The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

| Citation       | Kunjapur, Aditya et al. "Synthesis and Accumulation of Aromatic Aldehydes in an Engineered Strain of Escherichia coli." Journal of American Chemical Society 136, 33 (July 2014): 11644-11654 © 2014 American Chemical Society |
|----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| As Published   | http://dx.doi.org/10.1021/ja506664a                                                                                                                                 |
| Publisher      | American Chemical Society (ACS)                                                                                                                                 |
| Version        | Author’s final manuscript                                                                                                                                 |
| Citable link   | https://hdl.handle.net/1721.1/122618                                                                                                                                 |
| Terms of Use   | Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use. |
Synthesis and accumulation of aromatic aldehydes in an engineered strain of Escherichia coli

Aditya M. Kunjapur\textsuperscript{1,2}, Yekaterina Tarasova\textsuperscript{2,3}, Kristala L. J. Prather\textsuperscript{1,2,3}\textsuperscript{*}

\textsuperscript{1}Department of Chemical Engineering

\textsuperscript{2}Synthetic Biology Engineering Research Center (SynBERC)

\textsuperscript{3}Microbiology Graduate Program

Massachusetts Institute of Technology

Cambridge, MA 02139, USA

\textsuperscript{*} Corresponding author:

Department of Chemical Engineering

77 Massachusetts Avenue

Room E17-504G

Cambridge, MA 02139

Phone: 617.253.1950

Fax: 617.258.5042

Email: kljp@mit.edu
Abstract

Aromatic aldehydes are useful in numerous applications, especially as flavors, fragrances, and pharmaceutical precursors. However, microbial synthesis of aldehydes is hindered by rapid, endogenous, and redundant conversion of aldehydes to their corresponding alcohols. We report the construction of an *Escherichia coli* K-12 MG1655 strain with reduced aromatic aldehyde reduction (RARE) that serves as a platform for aromatic aldehyde biosynthesis. Six genes with reported activity on the model substrate benzaldehyde were rationally targeted for deletion: three genes that encode aldoketo reductases and three genes that encode alcohol dehydrogenases. Upon expressing a recombinant carboxylic acid reductase in the RARE strain and supplying the strain with benzoate during growth, benzaldehyde remained in the culture after 24 hours, with less than 12% conversion of benzaldehyde to benzyl alcohol. Although individual overexpression results demonstrated that all six genes could contribute to benzaldehyde reduction *in vivo*, additional experiments featuring subset deletion strains revealed that two of the gene deletions were dispensable under the conditions tested. The engineered strain was next investigated for the production of vanillin from vanillate and succeeded in preventing formation of the byproduct vanillyl alcohol. A pathway for the biosynthesis of vanillin directly from glucose was introduced and resulted in a 55-fold improvement in vanillin titer when using the RARE strain versus the wild-type strain. Finally, synthesis of the chiral pharmaceutical intermediate L-phenylacetylcarbinol (L-PAC) was demonstrated from benzaldehyde and glucose upon expressing a recombinant mutant pyruvate decarboxylase in the RARE strain. Beyond allowing accumulation of aromatic aldehydes as end products in *E. coli*, the RARE strain expands the classes of chemicals that can be produced microbially via aldehyde intermediates.
Introduction

Aromatic aldehydes are used widely in industrial applications, such as in flavors and fragrances.\textsuperscript{1-5} Vanillin, which is the primary ingredient in vanilla extract\textsuperscript{6-8}, and benzaldehyde, which is used to create almond and cherry flavors\textsuperscript{9-11}, are examples of aromatic aldehydes and are the two most widely used flavoring agents in food products.\textsuperscript{2,5} Biotechnological production of vanillin is of special interest given that less than 1\% of the 16,000 tons of vanillin sold annually originates from vanilla beans.\textsuperscript{12} Due to the limited supply and high price of extract from the vanilla bean, most of the market consists of vanillin that is chemically synthesized from either lignin or petroleum.\textsuperscript{12} Consumer preference for flavors in which no chemicals are used (i.e., natural flavors\textsuperscript{4}) has led to a price differential of $1,200-$4,000/kg for natural vanillin compared to $15/kg for artificial vanillin.\textsuperscript{12} Beyond their uses in flavoring, these and other aromatic aldehydes serve as intermediates for the production of industrial chemicals and pharmaceuticals.\textsuperscript{12,13} For example, benzaldehyde is used to produce L-phenylacetylcarbinol (L-PAC, also commonly referred to in the literature as R-PAC), which is a precursor to the pharmaceutical ephedrine.\textsuperscript{14-16}

The primary barrier to overproduction of aromatic aldehydes in engineered microorganisms is the rapid conversion of desired aldehydes into undesired alcohols by numerous endogenous enzymes.\textsuperscript{17-21} Previous work aimed at enabling biotechnological production of vanillin and L-PAC illustrate how long this obstacle has been recognized. Sixteen years have passed since a pathway from glucose to an immediate precursor of vanillin was constructed in \textit{E. coli}.\textsuperscript{22} In the same report, an argument was made that commercial relevance depended on engineering the ability to synthesize and accumulate vanillin in a single microbe.\textsuperscript{22} However, this feat has not been reported to date, prompting some researchers to search for organisms with intrinsically lower activity on aromatic aldehydes, such as \textit{Schizosaccharomyces pombe}.\textsuperscript{18} Similarly, L-PAC biotransformations performed by the addition of
exogenous benzaldehyde to yeast cultures suffer from low yield, largely due to benzyl alcohol byproduct formation. The use of purified enzymes to achieve higher yields has been explored but is likely to increase production costs.\textsuperscript{14} Alternatively, others have decreased benzyl alcohol formation by adding benzaldehyde to resting \textit{E. coli} cells that express a recombinant pyruvate decarboxylase (PDC) from \textit{Zymomonas mobilis}.\textsuperscript{15}

Several factors motivate the selection of \textit{E. coli} for identification and deletion of genes that encode aromatic aldehyde reductases. The superior tools and know-how established with this organism enable rapid evaluation of gene targets. Strategies to increase flux from central metabolism to the biosynthesis of aromatics in \textit{E. coli} are well-documented\textsuperscript{23-26} and thus expected to facilitate swift improvements in aromatic aldehyde production if their accumulation were realizable. Furthermore, \textit{E. coli} K-12 strains have been used to produce food additives designated as GRAS (Generally Recognized As Safe), such as chymosin.\textsuperscript{27} Knowledge of the \textit{E. coli} genes responsible for aromatic aldehyde reductase activity can also help inform efforts to engineer other microbial hosts.

We began this endeavor by exploring serial deletions of different combinations of genes that were reported to act on benzaldehyde, which is structurally the simplest aromatic aldehyde. Our search initially focused on aldo-keto reductases (AKRs), which form a superfamily of enzymes that have broad substrate specificity and convert aldehydes and ketones to alcohols in the presence of NADPH.\textsuperscript{28} In the few previously reported attempts at constructing microbial pathways to produce aldehydes, only genes encoding alcohol dehydrogenases (ADHs) were targeted for deletion.\textsuperscript{18,19} Unlike AKRs, ADHs typically use NADH as their co-factor, and under anaerobic conditions they perform the important function of recycling co-factors. We hypothesized that rational deletion of AKRs with activity on benzaldehyde, in addition to the deletion of select ADHs, would be a promising route to engineering the accumulation of aromatic aldehydes in \textit{E. coli}.
We report the construction of an *E. coli* MG1655 strain with reduced aromatic aldehyde reduction (RARE) that can serve as a platform for the synthesis of aromatic aldehydes with minimal or no conversion to their corresponding alcohols. We then use the RARE strain to enable the synthesis of vanillin and L-PAC, demonstrating the utility of this particular engineered strain and the general approach of rationally combining gene knockouts to overcome a highly redundant endogenous activity.

**Materials and Methods**

**Strains and plasmids**

*E. coli* strains and plasmids used in this study are listed in Table S1 (Supporting Information). Molecular biology techniques were performed according to standard practices unless otherwise stated. Molecular cloning and vector propagation were performed in DH5α. All targeted genes were deleted from *E. coli* K-12 MG1655(DE3). The genes *dkgB, yeaE, yahK, yjgB, endA,* and *recA* were deleted using donor strains from the Keio collection and P1 transduction. P1 bacteriophage was obtained from ATCC (25404-B1). The operon encoding *yqhC-dkgA* was deleted using the λ Red system. To generate homology, three pairs of oligonucleotides were used as PCR primers. These and other oligonucleotides are shown in Table S2. Oligonucleotides were purchased from Sigma. Q5 High Fidelity DNA Polymerase (New England Biolabs) was used for DNA amplification. In all cases of gene deletions, pCP20 was used to cure the kanamycin resistance cassette.

The *car* gene from *Nocardia iowensis*, the *sfp* gene from *Bacillus subtilis*, and the *Hs-S-COMT* gene from *Homo sapiens* were synthesized and codon-optimized for expression in *E. coli* (GenScript). The *asbF* gene from *Bacillus thuringiensis* was synthesized as a DNA string codon-optimized for expression in *E. coli* (GeneArt, Invitrogen). The *aroG* gene was kindly provided by Professor Jay D. Keasling at the University of California, Berkeley (USA). The gene encoding the
pyruvate decarboxylase (PDC) enzyme from *Zymomonas mobilis* was amplified from genomic DNA (ATCC 39676). The gene encoding the mutant PDC_E473Q enzyme was generated using Polymerase Incomplete Primer Extension (PIPE) cloning. All codon-optimized and mutant gene sequences are included in SI Text. *E. coli* AKR and ADH gene targets were amplified from MG1655(DE3) genomic DNA using PCR and cloned into the Duet vector system (Novagen) using PIPE cloning. Unless otherwise specified, all other genes were cloned into the Duet vector system (Novagen) using restriction digest-based cloning (see SI Text for more details). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Propagated constructs were purified using a QIAprep Miniprep Kit (QiaGen) and agarose gel fragments were purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research). All constructs were confirmed to be correct by nucleotide sequencing (Genewiz).

**Chemicals**

The following compounds were purchased from Sigma: sodium benzoate, benzaldehyde, benzyl alcohol, vanillic acid, vanillin, isovanillin, vanillyl alcohol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, and dimethyl sulfoxide (DMSO). 3,4-Dihydroxybenzyl alcohol was purchased from TCI America. L-phenylacetylcarbinol was purchased from Santa Cruz Biotechnology. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Denville Scientific. Ampicillin sodium salt, chloramphenicol, and kanamycin sulfate were purchased from Affymetrix.

**Culture conditions**

With the exception of growth rate experiments, all experiments were performed in 50 ml Pyrex VISTA screw-cap culture tubes (Sigma), which contained 5 ml of culture in order to maintain aerobic conditions and limit evaporation of volatile metabolites. Experimental cultures were initiated using 1% (v/v) inoculum volumes of overnight culture that were transferred into either LB medium or M9
minimal medium containing 1.2% glucose, incubated at 30°C, and agitated at 250 rpm. Overnight cultures were grown in 3 ml of the same medium in 14 ml round-bottom tubes (Corning). In general, experiments were performed in triplicates, and results are presented as averages with error bars representing one standard deviation.

For all substrate-feeding experiments excluding glucose as a substrate, filter-sterilized and pH-neutralized substrates were added to the cultures upon induction with IPTG. During 5 mM benzoate-feeding experiments, cultures were induced with 1 mM IPTG between optical densities (OD$_{600}$) of 0.7-1.0. During 5 mM vanillate-feeding experiments, cultures were induced between OD$_{600}$ of 1.0-1.3. Culture medium was supplemented with 50 mg/L ampicillin and 17 mg/L chloramphenicol to provide selective pressure for plasmid maintenance.

For experiments testing the production of vanillin directly from glucose, 1.2% (w/v) glucose was added prior to inoculation and cultures were induced with 0.5 mM IPTG between OD$_{600}$ of 0.8-1.1. Screw-caps remained tightly closed until sampling at final time points, but mass balances in the liquid phase did not always close, indicating some evaporation of volatile products. Either LB medium or M9 minimal medium was used. Culture medium was supplemented with 50 mg/L ampicillin and 17 mg/L chloramphenicol to provide selective pressure for plasmid maintenance.

For experiments testing the synthesis of L-phenylacetylcarbinol, 1.2% (w/v) glucose was added prior to inoculation and cultures were induced with 0.5 mM IPTG between OD$_{600}$ of 0.8-1.1. At induction, a filter-sterilized solution of 50 mM benzaldehyde in 10% DMSO was added to the cultures, resulting in an initial concentration of 5 mM benzaldehyde and 1% DMSO. For these experiments, culture medium was supplemented with either only 50 mg/L kanamycin or 25 mg/L kanamycin and 17 mg/L chloramphenicol depending on whether strains were expressing Car.
Growth rates for wild-type and RARE strains were determined from 50 ml shake flask cultures. Cultures were initiated with 1% inoculum volumes of overnight culture that were transferred into either LB medium containing 1.2% glucose or M9 minimal medium containing 1.2% glucose. Overnight cultures were grown in the same media. In both cases, cultures were incubated at 30°C and agitated at 250 rpm. The OD$_{600}$ was measured regularly during exponential growth using a DU800 UV/Vis spectrophotometer (Beckman Coulter).

Metabolite analysis

Culture samples were pelleted by centrifugation and aqueous supernatant was collected for HPLC analysis using either an Agilent 1100 series or 1200 series instrument equipped with a diode array detector. Wavelengths of 223, 242, and 192 nanometers were used to detect benzoic acid, benzaldehyde, and benzyl alcohol, respectively. The benzoate family of analytes was separated using an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories), with a mobile phase consisting of 70% 5 mM H$_2$SO$_4$ and 30% acetonitrile. All three compounds eluted within 35 minutes at a flow rate of 0.4 ml/min. Column temperature was maintained at 30°C. All chemicals reported in figures were quantified using calibration of standards on the HPLC instrument and linear interpolation.

Compounds used in vanillin experiments were separated using a Zorbax Eclipse XDB-C18 column (Agilent) and detected using a wavelength of 280 nm. A gradient method used the following solvents: (A) 50% acetonitrile + 0.1% trifluoroacetic acid (TFA); (B) water + 0.1% TFA. The gradient began with 5% Solvent A and 95% Solvent B. The setting at 20 minutes was 60% Solvent A and 40% Solvent B. The program restored the original ratio at 22 minutes and ended at 25 minutes. The flow rate was 1.0 ml/min and all compounds of interest eluted within 15 minutes. Column temperature was maintained at 30°C.
Phenylacetylcarbinol was detected using a Zorbax Eclipse XDB-C18 column (Agilent) and detected using a wavelength of 210 nm. A gradient method used the following solvents: (A) 100% acetonitrile + 0.1% trifluoroacetic acid (TFA); (B) water + 0.1% TFA. The gradient began with 5% Solvent A and 95% Solvent B. The setting at 20 minutes was 60% Solvent A and 40% Solvent B. The program restored the original ratio at 22 minutes and ended at 25 minutes. Column temperature was maintained at 30°C. The flow rate was 1.0 ml/min and the retention time of phenylacetylcarbinol was 8.3 minutes. Peak area corresponding to a co-eluting and static background peak in LB medium was subtracted in order to quantify concentrations of phenylacetylcarbinol produced. Although enantiomeric excess (ee) was not determined, it has been shown previously using chiral HPLC and near-UV circular dichroism spectroscopy that both wild-type PDC and PDC_E473Q catalyze the formation of the R stereoisomer in 98-99% ee.16

Quantitative Reverse Transcription PCR (qRT-PCR)

For isolation of RNA and generation of cDNA in biological duplicate, two cultures of MG1655(DE3) ΔendA ΔrecA were grown overnight in 3 ml of LB medium contained in 14 ml round-bottom tubes. Each overnight culture was used to inoculate two cultures in 4.5 ml of LB medium (1% v/v inoculum) contained in 50 ml PYREX VISTA tubes. Once cultures reached an OD₆₀₀ of 0.7-0.9, benzaldehyde was added to two out of the four cultures to a final concentration of 5 mM. After one hour of further incubation, 0.5 ml of cells were harvested for RNA isolation. RNA protect bacterial reagent (Qiagen) was added to cells prior to centrifugation and lysis. Total RNA was isolated using the illustra RNAspin Mini Isolation Kit (GE healthcare) with an on-column DNaseI treatment according to protocol. Turbo DNA-free reagents (Ambion) were used to further treat isolated RNA for removal of genomic DNA. Next, QuantiTect Reverse Transcriptase (Qiagen) was used to generate cDNA from 500
ng of total RNA for each isolation. Concentrations of RNA and DNA were measured using a NanoDrop 2000 (Thermo Scientific).

Quantitative PCR was performed using an ABI 7300 Real Time PCR System Instrument (Applied Biosystems). All samples analyzed by qPCR were performed in triplicate. 2 µl of cDNA from each RNA isolation was added to Brilliant II SYBR Green High ROX QPCR Mix (Agilent Technologies) and 0.5 µM of appropriate primers to a final volume of 25 µl per well. Amplification was performed according to the following program: an initial step of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. The number of cycles to reach the threshold (CT value) was measured for each primer pair in triplicate samples of each cDNA. The “Auto CT” option in the 7300 System SDS RQ Study Software was used to determine the threshold values. Sequences of primers used for analysis of dkgA, dkgB, yeaE, yqhD, yahK, and yjgB are listed in Table S2. Primer sequences were designed to have melting temperatures ranging from 56-60°C and to generate roughly 100 base pair amplicons. The specificity of primers was verified using gel electrophoresis using gDNA from MG1655(DE3) ΔendA ΔrecA as a template. No-template and no-RT controls confirmed that primer dimer formation was absent or negligible (i.e., CT values greater than 33). Plasmids containing gene deletion targets were diluted to concentrations ranging from roughly 10^-4 to 10^-8 ng/µl and analyzed in triplicate as standards during each respective run. Using linear standard curves, CT values were used to quantify absolute concentrations of reverse transcribed mRNA corresponding to each gene of interest, with and without benzaldehyde treatment. All PCR efficiencies were similar.

Results

A combination of rationally targeted gene deletions enables benzaldehyde accumulation in E. coli. Deletions of AKR genes were guided by literature reported activities of gene products on
benzaldehyde (Table S3). Protein BLAST was used to organize *E. coli* AKRs based on sequence similarity to DkgA, the AKR with the greatest reported activity on benzaldehyde (Table S4). The gene encoding DkgA is located downstream of *yqhD* in the same operon, and transcription of both genes is activated by the product of *yqhC*, which is located immediately upstream of the operon.\(^3^\) Given that YqhD is a broad substrate ADH that is also reported to act on benzaldehyde\(^3^9\)\(^-\)\(^4^1\), the entire operon was deleted. Benzaldehyde reductase activity was unaffected by the deletion of this operon and by the subsequent deletion of the two genes encoding AKRs with the greatest protein sequence similarity to DkgA (*dkgB* and *yeaE*). A recent report revealing the activity of two *E. coli* cinnamyl alcohol dehydrogenases (YahK and YjgB) on benzaldehyde was then discovered.\(^4^2\) A few months prior to that report, overexpression of the *yahK* and *yjgB* genes had also been reported to improve the production of aromatic alcohols in *E. coli*\(^4^3\). After deleting these two additional genes, we observed a significant change in the ability of *E. coli* to accumulate benzaldehyde. The final engineered strain (RARE ∆*endA* ∆*recA*) consists of nine gene deletions: *dkgB*, *yeaE*, *yqhC*, *yqhD*, *dkgA*, *yahK*, *yjgB*, *endA*, *recA*. The genes *endA* and *recA*, which encode an endonuclease and a recombinase, were deleted to increase plasmid stability. The deletion of *yqhC* forms a convenient but nonessential control. The remaining six genes constitute the rationally targeted set.

To compare the ability of the RARE strain to accumulate aromatic aldehydes, four additional strains containing complementary subsets of the total set of gene deletions were constructed (Table 1). In short, the “Subset 1” strain contained the *yqhC*-yqhD-dkgA operon intact, the “Subset 2” strain contained all gene targets that were not in the operon, the “Subset 3” strain contained only the targeted ADHs that were not in the operon, and the “Subset 4” strain contained only the targeted AKRs that were not in the operon. All strains were also built to express a recombinant and activated carboxylic acid reductase (Car). Car, from *Nocardia iowensis*, has broad substrate specificity and was previously used to
catalyze the formation of benzaldehyde and vanillin from their corresponding acids \textit{in vivo} in \textit{E. coli} and \textit{in vitro}.\textsuperscript{33} Expression of Car was paired with expression of Sfp from \textit{Bacillus subtilis}. Sfp is a phosphopanthetheine transferase that has been shown to activate Car.\textsuperscript{34,44} In these experiments, pH-neutralized acid substrates were added to the medium to obtain greater solubility and mimic product formation in an engineered pathway. The corresponding aldehydes were then generated intracellularly by the action of Car on the acid substrate.

Table 1. \textit{E. coli} strains featuring different combinations of gene deletions (“X” indicates deletion)

| Gene | Enzyme Family | MG endA-recA- | Subset 1 | Subset 2 | Subset 3 | Subset 4 | RARE endA-recA- |
|------|---------------|---------------|---------|---------|---------|---------|-----------------|
| \textit{dkgB} | AKR | X | X | X | X |
| \textit{yeaE} | AKR | X | X | X | X |
| \textit{dkgA} | AKR | | X | X | X | X |
| \textit{yqhC} | activator | | X | X | X | X |
| \textit{yqhD} | ADH | | X | X | X | X |
| \textit{yahK} | ADH | | | | | |
| \textit{yjgB} | ADH | | | | | |

Accumulation of benzaldehyde was investigated in the six different strains (Figure 1A). The wild-type strain and three out of the four strains containing subsets of gene knockouts converted all of the supplied 5 mM benzoate into benzyl alcohol within 24 hours, with no accumulation of benzaldehyde. On the other hand, the RARE strain accumulated 3.3 ± 0.1 mM benzaldehyde and displayed less than 12% conversion of benzaldehyde to benzyl alcohol (Fig. 1B). Similarly, the “Subset 4” strain also enabled the accumulation of benzaldehyde. The “Subset 4” strain contained the \textit{dkgB} and \textit{yeaE} genes intact in the genome, indicating that the deletions of \textit{dkgB} and \textit{yeaE} were not necessary for benzaldehyde accumulation under these conditions.
Figure 1. A combination of rational gene deletions enables benzaldehyde accumulation in *E. coli*. (A) Scheme depicting intracellular formation of benzaldehyde from benzoate and endogenous conversion to the byproduct benzyl alcohol. (B) Conversion of 5 mM benzoate after 24 hours in strains transformed with pETDuet-1 and pACYC-car-sfp. (C) Conversion of 5 mM benzoate after 24 hours in RARE strains transformed with pACYC-car-sfp and a pET plasmid harboring the gene indicated below the x axis.

Deleted genes were overexpressed individually alongside *car* in the RARE strain to determine whether each gene could contribute to benzaldehyde reductase activity *in vivo* (Fig. 1C). Benzoate was supplied and formation of aldehyde and alcohol products was monitored as before. Individual overexpression of each of the six target genes prevented detectable accumulation of benzaldehyde. Conversely, benzaldehyde accumulated when the control gene *yqhC* was overexpressed. For some
strains, lower conversion rates were observed in this experiment relative to the previous experiment, which may have been due to lower expression of car in the presence of native gene overexpression. Interestingly, individual overexpression of dkgB and yeaE also restored the inability to accumulate benzaldehyde, even though the deletion strain experiment demonstrated that these deletions were not required under these conditions. The results from these two experiments suggest that native expression of dkgB and yeaE may be minimal under these conditions and that, in general, overexpression experiments alone may mislead efforts to determine the significance of gene deletions. qRT-PCR results provide further support for our hypothesis of low levels of baseline expression of dkgB and yeaE compared to the other targeted genes (Fig. S1). Furthermore, expression of these two genes is not significantly different in the presence or absence of benzaldehyde, whereas expression of all other targeted genes increases upon benzaldehyde addition.

**In vanillate-feeding experiments, the RARE strain eliminates conversion of vanillin into vanillyl alcohol.** We next investigated the utility of the engineered strain by attempting to produce and accumulate other aromatic aldehydes. The RARE strain and subset deletion strains were next fed vanillate to assess the effect of the same set of gene deletions on the undesired conversion of vanillin to vanillyl alcohol (Fig. 2A). Our hypothesis was that a strain that displays minimal reductase activity on benzaldehyde would also display low reductase activity on vanillin. Although the probable physiological substrates of AKRs are smaller aldehydes such as methylglyoxal, benzaldehyde and vanillin are structurally similar, with vanillin differing only by the presence of additional hydroxyl and methoxy groups distant from the aldehyde group.

Cultures were supplied with vanillate and compared 48 hours after induction due to the slower kinetics of Car and endogenous enzymes on vanillate and vanillin relative to benzoate and benzaldehyde. After 48 hours, the RARE and “Subset 4” strains were the only strains that resulted in
no detectable formation of vanillyl alcohol (Fig. 2B). As before, in order to investigate whether each gene could contribute to vanillin reductase activity in vivo, individual overexpression of deleted genes in the RARE strain was examined for the presence of the alcohol after 24 hours. All gene products were active on vanillin, but surprisingly the overexpression of targeted AKRs (DkgB, YeaE, and DkgA) resulted in significantly more vanillyl alcohol production compared to the overexpression of targeted ADHs (YahK, YjgB, and YqhD) (Fig. 2C). As with benzaldehyde, these results suggest that dkgB and yeaE expression may remain minimal in the presence of vanillin and that overexpression results can mislead gene deletion efforts. These results also validate that the RARE strain can be used for the bioconversion of multiple acid substrates into their corresponding aldehydes.
Figure 2. In vanillate-feeding experiments, the RARE strain eliminates conversion of vanillin to vanillyl alcohol. (A) Scheme depicting intracellular formation of vanillin from vanillate and endogenous conversion to the byproduct vanillyl alcohol. (B) Conversion of 5 mM vanillate after 48 hours in strains transformed with pETDuet-1 and pACYC-car-sfp. (C) Conversion of 5 mM vanillate after 24 hours in RARE strains transformed with pACYC-car-sfp and a pET plasmid harboring the gene indicated below the x axis.

The RARE strain enables production of vanillin from glucose in *E. coli*. To investigate whether the RARE strain could enable the production of an aromatic aldehyde directly from glucose, a non-optimized pathway from glucose to vanillin was assembled in the RARE and wild-type strains. Previous reports established a route from glucose to vanillate in *E. coli*\(^{22}\), and a route featuring the same metabolites but different enzymes was also assembled in yeast\(^{18}\). In both cases, the native branch-point metabolite was 3-dehydroshikimate, which is part of the aromatic amino acid biosynthesis pathway (Fig. 3A and Fig. S2). A feedback-resistant form of *E. coli* *aroG* (*aroG*\(^\ast\))\(^{45}\), which encodes a 3-deoxy-D-arabinohexitulosonate 7-phosphate (DAHP) synthase, was included to ensure that flux enters this endogenous pathway.

The heterologous pathway constructed for our experiments consists of three genes: *asbF* from *Bacillus thuringiensis*\(^{35}\), *Hs-S-COMT* from *Homo sapiens*\(^{18,46}\), and *car* (Fig. 3B). Together with *aroG*\(^\ast\) and *sfp*, a total of five genes were overexpressed. The *asbF* gene encodes a 3-dehydroshikimate dehydrogenase, which efficiently converts 3-dehydroshikimate into protocatechuate. The *Hs-S-COMT* gene encodes a soluble *O*-methyltransferase (OMT) that has activity on catechols and related compounds. Depending on the relative enzyme kinetics and availability of co-factors, protocatechuate can either be converted into protocatechualdehyde by Car or be converted into vanillate by OMT. The final step in the pathway is either the conversion of protocatechualdehyde to vanillin by OMT or the
conversion of vanillate to vanillin by Car. Because this pathway can lead to the production of two possible alcohol byproducts (protocatechuic alcohol and vanillyl alcohol), we expected vanillin titers to be greater when using the RARE strain host rather than the wild-type host.

Figure 3. The RARE strain enables production of vanillin from glucose in *E. coli*. (A) Scheme depicting complete pathway from glucose to vanillin with overexpressed *E. coli* aroG* indicated in bold typeface. [PYR = pyruvate, F6P = fructose 6-phosphate, G3P = glyceraldehyde 3-phosphate, X5P = xylulose 5-phosphate, PEP = phosphoenolpyruvate, E4P = erythrose 4-phosphate, DAHP = 3-deoxy-D-arabinoheptulosonate 7-phosphate, DHQ = 3-dehydroquinate, DHS = 3-dehydroshikimate] (B) Scheme illustrating heterologous portion of pathway with two possible undesired alcohol byproducts. (C) Concentration profiles of the six heterologous metabolites of interest (including vanillin) when wild-type
and RARE hosts are transformed with pET-OMT-asbF and pACYC-car-sfp-aroG* plasmids and grown in LB + 1.2% glucose for 48 hours. (D) Concentration profiles of the six heterologous metabolites of interest (including vanillin) produced from glucose as a sole carbon source after 60 hours in the same strains grown in M9 + 1.2% glucose.

Fig. 3C displays the concentrations of the six metabolites of interest produced as a result of the vanillin pathway 48 hours after induction in wild-type and RARE strains. As expected, the dominant products generated using the wild-type host are the two alcohol byproducts, with minimal formation of vanillin (0.014 ± 0.001 mM). Conversely, the dominant products made by the RARE strain are vanillin and its precursor aldehyde, protocatechualdehyde. No detectable protocatechuic alcohol formed, revealing that the RARE host strain is capable of accumulating at least three different aromatic aldehydes (benzaldehyde, vanillin, and protocatechualdehyde). Although some vanillyl alcohol was detected, it represents less than 14% conversion of vanillin formed. Using the RARE strain, the average vanillin titer was 0.78 ± 0.02 mM (119 ± 3 mg/L), representing more than a 55-fold increase in production over the wild-type strain.

Finally, the same strains were cultivated in M9 minimal medium + 1.2% glucose instead of LB medium + 1.2% glucose to determine whether vanillin could be produced from glucose as a sole carbon source. Concentrations of the six metabolites of interest demonstrate that the RARE strain enables the accumulation of vanillin in minimal medium (Fig. 3D). Under these conditions, vanillin is the dominant product of the six metabolites of interest even after 60 hours. Compared to results obtained from growth in LB medium, the ratios of protocatechuate to protocatechualdehyde and vanillate to vanillin sharply increase in M9 minimal medium. Although the build-up of protocatechualdehyde in the cultures grown in LB indicated that the O-methyltransferase was limiting, the greater pool size of vanillate relative to protocatechualdehyde suggests that this enzyme was no longer limiting in M9. Given the documented
lack of specificity of the O-methyltransferase\textsuperscript{18}, the byproduct isovanillin was also produced in these experiments (Fig. S3).

**The RARE strain enables the synthesis of L-PAC in *E. coli.*** We next sought to demonstrate the utility of the RARE strain as a platform for the biocatalysis of products derived from aromatic aldehyde intermediates. Given the reactivity of aldehyde functional groups, there are numerous enzymatic chemistries that may be enabled by the accumulation of aromatic aldehydes. We were particularly interested in carboligations because these reactions are known to yield chiral products. We hypothesized that use of the RARE host strain could enhance synthesis of L-PAC. Wild-type and RARE strains were transformed to express a recombinant mutant PDC (PDC\textsubscript{E473Q}) with improved kinetic properties over the wild-type enzyme from *Z. mobilis*. Specifically, PDC\textsubscript{E473Q} displays inverted partitioning between aldehyde release and carboligation compared to the wild-type PDC, with an up to 100-fold preference for carboligation\textsuperscript{16}. In our case, the PDC\textsubscript{E473Q} enzyme was expected to catalyze the condensation of benzaldehyde supplied exogenously and pyruvate resulting from metabolism of glucose (Fig. 4A).

Expression of only PDC\textsubscript{E473Q} resulted in the synthesis of 2.59 ± 0.04 mM PAC in the RARE strain 24 hours after benzaldehyde addition, along with less than 4% reduction of benzaldehyde to benzyl alcohol (Fig. 4B). We also observed roughly 8% oxidation of benzaldehyde to benzoate. To prevent net oxidation of benzaldehyde, we expressed both Car and PDC\textsubscript{E473Q}. In this case, no benzoate was detected, and similar levels of PAC and benzyl alcohol were produced in the RARE strain. Surprisingly, wild-type strains transformed with the same constructs resulted in no synthesis of PAC (Fig. 4B). This result suggested that the timescale of benzaldehyde reduction is much shorter than 24 hours. To investigate this further, a time course study was performed to monitor the conversion of benzaldehyde to benzyl alcohol using the wild-type strain expressing Car and PDC\textsubscript{E473Q} (Fig. 4C).
Within just 2 hours of supplying 5 mM benzaldehyde, all of the benzaldehyde was either reduced to benzyl alcohol (~4 mM) or lost to the headspace (~1 mM). This result explains why the wild-type *E. coli* host strain failed to produce any PAC under these conditions. The RARE strain enables the synthesis of PAC in *E. coli* by extending the duration of benzaldehyde availability more than 10-fold, to the relevant timescale of PDC_E473Q kinetics.

Figure 4. The RARE strain enables the synthesis of L-phenylacetylcarbinol (L-PAC). (A) Scheme depicting the synthesis of L-PAC from the condensation of exogenously supplied benzaldehyde and metabolized pyruvate, catalyzed by either PDC or PDC_E473Q. (B) Concentration profiles of PAC, benzaldehyde, and benzaldehyde oxidation/reduction products 24 hours after addition of 5 mM benzaldehyde.
benzaldehyde to RARE and wild-type host strains. (C) Time course of benzaldehyde reduction using the wild-type host transformed with pRSF/PDC_E473Q and pACYC/Car/Sfp.

The deletion of all targeted genes to form the RARE strain has no effect on growth. To test whether the full set of gene deletions affects cell growth, the RARE and wild-type strains were grown in LB medium + 1.2% glucose and in M9 minimal medium + 1.2% glucose. In the absence of any particular stress, no effect on specific growth rate was observed under either condition (Fig. S4). The average specific growth rates were $\mu = 1.07 \text{ h}^{-1}$ and $\mu = 0.37 \text{ h}^{-1}$ in LB and M9, respectively. Furthermore, final OD$_{600}$ measurements were taken in the presence of aldehydes synthesized from experiments featuring the RARE and wild-type strains (Fig. S5). These measurements demonstrate that the presence of vanillin at concentrations explored in this study does not significantly affect the growth rate of the RARE strain relative to the wild-type strain. These results reveal the non-essential nature of the complete set of targeted genes and increase the industrial relevance of the engineered strain.

Discussion

AKRs are found in organisms ranging from vertebrates to archaeabacteria$^{47}$ and are believed to be responsible for catalyzing the conversion of methylglyoxal and related reactive metabolites into less toxic compounds$^{37}$. Microbial AKRs belong to ten families: AKR2, AKR3, AKR5, and AKR8-14.$^{28}$ Because AKRs consume the co-factor NADPH to reduce aldehydes, they are expected to be physiologically relevant under aerobic conditions. A total of nine open reading frames that encode AKRs in $E. coli$ have been identified using sequence similarity searches.$^{48}$ Unlike many other $E. coli$ AKRs, DkgA has been characterized and a crystal structure has been resolved.$^{49}$ Activity assays performed $\text{in vitro}$ constitute the majority of published data on $E. coli$ AKRs and demonstrate that most of these gene products act on overlapping sets of substrates.$^{37}$ Perhaps because of the known redundancy
of AKR activity in *E. coli*, no one has previously reported the intentional deletion of AKRs for the purpose of building up aldehyde pools. To our knowledge, there are no previous publications even describing the simultaneous deletion of two or more AKRs in *E. coli*. In our case, although we initially focused on AKRs as rational targets for deletion, we found that two out of the three targeted AKRs were not contributing to benzaldehyde or vanillin reduction under the conditions tested. Nevertheless, we also found that all AKRs were active on these substrates when overexpressed, and in the case of vanillin, they were significantly more active than targeted ADHs. From our experiments, it is still unclear what conditions, if any, may lead to significant expression of *dkgB* and *yeaE*, but there was no reduction in growth rate or other disadvantage incurred by their deletion.

Previously, yields of some aldehydes have been increased by deleting ADH genes responsible for reductase activity in model organisms such as *S. cerevisiae* and *E. coli*. ADHs are generally classified under the short-chain\textsuperscript{50-52} or medium-chain\textsuperscript{53,54} dehydrogenase/reductase families and, like AKRs, are known to have broad and redundant substrate specificity. Specifically, the ADH6 gene in *S. cerevisiae* was deleted in another study in order to produce 45 mg/L of vanillin from glucose.\textsuperscript{18} However, other genes known to convert aldehydes to alcohols in *S. cerevisiae* remained intact in that study and no further deletions have been reported to our knowledge. Another report described an effort to improve the production of isobutyraldehyde, which is a bulk chemical feedstock.\textsuperscript{19} In that study, eight genes were deleted (*yqhD, adhP, eutG, yiaY, yjgB, betA, fucO, eutE*). Unlike in our study, only five of these targeted genes (*yqhD, adhP, eutG, yiaY, yjgB*) were found to be capable of reducing isobutyraldehyde to isobutanol when individually overexpressed. Overall, the combination of all eight deletions resulted in an improvement in the ratio of isobutyraldehyde to isobutanol produced from 0.14 g\textsubscript{aldehyde}/L/OD\textsubscript{600} and 1.5 g\textsubscript{alcohol}/L/OD\textsubscript{600} to 1.5 g\textsubscript{aldehyde}/L/OD\textsubscript{600} and 0.4 g\textsubscript{alcohol}/L/OD\textsubscript{600}.\textsuperscript{19} However, the deletion of the *yqhD* gene alone led to a 1:1 ratio of aldehyde to alcohol, indicating a diminishing
marginal return on the deletions of the four other genes that may contribute to isobutryaldehyde reduction. Neither of the aforementioned studies described an attempt to delete genes encoding AKRs nor did they culture their strains under aerobic conditions.

By rationally evaluating three AKR deletions in combination with three ADH deletions, we constructed an *E. coli* host strain that displayed a significant step-change in the ability to accumulate aromatic aldehydes relative to several engineered strains containing subsets of these deletions. The brute-force method of rationally targeting and combining of several knockouts to overcome a redundant endogenous activity should become more accessible given significant advances in genome engineering and an increasing rate of functional gene annotation. Techniques that enable prompt construction of rational combinations of gene knockouts, such as Multiplex Automated Genome Engineering (MAGE)\textsuperscript{55}, or gene expression knockdowns, such as RNA or CRISPR interference\textsuperscript{56}, can be harnessed in future studies like this.

However, selection of the correct target genes to eliminate a highly redundant activity is not straightforward. Kinetic data from purified enzyme assays may be misleading given that enzyme activity and gene expression have no correlation but together affect endogenous activity. Similarly, we have shown that gene products displaying undesired activity *in vivo* when overexpressed may not need to be deleted in order to eliminate the undesired endogenous activity. A further complication is that not all of the necessary target genes may be fully characterized. Current genome modeling approaches for determining knockouts such as OptStrain\textsuperscript{57} would not predict these deletions. In fact, OptStrain has already been used in an attempt to optimize vanillin production in *E. coli* and did not include any of the aldehyde reductases targeted in our study.\textsuperscript{57} The alternative of transcriptional profiling is limited by the inability to account for redundant or constitutively expressed genes, as well as by off-target effects and secondary responses. Finally, combinatorial approaches to gene knockouts\textsuperscript{58,59} have led to the generation
of numerous strain improvements but are unlikely to surmount sufficiently redundant activities, especially in the absence of an effective selection strategy.

Although the creation of an *E. coli* strain that has minimal conversion of vanillin to vanillyl alcohol has been previously sought after\textsuperscript{22,33,60}, some groups have reported alternative strategies to use *E. coli* for vanillin production. At least one report documents the use of resting *E. coli* cells for the bioconversion of ferulic acid into vanillin with minimal vanillyl alcohol formation under these conditions.\textsuperscript{61} Under non-growing conditions, it is likely that the AKR and ADH genes identified in this study are not expressed to a high degree. However, the use of resting *E. coli* cells is not a viable strategy for producing vanillin from glucose, which is an order of magnitude more affordable as a starting substrate than ferulic acid ($0.3$/kg versus $5$/kg).\textsuperscript{18} Additionally, microbial cells in a non-growing state lose desired activity more rapidly than cells in a growing state, frequently resulting in lower overall productivity. Another group has published the use of growing *E. coli* cells for the bioconversion of ferulic acid into vanillin, asserting that *E. coli* has no degradation pathway from vanillin to vanillyl alcohol.\textsuperscript{62-64} It may be that no attempt to detect vanillyl alcohol was made in these particular studies. As illustrated throughout this report, even when performing simple bioconversions using vanillate as a substrate, we observe significant vanillyl alcohol formation. Whether for the purpose of bioconversions or utilization of glucose as a sole carbon source, the engineered strain reported in this study is expected to be a superior *E. coli* host strain for producing aromatic aldehydes.

The ability of the RARE strain to accumulate aromatic aldehydes has broad implications for the biosynthesis of products derived from aldehyde intermediates. The demonstration of L-PAC synthesis illustrates that aldehydes can be biologically converted into new chemical classes if they can avoid the fate of rapid reduction. This previously unattainable option may be more attractive than harvesting resting cells or using purified enzymes for many biocatalytic processes. Furthermore, it opens up the
potential for synthesis of end products directly from glucose as a sole carbon source. Besides pyruvate
decarboxylase, other carboligases have been used \textit{in vitro} to produce enantiopure products using
benzaldehyde as an intermediate, such as benzoins\textsuperscript{65} and 2-hydroxypropiophenones\textsuperscript{66}. However, as just
one enzyme class relying on a single aldehyde substrate, carboligations that utilize benzaldehyde offer a
small view into the range of microbial products potentially attainable from aromatic aldehyde
intermediates. For example, aromatic aldehydes can also serve as efficient amine acceptors, and there
are transaminases reported to catalyze the formation of aromatic primary amines.\textsuperscript{67}

\textbf{Conclusion}

Aromatic aldehydes are valuable chemicals, either as end products or as intermediates. Microbial
synthesis of aromatic aldehydes is ordinarily hindered by rapid, endogenous, and redundant conversion
to alcohol byproducts. We have constructed an \textit{E. coli} K-12 MG1655 strain that can accumulate a
variety of industrially relevant aromatic aldehydes, including benzaldehyde, protocatechualdehyde, and
vanillin. This result was achieved by rationally targeting six genes for deletion, spanning the aldo-keto
reductase and alcohol dehydrogenase superfamilies. This particular combination of deletions resulted in
a step-change in aromatic aldehyde accumulation, whereas several strains containing subsets of these
deletions did not deviate significantly from the wild-type strain. In spite of all of these deletions, the
growth rate of the engineered strain was unperturbed. The engineered strain was used to enable the
synthesis and accumulation of vanillin and L-phenylacetylcarbinol. Finally, the use of this engineered
strain may enable microbial synthesis of a variety of other compounds enzymatically derived from
aromatic aldehyde intermediates, expanding the boundaries of metabolic engineering and biocatalysis.

\textbf{Acknowledgements}
The authors would like to acknowledge Micah J. Sheppard for engaging in helpful discussions throughout the project. This work was supported by the Synthetic Biology Engineering Research Center (SynBERC, Grant No. EEC-0540879). A.M.K. is a recipient of a National Science Foundation Graduate Research Fellowship.

Supporting Information

Five figures, four tables, and text showing qRT-PCR results (Figure S1), chemical structures of select pathway metabolites (Figure S2), isovanillin titers (Figure S3), growth curves and growth rates of wild-type and RARE strains (Figure S4), final OD_600 measurements (Figure S5), strains and plasmids (Table S1), oligonucleotides (Table S2), reported enzyme activities on benzaldehyde (Table S3), results of protein sequence alignments (Table S4), and codon-optimized or mutant gene sequences (SI Text). Supporting information is available free of charge via the Internet at http://pubs.acs.org.
References

1. Hagedorn, S.; Kaphammer, B. *Annu. Rev. Microbiol.* **1994**, *48*, 773.

2. Krings, U.; Berger, R. G. *Appl. Microbiol. Biotechnol.* **1998**, *49*:1, 1.

3. Fahlbusch, K.; Hammerschmidt, F.; Panten, J.; Pickenhagen, W.; Schatkowski, D.; Bauer, K.; Garbe, D.; Surburg, H. In *Flavors and Fragrances*: Ullmann's Encyclopedia of Industrial Chemistry; Wiley-VCH Verlag GmbH & Co. KGaA: 2000.

4. Burdock, G. A. *Fenaroli's Handbook of Flavor Ingredients*; CRC Press: Boca Raton, Florida, 2005.

5. Feron, G.; Wache, Y. In *1.16 Microbial Biotechnology of Food Flavor Production*; Pometto, A., Shetty, K., Paliyath, G. and Levin, R., Eds.; Food Biotechnology, Second Edition; CRC Press: Boca Raton, FL, 2006; 407.

6. Li, T.; Rosazza, J. P. N. *Appl. Environ. Microbiol.* **2000**, *66*, 684.

7. Priefert, H.; Rabenhorst, J.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.* **2001**, *56*:3-4, 296.

8. Kaur, B.; Chakraborty, D. *Appl. Biochem. Biotechnol.* **2013**, *169*, 1353.

9. Duff, S. J. B.; Murray, W. D. *Biotechnol. Bioeng.* **1989**, *34*, 153.

10. Nierop Groot, M. N.; de Bont, J. A. M. *Appl. Environ. Microbiol.* **1998**, *64*, 3009.

11. Jain, A. N.; Khan, T. R.; Daugulis, A. J. *Biotechnol. Lett.* **2010**, *32*:11, 1649.

12. Walton, N. J.; Mayer, M. J.; Narbad, A. *Phytochemistry* **2003**, *63*, 505.

13. Pohl, M.; Lingen, B.; Muller, M. *Chem. -- Eur. J.* **2002**, *8*, 5288.

14. Tripathi, C. M.; Agarwal, S. C.; Basu, S. K. *J. Ferment. Bioeng.* **1997**, *84*, 487.

15. Yun, H.; Kim, B. *Biotechnol. Bioprocess Eng.* **2008**, *13*, 372.

16. Meyer, D.; Walter, L.; Kolter, G.; Pohl, M.; Muller, M.; Tittmann, K. *J. Am. Chem. Soc.* **2011**, *133*, 3609.

17. Li, T.; Rosazza, J. P. N. *Appl. Environ. Microbiol.* **2000**, *66*, 684.

18. Hansen, E. H.; Møller, B. L.; Kock, G. R.; Bünner, C. M.; Kristensen, C.; Jensen, O. R.; Okkels, F. T.; Olsen, C. E.; Motawia, M. S.; Hansen, J. *Appl. Environ. Microbiol.* **2009**, *75*, 2765.

19. Rodriguez, G.; Atsumi, S. *Microb. Cell Fact.* **2012**, *11*, 90.
20. Akhtar, M. K.; Turner, N. J.; Jones, P. R. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 87.

21. Howard, T. P.; Middelhaufe, S.; Moore, K.; Edner, C.; Kolak, D. M.; Taylor, G. N.; Parker, D. A.; Lee, R.; Smirnoff, N.; Aves, S. J.; Love, J. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7636.

22. Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1998**, *120*, 10545.

23. Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 9361.

24. Draths, K. M.; Pompliano, D. L.; Conley, D. L.; Frost, J. W.; Berry, A.; Disbrow, G. L.; Staversky, R. J.; Lievense, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 3956.

25. Snell, K. D.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 5605.

26. Berry, A. *Trends Biotechnol.* **1996**, *14*, 250.

27. Food and Drug Administration, Department of Health and Human Services 21 CFR 184.1685. **2012**.

28. Ellis, E. M. Microbial aldo-keto reductases. *FEBS Microbiol. Lett.* **2002**, *216*, 123.

29. Sambrook, J.; Russell, D. W. *Molecular cloning: a laboratory manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., 2001.

30. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H. *Mol Syst Biol* **2006**, *2*, 2006.0008.

31. Thomason, L. C.; Costantino, N.; Court, D. L. In *E. coli Genome Manipulation by P1 Transduction*; Current Protocols in Molecular Biology; John Wiley & Sons, Inc.: 2001.

32. Datsenko, K. A.; Wanner, B. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 6640.

33. He, A.; Li, T.; Daniels, L.; Fotheringham, I.; Rosazza, J. P. N. *Appl. Environ. Microbiol.* **2004**, *70*, 1874.

34. Nakano, M. M.; Corbell, N.; Besson, J.; Zuber, P. *Mol. Gen. Genet.* **1992**, *237*, 313.

35. Fox, D. T.; Hotta, K.; Kim, C.; Koppisch, A. T. *Biochemistry* **2008**, *47*, 12251.

36. Klock, H. E.; Lesley, S. A. In *The Polymerase Incomplete Primer Extension (PIPE) Method Applied to High-Throughput Cloning and Site-Directed Mutagenesis*; Doyle, S. A., Ed.; High Throughput Protein Expression and Purification; Humana Press: 2009; 91.

37. Ko, J.; Kim, I.; Yoo, S.; Min, B.; Kim, K.; Park, C. *J. Bacteriol.* **2005**, *187*, 5782.

38. Turner, P. C.; Miller, E. N.; Jarboe, L. R.; Baggett, C. L.; Shanmugam, K. T.; Ingram, L. O. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 431.
39. Sulzenbacher, G.; Alvarez, K.; van den Heuvel, R. H. H.; Versluis, C.; Spinelli, S.; Campanacci, V.; Valencia, C.; Cambillau, C.; Eklund, H.; Tegoni, M. J. Mol. Biol. 2004, 342, 489.

40. Pérez, J. M.; Arenas, F. A.; Pradenas, G. A.; Sandoval, J. M.; Vásquez, C. C. J. Biol. Chem. 2008, 283, 7346.

41. Miller, E. N.; Jarboe, L. R.; Yomano, L. P.; York, S. W.; Shanmugam, K. T.; Ingram, L. O. Appl. Environ. Microbiol. 2009, 75, 4315.

42. Pick, A.; Rühmann, B.; Schmid, J.; Sieber, V. Appl. Microbiol. Biotechnol. 2013, 97:13, 5815.

43. Koma, D.; Yamanaka, H.; Moriyoshi, K.; Ohmoto, T.; Sakai, K. Appl. Environ. Microbiol. 2012, 78, 6203.

44. Venkitasubramanian, P.; Daniels, L.; Rosazza, J. P. N. J. Biol. Chem. 2007, 282, 478.

45. Juminaga, D.; Baidoo, E. E. K.; Redding-Johanson, A. M.; Batth, T. S.; Burd, H.; Mukhopadhyay, A.; Petzold, C. J.; Keasling, J. D. Appl. Environ. Microbiol. 2012, 78, 89.

46. Zhang, J.; Klinman, J. P. J. Am. Chem. Soc. 2011, 133, 17134.

47. Jez, J. M.; Penning, T. M. Chem. Biol. Interact. 2001, 130–132, 499.

48. Lapthorn, A. J.; Zhu, X.; Ellis, E. M. Chem. Biol. Interact. 2013, 202, 168.

49. Jeudy, S.; Monchois, V.; Maza, C.; Claverie, J.; Abergel, C. Proteins: Struct., Funct., Bioinf. 2006, 62, 302.

50. Joernvall, H.; Persson, B.; Krook, M.; Atrian, S.; Gonzalez-Duarte, R.; Jeffery, J.; Ghosh, D. Biochemistry 1995, 34, 6003.

51. Oppermann, U.; Filling, C.; Hult, M.; Shafqat, N.; Wu, X.; Lindh, M.; Shafqat, J.; Nordling, E.; Kallberg, Y.; Persson, B.; Jörnvall, H. Chem. Biol. Interact. 2003, 143–144, 247.

52. Kavanagh, K. L.; Jornvall, H.; Persson, B.; Oppermann, U. Cell. Mol. Life Sci. 2008, 65, 3895.

53. Persson, B.; Zigler, J. S.; Jörnvall, H. Eur. J. Biochem. 1994, 226, 15.

54. Nordling, E.; Jörnvall, H.; Persson, B. Eur. J. Biochem. 2002, 269, 4267.

55. Wang, H. H.; Isaacs, F. J.; Carr, P. A.; Sun, Z. Z.; Xu, G.; Forest, C. R.; Church, G. M. Nature 2009, 460, 894.

56. Qi, L.; Larson, M.; Gilbert, L.; Doudna, J.; Weissman, J.; Arkin, A.; Lim, W. Cell 2013, 152, 1173.

57. Pharkya, P.; Burgard, A. P.; Maranas, C. D. Genome Res. 2004, 14, 2367.
58. Alper, H.; Miyaoku, K.; Stephanopoulos, G. Nat. Biotech. **2005**, 23, 612.

59. Santos, C. N. S.; Xiao, W.; Stephanopoulos, G. Proc. Natl. Acad. Sci. U.S.A. **2012**, 109, 13538.

60. Venkitasubramanian, P.; Daniels, L.; Das, S.; Lamm, A. S.; Rosazza, J. P. N. Enzyme Microb. Technol. **2008**, 42, 130.

61. Barghini, P.; Di Gioia, D.; Fava, F.; Ruzzi, M. Microb. Cell Fact. **2007**, 6, 13.

62. Yoon, S.; Li, C.; Kim, J.; Lee, S.; Yoon, J.; Choi, M.; Seo, W.; Yang, J.; Kim, J.; Kim, S. Biotechnol. Lett. **2005**, 27, 1829.

63. Yoon, S.; Lee, E.; Das, A.; Lee, S.; Li, C.; Ryu, H.; Choi, M.; Seo, W.; Kim, S. Biotechnol. Prog. **2007**, 23, 1143.

64. Lee, E.; Yoon, S.; Das, A.; Lee, S.; Li, C.; Kim, J.; Choi, M.; Oh, D.; Kim, S. Biotechnol. Bioeng. **2009**, 102, 200.

65. Dunkelmann, P.; Kolter-Jung, D.; Nitsche, A.; Demir, A. S.; Siegert, P.; Lingen, B.; Baumann, M.; Pohl, M.; Muller, M. J. Am. Chem. Soc. **2002**, 124, 12084.

66. Brandt, G. S.; Kneen, M. M.; Petsko, G. A.; Ringe, D.; McLeish, M. J. J. Am. Chem. Soc. **2010**, 132, 438.

67. Fuchs, M.; Tauber, K.; Sattler, J.; Lechner, H.; Pfeffer, J.; Kroutil, W.; Faber, K. RSC Adv. **2012**, 2, 6262.

68. Tseng, H.; Martin, C. H.; Nielsen, D. R.; Prather, K. L. J. Appl. Environ. Microbiol. **2009**, 75, 3137.

69. Tseng, H.; Harwell, C. L.; Martin, C. H.; Prather, K. L. J. Microb. Cell Fact. **2010**, 9, 96.

70. Salis, H. M.; Mirsky, E. A.; Voigt, C. A. Nat. Biotech. **2009**, 27, 946.
