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Identification of Conserved Pathways in *Bacillus* Strains Known for Plant Growth-Promoting Behavior Using a Multifaceted Computational Approach

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Abstract: *Bacillus* strains have long been recognized for their beneficial interactions with plants, enhancing growth, nutrient uptake, and stress resistance. Understanding their molecular mechanisms and plant-microbe interactions is crucial for harnessing their potential in sustainable agriculture. Here we used ten strains from the 5 *Bacillus* species namely *Bacillus velezensis*, *Bacillus subtilis*, *Bacillus atrophaeus*, *Bacillus altitudinis* and *Bacillus amylofaciens*, which are previously reported for PGPR activity. A comparative analysis of these strains was performed to determine their evolutionary relationships, which revealed that *Bacillus velezensis* and *Bacillus amyloliquefaciens* are closely related based on underlying genetic and proteomic similarities. *Bacillus altitudinis* strain LZP02 was the most distantly related to all the other selected strains. On the other hand, *Bacillus atrophaeus* strains GQJK17 and CNY01 are shown to be closely related to each other. Mauve alignment was performed to determine the genetic relationships between these strains. The LZP02 strain exhibited several unique inversions harboring important genes, such as betB, ftsW, and rodA, which are important for bacterial survival. Proteomic analysis highlighted important pathways that were conserved across these strains, including xenobiotic biodegradation and metabolism, biosynthesis of polyketides and nonribosomal pathways, and biosynthesis of secondary metabolites, all of which have been shown to be involved in plant growth promotion.

Keywords: plant growth promotion (PGP); evolutionary relationship; comparative genomic analysis; comparative proteomic analysis; gene inversion; pathway class

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1. Introduction

Plant growth-promoting (PGPR) bacteria are bacteria that reside in the rhizosphere, a narrow soil region influenced by root exudates [1]. They play a crucial role in enhancing plant growth. Due to their notable efficacy in stimulating plant growth and controlling diseases, PGPR are considered environmentally friendly alternatives to chemical fertilizers and pesticides. Various bacterial species, including *Bacillus*, *Burkholderia*, *Azospirillum*, *Azotobacter*, *Rhizobium*, and *Pseudomonas*, have been identified as PGPR, with *Bacillus*, *Rhizobium*, and *Pseudomonas* being the most prominent [2–9]. PGPR has been applied to a diverse range of plants, including chickpeas [10], maize [11], peas [12], peanuts [13], rice [14], soybeans [15], sugarcane, wheat [16], and sugarbeets [17]. Their positive impact on plant growth makes them valuable allies in sustainable agriculture practices.

In recent years, there has been increasing interest in harnessing the potential of plant growth-promoting rhizobacteria (PGPR) as sustainable and eco-friendly alternatives to enhance crop productivity. PGPRs have emerged as key players in this regard due to their multifaceted abilities to promote plant growth, confer stress tolerance, and mitigate the impact of various plant pathogens [18]. Among the myriad of mechanisms employed by PGPRs, the synthesis and secretion of auxins have been recognized as pivotal contributors

to enhanced plant development and nutrient uptake. Auxins, primarily indole-3-acetic acid (IAA), play a crucial role in regulating diverse physiological processes in plants, including cell elongation, root development, and stress responses [19].

Bacillus, belonging to the phylum Firmicutes, comprises gram-positive, rod-shaped, endospore-forming bacteria. *Bacillus* strains are known to produce spores, enabling them to survive even under harsh conditions, and they can release antimicrobial compounds that increase their chances of survival under biologically diverse conditions [20]. In addition, these bacteria can utilize a myriad of nutritional sources [21], further enabling them to survive in ecologically diverse habitats. Thus, these *Bacillus* strains are favored for inoculant preparation due to their extended viability, which supports the creation of long-lasting commercial products.

The strains selected for this study included *Bacillus altitudinis* LZP 02, *Bacillus amyloliquefaciens* subsp. *Plantarum* UCMB5036, *Bacillus atrophaeus* CNY01, GQJK17, *Bacillus* sp. C01-6, *Bacillus subtilis* SX01705, MBI 600, *Bacillus velezensis* S141, and sx01604, which have been reported to support PGPR activity. Previously published studies have tried to look at individual strains and underlying factors playing a significant role in bacterium's PGPR phenotype. One such study by Weihui Xu et al. [14] looked at the complete genome sequence of *B. altitudinis* LZP02 and found it to be a valuable resource for determining its ability to promote the growth of rice roots.

Similarly, it was previously reported that the UCMB5113 strain inhibits the proliferation of various fungal pathogens on oilseed rape, including *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans*, and *Verticillium longisporum* [22]. Another separate study concluded it has the ability to enhance the development of both subterranean and aerial tissues in various plants [23]. The *B. atrophaeus* has also been extensively investigated and recognized as a valuable group of bacteria. This is a plant growth-promoting rhizobacterium (PGPR) that significantly suppresses certain soil-borne diseases and enhances the growth of specific plants [7]. Studies have also confirmed its ability to be a prolific producer of known important biomolecules [24]. Morphological observations and phylogenetic analysis revealed a close relationship between *B. atrophaeus* GQJK17 and *B. atrophaeus* CPB072.

The genomic sequence of *Bacillus amyloliquefaciens* strain Co1-6 was identified as a plant growth-promoting rhizobacterium (PGPR) with extensive antagonistic efficacy against a range of plant-pathogenic fungi, bacteria, and nematodes [25]. The *B. subtilis* strains SRCM103689 and SX01705 stand out for their potential to enhance nutritional quality [26]. *Bacillus* spp. MBI 600, identified as a gram-positive bacterium, is a plant growth-promoting rhizobacterium (PGPR) with the ability to enhance plant growth [27]. *B. velezensis* S141 has also been suggested to play pivotal roles in promoting plant growth [15].

We investigated the plant growth-promoting phenotype of the aforementioned ten *Bacillus* strains using a holistic approach utilizing evolutionary relatedness, genetic data to understand conserved and unique genomic features, and proteomic data to understand conserved pathways and to identify key attributes responsible for the positive impact on plant growth rendered by these bacterial cohorts.

2. Materials and Methods

2.1. Genome Retrieval

For this work, the genomes were retrieved from the database of the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) [28,29]. The various bioinformatic tools developed by BV-BRC and used for this work are listed in Figure 1. We also used HeatMapper, which is a web server that generates the HeatMap [30].

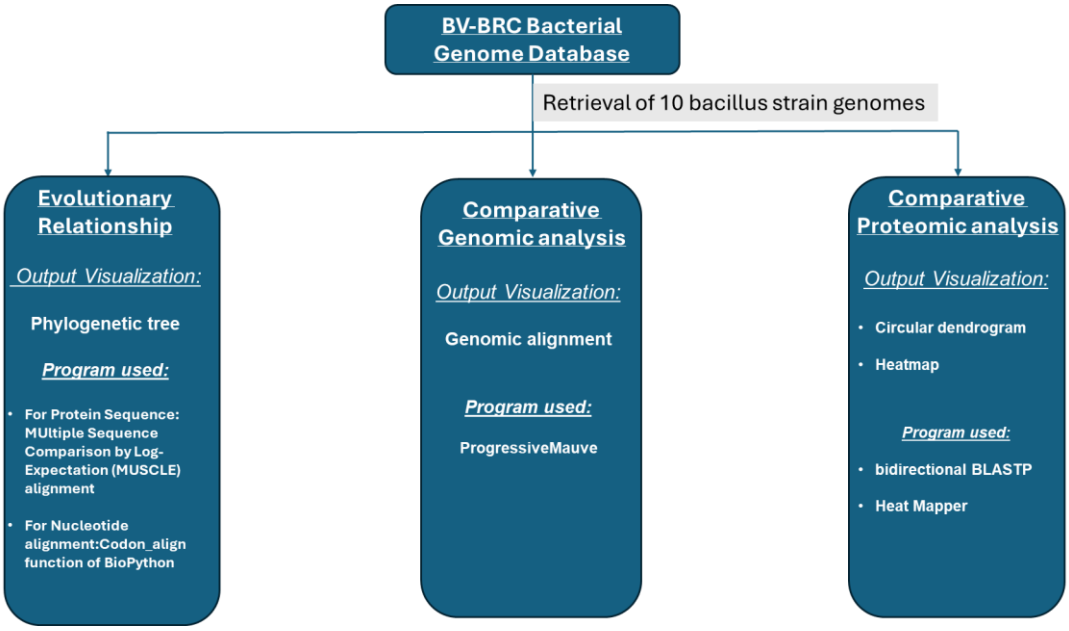

Figure 1. Depiction of the methodology and tools used in this study.

2.2. Evaluation Using a Phylogenetic Tree

Bacterial phylogenetic tree construction is a powerful technique used in microbiology to uncover evolutionary relationships between different bacterial species. This approach allows researchers to understand the diversity, connections, and evolutionary history of bacteria. The process used to generate the phylogenetic tree was as follows. Steps: 1. The protein sequences were aligned using MUSCLE Version 3.5 (Multiple Sequence Comparison by Log-Expectation) software [31], and step 2 involved aligning the nucleotide coding gene sequences using the BioPython Codon_align function [32]. Step 3 was to create a combined alignment of all proteins and nucleotides, which were subsequently formatted into a PHYLIP file [33]. Step 4: A partition file was generated, specifying the different data partitions (proteins and nucleotides) used in the analysis.

2.3. Comparative Genomic Analysis

ProgressiveMauve Version 2.3.1 [34] is a software application developed to align and visualize multiple genomes. Widely employed in comparative genomics, it serves to pinpoint both conserved and variable regions within related genomes. Embracing a progressive methodology, the tool systematically aligns sequences, proving especially valuable when confronted with sizable and intricate genomes. The regions of interest, such as conserved regions or the regions undergoing gene inversions, present in the genomes of the various strains analyzed here can be viewed using contig information. The contigs are labeled with numbers and have annotated lengths that can be used to visualize the genomic regions using the NCBI database, where in-depth genomic analysis can be performed to understand the different proteins (annotated and unannotated) that are harbored in the specific genomic regions.

2.4. Comparative Proteomic Analysis

To perform a comparative analysis involving the entire set of proteins from all ten bacterial strains, the BLASTP program [35,36] was used to find the protein similarity. For BLASTP analysis, a minimum coverage of 30%, a minimum identity of 10% and a BLAST E-value of 1×10^{-5} were used to generate hits that classified each gene into three categories: (1) unique, (2) unidirectional, and (3) bidirectional hits upon comparative analysis with the reference. Based on these three categories, the hits are colored, and a circular dendrogram is generated [37]. We further manually examined different protein categories, and any

protein that was missing from any strain was excluded. The resulting proteins were then visualized using HeatMap generated by HeatMapper [30].

We further analysed the different protein pathways that were classified based on their specific biological functions [36,38]. The analysis was performed using protein families that were generated using a previously published approach called PATtyFams [39]. This approach utilizes k-mers for functional assignment and family formation via RAST [36].

3. Results

3.1. Phylogenetic Tree Showing the Evolutionary Relationships between Selected Bacillus Strains with a Positive Impact on Plant Growth

To establish the evolutionary relationships between selected strains of *Bacillus*, we employed a previously published bacterial phylogenetic tree-based strategy [38]. This methodology helps in understanding the diversity, relatedness, and evolutionary history of bacterial strains.

The tree in Figure 2 is rooted with *Bacillus altitudinis* strain LZP 02. This means that this strain is the most distantly related to all the other strains in the tree. As the tree moves, the branches indicate how closely related the different strains are. The numbers along the branches represent the percentage of similarity between the 16S rRNA gene sequences of the two strains that are connected by that branch. For example, 100 bp between *Bacillus velezensis* strains AK-04 and S141 means that their 16S rRNA gene sequences are 100% identical.

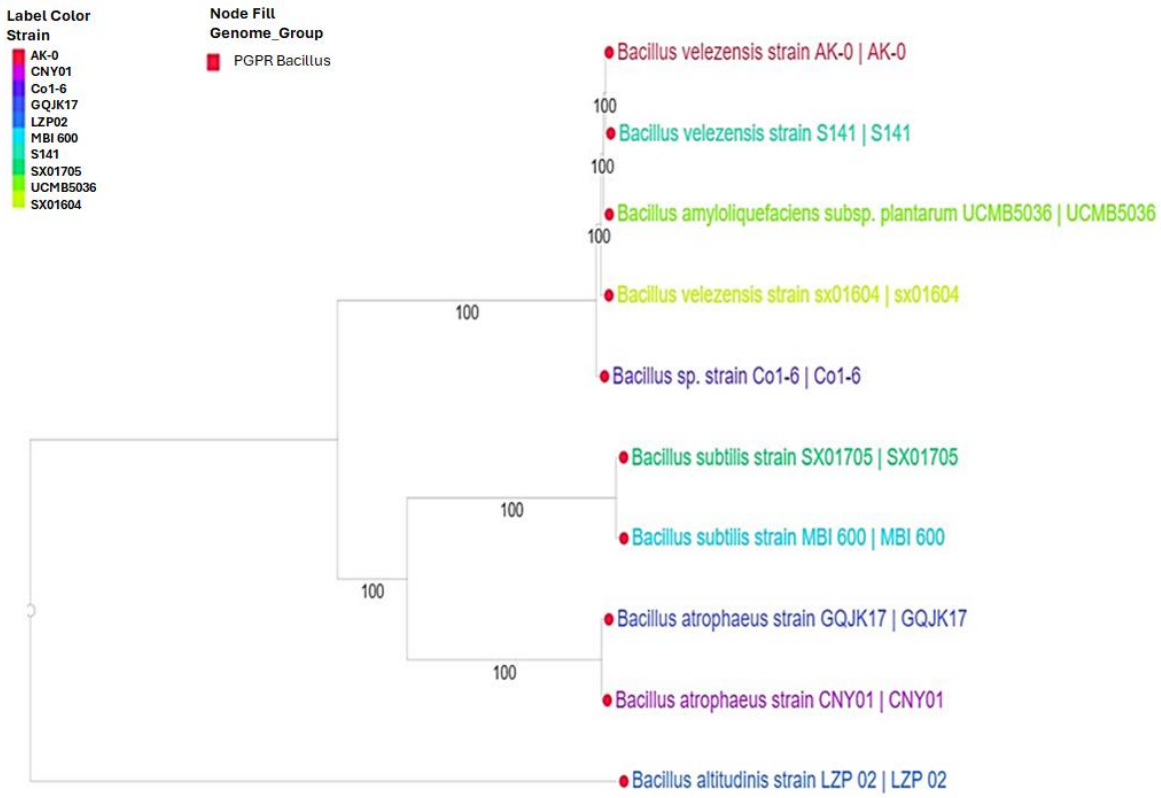

Figure 2. The phylogenetic tree shows the evolutionary relationships between ten strains of *Bacillus* spp. that are known for their PGPR activity.

The following are the main groups in the phylogenetic tree:

- *Bacillus altitudinis* group: This group consists of only *Bacillus altitudinis* strain LZP 02. As mentioned earlier, this strain is the most distantly related to all other strains in the tree.

- *Bacillus atrophaeus* group: This group consisted of *Bacillus atrophaeus* strains GQJK17 and CNY01. These two strains are more closely related to each other than any other strains in the tree.
- *Bacillus velezensis/Bacillus subtilis* group: This group is the largest and most diverse group in the tree. These strains included *Bacillus velezensis* strains sx01604, S141, and AK-04, *Bacillus subtilis* strains SX01705 and MBI 600, and *Bacillus sp.* strain Co1-6. The *Bacillus velezensis* and *Bacillus subtilis* strains are the most closely related strains in this group, while *Bacillus* sp. strain Co1-6 is more closely related to them than to the *Bacillus atrophaeus* group.

3.2. Comparative Genomic Analysis of the Ten Chosen Bacillus Strains

To investigate the genetic relationships, including conserved genetic regions and regions displaying variations, we compared the genomes of the ten selected *Bacillus* strains (Table 1). The genome sizes ranged from 3.9 M to 4.3 M across the ten chosen bacillus strains. Additionally, the number of encoded proteins varied among the strains. Interestingly, strains from *Bacillus velezensis* had the highest GC content among the chosen strains. The only strain with plasmids was *B. subtilis SX01705*, which had two plasmids. The range of RNA across the 10 genomes was from 12 to 30, and the range of TRNA was from 81 to 89.

Table 1. The different genomic features of the ten strains of *Bacillus*.

Species	Strain	Plasmids	Contigs	Size	GC Content	Contig L50	Contig N50	TRNA	RRNA	CDS
Bacillus amyloliq-uefaciens	UCMB5036		1	3,910,324	46.6	1	3,910,324	89	30	3914
Bacillus sp.	Co1-6		1	3,922,431	46.6	1	3,922,431	86	27	3997
Bacillus subtilis	SX01705	2	3	4,169,021	43.7	1	4,072,531	86	30	4365
Bacillus subtilis	MBI 600		1	4,076,736	43.8	1	4,076,736	86	20	4271
Bacillus atrophaeus	GQJK17		1	4,325,818	43.3	1	4,325,818	84	12	4507
Bacillus atrophaeus	CNY01		1	4,144,521	43.5	1	4,144,521	82	24	4332
Bacillus altitudinis	LZP 02		1	3,763,082	41.4	1	3,763,082	81	23	3911
Bacillus velezensis	sx01604		1	3,926,520	46.5	1	3,926,520	86	14	3987
Bacillus velezensis	S141		1	3,974,582	46.5	1	3,974,582	87	27	4028
Bacillus velezensis	AK-0		1	3,969,447	46.5	1	3,969,447	86	27	4017

To determine the genetic relationships between these ten selected genomes, the sequences were aligned using progressive Mauve (Figure 3). The progressive Mauve algorithm is designed to align genomes that have undergone rearrangements, such as insertions, deletions, and inversions [36]. Each genome is represented by a horizontal line, and different colored blocks represent different gene coding regions. The same-colored regions between the lines show regions of similarity between the different genomes.

The alignment clearly shows that there is some variation in the gene arrangements between the strains. This variation is likely due to mutations that have occurred since the strains diverged from their common ancestor. Also, looking at the alignment in Figure 3, we clearly see the lengths of same coding contigs differ among different genomes for the *Bacillus* strains.

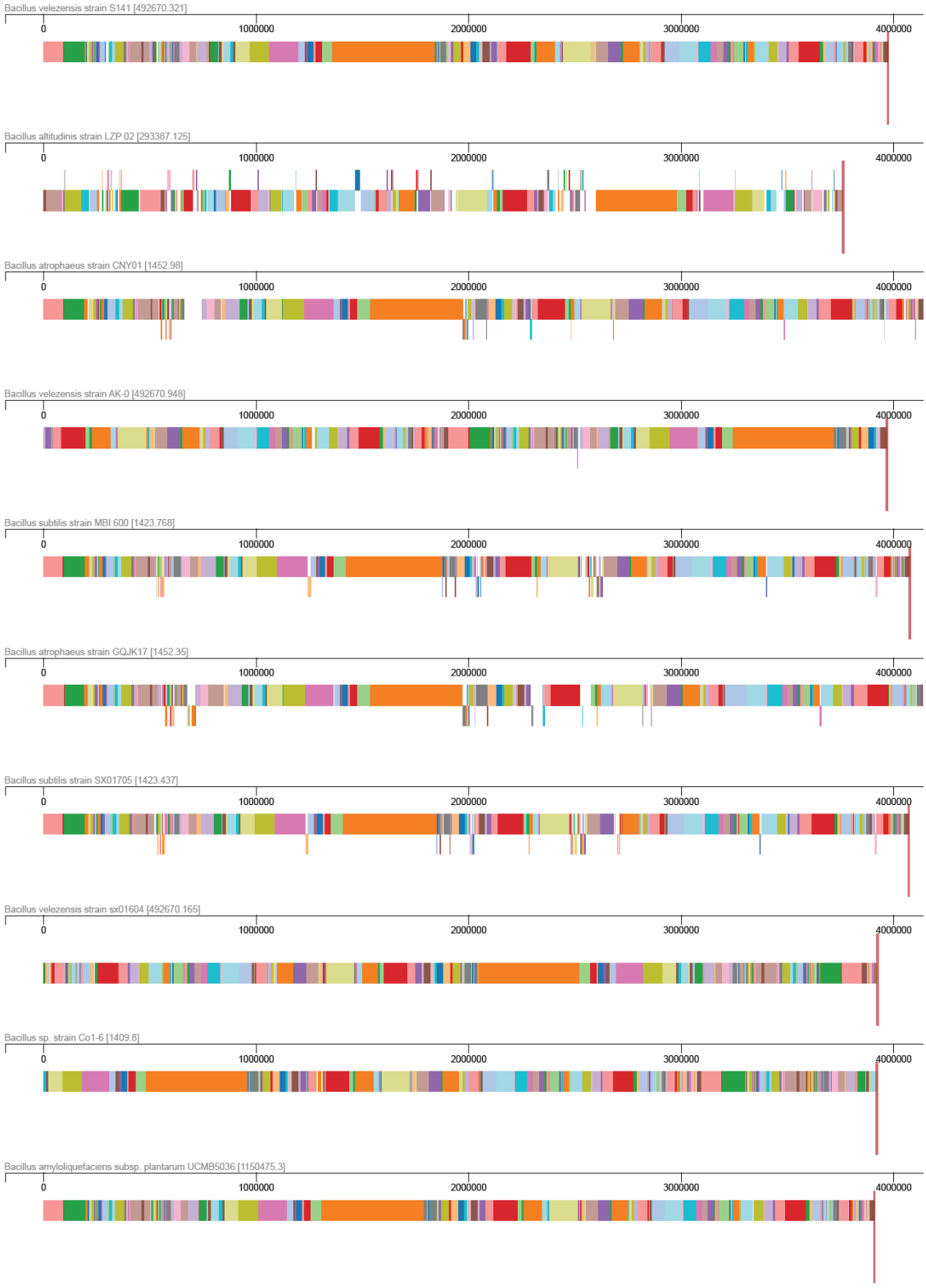

Figure 3. Alignment of *Bacillus* genomes using progressive Progressive Mauve. An example of a locally collinear block (LCB) identified by MAUVE (orange color-coded) linking between LCBs, as indicated by the thin orange-colored lines.

We then investigated whether there are regions unique in terms of characteristics such as inversions, and for that, we focused on the regions that showed inversions. We found that the strains LZP02, CNY01, MBI600, GQJK17, and SXO1705 had some inversions.

Interestingly, however, there were several regions that had undergone inversion only in the LZP02 strain, thus highlighting the genetic basis of its evolutionary distance from other Bacillus strains, as shown in the phylogenetic tree (Figure 2). We focused our analysis on the two major inverted regions.

As shown in Figure 4A, twelve genes were annotated in the LCB from position 595,455–608,432. These genes were dhbC and dnaB. In Figure 4B, position 1,482,268–1,502,365 had twenty-five genes, of which three genes were annotated. These genes were betB, ftsW and rodA. The ddhbC gene encodes dihydrolipoyl dehydrogenase, an enzyme involved in the pyruvate dehydrogenase complex, which is responsible for converting pyruvate to acetyl-CoA. Acetyl-CoA is a key intermediate in many metabolic pathways, including the citric acid cycle and fatty acid synthesis. Another important mechanism involved in the functionality of the dhbc gene is its important role in siderophore biosynthesis [40]. The second gene was dnaB, which encodes DNA polymerase III, the main enzyme responsible for DNA replication in bacteria. DNA replication is essential for bacterial growth and reproduction [41,42].

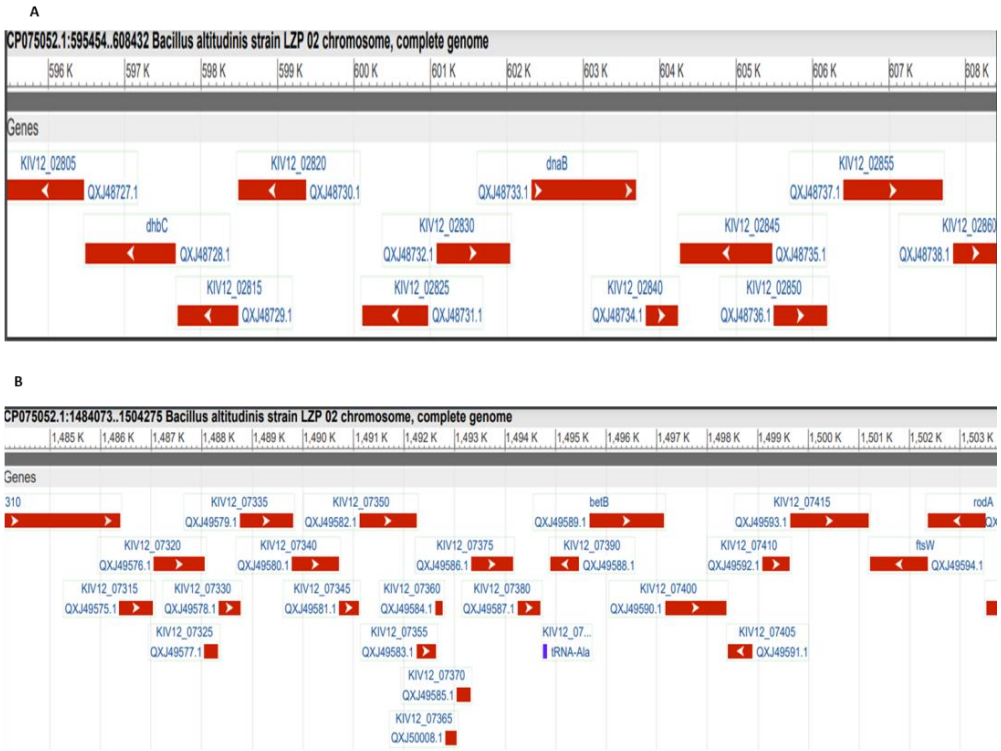

Figure 4. The region of inverted LCB in LZP02 was present at position (**A**) 595,455–608,432 and (**B**) 1,482,268–1,502,365.

The betB gene plays an important role in the biosynthesis of the osmoprotectant glycine betaine. Its involvement is crucial in cellular processes that render the adaptive ability of *Bacillus* [43]. Gene ftsW encodes the protein FtsW, which is essential for cell wall formation [44], and the rodA gene encodes DNA gyrase, an enzyme that is responsible for introducing negative supercoils into DNA. Negative supercoils are required for DNA replication and transcription [45]. It is an essential gene for maintaining the rod shape of cells, as well as maintaining cell viability [46].

Furthermore, upon closer examination of the gene arrangement pattern among the strains that were close in the phylogenetic tree cluster (Figure 2), it was observed in Figure 5 that the gene arrangement was comparable in these five *Bacillus* strains, in which two regions of conserved gene arrangement are highlighted. These regions are indicated by the dashed box, which shows that the genes in these regions are located in the same pattern in all five strains.

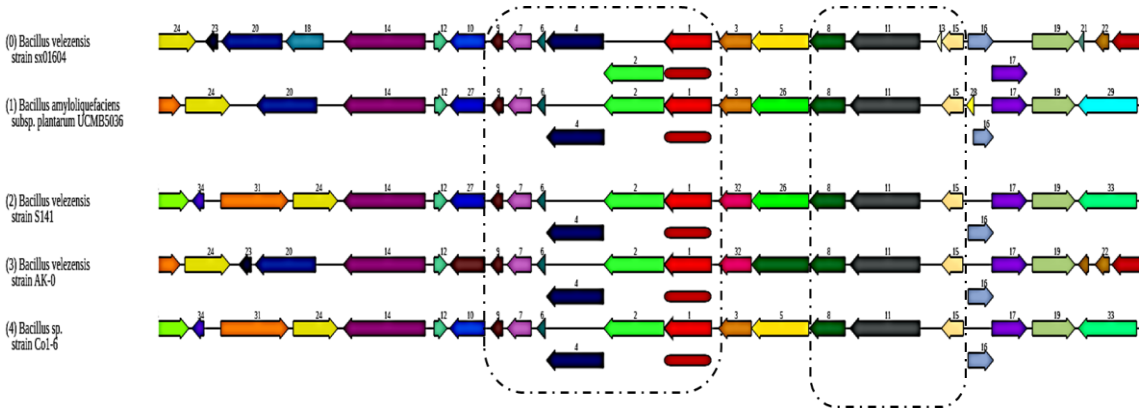

Figure 5. The alignment of *Bacillus* genomes that clustered together in the phylogenetic tree shows the conservation of genetic arrangements. The dashed box indicates two close regions of conserved gene arrangement. Color coding is used to represent the genes of the same protein family.

Taken together, these results not only shed light on the genomic structure differences but also on the underlying importance of these structural differences in the genes that are important for specific strains, and the mechanisms involved over the course of evolution to ensure that these genes are not lost but are utilized in characterizing the specific characteristics of the strains.

3.3. Comparative Proteomic Analysis of the Ten Chosen Bacillus Strains

Comparative analysis was performed for the 10 *Bacillus* strains, and mapping was performed for all the protein sets expressed in these strains. A circular dendrogram was constructed for the proteome of the 10 Bacillus strains (Figure 6). *Bacillus velezensis* strain S141 was randomly selected as a reference for protein alignment. The closeness of various protein hits has been depicted in Figure 6 using the color-coding scheme with dark blue, indicating that the gene was completely similar to the randomly selected reference genome of *Bacillus velezensis* strain S141. As blue became lighter, the protein sequence became increasingly less similar to that of the reference. As the protein sequence identity decreased, the color scheme changed from blue to green and eventually to shades of red, representing >30% sequence identity.

Using this methodology, we aimed to obtain a more holistic view of the proteomes of the different strains. The circular dendrogram not only shows the sequence identity matches ranging from very close matches (blue) to less similar (in yellow) and to more distinct identity matches (orange) but also shows multiple clear areas representing strains that did not express certain protein sets.

The data were thus processed to remove proteins that were absent in one or more strains. The processed data were used to generate a heatmap to show the differences between different *Bacillus* strains (Figure 7). The difference between the sequence is highlighted as we go from green to red. We found that *Bacillus altitudinis* is indeed farthest from the others in the group, thus providing us insight that the evolutionary distance is based on the genetic divergence that translates into proteomic diversity.

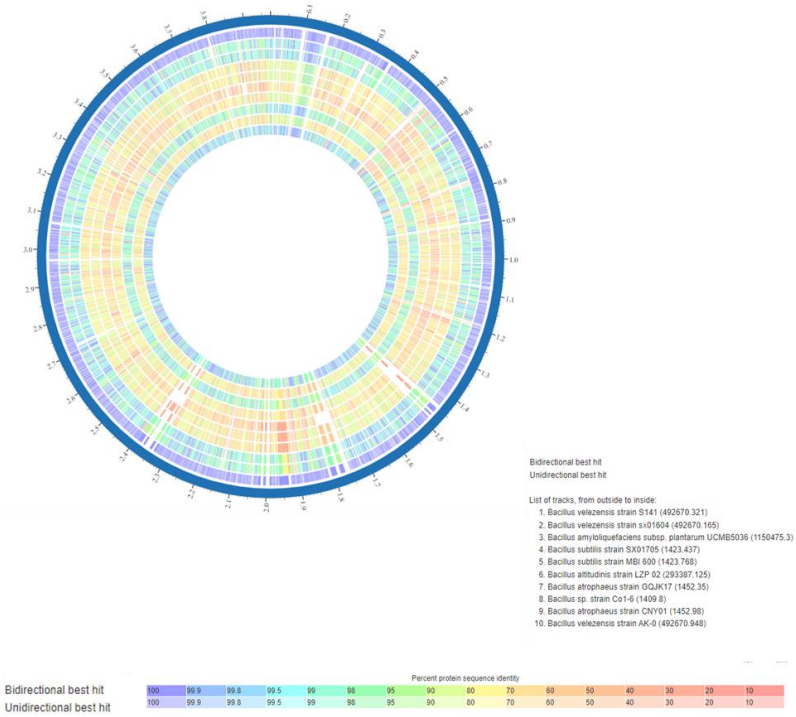

Figure 6. Visualization of percent protein sequence identity mapping for ten genomes of *Bacillus* strains using a circular dendrogram.

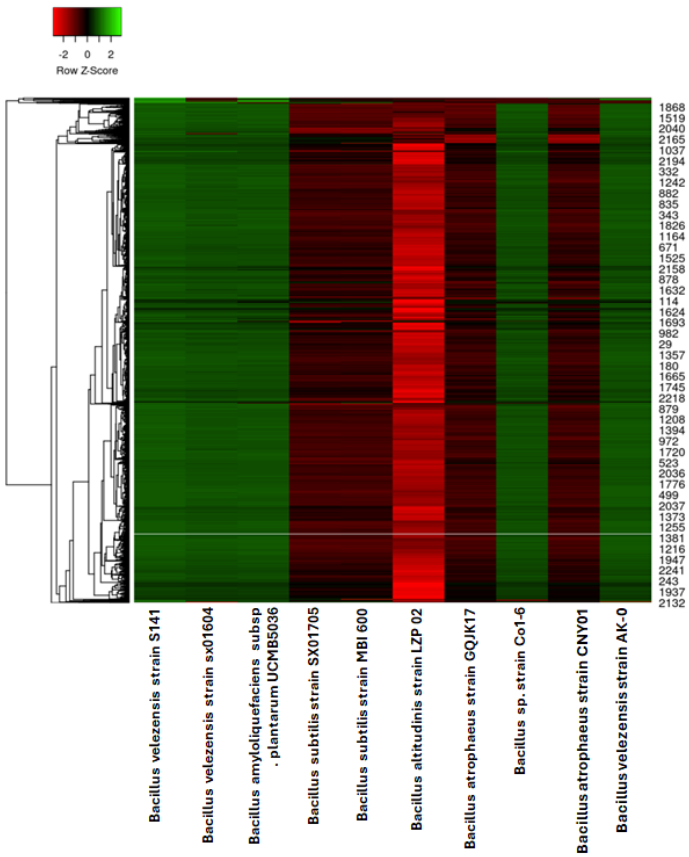

Figure 7. Comparison of different protein sequence identities across the ten genomes. A heatmap (generated using Heatmapper) was used to compare proteins that were present in all ten genomes.

We next investigated how the difference is seen at the macroscopic level in the proteomic data across different genomes of *Bacillus* strains and to what extent these differences appeared in proteomes. In order to find this, we looked at different protein families across the ten *Bacillus* strains and identified common protein families. Figure 8A shows a pie chart in which the total number of perfect protein families analyzed was 6791. These were the sum of all perfect protein families across the ten *Bacillus* strains. However, the number of common perfect protein families among all strains was only 1683. The extent of conservation of various proteins present in all the selected *Bacillus* strains was also evaluated (Figure 8B). Approximately 22% of the proteins (506 out of 2297 analyzed) across the ten *Bacillus* strains exhibited less than 50% sequence conservation across all *Bacillus* species. Approximately 34% of the proteins (778 out of 2297) exhibited between 50% and 70% sequence conservation. Most proteins (~38%, 873 out of 2297) exhibited between 70% and 90% sequence conservation. Approximately 6% (140 out of 2297) showed at least 90% or greater than 90% sequence conservation.

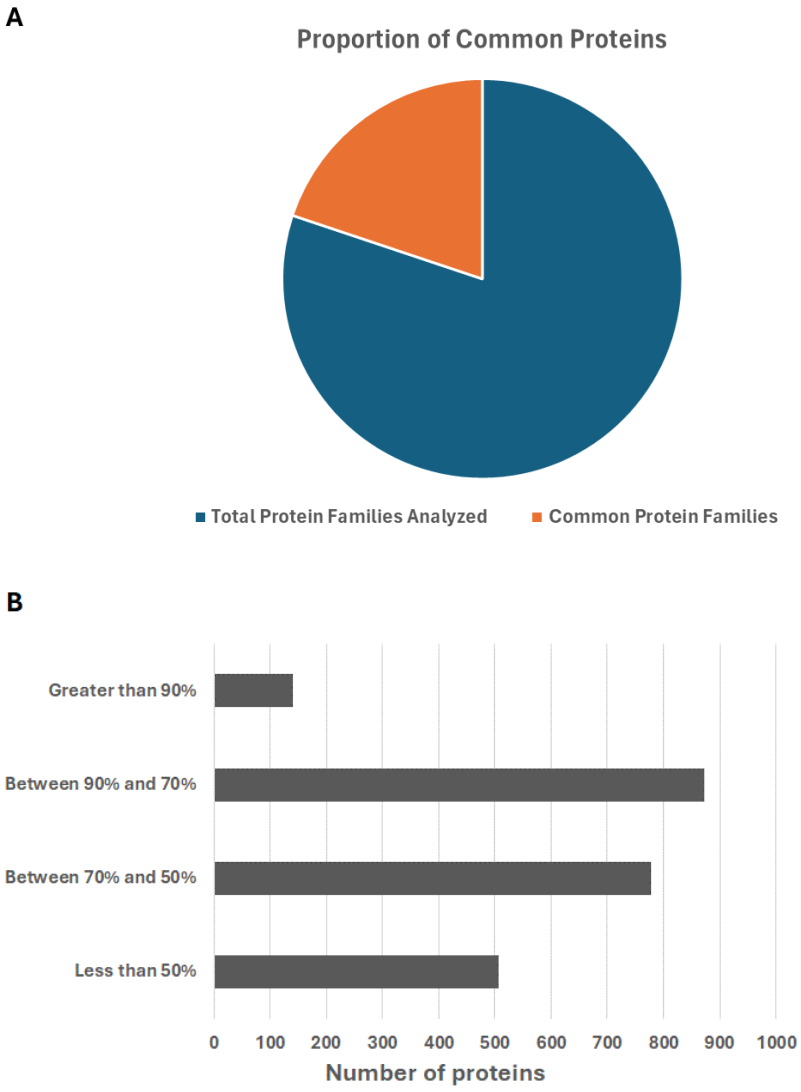

Figure 8. (**A**) Pie chart depiction of protein families across *Bacillus* strains. Blue represents the total number of perfect protein families (one protein per genome) present across at least one of the strains (the total number of such protein families is 6791), and orange represents common perfect protein families (the total number of such protein families is 1683) that are present in all the strains. (**B**) Bar graph showing the number of proteins and their percentage sequence conservation across all *Bacillus* strains.

We also investigated whether there were important pathways that were conserved and common to all the different strains. We focussed our analysis on pathway classes known to support plant growth and thus explored possible common mechanisms that contributed to the plant growth-promoting phenotypes of these different strains. Different pathway classes were analyzed, as shown in Figure 9.

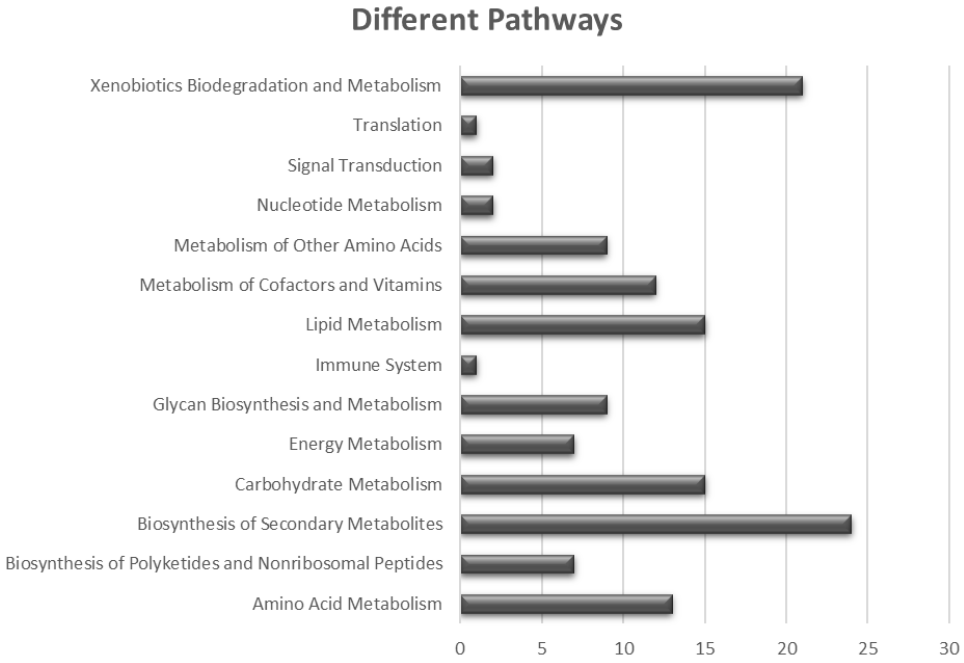

Figure 9. Bar chart showing various protein pathways analyzed across *Bacillus* strains.

The numbers at the end of the bars represent various pathways that were common across all the *Bacillus* strains for that particular class. For example, thirteen amino acid metabolism pathways included (1) alanine, aspartate and glutamate metabolism; (2) arginine and proline metabolism; (3) cysteine and methionine metabolism; (4) glycine, serine and threonine metabolism; (5) histidine metabolism; (6) lysine biosynthesis; (7) lysine degradation; (8) phenylalanine metabolism; (9) phenylalanine, tyrosine and tryptophan biosynthesis; (10) tryptophan metabolism; (11) tyrosine metabolism; (12) valine, leucine and isoleucine biosynthesis; and (13) valine, leucine and isoleucine degradation. The signal transduction and immune system classes each had a single pathway.

Next, we looked at the special genes that encode for virulence factors and antibiotic resistance, as shown in Figure 10A,B. These are key microbial indicators and result of adaptation in different ecological niches. After the analysis, it was found that at least four virulence factors were present in all *Bacillus* strains. The *Bacillus atrophaeus* strain GQJK17 had a maximum number of virulence genes with seven genes followed by *Bacillus subtilis* MBI600 which has 6. Furthermore, all strains of the *Bacillus* had antibiotic-resistance genes. *Bacillus altitudinis* LPZ02 had the lowest number of antibiotic resistance genes, with 45 genes among the group of *Bacillus* strains, while others ranged between 55 to 63 genes.

For the plant growth-promoting phenotype, we focused on pathway classes such as xenobiotic biodegradation and metabolism [47], biosynthesis of polyketides and nonribosomal peptides [48], and biosynthesis of secondary metabolites [49], which were previously reported to play a pivotal role in plant growth promotion. We further looked for the key genes that have been reported to play roles in PGP activity rendered by PGP bacteria.

We investigated various path through which plant growth enhancement have been facilitated by plant growth promoting bacteria. The results have been summarized in Table 2.

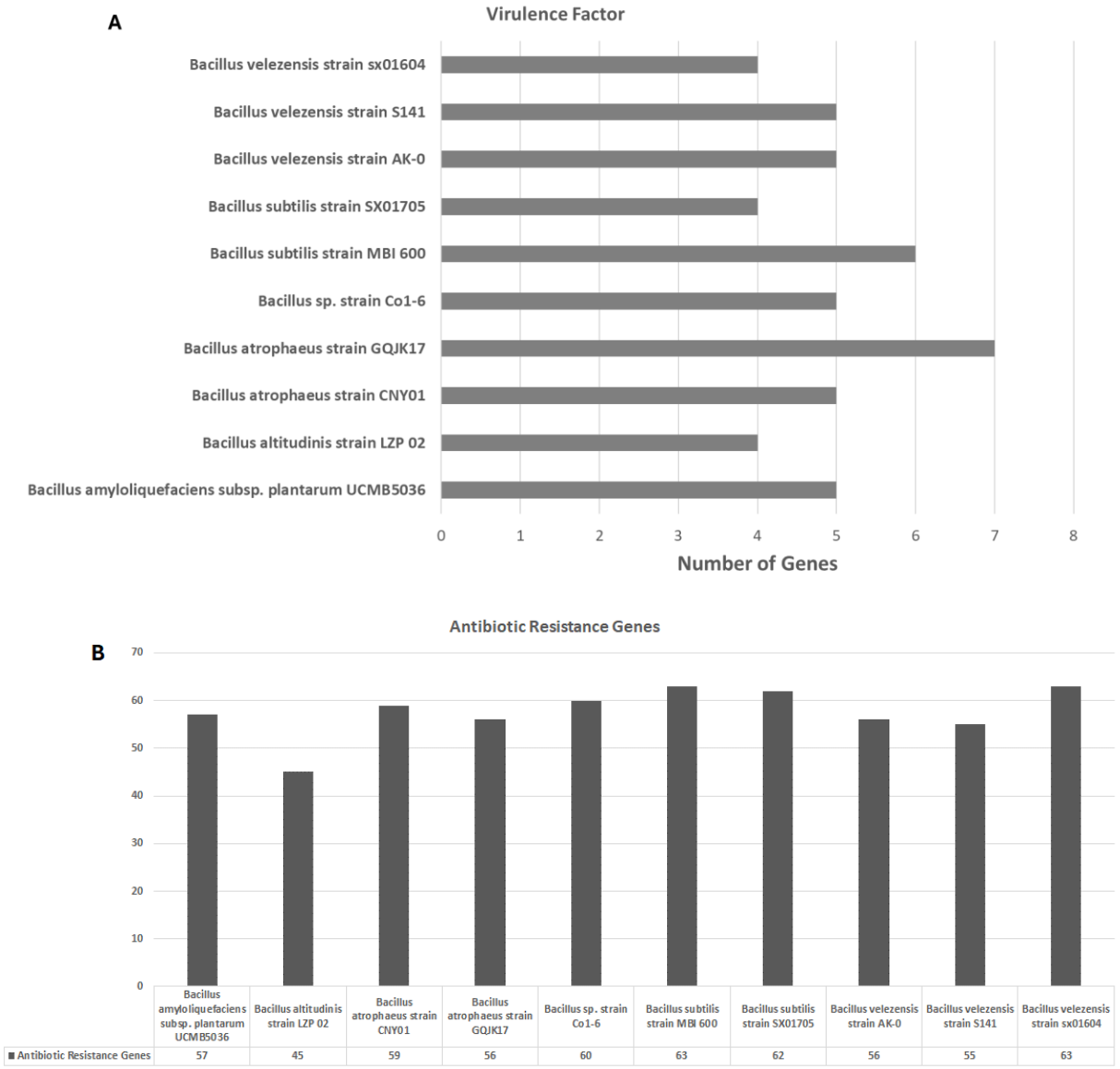

Figure 10. (**A**) Vertical Bar chart depiction of a number of Virulence factor (VF) genes across *Bacillus* strains (**B**) Bar graph showing the number of Antibiotic resistance genes across all *Bacillus* species.

It was found that most of the key pathways that play crucial roles in PGP activity, either in key metabolic activities or providing resistance from stress, such as heavy metals, antibiotics, etc., were present in almost all 10 *Bacillus* strains. Together, the results show that although there is variation in these strains and evolutionary differences, as expected from their speciation process, the plant growth-promoting phenotype between bacterial strains is rendered by evolutionarily conserved pathways.

Table 2. Qualitative summary of plant growth-promoting genes. Positive (+) depicts the presence of the gene, and Minus (−) depicts the absence of the genes in the respective strain.

Genes Reported to Support Plant Growth	**Plant Growth Promoting Property**	***B. amyloliquefaciens subsp. plantarum UCMB5036***	***B. sp. strain Co1-6***	***B. subtilis strain SX01705***	***B. subtilis strain MBI 600***	***B. atrophaeus strain GQJK17***	***B. atrophaeus strain CNY01***	***B. velezensis strain sx01604***	***B. velezensis strain S141***	***B. velezensis strain AK-0***	***B. altitudinis strain LZP 02***
Trilactone hydrolase [bacillibactin] siderophore	Improves Iron availability	+	+	+	+	+	+	+	+	+	+
2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (Siderphore biosynthesis)	Siderophore Biosynthesis	+	+	+	+	+	+	+	+	+	-
Nitrite-sensitive transcriptional repressor (NsrR)	stress resistance	+	+	+	+	+	+	+	+	+	+
Superoxide Dimutase	stress resistance	+	+	+	+	+	+	+	+	+	+
betaine aldehyde dehydrogenase	stress resistance	+	+	+	+	+	+	+	+	+	+
copC	copper resistance genes	+	+	+	+	+	+	+	+	+	+
copD	copper resistance genes	+	+	+	+	+	+	+	+	+	+
Fosfomycin	antibiotic resistance genes	+	+	+	+	+	-	+	+	+	-
ykkD	antibiotic resistance genes	+	+	+	+	+	+	+	+	+	+
ykkC	antibiotic resistance genes	+	+	+	+	+	+	+	+	+	-
norD	Nitrogen Metabolism	+	+	+	+	+	+	+	+	+	+
norQ	Nitrogen Metabolism	+	+	+	+	+	+	+	+	+	+
Tpx and related	Sulfate Metabolism	+	+	+	+	+	+	+	+	+	+
phoP	phosphate metabolism	+	+	+	+	+	+	+	+	+	+
tryptophan synthase	auxin biosynthesis	+	+	+	+	+	+	+	+	+	+
anthranilate phosphoribosyltransferase (trpD)	auxin biosynthesis	+	+	+	+	+	+	+	+	+	+
phosphoribosylanthranilate isomerase	auxin biosynthesis	+	+	+	+	+	+	+	+	+	+
gabR	γ-Aminobutyric Acid (GABA) Metabolism	+	+	+	+	+	+	+	+	+	+

4. Discussion

The relationship between certain bacterial strains present in the vicinity of plant roots that support plant growth has immense importance not only for plant growth but also for greener ways to harness such potential from nature's own microbial populations rather than using synthetic fertilizers and chemicals. However, the underlying mechanisms mediating these interactions between bacterial strains and plants remain unclear. To obtain a mechanistic understanding of the nature of the interaction that occurs and the resulting impact on the plant and the soil, a deeper understanding of the main player in such interaction is warranted. This need, coupled with advancements in the field of computational biology and improvements in computational capacity, has promoted studies that utilize genomic and proteomic data for comparative analysis of bacterial strains.

In the present study, we studied 10 *Bacillus* strains known to have a positive impact on plant growth using multifaceted computational analysis such as phylogenetic, genetic, and proteomic relationships to understand the underlying mechanisms towards the evolution of the growth-promoting phenotype of these bacteria.

We started by investigating how all these strains are evolutionarily related to each other. We constructed a phylogenetic tree, and our analysis revealed that these strains represented five species of the *Bacillus* genus. These species included strains from *Bacillus velezensis*, *Bacillus subtilis*, *Bacillus atrophaeus*, *Bacillus altitudinis*, and *Bacillus amyloliquefaciens*. These selected strains were PGPR strains with either known or predicted properties for promoting plant growth. Interestingly, the evolutionary relationships of *Bacillus velezensis* and *Bacillus amyloliquefaciens* were similar, showing their evolutionary closeness based on the underlying genetic and proteomic closeness [50]. *B. atrophaeus* and *B. subtilis* clustered closely as the nearest neighbors, as was also identified in a study by Gibbons et al. [51], where in a Bacillus-wide phylogenetic analysis, *B. subtilis* was found to be the nearest neighbor to *B. atrophaeus*. The *Bacillus altitudinis* strain LZP 02 was found to be the most distantly related to all the other strains in the tree.

Looking closely at *Bacillus altitudinis*, it was found that this strain has genetic features that were not shared with any of the other strains. We observed that some of the regions inverted in the *Bacillus altitudinis* strain LZP 02 were not inverted in the remaining nine Bacillus strains, which is consistent with the evolutionary analysis showing that this strain is farthest from all the other selected strains. Such inversions in bacterial genomes refer to the rearrangement of genetic material wherein a segment of DNA is flipped or reversed in orientation within the chromosome. It is widely known that these inverted elements often carry genes that contribute to bacterial adaptation and response to environmental stimuli, highlighting the significance of understanding the mechanistic aspects and influencing factors of gene inversion in bacteria [47]. We examined the segments of genomic regions that were inverted in the *Bacillus altitudinis* strain LZP 02 to understand the nature of the genes that are harbored in those regions.

We observed that the five annotated genes that were found in these regions were dhbC, dnaB, betB, ftsW, and rodA. A closer examination of the functions of these genes revealed that they are very important for the adaptation and survival of the *Bacillus altitudinis* strain LZP02. The dhbC gene encodes dihydrolipoyl dehydrogenase, an enzyme involved in the pyruvate dehydrogenase complex, which is responsible for converting pyruvate to acetyl-CoA. Acetyl-CoA is a key intermediate in many metabolic pathways, including the citric acid cycle and fatty acid synthesis. Another important mechanism involved in the functionality of the dhbc gene is its important role in siderophore biosynthesis [40]. The second annotated gene was dnaB, which encodes DNA polymerase III, the main enzyme responsible for DNA replication in bacteria. DNA replication is essential for bacterial growth and reproduction [41,42]. The third gene, betB, was reported to play an important role in the production of glycine betaine, which has been reported to act as an osmoprotectant protecting against abiotic stress conditions such as salinity [43,48]. The fourth gene candidate that was annotated was ftsW, which encodes the protein FtsW, which is essential for cell wall formation [44], and the rodA gene, which is known to encode

DNA gyrase, an enzyme that is responsible for introducing negative supercoils into DNA. Negative supercoils are required for DNA replication and transcription [45]. It is an essential gene for maintaining the rod shape of cells, and this gene is also essential for maintaining cell viability [46]. Overall, the set of annotated genes found in the inverted regions were found to be essential genes not only for survival but also for playing a potential role in PGPR activity. One of the postulated reasons for such inversions is collisions between the replisome and RNA polymerases (RNAPs). These conflicts collapse the replication fork, break DNA strands, and increase mutagenesis [47,49]. These "replication-transcription conflicts" can lead to stalled replication forks, broken DNA, and increased mutation rates, especially in essential genes [52–54]. To combat this threat, bacteria have evolved a clever strategy: co-orienting genes with DNA replication. This means that genes are arranged on the same DNA strand as in replication, minimizing head-on collisions. In fact, most bacterial genomes exhibit a strong bias toward co-orientation, with essential and highly transcribed genes such as rRNA operons almost exclusively coordinated [55,56].

Proteome comparisons for 10 selected *Bacillus* strains were performed using circular dendrograms. Interestingly, this pattern mimicked the pattern of relatedness observed in the phylogenetic tree, with *Bacillus altitudinis* strain LZP02 showing the most distant proteomic similarity. Additionally, gaps were observed, indicating that certain proteins were absent from certain strains. The data were processed to compare only the proteins that were present in all 10 *Bacillus* strains to remove any skewness in the analysis and to determine whether the pattern was preserved. Once we processed the dataset to eliminate all the missing data, we then used the percentage sequence identity to determine the relatedness using heatmap representation (see Figure 7). The results confirmed that the trend shown in Figure 6 was maintained, and in fact, the *Bacillus altitudinis* strain LZP 02 had the same annotated proteins but had a higher level of sequence diversity in those protein groups, thus making them more distant from other strains.

However, closer looking at the different protein pathway classes, it was found that despite the macroscopic differences observed, some important proteins were conserved. Overall, six percent of the proteins were conserved, with sequence conservation greater than 90%. Unexpectedly, nearly 40% of the remaining proteins exhibited high levels of conservation, ranging between 70% and 90%. We then looked at the protein pathways that were conserved. We found many important pathways that were conserved. Most of these played pivotal roles in sustaining the bacterium. We looked at the key microbial indicators, such as the presence of virulence factors and antibiotic resistance rendering genes. The presence of virulence factors and antibiotic resistance in bacteria plays a crucial role in their adaptation and survival in a competitive environment. Figure 10 shows that all the strains analyzed in this study had multiple of genes providing virulence and antibiotic resistance, thus ensuring the survival and adaption of these strains in different environments and ecological niches.

We focused our analysis on the three-pathway class that is shown to have relevance in rendering PGP activity to bacteria. These were xenobiotic biodegradation and metabolism [47], biosynthesis of polyketides and non-ribosomal peptides [48], and biosynthesis of secondary metabolites [49]. The presence of these common proteomic pathway classes indicates conservation at the pathway level, which renders important PGPR-related functionalities. This translated to the conservation of key genes such as gabR that encodes GABA aminotransferase. The amino acid GABA acts as a signal molecule in communication between plants and microorganisms in the rhizosphere [57]. Other key genes include genes involved in Auxin biosynthesis, nitrogen, sulfur, and phosphorus acquisition, as well as resistance against heavy metals and antibiotics. Table 2 summarizes the key genes that are present in almost all the strains playing important roles in different mechanisms that are associated with the PGP activity.

Other studies, such as Liu H et al. [58], carried out comparative genomic analysis of 96 strains of *Bacillus amyloliquefaciens* to shed light and provide valuable insights into the phylogenetic relationship, ecological niches, and functional differences of *B. amyloliq-*

uefaciens strains. Zhang N. et al. [59] also performed a comparative genomic analysis of *Bacillus amyloliquefaciens* (BA) and *Bacillus subtilis* (BS) strains. Their analysis showed that strains have evolved their genomes to better enable adaptation to different habitats, particularly plant-associated habitats. Shen et al. [60] also used comparative genomic-based analysis of four *Pseudomonas* strains involved in promoting plant growth being part of rhizobium. Their findings showed the presence of conserved sequences and gene order within *Pseudomonas* strains. Interestingly, they also observed the presence of major genomic inversions in the *Pseudomonas* strain upon comparing genomic landscapes as the differentiator between different related strains. Our study compared a larger, varied cohort of *Bacillus* strains. Furthermore, our analysis is based not only on genomic features but also on proteomic data derived from protein sequence conservation to evaluate the different strains used in this study.

Overall, this indicates that nearly half of the proteins across the selected strains had high levels of conservation, which may be related to functions that have high survival value; thus, significant changes could prove to be detrimental. In addition, all of these *Bacillus* strains have shown conservation of key genes that can play crucial roles in promoting plant growth through pathways ranging from affecting nutrient availability for plants to providing resistance to things like heavy metals and antibiotics.

5. Conclusions

Overall, this study aimed to adopt a multifaceted computational approach in which we examined evolutionary relationships, genomic features, and proteomic relatedness to see if important functions across the different strains are either conserved or unique to make these bacterial strains distant or more closely related. We found that in the selected cohort of *Bacillus* strains, *Bacillus altitudinis* was evolutionary most distant, which was further validated by looking at its genetic and proteomic features. We compared proteomic pathways and identified three important pathways that promote plant growth, which were largely conserved in all these strains in spite of being evolutionary distant. Such an in-depth analysis could help in the classification and identification of novel bacterial strains that can be used as green fertilizers and aid in a positive impact on crop cultivation.

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