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Short Communication

Analysis of Molecular Diversity of Bacterial Chitinase Genes in the Maize Rhizosphere Using Culture-Independent Methods

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DNA fragments of bacterial chitinase genes were successfully amplified from DNA extracts of maize rhizosphere and bulk soil. The molecular diversity of the bacterial chitinase genes in these soil samples was then evaluated by employing terminal-restriction fragment length polymorphism and sequencing analyses. Our results suggest the presence of novel groups of bacterial chitinase in both bulk and rhizosphere soils. Significant differences were observed, however, between the amplified chitinase genes from rhizosphere and bulk soils, and dominant clones obtained from the maize rhizosphere showed a high degree of similarity to the chitinases of *Streptomyces avermitilis* and close relatives there of. The current findings thus indicate the usefulness of culture-independent methods in the assessment of molecular diversity among the chitinolytic bacterial community in soils.

Key words: chitinase, maize, rhizosphere, *Streptomyces avermitilis*, T-RFLP

Chitin is one of the most abundant polysaccharides in nature and is widely distributed across diverse environments as a constituent of several organisms, including-fungal cell walls and the exoskeletons of insects[10]. Chitin-degrading enzymes, the chitinases, are also found in a wide variety of organisms, including fungi, plants, insects, crustaceans, and bacteria[7]. Fungi and bacteria are thought to be important degraders of chitin in soil and thereby contribute to the recycling of carbon and nitrogen resources in soil ecosystems. In bacteria, the primary role of the chitinases is thought to be the digestion and utilization of chitin as a carbon and energy source[3].

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The bacterial chitinases have generated considerable interest in recent years with regard to their possible roles in plant protection against fungal diseases, due mainly to their potential antifungal activity[10]. In addition, bacterial chitinase genes have been introduced into plants in an attempt to enhance their resistance to fungal pathogens[15]. The results of these studies indicate the potential importance of chitinolytic bacteria as biological agents that can confer increased plant protection in agricultural ecosystems. Furthermore, several reports have also now shown that phytospheres, such as leaves and rhizospheres, are important habitats for chitinolytic bacteria[1,9]. For technical reasons, however, most studies of chitinolytic bacteria in the phytosphere have utilized culturable bacteria that have been isolated from either leaves or rhizosphere soils.

To date, several bacterial chitinase genes have been cloned from diverse bacterial groups[8,17,26,27]. Suzuki *et al.*[23]

have proposed subdividing the bacterial family of 18 chitinases into groups A, B, and C, based on amino acid sequence similarity in the catalytic domain. So far, information on the diversity and distribution of the bacterial chitinases is most developed for the group A enzymes, and it has been speculated that this group A is more abundant than the group B or C enzymes[16]. To further elucidate the contribution of different groups of organisms to the degradation of chitin, and to provide additional valuable information concerning the diversity of chitinase genes in terrestrial and aquatic ecosystems, several PCR primer sets for the molecular detection of bacterial chitinase genes have been designed[3–5,20]. Furthermore, Williamson *et al.*[28] have developed a primer set for the group A bacterial chitinase genes that can be used in combination with denaturing gradient gel electrophoresis (DGGE) for the analysis of soil DNA. Subsequently, by using the chitin bag method, Metcalfe *et al.*[16] investigated bacterial chitinolytic communities in upland grassland sites that had been subjected to different soil management procedures.

Current advances in environmental microbiology have revealed that the majority of the microbes in nature are as yet non-culturable[2]. Moreover, there is increasing evidence to indicate that in soils, as other natural environments, complex microbial communities exist which contain both culturable and non-culturable entities[22]. Similarly, culture-independent methodologies have now revealed that the majority of rhizosphere-associated microbes have not been cultured in the laboratory. Thus, there is a need to reexamine the chitinolytic microbial community in the rhizosphere taking into consideration the presence of uncultured microbes. In the present study, we have applied culture-independent methodologies to the evaluation of the molecular diversity of bacterial chitinase genes in the maize rhizosphere, because maize is one of the most important crops and has been extensively used for analyzing microbial communities in the rhizosphere.

Seeds from the maize (*Zea mays*) cultivar 'Silver honey bantam' were sown in a field located within the Tsukuba city area on May 22, 2004. Sampling of the rhizospheres from three plants was subsequently conducted on July 3, 2004. The properties of the maize field soil (EM soil) were analyzed by Kankyo engineering co., Ltd. (Chiyoda-ku, Tokyo, Japan) as described previously[14] (Table 1). The root systems were carefully extracted from the ground and the bulk soils contained within the root structures were collected by shaking, and then sieved (2 mm diameter) and thoroughly mixed prior to DNA extraction. The sampled root systems were gently washed with distilled water, and any soil remaining on the roots was subjected to DNA extraction as a rhizosphere soil sample. Briefly, five root tips (5 cm in length and approximately 0.25 g in wet weight) were collected into a 2 ml screw-capped tube as a sub sample, and three sub samples from each plant were prepared. DNA extraction was subsequently performed using a FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's directions, except that skim milk (8 mg/g [soil weight]) was added to the extraction buffer before bead beating and the washing step was repeated three times instead of only once. The yields of DNA from approximately 0.25 g of wet weight of root sample were ranged from 2 to 3 μg. DNA concentrations were measured using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE, USA), and equal amounts of DNA from the three sub samples for each plant were then combined at a final concentration of 5 ng/μl. These combined DNA samples were used in the PCR analysis. Similarly, 0.5 g of bulk soil for each plant was subjected to DNA extraction and the samples were again prepared at a final concentration of 5 ng/μl.

The terminal restriction fragment length polymorphism (T-RFLP) analysis was carried out using the minor modifications previously reported by Ikeda *et al.*[13]. Briefly, the PCR primers used for the amplification of bacterial chitinase genes were GA1F (cgt cga cat cga ctg gga rtd bcc) and GA1R (acg ccg gtc cag ccn ckn ccr ta), as reported by Williamson *et al.*[28]. For fluorescence detection, the 5' end of the reverse primer was labeled with 6-carboxyrhodamine (Sigma Genosys, Ishikari, Japan). The PCR mixture (total 50 μl) contained 5 μl of 10×buffer, 10 μg of BSA, 0.5 μM of primers, 200 μM of each dNTP, and 4 U of Ex *Taq* HS DNA polymerase (Takara Bio, Otsu, Japan). One microliter

Table 1. Soil characteristics

Sample name	FAO soil grouping	pH	EC[a]	PAC[b]	%C[c]	%N[d]	%OC[e]	%HC[f]
EM	Andosol	4.4	18.9	1590	3.4	0.34	11.6	4.5

[a] Electrical conductivity, mS/cm. [b] Phosphate adsorption coefficient. [c] Total carbon level. [d] Total nitrogen level. [e] Organic content. [f] Humic acid content.

(5 ng of DNA) of soil DNA was used in each amplification reaction as the template. The PCR amplification program used for T-RFLP consisted of 2 min at 94°C, 35 cycles of 30 sec at 94°C and 30 sec at 68°C, and a final extension time of 7 min at 72°C.

The size of the PCR products (approximately 400 bp) was confirmed by 1% agarose gel electrophoresis in 0.5×TBE running buffer. The PCR products (50 µl) were then purified with NucleoSpin Extract II (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and eluted in 25 µl of elution buffer. Aliquots (2.5 µl) were digested with a cocktail containing 1 µl (1 U) each of *Acc*II, *Afa*I, *Hha*I and *Mbo*I (Takara Bio) and incubated at 37°C for 1 hour. The digested PCR products (3.5 µl) were mixed with 2.5 µl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, and 0.1% bromophenol blue). After incubation at 80°C for 2 min, the samples were chilled on ice for 5 min, and directly loaded onto a 6% polyacrylamide gel (19:1 ratio of acrylamide to bisacrylamide, 0.4 mm thick, 40 cm long) containing 7.7 M urea and 0.5×TBE. The electrophoresis was subsequently carried out at a constant 2000 V in 1×TBE for 1.5 hours. Duplicate samples were electrophoresed for both the rhizosphere and bulk soils. Following gel electrophoresis, digital fingerprinting images were then obtained using a fluorescent scanner (Molecular Imager FX, BIO-RAD Laboratories, Inc., Hercules, CA, USA).

PCR amplifications to enable the cloning of chitinase genes were also carried out. These reactions were performed essentially as described for the T-RFLP analysis, except that an unlabelled reverse primer was used. The PCR products were then purified with NucleoSpin Extract II (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and suspended in 15 µl of elution buffer. These purified PCR products were subsequently ligated to the plasmid vector pCR 2.1-TOPO and introduced into *E. coli* using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). After the transformants had been cultured at 37°C on LB agar plates containing 50 µg/ml kanamycin overnight, a total of 96 colonies were selected at random for each sample. Colony PCR was then conducted as described for the T-RFLP analysis of chitinase genes, except that each colony was used as the template DNA. Plasmid clones derived from rhizospheric soils were classified according to their T-RFLP fingerprinting patterns following digestion with *Afa*I and *Hha*I. Three clones corresponding to each fragment in the T-RFLP profiles for rhizosphere soils were then selected for sequencing. These clones were grown at 37°C in 4.5-ml cultures of LB medium containing 50 µg/ml kanamycin overnight, and plasmid DNA preparations and sequencing reactions were performed as described by Ikeda *et al.*[11]. Sequences were manually edited to remove the vector backbone, primer regions, and ambiguous sequences. Sequence data were compared to public database entries using BLASTX and sequence matches were considered to be significant for scores >50[18].

For the phylogenetic analysis, deduced amino acid sequences were aligned using the CLUSTAL W program[24] and the neighbor-joining method was used for building the trees[21]. The Phylip format tree output was applied using the bootstrapping procedure[6] (the number of bootstrap trials used was 1000). Bacterial chitinase genes sequenced in the present study have been deposited in the DDBJ database under the accession numbers AB243161, AB243211-AB243230, and AB274854-AB274881. The accession number for each clone are shown in Figs. 2 and 3.

A comparison of the T-RFLP profiles that we obtained for the bacterial chitinase genes identified in bulk and rhizospheric soils of maize revealed significant differences between them (Fig. 1). At least 17 detectable fragments in the T-RFLP profiles of the rhizosphere soil extracts were either of higher intensity or were specific when compared with the bulk soil profiles. Based on the T-RFLP patterns resulting from digestion with *Afa*I and *Hha*I, 28 and 21

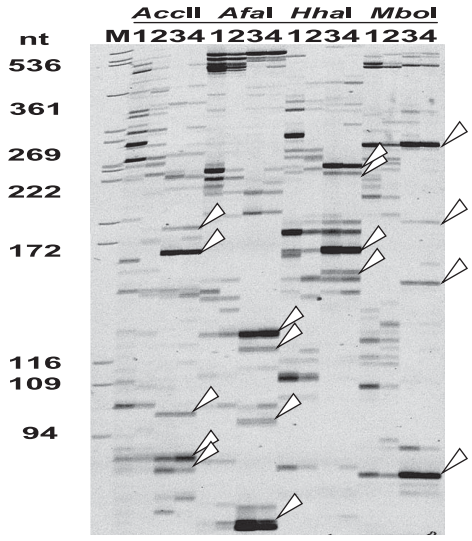

Fig. 1. The molecular diversity of bacterial chitinase genes in bulk and maize rhizosphere soil samples, as revealed by T-RFLP following digestion with five different restriction enzymes. Lane M, GeneScan-2500 ROX molecular size markers (Applied Biosystems); lanes 1 and 2 and lanes 3 and 4, results of duplicate samples for bulk and rhizospheric soils. Numbers indicate marker fragment lengths. Arrows indicate the presence of either dominant or specific DNA bands in rhizospheric soils, in comparison with bulk soils.

clones were selected from the bulk and rhizospheric maize soil samples, respectively, and subsequently subjected to sequencing. BLASTX searches of these clones revealed each of them to be similar to chitinases listed in current databases. As expected from the T-RFLP patterns, striking differences were observed between the bulk and rhizosphere clones in terms of their taxonomical properties.

For the bulk soil samples, most of the isolated clones showed a medium to low degree of similarity to chitinases of known species. Phylogenetic analyses further showed that most of these bulk soil clones are distantly related to the chitinase genes of *Streptomyces* (Fig. 2). Interestingly, three clusters were distinctly formed from known chitinases (B1, B2 and B3 in Fig. 2), suggesting the presence of novel subgroups within the non-*Streptomyces* chitinases. Only 3/28 clones were shown to be closely related to the *Streptomyces* chitinases, and these three clones also showed the highest degree of similarity to *Streptomyces avermitilis* among the

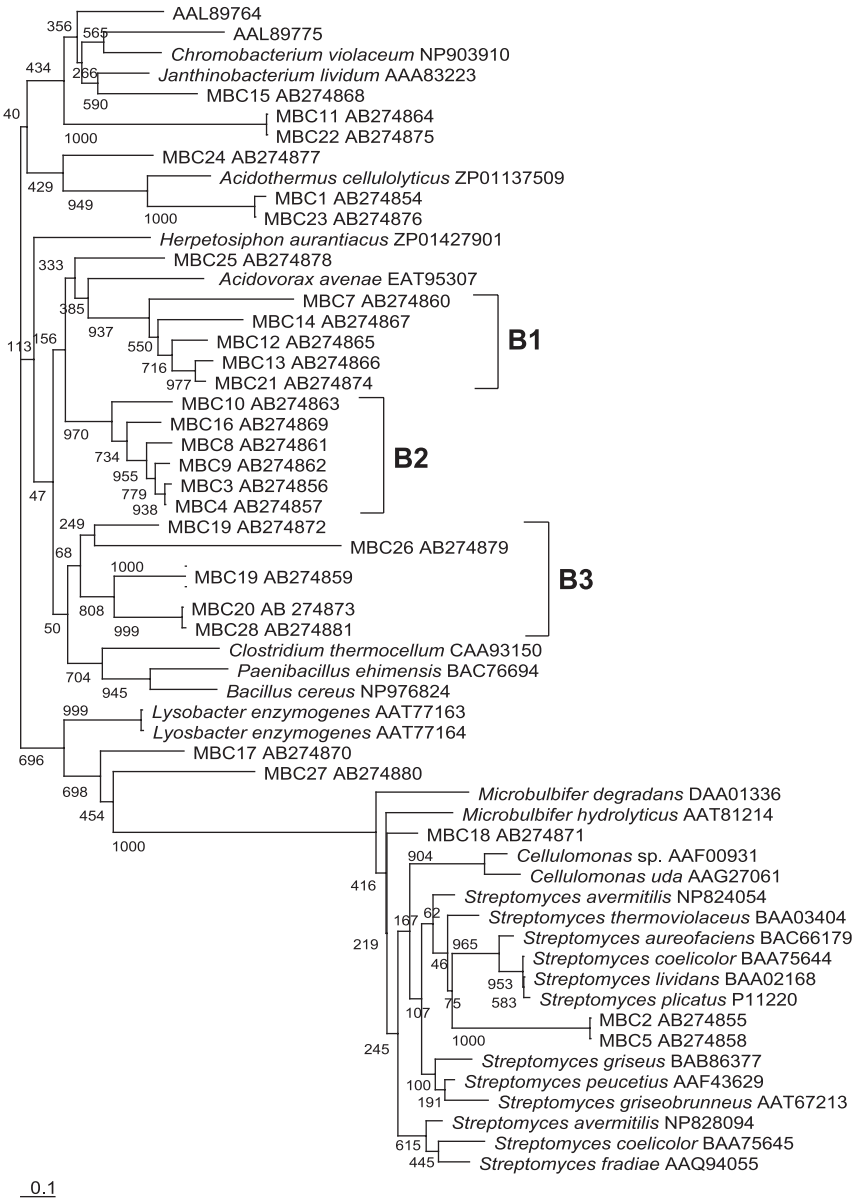

Fig. 2. Phylogeny of chitinases based on deduced amino acid sequences isolated from bulk soil. The clones isolated from bulk soil were designated as MBC with numbering, and the corresponding accession numbers are shown. The numbers at the nodes indicate levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. Trees were drawn with known chitinase sequences taken from GenBank. Bar represents the numbers of amino acid substitutions per site.

known species.

In contrast to the bulk soil samples, both sequencing and phylogenetic analyses of our maize rhizosphere samples revealed that 13/21 clones were similar to the *Streptomyces* chitinases, particularly in the case of *S. avermitilis* and its close relatives (Fig. 3). Interestingly, 12 of these 13 clones formed two distinct clusters (clusters R1 and R2 in Fig. 3). *Streptomyces* sp. is recognized as an efficient colonizer of plant roots[25], and is considered to be one of the most important bacterial groups in the rhizosphere due to its antifungal properties, including chitinase activity[9]. In addition, *S. avermitilis* has been shown to be an important industrial microorganism in the production of a group of antiparasitic agents, the avermectins, and its entire genome sequence has now been revealed[12]. However, although *S. avermitilis* possess several putative chitinase genes, they have not been

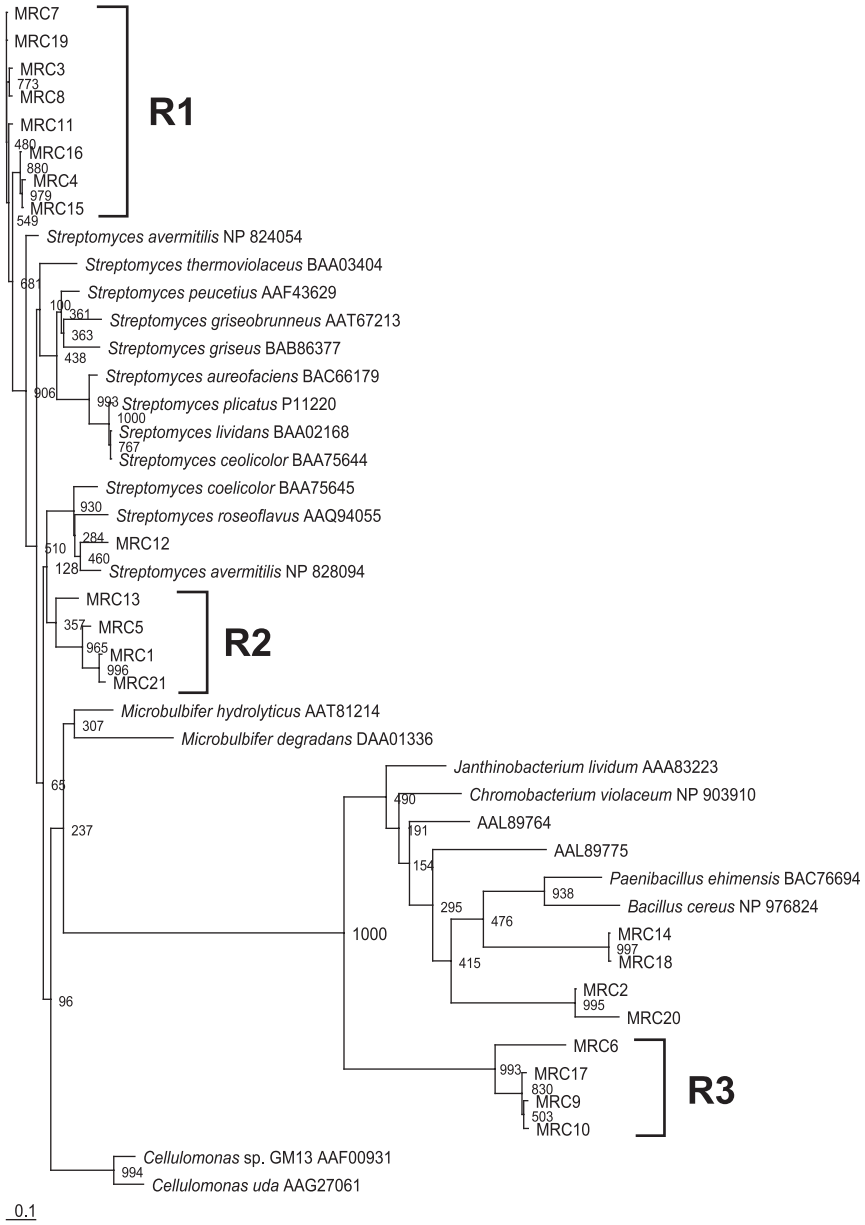

Fig. 3. Phylogeny of chitinases based on deduced amino acid sequences isolated from the maize rhizosphere. The clones isolated from bulk soil were designated as MRC with numbering, and the corresponding accession numbers are shown. The numbers at the nodes inidicate levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. Trees were drawn with known chitinase sequences taken from GenBank. Bar represents the numbers of amino acid substitutions per site.

characterized. Since many sequences that we identified in the maize rhizosphere show high levels of similarity, analysis of the chitinases of *S. avermitilis* may facilitate a better understanding of the chitinolytic bacterial community in such rhizospheric soils.

Williamson *et al.*[28] were the first to conduct the molecular analyses of bacterial chitinase genes in soil DNA using DGGE. Subsequently, Metcalfe *et al.*[16] demonstrated that the functional diversity of chitinase genes could be a useful indicator in the assessment of soil environments. However, the DGGE data presented in the former report had poor resolution for the purposes of community analysis, and no sequence information was provided following their profile analysis. Metcalfe *et al.*[16] could not amplify bacterial chitinase genes directly from soil DNA, even from the soils that were adjacent to the chitin bags used as bait for chitinolytic bacteria. In contrast to these previous studies, DNA fragments were successfully amplified at the predicted molecular size (about 400 bp) from both rhizosphere and bulk soil samples in the present study. This may be due to the purity of soil DNA[14]. Our current results also suggest that T-RFLP could be a useful tool for the assessment of molecular diversity among bacterial chitinase genes in ecosystems such as the rhizosphere (Fig. 1).

Although the same primers were employed, our results are noticeably different from those of Metcalfe *et al.*[16]. We identified a few clones (cluster B3 and R3 in Fig. 2 and Fig. 3, respectively) showing some homology to the chitinases of *Bacillus* sp. and relatives there of. *Bacillus* sp. is one of the major chitinase-producing bacterial groups among the culturable microbes and abundant in arable soil[19]. This finding was in contrast to the result reported by Metcalfe *et al.*[16] that no similarity with the chitinases of *Bacillus* sp. was obtained from sequence analyses. Moreover, only two (MBC11 and MBC22 in Fig. 2) of the bulk soil clones and four (MRC2, MRC14, MRC18, and MRC20 in Fig. 3) of the rhizosphere clones in the present study share any degree of similarity with the sequences reported by Metcalfe *et al.*[16]. Furthermore, none of the present clones show significant similarity to ArchiB of *Arthrobacter* sp., whereas Metcalfe *et al.*[16] identified several clones that were highly homologous to this gene. These differences may be due to the differences in the soils, or may reflect differences in the starting materials used for the cloning experiments in each study, since Metcalfe and co-workers isolated DNA from chitin bags instead of soils. Alternative explanations may lie in the PCR amplification conditions or in the screening procedures used to obtain the PCR clones. It is noteworthy, however, that the differences between the current finding and those of Metcalfe may well indicate that the molecular diversity of bacterial chitinases in natural ecosystems is considerably greater than the previous estimates that were based on culturable bacteria.

In conclusion, novel groups of chitinases in both bulk and rhizosphere soil were identified by the use of culture-independent methods. The potential usefulness of T-RFLP analysis in the assessment of the molecular diversity of bacterial chitinases in certain environments, such as the rhizosphere, was also demonstrated. The present results thus suggest that culture-independent methods are indispensable tools for fully analyzing chitinolytic bacterial communities in soils and show that the molecular diversity of bacterial chitinases in soils may be considerably greater than previous estimates based upon the analysis of culturable bacteria only.

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