Analyses of the Complete Genome Sequence of the Strain *Bacillus pumilus* ZB201701 Isolated from Rhizosphere Soil of Maize under Drought and Salt Stress

ZHONGBAO ZHANG1*, LONGFEI YIN1,2, XIANGLONG LI1, CHUN ZHANG1, HUAWEN ZOU1, CAI LIU1,2, and ZHONGYI WU1,2※

1Beijing Agro-Biotechnology Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China; 2Beijing College of Agriculture, Yangtze University, Hubei Collaborative Innovation Center for Grain Industry, Jingzhou 434023, Hubei, China; and 3College of Life Sciences, Capital Normal University, Beijing 10048, China

(Received June 25, 2018—Accepted June 14, 2019—Published online August 23, 2019)

*Bacillus pumilus* ZB201701 is a rhizobacterium with the potential to promote plant growth and tolerance to drought and salinity stress. We herein present the complete genome sequence of the Gram-positive bacterium *B. pumilus* ZB201701, which consists of a linear chromosome with 3,640,542 base pairs, 3,608 protein-coding sequences, 24 ribosomal RNAs, and 80 transfer RNAs. Genome analyses using bioinformatics revealed some of the putative gene clusters involved in defense mechanisms. In addition, activity analyses of the strain under salt and simulated drought stress suggested its potential tolerance to abiotic stress. Plant growth-promoting bacteria-based experiments indicated that the strain promotes the salt tolerance of maize. The complete genome of *B. pumilus* ZB201701 provides valuable insights into rhizobacteria-mediated salt and drought tolerance and rhizobacteria-based solutions for abiotic stress in agriculture.

Key words: *Bacillus pumilus*, genome sequence, salinity and drought, rhizobacteria

Soil salinity and drought stress are increasingly serious examples of abiotic stress worldwide that limit plant growth and productivity (5, 10). Selected beneficial rhizobacteria may play an important role in promoting plant growth and tolerance to drought and salinity stress, thereby increasing crop yields (10, 14). Plant growth-promoting bacteria (PGPB) are free-living bacteria that form specific symbiotic relationships with plants or bacterial endophytes colonizing some or a part of the interior tissues of plants (1). PGPB are generally used as inoculants for biostimulation, biocontrol, and biofertilization. These bacteria may improve plant growth under different environmental conditions (24).

*Bacillus* species are important rhizobacteria that may improve plant growth and development via different mechanisms (19). *Bacillus pumilus* strains exhibit increased resistance to environmental biotic and abiotic stress and produce a wide range of industrial metabolites (7). They are found in a range of environments, from stratospheric air to deep-sea sediments, and from soil to living organisms (3, 17). Different strains of *B. pumilus* have been isolated from different rhizospheres and organisms and have been shown to exert various effects on hosts. For example, *B. pumilus* isolated from the rhizosphere of alder exhibits strong growth-promoting activity (9), while *B. pumilus* isolated from the rhizosphere of drought-affected and saline soil in Bayan Nur of the Inner Mongolia Autonomous Region, China (40°13'–42°28', E105°12'–109°53'). Soil was collected from the maize rhizosphere at a depth of 5–10 cm. Five points were selected according to the “S” form five-spot sampling method (18). One hundred grams of soil was collected at each point and combined into one composite soil sample. To isolate bacteria, soil samples were placed in paper bags and stored at 4°C for approximately 1 d. One hundred milliliters of sterile water and 10 g soil were then transferred into a Waring blender. The sample was homogenized for 1 min and the supernatant was collected.

Supernatants were centrifuged at 5,000×g for 10 min and then added to 0.5 mL sterile water. Dilutions of 10−3, 10−4, and 10−5 were placed on agar plates (5 g L−1 beef extract, 10 g L−1 peptone, 5 g L−1 NaCl, 100 g L−1 mannitol, 1 L distilled water, and 15 g L−1 agar; pH 7.2). After a 2-d incubation at 30°C, the biggest colony was transferred to 1.5-mL frozen pipes and stored at −80°C.

Physiological characteristics and sequence similarity analysis

Physiological characteristics were identified based on Gram staining. A sequence similarity analysis was performed using 16S ribosomal RNA (rRNA) and two housekeeping genes (*recA* and *atpD*). A similarity search with the 16S rRNA gene nucleotide sequence (accession number MH368107) was conducted using EzBioCloud (https://www.ezbiocloud.net) (28) and average nucleotide identity (ANI) was calculated using JspeciesWS (25) to elucidate the interspecific relationships of the strain.

Genomic DNA preparation, genome sequencing, and assembly

To isolate genomic DNA, the strain was inoculated onto 50 mL liquid medium and cultivated overnight at 30°C in a shaker at 150 rpm. The overnight culture was used in the extraction of genomic DNA by a Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China).

Whole genome sequencing was performed using the PacBio RS and Illumina platforms. Illumina PE and PacBio (8–10 kb) libraries were constructed, and 52-Mb continuous long read (CLR)
Zhang et al.

L–1 NaCl was evenly applied to the soil. In the treatment group, the three plants in each group. In the control group, 100 mL of 0.1 mol

ZB201701) group, with

B. pumilus

once every 3 d. At the three-leaf stage, plants were divided into the

16 h of light; 22°C, 8 h of darkness; and 65% humidity) and watered

minated, and grown in pots (length×width×height=7×7×6.6 cm)

CK group×100. CK, 0 mol/L NaCl or D-sorbitol.

–1 and

D-sorbitol to a final concentration of 1, 2, or 3 mol L–1

achieve OD600 0.1. One hundred milliliters of this solution (0.1 mol

at 200×g at room temperature for 10–20 min. Pelleted cells were

strain was cultured in LB medium at 30°C for 36 h and centrifugated

incubating at 30°C for at least 12 h. Cell viability was calculated as

No. of colonies of the stress group/No. of colonies of the

corresponding genes were identified. BLAST results were analyzed

by Gene Ontology annotation (http://www.geneontology.org) with

kegg/genes.html), from which specific pathways involved in the

c镱ed genes were identified. BLAST results were analyzed

using the software Blast2go.

B. pumilus ZB201701 and resistance to abiotic stress

Luria-Bertani (LB) agar plates with NaCl (0, 1, 3, and 5 mol L–1) or D-sorbitol (0, 1, 2, and 3 mol L–1) were used to test stress

responses. In the salt and drought challenge, cultures were incubated at 30°C to a density of approximately 5×107 cells mL–1. At this

point, cell viability was assessed by plating appropriate dilutions

onto agar with NaCl to a final concentration of 1, 3, or 5 mol L–1 or D-sorbitol to a final concentration of 1, 2, or 3 mol L–1 and

incubating at 30°C for at least 12 h. Cell viability was calculated as follows: No. of colonies of the stress group/No. of colonies of

the CK group×100. CK, 0 mol/L NaCl or D-sorbitol.

Seeds of the maize inbred line B73 were surface-sterilized, ger-

minated, and grown in pots (length×width×height=7×7×6.6 cm)

filled with soil and vermiculite (1:1 [v/v]), with four seeds per pot. Pots were placed in a greenhouse under long-day conditions (30°C,

16 h of light; 22°C, 8 h of darkness, and 65% humidity) and watered

once every 3 d. At the three-leaf stage, plants were divided into the
control group and treatment (B. pumilus ZB201701) group, with

three plants in each group. In the control group, 100 mL of 0.1 mol L–1 NaCl was evenly applied to the soil. In the treatment group,

the strain was cultured in LB medium at 30°C for 36 h and centrifugated at 200×g at room temperature for 10–20 min. Pelleted cells were

resuspended in 0.1 mol L–1 NaCl and adjusted by further dilutions to achieve OD600 0.1. One hundred milliliters of this solution (0.1 mol

L–1 NaCl with OD600 0.1 B. pumilus ZB201701) was then evenly

applied to soil. All sampled tissues were washed by sterile distilled

water and 75% ethanol. The leaf tissues of four seedlings (300 mg)

were sampled after 0, 5, 10, 15, and 20 d. Maize seedling heights were measured every five d after the salt-stress treatment. Superoxide

dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)

activities were measured in all samples according to previously
described methods (2, 6, 23) (Table S1).

Table 1. Sequence similarity (%) with Bacillus pumilus ZB201701.

| Strains                  | 16S rRNA (%) | recA (%) | atpD (%) | MLSA (%) | ANI (%) |
|-------------------------|--------------|----------|----------|----------|---------|
| B. pumilus SH-B9        | 99.93        | 97       | 98       | ND       | 95.28   |
| B. zhangzhouensis DW5-4t| 99.93        | 94       | 97       | ND       | 91.46   |
| B. safensis KCTC 12796B | 99.86        | 92       | 98       | ND       | 92.40   |
| B. australimaris NH71 T  | 99.86        | 92       | 98       | ND       | 91.99   |
| B. altitudinis YNP4-TSU | 99.58        | 91       | 97       | ND       | 89.29   |
| B. atropphaeus BSS      | 97.32        | 80       | 82       | ND       | 85.33   |
| B. subtilis 168         | 97.03        | 81       | 82       | ND       | 84.99   |
| B. nakamura NNR B-41091 T| 97.03      | 81       | 81       | ND       | 84.73   |
| B. amylo芋quefaciens DSM7T | 96.82    | 80       | 81       | ND       | 85.70   |
| B. swezeyi NNR B-41282  | 96.40        | 80       | 81       | ND       | 84.53   |
| B. hayssini NNR B-41327T| 96.18        | 80       | 80       | ND       | 84.67   |
| B. gobiensis FIAT-4402T  | 95.41        | 76       | 78       | ND       | 85.28   |

Copyright 2019 by Japanese Society of Microbial Ecology / Japanese Society of Soil Microbiology / Taiwan Society of Microbial Ecology / Japanese Society of Plant and Microbe Interactions
Analyses of B. pumilus ZB201701 from Maize Root

of B. pumilus ZB201701 consisted of one circular chromosome of 3,640,542 bp (Fig. 1) and one plasmid sequence (Fig. S3) with no gaps. The G+C content of the genome was 41.86%.

A total of 3,712 predicted genes were detected, 3,608 (97.2%) of which were putative protein-coding genes; 86.21% were assigned a putative function (Table 2).

Fig. 1. The circular chromosome of Bacillus pumilus ZB201701.
Whole genome sequencing was performed using the PacBio RS and Illumina sequencing platforms. These sequencing data were assembled using Velvet assembler version 1.2.10 with a k-mer length of 99.
Based on COGs (26), the identified proteins were classified into 25 functional categories (Fig. 2). Some of the proteins were found to be involved in the response to salt and drought stress, including the amino acid transport and metabolism category (E), which contained the osmo-regulated proline transporter (27); the signal transduction mechanisms category (T) that contained the transcriptional regulatory protein DegU and signal transduction histidine-protein kinase/phosphatase DegS (16); and the transcription category (K) that contained the cold-shock protein CspB, which has been shown to improve grain yield in maize under water-limited conditions (4). A previous study indicated that the accumulation of stress-induced reactive oxygen species is counteracted by enzymatic antioxidant systems that include a number of scavengers, including SOD, CAT, and APX (21). COG functional analyses indicated several related genes, including some in the inorganic ion transport and metabolism category (P), which contains three SOD- and two CAT-related genes; some in the carbohydrate transport and metabolism category (G); and some in the function unknown category (S), which contained one APX-related gene.

Activity of the strain under salt and simulated drought stress

*B. pumilus* strains are resistant to environmental biotic and abiotic stress (7). A strain of *B. pumilus* isolated from the penaeid shrimp has been shown to exhibit high salt tolerance (12), and *B. pumilus* ES4 from arid land soils exhibits strong plant growth-promoting activity (11). We also identified several environmental biotic and abiotic stress-related genes based on COG analyses (Fig. 2). To identify the ability of *B. pumilus* ZB201701 to resist conditions of high salt and drought, we cultured the strain on medium with different concentrations of salt and D-sorbitol. The results obtained indicated that the strain has the ability to tolerate up to 3 M D-sorbitol (approximately 55% [w/v]) and 5 M NaCl (approximately 29% [w/v]), with Cell viability of 3.5 and 55.8%, respectively (Fig. 3a and b). These results indicated that *B. pumilus* ZB201701 has the ability to resist high salt and simulated drought.

Ability to promote the salt resistance of maize

The heights of maize in the *B. pumilus* ZB201701 groups were significantly greater (*P*<0.05) than those in the control group from days 5 to 30 (Fig. 4a). The results obtained also indicated that from days 25 to 30, SOD activity was significantly higher in the *B. pumilus* ZB201701 group than in the control group (*P*<0.05) (Fig. 4b). CAT activity was also markedly higher in the *B. pumilus* ZB201701 group than in the control group from days 0 to 30 (*P*<0.05) (Fig. 4c). APX activity in the *B. pumilus* ZB201701 group was higher than that in the control group on days 5, 15, 20, 25, and 30 (*P*<0.05) (Fig. 4d). These results indicate that *B. pumilus* ZB201701 promotes maize salt resistance by increasing the activities of SOD, CAT, and APX.
Acknowledgements

This work was supported in part by the Beijing Academy of Agriculture and Forestry Sciences (KJCX20170404, KJX20170203 and QNJ201724) and Beijing Association for Science and Technology.

References

1. Basu, S., R. Rabara, and S. Negi. 2017. Towards a better greener future—an alternative strategy using biofertilizers. I: Plant growth promoting bacteria. Plant Gene 12:43–49.

2. Beers, R.E., Jr., and I.W. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195:133–140.

3. Branquinho, R., E. Melrinhos-Soares, J.A. Carriço, M. Pintado, and L.V. Peixe. 2014. Phylogenetic and clonality analysis of *Bacillus pumilus* isolates uncovered a highly heterogeneous population of different closely related species and clones. FEMS Microbiol. Ecol. 90:689–698.

4. Castiglioni, P., D. Warner, R.J. Bensen, et al. 2008. Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiol. 147:446–455.

5. Forri, C., D. Duca, and B.R. Glick. 2017. Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. Plant Soil 410:335–356.

6. Giannopolitis, C.N., and S.K. Ries. 1977. Superoxide Dismutases I. Occurrence in Higher Plants. Plant Physiol. 59:309–314.

7. Gioia, J., S. Yerrapragada, X. Qin, et al. 2007. Paradoxical DNA repair and peroxide resistance gene conservation in *Bacillus pumilus* SAFR-032. PLoS One 2:e928.

8. Goris, J., K.T. Konstantinidis, J.A. Klappenbach, T. Coenye, P. Vandamme, and J.M. Tiedje. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. 57:81–91.

9. Gutiérrez-Mañero, F.J., B. Ramos-Solano, A. Probanza, J. Mehouchi, F.R. Tadeo, and M. Talon. 2001. The plant-growth promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol. Plantarum 111:206–211.

10. Han, Q.Q., X.P. Lu, J.P. Bai, et al. 2014. Beneficial soil bacterium *Bacillus subtilis* (GZ03) augments salt tolerance of white clover. Front. Plant Sci. 5:525.

11. Hernandez, J.P., L.E. de-Bashan, D.J. Rodriguez, Y. Rodriguez, and Y. Bashan. 2009. Growth promotion of the freshwater microalga Chlorella vulgaris by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. Eur. J. Soil Biol. 45:88–93.

12. Hill, J.E., J.C.F. Baiano, and A.C. Barnes. 2009. Isolation of a novel strain of *Bacillus pumilus* from penaeid shrimp that is inhibitory against marine pathogens. J. Fish Dis. 32:1007–1016.
13. Hyatt, D., G.L. Chen, P.F. Locascio, M.L. Land, F.W. Larimer, and L.J. Hauser. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119.

14. Kaushal, M., and S.P. Wani. 2016. Rhizobacterial-plant interactions: strategies ensuring plant growth promotion under drought and salinity stress. Agr. Ecosyst. Environ. 232:68–78.

15. Krzywinski, M., J. Schein, I. Birol, J. Connors, R. Gascoyne, D. Horsman, S.J. Jones, and M.A. Marra. 2009. Circos: an information aesthetic for comparative genomics. Genome Res. 19:1639–1645.

16. Kunst, F., and G. Rapoport. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. J. Bacteriol. 177:2403–2407.

17. Liu, Y., Q. Lai, C. Dong, F. Sun, L. Wang, G. Li, and Z. Shao. 2013. Phylogenetic diversity of the *Bacillus pumilus* group and the marine ecotype revealed by multilocus sequence analysis. PLoS One 8:e80097.

18. Lu, S., W. Quan, S.M. Wang, H.L. Liu, Y. Tan, G.P. Zeng, and X. Zhang. 2013. Correlation of soil microbes and soil micro-environment under long-term safflower (*Carthamus tinctorius* L.) plantation in China. J. Environ. Biol. 34:471–479.

19. Lugtenberg, B., and F. Kamilov. 2009. Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63:541–556.

20. Martin, C.F. 2011. A new repeat-masking method enables specific detection of homologous sequences. Nucleic Acids Res. 39:e23.

21. Mittler, R., S. Vanderauwerwa, M. Gollery, and F. Van Breusegem. 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9:490–498.

22. Morari, F., F. Meggio, A. Lunardon, E. Scudiero, C. Forestan, S. Farinati, and S. Varotto. 2015. Time course of biochemical, physiological, and molecular responses to field-mimicked conditions of drought, salinity, and recovery in two maize lines. Front. Plant Sci. 6:314.

23. Nakano, Y., and K. Asada. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22:867–880.

24. Numan, M., S. Bashir, Y. Khan, R. Mumtaz, Z.K. Shinwari, A.L. Khan, A. Khan, A. AL-Harrasi. 2018. Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: A review. Microbiol. Res. 209:21–32.

25. Richter, M., R. Rosselló-Móra, F.O. Glöckner, and J. Peplies. 2016. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32:929–931.

26. Tatusov, R.L., N.D. Fedorova, J.D. Jackson, et al. 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4:41.

27. Whatmore, A.M., J.A. Chudek, and R.H. Reed. 1990. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. J. Gen. Microbiol. 136:2527–2535.

28. Yoon, S.-H., S.-M. Ha, S. Kwon, J. Lim, Y. Kim, H. Seo, and J. Chun. 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67:1613–1617.

29. Zerbino, D.R., and E. Birney. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18:821–829.