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Publication Date

2014-09-01

DOI

10.1016/j.ymben.2014.07.012

Peer reviewed

HHS Public Access

Author manuscript

Metab Eng. Author manuscript; available in PMC 2015 September 01.

Published in final edited form as:

Metab Eng. 2014 September ; 25: 227–237. doi:10.1016/j.ymben.2014.07.012.

Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli*

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Abstract

Advances in synthetic biology and metabolic engineering have enabled the construction of novel biological routes to valuable chemicals using suitable microbial hosts. Aldehydes serve as chemical feedstocks in the synthesis of rubbers, plastics, and other larger molecules. Microbial production of alkanes is dependent on the formation of a fatty aldehyde intermediate which is converted to an alkane by an aldehyde deformylating oxygenase (ADO). However, microbial hosts such as *Escherichia coli* are plagued by many highly active endogenous aldehyde reductases (ALRs) that convert aldehydes to alcohols, which greatly complicates strain engineering for aldehyde and alkane production. It has been shown that the endogenous ALR activity outcompetes the ADO enzyme for fatty aldehyde substrate. The large degree of ALR redundancy coupled with an incomplete database of ALRs represents a significant obstacle in engineering *E. coli* for either aldehyde or alkane production. In this study, we identified 44 ALR candidates encoded in the *E. coli* genome using bioinformatics tools, and undertook a comprehensive screening by measuring the ability of these enzymes to produce isobutanol. From the pool of 44 candidates, we found five new ALRs using this screening method (YahK, DkgA, GldA, YbbO, and YghA). Combined deletions of all 13 known ALRs resulted in a 90–99% reduction in endogenous ALR activity for a wide range of aldehyde substrates (C2–C12). Elucidation of the ALRs found in *E. coli* could guide one in reducing competing alcohol formation during alkane or aldehyde production.

Keywords

Biofuel; Synthetic biology; Metabolic engineering; Aldehyde; Alkane

1. Introduction

Concerns regarding the future availability of petroleum and its adverse environmental effects have generated interest in developing alternative sources for fuels and chemicals. For these reasons, the biological production of fuels and chemicals is viewed as a promising method for curbing reliance on petroleum. Advances in synthetic biology and metabolic engineering have enabled the construction of novel biological routes to valuable chemicals by rationally combining genes from various natural sources into a suitable microbial host

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(Rabinovitch-Deere et al., 2013; Peralta-Yahya et al., 2012; Geddes et al., 2011). Metabolic engineering approaches then allow tuning of the metabolism by eliminating or reducing competing pathways in the host to achieve high carbon flux toward the desired end product while also maintaining cell fitness (Rabinovitch-Deere et al., 2013; Peralta-Yahya et al., 2012; Geddes et al., 2011). The marriage of these techniques has enabled the biological production of a multitude of fuels and chemicals in user-friendly hosts such as *Escherichia coli* (Rabinovitch-Deere et al., 2013; Peralta-Yahya et al., 2012; Geddes et al., 2011; Zhang et al., 2012).

Aldehydes serve as chemical feedstocks in the synthesis of rubbers, plastics, and other larger molecules. Aldehydes such as butanal and isobutyraldehyde are high volume chemicals, each chemically produced from petroleum substrates in quantities greater than 1 million ton per year (Raff, 2013). Producing these compounds using microbes would be a renewable alternative to current methods. However, microbial hosts such as *E. coli* are plagued by many highly active endogenous aldehyde reductases (ALR) that convert aldehydes to alcohols (Rodriguez and Atsumi, 2012; Zhu et al., 2011), which greatly complicates strain engineering for aldehyde production. As a result, *E. coli* production of only acetaldehyde (Zhu et al., 2011) and isobutyraldehyde (Rodriguez and Atsumi, 2012) has been shown. In contrast, alcohol and organic acid production spanning C2–C18 has been demonstrated in *E. coli* and other microbes (Rabinovitch-Deere et al., 2013; Zhang et al., 2012; Atsumi et al., 2008; Dellomonaco et al., 2011; Schirmer et al., 2010; Ingram et al., 1998; Cintolesi et al., 2014; Liu et al., 2014).

The presence of ALRs also impairs microbial production of alkanes, which has been a prominent area of interest with the discovery of the aldehyde deformylating oxygenase (ADO) (Schirmer et al., 2010; Li et al., 2012; Warui et al., 2011). Biological alkane production has been shown in the range from C3 to C20 alkanes (Schirmer et al., 2010; Khara et al., 2013; Harger et al., 2013; Choi and Lee, 2013; Akhtar et al., 2013; Howard et al., 2013; Andre et al., 2013), but overall titers and productivities remain lower than those of the corresponding alcohols. This biological pathway is dependent on the formation of a fatty aldehyde intermediate, which can be converted to an alkane or an alcohol by ADO or ALR, respectively (Schirmer et al., 2010; Li et al., 2012). It has been shown that the endogenous ALR activity outcompetes the ADO enzyme for fatty aldehyde substrate (Schirmer et al., 2010; Howard et al., 2013). This large degree of ALR redundancy coupled with an incomplete database of ALRs (Rodriguez and Atsumi, 2012) represents a significant obstacle in engineering *E. coli* for either aldehyde and alkane production. Thus, a comprehensive elucidation and characterization of ALRs would facilitate targeted reduction of endogenous ALR activity for the production of specific aldehydes or alkanes.

In a previous study, we engineered a strain of *E. coli* (AL626) capable of producing isobutyraldehyde at titers up to 35 g L^{-1} (Rodriguez and Atsumi, 2012). This was accomplished by deleting six ALR genes (*yqhD*, *adhP*, *eutG*, *yiaY*, *yjgB*, and *fucO*) from the genome. However, this strain still had a notable amount of isobutanol formation (~10 g L^{-1}) indicating that AL626 still contains enzymes with isobutyraldehyde reductase activity (Rodriguez and Atsumi, 2012). Owing to the high aldehyde to alcohol ratio exhibited by *E. coli* strain AL626, this strain can serve as an effective tool for *in vivo* screening of ALR

activity by overexpressing ALR gene candidates in the presence of aldehyde and measuring the resulting aldehyde and/or alcohol formation.

In this study, we sought to complete four major objectives. First, using our previously engineered isobutyraldehyde producing strain, AL626, we aimed to undertake a comprehensive screening of candidate ALRs encoded in the *E. coli* genome. Second, any positive hits for isobutyraldehyde reductase activity should be confirmed *in vitro* along with the acquisition of a broad substrate profile (C2–C10) of the elucidated ALRs. Third, we aimed to delete any ALR gene(s) we uncover from AL626, creating a strain with the potential to have complete or near abolishment of endogenous ALR activity. Lastly, we sought to characterize this strain for its ability (or inability) to convert a wide range of aldehydes (C2–C12) to their corresponding alcohols as compared to a strain without ALR deletions. The comprehensive elucidation of the ALRs in the *E. coli* genome could guide one in reducing competing alcohol formation pathways and achieve improved alkane and aldehyde production from *E. coli*.

2. Methods

2.1. Reagents

All enzymes were purchased from New England Biolabs (Ipswich, MA). All synthetic oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). DNA sequencing services were done by Davis Sequencing (Davis, CA). All chemicals for gas chromatography (GC) standards except for ethanol and 1-pentanol were purchased from Sigma-Aldrich (St. Louis, MO). 1-Pentanol was purchased from Acros Organics (Belgium). Ethanol (200 Proof) was purchased from VWR (Radnor, PA, Canada).

2.2. Plasmid and strain construction

All plasmids were constructed using sequence and ligation-independent cloning (SLIC) (Machado et al., 2012). Plasmids were verified by PCR, by digestion with restriction enzymes, and by sequencing. All oligonucleotides and plasmids are listed in Tables 1 and 2, respectively. To construct the plasmids listed in Table 2, the target gene(s) were amplified from the *E. coli* genome DNA with corresponding primers in Table 1 and pSA138 (Atsumi et al., 2008) vector was amplified with primers GR258 and GR259. The resulting fragments were purified and combined with SLIC (Machado et al., 2012; Rodriguez et al., 2014).

2.3. Aldehyde reductase activity assay

The strains were grown to OD_{600} of ~0.4 in 5 mL Luria Broth (LB) medium at 37 °C, followed by addition of 1 mM isopropyl-β-D-thio-galactoside (IPTG). Protein overexpression was performed at 30 °C for 2 h. Then 1.8 mL of cells were centrifuged at 17,000*g* for 10 min, resuspended in 300 μL BugBuster Protein Extraction Reagent (Novagen, San Diego, CA, USA), and incubated at room temperature for 20 min for cell lysis. The samples were centrifuged 16,000*g* for 20 min at 4 °C. Supernatants were taken for enzyme assays. ADH activities were measured by following the reduction of aldehyde with NADH or NADPH at 340 nm at 37 °C using a Synergy H1 Hybrid Plate Reader (BioTek Instruments, Inc., Winooski, VT). The enzyme assay was performed in a 96-well format as a

200 μL reaction. The reaction mixture was made up of 86 μL of H_2O, 10 μL of 1 M MOPS (pH 7.0), 4 μL of 10 mM NAD(P)H in 10 mM Tris–HCl (pH 7.0), 50 μL of 100 mM aldehyde, and 50 μL diluted enzyme (10 μL cell lysate in 40 μL phosphate buffer (pH 7.5)). One unit of activity is defined as the oxidation of 1 μmol of NAD(P)H per minute per mg protein. Protein concentrations were measured using Advanced Protein Assay Reagent (Cytoskeleton Inc., Denver, CO). Bovine Serum Albumin (NEB) was used to prepare a standard curve.

2.4. Culture conditions

Overnight cultures were grown in 5 mL LB containing appropriate antibiotics. Antibiotic concentrations were as follows: kanamycin (50 μg/mL), chloramphenicol (40 μg/mL), ampicillin (250 μg/ mL), and tetracycline (20 μg/mL). Production was carried out with modified M9 medium as used in previous studies (Rodriguez and Atsumi, 2012; Atsumi et al., 2008; Rodriguez et al., 2014) (hereby referred to as M9P medium): 33.7 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 g L^{-1} yeast extract, 50 g L^{-1} glucose, and 1000-fold dilution of A5 trace metal mix (2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 49.4 mg $Co(NO_3)_2 \cdot 6H_2O$ per liter water). Optical densities (OD) were measured at 600 nm with a Plate Reader (BioTek Instruments, Inc., Winooski, VT).

2.5. Isobutyraldehyde production for ALR candidate screening

Overnight cultures were inoculated 1% in 5 mL M9P in 15 mL screw-cap culture tubes. Cells were grown to an OD_{600} of ~ 0.4 at 37°C in a rotary shaker (250 rpm), followed by addition of 1 mM IPTG. Production was performed at 37 °C in a rotary shaker (250 rpm) for 24 h. Screw-cap tubes were tightly sealed to prevent evaporation of products. 1.5 mL of culture was taken for analysis every 24 h. 1.5 mL of the cultures was centrifuged at 17,000*g* for 3 min; then 1 mL of the supernatants was transferred to 2 mL GC vials for GC analysis.

2.6. Bioinformatics and literature search for candidate ALRs

To acquire the list of ALR candidate screened in this study, we first searched the *E. coli* genome database EcoCyc (Keseler et al., 2013) for genes annotated as oxidoreductase, reductase, or dehydrogenase which also bind NAD(P)H or FAD. Literature availability on any of these genes, especially regarding the enzyme activity, was used to determine likely and unlike candidates. For example, the methyl-glyoxal reductase class of enzymes (DkgA (Ko et al., 2005; Habrych et al., 2002; Yum et al., 1999; Jeudy et al., 2006), DkgB (Ko et al., 2005; Di Luccio et al., 2006), YeaE (Ko et al., 2005), YdjG (Di Luccio et al., 2006), YghZ (Ko et al., 2005; Totir et al., 2012; Grant et al., 2003)) are known to reduce methylglyoxal, but have not been characterized with primary aliphatic aldehydes. Overexpression of *ucpA* is known to increase furan tolerance in *E. coli* and potentially act on a range of other substrates (Wang et al., 2012). The glyoxylate reductases GhrA and GhrB reduce the beta-keto group to yield a beta-hydroxy acid (Nunez et al., 2001). Though unlikely to act on primary aldehydes, these two were selected for completion.

Most of the NAD(P)H-binding oxidoreductases in *E. coli* have not been characterized in the literature. In this case, protein BLAST searching against non-*Escherichia* taxids was used to

obtain information regarding conserved domains, family of the enzyme, and substrate preference of any homolog(s) characterized in other organisms (Supplementary Table S1). Specifically, BLAST searches that resulted in similarity (>80% sequence identity) to the short chain dehydrogenase family and alcohol dehydrogenase family were considered strong candidates (YahK, YgfF, YbbO, YohF, RspB, YbdR, YcjQ, YdjJ, YdjL, YphC, UcpA, YciK, YgcW, and YghA) (Supplementary Table S1). BLAST searches that identified enzymes as aldo/keto reductase or other families were also screened (YdbC, YdhF, Tas, YajO, YjhC, YkgE, GldA, Tdh, YbiC, YggP, YjjN, HdhA, and FrmA). In some cases, BLAST searching yielded little information about the enzyme, only identifying the candidate as NAD(P)H-binding oxidoreductases (YceM, YqiB, YhiN, YhhX, YhdH, YcjS, YgjR, and YdgJ). Such candidates were also included for the sake of completion.

The known ALRs (YqhD, AdhP, EutG, YiaY, YjgB, FucO, and BetA) were also blasted against the *E. coli* genome (Supplementary Table S1). Oxidoreductases that yielded an Expect value score less than ~E–20 (corresponding to a score= ~90 bits) were also screened, if not already selected for screening. This score threshold was chosen because it closely matched protein family and cofactor usage targeted in this study, and thus helped to narrow down the pool of candidates to test.

2.7. Deletion of all proven aldehyde reductases in E. coli

All gene deletions were carried out by the method developed by Datsenko and Wanner (2000). The deleted fragments were verified by PCR and sequencing. The method was repeated to delete all candidate genes. After each deletion, we have verified the deletions of the target genes and other previously deleted genes by PCR and sequencing.

All strains used in this study are listed in Table 2. During construction of AL1728, we found that AL626 and AL1728 had likely acquired multiple copies of the *yahK* gene. These genes were verifiably deleted from the target sites, but detected by PCR to still be present in another location. This rendered it difficult to construct a strain with a complete set of both the ALR gene deletions and those for competing pathways from AL626. This could have come about from the multiple iterations of P1 transduction (Thomason et al., 2007) used to create AL626 and its parent JCL260.

2.8. Ketoacid substrate feeding experiments

Overnight cultures were inoculated 1% in 5 mL M9P in 15 mL screw-cap culture tubes. Cells were grown to an OD_{600} of ~0.4 at 37°C in a rotary shaker (250 rpm), followed by addition of 1 mM IPTG. The cultures were incubated for 1 h after induction at 30 °C. Then metabolites of interest were added to the cultures. Production was performed at 30 °C in a rotary shaker (250 rpm) for 24 h. Screw-cap tubes were tightly sealed to prevent evaporation of products.

2.9. C10 and C12 aldehyde feeding experiments

Overnight cultures were inoculated 1% in 20 mL M9P in shake flasks. Cells were grown to an OD_{600} of ~0.4 at 37 °C in a rotary shaker (250 rpm). Then 10 or 15 mL of culture was added to a 250 mL screw cap flask, and 500 mg L^{-1} of C10 or C12 aldehyde was added to

the cultures. In the case of nonane bilayer experiments, 2 mL of nonane was also added along with aldehyde. Cultures were incubates at 30 °C in a rotary shaker (250 rpm) for 24 or 48 h. Screw-cap tubes were tightly sealed to prevent evaporation of products.

2.10. Gas chromatography sample preparation

For ALR candidate screening and keto acid feeding, 1.5 mL of the cultures were centrifuged at 17,000*g* for 3 min; then 1 mL of the supernatants was transferred to 2 mL vials for gas chromatography (GC) analysis.

For straight chain aldehyde feeding (C10 and C12), an equal volume of ethyl acetate (Sigma) as culture was added to extract products. These samples were mixed for 10 min in a rotary shaker (250 rpm) at 30 °C. Then each sample was centrifuged at 2750*g* for 5 min, and 1 mL of the ethyl acetate layer was transferred into a vial GC analysis. For nonane bilayer experiments, the entire culture and nonane layer was centrifuged at 2750*g* for 5 min. Then1 mL of both the nonane layer and culture layer was taken for analysis.

2.11. GC analysis

Concentrations of all products were analyzed by GC equipped with a flame ionization detector (FID). The GC system is a Shimadzu GC-2010 with an AOC-20 S autosampler and AOC-20i Auto Injector. The column used was a DB-Wax capillary column (30 m length, 0.32-mm diameter, 0.50-μm film thickness) from Agilent Technologies. GC oven temperature was initially held at 40 °C for 3 min, then increased at a rate of 45 °C min until 230 °C and held for 4 min. Injector temperature was held at 225 °C and an FID detector was held at 330 °C. Injection volume was 0.5 μL, injected at a 15:1 split ratio. Helium was used as the carrier gas. Retention times from samples were compared with external standards. Standard curves were prepared by diluting pure aldehyde or alcohol into water at concentrations of 0.01, 0.1, and 1 g L^{-1}. 100 mg L^{-1} of 1-pentanol was added to all samples and external standards as an internal standard.

In the case of C10 and C12 aldehydes and alcohols, the column used was a DB-FAPP capillary column (30 m length, 0.32-mm diameter, 0.50-μm film thickness) from Agilent Technologies. GC oven temperature was initially held at 40 °C for 3 min, then increased at a rate of 45 °C min until 250 °C and held for 10 min. Injector temperature was held at 225 °C and the FID detector was held at 330 °C. Injection volume was 1 μL, injected at a 15:1 split ratio. Helium was used as the carrier gas. Retention times from samples were compared with external standards. Standard curves were prepared by diluting purely the chemical into ethyl acetate at concentrations of 0.01, 0.1, and 1 g L^{-1}. 100 mg L^{-1} of dodecane was added to samples and external standards as an internal standard.

3. Results and discussion

3.1. Screening aldehyde reductase candidates in E. coli

Due to the high aldehyde to alcohol ratio exhibited by *E. coli* strain AL626 (Rodriguez and Atsumi, 2012) (Table 2), this strain can serve as an effective tool for screening ALR activity by overexpressing candidate ALRs and measuring the resulting alcohol formation. An

initial, larger pool of candidates (~70) was selected based on two initial requirements: Annotation of domains with 1. NAD(P)H/FAD dependency and 2. oxidoreductase activity. The initial pool of genes was further condensed by a BLAST (Altschul et al., 1990) analysis of those with high similarity to dehydrogenase-type enzymes (more details are in Section 2.6). This yielded 44 candidate ALRs genes encoded in the *E. coli* genome. We then undertook a comprehensive *in vivo* screening of the 44 candidate ALRs by measuring the ability of these enzymes to produce isobutanol from *E. coli* strain AL626 also expressing the isobutyraldehyde pathway (*alsS* (*Bacillus subtilis*), *ilvCD* (*E. coli*), *kivd* (*Lactococcus lactis*)) (Rodriguez and Atsumi, 2012) (Fig. 1a).

The candidate ALR genes were cloned onto a high copy plasmid (~50 copies per cell) downstream of *kivd* (De la Plaza et al., 2004) (Table 2). The well-known ALR, YqhD (Rodriguez and Atsumi, 2012; Sulzenbacher et al., 2004; Perez et al., 2008; Atsumi et al., 2010; Miller et al., 2009; Jarboe, 2011; Lee et al., 2010), was used as a positive control while a plasmid with only *kivd* was used as a negative control. The isobutyraldehyde and isobutanol formation was then measured from each of the 44 strains (Fig. 1b).

Five of the 44 enzymes tested (~10%) produced high amounts (>0.5 g L^{-1} OD_{600}^{-1}) of isobutanol: YahK, YbbO, GldA, DkgA, and YghA (Fig. 1b). During the course of this study, the YahK enzyme was also shown by Pick et al., with extensive kinetic data, to have activity for a multitude of substrates (Pick et al., 2013). Interestingly, the *dkgA* (also called *yqhE*) gene is coded directly downstream of the well-known ALR gene, *yqhD*. DkgA is known to act on methylglyoxal, glyceraldehyde, valeraldehyde, benzaldehdye, and 2,5-diketo-D-gluconate (Ko et al., 2005; Jeudy et al., 2006). *dkgA* and *yqhD* are transcribed from the same promoter (Turner et al., 2011), but *dkgA* may also have its own promoter as well (Lee et al., 2010). Several studies using YqhD for microbial production of chemicals have been published (Atsumi et al., 2010; Jarboe, 2011; Tang et al., 2009; Clomburg and Gonzalez, 2011; Lan and Liao, 2012; Atsumi et al., 2009), yet DkgA has seen few applications. Of the other ALR enzymes identified in our study, GldA is known to act on 1,2-propanedial in addition to glycerol (Gonzalez et al., 2008). Little is known about the two enzymes, YbbO and YghA, besides that they are both annotated as oxidoreductases that have a Rossmann-fold domain (Keseler et al., 2013). The sequence similarity of these five ALRs is quite different from that of other known ALRs in *E. coli* (YqhD, AdhP, EutG, YiaY, YjgB, and FucO) (Rodriguez and Atsumi, 2012; Dellomonaco et al., 2011).

3.2. Activity profile of aldehyde reductases

With five positive hits for *in vivo* aldehyde reductase activity, we further confirmed this activity *in vitro* (Fig. 2). We tested the five ALRs for activity toward a spectrum of C2–C10 aldehydes: acetaldehyde, isobutyraldehyde, hexanal, octanal, and decanal. Enzymes were also tested for cofactor specificity (NADH and NADPH), and all five appear to be strictly NADPH dependent with the substrates tested.

Both this work and the previously published work (Pick et al., 2013) confirm that YahK is strictly NAPDH dependent and has broad ALR activity (Fig. 2a). While acetaldehyde, isobutyraldehyde, and hexanal are known substrates for YahK (Pick et al., 2013), this study

adds octanal and decanal to the list of accepted substrates (Fig. 2a). YahK showed activity with all substrates tested, including activity toward acetaldehyde and octanal (~0.5 μg/min/mg protein), and highest activity with isobutyraldehyde (1.2 μg/min/mg protein).

DkgA showed activity toward the medium chain substrates isobutyraldehyde and hexanal (~0.95 μg/min/mg protein) and less with octanal (0.23 μg/min/mg protein) (Fig. 2b). No activity with acetaldehyde or decanal was detected. Since DkgA has no acetaldehyde activity, it may serve as a good candidate for medium-chain alcohol production.

The other three enzymes GldA, YbbO, and YghA showed about 10-fold lower activity for any of the tested substrates than DkgA and YahK. GldA has been shown to use NAD^+ for dehydrogenation of glycerol or 1,2-propanediol (Tang et al., 1979), but we did not detect any NADH dependent reductase activity with the substrates tested. GldA only had significant activity with isobutyraldehyde (0.12 μg/ min/mg protein), perhaps due to similar size compared to glycerol (Fig. 2c). YbbO showed broad substrate activity toward all the C2–C10 aldehydes with the highest activity toward hexanal and octanal (0.13 μg/min/mg protein) (Fig. 2d). YghA, on the other hand, showed activity toward isobutyraldehyde and decanal (0.05 μg/min/mg protein) and no activity toward hexanal and octanal (Fig. 2e).

3.3. Reduction in short and medium chain aldehyde reductase activity in E. coli

Now that the five candidates were shown to have ALR activity *in vivo* and *in vitro*, we were interested in the phenotype of an *E. coli* strain lacking all known ALRs. We deleted these five ALR genes from the *E. coli* AL626. All 13 targeted ALR genes (*adhE*, *yqhD*, *adhP*, *eutG*, *yiaY*, *yjgB*, *fucO*, *betA*, *yahK*, *dkgA* (*yqhE*), *ybbO*, *gldA*, and *yghA*) were deleted from the isobutanol production strain JCL260 (parent strain of AL626, Table 2), in which all major competing pathways have been deleted, creating AL1728. AL1728 showed ~60% reduction in isobutanol formation after 24 h as compared to AL626 (Fig. 3).

Next, we tested the ability of this strain to convert a wide range of aldehydes to alcohols, and compared it to JCL260. It has been shown that 2-ketoacids from branched chain amino acid biosynthesis can be decarboxylated by a ketoacid decarboxylase (Kdc) to form corresponding aldehydes (Atsumi et al., 2008; De la Plaza et al., 2004) (Fig. 4a). These aldehydes are subsequently converted to alcohols by endogenous ALRs in *E. coli* (Rodriguez and Atsumi, 2012; Zhu et al., 2011; Atsumi et al., 2010). Thus, we first tested the ability of AL1728 to convert 2-ketoacid-derived aldehydes to alcohols as compared to JCL260. We expressed *kivd* in JCL260 and AL1728 (strains AL1550 and AL1827 respectively (Table 2)), and separately fed the cultures 8 g L^{-1} of each ketoacid (2-oxobutyrate, 2-oxovalerate, 2-oxoisovalerate, 3-methyl-2-oxovalerate, 4-methyl-2-oxovalerate, or phenylpyruvate). The growth of AL1827 was similar with that of AL1550. We then measured the resulting aldehyde and alcohol after 24 h of incubation (Fig. 4). With AL1550, the majority of ketoacids were ultimately converted to alcohols by endogenous ALR activity (Fig. 4b) to efficiencies between 89% (2-methyl-1-butanol) and 99% (1-butanol). In contrast, only 2–15% alcohol conversion occurred in AL1827, with the majority of product remaining as aldehydes (Fig. 4c). These data suggest that we have successfully identified and removed nearly all branched chain ALR activity from *E. coli*. That there was

also a decrease in propanal and butanal as straight chain products from AL1827 *versus* AL1550 suggests that the majority of straight chain ALR activity has been removed as well.

3.4. Reduction in acetaldehyde reductase activity in E. coli

Ethanol can be a major byproduct in the biological production of fuels and chemicals. Although AdhE represents a major source of ethanol formation in *E. coli* during fermentation (Membrillo-Hernandez and Lin, 1999), complete elimination of ethanol formation is difficult (Zhu et al., 2011). To test the degree of ethanol formation in AL1827 as compared to AL1550, we fed 8 g L^{-1} pyruvate into the media (Fig. 5). Pyruvate is decarboxylated to acetaldehyde which can then be converted to ethanol by endogenous ALRs (Fig. 5a). AL1550 generated 842 mg L^{-1} of ethanol after 24 h, while AL1827 generated only 1.3 mg L^{-1} ethanol (Fig. 5b). This represents 99.8% less ethanol produced than AL1550, implying that we have achieved near abolishment of acetaldehyde reductase activity in *E. coli*. Thus, the 13 ALRs (or a combination there of) deleted in AL1827 likely encompass the vast majority of acetaldehyde reductase activity in *E. coli*. It is also important to note that additional ALR activity could still exist, but may not have been expressed by the genome under the conditions used in this study.

3.5. Reduced long chain aldehyde reductase activity in E. coli

Since *E. coli* strain AL1827 showed almost no ALR activity toward short or medium chain substrates after 24 h, we hypothesized it might also show reduced long-chain aldehyde reductase activity.

Thus, we tested AL1827 for its ability to convert long chain aldehydes (C10 and C12) to alcohols as compared to AL1550 (Fig. 6). We fed 500 mg L^{-1} of each aldehyde and observed the concentration of aldehyde and alcohol after 24 h (Fig. 6b and c). AL1550 was able to convert roughly 90% of C10 and C12 aldehyde after 24 h, while AL1827 showed 90:10 ratio of C10 aldehyde:alcohol and about 74:26 ratio of C12 aldehyde:alcohol. This low long chain alcohol phenotype is highly desirable for alkane production and may be useful for *in vivo* screening of ADO mutants since aldehydes remain in the culture with low alcohol conversion.

We observed a ~90% decrease of aldehyde presence in AL1827 cultures during the course of 24 h (Fig. 6c). This trend can also be seen in an 8 h time course with 500 mg L^{-1} C10 aldehyde and either with AL1550 or AL1827 (Fig. 6d and e). However, while aldehyde decreased in AL1827 cultures, little corresponding alcohol was detected. Another possibility is that the aldehyde was converted to an acid, but only a small amount of C10 or C12 acid was observed. Thus, the destination of C10 and C12 aldehydes in AL1827 cultures is unknown. Whatever the final product, its conversion is likely a less benign product than alcohols, since we also observed toxicity with both C10 and C12 aldehydes in AL1827 cultures.

When engineering *E. coli* for the production of toxic products, bilayer systems have been previously incorporated to extract the toxic products *in situ* (Rodriguez et al., 2014; Connor et al., 2010). Thus, to reduce the unknown fate of the long chain aldehydes as well as the

toxicity, we incorporated a nonane bilayer with C10 aldehyde substrate and measured conversion after 48 h (Fig. 7). AL1550 still displayed a high conversion rate of C10 aldehyde to alcohol, whereas AL1827 still showed little alcohol formation. In addition, the nonane layer protected the aldehyde from uptake or unknown degradation in AL1827, which retained 490 mg L^{-1} C10 aldehyde after 48 h. As demonstrated here and in previous studies, both bilayer systems (Rodriguez et al., 2014; Connor et al., 2010) as well as gas stripping systems (Rodriguez and Atsumi, 2012; Zhu et al., 2011) can be applied successfully to alleviate the toxic effects of aldehydes and other products in *E. coli*.

4. Conclusion

In this study, we make available the information necessary to reduce short, medium, and long chain alcohol formation which could lead to increased productivities of aldehydes and alkanes from an engineered *E. coli*. We elucidated five additional aldehyde reductases coded on the *E. coli* genome, bringing the total amount of ALRs to 13 (AdhE, YqhD, AdhP, EutG, YiaY, YjgB, BetA, FucO, YahK, DkgA, YbbO, YghA, and GldA). We also demonstrated that an *E. coli* strain lacking these genes leads to near abolishment of endogenous ALR activity. Strains with reduced ALR activity such as the ones used in this study can serve as a valuable tool for rapid and convenient *in vivo* screening of ALRs and their substrate profile. While we attempted to remove all 13 aldehyde reductases with proven activity from the *E. coli*'s genome for the sake of completion, such an exhaustive effort would be unnecessary for engineering useful production strains. Targeting certain ALRs for their specific endogenous expression, activity, and substrate profile could reduce the necessary gene deletions to 3–5 genes.

For future efforts in alkane production from *E. coli*, the activity of ADO remains a limiting factor. Several studies have been conducted to improve alkane production, yet overall productivities remain low (Schirmer et al., 2010; Khara et al., 2013; Harger et al., 2013; Choi and Lee 2013; Akhtar et al., 2013; Howard et al., 2013; Andre et al., 2013). Better enzymes need to be engineered through either rational or directed evolution approaches, as well as improving the recharge rate of the diiron enzyme system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by University of California-Davis startup fund and the Hellman Fellowship to S.A. G.M.R. was supported by a US National Institutes of Health Biotechnology Training Grant Fellowship (T32-GM008799) and a Sloan Fellowship. We thank Eric Walters and Yohei Tashiro for experimental assistance.

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Appendix A. Supporting information

Supplementary data associated with this paper can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.07.012.

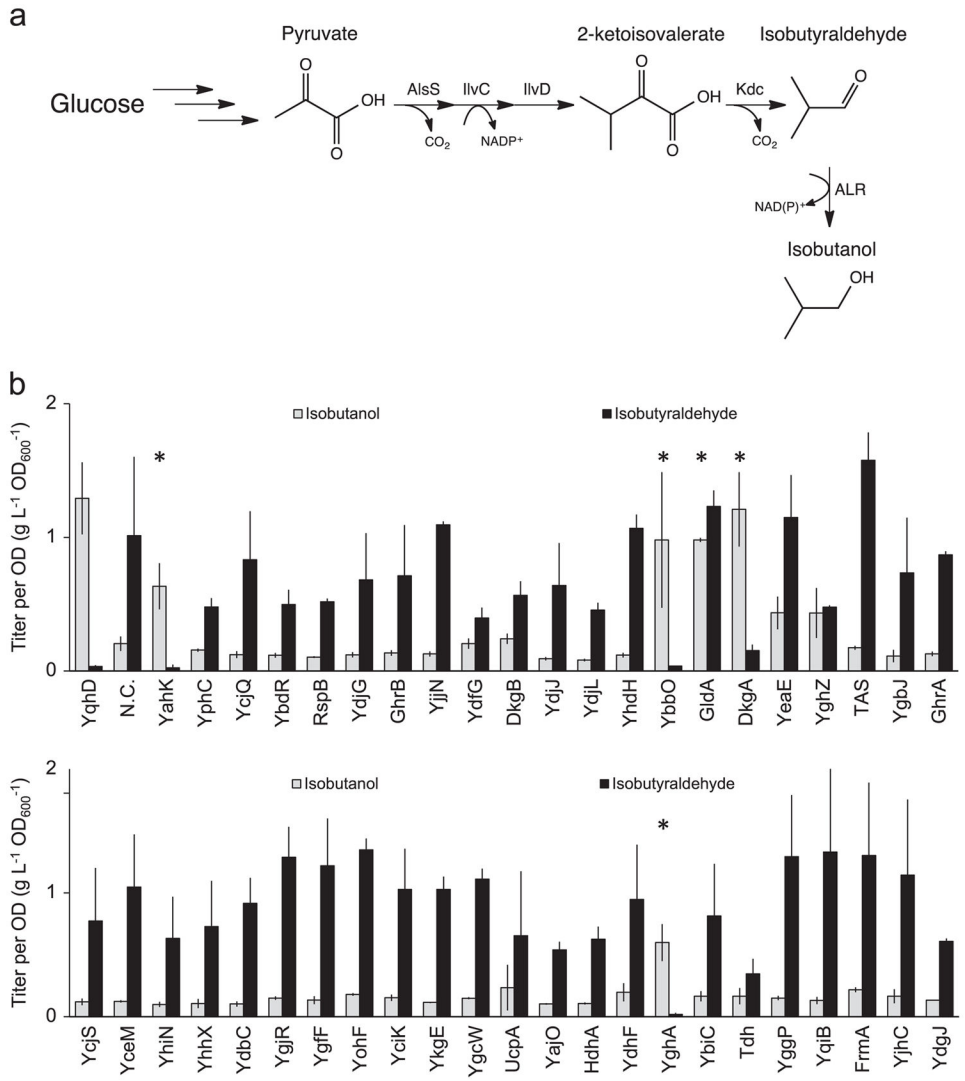

Fig. 1.

In vivo screening for ALR activity by isobutanol production. (a) The isobutanol pathway (AlsS, IlvCD, Kdc, and ALR). (b) Screening of 44 candidate ALRs from *E. coli* by measuring isobutanol production in strain AL626 which has greatly reduced ALR activity. Strain AL626 also expressing YqhD was used as a positive control and AL626 without any ALR was used as a negative control (N.C). Asterisks (*) indicates high ALR activity. Strains were grown in M9P media, then induced at OD_{600} ~ 0.4 with 1 mM IPTG and allowed to produce for 24 h at 37 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

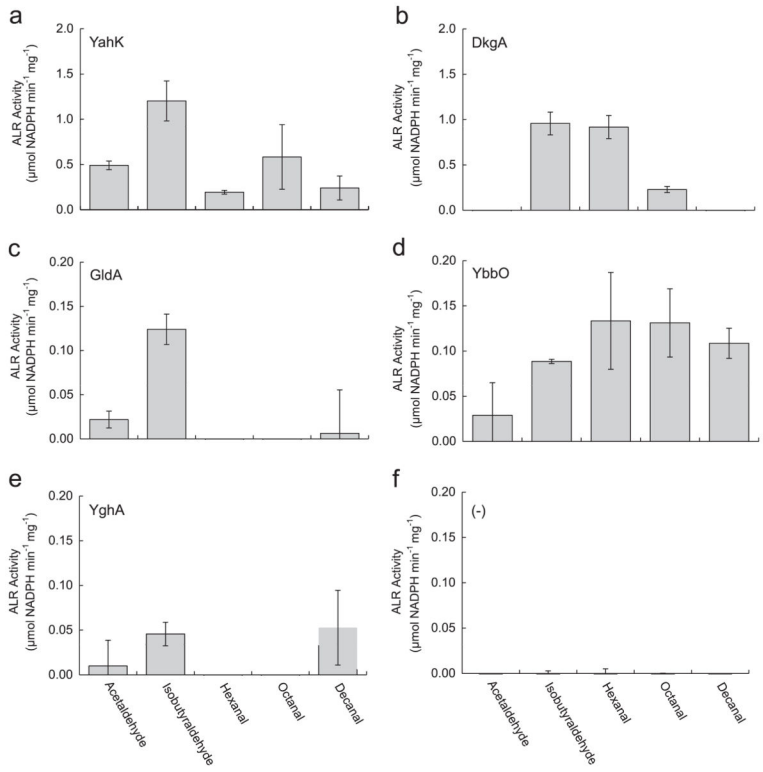

Fig. 2.

In vitro substrate profile of ALRs. ALR enzyme activity from *E. coli* AL626 cell lysates expressing each ALR: (a) YahK, (b) DkgA, (c) GldA, (d) YbbO, (e) YghA, and (f) no ALR. One unit of activity is defined as the oxidation of 1 μM NADPH per minute per mg protein. Error bars indicate SD (*n*=3).

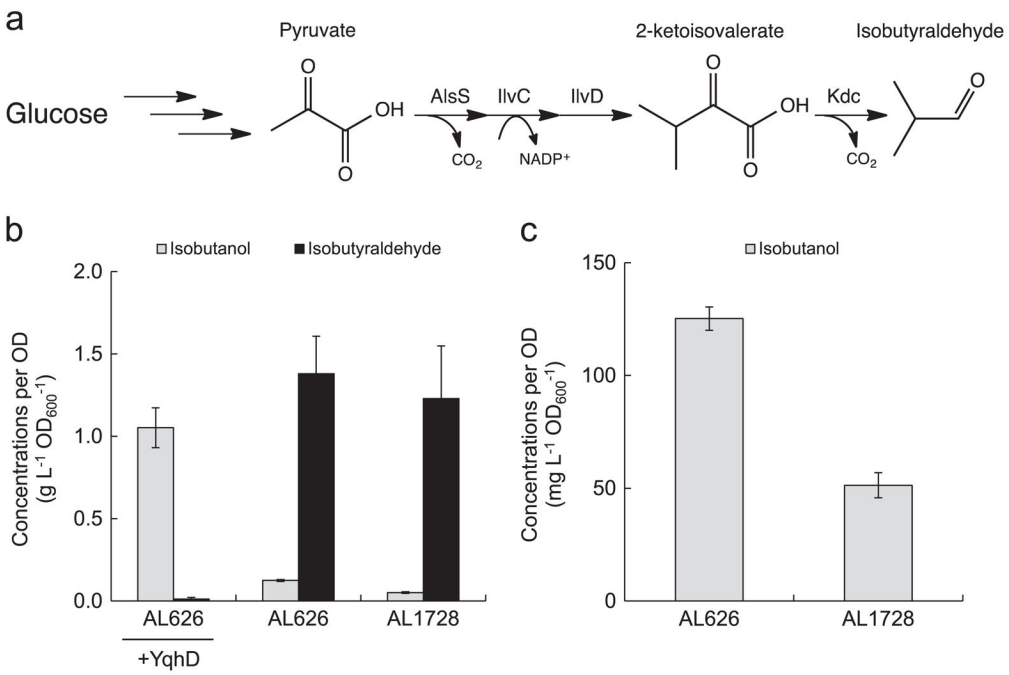

Fig. 3.

Comparison of isobutyraldehyde production from strain AL626 and AL1728. (a) Isobutyraldehyde pathway. (b) Isobutanol and isobutyraldehyde production from AL626 and AL1728 expressing the isobutyraldehyde pathway. AL626 also expressing *yqhD* was used as a positive control. (c) Isobutanol formation from AL626 and AL1728 (from (b)). Strains were grown in M9P media, then induced at OD_{600} ~ 0.4 with 1 mM IPTG and allowed to produce for 24 h at 37 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

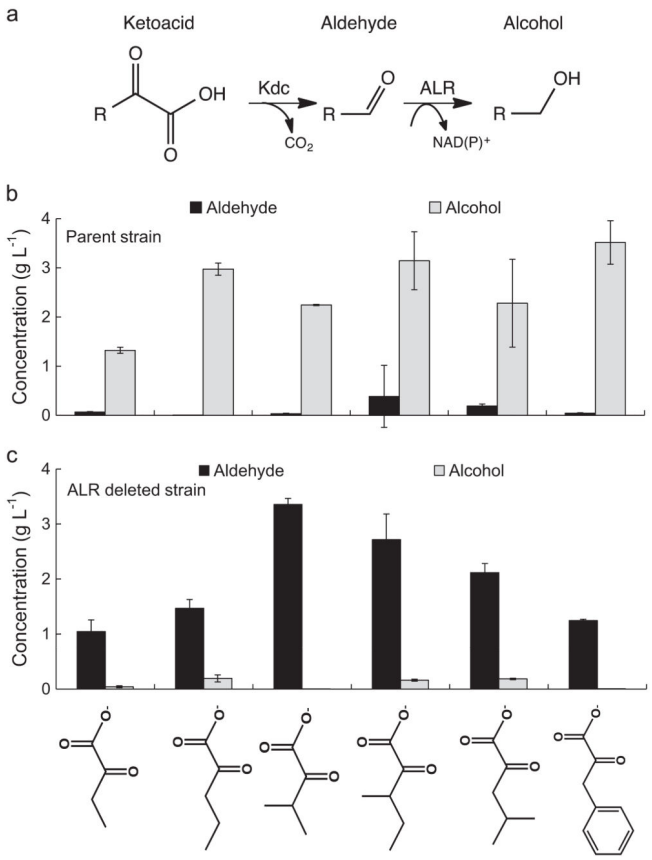

Fig. 4.

Comparison of ketoacid-based aldehyde and alcohol formation from the parent strain and ALR deleted strain. (a) Ketoacid based alcohol formation by Kdc and ALR. (b, c) Measurement of individual alcohol formation from ketoacids (2-ketobutyrate, 2-ketovalerate, 2-ketoisovalerate, 2-keto-3-methylvalerate, 2-keto-4-methylvalerate, phenylpyruvate) by AL1550 (b) and AL1827 (c). Strains were grown in M9P, then induced at OD_{600} ~0.4 with 1 mM IPTG and allowed to express for 1 h. After 1 h, 8 g L^{-1} ketoacid was added to the cultures and the strains were allowed to produce for 24 h at 37 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

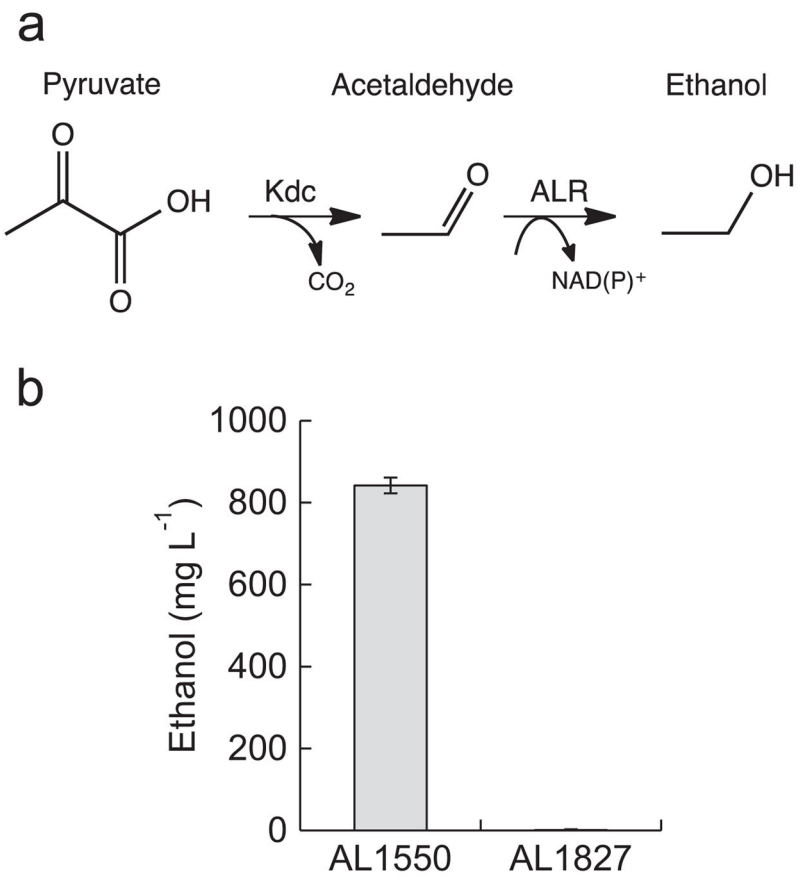

Fig. 5.

Comparison of ethanol formation from strain AL1550 and AL1827 by decarboxylation of pyruvate. (a) Biological route to ethanol from pyruvate. Pyruvate is decarboxylated to acetaldehyde by the ketoacid decarboxylase, Kdc (*L. lactis*), followed by conversion of acetaldehyde to ethanol by endogenous ALR activity. Feeding pyruvate into the media increases flux toward acetaldehyde. (b) Measurement of extracellular ethanol formation from *E. coli* strain AL1550 and AL1827 (Table 2) expressing *kivd* (*L. lactis*) after 24 h with 8 g L^{-1} pyruvate added to the media. Strains were grown in M9P media, then induced at OD_{600} ~ 0.4 with 1 mM IPTG and allowed to express for 1 h. After 1 h, 8 g L^{-1} pyruvate was added to the cultures and the strains were allowed to produce for 24 h at 37 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

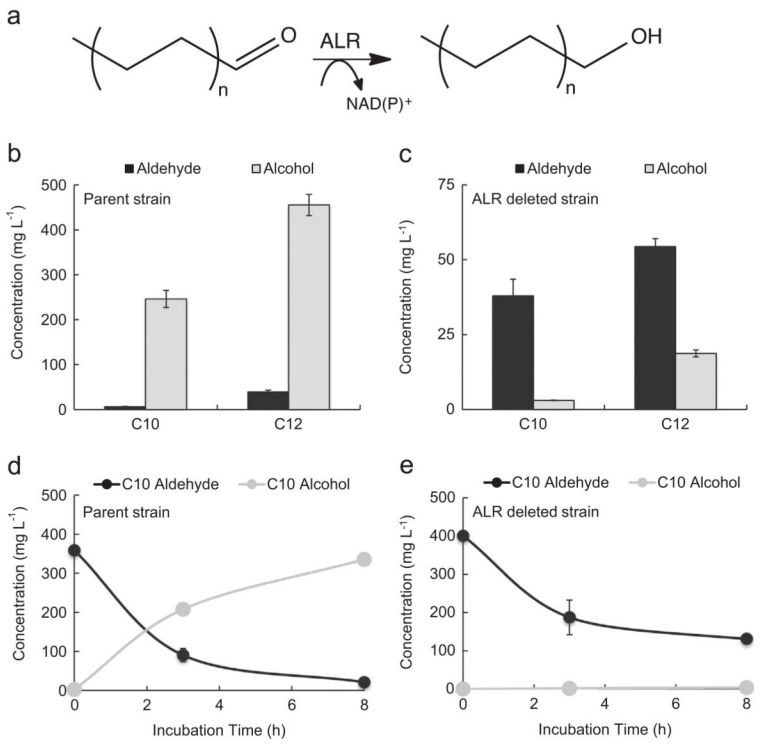

Fig. 6.

Comparison of C10 and C12 alcohol formation from strain AL1550 and AL1827 by aldehyde feeding. (a) Fatty aldehydes are converted to alcohols by endogenous ALRs. (b, c) Measurement of individual C10 and C12 aldehyde conversion to alcohol by *E. coli* strain AL1550 (b) and AL1827 (c) after 24 h with 500 mg L^{-1} of individual aldehydes added to the media. (d, e) Time course of C10 aldehyde conversion to alcohol in AL1550 (d) and AL1827 (e). Strains were grown in 15 mL M9P media. At OD_{600} ~ 0.4, 500 mg L^{-1} aldehyde was added to the cultures and the strains were incubated for 24 h at 30 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

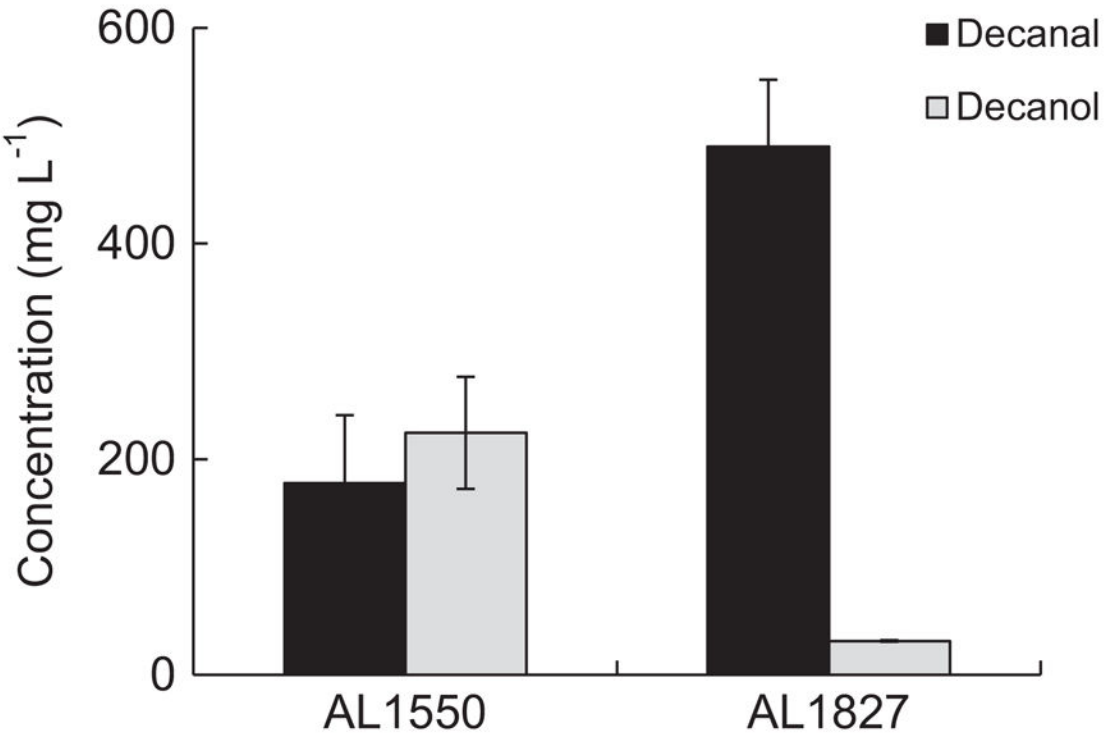

Fig. 7.

Comparison of C10 aldehyde conversion using a nonane bilayer from strain AL1550 and AL1827. Strains were grown in M9P media. At OD_{600} ~ 0.4, 500 mg L^{-1} aldehyde was added to the 10 mL of culture along with 2 mL nonane in a 250 mL screw cap flask, and the strains were incubated for 48 h at 30 °C in a rotary shaker (250 rpm). Error bars indicate SD (*n*=3).

Table 1

Oligonucleotides used in this study.

Name	Sequence 5′–>3′	Description
GR258	TCTAGAGGCATCAAATAAAACGAAA	Forward pSA138 Vector
GR259	GTGACCTTTCTCCTGCATGCTTATG	Reverse pSA138 Vector
GR260	CATAAGCATGCAGGAGAAAGGTCACATGAAGATCAAAGCTGTTGGTGCAT	Forward *yahK*
GR261	TTTCGTTTTATTTGATGCCTCTAGATCAGTCTGTTAGTGTGCGATTATCG	Reverse *yahK*
GR262	CATAAGCATGCAGGAGAAAGGTCACATGAAAACGATGCTGGCAGCTTATT	Forward *yphC*
GR263	TTTCGTTTTATTTGATGCCTCTAGATTAATCCGGGAAGTTAATCACAACT	Reverse *yphC*
GR264	CATAAGCATGCAGGAGAAAGGTCACATGAAAAAGTTAGTAGCCACAGCAC	Forward *ycjQ*
GR265	TTTCGTTTTATTTGATGCCTCTAGATTAAAACGTAACGCCCATTTTGATG	Reverse *ycjQ*
GR266	CATAAGCATGCAGGAGAAAGGTCACATGAAAGCATTGACTTATCACGGCC	Forward *ybdR*
GR267	TTTCGTTTTATTTGATGCCTCTAGATCATATTGTTCCCCCCGGCATCGCA	Reverse *ybdR*
GR268	CATAAGCATGCAGGAGAAAGGTCACATGAAAAGCATATTAATTGAAAAAC	Forward *rspB*
GR269	CTTTCGTTTTATTTGATGCCTCTAGATTATTCAGAAAAAGTGAGTAAGAC	Reverse *rspB*
GR270	CATAAGCATGCAGGAGAAAGGTCACATGAAAAAGATACCTTTAGGCACAA	Forward *ydjG*
GR271	CTTTCGTTTTATTTGATGCCTCTAGATTAACGCTCCAGGGCCTCTGCCAT	Reverse *ydjG*
GR272	CATAAGCATGCAGGAGAAAGGTCACATGAAGCCGTCCGTTATCCTCTACA	Forward *ghrB*
GR273	TTTCGTTTTATTTGATGCCTCTAGATTAGTCCGCGACGTGCGGATTCACA	Reverse *ghrB*
GR307	CATAAGCATGCAGGAGAAAGGTCACATGTCTACGATGAATGTTTTAATTT	Forward *yjjN*
GR308	TTTCGTTTTATTTGATGCCTCTAGATCAGAAAGTAATTACGCCTTTAATT	Reverse *yjjN*
GR309	CATAAGCATGCAGGAGAAAGGTCACATGATCGTTTTAGTAACTGGAGCAA	Forward *ydfG*
GR310	TTTCGTTTTATTTGATGCCTCTAGATTACTGACGGTGGACATTCAGTCCG	Reverse *ydfG*
GR311	CATAAGCATGCAGGAGAAAGGTCACATGGCTATCCCTGCATTTGGTTTAG	Forward *dkgB*
GR312	TTTCGTTTTATTTGATGCCTCTAGATTAATCCCATTCAGGAGCCAGACCT	Reverse *dkgB*
GR313	CATAAGCATGCAGGAGAAAGGTCACATGAAAAATTCAAAAGCAATATTGC	Forward *ydjJ*
GR314	TTTCGTTTTATTTGATGCCTCTAGATTAATCGCTAATTTTAATAACGCCT	Reverse *ydjJ*
GR315	CATAAGCATGCAGGAGAAAGGTCACATGAAAGCACTGGCTCGGTTTGGCA	Forward *ydjL*
GR316	TTTCGTTTTATTTGATGCCTCTAGATTATTCATCAAAGTCGTAAGTCATG	Reverse *ydjL*
GR317	CATAAGCATGCAGGAGAAAGGTCACATGCAGGCGTTACTTTTAGAACAGC	Forward *yhdH*
GR318	TTTCGTTTTATTTGATGCCTCTAGATTAGTTAACCTTCACCAGCGTGCGA	Reverse *yhdH*
GR319	CATAAGCATGCAGGAGAAAGGTCACATGACTCATAAAGCAACGGAGATCC	Forward *ybbO*
GR320	TTTCGTTTTATTTGATGCCTCTAGATCACCCCTGCAATATTTTGTCCATC	Reverse *ybbO*
GR321	CATAAGCATGCAGGAGAAAGGTCACATGGACCGCATTATTCAATCACCGG	Forward *gldA*
GR322	TTTCGTTTTATTTGATGCCTCTAGATTATTCCCACTCTTGCAGGAAACGC	Reverse *gldA*
GR491	GCATGCAGGAGAAAGGTCACATGGCTAATCCAACCGTTATTAAGCTACAGG	Forward *dkgA*
GR492	TTTTATTTGATGCCTCTAGATTAGCCGCCGAACTGGTCAGG	Reverse *dkgA*
GR493	GCATGCAGGAGAAAGGTCACATGCAACAAAAAATGATTCAATTTAGTGGCG	Forward *yeaE*
GR494	TTTTATTTGATGCCTCTAGATCACACCATATCCAGCGCAGTTTTTC	Reverse *yeaE*
GR495	GCATGCAGGAGAAAGGTCACATGGTCTGGTTAGCGAATCCCGAAC	Forward *yghZ*
GR496	TTTTATTTGATGCCTCTAGATCATTTATCGGAAGACGCCTGCC	Reverse *yghZ*
GR497	GCATGCAGGAGAAAGGTCACATGCAATATCACCGTATACCCCACAGTTC	Forward *tas*

Name	Sequence 5′–>3′	Description
GR498	TTTTATTTGATGCCTCTAGATTATGGTGCCGGATAAGTATAAACCTGATGC	Reverse *tas*
GR499	GCATGCAGGAGAAAGGTCACATGAAAACGGGATCTGAGTTTCATGTCG	Forward *ygbJ*
GR500	TTTTATTTGATGCCTCTAGATCATGATTTCGCTCCCGGTAGAGTG	Reverse *ygbJ*
GR501	GCATGCAGGAGAAAGGTCACATGGATATCATCTTTTATCACCCAACGTTCG	Forward *ghrA*
GR502	TTTTATTTGATGCCTCTAGATTAGTAGCCGCGTGCGCG	Reverse *ghrA*
GR511	GCATGCAGGAGAAAGGTCACGTGAAAAGTGCAATGACAAGCTCTCC	Forward *ycjS*
GR512	TTTTATTTGATGCCTCTAGATCATAATTCCACACGCGTCCCTG	Reverse *ycjS*
GR513	GCATGCAGGAGAAAGGTCACGTGAAAAAATTACGTATCGGCGTAGTGG	Forward *yceM*
GR514	TTTTATTTGATGCCTCTAGATTATTCACTCATCGCATCGCGC	Reverse *yceM*
GR515	GCATGCAGGAGAAAGGTCACGTGGAAAGGTTTGATGCCATTATTATAGGC	Forward *yhiN*
GR516	TTTTATTTGATGCCTCTAGATCAGGACGACTTTGCTGCAATC	Reverse *yhiN*
GR517	GCATGCAGGAGAAAGGTCACATGGTCATCAACTGCGCCTTTATTG	Forward *yhhX*
GR518	TTTTATTTGATGCCTCTAGATTACTTAGCGAGAGTTACTGTGGAGGGAG	Reverse *yhhX*
GR519	GCATGCAGGAGAAAGGTCACATGAGCAGCAATACATTTACTCTCGGTAC	Forward *ydbC*
GR520	TTTTATTTGATGCCTCTAGATTATTCTCGCGAAATACCATCCAACGTAG	Reverse *ydbC*
GR521	GCATGCAGGAGAAAGGTCACATGATACGTTTCGCTGTGATTGGTACG	Forward *ygjR*
GR522	TTTTATTTGATGCCTCTAGATTATAGTTTTACGCTATCTGCCGGAAAAATC	Reverse *ygjR*
GR523	GCATGCAGGAGAAAGGTCACATGGCTATAGCACTTGTGACTGGTGG	Forward *ygfF*
GR524	TTTTATTTGATGCCTCTAGATTATTTCCCGCCCGCCAAATC	Reverse *ygfF*
GR525	GCATGCAGGAGAAAGGTCACATGGCACAGGTTGCGATTATTACC	Forward *yohF*
GR526	TTTTATTTGATGCCTCTAGACTATTCTGGGTTGAACTGTGGATTCG	Reverse *yohF*
GR617	GCATGCAGGAGAAAGGTCACATGCATTACCAGCCAAAACAAGATTTAC	Forward *yciK*
GR618	TTTTATTTGATGCCTCTAGATCATTGGGAAATTCCTGGTTTACGG	Reverse *yciK*
GR619	GCATGCAGGAGAAAGGTCACATGAATGTCAATTTCTTTGTCACCTGTATTGG	Forward *ykgE*
GR620	TTTTATTTGATGCCTCTAGATCAGCGGCTCATCAACACTTCAG	Reverse *ykgE*
GR621	GCATGCAGGAGAAAGGTCACATGTCAATCGAATCTCTCAATGCGTTC	Forward *ygcW*
GR622	TTTTATTTGATGCCTCTAGATTAGCGCACTAAATAACCGCCATCAAC	Reverse *ygcW*
GR623	GCATGCAGGAGAAAGGTCACATGGGTAAACTCACGGGCAAGACA	Forward *upcA*
GR624	TTTTATTTGATGCCTCTAGATCAGATACCGACGCTAACCGTCTCC	Reverse *upcA*
GR625	GCATGCAGGAGAAAGGTCACATGCAATACAACCCCTTAGGAAAAACCG	Forward *yajO*
GR626	TTTTATTTGATGCCTCTAGATTATTTAAATCCTACGACAGGATGCGG	Reverse *yajO*
GR627	GCATGCAGGAGAAAGGTCACATGTTTAATTCTGACAACCTGAGACTCGAC	Forward *hdhA*
GR628	TTTTATTTGATGCCTCTAGATTAATTGAGCTCCTGTACCCCACCA	Reverse *hdhA*
GR629	GCATGCAGGAGAAAGGTCACATGGTTCAGCGTATTACTATTGCGCC	Forward *ydhF*
GR630	TTTTATTTGATGCCTCTAGATTACGGTACGTCGTACCCCAGTGC	Reverse *ydhF*
GR631	GCATGCAGGAGAAAGGTCACATGTCTCATTTAAAAGACCCGACCACG	Forward *yghA*
GR632	TTTTATTTGATGCCTCTAGATTAACCTAAATGCTCGCCGCCG	Reverse *yghA*
GR655	GCATGCAGGAGAAAGGTCACATGGAAAGTGGTCATCGCTTTGATG	Forward *ybiC*
GR656	TTTTATTTGATGCCTCTAGATTAGCTGGCTAACTGCTGACAGAAAG	Reverse *ybiC*
GR657	GCATGCAGGAGAAAGGTCACATGAAAGCGTTATCCAAACTGAAAG	Forward *tdh*
GR658	TTTTATTTGATGCCTCTAGATTAATCCCAGCTCAGAATAACTTTCC	Reverse *tdh*
GR659	GCATGCAGGAGAAAGGTCACATGAAAACCAAAGTTGCTGCTATTT	Forward *yggP*

Name	Sequence 5′–>3′	Description
GR660	TTTTATTTGATGCCTCTAGATCATTGCGCGGCCTCCC	Reverse *yggP*
GR661	GCATGCAGGAGAAAGGTCACATGAAGCGTTACACACCTGACTTTC	Forward *yqiB*
GR662	TTTTATTTGATGCCTCTAGACTAATAAACCGGAATCGCCATCGCT	Reverse *yqiB*
GR663	GCATGCAGGAGAAAGGTCACATGAAATCACGTGCTGCCGTTGCAT	Forward *frmA*
GR664	TTTTATTTGATGCCTCTAGATCAGTAACGAATTACGGTTCGAATG	Reverse *frmA*
GR665	GCATGCAGGAGAAAGGTCACATGATTAATTATGGCGTTGTTGGTG	Forward *yjhC*
GR666	TTTTATTTGATGCCTCTAGATTACATTACTGATGTATGTTTAATG	Reverse *yjhC*
GR667	GCATGCAGGAGAAAGGTCACATGAGCGACAACATCCGTGTTGGGT	Forward *ydgJ*
GR668	TTTTATTTGATGCCTCTAGATCATGCAAGGCACAAAGTCG	Reverse *ydgJ*

Table 2

Strains and plasmids used in this study.

***E. coli* strain**	**Genotype**	**Reference**
BW25113	*rrnB*$_{T14}$ Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LC78}$	(Datsenko and Wanner, 2000)
JCL16	BW25113/F′ [*traD* 36, *proAB*$^{+}$ *lacI*^{q} ZΔM15 Tn10(tet^{r})]	(Atsumi et al., 2008)
JCL260	Same as JCL16 but with Δ*adhE* Δ*frd-ldhA* Δ*pta* Δ*p3B* Δ*fnr*	(Atsumi et al., 2008)
AL626	Same as JCL260 but with Δ*yqhD* Δ*adhP* Δ*eutG* Δ*yiaY* Δ*yjgB* Δ*fucO*	(Rodriguez and Atsumi, 2012)
AL1728	Same as AL626 but with Δ*eutE* Δ*yahK* Δ*yqhE* Δ*gldA* Δ*ybbO* Δ*yghA*	This work
AL1550	JCL260 harboring pSA129	This work
AL1827	AL1728 harboring pSA129	This work
Plasmid name		
pGR03	p15A ori; Cm^{R}; P_{L}lacO$_{1}$: *alsS-ilvCD*	(Rodriguez and Atsumi, 2012)
pSA138	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yqhD*	(Atsumi et al., 2008)
pSA129	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd*	(Atsumi et al., 2010)
pZE12-luc	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *luc*	(Lutz and Bujard, 1997)
pAL337	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yahk*	This work
pAL338	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yphC*	This work
pAL339	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ycjQ*	This work
pAL340	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ybdR*	This work
pAL341	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-rspB*	This work
pAL342	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ydjG*	This work
pAL343	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ghrB*	This work
pAL358	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yjjN*	This work
pAL359	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ydfG*	This work
pAL360	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-dkgB*	This work
pAL361	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ydjJ*	This work
pAL362	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ydjL*	This work
pAL363	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yhdH*	This work
pAL364	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ybbO*	This work
pAL365	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-gldA*	This work
pAL504	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-dkgA*	This work
pAL505	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yeaE*	This work
pAL506	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yghZ*	This work
pAL507	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-tas*	This work
pAL508	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ygbJ*	This work
pAL509	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ghrA*	This work
pAL514	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-ycjS*	This work
pAL515	ColE1 ori; Amp^{R}; P_{L}lacO$_{1}$: *kivd-yceM*	This work

***E. coli* strain**	**Genotype**	**Reference**
pAL516	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yhiN*	This work
pAL517	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yhhX*	This work
pAL518	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ydbC*	This work
pAL519	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ygjR*	This work
pAL520	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ygfF*	This work
pAL521	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yohF*	This work
pAL553	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yciK*	This work
pAL554	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ykgE*	This work
pAL555	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ygcW*	This work
pAL556	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-upcA*	This work
pAL557	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yajO*	This work
pAL558	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-hdhA*	This work
pAL559	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ydhF*	This work
pAL560	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yghA*	This work
pAL580	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ybiC*	This work
pAL581	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-thd*	This work
pAL582	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yggP*	This work
pAL583	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yqiB*	This work
pAL584	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-frmA*	This work
pAL585	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-yjhC*	This work
pAL586	ColE1 ori; Amp^R; $P_L lacO_1$: *kivd-ydgJ*	This work