

Mutation in Acetate⁺ *aceK*Δ Strain Decreases IDH Activity in *Escherichia coli* Allowing for Growth on Acetate

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Abstract

The glyoxylate bypass via the enzymes isocitrate lyase and malate synthase allows bacteria to grow on acetate by bypassing the CO₂ producing steps and, therefore, accumulating carbon skeletons required for biosynthesis. In order to utilize isocitrate lyase in this bypass and subsequently allow growth on acetate, isocitrate dehydrogenase must be regulated via reversible phosphorylation completed by isocitrate dehydrogenase kinase. IDH activity was observed where the four acetate⁺ *aceK*Δ mutants selected for by growth on acetate media had significantly less IDH activity than the *aceK*Δ parent strain which had full IDH activity, but more than the *icd*Δ mutant strain which had approximately no IDH activity. The selected acetate⁺ *aceK*Δ mutant *MTM2* was chosen for further analysis because it contained <25% IDH activity relative to the parent strain *aceK*Δ. Sequencing of the *icd* gene in *MTM2* contained a SNP in the 5'UTR of the *icd*. Upon Western blot analysis, the relative fluorescent signal of the *MTM2* lane against the parent strain control lane was reduced indicating decreased protein abundance. Since the *MTM2* mutation is upstream of the coding region, decreased IDH protein levels were suspected. Future experimentation utilizing qRT-PCR, EMSA, and DNA footprinting may reveal an unknown transcription regulatory binding region upstream of the *icd* coding sequence.

Introduction

The tricarboxylic acid (TCA) cycle, composed of intermediate reactions in cellular respiration, remains vital for aerobic metabolism of any cell. The cycle utilizes several forms of carbon skeletons as energy sources that can be oxidized and eventually used in the production of ATP. Not only does the TCA cycle help regulate storage and usage of these intermediate metabolites, it also generates necessary precursors of essential, cellular molecules such as amino acids. Isocitrate dehydrogenase (IDH) acts at the center of the TCA cycle and converts isocitrate along with the cofactor NAD⁺ to oxalosuccinate and NADH. Oxalosuccinate, a β-keto acid, then undergoes a β-hydroxy decarboxylation to give the products of α-ketoglutarate and CO₂. When glucose, the initial carbon source for this cycle, is unavailable, plant and bacterial cells can utilize acetate as an additional source of carbon through the glyoxylate bypass.

The glyoxylate bypass skips two CO₂ producing steps in the TCA cycle and uses isocitrate lyase (IL) to produce glyoxylate and succinate and then malate synthase (MS) to produce malate via an additional acetyl-CoA. This bypass is essential for maintaining carbon skeletons and preventing loss of the acetate carbons (Laporte et al., 1989). In *E. coli*, regulation of the branchpoint is completed through several mechanisms. Since isocitrate is the substrate for both competing enzymes of IDH and IL, the low K_M of IDH and high K_M of IL allows IDH to become the

primary functioning enzyme under normal conditions. IDH activity must be reduced for IL to become the primary enzyme in the branchpoint, but not entirely depleted because α -ketoglutarate is a required intermediate for glutamate production and approximately 25% IDH activity is ideal during growth on acetate (Cozzone and El-Mansi, 2006; Thorsness and Koshland, 1987).

IDH activity is restricted through reversible phosphorylation on serine 113 via a bifunctional kinase/phosphatase known as IDH kinase/phosphatase (Thorsness and Koshland, 1987). The negatively charged phosphoserine 113 induces a conformational change that renders the enzyme inviable (Cozzone and El-Mansi, 2006). Decreased IDH activity causes an increase in cellular isocitrate levels which diverts isocitrate flow through IL and allows for growth on acetate. IDH kinase/phosphatase can keep up even with a drastically increased intracellular concentration of IDH and regulate the IDH levels appropriately to maintain biosynthetic precursor production (LaPorte et al., 1985). The protein kinase is regulated via cellular concentration of AMP as well as other metabolites and coded by the *aceK* gene on the *ace* operon (LaPorte et al., 1985). In *Escherichia coli*, the *ace* operon includes *aceB* which codes for malate synthase, *aceA* which codes for isocitrate lyase, and *aceK* which codes for IDH kinase/phosphatase (Cozzone and El-Mansi, 2006). Deletion of the IDH kinase/phosphatase gene (*aceKΔ*) prevents IDH from being phosphorylated and, therefore, impedes IDH regulation. The phenotypic consequence of this knockout includes bacteria that do not produce IDH kinase/phosphatase protein in order to regulate IDH. They do not exhibit growth on acetate because isocitrate concentration does not build and flux is not redirected through IL.

The purpose of this set of experiments was to identify mutations that compensate for the absence of *aceK* by selecting for reverse mutations that reduce IDH activity via growth on the selective media of acetate. Vital regions in the IDH enzyme that are required for it to function properly were expected to be a primary site for mutations that would reduce IDH activity and allow for growth on acetate. Mutations in untranslated, regulatory regions that affected transcription or translation were also expected to decrease overall cellular IDH activity. Mutants were selected for by growth on acetate media, the IDH activity of mutants that contained a change in the *icd* gene were measured for comparison against the *aceKΔ* parent strain, and the mutation was identified utilizing dideoxy sequencing. The acetate⁺ *aceKΔ* mutant phenotypic growth patterns were compared to that of the *aceKΔ* parent strain. These growth observations were completed under conditions that both did and did not supply additional glutamate in order to determine if some IDH activity was reserved to create sufficient α -ketoglutarate for survival and proliferation. Additionally, protein levels of IDH were determined via Western blot analysis to gain insight into the mutation's possible mode of reversion.

Materials and Methods

Strains

SL1R was the *aceKΔ* parent strain used to select suppressors of *aceKΔ* by growing them on acetate media. The genotype is as follows: *his4 lacBK1 rpsL31 sfiB thi1 aceKΔ::KmR*.

ST2010R was the wild-type *E. coli* used to assess IDH activity in glucose and acetate media. The genotype is as follows: *his4 lackBK1 rpsL31 sfiB thi1*.

JW1122-2 was the *icdΔ* strain used as a negative control in growth phenotype assessment, IDH assays, and Western blot analysis. The genotype is as follows: *(araD-araB)Δ567 lacZΔ4787 rph1 (rhaD-rhaB)Δ568 hsdR514 icdΔ::KmR*.

Mutant Selection and Cell Growth

The *aceKΔ* parent strain lacked IDH kinase/phosphatase activity and, therefore, could not decrease IDH activity via phosphorylation. Acetate⁺ *aceKΔ* mutants were selected for by growing the parent strain on acetate media. The *aceKΔ* parent strain was cultured initially in LB liquid, inoculated in M9 acetate liquid for two days, and then plated onto an M9 acetate plate followed by an LB plate. Following the creation of a stock plate, the colonies on the serial dilution plates were counted to show the 10⁻⁵ dilution at an original cell count of 2.15*10⁹ cells/mL and 2.1*10⁹ cells/mL, the 10⁻⁶ dilution at an original cell count of 1.9*10⁹ cells/mL and 2.5*10⁹ cells/mL, and the 10⁻⁷ dilution at an original cell count of 1*10⁹ cells/mL and 2*10⁹ cells/mL. The wild-type was grown to mid-log in the liquid M9 acetate and M9 glucose for use in IDH assays. *aceKΔ* parent, acetate⁺ *aceKΔ* suppressor, and *icdΔ* strains were grown overnight in LB liquid IDH assays as well as a Western blot. Growth phenotypes of the *aceKΔ* parent, acetate⁺ *aceKΔ* suppressor, and *icdΔ* strains were assessed on M9 glucose and M9 acetate plates with and without added glutamate.

Preparation of Cell Lysates

E. coli cells were harvested by centrifugation and resuspended in a buffer of 200μL 1X PBS. The cells were lysed mechanically via extensive vortex with 100μL of glass beads in the range of 425μm to 600μm in size. Cells were vortexed on high for exactly two minutes and subsequently centrifuged to pellet cell debris and glass beads to allow for the supernatant to be extracted as crude extract.

IDH Activity Assay

500μL of 1X IDH assay cocktail were added to cuvettes for use in a spectrophotometer. The cocktail contained 25mM MOPS (3-(N-morpholino) propanesulfonic acid) as a buffer at pH 7.5, 250μM NADP⁺, 500μM DL-isocitrate, 100mM NaCl, and 5mM MgCl₂. IDH activity was determined by the increase in absorbance measured at 340nm due to production of NADPH. The conversion of NADP⁺ to NADPH via IDH allowed for IDH activity to be measured through NADPH production. The assay was initiated through the addition of 40μL of crude extract to the assay cocktail with immediate repeated cuvette inversions while covered with parafilm for mixing. Absorbance readings were taken in three second intervals for 30 seconds. IDH activity was reported in units of Δabs at 340nm/min/grams of cell pellet.

PCR Amplification and DNA Purification

PCR was used for mutant screening and *icd* gene sequencing. The targeted DNA section was a 1685nt fragment that contained the 1251nt *icd* coding sequence along with some upstream and downstream flanking regions. The primer cocktail used contained the following:

icd F2: 5'AACTGCGGAAGAACATCGTA3'

icd R4: 5' TGGGCTTCATATAAATATGCTCG3'

Prepared cells were added to the primer cocktail along with Quick-Load Taq 2X Master Mix that contained Taq DNA polymerase, dNTPs, MgCl₂, KCl, tracking dyes, and stabilizers that allowed for direct loading of the product onto agarose gels. Initial denaturation was at 95°C for five minutes followed by 30 cycles of: 95°C denaturation (15 seconds), 49°C primer annealing (15 seconds), 68°C polymerase extension (1 minute 45 seconds), and the final extension was at 68°C for five minutes followed by cooling at 8°C to infinity. The PCR product was analyzed by agarose gel electrophoresis. PCR fragment purification was completed via a Qiagen spin column prior to sample sequencing.

DNA Sequence Analysis

Samples for direct *icd* sequencing were sent to Elim Biopharm and utilized the *icd* F2 and R4 primers as well as a third primer in the middle for stronger differentiation between bases in the data. Whole genomes of the parent strain and mutants that had been previously identified, including 19 acetate⁺ *aceKΔ* suppressors, were sequenced by Genewiz. All data was analyzed via Geneious Prime software and all mutant strains were compared to the *aceKΔ* parent strain.

Western Blot

Optical density of a diluted culture was measured at 600nm via Nanodrop instrumentation. OD₆₀₀ allowed for load normalization between strains. 30μL of 2X SDS sample buffer and Benzodase were added to the aliquoted culture for a 1:1 mixture. Samples were analyzed via SDS-PAGE and blotted to nitrocellulose. Probing and visualization was done with a 1:5,000 dilution of the primary antibody and a 1:20,000 dilution of the secondary antibody (LI-COR IRDye 680LT Goat anti-Rabbit IgG) via LI-COR Odyssey CLx.

Results

IDH Activity Assays on Wild-type *E. coli*

The IDH activity assay is based on the production of NADPH and the increase in absorbance seen at 340nm. The result provides evidence that the proposed reverse mutation was properly selected for and confers lower cellular IDH activity allowing for the observed growth on acetate. IDH activity was measured in wild-type *E. coli* grown on both M9 Glucose and M9 Acetate media. Two samples duplicated from a single extract of each type of growth media were taken for measuring the IDH activity.

Table 1. IDH Activity (Units) of Wild-type *E. coli* Grown on Glucose and Acetate Media

Average activity in the samples grown on acetate was 3.82% of the average activity in the samples grown on glucose.

Growth Media	IDH Activity (Units)	Average (Units)
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M9 Glucose 1	233.20	226.11
M9 Glucose 2	219.02	
M9 Acetate 1	9.86	8.63
M9 Acetate 2	7.39	

Mutant Isolation and Analysis

From selective plating, four acetate⁺ *aceKΔ* mutant strains showed decreased IDH activity and were chosen for further analysis. The mutant and control strains were compared to one another under growth restriction via acetate plates. Environmental glutamate was important in the differentiation between IDH mutants with partially reduced or fully reduced activity. Fully reduced IDH activity would not allow the cells to produce enough α -ketoglutarate to sustain proliferation. All four mutants showed growth under all conditions while the parent strain did not grow on acetate unless additional glutamate was provided. The *icdΔ* strain did not grow on acetate either with or without glutamate, nor did it grow on glucose media without glutamate.

Table 2. Growth Characteristics of *aceKΔ*, *icdΔ*, and Selected Acetate⁺ *aceKΔ* Mutant Strains (MTM1-4)
Growth phenotypes were determined via a +/- scale indicating growth or no growth, respectively. Strains were grown on M9 Acetate and M9 Glucose media with and without additional glutamate.

The mutant strains grew better than the parent strain on acetate and better than the *icdΔ* without glutamate.

Media	M9 Acetate		M9 Glucose	
	Added Glutamate	No Added Glutamate	Added Glutamate	No Added Glutamate
<i>aceKΔ</i>	+	-	+	+
<i>icdΔ</i>	-	-	+	-
<i>MTM1</i>	+	+	+	+
<i>MTM2</i>	+	+	+	+
<i>MTM3</i>	+	+	+	+
<i>MTM4</i>	+	+	+	+

The IDH activity assay was completed again on the *aceKΔ* and *icdΔ* strains as well as the four selected mutants. Based on the results of the assay along with PCR amplification of the *icd* gene, a single mutant was chosen for in-depth analysis that had an IDH activity of <25% of the parent strain but could still grow on acetate without additional glutamate. The knockout strain *icdΔ* showed greatly decreased IDH activity when compared to that of the parent strain. *MTM1*, *MTM2*, *MTM3* all showed lower levels of IDH activity (<10%); however, *MTM4* was just under half of the parent strain activity.

Table 3. IDH Activity of *aceKΔ*, *icdΔ*, and Selected Acetate⁺ *aceKΔ* Mutant Strains (MTM1-4)
IDH activity was measured for all four mutants and controls. Each strain's activity relative to the parent strain were calculated and reported.

Strain	IDH Activity (Units)	IDH Activity Relative to <i>aceKΔ</i> (% <i>aceKΔ</i>)
<i>aceKΔ</i>	365.88	100.00
<i>icdΔ</i>	9.25	2.53
<i>MTM1</i>	4.40	1.20
<i>MTM2</i>	16.09	4.40
<i>MTM3</i>	32.81	8.97
<i>MTM4</i>	161.68	44.19

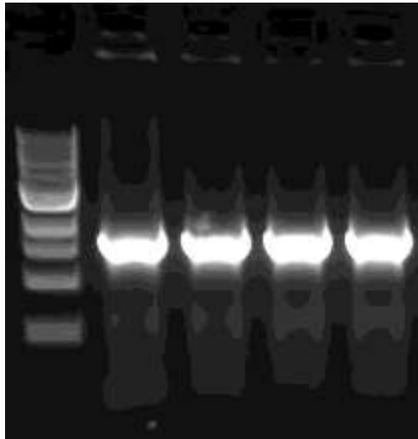


Figure 1. PCR for All Four Mutants
 PCR products analyzed via gel electrophoresis. Lanes from left to right: Ladder, *MTM1*, *MTM2*, *MTM3*, and *MTM4*. All four mutants had bands at approximately 1,685bp.

PCR was used as the first step in further characterization of the mutants by amplifying the *icd* gene from the acetate⁺ *aceKΔ* suppressor mutants. It was suspected that the reverse mutation occurred within or near the *icd* gene. The PCR products analyzed via gel electrophoresis showed strong bands for all four mutants near the 1.5kb ladder marker and the expected PCR product of 1,685bp was observed. Of the four mutants, *MTM2* was chosen for detailed analysis because it showed decreased IDH activity at 4.40% of the parent strain. This significant decrease was optimal for further analysis while maintaining enough IDH activity to produce its

own glutamate. It is important the selected mutant has retained the ability to produce its own α -ketoglutarate so it can proliferate without additional glutamate. *MTM2* sequencing revealed a single nucleotide polymorphism in the 5' untranslated region of the *icd* gene. C249T is a mutation -84 base pairs upstream of the translational start codon. While the SNP lies upstream of the

translational start codon, it is downstream of the only known activator and dual regulator as well as the two known transcriptional start sites (Prost et al., 1999).

Western Blot

A Western blot was run to determine if the mutation affected IDH protein levels in *MTM2* relative to the parent strain. The mutation in the upstream region may have influenced transcription or translation leading to a decrease in protein levels. The *icdΔ* strain had no IDH protein produced which inherently created no band on the gel whereas *aceKΔ* cell did create IDH protein which was observed via the Western blot. The mutant strain *MTM2* showed decreased IDH protein levels, but some protein was still observed.

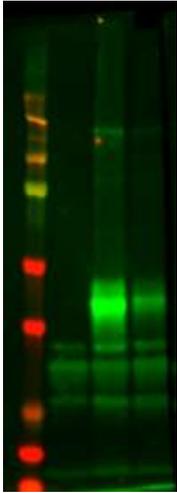


Figure 2. Western Blot of MTM2 Mutant and Controls

Analysis via LI-COR Odyssey CLx. Lanes from left to right: Ladder, *icdΔ*, *aceKΔ*, and MTM2. MTM2 IDH level lower than that of parent strain.

Genome Sequence Analysis

Whole genome sequences of the parent strain along with 19 acetate⁺ *aceKΔ* mutant strains were previously determined. Strain UW8 contained a mutation at the 955th nucleotide in the *sthA* gene sequence that contains a total of 1,401 nucleotides total. The 319th amino acid in the parent sequence was a nonpolar alanine and was altered to a polar threonine in the mutant strain.

Discussion

IDH Assays on Wild-Type *E. coli*

Decreasing IDH function via phosphorylation results in an increase in cellular isocitrate levels which diverts flow through IL. The decreased levels of IDH activity can be expected as the adaptive branchpoint mechanism allows for bacterial growth when either glucose or acetate are present in the environment (Cozzone and El-Mansi, 2006; Thorsness and Koshland, 1987). The wild-type samples grown on M9 Glucose plates contained high IDH activity due to fully intact *icd* genes that were able to produce appropriate amounts of functional IDH proteins. The samples had an observed IDH activity average of 226.11 units (Table 1). The wild-type samples grown on M9 Acetate that showed decreased IDH activity levels at an average of 8.63 units contained much more phosphorylated IDH to allow for growth on acetate without reducing IDH activity to a point where insufficient α -ketoglutarate is produced.

Mutation Isolation and Analysis

Mutants *MTM1*, *MTM2*, *MTM3*, and *MTM4* lacked *aceK*, but grew on acetate with and without additional glutamate. All showed significant decreases in IDH activity ranging between 1.20% and 44.19% of the total *aceKΔ* parent strain activity (Table 3). All mutations lowered IDH activity enough to result in growth on acetate via redirected flow of isocitrate through IL and MS. Since the PCR products of the mutants were all near the expected 1,685bp, a sample could

be chosen and sent off for sequencing. Mutant *MTM2* was chosen because it had <25% IDH activity relative to the parent but grew without additional glutamate (Table 2).

Direct sequencing of *icd* in mutant *MTM2* revealed a SNP at a critical location upstream of the coding sequence in the 5' UTR. Since the C249T mutation was located in the 5' UTR, it does not affect the coding region. It was hypothesized that the decreased IDH activity is a result of decreased transcription leading to lower protein levels and not due to decreased functionality of the IDH proteins produced. However, the mutation is not contained within the only known activator of *icd*, called Cra¹ or the dual regulator region, but is actually downstream (Prost et al., 1999). *MTM2* is also downstream of the two known transcriptional start sites, *icdAp1* and *icdAp2*, and, therefore, most likely does not influence σ^{70} binding at these locations (Prost et al., 1999). These observations may be evidence of an unknown promoter region, repressor region, or transcriptional start site at approximately -84bp upstream of the start codon of *icd*.

Western blot analysis proved that the IDH protein levels of *MTM2* were decreased and is consistent with an inhibitory effect on transcript production as hypothesized. The IDH proteins produced in *MTM2* may be fully functional, but the decrease in IDH activity observed is simply a result of lower protein levels relative to the parental control strain (Figure 2.)

Additional analysis of the mutant and controls could reveal more information as to how the mutation is causing decreased protein activity. qRT-PCR could be utilized to prove the hypothesized inhibition of transcription rates in *MTM2* by running mRNA transcripts of *icd* Δ , *aceKA*, and *MTM2* and comparing their relative mRNA levels. Secondary experiments could be run to determine the functionality and dimerization of the IDH proteins produced by *MTM2*. This would officially rule out decreased protein function as a possible cause of the decreased IDH activity observed. IDH morphology and dimerization could be determined via x-ray crystallography. If *MTM2* showed reduced IDH transcript levels, an electrophoretic mobility shift assay (EMSA) completed with the wild-type strain may reveal a binding site for a transcriptional regulatory protein at the location of the *MTM2* SNP. A follow up DNA footprinting experiment would further reveal the exact location of protein binding on the DNA.

Class wide, there were several types of mutations that affected IDH activity in different ways. Mutations in the coding sequence either directly affected vital residues of the active sites or disturbed hydrophobic interactions by drastically altering the molecule's shape. The mutations that resulted in changes from hydrophobic to hydrophilic residues, or vice versa, were most easily visualized utilizing the Shell 3D Visualization Center to address possible conformational changes. Mutations in the 5' UTR, such as *MTM2*, were suspected of altering transcript production. Interestingly, mutations discovered in the clamp region of IDH monomers were thought to decrease dimerization rates.

Genomic Sequence Analysis

Whole genome sequencing of the 19 acetate⁺ *aceKA* mutant strains from previous years revealed mutations in two distinct categories including mutations in or near *icd* as well as *sthA*. The two genes are vital to the metabolic pathway required for growth on acetate in *E. coli*. While mutations in *icd* directly affected IDH activity, mutations in *sthA* indirectly affected IDH

activity. *sthA* codes for soluble pyridine nucleotide transhydrogenase and oxidizes NADPH to form NADH. Disturbances in the ratio between NADPH and NADH alter the normal regulation of the TCA cycle and decrease IDH activity.

Class wide trends in mutations regarding the *icd* gene primarily included mutations in the coding region of *icd* and their encoded amino acids were located at the center of the active site near the Mg²⁺ that is utilized for stabilization. Other mutations occurred in areas that were upstream of the start codon such as *MTM2*. Some mutations were suspected of interrupting dimerization and there was even a mutation in the 3' untranslated region.

Mutations were identified in strains with various levels of IDH activity including strains with full activity. Strain UW12 contained a G to A nucleotide exchange that resulted in an alanine to arginine change. The sample depth was 190 and the allelic depth was 1,189. The UW8 strain with the mutation G955A in the *sthA* gene sequence contained a dramatic exchange of the hydrophobic residue alanine with the hydrophilic residue threonine. This most likely conferred a morphological alteration of the *sthA* protein inhibiting its function. Further analysis of this strain via x-ray crystallography would allow for any changes in morphology to be noted. Western blot analysis would provide information on protein levels and may give insight into its stability.

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