Deletion of *cg1360* Affects ATP Synthase Function and Enhances Production of L-Valine in *Corynebacterium glutamicum*^S

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Bacterial ATP synthases drive ATP synthesis by a rotary mechanism, and play a vital role in physiology and cell metabolism. *Corynebacterium glutamicum* is well known as an industrial workhorse for amino acid production, and its ATP synthase operon contains eight structural genes and two adjacent genes, *cg1360* and *cg1361*. So far, the physiological functions of Cg1360 (GenBank CAF19908) and Cg1361 (GenBank CAF19909) remain unclear. Here, we showed that Cg1360 was a hydrophobic protein with four transmembrane helices (TMHs), while no TMH was found in Cg1361. Deletion of *cg1360*, but not *cg1361*, led to significantly reduced cell growth using glucose and acetic acid as carbon sources, reduced F1 portions in the membrane, reduced ATP-driven proton-pumping activity and ATPase activity, suggesting that Cg1360 plays an important role in ATP synthase function. The intracellular ATP concentration in the $\Delta cg1360$ mutant was decreased to 72% of the wild type, while the NADH and NADPH levels in the $\Delta cg1360$ mutant were increased by 29% and 26%, respectively. However, the $\Delta cg1361$ mutant exhibited comparable intracellular ATP, NADH and NADPH levels with the wild-type strain. Moreover, the effect of *cg1360* deletion on L-valine production was examined in the L-valine-producing V-10 strain. The final production of L-valine in the V-10- $\Delta cg1360$ mutant reached 9.2 ± 0.3 g/l in shake flasks, which was 14% higher than that of the V-10 strain. Thus, Cg1360 can be used as an effective engineering target by altering energy metabolism for the enhancement of amino acid production in *C. glutamicum*.

Keywords: ATP synthase, *cg1360*, *C. glutamicum*, L-valine production

Introduction

The ATP synthase is a multi-subunit, membrane-bound enzyme complex that is found in all living organisms. It can rely on the proton electrochemical gradient to drive ADP and Pi to synthesize ATP by a rotational catalytic mechanism, and also hydrolyze ATP to create an ion gradient needed for other physiological activities [1–5]. The intact ATP synthase is typically composed of two major sub-complexes, the membrane-embedded Fo sector and the water-soluble F1 sector. The hydrophobic Fo domain consists of three subunits (a, b, and c) and can function as a proton channel. The hydrophilic F1 domain

consists of five subunits (α , β , γ , δ , and ϵ) and has three catalytic sites at the interfaces of α and β subunits [2, 6, 7]. In most bacteria, the *atp* operon contains nine open reading frames (ORFs), including eight structural genes (*atpBEFHAGDC*) and one preceded *atpI* gene [8, 9]. *AtpI*, also known as *UncI*, is a small membrane protein and is shown to have a role in the c-subunit ring formation during the assembly of some ATP synthases. In *Escherichia coli*, deletion of the *atpI* gene only shows a slight reduction in the biomass yield and ATPase activity [10]. Similarly, loss of the *atpI* gene in *Bacillus pseudofirmus* OF4 has no apparent effects on the nonfermentative growth [9]. Further investigation shows that *AtpI* of *B. pseudofirmus* OF4 is not

required for the c-ring assembly, but it supports the optimal ATP synthase stability [9]. However, the product of the *atpI* gene in *Propionigenium modestum* seems to play a necessary chaperone-like role in the formation of c-rotor ring and the subsequent assembly of ATP synthase [11, 12]. Overall, the physiological significance of AtpI in different species is still ambiguous, and further investigation on other microorganisms will provide more clues into the understanding of bacterial AtpI.

ATP is typically generated from oxidative phosphorylation with the membrane-bound F₁F₀-ATP synthase, or produced by substrate level phosphorylation (SLP). The regulation of ATP supply is considered as a promising strategy to improve cellular metabolism for enhanced bioproduction yields [13–15]. The H⁺-ATPase deficient mutants of *E. coli* have been successfully applied for increasing the yields of pyruvic acid [16], acetic acid [17], and alanine [18]. A deletion of the entire *atpBEFHAGDC* cluster encoding the F₁F₀-ATP synthase partially affects cell growth with glucose, and obviously increases pyruvate formation and proton motive force in the amino-acid producing bacterium *Corynebacterium glutamicum*. The defective process of oxidative phosphorylation in the ΔF₁F₀-ATP synthase mutant leads to a shortage of intracellular ATP, which in turn forces the mutant cells to increase glucose uptake and catabolism for more ATP generation. The results imply that the F₁F₀-ATP synthase and oxidative phosphorylation are not utterly indispensable for growth under glucose condition in *C. glutamicum* [19]. Consistent with these findings, a serine-to-proline mutation at residue 237 of γ subunit decreased ATP synthase activity to 25% of wild type in *C. glutamicum*, but it in turn leads to the enhancement of glucose consumption and respiration rate per cell. The increased glucose catabolism of the H⁺-ATPase-defective mutant causes an increase in the intracellular pyruvate pool, which is effective for more conversion from pyruvate to L-valine [20]. Moreover, a previous study on the transcriptional analysis of *C. glutamicum* ATP synthase reveals that the *atp* operon is expressed optimally at alkaline pH starting from the upstream of the *atpB* structural gene, and the sigmaH factor plays an important role in the regulation of the *atp* operon [21]. Although some studies have focused on the roles of the entire *atpBEFHAGDC* cluster in *C. glutamicum*, the physiological functions of two adjacent genes, *cg1360* and *cg1361*, located just at the upstream of the *atp* structural genes, had not yet been explored.

In this study, we investigated the potential roles of Cg1360 (encoded by the *cg1360* gene) and Cg1361 (encoded by the *cg1361* gene) for ATP synthase in *C. glutamicum*. The

data revealed that Cg1360, but not Cg1361, plays an important role in the maintenance of ATP synthase function and intracellular NADP(H) levels. Given that NADPH rather than ATP acts as an essential limiting factor for L-valine formation from the precursor pyruvate, we further examined the potential application of deletion of *cg1360* for efficient L-valine production. Our results supported that Cg1360 can be used as an effective engineering target for the enhancement of L-valine production in industrial biotechnology.

Materials and Methods

Strains and Growth Conditions

The plasmids and strains used in this study are listed in Table S1. *E. coli* DH5α was used as a host cell for general cloning. *C. glutamicum* ATCC 13032 was used as the parental strain for gene manipulation and the wild-type (WT) strain for functional analysis. The valine-producing *C. glutamicum* CGMCC 1.586 was purchased from the China General Microbiological Culture Collection Center (CGMCC, China). A mutant strain V-10, producing a high and stable yield of L-valine, was obtained from *C. glutamicum* CGMCC 1.586 through multiple rounds of random mutagenesis and screening. Briefly, the original *C. glutamicum* CGMCC 1.586 strain was treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as a mutagen, and the mutant was selected by flow cytometry measurements based on the Lrp-based amino acid biosensor according to the previous study [22]. The obtained *C. glutamicum* V-10 strain has been deposited into the China Center of Industrial Culture Collection (CICC, China) under the accession number CICC 24789. *E. coli* cells were routinely grown at 37°C in LB medium (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl), and *C. glutamicum* cells were grown at 32°C in LBHIS rich medium (2.5 g/l yeast extract, 5 g/l tryptone, 5 g/l NaCl, 18.5 g/l BHI powder and 91 g/l sorbitol) or modified CGXII minimal medium (20 g/l (NH₄)₂SO₄, 5 g/l urea, 1 g/l KH₂PO₄, 1 g/l K₂HPO₄, 0.25 g/l MgSO₄•7H₂O, 10 mg/l CaCl₂, 10 mg/l FeSO₄•7H₂O, 10 mg/l MnSO₄•H₂O, 1 mg/l ZnSO₄•7H₂O, 0.2 mg/l CuSO₄, 0.02 mg/l NiCl₂•6H₂O, 0.2 mg/l biotin, 4% (w/v) glucose or 100 mM acetic acid) [19]. During the shake-flask fermentation, *C. glutamicum* strains were cultivated in 250-ml Erlenmeyer flasks containing 30 ml fermentation medium at 32°C and 200 rpm for 72 hours. The fermentation medium consisted of 125 g/l glucose, 2 g/l corn steep liquor, 50 g/l (NH₄)₂SO₄, 1 g/l K₂HPO₄, 0.5 g/l MgSO₄•7H₂O, 100 μg/l VB₁, 50 μg/l biotin, 10 g/l potassium acetate, 0.2 g/l isoleucine, 0.2 g/l alanine and 20 g/l calcium carbonate. The initial pH of the above medium was usually adjusted to pH 7.0 and buffered with 200 mM MOPS (3-[N-morpholino]-propanesulfonic acid, pK_a = 7.20).

Strain Construction and Complementation

The primers used in the construction of plasmids and strains

are listed in Table S2. *C. glutamicum* mutant strains were achieved by a two-step homologous recombination using the temperature-sensitive plasmid pCRD206 as described previously [23]. In order to avoid polar effects in operon structures, markerless chromosomal in-frame deletion mutants were constructed (Fig. 2A). The $\Delta cg1360$ mutant contains an in-frame deletion of 127 amino acids of an open reading frame (ORF), while the $\Delta cg1361$ mutant contains an in-frame deletion of 69 amino acids of an ORF. Chromosomal complementation of the markerless in-frame deletions was also built by a two-step homologous recombination using an approach similar to the above. In order to distinguish the complementation strain ($\Delta cg1360+cg1360$ or $\Delta cg1361+cg1361$) with the wild-type strain in genome sequence, a synonymous point mutation at the Glu-4 site (GAA to GAG) of *cg1360* gene or at the Leu-4 site (CTG to CTC) of *cg1361* gene was introduced (Fig. 2A). The detailed procedures for the construction of the $\Delta cg1360$, $\Delta cg1361$ and two complementation strains were provided in the Supplemental Methods.

To detect the spatial distribution of ATP synthase β subunit (the product of *atpD* gene) in the WT, $\Delta cg1360$ and $\Delta cg1361$ strains, the hexa-histidine tag was introduced into its C-terminus according to an approach similar to the above. The L-valine-producing strain *C. glutamicum* V-10 was used as a starting strain for the fermentative analysis, and the *cg1360* gene was disrupted in the V-10 background strain to obtain the V-10- $\Delta cg1360$ strain, also according to an approach similar to the above.

Bioinformatics Analysis of AtpI-Like Protein

Multiple sequence alignment of the putative AtpI from different bacterial species was performed by the Clustal Omega and ESPript 3.0 server [24]. The prediction of transmembrane helices (TMHs) in proteins was performed by the TMHMM Server v. 2.0 [25]. The GenBank accession numbers of the proteins were listed as follows: *C. glutamicum* ATCC13032 Cg1360, No. CAF19908.1; *B. pseudofirmus* OF4 ATP synthase protein I, No. ADC49435.1; *E. coli* K-12 UncI polypeptide, No. AAA24730.1; *Pseudomonas putida* KT2440 ATP synthase protein I, No. P0A103.1; *Shigella boydii* Sb227 AtpI, No. ABB68220.1; *Clostridium kluyveri* DSM 555 AtpI, No. EDK35680.1; *Vibrio alginolyticus* ATP synthase protein I, No. P12983.1; *Pectobacterium atrosepticum*SCRI 1043 ATP synthase protein I, No. CAG77414.1.

Growth Assays and Glucose Consumption

The growth abilities of *C. glutamicum* WT, mutant and complementation strains were examined in liquid CGXII minimal medium containing 4% (w/v) glucose or 100 mM acetic acid as the sole carbon source. Briefly, the corresponding *C. glutamicum* strains were cultivated for 12 h at 32°C with shaking prior to growth experiments. Overnight cultures were then harvested, washed, and shifted to the CGXII minimal medium containing glucose or acetic acid with an initial optical density at 600 nm (OD_{600}) of 0.1. Growth curves were established by measuring the OD_{600} at the indicated time points. The residual glucose concentration

in the culture medium was determined by an SBA-40E biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China). Specific glucose consumption rate during the exponential growth phase was calculated from glucose consumption and biomass concentration using this formula: [decreased glucose mM]/[increased cell dry weight (g/l)]⁻¹ h⁻¹ [26]. The cell dry weight (CDW) was calculated from the OD_{600} value using the experimentally correlation factor of 0.25 g CDW per liter for an OD_{600} of 1.0 [19].

Determination of the Intracellular ATP and NAD(P)H Concentrations

C. glutamicum cells grown with glucose as a carbon source were harvested during the exponential growth phase at an OD_{600} of 3.0 for the determination of intracellular ATP, NADH and NADPH levels. To determine the intracellular ATP concentration, the collected cells were washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The ATP content was evaluated using a firefly luciferase-based ATP Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocols. To determine the intracellular NAD(P)H concentration, the collected cells were washed once with ice-cold phosphate-buffered saline (PBS, pH 7.5). Next, 300 μ l of 0.4 M NaOH was added to the cells for homogenization following a water bath for 10 min at 50°C. Then, 300 μ l of 0.4 M HCl was slowly added to neutralize the extract, and the mixture was shaken mildly and centrifuged at 8,000 $\times$ g for 5 min [27]. The levels of NADH or NADPH in extracts were quantified using a spectrophotometric enzyme cycle method as described previously [28, 29]. The reaction mixture for NADH determination consisted of 100 mM Bicine buffer (pH 8.0), 4 mM EDTA (pH 8.0), 3.32 mM phenazine ethyl sulfate, 0.42 mM thiazolyl blue, 5 U/ml alcohol dehydrogenase (ADH), 10% (v/v) ethanol and appropriate volume of cell extract. The reaction mixture for NADPH determination was the same as the NADH assay mixture except that ADH and ethanol were replaced with 1.75 U/ml glucose-6-phosphate dehydrogenase (G6PDH) and 3.8 mM glucose-6-phosphate. All the experiments were performed in triplicate and repeated three times with similar results.

Real-Time Quantitative PCR Analysis

Quantitative real-time reverse transcription-PCR (qPCR) was used to calculate the expression levels of the target genes. The primers used in this experiment are listed in Table S2. RNA extraction and reverse transcription were performed using a Bacterial Total RNA Extraction Kit (Tiangen Biotech, China) and a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). For relative qPCR analysis, the reaction mixture was prepared using SYBR Green Real-Time PCR Master Mix (Toyobo, Japan) with reference to the instructions, while data acquisition and analysis were realized using an Applied Biosystems 7500 Fast Real-Time PCR System. The 16S rRNA gene (encoding for RNA component of bacterial 30S ribosomal subunit) and a commonly used housekeeping *gyrB* gene (encoding for the B subunit of DNA gyrase) have been previously used as candidate reference genes

for qPCR analysis. The two genes are proved to be a relatively stable set of reference genes in many bacterial species, including *C. glutamicum* [30–32]. Similar experimental results were obtained according to these two reference genes in this study, and the 16S rRNA was used as a normalized reference gene for the present data. The relative fold changes in gene expression were calculated according to the delta delta threshold cycle method as described previously [33].

Assays for ATPase Hydrolysis Activity, ATP-Driven Proton Pumping Activity, and the Spatial Distribution of β Subunit

C. glutamicum cells grown with glucose as a carbon source were harvested during the exponential growth phase at an OD₆₀₀ of 3.0, and washed once with ice-cold PBS buffer. Then, cell pellets were resuspended in 35 ml of PS4 buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.3 mg/ml DNase I, one complete protease inhibitor tablet per 50 ml buffer). Inverted membrane vesicles were prepared as described previously [9, 34]. In brief, the cells were disrupted using a French press (15,000 p.s.i) and the crushed suspension was centrifuged at 15,000 $\times g$ for 15 min to remove cell debris and unbroken cells. The supernatant was centrifuged at 200,000 $\times g$ in an SW70 Ti Rotor, Optima LE-80K Ultracentrifuge (Beckman Coulter, USA) for 1 h at 4°C. The pellets were suspended in TCDG buffer (10 mM Tris-HCl, pH 7.5, 140 mM choline chloride, 0.5 mM dithiothreitol; 10% glycerol) using homogenizer, and stored at –80°C before use. The determination of ATPase hydrolysis activity was performed by measuring the amount of released inorganic phosphate from ATP [35]. The determination of ATP-driven proton-pumping activity was determined by the measurement of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching [9]. All the experiments were performed in triplicate and repeated at least three times.

To assess the total amount of the β subunit and its distribution between the cytoplasm and the membrane, the total protein, as well as the cytoplasmic fraction and membrane fraction were collected. Protein concentration was measured by the Bradford method (Bio-Rad, USA). Equal amounts of sample protein derived from different strains were loaded separately onto the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and examined by western blotting analysis using His-tag antibody. Protein bands were quantified using the ImageJ gel analysis software (Wayne Rasband, NIH).

Results and Discussion

Sequence Analyses of Cg1360 and Cg1361

Most bacterial ATP operons have 8 structural genes in the order *atp BEFHGADC* [8], and a ninth gene, named *atpI* gene, is located at the upstream of the *atp* operon (Fig. 1A). We first analyzed the complete genome sequence of

C. glutamicum ATCC 13032 [36]. Two unknown ORFs, with locus tags of *cg1360* and *cg1361*, were located at the upstream of the *atp* operon in *C. glutamicum* (Fig. 1A). The alignments of Cg1360 with some related AtpI protein were shown in Figs. 1B and S1. The *cg1360* gene was predicted to encode a hydrophobic protein with 4 TMHs, while no TMH was found in Cg1361. The AtpI protein from other microorganisms, such as *E. coli*, and *B. pseudofirmus* OF4, has also been predicted to have 4 TMHs, suggesting that Cg1360, rather than Cg1361, in *C. glutamicum* may play a role as AtpI in the *atp* operon. However, Cg1360 showed low sequence identity to the predicted AtpI from *E. coli* (23% identity, 34/149 aa), and to the AtpI from *B. pseudofirmus* OF4 (16% identity, 24/149 aa) (Table S3). Similarly, no apparent sequence homology was found for Cg1361 (Table S4). These findings indicated that the sequence of Cg1360/Cg1361 shares no obvious homology with the putative AtpI from different organisms.

Effects of Deletion of *cg1360* or *cg1361* on Cell Growth and Glucose Consumption

The $\Delta cg1360$, $\Delta cg1361$ and two related complementation strains were successfully constructed as described in Materials and Methods. The growth properties of the WT, $\Delta cg1360$ and $\Delta cg1361$ strains were compared under the conditions of different carbon sources (Fig. 2, Table 1). The $\Delta cg1360$ strain exhibited a large growth defect relative to the WT control (around 70% of WT) when glucose was used as the sole carbon source (Fig. 2B). In prokaryotic microorganisms, the metabolic utilization of acetate to produce acetyl-CoA requires the use of ATP. In the presence of acetate as a sole carbon source, most ATP is generated by oxidative phosphorylation while substrate-level phosphorylation provides a less efficient source of ATP [19, 37]. Here, we found that the $\Delta cg1360$ strain exhibited a more severe growth defect in the acetate-containing medium (Fig. 2C), which had only 31% biomass of the WT control (Table 1). As mentioned earlier, Cg1360 may belong to the homologous protein of bacterial AtpI, so we speculate that loss of *cg1360* may cause a deficiency of ATP synthase function, resulting in an insufficient ATP supply for acetate utilization. However, deletion of *cg1361* had no obvious impact on cell growth in either glucose or acetate carbon sources (Figs. 2B and 2C). In addition, there was no statistically significant difference in the growth parameters among the WT and two complementation strains (Fig. 2 and Table 1), indicating that the changes observed in the $\Delta cg1360$ mutant were indeed caused by gene deletion at the chromosomal level. Interestingly, the glucose consumption

Fig. 1. Bioinformatics analysis of bacterial AtpI-like protein. (A) Gene arrangements of the *atp* operon in *E. coli* K-12, *B. pseudofirmus* OF4 and *C. glutamicum* ATCC 13032. (B) Multiple sequence alignment of the putative AtpI from different bacterial species. The sequence alignment was performed by the Clustal Omega and ESPrict 3.0 server. The prediction of transmembrane helices in proteins was carried out by the TMHMM Server, and shown as black boxes.

rate of the $\Delta cg1360$ mutant during the exponential growth phase indeed showed 39% higher than the WT control (Fig. S2A). The enhancement of the glucose consumption rate by defective F1Fo-ATP synthase has also been reported for *E. coli* [16, 38], *Torulopsis glabrata* [39] and *C. glutamicum* [40], indicating a strategy enabling microorganisms to

survive in energy shortages caused by the ATP synthase defect.

Roles of *cg1360* or *cg1361* on the Assembly of ATP Synthase, ATP-Driven Proton-Pumping Activity and ATPase Activity

In order to confirm whether *cg1360* and *cg1361* were

Fig. 2. Growth assays of the wild-type, $\Delta cg1360$, $\Delta cg1361$, and two complementation strains.

(A) Sequence differences at the chromosomal locus of *cg1360* or *cg1361* gene among wild-type, $\Delta cg1360$, $\Delta cg1361$ and two complementation strains. * Indicates the synonymous point mutation in genome. (B and C) Growth abilities were examined in CGXII minimal medium containing 4% (w/v) glucose or 100 mM acetic acid as sole carbon sources. Overnight cultures were resuspended in the liquid medium with an initial OD₆₀₀ of 0.1, and growth curves were established by measuring the OD₆₀₀ at the indicated time points.

associated with the expression of the ATP synthase operon, the transcriptional levels of ATP synthase components were measured by qPCR. Given that the ATP synthase operon contains 8 different adjacent genes, we chose four representative genes (*atpB*, *atpE*, *atpH*, and *atpD*) to evaluate the transcription pattern of the whole operon. As shown in Fig. 3A, the transcription levels of *atpB*, *atpE*, *atpH*, and *atpD* in the $\Delta cg1360$ mutant were at least 55% lower than those of the WT control, indicating that *cg1360*

may play an important role in the transcriptional activation of the ATP synthase operon. However, lack of the *cg1361* gene had no significant influence on the expression of the ATP synthase operon.

AtpI from *P. modestum* is indispensable for the production of active ATP synthase when expressing a hybrid F1Fo in *E. coli* cells [12]. Functional c-rings can only be produced in the presence of both AtpI and c subunits [11]. Additionally, our previous study has shown that AtpI from *B.*

Table 1. Cell growth, β subunit distribution, ATPase activity and ATP-driven proton-pumping activity assays for *C. glutamicum* wild-type strain and its mutant derivatives.

Strain	Glucose (24 h)		Acetic acid (30 h)		β subunit content (% of WT)		ATPase activity (% of WT)	Proton-pumping activity (% of WT)
	Growth (OD ₆₀₀)	% of WT ^a	Growth (OD ₆₀₀)	% of WT	Membrane	Cytoplasm		
WT	7.1 ± 0.3	100	3.2 ± 0.06	100	100	100	100	100
$\Delta cg1360$	5.0 ± 0.3	70	1.0 ± 0.03	31	66 ± 5	235 ± 15	78 ± 4	55 ± 6
$\Delta cg1361$	7.0 ± 0.2	99	3.1 ± 0.09	96	97 ± 6	104 ± 9	99 ± 5	96 ± 4
$\Delta cg1360+cg1360$	7.2 ± 0.2	101	3.3 ± 0.08	103	ND ^b	ND	ND	ND
$\Delta cg1361+cg1361$	7.0 ± 0.4	99	3.2 ± 0.10	100	ND	ND	ND	ND

^aThe percentage (%) was calculated based on OD₆₀₀, β subunit content, ATPase activity or proton-pumping activity of recombinant strains compared to that of wild-type control, respectively.

^bND represents not detected.

Fig. 3. Effects of *cg1360* or *cg1361* deletion on the expression and assembly of the ATP synthase.

(A) Transcription levels of genes encoding ATP synthase subunits were quantified using real-time qPCR and fold changes were calculated relative to the wild-type levels (assigned a value of 1). Asterisks indicate significant differences between *C. glutamicum* wild type and its mutant derivatives according to a statistical Student's *t*-test (**, $p < 0.01$). (B) The total protein levels and spatial distribution of β subunit of ATP synthase in the wild type and its mutant derivatives. Image analysis was performed using the ImageJ software for quantitative purposes and the wild-type content was arbitrarily designated as 100%.

pseudofirmus OF4 is not absolutely required for c-ring assembly, but deletion of the *atpI* gene decreases the c-ring stability [9]. Therefore, we further investigated the effects of deletion of *cg1360* or *cg1361* in *C. glutamicum* on the assembly of ATP synthase by introducing a 6 $\times$ His tag into β subunit of ATP synthase F1 sector. If the peripheral F1 portions cannot properly be assembled with the hydrophobic Fo component to form an intact ATP synthase at the membrane surface, the increased levels of F1 (β subunit) will be detected in the cytoplasm. As shown in Fig. 3B, the total expression levels of ATP synthase β subunit in the $\Delta cg1360$ mutant was only 73% of the WT level, consistent

with the evidence of the reduced transcriptional levels of *atp* operon. Deletion of *cg1360* gene resulted in a 34% reduction, relative to the WT, in the content of ATP synthase β subunit in the membrane fraction, and a 235% increase of β subunit content was detected in the cytoplasm compared to that in the WT (Table 1). It should be noted that deletion of *cg1360* did not completely abolish the appearance of β subunit on cell membrane, indicating that *cg1360* can affect the assembly of intact ATP synthase, but it may not be an indispensable factor. In addition, deletion of *cg1361* had no obvious effects on the total protein levels of β subunit and its distribution between membrane and the cytoplasm. Taken together, the above findings suggested that Cg1360 plays important roles in controlling the expression and assembly of ATP synthase in *C. glutamicum*, which is inconsistent with the putative role of AtpI from other microorganisms [9, 12].

The effects of deletion of *cg1360* and *cg1361* on ATP-driven proton-pumping activity and ATPase hydrolysis activity were also investigated (Fig. 4 and Table 1). Deletion of *cg1360* resulted in a 45% decrease in proton-pumping activity compared to that in the WT, whereas deletion of *cg1361* slightly lowered the proton-pumping activity of ATP synthase in *C. glutamicum*. The ATPase hydrolysis activity of the $\Delta cg1360$ mutant only accounted for 78% of the WT, while the $\Delta cg1361$ mutant showed no obvious ATPase deficiency. The decreased proton-pumping activity and ATPase activity in the $\Delta cg1360$ mutant may be partly attributed to the findings that the absence of *cg1360* had a negative effect on the expression and assembly of ATP synthase complexes.

Comparison Analysis of the Intracellular ATP and NAD(P)H Concentrations

The decreased activity of ATP synthase, including ATP-driven proton-pumping activity and ATPase activity, in the $\Delta cg1360$ mutant prompted us to further analyze the levels of intracellular ATP and NAD(P)H. As shown in Fig. 5, deletion of *cg1360* resulted in a 28% decrease in the intracellular ATP concentrations, relative to the WT level. However, the levels of intracellular NADH and NADPH in the $\Delta cg1360$ mutant increased by 29% and 26%, respectively. As expected, re-introducing the native *cg1360* gene into the $\Delta cg1360$ mutant could restore the levels of intracellular ATP and NAD(P)H back to the WT level. The decreased ATP accumulation was in agreement with the growth phenotypes of the $\Delta cg1360$ mutant that exhibited an impaired growth under glucose condition and a more severe growth defect with acetate as the sole carbon source

Fig. 4. Effects of *cg1360* or *cg1361* deletion on the ATP-driven pumping and ATPase hydrolysis activities.

(A) ATP-driven proton-pumping activities in membrane vesicles from wild type and its mutant derivatives were monitored by fluorescence quenching of the pH-sensitive probe ACMA. NH_4Cl was added to the assay buffer to dissipate the proton gradient and establish a baseline. (B) ATPase hydrolytic activities of wild type and its mutant derivatives were evaluated by measuring the amount of inorganic phosphate produced by ATP substrate.

(Fig. 2), supporting the importance of ATP supply to cell growth. Moreover, the $\Delta cg1361$ mutant exhibited comparable levels of the intracellular ATP and NAD(P)H with the WT strain, suggesting its minor role in the regulation of ATP synthase.

In general, ATP synthase synthesizes ATP by a rotary mechanism driven by a transmembrane proton motive force (pmf) produced by the respiratory chain [6, 41, 42]. The NADH, as the electron donor, can transfer electrons to the respiratory chain, and is considered as the major donor to create the proton gradient for ATP synthesis. Therefore, it was reasonable that the deficiency of ATP production caused by deletion of *cg1360* would lead to the accumulation of intracellular reducing equivalents.

Enhancement of L-Valine Production with Deletion of *cg1360* in *C. glutamicum*

C. glutamicum is regarded as a generally-recognized-as-safe (GRAS) bacterium, and has been successfully engineered as an important platform for the production of various amino acids [43–45]. L-valine is synthesized from pyruvate without the requirement of ATP via the four-step enzymatic steps, and NADPH acts as a limiting cofactor in the second step (Fig. 6A) [20, 46]. In view of the above findings that deletion of *cg1360* led to a significant increase in the NAD(P)H levels, we further investigated the effect of deletion of *cg1360* on the production of L-valine. As shown in Fig. 6B, deletion of *cg1360* in the L-valine-producing strain of V-10 resulted in a 23% decrease in cell growth

Fig. 5. Effects of *cg1360* or *cg1361* deletion on the intracellular ATP (A), NADH (B), and NADPH (C) levels.

The ATP content was measured using a firefly luciferase-based ATP assay kit, and the NAD(P)H content was quantified using a spectrophotometric enzymatic cycling method as described previously.

Fig. 6. Effects of *cg1360* deletion on L-valine production in shake flask fermentation.

(A) The biosynthetic pathway of L-valine in *C. glutamicum*. (B) Growth curves of L-valine-producing *C. glutamicum* V-10 strain and its V-10 mutant lacking *cg1360* in shake flask cultures. (C and D) The intracellular NADH and NADPH levels after 48 h or 72 h of fermentation of V-10 and V-10-Δ*cg1360* strains. (E and F) The total L-valine titer and yield after 48 h or 72 h of fermentation of V-10 and V-10-Δ*cg1360* strains. Asterisks indicate significant differences between *C. glutamicum* V-10 and its mutant according to a statistical Student's t test (*, $p < 0.05$).

compared to that in V-10 strain, which was similar to the observations of the Δ*cg1360* mutant in the wild-type background (Fig. 2B). The glucose consumption rate of the V-10-Δ*cg1360* mutant during the exponential growth phase was 28% higher than that of the V-10 strain, implying that the mutant has a stronger carbon metabolism (Fig. S2B). As expected, the levels of intracellular NADH and NADPH in the V-10-Δ*cg1360* mutant increased by 40% and 42% after 72 h of fermentation as compared to the V-10 control (Figs. 6C and 6D), whereas deletion of *cg1360* in the V-10 background strain resulted in an approximately 24% decrease in intracellular ATP concentrations (Fig. S3). The final production of L-valine in the V-10-Δ*cg1360* mutant reached 9.2 ± 0.3 g/l with a yield of 0.078 ± 0.001 g/g

glucose after 72 h of fermentation in shake flasks, which was 14% higher than that of the starting V-10 strain (Figs. 6E and 6F). The improved L-valine titer of the V-10-Δ*cg1360* mutant could be attributed to an overall increase of the intracellular reducing equivalents. The effectiveness of the ATP synthase defects for the production of useful metabolites, such as pyruvate and amino acids, has also been reported for *E. coli* and *C. glutamicum* [16, 20, 40]. A previous study reveals that the combination of an H⁺-ATPase defect with the feedback-insensitive acetohydroxyacid synthase (*ilvBN* products) could enhance L-valine production from 2.54 g/l to 5.47 g/l in the shaking flasks [20]. Consistent with these above characterizations, our results showed the enhancement of L-valine production by deleting the *cg1360* gene in *atp* operon of *C. glutamicum*, suggesting that *cg1360* can be expected to offer a promising alternative candidate from the perspective of energy regulation for strain improvement. This energy deficiency strategy can potentially be applied for the fermentative production of other useful chemicals.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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