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A synthetic O₂-tolerant butanol pathway exploiting native fatty acid biosynthesis in *Escherichia coli*

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Abstract

Several synthetic metabolic pathways for butanol synthesis have been reported in *Escherichia coli* by modification of the native CoA-dependent pathway from selected *Clostridium* species. These pathways are all dependent on the O₂-sensitive AdhE2 enzyme from *Clostridium acetobutylicum* that catalyzes the sequential reduction of both butyryl-CoA and butyraldehyde. We constructed an O₂-tolerant butanol pathway based on the activities of an ACP-thioesterase, acting on butyryl-ACP in the native fatty acid biosynthesis pathway, and a promiscuous carboxylic acid reductase. The pathway was genetically optimized by screening a series of bacterial acyl-ACP thioesterases and also by modification of the physical growth parameters. In order to evaluate the potential of the pathway for butanol production, the ACP-dependent butanol pathway was compared with a previously established CoA-dependent pathway. The effect of (1) O₂-availability, (2) media and (3) co-expression of aldehyde reductases was evaluated systematically demonstrating varying and contrasting functionality between the ACP- and CoA-dependent pathways. The yield of butanol from the ACP-dependent pathway was stimulated by enhanced O₂-availability, in contrast to the CoA-dependent pathway which did not function well under aerobic conditions. Similarly, whilst the CoA-dependent pathway only performed well in complex media, the ACP-dependent pathway was not influenced by the choice of media except in the absence of O₂. A combination of a thioesterase from *Bacteroides fragilis* and the aldehyde reductase, *ahr*, from *E. coli* resulted in the greatest yield of butanol. A product titer of ~300 mg/L was obtained in 24 h under optimal batch growth conditions, in most cases exceeding the performance of the reference CoA-pathway when evaluated under equivalent conditions.

Key words: Butanol, *Escherichia coli*, synthetic pathway, fatty acid biosynthesis, biofuel

Introduction

As atmospheric CO₂ levels are continuously rising, the search for a complement and eventual replacement of fossil-derived fuels is an important global challenge. Simple alcohols, in the form of ethanol, are already used as substitutes for petroleum-derived fuel products. Longer chain-length alcohols such as 1-butanol (hereafter butanol) could also potentially be used in a similar way (Jin et al. 2011). Compared to ethanol, butanol offers improved technical specifications for conventional combustion engines with a higher energy density (29.2 vs. 19.6 MJ/liter), higher air-fuel ratio (11.1 vs. 9.0) and lower hygroscopicity, the latter resulting in less corrosion of fuel-storage vessels (Tornatore et al. 2011). In this regard, butanol is clearly a superior renewable fuel target for the existing transportation and fuel infrastructure compared to ethanol (Fortman et al. 2008).

At present, butanol is mainly used as a solvent in the cosmetic industry or as an intermediate for producing butyl esters and phthalates for the chemical industry (Lee et al. 2008; Mellan 1950). Butanol is currently produced in large quantities from propylene, derived from petroleum, as the traditional acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* became economically less feasible in the 1960's (Jones and Woods 1986; Zheng et al. 2009; Zverlov et al. 2006). Improvements to the biotechnological process, e.g. introducing highly productive biotechnological hosts, could potentially allow large-scale, economically sustainable production of butanol from renewable substrate to commence once again.

In recent years, clostridial species and *E. coli* have been routinely engineered to produce butanol via a CoA-dependent pathway (Cooksley et al. 2012; Shen et al. 2011; Sillers et al. 2008) or by reversal of the fatty acid beta-oxidation pathway (Dellomonaco et al. 2011). So far, the highest yielding CoA pathways utilize an oxygen sensitive clostridial enzyme, aldehyde/alcohol dehydrogenase (AdhE2). The pathway has been studied and engineered in various host organisms such as *Clostridium acetobutylicum* (Hou et al. 2013; Jang et al. 2012; Zheng et al. 2009), *Clostridium tyrobutyricum* (Yu et al. 2011), *Escherichia coli* (Bond-Watts et al. 2011; Shen et al. 2011), *Saccharomyces cerevisiae* (Steen et al. 2008) and *Synechococcus elongatus* PCC 7942 (Lan and Liao 2011; Lan and Liao 2012). By optimizing engineered butanol pathways and native host metabolism, high butanol titers (20-30 g/l; (Shen et al. 2011)) have been achieved under anoxic or partially oxic conditions in fermentors. Although impressive yield and productivity have been reported with the clostridial CoA-dependent pathways, they appear to be compromised in the presence of oxygen (Shen et al. 2011). Furthermore, such pathways have mainly been studied using rich complex media, adding to the cost of production and ruling out an evaluation of the stoichiometric yield.

We propose that butanol also can be produced by diversion of the bacterial fatty acid synthesis pathway. This idea was recently exploited to construct a synthetic pathway for the biosynthesis of propane, using an acyl-ACP thioesterase with a specificity for butyryl-ACP and an oxygen-insensitive carboxylic acid reductase (CAR) (Akhtar et al. 2013; Kallio et al. 2014). We observed that butanol accumulates as an undesirable by-product from this propane pathway and enquired whether the pathway instead could be used to effectively produce butanol. In order to enhance the flux through the butanol pathway, we evaluated the effect of alternative aldehyde reductases and thioesterases (Jing et al. 2011), in combination with CAR (Figure 1). The effect of aeration and culture media on pathway performance were also investigated and compared against a high-yielding CoA-dependent butanol pathway as a reference (Shen et al. 2011). Stoichiometric flux balance analysis was finally used to understand the available room for improvement and provide insights into how this may be achieved in future studies.

Materials and methods

Reagents

Restriction enzymes and Antarctic phosphatase were purchased from New England BioLabs (Ipswich, MA). Ligase and DNA polymerase enzyme were purchased from Thermo-Scientific Fermentas (Finland). Oligonucleotides were from Eurofins MWG Operon (Germany). All chemicals for culture media were obtained from Sigma-Aldrich (Finland).

Plasmid construction

E. coli DH5 α was used to propagate all plasmids and *E. coli* BL21 DE3 was used for expression studies. The oligonucleotide primers and plasmids used in the study are listed in Table 1. Acyl-ACP-thioesterase genes from *Bacteroides fragilis* (GenBank ID: CAH09236), *Marvinbryantia formatexigens* (GenBank ID: EET61113) and *Lactobacillus brevis* (GenBank ID: ABJ63754) were synthesized by GenScript (USA) and cloned into a pUC57 vector (GenScript, USA). Each thioesterase encoding gene was excised from the corresponding pUC57 plasmid with NcoI and HindIII and subcloned into a pET (Novagen, Amp^R) operon that already contained the carboxylic

acid reductase and its maturation factor phosphopantetheinyl transferase (Akhtar et al. 2013). The aldehyde reductases, Ahr from *E. coli* (GenBank ID: AAA97166.1) and slr1192 from *Synechocystis* sp. PCC6803 (GenBank ID: BAA18840.1) were selected based on their broad substrate specificity (Akhtar et al. 2013) and cloned into pCDF (Novagen, Sp^R) vectors.

Plasmids pIM8 (*ter*, Kan^R) and pEL11 (*atoB*, *adhE2*, *crt*, *hbd*, Amp^R) and the knock-out mutant JCL299 (*AldhA ΔadhE ΔfrdBC Δpta*) (Shen et al. 2011) were kindly provided by Claire R. Shen (Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, USA).

Media and cultivation

A 5 ml volume of LB liquid media was inoculated using *E. coli* glycerol stocks (-80°C), and incubated O/N at 37°C (150 rpm) in 12 ml Greiner polypropylene microbial culture tubes under aerobic conditions, with vent stoppers. A 50 ml volume of M9 minimal (240.8 mg MgSO₄, 11.1 mg CaCl₂, 1 mg thiamine, 6.78 g Na₂HPO₄, 3 g NaH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 20 g glucose per liter) or TB media (12 g tryptone, 24 g yeast extract, 4 ml glycerol, 12.5 g K₂HPO₄, 2.3 g KH₂PO₄, 20 g glucose per liter) was inoculated with a 1 v/v% (500 μl) inoculum ratio in 250 ml Duran-Schott, baffled Erlenmeyer flasks closed with GL-45 oxygen permeable screw caps for aerobic conditions. The cultures were allowed to grow (37°C, 200 rpm) until OD₆₀₀ = 0.4-0.5 (usually 3.5-4 h) when isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to all flasks at a final concentration of 0.5 mM. The induced cultures were thereafter grown (30°C, 200 rpm) until 24 h and samples were then taken for metabolite analysis. Cultivation under "micro-aerobic" conditions was carried out as described above with the following exceptions: main cultures were carried out in 20 mL of media in 160 ml serum bottles with a rotary shaking speed of 150 rpm. Induced cultures were sealed with gas tight rubber stoppers using aluminum seals and allowed to grow for 72 h before samples were taken for metabolite analysis. The oxygen amount was limited in the serum bottles, as no gas exchange was allowed through the rubber stopper.

Metabolite detection and growth rate measurement

The analytical system consisted of an Agilent 7890C gas chromatograph with autosampler and 5975C inert MS, the column was Supelco Equity-1 (300°C, 30 m x 0.32 mm x 1 μm). Supernatants of culture media were mixed with acetone and injected in split-less mode (1.9 ml/min He flow, injector at 250°C, rate of 20°C/min from 35-280°C, hold at 35°C for 4 min, hold at 280°C for 1 min). Butanol eluted typically at 5.0 min and was confirmed with commercial standards (Sigma-Aldrich, Finland); quantification was determined from a linear calibration curve of freshly prepared butanol standards prepared in acetone:water (1:1). The productivities of the strains were expressed as mg (of butanol)/liter (of culture)/h.

The concentration of residual glucose was quantified using a Megazyme total starch assay procedure modified for 96 well microtiter plates, as follows: 20 μl samples of the cultures were diluted with MQ water to a glucose concentration in the range between 25 and 500 mg/l. Then 10 μl of the diluted sample was mixed with a 190 μl reagent containing glucose oxidase/peroxidase and incubated at 37°C for 60 min. The absorbance was measured at 510 nm using the Tecan M200 Pro microplate reader in Perkin Elmer 96 MB transparent plates. Based on the remaining glucose concentration the product yield was calculated as mol(of butanol)/mol(of glucose). The

growth rates of the cultures were estimated by the optical density (600 nm) in 96 well microtiter plates using a Tecan M200 Pro microplate reader and 200 µl of liquid culture per well. Dry cell weight was determined gravimetrically for the stoichiometric analysis by drying cell pellets of 10 ml liquid culture at 80°C for 24h.

Table 1 – strains and plasmids.

Abbreviations used: BS - *Bacillus subtilis*; MM - *Mycobacterium marinum*; BF - *Bacteroides fragilis*; MF - *Marvinbryantia formatexigens*; LB - *Lactobacillus brevis*; EC - *Escherichia coli*; TD - *Treponema denticola*; CA - *Clostridium acetobutylicum*; S6803 - *Synechocystis sp.* PCC 6803

Stoichiometric analysis

Strain TPC4+Ahr was grown in M9 minimal media under aerobic conditions, as described in Media and cultivation. Flasks were sampled periodically for 24h and analyzed for glucose consumption and butanol production. Formation of biomass was monitored as cell density based on OD₆₀₀ values and later converted to gram per liter (Supplementary Figure 5).

Flux balance analysis was carried out with MATLAB® (MATLAB 2011) and COBRA toolbox v2.0 (Schellenberger et al. 2011) using Gurobi 5.5.0 solver (Gurobi Optimization 2013) for optimization. The *E. coli* stoichiometric model iAF1260 (Feist et al. 2007) was modified for the novel butanol pathway by inserting three catalytic reactions, one active transport through the cytoplasmic membrane and two additional transport reactions required by the model to function properly. For more detail see Supplementary Material.

Stoichiometric fluxes were individually calculated from experimental data for each time point (Figure 4). The approach applied here is similar to that called dynamic FBA (Mahadevan et al. 2002; Varma and Palsson 1994). In dFBA the batch time is divided into several time intervals. A standard FBA is carried out at the beginning of each interval followed by the integration of fluxes for that time step, assuming that the flux values are constant over that period. Here, the actual flux values were derived from the concentration change from one time point to the next, scaled to units of biomass and units of time. Therefore the flux values were supplied to the model in units of mmol per g DW per hour except for the biomass given in g per g DW per hour. The dry cell weights of the 24 h samples were determined and used to calculate the dry cell weight for each time point (see calibration curve on Supplementary Figure 3 and Metabolite detection and growth rate measurement chapter).

Results

Engineering a novel ACP-dependent pathway for biosynthesis of butanol

In order to utilize the *E. coli* host fatty acid synthase (FASII) pathway machinery for butanol biosynthesis (Figure 1A), we assembled three synthetic variant pathways, with each pathway harboring a distinct acyl-ACP thioesterase (E.C. 3.1.2.14; TPC4 – *Bacteroides fragilis* GenBank ID: CAH09236; TPC5 – *Marvinbryantia formatexigens* GenBank ID: EET61113; TPC6 – *Lactobacillus brevis* GenBank ID: ABJ63754). These thioesterases were selected based on their fatty acid substrate specificities in an *E. coli* K-27 host lacking the fatty acyl-CoA synthase FadD

(Jing et al. 2011). The thioesterases were assembled into a plasmid backbone carrying the *Bacillus subtilis* phosphopantetheinyl transferase maturation factor and a *Mycobacterium marinum* carboxylic acid reductase (hereafter, referred to as TPC4-6 with the number specifying the thioesterase; Table 1). Together, the TPC pathways catalyze the conversion of fatty acyl-ACP into corresponding fatty aldehydes as recently described (Akhtar et al. 2013). A previously described CoA-based butanol pathway (Figure 1B; plasmids pIM8 and pEL11 in Table 1) (Shen et al. 2011) was used as an external reference, in both the original host (JCL299) and BL21(DE3).

The assembled pathways were expressed in *E. coli* BL21(DE3), followed by quantitative and qualitative GC-MS analysis of alcohol products at specified time-points (Figure 2 and 3). In contrast to the fatty acid profile described by Jing and co-workers (Jing et al. 2011), the major observed product from the TPC pathways was butanol, although trace amounts of longer chain-length products (hexanol, octanol and decanol) also were detected in some cases (Kallio et al. 2014). Introduction of the TPC pathways did not appear to have any effect on the fitness of the host, even at relatively high product yields, as the growth rates remained constant for all the strains (Supplementary Figure 1 and 2).

The ACP- and CoA-dependent butanol pathways respond differently to environmental conditions

Given the substantial differences in the metabolic routes between the ACP- and CoA-dependent butanol pathways, we evaluated whether their performance may differ in response to environmental conditions. The pathways were first compared under micro-aerobic conditions as these were previously used for CoA-dependent pathways (Shen et al. 2011). In order to allow a precise quantification of the carbon incorporated into the products the cultivation was carried out in M9 minimal media with 20 g/l glucose. The TPC4 and TPC5 constructs produced 67 mg/l (0.026 mol butanol/mol glucose) and 5 mg/l (0.002 mol butanol/mol glucose) butanol, respectively, whereas no product was detected from TPC6 (Figure 2A). Under these conditions the butanol yield of the TPC4 system was 5 and 2 times higher in comparison to the CoA pathway strains JCL299/pIM8 + pEL11 and BL21 DE3/pIM8 + pEL11 respectively. The wild type strain, BL21 DE3, did not produce any detectable butanol.

The strains were then compared for butanol production yields in complex TB media with 20 g/l glucose and limited oxygen availability, conditions similar to those previously used to evaluate the CoA-dependent butanol producing pathways (Atsumi et al. 2008; Shen et al. 2011) (Figure 3). This time the highest titers were achieved by the control strain JCL299/pIM8 + pEL11 which produced 690 mg/l. The titer of TPC4 in TB media was 90% less than the CoA-dependent pathway (23 mg/l). On the contrary, the medium appeared not to have any significant effect on the performance of the TPC5 system.

The effect of increased aeration was evaluated using baffled Erlenmeyer flask and more intense shaking. The enhanced aeration resulted in increased butanol productivity of the TPC4 expressing strain by 90% in M9 minimal media and up to ten-fold in TB media (Figure 2C and 3). The productivity of the TPC5 construct did not change significantly in M9 minimal media, but it tripled in TB media (Figure 3). The higher aeration showed an opposite effect on both of

the reference strains in TB media; the productivity of JCL299/pIM8 + pEL11 and BL21 DE/pIM8 + pEL11 were still comparable to the TPC pathways, but dropped by 50% to 5.0 mg/l/h and 25% to 5.6 mg/l/h, respectively, in comparison to micro aerobic conditions (Figure 3).

Over-expression of aldehyde reductase enhances the productivity of the ACP-dependent butanol pathway

Under default conditions the conversion of butyraldehyde to butanol was accomplished by unknown native *E. coli* aldehyde reductases (AHR). Previous studies indicated (Rodriguez and Atsumi 2012) that the conversion of aldehyde to alcohol could be limited in the host organism, suggesting that over-expression of aldehyde reductases may improve the pathway flux towards butanol. Accordingly, two NADPH-dependent AHRs with a known specificity for butyraldehyde were evaluated: (1) Ahr from *E. coli* (Akhtar et al. 2013) and (2) the gene encoded by *slr1192* (Vidal et al. 2009) from *Synechocystis* sp. PCC 6803. Over-expression of Ahr resulted in an increase in butanol production in the TPC4 and TPC5 strains under aerobic conditions in TB media (Figure 3). The productivity of the TPC4 strain increased from 3.6 to 9.9 mg/l/h, whilst the productivity of TPC5 also increased from 1.5 to 1.8 mg/l/h. Also TPC6 was shown to produce butanol at 1 mg/l/h when Ahr was over-expressed (Figure 3). In contrast, over-expression of the aldehyde reductase, *slr1192*, appeared to have no effect on any of the pathways.

Stoichiometric evaluation of pathway performance

The highest producing strain, TPC4+Ahr, was chosen to evaluate butanol pathway performance in M9 minimal media (Supplementary Figure 5) and to compare it with theoretical modeling. A modified stoichiometric model *iAF1260* was constrained to experimentally quantified glucose (EGF), biomass and/or butanol (EBF) fluxes in order to calculate stoichiometric glucose flux (SGF), realistic and non-realistic maximum butanol (RMB and NMB, respectively), and realistic and non-realistic production efficiencies for butanol (RBE and NBE, respectively). For details see Materials and Methods and Supplementary Material.

The theoretical maximum of butanol production is achieved when the available carbon (i.e. glucose) is completely converted to alcohol whilst no other products are formed (see glucose consumption on Supplementary Figure 4A). In this sense there are two possible maxima: realistic, when biomass production is fixed at low level; and non-realistic, when butanol is the sole product. Figure 4A shows the calculated butanol production fluxes over time, when (1) biomass is constrained to experimentally measured levels (realistic maximum butanol, RMB), (2) the glucose influx is converted solely to butanol without biomass production (non-realistic maximum butanol, NMB) and (3) butanol is produced at levels experimentally determined (experimental butanol flux, EBF). The efficiency of butanol production peaks at 17 hours for both the realistic and non-realistic cases (see RBE and NBE on Figure 4B, respectively), although experimental butanol flux (EBF) reaches its highest value after 24 hours (Figure 4A). The peak of efficiency at 17 hours may be explained by a minimum in experimental glucose uptake (EGF, Supplementary Figure 4B) resulting in low values for both RMB and NMB. The transition from growth to stationary phase around 13h (Supplementary Figure 5A) may cause this significant drop in glucose uptake. At 24 hours, however, EGF increases again, and in a

much higher rate than would be expected from the increasing EBF (Supplementary Figure 4B and Figure 4A respectively). In fact, EBF is well below RMB and NMB throughout the 24 hours (Figure 4A) resulting in relatively low efficiencies as shown in Figure 4B. The low realistic (RBE) and non-realistic (NBE) butanol efficiency (Figure 4B), and the large difference between experimental and stoichiometric glucose fluxes (EGF, SGF; Figure 4A and B) together suggest that a significant amount of the consumed glucose is converted into an alternative byproduct that is not analyzed in the present study.

In complex TB media, a similar time course analysis of the TPC4+Ahr strain allowed higher butanol yields to be achieved compared to M9 minimal media. As predicted from stoichiometric analysis of M9 minimal media cultivation (Figure 4A) the majority of the consumed glucose was not utilized for biomass or butanol production. Instead almost 7 g/l acetic acid was formed while only 300 mg/l butanol was produced in 24h from 16 g/l consumed glucose (Figure 5).

Discussion

The present work describes several variations of an O₂-tolerant synthetic butanol pathway (TPC) which utilizes the endogenous fatty acid biosynthetic machinery of the *E. coli* host as a source of precursors. Throughout the work, the performance of the pathway was compared to a previously established CoA-dependent reference system, which has been reported to be sensitive to oxygen (Shen et al. 2011). The motivation here was to generate a pathway which would not be limited by the presence of O₂, thus allowing it to be utilized in a phototrophic biotechnological host such as microalgae (Ahmad et al. 2011) (Schenk et al. 2008) or cyanobacteria (Lan and Liao 2012). During the preparation of this study, Lan and co-workers described an alternative oxygen-tolerant pathway in cyanobacteria (Lan et al. 2013), but a direct comparison with the system presented here has not yet been carried out.

The three constructed TPC pathways, each expressing alternative acyl-ACP thioesterases, were all functional and produced butanol under aerobic conditions in *E. coli* BL21 (DE3). Thus, all the thioesterases were at least partially active towards the C4 butyryl-ACP substrate, although in the *E. coli* K27 strain the enzymes have previously been reported to be specific mainly towards C6 (TPC5 – *Marvinbryantia formatexigens*) and C8 (TPC4 – *Bacteroides fragilis*, TPC6 – *Lactobacillus brevis*) precursors (Jing et al. 2011). The most effective thioesterase for the release of butyrate was found to be the enzyme from *B. fragilis*, which has also recently been used for the production of propane in a pathway utilizing an aldehyde deformylating oxygenase (ADO) (Kallio et al. 2014). Unlike the reference butanol pathway, the availability of oxygen enhanced productivity in all cases; this is of specific interest for the design of autotrophic production platforms. Altogether, the TPC pathways were less sensitive to changes in environmental conditions (media and oxygen) than the CoA-dependent reference system, and considerable butanol production was observed under most of the tested conditions. Besides oxygen, the over-expression of aldehyde reductase *ahr* also had a clear positive impact on butanol synthesis. Together, all optimal modifications resulted in up to a thirty-fold improvement in productivity. The improvement afforded by aldehyde reductase over-expression was especially clear in complex media suggesting that under those conditions the conversion of the aldehyde intermediate to alcohol was insufficient in the native *E. coli* host, whereas in minimal medium other factors were limiting pathway flux.

Computational flux balance analysis of the TPC4 system highlighted that the performance of the pathway was far from optimal, and that the majority of the intermediates were directed towards products other than butanol. At least in part, this may be related to the kinetic properties of the enzymes involved, as for example the carboxylic acid reductase displays relatively low catalytic efficiency towards short chain length intermediates such as butyrate (approximately $0.01 \text{ k}_{\text{cat}}/\text{K}_{\text{m}}$, (Akhtar et al. 2013). In addition, the TPC operon has not yet been genetically optimized to balance the coupling between consecutive enzymatic steps. Such an imbalance is expected to result in a diversion away from the intended pathway towards undesired products. Altogether, the observed yields were markedly lower than those reported for industrial butanol production in *Clostridium acetobutylicum* (15 g/l in fermentors) (Liu et al. 2013) (Sillers et al. 2008) and *Escherichia coli* (30 g/l in fermentors) (Shen et al. 2011). These differences in yield can be explained by several factors. Firstly, the previous studies were carried out in controlled fermentors, and maximum productivity cannot be assessed nor compared with the small-scale shake flask culture systems used in the present proof-of-concept study. Secondly, there are clear fundamental differences between the TPC pathway and the CoA pathway in regards to (i) metabolic pathway structure and (ii) enzyme cofactor demand. For example, the *Clostridial* butanol pathway is an integral part of native fermentative metabolism, and linked directly to substrate level phosphorylation and growth. In comparison, the TPC pathway is a diversion of the fatty acid biosynthesis, without any specific metabolic pressure to generate butanol. Also, the TPC pathway is dependent on NADPH and ATP, whereas the reference pathway utilizes only NADH. The latter is an important consideration as the cofactor redox balance and potential metabolic bottlenecks may vary between different autotrophic or heterotrophic biotechnological systems.

In future studies, any optimization strategy should maximize the metabolic flux through the introduced pathway, while minimizing competition with other metabolic activities of the microbial host. Several metabolic engineering approaches have been shown to enhance fatty acid metabolism in general, which could also potentially be used to stimulate the TPC-based butanol pathway. Examples include over-expression of FadR (Zhang et al. 2012) or deletion of enzymes *fadD* (Lu et al. 2008) and *fadL* (Liu et al. 2012) associated with fatty acid β -oxidation. Knowing the enzymatic constraints of the following step, the kinetics of CAR for C4 substrates (Akhtar et al. 2013) (Kallio et al. 2014) would also be expected to contribute to enhanced production. The catalytic balance between the successive biosynthetic steps could further be optimized by modulating the expression profile of the individual enzymes involved (Zelcbuch et al. 2013), in combination with transcriptional down-regulation of genes (Na et al. 2013) involved in competing metabolic pathways, such as acetic acid formation. It has also been shown that modifications, which increase the availability of necessary cofactors, such as NADH, can be used to shift the metabolic homeostasis in favor of the target product (Shen et al. 2011). Finally, the maximum productivity of the ‘best’ genetically optimized strain should be evaluated in a controlled stirred fermentor system with regulated oxygen balance, pH and nutrient feeding in order to evaluate the biotechnological potential of the system.

Although isobutanol (Varman et al. 2013) and butanol (Lan and Liao 2011) have previously been produced in oxygenic cyanobacterial hosts, the yields are low, and the optimal strategy for

sustainable production of solar based biofuels is not yet known. As with all photosynthetic biotechnological platforms for the production of low value products, commercial applications are still hindered by relative inefficiency and challenges in scale-up and product recovery. In addition to advances to industrial scale biotechnologies using photosynthetic microalgae as production hosts, intensive fundamental research is currently being carried out to evaluate and overcome different limitations and technical obstacles.

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Table and Figure legends

Table I – Strains and plasmids

Abbreviations used: BS - *Bacillus subtilis*; MM - *Mycobacterium marinum*; BF - *Bacteroides fragilis*; MF - *Marvinbryantia formatexigens*; LB - *Lactobacillus brevis*; EC - *Escherichia coli*; TD - *Treponema denticola*; CA - *Clostridium acetobutylicum*; S6803 - *Synechocystis sp.* PCC 6803

Figure 1 – Scheme of 1-butanol synthesis in engineered *E. coli* BL21 DE3. (A) bacterial fatty acid synthesis with FASII multienzyme complex; (B) TPC pathway: acyl-ACP-thioesterase from *Bacteroides fragilis*, *Marvinbryantia formatexigens* or *Lactobacillus brevis*; carboxylic acid reductase from *Mycobacterium marinum* and aldehyde reductase either from the *E. coli* host, endogenously or overexpressing *slr1192* from *Synechocystis sp.* PCC 6803 or *ahr* from *E. coli*; (C) Previously described CoA-based reference pathway in plasmids pIM8 and pEL11 (Table 1), butanol production proceeds through butyraldehyde catalyzed by the oxygen sensitive aldehyde/alcohol dehydrogenase (AdhE2).

Figure 2 – A comparison of butanol production in M9 minimal media. (A) Butanol yield under micro aerobic conditions with limited oxygen availability, 72h incubation. (B) Butanol yield under aerobic conditions, 24h incubation. (C) Butanol productivity in mg butanol per liter per hour. Error bars show standard deviation (n=3).

Figure 3 – A comparison of butanol production in TB complex media. Butanol productivity in mg butanol/liter/hour; aerobic cultivation lasted for 24h while micro aerobic for 72h. Error bars show standard deviation (n=3).

Figure 4 – Stoichiometric evaluation of TPC4 butanol pathway. (A) Metabolic fluxes over time in M9 minimal media in mmol per g cell dry weight per hour. Both experimental (EBF) and *in silico* (RMB and NMB) butanol fluxes follow the pattern defined by experimental glucose (EGF, see Supplementary Figure 4B). Realistic and non-realistic maximum fluxes (RMB and NMB, respectively) tend the closest to their experimental counterparts at 17h rendering this point the highest in efficiencies (*cf.* panel B and main text). RMB gradually “reaches up” to NMB in accordance with the decrease in biomass flux (not shown here). (B) Realistic (RBE) and non-realistic (NBE) butanol production efficiencies calculated from the corresponding maximum fluxes (RMB and NMB, respectively) and the experimental butanol flux (EBF).

Figure 5 – Time-course profile of butanol and acetic acid production along with cell growth Dashed lines showing cell density measured in 96 well plates at 600 nm; unbroken black lines show butanol concentration; unbroken gray lines show acetic acid formation. Cultivation was performed in *E. coli* BL21 DE3 TPC4+Ahr under aerobic conditions in TB media. Error bars show standard deviation (n=3).

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For Peer Review

Table I – Strains and plasmids

Abbreviations used: BS - *Bacillus subtilis*; MM - *Mycobacterium marinum*; BF - *Bacteroides fragilis*; MF - *Marvinbryantia formatexigens*; LB - *Lactobacillus brevis*; EC - *Escherichia coli*; TD - *Treponema denticola*; CA - *Clostridium acetobutylicum*; S6803 - *Synechocystis sp.* PCC 6803

Strain/Plasmid	Description	Reference
<i>E. coli</i> strains		
DH5α	<i>fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Novagen
BL21 DE3	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS</i>	Novagen
JCL299	<i>ΔldhA ΔadhE ΔfrdBC Δpta</i>	[9]
Plasmids		
pET-TPC4	<i>lacI::aat_{BF}-ppt_{BS}-car_{MM} f1 ori Amp^R</i>	[19]
pET-TPC5	<i>lacI::aat_{MF}-ppt_{BS}-car_{MM} f1 ori Amp^R</i>	This study
pET-TPC6	<i>lacI::aat_{LB}-ppt_{BS}-car_{MM} f1 ori Amp^R</i>	This study
pCDF-ahr	<i>lacI::ahr_{EC} CDF ori Sp^R</i>	[18]
pCDF-slr1192	<i>lacI::slr1192_{S6803} CDF ori Sp^R</i>	[18]
pIM8	<i>lacO1::ter_{TD} Cola ori Kan^R</i>	[9]
pEL11	<i>lacO1::atoB_{EC}-adhE2_{CA}-crt_{CA}-hbd_{CA} Cole1 ori Amp^R</i>	[9]

Scheme of 1-butanol synthesis in engineered *E. coli* BL21 DE3. (A) bacterial fatty acid synthesis with FASII multienzyme complex; (B) TPC pathway: acyl-ACP-thioesterase from *Bacteroides fragilis*, *Marvinbryantia formatexigens* or *Lactobacillus brevis*; carboxylic acid reductase from *Mycobacterium marinum* and aldehyde reductase either from the *E. coli* host, endogenously or overexpressing *slr1192* from *Synechocystis* sp. PCC 6803 or *ahr* from *E. coli*; (C) Previously described CoA-based reference pathway in plasmids pIM8 and pEL11 (Table 1), butanol production proceeds through butyraldehyde catalyzed by the oxygen sensitive aldehyde/alcohol dehydrogenase (AdhE2).

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A comparison of butanol production in M9 minimal media. (A) Butanol yield under micro aerobic conditions with limited oxygen availability, 72h incubation. (B) Butanol yield under aerobic conditions, 24h incubation. (C) Butanol productivity in mg butanol per liter per hour. Error bars show standard deviation (n=3).
181x395mm (300 x 300 DPI)

A comparison of butanol production in TB complex media. Butanol productivity in mg butanol/liter/hour; aerobic cultivation lasted for 24h while micro aerobic for 72h. Error bars show standard deviation (n=3). 60x45mm (300 x 300 DPI)

Stoichiometric evaluation of TPC4 butanol pathway. (A) Metabolic fluxes over time in M9 minimal media in mmol per g cell dry weight per hour. Both experimental (EBF) and in silico (RMB and NMB) butanol fluxes follow the pattern defined by experimental glucose (EGF, see Supplementary Figure 4B). Realistic and non-realistic maximum fluxes (RMB and NMB, respectively) tend the closest to their experimental counterparts at 17h rendering this point the highest in efficiencies (cf. panel B and main text). RMB gradually “reaches up” to NMB in accordance with the decrease in biomass flux (not shown here). (B) Realistic (RBE) and non-realistic (NBE) butanol production efficiencies calculated from the corresponding maximum fluxes (RMB and NMB, respectively) and the experimental butanol flux (EBF).

63x24mm (300 x 300 DPI)

Time-course profile of butanol and acetic acid production along with cell growth Dashed lines showing cell density measured in 96 well plates at 600 nm; unbroken black lines show butanol concentration; unbroken gray lines show acetic acid formation. Cultivation was performed in *E. coli* BL21 DE3 TPC4+Ahr under aerobic conditions in TB media. Error bars show standard deviation (n=3).
51x34mm (300 x 300 DPI)

Butanol is an attractive biofuel for which sustainable production systems need to be developed. We constructed a novel synthetic pathway for butanol biosynthesis and carried out a systematic evaluation of its performance under varying environmental conditions. The pathway was found to be highly robust with features best suited for implementation in an oxygenic biotechnological host.

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Supplementary Material

A synthetic O₂-tolerant butanol pathway exploiting native fatty acid biosynthesis in *Escherichia coli*

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Contents

1. Stoichiometric evaluation of pathway performance 2

 1.1. Flux calculation 2

 1.2. Efficiency of butanol formation 3

 1.3. References 3

2. Supplementary figures 4

 Supplementary Figure 1 – Comparison of cell densities in M9 minimal media. 4

 Supplementary Figure 2 – Comparison of cell densities in TB media..... 5

 Supplementary Figure 3 – Correlation of optical density and cell dry weight. 6

 Supplementary Figure 4 – Glucose consumption and metabolic fluxes in M9 minimal media. 7

 Supplementary Figure 5 – Stoichiometric analysis in M9 minimal media..... 8

3. Oligonucleotide primers 9

1. Stoichiometric evaluation of pathway performance

For simulations and flux balance analyses the COBRA toolbox v2.0 (Schellenberger et al., 2011) was used in MATLAB® environment (MATLAB, 2011), using Gurobi 5.5.0 solver (Gurobi Optimization, 2013) for optimization. The *E. coli* stoichiometric model iAF1260 (Feist et al., 2007) was modified for the novel butanol pathway (TPC4) by adding the following reactions to the model:

- AATBF: acyl-ACP thioesterase from *Bacteroides fragilis*
- CARM: carboxylic acid reductase from *Mycobacterium marinum*
- AHR: aldehyde reductase (alcohol dehydrogenase) from *Escherichia coli* (missing from the original model)
- BTOHtp (transport between cytoplasm and periplasm), BTOHtex (diffusion between periplasm and the environment) and EX_btOH(e) (sink for excess butanol; not shown on the above graph) were only necessary for the model. Membrane transport by BTOHtp was hypothesized of being ATP-dependent towards periplasmic space and therefore unidirectional.

The following commands were sent to COBRA toolbox for execution (through MATLAB® command line interface; note that the model in this form includes the butanol pathway, but optimizes for biomass flux by default):

```
modelAAT = addReaction(model, 'AATBF', {'butACP[c]', 'h2o[c]', 'but[c]', 'ACP[c]', 'h[c]'}, [-1 -1 1 1 1], false)
modelCAR = addReaction(modelAAT, 'CARM', {'but[c]', 'nadph[c]', 'h[c]', 'atp[c]', 'btal[c]', 'nadp[c]', 'amp[c]', 'ppi[c]'}, [-1 -1 -1 -1 1 1 1 1], false)
modelAHR = addReaction(modelCAR, 'AHR', {'btal[c]', 'nadph[c]', 'h[c]', 'btoh[c]', 'nadp[c]'}, [-1 -1 -1 1 1], true)
modelBTOH = addReaction(modelCAR, 'BTOHtp', {'btoh[c]', 'atp[c]', 'adp[c]', 'pi[c]', 'btoh[p]'}, [-1 -1 1 1 1], false)
modelBTOH = addReaction(modelBTOH, 'BTOHtex', {'btoh[p]', 'btoh[e]'}, [-1 1], true)
modelBTOH = addReaction(modelBTOH, 'EX_btOH(e)', {'btoh[e]'}, [-1], true)
```

1.1. Flux calculation

Flux values were calculated using an approach similar to dynamic FBA (Mahadevan et al., 2002; Varma and Palsson, 1994). A standard FBA was carried out at the beginning of each time interval (i.e. at each time point) using concentrations in the previous step as input constraints. The flux values determined this way were assumed to be constant over the corresponding time interval and were integrated over the interval to get to the next time point. Using the fluxes calculated from experimental data for biomass and butanol production in oxic conditions the minimal glucose requirement was predicted for each time point. The model was constrained with all biomass-butanol flux pairs one by one and the objective function was set to the minimization of glucose flux (uptake). Since the glucose flux is a negative value from the model point of view, the mathematical maximization of the objective function was carried out instead. The following commands were executed by substituting the corresponding flux pairs for *fluxValueBiomass* and *fluxValueButanol*:

```
modelTest = changeObjective(modelBTOH, 'EX_glc(e)')
modelTest = changeRxnBounds(modelTest, 'Ec_biomass_iAF1260_core_59p81M', fluxValueBiomass, 'b')
modelTest = changeRxnBounds(modelTest, 'BTOHtex', fluxValueButanol, 'b')
```

```
solutionTest = optimizeCbModel(modelTest, 'max')
```

Stoichiometric glucose requirement of experimentally determined biomass and butanol has been calculated this way and plotted on Supplementary Figure 4A to compare with the actual glucose consumption rate. Flux values corresponding to both stoichiometric and experimental glucose requirement are shown on Supplementary Figure 4B.

1.2. Efficiency of butanol formation

Production efficiency measures the ability to convert carbon source (glucose) to desirable products (butanol). The theoretical maximum of butanol production is achieved when the available glucose is completely converted to alcohol, whilst no other products are formed. In this sense there are two possible maxima: realistic, when biomass production is allowed; and non-realistic, when butanol is the sole product. The COBRA toolbox was supplied with the following commands, while *fluxValueGlucose* and *fluxValueBiomass* were substituted for their actual experimental values, respectively:

```
modelTest = changeObjective(modelBTOH, 'BTOHtex')
modelTest = changeRxnBounds(modelTest, 'EX_glc(e)', fluxValueGlucose, 'b')
modelTest = changeRxnBounds(modelTest, 'Ec_biomass_iAF1260_core_59p81M', fluxValueBiomass, 'b')
solutionTest = optimizeCbModel(modelTest, 'max')
```

The above calculation was made with an assumption that the biomass flux in the cell cannot be zero at any time of its life cycle (realistic theoretical maximum). In the model, however, it is possible to optimize the solution for butanol production alone to get the stoichiometric or non-realistic maximum, when the glucose influx is being converted to butanol the most efficient way (see Figure 4A and B in the main text). The following commands were executed to simulate this behavior:

```
modelTest = changeObjective(modelBTOH, 'BTOHtex')
modelTest = changeRxnBounds(modelTest, 'EX_glc(e)', fluxValueGlucose, 'b')
solutionTest = optimizeCbModel(modelTest, 'max')
```

1.3. References

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2. Supplementary figures

Supplementary Figure 1 – Comparison of cell densities in M9 minimal media. Optical densities of various strains at 600 nm were compared from 200 μ l sample in a 96 well plate at the end of the production process in M9 minimal media, under aerobic conditions after 24h (green) and micro aerobic conditions after 72h (blue). Error bars show standard deviation (n=3).

Supplementary Figure 2 – Comparison of cell densities in TB media. Optical densities of various strains at 600 nm were compared from 200 μ l sample in a 96 well plate at the end of the production process in TB media, under aerobic conditions after 24h (violet) and micro aerobic conditions after 72h (orange). Error bars show standard deviation (n=3).

Supplementary Figure 3 – Correlation of optical density and cell dry weight. Cell optical density of strain TPC4+Ahr was measured at 600 nm after cultivation for 24h in M9 minimal media and calibrated for cell dry weight for stoichiometric evaluation of butanol production.

Supplementary Figure 4 – Glucose consumption and metabolic fluxes in M9 minimal media. The stoichiometric analysis of strain TPC4+Ahr was conducted in a 24h shake flask production process. **(A)** "Stoichiometric" glucose consumption (solid black) is calculated from experimentally measured biomass and butanol levels, whereas "experimental" glucose consumption (dashed grey) shows the actual, experimentally measured consumption rate. Cells consume more glucose throughout the experiment than they incorporate solely into biomass and butanol. **(B)** Both experimental glucose flux (EGF, solid light grey) and stoichiometric glucose flux (SGF, dashed dark grey) are calculated from their respective concentration change apparent on panel "A".

Supplementary Figure 5 – Stoichiometric analysis in M9 minimal media. Biomass (A) and butanol (B) production over time is shown in M9 minimal media with TPC4+Ahr strain for stoichiometric evaluation of butanol production. Error bars show standard deviation (n=3).

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3. Oligonucleotide primers

Primer name	Primer sequence
For_NcoI	ACT TCA <u>CCA TGG</u> GCT AAG GTA CCT AAT TAA TTA AAA AGG AG
Rev_BacFrag_TPC4_HindIII	ACC ATC <u>AAG CTT</u> TTT AAT TAA TTA AAC AAA TTT CAC TTT GGC GC
Rev_BryForm_TPC5_HindIII	ACC ATC <u>AAG CTT</u> TTT AAT TAA TTA TTC GGA AAA TTC AAC CAC GG
Rev_LacBrev_TPC6_HindIII	ACC ATC <u>AAG CTT</u> TTT AAT TAA TTA ACG GTT ATC CCA TTG AAT GG
For_Ahr_NdeI	ACC ATC <u>CAT ATG</u> TCG ATG ATA AAA AGC TAT GCC GCA AAA G
Rev_Ahr_XhoI	ACC ATC <u>CTC GAG</u> TCA AAA ATC GGC TTT CAA CAC CAC

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9 | **A synthetic O₂-tolerant butanol pathway exploiting native fatty acid biosynthesis in**
10 | ***Escherichia coli***
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Abstract

Several synthetic metabolic pathways for butanol synthesis have been reported in *Escherichia coli* by modification of the native CoA-dependent pathway from selected *Clostridium* species. These pathways are all dependent on the O₂-sensitive AdhE2 enzyme from *Clostridium acetobutylicum* that catalyzes the sequential reduction of both butyryl-CoA and butyraldehyde. We constructed an O₂-tolerant butanol pathway based on the activities of an ACP-thioesterase, acting on butyryl-ACP in the native fatty acid biosynthesis pathway, and a promiscuous carboxylic acid reductase. The pathway was genetically optimized by screening a series of bacterial acyl-ACP thioesterases and also by modification of the physical growth parameters. In order to evaluate the potential of the pathway for butanol production, the ACP-dependent butanol pathway was compared with a previously established CoA-dependent pathway. The effect of (1) O₂-availability, (2) media and (3) co-expression of aldehyde reductases was evaluated systematically demonstrating varying and contrasting functionality between the ACP- and CoA-dependent pathways. The yield of butanol from the ACP-dependent pathway was stimulated by enhanced O₂-availability, in contrast to the CoA-dependent pathway which did not function well under aerobic conditions. Similarly, whilst the CoA-dependent pathway only performed well in complex media, the ACP-dependent pathway was not influenced by the choice of media except in the absence of O₂. A combination of a thioesterase from *Bacteroides fragilis* and the aldehyde reductase, *ahr*, from *E. coli* resulted in the greatest yield of butanol. A product titer of ~300 mg/L was obtained in 24 h under optimal batch growth conditions, in most cases exceeding the performance of the reference CoA-pathway when evaluated under equivalent conditions.

Key words: Butanol, *Escherichia coli*, synthetic pathway, fatty acid biosynthesis, biofuel

Introduction

As atmospheric CO₂ levels are continuously rising, the search for a complement and eventual replacement of fossil-derived fuels is an important global challenge. Simple alcohols, in the form of ethanol, are already used as substitutes for petroleum-derived fuel products. Longer chain-length alcohols such as 1-butanol (hereafter butanol) could also potentially be used in a similar way (Jin et al. 2011). Compared to ethanol, butanol offers improved technical specifications for conventional combustion engines with a higher energy density (29.2 vs. 19.6 MJ/liter), higher air-fuel ratio (11.1 vs. 9.0) and lower hygroscopicity, the latter resulting in less corrosion of fuel-storage vessels (Tornatore et al. 2011). In this regard, butanol is clearly a superior renewable fuel target for the existing transportation and fuel infrastructure compared to ethanol (Fortman et al. 2008).

At present, butanol is mainly used as a solvent in the cosmetic industry or as an intermediate for producing butyl esters and phthalates for the chemical industry (Lee et al. 2008; Mellan 1950). Butanol is currently produced in large quantities from propylene, derived from petroleum, as the traditional acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* became economically less feasible in the 1960's (Jones and Woods 1986; Zheng et al. 2009; Zverlov et al. 2006). Improvements to the biotechnological process, e.g. introducing highly productive biotechnological hosts, could potentially allow large-scale, economically sustainable production of butanol from renewable substrate to commence once again.

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In recent years, clostridial species and *E. coli* have been routinely engineered to produce butanol via a CoA-dependent pathway (Cooksley et al. 2012; Shen et al. 2011; Sillers et al. 2008) or by reversal of the fatty acid beta-oxidation pathway (Dellomonaco et al. 2011). So far, the highest yielding CoA pathways utilize an oxygen sensitive clostridial enzyme, aldehyde/alcohol dehydrogenase (AdhE2). The pathway has been studied and engineered in various host organisms such as *Clostridium acetobutylicum* (Hou et al. 2013; Jang et al. 2012; Zheng et al. 2009), *Clostridium tyrobutyricum* (Yu et al. 2011), *Escherichia coli* (Bond-Watts et al. 2011; Shen et al. 2011), *Saccharomyces cerevisiae* (Steen et al. 2008) and *Synechocystis* ~~*sp.*~~ *Synechococcus elongatus*, PCC 68037942 (Lan and Liao 2011; Lan and Liao 2012). By optimizing engineered butanol pathways and native host metabolism, high butanol titers (20-30 g/l; (Shen et al. 2011)) have been achieved under anoxic or partially oxic conditions in fermentors. Although impressive yield and productivity have been reported with the clostridial CoA-dependent pathways, they appear to be compromised in the presence of oxygen (Shen et al. 2011). Furthermore, such pathways have mainly been studied using rich complex media, adding to the cost of production and ruling out an evaluation of the stoichiometric yield.

We propose that butanol also can be produced by diversion of the bacterial fatty acid synthesis pathway. This idea was recently exploited to construct a synthetic pathway for the biosynthesis of propane, using an acyl-ACP thioesterase with a specificity for butyryl-ACP and an oxygen-insensitive carboxylic acid reductase (CAR) (Akhtar et al. 2013; Kallio et al. 2014). We observed that butanol accumulates as an undesirable by-product from this propane pathway and enquired whether the pathway instead could be used to effectively produce butanol. In order to enhance the flux through the butanol pathway, we evaluated the effect of alternative aldehyde reductases and thioesterases (Jing et al. 2011), in combination with CAR (Figure 1). The effect of aeration and culture media on pathway performance were also investigated and compared against a high-yielding CoA-dependent butanol pathway as a reference (Shen et al. 2011). Stoichiometric flux balance analysis was finally used to understand the available room for improvement and provide insights into how this may be achieved in future studies.

Materials and methods

Reagents

Restriction enzymes and Antarctic phosphatase were purchased from New England BioLabs (Ipswich, MA). Ligase and DNA polymerase enzyme were purchased from Thermo-Scientific Fermentas (Finland). Oligonucleotides were from Eurofins MWG Operon (Germany). All chemicals for culture media were obtained from Sigma-Aldrich (Finland).

Plasmid construction

E. coli DH5 α was used to propagate all plasmids and *E. coli* BL21 DE3 was used for expression studies. The oligonucleotide primers and plasmids used in the study are listed in Table 1. Acyl-ACP-thioesterase genes from *Bacteroides fragilis* (GenBank ID: CAH09236), *Marvinbryantia formatexigens* (GenBank ID: EET61113) and *Lactobacillus brevis* (GenBank ID: ABJ63754) were synthesized by GenScript (USA) and cloned into a pUC57 vector (GenScript, USA). Each thioesterase encoding gene was excised from the corresponding pUC57 plasmid with NcoI and HindIII and subcloned into a pET (Novagen, Amp^R) operon that already contained the carboxylic

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acid reductase and its maturation factor phosphopantetheinyl transferase (Akhtar et al. 2013). The aldehyde reductases, Ahr from *E. coli* (GenBank ID: AAA97166.1) and slr1192 from *Synechocystis* sp. PCC6803 (GenBank ID: BAA18840.1) were selected based on their broad substrate specificity (Akhtar et al. 2013) and cloned into pCDF (Novagen, Sp^R) vectors.

Plasmids pIM8 (*ter*, Kan^R) and pEL11 (*atoB*, *adhE2*, *crt*, *hbd*, Amp^R) and the knock-out mutant JCL299 (*ΔldhA ΔadhE ΔfrdBC Δpta*) (Shen et al. 2011) were kindly provided by Claire R. Shen (Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, USA).

Media and cultivation

A 5 ml volume of LB liquid media was inoculated using *E. coli* glycerol stocks (-80°C), and incubated O/N at 37°C (150 rpm) in 12 ml Greiner polypropylene microbial culture tubes under aerobic conditions, with vent stoppers. A 50 ml volume of M9 minimal (240.8 mg MgSO₄, 11.1 mg CaCl₂, 1 mg thiamine, 6.78 g Na₂HPO₄, 3 g NaH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 20 g glucose per liter) or TB media (12 g tryptone, 24 g yeast extract, 4 ml glycerol, 12.5 g K₂HPO₄, 2.3 g KH₂PO₄, 20 g glucose per liter) was inoculated with a 1 v/v% (500 μl) inoculum ratio in 250 ml Duran-Schott, baffled Erlenmeyer flasks closed with GL-45 oxygen permeable screw caps for aerobic conditions. The cultures were allowed to grow (37°C, 200 rpm) until OD₆₀₀ = 0.4-0.5 (usually 3.5-4 h) when isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to all flasks at a final concentration of 0.5 mM. The induced cultures were thereafter grown (30°C, 200 rpm) until 24 h and samples were then taken for metabolite analysis. Cultivation under "micro-aerobic" conditions was carried out as described above with the following exceptions: main cultures were carried out in 20 mL of media in 160 ml serum bottles with a rotary shaking speed of 150 rpm. Induced cultures were sealed with gas tight rubber stoppers using aluminum seals and allowed to grow for 72 h before samples were taken for metabolite analysis. The oxygen amount was limited in the serum bottles, as no gas exchange was allowed through the rubber stopper.

Metabolite detection and growth rate measurement

The analytical system consisted of an Agilent 7890C gas chromatograph with autosampler and 5975C inert MS, the column was Supelco Equity-1 (300°C, 30 m x 0.32 mm x 1 μm). Supernatants of culture media were mixed with acetone and injected in split-less mode (1.9 ml/min He flow, injector at 250°C, rate of 20°C/min from 35-280°C, hold at 35°C for 4 min, hold at 280°C for 1 min). Butanol eluted typically at 5.0 min and was confirmed with commercial standards (Sigma-Aldrich, Finland); quantification was determined from a linear calibration curve of freshly prepared butanol standards prepared in acetone:water (1:1). The productivities of the strains were expressed as mg (of butanol)/liter (of culture)/h.

The concentration of residual glucose was quantified using a Megazyme total starch assay procedure modified for 96 well microtiter plates, as follows: 20 μl samples of the cultures were diluted with MQ water to a glucose concentration in the range between 25 and 500 mg/l. Then 10 μl of the diluted sample was mixed with a 190 μl reagent containing glucose oxidase/peroxidase and incubated at 37°C for 60 min. The absorbance was measured at 510 nm using the Tecan M200 Pro microplate reader in Perkin Elmer 96 MB transparent plates. Based on the remaining glucose concentration the product yield was calculated as mol(of butanol)/mol(of glucose). The

growth rates of the cultures were estimated by the optical density (600 nm) in 96 well microtiter plates using a Tecan M200 Pro microplate reader and 200 μ l of liquid culture per well. Dry cell weight was determined gravimetrically for the stoichiometric analysis by drying cell pellets of 10 ml liquid culture at 80°C for 24h.

Table 1 – strains and plasmids.

Abbreviations used: BS - *Bacillus subtilis*; MM - *Mycobacterium marinum*; BF - *Bacteroides fragilis*; MF - *Marvinbryantia formatexigens*; LB - *Lactobacillus brevis*; EC - *Escherichia coli*; TD - *Treponema denticola*; CA - *Clostridium acetobutylicum*; S6803 - *Synechocystis sp.* PCC 6803

Stoichiometric analysis

Strain TPC4+Ahr was grown in M9 minimal media under aerobic conditions, as described in Media and cultivation. Flasks were sampled periodically for 24h and analyzed for glucose consumption and butanol production. Formation of biomass was monitored as cell density based on OD₆₀₀ values and later converted to gram per liter (Supplementary Figure 5).

Flux balance analysis was carried out with MATLAB® (MATLAB 2011) and COBRA toolbox v2.0 (Schellenberger et al. 2011) using Gurobi 5.5.0 solver (Gurobi Optimization 2013) for optimization. The *E. coli* stoichiometric model iAF1260 (Feist et al. 2007) was modified for the novel butanol pathway by inserting three catalytic reactions, one active transport through the cytoplasmic membrane and two additional transport reactions required by the model to function properly. For more detail see Supplementary Material.

Stoichiometric fluxes were individually calculated from experimental data for each time point (Figure 4). The approach applied here is similar to that called dynamic FBA (~~dFBA~~) (Mahadevan et al. 2002; Varma and Palsson 1994). In dFBA the ~~experimental period~~ batch time is divided into several time intervals. A standard FBA is carried out at the beginning of each interval followed by the integration of fluxes for that time step, assuming that the flux values are constant over that period. Here, the actual flux values were derived from the concentration change from one time point to the next, scaled to units of biomass and units of time. Therefore the flux values were supplied to the model in units of mmol per g DW per hour except for the biomass given in g per g DW per hour. The dry cell weights of the 24 h samples were determined and used to calculate the dry cell weight for each time point (see calibration curve on Supplementary Figure 3 and Metabolite detection and growth rate measurement chapter).

Results

Engineering a novel ACP-dependent pathway for biosynthesis of butanol

In order to utilize the *E. coli* host fatty acid synthase (FASII) pathway machinery for butanol biosynthesis (Figure 1A), we assembled three synthetic variant pathways, with each pathway harboring a distinct acyl-ACP thioesterase (E.C. 3.1.2.14; TPC4 – *Bacteroides fragilis* GenBank ID: CAH09236; TPC5 – *Marvinbryantia formatexigens* GenBank ID: EET61113; TPC6 – *Lactobacillus brevis* GenBank ID: ABJ63754). These thioesterases were selected based on their fatty acid substrate specificities in an *E. coli* K-27 host lacking the fatty acyl-CoA synthase FadD

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(Jing et al. 2011). The thioesterases were assembled into a plasmid backbone carrying the *Bacillus subtilis* phosphopantetheinyl transferase maturation factor and a *Mycobacterium marinum* carboxylic acid reductase (hereafter, referred to as TPC4-6 with the number specifying the thioesterase; Table 1). Together, the TPC pathways catalyze the conversion of fatty acyl-ACP into corresponding fatty aldehydes as recently described (Akhtar et al. 2013). A previously described CoA-based butanol pathway (Figure 1B; plasmids pIM8 and pEL11 in Table 1) (Shen et al. 2011) was used as an external reference, in both the original host (JCL299) and BL21(DE3).

The assembled pathways were expressed in *E. coli* BL21(DE3), followed by quantitative and qualitative GC-MS analysis of alcohol products at specified time-points (Figure 2 and 3). In contrast to the fatty acid profile described by Jing and co-workers (Jing et al. 2011), the major observed product from the TPC pathways was butanol, although trace amounts of longer chain-length products (hexanol, octanol and decanol) also were detected in some cases (Kallio et al. 2014). Introduction of the TPC pathways did not appear to have any effect on the fitness of the host, even at relatively high product yields, as the growth rates remained constant for all the strains (Supplementary Figure 1 and 2).

The ACP- and CoA-dependent butanol pathways respond differently to environmental conditions

Given the substantial differences in the metabolic routes between the ACP- and CoA-dependent butanol pathways, we evaluated whether their performance may differ in response to environmental conditions. The pathways were first compared under micro-aerobic conditions as these were previously used for CoA-dependent pathways (Shen et al. 2011). In order to allow a precise quantification of the carbon incorporated into the products the cultivation was carried out in M9 minimal media with 20 g/l glucose. The TPC4 and TPC5 constructs produced 67 mg/l (0.026 mol butanol/mol glucose) and 5 mg/l (0.002 mol butanol/mol glucose) butanol, respectively, whereas no product was detected from TPC6 (Figure 2A). Under these conditions the butanol yield of the TPC4 system was 5 and 2 times higher in comparison to the CoA pathway strains JCL299/pIM8 + pEL11 and BL21 DE3/pIM8 + pEL11 respectively. The wild type strain, BL21 DE3, did not produce any detectable butanol.

The strains were then compared for butanol production yields in complex TB media with 20 g/l glucose and limited oxygen availability, conditions similar to those previously used to evaluate the CoA-dependent butanol producing pathways (Atsumi et al. 2008; Shen et al. 2011) (Figure 3). This time the highest titers were achieved by the control strain JCL299/pIM8 + pEL11 which produced 690 mg/l. The titer of TPC4 in TB media was 90% less than the CoA-dependent pathway (23 mg/l). On the contrary, the medium appeared not to have any significant effect on the performance of the TPC5 system.

The effect of increased aeration was evaluated using baffled Erlenmeyer flask and more intense shaking. The enhanced aeration resulted in increased butanol productivity of the TPC4 expressing strain by 90% in M9 minimal media and up to ten-fold in TB media (Figure 2C and 3). The productivity of the TPC5 construct did not change significantly in M9 minimal media, but it tripled in TB media (Figure 3). The higher aeration showed an opposite effect on both of

the reference strains in TB media; the productivity of JCL299/pIM8 + pEL11 and BL21 DE/pIM8 + pEL11 were still comparable to the TPC pathways, but dropped by 50% to 5.0 mg/l/h and 25% to 5.6 mg/l/h, respectively, in comparison to micro aerobic conditions (Figure 3).

Over-expression of aldehyde reductase enhances the productivity of the ACP-dependent butanol pathway

Under default conditions the conversion of butyraldehyde to butanol was accomplished by unknown native *E. coli* aldehyde reductases (AHR). Previous studies [indicated](#) (Rodriguez and Atsumi 2012) ~~indicated~~ that the conversion of aldehyde to alcohol could be limited in the host organism, suggesting that ~~the~~ over-expression of aldehyde reductases may improve ~~the~~ pathway flux towards butanol. Accordingly, two NADPH-dependent AHRs with a known specificity for butyraldehyde were evaluated: (1) Ahr from *E. coli* (Akhtar et al. 2013) and (2) the gene encoded by *slr1192* (Vidal et al. 2009) from *Synechocystis* sp. PCC 6803. Over-expression of Ahr resulted in an increase in butanol production ~~within~~ the TPC4 and TPC5 strains under aerobic conditions in TB media (Figure 3). The productivity of the TPC4 strain increased from 3.6 to 9.9 mg/l/h, whilst the productivity of TPC5 also increased from 1.5 to 1.8 mg/l/h. Also TPC6 was shown to produce butanol at 1 mg/l/h when Ahr was over-expressed (Figure 3). In contrast, over-expression of the aldehyde reductase, *slr1192*, appeared to have no effect on any of the pathways.

Stoichiometric evaluation of pathway performance

The highest producing strain, TPC4+Ahr, was chosen to evaluate butanol pathway performance in M9 minimal media (Supplementary Figure 5) and to compare it with theoretical modeling. A modified stoichiometric model *iAF1260* was constrained to ~~experimental~~ [experimentally quantified](#) glucose (EGF), biomass and/or butanol (EBF) fluxes in order to calculate stoichiometric glucose flux (SGF), realistic and non-realistic maximum butanol (RMB and NMB, respectively), and realistic and non-realistic production efficiencies for butanol (RBE and NBE, respectively). For details see Materials and Methods and Supplementary Material.

The theoretical maximum of butanol production is achieved when the available carbon (i.e. glucose) is completely converted to alcohol whilst no other products are formed, ~~except the co-factors involved directly in the butanol synthesis~~ (see glucose consumption on Supplementary Figure 4A). In this sense there are two possible maxima: realistic, when biomass production is ~~allowed~~ [fixed at low level](#); and non-realistic, when butanol is the sole product. Figure 4A shows the calculated butanol production fluxes over time, when (1) biomass is constrained to experimentally measured levels (realistic maximum butanol, RMB), (2) the glucose influx is converted solely to butanol without biomass production (non-realistic maximum butanol, NMB) and (3) butanol is produced at levels experimentally determined (experimental butanol flux, EBF). The efficiency of butanol production peaks at 17 hours for both the realistic and non-realistic cases (see RBE and NBE on Figure 4B, respectively), although experimental butanol flux (EBF) reaches its highest value after 24 hours (Figure 4A). The peak of efficiency at 17 hours may be explained by a minimum in experimental glucose uptake (EGF, Supplementary Figure 4B) resulting in low values for both RMB and NMB. The transition from growth to stationary phase around 13h (Supplementary Figure 5A) may cause this significant drop in

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9 283 glucose uptake. At 24 hours, however, EGF increases again, and in a much higher rate than it
10 284 would be expected from the increasing EBF (~~Figure 4A and Supplementary Figure 4B and~~
11 285 Figure 4A respectively). In fact, EBF is well below RMB and NMB throughout the 24 hours
12 286 (Figure 4A) resulting in relatively low efficiencies as shown in Figure 4B. The low realistic
13 287 (RBE) and non-realistic (NBE) butanol efficiency (Figure 4B), and the large difference between
14 288 experimental and stoichiometric glucose fluxes (EGF, SGF; Figure 4A and B) together suggest
15 289 that a significant amount of the consumed glucose is converted into an alternative byproduct that
16 290 is not analyzed in the present study.

17 291
18 292 In complex TB media, a similar time course analysis of the TPC4+Ahr strain allowed higher
19 293 butanol yields to be achieved compared to M9 minimal media. As predicted from stoichiometric
20 294 analysis of M9 minimal media cultivation (Figure 4A) the majority of the consumed glucose was
21 295 not utilized for biomass or butanol production. Instead almost 7 g/l acetic acid was formed while
22 296 only 300 mg/l butanol was produced in 24h from 16 g/l consumed glucose (Figure 5).

23 297
24 298 **Discussion**

25 299 The present work describes ~~a novel, O₂-tolerant synthetic pathway for butanol production that~~
26 300 ~~utilizes the endogenous fatty acid biosynthetic machinery of the *E. coli* host. The constructed~~
27 301 ~~CoA-independent TPC pathways are functional and synthesize butanol under aerobic conditions;~~
28 302 ~~the availability of O₂, in fact, has a consistently positive impact on productivity, enhancing~~
29 303 ~~butanol productivity up to thirty fold when co-expressed with the *ahr* aldehyde reductase. This is~~
30 304 ~~in agreement with the fact that none of the enzymes in the pathway have been reported in the~~
31 305 ~~literature to be sensitive to oxygen, a feature of specific interest for any future applications in~~
32 306 ~~oxygenic autotrophic hosts such as several variations of an O₂-tolerant synthetic butanol pathway~~
33 307 ~~(TPC) which utilizes the endogenous fatty acid biosynthetic machinery of the *E. coli* host as a~~
34 308 ~~source of precursors. Throughout the work, the performance of the pathway was compared to a~~
35 309 ~~previously established CoA-dependent reference system, which has been reported to be sensitive~~
36 310 ~~to oxygen (Shen et al. 2011). The motivation here was to generate a pathway which would not be~~
37 311 ~~limited by the presence of O₂, thus allowing it to be utilized in a phototrophic biotechnological~~
38 312 ~~host such as microalgae (Ahmad et al. 2011) (Schenk et al. 2008) or cyanobacteria (Lan and Liao~~
39 313 ~~2012). In contrast, the CoA-dependent reference pathway utilizes enzymes, like AdhE2 from the~~
40 314 ~~facultative/obligate anaerobe *Clostridium acetobutylicum*, that are sensitive to O₂. During the~~
41 315 ~~preparation of this study, another oxygen-tolerant butanol pathway was described (Lan et al.~~
42 316 ~~2013). Lan and co-workers developed a CoA-dependent pathway that produced butanol in the~~
43 317 ~~cyanobacteria *Synechococcus elongatus* PCC 7942 under aerobic conditions. A key factor in~~
44 318 ~~enhancing the productivity was to employ O₂-tolerant Bldh (butyraldehyde dehydrogenase) and~~
45 319 ~~YqhD (NADPH-dependent alcohol dehydrogenase) to catalyze the reduction of butyraldehyde to~~
46 320 ~~butanol, in order to overcome the O₂-sensitivity of AdhE2. A direct comparison between this~~
47 321 ~~pathway and the TPC pathway, in any organism. During the preparation of this study, Lan and~~
48 322 ~~co-workers described an alternative oxygen-tolerant pathway in cyanobacteria (Lan et al. 2013),~~
49 323 ~~but a direct comparison with the system presented here has not yet been carried out.~~

50 324
51 325 There are also other fundamental differences between the CoA- and ACP-dependent pathways.
52 326 The TPC pathways consume ATP and NADPH with an additional loss of CO₂ as a by-product

while the CoA pathway requires only NADH. The utilization of ATP and NADPH, as well as the formation of gaseous by products such as CO_2 , can serve as effective metabolic driving forces that can enhance the overall thermodynamics of the pathway in the direction of interest (Lan and Liao 2012). The supply of electrons and loss of CO_2 , however, is double edged. Although the ratio of reduced to oxidized co-factors is higher for NADP(H) than NAD(H), the oxidation of NADH can be more effectively coupled to glycolysis, and thereby growth (Veit et al. 2008). The loss of carbon dioxide, based purely on stoichiometric grounds, reduces the theoretical conversion of glucose to butanol. Which factors are most important for overall productivity is difficult to speculate upon and will depend also on other parameters such as growth conditions, host strain, substrate requirements and media composition.

Although the productivity of the CoA dependent reference pathway is similar to the TPC4 system under standard aerobic conditions, additional oxygenation had a negative effect on the CoA pathway and a positive effect on TPC4. The moderate yields achieved with the TPC pathways may possibly be explained by weaker driving forces via the acyl-ACP thioesterase, as the substrate of this enzyme is taken from the fatty acid synthesis machinery. It is also known that CAR has a relatively low catalytic efficiency towards shorter chain length intermediates such as butyrate (approximately $0.01 \text{ k}_{\text{cat}}/\text{K}_{\text{m}}$; (Akhtar et al. 2013) owing possibly to a lowered affinity. The conversion of butyraldehyde to butanol, however, has a relatively large negative free energy change under standard conditions. Accordingly, over-expression of the aldehyde-reductase Ahr had a clear positive impact on butanol production in TB media which suggests that the conversion of aldehyde intermediate to alcohol is insufficient in the native *E. coli* host in complex media. In minimal media, other limiting factors were likely more important determinants of pathway flux than aldehyde reduction.

Interestingly, the comparison between the ACP and CoA pathways suggest that the synthetic TPC pathways are less sensitive to environmental conditions, as considerable butanol production was observed under all but one condition. In contrast, the CoA dependent reference pathway only appears to function well in one condition; complex media and limiting O_2 availability. Under industrial heterotrophic conditions, O_2 availability is likely to be limiting rather than

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abundant, an O₂-tolerant butanol pathway may therefore mainly be relevant for photoautotrophic biotechnological hosts.

In all the TPC pathways the main detected biosynthetic alcohol product was butanol, and only trace amounts of longer chain alcohols were observed. No fermentation products (e.g. ethanol, lactate, succinate and formate) were measured except for acetate (Figure 5). The product specificity of the TPC pathway is determined by the substrate preference of the acyl ACP-thioesterases, which release the growing fatty acyl ACP intermediates from the FASII fatty acid synthase machinery *in vivo*. The high specificity for C₄ is surprising as the selected thioesterases have previously been characterized in relation to substrate specificity (Jing et al. 2011), where it was found that the chain length preferences are C₆ (TPC5—*Marvinbryantia formatexigens*) and C₈ (TPC4—*Bacteroides fragilis*, TPC6—*Lactobacillus brevis*). Notably, the work of Jing *et al* (Jing et al. 2011) was carried out in a K27 strain lacking the acyl CoA synthetase *fadD* whilst our work was carried out in BL21(DE3). Nevertheless, the product profile could, in theory, also be affected by imperfect coupling between the thioesterase and carboxylic acid reductase step. This may explain why less than half of the molar quantity of butyrate was recovered in the form of butanol when the *B. fragilis* thioesterase was complemented by co-expression with CAR. This suggests that other butyric acid consuming pathways also are present and that their removal may enhance butanol yield.

The flux balance analysis highlighted that the performance of the TPC4 pathway is far from optimal, and the titers were markedly lower than what has been achieved in industrial butanol production using *Clostridium acetobutylicum* (15 g/l in fermentors) (Liu et al. 2013; Sillers et al. 2008) and *Escherichia coli* (30 g/l) (Shen et al. 2011). As the TPC pathways were not evaluated using controlled bioreactors, it is difficult to assess the maximum performance of the TPC4 pathway. Further optimization will also be needed in order to fully evaluate the industrial potential of a TPC4 based butanol production system. *Escherichia coli* BL21 (DE3) does not have an endogenous butanol pathway, whereas *Clostridium acetobutylicum* has evolved the capacity to produce and excrete butanol as one of the main final electron acceptors under optimal environmental conditions. Under these circumstances, the production of butanol is effectively

coupled to the optimal production of biomass, e.g. by allowing NAD^+ to be recycled from NADH generated in glycolysis. A similar strategy was employed in the work of Shen and co-workers (Shen et al. 2011), whereby the removal of NADH oxidizing reactions enhanced the productivity of the engineered clostridial butanol pathway in *E. coli* (Shen et al. 2011). With the synthetic NADPH dependent TPC4 pathway, there is no such coupling, effective strategies to maintain metabolic homeostasis are already in place in the host organism and the TPC pathway only represents a diversion; this is the reason why no butanol production is predicted with the stoichiometric model when only biomass represents the objective function.

Several metabolic improvements have recently been demonstrated for fatty acid biosynthesis in general, and there is no reason to believe that they would not also stimulate the TPC-based butanol pathway. Examples include over-expression of FadR (Zhang et al. 2012), deletion of *fadD* (Lu et al. 2008), and the modulated expression of synthetic pathway components (Zelevuch et al. 2013). As a relatively large operon was used to study the TPC pathway, the regulation of the expression system could be enhanced by promoter and ribosomal binding site modifications. The transcriptional down-regulation of genes encoding for competing metabolic pathways, such as acetic acid formation, is also likely to improve butanol production, similar to that carried out with the CoA dependent butanol pathway (Shen et al. 2011). Another approach to increase productivity would be to further improve oxygen supply e.g. cultivation in aerated and stirred bioreactors. Of course, with any improvement in productivity, the issue of product tolerance will also escalate.

The three constructed TPC pathways, each expressing alternative acyl-ACP thioesterases, were all functional and produced butanol under aerobic conditions in *E. coli* BL21 (DE3). Thus, all the thioesterases were at least partially active towards the C4 butyryl-ACP substrate, although in the *E. coli* K27 strain the enzymes have previously been reported to be specific mainly towards C6 (TPC5 – *Marvinbryantia formatexigens*) and C8 (TPC4 – *Bacteroides fragilis*, TPC6 – *Lactobacillus brevis*) precursors (Jing et al. 2011). The most effective thioesterase for the release of butyrate was found to be the enzyme from *B. fragilis*, which has also recently been used for the production of propane in a pathway utilizing an aldehyde deformylating oxygenase (ADO) (Kallio et al. 2014). Unlike the reference butanol pathway, the availability of oxygen enhanced productivity in all cases; this is of specific interest for the design of autotrophic production platforms. Altogether, the TPC pathways were less sensitive to changes in environmental conditions (media and oxygen) than the CoA-dependent reference system, and considerable butanol production was observed under most of the tested conditions. Besides oxygen, the over-

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expression of aldehyde reductase *ahr* also had a clear positive impact on butanol synthesis. Together, all optimal modifications resulted in up to a thirty-fold improvement in productivity. The improvement afforded by aldehyde reductase over-expression was especially clear in complex media suggesting that under those conditions the conversion of the aldehyde intermediate to alcohol was insufficient in the native *E. coli* host, whereas in minimal medium other factors were limiting pathway flux.

Computational flux balance analysis of the TPC4 system highlighted that the performance of the pathway was far from optimal, and that the majority of the intermediates were directed towards products other than butanol. At least in part, this may be related to the kinetic properties of the enzymes involved, as for example the carboxylic acid reductase displays relatively low catalytic efficiency towards short chain length intermediates such as butyrate (approximately $0.01\text{ k}_{\text{cat}}/\text{K}_m$, (Akhtar et al. 2013). In addition, the TPC operon has not yet been genetically optimized to balance the coupling between consecutive enzymatic steps. Such an imbalance is expected to result in a diversion away from the intended pathway towards undesired products. Altogether, the observed yields were markedly lower than those reported for industrial butanol production in *Clostridium acetobutylicum* (15 g/l in fermentors) (Liu et al. 2013) (Sillers et al. 2008) and *Escherichia coli* (30 g/l in fermentors) (Shen et al. 2011). These differences in yield can be explained by several factors. Firstly, the previous studies were carried out in controlled fermentors, and maximum productivity cannot be assessed nor compared with the small-scale shake flask culture systems used in the present proof-of-concept study. Secondly, there are clear fundamental differences between the TPC pathway and the CoA pathway in regards to (i) metabolic pathway structure and (ii) enzyme cofactor demand. For example, the *Clostridial* butanol pathway is an integral part of native fermentative metabolism, and linked directly to substrate level phosphorylation and growth. In comparison, the TPC pathway is a diversion of the fatty acid biosynthesis, without any specific metabolic pressure to generate butanol. Also, the TPC pathway is dependent on NADPH and ATP, whereas the reference pathway utilizes only NADH. The latter is an important consideration as the cofactor redox balance and potential metabolic bottlenecks may vary between different autotrophic or heterotrophic biotechnological systems.

In future studies, any optimization strategy should maximize the metabolic flux through the introduced pathway, while minimizing competition with other metabolic activities of the microbial host. Several metabolic engineering approaches have been shown to enhance fatty acid metabolism in general, which could also potentially be used to stimulate the TPC-based butanol pathway. Examples include over-expression of FadR (Zhang et al. 2012) or deletion of enzymes *fadD* (Lu et al. 2008) and *fadL* (Liu et al. 2012) associated with fatty acid β -oxidation. Knowing the enzymatic constraints of the following step, the kinetics of CAR for C4 substrates (Akhtar et al. 2013) (Kallio et al. 2014) would also be expected to contribute to enhanced production. The catalytic balance between the successive biosynthetic steps could further be optimized by modulating the expression profile of the individual enzymes involved (Zelcbuch et al. 2013), in combination with transcriptional down-regulation of genes (Na et al. 2013) involved in competing metabolic pathways, such as acetic acid formation. It has also been shown that modifications, which increase the availability of necessary cofactors, such as NADH, can be used to shift the metabolic homeostasis in favor of the target product (Shen et al. 2011). Finally,

the maximum productivity of the 'best' genetically optimized strain should be evaluated in a controlled stirred fermentor system with regulated oxygen balance, pH and nutrient feeding in order to evaluate the biotechnological potential of the system.

Although isobutanol (Varman et al. 2013) and butanol (Lan and Liao 2011) have previously been produced in oxygenic cyanobacterial hosts, the yields are low, and the optimal strategy for sustainable production of solar based biofuels is not yet known. As with all photosynthetic biotechnological platforms for the production of low value products, commercial applications are still hindered by relative inefficiency and challenges in scale-up and product recovery. In addition to advances to industrial scale biotechnologies using photosynthetic microalgae as production hosts, intensive fundamental research is currently being carried out to evaluate and overcome different limitations and technical obstacles.

Acknowledgements

We thank Claire Shen (Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, USA) for help with the supply of both reference strains. The work was funded by the Tekes project 40194/10. The research leading to these results has also received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement number 317184 (DM). This material reflects only the author's views and the European Union is not liable for any use that may be made of the information contained therein.

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9	490 Table and Figure legends
10	491
11	492 Table I – Strains and plasmids
12	493 Abbreviations used: BS - <i>Bacillus subtilis</i> ; MM - <i>Mycobacterium marinum</i> ; BF - <i>Bacteroides</i>
13	494 <i>fragilis</i> ; MF - <i>Marvinbryantia formatexigens</i> ; LB - <i>Lactobacillus brevis</i> ; EC - <i>Escherichia coli</i> ;
14	495 TD - <i>Treponema denticola</i> ; CA - <i>Clostridium acetobutylicum</i> ; S6803 - <i>Synechocystis sp.</i> PCC
15	496 6803
16	497
17	498 Figure 1 – Scheme of 1-butanol synthesis in engineered <i>E. coli</i> BL21 DE3. (A) bacterial fatty
18	499 acid synthesis <u>with FASII multienzyme complex</u> ; (B) TPC pathway: acyl-ACP-thioesterase from
19	500 <i>Anaerococcus tetradus</i> , <i>Bacteroides fragilis</i> , <i>Marvinbryantia formatexigens</i> or <i>Lactobacillus</i>
20	501 <i>brevis</i> ; carboxylic acid reductase from <i>Mycobacterium marinum</i> and aldehyde reductase either
21	502 from the <i>E. coli</i> host, endogenously or overexpressing <i>slr1192</i> from <i>Synechocystis sp.</i> PCC 6803
22	503 or <i>ahr</i> from <i>E. coli</i> ; (C) Previously described CoA-based reference pathway in plasmids pIM8
23	504 and pEL11 (Table 1), butanol production proceeds through butyraldehyde catalyzed by the
24	505 oxygen sensitive aldehyde/alcohol dehydrogenase (<i>adhE2</i> <i>AdhE2</i>).
25	506
26	507 Figure 2 – A comparison of butanol production in M9 minimal media. (A) Butanol yield
27	508 under micro aerobic conditions <u>with limited oxygen availability</u> , 72h incubation. (B) Butanol
28	509 yield under aerobic conditions, 24h incubation. (C) Butanol productivity in mg butanol/ per liter
29	510 <u>per hour</u> . Error bars show standard deviation (n=3).
30	511
31	512 Figure 3 – A comparison of butanol production in TB complex media. Butanol productivity
32	513 in mg butanol/liter/hour; aerobic cultivation lasted for 24h while micro aerobic <u>for</u> 72h. Error
33	514 bars show standard deviation (n=3).
34	515
35	516 Figure 4 – Stoichiometric evaluation of TPC4 butanol pathway. (A) Metabolic fluxes over
36	517 time in M9 minimal media: <u>in mmol per g cell dry weight per hour</u> . Both experimental (EBF)
37	518 and <i>in silico</i> (RMB and NMB) butanol fluxes follow the pattern defined by experimental glucose
38	519 (EGF, see Supplementary Figure 4B). Realistic and non-realistic maximum fluxes (RMB and
39	520 NMB, respectively) tend the closest to their experimental counterparts at 17h rendering this point
40	521 the highest in efficiencies (<i>cf.</i> panel B and main text). RMB gradually “reaches up” to NMB in
41	522 accordance with the decrease in biomass flux (not shown here). (B) Realistic (RBE) and non-
42	523 realistic (NBE) butanol production efficiencies calculated from the corresponding maximum
43	524 fluxes (RMB and NMB, respectively) and the experimental butanol flux (EBF).
44	525
45	526 Figure 5 – Time-course profile of butanol and acetic acid production along with cell growth
46	527 Dashed lines showing cell density measured in 96 well plates at 600 nm; unbroken black lines
47	528 show butanol concentration; unbroken gray lines show acetic acid formation. Error bars show
48	529 standard deviation (n=3). Cultivation was performed in <i>E. coli</i> BL21 DE3 TPC4+Ahr under
49	530 aerobic conditions in TB media. <u>Error bars show standard deviation (n=3).</u>
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For Peer Review

Response to reviewer's comments BB 14-106.R1

Manuscript entitled:

A synthetic O₂-tolerant butanol pathway exploiting native fatty acid biosynthesis in *Escherichia coli*

Dear editor,

We would like to thank the reviewer for the second evaluation of the revised manuscript. The comments were addressed by corrections or additions to the manuscript. The discussion section was rewritten to address the productivity and host related questions. Small errors in the language were also corrected in a few places. Altogether, we believe that addressing the comments of the reviewer and the editor has improved the manuscript.

Below is a detailed response to all comments with reviewer's comments in bold, the response in regular font. A PDF version of the manuscript with highlighted changes is uploaded to Biotechnology & Bioengineering's website. We hope that our revision answers all concerns of the reviewers and that the manuscript now is suitable for publication in Biotechnology & Bioengineering.

Sincerely yours,

Patrik R. Jones
Corresponding author

Comments from the Editor

(1) Especially, you want to address the productivity/efficiency concerns in more rational way in Discussion part.

The Discussion section has been restructured accordingly for context and clarity. The text has been divided into separate paragraphs discussing (1) the reasons for relatively low yields of the TPC pathway, including comparison to the previous production systems (lines 329-351), and (2) possible ways to improve pathway flux and total productivity (lines 353-369).

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Comments from Reviewer #1

(1) However, in my version, I could not find that "butyricacid" was corrected in the figure.

We apologize for this mistake, Figure 1 has been revised.

(2) Nevertheless, I am not convinced that the technical obstacles (productivity) will be overcome.

As described in the previous comment to the Editor, in addition to the discussion on the relatively low yields of the TPC pathway, we have included plausible ways of developing the system further. Potential solutions in improving productivity (lines 353-369) include modification of the native fatty acid metabolism, enzyme engineering, modulation of relative enzyme expression profile and improvement of environmental conditions. Although various limiting factors have been identified, the true potential of the current pathway however cannot yet be estimated.

In this context it is important to note that direct quantitative comparison of the yields of the TPC system with reported industrial production strains is not possible: This would require detailed evaluation in controlled bioreactors, which is outside the scope of this study.

(3) I am also not at all convinced that cyanobacteria will be suitable for production of butanol. Utilizing of sun light for production processes sounds great, but this is by far not all!

It is true that there are major obstacles in using photosynthetic organisms for the biosynthesis of low value products, which would require extremely large scale and efficient throughput. However currently there are companies like Cellana Inc. and Joule Unlimited Inc. which are approaching towards industrial production of low value products (incl. alcohols) using photosynthetic microalgae as hosts. In addition there are numerous examples of proof-of-concept studies which suggest that the potential of photosynthetic microbial host should be evaluated further. Consequently we have now included eukaryotic microalgae as alternative hosts in addition to cyanobacteria, and raised the concern of the commercial viability of these systems in the last paragraph of the Discussion (lines 371-378).