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Functional Genomics Analysis of Free Fatty Acid Production under Continuous Phosphate Limiting Conditions

J. Tyler Youngquist^a, Travis C. Korosh^{a,c}, and Brian F. Pfleger^{a,b,*}

^aDepartment of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI, United States

^bMicrobiology Doctoral Training Program, University of Wisconsin-Madison, Madison, WI, United States

^cDepartment of Environmental Chemistry and Technology, University of Wisconsin-Madison, Madison, WI, United States

Abstract

Free fatty acids (FFA) are an attractive platform chemical that serves as a functional intermediate in metabolic pathways for producing oleochemicals. Many groups have established strains of *Escherichia coli* capable of producing various chain-length mixtures of FFA by heterologous expression of acyl-ACP thioesterases. For example, high levels of dodecanoic acid are produced by an *E. coli* strain expressing the *Umbellularia californica* FatB2 thioesterase, BTE. Prior studies achieved high dodecanoic acid yields and productivities under phosphate-limiting media conditions. In an effort to understand the metabolic and physiological changes that led to increased FFA production, the transcriptome of this strain was assessed as a function of nutrient limitation and growth rate. FFA generation under phosphate limitation led to consistent changes in transporter expression, osmoregulation, and central metabolism. Guided by these results, targeted knockouts led to a further ~11% in yield in FFA.

Keywords

Free fatty acid; microarray; *Escherichia coli*; phosphate-limitation; functional genomics; thioesterase

Introduction

Fatty acids are an attractive platform chemical that serves as a functional intermediate in metabolic pathways for producing oleochemicals (e.g. biodiesel, fatty alcohols, methylketones) [1]. *Escherichia coli* has been engineered by many groups as a means of converting renewable sugars into short-, medium-, and long-chain free fatty acids and oleochemicals [1–3]. In *E. coli* the key metabolic engineering steps are disruption of beta-oxidation and expression of an acyl-ACP thioesterase [3]. These steps provide a stable product sink and

*Corresponding author. 3629 Engineering Hall, 1415 Engineering Drive, Madison, WI 53706, United States. Phone: +1 608 890 1940. Fax: +1 608 262-5434. pfleger@engr.wisc.edu.

deregulate fatty acid biosynthesis by decreasing the accumulation of long-chain acyl-ACPs – a key regulatory signal in *E. coli* lipid metabolism. In past work, we and others, have shown that the FatB2 thioesterase from *Umbellularia californica* (a.k.a. BTE) confers upon *E. coli* the ability to produce high levels of medium chain length FFA (~80% C12/C12:1, ~20% C14/C14:1) [4, 5]. Three chromosomal copies of a gene cassette linking BTE to the P_{TRC} promoter generated a plasmid-free strain of *E. coli* (strain TY05) that could produce FFA at ~230 mg/L in minimal MOPS + 0.4% glucose [6]. Titters and yields (up to 30% of theoretical yield) were further improved by cultivating this strain in phosphate limited minimal media [7]. Chemostat studies of *E. coli* TY05 showed that FFA production was inversely correlated with dilution rate (cell growth), the majority of carbon was leaving the reactor as CO₂, and TY05 displays a higher maintenance energy than a comparable control strain TY06 harboring three copies of an inactive BTE active site variant (H204A) [6, 7].

To achieve the highest yields and titters, carbon flux to FFA biosynthesis must be maximized. The overall challenge can be subdivided into circumventing native regulation of lipid synthesis [8, 9], balancing the catalytic activity of each enzyme involved in fatty acid biosynthesis, maintaining high substrate uptake rates, balancing co-factor demand, and mitigating stresses caused by FFA production and export. Many approaches have been used to address these challenges and increase FFA yield on a given carbon source, including: transposon mutagenesis [10], targeted gene knockouts [11], altering transcriptional regulator levels [12], and alternative pathway engineering [13]. As strains are iteratively improved through a design, build, test, learn cycle, it becomes increasingly difficult to identify new limitations to increasing strain performance. Systems biology models can provide the framework for evaluating functional genomics (i.e. the combination of transcriptomics, proteomics, metabolomics, fluxomics) datasets in search of new hypotheses.

In order to better understand the advantages provided by *E. coli* strain TY05, particularly under phosphate-limited conditions, we collected transcriptomic data as a function of media and growth rate in continuous cultures. The microarray sets identified several sets of genes that were significantly differentially expressed under these high-FFA yielding conditions. Our findings reinforce past studies of FFA production in *E. coli* [14] and refine several key hypotheses. In general, FFA production in phosphate limited media increases the demand for NADPH, changes membrane composition and osmoregulation, and elevates expression of membrane transporters. With this knowledge we designed a set of gene deletion and overexpression strains in effort to increase FFA production. Of these, deletion of flagella had the greatest impact on FFA production.

Results and discussion

Transcriptional analysis of cells grown in carbon- and phosphate-limited media

In a past study, we found that as FFA producing *E. coli* enter stationary phase, there is a large number of gene expression changes relative to a non-producing culture [14]. Here, we performed similar experiments to determine the effects of FFA production on global gene expression as a function of growth rate and media composition in continuous culture. For the media composition studies, samples were taken from cultures of *E. coli* strain TY05 grown in continuous culture at a dilution rate of 0.1 h⁻¹ in minimal media formulated to be either

phosphate or carbon limited [7]. Biomass samples were collected at four time points per steady state for transcriptome analysis. Total RNA was isolated from each sample and the resulting cDNA was hybridized against custom *E. coli* oligo arrays. Data was reported as linear fold changes between mean intensities of carbon and phosphate limited TY05 chemostat cultures. After statistical analysis, 684 genes were found to have significant change between the two media. A brief table highlighting interesting sets of genes is shown in Table 1. A full data table for all statistically differentially expressed genes can be found in the Supplementary Material (Table S1).

To validate the collected data, we examined the expression values for *fadA*, *fadB*, *fadD*, and *fadE*. These genes were disrupted in *E. coli* TY05 (replaced with P_{TRC}-BTE cassettes) and therefore were expected to generate negligible expression in our samples. As expected, these transcripts showed extremely low expression values that were not statistically different than background (data not shown). The data was further validated by a significant increase in expression for the genes in the Pho regulon (selected genes in Table 1) under phosphate limitation. The Pho regulon is known to be upregulated under phosphate limitation. After normalization and statistical analysis, 602 genes were found to be significantly up- or down-regulated (fold change greater than 2 with a P value of less than 0.05) under phosphate limitation when compared to cells under carbon limitation. As a first filter, we focused on evaluating pathways where multiple genes leading to the production of a particular compound were up or down regulated together.

Genes involved in glycolysis and the pentose phosphate pathway were upregulated under phosphate limitation (Figure 1), which was expected based on increased biomass specific glucose uptake rates [7]. Upregulation of the pentose phosphate pathway also increases production of NADPH cofactor required for enzymatic activity in fatty acid biosynthesis [15]. Surprisingly, a majority of genes in the TCA cycle were not upregulated, many, including succinate dehydrogenase, were downregulated. The combined effects of high glycolytic flux and low TCA cycle flux suggested the cells responded to ATP demand during phosphate limitation via substrate level phosphorylation, rather than oxidative phosphorylation. This phenomena, also known as overflow metabolism, has recently benefited from systems biology studies [16, 17], and has been suggested to be related to saturation of the TCA cycle [18] or respiratory chain [19, 20] caused by differential proteomic resource allocation at high specific growth rates [17].

The comparison between phosphate limitation versus carbon limitation, revealed a number of transcriptional changes related to overflow metabolism, highlighting the role of acetyl-CoA partitioning, and thus free fatty acid biosynthesis, between several nodes in central metabolism in response to environmental cues. In particular, *acs* expression was downregulated, limiting the ability for excreted acetate to be re-assimilated into acetyl-coA. Similarly, *poxB* was upregulated, further enhancing acetate excretion into the medium and simultaneously allowing for quinone reduction. The enhanced overflow metabolism effect may have been exacerbated by reduced respiratory activity, caused by limited ATPase activity from a decreased P_i supply, as has been previously suggested [21]. This hypothesis is supported by a downregulation of H⁺ utilizing components of the electron transport chain,

such as *atpI* and *nuoA*, and upregulation of *mgo*, *ndh*, and *narWZ*, suggesting the cells are utilizing alternative respiratory pathways upon phosphate limitation.

Despite the significant increase in FFA biomass specific productivity under phosphate versus carbon limiting conditions, the differences in transcript level of fatty acid biosynthesis genes, such as *accA*, *accB*, *accC*, *accD*, *fabD*, *fabF*, *fabG*, *fabH*, *fabI*, and *fabZ*, were minimal or statistically insignificant. Genes encoding enzymes involved with unsaturated fatty acid biosynthesis were down-regulated under phosphate limitation (*fabA* and *fabB*, Table 1), consistent with prior studies [14]. FabR represses both *fabA* and *fabB* [22], however, it was not observed to be upregulated under phosphate limitation. As hypothesized by Lennen [14], such a decrease in expression could be due to the pool of saturated ACPs being depleted by the activity of BTE, leaving a higher proportion of unsaturated ACPs to ultimately make up the cell membrane. An increase in unsaturated content could lead to vastly different properties of the cell membrane and affect integrity [23]. The lower expression levels could be a way to combat such an effect by lowering the flux toward unsaturated fatty acid biosynthesis. The change in membrane saturation under phosphate limitation was also met with increased expression of several genes involved in osmoregulation, and biosynthesis of murein and colanic acid, which may act to enhance membrane integrity through cell wall reinforcement [24, 25] or acid resistance [26] (Table 1).

Several amino acid (e.g. glycine, threonine, glutamate, lysine) degradation pathways were found to be upregulated under phosphate limiting conditions (Table 1). Glycine and threonine degradation pathways lead to the generation of NADH and NADPH along with CO₂, indicating a cellular need for further reducing power under phosphate limitation. The glycine and threonine cleavage systems are induced by extracellular threonine and glycine, and negatively regulated in the presence of purines [27]. The glycine cleavage system (Figure 2) is also significant as it is used to donate one carbon units in the conversion of glycine to serine (while at the same time generating NADH and nitrogen in the form of ammonia), which can be degraded to pyruvate [28, 29]. However, as serine degradation genes are not upregulated under phosphate limitation, it is possible that the N⁵,N¹⁰-methylene tetrahydrofolate generated during glycine cleavage is converted to N⁵,N¹⁰-methenyl tetrahydrofolate (to eventually be converted to a purine) in the process generating an NADPH [28]. As FFA biosynthesis requires NADPH, it would make sense that more flux would go toward pathways capable of synthesizing NADPH, and it is possible that some of these amino acid degradation pathways are upregulated for the generation of reducing power in synthesizing FFAs.

Another possible reason behind the upregulation of amino acid degradation genes could have to do with restoring proton motive force, as the decarboxylation has the added effect of consuming an intracellular proton [30]. The high expression of the phage shock and significant upregulation of acid stress response genes suggest a loss of either membrane integrity and/or proton motive force [31, 32]. Removal of amino acid catabolic genes has been shown to lead to a significant reduction in growth, possibly owing to fewer options to regain proton motive force [33]. Chemostat cultured cells stain intact (when analyzed by SYTOX staining, Figure 3) during slow growth compared to the approximately 35% intact cells found in samples of cultures harvested in stationary phase [23]. Therefore, full loss of

membrane integrity is unlikely. Maintenance of the proton motive force is necessary to allow for ATP synthesis via the electron transport chain. Thus, it could be possible that FFA synthesis affects cellular pH and dissipates proton motive force as suggested by microarray data in this report and others [14].

Interestingly, not all genes observed by Lennen et al. to be up-regulated under FFA producing conditions [14] relative to wild type were observed to be differentially regulated under higher FFA producing conditions when cells were grown under phosphate limitation. For example, the genes encoding the phage shock proteins (*pspABCDEG*) were not observed to be significantly up-regulated (1.0–1.4 fold change) under phosphate limitation. However, normalized intensity values of *pspA*, *pspB*, *pspC*, and *pspD* transcripts were found to be similar to genes in the pentose phosphate pathway, indicating at least moderate expression. Additionally, the genes *marA*, *rob*, and *soxS* either had insignificant changes in expression (1.2 and –1.4 fold changes for *marA* and *rob*) or significant downregulation (–4.3 fold change with a P-value of 0.023 for *soxS*). Also of note were the low expression levels of each regulator under phosphate limitation (5-fold less than the phage shock transcript levels). However, these differences may be the result of different expression in stationary phase versus controlled slow growth.

In contrast, several genes demonstrated differential expression consistent with our prior transcriptomic results [14] and those of other FFA producing strains [12]. Genes encoding for the multidrug efflux pumps AcrAB, Cmr, MdtABC, MdtEF, and TolC were all increased in expression under phosphate limitation. Additionally, various membrane stress and acid shock proteins also increased in expression under phosphate limitation, including *hdeAB-yhiD* and genes in the glutamate dependent acid resistance. Surprisingly, *rpoE* (σ_E/σ^{24} , the extreme membrane/heat stress sigma factor) and *cadAB* were not observed to be up-regulated, despite *rpoE* expression being significantly associated with the cellular acid stress response [34].

The increase in expression of *mdtEF* (>8 fold) and *tolC* (~2 fold) under phosphate limitation could possibly explain the higher specific productivities. The inner membrane efflux pumps AcrAB, MdtEF, and EmrAB are likely involved in FFA export along with the outer membrane pump TolC [35]. Knocking out all three inner membrane efflux pumps essentially eliminated FFA production (unpublished results) and complementation of a knockout of one pump with overexpression of one of the other three leads to a restoration of FFA tolerance [35]. Thus, it is conceivable that the increased expression of *mdtEF* and *tolC* is contributing to increased FFA export allowing for a higher biomass specific FFA production rate without affecting the amount of intact cells.

Transcriptional analysis of cells grown in phosphate-limited media with varying dilution rates

In addition to a comparison of gene expression under phosphate versus carbon limitation, we collected mRNA samples from chemostats operating with varied dilution rates. FFA production was inversely correlated with growth rate and this data allowed us to examine the effect of growth rate on gene expression relevant to FFA production. Microarray analysis was performed on samples taken during TY05 steady states under phosphate limitation at

the growth rates of 0.05, 0.1, 0.2, and 0.3 h⁻¹. Samples were processed as described above. After statistical analysis, over 500 genes were found to have significant change between the highest and lowest tested growth rates tested. A brief table highlighting interesting sets of genes is shown in Table 2. A full data table for all statistically differentially expressed genes can be found in the Supplementary Material (Table S2).

Unlike the comparison between phosphate versus carbon limitation, many genes involved with glycolysis and the pentose phosphate pathway were not significantly upregulated at 0.3 h⁻¹ versus 0.05 h⁻¹ under phosphate limitation. At this high dilution rate, subunits of pyruvate dehydrogenase, *aceEF* were upregulated suggesting an increased acetyl-CoA flux. While not statistically significant, genes involved in the PEP-glyoxylate cycle (i.e. PEP carboxykinase and PEP synthase) were upregulated. Similarly, expression of isocitrate lyase, *aceA*, and malate synthase, *aceB* was increased suggesting that the cell was diverting the increased acetyl-CoA flux through the glyoxylate shunt. Enzymes in this cycle are thought to use one-third molar flux for anaplerosis, and two-thirds for oxidation to CO₂, in an effort to decouple catabolism from NADPH formation by isocitrate dehydrogenase in the TCA cycle [36]. In addition, expression of several components of oxidative phosphorylation and respiration were enhanced at 0.3 h⁻¹, including subunits of ATP synthase, cytochrome oxidase, and NADH:ubiquinone oxidoreductase (Table 2). Altogether, these data suggest differential control of several key cAMP-responsive transcriptional regulators at a dilution rate of 0.3 h⁻¹ during phosphate limitation, as shown with growth of *E. coli* on slowly-metabolized galactose versus quickly-metabolized glucose [37], thereby changing metabolic flux partitioning.

The data set showed significant up-regulation in flagella synthesis genes as a function of growth rate, with increased expression from 10 to 70 fold at the highest growth rate (Table 2), consistent with data showing high expression of flagella synthesis genes during mid log growth [38]. Increased expression of genes involved in flagella synthesis and chemotaxis may be explained by changes in acetyl-CoA metabolism at high growth rates. It has been proposed that acetyl-P, an intermediate in the acetogenic *pta-ackA* pathway, may act as a phosphodonator to a response regulator OmpR, negatively regulating flagella synthesis by binding to the *flhDC* promoter [39]. Upon depletion of glucose, acetyl-P levels would fall, thus derepressing *flhDC*. The *flhDC* operon, directly or indirectly acts as a global regulator [40], highlighting the role of acetyl-P as a signal for nutritional status in this high growth, low phosphate environment.

Of the genes with fold change differences greater than 5 (61), only genes associated with the Rac prophage (9 genes) and sulfonate utilization (3 genes) were of note. The increased transcript level of sulfonate utilization genes (*ssuEAD*) is odd as expression is related to consumption of aliphatic sulfonates as sulfur sources [41]. While *E. coli* has been shown to use MOPS as a sulfur source under conditions of sulfate starvation [42], the chemostat was not run under limiting sulfur conditions. Also, at the dilution rate most likely to have co-limitation conditions (0.05 h⁻¹), sulfonate utilization genes had expression levels similar to background. Despite this, other sulfur starvation proteins were observed to have increased expression, including a taurine transporter (*tauABC*, 3.5 fold increase) [43], indicating the possibility of sulfur co-limitation at the higher growth rate. The genes coding for the

multidrug efflux pump EmrAB were observed to have higher expression, which was surprising owing to the lack of difference in expression between carbon and phosphate limitation at the same growth rate. In addition to these genes, the ribosome modulation factor (*rmf*) was seen downregulated under higher growth conditions. Such a result is expected as expression has been shown to increase in growth regimes that are in a transitional region between exponential growth and stationary phase (such as 0.05 h^{-1}) [44]. However, the majority of expressed genes detected in the microarray study showed no clear pattern with respect to increasing growth rate. Despite this, it appears as though flagella synthesis/operation and chemotaxis show a clear pattern between higher growth and expression under phosphate limitation.

Using transcriptomic study to identify targets for modification

From prior studies, we hypothesized that a significant amount of carbon is being converted to CO_2 , around double that of what would be expected if the cell was maximizing FFA production [7]. In *E. coli*, glycolysis and the TCA cycle are likely sources of CO_2 production if the cell requires extra ATP for maintenance when producing elevated levels of FFA. In addition, over 70 enzymatic reactions encoded in the *E. coli* genome also produce CO_2 . Many of these were significantly expressed in TY05 and up-regulated when cells were grown in phosphate limited media. We therefore hypothesized that deletion of up-regulated CO_2 producing enzymes, deletion of unnecessary ATP sinks, and disruption of alternative carbon sinks could direct extra carbon flux to FFA. We used the functional genomics data collected above to prioritize a list of knockout targets that could potentially increase flux to FFA.

Considering the demand for NADPH in *E. coli* TY05 (for producing FFA), forcing flux through the NADPH generating pathways instead of glycolysis could improve FFA yields. For example, the pentose phosphate pathway (PPP) is a catabolic pathway that produces anabolic sugar-phosphates (precursors to nucleotides and aromatic amino acids), intermediates in glycolysis, and reducing power from glucose (Figure 1). The pathway converts three molecules of glucose-6-phosphate to two molecules of fructose-6-phosphate, one molecule of glyceraldehyde-3-phosphate, three molecules of CO_2 , and six NADPH. The cell uses the PPP to synthesize NADPH needed in anabolic pathways such as fatty acid biosynthesis. The cell is likely already partially using this strategy. From Table 1, genes in the PPP are up-regulated 2–4 fold under phosphate limitation versus carbon limitation. The disadvantage of the PPP relative to glycolysis is the loss of three carbons as CO_2 via the activity of 6-phosphogluconate dehydrogenase (Gnd) on 6-phosphogluconate. Alternatively, the 6-phosphogluconate can be acted upon by phosphogluconate dehydratase (Edd), which would send the compound through the Entner-Doudoroff (ED) pathway. The ED pathway is an alternative to the top of glycolysis, which produces one NADPH and one NADH (where glycolysis generates 2 NADH per glucose) at the expense of generating one less ATP. Directing flux through the ED pathway could help generate the needed NADPH without wasting carbon as CO_2 . Thus, knocking out *gnd* from TY05 could be a possible mechanism to reduce CO_2 as it would allow for NADPH production and eliminate a source of CO_2 production at the expense of an ATP. Similarly, blocking the isomerization of glucose-6-

phosphate to fructose-6-phosphate would force all carbon flux through either the PPP or ED pathway.

Another pathway identified under phosphate limiting conditions was the glutamate dependent acid resistance pathway (Figure 4). The pathway is typically expressed when a cell is exposed to extreme acid conditions and has the effect of increasing the proton motive force while consuming a proton while converting glutamate to 4-aminobutyrate (γ -aminobutyric acid, GABA) and CO₂ [32]. However, the system requires extracellular glutamate to function properly [45]. Up-regulation of pathway has also been observed when cells are exposed to osmotic stress, heat stress, and anaerobic phosphate starvation [46, 47]. In our functional genomics experiments, the genes for the decarboxylases (*gadA* and *gadB*), transporter (*gadC*), and transcriptional regulators (*gadX* and *gadE*) were upregulated 10–20 fold under phosphate limitation in TY05 versus carbon limitation. Given that the cells were not grown in the presence of extracellular glutamate and no extracellular GABA was detected, GABA must have been recycled. This can be achieved by using α -ketoglutarate (AKG) to convert GABA to glutamate and succinate via reactions catalyzed by PuuE and Sad. Overall, this set of reactions would be equivalent to steps in the TCA cycle (AKG to succinate) with the loss of one ATP (normally made by succinyl-CoA synthetase (SucCD)). We hypothesized that eliminating the glutamate dependent acid resistance pathway could reduce the maintenance energy of our FFA producing cells. Expression of *gadA* and *gadBC* requires the regulator GadE [45], so we decided to knock out *gadE*, as well as *gadA* and *gadBC*, from TY05. GadE has been shown to regulate other genes outside of the glutamate dependent acid resistance pathway [48], so direct disruption may be a better option.

A third attractive target for reducing ATP demand was flagella synthesis and operation. When FFAs were produced at the highest biomass specific rates, the genes involved in flagella synthesis and operation were significantly upregulated. As flagella synthesis and operation is very energy and biomass intensive [49], eliminating the ability of the cell to express these genes could allow for more cellular resources to be diverted to FFA production. While eliminating flagella function prevents cells from scavenging nutrients [50], such a concern would not be as significant in well mixed bioreactor. As FliA (σ^{28}) is the master regulator of flagella synthesis [51], we hypothesized that a disruption of *fliA* would eliminate flagella synthesis. Prior research has indicated significantly reduced motility in a *fliA* knockout strain of *E. coli* [52]. Alternatively, disrupting the gene encoding the primary filament of the flagellum (FliC) could prevent significant energy expenditure. This hypothesis is supported by the fact that *E. coli* mutants lacking *fliC* display a non-motile phenotype [53]. Other regulatory elements of flagella exist (FlgM, FlhC, and FlhD), however, overlapping regulation would require multiple knockouts to eliminate cellular motility [51].

A fourth target for improving FFA production is the removal of the acetate synthesis pathways. When *E. coli* TY05 was grown in chemostat cultures under phosphate limitation, significant levels of acetate (3–4% of total carbon processed) were observed [7]. Acetate production is used to generate additional ATP during rapid cell growth and is reincorporated when other carbon sources have been exhausted. Our FFA producing cells require acetyl-CoA for production of malonyl-CoA via acetyl-CoA carboxylase (AccABCD). This key

reaction, and regulatory point, competes with acetate production pathways for available acetyl-CoA. FFA production studies in our lab as well as research in other groups has shown that incorporation of an *ackA* (encoding acetate kinase) or *ackA-pta* (encoding acetate kinase and phosphate acetyltransferase) knockout to a FFA producing strain has a minimal impact on FFA titer and yield [54]. In addition to AckA-Pta, pyruvate oxidase (PoxB) can produce acetate and CO₂ from pyruvate in competition with synthesis of acetyl-CoA. The *poxB* transcript level was increased over four fold under phosphate limiting conditions versus carbon limiting conditions (Table 1). Therefore, it may be the dominant route for producing the acetate in our FFA producing cells.

Production results from targeted knockouts

Based on directions from the microarray study knockouts to the *fliA*, *fliC*, *gadA*, *gadB*, *gadC*, *gadE*, *gnd*, and *poxB* loci in *E. coli* TY05 were made using P1 transduction from the corresponding mutants in the Keio collection of single gene deletion mutants [55]. These strains were tested in triplicate shake flask production runs using phosphate limited modified MOPS minimal media. As each strain harbored no plasmids, no antibiotic was added to the media. Strains were cultivated for 48 hours after induction with IPTG, at which point they were harvested and the FFA titer was analyzed as described previously [7].

The only gene deletions that increased FFA yield on glucose were *fliA* and *fliC* (~11%) (Figure 5), with only TY05 *fliC* being statistically significant with high confidence (P value = 0.035). Conversely, knocking out genes involved in the glutamate dependent acid resistance system significantly decreased yield by 25–35%. Strains with knockouts of *gnd* and *poxB* showed no difference in final lauric acid yield on glucose compared to the TY05 control. Lauric acid titer was also reduced by 10–15% as TY05 *gnd* and TY05 *poxB* consumed only 85% of the fed glucose. All other strains consumed over 95% of fed glucose. Acetate production was around 0.1 g/L in most tested strains, with TY05 *gadB*, TY05 *gnd*, and TY05 *poxB* producing 0.02–0.03 g/L.

It is possible that the significant decrease in yield with the *gadE* knockout owes to its regulation of many genes outside those involved in glutamate dependent acid response [48]. Additionally, there could be polar effects on expression of the downstream transporter MdtEF, identified as a FFA exporter [35], owing to its proximity to *gadE*. Evidence linking the three genes to a single transcription unit, and data indicating that GadE activates expression of *mdtE* and *mdtF* have been reported [48, 56]. Individual knockouts of *gadA* and *gadB* likely showed no increase in FFA titer as each is capable of compensating for the loss of the other [57]. Additionally, a *gadA* disruption could have polar effects on *gadX*, which encodes for a transcriptional regulator affecting multiple genes [56, 58]. The lower FFA titer observed in the *gadC* mutant could be related to fact that GadA and GadB do not need GadC to decarboxylate glutamate [59]. The marginal effect seen with removal of *poxB* and *gnd* in TY05 could indicate a minimal shift in metabolic flux (pathways not previously consuming much carbon) or that flux has shifted to alternative pathways that still produce similar amounts of CO₂ (or direct carbon away from FFA biosynthesis). The increase in FFA yield due to the *fliC* knockout is likely due to retasking resources that are no longer being used on the synthesis and operation of the bacterial flagella to FFA synthesis or cellular

maintenance. As the flagella in *E. coli* are powered by proton pumps and very energy intensive [60, 61], their removal should allow for further ATP synthesis or maintenance of the cellular proton motive force. It is also likely that only a small increase in production occurred as the genes were only observed to be highly expressed under medium to high growth rates (Table 2) and are likely only using up a small percentage of the overall cellular resources, limiting the maximum benefit in FFA production that can be achieved from their removal.

Conclusions

A microarray study was performed comparing gene expression between FFA producing *E. coli* under carbon and phosphate limiting conditions in a continuous bioreactor. Upregulated non-essential pathways that generated CO₂ were identified, and used as a guide to modify *E. coli* TY05. Preliminary results indicated that eliminating flagella synthesis pathways increased FFA yield by ~10%. Multiple knockouts or downregulation of central metabolic pathways are likely needed before a significant impact in yield is achieved.

Materials and Methods

Strains, plasmids, enzymes, media, and bacterial cultivations

All strains used in the current study are listed in Table 3. Single gene deletions were transferred by P1 transduction of phage lysates from the collection of single gene knockouts obtained from the National BioResource Project (NIG, Japan) [55]. Cloning steps were performed with LB and FFA production was performed in carbon- or phosphate-limited modified MOPS minimal media described in Table 4 [6, 7]. Enzymes were purchased from New England Biolabs (Ipswich, MA). Nucleic acid purification materials were purchased from Qiagen (Venlo, Netherlands), Promega (Madison, WI), or Thermo Scientific (Waltham, MA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) unless otherwise specified.

RNA sample preparation and microarray analysis

RNA samples were taken from previously reported chemostat studies of *E. coli* TY05 FFA production [6, 7]. Carbon and phosphate-limited chemostats were run at a constant dilution rate of 0.1 h⁻¹. Phosphate-limited chemostats were also run at different dilution rates (0.05, 0.2, and 0.3 h⁻¹) to examine the impact of growth rate on gene expression. For each set of experiments, samples for RNA analysis were taken at 4 different time points per steady state condition.

RNA was isolated and prepared for microarray experiments using the approach described by Lennen [14]. Briefly, 10 OD-mL of culture was added to 1.25 mL of a solution of 5% phenol in absolute ethanol that had been sitting on ice. The solution underwent centrifugation at 5000 × g for 5 minutes at 4°C, after which the supernatant was removed and the remaining pellets were snap frozen in a dry ice ethanol bath before storage at -80°C. Total RNA was isolated using a Qiagen RNeasy minikit following manufacturer's instructions. Total RNA quality was assessed using an Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA) using the Prokaryote Total RNA Nano series II chip set.

10 µg of RNA was used to synthesize cDNA for microarray hybridization. 1 µg was labeled with Cy3 based on the NimbleGen array user guide instructions. The labeled cDNA samples were hybridized to Roche NimbleGen (Madison, WI) *Escherichia coli* K-12 microarray slides containing four arrays per slide and 72,000 probes per array. A GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) was used to scan arrays. The photomultiplier tube gain was set to achieve 10^{-5} normalized counts at a saturated intensity level. Images were then processed and .pair files were generated based on the intensities.

For analysis, the intensity data files were imported into ArrayStar software (DNASTAR, Madison, WI) and processed as described by Lennen [14]. The processed files were used to determine mean intensities across sample technical replicates, sample biological replicates, and the limitation condition as a whole. The reported values are linear changes in expression, with the statistical P-value calculated based on a modified t-test. Ratios with a change greater than 4 fold or pathways where a majority of the genes had a change greater than 3 fold and with P-values less than or equal to 0.05 were considered significant.

Batch shake flask cultivation of strain knockouts

The TY05 knockouts were cultivated in 250 mL shake flasks containing 50 mL of MOPS minimal medium [42] supplemented with 0.7% glucose, 0.276 mM potassium sulfate, and 9.5 mM ammonium chloride, but only containing 0.24 mM dibasic potassium phosphate. All cultures were grown in triplicate. For all studies, single colonies from fresh freezer stocks were used to inoculate 5 mL LB allowing for growth of at least 8 hours at 37°C. The initial outgrowth was used to inoculate the shake flask production cultures to an OD₆₀₀ of 0.04. Cultures were incubated with shaking at 37°C until each reached an OD of 0.2, at which point the cultures were induced with 1 mM IPTG. After 40 hours of incubation, samples for fatty acid-methyl-ester (FAME) analysis, glucose consumption, and cell density were taken, processed, and quantified.

6.4.4. Product analysis

Culture samples for analyzing fatty acids via GC/MS were taken and derivatized to methyl esters as described previously [5]. All C_{12:0}, C_{12:1}, C_{14:0}, and C_{14:1} were considered FFAs as past results indicate that less than 10% of these species are found in membrane fractions. Culture samples for SYTOX flow cytometry analysis were taken and analyzed following procedures described elsewhere [14]. SYTOX is a green nucleic acid dye that only can penetrate cells when the inner membrane is not intact. Intact cells show a lower fluorescence value that is associated with staining of the cell surface [62].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Changes in gene expression for central metabolism between carbon and phosphate limitation. ATP, CO₂ and reducing equivalents that are consumed or generated are shown as side products for each reaction where they are required/generated. Bold arrows indicate that transcripts that were upregulated under phosphate limitation. Small, dashed arrows indicate downregulation.

Figure 2.

The glycine cleavage system and recycling of tetrahydrofolate (THF) and N⁵,N¹⁰-methylenetetrahydrofolate (MTHF). When the glycine cleavage system genes are overexpressed the cell breaks down glycine to produce NADH (also generating MTHF). The MTHF can be recycled back to THF in the conversion of glycine to serine. Additionally, alternative pathways that generate NADPH (along with consuming ATP) can also help restore THF. Arrows connecting THF and MTHF represent multiple enzymatic steps in a metabolic pathway.

Figure 3. SYTOX staining study a.) Average green fluorescence (485nm excitation, 525 nm emission) histograms of SYTOX green stained cells and b.) averaged log scale forward scatter for *E. coli* TY05 cells taken from phosphate limited chemostat cultures at dilution rates of 0.05, 0.1, 0.2, and 0.3 h⁻¹. Non intact cells show log scale fluorescence values of greater than 500 [14], as SYTOX is a green nucleic acid dye that only can penetrate cells when the inner membrane is not intact. Intact cells show a lower fluorescence value that is associated with staining of the cell surface [62].

Figure 4.

The glutamate dependent acid resistance pathway. In the cytosol, **GadB** (or **GadA**) converts glutamate to GABA consuming a proton and giving off CO_2 . GABA export to the periplasm is then coupled to glutamate import through **GadC** (inner membrane transporter), which has the net effect of exporting a proton out of the cell. Enzymes are represented by bolded names.

Figure 5. Targeted knockout production results. a.) Normalized yield values of C₁₂ FFA on glucose in each of the TY05 knockout strains. The asterisk indicates where the difference was statistically significant (P value less than 0.05). b.) Final optical densities (OD₆₀₀) for each mutant at the time of harvest.

Table 1

Selected significantly up- and down-regulated genes in *E. coli* TY05 grown under phosphate limitation versus carbon limitation.

Category/ Locus	Gene name	Fold Change ^a	P-value	Description
Pho regulon				
b0383	<i>phoA</i>	59.7	2.5×10^{-7}	bacterial alkaline phosphatase
b0399	<i>phoB</i>	19.9	6.3×10^{-6}	DNA-binding response regulator in two-component regulatory system with PhoR
Acetate production				
b0871	<i>poxB</i>	3.7	4.5×10^{-3}	pyruvate oxidase
b4069	<i>acs</i>	-18.29	9.5×10^{-6}	acetyl-CoA synthetase
Acid stress				
b3517	<i>gadA</i>	11.8	3.2×10^{-2}	glutamate decarboxylase A
b1493	<i>gadB</i>	11.3	3.0×10^{-2}	glutamate decarboxylase B
b1492	<i>gadC</i>	10.3	1.9×10^{-2}	glutamate:gamma-aminobutyric acid antiporter
b3512	<i>gadE</i>	20.0	1.6×10^{-2}	DNA-binding transcriptional activator
b3509	<i>hdeB</i>	4.9	8.1×10^{-3}	acid resistance protein
b3511	<i>hdeD</i>	6.8	3.6×10^{-2}	acid-resistance membrane protein
b0186	<i>ldcC</i>	3.1	2.7×10^{-2}	lysine decarboxylase 2
b3506	<i>slp</i>	7.2	2.8×10^{-2}	starvation lipoprotein
b3508	<i>yhiD</i>	7.9	3.7×10^{-2}	Mg(2+) transport, acid resistance
Amino acid degradation^b				
b2903	<i>gcvP</i>	4.63	3.6×10^{-6}	glycine decarboxylase
b2905	<i>gcvT</i>	4.3	3.1×10^{-5}	aminomethyltransferase
b3616	<i>tdh</i>	2.2	2.4×10^{-2}	threonine dehydrogenase
b2965	<i>speC</i>	3.9	1.0×10^{-2}	ornithine decarboxylase
Fatty acid metabolism				
b0954	<i>fabA</i>	-3.0	4.8×10^{-2}	beta-hydroxydecanoyl thioester dehydrase
b2323	<i>fabB</i>	-2.1	3.4×10^{-2}	3-oxoacyl-[acyl-carrier-protein] synthase I
b2342	<i>fadI</i>	-11.1	3.0×10^{-2}	beta-ketoacyl-CoA thiolase, anaerobic, subunit
b2341	<i>fadJ</i>	-8.8	3.6×10^{-2}	fused enoyl-CoA hydratase and epimerase and isomerase - 3-hydroxyacyl-CoA dehydrogenase
b2344	<i>fadL</i>	-14.9	3.2×10^{-2}	long-chain fatty acid outer membrane transporter
Multi-drug efflux^b				
b2074	<i>mdtA</i>	3.0	1.2×10^{-3}	multidrug efflux system

Category/ Locus	Gene name	Fold Change ^a	P-value	Description
b3513	<i>mdtE</i>	19.6	1.8×10^{-2}	multidrug resistance efflux transporter
b3514	<i>mdtF</i>	8.4	2.7×10^{-2}	multidrug resistance efflux transporter
b0842	<i>cmr</i>	4.4	3.1×10^{-4}	multidrug efflux system protein
Membrane components^b				
b1782	<i>mipA</i>	2.4	3.9×10^{-3}	scaffolding protein for murein synthesizing machinery
b2701	<i>mltB</i>	2.1	8.4×10^{-3}	membrane-bound lytic murein transglycosylase B
b2963	<i>mltC</i>	3.9	5.1×10^{-4}	membrane-bound lytic murein transglycosylase C
b2047	<i>wcaJ</i>	8.1	3.2×10^{-2}	predicted UDP-glucose lipid carrier transferase
b2045	<i>wcaK</i>	11.8	6.4×10^{-4}	predicted pyruvyl transferase
b2044	<i>wcaL</i>	6.6	1.4×10^{-3}	predicted glycosyl transferase
b2043	<i>wcaM</i>	2.6	1.8×10^{-2}	predicted colanic acid biosynthesis protein
Oxidative Stress				
b1732	<i>katE</i>	3.8	5.7×10^{-2}	hydroperoxidase HP11(III) (catalase)
b0606	<i>ahpF</i>	2.5	3.9×10^{-5}	alkyl hydroperoxide reductase, F52a subunit, FAD/NAD(P)-binding
b1781	<i>yeaE</i>	2.7	9.8×10^{-6}	predicted oxidoreductase
Osmoregulation^b				
b0698	<i>kdpA</i>	11.8	2.3×10^{-2}	potassium translocating ATPase, subunit A
b0697	<i>kdpB</i>	17.1	2.8×10^{-2}	potassium translocating ATPase, subunit B
b0696	<i>kdpC</i>	14.5	2.9×10^{-2}	potassium translocating ATPase, subunit C
b4111	<i>proP</i>	8.2	2.7×10^{-4}	proline/glycine betaine transporter
b0050	<i>apaG</i>	2.0	1.8×10^{-2}	protein associated with Co ²⁺ and Mg ²⁺ efflux
Pentose phosphate^b				
b1852	<i>zwf</i>	2.4	8.0×10^{-4}	glucose-6-phosphate dehydrogenase
b0767	<i>pgl</i>	2.0	4.8×10^{-2}	6-phosphogluconolactonase
b2029	<i>gnd</i>	3.0	2.2×10^{-4}	gluconate-6-phosphate dehydrogenase, decarboxylating
b2465	<i>tktB</i>	4.4	3.4×10^{-2}	transketolase 2, thiamin-binding
b0008	<i>talB</i>	2.2	8.0×10^{-4}	transaldolase B
Glycolysis^b				
b4025	<i>pgi</i>	2.2	1.4×10^{-3}	glucosephosphate isomerase
b1723	<i>pfkB</i>	3.3	2.3×10^{-4}	6-phosphofructokinase II
b2097	<i>fbaB</i>	2.1	4.0×10^{-2}	fructose bisphosphate aldolase

Category/ Locus	Gene name	Fold Change ^a	P-value	Description
				class I
b3612	<i>gpmI</i>	4.2	2.6×10^{-7}	phosphoglyceromutase III, cofactor independent
b1676	<i>pykF</i>	3.1	2.6×10^{-4}	pyruvate kinase I
Respiration^b				
b1109	<i>ndh</i>	6.4	2.8×10^{-5}	respiratory NADH dehydrogenase 2/cupric reductase
b2288	<i>nuoA</i>	-2.7	2.6×10^{-4}	NADH:ubiquinone oxidoreductase, membrane subunit A
b3739	<i>atpI</i>	-3.4	6.9×10^{-3}	ATP synthase, membrane-bound accessory subunit
b1466	<i>narW</i>	2.5	3.3×10^{-2}	nitrate reductase 2 (NRZ), delta subunit (assembly subunit)
TCA cycle^b				
b0723	<i>sdhA</i>	-2.4	2.9×10^{-2}	succinate dehydrogenase, flavoprotein subunit
b0721	<i>sdhC</i>	-3.7	9.6×10^{-4}	succinate dehydrogenase, membrane subunit
b0722	<i>sdhD</i>	-2.8	3.2×10^{-2}	succinate dehydrogenase, membrane subunit
b1612	<i>fumA</i>	-2.9	2.2×10^{-2}	fumarate hydratase
b1611	<i>fumC</i>	-3.2	1.2×10^{-2}	fumarate hydratase
b2210	<i>mgo</i>	17.4	3.1×10^{-4}	malate dehydrogenase
b4015	<i>aceA</i>	-3.2	1.2×10^{-4}	isocitrate lyase
b4014	<i>aceB</i>	-3.3	2.0×10^{-5}	malate synthase A

^a. Fold change is calculated based on linear difference in average intensities between *E. coli* TY05 under phosphate versus carbon limitation.

^b. Only genes that were significantly up or down regulated (absolute value of fold change greater than 2, P value less than 0.05) in the indicated pathways are listed in this table

Table 2

Selected significantly up and down regulated genes in *E. coli* TY05 grown continuously at 0.3 h⁻¹ versus 0.05 h⁻¹ under phosphate limitation

Category/Locus	Gene name	Fold Change ^a	P-value	Description
Flagellar synthesis				
b1922	<i>fliA</i>	19.6	1.6×10 ⁻³	RNA polymerase, sigma 28 (F) factor
b1923	<i>fliC</i>	68.3	7.7×10 ⁻⁴	flagellar filament structural protein
b1076	<i>flgE</i>	58.0	1.8×10 ⁻³	flagellar hook protein
b1946	<i>fliN</i>	33.0	1.3×10 ⁻³	flagellar motor switching and energizing component
Chemotaxis				
b1888	<i>cheA</i>	41.2	7.6 ×10 ⁻⁴	fused chemotactic sensory histidine kinase in two-component regulatory system with CheB and CheY: sensory histidine kinase - signal sensing protein
b1883	<i>cheB</i>	15.9	2.5×10 ⁻³	fused chemotaxis regulator - protein-glutamate methylesterase in two-component regulatory system with CheA
b1884	<i>cheR</i>	17.5	3.6×10 ⁻⁴	chemotaxis regulator, protein-glutamate methyltransferase
b1882	<i>cheY</i>	20.3	2.3 ×10 ⁻³	chemotaxis regulator transmitting signal to flagellar motor component
b1881	<i>cheZ</i>	12.4	1.7 ×10 ⁻³	chemotaxis regulator, protein phosphatase for CheY
Multidrug efflux				
b2685	<i>emrA</i>	2.9	5.9×10 ⁻³	multidrug efflux system
b2686	<i>emrB</i>	2.7	5.5×10 ⁻⁴	multidrug efflux system
Acetyl-CoA Flux				
b0114	<i>aceE</i>	1.2	4.2×10 ⁻²	pyruvate dehydrogenase, decarboxylase component E1, thiamin-binding
b0115	<i>aceF</i>	1.2	5.5×10 ⁻⁴	pyruvate dehydrogenase, dihydrolipoyltransacetylase component E2
b4015	<i>aceA</i>	2.0	5.5×10 ⁻⁴	isocitrate lyase
b4014	<i>aceB</i>	2.4	5.9×10 ⁻⁵	malate synthase A
Respiration				
b0430	<i>cyoC</i>	1.8	7.8×10 ⁻⁴	cytochrome o ubiquinol oxidase subunit III
b2277	<i>nuoM</i>	2.2	7.0×10 ⁻³	NADH:ubiquinone oxidoreductase, membrane subunit M
b3734	<i>atpA</i>	2.0	4.7×10 ⁻³	F1 sector of membrane-bound ATP synthase, alpha subunit

Category/Locus	Gene name	Fold Change ^a	P-value	Description
b3738	<i>atpB</i>	1.9	1.5×10^{-4}	F0 sector of membrane-bound ATP synthase, subunit a
b3731	<i>atpC</i>	1.9	1.2×10^{-2}	F1 sector of membrane-bound ATP synthase, epsilon subunit
b3733	<i>atpG</i>	1.9	3.1×10^{-3}	F1 sector of membrane-bound ATP synthase, gamma subunit
b3735	<i>atpH</i>	1.8	2.5×10^{-3}	F1 sector of membrane-bound ATP synthase, delta subunit
Osmoregulation				
b2677	<i>proV</i>	3.2	4.5×10^{-3}	glycine betaine transporter subunit -ATP-binding component of ABC superfamily
b2678	<i>proW</i>	3.3	8.7×10^{-5}	glycine betaine transporter subunit - membrane component of ABC superfamily
b2679	<i>proX</i>	3.1	2.8×10^{-3}	glycine betaine transporter subunit - periplasmic-binding component of ABC superfamily
Sulfur transport (subset)				
b0936	<i>ssuA</i>	9.9	3.1×10^{-2}	alkanesulfonate transporter subunit -periplasmic-binding component of ABC superfamily
b0935	<i>ssuD</i>	5.2	1.3×10^{-2}	alkanesulfonate monooxygenase, FMNH(2)-dependent
b0366	<i>tauB</i>	3.5	1.3×10^{-3}	taurine transporter subunit - ATP-binding component of ABC superfamily
b3917	<i>shp</i>	3.9	2.4×10^{-3}	sulfate transporter subunit - periplasmic-binding component of ABC superfamily

^a. Fold change is calculated based on linear difference in average intensities between TY05 under phosphate limitation at a dilution rate of 0.3 h^{-1} to 0.05 h^{-1} .

Table 3

Strains and Plasmids used in this study

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
Strains		
<i>E. coli</i> K-12	F ⁻ λ^- <i>ilvG</i> ⁻ <i>rfb</i> -50 <i>rph</i> -1	ECGSC
MG1655		
TY05	K-12 MG1655 <i>fadD</i> ::trcBTE, <i>fadE</i> ::trcBTE, <i>fadAB</i> ::trcBTE	This work
MHS05	TY05 <i>gadA</i> :: <i>kan</i> ^R	This work
MHS06	TY05 <i>gadB</i> :: <i>kan</i> ^R	This work
MHS07	TY05 <i>gadC</i> :: <i>kan</i> ^R	This work
MHS08	TY05 <i>gadE</i> :: <i>kan</i> ^R	This work
MHS09	TY05 <i>fliA</i> :: <i>kan</i> ^R	This work
MHS10	TY05 <i>fliC</i> :: <i>kan</i> ^R	This work
MHS11	TY05 <i>poxB</i> :: <i>kan</i> ^R	This work
MHS12	TY05 <i>gnd</i> :: <i>kan</i> ^R	This work

Table 4

Conditions used for comparison of carbon and phosphate limitation at a dilution rate of 0.1 h^{-1} ^{**}.

Type of Limitation	Phosphate concentration (μM , from K_2HPO_4)	Glucose concentration	Nitrogen source minimum concentration (mM, from NH_4Cl) [*]	Sulfur source concentration (μM , from K_2SO_4) [#]
C	9240	0.4%	66.5	1932
C	9240	0.2%	66.5	1932
C	9240	1.0%	66.5	1932
P	185	1.0%	28.5	828
P	370	0.7%	28.5	828
P	370	1.0%	28.5	828
P	370	2.0%	28.5	828
P	528	1.0%	28.5	828

^{*} Additional nitrogen was added for pH control via the NH_4OH base

[#] A small additional amount of sulfur source was provided by FeSO_4

^{**} The growth rate studies all used the 370 μM P and 1.0% glucose media

The remaining media composition can be seen in [6, 7, 42].