Knockout of *pgdS* and *ggt* gene changes poly- γ -glutamic acid production in *Bacillus licheniformis* RK14-46

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Knockout of *pgdS* and *ggt* gene changes poly- γ -glutamic acid production in *Bacillus licheniformis* RK14-46

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ABSTRACT

Poly-gamma-glutamic acid (γ -PGA) is a water-soluble, nontoxic biocompatible polymer, which is extensively used in medicines, foodstuffs, cosmetics, and in water treatment. We previously isolated a novel γ -PGA producing strain *Bacillus licheniformis* RK14 from soil and developed a hyper-producing mutant strain RK14-46 by an ethyl methanesulfonate (EMS) treatment. In this study, endo-type (*pgdS*) and exo-type γ -PGA hydrolases (*ggt*) were disrupted by integrating plasmids into the genomic DNA of *B. licheniformis* RK14-46 strain. Unexpectedly, we observed strong inhibition of γ -PGA production following deletion of the *pgdS* gene, suggesting that *pgdS* is essential for γ -PGA biosynthesis in strain RK14-46, and in its parent strain RK14. In contrast, γ -PGA production increased by the deletion of the *ggt* gene and reached 39 g/L in the presence of 90 g/L glucose and elevated oxygen supply. Furthermore, γ -PGA from the *ggt*-disrupted mutant (Δggt) maintained a larger molecular mass throughout the culture period, whereas that from the original RK14-46 strain had degraded after glucose consumption. γ -PGA-containing culture supernatants from Δggt strain showed greater flocculation efficiency in sewage sludge than supernatants from the RK14-46 strain, reflecting greater production of γ -PGA with larger molecular mass by the Δggt strain. This is the first report concerning the deletion of *pgdS* and *ggt* genes in *B. licheniformis* strain and the properties of γ -PGA obtained from the mutant strain.

INTRODUCTION

Poly-gamma-glutamic acid (γ -PGA) is a water-soluble, biocompatible anionic polymer. Due its chemical properties, it is widely used in foods and cosmetics, in the medical industry, and as a water-soluble nontoxic and biodegradable flocculent for water treatment ([Shih and Van 2001](#); [Buescher and Margaritis 2007](#)). Several *Bacillus* strains have been studied for γ -PGA production ([Bajaj and Singhal 2011](#)). In particular, *B. subtilis* has been extensively researched as a model producing strain, and extremely high productivity of γ -PGA (101 g/L) was reported by the optimization of culture conditions ([Huang et al. 2011](#)). Moreover, the detailed mechanism of γ -PGA synthesis, secretion, and degradation have been clarified in recent years ([Luo et al. 2016](#)). In *B. subtilis*, γ -PGA is degraded via initial fragmentation of polymers by endo-type hydrolase (Pgds) followed by efficient degradation into glutamic acid monomers by the exo-type hydrolase γ -glutamyl transferase (GGT) ([Kimura et al. 2004](#); [Yao et al. 2009](#); [Kimura and Fujimoto 2010](#)). Recent work demonstrated that single-gene knockout of *pgdS* or *ggt* did not result in significant improvements of γ -PGA production in the laboratory strain *B. subtilis* 168; however, their simultaneous knockout significantly increased γ -PGA production ([Scoffone et al. 2013](#)).

Meanwhile, we previously isolated an RK14 strain of *B. licheniformis* with high γ -PGA productivity from soil ([Liu et al. 2017](#)). γ -PGA from RK14 cells had a large molecular mass, and its enantiomeric composition was different from those obtained from other *B. licheniformis* strains. To generate mutants with higher γ -PGA productivity, RK14 cells were treated with the mutagen ethyl methanesulfonate (EMS) ([Liu et al. 2017](#)). The resulting RK14-46 strain secreted 21.1 g/L γ -PGA at 48 h in a semi-synthetic medium. This productivity was significantly higher than that of the

RK14 strain (6.9 g/L). In combination with the cationic flocculent chitosan, γ -PGA containing culture medium was highly effective as a flocculation agent in real sewage sludge.

With respect to the γ -PGA degradation of *B. licheniformis* strains, genome analyses indicate the presence of *pgdS* genes ([King et al. 2000](#)). Recombinant PgdS protein was characterized by heterogenous expression using *E. coli* cells ([Tian et al. 2014](#)). With respect to γ -PGA production, overexpression of the *pgdS* gene in *B. licheniformis* WX-02 unexpectedly increased the γ -PGA production by 54% and decreased the molecular masses of γ -PGA molecules ([Tian et al. 2014](#)). GGT of *B. licheniformis* strain has been well characterized in heterogeneous expression systems ([Lin et al. 2006](#)) and has been applied in enzymatic reactions ([Chen et al. 2013](#)). However, the effects of *ggt* overexpression or disruption on γ -PGA production of *B. licheniformis* have not been described previously.

In this study, *ggt* and *pgdS* genes were disrupted in *B. licheniformis* RK14-46 strain and the effects on γ -PGA production were investigated. Quantities and molecular masses of γ -PGA in culture supernatants were compared between various knockout mutants. Finally, we compared flocculation efficacy of γ -PGA in culture supernatants from the Δggt strain with those of the parent strain using assays with sewage sludge.

Materials and Methods

Strains and culture condition

Bacterial strains and plasmids, and primers used in this study are listed in Table 1 and Table S1, respectively. The *B. licheniformis* RK14-46 strain, γ -PGA hyper-producing mutant strain of RK14, was used throughout this study ([Liu et al. 2017](#); [Liu et al. 2018](#)).

For cloning experiments, *B. licheniformis* and *E. coli* strains were cultured in Luria-Bertani (LB) medium (10 g/L of Bacto™ Tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl).

For γ -PGA production, the respective strains were precultured at 30°C for 16 h with a constant shaking speed in medium containing 30 g/L yeast extract (Oriental Yeast Co. Ltd.) and 20 g/L D-glucose. Cultures were then inoculated into 100 mL of γ -PGA production medium in 500 mL baffled conical flasks to an initial optical density of about 0.05 at 600 nm (OD₆₀₀). The production medium contained 60 g/L D-glucose, 4 g/L D-fructose, 60 g/L sodium L-glutamate·H₂O, 6 g/L yeast extract, 8 g/L sodium citrate·2H₂O, 4 g/L glycerol, 3.2 g/L NaNO₃, 6 g/L NaCl, 8 g/L (NH₄)₂SO₄, 1.3 g/L MgSO₄·7H₂O, and 0.48 g/L K₂HPO₄. Culture media for knockout mutants were supplemented with 5 mg/L chloramphenicol or 0.3 mg/L erythromycin when necessary. The medium was initially adjusted to pH 7.4 using NaOH solution. Cultures were then incubated at 37°C and 140 rpm on a rotary shaker (NR-30, Taitec Co., Ltd.) for prescribed culture times. Cell growth was recorded according to OD₆₀₀.

DNA manipulation and plasmid construction

The pBGSC6 and pMUTIN4 plasmids were obtained from the Bacillus Genetic Stock Center (BGSC) (Ohio State University, Columbus). For sequencing, the *pgdS* and *ggt* genes were amplified from genomic DNA of RK14 and RK14-46 strains using polymerase chain reactions (PCR). The designed primers for respective genes were as follows. For *pgdS* gene, 5'- TAAGAAAACCGCTCGAGTC -3' (sense) and 5'- ATCCCAGTTGAATGACGAT -3' (antisense); for *ggt* gene, 5'- ATGAAGACGGAAAGGTGAT -3' (sense) and 5'- CAACGTGTGAATCATGGTA -3'

(antisense). Obtained fragments were sequenced by a commercial service provider. In addition to the primers for fragment amplification, additional primers were used for sequencing; for *pgdS* gene 5'- ACATATTGGATAAACCCAGC -3'; for *ggt* gene 5'- ATCTCAGGATCGTTTCGAG -3'. Amino acid sequence alignments of Pgds and GGT proteins were performed using Jalview software ([Waterhouse et al. 2009](#)). To construct the plasmids for gene disruption, a part of *pgdS* and *ggt* genes were amplified by PCR. The primers for respective genes were as follows. For *pgdS* gene, 5'- AAGAATTCAACGGCTCGCATGGATAAAC -3' (sense) and 5'- AAGCGGCCGCAATCCGGATCTCGGATTTGA- 3' (antisense); for *ggt* gene, 5'- GGATCCCATGCTGCAGATGCTGAAATG -3' (sense) and 5'- GAATTCACAGTCAGCACCGGTTTTTC -3' (antisense). The *EcoRI*- and *NotI*-digested fragment of a partial *pgdS* gene was ligated into the plasmid pMUTIN4 ([Vagner et al. 1998](#)) to generate pMUTIN4- Δ *pgdS*, which was then propagated in *E. coli* DH5 α cells. Purified pMUTIN4- Δ *pgdS* was transformed into the RK14-46 strain using competent cells. Integration of pMUTIN4- Δ *pgdS* into genomic DNA was confirmed by resistance to erythromycin (0.3 mg/L) and by the appearance of a PCR product from DNA samples that was longer than the original amplicon. The primers for confirmation of pMUTIN4- Δ *pgdS* integration were 5'- GTTACACCGGCAGACAAGGT -3' (sense) and 5'- ATACAGCTACGGCGGAGAGA -3' (antisense). The following primers were also used for additional confirmation: 5'- ATAGCGCACCCCTGAAGAAGA -3' (sense) and 5'- AAATTCAGACGGCAAACGAC -3' (antisense). To disrupt the *ggt* gene, the *Bam*HI- and *Eco*RI-digested fragment of a partial *ggt* gene was ligated into the plasmid pBGSC6 to generate pBGSC6- Δ *ggt*. Competent cells of the RK14-46 strain were then transformed with purified pBGSC6- Δ *ggt*, and cells with genomic integration of the

plasmid were selected by their resistance to chloramphenicol (5 mg/L), as confirmed using PCR. The primers for confirmation of pBGSC6- Δggt integration were 5'-ATTCATTAATGCAGCTGGCA -3' (sense) and 5'-ACGGTCACAGCTTGTCTGTAA -3' (antisense). The following primers were also used for additional conformation: 5'-TTGGATGAAGACGGAAAGGT -3' (sense) and 5'-CATTTGCCAACGTGTGAATC -3' (antisense). The double knockout mutant $\Delta pgdS\Delta ggt$ was constructed by sequentially knocking out *pgdS* and *ggt* genes using the same procedure. The *pgdS* gene was also disrupted in the original RK14 strain.

γ -PGA recovery

γ -PGA was recovered using an ethanol precipitation method ([Wang et al. 2008](#)) with minor modifications. Briefly, 1 mL samples of culture broth were diluted 10-fold in pure water, and bacteria were removed by centrifugation at $20,000 \times g$ for 20 min. Supernatants were then added to two volumes of ethanol, and precipitates were recovered by centrifugation at $20,000 \times g$ for 20 min. After washing in 70% ethanol, precipitates were again centrifuged at $20,000 \times g$ for 20 min and samples were dissolved in pure water and dialyzed against pure water using a dialysis membrane tube with a molecular weight cutoff of 6–8 kD (Spectrum Devices Corp.). The inner dialysis liquid was finally lyophilized to obtain purified γ -PGA. Yields of γ -PGA were determined, and concentrations in culture broth were calculated.

Glucose and L-glutamate determinations

Supernatants from 10-fold diluted samples of culture broth were obtained by centrifugation at $20,000 \times g$ for 20 min. After filtration, glucose concentrations were

determined using HPLC (GL Sciences Inc. Tokyo, Japan) with an ULTRON PS-80N column using ultrapure water as fluent with 0.6 mL/min flow at 60°C. Glucose was detected using a refractive index detector. L-glutamate monomer concentrations were assayed using the enzymatic method of F-kit 139092 (JK International Inc.) according to the manufacturer's instructions.

Agarose gel electrophoresis

Molecular masses of γ -PGA from respective strains were compared using agarose gel electrophoresis as described previously ([Liu et al. 2017](#)). Briefly, supernatants of 10-fold diluted samples of culture broth were obtained as described above and 10 μ L aliquots were loaded onto and electrophoresed on 1% agarose gels in Tris–acetate–EDTA buffer containing 40 mM Tris(hydroxymethyl)aminomethane, 40 mM acetic acid, and 1 mM EDTA. After electrophoresis, γ -PGA was stained with 0.17% methylene blue ([Shimizu et al. 2007](#); [Scoffone et al. 2013](#)). A 0.2% solution of commercial γ -PGA (food level, Yakult Pharmaceutical Industry Co., Ltd.) was used as a standard.

Flocculation of sewage sludge

Sewage sludge solution was sampled from the domestic wastewater treatment facility of an agricultural commune in Hyogo prefecture (Japan). Sewage sludge primarily consists of an activated sludge in an aeration tank. In the present study, it was stored at 4°C until use. Sewage sludge was flocculated using purified γ -PGA, and flocculation efficacy was assayed as described previously ([Liu et al. 2017](#)). To achieve stable flocculation, sewage sludge in the same bottle was distributed to all tubes. Chitosan (Nippon Suisan Kaisha, Ltd.) was used as a co-flocculation agent with γ -PGA. In these

experiments, 60 μ L aliquots of 0.5% (w/v) chitosan were added to 1 mL sewage sludge solutions in test tubes (13 $\times$ 90 mm). Subsequently, 40 μ L aliquots of γ -PGA-containing culture supernatants at various dilutions were added and stirred with a vortex mixer for 10 s. The state of flocculation was recorded with photographs 5 min after vortex mixing.

Statistical analysis

Data are presented as the means $\pm$ standard deviations of more than three independent experiments.

Results and Discussion

Disruption of *pgdS* and *ggt* genes in the *B. licheniformis* RK14-46 strain

To design plasmids for gene disruption, sequences of the *pgdS* and *ggt* genes of the RK14-46 strain were analyzed. Furthermore, the obtained sequences were compared to the original RK14 strain and experimental strain of *B. licheniformis* ATCC14580 to understand the mutation derived due to EMS treatment or the difference from model strain of *B. licheniformis*. As shown in Fig. S1, the amino acid sequence of PgdS of the RK14 strain differs by only two amino acid residues from the reference PgdS of the ATCC14580 strain, suggesting that PgdS of the RK14 strain has high homology with that of the experimental strain. In addition, PgdS of the RK14-46 strain has two mutated residues compared to that of RK14 strain. In the case of GGT (Fig. S2), GGT of the RK14 and ATCC14580 strains differed only by two amino acid residues, and the EMS treatment did not modify the amino acid sequence of the GGT protein in the RK14-46 strain. In conclusion, PgdS and GGT sequences did not significantly differ between the

strains. Plasmids for each gene disruption were designed on the basis of the resulting sequences.

Next, gene disruption of *pgdS* and *ggt* was achieved using plasmids pMUTIN4- Δ *pgdS* and pBGSC6- Δ *ggt*. To confirm the disruption, genomic DNA was extracted from candidate knockout mutants. The target region of the *pgdS* gene was then amplified, and amplicons from PCR were electrophoresed to compare with RK14-46 strain (Supplementary Fig. S3). A fragment of approximately 900 bp was amplified from the RK14-46 strains (Outside PCR in Fig. S3), corresponding with the size of the target region of the *pgdS* gene. In contrast, a band of about 9000 bp was amplified from a candidate *pgdS*-mutant, indicating successful plasmid integration into the *pgdS* gene of genomic DNA in the RK14-46 strain. Another band of about 700 bp was amplified from *pgdS*-mutant DNA using primers that were targeted for a DNA region spanning the integrated plasmid and genomic DNA (Inside PCR in Fig. S3), supporting the conclusion of successful integration. To confirm *ggt* gene disruption in the *ggt* mutant, a band of about 5000 bp was amplified by Outside PCR and another of about 1000 bp was amplified by Inside PCR. These fragments had expected lengths only when pBGSC6- Δ *ggt* was successfully integrated into the genomic *ggt* gene. Sequential disruption of the *ggt* gene in the Δ *pgdS* strain was also confirmed as described above (Fig. S3).

Cell growth and γ -PGA production

Figure 1 shows cell growth and γ -PGA production of RK14-46 and respective knockout mutant strains. RK14-46, Δ *pgdS*, and Δ *ggt* strains showed similar growth profiles, reaching OD₆₀₀ of approximately 6 over 48 h post-inoculation. These results

suggest that single knockout of *pgdS* or *ggt* genes did not influence the growth of RK14-46 strain considerably. In contrast, cell growth was significantly repressed in the double knockout mutant $\Delta pgdS\Delta ggt$, with an OD₆₀₀ value of 3.4 ± 0.4 at 48 h, almost half those of the other strains. γ -PGA production by the RK14-46 strain was 12.0 ± 1.1 g/L at 24 h and increased to 18.6 ± 1.1 g/L by 48 h. These results correspond with those in previous studies ([Liu et al. 2017](#)). But after further culture until the 72 h time point, γ -PGA concentrations decreased to 15.4 ± 2.8 g/L. Numerous studies show that broth viscosity of *B. licheniformis* decreases at the late stationary phase due to γ -PGA degradation by depolymerases ([Bajaj and Singhal 2011](#)). This degradation likely decreased γ -PGA concentrations in the present RK14-46 cultures. Meanwhile, γ -PGA concentration of the Δggt strain was 15.4 ± 1.1 g/L at 24 h and increased to 23.5 ± 0.9 g/L at 48 h. These values were slightly higher than those of RK14-46 strain. However, γ -PGA concentration of Δggt strain kept increasing after 48 h and reached 29.0 ± 0.8 g/L at 72 h, whereas that of the RK14-46 strain decreased in the period between 48 h and 72 h. These results suggest that the deletion of *ggt* gene in the *B. licheniformis* RK14-46 strain improves γ -PGA production to prevent its degradation.

Unexpectedly, the $\Delta pgdS$ and $\Delta pgdS\Delta ggt$ strains failed to produce γ -PGA throughout the culture period. In the *B. subtilis* 168 strain, deletion of the *pgdS* gene did not significantly affect γ -PGA production ([Scoffone et al. 2013](#)), perhaps indicating the effects of EMS treatment on the RK14 strain, which may have abolished γ -PGA biosynthesis by disrupting the *pgdS* gene in the resulting RK14-46 strain. To resolve this disparity, we disrupted the *pgdS* gene in the original RK14 strain and confirmed the effect on γ -PGA biosynthesis (Supplementary Fig. S4). Successful *pgdS* disruption in the RK14 strain was confirmed by PCR (Fig. S4A). As shown in Fig. S4B, cell growth

of the RK14 strain was repressed by the deletion of the *pgdS* gene, suggesting that *pgdS* gene is important for the growth of wild type RK14 strain and the load of erythromycin resistance may also repress the cell activity. Although the RK14 strain produced 7.8 ± 1.1 and 6.9 ± 2.2 g/L γ -PGA at 24 and 48 h, respectively, RK14 Δ *pgdS* only produced 2.4 ± 0.4 g/L γ -PGA at 24 h, and this concentration did not increase thereafter. These results suggest that loss of γ -PGA production by *pgdS* disruption is not associated with EMS treatment. In previous reports, *pgdS* overexpression in the *B. licheniformis* WX-02 strain increased γ -PGA production even though PgdS is a γ -PGA degrading enzyme ([Tian et al. 2014](#)). Considering this report together with our results, we believe that PgdS plays an important role in γ -PGA biosynthesis of *B. licheniformis* strains.

Mechanisms underlying enhanced γ -PGA production following *ggt* disruption

Our results showed that the deletion of the *ggt* gene increased γ -PGA production of the *B. licheniformis* RK14-46 strain to prevent γ -PGA degradation. In further experiments, we compared the molecular mass of the γ -PGA produced by the RK14-46 and Δ *ggt* strains at every sampling time using agarose gel electrophoresis (Fig. 2). As previously reported, the molecular mass of all γ -PGA samples from cultures were relatively larger than that of commercial γ -PGA (average molecular mass 1,500 kDa) ([Liu et al. 2017](#)). In the case of RK14-46 strain, the largest molecular mass polymers were produced at 24 h after inoculation into media. The molecular mass of polymers gradually decreased after 48 h and 72 h, suggesting that γ -PGA is partially degraded by enzymes after production. In contrast, γ -PGA from the Δ *ggt* strain did not show a clear degradation over time. These results indicate that γ -PGA degradation in the RK14-46 strain was mainly conducted by exo-type hydrolases of GGT and not by the endo-type

hydrolase (Pgds). To support these results, we determined glucose and monomer L-glutamic acid concentrations in the culture media of each strain at each sampling time. As shown in Fig. 3, glucose concentrations (initial 60 g/L) decreased to less than half after 24 h culture of all strains. Furthermore, glucose concentrations in the RK14-46 and Δggt culture media were about 5.6 ± 0.5 and 2.3 ± 0.4 g/L, respectively, at 48 h. At 72 h, almost all glucose was consumed in both of these cultures. L-glutamic acid was gradually consumed in cultures of Δggt cells and was present at about 17.5 ± 0.1 g/L at 72 h. However, L-glutamic acid concentrations remained constant over 72 h in cultures of the RK14-46 strain, suggesting that after depleting glucose concentrations in the media, the cells degrade γ -PGA via the actions of the exo-type hydrolase GGT and release glutamic acid monomers.

Effects of glucose concentrations on γ -PGA production of the Δggt strain

As described above, glucose was completely consumed from the initial concentration of 60 g/L in cultures of the Δggt strain. To increase γ -PGA productivity of this strain, initial glucose concentrations were increased from 60 to 90 g/L and γ -PGA production was again determined under each culture condition (Fig. 4). In the presence of 90 g/L glucose, the Δggt strain produced 34.4 ± 2.0 g/L γ -PGA at 72 h, significantly more than that (29.0 ± 0.8 g/L) with 60 g/L glucose. Subsequently, γ -PGA concentrations did not increase further, and 19.8 ± 2.0 g/L glucose remained in the culture medium of Δggt cells at 96 h, suggesting that glucose concentration is sufficient for γ -PGA production. In general, highly viscous medium with γ -PGA production decreases the volumetric oxygen mass transfer, leading to oxygen limitation for γ -PGA production ([Cromwick et al. 1996](#)). For considering the effect of oxygen supply on γ -PGA production, culture

volume in shake flasks was changed from 100 to 50 mL to increase an aeration. As the result, γ -PGA concentration of Δggt reached 39.1 ± 1.2 g/L at 96 h (Fig. 4), suggesting that the increase in oxygen supply promotes γ -PGA production. Finally, γ -PGA production of our strain (39.1 ± 1.2 g/L) is comparable with the highest value reported using the *B. licheniformis* strain in flask cultures ([Bajaj and Singhal 2009](#); [Ogunleye et al. 2015](#)). Meanwhile, 43 g/L γ -PGA is reportedly the maximum production by *B. licheniformis* strain ([Cai et al. 2018](#)). In that research, recombinant *B. licheniformis* strain with increased ATP supply was cultured in a 1 L bioreactor.

Flocculation of sewage sludge using culture supernatant from the Δggt strain

Chitosan is a deacetylation product of chitin and is among the most promising of biological polymer flocculants ([Bhalkaran and Wilson 2016](#)). Under acidic conditions, chitosan chains are positively charged, suggesting that this biopolymer flocculates contaminants with negative surface charges. Our previous work showed that aliquots of γ -PGA-containing culture supernatant from the RK14-46 strain with 0.025% chitosan effectively induced flocculation of sewage sludge by the bridging effect ([Liu et al. 2017](#)). Herein we compared flocculation efficiencies of culture supernatant from RK14-46 and Δggt strains in sewage sludge. As shown in Fig. 5, addition of 0.025% chitosan did not induce effective flocculation of sewage sludge, yet in the presence of 10 times diluted culture supernatant (0.1%), all media samples from both strains induced flocculation at all sampling times. After continuous dilution of supernatants from 48 h cultures of the RK14-46 strain, 40-times dilution of media decreased flocculation efficiency. In contrast, 40-fold diluted Δggt supernatant still maintained the high flocculation efficiency. Supernatant from 72 h cultures of RK14-46 decreased the

flocculation efficiency with 30-fold dilution, although the 30-fold diluted supernatant from 48 h maintained the flocculation efficiency. These results support that γ -PGA in supernatant of RK14-46 was degraded from 48 to 72 h (Figs. 1, 2). In contrast, high flocculation efficacy remained in 40-times diluted samples from 72 h cultures of Δggt . Figure 5B shows the zoomed image of sewage sludge flocculated with 40-fold diluted culture supernatant at 72 h. Sewage sludge was diluted two times before flocculation for clear observation. Larger aggregate cluster was observed in the sewage sludge with the culture supernatants from Δggt , suggesting the stronger flocculation efficiency. These results correspond with the higher γ -PGA concentration and larger molecular masses of γ -PGA from the Δggt strain (Figs. 1, 2).

CONCLUSION

The *pgdS* and *ggt* genes were disrupted in the *B. licheniformis* RK14-46 strain by integrating plasmids into genomic DNA. The resulting *pgdS*-deficient mutant strain did not produce γ -PGA, suggesting that *pgdS* gene is essential for γ -PGA biosynthesis in the RK14 strain. In contrast, disruption of the *ggt* gene increased γ -PGA production due to the prevention of γ -PGA degradation. Changing the initial glucose concentration and aeration increased γ -PGA production to 39.1 ± 1.2 g/L. Finally, γ -PGA-containing culture supernatants from the Δggt strain showed greater flocculation efficiency in real sewage sludge than that from the RK14-46 strain.

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AUTHOR CONTRIBUTIONS

MA proposed the research concept and provided necessary tools for experiments and experimental instructions. YO designed the experiments and analyzed the data and wrote the manuscript. JK and TD conducted the experiments and analyzed the data. All authors read and approved the manuscript.

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Note	References
Strains		
<i>B. lichenforimis</i> RK14	high γ -PGA production strain isolated from soil	Liu et al., 2017
<i>B. lichenforimis</i> RK14-46	Derivative of RK-14 by EMS treatment	Liu et al., 2017
Δggt	RK14-46, $\Delta ggt::pBGSC6-\Delta ggt$	This study
$\Delta pgdS$	RK14-46, $\Delta pgdS::pMUTIN4-\Delta pgdS$	This study
$\Delta ggt\Delta pgdS$	RK14-46, $\Delta ggt\Delta pgdS$	This study
RK14 $\Delta pgdS$	RK14, $\Delta pgdS::pMUTIN4-\Delta pgdS$	This study
<i>E. coli</i> DH5 α	F ⁻ , $\Phi 80d$ lacZ Δ M15, Δ (lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK ⁻ mK ⁺), phoA, supE44, λ^- , thi-1, gyrA96, relA1	General cloning uses
Plasmids		
pBGSC6	Amp ^r Cm ^r	Fajardo-Cavazos et al., 1993
pMUTIN4	Amp ^r Erm ^r	Vagner et al., 1998
pBGSC6- Δggt	pBGSC64:: ggt	This study
pMUTIN4- $\Delta pgdS$	pMUTIN4:: $pgdS$	This study

Supplementary Table S1 Primers used in this study

Primers	Sequences
Sequencing	
<i>pgdS</i> -F	5'- TAAGAAAACCGCTCGAGTC -3'
<i>pgdS</i> -M	5'- ACATATTGGATAAACCCAGC -3'
<i>pgdS</i> -R	5'- ATCCCAGTTGAATGACGAT -3'
<i>ggt</i> -F	5'-ATGAAGACGGAAAGGTGAT -3'
<i>ggt</i> -M	5'- ATCTCAGGATCGTTTCGAG -3'
<i>ggt</i> -R	5'- CAACGTGTGAATCATGGTA -3'
Cloning for disruption	
<i>pgdS</i> -pMUTIN4-F	5'- AAGAATTCAACGGCTCGCATGGATAAAC -3'
<i>pgdS</i> -pMUTIN4-R	5'- AAGCGGCCGCAATCCGGATCTCGGATTTGA -3'
<i>ggt</i> -pBGSC6-F	5'- GGATCCCATGCTGCAGATGCTGAAATG -3'
<i>ggt</i> -pBGSC6-R	5'- GAATTCACAGTCAGCACCGGTTTTTC -3'
Confirmation of disruption	
<i>pgdS</i> -outside-F	5'- GTTACACCGGCAGACAAGGT -3'
<i>pgdS</i> -outside-R	5'- ATACAGCTACGGCGGAGAGA -3'
<i>pgdS</i> -inside-F	5'- ATAGCGCACCCCTGAAGAAGA -3'
<i>pgdS</i> -inside-R	5'- AAATTCAGACGGCAAACGAC -3'
<i>ggt</i> -outside-F	5'- ATTCATTAATGCAGCTGGCA -3'
<i>ggt</i> -outside-R	5'- ACGGTCACAGCTTGTCTGTAA -3'
<i>ggt</i> -inside-F	5'- TTGGATGAAGACGGAAAGGT -3'
<i>ggt</i> -inside-R	5'- CATTTGCCAACGTGTGAATC -3'

FIGURE LEGENDS

Fig. 1 Cell growth and γ -PGA production of *B. licheniformis* RK14-46 $\Delta pdgS$, Δggt , and $\Delta pdgS\Delta ggt$ strains at 24, 48, and 72 h; data were generated from more than three independent experiments. Vertical bars indicate standard deviations.

Fig. 2 Comparison of molecular masses using agarose (1%) gel electrophoresis with methylene blue staining; diluted culture supernatants (10 μ L) were analyzed using electrophoresis. The commercial γ -PGA with known average molecular mass (1,500 kDa) was used as a standard.

Fig2

Fig. 3 Glucose and L-glutamic acid concentrations in culture supernatants of *B. licheniformis* RK14-46 and Δggt strains at 24, 48, and 72 h; data were obtained from more than three independent experiments. Vertical bars indicate standard deviations.

Fig. 4 Production of γ -PGA by the Δggt strain with different glucose concentrations and culture volumes at 24, 48, 72, and 96 h; data were generated from more than three independent experiments. Vertical bars indicate standard deviations.

Fig. 5 Flocculation of sewage sludge. (A) Determination of efficacious concentrations of γ -PGA-containing culture supernatant for flocculation of sewage sludge; flocculation in 1 mL sewage sludge solutions was induced by the combined use of 60 μ L aliquots of 0.5% (w/v) chitosan and 40 μ L culture supernatant from RK14-46 and Δggt strains with different dilution. (B) Zoomed image of sewage sludge flocculated with 40-fold diluted culture supernatant at 72 h. Sewage sludge was two-fold diluted before flocculation for clear observation.

Fig5

Fig. S1 Amino acid sequence alignments of PgdS proteins from *B. licheniformis* ATCC14850, RK14, and RK14-46 strains. The different sequences between ATCC14850 and RK14 and between RK14 and RK14-46 are shown in blue and yellow colors, respectively.

Supplementary Fig.S1

PgdS

	1	60
B_licheniformis_ATCC14580	MIKKAANKKLVLFCGIAVLWMSLFLTNHNDVRADTIGEKIAETARQLEGAKYSYGGEKPK	
B_licheniformis_RK14	MIKKAANKKLVLFCGIAVLWMSLFLTNHNDVRADTIGEKIAETARQLEGAKYSYGGEKPK	
B_licheniformis_RK14-46	MIKKAANKKLVLFCGIAVLWMSLFLTNHNDVRADTIGEKIAETARQLEGAKYSYGGEKPK	
	61	120
B_licheniformis_ATCC14580	TGFDSSGFVQYVFQSLDITLPRTVKEQSTLGSSVGRQQLKGDLVFFKNAELES DGPTHV	
B_licheniformis_RK14	TGFDSPGFVRYVFQSLDITLPRTVKEQSTLGSSVGRQQLKGDLVFFKNAELES DGPTHV	
B_licheniformis_RK14-46	TGFDSPGFVRYVFQSLDITLPRTVKEQSTLGSSVGRQQLKGDLVFFKNAELES DGPTHV	
	121	180
B_licheniformis_ATCC14580	AIYLGNDQIIHSTKSNGVVVTKLEGSSYWSSGYFKAKRITKEPEISMDPVVQKAKSYVGV	
B_licheniformis_RK14	AIYLGNDQIIHSTKSNGVVVTKLEGSSYWSSGYFKAKRITKEPEISMDPVVQKAKSYVGV	
B_licheniformis_RK14-46	AIYLGNDQIIHSTKSNGVVVTKLEGSSYWSSGYFKAKRITKEPEISMDPVVQKAKSYVGV	
	181	240
B_licheniformis_ATCC14580	PYVFGGNSPDLGFDGSGLTQYVFREVLGVYLPRSAEQQWAVGQKVKLEDIRPGDVLFFSN	
B_licheniformis_RK14	PYVFGGNSPDLGFDGSGLTQYVFREVLGVYLPRSAEQQWAVGQKVKLEDIRPGDVLFFSN	
B_licheniformis_RK14-46	PYVFGGNSPDLGFDGSGLTQYVFREVLGVYLPRSAEQQWAVGQKVKLEDIRPGDVLFFSN	
	241	300
B_licheniformis_ATCC14580	TYKPGISHNGIYAGGGRFIHASRSNKVTISYLSASYWQKKFTGVRREFDNMSLPKNPIVSE	
B_licheniformis_RK14	TYKPGISHNGIYAGGGRFIHASRSNKVTISYLSASYWQKKFTGVRREFDNMSLPKNPIVSE	
B_licheniformis_RK14-46	TYKPGISHNGIYAGGGRFIHASRSNKVTISYLSASYWQKKFTGVRREFDNMSLPKNPIVSE	
	301	360
B_licheniformis_ATCC14580	AIRHIGEVGYQKGGTSPKEGFDTAGFIQYVYKTAAGVELPRYADKQYSTGKKITKQLEP	
B_licheniformis_RK14	AIRHIGEVGYQKGGTSPKEGFDTAGFIQYVYKTAAGVELPRYADKQYSTGKKITKQLEP	
B_licheniformis_RK14-46	AIRHIGEVGYQKGGTSPKEGFDTAGFIQYVYKTAAGVELPRYADKQYSTGKKITKQLEP	
	361	414
B_licheniformis_ATCC14580	GDIVFFKGTTVMNPAIYIGNGQVVLVTL SAGVTTADMETSAYWKDYAGSVRIE	
B_licheniformis_RK14	GDIVFFKGTTVMNPAIYIGNGQVVLVTL SAGVTTADMETSAYWKDYAGSVRIE	
B_licheniformis_RK14-46	GDIVFFKGTTVMNPAIYIGNGQVVLVTL SAGVTTADMETSAYWKDYAGSVRIE	

Fig. S2 Amino acid sequence alignments of GGT proteins from *B. licheniformis* ATCC14850, RK14, and RK14-46 strains. The different sequences between ATCC14850 and RK14 are shown in blue color.

Supplementary Fig.S2

GGT

	1	60
B_licheniformis_ATCC14580	MRRLAFLVVAFLAVGCFSPVSKAEGVMSGG	GDKVAVGKDGMMVATAHPLASKIGAEVL
B_licheniformis_RK14	MRRLAFLVVAFLAVGCFSPVSKAEGVMSGG	GDKVAVGKDGMMVATAHPLASKIGAEVL
B_licheniformis_RK14-46	MRRLAFLVVAFLAVGCFSPVSKAEGVMSGG	GDKVAVGKDGMMVATAHPLASKIGAEVL
	61	120
B_licheniformis_ATCC14580	KKGGNAIDAAIAIQYALNVTEPMSGIGGGFMMVYDGETKETS	IINSRERAPEGAKPDM
B_licheniformis_RK14	KKGGNAIDAAIAIQYALNVTEPMSGIGGGFMMVYDGETKETS	IINSRERAPEGAKPDM
B_licheniformis_RK14-46	KKGGNAIDAAIAIQYALNVTEPMSGIGGGFMMVYDGETKETS	IINSRERAPEGAKPDM
	121	180
B_licheniformis_ATCC14580	FLDE	GKVIPFSERSRHGNAVGPGLKGLEAAHKKWGTKKMEDLISPSIKLAEEGFPID
B_licheniformis_RK14	FLD	GKVIPFSERSRHGNAVGPGLKGLEAAHKKWGTKKMEDLISPSIKLAEEGFPID
B_licheniformis_RK14-46	FLD	GKVIPFSERSRHGNAVGPGLKGLEAAHKKWGTKKMEDLISPSIKLAEEGFPID
	181	240
B_licheniformis_ATCC14580	SVLADAIKDHQDKLSKTAAKDIFLPDGEPLKEGDILVQKDLAKTFKLIRKEGSKAFYDGE	
B_licheniformis_RK14	SVLADAIKDHQDKLSKTAAKDIFLPDGEPLKEGDILVQKDLAKTFKLIRKEGSKAFYDGE	
B_licheniformis_RK14-46	SVLADAIKDHQDKLSKTAAKDIFLPDGEPLKEGDILVQKDLAKTFKLIRKEGSKAFYDGE	
	241	300
B_licheniformis_ATCC14580	IGRAIADVVDFFGGSMTPDDL	SRYEVTDKPIWGEYHGYDIASMPPPSSGGVFMLQMLKL
B_licheniformis_RK14	IGRAIADVVDFFGGSMTPDDL	SRYEVTDKPIWGEYHGYDIASMPPLSSGGVFMLQMLKL
B_licheniformis_RK14-46	IGRAIADVVDFFGGSMTPDDL	SRYEVTDKPIWGEYHGYDIASMPPPSSGGVFMLQMLKL
	301	360
B_licheniformis_ATCC14580	IDDFHLSQYDPKSFKEYHLLAETMHL	SYADRAAYAGDPEFVDVPLRGLLDPDYIKERQKL
B_licheniformis_RK14	IDDFHLSQYDPKSFKEYHLLAETMHL	SYADRAAYAGDPEFVDVPLRGLLDPDYIKERQKL
B_licheniformis_RK14-46	IDDFHLSQYDPKSFKEYHLLAETMHL	SYADRAAYAGDPEFVDVPLRGLLDPDYIKERQKL
	361	420
B_licheniformis_ATCC14580	ISLDSMNRDVKEGDPWKYE	EEGEPNIEIVQPEDKTIGETTHFTVTDQWGNVVS
B_licheniformis_RK14	ISLDSMNRDVKEGDPWKYE	EEGEPNIEIVQPEDKTIGETTHFTVTDQWGNVVS
B_licheniformis_RK14-46	ISLDSMNRDVKEGDPWKYE	EEGEPNIEIVQPEDKTIGETTHFTVTDQWGNVVS
	421	480
B_licheniformis_ATCC14580	LFGTGILVPGYGLFLNNELTDFDAIPGGANEVQPNKRPLSSMTPTIVF	KDEKPVLTVGSP
B_licheniformis_RK14	LFGTGILVPGYGLFLNNELTDFDAIPGGANEVQPNKRPLSSMTPTIVF	KDEKPVLTVGSP
B_licheniformis_RK14-46	LFGTGILVPGYGLFLNNELTDFDAIPGGANEVQPNKRPLSSMTPTIVF	KDEKPVLTVGSP
	481	540
B_licheniformis_ATCC14580	GGTTIIASVFQTI	LNFEYGMSLQDAIEEPRIYTNLSYRYESGMPEDVRRKLNDFGHK
B_licheniformis_RK14	GGTTIIASVFQTI	LNFEYGMSLQDAIEEPRIYTNLSYRYESGMPEDVRRKLNDFGHK
B_licheniformis_RK14-46	GGTTIIASVFQTI	LNFEYGMSLQDAIEEPRIYTNLSYRYESGMPEDVRRKLNDFGHK
	541	586
B_licheniformis_ATCC14580	FGSNPVDIGNVQSIFIDRENKTFMGVADSSRNGTAVGVNIKTS	AK
B_licheniformis_RK14	FGANPVDIGNVQSIFIDRENKTFMGVADSSRNGTAVGVNIKTS	AK
B_licheniformis_RK14-46	FGANPVDIGNVQSIFIDRENKTFMGVADSSRNGTAVGVNIKTS	AK

Fig. S3 Electrophoresis of polymerase chain reaction (PCR) fragments after amplifying genomic DNA from *B. licheniformis* RK14-46, $\Delta pgdS$, Δggt , and $\Delta pgdS\Delta ggt$ strains; “Outside” samples are those in which the target region of plasmid integration was amplified with upstream and downstream regions and gave longer fragments than the original sequences, indicating plasmid integration into the target gene of the genomic DNA. “Inside” samples are those in which the fragment was amplified using the primer set, one primer is specific for the integrated plasmid region and another primer is specific for locations on genomic DNA. Successful amplification of these fragments indicates successful plasmid integration into genomic DNA; Lane M; commercial marker.

Supplementary Fig. S3

Fig. S4 Disruption of the *pgdS* gene from the originally isolated *B. licheniformis* RK14 strain; (A) confirmation of *pgdS* disruption in genomic DNA of the RK14 strain; (B) cell growth and γ -PGA production of the RK14 and RK14 Δ *pgdS* strains at 24, 48, and 72 h. Data were generated from more than three independent experiments. Vertical bars indicate standard deviations.

Supplementary Fig. S4

A

B