ORIGINAL ARTICLE

Functional GacS in *Pseudomonas* DSS73 prevents digestion by *Caenorhabditis elegans* and protects the nematode from killer flagellates

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The success of biocontrol bacteria in soil depends in part on their ability to escape predation. We explored the interactions between *Pseudomonas* strain DSS73 and two predators, the nematode *Caenorhabditis elegans* and the flagellate *Cercomonas* sp. Growth of the nematode in liquid culture was arrested when it was feeding on DSS73 or a DSS73 mutant (DSS73-15C2) unable to produce the biosurfactant amphisin, whereas a regulatory *gacS* mutant (DSS73-12H8) that produces no exoproducts supported fast growth of the nematode. The flagellate *Cercomonas* sp. was able to grow on all three strains. The biosurfactant-deficient DSS73 mutant caused severe dilation of the nematode gut. In three-species systems (DSS73, *Cercomonas* and *C. elegans*), the nematodes fed on the flagellates, which in turn grazed the bacteria and the number of *C. elegans* increased. The flagellates *Cercomonas* sp. usually kill *C. elegans.* However, DSS73 protected the nematodes from flagellate killing. Soil microcosms inoculated with six rhizobacteria and grazed by nematodes were colonized more efficiently by DSS73 than similar systems grazed by flagellates or without grazers. In conclusion, our results suggest that *C. elegans* and DSS73 mutually increase the survival of one another in complex multispecies systems and that this interaction depends on the *GacS* regulator.
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Introduction

Bacterial inoculants inevitably encounter predators in the soil environment. Therefore, the success of biocontrol bacteria in soil depends in part on their ability to escape predation. A more detailed understanding of the interactions between these bacteria and their predators is required to enhance the efficiency of bacterial inoculants for plant production purposes. The most important bacterial feeders in soil are protozoa and nematodes (England *et al.*, 1993; Ekelund and Rønn, 1994; Coleman and Crossley, 1996; Christensen *et al.*, 2007). The flagellate *Cercomonas* sp. and the nematode *Caenorhabditis elegans* are common representatives of fast-growing bacterial feeders that occur following pulses of carbon release in soil systems (Griffiths *et al.*, 1991; Ekelund *et al.*, 2001).

Pseudomonas biocontrol strains are able to suppress plant pathogenic fungi by a variety of mechanisms. For example, strain DSS73 produces the cyclic lipopeptide amphisin, a biosurfactant that increases surface motility and provides the bacteria with antibiotic properties (Sørensen *et al.*, 2001; Nielsen *et al.*, 2002; Andersen *et al.*, 2003). In DSS73 the two-component GacS/GacA regulatory system controls amphisin production as well as chitinase, protease and cyanide synthesis (Koch *et al.*, 2002). Nielsen *et al.* (2005) investigated the colonization potential of *Pseudomonas* sp. strains DSS73, the amphisin-negative mutant strain DSS73-15C2

(*amsY*) and regulatory mutant DSS73-12H8 (*gacS*) in bulk soil, in sugar beet rhizosphere and in dead organic matter (barley straw). Interestingly, colonization of sugar beet roots and decomposing barley straw was impaired for both mutants compared to the wild type (wt). This suggests that amphisin and/or other GacS/GacA-regulated traits are important for colonization of resources. The mechanism behind is yet to be unraveled but it can be hypothesized that the three strains differ in their susceptibility to predation by bacterial feeders. This idea is supported by Jousset *et al.* (2006) who showed that the biocontrol strain *Pseudomonas fluorescens* CHA0 is protected from protozoan grazers by secondary metabolites. CHA0 is a well-characterized biocontrol strain that is antagonistic due to production of a panel of Gac-regulated extracellular metabolites and enzymes including pyoluteorin, pyrrolnitrin, cyanide, 2,4-diacetylphloroglucinol and alkaline protease (Keel *et al.*, 1992; Raaijmakers *et al.*, 2002; Haas and Keel, 2003). A *gac* mutant of CHA0 was a poorer colonizer of cucumber roots (Jousset *et al.*, 2006) and rice (Jousset *et al.*, 2008) than the wt in the presence of protozoan grazers.

Production and release of metabolites is one among several mechanisms evolved among bacteria to protect them from protozoan predation (Matz and Kjelleberg, 2005). Likewise, growth of bacteria-feeding nematodes differs according to type of bacterial food source (Venette and Ferris, 1998; Bjørnlund *et al.*, 2006) and production of metabolites is of some importance. For example, phenazines produced by *P. aeruginosa* are lethal to nematodes (Tan *et al.*, 1999). Less effective compounds may also protect bacteria from grazing because toxins produced at sublethal levels may lead to reversible cessation of pharyngeal pumping and food intake in *C. elegans* (Jones and Candido, 1999).

Recently, we demonstrated that the bacterial feeders *C. elegans* and *Cercomonas* sp. have different effects on the outcome of interspecific competition among *Pseudomonas* strains in simple liquid systems (Pedersen *et al.*, 2009). Surprisingly, DSS73 performed better in the presence of nematode grazers than in the presence of the flagellate. However, the influence of these grazers on colonization of DSS73 in soil has not been established. Furthermore, we have recently reported that the flagellate *Cercomonas* is able to attack and kill the nematode *C. elegans* (Bjørnlund and Rønn, 2008). The possible influence of DSS73 on this interaction has not yet been examined.

The objective of this study was to supply a basic understanding of the trophic interactions between DSS73 and the two important bacterial feeders, *C. elegans* and *Cercomonas* sp. First, we tested if the function of the genes *amsY* and *gacS* affected the edibility of DSS73 for the two bacterial feeders. We found that *gac*-regulated traits cause DSS73 to be more toxic to *C. elegans* than to *Cercomonas*. On the basis of these results, we generated the hypothesis that three-species interactions between *Cercomonas*, *C. elegans* and DSS73 are influenced by these genes. This was tested in simple model systems with DSS73 along with one or two bacterial predators. Finally, we investigated whether the interactions observed in simple systems applied for colonization of soil by wt DSS73 and its interaction with predators in soil.

Materials and methods

Culture conditions and strains
An axenic culture of the flagellate *Cercomonas* sp. (ATCC 50334) was obtained from the American Type Culture Collection (Manassas, VA, USA). *C. elegans* N2 was provided by the Caenorhabditis Genetics Center, Twin Cities, MN, USA. Cultures were maintained as described in Brenner (1974), Bjørnlund *et al.* (2006) and Bjørnlund and Rønn (2008). The bacterial strains used in this study are listed in Table 1. Unless stated otherwise, the bacterial suspensions used as inoculum were stationary

Table 1 List of bacterial strains

		Phenotype	Mutations	Reference
Biocontrol strains				
DSS73	*Pseudomonas* sp.	Wild type	None	Sørensen *et al.* (2001)
DSS73-15C2	*Pseudomonas* sp.	Amphisin negative	*amsY*	Koch *et al.* (2001)
DSS73-12H8	*Pseudomonas* sp.	Deficient for multiple exoproducts	*gacS*	Koch *et al.* (2001)
Other strains used				
BEM453	*Sphingomonas* sp.	Wild type	None	Bjørnlund *et al.* (2006)
BEM494	*Brevundimonas vesicularis*	Wild type	None	Bjørnlund *et al.* (2006)
DSM50090	*Pseudomonas fluorescens*	Wild type	None	DSMZ
04.01.05	*Paenibacillus* sp.	Wild type	None	Danisco Seed
BEM28	*Rhodococcus* sp.	Wild type	None	Bjørnlund *et al.* (2006)
BEM447	*Arthrobacter* sp.	Wild type	None	Bjørnlund *et al.* (2006)
Na22	*Escherichia coli*	Wild type	None	CGC

Abbreviations: CGC, Caenorhabditis Genetics Center; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

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phase cultures in 1:10 Tryptic Soy Broth (TSB; $3 \, g \, l^{-1}$; Difco, Sparks, MD, USA) incubated at $28 \, ^{\circ}C$ at $100 \, r.p.m.$ for 48 h.

Two-species systems: growth of Cercomonas *sp. and* C. elegans *on bacterial strains*
The ability of bacterial strains to support growth of the bacterial feeders was determined in liquid culture according to Bjørnlund et al. (2006) with a few modifications. Wells of 96-well microtiter plates (Nunc, Roskilde, Denmark) were partly filled with $100 \, \mu l$ 1:300 TSB ($0.1 \, g \, l^{-1}$). For each bacterial strain, aliquots of 10^7 bacterial cells in $10 \, \mu l$ were added to 32 wells and the plates were incubated at $15 \, ^{\circ}C$ for 4 days. Then, approximately 100 cells of an axenic culture of Cercomonas sp. or one sterile newly hatched first-stage juvenile of C. elegans were added to each well. Plates were incubated at $15 \, ^{\circ}C$ and numbers of bacterial feeders were recorded for 14 days at 1–4 day intervals by inspection in an inverted microscope (Olympus CK2, Olympus, Ballerup, Denmark) at $\times 200$ magnification. The experiments were repeated twice.

Two-species systems: growth of C. elegans *in soil*
To evaluate the effect of soil on food quality of the bacterial strains, a soil microcosm experiment was performed in six-well culture plates (Nunc) with C. elegans as bacterial predator and six replicate wells per bacterial strain. Top soil (Sandy clay loam: 65% sand, 18% silt, 15% clay, 1.4% organic matter, in 0.01 M $CaCl_2$: pH between 5 and 6) was collected to a depth of 20 cm in a field located at an experimental farm in Tåstrup, Denmark. Soil was dried for 2 weeks, sieved and mixed with 50% quartz sand and β-irradiated (15 kGy) to kill indigenous soil organisms before use. Each soil microcosm consisted of 5 g soil added 1.5×10^8 bacterial cells in $150 \, \mu l$ and 1.5 ml 1:300 TSB to meet bacterial maintenance requirements. Microcosms were incubated for 4 days at $15 \, ^{\circ}C$. Then, first-stage juveniles of C. elegans (50 larvae in $100 \, \mu l$ M9) were added and the culture plates were incubated for 2 weeks at $15 \, ^{\circ}C$. When the experiment was terminated, nematodes were extracted from 1 g soil for 24 h by a modified Baermann funnel method and were immediately counted. The experiment was repeated once.

Nematode digestion of wild-type and mutant strains of DSS73
To examine how the nematodes ingest and digest the bacteria, we performed microscopy of C. elegans fed with *gfp*-tagged DSS73 (wt), DSS73-12H8 (*amsY*) and DSS73-15C2 (*gacS*). Bacteria were tagged according to Koch et al. (2001). Overnight cultures ($100 \, \mu l$) were plated onto 1:10 TSB agar, incubated for 48 h at $20 \, ^{\circ}C$ to produce thin bacterial lawns.

Young adult C. elegans were picked up with a glass hook and transferred from NG plates (Brenner, 1974) with *Escherichia coli* OP50 to the bacterial lawns. Plates were incubated at $15 \, ^{\circ}C$ and nematodes were recovered after 24 and 48 h, heat-killed by exposure to $60 \, ^{\circ}C$ for 5 s and the distribution of fluorescent protein in the nematodes was immediately examined by fluorescence microscopy at 470 nm (Axioplan2; Zeiss, Göttingen, Germany). Ten nematodes were inspected for each bacterial strain and the experiment was repeated once.

Model system for study of three-species interactions
The influence of the bacterial strains on the interactions between the two bacterial feeders was investigated in six-well plates (1.5 ml per well). Growth conditions were similar to those used for studies of two-species interactions. Each of the bacteria (DSS73, DSS73-15C2 (*amsY*) and DSS73-12H8 (*gacS*)) was added separately to wells as described above for the two-species systems. One of the two bacterial feeders, C. elegans and Cercomonas sp., or both were added to the wells. There were six replicates of each of the nine treatments. Numbers of Cercomonas were recorded after 1, 3, 7, 9, 13 and 15 days. Numbers of C. elegans were recorded after 0, 1, 7, 10 and 13 days.

The bacterial growth in this system was not high enough to support high densities of flagellates. To study the potential effect of high flagellate densities on nematodes as affected by the different bacterial strains, we increased growth potential for bacteria by increasing the strength of the growth media from 1:300 TSB to 1:100 TSB ($0.3 \, g \, l^{-1}$). In this experiment, flagellates were added to the systems at a high density according to surface area of the bottom of the wells (200 cells per mm^2). A flagellate cell density of 200 cells per mm^2 at the bottom of the well is normally sufficient to kill nematodes rapidly (Bjørnlund and Rønn, 2008). Two bacterial strains were included as reference strains to compare with DSS73 and its mutants. These were *P. fluorescens* DSM50090, which is of high food quality for protozoa (Andersen and Winding, 2004; Pedersen et al., 2009), and *Arthrobacter* sp. BEM447, which is of poor food quality for the flagellate (Bjørnlund et al., 2006). The nematode mortality was recorded after 4 days and the experiment was repeated once.

Effect of flagellates and nematodes on colonization of soil by DSS73
We established soil microcosms from the soil that was used for testing food quality of the bacterial strains for C. elegans and examined how the bacterial feeders, C. elegans and Cercomonas sp., influenced population levels of DSS73 in the presence of six other competing rhizosphere bacterial strains (Table 1). Each soil microcosm was prepared from 5 g soil and received 25 mg glucose

and 7.5 mg NH_4NO_3 in 1.5 ml water to ensure sufficient carbon and nitrogen supplies for bacterial growth. The bacteria were cultured for 4 days in 1:10 TSB at 15 C° before the experiment was started. Cultures were adjusted to approximately 2×10^7 cells per ml using optical density at 600 nm. The bacterial density of the cultures was confirmed by drop plate count. The bacterial cultures were mixed in equal proportions and 100 µl of the mixed culture was added to each microcosm resulting in inoculum densities of 4×10^5 bacterial cells per gram soil. The microcosms were either left without grazers or inoculated with flagellates or nematodes ($n = 3$). The microcosms subjected to flagellate grazers received 6000 Cercomonas sp. in 100 µl and those subjected to nematode grazers received 12–15 first-stage larvae of C. elegans in 20 µl. Microcosm were destructively sampled after 11 days and four 10-fold dilution series were prepared from each replicate and spread onto 1:10 TSB agar. DSS73gfp colonies were easily distinguished from the others by their green fluorescence and counted after 7 days of incubation at 15 °C using inverted fluorescence microscopy.

Data analysis

Densities of grazers in liquid systems were tested by repeated-measures analysis of variance (ANOVA) followed by Tukey's multiple comparisons. The density of nematodes and DSS73 in soil was tested by one-way ANOVA. All analyses were performed using SAS version 8e (SAS Institute Inc., Gary, NC, USA).

Results

Food quality of DSS73 for C. elegans in liquid medium

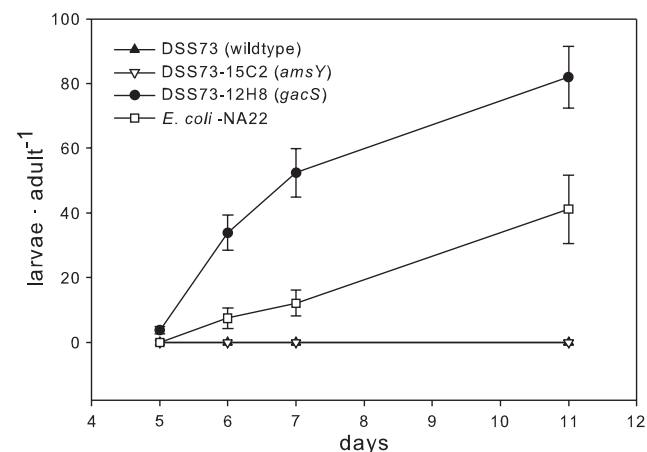The nematode was not able to reproduce on DSS73 or on the amphisin-negative mutant DSS73-15C2

($amsY$) (Figure 1). However, when fed DSS73-12H8 ($gacS$), the nematodes completed their life cycle within 5 days and initially each hermaphroditic worm produced on average 30 larvae per day. This was fourfold the initial larval production (7.5 larvae per individual) obtained when an E. coli strain (E. coli Na22) used routinely for growing C. elegans in liquid cultures was fed to C. elegans (Figure 1). Total larval production of C. elegans fed with E. coli Na22 was approximately 50% of that obtained with DSS73-12H8 ($gacS$) on day 11 (Figure 1). Two replicate experiments showed similar results. Hence, in this liquid system gacS-regulated traits decreased the food quality of strain DSS73 to C. elegans.

Food quality of DSS73 for C. elegans in soil

The findings from the liquid systems that the wt and the amphisin-negative mutant (DSS73-15C2) were of low food quality for the nematode were largely confirmed in the soil experiment. The number of nematodes recovered from soil inoculated with DSS73 and DSS73-15C2 ($amsY$) was lower than the number recovered from soil inoculated with DSS73-12H8 ($gacS$) and Na22 (Figure 2; $P < 0.05$, Tukey's). The number of nematodes in soil inoculated with DSS73-15C2 ($amsY$) matched with the number of individuals originally added. Apparently, nematodes had survived in soil treated with DSS73-15C2 ($amsY$) but no net reproduction had occurred. In soil with DSS73, the number of nematodes extracted was 4–5 times the inoculated number indicating that some reproduction had occurred (Figure 2). Finally, nematodes in soil with DSS73-12H8 ($gacS$) had multiplied by a factor 15. The experiment was replicated with essentially the same results. Hence, the results from these soil microcosm experiments were in accordance with the results obtained from the experiments in liquid culture.
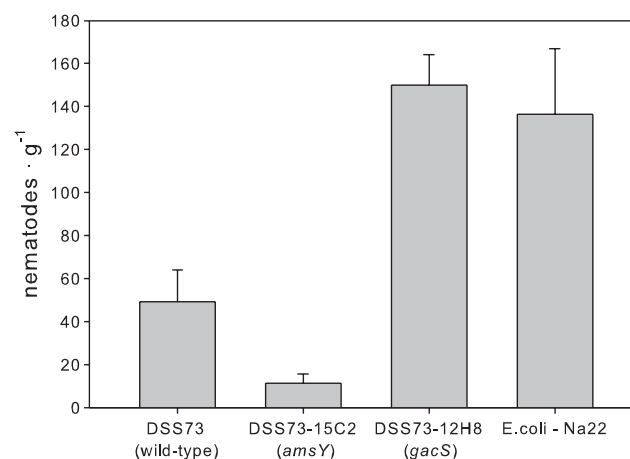

Figure 1 Larval production of C. elegans feeding on the wild type of Pseudomonas DSS73, the amphisin-deficient mutant DSS73-15C2 ($amsY$), the regulatory mutant DSS73-12H8 ($gacS$) or E. coli Na22 in liquid culture. There was no larval production when nematodes were fed DSS73 or DSS73-15C2 ($amsY$) ($N = 32$, experiment repeated twice).

Figure 2 Number of nematodes per gram extracted after 14 days from soil microcosms inoculated with the three strains of Pseudomonas, DSS73 (wild type), DSS73-15C2 ($amsY$), DSS73-12H8 ($gacS$) or E. coli Na22 ($N = 6$, experiment repeated once). Error bars correspond to 1 s.e.

Microscopy of C. elegans-*fed* gfp-*tagged bacteria*

All tested DSS73 *gfp*-tagged strains were ingested by the nematodes within 2 days after the worms were placed on bacterial lawns. Epifluorescence microscopy revealed that the digestibility of the three DSS73 bacterial strains differed considerably. Representative individuals are shown in Figure 3. After 48 h of feeding on DSS73, adult *C. elegans* exhibited accumulation of bacteria in the gut lumen. Some green-labeled vesicles were observed scattered across the intestinal epithelial cells indicating that some assimilation of the bacteria occurred (Figure 3a).

In contrast, *C. elegans* adults appeared to have normal gut morphology, with no visible accumulation of bacteria in the gut lumen, after 24 h of feeding on DSS73-12H8 (*gacS*) and numerous green-labeled

vesicles were seen in the epithelial cells of the intestine (Figure 3b). This indicates that DSS73-12H8 (*gacS*) cells were efficiently digested and assimilated. Apparently, the green fluorescent protein (GFP) is passed on intact to other tissues of the nematodes as eggs and gonads were GFP stained (Figure 3b). After 48 h, the nematodes fed DSS73-12H8 (*gacS*) were heavily stained by the GFP making it impossible to differentiate the various structures.

A dense mass of green fluorescent material caused the gut to be severely dilated in nematodes fed DSS73-15C2 (*amsY*) after 48 h. Assimilation of the bacteria in the mid-gut was very low as epithelial cells of the gut were hardly marked at all (Figures 3c and d). In one case, we observed that the dilated gut spontaneously forced itself through the vulva post
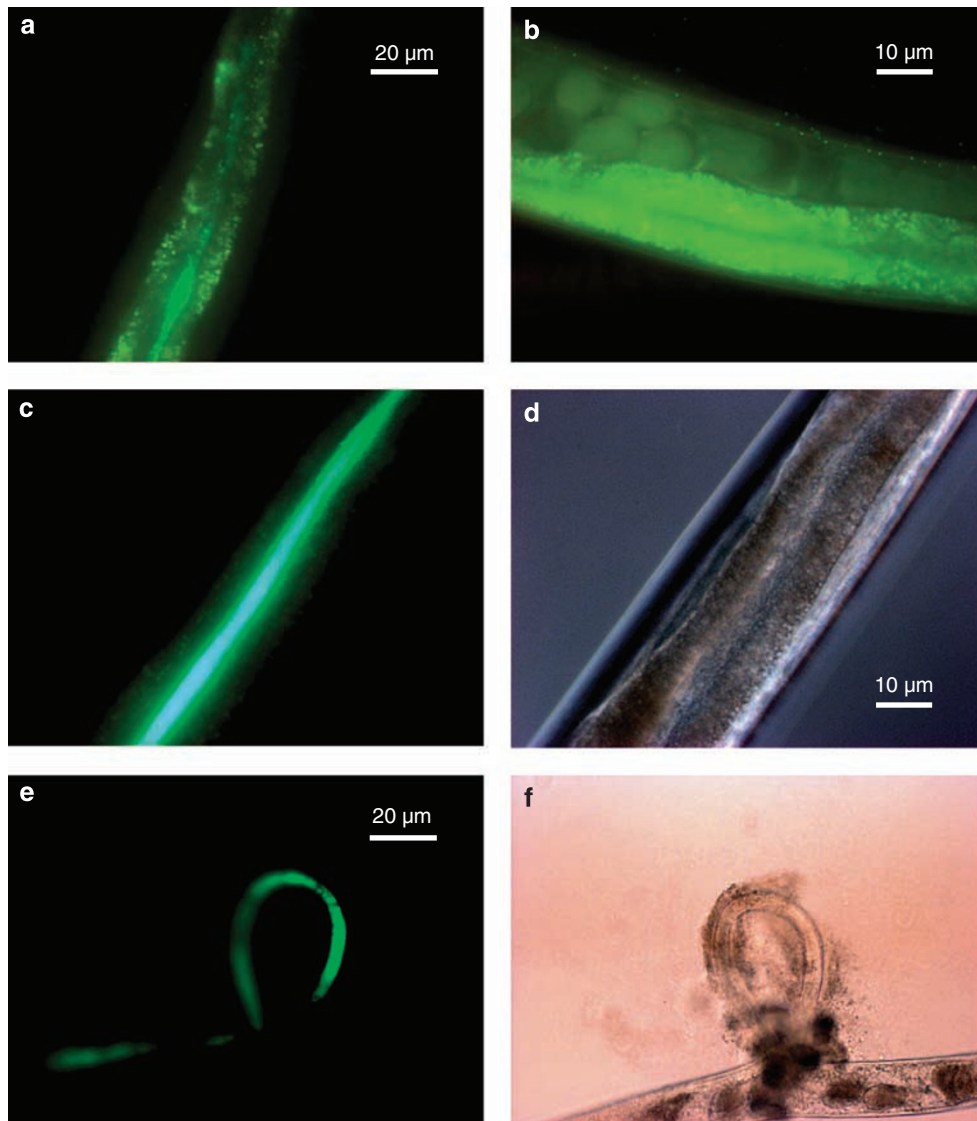

Figure 3 Epifluorescence microscopy of the mid-gut of representative young adult *C. elegans* fed the following *gfp*-tagged bacteria: DSS73 (**a**), DSS73-12H8 (*gacS*) (**b**), DSS73-15C2 (*amsY*) (**c** + **e**). Bright field microscopy of the specimens fed DSS73-15C2 (*amsY*) shows the position of the extended gut lumen and the vulva (**d** + **f**).
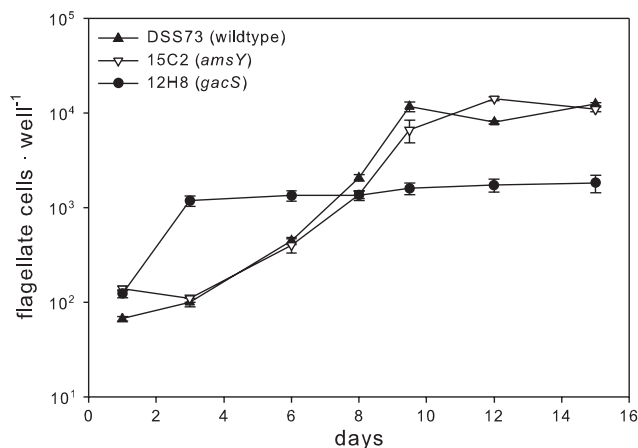
Figure 4 Numbers of the flagellate *Cercomonas* sp. per well in 96-well microtiter plates containing DSS73, DSS73-15C2 (*amsY*) or DSS73-12H8 (*gacS*) as food source ($N = 32$, experiment repeated once). Note the log scale. Error bars refer to 1 s.e.

mortem (Figures 3e and f). Hence these results, showing efficient uptake and assimilation exclusively of the DSS73 *gacS* mutant, were consistent with the high food quality of this strain to *C. elegans*.

Food quality of DSS73 for Cercomonas *sp. in liquid medium*
Similar growth curves for the flagellate *Cercomonas* sp. were obtained with DSS73 and DSS73-15C2 (*amsY)* as food source (Figure 4). The cell density of *Cercomonas* sp. was significantly higher when fed DSS73-12H8 (*gacS*) on days 3–7 and lower on days 13–15 as compared to the two other strains ($P < 0.01$, Tukey's). Hence, initial growth rate was lower on DSS73 and DSS73-15C2 (*amsY*) than on DSS73-12H8 (*gacS*) (Figure 4). The maximum cell density of flagellates fed DSS73-12H8 (*gacS*) was reached on day 3 whereas growth continued until day 10 when *Cercomonas* sp. was added DSS73 or DSS73-15C2 (*amsY*). Results of repeated experiments exhibited a similar pattern. Hence, in contrast to *C. elegans*, the flagellate *Cercomonas* sp. was able to multiply on all three strains of DSS73 in liquid cultures.

Three-species interactions between bacteria, flagellates and nematodes
The growth of *Cercomonas* sp. was independent of the type of added food bacteria from days 7–9 when *Cercomonas* was the only predator (Figure 5a). On average the system allowed for two cell divisions as the flagellates increased in cell number from approximately 2000 to 8000 per well. The nematode *C. elegans* died without producing offspring in the two-species situation (Figure 5b). However, *C. elegans* declined faster in systems inoculated with DSS73-15C2 (*amsY*) than in the systems

inoculated with DSS73 and DSS73-12H8 (*gacS*) from day 7 (ANOVA; $P = 0.022$, F = 4.49).

In the three-species systems, we observed by direct microscopy that *Cercomonas* sp. was predated on by *C. elegans* when DSS73 or DSS73-12H8 (*gacS*) were present as food bacteria. The additional food source supplied as flagellate cells supported reproduction of *C. elegans* and meanwhile *Cercomonas* sp. densities decreased by days 7–10 (Figure 5c and d). However, *C. elegans* was not able to reproduce in the three-species systems in wells containing DSS73-15C2 (*amsY*) even though *Cercomonas* sp. was available as an additional food source (Figure 5d). Hence, the response of the predators to the different bacterial strains depended on the presence/absence of the other predator. This was statistically underpinned by the significant interaction effects of food bacterium and grazing treatment on the densities of both grazers toward the end of the experiment (ANOVA; $P < 0.023$; F > 4.36).

Results from the three-species systems added 200 flagellates per mm^2 are presented in Figure 6. The high number of flagellates resulted in a high mortality of *C. elegans*. Only approximately 10% of the nematodes had survived after 4 days with flagellates in controls without bacteria or in model systems with *Pseudomonas* DSM50090 or *Arthrobacter* BEM447 (Figure 6). Interestingly, the wt DSS73 and the amphisin-deficient mutant DSS73-15C2 (*amsY*) rescued the nematodes from flagellate attack and killing. For these two bacteria nematode survival was above 75% and not different from the controls without flagellates (Figure 6). The ability to protect the nematodes from flagellate attack appeared to be associated with the *gacS* gene because survival of nematodes fed DSS73-12H8 (*gacS*) was similar to those fed with DSM50090 or BEM447.

Effect of grazing on colonization of DSS73 in soil microcosms inoculated with a bacterial model community
To test the effect of grazers on the colonization and survival of DSS73 in soil we used soil microcosms inoculated with a model community consisting of six rhizosphere bacteria selected to represent an even distribution of Gram-positive and -negative bacteria of low and high food quality, respectively (Table 1). DSS73 was *gfp*-tagged so that the number of DSS73*gfp* could be distinguished from the other bacteria on the CFU plates. The presence of the nematode predator increased the abundance of DSS73*gfp* after 11 days compared to the control or flagellate-treated soil microcosms (grazer: $P = 0.0026$, F = 18.7; Table 2). The relative abundance of DSS73 was 14.6% in nematode-grazed soil as compared to less than 1% in controls as well as flagellate-grazed microcosms. In other words, DSS73 depended on the presence of nematodes for efficient
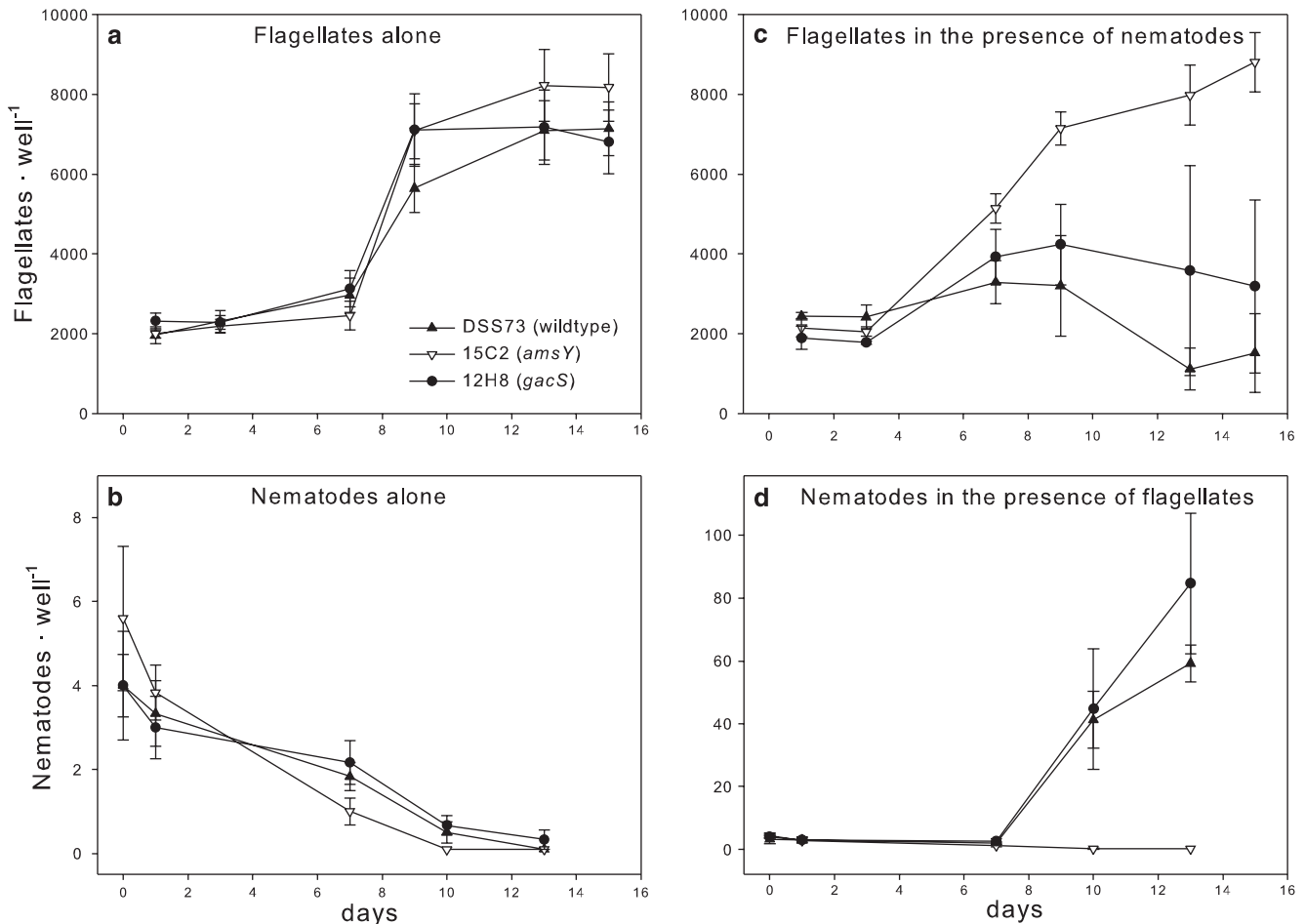
Figure 5 Number of *Cercomonas* sp. (**a**, **c**) and *C. elegans* (**b**, **d**) in the wells of six-well culture plates inoculated with either DSS73, DSS73-15C2 or DSS73-12H8 when present as single bacterial feeder (**a**, **b**) or in three-species systems with both bacterial feeders present (**c**, **d**) ($N = 6$).
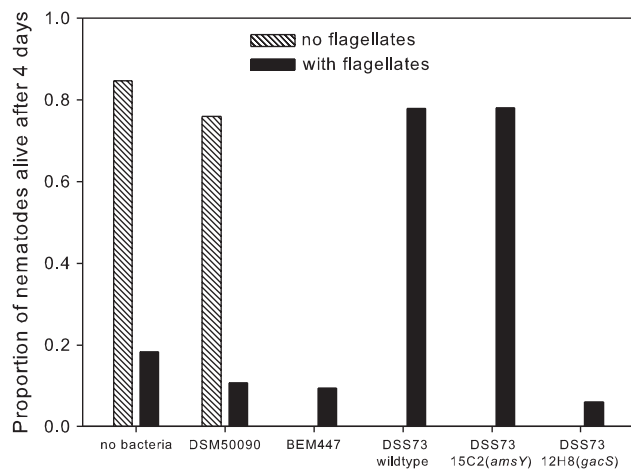

Figure 6 Survival of the nematode *C. elegans* after 4 days in the presence or absence of the flagellate *Cercomonas* and with different additions of food bacteria ($N = 3$, experiment repeated once). *Pseudomonas fluorescens* DSM50090 is a good food bacterium. *Arthrobacter* sp. BEM447 is a poor food bacterium.

colonization of these microcosms. A schematic summary of all the interactions analyzed in this paper is given in Figure 7.

Table 2 The effect of grazing on the total bacterial abundance and the proportion of *Pseudomonas* DSS73 in soil microcosms inoculated with a model bacterial community (standard error in brackets, $n = 3$)

Treatment	Number of total bacteria ($\times 10^6$ CFU per gram)	Relative abundance of DSS73 (% of total CFU)
No grazers	7.4 (1.7)	0.17 (0.17)[a]
Flagellates	4.6 (1.0)	0.25 (0.25)[a]
Nematodes	11.4 (5.3)	14.6 (1.3)[b]
P-value*	0.4008	0.0026

Each microcosm was inoculated with equal amounts of seven different bacterial strains (approximately 5.7×10^4 per gram of soil for each strain). The microcosms were destructively sampled after 11 days.
*The effect of grazer treatment was tested by one-way ANOVA. Grazing had a significant effect on the proportion of DSS73 in the soil. Means followed by different letters are significantly different ($P < 0.05$, Tukey's).

Discussion

DSS73 provides a source of energy and matter for proliferation of the flagellate, but not for the
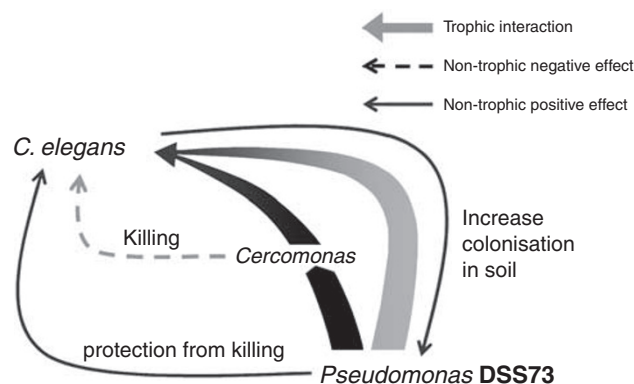
Figure 7 Schematic summary of the interactions between *Pseudomonas* DSS73 and two bacterial feeders, the nematode *C. elegans*, and the flagellate, *Cercomonas*. The interactions indicated by black arrows occur when the *gacS* gene of DSS73 is functioning. When DSS73 is mutated in *gacS*, exoproduct production is deficient, and the interactions indicated by the gray arrows predominate.

nematode in liquid cultures (Figure 7). This agrees with the observation that some α-proteobacteria support fast growth of *Cercomonas* and not *C. elegans* (Bjørnlund *et al.*, 2006). There was more assimilation and less accumulation of bacteria in the intestine of nematodes fed DSS73 than in nematodes fed DSS73-15C2 (*amsY*). Amphisin is a surfactant and may enhance the food flow through the intestine. As bacterial residues accumulated in the intestine when nematodes are fed DSS73-15C2 (*amsY*), the exposure of the intestinal epithelium to harmful exoproducts is most likely increased.

The biocontrol strain CHA0 is more toxic toward bacterial grazers than DSS73 (Pedersen *et al.*, 2009), perhaps because CHA0 produces a wider variety of metabolites with antibiotic effects than DSS73 (Keel *et al.*, 1992; Sørensen *et al.*, 2001). Still, production of exoproducts is disabled by the loss of a functional *gacS* regulatory gene and *C. elegans* exhibited similar growth on the *gacS* mutants of CHA0 and DSS73 on solid media (see Supplementary Material). We therefore suggest that the GacS regulator provides *Pseudomonas* strains with protection against nematode as well as protozoan grazers. We were not able to assign a protective function to the biosurfactant amphisin. Likewise, it appears that CHA0 is not protected against protozoa by a single excreted metabolite (Jousset *et al.*, 2006). Another biocontrol strain, *Pseudomonas* DR54, produces viscosinamid, which is also a biosurfactant with detrimental effects on amoebae (Andersen and Winding, 2004). However, viscosinamid is primarily membrane bound, not excreted. Likewise, violacein accumulates inside the cells of *Chromobacterium* and is a very potent toxin against flagellates (Matz *et al.*, 2004a). These studies indicate that defense metabolites targeted against bacterial feeders tend to be more effective when cell-associated in planktonic bacteria. In biofilms, however, *P. aeruginosa* use at least two defense strategies against amoebae:

quorum-sensing-dependent release of metabolites (Matz *et al.*, 2004b) as well as quorum-sensing-independent colonization of protozoa by the bacteria (Matz *et al.*, 2008).

Three-species interaction: C. elegans *uses flagellates as an alternative food source*
Observations from the three-species systems demonstrate that protozoa can act as a shunt that delivers an otherwise inaccessible energy source to a competitor/predator (Figure 7). This is consistent with a classic study by Elliott *et al.* (1980) who described how the nematode *Mesodiplogaster* fed on amoebae in soil that exploited bacteria confined in soil pores physically inaccessible to the nematodes themselves. Our results extend this principle to bacteria that are physically available but indigestible to the nematodes. However, the toxic effect of DSS73-15C2 (*amsY*) could not be overcome by the presence of an alternative food source in the form of flagellates in our gnotobiotic model system.

Three-species interactions: DSS73 protects C. elegans *from flagellate attack*
The three-species system revealed that nematodes, which were feeding on DSS73, avoided the attack of flagellates and hence survived at flagellate densities that usually kill the nematodes (Bjørnlund and Rønn, 2008; Figure 7). Such a phenomenon has never been described before for bacteria–bacterial feeder interactions. A possible parallel could be certain sea slugs (dorid nudibranchs) that sequester toxic metabolites from the sponges they feed on and use them for their own protection against predators (Faulkner and Ghiselin, 1983). Whether *C. elegans* use the metabolites produced by DSS73 or derivatives hereof to defend themselves against flagellate attack remains to be resolved.

DSS73 colonization of soil is aided by nematodes
Results from the soil systems containing seven bacterial strains showed that DSS73 is not a strong competitor as the proportion of DSS73 was disproportionately low in the two treatments without nematodes. However, when nematodes were present, DSS73 colonized the soil more efficiently (Table 2; Figure 7). This is consistent with a previous study, where DSS73 was also found to be a poor competitor compared to *P. fluorescens* strain CHA0 except when simple liquid systems were grazed by nematodes (Pedersen *et al.*, 2009). DSS73 aggregated in the intestine of the nematodes. Bacteria in soil are not very mobile across long distances. The volume of soil conquered by a bacterial strain will therefore increase if the bacteria are able to travel inside the intestine of a nematode. Accordingly, *Rhizobium* species can be transferred to the roots of legumes by *C. elegans* in response to

attractive volatiles released by the plants (Horiuchi *et al.*, 2005).

Conclusions

In summary, (1) Gac-regulated traits of DSS73 separate from amphisin production delayed or inhibited growth of the bacterial grazers *C. elegans* and *Cercomonas* sp. (2) The flagellates multiplied on DSS73 in liquid systems whereas *C. elegans* did not. However, the flagellate *Cercomonas* feeding on DSS73 can be used as an alternative food source for the nematode *C. elegans*. (3) The wt DSS73 protects the nematodes from flagellate attack and killing. This bacterial trait is associated with a functional Gac regulon. (4) The colonization of soil microcosms by DSS73 increased in the presence of nematodes. We conclude that the interaction between DSS73 and nematode predators is not adequately described as a simple predator–prey relation. On the contrary, the relation between these two organisms is mutually beneficial in complex systems. Although some of the ingested DSS73 are digested, others are harbored inside the nematode intestine and provide the nematodes with protection against their common fiend (*Cercomonas* sp.). In this way the nematodes can feed on the flagellates instead of being killed by them and meanwhile the bacteria gain a sheltered mean of transportation.

Acknowledgements

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