We have reported that a leguminous bacterial strain, *Bradyrhizobium* sp. strain 17-4, isolated from river sediment, phylogenetically very close to *Bradyrhizobium elkanii*, degraded methoxychlor through *O*-demethylation and oxidative dechlorination. In the present investigation, we found that *B. elkanii* (USDA94), a standard species deposited in the Culture Collection, degraded methoxychlor. Furthermore, *Bradyrhizobium* sp. strain 4-1, also very close to *B. elkanii*, isolated from Japanese paddy field soil, degraded methoxychlor. These *B. elkanii* and closely related strains degraded methoxychlor through almost identical metabolic pathways, and cleaved the phenyl ring and mineralized. In contrast, another representative *Bradyrhizobium* species, *B. japonicum* (USDA110), did not degrade methoxychlor at all. Based on these findings, *B. elkanii* and closely related strains are likely to play an important role not only in providing the readily biodegradable substrates but also in completely degrading (mineralizing) methoxychlor by themselves in the soil and surface water environment.

**Key words:** methoxychlor; *Bradyrhizobium elkanii*; leguminous bacterium; *O*-demethylation; dechlorination

Various elements are involved in the degradation of chemicals, such as pesticides, released into the natural environment. It is generally accepted that biodegradation by microbial activity plays a major role. To date, many microbial species capable of degrading specific pesticides have been isolated, in particular, bacteria and actinomycetes. With regard to pesticides that have long history of use and investigation, the degrading-microbes are known to belong to a wide range of phylogenetic classes. To date, for example, atrazine (and other triazine herbicide) degraders, *Pseudomonas*, *Rhodococcus*, *Ralstonia*, Agrobacterium, *Pseudomonas capreolica*, Nocardioidea, Chelatobacter, and Arthrobacter, have been reported from every corner of the world. Recently we reported on a methoxychlor-degrading bacterial strain, *Bradyrhizobium* sp. 17-4, that is closely related to the previously registered species *Bradyrhizobium elkanii*. The genus *Bradyrhizobium* originates from the genus *Rhizobium*, which includes all leguminous bacteria, and there have been few reports on pesticide-degraders. Thus, it is of interest that the genus *Bradyrhizobium* has been isolated as a methoxychlor-degrading bacterium.

In the present study, we newly isolated methoxychlor-degrading bacteria from paddy field soil, clarified their phylogenetic classification and metabolic pathways, and compared them with strain 17-4. The degradability of two standard *Bradyrhizobium* species deposited in the Culture Collection was also investigated.

Methoxychlor, an organochlorine insecticide, is used as a replacement for DDT. Although it is less persistent than DDT in the natural environment, its residual-prone is still of concern, but there are not many reports on its environmental fate in soils, or in sediments. We found in a preceding study that dissipation of methoxychlor occurs relatively rapidly in river sediment. That is, reductive dechlorination yielding dechlorinated methoxychlor (de-Cl-MXC) under microaerobic conditions in submerged environments, serves to increase the susceptibility of methoxychlor to biodegradation. In the present study, we also identified the possibility that *B. elkanii* and closely related strains are involved in the degradation of dechlorinated methoxychlor (de-Cl-MXC). Their total role in the degradation of methoxychlor in soil and surface water environments is discussed.

### Materials and Methods

**Chemicals.** Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] (99.5% purity) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). [Ring-U-14C]methoxychlor (specific radioactivity, 5.84 MBq mg⁻¹, >98% radiochemical purity, [14C]methoxychlor) was from BlyChem (Billingham, UK). Mono- and bis-demethylated methoxychlor (mono-OH [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane], >99% purity, and bis-OH [1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane], >99% purity) were obtained from Cedra (Austin, TX). Non-radiolabeled forms of the above-listed chemicals (methoxychlor, mono-OH, and bis-OH) were used as reference compounds. Methoxychlor was also used for isotopic dilution of [14C]methoxychlor. Dechlorinated methoxychlor [1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane] (de-Cl-MXC), mono-demethylated-dechlorinated methoxychlor [1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane] (de-Cl-Mono) and bis-demethylated-dechlorinated methoxychlor [1,1-dichloro-2,2-bis(4-hydroxyphenyl)ethane] (de-Cl-bis) were prepared from [14C]methoxychlor and described in the above-mentioned previous study, and were used as reference compounds. [14C]de-Cl-MXC was also used as initial substrate to estimate degradability by these strains compared to methoxychlor.

*Bradyrhizobium* species. Standard strains of *B. elkanii* (JCM 10832) and *B. japonicum* (JCM 10833) were purchased from the Japan Collection of Microorganisms (JCM) at the RIKEN Bioresource

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Preparation of methoxychlor-degrading bacterial source. Paddy field soil (Ushiku, Ibaraki prefecture, Japan) was used as source of degrading microbes. The soil was collected on April 25, 2011, to a depth of approximately 10 cm. After removal of gross debris, the soil was passed through a 2-mm sieve and stored at 4 °C in the dark. The physical and chemical properties of the soil were as follows: pH (H2O), 6.6; organic carbon content, 39.1 (g/kg of the dry soil); texture, clay loam (classified by the USDA method). To a glass bottle (inside diameter, 50 mm; capacity, 200 mL), fresh soil (corresponding to the 50 g of the dry weight) was transferred. An appropriate amount of pure water was then poured into the bottle, followed by agitation of the whole system. After precipitation of the soil, the depths of the water and of the soil were measured to be approximately 1 cm and 5 cm respectively. The bottle was allowed to stand for 15 d at 25 °C to stabilize the microflora (pre-incubation). Application was done by culture as the first step. Membrane pieces were used as inocula to obtain a mixed microbial population (approximately 3.5-mm square-shaped pieces) to 10–100 cells per piece. The bacterial cells were trapped, was cut into pieces (approximately 800 bases) of the PCR products were cycle-sequenced by Takara Bio (Shiga, Japan). We performed a similarity search by the BLAST search. This membrane filter, on which the bacterial cells were trapped, was cut into pieces (approximately 3.5-mm square-shaped pieces) to 10–100 cells per piece. The membrane pieces were used as inocula to obtain a mixed microbial culture as the first step.

Screening of methoxychlor-degrading bacteria. Isolation of degrading bacteria was done by the procedures described in our previous report. Of the 98 microbial mixed cultures in glass tubes, 89 degraded methoxychlor, if only to a slight extent. We selected 20 mixed bacterial cultures randomly and further screened them by a conventional spread plate method. Then we screened 38 single colonies that we thought might degrade methoxychlor. Of the 38 bacteria, 12 showing apparent degradability were further selected for phylogenetic analysis.

Phylogenetic and physiological characterization of methoxychlor-degrading isolates. The bacterial strains screened were grown on 10-fold diluted R2A agar without methoxychlor for characterization and classification. Eubacterial 16S rRNA genes of the pure isolates were amplified following a protocol previously described, with a slight modification: colony-direct PCR was used because it afforded a sufficient amount of target DNA fragments. The anterior half (approximately 800 bases) of the PCR products were cycle-sequenced by Takara Bio (Shiga, Japan). We performed a similarity search by the BLAST algorithm to compare the 16S rRNA gene sequence with those deposited in the Genbank/DDBJ/EMBL database. The 12 methoxychlor-degrading isolates selected as in the preceding section harbored 16S rRNA gene sequences that were 100% identical to each other, and these isolates showed high similarity to the species Bradyrhizobium elkanii. We further conducted conventional morphological and physiological examinations and almost full-length (1375 bases) 16S rRNA gene sequencing for one strain, referred to as strain 4-1, with the aid of the TechnoSuruga Laboratory (Shizuoka, Japan), or in our laboratory to ensure that the isolate was attributed to the genus identified on the basis of a BLAST search.

Degradation of 14C-methoxychlor and 14C-de-Cl-MXC. Ten-fold diluted R2A broth containing 0.29 mg/L of 14C-methoxychlor or 0.16 mg/L of 14C-de-Cl-MXC was prepared in a duplicate set of glass tubes (2.5 mL per tube). In each glass tube, a micro glass tube containing 1 mL of 1 M NaOH was mounted as to the collection of 14CO2 possibly released by cleavage of the radionuclide phenyl ring (mineralization). For inoculation, cultures of B. elkanii and strain 4-1 pre-grown on 10-fold R2A agar (0.1 mg/L methoxychlor) were harvested with a small amount of sterile water. An aliquot of the harvested cell suspension was then transferred to the medium (inoculation). The initial cell density was adjusted to 0.005 (O.D. 600) by adding a calculated amount of the cell suspension. All tubes were incubated at 25 °C for up to 82 d. Methoxychlor or de-Cl-MXC, and the degradation products in the medium, were analyzed periodically by TLC. The amount of 14CO2 trapped in the alkaline solution was measured at 49 and 82 d after inoculation. In addition, a non-inoculated control medium was prepared in monoplicate and analyzed in the same manner as the inoculated samples.

For an additional experiment, 10-fold diluted R2A broth containing 14C-methoxychlor was prepared in a triplicate set, and B. japonicum, B. elkanii, and strain 17-4 was inoculated into each set in the manner described above. The methoxychlor and the degradation products in the medium were analyzed by TLC 35 d after inoculation.

Identification of degradation products. Identification was done by HPLC and TLC co-chromatography for degradation products corresponding to the available reference standards: methoxychlor, de-Cl-MXC, mono-OH, bis-OH, de-Cl-mono, and de-Cl-bis. Carboxylic acids, mono-COOH and bis-COOH, were identified by comparing TLC co-chromatography developed with the metabolites produced by strain 17-4 obtained in our previous study.

Analytical methods. Radioactive compounds in the liquid medium were detected and quantified by TLC analysis. Aliquots of the liquid media were spotted onto an aluminum TLC plate pre-coated with silica gel 60F254 (Merck, Darmstadt, Germany), and the plate was developed by mixed solvent systems comprising n-hexane/ethyl acetate (75:25) for the screening of the degrading bacteria and by n-hexane/ethyl acetate/formic acid (60:40:1) for quantitative determination of the degradation products. Radioactive spots were detected with a Fujifilm BAS-2500 bio-imaging analyzer (Fujifilm, Tokyo) and quantified with the analyzer software. The undeveloped radioactivity was determined to be a mixture of trace amounts of multiple degradation products by comparison with HPLC analysis of the same sample. Accordingly we quantified this radioactivity collectively as “polar degradation products.” (Figs. 1 and 2).

For HPLC analysis, a Shimadzu LC-10A HPLC system (Shimadzu, Kyoto, Japan) with a Ramona Star radioactive flow scintillation analyzer (Raytest Isotopenmessgeraete, Straubenhardt, Germany) was used. Non-radioactive compounds were monitored at 240 nm. The radioactive compounds were separated using a Capcell Pak C18 ACR column (4.6 mm i.d. × 250 mm, 5 μm; Shiseido, Tokyo) with Atomlight(TM) or Hionic-Fluor(TM) (PerkinElmer) as scintillation cocktail.

Results

Characteristics of strain 4-1

Strain 4-1 is a gram-negative, short-to-long, rod-shaped (0.7–0.8 x 1.5–2.0 μm), non-spore-forming bacterium. Younger cells are highly motile. Colonies on R2A agar are circular (<1.0 mm in diameter), convex, entirely margined, smooth, opaque, butte-like, and pale yellow. Growth does not occur at temperatures ≥37 °C. The cells were positive for catalase and oxidase. Acid/ gas formation from glucose and anaerobic growth was negative. Growth did not occur in the medium at a concentration of ≥1.0% NaCl. Comparing the 16S rRNA partial gene sequence of strain 4-1 (1375 bp) to the sequence available in the Genbank database revealed 100% identity to B. elkanii (FJ025139) and several
other *B. elkanii* strains. Furthermore, the sequence was also 100% identical to strain 17-4. Most of the physiological properties tested were similar to those of the genus *Bradyrhizobium* and species *B. elkanii* type strain. Some properties, however, were distinct: growth did not occur in the presence of 1% NaCl, and the isolate reduced nitrate, and utilized L-arabinose, and D-mannitol, but not D-mannose or capric acid. Accordingly, we tentatively positioned the strain as a new species of the genus *Bradyrhizobium* and designated it strain 4-1 for further investigation.

**Degradation of methoxychlor by *B. elkanii* and strain 4-1**

The dissipation of [14C]methoxychlor and formation of the primary degradation product mono-OH in 10-fold diluted R2A broth after inoculation of *B. elkanii* is shown in Fig. 1A (left). In addition, the production of further degraded metabolites and the generation of radioactive carbon dioxide (14CO2) by ring-cleavage is shown in the right graph. Figure 1B represents degradation by strain 4-1. The graphs clearly indicate that *B. elkanii* and strain 4-1 transform methoxychlor through almost the same metabolic pathways. The half-life of methoxychlor by each strain was assumed to be about 20 d.

The right graphs in Fig. 1 show time-course quantification of degradation products other than mono-OH. As the graph indicates, the primary metabolic route probably was oxidative dechlorination forming the carboxylic acids mono-COOH. The polar degradation products that were left undeveloped on the TLC origin increased with time. DC-mono (de-Cl-mono) was also produced by both strains, but de-Cl-MXC was detected in a trace amount (<1% of applied radioactivity, not shown in the graph). As described in the following section, de-Cl-mono can be further metabolized relatively easily. Another main metabolite was bis-COOH. It increased with time, and particularly by strain 4-1, reached near 15% of applied radioactivity. Other than those metabolites described above, bis-demethylated methoxychlor (bis-OH) was detected in a small amount. The degradation products finally underwent ring-cleavage and mineralized to 14CO2.

**Degradation of de-Cl-MXC by *B. elkanii* and strain 4-1**

As shown in Fig. 2 (left graphs), when we used [14C]de-Cl-MXC as the initial substrate instead of [14C]methoxychlor, the degradation rates of [14C]de-Cl-MXC by the various strains, was higher than that of [14C]methoxychlor. Assuming that the initial degradation (0–15 d) followed first-order linear regression, the half-life of methoxychlor was calculated to be 8.1 d (r² = 0.9243) for *B. elkanii* and 7.3 d (r² = 0.9568) for strain 4-1. Moreover, as the graphs clearly indicate, de-Cl-mono is more susceptible to biodegradation by these strains than mono-OH. The further metabolic profile
(right graphs) was somewhat distinct from methoxychlor: “unknown” was the major degradation product. This compound appeared structurally to resemble bis-COOH, because it developed at the same position on TLC analysis, but did not match bis-COOH on HPLC analysis. The polar degradation products were also produced in large amounts as compared to methoxychlor-fed culture. Similar to the methoxychlor-fed culture, mono-COOH yielded more than 20% of applied radioactivity. A small amount of de-Cl-bis (DC-bis), which corresponds to bis-OH in methoxychlor-fed culture, was also detected. The degradation products were finally mineralized, but a cumulatively greater amount of $^{14}CO_2$ was released over 82 d as compared to the methoxychlor-fed culture.

Degradation of methoxychlor by B. japonicum, B. elkanii and strain 17-4

Figure 3 presents the pattern of degradation products by two *Bradyrhizobium* species and previously isolated strain 17-4.\(^ {19} \) Figure 4 shows the further progressed degradation (49 d after inoculation) patterns by *B. elkanii* and closely related strains, 4-1 and 17-4. These chromatograms indicate that the three strains transformed methoxychlor via common degradation products. *B. japonicum*, however, did not degrade methoxychlor (Fig. 3).
Discussion

Various microbial species capable of degrading specific pesticides have been isolated from the natural environment. They belong to a wide range of phylogenetic classes. For methoxychlor, several degrading-microbial species are known other than *Bradyrhizobium*. The details of the degradation route, however, have not been investigated. These microbes were isolated from the environment based on their capacity to degrade specific pesticides, so that their phylogenetic classification was not objective for the researchers. However, classification of them is necessary as basic information, and 16S ribosomal RNA gene sequences are often examined. In most cases, the isolates were classified at the genus level, but they hardly matched previously registered species. This indicates that most pesticide-degrading microbes isolated to date are to be assigned to new species. Considering these facts, it is rare that a standard strain deposited in a Culture Collection shows degradability against a specific pesticide. The degradation of methoxychlor by *B. elkanii* (USDA94) reported in this study is probably an exceptional case.

Two strains, *Bradyrhizobium* sp. 4-1 and 17-4, isolated in our study, were not to be classified as the same species as *B. elkanii* due to differences in physiological properties, but the partial sequences of the 16S ribosomal RNA gene are 100% identical to *B. elkanii* indicating that they are phylogenetically very close. To our knowledge, this is the first evidence that even a standard leguminous bacterium isolated from root nodule of soybean is able to degrade methoxychlor. Moreover, *B. japonicum*, which was originally the same species as *B. elkanii* (derived taxonomically from *B. japonicum* on 1993), showed no degradability for methoxychlor. In this study, we focused on the fact that the genus *Bradyrhizobium* has a greater number of cytochrome P450 genes than other bacteria. It is well known that *O*-demethylation of methoxychlor in higher organisms is mediated by cytochrome P450 enzymes. However, *B. japonicum*, which was originally the same species as *B. elkanii*, showed no degradability for methoxychlor. This suggests that *O*-demethylation of methoxychlor is not mediated by common cytochrome P450 enzymes in *Bradyrhizobium* species. To determine the relationship between microbial cytochrome P450 and the *O*-deme-

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Fig. 4. Degradation Profiles of [14C]Methoxychlor by *Bradyrhizobium elkanii*, and Closely Related Strains Visualized by TLC Radio-Chromatography 49 d after Inoculation.

The radio-chromatogram of strain 17-4 (right) is cited from reference 19.

Fig. 5. Summary of Main Degradation Pathways of Methoxychlor in the Natural Soil-Fresh Water Environment, Including Metabolism through *B. elkanii* and Closely Related Bacterial Strains.

The hollow arrow indicates that the reaction can occur abiotically.
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thylation of methoxychlor in detail, further analysis is needed.

Figure 5 summarizes the main degradation pathways of methoxychlor common to B. elkanii and B. sp. strain 4-1. In the surface water environment, methoxychlor has been found to be transformed to yield de-Cl-MXC relatively readily through both abiotic and microbial activity. Consequently, a pathway through de-Cl-MXC should facilitate faster degradation of methoxychlor by B. elkanii and closely related strains. Intermediary metabolites, such as carboxylic acid and the other polar metabolites, appear to serve as more available substrates, for ubiquitous microbes (cross-feeding), possibly resulting in faster dissipation of methoxychlor in the surface water environment. We also found in this study that B. elkanii and closely related strains themselves finally cleave the ring structure and mineralize methoxychlor. To our knowledge, this is the first report of bacteria that can mineralize methoxychlor individually. The three strains described here were isolated from root nodules of soybean plant in Florida (B. elkanii), river sediment in Japan (strain 17-4), and paddy field soil in Japan (strain 4-1). Although we need more samples from various locations, B. elkanii and closely related strains are probably distributed worldwide in the natural soil-water environment and probably play a major role in degrading methoxychlor. Although there is little evidence, many researchers concerned with the environmental fate of pesticides believe that biodegradation of a given pesticide passes through almost the same route everywhere in the world. Our findings are expected to prove to be typical evidence that phylogenetically close but geographically and ecologically discrete strains share a common degradation route for a pesticide.

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References