RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2010) 13:151-158
DOI: 10.2436/20.1501.01.120 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

Chemosensory response of marine flagellate towards L- and D- dissolved free amino acids generated during heavy grazing on bacteria

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Received 15 July 2010 · Accepted 20 August 2010

Summary. This study investigated the generation of dissolved free amino acids (DFAA) by the bacterivorous flagellate *Rhynchomonas nasuta* when feeding on abundant prey. Specifically, it examined whether this flagellate protist exhibits a chemosensory response towards those amino acids. The concentrations of glycine and the L- and D-enantiomers of glutamate, serine, threonine, alanine, and leucine were determined in co-cultures of the flagellate and bacteria. Glycine, L- and D-alanine, and L-serine were found to accumulate under these conditions in amounts that correlated positively with flagellate abundance, suggesting that protists are involved in their generation. Investigations of the chemotactic response of young and old foraging protists to the same amino acids, offered in concentrations similar to those previously generated, showed that glycine elicited the strongest attraction in both age groups. Young protists were strongly attracted to all the assayed amino acids, whereas older protists maintained a high level of attraction only for glycine. These results suggest that glycine generated by protists actively grazing in bacterially enriched patches functions as an infochemical, signaling to foraging protists the presence of available prey in the aquatic environment. [**Int Microbiol** 2010; 13(3):151-158]

Keywords: *Rhynchomonas nasuta* · marine flagellates · L- and D-amino acids · chemosensory response · grazing · bacterivore activity

Introduction

Bacterivorous protists are ubiquitous in marine ecosystems, where they are considered the primary grazers of bacteria. For these organisms, the efficient detection of abundant prey is relevant for successful feeding, as it increases the probability of predator-prey contact. Resource distribution in seawater is not homogeneous; rather, microscale patches of organic matter originate from processes such as the exudation of phytoplankton photosynthetic products [7,22], excretion by zooplankton [17], cellular lysis [6], and leakage of organic matter from particles [13]. These patches of organic matter attract and nurture bacteria [4,6,30], thus creating hotspots of bacterial production [34]. In these microzones, bacterial abundance is typically one to three orders of magnitude higher than in bulk seawater [8,13]. Not surprisingly then, bacterivorous protists are known to feed and grow at these sites [2,6], with some bacterivorous ciliates able to detect the colonizing bacteria on the basis of mechanoreception [36]. In addition, chemosensitivity may be important for detecting accumulations of molecules associated with bacterial growth or derived from protist grazing activity. In fact, bacterivore activity has been related to the generation of different types of chemicals, including undefined colloidal and dissolved organic matter [24,37], surfactants [15], and dissolved free amino acids (DFAA) [1,23].

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The grazing-derived generation of DFAA in aquatic systems was previously investigated to determine the dynamics of dissolved organic nitrogen [1,23,26]. However, DFAA generation in patches under heavy grazing pressure may influence the trophic dynamics of the microbial food web in other ways as well. For example, DFAA might provide a signal for foraging grazers in the detection of zones where bacterial prey are abundant. This hypothesis is supported by studies showing that some ciliates are able to detect amino acids and to exhibit chemosensory responses [19,38] as well as changes in swimming or feeding behavior [35]. Currently, flagellates are considered to be the most important bacterial grazers in seawater. While they are known to display chemosensory responses to amino acids [16,31], related studies are scarce.

Previous studies on the grazing-mediated production of DFAA and the ability of protists to detect them have focused on L-enantiomer amino acids and glycine but, to our knowledge, none has considered the D-enantiomers. D-Amino acids are derived primarily from degraded bacterial cell walls [21]; hence, they are expected to be abundant in bacterial zones under heavy grazing pressure. As such, they are good candidate chemical signals for protists in the detection of zones of abundantly available bacteria.

An additional factor that may modulate the response of protists to external stimuli is their physiological state under different environmental conditions. Actively growing and stationary protists may well differ in their behaviors with respect to a variety of biological activities, including the hunting, capture, digestion, and excretion of prey. However, the influence of the physiological state of protists in processes required, e.g., to hunt prey, has not been fully addressed.

The bodonid *Rhynchomonas nasuta* is a common seawater flagellate frequently found in marine waters [25]. In the present study, it was used as a model organism to investigate the chemosensory response of bacterivorous protists towards amino acids generated during heavy bacterial grazing. Specifically, it was hypothesized that the amino acids comprising the peptidoglycans of bacterial cell walls would be good candidates for eliciting chemosensory responses in the flagellate, since D-enantiomers are more resistant than L-enantiomers to bacterial uptake. Accordingly, the generation of glycine and the L- and D-enantiomers of alanine, aspartate, glutamate, and serine, associated with high bacterivore activities on dense bacterial communities, were qualitatively and quantitatively analyzed. Of particular interest were the chemosensory responses of this protist towards the generated amino acids and whether the age of the flagellate culture affects the nature and intensity of these responses.

Materials and methods

Microbial cultures and analysis. The *Rhynchomonas nasuta* strain used was isolated from coastal waters in the Gulf of Biscay. The clonal *R. nasuta* culture was maintained in the dark on 0.01% (w/v) cereal-leaves medium, prepared with filtered (0.22 µm) autoclaved natural seawater (CLMS; Sigma-Aldrich, Madrid, Spain) at 17ºC, and a mixed assemblage of marine bacteria derived from bacteria collected during isolation of the *R. nasuta* clone.

Culture experiments consisted of relatively low densities of the flagellate and its prey in 0.05% (w/v) CLMS. A well-grown maintenance culture of the flagellate was gravity filtered through a 0.8-µm pore polycarbonate filter and washed three times with 0.05% CLMS. Four co-culture experiments were carried out in which bacteria in the filtered suspensions were used as the food source for *R. nasuta* and their density adjusted to 10^6 cell/ml. Protists retained by the filter were inoculated in the co-culture experiments at an initial density of 10^2–10^3 cell/ml. Protist-free cultures of bacteria, thus without predation, served as controls. Cultures and controls were incubated for 180 h in the dark at 20ºC with shaking at 75 rpm. To determine microbial densities and concentrations of dissolved free amino acids, samples were taken every 4 h for the first 24 h of culture, and every 12–24 h thereafter.

Protists were counted immediately after sampling in triplicate samples of 20–500 µl with a Nikon Eclipse TE 2000-U inverted microscope (200× Nikon Instruments Europe B.V., Amstelveen, Netherlands). The bacterial samples were fixed with 2% (final concentration) sodium-tetraborate-buffered formalin, DAPI (4´, 6´, diamidino-2-phenylindole) stained and filtrated on black polycarbonate filters. Cell numbers were counted using an Nikon Optiphot epifluorescence microscope [27].

The concentrations of DFAA were measured by high-performance liquid chromatography. Samples of 10 ml were filtered through 0.22-µm pore filters (PVDF, Technochroma) under very gentle vacuum (<70 mmHg). To avoid cell disruption, care was taken to avoid cell exposure to the air at the end of the filtration. Primary amino acids were measured by precolumn derivatization with *o*-phthaldialdehyde [11,20]. To quantify the L- and D-enantiomers, *N*-isobutyryl-L-cysteine was used in the derivatization [10]. The amino acids were separated on a reversed-phase column (Nova-Pak Amino Acid Analysis Column, Waters) with a liquid-phase multistep gradient. They were eluted with three degasified eluents: eluent A was sodium acetate 25 nM, pH 7; eluent B, was 100% HPLC grade methanol; and eluent C was sodium acetate 25 nM, pH 5.3 [10]. Amino acids were detected with a Waters 474 scanning fluorescence detector, with excitation at 330 nm and emission at 445 nm. External standards were used for calibration. Glycine and the L- and D-enantiomers of glutamate, serine, threonine, alanine, and leucine were separated and quantified.

Positive or negative associations between the measured concentrations of DFAA and bacterial or flagellate abundances were estimated with Pearson's correlation coefficient at the $P < 0.05$ level. Statistical analysis was performed with SPSS v. 17.0 for Windows.

Chemosensory assays: selective migration and accumulation of *R. nasuta*.

Rhynchomonas nasuta was co-cultured with its accompanying bacteria in 0.05% CLMS. Under these conditions, the flagellate actively grew for 100–120 h, after which the cell density either remained constant or slightly decreased. Samples for use in the chemosensory assays were taken at 96 h, when the flagellates were actively growing, and at 144 h, when they were in the stationary phase of growth.

A fraction of these samples was carefully filtered through 0.22-µm pore filters, as previously described, with minimal cell disruption. This served as a control solution in the chemosensory assays. The remaining portions of the samples were also filtered but were then supplemented with the test amino acids glycine, both the L- and D-isoforms of glutamate, and serine, threonine, alanine, and leucine (Sigma, purity >99%) at final concentrations of 1 mM.

When necessary, the control solution was added to the flagellates in order to standardize the initial abundance to 10^4 cells/ml.

The responses of *R. nasuta* to the added amino acids were measured using the method described by Leick [18] and modified by Köhidai et al. [14]. Three to twelve tips of a 12-channel micropipette were filled with 100 µl of test (with amino acids) or control (no amino acids) solution and then carefully placed (without ejection) into a microtiter plate filled with the *R. nasuta* co-cultures. After 60 min, the contents of the tips were dispensed into separate microfuge tubes and the protists and bacteria that had migrated into the tips were counted as previously described.

Optimum exposure time to amino acids was initially examined by running a series of experiments with 1 mM glycine added to a *R. nasuta* co-culture, with contact times ranging from 10 to 240 min. The percentage of protists that migrated towards the control and test solutions inside the tips was then measured. An exposure time of 60 min was chosen because it was sufficiently long to obtain significant migration of the flagellate in both control and test tips and to detect significant differences between the percentages of migration, but sufficiently short to avoid a significant increase in flagellate densities due to cell division.

A preference index (PI) was used to quantify the flagellate response to the DFAA. The PI was defined as PI = T / (T + C), where T is the mean density of flagellates in the test tips and C the mean density of the flagellates in the control tips. A PI > 0.5 indicated attraction, or greater flagellate migration towards the test solution than towards the control solution. A PI < 0.5 indicated repulsion, or less flagellate migration towards the test solution than towards the control solution. A PI = 0.5 indicated no preference, or that there was no difference in flagellate migration towards the two solutions.

PIs for bacteria were also estimated, taking into account that bacteria exhibit chemotactic behavior towards several amino acids [5]; thus, the distribution of bacteria in control and test solutions may differ at the end of the assays. It was therefore determined whether migration towards the control or test solutions by the protists was due to previous bacterial migration. This required an analysis of covariation between the PI of bacteria and that of flagellates for each amino acid. No significant associations between these two PIs were detected for any of the tested amino acids, suggesting that changes in bacterial concentrations had only negligible effects on the swimming behavior of the flagellates.

Influences of the age of the protist culture were analyzed with Student's *t* test ($P < 0.05$). For each culture age, a PI significantly different from 0.5 was thus detected ($P < 0.05$). Significant differences in PI values among all the assayed DFAAs were determined with the Kruskal-Wallis test, with differences among mean values considered significant at $P < 0.05$. For multiple comparisons, differences among PIs were detected with the post-hoc Tamhane test ($P < 0.05$). All statistical analyses were performed with SPSS v. 17.0 for Windows.

Results

Strong bacterivore activity was observed in four experiments with co-cultures of mixed marine bacteria and the bodonid *Rhynchomonas nasuta*. The control experiment contained the same bacteria cultured without protists. The variation of bacterial abundance in the test and control experiments was similar: initially, bacterial abundance was in the range of 0.8–6.2 × 10^6 cells/ml; during the first 24–30 h, abundance increased to 1.0–1.4 × 10^8 cells/ml; after 30 h, the abundance slightly decreased until the end of the experiments, when it was 2.0–4.5 × 10^7 cell/ml. In the control experiment, the initial density of flagellates was <1 cell/ml. In the co-culture experiments, the flagellate density ranged from 90 to 5250 cells/ml. Active growth was detectable after the first 24 h of incubation and lasted for 72–100 h. Maximum flagellate densities ranged from 6 to 22 × 10^3 cell/ml. After the maximum

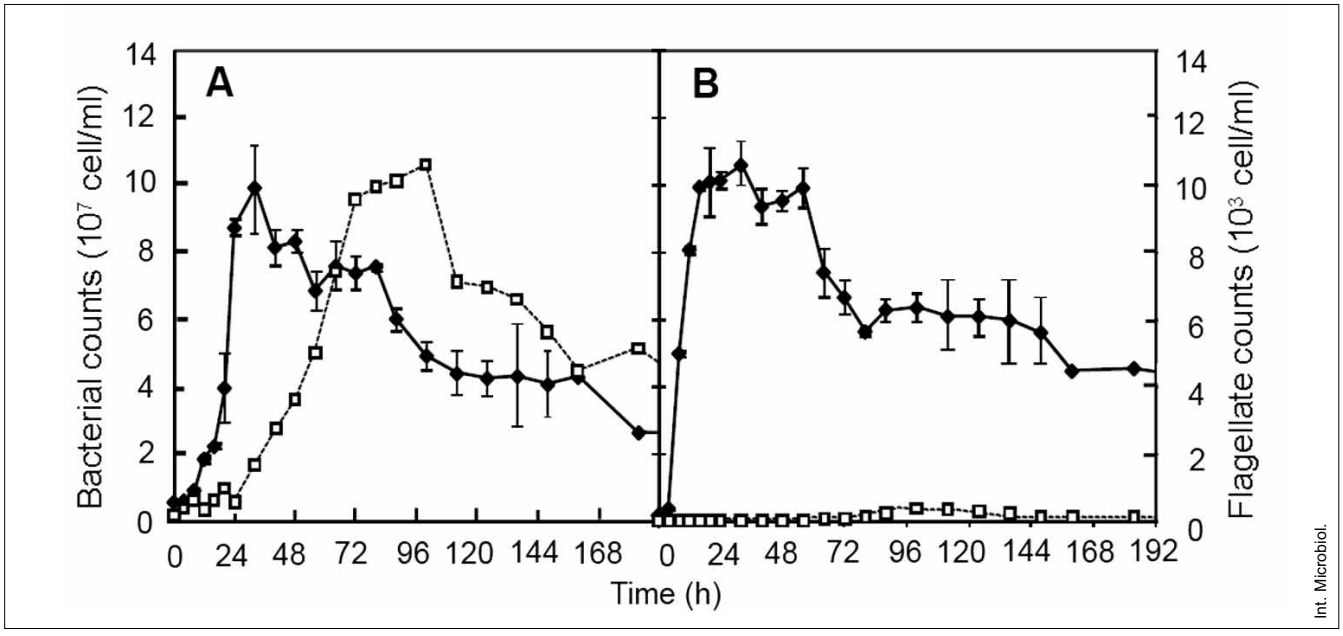

Fig. 1. Variation of microbial abundances (bacteria, closed diamonds, solid line; flagellates, open squares, dashed line) in a representative co-culture experiment (**A**) and in the control, protist-free experiment (**B**).

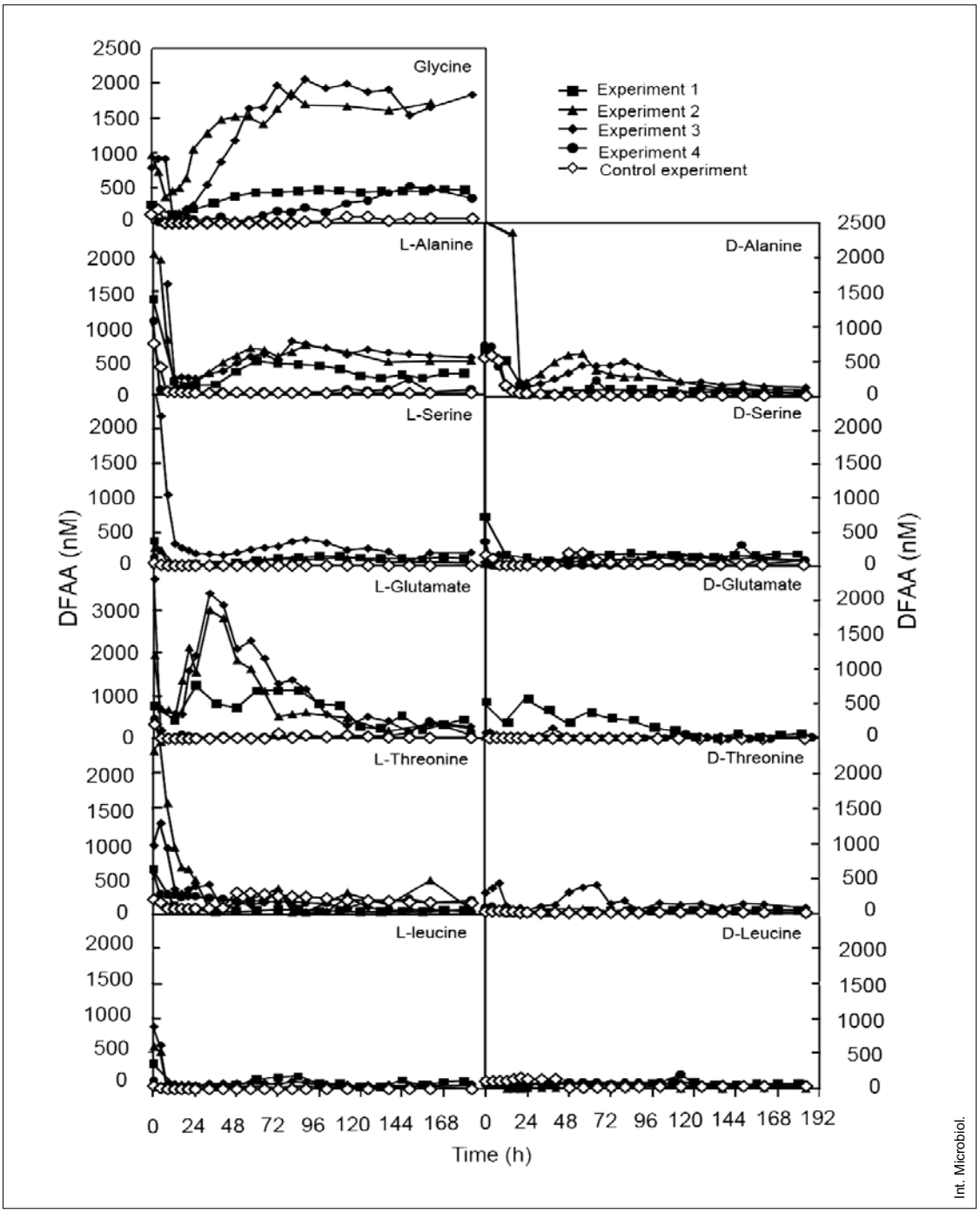

Fig. 2. Variation of DFAA concentrations in the four co-culture experiments and in the control, protist-free experiment. Note the different scale for L-glutamate.

was achieved, the flagellate density remained constant or decreased slightly. In the control experiment, maximum flagellate densities were approximately 350 cells/ml after 100 h of incubation. The variation of microbial abundance in one representative culture and in the control experiment is shown in Fig. 1.

The concentrations of glycine and the L- and D-enantiomers of serine, alanine, glutamate, threonine, and leucine showed different trends (Fig. 2). Initially, high concentrations of some DFAAs were detected in the culture media but after the first 24 h of incubation their levels dropped below 300 nM, probably due to consumption by growing bacteria. After

Table 1. Correlation coefficients among bacterial abundances or flagellate abundances and the measured DFAA concentrations. Significance levels are marked as (*) for $P < 0.050$ and (**) for $P < 0.010$, n = 73

	Bacterial abundance			Flagellate abundance		
	Pearsons's *r* coefficient		Significance	Pearsons's *r* coefficient		Significance
Gly	0.053		0.656	0.669	**	<0.001
L-Ala	0.017		0.889	0.694	**	<0.001
D-Ala	0.340	**	0.003	0.515	**	<0.001
L-Ser	–0.135		0.253	0.277	*	0.018
D-Ser	–0.119		0.316	0.213		0.071
L-Glu	0.276	*	0.018	0.170		0.151
D-Glu	–0.303	**	0.009	–0.130		0.273
L-Thr	0.427	**	<0.001	–0.112		0.344
D-Thr	0.000		0.998	0.057		0.635
L-Leu	–0.386	**	0.001	0.206		0.081
D-Leu	–0.750		0.526	–0.080	*	0.016

24 h, DFAA variation followed three primary patterns in the co-culture experiments: (i) the concentrations of glycine, L-alanine, and L-serine slightly increased during the first 100 h and then remained almost constant; (ii) the concentrations of L-glutamate and D-alanine sharply increased during the first 48–72 h and then decreased notably; (iii) the other measured DFAAs remained at very low concentrations throughout the experiments. None of the DFAAs accumulated during the control experiment.

Heterogeneous changes in several DFAA concentrations during the incubations suggested that the appearance or disappearance of these amino acids was related to the dynamics of microbial growth. To test this hypothesis, a correlation analysis (Table 1) was performed to determine whether bacteria or flagellate abundance was associated with the concentrations of DFAAs. Bacterial abundance was found to correlate positively with the concentrations of L-glutamate, D-alanine, and L-threonine but negatively with the concentrations of D-glutamate and L-leucine. Flagellate abundances correlated positively with the concentrations of glycine, L-alanine, and D-alanine, less positively with L-serine and negatively with D-leucine.

The PIs (Table 2) of younger cultures (96 h) were higher ($P < 0.01$) than that of older cultures (144 h). In addition, the PIs of *R. nasuta* in younger cultures were significantly higher than 0.5 ($P < 0.05$) towards all the DFAAs. This indicated that the flagellate was able to detect differences between the liquid it was growing in (control) and the same liquid supplemented with 1 mM DFAA (test). However, for protists in the older cultures, this was true only for 5 out of the 11 DFAAs tested (glycine, L-serine, D-serine, L-glutamate, and L-threonine).

The Kruskal-Wallis test showed significant differences only in the PIs of the younger cultures for the 11 DFAAs. The Tamhane test indicated significant differences ($P < 0.05$) between the DFAAs in the group that elicited the highest PIs (glycine, L-threonine, and L-leucine) and the group that elicited the lowest PIs (D-threonine and D-glutamate).

Discussion

The kinetoplastid flagellate *R. nasuta* is a bacterivorous protist mostly found in association with solid substrates, upon which it is able to glide and grasp in order to gather sufficient bacterial food. However, it has also been detected as a free-swimmer in aquatic systems [3]. Due to the significant differences in prey densities harbored in these two habitats, the probability of encountering prey is significantly higher near or over a particle than in a water column. Therefore, for a free flagellate in bulk seawater, any advantage in detecting zones enriched in bacterial prey would optimize food uptake. In this study, *R. nasuta* was used as an experimental model to study: (i) the generation of specific DFAAs under conditions of heavy bacterial grazing, and (ii) the abilities of other foraging flagellates to detect those molecules.

Microzones in nature that bear high bacterial and protist abundances were simulated in this study by using dense co-cultures of *R. nasuta* and a mixture of marine bacteria. In

Table 2. Preference indexes (PIs) showed by *Rhynchomonas nasuta* in cultures of 96 h and 144 h towards the 11 DFAA. PIs significantly different from 0.5 are marked as (*) for $P < 0.05$ and as (**) for $P < 0.01$

	Preference indexes (PIs)					
	96 h			144 h		
	Mean	SE[a]	n	Mean	SE	n
Gly	0.92**	0.016	5	0.83**	0.042	5
L-Ala	0.87**	0.038	4	0.58	0.099	5
D-Ala	0.80**	0.031	4	0.71	0.114	4
L-Ser	0.89**	0.014	4	0.67*	0.039	5
D-Ser	0.85**	0.014	4	0.74*	0.056	5
L-Glu	0.88**	0.025	3	0.73*	0.081	5
D-Glu	0.79**	0.012	3	0.63	0.078	5
L-Thr	0.92**	0.013	5	0.74*	0.075	5
D-Thr	0.78**	0.015	4	0.52	0.084	4
L-Leu	0.92**	0.038	3	0.57	0.091	5
D-Leu	0.91**	0.008	4	0.65	0.095	5

[a] SE, standard error.

these batch co-cultures, very high bacterial abundances were achieved; consequently, protist abundances increased from initial values of 10^2–10^3 cell/ml to maximum values of 22 × 10^3 cell/ml. DFAA concentration under these conditions significantly decreased during the first 24 h in both the co-culture and the control. This was associated with the high growth rates of the bacterial communities during this period. Afterwards, most of the detected amino acids were present in very low concentrations in both the co-cultures and the protist-free control, suggesting either that they were not produced by the bacteria or the flagellates or that they were indeed generated but then rapidly taken up by the rapidly growing bacterial community. However, after the initial sharp decrease of these amino acids in the co-culture experiments, the concentrations of some of them increased again, albeit with different patterns. Thus, the concentrations of glycine, L-alanine, and L-serine noticeably increased from 48 to 72 h and then remained relatively high; the concentrations of L-glutamate and D-alanine showed a pronounced peak from 48 to 72 h, but then noticeably decreased. These five DFAAs did not accumulate in the control experiments, suggesting that their appearance was associated with protist activity. Furthermore, DFAA production was not an artifact due to cell rupture during filtration, because the increase would have occurred with most or all of the amino acids; moreover, the amino acids would have accumulated during the entire incubation period.

Significant positive correlations between flagellate abundance and amino acid concentrations were noted for four of the five amino acids that accumulated (glycine, L-alanine, D-alanine, and L-serine), suggesting that these DFAAs were generated by bacterivorous flagellates. Similar results were previously reported in experiments conducted with different assemblages of bacterivorous protists and bacterial prey [1,23]. However, those studies lacked detailed information about the composition and quantity of DFAAs. Total DFAA concentrations ranging from 0.02 to 3.0 mM were reported in previous studies [1,9]. Those concentrations were slightly lower than the total DFAA concentrations detected in our study (0.3–16.2 mM). Andersson et al. [1] suggested that glycine, serine, and alanine are the dominant amino acids, but only the relative concentration of serine showed a good correlation with flagellate abundance. Nagata and Kirchman [23] detected the generation of different amino acids, depending on the offered prey. They found that alanine, tyrosine, glycine, threonine, and arginine accumulated when the prey was a mixed bacterial community, whereas glycine, alanine, asparagine, and aspartate accumulated when the prey was *Vibrio splendidus*. However, these studies did not provide information about the enantiomers present.

It can be assumed that the source of the amino acids is the bacterial prey. We initially hypothesized that glycine and the D-isomers of alanine, aspartate, glutamate, and serine, which are major components of the peptidoglycans of bacterial cell

walls, accumulate because they are more resistant than L-enantiomers to bacterial uptake [12]. However, our results indicated that, of those candidates, only glycine and, to a much lesser extent, D-alanine tended to accumulate. Another potential source could be a bacterial prey that is rich in the detected amino acids, including a large set of non-pathogenic environmental bacteria that have giant proteins on their cell surfaces [28,29]. These proteins (10,000 amino acids) are longer than average bacterial proteins (300 amino acids) [32]. They have a distinct amino acid composition and are very rich in glycine, alanine, threonine, and serine, which represent 30–40% of all their amino acids. This possibility fits well with our observations of glycine, L-alanine, and L-serine accumulations.

Following identification of the DFAAs in the first experiment, the differential response of *R. nasuta* towards these amino acids was analyzed. The tested flagellates came from 96-h cultures that comprised "young," actively growing cells and from 144-h cultures made up of older cells that had reached the stationary phase of growth. Culture age was found to affect the flagellate's ability to detect the assayed amino acids. Protists from young cultures were strongly attracted to all the assayed amino acids while protists from the older cultures were strongly attracted only to glycine. It seems that, when physiological conditions improve, non-growing protists undergo rearrangements in their cell-surface biochemistry that allow them to detect external chemical stimuli. The effect of age on the chemotactic abilities of bacterivorous protists is, nonetheless, unresolved. Snyder [33] observed that well-fed ciliates have a more rapid, intense response to bacterial surface compounds than starved cells. In contrast, Leick and Hellung-Larsen [19] reported that starved and growing *Tetrahymena* cells show similar responses to dissolved peptides and amino acids.

Our data suggested the following scenario: hotspots of bacterial growth resulted in the generation of amino acids, including mainly glycine, but also L- and D-alanine, L-serine, and L-glutamate, while supporting bacterivorous activity. A non-growing flagellate foraging in the vicinity of the hotspot would be able to detect the accumulated glycine and swim towards the source. Upon its arrival, it would encounter sufficient bacterial prey so as to grow and reproduce. Young flagellates could remain nearby, detecting not only glycine but also small quantities of other amino acids.

In summary, the amino acids glycine, L- and D-alanine, L-serine, and L-glutamate were found to accumulate during heavy feeding of the marine flagellate *R. nasuta*. In addition, there was a positive association between the abundance of the bacterivorous flagellate and the accumulation of glycine, L-and D-alanine, and L-serine, suggesting that flagellates are involved in their generation. Among these four amino acids, glycine always elicited the strongest response from *R. nasuta*; in contrast, the effects of L- and D-alanine were age-dependent and only significant in young flagellates. Based on these results, we propose that, in bacterially enriched patches, actively grazing bacterivorous protists generate glycine, a chemical signal able to attract foraging protists towards available prey in aquatic environments.

Acknowledgements. This work was supported by grants from UPV-EHU GIU 07/30, MEC BOS2003-06211 and CTM2006-08023 to J.I., A.T. and Z.B. were supported by predoctoral grants from UPV-EHU.

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