



Original Research

Sequence comparison of *phoR*, *gyrB*, *groEL*, and *cheA* genes as phylogenetic markers for distinguishing *Bacillus amyloliquefaciens* and *B. subtilis* and for identifying *Bacillus* strain B29

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Abstract: Given the close genetic relationship between *Bacillus amyloliquefaciens* and *B. subtilis*, distinguishing the two solely based on their physiological and biochemical characteristics and 16S rRNA sequences is difficult. Molecular identification was used to discover suitable genes for distinguishing the two bacteria, and to identify the bio-controlling strain B29, due to molecular identification has been paid more and more attention. The similarity of four genes, *cheA*, *gyrB*, *groEL* and *phoR*, of the two species was compared by the software BLASTN and MAGA, and phylogenetic tree was constructed. The B29 strain was re-identified by using the screened genes. The similarities of the four genes, *gyrB*, *groEL*, *cheA* and *phoR*, of the two species were 93-95%, 82-84%, 76-78% and 76-77%, respectively. The homologies of the four genes of the strain B29 and the strains of *B. amyloliquefaciens* strains were more than 95%. We determined how well the *phoR* and *cheA* genes could be used to differentiate *B. amyloliquefaciens* and *B. subtilis*. The previously isolated biological control strain B29, initially classified as *B. subtilis*, was re-classified as *B. amyloliquefaciens*. Our data indicate that other than the *phoR* gene, the *cheA* gene might be a useful phylogenetic marker for differentiating *B. subtilis* and *B. amyloliquefaciens*.

Key words: Biological control; *Bacillus subtilis*; *Bacillus amyloliquefaciens*; Phylogeny; Molecular classification.

Introduction

The genus *Bacillus* contains gram-positive bacteria and it is widely used in biological control given its convenient cultivation and storage (1). Two *Bacillus* species, *B. amyloliquefaciens* and *B. subtilis*, produce gibberellin, indole acetic acid (2), extracellular phytase (3), chitinases (4,5), and antifungal peptides (6-9), biologically active molecules that promote plant growth and thus improve crop production (10-13). *B. amyloliquefaciens* and *B. subtilis* share similar phenotypic characteristics and are genetically closely related. Previously, *B. amyloliquefaciens* was considered a subspecies of *B. subtilis* before it was identified as a separate species in 1967.

Given the close genetic relationship between *B. amyloliquefaciens* and *B. subtilis*, distinguishing the two based solely on their physiological and biochemical characteristics is very difficult; incorrect classification of related strains might affect their use in biological control. Currently, there are no sensitive methods/gene sequences for distinguishing *B. amyloliquefaciens* and *B. subtilis*, which hampers the classification of new isolated strains.

Analysis of the 16S rRNA gene sequence is the most frequently used method for constructing bacterial phy-

logenetic relationships and for distinguishing species with up to 98.7% sequence similarity (15). However, its use is limited in closely related species due to their high sequence similarity (99–100%) (16). Thus, many researchers are discovering more phylogenetic genes to distinguish bacteria at the species level. Generally, housekeeping genes encoding proteins involved in the central metabolism of organisms evolve faster than the 16S rRNA gene does, and can be used to distinguish closely related species (17). Presently, several genes are used to identify bacterial species or for identification within specific bacterial genera. The *gyrB* genes encode the subunit B protein of DNA gyrase, a type II DNA topoisomerase that plays an important role in DNA replication and prokaryotic transcription that is widely distributed in bacteria (18,19). The *gyrB* gene sequence has been used in the phylogenetic analysis of many bacterial genera, including *Bacillus* (20,21). In bacteria, the *groEL* gene encodes a 60-kD heat shock protein that is involved in maintaining normal physiological functions, and is a well-established phylogenetic marker for detecting many bacteria (22,23). The *phoR* gene encodes a histidine kinase belonging to the histidine kinase protein superfamily expressed in many bacteria (24); *phoR* is universally distributed in bacteria and is a single-copy gene (25) A recent study indicated

that the *phoR* gene is a useful marker for differentiating *B. subtilis* subspecies (25).

The *cheA* gene encodes a histidine kinase that couples environmental stimuli to bacterial swimming motions (26). CheA converts the sensory signal detected by transmembrane chemoreceptors into a cytosolic chemical signal via autophosphorylation. The phosphorylated CheA induces CheY phosphorylation; phosphorylated CheY is distributed in the cytoplasm and it interacts with flagellar motors, resulting in reversed flagellar rotation and cell chemotactic movement. Six proteins are involved in this action; CheA is the pivotal protein for chemotactic movement.

The biological control *Bacillus* strain B29 was isolated from major soybean varieties in Heilongjiang Province, China. B29 has biological activity against many soil-borne fungal diseases and exhibits >90% efficacy against *Fusarium oxysporum* (27). Based on its physiological and biochemical characteristics and 16S rRNA sequence, B29 was previously identified as *B. subtilis*. However, following whole-genome sequencing, we found that more genes in B29 exhibited high similarity with that of *B. amyloliquefaciens*. To classify the species for B29 accurately, we first compared the usefulness of several candidate markers for differentiating *B. amyloliquefaciens* and *B. subtilis*, and then determined the phylogenetic taxonomy for B29. As one gene may provide insufficient information on the genomic differences between closely related species, we used four gene sequences to distinguish *B. amyloliquefaciens* and *B. subtilis*.

Materials and Methods

Strains

We used two type strains: *B. subtilis* subspecies (subsp.) *subtilis* strain (str.) 168 (GenBank Accession No. GI: 728882887, CP010052.1) and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 (GenBank Accession No. GI: 154350369, CP000560.1), for the sequence comparison. The B29 experimental strain was isolated from major soybean varieties and preserved in our laboratory (preservation no.: CGMCC 0752). B29 is a highly effective broad-spectrum biological control strain that inhibits the growth of many fungi that infect plants, including *F. oxysporum* f. *cucumerinum*, *Rhizoctonia solani*, and *Pythium* spp. We sequenced the entire B29 genome and labeled its genes.

The control strains were the strains containing the whole-genome sequence of, which are available from the National Center for Biotechnology Information (NCBI) GenBank database, and specific genes had been labeled (Table 1 lists the NCBI accession numbers).

Comparison between genes

The 16S rRNA, *phoR*, *gyrB*, *cheA*, and *groEL* gene sequences of the above-mentioned strains were obtained from the NCBI GenBank database. We compared the sequence similarity among the five genes between *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 and among *B. subtilis* or *B. amyloliquefaciens* using BLASTN 2.2.30+ (NCBI). Then, we compared the sequence similarity of the five genes between *B. subtilis* subsp. *subtilis* str. 168

and other *B. amyloliquefaciens* strains and between *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 and *B. subtilis* strains (from NCBI) using BLASTN 2.2.30+.

Species identification and phylogenetic analysis of B29 based on *cheA* and *phoR* gene sequences

The sequence comparison between B29 and the *B. amyloliquefaciens* and *B. subtilis* strains was aligned using BLAST (NCBI). Phylogenetic analysis was performed using MEGA 6.0 (<http://www.megasoftware.net/>); the genetic distances were calculated and phylogenetic trees were constructed using the neighbor-joining method with bootstrap values based on 1000 replications.

Results

Comparison between *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42

There was only 80% genome-level similarity between *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 (Figure 1), further indication that *B. subtilis* and *B. amyloliquefaciens* are different species. Table 2 lists the analysis results of the similarities between the five genes in the two type strains. There was 99% similarity between the 16S rRNA genes in the two type strains, followed by 93% similarity between the *groEL* genes and 82% similarity between the *gyrB* genes; the *cheA* and *phoR* genes had the least similarity (both 77% similarity).

Comparison of genes among species and between type strains and control strains

Next, we compared the five genes among one species and between one type strain and other control strains. The genes all had 99% similarity in *B. subtilis* and 98–99% similarity in *B. amyloliquefaciens*. There was lower similarity between the *phoR*, *gyrB*, *cheA*, and *groEL* genes in *B. subtilis* subsp. *spizizenii* str.

Table 2. Similarity between five genes in *B. subtilis* str. 168 and *B. amyloliquefaciens* str. FZB42.

Gene	Similarity (%)
16sRNA	99
<i>phoR</i>	77
<i>gyrB</i>	82
<i>cheA</i>	77
<i>groEL</i>	93

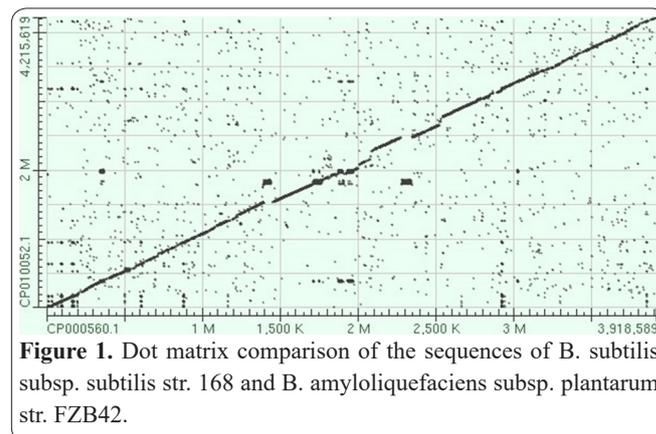


Figure 1. Dot matrix comparison of the sequences of *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42.

Table 1. (A). Comparison of five genes among species and between type strains and control strains. **(B).** Comparison of five genes among species and between type strains and control strains.

	NCBI accession number	Comparison among <i>B. amyloliquefaciens</i> (%)					Comparison with <i>B. subtilis</i> str. 168 (%)				
		16S rRNA	gyrB	groEL	cheA	phoR	16S rRNA	gyrB	groEL	cheA	phoR
<i>B. a.</i> str. FZB42	CP000560.1						99	82	92	77	77
<i>B. a.</i> str. L-H15	CP010556.1	100	99	99	98	98	99	82	93%	76	77
<i>B. a.</i> DSM7	FN597644.1	99	99	97	95	94	99	81	93%	76	77
<i>B. a.</i> subsp. <i>amyloliquefaciens</i> KHG19	CP007242.1	99	99	99	99	98	99	81	93	77	77
<i>B. a.</i> CC178, complete genome	CP006845.1	100	100	100	100	100	99	82	93	77	77
<i>B. a.</i> XH7, complete genome	CP002927.1	99	96	97	95	94	99	81	93	76	77
<i>B. a.</i> subsp. <i>plantarum</i> TrigoCor1448	CP007244.1	99	99	98	99	99	99	82	93	77	77
<i>B. a.</i> LFB112	CP006952.1	99	99	99	98	98	99	82	93	76	77
<i>B. a.</i> subsp. <i>plantarum</i> NAU-B3	HG514499.1	99	99	99	98	98	99	82	94	76	76
<i>B. a.</i> subsp. <i>plantarum</i> UCMB5033	HG328253.1	99	99	99	99	98	99	82	93	77	76
<i>B. a.</i> subsp. <i>plantarum</i> UCMB5036	HF563562.1	99	99	99	99	99	99	82	93	77	77
<i>B. a.</i> subsp. <i>plantarum</i> YAU B9601-Y2	HE774679.1	99	99	99	98	99	99	81	93	76	76
<i>B. a.</i> subsp. <i>plantarum</i> CAU B946	HE617159.1	99	99	98	98	98	99	82	93	76	77
<i>B. a.</i> IT-45	CP004065.1	99	99	99	98	98	99	82	93	76	77
<i>B. a.</i> subsp. <i>plantarum</i> AS43.3	CP003838.1	99	99	99	99	99	99	82	93	77	77
<i>B. a.</i> subsp. <i>plantarum</i> UCMB5113	HG328254.1	100	99	99	99	98	99	82	93	77	76
<i>B. a.</i> SQR9, complete genome	CP006890.1	99	99	99	99	99	99	82	93	76	77
<i>B. a.</i> TA208	CP002627.1	99	99	97	95	94	99	81	93	76	77
<i>B. a.</i> LL3 chromosome	CP002634.1	99	96	97	95	94	99	81	93	76	77
Average		99	99	99	98	98	99	82	93	76	77

	NCBI accession number	Comparison among <i>B. subtilis</i> 168 (%)					Comparison with <i>B. a.</i> FZB42 (%)				
		16S rRNA	gyrB	groEL	cheA	phoR	16S rRNA	gyrB	groEL	cheA	phoR
<i>B. s.</i> subsp. <i>subtilis</i> str. 168	CP010052.1						99	82	93	77	77
<i>B. s.</i> str. PS832	CP010053.1	100	100	100	100	100	99	82	93	77	77
<i>B. s.</i> subsp. <i>subtilis</i> 6051-HGW	CP003329.1	100	100	100	100	100	99	82	93	77	77
<i>B. s.</i> str. SG6	CP009796.1	99	99	99	99	99	99	82	93	77	77
<i>B. s.</i> BEST7613 DNA	AP012495.1	100	100	97	100	100	99	82	95	77	77
<i>B. s.</i> subsp. <i>spizizenii</i> str. W23	CP002183.1	99	94	98	95	94	99	82	93	78	77
<i>B. s.</i> subsp. <i>spizizenii</i> TU-B-10	CP002905.1	99	95	98	95	93	99	82	93	78	77
<i>B. s.</i> BEST7003 DNA	AP012496.1	100	100	97	100	100	99	82	95	77	77
<i>B. s.</i> subsp. <i>subtilis</i> str. 3NA	CP010314.1	100	100	100	100	100	99	82	93	77	77
<i>B. s.</i> PY79	CP006881.1	99	100	100	100	100	99	82	93	77	77
<i>B. s.</i> BSn5	CP002468.1	99	99	99	99	99	99	82	93	77	76
<i>B. s.</i> subsp. <i>subtilis</i> str. BAB-1	CP004405.1	99	99	98	99	99	99	82	94	77	76
<i>B. s.</i> subsp. <i>subtilis</i> RO-NN-1	CP002906.1	99	99	98	99	98	99	82	93	77	77
<i>B. s.</i> subsp. <i>subtilis</i> str. OH 131.1	CP007409.1	99	99	99	99	99	99	82	93	77	77
<i>B. s.</i> XF-1	CP004019.1	100	99	98	99	99	99	82	94	77	76
<i>B. s.</i> subsp. <i>natto</i> BEST195 DNA	AP011541.2	99	99	98	99	99	99	82	94	77	77
<i>B. s.</i> subsp. <i>subtilis</i> str. AG1839	CP008698.1	100	100	100	100	100	99	82	93	77	77
<i>B. s.</i> subsp. <i>subtilis</i> str. BSP1	CP003695.1	100	99	98	99	99	99	82	93	77	77
<i>B. s.</i> subsp. <i>spizizenii</i> str. NRS 231	CP010434.1	99	94	98	95	94	99	82	93	78	77
<i>B. s.</i> subsp. str. JH642	CP007800.1	100	100	100	100	100	99	82	93	77	77
<i>B. s.</i> QB928	CP003783.1	99	100	100	100	100	99	82	93	77	77
Average		99	99	99	99	99	99	82	93	77	77

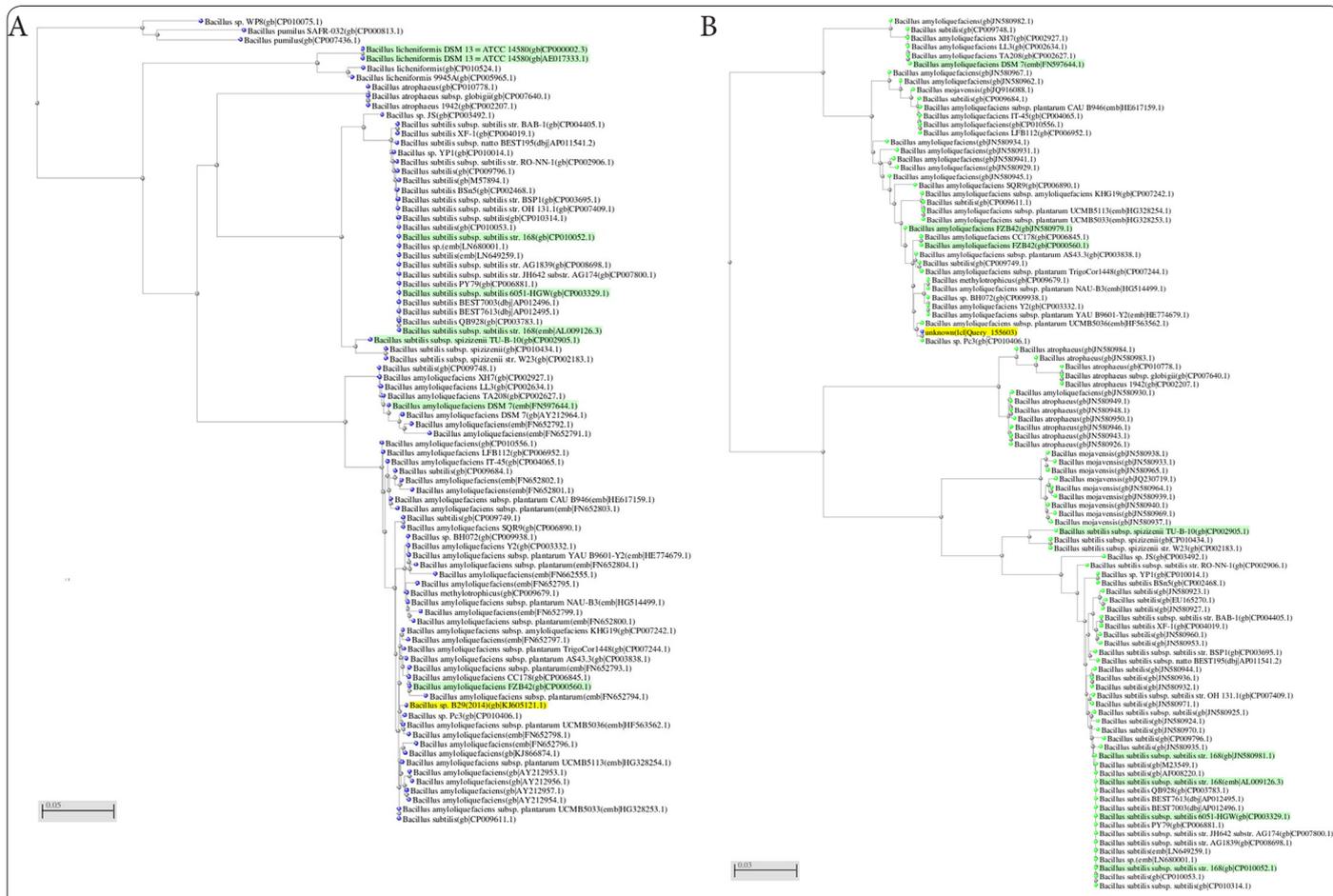


Figure 2. Phylogenetic trees constructed based on the *cheA* gene (A) and *phoR* gene (B). Identification of B29.

W23, *B. subtilis* subsp. *spizizenii* TU-B-10, and *B. subtilis* subsp. *spizizenii* strain NRS 231 than in the other strains. There was lower similarity between the *phoR*, *gyrB*, *cheA*, and *groEL* genes of *B. amyloliquefaciens* XH7, *B. amyloliquefaciens* TA208, and *B. amyloliquefaciens* LL3 chromosome as compared to that of the other *B. amyloliquefaciens* strains.

Comparison of the control strains with *B. subtilis* subsp. *subtilis* str. 168 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 revealed that the 16S rRNA gene in both type strains shared 99% similarity with the other *B. subtilis* and *B. amyloliquefaciens* strains. There was 76–77%, 82%, 77–78%, and 93–94% similarity between the *phoR*, *gyrB*, *cheA*, and *groEL* genes, respectively, of *B. subtilis* subsp. *subtilis* str. 168 and the other *B. amyloliquefaciens* strains.

There was 76–77%, 81–82%, 76–77%, and 93–95% similarity between the *phoR*, *gyrB*, *cheA*, and *groEL* genes, respectively, of *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 and the other *B. subtilis* strains.

Phylogenetic tree construction

The above data suggest that the 16S rRNA gene cannot be used to differentiate *B. amyloliquefaciens* and *B. subtilis*, nor is the *groEL* gene a good gene for phylogenetic tree construction, given the high level of similarity of the gene between the two species. Although there was 81–82% similarity between the *gyrB* genes of the two species, the 80% similarity between *B. amyloliquefaciens* subsp. *plantarum* str. FZB42 and *B. subtilis* subsp. *subtilis* str. 168 indicate that the gene is not ideal for distinguishing the two species. Therefore, we selected the *cheA* and *phoR* genes for the phylogenetic tree

construction.

We constructed phylogenetic trees based on the *cheA* (Figure 2A) and *phoR* genes (Figure 2B). *B. subtilis* strains and *B. amyloliquefaciens* were located in two clades in both trees. The *cheA* gene phylogenetic tree placed *B. pumilus*, *B. licheniformis*, and *B. atrophaeus* in three different clades; the *phoR* gene phylogenetic tree placed *B. atrophaeus*, *B. mojavensis*, and *B. subtilis* subsp. *spizizenii* in three distinct clades. Three *B. subtilis* strains: BS-916, ATCC13592, and ATCC19217, were in the same clade as *B. amyloliquefaciens* strains. The *phoR*, *gyrB*, *groEL*, and *cheA* genes from these strains shared close similarity with that of *B. amyloliquefaciens* subsp. *plantarum* str. FZB42. Thus, these three strains may warrant further re-classification.

Table 3 lists the comparison analysis of the *phoR*, *gyrB*, *groEL*, and *cheA* genes between B29 and the *B. subtilis* or *B. amyloliquefaciens* strains. There was >95% similarity among the four genes between B29 and the *B. amyloliquefaciens* strains, which was about 20% greater similarity than that between B29 and the *B. subtilis* strains. The *cheA* gene phylogenetic tree indicated a close genetic distance between B29 and *B. amyloliquefaciens* subsp. *plantarum* str. FZB42, a wide genetic

Table 3. Similarities between four genes from B29 and other strains available in the NCBI database.

Gene	<i>B. subtilis</i> (%)	<i>B. amyloliquefaciens</i> (%)
phoR	70–77	95–99
gyrB	81	98–99
groEL	93–97	98–99
cheA	70–78	95–99

distance between B29 and *B. subtilis* subsp. *subtilis* str. 168, and that B29 belonged to a different sub-cluster. Thus, B29 should be classified as *B. amyloliquefaciens*.

Discussion

To better distinguish *B. subtilis* and *B. amyloliquefaciens*, we compared the 16S rRNA, *phoR*, *gyrB*, *groEL*, and *cheA* genes from strains of the two species. Phylogenetic trees based on the *cheA* and *phoR* genes were constructed, enabling successful differentiation of *B. subtilis* and *B. amyloliquefaciens*. Using the *cheA* and *phoR* phylogenetic trees, we determined that B29 is a strain of *B. amyloliquefaciens*.

Generally, a phylogenetic gene is widely distributed in most microbes as a single copy of a certain length, can be cloned easily, and predicts whole-genome relationships accurately (28). The 16S rRNA gene is the most commonly used gene for such studies; however, it is not suitable for differentiating species within the same genus because of high inter-species similarity. Consequently, more sensitive genes should be used for distinguishing closely related species.

The *groEL* gene is a well-established and widely used phylogenetic marker in many bacteria (22,23). However, our data revealed 93–95% similarity between the *groEL* genes of *B. subtilis* and *B. amyloliquefaciens*, indicating it is not a good phylogenetic marker.

The *gyrB* gene is a 1.2–1.4-kb single-copy housekeeping gene that has an average base substitution rate of 0.7–0.8% every 100 million years; it evolves more quickly than the 16S rRNA gene, which has an average base substitution rate of 1% every 5000 million years (18,21,29). The *gyrB* gene is commonly distributed in bacteria without horizontal transfer. Moreover, it contains conserved sequences and variable regions, enabling amplification from different bacterial species and intra-species differentiation, respectively. Given these advantages, the *gyrB* gene is better suited for differentiating closely related species than are rRNA genes. In recent years, the *gyrB* gene has been widely used as a phylogenetic gene in different arenas (21,30). The *phoR* gene encodes a histidine kinase belonging to the superfamily of histidine kinase proteins expressed in many bacteria (24) and is a phylogenetic marker that differentiates *B. subtilis* subspecies (25). Our data revealed 82–84% similarity between the *gyrB* genes and 76–78% similarity between the *phoR* genes of *B. subtilis* and *B. amyloliquefaciens*, suggesting these two genes might be good phylogenetic markers for differentiating the two. The *cheA* gene is a single-copy housekeeping gene and has not been reported as a phylogenetic gene. However our data showed that there was 76–77% similarity between the *cheA* genes of *B. subtilis* and *B. amyloliquefaciens*, suggesting it may be a good phylogenetic marker for differentiating the two.

The International Committee on Systematic Bacteriology considers individuals with >70% similarity to be one species (31). However, a single gene may be insufficient for molecular differentiation. Combining ≥ 2 genes may be a future trend in genetic classification. Therefore, we used the *cheA* and *phoR* genes to differentiate *B. subtilis* and *B. amyloliquefaciens*.

There were similarities between the *phoR*, *gyrB*,

groEL, and *cheA* genes of *B. subtilis* and *B. amyloliquefaciens*. The *groEL* genes shared >93% similarity and were not suitable for distinguishing the two. There was 81–82% similarity between the *gyrB* genes of the two species, indicating it is a potential phylogenetic gene. However, the inter-species similarities of the *groEL* and *gyrB* genes closely resemble the intra-species similarities, and they cannot be used to distinguish between species effectively. There was <70% inter-species similarity and >95% intra-species similarity among the *cheA* and *phoR* genes, indicating they are potential phylogenetic genes. Further inter-species comparisons confirmed their potential as phylogenetic genes.

We know of no previous report involving the use of the *cheA* gene in taxonomy. In this study, we differentiated the closely related *B. subtilis* and *B. amyloliquefaciens* using a *cheA* gene-based phylogenetic tree. However, further studies are required before the *cheA* gene is confirmed as a phylogenetic marker for differentiating *B. subtilis* and *B. amyloliquefaciens*.

We spliced the *phoR*, *gyrB*, *groEL*, and *cheA* genes of the B29 strain based on their genome sequences, and identified B29 using *cheA* and *phoR* gene-based phylogenetic trees. We found that B29 belongs to *B. amyloliquefaciens* and that its previous classification was incorrect.

Microbial taxonomy is a rigorous subject: incorrectly classifying one strain can adversely affect its application. B29 is a biological control strain with high economic value and social benefit, thus it would be of great importance to classify it correctly. In China, many isolated biological control strains have been classified based only on their physiological and biochemical characteristics or the 16S rRNA gene (32), which might be inaccurate, thus classification might be incorrect. The rapid development of high-throughput sequencing techniques and bioinformatics analysis as facilitated the identification of bacterial strains, thus gene-based species identification is becoming increasingly important. Hence, discovering more housekeeping genes for taxonomy will provide a solid foundation for the further study of microorganisms.

In conclusion, we compared the similarities among five genes from *B. subtilis* and *B. amyloliquefaciens* and found that the *cheA* and *phoR* genes are potential phylogenetic genes. Phylogenetic trees based on the two genes successfully distinguished *B. subtilis* and *B. amyloliquefaciens*. Based on the phylogenetic trees, the B29 strain was classified as *B. amyloliquefaciens*. Other than the *phoR* gene, the *cheA* gene might be a useful phylogenetic gene for differentiating *B. subtilis* and *B. amyloliquefaciens*, although confirmation of this requires further study.

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