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Influence of age of aggregates and prokaryotic abundance on glucose and leucine uptake by heterotrophic marine prokaryotes

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Summary. The kinetics of glucose and leucine uptake in attached and free-living prokaryotes in two types of microcosms with different nutrient qualities were compared. Microcosm type M1, derived from unaltered seawater, and microcosm type M2, from phytoplankton cultures, clearly expressed different kinetic parameters (V_{max}/cell and K'_m). In aggregates with low cell densities (M1 microcosm), the attached prokaryotes benefited from attachment as reflected in the higher potential uptake rates, while in aggregates with high cell densities (M2 microcosm) differences in the potential uptake rates of attached and free-living prokaryotes were not evident. The aging process and the chemical changes in aggregates of M2 microcosms were followed for 15–20 days. The results showed that as the aggregates aged and prokaryotic abundance increased, attached prokaryotes decreased their potential uptake rate and their K'_m for substrate. This suggests an adaptive response by attached prokaryotes when aggregates undergo quantitative and qualitative impoverishment. [**Int Microbiol** 2007; 10(1):13-18]

Key words: marine prokaryotes · aggregates · glucose and leucine uptake

Introduction

In marine systems there are two different habitats, liquid phase and particle, with extremely different physical and chemical properties [1]. A large fraction of marine particulate matter consists of macroscopic aggregates, also known as "marine snow" [1]. The origin of these flocculent amorphous aggregates, while mainly phytoplanktonic, is diverse although they may also result from the aggregation of senescent diatom cells, coagulation of colloidal organic matter released by phytoplankton, and algal bacterial interactions [16]. In this heterogeneous system, detection and analysis of the adaptive responses of prokaryotes to the varying environmental conditions in the ocean remain of fundamental importance for understanding the oceanic carbon cycle.

Microbial diversity is expressed in terms of metabolism rather than structure, and prokaryotes have optimized their biochemistry for the uptake and utilization of a wide variety of nutrients [7,12]. In this context, transmembrane transport is a good indicator of organic-matter flux in marine prokaryotes. Glucose and leucine are low-molecular-weight compounds considered to be representative of the two main fractions of organic matter in the sea, carbohydrates and proteins [23], and most prokaryotes are able to transport and metabolize both compounds. The affinity (K_m) and capacity (V_{max}) of membrane transport systems can be regulated to optimize nutrient uptake in response to prokaryotic metabolic needs as well as nutrient availability in the environment. It is possible that attached prokaryotes function as high-capacity uptake systems as an adaptation to grow on particles, where nutrient-rich conditions prevail. Indeed, some authors [2,20] have

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reported higher uptake rates in attached prokaryotes than in their free-living counterparts. This proposed scenario fits well with speculations on the accumulation of prokaryotes with low-affinity high-capacity uptake systems in substrate production microzones [4] and with previous suggestions of "particle specialist prokaryotes" in the sea [3,27].

Nevertheless, particles seem to offer a range of very different conditions which at times could make them a less advantageous microhabitat than the liquid phase [17]. Müller-Niklas et al. [21] found that increasing size and age transformed the aggregates into poor habitats for prokaryotic growth, and Grossart and Ploug [11] showed a decrease in growth efficiencies in aging lab-made aggregates. These results demonstrate that for a better mechanistic understanding of prokaryotic growth and metabolic efficiency it is necessary to analyze substrate uptake by prokaryotes attached to aggregates of different ages.

There are several restrictions when working with natural aggregates, as these particles are very fragile and usually break up when collected, transported, and stored. Moreover, it is not possible to exactly determine the age and degree of decomposition of aggregates used as samples. Due to these constraints, an experimental system aimed at managing model particles under controlled conditions [28,30] constitutes a useful alternative for studying interactions between microbial communities and particulate matter with respect to colonization and decomposition processes.

Our aim in this study was to examine how prokaryotes modify the kinetic properties that characterize their uptake of organic compounds in order to cope with aggregates of different quality and age. To achieve this objective, we measured the kinetic parameters, i.e., cell-specific maximum uptake rate (V_{max}/cell) and the Michaelis-Menten constant (K'_m), for glucose as well as leucine uptake by prokaryotes attached to particles and by their free-living counterparts. Two types of lab-made artificial aggregates were used, and their aging processes followed and analyzed.

Materials and methods

Lab-made microcosms: design and sampling. The experimental system used to obtain aggregates under controlled conditions was based on that of Shanks and Edmondson [28], as adapted by Unanue et al. [30]. Aggregates were produced artificially using rolling tanks and served as a model of marine aggregates. To obtain aggregates of different quality, the basic procedure was modified as described below. Macroaggregates of M1 microcosms were created from unaltered seawater while those of M2 microcosms consisted of artificial seawater amended with phytoplanktonic material.

The source material for M1 microcosms was natural seawater collected from the coastal waters of the Bay of Biscay, 43º24.5′ N, 3º2.7′ W (north of Spain) at a depth of 3 m. Samples were taken using a Niskin bottle (PWS Hydro-Bios) and processed in the laboratory within 2 h after collection. Polypropylene cylindrical tanks (13 l) were filled with 12 l of seawater from the sampling location, placed on a roller table with a rotation velocity of 2.5 rpm, and incubated in the dark at room temperature. The maximum length of the particles was 0.2–8.7 mm, with a mean of 1.4 ± 0.01 mm (mean ± standard error). For the analysis, particles bearing minimal amounts of ambient water were carefully transferred from the tanks to Petri dishes using a pipette with a plastic tube at the end. Precise volumes of particles were taken from the Petri dishes with a digital micropipette, placing the tip directly on the particles. Subsamples of ambient water were directly taken from the tanks after sedimentation of the particles.

For the M2 microcosms, four species of diatoms (*Skeletonema costatum*, CCAP 1077/1B; *Navicula hanseni*, CCAP 1050/8; *Chaetoceros muelleri*, CCAP 1010/3; and *Nitzschia epithemoides*, CCAP 1052/18) commonly found in marine aggregates [5,26] and in the Bay of Biscay [24] were used to obtain phytoplankton-derived model particles [32]. A mixture of algal cells and phytoplankton-derived dissolved organic matter (DOM) from the four diatom species was added to 5-liter bottles filled with sterile artificial seawater (Sigma). The cells and phytoplankton-derived DOM were mixed so that the contribution of each species to the final algal abundance and dissolved organic carbon (DOC) concentrations in the mixture was the same. The bottles were autoclaved and placed on the roller table. After 2–3 days, the particles were allowed to sediment, the supernatant was removed, and the sterile particles were carefully transferred to polypropylene cylindrical tanks containing 13 l of sterile artificial seawater. The final DOC concentration was adjusted at the beginning of the experiments with autoclaved phytoplankton-derived DOM so that the levels were the same as those in the waters of the Bay of Biscay (1.5–2 mg C/l). Afterwards, the tanks were rolled (2.5 rpm) for 12 h to allow the particles to become uniform in size. At that point, the microcosms were inoculated with a microbial assemblage obtained by tangential-flow filtration (100,000 Da, Millipore) from the sampling location. Prokaryotic abundance was adjusted to that of natural seawater (10^9 cells/l). Microcosms were incubated in the dark at room temperature with rolling at 2.5 rpm. Particles and ambient water were sampled as above. The maximum length of the particles was 0.9–10.2 mm, with a mean of 2.2 ± 0.02 mm (mean ± standard error). The abundance of these particles ranged from 50 to 300 part/l, with a mean value of 185 part/l.

Prokaryotic counts. Three subsamples of aggregates (7 µl in 1 ml of sterile artificial seawater) and ambient water (10 ml) were fixed with formalin (2% v/v final concentration). Prokaryotic abundance was measured by acridine orange epifluorescence direct counting (AODC) [14]. Samples of aggregates were sonicated (100 W, six pulses of 5 s) immediately before staining to disperse prokaryotes attached to particles [31]. Aliquots were stained with acridine orange (0.01% w/v final concentration) for 2 min and filtered using 0.2-µm pore-size black polycarbonate filters (Millipore). The wet filters were placed on microscope slides, mounted in low-fluorescence immersion oil, and then examined under an epifluorescence microscope (Nikon) at a magnification of ×1250. Prokaryotes present in at least 30 randomly selected fields, with 20–30 prokaryotes per field, were counted.

Heterotrophic uptake of glucose and leucine by prokaryotes: kinetic parameters. Prokaryotic heterotrophic uptake rates were measured using D-[U-^{14}C]glucose (>230 mCi/mmol, Amersham) and [U-^{14}C]leucine (>50 mCi/milliatom carbon, Amersham) as substrates [32]. Labeled compounds at different concentrations (0.001, 0.01, 0.1, 0.5, 1, 2, 5, 10, 25, 50, 100, 200 µg C/l) were added to triplicate or duplicate subsamples of ambient water (10 ml) and aggregates (10 µl in 10 ml of ambient water). Samples were incubated 1–2 h at room temperature. Each bottle was sealed with a cap assemblage (rubber cap with a glass cup containing a Whatman N1 paper). After incubation, H_2SO_4 (final concentration, 0.02 N) was added through the cap in order to stop incorporation of the labeled compounds, and phenethylamine (0.2 ml) was injected into the glass cup to trap respired CO_2 [13]. After 12 h, the phenethylamine-soaked papers were placed in scintillation vials and radioassayed by liquid scintillation counting. The subsamples were filtered through 0.2-µm pore size filters (Millipore MF) by applying a

vacuum pressure of 150 mm Hg. The filters were rinsed three times with 10 ml of distilled water, placed in scintillation vials, and radioassayed by liquid scintillation counting. These measurements represented the assimilated ^{14}C-substrate. Controls for each substrate concentration were processed as above, but formaldehyde (final conc. 2% v/v) was added 1 h before addition of the radiolabeled substrates. Total uptake rates for each substrate were determined from the sum of the assimilation rate plus the respiration rate. Uptake rates of attached prokaryotes were calculated by subtracting the uptake value for ambient water from the uptake value obtained for ambient water plus aggregates.

Kinetic parameters were estimated by fitting the uptake rates to a Michaelis-Menten curve, $V=V_{max} \times S/ (K´_m + S)$, using a nonlinear regression program. V is the rate of the reaction, V_{max} is the maximum velocity of the reaction obtained at saturating concentration of substrate, S is the concentration of substrate added, and $K´_m$ (apparent K_m) is the concentration of substrate needed to obtain half V_{max}. $K´_m$ represents $K_m + S_n$; K_m is the affinity constant such that a lower value for K_m indicates a higher affinity of the enzyme for the substrate; S_n is the concentration of natural substrate in the system. Since the value of S_n was not measured, the real value of K_m could not be determined. Consequently $K´_m$ was considered to be a maximum estimation of K_m, although this assumption leads to an overestimation when the natural levels of substrate are close to the real affinity constant [2]. Average cell-specific potential uptake rates (V_{max}/cell) for attached and total prokaryotes were estimated by dividing V_{max} by the prokaryotic abundance and assuming uptake by all heterotrophic prokaryotic cells. To compare the kinetics of glucose and leucine uptake by attached and free-living prokaryotes, samples were taken on days 4–6 after the onset of the microcosms, when the aggregates were already highly colonized. Four and two experiments were performed with M1 and M2 microcosms, respectively (see below). The aging process of the aggregates was followed by measuring the kinetic parameters of attached prokaryotes on days 4, 9, and 19 for M1 aggregates and on days 4, 7, and 15 for M2 aggregates.

Hydrolytic activity. Prokaryotic α-glucosidase, β-glucosidase, and aminopeptidase activities in attached prokaryotes were measured using the fluorogenic substrates 4-methyl-umbellifeyl-α-D-glucopyranoside, 4-methyl-umbellifeyl-β-D-glucopyranoside, and L-leucyl 4-methylcoumarinyl-7-amide, respectively [15]. Fluorogenic substrates were added to triplicate 10.5-µl subsamples of aggregates in 3 ml of sterile artificial seawater at 250 and 350 µM for glucosidase and aminopeptidase activities, respectively; these were estimated in previous experiments to be saturating concentrations. Subsamples were incubated in the dark at room temperature on an orbital shaker (120 rpm). The fluorescence of methyl-umbelliferone (MUF) and 7-amino-4-methyl-coumarine (MCA), released by enzymatic cleavage of the substrates after 2–5 h for α-glucosidase and β-glucosidase, and after 1–3 h for aminopeptidase activity, was measured on a Perkin Elmer LS 50B spectrofluorometer at 360 nm excitation and 445 nm emission. Relative fluorescence units were calibrated with 100 nM MUF and MCA standards. Subsamples without substrate were used as blanks to determine the background fluorescence of the samples. Previous experiments showed that abiotic hydrolysis of the substrates was not significant.

Chemical analysis of aggregates. The concentrations of polysaccharides and combined amino acids were measured in 70 µl of aggregates in 100 ml of distilled water according to the method of Parsons et al. [25]. For amino acids, this technique actually measures the total concentration of primary amines, and the amino acid concentrations are therefore overestimated since they include ammonium. The evolution of chemical characteristics was followed (15–20 days) in four experiments carried out with M2 microcosms.

Results

Microbial parameters in aggregates with different nutritional qualities. The $K´_m$ values for glucose and amino acid uptake were higher (up to 100-fold; Wilcoxon test, $P < 0.05$) in particulate material than in bulk seawater for both microcosms (Table 1). However, and taking into account that the value of $K´_m$ is highly influenced by the natural substrate concentration (S_n), $K´_m$ did not seem to be a profitable parameter to compare prokaryotes living on habitats with extreme differences in natural concentrations of substrates, as is the case with aggregates and bulk water.

The average cell-specific potential uptake rate of leucine and glucose by prokaryotes in the different habitats of microcosms M1 and M2 is shown in Table 1. Attached prokaryotes were much more active than free-living prokaryotes in M1 microcosms (Wilcoxon test, $P < 0.05$) but, surprisingly, there were no differences between the two types of prokaryotes (Wilcoxon test, $P > 0.05$) in M2 microcosms. Although both types of aggregates were densely colonized, aggregates of M2 microcosms showed a higher cell abundance per unit volume of aggregates ($3.1 \pm 0.2 \times 10^{12}$ cell/l of aggregate) than aggregates of M1 microcosms ($2.0 \pm 0.3 \times 10^{12}$ cell/l of aggregate) (U-Mann Whitney test, $P < 0.05$). The cell abundance in ambient water ranged from 0.84×10^9 to 5.69×10^9 cell/l of ambient water in M1 microcosms and from 1.09×10^9 to 9.26×10^9 cell/l of ambient water in M2 microcosms.

Analysis of heterotrophic uptake by attached prokaryotes in aging aggregates. During the first interval (from 4 to 7–9 days), and in both microcosms, the prokaryotic abundance followed an antagonistic trend with

Table 1. V_{max} per cell and apparent Michaelis constant ($K´_m$) of leucine and glucose heterotrophic uptake in ambient water and aggregates in M1 and M2 microcosms (average value ± standard error)

		M1 microcosms		M2 microcosms	
Parameter	Substrate	Ambient water	Aggregate	Ambient water	Aggregate
V_{max}/cell (fg C/cell per h)	Glucose	0.36 ± 0.09	26.28 ± 10.03	0.65 ± 0.27	2.71 ± 2.20
	Leucine	0.35 ± 0.08	9.05 ± 4.49	0.45 ± 0.19	0.65 ± 0.27
$K´_m$ (µg C/l)	Glucose	2.62 ± 0.45	101.10 ± 31.83	13.62 ± 5.28	131.00 ± 0.40
	Leucine	1.18 ± 0.35	41.08 ± 12.10	1.70 ± 1.07	5.28 ± 0.40

Table 2. Prokaryotic abundance, V_{max} per cell ,and K'_m for leucine and glucose uptake during the aging of aggregates in M1 and M2 microcosms

	Age of M1 aggregates			Age of M2 aggregates		
Parameter	4 days	9 days	19 days	4 days	8 days	15 days
Prokaryotic abundance ($\times 10^{12}$ cell/l)	0.18	0.69	0.56	1.70	2.28	2.01
V_{max} Glu/cell (fg C/cell per h)	30.49	4.44	4.32	13.00	5.56	4.26
V_{max} Leu/cell (fg C/cell per h)	20.69	2.81	2.63	26.52	10.05	8.69
K'_m Glu (μg C/l)	116.30	60.87	64.16	15.53	131.81	12.45
K'_m Leu (μg C/l)	71.20	25.90	22.65	70.43	9.29	13.20

respect to the kinetic parameters for glucose and leucine uptake. An increase in the abundance of attached prokaryotes together with a decrease in cell-specific potential uptake rates and K'_m was observed, with the only exception being the K'_m of glucose uptake in M2 aggregates (Table 2). During the second interval (from 7–9 to 18 days), the above-mentioned parameters remained fairly constant, with the only exception again being the K'_m of glucose uptake in M2 aggregates.

The variations over time (13 days) of prokaryotic abundance, aminopeptidase and glucosidase activities of attached bacteria and carbohydrate and amino-acids concentrations in M2 microcosms were followed in four aging experiments. Since the results were quite similar, those from only one experiment are shown (Fig. 1A,B). During the first 6 days, the abundance of attached prokaryotes increased while polysaccharide and combined amino-acids concentrations decreased (Fig. 1A). From day 6 until the end of the aging process, these variables remained constant or decreased slowly. Prokaryotes attached to newly formed aggregates (2–4 days old) showed low aminopeptidase:glucosidases ratios and high α-glucosidase:β-glucosidase ratios (Fig. 1B). However, as the aggregates aged (4-6 days old) the ratio of the former increased while that of the latter decreased, and after 6 days both ratios were fairly constant.

Discussion

Free-living and attached prokaryotes in two different types of microcosms were analyzed with respect to the kinetics of glucose and leucine uptake. Previous studies in M1 and M2 microcosms showed that both types of aggregates were

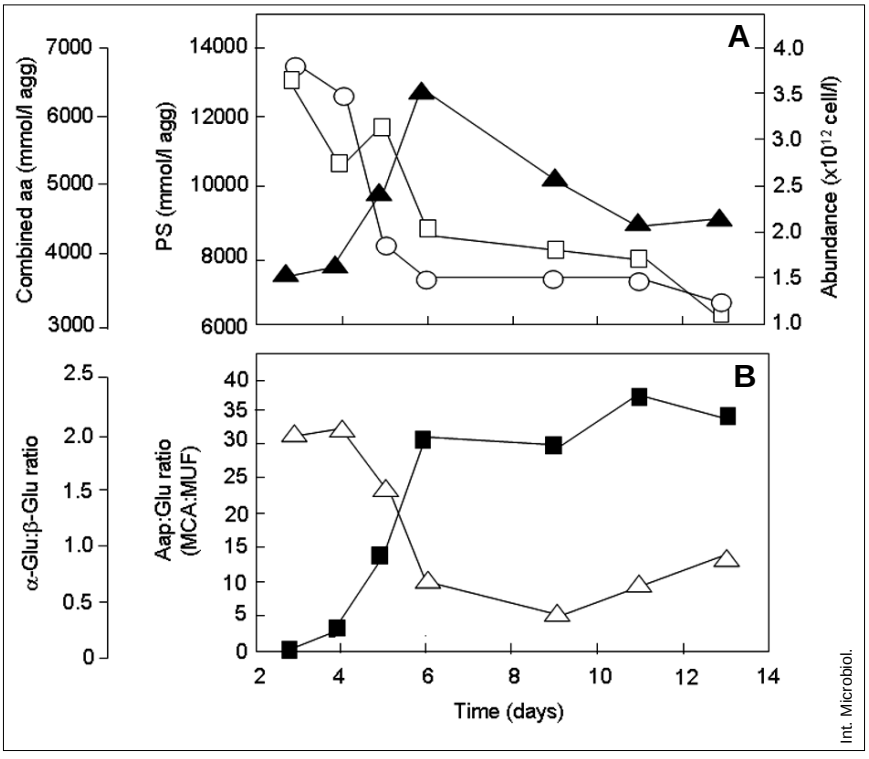

Fig. 1. Change of (**A**) prokaryotic abundance (closed triangles), combined amino acids (open squares) and polysaccharides (open circles) and (**B**) aminopeptidase:glucosidases ratio (closed square) and α-glucosidase:β-glucosidase ratio (open triangles) during the aging of aggregates in M2 microcosms. [α-Glu: α-Glucosidase; β-Glu: β-Glucosidase; aa: amino acids; PS: polysaccharides; Aap: aminopeptidase; agg: aggregate.]

enriched in carbohydrates and amino acids by a factor of 10^3 compared to the ambient water [30]. Thus, it was expected that in both microcosms K'_m values for glucose and amino-acid uptake would be higher in the particulate matter than in the bulk phase. However, since S_n was not measured for each substrate in each habitat, only a limit value and not the real degree of affinity can be established for the free-living and attached prokaryotes of the two substrates.

The average cell-specific potential uptake of leucine and glucose by free-living prokaryotes was similar in the two microcosms, but the same cannot be stated for their attached counterparts. In M1 microcosms, the values were much higher for attached prokaryotes than for free-living ones, while in M2 microcosms there were no significant differences. These results may have been due to the very high V_{max}/cell for leucine and glucose uptake by attached prokaryotes in M1 aggregates, which are characterized by lower prokaryotic abundances. The M1 microcosms were created from unaltered seawater, and organic materials present in natural seawater include a large fraction of aged, partially altered materials refractory to microbial attack [18]. By contrast, M2 microcosms were created from freshly produced phytoplankton material; consequently, the organic matter in them may contain more labile compounds that can be utilized by prokaryotes. Other sources of variability in prokaryotic abundance in the aggregates may be protistan grazing [19] and viral lysis [29], two biotic factors able to modify not only the abundance of prokaryotes but also their production rates and the structure of their communities.

Our results suggest that, in aggregates with a low nutritional quality and lower abundances (M1 microcosms), attached prokaryotes can take advantage of the accumulation of organic matter in the particulate material by incorporating low-molecular-weight compounds at a higher rate than free-living prokaryotes. Similar results have been observed in some marine systems [2,20]. Nevertheless, attached prokaryotes in M2 aggregates do not benefit from this attachment. Bell and Albright [6] studied different aquatic systems and also observed lower glucose-uptake rates in attached vs. free-living prokaryotes. The lower uptake rates in the attached community of M2 vs. M1 aggregates may reflect differences in the taxonomic structure of these communities. Different environmental conditions, such as those in the two microcosms, may lead to adaptations and genotypic selection of different communities and thus differences in their respective kinetic characteristics [7,9,10].

The age of the aggregates is another source of variability closely related to their nutritional value. In both types of microcosms, and in the first time-interval of the aging process, noticeable increases in attached prokaryotic abundance were observed, together with a decrease in the uptake rate and an increase in substrate affinities. Attached prokaryotes can modify their systems for glucose and leucine uptake over time due to changes in their community structure [7,9,10] or to phenotypic adaptation. This decrease in cell-specific potential uptake rates as prokaryotic abundance increases could also have been due to quantitative and/or qualitative changes in the chemical composition of the aggregates during the aging process. Our results show that aggregates undergo decreases in the concentrations of polysaccharides and combined amino acids as part of the aging process.

Moreover, as the aggregates aged, other processes, such as increases in C/N [21], recalcitrant organic matter [8], and refractory polysaccharides like chitin and cellulose [21] may occur, thereby impoverishing the aggregates qualitatively. The observed increase in the aminopeptidase:glucosidase ratio suggests that protein components are degraded faster than carbohydrates, leading to an increase in the C/N ratio. In addition, the α-glucosidase:β-glucosidase ratio in attached prokaryotes declined, indicating only minor degradation of polysaccharides [21,22]. Thus, during aging, the chemical composition of the aggregates changes not only quantitatively but also qualitatively. These changes could convert them to microhabitats less favorable for bacterial growth.

In summary, as aggregates are colonized and age, attached prokaryotes are able to adapt their uptake systems by decreasing the uptake rate and increasing substrate affinities. The decreased uptake rates could explain why in marine systems the growth rates of attached prokaryotes are similar or lower than those of free-living ones [17]. Thus, whilst source particles (highly enriched particles) are indeed suitable habitats, large sinking aggregates (aged and poorer particles) appear to be "inhospitable" for prokaryotic growth.

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