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To cite this version:

Naima Lemjiber, Khalid Naamani, Annabelle Merieau, Abdelhi Dihazi, Nawal Zhar, et al.. Identification and Genomic Characterization of Pathogenic Bacillus altitudinis from Common Pear Trees in Morocco. Agronomy, 2021, 11 (7), pp.1344. ⟨10.3390/agronomy11071344⟩. ⟨hal-03346160⟩

HAL Id: hal-03346160

https://normandie-univ.hal.science/hal-03346160v1

Submitted on 4 Jun 2024

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Identification and Genomic Characterization of Pathogenic *Bacillus altitudinis* from Common Pear Trees in Morocco

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Citation: Lemjiber, N.; Naamani, K.; Merieau, A.; Dihazi, A.; Zhar, N.; Jediyi, H.; Boukerb, A.M. Identification and Genomic Characterization of Pathogenic *Bacillus altitudinis* from Common Pear Trees in Morocco. *Agronomy* **2021**, *11*, 1344. https://doi.org/10.3390/agronomy11071344

Academic Editors: Muhammad Shahid and Ali Sarkhosh

Received: 20 May 2021
Accepted: 28 June 2021
Published: 30 June 2021

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Abstract: Bacterial burn is one of the major diseases affecting pear trees worldwide, with serious impacts on producers and economy. In Morocco, several pear trees (*Pyrus communis*) have shown leaf burns since 2015. To characterize the causal agent of this disease, we isolated fourteen bacterial strains from different parts of symptomatic pear trees (leaves, shoots, fruits and flowers) that were tested *in planta* for their pathogenicity on Louise bonne and Williams cultivars. The results showed necrotic lesions with a significant severity range from 47.63 to 57.77% on leaves of the Louise bonne cultivar inoculated with isolate B10, while the other bacterial isolates did not induce any disease symptom. 16S rRNA gene sequencing did not allow robust taxonomic discrimination of the incriminated isolate. Thus, we conducted whole-genome sequencing (WGS) and phylogenetic analyzes based on *gyrA*, *gyrB* and *cdaA* gene sequences, indicating that this isolate belongs to the *Bacillus altitudinis* species. This taxonomic classification was further confirmed by the Average Nucleotide Identity (ANI) and the *in silico* DNA-DNA hybridization (*is*DDH) analyzes compared to sixty-five *Bacillus* spp. type strains. The genome was mined for genes encoding carbohydrate-active enzymes (CAZymes) known to play a role in the vegetal tissue degradation. 177 candidates with functions that may support the *in planta* phytopathogenicity results were identified. To the best of our knowledge, this is the first data reporting *B. altitudinis* as agent of leaf burn in *P. communis* in Morocco. Our dataset will improve our knowledge on spread and pathogenicity of *B. altitudinis* genotypes that appears as emergent phytopathogenic agent, unveiling virulence factors and their genomic location (i.e., within genomic islands or the accessory genome) to induce trees disease.

Keywords: greenhouse; infiltration; next generation sequencing; pathovar; phylogeny; spray; virulence factors

1. Introduction

Common pear (*Pyrus communis*) is one of the main consumed fruits worldwide [1], with a production of around 25.2 million tons per year [2]. This fruit has a high nutritional value and represents a source of different vitamins and minerals [3]. In Morocco, pear trees were reported to occupy 3522 ha and the production varies according to climate variation, water supply and spread of diseases. In 2016, the production of pear fruit was

about 27,754 tons [4]. Unfortunately, this crop is prone to several diseases caused by several phytopathogens that affect growth and production of pear trees. This includes diseases such as the brown spot caused by *Stemphylium vesicarium* [5,6], the leaf scorch caused by *Xylella fastidiosa* [7], the soft rot caused by *Bacillus altitudinis* [8], the anthracnose disease caused by *Colletotrichum acutatum* [9] and the fire blight caused by *Erwinia amylovora* [10,11], which all are the most devastating bacterial or fungal diseases of pear worldwide with high economic losses [12].

In Morocco, little is known about pear diseases and the damages begotten by bacterial pathogens. Therefore, it becomes necessary to identify the causal agents in order to establish the appropriate strategies to control pear diseases. To the best of our knowledge, in Morocco only one study has been conducted in Ain Orma of the Meknes region [13]. During our survey, the burnt trees were observed with diseases in two pear farms in Asni province at El Haouz of the Marrakesh region. The disease symptoms were observed on leaves, fruits and shoot, similar to those caused by *Erwinia amylovora*. In a previous study, Kotan et al. [14] indicated that several bacterial species (i.e., *Erwinia amylovora*, *Pseudomonas. syringae* pv. *syringae*, *Erwinia rhapontici*, *Pseudomonas putida*, *Pseudomonas huttiensis*, *Chromobacterium violaceum*, *Pantoea agglomerans*, *Achromobacter piechaudii*, *Bacillus pumilus* GC subgroup B, *Echinococcus intermedius*, *Pseudomonas aeruginosa* and *Pseudomonas cichorii*) cause similar necrotic lesions on apple leaves.

The *Bacillus* genus brings together many of the most widely disseminated species in the environment. Bacteria belonging to this genus form spores that allow them to survive in extreme conditions [15]. Numerous studies have shown the role of biocontrol agents of few species. For instance, *B. subtilis* is well known for its antagonistic activities against several plant diseases caused by many fungi and bacteria [16,17]. Other species of the genus *Bacillus* were reported as responsible for infections in humans, e.g., *B. anthracis* and *B. cereus*. Other studies demonstrated that some *Bacillus* species are phytopathogens. For example, *B. polymyxa* and *B. circulans* were shown to cause soft rot in various types of vegetables and fruit crops [18,19]. In Egypt, *B. pumilus* was reported to cause leaf blight in mango trees [20], and a bacterial blotch of immature Balady peach [21]. On pome fruits, *B. pumilus* was also reported as responsible for Golden Delicious apple necrotic lesions in the Eastern Anatolia region of Turkey [14]. Furthermore, Li et al. [22] reported that the twig dieback disease on the Asian pear trees (*Pyrus pyrifolia*) was associated to *B. pumilus* infection.

The disease symptoms on pear trees described by local farmers were characterized by shoots drying and wilting, fruits browning and drying, branches and twigs burnt and secretion of exudate. Even though, in the absence of methods to contain this infectious disease, the cutting and destruction of infected parts remain the only strategy to mitigate the spread of the infection from one tree to another.

The diagnosis of plant diseases depends on the identification of the pathogens. While the use of biochemical tests to identify bacteria belonging to *Bacillus* genus is classical [23], molecular biology techniques have become a complementary tool to study bacterial diversity [24]. For example, the amplification and sequencing of the 16S rRNA gene is widely used as a biomarker for the identification and taxonomic classification of bacteria and archaea. This gene has a conserved sequence with a low mutation rate. The use of 16S rRNA gene in identifying closely related bacterial species, such as the species belonging to the *B. pumilus* group, may be difficult due to its highly conserved sequences within this group. In addition, these genes can undergo occasional lateral transfer or recombination [25]. The other disadvantage associated with the use of 16S rRNA gene is the presence of a variable number of copies. Therefore, other molecular markers would be helpful in discriminating the taxonomic affiliation of targeted species.

The symptoms observed in pear trees at Asni province in Morocco may be associated to diverse pathogenic bacteria. Thus, the aim of the present study was to isolate and identify the causative agent of these symptoms. Preliminary isolation suggests the presence of bacterial isolates in infected plants that were further investigated using (i) the in planta pathogenicity tests for disease severity estimation, (ii) the Whole Genome Sequencing for

taxonomic classification and (iii) the *in silico* pathogenicity evaluation to decipher potential virulence factors. These analyzes may unveil the association of a specific *B. altitudinis* genotype to pear trees bacterial burn disease. To the best of our knowledge, this is the first study on the identification of *B. altitudinis* as pear tree pathogen in Morocco.

2. Materials and Methods

2.1. Plants Sampling and Bacterial Strains Isolation

Samples of infected pear trees (leaves, shoots, fruits and flowers) showing bacterial burn symptoms were collected from Asni (Marrakesh region of Morocco; 31°14′41.93′′ N and 7°58′56.79′′ W). They were immediately sliced into small fragments, washed twice with sterile distilled water (SDW) and surface sterilized with Sodium Hypochlorite (0.5%) for 30 s, then placed in SDW for 30 min. Later, serial dilutions from 10^{-1} to 10^{-6} of the samples were realized in saline solution (water with 0.9% NaCl). King's B agar culture medium [proteose peptone 20 g/L, dipotassium hydrogen phosphate 1.5 g/L, magnesium sulphate 1.5 g/L, glycerol 15 mL/L and agar 15 g/L (Sigma Aldrich, USA)] was used for bacterial culture. After incubation at 30 °C for 24 h, fourteen isolates (named B1 to B14) were obtained and transferred to a new King's B agar medium. Each culture was presumed pure after four successive subcultures on the same medium.

2.2. Pathogenicity Test

2.2.1. Plant Material

The plant material was composed of two pear cultivars (cv. Louise bonne and cv. Williams). They were grown under natural photoperiod at 17 °C to 28 °C in a greenhouse.

2.2.2. Preparation of Bacterial Inoculum

This was achieved according to Peng et al. [26]. The isolates (n = 14) were grown at 30 °C on King's B agar medium for 24 h. The bacteria were collected and suspended in saline solution, and the concentration was adjusted to 10^9 CFU/mL that corresponds to an optical density (OD_{600}) of 0.5.

2.2.3. Pathogenicity Tests

The pathogenicity tests were performed according to Cesbron et al. [27]. After one month of growing in the greenhouse, the plants were sprayed with distilled water before inoculation and kept under a transparent plastic film shelter to maintain the humidity at saturation. Two inoculation methods were applied for each bacterial isolate: the spray inoculation and the infiltration method. In addition, control group of plants were inoculated with saline solution.

2.3. Infiltration Method

Bacterial inoculum was taken in a disposable injection syringe of 0.5 mL capacity. The syringe needle was placed on the leaf's underside, particularly at the interveinal region, then, the inoculum was softly injected. The leaf was firmly held by providing support on the opposite side at the injection point (mouth of the needle). The injection was carefully accomplished to avoid wounds at the point of injection. Plants were incubated in humid conditions, according to Patil et al. [28]. This method is the most common and fast, facilitating the penetration of bacteria inside the leaves without causing additional injuries.

2.4. Spraying Method

Plants were sprayed on the foliage using a hand sprayer.

After using the two methods, plants were first incubated inside the shelter for 2 h, then in normal greenhouse conditions outside the shelter, under natural photoperiod at 17 °C to 28 °C. These steps are necessary and may improve natural infection to achieve uniform disease pressure [29]. For each bacterial isolate, five pear plants of either Williams or Louise bonne variety were used for the pathogenicity test. Saline solution was used

as control within the two methods. To confirm Koch's postulate and to ensure that the observed symptoms were due to the same pathogen, we performed a re-isolation of the bacterium from infected leaves of cv. Louis bonne and identified the isolate by whole genome sequencing.

2.5. Disease Assessment

ImageJ v1.45 [30] was used to calculate the total area of each leaf, as well as the areas of the necrotic lesions. A digital image composed of pixels was converted to centimeters using the photo filter software version 7. After manually drawing the region of interest (total area and areas of necrotic lesion on each leaf), the area was measured and noted in the spreadsheet.

The percentage of infection was calculated according to the following formula:

$$\text{Percentage of infection} = [(\text{total area of the necrotic lesions})/(\text{total area of the leaf})] \times 100$$

The percentage of severity was evaluated according to the percentage of the infection and using a scale based on symptoms and ranged from 0 to 5 (0, no visible symptoms; 1, 1–25% of leaf area infection; 2, 26–50% of leaf area infection; 3, 51–75% of leaf area infection; and 5, >76% with highly blighted pear leaves). The following formula was used:

$$\text{Percentage of severity} = [(\text{number of infected leaves} \times \text{its grade})/(\text{number of observed leaves})] \times 100$$

Statistical analyzes were performed within R version 4.0.3 [31]. Non-parametric Kruskall-Wallis test was performed to investigate percentages of severity. Five repetitions were accomplished for each cultivar (i.e., Louise bonne and Williams), in addition to the controls exposed to saline solution. The statistical significance level was set at a p-value of 0.05.

2.6. Molecular Identification

The 16S rRNA gene of B10 isolate was amplified by PCR using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). Initial denaturation was set at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 90 s, followed by a final elongation step at 72 °C for 2 min and 4 °C forever. The polymerase used in this assay was the high-fidelity PCR enzyme (Thermo Fisher). The obtained amplicons were sent to Genewiz Los Angeles Metro (USA) for Sanger sequencing.

The 16S rRNA sequence was initially analyzed using the BLAST software on the NCBI (National Center Biotechnology Information, http//:www.ncbi.nml.nih.gov, accessed on 18 January 2021) and compared with the Reference RNA sequences (refseq rna) database.

2.7. Genomic DNA Extraction and Whole Genome Sequencing

The original isolate B10 and the B10-bis strain re-isolated from plants developing of disease were inoculated in 5 mL of nutrient broths and incubated overnight at 37 °C. Genomic DNA was extracted using the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Sunnyvale, CA, USA) according to the manufacturer's instructions for lysis of Gram-positive bacteria. The quality and concentration of DNA were determined on a Nanodrop spectrophotometer and a Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA). The DNA integrity was monitored through electrophoresis using a 0.8% agarose gel followed by Syber-safe staining (Thermo Fisher Scientific, USA) and visualized on a UV transilluminator. Libraries for both B10 and B10-bis were prepared using the Illumina DNA Prep kit (Illumina) and sequenced on a MiSeq platform (LMSM EA 4312, Rouen Normandy University, Evreux, France) according to the manufacturer's protocol, with the MiSeq reagent cartridge V3 (600 cycles, 300-bp dual-index paired-end reads).

2.8. Bioinformatics

2.8.1. Genome Assembly and Annotation

Default parameters were applied for each software unless otherwise specified. Sequence data quality was assessed using FastQC software v.0.11.9 [32]. Low-quality reads and adapters were trimmed using Trimmomatic v.0.39 [33]. Genome assembly was achieved *de novo* with Unicycler v.0.4.8 [34]. Quast v.5.0.2 [35] was used to check the consistency of the obtained assembly (i.e., genome size, number of contigs, N_{50} and G + C content). Mean coverage of contigs was determined by mapping trimmed reads onto the contigs using BBMap v.38.18 [36]. The assembled contigs were annotated using the NCBI prokaryotic genome automatic annotation pipeline (PGAP) [37].

2.8.2. Phylogenetic Analysis

The high-quality genome sequence of the B10 isolate was used to determine its taxonomic affiliation. The sequences of the 16S rRNA, *gyrA*, *gyrB* and *cdaA* genes were extracted from the annotated contigs. These sequences were further aligned and compared to the corresponding gene sequences from a selection of *Bacillus* species representing valid type strains of the genus: multiple sequence alignments were performed using clustalW, and inferring phylogenetic trees using the neighbor-joining method (with the Kimura 2-parameter model) and 1000 bootstrap iterations using MEGA 10.2.2 [38].

2.8.3. Genome Indexes

Whole-genome data from *Bacillus* sp. B10 and sixty-five taxonomically validated *Bacillus* species were compared against each other using average nucleotide identity analysis based on BLAST (ANIb) within Pyani [39], and the in silico DNA-DNA hybridization (*is*DDH) was calculated using the GGDC web server (http://ggdc.dsmz.de, accessed on 8 March 2021) [40].

2.8.4. Identification of Carbohydrate-Active Enzymes

The dbCAN annotation tool for automated carbohydrate-active enzymes (CAZyme) annotation, Run-dbcan v2.0.11 (https://github.com/linnabrown/run_dbcan, accessed on 10 May 2021) [41], was used with default parameters to predict CAZymes using CAZyDB version 07312020. CAZymes were grouped based on the substrates and the corresponding Merops families [42].

3. Results

3.1. Pathogenicity Tests

For all tested bacterial strains on Williams cv, no symptoms were observed, and no significant difference was noted compared to the controls ($p > 0.05$), regardless of the inoculation method (Figure 1e). However, on Louis bonne cv, the symptoms were observed only for the isolate B10 after two weeks of inoculation by spraying method. The severity ranged from 47.63% to 57.77% for 25% of B10-infected leaves (Figure 1a,c and Figure 2). Control plants or those treated with the other isolates (thirteen isolates) showed no significant symptoms *versus* B10 isolate ($p < 0.001$) (Figure 2).

For the infiltration method, small and limited necrotic spots on Louise bonne cv. appeared after 10 days of inoculation with B10 isolate (Figure 1b,d). In contrast, control plants exposed to saline solution were found asymptomatic (Figure 1f). The spray method using B10 isolate resulted in a clearly defined necrosis where leaves wilted rapidly, then turned to light brown and finally dried out. The bacterium re-isolated from symptomatic plant (cv. Louise bonne) was named strain B10-bis and thereafter characterized by whole-genome analysis compared to the inoculum B10.

Figure 1. Pathogenicity tests on pear plants grown in greenhouse after fifteen days of inoculation. For Louise bonne cultivar exposed to B10 isolate, panels (**a**,**c**) represent symptoms developed using the spray method, (**b**,**d**) symptoms observed by the infiltration method. Panel (**e**) shows no symptoms on Williams cultivar inoculated with B10 isolate, (**f**) shows no symptoms on control plants of Williams cv and (**g**,**h**) shows no symptoms on leaves inoculated by other isolates (B13 and B2) on Louise bonne cultivar.

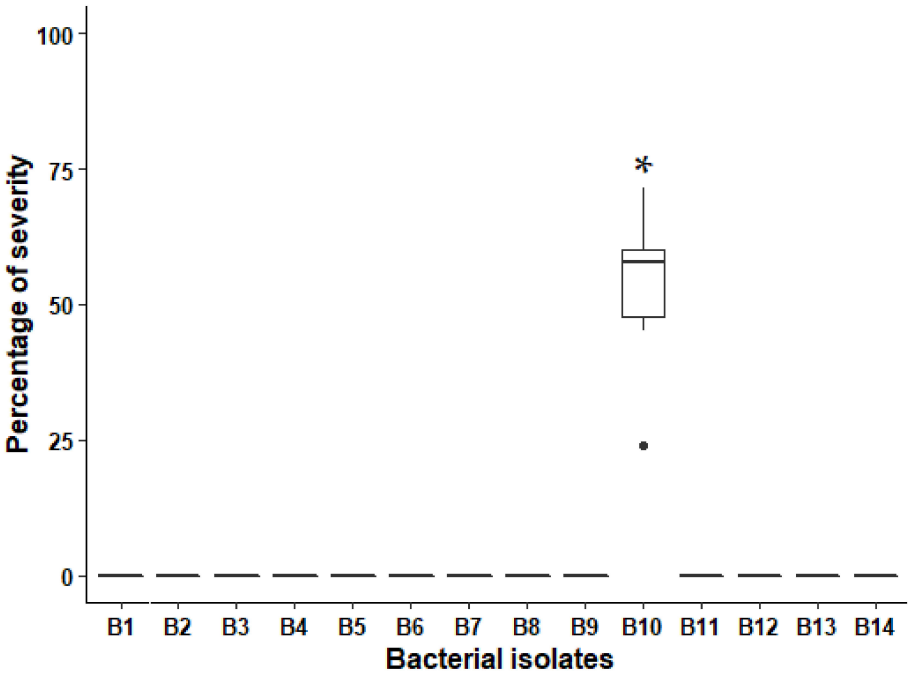

Figure 2. Boxplots of severity percentages calculated for exposed leaves to the fourteen bacterial isolates on Louise bonne cultivar using the spray method. Controls gave similar results as the non-pathogenic isolates (data not shown). Boxes represent the interquartile range, whiskers show the 10th and 90th percentiles and individual outliers are shown as points (Kruskal-Wallis test with * $p < 0.001$).

3.2. Taxonomic Identification of the Bacterial Isolate B10

The B10 isolate causing symptoms in pear trees in greenhouse conditions was subjected to molecular identification. The result of the 16S rRNA sequence alignment with BLASTn showed a very strong homology (99.84%) with *B. pumilus*, *B. safensis* and *Bacillus sp*. Thus, we conducted the whole-genome sequencing of both B10 and B10-bis (re-isolated from infected plants) isolates to acquire their precise taxonomic affiliation. For both strains, the DNA G + C content was 41.27 % and draft genome size of about 3,6 Mb (Table 1). The full size of the 16S rRNA gene sequences obtained by genome annotation of both strains were 1516 bp and shared 100% identity. The ANI and *is*DDH analyzes of both genomes gave scores of 100%, indicating that we were dealing with the same bacterial strain that was isolated twice. Thus, for the downstream analyzes, focus was made on WGS data from the B10 isolate. Details of assembly metrics and GenBank accession number for both isolates are provided in Table 1.

Table 1. Sequencing metrics and genomic data.

Parameter	Data for Strain	
	***Bacillus altitudinis* B10-bis**	***Bacillus altitudinis* B10**
Location	Greenhouse	Field
DNA library prep kit	DNA Prep kit (Illumina) (2 × 300)	DNA Prep kit (Illumina) (2 × 300)
Sequencing metrics		
No. Of total reads	2,030,980	2,169,478
Mean coverage (×)	123	136
Accession no.		
GenBank	na	JAGSNB000000000
Genomic data		
Genome size (bp)	3,649,248	3,648,564
G + C content (%)	41.27	41.27
No. of contigs	44	48
N50 (bp)	293,183	293,183

na. not available.

First, phylogenetic analysis of the 16S rRNA gene was achieved against selected type strains from *Bacillus* genus. The obtained neighbor-joining tree (Figure 3) shows B10 to be related to *B. altitudinis*, *B. aerophilus* and *B. xianmenensi*. This result suggests that B10 belong to the *B. pumilus* group, but the 16S rRNA phylogeny is not sufficient to discriminate the exact species. Thus, we used three other genes as alternative molecular markers: *gyrA*, *gyrB* and *cdaA*. Regarding the phylogenetic tree of the *cdaA* gene, strain B10 appears to have identity with strain *B. altitudinis* DSM21631^{T} with a bootstrap value of 79% (Figure 4). Similar result was obtained with the phylogenetic tree constructed from the *gyrA* gene sequences (Figure 5). The results of the identification based on the *gyrB* gene sequence analysis presented in Figure 6 also indicate a high identity with the *B. altitudinis* DSM21631^{T} strain with a bootstrap value of 100%.

To further investigate the taxonomic classification of the isolate B10, we calculated genome-related indexes between the genome sequence of this strain and the genomes of the closest species: the Average Nucleotide Identity (ANI) with values >97%, and the *in silico* DNA-DNA hybridization (*is*DDH) with values >70%, indicate belonging to the same species (Figure 7 and Table 2). Thus, the obtained results also classified the isolate B10 as *B. altitudinis,* confirming the results obtained by analyzing the *cdaA*, *gyrA* and *gyrB* phylogenies.

Figure 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of the B10 isolate belonging to *B. pumilus* group and the type strains of the genus *Bacillus.* Bootstrap values >70 are shown above branches. Bar: 0.01 substitutions per nucleotide position. Sequence accession numbers are given following strain IDs.

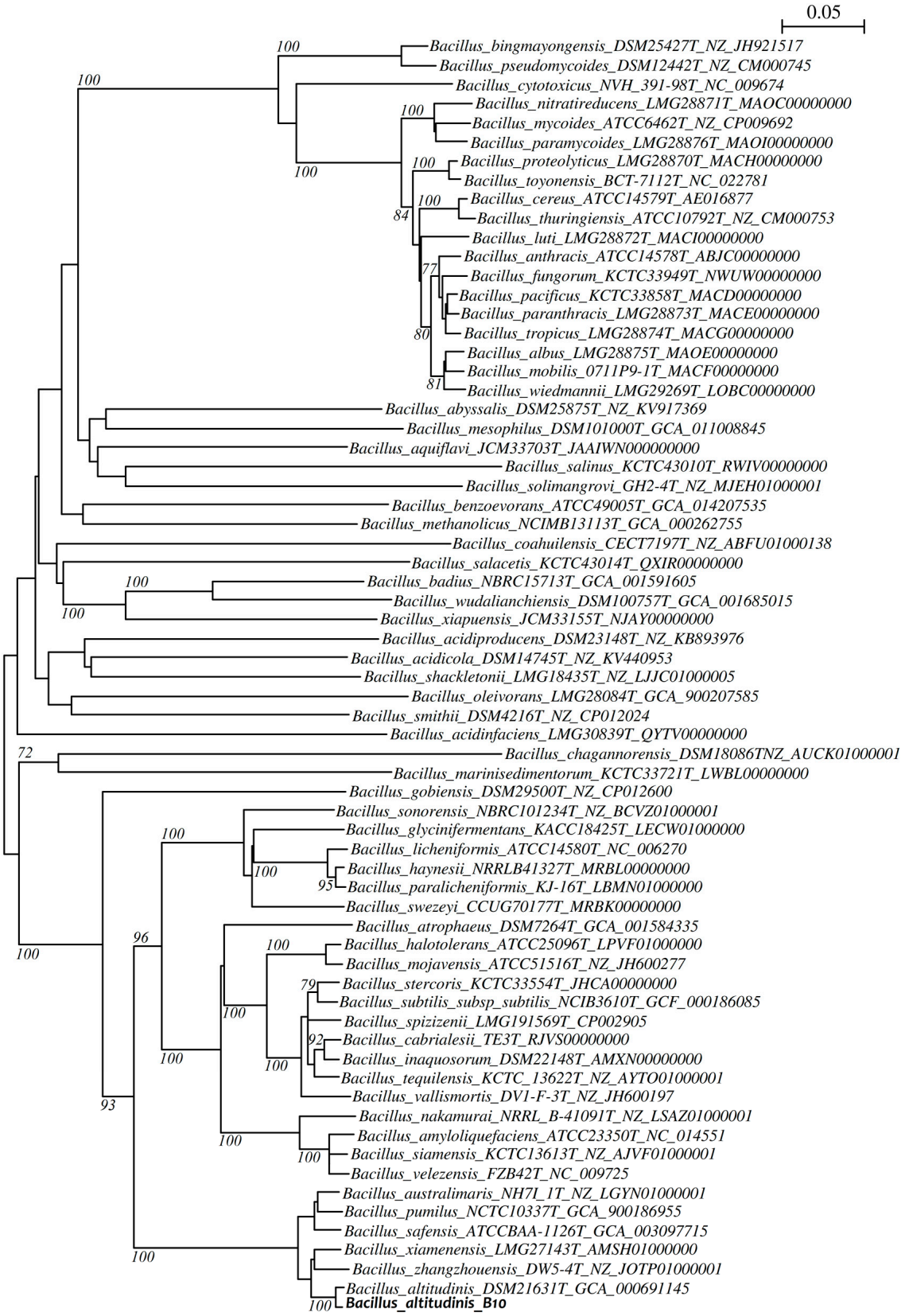

Figure 4. Neighbor-joining phylogenetic tree based on *cdaA* gene sequences showing the positions of the B10 isolate *B. altitudinis* and the type strains of the genus *Bacillus*. Bootstrap values >70 are shown above branches. Bar: 0.05 substitutions per nucleotide position. Sequence accession numbers are given following strain IDs.

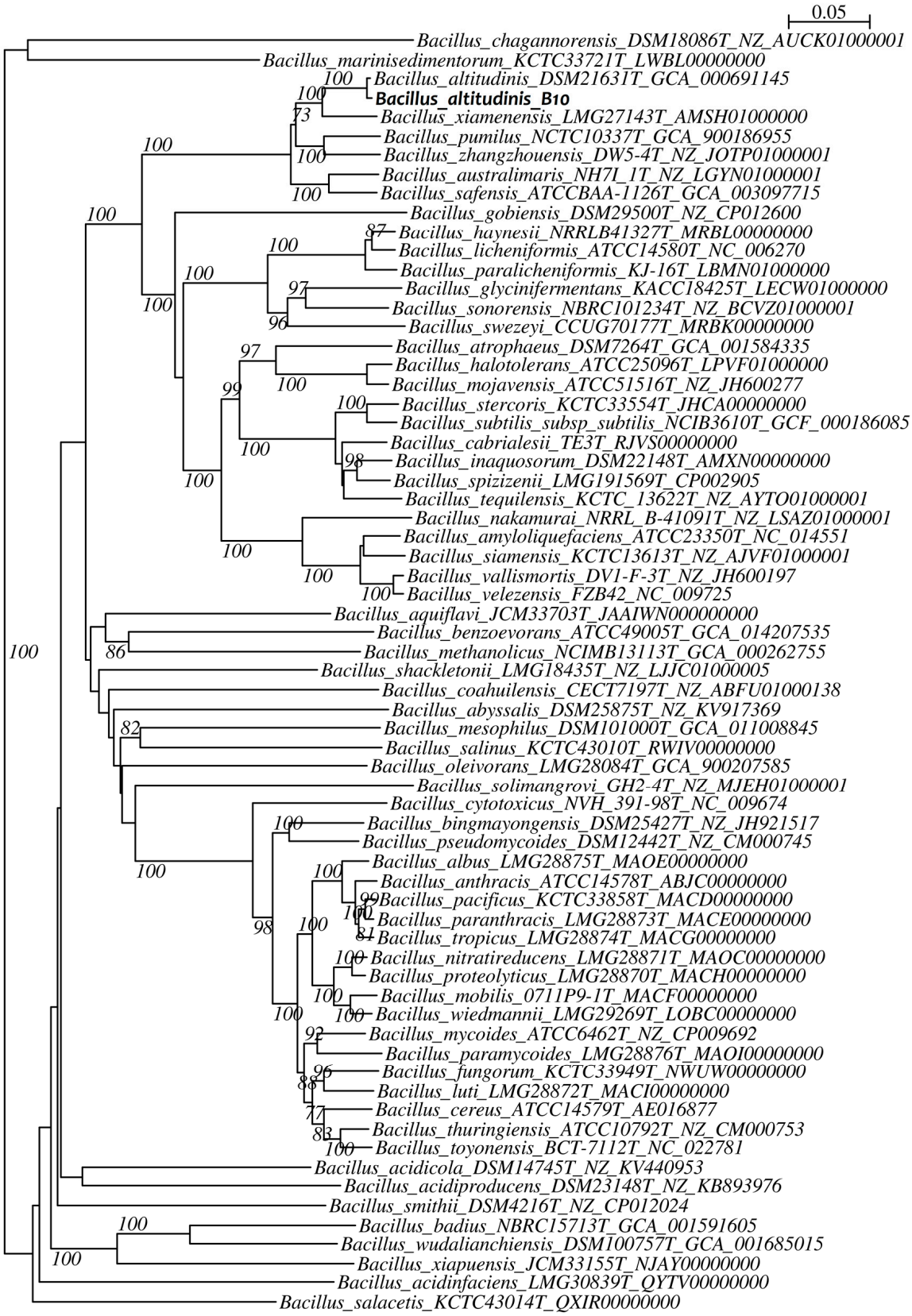

Figure 5. Neighbor-joining phylogenetic tree based on *gyrA* gene sequences showing the positions of the B10 isolate *B. altitudinis* and the type strains of the genus *Bacillus*. Bootstrap values >70 are shown above branches. Bar: 0.05 substitutions per nucleotide position. Sequence accession numbers are given following strain IDs.

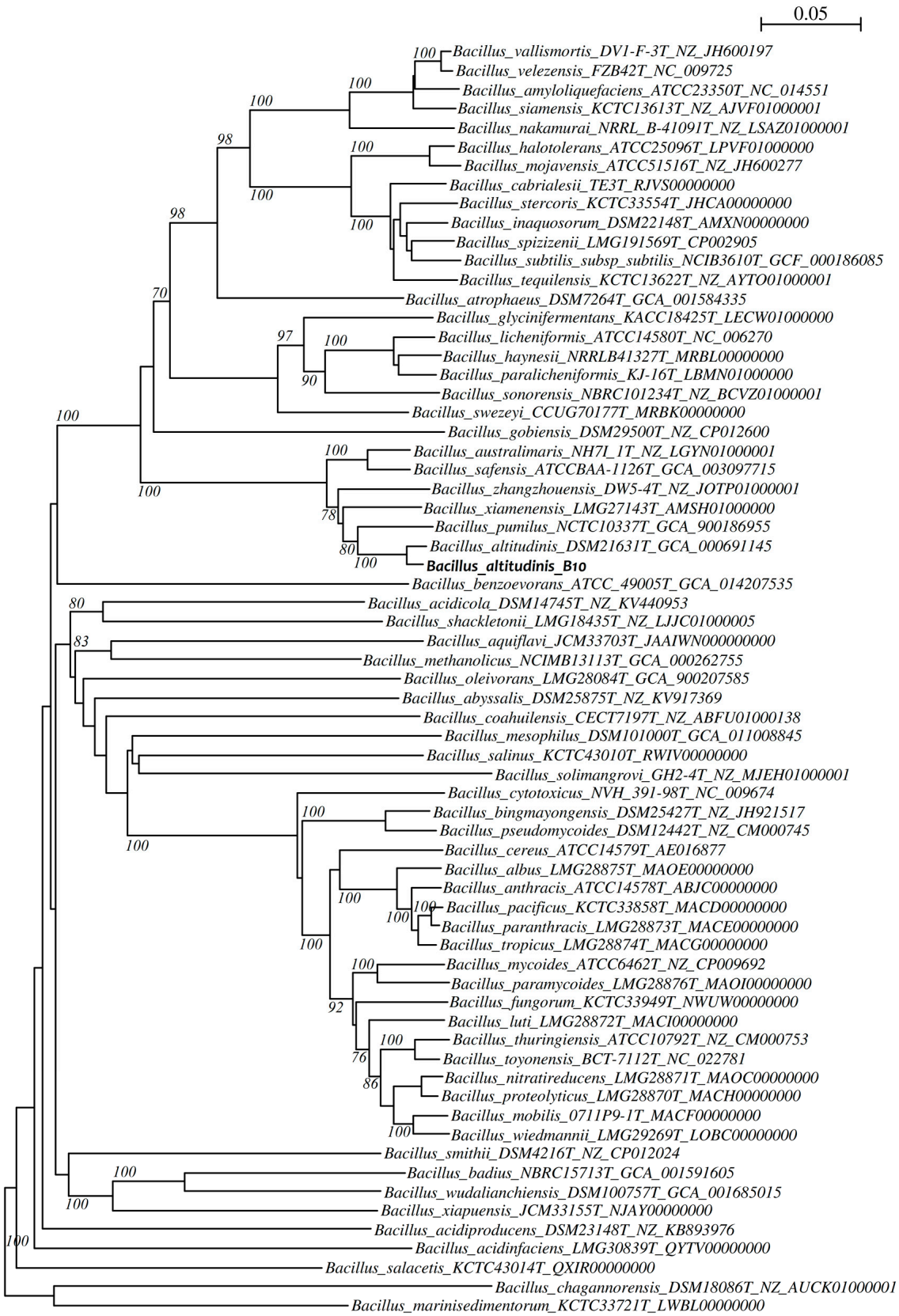

Figure 6. Neighbor-joining phylogenetic tree based on *gyrB* gene sequences showing the positions of the B10 isolate *B. altitudinis* and the type strains of the genus *Bacillus.* Bootstrap values >70 are shown above branches. Bar: 0.05 substitutions per nucleotide position. Sequence accession numbers are given following strain IDs.

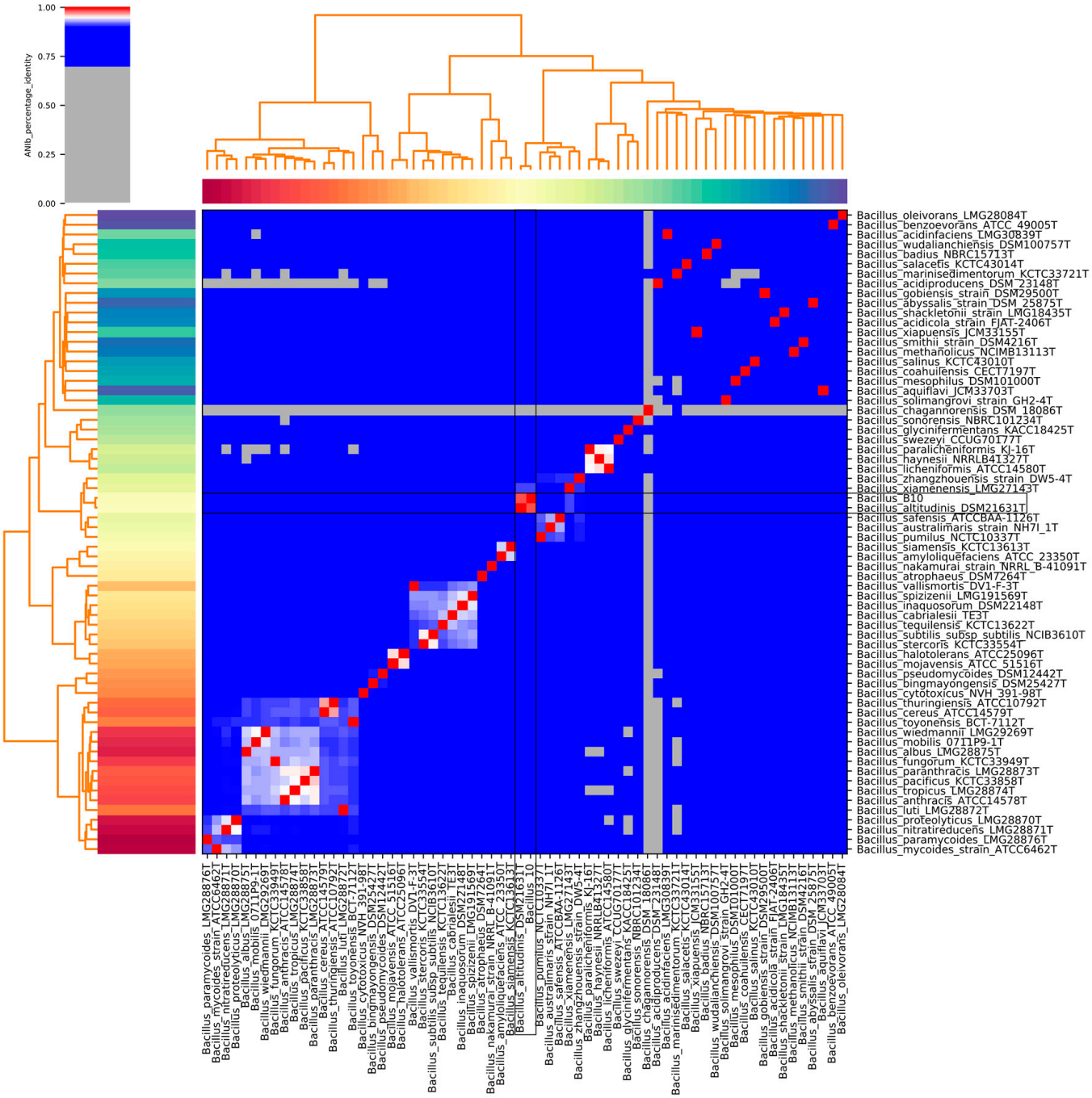

Figure 7. Average Nucleotide Identity (ANI) based on BLAST among *Bacillus* sp. B10 and type strains of the genus *Bacillus*. The ANI was calculated using the Pyani program after BLASTn alignment. The ANI above 97% between two genomes is an indication that they belong to the same species.

3.3. CAZyme Profiling

In silico screening of CAZymes within B10 genome sequence showed that this isolate encodes 177 putative genes (Table S1). Six classes were identified including carbohydrate esterases (CE, n = 21), glycoside hydrolases (GH, n = 34) and polysaccharide lyases (PL, n = 2) that are known as plant cell wall degrading enzymes. The three other classes with indirect roles in degrading carbohydrates are auxiliary activity (AA, n = 3), carbohydrate binding module (CBM, n = 19) and glycosyl-transferase (GT, n = 38).

Table 2. Average nucleotide identity based on BLAST (ANIb) and pairwise in silico DNA-DNA hybridization (*is*DDH) using GGDC formula 2 values for the *Bacillus* sp. B10 isolate and the most closely related members of the genus *Bacillus*. The numbers below the diagonal are ANI values predicted between pairs of genomes. The numbers above the diagonal are *is*DDH values between genomes. Strains: 1, *Bacillus* sp. B10; 2, *B. altitudinis* DSM21631^T; 3, *B. xiamenensis* LMG27143^T; 4 *B. zhangzhouensis* DW5-4^T; 5, *B. safensis* ATCCBAA-1126^T; 6, *B. pumilus*NCTC10337^T; 7, *B. australimaris* NH7I_1^T.

	1	**2**	**3**	**4**	**5**	**6**	**7**
1	-	83.8	44	39.2	36.4	36.3	35.9
2	98.1	-	44	39.2	36.4	36.1	35.8
3	91.3	91.3	-	42.2	37.6	36.7	37.2
4	89.8	89.8	90.9	-	42.3	41.7	41.6
5	88.9	88.8	89.3	90.8	-	45.1	52
6	88.7	88.7	88.9	90.6	91.6	-	43.5
7	88.6	88.6	89.1	90.6	93.3	91.2	-

4. Discussion

We isolated fourteen bacterial strains from pear plant samples showing burn symptoms. Pathogenicity test was performed in greenhouse under natural photoperiod conditions and validated B10 strain as responsible of the symptoms observed in the field.

The *Bacillus* genus is ubiquitous and can be found in soil (including plant rhizosphere), water, air and food as contaminant [43,44]. Decoding pathogen's taxonomy is required to understand its evolution and epidemiology. In this work, the taxonomic identification of B10 isolate was firstly achieved using the 16S RNA gene sequence. This gene has both conserved and variable regions, making it as the most widely used molecular marker for bacterial classification [45]. The sequence analysis indicated that B10 isolate belongs to *Bacillus* spp., with no evidence regarding the bacterial species (Figure 3). Thus, we performed whole genome sequence (WGS) analysis to acquire insights into the taxonomic affiliation and pathogenicity traits of this isolate. To obtain a finer identification, three genomic makers retrieved from the WGS dataset were used (i.e., *cdaA*, *gyrA* and *gyrB*). The *cdaA* gene (previously called *ybbP*) was proposed for a more discriminative classification of species within the *B. pumilus* group [46]. This gene is conserved in almost all Firmicutes (but not all bacteria) and was reported as responsible for the synthesis of cyclic di-AMP, a messenger necessary for cell wall homeostasis [47]. The two other genes, *gyrA* and *gyrB*, are universally distributed among bacterial species. They encode the A and B subunits of DNA gyrase, respectively, and play an essential role in DNA replication. The phylogenies obtained for these three genes allowed to classify the B10 strain as *B. altitudinis*. The higher bootstrap values were obtained for the *gyrA* and *gyrB* genes compared to 16S rRNA gene, providing a better resolution for taxonomic classification of species from the *Bacillus* genus. This gene-marker based classification was confirmed using the average nucleotide identity (ANI) and the in silico DNA-DNA hybridization analyzes applied for B10 genome sequence compared to sixty-five species from *Bacillus* genus (Figure 7 and Table 2).

Some species within the *Bacillus* genus were reported as phytopathogens such as *B. subtilis*, the causative agent of the post-harvest soft root [48]. *B. pumilus* was associated with the bacterial burn on pear in Turkey and with the ginger rhizome rot [14]. In the case of *B. altitudinis*, several reports describe it as a biocontrol agent used against fungal and bacterial pathogens [49,50], or as a plant growth-promoting bacteria (PGPB) able to produce phytohormones or to enhance mineral nutrition [51]. For its pathogenicity effect, the data available are scarce. In a previous study, Elbanna et al., reported *B. altitudinis* as responsible for the high soft rot on different apple and pear cultivars [8]. Similar results were reported on potatoes [52] and pomegranates [53]. However, so far no study has yet reported virulence factors in *B. altitudinis* and the host range of *B. altitudinis* is not yet determined, which suggests that the application of this bacterium as a biocontrol agent remains hazardous. Thus, we propose that specific genotypes (or pathovars) of

this bacterial species represent an emerging group of phytopathogens infecting, at least, *Rosaceae* family.

By comparing the two methods of inoculation, we concluded that the spraying method caused more damages compared to the infiltration method. This indicates that the external contact is sufficient and even more efficient to provoke the disease symptoms. In fact, under the spray inoculation method of B10 isolate, the necrotic lesions on Louise bonne cv. leaves appeared after two weeks, confirming that this bacterial isolate does not need wounds to induce such lesions, suggesting the involvement of hydrolytic enzymes that decompose vegetable cells and tissues. The WGS dataset screening for such enzymes confirmed that B10 genome sequence uncovers, at least, three classes known as plant cell wall degrading enzymes (i.e., carbohydrate esterases, glycoside hydrolases and polysaccharide lyases). Such traits will be investigated within in vitro assays in a future study to evaluate the expression of such enzymes and their potential to degrade plant cell tissues. In addition, a pan-genome analysis will help us to identify the pathovar clade regrouping such pathogenic strains of the *B. altitudinis* population, with the identification of virulence traits shared at the core and/or accessory genome levels.

5. Conclusions

Based on the phylotaxonomic analysis, the pathogenicity tests and the in silico CAZymes screening results, we report here the first genome sequence and characterization of a phytopathogenic *B. altitudinis* isolate in Morocco. Our study demonstrates that the spraying method remains the best method to test the pathogenicity of *B. altitudinis* on pear plants *in planta*. In addition, the whole genome sequencing and analysis is more accurate compared to 16S rRNA gene sequencing in identifying the tested bacterial isolates. Furthermore, the pathogenicity tests conducted here may be useful in acquiring insights into "pathovars" from this bacterial species. Future work is needed to study the expressed CAZymes and other virulence genes that induce bacterial burn symptoms in pear trees.

Supplementary Materials: The following is available online at https://www.mdpi.com/article/10.3390/agronomy11071344/s1, Table S1: Full list of carbohydrate active enzymes functionally annotated by HMMER/Hotpep analysis of the Bacillus altitudinis B10 genome.

Author Contributions: N.L. and N.Z. performed field sampling. N.L. and H.J. performed greenhouse pathogenicity experiments. A.M.B. performed whole genome sequencing and bioinformatics/biostatistics analyzes. A.M. performed 16S rRNA gene sequencing. N.L. and A.M.B. wrote the manuscript. N.L., K.N., A.M. and A.M.B. reviewed the manuscript. A.D. co-supervised the research work. K.N. supervised the research work. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by collaboration with the Laboratory of Microbiology, Signals and Micro-environment (LMSM EA4312), University of Rouen-Normandy, Evreux, France.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: We acknowledge the contribution of the Normandy Network of Microbial Metagenomics (RNAmb), supported by Région Normandie and European Union (FEDER). We thank Belarbi Lahcen for helping in samples collection in the farms. The authors thank Srinivasan Ramanathan for proofreading the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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