Chapter VII

Bacterial communities in chitin-amended soil as revealed by 16S rRNA gene pyrosequencing

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Abstract

Chitin and its derivatives are natural biopolymers that are often used as compounds for the control of soil-borne plant pathogens. In spite of recent advances in agricultural practices involving chitin amendments, the microbial communities in chitin-amended soils remain poorly known. The objectives of this study were (1) to investigate the bacterial diversity and abundance in an agricultural soil supplemented with chitin that turned disease-suppressive and (2) to assess the emergence of chitinolytic bacteria under conditions of raised soil pH.

Amplicon pyrosequencing based on the 16S rRNA genes was used to characterize the structures of bacterial communities in soil, chitin-amended or not, with native versus raised pH (5.7 vs 8.7), in microcosms and the field. As a result of chitin addition, changes in the relative abundances of Actinobacteria, Proteobacteria and Bacteroidetes were observed in the field soil. A large and significant increase of the relative abundance of Oxalobacteraceae (Betaproteobacteria, Burkholderiales) was found. At the genus level Duganella and Massilia revealed large increases. Moreover, responses of Alpha- and Gammaproteobacteria appeared shortly after alteration of the soil pH. A significant decrease in abundance of Actinobacteria was observed in amended field soil and in microcosm at high pH. Overall, the bacterial abundance decreased with the addition of chitin. Two groups, Actinobacteria and Oxalobacteraceae, were found to be most responsive to the amendment. These results enhance the understanding of responses and possible interactions within bacterial community to chitin that can be correlated to soil disease suppressiveness.
Introduction

Chitin (β-1,4-N-acetylglucosamine polymer) is broadly spread among organisms of all three domains of life, serving as a major component of their exoskeleton and structural elements (e.g. the exoskeletons of invertebrates, the cell wall of fungi). Given the prevalence of chitin in fungi as well as insects and the abundance of such organisms in soil, naturally chitin-free soil is probably inexisten on Earth (Veldkamp, 1955). Chitin is sensitive to natural degradation, and, in particular, bacterial chitinolytic enzymes are involved in the degradative process. Such chitinases may also be at the basis of the parasitism by bacteria on chitin-containing organisms, under which pathogens of different nature (Patil et al., 2000). Therefore, the amendment of soil with chitin has been proposed to represent a successful agricultural practice of defense against fungal and nematodal plant diseases (Cretoiu et al., 2013; Hjort et al., 2010; Kobayashi et al., 2002; Kotan et al., 2009). In a few previous studies, chitin has been shown to affect the soil microbiota in terms of its abundance and diversity (Hjort et al., 2010; Kielak et al., 2012; Manucharova et al., 2007; Manucharova et al., 2011; Metcalfe et al., 2002). However, the data so far obtained are rather limited. In the present study, we address the shifts in the bacterial community compositions of soil under chitin amendment, as compared to unamended soil, both taken from an agricultural field. We also studied the bacterial community changes upon chitin addition and a pH upshift in microcosms, in order to assess the immediate bacterial community changes.

Experimental set-up and methods used to assess bacterial community

The site chosen for sampling is an agricultural field located at the experimental farm De Vredepeel in the south-east of the Netherlands (51°32’ 27.10” N and 5°51’14.86” E). The chitin amendment experiment encompassed three replicate soil plots amended with chitin (thrice) next to unamended control plots. Soil samples were collected in June 2010, nine months after chitin amendment of the top 20 cm of the soil (Korthals et al., submitted). The soil was characterized as a sandy soil with pH 5.7 and 3.2% organic matter. Soil microcosms were also established on the basis of the unamended soil, as previously described (Kielak et al., 2013). Briefly, soil was amended with chitin purified from shrimp waste (Xu et al., 2008) and the pH was changed to 8.7 using Na₂CO₃. Control microcosms with unamended soil at native pH were also included. After 0, 1, 3, 7, 15, 30 and 60 days of incubation, approximately 5 g of soil was removed from each microcosm. Enzymatic measurements reported by Kielak et al. (2013) indicated that the chitin-treated soil at day three (three days incubation, further referred as T3) had maximal chitinolytic activity. On the basis of the field and microcosm data, the samples selected for in-depth analysis of the bacterial communities were (1) unamended and chitin-amended field soils, and (2) unamended and chitin-amended pH 5.7, as well as and chitin-amended pH 8.7 soils. Three biological replicates were used for each treatment. Following standard soil DNA extraction and purification, barcoded pyrosequencing of the 16S rRNA gene was performed as
previously described (Schluter et al., 2008) and carried out on a Roche 454 GS FLX system. The reads were processed (filtering, trimming, homopolymer and chimera removal) using Mothur (Schloss et al., 2009). All samples were then harmonized (randomly) to 2,257 sequences per sample and subjected to phylogenetic analyses. Phylotypes (operational taxonomic units – OTUs) were assigned at the 97% sequence similarity level and the taxonomic identity was determined using RDP classifier (http://rdp.cme.msu.edu/). Alpha-diversity indices were then calculated. ANOVA tests were applied to the relative abundance values obtained from the entire data set. All sequences from this study were deposited in the Sequence Read Archive (SRA) under numbers XXX.

**Bacterial community composition and ecological significance of selected groups**

Overall, 80% of the reads could be assigned to phylotypes, whereas approximately 20% of each sample remained unclassified. As we were interested in the identifiable phylotypes, we focused on the approximately 1,800 sequences for analysis. In these, a total of 17 bacterial phyla were found across all samples. Overall, the dominant phylum was **Proteobacteria**, (relative abundance 57.33±17.84%) followed by the phyla **Bacteroidetes** (relative abundance 12.51±6.09%), **Firmicutes** (relative abundance 8.75±3.74%), **Actinobacteria** (6.71±4.34%) and **Acidobacteria** (7.52±6.79%) (Figure 1). Phyla with minor or incidental occurrence were **Armatimonadetes**, **Gemmatimonadetes**, **Chloroflexi**, **Nitrospora** and **Verrucomicrobia** (minor, up to 2% relative abundance) and **Fibrobacter**, **Planctomycetes** and **Spirochetes** (incidental). The distribution of the major phyla was different per sample type. In field soil, the relative abundances of **Proteobacteria** and **Bacteroidetes** increased after chitin amendment, while those of **Actinobacteria**, **Acidobacteria** and **Firmicutes** decreased. The microcosm pH 5.7 unamended soil showed relative abundances akin to those observed in the unamended field soil, whereas the distribution changed towards a predominance of **Proteobacteria** (from 47.17% to 75.60%) upon a pH upshift to pH 8.7.

The highest number of sequences was affiliated to the phylum **Proteobacteria**. The relative abundance of this phylum was 46±1% in unamended soil (field and microcosm) as well as in the chitin-amended pH 5.7 soil, versus 70.14% and 75.6% in chitin-amended field and pH 8.7 microcosm soils, respectively (Figure 2).
Figure 1. Comparison of bacterial phyla diversity across the field soil samples. Average relative abundance data from three replicates were calculated as the ratio between the sequence type abundance and the total number of sequences. All calculation were performed on normalized data.

Figure 2. Distribution of Proteobacteria classes. Average relative abundance data from three replicates. Significant differences (P<0.05) are indicated with "*". 
Among the *Proteobacteria*, *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria* were found in all samples (Figure 2). On the basis of data from the *Proteobacteria*, the treatments were divided into two groups: one group was formed by unamended field and microcosm soils in addition to the chitin-amended (pH 5.7) microcosm soil, whereas the second group encompassed the chitin-amended field and pH 8.7 microcosm soils. In soils of the first group (unamended and chitin-amended pH 5.7 soils), *Alphaproteobacteria* showed the highest relative abundance (26±2%), followed by *Gammaproteobacteria* (10.60% - unamended field soil and ~7% - microcosm soil), *Betaproteobacteria* (4.77±1.67%) and *Deltaproteobacteria* (2.30±1.02%) (Figures 2, 3A, 3B). The insignificant differences between unamended and chitin-amended soils at native pH indicated that proteobacteria did not respond fast to the amendment under the prevailing conditions. In contrast, the chitin-amended field and pH 8.7 soils showed significant differences from the unamended soil (P<0.05) within the *Proteobacteria*. The pH raise selected for *Alpha- and Gammaproteobacteria*, mainly belonging to the families *Alcaligenaceae*, *Pseudomonadaceae* and *Xanthomonadaceae*. In this case, the highest number of sequences was typified as unclassified *Alphaproteobacteria* (Figure 3A). This observation was similar to previous reports on the diversity of bacteria in alkaline environments (Sorokin and Kuenen, 2005; Aislabie *et al*., 2009; Tripathi *et al*., 2012). In the chitin-amended field soil, the relative abundances of proteobacterial sequences were 18.27% for *Alphaproteobacteria*, 25.79% for *Betaproteobacteria*, 21.53% for *Gammaproteobacteria* and 1.12% for *Deltaproteobacteria*. Comparison of these values with those obtained for the unamended field soil revealed highly significant (P=0.0001) differences (upshifts due to the chitin addition) at the level of the *Betaproteobacteria* (Figure 2).

In particular, the family *Oxalabacteraceae* revealed a very strong increase, i.e. from 0.77% in the unamended to 20.63% in the chitin-amended soil (Figure 3A and 4). Deep taxonomic analyses revealed that two genera, i.e. *Duganella* and *Massilia*, were most responsive to the chitin amendment. The relative abundance of *Duganella* increased from 0.02% in unamended to 12.15% in amended soil, while that of *Massilia* went from 0.26% to 4.21% (Figure 4).
**Figure 3A.** Relative abundance (% of sequences) of representative *Alpha- and Betaproteobacteria* families.
Figure 3B. Relative abundance (% of sequences) of representative Delta- and Gammaproteobacteria families.
The high abundances resulting from the chitin amendment were in accordance with measurements of the relative abundances of Oxalobacteraceae by qPCR as previously communicated (Cretoiu et al., 2013). Interestingly, sequences affiliated with another oxalobacterial group, Collimonas, were not detected. Previous studies (Green et al., 2006; Ofek et al., 2012) have described the role of root-associated Oxalobacteraceae in biodegradation of complex compounds and showed a clear response of these organisms to amendment of soil with compost. Here, the significant increase in the abundance of particular types of oxalobacteraceae following soil amendment with chitin in the field is a possible indication of the involvement of such oxalobacteraceal community members in (1) the community response to chitin, and (2) the suppressiveness of the soil towards plant pathogens.
Considering the bacterial distributions in the field and microcosm soils, variations in relative abundances were observed for the phyla *Firmicutes* and *Bacteroidetes*.

*Firmicutes* amounted to relative abundances of 13.6% in unamended field soil, 5.30% in chitin-amended field soil, and 8.08±0.68% in microcosm soils. A statistical comparison across all samples revealed that the relative abundances in the amended field were significantly lowered as compared to those in the unamended field soil (P<0.05). *Firmicutes* were mainly represented by members of the *Bacillaceae*, amounting to 2.75±1.76% (Figure 5). Among the *Bacillaceae*, the following genera stood out as dominant ones: *Bacillus*, *Geobacillus* and *Virgibacillus*. In particular, the genus *Bacillus* revealed a significant decrease in the chitin-amended field soil. However, the drop in relative abundance was small and, overall, the level of *Firmicutes* across the samples was consistent with that in a previously reported metagenomic screening of forest soil for novel cellulases (Xia *et al.*, 2013).

The phylum *Bacteroidetes* (Figure 6) was also frequently found in the field soil (14% in unamended and 19.82% in chitin-amended soil). Members of this phylum were apparently lower in the microcosm soils, with relative abundances of 8% in unamended and 12.75% in chitin-amended pH 8.7 soils. Within *Bacteroidetes*, representatives of *Sphingobacteriaceae* were prominent. Their relative abundances were significantly higher (P<0.05) in chitin-amended field soil (17.01%) and pH-8.7 microcosm soil (11.58%) than in unamended field and microcosm (pH 5.7) soils (4.36%, 0.56%).

The phylum *Actinobacteria*, which contains many members that have previously been described as responsible for chitin degradation in soil (Gooday, 1990; Metcalfe *et al.*, 2002; Manucharova *et al.*, 2007; Manucharova *et al.*, 2011), was found to become significantly (P=0.001) less abundant in the field soil upon chitin addition as compared to the unamended soil (Figure 7). In the microcosm study, we observed an insignificant increase in the actinobacterial relative abundance upon chitin amendment of the soil at pH 5.7 and a significant decrease (P=0.001) in chitin-amended pH 8.7 soil (Figure 7).
**Figure 5.** Changes in the relative abundance of *Firmicutes* and representative families. Significant differences (P<0.05) are indicated with "*".
Figure 6. Changes in the relative abundance of *Bacteroidetes* and representative families. Significant differences (P<0.05) are indicated with **“*”**.
**Figure 7.** Changes in the relative abundance of *Actinobacteria* and representative families. Significant differences (P<0.05) are indicated with "*".
Throughout all samples, a considerable number (3.43±1.39%) of sequences from all samples was classified as affiliated with the *Micrococcaceae*. On the other hand and rather surprisingly, the relative abundances of well-known chitinase producers, such as members of the *Streptomycetaceae* and *Streptosporangiaceae* were lower. These were 0.21% in unamended (pH 5.7) soil, 0.15% in unamended field soil and in chitin-amended pH 5.7 soil, 0.09% in chitin-amended field soil and 0.01% in chitin-amended pH 8.7 soil. *Actinobacteria* may have been activated ephemerally by the chitin amendment, possibly resulting in a slight temporary outgrowth. However, beneficial conditions, e.g. reflecting liberated chitin oligomers, may have been created for the other bacterial groups like members of the oxalobacteraceae. The overall changes in the actinobacterial communities were concordant with data that were recently produced by Wellington (personal communication; family-19 chitinase pyrosequencing of amended soil), who also reported on chitinolytic communities in a chitin-amended test field.

**Conclusion**

Our results show that the bacterial communities in chitin-amended agricultural field soils are diverse and responsive to the chitin treatment (as compared to communities in chitin-untreated soil). The high abundance of *Oxalobacteraceae*, particularly of members of the genera *Duganella* and *Massilia*, indicate the necessity to deeply analyse soil microbial communities when soil suppressiveness is to be evaluated. Moreover, the selection of particular *Proteobacteria* in soil under alkaline conditions in the presence of chitin suggested that such treated soils may be good reservoirs of novel chitinolytic bacteria functioning under alkaline conditions.

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