Cofactor Engineering Redirects Secondary Metabolism and Enhances Erythromycin Production in Saccharopolyspora erythraea

Li, Xiaobo; Chen, Jun; Andersen, Joakim Mark; Chu, Ju; Jensen, Peter Ruhdal

Published in: ACS Synthetic Biology

Link to article, DOI: 10.1021/acssynbio.9b00528

Publication date: 2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Cofactor Engineering Redirects Secondary Metabolism and Enhances Erythromycin Production in *Saccharopolyspora erythraea*

Xiaobo Li, Jun Chen, Joakim M. Andersen, Ju Chu,* and Peter R. Jensen*

Cite This: ACS Synth. Biol. 2020, 9, 655−670

**ABSTRACT:** *Saccharopolyspora erythraea* is used for industrial erythromycin production. To explore the physiological role of intracellular energy state in metabolic regulation by *S. erythraea*, we initially overexpressed the F1 part of the endogenous F1F0-ATPase in the high yielding erythromycin producing strain E3. The F1-ATPase expression resulted in lower [ATP]/[ADP] ratios, which was accompanied by a strong increase in the production of a reddish pigment and a decreased erythromycin production. Subsequent transcriptional analysis revealed that the lower intracellular [ATP]/[ADP] ratios exerted a pleotropic regulation on the metabolism of *S. erythraea*. The lower [ATP]/[ADP] ratios induced physiological changes to restore the energy balance, mainly via pathways that tend to produce ATP or regenerate NADH. The F1-ATPase overexpression strain exhibited a state of redox stress, which was correlated to an alteration of electron transport at the branch of the terminal oxidases, and *S. erythraea* channeled the enhanced glycolytic flux toward a reddish pigment in order to reduce NADH formation. The production of erythromycin was decreased, which is in accordance with the net ATP requirement and the excess NADH formed through this pathway. Partial growth inhibition by apramycin increased the intracellular [ATP]/[ADP] ratios and demonstrated a positive correlation between [ATP]/[ADP] ratios and erythromycin synthesis. Finally, overexpression of the entire F1F0-ATPase complex resulted in 28% enhanced erythromycin production and markedly reduced pigment synthesis in E3. The work illustrates a feasible strategy to optimize the distribution of fluxes in secondary metabolism.

**KEYWORDS:** ATPase, synthetic biology, redox regulation, energy metabolism, secondary metabolism, *Saccharopolyspora erythraea*

Actinomycetes are well-known as prolific producers of a variety of bioactive compounds. The increasing occurrence of antibiotic-resistant microbial pathogens has sparked growing attention toward the discovery of novel antibacterial compounds from actinomycetes. However, some novel antibacterial compounds are isolated from actinomycetes which are non-culturable under laboratory conditions. Fortunately, due to the evolutionary homology, the biosynthesis of both traditional and novel antibacterial compounds by actinomycetes relies on accumulation of similar precursors, such as propionyl-CoA and malonyl-CoA. Heterologous expression of natural product biosynthetic gene clusters in well-studied actinomycetes provides access to full biosynthesis of new natural products. Therefore, studies of the biosynthesis of antibiotics in paradigm model organisms, e.g., erythromycin in *Saccharopolyspora erythraea* (*S. erythraea*), are important for elucidating the regulation mechanism involved in the biosynthesis of existing antibiotics and for future metabolic engineering of the heterologous production of new natural compounds.

Erythromycin A produced by *S. erythraea* is one representative of the macrolides antibiotics, and its derivatives also play vital pharmaceutical roles. The genome of *S. erythraea* contains at least 25 gene clusters for biosynthesis of known or predicted secondary metabolites, among which erythromycin and a
reddish pigment represent the major ones. In industry, the reddish pigment derived from malonyl-CoA is considered as a byproduct which may increase the cost of erythromycin purification. Some of the previous metabolic engineering attempts did lead to either up- or down-regulation of the synthesis of several different secondary products but always in concert.3 With respect to S. erythraea, previous research has not yet achieved the goal to reduce pigment production and simultaneously enhance erythromycin production.7,27 In general, there is a lack of knowledge about the transcriptional regulation of the erythromycin biosynthesis gene cluster and new strategies are required to optimize the production of secondary products by S. erythraea.

Secondary metabolism is linked tightly to primary metabolism through the requirement for specific precursors and cofactors.11 Over the past decades, metabolic engineering of microbial metabolism to increase the supply of precursors through deletion or overexpression of genes encoding key enzymes has improved the production of some of these secondary metabolites. For instance, deletion of mutB (encoding the \(\beta\) subunit of methylmalonyl-CoA mutase) in S. erythraea and deletion of pfkA2 (encoding phosphofructokinase) or over-expression of the acetyl-CoA carboxylase in Streptomyces coelicolor significantly increased the synthesis of erythromycin or actinorhodin, respectively.11,30 However, few studies to date have focused on the effects of cofactors, i.e., ATP and NADH, on the secondary metabolism, despite the fact that in S. erythraea more than 200 reactions were associated with ATP metabolism according to genome-scale metabolic models.14,15

In S. erythraea, ATP is predominantly generated via membrane-localized F1F0-ATPase at the expense of a proton-motive force due to the electron transfer to oxygen, and sufficient oxygen uptake is known to be important for erythromycin synthesis.17 Some studies have provided clues as to the important role of the cofactors in antibiotic biosynthesis. For instance, a comparative transcriptomic study of the wild-type strain and a high-erythromycin-producing strain isolated after random mutagenesis also showed that genes encoding F1F0-ATPase are among the wide spectrum of differentially expressed genes, and a highly oxidative metabolism was also observed in other antibiotic high-producing strains of Streptomyces.19 Another previous study showed that the addition of extracellular ATP to the culture medium had ambiguous effects on antibiotics production of S. coelicolor.20 However, the underlying mechanisms by which S. erythraea responds to changes in the cellular energy state are still not well understood.

The intracellular ATP level is well-known to play a regulatory role in controlling metabolic fluxes. For example, the intracellular ATP demand controls the glycolytic flux in E. coli and under certain conditions in S. cerevisiae and the success in enhancing glycolytic flux by increasing cellular ATP demand provided an alternative strategy to manipulate the flux through primary metabolism. When it comes to biotechnological applications, manipulation of intracellular ATP levels also affects the titer, productivity, yield, or even the spectrum of byproducts.23–26 These observations have prompted us to explore the physiological role of energy metabolism in secondary metabolism, with a view to find new ways to boost erythromycin production.

Figure 1. Overexpression of atpAGD caused lower [ATP]/[ADP] ratios. (a) Quantitative RT-PCR (qRT-PCR) analysis of the transcription profiles of F1F0-ATPase genes during the early exponential phase in minimal liquid medium. Relative transcript levels were obtained after normalization to the internal reference gene (16S rRNA). The relative expression values of each gene in parental strain E3 were set to 1.0 (arbitrary units). (b) Intracellular [ATP]/[ADP] ratio in two strains, E3 and E3::F1ATPase. Error bars show the standard deviation from three independent experiments. *, P value < 0.05.

The native F1F0-ATPase complex acts as an ATP synthase responsible for ATP production and consists of two main parts, the F0 and F1. The transmembrane F0 part serves as a proton channel through which H+ translocates, and the cytosolic F1 part catalyzes the synthesis of ATP from ADP and Pi. When the soluble F1 part is free from the membrane, it hydrolyzes ATP to ADP and it is therefore possible to alter the intracellular [ATP]/[ADP] ratio by changing the expression of the F1 part of the F1F0-ATPase.28,29 In fact, by tuning the expression of F1-ATPase, researchers found that the glycolysis could be uncoupled from biomass production without primary effects on other cellular processes.30 However, the earlier studies have focused solely on the effects on primary metabolism in E. coli and L. lactis, and less attention has been paid to organisms with extensive secondary metabolic routes. In this study, in order to investigate the role of intracellular ATP alteration on metabolism of S. erythraea, we initially...
decreased intracellular [ATP]/[ADP] ratios by overexpressing the soluble F1-ATPase. After characterization of the cellular response to the alteration of the intracellular energy state at multiple levels, we propose a model which may explain the metabolic shifts in secondary metabolism and verified the model by increasing the [ATP]/[ADP] ratio via inhibition with a sublethal concentration of an antibiotic. Finally, we showed that overexpressing the entire F1F0-ATPase complex resulted in increased intracellular [ATP]/[ADP] ratio and demonstrated a 28% enhancement of erythromycin production, as well as a marked decrease of pigment production, simultaneously.

## RESULTS AND DISCUSSION

**Overexpression of F1-ATPase Decreased the Intracellular [ATP]/[ADP] Ratios in S. erythraea.** In this study, we used a high-erythromycin-producing strain, E3,18 as the parental strain to explore the role of energy metabolism. E3 produces erythromycin at a comparable level with the one currently used in industry.13 A mutant strain, E3::F1ATPase, was constructed by overexpressing α, β, and γ (atpAGD, SACE_6282–6280) subunits of the native F1F0-ATPase in S. erythraea E3. Transcription of the additional copy of F1-ATPase was promoted by the constitutive promoter ermE’p.8 The transcription level of atpAGD was 1.9-fold in E3::F1ATPase compared to E3. The transcription of atpEFH was slightly higher, and that of atpb/atpc remained at the same level in E3 (Figure 1a). We then measured the intracellular concentrations of ATP and ADP over a time course in batch culture and calculated the [ATP]/[ADP] ratio. In general, [ATP]/[ADP] ratios in E3::F1ATPase were constantly lower than those in E3. In the early exponential phase (10 h), the ratio for E3::F1ATPase was one-third of that in E3 (Figure 1b). The ratio in E3::F1ATPase increased steadily by 2.5 times from 1.8 to 4.9 as cells entered the stationary phase (48 h). In contrast, the ratios in E3 remained around 6 throughout the experiment. The results confirmed the ATP hydrolysis due to expression of soluble F1-ATPase in S. erythraea.

**Overexpression of F1-ATPase Enhanced the Glycolytic Flux and [NADH]/[NAD+] Ratios.** In order to determine physiological changes caused by intracellular ATP perturbation, batch cultures in shake flasks were compared for both E3 and E3::F1ATPase in the minimal medium (Table 1). The growth rate of E3::F1ATPase decreased substantially by 22%, and the biomass yield also decreased by 27%. However, the specific glucose consumption rate for E3::F1ATPase increased by 24%. This indicates that the glycolysis in S. erythraea has been uncoupled from the biomass production in E3::F1ATPase due to the ATP deprivation. The enhanced glycolytic flux was in agreement with the response observed in E. coli and S.cerevisiae21,22 and may be caused by allosteric activation of some key enzymes, i.e., phosphofructokinase and pyruvate kinase in the glycolytic pathway at the low intracellular ATP level.26 For some obligately aerobic bacteria, the specific oxygen uptake rate (rO2) rises.34 Compared to E3, however, the rO2 value of E3::F1ATPase decreased by 38 and 9% in the exponential and stationary phases, respectively. A decreased oxygen uptake rate was correlated with a repressed NADH oxidation at the electron transport chain (ETC) which would indicate either a higher total amount of NADH generated or the existence of other pathways rather than ETC for NADH reoxidation in E3::F1ATPase with respect to E3. The decreased respiration is likely to result in a lower membrane potential, which in turn would mean that the capability of the individual molecule of membrane-anchored F1F0-ATPase to transfer H+ across the plasma membrane is expected to decrease.35

The decreased respiration in E3::F1ATPase prompted us to explore the intracellular redox environment in both strains which is predominantly reflected in the intracellular [NADH]/[NAD+] ratio.36 The concentrations of intracellular NADH and NAD+ were measured, and [NADH]/[NAD+] ratios were calculated over a time course (Figure 2). The [NADH]/

### Table 1. Growth and Physiological Parameters of Parental Strain E3 and atpAGD Mutant E3::F1ATPase as Determined in Batch Culture

<table>
<thead>
<tr>
<th>strain</th>
<th>μmax (h⁻¹)</th>
<th>Yx/gl⁻¹ (g of DCW/g)</th>
<th>qF⁻ (g/g of DCW-h⁻¹)</th>
<th>rO2 ( % O2/(s·g DCW))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3</td>
<td>0.12 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>0.076 ± 0.013</td>
</tr>
<tr>
<td>E3::F1ATPase</td>
<td>0.09 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.046 ± 0.009</td>
</tr>
</tbody>
</table>

“Standard deviations were calculated from biological triplicates. *Maximum specific growth rate. **Biomass yield on glucose. "Specific glucose uptake rate in the exponential phase. Specific oxygen uptake rate in the exponential phase.

Figure 2. F1-ATPase (atpAGD) overexpression in E3::F1ATPase induced a redox stress that was reflected in the increasing [NADH]/[NAD+] ratio in the exponential phase. Intracellular [NADH]/[NAD+] ratio in E3 (white) and in E3::F1ATPase (black). Error bars show the standard deviation from three independent experiments. *, P value < 0.01.

The [NAD+] ratio in E3 was approximately 3 before the midexponential phase and decreased to 1 in the stationary phase. The ratios in E3 were consistently and significantly lower than those in E3::F1ATPase. The ratio in E3::F1ATPase increased slightly from 3.5 to 4.3 in the exponential phase and then decreased. Reoxidizing NADH into NAD+ is essential to achieve a redox balance, and although there may be a fast turnover of NADH or NAD+,37 the persistently higher ratio indicates a state of redox stress until 72 h in E3::F1ATPase. These results are in accordance with the lower oxygen uptake rate (rO2) rises.34 Compared to E3, however, the rO2 value of E3::F1ATPase decreased by 38 and 9% in the exponential and stationary phases, respectively. A decreased oxygen uptake rate was correlated with a repressed NADH oxidation at the electron transport chain (ETC) which would indicate either a higher total amount of NADH generated or the existence of other pathways rather than ETC for NADH reoxidation in E3::F1ATPase with respect to E3. The decreased respiration is likely to result in a lower membrane potential, which in turn would mean that the capability of the individual molecule of membrane-anchored F1F0-ATPase to transfer H+ across the plasma membrane is expected to decrease.35

The decreased respiration in E3::F1ATPase prompted us to explore the intracellular redox environment in both strains which is predominantly reflected in the intracellular [NADH]/[NAD+] ratio.36 The concentrations of intracellular NADH and NAD+ were measured, and [NADH]/[NAD+] ratios were calculated over a time course (Figure 2). The [NADH]/
Figure 3. Concentration values of extracellular metabolites for E3 (white) and E3::F1ATPase (black): (a) pyruvate (PYR), (b) $\alpha$-ketoglutarate ($\alpha$-KG), (c) fumarate, (d) malate. Estimated relative flux modes around metabolites before 35 h were shown below each chart for E3 (white fill) and E3::F1ATPase (black fill). Experiments were repeated in duplicate.

Figure 4. Quantification of secondary metabolites in E3 and E3::F1ATPase. (a) Erythromycin titer in broth of E3 (white) or E3::F1ATPase (black). Error bars show the standard deviation from three independent experiments. (b) Reddish pigment (7-O-rahamnosyl flavinol) production at the middle exponential phase (24 h) and at the end (96 h) of culture in minimal liquid medium. Relative quantification of reddish pigment was represented by comparison of broth absorbance at 270 nm, for which values are shown below the pictures. (c) Reddish pigment production and spore formation on XM agar medium. The lowercase letters with or without single quotes are on plates for identification of the same regions on top sides and bottom sides.
rate in E3::F1ATPase (Table 1), which indicates that the respiration rate in E3::F1ATPase was insufficient to reoxidize the additional NADH produced.

Overexpression of F1-ATPase Changed the Pattern of Byproducts. Cells were subsequently cultured in 1 L fermenters, and pH was monitored to observe overflow of intermediates from primary metabolism, which would acidify the broth.38 The pH of the broth of both strains remained almost stable around the initial value 6.7 during the first 12 h due to the pH buffer in the starting medium (Figure S1). At 12.5 h, the pH in the E3::F1ATPase broth began to decrease, while the onset of the pH decrease was delayed to 15 h for E3. The early onset of pH drop for E3::F1ATPase correlated with its higher glucose uptake rate. The final pH of E3::F1ATPase broth was around 3.8, whereas it was about 4.1 for E3, suggesting changes in the extracellular metabolite profiles for the two strains.

We determined the concentration of intermediate organic acid overflow to the medium by HPLC analysis (Figure 3). In general, E3::F1ATPase secreted less pyruvate and fumarate, whereas more α-ketoglutarate and malate were released. Typical overflow metabolites/byproducts, e.g., acetate or glycerol in E. coli or S. cerevisiae,27,39 were not detected in broth of these strains.

The concentration of an extracellular metabolite results from the balance between its production flux and its consumption flux in the cell (Figure 3). As E3::F1ATPase exhibited a higher glycolysis flux (Table 1), the lower pyruvate overflow in the E3::F1ATPase broth meant that the consumption of intracellular pyruvate must be greater than that of E3. The increased overflow of α-ketoglutarate and the decreased overflow of fumarate indicate that the E3::F1ATPase strain had a reduced flux from α-ketoglutarate to fumarate via the tricarboxylic acid (TCA) cycle, which in turn results in less accumulation of several intermediate metabolites, such as succinate and succinyl-CoA. The lower concentration of fumarate and the higher concentration of malate in E3::F1ATPase indicated a higher flux through the glyoxylate shunt. The patterns of extracellular metabolite profiles show intermediates of the TCA cycle and of the glyoxylate shunt, from which excess NADH equivalents would be generated.

ATP Perturbation Affected the Production of Erythromycin and a Reddish Pigment. We also observed the morphological phenotype and measured the erythromycin production for both strains (Figure 4). When grown on agar medium with glucose as a carbon source, E3::F1ATPase obtained a reddish appearance from day 3, which was 2 days earlier compared to E3. On day 7, the production of the reddish

Figure 5. Addition of complex I (NADH dehydrogenase) inhibitor rotenone into E3 culture caused a same shifting trend as atpAGD overexpression. (a) Simplified synthesis pathways of the reddish pigment (polymer of 7-O-rhamnosyl flavilin). (b) Simplified synthesis pathways of erythromycin. NADH availabilities of pigment or erythromycin synthesis based on 1 mol of glucose consumption were calculated and shown below the pathways. (c) Erythromycin (blank) and pigment (diagonal fill) production of E3 in culture with addition of different concentrations of rotenone. Error bars show the standard deviation from three independent experiments.
pigment by E3::F1ATPase was markedly boosted compared to E3. When cultivated in minimal liquid medium, E3::F1ATPase started to produce pigment as early as from 24 h in the middle exponential phase, whereas for E3 production of the pigment and erythromycin began at 48 h, i.e., close to the end of the exponential phase (Figure 4b). Moreover, compared to E3, E3::F1ATPase showed a 12 h lag in erythromycin production onset (Figure 4a). The final erythromycin titer of E3::F1ATPase also decreased by approximately 20%. These results showed that the intracellular ATP perturbation altered both the onset of secondary metabolite production and flux distribution within secondary metabolism.

In the context of industrial fermentation with *S. erythraea* for the purpose of erythromycin production, the reddish pigment is considered a byproduct. Erythromycin is assembled from propanoyl-CoA and methylmalonyl-CoA, both of which can be derived either from succinyl-CoA or valine/leucine iso-leucine degradation, whereas the synthesis of the reddish pigment starts from chain extension of malonyl-CoA, a process which is catalyzed by acetyl-CoA carboxylase. Evidence suggested that malonyl-CoA cannot be used for the biosynthesis of erythromycin in *S. erythraea*, probably due to the lack of enzymes to catalyze the conversion from malonyl-CoA to 3-hydroxy-propionyl-CoA. Therefore, it is of interest to decipher the inverse alterations of the pigment and erythromycin production. During the stationary phase, the intracellular [NADH]/[NAD+] ratios remained about 3 times higher in E3::F1ATPase than in E3 (Figure 2). In principle, only half of the amount of NADH is generated along with the reddish pigment biosynthesis relative to erythromycin production with consuming equimolar glucose (Figure 5). We therefore hypothesize that the alteration of [NADH]/[NAD+] ratios induced by ATP perturbation plays a regulatory role in flux distribution at the branch of acetyl-CoA, resulting in an altered pattern of secondary metabolites. To validate this, an inhibitor of complex I of the ETC, rotenone, was added into E3 culture at increasing times that of E3, which could explain the lower pyruvate overproduction in broth of E3::F1ATPase. In contrast, the activity of pyruvate dehydrogenase complex (PDH), a key enzyme complex in supplying acetyl-CoA, in cell extracts of E3::F1ATPase was 2.8 times that of E3, which could explain the lower pyruvate overflow in broth of E3::F1ATPase. In contrast, the activity of citrate synthase (CIT) in E3::F1ATPase, which catalyzes the reaction from acetyl-CoA to citrate, was 1.9 times that of E3. This means that increase of the TCA cycle activity was lower than that in the glycolysis pathway, which corresponded to the hypothesis that [NADH]/[NAD+] ratios correlated with ATP perturbation play a regulatory role in flux distribution. Table 2 shows that the TCA cycle cannot entirely accommodate the enhanced glycolytic flux. Therefore, *S. erythraea* had to redirect the enhanced glycolytic flux to other alternative routes besides the TCA cycle. The stimulated pigment production we observed in E3::F1ATPase indicates that the total activity of acetyl-CoA carboxylase (ACC) is likely to be up-regulated in E3::F1ATPase.

### Table 2. Enzyme Activities in E3 and E3::F1ATPase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyruvate dehydrogenase</td>
<td>74.76</td>
</tr>
<tr>
<td>E3::F1ATPase</td>
<td>212.11</td>
</tr>
<tr>
<td>E3</td>
<td>623.30</td>
</tr>
<tr>
<td>ratio</td>
<td>2.84</td>
</tr>
<tr>
<td>citrate synthase</td>
<td>1208.17</td>
</tr>
<tr>
<td>E3::F1ATPase/E3</td>
<td>1.94</td>
</tr>
</tbody>
</table>

*Data were calculated from duplicates.*

Dehydrogenase complex (PDH), a key enzyme complex in supplying acetyl-CoA, in cell extracts of E3::F1ATPase was 2.8 times that of E3, which could explain the lower pyruvate overflow in broth of E3::F1ATPase. In contrast, the activity of citrate synthase (CIT) in E3::F1ATPase, which catalyzes the reaction from acetyl-CoA to citrate, was 1.9 times that of E3. This means that increase of the TCA cycle activity was lower than that in the glycolysis pathway, which corresponded to the hypothesis that [NADH]/[NAD+] ratios correlated with ATP perturbation play a regulatory role in flux distribution. Table 2 shows that the TCA cycle cannot entirely accommodate the enhanced glycolytic flux. Therefore, *S. erythraea* had to redirect the enhanced glycolytic flux to other alternative routes besides the TCA cycle. The stimulated pigment production we observed in E3::F1ATPase indicates that the total activity of acetyl-CoA carboxylase (ACC) is likely to be up-regulated in E3::F1ATPase.
Compared with E3 in E3:F1ATPase with respect to E3. It suggested that the higher SACE_0632 encoding citrate synthase in E3::F1ATPase was SACE_1218 encoding pyruvate dehydrogenase and the early exponential phase. For instance, transcription of (Table S1). The lower intracellular ATP level induced impact of ATP perturbation on central carbon metabolism −transcription of genes in the Embden Meyerhof−Parnas (EMP) pathway and in the TCA cycle when the cells were in the early exponential phase. For instance, transcription of SACE_1218 encoding pyruvate dehydrogenase and SACE_0632 encoding citrate synthase in E3:F1ATPase was 1.6 times and 1.4 times of those in E3, respectively. An important gene, SACE_5639 (mutB) encoding the β subunit of methylmalonyl-CoA mutase was transcriptionally repressed in E3:F1ATPase with respect to E3. It suggested that the higher α-ketoglutarate pool in E3:F1ATPase stimulated the nitrogen metabolism around glutamine/glutamate rather than accumulating more methylmalonyl-CoA for erythromycin synthesis. Transcription of most genes engaged in the pentose phosphate pathway was repressed, resulting in less NADPH accumulation, which exerted a negative effect on erythromycin biosynthesis. Transcription of genes involved in pigment biosynthesis including SACE_0500, SACE_3856, and SACE_7038 was significantly induced. SACE_0500 encodes a transcriptional regulator, DasR, which positively controls the pigment production. SACE_3856 and SACE_7038 encode acetyl-CoA carboxylase to convert acetyl-CoA into the pigment precursor malonyl-CoA. Transcriptional stimulation of these three genes was likely to be responsible for the pigment overproduction in E3:F1ATPase.

Table 3. Clusters of Orthologous Group (COG) Enrichment Analysis of Differentially Expressed Genes in E3::F1ATPase Compared with E3

<table>
<thead>
<tr>
<th>COG functional categories</th>
<th>early exponential phase up-regulated</th>
<th>down-regulated</th>
<th>erythromycin synthesis onset phase up-regulated</th>
<th>down-regulated</th>
<th>total gene number in this COG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C energy production and conversion</td>
<td>11</td>
<td>4</td>
<td>20</td>
<td>10</td>
<td>324</td>
</tr>
<tr>
<td>D cell cycle control and mitosis</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>E amino acid metabolism and transport</td>
<td>18</td>
<td>3</td>
<td>59</td>
<td>14</td>
<td>440</td>
</tr>
<tr>
<td>F nucleotide metabolism and transport</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>117</td>
</tr>
<tr>
<td>G carbohydrate metabolism and transport</td>
<td>11</td>
<td>2</td>
<td>15</td>
<td>12</td>
<td>429</td>
</tr>
<tr>
<td>H coenzyme metabolism</td>
<td>6</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>303</td>
</tr>
<tr>
<td>I lipid metabolism</td>
<td>4</td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>311</td>
</tr>
<tr>
<td>J translation</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>209</td>
</tr>
<tr>
<td>K transcription</td>
<td>8</td>
<td>6</td>
<td>34</td>
<td>18</td>
<td>555</td>
</tr>
<tr>
<td>L replication and repair</td>
<td>2</td>
<td>4</td>
<td>12</td>
<td>13</td>
<td>116</td>
</tr>
<tr>
<td>M cell wall/membrane/envelope biogenesis</td>
<td>4</td>
<td>1</td>
<td>16</td>
<td>6</td>
<td>190</td>
</tr>
<tr>
<td>O post-translational modification, protein turnover, chaperone functions</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>154</td>
</tr>
<tr>
<td>P inorganic ion transport and metabolism</td>
<td>1</td>
<td>6</td>
<td>12</td>
<td>11</td>
<td>216</td>
</tr>
<tr>
<td>Q secondary metabolites biosynthesis, transport, and catabolism</td>
<td>8</td>
<td>1</td>
<td>14</td>
<td>13</td>
<td>244</td>
</tr>
<tr>
<td>T signal transduction</td>
<td>6</td>
<td>2</td>
<td>15</td>
<td>12</td>
<td>217</td>
</tr>
<tr>
<td>V defense mechanism</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>115</td>
</tr>
<tr>
<td>S function unknown</td>
<td>29</td>
<td>19</td>
<td>118</td>
<td>63</td>
<td>185</td>
</tr>
<tr>
<td>TOTAL</td>
<td>116</td>
<td>57</td>
<td>352</td>
<td>211</td>
<td>4156</td>
</tr>
</tbody>
</table>

“Data is based on the IMG web resource (https://img.jgi.doe.gov/cgi-bin/main.cgi?section=TaxonDetail&page=cogs&cat=cat&taxon_oid=2576861650).

may have induced a fake nitrogen-rich signal, which would repress erythromycin biosynthesis from the point of signal transduction view. Among the top 15 metabolites, ubiquinol/ubiquinone and acceptor/acceptor (reduced) were all linked to NADH directly, which agreed well with the intracellular redox change we observed. From the metabolic point of view, the genome-scale model of S. erythraea and iPATH were utilized to illustrate the general impact of ATP perturbation on central carbon metabolism (Table S1). The lower intracellular ATP level induced transcription of genes in the Embden−Meyerhof−Parnas (EMP) pathway and in the TCA cycle when the cells were in the early exponential phase. For instance, transcription of SACE_1218 encoding pyruvate dehydrogenase and SACE_0632 encoding citrate synthase in E3:F1ATPase was 1.6 times and 1.4 times of those in E3, respectively. An important gene, SACE_5639 (mutB) encoding the β subunit of methylmalonyl-CoA mutase was transcriptionally repressed in E3:F1ATPase with respect to E3. It suggested that the higher α-ketoglutarate pool in E3:F1ATPase stimulated the nitrogen metabolism around glutamine/glutamate rather than accumulating more methylmalonyl-CoA for erythromycin synthesis. Expression of most genes engaged in the pentose phosphate pathway was repressed, resulting in less NADPH accumulation, which exerted a negative effect on erythromycin biosynthesis. Transcription of genes involved in pigment biosynthesis including SACE_0500, SACE_3856, and SACE_7038 was significantly induced. SACE_0500 encodes a transcriptional regulator, DasR, which positively controls the pigment production. SACE_3856 and SACE_7038 encode acetyl-CoA carboxylase to convert acetyl-CoA into the pigment precursor malonyl-CoA. Transcriptional stimulation of these three genes was likely to be responsible for the pigment overproduction in E3:F1ATPase.

**Overexpression of F₁-ATPase Alters the Electron Transport.** Under aerobic conditions, the respiration coupled with the transfer of electrons from NADH to O₂ would be the predominant way to reoxidize NADH in S. erythraea, as evidenced by the high rank of transcriptional stimulation of genes encoding electron carriers among all of the NAD(H)-related genes (Table S2). Moreover, the NADH dehydrogenase and the succinate dehydrogenase, i.e., complexes I and II in ETC, were up-regulated (Figure 7). There are two kinds of terminal oxidases with different oxygen affinities and different proton pumping abilities in the ETC of S. erythraea. In E3:F₁-ATPase, genes encoding bc₁-aa₃ oxidase were repressed, whereas genes encoding the bd-type oxidase were stimulated significantly. This indicates that the overexpression of F₁-ATPase caused electron transport shifting at the branch point of the terminal oxidases from the bc₁-aa₃ complex which exhibits a lower oxygen affinity to the bd-type terminal oxidase which exhibits a higher oxygen affinity. The switch of electron transport was correlated with the lower respiration rate in E3:F₁-ATPase, which implied a decreased NADH reoxidization by aerobic respiration. The two types of terminal oxidases exhibit different efficiencies in generating the electrochemical gradient of protons coupled to electron transport: bc₁-aa₃ (6H⁺/2e⁻) and bd (2H⁺/2e⁻). Therefore, E3:F₁-ATPase reoxidized only a portion of the excess NADH through ETC and probably without increasing the membrane potential.

The global physiological response in E3:F₁-ATPase was accompanied by energy deficiency and redox stress. Previous research showed that, when cellular ATP hydrolysis was enhanced in E. coli, the glucose consumption rate increased due to allosteric activation, which inevitably led to the elevation of NADH formation through the glycolytic pathway. Transcriptional alterations of the electron transport chain and the corresponding decreased respiration in E3:F₁-ATPase.
indicated that the excess NADH caused a persistent redox stress instead of an enhanced ATP generation through oxidative phosphorylation. It thus appears that metabolic shifts to avoid excess NADH formation occurred rather than an enhanced

Figure 6. Gene set analysis (GSA) of DEGs (E3 VS E3::F1ATPase) of samples taken in the early exponential phase based on gene ontology (GO) function annotation. Gene set consensus scores are shown in the heatmap. The consensus score is the mean rank of P-value given to each gene set by the different unique GSA runs. A gene set with a low consensus score value has a high significance of transcriptional change. The nondirectional class disregards the direction of gene regulation. In the mixed-directional class, a gene set can be found significant in both the up-regulated and down-regulated part. The distinct-directional class detects gene sets that are significantly affected by differential expression only in one distinct direction. The suffix "(dn)" means gene sets up-regulated in E3::F1ATPase, whereas "(up)" means gene sets down-regulated in E3.
respiration for NADH oxidation in *S. erythraea*. The excess NADH in E3::F1ATPase drives substantial acetyl-CoA to the pigment biosynthesis rather than to the TCA cycle or glyoxylate shunt pathway compared to E3. Similarly, most of the genes involved in the NADH-forming valine/leucin/isoleucin degradation were also repressed at the transcriptional level. These metabolic shifts all prevent more NADH formation. Consequently, despite the enhanced glycolytic flux induced by the lower [ATP]/[ADP] ratios in E3::F1ATPase, this did not result in a higher accumulation of precursors for erythromycin but rather the higher overflow of α-ketoglutarate and lower overflow of fumarate (Figure 3).

In this work, the addition of NADH dehydrogenase inhibitor, rotenone, repressed erythromycin production. Besides, structural alterations of ETC in *S. coelicolor* were shown to affect cell differentiation and antibiotic production, 48,50. Therefore, it suggests that tuning the intracellular redox environment by either engineering ETC or NADH-dependent reactions would be a feasible strategy to regulate secondary metabolism in *S. erythraea* as well. Noteworthy, the correlation or the balance

---

**Figure 7.** Transcriptional change of genes in ETC. (a) Transcription heatmap of genes encoding all complexes in ETC. (b) Schematic map of electron transport alteration in ETC. Arrows below the picture indicate the up-regulated (red) or down-regulated (green) electron transport route.
between redox environment and ATP generation must be taken into account.51

Enhancement of Intracellular [ATP]/[ADP] Ratios Stimulated Erythromycin Synthesis and Reduced Reddish Pigment Production. The observed physiological response in E3::F1F0-ATPase raises the question of whether increased [ATP]/[ADP] ratios might enhance erythromycin production. One way to increase intracellular [ATP]/[ADP] ratios is to reduce ATP consumption by the addition of sublethal concentrations of antibiotics to inhibit growth partially. Sublethal concentrations of apramycin were therefore added into the liquid culture of E3, which is sensitive to the apramycin, at the beginning of the fermentation. The growth of E3 was inhibited slightly (Figure 8a). Indeed, the intracellular [ATP]/[ADP] ratios increased gradually from 3.2 in culture without apramycin to 7.1 in culture with 0.3 μg/mL apramycin (Figure 8b). Interestingly, the pigment production was repressed (Figure 8c), while the specific erythromycin production relative to cell density was increased (Figure 9a).

The erythromycin titer decreased in different cultures, as the amount of apramycin increased which resulted in the lower production of biomass. Addition of antibiotics into the culture is therefore not the way forward for increasing erythromycin, but the results still inspired us to seek an industrially applicable way to boost erythromycin synthesis in the industrial strain. We therefore constructed a strain, E3::F1F0-ATPase, by over-expressing the entire F1F0-ATPase complex in an attempt to increase the intracellular [ATP]/[ADP] ratio. Indeed, the intracellular [ATP]/[ADP] ratio in E3::F1F0-ATPase was 11.4 in the exponential phase, which was twice that of E3 (Figure 9b). Intriguingly, compared to the parental strain E3, E3::F1F0-ATPase produced a less reddish pigment (Figure 9c) and the final erythromycin titers of E3::F1F0-ATPase were enhanced by around 28% both in the minimal liquid medium and in the complex medium (Figure 9d). The observed phenotype of the F1F0-ATPase overexpressing strain stressed the importance of high [ATP]/[ADP] ratios for erythromycin production. In addition to erythromycin production, the cell growth, maintenance, intracellular environment control, substrate transport, and product export also require an adequate supply of ATP.52 For instance, increasing ATP levels in baker’s yeast enhanced biosynthesis of S-adenosylmethionine,31 which is one of the essential precursors for erythromycin production in S. erythraea. Our results extend our knowledge about the correlation between energy level and antibiotic production in accordance with some previous research. For example, an energy deficiency due to disruption of polyphosphate kinase gene (ppk) exerted a positive effect on actinorhodin production in Streptomyces lividans,53,54 which appears to challenge the observations in our present study. However, rafamycin production was repressed when ppk was disrupted in Amycolatopsis mediterranei.55 It is noteworthy that
biosynthesis of rafamycin requires the same precursors, i.e., methylmalonyl-CoA and propanoly-CoA, which are also used for erythromycin synthesis. In addition, the model suggested in this study (Figure 10) is also consistent with results in previous research,56 in which lower intracellular [ATP]/[ADP] ratios induced by chemical supplementation stimulated the production of some antibiotics. Those antibiotics have a common precursor malonyl-CoA that is the same precursor of the reddish pigment in \textit{S. erythraea}. Thus, the pigment overproduction in E3::F1ATPase suggests that biosynthesis of some other secondary metabolites may benefit from lower intracellular [ATP]/[ADP] ratios. Together, these results suggest that manipulations of intracellular energy state to the reddish pigment synthesis in E3::F1ATPase appears to relieve the redox stress, as evidenced by slight [NADH]/[NAD\(^+\)] divergence which E3 and E3::F1ATPase exhibited at the end of culture (Figures 1 and 2). Eventually, the metabolic shift to pigment synthesis in response to ATP perturbation and a fake nitrogen-rich signal caused by the higher \(\alpha\)-ketoglutarate pool probably inhibiting the accumulation of (p)ppGpp\(^{44,57}\) contributed to the decrease of erythromycin production. The alteration of pigment and erythromycin synthesis indicated that manipulations of intracellular energy state could serve as strategies for simultaneous regulation of multiple secondary metabolism owing to the broad regulatory role of ATP perturbation. Synthesis of secondary metabolites is usually catalyzed by a series of enzymes encoded by genes in a cluster with transcriptional regulation in most cases. Manipulation of respective transcription regulators of pigment or erythromycin biosynthesis genes induced synergetic production of these two secondary metabolites.\(^{5,58}\) Even the disruption of the key gene for pigment synthesis, which resulted in eliminating pigment production totally, could not increase erythromycin produc-

**Figure 9.** Enhancement of the intracellular [ATP]/[ADP] ratio induced erythromycin production and repressed pigment production. The strain overexpressing the entire F\(_F\)F\(_0\)-ATPase is E3::F\(_F\)F\(_0\)ATPase. (a) Erythromycin titers in cultures with different initial concentrations of apramycin (Apr). (b) Intracellular [ATP]/[ADP] ratios and erythromycin titers of samples from liquid culture of E3, E3::F\(_F\)ATPase, and E3::F\(_F\)F\(_0\)ATPase at 34 h. Error bars show the standard deviation from three independent experiments. (c) Growth curve and erythromycin titers of samples from complex medium in 250 mL flasks. Experiments were repeated in duplicate. (d) Pigment production on XM medium after 6 days of incubation at 34 °C. Strain names are marked on plates. *, P-value < 0.05.

**CONCLUSIONS**

This study characterized the overall physiological response of an industrial strain of \textit{S. erythraea} toward the perturbation of intracellular ATP, and the overall response to lower [ATP]/[ADP] ratios was summarized in the schematic map (Figure 10). The results showed a positive correlation between intracellular energy level and erythromycin synthesis, which suggests novel metabolic engineering strategies to improve production of erythromycin and to repress pigment synthesis simultaneously in \textit{S. erythraea}. We hypothesized and also validated that the metabolic shift of enhanced carbon flux to the reddish pigment synthesis in E3::F\(_F\)ATPase appears to relieve the redox stress, as evidenced by slight [NADH]/[NAD\(^+\)] divergence which E3 and E3::F\(_F\)ATPase exhibited at the end of culture (Figures 1 and 2). Eventually, the metabolic shift to pigment synthesis in response to ATP perturbation and a fake nitrogen-rich signal caused by the higher \(\alpha\)-ketoglutarate pool probably inhibiting the accumulation of (p)ppGpp\(^{44,57}\) contributed to the decrease of erythromycin production. The alteration of pigment and erythromycin synthesis indicated that manipulations of intracellular energy state could serve as strategies for simultaneous regulation of multiple secondary metabolism owing to the broad regulatory role of ATP perturbation. Synthesis of secondary metabolites is usually catalyzed by a series of enzymes encoded by genes in a cluster with transcriptional regulation in most cases. Manipulation of respective transcription regulators of pigment or erythromycin biosynthesis genes induced synergetic production of these two secondary metabolites.\(^{5,58}\) Even the disruption of the key gene for pigment synthesis, which resulted in eliminating pigment production totally, could not increase erythromycin produc-
The secondary metabolic shifts in E3::F1ATPase and E3::F1F0ATPase indicate that manipulations of intracellular energy state are promising strategies to regulate secondary metabolism without considering the complex transcriptional regulation networks for the gene cluster, particularly for secondary products with an obscure regulation network.

**MATERIALS AND METHODS**

**Construction of Plasmids and Strains.** Strains and plasmids used in this study are listed in Table 4. Sequences of all of the primers were listed in Table 5. The native coding sequence of atpAGD (SACE_6282−6280) in S. erythraea E3 was amplified using primers, 6282F’ and 6282R’, digested by NdeI and EcoRI, and cloned into digested pIB139, yielding pIB-atpAGD. The entire coding sequence of ATPase atpBEFHAGDC (SACE_6286−6279) and its native promoter region were amplified into two fragments using primers, ATP8F2/atplink1-3 and atplink2-2/ATP8R2. The two fragments were cloned into pIB139 between NsiI and XbaI sites using the GA one-step cloning kit (Synthetic Genomics Inc.), yielding pIB-ATPase.

The overexpression mutants E3::F1ATPase and E3::F1F0ATPase were constructed by site-specific chromosomal integration, which is mediated by phiC31 integrase. E. coli top 10 cells were used for cloning, and ET12567/pUZ8002 was used for conjugation. Plasmids from E. coli ET12567 were then introduced into S. erythraea through transconjugation on ISP4.
Table 5. Primers Used in This Study

<table>
<thead>
<tr>
<th>designation</th>
<th>sequence (5’-3’)</th>
<th>description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6282F′</td>
<td>GTTGGTAGGATCCACATGGAGCCGTCTGTCGGA</td>
<td>for F1 part ATPase amplification</td>
</tr>
<tr>
<td>6282R′</td>
<td>CATAGCATATGATCCAGGCAATTTGAGCCGAT</td>
<td>for F1 part ATPase amplification</td>
</tr>
<tr>
<td>ATP8F2</td>
<td>TGCAGGTCGACTCTGATGAGTCAGTGGGAGGGCTTC</td>
<td>for up fragment of F1F0 part ATPase amplification</td>
</tr>
<tr>
<td>atpflink1-2</td>
<td>CTCTAGCAGCCTTTCTCACAT</td>
<td>for down fragment of F1F0 part ATPase amplification</td>
</tr>
<tr>
<td>atpflink2-2</td>
<td>GATTAGGTAAGGGCGCTGAGAG</td>
<td>for PCR identification of gene overexpression in S. erythraea</td>
</tr>
<tr>
<td>BF</td>
<td>ATAGTGGCTTGCTGCGCCGGA</td>
<td>for qPCR of atpB</td>
</tr>
<tr>
<td>BR</td>
<td>GCTGAAGTCTAGTAGTGACTCT</td>
<td>for qPCR of atpE</td>
</tr>
<tr>
<td>EF</td>
<td>ATCTGATCGTCGTCGATCT</td>
<td>for qPCR of atpF</td>
</tr>
<tr>
<td>ER</td>
<td>TAGACGACACAGCAGATCA</td>
<td>for qPCR of atpG</td>
</tr>
<tr>
<td>FF</td>
<td>CTGTTCGTCGTCTGAGGTA</td>
<td>for qPCR of atpH</td>
</tr>
<tr>
<td>FR</td>
<td>ACCGGTACGTGCTACGGGT</td>
<td>for qPCR of atpA</td>
</tr>
<tr>
<td>HR</td>
<td>TCCGACAGCAAGGCGCTC</td>
<td>for qPCR of atpD</td>
</tr>
<tr>
<td>AF</td>
<td>ATCTGATCGTCAGAGATCC</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>AR</td>
<td>GCAGCTTCTGGTCATGATGA</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>GP</td>
<td>AGAGGCTCTAGCTGCTACCT</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>GR</td>
<td>GCAGACGTCGGTCCGCGGGA</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>DP</td>
<td>ATGCCAAGACCTCTGACGGT</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>DR</td>
<td>TGCTCCTTGGACAGCTGACCGA</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>CP</td>
<td>TGGTGTCGTCGGTCCGCGGGA</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>CR</td>
<td>CGGGTACAGGTCTCCCTCT</td>
<td>for qPCR of atpC</td>
</tr>
<tr>
<td>8101F</td>
<td>GTGCGAGTCCGCGGGAAGGT</td>
<td>for qPCR of 16s rRNA, as inner reference</td>
</tr>
<tr>
<td>8101R</td>
<td>CCGGCTGTTACCACGTCTTCA</td>
<td>for qPCR of 16s rRNA, as inner reference</td>
</tr>
</tbody>
</table>

agar medium. A 30 µg portion of apramycin was overlaid on ISP4 plates for screening conjugators. Primers attBS/attPS and attBA/attPA were used for PCR identification of overexpression mutants.

**Medium and Culture Conditions.** *S. erythraea* was cultivated on XM agar medium at 34 °C for 6 days for spore collection. About 1 cm² XM agar medium covered with dense spores was picked into flasks with preculture medium. After the 48 h preculture, a 5 mL preculture was collected, resuspended in 1 mL of PBS buffer (pH 7.4), and inoculated into flasks with either 27 mL of modified minimal liquid medium for physiological study or complex fermentation medium for characterization under industrial conditions. The modified minimal liquid medium contained the following per 800 mL: 20 g of (NH₄)₂SO₄, 50 g of casamino acids (DifcoTM, BD), 0.6 g of MgSO₄·7H₂O, 1 g of ZnSO₄·7H₂O, 1 g of FeSO₄·7H₂O, 1 g of MnCl₂·4H₂O, 1 g of CaCl₂. A 800 mL portion of MMLM was then dispensed in 80 mL aliquots. A 15 mL portion of NaH₂PO₄/K₂HPO₄ buffer (0.1 M, pH 6.8) was added into 80 mL of liquid medium for pH buffering. After autoclaving, 25% glucose was injected into the medium to a final concentration of 20 g/L. Liquid culture was performed in shaking water baths (Julabo, Germany) with an agitation speed of 140 rpm at 34 °C.

To study the effects of a higher [NADH]/[NAD⁺] ratio on secondary metabolism in *S. erythraea*, various volumes of 50 mM rotenone were added in liquid culture of E3 in the middle exponential phase (24 h) and in the early stationary phase (48 h). In order to enhance intracellular [ATP]/[ADP] ratios, a gradient concentration of apramycin was supplemented in the minimal liquid medium at the start of fermentation. The initial OD₆₀₀ of fermentation culture with apramycin was controlled around 0.3–0.4 at inoculation.

**Fermentation Analysis.** Cell samples from cultures in duplicate or triplicate shake flask were collected over the time course. Dry cell weight (DCW) in 3 mL culture was measured for monitoring cell growth. The concentration of residual glucose in culture was determined by the salicylic acid method. Each culture sample was centrifuged at 8000 × g at 4 °C for 10 min, and extracellular organic acids in supernatants were determined using a HPLC (Thermo Scientific, USA). The HPLC detection system included an Aminex HPX-87H column (Bio-Rad, USA) and a Shodex RI-101 detector (Tokyo, Japan). The mobile phase consisted of 5 mM H₂SO₄, and the flow rate was set at 0.5 mL/min. The column oven temperature was 60 °C. The dissolved oxygen (DO) concentration was determined using a DO electrode (Endress+Hauser A/S, Denmark). A 25 mL portion of broth with cells was withdrawn from the flask, washed, and resuspended in 25 mL of PBS buffer (pH 7.4). Then, the DO value was monitored each 10 s for a total duration of 5 min. One L bioreactors (Sartorius, Germany) with 0.6 L working volume were used for monitoring online pH using electrodes (Endress+Hauser A/S, Denmark). For measuring the reddish pigment in liquid culture, the broth supernatant was diluted 100 times and then was relatively quantified by absorbance at 270 nm. The erythromycin titer was measured by the modified H₂SO₄-colorimetric method.

**Analysis of Intracellular Cofactors.** For [NADH]/[NAD⁺] determination, cold methanol quenching was applied. Previous reports on actinomycetes suggest leakage caused by cold shock. Each sample with 50 µL cell culture was quenched by immediate addition of 500 µL of methanol precooled at −80 °C.

---

ACS Synthetic Biology pubs.acs.org/synthbio  | Research Article

ACS Synth. Biol. 2020, 9, 655–670

https://dx.doi.org/10.1021/acssynbio.9b00528
Portions of 500 μL of 4 °C water and 500 μL of −20 °C chloroform were instantly added to the samples. Samples was vortexed vigorously and then stored at −20 °C for 1 h. Supernatant was reserved by centrifugation at 10000 × g at 4 °C for 10 min. A NAD/NADH-Glo assay kit (Promega, USA) was used for NADH/NAD⁺ measurement. For [ATP]/[ADP] ratio determination, cells were quenched by phenol as described previously.65 The amount of ATP or ADP was quantified with an ATP determination Kit (A22066) (Invitrogen, USA). ADP was assayed after ATP had been determined by adding pyruvate kinase and recording the increase in luminescence. The results were corrected for quenching of the signal by the addition of pyruvate kinase.

Activity Measurement of Enzymes. Cells in the exponential phase (12 h) were harvested by centrifugation, washed with an appropriate buffer, and kept at −20 °C until use. In order to standardize enzymatic activity values, a 3 mL culture was used for dry cell weight measurement. About 0.5 g of glass beads was added into another 1 mL cell culture. The cells were disrupted by a FastPrep (30 s × 4; M.P. Biomedical, USA) and centrifuged at 15,000 × g at 4 °C for 10 min. The supernatant was used for protein concentration measurement using a Nanodrop One® (Thermo Scientific, USA). The composition of the buffer system, reaction mixtures, and assay conditions for the respective enzymes were described previously.26 A 20 μL cell extract was added in 1 mL of reaction mixture. The specific activity of each enzyme under the assay conditions was expressed in nmol-min⁻¹·mg of protein⁻¹.

Sequencing of RNA. Two replicates from independent cultures in modified minimal liquid cultures were used. Cells were harvested at two time points, i.e., 10 h in the early exponential phase and 48 h at the onset point of erythromycin biosynthesis, for total RNA extraction. A 10 mL culture was centrifuged at 4000 × g at 4 °C for 10 min. The supernatant was discarded, and the cells were resuspended in 2 mL of RNAlater solution (Invitrogen, USA). Total RNA was extracted with an RNAeasy Plus Mini kit (Qiagen, Germany) using glass beads to mechanically disrupt cells with a FastPrep. DNase treatment by an RNase-Free DNase Set (Qiagen, Germany) aided to digest DNA in the samples. The RNA integrity was analyzed by 1% agarose gel electrophoresis and Bioanalyzer (Agilent, USA). The RNA concentration was also determined by Bioanalyzer (Agilent, USA). A 1 μg portion of total RNA was reverse transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) for RT-qPCR. Transcriptional levels were quantified in triplicate with 16s rRNA gene (SACE_8101) as the inner reference. Primers used for qPCR were listed in Table 2. The 2⁻ΔΔC₂ method was used for calculating the relative expression.66

RNA sequencing was accomplished using the BGiseq 500 next-gen sequencer by BGI, China. Data sets consisted of at least 30 M reads per sample. After sequencing, the raw reads were filtered, including removing adaptor sequences, contamination, and low-quality reads from raw reads.

Transcriptomic Analysis. FastQ files sequenced by BGiseq 500 were input into the software, Geneious Prime, for raw reads mapping into the S. erythraea NRRL2338 genome (GenBank accession number: NC_009142) and were trimmed afterward. The RPKM value of each gene was calculated by Geneious Prime. Differentially expressed genes (DEGs) were identified by using the DEseq2 method. Clusters of orthologous groups were identified in the EggNOG database.67 iPATH facilitates visualization of the distribution of DEGs upon different metabolic pathways.35 Gene set analysis and reporter metabolite analysis were conducted by R package Piano68 based on a genome-scale metabolic model.35 Hierarchical clustering of DEGs was carried out by a web-based tool, Morpheus (https://software.broadinstitute.org/GENE-E).

Data Availability. Raw reads of RNA sequencing used in this study can be obtained from the NCBI Gene Expression Omnibus (accession number: GSE134767).

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00528.

Figure S1, online pH curve for the parental strain E3 and atpAGD overexpression strain E3::F1ATPase batch-cultured in 1 L bioreactors; Figure S2, physiological characterization of E3 and E3::F1ATPase in 300 mL shake flasks with minimal liquid medium; Figure S3, reporter metabolite analysis based on transcriptomic data derived from the early exponential phase; Table S1, transcriptional response of some genes involved in the central carbon metabolism (PDF)
Table S2, expression change of NADH-related genes in E3::F1ATPase with respect to E3 (XLSX)

AUTHOR INFORMATION

Corresponding Authors
Ju Chu — State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, People’s Republic of China; Phone: (+86) 021-64253021; Email: juchu@ecust.edu.cn
Peter R. Jensen — National Food Institute, Technical University of Denmark, DK2800 Kongens Lyngby, Denmark; orcid.org/0000-0003-2080-2070; Phone: (+45) 20855601; Email: perj@food.dtu.dk

Authors
Xiaobo Li — State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, People’s Republic of China; National Food Institute, Technical University of Denmark, DK2800 Kongens Lyngby, Denmark
Jun Chen — National Food Institute, Technical University of Denmark, DK2800 Kongens Lyngby, Denmark
Joakim M. Andersen — National Food Institute, Technical University of Denmark, DK2800 Kongens Lyngby, Denmark

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.9b00528

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

X.L. and J.C. received funding from the National Natural Science Foundation of China (No. 21276081) and the National Scientific and Technological Major Special Project (Significant Creation of New drugs, No. 2011ZX09203-001-03). We are grateful for the financial support from the program of China Scholarships Council (No. 201706740055) to X.L. The funders had no role in study design, data collection, or interpretation or the decision to submit the work for publication. We thank Tine Suhr for assistance with laboratory work and Yufei Sui for assistance with the transcriptional analysis.
REFERENCES


