Short Communication

An Assessment of the Diversity of Culturable Bacteria from Main Root of Sugar Beet

KAZUYUKI OKAZAKI1, TAKAO INO2, YOSUKE KURODA1, KAZUNORI TAGUCHI1, HIROYUKI TAKAHASHI1, TAKUI OIHWA3, HIRO TSURUMARU4, TAKASHI OKUBO5, KIWAMU MINAMISAWA6, and SEIJI IKEDA1,*

1Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, 9-4 Shinsei-nami, Memuro-cho, Kasai-gun, Hokkaido 082-0081, Japan; 2Japan Collection of Microorganisms, RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan; 3Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan; and 4Graduate School of Life Science, Tohoku University, 2-1-1 Kathahra, Aoba-ku, Sendai, Miyagi 980-8577, Japan

(Received December 26, 2013—Accepted March 1, 2014—Published online April 30, 2014)

The partial sequences of the 16S rRNA genes of 531 bacteria isolated from the main root of the sugar beet (Beta vulgaris L.) were determined and subsequently grouped into 155 operational taxonomic units by clustering analysis (≥99% identity). The most abundant phylum was Proteobacteria (72.5–77.2%), followed by Actinobacteria (9.8–16.6%) and Bacteroidetes (4.3–15.4%). Alphaproteobacteria (46.7–64.8%) was the most dominant class within Proteobacteria. Four strains belonging to Verrucomicrobia were also isolated. Phylogenetic analysis revealed that the Verrucomicrobia bacterial strains were closely related to Haloferula or Verrucomicrobium.

Key words: Alphaproteobacteria, endophyte, Haloferula, sugar beet root microbiome, Verrucomicrobium

The sugar beet (Beta vulgaris L.) is one of the world’s most important crops as a source of sucrose and has recently drawn attention as an energy plant (10). However, the increasing cost of chemicals for fertilizers and pesticides in recent years has become a serious problem for agricultural production. Although the biomass productivity of sugar beet is high, the mechanisms supporting the productivity of this plant remain unclear. The high biomass of the sugar beet may be attributed to its high affinity with beneficial microbes, as has also been reported in the sweet potato (18).

The diversity and community composition of culturable bacteria from the main root of the sugar beet were assessed in the present study using 16S rRNA gene sequencing in order to provide basic ecological information and construct a resource for an efficient survey and utilization of plant-growth-promoting rhizobacteria (PGPR).

The seeds of the sugar beet (cultivar “Rycka”) were sown on 27 April 2011 in an experimental field of the Hokkaido Agricultural Research Center (Memuro, Hokkaido, Japan, 42°89.2’ N/143°0.7.7’ E). The width of the rows and spacing between plants were 60 cm and 22.5 cm, respectively. The field was dressed with S014 (150, 315, and 210 kg/ha for N, P2O5, and K2O, respectively; Hokuren Fertilizer Co., Darmstadt, Germany), and the supernatant was then stored as a 15% glycerol stock at −80°C until later bacterial isolation. Soil samples were collected from three sampling sites by an auger (between a depth of 5 cm to 15 cm) after removing surface soil on 12 October 2011, and were combined as a composite soil sample. The soil samples were determined by the Tokachi Nokyoren Agricultural Research Institute (Obihiro, Hokkaido, Japan) (Table S1).

Four bacterial isolate collections were constructed from the SU and CO parts of the main root of the sugar beet by R2A (BD, Franklin Lakes, NJ, USA) and HM media. The HM medium was modified Cole’s HM medium (3) by adding 0.1% L-arabinose and 0.03% yeast extract. The pH was adjusted to 6.8 with 2N NaOH prior to autoclaving. Homogenates of the SU and CO parts of the main root were serially diluted with 67 mM phosphate buffer (pH 7.0) and homogenized in a blender. The homogenate was filtered through a piece of Miracloth (Calbiochem, Darmstadt, Germany), and the supernatant was then stored as a 15% glycerol stock at −80°C until later bacterial isolation. Soil samples were collected from three sampling sites by an auger (between a depth of 5 cm to 15 cm) after removing surface soil on 12 October 2011, and were combined as a composite soil sample. The chemical characteristics of the soil sample were determined by the Tokachi Nayoren Agricultural Research Institute (Obihiro, Hokkaido, Japan) (Table S1).

Four bacterial isolate collections were constructed from the SU and CO parts of the main root of the sugar beet by R2A (BD, Franklin Lakes, NJ, USA) and HM media. The HM medium was modified Cole’s HM medium (3) by adding 0.1% L-arabinose and 0.03% yeast extract. The pH was adjusted to 6.8 with 2N NaOH prior to autoclaving. Homogenates of the SU and CO parts of the main root were serially diluted with 67 mM phosphate buffer (pH 7.0), and 100 µl of each dilution was inoculated on 1.5% agar plates of R2A medium containing 50 mg L−1 cycloheximide and HM medium containing 50 mg L−1 polymyxin B. After an incubation at 24°C in the dark for 7 d, colonies were randomly collected and subjected to single colony isolation twice by streaking them onto fresh medium. The purified bacterial strains were stored as a 15% glycerol stock at −25°C.

Regarding DNA extraction, strains were cultured on an agar plate of the R2A or HM medium for a few days at 24°C. An aliquot of bacterial cells was collected with an inoculation loop and total DNA was extracted from the cells using a
Main root-associated bacteria in the sugar beet

previously described DNA extraction method (7), PCR amplification for 16S rRNA gene sequencing, and the editing and analyses of sequences for the strains isolated in the present study were conducted as previously described (17).

A total of 531 strains were isolated from the surface and core parts of the main root of the sugar beet using two media (Table 1). Clustering analysis (≥99% identity) was used to group 531 strains into 155 OTUs, and library coverage was 83.1%. Statistical analysis revealed that the number of OTUs and both Shannon and Simpson diversity indexes were higher for the surface tissue collection than for the core tissue collection in R2A and HM media (Table 1). However, these differences were small, which was consistent with the findings reported by Lilley et al. (12). Differences were also attributed to the thickness of the surface tissue (10 mm), which may have led to a large physical overlap between the surface and core samples.

Proteobacteria was the most dominant phylum in all isolate collections (72.5–77.2%) (Table 2). In contrast to previous studies in which the dominance of Gammaproteobacteria, Actinobacteria, and Firmicutes was reported (8, 11, 12, 16), Alphaproteobacteria was the most abundant in all the collections in the present study (46.7–64.8%). These differences between the present and previous studies may be due to variations in the soil, fertilization conditions, or isolation methods. At the lower taxonomic ranks, three genera (Bosea, Devasia, and Mesorhizobium) in the order Rhizobiales were found to be stably present in all collections as the dominant genus (Table 2). Clustering analysis revealed that four genera (Devasia, Mesorhizobium, Rhizobium, and Sphingomonas) were highly diverse at the species level (Fig. S1). In Alphaproteobacteria, more than half of the OTUs (32 out of 63 OTUs) belonged to these four genera, which indicated a high microdiversity.

Actinobacteria was the secondary dominant phylum following Proteobacteria in all collections (9.8–16.6%) (Table 2). Microbacterium and Mycobacterium were stably detected in all collections (Table 2 and Table S2). Bacteroidetes was more abundant in collections with the HM medium than in collections with the R2A medium (Table 2). Clustering analysis revealed a high microdiversity at the species level for both Actinobacteria and Bacteroidetes (28 and 27 OTUs in a total 155 OTUs, respectively) (Fig. S1).

Four strains belonging to Verrucomicrobia were isolated from the surface part of the main root of the sugar beet using the R2A medium. These strains were closely related to two genera, Verrucomicrobiun and Haloferula (Fig. 1). The phylum Verrucomicrobia is known to be nearly ubiquitous and highly abundant in soils (1), but is generally considered to be recalcitrant for cultivation (4). To date, only three studies have reported the successful cultivation of Verrucomicrobiun bacteria from a rhizosphere (5, 6, 13). The members of this phylum have been phylogenetically classi-fi-
Okazaki et al.

Phylogenetic tree analysis of the partial 16S rRNA gene sequences of *Verrucomicrobia* bacteria isolated in the present study (BvORR085, BvORR071, BvORR034, and BvORR052). The strains isolated in the present study were indicated in a bold font. The accession numbers are given in parentheses. *Opitutus terrae* was used as an out group. The scale represents 0.1 substitutions per site. The numbers at the nodes are the proportions of 1,000 bootstrap resamplings, and values above 500 are shown.

Fig. 1. Phylogenetic tree analysis of 16S rRNA gene sequences of *Verrucomicrobia* strains isolated from the main root of the sugar beet. The tree was constructed by the neighbor-joining method with the reference sequences in subdivision 1 of *Verrucomicrobia* and the strains of *Verrucomicrobia* bacteria isolated in the present study (BvORR085, BvORR071, BvORR034, and BvORR052). The strains isolated in the present study were indicated in a bold font. The accession numbers are given in parentheses. *Opitutus terrae* was used as an out group. The scale represents 0.1 substitutions per site. The numbers at the nodes are the proportions of 1,000 bootstrap resamplings, and values above 500 are shown.
**Main root-associated bacteria in the sugar beet**

*Verrucomicrobium* and *Haloferula*, were obtained using a standard R2A medium. Functional analyses of these sugar beet-associated bacteria should be conducted in future studies in order to clarify their ecological roles in a rhizosphere.

The nucleotide sequences were deposited in the DDBJ/EMBL/GenBank database. The sequence data for main root-associated bacteria isolated from SU with R2A and HM media were deposited under the accession numbers AB851230–AB851416 and AB851138–AB851229, respectively. The sequence data from CO with R2A and HM media were deposited under the accession numbers AB850977–AB851137 and AB850886–AB850976, respectively. *Haloferula* spp. BvORR071 and BvORR085 and *Verrucomicrobium* sp. BvORR034 were deposited to the Japan Collection of Microorganisms at the RIKEN Bioresource Center (RIKEN-BRC JCM) under the culture collection accession numbers JCM 18780, JCM 18781, and JCM 18782, respectively.

**Acknowledgements**

We are very grateful to the following people for their technical assistance: Y. Ota, A. Yoshino, and M. Sasaki at Hokkaido Agricultural Center, NARO, Japan. This work was supported in part by the Ministry of Agriculture, Forestry and Fisheries, Japan through a research project entitled “Development of technologies for mitigation and adaptation to climate change in Agriculture, Forestry and Fisheries”, and by Grants-in-Aid for Scientific Research (C) 22580074 and (A) 23248052 from the Ministry of Education, Science, Sports and Culture of Japan.

**References**

1. Bergmann, G.T., S.T. Bates, K.G. Eilers, C.L. Lauber, J.G. Caporaso, W.A. Walters, R. Knight, and N. Fierer. 2011. The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities. Soil Biol. Biochem. 43:1450–1455.

2. Bibi, F., E.J. Chung, H.S. Yoon, G.C. Song, C.O. Jeon, and Y.R. Chung. 2011. *Haloferula luteola* sp. nov., an endophytic bacterium isolated from the root of a halophyte, *Rosa rugosa*, and emended description of the genus *Haloferula*. Int. J. Syst. Evol. Microbiol. 61:1837–1841.

3. Cole, M.A., and G.H. Elkan. 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother. 4:248–253.

4. da Rocha, U.N., L.S. van Overbeek, and J.D. van Elsas. 2009. Exploration of hitherto-uncultured bacteria from the rhizosphere. FEMS Microbiol. Ecol. 69:313–328.

5. da Rocha, U.N., F.D. Andreote, J.L. de Azevedo, J.D. van Elsas, and L.S. van Overbeek. 2010. Cultivation of hitherto-uncultured bacteria belonging to the *Verrucomicrobia* subdivision 1 from the potato (*Solanum tuberosum* L.) rhizosphere. J. Soils Sediments 10:326–339.

6. da Rocha, U.N., J.D. van Elsas, and L.S. van Overbeek. 2011. *Verrucomicrobia* subdivision 1 strains display a difference in colonization in the colonization of the leek (*Allium porrum*) rhizosphere. FEMS Microbiol. Ecol. 78:297–305.

7. Ikeda, S., T. Okubo, M. Anda, et al. 2010. Community- and genome-based views of plant-associated bacteria: plant-bacterial interactions in soybean and rice. Plant Cell Physiol. 51:1398–1410.

8. Jacobs, M., W.M. Bugbee, and D.A. Gabrielson. 1985. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. Can. J. Bot. 63:1262–1265.

9. Kinkle, B.K., J.S. Angle, and H.H. Keyser. 1987. Long-term effects of metal-rich sewage sludge application on soil populations of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 53:315–319.

10. Koga, N. 2008. An energy balance under a conventional crop rotation system in northern Japan: Perspectives on fuel ethanol production from sugar beet. Agric. Ecosyst. Environ. 125:101–110.

11. Lambert, B., P. Metre, H. Joos, P. Lens, and J. Swings. 1990. Fast-growing, aerobic, heterotrophic bacteria from the rhizosphere of young sugar beet plants. Appl. Environ. Microbiol. 56:3375–3381.

12. Lilley, A.K., J.C. Fry, M.K. Bailey, and M.J. Day. 1996. Comparison of aerobic heterotrophic taxa isolated from four root domains of mature sugar beet (*Beta vulgaris*). FEMS Microbiol. Ecol. 21:231–242.

13. Matsuura, H., Y. Tanaka, H. Yamakata, and K. Mori. 2010. Culture-dependent and independent analysis of microbial communities inhabiting the giant duckweed (*Spirodela polyrrhiza*) rhizosphere and isolation of a variety of rarely cultivated organisms within the phylum *Verrucomicrobia*. Microbes. Environ. 25:302–308.

14. Rivas, R., A. Willems, J.L. Palomo, P. Garcia-Beauvides, P.F. Mateos, E. Martínes-Molina, M. Gillis, and E. Velázquez. 2004. *Bradyrhizobium betae* sp. nov., isolated from roots of *Beta vulgaris* affected by tumor-like deformations. Int. J. Syst. Evol. Microbiol. 54:1271–1275.

15. Sameshima-Saito, R., K. Chiba, and K. Minamisawa. 2004. New method of denitrification analysis of *Bradyrhizobium* field isolates by gas chromatographic determination of 15*N*-Labeled N₂. Appl. Environ. Microbiol. 70:2886–2891.

16. Shi, Y., K. Lou, and H. Li. 2009. Isolation, quantity distribution and characterization of endophytic microorganisms within sugar beet. African Journal of Biotechnology 8:835–840.

17. Someya, N., Y. Ohdaira-Kobayashi, S. Tsuda, and S. Ikeda. 2013. Molecular characterization of the bacterial community in a potato rhizosphere. Microbes. Environ. 28:295–305.

18. Terakado-Tonooka, J., Y. Ohwaki, H. Yamakawa, F. Tanaka, T. Yoneyama, and S. Fujihara. 2008. Expressed *nifH* genes of endophytic bacteria detected in field-grown sweet potatoes (*Ipomoea batatas* L.). Microbes. Environ. 23:89–93.