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Metabolic engineering of *Escherichia coli* for high-level production of free lipoic acid

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ABSTRACT

L-Lipoic acid (LA) is an important antioxidant with various industrial applications as a nutraceutical and therapeutic. Currently, LA is produced by chemical synthesis. Cell factory development is complex as LA and its direct precursors only occur naturally in protein-bound forms. Here we report a rationally engineered LA cell factory and demonstrate *de novo* free LA production from glucose for the first time in *E. coli*. The pathway represents a significant challenge as the three key enzymes, native Octanoyltransferase (LipB) and Lipoyl Synthase (LipA), and heterologous Lipoamidase (LpA), are all toxic to overexpress in *E. coli*. To overcome the toxicity of LipB, functional metagenomic selection was used to identify a highly active and non-toxic LipB and LipA from *S. liquefaciens*. Using high throughput screening, we balanced translation initiation rates and dual, orthogonal induction systems for the toxic genes, LipA and LpA. The optimized strain yielded 2.5 mg free LA per gram of glucose in minimal media, expressing carefully balanced LipB and LipA, *Enterococcus faecalis* LpA, and a truncated, native, Dihydrolipoyllysine-residue acetyltransferase (AceF) lipoylation domain. When the optimized cell factory strain was cultivated in a fed-batch fermentation, a titer of 87 mg/L free LA in the supernatant was reached after 48 h. This titer is ~3000-fold higher than previously reported free LA titer and ~8-fold higher than the previous best total, protein-bound LA titer. The strategies presented here could be helpful in designing, constructing and balancing biosynthetic pathways that harbor toxic enzymes with protein-bound intermediates or products.

1. Introduction

Lipoic acid (LA) is a vitamin-like cofactor that is essential for all aerobically respiring organisms. It has been implicated in protecting against oxidative damage associated with various diseases and neurodegenerative disorders and is widely used in managing symptoms of diabetic neuropathy and health supplementation (Tibullo et al., 2017). Industrially, LA is produced by chemical synthesis. This chemical process is a costly multistep synthesis with toxic waste streams that generate a racemic mixture of bioactive (R-) and inactive (L-) enantiomers (Pawar et al., 2015). Efficient biological production of LA by a genetically engineered cell factory could provide a more sustainable, stereospecific, and potentially more profitable method of production.

In nature, several biosynthetic pathways exist for producing LA. With slight variations, all share the unique feature of building the LA moiety covalently bound to its cognate protein rather than as a free molecule (Cronan, 2016). The first elucidated and best-studied pathway is found in *E. coli* (Fig. 1|a). *De novo* production is initiated by Octanoyl-[Acyl Carrier Protein]: Protein N-Octanoyltransferase (LipB), which "hijacks" and transfers the C8 fatty acid precursor, octanoyl, from acyl-carrier protein (ACP) to a lipoylation domain (LD) of either pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) or the glycine cleavage system (GCS) (Jordan and Cronan, 2003). The activities of lipoylated P DH and OGDH are essential in *E. coli* during aerobic growth on glucose (Hermes and Cronan, 2014). In the last step of the pathway, the protein-bound octanoyl has two sulfur atoms inserted by the Radical SAM enzyme, lipoyl synthase (LipA), producing protein-bound LA (lipoyl-LD) (Miller et al., 2000). The final product is

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dihydrolipoyl which must be oxidized by dihydrolipoyl dehydrogenase (Lpd) to function as a swinging-arm co-factor (Cronan et al., 2005). Alternatively, LA or octanoic acid can be scavenged, activated with ATP, and attached to a lipoylation domain by Lipoate-protein ligase (LplA) (Green et al., 1995). There is currently no known mechanism for the regulation or transport of LA in *E. coli*, so these cannot be rationally targeted for cell factory purposes (Cronan, 2014). It is speculated that due to the unusual protein-bound nature of the pathway, the production of LA is auto-regulated simply by the number of available lipoylation domains and does not need additional regulatory elements (Cronan,

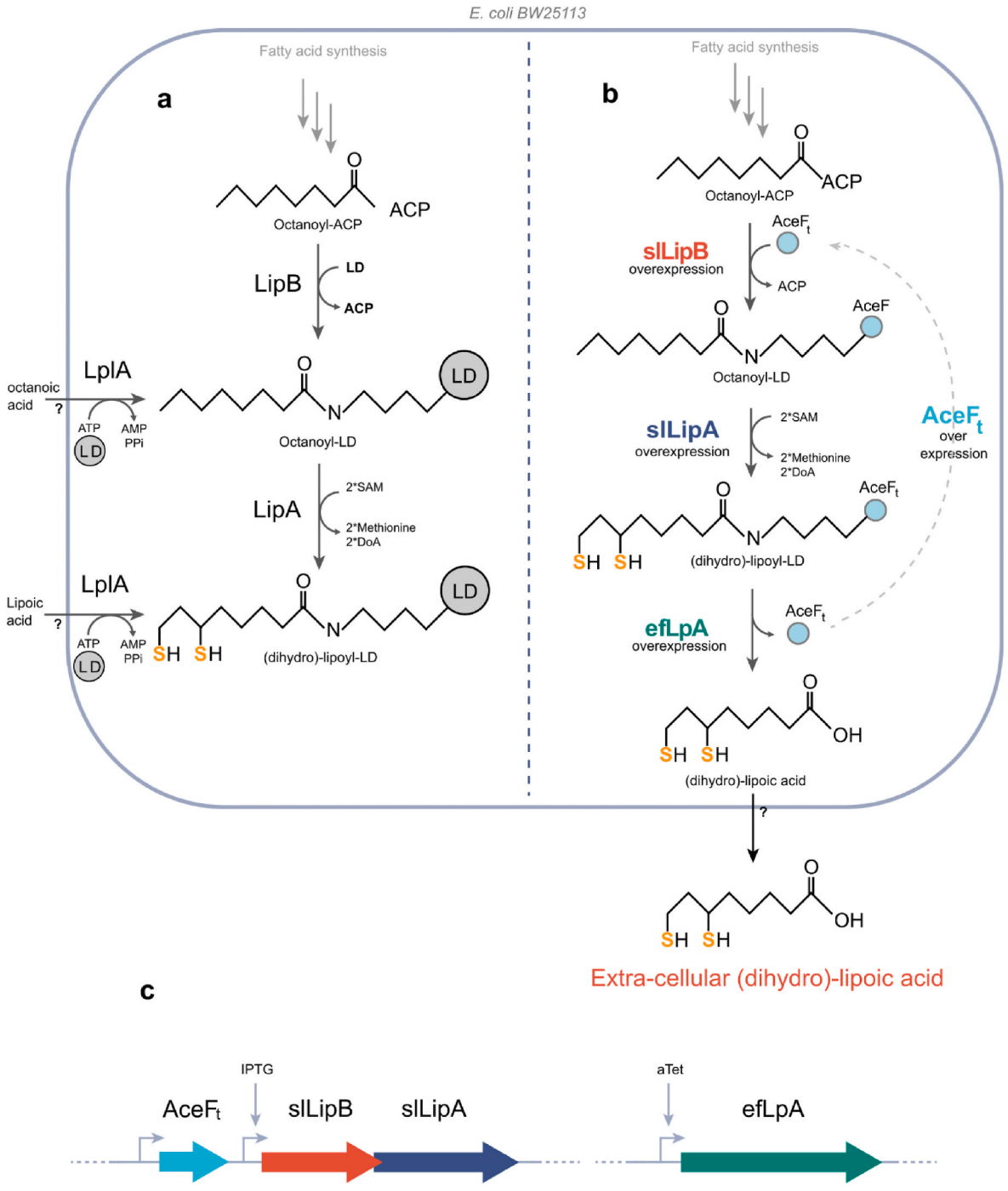

Fig. 1. a | Lipoic acid (LA) biosynthesis in *E. coli*, illustrating the LipB (octanoyl-transferase) dependent *de novo* pathway, starting from the fatty acid biosynthesis, and the LplA (lipoate-ligase) mediated scavenging of free octanoic acid or LA. The final step is the sulfur insertion by LipA (lipoyl synthase) at the C6 and C8 position of lipoylation-domain (LD) bound octanoyl. It should be noted that the final product of the LA biosynthetic pathway is dihydrolipoyl, which is then oxidized into lipoyl by Dihydrolipoyl dehydrogenase (Lpd) before it can function as a swinging-arm cofactor. Only the bioactive R-lipoic acid (or dihydro-lipoic acid) can be produced biosynthetically. **b** | The engineered cell factory pathway presented in this work utilizes over-expression of various heterologous genes to produce *de novo* free LA. These are LipA and LipB from *Serratia liquefaciens*, Lipoamidase (LpA) from *Enterococcus faecalis* and a native, truncated dihydrolipoyl lysine-residue acetyltransferase ($AceF_t$). **c** | The genes encoding the enzymes required for the cell factory are expressed via two plasmids in the final cell factory. One encodes constitutive transcription of AceFt and inducible slLipA and slLipB, while the second plasmid encodes efLpA the pTet promoter, inducible with anhydrous tetracycline (aTet).

2014).

While the LA biosynthetic pathway is relatively short, its distinctive protein-bound nature makes metabolic engineering difficult. Only a few projects aimed at engineering or optimizing a LA cell factory in bacteria have been reported (Ji et al., 2008; Moon et al., 2009; Sun et al., 2017). These studies resulted in the production of 74 μg, 94.5 μg, and 5.25 mg LA/g dry cell weight (g DCW). Rough conversion from the yield on biomass to titer puts the best attempt by Sun et al. (2017) (Sun et al., 2017) at 10.5 mg/L, but the specific titer is not reported in their work. None of these previous studies in bacteria aimed to produce free LA biologically but instead they chemically acid-hydrolyzed the amide-bound lipoyl after cell lysis, downstream of the production cultivation. They also utilized precursor feeding, in some cases coupled with LplA overexpression, only producing significant levels of LA when high concentrations of octanoic acid were present in the cultivation media. These strategies are not cost effective for developing an industrial fermentation process. Feedstock and downstream processing costs must be minimized while maximizing titers and productivity, i.e., without addition of precursors. Recently Chen et al. (2020) showed that functional heterologous expression of *Enterococcus faecalis* lipoamidase (efLpA), a hydrolase known to cleave lipoyl-lysine amide bonds (Jiang and Cronan, 2005; Spalding and Prigge, 2009), in the mitochondria of *S. cerevisiae,* can be used for cell factory production of *de novo* free LA (Chen et al., 2020). Coupling heterologous lipoamidase expression with overexpression of native yeast lipoyl synthase, octanoyl-transferase, H-protein (lipoylation domain), and SAM synthase, the authors demonstrated production of up to 30 μg/L free LA. This titer corresponds to 3 μg LA/g DCW, which is several orders of magnitude lower than the yields reported in bacteria above.

In this study we take an iterative metabolic engineering approach to develop a high *de novo* free LA producing *E. coli* cell factory. Overexpression of LipA, a truncated lipoylation domain $AceF_t$ (Sun et al., 2017; Ali and Guest, 1990), and efLpA led to significant production of free LA when fed with octanoic acid. As over-expression of native LipB and LipA caused toxicity, functional metagenomic selections were carried out to identify highly active heterologous LipB and LipA from *Serratia liquefaciens* (Fig. 1|b) (Jiang and Cronan, 2005; Spalding and Prigge, 2009). We further engineered libraries of synthetic ribosomal binding sites (RBSs) of all four over-expressed genes. By high-throughput screening of ~3000 combinations of plasmid architectures and expression levels, we identified the optimal translation initiation rates and induction levels for producing free LA, resulting in 2.5 mg/g glucose in small-scale. The cell factory was tested in fed-batch bioreactors, producing 87 mg/L of free LA in a 48-h fermentation. Finally, by regulating the Fe–S cluster capacities of the strain (Bali et al., 2020), we show that the bottleneck in the optimized cell factory is the catalytically challenging sulfur insertion reaction performed by LipA and discuss the next steps for maturing a free lipoic acid cell factory.

2. Results & discussion

2.1. Lipoamidase expression allows for free lipoic acid production from octanoic acid

No known microorganisms, including *E. coli*, produce any detectable amounts of free LA secreted into the growth media (Herbert and Guest, 1975). We previously described a basal LA cell factory that constitutively over-expresses a truncated AceF lipoylation domain and IPTG-inducible LipA, which we found to be toxic to cell growth if induced too highly based on a decrease of final ODs in liquid culture (Bali et al., 2020). This cell factory produced small amounts of free LA (~20 μg/L), which was strictly dependent on $AceF_t$ and LipA over-expression, and on high levels of octanoic acid being added to the media at just below its upper level of solubility in water (0.6 g/L) (Fig. S1). This free LA may be either spontaneously and non-enzymatically cleaved, or possibly produced through an endogenous amidase activity, for example, by the recently described native sirtuin lipoamidase, *cobB*, found in *E. coli* (Rowland et al., 2017). As the initial strain contained a genomic copy of *lplA* and *lipB*, it is unknown what fraction of LA was produced from the fed octanoic acid and what was *de novo* produced from glucose. We hypothesized that this strain had high levels of protein-bound LA, undetected by our quantification of only the extracellular product fraction.

In this work we aimed to increase the efficiency of production by cleaving the protein-bound fraction *in situ* by enzymatic activity. We initially over-expressed CobB but did not find this to significantly increase the production of free LA (Fig. S2). Lipoamidase from *Enterococcus faecalis* (efLpA) is a well-studied enzyme that can hydrolyze amide bonds linking LA to the lysine ε-amino groups of lipoylation domains in *E. coli* (Jiang and Cronan, 2005; Spalding and Prigge, 2009; Suzuki and Reed, 1963). While this enzyme is useful to release LA, it is also potentially toxic to the cell because its highly active catalysis easily cleaves all protein-bound LA from PDH and OGDH. Lacking the covalently attached LA cofactor, these enzyme complexes cease to function, and aerobic respiration shuts down, arresting growth (Reed, 2001; Perham, 2000).

Our initial attempts at cloning *E. faecalis* lipoamidase were unsuccessful, despite being cloned behind a very tightly regulated rhamnose-inducible promoter (Giacalone et al., 2006). Based on the previously described expression conditions by Jiang & Cronan (Jiang and Cronan, 2005), we grew clones in the presence of added acetate and succinate and were then able to isolate the cloned plasmid. These media additions allow *E. coli* to bypass the need for PDH and OGDH activity and regain partial function of the TCA cycle by activating acetate and succinate with Acetyl-CoA synthetase and Succinyl-CoA synthetase, respectively (Fig. S4). Combining the plasmid-based over-expression of LipA and $AceF_t$ with lipoamidase (efLpA), we were able to produce significant amounts of free LA from octanoic acid, which was secreted into the media (Fig. 2). Several versions of the lipoamidase expression plasmid were cloned and tested, varying the predicted strength of synthetic RBSs to test diverse translation initiation rates (TIRs) (Borujeni et al., 2017). Two strains expressing efLpA from pBS1189 and pBS1190, with synthetic RBS 1 and 2, were only able to grow in glucose minimal media with the addition of acetate and succinate during production, leading to very low final ODs. No growth or production was observed in minimal media with no added acetate or succinate (Fig. S3). In addition to the lower overall final OD, these strains, growing without the activities of PDH and OGDH due to depletion of intracellular lipoylation, were also more susceptible to toxic induction of LipA, leading to more severe IPTG-induced toxicity than those strains not requiring acetate and succinate supplementation and having a fully functioning TCA cycle, e.g. pBS993 and pBS1191 (Fig. 2) or indeed pBS993 alone (Fig. S1). The dysfunctional TCA cycle seemingly leading to higher sensitivity to the Fenton-chemistry based oxidative stress and Fe–S cluster depletion associated with LipA overexpression (Bali et al., 2020). One strain carrying a plasmid with efLpA RBS 3 (pBS1191) achieved expression of lipoamidase at a level where significant amounts of LA were released, but not so much as to become toxic to the cell (Fig. 2).

The best performing lipoamidase expressing strains produced ~200 μg/L free LA from fed octanoic acid in the presence of $AceF_t$ and LipA overexpression, representing a ~10-fold increase compared to the spontaneously released LA in the previous best strain, *BS1995*, shown in Fig. S1. 200 μ g/L free LA was produced in the presence of both efLpA expression from pBS1190 and pBS1191, the former releasing all protein-bound lipoic acid resulting in toxicity but leading to a very high free LA production per unit of biomass/OD. The latter, pBS1191, expressed efLpA at a more 'balanced' level, where significant free LA was produced but efLpA toxicity was not observed as seen from the OD values and ability to grow on glucose only minimal media. It should be noted that all efLpA expressing plasmids, including pBS1191, were toxic when not in the presence of pBS993 due to a much lower wild-type protein-bound lipoic acid pool. The ability of pBS933 (dihydrolipoyl-LD producing) to

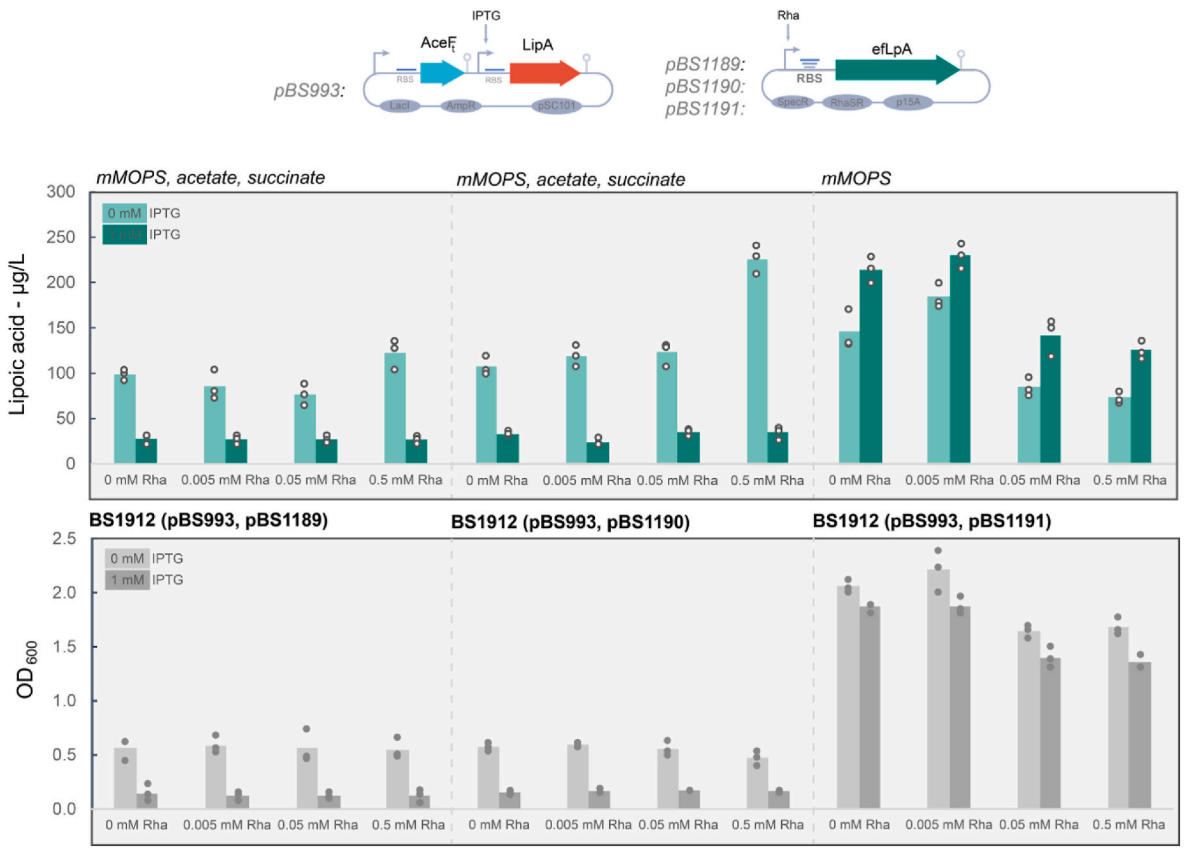

Fig. 2. Production of free LA from octanoic acid, with three RBS variants of inducible efLpA. The bars indicate median LA titer and final OD_{600} of three biological replicates (circles). Rhamnose was added to the production media as indicated below bars and IPTG (indicated by bar color intensity) was added to induce LipA. 0.6 g/L octanoic acid fed in all experiments and 10 mM acetate and succinate were added where indicated. pBS1189 and pBS1190 containing strains were only able to grow with addition of succinate and acetate to the media.

'rescue' the toxicity of pBS1191 (dihydrolipoyl-LD to free LA cleaving) is cause by this strain still retaining a healthy pool of intracellular protein-bound LA fraction, allowing some remaining functional AceF and SucB with bound LA and thereby letting PDH and OGDH retain functionality despite the over-expression of efLpA. This better balanced and healthy free LA producing strain, not needing additional media additions for growth, is of the greatest interest for further cell factory development as cellular metabolic health and reducing media cost is important for final bioprocess development. It should be said that the intracellular fraction of lipoic acid was not quantified, and there may still be room for more optimization of lipoamidase expression leading to further increases in the measurable free lipoic acid fraction before reaching toxicity caused by over-cleavage.

Hydrolyzing the protein-bound LA *in situ* during cultivation, rather than downstream, means that lipoylation domains can be recycled for multiple rounds of catalysis by LipB and LipA (Fig. 1 |b). For comparison if LA is only released downstream, each lipoylation domain is sequestered for the duration of the cultivation, until downstream processing. Therefore, it is only possible to produce the same number of LA molecules as there are lipoylation-domain protein subunits available. $AceF_t$ contains one lipoylation domain and has a molecular weight of 8918 g/mol, while LA has a molecular weight of 206.33 g/mol. This means that for each gram of LA, a corresponding 43.2 g of $AceF_t$ must be synthesized by the cell factory. With this basic calculation, the yield of 5.25 mg (g DCW)$^{-1}$ reported by Sun et al. (2017) would require 227 mg lipoylation-domain protein (g DCW)$^{-1}$, corresponding to ~50% of the protein content of the cell under the assumption that ~50% of *E. coli* DCW is protein (Stouthamer, 1973). With this in mind, the production of protein-bound LA as the final product does not seem feasible for producing the high titers needed for developing an economically attractive bioprocess.

2.2. Metagenomic selection of improved LipB and LipA enzymes

Our initial efforts in producing LA were performed with the addition of the precursor octanoic acid in the media, to be attached by LplA. Besides adding the cost of an additional media component, the use of octanoic acid feeding with LplA attachment is not compatible with free LA production owing to the promiscuity of LplA, which prefers free LA to octanoic acid as a substrate (Morris et al., 1994). To overcome this and produce LA, from glucose we initially attempted to utilize *E. coli*'s native LipB and LipA. Our previous work showed that excessive overexpression of LipA is highly toxic to *E. coli* (Bali et al., 2020). Similarly, we found strains over-expressing *E. coli* LipB to have low viability, often not growing when transferred from agar plates to liquid media. They showed extremely long lag-phases in production in liquid culture and resulted in no significant increase in LA titers compared to the control with native LipB expression only from the genome (Fig. S5). Toxicity related to the over-expression of *E. coli* LipB has been reported previously, with authors assigning LipB a regulatory role associated with DNA-methylation (Vaisvila et al., 2000). This could explain why none of the previously reviewed attempts at engineering a bacterial LA cell factory utilized LipB for *de novo* production, instead opting for octanoic acid feeding via LplA, which superficially seems a less efficient route.

Rather than engineering strains to use the toxic *E. coli* LipB, we hypothesized that a heterologous LipB could potentially alleviate toxicity by abolishing disadvantageous regulatory functions in *E. coli*. To this end, we set up an auxotrophic complementation-based selection system to identify functional LipB-LipA operons from previously constructed metagenomic plasmid libraries. These metagenomic libraries

constitutively express random fragments of sheared metagenomic DNA from soil and gut microbiomes (Fig. 3). We chose to initially select for both LipB and LipA in one selection as many bacteria encode these two genes in one operon or separated by one other gene as in the case of *E. coli* (Fig. S6). Using a double knock-out strain lacking LipB and LipA, which we transformed with five libraries of metagenomic plasmids, we selected the transformants on minimal media with no added LA. This approach allowed cells to grow only when expressing both functional LipB and LipA.

A few transformants grew on the selective plates, and their metagenomic inserts were sequenced. This revealed that all hits bore metagenomic plasmids with a LipB-LipA operon, as targeted by this selection. The phylogenetic diversity of organisms from which the hits originated was relatively low compared to the known diversity of the utilized plasmid libraries, only providing LipB-LipAs from the *Serratia* and *Pseudomonas* genera (Fig. 3|b). These are all relatively closely related to *E. coli*, being of the gammaproteobacterial class. The isolated *S. liquefaciens* and *Pseudomonas* sp. LipB-LipA operon plasmids were co-transformed with a lipoamidase plasmid into *E. coli* and production of free LA was assayed. Unlike the combination of native LipB and LipA, the heterologous LipB-LipA operons enable *E. coli* to produce significant amounts of free LA from glucose, with *S. liquefaciens* LipB-LipA producing double that of *Pseudomonas* (Fig. 3|c). The *S. liquefaciens* LipB-LipA operon was able to produce 20-fold higher levels of free LA than *E. coli's* native machinery, up to 2 mg/L. It should be noted that the strain from which the metagenomic *S. liquefaciens* LipB-LipA operon originates has not been genome sequenced. The closest available sequence in NCBI is that of *Serratia liquefaciens* strain JL02 (99% identity).

We know from previous work that these metagenomic libraries contain a significantly higher sequence diversity than that obtained in our genetic selection (Bali, 2019; Buerger, 2018; Genee, 2015; Genee et al., 2016; Forsberg et al., 2012; Sommer et al., 2009). The lack of diversity identified in the screen may be due to the additional layer of selection provided by LipB toxicity, or the need for protein-protein interaction with *E. coli's* native lipoylation domains. The utilized metagenomic libraries contain a high copy-number, ColE1, origin of replication, and the gene(s) of interest are expressed from a strong and non-repressed pTet promoter. The relatively high copy number and transcription would likely de-select any toxic LipBs. While LipB was the main target, the initial selection was performed for LipB and LipA simultaneously. Obviously, this limits the potential hits to those organisms in which these are co-localized on the genome. This was done for two reasons: naturally occurring operons could provide optimal expression balance between the two genes, and it allowed for easy screening by direct transformation of the lipoamidase plasmid into the selected strain, without the need for sub-cloning every hit into a new plasmid with ecLipA. Based on recent observations on the difficulty of expressing heterologous radical SAM enzymes in *E. coli* that are active *in vivo* (Lennox-Hvenekilde et al., 2021; Lennox-Hvenekilde, 2021; Shomar et al., 2021), the inclusion of LipA functionality in the functional selection was also likely a limiting factor for diversity.

2.3. High-throughput optimization and balancing of LA production

The metagenomic plasmids express their inserts constitutively. We cloned the newly identified *S. liquefaciens,* slLipB-slLipA, operon behind an IPTG inducible promoter to allow for inducibility. To ensure optimal testing of expression space, especially of slLipA, we sought to induce this to toxicity as we previously observed for ecLipA. When initially expressed from the T5lacO promoter, the slLipB-slLipA operon with the native RBS could be expressed to a toxic level (Fig. S7). In the native operon structure, the open reading frame of slLipB and slLipA overlap, limiting the possibility of editing the RBS of slLipA without influencing the C-terminal translation of slLipB. Various synthetic RBSs in front of slLipB were calculated, created, and tested, from which one construct (TIR: 10k AU, pBS1506) allowed for the sought-after toxic effects on cellular growth. To determine whether LipA of LipB caused the toxicity, LipA was removed from the plasmid, leaving only LipB, which was not inducible to toxicity, indicating that the toxic element was LipA, the expression of which was apparently highly dependent on the RBS in front of LipB (Fig. S7).

We now sought to optimize and balance the expression of the pathway, focusing on the two toxic genes: slLipA and efLpA. Based on previous observations, LipA requires a carefully balanced expression to a level that maximizes conversion of the octanoyl-AceF$_t$ precursor to lipoyl-AceF$_t$ but is not so high that LipA misfolds into inclusion bodies (Reed and Cronan, 1993a) or is toxic due to Fe–S cluster drainage, oxidative stress, or other unknown causes (Bali et al., 2020). At the same time, the other toxic component of the pathway, efLpA, needs to be expressed at a level that allows for optimal release of LA without releasing so much that essential LA-dependent enzymes cease to function or that efLpA misfolds (Spalding and Prigge, 2009). This requires a careful balancing of the two components.

In addition to the previously utilized rhamnose inducible plasmids, efLpA was cloned behind a tetracycline-inducible promoter. This system

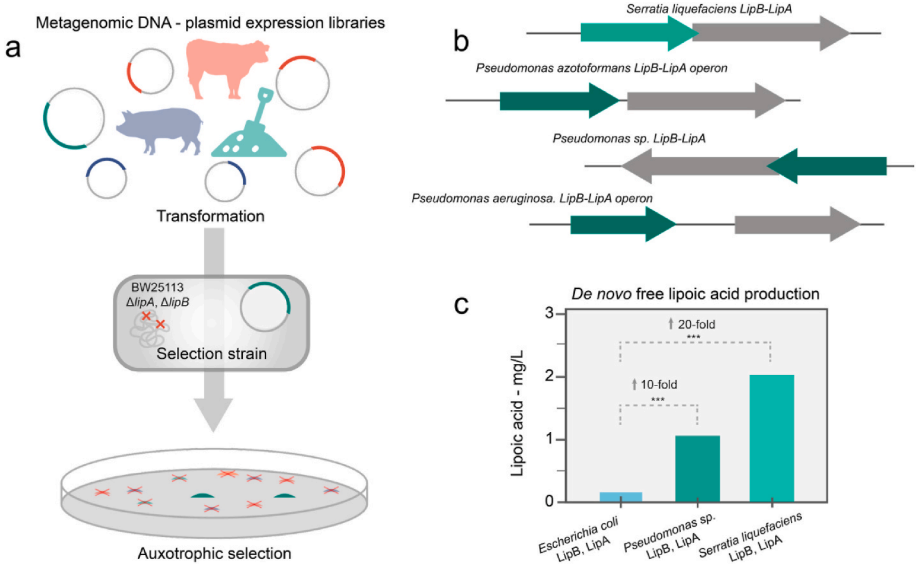

Fig. 3. a | Metagenomic selections were performed with plasmid libraries expressing randomly sheared DNA from various sources (Table S7). A Δ*lipB,* Δ*lipA,* LA auxotrophic strain (*BS2548*) was used to select on minimal media, allowing only strains expressing functional but non-toxic LipB and LipA to grow. Hits were only identified from the soil metagenomic plasmid library (green). **b** | Sequencing of plasmid hits revealed that almost all carried genes originating from the genus *Serratia* or *Pseudomonas.* A full list of hits is shown in Table S1. **C** | Two of these plasmid hits containing the LipB-LipA operons from *Pseudomonas* sp. And *Serratia liquefaciens* were tested for LA production in an *E. coli* cell factory also expressing efLpA from pBS1190 (strains: *Bsmeta1* and *Bsmeta2*). Compared to the expression of *E. coli* native LipB and LipA, the metagenomic hits showed significant improvements in producing *de novo* LA (mean of 3 biological replicates).

is orthogonal to IPTG induction and is better suited for titratable expression in the presence of glucose than the glucose repressed rhamnose induction system (Bertram and Hillen, 2008). Next, expression levels were modulated by small libraries of synthetic RBSs with wide ranges of predicted TIRs. These parts were all designed in an easily interchangeable and modular way using USER-cloning with complementary overhangs that included the RBS sequences. Finally, we also tested different origins of replication and an (anhydrous)-tetracycline efflux pump (TetA) for improved aTet induction response range. To sample this system's large expression space, we set up a high-throughput 96-well microtiter plate-based workflow for cultivation, induction, and production followed by semi-automated quantification of free LA in the supernatant by a LA bioassay (Herbert and Guest, 1970). This setup was used to rapidly test ~3000 individual production experiments (i.e. wells), allowing us to identify the optimal plasmid configuration and induction levels for the highest free LA titers without cellular toxicity (Fig. 4).

After several iterations of testing, we identified the best performing strain, *BS3804*, that produced high LA levels as a function of IPTG and aTet induction, up to ~7 mg/L in the initial library screening (Fig. 4c). When validated with multiple biological replicates, it consistently reached an average of 5 mg/L LA in small-scale batch screening conditions (Fig. 4d). We observed that in the best performing strain, no aTet induction of efLpA was needed for high level production of free lipoic acid. This is not because efLpA is not expressed in this strain, without efLpA almost no free LA is produced (Fig. S1), but rather because the basal expression of from the uninduced pTet promoter is in this case enough to provide the efLpA enzyme levels needed. Indeed, when slightly induced, at nM levels of aTet, the expression becomes toxic (Fig. 4d). LipA and LipB induction with IPTG is needed to reach maximum free LA titers, but likewise becomes toxic when induced too highly. The drop in production titers seen at high induction with both IPTG and aTet was caused by growth inhibition.

2.4. Testing of LA cell factory in fed-batch fermentations

Having constructed and identified a high-producing *de novo* free LA cell factory strain, we tested the best performer, *BS3804*, in benchtop fermenters of 0.4-liter working volumes. Based on the CO_2 evolution rate, the production strain grew at the same rates observed in small-scale screening, ~0.5 h^{-1}, during the ~8-h batch-phase of the fermentation, during which they produced up to 20–40 mg/L free LA from 20 g/L glucose (Fig. 5). However, a few hours into the fed-batch phase, all fermentations showed reduced CO_2 production, increased acidification

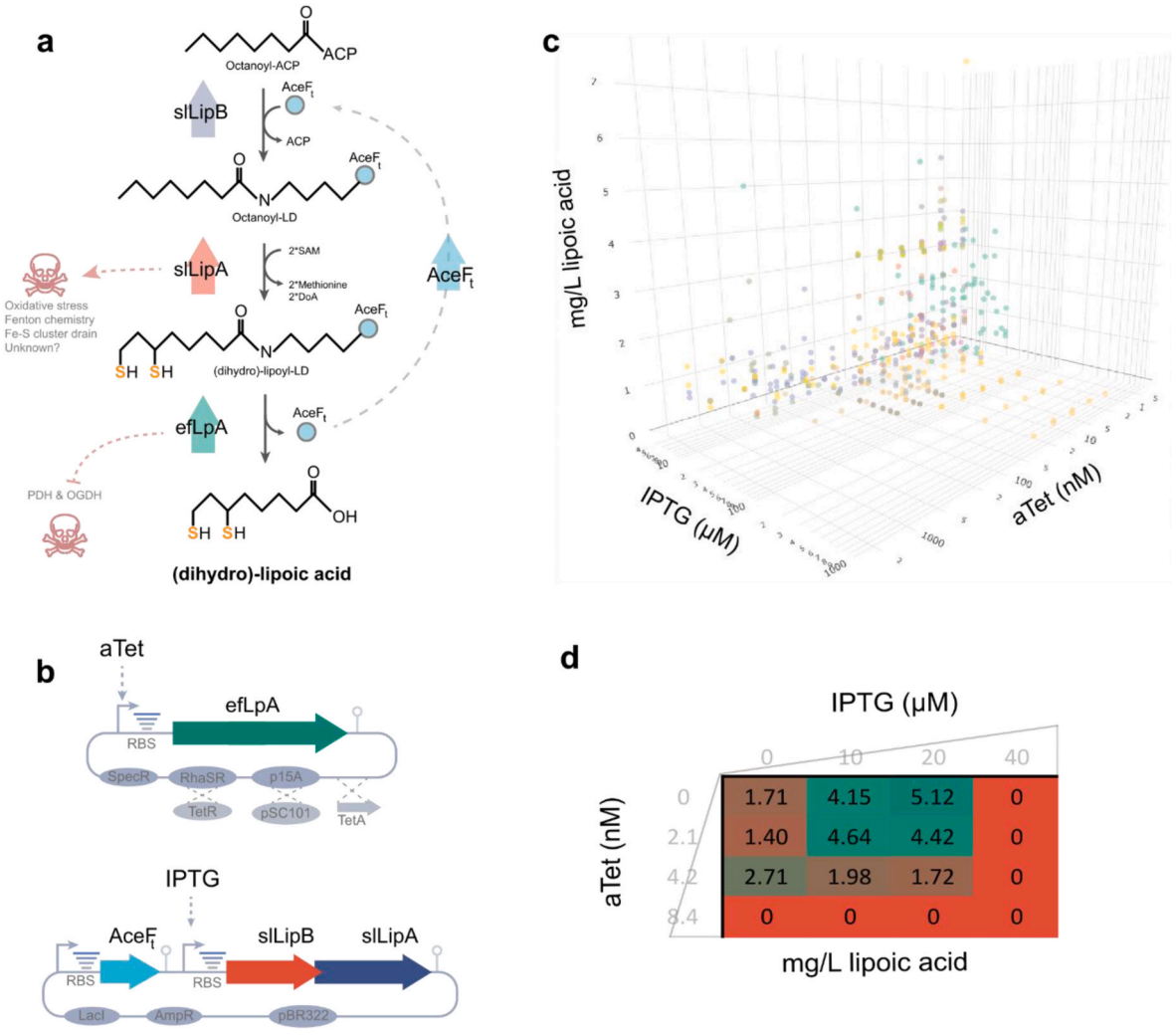

Fig. 4. **a** | The *de novo* biosynthetic pathway of free LA, indicating genes over-expressed (colored arrows) and toxicity associated with over-expression of slLipA and efLpA. **b** | Many iterations of plasmids were constructed, with synthetic RBS libraries, different inducible promoters, origins of replication, etc. **c** | Approximately 3000 distinct replicate experiments of various induction levels across different plasmid constructs was carried out. Those that resulted in a measurable free LA titers are shown as function as IPTG and aTet induction. Each discrete color indicates a different strain. **D** | The highest producing strain, *BS3804*, was validated for production (average titer, n = 4).

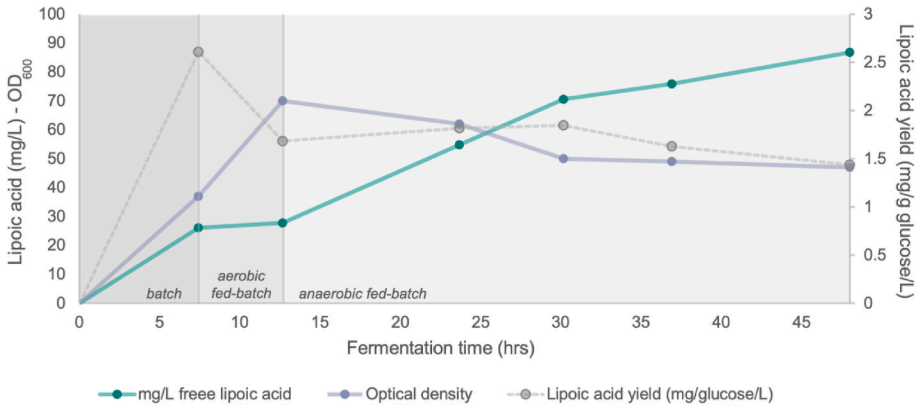

Fig. 5. Three-phase fermentation of the best performing LA production strain, *BS3804*. This strain is a *BS2548* (Δ*lipA*, Δ*lipB*, *iscR* C92Y) background with two plasmids: pBS1984 (tetracycline-inducible efLipA) and pBS1506 (constitutive $AceF_t$, IPTG inducible slLipB and slLipA). The first phase is the batch culture of ~8 h, while the second and third phases are fed-batch under aerobic and anaerobic conditions, respectively, of short (6 h) and long (>30 h) duration. The LA titer, OD, and LA yield on glucose were measured at 6 time points over a 48-h fermentation.

of the media, and foaming, no matter the induction levels of slLipA or efLpA applied (Table S2). This indicated arrest of cell growth, disruption of the metabolism, and cell lysis, accompanied by a complete disruption of further LA production.

In order to troubleshoot the toxicity appearing in the fed-batch phased, we performed 24 fed-batch fermentations varying strain and process parameters slightly (Table S2). We utilized earlier iterations of lower producing slLipA and efLpA plasmids in fermentation which displayed no toxicity in small-scale, hoping that these would allow for normal growth and elucidate the toxic component. The background strain was switched to avoid potentially unwanted mutations, and a strain that escaped toxicity but also stopped production during the fed-batch phase was whole-genome sequenced. Sequencing of the toxicity escapee did not indicate any genomic reason why this specific replicate could resume growth when compared to the sequenced background strain. When swapping plasmids to versions not predicted to be toxic, we found indications that both efLpA and slLipA caused toxicity in the fed-batch fermentation phase. A lower expression of efLpA partially reduced toxicity, and replacement of the slLipB-slLipA production plasmid with IPTG-inducible GFP also allowed for better growth (Table S2). We also changed various process parameters, including temperature, feeding rate, batch glucose concentration, and oxygen levels, but the most relevant factor seemed to be dissolved oxygen levels. We hypothesized that dissolved oxygen could influence LipA performance and likely toxicity since this enzyme's activity is dependent on two iron-sulfur [4Fe–4S] clusters, cofactors that are notoriously sensitive to oxygen (Imlay, 2006). Similarly, lipoamidase activity cannot cause toxicity under anaerobic conditions, as PDH and OGDH only function in the aerobic metabolism of *E. coli*, being functionally replaced by pyruvate-lyase formate lyase and fumarate reductase, respectively, under anaerobic growth (Reed and Cronan, 1993b).

To this end, we performed the batch phase as in the previous fermentations. To reach high biomass, we allowed for aerobic growth during the first hours of the fed-batch until the exhaust CO_2 ceased increasing, indicating the metabolic shift to toxicity as previously observed. At this point, the fermentation was switched to anaerobic/micro-aerobic by turning off sparging for the rest of the fed-batch phase (Fig. 5). This strategy was successful and produced the most LA of any tested fermentation: 87 mg/L. To our knowledge, this is also by far the highest titer reported of any LA cell factory, approximately 3000-fold higher than the previously reported best free LA titer (Chen et al., 2020) and ~8-fold higher than the previous best total protein-bound LA titer (Sun et al., 2017). While we were able to adjust fermentation process parameters to improve the final LA titer, this can be likened to treating the symptoms of toxicity rather than the underlying problem.

Further work would be needed to resolve this issue and improve the current cell factory for an industrial bioprocess. As discussed, the pathway relies on the careful balancing of the different toxic elements in the pathway. Scaling up can have a negative effect on the performance of a strain. This can happen as the population becomes more heterogeneous over longer cultivation time scales and is exposed to different process parameters than those in small-scale batch cultivations (Rugbjerg et al., 2018; Rugbjerg and Sommer, 2019).

2.5. LipA catalysis is the bottleneck in the optimized cell factory

The unique protein-bound nature of the pathway makes feeding or quantifying precursors highly challenging. We could not feed or quantify octanoyl-ACP, octanoyl-D*, or lipoyl-D* in a simple way to assess whether LipB, LipA, or LpA was the respective bottleneck. However, we do not believe that the availability of octanoyl-ACP precursor limits LA production: Previous studies have shown that the capacity of wild-type *E. coli* C8 fatty acid synthesis exceeds by far the levels of LA produced here. Hernández Lozada et al. (2018) showed that *E. coli* expressing only an engineered C8-specific thioesterase could produce 1.7 g/L octanoic acid (170 mg/g glucose) using similar minimal media and cultivation parameters with which we produced 3 mg/g glucose LA (Hernández Lozada et al., 2018).

The last and most critical step of the LA synthesis is performed by lipoyl synthase (LipA), which is a SAM-radical enzyme that inserts two S-atoms into octanoic acid. This reaction is catalytically challenging and sacrifices a 4Fe–4S cluster at each turnover. We previously reported that a point mutation in the global regulator, IscR, improved Fe–S cluster biosynthesis, reducing the toxicity of ecLipA over-expression and improving LA production in an early version of the LA cell factory described here (Bali et al., 2020). All small-scale screening and fermentations in this work were done using a strain containing an *iscR* C92Y mutation. To assess whether LipA was still limiting production in the optimized strain, we tested the effect of the presence or lack of the *iscR* C92Y genomic mutation combined with a high performing LA producing plasmid setup. The *iscR* mutant showed a 2-fold improvement of free LA production compared to the same strain with wild-type *iscR* (Fig. S9).

While there is no previously published data directly relating to LipA as the limiting element in cell factories, many such observations have been made for the highly similar Biotin synthase (BioB). BioB is well characterized as the bottleneck enzyme in biotin cell factories (Bali et al., 2020; Ikuku et al., 1995; Van Arsdell et al., 2005; Xiao et al., 2019). LipA functions by almost the same mechanism as BioB, and as such, LipA acting as the bottleneck in the engineered cell factory is unsurprising. It has recently been shown that the Fe–S cluster carrier, NfuA, allows efficient reconstitution of the sacrificial 4Fe–4S cluster of *E. coli* LipA *in vitro*, allowing each molecule of LipA to perform continuous catalysis rather than a single turn-over (McCarthy and Booker, 2017). However, upon over-expressing NfuA in our LipA limited cell factory strain, we found no significant improvement in LA production (Fig. S8). The catalysis of LipA and the biogenesis of Fe–S clusters, in general, is highly complex, and there are several theories as to the cause

of toxicity and bottleneck status of Fe–S cluster enzymes, making this class of enzymes a problematic target for metabolic and protein engineering (Py and Barras, 2010).

There have previously been reports of *E. coli* LipA over-expression resulting in a significant fraction of the protein in the insoluble fraction (Reed and Cronan, 1993a). We analyzed the level of LipA protein by whole cell-lysate SDS-PAGE and found this to be the case in our system as well (Fig. S10), with most of the LipA protein visible in the insoluble fraction. One study showed that the co-overexpression of the *isc* operon increased the yield of soluble LipA protein by 3-fold (Kriek et al., 2003). This indicates that the insoluble fraction of LipA arises due to lack of Fe–S cluster supply causing high levels of apo-protein, more prone to aggregation than the Fe–S cluster loaded holo-protein. However, we could not see a major difference between an *iscR* mutant, which has higher Fe–S cluster biosynthesis capabilities, and a wild-type *iscR* strain on the solubility of LipA (Fig. S10).

3. Conclusions and future perspectives

Lipoamidase and LipA must be addressed in terms of their toxicity and bottleneck roles in the free LA biosynthetic pathway to develop the cell factory further. It is counter-intuitive for an organism to express an intracellular, toxic lipoamidase. There has been some speculation that the natural role of lipoamidase is for scavenging LA from the environment (Jiang and Cronan, 2005; Spalding and Prigge, 2009). However, lipoamidase is not an exported enzyme, and extracellular protein-bound LA would not easily pass from the environment into the cytoplasm. Since *E. faecalis* does not encode *de novo* LA biosynthetic machinery, it requires LA from a different source. One could speculate that *E. faecalis* must have some inherent defense against the hydrolysis of its own protein-bound lipoic acid. There are some distinct sequence features of the *E. faecalis* lipoylation domain, variations in otherwise conserved residues of the lipoylation domain pointed out by Spalding and Prigge (2009) (Spalding and Prigge, 2009). To overcome the toxicity and need for fine-tuning lipoamidase expression, one could envision generating a strain that does not require LA, using lipoamidase-resistant lipoylation domains for its essential enzymes. One could even imagine a LipA that functions directly on free octanoic acid to produce free LA, thus eliminating the need for lipoamidase altogether. It is not unlikely that this mechanism potentially already exists in nature or that it is engineerable as almost the exact same radical SAM sulfur insertion already exists in the form of biotin synthase (BioB) (McLaughlin et al., 2016). BioB performs its catalysis in the same way as LipA, but on the free substrate of desthiobiotin, producing biotin, which must be attached by a ligase (BirA) before it functions as a cofactor in a very similar manner to LA (Berkovitch et al., 2010). The significant difference between the function of LipA and BioB is the fact that LipA must undergo a major conformational change to perform its catalysis. This conformational change is initiated by the interaction between LipA and the lipoylation domain, to which the octanoyl substrate is bound (McLaughlin et al., 2016).

As discussed, the general catalytic efficiency of LipA also needs to be improved further. While we know that it is possible to improve LipA catalysis (Bali et al., 2020), the complexity and lack of systematic understanding of the biology related to oxidative stress, electron transfer, and Fe–S cluster biosynthesis and delivery makes targeted engineering difficult (Shomar et al., 2021). More untargeted and multiplexed strategies may be needed to engineer this. Furthermore, based on the LA yield on glucose observed in small-scale, we would expect a 48-h fed-batch fermentation to produce at least two-fold higher lipoic acid titers than the 87 mg/L observed here. The cause of toxicity in fermentation needs to be further explored and hopefully eliminated for future process development.

Despite the yet unresolved bottlenecks, we were able to engineer a state-of-the-art lipoic acid cell factory producing significant levels of *de novo* and free lipoic acid. Using a combination of rational cell factory engineering, functional metagenomic selections, multiplexed plasmid cloning coupled with high-throughput screening, and process optimization, we produced 87 mg/L lipoic acid in a bench-scale fed-batch fermentation. This is a good starting point for the further development of an *E. coli* lipoic acid cell factory with the potential to replace the current petrochemical synthesis of lipoic acid currently in use today.

4. Materials and methods

General methods - All work was carried out in *Escherichia coli* BW25113 derived strains. Chemicals were bought from Sigma Aldrich or Carl Roth unless otherwise stated. All enzymes and buffers used for molecular biology methods were acquired from Thermo Fischer Scientific, and the manufacturer's protocol was followed unless otherwise stated. All LA titers reported were quantified by lipoic acid bioassay. All selections, testing of complementation, or small-scale production of free LA was done in minimal synthetic media, mMOPS, containing 2 g/L glucose, and other additives if indicated (Table S3). All growth of plasmid containing strains was done with relevant antibiotic added to the media, as specified by the resistance markers of plasmids (ampicillin: 100 μg/L, kanamycin: 50 μg/L; spectinomycin: 50 μg/L, zeocin: 40 μg/L and/or chloramphenicol: 30 μg/L). All incubation for molecular biology was done aerobically overnight (16–24 h), at 37 °C, with 300 rpm shaking for liquid cultures. Strains auxotrophic for LA were grown in the same way but in LB or 2xYT media. Strains expressing potentially toxic levels of lipoamidase were grown in 2xYT with added 10 mM K-acetate and 10 mM Na-Succinate unless used for production assays. Strains were stored at −80 °C in a 40% (vol/vol) glycerol solution. Synthetic DNA oligos for PCR amplification and MAGE were ordered from IDT, and Sanger and NGS sequencing was carried out by Eurofins Genomics (Germany). All PCR products and plasmids used were purified with E.Z.N.A.® Cycle Pure Kit (V-spin) and E.Z.N.A.® Plasmid Mini Kit I (V-spin) from Omega Bio-Tek, respectively.

Cloning of production plasmids - All plasmids were built by USER cloning with PhusionU Hot Start DNA Polymerase and New England Biolabs USER® enzyme, according to manufacturer protocols (Cavaleiro et al., 2015). Primers were designed with 5′ end USER-excision compatible overhangs. If applicable, a synthetic RBS with a specific predicted TIR, designed and predicted with the De Novo DNA© webserver (Reis and Salis, 2020), and a complementary sequence for amplifying the gene, part, or plasmid backbone of interest. Constitutive "apFAB" promoters were used, as reported by Mutalik et al. (2013) (Mutalik et al., 2013). The plasmids and oligos used for PCR amplification and USER-cloning are listed in Table S7 and Table S8. After PCR, the reaction mixtures were directly treated with DpnI enzyme for 1 h, purified, treated with USER-enzyme, and then with T4-ligase. Strains were then transformed with ligated plasmids by electroporation using homemade electro-competent cells of the background strain in question, using the washing and electroporation protocol as reported by Dower et al. (1988) (Dower et al., 1988). After transformation, cells were rescued in SOC media for 2 h, then washed twice in mMOPS and plated on selective minimal media plates if LipB/LipA containing plasmids were inserted to a ΔLipB/ΔLipA background strain. If necessary, cells were cultured with relevant antibiotics and possibly acetate and succinate and octanoic acid for growth overnight. If no auxotrophic selection was performed, cells were not washed and were plated on LB.

Genome editing and recombineering of *E. coli* - The starting strain was a Δ*lipA* obtained from the Keio collection, which was cured of the inserted *kanR* marker by FLPase (Baba et al., 2006). The C92Y mutation in the *iscR* was introduced as previously published (Bali et al., 2020). Translational knock-out of *lipB* was constructed by MAGE (Gallagher et al., 2014). BS1912 holding pBS136 was grown to 0.5 OD in 4 mL 2xYT ampicillin media, and L-Arabinose was added to a final concentration of 2% to induce the λ-Red system for 30 min of additional growth. For making electrocompetent cells, cultures were placed on ice for 20 min, centrifuged at 5000G for 5 min, and washed in 4 mL ice-cold water, the

washing was repeated twice, and cells were resuspended in 200 μl ice-cold 10% glycerol. 45 μl cells were mixed with 5 μl of 100 μM mOBS441 and electroporated at 1800 V. The cells were rescued in 950 μl prewarmed 2xYT media with ampicillin, and after 30 min, this was inoculated into a final volume of 4 mL 2xYT ampicillin media and incubated to 0.5 OD whereafter another round of MAGE was carried out. This was repeated to a total of 4 times, whereafter cells were plated on LB ampicillin plates and incubated overnight. Single colonies were colony PCR'ed using DreamTaq PCR Master Mix (2X) with oBS2394 and oBS2367, amplifying the *lipB* region of the genome. The presence of the translational knock-out of LipB was confirmed by Sanger sequencing. Confirmed knock-out strains were transformed with pBS848, the CRISPR/Cas9 pFREE plasmid curing system, and cured of all plasmids according to published protocol (Lauritsen et al., 2017). Successful plasmid during was confirmed by loss of ampicillin and kanamycin resistance by screening of LB plates. The MAGE oligo for knocking out genomic *lipB* was designed with the MODEST web-server by selecting "E_coli_K12_MG1655", "lipB", and "translational-knockout" operation (Bonde et al., 2014).

Metagenomic plasmid library selections - The double knock-out strain, *BS2548*, was used for selection. This strain has neither functional *lipB* nor *lipA* on the genome and requires LA supplementation or functional complementation of these two enzyme activities. 2.5 mL electrocompetent BS2548 were prepared from 125 mL exponentially growing cell culture as described above. Each of the five metagenomic plasmid libraries was transformed in five transformations by adding 95 μl competent cells to 1 μl plasmid and electroporating. The five transformations for each library were pooled and rescued in SOC for 2 h, whereafter they were washed in mMOPS twice and plated on mMOPS kanamycin plates at 1000x, 100x, 10x, and 1x dilutions. Additionally, a 10.000x dilution was plated on non-selective LB kanamycin plates as a control to calculate the total number of transformants obtained. All libraries had ~10^6- 10^7 transformants. Single colonies growing on the selection plates, the "hits", were incubated overnight in mMOPS kanamycin media, and plasmids were purified. To identify the inserted fragment of metagenomic DNA, sequencing oligos oBS0265 and oBS1223 were used for PCR with DreamTaq PCR Master Mix (2X), purified, and sequenced via Sanger method using oBS0265. Sequencing results were analyzed with the NCBI Open Reading Frame Finder (Wheeler et al., 2007) to identify the enzymes and their organism of origin. Hits are reported in Table S1.

Small-scale production of LA- All small-scale production tests of LA were carried out in the same way. Individual colonies (biological replicates) of each strain were transferred from plates of freshly transformed production strains or cryostock streak-outs and inoculated into 400 μl mMOPS with relevant antibiotics depending on strains/plasmids used (Table S7), and 10 mM K-acetate and 10 mM Na-succinate if needed for growth. After incubation for ~16 h in 96-well deep-well plates, these precultures were diluted 100-fold into 400 μl of the same media, but with the indicated addition of IPTG, rhamnose, anhydrous-tetracycline or octanoic acid for induction of LipB, LipA, and Lipoamidase and precursor supply. The deep-well plates were sealed with non-breathable aluminum seals and incubated for 20 h, the production phase. If the production experiment was assayed for growth rates, 600 μl production volumes were set up in the deep-well plates, whereafter 200 μl were transferred to a 96-well microtiter plate, which was incubated in a Multiskan™ FC Microplate Photometer with medium shaking, measuring OD_{620} every 20 min for at least 20 h. After the production phase, cultures were diluted 5-fold into microtiter plates, and the final OD_{620} was measured on Multiskan™ FC Microplate Photometer. Multiskan OD_{620} was converted to standard cuvette OD_{600} by dividing by 0.2569. The deep-well plates were centrifuged for 10 min at 4000×*g* to pellet cells, and 200 μl supernatant was aspirated into microtiter-plates, which was used directly for bio-assaying of free LA concentration or saved at −80 °C for later quantification. A data frame of all carried out production experiments with metadata, final ODs, and LA titers, as well as the scripts used to analyze/visualize this can be found at: https://github.com/DavidL-H/LA_cell_factory.

Fed-batch bioreactor production of LA- A single colony of the indicated strain was inoculated into 50 mL mMOPS with relevant antibiotics and grown to full cell density (cuvette OD_{600} ~2) overnight in a 250 mL baffled shake-flask. 200 ml Fermentation Batch medium (Table S4) supplemented with antibiotics, and 1 mL of a 1% (v/v) antifoam solution were added to Applikon 500 ml MiniBio Reactors with the temperature set to 37 °C, pH-controlled to pH = 7 by addition of 5 M NH_4OH and dissolved oxygen (DO) set-point to DO = 15% by agitation speed; sparging was fixed at 1 vol/vol/minute unless stated otherwise. 10 ml of each strain preculture was used to inoculate reactors, resulting in a starting cuvette OD_{600} = 0.10 for all reactors and strains/conditions. Once the CO_2 in the outlet gas reached greater than 0.4%, ~4–6 h into the exponential batch-phase, each fermentation was induced by the addition of IPTG, aTet, and/or Rha to the indicated concentrations to induce expression of LipB, LipA, and Lipoamidase. Following the depletion of glucose in the medium, as seen by a drop in CO_2, a fed-batch phase was initiated by the addition of the Feed Medium (Table S5) to each fermentation at a constant feed rate of 0.04 ml/min. This transition took place between ~8 and 10 h. The DO control continued to operate at DO = 15%, with the agitation increasing in all reactors to between 1250 and 1800 rpm. For anaerobic/microaerobic fermentation phases, with sparging terminated, the DO was 0%. Additional 1 ml 1% (v/v) antifoam solution was added at 7 and 24 h. The fermentations were terminated at the time indicated by the last sampling, ~48 h. Culture samples were taken from the fermenter at up to five various time points after inoculation, as indicated in fermentation profile(s). From these samples, optical density (cuvette OD_{600}) was measured after diluting samples into the range of 0.2–2 OD. Supernatants were obtained by spinning biomass down in a microcentrifuge at 13,000×*g* for 1 min. Supernatants were diluted 10-fold and stored at −20 °C for later quantification of LA by bioassay. Glucose feed bottles were weighed before and after fermentation to accurately calculate the total glucose fed. From this data, the LA yield on glucose was calculated.

Bioassay quantification of free LA - We used a modified version of a published liquid bioassay by Herbert and Guest (1970) (Herbert and Guest, 1970) using *E. coli* BW25113 Δ*lipA* (BS1912) with *zeoR* plasmid pBS451 as the bioassay strain that was unable grown without supplementation of exogenous LA. A single colony from an LB plate with zeocin was used to inoculate LB media with zeocin overnight. We found this strain to be limited in growth by LA in LB and therefore did not reach full OD overnight, but only ~1 OD_{600}. The overnight LB culture was centrifuged at 5.000×*g* for 5 min, the partially spent LB was decanted, and the cell pellet was resuspended in mMOPS succinate medium (Table S3) with zeocin to a final OD_{600} of 0.01, giving the Bioassay Medium. In a microtiter plate, 15 μL production sample supernatant was mixed with 135 μL Bioassay Media. Each plate contained an internal standard curve with known concentrations of LA, added in the same way to the Bioassay Medium. Plates were sealed with breathable seals and incubated for 20 h. OD_{620} was measured, and LA concentrations were calculated based on linear or logarithmic regression of the standard curve. If necessary, sample supernatants were diluted to be in the range of the bioassay. A linear range of growth between 0.2 and 2 μg/L LA was generally obtained and used for sample quantification using the described method. 24 standards were used for each 96-well plate, between the concentrations of 0–40 mg/L, allowing quantification of LA concentrations in this range.

SDS-page of whole-cell lysates - The expression of ecLipA was induced with IPTG from pBS1037 when cells were in early exponential phase growth (OD 0.2–0.4). Protein expression was done in 24 well deep-well plates in volumes of 2 ml mMOPS for 6 h at 37 °C or 18 h at 22 °C (room temperature) and constant shaking. After protein expression, samples were normalized, corresponding to 1 ml of 1 OD culture broth. Samples were centrifuged, supernatants were removed, and pellets were resuspended in 100 μl Millipore Bugbuster® 1x and incubated

for 1 h at room temperature. Samples were spun down, and supernatants, the soluble protein fractions, were removed. The pellets were resuspended in 100 μl 8 M urea, yielding the insoluble protein fractions. Each sample was resuspended in 100 μl 2x Laemmli Sample Buffer, and samples were stored at −20 °C for up to one month before running. Thawed samples were centrifuged at 5 min at 13000 G and loaded in Bio-Rad Mini-PROTEAN® TGX™ precast gels along with Bio-Rad Precision Plus Protein™ Dual Color Standards. Gels were run at 200 V for 40–60 min in Bio-Rad Tris/Glycine/SDS buffer. Gels were stained with EZBlue™ Gel Staining Reagent (Coomassie) according to protocol.

Author contributions

David Lennox-Hvenekilde: Conceptualization, Investigation, Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing. **Hans Genee, Luisa Gronenberg, Anne Bali:** Conceptualization, Supervision, Writing - Review & Editing. **Morten Sommer, Carlos Rocha**: Supervision, Writing - Review & Editing.

Declaration of competing interest

All authors are or have been employed at Biosyntia ApS, which has a financial interest in the cell factory production of vitamins and nutraceuticals, including lipoic acid.

Data availability

Data not in manuscript of supplementary file, has been shared via a github repository link.

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Appendix A. Supplementary data

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