**Comprehensive analysis of glucose and xylose metabolism in
Escherichia coli under aerobic and anaerobic conditions by ^{13}C
metabolic flux analysis**

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ABSTRACT

Glucose and xylose are the two most abundant sugars derived from the breakdown of lignocellulosic biomass. While aerobic glucose metabolism is relatively well understood in *E. coli*, until now there have been only a handful of studies focused on anaerobic glucose metabolism and no ^{13}C -flux studies on xylose metabolism. In the absence of experimentally validated flux maps, constraint-based approaches such as MOMA and RELATCH cannot be used to guide new metabolic engineering designs. In this work, we have addressed this critical gap in current understanding by performing comprehensive characterizations of glucose and xylose metabolism under aerobic and anaerobic conditions, using recent state-of-the-art techniques in ^{13}C metabolic flux analysis (^{13}C -MFA). Specifically, we quantified precise metabolic fluxes for each condition by performing parallel labeling experiments and analyzing the data through integrated ^{13}C -MFA using the optimal tracers [1,2- ^{13}C]glucose, [1,6- ^{13}C]glucose, [1,2- ^{13}C]xylose and [5- ^{13}C]xylose. We also quantified changes in biomass composition and confirmed turnover of macromolecules by applying [U- ^{13}C]glucose and [U- ^{13}C]xylose tracers. We demonstrate that under anaerobic growth conditions there is significant turnover of lipids and that a significant portion of CO_2 originates from biomass turnover. Using knockout strains, we also demonstrate that β -oxidation is critical for anaerobic growth on xylose. Quantitative analysis of co-factor balances (NADH/FADH₂, NADPH, and ATP) for different growth conditions provided new insights regarding the interplay of energy and redox metabolism and the impact on *E. coli* cell physiology.

KEYWORDS

Parallel labeling experiments; metabolism; model validation; flux estimation; co-factor balances.

1. INTRODUCTION

Biological conversion of lignocellulosic biomass into fuels and other chemicals has gained much attention in recent years (Choi et al., 2015). Lignocellulosic biomass is composed mainly of cellulose and hemicellulose, both of which can be broken down to carbohydrate monomers such as glucose, xylose, mannose, and galactose (Girio et al., 2010). With glucose and xylose as the major products of lignocellulose breakdown, conversion of these two sugars into value-added products has been the focus of many metabolic engineering efforts (Hasona et al., 2004; Liu et al., 2012; Tao et al., 2001). While progress in metabolic engineering has allowed the generation of unique strains for improved glucose and xylose fermentations, detailed knowledge regarding changes in cellular metabolism as a result of these modifications is lacking and this limits further rational strain design (Long and Antoniewicz, 2014a; Woolston et al., 2013).

Constraint-based reconstruction and analysis (COBRA) methods have been widely used in metabolic engineering for strain design (Becker et al., 2007; King et al., 2015). These methods include flux balance analysis (FBA) (Edwards et al., 2002), minimization of metabolic adjustment (MOMA) (Segre et al., 2002), regulatory on/off minimization of metabolic flux changes (ROOM) (Shlomi et al., 2005), and relative optimality in metabolic networks (RELATCH) (Kim and Reed, 2012), which can be implemented for strain design in algorithms such as OptKnock (Burgard et al., 2003). An important requirement for COBRA methods is the presence of experimentally validated reference flux maps. ^{13}C -Metabolic flux analysis (^{13}C -MFA) is the most robust technique for determining precise intracellular metabolic fluxes (Antoniewicz, 2015a; Antoniewicz et al., 2006b; Crown and Antoniewicz, 2013). In the past decade, ^{13}C -MFA has been applied extensively to investigate aerobic metabolism of glucose in

E. coli (Chen et al., 2011; Perrenoud and Sauer, 2005; Toya et al., 2012); several ^{13}C -MFA studies have also focused on elucidating anaerobic glucose metabolism in *E. coli* (Chen et al., 2011; Choudhary et al., 2011), and other microbes (Au et al., 2014). In contrast, relatively little is known about xylose metabolism. To our knowledge, there have been no prior ^{13}C -MFA studies on xylose metabolism in *E. coli*, and only a handful of ^{13}C -MFA studies have been published on xylose metabolism in other organisms (Cordova and Antoniewicz, 2016; Cordova et al., 2016; Feng and Zhao, 2013; Liu et al., 2012; Wasylenko and Stephanopoulos, 2015).

To address this gap in current knowledge, in this work we have applied advanced methods for ^{13}C -flux analysis based on parallel labeling experiments and integrated ^{13}C -MFA (Antoniewicz, 2015b; Leighty and Antoniewicz, 2013) to comprehensively quantify metabolism of glucose and xylose in *E. coli* under aerobic and anaerobic growth conditions. Fully ^{13}C -labeled tracers were also applied to measure changes in biomass composition and turnover of macromolecules under all growth conditions. Results from this work provide valuable new insights into metabolism of *E. coli* that can serve as the basis for future model building efforts and new strain designs using COBRA approaches.

2. MATERIALS AND METHODS

2.1. Materials

Media and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Tracers were purchased from Cambridge Isotope Laboratories: [1,2- ^{13}C]glucose (99.9 atom% ^{13}C), [1,6- ^{13}C]glucose (99.6%), [U- ^{13}C]glucose (99.3%), [1,2- ^{13}C]xylose (99.3%), [5- ^{13}C]xylose (99.6%),

and [U-¹³C]xylose (99.3%). The isotopic purity of all glucose tracers was determined by GC-MS (Long et al., 2016a). MOPS minimal medium was used for all experiments.

2.2. Strains and growth conditions

For all labeling experiments, a single colony of *E. coli* BW21135 (GE Healthcare Dharmacon) was first suspended in MOPS medium containing either 40 mM glucose or 40 mM xylose and grown overnight at 37°C in a shaker flask. The pre-culture was then re-suspended in fresh MOPS medium containing a particular tracer (40 mM initial concentration). For labeling experiments with [U-¹³C]glucose and [U-¹³C]xylose the cells were first washed with glucose-free and xylose-free medium. The initial OD₆₀₀ of inoculated cultures was 0.15 ± 0.02 (an inoculation ratio of approximately 1:10 was used). Cells were grown at 37°C in parallel mini-bioreactors with a working volume of 10 mL, as described before for aerobic *E. coli* cultures (Crown et al., 2015a). Air was sparged into the liquid at a rate of 12 mL/min to provide oxygen and to ensure sufficient mixing of the culture by the rising gas bubbles. For anaerobic cultures, nitrogen was sparged into the headspace of the mini-bioreactors at 5 mL/min to maintain anaerobic conditions (Au et al., 2014), and mixing was achieved by continuous stirring with a stirrer bar. The pH of the anaerobic cultures was maintained at $\text{pH } 7.0 \pm 0.15$ through automatic addition of 1N NaOH. In all cases, glucose pre-cultures were used for glucose tracer experiments, and xylose pre-cultures for xylose tracer experiments. The following tracers were used for ¹³C-MFA (40 mM initial concentration): [1, 2-¹³C]glucose, [1, 6-¹³C]glucose, [1, 2-¹³C]xylose, [5-¹³C]xylose. Labeling experiments were also performed with 40 mM of [U-¹³C]glucose and [U-¹³C]xylose to determine turnover of biomass macromolecules (including proteins, lipids and RNA) for aerobic and anaerobic growth of *E. coli*. Growth characterization of knockout strains ΔfadD , ΔfadK , and

$\Delta fadD\Delta fadK$ was performed as described above for wild-type *E. coli*. The strains $\Delta fadD$ and $\Delta fadK$ were obtained from the Keio collection (GE Healthcare Dharmacon) and $\Delta fadD\Delta fadK$ strain RMK58 was obtained from Dr. John Cronan (University of Illinois at Urbana-Champaign) (Campbell et al., 2003).

2.3. Analytical methods

Samples were collected at multiple times during the exponential growth phase to monitor cell growth, substrate uptake and product formation. Cell pellets and supernatants for isotopic labeling analysis were collected at an OD₆₀₀ of 0.85 ± 0.02 . Cell growth was monitored by measuring the optical density at 600nm (OD₆₀₀) using a spectrophotometer (Eppendorf BioPhotometer). The OD₆₀₀ values were converted to cell dry weight concentrations using previously determined OD₆₀₀-dry cell weight relationship for *E. coli* ($1.0 \text{ OD}_{600} = 0.32 \text{ g}_{\text{DW}}/\text{L}$; molecular weight of dry biomass = $24.6 \text{ g}_{\text{DW}}/\text{C-mol}$) (Long et al., 2016b). After centrifugation of the samples, the supernatant was separated from the biomass pellet. Acetate, formate, succinate, ethanol, and xylose concentrations in the supernatant were determined using an Agilent 1200 Series HPLC (Au et al., 2014). Glucose and lactate concentrations were determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH).

2.4. Biomass composition analysis

The methods used for quantifying biomass composition were described in (Long and Antoniewicz, 2014b). Briefly, samples were prepared by three respective methods: hydrolysis of protein and subsequent TBDMS derivatization of amino acids; hydrolysis of RNA and glycogen and subsequent aldonitrile propionate derivatization of sugars (ribose and glucose, respectively);

and fatty acid methyl ester derivatization for analysis of fatty acid. In total, 17 amino acids were quantified. The amino acids arginine, cysteine and tryptophan are degraded during hydrolysis and were thus not detected. For total protein quantification, we assumed the values previously reported for these three amino acids (Neidhardt, 1987). Glutamine and asparagine were deaminated to glutamate and aspartate, respectively, during hydrolysis; thus, we report the combined pools of each. Quantification of all components was achieved by isotope ratio analysis using an isotopically labeled standard and a naturally labeled sample. In this study, the standard was generated by growing wild-type *E. coli* on [U-¹³C]glucose and aliquoting identical (1 mL of an OD₆₀₀ = 1.0) samples of this “fully labeled” biomass. These were centrifuged and washed twice with M9 medium. The composition of the fully labeled biomass was characterized using unlabeled chemical standards, and subsequently these were used as standards by co-dissolving with the unlabeled samples at the beginning of each respective analytical method.

2.5. Gas chromatography-mass spectrometry

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 250°C. GC-MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatized proteinogenic amino acids was performed as described in (Antoniewicz et al., 2007a). Labeling of glucose and xylose were determined after aldonitrile propionate derivatization as described in (Antoniewicz et al., 2011; Sandberg et al., 2016). Labeling of fatty acids was determined after

derivatization to fatty acid methyl esters (FAME) (Crown et al., 2015b). Labeling of glucose (derived from glycogen) and ribose (derived from RNA) were determined as described in (Long et al., 2016a; McConnell and Antoniewicz, 2016). In all cases, mass isotopomer distributions were obtained by integration (Antoniewicz et al., 2007a) and corrected for natural isotope abundances (Fernandez et al., 1996).

2.6. Metabolic network model and ^{13}C -metabolic flux analysis

The metabolic network models used for ^{13}C -MFA for all four growth conditions, i.e. aerobic and anaerobic growth with glucose and xylose as carbon source, are provided in Supplemental Materials. The models are based on the *E. coli* model described in (Crown et al., 2015a). The models include all major metabolic pathways of central carbon metabolism, lumped amino acid biosynthesis reactions, and a lumped biomass formation reaction. Because we cannot distinguish between the NAD-dependent malic enzyme (meaA) and NADP-dependent malic enzyme (meaB), we included only one of the two malic enzymes in the ^{13}C -MFA models, namely the NADP-dependent malic enzyme. Previous studies have demonstrated that neither enzyme is expressed during growth on glucose, and that only the NADP-dependent (meaB) malic enzyme is significantly expressed during growth on xylose (Schmidt et al., 2016). The stoichiometries for the biomass formation reactions were derived using the measured biomass composition for each growth condition (Supplemental Materials). The models also accounted for dilution of intracellular labeling from unlabeled CO_2 (Leighty and Antoniewicz, 2012), and dilutions resulting from the turnover of macromolecules as described in the text.

All ^{13}C -MFA calculations were performed using the Metran software (Yoo et al., 2004) which is based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007b). Fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the experimentally measured and model predicted external rates and mass isotopomer distributions of biomass amino acids, glucose derived from glycogen, ribose derived from RNA, and external succinate (anaerobic cultures only), using non-linear least-squares regression (Antoniewicz et al., 2006a). All measured mass isotopomers are provided in Supplemental Materials. For integrated analysis of parallel labeling experiments, the data sets were fitted simultaneously to a single flux model as described previously (Leighty and Antoniewicz, 2013). Flux estimation was repeated 10 times starting with random initial values for all fluxes to find a global solution. At convergence, accurate 95% confidence intervals were computed for all estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations. Precision of estimated fluxes was determined as follows (Antoniewicz et al., 2006a):

$$\text{Flux precision (stdev)} = [(\text{flux}_{\text{upper bound 95\%}}) - (\text{flux}_{\text{lower bound 95\%}})] / 4 \quad (7)$$

To describe fractional labeling of metabolites, G-value parameters were included in ^{13}C -MFA. As described previously (Antoniewicz et al., 2007c), the G-value represents the fraction of a metabolite pool that is produced during the labeling experiment, while 1-G represents the fraction that is naturally labeled, i.e. from the inoculum. By default, one G-value parameter was included for each measured metabolite in each data set. Reversible reactions were modeled as separate forward and backward fluxes. Net and exchange fluxes were determined as follows: $v_{\text{net}} = v_f - v_b$; $v_{\text{exch}} = \min(v_f, v_b)$.

2.7. Goodness-of-fit analysis

To determine the goodness-of-fit, ^{13}C -MFA fitting results were subjected to a χ^2 -statistical test. In short, assuming that the model is correct and data are without gross measurement errors, the minimized SSR is a stochastic variable with a χ^2 -distribution (Antoniewicz et al., 2006a). The number of degrees of freedom is equal to the number of fitted measurements n minus the number of estimated independent parameters p . The acceptable range of SSR values is between $\chi^2_{\alpha/2}(n-p)$ and $\chi^2_{1-\alpha/2}(n-p)$, where α is a certain chosen threshold value, for example 0.05 for 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1. Growth characterization

Growth characteristics of wild-type *E. coli* grown in MOPS minimal medium at 37°C were determined for four growth conditions: aerobic and anaerobic growth with glucose and xylose as the carbon source. For the anaerobic cultures, controlling the pH at 7.0 was necessary to maintain exponential growth; no pH control was required for the aerobic cultures (up to OD₆₀₀ of about 1.0). Table 1 shows the measured growth rates, biomass and product yields, and the corresponding biomass specific product secretion rates for all conditions. The aerobic growth rates for both substrates (0.70 ± 0.01 and $0.50 \pm 0.02 \text{ h}^{-1}$ for glucose and xylose, respectively) were more than 2-fold higher than the corresponding anaerobic growth rates (0.33 ± 0.02 and $0.13 \pm 0.02 \text{ h}^{-1}$, respectively). The aerobic biomass yields (0.44 ± 0.02 and $0.35 \pm 0.03 \text{ g}_{\text{DW}}/\text{g}$ for glucose and xylose, respectively) were also several-fold higher compared to the anaerobic biomass yields (0.14 ± 0.01 and $0.08 \pm 0.01 \text{ g}_{\text{DW}}/\text{g}$, respectively). Under aerobic growth

conditions, acetate was the only secreted product. Under anaerobic conditions, acetate, ethanol, formate and succinate were produced. Acetate, ethanol and formate were secreted at a relatively constant ratio of about 1:1:2. No lactate was detected in any of the cultures. The biomass specific glucose uptake rate was 50% higher during anaerobic growth (13.1 ± 1.0 mmol/g_{DW}.h) compared to aerobic growth (8.8 ± 0.5 mmol/g_{DW}.h), consistent with previous reports (Chen et al., 2011). In contrast, the biomass specific xylose uptake rate was only 13% higher during anaerobic growth (10.8 ± 1.1 mmol/g_{DW}.h) compared to aerobic growth (9.5 ± 0.5 mmol/g_{DW}.h). The carbon and electron recoveries (not accounting for CO₂ and O₂, which were not measured) for the aerobic cultures were 77% for glucose and 57% for xylose (Table 1), which suggests that a larger fraction of xylose was catabolized to CO₂ compared to glucose. The carbon and electron recoveries for the anaerobic cultures were 87% for glucose and 81% for xylose, suggesting that a non-negligible fraction of both substrates must have been converted to e.g. CO₂ and H₂. Indeed, in sealed flask cultures we detected net accumulation of CO₂ and H₂ in the head-space that could account for the missing 13-19% of carbons and electrons.

3.2. Biomass composition analysis

In nearly all flux studies performed to date, a constant *E. coli* biomass composition has been assumed. However, it is well known that the composition of biomass can change in response to environmental and genetic perturbations (Long et al., 2016b). This in turn can have a non-negligible impact on the accuracy of flux predictions. To ensure that flux results generated in this study are as accurate as possible, biomass composition was measured for all growth conditions using the methods described in (Long and Antoniewicz, 2014b). This information was then used to generate condition-specific growth stoichiometries for ¹³C-MFA (Supplemental Materials).

The results of the biomass composition analyses are summarized in Figure 1 (complete results are provided in Supplemental Materials). Proteins were the most abundant component of biomass for all conditions. The protein content of dry biomass was slightly higher for xylose as the substrate (57 wt% and 61 wt% for aerobic and anaerobic cultures, respectively) compared to glucose as the substrate (51 wt% for both aerobic and anaerobic cultures). RNA was the next most abundant component of biomass. For both substrates, the RNA content was higher during aerobic growth (18 wt% for both substrates) compared to anaerobic growth (11 wt% for glucose and 9% for xylose). It is well known that the RNA content of biomass positively correlates with the growth rate of cells (Long et al., 2016b), which is consistent with the results obtained here. The lipid content was relatively constant at about 6 wt% for all growth conditions. Glycogen content varied significantly, ranging from 2 wt% for aerobic growth on glucose to 9 wt% for anaerobic growth on glucose. Figure 1B shows the distribution of fatty acids in biomass, which varied notably depending on the specific growth condition. For example, the relative abundance of C18:1 was reduced by more than 2-fold when cells were grown anaerobically on xylose, compared to the other three growth conditions. No significant changes were observed in the relative amino acid profiles (Supplemental Materials). The composition for the aerobic glucose condition is consistent with previous reports (Long and Antoniewicz, 2014b; Long et al., 2016b). Small differences in the fatty acid distribution, particularly higher C16:1 levels here, may be the result of differences in media compositions (MOPS here, M9 previously).

3.3. Turnover of biomass macromolecules

A common assumption in ^{13}C -MFA is that turnover of biomass macromolecules such as proteins, lipids and RNA can be neglected; however, this assumption has not been validated rigorously under all relevant growth conditions. To investigate the presence or absence of biomass turnover, cells were first pre-cultured in medium containing unlabeled glucose or xylose (i.e. natural abundance of ^{13}C), washed with sugar-free medium, and then transferred to medium containing fully labeled glucose $[\text{U-}^{13}\text{C}]\text{glucose}$, or fully labeled xylose $[\text{U-}^{13}\text{C}]\text{xylose}$. The initial OD_{600} of the cultures was 0.15 ± 0.02 and cells were harvested for GC-MS analysis when OD_{600} reached 0.85 ± 0.02 . Assuming that no turnover of biomass occurs, the expected labeling profile of biomass components at the time of harvesting would be: 18% ($=0.15/0.85$) fully unlabeled ($\text{M}+0$), and 82% fully labeled ($\text{M}+N$, where N is the number of C-atoms in the measured compound). Since isotopic tracers are not 100% ^{13}C -labeled (for example, here, we determined that $[\text{U-}^{13}\text{C}]\text{glucose}$ and $[\text{U-}^{13}\text{C}]\text{xylose}$ tracers both had an isotopic purity of about 99.3 atom% ^{13}C), some incompletely labeled mass isotopomers ($\text{M}+N-1$) are also expected. On the other hand, if biomass turnover does occur, then we would expect unlabeled carbon from the initially unlabeled biomass to enter central carbon metabolism, which would subsequently result in increased abundances of incompletely labeled mass isotopomers. Figure 2 shows the expected and measured mass isotopomer distributions (after correction for natural isotope abundances), for five representative metabolites: valine, serine, phenylalanine, aspartate and palmitate. The complete set of GC-MS measurements are provided in Supplemental Materials.

For aerobic glucose and xylose cultures, the measured mass isotopomers matched well with the expected mass isotopomers assuming no biomass turnover (Fig. 2A and 2B). The notable exceptions were aspartate, glutamate and related amino acids (see Supplemental materials),

which displayed higher than expected abundances of M+N-1 mass isotopomers. In a previous study using [U-¹³C]glucose (Leighty and Antoniewicz, 2012), we noted similar dilutions of labeling which could be explained by the incorporation of unlabeled (atmospheric) CO₂ via the anaplerotic reaction: phosphoenolpyruvate + CO₂ → oxaloacetate (i.e. oxaloacetate is the precursor for aspartate, glutamate and related amino acids). The inlet air in this study contained ~0.04% CO₂. The partial labeling observed here for the aerobic cultures is thus not related to biomass turnover, but results from the incorporation of unlabeled atmospheric CO₂.

For anaerobic glucose and xylose cultures, the measured mass isotopomers differed more significantly from the expected mass isotopomers (Fig 2C and 2D). Most striking were the very high abundances of M+N-1 mass isotopomers of aspartate, glutamate and related amino acids, suggesting significant incorporation of unlabeled CO₂. For example, for the anaerobic [U-¹³C]glucose experiment, the M+N-1 mass isotopomer of aspartate (M+3) was the most abundant mass isotopomer in the mass spectrum (>50% relative abundance). We validated that nitrogen gas used in the anaerobic cultures did not contain any CO₂ (less than 0.001%). Thus, all of the unlabeled CO₂ must have originated from turnover of unlabeled biomass. These results further suggest that under anaerobic conditions there is not enough CO₂ generated in central carbon metabolism and amino acid biosynthesis pathways to support anaplerosis (phosphoenolpyruvate + CO₂ → oxaloacetate), but instead a large fraction of CO₂ originates from biomass turnover.

For the anaerobic cultures, we also noted that palmitate had significantly higher than expected abundances of incompletely labeled mass isotopomers. This was especially pronounced for the anaerobic xylose culture, where we detected significant abundances of M+10, M+12, and M+14

mass isotopomers, in addition to the expected M+16 mass isotopomer of palmitate. The presence of M+N-2, M+N-4, and M+N-6 mass isotopomers suggests incorporation of unlabeled AcCoA that originated from biomass turnover, e.g. via β -oxidation of initially unlabeled fatty acids.

Moreover, we found evidence that unlabeled phosphoenolpyruvate (PEP) was present. For example, we observed significant M+6 (=M+N-3) and M+7 (=M+N-2) mass isotopomers in the mass spectrum of phenylalanine for the anaerobic xylose culture. Phenylalanine is produced from the condensation of erythrose 4-phosphate, PEP_{C1-C2} and PEP_{C1-C3}. If unlabeled PEP (M+0) is present, then we would expect to observe equal abundances of M+6 and M+7 mass isotopomers for phenylalanine, consistent with our GC-MS measurements (Fig 2). To further validate that unlabeled PEP was present, we measured directly the labeling of three intracellular metabolites in glycolysis, 3PG, PEP and pyruvate. For the anaerobic xylose culture, 7% of 3PG was fully unlabeled (M+0), and 5% of PEP and pyruvate were fully unlabeled (Fig 3). For the other three growth conditions, the M+0 abundances of these metabolites were less than 1%. A possible explanation for the presence of unlabeled PEP is that it originated from the glycerol moiety of unlabeled lipids that turned over. Taken together, the labeling data presented here provide strong evidence that biomass turnover occurs under anaerobic growth conditions and that this cannot be neglected when analyzing labeling data.

3.4. Growth of β -oxidation knockouts

The results described above suggest that lipid turnover could be a characteristic feature of anaerobic growth on xylose. To determine if β -oxidation is strictly necessary for cell growth under this condition, the effect of gene knockouts of key enzymes in the β -oxidation pathway

were investigated. Specifically, we determined growth characteristics for the strains $\Delta fadD$, $\Delta fadK$, and $\Delta fadD\Delta fadK$ under aerobic and anaerobic conditions for both substrates. The genes *fadD* and *fadK* encode for acyl-CoA synthetase, the first step in the β -oxidation pathway (Fig. 4A). It is believed that *fadD* is mainly involved in aerobic fatty acid oxidation, while *fadK* is active during anaerobic fatty acid oxidation (Campbell et al., 2003). Figure 4B compares the growth rates of wild-type *E. coli* and each of the knockout strains for the four growth conditions. No significant differences in growth rates were observed for the aerobic cultures and for the anaerobic glucose culture. However, for the anaerobic xylose culture, the growth rates of the single gene knockout strains were significantly lower compared to wild-type (~40-70% reduction in growth rate). Moreover, the double-knockout $\Delta fadD\Delta fadK$ failed to grow on xylose. These results provide additional support that β -oxidation is indeed necessary for anaerobic growth on xylose.

3.5. Validation of metabolic network models

To statistically validate the proposed metabolic network models for ^{13}C -MFA (Leighty and Antoniewicz, 2012), labeling data from $[\text{U-}^{13}\text{C}]\text{glucose}$ and $[\text{U-}^{13}\text{C}]\text{xylose}$ experiments were fitted to four models accounting for various dilution effects: i) a base model that did not account for any dilution; ii) an extended model that accounted for the dilution of CO_2 from unlabeled sources (either external or internal); iii) an extended model that accounted for dilutions resulting from lipid turnover, modeled here as dilutions of intracellular glyceraldehyde 3-phosphate (GAP) and AcCoA; and iv) an extended model that accounted for CO_2 dilution and dilutions resulting from lipid turnover. Figure 5 shows the determined sum of squares residuals (SSR) values from fitting each model to each set of labeling data. For the aerobic cultures, the only dilution effect

that had to be accounted for to get a statistically acceptable fit was CO₂ dilution. For the anaerobic cultures, CO₂ dilution and lipid turnover had to be included in the model to obtain statistically acceptable fits. When carbohydrate and amino acid turnover was included in the models, there was no significant effect on the SSR values (Supplemental Materials), which suggests that turnover of proteins, glycogen and RNA was minimal.

3.6. ¹³C-Metabolic Flux Analysis

Next, we quantified precise metabolic fluxes for all four growth conditions using state-of-the-art techniques in ¹³C-MFA. Specifically, we performed parallel labeling experiments using the optimal isotopic tracers [1,2-¹³C]glucose, [1,6-¹³C]glucose, [1,2-¹³C]xylose and [5-¹³C]xylose, which were identified using the approaches described in (Antoniewicz, 2013; Crown et al., 2012; Crown and Antoniewicz, 2012; Crown et al., 2016), and measured isotopic labeling of biomass amino acids, biomass glycogen and RNA, and external succinate (anaerobic cultures only). The measured mass isotopomer distributions together with the measured external rates (Table 1) were fitted to the validated metabolic network models, which were updated with condition-specific growth stoichiometries based on the measured biomass compositions. Statistically acceptable fits were obtained in all cases. The minimized SSR values were lower than the maximum statistically acceptable SSR values at 95% confidence level, assuming a constant measurement error of 0.4 mol% for all GC-MS measurements (Antoniewicz et al., 2007a). The estimated metabolic fluxes and 95% confidence intervals are provided in Supplemental Materials.

Figure 6 shows the estimated flux maps for the four growth conditions. During aerobic growth on glucose and xylose, the classical central metabolic pathways were active, including

glycolysis, pentose phosphate pathway (PPP), and TCA cycle (Fig 6A and 6B). The results for glucose matched well with previous studies (Chen et al., 2011; Crown et al., 2015a). About 24% of glucose was metabolized via oxPPP and the remaining 76% was metabolized via glycolysis. The Entner–Doudoroff pathway, glyoxylate shunt and malic enzyme were all inactive during growth on glucose, consistent with previous reports. During growth on xylose, about 24% of fructose 6-phosphate (F6P) that was produced from xylose via non-oxidative PPP was metabolized via oxPPP and the remaining 76% via glycolysis. The Entner–Doudoroff pathway and glyoxylate shunt were inactive; however, in contrast to growth on glucose, malic enzyme was active during growth on xylose. For both substrates, phosphoenolpyruvate carboxylase was the only active anaplerotic reaction. The TCA cycle fluxes were notably higher during growth on xylose compared to growth on glucose. For example, during growth on xylose the citrate synthase flux corresponded to 35% of xylose uptake rate (or 3.3 ± 0.2 mmol/gDW.h), while during growth on glucose the citrate synthase flux was 26% of glucose uptake rate (or 2.3 ± 0.1 mmol/gDW.h)

The anaerobic flux maps were strikingly different from the aerobic flux maps (Fig 6C and 6D). Most notably, the flux through oxPPP was significantly reduced and the TCA cycle became bifurcated. Under anaerobic conditions, only 8% of glucose was metabolized via oxPPP, and oxPPP was inactive during anaerobic growth on xylose. For both substrates, the TCA cycle was disconnected between α -ketoglutarate (AKG) and succinyl-CoA. The oxidative branch of the TCA cycle served to supply AKG, a precursor for several amino acids, while the reductive branch of the TCA cycle produced the by-product succinate. Similar to aerobic cultures, the Entner–Doudoroff pathway and glyoxylate shunt were inactive for both substrates, and malic

enzyme was only active during growth on xylose. Similar results have been previously reported regarding the low flux of the TCA cycle under anaerobic conditions (Chen et al., 2011). However, fermentation product profiles varied from our results. This difference is possibly due to experimental setup (i.e. pH control vs. no pH control).

During aerobic growth on both substrates, sufficient CO₂ was produced in central carbon metabolism (via oxPPP, glycolysis, and TCA cycle) to support anaplerosis via phosphoenolpyruvate carboxylase ($\text{PEP} + \text{CO}_2 \rightarrow \text{oxaloacetate}$). However, during anaerobic growth, the amount of CO₂ produced was reduced due to lower oxPPP and TCA cycle fluxes. Moreover, pyruvate formate lyase was mainly used to convert pyruvate to AcCoA under anaerobic conditions (which does not generate CO₂), compared to pyruvate dehydrogenase during aerobic growth (which does generate CO₂). For example, for the anaerobic glucose culture, the net CO₂ production rate via oxPPP and TCA cycle was reduced to 0.13 mmol/gDW.h, which lower than 0.28 mmol/gDW.h of CO₂ needed for anaplerosis. For xylose, the net CO₂ production via oxPPP and TCA cycle was only 0.04 mmol/gDW.h, much less than 0.18 mmol/gDW.h of CO₂ needed for anaplerosis. Thus, for both substrates additional CO₂ must have been generated via other pathways. This result is in agreement with our findings described in previous sections, where we concluded that a large fraction of CO₂ during anaerobic growth must originate from biomass turnover.

3.7. Quantitative analysis of co-factor balances

To provide additional insights into the physiology of *E. coli* grown aerobically and anaerobically on glucose and xylose, we calculated for each condition the production and consumption rates of

key co-factors in metabolism NADH/FADH₂, NADPH, and ATP, using the ¹³C-MFA estimated fluxes. The results are summarized in Fig. 7 (see Supplemental Materials for details).

For the aerobic cultures, the production and consumption rates of NADH/FADH₂ and NADPH were very similar during growth on glucose (32.0 mmol/gDW.h for NADH/FADH₂, and 10.9 mmol/gDW.h for NADPH) and growth on xylose (29.8 mmol/gDW.h for NADH/FADH₂, and 10.9 mmol/gDW.h for NADPH). The co-factors NADH/FADH₂ were produced about equally via glycolysis (~50% contribution) and the TCA cycle (~50% contribution) for both substrates. The vast majority of NADH/FADH₂ was oxidized to generate ATP via oxidative phosphorylation. Based on our flux results, we estimated that the oxygen consumption rates were 13.8 and 13.2 mmol/gDW.h during growth on glucose and xylose, respectively. During growth on glucose, NADPH was produced mainly via oxPPP (39%) and transhydrogenase (39%), and to a lesser extent in the TCA cycle (21%). During growth on xylose, NADPH was produced about equally via transhydrogenase (31%), the TCA cycle (30%), and oxPPP (27%), and to a lesser extent by malic enzyme (12%).

For the anaerobic cultures, the total production and consumption rates of NADH and NADPH were significantly reduced, especially during growth on xylose. For the anaerobic glucose culture, the production rates of NADH and NADPH were 25.6 mmol/gDW.h and 9.5 mmol/gDW.h, respectively; and for the anaerobic xylose culture, the production rates of NADH and NADPH were 18.0 mmol/gDW.h and 3.4 mmol/gDW.h, respectively. For both substrates, nearly all NADH was produced in glycolysis (>95%), and the majority of the generated NADH was used for ethanol production. During growth on glucose, NADPH was mainly produced via

transhydrogenase (72%), and to a lesser extent via oxPPP (21%). During growth on xylose, NADPH was produced about equally by malic enzyme (50%) and transhydrogenase (41%). The TCA cycle did not contribute significantly to NADPH production.

Biological energy (in the form of ATP) is needed for three key cellular processes: i) transport of substrates and nutrients into the cells; ii) cell growth (anabolism); and iii) maintenance. During aerobic growth, ATP is mainly produced via respiration and substrate-level phosphorylation, and to a lesser extent from acetate production. For example, our flux analysis results suggest that the majority of ATP was produced via oxidative phosphorylation (68% for glucose and 70% for xylose); here, we assumed a value of 2.0 for the P/O ratio. Even if we assume a more conservative value for the P/O ratio of 1.5 (Noguchi et al., 2004; Taymaz-Nikerel et al., 2010), the oxidative phosphorylation is still the major contributor to ATP production during aerobic growth on glucose (61%) and xylose (63%). The total ATP production rate was slightly higher during growth on glucose (79.7 mmol/gDW.h) compared to growth on xylose (72.0 mmol/gDW.h). During growth on glucose, 11% of ATP was used for glucose transport (8.8 mmol/gDW.h), 34% for cell growth (26.9 mmol/gDW.h), and 55% for maintenance (43.9 mmol/gDW.h). We define maintenance cost to be the difference between the total rate of ATP production and ATP consumption for substrate uptake and cell growth. If we assume $P/O = 1.5$, the estimated ATP maintenance cost is reduced to 30.1 mmol/gDW.h. For glucose transport, we equated the donated phosphate via PTS transport with one ATP equivalent. During growth on xylose, a relatively larger fraction of ATP was used for substrate transport and phosphorylation (26%, or 19.0 mmol/gDW.h), 38% for cell growth (27.4 mmol/gDW.h), and 35% for

maintenance (25.5 mmol/gDW.h). Assuming $P/O = 1.5$, the estimated ATP maintenance cost is reduced to 12.3 mmol/gDW.h.

During anaerobic growth, the total ATP production rate was reduced for both substrates. We estimated that the ATP production rate was higher during anaerobic growth on glucose (38.1 mmol/gDW.h) compared to anaerobic growth on xylose (30.5 mmol/gDW.h). For both substrates, ATP was mainly produced via substrate-level phosphorylation in the glycolysis (~80% for both substrates), with acetate production contributing the remaining ~20%. During growth on glucose, 34% of ATP was used for glucose transport (13.1 mmol/gDW.h), 59% for cell growth (22.5 mmol/gDW.h), and 6% for maintenance (2.2 mmol/gDW.h). During growth on xylose, 71% of ATP was used for xylose transport and phosphorylation (21.6 mmol/gDW.h), 26% for cell growth (7.8 mmol/gDW.h), and 3% for maintenance (1.0 mmol/gDW.h). These results suggest that the higher ATP cost for xylose transport and phosphorylation (2 ATP equivalents), compared to glucose (1 ATP equivalent), significantly affects cell growth and biomass yield on xylose. Taken together, these results illustrate that key differences exist between glucose and xylose metabolism at the level of co-factor production and utilization, and that these differences play a role in determining cell physiology of *E. coli*.

4. CONCLUSION

E. coli is the most widely used microorganism in both industry and academia. Previous studies on *E. coli* metabolism have been limited mainly to aerobic growth on glucose. Only a few studies have focused on elucidating fermentative metabolism, and no ^{13}C -MFA studies exist on xylose metabolism in *E. coli*. To address this critical gap in current understanding, here, we have

applied state-of-the-art methods in ^{13}C -MFA to determine precise metabolic fluxes for wild-type *E. coli* grown aerobically and anaerobically on glucose and xylose. We measured condition-specific changes in biomass composition, quantified biomass turnover, and determined that β -oxidation was strictly required for anaerobic growth on xylose. By analyzing co-factor balances, we identified important differences in pathway utilization for the production of consumption of energy and redox cofactors. These validated flux maps can now be used as references for COBRA predictions. In particular, the elucidation of biomass turnover under various conditions will further improve these predictive tools. If biomass turnover is not included, it is likely that predictions will be incorrect. Taken together, this study provides a wealth of new information on aerobic and anaerobic metabolism in *E. coli* that can be used in future metabolic engineering studies to improve predictive strategies for new strain designs.

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FIGURE LEGENDS

Figure 1. Biomass composition analysis of wild-type *E. coli* grown aerobically and anaerobically on glucose and xylose.

Figure 2. Expected (black bars) and measured (red bars) mass isotopomer distributions for five metabolites (valine, serine, phenylalanine, aspartate, and palmitate) from tracer experiments with [U-¹³C]glucose and [U-¹³C]xylose. Presence of incompletely labeled mass isotopomers, especially under anaerobic conditions, indicates significant biomass turnover.

Figure 3. Mass isotopomer distributions for the glycolytic intermediates 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP), and pyruvate (Pyr) from tracer experiments with [U-¹³C]glucose and [U-¹³C]xylose. Presence of unlabeled mass isotopomers (M+0), especially during anaerobic growth on [U-¹³C]xylose, indicates that significant biomass turnover occurs.

Figure 4. (A) β -oxidation pathway with genes encoding each reaction. (B) Growth rates of wild-type *E. coli* and knockout strains $\Delta fadD$, $\Delta fadK$, $\Delta fadD\Delta fadK$ grown aerobically and anaerobically on glucose and xylose as substrates. The double-knockout strain $\Delta fadD\Delta fadK$ did not grow on xylose under anaerobic conditions.

Figure 5. Validation of metabolic network models for ¹³C-MFA. Sum of squared residual (SSR) values are shown for models containing various dilution reactions. For the aerobic cultures, inclusion of CO₂ dilution was necessary to obtain an acceptable SSR value (below the red dotted line). For the anaerobic cultures, CO₂ and lipid dilution were necessary to achieve an acceptable SSR value.

Figure 6. Metabolic flux maps for *E. coli* grown in batch culture at four growth conditions: aerobic and anaerobic growth on glucose and xylose, respectively. Fluxes were determined using integrated ^{13}C -MFA by simultaneously fitting labeling data from two tracers for each substrate. For glucose, $[1,2-^{13}\text{C}]$ glucose and $[1,6-^{13}\text{C}]$ glucose tracers were used. For xylose, $[1,2-^{13}\text{C}]$ xylose and $[5-^{13}\text{C}]$ xylose tracers were used. Complete flux results are provided in Supplemental Materials.

Figure 7. Production and consumption rates of key co-factors in metabolism NADH//FADH_2 , NADPH , and ATP , during aerobic and anaerobic growth on glucose (Gluc) or xylose (Xyl). “Other” in NADPH panel represents the contribution of malic enzyme to NADPH production. “Other” in ATP panel represents the estimated ATP maintenance cost (here, assuming P/O ratio = 2.0).

TABLES

Table 1. Physiological characteristics of *E. coli* grown in batch culture on glucose and xylose under aerobic and anaerobic conditions in MOPS minimal medium at 37°C.

	Aerobic Glucose	Aerobic Xylose	Anaerobic Glucose	Anaerobic Xylose
μ (1/h)	0.70 ± 0.01	0.50 ± 0.02	0.33 ± 0.02	0.13 ± 0.02
Y_X (g _{DW} /g)	0.44 ± 0.02	0.35 ± 0.03	0.14 ± 0.01	0.08 ± 0.01
Y_{Ac} (mol/mol)	0.65 ± 0.01	0.33 ± 0.01	0.56 ± 0.02	0.51 ± 0.02
Y_{Form} (mol/mol)	-	-	1.25 ± 0.05	1.00 ± 0.03
Y_{EtOH} (mol/mol)	-	-	0.61 ± 0.04	0.56 ± 0.01
Y_{Suc} (mol/mol)	-	-	0.15 ± 0.02	0.10 ± 0.01
q_{Gluc} (mmol/g _{DW} .h)	8.8 ± 0.5	-	13.1 ± 1.0	-
q_{Xyl} (mmol/g _{DW} .h)	-	9.5 ± 0.5	-	10.8 ± 1.1
q_{Ac} (mmol/g _{DW} .h)	5.7 ± 0.2	3.1 ± 0.2	7.3 ± 0.7	5.5 ± 0.8
q_{Form} (mmol/g _{DW} .h)	-	-	16.4 ± 0.9	10.8 ± 0.8
q_{EtOH} (mmol/g _{DW} .h)	-	-	8.0 ± 0.5	6.1 ± 0.5
q_{Suc} (mmol/g _{DW} .h)	-	-	2.0 ± 0.1	1.1 ± 0.1
Carbon recovery (%)	$75\% \pm 7\%$	$56\% \pm 9\%$	$87\% \pm 5\%$	$81\% \pm 7\%$
Redox recovery (%)	$78\% \pm 8\%$	$58\% \pm 9\%$	$86\% \pm 6\%$	$81\% \pm 8\%$

* The data shown are: biomass yield (Y_X), yields of secreted products (Y_i), specific growth rate (μ), specific substrate uptake rate (q_{Gluc} or q_{Xyl}), and specific production rates (q_i) for secreted metabolites acetate (Ac), formate (Form), ethanol (EtOH) and succinate (Suc).

Figure 1

Figure 2

A) Aerobic [$U-^{13}C$]Glucose

B) Aerobic [$U-^{13}C$]Xylose

C) Anaerobic [$U-^{13}C$]Glucose

D) Anaerobic [$U-^{13}C$]Xylose

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Aerobic Growth

Anaerobic Growth