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Proliferation of diversified clostridial species during biological soil disinfestation incorporated with plant biomass under various conditions

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Abstract

Biological soil disinfestation (BSD) involves the anaerobic decomposition of plant biomass by microbial communities leads to control of plant pathogens. We analyzed bacterial communities in soil of a model experiment of BSD, as affected by biomass incorporation under various conditions, to find out the major anaerobic bacterial groups emerged after BSD treatments. The soil was treated with *Brassica juncea* plants, wheat

bran or *Avena strigosa* plants, irrigated at 20% or 30% moisture content, and incubated at 25-30°C for 17 days. The population of *Fusarium oxysporum* f. sp. *spinaciae* incorporated at the start of the experiment declined markedly for some BSD conditions and rather high concentrations of acetate and butyrate were detected from these BSD-treated soils. The polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis based on the V3 region of 16S rRNA gene sequences from the soil DNA revealed that bacterial profiles greatly changed according to the treatment conditions. Based on the clone library analysis, phylogenetically diverse clostridial species appeared exceedingly dominant in the bacterial community of BSD soil incorporated with *Brassica* plants or wheat bran, in which the pathogen was suppressed completely. Species in the class *Clostridia* such as *Clostridium saccharobutylicum*, *Clostridium acetobutylicum*, *Clostridium xylanovorans*, *Oxobacter pfennigii*, *Clostridium pasteurianum*, *Clostridium sufflavum*, *Clostridium cylindrosporium*, etc. were commonly recognized as closely related species of the dominant clone groups from these soil samples.

Keywords

Anaerobic bacteria, Biological soil disinfestation (BSD), Clone library, Clostridial

group, *Fusarium oxysporum*

Introduction

Agricultural producers often apply some form of soil disinfestation before planting high-value cash crops, to reduce soil-borne crop pests including fungal, bacterial and nematode pathogens, weeds, and insects (Spadaro and Gullino 2005). For the last few decades, methyl bromide was the major fumigant used for pre-planting soil disinfestation, but it has been shown that gaseous methyl bromide may destroy stratospheric ozone (Prather and McElroy 1984). The search for alternatives to this effective soil fumigant has recently been emphasized in light of the phasing-out of methyl bromide (Ristaino and Thomas 1997), giving preference to non-chemical fumigation methods. Instead of the chemical disinfestants, steam disinfestation (Katan 2000), soil solarization (Katan 1981) or flooding (Stover 1979) has been mentioned, but may have some practical limitations (Goud et al. 2004).

Biological soil disinfestation (BSD) is one of the alternative methods of soil disinfestation using anaerobic decomposition of organic matter in soil, which has been developed in the Netherlands (Blok et al. 2000) and Japan (Shinmura 2004; Shinmura et al. 1999) as an environmentally friendly approach. BSD can be a promising alternative

for all other fumigation methods due to its broad spectrum for suppression of soil-borne pathogens at various incubation duration, temperature, and location (Goud et al. 2004; Shinmura 2004; Messiha et al. 2007). The principles of BSD conducted in Japan include three steps: (1) incorporating the plant biomass into soil, (2) flooding the soil by irrigation, and (3) covering the soil surface with a plastic film to induce reducing soil conditions (Shinmura 2000, 2004). The process lasts for about three weeks and becomes suitable for crop cultivation soon after removing the plastic film followed by plowing the field. A number of plant biomass sources such as *Brassica* spp., wheat bran, rice bran, grasses, or other organic substances have been used successfully as BSD materials for controlling soil borne pests and diseases (Shinmura 2004; Goud et al. 2004; Momma 2008).

It has been reported that survival of many plant pathogens is significantly decreased under anaerobic soil conditions (Blok et al. 2000; Messiha et al. 2007), and the bacterial communities developed due to anaerobiosis may enhance the pathogen elimination efficacy. In a previous report, we analyzed the bacterial communities in soil of a BSD experiment using *Brassica juncea* plants or wheat bran (Mowlick et al. 2012). The BSD treatment with these plant biomass successfully decreased the population of the pathogen (*Fusarium oxysporum* f. sp. *lycopersici*, fusarium wilt pathogen of tomato)

incorporated in the soil before the treatment, and it was found by clone library analysis based on the 16S rRNA gene sequences that anaerobic bacteria including especially the members of the class *Clostridia* in the phylum *Firmicutes* became the major and dominant bacterial groups in the soil. The members in the class *Clostridia* are known to produce some harmful compounds to pathogens, such as skatole, indole, cresol or some phenolic compounds, by the fermentation of amino acids such as tryptophan and tyrosine under anaerobic conditions (Macfarlane and Macfarlane 1995). Besides, fatty acids like acetate or butyrate produced by these bacterial groups were supposed to contribute to pathogen inactivation (Momma et al. 2006). These acids were actually detected in the BSD soil (Mowlick et al. 2012), and it was strongly suggested that acetate, butyrate or other substances could be the products of the clostridial groups responsible for the suppression of some soil borne pathogens. Thus, it seems that proliferation of clostridial species in BSD treated soil may be important to make the treatment more effective.

Considering the facts stated, the aim of this study was to confirm the effects of various plant biomass sources and incubation conditions on the suppression of pathogens and to identify common bacterial groups that proliferated during the BSD treatment. We analyzed the bacterial communities of BSD-treated soils amended with

three types of biomasses, mustard (*B. juncea* L.) plants, wheat (*Triticum aestivum* L.) bran, and lopsided oat (*Avena strigosa* L.) plants under different incubation conditions (temperature and moisture content) of a model experiment using closed vessels. Molecular techniques such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer et al. 1993) and clone library analysis (Maidak et al. 1999) were mainly used to determine the changes in the major bacterial populations and detailed community composition in the BSD soil. To compare with our previous results (Mowlick et al. 2012), soil was obtained from a different district of Japan and a different pathogen was used in the experiment.

Materials and methods

BSD experiments using closed vessels

The model experiment of BSD was carried out by using 640 ml plastic pots. Soil obtained from a field (gray lowland soil at pH 5.18) in the NARO Western Region Agricultural Research Center, Fukuyama, Hiroshima, Japan (34.5°N, 133.4°E) was air-dried and passed through a sieve with 2 mm opening. The nitrate-non utilizing (*nit*)

mutant of *F. oxysporum* f. sp. *spinaciae* cultivated in potato sucrose broth (PSB) medium (Takehara and Kuniyasu 1994) was incorporated into the bulk soil (5×10^5 CFU/g) and mixed thoroughly to make uniformly pre-infested soil. The *nit* mutant strain (M2-1) was generated from the parent strain Spin-2 (wild type, the NIAS Genebank accession No. MAFF103060), by incubating mycelial plugs of the wild type strain on a chlorate-containing medium for 12 days according to the method described by Puhalla (1985). Strain M2-1 was comparable to strain Spin-2 in growth and pathogenicity. The pots were filled with the *F. oxysporum* infested soil (450 g dry soil per pot) and treated with three types of plant biomass, namely, mustard (*B. juncea*) plants, wheat bran, and lopsided oat (*A. strigosa*) plants. The incorporation amounts of plant biomass were determined based on the standard methods for BSD treatment (Goud et al. 2004; Shinmura 2004). Leaves of the *B. juncea* plants were taken from the field before the flowering stage, dried at 40°C for 2 days and incorporated into pot soil at the rate of 4.0 g of dry matter/kg of soil (1.8 g/pot). Wheat bran was applied at the rate of 10.9 g /kg of soil (4.9 g/pot). Grass plants of *A. strigosa* were cut above the ground at the flowering stage, dried at 40°C for 4 days and incorporated into pot soil at a rate of 7.3 g of dry matter/kg of soil (3.3 g/pot). For the control treatment, none of plant material or any other substances was incorporated into the soil.

The field capacity for moisture content (MC) measured for the original soil was 24.5% (w/w of wet soil). Soil in each pot was irrigated at 20 or 30% MC and the pots were covered with lids tightly to avoid penetration of oxygen. The pots containing 30% MC soil were incubated at three different temperatures (25°C, 27.5°C, and 30°C) for each biomass condition as well as the control, while all pots with 20% MC soil were incubated at 30°C. Each treatment condition was applied in duplicate resulting 32 pots in total. The names of the treatments were designated considering the biomass (or control), incubation temperature, and MC, e.g., Br30-20 for *Brassica*-treated soil at 30°C with 20% MC (Table 1). Soil samples were collected from all the pots using sterile spatulas after 17 days of incubation. In addition to these treated soil samples, the original field soil sample without any treatment was also collected in duplicates (Y1 and Y2). The oxidation-reduction potential (ORP) of soil was measured for all treatments by electrodes (Ag/AgCl) inserting into the soil directly. The number of *nit* mutant of the pathogen incorporated into the soil was determined by the dilution plate technique using a selective medium for the mutant (CMP medium) (Takehara et al. 2003) for all soil samples.

Determination of concentrations of volatile fatty acids 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 volatile fatty acids (VFAs) and alcohols in the supernatants were analyzed by gas chromatography (Hitachi G-3500 and G-5000) as described previously (Ueki et al. 1986). Concentrations of VFAs and alcohols are expressed in the text as those determined in the supernatant of slurry samples.

DNA extraction and PCR amplification

About 1 g soil from each sample was taken and DNA was extracted using 'Ultra Clean™ Soil DNA Isolation kit' (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Finally, 50 µl volume was extracted for each sample in which the DNA was eluted from the spin column at a concentration of 48-72 ng/µl. 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 μ l) contained 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems, Foster, CA, USA), 15 mM Tris-HCl (tris(hydroxymethyl)aminomethane-HCl) (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% bovine serum albumin, each deoxynucleotide triphosphate mixtures (dNTPs) at a concentration of 200 μ M, 0.25 μ M of each primer, and 60-100 ng of template DNA. The amplification conditions for the primer set B341fGC and 534r or B27f and U1492r were followed as described in the previous study (Mowlick et al. 2012). Amplified DNA fragments were confirmed after agarose gel electrophoresis staining with ethidium bromide.

DGGE analysis

For the DGGE analysis, PCR products of the 16S rRNA gene were separated by DGGE using a DCodeTM system (Bio-Rad Laboratories, Hercules, CA, USA). A total of 10 μ g DNA sample was applied in each lane representing the soil samples. The DNA samples

were loaded 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 images were transformed into digital data and the positions of major DNA bands in the DGGE profiles were numerically designated for performing cluster analysis.

Clone library and nucleotide sequencing

The following ten soil samples were selected based on the various data obtained (soil parameters, DGGE, etc) as representatives for the clone library analysis of the bacterial community in the soil samples: Br30-20 (pot no. A1), Br25-30 (B2), Br30-30 (E1), Wh25-30 (H1), Wh30-30 (K1), Av25-30 (M2), Av30-30 (P2), Co25-30 (R1), Co30-30 (U1), and Orginal (Y2) (Table 1). The PCR products of DNA 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). Sequence analysis (about 600 bp) was done for a total of 96 clones from each soil sample using a sequence primer U515f (5' GTG YCA GCM GCC GCG GTAA-3') according to the Dye Terminator method using a capillary sequencer at Takara Co. Ltd.

Statistical analysis

The major DGGE bands detected on the profiles were numbered and classified into four categories for the cluster analysis depending on their intensities (0 = no band; 1 = weak; 2 = moderate; 3 = strong) (Watanabe et al. 2009). The cluster analysis with normalized data was carried out using the PAST program and Ward method (Hammer et al. 2001). 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 97% similarity of nucleotide sequences. All the representative clones of the OTUs were analyzed to determine the taxonomic affiliations 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, non-chimeric 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>). The coverage of the clone libraries (C) was calculated (Good 1953) from the equation $C = 1 - (n_1/N)$; where n_1 is the number of clones that occurred only once (frequency 1), and N is the total number of clones examined. The richness (chao 1) was determined for all the clone libraries using an online grouping method 'Fastgroup' (http://biome.sdsu.edu/fastgroup/fg_tools.htm). Bacterial diversity was calculated using the Simpson's diversity index (D) by the function, $D = 1 - \sum n(n-1)/(N(N-1))$, where n = the total number of clones of a particular OTU and N = the total number of clones of all OTUs. Besides, Shannon-Wiener diversity index (H') was determined to compare the changes in diversity of bacterial communities within the libraries by the function: $H = -\sum P_i \log P_i$, where the proportion of

OTU i relative to the total number of OTU (p_i) was calculated. All those diversity indexes were calculated using online biodiversity calculator (http://www.ayoung.com/labs/biodiversity_calculator.html).

The nucleotide sequences obtained from the clone library analyses have been reported in DDBJ/GenBank under the accession numbers AB642681-AB643454 (774 entries).

Results

Status of the treated soil samples

The incorporated pathogen (the *nit* mutant of *F. oxysporum*) was enumerated at 5×10^5 CFU/g of dry soil at the start of the incubation. Densities of the pathogen in all treated soils after 17 days of incubation were determined along with the measurements of ORP and concentrations of VFAs and alcohols (Table 1). A high density of the fungal pathogen (higher than 1×10^3 CFU/g) was detected from all the control soil samples (without plant biomass), indicating inability to kill the pathogen effectively without biomass incorporation. In contrast, the *Fusarium* population was eliminated completely

from all wheat bran-treated soil irrespective of the soil conditions. For *Brassica*- and *Avena*-treatments, the pathogen was not detected at all from soil treated at 30°C for both 20% and 30% MC, while it was still alive in soil treated at 25°C and 27.5°C.

The ORP values for all biomass-treated (BSD) soil dropped considerably as compared to the values of the original or the control soils. The results indicated the development of much reduced condition for all BSD-treated soils compared with the control soil. No VFAs was detected from the control as well as the original soil samples, while considerable amounts of acetate were detected from all conditions of BSD-treated soils followed by butyrate (for wheat bran-treated soils mainly) together with traces of propionate. In case of wheat bran-treated soil, when compared with other BSD soils, the concentrations of acetate were almost double and those of butyrate were much higher. Small amounts of ethanol (3.2-4.0 mmol/l) were detected from some BSD conditions.

PCR-DGGE analysis

The PCR-DGGE analysis was carried out to know the differences in the bacterial community profiles of these differently treated soil samples. The DGGE banding patterns and cluster analysis of the *Brassica*-treated soils showed almost similar

community profiles between duplicate samples with an exception (B1 and B2 for Br25-30), indicating that similar bacterial communities usually developed in soil treated with the same condition (Fig. 1). The differences of incubation temperature or MC, however, clearly resulted in distinct profiles.

The DGGE profiles for other soil samples (wheat bran-treated, *Avena*-treated and control soils) with the cluster analysis (Fig. 2) revealed that banding patterns for soil samples treated at 25°C and 27.5°C were rather similar for each treatment, while those were distinctive from soil treated at 30°C for all cases irrespective of MC. This indicates that the rise in the incubation temperature from 27.5°C to 30°C affected the bacterial communities more strongly as compared with the rise from 25.0°C to 27.5°C.

The DGGE analysis was carried out for selected soil samples including the original soil to know the effects of different plant biomass sources and MC on the bacterial community compositions (Supplementary Fig. 1). Clear changes were shown in the profiles for all treated soil as compared with the original soil. Amendment with *Brassica* plants or wheat bran resulted in greater differences from the control soil than the amendment with *Avena* plants. Although the variation in plant biomass sources resulted in rather different DGGE profiles, the difference of MC (20% or 30%) for each biomass condition did not bring about such clear differences in the profiles (Supplementary Fig.

1b).

Clone library analysis

Soil samples treated at 25°C and 30°C under 30% MC as well as the original soil were selected for the clone library analysis. In addition, for *Brassica*-treated soil, the soil sample treated at 30°C under 20% MC (Br30-20) was also selected, because it effectively eliminated the pathogen. One of soil samples from the duplicate pots was selected for each treatment based on all results shown above.

The clones from all libraries appeared to be assigned to at least six major phyla of the domain *Bacteria*, namely *Firmicutes*, *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi*. However, the phylogenetic compositions and proportions of the major phyla or classes varied markedly depending upon the type of biomass used and the treatment conditions applied. The affiliations of clone sequences from each soil sample are shown in Fig. 3 in relation to the percentages of number of clones belonging to each phylum or class. Out of the phylogenetic trees constructed for all ten libraries, those for Br30-30 (E1) and Wh25-30 (H1) were selected as representative trees from the soil samples that eliminated the pathogen completely (Fig.

4). In addition, major OTUs (consisting of more than two clones) and their closest relatives are presented in Table 2 for each library.

Phylogenetic groups in the original and the control soil

The clone library for the original soil (Y2) showed much diversified populations of different phylogenetic groups. The most abundant taxonomic group of the original soil was allocated to the phylum *Proteobacteria* (about 48% of the total number of clones) (*Alpha*- and *Gammaproteobacteria* classes mainly). Other clone sequences belonged to the phyla *Firmicutes* (7% from the *Clostridia* and 8% from the class *Bacilli*), *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, etc.

In case of the control library Co25-30 (R1), diversified bacterial populations were detected. An OTU relating to the unique clade “*Symbiobacterium*”, a facultatively anaerobic group in the *Firmicutes*, at about 90-91% of sequence similarity was the most dominant (24%), and a few clones were retrieved from the *Bacilli* and *Clostridia* (Fig. 3). Other phylogenetic groups detected were almost similar with those from the Original clone library. For another control library Co30-30 (U1), the “*Symbiobacterium*” related bacterial groups were also dominant with a few number of clostridial clones. Besides,

Rhodanobacter terrae in the *Gammaproteobacteria* was assigned to the closest relative of the major OTUs commonly appeared in both control libraries as well as in the original soil (Table 2).

Phylogenetic groups in the BSD-treated soil

Clone libraries for all BSD-treated soil samples showed substantial differences as compared with those of the original and control libraries (Fig. 3). The BSD libraries irrespective of the treatment conditions showed the presence of exclusively dominant bacterial taxonomic groups in the communities. In case of the *Brassica*-treated libraries, about 80% clones were assigned to the phylum *Firmicutes*, of which the majority belonged to the class *Clostridia* (strictly anaerobic spore-formers), with relatively minor clones in the class *Bacilli* (aerobic or facultatively anaerobic spore-formers). The clone libraries for the *Brassica*-treated soils, that is, Br30-20 (A1), Br25-30 (B2), and Br30-30 (E1), contained 54%, 50%, and 75% clostridial clones, whereas, 41%, 27%, and 6% clones from the class *Bacilli*, respectively (Fig. 3). The other clones were mainly affiliated with the different classes of the phylum *Proteobacteria*.

The compositions of the major *Firmicutes* groups for the *Brassica*-treated three

clone libraries were compared (Table 2). The clones closely related at high similarities (about 99%) to *Clostridium saccharobutylicum* occupied major portions of those clone library, especially for the Br30-20 library. A number of clones related to diverse species in clostridial groups such as *Oxobacter pfennigii*, *Zymophilus raffinovorans*, *Clostridium xylanovorans*, *Clostridium cylindrosporium*, *Clostridium sufflavum*, etc. increased with the rise of MC and temperature (Fig. 4). The clones closely related to *Bacillus azotoformans* and *Bacillus niacini* in the class *Bacilli* were also the dominating OTUs in both Br30-20 and Br25-30 libraries.

For the wheat bran-treated two clone libraries Wh25-30 (H1) and Wh30-30 (K1), about 76% and 58% clones were detected from the class *Clostridia* with a few number of clones from the class *Bacilli* (Fig. 3). Other phylogenetic groups retrieved from the two libraries were placed in the phyla *Proteobacteria* and *Actinobacteria* mainly. The closely related species of the major bacterial OTUs in the wheat bran-treated clone libraries showed that the lower temperature (25°C) stimulated the clostridial ratios as well as diversification consisting of *Clostridium pasteurianum*, *Clostridium acetobutylicum*, *C. xylanovorans*, *C. saccharobutylicum*, *Clostridium tetanomorphum* etc (Table 2, Fig. 4) in the community. Instead, higher temperature (30°C) induced two dominant groups closely related to *B. niacini* and *Azotobacter chroococcum* as well as

diversified clostridial clones (Table 2).

For the *Avena*-treated clone libraries, members of the *Firmicutes* also occupied the dominant position. From the library Av25-30 (M2), 51% clones belonged to the *Firmicutes* phylum (43% from the *Clostridia* and 8% from the *Bacilli*) (Fig. 3). Clones relating to *C. saccharobutylicum*, *C. cylindrosporum*, *C. xylanovorans*, and *B. niacini*, detected in other BSD libraries, were also the most dominant groups in the library. A number of clones from the phyla *Proteobacteria* and *Bacteroidetes* were also detected. On the other hand, the Av30-30 (P2) library contained the classes *Clostridia* and *Bacilli* in the *Firmicutes* at almost the same percentage. Clones closely related to *Veillonella rogosae* in the *Clostridia* and *Streptococcus parasanguinis* in the *Bacilli* occupied extraordinarily dominant positions in the library, respectively (Table 2).

Bacterial diversity in the clone libraries

Rarefaction analysis based on the OTU clustering (97% sequence similarity cut-off level) (Fig. 5) suggests that the curve for the Original library was far from the saturation. Among the rarefaction curves for the BSD-treated libraries, the curves for Br30-20 and Av30-30 seemed to almost reach the plateau, whereas others were far from it and

showed similar curves as those for the Co25-30 and Co30-30 libraries. The results suggest that bacterial richness in these samples was not fully recovered in these clone libraries with some exceptions.

Table 3 shows estimated bacterial richness and diversity for each clone library. A total of 49 OTUs were recognized in the Original library, whereas the numbers decreased for all the treated soil libraries. The estimates of richness and diversity in the communities suggest that the original soil harbors the most diverse communities. All treatments reduced the diversity as compared with the original soil, and especially the bacterial diversities in the libraries Br30-20 and Av30-30 were decreased considerably. However, high bacterial diversities were still maintained for other BSD treatments, especially in case of Br25-30, Br30-30, and Wh25-30 communities. The result suggests that although the BSD treatment increased the percentages of clostridial clones considerably, bacterial diversity at the species (97% sequence similarity) level was not usually reduced so much.

Phylogenetic diversity of clostridial groups after BSD

Based on the suppression of *F. oxysporum* population incorporated, compositions of

clostridial groups retrieved from the four libraries, Br30-20, Br30-30, Wh25-30, and Wh30-30, were further analyzed phylogenetically to identify the major common clostridial species and to compare the clostridial diversities among the BSD samples.

A phylogenetic tree consisting of all OTUs assigned to the class *Clostridia* from these BSD samples was generated (Fig. 6). All OTUs related to the clostridial groups were divided into six clusters (Collins et al. 1994) including *Oxobacter*, *Caloramator* and *Pelotomaculum* groups, indicating occurrence of extraordinarily diverse species in the class under these BSD conditions. Majority (63%) of the clostridial clones from the four libraries were classified into the cluster I (*Clostridium sensu stricto*) including the *Oxobacter* and *Caloramator* groups, and the remaining clones (37%) were affiliated with other miscellaneous clusters (Cluster III, IV, VI, IX, and XIVa). For the wheat bran-treated libraries, almost two thirds (66%) of the clostridial clones were assigned to the cluster 1 group. On the other hand, clostridial groups in the *Brassica*-treated soil, especially for the Br30-30 library, were distributed to cluster 1 and other clusters almost in an equal proportion. The result indicates that the phylogenetic composition of clostridial communities changed with the influence of biomass and treatment conditions.

From the combined clostridial phylogenetic tree, it was revealed that OTUs closely

related to *C. saccharobutylicum* (about 99% sequence similarity) or *C. xylanovorans* (about 95-96%) were recognized in all four libraries as the dominant groups, and the clones relating to *C. cylindrosporum*, *O. pfennigii* or *Pelotomaculum shinkii* were also detected commonly as relatively minor groups. The clostridial clones assigned to the cluster III (e.g., *C. sufflavum*) appeared only in the *Brassica*-treated libraries (Table 2).

The rarefaction analysis for the clostridial clones (Supplementary Fig. 2) almost corresponded with it for all communities (Fig. 5), and also did not reach the plateau except for the Br30-20 library. The result of richness and diversity estimates of OTUs (Supplementary Table 1) suggests that diversities of clostridial groups were higher in the Br30-30 and Wh25-30 BSD samples, and the diversity was much reduced in case of *Brassica*-treated soil of lower MC (Br30-20) and wheat bran-treated soil at higher temperature (Wh30-30).

Discussion

Achieving a reducing state or a decrease in ORP in soil is the most important aspect during BSD (Shinmura et al. 1999; Blok et al. 2000). Microbial activities can affect soil ORP by influencing redox couples including $\text{NO}_3^-/\text{NO}_2^-$, $\text{Fe}^{3+}/\text{Fe}^{2+}$, H^+/H_2 , $\text{SO}_4^{2-}/\text{HS}^-$,

and CO₂/CH₄ along with the partial or complete consumption of oxygen by soil microbes under the higher moisture content (Peters and Conrad 1996; Savant and Ellis 1964). In this study, the ORP decreased for most of BSD treatments as compared with the soil without biomass incorporation (control) and it should stimulate growth and multiplication of anaerobic bacteria in the BSD soil. In fact, a large number of bacterial species relating to the strictly anaerobic clostridial groups were detected as the closest relatives for the clone libraries of the BSD-treated soil samples.

The antagonistic activity of some soil microorganisms may be involved during BSD against soil-borne pathogens (Bailey and Lazarovits 2003; Kubo et al. 2005; Larkin and Griffin 2007). It has been pointed out that accumulation of VFAs such as acetate and butyrate would be the important factors for killing the plant pathogens during the process of disinfestation (Momma et al. 2006). In our study, rather high amounts of acetate were detected from all BSD soil samples together with butyrate especially for the wheat bran-treated soils, while no VFAs was detected from the control soils. Since the incorporation of plant biomasses to the soil strongly enhanced growth of clostridial species as shown by the clone library analysis, VFAs might be produced by these anaerobic bacteria through decomposition of the biomass. These results coincided with the results of our previous study (Mowlick et al. 2012). As mentioned below, we have

already isolated many strains of anaerobic bacteria from both *B. juncea*- and wheat-bran-treated soil used in this study and confirmed formation of acetate and butyrate as fermentation products for many of the isolates (data not shown).

It is reported that BSD using *Brassica* plants, wheat bran, grasses or molasses effectively killed a wide range of soil-borne pathogens including *Botrytis cinerea*, *Fusarium* spp., *Rhizoctonia solani*, *Phomopsis scleroides*, *Verticillium dahliae*, *Ralstonia solanacearum*, *Meloidogyne incognita*, etc. (Shinmura 2000, 2004; Takeuchi 2004; Messiha et al. 2007). Although the incorporated pathogen seemed to be completely eliminated from the wheat bran-treated soils for all conditions in this study, the effects of BSD on the pathogen were different depending on the incubation conditions for *Brassica*- and *Avena*-treatments. For these *Brassica*- and *Avena*-treated soils, the variations in the incubation temperature greatly influenced the suppression of the pathogenic population during BSD. The result indicates that temperature control is very important to conduct the BSD treatment successfully. However, the temperature effect should be verified in the further studies by considering other factors including the amount of plant material incorporated.

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

or of different phylogenetic groups at the phylum or class level (Janssen 2006). The clone library for the original soil showed that the soil contained diversified bacterial groups of different phyla or classes. The bacterial compositions of both control libraries (Co25-30 and Co30-30) were somewhat similar with that from the original soil in containing clones relating to the *Proteobacteria* as major groups. The similarities of DGGE banding patterns between the original and the two control soils coincided with the results of the clone library analysis. We tried to know the differences in the recovery of the bacterial diversities among treatments analyzing the rarefaction curves. Based on the results, we think that, in the future studies, we need to analyze more number of clones for most of the soil samples to know the saturation point of the bacterial diversities.

In both control soils, a group relating to *Symbiobacterium thermophilum*, a very deep group within the *Firmicutes* or, possibly, a novel phylum (Beppu and Ueda 2009), proliferated, however, the strict anaerobes relating to the typical clostridial groups did not occupy a major position. In the BSD-treated libraries, clones affiliated with the general *Firmicutes* were exceedingly dominant and the ratios of the clostridial clones (50-75%) and *Bacillus* group (6-50%) varied considerably. Soil microbial communities can be influenced by many factors like temperature (Pietikänen et al. 2005), water

availability (Fierer et al. 2003), ORP status, and others. In this study, it appeared that both temperature and MC contributed to the changes in bacterial communities in the BSD-treated soils. As shown in Fig. 3, increase in the incubation temperature stimulated the clostridial proliferation, but decreased the *Bacillus* group for *Brassica*-treated soil, while the opposite results were obtained for wheat bran- and *Avena*-treated soils. The effect of MC was very distinct in case of *Brassica*-treated soil, where the ratio of clones related to the class *Bacilli* was declined markedly with an increase in MC. Most of the species in the *Bacilli* produce spores and they should be alive as spores even in dry soil. Although they might start to grow under the humid condition immediately after the start of the treatment, earlier exhaustion of oxygen under the higher MC should suppress aerobic growth.

It was found that the members related to the *Clostridia* class appeared as major and important taxonomic groups in the bacterial communities in the BSD soils. The results agreed well with our previous results (Mowlick et al. 2012). Among the plant biomass incorporated in this study, *Brassica* or wheat bran was considered to stimulate the development of clostridial community during BSD. The clones assigned to the *Clostridia* class actually contained phylogenetically diverse members and it was indicated that the growth of abundant clostridial species might contribute to the total

diversity of the bacterial communities in the BSD soil. Diverse species related to the class *Clostridia* should be also present as spores even in the aerobic field soil, and start to grow using plant biomass as growth substrates soon after the development of reduced condition. The result indicates that field soil, even when the soil has been maintained under the aerobic condition for a long time, harbors exceedingly diverse, strictly anaerobic, clostridial species, and the introduction of reduced condition to the soil induces drastic changes in the bacterial community composition in the soil along with their active growth.

In case of the five clone libraries (Br30-20, Br30-30, Wh25-30, Wh30-30, and Av30-30), where the pathogen was effectively eliminated, the abundance of the members of the *Firmicutes* was almost 70-90% of the total clones, of which the majorities were related to the strictly anaerobic, clostridial species. Therefore, it seems likely that the dominant clostridial groups in these treatment conditions might have an important role to suppress the pathogen during BSD. Bacterial groups related to *C. acetobutylicum*, *C. saccharobutylicum*, *C. xylanovorans*, *C. sufflavum*, *C. pasteurianum*, *O. pfennigii*, *Z. raffinovorans*, *C. cylindrosporum*, *Caloramator mitchellensis*, *V. rogosae*, etc. seemed to be commonly proliferated anaerobes by the BSD treatment.

It is known that most of the closely related clostridial species of the clones detected

in this study produce various products including acetates, butyrate, alcohol, and H₂ gas as well as indole or skatole (Krumholz and Bryant 1985; Rainey et al. 2009; Wiegel 2009; Schleifer 2009). Since the similarity levels of 16S rRNA gene sequences between the major clone groups and their closest relatives are different depending on the clones, physiological properties and fermentation products of the species in the BSD soil represented by the clone sequences should be different from those of the related species. However, some species such as *C. acetobutylicum*, *C. saccharobutylicum*, *C. pasteurianum*, and *C. xylanovorans* were closely related with the major OTUs at considerably high similarity values (95-99%), respectively, suggesting that bacterial groups represented by the clones have similar properties as these recognized species.

Besides the clostridial population, the clones of the classes *Bacilli* and *Gammaproteobacteria* also proliferated in the BSD soils. The major *Bacillus* species included *B. azotoformans* (nitrogen-forming bacterium) and *B. niacini* (nicotinic acid-metabolizing) (Logan and Vos 2009; Priest 2009), and the *Gammaproteobacteria* group contained *A. chroococcum* (nitrogen fixer) (Kumar and Singh 2001). *B. niacini* and *A. chroococcum* were also detected as the closest relatives for the clones in our previous BSD soils (Mowlick et al. 2012). Thus, it is possible that species relating to these species might contribute partly for changing the soil status during BSD. In

addition, in our previous study, nitrogen-fixing bacteria such as *C. acetobutylicum*, *C. pasteurianum*, and *A. chroococcum* were also appeared as the closely related species of the major groups in the BSD communities. It is of interest to know their roles in nitrogen supply to the soil microbial communities.

We have isolated many clostridial strains relating to the clone sequences shown above from the BSD-treated soil samples examined in this study. We are now investigating the physiological properties of these isolates to confirm the effects of clostridial species on the pathogens during the BSD treatment. Especially it should be important to clarify the products to suppress the pathogens or to find out some other effective functions during the BSD treatment for these anaerobes. We have used *F. oxysporum* (fusarium wilt pathogen of tomato or spinach) as pathogenic agents in the present study as well as in our previous study (Mowlick et al. 2012). Other plant pathogens including fungi, bacteria, etc. should be also considered in the future studies. Moreover, BSD experiments should be applied in field conditions to confirm the suppression of plant pathogens and proliferation of clostridial communities.

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References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997)

Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402

Bailey KL, Lazarovits G (2003) Suppressing soil borne diseases with residue management and organic amendments. *Soil Till Res* 72:169-180

Beppu T, Ueda K (2009) Genus I. *Symbiobacterium* Ohno, Shitatori, Park, Saitoh, Kumon, Yamshita, Hirata, Nishida, Ueda and Beppu 2000, 1832^{VP}. In: Whitman WB, Parte AC (eds) *Bergey's manual of systematic bacteriology*, Vol. 3, Springer, New York, pp 1188-1190

Blok WJ, Lamers JG, Termorshuizen AJ, Bollen GJ (2000) Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90:253-259

Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P,

- Cai J, Hippe H, Farrow JAE (1994) The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44:812-826
- Fierer N, Schimel JP, Holden PA (2003) Influence of drying rewetting frequency on soil bacterial community structure. *Microb Ecol* 45:63-71
- Gans J, Wolinsky M, Dunbar J (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309:1387-1390
- Good IJ (1953) The population frequencies of species and the estimation of population parameters. *Biometrika* 40:237-262
- Goud JKC, Termorshuizen AJ, Blok WJ, van Bruggen AHC (2004) Long-term effect of biological soil disinfestation on *Verticillium* wilt. *Plant Dis* 88:688-694
- Hammer Ø, Harper DQAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4:4. http://palaeo-electronica.org/2001_1/past/issue1_01.htm
- Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* 72:1719-1728
- Kaku N, Ueki A, Ueki K, Watanabe K (2005) Methanogenesis as an important terminal electron accepting process in estuarine sediment at the mouth of Orikasa River.

Microbes Environ 20:41-52

Katan J (1981) Solar heating (solarization) of soil for control of soil borne pests. Annu Rev Phytopathol 19:211-236

Katan J (2000) Physical and cultural methods for the management of soil borne pathogens. Crop Prot 19:25-31

Krumholz LR, Bryant MP (1985) *Clostridium pfennigii* sp. nov. uses methoxyl groups of monobenzenoids and produces butyrate. Int J Syst Bacteriol 35:454-456

Kubo C, Ushio S, Katase M, Takeuchi T (2005) Analysis of factors involved in sterilization effect by soil reduction. Jpn J Phytopathol 71:281-282 (abstract in Japanese)

Kumar V, Singh KP (2001) Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. Biores Technol 76:173-175

Larkin RP, Griffin TS (2007) Control of soil borne potato diseases using *Brassica* green manures. Crop Prot 26:1067-1077

Logan NA, Vos PD (2009) Genus I. *Bacillus* Cohn 1872, 174AL. In: Whitman WB, Parte AC (eds) Bergey's manual of systematic bacteriology, Vol. 3, Springer, New York. pp 21-111

Macfarlane J, Macfarlane GT (1995) Proteolysis and amino acid fermentation. In:

- Gibson GR, Macfarlane GT (eds) *Human Colonic Bacteria*, CRC Press, New York, pp 75-100
- Maidak BL, Cole JR, Parker CTJr, Garrity GM, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* 27:171-173
- Messiha NAS, van Diepeningen AD, Wenneker M, van Beuningen AR, Janse JD, Coenen TGC, Termorshuizen AJ, van Bruggen AHC, Blok WJ (2007) Biological soil disinfestation (BSD), a new control method for potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2. *Eur J Plant Pathol* 117:403-415
- Momma N (2008) Biological soil disinfestation (BSD) of soil-borne pathogens and its possible mechanisms. *JARQ* 42:7-12.
- Momma N, Yamamoto K, Simandi P, Shishido M (2006) Role of organic acids in the mechanisms of biological soil disinfestation (BSD). *J Gen Plant Pathol* 72:247-252
- Mowlick S, Hirota K, Takehara T, Kaku N, Ueki K, Ueki A (2012) Development of anaerobic bacterial community consisted of diverse clostridial species during biological soil disinfestation amended with plant biomass. *Soil Sci Plant Nutr* 58:273-287

- Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695-700
- Peters V, Conrad R (1996) Sequential reduction processes and initiation of CH₄ production upon flooding of oxic upland soils. *Soil Biol Biochem* 28:371-382
- Pietikäinen J, Pettersson M, Bååth E (2005) Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiol Ecol* 52:49-58
- Prather MJ, McElroy MB, Wofsy SC (1984) Reductions in ozone at high concentrations of stratospheric halogens. *Nature* 312:227-231
- Priest FG (2009) Genus I. *Paenibacillus* Ash, Priest and Collins 1994, 852VP. In: Whitman WB, Parte AC (eds) *Bergey's manual of systematic bacteriology*, Vol. 3, Springer, New York, p 280
- Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Can J Bot* 63:179-183
- Rainey FA, Hollen BJ, Small A (2009) Genus I. *Clostridium* Prazmowski 1880, 23^{AL}. In: Whitman WB, Parte AC (eds) *Bergey's manual of systematic bacteriology*, Vol. 3, Springer, New York, pp 738-828

- Ristaino JB, Thomas W (1997) Agriculture, methyl bromide, and the ozone hole: Can we fill the gaps. *Plant Dis* 81:954-975
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425
- Sandaa RA, Torsvik V, Enger O, Daae FL, Castberg T, Hahn D (1999) Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. *FEMS Microbiol Ecol* 30:237-251
- Savant NK, Ellis R (1964) Changes in RP and phosphorus availability in submerged soil. *Soil Sci* 98: 388-394
- Schleifer KH (2009) Genus XXVI. *Zymophilus* Schleifer, Leuteritz, Weiss, Ludwig, Kirchhof and Seidel-Rüfer 1990, 26^{VP}. In: Whitman WB, Parte AC (eds) *Bergey's manual of systematic bacteriology*, Vol. 3, Springer, New York, p 1119
- Shinmura A (2000) Causal agent and control of root rot of welsh onion. *PSJ Soilborne Disease Workshop Report* 20:133-143(in Japanese with English Summary)
- Shinmura A (2004) Principle and effect of soil sterilization method by reducing redox potential of soil. *PSJ Soilborne Disease Workshop Report* 22:2-12 (in Japanese with English Summary)
- Shinmura A, Sakamoto N, Abe H (1999) Control of *Fusarium* root rot of welsh onion

- by soil reduction. Jpn J Phytopathol 65:352-353
- Spadaro D, Gullino ML (2005) Improving the efficacy of biocontrol agents against soil borne pathogens. Crop Prot 24:601-613
- Stover RH (1979) Flooding of soil for disease control. In: Mulder D (ed) Soil disinfection. Elsevier Scientific Company, Amsterdam, The Netherlands, pp 19-28
- Takehara T, Kuniyasu K (1994) Use of nitrate-nonutilizing mutants in ecological studies of Fusarium diseases. II. Isolation of nitrate non-utilizing mutants of *Fusarium oxysporum* on selective media. Ann Phytopathol Soc Jpn 60:705-710 (In Japanese with English summary)
- Takehara T, Kuniyasu K, Mori M, Hagiwara H (2003) Use of a nitrate-nonutilizing mutant and selective media to examine population dynamics of *Fusarium oxysporum* f. sp. *spinaciae* in soil. Phytopathology 93:1173-1181
- Takeuchi T (2004) Effect of sterilization by soil reduction on soil borne diseases in Chiba Prefecture. PSJ Soilborne Disease Workshop Report, 22:13-21 (In Japanese with English summary)
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res

22:4673-4680

Ueki A, Matsuda K, Ohtsuki C (1986) Sulfate reduction in the anaerobic digestion of animal waste. *J Gen Appl Microbiol* 32:111-123

Watanabe T, Cahyani VR, Murase J, Ishibasi E, Kimura M, Asakawa S (2009) Methanogenic archaeal communities developed in paddy fields in the Kojima Bay polder, estimated by denaturing gradient gel electrophoresis, real time PCR and sequence analyses. *Soil Sci Plant Nutr* 55:73-79

Wiegel J (2009) Family I. *Clostridiaceae* Pribram 1933, 90^{AL}. In: Whitman WB, Parte AC (eds) *Bergey's manual of systematic bacteriology*, Vol. 3, Springer, New York. p 737

FIGURE LEGENDS

Fig. 1. (a) DGGE patterns of bacterial populations in soil treated with *Brassica juncea* plants under different conditions. (b) Cluster analysis of DGGE band pattern for similarity index. 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 A1 and A2 are from soil samples at 30°C and 20% MC; whereas others are from samples treated at 30% MC under different temperatures: 25°C (B1, B2); 27.5°C (C1, C2); 30°C (E1, E2). The numbers appearing aside of bands indicate the band numbers and positions for performing cluster analysis. The numbering was done chronologically starting from the upper position of the left sided DGGE lane.

Fig. 2. (a) DGGE patterns for control (U1, S2, R1, Q2), *Avena*-treated (P2, N2, M2, L1), and wheat bran-treated (K1, J1, H1, G2) soil samples at different conditions. (b) Cluster analysis of DGGE band pattern for similarity index. The lanes Q2, L1, and G2 represent samples treated at 30°C with 20% MC, whereas others were treated at 30% MC under different temperatures: 30°C (U1, P2, K1); 27.5°C (S2, N2, J1); 25°C (R1, M2, H1).

Other notifications of DGGE were similar as described in Fig. 1.

Fig. 3. Phylogenetic composition (phylum or class) of bacteria based on 16S rRNA gene sequences from different clone libraries. The compositions are represented as relative abundances in relation to the percentages of number of clones belonging to each phylum or class. Clone libraries: A1, Br30-20; B2, Br25-30; E1, Br30-30; H1, Wh25-30; K1, Wh30-30; M2, Av25-30; P2, Av30-30; R1, Co25-30; U1, Co30-30; Y2, Original.














Symbols:  , *Alphaproteobacteria*;  , *Betaproteobacteria*;  , *Gamma*proteobacteria;  , *Deltaproteobacteria*;  , *Acidobacteria*;  , *Bacteroidetes*;  , *Planctomycetes*;  , *Firmicutes* (*Clostridia*);  , *Firmicutes* (*Symbiobacterium*);  , *Firmicutes* (*Bacilli*);  , *Actinobacteria*;  , *Chloroflexi*;  , Others.

Fig. 4. Neighbor-joining trees showing the phylogenetic relationships of all OTUs derived from *Brassica*-treated (30°C-30% MC) and wheat bran-treated (25°C-30% MC) soil samples 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 abbreviation *C.* indicates the genus *Clostridium*. The name of each clone starts with the pot number for each treatment (E1 for Br30-30 and

H1 for Wh25-30). 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. Abbreviations: α -, and γ -Prot, *Alpha*-, and *Gammaproteobacteria*, respectively; Actino, *Actinobacteria*; Acido, *Acidobacteria*.

Fig. 5. Rarefaction curves for the bacterial communities from all clone libraries. A1, Br30-20; B2, Br25-30; E1, Br30-30; H1, Wh25-30; K1, Wh30-30; M2, Av25-30; P2, Av30-30; R1, Co25-30; U1, Co30-30; Y2, Original.

Fig. 6. Neighbor-joining tree showing the phylogenetic relationships of clostridial clones from some effective BSD samples based on 16S rRNA gene sequences (according to the clostridial cluster analysis by Collins et al. 1994). 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 pot number for each treatment: A1, Br30-20; E1, Br30-30; H1, Wh25-30; K1, Wh30-30. 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. Tree construction and other notifications are similar as described in Fig. 4.

Fig. 1

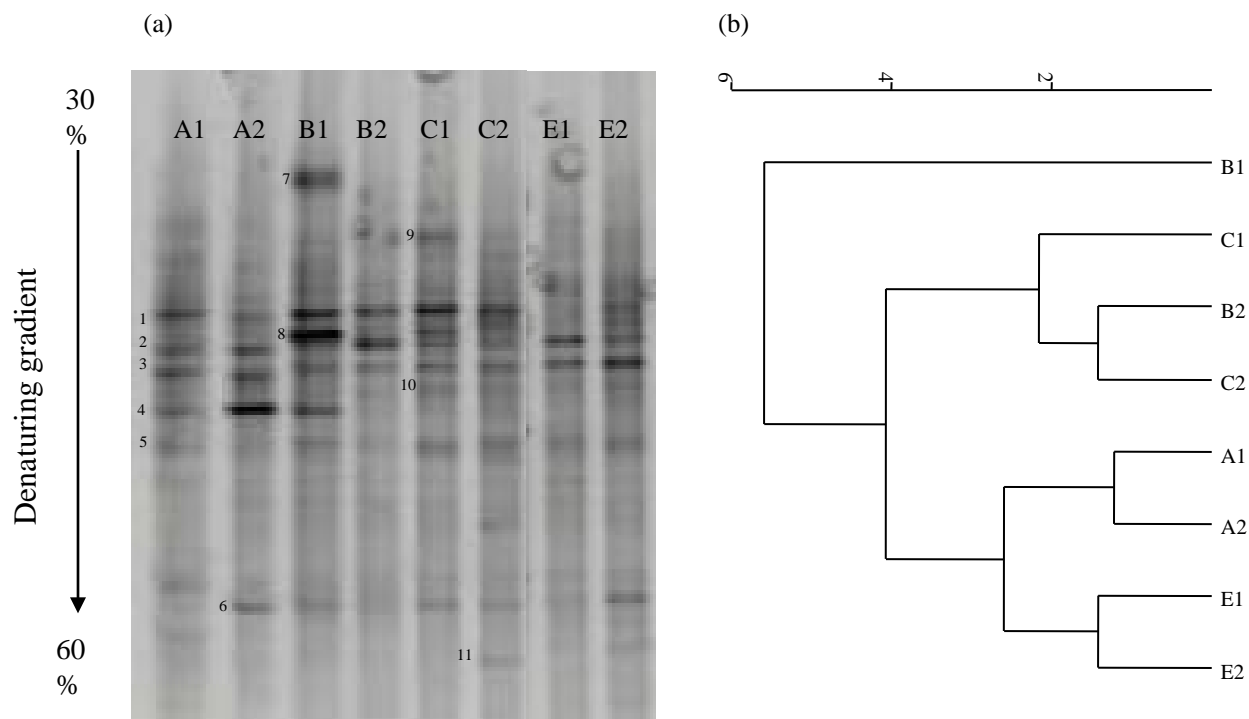


Fig. 2

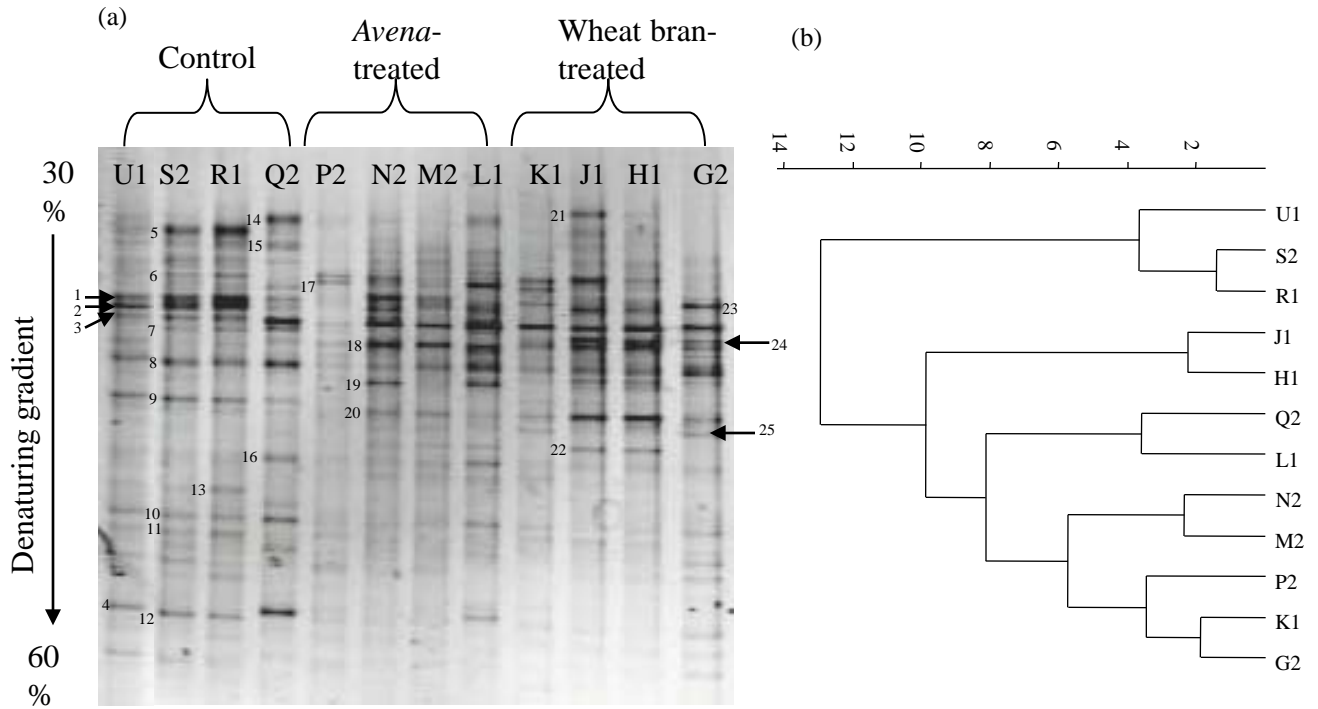


Fig. 3

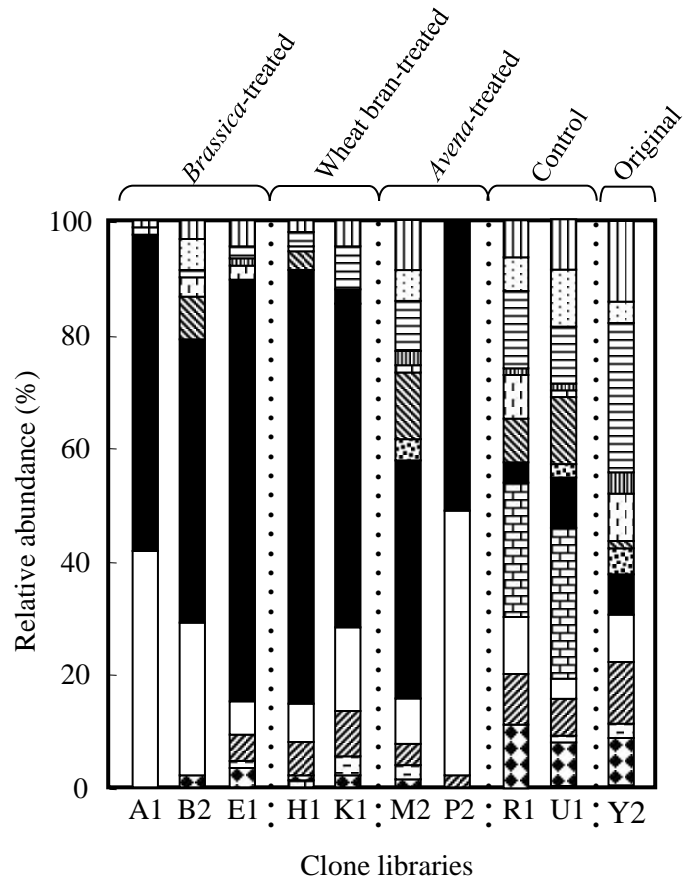


Fig. 4

Brassica-treated

Wheat bran-treated

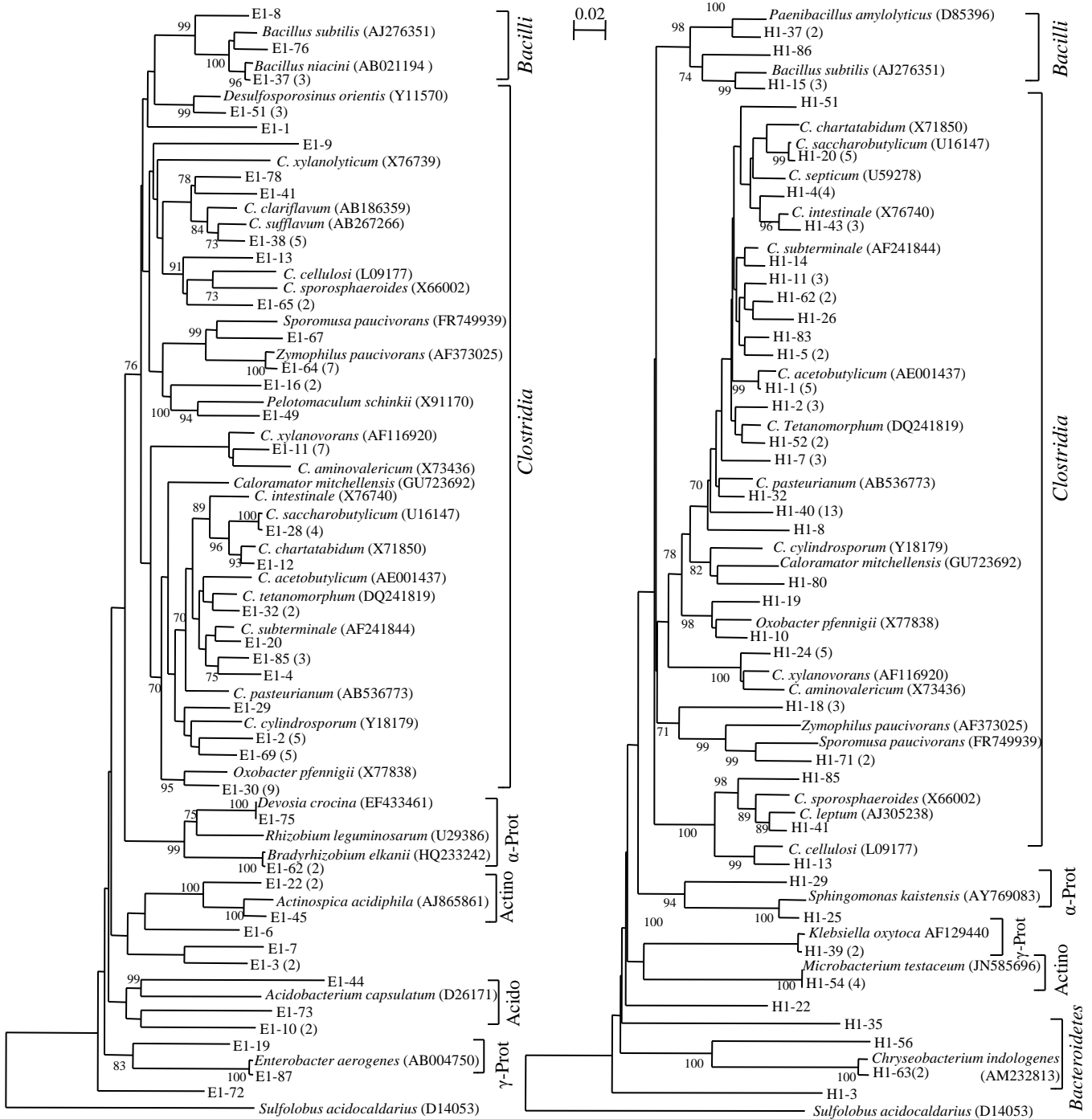


Fig. 5

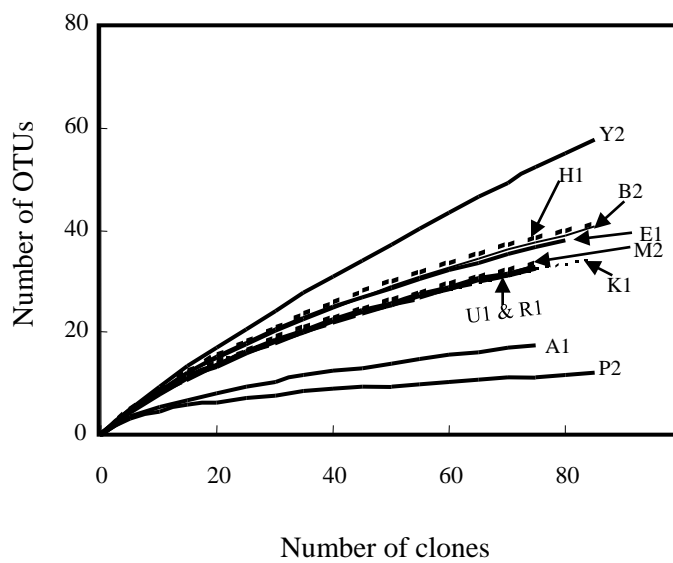


Fig. 6

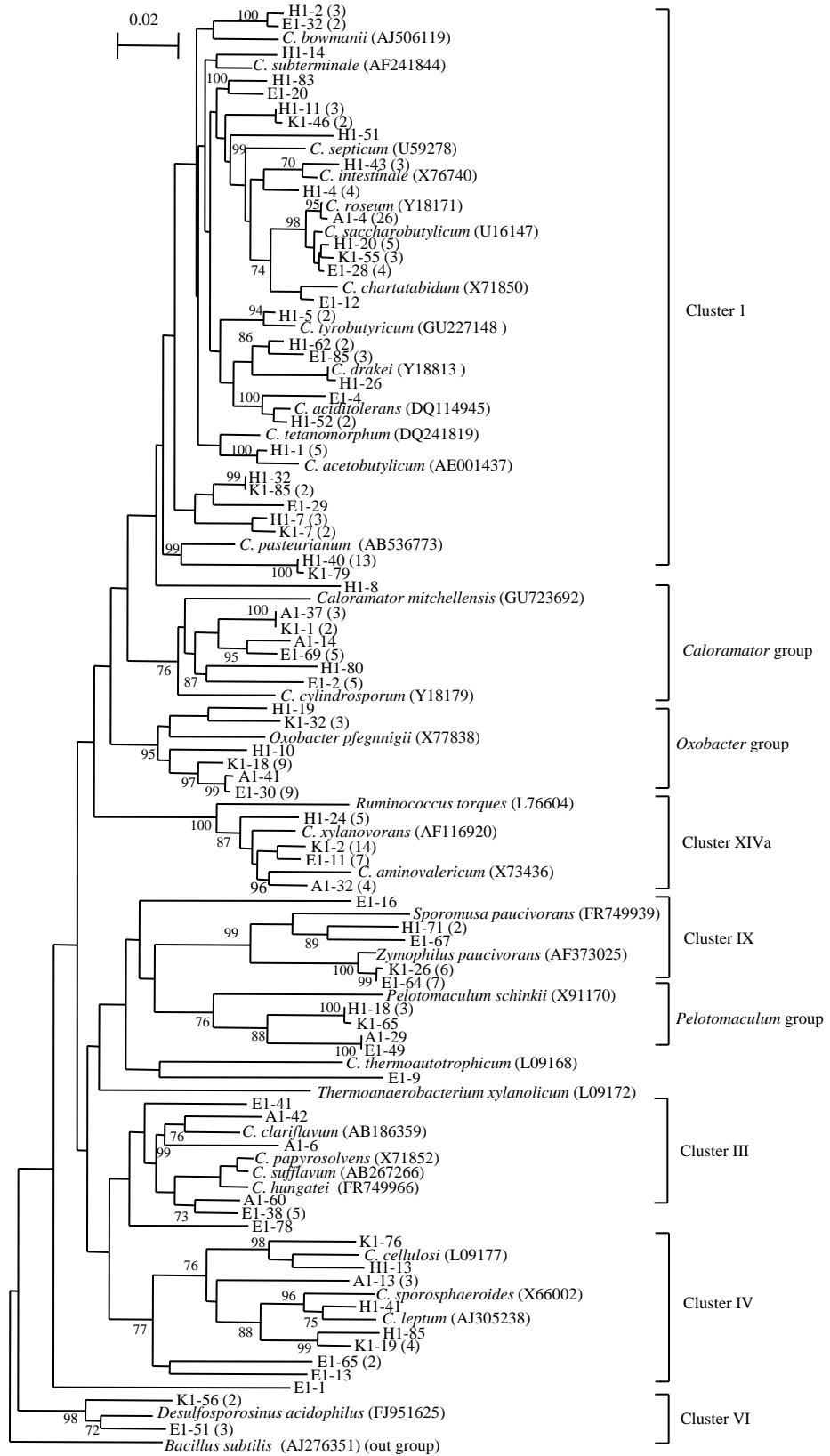


Table 1. Status of soil as affected by biomass incorporation and treatment conditions

| Designation of treatment | Pot No. (Duplicates) | Plant biomass incorporated | Incubation conditions | | After 17 days of incubation | | | | | |
|-----------------------------|----------------------|----------------------------|-----------------------|--------|---|-----------|----------------------|------------|-----------|-----------|
| | | | Temp. (°C) | MC (%) | No. of <i>Fusarium</i> (CFU/g dry soil) | ORP (mV) | Volatile fatty acids | | | Ethanol |
| | | | | | | | Acetate | Propionate | Butyrate | |
| Br30-20 | A1, 2 | <i>Brassica juncea</i> | 30 | 20 | nd | -232 ± 4 | 7.0 ± 2.0 | tr | tr | nd |
| Br25-30 | B1, 2 | | 25 | 30 | (7.75 ± 10.0) x 10 | -173 ± 16 | 5.9 ± 0.6 | 1.0 | tr | nd |
| Br27.5-30 | C1, 2 | | 27.5 | 30 | (2.70 ± 0.32) x 10 ² | -170 ± 1 | 5.2 ± 0.7 | 1.0 | tr | nd |
| Br30-30 | E1, 2 | | 30 | 30 | nd | -169 ± 6 | 8.0 ± 0.7 | tr | tr | tr |
| Wh30-20 | G1, 2 | Wheat bran | 30 | 20 | nd | -56 ± 64 | 17.3 ± 2.2 | nd | 8.1 ± 0.8 | nd |
| Wh25-30 | H1, 2 | | 25 | 30 | nd | -35 ± 105 | 14.6 ± 0.1 | nd | 6.3 ± 1.1 | nd |
| Wh27.5-30 | J1, 2 | | 27.5 | 30 | nd | -149 ± 69 | 12.1 ± 0.4 | nd | 4.8 ± 0.7 | nd |
| Wh30-30 | K1, 2 | | 30 | 30 | nd | -201 ± 7 | 13.4 ± 1.8 | nd | 5.5 ± 1.7 | 4.0 ± 0.4 |
| Av30-20 | L1, 2 | <i>Avena strigosa</i> | 30 | 20 | nd | -268 ± 4 | 8.4 ± 0.6 | tr | 1.0 | nd |
| Av25-30 | M1, 2 | | 25 | 30 | (1.02 ± 5.02) x 10 | -164 ± 16 | 6.6 ± 0.6 | tr | tr | nd |
| Av27.5-30 | N1, 2 | | 27.5 | 30 | 3.50 ± 4.90 | -176 ± 17 | 8.3 ± 0.9 | 1.0 | tr | nd |
| Av30-30 | P1, 2 | | 30 | 30 | nd | -170 ± 10 | 9.6 ± 1.9 | tr | tr | 3.2 ± 0.3 |
| Co30-20 | Q1, 2 | None (Control) | 30 | 20 | (9.50 ± 1.10) x 10 ⁴ | 318 ± 28 | nd | nd | nd | nd |
| Co25-30 | R1, 2 | | 25 | 30 | (1.50 ± 0.53) x 10 ³ | 299 ± 44 | nd | nd | nd | nd |
| Co27.5-30 | S1, 2 | | 27.5 | 30 | (1.30 ± 0.15) x 10 ³ | 263 ± 18 | nd | nd | nd | nd |
| Co30-30 | U1, 2 | | 30 | 30 | (1.40 ± 0.02) x 10 ³ | 257 ± 18 | nd | nd | nd | nd |
| Original soil (non-treated) | | Y1, Y2 ^{a)} | | | (5.04 ± 0.08) x 10 ⁵ | 403 ± 4 | nd | nd | nd | nd |

MC, moisture content; ORP, oxidation-reduction potential; nd, not detected; tr, trace.

^{a)}, Values at the start.

Table 2. Major bacterial OTUs and their closest relatives from different clone libraries

| Library | Representative clone | Number of clones | Closest relative (accession no.) | Taxonomic affiliation | | Sequence* similarity |
|---------------|----------------------|------------------|---|-----------------------|-----------------------|----------------------|
| | | | | Phylum | Class | |
| Br30-20 (A1) | A1-4 | 26 | <i>Clostridium acetobutylicum</i> (X81021) | Firmicutes | Clostridia | 99.5 |
| | | | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 98.3 |
| | A1-2 | 22 | <i>Bacillus azotoformans</i> (AB363732) | Firmicutes | Bacilli | 99.1 |
| | A1-50 | 7 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 99.3 |
| | A1-61 | 4 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 95.5 |
| | A1-13 | 3 | <i>Clostridium leptum</i> (AJ305238) | Firmicutes | Clostridia | 92.1 |
| Br25-30 (B2) | B2-9 | 13 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 99.3 |
| | B2-11 | 8 | <i>Bacillus azotoformans</i> (AB363732) | Firmicutes | Bacilli | 99.0 |
| | B2-74 | 7 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 94.3 |
| | B2-5 | 6 | <i>Clostridium cylindrosporium</i> (Y18179) | Firmicutes | Clostridia | 92.8 |
| | B2-78 | 3 | <i>Clostridium subterminale</i> (AF241844) | Firmicutes | Clostridia | 97.7 |
| | B2-28 | 3 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 95.1 |
| | B2-20 | 2 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 99.1 |
| Br30-30 (E1) | E1-30 | 9 | <i>Oxobacter pfennigii</i> (X77838) | Firmicutes | Clostridia | 91.5 |
| | E1-11 | 7 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 94.6 |
| | E1-64 | 7 | <i>Zymophilus raffinovorans</i> (DQ217599) | Firmicutes | Clostridia | 99.8 |
| | E1-38 | 5 | <i>Clostridium sufflavum</i> (AB267266) | Firmicutes | Clostridia | 93.5 |
| | E1-69 | 5 | <i>Clostridium cylindrosporium</i> (Y18179) | Firmicutes | Clostridia | 93.5 |
| | E1-2 | 5 | <i>Clostridium cylindrosporium</i> (Y18179) | Firmicutes | Clostridia | 91.9 |
| | E1-28 | 4 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 99.3 |
| | E1-68 | 3 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 93.6 |
| | E1-51 | 3 | <i>Desulfosporosinus acidophilus</i> (FJ951625) | Firmicutes | Clostridia | 94.6 |
| Wh25-30 (H1) | H1-40 | 13 | <i>Clostridium pasteurianum</i> (AB536773) | Firmicutes | Clostridia | 95.0 |
| | H1-1 | 5 | <i>Clostridium acetobutylicum</i> (AE001437) | Firmicutes | Clostridia | 98.2 |
| | H1-24 | 5 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 95.2 |
| | H1-20 | 5 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 99.0 |
| | H1-4 | 4 | <i>Clostridium intestinale</i> (X76740) | Firmicutes | Clostridia | 94.4 |
| | H1-54 | 4 | <i>Microbacterium testaceum</i> (JN585696) | Actinobacteria | Actinobacteria | 99.8 |
| | H1-7 | 3 | <i>Clostridium tetanomorphum</i> (DQ241819) | Firmicutes | Clostridia | 94.9 |
| | H1-43 | 3 | <i>Clostridium intestinale</i> (X76740) | Firmicutes | Clostridia | 96.7 |
| | H1-2 | 3 | <i>Clostridium subterminale</i> (AF241844) | Firmicutes | Clostridia | 94.7 |
| | H1-18 | 3 | <i>Desulfosporosinus acidophilus</i> (FJ951625) | Firmicutes | Clostridia | 89.6 |
| | H1-15 | 3 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 99.0 |
| Wh30-30 (K1) | K1-2 | 14 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 95.8 |
| | K1-4 | 11 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 98.4 |
| | K1-18 | 9 | <i>Oxobacter pfennigii</i> (X77838) | Firmicutes | Clostridia | 90.3 |
| | K1-26 | 6 | <i>Zymophilus raffinovorans</i> (DQ217599) | Firmicutes | Clostridia | 99.8 |
| | K1-6 | 7 | <i>Azotobacter chroococcum</i> (EF634038) | Proteobacteria | Gammaproteobacteria | 100 |
| | K1-19 | 4 | <i>Clostridium leptum</i> (AJ305238) | Firmicutes | Clostridia | 92.8 |
| | K1-55 | 3 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 98.8 |
| Av25-30 (M2) | M2-47 | 10 | <i>Clostridium cylindrosporium</i> (Y18179) | Firmicutes | Clostridia | 92.9 |
| | M2-34 | 9 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 88.7 |
| | M2-55 | 6 | <i>Prolixibacter bellariivorans</i> (AY918928) | Bacteroidetes | Bacteroidia | 89.3 |
| | M2-18 | 5 | <i>Clostridium xylanovorans</i> (AF116920) | Firmicutes | Clostridia | 96.3 |
| | M2-3 | 5 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 99.5 |
| | M2-40 | 4 | <i>Pelotomaculum schinkii</i> (X91170) | Firmicutes | Clostridia | 87.1 |
| Av30-30 (P2) | P2-81 | 32 | <i>Veillonella rogosae</i> (EF108446) | Firmicutes | Clostridia | 99.5 |
| | P2-10 | 30 | <i>Streptococcus parasanguinis</i> (GU561390) | Firmicutes | Bacilli | 99.8 |
| | P2-68 | 4 | <i>Clostridium saccharobutylicum</i> (U16147) | Firmicutes | Clostridia | 98.0 |
| Co25-30 (R1) | R1-16 | 15 | <i>Symbiobacterium thermophilum</i> (AB004913) | Firmicutes | Symbiobacterium clade | 91.2 |
| | R1-20 | 9 | <i>Rhodanobacter terrae</i> (FJ405366) | Proteobacteria | Gammaproteobacteria | 99.8 |
| | R1-5 | 7 | <i>Alicyclobacillus aeris</i> (FM179383) | Firmicutes | Bacilli | 95.5 |
| | R1-10 | 4 | <i>Bordetella petrii</i> (EF212440) | Proteobacteria | Betaproteobacteria | 97.6 |
| | R1-19 | 4 | <i>Sediminibacterium salmoneum</i> (AB682145) | Bacteroidetes | Sphingobacteria | 93.0 |
| | R1-23 | 3 | <i>Terrabacter tumescens</i> (JQ342911) | Actinobacteria | Actinobacteria | 100 |
| Co30-30 (U1) | U1-5 | 17 | <i>Symbiobacterium thermophilum</i> (AB004913) | Firmicutes | Symbiobacterium clade | 91.4 |
| | U1-14 | 6 | <i>Sphingobacterium multivorum</i> (AY787820) | Bacteroidetes | Sphingobacteria | 99.2 |
| | U1-60 | 6 | <i>Achromobacter spanius</i> (JN629043) | Proteobacteria | Betaproteobacteria | 99.6 |
| | U1-1 | 5 | <i>Sphingomonas kaistensis</i> (AY769083) | Proteobacteria | Alphaproteobacteria | 96.3 |
| | U1-35 | 5 | <i>Rhodanobacter terrae</i> (FJ405366) | Proteobacteria | Gammaproteobacteria | 99.8 |
| | U1-21 | 4 | <i>Symbiobacterium thermophilum</i> (AB004913) | Firmicutes | Symbiobacterium clade | 89.7 |
| | U1-6 | 3 | <i>Zymophilus raffinovorans</i> (DQ217599) | Firmicutes | Clostridia | 100 |
| Original (Y2) | Y2-12 | 10 | <i>Rhodanobacter terrae</i> (FJ405366) | Proteobacteria | Gammaproteobacteria | 99.5 |
| | Y2-2 | 10 | <i>Dokdonella fugitiva</i> (AJ969432) | Proteobacteria | Gammaproteobacteria | 94.9 |
| | Y2-8 | 5 | <i>Zymophilus paucivorans</i> (AF373025) | Firmicutes | Clostridia | 98.5 |
| | Y2-41 | 3 | <i>Bacillus niacini</i> (AB021194) | Firmicutes | Bacilli | 99.6 |
| | Y2-62 | 3 | <i>Sphingomonas azotifigens</i> (AB680881) | Proteobacteria | Alphaproteobacteria | 92.5 |
| | Y2-84 | 2 | <i>Sphingomonas azotifigens</i> (AB680881) | Proteobacteria | Alphaproteobacteria | 99.8 |

*, Sequence similarity (%) of 16S rRNA gene sequence between each representative clone and the closest relative.

Table 3. Estimates of richness and diversity of bacterial community for each soil sample

| Soil sample | A1 | B2 | E1 | H1 | K1 | M2 | P2 | R1 | U1 | Y2 |
|--------------------------------|------|------|------|------|------|------|------|------|------|------|
| No. of total clones | 79 | 89 | 87 | 88 | 89 | 76 | 82 | 80 | 79 | 82 |
| No. of total OTUs | 18 | 42 | 39 | 38 | 32 | 34 | 13 | 34 | 34 | 49 |
| No. of phyla | 3 | 5 | 6 | 7 | 5 | 8 | 2 | 7 | 9 | 9 |
| Coverage (%) | 87.3 | 71.9 | 74.7 | 76.1 | 82.0 | 76.3 | 91.4 | 76.2 | 73.4 | 57.3 |
| Richness (Chao 1) | 318 | 380 | 468 | 270 | 386 | 272 | 40 | 667 | 163 | 996 |
| Shimpson's diversity index | 0.80 | 0.96 | 0.96 | 0.96 | 0.94 | 0.95 | 0.75 | 0.94 | 0.93 | 0.96 |
| Shannon-Wiener diversity index | 2.99 | 4.86 | 4.85 | 4.84 | 4.40 | 4.45 | 2.50 | 4.47 | 4.42 | 5.01 |

Soil sample: A1, Br30-20; B2, Br25-30; E1, Br30-30; H1, Wh25-30; K1, Wh30-30; M2, Av25-30; P2, Av30-30; R1, Co25-30; U1, Co30-30; Y2, Original.

Supplementary material:

Name of journal: Applied Microbiology and Biotechnology

Title: Proliferation of diversified clostridial species during biological soil disinfection incorporated with plant biomass under various conditions

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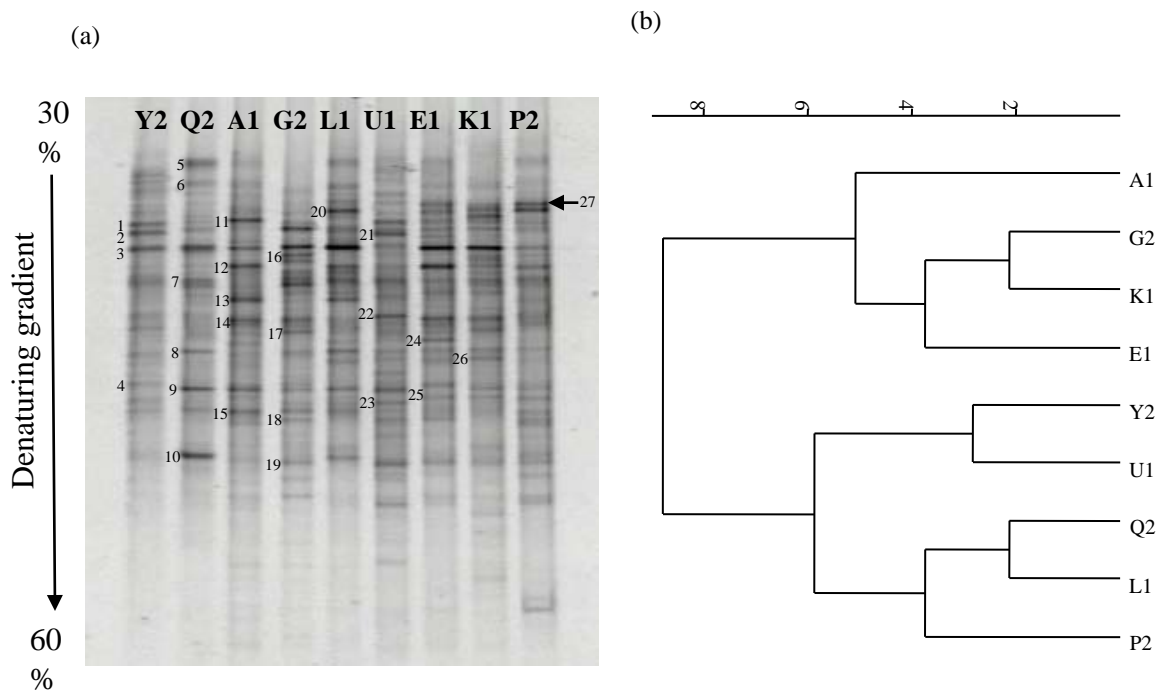
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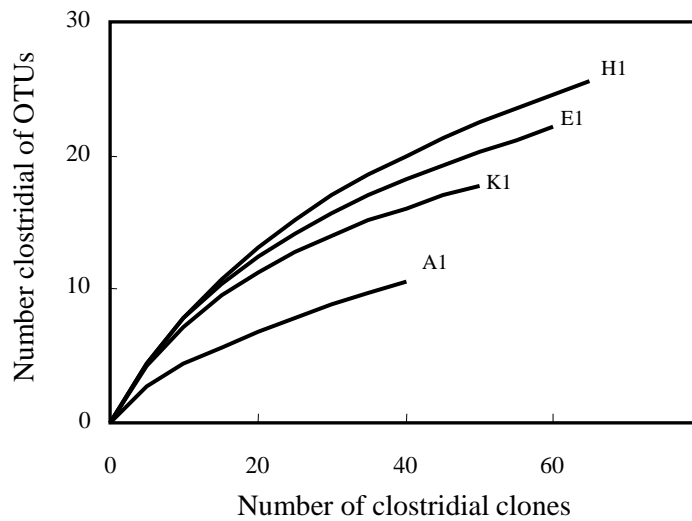
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Supplementary Fig. 1



Supplementary Fig. 1. (a) DGGE patterns for the original (non-treated) and the treated soil samples. (b) Cluster analysis of DGGE band pattern for similarity index. The lane Y2 indicates for the original soil, whereas other lanes for the treated soil at 30°C under different MC: control (Q2 for 20%, U1 for 30%), *Brassica*-treated (A1, E1) wheat bran-treated (G2, K1), and *Avena*-treated soils (L1, P2). Other notifications of DGGE were similar as described in Fig. 1.

Supplementary Fig. 2



Supplementary Fig 2. Rarefaction curves for the clostridial groups of the libraries of soils that effectively suppressed the pathogen. Clone library: A1, Br30-20; E1, Br30-30; H1, Wh25-30; K1, Wh30-30.

Supplementary Table 1. Estimate of richness and diversity of clostridial community for the soil samples suppressed the pathogen effectively

| Soil sample | A1 | E1 | H1 | K1 |
|---------------------------------|------|------|------|------|
| No. of total clostridial clones | 42 | 64 | 67 | 52 |
| No. of total clostridial OTUs | 10 | 23 | 26 | 14 |
| Coverage (%) | 83.3 | 82.8 | 80.6 | 94.2 |
| Chao1 (richness) | 114 | 281 | 176 | 197 |
| Simpson's diversity index | 0.61 | 0.94 | 0.94 | 0.88 |
| Shannon-Wiener diversity index | 2.07 | 4.06 | 4.23 | 3.30 |

Soil sample: A1, Br30-20; E1, Br30-30; H1, Wh25-30; K1, Wh30-30.