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Short Communication

The aldehyde dehydrogenase, AldA, is essential for L-1,2-propanediol utilization in laboratory-evolved *Escherichia coli*



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ABSTRACT

Most *Escherichia coli* strains are naturally unable to grow on 1,2-propanediol (PDO) as a sole carbon source. Recently, however, a K-12 descendent *E. coli* strain was evolved to grow on 1,2-PDO, and it was hypothesized that this evolved ability was dependent on the aldehyde dehydrogenase, AldA, which is highly conserved among members of the family Enterobacteriaceae. To test this hypothesis, we first performed computational model simulation, which confirmed the essentiality of the *aldA* gene for 1,2-PDO utilization by the evolved PDO-degrading *E. coli*. Next, we deleted the *aldA* gene from the evolved strain, and this deletion was sufficient to abolish the evolved phenotype. On re-introducing the gene on a plasmid, the evolved phenotype was restored. These findings provide experimental evidence for the computationally predicted role of AldA in 1,2-PDO utilization, and represent a good example of *E. coli* robustness, demonstrated by the bacterial deployment of a generalist enzyme (here AldA) in multiple pathways to survive carbon starvation and to grow on a non-native substrate when no native carbon source is available.

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1. Introduction

Advances in sequencing technology and accumulation of genomic information accelerated the discovery and exploitation of novel pathways in medically and industrially important microorganisms. Nevertheless, even 20 years after the first microbial genome was sequenced (Fleischmann et al., 1995), the encoded functions of over 40% of sequenced microbial genes remain undetermined (Aziz et al., 2012). Even more intriguingly, novel functions are still being discovered for genes with previously established roles, as revealed by systematic gene deletion investigations (e.g., Baba et al., 2006) and adaptive laboratory evolution (ALE) experiments (Conrad et al., 2011).

As an example, one experimentally well-studied pathway that is still being explored on the genomic level is the propanediol utilization pathway. 1,2-propanediol (PDO; PubChem ID: 1030, CAS# 57-55-6), also known as propylene glycol, is a water-miscible organic solvent commonly used in industrial and medical applications (O'Neil, 2006). In industry, 1,2-PDO is a substrate in polymer production and an approved food additive (E# 1520) (Niu and Guo, 2015). In medicine, it is used as an antiseptic, a vehicle for intravenous and dermatological formulations, as well as a hygroscopic agent in respiratory inhalants (Bennett and San, 2001). Moreover, there is mounting evidence that 1,2-PDO plays a key role in the metabolic activities of human gut microbiota (Pacheco et al., 2012; Staib and Fuchs, 2014).

Theoretically, *E. coli* has the potential to catabolize 1,2-PDO through one of two pathways. The first involves the propanediol-utilization (PDU) locus, which only some strains possess (Monk et al., 2013). The second has been hypothesized to involve the aldehyde dehydrogenase, *aldA*. Several research groups have suggested a key role for this gene since AldA is the only enzyme in the *E. coli* genome annotated to oxidize the intermediate product L-lactaldehyde to L-lactate (Hacking et al., 1978; Chen et al., 1987; Zhang et al., 2006). No definitive experimental proof, however, has

Abbreviations: 1,2-PDO, 1,2-propanediol; ALE, Adaptive laboratory evolution; K12PDO, Evolved *E. coli* K-12 capable of growth on L-1,2-PDO.

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been provided to support this hypothesis, in large part because this AldA-associated pathway is not active in wild-type *E. coli* isolates, which naturally fail to utilize 1,2-PDO as a carbon source.

A recent study reported the use of ALE to generate a strain of *E. coli* K-12 MG1655 that can grow in defined media with L-1,2-PDO as the sole carbon source and in the absence of the PDU locus (Lee and Palsson, 2010); yet, the biochemical basis of the evolved mechanism was not fully deciphered. Here, we used this laboratory-evolved strain to test and confirm the hypothesis that *aldA* does indeed play a key role in 1,2-PDO utilization through computational modeling and experimentation

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two *Escherichia coli* K-12 MG1655 strains were used in this study: the wild type and a descendant that had been evolved to utilize 1,2-PDO as the primary carbon source (Lee and Palsson, 2010). Bacteria were maintained on Luria Bertani (LB) broth or plates. Carbon source and growth curve experiments were performed in M9 minimal media (Fong et al., 2013) supplemented with glucose (Sigma, St. Louis, MO) M9-glucose, or with L-1,2-propanediol (Sigma, St. Louis, MO), M9-PDO. In M9-glucose, glucose was added at 2 g/L concentrations, whereas L-1,2-PDO was added to M9-PDO at 1, 2, 4, or 8 g/L according to the experimental design. Ampicillin, kanamycin, and chloramphenicol (Sigma, St. Louis, MO) were added for selection of transformed strains to final concentrations of 100, 50, and 25 µg/mL, respectively.

Cultures were routinely tested for purity on MacConkey or Eosin-Methylene Blue agar plates.

2.2. Plasmids

pKD46, pKD13, pCP20 were used for precise gene deletion by the Lambda Red system according to Baba and coworkers' modification (Baba et al., 2006) of the original protocol described by Datsenko and Warner (Datsenko and Wanner, 2000). pASK1988 was used for gene complementation exactly as previously described (Fong et al., 2013).

2.3. Computational genomic screening and pathway analysis

Genomic screening for AldA homologs and paralogs was performed on the SEED genome analysis servers (Aziz et al., 2012). Specifically, the "Compare Region" tool in the SEED server was used to find major homologs of the *E. coli* AldA-encoding gene; then all confirmed orthologs of the gene (defined as bidirectional best hits) were downloaded from the SEED database. Absence of other *aldA* copies in *E. coli* and absence of the gene was investigated by the "Browse Subsystems" feature in the SEED database (Aziz et al., 2012). Further sequence analysis and confirmation of gene presence/absence were performed by BlastP and tBLastN (Altschul et al., 1997).

The COBRApy toolbox (Ebrahim et al., 2013) and Escher visualization tool (King et al., 2015) were used for pathway investigation of the role of AldA in PDO utilization.

2.4. Construction of deletion mutants and genetic complementation

The Lambda Red system, originally developed by Datsenko and Warren (Datsenko and Wanner, 2000), was used for precise gene deletion according to the modified protocol used in creating the Keio collection (Baba et al., 2006). Primers used for construction of the knockout strain and confirmation of *aldA* deletion have been

described in our recent work (Aziz et al., 2015b). Genetic complementation with *aldA*-carrying pASK plasmid was used exactly as described (Fong et al., 2013).

2.5. Analysis of *aldA* transcription by real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

RT-PCR experiments and measurements were carried out in a CFX96 C1000 Touch instrument running CFX Manager software version 3.0.1224.1015 (Bio-Rad Hercules, CA). All experiments were in accordance with the MIQE guidelines (Bustin et al., 2009; Bustin et al., 2013).

RNA was extracted by the RNeasy kit (Qiagen, Valencia, CA) from three biological replicates of each strain grown to mid-log phase. RNA concentration and quality were checked by a NanoDrop instrument (Thermo Fisher, Waltham, MA).

SuperScript III reverse transcriptase was used for first strand cDNA synthesis, in presence of 10 mM dNTPs, random primers, DTT, 1 st strand buffer, the RNase inhibitor, Superase-In, and DEPC-treated water, all purchased from Life Technologies (Carlsbad, CA). Thermocycler conditions for annealing of random primers were 70 °C for 10 min; 25 °C for 10 min; 4 °C hold. The mixture was subsequently added in its entirety to reagents for first strand synthesis, for which the thermocycler conditions were: 25 °C for 10 min; 37 °C for 60 min; 42 °C for 60 min; 70 °C for 10 min; 4 °C hold. Next, 20 µL of a 1 N NaOH solution was added to the mixture and incubated at 65 °C for 30 min, after which 20 µL of a 1 N HCl solution was added to neutralize. The cDNA was then purified with the Qiagen PCR purification kit. The quantity and purity were again measured in Nanodrop and Qubit instruments (Thermo Fisher, Waltham, MA), and the integrity was measured with a Bioanalyzer (Agilent Technologies, Santa Clara, CA).

The housekeeping genes used for normalization were *ihfB*, *hcaT*, and *idnT* (Zhou et al., 2011). Primer sequences and product lengths for these three genes, plus that for *aldA*, are provided in Table 1.

The RT-PCR cycling conditions were: 98 °C for 6 min followed by 65 cycles of 98 °C for 15 s, 55 °C for 15 s. The reagents were 1 µL each of the forward and reverse primers in which the stock concentrations were 1 µM, 5 µL of SsoAdvanced SYBR green mix (Bio-Rad, Hercules, CA), 10 ng of cDNA, and water up to 10 µL final volume.

A similar mixture was used to determine the efficiency for each primer set, but genomic DNA (gDNA) replaced the cDNA and a series of five serial dilutions were used. The highest concentration of gDNA was 10 ng, and each of the dilutions differed in concentration by 1/10. An additional well containing no gDNA was also added to the melt curve analysis.

The threshold cycle (C_t) for each sample was calculated using the single threshold, baseline subtracted curve fit algorithm supplied with the CFX Manager software. C_t values for no template control (NTC) wells were routinely 35 and higher, whereas for sample wells, these values ranged between 10 and 20. Normalization of the *aldA* transcript levels to the housekeeping genes was also calculated with the same software.

2.6. Phenotypic screens and growth experiments

Phenotypic screens were performed on LB, M9, M9-glucose, or M9-PDO. All growth experiments were performed in replicates (3–6 technical replicates and at least two biological replicates) in 100-well plates that were loaded into the Bioscreen C instrument (Oy Growth Curves Ab Ltd, Finland) and measured automatically every 20 min at 37 °C for up to 48 h, after 10-s shaking prior to each measurement. Readings were copied to Microsoft Excel, and the growth rate was calculated as the slope of the logarithmic best-fit line during exponential growth.

Table 1
Primer sequences used for RT-PCR assays.

Target gene	Primer name	Primer sequence	Product length
<i>aldA</i>	aldA-RT-FaldA-RT-R	5'-GAACGTGCACAACCAGAATG-3' 5'-CCTGGACGATCGCTTTGAATA-3'	239
<i>ihfB</i>	ihfB-RT-F ihfB-RT-R	5'-GATAGAAAGACTTGCCACCCA-3' 5'-CCAGTTCTACTTTATCGCCAG-3'	200
<i>hcaT</i>	hcaT-RT-FhcaT-RT-R	5'-CGTTGCCGTGGTTGATAGT-3' 5'-CGTGCCAGATATTGATACA-3'	201
<i>idnT</i>	idnT-RT-FidnT-RT-R	5'-CCCTCTTCTGGAACAGTATT-3' 5'-TCGATGGTGCCTCCATTAC-3'	200

3. Results

3.1. *aldA* is highly conserved in *E. coli* and enterobacteria

A survey of *aldA* orthologs in bacterial genomes showed the high conservation of this gene in *E. coli* strains as well as in other enterobacteria. Overall, 1742 putative orthologs of *E. coli aldA* have been detected across different bacterial genomes, including some of the closest *E. coli* phylogenetic relatives such as *Shigella*, *Citrobacter*, *Klebsiella*, and *Enterobacter*. AldA (accession # NP_415933.1) is highly conserved among enterobacteria, and the amino acid sequence identity among enterobacterial AldA homologs ranges from 71 to 100% (with tBlastNE-values $<10^{-100}$). Curiously, no AldA ortholog was found in any *Salmonella* species, which are among *E. coli*'s closest phylogenetic neighbors (confirmed by tBlastN (Altschul et al., 1997) search for any unannotated or pseudogenes within all available *Salmonella* genomes). Alternatively, the *S. enterica* protein with highest similarity to AldA was found to be NADP-dependent succinate-semialdehyde dehydrogenase (accession # WP_001176519.1), a paralog of AldA that is conserved among enterobacteria, including *E. coli* (Accession # WP_000772831.1).

3.2. Computational pathway simulation suggests a role for AldA in L-1,2-PDO utilization

To test the possibility that AldA plays a role in the evolved ability of *E. coli* to grow on 1,2-PDO (Lee and Palsson, 2010), we applied the most recent genome-scale models for *E. coli* (Monk et al., 2013) to computationally investigate whether the *aldA* gene product would be essential when the laboratory-evolved *E. coli* K12PDO strain is simulated to grow in M9 medium with L-1,2-PDO as the sole carbon source. Model simulations suggested that it is indeed essential in this background. The mechanism for lethality according to the simulations is shown schematically in Fig. 1. Specifically, this strain is posited to metabolize L-1,2-PDO by first converting it to lactaldehyde. Next, the AldA enzyme converts lactaldehyde to L-lactate, after which L-lactate is converted to pyruvate by LldD and enters central metabolism. The *aldA* gene product is predicted to be essential because, without it, an infinite amount of lactaldehyde would accumulate, an infeasible model solution that corresponds to cell death.

3.3. Evolved K12 (K12PDO) growth on L-1,2-PDO is dose-dependent

We next sought to confirm these computational predictions experimentally, and started by testing whether the evolved L-1,2-PDO strain grows in L-1,2-PDO in a concentration-dependent manner. To this end, we grew the K12PDO strain on increasing concentrations of L-1,2-PDO (1, 2, 4, and 8 g/L). Both the rate and extent of bacterial growth increased with increasing L-1,2-PDO concentration in a linear dose-response manner (Fig. 2): the growth rate positively correlated with L-1,2-PDO concentration (Pearson

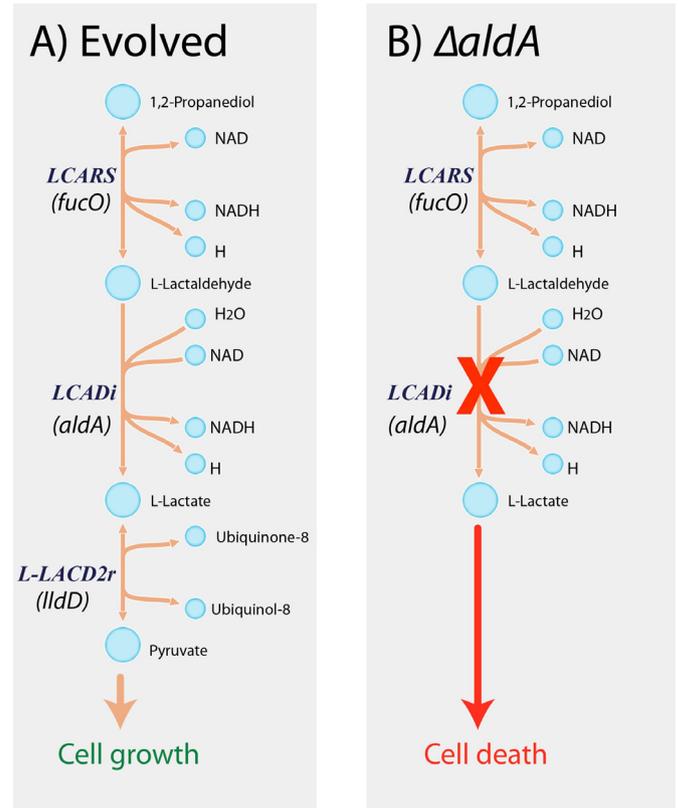


Fig. 1. Model-based hypothesis about the role of AldA in L-1,2-PDO utilization. In the evolved strain (A), L-1,2-PDO is oxidized to L-lactaldehyde, which can be further converted into pyruvate allowing cell growth. The hypothesis is that deleting *aldA* (B) will lead to L-lactaldehyde accumulation, which will halt cell growth.

$r=0.990$, $R^2=0.979$); and so did the biomass (Pearson $r=0.999$, $R^2=0.997$).

3.4. Loss of function analysis: *aldA* deletion abolishes the ability of the evolved K12PDO to grow on L-1,2-PDO

Using the Lambda Red strategy (Datsenko and Wanner, 2000; Baba et al., 2006), we precisely deleted *aldA* from the evolved K12PDO strain and confirmed the gene deletion with polymerase chain reaction (PCR). The resulting knockout strain (K12PDO Δ *aldA*) readily grew on rich media as well as minimal media supplied with glucose (M9-glucose); however, unlike the parent K12PDO strain, the Δ *aldA* isogenic mutant failed to grow on M9 media supplemented with 1,2-PDO as a sole carbon source (Fig. 3). To confirm that *aldA* loss has not affected the natural growth of *E. coli* in any other aspects, we deleted *aldA* in the unevolved K-12 strain. The generated K12 Δ *aldA* knockout was able to grow on LB, M9-glucose, but—as expected—did not grow in M9-PDO.

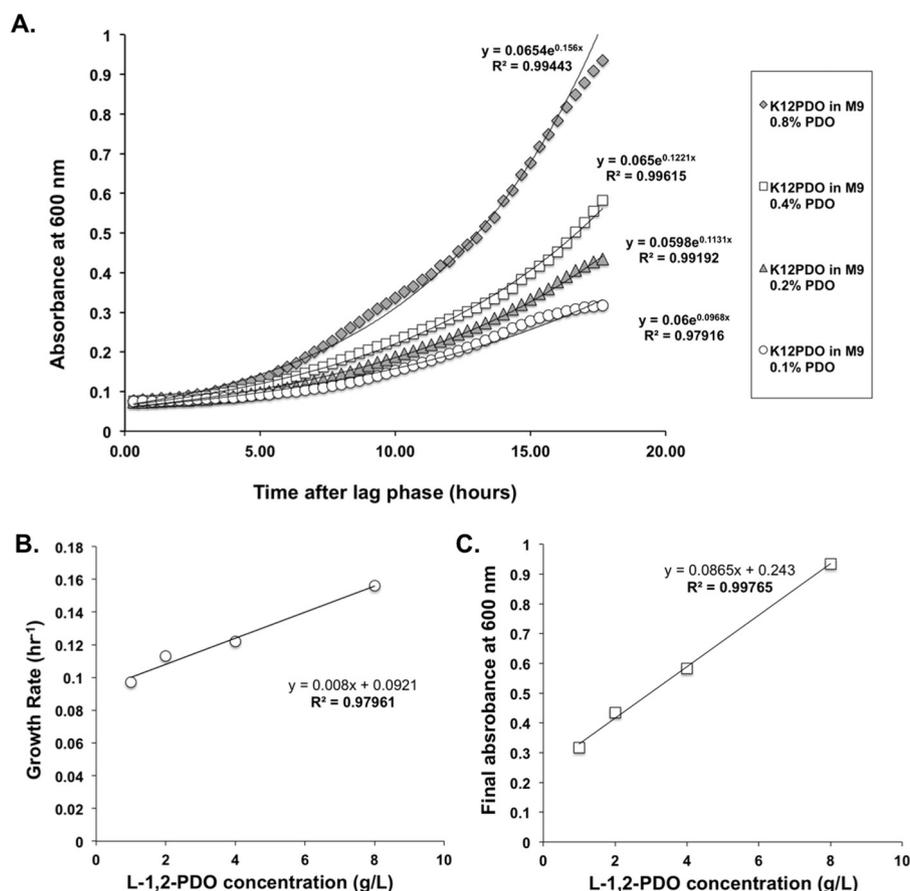


Fig. 2. Substrate concentration-dependent growth of K12PDO at different L-1,2-PDO concentrations.

L-1,2-PDO was added to the minimal M9 media at increasing concentrations (0.1, 0.2, 0.4, 0.8% w/v). A. Growth curves of K12PDO in M9 supplemented with different L-1,2-PDO concentrations. The curves represent OD600 values recorded every 20 min right after lag phase. Best-fitted natural logarithmic slopes are computed and shown next to each growth curve indicating the growth rate. B-C. Growth rates (B) and final biomass, expressed as final OD600 values (C) at each of the four L-1,2-PDO concentrations are positively correlated with the substrate concentration, with slopes of 0.008 and 0.08, respectively.

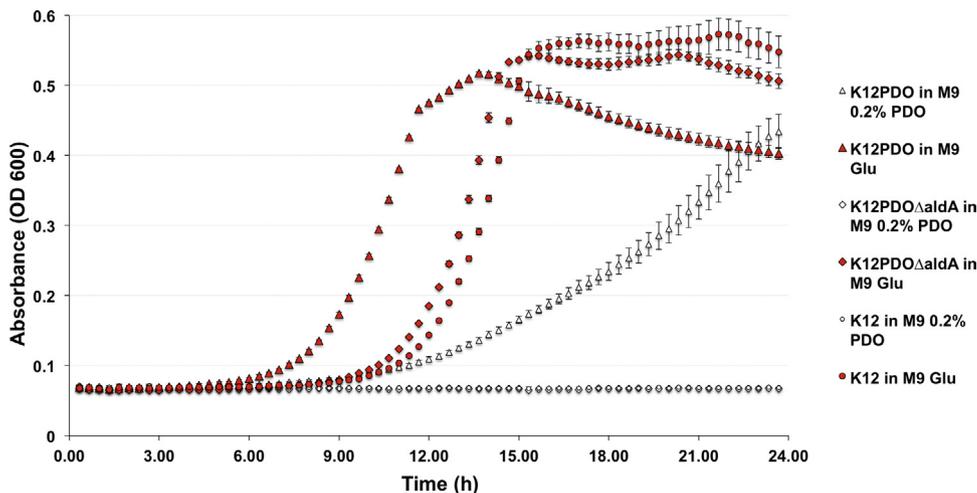


Fig. 3. Loss of function analysis of *aldA*.

Growth curves for K12, K12PDO, and K12PDOΔ*aldA* in M9 supplemented with 0.2% glucose (M9 Glu, filled symbols) or 0.2% L-1,2-PDO (M9 0.2% PDO, open symbols) indicating that K12PDO can only grow on L-1,2-PDO as a sole carbon source when the *aldA* gene is intact, while both the wild-type and *aldA*-knockout strains readily grow on M9-glucose. Each data point represents the mean of four replicates; error bars represent the standard error of the means.

3.5. Complementing the K12PDOΔ*aldA* strain with a plasmid-borne copy of *aldA* restores its ability to grow on L-1,2-PDO

To confirm that the failure of K12PDOΔ*aldA* to grow on L-1,2-PDO is due to the absence of *aldA* and not to a secondary mutation

generated during storage, subculture or genetic manipulation, we reintroduced a plasmid-borne copy of *aldA* into the knockout strain. The resulting K12PDOΔ*aldA*:pASK-*aldA* strain was able to grow on M9 supplemented with L-1,2-PDO (at 2 g/L) as a sole carbon source.

Bacteria transformed with plasmid-borne genes sometimes fail to express the genes or express them at very low levels, since

some cis-regulatory elements may be needed for full gene expression. To verify that *aldA* was expressed in the complemented strain, we tested for the presence of the gene's transcript using reverse transcriptase PCR. We also performed the same analysis in the evolved strain, which hasn't been previously tested for *aldA* expression. The evolved K12PDO and the complemented K12PDO Δ *aldA*:pASK-*aldA* successfully transcribed *aldA*, unlike the knockout strain K12PDO Δ *aldA*, in which no *aldA* transcript could be detected.

4. Discussion

The hypothesis about the possible involvement of an aldehyde dehydrogenase in L-1,2-PDO utilization by *E. coli* dates back to the pre-genomic era (Hacking et al., 1978). Genetic and biochemical studies based on *hfr* mapping (Chen et al., 1987) and enzymatic activity analysis (Hacking et al., 1978) suggested *aldA* as one possible key gene in spontaneous or laboratory-evolved *E. coli* mutant strains that grew on L-1,2-PDO. Additionally, AldA and its orthologs have been manipulated or exploited in biotechnology for their involvement in the propanediol biosynthesis (but not utilization) pathways in *E. coli*, *Klebsiella*, and *Citrobacter* (Zhang et al., 2006; Clomburg and Gonzalez, 2011; Lawrence et al., 2014). In spite of these rather old hypotheses, no direct evidence has been presented on the role of AldA in the L-1,2-PDO utilization pathway.

Here, we provide direct genetic evidence for this long-hypothesized function, showing that *aldA* is both essential and sufficient for the L-1,2-PDO utilization phenotype in the laboratory-evolved *E. coli* K12PDO.

AldA has been known for its broad spectrum as a glycoaldehyde dehydrogenase that oxidizes α -hydroxyaldehyde in several pathways involved in the utilization of monosaccharides such as L-fucose, L-rhamnose, D-arabinose, and L-xylose (Baldoma and Aguilar, 1987; Badia et al., 1991; Quintilla et al., 1991). It has been also classified as a generalist enzyme in a systems-level analysis of *E. coli* enzyme specificity (Nam et al., 2012). We recently showed that its involvement in the folate biosynthesis pathway is dispensable and complemented by another *E. coli* enzyme, PrpC (Aziz et al., 2015b). In the current study, the broad-spectrum of AldA is further emphasized, as it was confirmed to play an essential role in the utilization of L-1,2-PDO by *E. coli* when no other carbon source is present. This finding assigns to this enzyme one more pathway than previously expected (Baldoma and Aguilar, 1987).

More generally, this outcome represents a good example of the robustness of *E. coli* metabolism, demonstrated by the bacterial deployment of a possibly broad-spectrum enzyme (here, AldA) in multiple pathways to survive carbon starvation and to grow on a non-native substrate when no native carbon source is available. Moonlighting and generalist enzymes often play extra roles, notably when the primary enzymes that catalyze a specific reaction damaged or mutated. Nam, Lewis, and coworkers showed that generalist enzymes have been evolutionarily preserved even when specific alternatives are available (Nam et al., 2012). We believe that these generalists may not only serve as a "backup" when specialists are lost, damaged, or inhibited, but may also act as a "reserve" for adaptive functions, when the organism is exposed to an unusual stress, such as being forced to survive on a nonnative substrate. The availability of genes encoding such enzymes with dual/multiple functions or with broad-spectrum is likely to increase the fitness and robustness of a versatile organism, such as *E. coli*, and may explain why genome-scale models often fail to predict gene essentiality or synthetic lethality (Aziz et al., 2015a; Guzmán et al., 2015).

In conclusion, using genome-scale metabolic models and simulation experiments, we hypothesized that the gene encoding AldA plays a key role in *E. coli*'s adaptively acquired utilization of

nonnative L-1,2-PDO, and we used (i) loss of function, (ii) complementation, and (iii) gene expression analyses to prove the hypothesis. Future studies may exploit this finding in strain engineering to improve L-1,2-PDO utilization via modulating AldA activity or improving its efficiency by site-specific mutagenesis. Additionally, given the apparent role of L-1,2-PDO and its precursors, fucose and rhamnose, in bacterial-host sensing and bacterial-bacterial communication in the human gut (Pacheco et al., 2012), the experimentally validated role of AldA in PDO utilization may provide a foundation for modulation of this enzyme and pathway and, in turn, manipulation of the complex sensing and signaling pathway between resident gut microbiota and invading outsiders.

Authors' contributions

RKA and PC: Conceived and designed the study.

JMM: Performed computational pathway analysis.

RKA, JMM, KA, JN, VK and HW: Performed the experiments.

BOP and PC: Provided strains or reagents.

RKA, JMM, and PC: Drafted the paper.

All authors contributed to and approved the manuscript in its final format.

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