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Identification of microbial communities that assimilate substrate from root cap cells in an aerobic soil using a DNA-SIP approach

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ABSTRACT

Although root cap cells are an important substrate for microorganisms in the rhizosphere, little attention has been paid to the decomposition of sloughed root cap cells by microorganisms. This study used rice plant callus cells grown on medium containing ^{13}C-labelled glucose as a model material for rice plant root cap cells. Harvested ^{13}C-labelled callus cells (78 atom % ^{13}C) were subjected to decomposition in an aerobic soil microcosm for 56 days. The low cellulose and lignin levels and the disaggregated nature of the callus cells indicated that these cells were an appropriate model material for root cap cells. DNA was extracted from a soil incubated with ^{12}C- and ^{13}C-callus cells and subjected to buoyant density gradient centrifugation to identify bacterial species that assimilated carbon from the callus cells. The stability of the total bacterial communities during the incubation was estimated. Many DGGE bands in light fractions of soil incubated with ^{13}C-callus cells were weaker in intensity than those from soil incubated with ^{12}C-callus cells, and those bands were shifted to heavier fractions after ^{13}C-callus treatment. ^{13}C-labelled DNA was detected from Day 3 onwards, and the DGGE bands in the heavy fractions were most numerous on Day 21. DGGE bands from heavy and light fractions were sequenced, revealing more than 70% of callus- C incorporating bacteria were Gram-negative, predominantly α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria*, *Sphingobacteria* and *Actinobacteria*. These species were phylogenetically distinct from the bacteria reported to be present during plant residue decomposition and resident in rice roots. This study indicates that root cap cells are decomposed by specific bacterial species in the rhizosphere, and that these species augment the diversity of rhizospheric bacterial communities.

1. Introduction

Plant roots foster microorganisms in the rhizosphere by supplying various organic substrates such as exudates, mucilages and sloughed-off root tissues. Root cap cells are also a substrate for rhizospheric microorganisms. Sloughing of root cap cells and the exudation of mucilages are thought to play an important role in the penetration of roots into compacted soil (Bengough and McKenzie, 1997; Iijima et al., 2003).

Many cells are generated in the root cap meristem and sloughed into the rhizosphere (Clowes, 1976). For example, Iijima et al. (2004) showed that there were 1400 to 1700 cells in the root cap of maize grown in non-sterile sandy loam soil, and the number of root cap cells sloughed into sand reached 3320 per day per primary root (Iijima et al., 2000). Up to 1610 cells per root were isolated from rice root cap cells simply by gentle agitation in water (Hawes and Pueppke, 1986).

Although these findings indicate the importance of root cap cells as a substrate for microorganisms inhabiting the rhizosphere, the microorganisms responsible for the decomposition of sloughed root cap cells have not been identified. This is probably because of the methodological difficulty of discriminating the microorganisms responsible from those growing on other organic substrates prevalent in the rhizosphere. This study was aimed at identifying the bacteria that decompose sloughed root cap cells from rice plants under aerobic soil conditions.

Linking organisms or groups of organisms to specific functions within natural environments is a fundamental challenge in microbial ecology. Stable isotope probing (SIP) of DNA and RNA can make it easier to link the identity of organisms to their function. DNA- and RNA-SIP involve the incorporation of isotope-labelled substrates into nucleic acids that can be used to identify organisms incorporating the substrate. This study used rice callus cells as a model material for root cap cells, after growing the rice callus on medium containing ^{13}C-labelled glucose (Lee et al., 2011).

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Harvested ^{13}C-labelled callus cells were allowed to decompose in an aerobic soil microcosm. DNA was extracted from the soil periodically, and buoyant density (BD) gradient centrifugation was used to identify the bacterial communities that incorporate rice callus cells.

2. Materials and methods

2.1. Callus induction from rice seeds

Mature rice seeds (*Oryza sativa L.* cv, Yukihikari) were sterilised in 70% ethanol for 1 min and in 4% sodium hypochlorite for 15–18 min, followed by three washes with sterilised distilled water. N6D medium was used as the basal medium for callus induction (Chu et al., 1975; Toki, 1997). After we confirmed that callus cells proliferated just as well with glucose as with sucrose, glucose was added to the N6D medium instead of sucrose. The sterilised seeds were placed on two kinds of N6D agar medium: non-labelled glucose medium and ^{13}C-labelled glucose medium (^{13}C 99%: SI Science Co., Tokyo, Japan). The cultures were incubated at 28 °C. After 3 weeks, callus cells that had proliferated from the rice seed scutella were subcultured on new N6D agar medium and incubated for an additional 3 weeks. After washing with sterilised deionised water, the lump of callus cells was disaggregated by pressing with a spatula, and the cells were used for the soil incubation experiments. The ^{13}C concentration in the ^{13}C-labelled calluses was 78 atom %, as measured by mass spectrometry (Hitachi RMI-2, Tokyo, Japan). The carbon and nitrogen contents of the calluses were 39.3% and 6.7%, respectively, and the composition of the callus cells, as determined by proximate analysis (Waksman and Stevens, 1930; Watanabe et al., 1993), was 10.4% lipid, 34.6% water soluble organic materials, 32.8% hemicelluloses, 6.6% cellulose, 3.8% lignin and 11.8% ash (based on dry weight, Lee et al., 2011). The moisture content of the calluses was 91%.

2.2. Soil sample and preincubation

The soil sample used in this study was collected from a paddy field (D2 field) at Aichi-ken Anjo Research and Extension Center (34°48′N, 137°30′E; Oxyaquic Dystrudept) on 22 April, 2009. The field had been fallow since the previous harvest, six months earlier. Soil from the plough layer (1–15 cm deep) was sampled from three randomly chosen sites in the field, and these samples were mixed well in a polyethylene bag to make a composite sample and stored at 4 °C until use. After sieving through a 2 mm screen on 27 November 2009, the soil sample was adjusted to 55% of the maximum water holding capacity (230 g H_2O per kg dry soil) and preincubated at 25 °C for 14 days. The soil sample had the following characteristics: 12 g kg^{-1} total C, 1.3 g kg^{-1} total N and pH(H_2O), 5.8.

2.3. Soil incubation

Three soil treatments were prepared, as follows: 1) soil without rice callus cells, 2) soil with non-labelled callus cells (referred to as ^{12}C-callus), and 3) soil with ^{13}C-labelled callus cells. Callus cells were added to the preincubated soil at the rate of 20 g kg^{-1} soil (0.87 g callus carbon kg^{-1} dry soil). For each treatment, 10 g of soil was put in a test tube (3 cm in diameter and 20 cm in height) and closed with a silicon plug. Test tubes were placed in a dark room at 25 °C for 8 weeks. Three test tubes were subjected to microbial analysis at each sampling time; samples were taken after incubating for 0 (immediately after mixing the soil with the rice calluses), 3, 7, 14, 21, 28, 42 and 56 days. In addition, the pH was measured in one test tube from the no callus and ^{12}C-callus treatments at each sampling time. The measurement of soil pH was conducted immediately and the soil samples for microbial analysis were stored at −30 °C until use.

2.4. Soil pH

The soil pH was measured from a soil suspension at a soil:water ratio of 1:2.5 with a pH meter (F-7ssII, HORIBA, Kyoto, Japan) after 30 min of occasional stirring.

2.5. DNA extraction and isopycnic centrifugation

DNA was extracted using a FastDNA SPIN kit for soil (MP Biochemicals; Solon, OH, USA), according to the manufacturer's instructions. For each sample, 500 mg of soil was mixed with 978 μL sodium phosphate buffer and 122 μL MT buffer in a Lysing Matrix E tube and homogenised in a Mini-Beadbeater (Biospec Products; Bartlesville, OK, USA) at 2,200 × rpm for 30 s. Purification of the extracted DNA was conducted according to the methods of Jackson et al. (1997) and Cahyani et al. (2003) by loading the DNA extract (100 μL) onto a Sephadex G-200 column in a 1.5 mL tube (spin column). The eluted DNA solution was collected by ethanol precipitation, and the DNA pellet was dissolved in 100 μL of TE buffer and stored at 4 °C.

Density gradient centrifugation was performed on DNA extracted from the ^{12}C-callus and ^{13}C-callus treatments as described by Lueders et al. (2004), with a minor modification in scale; 12 mL polyallomer seal tubes (Hitachi, Tokyo, Japan) and a RPV45 T rotor (*Hitachi,* Tokyo, Japan) were used. The centrifugation conditions were 144,000 × *g* at 20 °C for 36 h. The DNA was resolved in CsCl gradients (4.3 mol l^{-1} CsCl gradient buffer with an average density of 1.720 g mL^{-1}). Centrifugation media were prepared by mixing 10.34 mL of a 1.875 g mL^{-1} CsCl solution with 5 μg of DNA diluted in 2.16 mL of gradient buffer (0.1 M Tris–HCl, pH 8.0, 0.1 M KCl, 1 mM EDTA). Prior to centrifugation, the average density of all prepared gradients was checked with an AR200 digital refractrometer (Reichert, New York, USA) and adjusted by adding small volumes of CsCl solution or gradient buffer, if necessary.

Centrifuged gradients were fractionated from bottom to top into 16 equal fractions (750 μL each). A precisely controlled flow rate was achieved by displacing the gradient medium with water at the top of the tube (Manefield et al., 2002) using an NE-1000 programmable syringe pump (New Era Pump Systems, New York, USA). The mass density of a small aliquot (60 μL) of each collected fraction was determined using the refractometer. DNA was precipitated from the CsCl fractions with polyethylene glycol. The precipitates from the gradient fractions were washed once with 70% ethanol and re-eluted in 30 μL of TE buffer.

2.6. PCR amplification

The variable region (V3 region) of 16S rDNA was PCR-amplified using the bacteria-specific primers 357f-GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG CTT ACG GGA GGC AGC AG-3′, the underlined sequence corresponds to the GC clamp) and 517r (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al., 1993). PCRs were performed in a total volume of 50 μL in 200 μL microtubes; each reaction contained 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mM dNTP mixture, 5 μL of 10 × *Ex Taq* DNA buffer (20 mM Mg^{2+}; TaKaRa, Tokyo, Japan), 0.5 μL of *Ex Taq* DNA polymerase (TaKaRa, Bio), 1 μL of DNA template and 37.75 μL milli-Q water. The cycling conditions for PCR amplification were as follows: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min using a TaKaRa PCR Thermal Cycler Dice (TaKaRa) (Cahyani