Taxonomic note: speciation within the operational group *Bacillus amyloliquefaciens* based on comparative phylogenies of housekeeping genes

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Abstract. Many of the publically available *Bacillus* 16S rRNA genes and genomes in the NCBI database are inconsistently assigned as *B. amyloliquefaciens*. The highly conserved nature of the 16S rRNA gene makes it fail to differentiate species within the operational group *B. amyloliquefaciens*. Here, comparative phylogenies of the complete 16S rRNA, *gyrB*, *rpoB*, *trpB*, *recA*, and *cheA* nucleotide sequences of bacterial strains within the operational group were analyzed. As the result, the *gyrB*, *rpoB*, and *trpB* phylogenetic analyses showed stable topology that comprised three monophyletic clades: (i) *B. amyloliquefaciens*; (ii) *B. siamensis*; and (iii) *B. velezensis*. Phylogenies derived by comparison of the *gyrB*, *rpoB*, *trpB*, *recA*, and *cheA* with the 16S rRNA gene-derived phylogeny was significant as evaluated by the likelihood ratio test. The *trpB*, *rpoB*, and *trpB* gene-derived phylogenies provide a tool for speciation within the operational group *B. amyloliquefaciens*.

Keywords: *B. amyloliquefaciens* identification; phylogenetic analyses of *gyrB*, *rpoB*, *trpB*, *recA*, *cheA* and 16S rRNA genes

Bacillus amyloliquefaciens was first discovered by a Japanese scientist, Juichiro Fukumoto, in the soil (Fukumoto, 1943). The species is named after its unique character as it produces (*faciens*) a liquefying (*lique*) amylase (*amyl*) (Priest *et al.*, 1987). *B. amyloliquefaciens* strains are capable of producing different bioactive compounds useful in bio-control of plant and human pathogens (Balhara *et al.*, 2011). This bacterial species is widely distributed in various niches including soil (Lončar *et al.*, 2013), foods (Krawczyk *et al.*, 2016), fruits (Wu *et al.*, 2017), bee products (Ngalimat *et al.*, 2019), animal faeces (Singh *et al.*, 2013), and aquatic environments (Daas *et al.*, 2018).

Taxonomically, the operational group *B. amyloliquefaciens* belonged to the group *B. subtilis* species complex, family Bacillaceae, class Bacilli, and phylum Firmicutes (Berkeley *et al.*, 1984; Fritze, 2004). This operational group is comprised of three species: *B. amyloliquefaciens*, *B. siamensis*, and *B. velezensis* (Fan *et al.*, 2017). Moreover, *B. amyloliquefaciens* has been sub-divided into two subspecies: *B. amyloliquefaciens* subsp. *plantarum* and subsp. *amyloliquefaciens*. The subspecies division was based on phylogenetic analysis and physiological characteristics i.e. production of plant growth hormones/antibiotics and abilities for plant root colonization (Borriss *et al.*, 2011).

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Therein, some strains of *B. amyloliquefaciens* subsp. *plantarum*, such as FZB42 (Borriss *et al.*, 2011), was reported as being synonymous to *B. velezensis* based on phylogenomic analysis (Dunlap *et al.*, 2016). Therefore, it is a cryptic controversy to the sequences deposited in the NCBI database could be incorrectly assigned as *B. amyloliquefaciens*.

For many years, it has been recognized that the group *B. subtilis* species complex is hard to be distinguished using classical taxonomy parameters: morphological and physiological characteristics, cell wall compositions, 16S rRNA sequence, fatty acid methyl ester analysis (FAME), and G+C content (Rooney *et al.*, 2009). The determination of DNA-DNA relatedness (DDH) also often shows erroneous and variable results (Auch *et al.*, 2010). Due to these limitations, the taxonomic status of *Bacillus* spp. is constantly bringing confusion to researchers, especially for non-professional taxonomy researchers. Thanks to the new approaches in the next-generation sequencing (NGS) technology, bacterial genome sequencing has helped to identify bacterial species through phylogenomic analysis (Chan & Ragan, 2013; Fan *et al.*, 2017). Furthermore, a multilocus sequence typing (MLST) also could be helpful for typing *Bacillus* spp. by sequence analysis of housekeeping genes (Helgason *et al.*, 2004). Noteworthy, these phylogenomic and MLST analyses could be time-consuming and expensive for small laboratories in research institutions (Boers *et al.*, 2012; Haas *et al.*, 2011). Owing to these limitations, comparative phylogenies of the 16S rRNA gene and the housekeeping genes were analyzed to improve speciation within the operational group *B. amyloliquefaciens*.

To date, the operational group *B. amyloliquefaciens* is consisted of 337 strains with validly published on the NCBI Entrez Genome Project database (<https://www.ncbi.nlm.nih.gov/genome>). The *B. amyloliquefaciens* 16S rRNA gene clade contains species with identical 16S rRNA gene sequences. Dendrograms based on complete *rpoB* nucleotide sequences and on core genome sequences have divided the species into the *B. amyloliquefaciens*, *B. siamensis*, and *B. velezensis* clades, respectively (Fan *et al.*, 2017). To aid in the comparison in the present study, comparative phylogenies of the complete 16S rRNA, DNA gyrase subunit B (*gyrB*), RNA polymerase β -subunit (*rpoB*), tryptophan synthase β -subunit

(*trpB*), RecA protein (*recA*), and signal transduction histidine kinase CheA (*cheA*) nucleotide sequences of 40 bacterial strains within the operational group were analyzed (Table 1). Type strains including *B. amyloliquefaciens* subsp. *plantarum* FZB42, *B. velezensis* CBMB205, *B. siamensis* KCTC 13613, and *B. amyloliquefaciens* DSM7 were included. All the nucleotide sequences were retrieved from the NCBI Entrez Genome Project database. Multiple sequence alignment has been generated using the MEGA 7.0 software (Kumar *et al.*, 2016) and programme ClustalX (Thompson *et al.*, 1997). The phylogenetic tree was created according to the neighbor-joining method (Saitou & Nei, 1987) with the *p*-distance method (Nei & Kumar, 2000). Bootstrap analysis was based on 1,000 replications (Felsenstein, 1985). *B. subtilis* subsp. *subtilis* 168 was chosen as an outgroup. The likelihood ratio test was used for the statistical analysis (Knudsen & Miyamoto, 2001).

As a result, it is evident that the complete 16S rRNA nucleotide sequences were not sufficient for the speciation within the operational group (Figure 1). The presence of unstable taxa was observed that makes cladistic analysis undefinable. Moreover, *B. subtilis* subsp. *subtilis* 168 failed to act as the outgroup. To elucidate the phylogenetic relationship within the operational group more precisely, the complete sequences of *gyrB*, *rpoB*, *trpB*, *recA*, and *cheA* were used in the phylogenetic analyses. Phylogenies derived from the comparison of the housekeeping genes with the 16S rRNA gene-derived phylogeny was significant ($P \leq 0.05$), showing that the tree topology was optimum. The *gyrB*, *rpoB*, and *trpB* gene-derived phylogenies obtained (Figure 2), have the same topology as reported by Fan *et al.* (2017) whereas, the root of genome-based phylogeny indicated that an ancestral lineage within the operational group started by the *B. amyloliquefaciens* clade. However, the tree topologies of *recA* and *cheA* gene-derived phylogenies were different (Figure S1). The ancestral lineage of *recA* gene-derived phylogeny started by *B. velezensis* clade, while, *cheA* gene-derived phylogeny started by *B. siamensis* clade.

Table 1. The complete sequenced 16S rRNA, *gyrB*, *rpoB*, *trpB*, *recA*, and *cheA* of 40 bacterial strains within the operational group *B. amyloliquefaciens* and *B. subtilis* subsp. *subtilis* 168 obtained from the NCBI database.

Bacteria	Accession	16S rRNA	Number of nucleotides (bp)					Isolation source
			<i>gyrB</i>	<i>rpoB</i>	<i>trpB</i>	<i>recA</i>	<i>cheA</i>	
<i>B. subtilis</i> subsp. <i>subtilis</i> 168	AL009126	1550	1917	3582	1203	1047	2019	Unknown
<i>B. siamensis</i> SCSIO 05746	CP025001	1552	1923	3582	1203	1044	2019	Sea mud
<i>B. siamensis</i> XY18	LAGT000000000	1525	1923	3582	1203	1044	2019	Cured vanilla beans
<i>B. siamensis</i> KCTC 13613	AJVF000000000	1525	1836	3015	1203	1044	2019	Sugar cane
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> FZB42	CP000560	1550	1917	3582	1203	1044	2019	Infected sugar beet
<i>B. velezensis</i> NJN-6	CP007165	1540	1923	3582	1203	1044	2019	Soil
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> YAU B9601-Y2	HE774679	1538	1923	3441	1203	1044	2019	Wheat rhizosphere
<i>B. velezensis</i> SRCM103691	CP035393	1554	1923	3582	1203	1044	2019	Food
<i>B. velezensis</i> KCTC 13012	LHCC010000000	1550	1923	3582	1203	1044	2019	River mouth
<i>B. velezensis</i> SYBC H47	CP017747	1554	1923	3582	1203	1044	2019	Honey
<i>B. velezensis</i> CBMB205	CP011937	1555	1923	3582	1203	1044	2019	Rice rhizosphere soil
<i>B. amyloliquefaciens</i> DSM7	FN597644	1538	1917	3582	1203	1044	2028	Soil
<i>B. amyloliquefaciens</i> FS1092	CP038028	1553	1923	3582	1203	1044	2019	Food processing environment
<i>B. amyloliquefaciens</i> Y2	CP003332	1555	1917	3441	1203	1044	2019	Wheat rhizosphere
<i>B. amyloliquefaciens</i> SRCM101267	CP021505	1550	1923	3582	1203	1044	2019	Food
<i>B. amyloliquefaciens</i> ZJU1	CP041691	1553	1917	3582	1203	1044	2019	<i>Morus alba</i>
<i>B. amyloliquefaciens</i> ALB69	CP029070	1553	1917	3582	1203	1044	2019	Almond drupe
<i>B. amyloliquefaciens</i> SH-B74	CP030097	1554	1917	3582	1203	1044	2019	Marine-sediment
<i>B. amyloliquefaciens</i> ALB65	CP029069	1553	1917	3582	1203	1044	2019	Alfalfa silage
<i>B. amyloliquefaciens</i> UMAF6614	CP006960	1554	1917	3582	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> YP6	CP032146	1547	1923	3441	1203	1044	2019	Rhizosphere of <i>Lolium perenne</i> L.
<i>B. amyloliquefaciens</i> B15	CP014783	1555	1917	3582	1203	1044	2019	Grape skin
<i>B. amyloliquefaciens</i> UMAF6639	CP006058	1554	1923	3582	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> HK1	CP018902	1538	1923	3582	1203	1044	2019	Corn stalk residue compost
<i>B. amyloliquefaciens</i> LL3	CP002634	1538	1923	3246	1203	1044	2019	Korean fermented food (bibimbap)
<i>B. amyloliquefaciens</i> LM2303	CP018152	1555	1917	3582	1203	1044	2019	Wild yak dung
<i>B. amyloliquefaciens</i> ALB79	CP029071	1553	1917	3582	1203	1044	2019	Grape
<i>B. amyloliquefaciens</i> MBE1283	CP013727	1556	1917	3582	1203	1044	2019	Korean traditional alcoholic beverage
<i>B. amyloliquefaciens</i> Y14	CP017953	1556	1917	3582	1203	1044	2019	Rhizosphere of peanut

Bacteria	Accession	16S rRNA	Number of nucleotides (bp)					Isolation source
			<i>gyrB</i>	<i>rpoB</i>	<i>trpB</i>	<i>recA</i>	<i>cheA</i>	
<i>B. amyloliquefaciens</i> H	CP041693	1554	1917	3582	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> subsp. <i>amyloliquefaciens</i> KHG19	CP007242	1538	1923	3441	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> LFB112	CP006952	1554	1917	3582	1203	1044	2019	Chinese herbs
<i>B. amyloliquefaciens</i> XH7	CP002927	1539	1923	3246	1203	1044	2028	Unknown
<i>B. amyloliquefaciens</i> TA208	CP002627	1559	1917	3582	1203	1044	2019	Soil
<i>B. amyloliquefaciens</i> WS-8	CP018200	1555	1917	3582	1203	1044	2019	Soil
<i>B. amyloliquefaciens</i> IT-45	CP004065	1559	1917	3582	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> S499	CP014700	1555	1917	3582	1203	1044	2019	Soil
<i>B. amyloliquefaciens</i> B-4	CP031424	1554	1917	3582	1203	1044	2019	Unknown
<i>B. amyloliquefaciens</i> CC178	CP006845	1543	1917	3441	1203	1044	1860	Cucumber phyllosphere
<i>B. amyloliquefaciens</i> MT45	CP011252	1555	1917	3582	1203	1044	2019	Daqu
<i>B. amyloliquefaciens</i> RD7-7	CP016913	1550	1923	3582	1203	1044	2019	Fermented soybean paste

Figure 1. The 16S rRNA gene phylogenetic analysis within the operational group *B. amyloliquefaciens*. Bootstrap values are indicated at branch points. Accession numbers for the bacterial strains are indicated within the bracket. Type strains are in bold. Noteworthy, the 16S rRNA phylogenetic analysis failed to differentiate species within the operational group due to the highly conserved nature of the gene. *B. subtilis* subsp. *subtilis* 168 (in a box) failed to act as an outgroup.

Figure 2. The phylogenetic tree analyses from 40 complete *gyrB* (a), *rpoB* (b), and *trpB* (c) nucleotide sequences within the operational group *B. amyloliquefaciens*. Bootstrap values > 90 % are indicated at branch points. Type strains are in bold. *B. subtilis* subsp. *subtilis* 168 was chosen as an outgroup.

In this study, *gyrB*, *rpoB*, and *trpB* gene-derived phylogenies were robust showing high bootstrap values (> 98 %) for all the three clades. Based on this data, it can be concluded that the speciation of *B. amyloliquefaciens*, *B. siamensis*, and *B. velezensis* within the operational group can be confirmed by phylogenetic analysis of *gyrB*, *rpoB*, and *trpB*. Interestingly, *trpB* nucleotide sequences for all the strains chosen in this study were perfectly similar in size (1,203 bp) as compared to *gyrB* (1,836 - 1,923 bp) and *rpoB* (3,015 - 3,582 bp) nucleotide sequences (Table 1). Based on the phylogenetic analyses, *gyrB*, *rpoB*, and *trpB* have allowed speciation within the operational group. As *gyrB*, *rpoB*, and *trpB* are found in the bacteria (Jablonski *et al.*, 1996; Ogier *et al.*, 2019; Watanabe *et al.*, 2001), speciation within the operational group through the *gyrB*, *rpoB*, and *trpB* gene-derived phylogenies are recommended.

In summary, *gyrB*, *rpoB*, and *trpB* gene-derived phylogenies in this analysis supported the whole genome-based phylogeny as proposed by Fan *et al.* (2017) but not 16S rRNA, *recA*, and *cheA* gene-derived phylogenies. Thus, 16S rRNA, *recA*, and *cheA* cannot be used for speciation within the operational group. The *gyrB*, *rpoB*, and *trpB* phylogenetic analyses are the promising approach for the ongoing revision on the systematic taxonomy of the operational group *B. amyloliquefaciens*. Identification of bacteria that belongs to the operational group through phylogenetic analyses of *gyrB*, *rpoB*, and *trpB* are strongly recommended for reliably assign novel strains or isolates to bacterial species.

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