ACTA
PROTOZOOLOGICA
Special issue:
Protists in Soil Processes

Acta Protozool. (2012) 51: 249–258
http://www.eko.uj.edu.pl/ap
doi:10.4467/16890027AP.12.020.0766

Effects of the Secondary Metabolite Producing *Pseudomonas fluorescens* CHA0 on Soil Protozoa and Bacteria

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Abstract. Bacteria producing secondary metabolites with antagonistic effects on fungal pathogens have received attention during the last decades as an alternative to chemical pesticides. They, however, might also have effects on indigenous soil organisms like bacteria and protozoa, the latter ones being among the most important grazers of bacteria in soil. The present study reports on the effect of the potential biocontrol agent *Pseudomonas fluorescens* CHA0 and its genetically modified derivative CHA0/pME3424 on indigenous soil bacteria and protozoa in a soil system. CHA0/pME3424 overproduces two of the secondary metabolites produced by CHA0: the polyketide antibiotics pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (DAPG). *P. fluorescens* CHA0/gfp1 and CHA0/pME3424 both negatively affected the abundance of soil bacteria and protozoa and the genetic community structure of Kinetoplastida studied by PCR-DGGE. The negative effects were detectable after 14 days but were decreasing and are expected to be temporary. The overproducer of secondary metabolites did not differ in effect from the wild type. The soil respiration and bacterial genetic community structure were not significantly affected. The study shows the soil bacteria and protozoa to be temporary affected by bacteria producing secondary metabolites, which can have implications for nutrient-cycling in soil and environmental risks of biocontrol agents.

Key words: BCA, secondary metabolite, *Pseudomonas fluorescens* CHA0, soil, protozoa, bacteria, PCR-DGGE.

INTRODUCTION

Together with nematodes, protozoa are the most important grazers of bacteria in soil and rhizosphere (Bonkowski *et al.* 2009). Bacteria have an interest in avoiding predation and do so by many different means such as escaping into small soil structures inaccessible to the larger protozoa, modifying size and external structures, forming microcolonies and biofilms hard to ingest and by producing secondary metabolites (Matz and Kjelleberg 2005, Wright *et al.* 1995). The production of secondary metabolites often involves compounds with negative effects not only on predators but also on fungi and plants (Hass and Keel 2003). Concomitantly, bacteria are being increasingly recognized as plant protection agents as some bacteria inhibit pathogenic fungi, nematodes and insects, acting as biocontrol agents (BCA) (Whipps 2001) as an alternative to chemical pesticides.

The mode of action of antagonistic bacteria may be competition for space and nutrients or production

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of various growth-inhibiting secondary metabolites (Whipps 2001, Winding *et al.* 2004). Not surprising, such inhibition may not be restricted to the target organisms, but may also affect other members of the soil microbiota (Girlanda *et al.* 2001, Winding *et al.* 2004, Johansen *et al.* 2005). Especially the production of secondary metabolites by *Pseudomonas* species has received attention and several different strains and species are being investigated for commercial use though only a few have so far been marketed (Winding *et al.* 2004 and references therein). It is known that different groups of bacteria are grazed differently by protozoa (Matz and Kjelleberg 2005, Rønn *et al.* 2002), depending on e.g. ability to ingest and digest the bacterial prey (Jezbera *et al.* 2005), nutritional status (Shannon *et al.* 2007), structures preventing predation (Pernthaler 2005) and community composition (Hahn and Höfle 2001). High sensitivity of protozoa to antifungal secondary metabolites produced by pseudomonads has been demonstrated both in studies including complex microbial decomposer communities in soil (Johansen *et al.* 2005) and in studies with pure cultures of amoebae, flagellates and a ciliate (e.g. Schlimme *et al.* 1999, Matz *et al.* 2004, Jousset *et al.* 2006, Pedersen *et al.* 2010). Based on these studies and the mode of action of the secondary metabolites, focus has turned towards prey-predator interaction between bacteria and protozoa (Jousset *et al.* 2008, Pedersen *et al.* 2011).

The present study aim to explore the results obtained in laboratory studies with a few prey and predator organisms further in a semi natural soil system. The objectives were to study the effect of the potential BCA *Pseudomonas fluorescens* CHA0 and its genetically modified derivative CHA0/pME3424 on indigenous soil bacteria and protozoa in a soil system. CHA0/pME3424 carries an additional copy of *rpoD* encoding the housekeeping factor σ^{70} and overproduces two of the secondary metabolites produced by CHA0: the polyketide antibiotics pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (DAPG), and other pleiotropic effects of the genetic modification cannot be excluded (Schnider *et al.* 1995). Growth inhibiting effects have not only been found for fungi and protozoa but also for plants (Maurhofer *et al.* 1995, Haas and Keel 2003), indicating low specificity of secondary metabolites produced by CHA0. We hypothesize that CHA0 will have a negative effect on abundance of protozoa and change genetic community of Kinetoplastida with the overproducer CHA0/pME3424 showing a higher negative effect. The protozoan subphylum of Kinetoplastida was chosen as a model group due to availability of primers and genetic tool and as this technique previously has shown effects of carbon-nanomaterials in the same agricultural soil (Johansen *et al.* 2008) and used to discover effects on the genetic composition of protozoa communities of soil amendments (Edel-Hermann *et al.* 2008). The overproducer CHA0/pME3424 is hypothesized to be less predated by the protozoa and showing better survival in the soil microcosms compared to CHA0/gfp1. Finally, we expect no long term effects on the indigenous bacteria due to the stability and resilience of the intact microbial community. The CHA0 was chosen as a model organism as it is a well-characterised potential BCA and derivatives with varying production of secondary metabolites were available.

Soil microcosms amended with barley straw and one of the following bacteria were introduced: either of the two derivatives of CHA0, the bacteria *Enterobacter aerogenes* or without addition of bacteria. Soil microbial respiration, culturable soil bacteria, survival of CHA0/gfp1 and CHA0/pME3424, and culturable protozoa were followed and the genetic diversity of the Eubacterial community and the protozoan order of Kinetoplastida in the soil were determined by PCR-DGGE.

The outcome of the study will provide input to the bridging of results obtained in liquid culture systems with few prey and predator species to the complex processes in soil ecosystems. Furthermore, the results are valuable for environmental risk assessment of BCA.

MATERIAL AND METHODS

Bacteria

Two derivatives of *Pseudomonas fluorescens* CHA0 were used: *P. fluorescens* CHA0/gfp1 that is resistant against kanamycin and ampicillin while the production of secondary metabolites is unchanged (Koch *et al.* 2001, Natsch *et al.* 1994) and *P. fluorescens* CHA0/pME3424 that carries an additional copy of *rpoD* encoding the housekeeping factor σ^{70} and overproduces the antibiotics pyoluteorin (Plt) and 2,4-diacetylphloroglucinol (Phl) and is resistant against tetracycline and kanamycin (Schnider *et al.* 1995). CHA0 has been isolated from a suppressive soil and has been shown to inhibit a range of plant pathogenic fungi (e.g. Keel *et al.* 1990). *Enterobacter aerogenes* SC (Christensen and Bonde 1985) without known antagonistic activity was used as a positive control, as it is a good food source for soil protozoa (Pedersen *et al.* 2009).

Soil

The soil was a sandy loam soil from Snubbekorsgård (field 26), Faculty of Life Sciences, Copenhagen University, Taastrup, Denmark. The soil was sampled in August 2005, sieved (2 mm mesh)

and stored less than 2 weeks before the experiment. The dry matter content of the soil after sieving was 88.6%.

Microcosms

The experiment included treatments varying in introduction of bacteria: (A) control with no added bacteria, (B) introduction of *Enterobacter aerogenes* SC, (C) introduction of *Pseudomonas fluorescens* CHA0/gfp1 and (D) introduction of *Pseudomonas fluorescens* CHA0/pME3424. Bacteria were grown over night in 25 ml of Tryptic Soy Broth (3.0 g l^{-1} TSB) at 200 rpm and room temperature, washed twice in Amoebae Saline (AS, Darbyshire *et al.* 1974) and resuspended in 10 ml soil extract (1 g dw soil and 100 ml MQ water, blended 1 min. at maximum speed in a Waring blender, centrifuged 10 min. at high speed).

Bacterial suspensions, shredded barley straw (1.125 mg g^{-1} dry soil) and additional sterile MQ water to adjust the dry matter content to 77.7% were added to soil portions of 100 g dw and mixed carefully. The equivalent of 10 g dry weight soil was weighed into 116 ml serum bottles. After capping with Teflon-lined rubber stoppers the bottles were incubated at 18°C in the dark.

At harvest a soil suspension was prepared by mixing 5 g dw^{-1} soil with 100 ml AS for 1 min. in a Waring blender (Waring, NY, USA) at maximum speed and used for analysis of bacteria and protozoa. Nine replicates of each treatment were set up, totalling 36 microcosms. Triplicates of each treatment were harvested after 1, 7 and 14 days. At the start of the experiment, protozoa were enumerated only in the control without additions of bacteria.

Soil microbial respiration

Accumulated microbial respiration was measured several times during the 14 day incubation period by sampling 100 µl of head space gas and analyzing its content of CO_2 on a Shimatzu GC-8A gas chromatograph equipped with a Porapack-Q column and a thermal conductivity detector at an oven temperature of 40°C (Hendriksen 1997). The concentration of CO_2 was determined by comparing the obtained areas to a CO_2 standard curve.

Enumeration of protozoa

The number of protozoa able to grow on indigenous soil bacteria was determined by the most probable number method as described by Darbyshire *et al.* (1974) and modified by Rønn *et al.* (1995). In brief, triplicate micro-titre plates (Nunc A/S, Roskilde, Denmark, cat. no. 260860) were filled with 100 µl 0.3 g/L TSB in each well. Fifty µl soil suspensions with protozoa were added to the initial wells and the subsequent wells inoculated with a threefold dilution series of this suspension. The plates were incubated at 18°C in darkness and the wells inspected for positive growth of protozoa after one week (fast-growing protozoa) and three weeks (total number of growing protozoa) using a microscope (Olympus, inverted microscope, 40 ×, Olympus Europe, Hamburg, Germany) as described by Pedersen *et al.* (2009). Numbers of protozoa (active and cysts) were estimated using a MPN calculation method (Briones and Reichardt 1999). The limit of detection, based on one well with detectable growth at the lowest dilution, was calculated to 157 protozoa g^{-1} dw soil.

Enumeration of bacteria

At each sampling after 1, 7, and 14 days, three dilutions of each soil suspension were plated (in triplicate) on Tryptic Soy Agar (TSA) containing TSB, Delvocid (100 mg/L containing 50% natamycin and 50% lactose (Delvocid® Instant, DSM Food Specialties, Delft, The Netherlands), dissolved in 10 ml methanol l^{-1}) and solidified with agar (15 g l^{-1}). The plates were incubated at 18°C in the dark. The number of new emerging colonies was counted by inspecting the plates repeatedly during 22–35 days. *P. fluorescens* CHA0/gfp1 and CHA0/pME3424 were selectively cultured on the same media with either kanamycin (25 µg ml^{-1}) and ampicillin (100 µg ml^{-1}) or tetracycline (125 µg ml^{-1}), respectively, by drop-plating, and the CFU were counted after 3–4 days at 18°C. The number of introduced bacteria was also counted prior to introduction to soil.

DNA extraction

At all sampling events, 3 × 1 g moist soil from each experimental unit were weighed into 2-ml Eppendorf tubes and 1ml TE buffer was added. The samples were stored at –80°C. DNA was extracted with FastDNA®SPIN®Kit for soil (Q-Bio gene, catalogue number 6560-200; B10101, Carlsbad, CA, USA).

PCR-DGGE of Eubacteria and Kinetoplastida

In separate runs, PCR was performed with the primers GC341F and 907R (DNA Technology A/S, Århus, Denmark) (Muyzer *et al.* 1998) amplifying 16S rDNA of Eubacteria and the primers Kin24SF and Kin24SR (TAG Copenhagen A/S, Copenhagen, Denmark) (Rasmussen *et al.* 2001) amplifying 18S rDNA from the phylogenetic group of Kinetoplastida. The primers GC341F and Kin24SR each had a GC-clamp added. Each PCR tube contained a total volume of 25 µl, with 12 µl DNase-free water, 2 µl of $MgCl_2$ (25 m*M*), 2.5 µl 10 × PCR buffer ($+NH_4SO_4$ – $MgCl_2$), 2.5 µl bovine serum albumin (30 mg/ml), 2.5 µl deoxynucleoside triphosphate (0.25 m*M*), 1 µl each of primer, either GC341F and 907R (5 pmol/µl) or Kin24SF and Kin24SR (5 pmol/µl), 0.5 µl Taq DNA polymerase (1 U/µl), 0.5 µl Taq DNA polymerase (1U/µl, Fermentas, Helsingborg, Sweden) and 1 µl sample as template. For the Eubacteria the PCR conditions were based on Muyzer *et al.* (1998) and optimized for our conditions: 5 min. at 94°C; 35 cycles of 1 min. at 94°C, 1 min. at 56.5°C, 3 min. at 72°C; 10 min. at 72°C; and final cooling at 4°C. For the Kinetoplastida the PCR conditions were based on Rasmussen *et al.* (2001) and optimized to our conditions: 5 min. at 94°C; 35 cycles of 1 min. at 94°C, 1 min. at 60°C, 1 min. at 72°C; 10 min. at 72°C; and final cooling at 4°C. The size of PCR products was checked on 1.5% agarose gels. The PCR product was purified using the kit QIAquick®PurificationKit (Qiagen, Hilden, Germany). DNA was quantified after staining with the epifluorescent dye PicoGreen in a Fluostar microplate reader (BMG Labtech, Offenburg, Germany) (Riemann and Winding 2001).

A denaturing gradient gel electrophoresis was performed using a 7.5% acrylamide gel (ratio of acrylamide to bisacrylamide 19:1) with a 30 to 70% denaturing gradient for the Eubacteria and a 30 to 50% denaturing gradient for the Kinetoplastida, using urea and formamide as denaturing agents according to the Bio-Rad manual (Bio-Rad Lab, CA, USA). Forty ng PCR product of Eubacteria and 50 ng PCR product of Kinetoplastida were loaded into each well on the respective gels. Denaturing gel electrophoresis was performed at 60°C and 70 V for 17 h by using 1 × TAE buffer

and the D Gene System (Bio-Rad Lab). The gels were stained with SYBR Gold (10000 × concentrate, Molecular Probes, Taastrup, Denmark) for 1 h and photographed. The number of bands and their position on the DGGE gels were analyzed and dendrograms were created based on Dice coefficient of similarity (Dice 1945) and the unweighted pairwise grouping method using arithmetic means (UPGMA) by Dendron® (Solltech, Oakdale, IA, USA).

Statistics

Statistical significant differences ($p < 0.05$) in respiration, abundance of colony forming units and protozoa were tested by One Way ANOVA followed by all pairwise multiple comparison procedure (Student-Newman-Keuls method) or by a General Linear Model Two-way ANOVA using SigmaStat 1.0 (Jandel Sci., Erkrath, Germany). Statistical significant differences between number of bands on the DGGE gel were tested by Two Way ANOVA followed by all pairwise multiple comparison procedure (Holm-Sidak Method) using Sigma Plot 11.0 (Jandel Sci., Erkrath, Germany). When necessary the data were log transformed to obtain normal distribution. The increase in CFU during plate incubation was not normal distributed even after transformation. Therefore only the normal distributed final number of CFU was tested for significant differences.

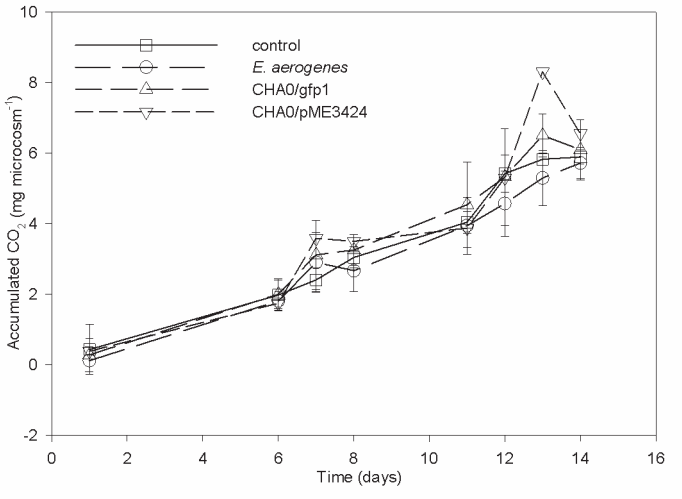

Fig. 1. Soil respiration measured as accumulated CO_2 during the incubation of microcosms consisting of soil, shredded barley straw and either of three bacterial inoculants: *E. aerogenes, P. fluorescens* CHA0/gfp1, *P. fluorescens* CHA0/pME3424. Control treatment did not receive any bacteria.

RESULTS

Soil respiration

Soil respiration estimated as accumulated CO_2 in the headspace during 14 days of incubation was generally unaffected by introduction of bacteria and after 14 days the CO_2 developed in the microcosms was not significantly different when tested by one-way ANOVA (Fig. 1).

Culturable bacteria

The number of introduced bacteria in the microcosms was 4.1×10^7 CFU g^{-1} dw soil of *E. aerogenes*, 7.3×10^7 CFU g^{-1} dw soil of *P. fluorescens* CHA0/gfp1, and 6.4×10^7 CFU g^{-1} dw soil of *P. fluorescens* CHA0/pME3424. Already after 1 day the CFU of CHA0/gfp1 had declined to half while the CFU of CHA0/pME3424 was unchanged. After 7 days the abundance of culturable CHA0 of both strains had declined to 9–33% of inoculum declining further to 4–10% of the inoculum density to 3–7 × 10^6 CFU g^{-1} dw soil after 14 days (Fig. 2). Linear regression analysis followed by t-testing of the surviving numbers (log-transformed) of the two CHA0 strains extracted from soil between day one and 14 showed no significant difference in the decay rates (The decay rate of CHA0/gfp1 was –0.0815 ± 0.0098, and of CHA0/pME3424 –0.0768 ± 0.0049). Selective culturing of *E. aerogenes* was not possible.

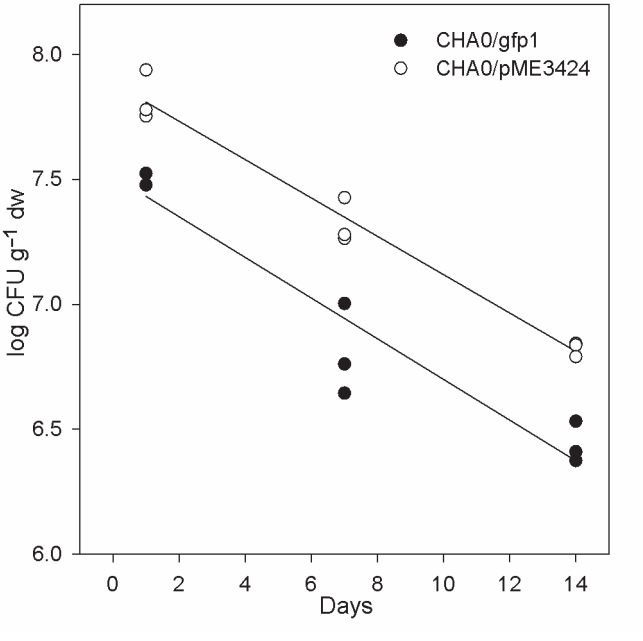

Fig. 2. Fate of inoculated *P. fluorescence* CHA0/gfp1 and *P. fluorescens* CHA0/pME3424 during incubation in soil microcosms determined as CFU on selective agar media (see Materials and Methods for selective agents). The individual data points for each replicate are shown along with the linear regression line for each strain.

The total number of CFU was counted repeatedly during incubation and the colony forming curves showed the control soil having slower colony appear-

ance at the harvest after 1 day (Fig. 3). We believe the faster colony appearance in microcosms with introduced bacteria to be due to the introduced bacteria being readily culturable and forming colonies on the non-selective medium. At the final counting after 36 days there was no significant difference in CFU. To reduce effect of inhibition on the plate, the same dilution was spread on all plates and the number of colonies on each agar plate varied from 33–69 colonies on plates from the control treatments and from 13–38 colonies on plates from CHA0 treatments. This indicates that competition on the plates would be higher in the control treatments. Despite no statistical testing could be performed on the colony forming curves there was a tendency of the colony forming curves to be different in microcosms receiving CHA0 strains and harvested after 7 days and in the microcosm receiving CHA0/pME3424 harvested after 14 days. At the harvest after 7 days, the CFU in the control soil and the soil with *E. aerogenes* were not significantly different in final number, while CFU in soils with either CHA0 strain were significantly lower. At harvest after 14 days, still CFU in the soil with CHA0/pME3424 was significantly lower at the final counting than the other three soils which were not significantly different.

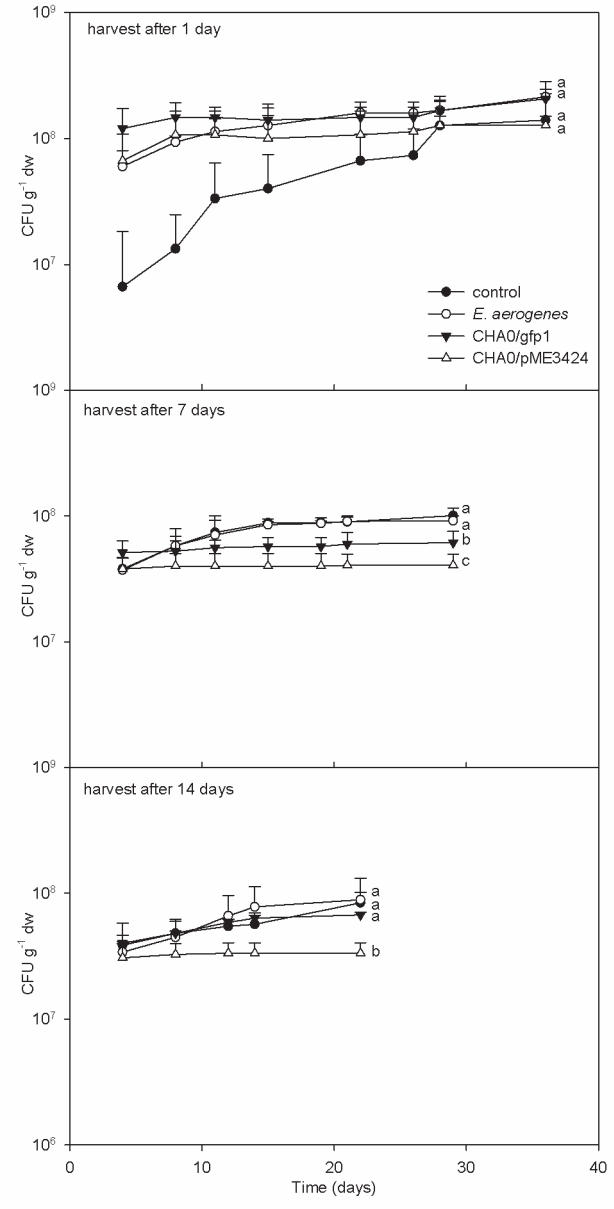

Fig. 3. Colony forming curves of culturable bacteria in soil microcosms harvested after 1, 7, and 14 days on non-selective agar media. For each harvest event the same plates were counted repeatedly. Statistical significant differences between the treatments at the last counting event of each harvest are indicated by different letters.

Enumeration of protozoa

The number of fast-growing protozoa was counted after 1 week incubation and the total number of culturable protozoa was counted after 3 weeks incubation in all microcosms harvested after 7 and 14 days (Fig. 4). Protozoa in the control microcosm was also counted at the harvest at 1 day. Both the fast-growing and the total number of protozoa increased significantly ($p < 0.05$) in the control microcosm without introduced bacteria between day 1 and day 7 without any further significant increase during the second week. This shows that the protozoa thrived in the microcosms, probably due to increased food supply based on the shredded barley straw added to all microcosms. The total number of protozoa was at all times higher in the control microcosm without any introduced bacteria compared to the microcosms with introduced bacteria. The introduction of both pseudomonads had significant detrimental effects on viable protozoa as after 7 days no fast growing protozoa was detected and only few total culturable protozoa was detected in the microcosm with CHA0/gfp1. Interestingly, after 14 days the viable fast-growing protozoa had caught up and were no longer significantly different from the control and the microcosm inoculated with *E. aerogenes,* while after 14 days the total number of protozoa was significantly different from the control microcosms without introduced bacteria but not different from the microcosms inoculated with *E. aerogenes*.

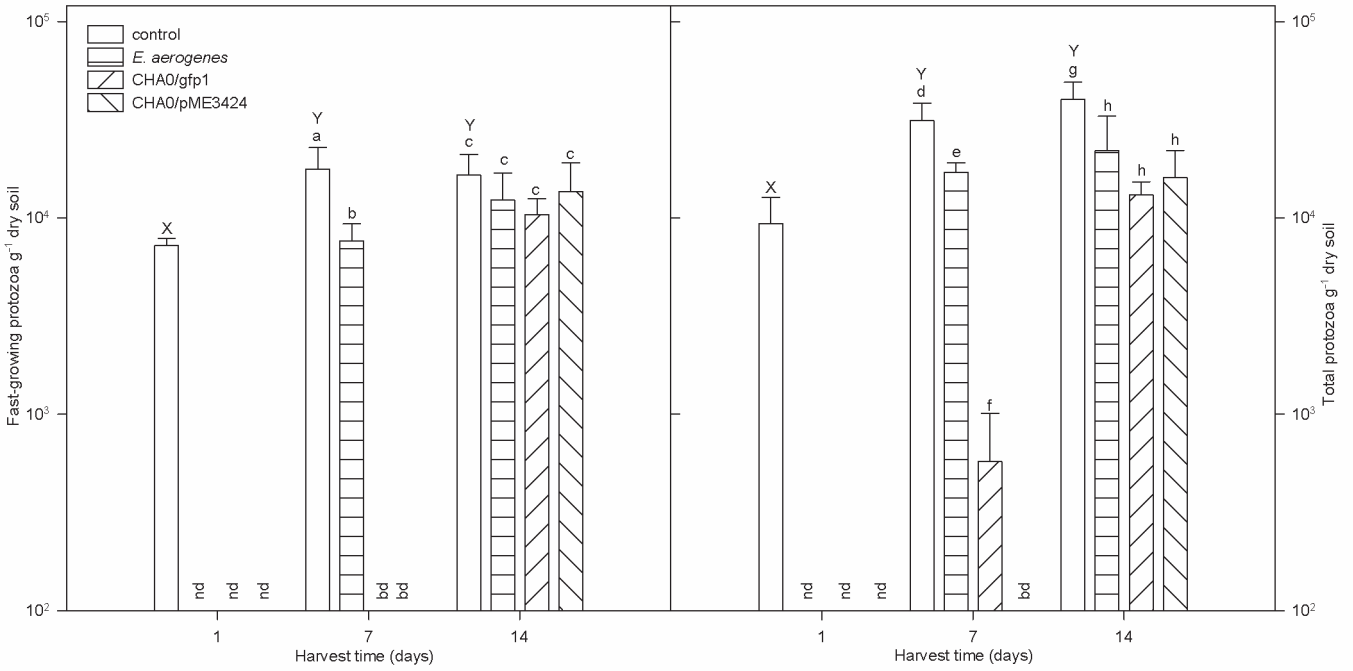

Fig. 4. Abundance of culturable protozoa in the four different soil microcosms. The protozoa were counted by MPN as fast-growing protozoa after 1 week of incubation and as total protozoa after 3 weeks of incubation by inspecting the same plates twice. Significant differences of treatments within each sampling time and incubation time are shown as different small letters above the bars. After one day protozoa was only counted in the control microcosm. Significant differences between the abundance of protozoa in the control microcosm are shown as capital letters. bd: below detection limit of 157 protozoa g^{-1} dw. nd: not determined.

Genetic diversity

Analysis of bacterial genetic diversity by PCR-DGGE showed numerous bands but no changes as a function of incubation time or introduction of bacteria (data not shown). The genetic diversity of Kinetoplastidae, a phylum of protozoa, showed a high similarity (> 80%) of all samples (Fig. 5). Introduction of either CHA0 strain immediately increased the similarity of the protozoa after one day in the soil microcosms. After 7 and 14 days the similarity of the Kinetoplastidae communities in all samples were increased and the effect of CHA0 strains reduced. No difference was observed between the effects of CHA0/gfp1 and CHA0/pME3424. Two way ANOVA of number of bands on the DGGE gel showed the control microcosm to have significantly more bands after 1 day of incubation compared to the microcosms with introduced bacteria while these were not significant different from each other (Fig. 6). This difference between treatments was not evident after 7 and 14 days. The number of bands in the control microcosm without inoculated bacteria decreased over time, increased when *E. aerogenes* was inoculated, and increased for the treatment with the two pseudomonads after 7 days followed by a decrease after 14 days not significantly different from 1 day. Each band is supposed to represent a genetic entity, so the Kinetoplastidae community has a higher number of genetic entities in the control microcosm at the start of the experiment, generally followed by an increase in genetic entities after 7 days when bacteria were inoculated and finally ended at a number after 14 days which showed no differences between treatments.

DISCUSSION

The soil respiration measured as CO_2 developed during the experiment reached approximately 3%, corresponding to 6.5 mg CO_2 or 1.8 mg C-CO_2 per microcosm. In similar soil microcosms with added barley straw Ekelund *et al.* (2003) found 1.4 mg C-CO_2 being produced during 15 days. The produced CO_2 cor-

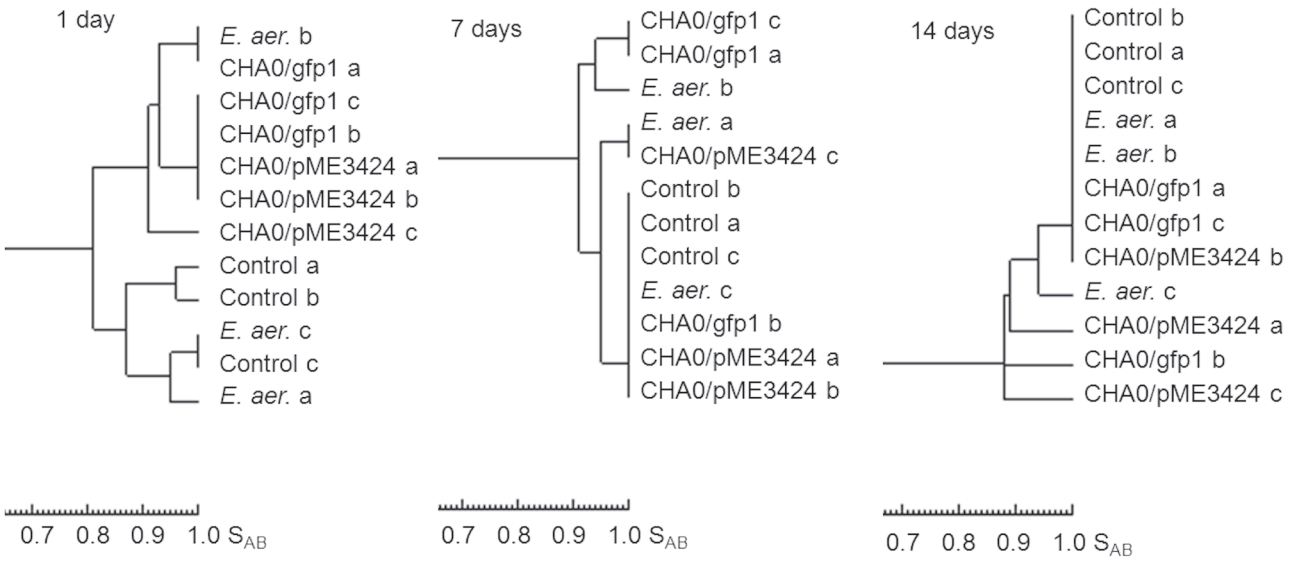

Fig. 5. Genetic diversity of the protozoa belonging to the Kinetoplastida. A dendrogram is constructed for each incubation time of the soil microcosms. The scale is the similarity index (S_{AB}). *E. aer.*: *Enterobacter aerogenes*.

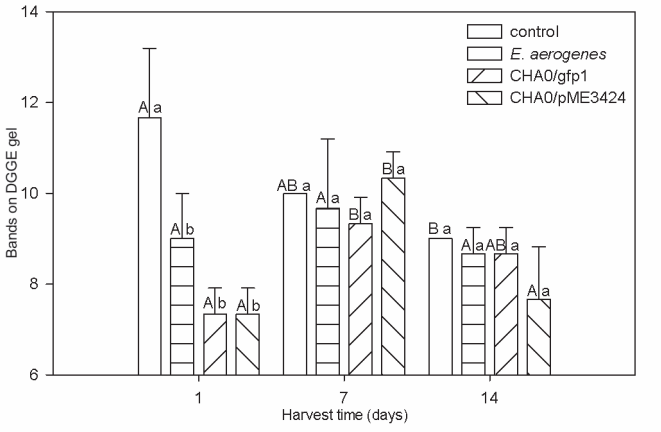

Fig. 6. The average number of bands detected on the PCR-DGGE gel of Kinetoplastida. Capital letters indicate significant differences between sampling time of the same treatment. Lower case letters indicate significant differences between treatments within the same sampling time.

responded to 36% of added C in barley straw assuming 0.5 g C g^{-1} straw, or 0.8% of C in introduced bacteria assuming an average bacterial cell weight of 3×10^{-13} g and 20×10^{-15} g C $cell^{-1}$. Hence, it is not surprising that developed CO_2 was largely independent of introduction of bacterial biomass (Fig. 1).

The abundance of *P. fluorescens* CHA0/gfp1 and *P. fluorescens* CHA0/pME3424 declined during incubation as is often seen for introduced bacteria generally and as also reported earlier for CHA0 (e.g. Natsch *et al.* 1998). This is generally believed to be due to lack of nutrients compared to the relatively rich bacterial growth medium used for preculturing. In addition, predation by e.g. protozoa and nematodes and competition with indigenous bacteria in the soil ecosystem might also lead to a decline. CHA0/pME3424 producing extra secondary metabolites survived just as well as the CHA0/gfp1 which was also found by Natsch *et al.* (1998) and Schnider *et al.* (1995). Jousset *et al.* (2008) showed that CHA0 wild type survived better in the presence of a single predator than a mutant deficient in production of secondary metabolites. Our results also show that CHA0 directly or indirectly negatively affected the abundance of culturable bacteria as well as the abundance of protozoa. Protozoa have been shown generally to grow poorly on CHA0 and selectively not to graze on CHA0 due to production of secondary metabolites, whereas the nematode *Caenorhabditis elegans* has been shown to predate CHA0 (Jousset *et al.* 2006; Pedersen *et al.* 2009, 2011). In our experiment with intact soil communities bacterial predators as nematodes will be present. It is a possibility that the

reduction of CHA0 can partly be due to predation by protozoa or other predators resistant to secondary metabolites in addition to death due to the harsh conditions for survival in soil.

The genetically modified *P. fluorescens* CHA0/pME3424 led to a higher decrease in total culturable bacteria and protozoa compared to CHA0/gfp1. Differential or increased antifungal effects of CHA0/pME3424 compared to the wild type of CHA0 have been reported earlier (e.g. Girlanda *et al.* 2001) while others did not find different effects (e.g. Natsch *et al.* 1997, 1998). The effect of CHA0 on CFU after 7 and 14 days (Fig. 3) is comparable to Naseby *et al.* (2001) while Natsch *et al.* (1998) found no effects on culturable rhizosphere bacteria after 10 and 52 days of inoculation with CHA0. Effects of inoculated BCA on colony forming curves have been reported (Naseby and Lynch 1998) while reports of effects on bacterial community structure are variable (Winding *et al.* 2004). As found earlier with other potential BCAs (Sigler *et al.* 2001, Thirup *et al.* 2003), we found no effect of the BCA on the bacterial community by PCR-DGGE. Hence, the PCR-DGGE of bacteria appears to be less sensitive to changes compared to CFU.

The most drastic negative effect of introduced bacteria found in our study was on the abundance of the viable protozoa. All three introduced bacteria had a significant negative effect on the number of culturable protozoa and number of genetic entities of Kinetoplastida in the soil microcosms with the two CHA0 strains showing a more drastic effect than *E. aerogenes.* Rønn *et al.* (2001, 2002) also found effects of introduced bacteria on the microbial communities. However, the negative effects seemed to be temporary. After 14 days, the effect on the culturable bacteria and the fast-growing protozoa was insignificant, while the negative effect on the total culturable protozoa was still significant compared to the control, while not significant different compared to the effect of *E. aerogenes.* The latter might be due to low palatability of *E. aerogenes* in situ despite the high palatability found in pure culture studies (Pedersen *et al.* 2009, 2011). Both CHA0 strains also seemed to initially reduce the genetic diversity of the Kinetoplastida in soil while no effect was found on the bacterial genetic diversity. This effect was not evident after 7 and 14 days.

In previous experiments studying the effect of CHA0 and other potential BCA of the genus *Pseudomonas* on protozoa in liquid cultures with individual protozoa and a few test bacteria comparable results were found (Andersen and Winding 2004; Jousset *et al.* 2006; Pedersen *et al.* 2010, 2011). We believe it is an effect of the specific features of the strains producing secondary metabolites or pleiotropic effects as other *P. fluorescens* strains have been shown to be good food bacteria for soil protozoa in pure culture (Andersen and Winding 2004; Pedersen *et al.* 2010, 2011). The negative effect of CHA0 in our experiment was followed by growth of the indigenous protozoa. These protozoa must either be tolerant to the secondary metabolites or produced by CHA0, or able to avoid the metabolites and bacteria in the soil matrix as the number of CHA0 decreased. Growth of tolerant protozoa and bacteria as well as reduction in secondary metabolite production due to decrease in abundance of CHA0 might explain the decreasing effect of the CHA0 and the apparent resilience of the soil bacterial genetic community structure and soil respiration. In contrast, Johansen *et al.* (2005) found the introduction of *P. fluorescens* DR54 to promote the abundance of soil and rhizosphere protozoa in plant microcosms. This might be due to differences in the secondary metabolites of the specific *P. fluorescens* strains as DR54 produces a cell bound viscosinamide (Nielsen *et al.* 1999) while CHA0 produces a range of metabolites both free and membrane bound (Haas and Keel 2003).

The observed effect of CHA0 on the genetic diversity of the community of Kinetoplastida one day after introduction indicates immediate negative effects on this subphylum of protozoa and corresponds with the findings of Pedersen *et al.* (2011) of Bodonidae being sensitive to secondary metabolite producing bacteria. These negative effects were relieved after 7 and 14 days or dominated by other effects like increased protozoan growth based on microbial activity associated with the introduced shredded barley straw. This is out of phase with the negative effect observed on the abundance of culturable protozoa after 7 and 14 days. With the major impact on the culturable protozoa it is surprising that the effect on the genetic diversity of Kinetoplastida was so small and might be due to Kinetoplastida being only a smaller part of the total diversity of bacterial feeding protozoa in soil. Other culturable protozoa seem to have been affected for a longer period by the secondary metabolite producing bacteria.

As discussed by Standing *et al.* (2007) the scaling up of experiments from laboratory cultures to the field is difficult. We consider the used soil microcosms to be a useful step on the way and showed that even in the more complex soil system of our microcosms CHA0 negatively affected the soil protozoa.

CONCLUSION

P. fluorescens CHA0/gfp1 and the overproducer CHA0/pME3424 both negatively affected the abundance of soil bacteria and protozoa and the genetic community structure of Kinetoplastida in the soil microcosms while soil respiration and bacterial genetic community structure were unaltered. The overproducer of antagonistic compounds did not differ in effect from the wild type. The negative effects were detectable after 14 days but were decreasing and we anticipate they will further decrease with time. The study shows the soil bacteria and protozoa to be sensitive to bacteria producing secondary metabolites, which can have implications for nutrient-cycling in soil and environmental risks of biological control agents.

Acknowledgements. This research was supported by the Danish Research Council of Technology and Production, grant no. 23-04-0089. We thank Dr. C. Keel for providing the *P. fluorescens* CHA0 strains. Anne Grethe Holm-Jensen and Tina Thane are acknowledged for skilful technical assistance.

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Received on 25th June, 2012; revised on 25th October, 2012; accepted on 25th October, 2012