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Microbes Enriched in Seawater after Addition of Coral Mucus▽†

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We investigated which microbial taxa in coastal Red Sea water were stimulated by addition of mucus from the coral *Fungia* sp. Decreases in the concentration and C/N ratio of particulate organic material during short-term incubations (50 h) were paralleled by a steep rise in the number of *Gammaproteobacteria*, particularly *Alteromonadaceae*, followed by *Vibrionaceae*. Two almost identical genotypes affiliated with *Alteromonas macleodii* accounted for up to >85% of all *Alteromonadaceae* (45% of the total cells) in the mucus-amended enrichments but were rare in unamended control incubations and in ambient seawater. *A. macleodii*-like bacteria might thus be important in the transfer of organic carbon from coral mucus to the pelagic microbial food webs of coral reefs.

Coral reefs are located in some of the most nutrient-depleted marine areas but are nevertheless ecosystems with high primary production (20). Some corals secrete mucopolysaccharide material in such quantities that it can dominate the suspended matter in reefs (38). Mucus plays an important role as a carrier of energy and nutrients to a range of planktonic and benthic consumers (36). Moreover, it represents an important resource for microbial growth in reef ecosystems (reference 10 and references therein).

We therefore, investigated which bacteria in seawater are favored by the release of freshly detached mucus material into seawater. Short-term changes in organic C and N and microbial community composition were simultaneously analyzed in enrichment cultures of coastal seawater amended with mucus from one of the most common Red Sea scleractinian corals (*Fungia* sp.).

Coral mucus and seawater were collected from the lagoon of Dahab in the southern part of the Gulf of Aqaba, northern Red Sea, in May 2004. Polyps of *Fungia* sp. (diameter, 5 to 10 cm) were collected from water depths of 3 to 8 m using SCUBA and exposed to air for 3 min to trigger mucus production. Mucus released during the first 1 min was discarded to reduce contamination with bacteria from the coral surface. The mucus produced during the following 2 min was aseptically collected and placed into sterile glass bottles. The coral mucus was homogenized, mixed 1:10 (vol/vol) with seawater obtained from the same site within 60 min after collection, and placed in triplicate sterile 1-liter glass bottles. Triplicate bottles of unamended seawater served as controls. All bottles were incubated for 50 h at the in situ temperature (24°C) and light conditions (500 to 800 μmol quanta m^{-2} day^{-1} at a water depth of 1 m [C. Jantzen, personal communication]).

Substrate consumption and microbial growth. Portions (50 ml) from all bottles were filtered onto precombusted GF/F filters (diameter, 25 mm; Whatman) at 0, 26, and 50 h. The concentrations of particulate organic C (POC) and particulate N (PN) and stable isotope ratios of C to N were determined using dried filters (48 h at 40°C) with a THERMO NA 2500 elemental analyzer coupled to a THERMO/Finnigan MAT Delta plus isotope ratio mass spectrometer at GeoBio Center (Munich, Germany).

The POC and PN concentrations at time point zero were 0.71 ± 0.23 mg C $liter^{-1}$ and 0.07 ± 0.03 mg N $liter^{-1}$, respectively, in the controls and were two- to threefold higher in the mucus-amended incubations (Table 1). The total POC concentration did not significantly decrease in either treatment during the incubation period (one-sided Mann-Whitney U test). The C/N ratio in the mucus incubations significantly decreased over the duration of the experiment ($\alpha = 0.05$). The average $\delta^{13}C$ values for particulate organic matter in mucus incubations at time point zero were significantly less negative than those for the controls ($\alpha = 0.05$), and there were no significant changes over the entire incubation time (Table 1). The $\delta^{15}N$ values for particulate organic matter in the mucus incubations were always higher than those for the control treatments and were positive at 26 and 50 h (Table 1).

Samples (15 ml) for bacterial cell counting and fluorescence in situ hybridization (FISH) (see below) (29) were taken after 0, 2, 4, 6, 10, 26, and 50 h. Portions (0.5 to 10 ml) were filtered onto polycarbonate membrane filters (GTTP; pore size, 0.2 μm; diameter, 25 or 47 mm; Millipore, Eschborn, Germany) after 24 h of fixation in buffered paraformaldehyde solution (final concentration, 1%; 4°C). Total cell numbers were determined by staining with 4′,6-diamino-2-phenylindole (DAPI) (final concentration, 1 μg ml^{-1}) and automated epifluorescence microscopy (30).

The initial cell numbers in the mucus-seawater mixture were only marginally higher than the control cell numbers (Fig. 1). Addition of mucus led to an almost fourfold increase in bacterial abundance after 10 h of incubation, whereas the cell numbers in the seawater control only approximately doubled over the whole incubation period. Exponential growth of the

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TABLE 1. Changes in the concentrations and isotopic signatures of PN and POC in enrichments with coral mucus and unamended controls during 50 h of incubation[a]

Sample	Time (h)	PN concn (mg liter^{-1})	POC concn (mg liter^{-1})	C/N ratio (molar)	δ^{15}N (‰)	δ^{13}C (‰)
Control	0	0.07 ± 0.03	0.71 ± 0.23	13.68	−0.81 ± 5.96	−24.75 ± 1.48
	26	0.06 ± 0.04	1.02 ± 0.37	21.95	−0.56 ± 1.97	−25.29 ± 1.02
	50	0.05 ± 0.02	0.69 ± 0.06	17.24	−2.05 ± 6.84	−24.79 ± 0.74
Mucus	0	0.17 ± 0.02	2.34 ± 1.01	15.82	−0.45 ± 1.94	−19.97 ± 1.09
	26	0.24 ± 0.04	2.13 ± 0.19	10.30	2.16 ± 1.60	−19.59 ± 0.36
	50	0.24 ± 0.03	2.05 ± 0.15	9.99	1.82 ± 0.96	−19.45 ± 0.12

[a] The values are means ± standard deviations of three replicates.

bacterial community in the mucus-amended preparations ended within the first 24 h. This was probably due to substrate or nutrient limitation, since no growth of bacteriovorous protists was observed (data not shown).

The rapid enrichment of bacteria in the mucus-amended incubations confirms previous observations of enhanced bacterial growth in similar experimental setups (36). The increasing δ^{15}N values for PN (Table 1) in these treatments indicate that there was isotopic enrichment and microbial decomposition of PN (23). Moreover, the significant decrease in the POC/PN ratio in mucus-amended enrichments suggests that there was efficient microbial carbon mineralization, probably due to degradation of the complex polysaccharides that represent a major fraction of the particulate mucus material (10).

Identification of enriched bacteria. Microbial diversity in the enrichment cultures was assessed by 16S rRNA gene sequence analysis. For construction of rRNA gene clone libraries, mucus-amended incubation mixtures were sampled after 10 and 26 h (libraries M10 and M26), and the control incubation mixtures were sampled after 50 h (library C50). Ten milliliters of unfixed sample was filtered onto white membrane filters (GTTP; pore size, 0.2 μm; diameter, 25 mm; Millipore). 16S rRNA genes were directly PCR amplified from cells on filter pieces (ca. 4 mm^2) after freeze-thaw cycles (21) with primers GM3F and GM4R (28) under conditions described previously (16). The amplified fragments were purified (QIAquick; Qiagen, Hilden, Germany) and cloned using the TOPO TA cloning system (Invitrogen, Karlsruhe, Germany). Insert-bearing clones were PCR screened with vector primers M13F and M13R, and partial sequences were generated from the PCR products with primer GM1F (5′-CCA GCA GCC GCG GTA AT-3′; modified from Muyzer et al. [27]). Sequence analysis was carried out with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Plasmids from selected clones were prepared with a QIAprep kit (Qiagen), and almost complete 16S rRNA gene sequences were obtained from plasmid DNA using primers GM1F, GM3, and GM4. After assembly (Sequencher; Gene Codes Corp., Ann Arbor, MI) sequence types were tentatively identified by BLAST (3).

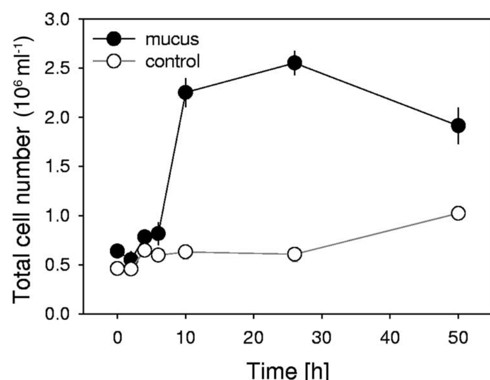

FIG. 1. Changes in the total abundances of prokaryotic cells in mucus-amended and control incubations.

Ninety-five distinct (≤98% identity) 16S rRNA genotypes were obtained from the three samples. Fifty-nine percent of the sequences in the C50 library (n = 27) and more than 90% of the sequences in both the M10 (n = 24) and M26 (n = 44) libraries were affiliated with the *Gammaproteobacteria* (see Table S1 in the supplemental material). Good's coverage (18) and the S_{Chao1} estimator (11) were calculated from clone frequencies using a tool described by Kemp and Aller (19). Both indicators suggested that most gammaproteobacterial diversity in the individual libraries was covered by the number of sequenced clones (Good's coverage for M10 was 0.8, for M26 was 0.7, and for C50 was 0.7) (see Fig. S1 in the supplemental material). The asymptotic behavior of the number of predicted phylotypes indicated that the M10 and M26 libraries were large enough to yield stable estimates of gammaproteobacterial phylotype richness (see Fig. S1, left panel, in the supplemental material).

Phylogenetic analysis was carried out using the ARB software package and database (25), which was supplemented by sequences from RDP (12). A maximum parsimony tree of *Alteromonas* phylotypes was calculated using sequences >1,200 nucleotides long. Shorter sequences were subsequently added by the ARB QUICK_ADD option. All gammaproteobacterial sequences in the mucus-derived M10 and M26 clone libraries were affiliated with *Alteromonadaceae*, *Pseudoalteromonas* spp., and *Vibrio* spp. Twenty-one distinct genotypes from these groups were fully sequenced (Table 2 and Fig. 2). Sequence types of the *Alteromonadaceae* were affiliated with the species *Alteromonas macleodii* and *Alteromonas marina* and with uncultured *Alteromonas* spp. (Fig. 2). One sequence type of *Vibrionaceae* (M26-030) was closely affiliated with the coral pathogen *Vibrio coralliilyticus* (7) (Table 2). Cultivation-based studies have shown that particular *Gammaproteobacteria* (*Halomonas* spp., *Vibrio* spp., *Pseudoalteromonas* spp., and *Alteromonas* spp.) can be "residents" and/or "visitors" of the surface mucus of, e.g., *Fungia* and *Acropora* (22, 32).

Enumeration of gammaproteobacterial populations. Changes in the bacterial community structure were analyzed by FISH and

TABLE 2. 16S rRNA gene sequence types affiliated with *Pseudoalteromonas* spp. and *Vibrio* spp. from enrichments with coral mucus (M10 and M26; 10 and 26 h of incubation)

Designation	Closest relative/closest cultivated relative	% Identity	Accession no.
M10-001	Coral mucus clone M26-003/*Pseudoalteromonas* sp.	99.9/99.9	AM941175/AJ874345
M10-022	*Pseudoalteromonas* sp. strain EBD	99.8	DQ218321
M26-003	Coral mucus clone M10-001/*Pseudoalteromonas* sp. strain A28	99.9/99.8	AM941169/AF227238
M26-004	*Thalassomonas ganghwensis* JC2041	96.8	AY194066
M26-031	*Pseudoalteromonas* sp. strain 03/034	99.8	AJ874351
M26-041	Marine bacterium Tw-2/*Alteromonas* sp. strain KT0232	95.5/95.1	AY028197/AF235125
M26-005	*Vibrio fortis* LMG 21562	99.4	AJ514915
M26-029	*Vibrio brasiliensis* LMG 20546	98.6	AJ316172
M26-030	Bacterium CWISO20/*Vibrio coralliilyticus* LMG 21349	99.3/99.2	DQ334358/AJ440004
M26-032	*Vibrio* sp. strain LMG 20548	98.9	AJ316170
M26-036	Coral mucus clone M26-032/*Vibrio* sp. strain LMG 19270	98.7/98.6	AM941182/AJ316169
M26-037	*Vibrio chagasii* LMG 13237	97.3	AJ490157

signal amplification (catalyzed reporter deposition) (29). Cells on membrane filters (see above) were pretreated as described by Teira et al. (34), with slight modifications (permeabilization by proteinase K; concentration, 0.15 μl/ml [2,129 U/mg; Fluka]; incubation time, 15 min). Catalyzed reporter deposition-FISH on filter sections was performed as described previously (29) with probes targeted to *Bacteria* (EUB I-III mixture) (13), *Gammaproteobacteria* (GAM42a) (26), *Vibrionaceae* (GV) (17), *Alteromonadaceae*/*Colwelliaceae* (ALT1413), and *Pseudoalteromonadaceae* (PSA184) (15). The formamide concentrations in hybridization buffers were 55% (EUB I-III, PSA184, and GV) and 60% (ALT1413). The fractions of hybridized cells of all DAPI-stained objects were determined by automated epifluorescence microscopy (30).

The detection rates with EUB I-III were 68% of the total DAPI counts in the original water sample and 83% ± 5% in the incubations (data not shown). Addition of mucus to seawater clearly favored the growth of *Gammaproteobacteria* (Fig.

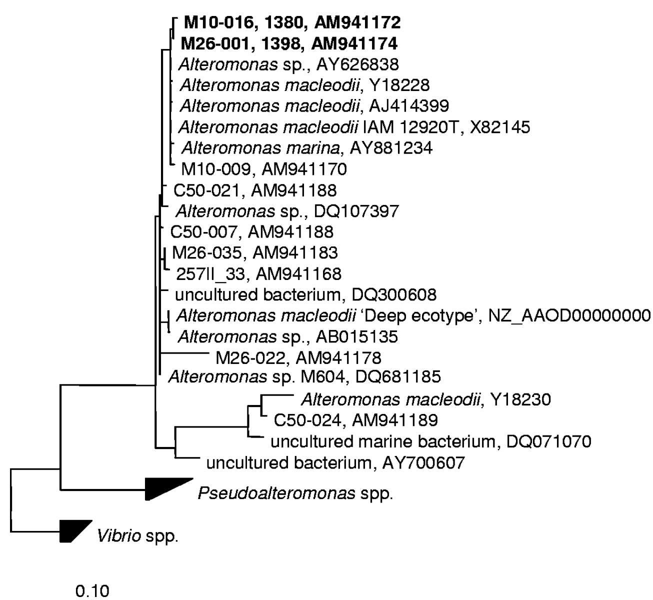

FIG. 2. Phylogenetic relationship of genotypes affiliated with *Alteromonas* from mucus-amended (M10 and M26) and control (C50) incubations. Bold type indicates sequence types targeted by oligonucleotide probe AMAC83.

3). The relative abundances of these microorganisms initially developed similarly in the mucus and control incubations (Fig. 3). Between 4 and 10 h, the proportions of *Gammaproteobacteria* in the mucus incubations rose steeply, to 60% ± 6% of all cells after 10 h and to 72% ± 2% toward the end of the experiment. The *Gammaproteobacteria* never exceeded 40% of the total cell counts in the controls.

Pseudoalteromonadaceae accounted for <10% of the total counts in mucus-amended incubations and were even rarer in the untreated controls (data not shown). *Alteromonadaceae* were the most successful of the studied bacterial populations in mucus-amended seawater during the initial phase of the incubation and accounted for most *Gammaproteobacteria* (Fig. 3). The relative abundances of these bacteria increased from <1% to almost 50% ± 1% of the total counts within 10 h (from $<5 \times 10^3$ to $>10^6$ cells ml^{-1}) and declined thereafter. Their relative abundances in the controls rose steadily but more slowly to a maximum of 8% ± 4% at the end of the incubation period. Rapid growth of *Alteromonadaceae* upon confinement has been observed for incubation of marine waters from habitats as different as the North Sea, the Mediterranean Sea, and the Red Sea (2, 14, 31). Here we show that the growth of these bacteria is particularly favored by addition of coral mucus to seawater.

The *Vibrionaceae* was another successful bacterial group in mucus-amended enrichments (Fig. 3). The relative abundances of these bacteria increased to 21% ± 6% of the total DAPI counts in these treatments, whereas they formed only small populations (<2%) in the controls. The largest population of *Vibrionaceae* (0.6×10^6 cells ml^{-1}) formed only after 26 h. This delay might indicate that *Vibrionaceae* were not able to directly consume organic material in mucus but profited from the exoenzymatic activity of *Alteromonadaceae*. Alternatively, antagonistic effects between the two populations are conceivable. Both *Alteromonas* spp. and *Vibrio* spp. can inhibit the growth of strains belonging to their own genus and strains belonging to the other genus (24).

Growth of *A. macleodii*-like bacteria favored by addition of coral mucus. To further identify the dominant bacterial genotype in the enrichments, a specific probe for FISH was designed (ARB tool PROBE_DESIGN). Probe AMAC83 (5′ CGT AAC GCC ACT CGT CAT CTT 3′; *Escherichia coli* positions 83 to 104 [9]) is targeted to two newly obtained sequence types affiliated with *A. macleodii* (clones M10-16 and

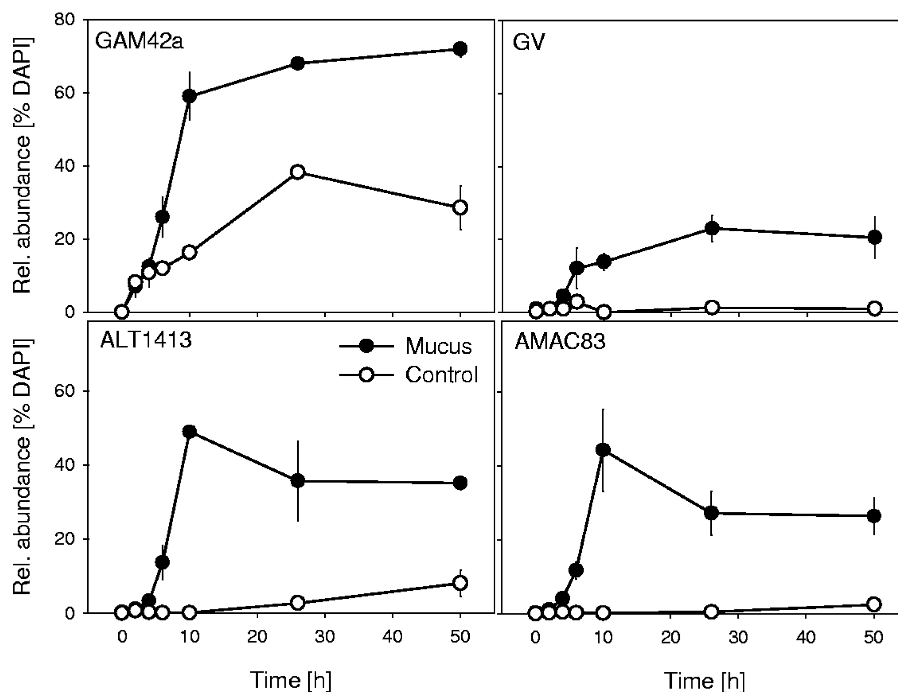

FIG. 3. Relative abundances of *Gammaproteobacteria* (probe GAM42a), *Vibrionaceae* (probe GV), *Alteromonadaceae* (probe ALT1413), and *A. macleodii* (probe AMAC83) in mucus-amended and control incubations.

M26-001) (Fig. 2). This probe was used in combination with unlabeled helper probes h-AMAC60 (5′ CTA GCA AGC TAG AAA TGT TAC CG 3′) and h-AMAC104 (5′ CAA GTT CCC AAG CAT TAC TCA CC 3′) (6). The specificity of the AMAC83 probe was verified in silico using the ARB and RDP (release 51) databases. Stringent hybridization conditions were established with 55% of formamide in the hybridization buffer (see Fig. S2 in the supplemental material).

The vast majority of cells detected by probe ALT1413 in the mucus-amended enrichments also hybridized with the newly designed specific probe AMAC83 (Fig. 3). Cells targeted by AMAC83 accounted for up to 44% ± 11% of the bacterial community. Thus, bacterial genotypes closely related to *A. macleodii* (Fig. 2) were among the dominant genotypes enriched by addition of mucus. Moreover, the observed changes in cell numbers during the first hours of the incubations (zero time, 1.8×10^3 cells ml^{-1}; 10 h, 9.9×10^5 cells ml^{-1}) suggested that the doubling times of these bacteria were extremely short (approximately 1 h).

The closest cultivated relative of M10-016 and M26-001, *A. macleodii* IAM 12920 (sequence identity, 99.8 and 99.9%; not targeted by probe AMAC83), exhibits hydrolytic exoenzymatic activities of, e.g., amylases, gelatinases, and lipases (4). Thus, it should be well equipped to degrade major components of coral mucus (10). Moreover, *Alteromonas* spp. utilize the resulting monomers, such as hexoses, disaccharides, sugar acids, amino acids, and ethanol (4). The versatile metabolism of these microorganisms may help them exploit rapid changes in the supply of a complex substrate source, such as coral mucus. *A. macleodii*-like bacteria in our samples were present as free-living single cells but also formed aggregates, likely on coral mucus particles. A transition from dissolved matter to particulate matter is a typical aspect of the coral mucus cycle in coral reefs (37). *A. macleodii*, which thrives both on particles (1) and in the water phase (14), is probably favored in an organic matter field with a pronounced gradient of dissolved organic matter to particulate organic matter.

Trophic link to pelagic food webs? We did not attempt to comprehensively describe the community composition of coral mucus. Instead, we assessed which bacterial taxa were enriched first on mucus material freshly released into ambient water irrespective of their origin (mucus or seawater). Genotypes closely related to *A. macleodii* have been detected both in coral tissue (8) and in mucus (32). It is, therefore, likely that *A. macleodii*-like bacteria are also common in the bacterium-rich layer of the coral-water interphase (33).

Culturable gammaproteobacterial lineages, such as *Alteromonadaceae* and *Vibrionaceae*, exhibit higher sensitivity to grazing by heterotrophic nanoflagellates than other members of the marine bacterioplankton (2, 5). A substantial fraction of the organic carbon released as mucus in coral reef systems might thus be channeled to higher trophic levels via such tight predator-prey interactions. Moreover, selective protistan grazing might help to explain why cells detected by probe AMAC83 were virtually absent from the ambient bacterioplankton (Fig. 3). On the other hand, elevated viral concentrations have also been reported in the close vicinity of corals (33), and the rapid enrichment of only a limited number of genotypes would allow efficient population control by viral lysis (35).

Nucleotide sequence accession numbers. All sequences determined in this study have been deposited in the GenBank database under accession numbers AM941168 to AM941189.

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