

Involvement of Cell Surface Structures in Size-Independent Grazing Resistance of Freshwater *Actinobacteria*^{∇†}

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We compared the influences of grazing by the bacterivorous nanoflagellate *Poterioochromonas* sp. strain DS on ultramicrobacterial *Actinobacteria* affiliated with the Luna-2 cluster and ultramicrobacterial *Betaproteobacteria* of the species *Polynucleobacter cosmopolitanus*. These bacteria were almost identical in size (<0.1 μm³) and shape. Predation on a *Polynucleobacter* strain resulted in a reduction of >86% relative to the initial bacterial cell numbers within 20 days, while in comparable predation experiments with nine actinobacterial strains, no significant decrease of cell numbers by predation was observed over the period of ≥39 days. The differences in predation mortality between the actinobacterial strains and the *Polynucleobacter* strain clearly demonstrated size-independent grazing resistance for the investigated *Actinobacteria*. Importantly, this size-independent grazing resistance is shared by all nine investigated Luna-2 strains and thus represents a group-specific trait. We investigated if an S-layer, previously observed in an ultrastructure study, was responsible for the grazing resistance of these strains. Experiments aiming for removal of the S-layer or modification of cell surface proteins of one of the grazing-resistant strains by treatment with lithium chloride, EDTA, or formaldehyde resulted in 4.2- to 5.2-fold higher grazing rates in comparison to the levels for untreated cells. These results indicate the protective role of a proteinaceous cell surface structure in the size-independent grazing resistance of the actinobacterial Luna-2 strains, which can be regarded as a group-specific trait.

Predation by phagotrophic flagellates is considered (besides the effect of viruses and sedimentation) one of the major mortality factors affecting planktonic bacteria in freshwater and marine environments (13, 24, 28). A large number of investigations demonstrated that bacteria with small cell sizes are less susceptible to grazing mortality caused by flagellates than medium-sized cells (e.g., references 9 and 34). However, even bacterial cells with ultramicrobacterial (<0.1 μm³) cell sizes can be ingested and probably processed by bacterivorous flagellates (4). Interestingly, an increasing number of observations indicate that planktonic *Actinobacteria* indigenous to freshwater systems are less vulnerable to protistan predation than other taxa of freshwater bacterioplankton. Pernthaler and colleagues observed in a two-stage continuous cultivation system that freshwater *Actinobacteria* increased in relative and absolute abundance in the presence of a bacterivorous flagellate (26). Other investigations also indicate at least a low level of vulnerability of freshwater *Actinobacteria* to protistan grazing (17, 29). Experiments with a single actinobacterial strain affiliated with the Luna-2 cluster indicated complete grazing resistance for this freshwater strain against predation by a bacterivorous flagellate (14). Incubation of the strain in the presence of the flagellate over a period of 20 days resulted only in minor changes of bacterial cell numbers. Addition of heat-

killed cells of another bacterial species serving as alternative prey resulted even in simultaneous increases of actinobacterial and flagellate numbers. Thus, even the rapidly growing flagellate population was not able to efficiently reduce the actinobacterial cell numbers. Recently, experiments with natural bacterial and protist communities demonstrated a strong negative selection of planktonic *Actinobacteria* by bacterivorous flagellates (18). In this investigation, the relative abundances of several phylogenetic groups of bacteria inside food vacuoles of flagellate cells and in the surrounding water have been determined. Actinobacterial cells were strongly underrepresented in the food vacuoles compared to their presence in the surrounding water. Currently, it is not known if the observed strong predation resistance of freshwater *Actinobacteria* is the result of postingestional protection or digestion protection taking place after ingestion of cells by a predator. Furthermore, it is unknown if this grazing protection is a common trait shared by all planktonic *Actinobacteria* present in freshwater systems or if only some taxa are protected.

Importantly, *Actinobacteria* constitute large fractions (up to 60%) of bacterioplankton in freshwater systems (8) and seem to present a ubiquitous component of freshwater bacterioplankton (8, 23, 37). Almost all actinobacterial taxa identified by cultivation-independent methods so far represent indigenous taxa exclusively known to occur in freshwater habitats (8, 37, 39), and only some of these indigenous actinobacterial taxa are represented by cultivated strains (7, 14–16).

The cultivated strains representing indigenous freshwater *Actinobacteria* provide excellent opportunities for detailed studies of the grazing resistance mechanisms of this ecologically important group of bacteria. Cultivated strains affiliated with the Luna cluster (14), also known as the acII clade (37),

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currently represent the best-characterized planktonic *Actinobacteria* indigenous to freshwater systems. Many of the cultivated Luna strains are characterized by C-shaped (selenoid) cells with ultramicrobacterial sizes of $<0.07 \mu\text{m}^3$. An electron microscopy investigation revealed a so-called S-layer at the surfaces of their cells (14). S-layers are monomolecular arrays composed of identical protein or glycoprotein subunits forming crystalline layers at the cell surfaces of many *Bacteria* and *Archaea* (30, 35). We hypothesize that this S-layer is involved in the grazing protection of the previously investigated freshwater *Actinobacteria*.

In the study presented here, we investigated (i) if grazing resistance is a common trait among strains of the Luna-2 cluster, (ii) if the grazing protection previously demonstrated for an actinobacterial strain affiliated with the Luna-2 cluster is independent of the small size and the C shape of the cells, and (iii) if the cell surface structure is involved in the grazing resistance mechanism of Luna-2 strains.

MATERIALS AND METHODS

Microorganisms. Nine actinobacterial strains belonging to the Luna-2 cluster (14, 15), namely, strains MWH-Mo1, MWH-Wo1, MWH-Aus1, MWH-Uga2, MWH-Po1, MWH-Bo1, MWH-Ta4, MWH-Ta3y, and MWH-Uga1 (see Fig. S1 in the supplemental material), and the betaproteobacterial strain *Polynucleobacter cosmopolitanus* MWH-MoNR1 (formerly known as the D subcluster [PncD] of the *Polynucleobacter* cluster [4, 10]) were used for grazing experiments. All actinobacterial strains, as well as *P. cosmopolitanus* MWH-MoNR1, possess C-shaped cells with ultramicrobacterial sizes ($<0.1 \mu\text{m}^3$) (4, 10, 14). Prior to the experiments, bacteria were cultivated in 3 g liter⁻¹ NSY medium (16) at room temperature with rotary shaking.

The mixotrophic bacterivorous nanoflagellate *Poteroiochromonas* sp. strain DS (11) was used as a predator organism. This flagellate strain was previously assigned to the genus *Ochromonas*, but the recent sequencing of the 18S rRNA gene revealed its affiliation with the genus *Poteroiochromonas*, which is closely related to the genus *Ochromonas*. This flagellate strain was also used in previous predation experiments (4, 11, 12, 14, 26, 29). Prior to the experiments, *Poteroiochromonas* cells were cultivated axenically in inorganic basal medium (IBM) (16) at 15°C under permanent illumination and fed with heat-killed cells of bacterial strain *Listonella pelagia* CB5 (*Gammaproteobacteria*).

Long-term grazing experiments with single actinobacterial strains and *Poteroiochromonas* cells. A total of nine actinobacterial strains were separately cocultivated with *Poteroiochromonas* cells for 39 or 45 days. For the setup of the experiments, a 50-ml stationary-phase culture of each actinobacterial strain was centrifuged at $5,000 \times g$ for 15 min. The cell pellets obtained were resuspended in 50 ml of IBM and incubated with rotary shaking at room temperature overnight. This procedure was performed for washing of the cells and acclimatization to IBM prior to the experiment. Then, the respective bacterial strain and *Poteroiochromonas* cells were inoculated into 50 ml of IBM in 100-ml Erlenmeyer flasks to give final numbers of ca. 1.5×10^7 and ca. 2×10^3 cell ml⁻¹, respectively. Triplicate cocultures were incubated at 15°C in the dark without shaking except for gentle stirring once a day. At each sampling date, 3 ml of samples were taken, preserved immediately with formaldehyde (2% final concentration), and stored in a refrigerator until staining with 4',6-diamidino-2-phenylindole (DAPI). Monocultures of bacteria or *Poteroiochromonas* cells were also prepared as controls.

Long-term grazing experiment with a mixed bacterial culture and *Poteroiochromonas* cells. A one-to-one mixture of the actinobacterial strain MWH-Mo1 and the betaproteobacterial strain MWH-MoNR1 was cocultured with *Poteroiochromonas* cells for 20 days in triplicate batches. The experiment was set up as described above for the long-term experiments with single actinobacterial strains. Cell numbers for the two bacterial strains were determined by whole-cell fluorescence in situ hybridization (FISH) (25). Subsamples for FISH were fixed with paraformaldehyde (2% final concentration) at room temperature for 2 h, filtered onto 0.2- μm -pore-sized Isopore membrane filters (type GTTP; Millipore Corp.), rinsed with 1 ml phosphate-buffered saline and 1 ml sterile Milli-Q water, and stored at -20°C until hybridization. The Cy3-labeled probes HGC69a (specific for *Actinobacteria*; 23S rRNA targeted) (1) and BET42a (specific for *Betapro-*

tobacteria; 23S rRNA targeted) (22) were used for enumeration of MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1, respectively.

Detection of bacteria in food vacuoles of *Poteroiochromonas* cells. Strain-specific detection of bacterial cells in food vacuoles of *Poteroiochromonas* cells fed with a mixture of MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1 was performed by means of catalyzed reporter deposition FISH according to the protocol of Jezbera et al. (18). The experiment was performed in two parallel batches. *Poteroiochromonas* cells were inoculated into 50 ml of IBM to give a final abundance of ca. 5×10^3 cell ml⁻¹ and incubated at 15°C in the dark for 2 h in order to eliminate remaining heat-killed bacteria in food vacuoles, which was microscopically confirmed. Then, MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1 were inoculated to give final abundances of $1.1 \times 10^7 \pm 0.2 \times 10^7$ and $1.0 \times 10^7 \pm 0.2 \times 10^7$ cells ml⁻¹ in the first coculture and $1.4 \times 10^7 \pm 0.3 \times 10^7$ and $0.9 \times 10^7 \pm 0.2 \times 10^7$ cells ml⁻¹ in the second coculture, respectively, and incubated at room temperature (20 to 22°C). In order to follow the uptake of bacteria by *Poteroiochromonas* cells, 1-ml samples were taken at 0, 5, 10, 20, 30, and 60 min after the inoculation of bacteria and preserved with neutral Lugol's solution (5% final concentration), followed by addition of formaldehyde (2% final concentration), and within 2 to 3 min, several drops of 3% sodium thiosulfate were added to clear the Lugol's color (33). Sixty minutes after the inoculation of bacteria, a 100-fold dilution of the culture with IBM was performed (18). This resulted in a 100-fold decrease in the probability of encounter of bacterial and *Poteroiochromonas* cells and enabled direct observation of the decrease in the concentration of digested bacterial prey inside the food vacuoles. In order to observe the strain-specific digestion of bacteria by *Poteroiochromonas* cells, 20-ml subsamples were taken at 5, 10, 20, and 30 min after the dilution and preserved as described above. Probes HGC69a and BET42a, labeled with horseradish peroxidase, were used for detection of cells of the two bacterial strains in the protistan food vacuoles. A minimum of 40 *Poteroiochromonas* cells were inspected for the presence of the two bacterial strains in the food vacuoles.

Effect of cell surface treatments on rates of *Poteroiochromonas* grazing on *Actinobacteria*. Three different treatments for removal of the S-layer or modification of cell surface proteins of the actinobacterial strain MWH-Po1 were performed. The cell surface-treated bacteria were offered to *Poteroiochromonas* cells, and grazing rates were measured. Removal of the S-layer was performed by treatment with EDTA or lithium chloride according to protocols for extraction of an S-layer from prokaryotic cells (27, 36). A 10-ml portion of culture of MWH-Po1 cultivated at 15°C was centrifuged at $5,000 \times g$ for 15 min at 15°C. The cell pellet was resuspended in 10 ml of extraction solution, namely, IBM containing 0.2 M EDTA or 0.5 M lithium chloride. After gentle shaking at 15°C for 30 min., cell suspensions were centrifuged as described above and supernatants were removed. After the extraction procedure was performed twice, cell pellets were resuspended in 10 ml of IBM for washing and centrifuged as mentioned above. Cell pellets were used for the experiment after being washed three times. Modification of cell surface proteins was performed by a treatment with formaldehyde (5, 6). Formaldehyde was added to a 10-ml portion of culture of MWH-Po1 to give a final concentration of 2%. After the centrifugation and the wash with IBM, as mentioned above, cell suspensions were used for the experiment. The influence of the performed treatments on the viability of the treated cells was examined by plating of samples on NSY agar plates.

For determination of grazing rates, MWH-Po1 cells were inoculated into the culture of *Poteroiochromonas* sp. strain DS cultivated at 25°C, and the experiment was performed at 25°C in the dark for 2 h. At the beginning and the end of the incubations, 2 ml of the samples was taken for determination of bacterial numbers and sizes. The initial numbers of *Poteroiochromonas* cells were in the range of 1.5×10^5 to 1.8×10^5 cell ml⁻¹. Only a few heat-killed bacteria were detected in the culture of *Poteroiochromonas* cells before the experiment.

Grazing rates of *Poteroiochromonas* cells were calculated according to the following equation: grazing rate = (initial abundance of bacteria - final abundance of bacteria)/*Poteroiochromonas* abundance/incubation period.

Determination of microbial numbers and sizes. The numbers of bacteria and flagellates were determined after DAPI staining by epifluorescence microscopy as described previously (11). Samples for determination of actinobacterial cell numbers were stained at least overnight, because shorter staining periods frequently resulted in weak signals.

Bacterial cell volumes were measured according to the method of Hahn et al. (14). In brief, images of 100 DAPI-stained cells were captured with a monochrome charge-coupled-device camera (Hitachi Denshi Ltd., Japan), and sizes were determined with the LUCIA D image analysis systems (Laboratory Imaging, Czech Republic). Bacterial cell volumes were calculated by using the formula of Andersson et al. (2).

Statistical analysis. The data for cell numbers, cell volumes, and grazing rates were analyzed by using the Friedman test, a one-way analysis of variance

(ANOVA), or Student's *t* test with the software package Kaleida Graph for Macintosh, version 3.6.4 (Synergy Software). Tukey's honestly significant difference test was performed when appropriate.

Nucleotide sequence accession numbers. The almost complete sequence of the 16S rRNA gene of the new isolate MWH-Ta3y and the almost complete 18S rRNA sequence of *Poteroiochromonas* sp. strain DS have been deposited in GenBank under accession numbers AM183217 and AM981258, respectively. The 16S rRNA sequences of all other strains were deposited previously (10, 14, 15).

RESULTS

Long-term grazing experiments with single actinobacterial strains and *Poteroiochromonas* cells. The cell volumes of all actinobacterial strains at the beginning of the incubations, except for MWH-Uga2, were in the range of 0.042 to 0.045 μm^3 , and the size differences among strains were insignificant ($P > 0.01$; one-way ANOVA, data not shown). The cell volume of MWH-Uga2 was 0.040 μm^3 , which was significantly smaller than that for the other strains ($P < 0.001$; one-way ANOVA). None of the actinobacterial strains decreased in cell number significantly when cocultured with *Poteroiochromonas* cells for 39 or 45 days (Fig. 1). The changes in cell numbers for each actinobacterial strain during the incubation periods were insignificant ($P > 0.01$), except for MWH-Ta4 ($P < 0.005$), according to the Friedman test (data not shown).

The numbers of *Poteroiochromonas* cells increased slowly but significantly ($P < 0.02$; Friedman test) in all cocultures with *Actinobacteria*, contrary to what was found for the treatments where bacteria were absent (Fig. 1). Interestingly, the increase of flagellate numbers was always linear and not exponential (Fig. 1). Pronounced differences in bacterial cell sizes between treatments with and without flagellates were not observed at the end of incubation period (data not shown).

Long-term grazing experiment with a mixed bacterial culture and *Poteroiochromonas* cells. When both the actinobacterial strain MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1 were offered to *Poteroiochromonas* cells simultaneously, MWH-Mo1 cells did not decrease in number ($P > 0.1$; Friedman test), but MWH-MoNR1 decreased markedly ($P < 0.02$; Friedman test) (Fig. 2A). These changes in number were almost identical to the changes observed in the absence of the respective second bacterial strain. Specifically, MWH-Mo1 cells did not decrease in number when cocultured with *Poteroiochromonas* cells (Fig. 2B), but MWH-MoNR1 cells decreased markedly when cocultured with *Poteroiochromonas* cells alone (Fig. 2C). Neither MWH-Mo1 nor MWH-MoNR1 cells decreased in number when cocultured in the absence of the flagellates (Fig. 2D). *Poteroiochromonas* cells fed with both MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1 or with MWH-MoNR1 alone increased in number markedly (Fig. 2A and C). In contrast, *Poteroiochromonas* cells did not increase in number in coculture with MWH-Mo1 (Fig. 2B) or in the absence of any bacteria (data not shown).

The cell volume of MWH-Mo1 was slightly lower than *P. cosmopolitanus* MWH-MoNR1 at the beginning of the experiment, while the difference of cell volumes was insignificant at the 5th and 11th day of incubation (Table 1).

Detection of bacteria in food vacuoles of *Poteroiochromonas* cells. Observation of bacteria in food vacuoles of *Poteroiochromonas* cells revealed that the flagellate strongly preferred

grazing on *P. cosmopolitanus* MWH-MoNR1 to the actinobacterial strain MWH-Mo1 (Fig. 3). The number of MWH-Mo1 in food vacuoles was less than 0.2 cells flagellate⁻¹ during the incubation period before dilution, and reliable uptake rates could not be determined. In contrast, the increase in number of *P. cosmopolitanus* MWH-MoNR1 cells was significant over the first 20 to 30 min of incubation. The uptake rates calculated with linear regression for the first 10 min of incubation for the two cocultures were 3.1 ($r^2 = 0.93$) and 2.8 ($r^2 = 0.94$) cells flagellate⁻¹ h⁻¹, respectively. After dilution, MWH-Mo1 cell numbers in food vacuoles dropped during the first 5 min, but a very small number of cells could be detected during the further incubation period. On the other hand, *P. cosmopolitanus* MWH-MoNR1 cells detected in food vacuoles decreased in number exponentially.

Effect of cell surface treatments on rates of *Poteroiochromonas* grazing on *Actinobacteria*. The rates of *Poteroiochromonas* sp. strain DS grazing on actinobacterial strain MWH-Po1 treated with EDTA, lithium chloride, or formaldehyde were 4.2- to 5.2-fold higher than those for untreated cells ($P < 0.02$; Student's *t* test) (Table 2). *Poteroiochromonas* cells grazed $2.2 \times 10^6 \pm 0.26 \times 10^6$ (18% \pm 2.3% of the initial level), $1.8 \times 10^6 \pm 0.35 \times 10^6$ (14% \pm 2.0% of the initial level), or $2.3 \times 10^6 \pm 0.57 \times 10^6$ (18% \pm 3.9% of the initial level) cells ml⁻¹ of EDTA-, lithium chloride-, or formaldehyde-treated cells during the 2 h of incubations. However, the decrease in the number of untreated MWH-Po1 cells in the presence of the flagellate during the same period was negligible ($0.43 \times 10^6 \pm 0.35 \times 10^6$ cells ml⁻¹ [3.6% \pm 3.0% of the initial level]). In monocultures of treated or untreated MWH-Po1, changes in cell numbers were in the range of -0.5×10^6 to 0.4×10^6 cells ml⁻¹ (-3.9 to 3.7% of the initial level), irrespective of treatments (data not shown). The cell volumes of treated cells were identical to those of untreated cells (Table 2). MWH-Po1 cells treated with EDTA or lithium chloride were viable, i.e., they formed colonies on agar plates (data not shown). *Poteroiochromonas* cells grazed $1.3 \times 10^6 \pm 0.37 \times 10^6$ ml⁻¹ (13% \pm 2.8% of the initial level) of untreated *P. cosmopolitanus* MWH-MoNR1 cells.

DISCUSSION

In this study, we clearly demonstrated that resistance to flagellate grazing is a common trait among freshwater *Actinobacteria* affiliated with the Luna-2 cluster and, more importantly, that this trait is based on a size-independent mechanism. Our experiments with Luna-2 cells chemically treated by substances known to modify S-layers or proteinaceous cell surface structures strongly indicated the involvement of such cell surface structures in the grazing resistance mechanism of these bacteria.

The nanoflagellate *Poteroiochromonas* sp. strain DS has been used in a number of predation experiments of flagellates on single bacterial strains (e.g., references 4, 14, and 38), on bacterial assemblages consisting of two strains (11), or on bacterial communities consisting of an unknown number of strains (e.g., references 12, 26, and 29). In almost all experiments, predation by this flagellate resulted in a strong decrease in total bacterial numbers by more than 90%. The observed overall reductions of cell numbers were independent of the exper-

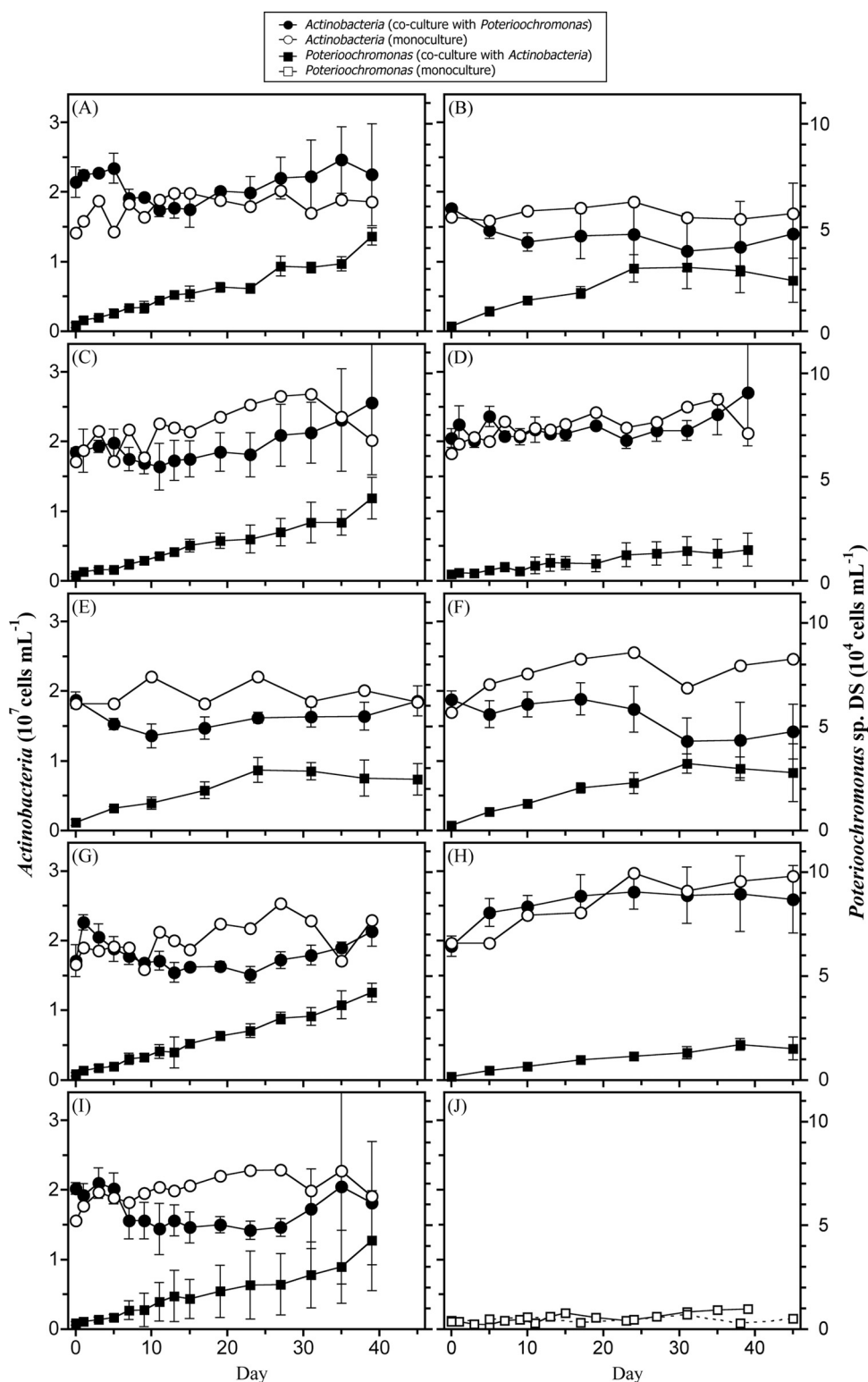


FIG. 1. Changes in abundance of actinobacterial strains or *Poterioochromonas* sp. strain DS in coculture or monoculture in mineral medium. (A) Actinobacterial strain MWH-Mo1; (B) MWH-Wo1; (C) MWH-Aus1; (D) MWH-Uga2; (E) MWH-Po1; (F) MWH-Bo1; (G) MWH-Ta4; (H) MWH-Ta3y; (I) MWH-Uga1; (J) monoculture of *Poterioochromonas* sp. strain DS was performed with duplicate cultures (solid and dotted lines).

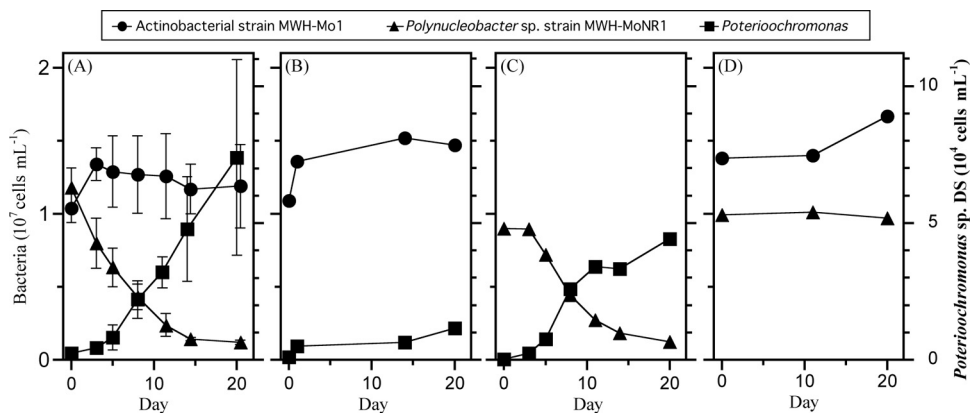


FIG. 2. Changes in abundance of actinobacterial strain MWH-Mo1, *P. cosmopolitanus* MWH-MoNR1, and the *Poterioochromonas* sp. strain DS flagellate. (A) Coculture of MWH-Mo1, MWH-MoNR1, and *Poterioochromonas* cells. (B) Coculture of MWH-Mo1 and *Poterioochromonas* cells. (C) Coculture of MWH-MoNR1 and *Poterioochromonas* cells. (D) Coculture of MWH-Mo1 and MWH-MoNR1. Error bars in panel A indicate standard deviations of results for triplicates. Cultures for panels B, C, and D were performed as a single experiment. Bacterial cell size data are presented in Table 1.

imental system (i.e., batch culture experiments or continuous cultivation systems), but the dynamics of the reductions were related to cell size and the morphological traits of the investigated bacteria. That means that larger (i.e., filamentous bacteria [11, 38]) and smaller (i.e., ultramicrobacterial [$<0.1\text{-}\mu\text{m}^3$] cells [4, 12]) bacteria were less efficiently grazed than medium-sized bacteria (12). In all but one of these experiments, the flagellate was able to strongly decrease the bacterial numbers. The only exception was a predation experiment with the actinobacterial strain MWH-Mo1 affiliated with the Luna-2 cluster (14).

In this study, we demonstrated that the observed grazing resistance of strain MWH-Mo1 is a common feature of strains forming the species-like Luna-2 cluster (see Fig. S1 in the supplemental material). In contrast, in a previous investigation of the thermal adaptation of six Luna-2 strains, pronounced differences in phenotypic adaptation were demonstrated (15). Interestingly, a gene encoding an actionobacterial variant of proteorhodopsin was recently detected in one of the Luna-2 strains (strain MWH-Uga1) (32), which was also investigated here. However, other strains seem to lack the gene for this light-driven proton pump (32). Thus, with regard to some ecological traits, the Luna-2 cluster represents a heterogeneous group, but on the other hand, it represents a highly homogeneous group with regard to its grazing resistance. This indicates an important role for grazing resistance in the ecology of these bacteria.

TABLE 1. Cell volumes of *Actinobacteria* strain MWH-Mo1 and *P. cosmopolitanus* MWH-MoNR1 in coculture with bacteria and *Poterioochromonas* sp. strain DS^a

Strain	Cell vol \pm SD (μm^3) for indicated incubation period		
	0 days	5 days	11 days
MWH-Mo1	0.051 \pm 0.004 ab	0.051 \pm 0.005 ab	0.050 \pm 0.006 b
MWH-MoNR1	0.057 \pm 0.010 c	0.055 \pm 0.009 ac	0.051 \pm 0.008 ab

^a Compare Fig. 2. FISH-hybridized cells were measured. Values with the same letter do not show significant difference ($P < 0.001$) by Tukey's honestly significant difference test ($n = 100$).

The flagellate could not significantly decrease the numbers of the investigated actinobacterial strains, whether in the presence or in the absence of alternative prey (Fig. 1 and 2). Even well-growing and actively grazing *Poterioochromonas* populations had no significant effect on the numbers of *Actinobacteria* (Fig. 2A). However, we cannot exclude the possibility of a very small predation mortality of the investigated *Actinobacteria* caused by the *Poterioochromonas* flagellate. The slight decrease of actinobacterial numbers in some experiments and the rare detection of *Actinobacteria* inside *Poterioochromonas* cells (Fig. 3) may hint toward a very small grazing mortality of these bacteria, which might probably be considered an incidental uptake.

The pronounced increase of *Poterioochromonas* cell numbers in the presence of actinobacterial cells compared to what

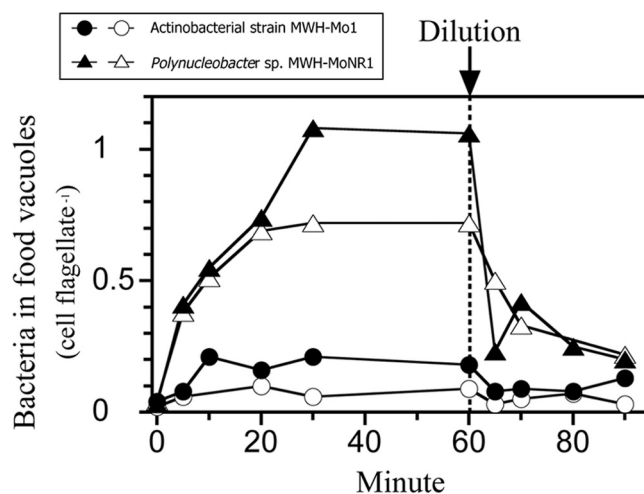


FIG. 3. Changes in number of actinobacterial strain MWH-Mo1 or *P. cosmopolitanus* MWH-MoNR1 cells in food vacuoles of *Poterioochromonas* sp. strain DS in duplicated cultures (closed and open symbols, respectively) in which equal abundances of MWH-Mo1 and MWH-MoNR1 were offered to the flagellate. The vertical break line represents a 100-fold dilution with mineral medium.

TABLE 2. Rates of *Poteroiochromonas* sp. strain DS grazing on surfaces of treated or untreated cells of actinobacterial strain MWH-Po1 as well as on *P. cosmopolitanus* MWH-MoNR cells^a

Strain	Treatment	Grazing rate ^b ± SD (no. of cells/flagellate/h)	Cell vol ^c ± SD (μm ³)
MWH-Po1	None	1.3 ± 1.1	0.048 ± 0.006
MWH-Po1	EDTA (0.2 M)	6.2 ± 0.7*	0.047 ± 0.006
MWH-Po1	LiCl (0.5 M)	5.5 ± 0.6*	0.047 ± 0.007
MWH-Po1	Formaldehyde (2%)	6.7 ± 1.9**	0.046 ± 0.007
MWH-MoNR1	None	3.8 ± 1.3**	0.046 ± 0.007

^a * and ** indicate significant differences from the values for untreated MWH-Po1 cells ($P < 0.005$ and $P < 0.05$, respectively; Student's *t* test). The cell volumes for treated MWH-Po1 and MWH-MoNR1 were insignificantly different from those for untreated MWH-Po1 cells ($P > 0.01$; Student's *t* test).

^b Grazing rate = (initial abundance of MWH-Po1 – final abundance of MWH-Po1)/abundance of *Poteroiochromonas* cells/incubation period. Prior to the experiment, strain MWH-Po1 and *Poteroiochromonas* sp. strain DS were cultivated at 15 and 25°C, respectively. The experiments were performed at 25°C for 2 h ($n = 3$).

^c DAPI-stained cells were measured ($n = 100$).

was found in their absence (Fig. 1) seems to be inconsistent with the lack of decrease in actinobacterial cell numbers. This might be explained by the availability of essential lipids, usually provided by the bacterial prey, that are crucial for the growth of *Poteroiochromonas* cells (M. W. Hahn, unpublished data). Starved flagellate cells are in principle able to undergo reductive cell divisions, which may be limited by the availability of such lipids. Thus, it is conceivable that the flagellates increased in number by grazing on actinobacterial cells at very low, immeasurable rates, which could be sufficient for providing the essential lipids. Although it has not yet been evidenced whether *Poteroiochromonas* cells are capable of absorbing dissolved organic compounds, the effect of pinocytosis on the growth of the flagellate in our experiment is unlikely, because the amount of the dissolved organic compounds present in the cultures was too low to support the growth of the flagellate.

The comparative grazing experiment with *Poteroiochromonas* cells preying on a Luna-2 strain and a *Polynucleobacter* strain (Fig. 2), displaying almost identical cell sizes (Table 1), showed strong negative selection against the actinobacterial strain and also demonstrated size independence for the grazing protection.

The observed grazing vulnerability of the *Polynucleobacter* strain was comparable to the size-dependent vulnerability previously observed for *Polynucleobacter* strains also affiliated with the PnecD subcluster (4). In their study, Boenigk and his co-workers proved that the cell volume of the grazing-resistant actinobacterial strains were clearly in the prey size range for which *Poteroiochromonas* cells exhibit significant grazing activity.

The observed strong increase of grazing efficiency after chemical removal of the S-layer by EDTA or lithium chloride (27, 36) or modification of cell surface proteins by formaldehyde (5, 6) (Table 2) clearly indicates that the grazing resistance of the investigated Luna-2 bacteria is related to/depends on their cell surface structures. The involvement of the S-layer in grazing protection had already been suggested, e.g., for *Synechococcus* cells (*Cyanobacteria*) ingested by the ciliate *Tetrahymena thermophila* (20). Other investigations, however, in-

dicated that S-layers do not provide an overall protection against predation by bacterivorous protists (21).

An S-layer may prevent the ingestion of a prey cell due to surface hydrophobicity (21) or due to shielding prokaryotic surface structures serving as ligands in receptor-mediated prey recognition. It is assumed that such receptor-mediated mechanisms are involved in predator-prey interactions of protists and prokaryotes; however, details of the molecular mechanisms were not revealed yet (19). Instead of a preingestional protection mechanism, S-layers may have a postingestional protection function, as demonstrated for *Synechococcus* (20). It is not clear whether the investigated actinobacterial strains were protected by a pre- or postingestional mechanism. Immediate egestion of consumed actinobacterial cells, as observed by Boenigk et al. for cyanobacterial cells (3), as well as very low uptake rates caused by a preingestional protection mechanism may be responsible for the very low numbers of Luna-2 cells observed in food vacuoles of the flagellate (Fig. 3) as well as for the very low grazing rates measured for untreated Luna-2 cells (Table 2). On the other hand, both results could represent experimental artifacts, since both employed methods work close to their detection limits, which may result in certain overestimation of actinobacterial uptake by the flagellate.

Important future questions to be answered include whether the observed grazing protection of Luna-2 bacteria is a general trait holding also for other actinobacterial lineages indigenous to freshwater habitats and whether these bacteria are resistant against predation by other bacterivorous protists. Previous investigations with natural bacterioplankton communities indicated that the majority of freshwater *Actinobacteria* possess resistance against protistan predation (18). Such resistance could be a key trait of freshwater *Actinobacteria*. Another key trait could be light-harvesting rhodopsins (actinorhodopsins) encoded at least by some strains of two important lineages of freshwater *Actinobacteria* (31, 32). Future investigations have to reveal the particular contributions of these two potential ecological key traits to the enormous ecological success of planktonic *Actinobacteria* in the pelagic zones of freshwater systems (8, 23, 37).

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