The Israeli Journal of Aquaculture – Bamidgeh • ISSN 0792-156X • IJA.74.2022.1812637, 12 pages
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Antibacterial characterization of *Bacillus velezensis* LG37 and mining of genes related to biosynthesis of antibacterial substances

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(Received Sep 25, 2022; Accepted Oct 14, 2022; Published Nov 07, 2022)

Keywords: *Bacillus velezensis*; bacteriocin; gene cluster; non-ribosomal peptide; ribosomal peptide

Abstract

Bacillus velezensis LG37 secretes various antibacterial substances and inhibits the growth of other bacteria. Here, we analyzed the antibacterial characteristics and the screening and verification of genes related to the synthesis of the antibacterial substance of LG37 by antibacterial activities experiment, Local BLAST+, and RT-PCR. LG37 was isolated from aquaculture water and preserved in our laboratory. The phylogenetic tree was used to analyze the genetic relationship between LG37 and the bacteriostatic test indicator strain. LG37 had a more substantial inhibitory effect on closely related strains, while the inhibitory effect on the more distantly related strains was weak. Combined with genome sequencing results, the ribosomal peptide (RP) bacteriocin gene and non-ribosomal peptide synthetase (NRPSs) related gene clusters were screened and analyzed. A total of six gene-coding RP bacteriocins and two genes coding surfactins and fengycin A NRPSs gene cluster were screened. Local BLAST+ analysis revealed a total of 11 NRPSs gene clusters. RT-PCR further validated the active expression of the NRPSs and RP encoding genes. The findings revealed various genes and gene clusters encoding RP bacteriocins and NRPSs in *B. velezensis* LG37. The bacterium is potentially valuable in diverse applications in aquaculture.

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Introduction

In recent years, with the continuous development and promotion of high-density culture, the aquaculture industry in China has developed rapidly. The mu yield has risen annually (Chang et al., 2020). The increased mu yield has been accompanied by increased use of feed. This has led to the accumulation of feed residues, aquatic animal excreta, and large amounts of nitrogenous organic matter in the aquaculture water. These increases have resulted in the proliferation of pathogenic microorganisms and disease outbreaks in the aquaculture water, with huge economic losses to the aquaculture industry (Liu et al., 2022; Zhao et al., 2019). The development of antibiotics and their application have effectively reduced the risk of disease in aquaculture by effectively reducing the content of pathogenic microorganisms in the water column and preventing outbreaks of various aquaculture animal diseases (Balcázar et al., 2006; Xiang et al., 2019). However, the improper use of antibiotics has led to increasing problems of residual antibiotics in the water column and antibiotic resistance of pathogenic bacteria. Research and development of effective antibiotic replacement products has become increasingly urgent (Shao et al., 2021; Santos et al., 2018).

Bacillus velezensis was isolated from water samples by Ruiz-García et al. in 2005 and was validated by DNA hybridization (Ruiz-Garcia et al., 2005). *B. velezensis* (also designated as CR-502T) is appealing because of its fast growth rate, a rich variety of secondary metabolites, high tolerance, broad spectrum of bacterial inhibition, significant inhibition effect, and excellent safety. *B. velezensis* has significant inhibitory effects against many common aquatic pathogens that include *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *V. harveyi*, and *Edwardsiella tarda* (Maung et al., 2021; Ye et al., 2018). In addition, *B. velezensis* has been studied concerning its effective inhibition of various crop fungal pathogens, such as *Pseudocercospora musae*, *Botrytis cinerea*, and *Ascochyta citrullina*, and has achieved significant results in field biological control practices (Balthazar et al., 2022; Tapi et al., 2010). *B. velezensis* shows excellent potential for application in preventing and controlling aquatic animal diseases. Before this is realized, it is necessary to comprehensively study the inhibition properties and mechanisms of *B. velezensis* to provide a theoretical basis for its application in aquaculture biocontrol.

B. velezensis can secrete a variety of secondary metabolites with antibacterial properties. The synthesis of these metabolites involves two main pathways: the ribosomal peptide (RP) and non-ribosomal peptide (NRP) synthesis pathways (Rabbee et al., 2019; Yang et al., 2018; Zheng and Sonomoto, 2018). The RP synthesis pathway refers to polypeptides or precursor polypeptides formed by condensation, modification, and cyclization of polypeptides through ribosomes. The structures are primarily cyclic peptides (Kaniusaite et al., 2020). RP bacteriocins are very safe and mainly used in food, medicine, and farming. The NRP synthesis pathway refers to the cyclization of amino acids by modular non-ribosomal peptide synthetases (NRPSs), the introduction of cyclized and other molecules, and re-modification by acylation, glycosylation, lipidation, and other reactions. The generated NRP antibacterial substances are characterized by a complex structure, variety, high inhibitory activity, and broad spectrum of inhibition (Ahmad et al., 2017; Musiol et al., 2007). Most of the NRP antibacterial substances are based on bacterial inhibition and eradication caused by the destruction of the cell membrane structure of pathogenic bacteria. The cell membrane is relatively stable and resists large changes. Therefore, tolerance to NRP antibacterial substances is uncommon.

B. velezensis LG37 was isolated from pond culture water in the laboratory. Previous experiments showed that LG37 could assimilate inorganic nitrogen efficiently and secret secondary metabolites to inhibit the growth of various microorganisms. The antibacterial properties of *B. velezensis* have been confirmed by many studies (Liu et al., 2020). However, the composition of the secondary metabolites secreted by bacteria is complex, and external

microorganisms and nutrients influence the secretion of inhibitory metabolites. Therefore, inhibition varies greatly.

Sequencing and bioinformatics technologies have become important technical means to reveal biological properties in depth. In this study, we analyzed the types of RP and NRP bacteriocins secreted by LG37 at the genetic level. Bioinformatics analysis of genome sequencing further revealed the types of secreted NRP bacteriocins and the coding genes and gene clusters. The findings laid the theoretical foundation for future studies of bacteriocins.

Materials and Methods

LG37 morphology

LG37 was isolated, identified, and stored in our laboratory. Colony morphology was observed on streak plates using Luria-Bertani (LB) solid medium. The genome sequencing results have been uploaded to NCBI Genome Database as NCBI GenBank registration number CP023341 (Liu et al., 2020, Liu et al., 2022).

Phylogenetic tree of LG37

LG37 16S rDNA sequence was compared with the 16S rDNA sequences of 20 indicator strains downloaded from NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/). The phylogenetic tree was generated by MEGA6 (Liu et al., 2022).

Bacteriostasis testing

LG37 was used as the target strain. The 20 indicator strains were *B. subtilis* 168, *B. licheniformis* AB92069, *B. altitudinis* AB205059, *B. coagulans* AB93247, *B. firmus* AB94028, *B. soli* AB205457, *B. jeotgali* AB205079, *B. boroniphilus* AB205074, *B. thurisngiensis* AB94047, *Staphylococcus aureus* RN4220, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* UA159, *S. pneumoniae PUS084*, *S. suis* sp, *S. pyogenes* 12344, *S. dysgalactiae* 35666, *S. agalactiae* S12, *Mycobacterium smegmatis* MC2155, *Pseudomonas putida* KT2440, and *Escherichia coli* O157. The indicator strains were from the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University.

After activation, LG37 and the indicator strains were inoculated in 250 mL triangular flasks containing 100 mL of LB and BHI liquid medium, respectively, and incubated to an optical density at 600 nm of 0.6-0.8 at 32ºC with a shaker speed of 160 rpm. The solid medium containing 1% agar powder was sterilized. The medium was cooled to 45−48ºC followed by the addition of bacteria 1:1 with the solid medium. The suspension was poured into the solid plate to complete the preparation of the semi-solid plate containing each indicator bacterium. Rice paper sheets 0.5 cm in diameter prepared by the puncher were mixed with LG37 bacterial solution. They were placed in the center of the semi-solid plate and incubated in a constant temperature incubator at 32ºC for 48 h. The aseptic operation was carried out in a sterile operation table.

Screening for genes encoding bacteriocins

Genome sequencing, annotation information, and online prediction results were combined to screen RP bacteriocin-encoding genes and NRPSs-encoding gene clusters. The clusters were analyzed. The coding genes obtained by screening were matched by NCBI, UniProt, and KEGG (Kyoto Encyclopedia of Genes and Genomes; https://www.kegg.jp/) databases and online prediction results and compared one by one.

Verification of expression of genes encoding bacteriostatic substances

LG37 was inoculated and cultured in LB liquid medium. Total RNA was extracted and reverse transcribed into cDNA by the SYBR Premix Ex Taq kit (TaKaRa Bio, Shiga, Japan) as previously described (Liu et al., 2022). The cDNA was used as the template for PCR reactions

in a 20 μL reaction system as follows: 1 μL of each 10 μmoL/L primer, 10 μL of 2× Taq PCR Master Mix, 1 μL of template, and 8 μL of distilled deionized water. PCR amplification conditions were 95°C for 5 min; 35 cycles of 95°C for 30 s, 57°C for 30 min, and 72°C for 1 min; 72°C extension for 2 min, and storage at 4°C. The specific primers for the target genes were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Primer synthesis was performed by Shanghai Oebiotech Co., Ltd., Shanghai, China.

Screening of gene clusters encoding NRP bacteriocins

BLAST+ (Basic Local Alignment Search Tool; https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/) was used to deeply analyze and mine the LG37 genome for NRPSs-related encoding gene clusters. The identified gene clusters related to the synthesis of NRP bacteriocins in the NCBI and UniProt databases were downloaded and compared with the genome sequencing results one-by-one to determine whether there were deletions or mutations in the LG37 genome. In addition, based on the information of the screened gene clusters, the location of their genomes and the proportion of the whole genome sequence were determined.

Results

The morphological observation of LG37 was carried out by inoculating LB solid medium and incubating them in a constant temperature incubator at 32°C for 24 h and 48 h. At 24 h, the colonies were irregular, yellowish, translucent, elevated, glossy, and very hydrated, with a single colony diameter of 2−7 mm (**Figure 1A**). After 48 h of incubation, the colonies were dehydrated, irregular in shape, creamy white, opaque, and wrinkled, with no bubble production (**Figure 1B**).

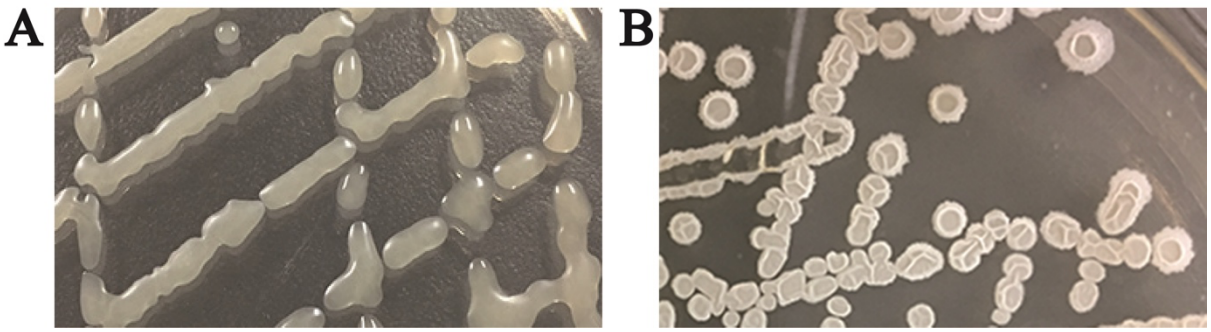

Figure 1 Morphological analysis of LG37. A. 37 ºC culture for 24 hours with LB medium. B. 37 ºC culture for 48 hours with LB medium.

The results of the bacteriostasis test are shown in **Figure 2**. LG37 had a significant inhibitory effect on all strains of *Bacillus* indicator bacteria, except *B. subtilis* 168, with clear inhibition zones. The top three strains with significant inhibition effects were *B. coagulans* AB93247, *B. firmus* AB94028 and *B. thuringiensis* AB94047. None of the non-*Bacillus* Gram-positive indicator strains showed significant inhibition effects. The results in **Figures 2−3, 2−5, 2−6, 2−7, 2−8, and 2−9** show that the target strain LG37 produced a clear "safe zone" around the bacteria. The concentration of extracellular secondary metabolites secreted by bacteria should gradually decrease from the inner to the outer circle, significantly different from the results in 2−3 and 2−4. The strain identification analysis showed that the strains in the "safe zone" were all indicator strains.

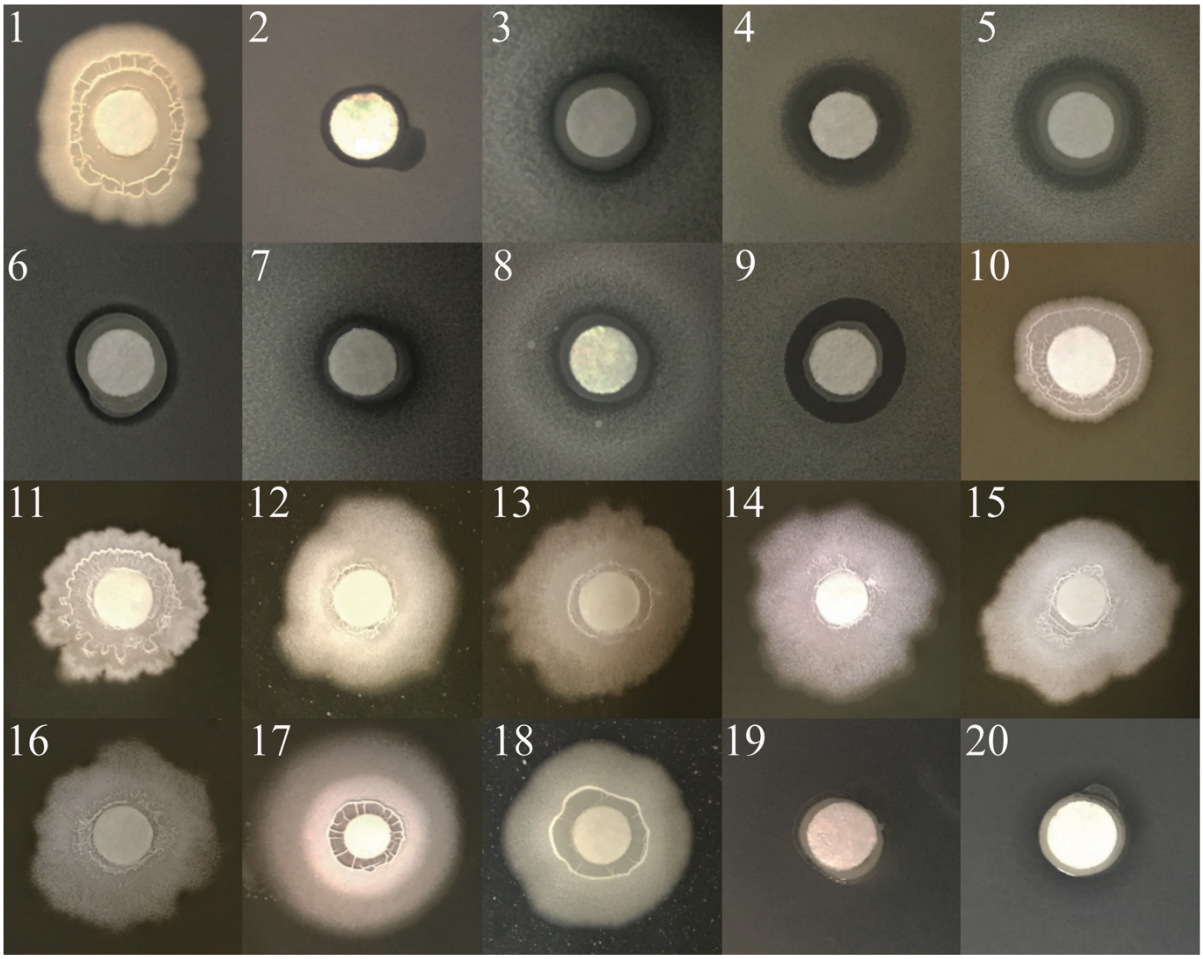

Figure 2 Antibacterial activity assay of LG37 against Gram-positive (1-18) and Gram-negative bacteria (19-20).
1. *Bacillus subtilis* 168; 2. *B. licheniformis* AB92069; 3. *B. altitudinis* AB205059; 4. *B. coagulans* AB93247; 5. B. firmus AB94028; 6. *B. soli* AB205457; 7. *B. jeotgali* AB205079; 8. *B. boroniphilus* AB205074; 9. *B. thurisngiensis* AB94047; 10. *Staphylococcus aureus* RN4220; 11. *Enterococcus faecalis* ATCC 29212; 12. *Streptococcus mutans* UA159; 13. *S. pneumoniae* PUS084; 14. *S. suis* sp; 15. *S. pyogenes* 12344; 16. *S. dysgalactiae* 35666; 17. *S. agalactiae* S12; 18. *Mycobacterium smegmatis* MC2155; 19. *Pseudomonas putida* KT2440; 20. *Escherichia coli* O157.

The phylogenetic tree constructed based on LG37 and 20 indicator strains showed that LG37 was most closely related to *B. subtilis* 168, followed by *B. licheniformis* AB92069, *B. altitudinis* AB205059, and *B. jeotgali* AB205079. LG37 was more distantly related to *Streptococcus* spp, Gram-negative *P. putida* KT2440, and LG37 was more distantly related to *Streptococcus* spp, Gram-negative *P. putida* KT2440, and *E. coli* O157 (**Figure 3**). These results, combined with the results of the bacteriostasis test, indicated that the bacteriocins secreted by LG37 could inhibit closely related strains but not the more distantly related strains. In the bacteriostasis test between LG37 and *B. subtilis* 168, a clear circle of inhibition was not evident (**Figure 2-1**).

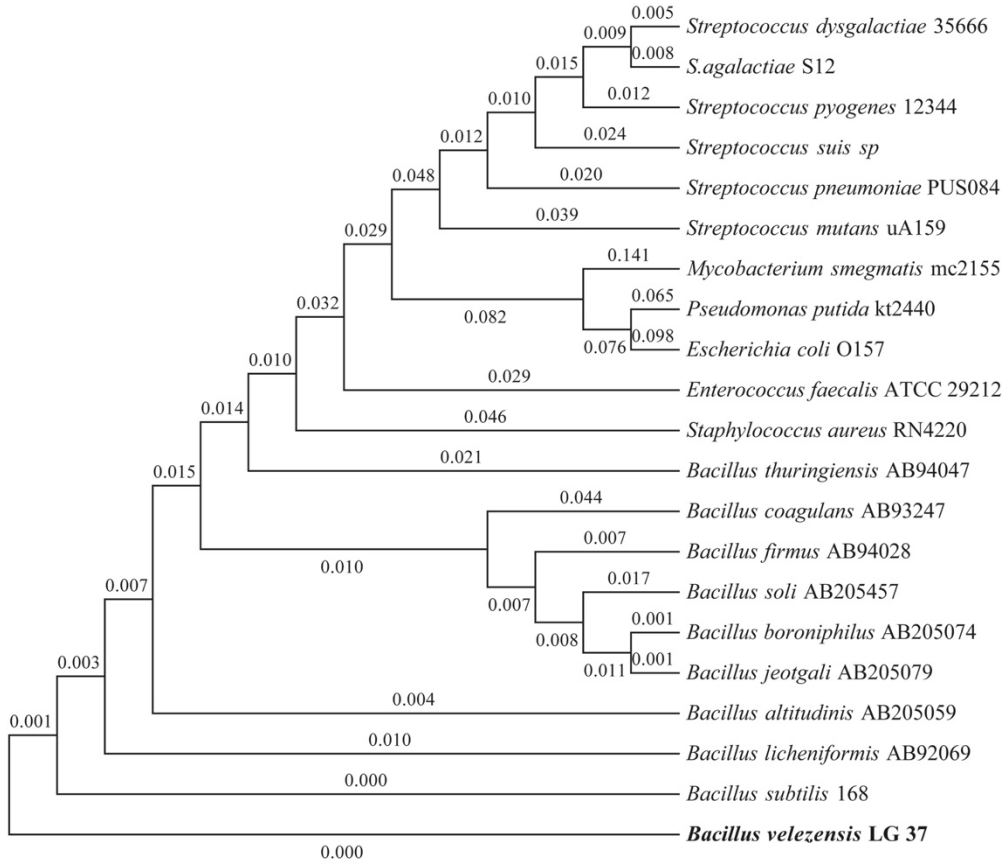

Figure 3 The neighbor-joining tree of LG37 based on 16S rDNA sequences

The gene names, translated proteins, gene ontology molecular functions, and genomic locations of the six genes encoding RP bacteriocins (*orf01068*, *orf01115*, *orf01145*, *orf01176*, *orf03147* and *orf03824*) were obtained by analyzing the genome sequencing results (**Table 1**) (Liu et al., 2022).

Table 1 RP bacteriocin candidate genes in *B. velezensis* LG37.

Bacteriocin gene	Annotation of database information	Similarity (%)
orf01068	SDJ56094.1 antimicrobial peptide, SdpC family	100
orf 01115	AKF31937.1 bacteriocin	100
orf 01145	AKL77573.1 bacteriocin YukD	100
orf 01176	AKL77545.1 putative bacteriocin	95.8
orf 03147	AKD29160.1 bacteriocin	100
orf 03824	ANF35207.1 antimicrobial peptide, Lci	100

Genome sequencing KEGG data showed that the LG37 genome contains a cluster of genes encoding *surfactin* and *fengycin* A NRPSs. Four genes encoding synthetic surfactin NRPSs were identified (*srfAA*, *srfAB*, *srfAC*, and *srfAD*). Of these, *srfAA*, *srfAB*, and *srfAC* synthesized seven amino acid polypeptides, cyclized and assembled by SrfAD to form mature surfactin. Fengycin

A is synthesized by six NRPS genes: *fenA*, *fenB*, *fenC*, *fenD1*, *fenD2*, and *fenE*. The gene cluster information is shown in **Figure 4**, and the structures are shown in **Figure 5**.

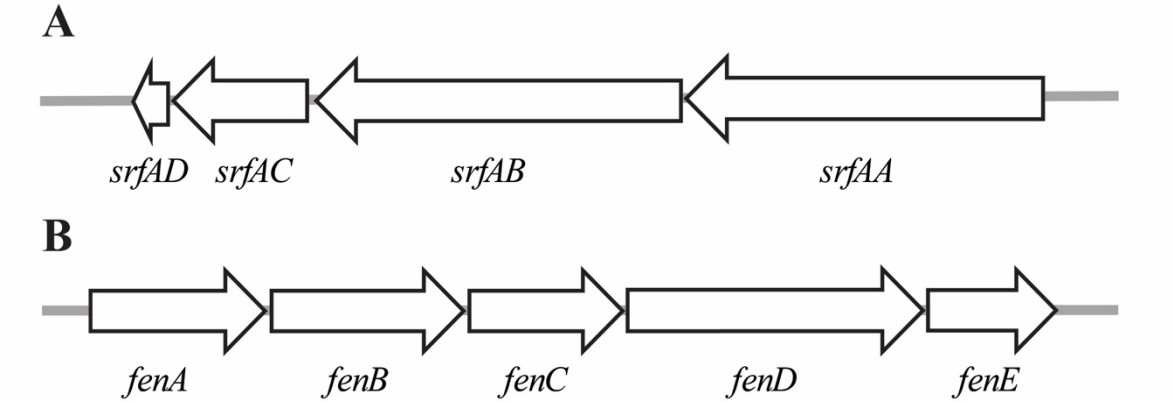

Figure 4 The gene clusters encoding for the two NRPSs. A. *srfAA* (*orf03794*), *srfAB* (*orf03793*), *srfAC* (*orf03792*), and *srfAD* (*orf03791*) encode a NRPS to catalyze the synthesis of surfactin. B. *fenA* (*orf02241*), *fenB* (*orf02242*), *fenC* (*orf02243*), *fenD1* (*orf02244*), *fenD2* (*orf02245*) and *fenE* (*orf02246*) encode another NRPS to catalyze the synthesis of fengycin A. The orientations of genes in genome are indicated by arrows.

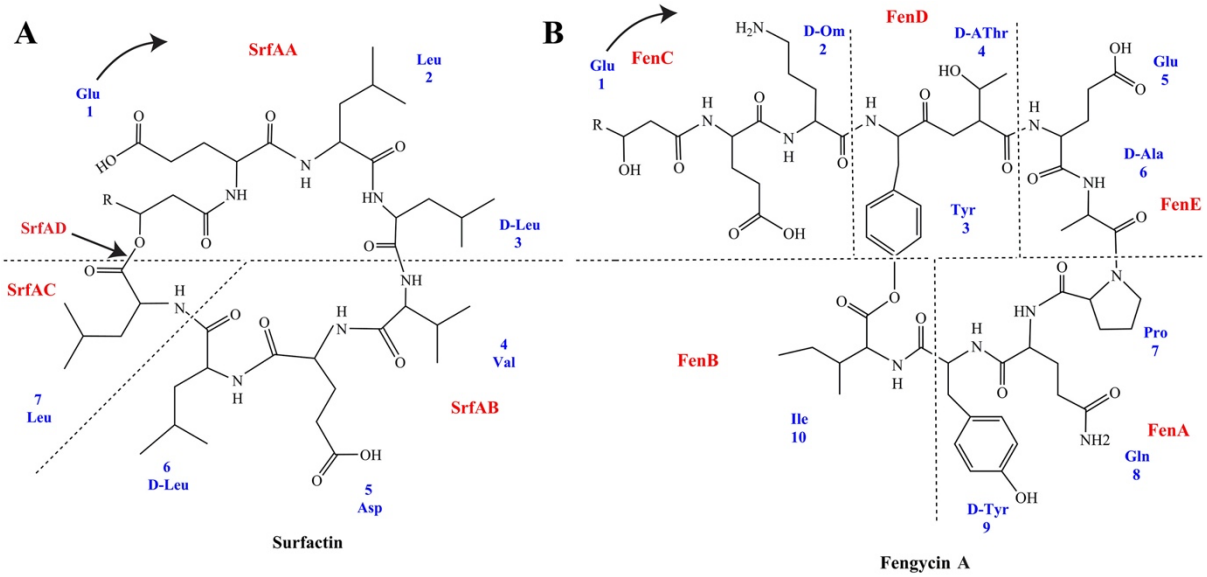

Figure 5 The chemical structures and directions for the biosynthesis of surfactin and fengycin A by NRPS complexes in LG37. **A**. Surfactin synthetase complex comprises SrfAA, SrfAB, SrfAC and SrfAD; SrfAA, SrfAB and SrfAC are used to synthesize a seven amino acid peptide, which is cyclized by SrfAD.
B. The fengycin A synthetase complex comprises FenA, FenB, FenC, FenD, and FenE, which synthesize a ten amino acid cyclopeptide of fengycin A. Arrows and Arabic numerals indicate the initial direction and the order of bacteriocins synthesis, respectively.

The transcript levels of the 16 genes obtained by screening were analyzed by RT-PCR using specific primers (**Table 2**, **Figure 6**). These included six RP bacteriocins encoding genes (*orf01068*, *orf01115*, *orf01145*, *orf01176*, *orf03147,* and *orf03824*) and two clusters of NRPs encoding genes containing a total of 10 genes (*srfAA*, *srfAB*, *srfAC*, *srfAD*, *fenA*, *fenB*, *fenC*,

fenD1, *fenD2*, and *fenE*). The experimental results showed that the expression of all 16 screened genes was high during the logarithmic growth of LG37.

The LG37 genome was compared with the NCBI and UniProt databases by local BLAST. Bacilysin, Bacillibactin, Difficidin, Iturin A, Mycosubtilin A, Bacillomycin D, Bacillaene, Macrolactin, and amylolicin A, were encoded in the LG37 genome, in addition to surfactin and fengycin A. The core gene fragments of the RP and NRPs gene clusters were 11 NRPs encoding different-sized gene clusters. The core gene fragment size of the RP bacteriocins and NRPs gene cluster was approximately 449.6 kb, which accounted for approximately 11.4% of the total genome length.

Table 2 The specific primers used in RT-PCR for amplifying the NRPSs and RP bacteriocin candidate genes.

Name	Primer	Primer sequence	Product size (bp)
srfAA (orf03794)	Forward	TGATGCGTCCGTCAAACAGA	327
	Reverse	GACGCATCTACGCACGTTTC	
srfAB (orf03793)	Forward	CGTCATGCTGGATGAAAGCG	278
	Reverse	GATGCAATGACGCTCCGTTC	
srfAC (orf03792)	Forward	CAATACTGAATGCGGCACGG	213
	Reverse	TTCTGACGTGAGGCACAGAC	
srfAD (orf03791)	Forward	CAAATGTCCCCCGTTGAGGA	337
	Reverse	CGCGGTAGTCGGATCTGAAA	
fenA (orf02241)	Forward	TTGCAGAACTCGCAAAGCAC	363
	Reverse	CAGCGGCTATCCGTTTTTCG	
fenB (orf02242)	Forward	GGAATCGGCGGAGAATTTGC	344
	Reverse	GCGGATTGCGATTGACTTCC	
fenC (orf02243)	Forward	CTGTGCGGATGATTGAAGCG	565
	Reverse	TTCACCTGTCCGTCTGAACG	
fenD1 (orf02244)	Forward	CGTGCAAAAGGACGCTTTGA	345
	Reverse	TCGAGAACCTGGGAGACGAT	
fenD2 (orf02245)	Forward	GACCGTTTCGCGTTTGTTCA	343
	Reverse	TCTTGCCAAATCTCCGGTCC	
fenE (orf02246)	Forward	GCTGATCGACATTTGGACGC	497
	Reverse	CTAAGCCGCGCTTTTCCATC	
orf01068	Forward	CCGTCGGTCTCTTATTCGGG	431
	Reverse	TCGTCAACGCTTGCTACTGT	
orf01115	Forward	CTTTCATTTTGTTTGGCGCGG	245
	Reverse	TGCATAAGAAATCGCGCCTG	
orf01145	Forward	ATAACGGCAGCGTCTTCGAT	201
	Reverse	AAATTTCAAGGCGGTCTCCG	
orf01176	Forward	AAAGGCGGCGGCCTGTATT	217
	Reverse	ACGATTCTGATATACTCACGCGG	
orf03147	Forward	CAACTTCTTGGCATTCGGGC	380
	Reverse	TCAGACATGATTTGGGCGCT	
orf03824	Forward	CTGCGCTGTCTCTTGCCTTA	222
	Reverse	ACCCTTTGCTGCTGTCATAGT	

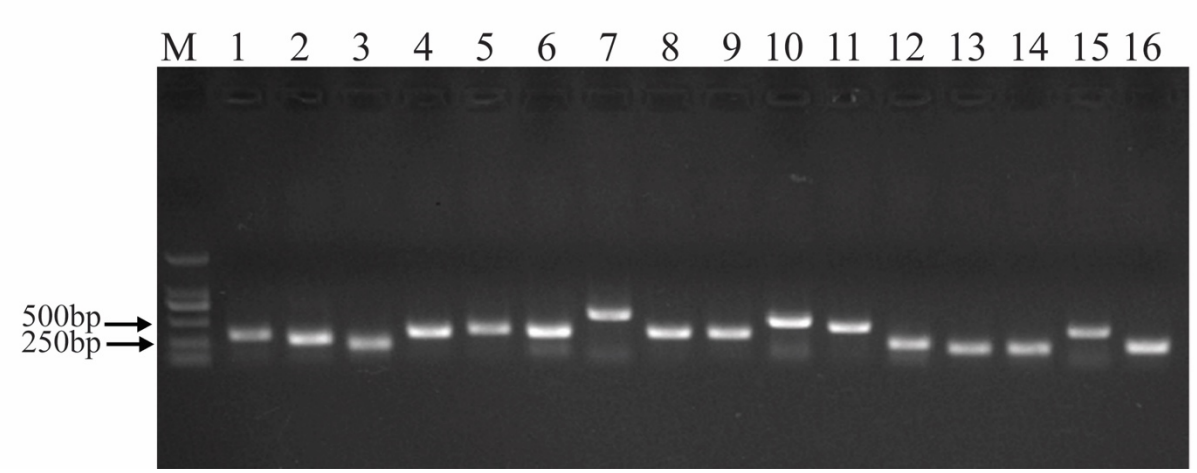

Figure 6 Validation of the transcriptions of bacteriocin synthetase encoding genes from LG37 by RT-PCR. M: Marker DL 2000; NRPs encoding genes of surfactin 1-4: *srfAA*, *srfAB*, *srfAC* and *srfAD*; NRPs encoding genes of fengycin A, 5-10: *fenA*, *fenB*, *fenC*, *fenD1*, *fenD2* and *fenE*; RP encoding genes, 11-16: *orf01068*, *orf01115*, *orf01145*, *orf01176*, *orf03147* and *orf03824*.

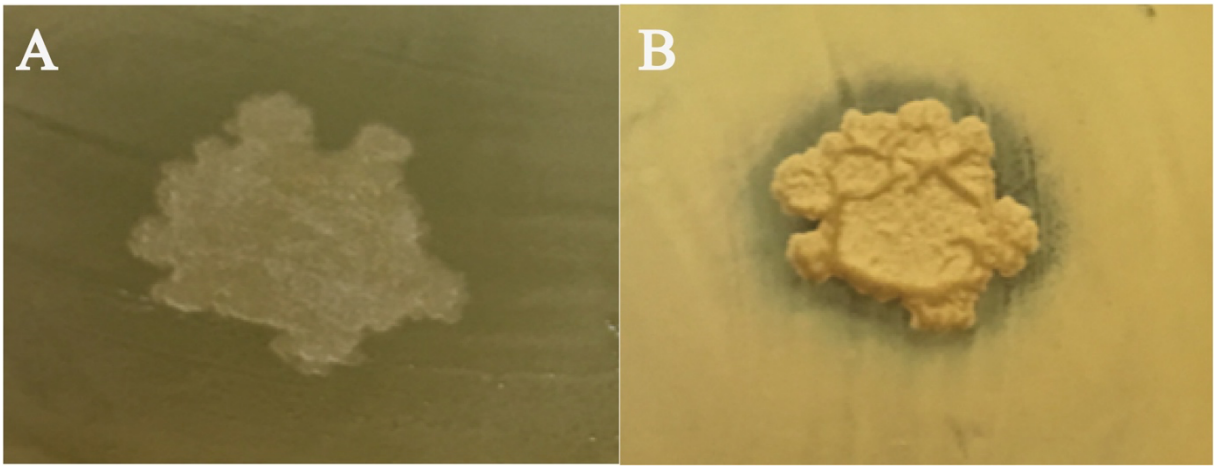

Figure 7 Antibacterial activity analysis of LG37 against *S. aureus* RN4220. A: culture for 12 h; B: culture for 48 h.

Discussion

In recent years, with the promotion of the "green, ecological, safe, and healthy" development model in China, the adverse effects and secondary pollution caused by the irregular use of chemical drugs and antibiotics in the aquaculture industry have been gradually recognized (Mo et al., 2009). *B. subtilis* has been widely used in agriculture, medicine, animal husbandry, biofertilizer, and other fields and has achieved significant benefits in biofertilization (Fasusi et al., 2021). The application of biocontrol bacteria in aquaculture water has more significant advantages in terms of water temperature, pH, and nutrient elements compared with other fields. These advantages have greatly improved the application effects of biocontrol bacteria. However, relatively little is known concerning the biological control of aquatic animal diseases (Brooker et al., 2018). In this study, we analyzed the potential of LG37 as a green

biocontrol strain through bacteriostasis testing. To analyze the inhibition characteristics of LG37 at the genetic level, we combined genome-wide data analysis and local BLAST+ bioinformatics analysis technology to mine the genes and gene clusters encoding the secreted inhibition substances of LG37 and lay the foundation for the development and utilization of LG37 in the field of aquatic animal disease biocontrol (Liu et al., 2022).

LG37 genome sequencing showed that the genome size was 3,929,697 bp, with 3,057 protein-coding genes with precise biological functions. Homology matching analysis revealed that LG37 was most closely related to *B velezensis* FZB42 (78.4%), which contains a gene cluster encoding an NRP bacteriophage synthase (Liu et al., 2022). This strain has a strong inhibitory effect on both fungi and bacteria. The size of the six repressor-encoding gene clusters in FZB42 accounted for 7.5% of the entire genome. The 11 gene clusters in LG37 were 449.6 kb in size, accounting for 11.4% of the entire genome (Liu et al., 2022; Shen et al., 2022), suggesting that the LG37 strain produced a wider variety of bacteriocins than FZB42 and may have a broader spectrum of repression.

In the LG37 antagonism assay, the results of the antagonism pre-test with the *S. aureus* RN4220 indicator strain showed that LG37 effectively inhibited the growth of *S. aureus* RN4220 (**Figure 7**). The subsequent positive antagonism assay results were inconsistent with the observation. RT-PCR results for LG37 revealed high transcript levels of surfactin, fengycin A synthetase coding gene cluster, and RP bacteriocin coding gene during incubation (**Figure 6**). The remaining NRP bacteriocins showed corresponding transcriptional instability. Iturin A, Mycosubtilin A, and the three gene clusters Iturin A, Mycosubtilin A, and Bacillomycin D showed a high degree of overlap. These findings suggest that LG37 can secrete a variety of bacteriocins. Still, the process of bacteriocins synthesis and secretion varies depending on the external indicator bacteria and environmental factors, resulting in the instability of LG37 synthesis and secretion of bacteriocins. Thus, LG37 has potential value for novel drug development and application. The study findings provided a basis for the screening of targeted stimulation of LG37 secretion of inhibitory substances. They provided a direction for the targeted screening of biocontrol bacteria that inhibited aquaculture pathogenic bacteria.

This study indicated that *B. velezensis* could be applied as a biocontrol bacterium in aquaculture waters. Further comprehensive studies are warranted. Future research will focus on the inducing conditions and stimulating factors that affect the secretion of LG37 inhibitory substances. These findings will inform the inhibition of pathogenic bacteria in aquaculture water, biosafety of economic animals, and biocontrol mechanism of action.

Acknowledgments

This study was jointly supported by a fund from the Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (No. 2020TS04); Central Public-interest Scientific Institution Basal Research Fund South China Sea Fisheries Research Institute CAFS (NO.2021SD15); the Guangdong Provincial Rural Science and Technology Commissioner Fund for Stationed in the town to help the village (KTP20210318); and the Guangdong Provincial Special Fund For Modern Agriculture Industry Technology Innovation Teams（2019KJ141）.

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