Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol
Retro-biosynthetic screening of a modular pathway design achieves selective route for microbial synthesis of 4-methyl-pentanol

Micah J. Sheppard, Aditya M. Kunjapura, Spencer J. Wenck, and Kristala L. J. Prather

Department of Chemical Engineering, and Synthetic Biology Engineering Research Center, Massachusetts Institute of Technology, Cambridge, MA 02139

Abstract

Recently, increasingly complex metabolic pathways have been engineered by modifying natural pathways and establishing de novo pathways with enzymes taken from a variety of organisms. Here, we have applied retro-biosynthetic screening to a modular pathway design in order to identify a redox neutral, theoretically high yielding route to a branched C6 alcohol. Enzymes capable of converting natural E. coli metabolites into 4-methyl-pentanol via CoA-dependent chemistry were taken from 9 different organisms to form a ten step de novo pathway. Selectivity for 4-methyl-pentanol was enhanced through the use of key enzymes acting on acyl-CoA intermediates, a carboxylic acid reductase from Nocardia iowensis, and an alcohol dehydrogenase from Leifsonia sp. Strain S749. One implementation of the full pathway from glucose demonstrated selective carbon chain extension and acid reduction with 4-methyl-pentanol constituting 81% (90 ± 7 mg/L) of the observed alcohol products. In this initial demonstration, the highest observed 4MP titer was 192 ± 23 mg/L.
Introduction

Increasing interest in sustainable production of a wide range of chemical products has encouraged development of microbial catalysts for the conversion of renewable feedstocks to specialty and bulk chemicals as well as transportation fuels. While natural hosts and metabolic pathways have been used for decades in the production of chemical products, the desire to synthesize direct replacements for current petroleum products has led to appropriation of natural pathways for production of noncognate chemicals. Early examples of this new paradigm have focused on introducing or combining portions of natural pathways in alternative host organisms or creating new products by altering the termination of natural pathways of a host organism with promiscuous enzymes. Recent work has been focused on improving modified natural pathways by substituting new enzymes to improve kinetics, improve expression, or utilize alternate cofactors. In some cases, pathways have been repurposed to synthesize new products by capitalizing on the natural capacity of enzymes to accept closely related substrates or by engineering protein specificity. The boldest designs have utilized previously undescribed pathways created by combining the natural chemistry of individual enzymes from multiple hosts.

Liquid transportation fuels are one class of chemical targets for which natural pathways to exact replacements have not been discovered. Recent interest in microbial production of renewable fuels has led to successful synthesis of a variety of next-generation biofuels with improved properties over ethanol. Microbial synthesis of these reduced chemical species by de novo designed pathways can potentially lead to more efficient production strains which are necessary for next-generation targets to achieve commercial relevance. Predominantly these pathways have employed reconstitution of natural pathways in new hosts (acetone-butanol-ethanol (ABE) or...
modified termination of natural pathways (fatty acid synthesis (FAS)\textsuperscript{11, 23, 24}, amino acid synthesis\textsuperscript{12, 29}, isoprenoid synthesis\textsuperscript{22}).

While natural metabolites of lipid and terpene synthesis closely resemble biodiesel, there are few natural metabolites that closely mimic gasoline. Because gasoline constitutes 40% of total U.S. petroleum consumption, a bio-based gasoline alternative would be useful for alleviating petroleum reliance. Two synergistic approaches are required for making a bio-based gasoline more economical: utilization of less expensive feedstocks and implementation of high efficiency pathways\textsuperscript{30, 31, 32, 33}. The presented work focuses on development of a high efficiency pathway for a bio-alternative alcohol for spark ignition engines. Such an alcohol would ideally fall in the C6-C7 range\textsuperscript{25, 26, 34}. These medium-chain length alcohols achieve energy density equal to that of petroleum derived gasoline (32 MJ/L) (Fig. S1A)\textsuperscript{35, 36, 37, 38}. Branched alcohols have the additional desired property of improved octane rating (Fig. S1B)\textsuperscript{34}. To date, the best examples of synthesizing such compounds have come from modified termination of branched amino acid synthesis or from isoprenoid synthesis\textsuperscript{22, 29}. The amino acid based pathway utilizes carbon chain extension by engineering $\alpha$-ketoacid elongation ($\alpha$KAE) enzymes and an $\alpha$-ketoacid decarboxylase to produce a blend of alcohols (Fig. 1A). The inefficient carbon chain extension mechanism creates a redox imbalance for medium-chain products, which limits maximum pathway efficiency\textsuperscript{39}. The isoprenoid pathway can be used to produce C5 isopentenol, but it is limited to forming carbon chain lengths in multiples of five because it uses isopentenyl diphosphate (IPP) as a C5 building block, and it is limited by the inherent redox imbalance of the pathway.

Here, we present a pathway design which combines a portion of a native pathway (valine biosynthesis) with a ten step \textit{de novo} pathway to produce 4-methyl-pentanol. In order to identify specific pathway variants, we utilized a conceptual modular framework based on general natural chemistries. Biosynthetic routes to alkyl chains most commonly employ a system of precursor generation followed by
chain elongation through carbon-carbon bond forming reactions\textsuperscript{40, 41, 42}. We structured our conceptual modules to correspond to this pathway structure. We envisioned precursor generating modules (Glycolysis and Modules 1 and 2) and carbon chain elongation modules (Module 3) coupled to alcohol terminating modules (Module 4).

This approach produced a pathway with enzymes selected from nine different metabolic contexts (organisms and/or pathways) (Fig. 1B and 1C). Four enzymes were selected to act on their presumed cognate substrates and 6 were applied to presumed noncognate substrates in the engineered pathway.

The core Module 3 pathway architecture is based on synthetic CoA-dependent chemistry first understood in the ABE pathway of \textit{Clostridium acetobutylicum}. This CoA-dependent architecture has several advantages compared to the αKAE and isoprenoid pathways described above. Biosynthetic CoA-dependent pathways typically utilize acetyl-CoA building blocks and extend carbon chains through condensation reactions without release of CO\textsubscript{2}. The potential for generation of two reducing equivalents per acetyl-CoA generated from glycolysis perfectly balances with those consumed for reduction to a primary alcohol product\textsuperscript{15, 43}. Indeed, the presented pathway achieves redox neutrality and a maximum theoretical yield of 0.67 mol 4MP/mol glucose (0.38 g/g, 100% maximum pathway energy efficiency). β-oxidation chemistry has been used for synthesis of several straight-chain acids and alcohols\textsuperscript{5, 6, 16, 21, 44}. Unlike these previous demonstrations of CoA-dependent pathways, here we present the expansion of potential products to branched alcohols of medium chain length using independently selected enzymes chosen to enhance specificity for our desired intermediates.

**Results**

**4MP Pathway Description.** The 4MP pathway design does not rely on the simple transfer of a single recombinant pathway; rather, it relies on a patchwork of enzymes from multiple organisms and multiple natural pathways (Fig. 1A and 1B). Figure 1 presents the overall pathway as a composite of 4 modules.
Module 1, an adaptation of previously described routes to isobutanol and isobutyrate\textsuperscript{12,45}, converts pyruvate, from glycolysis, to $\alpha$-keto-isovalerate ($\alpha$-KIV) via valine biosynthesis using \textit{Bacillus subtilis} acetolactate synthase Als\textsubscript{Bs}, \textit{Escherichia coli} acetoxyhydroxy acid isomeroreductase Ilv\textsubscript{Ec}, and \textit{E. coli} dihydroxy acid dehydratase IlvD\textsubscript{Ec}. $\alpha$-KIV is further converted to isobutyrate by the \textit{Lactococcus lactis} decarboxylase KivD\textsubscript{Ll} and an isobutyraldehyde preferring aldehyde dehydrogenase from \textit{Flavobacterium johnsoniae} (Fjoh\_2967)\textsuperscript{46}. Module 2 consists of the ATP-dependent activator \textit{Rhodopseudomonas palustris} isobutyryl-CoA ligase IbuA\textsubscript{Rp}, which converts isobutyrate to isobutyryl-CoA\textsuperscript{47}. Acetyl-CoA, generated by the endogenous pyruvate decarboxylase complex, condenses with isobutyryl-CoA in the first reaction of Module 3, mediated by the \textit{Cupriavidus necator} thiolase BktB\textsubscript{Cn}. The subsequent reactions of Module 3 reduce the branched 3-keto-4-methylvaleryl-CoA intermediate to 4-methylvaleryl-CoA by \textit{C. necator} acetoacetyl-CoA reductase PhaB\textsubscript{Cn}, \textit{C. necator} enoyl-CoA hydratase PhaJ4\textsubscript{Cn}, and \textit{Treponema denticola} enoyl-CoA reductase Ter\textsubscript{td}\textsuperscript{14,15,48,49,50,51,52}. Endogenous thioesterase activity (potentially from TesB and Ydi\textsubscript{S}\textsuperscript{53}) generates free 4-methyl-valerate (4MV). Module 4 reduces the free 4MV to 4MP by the \textit{Nocardioides iowensis} carboxylic acid reductase Car\textsubscript{Ni} and either \textit{Saccharomyces cerevisiae} alcohol dehydrogenase Adh6p\textsubscript{Sc} or \textit{Leifsonia} sp. Strain S749 alcohol dehydrogenase Lsadh\textsuperscript{54,55,56,57,58} (see Supplementary Fig. S2 for enzyme cofactor requirements and byproduct reactions).

Throughout this manuscript, strain names indicate the modules present in the strain (i.e., M1F2I34 includes “M” for modules, “1F” for Module 1 with fea\textsubscript{Ec}, “2I” for Module 2 with ibuA\textsubscript{Rp}, “3” for Module 3, and “4” for Module 4). Strain names with “( )” contain abbreviations for operon structure indicating the order of als\textsubscript{Bs} and ilv\textsubscript{Ec} (i.e., M1F(IA)2I34 includes (IA) indicating an ilv\textsubscript{Ec}-als\textsubscript{Bs} operon order). See Table 1 and Supplementary Table S1 for descriptions of all strains used and Supplementary Table S2 for relevant descriptions of enzymes used in all pathway variants.

**Identification of Acid and Aldehyde Reductases for Synthesis of 4MP (Module 4).** Adoption of a CoA-dependent synthesis route required identification of pathways to link a saturated CoA thioester to the
final alcohol product. The CoA thioester could be reduced by either a CoA-dependent aldehyde
dehydrogenase or thioesterase/carboxylic acid reductase pairing. The resulting aldehyde could then be
reduced by an alcohol dehydrogenase to generate the primary alcohol product. The wide array of
identified alcohol dehydrogenases created a high probability that an alcohol dehydrogenase could be
found for conversion to the final alcohol. S. cerevisiae Adh6pSc was initially selected because it
was previously found to be a broad specificity alcohol dehydrogenase with high activity on medium- and
branched-chain aliphatic aldehydes.

A smaller number of acid or CoA-thioester reductases have been characterized in the literature. From
these previously identified enzymes, we looked to identify candidates with the potential to selectively
convert 4MV to 4-methyl-valeraldehyde. Recently, a carboxylic acid reductase (Car) from
Mycobacterium marinum was shown to convert a range of straight-chain fatty acids to fatty aldehydes,
but with increasing catalytic efficiency \((k_{\text{cat}}/K_{M})\) for longer chain lengths. A previously studied
homolog, Car from N. iowensis, was found to have activity on a broad array of acids, but its activity on a
range of aliphatic acids was not examined. CarNi from N. iowensis was selected for further study to
determine if it had specificity for targeted medium-chain branched acids.

Assays were devised to confirm activity on desired substrates in vitro and in vivo. First, N-terminal His-
tagged CarNi was purified and assayed for relative activity on 13 straight and branched acid substrates
from C2-C8. CarNi showed a peak in activity for acids with a primary chain-length of 5-6 carbons (Fig.
2A). The highest CarNi activity was found for the branched species 4MV and 4-methyl-hexanoate. Given
the need to reduce flux of precursors to undesired byproduct alcohols, CarNi was a logical selection
because of its preference for 4MV over the short-chain acids acetate, isobutyrate, and butyrate. A
complementary in vivo assay was designed to examine the effectiveness of the Module 4 pairing.

Butyrate, valerate, 3-methyl-valerate, 4MV, and hexanoate were fed to E. coli cultures expressing
CarNi/Adh6pSc and conversion was monitored by sampling the culture media. OD_{600}-normalized conversions followed a trend similar to observed *in vitro* results with maximal conversion for substrates with C5 primary chain length, as desired (Supplementary Fig. S3).

Strong specificity of CarNi for 4MV over isobutyrate was desired because isobutyrate is produced as an upstream intermediate. The Michaelis-Menten kinetic parameters of CarNi were found using these two key intermediates to confirm the desired specificity (Fig. 2B). The $k_{cat}/K_m$ ratio with 4MV was found to be 450 times higher than with isobutyrate, indicating the significant preference of CarNi for 4MV over other acid substrates generated by the pathway. With a $K_m$ of 78 ± 9 mM with isobutyrate, CarNi is expected to convert isobutyrate poorly under physiologically relevant concentrations, which limits shunting of the precursor to isobutyraldehyde.

**Identification of Dehydratases and Reductases for Synthesis of 4MV (Module 3).** Enzymes of the *Clostridium acetobutylicum* butanol pathway and enzymes from polyhydroxyalkanoate pathways have previously been used to synthesize straight chain butanol and pentanol. Previous work from our group found the bktBCn/phaBCn combination is capable of synthesizing 3-hydroxy-4-methylvaleryl-CoA from isobutyryl-CoA and acetyl-CoA. To the best of our knowledge, 3-hydroxy-acyl-CoA dehydratases and trans-enoyl reductases with activity on the subsequent branched intermediates have not been identified. From enzymes documented to have activity on straight medium-chain CoA substrates, 4 phaJ and 6 ter homologs were selected for further screening (Supplementary Table S1). An assay was developed to screen for enzymes with the desired activity by isolating Modules 2 and 3 of our pathway *in vivo* with different combinations of dehydratases and reductases. Isobutyrate (10 mM) and glucose (1%) were supplied in LB medium, and active gene combinations were identified by detecting 4MV secretion. The propionyl-CoA transferase from *Megasphera elsdenii*, pct_{Me}, was used to activate isobutyrate. Of the 24 combinations tested, phaJ4 homologs from *Pseudomonas syringae*,
Pseudomonas aeruginosa, and C. necator in combination with ter homologs from Vibrio parahaemolyticus and T. denticola produced 4MV (Supplementary Fig. S4A). The high producer, C. necator phaJ4bCn/T. denticola terTd (Strain M3Sc-TdCn), yielded 297 ± 45 mg/L 4MV and was selected for Module 3 moving forward. The previously used dehydratase hbdCa and reductase crtCa from the Clostridium acetobutylicum butanol pathway were also tested in place of phaBCn and phaJ4bCn (Strain M3Sc-Ca) (Supplementary Fig. S4B). While some butyrate was produced by Strain M3Sc-Ca, 4MV was not detected.

With Module 4 in vivo activity previously confirmed, 4MP production from glucose and isobutyrate was tested with a strain expressing Module 2, 3, and 4 genes (Strain M2P34). As predicted by observed activities for CarNi and Adh6pSc, Strain M2P34 preferentially produced 4MP (272 ± 7 mg/L) over isobutanol (111 ± 7 mg/L) and butanol (142 ± 9 mg/L) even while feeding 10 mM (870 mg/L) isobutyrate (Fig. 2C).

**Incorporation of a Pathway from Glucose to Isobutyryl-CoA (Modules 1 & 2).** Combining isobutyrate synthesis with CoA activation supplies the necessary isobutyryl-CoA precursor for 4MP synthesis from glucose or other simple carbon sources. The Module 1 pathway was identified by building from the previous work of Zhang et al., which synthesized isobutyrate in E. coli by combining valine biosynthesis with the L. lactis kivDLl decarboxylase and various native aldehyde dehydrogenases, including PuuC and FeaB. In previous work, our group has utilized the M. elsdenii transferase PctMe for CoA activation of carboxylic acids including isobutyrate. The ATP-dependent isobutyryl-CoA ligase (IbuArp) from R. palustris provides an alternative activation mechanism for Module 2 which creates a redox neutral overall pathway.

Multiple module combinations were used to explore the activities of the two E. coli aldehyde dehydrogenases selected for the final oxidation of isobutyraldehyde to isobutyrate. Module 1
expression with Modules 2 (pctMe) and 3, led to production of 4MV from glucose with titers up to 111 ± 185 mg/L (puuCEc) and 90 ± 9 mg/L (feaBEc) (Supplementary Fig. S5A). Module 1 with puuCEc led to higher 4MV titers when coupled to Modules 2 and 3, but it was possible that the *E. coli* aldehyde dehydrogenases had activity on the Module 4 intermediate 4-methyl-valeraldehyde which could regenerate 4MV and decrease reduction to 4MP when Module 4 is added. Both aldehyde dehydrogenases were used for alternate versions of the full pathway, and only the feaBEc strain (Strain M1F2P34) produced 4MP (67 ± 13 mg/L) while the puuCEc strain (Strain M1P2P34) produced 4MV (67 ± 11 mg/L) (Supplementary Fig. S5B). Additionally the puuCEc strain produced more butyrate (156 ± 4 mg/L) and less butanol (15 ± 6 mg/L) than the feaBEc strain (62 ± 15 mg/L butyrate, 49 ± 17 mg/L butanol).

While a demonstration of 4MP synthesis from glucose was made, relatively low 4MP titers and high isobutyrate (1113 ± 34 mg/L) and isobutanol (2205 ± 225 mg/L) titers suggested there were possible bottlenecks in the initial strain, Strain M1F2P34. The ATP-dependent isobutyrate activator, ibuARP, was used in place of the CoA transferase pctMe in order to relieve acetyl-CoA requirements and create redox neutrality for the pathway. New plasmid constructs were made to reorganize genes onto plasmids by module (Supplementary Table S2 and S4). It was anticipated that operon construction would reduce enzyme expression, especially for genes in the second position, but it was unknown if the effect would be detrimental to overall production without knowledge of the rate limiting enzyme64. Two Module 3 plasmid variants were tested to explore whether PhaBRe activity could become limiting when expressed from an operon used in the new constructs (Supplementary Fig. S6A). The variant with phaBRe in the first position of a two gene operon generated higher titers supporting the theory that phaBRe could be the limiting activity within Module 3. Comparison of phaBcn expression from the two operon variants by SDS-PAGE confirmed higher phaBcn expression when placed in the first position (Supplementary Fig. S6B). Additionally, operon variants for alsSRe and ilvCEc expression were tested to examine if better balancing
of flux between Module 1 and the native acetyl-CoA pathway could improve 4MP production (Supplementary Fig. S6A). Placing alsSBs in the second position while using the new plasmid constructs (Strain M1F(IA)2I34) increased 4MP titers (168 ± 31 mg/L) while reducing isobutyrate (290 ± 24 mg/L) and isobutanol (1046 ± 45 mg/L) titers (See Supplementary Text for further details).

Based on available in vitro data and the presence of 4MV (42 ± 7 mg/L) even for improved Strain M1F(IA)2I34, it was possible that FeaBEc could be oxidizing 4-methyl-valeraldehyde into 4MV creating a futile cycle with CarNi (Fig. 3A, Supplementary Fig. S6C). An aldehyde dehydrogenase, Fjoh2967fj from Flavobacterium johnsonae had been found to prefer an isobutyraldehyde substrate over other aldehyde substrates when tested in vitro. Replacing feaBEc with Fjoh2967fj in Strain M1Ffj(IA)2I34 led to increased 4MP (193 ± 23 mg/L, 0.033 ± 0.005 mol/mol glucose) and elimination of detectable 4MV (Fig. 3B). Isobutyrate titers (424 ± 9 mg/L, 0.084 ± 0.004 mol/mol glucose) were increased and isobutanol titers (797 ± 7 mg/L, 0.187 ± 0.006 mol/mol glucose) were reduced (Fig. 3C).

In order to examine if alcohol toxicity could be limiting product titers, toxicities of the dominant byproduct isobutanol and desired product 4MP were assayed through exogenous addition of alcohols to the growth medium at concentrations from 1-10 mM. Isobutanol and 4MP concentrations up to 5 mM did not inhibit the exponential growth rate (Supplementary Fig. S7). A combination of 10 mM (741 mg/L) isobutanol and 2 mM (204 mg/L) 4MP (comparable to titers observed for Strain M1Ffj(IA)2I34) only reduced the exponential growth rate by 10%, the same reduction observed with 10 mM isobutanol alone. While endogenously produced alcohols may be involved in alternative toxicity mechanisms, this result suggests that current titers are likely not limited by product toxicity.

Achieving Pathway Selectivity through Enzyme Selection (Revisiting Module 4). With knowledge of the specificity of CarNi for 4MV over isobutyrate, the continued high isobutanol titers suggested Adh6pSc was converting the isobutyraldehyde intermediate to isobutanol (Fig 4A). Removing ADH6pSc from Strain
M1F(IA)2I34 produced Strain M1F(IA)2I34a which generated an isobutanol titer of 27 ± 3 mg/L with nearly undetectable butanol and 4MP titers (Fig. 4B). An alternative to ADH6sc was identified from *Leifsonia* sp. Strain S749. The new alcohol dehydrogenase LsadhLs was hypothesized to have improved specificity for 4-methyl-valeraldehyde based on substrates that were assayed *in vitro* [58]. When LsadhLs was combined with the isobutyraldehyde specific dehydrogenase Fjoh2967fj in Strain M1Fj(IA)2I34L, selective synthesis of 4MP was achieved over other alcohol byproducts (Fig 4C). Isobutanol titers were reduced to 21 ± 3 mg/L similar to those observed with the no alcohol dehydrogenase control. 4MP was produced at 90 ± 7 mg/L (0.016 ± 0.001 mol/mol glucose) making up 81% of all alcohol products. The dominate byproducts were the 4MP precursors acetate (592 ± 34 mg/L, 0.177 ± 0.010 mol/mol glucose) and isobutyrate (1128 ± 34 mg/L, 0.229 ± 0.002 mol/mol glucose), suggesting that byproduct shunts were reduced and further improvement could be made by relieving a downstream rate limitation in Module 2, 3, or 4 (Supplementary Fig. S8). While 4MP titers were lower with LsadhLs, SDS-PAGE analysis of LsadhLs confirmed strong overexpression in *E. coli* (Supplementary Fig. S8C). The reduction in titer is likely due to the change from an NADPH-dependent dehydrogenase (Adh6pSc) to an NADH-dependent dehydrogenase (LsadhLs) under the aerobic conditions used. The ratio of NADH/NAD⁺ has been observed to be lower than that of NADPH/NAD⁺ under similar culture conditions [65].

**Discussion**

Recent efforts to develop microbial pathways for chemical synthesis have moved beyond upregulation of native pathways to include transfer and modification of heterologous pathways to new hosts and modified termination of native host pathways. Only a small number of truly *de novo* pathway designs have been published and most use isolated heterologous enzymes acting on their cognate substrates [18, 19, 51]. Engineered pathways to liquid fuels, in particular, have predominantly relied on entirely natural (ethanol, butanol, isoprenoid) or terminally modified natural pathways (fatty acid synthesis, amino acid
αKAE, isoprenoid). The presented work moves beyond modification of natural pathways by successfully demonstrating synthesis of a C6 branched alcohol via an extended de novo pathway which maintains selectivity while utilizing multiple naturally occurring enzymes outside their native pathway contexts.

While one set of Modules has been presented in the current work, alternate chemistries could be substituted for or combined with the selected modules to create new pathways to the same or alternate products. For example, an isobutyryl-CoA mutase or branched α-keto-acid decarboxylase route could be used to generate the isobutyryl-CoA precursor in place of Modules 1 and 2. Similarly, a FAS route could be substituted for Module 3 to generate the longer saturated acid substrate for Module 4. Using this design, individual alternative modules or module combinations can be directly compared to the existing pathway in vivo. In addition, entirely new classes of branched products (e.g., aldehydes, alkanes) could be made by using different Module 4 enzymes.

For the presented pathway, an iterative screening approach identified the enzymes catalyzing conversion of the downstream 4-methyl-valeraldehyde and upstream isobutyraldehyde intermediates as key components controlling selectivity of the pathway. Our initial Module 4 alcohol dehydrogenase selection, Adh6pSc, proved to be highly active, but non-selective in the full pathway context. Module 4 displayed high activity on our desired substrate, but in vivo results with the full pathway suggested this module had a broad substrate range. Persistent high isobutanol titers from strains expressing Modules 1-4 suggested that Module 4 enzymes were interacting with isobutyrate and/or isobutyraldehyde. In vitro and in vivo data from Module 4 testing implicated the alcohol dehydrogenase, Adh6pSc, as the non-selective enzyme (Fig. 2 and 4). By replacing Adh6pSc with the isobutyraldehyde specific and NADH-dependent alcohol dehydrogenase, LsadhlS, pathway selectivity and overall cofactor utilization were improved.
As with alcohol dehydrogenase candidates, we initially selected aldehyde dehydrogenases previously validated for an isobutyraldehyde substrate in an engineered pathway. Two endogenous enzymes, PuuEc and FeaEc, were previously identified as the most effective *E. coli* aldehyde dehydrogenases for isobutyraldehyde oxidation to isobutyrate. Of the two *E. coli* aldehyde dehydrogenases, FeaEc proved to successfully synthesize 4MP from glucose in Strain M1F2P34 expressing Modules 1, 2, 3, and 4 (Fig. 3B). Based on *in vitro* data one may predict PuuEc to function more effectively because its $k_{cat}/K_m$ is more consistent across substrate lengths while the $k_{cat}/K_m$ of FeaEc actually increases by an order of magnitude between propionaldehyde and hexanaldehyde substrates. *In vivo* results disproved this prediction with only FeaEc producing 4MP (Fig. 3A). The better performance of FeaEc in the context of the full pathway may be explained by reported $K_m$ values for the two dehydrogenases. FeaEc has $K_m$ values below 100 $\mu$M for relevant substrates while the $K_m$ values for PuuEc are 1 mM. PuuEc and FeaEc were tested in strains expressing ADH6Sc. Like FeaEc, Adh6pSc has reported $K_m$ values for relevant substrates in the 100-200 $\mu$M range. Adh6pSc was observed to have $k_{cat}$ values ($\sim 100$ sec$^{-1}$) an order of magnitude higher than values observed for FeaEc and PuuEc ($\sim 10$ sec$^{-1}$) for related aliphatic aldehydes. Together these observed kinetics support the hypothesis that Adh6pSc out-competes PuuEc and FeaEc for the isobutyraldehyde substrate. Isobutyraldehyde and reducing equivalents are diverted to isobutanol, lowering 4MP titers (Supplementary Fig. S5). Strain M1P2P34 with PuuEc produces significantly more isobutanol than Strain M1F2P34 with FeaEc, as expected based on observed $K_m$ values.

In addition, *in vitro* data suggested that even though FeaEc functioned as an isobutyraldehyde dehydrogenase, it may also favor a 4-methyl-valeraldehyde substrate. The potential futile cycle created by activity on 4-methyl-valeraldehyde was avoided by using the isobutyraldehyde specific dehydrogenase *Fjoh*$_{2967Fj}$ from *Flavobacterium johnsonae*'. Replacing feaEc with *Fjoh*$_{2967Fj}$ led to increased isobutyrate and eliminated detectable 4MV production (Fig. 3). Combining more selective
alcohol and aldehyde dehydrogenases led to a highly selective overall pathway with the major byproduct being overflow of the upstream intermediate isobutyrate (Supplementary Fig. S6). Together the results from alcohol and aldehyde dehydrogenase selection highlight the importance of considering both high activity and required selectivity when utilizing retro-biosynthetic screening. Proposing potential upstream pathways is required to identify intermediates which could have cross-reactivity with downstream enzymes.

Further engineering of the CoA-dependent 4MP pathway is warranted given the potential high energy yield. Dugar and Stephanopoulos have outlined the importance of balancing reducing equivalents generated and consumed in a recombinant pathway if high yields are desired. Using the current 4MP pathway enzymes the overall reaction can be written as:

\[
1.5 \text{C}_6\text{H}_{12}\text{O}_6 + 3 \text{ADP} + 3 \text{NAD}^+ + 3 \text{NADPH} \rightarrow \text{C}_6\text{H}_{14}\text{O} + 3 \text{CO}_2 + 1 \text{H}_2\text{O} + 2 \text{AMP} + \text{P}_i + 1 \text{ATP} + 3 \text{NADH} + 3 \text{NADP}^+ + 1 \text{H}^+ \\
\]

The reducing equivalents of the pathway are balanced, but some are contained in different cofactors. The maximum pathway energy efficiency \( \gamma^\eta \) can be calculated using the degrees of reductance and pathway stoichiometry for a glucose substrate and 4MP product. Maximum pathway energy efficiency for the \( \alpha \)KAE pathway and the presented CoA-dependent pathway are 75% and 100%, respectively. Accounting for cofactor requirements, the adjusted pathway energy efficiencies \( \eta_{\text{CI}}^{\mu,G} \) are 24% and 45% for the \( \alpha \)KAE and CoA pathways respectively (Supplementary Methods). If alternative enzymes are identified or engineered to accept NADH in place of NADPH, maximum pathway yields could be achieved under anaerobic fermentation. The maximum adjusted efficiency values for these pathway architectures then become 28% (\( \alpha \)KAE) and 100% (CoA). The yield calculations highlight how our rational
design approach leads to a pathway architecture with high yield potential unlike inherently limited pathways utilizing modification of amino acid synthesis.

This work has identified a novel pathway for the selective synthesis of the branched medium-chain length alcohol 4MP. The highest titers (193 ± 23 mg/L) were achieved with Strain M1Fj(IA)2I34 which expresses both Fjoh2967Fj and ADH6Sc. Selectivity was achieved by replacing ADH6Sc with IsadhLs in Strain M1Fj(IA)2I34L. The 90 ± 7 mg/L of 4MP produced by M1Fj(IA)2I34L represented 81% of observed alcohol products. In comparison, of the 9 alcohols generated in the previous demonstration of microbial 4MP synthesis using α-KAE, 4MP (202.4 ± 1.1 mg/L) makes up 14% of the total alcohol product. High potential efficiency and selectivity make our CoA pathway a preferred candidate for future engineering. Currently, the major byproducts of the CoA-dependent route are the acids acetate, isobutyrate, and butyrate (Fig. S8). We expect that a combination of tuning thioesterase/transferase activities of the host to selectively cleave the longer 4-methyl-valeryl-CoA intermediate and relieving Module 3 rate limitations will further enhance titers. Ultimately, screening or engineering for NADH-dependent enzymes should produce a high yielding fermentative pathway. Our existing pathway can also be adapted to produce other branched medium-chain products by testing new downstream modules. Finally, we believe the pathway design approach described here can be useful for creation of new metabolic pathways which rely on long de novo routes. Using retro-biosynthetic screening within a proposed pathway framework allows exploration of diversity using a small number of assays while constraining enzyme space to a chemical route which is maximally efficient for a given product.

Methods

Bacterial Strains and Plasmids: E. coli MG1655(DE3) ΔendA ΔrecA described previously was the host strain for production experiments, alcohol toxicity experiments and for protein expression analysis using
cell lysates\textsuperscript{70}. \textit{E. coli} DH10B (Invitrogen, Carlsbad, CA) and ElectroTen-Blue (Stratagene, La Jolla, CA) were used in plasmid cloning transformations and for plasmid propagation. \textit{E. coli} BL21Star(DE3) (Life Technologies, Grand Island, NY) was used for expression of \textit{car}$_{Ni}$ for purification. (See Supplementary Tables S1 & S2 for strain details)

A codon optimized version of \textit{S. cerevisiae ADH6} was purchased from DNA 2.0 (Menlo Park, CA) and codon optimized versions of \textit{N. iowensis car} and \textit{B. subtilis sfp} were purchased from GenScript (Piscataway, NJ) (See Supplementary Methods for codon optimized sequences). \textit{T. denticola ter} and \textit{E. gracilis ter} were purchased from GenScript (Piscataway, NJ) as described previously \textsuperscript{15}. \textit{Leifsonia} sp. Strain S749 \textit{Isad}h was purchased as a codon optimized GeneArt String from Life Technologies (Grand Island, NY). All other genes were amplified from gDNA. \textit{B. subtilis PY79}, \textit{E. coli MG1655}, \textit{P. putida KT2440}, \textit{C. necator} (formerly \textit{R. eutropha}) H16, \textit{M. elsdenii}, \textit{R. palustris} CGA009, \textit{P. syringae} DC3000, \textit{C. acetobutylicum} ATCC 824, and \textit{S. oneidensis} MR-1 gDNA were prepared using the Wizard Genomic DNA purification Kit (Promega, Madison, WI). \textit{P. aeruginosa} PAO1-LAC (ATCC# 47085), \textit{F. johnsoniae} (ATCC# 17061) and \textit{V. parahaemolyticus} EB 101 (ATCC# 17802) gDNA were purchased from American Type Culture Collection (Manassas, VA). Custom oligonucleotide primers were purchased (Sigma-Genosys, St. Louis, MO) for PCR amplification of genes from gDNA using either Phusion High-Fidelity DNA polymerase (Finnzymes, Thermo Scientific Molecular Biology) or Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). Synthetic operons were constructed using a modified Splice by Overlap Extension (SOE) PCR method.

The compatible vector set pETDuet-1, pCDFDuet-1, pACYCDuet-1, and pCOLADuet-1 was used to express single genes or synthetic operons under control of a T7\textit{lac} promoter and individual ribosome binding sites. Plasmids were constructed using standard molecular biology techniques with restriction enzymes and T4 DNA ligase purchased from New England Biolabs. Ligation products in pETDuet-1, pACYCDuet-1,
and pCOLADuet-1 were used to transform *E. coli* DH10B and pCDFDuet-1 products were used to transform *E. coli* ElectroTen-Blue. Propagated constructs were purified using a QIAprep Miniprep Kit (Qiagen, Valencia, CA) and agarose gel fragments were purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Completed constructs were used to co-transform *E. coli* MG1655(ΔendA ΔrecA). (See Supplementary Table S3 for plasmid details).

**Splice by Overlap Extension:** Initial PCR products with homologous ends were added to a PCR mixture without additional primers and cycled through a standard PCR cycle 4 times with annealing temperatures set at 6°C above, 3°C above, and at the designed melting temperature for the homology. The upstream primer for the upstream gene and the downstream primer for the downstream gene in the designed operon were then added to amplify the full length product. A standard PCR method using the annealing temperature for the primer pair was used for final amplification.

**Culture Conditions:** For all production experiments triplicate seed cultures were grown from isolated colonies at 30°C overnight in 3 ml LB medium in a 14 ml culture tube on a rotary shaker at 250 rpm. All production cultures were inoculated with 1% inoculum from overnight seed culture and grown at 30°C on a rotary shaker at 250 rpm. Cultures were induced with 0.5 mM IPTG when OD_{600} values reached 0.6-1.0 corresponding to mid-exponential phase. For constructs designed for 4MV production 50 ml cultures in 250 ml shake flasks were used, and for constructs designed to produce 4MP 3 ml cultures in 1 inch diameter 50 ml screw-cap culture tubes (Pyrex VISTA) were used. Unless otherwise stated, 1 ml culture samples were taken 48 hours post induction, centrifuged to pellet cells, and the supernatant was removed for analysis.

For production of 4-methyl-valerate and 4-methyl-pentanol from glucose and isobutyrate, LB medium supplemented with 1% glucose and 10 mM isobutyrate was used. For production of 4-methyl-valerate and 4-methyl-pentanol from glucose, LB medium supplemented with 1.2% glucose was used. Samples
were taken 48 hours post induction except for initial experiments with Strains M1F2P34 and M1P2P34
when samples were taken 72 hours post induction.

For assessing toxicity of isobutanol and 4MP, an MG1655(DE3) ΔendA ΔrecA seed culture was grown
overnight in LB medium. Duplicate 3 ml cultures in LB + 1.2% glucose + alcohol were inoculated to an
initial OD$_{600}$ of 0.1. Cultures were contained in the same 50 ml screw-cap tubes used in production
experiments. Cultures contained 1 mM isobutanol, 5 mM isobutanol, 10 mM isobutanol, 1 mM 4MP, 5
mM 4MP, 10 mM 4MP, or no alcohol. Growth was monitored by optical density and the growth rate
was calculated from a linear regression of the natural log of the OD$_{600}$ values for the 1.5, 2, and 2.5 hour
post-inoculation time points.

Relative Activity Assay for Purified his-Car: An overnight culture of BL21 Star (DE3) (Invitrogen)
harboring pET/His-Car-RBS2-Sfp was used as 10% (v/v) inoculum in 2 L of LB Broth. The culture was
incubated at 30°C and 250 rpm, and expression was induced using a final concentration of 1 mM IPTG at
OD 0.6. Cells were harvested after 20 hours using centrifugation and resuspended in a buffer containing
50 mM Tris-HCl pH 8.0, 300 mM NaCl, and 10% glycerol. Cells were subsequently lysed using sonication.
The supernatant was collected and applied to a column containing Ni-NTA resin (Qiagen). Affinity
chromatography was performed using step-wise increasing concentrations of imidazole. Fractions
containing purified His6-Car were dialyzed overnight at 4°C into 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1
mM DTT, and 10% glycerol. Dialyzed enzyme was then flash frozen using liquid nitrogen and stored at -
80°C.

The activity of His-Car on various substrates was determined by measuring changes in absorbance at 340
nm for up to 5 minutes in 96-well microplates (Tecan Infinite F200 Pro). Reactions were prepared as
follows: 100 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 0.6 mM NADPH, 1 mM ATP, 224 nM His-Car, and 50 mM
pH neutralized acid substrate. All substrates were assayed in triplicate. For $K_m$ and $V_{max}$ determinations, substrates were assayed at 5 different concentrations.

**SDS-PAGE Analysis:** *E. coli* MG1655(DE3) $\Delta$endA $\Delta$recA was transformed with empty pETDuet-1, pET-(bktBCn-terTd)-(phaJ4bCn-phaBCn), pET-(bktBCn-terTd)-(phaBCh), pACYC-(carNi-sfpBs)-ADH6Sc, or pACYC-(carNi-sfpBs)-lsadhLs. Single colonies from plates of each transformation were grown overnight in 3 ml of LB with appropriate antibiotic. Shake flask cultures (250 ml flasks) containing 50 ml LB + 0.6% glucose were inoculated at 1% inoculum from overnight LB cultures and incubated with agitation at 30°C and 250 rpm. Shake flasks were induced with 0.5 mM IPTG OD$_{600}$ values between 0.5 and 0.6.

Five and a half hours after induction 7 ml of each culture were sampled and pelleted by centrifugation. Cell pellets were resuspended in 1 ml of 10 mM Tris-HCl at pH 8.0 and added to 1.7 ml microcentrifuge tubes containing 500 ul of 0.1 mm diameter glass beads (Scientific Industries, Inc. Disruptor Beads, SI-BG01). Samples were then vortexed for 10 minutes.

After lysis, samples were pelleted by centrifugation (6,000g, 4°C, 10 min) and the supernatant was removed as soluble lysate. Total protein was quantified by a previously described Bradford assay method using Bio-Rad Protein Assay Dye Reagent (Cat #500-0006). A Bio-Rad 10% Mini-PROTEAN TGX gel (Cat #456-1034) was run using the Mini-PROTEAN Tetra Cell electrophoresis set up. Bio-Rad Precision Plus Protein All Blue Standard (Cat #161-0373) and 10 μg of total protein for each sample was loaded on the gel. After running at 200 volts for 33 minutes, the gel was washed with deionized water before staining with Bio-Rad Bio-Safe Coomassie Stain (Cat #161-0786).

**Metabolite Analysis:** Culture samples were pelleted by centrifugation and supernatant was removed for HPLC analysis with an Agilent 1200 series instrument with a refractive index detector. Analytes were separated using the Aminex HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA) with a 5mM sulfuric acid mobile phase at 35°C and a flowrate of 0.6 ml/min. Commercial standards of
glucose, α-ketoisovalerate, acetate, acetoin, isobutyrate, butyrate, isobutanol, butanol, 4-methylvalerate, and 4-methyl-pentanol were used for quantification of experimental samples by linear interpolation of external standard curves.

References:


690 **Acknowledgements**

This research was supported in part by an award from the Department of Energy (DOE) Office of Science Graduate Fellowship Program (DOE SCGF). The DOE SCGF Program was made possible in part by the American Recovery and Reinvestment Act of 2009. The DOE SCGF program is administered by the Oak Ridge Institute for Science and Education for the DOE. ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract number DE-AC05-06OR23100. All opinions expressed in this paper are the author’s and do not necessarily reflect the policies and views of DOE, ORAU, or ORISE.

This research was also supported by the Institute for Collaborative Biotechnologies through grant W911NF-09-0001 from the U.S. Army Research Office. The content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

**Author contributions**

K.L.J.P. supervised the research. All authors wrote, reviewed, and edited the manuscript.

Additional information

Competing financial interests: The authors declare competing financial interests in the form of a pending patent application.
Tables:

Table 1: Strains Used for Module and Full Pathway Evaluation. Host strains and plasmids are listed. Strain names indicate the modules present in the strain, i.e. M1F2P34 includes “M” for modules, “1F” for Module 1 with feaBEc, “2P” for Module 2 with pctMe, “3” for Module 3 and “4” for Module 4. Strains with “( )” contain abbreviations for operon structure indicating the order of alsS8s and ilvCec, i.e. M1F(IA)2I34 indicates that it contains an ilvCec-alsS8s operon. Key strains indicated in bold.

Figures:

Figure 1: 4-methyl-pentanol (4MP) pathway schematic and alternative biofuel pathways. (A) The \( \alpha \)-ketoacid elongation (\( \alpha \)KAE) pathway was previously used to synthesize 4MP among other products. The \( \alpha \)KAE route utilizes relatively inefficient single-carbon extension and non-specific decarboxylation and reduction of upstream precursors resulting in a redox imbalance and a mix of products, three of which are shown. (B) The presented CoA-dependent pathway to 4MP is assembled with genes from 9 organisms taken from 10 different pathway contexts. Pathway genes are shown with known native pathways or putative metabolic roles. Selectivity for 4MP was achieved while requiring enzymes for Modules 3 and 4 to act on noncognate substrates. (C) The 4MP pathway can be organized into 4 modules which were used to identify better performing enzymes for individual steps and validate portions of the overall pathway independently \textit{in vivo}: Module 1, modified valine biosynthesis to isobutyrate; Module 2, isobutyrate activation to isobutyryl-CoA; Module 3, CoA-dependent condensation and reduction of isobutyryl-CoA and acetyl-CoA to 4-methyl-valerate (4MV); Module 4, reduction of 4MV to 4MP. Genes in italics were overexpressed from plasmid sets. Modules were constructed working backwards from the 4MP product. A potential byproduct shunt to butyrate and butanol was monitored during pathway construction.

Figure 2: Tuning of pathway selectivity by the carboxylic acid reductase Car\( _{ni} \). (A) \textit{In vitro} analysis of his-purified Car\( _{ni} \) reveals a dependence on acid primary-chain length with maximum activity at a chain length of five and six carbons. Branching at the C4 position is preferred significantly over straight acid species. The potential substrates for byproduct formation, butyrate and isobutyrate, are seen to have 56% and 25% of the observed activity on 4-methyl-valerate (4MV) respectively. (B) Michaelis-Menten kinetics for isobutyrate and 4MV reveal that Car\( _{ni} \) has a strong preference for the latter intermediate. (C) Car\( _{ni} \) substrate preference influences product selectivity \textit{in vivo} generating 1.9 times as much 4-methyl-pentanol (4MP) (272 mg/L, 2.7 mM) as butanol (142 mg/L, 1.9 mM) in Strain M2P34 (expressing Module 2(pct), 3, and 4 genes) supplied with both glucose and isobutyrate. Even though 10 mM isobutyrate is supplied to the cultures of Strain M2P34 only 111 mg/L (1.5 mM) isobutanol is observed. All data are presented as the mean ± s.d. (n=3) with \textit{in vivo} data generated using biological triplicates.

Figure 3: 4MP synthesis from glucose improved through aldehyde dehydrogenase selection. (A) Key reactions involving aldehydes can generate futile cycles (aldehyde dehydrogenase feaB\( _{ec} \) with the carboxylic acid reductase car\( _{ni} \)) or byproduct shunts (alcohol dehydrogenase ADH\( _{6ec} \)). The desired pathway route is shown in bold arrows within the shaded box. Undesired reactions are shown with dashed arrows. Enzymes with insufficient selectivity have dashed outlines. (B) When feaB\( _{ec} \) (Strain M1F(IA)2I34) was replaced with the isobutyraldehyde specific aldehyde dehydrogenase \( Fjoh2967_{fi} \) in Strain M1F(IA)2I34 4MV titers were reduced and 4MP titers were increased. (C) Complementarily, Strain M1F(IA)2I34 (\( Fjoh2967_{fi} \)) produced lower isobutanol and higher isobutyrate titers relative to strain M1F(IA)2I34 (feaB\( _{ec} \)).
Figure 4: Improved alcohol dehydrogenase selectivity with *lsadh*<sub>ls</sub>. (A) The desired pathway reactions to 4MP are indicated by bold arrows with the byproduct shunt to isobutanol indicated by the dashed arrow. High activity of *Adh*<sub>6pSc</sub> on isobutyraldehyde diverts isobutyrate flux to isobutanol. The *Lsadh*<sub>ls</sub> alcohol dehydrogenase’s selectivity for 4-methyl-valeraldehyde greatly reduces flux to the isobutanol shunt. (B) The alcohol profile of Strain M1F(IA)2I34 expressing *feaBEc* and *ADH6Sc* contains 168 ± 31 mg/L of 4MP but is dominated by 1.046 ± 45 g/L of isobutanol. The M1F(IA)2I34a control without *ADH6Sc* expression produces low to undetectable levels of all three alcohols. (C) Replacing *feaBEc* with *Fjoh2967f* in Strain M1Fj(IA)2I34 reduces isobutanol (797 ± 20 mg/L) and increases 4MP (192 ± 23 mg/L) marginally. Replacing *ADH6Sc* with *lsadh*<sub>ls</sub> greatly enhanced alcohol selectivity producing 90 ± 7 mg/L 4MP with only 20 ± 5 mg/L isobutanol.
Figure-1 (Prather)
Figure 2 (Prather)
Figure 3 (Prather)
Figure 4 (Prather)
<table>
<thead>
<tr>
<th>Plasmid 1</th>
<th>Plasmid 2</th>
<th>Plasmid 3</th>
<th>Strain name</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td></td>
<td></td>
<td>M4</td>
</tr>
<tr>
<td>pET-ter1Cr(bktBCn-pctM6)</td>
<td>pCDF-phaJ4bCn-phaBCn</td>
<td></td>
<td>M2P34</td>
</tr>
<tr>
<td>pET-(bktBCn-pctM6) - (phaJ4bCn-phaBCn)</td>
<td>pCDF-(ilvDEc-ter1Cr)-(alsSbs-ilvCec)</td>
<td>pCOLA-kivDf-feaBeC</td>
<td>M1F2P3</td>
</tr>
<tr>
<td>pCOLA-kivDf-puuCec</td>
<td></td>
<td></td>
<td>M1P2P3</td>
</tr>
<tr>
<td>Plasmid 1</td>
<td>Plasmid 2</td>
<td>Plasmid 3</td>
<td>Plasmid 4</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>pET-(bktBCn-pctM6) - (phaJ4bCn-phaBCn)</td>
<td>pCDF-(ilvDEc-ter1Cr)-(alsSbs-ilvCec)</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>pCOLA-kivDf-feaBeC</td>
</tr>
<tr>
<td></td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>M2P3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-puuCec</td>
<td></td>
<td>M1P2P34</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-puuCec</td>
<td></td>
<td>M2P3b</td>
</tr>
<tr>
<td>pET-(bktBCn-ter1Cr)-(phaBcr-phaJ4bCn)</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>pCOLA-kivDf-feaBeC</td>
<td>pCDF-(ibuARp-ilvDEc)-(alsSbs-ilvCec)</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-feaBeC</td>
<td>M1F(AJ)234</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCDF-(ibuARp-ilvDEc)-(ilvCec-alsSbs)</td>
<td></td>
<td>M1F(AJ)234</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-Fjoh_2967Fj</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>M1F(AJ)234a</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-FeaBf</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>M1F(I)234</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-FeaBf</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>M1F(I)234</td>
</tr>
<tr>
<td></td>
<td>pCOLA-kivDf-FeaBf</td>
<td>pACYC-(carNi-sfpBs)-ADH6Sc</td>
<td>M1F(I)234L</td>
</tr>
</tbody>
</table>