Chitinase genes revealed and compared in bacterial isolates, DNA extracts and a metagenomic library from a phytopathogen suppressive soil.

Karin Hjort[1,2], Maria Bergström[1], Modupe F Adesina[3], Janet K Jansson[2,4], Kornelia Smalla[3], and Sara Sjöling[1]

[1] *Södertörn University, School of Life Sciences, SE-141 89 Huddinge, Sweden*

[2] *Swedish University of Agricultural Sciences, Department of Microbiology, SE-750 07 Uppsala, Sweden*

[3] *Julius Kühn-Institut - Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, D-38104 Braunschweig, Germany*

[4]*Lawrence Berkeley National Laboratory, Division of Earth Sciences, 1 Cyclotron Rd., Berkeley, CA 94720*

For correspondence: e-mail: sara.sjoling@sh.se; Tel. +46-8-6084767; Fax. +46-8-6084510

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ABSTRACT

Soil that is suppressive to disease caused by fungal pathogens is an interesting source to target for novel chitinases that might be contributing towards disease suppression. In this study we screened for chitinase genes, in a phytopathogen-suppressive soil in three ways: 1) from a metagenomic library constructed from microbial cells extracted from soil, 2) from directly extracted DNA and 3) from bacterial isolates with antifungal and chitinase activities. Terminal-restriction fragment length polymorphism (T-RFLP) of chitinase genes revealed differences in amplified chitinase genes from the metagenomic library and the directly extracted DNA, but approximately 40% of the identified chitinase terminal-restriction fragments (TRFs) were found in both sources. All of the chitinase TRFs from the isolates were matched to TRFs in the directly extracted DNA and the metagenomic library. The most abundant chitinase TRF in the soil DNA and the metagenomic library corresponded to the TRF^{103} of the isolate, *Streptomyces mutomycini* and/or *Streptomyces clavifer*. There were good matches between T-RFLP profiles of chitinase gene fragments obtained from different sources of DNA. However, there were also differences in both the chitinase and the 16S rRNA gene T-RFLP patterns depending on the source of DNA, emphasizing the lack of complete coverage of the gene diversity by any of the approaches used.

INTRODUCTION

Exploitation of the previously hidden members of the soil microbiota is a focus of current research interest in the hunt for novel bioactive molecules beneficial in medicine, industry and agriculture, for reviews see (Handelsman, 2004; Sjöling *et al.*, 2007).For example, some soils are naturally suppressive towards plant diseases and microorganisms in these soils are often proposed to be the cause of suppressiveness (Borneman and Becker 2007; Steinberg *et al.*, 2007). Therefore, there is considerable commercial and research interest in isolation of the microorganisms, or the bioactive compounds that might contribute to disease suppression. Biological control methods have been recommended to replace chemical control methods since these are more economical and environmentally sustainable (Fravel, 2005; Herrera-Estrella & Chet, 1999). One example of a biological mechanism for suppression of fungal pathogens in suppressive soils is that of microbial chitinase activity (Chernin *et al.*, 1997; Downing and Thomson, 2000; Kobayashi *et al.*, 2002) and chitinases (or chitinase-producing microorganisms) have a potential application for biocontrol of plant diseases.

Chitinases belong to the group of glycosyl hydrolases, either family 18, or 19. Family 18 is further subdivided into A, B or C based on amino acid sequence similarities of the catalytic domains (Henrissat & Bairoch, 1993; Karlsson & Stenlid, 2009). Chitinases hydrolyze chitin, which otherwise is rather resistant to degradation, to enable utilization of the end products as an energy-, carbon- and/or nitrogen source (Gooday, 1990; Williamson *et al.*, 2000; Lindahl & Finlay, 2006). This is an important step in the biogeochemical cycling of carbon and nitrogen in the environment. In soil, chitin is widely distributed within insect bodies and fungal cell walls (Gooday, 1990). Conventional molecular screening approaches have identified chitinase genes within aquatic (Ramaiah *et al.*, 2000; Hobel *et al.*, 2005) and soil environments (Metcalfe *et al.*, 2002; Williamsson *et al.*, 2000; Uchiyama & Watanabe,

2006; LeCleir *et al.,* 2004). However, only a few studies have used a metagenomic approach to identify chitinase genes (Cottrell *et al.,* 1999; LeCleir *et al.,* 2007).

Metagenomics offers access to functional genes in uncultured representatives of the microbiota and has previously facilitated the characterization of large genomic regions or even complete genomes of uncultured bacteria (Rondon *et al.,* 2000; Gillespie *et al.,* 2002; Tringe *et al.,* 2005) and access to novel bioactive products (Sjöling *et al.,* 2007; Hårdeman & Sjöling, 2007). Soil metagenomics typically involves the isolation and purification of high molecular weight (HMW) DNA followed by cloning into a library and sequencing, or alternatively direct sequencing using 2nd generation sequencing platforms. A clone library has the advantages of facilitating functional, expression-based screening and sequencing of long contigs (Rondon *et al.,* 2000; Sjöling *et al.,* 2007).

We have previously (Hjort *et al.,* 2007) used the molecular fingerprinting techniques, terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) to study changes in bacterial communities in response to chitin amendment in a soil reported to be suppressive towards clubroot disease caused by *Plasmodiophora* (Worku & Gerhardson, 1996). We found that after chitin addition to the soil the relative abundances of known chitin-degrading genera such as *Oerskovia*, *Kitasatospora*, and *Streptomyces* species increased dramatically and became dominant both in the total and in the actively growing bacterial community. Also, a number of isolates with antifungal and chitinase activity were obtained from this soil (Adesina *et al.,* 2007).

The aim of this study was to screen for chitinase genes in the suppressive soil using a combination of molecular approaches. To begin with we searched for chitinase genes in the bacterial isolates previously obtained from the soil with known chitinase and antifungal activities (Adesina *et al.,* 2007). We also used T-RFLP (Liu *et al.,* 1997; Braker *et al.,* 2001) to screen DNA directly extracted from the same soil. Finally, we prepared a fosmid library

and screened the resulting soil metagenome for chitinase genes. This study is the first to compare these different sources of DNA from the same soil. We found surprisingly good agreement between the different sources of material for the dominant chitinase genes detected, but some differences were also found indicating that specific biases need to be taken into account for each method. These results should lay the groundwork for making informed decisions about the appropriate source material to use in other studies that aim to screen for specific functional genes in environmental samples.

MATERIALS AND METHODS

Soil sampling

The soil (clay loam, pH 6.9 and an organic carbon content of 1.48%) was sampled from a field at the Swedish University of Agricultural Sciences in Uppsala, Sweden in October 2003 and July 2004 as previously described (Hjort *et al.*, 2007). The field was previously characterized as suppressive to clubroot disease of cabbage (Worku & Gerhardson, 1996). Twenty soil core samples from the top 20 cm were randomly collected from 4 sites (each 5 m by 5 m) using a core sampling device. All 20 core samples from an individual site were mixed to one composite sample, sieved through a 4 mm mesh and high molecular weight (HMW) DNA was immediately extracted from the soil as described below.

Metagenomic library construction

The metagenomic library was constructed in fosmids using a modification of the procedure described by Hårdeman & Sjöling (2007). The cells were extracted from 100 g of freshly collected soil as previously described (Gabor *et al.*, 2003) with the following modifications. The soil was mixed with 100 mL 0.5% pyrophosphate buffer, pH 8.0 in a Waring blender

(Robert Bosch GmbH, Germany) for 3 times 30 s, followed by incubation at 4°C for 30 min. Soil particles were sedimented by low speed centrifugation at 500 x *g* for 20 min, at 10°C. The supernatant containing the cells was collected and transferred to a different tube. The soil pellet was re-suspended and mixed in 50 mL CTAB (hexadecyltrimethylammonium bromide) buffer pH 8.5 (100 mM EDTA, 100 mM Tris-HCl, 0.1% SDS, 1% CTAB) using the blender for 30 s, followed by an additional centrifugation at 500 x g for 20 min at 10°C. This step was repeated. Finally all supernatants containing microbial cells extracted from the soil were pooled and cells were collected by centrifugation at 10,000 x *g* for 30 min at 10°C. The cells were resuspended in 2 mL TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0). 12 μL proteinase K (20 mg/mL) and 120 μL 10% SDS were added to lyse the cells during incubation at 37°C for 60 min. This was followed by the addition of 400 μL 5 M NaCl and 320 μL (10% CTAB, 0.7 M NaCl) and incubation at 65°C for 10 min. The DNA was recovered by gentle phenol/chloroform/isoamylalcohol extraction (25:24:1) and precipitated by 1:10 volume of sodium acetate and 2.5 volumes of ethanol.

High molecular weight (HMW) DNA was separated on a 1% low-melting-point agarose gel (GE Healthcare, Sweden) by pulsed field gel electrophoresis (PFGE), 20 V x 40 s, at 6 V cm^{-1}, 5-15 s switch at 14°C for 18 h (CHEF-DR II, BIO RAD, Laboratories, UK). DNA fragments ranging from 25 to 300 kb were excised from the gel and extracted using β-agarase I according to the manufacturer's instructions (New England Biolabs, Ipswich, MA) to avoid shearing. The DNA was gently precipitated with ethanol as above. Approximately 500 ng of DNA was cloned into a CopyControl Fosmid vector (Epicentre, Madison, WI) according to the manufacturer's instructions. Fosmid clones were picked into 96-well microtitre-plates and grown in LB (Lauria Bertani), supplemented with 12.5 μg/mL chloramphenicol (Cm) and 7% glycerol, overnight at 37°C. The 7800 clones of the original

library, stored at -80°C, were pooled into one sample which was used for chitinase screening and 16S rRNA gene analysis.

The average insert size was analysed by randomly selecting 20 clones where the vector was isolated by means of standard alkaline lysis and plasmid mini preparation (Qiagen, Hilden, Germany) and the insert sizes were determined by *Not*I digestion (Fermentas, Ontario, Canada). The sizes of the inserts were estimated from 1% agarose gels.

Subsequent PCR screenings of the library for the presence of chitinase and 16S rRNA genes were made after extraction of vector DNA from the pooled fosmid library using the plasmid midi prep kit following the manufacturer's instructions (Qiagen).

Direct soil DNA extraction

Triplicate soil DNA samples were directly extracted from 400 mg frozen soil (collected October 2003) by bead beating using the FastPrep for soil kit, Bio101 (Qbiogene Carlsbad, CA) and a FastPrep bead beating machine (BIO101, Qbiogene) according to the manufacturer's instructions. The extracted DNA had a lower average molecular weight (<20 kb) compared to the HMW DNA prepared for the metagenomic library.

DNA extraction from isolates

Genomic DNA was extracted from 18 bacterial isolates previously obtained from the same soil batch used for DNA extractions described above, with demonstrated chitin degrading capacities based on an agar plate assay (Adesina *et al.*, 2007). The isolates were also previously demonstrated to have antifungal activity towards *Rhizoctonia solani* and/or *Fusarium oxysporum* (Adesina *et al.*, 2007). Cells were lysed with 0.1 mm silica beads (Biospec production inc., Bartlesville, OK) and two executive bead beating steps at a speed of 5.5 m s^{-1} for 45 sec each in the Fast prep bead beating machine and DNA was extracted using

the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the manufacturer's instructions, except for the addition of the additional lysis step.

T-RFLP of 16S rRNA genes and chitinase genes

Partial 16S rRNA genes were amplified in triplicate from DNA (pooled metagenomic library and directly extracted from soil) using bacterial forward primer fD1-FAM (5´-AGAGTTTGATCMTGGCTCAG-3´) 5´ end-labelled with 5´6-FAM (phosphoramidite fluorochrome 5-carboxy-fluorescein) and reverse primer 926r (5´-CCGTCAATTCCTTTRAGTTT-3´) (Weisburg *et al.,* 1991; Muyzer *et al.,* 1995), as described in Edlund *et al.* (2006). All primers were synthesized by Invitrogen (Carlsbad, CA).

Partial family 18 chitinase genes were amplified in triplicate from each DNA sample of the same source as above and in single amplifications from DNA of bacterial isolates using forward primer ChiA_F2 (5´-CGT GGA CAT CGA CTG GGA RTW YCC-3´) 5´ end-labelled with 5´6-FAM, and reverse primer ChiA_R2 (5´-CCC AGG CGC CGT AGA RRT CRT ARS WCA-3´) (Hobel *et al.,* 2005). The PCR reactions were set up according to Hobel *et al.* (2004) with a few modifications; primer concentration 20 pmol, *Taq* polymerase 2.5 U (GE Healthcare, Chalfont St. Giles, UK) (Hobel *et al.,* 2004) and the annealing temperature was increased to 47°C from 42°C.

For the analysis of bacterial community structures, duplicate PCR reactions were amplified and pooled from each of the triplicate DNA extracts from a composite soil sample of 4 sampled sites. The duplicate amplicons were pooled, digested in parallel with *Hae*III, *Hha*I and *Msp*I (GE Healthcare) and analysed by T-RFLP (Cybergen, Huddinge, Sweden) as described by Edlund *et al.* (2006). The relative abundance of each terminal restriction fragment (TRF) was determined by dividing the area of the electropherogram fluorescent signal for the peak of interest by the total fluorescent signal area of peaks within the following

threshold values: lower threshold, 60 bp; upper threshold, 500 bp and a fluorescent threshold of 50. The TRF value corresponding to *E. coli* (TRF 498, using *Msp*1) was excluded because *E. coli* was the host for the fosmid vector used, and the relative abundances of the remaining peaks were then re-calculated for both the soil and the metagenomic library. TRFs were only included in the analyses if they were present in at least two of the three replicates.

For the analysis of chitinase genes, triplicate PCR products were digested in parallel with *Hae*III, *Hha*I and *Msp*I and analysed by T-RFLP (Uppsala Genome Centre, Uppsala, Sweden) as described by Hjort *et al.* (2007). Threshold values: lower threshold, 60 bp; upper threshold, 245 bp and a fluorescent threshold of 50 were applied. For assignment of possible chitinase genes, data from all three restriction enzyme digests were combined. The sizes of TRF's from T-RFLP analysis of chitinase genes of 18 bacterial isolates (antifungal and chitin degrading) from the suppressive soil described in the following section, were used as references for comparison of TRFs in the T-RFLP analysis of chitinase genes, in DNA from directly extracted soil and DNA from pooled metagenomic library. TRFs were only included in the analyses if they were present in at least two of the three replicates.

Sequencing of 16S rRNA genes and chitinase genes

16S rRNA genes were amplified from the bacterial isolates with the forward primer 27f (5´-AGA GTT TGA TCM TGG CTC AG-3´) and the reverse primer 1492r (5´-GGY TWC CTT GTT ACG ACT T-3´) using the same PCR conditions as for T-RFLP according to Hjort *et al.* (2007). The PCR products were sequenced with the 27f, 1492r and the 1378R (5´-CGG TGT GTA CAA GGC CCG GGA ACG-3´) primers. The partial chitinase gene was amplified with the same primer set as mentioned above for chitinase T-RFLP except that the forward primer was unlabelled. The chitinase PCR products from all three sources (directly extracted from soil, pooled metagenomic library and bacterial isolates) were separately purified (according to

manufacturer's instructions, Qiagene), ligated into a pCR 4-TOPO vector (Invitrogen) and transformed into competent cells (TOP10 Chemically Competent *E. coli*) as described by the manufacturer (Invitrogen). All sequencing was performed at the Uppsala Genome Centre, Sweden. The cloned partial chitinase genes were sequenced using the T7 primer (5´-TAATACGACTCACTATAGGG-3´). Sequence identities of 16S rRNA and chitinase genes were determined with Blast searches in GenBank (NCBI database). The chitinase sequences were aligned with ClustalW and a Neighbor Joining, best tree was constructed with the use of the software Macvector (http://www.macvector.com/index.html).

RESULTS

Soil microbiome metagenomic library

The soil microbiome metagenomic library comprised 7800 fosmid clones with insert sizes ranging between 20-40 kb with an average insert length of 30 kb covering an estimated 230 Mbp, calculated from the average insert sizes of the clones. It has been estimated that 1 g of soil may contain 4000 (Torsvik *et al*., 1990) to 50,000 species (Roesch *et al.,* 2007). Given an average genome size of at least 3.8 Mb (calculated from 220 fully sequenced bacterial genomes randomly selected from the Genomes OnLine Database), our metagenomic library could cover approximately 0.1% to 1.5% of the diversity in a typical soil.

16S rRNA gene sequences from bacterial isolates

16S rRNA genes (approximately 1310-1440 nt) of 18 bacterial isolates from the suppressive soil, that were previously shown to have the combined features of antifungal activity and the ability to degrade chitin (Adesina *et al.,* 2007), were PCR amplified and sequenced (Table 2,

Fig. 1). Sequence alignment to known sequences in GenBank showed that 11 of the isolates had closest identities to *Streptomyces spp.* and 9 of these isolates (Nr. IX to XVII) matched most closely to *S. clavifer* and/or *S. mutomycini*. The rest of the isolates showed closest matches to *Pseudomonas* (Nr. III and IV), *Stenotrophomonas* (Nr. V, VI and VII), *Bacillus pumilus* (Nr. I) and *Brevibacterium antarcticum* (Nr. II).

Chitinase gene sequences from isolates, the pooled metagenomic library and direct extracted soil DNA

From all isolates, except the strain identified as *Pseudomonas* sp. (Table 2; Nr. III), a chitinase specific PCR product could be amplified using specific bacterial chitinase primers. Most of the amplified PCR products were approximately 240 bp long, except for amplicons of 277 bp from two isolates with closest 16S rRNA gene identities to *B. antarcticum* (Nr. II) and *Pseudomonas* sp. (Nr. IV). Not unexpectedly, all chitinase gene sequences from *Streptomycetes* isolates showed closest identities to chitinase genes from *Streptomycetes*. Phylogenetic analyses showed that chitinase genes from the *S. mutomycini* and/or *S. clavifer* isolates clustered together with three different sequences in GenBank (uncultured bacterium clone control1, uncultured bacterium gene for chitinase and *Streptomyces* sp. An26) of chitinase genes (Fig. 1). All three *Stenotrophomonas* isolates (Nr. V, VI and VII) contained chitinase gene fragments with closest identities to a *Myxococcus xanthus* chitinase gene (Fig. 1). However, the chitinase genes from these isolates were also very similar (85, 95 and 96%) to a chitinase gene from a *Stenotrophomonas maltophilia* strain.

Clone libraries of chitinase gene fragments (240 bp) were also constructed from amplified PCR fragments of directly extracted soil DNA and pooled metagenomic library DNA. Sequences from both the clone library from directly extracted soil DNA (35 sequences) and the pooled soil metagenomic library (29 sequences) showed similarities to a diversity of

chitinase genes when aligned with known sequences in GenBank. None of the sequenced chitinase gene fragments were identical on a nucleotide level to each other or to any of the isolates.

Phylogenetic analysis (Fig. 1) showed that one sequence cluster contained only chitinase sequences from the metagenomic DNA library and sequences from isolates V, VI and VII (*Stenotrophomonas* spp.), all similar to a chitinase gene of *M. xanthus* USC7 (AY033407), and contained no soil derived chitinase genes. By contrast, another cluster contained only sequences from the directly extracted soil DNA and sequences from isolates I (*Bacillus*) and XII (*Streptomyces*) that showed highest similarities to a chitinase gene previously sequenced from an uncultured bacterium (AB361986) amplified from arable soil DNA (Terahara *et al.,* 2009). However, a third cluster contained chitinase gene sequences from the metagenomic library, the directly extracted soil DNA, the most dominant bacterial isolate *S. clavifer* and/or *S. mutomycini* and from two isolates of *Streptomyces viridochromogenes*. These sequences were similar (81-88% identity in nucleotide sequence) to the chitinase gene described as originating from an uncultured bacterium clone (AF455091), initially detected by molecular analysis of a chitinolytic bacterial community in chitin-containing bags buried in grassland sites (Metcalfe *et al.,* 2002). The distribution of the sequences within this latter cluster was relatively even and the cluster also contained chitinase sequences of the most dominant bacterial isolates, *S. clavifer* and/or *S. mutomycin* (Table 2). A fourth cluster was smaller and contained chitinase sequences from both directly extracted soil DNA and the metagenomic library with highest similarities (82-84% nucleotide identity) to a chitinase gene described as a different uncultured bacterial clone (AF484821) from the same chitinolytic community as that mentioned above (Metcalfe *et al.,* 2002).

T-RFLP of 16S rRNA genes and chitinase genes

A rapid screening of chitinase genes in the pooled metagenomic library (HMW DNA) and the directly extracted soil DNA was performed by T-RFLP analysis. The results showed a difference in TRF profiles between the DNA directly extracted from soil and the pooled metagenomic library DNA with an average of 42% shared TRFs between the T-RFLP profiles for all three enzymes (*Hha*I, *Hae*III and *Msp*I; not shown) (Table 1 and Fig. 4).

The most dominant TRF identified in the T-RFLP profiles of both directly extracted soil DNA and the pooled metagenomic library was TRF^{103} using *Hha*I (Fig. 4). A comparison of the T-RFLP profiles (*Hha*I) from the chitinase genes of the antifungal isolates showed that TRF^{103} corresponded to chitinase genes from the most common isolates from the suppressive soil (*S. mutomycini* and/or *S. clavifer*), that were previously demonstrated to have both antifungal and chitin degrading activity (Fig. 1). However, this pattern was not consistent for all 9 of these isolates (Table 2; Nrs. IX to XVII): two of the isolates did not have any detectable TRFs (Nrs. XII and XIV), and Nr. XI had a shorter TRF (TRF^{85}).

Other TRFs could be matched to some additional isolates. For example, TRF^{181} could be matched to chitinase genes from three isolates with closest identities to a *Stenotrophomonas* sp. (Nrs. V to VII). In addition, TRF^{75} could be matched to the chitinase genes of the *Pseudomonas* sp. (Nr. IV) and the *B. antarcticum* (Nr. II) isolates and these sequences were nearly identical (over 99% identical).

T-RFLP was also used to analyse the dominant 16S rRNA genes in the pooled fosmid library (Figs. 2 and 3). The results were compared with analyses of the bacterial community structure in directly extracted soil DNA and with the 16S rRNA gene sequences of the 18 isolates from the soil. The dominant 16S rRNA gene TRFs of the pooled metagenome indicated the presence of common soil bacteria such as *Bacillus*, *Paenibacillus*, *Nitrosomonas*, *Rhizobium* and *Clostridium* (Fig. 2). Representative TRFs of all of the bacterial isolates (based on *in vitro* digestion of their cloned 16S rRNA genes) could be

identified in both sources of DNA (pooled fosmid library and directly extracted soil DNA) with the exception of TRFs for the *Stenotrophomonas* isolates that were not detected in the directly extracted soil DNA. Although there were differences in many of the 16S rRNA gene TRFs detected in the DNA from the pooled fosmid library compared to the directly extracted DNA (Fig. 2B), approximately 30% of the TRFs were detected in both sources of DNA (Table 1, Fig. 3). This finding was enforced by the high agreement (high reproducibility) in the T-RFLP results obtained from replicate DNA samples that were obtained using both approaches. We could also conclude that the expected high level of *E. coli* contamination (host cell for the metagenomic library) although present, did not interfere with the analysis after subtraction (Figs. 2 and 3).

Nucleotide sequence accession numbers

The 16S rRNA gene fragment sequence data was submitted to GenBank under accession numbers EU864323 to EU864340 and the chitinase gene fragment data under accession numbers EU864341 to EU864421.

DISCUSSION

In this study we used a combination of approaches to screen for chitinase genes in a Swedish soil that was previously characterized to be suppressive to phytopathogens. We compared results obtained from a metagenomic DNA library to those obtained from direct extraction of DNA from soil. In addition, we investigated a number of isolates previously obtained from the same suppressive soil that were demonstrated to be antagonistic to phytopathogens and to have chitinase activity on agar plates (Adesina *et al.*, 2007). The different DNA sources

(pooled fosmid library, directly extracted soil DNA and bacterial isolate DNA), were screened for chitinase genes and 16S rRNA genes by T-RFLP and cloning and sequencing. To our knowledge, our study is the first to employ such a comprehensive set of comparisons to assess a specific function in soil.

Previously Ikeda *et al*. (2007) used T-RFLP and clone library analysis to assess chitinase genes in bulk and rhizosphere soil from a maize field. They found novels groups of bacterial chitinase genes and large differences in chitinase gene diversity between the bulk and rhizosphere soil. Metagenomics has previously been used to identify chitinase genes in aquatic environments (Cottrell *et al.,* 1999; LeCleir *et al.,* 2007). Cottrell *et al*. found that culture-dependent methods were inline with metagenomic estimations of bacterial communities capable of chitin degradation. This is in line with our results in soil where the chitinase genes of the isolates were well distributed among the clusters of sequences from both metagenomic and directly extracted soil DNA. Also, all of the isolate's 16S rRNA gene sequences corresponded to TRFs and were either represented in directly extracted soil DNA or in the pooled fosmid DNA.

Nearly all of the chitin-degrading isolates belonged to known genera with chitinase producing capacity, such as *Streptomyces* (Joo 2005), *Stenotrophomonas* (Zhang *et al.,* 2001), *Pseudomonas* (Kitamura & Kamei, 2003) and *Bacillus* (Watanabe *et al.,* 1990). The most common chitinase producing isolates (Adesina *et al.,* 2007) corresponded to *S. mutomycini* and/or *S. clavifer* and these bacteria also contained the most dominant chitinase gene variant (Fig. 4; TRF^{103}). In a previous T-RFLP analysis of 16S rRNA genes from the same suppressive soil we found that representatives of *Pseudomonas* and *Streptomyces* increased significantly in abundance after chitin was added to the soil (Hjort *et al.,* 2007) and *S. mutomycini* and/or *S. clavifer* was predicted to be the dominant species in both the total and active bacterial communities after chitin addition. In the present study we also found that

TRF^{159} that correlates to a *S. mutomycini* and/or *S. clavifer* 16S rRNA gene was present in and highly abundant in the soil. Taken together these combined results strongly suggest that the *S. mutomycini* and/or *S. clavifer* chitinase and 16S rRNA genes that we detected using molecular approaches correspond to some of the *Streptomyces* spp. isolates that we obtained from the same soil. These isolates, therefore, were most likely responsible for chitinase-production in the suppressive soil and they may have potential for biocontrol of some soil-borne fungal diseases.

Previous studies have shown that the soil we studied here contains bacteria that have the dual effect of growth inhibition of *Rhizoctonia* and production of chitinolytic activity (Adesina *et al.*, 2007). In addition, the same soil was previously classified as suppressive to clubroot disease caused by *Plasmodiophora*. Both the cell wall of *Plasmodiophora* spores and mycelia of *Rhizoctonia* contain chitin (Moxham & Buczacki, 1983, Bartnicki-Garcia, 1968), suggesting that chitinase activity would be a relevant tool in the antagonistic arsenal used against these phytopathogens. However, abiotic or other unknown biological factors could also be the cause for the suppressiveness.

Interestingly, the clone library analyses showed that some chitinase gene groups were specifically detected in different sources of DNA. For example, chitinase genes from two of our *Stenotrophomonas* sp. isolates were only detected in the metagenomic library whereas another group of chitinases were only detected in the directly extracted soil DNA. However, all bacterial isolates, except the *Stenotrophomonas* sp. isolates that were only detected in the metagenomic library, were represented in T-RFLP profiles from both sources of DNA.

The species prediction based on TRF length is not conclusive because more than one species can have the same TRF length, although three different restriction enzymes were used in this study to increase the predictive power of the analysis. In addition, previous

studies have shown that the T-RFLP technique does not detect all 16S rRNA genes present in a complex sample but identifies the most dominating populations, limiting the detection of rare populations (Benítez *et al.*, 2007; Bankhead *et al.*, 2004; Engebretson & Moyer,2003). However, the T-RFLP method is very reproducible and we have previously observed that this soil has a very similar T-RFLP temporal profile of 16S rRNA genes over different seasons (Hjort *et al.*, 2007; Hjort unpublished results).

Optimally, both sources (metagenome and directly extracted soil DNA) should contain the same chitinase and 16S rRNA gene profiles for the same soil samples. However, the DNA preparation procedure differed for these two approaches: i.e. harsh but efficient direct extract of DNA versus a gentle HMW extraction from extracted microbial cells for the metagenomic library construction. Also, there is more loss of DNA during preparation of the metagenomic library compared to directly extracted DNA. In addition, the efficiency of cloning of different sources of DNA, the ability of the vector and host to stably replicate the foreign DNA (Hårdeman & Sjöling, 2007; Riesenfeld *et al.*, 2004) or the potential toxicity of a cloned insert encoding molecules harmful to the host, if expressed, may be some of the factors contributing to the differences in composition of the DNA cloned into the fosmid library compared to the directly extracted DNA. Undoubtedly, we were primarily limited by low coverage with all sampling methods used and have only screened a small fraction of the diversity of the soil community.

Regardless of these technical limitations we demonstrated for the first time an impressive agreement between three very different screening techniques all of which pointed towards specific *Streptomyces* species that could play a role in suppression of fungi by chitinase production in soil. At the same time, due to different biases in the methods used we found different clusters of chitinase genes that were represented depending on the approach used. Therefore we can conclude that the combination of targeted molecular approaches

increases the information obtained and the reliability of the data. These results should lay the groundwork for making informed decisions about the appropriate source material to use in other studies that aim to screen for specific functional genes in environmental samples.

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Figure legends

Figure 1. Neighbour-joining tree based on ClustalW sequence alignment of the partial chitinase genes from clone library analyses of the soil, the pooled metagenomic library and from bacterial isolates. Clones from the pooled soil metagenomic library (blue) and clones from directly extracted soil DNA (red).

Figure 2. A. 16S rRNA gene analysed by T-RFLP (*Msp*I). TRFs from 16S rRNA genes amplified from DNA directly extracted from soil (red) compared to DNA from the pooled metagenomic library (blue). B. The TRF values corresponding to *E. coli* (host) were excluded and the relative abundance was recalculated. The graphs represent the average relative abundance from three replicates for each TRF.

Figure 3. 16S rRNA gene analysed by T-RFLP (*Msp*I). Comparison showing only those TRFs that were identified in both DNA sources; DNA from directly extracted soil (red) and extracted from the pooled metagenomic library (blue) without *E. coli* (host) TRFs. The graph represents the average relative abundance from three replicates for each TRF.

Figure 4. Chitinase genes detected by T-RFLP (*Hha*I). DNA from direct extracted soil (red) and the pooled metagenomic library (blue). The graph represents the average relative abundance from three replicates for each TRF.

Table 1. 16S rRNA and chitinase gene T-RFLP analyses of bacterial isolates, DNA from the metagenomic library and from directly extracted soil. Number of TRFs detected using different restriction enzymes. Number of isolates with their 16S rRNA or chitinase gene TRF size represented in the T-RFLP analysis of metagenomic library and directly extracted soil.

	16S rRNA gene			*Chitinase gene*		
	*Msp*I	*Hha*I	*Hae*III	*Msp*I	*Hha*I	*Hae*III
Soil metagenomic library (DNA of pooled clones)	98	24	34	53	45	42
Number of isolates represented in the metagenomic library	18/18	18/18	18/18	6/6[a]	13/13[b]	12/12[c]
Soil DNA (directly extracted)	93	62	82	54	77	58
Number of isolates represented in the soil DNA (directly extracted)	15/18	15/18	15/18	6/6[a]	11/13[b]	12/12[c]
Common TRFs between metagenomic and direct extracted DNA	32 33%	11 23%	20 34%	18 34%	31 51%	20 40%

[a]The total number of isolates was 6 due to two isolates without detected chitinase genes and ten isolates of the remaining were without TRF fragments in the detectable range of 60-240nt

[b]The total number of isolates was 13 due to two isolates without detected chitinase genes and three isolates of the remaining were without TRF fragments in the detectable range of 60-240nt

[c]The total number of isolates was 12 due to two isolates without detectable chitinase genes and four isolates of the remaining were without TRF fragments in the detectable range of 60-240nt

Table 2. 16S rRNA and chitinase genes from bacterial isolates with their respective similarities (nucleotide) to sequences in GenBank and TRF sizes of their chitinase genes

Isolate	Closest identity 16S rRNA gene	(% identity, over nt)	Closest identity chitinase gene	(% identity, over nt)	T-RF (*Hha*I) chitinase gene
I	*Bacillus pumilus* (DQ523500)	100%, 1434	Uncultured bacterium gene for chitinase (AB361986)	85%, 166	No fragment
II	*Brevibacterium antarcticum* (AJ577724)	99%, 1405	Uncultured organism clone ChiCSR29 (AY699345)	88%, 238	75
III	Pseudomonas sp (AY599710)	100%, 1414	No chitinase gene detected with PCR		
IV	Pseudomonas sp. (DQ279324)	99%, 1404	Uncultured organism clone ChiCSR29 (AY699345)	88%, 237	75
V	Stenotrophomonas sp. (AY131216)	99%, 1422	*Myxococcus xanthus* USC7-1p (AY033407)	86%, 239	181
VI	*Stenotrophomonas maltophilia* (AJ293470)	99%, 1431	*Myxococcus xanthus* USC7-1p (AY033407)	97%, 220	181
VII	*Stenotrophomonas maltophilia* (AJ293470)	99%, 1439	*Myxococcus xanthus* USC7-1p (AY033407)	97%, 219	181
VIII	*Streptomyces viridochromogenes* (AB184088)	100%, 1353	Uncultured bacterium clone control1 (AF455091)	87%, 196	103
IX	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1353	Uncultured bacterium clone control1 (AF455091)	88%, 200	103
X	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1354	Uncultured bacterium clone control1 (AF455091)	87%, 208	103
XI	*Streptomyces clavifer/ S.*	100%, 1378	Uncultured bacterium clone	87%, 162	85

	mutomycini (DQ026670, AB249951, AJ781357)		control1 (AF455091)		
XII	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1354	Uncultured bacterium gene for chitinase (AB361986)	85%, 198	No fragment
XIII	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1345	Uncultured bacterium clone control1 (AF455091)	87%, 172	103
XIV	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1353	Streptomyces sp. An26 (AJ968655)	89%, 196	No fragment
XV	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1353	Uncultured bacterium clone control1 (AF455091)	85%, 236	103
XVI	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1353	Uncultured bacterium clone control1 (AF455091)	86%, 199	103
XVII	*Streptomyces clavifer/ S. mutomycini* (DQ026670, AB249951, AJ781357)	100%, 1377	Uncultured bacterium clone control1 (AF455091)	85%, 175	103
XVIII	*Streptomyces viridochromogenes* (AB184088)	100%, 1352	Uncultured bacterium clone control1 (AF455091)	86%, 175	103

Figure 1

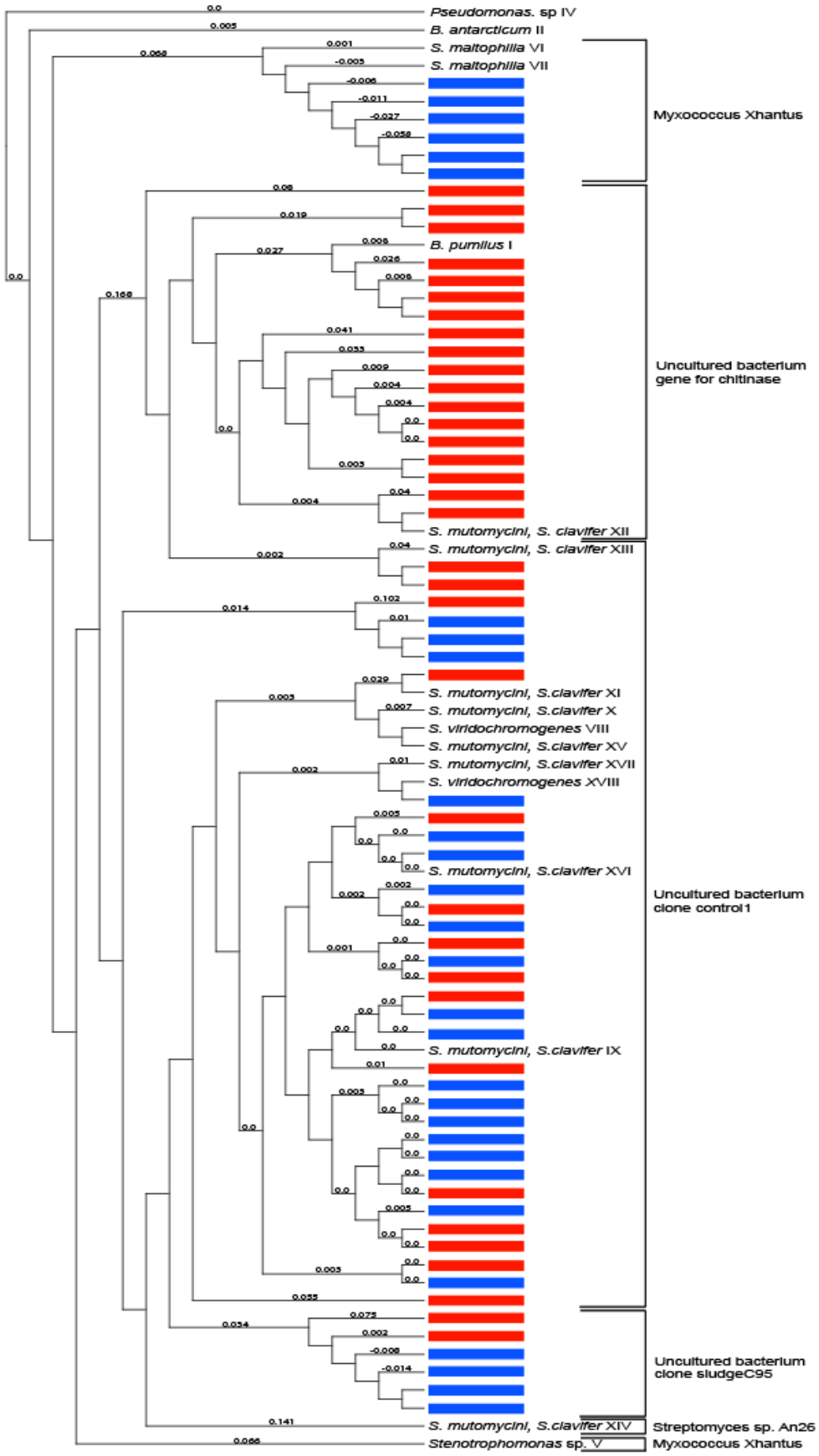

Pseudomonas. sp IV
B. antarcticum II
S. maltophilia VI
S. maltophilia VII
Myxococcus Xhantus
B. pumilus I
Uncultured bacterium gene for chitinase
S. mutomycini, S. clavifer XII
S. mutomycini, S. clavifer XIII
S. mutomycini, S.clavifer XI
S. mutomycini, S.clavifer X
S. viridochromogenes VIII
S. mutomycini, S.clavifer XV
S. mutomycini, S.clavifer XVII
S. viridochromogenes XVIII
S. mutomycini, S.clavifer XVI
Uncultured bacterium clone control1
S. mutomycini, S.clavifer IX
Uncultured bacterium clone sludgeC95
S. mutomycini, S.clavifer XIV
Streptomyces sp. An26
Stenotrophomonas sp. V
Myxococcus Xhantus

Figure 2A

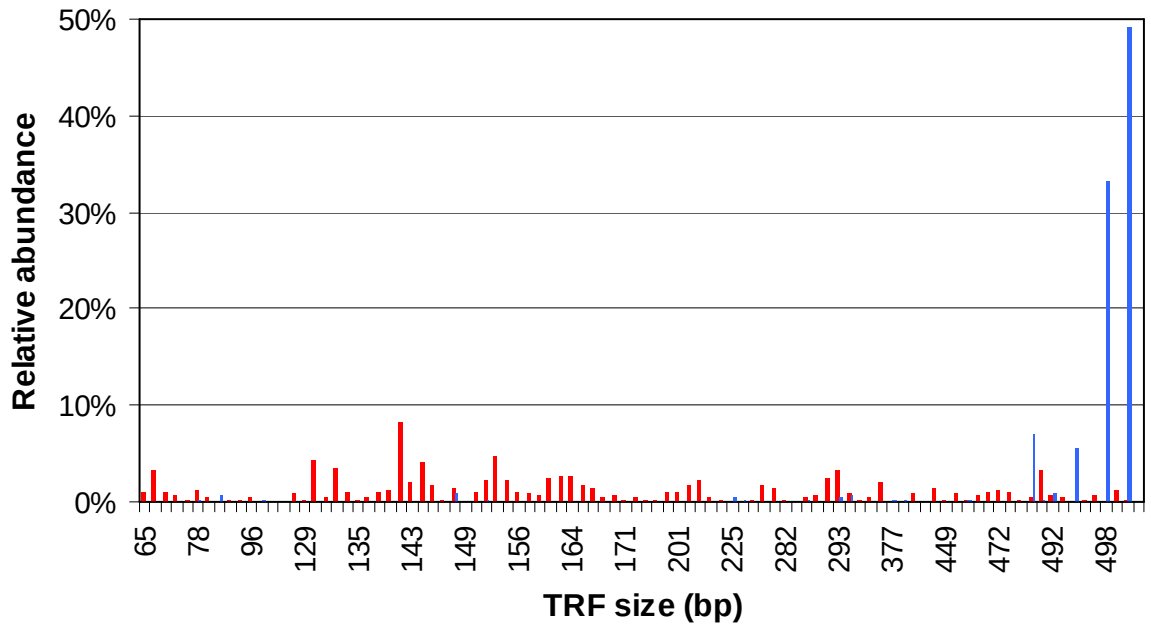


Figure 2B

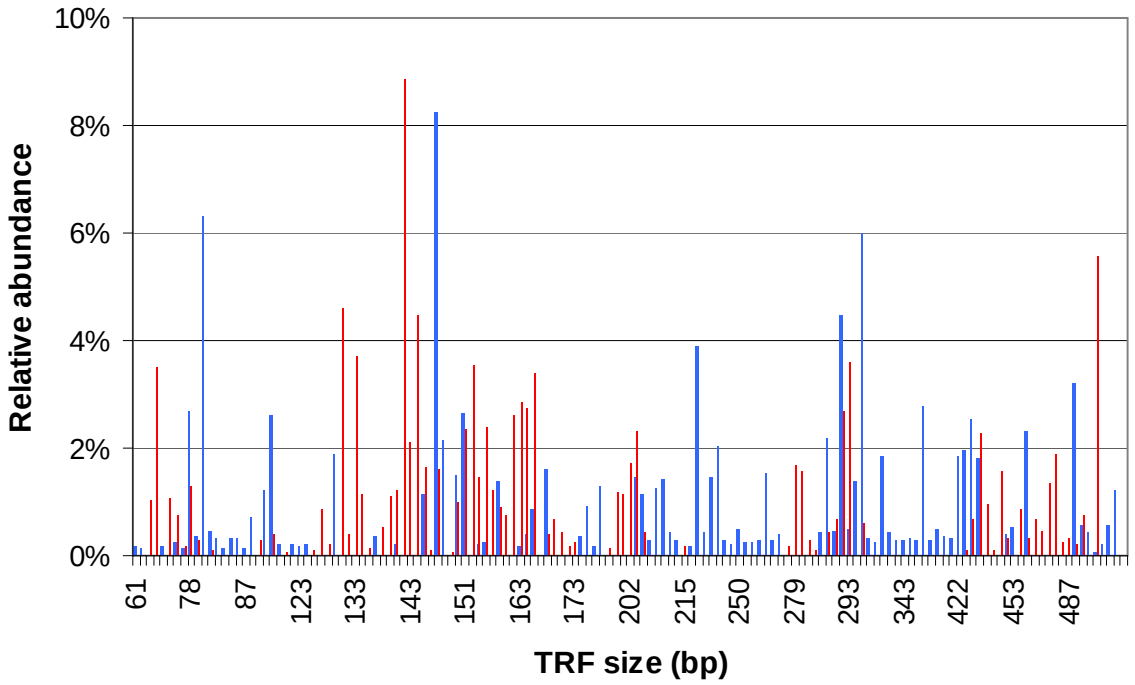


Figure 3

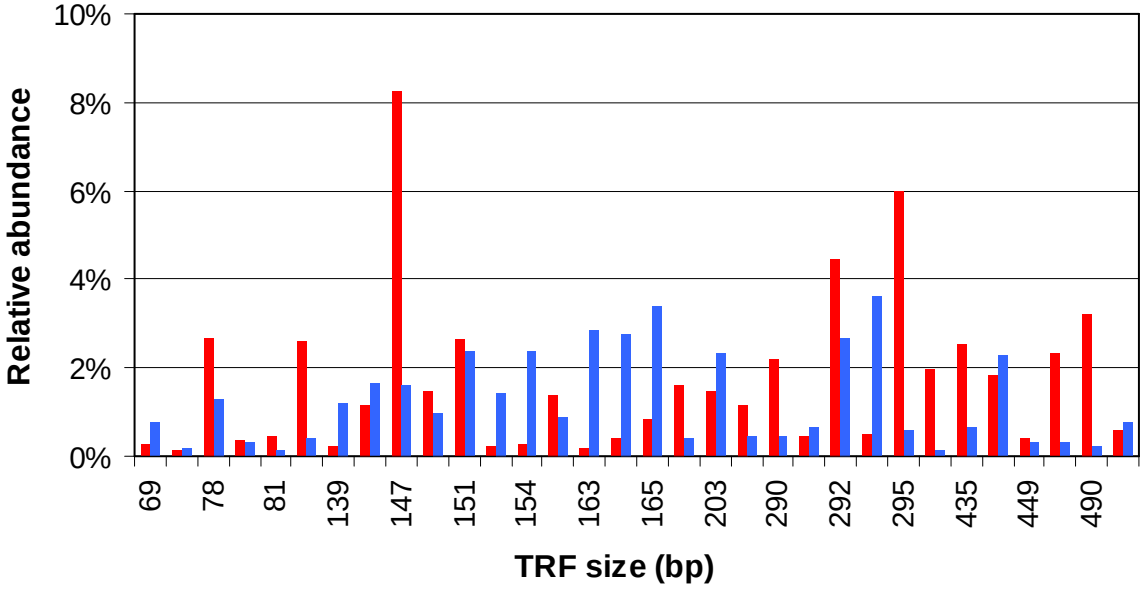
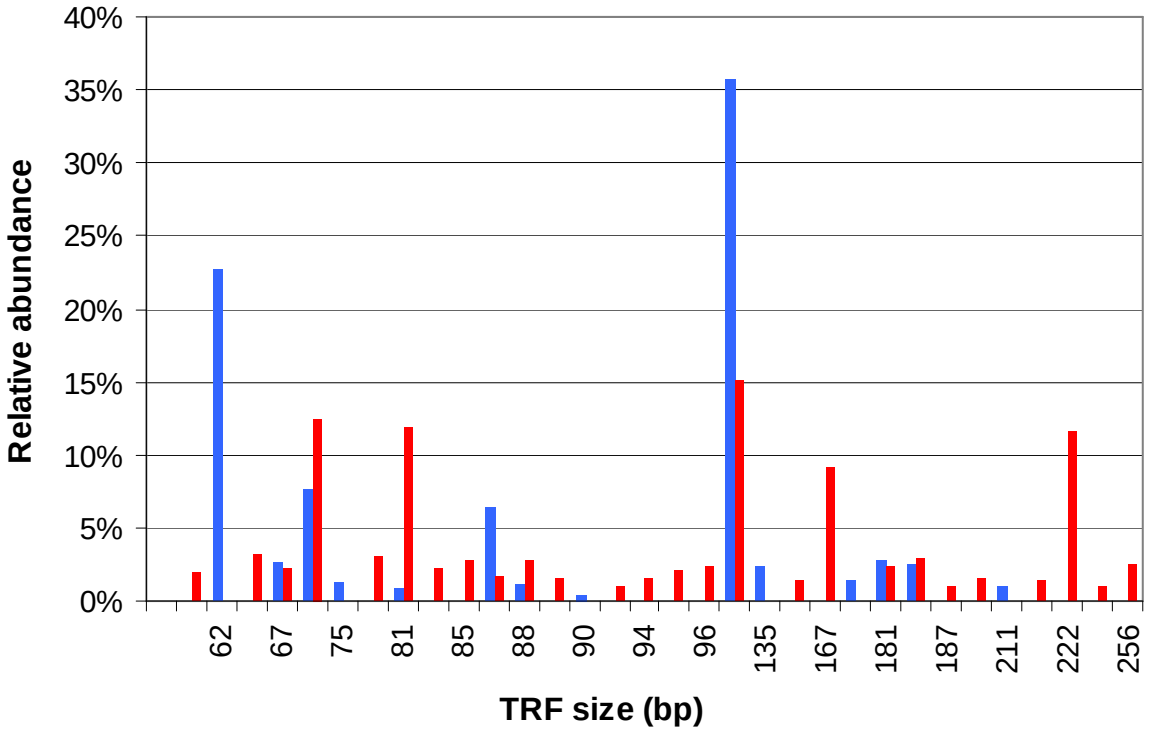

10%
8%
6%
4%
2%
0%
Relative abundance
69
78
81
139
147
151
154
163
165
203
290
292
295
435
449
490
TRF size (bp)

Figure 4.


40%
35%
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15%
10%
5%
0%
Relative abundance
62
67
75
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90
94
96
135
167
181
187
211
222
256
TRF size (bp)