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Bacterial communities in chitin-amended soil as revealed by 16S rRNA gene based 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 of soil-extracted DNA 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. Within the *Oxalobacteraceae*, the genera *Duganella* and *Massilia* showed large increases. Moreover, responses of the *Alpha-* and *Gammaproteobacteria* appeared shortly after the alteration of the soil pH in the microcosms. A significant decrease in the abundance of *Actinobacteria* was observed in the chitin-amended field soil and in the microcosm at high pH. Overall, the bacterial abundance in soil tended to decrease 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 to chitin and possible interactions within bacterial communities in soil that can be correlated to soil disease suppressiveness.

1. 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 inexistent 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, such as pathogenic fungi and nematodes (Patil et al., 2000). Thus, in the presence of chitin and its oligomers, a temporary decrease of the rate of infection of plant roots by nematodes has been observed (Sarathchandra et al., 1996; Green et al., 2006; Radwan et al., 2012). This enhancement of disease suppression could be related to changes in the activity of the soil microbiota. Chitinolytic organisms capable of degrading the chitinous structures of (pathogenic) fungi, nematodes and insects were favoured by the chitin addition (Weller et al., 2002; Mendes et al., 2011), and so chitinolytic enzymes that affect chitin-containing organisms may have been released to a larger extent.

Therefore, the amendment of soil with chitin has been proposed to represent a successful agricultural practice of defence against fungal and nematodal plant diseases (Kobayashi et al., 2002; Kotan et al., 2009; Hjort et al., 2010; Cretoiu et al., 2013).

In previous studies, chitin has been shown to affect the soil microbiota in terms of its abundance and diversity (Metcalfe et al., 2002; Manucharova et al., 2007, 2011; Hjort et al., 2010; Kielak et al., 2013). Moreover, other reports discuss the bioexploration and use of chitinases, in particular those that work well at raised pH, in agriculture, industry and medicine (Gooday, 1990; Felse and Panda, 1999; Olander and Vitousek, 2000; Dutta et al., 2002, 2004; Krajewska, 2004; Qiu et al., 2009). However, at this point in time,

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only few bacterial chitinases are commercially available and these have limited optimal activities, working mostly at acidic to neutral pH.

Given the great interest in promoting our understanding of soil bacterial responses to chitin, both to foster our understanding of the effects of this ecological shift on disease suppression and to enhance the chances of bioexploratory success, we here examined the shifts in the bacterial community compositions of soil under chitin amendment, as compared to unamended soil, in an agricultural field. In addition, we studied the bacterial community changes upon chitin addition and a pH upshift in microcosms using the same field soil, in order to assess the immediate bacterial community changes, as a strategy to enable understanding the short-term consequences of the amendment for bioexploration and the ecology of the microbial community.

2. Material and methods

2.1. 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 next to three unamended control plots. Soil samples were collected in June 2010, nine months after chitin amendment of the top 20 cm of the soil (Cretoiu et al., 2013; Korthals et al., personal communication). 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 reported and described (Kielak et al., 2013). Briefly, soil was amended with chitin purified from shrimp waste (Xu et al., 2008) and for one treatment (three replicates) the pH was changed to 8.7 using Na_2CO_3. 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 (Schlüter 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 2257 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.

3. Results and discussion

3.1. Bacterial community composition and ecological significance of selected groups

Overall, 80% of all reads obtained could be assigned to phylotypes, whereas approximately 20% of the reads in each sample remained unclassified. As we were interested in the identifiable phylotypes, we focused on the approximately 1800 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 *Bacteriodetes* (12.51 ± 6.09%), *Firmicutes* (8.75 ± 3.74%), *Actinobacteria* (6.71 ± 4.34%) and *Acidobacteria* (7.52 ± 6.79%) (Fig. 1). Phyla with minor or incidental occurrence were *Armatimonadetes*, *Gemmatimonadetes, Chloroflexi*, *Nitrospira* and *Verrucomicrobia* (minor, meaning consistently present up to 2% relative abundance) and *Fibrobacter*, *Planctomycetes* and *Spirochetes* (incidental, meaning present in some samples but not in others). The distribution of the major phyla was different per sample type. In field soil, the relative abundances of *Proteobacteria* and *Bacteroidetes* showed an increase due to the chitin amendment, while those of *Actinobacteria, Acidobacteria* and *Firmicutes* decreased. The microcosm pH-5.7 unamended soil showed relative abundances of the different groups that were 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 an upshift of the pH to 8.7. Overall, the highest number of sequences was affiliated with the phylum *Proteobacteria*. The relative abundance of this phylum was 46 ± 1% in unamended soil (field as well as microcosm) as well as in the chitin-amended pH-5.7 soil, versus 70.14% and 75.6% in the chitin-amended field and pH-8.7 microcosm soils, respectively (Fig. 2).

Among the *Proteobacteria, Alpha-*, *Beta-*, *Gamma-* and *Deltaproteobacteria* were found in all samples (Fig. 2). On the basis of the data on the *Proteobacteria*, the treatments were divided into two groups. Of these, 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%) (Fig. 2). The insignificant differences between the native-pH unamended and chitin-amended soils at this level indicated that proteobacteria did not respond (increase) fast to the chtin amendment under the prevailing conditions. In contrast, the chitin-amended field and pH-8.7 (microcosm) soils showed significant differences from the unamended soil ($P < 0.05$) within the *Proteobacteria*. The pH elevation selected for *Alpha-* and *Gammaproteobacteria*, mainly belonging to the families *Alcaligenaceae*, *Pseudonomadaceae* and *Xanthomonadaceae*. In this case, the highest number of sequences were typified as unclassified *Alphaproteobacteria* (Fig. 3). This observation was consistent with data reported in previous studies 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* (Fig. 2). In particular, the family *Oxalabacteraceae* showed a very strong increase, i.e. from 0.77% in the unamended to 20.63% in the chitin-amended field soil (Figs. 3 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 the unamended to 12.15% in the amended soil, while that of *Massilia* went from 0.26% to 4.21% (Fig. 4).