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FEATURE ARTICLE

Species-specific patterns in the vulnerability of carbon-starved bacteria to protist grazing

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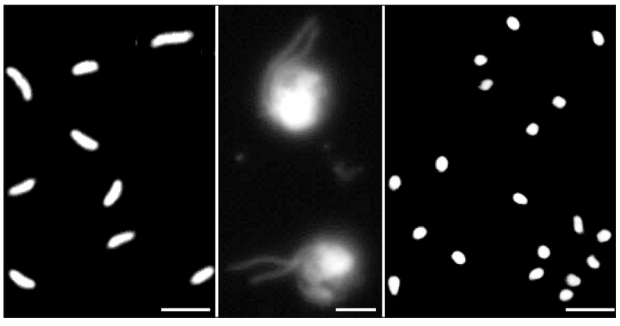
ABSTRACT: Many heterotrophic bacteria possess adaptations for prolonged survival under carbon and energy limitation, generally involving a reduction in cell size and an increased resistance to environmental stress factors. In order to reveal whether carbon-starved bacteria also become less vulnerable to protist grazing, we compared the growth of a bacterivorous nanoflagellate, *Cafeteria roenbergensis*, on different physiological states of 3 bacterial strains with well-studied starvation responses (*Vibrio vulnificus, Photobacterium angustum* and *Sphingopyxis alaskensis*). Protists achieved high growth rates on all 3 bacterial strains when they were provided in a non-starved state. However, for carbon-starved bacteria, pronounced differences in the response of the flagellates were observed. *P. angustum* provided similar protist growth for an equal biomass of non-starved and starved cultures, indicating no change in food quality or grazing resistance for carbon-starved cells, despite smaller cell size. In contrast, starved *V. vulnificus* did not support protist growth, even resulting in a strong decrease in flagellate numbers at most concentrations tested; and starved *S. alaskensis* provided only reduced growth rates. Our results demonstrate that (1) feeding on bacteria of smaller cell size does not necessarily impose energy constraints on a flagellate grazer, and (2) a pronounced species-specific variability exists in the susceptibility of carbon-starved bacteria to protist grazing.

KEY WORDS: Grazing vulnerability · Carbon starvation · Growth rate · Protist · Bacteria



Epifluorescence microscopy photographs of the flagellate *Cafeteria roenbergensis* (centre) and a bacterial prey (*Vibrio vulnificus*) in its non-starved (left) and starved (right) physiological states. Scalebars = 2.5 µm

Photos: R. Anderson

INTRODUCTION

In marine pelagic environments, suspended bacteria are strongly influenced by both top-down (predation) and bottom-up (resources) controlling forces. Concentrations of organic substrates and inorganic nutrients are generally very low, requiring physiological adaptations for efficient uptake and utilization of substrates as well as for long-term survival under carbon and energy limitation. In addition, pelagic environments offer little refuge against predation by bacterivorous protists, making it necessary for bacteria to develop strategies to decrease their vulnerability. How bacterial commu-

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nities cope with these 2 selective forces has been the focus of numerous studies, revealing a wide range of mechanisms of resistance to protist grazing (Hahn & Höfle 2001, Jürgens & Matz 2002, Pernthaler 2005) and a well-characterized carbon-starvation response (Kjelleberg 1993, Morita 1997). However, few studies have analyzed the response to the 2 forces jointly, centring on the potential ideal for planktonic prokaryotes, an adaptive response able to cope simultaneously with both substrate limitation and grazing pressure.

To date, the physiological and molecular mechanisms of the starvation response have been studied in detail for a number of bacteria, such as *Vibrio* spp. (Kjelleberg 1993, McDougald et al. 2001, 2003), Enterobacteria (Jenkins et al. 1988, Galdiereo et al. 1994, Brauer et al. 2006) and others (Wrangstadh et al. 1986, Redon et al. 2005, Johnson et al. 2006). For these model organisms, the final outcome is so-called non-growing 'ultramicrobacteria' which are highly resistant to diverse stress factors (e.g. ultraviolet light, chemical oxidants and high temperatures); they remain viable for extended periods (from weeks to months or years), and they can recover to normally growing cells given the addition of appropriate substrates. Interestingly, one of these adaptations — miniaturization of the cells — is known to be a major factor leading to decreased vulnerability towards protist predation, due to a lowered grazer feeding efficiency on smaller bacterial cells (Gonzalez et al. 1990, Šimek et al. 1994, Posch et al. 1999). This could therefore constitute a mechanism — directly developed or as an indirect benefit of the starvation survival response — for carbon-starved bacteria to decrease their vulnerability towards grazers. Additionally, it becomes plausible to consider that the dominance of small or ultramicrobacterial cells among planktonic prokaryotic communities could be due partly to the combination of a high abundance of cells in the starvation survival state and a preferential elimination of bacteria of larger cell size by predators, particularly bacterivorous protists.

However, despite the potential importance of small cell size, it alone may not be sufficient for long-term survival of starvation-adapted, non-growing cells in the presence of protist predators. For most interception-feeding flagellates there is no physical limit to the uptake of small particles, as these organisms can ingest even viruses (Gonzalez & Suttle 1993) and colloids (Sherr 1988, Tranvik et al. 1993), though not at the same rates as larger particles. Geometric models predict that the clearance rate of interception feeders decreases approximately with the square of the particle radius (Spielman 1977, Fenchel 1982a). This encounter-based size selectivity has been confirmed in feeding studies with fluorescent beads; these studies revealed that particles < 0.5 µm are removed by typical bacterivorous nanoflagellates with an efficiency 4 to 6 times lower than that for particles of 1 µm (Jürgens & Matz 2002). Extrapolated to the natural environment, this would imply that — in situations where protist grazing accounts for the removal of bacterial standing stocks within 1 or a few days — small cell size would prolong survival to only 1 to 2 wk. These first-order estimates show that miniaturization of cell size alone would not enable long-term survival in the presence of bacterial grazers.

The aspects considered above, combined with the fact that the starvation survival programme confers a high, long-term resistance to abiotic stress factors, raises the question as to whether carbon-starved cells are also capable of prolonged survival in the presence of protist grazers. It has been speculated that the starvation-induced differentiation programme, which results in cross-protection against different stress factors, might also involve an increased protection against predators, e.g. by resisting the digestive enzymes inside protist food vacuoles (Jürgens & Matz 2002). Similar effects have been observed for bacteria with certain cell wall structures, such as Gram-positive bacteria (Gonzalez et al. 1990, Iriberri et al. 1994, Tarao et al. 2009), for certain pathogens (e.g. *Legionella*) inside protists and macrophages (Barker & Brown 1994), and for bacteria that secrete certain macromolecules and proteins (Greub & Raoult 2004). Additionally, other resistance mechanisms could act at different stages of the interaction between bacteria and protists (i.e. avoiding capture or ingestion) (Matz et al. 2002, Montagnes et al. 2008).

In the present study, we aimed to take the first step of assessing whether the potential exists for carbon-starved bacteria to become less vulnerable to protist grazing, thus creating a basis for future studies on long-term survival and potential resistance mechanisms. To this end, we selected 3 model bacteria with a clearly characterized carbon-starvation respons, but which nevertheless differ in their lifestyle strategies. *Vibrio vulnificus*, found mostly in coastal temperate waters and often associated with plankton, shellfish and fish (Oliver 2006), and *Photobacterium angustum*, which was isolated from surface coastal waters, are both copiotrophic organisms with relatively large and fast growing cells; these organisms quickly react to carbon limitation by forming the aforementioned starvation survival ultramicrobacterial cells. Conversely, *Sphingopyxis alaskensis* is a model oligotroph that grows with a constant maximum growth rate on low concentrations of substrates and maintains a relatively small cell volume (Lauro et al. 2009). We used the simple but effective approach of comparing protist growth rates on starved vs. non-starved cells. This provides an

integrated measure of the grazers' ability to capture, ingest and digest bacterial prey and the subsequent efficiency in transforming it into protist biomass. Therefore, this methodology, though not revealing specific processes, encompasses both the possible resistance mechanisms of these bacteria, and the potential differences in nutritional value between starved and non-starved cells.

MATERIALS AND METHODS

Bacterial strains and pre-cultures. The 3 bacterial strains used in this study, their specific starvation responses, and their subsequent increase in resistance to abiotic stress factors, have been described previously: *Sphingopyxis alaskensis* RB2256 (Schut et al. 1993, Schut 1994, Cavicchioli et al. 2003); *Photobacterium angustum* S14 (formerly *Vibrio angustum*) (Humphrey et al. 1983, Albertson et al. 1990, Nyström et al. 1992) and *Vibrio vulnificus* M06-24/O (Wright et al. 1990, Morton & Oliver 1994). All strains were obtained from the CMB (Centre for Marine Bio-Innovation, UNSW, Sydney) and were maintained as glycerol stocks at −80°C.

Vibrio vulnificus and *Photobacterium angustum* were routinely grown from glycerol stocks on Luria Broth agar with 2% NaCl (LB20) and MMM2000 (Marine Minimal Medium (MMM) with 2 g l^{-1} of glucose (Östling et al. 1991); for all our solutions, 50 ng l^{-1} of vitamin B_{12} were added after autoclaving (Cavicchioli et al. 1999). *Sphingopyxis alaskensis* did not grow well on LB20, so VNSS agar (Mårdén et al. 1985) was used instead. In all cases, incubation was at 30°C, with orbital shaking at 150 rpm for liquid cultures.

Starved bacterial cultures were obtained as previously described (Holmquist & Kjelleberg 1993), with some modification. Bacteria were grown in MMM2000 until an optical density corresponding to mid-exponential phase was reached — OD_{610} = 0.2 to 0.3 for *Vibrio vulnificus* and *Photobacterium angustum*, and OD_{433} = 0.3 for *Sphingopyxis alaskensis* (D. McDougald &. L. Ting pers. comm.). Cultures were then washed with MMM, resuspended in the same medium, and allowed to starve for 72 h to ensure that the cells were fully adapted to long-term starvation conditions (Kjelleberg 1993).

Non-starved bacterial cultures were obtained from overnight cultures grown in MMM2000, corresponding to mid- to late-exponential growth phase. Starved and non-starved bacterial cultures to be used in a given experiment were inoculated from the same plate. With *Sphingopyxis alaskensis*, additional cleaning steps with MMM proved to be necessary, for both the non-starved and starved cultures, because strong bacterial growth was observed in the first grazing experiment with *Cafeteria roenbergensis*. This was likely due to the fact that this bacterium has broad-specificity, high-affinity uptake systems, enabling efficient substrate scavenging (Cavicchioli et al. 2003), and it can grow on low levels of nutrients (Williams et al. 2009) such as those present in the inoculum.

***Cafeteria roenbergensis* pre-cultures.** The widespread marine bicosoecid nanoflagellate *C. roenbergensis* (Fenchel & Patterson 1988) was selected as a model predator in tests prior to the experiments because of its ability to grow well on the selected prey and under the growth conditions used in this study (data not shown). Two different stocks of *C. roenbergensis* were used. For the first experiment with *Photobacterium angustum* and the experiment with *Vibrio vulnificus*, we used an axenic culture of *C. roenbergensis* from the CMB (described in Matz et al. 2005), while in all other experiments we used a culture from the Leibniz Institute for Baltic Sea Research (reference IOW23). Both were isolated from the Baltic Sea by A. P. Mylnikov. Cultures were routinely grown in MMM at room temperature (23 to 25°C) with moderate orbital shaking, using the bacterium to be tested in the experiment as food source.

To eradicate indigenous bacteria in the IOW23 cultures and obtain a protist culture with the desired bacteria as sole prey, we carried out serial dilutions in multiwell plates containing MMM as the medium. Protists were allowed to grow for 1 to 2 d at room temperature, with light orbital shaking and the desired bacteria as added food source. New multiwell plates were then inoculated from the highest dilutions at which growth of the protist was observed. This process was repeated until only the desired bacterial strain was detected (inspected by fluorescence microscopy and/or colony morphology on plates) and the final cultures were then used to inoculate the protist pre-cultures. The efficiency of the method was tested in the second experiment with *Photobacterium angustum* with an immunofluorescence assay using polyclonal antibodies against *P. angustum*, as previously described (Christoffersen et al. 1997).

Growth experiments. For each experiment, batch cultures were inoculated in tissue culture flasks (Sarstedt) containing MMM as medium, using a standard protist concentration of 10^3 flagellates ml^{-1} (obtained from the same protist pre-culture for both treatments), and 6 different initial bacterial concentrations of starved or non-starved cells, ranging from 10^6 to 10^8 bacteria ml^{-1}. As controls, we prepared treatments with an intermediate bacterial concentration of ~10^7 cells ml^{-1} and no protists. Flasks were incubated at room temperature (23 to 25°C), with moderate orbital shaking, for 60 to 72 h. Samples were taken every 4 h

during the day and immediately fixed with 2% formaldehyde.

In total, 5 experiments were carried out: 2 with *Photobacterium angustum* (the first of which tested only growth on starved bacteria); 1 with *Vibrio vulnificus*; and 2 with *Sphingopyxis alaskensis*. Hereafter, these experiments are referred to, respectively, as Expts P.a.1 and P.a.2; Expt V.v; and Expts S.a.1 and S.a.2. For Expt S.a.1, due to the active bacterial growth during incubation with flagellates, only data for the growth of the flagellate on the non-starved bacterial treatment are shown, and this is referred to as flagellate growth on actively growing bacteria. Data from the starved bacterial treatment were excluded from further analysis because the fact that there was bacterial growth was an obvious indication that they were no longer in the starvation survival state.

Enumeration of organisms and biovolume measurements. For bacterial enumeration, fixed subsamples were filtered onto black polycarbonate filters (0.2 µm pore size; 25 mm diameter; Whatman) and stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.01 mg ml^{-1}). For protist enumeration, the same procedure was employed using filters of pore size 0.8 µm (Whatman). Samples were observed under a Zeiss Axioskop 2 mot *plus* microscope (Carl Zeiss), and a minimum of 200 bacterial cells (1000× magnification) and 100 flagellate cells (630× magnification) were counted per sample using filter set 02 (Carl Zeiss).

For measurements of bacterial cell size, image acquisition and analysis were performed using the CellP Image analysis software (Soft Imaging System) and an F-View camera (Soft Imaging System). Images were processed as previously described (Posch et al. 2009), with a modification of the morphological filters: Erosion, Morphological Opening and Dilatation. A total of 200 to 250 cells were analyzed (Massana et al. 1997), and cell volumes were calculated using the formula described by Bjørnsen (1986). The accuracy of the method was tested by calibration with fluorescent beads of known size.

Size distributions, based on cell volumes, were determined for each bacterial population, and the average length-to-width ratio was calculated for larger size classes as Ferret max/Ferret min (respectively, the maximum and minimum values between tangents circulating at angle α 0, 10, 20… 180° around the particle). Size distributions were determined for control treatments without flagellates at the start of the experiment, and for treatments with protists, at intermediate to high bacterial concentrations, for later time points during the experiment. This should reveal possible changes in bacterial morphology, e.g. due to nutrient regeneration by the grazers. The time points studied were selected to cover the whole exponential phase of flagellate growth, in some cases extending beyond this time period. Statistical comparison was done by means of parametric tests when possible (*t*-test or analysis of variance (ANOVA), see Table 1), but, on occasion, the distribution of bacterial cell sizes was not normal, requiring the use of non-parametric tests (Mann Whitney *U*-test)

Data analysis and statistics. For all experiments and treatments, flagellate and bacterial numbers were followed over time at all bacterial concentrations. Analysis was conducted with data obtained from the first 36 to 40 h of the experiment, and initial bacterial numbers were determined from samples taken immediately after inoculation of the experimental flasks, to avoid errors arising from the carry over of the bacterium from the flagellate inoculum. Total initial biovolumes inoculated in each experimental flask were determined by multiplying the initial concentration by multiplying the initial bacterial concentration by the mean cell volume calculated for the corresponding physiological state.

Net flagellate growth rates were obtained by linear regression of the natural logarithm of the *Cafeteria roenbergensis* concentration against time for each inoculated concentration of bacterial prey (starved and non-starved) (Fenchel 1982b). Data for flagellate growth rate and initial bacterial concentration or biovolume were fitted by iteration to a hyperbolic function:

$$\mu = x\,\mu_{max}/(K_s + x)$$

where µ = growth rate (d^{-1}); μ_{max} = maximum growth rate (d^{-1}); x = prey concentration or biovolume available; and K_s = half saturation constant for growth. Uncertainties in the estimates of the regression coefficients are expressed by the corresponding 95% confidence intervals and asymptotic standard errors (see Table 2).

As an indication of the minimum number of bacteria needed for flagellate growth, theoretical threshold bacterial concentrations were calculated as previously described (Eccleston-Parry & Leadbeater 1994).

Finally, as a measure of the decrease in bacterial populations in the experimental flasks, bacterial loss rates were determined during the exponential phase of bacterial decrease (coinciding with the flagellate exponential growth phase) for each treatment and bacterial concentration as follows:

$$[(\ln N_t - \ln N_0)/(t_t - t_0)] - [(\ln C_t - \ln C_0)/(t_t - t_0)]$$

where N_t and N_0 are, respectively, the final and initial concentrations of bacteria in the test flasks (with protists); C_t and C_0 are, respectively, the final and initial concentrations of bacteria in the control flasks (without protists); and t_t and t_0 are, respectively, the final and initial time points.

Statistical analyses were performed with the SPSS 15.0 (SPSS) and SigmaPlot10.0 (Systat Software) software packages.

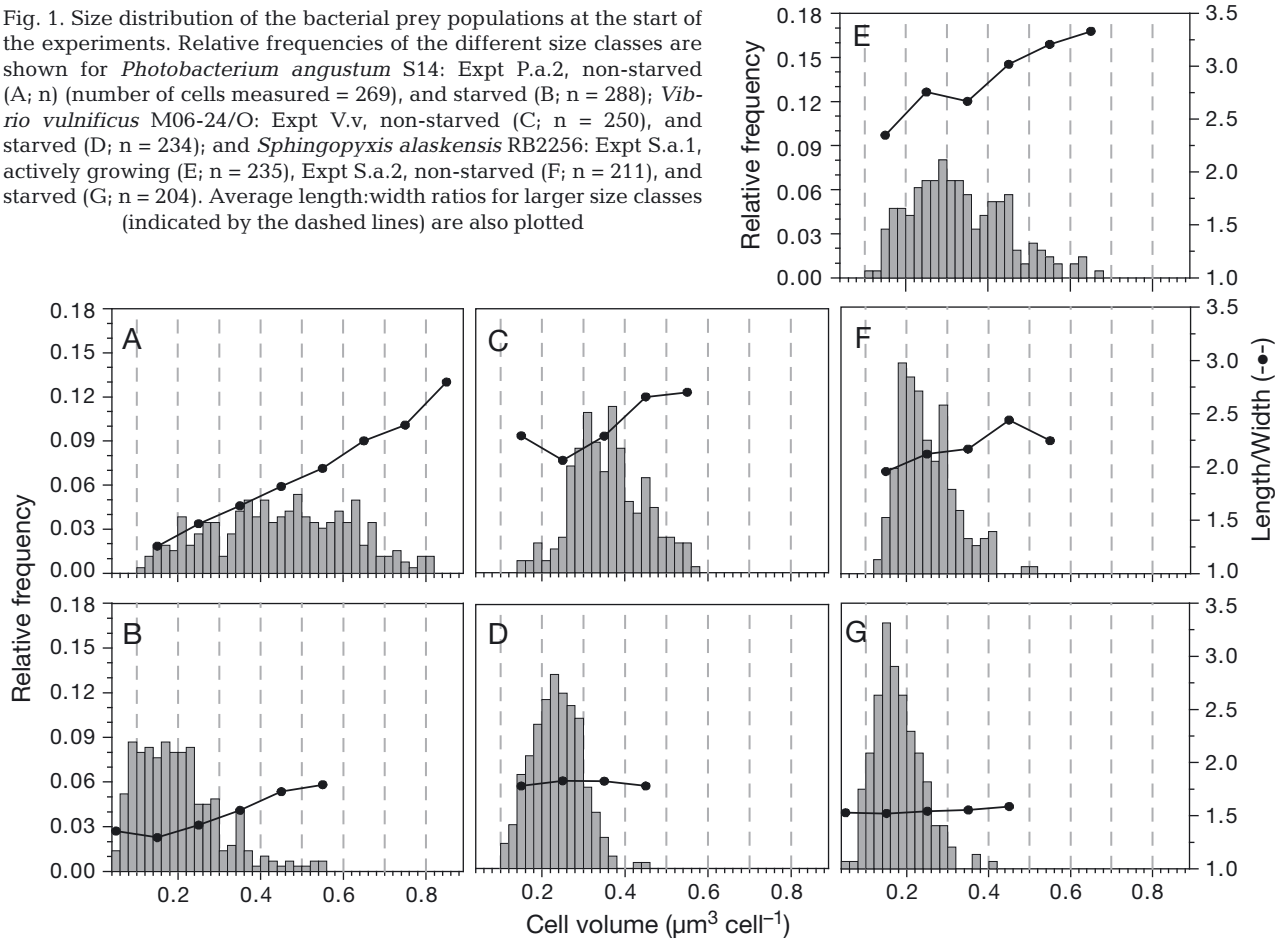

Fig. 1. Size distribution of the bacterial prey populations at the start of the experiments. Relative frequencies of the different size classes are shown for *Photobacterium angustum* S14: Expt P.a.2, non-starved (A; n) (number of cells measured = 269), and starved (B; n = 288); *Vibrio vulnificus* M06-24/O: Expt V.v, non-starved (C; n = 250), and starved (D; n = 234); and *Sphingopyxis alaskensis* RB2256: Expt S.a.1, actively growing (E; n = 235), Expt S.a.2, non-starved (F; n = 211), and starved (G; n = 204). Average length:width ratios for larger size classes (indicated by the dashed lines) are also plotted

RESULTS

Bacterial population characteristics

Photobacterium angustum in its non-starved state is a rod-shaped bacterium (0.82 × 1.4 µm, median width and length respectively) with a median volume of 0.46 µm³. Starved cells are smaller, with a median volume of 0.18 µm³ and a more coccus-like morphology (0.70 × 0.89 µm). Observing the populations as whole, non-starved bacteria had a more heterogeneous size distribution, covering a wide range of size classes, and possessed higher and more variable length-to-width ratios (Fig. 1A,B; only data from Expt S.a.2 is shown for starved bacteria because cell size distributions were very similar in both experiments).

Vibrio vulnificus has a smaller volume (median 0.35 µm³) and forms longer but thinner rods compared with *Photobacterium angustum* (0.73 × 1.69 µm). As a result of starvation conditions it forms shorter rods (0.66 × 1.19 µm), with a median volume of 0.23 µm³. Cell size distributions of both populations tended towards a normal distribution, with a maximum in the median value (Fig. 1C,D). Length-to-width ratios were higher for the non-starved bacterial population.

Sphingopyxis alaskensis is a rod-shaped bacterium with decreasing sizes in its 3 physiological states, passing from a median cell volume of 0.32 µm³ (0.65 × 1.70 µm) when actively growing, to 0.23 µm³ (0.62 × 1.27 µm) when non-starved (but not growing), and, finally, to 0.17 µm³ (0.58 × 1.02 µm) when starved. The reduction in length between the 3 physiological states was more marked than the reduction in width, as shown by the decreasing length-to-width ratios (Fig. 1E–G). Cell size distributions of the populations showed a clearer dominance of certain size classes for non-starved bacteria (Fig. 1F) and starved bacteria (Fig. 1G), while the actively growing population had a more widely spread heterogeneous distribution (Fig. 1E).

For all 3 bacterial strains, an overlap was observed between the initial cell size distributions in the different physiological states (Fig. 1). However, a significant difference was always maintained between the 2 populations, starved and non-starved, both at the start of the experiment and at later time points (Table 1; $p <$

Table 1. Mean and median cell volume (μm^3), with the corresponding standard deviation (SD), measured at different time points for each type of bacterium and physiological state (n = number of measured cells). Data are shown for only 1 experiment with each type of bacterium, and in each case it proceeds from 1 experimental flask per physiological state, inoculated with an intermediate to high initial concentration of bacteria

Time (h)	n	Median	Mean	SD
Non-starved				
Photobacterium angustum Expt P.a.2				
0[a,b]	269	0.46	0.47[a]	0.19
19	185	0.46	0.49[a]	0.18
36[a,b]	178	0.46	0.48[a]	0.18
Vibrio vulnificus Expt V.v				
0[b]	250	0.35	0.36	0.09
27[a,b]	240	0.36	0.39[a]	0.15
31	199	0.56	0.56*	0.22
Sphingopyxis alaskensis Expt S.a.2				
0[b]	211	0.23	0.25	0.06
20	185	0.23	0.25[a]	0.07
24	225	0.20	0.21*	0.06
36[a,b]	212	0.19	0.20[a,*]	0.06
Starved				
Photobacterium angustum Expt P.a.2				
0[a,b]	288	0.18	0.19[a]	0.08
23	233	0.17	0.18[a]	0.07
36[a,b]	191	0.20	0.25[a]*	0.13
Vibrio vulnificus Expt V.v				
0[b]	234	0.23	0.23	0.06
27[a,b]	211	0.21	0.21[a,*]	0.06
56	196	0.18	0.19[a,*]	0.07
Sphingopyxis alaskensis Expt S.a.2				
0[b]	204	0.17	0.18	0.05
20	177	0.16	0.17	0.04
36[a,b]	169	0.15	0.16*	0.04
44	147	0.16	0.17	0.05

*Significant difference compared with the 0 h value (analysis of variance (ANOVA) or Mann Whitney *U*-test ([a]), $p < 0.05$)

[b]Time points at which the cell volume of starved and non-starved bacterial cells were compared. In all cases significant differences were found (*t*-test or Mann Whitney *U*-test ([a]), $p < 0.05$)

0.05). As a whole, the measured median cell volumes remained constant with time, though some small shifts were observed, i.e. the tendency towards smaller cells in the starved *Vibrio vulnificus* treatment or the appearance of larger cells at the final time point for non-starved *V. vulnificus* (Table 1). Mean and median cell volumes were normally equal, or very similar, though in some cases, such as the final time point for the starved *Photobacterium angustum* treatment, divergences between the two occurred due to the appearance of small subpopulations of cells with larger or smaller cell volumes (observed in histograms; data not shown).

In Expt P.a.2, the percentage of total bacteria corresponding to *Photobacterium angustum* was determined by immunofluorescence staining, allowing the quantification of potential contaminant bacteria in the test treatments. These were detected, but they accounted for only ≤2% of the initial total bacterial numbers in most treatments, though slightly higher in those inoculated with the lowest bacterial concentration (data not shown). No contaminant bacteria were detected in control treatments, confirming that contaminants came from the protist pre-cultures. The relative proportion of these bacteria did increase as the experiment proceeded, and total bacterial numbers were reduced due to grazing, but they constituted a high percentage only towards the end of the experiment, when total bacterial numbers were ≤10% of the original values.

Flagellate growth on starved and non-starved bacteria

Cafeteria roenbergensis grew on all offered concentrations of the 3 bacterial strains in their non-starved or actively growing states, achieving maximal growth rates in the range of 0.2 to 0.3 h^{-1} (Table 2), which correspond to doubling times of approximately 2.5 to 3.5 h. In contrast, growth on carbon-starved bacterial cultures varied with the different bacterial strains (Fig. 2, Table 2). The exponential growth phase for the flagellates was observed in most cases between 12, and 24 to 36 h, with the exception of the treatments in Expt S.a.2 supplied with starved bacteria, where it was delayed until 20 h and then continued until 40 h (data not shown).

When fed with *Photobacterium angustum*, the increase in flagellate growth with bacterial concentration and biovolume was the same for both experiments and treatments (Fig. 2A,B). The hyperbolic fits tended to fall together, and the corresponding estimated growth parameters (μ_{max} and K_s values) were similar, presenting in all cases overlapping standard errors and 95% confidence intervals (Table 2). Only theoretical threshold values differed, being higher for flagellates fed on non-starved bacteria (Table 2).

In the experiment with *Vibrio vulnificus* as a food source, marked differences were observed between the growth on starved and non-starved bacteria (Fig. 2C,D, Table 2). An increase in flagellate growth rates with increasing prey concentration and biovolume, equal to that obtained for *Photobacterium angustum*, was observed when *Cafeteria roenbergensis* was supplied with non-starved cells. However, only the highest concentration of starved bacteria supported flagellate growth, and a marked decrease in flagellate numbers

Table 2. Theoretical threshold values for flagellate growth, maximum growth rates (μ_{max}) and half-saturation constants (K_s) (in terms of concentration and biovolume) for *Cafeteria roenbergensis* fed on the 3 bacterial species tested: *Photobacterium angustum* (Expts P.a.1 and P.a.2), *Vibrio vulnificus* (Expt V.v) and *Sphingopyxis alaskensis* (Expts S.a.1 and S.a.2). The last 2 parameters were calculated by iteration to a hyperbolic function based on the growth rate of the flagellate on the different bacterial concentrations and treatments (n = 6; see Fig. 2); the R^2, 95% confidence intervals (CI) and computed asymptotic standard errors (SE) for the estimated values are also shown

Expt and treatment	Threshold (10^5 cells ml^{-1})	μ_{max}			K_s (conc.)			K_s (biovolume)			R^2
		h^{-1}	SE	CI	10^6 cells ml^{-1}	SE	CI	10^6 µm^3 ml^{-1}	SE	CI	
Expt P.a.1											
Starved	4.79	0.19	0.01	[0.17; 0.21]	6.45	1.07	[4.35; 8.55]	1.29	0.21	[0.88; 1.7]	0.99
Expt P.a.2											
Non-starved	6.77	0.26	0.04	[0.18; 0.34]	5.85	2.92	[0.13; 11.6]	2.93	1.53	[−0.1; 5.93]	0.88
Starved	3.57	0.25	0.05	[0.15; 0.35]	7.45	4.34	[−1; 15.9]	1.58	0.89	[−0.2; 3.32]	0.88
Expt V.v											
Non-starved	4.26	0.21	0.02	[0.17; 0.25]	2.72	1.29	[0.19; 5.25]	0.99	0.47	[0.07; 1.91]	0.86
Starved	–	–	–	–	–	–	–	–	–	–	–
Expt S.a.1											
Actively growing	0.05	0.3	0.00	[0.30; 0.30]	0.69	0.08	[0.53; 0.85]	0.23	0.03	[0.17; 0.29]	0.98
Expt S.a.2											
Non-starved	7.54	0.24	0.02	[0.20; 0.27]	7.40	2.41	[2.68; 12.1]	1.86	0.60	[0.68; 3.04]	0.86
Starved	20.7	0.12	0.02	[0.08; 0.16]	2.39	2.07	[−1.7; 6.45]	0.43	0.37	[−0.3; 1.15]	0.35

was observed for almost all other concentrations tested. From the lowest to the highest initial bacterial concentration inoculated, a respective decrease in flagellate numbers of 60, 70, 35, 84 and 5% was observed within the first 27 h of incubation (data not shown).

Finally, when fed with *Sphingopyxis alaskensis*, clear differences were observed in the growth of the flagellates on actively growing, non-starved and starved cells (Fig. 2E,F). Calculated μ_{max} were highest for non-starved bacteria and lowest for starved bacteria (Table 2), with standard errors and 95% confidence intervals in no case overlapping. Theoretical threshold values correspondingly showed the inverse pattern. K_s values, however, were conversely higher for the non-starved treatment than for the starved one, but data from the latter bacterial treatment should be treated with caution because the data points tended to cluster together, gave the only hyperbolic fit with low R^2, and presented estimated K_s values with very high standard errors (Table 2).

Overall, protist growth resulted in a corresponding decline in bacterial numbers, with bacterial loss rates increasing with protist growth rates (Fig. 3). As an exception, in the treatment with actively growing *Sphingopyxis alaskensis*, bacterial growth exceeded mortality, though a tendency towards lower bacterial growth with increased protist growth was still observed (Fig. 3C,F). In control treatments, with the exception of actively growing bacteria, and test treatments in which no protist growth was detected, bacterial loss rates remained around 0 h^{-1} (the average value for all control treatments was 0.0009 ± 0.005 h^{-1}, and 0.004 ± 0.01 h^{-1} for treatments where no protist growth was detected).

DISCUSSION

Methodological aspects

In the present study, we compared the growth of a bacterivorous flagellate, *Cafeteria roenbergensis*, on 3 model bacterial strains harvested in different physiological states: carbon-starved, non-growing and exponentially growing. To ensure that bacteria had remained in the desired physiological state throughout the duration of the experiment, analysis was restricted to the first 36 to 40 h sampled, and cell size was employed as an indicator of change in the physiological state during the experiment. We recognize that, by employing this measure, we potentially ignore certain effects that may occur prior to an increase in cell size (i.e. changes in cell-surface characteristics or excretion of certain substances). However, the response of carbon-starved bacteria to the appearance of a new carbon source, for example by the regeneration of nutrients through protist feeding, is very fast (Kjelleberg 1993, Morita 1997), and thus would soon be detected as shifts in the distribution of cell size in the population. This measure showed that the bacterial populations in most cases retained a constant cell volume throughout the experiments and that a significant difference was always maintained between the starved and non-starved treatments (Table 1).

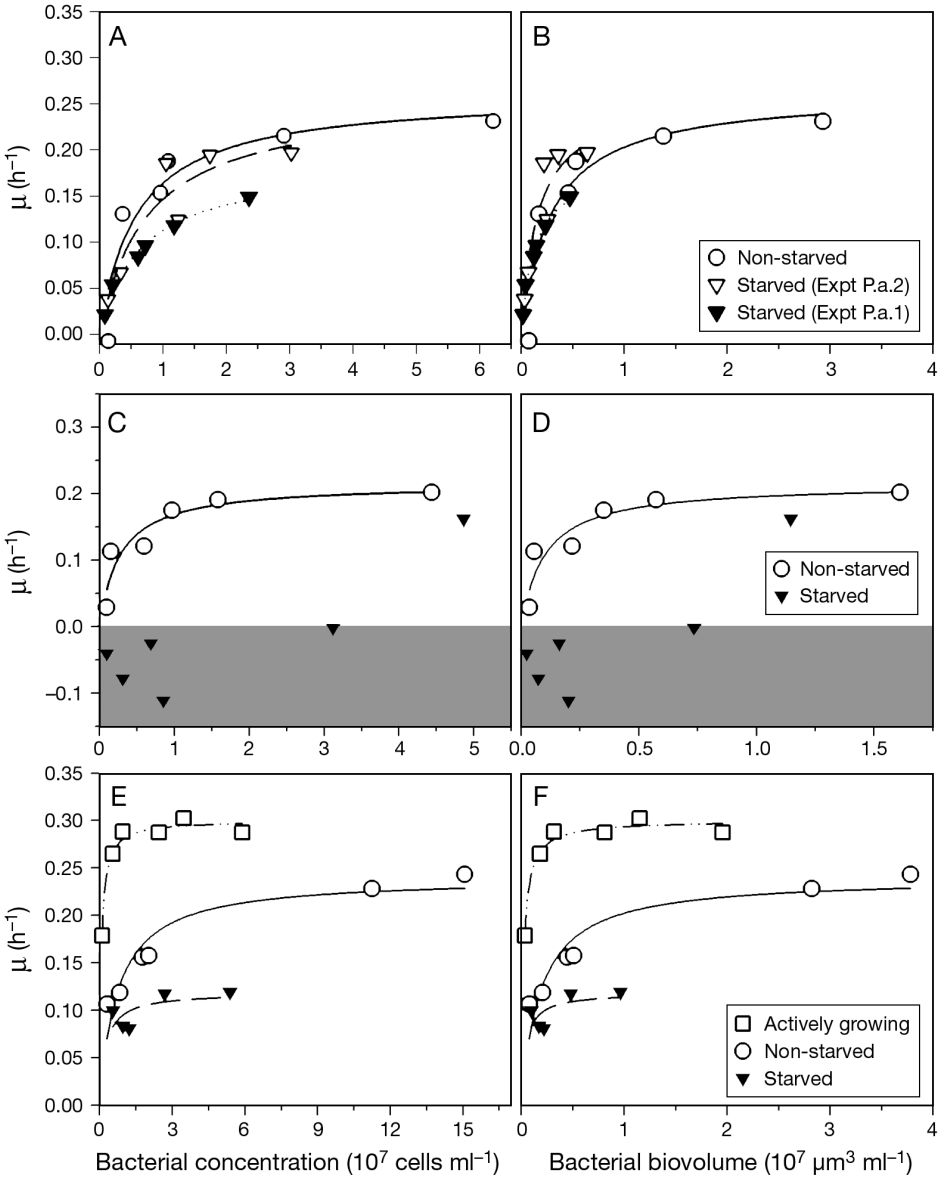

Fig. 2. Growth rate (μ) of *Cafeteria roenbergensis* fed on different initial bacterial concentrations, and corresponding biovolumes, of the 3 model bacteria in their different physiological states: (A,B) *Photobacterium angustum* S14 (Expts P.a.1 and P.a.2); (C,D) *Vibrio vulnificus* M06-24/O (Expt V.v); and (E,F) *Sphingopyxis alaskensis* RB2256 (Expts S.a.1 and S.a.2). Shaded zones indicate negative growth rates for the flagellate. Hyperbolic fits are represented by the straight and dashed lines (further information is given in Table 2)

At a bacterial strain level, the mean values of cell volume from our experiments were comparable to those reported previously, with the exception of *Sphingopyxis alaskensis*. This strain was shown to have a constant small size of <0.1 μm^3 in prior studies (Schut et al. 1993, Eguchi et al. 1996), whereas we observed a change from 0.16 μm^3 in its carbon-starved state to 0.32 μm^3 when growing. Nevertheless, the identity of the strain used was positively confirmed by the 16S rRNA gene sequences (100% similarity), and similar increases in cell size were also observed in other recent studies with this bacterium (L. Ting pers. comm.), indicating that there may be certain phenotypic changes in this strain compared to studies performed in earlier years.

In the second experiment with *Photobacterium angustum* S14 as the food source (Expt P.a.2), small levels of contaminant bacteria were detected at the start of the experiments. However, this contaminant remained after a final dilution of 10^{10} of the initial protist culture (after successive dilution steps) and the

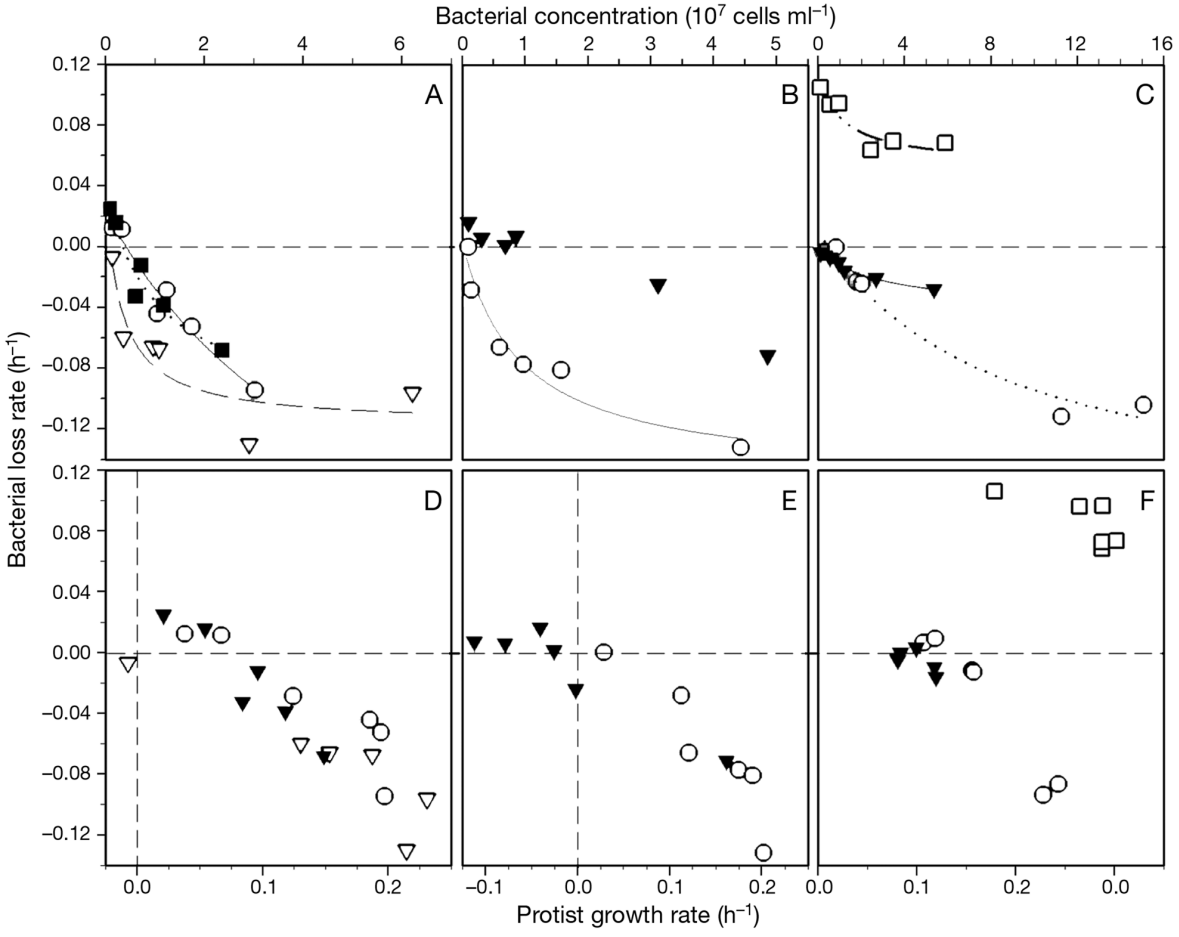

Fig. 3. Bacterial loss rates in relation to (A–C) the initial bacterial concentration inoculated (hyperbolic fits are also plotted (R^2 > 0.81 in all cases)), and to (D–F) the protist growth rate. Data are shown for experiments with (A,D) *Photobacterium angustum*, (B,E) *Vibrio vulnificus* M06-24/O, and (C,F) *Sphingopyxis alaskensis* RB2256. ○: non-starved bacteria; ▼: starved bacteria; □: actively growing bacteria. For *P. angustum*, where 2 experiments with starved bacteria were conducted: ▼, data from Expt P.a.1; ▽, data from Expt P.a.2. Dashed vertical and horizontal lines serve as reference to 0 h^{-1} protist growth rates or bacterial loss rates

experimental results did not differ from those obtained in Expt P.a.1, which employed an axenic culture. Therefore, we concluded that the contaminant bacteria were probably not preferentially grazed upon by *Cafeteria roenbergensis*, and appeared to have no effect on the experimental outcome.

Protist growth on starved vs. non-starved bacteria

The results obtained in this study indicate species-specific differences in the vulnerability of carbon-starved bacteria, even among the closely related strains *Photobacterium angustum* and *Vibrio vulnificus* (Ruimy et al. 1994). *Cafeteria roenbergensis* grew well on all 3 strains of bacteria in their non-starved state, achieving maximal growth rates which were in the range of those previously reported for this flagellate (Gonzalez et al. 1993). In contrast, marked differences were observed when comparing protist growth on starved vs. non-starved cells of each bacterial strain.

Cafeteria roenbergensis exhibited equal growth when offered a comparable amount of starved and non-starved cells of *Photobacterium angustum* (Fig. 2A,B), indicating that this bacterium was an equally good food source for the flagellate in both physiological states, and that no apparent resistance mechanisms existed. Higher threshold values for starved bacteria and the tendency towards higher K_s values (Table 2) could even point towards a slightly better nutritional quality in this physiological state. A higher growth efficiency of flagellates when feeding on starved bacteria was also demonstrated in a previous study (Gonzalez et al. 1993). Furthermore, the results obtained with *P. angustum* are also evidence that there is not necessarily an energy constraint for flagellates feeding on smaller prey particles. This is

contrary to observations from an earlier study (Boenigk et al. 2006), which found lower growth rates for flagellates feeding on smaller sized bacteria. However, in their study, different bacterial strains were compared instead of different sized cells of the same strain, which makes other effects possible, such as inter-specific variations in food quality and grazing resistance. Also, differences in the behaviour of the protist species studied should be considered.

In contrast to *Photobacterium angustum*, starved *Vibrio vulnificus* did not support flagellate growth, and protist numbers declined strongly in the first 27 h, except at the highest bacterial concentration tested. This drastic negative response at low prey concentrations is too strong to be due to a simple starvation response caused by a decrease in food quality (Fenchel 1987) and suggests the presence of bacterial resistance or repellent mechanisms. As we did not analyze the bacteria–protist interactions in detail, e.g. by live video microscopy (Boenigk & Arndt 2000), we do not know at which stage of the feeding process *V. vulnificus* resisted grazing. Potentially, ingestion of bacteria could be avoided due to receptor-mediated repellent effects (Matz et al. 2002); there could be a post-ingestional mechanism involved, causing ingested bacteria to be egested after entering the food vacuoles (Boenigk et al. 2001) or allowing them to undergo the vacuole passage without being harmed by digestive enzymes (King & Shotts 1988, Barker & Brown 1994); or antiprotozoal factors could be produced at different stages of the feeding process, as has been seen for *Vibrio cholerae* during biofilm formation (Matz et al. 2005).

The observed exception to the overall pattern with *Vibrio vulnificus* as prey, namely, the positive protist growth at the highest bacterial concentration, would need further examination. The drastic negative response of the flagellates at lower bacterial concentrations does not support the explanation of a compensation of low food quality by a much higher food concentration. As a possible alternative, it remains to be examined whether high concentrations of this bacterial strain trigger regulatory shifts, e.g. by components involved in quorum sensing, known to affect both virulence and the starvation survival response in *Vibrio* strains (McDougald et al. 2000, 2001, 2003, Brackman et al. 2009). Nevertheless, this is, to our knowledge, the first documented case of a bacterial strain which can change from a highly edible to an unpalatable food when entering the starvation state.

Finally, *Cafeteria roenbergensis* also exhibited differences in growth when fed on different concentrations and biovolumes of the 3 physiological states of *Sphingopyxis alaskensis*. However, in contrast to the experiment with *Vibrio vulnificus*, no deleterious effect on the protists was observed. The observed reduction in growth probably points to a gradual decrease in food quality from actively growing to carbon-starved cells. It has been shown that both ingestion (Shannon et al. 2007) and growth rates (Grover & Chrzanowski 2009) of bacterivorous flagellates are affected by the nutritional quality of the bacterial prey, although, in these studies, mainly a variable C:N:P stoichiometry of the offered bacterial strains was taken into account.

Conclusions and outlook

In the present study, using predator–prey model systems, we show (1) that miniaturization of cell size alone is not sufficient to lower grazing vulnerability, and (2) that some bacterial strains could possess efficient (yet to be examined) defence mechanisms that accompany the starvation survival programme and result in drastic changes in their edibility. As future work, the following points remain to be elucidated in order to determine the relevance and universality of the model systems used in this study:

- Further model systems should be tested, including different bacterial phylogenetic groups and grazers, in order to discern general patterns in the effect of bacterial carbon starvation on grazing vulnerability;
- Comprehensive studies of the underlying mechanisms should be carried out in cases where decreased vulnerability of carbon-starved bacteria is observed, such as in the present study with *Vibrio vulnificus* as food source;
- Adequate methodologies should be developed to assess the prevalence of the carbon starvation state in natural bacterial communities and its impact on grazer vulnerability. Furthermore, the effect of a varied prey pool (different bacterial strains in different physiological states) on prey vulnerability and prey selectivity should be analyzed in detail to obtain an accurate vision of what occurs in natural environments.

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