1 Root endophytic bacteria of a ^{137}Cs and Mn accumulator plant, *Eleutherococcus*
2 *sciadophylloides*, increase ^{137}Cs and Mn desorption in the soil

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6 7 Abstract

8 We found that root endophytes of ^{137}Cs accumulator plant produce
9 siderophores, resulting in the desorption of ^{137}Cs from the contaminated soil collected at
10 Fukushima, Japan. We selected an endemic Japanese deciduous tree, *Eleutherococcus*
11 *sciadophylloides* (Franch. et Sav), that accumulates high concentrations of ^{137}Cs and Mn.
12 Root endophytic bacteria were isolated from *E. sciadophylloides* and microbial
13 siderophore production was evaluated via chrome azurol S (CAS) Fe and CAS Al
14 assays. Of the 463 strains that we isolated, 107 (23.1%) produced the siderophores.
15 Using eight strains that showed high siderophore production in our assays, we examined
16 desorption of ^{137}Cs , Mn, Fe and Al by the bacterial culture filtrates from
17 ^{137}Cs -contaminated soil after decomposing the soil organic matter using hydrogen
18 peroxide. We found ^{137}Cs and Mn desorption concomitant with Al and Fe desorption, as
19 well as a decrease of pH. We also detected succinic acid, a well-known siderophore, in
20 the bacterial culture filtrates of our two root endophytic bacteria. Our results strongly
21 suggest that the root endophytic bacteria of *E. sciadophylloides* produce the
22 siderophores that enhance ^{137}Cs and Mn desorption in the rhizosphere, making the
23 resulting ^{137}Cs and Mn ions easier for *E. sciadophylloides* to absorb from the
24 rhizosphere.

Keywords: Root endophytic bacteria, CAS assay, siderophore, ^{137}Cs desorption, Mn desorption

Abbreviations: CAS, chrome azurol S; CEC, carbon exchange capacity; GC-MS, gas chromatography–mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; NBA, nutrient broth agar; PTFE, polytetrafluorethylene; RSM, rhizosphere liquid medium; SIM, selected ion monitoring; TSA, tryptic soy agar

1. Introduction

Damage to Japan's Fukushima Daiichi Nuclear Power Station caused by a giant tsunami in March 2011 resulted in widespread contamination of the environment via the release of radionuclides. Radiocesium (^{137}Cs) is an especially serious environmental contaminant because of its long half-life (30.2 years) compared with other radionuclides such as ^{131}I (8.04 days) and ^{134}Cs (2.06 years). Because Cs ions are adsorbed on negatively charged sites of soil organic matter and clay minerals (Cornell, 1993; Wahlberg and Fishman, 1962; Yamaguchi et al., 2012; Ohnuki and Kozai, 2013), ^{137}Cs is retained in the surface soil (Fujii et al., 2014; Kato et al., 2012; Tanaka et al., 2012). Although adsorbed ^{137}Cs is not considered to be a bioavailable form in the soil, there are many reports that crops (Harada et al., 2014; Sakai et al., 2014), trees (Kuroda et al., 2013), weeds (Yamashita et al., 2014), and mushrooms (Miyazaki et al., 2013) have absorbed ^{137}Cs from the soil since the Fukushima accident. However, for plants and

microbes to absorb ^{137}Cs from soil, the ^{137}Cs must be desorbed from the adsorption sites of clay minerals and organic matter.

Rhizosphere microorganisms, such as those at the root–soil interface, generally enhance nutrient uptake by plant roots via the production of chemical substances such as siderophores (Brimecombe et al., 2001; Nannipieri et al., 2008; Schultz and Boyle, 2006), thereby promoting plant growth. Microbial siderophores, including organic acids, can chelate Fe and other metals and make them bioavailable (Bultreys, 2007). Root endophytes, which are members of the rhizosphere microorganism community, were isolated from plant species that accumulate metals and found to produce siderophores (Whiting et al., 2001), indicating the possibility of a microbial role in the uptake of metals into plant tissues (Nagata et al., 2013).

Similar microbial support might apply to ^{137}Cs uptake by plant species that accumulate Cs. For example, oxalic acid is probably released from plant roots and microbes (Brimecombe et al., 2001), and can enhance Cs desorption from illite by weathering the illite (Wendling et al., 2004). In addition, Wendling et al. (2005) found that bacterial metabolites of *Bacillus* sp. significantly enhanced Cs desorption from frayed edge sites (FES; strong surface association sites of Cs in illitic minerals) of illite due to the Al-complexing abilities of the metabolites, which enhanced weathering at FES of illite. Chiang et al. (2011) showed that representative siderophores such as acetic, succinic, oxalic, tartaric, and citric acids, which are likely released from microorganisms, weather clay minerals and result in desorption of ^{137}Cs from strongly adsorbed sites, such as interlayer and FES of clay minerals via their abilities to supply H^+ ions to weaken Al-O and Fe-O bonds on clay minerals, and to chelate Al and Fe to weaken the surface structure of clay minerals. Soil is composed of many constituents,

including minerals and organic matter. This indicates that the fallout ^{137}Cs in the soil of Fukushima is sorbed not only by illitic minerals, but other minerals (Kozai et al., 2012).

In this study, we examined the dissolution of ^{137}Cs from the contaminated soil samples collected in Fukushima, Japan by using root endophytes in ^{137}Cs accumulator plants. One of the important problems in-with radiocesium-Ces pollution in, Japan is that ^{137}Cs is strongly adsorbed to clay minerals in soil (Yamaguchi et al., 2012), therefore, as a first step, we focused on ^{137}Cs strongly adsorbed to clay minerals. We selected an endemic Japanese deciduous tree, *Eleutherococcus sciadophylloides* (Franch. et Sav), that accumulates high concentrations of ^{137}Cs (Kiyono and Akama, 2013). This plant is also interesting because it is a known hyperaccumulator of Mn (Memon and Yatazawa, 1982, 1984), indicating that specific root endophytes might also enhance Mn desorption. We isolated root endophytic bacteria from *E. sciadophylloides* and examined microbial siderophore production via the chrome azurol S (CAS) Fe and CAS Al assays (Alexander and Zuberer, 1991).

Using eight strains that showed high siderophore production in the CAS assays, we analyzed ^{137}Cs , Fe, Al, and Mn desorption using ^{137}Cs -contaminated soil after decomposing the soil organic matter and obtaining bacterial culture filtrates. Manganese desorption from soil after decomposition of organic matter was also examined. Finally we identified possible siderophores in the bacterial culture filtrates. Our research provides important information on the mechanisms of ^{137}Cs absorption that occur due to plant-microbe interactions.

2. Materials and methods

2.1 Collection of *Eleutherococcus sciadophylloides* and ^{137}Cs analysis

Five individuals of *E. sciadophylloides* (3.4 ± 0.2 years old, confirmed by means of annual ring analysis) were collected in September 2013 from a forest ($37^{\circ}41'N$, $140^{\circ}27'E$) at Fukushima University. According to our preliminary analysis, the collection site was not contaminated by metals compared with the data of non-polluted forest soil in Japan (Asami, 2001), and *E. sciadophylloides* contained an extremely high concentration of Mn in its leaves (1280 ± 388 mg/kg dry weight, $n=6$) in our analysis performed 1 month before the sample collection. Collected plants were used for ^{137}Cs analysis and root-endophyte isolation. Simultaneously, root-zone soil ($100 \times 100 \times 50$ mm volume excluding litter was collected from the observation area of the root system, which was shallow-rooted, $100 \times 100 \times 50$ mm in depth) removing litters was collected from each individual tree. After soil collection, the roots were dug out with digging roots by trowels so that the whole trees were collected. The collected trees were carefully washed with tap water followed by deionized water, and were then separated into leaves and the root system. In the root system, there were tap roots, which were shallowly sinuous, and few fine roots emerging from tap roots in accordance with Karizumi (1979); therefore, we used only tap roots in this study. Leaves and subsamples of tap roots were dried at $80^{\circ}C$ for 48 h and the dried plant samples were then weighed and ground with electric mill (IFM-650D, Iwatani, Tokyo, Japan). Root-zone soil was air-dried and passed through a sieve (<2 mm). Concentrations of ^{137}Cs in the plant powder and soil were analyzed at 662 keV for 3000 seconds using a germanium semiconductor detector (GMX-15190-P, ORTEC, Oak Ridge, Tennessee, USA). The remaining subsamples of tap roots were used for observation and isolation of root endophytes shown as in section 2.2.

2.2 *Observation and isolation of root endophytes*

To measure infection rates by arbuscular mycorrhizal fungi (*Paris* types) (Oba et al., 2006) or fungal endophytes (microsclerotia) (Jumpponen and Trappe, 1998), subsamples of tap roots, which were cut into round slices and stained with trypan blue, were observed under the microscope. Infection percentage of root length colonized was calculated according to the gridline-insect method (Giovannetti and Mosse, 1980; McGonigle et al., 1990). We focused on the bacterial root endophytes because arbuscular mycorrhiza (*Paris* type) and other fungal structures were rarely observed in epidermal cells of the tap roots; the percentage of roots infected by arbuscular mycorrhizal fungi and other fungi was $0.67 \pm 0.38\%$ (mean $\pm$ SE, $n = 5$). Root bacterial endophytes were isolated from the tap roots of *E. sciadophylloides* by means of the sterile procedure described by Hata (1997). Briefly, subsamples of tap roots were carefully washed with running tap water to remove soil, followed by rinsing with deionized water, then the root surfaces were sterilized in 70% ethyl alcohol for 1 min, in 15% hydrogen peroxide solution for 15 min, and in 70% ethyl alcohol for 1 min. After the surface sterilization, roots were rinsed with sterile deionized water to remove the solvents. The sterilized roots were cut into round slices including epidermal cells (about $10 \times 10 \times 5$ mm pieces), using sterile pruning scissors and a sterile scalpel. We obtained a total of 1193 pieces from the five individuals, and placed half of this sample (597 pieces) on 2% agar plates containing nutrient broth (Difco) (at 1% nutrient broth agar [NBA]) and the remaining 596 pieces on 2% agar plates containing tryptic soy broth (Difco) (at 1% tryptic soy agar [TSA]); thus, about 119 pieces per individual plant were placed on 1% NBA and another 119 were placed on 1% TSA. The agar plates were incubated at 23°C for 20 days in the dark. After 20 days, root bacterial endophytes were

purified by means of serial plating on each medium (191 strains on 1% NBA; 272 strains on 1% TSA).

2.3 Bacterial siderophore detection using the CAS assay

The bacterial siderophores capable of chelating Fe and Al were detected using CAS assay agar plates (90-mm i.d.) using FeCl₃ (final concentration, 10 µM) in the CAS Fe assay (Alexander and Zuberer, 1991; Gascoyne et al., 1991) and AlCl₃ (final concentration, 10 µM) in the CAS Al assay (Alexander and Zuberer, 1991). Bacterial strains were incubated on CAS Fe and CAS Al assay agar plates for 7 days at 23°C in the dark. When bacteria produced siderophores, orange halos formed around the bacterial colonies. We measured the diameter of the orange halos and bacterial colonies, and defined siderophore production as the ratio of the orange-halo diameter to the bacterial-colony diameter. A ratio of more than 1.0 was considered to indicate significant siderophore production. We used three plates for each bacterial strain, and all reported values represent the mean ± standard error (SE).

2.4 Test of ¹³⁷Cs, Fe, Al, and Mn desorption from ¹³⁷Cs-contaminated soil after decomposition of organic matter

We used eight bacterial strains (n142, n164, n585, n1101, n3172, t114, t142, and t354) that showed high siderophore production in the CAS Fe and CAS Al assays in the desorption test. Each bacterial strain was inoculated at a density of 1×10⁶ cfu/mL into 100 mL of rhizosphere liquid medium (RSM; Buyer et al., 1989). The inoculated media were then incubated in an SI-600R Incubator (As-One, Osaka, Japan) with shaking at 150 rpm at 23°C for 48 h in the dark. After the incubation, the bacterial

cultures were centrifuged at $20\,000 \times g$ for 30 min (himac CR22E, Hitachi, Tokyo, Japan). The supernatants were filtered first through 0.4- μm mixed cellulose–ester filters, then through 0.2- μm polytetrafluorethylene (PTFE) filters (Advantec, Tokyo, Japan). The resulting culture filtrates were used for the desorption test. The culture filtrate pH was measured using an AF-22 meter (Horiba, Tokyo, Japan).

Samples of a highly ^{137}Cs -contaminated muck soil (soil classification of NIAES, 2010; Takata et al., 2009, 2011) were randomly collected to a depth of 5 cm from 10 points of a paddy field (10 $\times$ 25 m) (about 1 L of soil was collected from each point) in Fukushima Prefecture (37°23'17.5"N, 141°01'27.4"E) in July 2014 and fully mixed. The clay mineralogical composition in the area around the collection site (the Hama-dori region) was semi-smectitic or smectitic (Nakao et al., 2014; Sano et al., 2010). The soil was air-dried and passed through a sieve (<2 mm). In order to evaluate ^{137}Cs desorption from mainly clay minerals, organic matter in the air-dried soil was fully decomposed by adding excess 30% hydrogen peroxide (CSEA, 1997) and fully washed with deionized water to remove exchangeable and absorbed ^{137}Cs on organic matter. We used four replicates for the following each analysis: Table 1 summarizes the ^{137}Cs , Fe, and Al concentrations, pH (H₂O) and exchangeable Na, K, Ca and Mg of the soil after decomposition of the organic matter. Total Fe and total Al were analyzed by means of inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7300DV, PerkinElmer, Waltham, MA, USA) after digestion of the post-decomposition soil in concentrated HNO₃-HClO₄ (1:4 v/v). Table 1 also shows the proportion of the ^{137}Cs (%) in the residual fraction after dithionite-citrate extraction (Holmgren, 1967) and the concentrations of the dithionite-citrate-extractable Fe and Al (Table 1). ^{137}Cs (%) in the residual fraction shows the percentage of ^{137}Cs concentration in the post-decomposition

soil. For the calculation of ^{137}Cs (%) in the residual fraction, data determined by a gamma ray scintillation counter (TDC-521, Hitachi Aloka Medical Ltd., Tokyo, Japan) were used. We used two replicates for the analysis of CEC, organic C and total N of the soil after decomposition of the organic matter. CEC was analyzed according to the method of FAO (2014). Organic C and total N were analyzed by NC analyzer (NC-900, Sumika Chemical Analysis Service, Ltd.) using soil after decomposition of the organic matter (<500 μm).

In the ^{137}Cs , Fe, Al, and Mn desorption analysis, 20 mL of each bacterial culture filtrate was mixed with 10 g of soil after decomposition of the organic matter, and was then shaken at 150 rpm for 24 h at room temperature. After the shaking, the mixed solution was filtered through No. 6 filter paper and 0.2- μm PTFE filters (Advantec). As a control, RSM medium was mixed with the post-decomposition soil and shaken in the same way. ^{137}Cs in each culture filtrate was analyzed at 662 keV for 3000 seconds using a germanium detector (GEM40-76, ORTEC) with a sample changer (model KS-100mk10, Shinwa, Kanagawa, Japan). Fe, Al, and Mn in each culture filtrate were analyzed using ICP-OES. Three replicates were used for each bacterial strain, and values are reported as the mean $\pm$ standard error (SE).

2.5 Qualitative and quantitative analyses of bacterial culture filtrates

Four bacterial strains (n142, n164, n1101, t142) that showed high siderophore production in the CAS Fe and CAS Al assays and high desorption of ^{137}Cs , Fe, Al, and Mn in the desorption assays were analyzed to detect organic acid production. Each bacterial strain was inoculated at a density of 1×10^6 cfu/mL into 100 mL of RSM, and the inoculated media were incubated in the SI-600R incubator with shaking at 150 rpm

at 23°C for 48 h in the dark. After the incubation, each medium was filtered through a 0.2- μ m PTFE filter (Advantec) and part of the filtrate (100 μ L) was applied to a formic acid ion-formeD Toyopak-DEAE M column (1 mL; Tosoh Corporation, Tokyo, Japan) that was pre-equilibrated using de-ionized water. After washing the column with de-ionized water (5 mL), organic acids from the filtrate samples were eluted using 5 mL of 6 M formic acid and dried *in vacuo* at 40°C, followed by freeze-drying (VD-250F; Taitec Corporation, Saitama, Japan) to remove the formic acid. Formic acid ion-formeD DEAE resin is a weakly basic anion exchanger, and it can adsorb weakly acidic compounds from water.

The dried eluate was dissolved in 100 μ L of pyridine and trimethylsilylated using 100 μ L of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Thermo Scientific, Bellefonte, PA, USA), and the mixture was incubated at 37°C for 30 min. Trimethylsilylated samples were analyzed by means of gas chromatography-mass spectrometry (GC-MS; GC/MS-QP2010 equipped with a GC-2010 electron ionization (EI)-MS detector; Shimadzu, Kyoto, Japan), using a low-polar InertCap 5MS/Sil capillary column (30 m $\times$ 0.25 mm i.d., 0.25- μ m film thickness; GL Sciences Inc., Tokyo, Japan). Oven temperature was kept at 80°C for 2 min, increased to 250°C at a rate of 5°C/min, and then kept at 250°C for 5 min. The injector temperature (split ratio, 25) was set at 250°C and the flow rate of the carrier gas (He, 99.999% purity) was set at 1.77 mL/min. The injection volume was 1 μ L. For the EI-MS detector, the interface temperature and ion source temperature were set at 250°C and 200°C, respectively. Ions were generated by a 70 eV beam and masses were detected at mass/charge (m/z) ratios ranging from 50 to 500. Mass spectra of all detected peaks were compared with the spectra shown in the 9th edition of the Wiley Registry of Mass Spectral Data library

(Wiley, Hoboken, NJ, USA). Main peaks were identified based on the retention time and mass fragmentograms of a purchased laboratory standard solution of succinic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which was trimethylsilylated using MSTFA. For the quantification analysis of succinic acid in the bacterial culture filtrate of n1101 and t142, GC-MS-SIM was used (monitoring ion; m/z 147), after purification of organic acids and trimethylsilylation as described above. Three replicates were used for each bacterial strain, and values are reported as the mean $\pm$ standard error (SE).

2.6 Identification of the bacterial root endophytes using 16 rRNA analysis

Four bacterial strains (n142, n164, n1101, t142) that exhibited high siderophore production and ^{137}Cs desorption were identified. Bacterial DNA was extracted from a single colony isolated on a plate using PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The bacterial 16S rRNA gene was amplified using the primer pairs 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991) with Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan), as described previously (Fukuda et al., 2010). After purification, the PCR products were sequenced using a 3730XL DNA Analyzer (Applied Biosystems) with a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) using 341F (5'-CCTACGGGAGGCAGC AG-3') (Muyzer et al., 1993) as a sequencing primer. We then performed a homology search using the BLAST program provided by the DNA Data Bank of Japan (<http://blast.ddbj.nig.ac.jp/>).

2.7 Statistical analysis

We used one-factor ANOVA followed by a *post hoc* Scheffé test to compare desorption of Al, Fe, ^{137}Cs , and Mn among the bacterial strains, with significance at $P < 0.05$. We also performed Spearman's rank-correlation test (both with significance at $P < 0.05$) to test for correlations among Al, Fe, ^{137}Cs , Mn, and pH in the desorption test. Statistical analysis was conducted using version 21.0.0.0 of the SPSS statistics software for Macintosh (IBM, Armonk, New York USA).

3. Results and discussion

In plants, radiocesium can be distributed to the leaves, flowers, and fruits because of its high mobility in plant cells, which is similar to that of K (Zhu and Smolders, 2000). In contrast, the radiocesium concentration is often higher in roots than in leaves and stems (Cline and Hungate, 1960; Smolders and Shaw, 1995). In our study, we detected a high concentration of ^{137}Cs in *E. sciadophylloides* (Table 2) 3.5 years after the Fukushima accident. Because of this time lag and the fact that the leaves are annually deciduous, adherence of radioactive fallout to leaves would not be a significant source of ^{137}Cs in the leaves. The much higher transfer factor for leaves than for roots indicates that ^{137}Cs was transferred to the leaves from the roots in *E. sciadophylloides* (Table 2). Compared with ^{137}Cs concentrations (22 to 4,870 Bq/kg dry weight) of 20 wild plants, including *E. sciadophylloides* (Kiyono and Akama, 2013), the ^{137}Cs concentration in *E. sciadophylloides* in the present study is clearly high. This suggests that the samples were suitable candidates for isolation of root endophytes capable of increasing ^{137}Cs desorption in the soil.

Although arbuscular mycorrhizal fungi and fungal endophytes are known to

enhance nutrient uptake via their hyphae and infection structures (Smith and Read, 2008), microscopic observation of Trypan-stained roots in the present study revealed very low infection rates by arbuscular mycorrhizal fungi (*Paris* types) and fungal endophytes (microsclerotia; O'Dell et al., 1993). Therefore, these soil microorganisms probably had little effect on ^{137}Cs uptake by *E. sciadophylloides*. For this reason, our analyses focused on bacterial symbiotes. We isolated 191 and 272 bacterial strains on 1% NBA and 1% TSA, respectively, and examined siderophore production in all strains using CAS Fe and CAS Al assays. Among the 463 strains that we isolated, 107 (23.1%) produced siderophores capable of chelating Fe and Al. Among these 107 strains, 59 (55.1%) and 48 strains (44.9%) were isolated on 1% NBA and 1% TSA, respectively.

Eight strains (n142, n164, n585, n1101, n3172, t114, t142, and t354) showed especially high siderophore production (Table 3), so we used these strains in the desorption test with ^{137}Cs -contaminated soil after decomposition of the soil organic matter. After incubation, the pH of the bacterial culture filtrates ranged from 4.91 to 6.87 (Table 3). For comparison, the pH of the RSM medium alone was 6.86; thus, most but not all of the eight strains acidified the culture medium. Four of the eight strains (n142, n164, n1101, and t142) showed significantly higher desorption of Fe, Al, ^{137}Cs , and Mn (Fig. 1a-d) and a greater pH decrease (Table 3) than in the control and compared with most of the other strains.

Spearman's rank-correlation test using data from these eight strains revealed significant ($P < 0.05$) positive correlations between ^{137}Cs desorption and Fe or Al desorption: $r = 0.762$ for ^{137}Cs vs. Fe and $r = 0.714$ for ^{137}Cs vs. Al (Fig. 2a). We also found significant ($P < 0.05$) positive correlations between Mn, Fe, and Al desorption: $r = 0.905$ for Mn vs. Fe and $r = 0.762$ for Mn vs. Al (Fig. 2b). In addition, the pH of the

bacterial culture filtrate was significantly ($P < 0.05$) negatively correlated with desorption of Al and Mn: $r = -0.833$ for pH vs. Al, and $r = -0.881$ for pH vs. Mn, but the correlation between pH and Fe desorption was not significant (Fig. 2c). The correlation between pH and ^{137}Cs desorption was weak, negative, and only marginally significant ($r = -0.690$, $P = 0.058$; Fig. 2d). These results indicate that ^{137}Cs desorption occurred concomitantly with Fe or Al desorption, and that the pH decrease caused by the bacteria was an important factor for Al desorption, but possibly not for Fe and ^{137}Cs desorption. **Manganese** desorption would also be concomitant with Fe and Al desorption and the pH decrease. Based on these results, bacterial root endophytes of *E. sciadophylloides*, which is known to be a ^{137}Cs and Mn accumulator (Kiyono and Akama, 2013; Memon and Yatazawa, 1984), appear to produce siderophores that are capable of chelating Fe and Al, and capable of enhancing desorption of ^{137}Cs and Mn in the rhizosphere: ^{137}Cs and Mn ions would be easily absorbed by *E. sciadophylloides* in the rhizosphere.

Our GC-MS analysis detected succinic acid in the bacterial culture filtrates of strains n1101 and t142 (Table 4), and **the concentrations seemed to be enough to show ^{137}Cs desorption from the soil according to previous research of Wendling et al. (2005): 0.04 mM citrate and oxalate increased FES density on illite, resulting Cs desorption.** In the culture filtrates of n142 and n164, we did not detect any common organic acids and these strains might produce species-specific siderophores. Although organic acids, which possess carboxylic and hydroxyl functional groups, have been reported to supply H^+ ions and chelate Fe and Al in the soil (Jones, 1998), organic acids weakly form complexes with alkali metals such as Cs (Daniele et al., 2008). Chiang et al. (2011) clarified the mechanism of ^{137}Cs desorption by five organic acids (acetic, succinic,

oxalic, tartaric, and citric acids): protonation of the hydroxyl groups and oxygen atoms at the broken edges or surfaces of clay minerals weakens Fe–O and Al–O bonds, and organic ligands attack the OH and OH₂ groups in the minerals to chelate Al and Fe, resulting in weathering clay mineral structures and ¹³⁷Cs desorption from interlayer and FES of clay minerals.

The post-decomposition soil used in our study mainly contained ¹³⁷Cs in the residual fraction after dithionite-citrate extraction (Table 1), and the ¹³⁷Cs is not easily exchangeable in this fraction. However, for the strains that produced organic acids (Table 4), the amounts of ¹³⁷Cs desorption by n1101 and t142 (3.21 and 3.44 Bq/g, respectively; Fig. 1c) were less than the dithionite-citrate extractable ¹³⁷Cs (11.4 Bq/g) calculated from the percentage of ¹³⁷Cs in the residual fraction (93.3%; Table 1). Therefore, strains n1101 and t142 would release ¹³⁷Cs that is weakly bound to clay minerals, but not strongly bonded ¹³⁷Cs on clay minerals (e.g., at FES) via succinic acid production. In contrast, the amounts of Al desorption by n1101 and t142 (5.70 and 4.79 mg/kg, respectively) were much greater than the dithionite-citrate extractable amount (2.43 mg/kg, Table 1), indicating that succinic acid production might release Al from clay minerals, as Chiang et al. (2011) proposed. Based on the mechanism as described above, Al desorption by the root endophytic bacteria found in our study might affect ¹³⁷Cs desorption from the strongly adsorbed sites on clay minerals in the long term. According to the clay mineralogical composition in the area around our collection site (the Hama-dori region; Nakao et al., 2014; Sano et al., 2010), minerals in our soil would belong to smectite group and we should clarify the detailed content of minerals such as montmorillonite, beidellite, and nontronite. It should be noted that ¹³⁷Cs adsorption mechanisms are different between them; montmorillonite weakly adsorbs ¹³⁷Cs swollen

interlayer sites, and beidellite strongly adsorbs ^{137}Cs on interlayer sites and FES (Yamaguchi et al., 2012).

Manganese exists as bioavailable Mn^{2+} or as insoluble Mn^{3+} and Mn^{4+} oxides in the soil (Teboet al., 2005; Thompson et al., 2005). Mizuno et al. (2006) clarified the Mn-accumulation mechanism of *E. sciadophylloides* using plants regenerated from callus: H^+ release via plasma H^+ -ATPase into rhizosphere enhances MnO_2 solubilization, and would accumulate Mn in the plant cells. Many microorganisms are known to produce metabolites such as formic, pyruvic, and oxalic acids, which can indirectly reduce Mn oxides, converting the Mn oxides into soluble bioavailable forms (Gadd, 2010; Duckworth, 2007; Konhauser, 2007; Wei et al., 2012). In our study, siderophores of bacterial root endophytes would supply H^+ ions from carboxyl group and then reduce Mn oxides, resulting Mn desorption. This Mn desorption by bacterial root endophytes might therefore enhance Mn accumulation in the Mn hyperaccumulator *E. sciadophylloides*.

According to our 16S rRNA analysis, strains n1101 and t142 were identified as *Pseudomonas citronellolis* (homology, 100%) and *Stenotrophomonas maltophilia* (homology, 99%), respectively. Strains n142 and n164 were identified as *Pseudomonas denitrificans* (homology, 99%). These bacteria species are not specific to tree species. *Pseudomonas* species (Dimkpa et al., 2009; Glick, 1995; Kloepper et al., 1989) and *Stenotrophomonas* species (Berg et al., 2005; Suckstorff and Berg, 2003; Tsavkelova et al., 2007) are known to be root endophytes that promote plant growth and plant defenses against biotic and abiotic stresses. *Pseudomonas* species are particularly well known as producers of siderophores (Baysse et al., 2000; Cornelis and Matthijs, 2007; Visca et al., 1992). *Pseudomonas citronellolis* produces pyoverdine, which is a

well-known siderophore (Meyer, 2010). *Stenotrophomonas maltophilia* was also reported to produce catechol-type siderophores to chelate Fe based on a CAS assay (Garcia et al., 2012). Microbial siderophores affect nutrient uptake both by plant cells and by microbial cells. Cs⁺ released from the soil by siderophores can be absorbed into microbial cells via the monovalent cation transport system, as is the case for other cations such as K⁺ (Avery, 1995; Kuwahara et al., 2011; Tomioka et al., 1992). This would result in Cs accumulation in the microorganisms themselves. In addition, Cs accumulation in *Pseudomonas fluorescens* cells was influenced by reversible adsorption on the cell walls (Nakao et al., 2005), which might cause decreases and increases of bioavailable ¹³⁷Cs in the rhizosphere soil and in the roots, accompanied by ¹³⁷Cs uptake by the host plants. We did not examine ¹³⁷Cs accumulation in the cells of the root endophytic bacteria in this study, and further study will be needed to clarify the mechanism of ¹³⁷Cs uptake via plant–microbe interactions.

4. Conclusions

We found that bacterial root endophytes isolated from *E. sciadophylloides*, which accumulates unusually high quantities of ¹³⁷Cs and Mn, produce siderophores capable of chelating Fe and Al, resulting in enhanced ¹³⁷Cs and Mn desorption from ¹³⁷Cs-contaminated soil in Fukushima prefecture, Japan. These endophytes probably affect ¹³⁷Cs and Mn uptake by this plant as a result of these chemical reactions. In the natural world, plants seem unable to thrive without symbiotic microorganisms because of the strong associations between the two life forms. Thus, we should give considerable thought to these relationships because, as the present study suggests, they may increase the risk to human health by mediating the uptake of radioisotopes,

especially in **wild vegetables**. Furthermore, our results would give helpful suggestions to clarify ^{137}Cs uptake mechanisms in plants as well as to improve phytoremediation of radioisotopes.

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Figure captions

Figure 1 Desorption of ¹³⁷Cs, Fe, Al, and Mn using bacterial culture filtrates and soil after decomposition of the organic matter. Note that the y-axis scale differs among the

graphs.

(a) Desorption of Fe. (b) Desorption of Al. (c) Desorption of ^{137}Cs . (d) Desorption of Mn. Values were compared using one-factor ANOVA followed by Scheffé's *post hoc* test; bars labeled with different letters differed significantly between bacterial strains ($P < 0.05$). Values are means $\pm$ SE ($n = 3$).

Figure 2 Spearman's rank correlation test using data from the eight bacterial strains in Figure 1 for the relationships among Fe, Al, ^{137}Cs , Mn, and pH in the desorption test.

(a) Correlations between ^{137}Cs , Fe, and Al desorption. (b) Correlations between Mn, Fe, and Al desorption. (c) Correlations between the pH of the bacterial culture filtrates and Fe, Al, and Mn desorption. (d) Correlation between the pH of the bacterial culture filtrates and ^{137}Cs desorption. Correlation coefficients (r values) are significant at $**P < 0.01$ and $*P < 0.05$.

Figure 1

Figure 2

Table 1 ¹³⁷Cs, Fe, and Al concentrations and pH (H₂O) of the paddy muck soil used for the desorption analysis after decomposition of the organic matter using H₂O₂. Values represent means ± SE (n=4). The residual fraction represents the amount in the post-decomposition soil after dithionite-citrate extraction.

¹³⁷ Cs (kBq/kg)	169.7 ± 1.9
Total Fe (mg/kg)	18000 ± 133
Total Al (mg/kg)	27500 ± 237
¹³⁷ Cs (%) in the residual fraction	93.3 ± 1.5
Dithionite-citrate extractable Fe (mg/kg)	4.43 ± 0.02
Dithionite-citrate extractable Al (mg/kg)	2.43 ± 0.01
pH (H ₂ O)	4.79 ± 0.00
Exchangeable Na (mg/kg)	77.8 ± 9.0
Exchangeable K (mg/kg)	2800 ± 71
Exchangeable Ca (mg/kg)	966.5 ± 18.0
Exchangeable Mg (mg/kg)	235.8 ± 4.3
CEC (cmol/kg)	29.8
Organic C (%)	1.21
Total N (%)	0.14

Table 2 ^{137}Cs concentration in *E. sciadophylloides* and in the root-zone soil. Values are means $\pm$ SE ($n = 5$).

	^{137}Cs concentration (Bq/g) of each individual tree					Average $\pm$ SE
	1	2	3	4	5	
Leaves	8.71	2.00	5.66	2.00	1.24	3.92 ± 1.42
Roots	3.41	0.97	2.11	0.57	0.41	1.49 ± 0.56
Root-zone soil	8.47	2.05	1.56	3.45	1.46	3.40 ± 1.32
Transfer factor Ratio of leaves content to soil content	1.03	0.98	3.63	0.57	0.85	1.41 ± 0.56
Transfer factor Ratio of roots content to soil content	0.40	0.47	1.35	0.17	0.28	0.53 ± 0.21

Table 3 Siderophore production by eight bacterial strains detected by means of the CAS Fe and CAS Al assays, and the culture filtrate pH in RSM medium used for test of ^{137}Cs , Fe, Al, and Mn desorption. Values are means $\pm$ SE ($n = 3$).

Strain	Medium for isolation	CAS Fe assay [†]	CAS Al assay [†]	pH of culture filtrate in RSM medium
n142	NBA	3.0 ± 0.30	2.0 ± 0.10	4.91
n164	NBA	2.2 ± 0.20	2.3 ± 0.40	4.94
n585	NBA	2.0 ± 0.04	3.8 ± 0.10	6.87
n1101	NBA	3.2 ± 0.20	2.5 ± 0.40	4.90
n3172	NBA	1.2 ± 0.03	1.3 ± 0.04	6.41
t114	TSA	2.0 ± 0.04	2.2 ± 0.20	6.74
t142	TSA	2.0 ± 0.30	2.1 ± 0.10	4.92
t354	TSA	4.3 ± 0.30	3.3 ± 0.20	6.70

[†]Values represent the ratio of the clear-zone diameter to the bacterial colony diameter. NBA, nutrient broth agar; TSA, tryptic soy agar; RSM, rhizosphere liquid medium.

Table 4 Species identification of bacterial strains and organic acids in bacterial culture filtrates. n.d., common organic acids were not detected in our analysis. Values are means $\pm$ SE ($n = 3$).

Strain	Species	Organic acid	Succinic acid concentration (mM)
n142	<i>Pseudomonas denitrificans</i>	n.d.	-
n164	<i>Pseudomonas denitrificans</i>	n.d.	-
n1101	<i>Pseudomonas citronellolis</i>	succinic acid	0.99 $\pm$ 0.13
t142	<i>Stenotrophomonas maltophilia</i>	succinic acid	1.00 $\pm$ 0.03