sites located at the subunit interface (Figure 7C) may also be perturbed.

AarCH6-C357Y was heterologously overproduced in *E. coli*

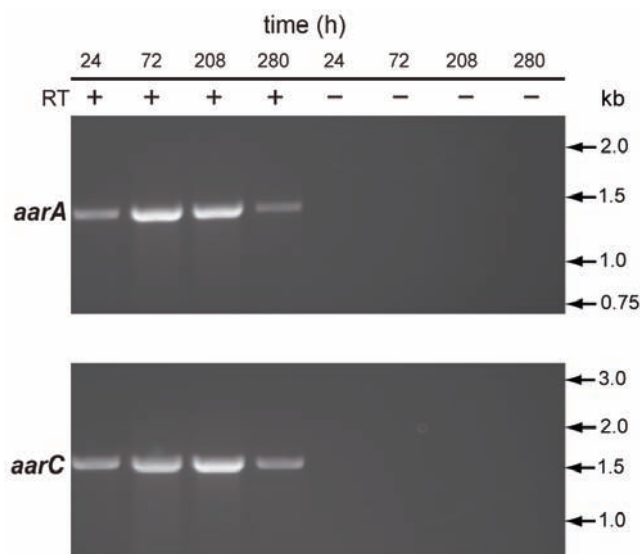


Figure 5. Analysis of *aarA* and *aarC* transcripts by reverse transcription-polymerase chain reaction. Size standard positions are indicated.

C41(DE3). While AarCH6-C357Y was abundant in the total lysate, no soluble protein remained after solids were removed by centrifugation (*data not shown*). Furthermore, no SCACT activity was detected in recombinant *E. coli* C41(DE3) lysate. Profound insolubility of AarCH6-C357Y would account for the lack of SCACT activity in the lysate of recombinant *E. coli* C41(DE3) and the Ace^S phenotype of *A. aceti* strain AS10.

Discussion

Favored vinegar production strains accumulate and tolerate high concentrations of acetic acid, while minimizing overoxidation of acetate to carbon dioxide.² This study examined the relationship between acetate metabolism and the functional expression of *aar* genes in the industrial vinegar strain *A. aceti* 1023. In agreement with previous reports,^{10,11} strain 1023 rapidly produced acetic acid in the first two days of growth as cell density increased and then maintained and tolerated a high concentration of acetic acid (~280 mM from 340 mM ethanol) for six days as cell density remained constant. On the

eighth day, acetic acid depletion began as cell density once again increased. AarA and AarC were abundant during both log phases (Figure 3C,E), indicating the diauxic shift was not a simple consequence of CAC enzyme induction at the onset of the second log phase.

Functional production of AarA and AarC

CAC enzyme levels are correlated with acetic acid production and resistance.²⁵ *A. aceti* strain 1023 (and its derivatives) requires AarA and AarC for both CAC flux and acetic acid resistance on solid medium.^{6,26} Transcription of *aarA* and *aarC* was highest during the second log phase (Figure 3D), consistent with a role for the variant CAC in dissimilatory acetate metabolism.⁸ mRNA abundance correlated well with functional production of AarA and AarC. Based upon maximal enzyme specific activities,^{7,8} AarA and AarC represented about 2% and 1%, respectively, of the soluble proteins present during the second log phase, about twice the level reached during the first log phase.

YPDE growth curves for *aarC*⁺ and *aarC*⁻ strains demonstrate that AarC is not required for ethanol oxidation, but is required for acetate overoxidation (Figure 3A,B). The first finding is consistent with the expectation that ethanol oxidation serves as the main energy source during the first log phase, while the CAC plays a supporting or

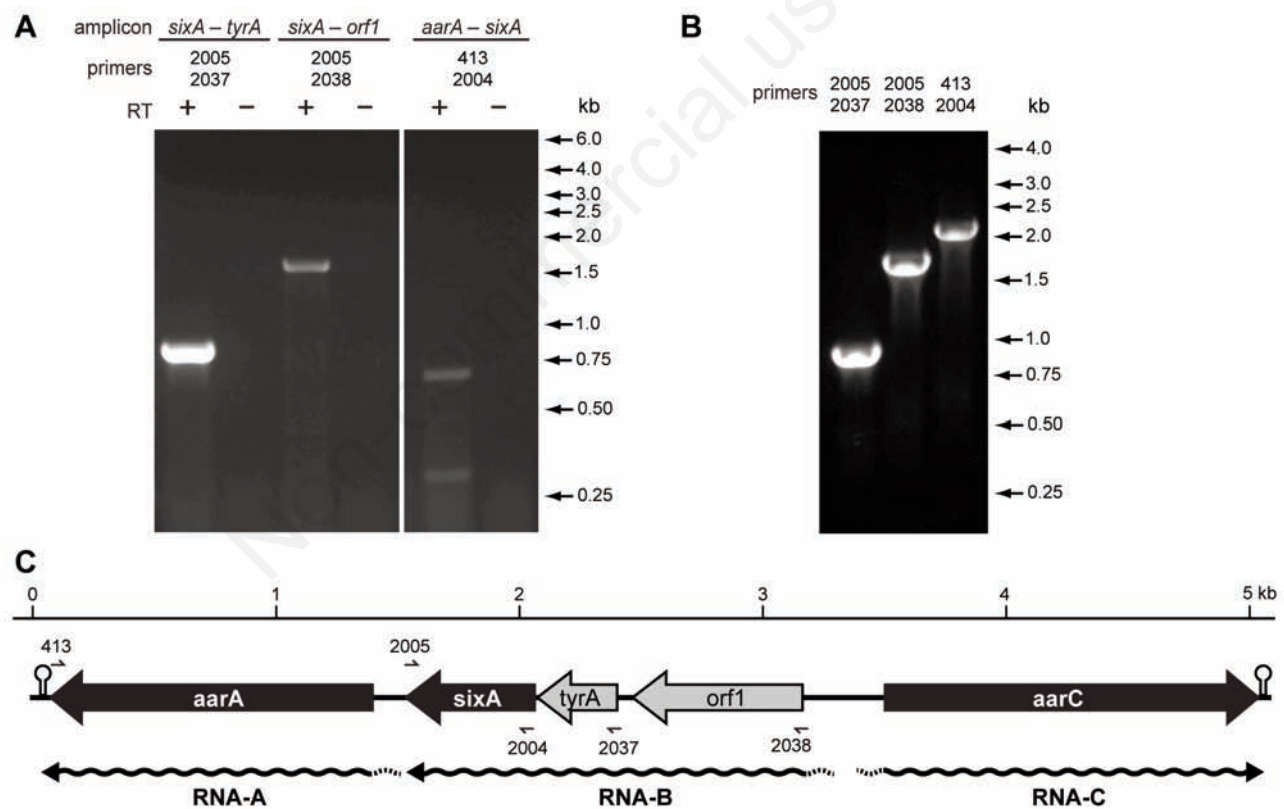


Figure 6. Mapping of transcripts produced from the *aar* gene cluster of *A. aceti* strain 1023. (A) Amplification of intergenic regions by reverse transcription-polymerase chain reaction (RT-PCR). Oligodeoxynucleotide primers were selected that would produce amplicons spanning *sixA* – *tyrA*, 833 bp expected; *sixA* – *orf1*, 1639 bp expected; and *aarA* – *sixA*, 2049 bp expected. The faint bands present in the *aarA* – *sixA* lane correspond to significantly shorter products than expected for the *aarA* – *sixA* amplicon. Intermediate lanes are omitted. Size standard positions are indicated. (B) Corresponding PCR controls containing gDNA template. Size standard positions are indicated. (C) Transcripts produced by the *aar* gene cluster. Five proteins are encoded on three transcripts (wavy lines with 5' start sites shown as dotted lines). The polycistronic transcript RNA-B includes *sixA*, *tyrA*, and *orf1*. The monocistronic transcripts RNA-A and RNA-C include *aarA* and *aarC*, respectively. Putative rho-independent terminator sequences (stem-loops) downstream of *aarA* and *aarC* define the *aar* gene cluster. Small arrows indicate the positions and polarities of ODN primers (Table 2).

anaplerotic role. The second finding confirms that the *aar* genes play a role in diauxic growth in liquid medium. These data also demonstrate that *A. aceti* strain 1023 (and its derivatives) lacks a functional alternative to AarC, which confirms and extends a finding that none of the proteins produced by the other four CoA-transferase genes identified in the genome of *A. aceti* strain 1023²³ has significant *in vitro* SCACT activity.⁸ Further study will be needed to determine if the new *aarA* alleles identified in *A. aceti* strains 10-8 and AS10 (Table 3) are catalytically impaired or downregulated.

Acetic acid sensitivity of *A. aceti* strain AS10

A. aceti cells counteract a constant influx of acetic acid. While passive acidification of the cytoplasm²⁷ should suppress intracellular acetate accumulation,²⁸ an energy source is required to support active acetic acid export by a proton gradient-dependent transporter²⁹ or the ABC transporter AatA,³⁰ each of which contributes to acetic acid resistance.³¹ AatA overexpression has been shown to improve acetic acid yields³⁰ under moderate acetic acid levels similar to those used in this study. (Cultures used in the vinegar industry routinely reach much

higher ethanol and acetic acid levels⁵). The importance of vigorous aeration^{32,33} and periplasmic alcohol dehydrogenase activity^{34,35} in *A. aceti* and highly acetic acid resistant AAB underscores the critical link between aerobic energy metabolism and acetic acid resistance.

Under the conditions examined, AarC does not appear to be required prior to the onset of acetic acid consumption (Figure 3A,B). CS and SCACT activities were also greatly reduced in the first log phase of *A. aceti* strain 10-8, possibly due to a mutation outside the *aar* gene cluster. CAC flux in *A. aceti* strain AS10 was apparently insufficient to support substantial acetate overoxidation on solid⁶ or in liquid medium (Figure 3B), likely resulting from SCACT deficiency due to a mutation that prevents proper folding of AarC (Figure 7C,F). The lack of a second log phase suggests that aerobic acetate overoxidation and diauxic growth are linked. We suggest that AarC is required during the second log phase for two reasons: to provide the energy needed for acetic acid resistance in otherwise depleted growth medium and to eliminate cytoplasmic acetate by dissimilation. In contrast, the assimilation function originally attributed to AarC^{6,26} is unlikely to significantly contribute to acetic acid resistance.⁹

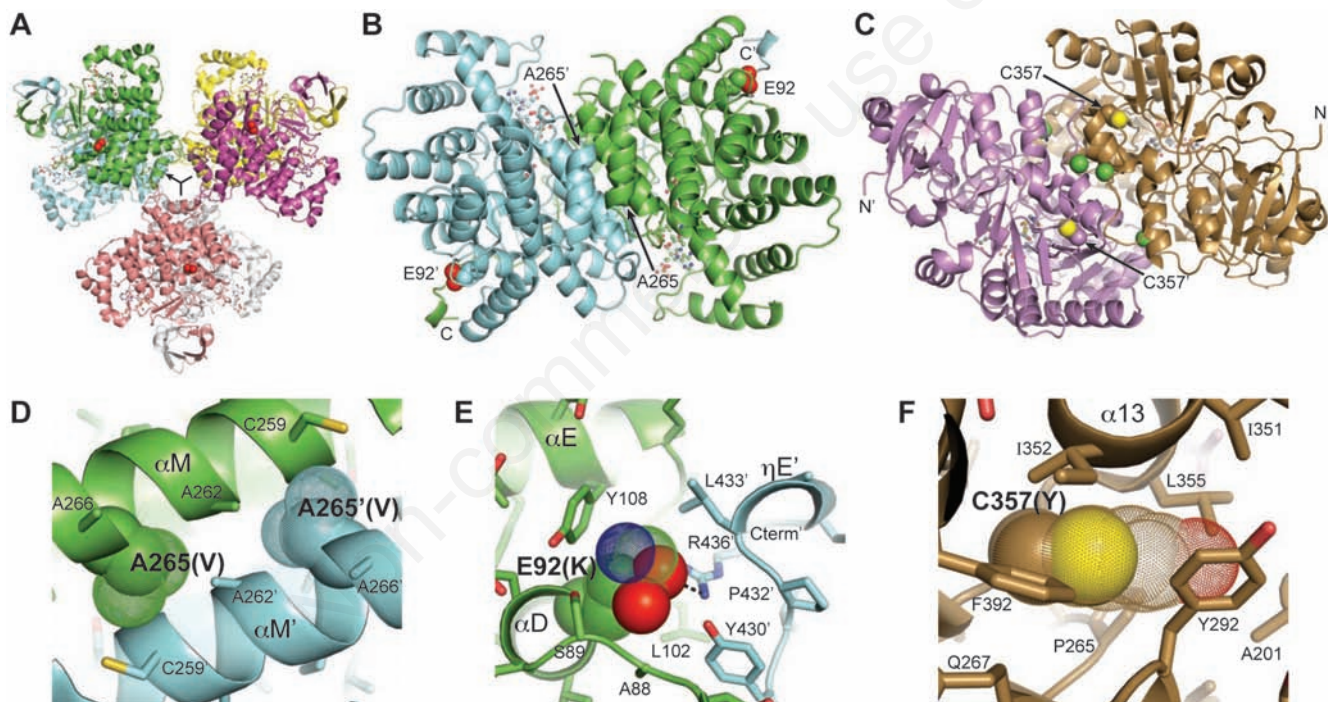


Figure 7. Modeled mutations in AarA and AarC from *A. aceti* strains 10-8 and AS10. In all panels, residues and secondary structural elements in subunit B are indicated with primes. (A) AarA hexamer (PDB id 2h12). The shape at the center indicates the pseudo-threefold symmetry axis. (B) AarA dimer viewed from the center of the hexamer (along the arrow at the center of panel A) showing Glu92 and Ala265 side chains rendered as spheres. Active site ligands are shown in ball-and-stick rendering; sulfate ions from the crystallization medium are omitted. Subunit A is green and subunit B is light blue. The C-terminus of each AarA subunit (C or C') wraps around its partner in the dimeric unit. (C) AarC dimer (PDB id 4eu7) showing Cys357 side chains rendered as spheres. Active site ligands are shown in ball-and-stick rendering; four chloride ions at the subunit interface are shown as green spheres. Subunit A is light brown and subunit B is violet. (D) Location of the AarA-A265V mutation identified in strain 10-8. Ala265 and Ala265' (solid spheres) are situated between the pseudosymmetric helices α M and α M'. The Ala265 \rightarrow Val substitution (dotted spheres) may wedge apart these interfacial secondary structural elements. (E) Location of the AarA-E92K mutation identified in strain AS10. A solvent-accessible salt bridge formed between Glu92 (solid spheres) and Arg436' would be disrupted by the Glu92 \rightarrow Lys substitution (dotted spheres). (F) Location of the AarC-C357Y mutation identified in strain AS10. Cys357 (solid spheres) is buried near a central β -sheet. The Cys357 \rightarrow Tyr substitution (dotted spheres) would clash with several hydrophobic residues and disrupt a tightly packed core. Helix α 13 contains four affected residues: Ile351, Ile352, Arg354 (not shown), and Leu355. Arg354 contacts two chloride ions located at the center of the subunit interface (the average Cl - SY distance is 13.6 Å).

Modulation of citric acid cycle enzyme synthesis by environmental inputs

Genes involved in ethanol oxidation are regulated by quorum sensing in *Gluconacetobacter* strains^{36,37} and perhaps other AAB. Residual ethanol suppresses acetate overoxidation in AAB by an undefined mechanism.^{2,3,38} The role of SixA in the *aar* system may be to regulate CAC gene expression in one or both log phases. The relatively low abundance of SixA (Figure 4) and its production from a separate mRNA (Figure 6) are consistent with a regulatory role.

In facultative anaerobes like *E. coli*, CAC enzyme synthesis is regulated by the Arc two-component system.³⁹ Oxygen deficiency causes the receptor kinase ArcB to autophosphorylate and then transphosphorylate the response regulator ArcA, which represses several operons involved in aerobic respiration.³⁹⁻⁴¹ Anaerobic repression of the *sdh* – *suc* operon is particularly strong.⁴² The anaerobic metabolites D-lactate and acetate enhance anaerobic repression by increasing ArcB autophosphorylation.^{43,44} Under anaerobic conditions, SixA attenuates repression by dephosphorylating ArcB.⁴⁵

The primary role of SixA in thoroughly aerated *A. aceti* cultures may therefore be to derepress CAC enzyme synthesis at moderate to high acetic acid levels, thereby ensuring the presence of anaplerotic pathways during both log phases and a continuous energy supply during the second log phase when other nutrients are depleted.

Metabolic streamlining of industrial vinegar production strains

AAB with a complete CAC contain genes for succinyl-CoA synthetase, SCACT, or both.⁴⁶ Some *Acetobacter* strains, including *A. aceti* strain NBRC 14818, also possess a functional glyoxylate shunt.^{4,47} Others, including *A. aceti* strain 1023, do not.^{8,48} In conjunction with enzymes that interconvert acetate and acetyl-CoA, these alternatives (Figure 1) support acetate assimilation, dissimilation, or both. Vinegar production strains like *A. aceti* strain 1023 that possess streamlined central carbon metabolism provide a comparatively straightforward link between metabolic flux control and acetic acid production.

Diverse metabolic pathways would equip wild *Acetobacter* strains to exploit the complex nutritional environment present in fruit and other natural niches.¹ For industrial strains adapted to defined ethanol medium, however, a glyoxylate shunt may be superfluous and could decrease acetic acid yields. *A. aceti* strain GP, an *aceA/glcB* mutant of NBRC 14818 that lacks a glyoxylate shunt, furnishes a higher yield of acetic acid than the parent strain.⁴⁹ Selection for high vinegar yields may have favored strains like *A. aceti* strain 1023 that have a limited ability to assimilate acetate.

Conclusions

Diauxic growth in YPDE medium is accompanied by distinct stages of acetic acid accumulation, conservation, and depletion. AarC is abundant during both acetic acid accumulation and acetic acid depletion, suggesting that modulation of AarC levels is not the method by which acetate catabolism is regulated. AarC is not required for the conversion of ethanol to acetic acid, indicating that acetic acid resistance is supported by other energy sources during the first log phase. The *aar* genes enable aerobic growth in otherwise depleted medium during the second log phase, consistent with a dual role for the variant CAC in energy harvesting and acetate dissimilation. We speculate that acetate overoxidation is controlled by cytoplasmic acetate levels and linked to active acetic acid export, while SixA ensures that aerobic acetate metabolism proceeds in conditions that would otherwise suppress CAC flux.

References

- Asai T. Acetic acid bacteria. Classification and biochemical activities. Tokyo: University of Tokyo Press; 1968.
- Saeki A, Taniguchi M, Matsushita K, et al. Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. *Biosci Biotechnol Biochem* 1997;61:317-23.
- Saeki A, Matsushita K, Takeno S, et al. Enzymes responsible for acetate oxidation by acetic acid bacteria. *Biosci Biotechnol Biochem* 1999;63:2102-9.
- Sakurai K, Arai H, Ishii M, Igarashi Y. Transcriptome response to different carbon sources in *Acetobacter aceti*. *Microbiology* 2011;157:899-910.
- García-García I, Santos-Dueñas IM, Jiménez-Ot C, et al. Vinegar engineering. In: Solieri L, Giudici P, eds. *Vinegars of the world*. 1st ed. New York: Springer; 2008. pp 97-120.
- Fukaya M, Takemura H, Okumura H, et al. Cloning of genes responsible for acetic acid resistance in *Acetobacter aceti*. *J Bacteriol* 1990;172:2096-104.
- Francois JA, Starks CM, Sivanuntakorn S, et al. Structure of a NADH-insensitive hexameric citrate synthase that resists acid inactivation. *Biochemistry*. 2006;45:13487-99.
- Mullins EA, Francois JA, Kappock TJ. A specialized citric acid cycle requiring succinyl-coenzyme A (CoA):acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile *Acetobacter aceti*. *J Bacteriol* 2008;190:4933-40.
- Steiner P, Sauer U. Proteins induced during adaptation of *Acetobacter aceti* to high acetate concentrations. *Appl Environ Microbiol* 2001;67:5474-81.
- Ohmori S, Uozumi T, Beppu T. Loss of acetic acid resistance and ethanol oxidizing ability in an *Acetobacter* strain. *Agric Biol Chem* 1982;46:381-9.
- Ohmori S, Masai H, Arima K, Beppu T. Isolation and identification of acetic acid bacteria for submerged acetic acid fermentation at high temperature. *Agric Biol Chem* 1980;44:2901-6.
- Miroux B, Walker JE. Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 1996;260:289-98.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
- Ewing B, Green P. Base-calling of automated sequencer traces using *Phred*. II. Error probabilities. *Genome Res* 1998;8:186-94.
- Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using *Phred*. I. Accuracy assessment. *Genome Res* 1998;8:175-85.
- Gordon D, Abajian C, Green P. Consed: A graphical tool for sequence finishing. *Genome Res* 1998;8:195-202.
- Naville M, Ghuillot-Gaudeffroy A, Marchais A, Gautheret D. ARNold: a web tool for the prediction of Rho-independent transcription terminators. *RNA Biol* 2011;8:11-3.
- Chinnawirotpisan P, Theeragool G, Limtong S, et al. Quinoprotein alcohol dehydrogenase is involved in catabolic acetate production, while NAD-dependent alcohol dehydrogenase in ethanol assimilation in *Acetobacter pasteurianus* SKU1108. *J Biosci Bioeng* 2003;96:564-71.
- Ausubel FM, Brent R, Kingston RE, et al. Short protocols in molecular biology: A compendium of methods from *Current protocols in molecular biology*. 4th ed. New York, NY: Wiley; 1999.
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 2012;9:671-5.
- DeLano WL. The PyMOL molecular graphics system. Palo Alto, CA:

- DeLano Scientific; 2002.
22. Grossman TH, Kawasaki ES, Punreddy SR, Osburne MS. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene* 1998;209:95-103.
 23. Francois JA, Kappock TJ. Alanine racemase from the acidophile *Acetobacter aceti*. *Protein Expression Purif* 2007;51:39-48.
 24. Mullins EA, Kappock TJ. Crystal structures of *Acetobacter aceti* succinyl-coenzyme A (CoA):acetate CoA-transferase (AarC) reveal specificity determinants and illustrate the mechanism used by class I CoA-transferases. *Biochemistry* 2012;51:8422-34.
 25. Nakano S, Fukaya M, Horinouchi S. Enhanced expression of aconitase raises acetic acid resistance in *Acetobacter aceti*. *FEMS Microbiol Lett* 2004;235:315-22.
 26. Fukaya M, Takemura H, Tayama K, et al. The *aarC* gene responsible for acetic acid assimilation confers acetic acid resistance on *Acetobacter aceti*. *J Ferment Bioeng* 1993;76:270-6.
 27. Menzel U, Gottschalk G. The internal pH of *Acetobacterium wieringae* and *Acetobacter aceti* during growth and production of acetic acid. *Arch Microbiol* 1985;143:47-51.
 28. Russell JB. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J Appl Microbiol* 1992;73:363-70.
 29. Matsushita K, Inoue T, Adachi O, Toyama H. *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. *J Bacteriol* 2005;187:4346-52.
 30. Nakano S, Fukaya M, Horinouchi S. Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. *Appl Environ Microbiol* 2006;72:497-505.
 31. Nakano S, Fukaya M. Analysis of proteins responsive to acetic acid in *Acetobacter*: Molecular mechanisms conferring acetic acid resistance in acetic acid bacteria. *Int J Food Microbiol* 2008;125:54-9.
 32. Muraoka H, Watabe Y, Ogasawara N. Effect of oxygen deficiency on acid production and morphology of bacterial cells in submerged acetic fermentation by *Acetobacter aceti*. *J Ferment Technol* 1982;60:171-80.
 33. Park YS, Ohtake H, Fukaya M, et al. Effects of dissolved oxygen and acetic acid concentrations on acetic acid production in continuous culture of *Acetobacter aceti*. *J Ferment Bioeng* 1989;68:96-101.
 34. Trcek J, Toyama H, Czuba J, et al. Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. *Appl Microbiol Biotechnol* 2006;70:366-73.
 35. Trcek J, Jernejc K, Matsushita K. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. *Extremophiles* 2007;11:627-35.
 36. Iida A, Ohnishi Y, Horinouchi S. Control of acetic acid fermentation by quorum sensing via *N*-acylhomoserine lactones in *Gluconacetobacter intermedius*. *J Bacteriol* 2008;190:2546-55.
 37. Iida A, Ohnishi Y, Horinouchi S. Identification and characterization of target genes of the GinI/GinR quorum-sensing system in *Gluconacetobacter intermedius*. *Microbiology* 2009;155:3021-32.
 38. Kornmann H, Duboc P, Niederberger P, et al. Influence of residual ethanol concentration on the growth of *Gluconacetobacter xylinus* I 2281. *Appl Microbiol Biotechnol* 2003;62:168-73.
 39. Iuchi S, Lin ECC. *arcA* (*dye*), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. *Proc Natl Acad Sci U S A* 1988;85:1888-92.
 40. Iuchi S, Matsuda Z, Fujiwara T, Lin ECC. The *arcB* gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the *arc* modulon. *Mol Microbiol* 1990;4:715-27.
 41. Gunsalus RP, Park SJ. Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. *Res Microbiol* 1994;145:437-50.
 42. Park SJ, Chao G, Gunsalus RP. Aerobic regulation of the *sucABCD* genes of *Escherichia coli*, which encode α -ketoglutarate dehydrogenase and succinyl coenzyme A synthetase: Roles of ArcA, Fnr, and the upstream *sdhCDAB* promoter. *J Bacteriol* 1997;179:4138-42.
 43. Georgellis D, Kwon O, Lin ECC. Amplification of signaling activity of the Arc two-component system of *Escherichia coli* by anaerobic metabolites. An *in vitro* study with different protein modules. *J Biol Chem* 1999;274:35950-4.
 44. Rodriguez C, Kwon O, Georgellis D. Effect of D-lactate on the physiological activity of the ArcB sensor kinase in *Escherichia coli*. *J Bacteriol* 2004;186:2085-90.
 45. Matsubara M, Mizuno T. The SixA phospho-histidine phosphatase modulates the ArcB phosphorelay signal transduction in *Escherichia coli*. *FEBS Lett* 2000;470:118-24.
 46. Matsutani M, Hirakawa H, Yakushi T, Matsushita K. Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*. *FEMS Microbiol Lett* 2011;315:122-8.
 47. Stouthamer AH, van Boom JH, Bastiaanse AJ. Metabolism of C₂ compounds in *Acetobacter aceti*. *Antonie Van Leeuwenhoek* 1963;29:393-406.
 48. Azuma Y, Hosoyama A, Matsutani M, et al. Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. *Nucleic Acids Res* 2009;37:5768-83.
 49. Sakurai K, Yamazaki S, Ishii M, et al. Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. *J Biosci Bioeng* 2013;115:32-6.