Short Communication

Presence of 2,4-D-catabolizing Bacteria in a Japanese Arable Soil that Belong to BANA (*Bradyrhizobium-Agromonas-Nitrobacter-Afipia*) Cluster in α -*Proteobacteria*

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We isolated the 2,4-D-catabolizing bacterial strain RD5-C2 from Japanese upland soil that had no history of 2,4-D exposure. RD5-C2 could use 2,4-D as the sole carbon and energy sources for their growth. Phylogenetic analyses based on 16S rDNA sequencing revealed that RD5-C2 was a member of the BANA (*Bradyrhizobium-Agromonas-Nitrobacter-Afipia*) cluster in the α subdivision of the class *Proteobacteria*. The gene sequence of RD5-C2 most closely matched that of *Bradyrhizobium* sp. and also closely related to those of 2,4-D-catabolizing α -*Proteobacteria* previously isolated from pristine environments of Hawaii, northern Canada and central Chile. This is the first instance that 2,4-D-catabolizing bacteria in that phylogenetic group was isolated from an arable soil environment.

Key words: 2,4-D-catabolizing bacteria, α -*Proteobacteria*, BANA (*Bradyrhizobium-Agromonas-Nitrobacter-Afipia*) cluster

The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) has been used as a model compound to study the evolution of bacterial catabolic genes for chloro-aromatic compounds because no compounds analogous to 2,4-D have been found in nature to date^{4,7,10,15}. A number of bacteria capable of catabolizing 2,4-D have been isolated and were shown to be distributed over various genera in the α , β and γ subdivisions of the class *Proteobacteria*^{1,3,7,9,10}. Most of the strains have been isolated from environments that have encountered chlorinated compounds,

and known to be copiotrophic. Recently, a new group of oligotrophic 2,4-D-catabolizing bacteria were isolated from pristine environments such as Hawaii, northern Canada and central Chile, and it was revealed that these belong to the BANA (*Bradyrhizobium-Agromonas-Nitrobacter-Afipia*) cluster of α -*Proteobacteria*⁸. Because the Hawaiian 2,4-D-catabolizing bacterial strain was isolated from soil that appears to have been isolated from both immigrant microorganisms and synthetic chemicals for about 4800 years by a solid lava cap, it was suggested that these bacteria existed independent of 2,4-D before the volcanic activity⁸. As 2,4-D-catabolizing bacteria that belong to the BANA cluster have previously been

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isolated only from pristine environments, it is important to survey their geographical distribution and genetic characterization to determine the evolution of 2,4-D-catabolizing genes.

Kawatsu diluvial upland soil (CL, T-C: 1.4%, T-N: 0.1%, pH (H₂O): 6.4)¹⁴ collected from the experimental farm in Shimane University (Matsue, Japan) was used in this experiment. The soil had not been treated with any pesticides for 30 years since the farm was set up in 1968 using subsoil from a diluvial hill. The soil sample was partially dried and passed through a 2-mm sieve before use. Approximately 50 g of soil was mixed with 200 ml of a basal solution [(in grams per liter) 2,4-D (0.050), K₂HPO₄ (0.5), and NH₄NO₃ (0.25)] in a 500-ml Erlenmeyer flask, and mechanically shaken at 200 rpm for 20 min. Ten 25 ml aliquots of the soil suspension were separately mixed with 25 ml of the basal solution in a 200-ml Erlenmeyer flask (A-J), and incubated without shaking at 25°C in the dark. An aliquot was removed from the soil suspensions every one week, and subjected to 2,4-D quantification. The sample was centrifuged (8060 × g, 30 min) to remove soil particles, and UV-VIS spectra were measured in the range 200 to 400 nm using a UV-VIS spectrophotometer (model UV-160A; Shimadzu Co., Kyoto, Japan). The amount of 2,4-D in the supernatant was determined by subtracting A₃₂₀ from A₂₈₄ (λ_{max} of 2,4-D). There was no interference from other compounds in the soil solution. When 2,4-D disappeared from the soil suspension, one milliliter of 0.25% aquatic solution of 2,4-D sodium salt (pH 7.0) was added to obtain the initial concentration of 2,4-D.

When the first and second disappearance of 2,4-D were observed, a one milliliter aliquot of each soil suspension was taken and diluted with sterilized distilled water to prepare a series of dilutions from 10⁻¹ to 10⁻⁸. A portion of the dilutions was plated onto a 2,4-D-containing basal agar medium [(in grams per liter) 2,4-D (1.0), K₂HPO₄ (0.5), KH₂PO₄ (0.5), NH₄NO₃ (0.25), MgCl₂·6H₂O (0.2), CaCl₂·2H₂O (0.02), Na₂MoO₄·2H₂O (0.002), FeSO₄·7H₂O (0.001), MnSO₄·7H₂O (0.001), and agar (15)]. After a 10- to 20- day incubation at 25°C, a plate with an adequate number of colonies was selected, and 2,4-D-degrad-

ing ability of all the isolated colonies was determined using the basal liquid medium containing 2,4-D at 20 mg l⁻¹.

After several disappearances of 2,4-D, a population of 2,4-D-degrading microorganisms in the soil suspension was estimated. A portion of the soil dilutions prepared as described above was inoculated into the basal medium containing 2,4-D at 50 mg l⁻¹. After a 3-week incubation without shaking at 25°C, concentration of 2,4-D in the medium was spectrophotometrically determined as described above.

For phylogenetic analysis of the isolates, whole DNA was extracted according to the method described previously⁶, and almost the entire 16S rDNA gene of each isolate was amplified using fD1 and rP2 as primers¹⁶. The amplified gene fragment was digested with Hae III restriction enzyme (Takara) according to the supplier's instruction in order to compare the 2,4-D-degrading bacteria isolated from different soil suspensions. The amplified 16S rDNA fragments were directly sequenced with an ABI 377 DNA sequencing system (Perkin-Elmer Japan) using primers described previously⁶. The 16S rDNA gene sequences were compared with sequences in the DNA Data Bank of Japan (DDBJ/EMBL/GenBank) using the FASTA algorithm¹¹. Sequences were aligned manually using the CLUSTAL W (1.8) program, and sites where nucleotides had not been resolved for all sequences were deleted from the alignments. Phylogenetic analysis was performed with the CLUSTAL W (1.8) program using the neighbor-joining method¹³ and the bootstrap analysis based on 1000 replicates was used to place confidence estimates on the tree.

Degradation of 2,4-D started in all soil suspensions after a 2-week lag period, and degradation without a lag period were observed with repeated applications of 2,4-D. There were few differences in the apparent fates of 2,4-D among the ten soil suspensions, however, 2,4-D-degrading bacteria were successfully isolated from only three soil suspensions after the first degradation of 2,4-D. There were no additional 2,4-D-degrading bacteria isolated from the other soil suspensions after the second degradation. All the colonies (18 to 19) on each plate from the

three soil suspensions were examined for their 2,4-D-degrading ability, and 79 to 84% appeared to be 2,4-D-degrading bacteria. After repeated applications of 2,4-D, degradation of 2,4-D was observed in 10^{-6} to 10^{-7} soil dilutions prepared from the three soil suspensions from which 2,4-D-degrading bacteria were isolated. In contrast, there was no degradation of 2,4-D observed in the soil dilutions even at 10^{-1} prepared from the other soil suspensions.

The isolated 2,4-D-degrading bacterial strains were motile gram negative rods (ca. 2–3 μm long and ca. 0.7 μm in diameter). The isolates could use 2,4-D as sole carbon and energy sources for their growth. They are slow-growing bacteria on 2,4-D-containing basal medium, 0.1 $\times$ Nutrient broth (Difco) and 0.1 $\times$ PTYG as previously isolated 2,4-D-catabolizing bacteria from pristine environments⁸). Their doubling time on the both media was about 24 h. It was supposed that the genes involved in the 2,4-D degradation could be located on the chromosome since the 2,4-D degradation trait was not lost even after sever-

al transfers of the strains on a 0.1 $\times$ Nutrient broth non-selective medium.

The isolated 2,4-D-catabolizing bacterial strains from the three soil suspensions appeared to belong to the same phylogenetic group based on the identical PCR-RFLP patterns of their 16S rDNA gene fragments (data not shown). The 16S rDNA gene sequence of the isolate (RD5-C2) (GenBank accession no. AB035489) was found to be similar to those of members of the α subdivision of the class *Proteobacteria*. The gene sequence of RD5-C2 most closely matched that of *Bradyrhizobium* sp. LMG9980 (99.7% similarity) isolated from a soil sample under *Acacia albida* trees in Senegal²) (GenBank accession no. X70404), and was also closely related to those of 2,4-D-catabolizing bacteria [HWK12 (99.5%), HW13 (99.2%), BTH (97.9%) and RCO (96.2%)] isolated from the pristine environments of Hawaii, Canada and Chile⁸). Phylogenetic position of RD5-C2 is shown in Fig. 1. All of the 2,4-D-catabolizing bacteria appeared to be members of the BANA

Fig. 1. Phylogenetic position of the isolated 2,4-D-catabolizing bacterial strain RD5-C2. Designations in parentheses are GenBank accession numbers. HWK12, HW13, BTH and RCO were 2,4-D-catabolizing bacteria isolated from pristine environments⁷). The phylogenetic tree was constructed using the neighbor-joining method¹²). Bootstrap values above 50% are shown at nodes. The scale bar indicates substitutions per site.

(*Bradyrhizobium-Agromonas-Nitrobacter-Afipia*) cluster⁵⁾.

We could isolate the 2,4-D-catabolizing bacteria from only three of ten soil suspensions (ca. 5 g soil in 50 ml basal solution), which indicates that the isolated 2,4-D-catabolizing bacterial strain, RD5-C2, was originally present in the soil at a low level of population. The 2,4-D-catabolizing α -*Proteobacteria* might be spread worldwide at quite a low population with no relation to 2,4-D exposure. In that case, the 2,4-D-catabolizing bacteria must have a series of enzymes to transform several chlorinated compounds independent of the presence of 2,4-D, and it would be important to determine the original substrate for the bacteria. It would be interesting to note that Kamagata *et al.*⁸⁾ had to transfer the cultures to fresh 2,4-D medium more than 10 times for enrichment and isolation of the 2,4-D-degrading bacteria from pristine environments.

In the dilution plating method used in this study, a relatively fast grower among 2,4-D-catabolizing bacteria under the test conditions increased in number in the soil suspensions and could be isolated. It is reported that the BANA cluster generally consists of slow-growing oligotrophic bacteria^{5,12)}, and RD5-C2 was also slow-growing bacteria. However, the 2,4-D-catabolizing bacteria isolated from the environments with a history of 2,4-D exposure have usually been fast-growing bacteria⁹⁾. This might be the reason why the 2,4-D-catabolizing bacteria in the BANA cluster have not been isolated from the contaminated environments with 2,4-D. As no growth of 2,4-D-catabolizing bacteria was observed in soil suspensions from which these bacteria could not be isolated, the other groups of 2,4-D-catabolizing bacteria could not be present in the soil sample or grew very slowly under the conditions examined. It is not clear why the apparent fates of 2,4-D were almost the same among the ten soil suspensions with and without increased numbers of the 2,4-D-catabolizing bacteria.

In contrast to the 2,4-D-catabolizing β - and γ -*Proteobacteria*, sequence information of the 2,4-D-catabolizing genes in α -*Proteobacteria* is unknown. To understand the origin and evolutionary relationship of the 2,4-D-catabolizing genes, it would be im-

portant to characterize the genes involved in 2,4-D degradation by α -*Proteobacteria*.

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