




Phenotypic features and analysis of genes supporting probiotic action unravel underlying perspectives of *Bacillus velezensis* VTX9 as a potential feed additive for swine

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Abstract

Purpose: To date, a total of 13 probiotic *Bacillus* species are considered as a Generally Recognized as Safe organism (GRAS) approved by the US Federal Food, Drug, and Cosmetic Act (FDCA), which are used for food and feed additives. However, *Bacillus velezensis* is not considered as a probiotic candidate in swine farming due to a lack of genetic basis of probiotic action-related traits. Therefore, the present study was undertaken to exploit the genetic basis underlying the probiotic traits of *B. velezensis* VTX9.

Methods: The genome sequencing of *B. velezensis* VTX9 was performed on a PacBio Sequel platform. The probiotic properties including biosafety, antioxidative capacity, and riboflavin and exopolysaccharide production were evaluated by using genotypic and phenotypic analysis. The secondary metabolite potentials were also predicted.

Results: Strain VTX9 isolated from swine feces proved some probiotic properties including resistance to 3 mM H₂O₂, 0.6 mM bile salt, low pH, and antipathogenic activity. The complete genome of *B. velezensis* VTX9 consists of a 3,985,800 bp chromosome that housed 3736 protein-coding genes and 5 plasmids with the size ranging from 7261 to 20,007 bp. Genome analysis revealed no functional genes encoding enterotoxins and transferable antibiotic resistance, which confirmed the safety of VTX9. A total of 82 genes involved in gastrointestinal stress tolerance were predicted, which has not been reported previously. The maximum production of riboflavin reached 769 ± 7.5 ng/ml in LB medium after 72 h, which was in agreement with the complete de novo riboflavin biosynthetic pathway exploited for the first time in the *B. velezensis* genome. Antagonistic activity against pathogenic bacteria was attributed to 10 secondary metabolites clusters. The presence of a large gene cluster involved in biosynthesis of exopolysaccharides underscored further the adhesion and biofilm-forming capabilities of VTX9 in swine intestines.

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Conclusion: Our results revealed for the first time that *B. velezensis* VTX9 has the potential to be a probiotic candidate. The information provided here on the genome of *B. velezensis* VTX9 opens new opportunities for using *B. velezensis* as a feed additive for swine farming in the future.

Keywords: Antibiotic resistance, *Bacillus velezensis*, Biofilm formation, Complete genome sequencing, Probiotic, Riboflavin

Introduction

Probiotics are considered a safe alternative for the increasing use of antibiotics to improve the performance of livestock, leading to unprecedented attention over the world. Known as live microorganisms, the administration of probiotics has been demonstrated to improve growth performance and nutrient digestibility, prevent pathogen colonization, maintain mucosal and systemic immunity, and especially reduce antibiotic usage (Saulnier et al. 2009, Robles Alonso and Guarner 2013, Popova 2017). To date, the bacterial genera most frequently used as probiotics for animals are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and *Bacillus* (Holzapfel et al. 2001, Oliveira et al. 2017). Particularly, due to its cost-effective benefits, stability, and strong resistance to harsh gastric environments (Elshagabee et al. 2017, Wu et al. 2019), the genus *Bacillus* is an outstanding candidate in animal farming.

Members of the genus *Bacillus* are Gram-positive endospore-forming bacilli that are widely distributed in human and animal gut commensals. *Bacillus* species have an indefinite shelf life and are strongly resistant to enormous stresses generated in the gastrointestinal tract, earning it a rising interest in the probiotic industry (Hong et al. 2005, Li et al. 2018). Many *Bacillus* spp.-based products such as BioGrow[®], BioPlus[®]2B, AlCare[™], and Sporlac[®] are available on the market, which prove the efficacy of *B. subtilis*, *B. licheniformis*, *B. clausii*, and *B. coagulans* for animal feed and aquaculture (Khatri et al. 2016, Elshagabee et al. 2017). With proposed scientific evidence, the safety, bile and acid tolerance, mucin-binding ability, and production of bacteriocins and vitamins contribute to the probiotic efficiency of the genus *Bacillus* (Khatri et al. 2016, Lee et al. 2019). *B. subtilis*, a Generally Recognized as Safe organism (GRAS), was reported to produce many bacteriocins, such as subtilin and subtilisin, to prevent the growth of livestock-associated pathogens including *Salmonella enterica*, *Clostridium perfringens*, and *Escherichia coli* (La Ragione et al. 2001, La Ragione and Woodward 2003, Khatri et al. 2016). The administration of *B. subtilis* resulted in the efficiency in treating acute infectious diarrhea, which is involved in adhesion and biofilm-forming capabilities in the mucosal host (Allen et al.

2010). Exopolysaccharide (EPS) is one of the main factors contributing to these probiotic properties, protecting bacterial cells against gastrointestinal stress conditions (Caggianiello et al. 2016). EPS produced by probiotics are able to inhibit biofilm formation by pathogenic bacteria and harmful fecal enzymes such as β -glucosidase, β -glucuronidase, and tryptophanase (Bujnakova et al. 2014, Caggianiello et al. 2016). Another important probiotic property is the ability to produce riboflavin (vitamin B2) that is supplied to diets for improving pig production. To date, only *B. subtilis*, *Ashbya gossypii*, and *Candida famata* have been used for industrial riboflavin production (Averianova et al. 2020). The use of probiotics as in situ riboflavin supply is recognized as an attractive alternative to food fortification. Recent studies showed that probiotic *B. clausii* is also a producer of riboflavin (Paracchini et al. 2017, Khatri et al. 2019).

To date, *B. velezensis* is only known as ecologically safe biopesticides, fungicides, and plant-growth promoting rhizobacteria. Despite being reported firstly in 2008 as a heterotypic synonym of *B. amyloliquefaciens*, *B. velezensis* was re-classified as a big species by the addition of several species including *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. oryzicola* (Dunlap et al. 2016). Plant growth promotion, production of a wide array of secondary metabolites against phytopathogens, and efficient colonization on plants are the outstanding characteristics of *B. velezensis* that makes itself highly cited as a biocontrol agent (Cai et al. 2017, Balderas-Ruiz et al. 2020, Zhu et al. 2020). A recent report demonstrated that the use of *B. velezensis* as a feed additive enhanced the production performance, egg quality, and especially replaced the feed antibiotics such as flavomycin in laying hen farming (Ye et al. 2020). In 2020, only *B. velezensis* DSM 15544 was listed for the first time on the Qualified Presumption of Safety (QPS) approved by The European Union Food Safety Authority (EFSA) and Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration, which confirmed the safety and efficacy of this species in swine farming (Additives et al. 2020). However, no report was done to demonstrate systematically the probiotic properties of *B. velezensis* at both phenotypic and genetic levels.

Sequencing and functional annotation of the genome is an effective approach to rapidly evaluate genetic determinants involved in probiotic potential. Although a total of 348 genome sequences have been deposited on the GenBank database, no report exploits the genetic basis underlying the probiotic characteristics of *B. velezensis* in swine farming. In this study, we carried out the complete genome sequencing of *B. velezensis* VTX9 isolated from swine feces. Probiotic traits including safety, oxidative stress, low pH and bile salt tolerance, secondary metabolite and vitamin production, and adhesive ability were evaluated through genotypic and phenotypic analysis in order to gain insights into the molecular basis of probiotic function exerted by the probiotic candidate.

Materials and methods

Isolation and characterization of the strain VTX9

The fecal samples collected at several swine farms in Hanoi, Vietnam were heat-treated (80 °C for 10 min) to kill all vegetative cells and spread on *Bacillus* agar medium (Himedia, India). The plates were inoculated at 37 °C for 24 h under aerobic conditions. The isolate was subsequently purified by streaking on the fresh *Bacillus* agar medium and then stored at – 80 °C as glycerol (40%) stock for further studies. Cell morphology and spore of strain VTX9 were visualized by scanning electron microscopy (SEM) JSM-5410 (JEOL, Tokyo, Japan). The effects of different conditions, including pH, temperature, NaCl, carbon, and nitrogen sources, on the growth of strain VTX9 were performed as described previously (Fan et al. 2017). The ability to produce extracellular enzymes including catalase, oxidase, amylase, protease, and cellulase, was performed as described formerly by Gopalakrishnan et al. 2011. The antibiotic susceptibility patterns of strain VTX9 were examined against 12 antibiotics by using the disc diffusion test (Rechenchoski et al. 2017). The test discs were amikasin (30 µg), nalidixic acid (30 µg), kanamycin (30 µg), neomicin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), meropenem (10 µg), ceftazime (30 µg), fosfomycin (200 µg), tetracycline (30 UI), and levofloxacin (5 µg).

DNA extraction, PCR amplification, and 16S rRNA gene sequence analysis

Total genomic DNA was extracted from VTX9 bacterial suspension using G-spin™ Total DNA Extraction Mini Kit (Intron Bio) according to the manufacturer's instructions. The 16S rRNA gene was amplified by using the universal primer pair 27F (5'-TAACACATGCAAGT CGAACG-3') and 1429R (5'-GGTGTGACGGGCGG TGTGTA-3'). About 100 ng of DNA template was added to a PCR mixture (50 µl) containing 1× Taq Master, 1× PCR buffer, 2.75 mM MgCl₂, 0.06 mM dNTP, 20

pm of each primer, 2 U Taq DNA polymerase (Eppendorf, Westbury, NY). The PCR conditions composed of an initial denaturation at 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 55 °C for 1.5 min, and 72 °C for 1 min; and a final extension step consisting of 72 °C for 10 min. The PCR products were run on 1% agarose gel, stained with ethidium bromide, and visualized with the Gel Doc EZ Imager (Bio-Rad Laboratories Inc.). Afterwards, the purified PCR products were sent to First BASE Laboratories Sdn. Bhd. (Malaysia) for Sanger sequencing. The obtained sequence of strain VTX9 was compared with the representative 16S rRNA gene sequences of related type strains using the Basic Local Alignment Search Tool program (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was computed by using the neighbor-joining method with 1000 bootstrap in MEGA version 7.0. Numbers at nodes indicate the percentages of 1000 bootstrap re-samplings and *Lactobacillus paracasei* ATCC 25302 (NR 117987) was used as the outgroup branch. The 16S rRNA gene sequence of strain VTX9 was deposited onto the GenBank (NCBI) under accession number MZ127796.

Genome sequencing, de novo assembly, and annotation

Whole-genome sequencing of strain VTX9 was implemented by using the Pacific Bioscience SEQUEL platform (Menlo Park, CA, USA) with single-molecule real-time (SMRT) sequencing by the Institute of Biotechnology, Vietnam Academy of Science and Technology. The raw reads were generated, and de novo assembled into one contig using the CLC Genomics Workbench version 7.5.1 (CLC Bio, Aarhus, Denmark) with the hierarchical genome-assembly process (HGAP) algorithm in SMRT analysis. The complete genome was annotated using Prokaryotic Genomes Annotation Pipeline (PGAP) at NCBI and Rapid Annotations using Subsystem Technology (RAST) (Overbeek et al. 2014, Tatusova et al. 2016). Orthologous genes were analyzed using clusters of orthologous genes (COGs) (Galperin et al. 2015). Annotation indicating the biological processes, molecular functions, and cellular components were evaluated using InterProScan 5 (Jones et al. 2014). The complete sequences of the chromosome and five plasmids of VTX9 were deposited in the NCBI GenBank database.

ANI comparison and identification of the antibiotic resistome, virulome, secondary metabolite clusters, riboflavin, and EPS biosynthetic pathways

In order to identify strain VTX9 at genomic levels, average nucleotide identity (ANI) was conducted to measure the overall similarity between *Bacillus* genome sequences using OrthoANI with default parameters (Lee et al. 2016). Heat map was generated with OrthoANI values that were calculated from Orthologous Average

Nucleotide Identity Tool. Antimicrobial resistance and virulence genes residing in the genome of strain VTX9 were screened via the functional annotation data generated from Pathosystems Resource Integration Center (PATRIC) platform (Snyder et al. 2007) and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al. 2017). The CRISPRCasFinder, a web tool, was used for the prediction of the Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) sequence in the chromosome of the strain VTX9 (Couvin et al. 2018). Regarding bioactive potential, the genome of VTX9 was analyzed for the presence of secondary metabolites BGCs using antibiotic and Secondary Metabolite analysis shell (antiSMASH) version 4.0 (Blin et al. 2017) and BAGEL3 (Hart and Moffat 2016). The genes involved in riboflavin and EPS biosynthesis were determined by using BLAST of NCBI and Rapid Annotations using Subsystems Technology (RAST) (Aziz et al. 2008).

Antibacterial activity

The antibacterial activity of the VTX9 culture supernatant was investigated using the agar diffusion method as described previously (Vu et al. 2018, Quach et al. 2021). Human pathogenic bacteria, including *Escherichia coli* ATCC 11105, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341, and *Staphylococcus aureus* ATCC 6538 were used in this study. Diameters of the inhibition zones were measured using a zone scale and expressed in millimeters. The experiment was carried out in triplicate.

Oxidative stress, pH, and bile salt tolerance

The strain VTX9 was grown as overnight culture in LB medium at 37 °C under vigorous agitation. To evaluate the response of VTX9 to stress conditions, an optical density at 600 nm (OD_{600}) of 0.1 of bacterial cells was grown in the fresh LB medium containing different concentration of H_2O_2 (0, 0.5, 1, 2, 3 mM), pH (pH 2.0, pH 3.0, pH 4.0, pH 5.0), and ox-bile (0, 0.2, 0.4, 0.6%). The cultures were incubated in the shaking machine at 37 °C with continuous shaking, and cell growth was observed spectrophotometrically at 600 nm every hour.

Quantification of riboflavin production

Bacillus velezensis VTX9 was propagated in the LB medium at 37 °C overnight. The overnight culture was transferred to a new riboflavin production medium (yeast extract 4.0 g, fructose 38.1 g, $MgSO_4$, 0.85 g, K_2HPO_4 , 2.2 g, $FeSO_4$ 0.02 g, pH 6.8–7.0 in a liter) and adjusted to an optical density at 600 nm of 0.1. At intervals, the culture was centrifuged at 4 °C, 10,000 rpm for 10 min to obtain the supernatant. Cell-free supernatant

was used to determine riboflavin production. Quantification of riboflavin level was performed by using HPLC with a Fluorescence detector (Shimadzu, Japan) (Juarez del Valle et al. 2014). Detection was conducted by a fluorescent detector. The excitation and emission wavelengths were 445 and 530 nm, respectively. Riboflavin was eluted in isocratic conditions using a mobile phase consisting of 0.05 M sodium acetate/methanol (30:70, v/v). A standard curve was constructed with different dilutions of riboflavin.

EPS production and isolation

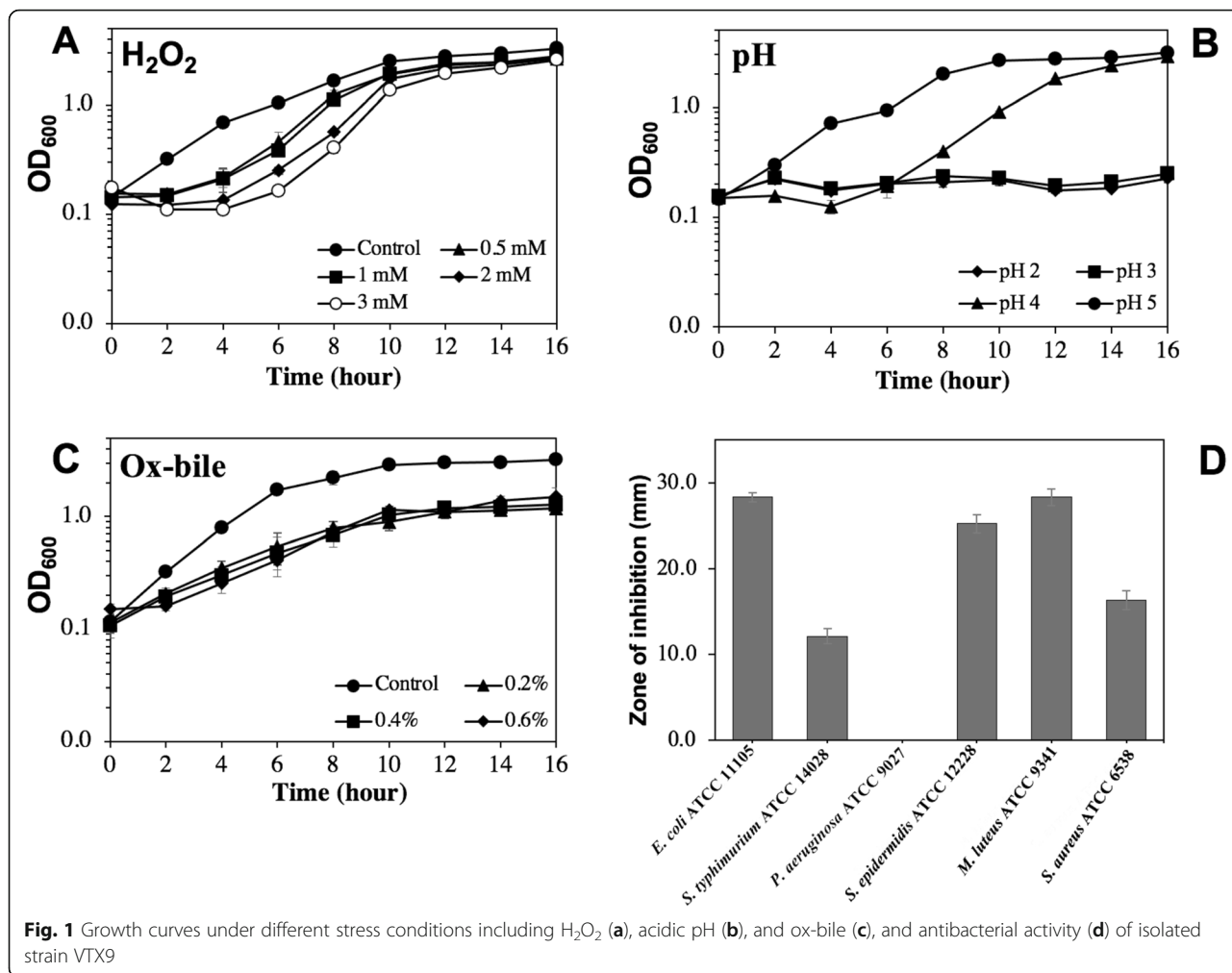
B. velezensis VTX9 was grown as an overnight culture in the LB medium at 37 °C. The overnight culture was then transferred to EPS medium (sucrose/glucose source, yeast extract 2.0 g, $(NH_4)_2SO_4$ 1.5 g, K_2HPO_4 2.5 g, pH 7.0 per liter) as described (Ruas-Madiedo and de los Reyes-Gavilán 2005) and adjusted to an optical density at 600 nm (OD_{600}) of 0.1. Effect of sucrose/glucose concentrations on EPS production was studied by the supplement of 0–150 g/L sugar source in the EPS medium. After 48 h, EPS was isolated from cell-free supernatant through the addition of 3-fold cold ethanol, which subsequently was chilled at 4 °C overnight for complete precipitation. After centrifugation at 10,000 rpm for 20 min, precipitated EPS was washed twice with chilled ethanol, then was freeze-dried, and weighed to determine raw EPS concentration, expressed as g/l.

Results

Identification of the strain VTX9

A total of 12 spore-forming isolates with distinct morphology were isolated from swine feces. The growth of these spore formers at different H_2O_2 , pH, and ox bile were performed to screen for potential probiotic isolates. Out of 12 isolates, only isolate VTX9 was able to grow on LB medium supplemented with 3 mM H_2O_2 and 0.6% ox bile (Fig. 1a, c), and preferred acidic pH such as pH 4 (Fig. 1b). Moreover, VTX9 proved to have significant activity against all the pathogenic bacteria, including *E. coli* ATCC 11105 (28.3 ± 0.5 mm), *S. typhimurium* ATCC 14028 (12.1 ± 0.9 mm), *S. epidermidis* ATCC 12228 (22.2 ± 1.1 mm), *M. luteus* ATCC 9341 (28.3 ± 1.0 mm), *S. aureus* ATCC 6538 (16.3 ± 1.1 mm) (Fig. 1d).

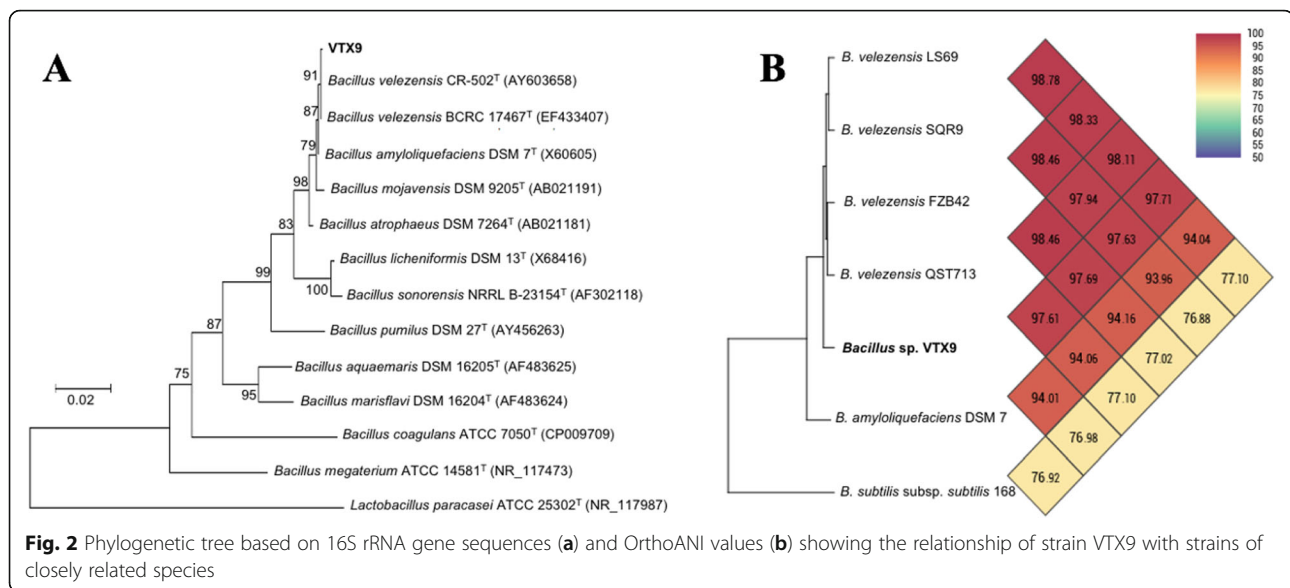
The isolate VTX9 was observed as Gram-positive, aerobic, and spore-forming bacterium. As viewed under SEM, the cells were rod-shaped with rough outer surfaces and produced sticky material when grown on EPS liquid medium (Fig. S1). When heated at 80 °C for 20 min, the isolate VTX9 produced spores that were ellipsoidal. The colonies grown on LB agar were circular and flat with a serrated edge, rough surface, and viscous. The temperature range of 25–45 °C, pH range of 2–9, and



NaCl range of 1–8% were sufficient for the growth of VTX9 (Table S1). The biochemical tests of isolate VTX9 included the ability to produce an array of extracellular enzymes, including catalase, oxidase, amylase, protease, cellulase, and utilize various sole carbon sources, such as glucose, lactose, inositol, D-raffinose, D-fructose, D-sorbitol, and methyl- α -D-glycoside. In addition, this isolate gave negative tests for mannitol, melibiose, and D-turanose (Table S1). The strain VTX9 showed similar morphological and biochemical characteristics to the type strain *Bacillus velezensis* CR-502^T. Utilization of mannitol and susceptibility to ceftazidime were the differences between VTX9 and *Bacillus velezensis* CR-502^T.

The 16S rRNA of strain VTX9 was around 99.7% identical to that of *B. velezensis* CR-502^T (AY603658) and *B. velezensis* BCRC 17467^T (EF433407). Moreover, the 16S rRNA gene sequence of VTX9 also shared high similarities with other *Bacillus* species such as *B. amyloliquefaciens* DSM 7^T (99.6%), *B.*

mojavensis DSM 9205^T (99.4%), *B. atrophaeus* DSM 7264^T (99.4). Furthermore, the neighbor-joining phylogenetic tree indicated that VTX9 and *B. velezensis* were in the same branch (Fig. 2a). To make the identification more accurate, the OrthoANI software was used to determine the OrthoANI values among the isolate VTX9 and six closely related *Bacillus* species. Analysis of OrthoANI values indicated that the strain VTX9 had 76.9–97.7% genome sequence identities with other species (Fig. 2b). VTX9 showed the highest OrthoANI value of 97.71% with *B. velezensis* LS69 (accession number CP015911) and 97.69% with *B. velezensis* FZB42 (accession number NC_009725), which was significant enough for species demarcation. Based on the phenotypic characteristics, the 16S rRNA gene sequence and ANI analysis, strain VTX9 should be classified as *B. velezensis* VTX9, which then was deposited at VAST-Culture Collection of Microorganisms (VCCM, www.vccm.vast.vn) with the accession number VCCM 14174.



Genome sequence and general features of the *B. velezensis* strain VTX9

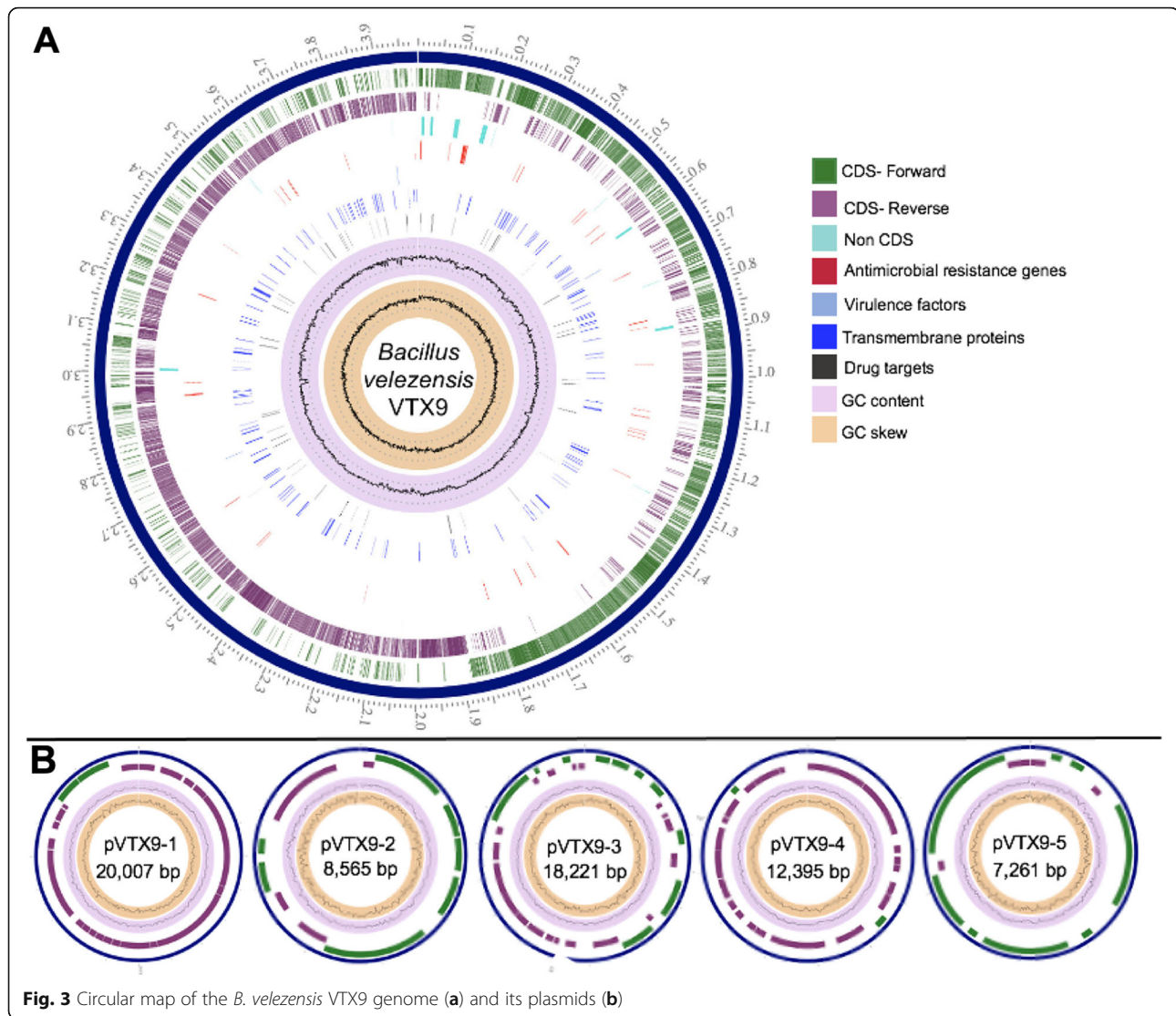
Using the PacBio platform, the complete genome of *B. velezensis* VTX9 was found to contain a circular chromosome of 3,985,800 bp with the average GC content of 46.1% (Fig. 3a) and five distinct plasmids whose length ranges from 7261 bp to 20,007 bp (Fig. 3b). Five circular plasmids include pVTX9-1 (20,007 bp with 37.6% G + C), pVTX9-2 (8565 bp with 39.9% G + C), pVTX9-3 (18,221 bp with 35.3% G + C), pVTX9-4 (12,395 bp with 38.1% G + C), pVTX9-5 (7261 bp with 42.2% G + C) (Table S2). Additionally, the annotation pipeline generated a total of 3736 protein-coding genes (CDSs), 86 tRNA genes, 27 rRNA genes, and 110 pseudogenes. The genomic feature of strain VTX9 falls in the size range reported for *B. velezensis* species. In addition, this is the first report showing the largest number of plasmids found in a single *B. velezensis* strain. The largest plasmid contains 28 CDSs whose the average GC content of 37.6% that is higher than most *B. velezensis* strains that were reported around 8 kb in size (Chen et al. 2018), except for *B. velezensis* GH1-13 comprising of a unique plasmid with the size of 71.6 kb (Kim et al. 2017).

Functional annotation of the entire genome of *B. velezensis* VTX9 showed that primary virulence factors of food-borne and food-poisoning pathogens, including hemolysin (*hblA*, *hblC*, *hblD*), non-hemolytic (*nheA*, *nheB*, *nheC*), emetic toxin (*cesA*, *cesB*, *cesC*, *cesD*), and one-component cytotoxin K (*cytK*) were absent. The transferable antibiotic resistance genes were not found in chromosome and five plasmids using the ResFinder software.

Lifestyle adaption and antioxidative capacity

Probiotic *Bacillus* species must survive under oxidatively hostile environments in the gastrointestinal tract due to prolonged luminal oxidant exposure, exogenous stimuli, or imbalanced microbiota (Wu et al. 2019). Four important genes responsible for the biosynthesis of bacillithiol (BSH), a low-molecular-weight thiol involved in the defense against oxidative stress were identified, which includes glycosyltransferase BshA (*orf_815*), deacetylase BshB1 (*orf_814*) and BshB2 (*orf_1004*), and cysteine ligase BshC (*orf_1517*) (Table 1). In response to excessive reactive oxygen species (ROS), BSH forms mixed disulfides with redox proteins, named *S*-bacillithiolations, which are reduced by bacilliredoxins such as BrxA (*orf_139*), BrxB (*orf_632*), and BrxC (*orf_939*). NADPH-dependent flavin disulfide reductase YpdA (*orf_763*) acts together with the BrxAB pathway in the de-bacillithiolation of proteins. In line with BrxABC/BSH/YpdA determined, 5 genes (*orf_132*, *orf_280*, *orf_1472*, *orf_2778*, *orf_3892*) and 4 genes (*orf_763*, *orf_2717*, *orf_3665*, *orf_3963*) annotated as thioredoxin (*trx*) and thioredoxin reductase (*trxR*) (Table 1), respectively play an important role in maintaining a reducing intracellular environment for cell viability (Arnér and Holmgren 2011).

Regarding enzymatic antioxidant, *B. velezensis* VTX9 harbors three copies of catalase (*kat*; *orf_2172*, *orf_3239*, *orf_3266*) and two copies of alkyl hydroperoxide reductase (*ahp*; *orf_3146*, *orf_3147*) strictly regulated by peroxide stress regulator PerR (*orf_2188*) (Table 1). Given that organic hydroperoxide resistance *ohr* and its regulator *ohrR* contribute mainly to detoxification of organic hydroperoxide stress, two operons including *ohrB-ohrR-ohrA1* and *ohrR-ohrA2* were present in the chromosome, which is unusual in *Bacillus* species.



When exposed to an acidic environment, *B. velezensis* VTX9 employs 8 ATP synthase *atp* genes clustered together in an operon (*atp* operon) to maintain H⁺ homeostasis via hydrolyzing ATP to pump protons (H⁺) from the cytoplasm. Various genes involved in glucose catabolism were attributed to pH resistance-related genes such as three glucose transport proteins (*orf_3271*, *orf_3283*, *orf_3299*) and one glucose-6-phosphate isomerase (*orf_2*), pyruvate kinase (*orf_203*), and phosphoglycerate kinase (*orf_3742*) (Table 1). As known that bile stress induces intracellular acidification and disrupt bacterial membranes (Begley et al. 2005), 15 gene encoding proteins annotated as oligopeptide ABC transporter, ribosomal protein, glucosamine-6-phosphate deaminase, CTP synthase, arginyl-tRNA synthetase, choloylglycine hydrolase, and inorganic pyrophosphatase were predicted to be involved in bile tolerance.

Riboflavin production

Another important property of *B. velezensis* VTX9 was its capability to synthesize riboflavin. All genes responsible for *de novo* riboflavin biosynthesis are present in the genome of VTX9. The set of genes consists of a complete *rib* operon including *ribD* (*orf_729*), *ribE* (*orf_730*), *ribBA* (*orf_731*), *ribH* (*orf_732*), and *ribT* (*orf_733*), in which the function of the *ribT* gene remains unclear to date, clustered in the same orientation in a predicted 3.9 kb operon structure (Fig. 4a). Four genes encode the key enzymes for riboflavin biosynthesis from guanosine triphosphate and ribulose-5-phosphate. The genome also contains unconnected *ribF* (*orf_1356*) homologue encoding a bifunctional riboflavin kinase/FMN adenylyltransferase catalyzing the conversion of riboflavin to FMN and FAD. Of note, VTX9 employs 3 genes encoding putative riboflavin transporters *ribZ* (*orf_438*, *orf_3487*,

Table 1 Stress-related genes predicted in the genome of *B. velezensis* VTX9

Stress	Stress-related protein	Gene	Locus tag
Oxidative stress	Superoxide dismutase	<i>sod</i>	<i>orf_522, orf_1012, orf_1021</i>
	N-acetylglucosaminyl L-malate synthase	<i>bshA</i>	<i>orf_815</i>
	N-acetylglucosaminyl-L-malate deacetylase	<i>bshB12</i>	<i>orf_814, orf_1004</i>
	Glucosaminyl-malate cysteine ligase	<i>bshC</i>	<i>orf_1517</i>
	Bacilliredoxin	<i>brxABC</i>	<i>orf_139, orf_632, orf_939</i>
	Bacillithiol dilsulfide reductase	<i>ypdA</i>	<i>orf_763</i>
	NADH- oxidoreductase	-	<i>orf_608, orf_651, orf_2543</i>
	Catalase	<i>kat</i>	<i>orf_2172, orf_3239, orf_3266</i>
	Thiol peroxidase	<i>tpx</i>	<i>orf_180, orf_2189</i>
	Glutathione peroxidase	<i>gpx</i>	<i>orf_935</i>
	Alkyl hydroperoxide reductase	<i>ahp</i>	<i>orf_3146, orf_3147</i>
	Bacillopeptidase	<i>bpr</i>	<i>orf_1500, orf_1501</i>
	Manganese catalase	<i>ydbD</i>	<i>orf_2341, orf_2441, orf_2588</i>
	Peroxide stress regulator	<i>perR</i>	<i>orf_2188</i>
	Organic hydroperoxide regulator	<i>ohrR</i>	<i>orf_1732, orf_2546</i>
	Organic hydroperoxide resistance	<i>ohr</i>	<i>orf_1731, orf_1733, orf_2547</i>
	Nitric oxide dioxygenase	<i>hmp</i>	<i>orf_25, orf_159</i>
	Thioredoxin	<i>trx</i>	<i>orf_132, orf_280, orf_1472, orf_2778, orf_3892</i>
	Thioredoxin reductase	<i>trxR</i>	<i>orf_763, orf_2717, orf_3665, orf_3963</i>
	Acid stress	Glyceraldehyde-3-phosphate dehydrogenase	<i>gapDH</i>
ATP synthase		<i>atp</i>	<i>orf_3445, orf_3446, orf_3447, orf_3448, orf_3449, orf_3450, orf_3451, orf_3452</i>
PTS system		<i>ptcC</i>	<i>orf_3271, orf_3283, orf_3299</i>
Glucose-6-phosphate isomerase		<i>pgi</i>	<i>orf_2</i>
Pyruvate kinase		<i>pyk</i>	<i>orf_203</i>
Phosphoglycerate kinase		<i>pgk</i>	<i>orf_3742</i>
RecA protein		<i>recA</i>	<i>orf_1326</i>
ATP-dependent Clp protease		<i>clp</i>	<i>orf_1673</i>
GTP pyrophosphokinase		<i>rel</i>	<i>orf_370, orf_1899, orf_3293</i>
Enolase		<i>eno</i>	<i>orf_3745</i>
Bile stress	Chaperone protein	<i>dnaK</i>	<i>orf_474</i>
	Glucosamine-6-phosphate deaminase	<i>nagB</i>	<i>orf_3643</i>
	CTP synthase	<i>pyrG</i>	<i>orf_3414</i>
	Arginyl-tRNA synthetase	<i>argS</i>	<i>orf_3397</i>
	Oligopeptide ABC transporter	<i>oppA</i>	<i>orf_1917, orf_1918, orf_1925, orf_1926, orf_2177</i>
	Ribosomal protein	<i>rps/rpl</i>	<i>orf_2895, orf_2897, orf_2900, orf_2906, orf_2911,</i>
	Choloylglycine hydrolase	<i>chb</i>	<i>orf_3265</i>
	Inorganic pyrophosphatase	<i>PPase</i>	<i>orf_3083</i>

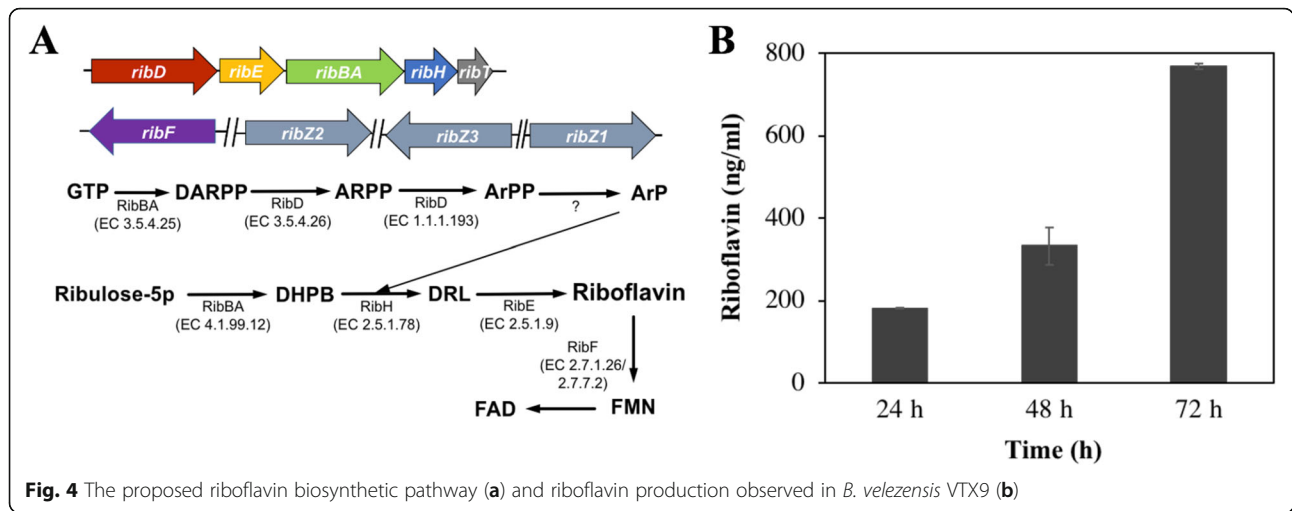
orf_3628) that facilitate riboflavin uptake from outside of the cell membrane (Fig. 4a).

Riboflavin production of *B. velezensis* VTX9 was evaluated after 24 h, 48 h, and 72 h. The riboflavin concentration reached 182 ± 1.0 ng/ml and 333 ± 44.9 ng/ml in the un-optimized medium after 24 h and 48 h, respectively (Fig. 4b). Surprisingly, the maximum riboflavin production

was achieved after 72 h (769 ± 7.5 ng/ml) which was about 4.2-fold higher than that of 24 h of incubation.

Secondary metabolite biosynthetic gene clusters in strain VTX9

B. velezensis is known as the producer of enormous bioactive secondary metabolites (Reva et al. 2019, Zhu et al.



2020). Using antiSMASH, a total of 10 such clusters were predicted in the genome of strain VTX9, including four coding non-ribosomal peptide synthetase (NRPS), two trans-acyl transferase polyketide synthetase (TransAT PKS), two transATP KS-NRPS, one keto-synthase (KS), and one saccharide (Table 2). Indeed, eight clusters were clearly involved in the biosynthesis of macrolactin, bacillaene, fengycin, difficidin, bacillibactin, surfactin, teichuronic acid, and bacilysin due to high similarity BGC (> 82%). By contrast, cluster 5 showed a 17% amino acid similarity with a molybdenum cofactor synthetase gene cluster, and cluster 10 showed a 7% amino acid similarity with a butirosin gene cluster. Moreover, the VTX9 genome possesses 10 unexplored silent secondary metabolite biosynthetic gene clusters. Among predicted clusters, 8 clusters were known to confer resistance against pathogenic bacteria (difficidin, bacillaene, macrolactin, butirosin, bacilysin, teichuronic acid, bacillibactin, and molybdenum cofactor). In support of this finding, *B. velezensis* VTX9 exhibited strong

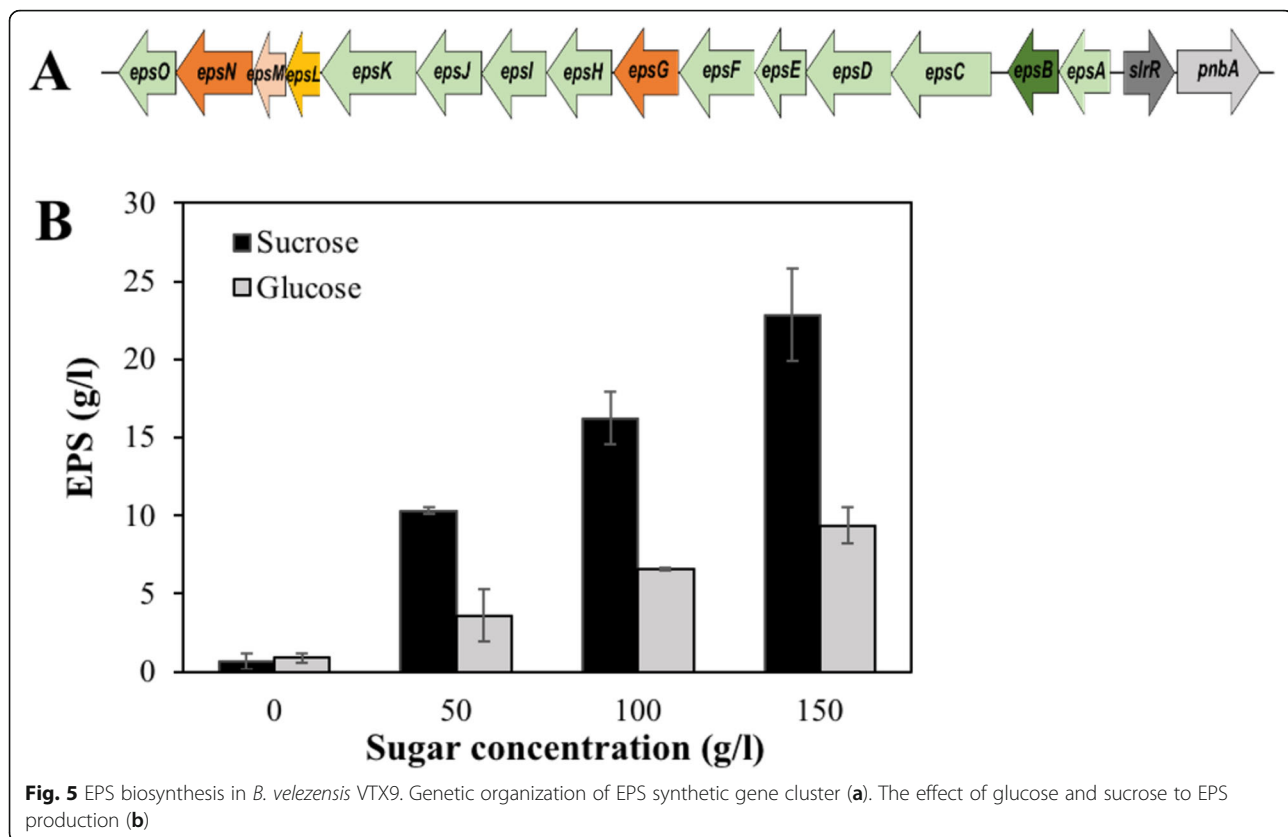
antibacterial activity against some Gram-negative pathogens, including *Escherichia coli* ATCC 11105, *S. typhimurium* ATCC 14028, and Gram-positive pathogens, including *S. epidermidis* ATCC 12228, *M. luteus* ATCC 9341, *S. aureus* ATCC 6538 (Fig. 1d).

Production of EPS

EPS is one important factor contributing to the adhesive ability of probiotic bacteria to the intestinal mucosa through biofilm formation (Caggianiello et al. 2016, Wu et al. 2020). Genome analysis revealed the complete EPS biosynthetic gene clusters containing 15 *eps* genes (*epsO*, *epsN*, *epsM*, *epsL*, *epsK*, *epsJ*, *epsI*, *epsH*, *epsG*, *epsF*, *epsE*, *epsD*, *epsC*, *epsB*, *epsA*) and one transcriptional factor *slrR* (Fig. 5a). All *eps* genes are located in the same orientation. This cluster is conserved across *B. velezensis* strains and contains four types of functional proteins including regulation, biosynthesis of repeating units, polymerization and chain-length determination, and export (Wu et al. 2020). The presence of transcriptional

Table 2 Secondary metabolite biosynthetic gene clusters determined in *B. velezensis* VTX9

Metabolites	Synthetase	MIBiG BGC (similarity)	Bioactive spectrum
Difficidin	TransATPKS	BGC0000176 (100%)	Bacteria
Fengycin	TransATP KS- NRPS	BGC0001095 (100%)	Fungi
Bacillaene	TransATP KS-NRPS	BGC0001089 (100%)	Bacteria
Macrolactin	TransATPKS	BGC0000181 (100%)	Bacteria
Butirosin	Other KS	BGC0000693 (7%)	Bacteria
Surfactin	NRPS	BGC0000433 (82%)	Virus, fungi
Bacilysin	NRPS	BGC0001184 (100%)	Bacteria, yeast
Teichuronic acid	Saccharide	BGC0000868 (100%)	Bacteria
Bacillibactin	NRPS	BGC0000309 (100%)	Microbes
Molybdenum cofactor	NRPS	BGC0000916 (17%)	Bacteria



factor *slrR* confirmed that EPS biosynthesis is directly regulated by SlrR. Both *epsA* and *epsB* are responsible for the polymerization and chain length determination process. The genes *epsF*, *epsG*, *epsH*, *epsI*, *epsJ*, and *epsK* function in transferring a variety of nucleotide sugars, while *epsC*, *epsI*, *epsO*, *epsM*, and *epsN* catalyze the modification of polysaccharide repeating units.

Using glucose as a carbon source, strain VTX9 started EPS production at 50 g/l glucose, and yielded the highest EPS of 9.4 ± 1.16 $\mu\text{g/l}$ when supplemented with 150 g/l glucose (Fig. 5b). Different from glucose, the use of sucrose is more advantageous for the production of EPS. The biosynthesis of EPS in the strain VTX9 reached 10.3 ± 1.16 g/l at sucrose concentration of 50 g/l, which was equal to the highest EPS concentration recorded when using glucose. The greatest EPS yield of 22.8 ± 2.97 g/l was observed in a medium with 150 g/l sucrose.

Discussion

Due to the ban on antibiotics as growth promoters in animal farming, the supplementation of probiotic bacteria as a feed additive has been enticing attention over the past decades. *B. velezensis* is only known as a bio-control and plant-growth promoting bacterium. Although *B. velezensis* has been recently applied as a feed

additive for improving the growth and health status of livestock, the biosafety and probiotic properties have not been fully reported at the phenotypic and genetic levels. In this study, we, for the first time, shed light on genome determinants involved in probiotic traits of *B. velezensis* VTX9 applicable in swine farming.

Despite various beneficial effects in animal production, biosafety for host and the environment is frequently cited as a concern. According to the latest classification, *B. amyloliquefaciens* subsp. *plantarum*, *B. methylotrophicus*, and *B. oryzicola* are members of *B. velezensis*. Previously, *B. amyloliquefaciens*, which was used for food processing industries, was recognized as a GRAS organism approved by FDA (de Boer Sietske and Diderichsen 1991). However, *B. velezensis* has not yet been considered. To be a safe *Bacillus* candidate, enterotoxin genes, including four *hblA*, *hblC*, *hblD*, three *nheA*, *nheB*, *nheC*, four *cesA*, *cesB*, *cesC*, *cesD*, and one *hlyII*, *bceT*, *cytK*, should be absent in the genome (Fu et al. 2019), which matched the genomic analysis of *B. velezensis* VTX9. In support of this result, this strain did not produce α - or β -hemolytic activity after incubation on blood agar plates for 24 h (unshown data). Furthermore, the possibility of passing antibiotic resistance genes from probiotic bacteria to commensals and pathogens through

horizontal gene transfer is a concerning issue (Selvin et al. 2020). In this study, the transferrable antibiotic resistance genes were not found in the chromosome and five plasmids. Additionally, *B. velezensis* VTX9 is susceptible to 10 antibiotics, including kanamycin, levofloxacin, neomycin, ciprofloxacin, gentamicin, amikacin, nalidixic acid, ceftazidime, tetracycline, and fosfomycin (Table S1). A latest study proved that the supplementation of *B. velezensis* in diets of laying hens significantly enhanced egg production rate, egg quality, and plasma biochemical index (Ye et al. 2020). Thus, *B. velezensis* could replace flavomycin that is a growth promoter in livestock production (Limede et al. 2021). EFSA also approved the use of Calsporin® containing viable spore of *B. velezensis* DSM 15544 as a feed additive for weaned piglets and fattening pigs. These findings further strengthened the biosafety of *B. velezensis* VTX9 in swine farming.

Genomically, *B. velezensis* VTX9 encodes 43 genes related to resistance to oxidative stress, among which genes involved in the functioning of BrxABC/BSH/YpdA and Trx/TrxR systems were fully described for the first time in *B. velezensis*. *trx* and *trxR* exist as one copy in the chromosome of *B. subtilis*, contributing to high sensitivity to 1 mM H₂O₂ (Zheng et al. 2019, Arias Padró et al. 2021). By contrast, *B. velezensis* VTX9 conferred resistance to 3 mM H₂O₂ by harboring 9 genes involved in Trx/TrxR system. *ohrB-ohrR-ohrA1* and *ohrR-ohrA2* operons responsible for detoxification of organic hydroperoxide and hydrogen peroxide stress were observed in the VTX9 genome, while only one *ohrR-ohrA* operon was identified in *B. subtilis* (Chi et al. 2011). Since exposure to bile salts and acidic pH cause ROS and oxidative DNA damage, leading to cell death (Begley et al. 2005), the distribution of nonenzymatic and enzymatic antioxidants suggested the potential capability of strain VTX9 to survive in harsh conditions encountered in the gastrointestinal tract of pigs.

The commensal colonic bacteria are known to be a significant source of water-soluble vitamins such as riboflavin. Due to the inability to synthesize vitamins, humans and animals have to absorb riboflavin from exogenous sources such as the diet and gut-associated microorganisms (Rossi et al. 2011). In pig production, deficiency of this vitamin resulted in a reduced feed intake, impaired reproduction, retarded growth, and nervous disorders (Pettigrew et al. 1996, Khan et al. 2020). Feeding diets supplemented with riboflavin led to decreased stress effects, increased growth performance, and improvement of the lean meat percentage of growing-finishing pigs (Shi et al. 2018). In the *Bacillus* genera encompassing 266 named species, only *B. subtilis* and *B. tequilensis* are so far reported to produce riboflavin (Averianova et al. 2020). Riboflavin used as a feed additive occupies approximately 70% on global market,

which is mainly yielded by genetically modified microorganisms such as *A. gossypii*, *C. famata* var. *flareri*, and *B. subtilis*. In this present study, *B. velezensis* VTX9 harbors 5 genes organized in the *rib* operon, leading to riboflavin production observed in the supernatant. Interestingly, 3 riboflavin transporters *ribZ* are used to transport riboflavin from outside of the cell membrane to the cytoplasm. Under un-optimized conditions, the strain VTX9 produced higher riboflavin levels (769 ± 7.5 ng/ml) compared to *L. plantarum* CRL 725 (309 ± 9 ng/ml), *Lactococcus lactis* subsp. *lactis*. C173 (223.3 ± 0.02 ng/ml) (Sabo et al. 2020). However, it was 2.5-fold lower than *B. subtilis* subsp. *subtilis* ATCC 6051 (Bacher and Mailänder 1978). This is the first report demonstrating the ability to produce riboflavin of the *B. velezensis* at the genotypic and phenotypic levels. Given that *B. velezensis* VTX9 was isolated from swine feces, this strain could be an alternative means to increase available riboflavin in the digestive tract of pig.

Genome analysis identified the presence of *eps* operon in *B. velezensis* VTX9. It is worth noting that this operon is highly conserved *B. velezensis* and *B. licheniformis* strains (Chen et al. 2017, Wu et al. 2020). EPS is one of the important metabolites providing health benefits to the host. A recent study proved that EPS produced by probiotics had strong immunomodulatory, antipathogen, and antioxidant effects in the host's gut (Castro-Bravo et al. 2018, Khalil et al. 2018). Moreover, EPS is required to protect bacterial cells from environmental hazards and improve adhesion and biofilm formation (Khalil et al. 2018). The use of glucose yielded low EPS concentration, while the supplement of sucrose significantly promoted EPS production in strain VTX9. This result suggested that EPS production of *B. velezensis* VTX9 depends on sugar source supplemented in the liquid medium. The highest EPS production of 22.8 ± 2.97 g/l was recorded after 24 h, which is around 5-fold and 53-fold higher than EPS produced by *B. licheniformis* MS3 and *L. plantarum* WLPL04, respectively (Asgher et al. 2020). Under observation by SEM, the cells were surrounded by sticky material that is supposed to be EPS (Fig. S1). These findings proved that the ability to produce high EPS concentration is one of the outstanding probiotic properties of *B. velezensis* VTX9.

Conclusion

This study provided valuable insights into essential properties of probiotic *B. velezensis* VTX9 at the genomic level, which has not been previously reported. The identified genes contributing to desirable probiotic traits such as oxidative stress, acid pH, bile tolerance, and anti-pathogenic activity were revealed through genome analysis. Moreover, this strain possesses the entire metabolic pathways of riboflavin and EPS biosynthesis that

strengthen its potential application in swine nutrition. These findings presented here provide solid evidence to demonstrate that *B. velezensis* VTX9 is a suitable probiotic candidate that can be approved by EFSA in the future and could be a new candidate for the industrial application of riboflavin production.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13213-021-01646-4>.

Additional file 1: Table S1. Phenotypic characteristics and antibiotic sensitivity of *Bacillus* sp. VTX9 and the type strain *B. velezensis* CR-502^T. Table S2. Genomic features predicted in the chromosome and plasmids of *B. velezensis* VTX9. Fig. S1. Scanning electron microscopic images showing the cell morphology (a) and spore (b) of *Bacillus* sp. VTX9. *Bacillus* sp. VTX9 was grown as an overnight culture in the EPS medium at 37 °C. Scale bar is shown in each figure.

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Authors' contributions

NTQ and THNV conceived of this study. NTQ, NAN, THNV, VTN, TLL, and TLB designed and performed the experiments. TTML, HH, CCN, and TNN supervised and implemented the statistical analysis. NTQ, THNV, and QTP wrote the manuscript. HHC, SCK, and QTP improved the writing of the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

The participant has consented to the submission of this article to the journal. We confirm that the manuscript, or part of it, has neither been published nor is currently under consideration for publication. This work and the manuscript were approved by all co-authors.

Competing interests

The authors declare that they have no competing interests.

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