

Title: Genome engineering allows selective conversions of terephthalaldehyde to multiple valorized products in bacterial cells

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Abstract:

Deconstruction of polyethylene terephthalate (PET) plastic waste generates opportunities for valorization to alternative products. We recently designed an enzymatic cascade that could produce terephthalaldehyde (TPAL) from terephthalic acid. Here, we showed that the addition of TPAL to growing cultures of *Escherichia coli* wild-type strain MG1655 and an engineered strain for reduced aromatic aldehyde reduction (RARE) strain resulted in substantial reduction. We then investigated if we could mitigate this reduction using multiplex automatable genome engineering (MAGE) to create an *E. coli* strain with 10 additional knockouts in RARE. Encouragingly, we found this newly engineered strain enabled a 2.5-fold higher retention of TPAL over RARE after 24h. We applied this new strain for the production of *para*-xylylenediamine (pXYL) and observed a 6.8-fold increase in pXYL titer compared to RARE. Overall, our study demonstrates the potential of TPAL as a versatile intermediate in microbial biosynthesis of chemicals that derived from waste PET.

22 **Topical Heading:**

23 Biomolecular Engineering, Bioengineering, Biochemicals, Biofuels, and Food

24 **Key words:**

25 Aldehyde, amine, biosynthesis, oxidoreductases, genome engineering

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Introduction

The increasing societal dependence on plastics derived from petroleum and natural gas feedstocks has generated a demand to divert plastic waste from landfills to alternative products¹⁻³. As such, tremendous efforts have been made to enhance the sustainability and renewability in polymer life cycles. Particularly, polyethylene terephthalate (PET) plastic, one of the most commonly produced polyesters, has garnered increasing attraction with the development of chemical deconstruction through synthetic approaches⁴⁻⁷ or use of microbial PET-degrading enzymes⁸⁻¹¹. Both enzymatic and chemical means of PET deconstruction toward the monomer unit terephthalic acid (TPA) generate opportunities to create higher value products or monomers for use in other classes of materials. In recent years, there have been several examples of platform chemicals produced biocatalytically from TPA, including gallic acid, pyrogallol, catechol, muconic acid, vanillic acid and vanillin made by live cells^{12,13}. We recently identified carboxylic acid reductase enzymes that can efficiently generate the versatile dialdehyde terephthalaldehyde (TPAL) from TPA¹⁴. Our first step was to report the use of these enzymes *in vitro*, in part because it is unclear if these steps would function well in live cells given the potential instability and reactivity of TPAL¹⁵. However, the use of live cells to convert TPAL to useful chemical building blocks could provide a more cost effective and efficient biosynthetic process compared to the use of purified enzymes¹⁶⁻¹⁸.

Aldehydes can readily undergo numerous biological transformations to generate a large variety of potential products. Here, we envision utilizing the dialdehyde functionality of TPAL to access potential new opportunities in biosynthetic pathways, including asymmetrically functionalized products. Aldehyde-derived biosynthetic targets have ranged broadly¹⁹, including diamine polymer building blocks^{14,20}, hydroxylated non-standard amino acids²¹⁻²⁵, nitro alcohols²⁶, and pharmaceutical mono-amine precursors²⁷⁻²⁹. Despite the biosynthetic possibilities available from

aldehydes, their redox instability in cellular environments due to endogenous oxidoreductase activity remains a key challenge³⁰. First, we were curious to learn if we could take advantage of the endogenous enzymes that reduce TPAL in growing wild-type *E. coli* K-12 MG1655 cells to form the target diol 1,4-benzenedimethanol (BDM), a valuable building block that can be utilized for the production of pesticides, perfumes, or dyes^{31–34}. While this instability can be utilized to rapidly reduce aldehydes into alcohols products like BDM, the strongly reductive natural cellular environment mitigates the use of aldehydes as a platform intermediate. To address this challenge, alcohol dehydrogenases (ADHs) or aldo-keto reductases (AKRs) in *E. coli* can be deleted and have resulted in sustainable improvements in stability for a wide set of aromatic and aliphatic aldehydes under aerobic conditions^{35–37}. Of particular interest, the RARE.Δ6 strain (more commonly known as the RARE strain), an *E. coli* MG1655 strain named for its reduced aromatic aldehyde reduction, has alleviated the issue of aldehyde stability for many aromatic aldehydes³⁷. Thus, we were also curious to learn if the RARE.Δ6 strain would allow us to convert TPAL to higher value compounds.

In this study, we investigated the stability of TPAL when supplemented to *E. coli* MG1655 and the RARE.Δ6 strain for the potential reduction by endogenous enzymes to the corresponding mono- or di- alcohols. We showed that TPAL is reduced by growing cell cultures of the MG1655 strain and, to our surprise, TPAL is also reduced by growing cell cultures of the RARE.Δ6 strain. Interestingly, we discovered that we could use the RARE.Δ6 strain to stably accumulate the mono-aldehyde mono-alcohol 4-(hydroxymethyl) benzaldehyde (4HMB), which can be used in polymer applications as a precursor that is converted to an aryl bromide on route to polymers bearing an aldehyde at their chain ends³⁸. To determine if we could overcome reduction of either aldehyde functional group by identifying and inactivating additional aldehyde reductases, we used multiplex

automatable genome engineering (MAGE) to perform translational gene inactivation of up to 10 additional ADH and AKR genes. These gene inactivations were partly guided by RNA-seq that revealed a previously unreported target whose expression was elevated by TPAL challenge. After performing these additional gene inactivations, we created a strain (RARE.Δ16) that achieved significant retention of TPAL under aerobic growth conditions after 24 h. Through plasmid-based reinsertion of targeted genes, we created an additional strain (RARE.Δ10) with only select knockouts still capable of significant TPAL stability. Finally, we showed that the use of both of these strains can lead to large improvements in the biosynthesis of the diamine pXYL from TPAL. Our study thus exploits genome engineering and heterologous expression to demonstrate selective routes to three distinct building blocks from TPAL.

Materials and Methods

Strains and plasmids

Escherichia coli strains and plasmids used are listed in **Table S1**. Molecular cloning and vector propagation were performed in DH5α (NEB). Polymerase chain reaction (PCR) based DNA replication was performed using KOD XTREME Hot Start Polymerase (MilliporeSigma) for plasmid backbones. Cloning was performed using Gibson Assembly. Oligos for PCR amplification and translational knockouts are shown in **Table S2**. Oligos were purchased from Integrated DNA Technologies (IDT). The DNA sequence and translated sequence of proteins overexpressed in this paper are found in **Table S3**. The pORTMAGE-Ec1 recombineering plasmid was kindly provided by Timothy Wannier and George Church of Harvard Medical School.

Chemicals

96 The following compounds were purchased from MilliporeSigma: sodium borate decahydrate,
97 sodium phosphate dibasic anhydrous, chloramphenicol, kanamycin sulfate, dimethyl sulfoxide
98 (DMSO), boric acid, L-alanine, and HEPES. D-glucose and m-toluic acid. The following
99 compounds were purchased from Alfa Aesar: agarose and ethanol were purchased. The following
100 compounds were purchased from Fisher Scientific: isopropyl β -D-1-thiogalactopyranoside
101 (IPTG), acetonitrile, sodium chloride, trifluoroacetic acid, LB Broth powder (Lennox), and LB
102 Agar powder (Lennox). A MOPS EZ rich defined medium kit was purchased from Teknova. Taq
103 DNA ligase was purchased from GoldBio. Anhydrotetracycline (aTc) was purchased from
104 Cayman Chemical. Phusion DNA polymerase and T5 exonuclease were purchased from New
105 England BioLabs (NEB). Sybr Safe DNA gel stain was purchased from Invitrogen. The following
106 compounds were purchased from TCI America: Pyridoxal 5'-phosphate (PLP), *ortho*-
107 phthalaldehyde and 3-mercaptopropionic acid

108 **Cloning of TA and AlaDH**

109 Molecular cloning and vector propagation were performed in *E. coli* DH5 α . Codon-optimized
110 genes were PCR amplified using KOD Xtreme polymerase The ω -transaminase from
111 *Chromobacterium violaceum* (CvTA, WP_011135573.1) and alanine dehydrogenase from
112 *Bacillus subtilis* (BsAlaDH, WP_003243280.1) were purchased as gene fragments from IDT and
113 cloned using Gibson Assembly into a pACYC vector harboring an N-terminal His₆-tag for
114 purification, p15a ori, and chloramphenicol acetyltransferase gene for antibiotic selection. All
115 plasmids were verified by Sanger sequencing.

116 **Cloning of RARE. Δ 16 gene KO**

The targeted genes for inactivation in RARE.Δ16 (*adhP*, *fucO*, *eutG*, *viaY*, *adhE*, *eutE*, *gldA*, *gpr*, *ybbO* and *yghA*) were amplified by PCR from the *E. coli* MG1655 genome and were cloned into a pACYC p15a ori, and chloramphenicol acetyltransferase gene for antibiotic selection by Gibson Assembly.

Culture Conditions

Cultures were grown in LB-Lennox medium (LB: 10 g/L bacto tryptone, 5 g/L sodium chloride, 5 g/L yeast extract), M9-glucose minimal media³⁹ with Corning® Trace Elements A (1.60 µg/mL CuSO₄ • 5H₂O, 863.00 µg/mL ZnSO₄ • 7H₂O, 17.30 µg/mL Selenite • 2Na, 1155.10 µg/mL ferric citrate) and 1.5% glucose, or MOPS EZ rich defined media (Teknova M2105) with 2% glucose (MOPS EZ Rich-glucose).

Stability Assays

For testing stability in metabolically active cells, cultures of each *E. coli* strain to be tested were inoculated from a frozen stock and grown to confluence overnight in 5 mL of LB media. Confluent overnight cultures were then used to inoculate experimental cultures to an initial starting OD₆₀₀ of 0.01 in 400 µL volumes in a 96-deep-well plate (Thermo Scientific™ 260251). Cultures were supplemented with 5 mM of TPAL (prepared in 100 mM stocks in 100% DMSO) at mid-exponential phase (OD₆₀₀ = 0.5-0.8). Cultures were incubated at 30 °C with shaking at 1000 RPM and an orbital radius of 3 mm. Samples were taken by pipetting 200 µL from the cultures, centrifuging in a different 96-deep-well plate and collecting the extracellular broth. Compounds were quantified over a 24 h period using HPLC with samples collected at 4 h and 24 h. For experiments testing the effect of 4 h sampling, samples with and without 4 h sampling were taken, and compounds were quantified using HPLC with collection at 24 h.

For metabolically active cell stability testing with targeted single gene knockouts plasmids, cultures of *E. coli* RARE.Δ6 and RARE.Δ16 harboring a gene from the 10 targeted knockouts were inoculated from a frozen stock and grown to confluence overnight in 5 mL of LB media with 34 μg/mL chloramphenicol. Confluent overnight cultures were then used to inoculate experimental cultures with cm to an initial starting OD₆₀₀ of 0.01 in 400 μL volumes in a 96-deep-well plate. Cultures were supplemented with 5 mM of TPAL at mid-exponential phase (OD₆₀₀ = 0.5-0.8). Cultures were incubated at 30 °C with shaking at 1000 RPM and an orbital radius of 3 mm. Samples were centrifuged and the extracellular broth was collected at 24 h.

For TPAL stability testing, LB media with 34 μg/mL chloramphenicol was supplemented with 5 mM of TPAL. For experiments testing effect of incubation temperature, supplemented media was incubated in 400 μL volumes in a 96-deep-well plate at 4 °C, 30 °C and 37 °C. For experiments testing effect of head space, supplemented media was incubated in 200 μL, 400 μL and 1 mL volumes in a 1 mL 96-deep-well plate at 30 °C. Samples were taken by collecting 25 μL from the media. Compounds were quantified over a 24 h period using HPLC with samples collected at 0 h, 4 h, 12 h and 24 h.

RNA-seq

Total RNA was sampled from duplicate cultures for each condition. To prepare samples for RNA extraction, cultures of *E. coli* RARE.Δ6 were inoculated at a 1:50 dilution from a confluent overnight culture into 3 mL of M9-glucose minimal media in 14 mL culture tubes. Cultures were then grown at 37 °C for 2 h (until OD₆₀₀~0.4 was reached). After 2 h, 1 mM of TPAL was supplemented to cultures from 400 mM stocks prepared in DMSO. Culture tubes were then snapped shut and sealed with parafilm to limit loss of aldehyde due to volatility. Cultures were then grown at 37 °C for an additional 1.5 h. Then, 5 x 10⁸ cells (as measured by optical density)

were mixed with RNAprotect Bacteria Reagent (1:2 volume ratio). Then, bacterial lysis and RNA purification were performed using a Qiagen RNeasy purification kit (Handbook 2020). Following purification to ensure better purity, the RNA was precipitated by adding 6 μ L of 5 M sodium acetate with 180 μ L of ethanol. Then, the samples were placed at -20 °C. After 18 h, the samples were centrifuged at 18,000 x g at 4 °C for 10 min and then the supernatant was decanted. After the samples dried, they were resuspended in RNase free water and frozen at -80 °C until submission to Novogene for total RNA sequencing. Transcriptional fold change was performed with TPAL supplementation compared to a RARE. Δ 6 baseline in duplicates (**Table S4**).

Translational genomic knockouts

Translational knockouts to the strain were performed using 10 rounds of multiplexed automatable genome engineering (MAGE) where stop codons were introduced into the genomic sequence for each aldehyde dehydrogenase target in the upstream portion of the gene (**Table S5**). Construction of RARE. Δ 16 strain was done with two subsets of knockouts (subset #1: *adhP*, *fucO*, *eutG*, *yiaY* and *adhE*, subset #2: *eutE*, *gldA*, *gpr*, *ybbO*, and *yghA*) performed in the RARE. Δ 6 strain³⁷. Construction of RARE. Δ 10 was done using the same oligos that were used for the RARE. Δ 16 strain. MAGE was performed using the pORTMAGE-Ec1 recombineering plasmid⁴⁰. Briefly, bacterial cultures were inoculated with 1:100 dilution in 3 mL of LB media with 30 μ g/mL kanamycin (Kan) and grown at 37 °C until OD₆₀₀ of 0.4 - 0.6 was reached. Then, the proteins responsible for recombineering were induced with 1 mM m-toluic acid and cultures were grown at 37 °C for an additional 15 min. Cells were then prepared for electroporation by washing 1 mL three times with refrigerated 10% glycerol and then resuspending in 50 μ L of 10% glycerol with each knockout oligo within a subset added at 1 μ M. Cells were then electroporated and recovered in 3 mL of LB with 30 μ g/mL kanamycin to be used for subsequent rounds. Preliminary assessment

of knockouts was performed using mascPCR (multiplexed allele specific colony PCR)⁴¹ and confirmed using Sanger Sequencing. RARE.Δ11, RARE.Δ16 and RARE.Δ10 were cured of the pORTMAGE-Ec1 plasmid following confirmation of genomic knockouts.

Growth rate and protein production assay

For growth rate testing, confluent overnight cultures were used to inoculate experimental cultures in LB and MOPS EZ Rich media at 100x dilution in 200 μL volumes in a Greiner black clear bottom 96 well plate (Greiner 655090). Cultures were grown for 12 h in a Spectramax i3x plate reader with medium plate shaking at 37 °C with absorbance readings at 600 nm taken every 7 min to determine growth rate of each strain. For protein expression testing, strains were transformed with a pZE-Ub-sfGFP reporter plasmid. Confluent cultures of cells harboring the pZE-Ub-sfGFP were grown as described above with 30 μg/mL kanamycin and 0.2 nM aTc added at inoculation. For shake flask experiments, confluent overnight cultures were used to inoculate experimental cultures in 50 mL LB in 250 mL shake flasks. Cultures were grown for 7 h at 37 °C with absorbance readings taken using a Biomate 160 spectrophotometer every 30 minutes with 1 mL samples at 600 nm.

***In vivo* transaminase activity**

For metabolically active ω-transaminase testing, cultures of MG1655, RARE.Δ6, RARE.Δ10 and RARE.Δ16 harboring a plasmid containing both CvTA and AlaDH or just AlaDH were inoculated from a frozen stock and grown to confluence overnight in 5 mL of LB media with 34 μg/mL chloramphenicol. Confluent overnight cultures were then used to inoculate experimental cultures in a 96-deep-well plate initial starting OD₆₀₀ of 0.01 in 300 μL in LB with 34 μg/mL chloramphenicol, 10 mM L-alanine (amine donor), 60 mM ammonium chloride at pH of 7.4.

Cultures were supplemented with 1 mM of IPTG at mid-exponential phase ($OD_{600} = 0.5-0.8$) and with 5 mM of TPAL (prepared in 100 mM stocks in DMSO) after 24 h. Cultures were incubated at 30 °C with shaking at 1000 RPM and an orbital radius of 3 mm for 4 hours. Samples were taken by centrifuging in a 96-deep-well plate and collecting the extracellular broth. Compounds were quantified after 24 h using HPLC.

HPLC analysis

TPAL, 4HMB, 4HMBA, BDM and pXYL were quantified using reverse-phase high-performance liquid chromatography (HPLC) with an Agilent 1260 Infinity with a Zorbax Eclipse Plus-C18 column with a guard column installed. Amines were derivatized with *ortho*-phthalaldehyde and 3-mercaptopropionic acid and identified using reverse-phase high-performance liquid chromatography (RP-HPLC) with an Agilent 1260 Infinity with a Zorbax Eclipse Plus-C18 column with a guard column installed. The methods for detection of TPAL, 4HMB, 4HMBA, BDM and pXYL used were described in Gopal et al¹⁴.

Results

TPAL is reduced in MG1655 and RARE.Δ6 under aerobic conditions

We first sought to measure the stability of TPAL and the potential to produce a diol product under aerobic growth conditions (**Fig. 1A**). To evaluate whether reduction occurred during fermentation, we supplemented 5 mM TPAL to cultures of the *E. coli* MG1655 strain in LB media at mid-exponential phase. We grew cells in 96-deep well plates with 400 μL of media and used non-breathable aluminum seals to limit loss due to aldehyde volatility. We sampled the culture broth at three time points (4 h, 12 h and 24 h after aldehyde addition) to gain some insight on the kinetics of aldehyde stability. In cultures of the wild-type *E. coli* MG1655 strain, we observed complete

reduction of TPAL to BDM within 12 h, with this condition persisting after 24 h (**Fig. 1B**). We expect that this reduction is catalyzed by several endogenous aldehyde reductases in *E. coli* that in prior studies we have worked to identify and eliminate for the stabilization of aldehydes^{35–37,42}.

We were also curious about whether we could improve or enable the conversion of TPAL to other products by inactivating endogenous ADHs and AKRs. As a first step towards testing whether inactivation of aldehyde reductases could eliminate TPAL reduction, we evaluated TPAL stability in the previously engineered RARE.Δ6 strain under aerobic growth. Although we recently verified that the RARE.Δ6 strain reliably stabilizes a broad range of aromatic aldehydes under these conditions⁴³, we were surprised to observe TPAL reduction at our first time point of 4 h with greater reduction seen at 12 h and 24 h (**Fig. 1C**). However, we observed that cultures of the RARE.Δ6 strain at all timepoints were able to stabilize the mono-aldehyde 4HMB, eliminating the complete reduction of TPAL to BDM. Thus, we can select production of BDM and 4HMB through the use of MG1655 and RARE.Δ6 respectively. While the RARE.Δ6 strain was able to provide enhanced TPAL stability over that of the wild-type MG1655 strain at 4 h, there is still significant reduction within our system at 24 h (0.22 ± 0.07 mM TPAL, 2.70 ± 0.27 mM 4HMB).

Rational targeted gene inactivation enables TPAL stability

Given the effectiveness of combinatorial gene deletions at limiting the reduction of aldehydes, we next investigated whether we could mitigate the reduction of TPAL observed using additional genome engineering. We used MAGE to inactivate potential genes responsible for the reduction of TPAL (**Fig. 2A**). Given the partial success of the RARE.Δ6 strain at increasing TPAL stability, we sought to identify additional ADH or AKR targets that could contribute to TPAL reduction. We first looked at additional ADH candidates reported in the previously engineered *E. coli* strain AL1728 which reported 13 aldehyde reductase deletions, several of which were not included in

252 the RARE.Δ6 strain³⁶. We generated an initial subset of 5 targeted gene deletions (S1: Δ*adhP*
 253 Δ*fucO* Δ*eutG* Δ*yiaY* Δ*adhE*) to test if additional ADH knockouts could provide enhanced TPAL
 254 stabilization. In addition to investigating aldehyde reducing enzymes that had been deleted in
 255 previous studies, we also sought to determine whether other, nonobvious targets existed. To do so,
 256 we performed an RNA-seq experiment comparing conditions with and without addition of TPAL.
 257 We grew RARE.Δ6 in culture tubes in M9-glucose media, where media composition was defined.
 258 After cultures reached mid-exponential phase, we then either added 1 mM of TPAL and sealed
 259 culture tubes or simply sealed culture tubes and grew cultures for an additional 1.5 h. We then
 260 harvested and submitted total RNA for sequencing at Novogene. We then examined the
 261 sequencing results for upregulated ADH and AKR transcripts in the TPAL addition case. We
 262 selected *gpr*, *eutE* and *gldA* as targeted genes for deletion as all had relatively high fold changes
 263 (> 1) (**Fig 2B**). We also selected *ybbO* as we observed a small positive fold change. Additionally,
 264 we selected *yghA*, for deletion despite the down regulation as we still observed relatively high
 265 transcript levels. With this, we then constructed a second subset of 5 additional ADHs and AKRs
 266 for deletions (S2: Δ*eutE* Δ*gldA* Δ*gpr* Δ*ybbO* Δ*yghA*).

267 We utilized MAGE to inactivate S1 and S2 for a total of 10 gene deletions. We used 10 total rounds
 268 of MAGE to inactivate each subset of targeted genes in the *E. coli* RARE.Δ6 strain via introduction
 269 of in-frame stop codons within the first 100 codons. We then used multiplex allele-specific colony-
 270 PCR and confirmation by Sanger sequencing to obtain a variant containing the first subset of
 271 knockouts denoted as RARE.Δ11 (RARE.Δ6, S1) and a variant with all 10 knockouts denoted
 272 RARE.Δ16 (RARE.Δ11, S2) (**Fig 2C**). Next, we sought to determine the stability of 5 mM TPAL
 273 under aerobic growth conditions in both new strains along with their progenitor strains at 4 h and
 274 24 h. We observed that RARE.Δ11 had similar TPAL stability to that of RARE.Δ6 at 4 h and 24

h, indicating that just S1 knockouts alone do not yield TPAL stability. However, we were excited to see no detectable reduction of TPAL after 4 h in RARE.Δ16 (**Fig. 2D**). When compared to RARE.Δ6 at 24 h, we observed a 2.5-fold increase in TPAL concentration and a near 10-fold decrease in the 4HMB concentration (**Fig. 2E**).

Contribution of ALR knockouts to TPAL reduction

We set out to determine the impact of each of the additional 10 KOs on TPAL stability because of the potential tradeoffs on fitness and heterologous expression associated with multiple gene knockouts. To reintroduce each gene, we transformed RARE.Δ16 with a plasmid of each gene cloned from the wild-type MG1655. We then cultured RARE.Δ6, RARE.Δ16, and distinct RARE.Δ16 transformants that overexpressed each gene that had been targeted for inactivation under aerobic conditions at 37 °C. At mid-exponential phase, we induced each culture and supplied 5 mM TPAL. To our surprise, here we observed that when each targeted gene was individually overexpressed, only 4 (*ybbO*, *gpr*, *yiaY*, *yghA*) out of the 10 resulted in TPAL reduction (**Fig. 3A**). Because MAGE is a rapid and combinatorial genome engineering strategy, we then chose to create a new strain to test the effect of introducing the translational knockouts of only these four additional genes in RARE.Δ6, denoted as RARE.Δ10 (RARE.Δ6, Δ*ybbO* Δ*gpr* Δ*yiaY* Δ*yghA*) (**Fig. 3B**). We then evaluated the stability of TPAL in aerobic conditions with RARE.Δ10 and observed comparable stability to that of RARE.Δ16 (**Fig. 3C**). This result indicated that the inactivation of only *ybbO*, *gpr*, *yiaY* and *yghA* were required to achieve the increased TPAL stability.

Stability of TPAL in culture media

With TPAL stability achieved in metabolically active cells, we then investigated stability in LB media to address the loss of material observed in **Figs. 1-3**. We evaluated whether the potential

loss of material could be exacerbated by multiple samplings of the culture media. We measured TPAL concentrations in aerobic conditions when supplemented to LB media or cultures of MG1655, RARE.Δ6, RARE.Δ10 and RARE.Δ16 strains, with and without the 4 h sampling timepoint (**Fig. S1**). We observed little to no change in TPAL stability, indicating multiple sample points does not lead to increase material loss within our system. We then hypothesized that the potential loss of material could be due to the volatility of TPAL. We tested the stability of TPAL at different temperatures (4 °C, 30 °C and 37 °C) (**Fig. S2A**) and with variations in head space (200 μL, 400 μL and 1 mL of media in a 1 mL 96-deep-well plate) (**Fig. S2B**) to explore potential TPAL evaporation. We observed that TPAL stability increased as incubation temperature and head space were lowered. However, we selected conditions that exhibited lower TPAL stability to ensure aerobic growth in our cultures were maintained. We observed that under our selected conditions for our stability assays with cultures (30 °C and 200-400 μL) that TPAL is naturally evaporated, and the material loss matched that observed within **Fig. 1-3**.

Characterization of the RARE.Δ10 and RARE.Δ16 strains for biocatalysis applications

We then investigated whether the genome engineering performed impacted important parameters for functional application of these strains, namely effects on the cellular growth rate and protein overproduction. Thus, we investigated the growth rates across MG1655, RARE.Δ6, RARE.Δ10 and RARE.Δ16. We observed in both LB, a complex media, as well as MOPS EZ Rich-glucose, a defined media, there was no significant differences in the doubling time between the progenitor MG1655 strain and the engineered strains (**Fig. 4A, B, Table 1**). However, we noticed there were small differences in growth rates towards late exponential and stationary phase, which resulted in lower final biomass concentrations of RARE.Δ16 for both media conditions. As cellular growth rate can be hindered in 96-well plates, we observed the growth rate of these strains in shake flasks

in LB media to ensure that potential impacts on growth rates were captured. We noticed that in the more favorable conditions, all strains exhibited no significant differences in growth rates or final OD₆₀₀ (**Fig. S3**). Furthermore, we used expression of a superfolder green fluorescent protein (sfGFP) reporter to compare protein production capacity of each previously mentioned strain. Each strain was transformed with a plasmid that harbored sfGFP and was grown in LB and MOPS EZ Rich-glucose media. We observed similar production of sfGFP when normalized by OD₆₀₀ during exponential growth (**Fig. 4 C, D**). Additionally, we noted that RARE.Δ16 had slightly increased normalized sfGFP in both media conditions. With the comparable levels in RARE.Δ10 and RARE.Δ16 to that of MG1655, our engineered strains show limited deleterious impact to growth rate and ability to overexpress desired protein despite the inactivation of up to 16 total genes.

Application of the RARE.Δ16 strain for improved amine synthesis

We anticipate that the enhanced stability of TPAL in RARE.Δ10 and RARE.Δ16 affords opportunities to design potential biosynthesis pathways to convert TPAL to products besides BDM or 4HMB under aerobic fermentation conditions (**Fig. 5A**). To do so, we looked into the enzyme class of ω-transaminases (TAs), which are reversible pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyze the transfer of an amino group between donor and acceptor⁴⁴. Here, we evaluated the capability of RARE.Δ10 and RARE.Δ16 strains for improved biosynthesis of the diamine *para*-xylylenediamine (pXYL). pXYL can be utilized as a component in a wide variety of materials including polyamides, polyimides, or non-isocyanate polyurethanes^{45,46}. We transformed MG1655, RARE.Δ6, and RARE.Δ16 with a plasmid construct that inducibly expresses a His6x-tagged TA from *Chromobacterium violaceum* (CvTA) and an L-alanine dehydrogenase for amino donor recycling from *Bacillus subtilis* (BsAlaDH). We additionally transformed these strains with a plasmid construct that inducibly expresses only BsAlaDH to

observe its potential effect on TPAL stability. We cultured these strains under aerobic conditions in LB media with 400 μ M PLP, 60 mM ammonium chloride and 10 mM L-alanine (amine donor). At mid-exponential phase ($OD_{600} = 0.5-0.6$), we induced each culture then supplied 5 mM TPAL after 2 h. When only BsAlaDH was expressed, we observed TPAL stability increased in all strains, with TPAL still being detected in MG1655 after 24 h (**Fig. S5**). When both CvTA and BsAlaDH are expressed in MG1655, we found no detectable amination to pXYL after 24 h but observed the similar unexpected partial reduction of TPAL to 4HMB with minimal reduction to BDM when only BsAlaDH was expressed (**Fig. 5B**). We detected a similar result when both CvTA and BsAlaDH are expressed in RARE. Δ 6 with the majority of TPAL reduced to 4HMB, however a small amount of pXYL was produced. We observed that the RARE. Δ 10 and RARE. Δ 16 strains achieved nearly 7-fold enhancement in pXYL production compared to the RARE. Δ 6 strain after 24 h (4.12 ± 0.10 mM RARE. Δ 10, 4.08 ± 0.12 mM RARE. Δ 16 and 0.63 ± 0.03 mM RARE. Δ 6). Thus, we have shown that the genetic engineering approach that led to the creation of both RARE. Δ 10 and RARE. Δ 16 are able to unlock avenues to convert TPAL to multiple sets of valuable building block chemicals, including pXYL.

Discussion

In this work, we identified that the wild-type MG1655 *E. coli* strain rapidly reduces TPAL to BDM in metabolically active cells. We then evaluated TPAL stability in the engineered RARE. Δ 6 strain. Here, we showed that the RARE. Δ 6 strain provided a 17.6-fold change in TPAL stability over that of the wild-type MG1655 after 4 h. However, at the later time point of 24 h, we observed the RARE. Δ 6 strain had less than $8 \pm 2\%$ retention of TPAL. The RARE. Δ 6 strain contains knockouts of six different genes that encode aldehyde reductases from two distinct families of enzymes, the AKR family and the NADH-dependent ADH family³⁷. To determine if we could further improve

366 TPAL stability, we utilized the combinatorial genome engineering of MAGE to inactivate the
 367 translation of additional full-length ADHs and AKRs in the RARE.Δ6 strain. To identify potential
 368 genes that may be active on TPAL, we performed an RNA-seq comparing conditions with and
 369 without supplementation of TPAL. With these genes identified and others identified in prior
 370 literature, we utilized MAGE to inactivate either 5 additional genes (RARE.Δ11: RARE.Δ6,
 371 *ΔadhP*, *ΔfucO*, *ΔeutG*, *ΔyiaY*, *ΔadhE*) or 10 additional genes (RARE.Δ16 RARE.Δ11, *ΔeutE*,
 372 *ΔgldA*, *Δgpr*, *ΔybbO*, *ΔyghA*) in the RARE.Δ6 strain. We observed that the RARE.Δ16 strain had
 373 increased TPAL stability over MG1655 and RARE.Δ6 at short (4 h) and even at long (24 h) time
 374 scales. We then found that RARE.Δ16 contained 6 knockouts that were not required for TPAL
 375 stability and further showed that the inactivation of only *ybbO*, *gpr*, *yiaY* and *yghA* combined with
 376 the RARE.Δ6 knockouts are necessary to achieve the TPAL stability exhibited in RARE.Δ16.
 377 Interestingly, RARE.Δ11 only contained one (*yiaY*) out of these four critical knockouts that were
 378 made in both RARE.Δ10 and RARE.Δ16, and we observed that RARE.Δ11 had similar TPAL
 379 stability to that of RARE.Δ6. This study indicates that the presence of the remaining critical
 380 knockouts (*ybbO*, *gpr* and *yghA*) in both RARE.Δ10 and RARE.Δ16 are vital to TPAL stability
 381 and suggest *yiaY* could potentially play a lesser role in TPAL stabilization. We can attribute the
 382 limited effect of RARE.Δ11 to *yiaY* having significantly lower relative expression level as
 383 determined through RNA-seq (2.12 Fragments Per Kilobase of transcript sequence per Millions
 384 base pair-sequenced or FPKM) compared to *ybbO* (515.64 FPKM), *gpr* (3239.71 FPKM) and *yghA*
 385 (484.67 FPKM) (**Table S4**). As our differential over-expression analysis masks low basal
 386 expression levels, *yiaY* deletion may not be required for TPAL stability under our tested condition.
 387 By performing differential expression analysis after an aldehyde challenge experiment, our study
 388 is the first to reveal the relevance of *gpr* to the reduction of aldehydes featured in engineered

389 biosynthetic pathways. The *gpr* gene encoded the only AKR from our targeted knockouts. It has
390 been shown to have high activity on methylglyoxal and has been utilized for glyoxal and
391 methylglyoxal detoxification in *E. coli*^{47–49}. Interestingly, *gpr* has been shown to have slight
392 activity towards benzaldehyde and relatively high activity towards 4-nitrobenzaldehyde^{47,48}. The
393 *yiaY*, *yghA* and *ybbO* genes have been knocked out for a variety of short and long chain aliphatic
394 reductions^{35,36,50–53}. However, the activity of *yiaY* and *yghA* on aromatic aldehydes has not been
395 shown, with *ybbO* very recently shown to contribute to the reduction of the aromatic aldehyde
396 cinnamaldehyde⁴². Interestingly, we noticed *yghA* was the only gene during our differential over
397 expression analysis to yield the acid-alcohol 4HMBA as the main product (**Fig. S4**). Besides the
398 oxidoreductase capabilities highlighted previously, little is known about this enzyme that may
399 yield 4HMBA production. Further studies into *yghA* may yield capabilities for higher production
400 of 4HMBA, which is an important intermediate in the synthetic process of the antihypertensive
401 drug Eprosartan^{54,55}.

402 Given what previous literature suggests, our work highlights the ability of RNA-seq to reveal
403 potential ADH and AKR that otherwise would not have been clear targets for gene inactivation for
404 TPAL stability. We selected *gpr* for deletion as it had the highest log₂ fold change (1.55) of any
405 ADH or AKR class enzymes identified by RNA-seq after a TPAL challenge. Additionally, we
406 targeted *ybbO* as a targeted candidate as it was also upregulated during the TPAL challenge
407 experiment, but lower than of *gpr* (0.17). However, the approach of relying on transcriptomic
408 analysis is limited by the fact that many genes, such as some targeted ADHs in this study (*eutE*,
409 *eutG*, *gldA*, *fucO*), may be upregulated upon addition of a toxic product like TPAL but do not
410 directly contribute to its removal. We additionally found multiple genes that play a significant role
411 in TPAL reduction that were not upregulated during a TPAL challenge. The other two critical

412 knockouts (*yghA* and *yiaY*) were both downregulated (-0.76 and -1.48 respectively). We selected
413 *yghA* as it had high expression levels despite being downregulated (**Table S4**) and *yiaY* based on
414 previously reported aldehyde retaining strains³⁶. With this, our work demonstrates for the case of
415 TPAL that challenges experiments could be used to uncover new oxidoreductase targets for
416 deletion.

417 When only BsAlaDH was expressed, we observed that TPAL stability increased in all strains.
418 During aerobic growth on glucose, cells typically contain large concentrations of both NADH and
419 pyruvate. It is known that both ADHs and AKRs utilize NAD(P)H as cofactors for the formation
420 of alcohols⁵⁶. Additionally, BsAlaDH consumes NADH during the reductive amination of
421 pyruvate to form L-alanine, which is the physiological direction⁵⁷. The co-expression of BsAlaDH
422 could decrease the NADH pool in engineered cells relative to wild-type cells under these
423 conditions, which could then change the equilibrium of the 4HMB reduction catalyzed by the
424 NADH-dependent ADHs and AKRs⁵⁸.

425 The improved stability of TPAL using live microbial cells can provide a more cost effective and
426 efficient biosynthesis pathway over purified enzymes to other useful chemical building blocks^{16–}
427 ¹⁸. A fermentative process circumvents the need for cell lysis and enzyme purification that can
428 lower biocatalyst cost compared to purified enzymes^{44,59–61}. This difference in biocatalyst cost can
429 increase with multi-enzyme cascades and the need for additional co-factor regenerating enzymes.
430 However, fermentative processes are limited by aldehyde toxicity to microbial cells. While
431 aldehyde products could inhibit cellular growth, aldehyde intermediates could be kept at a low
432 steady-state concentration with downstream enzymes.

433 Here, we demonstrated that the RARE.Δ10 and RARE.Δ16 strains can offer a significant
434 improvement for TPAL biocatalysis using cells expressing CvTA. We observed both strains

outperformed the RARE.Δ6 strain by nearly 7-fold after 24 h. Despite the 6 additional knockouts made in RARE.Δ16, we have shown that both strains have similar TPAL stability, growth kinetics, and biocatalytic capabilities. While either strain could be used for future work, both strains contain potential advantages depending on their application. RARE.Δ16 contains additional knockouts that may provide enhanced stability for additional aldehydes other than TPAL while RARE.Δ10 provides a less engineered chassis for additional engineering or synthetic pathway development. There are several synthetic pathways that involve potential biosynthetic pathways involving TPAL as a substrate, intermediate, or product. In our previous work, we have shown potential biosynthesis pathways from PET deconstruction products like terephthalic acid or even mono-(2-hydroxyethyl) terephthalic acid to TPAL with the use of purified carboxylic acid reductases and a lipase. It is possible that other PLP-dependent enzymes could also utilize TPAL as a potential substrate. Our future work will look to identify whether RARE.Δ10 and RARE.Δ16 can serve as platform strains to take advantage of the reactivity of aldehydes for the production of building blocks of diverse macromolecules, including non-standard amino acids⁶².

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Author Contributions: R.M.D designed and performed the MAGE to create RARE.Δ11, RARE.Δ10 and RARE.Δ16, designed and conducted all stability, biocatalysis and growth experiments, analyzed data, prepared figures, and wrote the manuscript; M.A.J. initially documented ADH activity on TPAL, designed and performed MAGE to create RARE.Δ11. N.D.B performed the TPAL challenge and RNA-seq experiment as well as genome sequencing of RARE.Δ16. I.G. helped designed and performed MAGE to create RARE.Δ11. A.M.K. contributed to writing the manuscript, secured funding, provided oversight, and reviewed the manuscript.

Conflict of Interest Statement: None

Data Availability Statement: The data that support the findings of this study are openly available in Dryad at:

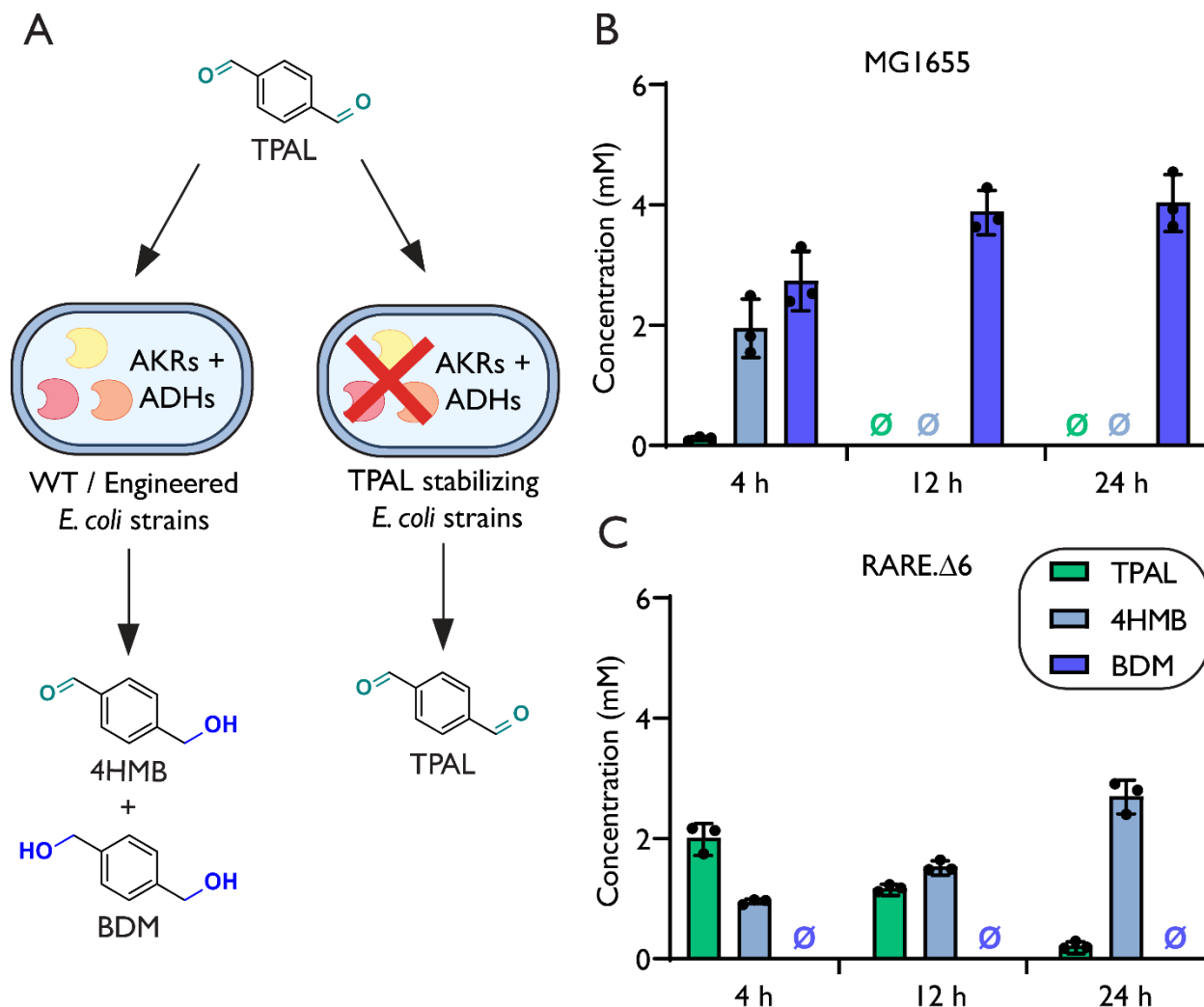
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Dickey, Roman et al. (2023), Genome engineering allows selective conversions of terephthalaldehyde to multiple valorized products in bacterial cells, Dryad, Dataset,

<https://doi.org/10.5061/dryad.6t1g1jx45>

Additional information including Materials and Methods can be found in the Supporting Information document available online.

470 **Figures**



471

472 **Figure 1. Evaluation of the stability of TPAL when supplemented to aerobic cultures of *E.***

473 ***coli*.** (A) TPAL was added to culture media to determine its fate in the presence of growing *E. coli*

474 cells over time. (B) Cultures of wild-type *E. coli* MG1655 were grown in LB media at 37°C and

475 supplemented with 5 mM TPAL at mid-exponential phase. TPAL metabolites were tracked via

476 HPLC at 4 h, 12 h and 24 h. (C) Cultures of the previously reported *E. coli* RARE.Δ6 strain were

477 grown under identical conditions and TPAL metabolites were tracked via HPLC at 4 h, 12 h and

478 24 h. Data represents technical triplicates (n=3) where error bars represent the standard deviation
479 across triplicates. Null sign indicates no detectable quantities were observed.

480

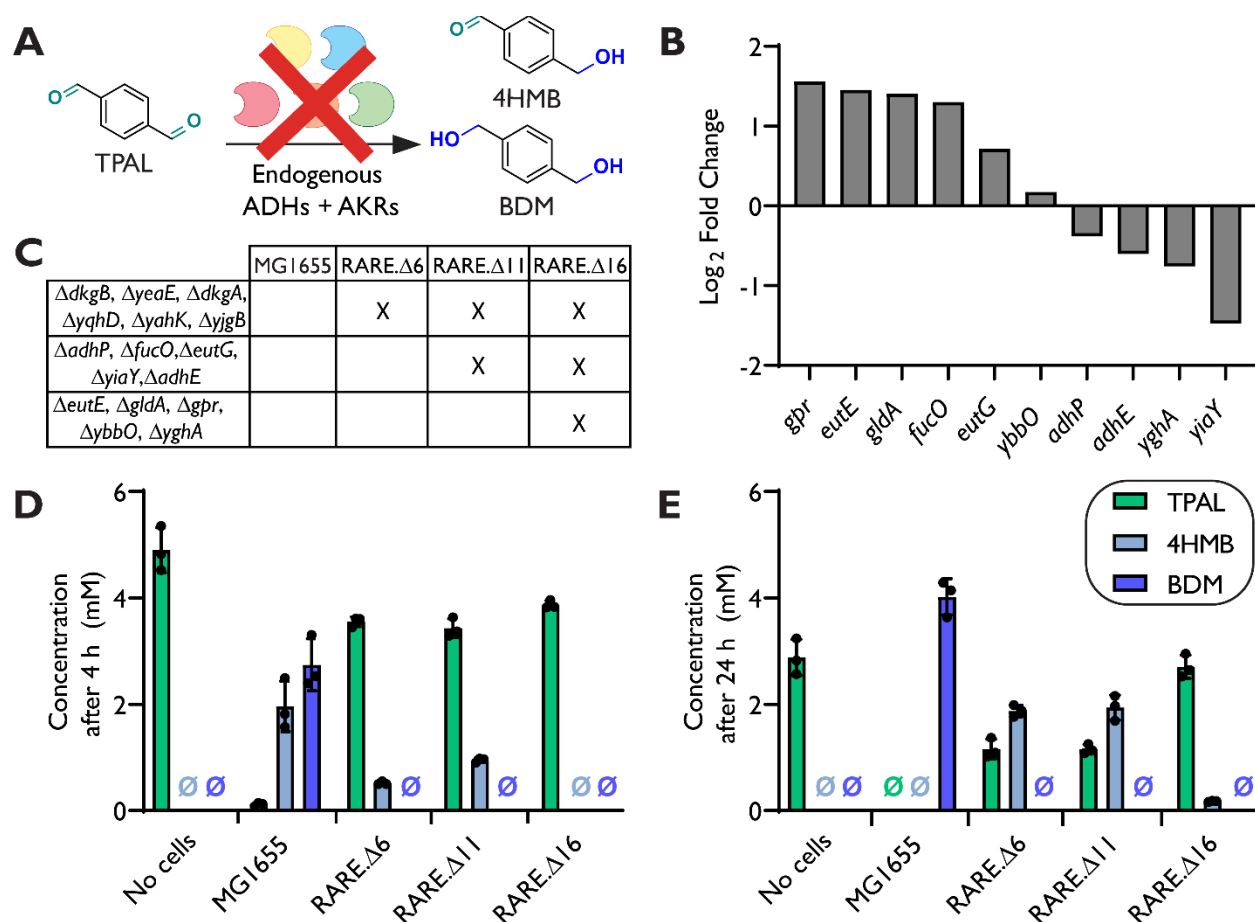


Figure 2. Genomic knockout of aldehyde reductases (ALR) toward improved TPAL stability in aerobic conditions. (A) Using the *E. coli* RARE.Δ6 strain as a basis, MAGE was performed to translationally knockout ADHs and AKRs to generate two strains RARE.Δ11 (RARE.Δ6, ΔadhP ΔfucO ΔeutG ΔyiaY ΔadhE) and RARE.Δ16 (RARE.Δ11, ΔeutE ΔgldA, Δgpr ΔybbO ΔyghA). (B) Log₂ fold change of targeted genes were determined through a TPAL challenge RNA-seq. Data represents TPAL supplementation in RARE.Δ6 compared to a RARE.Δ6 baseline in technical duplicates (n=2). (C) Gene knockouts contained within each strain. (D&E) Cultures of wild-type *E. coli* MG1655, RARE.Δ6, RARE.Δ11 and RARE.Δ16 were grown in LB media at 37°C and supplemented with 5 mM TPAL at mid-exponential phase. TPAL metabolites were tracked via

492 HPLC at 4 h (**D**) and 24 h (**E**). Data represents technical triplicates (n=3) where error bars represent
493 the standard deviation across triplicates. Null sign indicates no detectable quantities were
494 observed.

495

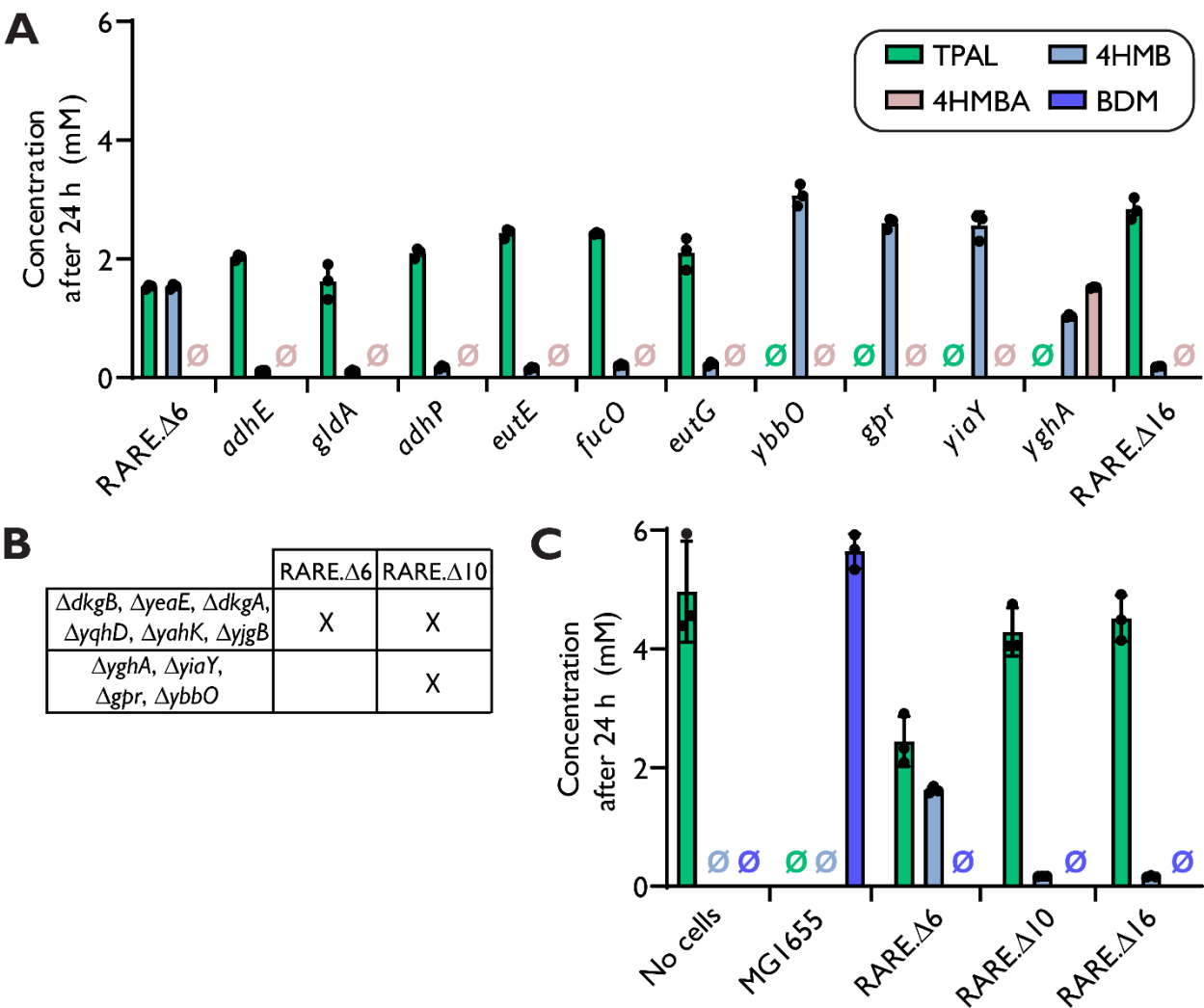
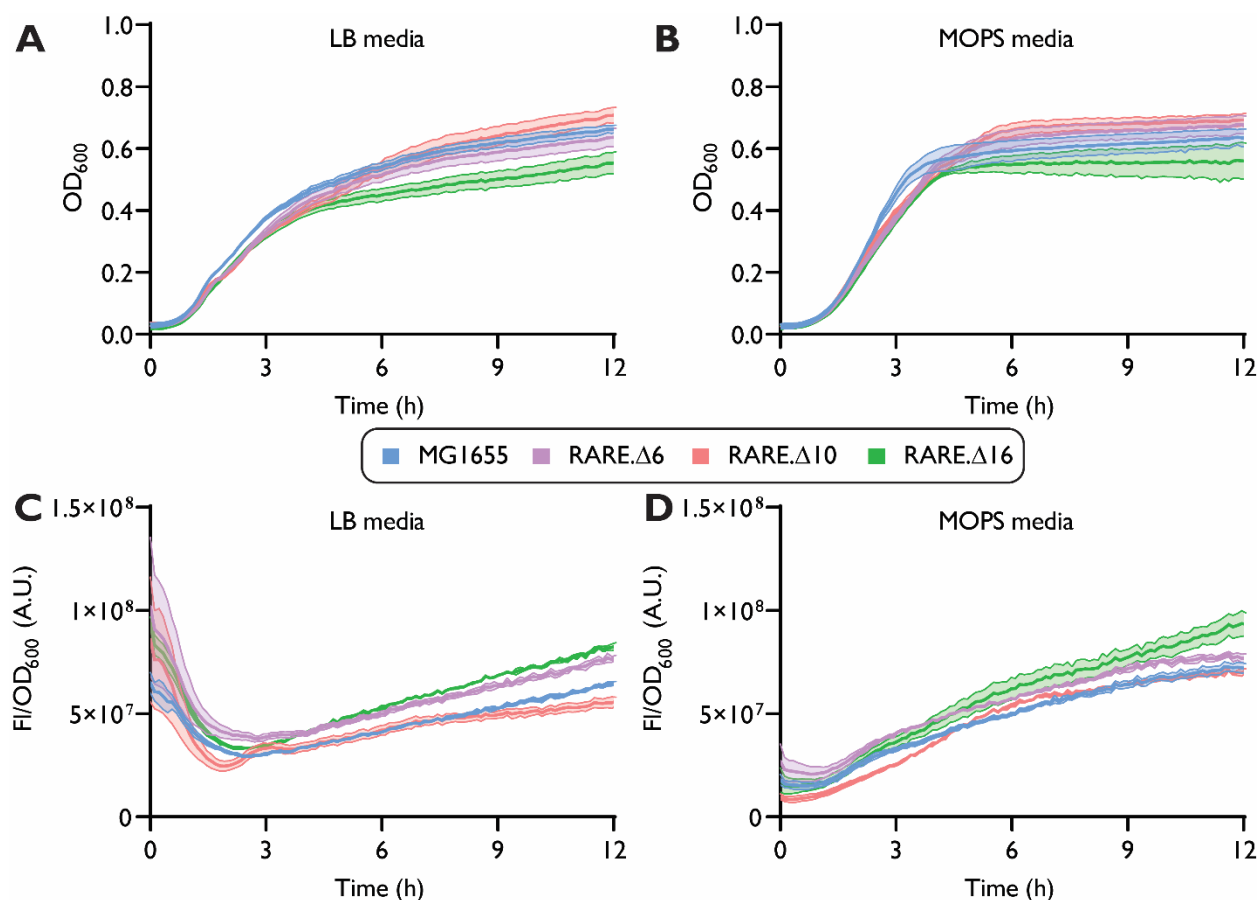


Figure 3. Evaluation of overexpressed ALR activity on TPAL stability. (A) Cultures of RARE.Δ6, RARE.Δ10 and RARE.Δ16 transformed with a single plasmid for each individual ALR gene from this study were grown in LB media at 37°C and supplemented with 5 mM TPAL and then induced of targeted gene at mid-exponential phase and tracked via HPLC after 24 h (4HMBA indicates 4-hydroxymethyl benzoic acid). (B) Gene knockouts contained within RARE.Δ10 strain. Using the *E. coli* RARE.Δ6 strain as a basis, MAGE was performed to translationally knockout the 4 ALRs that showed reduction of TPAL when overexpressed to generate the RARE.Δ10 strain

505 (RARE.Δ6, ΔybbO Δgpr ΔyiaY ΔyghA). (C) Cultures of wild-type *E. coli* MG1655, RARE.Δ6,
506 RARE.Δ10 and RARE.Δ16 were grown in LB media at 37°C and supplemented with 5 mM TPAL
507 at mid-exponential phase. TPAL metabolites were tracked via HPLC at 24 h. Data represents
508 technical triplicates (n=3) where error bars represent the standard deviation across triplicates. Null
509 sign indicates no detectable quantities were observed.

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Figure 4. Growth and protein production performance of engineered TPAL retaining strains. Growth was monitored via optical density at 600 nm (OD₆₀₀) in 96-well plate for 12 h in LB media (A) and in MOPS EZ Rich media (B). Plasmid-based protein overexpression of superfolder green fluorescent protein (sfGFP) was monitored via 96-well plate in a plate reader for 12 h by measuring fluorescence (ex: 488 nm, em: 525 nm) normalized by OD₆₀₀ in both LB media (C) and MOPS EZ Rich media (D) (A.U. indicates arbitrary units). Data represents technical triplicates (n=3) where error bars represent the standard deviation across triplicates.

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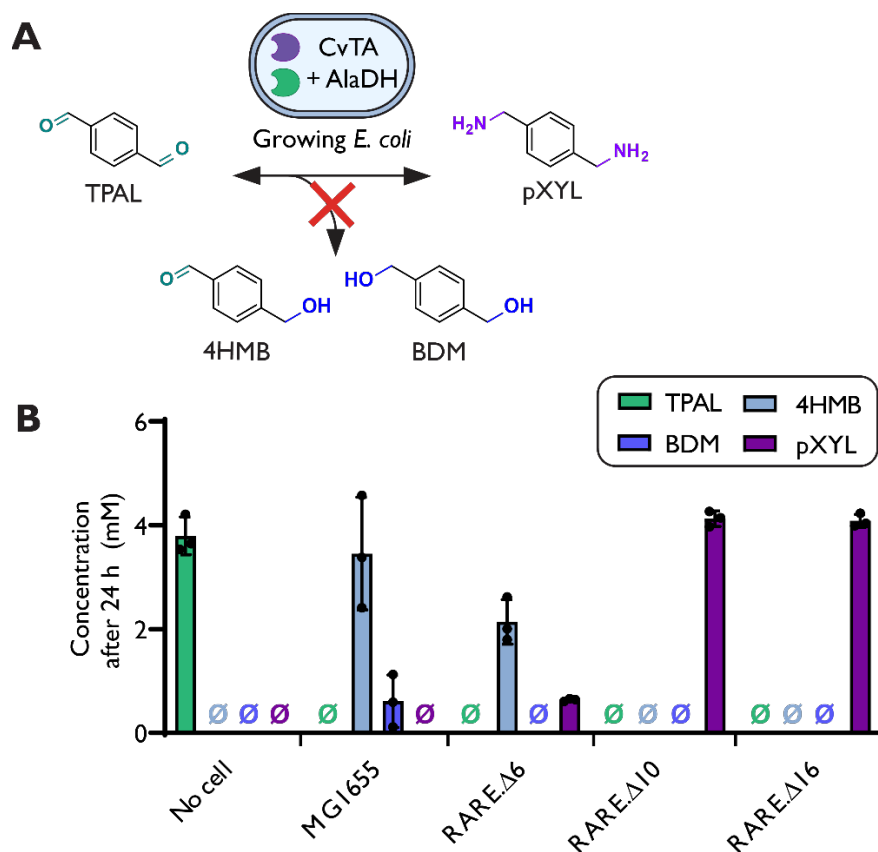
522 **Table 1.** Growth performance of engineered TPAL retaining stains. Data represents average of
 523 technical triplicates (n=3) where error represent the standard deviation across triplicates.

	LB MEDIA		MOPS MEDIA		524
	Doubling time (min)	Final OD ₆₀₀	Doubling time (min)	Final OD ₆₀₀	
MG1655	22.6 ± 1.7	0.63 ± 0.01	22.1 ± 0.7	0.61 ± 0.03	
RARE.Δ6	25.6 ± 0.5	0.61 ± 0.03	23.3 ± 1.1	0.65 ± 0.03	
RARE.Δ10	24.5 ± 1.7	0.68 ± 0.02	22.8 ± 0.8	0.66 ± 0.02	
RARE.Δ16	24.1 ± 1.1	0.53 ± 0.04	24.0 ± 0.7	0.54 ± 0.05	

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Figure 5. Biosynthesis of pXYL in engineered strains expressing CvTA. (A) We created *E. coli* strains containing CvTA and AlaDH that can convert TPAL to pXYL without additional reduction of TPAL. (B) Cultures of MG1655, RARE.Δ6, RARE.Δ10, and RARE.Δ16 expressing CvTA were grown in LB media at 37°C and were supplemented with 10 mM L-alanine (amino donor) and 5 mM of TPAL at mid-exponential phase. TPAL, 4HMB, and pXYL concentrations were tracked via HPLC at 24 h. Data represents technical triplicates (n=3) where error bars represent the standard deviation across triplicates. Null sign indicates no detectable quantities were observed.

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