

Truncating the Structure of Lipopolysaccharide in *Escherichia coli* Can Effectively Improve Poly-3-hydroxybutyrate Production

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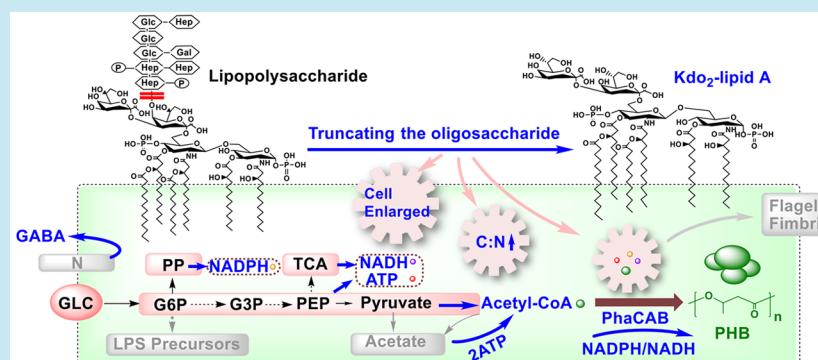


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ABSTRACT: Poly-3-hydroxybutyrate is an environmentally friendly polymer with many promising applications and can be produced in *Escherichia coli* cells after overexpressing the heterologous gene cluster *phaCAB*. In this study, we found that truncating the structure of lipopolysaccharide in *E. coli* can effectively enhance poly-3-hydroxybutyrate production. *E. coli* mutant strains WJW00, WJD00, and WJJ00 were constructed by deleting *rfaD* from *E. coli* strain W3110, DHSa, and JM109, respectively. Compared to the controls W3110/pDXW-8-*phaCAB*, DHSa/pDXW-8-*phaCAB*, and JM109/pDXW-8-*phaCAB*, the yield of poly-3-hydroxybutyrate in WJW00/pDXW-8-*phaCAB*, WJD00/pDXW-8-*phaCAB*, and WJJ00/pDXW-8-*phaCAB* cells increased by 200%, 81.5%, and 75.6%, respectively, and the conversion rate of glucose to poly-3-hydroxybutyrate was increased by ~250%. Further analysis revealed that LPS truncation in *E. coli* rebalanced carbon and nitrogen metabolism, increased the levels of acetyl-CoA, γ-aminobutyric acid, NADPH, NADH, and ATP, and decreased the levels of organic acids and flagella, resulting in the high ratio of carbon to nitrogen. These metabolic changes in these *E. coli* mutants led to the significant increase of poly-3-hydroxybutyrate production.

KEYWORDS: *Escherichia coli*, poly-3-hydroxybutyrate, PHB, lipopolysaccharide, LPS, acetyl-CoA, C/N ratio, metabolic rebalance

Biodegradable polymer poly-3-hydroxybutyrate (PHB), produced by bacteria from a renewable source, can be used as bioplastic, fine chemical, biomaterial, medicine, and biofuel.^{1–4} *Escherichia coli* is an important microbial cell factory and it has been used for PHB production by overexpressing the heterologous gene cluster *phaCAB*.^{5–7} Because of the many uses of PHB, the improvement of its production has become more and more important. Many studies have focused on metabolic engineering to yield genetically defined PHB overproducers.^{8,9} The intracellular PHB particles are attached to the cell membrane,^{10,11} and the intracellular metabolism, substrate availability, and cell size^{3,12} could significantly affect PHB yield. Therefore, attempts have been made to increase PHB production by growth pattern tuning for rapid proliferation,¹³ redirecting metabolic flux,¹⁴ enhancing cofactor supply,¹⁵ and engineering cell division^{16,17} and membrane thickness to enlarge cell sizes.⁶

Located on the outer layer of the outer membrane, lipopolysaccharides (LPSs) are major components in most Gram-negative bacteria. Each LPS molecule contains a hydrophobic anchor domain Kdo₂-lipid A and a hydrophilic domain polysaccharide.^{18–20} Since it is a crucial component, the biosynthetic pathway,²¹ structural modification,¹⁹ sRNA regulation,²² and impact on immunopharmacology²³ of LPS have been studied thoroughly, while few studies focused on metabolism alteration responding to the change of LPS structure. Several reports claimed that LPS biosynthesis

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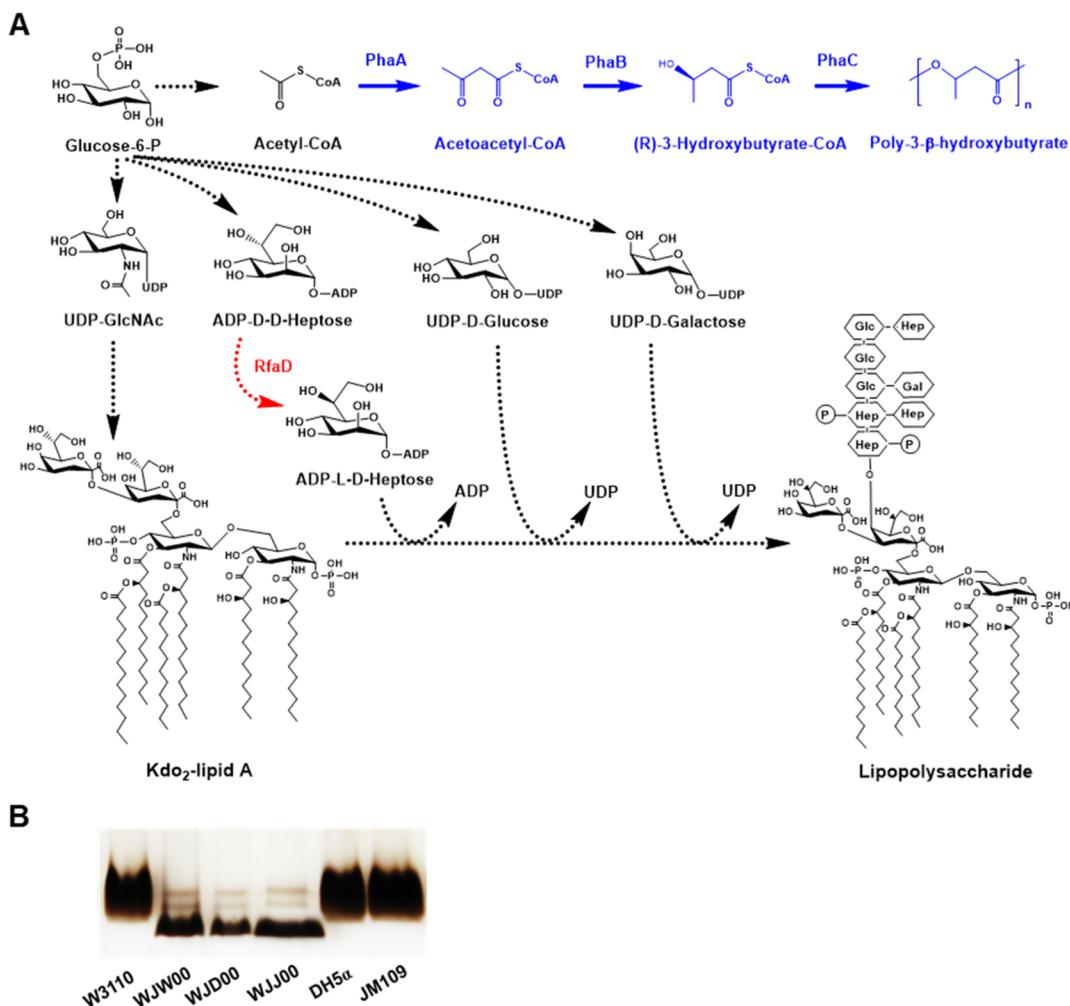


Figure 1. Biosynthesis of lipopolysaccharide and PHB in *E. coli* cells. (A) The carbon flux changed after overexpressing the heterologous gene cluster *phaCAB* and deleting the gene *rfaD* in *E. coli*. Kdo, 3-deoxy-D-manno-oct-2-ulose; Hep, heptose; Glc, glucose; Gal, galactose; Rha, rhamnose. (B) SDS-PAGE of silver stained LPS samples extracted from W3110, WJW00, DH5 α , WJD00, JM109, and WJJ00.

might be related to the intracellular PHB accumulation, for example, PHB accumulation negatively affected by LPS in *Pseudomonas putida* CA-3²⁴ and *Ralstonia eutropha* H16.²⁵

Kdo₂-lipid A is the minimum LPS structure of *E. coli*, and ADP-L-D-heptose synthesized by RfaD is required for forming complete LPS (Figure 1).^{23,26,27} In this study, the LPS in *E. coli* W3110, DH5 α , and JM109 was truncated to Kdo₂-lipid A by deleting *rfaD*, and the intracellular metabolism reactions were rebalanced by LPS truncation, further influencing the PHB productivity. Our results indicated the LPS truncation is an effective strategy to improve PHB production.

RESULTS AND DISCUSSION

Truncation of LPS Structure Facilitates PHB Biosynthesis. To obtain the mutant biosynthesizing the simplest LPS, the nonessential structure of polysaccharides in the LPS molecule need to be truncated. *E. coli* mutant WJJ00 was constructed from *E. coli* W3110 by deletion of *rfaD*, and it can only produce the truncated LPS, Kdo₂-lipid A.²⁷ To investigate the universality, we also constructed the *rfaD* gene mutant WJD00 and WJJ00, from *E. coli* DH5 α and JM109, respectively. DH5 α and JM109 share the same LPS biosynthetic pathway with W3110; therefore, WJD00 and WJJ00 also produced the truncated LPS, Kdo₂-lipid A (Figure

1B). To comprehensively study the impact on PHB production by LPS simplification and confirm the universality, we studied it not only in *E. coli* W3110, but also in *E. coli* DH5 α and JM109. To investigate whether LPS truncation influences PHB accumulation in *E. coli*, we used the plasmid pDXW-8-*phaCAB*²⁸ overexpressing the operon *phbCAB* from *Ralstonia eutropha*²⁹ under a *tacM* promoter.³⁰ This was introduced into *E. coli* W3110, WJW00, DH5 α , WJD00, JM109, and WJJ00, resulting in W3110/pDXW-8-*phaCAB*, WJW00/pDXW-8-*phaCAB*, DH5 α /pDXW-8-*phaCAB*, WJD00/pDXW-8-*phaCAB*, JM109/pDXW-8-*phaCAB*, and WJJ00/pDXW-8-*phaCAB*, respectively. These cells were grown in M9 medium supplemented with 20 g/L glucose for 24 h, harvested, stained with Nile Red, and observed under fluorescence microscopy to evaluate the PHB accumulation. Figure 2 shows differential interference contrast (DIC), fluorescence contrast (FITC), and the merged images of each sample. PHB was produced in most of the WJW00/pDXW-8-*phaCAB* cells but only in a small amount of W3110/pDXW-8-*phaCAB* cells; furthermore, the size of the WJW00/pDXW-8-*phaCAB* cells that produced PHB tended to be larger than that of the W3110/pDXW-8-*phaCAB* cells (Figure 2B 2A). A similar situation was observed for WJJ00/pDXW-8-*phaCAB* and JM109/pDXW-8-*phaCAB* cells; the former produced much more PHB than the latter, and its

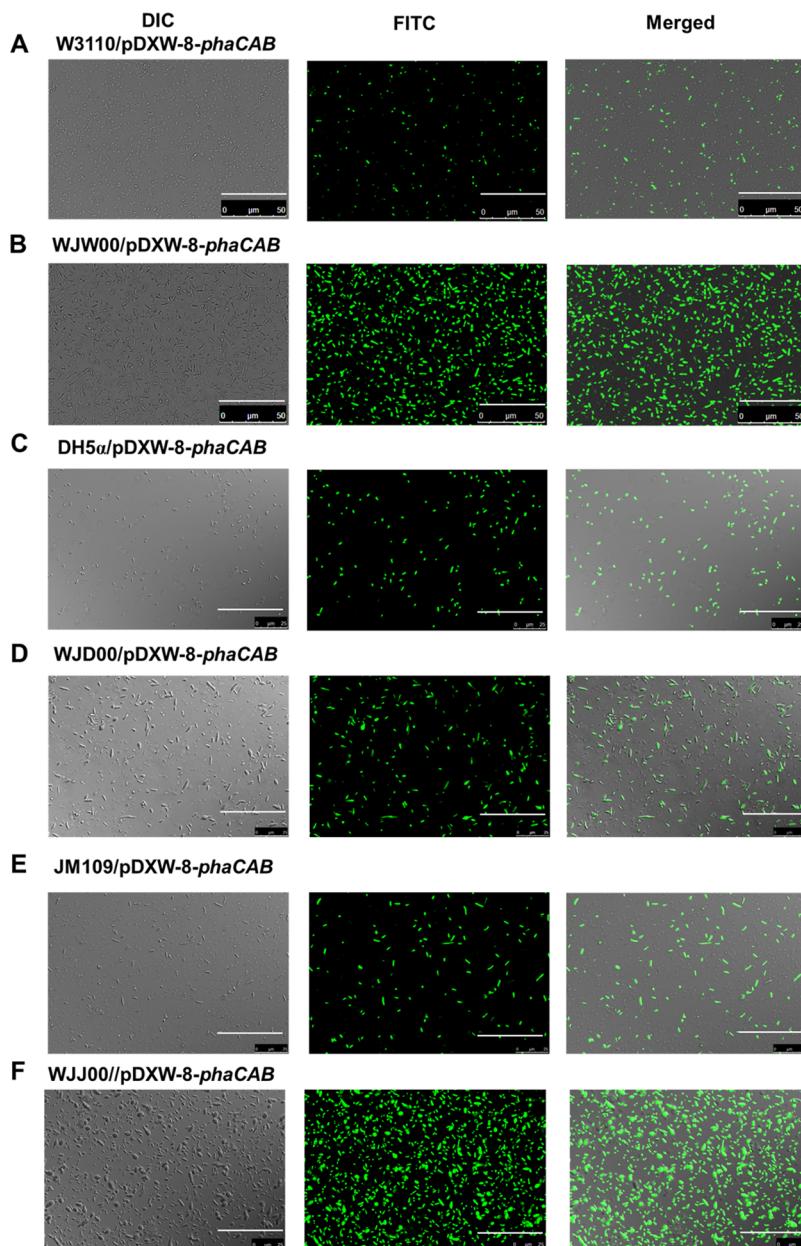


Figure 2. Fluorescence microscopy images of different *E. coli* cells. (A) W3110/pDXW-8-*phaCAB*; (B) WJJW00/pDXW-8-*phaCAB*; (C) DH5 α /pDXW-8-*phaCAB*; (D) WJD00/pDXW-8-*phaCAB*; (E) JM109/pDXW-8-*phaCAB*; (F) WJJ00/pDXW-8-*phaCAB* cells. Cells were cultured after 24 h fermentation in M9 medium supplemented with 20 g/L glucose, then stained with the fluorescent dye Nile red, and were visualized by fluorescence microscopy under excitation of 488 nm and emission of 530 nm. Since PHB stained with Nile Red can emit green fluorescence, the number and size of the cells that can produce PHB can be detected by the green spots in the images. DIC, differential interference contrast; FITC, fluorescence contrast; Merged, overlapped contrast of both DIC and FITC. Scale bar in white is 50 μ m.

cell size was also larger (Figure 2F vs 2E). However, WJD00/pDXW-8-*phaCAB* cells produced only slightly more PHB than the control DH5 α /pDXW-8-*phaCAB* cells, but the cell size of the former was larger than the latter (Figure 2D vs 2C). These results indicate that the truncation of LPS in *E. coli* can facilitate PHB production.

Further TEM analysis showed the details of intracellular PHB accumulation (Figure 3). Most of the WJJW00/pDXW-8-*phaCAB* cells produced PHB, and some of them were fully filled with PHB (Figure 3B), but only a few W3110/pDXW-8-*phaCAB* cells produced PHB (Figure 3A); in addition, many PHB-producing WJJW00/pDXW-8-*phaCAB* cells became much larger (Figure 3B). DH5 α /pDXW-8-*phaCAB* cells could

produce more PHB than W3110/pDXW-8-*phaCAB* cells (Figure 3C vs 3A), but almost all WJD00/pDXW-8-*phaCAB* cells were fully filled with PHB (Figure 3D); and even though some DH5 α /pDXW-8-*phaCAB* cells could fully filled with PHB, they were much smaller than the PHB-producing WJD00/pDXW-8-*phaCAB* cells (Figure 3D). JM109/pDXW-8-*phaCAB* cells could produce PHB and also fully fill with PHB, but the PHB granules were usually small (Figure 3E), whereas WJJ00/pDXW-8-*phaCAB* cells were fully filled with much larger PHB granules and cells became larger (Figure 3F). This suggests that LPS truncation could increase the cell sizes when filled with PHB granules in *E. coli*, possibly because the cell envelope elasticity was increased and the outer membrane

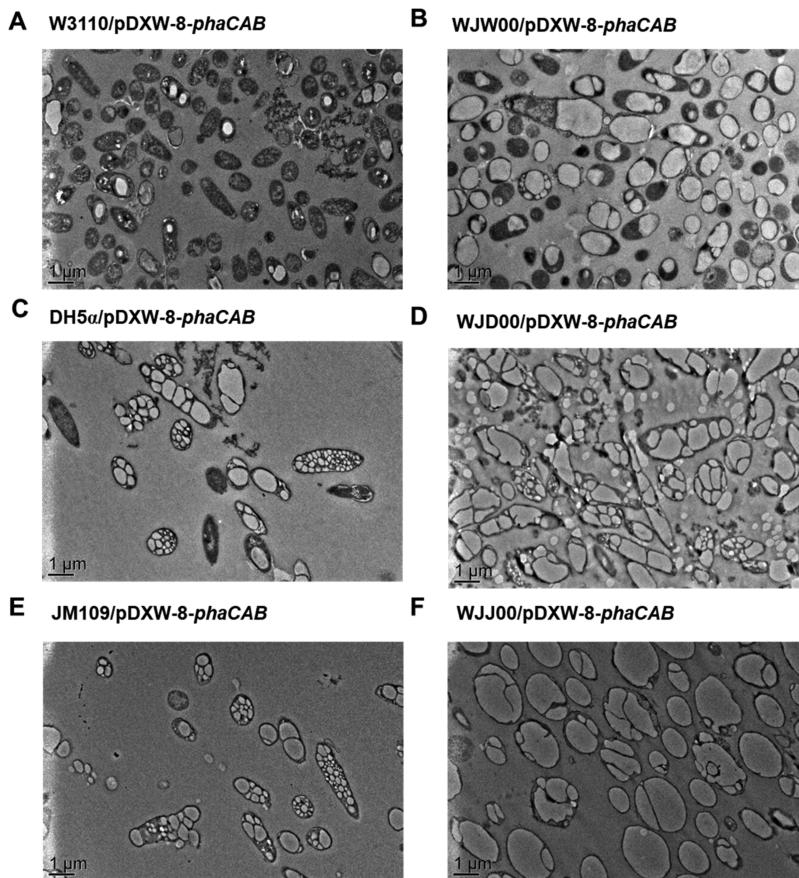


Figure 3. Ultrathin section TEM images of different *E. coli* cells. (A) W3110/pDXW-8-*phaCAB*; (B) WJJ00/pDXW-8-*phaCAB*; (C) DH5 α /pDXW-8-*phaCAB*; (D) WJD00/pDXW-8-*phaCAB*; (E) JM109/pDXW-8-*phaCAB*; (F) WJJ00/pDXW-8-*phaCAB* cells.

rigidity was decreased by LPS truncation. The results showed that the mutant cells with the simplest LPS structure could expand more easily to accumulate more PHB granules.

When filled with PHB particles, LPS truncation mutant cells WJJ00/pDXW-8-*phaCAB*, WJD00/pDXW-8-*phaCAB*, and WJJ00/pDXW-8-*phaCAB* showed much larger sizes than W3110/pDXW-8-*phaCAB*, DH5 α /pDXW-8-*phaCAB*, and JM109/pDXW-8-*phaCAB*, respectively. Recently, a study published on nature proved that the stiffness and strength of *E. coli* cells are largely due to the outer membrane, which can be stiffer than the cell wall.³¹ LPS is the outer leaflet of the outer membrane in *E. coli*²³ and contributes to the rigidity and stiffness of the outer membrane.³¹ The truncation of LPS could decrease the stiffness of the cell envelope in *E. coli*.³¹ The decreased rigidity of the cell envelope benefits the enlargement of cell volume when PHB granules accumulate in cells.^{6,16,17} Hence, the LPS simplification significantly benefits PHB granules accumulation in *E. coli*. *E. coli* does not contain PhaP; therefore, lots of small PHB granules observed in some cells have nothing to do with PhaP. The cytoplasm is a hydrophilic environment. At the beginning, the newly synthesized PHB might gather into many small granules. With the increase of PHB amount, the small granules might gather together through hydrophobic interaction to form large granules. This explanation is consistent with the fact that large PHB granules were observed in the mutant strains with higher PHB production, while lots of small PHB granules were formed in the wild type strains with low PHB production (Figure 3). Other unknown factors in the cytoplasm might also

affect the gathering of PHB, because the number of PHB granules formed in different wild-type strains was quite different (Figure 3).

We also quantify the PHB production of the above six *E. coli* strains in the minimal medium containing 20 g/L glucose after 24 and 48 h fermentation. As expected, the DCW, PHB concentration, and conversion efficiency of the LPS-truncated mutants significantly increased (Figure 4). The DCW, PHB content, and glucose conversion efficiency of WJJ00/pDXW-8-*phaCAB* reached 3.30 g/L (Figure 4A), 67.8% (Figure 4B), and 0.22 g/g (Figure 4D) after 24 h, respectively, increasing 73.6%, 2.0-fold, and 2.7-fold than that of W3110/pDXW-8-*phaCAB*, only reaching 1.9 g/L, 22.4%, and 6%. Since DCW includes the PHB, the residual biomass was obtained by subtraction of PHB from DCW (DCW – PHB). As shown in Figure 4C, the residual biomass of the LPS truncated *E. coli* cells was always smaller than that of their corresponding wild type cells. This could be due to the slow growth caused by LPS truncation and the growth stress induced by PHB accumulation. The biomass is related to the concentration of soluble proteins which could be significantly decreased in the PHB producing strains.³²

Moreover, WJD00/pDXW-8-*phaCAB* cells and WJJ00/pDXW-8-*phaCAB* cells showed better DCW, PHB content, and glucose conversion efficiency. WJD00/pDXW-8-*phaCAB* cells could accumulate 78.6% PHB of DCW after 48 h fermentation, and produced 0.39 g of PHB from 1 g of glucose; increasing 81.5% and 2.3-fold than that of DH5 α /pDXW-8-*phaCAB*, respectively; WJJ00/pDXW-8-*phaCAB* cells could

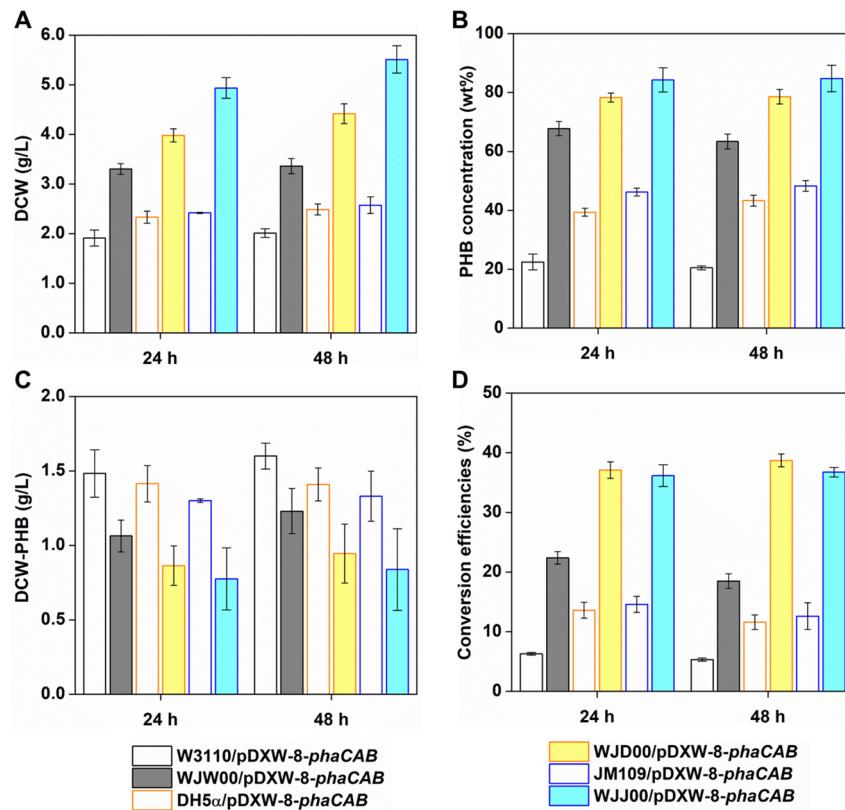


Figure 4. PHB productions in W3110/pDXW-8-phaCAB, WJW00/pDXW-8-phaCAB, DH5α/pDXW-8-phaCAB, WJD00/pDXW-8-phaCAB cells, JM109/pDXW-8-phaCAB, and WJJ00/pDXW-8-phaCAB cells. *E. coli* cells were cultivated in the minimal medium containing 20 g/L glucose at 37 °C, and samples were taken at 24 and 48 h. A. Dry cell weight (DCW). (B) PHB concentration. (C) The residual biomass after removing PHB weight (DCW-PHB). (D) Conversion efficiency from glucose to PHB. Error bars show standard deviation from the mean of three independent experiments.

accumulate the highest PHB content (84.8%) of DCW after 48 h fermentation, and produced 0.37 g of PHB from 1 g of glucose; increasing 75.6% and 2.3-fold than that of JM109/pDXW-8-phaCAB, respectively. The highest PHB production (84.8%) was obtained in WJJ00/pDXW-8-phaCAB cells after 48 h. The highest conversion efficiency of glucose to PHB (38.7%) was obtained in WJD00/pDXW-8-phaCAB cells after 48 h. The PHB content was determined by collecting the cells, trying them, and analyzing the PHB content with gas chromatography; therefore, it represents the average PHB content in all cells. The TEM images, however, show the typical PHB content in single cells; therefore, higher than 85% PHB content could be observed in some cells, but lower than 85% PHB content could be also observed in other cells (Figure 3).

These results confirmed that LPS truncation in *E. coli* facilitates PHB production in various *E. coli* strains. To understand what is behind the mechanism, further investigation was performed mainly on WJW00, using W3110 as the control.

LPS Truncation in *E. coli* Caused the Increase of Acetyl-CoA Accumulation. Since acetyl-CoA is the direct precursor for PHB biosynthesis in *E. coli* strains harboring pDXW-8-phaCAB (Figure 1), its level in mutant WJW00 is the key factor for PHB biosynthesis. Therefore, the intracellular acetyl-CoA of WJW00 together with the wild-type W3110 grown at mid-log phase was determined (Figure 5A). Interestingly, 9.9 μg/L acetyl-CoA was detected in WJW00 cells, but only 2.2 μg/L acetyl-CoA was detected in W3110

cells. Then, we studied the transcriptional level of the Entner–Doudoroff pathway (EMP) for the biosynthesis of acetyl-CoA, the results showed that almost all the relevant genes were up-regulated in WJW00 as compared with the control W3110 (Figure 5B). Compared to the wild-type W3110, glucose could be increasingly fluxed into the glycolysis due to up-regulated *ptsGHI* in WJW00. The metabolic flux of the EMP pathway was strongly directed into the biosynthesis of acetyl-CoA. WJW00 could biosynthesize more Acetyl-CoA, which benefits the PHB production.¹⁴

Meanwhile, we also determined the regulation of carbon flux pathway to other sugars, the transcriptional analysis showed that the genes involved in reactions of glucose to other sugars were significantly down-regulated (Figure 5C) in WJW00 compared to W3110. The significantly weakened pathways of glucose flux to other sugars including the biosynthesis of D-fructose, D-galactose, D-ribulose, D-xylulose, L-xylulose, L-ribulose, L-arabinose, and L-rhamnose (Figure 5A). Genes *araA*, *araB*, *rhaA*, and *rhaB* for L-arabinose, ribulose, xylulose, and rhamnose synthesis were significantly down-regulated with a \log_2^{RI} more than 13 (Figure 5C). The above sugars are usually used to biosynthesize the LPS core, LPS O-antigen, and exopolysaccharides. There are about 2×10^6 LPS molecules on the *E. coli* cell envelope, and they consume much of the carbon source. This demonstrated that more carbon sources could be saved by LPS simplification. Indeed, the LPS truncation leads to the up-regulation of the EMP pathway to form more acetyl-CoA, guaranteeing more sufficient precursor for PHB biosynthesis. Moreover, the LPS truncation significantly down-

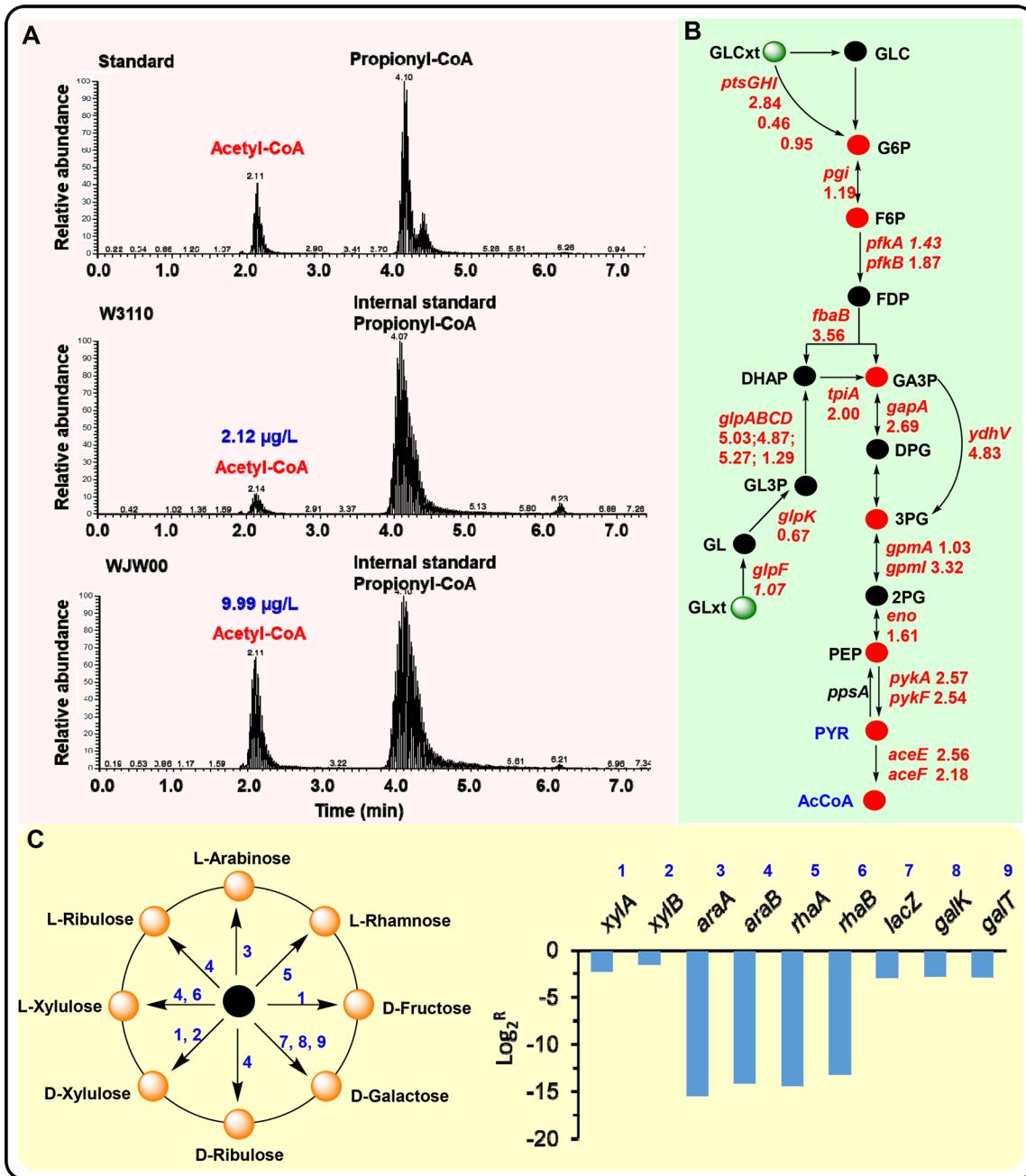


Figure 5. Comparison of the acetyl-CoA production and transcriptional levels of carbon metabolism pathway in *E. coli* strains WJW00 and W3110. (A) Comparison of the acetyl-CoA production of *E. coli* WJW00 and W3110. (B) Transcriptional analysis for Entner–Doudoroff pathway (EMP) in *E. coli* WJW00 using W3110 as a control. Up-regulated genes with \log_2 ratio (WJW00/W3110) were shown in red. (C) Genes relevant to glucose metabolism to other sugars. The black solid circle represents glucose molecule. Down-regulated genes with \log_2 ratio (WJW00/W3110) were shown in blue. The gene numbers in blue represent down-regulated genes and \log_2^R . GLC, glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate; GL3P, glycerate-phosphate; DPG, glycerate-1,3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenol pyruvate; PYR, pyruvate; LAC, lactate. AcCoA, acetyl-CoA.

regulated the carbon flux to other sugars from glucose due to the one need of LPS polysaccharide biosynthesis. It indicated that LPS simplification contributes to shift more carbon sources to acetyl-CoA biosynthesis and PHB accumulation.

LPS-Truncation Caused the Decrease of Organic Acids but Increase of γ -Aminobutyric Acid. During the fermentation of *E. coli* strains in M9 medium with glucose, we found that LPS truncation caused the higher culture pH as compared to the wild-type controls. Compared to W3110, WJW00

showed approximately 0.3 higher culture pH at 24 h and 0.2 higher at 48 h (Figure 6A). In *E. coli* fermentation, the pH regulation is closely related to the accumulation of organic acids and acid resistance systems (ARs), especially AR II (γ -aminobutyric acid biosynthesis from glutamate). Therefore, the levels of these organic acids and γ -aminobutyric acid in WJW00 and W3110 were determined when grown in M9 medium with 20 g/L of glucose (Figure 6B,C).

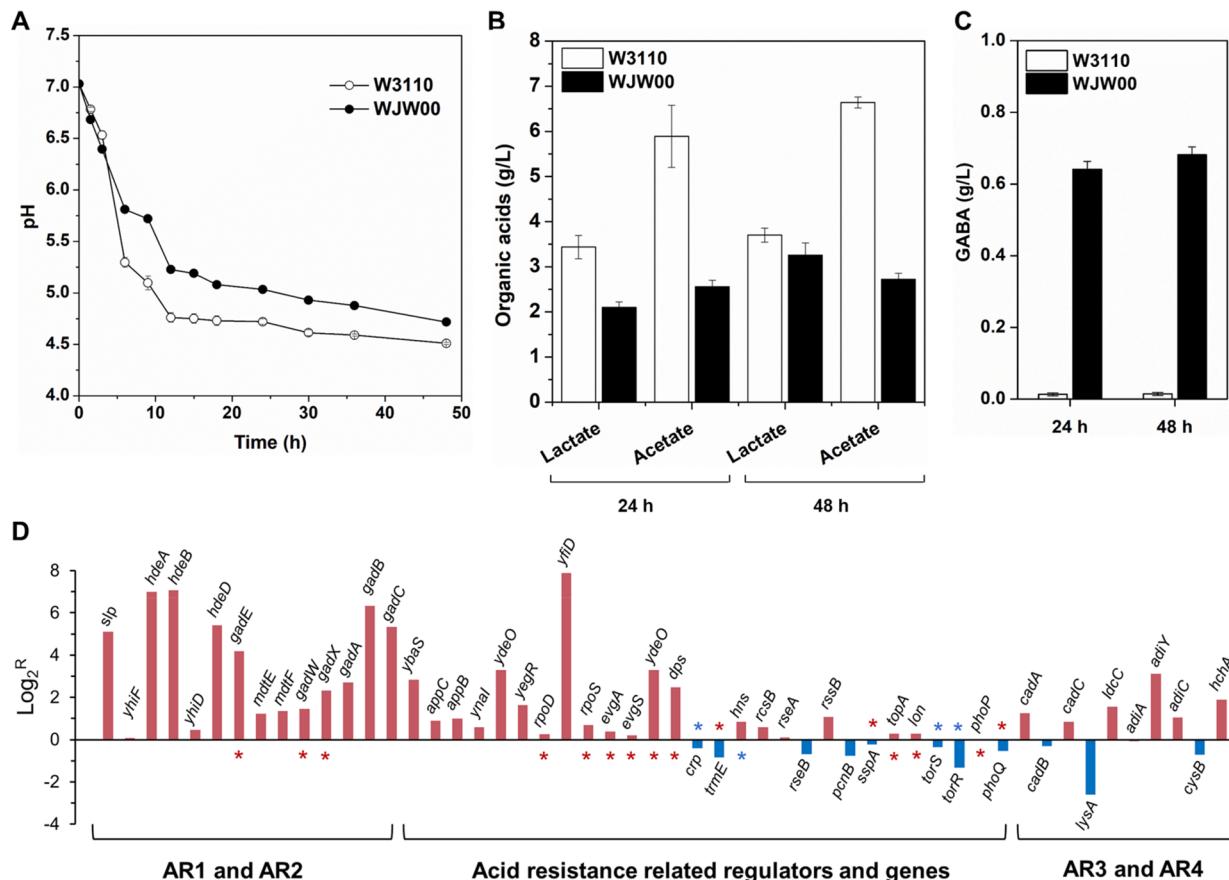


Figure 6. The batch fermentation profiles of *E. coli* W3110 and WJW00 cells. (A) Comparison of culture pH. (B) Comparison of organic acids levels. Acetic acid and lactic acid levels of W3110 and WJW00 cells were determined after 24 and 48 h fermentation. (C) Comparison of extracellular γ -aminobutyric acid (GABA) levels of W3110 and WJW00 cells was performed after 24 and 48 h fermentation. Error bars show standard deviation from the mean of three independent experiments. (D) Regulations of genes relevant to acid resistance in WJW00 using W3110 as the control. There are acid resistant systems AR1, AR2, AR3, and AR4 in *E. coli* cells. AR1 function in cells, AR2 to AR4 are transmembrane distributions on the cell envelope. Genes marked with red asterisk are positive regulators, and genes marked with blue asterisk are negative regulators for acid resistance. Red bars denote up-regulated genes, and blue bars denote down-regulated genes.

W3110 produced 9.7 g/L and 11.2 g/L of organic acids after 24 and 48 h, respectively, but WJW00 only produced 4.8 g/L and 6.2 g/L, decreasing 51% and 45% than that of W3110 after 24 and 48 h fermentation, respectively. Organic acids are mainly composed of acetate and lactate, which are usually identified as the major byproducts in PHB fermentation, because the lactate and acetate could compete with acetyl-CoA for carbon flux. So, the level of acetyl-CoA in *E. coli* could be weakened by a greater accumulation of lactate and acetate. During fermentation, lactate and acetate production were slightly higher after 48 h than 24 h (Figure 6B). After 48-h fermentation, WJW00 accumulated only 2.7 g/L acetate and 3.3 g/L lactate, decreasing 59% and 13% of that for W3110, which produced 6.6 g/L acetate and 3.7 g/L lactate. The results indicated that the LPS truncation contributes to the decrease of acetate and lactate, byproducts for PHB production. Thus, the less organic acids produced by the LPS simplified mutant could facilitate more carbon sources to be saved, carbon pool enlarged to acetyl-CoA, and more carbon flux to PHB biosynthesis.^{14,33}

In addition, the amino acids measurement showed that the higher pH is also related to the significantly higher level of GABA (Figure 6C). After 24 and 48 h fermentation, WJW00 produced 0.64 g/L and 0.68 g/L of GABA, an increase of both of 46-fold of those for W3110 which only produced 0.014 g/L

and 0.015 g/L of GABA, respectively. Other amino acids levels were very low and did not show significant differences between WJW00 and W3110. GABA is biosynthesized from glutamate and transferred outside the cell, and the process is not reversible.³⁴ The results suggest that the intracellular metabolic pathway of glutamate to GABA in *E. coli* is significantly enhanced by LPS simplification. GABA consumed many nitrogen sources and led to more nitrogen sources fixed in the form of GABA and then transferred outside the *E. coli* cells. As we know, a decrease of the available nitrogen source concentration could obviously benefit PHB synthesis. In conclusion, the rebalance of carbon and nitrogen metabolism by LPS truncation facilitates the increase of the intracellular C/N ratio, which could effectively facilitate PHB production.

The pH values decreased with time in either W3110 or WJW00 during fermentation, and the pH value in WJW00 was usually higher than that in W3110 at the same time points (Figure 6A). After either 24 or 48 h, organic acids, such as lactate and acetate, were accumulated in W3110 and WJW00 cells, and more were accumulated in W3110 than in WJW00 (Figure 6B). This explains why pH values decreased with time in either W3110 or WJW00, and why at the same time points out that the pH value was higher in WJW00 than in W3110. In addition, the concentration of GABA, the synthesis of which requires consumption of H⁺, was significantly higher in

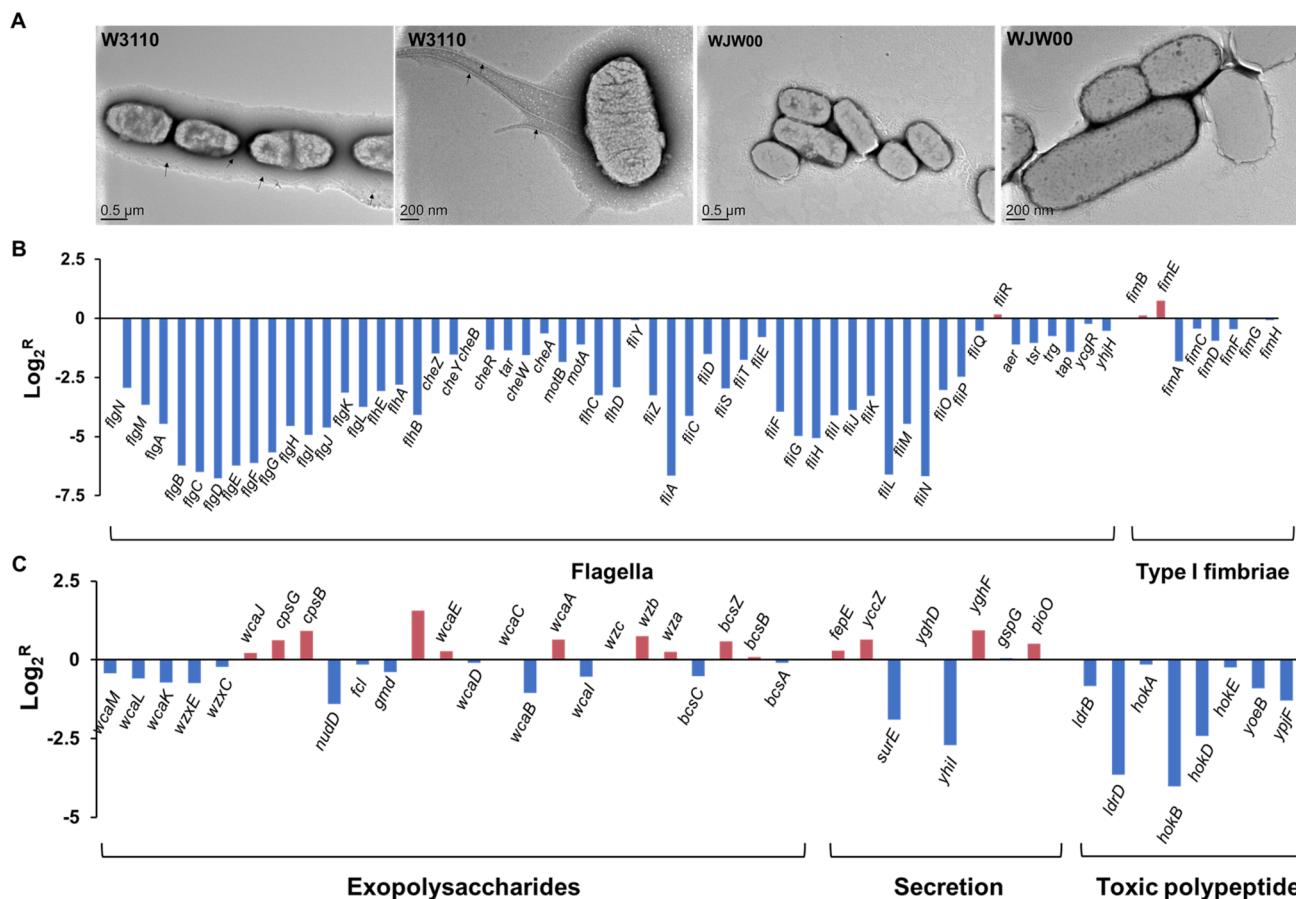


Figure 7. LPS truncation in *E. coli* led to the significantly decreased extracellular secretion and flagellar formation. (A) TEM image of W3110 and WJW00 cells. Flagella are pointed by black arrows. (B) Transcriptional analysis of genes relevant to flagella and type I fimbriae in WJW00. (C) Transcriptional analysis of genes relevant to secretions in WJW00.

WJW00 than in W3110 (Figure 6C). This might be another reason why the pH value was higher in WJW00 than in W3110 at the same time points during fermentation.

LPS-Truncation Activates the pH Regulation Systems. The above influences are complexed and maybe caused by the global regulations when the LPS structure was truncated in *E. coli*. Compared to W3110, WJW00 grew at the similar rate under pH 7.0, but much slower under pH 4.5. Transcriptional analysis further confirmed our above analysis, showing that LPS simplification significantly influences the intracellular metabolism and pH regulation systems in *E. coli*. We found that acid resistance genes were almost up-regulated in WJW00 compared to W3110 (Figure 6D), which resulted in the higher pH of WJW00 during fermentation. There are four acid resistance (AR) systems AR1 to AR4 in *E. coli*.³⁵ AR1 and AR2 are the main AR systems and could be regulated by sigma S (RpoS), which can activate HdeABD and GadX, further activating GadE and GadABD proteins to regulate intracellular pH. Compared to W3110, the key global regulator *rpoS* was up-regulated by 1.6-fold and 2.0-fold in WJW00 at the log phase and stationary phase, respectively. As expected, the genes in AR1 and AR2 were all up-regulated, and most genes in AR3 and AR4 were also up-regulated in WJW00 (Figure 6D). Notably, the significant up-regulations of the positive regulators GadE, GadW, and GadX also benefit the up-regulations of GadA, GadB, and GadC, which promote the GABA biosynthesis from glutamate and transport to the extracellular environment. In addition to the ARs, other

regulators and genes relevant to acid resistance in WJW00 were also determined as up-regulation trends. Most positive regulators such as *rpoD*, *rpoS*, *evgD*, *evgS*, *ydeO*, and *dps* were up-regulated and most negative regulators such as *crp*, *tors*, and *torR* were down-regulated (Figure 6D). These regulations caused by LPS simplification led to higher pH, less organic acids, and more GABA. All the regulations are helpful to increase PHB accumulation.

The regulations for acid resistance was related to σ^E stress-response system.³⁶ As we know, in *E. coli*, outer-membrane integrity is monitored by the σ^E stress-response system, which initiates damage-repair pathways including acid resistance systems.³⁶ Kdo₂-lipid A could activate σ^E to the highest levels.³⁷ Therefore, LPS truncation activates some global regulators and pH regulation systems, resulting in the decrease of acetate and lactate, and the significant increase of GABA formation. All these results benefit the C/N ratio increasing, and a higher C/N ratio benefits PHB production.³⁸

LPS Truncation in *E. coli* Influences Secretions, Flagella, and Fimbria Formation. WJW00 and W3110 cells were analyzed by TEM (Figure 7A). A large amount of extracellular secretion and several flagella were observed around W3110 cells (Figure 7A), but not around WJW00 cells (Figure 7A). This indicates that LPS truncation in *E. coli* could lead to a decrease of the extracellular secretion and flagellar formation. Further transcriptomic analysis of WJW00 and W3110 cells supported the above result and showed that many genes relevant to flagellar formation and type I fimbriae were

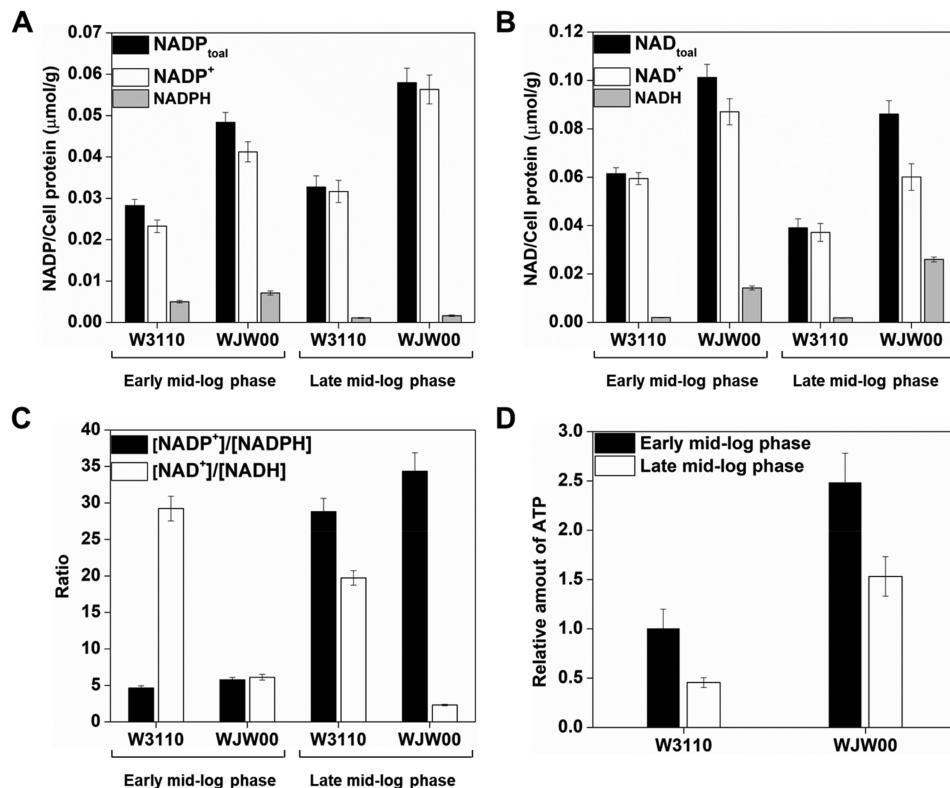


Figure 8. LPS truncation leads to increased levels of NADP, NAD, and ATP. (A) Determination NADP_{total}, NADP⁺ and NADPH levels in WJW00 cells and W3110 cells at early and late midlog phase. (B) Determination NAD_{total}, NAD⁺, and NADH levels in WJW00 cells and W3110 cells at early and late midlog phase. (C) Ratio of [NADP⁺]/[NADPH] and [NAD⁺]/[NADH]. (D) Determination of ATP levels of WJW00 cells and W3110 cells at early and late midlog phase.

significantly down-regulated in WJW00, compared with W3110 (Figure 7B). While much less influences on secretions were determined, transcriptional analysis showed that most genes relevant to the secretions and toxic polypeptide were down-regulated, which may be closely related to the secretions detected around W3110 cells, while no obvious influence on the genes relevant to exopolysaccharides biosynthesis was seen.

E. coli K-12 exhibits huge flagella and type I fimbriae on the cell envelope,³⁹ which consumes much of the resources and energy sources. Especially, flagellar synthesis imposes a cost of approximately 2% of the biosynthetic energy expenditure of the cell in *E. coli*.^{40,41} Our previous study also showed that deletion of 76 genes involved in flagella and pili in *P. putida* significantly enhanced PHA production.⁴² The reduction of LPS polysaccharides together with the lack of flagella could also be helpful to save more resources and energy; both benefit the enhancement of PHB production.^{3,14}

LPS Truncation in *E. coli* Increases the Levels of Cofactors NADP, NAD, and ATP. The significant decrease of flagella caused by LPS truncation benefits the save of sources and energy. Therefore, we analyzed the intracellular NADPH, NADP⁺, NADH, NAD⁺, and ATP levels in *E. coli* WJW00 and W3110 cells both grown to early midlog phase (OD₆₀₀ = 1.0) and late midlog phase (OD₆₀₀ = 2.5). We found that WJW00 showed a significantly enhanced pool of NADP, NAD, and ATP (Figure 8). At early midlog phase, WJW00 biosynthesized 0.0484 μmol NADP_{total}/g protein, 0.0412 μmol NADP⁺/g protein, and 0.007 μmol NADPH/g protein, increasing 71.2%, 77.3%, and 42.4% than that of W3110 cells (Figure 8A); at late midlog phase, *E. coli* cells showed a slight increase of NADP_{total} and NADP⁺, but obvious decrease of NADPH; despite this,

WJW00 cells still showed a similarly increased NADP_{total} (77.0%), NADP⁺ (77.9%), and NADPH (49.2%) than W3110, respectively. The influence on the levels of NADP resulted in the slightly increased ratio of NADP⁺/NADPH in WJW00 than W3110 (Figure 8C), increasing 24.5% and 19.2% at early midlog phase and late midlog phase, respectively. Even though the ratio slightly increased, the much higher [NADPH] supply in WJW00 cells is more conducive to PHB synthesis.

In addition to NADP, NAD is also an important cofactor and could be synthesized more than NADP in *E. coli* cells. At early midlog phase, WJW00 cells showed 0.101 μmol NAD_{total}/g protein, 0.087 μmol NAD⁺/g protein, and 0.014 μmol NADH/g protein, increasing 64.8%, 46.5%, and 6-fold than that of W3110, respectively (Figure 8B); at the late midlog phase, *E. coli* cells showed decreased levels of NAD, but WJW00 cells showed greater increase of NAD_{total} (1.2-fold), NAD⁺ (61.6%), and NADH (12.8-fold) than W3110. The significant increase of NADH levels resulted in sharply decreased ratio of [NAD⁺]/[NADH] in WJW00 cells, showing 79.1% and 88.3% less than W3110, at early midlog phase and late midlog phase, respectively (Figure 8C). This suggested that WJW00 could produce 3.8-fold and 7.5-fold more [NADH]/[NAD⁺] than W3110. The much higher [NADH] supply provides more sufficient reductants and facilitates PHB biosynthesis.

Moreover, WJW00 cells showed significantly increased ATP levels, and increased 1.7-fold and 2.35-fold at early midlog phase and late midlog phase, respectively, comparing with W3110 (Figure 8D). The results suggested that LPS truncation leads to enhanced pools and supply of cofactors and ATP, which may be due to the preservation of sources and energy in

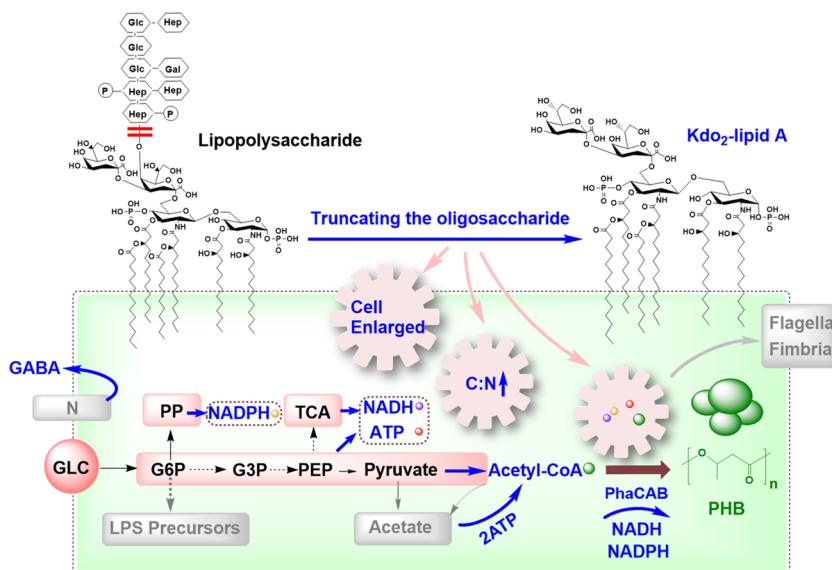


Figure 9. LPS truncation makes a better intracellular environment for PHB accumulation. GLC, glucose; N, nitrogen source; GABA, γ -aminobutyric acid; G6P, glucose-6-phosphate; G3P, 3-phosphoglycerate; PEP, phosphoenol pyruvate; PP, pentose phosphate pathway; TCA, tricarboxylic acid cycle. Down-regulated pathways and metabolites or structures were shown in gray arrows and gray boxes.

Table 1. Fermentation Comparison of the LPS Truncated Strains, Using the Wild-Type Strains as the Control. Data Were Collected after 24 h Fermentation

strains	DCW (g/L)	PHB (wt %)	PHB (g/g glucose)	biomass (g/g glucose)	pH
W3110/pDXW-8-phaCAB	1.9 \pm 0.1	22.4 \pm 2.7	0.06 \pm 0.01	0.21 \pm 0.01	4.72 \pm 0.02
WJW00/pDXW-8-phaCAB	3.3 \pm 0.1	68.7 \pm 2.4	0.22 \pm 0.01	0.10 \pm 0.01	5.03 \pm 0.01
DH5 α /pDXW-8-phaCAB	2.3 \pm 0.1	39.4 \pm 1.3	0.14 \pm 0.01	0.22 \pm 0.01	4.89 \pm 0.01
WJD00/pDXW-8-phaCAB	4.0 \pm 0.1	78.6 \pm 1.5	0.37 \pm 0.01	0.10 \pm 0.01	5.38 \pm 0.02
JM109/pDXW-8-phaCAB	2.4 \pm 0.1	46.2 \pm 1.4	0.15 \pm 0.01	0.27 \pm 0.01	4.91 \pm 0.01
WJJ00/pDXW-8-phaCAB	4.9 \pm 0.2	84.3 \pm 4.1	0.36 \pm 0.02	0.07 \pm 0.01	5.05 \pm 0.02

view of no need to biosynthesize the LPS oligosaccharide structure, flagella, and fimbria. As we know, a more sufficient NADPH, NADH, and ATP supply benefits the production of PHB.^{43,44} *E. coli* cells can produce NADPH by the PP pathway, and NADH and ATP by the EMP pathway and TCA cycle, then the PHB biosynthesis needs NADH or NADPH, and the precursor acetyl-CoA formation from acetate consumes 2ATP (Figure 9). Therefore, enhancement of cofactors and ATP supply by LPS truncation is very beneficial to PHB accumulation. The results also indicated that additional acetate could be more easily redirected to acetyl-CoA in WJW00 cells as compared with W3110 cells. This was highly consistent with the increase of acetyl-CoA and decrease of acetate detected in WJW00 cells. Moreover, the significant reduction of flagella and fimbria by LPS truncation could save more sources and energy, and the EMP pathway was up-regulated, so both benefit the significant increase of NADPH, NADH, and ATP levels all benefit PHB production.^{15,43,44} The results further proved that LPS truncation could rebalance the intracellular metabolism and provide much better cellular environment and metabolism conditions to produce PHB.

As compared to W3110, the genes *osmY*, *osmE*, *osmB*, and *osmC* involved in the osmotic resistance in WJW00 were up-regulated with 2^{4.3}-, 2^{3.6}-, 2^{2.5}-, and 2^{1.9}-fold, respectively. WJW00 cells were more sensitive than W3110 cells to antibiotics streptomycin, neomycin, gentamycin, kanamycin, tetracycline, clindamycin, erythromycin, and clarithromycin.

Moreover, our previous study showed that LPS simplification dramatically increased outer membrane permeability and autoaggregation ratio, but decreased the biofilm formation.²⁷ Higher cell permeability may facilitate the efficient uptake of carbon sources, and stronger autoaggregation ability benefits centrifugal collection of cells.^{17,45} Biofilm can impinge significantly upon our industrialized world.^{46,47} This is a problem for industrial fermentation due to the possibility of contamination,⁴⁸ insecurity,^{49,50} and biofouling.^{51,52} These phenotypes caused by LPS truncation would benefit the PHB production industry. The results strongly suggest that engineering LPS not only affects the cell envelope, but also affects intracellular metabolism and significantly enhances the PHB production. Thus, LPS simplification might have the potential to be an effective strategy to improve productivity in Gram-negative bacteria.

CONCLUSIONS

The major fermentation characteristics of the wild type and LPS truncated *E. coli* strains are listed in Table 1. The results showed that LPS truncation significantly enhanced the PHB biosynthesis with higher DCW, PHB%, and PHB yield on glucose. However, LPS truncated *E. coli* showed lower residual biomass yield on glucose. Thus, further study to improve the biomass or growth should be considered. PHB biosynthesis could not be separated from the adequate pH regulation response caused by the LPS truncation. The results suggest

that the mechanism of PHB production caused by LPS truncation is comprehensive and complex.

In conclusion, simplifying LPS to the Kdo₂-lipid A structure is an effective strategy to enhance PHB production in *E. coli* (Figure 9). Compared to W3110/pDXW-8-phaCAB, DH5α/pDXW-8-phaCAB, and JM109/pDXW-8-phaCAB cells, the cell sizes of WJW00/pDXW-8-phaCAB, WJD00/pDXW-8-phaCAB, and WJJ00/pDXW-8-phaCAB cells were much larger, and poly-3-hydroxybutyrate contents and conversion efficiencies were improved approximately 100% and 200%, respectively. The mechanism was concluded to be two factors: (i) The decreased membrane stiffness by truncating LPS polysaccharide³¹ and (ii) better balanced intracellular metabolism with increased C/N ratio, and more sources and energy supply for PHB accumulation by LPS truncation.

MATERIALS AND METHODS

Bacterial Growth Condition. Bacteria and plasmids used in this study are listed in Table 2.

Table 2. Strains and Plasmids Used in This Study

strains or plasmids	description	source
Strains		
W3110	Wild-type <i>E. coli</i> , F ⁻ , λ ⁻	NEB
DH5α	<i>E. coli</i> , deoR, recA1, endA1, hsdR17 (rK ⁻ , mK ⁺), phoA, supE44, λ ⁻ , thi ⁻¹ , gyrA96, relA1, F ⁻ , φ 80d lacZ ΔM15, Δ(lacZYA -argF)U169,	NEB
JM109	<i>E. coli</i> , endA1, recA1, gyrA96, thi-1, hsdR17 (rK ⁻ , mK ⁺), relA1, supE44, Δ(lac-proAB)/F ^[traD36 proAB lacIq lacZΔM15]	NEB
WJW00	W3110 mutant with a deletion of <i>rfaD</i> gene	27
WJD00	DH5α mutant with a deletion of <i>rfaD</i> gene	this study
WJJ00	JM109 mutant with a deletion of <i>rfaD</i> gene	this study
W3110/pDXW-8-phaCAB	W3110 harboring pDXW-8-phaCAB	this study
WJW00/pDXW-8-phaCAB	WJW00 harboring pDXW-8-phaCAB	this study
DH5α/pDXW-8-phaCAB	DH5α harboring pDXW-8-phaCAB	this study
WJD00/pDXW-8-phaCAB	WJD00 harboring pDXW-8-phaCAB	this study
JM109/pDXW-8-phaCAB	JM109 harboring pDXW-8-phaCAB	this study
WJJ00/pDXW-8-phaCAB	WJJ00 harboring pDXW-8-phaCAB	this study
Plasmids		
pBS- <i>rfaD</i> -FRTkan	plasmid for deleting <i>rfaD</i> in <i>E. coli</i> red cloning vector	27
pKD46	λ _R Rep, Cam ^R , Amp ^R	53
pCP20	FLP, λ _{C1857} , λ _P Rep, Cam ^R , Amp ^R	53
pDXW-8-phaCAB	pDXW-8-phaCAB genes from <i>Ralstonia eutropha</i> , Kan ^R	28

E. coli cells were usually grown at 37 °C in the Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.2), but the strains containing plasmids pKD46 and pCP20 were grown at 30 °C. For fermentation, the seed cultures were incubated in 25 mL of LB medium in a 100 mL flask at 37 °C with 200 rpm, and then incubated in 100 mL of minimal medium (M9, pH 7.0) (17.1 g/L Na₂HPO₄·12H₂O, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, 10 mg/mL VB₁) supplemented with 20 g/L glucose in a 500 mL flask at 37 °C with 200 rpm. Media were supplemented with

30 mg/mL kanamycin, 100 mg/mL ampicillin, 30 mg/mL chloramphenicol, or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when necessary.

Construction of *E. coli* Mutants. The WJD00 and WJJ00 were constructed by sites recombination according to previous study.^{27,53} Briefly, the *rfaD*:FRTkan mutant fragment was amplified by PCR with primers *rfaD*-U-F (5'-TCCGT-TACACCTTCAGCA-3') and *rfaD*-D-R (5'-GCTTTA-TCGGCAGCAACA-3'), then moved into strain DH5α/pKD46 and JM109/pKD46 by transduction. Then the Flp was expressed by pCP20 to delete the FRTkan, and the pCP20 was eliminated by culture at 42 °C to form mutants WJD00 and WJJ00 which contain no plasmids and antibiotic resistance genes. Plasmid pDXW-8-phaCAB was transferred into *E. coli* cells to construct recombinants to produce PHB. DNA purification and plasmid isolation kits were purchased from Qiagen (Shanghai, China). DNA manipulation enzymes and restriction enzymes were provided by New England BioLabs (USA).

Extraction and Exhibition for LPS. LPS were isolated from *E. coli* strains W3110, WJW00, DH5α, WJD00, JM109, and WJJ00, using the hot phenol–water extraction method,²⁷ separated on 15% SDS–polyacrylamide gels, and visualized after silver staining.⁵⁴

Antibiotic Disk Assay. *E. coli* strains were grown to the midlog phase with an OD₆₀₀ of 1.0. A lawn of cells (200 μL culture) was spread onto a LB broth agar plate. Different sterile antibiotic drug sensitive papers (6 mm in diameter) were placed on the top of the lawn. Plates were incubated overnight (18 h) at 37 °C, and zone diameters around disks were measured to assess the different antibiotics resistance. Different sterile antibiotic drug sensitive papers were purchased from Hangzhou Binhe Microbial Reagent Co., Ltd.

Acid Osmotic Shock Assay. Overnight cultures were transferred to 50 mL of LB medium with the initial OD₆₀₀ = 0.025, under pH 7.0 and 4.5, adjusted with hydrochloric acid (HCl), incubated at 30 °C, 200 rpm. The values of OD₆₀₀ were measured every 2 h to obtain the growth curve.

Fluorescence Microscopy Analysis of PHB Granules. *E. coli* cells were cultured in liquid minimal medium supplemented with 20 g/L of glucose for PHB fermentation. After 24 h, 1 mL of culture was harvested by centrifugation at 10 000g for 1 min and washed with phosphate-buffered saline (PBS), and resuspended in PBS, then mixed with 1 μmol Nile Red dissolved in 1 g/L dimethyl sulfoxide (DMSO), and incubated in the dark at 37 °C for 30 min. The stained cells were washed three times with deionized water (ddH₂O) to remove any residual fluorescent dye, and then resuspended in PBS. A 10 μL sample was put on the slides with a cover glass and visualized with the use of a fluorescence microscope (Leica TCS SP8, Leica, Germany). Cell excitation was accomplished by using a 488 nm argon laser. Photographs were captured with Leica TCS SP8 software (CellSens Standard 1.9).

TEM Analysis and Ultrathin Section TEM Analysis. To analyze the impact on cell morphology, *E. coli* cells were grown on solid LB medium for 10 h and used for TEM analysis.⁵⁵ The cells were negative-stained with 2.5% valeric acid phosphate. The specimens were imaged with a JEOL JEM 2100 instrument (JEOL Ltd., Tokyo, Japan).

For PHB synthesis of *E. coli* cells, after 24 h fermentation in liquid minimal medium supplemented with 20 g/L of glucose, *E. coli* cells were harvested by centrifugation at 10 000g for 1 min, washed three times with PBS, fixed with 2.5%

glutaraldehyde (pH 7.2), and prepared for ultrathin section TEM analysis.²⁸ The specimens were imaged with a JEOL JEM 2100 instrument (JEOL Ltd., Tokyo, Japan).

Determination of Intracellular PHB. The determination of intracellular PHB was accorded to the method in our previous study.²⁸ For PHB quantification, a GC-2010 plus system (Shimadzu, Japan) employed with a DBWAX column (30 m × 0.32 mm) (Agilent Technologies, Waldbronn, Germany) with a flame ionization detector was used, and the injection temperature was 250 °C. *E. coli* cells were fermented in minimal medium added with 20 g/L glucose for 48 h. The cells were harvested by centrifugation at 10 000 rpm for 2 min, washed with pH 7.2 PBS, and then lyophilized for 48 h. The dry cell weight was calculated, and about 20 mg of lyophilized cells were weighed into esterification tubes to determine the intracellular PHB. Then, 2 mL of methanol containing 3% H₂SO₄ and 2 mL of chloroform were added into the esterification tubes, and then treated in boiled water for 6 h. After the mixture was cooled, 1 mL of ddH₂O was added, and the mixture was rotary vibrated for 5 min. When the mixture completely phased, 600 μL of organic phase was collected and filtrated with 0.22 μm filters (Sartorius, Germany). The samples were immediately subjected to gas chromatography (GC) analysis for PHB quantification.⁵⁶

Extraction and Determination of Central Intermediate Acetyl-CoA. The level of intracellular acetyl-CoA was determined according the published method.²⁸ Briefly, *E. coli* cells were grown in 25 mL of LB medium in a 100 mL flask for 6 h to an OD₆₀₀ of 1.5, then 1 mL of culture was transferred to a prechilled Eppendorf tube and centrifuged at 10 000 rpm and 0 °C for 30 s. The cellular debris was quickly washed twice with cold 0.9% NaCl solution, and resuspended in 1 mL of a mixture of methanol, acetonitrile, and ddH₂O (45:45:10, v/v/v) at -20 °C,⁵⁷ and the mixture also contained 0.1 M formic acid for quenching.⁵⁸ The resuspension was ultrasonicated at 0 °C for 5 min (60 cycles of 2 s running and 3 s interval), and then was centrifuged at 10 000 rpm and 0 °C for 30 s. The supernatant was filtrated through the 0.22-μm filter and directly injected into a TSQ Quantum Ultra instrument (Thermo Scientific, San Jose, CA, USA). The commercially available standard acetyl-CoA and internal standard propionyl-CoA were purchased from Sigma-Aldrich (Saint Louis, Missouri). The acetyl-CoA standard with different concentrations of 5, 10, 50, 100, 200 ppm was measured to obtain a standard curve, and 100 ppm of propionyl-CoA was supplemented to standards and samples for the standard curve calibration. Samples were eluted at a flow rate of 0.4 mL/min, and acetyl-CoA was detected and quantified in multiple reaction monitoring mode according to the calibration curve. The data were analyzed with Xcalibur software.

Determination of Glucose, Organic Acids, and Amino Acids. *E. coli* cells were fermented in minimal medium with 20 g/L glucose added. During the fermentation, the supernatants were collected by centrifugation at 10 000g for 1 min for extracellular glucose, organic acids, and amino acids determination.²⁸ For glucose determination, SBA-40C immobilized enzyme biosensor (Shandong Province Academy of Sciences, China) was employed. Supernatants were diluted 10 times with distilled water, and 25 μL was injected.

For organic acids determination, the collected supernatant was filtrated through the 0.22-μm filter and subjected to reverse phase high-pressure liquid chromatography (HPLC) analysis. An Agilent 1260 HPLC equipped with Diamonsil C18

column (5 μm, 250 mm × 4.6 mm No. 99603) (DiKMA technology, Beijing, China) was employed for extracellular organic acids quantification. A linear gradient elution procedure was used with methanol/H₂O/phosphate from 5:95:0.05 to 60:40:0.05 in 20 min. Samples were detected with a flow rate of 0.9 mL/min under an ultraviolet detector at an emission wavelength of 210 nm. The temperatures of injector, detector, and column were maintained at 120 °C, 120 °C, and 80 °C, respectively.

For amino acids determination, the collected supernatant was also filtrated through the 0.22-μm filter and subjected to HPLC analysis. An Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with Thermo Hypersil ODS-2 column (5 μm particle, 250 mm × 4.6 mm) (Cheshire, UK) and diode array detection system was used. The supernatants were subsequently derivatized with *o*-phthalaldehyde reagent solution (Agilent Technologies, Waldbronn, Germany) and then detected at 338 nm with a flow rate of 1 mL/min.

Transcriptome Analysis and qRT-PCR Analysis. The total RNA from *E. coli* cells was isolated using the Qiagen RNeasy Total RNA kit. Reverse transcription was carried out using random hexamer-primers and Oligo dT with the RevertAid First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer instruction. RT-PCR was performed with SYBR Premix Ex TaqII (Takara, Japan) following the protocol of the ABI Step One real-time PCR system (Applied Biosystem, Foster City, CA). All the measurements were performed in triplicate. The primers RT-*rpoS*-F (5'-TCCGCACTTGGTTCATGGT-3') and RT-*rpoS*-R (5'-CTCAACATACGCAACCTGG-3') were used for RT-PCR, with the internal reference gene *rrnG* primers RT-*rrnG*-F (5'-CAAGGGCACAAACCTCCAAGT-3') and RT-*rrnG*-R (5'-TGTAGCGGTGAAATGCGTAG-3').

The whole genome transcriptome profile of *E. coli* strains W3110 and WJW00 were established according to a published method.⁵⁵ Overnight cultures of *E. coli* cells were diluted 100 times into 100 mL of LB medium with a 500 mL flask and grown at 37 °C to the early exponential phase (OD₆₀₀ around 1.3). Then cells were harvested by centrifugation at 4000 rpm and 4 °C for 10 min, washed twice with PBS pH 7.2, and then resuspended in 2 mL of PBS (0 °C), with 5 mL of RNA safer stabilizer reagent from Wegene (Shanghai, China) added. The samples were delivered to Huada (BGI Shenzhen, China) with dry ice. Illumina TruSeq Ribo-Zero Stranded Total RNA Sample Preparation kit was used for cDNA library preparation, and biotinylated oligos combined with Ribo-Zero rRNA removal beads were used to remove ribosome RNA.²⁸ The libraries were sequenced using an Illumina HiSeq 2000 (BGI Shenzhen, China). The complete genome of *E. coli* W3110 was used as the reference genome for RNA-sequence reads sequence alignment. The expression level of each gene was calculated using the reads per kilobases per million reads, the *P*-value corresponding to the differential gene expression test. The calculated *P*-value goes through a Bonferroni Correction,⁵⁹ and the corrected *P*-value ≤ 0.05 is taken as a threshold.

Determination of NADP, NAD, and ATP. *E. coli* cells were fermented in minimal medium with 20 g/L glucose added. The cells grown to the early midlog phase (OD₆₀₀ = 1.0) and late midlog phase (OD₆₀₀ = 2.5) were both collected for the determination of NADP, NAD, and ATP levels, according to the NADP⁺/NADPH Assay Kit with WST-8 (S0179, Beyotime), NAD⁺/NADH Assay Kit with WST-8

(S0175, Beyotime), and Enhanced ATP Assay Kit (S0027, Beyotime), respectively. First, three replicates of 1 mL of culture were prepared for every determination of NADP, NAD, or ATP. Cultures were collected at 10000g for 1 min at 4 °C. The supernatants were immediately thoroughly removed to guarantee the determination accuracy and then immediately frozen with liquid nitrogen to quench the metabolism of the cells. The prepared samples were used for the determinations. Meanwhile, the standards with different conditions (0 to 10 μ M) were also prepared. All samples and standards determinations required consistent timeliness with the use of a townhouse pipet. For NADP and NAD determination, the reactions were performed with a transparent 96-well plate, and the absorbance values at λ 450 nm were read after 30 min color rendering with Microplate reader. For ATP, the reactions were performed with a 96-well opaque plate in addition to the bottoms, and the absorbance values were all read after a 2-s waiting period and a 10-s detection time in luminescence mode with a Microplate reader. Meanwhile, the concentration of the protein cells of the extraction mixture was also determined.

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Author Contributions

J.W. and X.W. conceived and designed the experiments. J.W. and W.M. conducted all the experiments. Y.F. and H.L. participated in the determination of intracellular organic acids and cofactors levels. J.W., W.M., Y.F., H.Z., Y.L., and X.W. analyzed the results. J.W., W.M. and X.W. drafted the manuscript.

Notes

The authors declare no competing financial interest.

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