농학석사학위논문

*Burkholderia glumae*의 quorum sensing에 의한 methionine 생합성 유전자의 발현 조절

Regulation of methionine biosynthesis genes by quorum sensing in *Burkholderia glumae*

2014년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

정 혜 성

A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

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BY

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The Graduate School of Seoul National University
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지도교수 황 인 규

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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Regulation of methionine biosynthesis genes by quorum sensing in *Burkholderia glumae*

UNDER THE DIRECTION OF DR. INGYU HWANG

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

BY
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Regulation of methionine biosynthesis genes by quorum sensing in *Burkholderia glumae*

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ABSTRACT

Burkholderia glumae is a Gram-negative bacterium that produces a broad-spectrum phytotoxin, called toxoflavin. Toxoflavin production is dependent on the TofI/TofR quorum sensing system, which is mediated by *N*-octanoyl homoserine lactone (C8-HSL). C8-HSL is biosynthesized by TofI and its cognate receptor is TofR. The TofR/C8-HSL complex activates the expression of toxoflavin biosynthesis and an IclR-type transcriptional regulator gene, *qsmR*.

Quorum sensing is a cell-to-cell signaling mechanism that refers to the

ability of bacteria to respond to small diffusible autoinducers. When an autoinducer reaches a critical threshold, the bacteria detect and respond to the signals to alter gene expressions. Autoinducer is a byproduct of the activated methyl cycle (AMC), which is a central metabolic pathway responsible for the methylation of cellular components and the recycling of certain sulfur-containing amino acids. The last step of AMC involves the methylation of homocysteine to produce methionine. In *Escherichia coli*, there are two different enzymes, MetH and MetE, that catalyze methionine biosynthesis. MetH is dependent on vitamin B_{12} whereas MetE is not. In *B. glumae*, there are two *metE* homologs, *metE1* and *metE2*, and a *metH* gene is split into two genes, *metH1* and *metH2*.

The objectives of this study are to determine if methionine biosynthesis is regulated by quorum sensing and identify regulation mechanisms of quorum sensing-dependent methionine biosynthesis genes in *B. glumae*. The *metE1, metE2,* or *metH1-2* mutant showed similar growth rates as the wild type strain BGR1 in M9 minimal medium supplemented with 0.2% glucose. The *metE1, metE2,* and *metH1-2* triple mutant was a methionine auxotroph. Autoinducer assay using a *Chromobacterium violaceum* biosensor on TLC plates indicated that there was no significant difference in C8-HSL production between

the methionine biosynthesis gene mutants and the wild-type strain. Vitamin B_{12} bioassay provided indirect evidence for the vitamin B_{12} biosynthesis in *B. glumae*. Expression of *metE1* is up-regulated by quorum sensing as proved by reverse transcription and real-time PCR experiments. Expression of *metE2* was not expressed at a detectable level; however, its expression was activated in the absence of *metH* in a QS-dependent manner. These results indicate that methionine biosynthesis is controlled by quorum sensing; however, its biological meaning is still under investigation.

KEY WORDS : *Burkholderia glumae,* Quorum sensing, Methionine biosynthesis gene, Activated methyl cycle

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Burkholderia glumae is a Gram-negative bacterium which is the causal agent of rice grain rot and seedling rot diseases. Depending on the rice growth stage and environmental conditions such as high temperature and humidity, the disease spreads more easily. This bacterium produces a broad-host range phytotoxin, called toxoflavin, which is essential for virulence (Kim *et al*., 2004). Toxoflavin production is dependent on the TofI/TofR quorum sensing system, which is mediated by *N*-octanoyl homoserine lactone (C8-HSL). C8-HSL is biosynthesized by TofI and its cognate receptor is TofR. The TofR and C8-HSL complex activates the expression of toxoflavin biosynthesis and an IclR-type transcriptional regulator gene, *qsmR* (Kim *et al*., 2007).

Quorum sensing (QS) is a cell dentisity-dependent communication mechanism which is ability of bacteria to respond to the small diffusible molecules, called autoinducer (Fuqua *et al*., 2002). The accumulation of these autoinducer results in signaling that alter gene expression and lead to adaptive response to environment (Winzer *et al*., 2002). Autoinducer is a byproduct of the activated methyl cycle (AMC), which is a central

metabolic pathway responsible for the methylation of cellular components and the recycling of certain sulfur-containing amino acids such as methionine (Parveen *et al*., 2011). Therefore, the AMC plays an important role in bacterial intercellular communication and coordinated gene expression associated with QS-dependent characteristics, including virulence (Winzer *et al*., 2002), motility (Daniels *et al*., 2004), biofilm maturation (Kievit *et al*., 2009), and bioluminescence (Anetzberger *et al*., 2009). The methylation of essential biological molecules is of crucial importance for many key biochemical processes, which have a profound effect on the development and function of an organism (Halliday *et al*., 2010). And methionine is an essential amino acid and is required for a number of important cellular functions, including the initiation of protein synthesis and the biosynthesis of phospholipids and polyamines (Figge, 2006).

The last step of AMC involves the methylation of homocysteine to form methionine, which is a reaction catalyzed by cobalamin-independent methionine synthase or cobalamin-dependent methionine synthase that consequently result in distinctly different responses. The cobalamin-independent methionine synthase, MetE, is a gene product of *metE*, and the cobalamin-dependent methionine synthase,

MetH, is a gene product of *metH* (Cai *et al*., 1989). Both MetE and MetH exist in bacteria, but mammals only possess MetH whereas fungi and plants only utilize MetE (Gruber *et al*., 2001). In *E. coli*, the MetE catalyzes methionine biosynthesis pathway and utilizes 5-methyltetrahydropteroyl-tri-L-glutamate as a methyl donor, whereas the MetH utilizes 5-methyltetrahydrofolate as a methyl donor (Figge, 2006).

The regulation system and several regulatory factors of methionine biosynthesis genes are well documented in *E. coli*. The *metE* and *metH* genes require the MetR regulator protein, the *metR* gene product, in order to be efficiently expressed in *E. coli* (Urbanowski *et al*., 1987). Cai *et al*., previously reported that the MetR protein stimulate the expression of *metE* 16-fold and *metH* 8-fold (Cai *et al*., 1989). It was also noted that the MetR protein autoregulates its own synthesis, and that this reaction is stimulated by homocysteine (Cai *et al*., 1989). In the presence of the MetR protein, homocysteine had different effects on *metE* and *metH* expression. Homocysteine activates *metE* expression, but a negative regulator for *metH* expression (Cai *et al*., 1989). In addition, it is known that the MetJ protein and S-adenosylmethionine repress the expression of *metE*, but they had little or no effect *metH* expression in *E. coli* (Cai *et al*.,

1989). However, the MetJ protein is not present in *B. glumae* and little is known about the regulation of methionine biosynthesis genes in *B. gluame*.

In this study, we focused on how the methionine biosynthesis gene is regulated by quorum sensing in *B. glumae*.

MATERIALS AND METHODS

1. Bacterial strains, plasmids and growth conditions

The bacterial strains used in this study are listed in Table 1. All the strains were cultured in Luria-Bertani (LB) medium and M9 minimal medium with 0.1% of 0.2% D-glucose. The *B.glumae* strain BGR1 and *E. coli* were cultured at 37℃. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; Gentamycin, 20 μg/ml; kanamycin, 50 μg/ml; rifampicin, 50 μg/ml; spectinomycin, 25 μg/ml; and tetracycline, 10 μg/ml.5-Bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-Gal) was used at 50 μg/ml when necessary.

2. Enzyme and DNA manipulation

2. 1. Small-scale plasmid DNA isolation

Small-scale preparation of plasmid DNA from *E. coli* was performed by alkaline lysis method (Sambrook *et al*., 1989). *E. coli* cells were grown in 2 ml LB medium containing the appropriate antibiotics at 37 ℃ with shaking at 250 rpm. The bacterial cells were harvested by centrifugation at 13000 rpm for 1 min. The supernatant was removed and

the bacterial pellet was resuspended with 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl [pH8.0]. 10 mM EDTA [pH8.0]) and 5 µl of RNaseA solution (20 µl/ml) was added. 200 µl of solution II (0.2 N NaOH, 1 % SDS) was added and mixed the contents by inverting the tube several times. Ice-cold solution III (5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, sterile distilled water 28.5 ml) of 150 µl was added and mixed well. Then 500 ul of PCI was added and mixed well, then microcentrifuged for 5 min. After the phenol extraction, 350 ul of the upper aqueous layer was transferred to a clean tube and 700 ul of 95% ethanol was added and mixed well. After incubation at 4 ℃ for 15 min, the DNA pellet was precipitated by centrifugation at 14,000 rpm for 20 min at 4 ℃. The pellet was washed with 70% ethanol and dissolved in 40 µl of TERNase (100 mg/ml RNaseA in Tris-EDTA) and the DNA was stored at -20 ℃.

2. 2. Restriction Enzyme Digestion and agarose gel-electrophoresis

Restriction enzymes, T4 DNA ligase and other related reagents were purchased from Takara (Japan). Assay conditions were as recommended by descriptions of manufacturer. Bacterial DNA was digested with various endonucleases and splited in suitable percentage (0.7 ~ 1.5 %)

of agarose gel (Lonza Seakem) in 0.5×TBE (45 mM Tris-borate, 1 mM EDTA) buffer. DNA was mixed and loaded with gel loading buffer (0.05 % bromophenol blue, 50 % glycerol, 0.9 % SDS). After the electrophoresis, the gel was stained for 15min in 0.5 μl/ml ethydium bromide solution and visualized with ChemiDoc™ XRS+ using Image Lab™ software.

2. 3. Isolation of DNA fragment from agarose gel

DNA elution was performed to get a specific DNA fragment. A AccuPrep® Gel purification Kit (BIONEER, Republic of Korea) was used as described by the manufacturer after the target band had been isolated.

2. 4. Preparation of genomic DNA

Extraction of genomic DNA from *B.glumae* strain BGR1 was performed by the modified lysozyme-sodium dodecyl sulfate (SDS) lysis procedure (Leach *et al*. 1990). Bacteria were cultured in 2 ml of Luria-Bertani medium containing the appropriate antibiotic at 37 ℃ with shaking at 250 rpm. The bacterial cells were harvested by centrifugation. The bacterial pellet was resuspended in 567 μl Tris-EDTA solution (25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]). 30 μl 10 % SDS, 6 μl

proteinase K solution (20 mg/ml) was added and incubated for minimum 1 hour at 37 ℃. 100 μl of 5 M NaCl was added then mixed throughly. 80 μl of CTAB/NaCl solution was added and mixed then incubated at 65 ℃ for 20 min. Extraction was done with 800 μl of 24:1 (v:v) chloroform: isoamyl alcohol then microcentrifuged for 5 min. 650 μl of the aqueous phase was transferred to a fresh tube then another extraction was done with 800 μl of 25:24:1 (v:v:v) phenol: chloroform: isoamyl alcohol then mixed by vigorous shaking during 5min. After microcentrifugation at 14,000 rpm for 5 min, the 500 μl upper aqueous layer was transferred into a clean microcentrifuge tube, then 300 μl of isopropanol was added to extract DNA. The extracted DNA was washed with 70% ethanol and then the pellet was air dried. This pellet was dissolved in 50 ~ 80 μl of TERNase (100 mg/ml RNaseA in Tris-EDTA) and the DNA was stored at -20 ℃.

3. Transposon mutagenesis

The *metE1*, *metE2*, and *metH1-2* which encode methionine biosynthesis genes in *B. glumae* were mutagenized with Tn*3-gusA*, as described by Bonas *et al.* (Bonas *et al.*, 1989). The insertion site and orientation of Tn*3-gusA* in each mutant were mapped using restriction

enzyme digestion analysis and direct sequencing of the plasmid using the primer Tn3gus (5'-CCGGTCATCTGAGACCATTAAAAGA-3'), which allows sequencing out of the Tn*3-gusA*. The mutagenized plasmids that carried Tn*3-gusA* insertions were introduced individually into the parent strain BGR1 by conjugation and marker-exchanged into wild type strain BGR1, and QS-defective mutants BGS2, as described previously (Fellay *et al.*, 1989). All marker-exchanges were confirmed by Southern hybridization analysis.

4. DNA sequencing and data analysis

The DNA sequencing analysis was order to the MACROGEN with appropriate primers. Then the DNA sequence data were analyzed using the BLAST program at the National Center for Biotechnology Institute (Gish and States, 1993), MEGALIGN software (DNA STAR), and GENETYX-WIN software (Software Development, Tokyo, Japan).

5. Autoinducer assay

The QS signal molecule production assay was performed as described previously, with some modification (Shaw *et al*., 1997). All the *B. glumae* strains were cultured in 2 ml LB broth at 37 ˚C for 12 h,

centrifuged, washed twice with sterile water, and subcultured in the M9 + 0.1% glucose medium for 24 h with vigorous shaking. The 1 ml of bacterial cultures were centrifuged at 13,000 rpm for 1 min. 500 μl of supernatant was transferred into fresh tube then 500 μl of ethyl acetate was added to the transferred supernatant. The mixture was then vortexed and centrifuged for 10 min at 13,000 rpm. 450 μl of supernatant was transferred to fresh tube. The samples were dried until the pellet of extracted autoinducer was observable on the bottom of the tube and melt the pellet with 10 μl of dimethyl sulfoxide (DMSO). The extracts were then spotted on a thin-layer chromatography (TLC) plates (Merck), and the chromatograms were developed with methanol/water (70:30, v/v). After development, the solvent was evaporated, and the dried plates were overlaid with soft agar that contained the indicator strain. For the *Chromobacterium violaceum* CV026 indicator strain, a 1 ml overnight culture was used to inoculate 50 ml of LB. After the agar solidified, the overlaid plates were incubated at 28 ˚C in a closed plastic container. Then the plates were air-dried at room temperature for 48 hours. The concentration of C8-HSL produced by each strain was determined by comparison with the standard curve for synthetic C8-HSL at different concentrations.

6. Vitamin B_{12} bioassay

Vitamin B_{12} (cobalamin) production was determined using a modified version of a previously bioassay (Xie *et al.*, 2013). The *B. glumae* strains BGR1 and BGK59 (*toxA* :: Km^r) were cultured in 2 ml LB broth at 37˚C for 12 h, centrifuged, washed twice with sterile water, and subcultured in the M9 + 0.2% glucose medium for 24 h. From this strain culture, the supernatant and cell extract were obtained, respectively. First, the 1 ml of bacterial cultures were centrifuged at 13,000 rpm for 1 min and 100 μl of filter sterilized supernatant was obtained. Second, for cell extract, the 1 ml of bacterial cultures were centrifuged, washed 3 times with sterile water, and re-suspended in 500 μl sterile water prior to sonication to lyse cells. Cellular debris was removed by centrifugation and the cell lysate supernatant was filter sterilized. Then 100 μl of *B. glumae* strain supernatant and cell extract were inoculated to the *Escherichia coli metE* mutant strain JW3805 culture in M9 + 0.2% glucose medium, respectively. For positive control, 5 μl/ml of vitamin B_{12} was added to JW3805 culture and 5 μl/ml of vitamin B_1 was added to all *E. coli* culture.

7. Overexpression and purification of TofR and QsmR

The His-TofR was overexpressed in *E. coli* strain BL21 (DE3), which carries pLysS, with the addition of 1 μM C8-HSL, as described by the manufacturer (Novagen) as described previously (Kim *et al*., 2007). Soluble His-TofR was purified in a buffer that contained 20 mM Tris-HCl (pH 7.7), 1 mM EDTA, 1 mM DTT, 10% glycerol and 100 mM NaCl, using a Ni-NTA spin column as described by the manufacturer (Qiagen). The QsmR-His was overexpressed and purified as for His-TofR overexpression but without C8-HSL.

8. Gel mobility shift assay

The 181-bp upstream region of *metE1* was amplified by PCR using MetE1-F (5'-AGCGCCGTGAGGGTCTGCAG-3') and MetE1-R (5'-TGGTGTCCTCCAGATATGGGCT-3') primers. The DNA fragments were eluted from the agarose gel and labeled with biotin for chemiluminescence using a light-shift chemiluminescent electrophoretic mobility shift assay kit (Pierce Biotechnology). For specific competitor DNA, we used the 242-bp upstream region of a catalase gene E (*katE*) of *B. glumae*, which was amplified using the KEN1 and KEN2 primers as described previously (Kim *et al*., 2004). Purified QsmR-His and

His-TofR (500 nM) were incubated with 2 nM labeled DNA in binding buffer [10 mM Tris-HCl (pH 7.6), 10 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5% (vol vol^{-1}) glycerol, and 1 μg $μl^{-1}$ poly(dI-dC)] for 15 min at 28 ˚C. For the competitor DNA, a 20-fold molar excess of unlabeled target DNA was added to the reaction mixture, along with the extract, before the labeled DNA target was added. The mixture were size fractionated in a nondenaturing 4% polyacrylamide gel, followed by transfer to nitrocellulose membranes and detection of biotin-labeled probes by streptavidin-horseradish peroxidase chemiluminescence.

9. Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR analysis

A total 1 μg of RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Medison, WI) for 3 h at 42 ˚C. PCR reactions were performed using rTaq polymerase (Takara, Shiga, Japan) on a PTC-200 Thermo Cycler (MJ Research, Walthan, MA) with the following conditions : 95 ˚C for 2 min, followed by 25 cycle of 94 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 30 s. The primer pairs used for RT-PCR and real-time RT-PCR are MetE1-F (5'-GAGAACTG

GCGGATGCAGCG-3'), MetE1-R (5'-ATTCCGGCACCACGTAACG A-3'), and MetE1-C (5'-GCCTCCGCCACCTCGTCGAA-3'). RT-PCR products from all samples were analyzed on agarose gels. The 16S rRNA was used as a positive control. Transcriptional levels were determined by SsoFast^{TM} EvaGreen Supermix (BIO-RAD). The thermal cycling parameters were : 95 ˚C for 10 min, followed by 39 cycles of 94 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 30 s. Real-time RT-PCR were repeated three times, and all data were normalized based on the 16S rRNA gene, as a reference, using a Bio-Rad CFX Manager software version 1.6 (BIO-RAD).

10. Viable cell counts

All *B. glumae* derivatives were grown in LB medium at 37 ˚C for 12 hours with shaking and transferred to M9 minimal medium supplemented with 0.2% glucose, and then viable cells were counted every 6 hours up to 24 hours.

RESULTS

1. The *metE1*, *metE2*, and *metH1-2* triple mutant is a methionine auxotroph in *B. glumae*

To identify the importance of methionine biosynthesis genes in viability of *B. glumae*, we have monitored growth of the wild-type and the *metE1*, *metE2*, or *metH1-2* mutants in M9 minimal medium supplemented with 0.2% glucose. The results showed that the single and double mutants of *metE1*, *metE2*, and *metH1-2* have a similar growth rates to the wild-type strain BGR1 (Figure 2). The *metE1*, *metE2*, and *metH1-2* triple mutant was not able to grow in minimal medium (Figure 2), and had growth recovery to the wild-type strain when supplemented with 0.3 mM methionine. This data suggests that only the *metE1*, *metE2*, and *metH1-2* triple mutant can be considered as methionine auxotroph.

2. C8-HSL production of mutants involved in methionine biosynthesis in *B. glumae*

To determine whether the methionine biosynthesis genes were

involved in C8-HSL production in *B. glumae*, we have performed autoinducer extraction and the autoinducer assay of the wild-type strain BGR1 and the *metE1*, *metE2*, or *metH1-2* single and double mutants. The extracted autoinducer from *B. glumae* strains were spotted at TLC plate and the amount of autoinducer was identified by comparing the size of those spots. In M9 + 0.1% glucose medium, the amount of autoinducer of the *metE1 metH1-2* mutant was slightly less than the wild-type and other mutants when grown for 24 hours (Figure 3). This may be an indication that, the *metE2* cannot function as efficiently in autoinducer biosynthesis than that of the other methionine biosynthesis genes.

3. Indirect evidence of the vitamin B_{12} biosynthesis in *B. glumae*

To compare the growth of methionine biosynthesis mutants in *B. glumae* and *E. coli*, we have performed viable cell counts. In the case of *E. coli*, as we have expected, the *metH* mutant strain JW3979 was able to grow on M9 + 0.2 % glucose minimal medium and showed similar growth rate as the wild-type strain BW28357. Inversely, the *metE* mutant strain JW3805 was only able to grow when minimal medium was supplemented with vitamin B_{12} (cobalamin). On the other hand, the

metE1 metE2 double mutant in *B. glumae* showed no differential growth rate in M9 + 0.2 % minimal medium compared to the wild-type strain BGR1 (Figure 4). This results led us to carry out vitamin B_{12} bioassay to identify whether the *B. glumae* can biosynthesize cobalamin or not. The wild-type strain BGR1 and the *toxA* mutant strain BGK59 were cultivated in M9 + 0.2% glucose minimal medium to obtain cell extract and culture supernatant. Then cell extract and culture supernatant were inoculated to the *E. coli metE* mutant strain JW3805 culture. As a result, the *E. coli metE* mutant strain JW3805 culture that supplemented with cell supernatant were not able to grow. In contrast, the *E. coli metE* mutant strain JW3805 culture with cell extract addition showed moderate growth (Table 3). This result can be explained by the ability of methionine biosynthesis in *B. glumae*.

4. Quorum sensing activates the *metE1* expression at transcriptional level

As RNAseq analysis data showed that the expression of *metE1* is quorum sensing-dependent, real-time PCR was performed to compare gene expression levels in the wild-type and the quorum sensing dificient

mutants. The *metE1* was expressed approximately 13-fold more in the wild-type strain BGR1 than the *tofI* mutant strain BGS2 and the *qsmR* mutant strain BGS9 in Luria-Bertani (LB) medium. When the *tofI* mutant strain BGS2 was supplemented with 1 μM C8-HSL, the expression level of *metE1* was recovered to the level of the wild-type strain BGR1. And BGS9C, complemented with *qsmR*, recovered *metE1* expression level about 70% to the wild-type strain BGR1 level. The expression level of *tofI qsmR* double mutant, QGMS9, was lower than that of the *tofI* mutant strain BGS2 and the *qsmR* mutant strain BGS9. The *tofI qsmR* double mutant strain QGMS9 recovered *metE1* expression level to the *qsmR* mutant strain BGS9 when 1 μM C8-HSL was supplemented (Figure 5A). This indicated that the *metE1* expression is regulated by quorum sensing.

5. TofR and QsmR bind to the *metE1* promoter region

To determine whether TofR and QsmR bind directly to the *metE1* promoter region to regulate its expression, gel mobility shift assay was performed with the purified His-TofR, QsmR-His, and the *metE1* promoter region. The His-TofR at 500 nM bound to the promoter region of the *metE1*, but not to the *katE* promoter region (Figure 5B). It was the

same in the case of QsmR-His binding to *metE1* promoter region at 500 nM (Figure 5C). These results suggest that TofR and QsmR are specifically bind to the *metE1* promoter region and consequently end up with a direct regulation of its expression.

6. Expression of *metE2* is dependent on the presence of *metH*

Since RNAseq analysis data indicated a low expression of *metE2*, a polymerase chain reaction with reverse transcription (RT-PCR) analysis were carried out to investigate *metE2* expression level *in vitro*. The results demonstrated that *metE2* was not expressed at a detectable level in wild-type strain BGR1 and *metE1* mutant strain though, its expression was activated in the *metH* mutant strain. In addition, an expression of *metE2* in the *metH* mutant strain is higher than that of the *tofI metH* double mutant strain (Figure 6). This data proves that the expression of *metE2* is activated in the absence of *metH* in a QS-dependent manner.

DISCUSSION

Methionine is one of the sulfur-containing proteinogenic amino acids that is essential in mammals, plants, and bacteria and is involved in an activated methyl cycle (AMC) which is important in many biosynthetic steps as a methyl group donor (Figge, 2006 ; Parveen *et al*., 2011). The reaction of methionine biosynthesis is catalyzed by two different enzymes, one of which is cobalamin-independent enzyme MetE and the other is cobalamin-dependent enzyme MetH (Cai *et al*., 1989). The studies of methionine biosynthesis and its regulation in *E. coli* are well-documented and characterized, but little is known about the regulatory mechanism of methionine biosynthesis genes in *B. glumae*. In this study, we showed the regulation of methionine biosynthesis gene, *metE1*, in *B. glumae* is regulated by QS and a binding of TofR and QsmR to *metE1* promoter region in order to regulate its expression. In real-time PCR, the expression level of *metE1* of the wild-type strain BGR1 was approximately 15-fold higher than the QS-defective mutants BGS2 and BGS9. To determine which regulator binds to the *metE1* promoter region, we carried out the gel mobility shift assay and concluded that TofR and

QsmR bind to the *metE1* promoter region, respectively. However, the condition where both of the TofR and QsmR present *in vivo* is not clear to draw a conclusion in its reaction pattern. For that reason, an extensive study in determining specific mode of action is needed. Moreover, a fundamental reason why an expression of *metE1* is regulated by QS in *B. glumae* is yet to be discovered.

The expression level of *metE2* was activated in *metH* mutant background in a QS-dependent manner. In agarose gel electrophoresis of RT-PCR, known as RNAseq data, the expression level of *metE2* was not detectable in the wild-type strain BGR1, the QS-defective mutant strain BGS2, and the *metE1* mutant strain. But in the *metH* mutant background, the *metE2* had a significant increase in its expression. González *et al*., who examined the activity of MetE and MetH in *E. coli* showed that MetH activity is approximately 50-times higher compared to the MetE enzyme (González *et al*., 1996). Hence, we may hypothesize as follow : without highly efficient *metH*, methionine biosynthesis reaction time will be slow and consequently, the methionine will be insufficient. At that point, such limitation will lead to an increase in homocysteine as a substrate in methionine biosynthesis pathway and the *metE2*, which has low efficiency, will maximize its usage in methionine biosynthesis

pathway. As a result, the *metE2* expression seems to be increased, as observed. To elucidate the activation of the *metE2* expression in *metH* mutant background more clearly, it is necessary to measure the homocysteine level in the wild-type strain BGR1 and the *metH* mutant strain.

As discussed in the above, the *metE* in *B. glumae* is constituted by two homologs and the *metH* is split into two pieces. Unlike *B. glumae*, *Burkholderia mallei*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* have a single *metE* and *metH* gene. The reason why methionine biosynthesis genes are present in multiple copies in the genome of *B. glumae* is yet to be found. There is only a supposition that the construction of methionine biosynthesis genes is involved in conserving energy to adapt environment efficiently. Therefore an investigation is needed to find out its biological meaning and its relevant advantages that generated by splitting methionine biosynthesis genes.

In conclusion, we have constructed the mutants of *metE1*, *metE2*, or *metH1-2* to identify regulation of methionine biosynthesis genes in *B. glumae* in this study. We have established that only *metE1*, *metE2*, and *metH* triple mutant was methionine auxotroph in M9 minimal medium

supplemented with 0.2% glucose. The *metE2* was less efficient in synthesis of autoinducer than the other methionine biosynthesis genes. The expression of *metE1* was regulated by QS and the expression of *metE2* was dependent on the presence of *metH*. To identify additional biological meanings of regulation of methionine biosynthesis genes in *B. glumae*, studies of mechanisms and its biochemical analysis are needed.

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Table 1. Bacterial strains

Strain or plasmid	Characteristics	Source or reference
Escherichia coli		
DH5α	F^- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *end*A1 *recA1*	Gibco BRL
	*hsd*R17 ($r_K^- m_K^+$) *deo*R *thi*-1 *sup*E44 λ^- *gyr*A96 *rel*A1	
S17-1	Tra^+, *recA*, Sp^R	Simon *et al*. (1983)
C2110	*polA*, Nal^R	Stachel *et al*. (1985)
HB101	F^- *mcrB mrr hsd*S20($r_B^- m_B^-$) *rec*A13 *leu*B6 *ara*-14 *pro*A2	Gibco BRL
	*lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^R) *sup*E44 λ^-	
BW28357	Wild-type, Δ(*ara*D-*ara*B)567 Δ*lac*Z4787(::*rrn*B-3) λ^- Δ(*rha*D-*rha*B)568 *hsd*R514	Zhou *et al*. (2003)
JW3805	Δ(*ara*D-*ara*B)567 Δ*lac*Z4787(::*rrn*B-3) λ^- rph^{-1} Δ*metE*774::kan Δ(*rha*D-*rha*B)568 *hsd*R514, Km^R	Baba *et al*. (2006)
JW3979	Δ(*ara*D-*ara*B)567 Δ*lac*Z4787(::*rrn*B-3) λ^- rph^{-1} Δ*metH*786::kan Δ(*rha*D-*rha*B)568 *hsd*R514, Km^R	Baba *et al*. (2006)

Table 1-*Continued*

Burkholderia glumae		
BGR1	Wild-type, Rif^R	Jeong *et al*. (2003)
BGS2	BGR1 *tofI*::Ω, Sp^R	Kim *et al*. (2004)
BGS9	BGR1 *qsmR*::Ω, Sp^R	Kim *et al*. (2007)
BME1	BGR1 *metE1*::Tn*3-gusA* 36, Km^R	This study
BME3	BGR1 *metE2*::Tn*3-gusA* 111, Km^R	This study
BMH1	BGR1 *metH1-2*::Ω, Sp^R	This study
BME12	BGR1*metE1*::Tn*3-gusA* 36 / *metE2-metR2*::Gm^R, Km^R, Gm^R	This study
BE1H12	BGR1 *metE1*::Tn*3-gusA* 36 / *metH1-2*::Ω, Km^R, Sp^R	This study
BE2H12	BGR1 *metE2*::Tn*3-gusA* 111 / *metH1-2*::Ω, Km^R, Sp^R	This study
BERH12	BGR1*metE1*::Tn*3-gusA* 36 / *metE2-metR2*::Gm^R / *metH1-2*::Ω, Km^R, Sp^R, Gm^R	This study
BGK59	BGR1 *toxA* :: Tn*3-gusA* 59, Km^R	Kim *et al*. (2004)

Table 2. Growth of methionine biosynthesis mutants in M9 + 0.2% glucose medium in the presence and absence of methionine

Strains	M9 + 0.2% glucose	M9 + 0.2% glucose + 0.3mM Met
BGR1 (wild-type)	Full growth	Full growth
BGR1 (*metE1* :: Tn*3*-*gusA* 36)	Full growth	Full growth
BGR1 (*metE2* :: Tn*3*-*gusA* 111)	Full growth	Full growth
BGR1 (*metH1-2 :: Ω)*	Full growth	Full growth
BGR1 (*metE1* :: Tn*3*-*gusA* 36 / *metE2 – metR2* :: Gm^r)	Full growth	Full growth
BGR1 (*metE1* :: Tn*3*-*gusA* 36 / *metH1-2 :: Ω)*	Full growth	Full growth
BGR1 (*metE2* :: Tn*3*-*gusA* 111 / *metH1-2 :: Ω)*	Full growth	Full growth
BGR1 (*metE1* :: Tn*3*-*gusA* 36 / *metE2 – metR2* :: Gm^r / *metH1-2 :: Ω)*	No growth	Full growth

Table 3. Vitamin B_{12} bioassay

Strain	Growth rate in M9 + 0.2% glucose
E. coli JW 3805 (∆ *metE* :: Km^r) (negative control)	-
E. coli JW 3805 (∆ *metE* :: Km^r) + BGR1 supernatant 100 ul	-
E. coli JW 3805 (∆ *metE* :: Km^r) + BGR1 cell extract 100 ul	+
E. coli JW 3805 (∆ *metE* :: Km^r) + BGK59 *(toxA* :: Km^r) supernatant 100 ul	-
E. coli JW 3805 (∆ *metE* :: Km^r) + BGK59 (*toxA* :: Km^r) cell extract 100 ul	+ +
E. coli JW 3805 (∆ *metE* :: Km^r) + Vitamin B_{12} (positive control)	+ + +

- : no growth
+ : very slow growth
++ : slow growth
+++ : fast growth

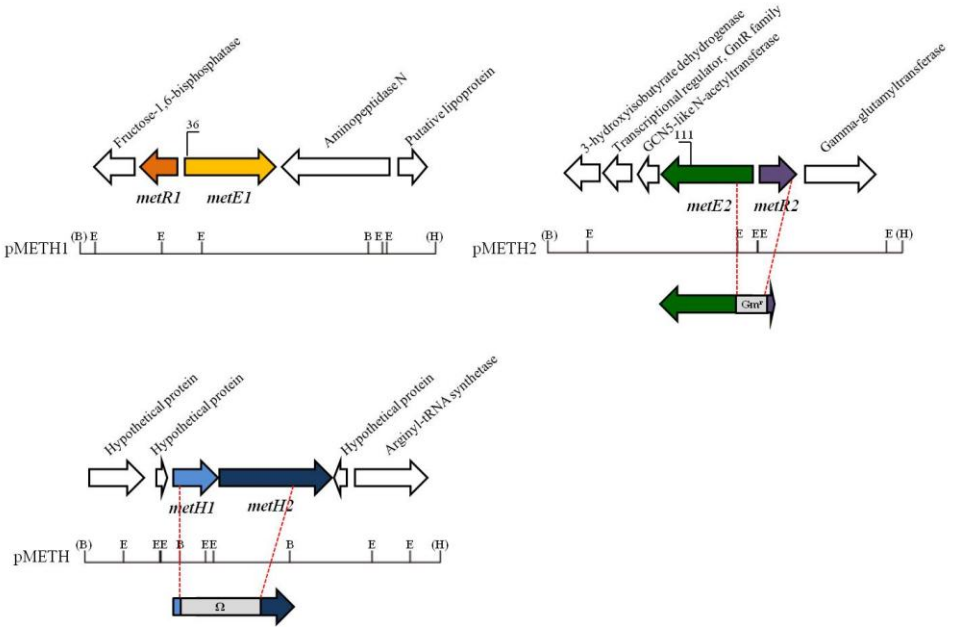


Figure 1. Genetic organization of methionine biosynthesis genes in *B. glumae.* Vertical bars in the maps indicate the positions and orientations of the Tn*3-gusA* insertions. B, *Bam*HI ; E, *Eco*RI ; H, *Hin*dⅢ.

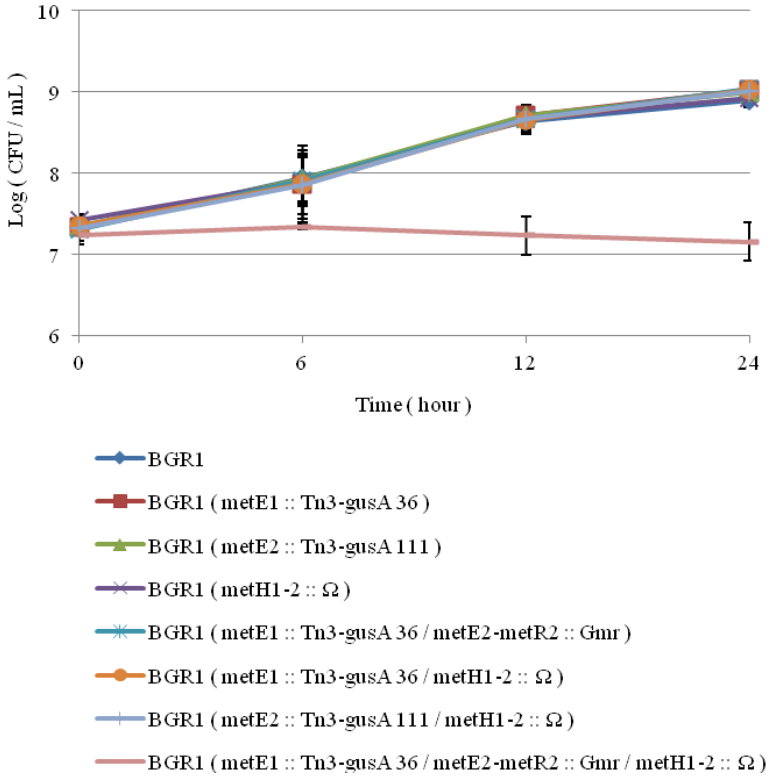
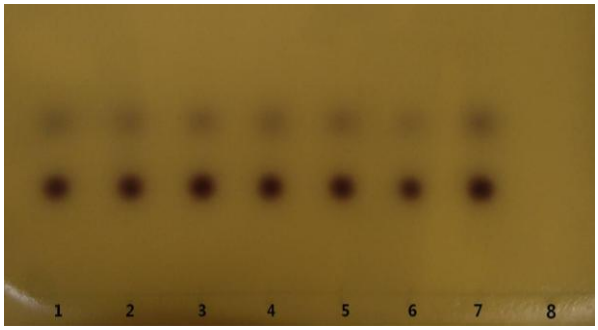
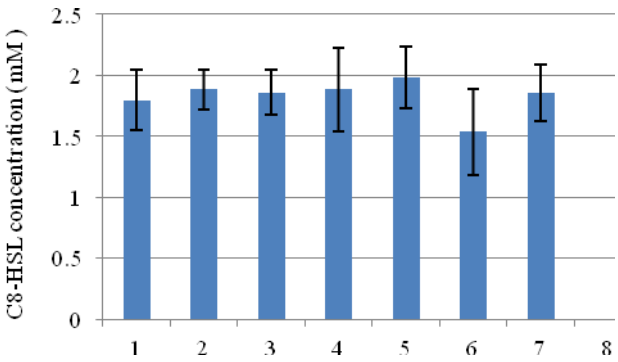


Figure 2. Growth curves of BGR1 and methionine biosynthesis mutants in M9 minimal medium supplemented with 0.2% glucose. The *metE1*, *metE2*, or *metH1-2* mutants showed similar growth rates as the wild-type strain BGR1. Only the *metE1*, *metE2*, and *metH1-2* triple mutant was not able to grow in minimal medium.

A



B



1. BGR1
2. BGR1 (*metE1* :: Tn*3*-*gusA* 36)
3. BGR1 (*metE2* :: Tn*3*-*gusA* 111)
4. BGR1 (*metH1-2* :: Ω)
5. BGR1 (*metE1* :: Tn*3*-*gusA* 36 / *metE2*-*metR2* :: Gm^r)
6. BGR1 (*metE1* :: Tn*3*-*gusA* 36 / *metH1-2* :: Ω)
7. BGR1 (*metE2* :: Tn*3*-*gusA* 111 / *metH1-2* :: Ω)
8. BGS2

Figure 3. C8-HSL production of mutants involved in methionine biosynthesis in *B. glumae*. (A) Thin layer chromatography analysis using *C. violaceum* CV026. (B) Based on the standard curves, the amount of autoinducer was identified by comparing the size of spots.

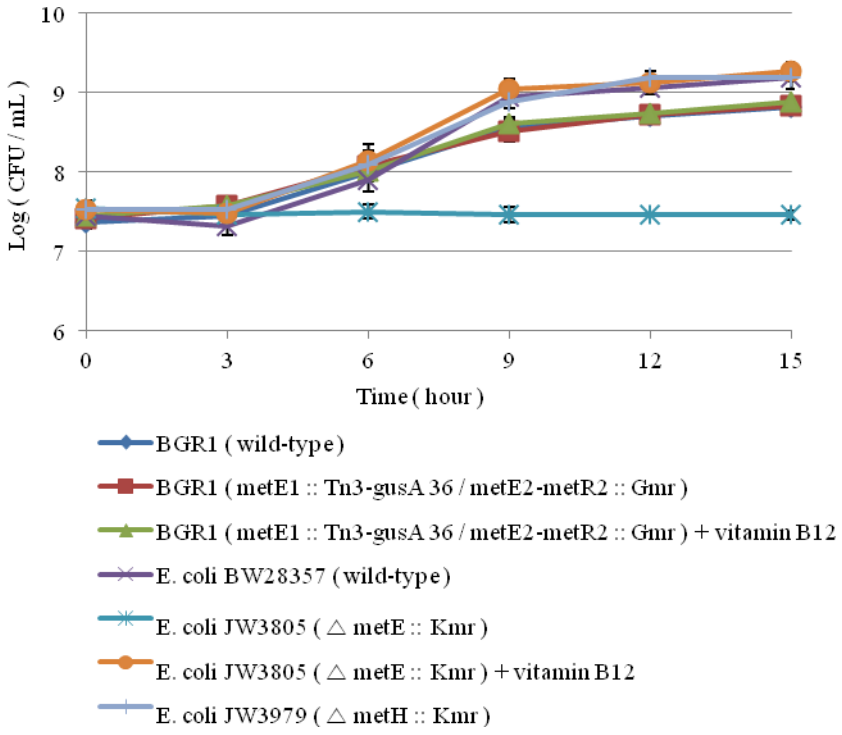


Figure 4. Growth of the *metE* mutants in *B. glumae* and *E. coli*. Unlike the *E. coli*, the *metE* mutant in *B. glumae* showed no differential growth rate in M9 + 0.2% minimal medium compared to the wild-type strain BGR1.

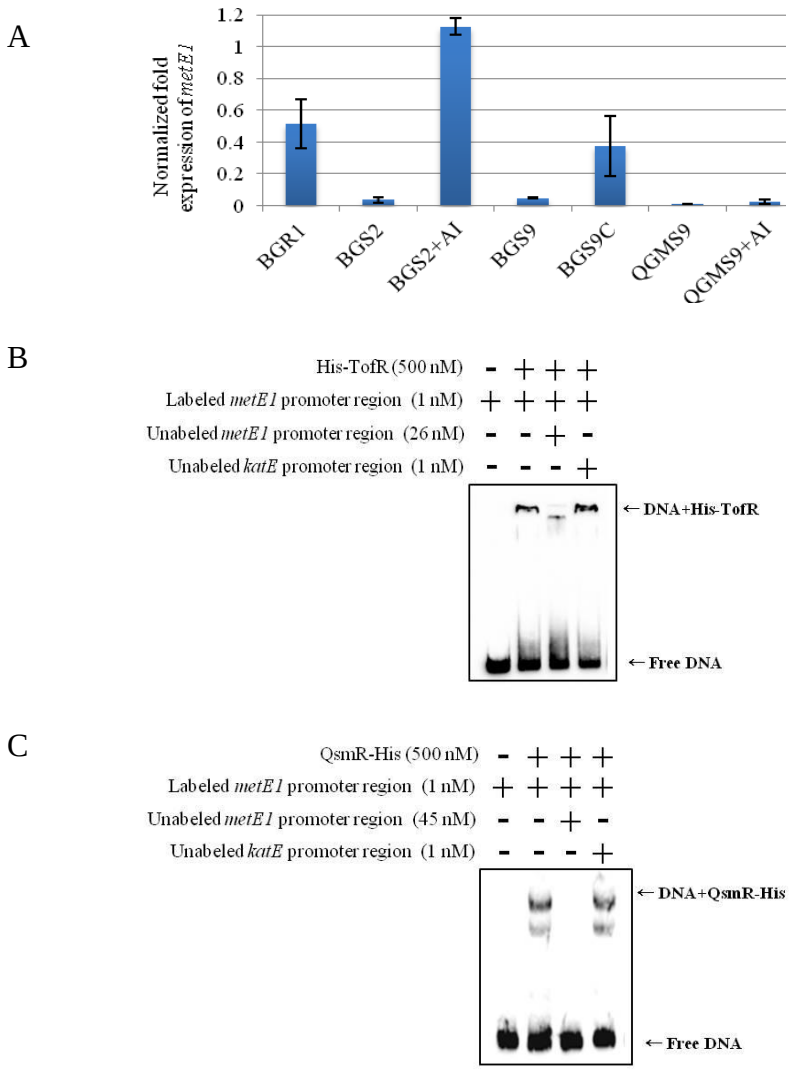


Figure 5. Real-time PCR and gel mobility shift assay. (A) Real-time PCR *metE1* expression level quantification. (B) Gel mobility shift assay with purified His-TofR and the DNA fragment of *metE1* promoter region. (C) Gel mobility shift assay with purified QsmR-His and the DNA fragment of *metE1* promoter region.

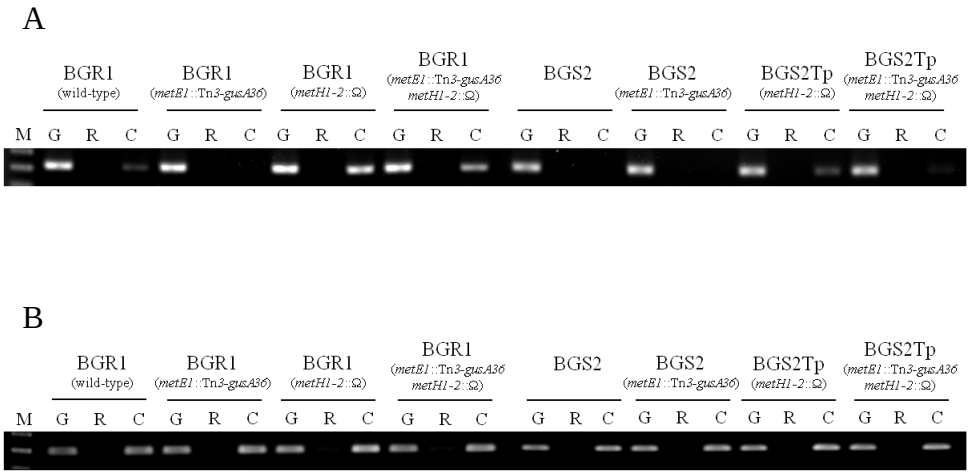


Figure 6. Agarose gel electrophoresis of RT-PCR product. (A) RT-PCR analysis of *metE2*. Expression of *metE2* is dependent on presence of *metH*. (B) RT-PCR analysis of 16S rRNA as positive control.

Burkholderia glumae 의 quorum sensing 에 의한 methionine 생합성 유전자의 발현 조절

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초록

Burkholderia glumae 는 LuxI-LuxR type 의 세균밀도인식기작 (Quorum sensing) 을 가지며 TofI 에 의해 만들어지는 *N*-octanoyl-homoserine lactone (C8-HSL) 이라는 신호물질과 receptor 인 TofR 을 사용하여 유전자의 발현을 조절한다. C8-HSL 은 activated methyl cycle (AMC) 이 돌면서 만들어지고, AMC 의 중간산물은 생물학적으로 중요한 아미노산인 methionine 이다.

B. glumae 는 methionine 생합성 유전자로 vitamin B_{12} 에 비의존적인 *metE1*, *metE2* 와 vitamin B_{12} 에 의존적인 *metH1*, *metH2* 를 가진다. 본 연구 에서는 methionine 생합성 유전자가 *B. glumae* 의 quorum sensing 에 의해 어떻게 조절되는지를 알아보았다. 실험 결과, *metE1*, *metE2*, *metH1-2* triple mutant 만이 methionine auxotroph 가 되었으며, *metE1 metH1-2* mutant 가 내는 autoinducer 는 M9 + 0.1% glucose medium 에서 wild-type 이나 다른 mutants 보다 약간 적을 뿐 큰 차이가 없었다. Bioassay 를 통해서는 *B. glumae* 가 vitamin B_{12} 를 생합성 할 수 있다는 간접적인 증거를 얻을 수 있었다. 또한 real-time PCR 과 gel mobility shift assay 를 통해 *metE1* 의 발현은 quorum sensing 에 의해 up-regulation 되고 *metE2* 의 발현은 *metH* 의 유무에 의존적이라는 것을 확인하였다.

주요어 : *Burkholderia glumae*, Quorum sensing, Methionine biosynthesis gene, Activated methyl cycle

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