Complete genome sequence of *Bacillus velezensis* LPL-K103, an antifungal cyclic lipopeptide bacillomycin L producer from the surface of lemon

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Received: 30 April 2019 / Accepted: 18 November 2019 / Published online: 2 December 2019
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Abstract

Bacillus velezensis LPL-K103, which shows strong antifungal function, was isolated from the surface of lemon in Beijing, China. The complete genome of *B. velezensis* LPL-K103 contains a circular chromosome of 3,933,292 bp (46.61% G+C content). According to genomic analysis, 4080 protein-coding genes, 113 RNAs (27 rRNAs + 86 tRNAs), and a non-ribosomal peptide synthase gene cluster involved in antifungal cyclic lipopeptide bacillomycin L biosynthesis were identified. Here, we propose that the biosynthesis pathway of bacillomycin L in LPL-K103 depends on its genome information.

Keywords Bacillomycin L · *Bacillus velezensis* · Biosynthesis · Genome sequence

Introduction

Bacillus species are Gram-positive, rod-shaped, endospore-forming bacteria that produce a vast array of structurally diverse antifungal compounds (Emmert and Handelsman 1999). Among these species, *Bacillus subtilis* and *Bacillus amyloliquefaciens* are regarded as high-quality biocontrol agents owing to their broad inhibitory spectrum (Ongena and Jacques 2008). *Bacillus velezensis* was previously reported to be a later heterotypic synonym of *B. amyloliquefaciens* (Wang et al. 2008) and has recently been reclassified as a synonym of *B. amyloliquefaciens* subsp. *plantarum*, *Bacillus methylogrophicus* and *Bacillus oryzicola* (Dunlap et al. 2015). In the well-studied commercial biocontrol strain *B. velezensis* FZB42, 8.5% of the genome devoted to peptide-based antibiotics and siderophore synthesis. In its genome, several gene clusters are involved in the non-ribosomal

synthesis of cyclic lipopeptides and polyketides, which are highly effective anti-fungal agents (Chen et al. 2007).

B. velezensis LPL-K103, which was isolated from the surface of lemon in Beijing, China, has been deposited at the China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC No. 7747. Our preliminary results showed that *B. velezensis* LPL-K103 can produce bacillomycin L, an antifungal lipopeptide with a molecular mass of 1034.55 Da (Zhang et al. 2013a). This lipopeptide belongs to the iturin family, which consists of iturin A, C, D, and E, bacillomycin D, F, L, and LC, and mycosubtilin, which exhibit strong antifungal activity against a wide range of pathogenic yeasts and fungi and shows hemolytic activity and limited antibacterial activity toward bacteria such as *Micrococcus luteus* (Jacques 2011; Maget-Dana and Peypoux 1994; Mnif and Ghribi 2015). Bacillomycin L produced by *B. velezensis* LPL-K103 contains seven amino acid residues bonded to a β -hydroxy fatty acid, and the chiral amino acid sequence of the peptide chain is “L-Asp—D-Tyr—D-Asn—L-Ser—L-Gln—D-Ser—L-Thr” (Zhang et al. 2013a). Furthermore, we found that some components of the inhibition mechanism of bacillomycin L against *Rhizoctonia solani* Kühn were due to its membrane permeabilization and intracellular DNA interaction capacities (Zhang et al. 2013b). To further investigate its antifungal mechanisms, we proposed a model of the action of bacillomycin L against intracellular targets of *R. solani* Kühn hyphal cells based on proteomic analysis (Zhang et al. 2016).

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-019-1995-y>) contains supplementary material, which is available to authorized users.

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In this study, to screen the seven coding genes involved in the substrate selective adenylation domain biosynthesis of bacillomycin L in LPL-K103, PCR amplification was performed using seven pairs of specific primers based on the reported sequence (Luo et al. 2015) (Table S1). Among the seven pairs of primers, only five exhibited positive results for the isolate showing the presence of a gene (Fig. S1). Therefore, to obtain more information about the bacillomycin L biosynthesis gene cluster, we present the complete genome sequence of *B. velezensis* LPL-K103. Combining the genome analysis with our previously published results, the synthesis pathway of bacillomycin L in LPL-K103 is now better understood.

Materials and methods

Genomic DNA of *B. velezensis* LPL-K103 was extracted with the Gentra Puregene Yeast/Bact. Kit (Qiagen, Germany) according to the manufacturer's instructions and sequenced on the Pacific Biosciences RS II single-molecule real-time sequencing (SMRT) platform (Eid et al. 2010). The 20 kb SMRTbell insert library was sequenced using a SMRT cell. The assembly of the resulting sequence was performed using hierarchical genome assembly process (HGAP) 3.0 (Chin et al. 2013). Genome annotation was accomplished by rapid annotation using subsystem technology (RAST) (Aziz et al. 2008). The Cluster of Orthologous Groups (COG) database (Tatusov et al. 2003) was utilized for protein function annotation of expressed genes. rRNAs and tRNAs were identified by using RNAmmer 1.2 (Lagesen et al. 2007) and tRNAscan-SE 1.23 (Lowe and Eddy 1997), respectively. A phylogenetic tree based on the *gyrB* gene was constructed from *B. velezensis* K103 and close relatives in MEGA 6 (Tamura et al. 2013). In addition, genomic islands were analyzed with IslandViewer (Dhillon et al. 2015). Secondary metabolite biosynthesis gene clusters were identified and annotated using antiSMASH (Medema et al. 2011).

Results and discussion

Genome features of *B. velezensis* LPL-K103

The sequencing run of *B. velezensis* LPL-K103 yielded 80,411 reads for 1,000,961,364 bases of raw sequencing data. The complete genome of LPL-K103 contains a single circular chromosome of 3,933,292 bp with a G + C content of 46.61% (Fig. 1). A total of 4080 coding DNA sequences (CDSs) were predicted and classified into 23 functional categories based on the COGs of the proteins (Table S2). In addition, 27 rRNAs and 86 tRNAs were identified. The phylogenetic tree based on *gyrB* gene sequences showed that

LPL-K103 presented approximately 100% sequence similarity to *Bacillus velezensis* (Fig S2).

Five genomic islands were identified in the genome of *B. velezensis* LPL-K103 (Table S3), and no pathogenic islands were found. Functional genes encoding transcriptional regulators, translocase, conjugal transfer proteins, endopeptidase, transporters and transposase were identified in the LPL-K103 genomic islands. In addition, genes encoding the cold shock protein CspC and an arsenic resistance protein related to the stress response and detoxification were identified in the genomic islands.

B. velezensis acts as a plant growth-promoting rhizobacterium (PGPR) by stimulating plant growth and inhibiting the growth of phytopathogens, which is related to the production of a vast array of biologically active secondary metabolites (Arguelles-Arias et al. 2009). There were nine non-ribosomal peptide synthetase (NRPS) and polyketide synthetase (PKS) gene clusters identified in the genome of *B. velezensis* LPL-K103 (Fig. 2; Table 1), which included the bacillomycin synthetase gene cluster. All of the secondary metabolite gene clusters shared high identity (up to 99%) with *B. velezensis* FZB42. However, no novel secondary metabolite gene clusters were identified in the genome of *B. velezensis* LPL-K103.

Analysis of the bacillomycin L synthetase gene cluster

Comparative genomic analysis with FZB42 revealed that the genome of *B. velezensis* LPL-K103 contains the complete bacillomycin L synthetase gene cluster, including *bmyD*, *bmyA*, *bmyB* and *bmyC* (Fig. 3). The first gene of the bacillomycin L operon is *bmyD*, which encodes an [acyl-carrier-protein] malonyl-CoA transacylase of 45.02 kDa. The second gene is *bmyA*, which encodes a bacillomycin L synthetase subunit of 448.56 kDa that combines functional domains of fatty acid synthases, amino acid transferase and peptide synthetase (Tsuge et al. 2001). *bmyB* and *bmyC* encode peptide synthetase subunits B (608.36 kDa) and C (299.54 kDa), which are used to assemble characteristic conserved condensation, adenylation, and thiolation domains, respectively (Stein 2005).

The bacillomycin L synthetase multienzyme system is composed of modularly arranged catalytic domains that are responsible for the selection and ordered condensation of amino acid residues (Shaligram and Singhal 2010). Each amino acid-activating module requires three core domains: (1) an adenylation domain (A domain), which selects one specific amino acid and generates an aminoacyl adenylate; (2) a thiolation or peptidyl carrier protein domain (PCP domain), which transports the activated amino acid covalently bonded to its 4'-phosphopantetheine as a thioester; and (3) a condensation domain (C domain), which

Fig. 1 Circular genome map of *B. velezensis* LPL-K103. From inner to the outer: ring 1 represents the GC skew (green, positive skew; purple, negative skew); ring 2 represents the G+C content (red, positive deviation from the average; black, negative deviation from the average); ring 3 represents tRNA and rRNA genes; ring 4 represents

forward and reverse CDSs (colored according to COG categories); rings 5 and 6 represent forward and reverse methylation modification in genetic regions and intergenic regions, respectively. The genome map was drawn using Circos v0.64 (<http://circos.ca/>)

catalyzes the direct condensation of activated amino acids bound upstream and downstream in a growing chain (Finking and Marahiel 2004). In addition to the core domains, bacillomycin L synthetase comprises other significant functional domains. For lipopeptide synthesis, the cyclization reaction of the linear peptide chain is performed by a thioesterase domain (TE domain), which is the terminus of the last module of the enzyme (Schneider and Marahiel 1998). Bacillomycin L is composed of three amino residues with D-configurations, resulting from the epimerization of PCP-bound L-amino acid catalyzed by the epimerization domain (E domain) (Linne et al. 2001). In contrast to other lipopeptides such as surfactin and fengycin, lipopeptides from the iturin family are synthesized by a polyketide synthase and non-ribosomal peptide synthetase (PKS–NRPS) hybrid complex multienzyme system. *bmyA* contains genes related to PKS that are responsible for the biosynthesis of the fatty acid chain before it binds the first amino acid of the growing lipopeptide chain. Fatty acids are loaded by the acyl-CoA ligase domain (AL domain) onto the approximal acyl carrier protein domain (ACP domain), while malonyl-CoA is loaded onto another ACP domain in trans by the malonyl-CoA transferase domain (MCT domain). Then, they

are condensed via the ketosynthase domain (KS domain) (Hansen et al. 2007). Finally, the amino transferase domain (AMT domain) transfers an amine to the β -position of the growing acyl chain (Aron et al. 2005).

The high structural conservation of the A domain supports the predictability of amino acids, which means that the putative binding pocket determines substrate specificity (Challis et al. 2000). As shown in Fig. 3, according to the sequence analysis of the A domains in the bacillomycin L operon at the PKS/NRPS Analysis website (<http://nrps.igs.umaryland.edu/>), a total of seven amino acid-activating modules were detected: one in bacillomycin L synthetase subunit A (BmyA), four in BmyB, and two in BmyC. The amino acid sequence of bacillomycin L predicted by the PKS/NRPS Analysis software was in good agreement with the actual results based on amino acid analysis and Q-TOF–MS from previous studies (Zhang et al. 2013a).

Although several lipopeptides belonging to the bacillomycin family produced by *Bacillus* species have been identified and reported (Table 2), there has been no bacillomycin L with the amino acid residue sequence of “L-Asp—D-Tyr—D-Asn—L-Ser—L-Gln—D-Ser—L-Thr” in the synthetase gene cluster reported from other relevant producers. The

Fig. 2 The secondary metabolite gene clusters identified in *B. velezensis* LPL-K103

Table 1 Gene clusters associated with secondary metabolites in *B. velezensis* LPL-K103 and their identity with *B. velezensis* FZB42

Compound	Enzyme	Gene clusters (core)	Position	Size (kb)	Identity (%)
Surfactin	NRPS	<i>srfABCD</i>	334,194–360,351	26.16	99.01
Macrolactin	PKS	<i>mlnABCDEFGHI</i>	1,414,319–1,467,572	53.25	98.84
Bacillaene	PKS/NRPS	<i>baeBCDEGHIJLMNRS</i>	1,723,776–1,796,228	72.45	98.61
Bacillomycin	NRPS/PKS	<i>bmyDABC</i>	1,897,630–19,349,11	37.28	98.52
Fengycin	NRPS	<i>fenABCDE</i>	1,957,817–1,995,485	37.67	98.37
Difficidin	PKS	<i>dfnAYXBCDEFGHIJKLM</i>	2,284,151–2,353,656	69.51	98.67
Unknown peptide	NRPS	<i>nrsABCDEF</i>	2,872,960–2,890,474	17.52	99.53
Bacillibactin	NRPS	<i>dhbACEBF</i>	3,025,570–3,037,290	11.72	98.98
Bacilysin	NRPS	<i>bacABCDE</i>	3,621,945–3,626,630	4.69	99.34

comparative analysis of biosynthetic gene clusters from different species illustrated that bacillomycin synthesis in *Bacillus* is highly conserved (Fig. S3). Multiple sequence alignment analysis of the protein sequences of bacillomycin synthetases also showed high identity (Fig. S4). However, major differences between LPL-K103 and the protein sequences of BmyB from other strains were found. The amino acid sequences of BmyB from positions 1910 to 2050 showed differences between *B. subtilis* and *B. velezensis*, but the function was very conservative. Furthermore, the differences in the amino acid sequences from 3250 to 3780 indicated differences between bacillomycin L and bacillo-mycin D at the fifth and sixth amino acid residues.

Conclusion

Together with our previous results related to the structure and partial characterization of bacillomycin L, this study further enriches the knowledge of the biosynthesis mechanism of bacillomycin L in LPL-K103. Based on the complete genome sequence of *B. velezensis* LPL-K103, genes involved in bacillomycin L biosynthesis were annotated, and the mechanism of bacillomycin L biosynthesis was analyzed. These results provide possibilities for us to increase the yield and antifungal activity of bacillomycin L produced by LPL-K103 using genetic engineering

Fig. 3 Bacillomycin L biosynthesis by the modular synthetase multienzyme system of *B. velezensis* LPL-K103. The bacillomycin L synthetase subunits are encoded by the four-gene *bmy* cluster (top), which contains *bmyD*, *bmyA*, *bmyB* and *bmyC* (bottom). The individual domains of the synthetase subunits are shown with colored bars. The activated amino acid bonding of the 4'-phosphopantetheinyl

prothetic group of the PCP domain via a thioester linkage is shown with the current synthesis states. The growing peptide chain is passed from left to right, and the linear product at the last PCP domain is cyclized by the TE domain. The β -hydroxy fatty acid (FA) side chain of 12 carbon atoms was confirmed by MALDI-TOF-MS (Zhang et al. 2013a)

Table 2 Some characteristics of bacillomycin group lipopeptides

Lipopeptide	Peptide sequence	Molecular weight (C15) (Da)	Strain	GenBank ID	References
Bacillomycin D	N-Y-N-P-E-S-T	1.045	<i>B. velezensis</i> FZB42	CP000560.1	Koumoutsis et al. (2004)
	D-Y-N-P-E-S-T	1.039	–	–	Peypoux et al. (2010)
	–	–	<i>B. velezensis</i> QST713	CP025079.1	Pandin et al. (2018)
Bacillomycin L	D-Y-N-S-Q-S-T	1.034	–	–	Peypoux et al. (1984)
	N-Y-N-S-E-S-T	1.034	<i>B. subtilis</i> 916	CP009611.1	Luo et al. (2015)
Bacillomycin F	N-Y-N-Q-P-N-T	1.071	–	–	Peypoux et al. (1981)

techniques. Moreover, the genome information of *B. velezensis* LPL-K103 will be helpful for the identification of more active secondary metabolites that might show great antibacterial and antifungal activities against different kinds of pathogens, indicating that this strain is a potential biological control agent for use in the future.

Genome sequence accession number

The complete genome sequence of *B. velezensis* LPL-K103 has been deposited at GenBank/EMBL/DBJ under the accession number CP039380.

Acknowledgements This work was funded by the Special Fund for Agro-scientific Research in the Public Interest of China (201303014) and the National Key R&D Program of China (2018YFD0401200).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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