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Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues

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Summary

We investigated the induction of aggregate formation in the freshwater bacterium *Sphingobium* sp. strain Z007 by growth state and protistan grazing. Dialysis bag batch culture experiments were conducted in which these bacteria were grown spatially separated from bacteria or from co-cultures of bacteria and predators. In pure cultures of *Sphingobium* sp. strain Z007, the concentrations of single cells and aggregates inside and outside the dialysis membranes developed in a similar manner over 3 days of incubation, and the proportions of aggregates were highest during the exponential growth phase. Cell production of *Sphingobium* sp. strain Z007 was enhanced in the presence of another isolate, *Limnohabitans planktonicus*, from an abundant freshwater lineage (R-BT065) outside the bags, and even more so if that strain was additionally grazed upon by the bacterivorous flagellate *Poterioochromonas* sp. However, the ratios of single cells to aggregates of *Sphingobium* sp. strain Z007 were not affected in either case. By contrast, the feeding of flagellates on *Sphingobium* sp. strain Z007 outside the dialysis bags led to significantly higher proportions of aggregates inside the bags. This was not paralleled by an increase in growth rates, and all cultures were in a comparable growth state at the end of the experiment. We conclude that two mechanisms, growth state and the possible release of infochemicals by the predator, may induce aggregate formation of *Sphingobium* sp. strain Z007. Moreover, these infochemicals only appeared to be generated by predation on cells from the same species.

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Introduction

Free-living bacteria in aquatic habitats typically suffer high mortality rates because of grazing by interception-feeding flagellated protists (Pace, 1988; Pernthaler, 2005). The formation of filaments or of cell aggregates that are too large to be ingested is believed to be an effective bacterial defense strategy against flagellate predation. A rapid increase of such grazing-resistant morphotypes at high protistan densities has been demonstrated both in laboratory investigations and in the field (Jürgens *et al.*, 1999; Hahn *et al.*, 2000; 2004; Šimek *et al.*, 2001; Pernthaler *et al.*, 2004). Some bacterial genotypes in freshwaters may be permanently grazing-resistant [e.g. the filamentous SOL bacteria (Schauer and Hahn, 2005)], whereas others possess inherent phenotypic plasticity, i.e. a fraction or the total population may morphologically shift from readily consumable rod-shaped single cells to large complex morphologies, aggregates or microcolonies (Pernthaler *et al.*, 1997; Šimek *et al.*, 1997; 1999; Hahn and Höfle, 1999; Salcher *et al.*, 2005).

Two mechanisms are held responsible for inducing such changes of cell shape or aggregation behaviour in bacterial strains exposed to protistan predation. For one, it has been argued that flagellate foraging will release surplus substrates and nutrients into the surrounding medium (Caron *et al.*, 1988; Verhagen and Laanbroek, 1992; Nagata, 2000), thus inducing morphological shifts in bacteria by providing better growth conditions. A direct relationship between growth rate and the emergence of filamentous morphotypes in a bacterial strain has been demonstrated in continuous culture (Hahn *et al.*, 1999). By contrast, no growth rate-related microcolony formation was found in another strain (Hahn *et al.*, 2000). On the other hand, there have been speculations about the action of specific chemical cues (Pernthaler *et al.*, 1997), in analogy with morphogenetic factors that modify other predator–prey interactions in the plankton (Pohnert *et al.*, 2007). Evidence for this hypothesis has recently been produced in chemostat experiments with a pleiomorphic

Fig. 1. Experimental setup: *Sphingobium* sp. strain Z007 inside the dialysis bags was amended with (i) *Sphingobium* sp. strain Z007 only (SP), (ii) *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS (SP + P), (iii) *Limnohabitans planktonicus* only (LH) and with (iv) *L. planktonicus* and *Poterioochromonas* sp. strain DS (LH + P) outside of the dialysis bags. Additionally, aggregate formation of *Sphingobium* sp. strain Z007 was studied without competing organisms outside the dialysis bags (NC).

bacterial strain (Corno and Jürgens, 2006): the formation of filamentous cells was observed in the presence of bacterivorous flagellates that were feeding on these bacteria and were spatially separated by a dialysis membrane. However, it remains unknown if comparable morphological shifts would have also been induced by protists feeding on a different bacterial species.

Interestingly, the above-described cases of cell filamentation triggered by either growth rate or by infochemicals were observed in two phylogenetically closely related bacterial strains (Hahn *et al.*, 1999; Corno and Jürgens, 2006). This raises the question if the proposed reasons for the induction of phenotypic changes during predation are indeed mutually exclusive. The formation of protist inedible bacterial morphotypes in grazed microbial assemblages appears to be related to growth limiting factors or overall community productivity (Matz *et al.*, 2005; Corno and Jürgens, 2008). Additionally, a variety of conditions may stimulate bacterial cell aggregation or elongation, e.g. intraspecific cell signalling (Gram *et al.*, 2002), starvation (Kolter *et al.*, 1993), hydrostatic pressure (Zobell and Cobet, 1964) or a combination of virus- and grazing-induced mortality of bacterial competitors (Šimek *et al.*, 2007). It is, thus, conceivable that a phenotypic change conferring grazing protection may be constitutively triggered by high growth rates, but additionally (e.g. at suboptimal growth conditions) also by a predator-mediated chemical cue.

While it seems that the causes for phenotypical changes in bacteria can be readily studied in static batch culture because of the rapid transition from optimal to stagnant growth conditions, it has so far not been possible to demonstrate the involvement of a chemical factor in cell aggregation or filamentation by this approach (Hahn *et al.*, 2000; Corno, 2006; Corno and Jürgens, 2006). This may in parts be related to the choice of model bacteria. We recently isolated a bacterial strain (*Sphingobium* sp. strain Z007) that forms cell aggregates in pure culture but also appears to phenotypically respond to predators during batch growth (J.F. Blom, Y.S. Zimmermann, T. Ammann and J. Pernthaler, unpublished). Aggregate formation by this strain was studied during different growth phases with and without predation, and keeping bacteria spatially separated from predators inside dialysis bags (Fig. 1). In addition, a second bacterial strain with no morphological plasticity was used either as competitor or as alternative prey in order to assess if cell aggregation in *Sphingobium* sp. strain Z007 was also affected by interspecific interactions. In order to account for differences in nutrient availability on aggregate formation *Sphingobium* sp. strain Z007 was additionally studied without competing bacteria outside of the dialysis bags.

Results

Experiment 1: bacterial and flagellate abundances

Single cells and aggregates of *Sphingobium* sp. strain Z007 could be readily discriminated and counted by flow cytometry (Fig. 2). The sorting of events from the different gates and microscopic inspection confirmed that the cytograms had been appropriately divided for a quantification of the two subpopulations.

The abundances of single cells of *Sphingobium* sp. strain Z007 in the control treatment (SP; Fig. 1) continuously increased throughout the experiment and reached maximal values of 0.4×10^8 cells ml^{-1} both inside and outside of the dialysis bags (Fig. 3). In treatments featuring both *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. outside the bags (SP + P), bacteria

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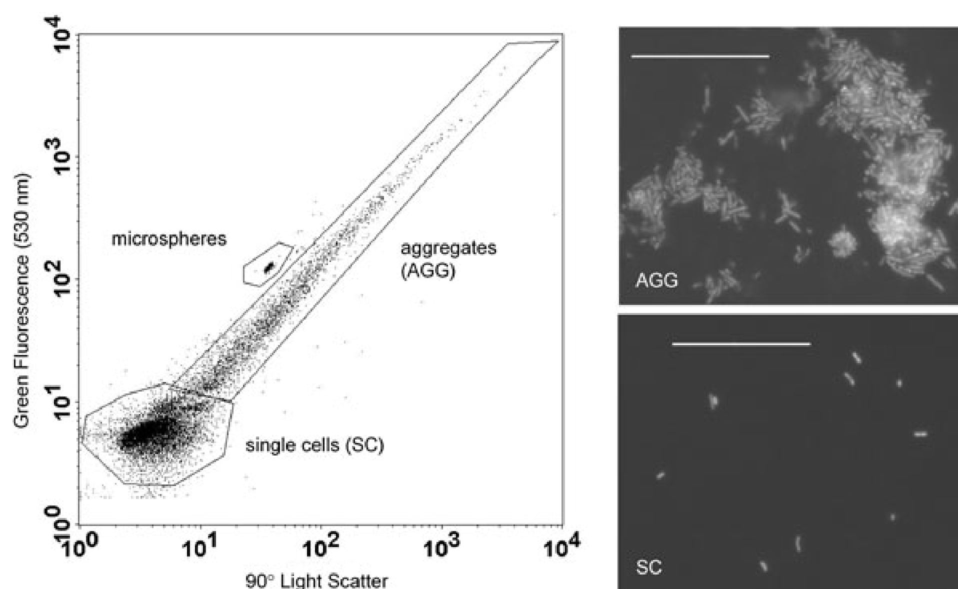

Fig. 2. Left panel: example of a cytogram (90° light scatter vs. green fluorescence, after staining with SYTO 13) of cells in treatment SP + P inside the dialysis bag at the end of the experiment (72 h). The three polygons (gates) define the populations of aggregates (AGG) and single cells (SC) of *Sphingobium* sp. strain Z007 and microspheres (1 μm diameter, internal standard). Right panels: micrographs of aggregates and single cells from the same treatment after sorting and DAPI staining. Bar = 20 μm.

inside the dialysis bags reached virtually the same abundances as in the control treatments. By contrast, they were heavily reduced outside the bags because of flagellate grazing, and the numbers of *Poterioochromonas* sp. increased exponentially over the whole study interval (Fig. 3).

Sphingobium sp. strain Z007 grew significantly better if *Limnohabitans planktonicus* was inoculated outside the bags (treatment LH) than in control treatments, reaching more than twice as high maximal cell densities. The growth of *Sphingobium* sp. strain Z007 inside the dialysis bags with no other competing organism outside (NC) was comparable with treatment LH, in both treatments single cell concentrations reached values around 0.9×10^8 cells ml^{-1} (Fig. 3). An insignificantly stronger stimulating effect on *Sphingobium* sp. strain Z007 inside dialysis bags was observed in treatments simultaneously amended with *L. planktonicus* and *Poterioochromonas* sp. (LH + P). Again, flagellates efficiently controlled the development of *L. planktonicus* outside the bags and grew exponentially until the end of the experiment (Fig. 3). Interestingly, *Poterioochromonas* sp. reached almost the identical final abundances when grazing on either *Sphingobium* sp. strain Z007 or *L. planktonicus*.

Experiment 1: growth rates

Growth rates for the single cells of *Sphingobium* sp. strain Z007 were calculated per unit time for the exponential growth phase and for the early stationary phase (Table 1). The increase in aggregate formation during the first 17 h could be unambiguously assigned to the growth rate that was comparable in all four treatments as well as the increase in the percentage of aggregates (Fig. 3). Towards the end of the experiment the higher percentage of aggregates in the SP + P treatment only was not accompanied by an increase in growth rate (Table 1).

Experiment 1: aggregate formation

The formation of new aggregates inside the dialysis bags stopped after 48 h in the control treatment (SP, Fig. 3, middle panels). By contrast, the numbers of aggregates of *Sphingobium* sp. strain Z007 in the bags continuously increased throughout the experiment in all other treatments harbouring the flagellate predator or *L. planktonicus* or without any competing organism, with maximal values after 72 h. Aggregate numbers outside the bags decreased towards the end of the experiment both in ungrazed and grazed *Sphingobium* sp. strain Z007 populations (Fig. 3, middle panels). The relative proportions of aggregates (ratio of aggregates to single cells) rapidly increased over the first 24 h in all treatments (Fig. 3, lower panels) and tended to decrease thereafter. Only the SP + P treatment showed a significantly higher percentage of aggregate formation compared with the SP control at the 72 h time point.

Aggregate area generally varied within a range of 10–1000 μm^2, with median values of approximately 80 μm^2. The sizes of aggregates in treatments SP + P and

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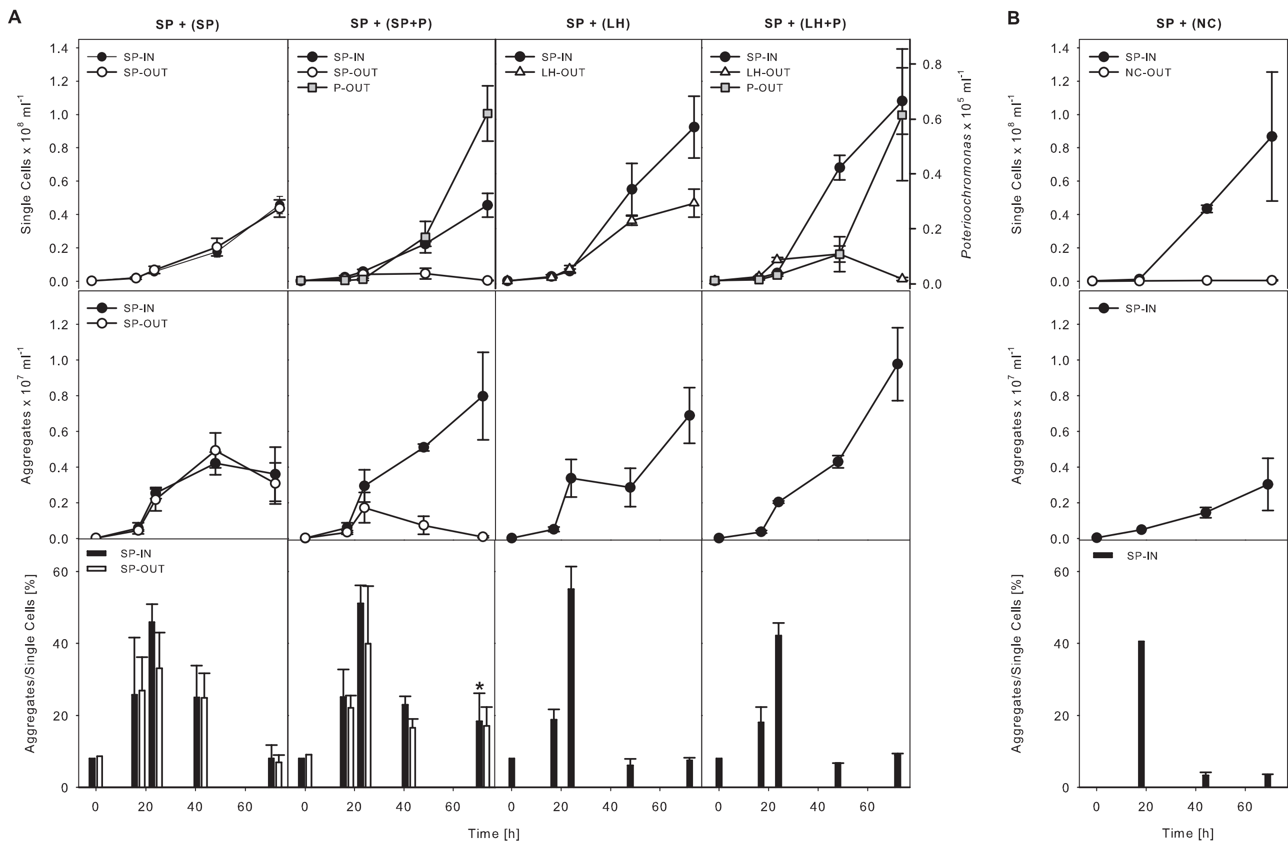

Fig. 3. Experiment 1 (A) and experiment 3 (B): changes in the abundance of single cells of *Sphingobium* sp. strain Z007 (SP), *Limnohabitans planktonicus* (LH) and *Poterioochromonas* sp. strain DS (P) (upper panels); changes in aggregate numbers (middle panels); changes of the ratios of aggregates to single cells (lower panels) of *Sphingobium* sp. strain Z007 (SP) inside (IN) and outside (OUT) of the dialysis bags. Asterisk above bar indicates that the ratio of aggregates to single cells inside the bags after 72 h was significantly different from the control ($P < 0.05$; tested by ANOVA, followed by Dunnett's post hoc tests).

LH + P at 72 h did not significantly differ (as estimated from aggregate areas by epifluorescence microscopy after flow cytometric sorting).

Experiments 1 and 2: differences and similarities

Higher initial proportions of aggregates of *Sphingobium* sp. strain Z007 were observed in experiment 2 (Fig. S1) than in experiment 1, likely because of differences in bacterial pre-cultivation conditions (i.e. starter cultures were harvested after 72 and 24 h, respectively, in experiments 1 and 2). Apart from this difference, the patterns of total population development and of aggregate formation in experiment 2 were comparable to those of experiment 1. Specifically, higher bacterial growth inside dialysis bags was again observed in treatments LH and LH + P than in the controls. In addition, significantly elevated proportions of aggregates inside the dialysis bags were found on the final time point (70 h) only if *Poterioochromonas* sp. was feeding on *Sphingobium* sp. strain Z007 outside the bags (Fig. S1).

Table 1. Growth rates (h^{-1}) of single cells of *Sphingobium* sp. strain Z007 inside the dialysis bags over two periods (0–17 h and 48–72 h).

	(0–17 h)		(48–72 h)	
Treatment	Mean	SD	Mean	SD
SP + (SP)	0.156	0.01	0.040	0.01
SP + (SP + P)	0.157	0.02	0.029	0.01
SP + (LH)	0.167	0.02	0.022	0.01
SP + (LH + P)	0.151	0.01	0.019	0.01

SD indicates the standard deviation of three replicates.

Impact of grazing and competition on Sphingobium *sp. strain Z007*

To better assess the effects of flagellate predation and a competing bacterial strain on the growth patterns of *Sphingobium* sp. strain Z007 the abundances of single cells and aggregates inside the dialysis bags in the various treatments at 72 h were set in relationship with the corresponding values of these parameters in the control treatment (Fig. 4). While the presence of

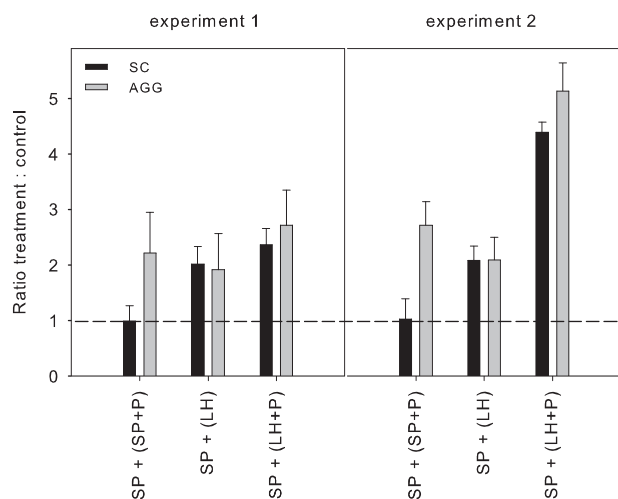

Fig. 4. Ratio of the mean abundances of single cells (SC, black columns) and aggregates (AGG, grey columns) of *Sphingobium* sp. strain Z007 in the different treatments to the corresponding values in the control treatment (SP) at the last sampling time point (72 h for experiment 1; 70 h for experiment 2). Standard deviations were calculated as the summed coefficients of variations of the two corresponding values, assuming error propagation. As the median size of aggregates was similar in all treatments, the numbers of aggregates was related to the numbers of single cells.

Poterioochromonas sp. did not visibly affect the abundances of single cells inside the bags, it substantially stimulated aggregate formation, as reflected in a treatment to control ratio of 2–3 in experiments 1 and 2 respectively. In the presence of *L. planktonicus*, both the ratios of single cell and aggregate abundances increased equally. The combined effects of *L. planktonicus* being grazed by *Poterioochromonas* sp. again stimulated the growth of both single cells and aggregates to a similar extent (cf. Fig. 3, lower panels).

Discussion

Aggregate formation induced by growth conditions

Cell aggregation, ranging from the formation of small aggregates or microcolonies to the development of large cell clusters covered by exopolymerous material, is a commonly encountered phenotypic property of freshwater microbes (Jürgens and Güde, 1994; Hahn and Höfle, 1999). A formation of large flocks of cells during the stationary growth phase in pure cultures has been interpreted as a result of suboptimal growth conditions (Kolter *et al*., 1993). However, aggregation may also be a specific strategy in some bacteria. Growth within single or multispecies aggregates may provide additional nutrients, substrates or enzymes (Simon *et al*., 2002), and the exopolymeric matrix around aggregated cells may act as a physical barrier to protect bacteria from various adverse chemical or biological influences (Costerton, 1999; Matz *et al*., 2005). For example, *Azospirillum brasiliensis* Sp7 forms the highest fractions of aggregates during the early exponential growth phase in static culture and releases autogenous lectins that specifically bind to its own extracellular polysaccharide-containing complexes only (Nikitina *et al*., 2001).

Sphingobium sp. strain Z007 in pure culture showed highest proportions of cell aggregates during the early exponential growth phase, whereas the population shifted towards single cells during the later growth stage (Figs 3 and S1). This suggests that aggregation in this strain is enhanced during optimal growth. Schleheck and colleagues (Schleheck *et al*., 2009) described similar phenomenon in a population of *Pseudomonas aeruginosa* biofilms, which rapidly dispersed and released single cells into the medium under suboptimal growth conditions, e.g. starvation. Several reasons can be envisaged why higher aggregate formation at favourable growth conditions might be advantageous for aquatic bacteria. For one such behaviour could be interpreted as indirect anti-predation strategy (Hahn *et al*., 1999): intense flagellate grazing will result in the recycling of essential nutrients (Caron *et al*., 1988; Verhagen and Laanbroek, 1992; Nagata, 2000) and the removal of competing bacteria, allowing for faster growth of grazing-resistant bacteria (Šimek *et al*., 2007). Our results thus provide indirect support for the growth-related induction of morphological protection against protistan grazing (Hahn *et al*., 1999).

However, the advantage of cell aggregation during early exponential phase need not be limited to predator–prey interactions. The pelagic zone of freshwaters is no chemically uniform environment but a patchwork of substrate and nutrient gradients that are steepest, e.g. in the phycosphere of senescent algal cells (Grossart and Simon, 1998). In this context, the enhanced formation of aggregates by *Sphingobium* sp. strain Z007 at favourable conditions could represent a beneficial strategy for exploiting point sources of organic matter in the water column. Members of the closely related genus *Sphingomonas* have been reported to occur in high abundances in newly formed (Knoll *et al*., 2001) and mature (Schweitzer *et al*., 2001) freshwater organic aggregates (lake snow) or in enrichment cultures after addition of dissolved organic matter (Eiler *et al*., 2003). At the same time, there are also metabolic and ecological disadvantages of such a growth strategy for planktonic bacteria, e.g. vulnerability to larger zooplankton, as discussed in detail elsewhere (Jürgens and Güde, 1994; Hahn *et al*., 2000).

Interestingly, the overall densities of *Sphingobium* sp. strain Z007 inside dialysis bags were higher in the presence of another bacterial strain (*L. planktonicus*) rather than itself outside the dialysis bags (Fig. 3). This at a first glance counterintuitive finding is probably best interpreted in the context of interspecific versus intraspecific compe-

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tition: it is conceivable that the two strains did not entirely overlap in their respective substrate spectra, and that the consequently higher availability of particular essential organic compounds might explain the higher growth of *Sphingobium* sp. strain Z007.

Alternatively, an active, e.g. auxotrophic, relationship might be assumed. However, as the competition between the two strains was not in the focus of our investigation, additional experiments on defined media would be required to specifically address the nature of this interaction.

Evidence for chemically induced aggregate formation

Our results go beyond providing evidence for growth-related cell aggregation in *Sphingobium* sp. strain Z007. A second phase of high percentage of aggregates could be discerned in two independent experiments (Figs 3A and S1): while the proportions of aggregates inside the dialysis bags declined after 72 h in the absence of competing bacteria (Fig. 3B) or predators (SP and LH) or during grazing on *L. planktonicus* (LH + P), a significantly higher fraction of aggregates was found at that time point if flagellates were feeding on *Sphingobium* sp. strain Z007 outside the dialysis bags (SP + P, Fig. 3A). At the same time, the final abundances of single cells formed inside the bags were identical irrespective of whether these bacteria were grazed upon or not (Fig. 3, SP and SP + P). Because better growth conditions always lead to a parallel increase of both single cells and aggregates in the other treatments (LH, LH + P) (Figs 3 and 4), the higher proportions of aggregates only in SP + P were most likely not a consequence of predation-induced nutrient recycling (Caron *et al*., 1988). Moreover, the increase in the proportions of aggregates in SP + P towards the end of the experiment was not accompanied by an increase in growth rate of single cells (Table 1): growth rates were comparably low in all treatments and even slightly (but not significantly) higher in treatments without flagellate grazing. Therefore, a chemical stimulus most likely accounts for the higher final proportions of aggregates in the SP + P treatment, which was moreover only observed when protists were feeding on bacteria of the same species.

These findings extend earlier reports of cell filamentation induced by chemical cues (Corno and Jürgens, 2006) to a different type of morphological response. Moreover, the involved factor appeared to be conspecific, because cell aggregation was only triggered upon feeding on *Sphingobium* sp. strain Z007, but not on *L. planktonicus*. This might in fact be a reason why no comparable reaction was observed in other aggregate-forming bacteria after addition of supernatants from a predator culture (Hahn *et al*., 2000).

Various organisms exhibit morphological defence against grazers and predators that are triggered by infochemicals (Pohnert *et al*., 2007). The term 'kairomone' was originally proposed to denote substances that confer an advantage upon the receiver and a disadvantage for the emitter (Brown *et al*., 1970). It is often used in the literature to describe substances released by a predator, which lead to changes in morphology, behaviour or life history traits of the prey. In many predator–prey systems the chemical cues inducing the defence response are produced during feeding. As a consequence, the prey potentially receives complex chemical information including specifications about type and density of the predator present and the identity of the prey being consumed (Larsson and Dodson, 1993). Both types of information may be relevant to identify the predator and to establish the identity of the prey. Indeed, many studies could show differences in defence respond when conspecific or heterospecific prey was consumed. For example, brook stickleback decreased activity only when exposed to chemical cues from predatory pike that was fed with conspecific sticklebacks but not with heterospecific fish species (Gelowitz *et al*., 1993). Frog tadpoles reduced movements only when exposed to predatory newts that had eaten conspecific frogs but not to newts that had eaten insects (Wilson and Lefcort, 1993), and morphological changes in *Daphnia* were only induced by infochemicals from fish fed with *Daphnia*, but not with earthworms (Stabell *et al*., 2003).

It is interesting to note that aggregate formation did not protect the *Sphingobium* sp. strain Z007 population outside the dialysis bags from being affected by predation (Fig. 3, middle panels). This might indicate that morphological change in *Sphingobium* sp. strain Z007 does not represent a specific strategy against our model predator only but is a rather general response that might be more or less effective for different flagellate species. Additional experiments using other axenic predator species would be required to test this hypothesis.

Conclusions

Our data suggest that aggregate formation could be induced in *Sphingobium* sp. strain Z007 either via substrate availability or through the action of infochemicals, and that the two triggers were temporally distinguishable during growth in static culture. From an ecological view point it could be envisaged that *Sphingobium* sp. strain Z007 may tend to aggregate or form biofilms under optimal growth conditions to escape predation, but also to gain access to inhomogeneously distributed substrates and nutrients. In addition, such morphological change might be also useful at suboptimal conditions if induced in the presence of protistan predators. While the here pre-

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sented results strongly indicate that this was due to a conspecific chemical cue, the establishment of an appropriate bioassay will be a precondition for eventually elucidating its chemical nature.

Experimental procedures

Bacterial strains and axenic flagellate predator

Sphingobium sp. strain Z007 is a rod-shaped bacterium forming yellow coloured colonies, which has been isolated from the surface layer of mesotrophic Lake Zürich in 2006 (Blom and Pernthaler, 2010). It is a moderately fast-growing strain with the ability to form aggregates. The partial sequence of the 16 rRNA gene has been deposited in the EMBL Nucleotide Sequence Database (accession number FN293045). *Limnohabitans planktonicus* strain II-D5 is a fast-growing rod-shaped bacterium, never forming aggregates or filaments affiliated with the R-BT065 subcluster of β-proteobacteria (Šimek *et al*., 2001). The strain was isolated from the surface layer of the freshwater meso-eutrophic Římov reservoir (Czech Republic). It is under description as a member of a new genus, *Limnohabitans* (Kasalický *et al*., 2010), suggested by Hahn and colleagues (Hahn *et al*., 2010). The partial sequence of the 16S rRNA gene of *L. planktonicus* strain II-D5 has been deposited in the GenBank (accession number FM165535). Axenic cultures of the facultatively mixotrophic flagellate predator *Poterioochromonas* sp. strain DS (formerly *Ochromonas* sp. DS) (Hahn and Höfle, 1998; Boenigk *et al*., 2006), originally isolated from Lake Constance, were produced as described elsewhere (Blom and Pernthaler, 2010). Briefly, mixtures of antibiotics (chloramphenicol, streptomycin and gentamycin) were added in different amounts to the flagellate cultures, which were kept in the dark and fed with heat-killed cells of *Flectobacillus major* DSMZ 103. Prior to experiments, the flagellate culture was grown in 'undefined *Ochromonas*-medium' [yeast extract (1 g l^{-1}), meat extract (1 g l^{-1}), glucose (1 g l^{-1}) and peptone (1 g l^{-1}); Culture Collection of Algae (SAG) at the University of Göttingen, Germany] at 18°C at a light/dark cycle of 14:10 h without addition of heat-killed bacteria.

Experimental design

Prior to both experiments, *Sphingobium* sp. strain Z007 and *L. planktonicus* were separately pre-cultured in 10 mg l^{-1} of NSY medium (Hahn *et al*., 2004) and then re-inoculated a second time into fresh NSY medium of the same concentration. Before starting the experiments light-adapted cultures of *Poterioochromonas* sp. strain DS were kept in darkness at 18°C for 2 days. Bacterial inoculates from the late exponential phase/early stationary growth phase (after 72 h) were used in experiment 1 and 3, and from mid-exponential phase/early stationary phase (after 24 h) in experiment 2. At the start of the experiment, bacteria and *Poterioochromonas* sp. were first counted by flow cytometry. Both bacterial strains were subsequently inoculated into fresh NSY medium (10 mg l^{-1}) to yield initial densities of approximately 2×10^5 cells ml^{-1} and 3×10^5 cells ml^{-1} for experiments 1 and 2 respectively. The initial cell concentration in experiment 3 was 1×10^5 cells ml^{-1}.

To follow the growth and the aggregate formation of *Sphingobium* sp. strain Z007 in response to a flagellate predator (*Poterioochromonas* sp.) and a competing bacterial strain (*L. planktonicus*), the following setup was established in all three experiments (Fig. 1): *Sphingobium* sp. strain Z007 was incubated inside dialysis bags, each containing 20 ml of bacterial culture at the densities reported above (diameter of dialysis tubes: 29 mm; molecular weight cut-off: 12–14 kDa; pre-incubated 3 times in Milli-Q water at 80°C for 60 min; Spectra/Por®, Spectrum Laboratories, USA). The bags were aseptically transferred inside sterile 500 ml Erlenmeyer flasks filled with 200 ml of NSY medium (10 mg l^{-1}) containing either: (i) *Sphingobium* sp. strain Z007 (control treatment SP) or (ii) *L. planktonicus* (treatment LH) at the same starting abundances. A second set of flasks containing the above bacterial strains were additionally inoculated with *Poterioochromonas* sp. (treatments SP + P and LH + P) at approximate initial abundances of 0.5×10^3 and 1×10^3 cells ml^{-1} for experiments 1 and 2 respectively. To ensure the purity of the treatments depicted in Fig. 1, all the above steps of setting up of the respective experimental treatments were conducted aseptically inside of a laminar flow box (Holten Laminair, Therma, Electron). The differently amended flasks were incubated under moderate shaking (60 r.p.m.) at 18°C in the dark for 72 h. Each treatment was run in triplicates. Samples of 15 ml from inside as well as from outside the dialysis bags were taken at the start of the experiments and at four subsequent time points, fixed with glutaraldehyde (final concentration 1.5%) for 30 min and stored at 4°C until further processed.

Flow cytometric enumeration of microbes

Immediately after sampling, subsamples of 1 ml were stained for flow cytometry with the fluorochrome Syto13 (10× diluted in dimethylsulfoxide, final concentration 5 μM, Molecular Probes) for 10 min in the dark and analysed on a FACS Calibur flow cytometer (Becton Dickinson). Samples were diluted with fresh 0.2 μm filtered Milli-Q water if required to avoid particle coincidence. The rate of particle passage was kept at < 1500 events s^{-1}. To convert flow cytometric counts to cell numbers, flow rates determined using the Trucount tubes (Becton Dickinson) with a defined amount of fluorescent microspheres were used. Alternatively, yellow-green latex microspheres (diameter 1 μm, Polysciences) were added as an internal standard. Bacterial strains and *Poterioochromonas* sp. were identified using 90° angle light scatter versus green fluorescence (530 nm) or green versus red fluorescence (630 nm) plots (Gasol and Del Giorgio, 2000). Because of the markedly larger cell size and up to three orders of magnitude lower abundances of *Poterioochromonas* sp. compared with bacteria, flagellates were counted separately applying different cytometric settings. Aggregates of *Sphingobium* sp. strain Z007 were clearly separated from the single cells based on their 90° angle light scatter (Fig. 2, left panel). Abundance of aggregates was then subtracted from total bacterial counts. At the end of the experiments, aggregates and single cells of *Sphingobium* sp. incubated inside the dialysis bags were sorted out from the SP + P and LH + P treatments, stained with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI,

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final concentration 1 μg ml^{-1}), concentrated on 0.2 μm black membrane filters (Poretics) and inspected under an epifluorescence microscope (AX 70, Olympus, Fig. 2, right panels). Average aggregate area (μm^2) was estimated by using the image analysis system LUCIA (Laboratory Imaging). At least 50 aggregates were measured per sample. Growth rates of single cells of *Sphingobium* sp. strain Z007 were calculated per unit time by following equation: $\mu = \ln(N_t/N_0)/\Delta t$, whereas μ is the calculated growth rate, N_0 is the population size at the beginning and N_t the abundance at the end of the interval, and Δt the length of the time interval. Towards the end of the experiment (last time point) a small proportion of *Sphingobium* sp. cells (always < 5% of total bacterial counts) contaminating the *L. planktonicus* culture outside the dialysis bags were detected in a minority of cases. However, the cells of the contaminating strain could be unambiguously distinguished by their flow cytometric signature and the cell numbers of *L. planktonicus* were corrected accordingly. In experiment 3 only those replicates with no detectable growth outside the dialysis bags were taken into account.

Statistical analysis

Data on the relative proportions of aggregates (ratios of aggregates to single cells) of *Sphingobium* sp. strain Z007 inside the dialysis bags in different treatments were normalized by arcsine transformation. A one-way analysis of variance (ANOVA), using replicate data ($n = 3$) from every treatment, was applied to test for significant differences between treatments at the final time point of the two experiments (72 and 70 h, respectively). Subsequently, Dunnett's post hoc tests were performed for the pair-wise comparison of the fractions of aggregates in different treatments with the controls (SP). All statistical analyses were performed using GaphPad Prism (GraphPad Software).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Experiment 2: changes in the abundance of single cells of *Sphingobium* sp. strain Z007 (SP), *Limnohabitans*

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planktonicus (LH) and *Poterioochromonas* sp. strain DS (P) (upper panels); changes in aggregate numbers (middle panels); changes of the ratios of aggregates to single cells (lower panels) of *Sphingobium* sp. strain Z007 (SP) inside (IN) and outside (OUT) of the dialysis bags. Asterisk above bar indicates that the ratio of aggregates to single cells inside the bags after 70 h was significantly different from the control ($P < 0.05$; tested by ANOVA, followed by Dunnett's post hoc tests).

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