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Genome Sequence and Characterisation of *Peribacillus* sp. Strain AS_2, a Bacterial Endophyte Isolated from *Alectra sessiliflora*

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Abstract: *Peribacillus* sp. AS_2, a leaf endophytic bacterium isolated from the medicinal plant *Alectra sessiliflora*, was previously identified using the 16S rRNA gene sequence. The draft genome of AS_2 had a 5,482,853 bp draft circular chromosome, 43 contigs, N_{50} of 360,633 bp and an average G + C% content of 40.5%. Whole genome sequencing and phenotypic analysis showed that AS_2 was Gram-positive, endospore-forming, motile, and rod-shaped and it showed a high sequence similarity with *P. frigoritolerans* DSM 8801^T. Genomic digital DNA–DNA hybridisation (dDDH) between strain AS_2 and *Peribacillus frigoritolerans* DSM 8801^T and *P. castrilensis* N3^T was 84.8% and 79.2%, respectively, and the average nucleotide identity (ANI) of strain AS_2 with *P. frigoritolerans* DSM 8801^T and *P. castrilensis* N3^T was 97.0% and 96.7%, respectively. The antiSMASH software predicted a total of eight secondary metabolite gene clusters comprising non-ribosomal peptide synthetase (NRPS) type koranimine, terpenes, and siderophore clusters. Strain AS_2 also displayed genes involved in endophytic lifestyle and antibiotic resistance gene clusters such as small multidrug resistance antibiotic efflux pumps (*qacJs*). Using the multilocus sequence analysis (MLSA), together with the phenotypic data and genomic analysis, we demonstrated that strain AS_2 is a subspecies of *P. frigoritolerans* DSM 8801^T. Genome sequencing of *Peribacillus* sp. AS_2 from medicinal plants provides valuable genomic information and allows us to further explore its biotechnological applications.

Keywords: *Peribacillus*; endophyte; *Alectra sessiliflora*; whole genome sequencing

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1. Introduction

Bacterial endophytes are symbiotic microorganisms that colonise the internal tissues of plants without posing a threat to the plants' health [1]. Bacterial endophytes are present in all plants and have been identified as a promising source of biological raw materials for the agricultural, pharmaceutical, cosmetic, and food sectors [2]. Moreover, research has been focused mostly on bacterial endophytes isolated from medicinal plants due to their therapeutic importance, yet little is known about the mechanisms through which endophytes interact with their host plants. Numerous studies have demonstrated that bacterial endophytes synthesise bioactive compounds that assist plants by enabling them to withstand harsh conditions like high temperatures, low pH levels, salt, and heavy metals [3,4]. Additionally, their secondary metabolites offer resistance against biotic and abiotic stressors by stimulating the formation of immunological or antimicrobial compounds [5]. Due to their lower toxicity towards humans, animals, and the environment, these endophyte-produced secondary metabolites have attracted more attention globally [6].

The genus *Peribacillus* was first grouped with over 100 species and subspecies of the genus *Bacillus*, which belonged to the Firmicutes phylum [7]. However, based on phenotypic, chemotaxonomic, and genetic differences, some of the species of *Bacillus* have recently been reclassified into new genera, *Alkalihalobacillus*, *Cytobacillus*, *Metabacillus*, *Mesobacillus*, *Neobacillus*, *Peribacillus* [8–10].

Members of the genus *Peribacillus* have been isolated from various sources including soil [11], cow faeces [12], plant tissues [13,14], and river water [15]. Currently, within the

genus *Peribacillus* there are 17 species which have been validly published [16,17]. From the 17 published species, only 11 have whole-genome sequences available, which vary in size from 4.1 to 5.7 Mbp. The G + C composition of genomic DNA ranges from 37.5 to 43.0% [15]. According to [9], *Peribacillus* species are motile, rod-shaped, Gram-positive, aerobic, or facultatively anaerobic and can grow in different temperatures ranging from 3 to 45 °C, with their optimum temperature ranging between 25 and 37 °C.

Some *Peribacillus* species such as *Peribacillus butanolivorans* are used industrially for the remediation of butanol [9]. Similarly, other *Peribacillus* and *Bacillus* strains, such as *Bacillus thuringiensis* SG4 and *Bacillus* sp. SG2, have been shown to have biodegradation abilities for a variety of pesticides, including cypermethrin [18]. The present study aims to characterise *Peribacillus* sp. strain AS_2, a bacterial endophyte isolated from the medicinal plant, *Alectra sessiliflora* [19]. To the best of our knowledge, this is the first study to characterise a bacterial endophyte from the genus *Peribacillus* isolated from a medicinal plant. Strain AS_2 was previously isolated and initially identified using the 16S rRNA sequence by [19] and it showed promising antibacterial and antitumour activity against human clinical pathogens and three cancer cells. Therefore, further studies of strain AS_2 may be required to better understand and shed light on its possible applications in the industrial, medicinal, and agricultural sectors.

2. Materials and Methods

2.1. Isolation of the Bacterial Peribacillus sp. Strain AS_2

Peribacillus sp. strain AS_2 was isolated from sterilised leaves of *A. sessiliflora*, obtained from Eisleben, Botlokwa (23°31′49.5″ S 29°49′27.1″ E) in the Limpopo province, South Africa, as described by [19]. The 16S ribosomal RNA gene (GenBank accession number MZ976847) sequencing was used to initially identify the strain. Glycerol stocks (30%, *v*/*v*) of AS_2 were inoculated on nutrient agar (NA) plates and incubated at 28 °C for 24–48 h. For maintenance, strain AS_2 was sub-cultured and incubated at 28 °C continuously and preserved in 30% glycerol stocks at −80 °C for long-term storage.

2.2. Genome Extraction, Library Preparation, and Sequencing

The Zymo Research Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) was used to extract whole genomic DNA from solid colonies of strain AS_2. The Implen Nanophotometer N60 (Implen GmbH, Munich, Germany) was used to quantify the concentration of strain AS_2 DNA. The full genome sequence of strain AS_2 was completed by a commercial service provider, the Agricultural Research Council (ARC) Biotechnology platform, Onderstepoort, Pretoria, South Africa, using the HiSeq 2500 platform. Paired-end libraries (2 × 150 bp) were prepared using the Illumina HiSeq instrument v3 and the NextEra DNA library preparation kit (Illumina, San Diego, CA, USA).

2.3. Genome De Novo Assembly and Annotation

The raw sequence reads of strain AS_2 were uploaded to the Galaxy (www.usegalaxy.org) platform [20] accessed on 12 July 2023, where all pre-annotation analysis was carried out. FastQC (version 0.72.0) was used to examine the quality of the raw reads [21]. This was followed by *de novo* assembly with Unicycler version 0.4.8.0 [22], and the assembled genome was evaluated using the Quality Assessment Tool for Genome Assemblies (QUAST) version 5.0.2 [23]. The draft genome of strain AS_2 was annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) [24] and Rapid Annotations utilising Subsystems Technology [25]. The genome completeness of strain AS_2 was assessed using BUSCO (Benchmarking Universal Single-Copy Orthologs) version 5.0 [26], whereas contamination was assessed using CheckM on Kbase (https://www.kbase.us/) [27], accessed on 28 November 2023.

2.4. Phylogenome Analysis

The draft genome of strain AS_2 was assembled into contigs and uploaded to the Type Strain Genome Server (TYGS) (available at https://tygs.dsmz.se, accessed on 12 August 2023) for whole genome-based taxonomic analysis with other type strain species of the genus *Peribacillus* [28]. The Orthologous Average Nucleotide Identity (OrthoANI) software tool (version 0.93.1) [29] was used to compute the ANI values between strain AS_2 and other closely related *Peribacillus* species. Multilocus sequencing analysis (MLSA) was performed using four housekeeping genes, *gyr*A (DNA gyrase subunit A), *gyr*B (DNA topoisomerase ATP-hydrolysing), *rpo*B (DNA-directed RNA polymerase subunit beta), and *rpo*C (DNA-directed RNA polymerase subunit beta). The sequences of the four housekeeping genes were aligned using the ClustalW logarithm on MEGA X [30], after which a protein concatenated phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap repetitions. The CRISPRCas finding software (available at https://crisprcas.i2bc./CrisprCasFinder/Viewing/, accessed on 15 August 2023) was used to predict the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) [31–33]. Genomic islands (GIs) were identified and analysed using the IslandViewer 4 online database (version 4.0) at http://www.pathogenomics.sfu.ca/islandviewer/ [34], accessed on 15 August 2023. Furthermore, additional annotation was performed on strain AS_2 using the following databases: The antiSMASH (version 5.0) tool (available at https://antismash.secondarymetabolites.org, accessed on 15 August 2023) [35] and PRISM 4 (Prediction Informatics for Secondary Metabolomes) (available at https://magarveylab.ca/prism, accessed on 15 August 2023) were used to predict gene clusters for secondary metabolites. The Comprehensive Antibiotic Resistance Database (CARD), (https://arpcard.mcmaster.ca, accessed on 15 August 2023) was used to identify antibiotic resistance genes [36].

2.5. Phenotypic Characterisation

To differentiate *Peribacillus* sp. strain AS_2 from other closely related *Peribacillus* species, characteristics recommended by [37] were applied. Cells of strain AS_2 were stained with a Gram stain kit (Sigma-Aldrich, Eschenstr, Taufkirchen, Germany) according to the manufacturer's protocol. Scanning electron microscopy (TESCAN VEGA SEM) was used to examine cell morphology, while light microscopy was used for the observation of spore production. The motility of the cells was observed with light microscopy using the hanging drop method after growing the cells for 24 h. Strain AS_2 was inoculated on tryptone soy agar (TSA) and incubated at 30 °C for 48 h, after which the form, size, and color of colonies were studied. Anaerobic growth on TSA was investigated for 48 h in an anaerobic chamber at 30 °C. The optimum growth and growth range of *Peribacillus* sp. strain AS_2 were determined in a tryptone soy broth (TSB) medium at varied NaCl concentrations ranging from 0 to 15% (w/v) with 1% intervals at 30 °C for 72 h. Growth at several temperature ranges (4, 15, 20, 25, 30, 37, 40, 45, 50 and 55 °C) was studied in TSB for 72 h. The pH growth range and optimum were determined in TSB media at 30 °C for 72 h, at pH 3 to 11 with increments of 1 pH units. Catalase activity was evaluated by observing bubble formation after adding a 3% (v/v) hydrogen peroxide (H_2O_2) solution. The presence of bubbles indicated a positive result. The oxidase test reagent (Thermo Scientific, Waltham, MA, USA) was used to determine oxidase activity. Hydrolysis of starch and protein were examined on modified NA enriched with 1% skimmed milk and 1% soluble starch. Other biochemical characteristics were validated using API 20E and API 50CHB strips (bioMérieux, Midrand, South Africa) according to the manufacturer's protocol.

2.6. Statistical Analysis

All genetic studies were carried out in accordance with the manufacturer's instructions. The experiments were conducted in triplicates, and the findings were expressed as mean standard deviations. The data was analysed in Microsoft Excel version 2016, using one-way analysis of variance with post-hoc *t*-test (Bonferroni Correction), and the $p \leq$ was declared statistically significant.

3. Results and Discussion

3.1. Genome Characteristics of Peribacillus sp. Strain AS_2

A total of 2,646,598 paired-end reads at 72× coverage were obtained from this workflow. Sequencing and *de novo* assembly of *Peribacillus* sp. strain AS_2 resulted in a draft genome sequence with 43 contigs. The genome size of strain AS_2 was 5,482,853 bp with a G + C content of 40.5%. The size of the genome and G + C% content of strain AS_2 falls within the range of most reported *Peribacillus* species genomes [15]. The QUAST software was used to assess the quality of the assembly. The N_{50} value of strain AS_2 was 360,633, whereas the L_{50} value was six. The PGAP annotation identified a total of 5290 genes, of which 5072 are protein coding sequences (CDS) and 130 are pseudogenes. A total of 88 RNAs were predicted from the draft genome and 79 of these coded for tRNA genes, three coded for rRNA genes comprising three operons (5S, 16S, 23S), and six genes were non-coding RNA (ncRNA). The BUSCO analysis showed a completeness of 99.8% (445 completed and single-copy genes, and four duplicated genes). CheckM showed a genome completeness of 98.91% and a contamination of 2.21%. We identified four CRISPR repeats and one Cas cluster in strain AS_2 (Table S1). The genomic characteristics of strain AS_2 are summarised in Table 1. The draft genome sequence of strain AS_2 was deposited in the GenBank/EMBL/DDBJ database under the accession number JAPTGA000000000.

Table 1. Genome characteristics of *Peribacillus* sp. strain AS_2.

Genome Characteristic	Value
Genome size (bp)	5,482,853
G + C content (%)	40.5
Total number of genes	5290
Protein coding genes	5072
Number of RNAs	88
rRNA genes	1, 1, 1 (5S, 16S, 23S)
tRNA genes	79
ncRNAs	6
Pseudogenes	130
CRISPR repeats	4
Cas cluster	1

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; rRNA: ribosomal RNA, tRNA: transfer RNA.

3.2. Functional Annotation

The TYGS (https://tygs.dsmz, accessed on 12 August 2023), a bioinformatics platform which is used for whole genome taxonomy analysis [28], was used for phylogenomic classification of strain AS_2 with closely related *Peribacillus* species. Furthermore, the OrthoANI tool was used to calculate the ANI value of *Peribacillus* AS_2 with other closely related species [29]. Strain AS_2 was found to be closely related to *P. frigoritolerans* DSM 8801^T with a dDDH value of 84.8% and a 0.13% difference in G + C% content, and to *P. castrilensis* N3^T with a dDDH value of 79.2% and a difference of 0.17 G + C% content (Table S2). The dDDH values of both *Peribacillus* species exceeded the dDDH > 70% threshold recognised as the cut-off point for species delineation [38,39]. The ANI values of strain AS_2 was 97.0% with *P. frigoritolerans* DSM 8801^T and 96.7% with *P. castrilensis* N3^T, as shown in Figure 1. Both ANI values were above the species boundary value (ANI, >95–96%) used to distinguish species [40,41], confirming that strain AS_2 is a *Peribacillus* species. Surprisingly, DSM 8801^T and N3^T had an ANI value of 96.7%, which was congruent with the dDDH value of 75.1% (results not shown), because the latter was described by [15]

later than strain DSM 8801^T [42], which was reclassified by [43,44]. We thus recommend reclassification of *P. castrilensis* N3^T's position within the *Peribacillus* genus.

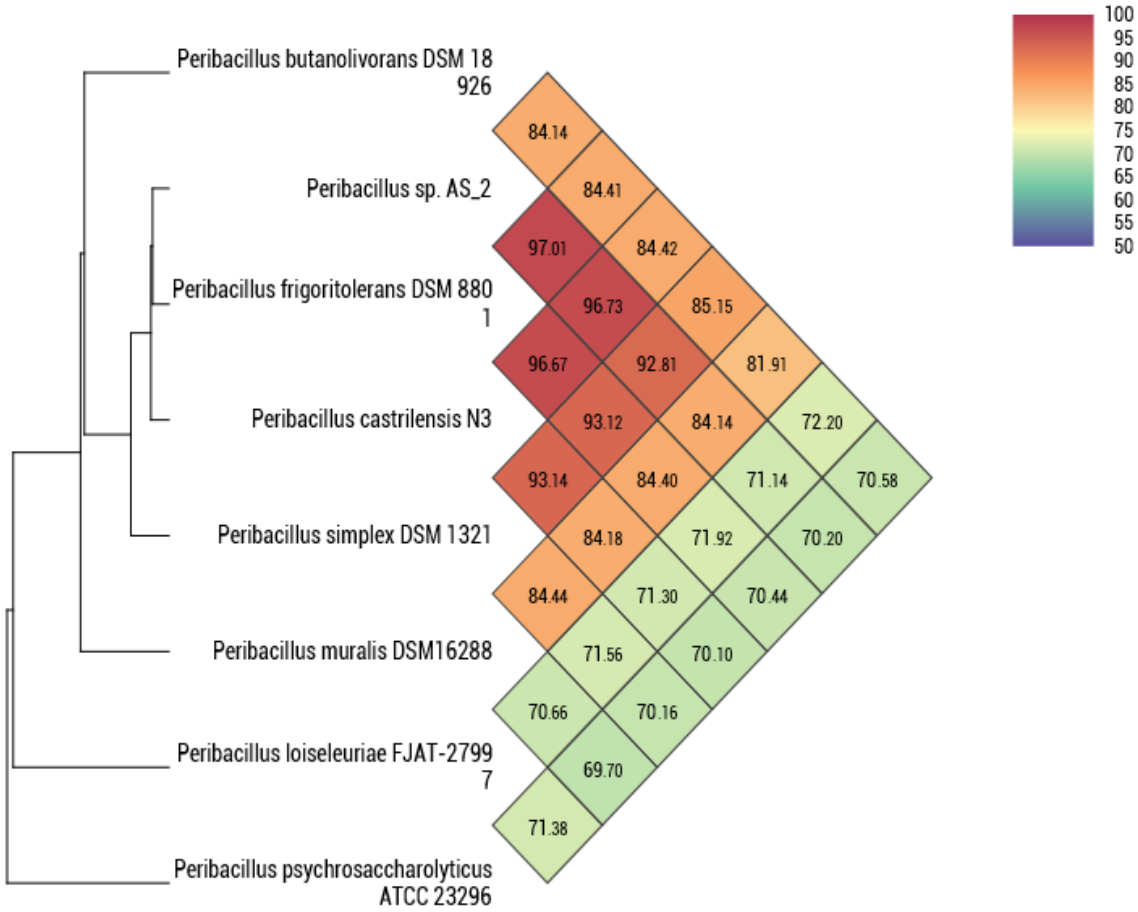

Figure 1. Heatmap generated with OAT software (version 0.93.1) showing the OrthoANI values of *Peribacillus* sp. strain AS_2 with other closely related *Peribacillus* species.

The RAST server was used to further annotate the functional categories of genes encoded by strain AS_2 using default parameters [25]. A broad spectrum of housekeeping genes is often required for an endophytic lifestyle for bacterial growth and reproduction, including the uptake and transport of nutrients [44]. The RAST server predicted functional subsystems (Figure 2) which comprised genes involved in the metabolism of carbohydrates, amino acids and derivatives, fatty acids, lipids, co-factors, and vitamins of which the majority are important in the formation of endophytic relationships. Overall, the functional subsystems identified in the draft genome of strain AS_2 may also pave the way for further exploration in biotechnological applications, including genetic engineering and converting aromatic compounds to be used in bioremediation [45].

Acquisition of GIs in bacterial endophytes plays an important role, as they code for multiple accessory genes and may augment the diversification and adaption of a bacterium to its host or environment [46]. The GI Viewer tool was used to identify GIs present in strain AS_2, in which several sets of genomic regions revealed evidence of horizontal gene transfer. A total of 16 (Figure 3) GIs were identified in the strain AS_2 genome when aligned to *P. butanolivorans* strain KJ40, which was used as a reference genome. The 16 GIs comprised genes encoding penicillin-binding transpeptidase, ornithine cyclodeaminase family protein, glycosyltransferase, phage major capsid protein, cupin, and putative quinol monooxygenase. Cupin has been found to be present in a variety of endophytes and is known to play a role in the modification of plant cell wall polysaccharides [47].

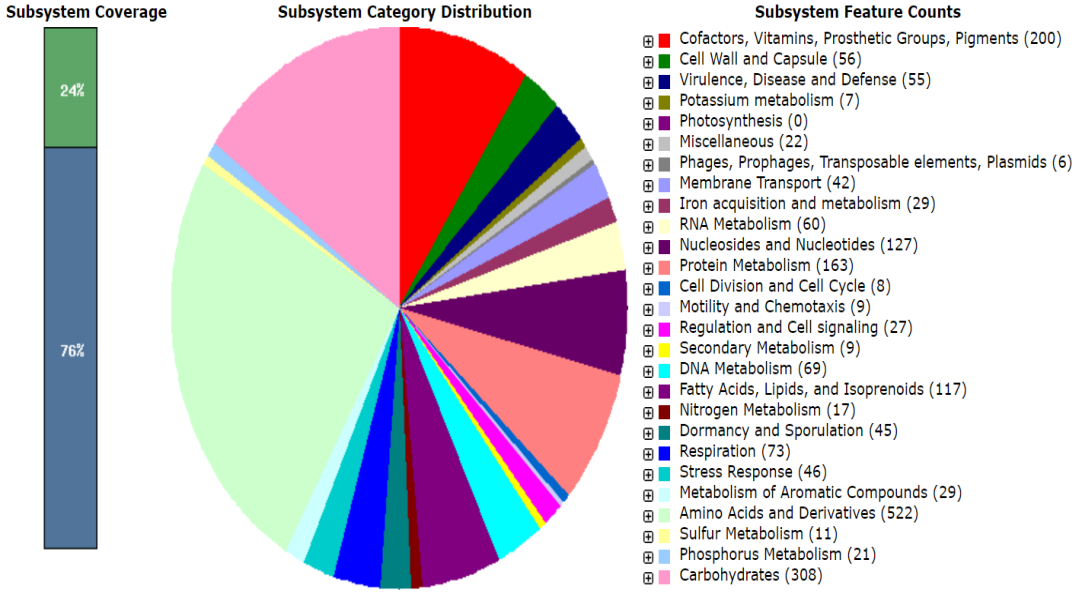

Figure 2. Pie chart showing an overview of RAST subsystems assigned to genes in *Peribacillus* AS_2 genome. Each subsystem (**left**) is represented by a particular colour (**right**).

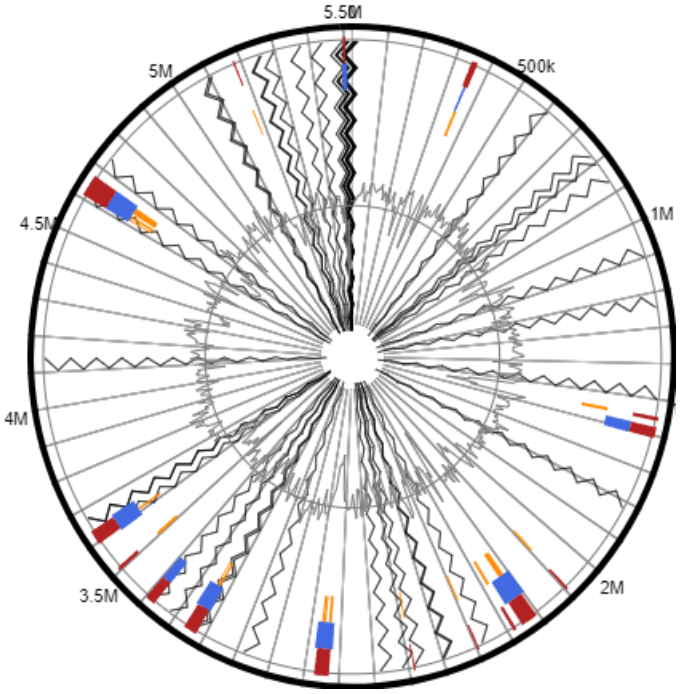

Figure 3. Circular plot of genomic islands identified from the *Peribacillus* sp. strain AS_2 genome aligned against the reference genome *Peribacillus butanolivorans* strain KJ40 using IslandViewer 4. The black outer circle represents the scale line of the genome in Mbps, and the identified genomic islands are represented in three colours: red (integrated detection), blue (IslandPath-DIMOB), and orange (SIGI-HMM).

In our previous study, *Peribacillus* sp. AS_2 was isolated and 16S rRNA gene sequencing revealed a polytomy relationship with *P. simplex* NBRC 15720^{T} and *P. muralis* DSM 16288^{T}, supported by an 83% bootstrap value [19]. The 16S rRNA is primarily utilised for the initial identification of bacterial species, although it cannot distinguish closely related species [48], necessitating further identification using other methods such as multilocus sequence analysis (MLSA). To investigate the genetic diversity of strain AS_2 at the species

level with closely related *Peribacillus*-type strains, multilocus sequencing analysis was used. Multilocus sequencing analysis plays a key role in the phylogenetic classification of prokaryotic taxonomy, as it can obtain a higher resolution of the phylogenetic relationships between species at the species level [49,50]. Four housekeeping genes *gyr*A-*gyr*B-*rpo*C-*rpo*B were used for phylogenetic analysis and based on the concatenated phylogenetic tree, strain AS_2 clustered with *P. frigoritolerans* DSM 8801^T and *P. castrilensis* N3^T, supported by a 100% bootstrap value (Figure 4). This was congruent with dDDH and ANI value results. The results based on the MLSA, ANI and dDDH suggest that *Peribacillus* sp. AS_2 is a subspecies of *P. frigoritolerans* DSM 8801^T.

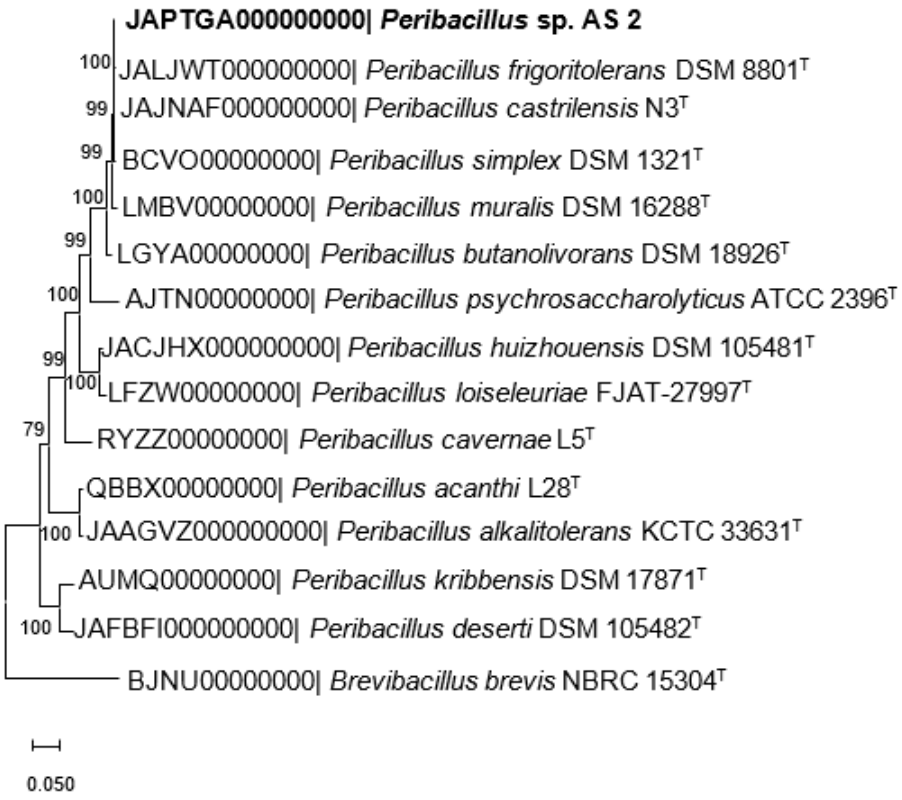

Figure 4. Phylogenetic analysis of strain AS_2 and closely related species of the *Peribacillus* group based on MLSA of four housekeeping genes, *gyr*A-*gyr*B-*rpo*B-*rpo*C. *Brevibacillus brevis* NBRC 15304^T was used as an outgroup.

3.2.1. *In Silico* Analysis of Biosynthetic Gene Clusters

Bioactive compounds extracted from endophytic bacteria have gained attention due to their benefits to plants and humans in various sectors including agriculture, food, and pharmaceuticals [51]. Secondary metabolites in bacterial endophytes are mainly produced by biosynthetic gene clusters, which code for vital enzymes such as non-ribosomal peptide synthetases (NRPs), polyketide synthases (PKSs), transporters, and genes which code for tailoring enzymes [52,53]. The genome of the bacterial strain AS_2 was analysed using the antiSMASH 5.0 and PRISM online databases to explore the biosynthetic gene clusters of secondary metabolites. The antiSMASH database predicted a total of eight biosynthetic gene clusters in the genome, comprising NRPS-type koranimine, two terpenes, one siderophore cluster, one type III polyketide synthase (T3PKS), one linear azol(in)e-containing peptide (LAPS), and beta-lactone-type fengycin, whereas the PRISM database predicted three clusters which included two non-ribosomal peptides and NRPS-independent siderophore synthase gene clusters (Table 2). When comparing the predicted gene clusters, the koranimine-encoded gene cluster had a similarity percentage of 87% to *Bacillus* sp., whereas the Schizokinen and fengycin-encoded gene clusters had a similarity

percentage of 60 (*Nostoc* sp.) and 46% (*Bacillus velezensis*), respectively. However, no similarity was detected in the other biosynthetic gene clusters (Table 2), terpene, TSPKS, NSPS, and LAPs, suggesting that some secondary metabolites may be uniquely produced by strain AS_2. Similar biosynthetic gene clusters were also identified in the *P. frigoritolerans* strain Q2H1, a bacterial endophyte in potato plants with a growth-promoting mechanism [54]. Koranimine, a biosynthetic gene cluster isolated from the bacterial endophyte *P. frigoritolerans* EB93, was discovered to have nematicidal action against *Bursaphelenchus xylophilus*, a common pine wilt nematode [55].

Table 2. Biosynthetic gene clusters involved in the production of secondary metabolites in *Peribacillus* sp. strain AS_2.

From (bp)	To (bp)	Type	Production	Similarity (%)	Reference
236,408	296,726	NRPs	Koranimine	87	*Bacillus* sp. NK2003 [56]
140,936	165,105	Beta-lactone	Fengycin	46	*Bacillus velezensis* FZB42 [57]
50,380	65,892	NI-siderophore	Schizokinen	60	*Nostoc* sp. PCC 7120 [58]
380,256	402,151	Terpene	-	-	
389,596	430,684	T3PKS	-	-	
87,936	132,003	NRPS	-	-	
8889	32,424	LAP	-	-	
7431	28,249	Terpene	-	-	

3.2.2. Antibiotic Resistance Genes

Antimicrobial resistance is still a major public concern, topping the World Health Organization's (WHO) list of health concerns [59,60]. Recent studies have demonstrated that antimicrobial resistance genes (ARGs) are frequently acquired via horizontal gene transfer (HGT) using mobile genetic elements, resulting in the establishment and evolution of antimicrobial resistance bacteria [61,62]. Bacteria use HGT to transfer antibiotic resistance genes on plasmids using three primary mechanisms, which are conjugation, transduction, and natural transformation, and which occur in any environment including but not limited to the gut microbiome of humans and animals, soil, and water [63,64]. The transfer of plasmids between pathogenic microorganisms has resulted in a wide distribution of AGRs encoding resistance to a variety of drug classes including beta-lactams, tetracyclines, and quinolones [65]. A study by [66] revealed that the HGT of a plasmid encoded ARG was responsible for the outbreak of *Shigella* in the United Kingdom. The broad dissemination of ARGs in different host species and environments indicates that these genes can function well immediately after HGT [67]. Antibiotic resistance genes have been linked to the methylation of 16S rRNA [68], ribosomal proteins [69], lysyl-phosphatidylglycerols [70], and the encoding of the beta-subunit of RNA polymerase [71].

The genome of AS_2 was mapped onto the CARD database [72], which revealed gene clusters involved in small multidrug resistance antibiotic efflux pumps (*qac*Js), glycopeptide resistance (*van*Y, *van*W, *van*T, *van*R), and fosfomycin thiol transferase (*fos*Bx1). A class A *Bacillus cereus* Bc beta-lactamase gene cluster was also discovered, suggesting that the *Peribacillus* sp. strain AS_2 may be resistant to cephalosporin and penem (*Bc*III). Penem compounds are antimicrobials that are effective against a wide range of Gram-positive and Gram-negative bacteria [73].

3.2.3. Genes Involved in Endophytic Lifestyle

To better understand the factors that influence the *Peribacillus* sp. strain AS_2's endophytic colonisation and lifestyle, we identified putative genes involved in the bacterial endophytic lifestyle (Table S3). Genes putatively involved in membrane transport proteins, detoxification, adhesion, substrate utilisation, secretion and delivery systems, transport,

and transcriptional regulators were identified in AS_2. In addition, motility and chemotaxis are well known mechanisms which bacteria use to colonise their host plants [74]; consistent with this, two chemotactic signal-transducing proteins (CheC and CheD) were also identified.

Transcriptional regulators play an important role in the biological functions of bacterial endophytes, such as responding to environmental changes and colonising their host plants [75,76]. The TetR/AcrR family transcriptional regulators are known to influence a wide range of cell functions, including multidrug resistance, virulence, antibiotic production, osmotic stress, and bacterial pathogenicity [77]. In addition, the PadR transcriptional regulators are known to function as environmental sensors and assist in detoxifying harmful phenolic compounds [78]. Similarly, LysR family proteins were identified in the genome of strain AS_2, and are known to regulate the expression of genes mainly involved in the expression of motility, metabolism, and quorum sensing [79]. These have similarly been identified in other bacterial endophytes with plant-promoting, activities such as *Burkholderia phytofirmans* PsJN [47] and *Bacillus paranthracis* strain MHSD3 [80].

Evolutionary conserved antibiotic and virulence factors acquired by natural mutation and/or HGT may have the potential to serve as therapeutic new drug targets or vaccine antigens [81,82]. Lòpez-Fernàndez and colleagues hypothesised in 2015 that bacterial endophytes have the potential to express and share the same virulence-related genes as pathogens at the genomic level [83]. One example is the analysis of the Gram-negative colonising genome of *B. phytofirmans* PsJN, in which different mechanisms including siderophores, protein-secreting enzymes and polymer-degrading enzymes were identified, which are the same colonisation mechanisms that pathogens use to infect plants [84].

We discovered genes that encode the YihY/virulence factor BrkB family protein, a new virulence factor discovered in *Bordetella pertussis* that is relatively resistant to complement-dependent death via normal human serum [85]. The antibiotic biosynthesis monooxygenases were also identified, which are involved in the biosynthetic pathways for polyketide antibiotics, including tetracenomycin [86], alnumycin [87], and daunomycin [88]. The class D beta-lactamase antibiotic factors were also identified in strain AS_2, which are a group of antimicrobial agents that are mainly used to treat infections caused by bacterial pathogens [89]. Three genes encoded for a type III secretion system (*EscU,YscU,HrcU*) were also identified in the strain AS_2 genome, which is commonly found in Gram-negative pathogens including *Pseudomonas*, *Escherichia coli*, *Shigella* and *Salmonella* [90,91]. Although strain AS_2 was isolated as an endophyte, summing up these observations, strain AS_2 may have utilised these virulence genes to achieve colonisation in *A. sessiliflora* using the same mechanisms as pathogens.

3.2.4. Genes Involved in Biodegradation

Genomic studies have demonstrated that bacteria belonging to the genera *Alcaligenes, Burkholderia, Mycobacteria, Rhodococcus* and *Pseudomonas* [92] are capable of degrading organic contaminants such as aliphatic and aromatic compounds and converting them into either carbon dioxide, water, or biomass, which is essential for their growth [93]. In addition, synthetic chemicals used in pesticides, dyes, solvents, and refrigerators are often persistent in nature and hence are considered hazardous to the environment and humans, as they are toxic and insoluble and are often accumulated in high concentrations [94,95]. The biotransformation of these organic compounds can be further exploited for the treatment of contaminated water and soils through DNA recombinant techniques by constructing strains with an enhanced ability to degrade several toxic compounds.

Genes involved in the degradation of nitroalkanes (nitromethane, nitroethane, nitropropane)—-which are a group of toxic compounds used in the chemical industries as intermediates to develop pharmaceutical compounds, insecticides, and dyes—-were identified in the genome of strain AS_2 [96]. These nitroalkanes have been reported to be hazardous as they are toxic and carcinogenic [97]. Genes that code for the degradation of nitroalkanes, known as nitronate monooxygenases, were identified in strain AS_2, indicating

its ability to catabolise toxic nitroalkane compounds. Nitronate monooxygenases have also been identified in other bacterial species, including *Streptomyces ansochromogenes* [98]. We further identified *ssu*D and *tau*D genes, which are mainly responsible for the degradation of organosulphonates such n-hexadecane, as the removal of this by-product is important for bacterial survival during oxidative stress in sulphur-limited conditions [99].

Pollution caused by organic contaminants coupled with heavy metals produced by industrial activities such as mining is a matter of great concern due to their toxic and carcinogenic effects on the environment and their position at top of the list of hazardous waste [100]. Research studies have demonstrated that genes involved in heavy metal resistance are often found in bacterial plasmids [101]. Several genes involved in heavy metal resistance were identified in the genome of strain AS_2, such as genes responsible for copper resistance, the CopC/CopD family protein, the heavy metal translocating P-type ATPase, and a magnesium transporter. In a similar study by [102], the bacterium *Sphingobium yanoikuye* strain SJTF8 was shown to be resistant to heavy metals such as copper, cadmium, and zinc and further showed degrading capabilities when co-cultured with these heavy metals with polycyclic aromatic hydrocarbons (PAHs), which are a group of hydrophobic compounds known for their toxic, carcinogenic, and mutagenic effects [103]. Other key enzymes required for initiating the degradation-like process, such as dioxygenases, have also been identified in the genome of AS_2, expanding the potential of this strain for bioremediation processes.

3.3. Phenotypic Characterisation

The strain AS_2 was a Gram-positive, spore-forming, and motile bacterium. Colonies of strain AS_2 on TSA plates were creamy, white, and circular with regular margins of 5–6 mm in diameter following incubation at 30 °C for 48 h. The morphology of strain AS_2 was observed using the SEM (Figure 5).

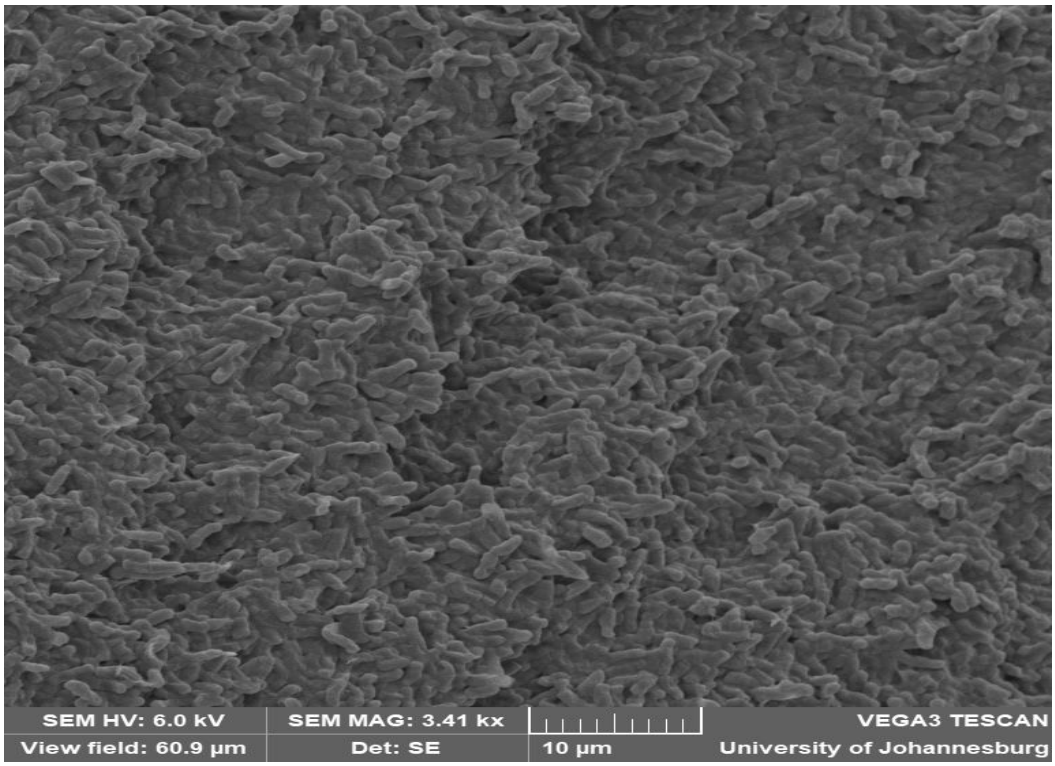

Figure 5. Scanning electron micrograph of *Peribacillus* sp. strain AS_2 cells grown for 24 h at 30 °C.

Peribacillus sp. strain AS_2 was catalase and oxidase positive, with growth temperatures ranging from 15 to 50 °C and a 30 °C optimum temperature. The cells of strain AS_2 were able to grow in NaCl concentrations ranging from 1 to 7% (*w*/*v*); the optimum

was at 1% (*w*/*v*), demonstrating that it is a halotolerant bacterium [104]. The pH range in which strain AS_2 grew was 4–12, with an optimal pH of 7. Table 3 displays the phenotypic characteristics of strain AS_2 compared to closely related type strain species, *P. frigoritolerans* DSM 8801^T, P. *simplex* DSM 1321^T, *P. muralis* DSM 16288^T, *P. butanolivorans* DSM 18926^T, and *P. loiseleuriae* DSM 101776^T. Strain AS_2 differs from some of the type strain species in the following characteristics: the ability to grow in anaerobic conditions and at 50 °C, the hydrolysis of casein, and being positive for oxidase and Voges–Proskauer. Strain AS_2 tested positive for eleven sugars in API 50CH, while some of the type strain species could not produce acids from the sugars tested. In contrast, AS_2 grew in a wider range of pH (4–12) and NaCl (1–7%) and it tested negative for hydrolysis of gelatine, arginine dihydrolase, indole production, and tryptophan deaminase.

Table 3. Differential characteristics between the *Peribacillus* sp. strain AS_2 and closely related type strains.

Characteristic	1	2$^+$	3$^+$	4$^+$	5$^+$
Anaerobic growth	+	-	v	w	-
Growth at 4 °C	-	+	-	-	+
Growth at 45 °C	+	-	-	-	+
NaCl (*w*/*v*, %)	0–7	0.5–7.5	<5	<7	0.5–5
Optimum NaCl (*w*/*v*, %)	1	0	0	0	1
pH range	3–12	5–10	6–9	6–9	6–9
Optimum pH	7	7	8	7	7
Oxidase	+	-	-	+	+
API 20E:					
ONPG	-	-	-	+	-
Arginine dihydrolase	-	-	-	-	-
Citrate utilisation	-	-	-	-	-
Voges– Proskauer	+	-	-	-	-
Urease	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-
Indole production	-	-	-	-	-
Gelatine hydrolysis	-	-	-	-	-
API 50:					
L-arabinose	+	+	-	+	-
Ribose	+	-	-	+	-
Glucose	+	+	w	+	-
Fructose	+	+	w	+	-
Mannitol	+	-	-	+	-
N-acetylglucosamine	+	-	w	+	w
Salicin	+	-	*w*/*v*	+	*w*/*v*
Saccharose	+	-	-	-	-
Trehalose	+	-	w	+	w
Inulin	+	-	w	-	w
Raffinose	+	+	-	+	-
Hydrolysis of:					
Starch	-	-	+	+	-
Casein	+	+	v	v	-
DNA G + C content (mol %)	40.45	40.6	39.9	41.2	37.4

Strains: 1, *Peribacillus* sp. AS_2; 2, *Peribacillus frigoritolerans* DSM 8801^T [40]; 3, *P. simplex* DSM 1321^T [105]; 4, *P. muralis* DSM 16288^T [105]; 5, *P. butanolivorans* DSM 18926^T [106]. +, positive; -, negative; w, weak; v, variable; *w*/*v*, weak and variable.

4. Conclusions

The phenotypic and genomic analyses showed that strain AS_2, isolated from the medicinal plant *Alectra sessliflora*, belongs to the *Peribacillu*s genus and is a subspecies of

P. frigoritolerans DSM 8801^T. Further studies are currently underway to describe it as a subspecies of *P. frigoritolerans* DSM 8801^T. Genes involved in endophytic lifestyle were identified along with biosynthetic gene clusters. The genomic analysis has provided an initial step towards fully understanding the characteristics of AS_2 and its potential for broader application in the biotechnology sector. Furthermore, comparisons with other closely related *Peribacillus*-sequenced genomes will aid in delineating the distinct and shared characteristics of different *Peribacillus* species, shedding light on the evolutionary changes that may have occurred.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15010004/s1, Table S1: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences present within *Peribacillus* sp. strain AS_2 identified using CRISPRCasFinder; Table S2: Pairwise dDDH and ANI values between *Peribacillus* sp. AS_2 and the *Peribacillus* group strains; Table S3: Putative genes of *Peribacillus* sp. strain AS_2 involved in endophytic lifestyle; Table S4: Genes involved in biodegradation.

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