

Original Research

Antibiotic Resistance Patterns in Cold-Adapted *Bacillus subtilis* From the Southern Ocean and Comparative Genomic Insights

Shikha Sharma¹, Abhishek Chauhan^{2,*}, Anuj Ranjan², Rajpal Srivastav³,
Ritu Chauhan⁴, Vivek Narayan Singh⁵, Tanu Jindal²

¹Amity Institute of Environmental Sciences, Amity University, 201313 Noida, Uttar Pradesh, India

²Amity Institute of Environmental Toxicology, Safety and Management, Amity University, 201313 Noida, Uttar Pradesh, India

³Amity Institute of Biotechnology, Amity University, 201313 Noida, Uttar Pradesh, India

⁴Department of Biotechnology, Graphic Era (Deemed to be University), 248002 Dehradun, Uttarakhand, India

⁵Department of Environment Protection, Shriram Institute for Industrial Research, 110007 Delhi, New Delhi, India

*Correspondence: akchauhan@amity.edu (Abhishek Chauhan)

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Abstract

Background: Antibiotic resistance is a contemporary public health issue that poses significant environmental and public health concerns. The presence of antimicrobial-resistant (AMR) microbes has been reported across media irrespective of geography and landscape. This study aimed to analyze the antibiotic susceptibility of *Bacillus subtilis* obtained from the Indian Sector of the Southern Ocean (39°19' S, 57°30' E to 66°38' S, 76°22' E). **Methods:** *Bacillus subtilis* was revived and cultured on Mannitol Yolk Polymyxin Agar. Antibiotic susceptibility was assessed via the agar well diffusion assay against 10 therapeutically significant antibiotics. Whole-genome sequencing was performed to identify the presence of AMR genes. A total of 12 AMR genes were identified via the Comprehensive Antibiotic Resistance Database (CARD). A comparative genomics approach was employed to investigate the global distribution of AMR genes from 2014 to 2024. **Results:** Antibiotic susceptibility testing indicated complete resistance to metronidazole, while the isolates remained susceptible to ampicillin, doxycycline, tetracycline, ciprofloxacin, norfloxacin, cefixime, azithromycin, meropenem, and cotrimoxazole. Whole-genome sequencing and comparative analysis identified 12 AMR genes, including *aadK*, *vanT* (within the *vanG* cluster), *ykkC*, *ykkD*, *vanW* (within the *vanI* cluster), *FosBx1*, *qacJ*, *qacG*, *tet(45)*, *vanY* (within the *vanM* cluster), and *blt*. The observed resistance mechanisms included antibiotic efflux, target modification, and enzymatic inactivation. Comparative genomic analysis of 15 closely related strains revealed variability in the distribution of AMR genes, with *B. subtilis* strain MB415 carrying all 12 resistance genes. **Conclusion:** The detection of antibiotic-resistant *B. subtilis* in the Southern Ocean suggests potential anthropogenic influences on microbial communities, underscoring the need for continuous surveillance of AMR in remote marine environments to prevent its proliferation and mitigate its ecological consequences.

Keywords: antimicrobial resistance; *Bacillus subtilis*; antibiotics; comparative genomics; resistant genes

1. Introduction

Drug resistance or antimicrobial resistance (AMR) has become an urgent global issue that poses serious risks to both the environment and public health. The excessive and unregulated use of antibiotics has resulted in their widespread presence in ecosystems, particularly in soil and water. Recognizing its severity, the United Nations Environment Programme (UNEP) has identified AMR as one of the six most pressing environmental concerns, alongside climate change, ecological degradation, and water scarcity. According to experts, this is one of the greatest problems humanity will encounter in the decades that follow [1]. The World Health Organization (WHO) has also emphasized the growing threat of AMR in pathogenic microbes and has been actively raising awareness of this issue. In a report published in May 2024, the WHO classified 15 families of antibiotic-resistant bacteria into three priority levels, namely, critical, high, and medium, to prioritize re-

search and development efforts for new antibiotics. Among the most concerning pathogens identified are *Acinetobacter baumannii* (resistant to carbapenems), *Enterobacterales* (resistant to third-generation cephalosporins and carbapenems), *Salmonella typhi* (fluoroquinolone resistant), *Pseudomonas aeruginosa* (carbapenem resistant), *nontyphoidal Salmonella* (fluoroquinolone resistant), and *Staphylococcus aureus* (methicillin resistant), all of which present significant public health risks [2]. In this context, various studies have documented the emergence of AMR in numerous pathogenic microorganisms worldwide, resulting in resistance to several broad-spectrum antibiotics [3–5].

The Southern Ocean, bordering southern China by the Antarctic continent [6], surrounds one of the remaining untouched regions on Earth. This continent is home to a vast array of marine life and significantly contributes to global climate regulation through the albedo effect [7]. Maintaining pristine conditions is essential for preserving



critical oceanic currents that support marine ecosystems and influence global climate patterns. However, human-associated microorganisms have been introduced into this isolated habitat as a result of increasing human activities, such as the construction of research stations and tourism. Antimicrobial resistance (AMR) is thought to be low in these remote areas despite the small number of people living there. However, because of their possible danger to biodiversity, the presence of AMR bacteria in aquatic environments, especially in marine ecosystems, has drawn increasing attention in the last 10 years. Marine waters and sediments close to these areas are impacted by aquaculture, industrial processes, and municipal garbage discharge, which frequently include multidrug-resistant microorganisms [8]. The prevalence of AMR genes is significantly greater in environments impacted by human activity and wildlife than in remote locations. In contaminated ecosystems, human activity directly contributes to the persistence and spread of antibiotic-resistant microorganisms [9]. Additionally, Article 5 of Annex III of the Protocol on Environmental Protection to the Antarctic Treaty permits the discharge of liquid waste and sewage into ocean waters [10,11]. This practice poses a substantial risk of introducing mobile genetic elements and antibiotic-resistant bacteria (ARB) linked to human activity into Antarctic waters, potentially facilitating their spread to local wildlife [12].

Understanding the extent to which human activities contribute to the dissemination of antibiotic resistance genes (ARGs) and AMR is crucial, particularly in remote environments [13]. These isolated regions offer valuable model ecosystems for investigating the fundamental processes of AMR evolution, including its *de novo* emergence, acquisition, and transmission. Furthermore, microorganisms that thrive in such extreme conditions have naturally developed resistance to various environmental stressors, including radiation, high salinity, nutrient scarcity, and low temperatures [14]. Research has demonstrated that human interventions can alter natural microbial communities, leading to the selection of antibiotic-resistant strains against multiple antibiotics [15–18]. For example, an analysis of Antarctic soil samples revealed the presence of 79 ARG subtypes associated with 12 distinct antibiotic classes, with polypeptide and multidrug resistance genes being the most prevalent. The composition, abundance, and potential for horizontal gene transfer of ARGs in these microorganisms differed significantly from those reported in activated sludge, poultry waste, and swine feces. Notably, ARG subtypes such as *bacA*, *ceoB*, *dfrE*, *mdtB*, *amrB*, and *acrB* were detected at higher frequencies than other ARG variants in Antarctic soils. This study further revealed that approximately 60% of ARGs are resistant through efflux mechanisms, whereas only approximately 16% are likely to be located on plasmids [19].

In a study by Calisto *et al.* [20] in 2024, they examined three *Pseudomonas* spp. isolates, C1-4-7, D2-4-6, and M1-

4-11, from Antarctic soil, and all the isolates exhibited resistance to ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, ertapenem, trimethoprim, chloramphenicol, and bacitracin. Additionally, two-thirds of the isolates were resistant to ceftriaxone, sulfafurazole, and cotrimoxazole. In another recent study focusing on the most human-impacted areas of the Antarctic Peninsula, an analysis of 137 fresh and cloacal fecal samples from various bird and marine mammal species revealed antibiotic resistance in 80% of the isolates. Most of these resistant bacteria belong to the gastrointestinal microbiota, including *Enterobacteriaceae* and *Enterococcus* species. The study confirmed that antibiotic-resistant bacteria were prevalent among both penguins and pinnipeds in the region [12].

Bacillus spp. are utilized in several biotechnological applications, including probiotic dietary supplements for people and inoculants for animal feed, owing to their capacity to enhance the immune system [21,22], and they generate antibacterial chemicals that suppress harmful germs [23,24]. There is increasing public health concern over the potential of microbial cultures utilized as dietary supplements or in food production to serve as sources for the transmission of antibiotic resistance genes. Concern over the spread of antibiotic resistance genes is increasing, and little is known about their resistance to different antibiotics. In a study by Adimpong *et al.* (2012) [21], 85 strains of *Bacillus* species, including *Bacillus subtilis* subsp. *subtilis* (n = 29), *Bacillus licheniformis* (n = 38), and *Bacillus sonorensis* (n = 18), were recovered from starters used to produce Sudanese bread. The MICs of eight antibiotics were calculated for these bacteria, where the strains were all resistant to streptomycin but susceptible to gentamicin, vancomycin, and tetracycline [24]. Two different methods are used by *B. subtilis* cells to adapt their membranes to low temperatures. One is when *Bacillus subtilis* is cultivated aerobically, it uses the fatty acid desaturase gene (*Des*) to fluidize its membrane when exposed to a decrease in temperature. *Des* requires molecular oxygen to function, and its expression is controlled by *DesK-DesR*, a two-component system. *Des* transcription increases with decreasing temperature and decreases when membrane fluidity is restored [25]. In addition, an increase in low-melting-point anteiso-branched FAs (mostly a-15:0 and a-17:0) is used in long-term membrane adaptation to efficiently fluidize the membrane [26,27].

The detection of AMR and ARGs in the farthest ocean and pristine environments raises concerns about potential threats to preserved biodiversity, environmental stability, and human health. With these concerns, the present study aimed to assess the presence of AMR bacteria isolated from the Indian sector of the Southern Ocean within the Antarctic Circle. The research involved profiling the isolate against ten commonly prescribed antibiotics, characterizing them through 16S rRNA sequencing and whole-genome sequencing, and conducting a comparative genome-wide

analysis with closely related strains to examine the ARG distribution and resistance mechanisms reported between 2014 and 2024.

2. Methods and Materials

2.1 Bacterial Culture, Growth Conditions and Characterization

The *Bacillus* strain utilized in this study was originally isolated from water samples collected during the 10th Indian Scientific Expedition to the Southern Ocean (39°19' S, 57°30' E to 66°38' S, 76°22' E). For the present study, the strain was stored at –80 °C and cultured in buffered peptone water, followed by the addition of mannitol yolk polycyxin agar, a nutrient-rich medium conducive to *Bacillus* sp. growth at 37 °C. A compound microscope (Model no. Olympus BX41, Olympus Corporation, Tokyo, Japan) was used to view the form, size, edge, color, elevation, and surface texture of a colony, and biochemical tests, such as Gram staining, catalase tests, urease tests, coagulase tests, and glucose fermentation, were performed.

2.2 Antibiotic Susceptibility Profiling

The antibiotics tetracycline, ampicillin, norfloxacin, doxycycline, ciprofloxacin, metronidazole, azithromycin, cefixime, cotrimoxazole and meropenem were used in the study procured from Sigma Aldrich (Bangalore, India). The agar well diffusion method was used [28] at a concentration of 5 µg/mL.

2.3 Molecular Characterization and Phylogenetic-Based Study

2.3.1 16S rRNA Sequencing

For bacterial characterization, DNA extraction was carried out via the Xploreagen Soil Kit (manufactured by Xploreagen Discoveries Pvt Ltd., Bangalore, India). An ~1.5 kbp segment of the 16S *rRNA* gene was amplified via universal primers adopted from a previous study [29]: 16S forward (5'-GGATGAGCCCGCGGCTA-3') and 16S reverse (5'-CGGTGTGTACAAGGCCCGG-3'), and polymerase chain reaction (PCR) was conducted. Each PCR (50 µL total volume) contained 1 µL of bacterial DNA template, 30 µL of water, 10 µL of Taq DNA polymerase (3 U/mL), 4 µL of dNTPs (2.5 mM), 10 µL of 10X assay buffer, and 2 µL of each 16S forward and reverse primer (10 pM). The resulting PCR amplicons were purified to remove contaminants and subsequently analyzed via agarose gel electrophoresis. DNA sequencing was performed via the BDT V3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Mumbai, Maharashtra, India) and an ABI 3130xl Genetic Analyzer for both forward and reverse sequencing reactions. The genomic sequence of the strain was determined via the National Centre for Biotechnology Information (NCBI) database, and BLAST-n (BLAST+ 2.15.0) was employed to identify closely related strains [29–31].

2.3.2 Whole-Genome Sequencing

For whole-genome sequencing, a Kapa Hyper Plus Kit (Roche Dia, Cape Town, South Africa) was used, and the extracted DNA samples were fragmented via the KAPPA fragmentation system (Roche Dia, Cape Town, South Africa). The fragmented DNA underwent end repair and A-tailing via the Hyper plus ERAT enzyme mixture (Roche Dia, Cape Town, South Africa). Adapter-ligated samples were subjected to library amplification via Illumina primers, followed by purification with AMPure beads (Beckman Coulter, Brea, CA, USA). The libraries were quantified via the Qubit dsDNA High Sensitivity Assay Kit (Fisher Scientific (Waltham, MA, USA). Sequencing was performed on the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA), and genome assembly was conducted via Unicycler software v. 0.4.8 [32]. The annotation of the assembled genome was performed via the online tool Rapid Prokaryotic Genome Annotation (PROKKA) v. 1.14.6 [33]. The whole-genome sequence of *Bacillus subtilis* was submitted to the GenBank database, NCBI with SOI-28 as the strain name and accession number JB-DUTP000000000. The phylogenetic tree was constructed by retrieving whole-genome sequences of *Bacillus subtilis* via the online database Pathosystems Resource Integration Center (PATRIC), which was last accessed on August 6, 2024. Additionally, Reference Alignment-Based Phylogeny Builder (ReaLPHY) v1.12 [34], which utilizes Bowtie2 (v2.2) for sequence alignment, was employed to construct the phylogenetic tree [35].

2.4 Comparative Genomic Analysis: Retrieval of Genome Sequence Data and AMR Gene Identification

BLASTn analysis was performed on the isolated *B. subtilis* SOI-28 to evaluate its genetic similarity with other strains. A total of 10,763 high-quality genome sequences of *B. subtilis* isolates submitted between 1887 and 2024 are available in the Pathosystems Resource Integration Center (PATRIC) database. Among these, 233 sequences were from isolates submitted between 2014 and 2024. The good-quality assembled FASTA sequences for these 233 *B. subtilis* strains were retrieved, and 62 genomes were further selected on the basis of specific criteria, including sequencing platforms (Illumina MiSeq, HiSeq, and NovaSeq) and assembly methods (Unicycler, SPAdes, and Shovill). The traces of AMR genes in the retrieved genomes, including 15 closely related and high-quality genomes identified from the phylogenetic tree, were examined via the Comprehensive Antibiotic Resistance Database (CARD) 3.3.0. Only strict and perfect hits were considered to ensure result accuracy [36]. Additionally, a comprehensive genome-wide comparison was conducted via the Circular Gene Viewer (CGView) online tool (last accessed on August 22, 2024) to analyze genomic similarities and differences. Particular attention has been given to the distribution and spread of resistance genes among *B. subtilis* strains [37,38].



Fig. 1. Zone of inhibition (in mm) for *Bacillus subtilis* SOI-28. This image depicts the zone of inhibition for *B. subtilis* SOI-28 (NCBI Accession Number: JBDUTP000000000) against ten therapeutically used antibiotics obtained by agar well diffusion. The inhibition zones are cefixime, 20.6 mm; metronidazole, 0 mm; cotrimoxazole, 16.4 mm; norfloxacin, 20.2 mm; doxycycline, 36.2 mm; ciprofloxacin, 34.6 mm; ampicillin, 44.4 mm; tetracycline, 10.2 mm; azithromycin, 10.4 mm; and meropenem, 28.6 mm.

Table 1. Morphological and biochemical characteristics of *Bacillus subtilis* SOI-28.

S. No.	Morphological characteristics	Observed characteristics	Biochemical characteristics	Observations
1.	Colony shape	Rods	Indole test	Negative
2.	Margin	Irregular	Catalase test	Positive
3.	Size of colony	0.7–3.0 μ m long	Urease utilization	Negative
4.	Elevation	Flat	Citrate utilization	Positive
5.	Surface	Rough & opaque	Coagulase test	Positive
6.	Color	Purple-pink color on MYPA	Glucose fermentation	Positive
7.	Motility	Nonmotile	Mannitol fermentation	Positive
8.	Spore formation	Spore forming	Fructose fermentation	Positive
9.	Gram Staining	gram-positive	-	-

MYPA, Mannitol Yolk Polymyxin Agar.

3. Results and Discussion

3.1 Antibiotic Susceptibility Profiling and Phylogenetic Analysis

Antibiotics are introduced into the environment through pharmaceutical industrial waste [39] and via human and animal excreta from individuals undergoing antibiotic treatments [40]. Traces of antimicrobial residues in water pose significant environmental and public health risks, as these compounds can accumulate in aquatic organisms. Additionally, the widespread use of antimicrobials in the fisheries, poultry, and dairy industries contributes to antibiotic contamination, creating potential pathways into various food chains and, ultimately, the human body [41]. Aquatic environments serve as reservoirs for antibiotic-resistant microorganisms, facilitating their proliferation and dissemination. Furthermore, resistance genes can integrate into naturally occurring bacterial ecosystems, increasing the risk of horizontal gene transfer among human, animal, and environmental microbiomes [42].

Morphologically, the strain exhibits characteristics typical of *B. subtilis*, forming cylindrical rod cells, with individual cells measuring approximately 0.7 to 3.0 μ m long.

Colonies appear opaque in a creamy-white color on nutrient agar and develop a characteristic purple color on Mannitol Yolk Polymyxin Agar due to mannitol fermentation. Biochemically, the strain is gram-positive, displays positive catalase activity, and is coagulase positive. These features confirmed the identity of the isolate as a cold-adapted strain of *Bacillus subtilis* capable of thriving in the unique conditions of the Southern Ocean (Table 1). Antibiotic susceptibility testing for *Bacillus subtilis* SOI-28 was performed via the agar well diffusion method against ten commonly prescribed antibiotics. The results indicated that the isolate displayed resistance to certain antibiotics. The average diameter of the inhibition zones for *B. subtilis* SOI-28 was measured at a concentration of 5 μ g/mL for each antibiotic. These findings suggest that *B. subtilis* SOI-28 exhibited 100% antibiotic resistance to metronidazole (Fig. 1). The susceptibility patterns of *B. subtilis* SOI-28 were as follows: ampicillin (zone—44.4 mm) > doxycycline (zone—36.2 mm) > ciprofloxacin (zone—34.6 mm) > meropenem (zone—28.6 mm) > cefixime (zone—20.6 mm) > norfloxacin (zone—20.2 mm) > cotrimoxazole (zone—16.4 mm) > azithromycin (zone—10.4

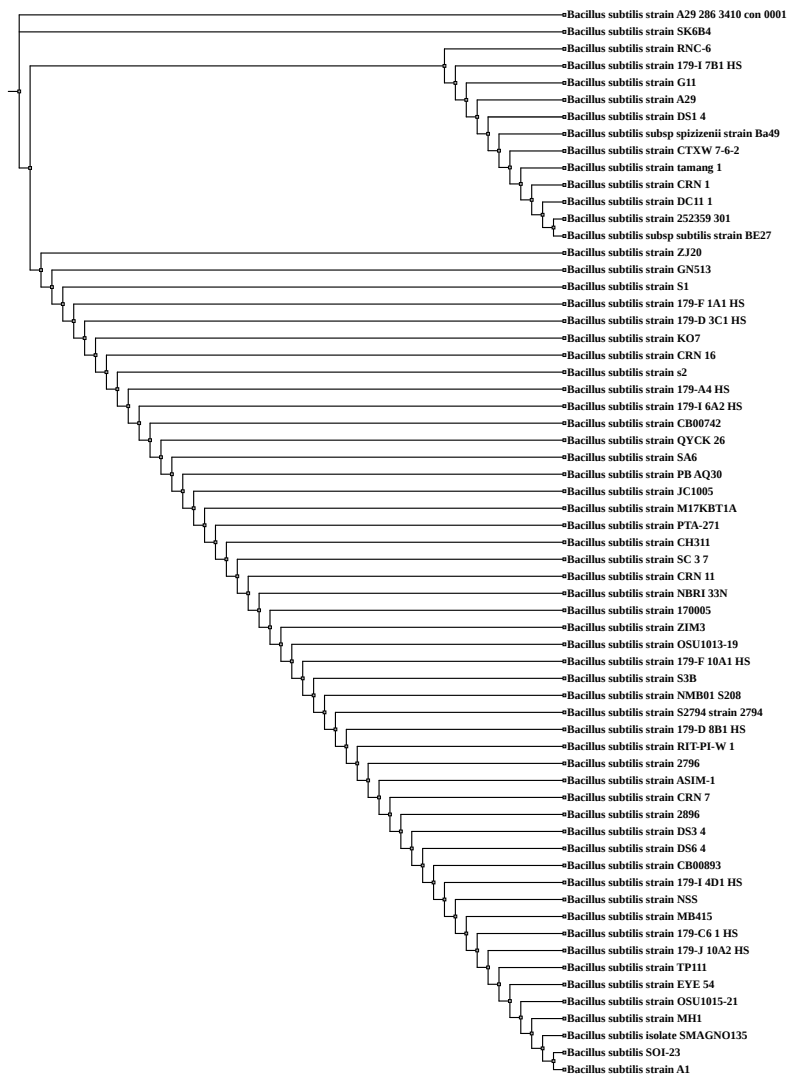


Fig. 2. Whole-genome sequence-based phylogenetic tree for *Bacillus subtilis* SOI-28. The figure shows the phylogenetic tree generated using the RealPHY tool based on whole-genome alignment of 62 *Bacillus subtilis* genomes, including the isolate SOI-28 and was further visualized and annotated using the Interactive Tree of Life (iTOL) tool. *Bacillus subtilis* SOI-28 is highlighted within the tree to indicate its phylogenetic position.

mm) > tetracycline (zone—10.2 mm). *B. subtilis* SOI-28 is highly resistant to antibiotics belonging to different antibiotic classes, including fluoroquinolones, glycopeptides, aminoglycosides, and tetracycline and phenolic antibiotics.

Whole-genome sequencing (WGS) data for *Bacillus subtilis* SOI-28, along with genomes retrieved from the PATRIC database, were utilized for phylogenetic analysis. The whole-genome-based phylogenetic assessment revealed that *B. subtilis* SOI-28 exhibited close genetic similarity to the reference strain SHOB000000000 *Bacillus subtilis* A1, which was isolated from the maize rhizosphere in South Africa, and JBCNOU000000000 *Bacillus subtilis* SMAGNO135, which was obtained from sugarcane leaves, stems, roots, and rhizosphere soil in China (Fig. 2).

3.2 Retrieval of Genomic Data for *Bacillus subtilis* Isolates

The *Bacillaceae* family is widely distributed across diverse natural environments, with habitats ranging from soil and sediment to extreme and unconventional locations, including clean rooms at the Kennedy Space Center, vaccine production facilities, and even human blood [43–45]. *Bacillus* species are rod-shaped, aerobic bacteria capable of staining as either gram-positive or gram-negative bacteria and are widely recognized for their ability to produce resilient spores, which opposes cold, heat and common disinfectants, enabling survival in diverse ecosystems [46]. The *Bacillus* genus includes more than 200 species, most of which are nonpathogenic and have been widely employed in biotechnological and industrial processes [47,48]. Only a small number of species in this genus are known to be pathogenic to humans and animals [49]. Numerous stud-

Table 2. PATRIC* Database metadata for *Bacillus subtilis* strains.

S. No.	Strain	GenBank Accession No.	Genome Size (bp)	Contigs	GC Content (in %)
1.	<i>Bacillus subtilis</i> strain A1	SHOB00000000	4,060,396	22	43.75%
2.	<i>Bacillus subtilis</i> 2896	JANLFH000000000	4,291,432	54	43.05%
3.	<i>Bacillus subtilis</i> strain DS3_4	JAHFZW000000000	4,000,557	65	43.79%
4.	<i>Bacillus subtilis</i> strain DS6_4	JAHFZV000000000	4,134,799	142	43.56%
5.	<i>Bacillus subtilis</i> CB00893	JAXKH000000000	4,215,674	42	43.35%
6.	<i>Bacillus subtilis</i> strain 179-I 4D1 HS	JAHHXX000000000	3,996,990	27	43.72%
7.	<i>Bacillus subtilis</i> strain NSS	WVRO000000000	4,021,428	150	43.70%
8.	<i>Bacillus subtilis</i> strain MB415	MQSR000000000	4,266,089	244	43.09%
9.	<i>Bacillus subtilis</i> strain 179-C6.1 HS	JAHHZA000000000	4,211,076	14	43.35%
10.	<i>Bacillus subtilis</i> strain 179-J 10A2 HS	JAHHXN000000000	3,995,942	12	43.76%
11.	<i>Bacillus subtilis</i> TP111	JAXIVG000000000	4,174,638	26	43.47%
12.	<i>Bacillus subtilis</i> EYE_54	JAHXNR000000000	4,172,904	16	43.41%
13.	<i>Bacillus subtilis</i> OSU1015-21	JARKHW000000000	4,639,106	346	42.56%
14.	<i>Bacillus subtilis</i> strain MH1	PYBK000000000	4,091,387	60	43.49%
15.	<i>Bacillus subtilis</i> SMAGNO135	JBCNOU000000000	3,910,489	163	43.85%

*. PathoSystems Resource Integration Center (PATRIC) Bioinformatics Resource Center (Chicago, IL, USA). GC stands for Guanine-Cytosine content.

ies have reported the presence of *Bacillus* species in deep-sea environments [50–54]. Furthermore, members of the *Bacillaceae* family have been found in marine and freshwater environments, human and animal systems, activated sludge and diverse food sources, including fermented products. They have also been detected in inhospitable settings such as hot solid and liquid systems (e.g., hot springs and compost), salt lakes, and salterns [55–58]. While *Bacillus* species are capable of producing heat-resistant spores, they are typically not associated with human infections. Notably, *Bacillus subtilis* and *Bacillus velezensis* have garnered considerable attention in the food industry because of their established safety and competitive ability against other microorganisms in natural environments, which may influence microbiota selection [59]. The whole-genome sequence of *Bacillus subtilis* SOI-28 revealed that the strain had 43.44% Guanine-Cytosine (GC) content and a genome size of ~3,990,483 bp. genome coverage—422.38 × and 71 contigs, contig L50: 10 and contig N50: 129780.

Table 2 in the PATRIC metadata provides detailed information on 15 *Bacillus subtilis* strains, including their strain names, isolation sources, sequencing platforms, assembly methods, genome sizes, and GC contents. The strains were isolated from diverse sources, including the maize rhizosphere ($n = 1$), cell culture ($n = 1$), commercial dietary supplements ($n = 2$), insect gut ($n = 1$), fish gut ($n = 1$), plant leaves ($n = 2$), cleanroom floors ($n = 3$), soil ($n = 2$), ocular surface of a healthy individual ($n = 1$), and sourdough ($n = 1$). In accordance with the PATRIC database, these genomes were sequenced primarily via the Illumina NovaSeq platform, with some also processed via Illumina MiSeq and HiSeq. The genome sizes of these 15 strains ranged from 3.9 Mb to 4.6 Mb, with the GC content varying between 42.56% and 43.85%.

3.3 Antimicrobial Resistance Genes

The ecosystem of Antarctica is fragile and relatively simple due to its extreme climate and nutrient scarcity, supporting only a limited number of resilient species [60,61]. Despite these harsh conditions, the region is home to remarkable megafauna, including whales, seals, penguins, and albatrosses, which migrate over vast distances [62]. However, environmental changes and human activities pose significant threats to this unique biodiversity, with humans acting as vectors for diseases and antibiotic-resistant bacteria [63–66]. The widespread presence of antibiotic residues in the ocean, largely due to human activities, may accelerate the emergence of antibiotic-resistant *Bacillus* species, posing serious risks to human health [67]. *Bacillus subtilis* is one of the most extensively studied gram-positive bacteria, primarily serving as a model for cell differentiation and industrial applications. However, its potential virulence remains relatively unexplored [54]. Human activities contribute to the introduction of pharmaceutical residues and ARGs into marine ecosystems through wastewater discharge from healthcare facilities, agriculture, and aquaculture [68–70]. Many aquatic bacteria possess mobile genetic elements that facilitate the horizontal transfer of ARGs, potentially spreading resistance traits among environmental, commensal, and pathogenic bacteria, including *Staphylococci*, *Bacillus* spp., and *Escherichia* spp. [71]. Research on deep-sea environments has revealed the significant presence of spore-forming *Bacillus* species. One study reported that nearly 90% of bacterial isolates from deep-sea sediments were associated with *Bacillus* [72]. Similarly, findings from deep-sea floor samples collected at Site C0020, which is off the Shimokita Peninsula in Japan, revealed that spore-forming gram-positive bacteria from the *Bacillales* order were dominant in the microbial community [73]. In

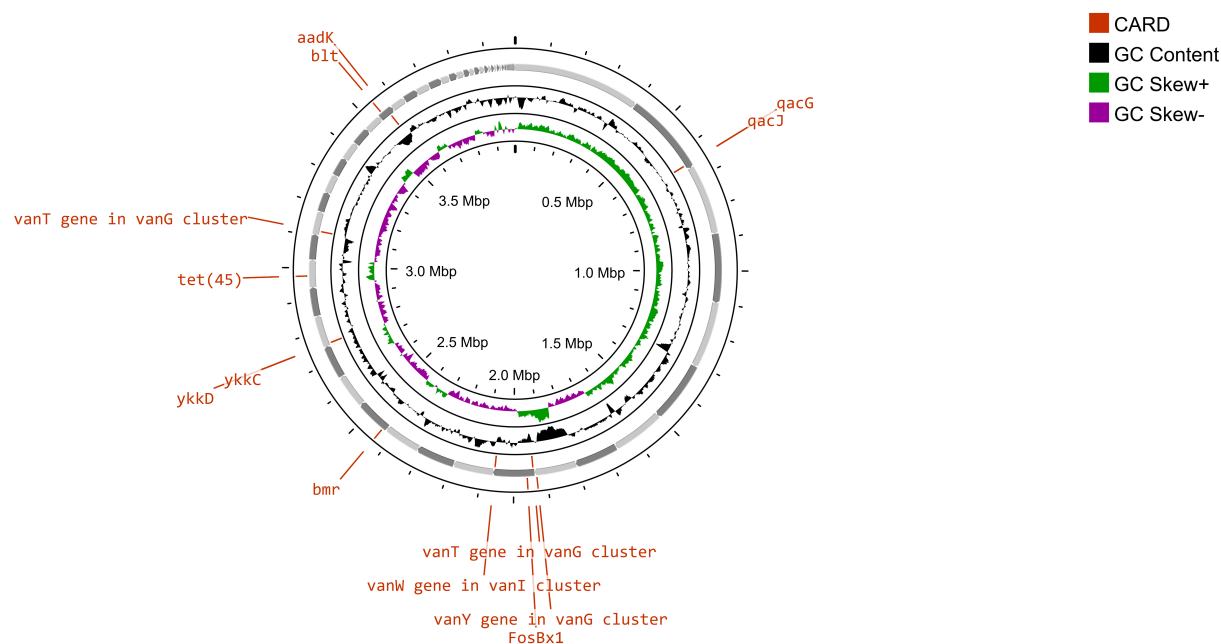


Fig. 3. Circular gene map of AMR genes in *Bacillus subtilis* SOI-28. The figure shows the presence of 12 ARGs—*blt*, *qacG*, *qacJ*, and *vanY* in the *vanM* cluster; *vanT* in the *vanG* cluster; *FosBx1* and *vanW* in the *vanI* cluster; and *ykkD*, *ykkC*, *tet(45)*, and *addK* in *Bacillus subtilis* SOI-28 (NCBI accession number: JBDUTP000000000) constructed using Circular Genome (CG) viewer.

addition to sediment and water, *Bacillus* species have also been detected in marine organisms, including sponges, ascidians, and crabs, further underscoring their adaptability and ecological significance in oceanic environments [74].

A study has revealed a limited presence of antibiotic resistance in the North Sea, particularly in areas where offshore oil and gas platforms are located [75]. Additionally, previous research has revealed that ballast water from ships can serve as a vehicle for the spread of AMR bacteria and ARGs [76,77]. The traces of antibiotic resistance genes (ARGs) in *Bacillus subtilis* SOI-28 were identified via the Comprehensive Antibiotic Resistance Database (CARD), which considers only perfect and strict hits. *Bacillus subtilis* SOI-28, which was isolated from the Indian Sector of the Southern Ocean, exhibited complete phenotypic resistance to metronidazole, a nitroimidazole drug caused by the presence of *nim* gene(s). The analysis identified a total of 12 ARGs—*blt*, *qacG*, *qacJ*, and *vanY* in the *vanM* cluster; *vanT* in the *vanG* cluster; *FosBx1* and *vanW* in the *vanI* cluster; and *ykkD*, *ykkC*, *tet(45)*, and *addK* spanning multiple drug classes and resistance mechanisms (Supplementary Table 1) in which *nim* gene(s) were not identified. AMR genes have antibiotic resistance mechanisms, such as antibiotic efflux, antibiotic target alteration and antibiotic inactivation. Gene mapping of *B. subtilis* SOI-28 revealed three key gene clusters: {*qacG*, *qacJ*}, {*ykkC*, *ykkD*}, and {*vanY* (*vanM* cluster), *vanW* (*vanI* cluster)}. Furthermore, the *vanT* gene from the *vanG* cluster was found in two distinct locations within the genome, separated by 6,542 base pairs (Fig. 3). In the present study, the identifica-

tion of AMR genes was conducted via the protein homolog model, which detects protein sequences by comparing their similarity to curated reference sequences via predefined BLASTP bitscore cutoffs. This model applies to any gene that confers resistance solely by its presence in an organism, such as a beta-lactamase gene located on a plasmid. The inclusion of a reference sequence and a bitscore cut-off ensures accurate detection through BLASTP. However, the phenotypic AMR results of *Bacillus subtilis* SOI-28 were not 100% in accordance with the genotypic results of *Bacillus subtilis* SOI-28 (Table 3), as *Bacillus subtilis* SOI-28 showed signs of phenotypic resistance to metronidazole, although no known mutations or resistance genes were found. On the other hand, the *tet(45)* gene is linked to tetracycline resistance, and the *blt* gene is linked to fluoroquinolone resistance (ciprofloxacin), even though *Bacillus subtilis* SOI-28 shows phenotypic sensitivity to tetracycline and ciprofloxacin antibiotics. These results show 70% concordance and 30% discrepancy between the phenotypic and genotypic results.

3.4 Comparative Genomics Approach for AMR Gene Variability Study

The whole-genome sequence of *Bacillus subtilis* SOI-28 (reference genome) along with 15 good-quality whole-genome sequences of *Bacillus subtilis* strains retrieved from the PATRIC database were used for comparative analysis (Fig. 4).

Heatmapping was performed to analyze the distribution of AMR genes in 15 selected genomes. Through

Table 3. Differences in the inhibition zones and CARD results between phenotypic resistance and genotypic resistance between *Bacillus subtilis* SOI-28.

S. No.	Drugs class	Antibiotics	Phenotypic Resistance (<i>Bacillus subtilis</i> SOI-28)	Genotypic Resistance (<i>Bacillus subtilis</i> SOI-28)
1.	Cephalosporin	Cefixime	No	No
2.	Quinolone	Norfloxacin	No	No
3.	Penicillin	Ampicillin	No	No
4.	Tetracycline	Doxycycline	No	No
5.	Protein inhibitor	Tetracycline	No	Yes
6.	Macrolides	Azithromycin	No	No
7.	Fluoroquinolone	Ciprofloxacin	No	Yes
8.	Sulphonamides	Cotrimoxazole	No	No
9.	Nitroimidazole	Metronidazole	Yes	No
10.	Carbapenem	Meropenem	No	No

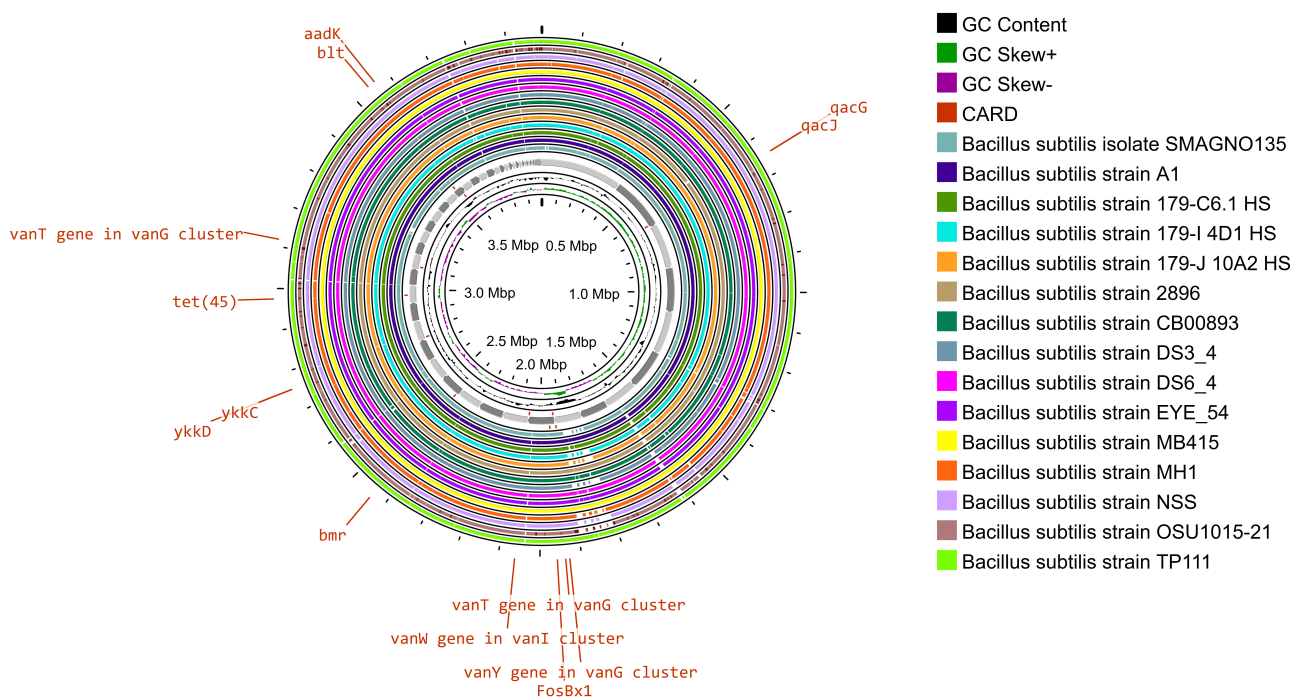


Fig. 4. Gene map providing a comparative analysis of *Bacillus subtilis* SOI-28 against other *Bacillus subtilis* genomes. The innermost ring represents the reference genome sequence of *B. subtilis* SOI-28. The next 15 concentric rings illustrate regions with sequence similarity, as determined through BLAST comparisons between *Bacillus subtilis* SOI-28 (query genome) and 15 selected *B. subtilis* genomes. The outermost ring highlights the antibiotic resistance (AMR) genes identified in the reference genome on the basis of the comprehensive antibiotic resistance database (CARD) results.

CARD database analysis, *Bacillus subtilis* SOI-28 was found to have twelve antibiotic resistance genes, which were selected for AMR profiling across the other 15 genomes. The highest resistance genes found were *aadK* 100% (n = 15); the *vanT* gene in vanG cluster 100% (n = 15) in two regions; *ykkC* 100% (n = 15); *ykkD* 100% (n = 15); the *vanW* gene in vanI cluster 100% (n = 15); *FosBx1* 100% (n = 15); *qacJ* 100% (n = 15) and *qacG* 100% (n = 15) followed by *tet(45)* 66.67% (n = 10); and the *vanY* gene in vanM cluster 53.34% (n = 7) and *blt* 40% (n = 6). The

findings indicated that the genomes included at least nine (n = 3) and a maximum of twelve (n = 1) AMR genes (Fig. 5).

According to the CARD database results, the most common drug resistance mechanisms are antibiotic efflux (100%, n = 15), antibiotic inactivation (100%, n = 15), antibiotic target alteration (100%, n = 15), antibiotic target protection (93.34%, n = 14), and reduced permeability (80%, n = 12) (Fig. 6). The primary gene families associated with the antibiotic efflux resistance mechanism are the MFS and the SMR antibiotic efflux pump. Similarly,

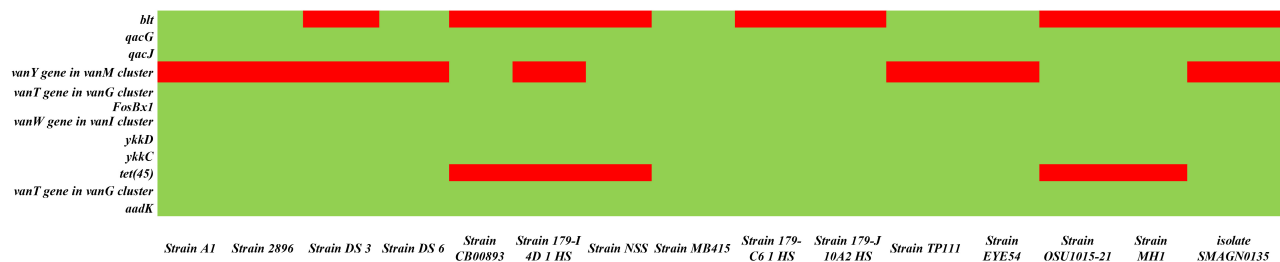


Fig. 5. Heatmap depicting the presence of 12 AMR genes, including *blt*, *qacG*, *qacJ*, and *vanY* in the *vanM* cluster; *vanT* in the *vanG* cluster; *FosBx1* and *vanW* in the *vanI* cluster; *ykkD*, *ykkC*, and *tet(45)*; and *addK* across different *Bacillus subtilis* strains. The green color depicts the existence of the gene, and the red color depicts the absence of the gene (The X-axis represents the different *Bacillus subtilis* strains, and the Y-axis represents AMR genes present in *Bacillus subtilis* SOI-28).

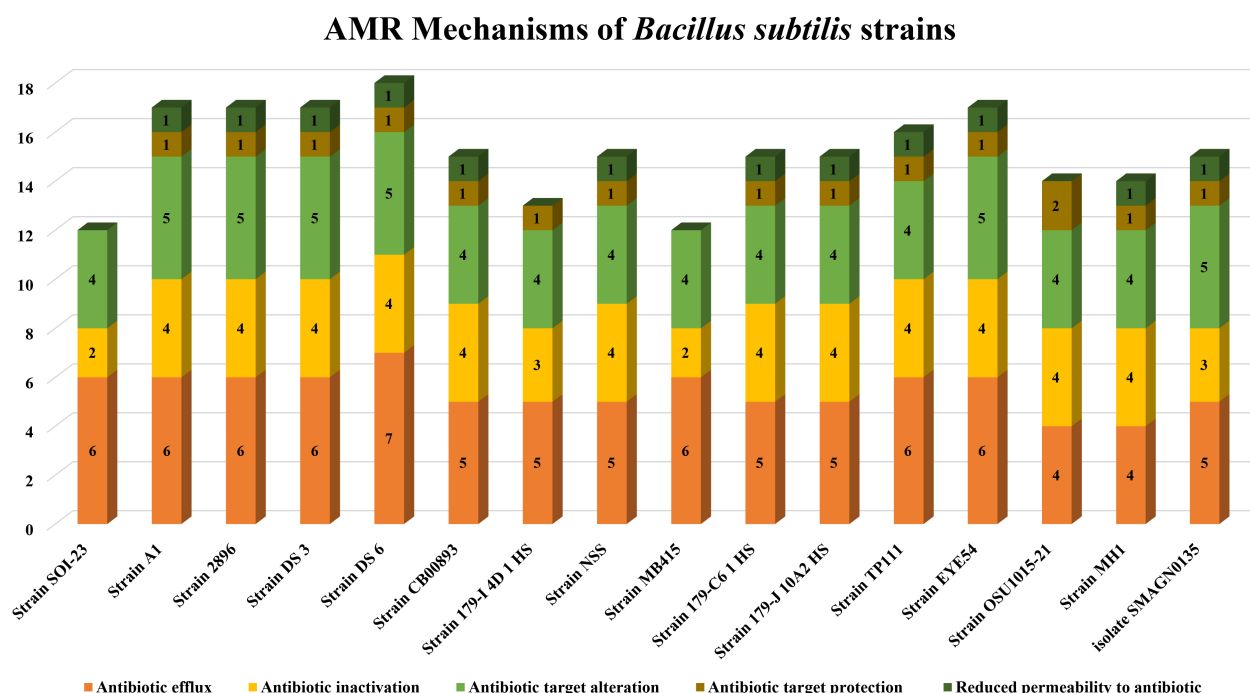


Fig. 6. AMR genes variation. The figure shows variations in the presence of AMR genes with antibiotic resistance mechanisms-antibiotic efflux; target alteration, replacement and protection; and antibiotic inactivation present in *Bacillus subtilis* SOI-28 (reference sequence) across 15 different *Bacillus subtilis* genomes retrieved from PATRIC (The X-axis represents the different *Bacillus subtilis* strains, and the Y-axis represents the number of AMR genes).

in the case of antibiotic alterations, *vanY*, the glycopeptide resistance gene cluster, *vanT*, and *vanG* were the top gene families observed across all the genomes ($n = 15$). The AMR genes identified in *Bacillus subtilis* SOI-28 included *blt*, *qacG* and *qacJ*; the *vanY* gene in the *vanM* cluster; the *vanT* gene in the *vanG* cluster; *FosBx1*; the *vanW* gene in the *vanI* cluster; *ykkD* and *ykkC*; and *tet(45)* and *aadK*, which were screened for their prevalence among 15 PATRIC-retrieved genomes. The *B. subtilis* strain MB415 was found to have all twelve AMR genes belonging to different antibiotic classes, representing 6.67% of the studied genomes.

Saikat *et al.* [78] in 2024 conducted a comprehensive phenotypic characterization and whole-genome analysis of *Bacillus subtilis* BDSA1 obtained from polluted river water in Dhaka, Bangladesh, and the findings revealed that *B. subtilis* BDSA1 exhibited resistance to streptomycin, cefotaxime, and ceftriaxone while remaining susceptible to imipenem, meropenem, trimethoprim, gentamycin, and ampicillin. Similarly, in 2021, Cardoso *et al.* [79] conducted a study to isolate and characterize bacterial species from deep water samples collected from three artesian wells, and the susceptibility profile was assessed against antinicrobials. This study identified four bacterial isolates:

Bacillus pumilus, *Bacillus subtilis*, and two strains of *Bacillus megaterium*. All the isolates demonstrated sensitivity to all the tested antimicrobials, including amikacin, vancomycin, meropenem, oxacillin, imipenem, cefoxitin, and sulbactam with ampicillin. These findings suggest that microorganisms from natural environments tend to be inherently sensitive to antibiotics, reinforcing the ecological role of these bacteria in antimicrobial interactions [79].

Similarly, in an investigation by Haque *et al.* [80] in 2024, the phenotypic and genotypic resistance profiles of *Bacillus* spp. were evaluated, and the phenotypic analysis revealed that all *Bacillus* isolates (n = 191) were 100% sensitive to gentamicin. However, reduced sensitivity was observed for levofloxacin, ciprofloxacin, clindamycin, amoxicillin-clavulanic acid, azithromycin, tetracycline, nitrofurantoin, cotrimoxazole, and erythromycin. The isolates exhibited complete resistance to penicillin, amoxicillin-clavulanic acid, cefixime, ceftriaxone, vancomycin, and cotrimoxazole. A distinct species-specific pattern was evident in both the phenotypic and genotypic resistance profiles. The genotypic analysis revealed the presence of multiple resistance genes, with *blaI* detected in 71.5% of the isolates, *tetA* in 33%, *ermI* in 27%, *blaTEM* in 13.1%, *blaCTX-M-1/blaCTX-M-2/sulI* in 10.1%, *blaSHV* in 9.6%, and *qnrS* in 4.1%. Notably, the *blaI* gene was present in all the isolates that were resistant to penicillin. Among the 191 isolates analyzed, 89.6% were classified as multidrug resistant (MDR), with the MDR prevalence reaching 100% in isolates from diarrheal cases, 90.3% in food-derived isolates, and 88.7% in animal feed-derived isolates [80]. AMR in psychrophilic bacteria has received relatively little research attention to date, and *B. subtilis* isolated from the Southern Ocean has not yet been studied.

The presence of AMR *Bacillus subtilis* in the Indian sector of the Southern Ocean, Antarctica, highlights the potentially alarming intrusion of anthropogenic contaminants into one of the most pristine environments on Earth. The detection of AMR bacteria and associated resistance genes (ARGs) in this remote ecosystem suggests multiple pathways of pollution, including human activities at research stations, wastewater discharge, and the long-range transport of resistant microbes through ocean currents and migratory species. The persistence and dispersal of AMR in such isolated regions pose significant ecological risks, potentially disrupting native microbial communities and altering biogeochemical cycles. To mitigate this emerging threat, strict biosecurity measures must be enforced, including enhanced decontamination protocols for research personnel and equipment, improved waste management strategies, and rigorous monitoring of microbial resistance threats in Antarctic ecosystems. Investigating the pathways through which AMR spreads in aquatic environments and the potential for horizontal gene transfer among different bacterial species is essential for understanding its broader ecological impact. Regular monitoring of antibiotic-resistant

organisms in drinking and potable water supplies is critical to safeguarding public health and minimizing AMR-related infections. Additionally, international cooperation is crucial for establishing comprehensive AMR surveillance programs and promoting responsible antibiotic use worldwide. These proactive interventions are essential for safeguarding the ecological integrity of Antarctica and preventing the unchecked spread of AMR in the Southern Ocean.

4. Conclusion

The widespread use of antibiotics in the healthcare, poultry, fisheries, and dairy industries has contributed to their environmental dissemination, not only in local water bodies but also in distant and pristine marine ecosystems. The emerging threat of antibiotic resistance in these remote regions poses a significant ecological threat. Previous studies reported antimicrobial resistance in various *Bacillus* species, such as *B. cereus*, *B. pumilus*, and *B. subtilis*. In our study, the isolate *B. subtilis*, strain SOI-28, was approximately 0.7–3.0 µm in length and had an opaque, creamy-white appearance on nutrient agar and a pink color colony on MYPA. Biochemically, the isolate was gram-positive, catalase-positive, and coagulase-positive. Antibiotic susceptibility testing revealed complete phenotypic resistance to metronidazole, a nitroimidazole-class drug, and susceptibility to cefixime, tetracycline, ampicillin, azithromycin, cotrimoxazole, meropenem, doxycycline, ciprofloxacin and norfloxacin. However, genotypic analysis revealed the absence of *nim* gene(s), which are responsible for metronidazole resistance, and the presence of the *ykkD*, *ykkC* and *tet(45)* genes, which are responsible for tetracycline resistance. Genome-wide analysis of *Bacillus subtilis* SOI-28 revealed twelve resistance genes, namely, the *aadK* and *vanT* genes in the *vanG* cluster; the *ykkC*, *ykkD*, and *vanW* genes in the *vanI* cluster; and the *FosBxI*, *qacJ*, *qacG*, *tet(45)*, and *vanY* genes in the *vanM* cluster and the *blt*. Additionally, this study revealed the presence of twelve resistance genes in other closely related *B. subtilis* strains, with genomes containing a minimum of nine AMR genes and a maximum of twelve AMR genes. Among the resistance mechanisms identified, antibiotic efflux and target modification were the most prevalent, while antibiotic inactivation was also observed. Our results highlight the need for AMR monitoring in distant maritime areas and the possible human effects on microbial resistance in these settings. The identification of AMR genes in our *Bacillus subtilis* isolate SOI-28 suggests potential pathways for resistance gene transfer, which can affect marine biodiversity and pose unexpected risks to public health through ecological and food chain interactions. The current study also highlights the need for continued research on AMR mechanisms, its introduction into untouched environments, and its evolutionary behavior, which is crucial for controlling the spread of resistance and minimizing its effects on both environmental and human health.

Availability of Data and Materials

This research paper presents a comprehensive overview of the experiments conducted and the findings obtained only from the current study. The whole genome sequence of the resistant *Bacillus subtilis* strain SOI-28 has been deposited in the National Centre for Biotechnology Information (NCBI) GenBank database under the accession number JBDUTP000000000. Additional details are provided in **Table 1** of the **Supplementary Material**. Further details can be obtained upon reasonable request from the corresponding author.

Author Contributions

Conceptualization: SS, AC, AR and VNS; methodology, data collection and original data analysis and data presentation: SS, AC, AR, RS, RC and TJ; writing, reviewing and editing: SS, AC, AR, VNS and TJ. All authors have read and agreed to the final version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

According to Indian regulations and policies, this study does not involve human or animal subjects; therefore, ethical committee approval is not necessary.

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Conflict of Interest

All the authors declare no conflict of interests. Dr. Anuj Ranjan, given his role as the Editorial Board member in the journal FBE, had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Baohong Zhang.

Declaration of AI and AI-Assisted Technologies

The writers used ChatGpt to proofread their work for grammatical and spelling errors. The writers take full responsibility for the publication's content after using this tool, reviewing and editing it as necessary. The tool was used in the introduction and discussion sections.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBE38809>.

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