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Original Paper

Bioinformatic insights into the xenobiotic degradation potential gene clusters of fish-associated novel *Bacillus velezensis* SNR14-4

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Abstract

Bacillus velezensis is a member of the genus *Bacillus*, which harbours useful, novel, and efficient secondary metabolites that can be utilized in the disruption of xenobiotics. Although a few strains of *B. velezensis* and related species are reported every year as having xenobiotic metabolism potential, several novel gene clusters are still unexplored, which could be more potent than those already discovered. The current *B. velezensis* strain was isolated from gills of healthy *Oreochromis niloticus* and the novelty of the strain was assessed through whole genome sequence analysis. Prokka, DFAST, BAKTA, and RASTtk were computational tools utilized for genome elucidation following the genome assembly. Protein and protein pathway prediction was achieved through the PATRIC database. Additionally, the resistance genes against microorganisms were examined through CARD (via Proksee), bacteriocin, and RiPPs using BAGEL4, and forecast of virulence factors using VFDB in PATRIC. The analysis led to identification of indicator genes for xenobiotic breakdown. The results were compared to pre-existing strains of *B. velezensis* and it was compelling to conclude the high biotechnological potential and the candidacy of the strain in xenobiotic degradation.

Key words: *Bacillus velezensis* SNR14-4, bioinformatics, gene clusters, whole genome sequencing, xenobiotics.
Abbreviations: CDS, coding sequences; GC, guanine-cytosine; OGBa, operational group *Bacillus amyloliquefaciens*; PBS, phosphate buffered saline.

Introduction

Aquaculture has garnered significant attention within the food sectors globally, being capable of supplying over half of the world's food resources exclusively. The primary cause of losses in aquaculture can be attributed to pathogens, resulting in reduced economic gains and food output. Various chemicals are utilized in aquaculture to either prevent or address disease outbreaks. These substances, administered through prolonged immersion or dietary incorporation, exhibit high efficacy against most protozoa, albeit being associated with genotoxic and cytotoxic potential (Jerbi et al. 2011). A prominent global concern linked to aquaculture pertains to the management and treatment of xenobiotics, given their adverse impact on aquatic ecosystems. These chemicals can accumulate in food chains through the consumption of aquaculture products, subsequently accumulating in the human body and potentially leading to the development of cancers. Furthermore, factors that impact the aquatic sources also impact the soil. On a broader scale, the toxins and harmful xenobiotic compounds can find their way into the food and water sources of humans and animals. The primary strategy for xenobiotic management involves their degradation by diverse bacteria (Iqram ul Haq et al. 2023).

One of the most promising genera in this regard is *Bacillus*, due to its ubiquitous nature and ease of cultivation. *Bacillus velezensis* is closely related to non-pathogen members of the genus like *Bacillus substilis, Bacillus licheniformis* and *Bacillus amyloliquefaciens*, which house numerous secondary metabolite synthesizing gene clusters and fall under the Operational Group *B. amyloliquefaciens* (OGBa) (Fan et al. 2017; Ngalimat et al. 2021). *B. velezensis* shares 99% 16SrRNA sequence similarity with *B. amyloliquefaciens*. Different strains of *B. velezensis* are discovered annually from various sources, with their classification within OGBa confirmed through whole genome sequencing. The potential of *B. velezensis* in

plant growth promotion, screening of new antimicrobial compounds, and other biotechnologically relevant areas has been explored extensively (Adeniji et al. 2019; Alenezi et al. 2021). Yet, the information that lies within the genome of the *Bacillus* members is still to be uncovered. Bioinformatic advancements have drastically improved the exploration of the potential of many species. Several genome mining and protein pathway prediction tools improved the evaluation of of several strains that can be effectively employed in agriculture, medicine, therapeutics, and bioremediation (Xu et al. 2019; Mohan et al. 2020; Ding et al. 2024). With its unique adaptability and high growth rate, *B. velezensis* is a candidate for many xenobiotic degradation processes (Vörös et al. 2019; Song, Hwang 2023).

The research community has begun to specifically target cryptic gene clusters present within different strains that have been sufficiently evaluated (Ochi, 2017; El-Hawary et al. 2023). Cryptic genes require specific conditions to be triggered and most of them remain silent during *in vitro* tests (Zarins-Tutt et al. 2016). Their existence can be uncovered with the use of bioinformatics tools such as open reading frame readers and gene sequence identifiers. There are several limitations concerning cryptic genes and their activation, but recent studies have shown that some members of the *Bacillus* genus can be applied in biotechnological sectors. Only a few studies have focused on xenobiotic degradation using *B. velezensis* (Cheffi et al. 2019; Sultana et al. 2021; Ding et al. 2024) and related species (López-Moreno et al. 2021; Gangola et al. 2023; Lesanavičius et al. 2024).

The ongoing investigation had a dual purpose, initially centred on the extraction of a beneficial strain of *B. velezensis* from healthy *Oreochromis niloticus*, which is known for its notable xenobiotic degradation capability and other practical values. Subsequently, the focus of the study shifted towards the utilization of a 'gene-before-lab' approach. Through the utilization of complete genome analysis, the study aimed to pinpoint specific genes that could potentially assist in the degradation of xenobiotics such as dichlorodiphenyltrichloroethane, atrazine, and various biphenyl contaminants found in soil and water. Gene prediction tools and a profound understanding of the genome allow for more accurate *in vitro* evaluation of a strain, thus enhancing the precision of the assessment process. With the 'gene-before-lab' approach, the search for novel compounds and pathways for xenobiotic degradation and bioremediation can be understood better, rather than exhausting laboratory resources and time on tests with low probability of results.

Materials and methods

Sample collection

Three healthy *Oreochromis niloticus* (Nile tilapia) individuals were obtained from the hatchery of Kerala University of Fisheries and Ocean Studies, Kochi, Kerala, India. The fish were confirmed to be healthy and free of any viral, bacterial, or fungal diseases. No signs of haemorrhage, sepsis, or lethargy were present. The fish had mean weight 250 ± 2 g. They were collected and transported in a polythene bag to the laboratory.

Screening and isolation

The collected fish were treated with a few drops of clove oil to cause death by anaesthetic overdose (Von Krogh et al. 2021). Within a few minutes, the fish were killed and cleaned using 70% ethanol to remove any bacteria present on the surface. Fish gills were dissected carefully and placed on a sterile petri plate. About 5 g sample of gills was obtained and washed with an equal proportion of phosphate buffered saline (PBS). The washing process was repeated three times to guarantee the elimination of all dirt and potential contaminants. The gills were then homogenized in 3 mL PBS using a sterile mortar and pestle. One mL of the homogenized sample was mixed with 9 mL deionized water in a sterile 10 mL screw cap tube to make a 10^{-1} dilution. The sample solution was then serially diluted to 10^{-4} and 100 µL was transferred to nutrient agar plates and incubated for 24 h at 37 °C.

Cultural and morphological characterization

The most prominent bacterial strain was sub-cultured several times to isolate pure cultures, which were identified initially through microscopic observation and Gram staining. The procedure of identification followed Bergey's Manual of Bacterial Identification and other literature (Ramesh et al. 2015; Santos et al. 2021). The bacteria was identified to be a member of genus *Bacillus*.

16s rRNA sequencing

16S rRNA sequencing was performed to accurately identify the species and strain that was isolated (Ray et al. 2009; Reda et al. 2018). The PCR amplification was conducted by using a PCR master mix (2X) of Emerald, with purified and spooled-out isolate DNA. PCR was initiated by denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 35 cycles, annealing at 50 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C by holding the reaction mixture for 7 min. The PCR product was resolved by running in 1% agarose gel electrophoresis. The product was later sequenced using the universal bacterial primers: forward primer 27F (AGAGTTTGATCCTG GCTCAG) and reverse primer 1492R (GGTTACCTTGTTACGACTT) through PCR. The 16s rRNA sequence obtained was comparatively evaluated based on its homology with 16S rRNA sequences of other *B. velezensis* strains available in the National Centre for Biotechnology Information (NCBI), using Basic Local Alignment Search Tool (BLAST) similarity analysis to specifically identify the strain isolated. A phylogenetic tree was created with MEGA11 software

(https://www.megasoftware.net/) using the neighbor-joining tree reconstruction method for the core genomes of other strains of *Bacillus* species.

Whole genome sequencing combined with de novo *assembly*

Whole genome sequencing of the strain isolated was performed with the aid of Medgenome Labs Ltd., Bangalore, India. Raw data was assessed for quality using the fastq-mcf tool (version 1.04.8030) and generated a 2.2 Gb file. The sample was sequenced using NovaSeq after the removal of all human contaminants using the BWA-mem tool (version 0.7.12). *De novo* assembly was performed using the SPAdes assembler (version. 3.11.1). Primarily, the open reading frame prediction followed by annotation of the WGS data was carried out using Prodigal software. Reference guide assembly was carried out and consensus genome FASTA sequences were developed from the aligned bam files.

Functional gene annotation

The consensus genome FASTA file additionally was used for gene annotation and gene function prediction using several different web-based and online gene annotation pipelines such as Prokka in Proksee (https://proksee.ca/), BAKTA (https://bakta.computational.bio/) (Schwengers et al. 2021), and DFAST (https://dfast.ddbj.nig.ac.jp/dfc/) (Tanizawa et al. 2018). The results were further cross-checked with the comprehensive database of Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org/) (Olson et al. 2023) that combines Pathosystems Resource Integration Center (PATRIC) (Wattam et al. 2014), the Influenza Research Database (IRD) and the Virus Pathogen Database and Analysis Resource (ViPR) bioinformatic resource centers. A comparative analysis was conducted to assess the effectiveness of each tool. A circular view of the entire genome was created using web-based CGview in Proksee (Grant et al. 2023).

Results

Phylogenetic analysis

Following the morphological classification, the construction of a phylogenetic tree through 16S rRNA sequencing using MEGA11 software revealed similarity of the current strain to *B. amyloliquefaciens* and *B. velezensis* (Fig. 1). Typically, such resemblance is addressed through comprehensive genome sequencing (Liu et al. 2017; Kang et al. 2022), since these species of the *Bacillus* genus are genetically related by almost 99%.

Complete genome sequencing and assembly of the isolate

De novo assembly was completed using SPAdes assembler (v3.11.1) and the assembly was executed with k-mer size 55 using the de-Bruijn graph method, which showed that the number of scaffolds (≥ 0 bp) was 343, N50 of 166965 bp,

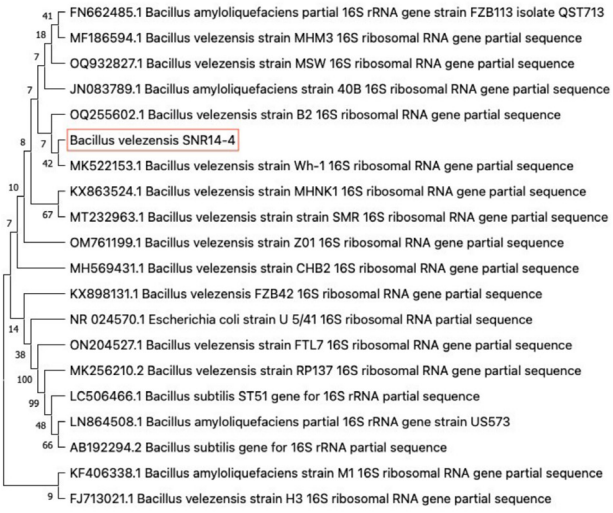

Fig. 1. Phylogenetic tree of the isolated strain SNR14-4. Phylogenetic tree built by the neighbour-joining method using MEGA 11 software, showing the relatedness of *B. velezensis* SNR14-4 to other *Bacillus* species.

N75 of 117388 bp, L50 of 8bp, L75 of 16 bp. The assembly produced a 4.1 Mb file with a single circular chromosome, one contig with a genome length of 41,83,910 bp, with an average guanine-cytosine (GC) content of 46.52%, which is consistent with the GC ratio of other *B. velezensis* strains. The sample was aligned over the reference genome using NCBI BLAST and the closest match was with *B. velezensis* strain SGAir0473 with an alignment percentage of 93.64%. The strain was proposed as novel as the ratio of unrecognized nucleotide sequences was high. The sequence was then named *B. velezensis* strain SNR14-4 and deposited in the SRA-NCBI database with Bioproject accession ID: PRJNA994302 (https://www.ncbi.nlm.nih.gov/sra/PRJNA994302).

Comprehensive whole genome analysis of B. velezensis *SNR14-4*

The RASTtk bioinformatic tool in BV-BRC produced more detailed analytical data of the isolate's genome; the contig data processed showed a total of 4163 protein coding sequences (CDS), which included coding sequences that code for 882 hypothetical proteins, 205 repeat regions, 84 tRNA, and 27 rRNA. The data was cross-reviewed with other gene annotation pipelines such as DFAST and BAKTA that were available online (Table 1). Detailed protein data was generated using PATRIC and summarized in Table 2. The annotated genome data was represented as a circular diagram using both Proksee and PATRIC visualization tools (Fig. 2 and Fig. 3). The VFDB analysis through PATRIC showed no potent virulence factors present in the isolated strain. There were an overall 50 drug targets and 197 transporters.

Table 1. Cross-reference of WGS data of *B. velezensis* SNR14-4 through DFAST, BAKTA, and RAStk. The presented data is the comparative features of the whole genome of sequence of SNR14-4 to authenticate the quality

Features	DFAST	BAKTA	RASTtk (BV-BRC)
Contig length read (bp)	4183857	4183910	4183910
GC content (%)	46.5	46	46.52
No. of PLFAM CDS	3646	3670	3657
No. of rRNA	27	27	27
No. of tRNA	82	85	84
No. of CRISPRs	0	0	0
Coding ratio (%)	80.9	–	87.0
Read quality	Good	Good	Good

SNR14-4 shows xenobiotic degradation pathways

Table 3 presents several xenobiotic degradation pathways, along with their corresponding Enzyme Commission numbers and resulting products. A total of 68 genes that are responsible for facilitating xenobiotic degradation have been identified,. Some of these genetic sequences remain unidentified, as they did not yield any significant matches when aligned across various databases. Though many gene clusters have been recognized for their involvement in xenobiotic degradation, the extent of their activity in *B. velezensis* and closely related species has not been thoroughly explored. The unknown coding sequences identified by RASTtk could also harbour gene clusters of xenobiotic degradation potential.

Table 2. Protein annotation of SNR14-4 using PATRIC. The data shows the potent virulence factors if present in the isolated strain

Protein features PATRIC	Number
Protein with functional assignments	3282
Proteins with EC number assignments	1000
Proteins with GO assignments	837
Proteins with subsystem assignments	744
Proteins with PATRIC genus-specific family (PLfam) assignments	1213
Proteins with FIGfam assignments	3733

Discussion

Over the past years, several *Bacillus* strains have been collected from marine sources such as plants, fish, soil sediments, etc. Metagenomics has also improved the screening process for desired strains that can be used as a probiotic supplement. Kerala waters are reservoirs for numerous pollutants and xenobiotics, due to the lack of routine assessment and water quality evaluation (Jyothi et al. 2021). The experiment conducted was of a two-fold nature. The initial step of isolating potential strains from healthy *O. niloticus* was carried out, as this fish is popular and commercially relevant across different countries in the world. Owing to the widespread prevalence and non-pathogenic characteristics, the likelihood of encountering a *Bacillus* member was notably high. If such a member was indeed present, one could reasonably infer the possibility of a symbiotic relationship between the fish and the bacteria.

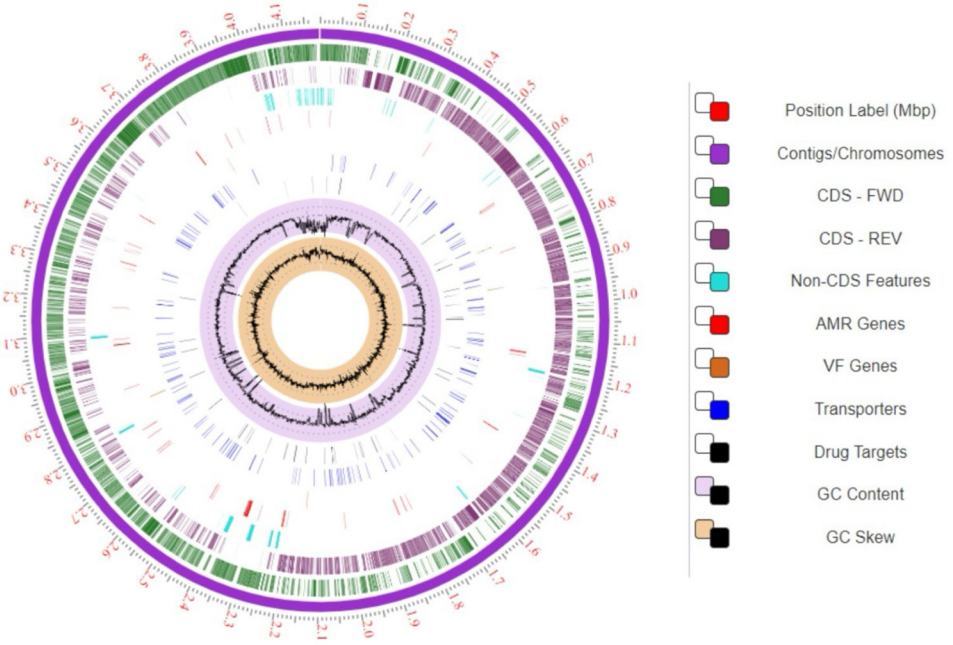

Fig.2. Circular genome assembly of *B. velezensis* SNR14-4 visualized using PATRIC-BV-BRC. Arrangement of rings from outer to inner side – contigs, coding sequences (CDS) on forward strand, coding sequences on reverse strand, non-coding sequence features, antimicrobial resistant (AMR) genes, CDS with homology to known virulence factors, GC content, GC skew.

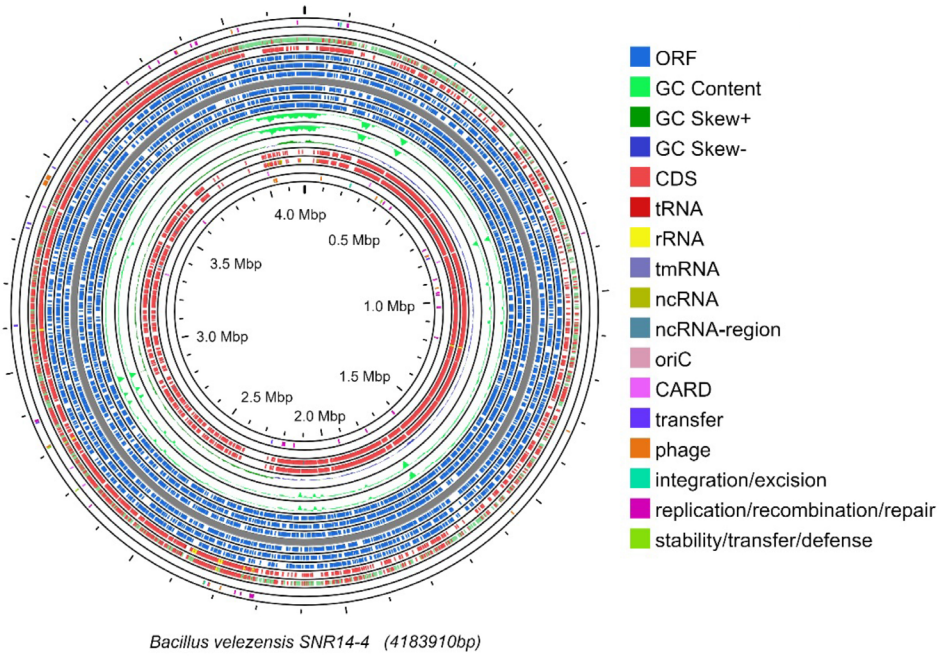

Fig. 3. Assembled genome evaluated using Proksee-CGview.js. The genome representation using Proskee softaware with default features. The central circle represents the scale of the genome in megabases, followed by circles of CDS, ORF and tRNA and other genetic features of the strain.

The isolation and culturing of *B. velezensis* isolate from the gill specimens exhibited rapid and robust proliferation in the laboratory setting, thus designating this particular isolate for subsequent analysis. Using 16SrRNA sequencing and construction of a phylogenetic tree using MEGA11 software, the relatedness of the species in question with other members of the *Bacillus* genus was determined (Liu et al. 2017; Kang et al. 2022). Instead of assessing the capacity for xenobiotic degradation through time-consuming and laborious *in vitro* techniques, the focus shifted towards the

Table 3. Xenobiotics degradation pathways present in the SNR14-4 genome. Data is the representation of pathways pointing to xenobiotic degradation in the strain.

Pathway name	EC number	Description
Cytochrome P450: metabolism of xenobiotics	1.1.1.1	Alcohol dehydrogenase
Cytochrome P450: metabolism of xenobiotics	3.3.2.9	Microsomal epoxide hydrolase
Cytochrome P450: metabolism of xenobiotics	1.14.14.1	Unspecific monooxygenase
Caprolactam degradation	3.1.1.17	Gluconolactonase
Caprolactam degradation	4.2.1.17	Enoyl-CoA hydratase
Atrazine degradation	3.5.1.5	Allophanate hydrolase 2 subunit 2
Tetrachloroethene degradation	1.2.1.3	Aldehyde dehydrogenase
Styrene degradation	3.5.1.4	Aliphatic amidase AmiE
Biphenyl degradation	1.14.13.1	Salicylate hydroxylase
1,4-Dichlorobenzene degradation	3.1.3.1	Alkaline phosphatise
Geraniol degradation	4.1.3.4	Hydroxymethylglutaryl-CoA lyase
Toluene and xylene degradation	1.13.11.2	Catechol-2,3-dioxygenase
Benzoate degradation via hydroxylation	4.1.1.44	Carboxymuconolactone decarboxylase
Ethylbenzene degradation	2.3.1.16	3-Ketoacyl-CoA thiolase
2,4-Dichlorobenzoate degradation	1.1.1.35	3-Hydroxyacyl-CoA dehydrogenase
Biphenyl degradation	4.1.1.-	Hydro-lyases
Bisphenol A degradation	4.2.1.-	Carboxy-lyases
Other enzymes	3.3.2.9	Microsomal epoxide hydrolase
Other enzymes	2.4.2.10	Ortate phosphoribosyltransferase
Other enzymes	3.1.1.1	Carboxylesyerase

sequencing and genetic examination, mining, and profiling of the isolate.

Using the methods employed, genomic data is relegated to a supplementary role rather than a primary one. The objective of the present methodology was to restructure the evaluation framework to enhance comprehension of the relevant species. Despite the numerous *B. velezensis* strains that have been isolated, a comprehensive scrutiny of the entire genetic repertoire is seldom documented. The current study addresses the proposition that a genome-guided assessment of an isolated strain can yield significantly more precise deductions. The isolated SNR14-4 strain hence was used as a model for stating using the potential of the 'gene-before-lab' technique in evaluating the capabilities of any isolated strain. Regarding use of SNR 14-4 as a model, the similarity it shares with other strains of *B. velezensis* and other members of OGBa can provide insight into the potential of SNR14-4 itself. In research conducted previously using 24 *B. velezensis* genomes, there was a universal similarity in genes encoding cellulose and hemicellulose degradation enzymes (Chen et al. 2018). Previously, reserahers successfully isolated *B. velezensis* WRN014 from the banana fields in Hainan, which had the potentiality to be used as plant growth-promoting rhizobacteria and biopesticide (Wang et al. 2019). Our strain from aquatic sources had a larger genome with 4183910 bp as compared with *B. velezensis* WRN014 isolated for the plants with a genome 4 063 541 bp.

Studies on another strain, CMRP 4490, found antifungal activity and plant growth promotion activity, by comparing genetic similarity with two other strains of *B. velezensis*: S141 (Teixeira et al. 2021) and FZB42 (Chen et al. 2009). All these strains showed above 97% average nucleotide identity. This high level of genetic similarity can be used to confirm the obvious capability of SNR14-4 in xenobiotic degradation (Arora 2020), plant growth promotion, biocontrol, and antifungal activity. For instance, the SNR 14-4 strain has the gene cluster for bisphenol A degradation (Table 3). Due to the genetic similarity between *B. velezensis* and *B. subtilis*, the candidacy of SNR14-4 in degrading bisphenol A can suggested by earlier work carried out on *B. subtilis* P74 (Park et al. 2023). The ability of SNR 14-4 in degrading atrazine can be evaluated by the results of the study done on *B. velezensis* MHNK1 (Jakinala et al. 2019). Similarly, for other applications, the probiotic effect of the strain can be suggested by work on *B. velezensis* strains like LF01 (Zhang et al. 2019) and JW (Yi et al. 2018).

Even with the immense genetic similarity, not all genes and gene clusters evaluated are expressive, and they may be inactive (Onaka 2017; Hur et al. 2023). In total, 882 putative proteins identified through protein prediction tools indicated the presence of new gene clusters, some of which remain dormant or unexpressed. Various strategies have been employed to activate these silent genes, as discussed in a recent review (Zarins-Tutt et al. 2016). Techniques such as co-culturing, ribosomal engineering, manipulation of protein pathways using transcriptional repressors and regulators, as well as deletion of known genetic elements are currently under exploration.

Considering its aquatic origin, the potential practical significance of this strain in aquaculture must be considered. A comprehensive analysis of the entire genome of the current isolate reveals numerous gene clusters that contribute to the survival of the strain under conditions of elevated temperature, acidity, and bile concentrations (Nevin et al., unpublished results). While probiotic strains are continually being identified, leveraging whole genome sequencing as an initial step can uncover latent properties associated with probiotics. Employing genome-guided assessments of strains can serve as a foundational step in establishing a consortium, leading to the development of strategic laboratory and in vivo protocols over time. Furthermore, gene clusters responsible for promoting plant growth through siderophore production, indole acetic acid and other phytohormone production, phosphate solubilization, nitrogen fixation, and ammonia production were identified via Basic Local Alignment Search Tool (BLASTp) analysis of the whole genome sequence, which showed that the strain SNR14-4 was a promising candidate for probiotics in fish (Nevin et al., unpublished results), bioremediation of soil and water, and also served as a potential source of novel compounds for plant growth promotion and the need for further investigation in the future.

Conclusions

The current study showed that the 'gene-before-lab' approach has immense benefits in terms of saving time and resources. The approach also showed how strain-specific profiling can be carried out using bioinformatics tools. The overall assessment and examination were conducted on the highly significant constitution of the novel *B. velezensis* strain SNR14-4, employing advanced genome-assisted bioinformatics tools. With comparative genomic analysis from previously reported work on similar strains, the conclusion of defining the candidacy of SNR14-4 in xenobiotic degradation is undeniable. The data derived from this analysis exhibits great promise for further exploration, indicating the necessity for the initiation of numerous experiments aimed at the development and optimization of this strain for the specific purpose of bioremediation.

Acknowledgements

The sequence was deposited to SRA-NCBI database with Bioproject accession ID: PRJNA994302 (https://www.ncbi.nlm.nih.gov/sra/PRJNA994302). Other data sets produced in the course of the current study are available from the corresponding author based on reasonable request. The authors are grateful to the Center for Bioactive Compounds from Marine Organisms,

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