Growth promotion in *Corynebacterium glutamicum* by overexpression of the NCgl2986 gene encoding a protein homologous to peptidoglycan amidases

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We previously reported the extracellular production of antibody fragment Fab by *Corynebacterium glutamicum*. In the course of searching for genes which improve the secretion efficiency of Fab, we coincidentally found that the final growth increased significantly when the NCgl2986 gene encoding an amidase-like protein was overexpressed. This effect was observed when cells were grown on the production medium MMTG, which contains high concentrations of glucose and neutralizing agent CaCO₃, but not on MMTG without CaCO₃ or Lennox medium. Not only turbidity but also dry cell weight was increased by NCgl2986 overexpression, although the growth rate was not affected. It was recently reported that the *Mycobacterium tuberculosis* homolog Rv3915 functions as an activator of MurA protein, which catalyzes the initial step of peptidoglycan synthesis. Growth promotion was also observed when the MurA protein was overproduced. His-tagged NCgl2986 protein was purified, but its peptidoglycan hydrolyzing activity could not be detected. These results suggest that NCgl2986 promotes cell growth by activating the peptidoglycan synthetic pathway.

Key Words: amidase-like protein; *Corynebacterium glutamicum*; growth promotion; MurA; NCgl2986; Rv3915

Introduction

The demand of amino acids has been rising annually in many fields for use in the production of human food and dietary supplements, animal feed, cosmetics, and pharmaceuticals. In 1956, researchers at Kyowa Hakko Kogyo Co., Ltd. isolated a new bacterial species named *Micrococcus glutamicus*, which produced the highest levels of glutamate (Kinoshita et al., 1957; Udaka, 1960); it was later renamed as *Corynebacterium glutamicum* (Liebl et al., 1991). The industrial production of glutamic acid by fermentation using *C. glutamicum* was established in 1960 (Hirasawa and Wachi, 2017), followed by the fermentative production of other amino acids including lysine and threonine (Nakayama et al., 1961; Sahm et al., 1996; Sano and Shiio, 1970; Shiio and Nakamori, 1970). *C. glutamicum* has also been widely used for the production of various other metabolites, including D-amino acids, organic acids, diamines, fuels, and aromatic compounds (Becker and Wittmann, 2012; Kogure et al., 2016; Kubota et al., 2016).

Recently, a recombinant protein expression system using *C. glutamicum* strains as an expression host (CORYNEX®) was developed by Ajinomoto Co., Ltd. (Date et al., 2006; Kikuchi et al., 2002, 2003, 2009). Several host strains, which showed improved protein secretion ability, were developed using conventional genetic breeding. One of these strains was YDK010 (Kikuchi et al., 2002, 2009). Using YDK010 as a host, we successfully produced the active form of antibody fragment Fab into the culture medium (Matsuda et al., 2014). We also performed gene screening to improve the productivity of Fab when overexpressed or deleted (Matsuda et al., 2014). During this screening, it was found that the cell growth of the Fab producer strain YDK010/pPKStrastFabHL was significantly promoted when the NCgl2986 gene was overexpressed. NCgl2986 encodes an amidase-like protein (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003;
Nishio et al., 2017; CoryneRegNet: https://coryneregnet.compbio.sdu.dk/v6/index.html), but its precise function has not yet been analyzed in C. glutamicum. In this study, we investigated the effect of NCgl2986 overproduction on the cell growth of a wild-type C. glutamicum strain ATCC 13869.

C. glutamicum has a unique cell wall organization, composed of peptidoglycan, arabinogalactan, and mycolic acid. The peptidoglycan is composed of repeating sugar units, N-acetylmuramic acid (GlcNAc) and N-acetylmuramic acid (MurNAc), linked by \( \beta \)-1,4-bonds and crosslinked via a peptide bridge. There are two main types of peptidoglycan hydrolases: peptide-cleaving enzymes, such as N-acetylmuramoyl-L-alanine amidase, carboxypeptidase, and glycan-cleaving enzymes, such as N-acetylmuramosidases and N-acetylt-\( \delta \)-o-muramidases. These hydrolase enzymes are involved in the regulation of cell growth, cell division, and cell lysis (Fireczuk and Bochtler, 2007; Foster, 1992; Vollmer et al., 2008). The roles of amidase enzymes have already been characterized in several bacteria. In E. coli, amidases are reportedly involved in the separation of daughter cells after septum formation. E. coli possesses six amidase genes: amiA, amiB, amiC, ampD, amiD, and one biochemically-characterized 39-kDa protein (van Heijenoort, 2011). Studies showed that amiA amiB amiC triple mutant formed chain-like filaments (Heidrich et al., 2001; Priyadarshini et al., 2006). This shape formation is considered to result from failed daughter cell separation. It has also been reported that amidases are involved in mother cell lysis for the release of mature endospores in Bacillus subtilis (Nugroho et al., 1999; Smith and Foster, 1995). Mother cells are unable to lyse at the end of sporulation when the cwlC and lycC genes are inactivated.

C. glutamicum possesses only one peptidoglycan amidase-like protein, which is encoded by the NCgl2986 gene. In this report, the role of the NCgl2986 gene in C. glutamicum cell growth was investigated. We found that overexpression of NCgl2986 promotes cell growth by enhancing cell mass synthesis, most likely by a mechanism other than amidase activity.

### Materials and Methods

#### Bacterial strains and culture conditions.

C. glutamicum wild-type strain ATCC 13869 was used in this study. The E. coli strain JM109 (endA1 recA1 gyrA96 thi-1 hsdR17 (rK-, mK-)] relA1 supE44 Δ(lac-proAB)F’ [traD36 proA’ B’ lacI’ lacZAM15]) was used for genetic manipulation and BL21(DE3) (F’ ompT gal dcm lon hsdSB (rK-, mK-)] DE3) for protein production. Cells were grown in either MMTG medium, which is conventionally used for the production of recombinant proteins by the CORNEX system (Kikuchi et al., 2003), or Lennox (L) medium. MMTG medium contained 80 g/L of glucose, 1.2 g/L of soybean hydrolysate (as total nitrogen), 1 g/L of KH₂PO₄, 30 g/L of (NH₄)₂SO₄, 0.4 g/L of MgSO₄·7H₂O, 0.01 g/L of MnSO₄·5H₂O, 0.01 g/L of FeSO₄·7H₂O, 0.2 mg/L of vitamin B₁, and 0.3 mg/L of biotin. CaCO₃ (0.25 g in 5 mL culture) was added as a pH buffer when indicated. L medium was composed of 1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.2). Cells were grown at 30°C and 37°C for C. glutamicum and E. coli, respectively. The appropriate antibiotic was added to the culture when needed: 5 μg/mL of chloramphenicol or 10 μg/mL of kanamycin for C. glutamicum, and 10 μg/mL of chloramphenicol or 20 μg/mL of kanamycin for E. coli. Cell growth was monitored by measuring OD₆₆₀. Samples from MMTG medium containing CaCO₃ were diluted 50-fold with 0.1 M HCl to dissolve CaCO₃ prior to OD₆₆₀ measurement. Counting of cell number was done by use of a Thoma cell counting chamber after appropriate dilution.

#### Growth assay in a jar fermenter.

Cells were grown at 30°C with agitation in 400 mL of MMTG medium in a 1 L vessel of a jar fermenter (Able BMZ-P, Able & Biott, Tokyo, Japan). Then, 8 mL of overnight culture was inoculated into the fresh MMTG medium. The pH was maintained at 7.2 by adding 2 M NaOH solution using a pH-stat apparatus. Samples were collected every 24 h to analyze several parameters, including OD₆₆₀, glucose concentration, and dry cell weight. Glucose concentrations were measured using a Biotech Analyzer with a glucose oxidase sensor (AS-310, Sakura Seiki, Tokyo, Japan).

#### Plasmid construction.

A 1295-bp DNA fragment containing the NCgl2986 gene was PCR-amplified using a primer set 5’-TAACTGGTATTCTTACTCGAGTCA-3’ and 5’-GACTCTTCAAGAGATGAGCGCGGAT-3’. The amplified fragment was digested with SmaI and then cloned at the SmaI site of pVC7 (Kikuchi et al., 2003). The resulting plasmid was named pVC7-NCgl2986.

A 1212-bp DNA fragment containing the NCgl2986 gene was PCR-amplified using a primer set 5’-AGAGTCGACATGAATGATGGAAGGAG-3’ and 5’-ATTTGAATCCGCGCCGCTCTGATTG-3’, and ATCC 13869 genomic DNA as a template. A 5227 bp DNA fragment was PCR-amplified using a primer set of 5’-CGGTCGACGGTGATATCTCTCTCTTSTAAG-3’ and 5’-TATGATCCACCCACACCCACACCACCAAC-3’, and a pET28b(+)-plasmid (Novagen, Darmstadt, Germany) as a template. The amplified NCgl2986 fragment was digested with Sall and EcoRI, and then cloned at the Sall site of pET-28b(+). The resulting plasmid was named pET-NCgl2986-His, with the His-tag sequence fused at the C-terminal of NCgl2986.

A 1257-bp DNA fragment containing the murA gene was PCR-amplified using a set of primers 5’-G G G C G A A T T C A G G A G A A C C G TGTAAGAGCAGAGTTTTAGTCAGT-3’ and 5’-CGTGTACGTAGAGGAAACCCCTTTCCGATGG-3’. The amplified DNA fragment was digested with EcoRI and Sall and inserted into the EcoRI-Sall site of the pECo plasmid (Sato et al., 2008). The resulting plasmid was named pECo-MurA.

#### Scanning electron microscopic observation.

Cells were fixed with 2.5% glutaraldehyde and applied on a cover slip coated with 0.1% poly-L-lysine. Samples were then stained with osmium tetroxide (1% OsO₄ in 0.1 M sodium phosphate buffer, pH 7). Serial dehydration was then carried out using 60%, 70%, 80%, 90%, and 99.5% ethanol. Finally, ethanol was substituted with isoamyl acetate. Samples were dried by supercritical drying method (JCPD-5,
PEPTIDOGLYCAN HYDROLYZING ASSAY IN VITRO.

50 mM Tris-HCl (pH 7).

Peptidoglycan hydrolyzing assay in vitro. Micrococcus luteus cells grown in L medium overnight were harvested and suspended in 50 mM Tris-HCl buffer (pH 7), 300 mM NaCl, and 500 mM imidazole. The protein was eluted with a buffer containing 50 mM Tris-HCl (pH 7), 0.01 mM ZnCl2, and OD555 was adjusted at 1.2. Then, 20 μL of NCgl2986 protein solution (0.3 μg/μL) was added to 80 μL of cell suspension. A positive control of 1 μg/mL of lysozyme solution was used. The mixture was incubated at 30°C and the decrease in OD555 was measured after incubation for 60 min.

Purification of His-tagged NCgl2986 protein. The BL21(DE3) cells carrying the pET-NCgl2986-His plasmid were grown in L medium for 2–3 h until OD660 reached 0.4–0.8. Production of NCgl2986-His was induced by 1 mM IPTG for 2 h at 25°C. Bacterial cells were harvested and suspended in 50 mM Tris-HCl buffer (pH 7). Cells were then disrupted by sonication. Unbroken cells were removed by centrifugation (5,000 × g for 10 min). The cytoplasmatic fraction was then separated from the membrane fraction by ultracentrifugation (40,000 × g for 30 min). His-tagged NCgl2986 was purified using a Ni-NTA spin column (QIAGEN, Venlo, Netherlands) and the protein was eluted with a buffer containing 50 mM Tris-HCl (pH 7), 300 mM NaCl, and 500 mM imidazole. The protein solution was then subjected to a PD-10 gel filtration column (GE healthcare, Chicago, IL) and was eluted with 50 mM Tris-HCl (pH 7).

Peptidoglycan hydrolyzing assay in vitro. Micrococcus luteus cells grown in L medium overnight were harvested and suspended in 50 mM Tris-HCl buffer (pH 7) containing 0.01 mM ZnCl2, and OD555 was adjusted at 1.2. Then, 20 μL of NCgl2986 protein solution (0.3 μg/μL) was added to 80 μL of cell suspension. A positive control of 1 μg/mL of lysozyme solution was used. The mixture was incubated at 30°C and the decrease in OD555 was measured after incubation for 60 min.

Results

Growth promotion by overexpression of the NCgl2986 gene

We previously reported that the extracellular production of antibody fragment Fab increased when a deletion of the pphla gene encoding a penicillin-binding protein (PBP) 1a, which is involved in cell wall peptidoglycan synthesis, was introduced into the Fab producer strain YDK010/pPKStrastFabHL (Matsuda et al., 2014). Therefore, the effect of genes involved in the peptidoglycan metabolism on Fab production was investigated systematically in this study. A deletion or expression plasmid of a target gene was introduced into the Fab producer strain. Then, cell growth and Fab production were monitored in the MMGT production medium.

Over the course of this screening, it was coincidentally found that the final cell growth increased significantly as the concentration of NCgl2986 seemed to have no significant effect on cell morphology (S-5500, Hitachi Ltd.). Cell length and width were measured manually on photographs.

Effect of NCgl2986 overexpression on C. glutamicum growth in various media.

Cells were grown in MMGT medium with CaCO3 (A), L medium without CaCO3 (B), MMGT without CaCO3 (C), or L medium with CaCO3 (D). Open circle (○), C. glutamicum/pVC7: closed circle (●), C. glutamicum/pVC7-NCgl2986. Cell growth was monitored by measuring OD660. Samples from media containing CaCO3 were diluted 10-fold with 0.1 M HCl to dissolve CaCO3 prior to OD660 measurement. Values represent the means with standard deviations of three independent experiments.

Fig. 1. Effect of NCgl2986 overexpression on C. glutamicum growth in various media.

Growth promotion by NCgl2986 in C. glutamicum

growth promotion was also observed in ATCC 13869. The NCgl2986 overproducer strain and the control strain grew almost similarly during the first 24 h. The growth rate of the control strain then gradually reduced, but the overproducer strain continued to grow over the next 24 h. The final OD660 of the overproducer strain was approximately 1.3 times higher than that of the control strain at 72 h. These results indicate that the overproduction of NCgl2986 enhances cell growth regardless of Fab production. Hence, the wild-type strain ATCC 13869 was used for analysis.

Then a growth assay was performed with a conventional complex medium, L medium, instead of MMGT. Unexpectedly, no growth promotion was observed when cells were grown in L medium (Fig. 1B). When comparing the compositions of MMGT and L, MMGT contained 80 g/L of glucose and CaCO3 as pH buffer, while L contained 1 g/L of glucose and no CaCO3. Therefore, the growth assay was carried out with MMGT medium without CaCO3 and L medium with CaCO3. As a result, it was shown that growth promotion was observed only when cells were grown in MMGT with CaCO3 (Fig. 1).
control and 2.36 ± 0.11 μm in the overproducing strain. The average cell width was almost the same: 0.86 ± 0.02 μm in the control and 0.86 ± 0.01 μm in the overproducing strain. These results indicate that the increase in turbidity by NCgl2086 overproduction was due to the increase in both the cell number and cell size.

A growth assay was also performed using a jar fermenter. Cells were grown at 30°C with agitation in 400 mL of MMTG medium in a 1 L vessel of a jar fermenter. pH was maintained at 7.2 by adding 2 M NaOH solution using a pH-stat apparatus. A. Cell growth (OD$_{660}$) and glucose consumption. Open circle (●), OD$_{660}$ of _C. glutamicum_/pVC7; closed circle (○), OD$_{660}$ of _C. glutamicum_/pVC7-NCgl2986; open triangle (▲), glucose concentration of _C. glutamicum_/pVC7; closed triangle (▼), glucose concentration of _C. glutamicum_/pVC7-NCgl2986. B. Dry cell weight. Open circle (●), _C. glutamicum_/pVC7; closed circle (○), _C. glutamicum_/pVC7-NCgl2986. Values represent the means with standard deviations of three independent experiments.
Growth promotion by NCgl2986 in *C. glutamicum*

at 7.2 by adding a 2 M NaOH solution using a pH-stat apparatus. OD$_{660}$, residual glucose concentration, and dry cell weight were monitored for 72 h. As shown in Fig. 3, growth promotion was also observed when the pH was controlled with NaOH instead of CaCO$_3$. The final OD$_{660}$ of the overproducer strain was approximately 1.6 times higher than that of the control. The final dry cell weight was also approximately 1.8 times higher in the overproducer strain than in the control according to the increases in OD$_{660}$. Interestingly, the glucose consumption rates were almost the same between the two strains throughout cultivation. The final growth yields [dry cell mass (g)/glucose consumed (g)] were 0.13 ± 0.02 for the overproducer and 0.06 ± 0.01 for the control.

**Peptidoglycan hydrolyzing activity of NCgl2986**

In order to examine whether *C. glutamicum* NCgl2986 has amidase activity, the His-tagged NCgl2986 protein was purified. NCgl2986-His was successfully purified by Ni affinity chromatography nearly homogenously (Fig. 4A). Using *M. luteus* cells as a substrate, peptidoglycan hydrolyzing activity was assayed, but no activity was detected (Fig. 4B).

**Growth promotion by overproduction of MurA**

During the course of our analysis, Boutte et al. (2016) reported that Rv3915, the most closely related gene of NCgl2986, interacts physically and genetically with MurA, a protein involved in the first step of peptidoglycan synthesis (Du et al., 2000). Thus, we assumed that *C. glutamicum* MurA overexpression could mimic NCgl2986 overexpression. To test this hypothesis, the MurA gene was overexpressed in *C. glutamicum* ATCC 13869. As expected, MurA overproduction caused a similar growth promotion when cells were grown in MMTG medium, but not in L medium, as with NCgl2986 overproduction (Fig. 5). An increase in cell number (16%) and cell length (14%) was also observed, as in the case of NCgl2986 overexpression (data not shown).

It has also been reported that the over-activation of MurA causes increased sensitivity to multiple classes of antibiotics in *M. tuberculosis* (Boutte et al., 2016). Therefore, the antibiotic sensitivity of NCgl2986- and MurA-overproducing strains was examined. As shown in Fig. 6, the growth promoting effects were almost completely counteracted by the addition of lower concentrations of ampicillin, an inhibitor of peptidoglycan synthesis, in the overproducers (Figs. 6B and 6E), and growth was more...
severely suppressed in the overproducers than in the controls by higher concentrations of ampicillin (Figs. 6C and 6F). A similar effect was also observed by rifampicin (RNA synthesis inhibitor) and sparfloxacin (DNA synthesis inhibitor) (data not shown).

Discussion

In this study, we investigated the effect of NCgl2986 overproduction on C. glutamicum cell growth. We demonstrated that the overproduction of this protein significantly increased final cell growth. The increase in turbidity was accompanied with the increase in both cell number and cell size. Consequently the final dry cell weight increased. This suggests that the overproduction of NCgl2986 promotes cell growth by enhancing cell mass synthesis.

The NCgl2986 gene encodes a protein homologous to peptidoglycan amidases; that is, N-acetylmuramoyl-L-alanine amidase. The most closely related gene of NCgl2986 is the Rv3915 of M. tuberculosis (52% identity), which is also designated as cwlM (cell wall lytic enzyme of M. tuberculosis). NCgl2986 and Rv3915 have two peptidoglycan binding domains in their N-terminal halves and an amidase domain in the C-terminal halves. Since amidase hydrolyzes peptidoglycan outside of the cytoplasmic membrane, it should contain a signal sequence for protein secretion. However, no such signal sequence was predicted for Rv3915 or NCgl2986.

The study of Rv3915 has provided contradictory findings, where one study showed that the Rv3915 had peptidoglycan hydrolyzing activity (Deng et al., 2005), while another reported that the Rv3915 was a cytoplasmic protein, which regulates the enzymatic activity of the MurA protein (Boutte et al., 2016). In this study, we could not detect the peptidoglycan hydrolyzing activity of NCgl2986, at least under our experimental conditions. NCgl2986 lacks two residues out of four conserved Zn$^{2+}$-coordinating residues, which have been shown to be required for amidase activity in related proteins (Shida et al., 2001) (see Supplementary Fig. S1). This is a similar situation to M. tuberculosis Rv3915. It has been reported that Rv3915 is essential for M. tuberculosis growth (Sassetti et al., 2003; Zhang et al., 2012). To determine if Rv3915 requires amidase activity for its essential function, Boutte et al. (2016) mutated the other two residues that coordinate the Zn$^{2+}$. The mutant Rv3915 lacking all four Zn$^{2+}$-coordinating residues could complement the M. tuberculosis cell growth, suggesting that amidase activity is not required for its essential function. Our attempts to construct a disruptant of NCgl2986 failed (data not shown), suggesting that NCgl2986 is also essential for C. glutamicum growth. Further investigation is necessary to determine if NCgl2986 has amidase activity or not.

Rv3915 was found to be phosphorylated by a protein kinase PknB when cells have plenty of nutrients, and the phosphorylated form of Rv3915 bound to MurA and activated its enzymatic activity (Boutte et al., 2016). The essentiality of Rv3915 could be attributed to its regulatory function on MurA. MurA is also reported to be essential for Mycobacterium growth (Griffin et al., 2011; Xu et al., 2014).

Moreover, the overproduction of MurA and NCgl2986 caused a similar growth promotion in C. glutamicum when grown in MMTG medium, which contains high concentrations of glucose. In addition, NCgl2986- and MurA-overproducing strains showed increased sensitivity to antibiotics. These findings suggest that NCgl2986 promotes cell growth by activating MurA rather than functioning as an amidase.

Cell growth is mainly characterized by two parameters: growth rate and growth yield. Whether these two are independent of each other, or are correlated in some way, has not yet been fully elucidated (Lele and Watve, 2014). In our study, growth yield increased significantly as a result of NCgl2986 overproduction; however, the growth rate was not affected. Both the NCgl2986 overproducer strain and the control strain grew at similar levels during the first 24 h. Thereafter, the growth rate of the control strain gradually fell while the overproducer strain continued to grow over the course of the following 24 h (Figs. 1 and 3). NCgl2986 overproduction appears to delay the timing of the transition from the exponential growth phase to the stationary phase and may also cause the continuous biosynthesis of peptidoglycan via the activation of MurA at the same time that the wild-type cells transit to the stationary phase, which consequently results in continuous cell division. That is, growth rate and growth yield are independently regulated in C. glutamicum. However, the molecular mechanism by which the overproduction of NCgl2986 or MurA promotes cell growth in C. glutamicum remains to be elucidated.

Carbon flux from substrates to cell mass synthesis is often the greatest cause of reduced productivity during the fermentation processes. In the case of Fab production, NCgl2986 overproduction increased the cell mass synthesis but decreased Fab productivity (data not shown). Controlling the expression levels of NCgl2986 could be a novel technique for improving productivity by suppressing cell mass synthesis.

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Supplementary Materials

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/gam).

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