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Engineering a Coenzyme A Detour To Expand the Product Scope and Enhance the Selectivity of the Ehrlich Pathway

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Abstract

The Ehrlich pathway is a major route for the renewable production of higher alcohols. However, the product scope of the Ehrlich pathway is restricted, and the product selectivity is suboptimal. Here, we demonstrate that a Coenzyme A (CoA) detour, which involves conversion of the 2-keto acids into acyl-CoAs, expands the biological toolkit of reaction chemistries available in the Ehrlich pathway to include the gamut of CoA-dependent enzymes. As a proof-of-concept, we demonstrated the first biosynthesis of a tertiary branched-alcohol, pivalcohol, at a level of ~10 mg/L from glucose in *Escherichia coli*, using a pivalyl-CoA mutase from *Xanthobacter autotrophicus*. Furthermore, engineering an enzyme in the CoA detour, the *Lactobacillus brevis* CoA-acylating aldehyde dehydrogenase, allowed stringent product selectivity. Targeted production of 3-methyl-1-butanol (3-MB) in *E. coli* mediated by the CoA detour showed a 3-MB:side-product (isobutanol) ratio of >20, an increase over the ratios previously achieved using the conventional Ehrlich pathway.

Graphical Abstract

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Author Contributions

H.L. and R.L. conceived the study. W.B.B., E.K., Y.W., A.J., A.T.R., and K.S. performed the experiments. W.B.B., E.K., R.L., and H.L. wrote the manuscript.

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00358. Plasmid and strain construction; Production conditions and analytic methods for higher alcohols; Enzyme assays for ALDHs, ADHs, and PCMs; Specific activity of ALDHs toward 3-MBA and isobutyraldehyde (Figure S1 and S2); Catalytic efficiency (k_{cat}/K_M) of the engineered CoA-acylating aldehyde dehydrogenase (Table S1); 3-MB to isobutanol ratios of previous efforts aimed at producing 3-MB using the conventional Ehrlich pathway (Table S2); Computational modeling of protein structures. Activity of various pivalyl-CoA mutases (PCMs) (Figure S3) (PDF)

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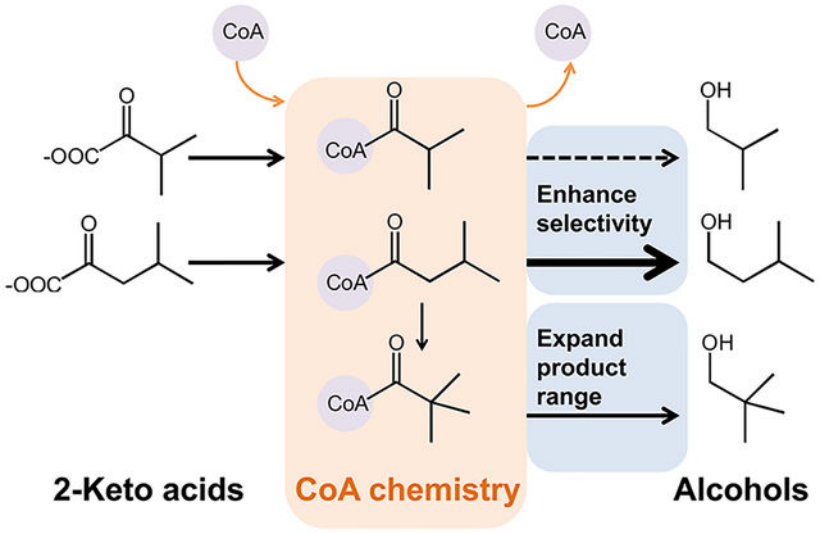

Keywords

metabolic engineering; Ehrlich pathway; higher alcohol; tertiary branched-chemicals; pivalyl-CoA mutase

The Ehrlich pathway, which converts 2-keto acids into alcohols *via* aldehydes (Figure 1), has been explored extensively to produce fuels, commodities, and value-added chemicals.[1–7] However, the scope of the Ehrlich pathway is limited by the types of 2-keto acid precursors available. Biosynthetic pathways of amino acids provide a natural source of 2-keto acids, and recent efforts have substantially expanded the repertoire of products by elongating natural 2-keto acids with a synthetic "+1" cycle.[3,4] Nevertheless, many product configurations are still inaccessible, mainly due to the limited chemistries catalyzed by 2-keto acid-utilizing enzymes. For example, the α,β-unsaturated and 3-functionalized alcohols have only been produced through the isoprenoid pathway or the reversed β-oxidation pathway.[8,9] Furthermore, some configurations, such as the tertiary branched-alcohols, have not been produced in biomanufacturing, despite their important applications as fuel additives and solvents.[10] Due to their bulky and hydrophobic nature, tertiary branched-compounds, such as *tert*-leucine, are essential reagents for molecular conformational control in chemical synthesis.[11] However, *de novo* formation of tertiary branched-carbon chains has been elusive in biomanufacturing.

To overcome this limitation, a CoA-dependent detour is proposed (Figure 1), which is composed of three steps. First, 2-keto acids are converted to acyl-CoAs through the function of keto acid dehydrogenase complexes (KDHCs).[12–16] Second, a range of acyl-CoA utilizing enzymes in natural product biosynthesis and various degradation pathways can be employed to diversify the carbon chains by conducting chemistries such as rearrangement,[17] desaturation,[18] and 3-functionalization.[19] Third, the CoA-derivatives are converted to aldehydes by CoA-acylating aldehyde dehydrogenases (ALDHs), and re-enter the Ehrlich pathway. The aldehydes can then be turned into alcohols by alcohol dehydrogenases (ADHs). These three steps have been explored individually in different contexts;[13–16,21] here

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we show that optimizing their compatibility is the key to successfully establishing the detour. Importantly, by using a recently discovered pivalyl-CoA mutase (PCM) from *Xanthobacter autotrophicus* as the rearrangement enzyme[17] and matching it with an appropriate KDHC, ALDH, and ADH, we achieved the first bioproduction of a tertiary branched-alcohol, pivalcohol, at ~10 mg/L from glucose in *Escherichia coli*.

In addition, it has been a challenge to tune the product selectivity of the Ehrlich pathway.[4,22] Since many 2-keto acids are present inside the cells, the Ehrlich pathway often produces a mixture of alcohols.[2,4,22,23] Targeted production of long chain alcohols is especially difficult.[22] Our hypothesis is that the attachment of the CoA handle can aid enzymes in substrate recognition, which may provide opportunities for engineering highly specific enzymes. The model system we studied here is the selective production of 3-methyl-1-butanol (3-MB) *versus* the side-product isobutanol, which only differ by one carbon in length. By employing the CoA detour and engineering the ALDH enzyme, we achieved a 3-MB:isobutanol ratio of >20 (~1250 mg/L 3-MB *versus* ~57 mg/L isobutanol produced from glucose by engineered *E. coli*), which exceeds the highest ratio (~12) achieved to date by the conventional Ehrlich pathway.[23]

In summary, we demonstrated that the engineered CoA detour can effectively expand the scope and enhance the selectivity of the Ehrlich pathway. We systematically compared a panel of KDHCs, ALDHs, and ADHs in higher alcohol production. We previously applied the vitamin B12-dependent carbon chain rearrangement chemistry to produce a secondary branched-carbon chain from a linear precursor.[24] Here, we demonstrated that this method can be further extended to produce tertiary branched chemicals, which has not been achieved before.

RESULTS AND DISCUSSION

First, we sought to establish a functional CoA detour of the Ehrlich pathway in *E. coli* and demonstrate higher alcohol production (Figure 2A). The initial construction of the CoA detour was achieved by overexpressing the KDHC encoded by the *bkdAA-bkdAB-bkdB-lpdV* operon from *Pseudomonas putida*[13] and the ALDH encoded by *pduP* from *Klebsiella pneumoniae*[20] on a high-copy plasmid (pWB102). This plasmid was transformed into the ADH-defective *E. coli* strain AL707[25] together with a second plasmid (pWB101) containing a wide substrate range ADH, YqhD from *E. coli*.[26] The resulting strain was named WB101. Use of the AL707 strain as the host, which has all seven annotated ADHs disrupted,[25] allowed us to study the effect of different ADHs on substrate selectivity, which will be discussed below. In rich medium, strain WB101 produced C3–C5 linear and C4–C5 branched alcohols, with titers ranging between 20 and 30 mg/L (Figure 2B), which can be used as gasoline substitutes. These results showed that a wide range of 2-keto acids naturally existing in *E. coli* can serve as inputs of the CoA detour. Upon further overexpressing the feedback-resistant LeuA and LeuBCD of *E. coli*[23] on a third plasmid (pWB103), the resulting strain WB102 produced significantly increased titers of longer-chain higher alcohols such as 1-butanol, 1-pentanol, and 3-MB (Figure 2B), the production of which requires more cycles of chain elongation (Figure 2A). Interestingly, the titer of 1-propanol also increased in WB102. It might be possible that the LeuABCD were active in converting

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pyruvate to 2-ketobutyrate,[27] the 2-keto acid precursor of 1-propanol. Alternatively, overexpression of the feedback-resistant LeuA may result in accumulation of branched chain amino acids, which have been shown to activate the *P. putida* KDHC complex.[28]

When *P. putida* KDHC in WB102 was replaced with its homologue from *B. subtilis*[12] (on plasmid pWB104), the resulting strain WB103 showed a distinct product spectrum. While the *P. putida* KDHC strongly favored the production of linear alcohols, the *B. subtilis* counterpart generally had higher preference toward the branched alcohols, as well as the long linear alcohol 1-pentanol (Figure 2B). These results suggested that *B. subtilis* KDHC might have a wider substrate binding pocket, and thus, it can better accommodate bulkier substrates (or wrapped long linear substrates). On the other hand, WB102 had a much higher total alcohol titer (~1000 mg/L) than WB103 (~224 mg/L), suggesting that *P. putida* KDHC has a higher activity *in vivo* in *E. coli*. KDHCs have been used to produce acyl-CoA precursors for ester and fatty acid production.[12,13] Here, we report the direct comparison of the two commonly used KDHCs, which may guide future pathway construction involving these enzymes.

Interestingly, when AlsS from *B. subtilis* and IlvCD from *E. coli* were overexpressed[2] (on plasmid pWB105), the strains harboring *P. putida* or *B. subtilis* KDHC (WB104 and WB105, respectively) almost exclusively produced branched alcohols (Figure 2B). These results suggested that the overexpression of the upstream enzymes in branched-chain amino acid biosynthetic pathway (Figure 2A) substantially shifted the intracellular distribution of 2-keto acids. Despite different compositions, the total alcohol titers (~1136 mg/L for WB104 and ~260 mg/L for WB105) remained comparable to those before AlsS and IlvCD were overexpressed (strains WB102 and WB103), indicating that the pathway flux was mainly determined by the choice of KDHC. Given the high activity of *P. putida* KDHC, this enzyme was used for all further studies.

Next, we tested the production capacity of the CoA detour. In the study by Connor *et al.*,[23] 3-MB production in *E. coli* using the conventional Ehrlich pathway was performed using strain JCL260 as the host, which is an engineered *E. coli* strain with major native fermentation pathways disrupted to conserve carbon and reducing equivalents in higher alcohol production. When we switched the host of the CoA detour-mediated 3-MB production pathway from AL707 to JCL260, the resulting strain WB106 produced ~2500 mg/L isobutanol and ~1900 mg/L 3-MB, roughly a 5-fold increase compared to WB104 (Figure 3). With the conventional Ehrlich pathway in JCL260, the final titer was ~120 mg/L isobutanol and ~1280 mg/L 3-MB.[23] These data indicated that the CoA detour has a similar capacity to the conventional Ehrlich path way in higher alcohol production. Connor *et al.*[29] also showed that two-phase extractive fermentation increased 3-MB production titer, presumably by removing toxic alcohol products from the medium. Using an organic layer of oleyl alcohol in the shake flasks, we also achieved higher production titers with WB104 (~2500 mg/L isobutanol and ~1880 mg/L 3MB) (Figure 3). To further increase the titers, extractive fermentation may be conducted with WB106.

After successfully establishing the CoA detour of the Ehrlich pathway in *E. coli*, we aimed to test if the detour would allow tuning of the product selectivity. We examined whether the

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enzymes in the CoA detour could serve as molecular sieves in chain length selection to specifically produce 3-MB (C5) while minimizing isobutanol (C4) formation. In the conventional Ehrlich pathway, the committing enzymes, keto acid decarboxylases (KDCs), have been engineered to select for longer chain lengths[3,4,22] to prevent premature termination of the reiterative chain elongation cycle. However, KDCs are highly promiscuous, which makes narrowing their substrate range difficult. Our results (Figure 2B) indicate that similar challenges may exist in engineering the committing enzymes of the CoA detour, namely KDHCs. Therefore, we focused on the other enzyme in the detour, the ALDH. The broad substrate range ALDH that we used initially, PduP from *K. pneumoniae*, is highly active (Figure S1 and S2), but its specific activity ratio for 3-methylbutyraldehyde:isobutryaldehyde (3-MBA:IBA) is ~1 (Figure 4A), as assayed *in vitro* with purified proteins. Note, since the acyl-CoAs are not commercially available, we assayed the enzymes in the reverse direction using aldehydes as the substrates. We identified five additional ALDHs from the literature, which have been shown to use long or bulky substrates: *Salmonella enterica* PduP,[20] *Pseudomonas* sp. DmpF,[30] *Lactobacillus brevis* PduP,[20] *E. coli* MphF,[19] and *Burkholderia xenovorans* BphJ.[31] However, the specific activity ratios (3-MBA:IBA) for these ALDHs also did not exceed 1 (Figure 4A). These findings necessitated protein engineering to create a 3-MBA favoring ALDH. We chose the *L. brevis* PduP to engineer for its high expression level. Through homology modeling of *L. brevis* PduP, we predicted that the methyl group of T428 extends into the active site, creating steric hindrance that potentially limits the maneuverability of larger substrates from aligning with catalytic residues to attain active geometry (Figure 4B). This finding is consistent with a recent study on an ALDH from *Clostridium phytofermentans*.[32] After we changed the T428 to A, the resulting enzyme demonstrated a specific activity ratio (3-MBA:IBA) of ~3, which is higher than all native enzymes tested (Figure 4A). The catalytic efficiency (k_{cat}/K_M) of T428A *L. brevis* PduP toward 3-MBA was significantly increased compared to that of the wild type (Table S1). Computationally generated structure models also suggest that the engineered substrate binding pocket may better accommodate 3-methylbutyraldehyde (Figure 4C).

Following replacement of *K. pneumoniae* PduP with *L. brevis* PduP T428A, the resulting strain WB108 produced ~1110 mg/L 3-MB but only ~50 mg/L isobutanol (Figure 4D). The ratio of 3-MB to isobutanol was greater than 20 (Figure 4E), which was higher than those achieved using the conventional Ehrlich pathway in various organisms (Table S2. The highest ratio of ~12 was reported by Connor *et al.*[23]). In comparison, both the strains harboring *K. pneumoniae* PduP (WB104) and wild type *L. brevis* PduP (WB107) showed the 3-MB/isobutanol production ratio of ~1 (Figure 4E). These results highlight the effectiveness of chain length selection using the engineered CoA detour. Although the specificity was much higher, the 3-MB titer of WB108 was lower than that of WB104, presumably because the activity of *L. brevis* PduP T428A toward 3-MBA is relatively low (Figure S1), as assayed using purified proteins. Future work will focus on increasing the expression level of the engineered ALDH using stronger promoters, as well as engineering the highly active *K. pneumoniae* PduP to favor 3-MBA.

After characterizing the simplest CoA detour, which is only comprised of the entering enzyme KDHC and the exiting enzyme ALDH, we next sought to insert the diversifying step

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(Figure 1). We chose the rearrangement chemistry which can enable the formation of a tertiary branched-alcohol, pivalcohol (Figure 5A). The degree of branching has been shown to greatly affect the properties of fuels.[12,16,33] Prior to this study, only linear and secondary branched-biofuels have been produced. Recently, a pivalyl-CoA mutase (PCM) was identified in *X. autotrophicus* that catalyzes the interconversion of isovaleryl CoA and pivalyl-CoA.[17] We cloned the *X. autotrophicus* PCM as a fusion protein of the large and small subunits (PCM-F),[17] and overexpressed it in *E. coli*. We detected significant activity in converting isovaleryl-CoA to pivalyl-CoA in crude cell lysates (Figure 5B), demonstrating functional overexpression of the enzyme. Since the vitamin B12-dependent acyl-CoA mutase family of proteins rely on chaperones to protect them from inactivation during catalysis,[17] we next coexpressed the predicted G-protein chaperone with the PCM fusion protein. The G-protein chaperone, encoded by *Xaut_5042* of *X. autotrophicus*, is in the same operon as the PCM catalytic subunits, but its function has not been characterized.[17] Using crude lysate-based enzymatic assays, we showed that the chaperone significantly increased the PCM activity (Figure 5B).

However, when the plasmid containing *X. autotrophicus* PCM-F and G-protein chaperone (pWB108) was added to the 3-MB production strain WB104, the resulting strain WB109 did not produce pivalcohol (Figure 5C, D). We hypothesized that the downstream enzymes, ALDH and ADH, might be incapable of accepting the sterically demanding tertiary branched carbon chain. Since we showed that the ALDH used in this strain, namely *K. pneumoniae* PduP, has positive activity with the tertiary branched-substrate (highest activity among all six tested ALDHs, Figure 5E), the ADH enzyme *E. coli* YqhD was likely the culprit. Indeed, YqhD showed virtually no activity in reducing pivaldehyde to pivalcohol (Figure 5F). We then characterized four ADHs from other organisms which were shown to have broad substrate-ranges or prefer bulky substrates. These ADHs include: ADH6[34] and Ypr1P[35] from *Saccharomyces cerevisiae*, ADH from *L. brevis*,[36] and ADH from *Ralstonia* sp.[37] From *in vitro* enzyme assays with purified proteins, *Ralstonia* sp. ADH showed the highest activity toward pivaldehyde (Figure 5F). When YqhD was replaced with *Ralstonia* sp. ADH, the resulting strain WB110 was able to produce ~10 mg/L pivalcohol from glucose in rich medium in two-phase fermentation with oleyl alcohol as the extractive solvent[23] (Figure 5C,D). The bioproduction of a tertiary-branched alcohol significantly expands the chemical inventory that can be accessed through metabolic engineering.

The pivalcohol production titer is still low. One of the major causes could be the relatively low activity of PCM, which is consistent with the observation that WB110 still produced significant amounts of 3-MB (data not shown). On the basis of sequence homology and literature,[17] we tested another five PCMs from different organisms (Figure S3). Unfortunately, none of these PCMs performed better than the *X. autotrophicus* one, as assayed *in vitro* using crude cell lysates. Future work will focus on identifying highly active PCMs using bioinformatics tools[17] from the ever-expanding genomic sequence database. In addition to the G-protein chaperone, the acyl-CoA mutase family of enzymes has been shown to require other auxiliary proteins, such as adenosyltransferases.[38] Our ongoing work will explore the effects of these auxiliary proteins, as well as the effect of the coenzyme B12 biosynthesis system[39] to better support PCM activity *in vivo*.

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In conclusion, the CoA detour of the Ehrlich pathway shows promise to be a highly specific and versatile biosynthetic route. By leveraging both the capability of the 2-keto acid pathway to supply odd and even chain-length, linear, branched, and aromatic starter units and the extensive diversity of CoA-dependent carbon chain modifying chemistries, the CoA detour of the Ehrlich pathway may open up new compounds as biomanufacturing targets. Our results also suggest that KDHC, ALDH, and ADH all need to be carefully chosen to optimize the performance of the pathway, and their substrate specificities may be tuned simultaneously to offer a multidimensional control on product selectivity.

MATERIALS AND METHODS

The plasmids and strains used in this study are summarized in Table 1. The conditions used for higher alcohol production, enzyme assays, and computational modeling of protein structures are detailed in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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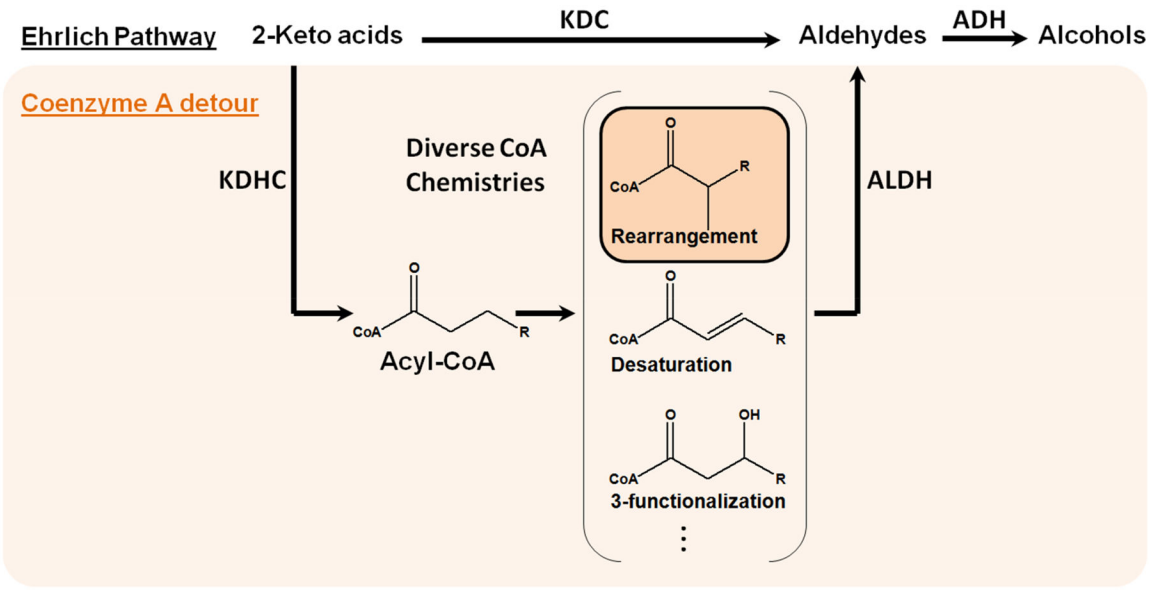

Figure 1.

Coenzyme A (CoA) detour of the Ehrlich pathway. The conventional Ehrlich pathway converts 2-keto acids to alcohols *via* aldehydes. The CoA detour first converts 2-keto acids into acyl-CoAs, which allows the application of diverse CoA-dependent chemistries (The rearrangement chemistry explored in this study is highlighted). Finally, the CoA-derivatives are converted to aldehydes, re-entering the Ehrlich pathway. KDC, keto acid decarboxylase; KDHC, keto acid dehydrogenase complex; ALDH, CoA-acylating aldehyde dehydrogenase; ADH, alcohol dehydrogenase.

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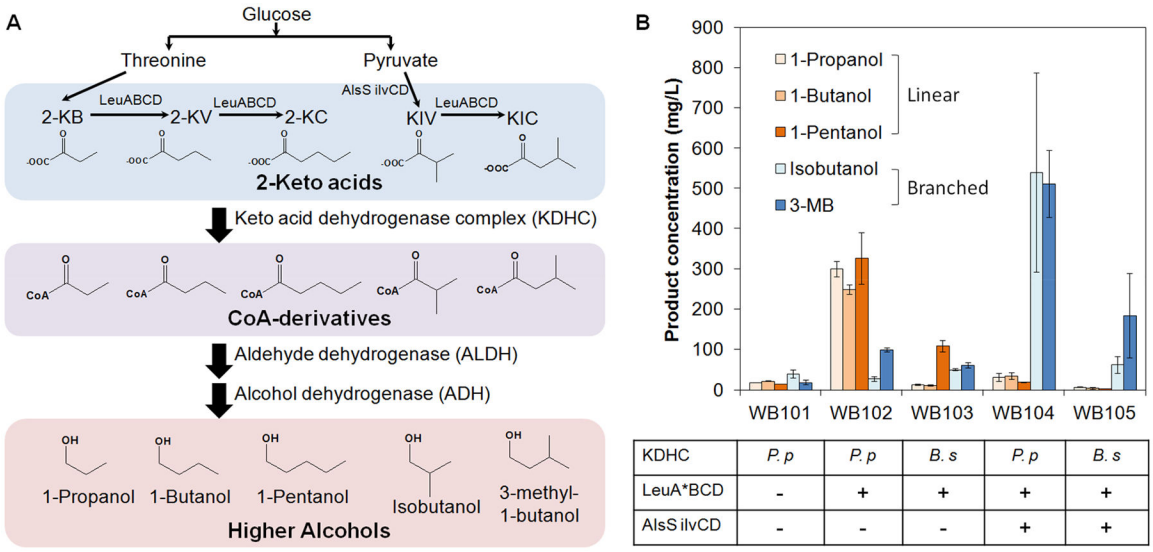

KDHC	*P. p*	*P. p*	*B. s*	*P. p*	*B. s*
LeuA*BCD	-	+	+	+	+
AlsS ilvCD	-	-	-	+	+

Figure 2.

CoA detour of the Ehrlich pathway enables the production of various higher alcohols in *Escherichia coli*. (A) The production pathways for linear and branched alcohols. (B) Choice of KDHC and overexpression of upstream genes altered the product distribution and titers. 2-KB, 2-ketobutyrate; 2-KV, 2-ketovalerate; 2-KC, 2-ketoisocaproate; KIV, ketoisovalerate; KIC, ketoisocaproate; 3-MB, 3-methyl-1-butanol. KDHC, keto acid dehydrogenase complex; ALDH, CoA-acylating aldehyde dehydrogenase; ADH, alcohol dehydrogenase. LeuA*, the feedback-resistant variant (G462D) of *E. coli* LeuA. Pp, *Pseudomonas putida*; Bs, *Bacillus subtilis*. Alcohol titers were quantified at 72 h. Error bars represent one standard deviation of three replicates ($n = 3$).

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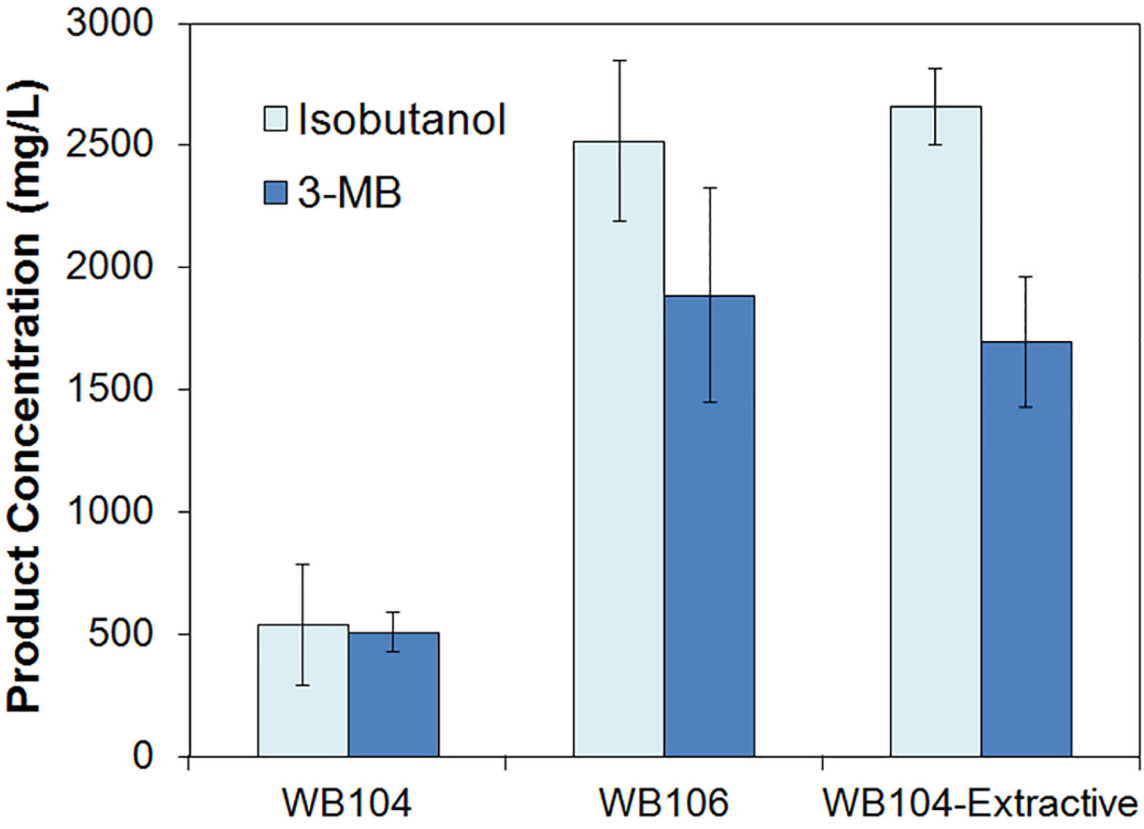

Figure 3.

Optimization of isobutanol and 3-MB production through the CoA detour of the Ehrlich pathway. 3-MB, 3-methyl-1-butanol. Alcohol titers were quantified at 72 h. Error bars represent one standard deviation of three replicates ($n = 3$).

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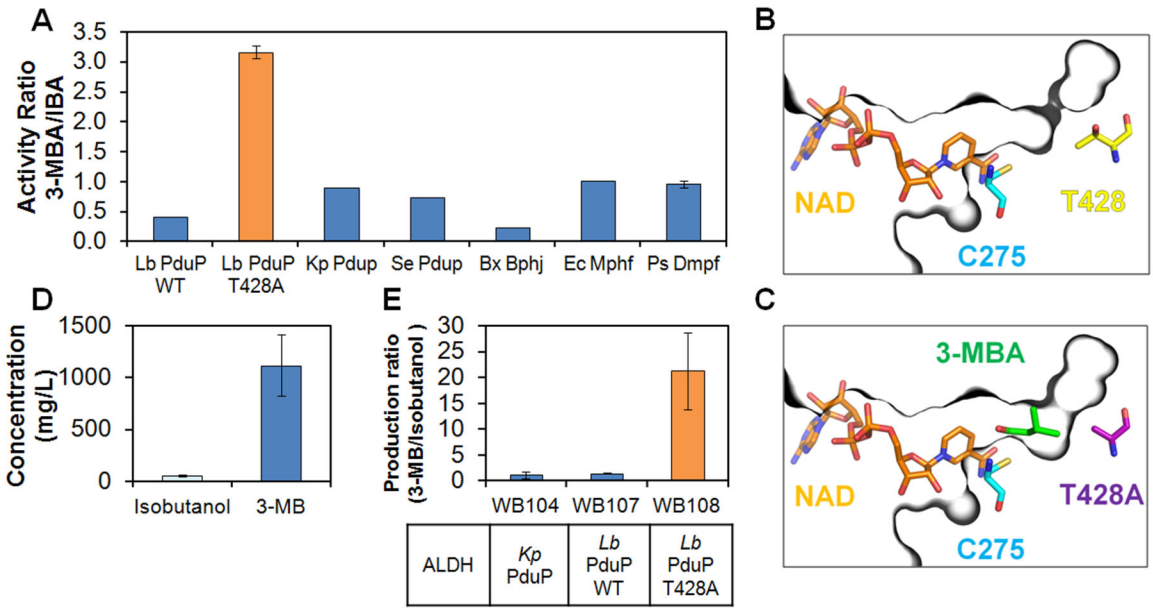

Figure 4.

Targeted production of 3-methyl-1-butanol (3-MB) by the CoA detour of the Ehrlich pathway. (A) The native and engineered CoA acylating aldehyde dehydrogenases (ALDHs) showed different ratios of specific activities toward 3-methylbutyraldehyde and isobutryaldehyde (3-MBA:IBA). The specific activities were measured using enzyme assays *in vitro* with purified enzymes. (B,C) Computational structure modeling revealed the role of the T428A mutation in accommodating 3-MBA. Contours of the binding pocket are shown in black. The T428A mutation allows the bulky 3-MBA (green) to orient in a catalytic geometry, with the carbonyl carbon positioned for nucleophilic attack by Cys275 (cyan) and hydride transfer to NAD (orange). (D) Isobutanol and 3-MB production titers by WB108 strain. (E) Comparison of the 3-MB/isobutanol production ratio among different strains harboring different ALDHs. Alcohol titers were quantified at 72 h. Error bars represent one standard deviation of three replicates ($n = 3$).

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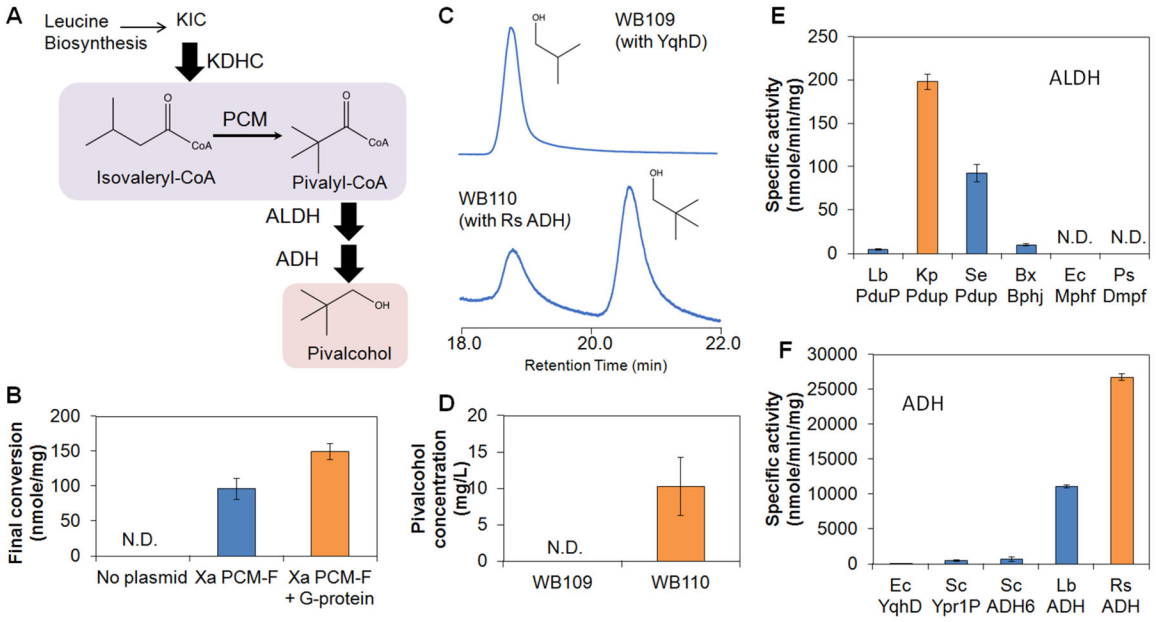

Figure 5.

Production of pivalcohol using the CoA detour of the Ehrlich pathway. (A) The pivalcohol production pathway in *Escherichia coli*.(B) Conversion of isovaleryl-CoA to pivalyl-CoA *in vitro* by crude cell lysates containing the pivalyl-CoA mutase fusion protein (PCM-F) from *Xanthobacter autotrophicus*, and with its G-protein chaperone. (C) Typical gas chromatography-flame ionization detector (GC-FID) traces showing the absence of pivalcohol in the production medium of WB109 (which has the *E. coli* YqhD), and the presence of pivalcohol in that of WB110 (which has *Ralstonia* sp. ADH). (D) Pivalcohol titers of WB109 and WB110. (E) Specific activities of various CoA-acylating aldehyde dehydrogenases (ALDHs) toward pivaldehyde, as measured *in vitro* using purified proteins. (F) Specific activities of various alcohol dehydrogenases (ADHs) toward pivaldehyde, as measured *in vitro* using purified proteins. N.D. not detected. See text for the sources of ALDHs and ADHs. Alcohol titers were quantified at 72 h. Error bars represent one standard deviation of three replicates ($n = 3$).

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Table 1.

Plasmids and Strains[a]

	comments	reference
Strains		
JCL260	BW25113/F′ [*traD36, proAB+, lacIq ZΔM15*, Δ*adhE*, Δ*ldhA*, ΔfrdBC, Δ*fnr*, Δ*pta*, Δ*pflB*]	2
AL707	JCL260: Δ*adhP*, Δ*eutG*, Δ*yiaY*, Δ*yjgB*, Δ*betA*, Δ*fucO*, Δ*eutE*	25
WB101	AL707 transformed with pWB101 and pWB102	this study
WB102	WB101 transformed with pWB103	this study
WB103	AL707 transformed with pWB101, pWB103, and pWB104	this study
WB104	AL707 transformed with pWB102, pWB103, and pWB105	this study
WB105	AL707 transformed with pWB103, pWB104, and pWB105	this study
WB106	JCL260 transformed with pWB102, pWB103, and pWB105	this study
WB107	AL707 transformed with pWB103, pWB105, and pWB106	this study
WB108	AL707 transformed with pWB103, pWB105, and pWB107	this study
WB109	WB104 transformed with pWB108	this study
WB110	AL707 transformed with pWB102, pWB103, and pWB108, pWB109	this study
Plasmids		
pWB101	P_{LlacO1}*::Ec yqhD*, SC101 *ori*, $Spec^R$	this study
pWB102	P_{LlacO1}*::Kp pdup-Pp KDHC*, ColE1 *ori*, Amp^R	this study
pWB103	P_{LlacO1}*::Ec leuA G462D-leuBCD*, p15A *ori*, Kan^R	this study
pWB104	P_{LlacO1}*::Kp pdup-Bs_KDHC*, ColE1 *ori*, Amp^R	this study
pWB105	P_{LlacO1}*::Bs alsS-Ec ilvCD-Ec yqhD*, SC101 *ori*, $Spec^R$	this study
pWB106	P_{LlacO1}*::Lb pdup-Pp KDHC*, ColE1 *ori*, Amp^R	this study
pWB107	P_{LlacO1}*::Lb pduP T428A-Pp KDHC*, ColE1 *ori*, Amp^R	this study
pWB108	P_{BAD}*::-Xa PCM-F-Xaut 5042*, RSF *ori*, Cm^R	this study
pWB109	P_{LlacO1}*::Bs alsS-Ec ilvCD-Rs ADH*, SC101 *ori*, $Spec^R$	this study

[a]Abbreviations indicate source of the genes: Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Bs, *Bacillus subtilis*; Pp, *Pseudomonas putida*; Lb, *Lactobacillus brevis*; Xa, *Xanthobacter autotrophicus*; Rs, *Ralstonia* sp.