RESEARCH LETTER

Protozoan growth rates on secondary-metabolite-producing *Pseudomonas* spp. correlate with high-level protozoan taxonomy

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Received 10 May 2010; revised 29 November 2010; accepted 29 November 2010.
Final version published online 12 January 2011.

DOI:10.1111/j.1574-6968.2010.02182.x

Editor: Steve Diggle

Keywords
protozoa; grazing; secondary metabolites; *Pseudomonas*; flagellates; amoebae.

Abstract

Different features can protect bacteria against protozoan grazing, for example large size, rapid movement, and production of secondary metabolites. Most papers dealing with these matters focus on bacteria. Here, we describe protozoan features that affect their ability to grow on secondary-metabolite-producing bacteria, and examine whether different bacterial secondary metabolites affect protozoa similarly. We investigated the growth of nine different soil protozoa on six different *Pseudomonas* strains, including the four secondary-metabolite-producing *Pseudomonas fluorescens* DR54 and CHA0, *Pseudomonas chlororaphis* MA342 and *Pseudomonas* sp. DSS73, as well as the two nonproducers *P. fluorescens* DSM50090^T and *P. chlororaphis* ATCC43928. Secondary metabolite producers affected protozoan growth differently. In particular, bacteria with extracellular secondary metabolites seemed more inhibiting than bacteria with membrane-bound metabolites. Interestingly, protozoan response seemed to correlate with high-level protozoan taxonomy, and amoeboid taxa tolerated a broader range of *Pseudomonas* strains than did the non-amoeboid taxa. This stresses the importance of studying both protozoan and bacterial characteristics in order to understand bacterial defence mechanisms and potentially improve survival of bacteria introduced into the environment, for example for biocontrol purposes.

Introduction

Protozoan grazing increases bacterial turnover of organic matter and reduces bacterial biomass (Rønn *et al.*, 2002; Bonkowski, 2004; Christensen *et al.*, 2006). Furthermore, particular protozoa consume different bacteria to different extents (Rønn *et al.*, 2001, 2002; Mohapatra & Fukami, 2004; Pickup *et al.*, 2007). Factors that presumably affect bacterial susceptibility to grazing include cell size, speed of movement, extent of biofilm production, and the composition of the bacterial envelope (Matz & Kjelleberg, 2005). Bacteria that produce secondary metabolites may likewise be less suitable as protozoan food (Rønn *et al.*, 2001; Andersen & Winding, 2004; Matz *et al.*, 2004; Jousset *et al.*, 2006; Pedersen *et al.*, 2009). The genus *Pseudomonas* is interesting in this context as it includes strains that produce a wide range of secondary metabolites (Haas & Défago, 2005).

Protozoa can discriminate between different food items (e.g. Jürgens & DeMott, 1995; Boenigk *et al.*, 2001; Jezbera *et al.*, 2006; Pedersen *et al.*, 2009) and therefore only ingest some bacterial strains. Hence, protozoa graze different taxonomic groups of bacteria differently (Matz *et al.*, 2004). Still, we know only little about how protozoan features correlate with which bacteria they can ingest and hence digest. Here, we focus on protozoan characteristics; thus, we hypothesize that protozoan taxonomic affiliation (Adl *et al.*, 2007) can be used to predict which bacteria they can subsist on, depending upon the bacterial production of secondary metabolites. Thus, we hope to find protozoan characteristics that correlate with their ability to grow on specific bacteria. Moreover, we hypothesize that *Pseudomonas* production of secondary metabolites will affect protozoan growth compared with non-metabolite producers, and, that this effect may be related to the nature of the toxin (Pedersen *et al.*, 2010).

Materials and methods

Bacterial food organisms

We used six different *Pseudomonas* strains, four of which produce well-characterized secondary metabolites that inhibit root-pathogenic fungi. *Pseudomonas fluorescens* DR54

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produces viscosinamide: a membrane-bound cyclic lipopeptide with biosurfactant properties and broad antifungal activity (Nielsen *et al.*, 1999; Thrane *et al.*, 2000). *Pseudomonas fluorescens* CHA0 produces various extracellular metabolites, two of them being DAPG (2,4-diacetylphloroglucinol), which causes membrane damage in fungi (*Pythium*) and inhibits zoospores, and pyoluteorin, which inhibits the fungal respiratory chain (Keel *et al.*, 1992; Laville *et al.*, 1992). *Pseudomonas* sp. DSS73 produces amphisin, an extracellular cyclic lipopeptide with biosurfactant properties and broad antifungal activity (Sørensen *et al.*, 2001; Nielsen & Sørensen, 2003), and *Pseudomonas chlororaphis* MA342 produces DDR (2,3-de-epoxy-2,3-didehydro-rhizoxin), a membrane-bound compound that inhibits mitosis in eukaryotic cells (Hökeberg *et al.*, 1997; Brendel *et al.*, 2007). Two *Pseudomonas* strains, *P. fluorescens* type strain DSM50090^T (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and *P. fluorescens* ATCC43928 (American Type Culture Collection), produce no known antagonistic secondary metabolites. We further included the well-suited food bacterium *Enterobacter aerogenes* SC (Christensen & Bonde, 1985) as a positive control, and a treatment only with phosphate buffer, but without bacteria, as a negative control.

The bacteria for the protozoan growth experiments were pure cultures grown on tryptic soy broth (TSB) medium (3 g L^{-1}, Difco Bacto, Detroit) at 22 °C for 24 h. Bacteria were then diluted 1/10 in weak phosphate buffer ('Neff's modified amoeba saline'; Page, 1988), which yields bacterial cultures with 5–10 $\times$ 10^7 cells mL^{-1}. This approach yields more reproducible results than if a fixed cell number (e.g. 5 $\times$ 10^7 cells mL^{-1}) is used for standard comparison between cultures. This is because different bacterial cultures with similar cell numbers may vary considerably with regard to carbon content, because cell sizes differ (Lekfeldt & Rønn, 2008). Bacterial cell size depends on the growth medium. Here, all bacteria were cultivated on the same medium and microscopic evaluation demonstrated that differences between cell sizes were negligible. No biofilm formation was observed in the current set-up, even though both bacteria and protozoa settled at the bottom of the experimental units (data not shown).

Protozoa

The protozoa used in the experiments belong to several very distantly related protozoan lineages (Adl *et al.*, 2007). They included three amoeboid Rhizaria (*Cercomonadida*) *Cercomonas longicauda* (SCCAP C 1), *Neocercomonas jutlandica* (SCCAP C 161), and *Heteromita globosa* (SCCAP H 251), three non-amoeboid Excavata (*Bodonidae*) *Bodo caudatus* (SCCAP BC 330), *Bodo designis* (UJ), and *B. designis* (SCCAP BD 23), the non-amoeboid Chromalveolata *Spumella* sp. SCCAP S 352, and the two Amoebozoa *Hartmannella vermiformis* and *Phalansterium solitarium* (SCCAP Ph 185). To make sure, we notice that our *B. caudatus* and *B. designis* are synonymous with *Parabodo caudatus* and *Neobodo designis*, respectively (Moreira *et al.*, 2004), and, likewise, our *C. longicauda* (SCCAP C 1) and *N. jutlandica* (SCCAP C 161) are synonymous with *Paracercomonas ekelundi* and *Cercomonas jutlandica* (Karpov *et al.*, 2006). All strains were originally isolated from Danish soils, and are now deposited in the Scandinavian Culture Centre for Algae and Protozoa (SCCAP), except for *B. designis* UJ and *H. vermiformis* that, regrettably, passed away. The origin of *H. vermiformis* is described by Vestergård *et al.* (2007); it was identified according to Page (1988). Origin and identification of the other strains are accounted for by Ekelund (2002a, b), Ekelund *et al.* (2004), and Koch & Ekelund (2005). Clonal cultures were originally established by repeated dilution and growth on TSB (0.1 g L^{-1}, Difco Bacto) (Ekelund, 1996). This method provides protozoan cultures on assemblages on their original food bacteria. Before experiments were begun, we used the stepwise dilution technique (Pelegri *et al.*, 1999; Mohapatra & Fukami, 2004) to provide monoxenic cultures of our nine protozoan strains. In short, we repeatedly transferred 600 μL protozoan culture material to 9.4-mL *E. aerogenes* SC culture produced as described above, and left the culture at 15 °C for 8–16 days. We repeated this procedure until no bacteria, but *E. aerogenes* were detectable on agar plates (0.3 g TSB mL^{-1} solidified with 15 g L^{-1} agar, detection level: 10^2 cells mL^{-1}).

Grazing experiment

We cultivated the previously produced monoxenic protozoan cultures on *E. aerogenes* for 10–14 days in cell culture flasks (Nunc A/S, Roskilde, Denmark, # 156367, 25 cm^3) in darkness, at 15 °C, until late exponential phase. We then diluted the protozoan cultures in phosphate buffer to obtain final concentrations of 2–5 $\times$ 10^3 protozoa mL^{-1}.

We conducted the growth experiments in 96-well microtiter plates (Costar® 3598, Corning Inc.). We amended the wells with 125 μL bacterial and 25 μL protozoan culture, produced as described above. Each particular combination of bacteria and protozoa was set up in four replicates. The microtiter plates were incubated in darkness at 15 °C and counted at regular intervals until the cell number stabilized after 8–16 days. Stabilization occurred either because the culture entered the stationary phase, in case of good food-quality bacteria, or because the protozoa stabilized without growth or simply died out.

Growth rates

We used an inverted microscope (Olympus CK X31) equipped with a 10 $\times$ 10 counting grid to estimate protozoan cell numbers at $\times$ 200 or $\times$ 400 magnification. At each counting, we counted a minimum of 200 cells in nine to 17

microscopic fields distributed widely over the bottom of the well. When < 200 protozoa occurred in 17 fields, we searched the entire bottom of the well for protozoa, which yields a detection limit of 1.7 cells mL^{-1} for the four replicates. Determination of intrinsic growth rates was as in Koch & Ekelund (2005).

To evaluate the overall food quality of the seven bacteria tested, we calculated, for each bacterial strain, the average growth rate for the nine protozoa. Likewise, to evaluate the individual protozoa's ability to cope with metabolite-producing bacteria, we calculated, for each protozoan strain, the ratio between the average growth rate on the four metabolite-producing bacteria and the three well-suited food bacteria. We calculated each of these compound parameters separately for the four individual replicates as to allow the application of statistics.

Statistics

We used a two-way GLM (SAS program package, Statistical Analysis System Institute, version 9.1) with protozoan and bacterial strains as factors for preliminary analysis of the data set (Table 1). For each flagellate strain, differences in growth rate on the different bacterial strains were tested using a one-way ANOVA, followed by a Tukey pair-wise comparison ($\alpha = 0.05$). Similarly, the resulting average growth rate for each bacterial strain when fed to the nine different protozoa (Fig. 1), and the ratio between the average growth rates for the nine different protozoa, on the four metabolite-producing bacteria and the three nonproducers (Fig. 2), were tested using a one-way ANOVA followed by Tukey's pair-wise comparison ($\alpha = 0.05$). When needed, data were log transformed before analyses.

Results

Bodo designis UJ illustrates in an exemplarily manner the different possible outcomes of the protozoan–bacterial combinations (Fig. 3). Protozoa fed with suitable food bacteria generally followed a regular pattern with an exponential phase that gradually levelled out into a stationary phase (Fig. 3: *P. fluorescens* DSM50090) and displayed a positive growth rate (Table 1). Protozoa exposed to bacteria that did not support growth, or to phosphate buffer without bacteria, either lysed (Fig. 3: *P. fluorescens* CHA0) and were thus assigned the growth rate 0 or remained at an almost constant level with little or no growth (Fig. 3: no bacteria added). In some cases, protozoa transferred to a medium without bacteria performed a few reductive cell divisions before entering a constant cell level (Fig. 3: no bacteria added). In order to follow a consistent procedure, we assigned such outcomes a positive growth rate, even though the initial cell divisions yielded no extra biomass, but just more, smaller bacteria.

Table 1. Growth rates (day^{-1}) of soil protozoa grown on different bacteria

	Cercomonadidae, Rhizaria			Excavata, *Bodonidae*			Chromalveolata	Amoebozoa	
	Cercomonas longicauda	*Neocercomonas jutlandica*	*Heteromitaglobosa*	*Bodo caudatus*	*Bodo designis* 23	*Bododesignis* UJ	*Spumella* sp.	*Phalansterium solitarium*	*Hartmannella vermiformis*
No bacteria added	0 (0)	2.05 (0.30) A	0.84 (0.06) F	0 (0)	0.73 (0.06) C	0.90 (0.11) C	0 (0)	0 (0)	0 (0)
E. aerogenes	1.82 (0.04) A	1.72 (0.10) AB	3.76 (0.37) D	1.97 (0.05) A	1.31 (0.05) B	2.13 (0.06) AB	1.04 (0.12) B	1.34 (0.04) B	1.75 (0.09) A
P. chlororaphis ATCC43928	1.96 (0.08) A	1.61 (0.03) AB	4.82 (0.16)B	1.90 (0.07) AB	2.00 (0.10) A	2.66 (0.20) A	0.94 (0.07) B	0.72 (0.03) C	1.39 (0.03) B
P. fluorescens DSM50090^{T}	1.81 (0.03) A	1.63 (0.03) AB	4.31 (0.08) C	1.76 (0.03) B	1.89 (0.05) A	2.61 (0.09) A	2.22 (0.02) A	1.10 (0.14) B	1.69 (0.08) A
P. fluorescens DR54	1.96 (0.11) A	1.73 (0.07) AB	5.62 (0.10) A	0 (0)	0.60 (0.04) C	0 (0)	0 (0)	*1.82 (0.07) A	0.71 (0.03) DE
P. fluorescens CHA0	*1.84 (0.16) A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	†1.26 (0.01) BC
Pseudomonas sp. DSS73	1.73 (0.09) A	0.46 (0.02) C	2.70 (0.09)E	0.72 (0.04) D	1.25 (0.15) B	0 (0)	0 (0)	0 (0)	*0.61 (0.01) E
P. chlororaphis MA342	1.55 (0.14) A	1.36 (0.05) B	1.30 (0.19) E	1.52 (0.03) C	0 (0)	1.79 (0.18) B	0 (0)	0.24 (0.01) D	0.95 (0.06) CD

Numbers in parentheses are SE ($n = 4$).
Grey-shaded background: bacteria produce secondary metabolites. Only flagellate populations increasing in size were tested statistically. Each flagellate was tested separately. Different letters indicate significant different growth rates ($P < 0.05$).
*The population size decreased the first 1–2 days followed by growth.
†Values based on two replicates where *Hartmannella vermiformis* proliferated; in the other two replicates, the organisms died.

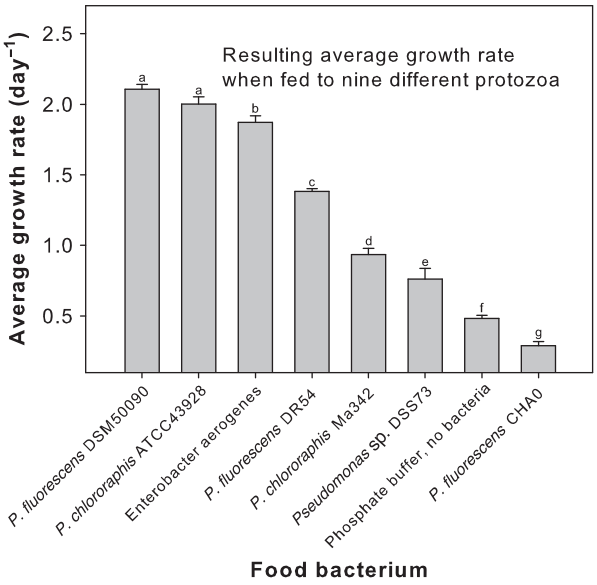

Fig. 1. The resulting average protozoan growth rate when seven different bacteria, and a bacteria-free control, were fed to nine different protozoa. Error bars indicate SE ($n = 4$); columns with similar letters do not differ significantly ($P < 0.05$, one-way ANOVA, Tukey's pair-wise comparison).

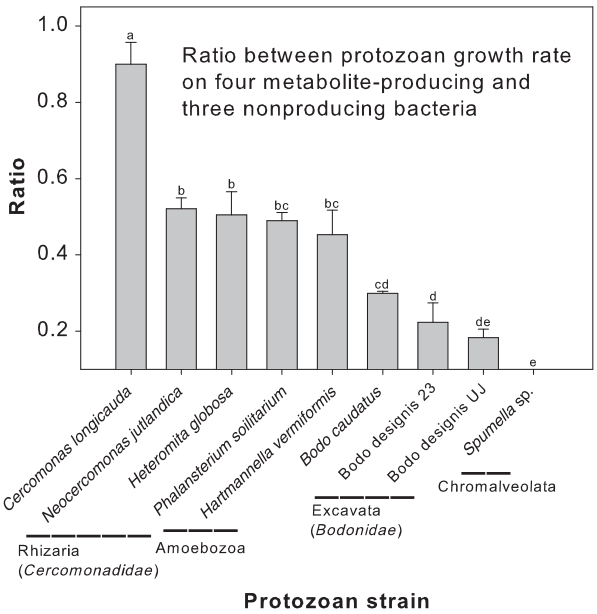

Fig. 2. The ratio between the average growth rates of nine different protozoa on four metabolite-producing bacteria and three nonproducers. Error bars indicate SE ($n = 4$); columns with similar letters do not differ significantly ($P < 0.05$, one-way ANOVA, Tukey's pair-wise comparison).

Growth rates for the protozoan cultures

The protozoan and bacterial strain as well as their interaction significantly affected protozoan growth rate

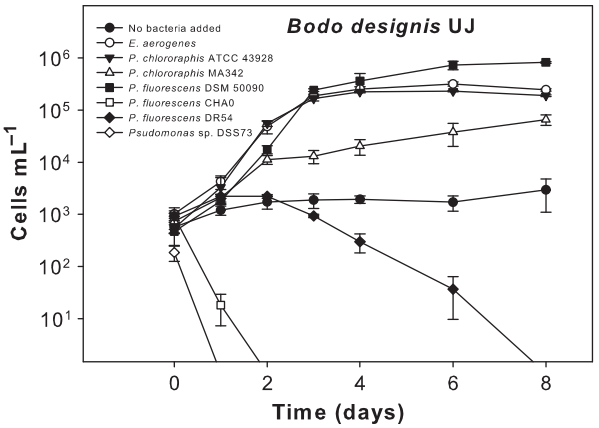

Fig. 3. Growth curves for the flagellate *Bodo designis* UJ on the seven different bacterial strains used in the experiment. Please notice that a treatment without bacteria (no bacteria added) was included as a negative control.

($P < 0.0001$). *Pseudomonas fluorescens* DSM50090^T yielded the highest average growth rates (Fig. 1). For all tested protozoan strains, except *B. caudatus*, the growth rates for this strain were similar to, or higher than, on *E. aerogenes* (Table 1). The two *Pseudomonas* strains without any known production of secondary metabolites, i.e. DSM50090^T and ATCC43928, supported equal growth statistically (Fig. 1). Five out of nine protozoan strains displayed similar growth rates on these strains (Table 1). Three strains, however, had significantly lower growth rates on ATCC43928 than on DSM50090^T, and one had a higher growth rate.

All *Pseudomonas* strains producing secondary metabolites affected protozoan growth negatively (Table 1, Figs 1–3). Only *C. longicauda* displayed similar growth rates on all bacterial strains. Likewise, *C. longicauda* was the only one of the nine tested protozoa that did not display inhibited growth on MA342 and DSS73 as compared with the bacterial strains without known production of secondary metabolites. *Pseudomonas fluorescens* CHA0 was the least suited food bacterium of the tested strains (Fig. 1). It supported growth of none of the tested protozoa, but *C. longicauda* and *H. vermiformis* (Table 1).

Discussion

Secondary-metabolite-producing bacteria supported protozoan growth poorly as compared with nonproducers (Fig. 1). Thus, eight of the nine tested protozoa displayed lower growth rates when grown on secondary metabolite producers than on the nonproducers (Fig. 2, Table 1). This clearly indicates that the metabolites protect bacteria against grazing. This inhibition of protozoan growth was also observed in experiments using other protozoa and in a set-up investigating potential negative effects of antagonistic bacteria in soil (Schlimme *et al.*, 1999; Johansen *et al.*, 2005;

Jousset *et al.*, 2006; Pedersen *et al.*, 2010). Further, growth of different protozoa increased considerably when grown on mutants where synthesis of secondary metabolites was blocked completely compared with wild-type bacteria, which produce the secondary metabolites (Jousset *et al.*, 2006).

To examine further as to how differences in the mode of action of *Pseudomonas* secondary metabolites relate to their effect on protozoa, Pedersen *et al.* (2010) incubated the protozoan *C. longicauda* in batch cultures with three different *P. fluorescens* strains that we also used in the experiments reported here. These three *P. fluorescens* strains have contrasting secondary metabolite properties. Thus, the type strain DSM50090^T produces no known secondary metabolites, DR54 produces a membrane-bound cyclic lipopeptide, and CHA0 produces various extracellular metabolites. For all three *Pseudomonas* strains, Pedersen *et al.* (2010) set up batch cultures with washed bacterial suspensions, presumed to be devoid of extracellular metabolites, as well as unwashed cultures retaining potential extracellular metabolites. In accordance with their assumptions, Pedersen *et al.* (2010) found that when offered washed CHA0, *C. longicauda* was able to multiply, whereas for the two other *Pseudomonas* strains washed and unwashed bacteria affected *C. longicauda* similarly. Likewise, Andersen & Winding (2004) found that cell extract from *P. fluorescens* DR54 inhibited a mixed community of soil protozoa. These findings provide further proof that *Pseudomonas* anti-protozoan toxicity is related to toxin production, and that toxicity to fungi can be used to make predictions about toxicity to protozoa.

Both protozoan and bacterial strain, as well as their particular combinations, significantly influenced the outcome of their interactions (Table 1). *Pseudomonas fluorescens* CHA0 was especially harmful (Figs 1 and 2, Table 1). This strain efficiently restrains growth of various plant-pathogenic fungi, inhibits egg hatch and cause mortality of plant-pathogenic nematode juveniles, (Keel *et al.*, 1992; Siddiqui *et al.*, 2006) and inhibits several nontarget fungi (Winding *et al.*, 2004). Jousset *et al.* (2006) found that only mutants completely devoid of metabolite production (GacA/GacS-negative) supported protozoan growth, which suggests that the high toxicity of CHA0 is linked to the production of a broad range of different secondary metabolites.

We observed that the strains producing extracellular metabolites, i.e. CHA0 and DSS73, were more harmful to protozoa than strains that mainly produce membrane-bound metabolites, i.e. DR54 and MA342 (Fig. 1). To analyze this matter further, we arranged our *Pseudomonas* strains into three groups: those without secondary metabolites, those that produce membrane-bound secondary metabolites, and a group of bacteria producing extracellular secondary metabolites. We then correlated growth rates of each of these three groups to the growth rates of *E. aerogenes*. We found a very high correlation between the growth rates of *E. aerogenes* and the supposedly harmless *Pseudomonas* ($r^2 = 0.85$, $P = 0.0002$); we obtained no correlation at all between *E. aerogenes* and the *Pseudomonas* with extracellular metabolites ($r^2 = 0.02$, $P = 0.36$), whereas *Pseudomonas* with membrane-bound metabolites correlated better and almost significantly ($r^2 = 0.26$, $P = 0.08$). We suggest that the relatively increased ability to cope with membrane-bound toxins in organisms with higher growth rates can be attributed to egestion of harmful remnants enclosed in the food vacuole (membrane parts) whereas extracellular metabolites are in contact with the cell surface and are difficult to avoid. This is in accordance with the mechanism discussed by Deines *et al.* (2009). They elegantly showed that volume-specific clearance rate correlated positively with toxin tolerance; probably because organisms with a relative higher clearance rate use their food less efficiently, and egest cell remnants that contain harmful substances. Everything else being equal, volume-specific clearance rate and intrinsic growth rate will correlate. Hence, we suggest that egestion of harmful remnants can explain the higher tolerance.

The ability of protozoa to grow on specific bacteria did not correlate particularly well with low-level taxonomic group (Table 1). For example, the two strains of *B. designis* reacted quite differently to the presented bacteria. Likewise, toxic effects of the bacteria were not related to bacterial species, but rather to strain-specific toxin production. This is in accordance with Koch & Ekelund (2005), who observed that different *B. designis* strains varied considerably in physiological parameters such as salt tolerance and growth rate. In fact, growth rate varied almost as much between different strains of *B. designis* as the whole range reported for heterotrophic flagellates. By contrast, overall average effects seemed to correlate extremely well with high-level taxonomy. Hence, the average protozoan response to metabolite-producing bacteria simply grouped them taxonomically in accordance with Adl *et al.* (2007) (Fig. 2). We emphasize that this correlation must be considered a preliminary hypothesis, and that more protozoan groups must be examined to confirm or reject this.

In some cases, only a minor fraction of the protozoan cells survived and divided when transferred to a harmful bacterium. In case of some of the tested bacteria, the populations of *C. longicauda*, *P. solitarium*, and *H. vermiformis* decreased for a period before the growth phase, and in case of the latter, only some of the replicates proliferated when grown on *P. fluorescens* CHA0. A possible explanation is that genetically based enzymatic detoxification mechanisms must be induced before growth as discussed by Liu (2006).

We notice that the taxonomic ranking in Fig. 2 largely reflects a division of the strains in two sets: the less susceptible, largely amoeboid Rhizaria and Amoebozoa and the more susceptible, non-amoeboid Excavata and

Chromalveolata. Thus, we suggest that the property amoeboid or non-amoeboid may correlate with tolerance to metabolite-producing bacteria. Several highly motile, non-amoeboid protozoa, including *Bodo* and *Spumella*, can discriminate between different bacteria (Jürgens & DeMott, 1995; Boenigk *et al*., 2001; Pedersen *et al*., 2009). We thus put forward the hypothesis that the less-motile amoeboid forms must depend on the bacteria at their disposal to a higher degree, as they cannot easily move to new patches, and thus must have a better-developed enzymatic detoxification. Therefore, they can proliferate on a larger number of different food bacteria. This agrees with the prolonged lag phases that we observed in some of the Rhizaria and Amoebozoa. Further, it agrees with previous studies on pesticide tolerance in protozoa, where amoeboid protozoa proved less susceptible to toxic compounds (Ekelund *et al*., 1994, 2000; Ekelund, 1999). This hypothesis could be tested by feeding an amoeboid and a non-amoeboid protozoan with a mixture of two bacterial strains: one with and the other without secondary metabolites.

Because protozoa perform important soil functions such as stimulation of nutrient turnover and plant growth (Ekelund & Rønn, 1994), it is essential to consider the potential harmful side effects of soil amendments on protozoa (Ekelund, 1999). Antifungal agents, chemical as well as biological, may drastically affect the trophic interactions between protozoa and bacterial prey including *Pseudomonas* spp. (Thirup *et al*., 2000), and thus change the nutrient turnover patterns. Conversely, bacteria with secondary metabolite production will resist predation better, which is a serious problem with artificially introduced bacteria (Ekelund & Rønn, 1994). Our results demonstrate that metabolite-producing *Pseudomonas* affect some protozoan groups more than others and that the most mobile protozoan groups are the most vulnerable. Hence, when considering administration of bacteria to protect plants against fungi, it is preferable to use bacteria with membrane-bound metabolites as protozoa can better cope with them, and, in nature, the protozoa can avoid them simply by moving to another location.

Acknowledgements

The Danish Research Council for Technology and Innovation grant no. 23-04-0089 financed the project. Mette Vestergaard and Trine Koch, Biological Institute, Copenhagen University kindly provided us with *H. vermiformis* and *B. designis* UJ, respectively. C. Keel provided *P. fluorescens* CHA0.

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