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Bacillus velezensis LG37: Functional verification of GlnL and analysis of the ammonia assimilation metabolic pathway

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Abstract

High concentrations of ammonia have toxic effects on bred animals. In aquaculture, the possibility of using *Bacillus* preparations to remove ammonia nitrogen in aquaculture water through assimilation has been generally recognized. In this study, to analyze the metabolic pathways of ammonia assimilation in *Bacillus*, the characteristics and pathways of ammonia assimilation of *Bacillus velezensis* LG37 stored in the laboratory were analyzed. The results showed that the rate of ammonia assimilation by LG37 in a minimal medium could reach 94.2% at 20 h, and the highest assimilation rate was 95.4% within 60 h. In a minimal medium, the growth rate of LG37 in the glutamine group was significantly faster than that in the ammonia group, but the expression of *glnL* showed opposite results. The gene expression level of *glnL* in the ammonia group was significantly higher than in the glutamine group. *glnL* overexpression (OE*glnL*) and deletion (Δ*glnL*) strains were constructed by CRISPR/Cas9 technology and using the pHT1K vector, respectively. The expression levels of *glnL* in LG37, OE*glnL*, and Δ*glnL* strains were determined by RT-qPCR. The *glnL* gene expression levels were ordered as follows: OE*glnL* > LG37 > Δ*glnL*. In all three strains (LG37, Δ*glnL*, and OE*glnL*) and at different ammonia concentrations, the expression levels of *glnL* were consistent with *glnA* and *glnB* levels, showing a positive correlation. However, the expression levels of *glnK* and *glnR* in different strains hardly changed significantly.

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Introduction

The rapid upscaling and intensification of aquaculture, with increased aquaculture density and continuous feed input, caused the accumulation of inorganic nitrogen and ammonia nitrogen in aquaculture water, which in turn exert toxic effects on bred animals (Maarefian et al., 2019; Xu et al., 2021). Ammonia nitrogen is a toxic substance that can enter the blood through the gills and is transported to other tissues and organs, resulting in hyperammonemia, which causes a decrease in blood oxygen-carrying capacity, damages various organs, disorganizes the internal environment, reduces immunity, and in severe cases even causes death, causing huge economic losses (Kim et al., 2019; Zhang et al., 2022). The biological treatment of ammonia nitrogen with *Bacillus* has become a hotspot in the aquaculture research field because of its safety, high efficiency, and low cost, and its effect on aquaculture has been recognized by many aquaculturists and scholars (John et al., 2020; Kuebutornye et al., 2019).

Regulatory systems are diverse signaling modalities that have evolved in microorganisms to adapt to changes in the external environment and to ensure their survival and reproduction (Bhagirath et al., 2019). By sensing changes in extracellular nutrients and concentrations as well as changes in physicochemical factors, they transmit downstream signals to regulate the expression and activity of relevant proteins and cellular processes in the host, thereby adapting to changes in the external environment (Laventie et al., 2020; Zhao et al., 2020). The *Bacillus* GlnK/GlnL two-component regulatory system plays an important role in ammonia nitrogen assimilation metabolism. The histidine kinase GlnK senses extracellular NH_4^+/NH_3 concentrations and concentration changes and activates the response regulator GlnL by GlnK autophosphorylation and phosphorylation of GlnL, which regulates the transcription and activity of the transporter MnrA and the regulator GlnB to achieve ammonia uptake (Liu et al.,2020).

The uptake of NH_3 and NH_4^+ by *Bacillus* is carried out in different ways. At high pH and high NH_4^+/NH_3 concentrations, NH_3 is mainly taken up by free diffusion. At low pH and low NH_4^+/NH_3 concentrations, *Bacillus* mainly transports and absorbs NH_4^+ through MnrA. The intracellular NH_4^+ and NH_3 that are transported to the cell are assimilated by TnrA and GlnR (a transcriptional regulator), GlnA (a glutamine synthetase), GlnB (an assimilation regulator), GDH (glutamate dehydrogenase), and other proteins, and finally the nitrogenous organic matter is synthesized into the organic nitrogen form required for microbial growth, thus completing the process of ammonia nitrogen uptake and utilization (Dai et al., 2022; Wang et al., 2020; Liu et al., 2020).

Based on the results of previous studies, we found that *Bacillus velezensis* LG37 could regulate the efficiency of ammonia assimilation through GlnK and identified a new ammonia-specific transporter, MnrA. Based on this, we constructed the overexpression (OE*glnL*) and knockdown (Δ*glnL*) strains of *glnL* using CRISPR/Cas9 technology and the pHT1K vector, respectively. To validate the specific regulatory mechanism, we analyzed (i) the bacterial growth curves and GlnL expression in LG37, OE*glnL*, and Δ*glnL* and (ii) the interrelationship with GlnL and downstream ammonia assimilation-related proteins (Liu et al., 2020).

It was reported that GlnK/GlnL plays a crucial role in ammonia assimilation metabolism, and the glutamine assimilation two-component system response regulator GlnL is not specific to the process of glutamine assimilation. Still, the role of GlnL in ammonia assimilation metabolism is not negligible. Compared with glutamine metabolism, GlnK/GlnL was shown to be specific to ammonia nitrogen assimilation metabolism. Meanwhile, we verified the interaction between GlnL and downstream ammonia assimilation proteins at the transcriptional level by overexpressing and knocking out *glnL*, which effectively verified its regulatory function in ammonia transport and the assimilation metabolic pathway and provided a theoretical basis for the in-depth study of the molecular mechanism of ammonia assimilation in *Bacillus*.

Materials and Methods

Strains

The wild-type strain and mutant strains of LG37 were preserved in our laboratory.

Determining the growth characteristics of LG37 and mutant strains

Conical flasks were filled with 100/250 mL minimal medium in which the nitrogen source was 10 mM glutamine or ammonia and LG37 initial inoculum (OD_{600} 0.02) and incubated at 30°C for 60 h. Minimal medium without LG37 was used as a blank control, and the OD_{600} was measured every 4 h. The data were recorded and the growth curves were plotted. Three biological replicates were included per experimental group.

Determination of the efficiency of ammonia assimilation of LG37

LG37 was inoculated in 100/250 mL minimal medium with an initial ammonia nitrogen concentration of 10 mM (initial OD_{600} 0.02) and incubated at 160 r/min at 30°C. The ammonia content in the medium was measured every 4 h. The ammonia assimilation rate was calculated using the nano reagent photometric method (Gao et al., 2018) as follows:

$W\ (\%) = C_0 - C_1/C_0 \times 100\%$,

Where W is the ammonia assimilation rate, C_0 is the initial ammonia concentration (mg/L), and C_1 is the residual ammonia concentration (mg/L). Finally, the ammonia nitrogen assimilation rate curve was plotted.

DNA/RNA extraction and PCR amplification

LG37 was cultivated in LB and minimal medium. The concentration of nitrogen was set at 10 mM with NH_4^+ (treatment) or Gln (control). Total DNA and RNA were extracted by TRIzol reagent (Invitrogen, USA), following the recommendations. The obtained LG37 genomic DNA was used as a template for PCR. PCR amplification conditions were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min and a final extension step at 72°C for 2 min (Liu et al., 2020). All primers used for PCR and RT-qPCR were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized by Sangong Bioengineering (Shanghai) Co., Ltd. The primer sequences are shown in **Table 1**.

Construction of a glnL knockout strain

The Δ*glnL* strain was constructed with chimeric single guide RNA (sgRNA) with plasmid pJOE8999 and the homologous recombination method. The 20-nt protospacer adjacent motif sequence was cloned upstream of 5′-NGG-3′ in the *glnL* sequence of LG37. The primers were designed using Primer-BLAST. The pJOE8999 plasmid and sgRNA-*glnL* were digested by the *Bsa*I restriction endonuclease and then ligated to generate the pJOE8999_sgRNA-*glnL* plasmid.

The 600-bp homologous exchange fragments (HEFs) upstream and downstream of *glnL* were amplified. The complete *glnL* HEFs were obtained by overlap extension PCR. The obtained complete GlnL-HEFs was inserted into the pJOE8999 plasmid by digestion with the *Sfi*I restriction enzyme followed by ligation to generate the plasmid pJOE8999-sgRNA-*glnL*UD. Successful cloning of pJOE8999-sgRNA-*glnL*UD was verified by sequencing and restriction enzyme digestion analysis (Altenbuchner et al., 2016).

Generation of glnL overexpression LG37 strains

PCR amplified products of *glnL* were cloned into the integrating plasmid pHT1K-P_{xyl} between the *BamH*I and *Kpn*I restriction endonuclease sites to obtain pHT1K-P_{xyl}-*glnL* recombinant plasmids. The primers are listed in Additional File 11. The recombinant plasmids were transformed into *Escherichia coli* DH5α cells for cultivation on LB plates with ampicillin for 24 h. Positive clones of pHT1K-P_{xyl}-*glnL* recombinant plasmids were

verified by sequencing and restriction enzyme digestion analysis. The recombinant plasmid was transformed into LG37 by electroporation, and successful transformation was screened with 25 μg/mL erythromycin (Liu et al., 2020).

Gene expression analysis by quantitative RT-PCR

To validate the expression of genes related to the ammonia nitrogen assimilation metabolic pathway, *glnK*, *glnL*, *mnrA*, *glnA*, and *glnB* were selected for quantitative real-time RT-PCR (RT-qPCR) analysis. The RT-qPCRs were carried out using the SYBR Premix Ex Taq kit (Takara, Japan) with StepOne Real-Time PCR (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instruction. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). The 16S rDNA gene was used for normalization.

Statistical analysis

Statistical analyses were conducted by one-way analysis of variance (ANOVA) with SPSS 18.0. All analysis results are represented as mean ± standard deviation of three independent assays.

Results

Glutamine and ammonia were added to a minimal medium at a concentration of 10 mM. The growth curves are shown in **Figure 1**. LG37 could grow rapidly in a minimal medium with both glutamine and ammonia as the only nitrogen source. In a minimal medium, LG37 grew more rapidly with glutamine than with ammonia as the only nitrogen source, which effectively shortened the period of the beginning of the growth curve.

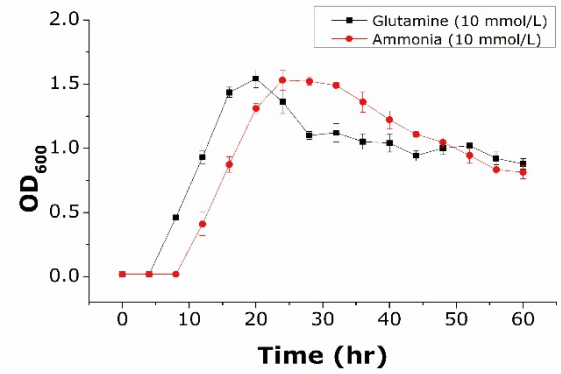

Figure 1 Growth curves of LG37 in minimal media with 10 mM glutamine and ammonia as the sole nitrogen source.

The ammonia nitrogen utilization efficiency of LG37 is shown in **Figure 2**. At 0–4 h, LG37 was at the beginning of the growth period, when the assimilation efficiency of ammonia nitrogen gradually increased. The assimilation rate increased rapidly from 4 to 20 h. At 20 h, the assimilation rate was stable, reaching 94.2%. The assimilation rate maintained 94.1–95.4% during 20–60 h.

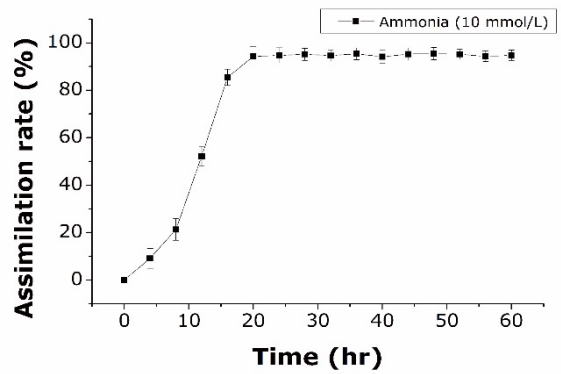

Figure 2 Determination of ammonia assimilation rate of LG37, initial NH_4^+ concentration 10 mM.

The expression of *glnL* was determined by RT-PCR in different strains cultured in minimal medium with 10 mM ammonia as the nitrogen source. The results show that *glnL* expression was significantly higher in OE*glnL* than in the wild-type and knockout strains. The expression of *glnL* was lowest in the knockout strain (**Figure 3a**). The growth curves revealed that OE*glnL* and Δ*glnL* showed different growth trends compared with the wild-type LG37 strain, with OE*glnL* showing shorter initiation and logarithmic phases compared with LG37 and Δ*glnL* showing slower growth and a significant prolongation of the growth cycle (**Figure 3b**).

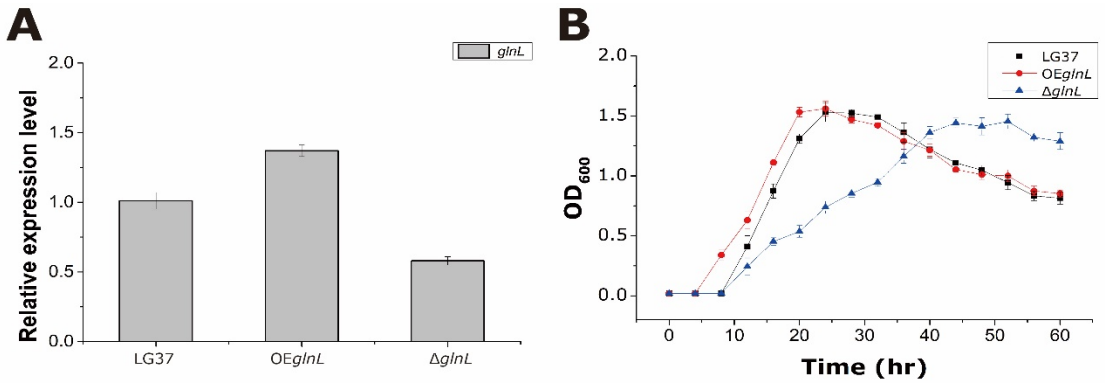

Figure 3 Analysis of *glnL* expression and growth curve of LG37, OE*glnL* and Δ*glnL* strains. (A) The relative expression level of *glnL* in LG37, OE*glnL* and Δ*glnL* strains. (B) Growth curve of LG37, OE*glnL* and Δ*glnL* strains.

In LG37, *glnL*, *glnA*, and *glnB* expression is significantly correlated with the ammonia concentration (**Figure 4**). In OE*glnL*, we observed a significant increase in the expression of *glnL*, *glnA* and *glnB*, and a significant decrease in the expression of *glnL*, *glnA* and *glnB* in Δ*glnL* (**Figure 5**). The expression of *tnrA* and *glnR* was examined at different ammonia nitrogen concentrations, the expression of *tnrA* showed a positive correlation with the increase of concentration, however, there was no significant difference in the expression of *glnR* (**Figure 6A**). In both OE*glnL* and Δ*glnL*, *tnrA* was significantly upregulated upon culture in 10 mM ammonia nitrogen, while the expression of *glnR* did not show significant changes in any of the three strains (**Figure 6B**).

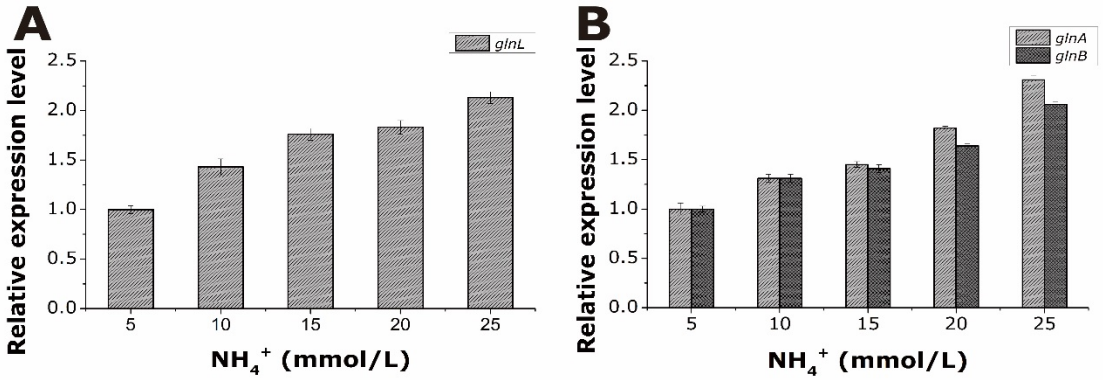

Figure 4 Relative expression of *glnL*, *glnA* and *glnB*. (A) Relative expression of *glnL* with NH_4^+ (5, 10, 15, 20 and 25 mM) of LG37, 5 mM as control. (B) Relative expression of *glnA* and *glnB* with NH_4^+ (5, 10, 15, 20 and 25 mM) of LG37, 5 mM as control.

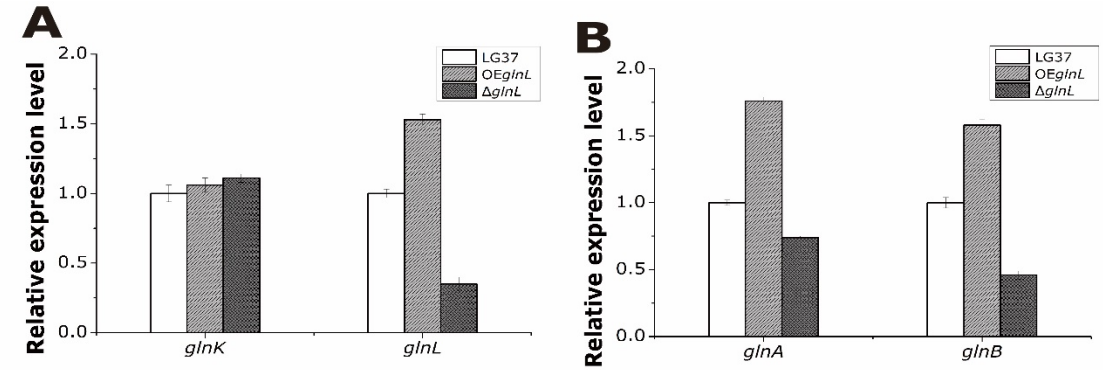

Figure 5 Relative expression of *glnK*, *glnL*, *glnA* and *glnB* in LG37, OE*glnL* and Δ*glnL* strains. (A) Relative expression of *glnK* and *glnL* in LG37, OE*glnL* and Δ*glnL* strains with 10 mM NH_4^+. (B) Relative expression of *glnA* and *glnB* in LG37, OE*glnL* and Δ*glnL* strains with 10 mM NH_4^+.

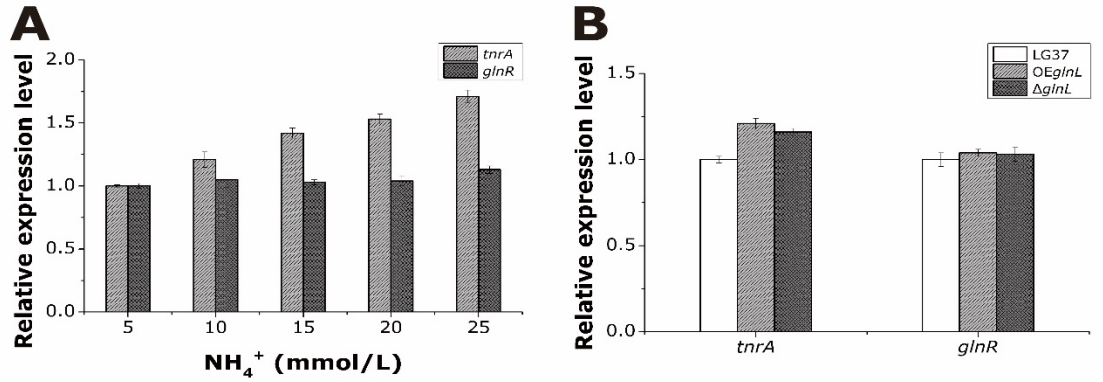

Figure 6 Relative expression of *tnrA* and *glnR*. (A) Relative expression of *tnrA* and *glnR* with NH_4^+ (5, 10, 15, 20 and 25 mM) of LG37, 5 mM as control. (B) Relative expression of *tnrA* and *glnR* in LG37, OE*glnL* and Δ*glnL* strains with 10 mM NH_4^+.

Discussion

The accumulation of nitrogenous compounds such as excrement and residual bait in aquaculture water bodies with the prolongation of the culture cycles results in excessive ammonia nitrogen, which in turn affects the growth of aquatic animals and even leads to the outbreak of diseases (Zhao et al., 2020). *Bacillus* can rapidly utilize ammonia in aquaculture water through assimilation metabolism, thus reducing the environmental burden of aquaculture. *Bacillus* has its own specific ammonia sensing, transport, and

assimilation proteins to assist in its proliferation and metabolic reactions (Richts et al., 2021).

GlnK/GlnL is a two-component regulatory system which plays a key role in the assimilation of ammonia nitrogen. GlnK, as a signal-sensing protein, senses the presence of extracellular ammonia and activates the downstream transcriptional regulators GlnL through phosphorylation (Chuang et al., 2022; Liu et al., 2020). In the present study, the growth rate of LG37 in the glutamine group was significantly higher than that of LG37 in the ammonia group. However, combined with the previous experimental results and transcriptome data analysis, it was found that the expression of GlnL in the ammonia group was significantly higher than that in the glutamine control group. GlnL is a glutamine assimilate two-component system response regulator whose expression in the glutamine group should have been higher than that in the ammonia group (Satomura et al., 2005), but the opposite results were obtained, indicating that GlnL played a greater role in ammonia assimilation metabolism than in glutamine assimilation metabolism and that it played an important role in the process of ammonia assimilation. Subsequently, LG37 was cultured in minimal medium and the assimilation rate of ammonia nitrogen was measured. The results indicated that LG37 could efficiently use ammonia as its own nitrogen source for growth and proliferation.

To further prove the role of GlnL in the process of ammonia assimilation and its relationship with other assimilation proteins, OE*glnL* and Δ*glnL* were generated through the CRISPR/Cas9 system and the pHT1K vector, respectively, and cultured in minimal medium with an ammonia concentration of 10 mM. The expression of *glnL* was determined by RT-qPCR. The order of *glnL* expression was as follows: OE*glnL* > LG37 > Δ*glnL*. Combined with the growth curves, the experiments showed that the overexpression and knockout strains were successfully established and that GlnL played an important role in the assimilation and metabolism of ammonia nitrogen.

In minimal medium, with increasing ammonia concentrations, the expression of *glnL* increased significantly, and the expression of downstream *glnA* and *glnB* assimilation genes also increased. Under 10 mM ammonia culture, in OE*glnL*, both *glnA* and *glnB* showed a significant upregulation, while in Δ*glnL*, the expression levels of *glnA* and *glnB* both showed a downward trend. There was a positive correlation with assimilation metabolism. However, the expression of *glnK* in different strains hardly changed, indicating that GlnK sensed the changes in ammonia nitrogen concentrations in the outside world and then activated GlnL, but did not participate in regulating the expression of downstream assimilation protein-encoding genes.

There was no significant difference in the expression of *glnR* under different ammonia concentrations or between the three different strains, indicating that under the restriction of ammonia concentration, GlnR does not play a role in ammonia assimilation and metabolism. However, *tnrA* expression showed a positive correlation with *glnL* expression, indicating that the specific role of GlnL in ammonia assimilation metabolism is also regulated by the global transcriptional regulator TnrA under the condition of ammonia nitrogen limitation. The results of this study and previously published results indicate that in the ammonia-specific assimilation metabolic signaling pathway, in which GlnK senses ammonia and activates the transcriptional regulator GlnL, under the condition of ammonia limitation, GlnL and TnrA play a synergistic role, positively regulating MnrA, GlnA, and GlnB. The role of GlnR in the metabolic process is silent.

Acknowledgments

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