Development of anaerobic bacterial community consisted of diverse clostridial species during biological soil disinfestation amended with plant biomass

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Abstract

Biological soil disinfestation (BSD) using plant biomass incorporation is an effective method

and a good alternative of chemical fumigants for controlling soil-borne plant pathogens. In this study the bacterial communities in pot soil treated with three different BSD conditions (without plant biomass and with *Brassica juncea* plants or wheat bran) were analyzed using mainly molecular techniques. Earlier dropping of redox potential of the both biomass-treated soil indicated rapid development of anaerobic condition in the soil. The population of *Fusarium oxysporum* pathogen incorporated in the soil at the starting was decreased considerably during the treatment, and the number of culturable anaerobic bacteria increased in both biomass-treated soils. Rather high concentrations of acetate and butyrate were detected from the biomass-treated soils. The polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis for the V3 region of 16S rRNA gene sequences revealed that the profiles of both biomass-treated soils were initially represented by similar and dominant groups, many of which were closely related to the species in the classes *Clostridia* and *Bacilli* of the phylum *Firmicutes*. Based on the clone library analysis, the control soil samples showed diverse bacterial groups with a few number of anaerobic clones. In contrast, for both biomass-treated libraries, clones belonging to the class *Clostridia*, a strictly anaerobic spore-forming bacterial group, appeared exceedingly dominant. The clostridial group detected was composed of phylogenetically diverse members, and it seemed likely that the diverse clostridial species were responsible for suppression of pathogens by making various compounds including volatile fatty acids and other

compounds during anaerobic decomposition of plant biomass.

Keywords: Biological soil disinfestation, Soil-borne plant pathogens, Anaerobic bacteria, PCR-DGGE, Clone library, Nucleotide sequencing, Clostridial groups.

INTRODUCTION

Soil disinfestation is the process of removing pests and diseases from soil prior to planting. Among the methods of soil disinfestation, biological soil disinfestation (BSD) is recently developed, which involves application of easily decomposable organic materials to the soil and allowing them to be decomposed under wet and anaerobic circumstances (Goud *et al.* 2004; Momma 2008). The treatment includes following four steps; (1) incorporating biomass materials into field soil, (2) flooding the soil by irrigation, (3) covering the soil surface with a plastic film for about 15-18 days to establish reducing soil conditions and decomposing the biomass, and then (4) plowing the field and planting crops (Shinmura 2000, 2004). The method is becoming popular, especially in the organic farming, due to its broad spectrum suppression of soil-borne pathogens in an environmental friendly manner within a rather short time.

Researches on the BSD has been emphasized recently due to the restrictions on several well recognized synthetic soil fumigants for disinfestation such as methyl bromide, ethylene bromide,

etc. because of their harmful effects on human health and destructive impact on the stratospheric ozone layer (Kirkegaard *et al.* 1996; Subbarao 2002). Other chemical disinfestants for soil treatment such as chloropicrin, 1,3-dichloropropene, dazomet, metham sodium, metham potassium, methyl isothiocyanate, etc. are available as not so toxic (Mattner *et al.* 2008; Porter *et al.* 2006), but these chemicals may not be compatible with sustainable agriculture.

Since it has been well documented that survival of many plant pathogens is considerably decreased under anaerobic soil conditions (Blok *et al.* 2000), soil anaerobiosis may cause death of soil-borne pathogens during the reductive treatment. In addition, anaerobic bacteria such as *Clostridium* spp. and some other *Firmicutes* species are known to produce skatole, indole, cresol or some phenolic compounds by decomposition of amino acids such as tryptophan and tyrosine under anaerobic conditions (Macfarlane and Macfarlane 1995). These substances may be involved in the BSD treatment leading to kill pathogens. Furthermore, volatile fatty acids (VFAs) like acetate or butyrate evolved in the treated soil have been also suggested to contribute pathogenic inactivation (Momma *et al.* 2006). Many species of anaerobic bacteria, especially *Clostridium* spp. are known to produce these organic acids as fermentation products from saccharides as well as amino acids. Considering the facts stated, the anaerobic bacterial community should take a very important role in the BSD treatment for soil-borne disease management. However, the composition of bacterial communities in the soil under the treatment

and the effects of bacterial activities on pathogens have not been investigated in detail at all.

Wheat bran is one of the popular plant materials incorporated in soil for the treatment (Momma *et al.* 2006). Other plant biomass such as *Brassica* spp. or oats (*Avena sativa*) also has been used successfully for the treatment (Mojtahedi *et al.* 1991; Sarwar and Kirkegaard 1998). Out of these plant materials, *Brassica* spp. plants release specific substances (isothiocyanates, ITCs) having biocidal activities, when the enzyme myrosinase hydrolyses glucosinolates in the plants following tissue damage (Mattner *et al.* 2008). Thus, application of *Brassica* spp. plants for the BSD treatment seems to have the advantage to suppress soil-borne plant pathogens.

The objective of the present research was to analyze bacterial communities during BSD incorporated with *Brassica juncea* plants as well as wheat bran as a model experiment and to identify the major bacterial groups involving with the treatment. Molecular methods based on the PCR-DGGE technique (Muyzer *et al.* 1993) and the clone library method (Maidak *et al.* 1999) were mainly used.

MATERIALS AND METHODS

Model experiment by using pot soil

A model experiment of BSD using polypropylene pots (160 x 160 x 154 mm) with soil (6.5 kg per pot) was carried out. Rather large single pot was used for each treatment as described below

to make it possible to get soil samples from the same pot throughout the experiment. The soil (gray lowland soil) was obtained from a field of the Tokushima Agricultural Research Centre (34°07′N, 134°36′E) during 2009, which had been kept bare for about three years without planting, with eggplant (*Solanum melongena*) growing in the adjacent field. *Fusarium oxysporum* R3-1-2 (wilt pathogen of tomato) cultivated in Soil-Wheat bran medium (100 g of commercial soil for nursery, 25 g of wheat bran, and 15 g of water) was incorporated into the pot soil together with the medium (180 g per pot) for all treatments. We used the bare soil for the experiment considering the uniform distribution of the artificially induced pathogen for all treatments, since pathogenic populations are often distributed heterogeneously in soil. *Brassica juncea* plants were cut into pieces and incorporated into the pot soil at the rate of 6 kg m^{-2} (153.6 g fresh weight per pot). Wheat bran was applied at the rate of 1 kg m^{-2} (25.6 g per pot). For the control soil, none of plant materials or substances was incorporated. For all pots, water (0.5 l per pot) was irrigated to provide reducing conditions in the soil. The pots were closed with lids equipped with packing to avoid penetration of oxygen and incubated at 30°C for 18 days. Small holes were opened in the lids before the beginning of the experiment and used for sampling of soil and insertion of electrodes to measure the oxidation reduction potential (Eh) and pH. The electrodes were fixed in the soil throughout the experiment and the values were read every three days. Soil samples were also collected every three days from all three pots through the holes,

which were usually closed with silicon seals.

Determination of concentrations of VFAs in soil samples

A five g soil sample of each pot soil collected was suspended in 5 ml deionized water and shaken for 20 min with a reciprocating shaker. The slurry samples were centrifuged at 2,500 rpm for 10 minutes and the concentrations of VFAs in the supernatants were analyzed by gas chromatography (Hitachi G-5000) as described previously (Ueki *et al.* 1986). Concentrations of VFAs are expressed in the text as those in the supernatant of slurry samples.

Enumeration of populations of *Fusarium* and bacteria in the soil samples

The number of wilt pathogen (*Fusarium oxysporum*) was determined by the dilution plate technique by using a selective medium for *Fusarium oxysporum* (FD-G1 medium) (Nishimura 2007; Takehara *et al.* 2003) for all soil samples collected every three days. Both aerobic and anaerobic bacteria were enumerated for soil samples collected at 18 days of all three pots as well as the original soil sample by the dilution plate technique and the anaerobic roll tube method, respectively. The diluted samples treated at 80°C for 10 min were also used to enumerate bacteria present as spores in the soil samples. Diluted nutrient broth (DNB) agar (0.1 g each of meat extract and peptone, 0.05 g of NaCl, and 15 g of agar per liter) was used for enumeration of aerobic bacteria. For enumeration of anaerobic bacteria, 1/10 PY4S medium that

contained one-tenth amounts of peptone (Trypticase, BBL) and yeast extract in PY4S agar (Nishiyama *et al.* 2009) was used with oxygen-free 95% N_2/5% CO_2 mixed gas as a headspace. All the petri dishes and roll tubes inoculated were incubated at 30°C for two weeks and each viable count (CFU g^{-1} = colony forming units per g of dry soil) was determined. The number of CFU for the selected soil sample was analyzed statistically using ANOVA two-way analysis without replication.

DNA extraction and PCR amplification

DNA was isolated from each soil sample (about 1 g) using 'Ultra Clean™ Soil DNA Isolation kit'(MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. About 50 µl volume was extracted for each sample in which the DNA was eluted from the spin column. For PCR-DGGE, the V3 region of 16S rRNA gene from DNA samples was PCR-amplified using a primer set B341fGC (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG- 3', with underlined GC clamps) and 534r (5'- ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.* 1993). For clone library analysis, bacterial 16S rRNA genes were amplified using a primer set B27f (5'-AGA GTT TGA TYM TGG CTC AG-3') and U1492r (5'-GGY TAC CTT GTT ACG ACT T -3'). The PCR mixture (50 µl) contained 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems,

Foster, CA, USA), 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% bovine serum albumin, each deoxynucleotide triphosphate mixtures (dNTPs) at a concentration of 200 μM, 0.25 μM of each primer, and 60-100 ng of template DNA. The amplification conditions were as follows; for the primer set B341fGC and 534r: 10 min of activation of the polymerase at 94°C, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, 1 min elongation at 72°C, and finally 10 min of extension at 72°C; for the primer set B27f and U1492r: 12 min of activation of the polymerase at 95°C, followed by 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, 1.5 min elongation at 72°C, and finally 2 min of extension at 72°C. Amplified DNA fragments were confirmed after agarose gel electrophoresis staining with ethidium bromide.

DGGE analysis and nucleotide sequencing

The PCR products were separated by DGGE using a DCode™ system (Bio-Rad Laboratories, Hercules, CA, USA). A total of 9 μg DNA sample was applied for each lane irrespective of treatment and sampling date. The samples were applied to 10% acrylamide gels with a urea-formamide denaturing gradient of 30-60% (100% denaturant was defined as 7 M urea and 40% formamide) at an electrophoretic movement for 3.5 h and 200 V. The gels were stained in SYBR Gold solution and viewed by a UV transilluminator. The photographic image was

transformed into digital data and the position of some major DNA bands in the DGGE profiles was numerically designated. The selected DGGE bands were excised from the gels, and DNA was extracted in TE buffer (10mM Tris-HCl and 1 mM EDTA-2Na, pH 8.0). After purification by ethanol precipitation, the DNA samples were subjected to PCR amplification for the second DGGE analysis. The single bands of the second DGGE profile were excised again and purified as described above. The DNA samples obtained were used for reamplification with a primer set T7W-341f (5'-TAA TAC GAC TCA CTA TAG GGC CTA CGG GAG GCA GCA-3') and SP6W-534r (ATT TAG GTG ACA CTA TAG AAT ACT CAT TAC CGC GGT GCT GG -3') and sequenced (about 180 bp) by the standard methods (Crump *et al.* 2004) using a primer set T7W (TAA TAC GAC TCA CTA TAG GGC) and SP6W (ATT TAG GTG ACA CTA TAG AAT ACT C) and a DNA sequencer (4000L, Li-COR).

Clone library and nucleotide sequencing

The following seven soil samples were selected as representatives for the clone library analysis of the bacterial community in the treated soil: the original field soil sample without treatment (referred to as Control 0 in the clone library), 9 and 18 days of treated soil without plant biomass (Control 9 and Control 18), 9 and 18 days of *Brassica*-treated soil (*Brassica* 9 and *Brassica* 18), and 9 and 18 days of wheat bran-treated soil (Wheat bran 9 and Wheat bran 18).

PCR products from these samples were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, and USA) and cloned into *Escherichia coli* JM109 competent cells following the instructions of pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). The vector-harboring clones containing an insert of appropriate sizes (about 1500 bp) were obtained in the Luria-Bertani (LB) plates by the standard methods (Kaku *et al.* 2005). A total of 96 clones were subjected to undergo sequence analysis (about 900 bp) for each soil sample with a sequence primer set U515f (5' GTG YCA GCM GCC GCG GTAA-3') and U1492r (5'-GGY TAC CTT GTT ACG ACT T -3') according to the Dye Terminator method using a capillary sequencer at TaKaRa Co. Ltd.

Analysis of nucleotide sequencing

Database searches for related 16S rRNA gene sequences were conducted using BLAST program and GenBank database (Altschul *et al.* 1997). The profile alignment function of ClustalW program was used to align the sequences. The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei 1987) with Njplot program in ClustalW package (Thompson *et al.* 1994). Bootstrap resampling analysis for 1000 replicates was performed to determine the confidence of the tree topology. An OTU (operational taxonomic unit) was designated as a phylogenetic group or unit consisted of the resulting clones showing almost

98% similarity of nucleotide sequences. All the representative clones of the OTUs were analyzed to determine the taxonomic affiliation of the clones of all libraries. All 16S rRNA gene sequences obtained in the present study were checked for possible chimeras using the chimera check online analysis program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) of the Bellerophon server. Finally, 69-89 clonal sequences were validly used for the analysis of the bacterial community for each soil sample. A rarefaction analysis of the 16S rRNA gene sequences in the clone libraries was carried out with the software aRarefactWin (http://www.uga.edu/strata/software/Software.html).

Accession numbers of nucleotide sequences

The nucleotide sequences determined from the DGGE and clone library analyses have been reported in DDBJ /GenBank under the accession numbers AB627045-AB627070 (26 entries) and AB589501-AB590035 (535 entries), respectively.

RESULTS

Conditions of pot soil

Water contents of the pot soil were about 30% (w/w) throughout the experiment irrespective of the treatments (data not shown). Eh potential for *Brassica*- and wheat bran-treated soil dropped

earlier than the control soil. It declined very rapidly from +100 to -200 mV within six days of incubation, and then gradually reached to -250 to -300 mV until nine days (Fig. 1A), indicating the development of strongly reduced conditions in these pot soils. Eh value in the control soil also decreased slightly later than the biomass-treated soils. The initial pH of the pot soil was 6.0-6.1. The pH values decreased to 4.9-5.3 within three days, and the final pH values of the control, *Brassica*-treated, and wheat bran-treated soils were 5.9, 5.3, and 5.2, respectively.

None of VFAs was detected from the control soil samples throughout the treatment. On the other hand, considerable amounts of VFAs were detected in both *Brassica*- and wheat bran-treated soils. Acetate was the major component followed by butyrate and traces of propionate, and the amounts rapidly increased until six days of incubation and gradually decreased in the later stages (Fig. 2). The maximum concentrations of acetate and butyrate in *Brassica*-treated soil were 7.3 and 2.8 mmol l^{-1} (as the concentrations in the slurry supernatant as shown above) at six days, respectively, whereas those in wheat bran-treated soil were 6.0 and 2.0 mmol l^{-1} at both three and six days.

Enumeration of pathogenic fungi and bacteria

Both *Brassica*- and wheat bran-treated soils showed similar performances for the reduction of pathogenic propagules (*Fusarium oxysporum* R3-1-2) incorporated into the soil at the start of

the experiment (Fig. 1B). The fungal population declined from 10^6 to 10^2 CFU g^{-1} in both biomass-treated soils until 18 days, and the numbers of pathogen were 100 times lower as compared with that in the control soil.

For the original field soil, the culturable anaerobic bacterial population was 4.6 x 10^6 CFU g^{-1} and almost the same number of heat tolerant cells (spores) was detected from it (Table 1). The result indicated that most of anaerobes were present as spores in the original aerobic field soil at a rather high number. For all pot soils at 18 days, the number of anaerobes was considerably higher (about four to seven times) than that in the original soil. The number of spores in both biomass-treated soils was lowered similar to the original soil, whereas it was higher in the control soil. The results also revealed that biomass incorporation or heat treatment affected the numbers of aerobic bacteria significantly. The number of aerobic bacteria at 18 days was not so different from that in the original soil including heat tolerant cells for all soil samples.

PCR-DGGE analysis

The PCR-DGGE results showed clear differences in the bacterial communities depending upon the soil samples and sampling dates (Fig. 3). The zero day sample of the control soil represents the microbial population from the original field soil that was used as a starting soil for all

treatments. All bands developed from the sample were very thin and no dominant band was detected in the profile. Almost similar results were obtained for all stages of the control soil (3, 6, 9, 12, 15, and 18 days).

For both *Brassica*- and wheat bran-treated soils, the DGGE profiles after three days of the starting changed remarkably as compared with that of the original soil sample (0 day of the control). Some distinct and major bands (B1, B2, B3, and B4) were detected at the same positions from both *Brassica*- and wheat bran-treated soils. The profiles were somewhat similar thereafter up to nine days for both treatments, although the intensity of some bands (W5, W6, W13, W14, W15, and W16) in the wheat bran-treated profile was obviously higher than that in the *Brassica*-treated profile. Later on, the profiles changed again, and new and different profiles were developed gradually (15 to 18 days) for both soils. Some distinct bands for wheat bran-treated soil at 15-18 days (W1, W2, W3, and W4) were not recognized in the profiles of *Brassica*-treated soil. Some other weak and temporal bands (B5, B6, W11, and W12) also appeared in both biomass-treated profiles.

Sequences of the numbered bands were determined and each closely related species was searched (Table 2). Of 23 sequences determined, 15 sequences showed the highest similarities with the 16S rRNA gene sequences from the members in the phylum *Firmicutes*, affiliating nine sequences to the class *Bacilli* and six to the *Clostridia*. All nine sequences assigned to the class

Bacilli appeared to be closely related to *Bacillus senegalensis* or *Bacillus niacini*. Three sequences were closely related to the *Azotobacter* species of the class *Gammaproteobacteria,* and the remaining sequences showed similarities with the different species belonging to the phylum *Bacteroidetes*. Thus, the DGGE profiles for both biomass-treated soils indicated that both aerobic (the *Bacilli*) and anaerobic (the *Clostridia*) spore-forming bacteria in the *Firmicutes* phylum proliferated rapidly at the initial stage of treatment and these groups became comparatively less abundant at the later stages (15-18 days). Instead, several species in the *Bacteroidetes* and the *Gammaproteobacteria* became abundant at the last stage especially in the wheat bran-treated soil. Out of the bands determined for *Brassica*-treated soil at 18 day (B7, B8, B9, and B10), only B9 appeared to be a newly developed band.

Clone library analysis

Based on the results of DGGE profiles, seven soil samples were selected for the clone library analysis. On the basis of the taxonomic affiliations of closely related organisms, the clone sequences from all libraries were found to be affiliated with at least eight major phyla of the domain *Bacteria,* namely *Proteobacteria*, *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia,* and *Chloroflexi*. However, the phylogenetic compositions and proportions of the major phyla or classes varied depending upon the soil

samples. The affiliations of clone sequences in each soil sample are shown in Fig. 4 in relation to the percentages of number of clones belonging to each phylum or class. In addition, out of the phylogenetic trees constructed for all libraries, those for Control 0, *Brassica* 9 and Wheat bran 9 were shown in Fig. 5 and 6, respectively. Besides, rarefaction curves were calculated for all clone libraries to evaluate and compare the diversities of sequences (Fig. 7).

Library for control soil

The Control 0 library showed much diversified populations of different phylogenetic groups and there was not any exceedingly dominant bacterial phylum or class in the profile. The most abundant taxonomic group of the Control 0 library was allocated to the phylum *Proteobacteria* (about 35% of the total number of clones) and the clones assigned to the phylum were distributed into four classes, that is, *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria* (16, 12, 3, and 4%, respectively). Other sequences belonged to the phyla *Acidobacteria* (15%), *Actinobacteria* (13%), *Bacteroidetes* (11%), *Planctomycetes* (11%), *Verrucomicrobia* (7%), *Chloroflexi* (2%), and only one clone to the class *Bacilli* of the *Firmicutes* phylum (Fig. 4, 5).

Similarly to the Control 0 composition, the Control 9 library also demonstrated diversified populations. About one third (36%) of clones were affiliated to the phylum *Proteobacteria* (*Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*; 11, 11, 8, and 6 %, respectively) followed by 15% to the phylum *Acidobacteria*, and 12% to the *Firmicutes* phylum. In the *Firmicutes* group,

about 5% were clostridial clones, whereas the remaining 7% were in the class *Bacilli.* The Control 18 library also showed diversified populations that were almost similar to other Control libraries. The phylum *Proteobacteria* was also the most abundant (about 34%) taxonomic group (*Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*; 10, 14, 8, and 2%, respectively). The phylum *Acidobacteria* contributed the second most abundant group (23%) followed by the phylum *Bacteroidetes* (8%). Two clones (about 2%) belonging to the clostridial groups in the *Firmicutes* phylum were detected. The numbers of OTUs (at the 98% sequence similarity level as shown above) recognized for the Control libraries were 57 for Control 0, 58 for Control 9, and 65 for Control 18 libraries. The rarefaction curves of the three libraries almost overlapped each other and did not reach saturation, indicating highly and equally diversified bacterial communities in these soils (Fig. 7).

Libraries for biomass-treated soil

Unlike the Control libraries, the biomass-treated libraries showed the presence of exclusively dominant bacterial taxonomic groups (phyla or classes) in the profiles (Fig. 4). In case of the *Brassica* 9 library, more than half of clone sequences were affiliated with the phylum *Firmicutes* (58%), and the clones in the phylum were almost equally distributed to the classes *Clostridia* and *Bacilli* (31 and 27%, respectively). The next abundant group was represented by 16% clones from the different classes of the phylum *Proteobacteria.* For the *Brassica* 18 library,

the major phyla detected were from the *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. In the phylum *Firmicutes*, a large number of clones were also affiliated with the class *Clostridia* (22%), whereas the number of clones in the *Bacilli* significantly decreased (4%) as compared with that in the *Brassica* 9 library. As shown in Fig. 7, bacterial diversity in the soil much decreased until nine days of *Brassica*-treatment as compared with the starting soil, while the community was much diversified again until 18 days. A total of 32 and 65 OTUs were recognized from the *Brassica* 9 and *Brassica* 18 libraries, respectively.

In the *Brassica* 9 library, the largest OTU contained 21 clones, which was closely related to *Bacillus niacini* (99% of sequence similarity with the closest clone) of the class *Bacilli*, that is, the closest relatives of the major bands of both biomass-treated DGGE profiles. The closely related described species of clostridial OTUs containing at least two clones in the *Brassica* 9 library were *Clostridium sporogenes* or *Clostridium subterminale* (10 clones, 93-94% similarity), *Clostridium saccharobutylicum* (4 clones, 98%), *Pelotomaculum schinkii* (4 clones, 90%), *Clostridium xylanovorans* (2 clones, 95%), and *Clostridium paraputrificum* (2 clones, 93%) (Fig. 6). The largest OTU (10 clones) in the *Brassica* 18 library was closely related to *Azotobacter beijerinckii* (99%), corresponding to the DGGE band B9, from the *Gammaproteobacteria*, and the closest relative of the second largest OTU (7 clones) was *Prolixibacter bellariivorans* (91%) (band W9) in the *Bacteroidetes*. The following species were

the closest relatives of five OTUs containing two clones; *Bacillus niacini* (99%), *Clostridium magnum* (97%) (bands W15 and 16), and *Clostridium chartatabidum* (98%) in the *Firmicutes*, and *Sphingomonas jaspsi* (96%) and *Sphingomonas wittichii* (98%) in the *Alphaproteobacteria*.

For the Wheat bran 9 library, 74% of the total number of clones were assigned to the *Firmicutes* phylum, most of which (62%) were to the class *Clostridia*. The number of clones in the phylum *Proteobacteria* was only 10% (Fig. 4, 6). The Wheat bran 18 library included 35% clones from the *Firmicutes* (33% from the *Clostridia* and 2% from the *Bacilli*) and 26% from the phylum *Proteobacteria*. Many clones from the *Bacteroidetes* phylum (17%) were also found in the library. Almost the same curves were obtained with the rarefaction analysis of the two wheat bran libraries, indicating that the diversity of the community did not recover until 18 days of the treatment in contrast to the *Brassica* 18 library (Fig. 7). The number of OTUs recognized for the Wheat bran 9 and Wheat bran 18 libraries were 35 and 42, respectively.

The closely related species of major clostridial OTUs in the Wheat bran 9 library were *Clostridium septicum* (9 clones, 95%), *Clostridium acetobutylicum* (7 clones, 93%) (band W13), *Clostridium saccharobutylicum* (6 clones, 99%), *Pelotomaculum schinkii* (5 clones, 90%) *Clostridium papyrosolvens* (3 clones, 97%), and *Clostridium paraputrificum* (3 clones, 89%) (Fig. 6). For the Wheat bran 18 library, the largest OTU (15 clones) was closely related to *Azotobacter chroococcum* (99%) (band W4 and 7) from the *Gammaproteobacteria*. Besides,

Prolixibacter bellariivorans (91%) (band B9) and *Bacteroides eggerthii* (94%) in the *Bacteroidetes* were those of the second (7 clones) and the third (6 clones) largest OTUs, respectively. The closely related species of major clostridial groups for the Wheat bran 18 library were *Clostridium subterminale* (4 clones, 94%), *Clostridium sufflavum* (3 clones, 98%), *Clostridium septicum* (2 clones, 95%), and *Clostridium diolis* (2 clones, 98%).

Phylogenetic diversity of clostridial group

A phylogenetic tree consisting of all OTUs assigned to the class *Clostridia* from all samples was generated (Fig. 8), of which almost all sequences were derived from both biomass-treated soils. Although some major OTUs affiliated with the class consisted of more than two clones as shown above, many of OTUs contained only one clone, indicating occurrence of extraordinarily diverse species in the class in the biomass-treated soil. About two thirds of clones (68%) from all 9 day libraries (Control 9, *Brassica* 9, and Wheat bran 9) were classified into the cluster I (*Clostridium sensu stricto*) including the *Oxobacter* group, and the remaining clones were affiliated with other miscellaneous clusters (Cluster III, IV, VI, VII, XIVa, etc.) (Collins *et al.* 1994). On the contrary, for 18 day libraries (Control 18, *Brassica* 18, and Wheat bran 18), 40% of clones were assigned to the cluster I. The result indicates that the phylogenetic composition of clostridial communities greatly changed during the later stages of the treatment.

A total of nine closely related groups of OTUs derived form different libraries (indicated by

curly brackets with asterisks) were detected in the phylogenetic tree. It appeared that most of major OTUs in the cluster I (five groups) were commonly recovered from both biomass-treated soils. A group of OTUs remotely related to *Pelotomaculum shinkii* (cluster VI) was also recognized in all four libraries from the biomass-treated soils.

DISCUSSION

Effect of treatment on soil conditions and microbes

Eh decrease in soil for all treatments implied the successful consumption of total oxygen within the soil and avoidance of penetration of oxygen from outside. Thus, it favored the growth and multiplication of anaerobic bacteria in the soil. In fact, anaerobic bacteria were enumerated at much higher populations in all treated soils at 18 days as compared with that in the original soil, although the levels of aerobic bacterial population were almost the same. Rather high amounts of acetate and butyrate together with traces of propionate were detected in all the biomass-treated soils, while none of VFAs was detected in the control soil throughout the experiment. As shown by the molecular analyses of the bacterial communities, since the incorporation of *Brassica* plants or wheat bran to the soil strongly enhanced growth of anaerobic bacteria, VFAs should be produced by these anaerobic bacteria through

decomposition of biomass in the anoxic condition.

It has been reported that BSD using *Brassica* plants, wheat bran, grasses or molasses effectively killed a wide range of soil-borne pathogens (Shinmura 2000, 2004; Takeuchi 2004; Urbasch 1984). In this study, the population of *Fusarium oxysporum* pathogen was also declined markedly during BSD. The result coincided with these reports and confirmed the effects of biomass incorporation on suppression of the plant pathogen, although the differences between *Brassica* plants and wheat bran in the effects on the pathogen were not presented clearly.

Analysis of bacterial communities by the molecular techniques

The results obtained by the two molecular techniques (DGGE and clone library) coincided well with each other. Soil harbors highly diverse bacterial communities with up to 50,000 (Sandaa *et al.* 1999) or even up to millions (Gans *et al.* 2005) of different 16S rRNA gene sequences. It is known that the high bacterial diversity may result too low PCR products per species to give less visible bands in DGGE profiles (Dar *et al.* 2005). The absence of any dominant band or presence of many thin bands in the DGGE profile of the original soil suggested the high diversity in the bacterial communities, and the bacterial community shown in the Control 0 library confirmed it. It is generally said that soil bacteria are affiliated with 32 bacterial phyla, however, an average of 92% are members of nine major groups constituting *Proteobacteria*

(39%), *Acidobacteria* (20%), *Actinobacteria* (13%), *Verrucomicrobia* (7%), *Bacteroidetes* (5%), *Chloroflexi* (3%), *Planctomycetes* (2%), *Gemmatimonadetes* (2%), and *Firmicutes* (2%) (Janssen 2006). The composition is very similar to the result obtained for the Control 0 library (34%, 15%, 13%, 7%, 11%, 2%, 11%, 0%, and 1%, respectively). Both clone libraries for the control soil (Control 9 and Control 18) revealed also highly diversified bacterial communities. Although some changes in the bacterial population were shown and some of strict anaerobes from the class *Clostridia* or facultative anaerobes from the *Gammaproteobacteria* increased, the result indicated that irrigation of soil itself without incorporation of organic matter did not change the composition of the bacterial community so much.

Although the sequence length determined for each DGGE band was rather short (about 170-190 bp) and the closest species names were not necessarily the same, the closely related species of the major DGGE bands almost corresponded with those of major OTUs consisted of at least four clones in each library. Thus, it was shown that the distinct and major bands recognized in the DGGE profiles might represent the most abundant species present in the bacterial community in each soil sample (more than about 5%).

The DGGE profiles from three to nine days for the *Brassica*-treated soils demonstrated correlation with the composition of the *Brassica* 9 clone library. The *Brassica* 9 clone library contained the members of the *Firmicutes* phylum including those from both classes *Clostridia*

and *Bacilli* as the most abundant groups. The detection of major clostridial clones, or increasing number of clones in the *Gammaproteobacteria* or *Bacteroidetes* in the *Brassica* 18 clone library also correlated with the appearance in the DGGE profile of *Brassica*-treated soil at 18 days. The Wheat bran 9 clone library showed the members of the *Firmicutes* phylum as the exclusively most dominant group including majority of the clones from the class *Clostridia*. Similarly to the DGGE profiles for the *Brassica*-treated soil, the bacterial community of wheat bran-treated soil differed at the later stages.

According to the results of the molecular analyses, it assumed that some *Bacillus* species present as spores in the original soil might grow rapidly at the initial stage of the treatment by using oxygen remained in the soil and decreasing Eh. After the initial drop of Eh, the plant biomass might support growth of some common rapidly-growing clostridial groups as easily decomposable substrates for both *Brassica*- and wheat bran-treatments. The development of the similar banding patterns of the DGGE profiles from three to nine days might explain proliferation of these limited bacterial species, which could decrease the bacterial diversities in the soil (Fig. 7). Both cellulose and xylan are major components of plant biomass and the class *Clostridia* includes many anaerobic species decomposing these compounds (Bergquist *et al.* 1999; Carere *et al.* 2008; Rainey *et al.* 2009). Thus, these slowly decomposable plant materials might support growth of some relatively slow-growing clostridial species, including cellulolytic

or xylanolytic species (e.g., *Clostridium sufflavum*) (Nishiyama *et al.* 2009), which represented new and different profiles at the later stages (15-18 days). In fact, the phylogenetic composition of major groups assigned to the *Clostridia* in both biomass-treated libraries largely changed at 18 days for both biomass-treated soils. The diversity of clostridial populations was much pronounced in case of the *Brassica* 18 library, which should contribute to the total bacterial diversity (Fig. 7).

Thus, it was found that the *Firmicutes*, especially species in the *Clostridia* class appeared as exceedingly dominant taxonomic groups in the bacterial communities in the soils treated with plant biomass. Although the clones assigned to the *Clostridia* class were dominant based on the phylogenetic classification of the phylum or class level, the clostridial group detected actually contained phylogenetically diverse members (Fig. 8). The result indicates that diverse species of anaerobic bacteria, especially relating to the class *Clostridia* present as spores in the field soil, start to grow soon after the beginning of the treatment and proliferate actively under the favored anaerobic conditions using plant biomass as growth substrates. As shown by the enumeration of *Fusarium* pathogen in the treated soil, in addition to the development of anaerobiosis, rapid growth of these various strictly anaerobic bacteria should play an important role in controlling soil-borne plant pathogens. It is known that the closely related clostridial species of the clones detected in this study form various products including VFAs and alcohols as well as indole or

skatole (Rainey *et al.* 2009; Wiegel 2009). Thus, it was considered that the diverse clostridial populations in the biomass-treated soils also produced various compounds during the decomposition of plant material.

The clostridial group includes some dangerous pathogens for the human like *Clostridium tetani* of Tetanus disease. Thus, applying BSD in practice may grow some public concerns, although no such harmful clostridial groups were detected in this study. When the treated soil would be faced under aerobic condition for crop cultivation after the treatment, the biofumigation effect must be terminated and the proliferation of all anaerobic clostridial species should be also eliminated. However, analysis of bacterial communities after the BSD treatment or during the cropping stage might be necessary to know the behavior of clostridial species of concern.

Besides the clostridial species, the populations of the *Gammaproteobacteria* and the *Bacteroidetes* also increased in the BSD soils, especially at the later stages (18 days). The two major OTUs in the *Gammaproteobacteria* were closely related to nitrogen-fixing species, *Azotobacter chroococcum* and *Azotobacter beijerinckii*, respectively (Kennedy *et al.* 2005). The species of *Gammaproteobacteria* relating to *Azotobacter chroococcum* were reported to produce indole and other antifungal antibiotics (Gowariker *et al.* 2009). In addition, many of *Bacteroides* species are known to be xylanolytic (Dodd *et al.* 2011) and produce fatty acids such as acetate

and succinate as the fermentation products (Shah 1992). *Bacteroides eggerthii*, the closest relative of one of the large OTUs in the Wheat bran 18 library, is a representative of such *Bacteroides* species. Thus, species relating to the genera *Azotobacter* and *Bacteroides* might contribute partly for pathogenic elimination in the treated soils by decomposition of plant materials and their products. It is of interest that clones closely related to aerobic *Azotobacter* species were detected as one of dominant groups in the highly reduced soil.

We have isolated many clostridial strains from the BSD soils during this study. We are now examining their physiological characteristics to know their roles in suppression of pathogens. These isolates may be used in BSD experiments under gnotobiotic conditions to find out the potentiality of these bacterial groups during BSD. Furthermore, community compositions of bacteria in BSD soils conducted in fields should be analyzed to confirm the results obtained in this study.

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FIGURE LEGENDS

Figure 1. Time course of changes in redox potential (A) and the number of *Fusarium oxysporum* (wilt of tomato) (B) in soil under the treatment. Symbols: ●, control; ▲, *Brassica*-treated; ■, wheat bran-treated.

Figure 2. Changes in concentrations of volatile fatty acids (VFAs) in *Brassica*-treated and wheat bran-treated soil. Concentrations of VFAs are expressed as those in the supernatant of slurry samples as shown in the text. For the control soil, none of them was detected (data not shown). Symbols: ●, acetate; ▲, butyrate ; ■, propionate.

Figure 3. DGGE patterns of bacterial populations in soil treated with different conditions. V3 region of bacterial 16S rRNA gene was PCR-amplified with a primer set of B341fGC/B534r. Amplified products were separated on a gradient gel of 30-60% denaturant. The lanes for each soil sample represent the sampling dates (3 to 18 days), whereas 0 day for the control soil represents the starting soil sample before the treatment. All labeled bands were excised from the gel, reamplified, and subjected to sequence analysis.

Figure 4. Composition profiles of phylogenetic groups (phylum or class) of bacteria based on 16S rRNA gene sequences from different clone libraries. Compositions are represented as

relative abundances in relation to the percentages of number of clones belonging to each phylum or class. Symbols: ◫ , *Alphaproteobacteria*; ◻ , *Betaproteobacteria*; ⊟ , *Gammaproteobacteria*; ▧, *Deltaproteobacteria*; ▨, *Acidobacteria*;⊡, *Verrucomicrobia*; ⊟, *Bacteroidetes*; ▨, *Planctomycetes*; □, *Firmicutes* (*Clostridia*); ■, *Firmicutes* (*Bacilli*); ⊡, *Actinobacteria*; ▥, *Chloroflexi*; ⊠, Others.

Figure 5. Neighbor-joining tree showing the phylogenetic relationships of all OTUs derived from Control 0 library based on 16S rRNA gene sequences. Bootstrap values (n = 1,000) above 70% are indicated at branch nodes. The scale bar represents 2% estimated difference in nucleotide sequence position. The name of each clone starts with the clone library designation and C0 represents Control 0 library. As the outgroup, *Sulfolobus acidocaldarius* (D14053) (the domain *Archaea*) 16S rRNA gene sequence was used. Accession numbers of the species are shown in the parentheses. Numbers in the parentheses aside each clone name denote the number of clones assigned to the OTU. Each clone name without parenthesis represents one OTU with one clone.

Figure 6. Neighbor-joining trees showing the phylogenetic relationships of all OTUs derived from *Brassica* 9 and Wheat bran 9 libraries based on 16S rRNA gene sequences. The

abbreviation *C.* indicates the genus *Clostridium*. The name of each clone starts with the clone library designation: BR9 and WB9 represent the *Brassica* 9 and Wheat bran 9 libraries, respectively. Abbreviations: α-, β-, and γ-Prot, *Alpha-*, *Beta-*, and *Gammaproteobacteria*, respectively; Actino, *Actinobacteria*; Bacter, *Bacteroidetes*; Acido, *Acidobacteria*; Verruco, *Verrucomicrobia*; Plancto, *Planctomycetes*. Tree construction and other notifications are similar as described in Fig. 5.

Figure 7. Rarefaction curves for the 16S rRNA gene sequences from all clone libraries. Libraries: C0, Control 0; C9, Control 9; C18, Control 18, BR9, *Brassica* 9; BR18, *Brassica* 18; WB9, Wheat bran 9, WB18, Wheat bran 18.

Figure 8. Neighbor-joining tree showing the phylogenetic relationships of clostridial clones (all OTUs from each library) based on 16S rRNA gene sequences (according to the clostridial cluster analysis by Collins *et al.* 1994). Bootstrap values (n = 1,000) above 70% are indicated at branch nodes. The scale bar represents 1% estimated difference in nucleotide sequence position. As the outgroup, *Bacillus subtilis* DSM10 16S rRNA gene sequence was used. The abbreviation *C.* indicates the genus *Clostridium*. The name of each clone starts with the clone library designation: C9, C18, BR9, BR18, WB9, and WB18 represent the Control 9, Control 18,

Brassica 9, *Brassica* 18, Wheat bran 9, and Wheat bran 18 libraries, respectively. For the Control 0 library, none of clostridial clones was detected. Accession numbers of the species are shown in the parentheses. Numbers in the parentheses aside each clone name denote the number of clones in the OTU of each library. The curly brackets with asterisks aside some clonal groups show the closely related OTUs from the respective clone libraries.

Figure 1

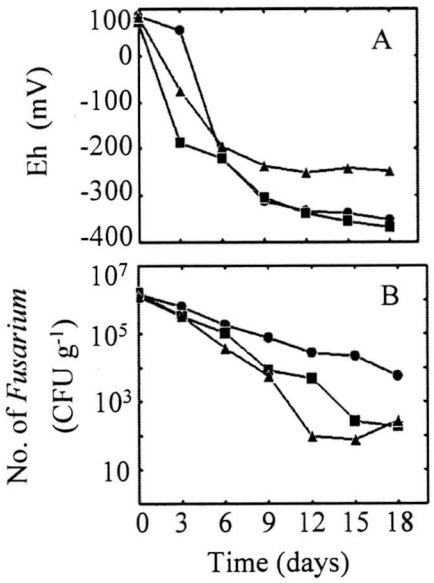
A
B
Eh (mV)
100
0
-100
-200
-300
-400
No. of Fusarium (CFU g-1)
10^7
10^5
10^3
10
0 3 6 9 12 15 18
Time (days)

Figure 2

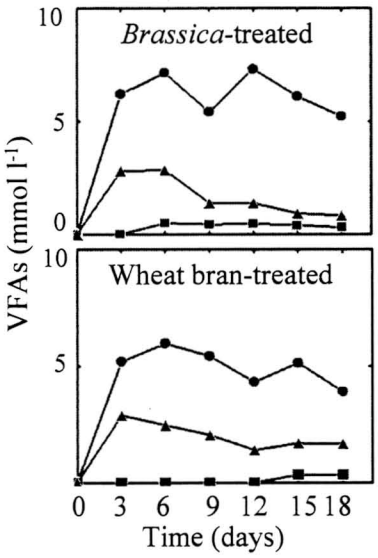
10
Brassica-treated
5
0
10
Wheat bran-treated
5
VFAs (mmol l-1)
0 3 6 9 12 15 18
Time (days)

Figure 3

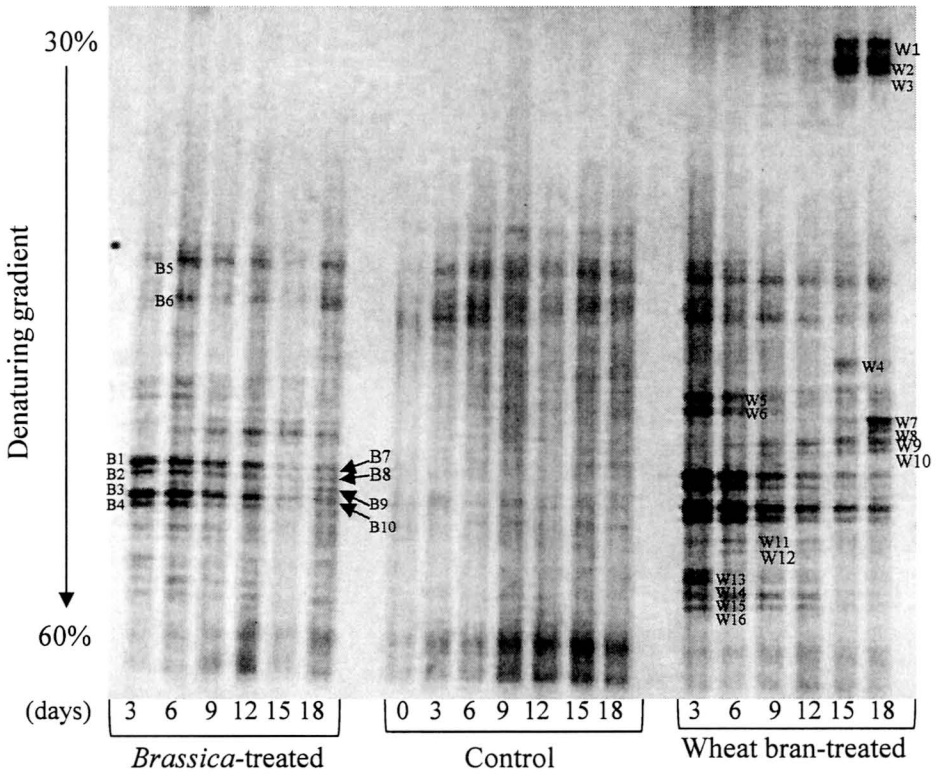
30%
Denaturing gradient
60%
B1
B2
B3
B4
B5
B6
B7
B8
B9
B10
W1
W2
W3
W4
W5
W6
W7
W8
W9
W10
W11
W12
W13
W14
W15
W16
(days)
3 6 9 12 15 18
0 3 6 9 12 15 18
3 6 9 12 15 18
Brassica-treated
Control
Wheat bran-treated

Figure 4

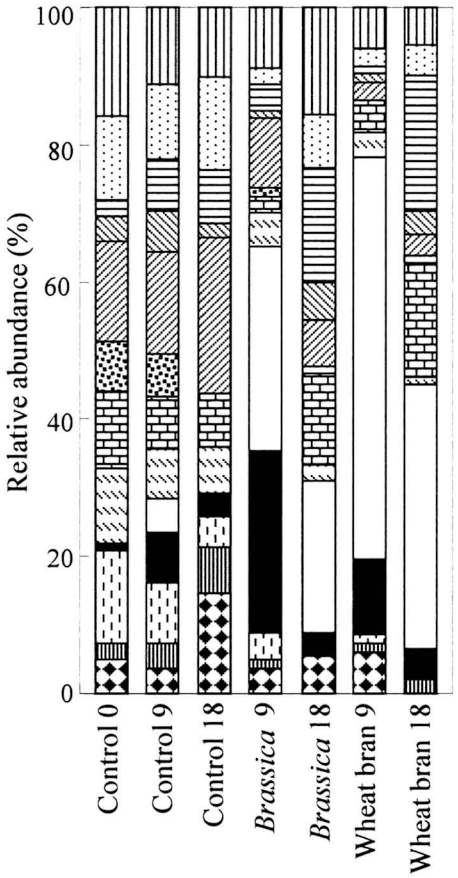
Relative abundance (%)
100
80
60
40
20
0
Control 0
Control 9
Control 18
Brassica 9
Brassica 18
Wheat bran 9
Wheat bran 18

Figure 5

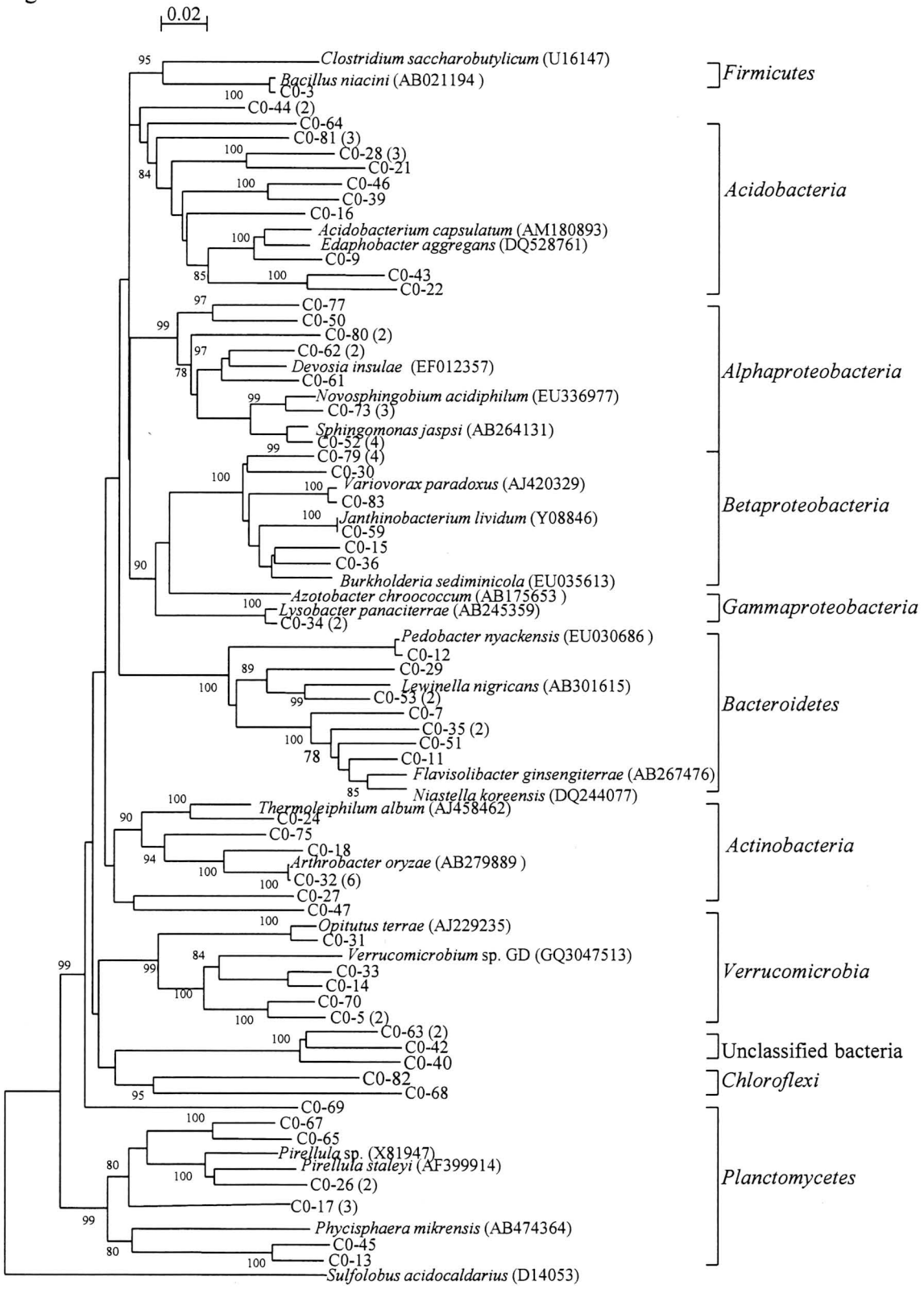
0.02
Clostridium saccharobutylicum (U16147)
Bacillus niacini (AB021194)
C0-3
C0-44 (2)
C0-64
C0-81 (3)
C0-28 (3)
C0-21
C0-46
C0-39
C0-16
Acidobacterium capsulatum (AM180893)
Edaphobacter aggregans (DQ528761)
C0-9
C0-43
C0-22
C0-77
C0-50
C0-80 (2)
C0-62 (2)
Devosia insulae (EF012357)
C0-61
Novosphingobium acidiphilum (EU336977)
C0-73 (3)
Sphingomonas jaspsi (AB264131)
C0-52 (4)
C0-79 (4)
C0-30
Variovorax paradoxus (AJ420329)
C0-83
Janthinobacterium lividum (Y08846)
C0-59
C0-15
C0-36
Burkholderia sediminicola (EU035613)
Azotobacter chroococcum (AB175653)
Lysobacter panaciterrae (AB245359)
C0-34 (2)
Pedobacter nyackensis (EU030686)
C0-12
C0-29
Lewinella nigricans (AB301615)
C0-53 (2)
C0-7
C0-35 (2)
C0-51
C0-11
Flavisolibacter ginsengiterrae (AB267476)
Niastella koreensis (DQ244077)
Thermoleiphilum album (AJ458462)
C0-24
C0-75
C0-18
Arthrobacter oryzae (AB279889)
C0-32 (6)
C0-27
C0-47
Opitutus terrae (AJ229235)
C0-31
Verrucomicrobium sp. GD (GQ3047513)
C0-33
C0-14
C0-70
C0-5 (2)
C0-63 (2)
C0-42
C0-40
C0-82
C0-68
C0-69
C0-67
C0-65
Pirellula sp. (X81947)
Pirellula staleyi (AF399914)
C0-26 (2)
C0-17 (3)
Phycisphaera mikrensis (AB474364)
C0-45
C0-13
Sulfolobus acidocaldarius (D14053)
Firmicutes
Acidobacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Bacteroidetes
Actinobacteria
Verrucomicrobia
Unclassified bacteria
Chloroflexi
Planctomycetes

Figure 6

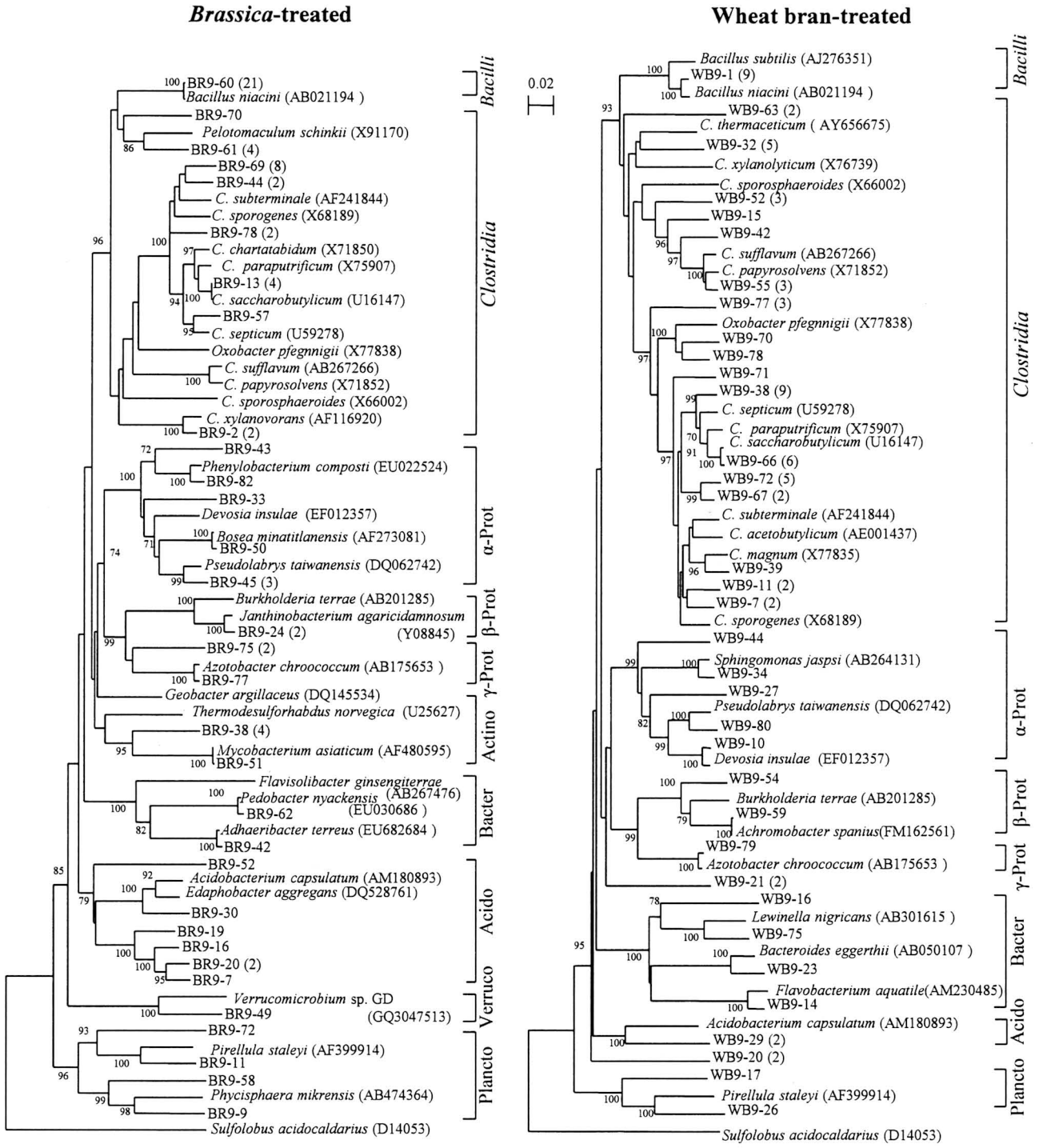
Brassica-treated
BR9-60 (21)
Bacillus niacini (AB021194)
BR9-70
Pelotomaculum schinkii (X91170)
BR9-61 (4)
BR9-69 (8)
BR9-44 (2)
C. subterminale (AF241844)
C. sporogenes (X68189)
BR9-78 (2)
C. chartatabidum (X71850)
C. paraputrificum (X75907)
BR9-13 (4)
C. saccharobutylicum (U16147)
BR9-57
C. septicum (U59278)
Oxobacter pfegnnigii (X77838)
C. sufflavum (AB267266)
C. papyrosolvens (X71852)
C. sporosphaeroides (X66002)
C. xylanovorans (AF116920)
BR9-2 (2)
BR9-43
Phenylobacterium composti (EU022524)
BR9-82
BR9-33
Devosia insulae (EF012357)
Bosea minatitlanensis (AF273081)
BR9-50
Pseudolabrys taiwanensis (DQ062742)
BR9-45 (3)
Burkholderia terrae (AB201285)
Janthinobacterium agaricidamnosum
BR9-24 (2)
(Y08845)
BR9-75 (2)
Azotobacter chroococcum (AB175653)
BR9-77
Geobacter argillaceus (DQ145534)
Thermodesulforhabdus norvegica (U25627)
BR9-38 (4)
Mycobacterium asiaticum (AF480595)
BR9-51
Flavisolibacter ginsengiterrae
Pedobacter nyackensis (AB267476)
BR9-62
(EU030686)
Adhaeribacter terreus (EU682684)
BR9-42
BR9-52
Acidobacterium capsulatum (AM180893)
Edaphobacter aggregans (DQ528761)
BR9-30
BR9-19
BR9-16
BR9-20 (2)
BR9-7
Verrucomicrobium sp. GD
BR9-49
(GQ3047513)
BR9-72
Pirellula staleyi (AF399914)
BR9-11
BR9-58
Phycisphaera mikrensis (AB474364)
BR9-9
Sulfolobus acidocaldarius (D14053)
Bacilli
Clostridia
α-Prot
β-Prot
γ-Prot
Actino
Bacter
Acido
Verruco
Plancto
Wheat bran-treated
0.02
Bacillus subtilis (AJ276351)
WB9-1 (9)
Bacillus niacini (AB021194)
WB9-63 (2)
C. thermaceticum (AY656675)
WB9-32 (5)
C. xylanolyticum (X76739)
C. sporosphaeroides (X66002)
WB9-52 (3)
WB9-15
WB9-42
C. sufflavum (AB267266)
C. papyrosolvens (X71852)
WB9-55 (3)
WB9-77 (3)
Oxobacter pfegnnigii (X77838)
WB9-70
WB9-78
WB9-71
WB9-38 (9)
C. septicum (U59278)
C. paraputrificum (X75907)
C. saccharobutylicum (U16147)
WB9-66 (6)
WB9-72 (5)
WB9-67 (2)
C. subterminale (AF241844)
C. acetobutylicum (AE001437)
C. magnum (X77835)
WB9-39
WB9-11 (2)
WB9-7 (2)
C. sporogenes (X68189)
WB9-44
Sphingomonas jaspsi (AB264131)
WB9-34
WB9-27
Pseudolabrys taiwanensis (DQ062742)
WB9-80
WB9-10
Devosia insulae (EF012357)
WB9-54
Burkholderia terrae (AB201285)
WB9-59
Achromobacter spanius(FM162561)
WB9-79
Azotobacter chroococcum (AB175653)
WB9-21 (2)
WB9-16
Lewinella nigricans (AB301615)
WB9-75
Bacteroides eggerthii (AB050107)
WB9-23
Flavobacterium aquatile(AM230485)
WB9-14
Acidobacterium capsulatum (AM180893)
WB9-29 (2)
WB9-20 (2)
WB9-17
Pirellula staleyi (AF399914)
WB9-26
Sulfolobus acidocaldarius (D14053)
Bacilli
Clostridia
α-Prot
β-Prot
γ-Prot
Bacter
Acido
Plancto

Figure 7

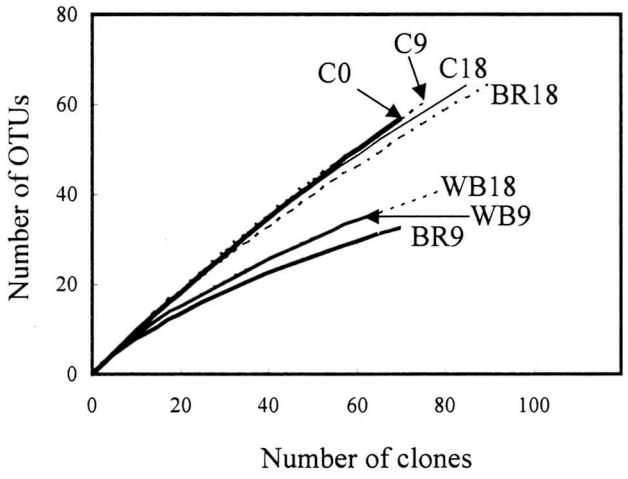
80
60
40
20
0
Number of OTUs
0
20
40
60
80
100
Number of clones
C0
C9
C18
BR18
WB18
WB9
BR9

Figure 8

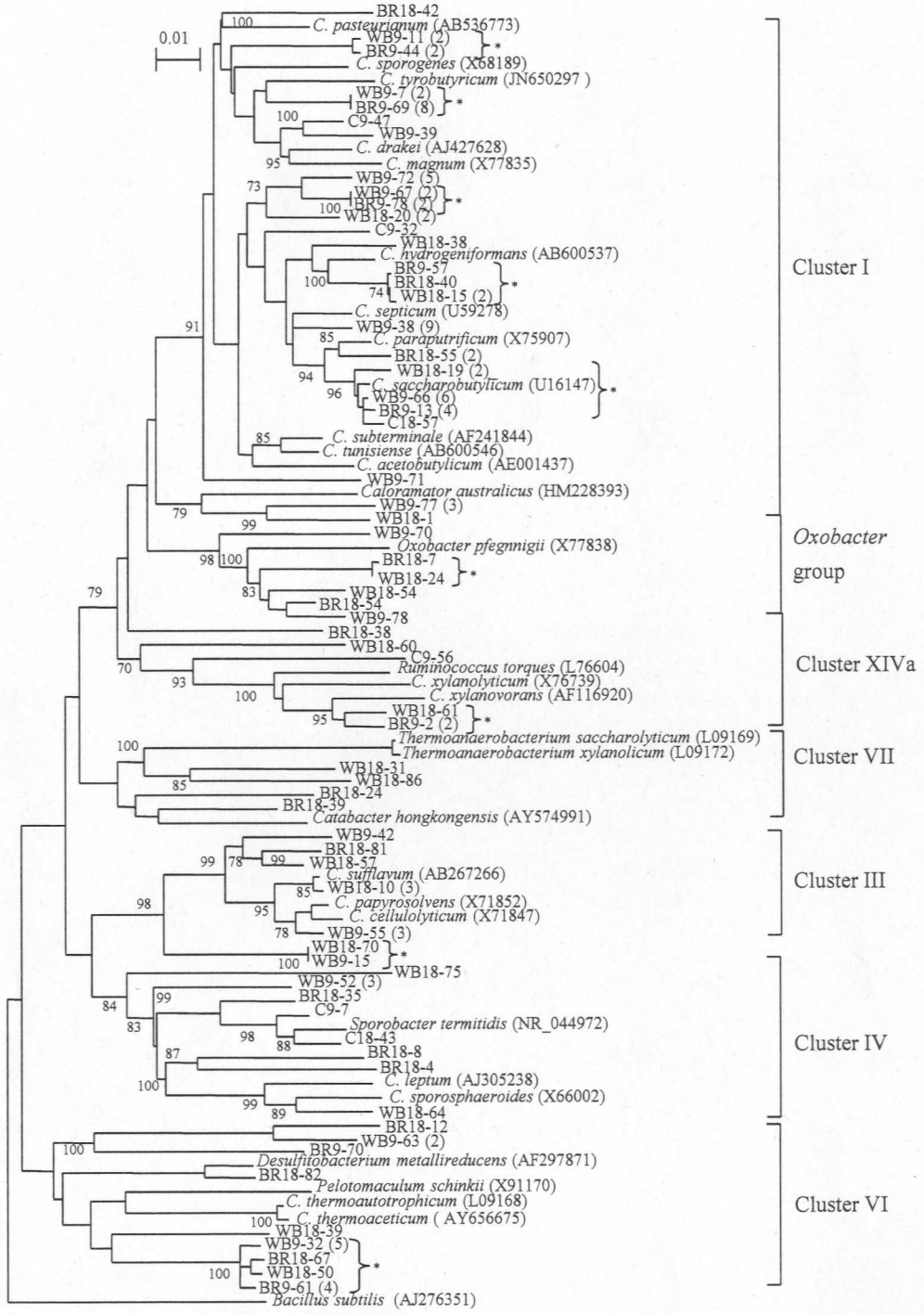
0.01
BR18-42
C. pasteurianum (AB536773)
WB9-11 (2)
BR9-44 (2)
C. sporogenes (X68189)
C. tyrobutyricum (JN650297)
WB9-7 (2)
BR9-69 (8)
C9-47
WB9-39
C. drakei (AJ427628)
C. magnum (X77835)
WB9-72 (5)
WB9-67 (2)
BR9-78 (2)
WB18-20 (2)
C9-32
WB18-38
C. hydrogeniformans (AB600537)
BR9-57
BR18-40
WB18-15 (2)
C. septicum (U59278)
WB9-38 (9)
C. paraputrificum (X75907)
BR18-55 (2)
WB18-19 (2)
C. saccharobutylicum (U16147)
WB9-66 (6)
BR9-13 (4)
C18-57
C. subterminale (AF241844)
C. tunisiense (AB600546)
C. acetobutylicum (AE001437)
WB9-71
Caloramator australicus (HM228393)
WB9-77 (3)
WB18-1
WB9-70
Oxobacter pfegnnigii (X77838)
BR18-7
WB18-24
WB18-54
BR18-54
WB9-78
BR18-38
WB18-60
C9-56
Ruminococcus torques (L76604)
C. xylanolyticum (X76739)
C. xylanovorans (AF116920)
WB18-61
BR9-2 (2)
Thermoanaerobacterium saccharolyticum (L09169)
Thermoanaerobacterium xylanolicum (L09172)
WB18-31
WB18-86
BR18-24
BR18-39
Catabacter hongkongensis (AY574991)
WB9-42
BR18-81
WB18-57
C. sufflavum (AB267266)
WB18-10 (3)
C. papyrosolvens (X71852)
C. cellulolyticum (X71847)
WB9-55 (3)
WB18-70
WB9-15
WB18-75
WB9-52 (3)
BR18-35
C9-7
Sporobacter termitidis (NR_044972)
C18-43
BR18-8
BR18-4
C. leptum (AJ305238)
C. sporosphaeroides (X66002)
WB18-64
BR18-12
WB9-63 (2)
BR9-70
Desulfitobacterium metallireducens (AF297871)
BR18-82
Pelotomaculum schinkii (X91170)
C. thermoautotrophicum (L09168)
C. thermoaceticum (AY656675)
WB18-39
WB9-32 (5)
BR18-67
WB18-50
BR9-61 (4)
Bacillus subtilis (AJ276351)
Cluster I
Oxobacter group
Cluster XIVa
Cluster VII
Cluster III
Cluster IV
Cluster VI

Table 1. Enumeration of bacterial population from different soil samples

Soil	Day	Heat [1] treatment	x 10^6 CFU g^{-1} *	
			Anaerobes	Aerobes
Control	0 #	-	4.6 ± 0.47	31.4 ± 5.90
		+	5.1 ± 2.65	9.1 ± 2.74
Control	18	-	18.2 ± 9.96	24.1 ± 7.10
		+	28.2 ± 5.87	8.5 ± 1.30
Brassica-treated	18	-	18.9 ± 8.60	15.6 ± 2.70
		+	2.7 ± 1.21	7.5 ± 1.36
Wheat bran-treated	18	-	28.8 ± 9.50	19.0 ± 7.21
		+	5.8 ± 2.48	2.7 ± 2.40

Each value represents the means (± SD) of three repetitions.

Plant biomass and heat treatment affected the number of aerobes significantly at $p < 0.05$ using ANOVA 2-way analysis without replication

[1] Diluted soil samples were treared at 80°C for 10 minutes before inoculation to the media for enumeration of viable counts of heat tolerant cells.

* Media for enumeration: anaerobes, 1/10PY4S; aerobes, DNB agar.

Original soil before the treatment.

Table 2. Closely related species of sequences from DGGE bands of biomass-treated soils

Band No.	Closely related species (Accession no.)	Taxonomic affiliation		Similarity (%)
		Phylum	Class	
B1, B2, B3, B4, B7, B8, B10, W11, W12	*Bacillus senegalensis* (AB110415) or *Bacillus niacini* (EU221375)	*Firmicutes*	*Bacilli*	97-100
W5	*Clostridium puniceum* (X71857)	*Firmicutes*	*Clostridia*	98
W6	*Clostridium akagii* (AJ237755)	*Firmicutes*	*Clostridia*	90
W13	*Clostridium acetobutylicum* (AE001437)	*Firmicutes*	*Clostridia*	98
W14	*Clostridium intestinale* (X76740)	*Firmicutes*	*Clostridia*	98
W15, W16	*Clostridium magnum* (X77835)	*Firmicutes*	*Clostridia*	97
B9	*Azotobacter beijerinckii* (EF100152)	*Proteobacteria*	*Gammaproteobacteria*	94
W4, W7	*Azotobacter chroococcum* (EF634040)	*Proteobacteria*	*Gammaproteobacteria*	97-98
B5, B6	*Pedobacter cryoconitis* (EU169155)	*Bacteroidetes*	*Sphingobacteria*	98-100
W1	*Bacteroides massiliensis* (AY126616)	*Bacteroidetes*	*Bacteroidia*	92
W2, W3	*Bacteroides coprocola* (AB200225)	*Bacteroidetes*	*Bacteroidia*	93
W8	*Flavobacterium denitrificans* (AJ318907)	*Bacteroidetes*	*Flavobacteria*	87
W9	*Prolixibacter bellariivorans* (AY918928)	*Bacteroidetes*	*Flavobacteria*	90
W10	*Flavobacterium johnsoniae* (DQ530149)	*Bacteroidetes*	*Flavobacteria*	90