A manuscript for Journal of Bioscience and Bioengineering. (Regular paper)

Title: Analysis of a change in bacterial community in different environments with addition of chitin or chitosan

Running title: Change of bacterial community after chitin addition

Authors: Kazuaki Sato[1], Yasuhito Azama[1], Masahiro Nogawa[1], Goro Taguchi[1], and Makoto Shimosaka[1*]

Affiliation: Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan[1]

Key words: bacterial community, PCR-DGGE, 16S rRNA gene, chitin, chitosan, unculturable bacteria

[*]Corresponding author. e-mail: mashimo@shinshu-u.ac.jp
phone/fax +81-(0)268-21-5341

Abstract

The temporal changes of a bacterial community in soil with chitin or chitosan added were analyzed by PCR-denaturing gradient gel electrophoresis (DGGE) targeting the 16S rRNA gene using total DNAs prepared from the community. Band patterns of PCR-DGGE confirmed that 31 species become predominant after the addition of chitin or chitosan. The determination of the nucleotide sequences of the bands of the 31 species indicated that 20 species belonged to the division Proteobacteria, and that the genus *Cellvibrio* was apparently predominant among them (7/20). The 16S rRNA sequences of the 16 deduced species (16/31) showed less than 98% similarities to those of previously identified bacteria, indicating that the species were derived from unidentified bacteria. The total community DNAs extracted from bacterial cells adsorbed on the surface of flakes of chitin and chitosan placed in a river, a moat, or soil were subjected to PCR-DGGE to examine the extent of diversity of chitinolytic bacteria among different environments. The predominant species significantly differed between the chitin and chitosan placed in the river and moat, but not so much between those placed in the soil. The large difference between the diversities of the three bacterial communities indicated that a wide variety of bacteria including unidentified ones are involved in the degradation of chitin and chitosan in the above-mentioned natural environments.

Introduction

Chitin, a linear polysaccharide constituting of β-1,4-linked *N*-acetyl-$_D$-glucosamine, is widely distributed in nature, such as in the exoskeletons of crustaceans, insects, and mollusks and in the cell walls of fungi (1, 2). More than $1x10^{11}$ tons of chitin is synthesized annually on the earth, and it is the most abundant biomass next to cellulose. Chitosan, a deacetylated derivative of chitin, exists in the cell walls of a limited group

of fungi (e.g., the genera *Rhizopus*, *Absidia*, and *Fusarium*) in nature (3, 4). Chitosan is industrially produced by the chemical *N*-deacetylation of chitin using a strong base. Chitin, chitosan, and their oligomers have attracted considerable attention because of their various biological properties, and are widely used in various fields such as health care, food industry, agriculture, chemical industry, and environmental engineering (5-8).

In agriculture, crab- or shrimp-shell-derived chitin has been used as a soil conditioner over many years (9, 10). Chitin addition reduces the population of fungal plant pathogens in soil, resulting in an increase in crop yield. This favorable effect can be postulated as follows. First, chitin added to soil activates the growth of soil-borne chitinolytic bacteria. These bacteria secrete chitinolytic enzymes to degrade and utilize chitin. These chitinolytic enzymes attack chitin contained in cell walls of plant pathogenic fungi, and then impair the growth of fungi living in soil (11, 12). As a consequence, the population of soil-borne plant pathogens is so markedly reduced that the onset of plant diseases can be suppressed. During this process, chitin oligomers are produced by chitinolytic enzymes and accumulate in soil. Chitin oligomers have an elicitor activity, which induces a defense mechanism in plants against a wide range of plant pathogens (13, 14). In this scenario, communities of soil-borne chitin-degrading bacteria play an important role.

Over 99% of microorganisms in nature are difficult to culture in the laboratory by conventional culture methods (15-17). Although a large number of chitin-degrading bacteria have been isolated from soil to date, there must still be a limited portion of yet unidentified greatly diverse chitinolytic bacteria actually living in soil. To fully understand the effect of chitin added to soil, a microbial community including unculturable microorganisms must be investigated comprehensively. Environmental DNA, prepared from whole microbial cells living in different environments without cultivation, has recently been used to obtain an overview of the structure of a microbial community such as the diversity and proportion of constituent microorganisms (18, 19).

For this purpose, the nucleotide sequences of 16S ribosomal RNA (rRNA) genes are often used to reveal the structure of a bacterial community since the genes are conserved in all bacteria and give helpful criteria for the elucidation of phylogenetic relationships among bacteria (20, 21).

In this article, we report the effect of chitin addition on the diversity of a bacterial community in soil, which would explain how chitin works as a soil conditioner. Chitosan, a deacetylated derivative of chitin, was also examined for its effect when added to soil. Furthermore, bacterial communities grown on the surface of flakes of chitin or chitosan placed in soil, a river, and a moat were analyzed similarly to determine the extent of diversity of chitinolytic bacteria among different environments.

Materials and Methods

Placing of chitin and chitosan in natural environments

Flakes of chitin (degree of acetylation, 94%) and chitosan (degree of acetylation, 38%) were kindly gifted by Kyowa Technos Co., Ltd., Chiba, Japan. They were filtered using a 2 mm mesh sieve to remove small particles less than 2 mm in diameter. Then, the flakes (3 kg each) were forked in soil (1 meter square, 30 cm in depth) in the test locations in an experimental farm of the Faculty of Textile Science and Technology, Shinshu University. At the same time, nylon nets containing 5 g each of flakes of chitin or chitosan were buried in soil at the test locations. The test was started in May 2006, and soil samples were collected at one to two week intervals until November, 2006. The collected soil samples were passed through a 2 mm mesh sieve, and stored at -20°C until use. Samples were also collected from untreated soil near the test locations as a control. The nylon nets were sequentially retrieved, and then the flakes of chitin or chitosan inside them were washed with tap water, dried at 70°C for 4 hr, and weighed.

Similarly, nylon nets were also placed in a stream of the Chikuma River, Ueda, Japan, in the moat of Ueda Castle, Japan, and in the experimental farm. The flakes were sequentially recovered, washed with tap water, and stored at -20°C until use.

Preparation of environmental DNA

Environmental DNA was prepared from soil samples using a DNA isolation kit, ISOIL for Beads Beating (Nippon Gene Co., Ltd., Tokyo), in accordance with the manufacturer's instructions. To prepare total DNA from microbial cells tightly bound on the surface of flakes of chitin or chitosan, 2 g of flakes was suspended in 4 ml of lysis buffer (100 mM Tris-Cl pH 8.0, 100 mM EDTA, 100 mM NaCl, 10% SDS), vortexed vigorously, and incubated at 70°C for 30 mins. Then, the sample was immediately frozen in liquid nitrogen, and successively thawed at 70°C. This freeze-thawing step was repeated three times. Then, a clear lysate was recovered with centrifugation (15,000 rpm, 20 min), and extracted with chloroform:isoamyl alcohol (24:1). After adding of 0.1 volumes of 3 M sodium acetate, total DNAs were precipitated with an equal volume of isopropanol, washed with 70% (v/v) ethanol, and dissolved in TE buffer.

PCR primers

The set of oligonucleotides used for PCR amplification of the eubacterial 16S rRNA gene consisted of a forward primer (5'-AACGCGAAGAACCTTAC-3') and a reverse primer (5'-CGGTGTGTACAAGGCCC-3'), which was designed for amplifying the fragment including the variable regions V6, V7, and V8 of the 16S rRNA gene (22, 23). When the amplified fragments were used in denaturing gradient gel electrophoresis (DGGE), a 40-bp GC-rich clamp (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3') was added at the 5'-end of the forward primer to prevent the complete denaturation of the PCR products on the DGGE gel.

PCR

PCR amplification was performed with a PTC-100 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., CA, USA). The standard reaction mixture (10 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100, 0.25 μM each of the primers, 0.05 mM each of the dNTPs, 25 U/ml rTaq DNA polymerase, and 0.01% bovine serum albumin. An initial denaturation step of 4 min at 96°C was followed by 35 cycles of amplification (1 min at 96°C, 1 min at 62°C, and 1 min at 72°C), and a final elongation step of 4 min at 72°C.

DGGE

A DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc.) was used for DGGE. The PCR products were separated on 16-cm-long polyacrylamide gels (8% [w/v] acrylamide:bisacrylamide at 37.5:1) with a 40% to 60% denaturant gradient (7 M urea and 40% [v/v] formamide were regarded as 100% denaturant). Electrophoresis was performed in TAE buffer (40 mM Tris-Cl (pH 8.0), 20 mM acetic acid, 1 mM EDTA) at 60°C and 200 V (constant voltage) for 360 min using Power Pac 300 (Bio-Rad Laboratories, Inc.) as an electric power supply. After the electrophoresis, the gels were stained for 60 min in TAE buffer containing Gelstar Nucleic Acid Stain (Takara Bio Inc., Shiga), and then fluorescent bands were photographed using a Storm 860 Gel and Blot Imaging System (GE Healthcare Ltd., CT, USA).

Determination of nucleotide sequences

Sequencing reactions were performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). DNA fragments recovered from each band of the DGGE gels were reamplified by PCR using a primer set without a GC clamp. The PCR

products were directly used as templates for sequencing reactions.

Phylogenetic analysis

To infer the taxonomic position of the bacterial strains to which the determined sequences of the 16S rRNA gene should be ascribed, the sequences were subjected to BLAST search at the website of DDBJ (http://blast.ddbj.nig.ac.jp/top-j.html). The 16S rRNA gene sequences of related taxa were retrieved from the DDBJ databases. To construct multiple alignments, the ClustalX2 program (24) was used, and alignment positions with gaps and unidentified bases were excluded with the BioEdit program (25). A phylogenetic tree was constructed by the neighbor-joining method using the Neighbor program in PHYLIP (PHYLogeny Inference Package). Branching patterns of the tree were evaluated by bootstrapping with 1,000 resamplings, and evolutionary distance was computed using Kimura's 2-parameter model. The tree was illustrated using the TreeExplorer program (26).

Results

Addition of chitin and chitosan to soil

A large number of chitinolytic bacteria belonging to a wide range of genera have been isolated from soil to date. To assess an actual population of chitinolytic bacteria in soil, we analyzed the variation in a bacterial community caused by chitin or chitosan addition. Flakes of chitin or chitosan were forked in soil at the test locations (1% by weight) as described in Materials and Methods. Nylon nets containing flakes of chitin or chitosan were buried at the same test locations to monitor their degradation rates. As a result, both chitin and chitosan decreased by 60% in weight for the experimental period of 180 days (from May to November 2006) (Fig. 1). This decrease could be ascribed to

microbial degradation since both chitin and chitosan are not soluble in water, and the flakes used in this experiment (more than 2 mm in average diameter) could not pass through the nylon net.

Analysis of diversity of bacterial community by PCR-DGGE

Total DNAs of a microbial community were prepared from soil samples that were temporally collected from the test locations. DNA fragments (approximately 470 bp in length) corresponding to a portion of a eubacterial 16S rRNA gene including the variable regions V6, V7, and V8 were PCR-amplified (22, 23). The addition of bovine serum albumin (BSA) to the PCR mixture at a concentration of 0.01% was effective for obtaining a uniform amount of amplified products, since they could not often be amplified, possibly because unknown impurities from the soil might inhibit the reaction (27).

The amplified PCR products were separated by DGGE based on differences in their nucleotide sequences (Fig. 2). In the untreated soil, no significant change in the pattern of the bands was observed throughout the test period. This result indicates that the population of bacterial community in soil from the test location was very stable despite the seasonal change from spring to fall. On the other hand, the addition of either chitin or chitosan resulted in more bands with higher fluorescence intensities. They were likely to be derived from bacterial species that become predominant in soil with chitin or chitosan added through the degradation and utilization of these polysaccharides. Note that some distinct bands appeared at the early stage of the experimental period (28 days after chitin or chitosan addition) but others appeared at the late stage (84 days after addition). This result may explain the complicated interaction among bacterial species that appeared predominantly, and some of them could have grown as a result of secondary effects such as the accumulation of oligosaccharides produced from chitin and chitosan by the action of bacterial species appearing at the early stage.

Phylogenetic analysis of bacterial species predominant in soil with chitin or chitosan added

Nucleotide sequences of partial 16S rRNA genes were determined using the DNA fragments extracted from the 31 distinct bands (16 fragments from chitin and 15 fragments from chitosan) on the DGGE gel. To determine the bacterial species from which the 16S rRNA gene fragments originated, a BLAST search was carried out using the determined sequences as query sequences (Table 1). Of the 31 species deduced from the 16S rRNA sequences, 23 (74%) are estimated to belong to the phylum Proteobacteria (the class γ-Proteobacteria was the most prevalent, 20/23). Note that species belonging to the genus *Cellvibrio* were most abundant (7 species). *Cellvibrio mixtus*, the type species of the genus *Cellvibrio*, was previously reported to possess various glycoside hydrolases including chitinase (28), although no species of *Cellvibrio* have been reported to produce chitosan-degrading enzymes. When the deduced species were compared between the soil samples with chitin and chitosan added, they were almost similar at the phylum level.

As for the taxonomic identification of bacterial strains, generally, the phylogenetic definition of a species would include strains with approximately 70% DNA-DNA relatedness or greater (29), and this threshold value corresponds to 98.7-99.0% similarity in the case of 16S rRNA gene sequences (30). By applying this criterion to the 31 sequences of 16S rRNA genes obtained in this study, the 16 sequences (52%) showed similarities of less than 98.7% to any known bacterial species deposited in the public database. This indicates that they are derived from unidentified bacterial species, which might be unculturable.

Phylogenetic relationships of the 31 deduced species were investigated on the basis of the 16S rRNA sequences in comparison with those of the type strains of the related genera deposited in the database. A neighbor-joining phylogenetic tree inferred from the

sequences is shown in Fig. 3. The species with the sequences showing similarities of more than 94% to those of known bacterial species were located in the cluster composed of these relatives. In contrast to this, the three species with the sequences i05, o10, and o12 with lower similarities (91-92%) were located relatively far from the clusters composed of the most closely related genera *Chondromyces* (class δ-Proteobacteria), *Devosia* (class α-Proteobacteria), and *Streptacidiphilus* (phylum Actinobacteria), respectively. These three species were considered to be novel species of a novel genus. The species with the sequence i16 showed a similarity of 91% to *Methylococcus capsulatus* UNIQEM1^T belonging to the class γ-Proteobacteria. It was distantly located from the most closely related strain *M. capsulatus*, suggesting that the species is an unidentified bacterium that can form a new taxon at a level higher than the genus.

The PCR-DGGE revealed that several or more bacterial species became predominant in the soil with chitin or chitosan added; however, not all of these species might be involved in the degradation of chitin and chitosan since some of the species appeared at the later stage of the experimental period, as described before. Flakes of chitin or chitosan in the nylon bags gradually changed color during the experimental period. This change in color would explain the presence of bacterial cells tightly bound on the surface of the flakes and would be directly involved in degradation. To determine the extent of variety of chitinolytic bacteria among different environments, the diversity of a bacterial community bound on the surface was examined by PCR-DGGE similarly using flakes of chitin and chitosan placed in soil, a river, and a moat.

Analysis of bacterial communities bound on the surface of flakes of chitin and chitosan

Nylon nets containing flakes of chitin or chitosan were placed in a river, a moat, and soil. They were collected at appropriate intervals, washed with tap water, and used for preparing total DNAs from microbial cells bound on the surface. During this examination period, flakes of chitin and chitosan gradually changed color from coral

pink or yellow to sepia, and lost weight (data not shown). The degradation rates of chitin and chitosan in the hydrosphere (the river and moat) were much higher than that in the soil, since most of the chitin and chitosan disappeared within 2 months of the examination period.

PCR-DGGE of 16S rRNA genes was performed on the bacterial communities tightly bound to the surface of flakes of chitin and chitosan that were placed in three different environments. When the band patterns of DGGE gels were compared between chitin and chitosan, common bands appeared at the early stage (on days 1-2 in the hydrosphere and on day 14 in soil) of the experimental period (data not shown). Interestingly, this phenomenon was observed in all three environments. Sequence analysis of the common bands revealed that most of them were derived from bacterial species belonging to the class γ-Proteobacteria, although the sequences (hence deduced species) differed among the three environments. They might be bacterial strains capable of easily binding to any surface of solid materials rather than degrading both chitin and chitosan. Throughout the experimental period (on days 7-52 in the hydrosphere and on days 14-80 in the soil), new bands specific to either chitin or chitosan appeared in each of the three environments. These bands corresponded to bacterial species that could degrade and utilize either chitin or chitosan. Results of the phylogenetic analysis of the bacterial species deduced from 16S rRNA sequences from each of the three environments are summarized below.

In the flakes placed in the soil, seven species were detected from chitin (Si1-Si7) and seven species from chitosan (So1-So7) (Table 2). Thirteen species were estimated to belong to the class γ-Proteobacteria, while only one species (So7) to the class α-Proteobacteria. Six species (Si1, Si2, Si5, So1, So2, and So5) were considered to be closely related to known species since the similarities of their 16S rRNA sequences were more than 98.0% to those of known bacterial species deposited in the database. Hence, the remaining eight species (57%) could be unidentified species including

unculturable ones.

In the flakes placed in the river, seven species were detected from chitin (Ri1-Ri7) and six species from chitosan (Ro1-Ro6) (Table 2). Of interest is that the constituents of the community were totally different at the phylum level between chitin and chitosan. Six species (Ri1-Ri5 and Ri7) obtained from chitin were considered to belong to the phylum Firmicutes, while five species (Ro2-Ro6) obtained from chitosan to the class γ- or δ-Proteobacteria. Ten (Ri1-Ri6 and Ro1-Ro4) of the thirteen species (77%) were likely to be unidentified ones, as judged from the threshold of 16S rRNA sequence similarity (98.0%).

In the flakes placed in the moat, seven species (Mi1-Mi7) were detected from chitin and six species (Mo1-Mo6) from chitosan (Table 2). The species of each community markedly differed at the phylum level. The chitin community was composed of the phyla Firmicutes (Mi1-Mi3), α- or β-Proteobacteria (Mi4-Mi6), and Actinobacteria (Mi7), whereas the chitosan community was composed of γ- or α-Proteobacteria (Mo1-Mo4), Firmicutes (Mo5), and Cyanobacteria (Mo6). Eleven (except Mi5 and Mi7) of the thirteen species (85%) could be unidentified species. Note that the five species (Mi1, Mi2, Mo2, Mo3, and Mo5) showed less than 90% similarities, suggesting that they are strains classified under novel genera.

Discussion

In this study, we identified bacterial species that had become predominant after chitin or chitosan addition in three environments (soil, a river, and a moat) by PCR-DGGE targeting 16S rRNA genes. The resulting view is expected to reflect the actual bacterial community more correctly than those obtained by other methods dependent on cultivation, although we cannot avoid some biases caused by the preparation of total community DNA and PCR amplification (31-33).

Most bacterial species predominant in soil with chitin or chitosan added, and those tightly bound on the surface of chitin or chitosan buried in soil belong to the class γ-Proteobacteria (Table 1). This result implies that the species in the γ-Proteobacteria must play an important role in chitin degradation in soil. We screened microbial strains capable of utilizing chitin as a carbon source from the same soil samples by directly spreading microbial cells washed out from soil on synthetic agar medium containing colloidal chitin. As a result, all eight strains isolated were members of the phylum Actinobacteria (consisting of the genera *Streptomyces* and *Amycolatopsis*) (data not shown). In this work, the species belonging to the phylum Actinobacteria accounted for only a small percentage of the deduced predominant species in the tested soil (Table 1), although they have been well known as a representative decomposer of chitin in soil (34-38). Of the deduced species belonging to γ-Proteobacteria, the genus *Cellvibrio* was most predominant in the early phase of the experimental period in the tested soil for both chitin and chitosan (Table 1). We are now trying to isolate *Cellvibrio* species that were predominant in the tested soil to clarify their potential activity for the degradation of chitin and chitosan.

Bacterial species tightly bound on the surface of the flakes markedly differed between chitin and chitosan that were placed in the river and moat (Table 2). In the hydrosphere, species belonging to the phylum Firmicutes were predominant on chitin flakes, while those belonging to the phylum Proteobacteria were predominant on chitosan flakes (Table 2). In nature, chitosan (a deacetylated derivative of chitin) is found only in the cell walls of a limited group of fungi belonging to the phylum Zygomycota, while chitin is distributed in various organisms (1-4). This marked difference in distribution may explain why a wider variety of chitin-degrading bacteria than of chitosan-degrading bacteria could exist in various environments.

Most of the deduced bacterial species tightly bound on the surface of flakes of chitin or chitosan in the river and moat showed low similarities (less than 98.0%) when

their 16S rRNA sequences were compared with those of known bacterial species (Table 2). Most of them are likely to be unidentified species, which might be unculturable. Traditional methods depending on cultivation are insufficient to fully understand the actual structure and function of a microbial community, leading to the circulation of organic and inorganic compounds in natural environments.

In this work, we report the possible participation of a wide variety of bacterial species including a large number of unidentified ones in chitin and chitosan degradation in natural environments. The analysis of this bacterial community will be helpful in elucidating the actual process of chitin or chitosan degradation. Moreover, it can result in the isolation of genes coding for a novel type of chitinolytic enzymes from total community DNAs, which can be applicable to the efficient degradation of biomass chitin.

Acknowledgments

This work was supported by a Grant-in-Aid from the Global COE Program of the Ministry of Education, Culture, Sports, Science and Technology, and by The Institute for Fermentation, Osaka (IFO) Research Grant. We are indebted to the Division of Gene Research of the Research Center for Human and Environmental Sciences, Shinshu University, for providing facilities. We thank Ms. Ritsuko Kodaira, Ms. Kimi Kashiwagi, Ms. Eriko Takahashi, Ms. Ayabi Fukamachi, and Mr. Yuichi Kato for their technical assistance.

References

1. **Tharanathan, R. N. and Kittur, F. S.**: Chitin--the undisputed biomolecule of great potential. Crit. Rev. Food Sci. Nutr., **43**, 61-87 (2003).

2. **Synowiecki, J. and Al-Khateeb, N. A.**: Production, properties, and some new applications of chitin and its derivatives. Crit. Rev. Food Sci. Nutr., **43**, 145-171 (2003).

3. **Chandy, T. and Sharma, C. P.**: Chitosan--as a biomaterial. Biomater. Artif. Cells Artif. Organs, **18**, 1-24 (1990).

4. **Raafat, D., von Bargen, K., Haas, A., and Sahl, H. G.**: Insights into the mode of action of chitosan as an antibacterial compound. Appl. Environ. Microbiol., **74**, 3764-3773 (2008).

5. **Cohen-Kupiec, R. and Chet, I.**: The molecular biology of chitin digestion. Curr. Opin. Biotechnol., **9**, 270-277 (1998).

6. **Ueno, H., Mori, T., and Fujinaga, T.**: Topical formulations and wound healing applications of chitosan. Adv. Drug Deliv. Rev., **52**, 105-115 (2001).

7. **Masotti, A. and Ortaggi, G.**: Chitosan micro- and nanospheres: fabrication and applications for drug and DNA delivery. Mini Rev. Med. Chem., **9**, 463-469 (2009).

8. **Alves, N. M. and Mano, J. F.**: Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications. Int. J. Biol. Macromol., **43**, 401-414 (2008).

9. **Kobayashi, D. Y., Reedy, R. M., Bick, J., and Oudemans, P. V.**: Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. Appl. Environ. Microbiol., **68**, 1047-1054 (2002).

10. **Hjort, K., Lembke, A., Speksnijder, A., Smalla, K., and Jansson, J. K.**: Community structure of actively growing bacterial populations in plant pathogen suppressive soil. Microb. Ecol., **53**, 399-413 (2007).

11. **Dahiya, N., Tewari, R., and Hoondal, G. S.**: Biotechnological aspects of chitinolytic enzymes: a review. Appl. Microbiol. Biotechnol., **71**, 773-782 (2006).

12. **Waniska, R. D., Venkatesha, R. T., Chandrashekar, A., Krishnaveni, S., Bejosano, F. P., Jeoung, J., Muthukrishnan, S., and Liang, G. H.**: Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold. J. Agric. Food Chem., **49**, 4732-4742 (2001).

13. **Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N.**: Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc. Natl. Acad. Sci. U. S. A., **103**, 11086-11091 (2006).

14. **Ebel, J.**: Oligoglucoside elicitor-mediated activation of plant defense. Bioessays, **20**, 569-576 (1998).

15. **Riesenfeld, C. S., Schloss, P. D., and Handelsman, J.**: Metagenomics: genomic analysis of microbial communities. Annu. Rev. Genet., **38**, 525-552 (2004).

16. **Singh, J., Behal, A., Singla, N., Joshi, A., Birbian, N., Singh, S., Bali, V., and Batra, N.**: Metagenomics: Concept, methodology, ecological inference and recent advances. Biotechnol. J., **4**, 480-494 (2009).

17. **Rappe, M. S. and Giovannoni, S. J.**: The uncultured microbial majority. Annu. Rev. Microbiol., **57**, 369-394 (2003).

18. **Schloss, P. D. and Handelsman, J.**: Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. Genome Biol., **6**, 229 (2005).

19. **Rajendhran, J. and Gunasekaren, P.**: Strategies for accessing soil metagenome for desired applications. Biotechnol. Adv., **26**, 576-590 (2008).

20. **Streit, W. R. and Schmitz, R. A.**: Metagenomics--the key to the uncultured microbes. Curr. Opin. Microbiol., **7**, 492-498 (2004).

21. **Rodriguez-Valera, F.**: Approaches to prokaryotic biodiversity: a population genetics perspective. Environ. Microbiol., **4**, 628-633 (2002).

22. **Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S., and De Wachter, R.**: Comparison of small ribosomal subunit RNA structures. Nucleic Acids Res., **21**,

3025-3049 (1993).

23. **Woodson, S. A. and Leontis, N. B.**: Structure and dynamics of ribosomal RNA. Curr. Opin. Struct. Biol., **8**, 294-300 (1998).

24. **Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G.**: The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., **25**, 4876-4882 (1997).

25. **Hall, T. A.**: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser., **41**, 95-98 (1999).

26. **Kumar, S., Tamura, K., Jakobsen, I. B., and Nei, M.**: MEGA2: molecular evolutionary genetics analysis software. Bioinformatics, **17**, 1244-1245 (2001).

27. **Rohland, N. and Hofreiter, M.**: Comparison and optimization of ancient DNA extraction. Biotechniques, **42**, 343-352 (2007).

28. **Wynne, E. C. and Pemberton, J. M.**: Cloning of a gene cluster from *Cellvibrio mixtus* which codes for cellulase, chitinase, amylase, and pectinase. Appl. Environ. Microbiol., **52**, 1362-1367 (1986).

29. **Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Truper, H. G.**: Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol., **37**, 463-464 (1987).

30. **Stackebrandt, E. and Ebers, J.**: Taxonomic parameters revisited: tarnished gold standards. Microbiology Today, **33**, 152-155 (2006).

31. **Frostegard, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X., and Simonet, P.**: Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol., **65**, 5409-5420 (1999).

32. **Polz, M. F. and Cavanaugh, C. M.**: Bias in template-to-product ratios in

multitemplate PCR. Appl. Environ. Microbiol., **64**, 3724-3730 (1998).

33. **von Wintzingerode, F., Gobel, U. B., and Stackebrandt, E.**: Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev., **21**, 213-229 (1997).

34. **Vionis, A. P., Niemeyer, F., Karagouni, A. D., and Schrempf, H.**: Production and processing of a 59-kilodalton exochitinase during growth of *Streptomyces lividans* carrying pCHI012 in soil microcosms amended with crab or fungal chitin. Appl. Environ. Microbiol., **62**, 1774-1780 (1996).

35. **Krsek, M. and Wellington, E. M.**: Assessment of chitin decomposer diversity within an upland grassland. Antonie Van Leeuwenhoek, **79**, 261-267 (2001).

36. **Gomes, R. C., Semedo, L. T., Soares, R. M., Alviano, C. S., Linhares, L. F., and Coelho, R. R.**: Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Lett. Appl. Microbiol., **30**, 146-150 (2000).

37. **Gomes, R. C., Semedo, L. T., Soares, R. M., Linhares, L. F., Ulhoa, C. J., Alviano, C. S., and Coelho, R. R.**: Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. J. Appl. Microbiol., **90**, 653-661 (2001).

38. **Williamson, N., Brian, P., and Wellington, E. M.**: Molecular detection of bacterial and streptomycete chitinase in the environment. Antonie Van Leeuwenhoek, **78**, 315-321 (2000).

39. Garrity, G. M. (ed.): Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Williams & Wilkins, Baltimore, USA (2005)

Figure legends

Fig. 1. Degradation rates of flakes of chitin or chitosan in soil

Flakes of chitin or chitosan (5 g each) were placed in nylon nets and buried in soil in the test field. At appropriate intervals, they were collected, washed with tap water, and weighed after drying. The test period was from May to November in 2006. Closed circles, chitin; open circles, chitosan.

Fig. 2. PCR-DGGE analysis of 16S rRNA genes for determining structure of soil-borne bacterial community

Total DNAs were prepared from whole microbial cells in untreated soil (A), and in soil with chitin (B) or chitosan (C) added. Lane numbers indicate days after addition of chitin or chitosan. Arrowheads indicate the fluorescent bands that were used in the determination of nucleotide sequences. Electrophoresis was performed on 8% polyacrylamide gels (40% - 60% denaturant) at 60°C and 200 V for 360 min.

Fig. 3. Rooted neighbor-joining distance matrix tree among 16S rRNA sequences

The sequences determined in this work are shown by symbols corresponding to the bands on DGGE gels (Fig. 2). The authentic sequences obtained from type culture strains of related taxa were also included. Bootstrap values (%) obtained with 1,000 bootstrap resamplings are shown at branching points; only values >50% are shown. Scale bar represents 5% nucleotide substitution rate according to Kimura's 2-parameter model.

Table 1. Similarities of 16S rRNA gene sequences determined from each of bands on DGGE gels

Band[a]	Highest similarity[b]	Identity	Phylum (class)	Chitin utilization		Amino sugar metabolism[e]
				genes[c]	enzymes[d]	
i01	*Cellvibrio vulgaris* NCIMB8633^T	98%	Proteobacteria (γ)	-	-	-
i02	*Cellvibrio vulgaris* NCIMB8633^T	97%	Proteobacteria (γ)	-	-	-
i03	*Cellvibrio mixtus* ACM2601^T	100%	Proteobacteria (γ)	+	+	+
i04	*Cellvibrio vulgaris* NCIMB8633^T	98%	Proteobacteria (γ)	-	-	-
i05	*Chondromyces lanuginosus* TC4494^T	92%	Proteobacteria (δ)	-	-	NA
i06	*Pseudoxanthomonas mexicana* NBRC101034^T	99%	Proteobacteria (γ)	-	-	NA
i07	*Pseudoxanthomonas mexicana* NBRC101034^T	99%	Proteobacteria (γ)	-	-	NA
i08	*Lysobacter oryzae* KCTC22249^T	98%	Proteobacteria (γ)	-	-	NA
i09	*Dyella ginsengisoli* KCTC12599^T	97%	Proteobacteria (γ)	-	-	NA
i10	*Kitasatospora niigatensis* NBRC16453^T	100%	Actinobacteria	-	-	NA
i11	*Kitasatospora niigatensis* NBRC16453^T	99%	Actinobacteria	-	-	NA
i12	*Dyella koreensis* NBRC100831^T	99%	Proteobacteria (γ)	-	-	+
i13	*Lysobacter daejeonensis* KACC11406^T	99%	Proteobacteria (γ)	-	-	-
i14	*Streptomyces bluensis* NBRC13460^T	96%	Actinobacteria	-	-	NA
i15	*Kitasatospora arboriphila* NCIMB13973^T	98%	Actinobacteria	-	-	NA
i16	*Methylococcus capsulatus* UNIGEM1^T	91%	Proteobacteria (γ)	+	-	NA
o01	*Klebsiella pneumoniae* ATCC13884^T	99%	Proteobacteria (γ)	+	-	NA
o02	*Cellvibrio vulgaris* NCIMB8633^T	99%	Proteobacteria (γ)	-	-	-
o03	*Cellvibrio ostraviensis* LMG19434^T	99%	Proteobacteria (γ)	-	-	-
o04	*Enterobacter aerogenes* NCTC10006^T	99%	Proteobacteria (γ)	-	-	NA

o05	*Cellvibrio mixtus* ACM2601^{T}	99%	Proteobacteria (γ)	+	+	+
o06	*Opitutus terrae* JCM15787^{T}	94%	Verrucomicrobia	+	-	NA
o07	*Pantoea agglomerans* NCTC9381^{T}	99%	Proteobacteria (γ)	-	-	+
o08	*Luteimonas aestuarii* KCTC22048^{T}	98%	Proteobacteria (γ)	-	-	+
o09	*Devosia insulae* KCTC12821^{T}	95%	Proteobacteria (α)	-	-	-
o10	*Devosia riboflavina* NBRC13584^{T}	91%	Proteobacteria (α)	-	-	+
o11	*Dokdonella fugitiva* LMG23001^{T}	95%	Proteobacteria (γ)	-	-	+
o12	*Streptacidiphilus jiangxiensis* NBRC100920^{T}	91%	Actinobacteria	-	-	NA
o13	*Dokdonella fugitiva* LMG23001^{T}	95%	Proteobacteria (γ)	-	-	+
o14	*Amycolatopsis saalfeldensis* HKI0457^{T}	99%	Actinobacteria	-	-	NA
o15	*Actinomadura hibisca* NBRC15177^{T}	98%	Actinobacteria	-	-	NA

[a] Each number corresponds to the bands indicated in Fig. 2.

[b] The type culture strain to which the highest similarity is shown.

[c] The presence (+) or absence (-) of genes coding for chitinolytic enzymes in public databases available.

[d] The presence (+) or absence (-) of chitinolytic enzymes in public databases available.

[e] Ability of *N*-acetylglucosamine metabolism depicted in reference 39. +, positive; -, negative; NA, no data is available.

Table 2. Similarity of 16S rRNA gene sequences determined from bacterial cells bound on the surface of flake chitin or chitosan

Band[a]	Highest similarity[b]	Identity	Phylum (class)
Si1	*Cellvibrio fulvus* NCIMB8634^T	99%	*Proteobacteria (γ)*
Si2	*Cellvibrio mixtus* ACM2601^T	99%	*Proteobacteria (γ)*
Si3	*Stenotrophomonas maltophilia* NBRC14161^T	90%	*Proteobacteria (γ)*
Si4	*Methylobacter psychrophilus* Z-0021^T	91%	*Proteobacteria (γ)*
Si5	*Pseudomonas fluorescens* NCIMB9046^T	99%	*Proteobacteria (γ)*
Si6	*Pseudomonas fluorescens* NCIMB9046^T	94%	*Proteobacteria (γ)*
Si7	*Stenotrophomonas acidaminiphila* DSM13117^T	92%	*Proteobacteria (γ)*
So1	*Grimontella senegalensis* C1p^T	99%	*Proteobacteria (γ)*
So2	*Cedecea davisae* LMG7862^T	99%	*Proteobacteria (γ)*
So3	*Cellvibrio mixtus* ACM2601^T	95%	*Proteobacteria (γ)*
So4	*Pseudomonas fluorescens* NCIMB9046^T	96%	*Proteobacteria (γ)*
So5	*Lysobacter gummosus* LMG8763^T	98%	*Proteobacteria (γ)*
So6	*Pseudomonas fluorescens* NCIMB9046^T	95%	*Proteobacteria (γ)*
So7	*Mesorhizobium amorphae* NBRC102496^T	93%	*Proteobacteria (α)*
Ri1	*Clostridium sporosphaeroides* DSM1294^T	88%	*Firmicutes*
Ri2	*Lactococcus lactis* ATCC19435^T	97%	*Firmicutes*
Ri3	*Clostridium sporosphaeroides* DSM1294^T	90%	*Firmicutes*
Ri4	*Lactobacillus plantarum* NBRC15891^T	89%	*Firmicutes*
Ri5	*Pelosinus fermentans* DSM17108^T	86%	*Firmicutes*
Ri6	*Desulfovibrio desulfuricans* NCIMB8307^T	99%	*Proteobacteria (δ)*
Ri7	*Anaerovibrio burkinabensis* DSM6283^T	91%	*Firmicutes*
Ro1	*Holophaga foetida* DSM6591^T	93%	*Acidobacteria*
Ro2	*Cellvibrio mixtus* ACM2601^T	95%	*Proteobacteria (γ)*
Ro3	*Cellvibrio mixtus* ACM2601^T	96%	*Proteobacteria (γ)*
Ro4	*Desulfovibrio putealis* DSM16056^T	95%	*Proteobacteria (δ)*
Ro5	*Aeromonas hydrophila* BCRC13018^T	99%	*Proteobacteria (γ)*
Ro6	*Desulfovibrio putealis* DSM16056^T	99%	*Proteobacteria (δ)*
Mi1	*Clostridium tertium* NCIMB10697^T	90%	*Firmicutes*
Mi2	*Clostridium cellobioparum* LMG5589^T	85%	*Firmicutes*
Mi3	*Clostridium sporosphaeroides* DSM1294^T	93%	*Firmicutes*
Mi4	*Caenimicrobium bisanense* K92^T	94%	*Proteobacteria (α)*
Mi5	*Chitinibacter tainanensis* DSM15459^T	98%	*Proteobacteria (β)*
Mi6	*Zymomonas mobilis* ATCC10988^T	91%	*Proteobacteria (α)*

Mi7	*Streptomyces longispororuber* NBRC13488^T	100%	*Actinobacteria*
Mo1	*Rheinheimera texasensis* DSM17496^T	91%	*Proteobacteria (γ)*
Mo2	*Bartonella washoensis* NVH1^T	89%	*Proteobacteria (α)*
Mo3	*Pseudomonas aeruginosa* NCIMB8295^T	86%	*Proteobacteria (γ)*
Mo4	*Bartonella washoensis* NVH1^T	93%	*Proteobacteria (α)*
Mo5	*Clostridium aldrichii* OCM112^T	80%	*Firmicutes*
Mo6	*Oscillatoria boryana* BDU92181^T	95%	*Cyanobacteria*

[a] Each symbol indicates the bands of DGGE gels obtained from bacterial cells bound on the surface of flake chitin (i) or chitosan (o) which were placed in soil (S), river (R) or moat (M).

[b]The type culture strain to which the highest similarity was shown.

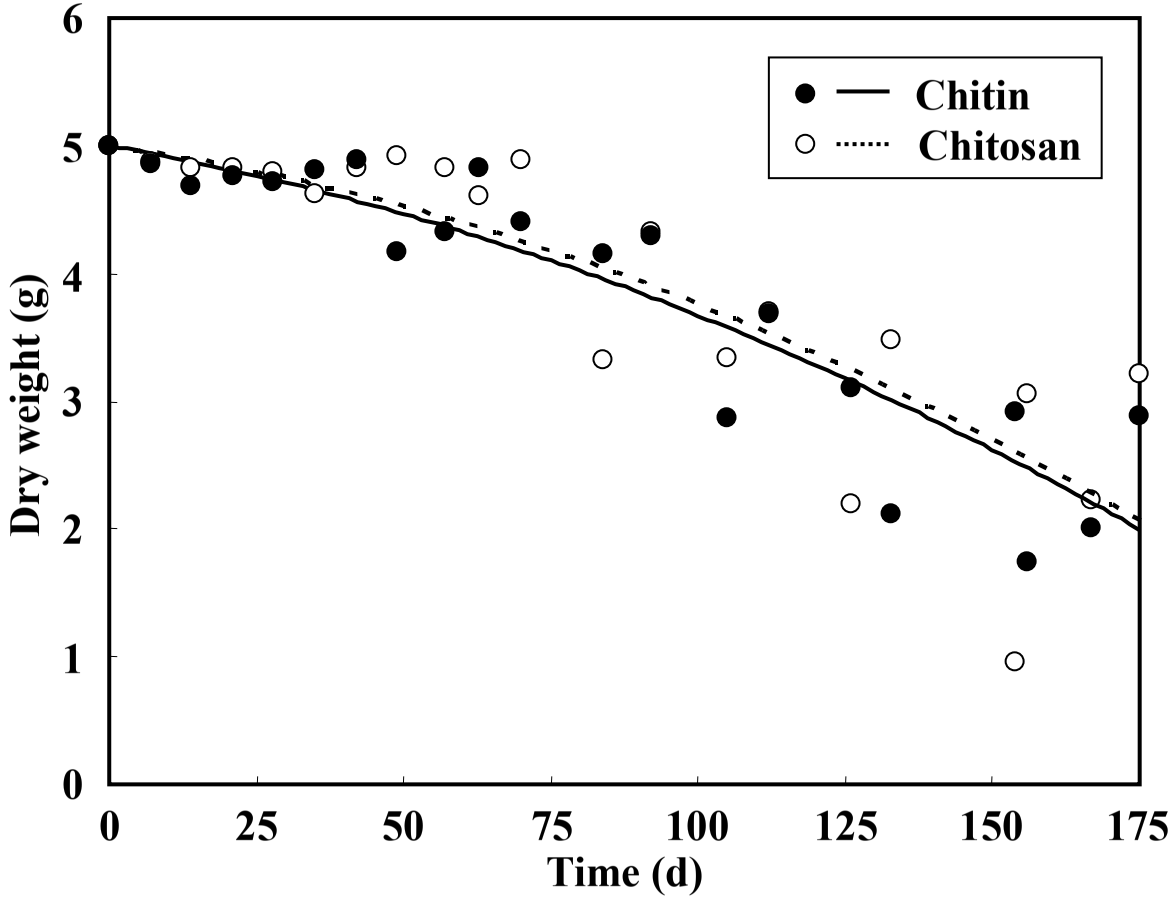
Chitin
Chitosan
Dry weight (g)
Time (d)
0
1
2
3
4
5
6
0
25
50
75
100
125
150
175

Fig. 1

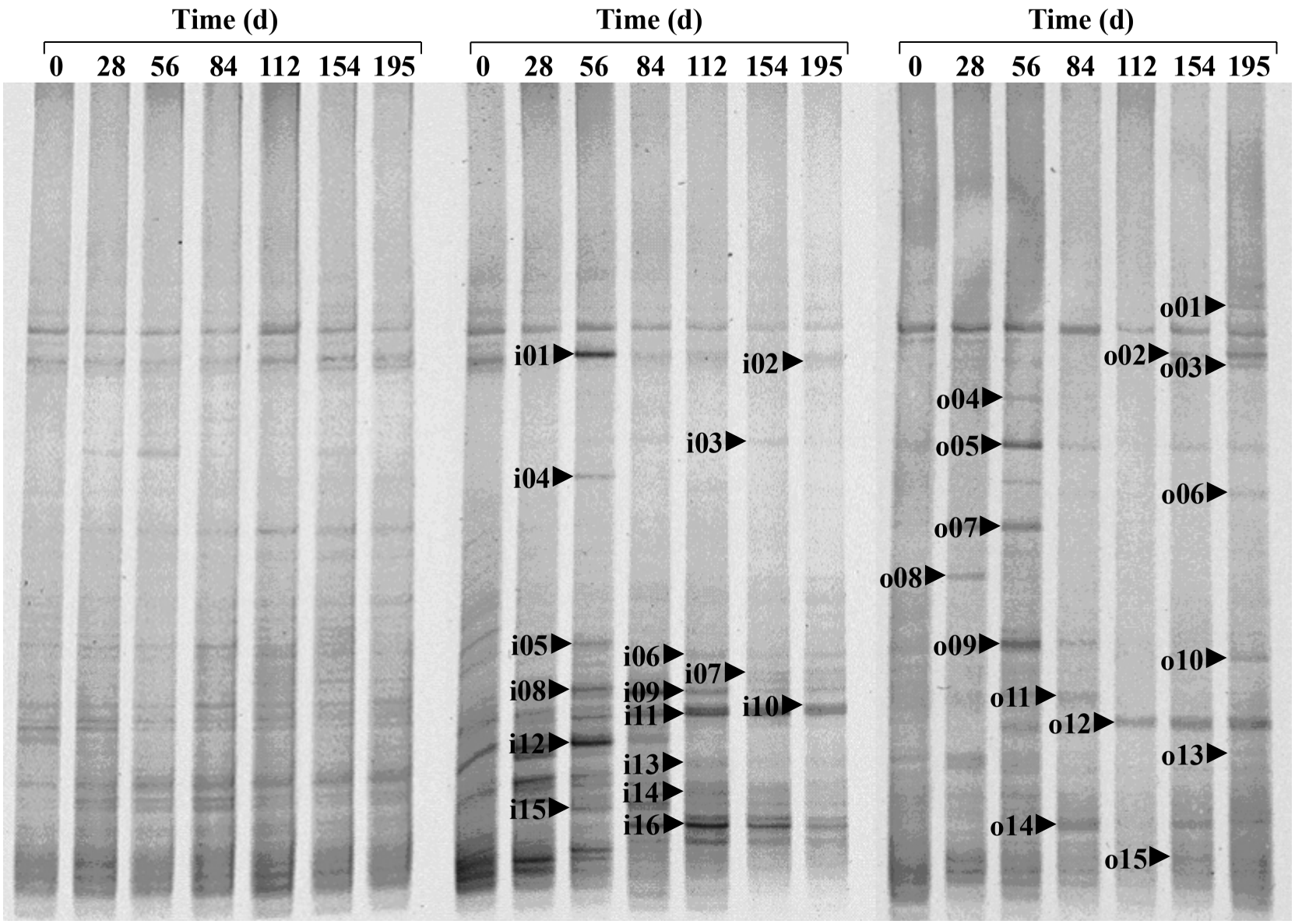
Time (d)
0 28 56 84 112 154 195
Time (d)
0 28 56 84 112 154 195
Time (d)
0 28 56 84 112 154 195
i01
i02
i03
i04
i05
i06
i07
i08
i09
i10
i11
i12
i13
i14
i15
i16
o01
o02
o03
o04
o05
o06
o07
o08
o09
o10
o11
o12
o13
o14
o15
Untreated soil
Soil amended with chitin
Soil amended with chitosan

Fig. 2

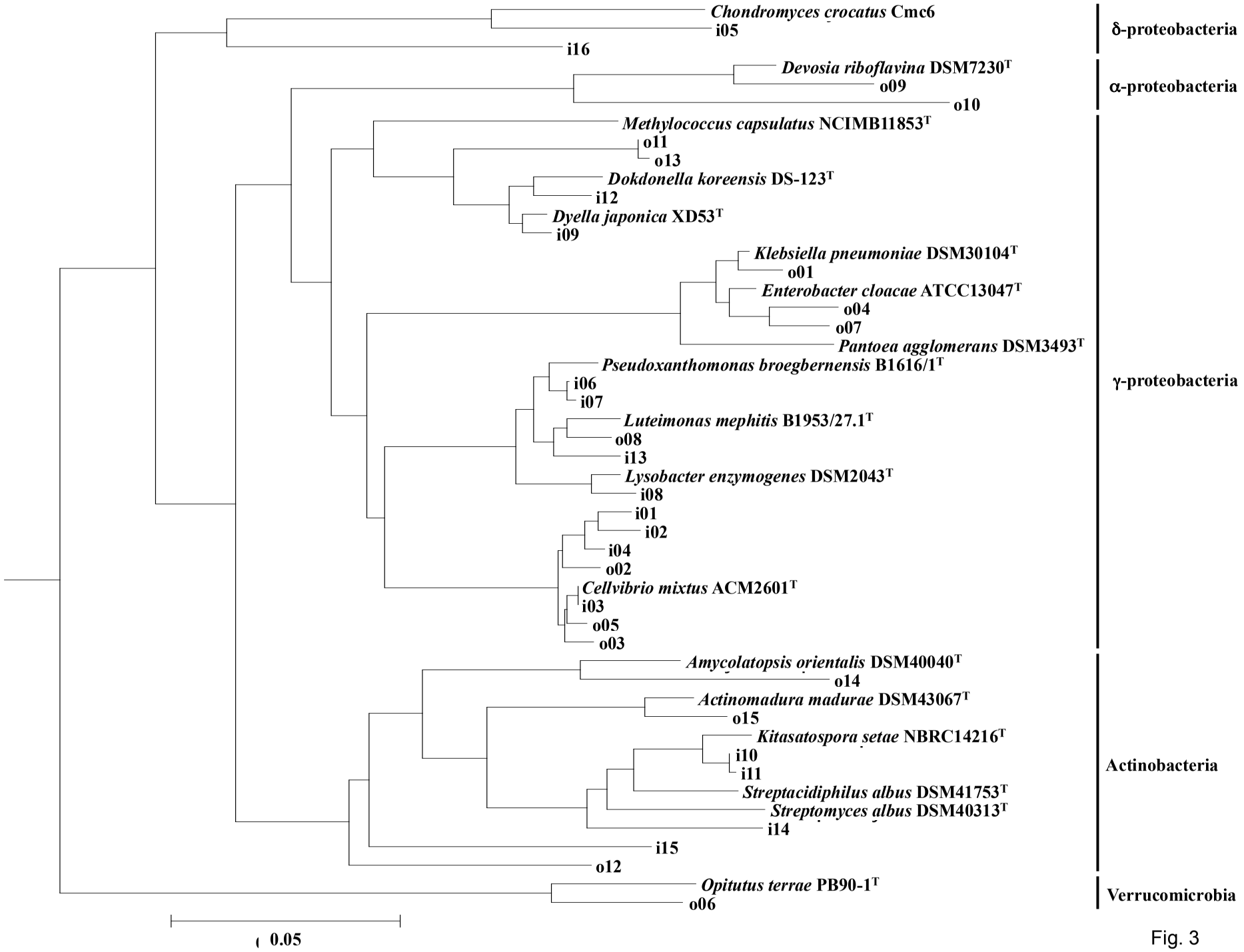
Chondromyces crocatus Cmc6
i05
i16
Devosia riboflavina DSM7230T
o09
o10
Methylococcus capsulatus NCIMB11853T
o11
o13
Dokdonella koreensis DS-123T
i12
Dyella japonica XD53T
i09
Klebsiella pneumoniae DSM30104T
o01
Enterobacter cloacae ATCC13047T
o04
o07
Pantoea agglomerans DSM3493T
Pseudoxanthomonas broegbernensis B1616/1T
i06
i07
Luteimonas mephitis B1953/27.1T
o08
i13
Lysobacter enzymogenes DSM2043T
i08
i01
i02
i04
o02
Cellvibrio mixtus ACM2601T
i03
o05
o03
Amycolatopsis orientalis DSM40040T
o14
Actinomadura madurae DSM43067T
o15
Kitasatospora setae NBRC14216T
i10
i11
Streptacidiphilus albus DSM41753T
Streptomyces albus DSM40313T
i14
i15
o12
Opitutus terrae PB90-1T
o06
0.05
δ-proteobacteria
α-proteobacteria
γ-proteobacteria
Actinobacteria
Verrucomicrobia

Fig. 3