APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1996, p. 4521–4528 Vol. 62, No. 12
0099-2240/96/$04.00+0

Relationship between the Intracellular Integrity and the Morphology of the Capsular Envelope in Attached and Free-Living Marine Bacteria

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Received 5 August 1996/Accepted 9 October 1996

The integrity of the intracellular structures and the presence and dimension of the capsular envelope were investigated in marine snow-associated and marine free-living bacteria by transmission electron microscopy and special fixation techniques. Three categories depending on the presence of internal structures were differentiated. In marine snow, 51% of the marine snow-associated bacterial community was considered intact, 26% had a partly degraded internal structure, and 23% were empty with only the cell wall remaining. For the free-living bacterial community, 34% were intact cells, 42% exhibited damage, and 24% of the cells were lacking any internal structure. We also investigated the morphology and the extent of the bacterial capsular envelope. More than 95% of all intact marine snow-associated bacteria were surrounded by a capsule while ≈55% of empty marine snow-associated bacteria had no capsule. For free-living bacteria, ≈65% of the intact cells had a capsule while ≈80% of the empty free-living bacteria lacked a capsule. Thus there is a clear trend from intact cells which are commonly surrounded by a capsular envelope to empty bacteria for which only the cell wall is remaining. Since bacterioplankton represent the largest living surface in the ocean, it is concluded that the release of intracellular material from bacteria into the environment as well as the release of extracellular capsular material might fuel the dissolved organic matter pool of the ocean.

Bacterioplankton represent the largest living surface area in the oceanic environment because of their high abundance and small size. Standard enumeration techniques for bacteria using epifluorescence microscopy and acridine orange or DAPI (4′,6-diamidino-2-phenylindole) staining (22, 37), however, do not distinguish between metabolically active and inactive bacteria; therefore, these enumeration techniques result in bacterial abundance estimates which are higher than the abundance of metabolically active bacteria.

Several methods have been developed to determine the metabolic activity of individual cells. The most frequently applied technique is microautoradiography in which radiolabeled substrates are incorporated into the cell and the development of silver grains on a photographic film is observed by light microscopy often in combination with epifluorescence (23, 29, 44). The major drawbacks of this method are the long incubation time (up to several weeks) and the sometimes erroneous images due to adsorption of the radiolabeled substrate onto nonliving particles. Another frequently applied method uses the development of dye crystals in a reduced environment such as in respiring bacteria; traditionally the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) has been used widely (24). Recently, the more sensitive 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was applied in different environments (39) although the toxicity of the dye at higher concentrations (39) leads most likely to an underestimation of the percentage of active bacteria in natural bacterial consortia.

Most recently, DAPI staining and subsequently isopropanol rinsing to wash off the nonspecifically bound DAPI from the bacterial cell wall revealed a large fraction of non-nucleoid-containing bacteria, i.e., bacteria not containing DNA (48). Although this method does not directly indicate activity on a cellular level, it is assumed that bacteria not containing any DNA are not metabolically active.

Electron microscopy has been frequently used to study the morphology of bacterial cells or cell compartments in cultures and natural samples (1, 2, 15, 32). Despite the enormous structural heterogeneity of bacterial cells documented in these studies, there are several morphological features such as the ultrastructure of the cell wall common to all prokaryotes or, at least, to the vast majority of the bacterioplankton community, the gram-negative bacteria. Fixation has been shown to be the most crucial step in sample preparation for transmission electron microscopy (TEM) potentially causing artifacts. This problem can be overcome by applying nonperturbing fixation and embedding techniques (35) using glutaraldehyde as fixative. It has been shown that observations made on well-preserved samples correlate well with results obtained by biochemical analysis (6). Careful examination of the morphology of the internal and external structures of the bacterial cells should therefore reveal information about the integrity of the cell and therefore allow us to determine whether a single cell can be potentially active.

Bacterioplankton as the major living component of the microbial loop are the main consumers of the dissolved organic matter (DOM) pool which represents the largest organic carbon reservoir in the ocean (12). Bacterioplankton carbon, in turn, is transferred to protists via bacterivorous grazing (41). In this concept, the bacterial compartment converts DOM into living particulate organic matter, i.e., bacterial biomass. While this pathway represents an important carbon and energy flux, viral activity might modify this pathway by converting a part of the bacterial particulate organic matter into DOM again. Indeed, Fuhrman and Noble (14) have recently shown that

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flagellates and viruses are equally important in controlling bacterial production. Another potentially important flux from bacterial particulate organic matter into the oceanic DOM pool is the release of polymeric substances by bacteria. Bacteria have been shown to produce copious amounts of polymeric substances, and it has also been shown that the production of these substances is related to specific environmental conditions. Although some specific reactions for the biosynthesis of specific exopolymers are well known and frequently used in biotechnology, the knowledge on the factors regulating capsule or exopolysaccharide formation is limited. The bacterial capsule has many functions (reference 10 and references therein), like adsorption of nutrients or protection against predators. Furthermore, it plays an important role in the biofilm formation on surfaces (8).

Although the ability to produce capsules seems to be a common feature of bacteria in all environments, there is a remarkable lack of information concerning marine bacterioplankton. Cowen (9) and, more recently, Heissenberger et al. (20) have shown that capsular envelopes are widely distributed in marine free-living and particle-associated bacteria. These capsules consist mainly of high-molecular-weight polysaccharides (43). It has been shown that about 6% of leucine is converted by bacterioplankton into refractory compounds with molecular masses of >50,000 Da within 10 days (19) and secreted into the surrounding medium, possibly as capsular material.

The aim of this study was to examine the relationship between the integrity of the intracellular structures and the extent of the extracellular capsular envelope in free-living and marine snow-associated bacteria. Marine snow is a collective term for particles larger than 0.5 mm in diameter with a mucopolysaccharide matrix in which particles are embedded (21). It has been shown that attachment stimulates exopolysaccharide production in bacteria (46). We assumed that this high polysaccharide production in particle-associated bacteria is, at least partly, due to production of capsular material leading consequently to larger capsular envelopes in particle-associated than in free-living bacteria. We hypothesized that only bacteria with intact internal structures exhibit a pronounced capsular envelope while obvious internal destruction, indicative for a nonactive state of the cell, is accompanied by a less developed or even a missing capsular envelope. This scenario in which active cells possess a distinct capsule while inactive cells lack a capsular envelope would indicate that bacteria release a significant part of the capsular material into the ambient water if they become inactive, thereby fueling the oceanic DOM pool with high-molecular-weight DOM.

(This work was done in partial fulfillment of the requirements for a Ph.D. degree from the University of Vienna for A.H.)

MATERIALS AND METHODS

Sampling of ambient water and marine snow and preparation for TEM. Water and marine snow were collected along a trophic gradient across the northern Adriatic Sea from 1.5 km off Rovinj (Croatia) to the Po estuary (Italy) in July 1993 (details of the sampling stations are given in the work of Müller-Niklas and Herndl [30]). Samples were taken from four depth layers with cleaned and rinsed Niskin bottles as well as by scuba diving using 60-ml disposable syringes as described in the work of Herndl and Peduzzi (21). On board the RV *Vila Velebita*, three replicates of each sample were immediately fixed in glutaraldehyde-ruthenium red fixative (2.6% electron microscopy-grade glutaraldehyde and 0.05% electron microscopy-grade ruthenium red in 67 mmol of Na-cacodylate buffer liter^{-1} [pH 7]) to prevent storage artifacts (25, 35). Marine snow samples were transferred into the fixative with a minimum of ambient water (<200 μl). Water samples were added 1:2 to the fixative and gently mixed. After at least 2 h of fixation, the samples were washed, fixed with osmium tetroxide, dehydrated, and embedded in Spurr's epoxy resin (42) according to the protocol of Leppard et al. (26).

Ultrathin sections (50 to 100 nm) were obtained from the resin blocks by sectioning with a diamond knife mounted in an ultramicrotome (RMC Ultramicrotome MT-7). The sections were mounted on Formvar-covered copper grids and counterstained with uranyl acetate (27) and subsequently with lead citrate (38). All samples were examined with a JEOL 1200 EXII TEMSCAN scanning transmission electron microscope operated at 80 keV. For energy-dispersive spectroscopy (4), the same scanning transmission electron microscope equipped with a Princeton GammaTech Si (Li) X-ray detector was used.

Examination of intracellular integrity. At least three randomly chosen sections of each replicate were examined. The intracellular integrity of each bacterial cell was examined and assigned to one of three categories. (i) Intact bacteria are not deformed, possess an intact cytoplasmic membrane, and have a fully developed (not degraded) cytoplasm and nucleoplasm (3, 15, 18). (ii) Damaged bacteria lack at least one of the above criteria or are infected by viruses. (iii) Empty bacteria lack any plasma inside the cell wall.

Because of the thickness of the sections (50 to 100 nm) compared with the cell size (0.25 to 1.5 μm), it was possible to clearly detect all cellular components (membrane, cytoplasm, nucleoplasm) by focusing through the object even if the plasma was not regularly distributed within the cell. We consider only intact cells as potentially active while damaged and empty cells are considered inactive.

The spines of *Chaetoceros* sp., diatoms which were the most frequent algae in the samples (20), are very similar in size and shape to empty bacteria if vertically cross-sectioned. In order to ensure that only those bacteria which had a real bacterial cell wall were counted as empty bacteria, energy-dispersive spectroscopy spectra of presumably empty cells were frequently recorded. *Chaetoceros* spines showed a clear silica peak, while bacterial cell walls which are usually devoid of silica gave no comparable signal.

The morphology of bacterial capsular envelopes. Simultaneously with the assessment of the intracellular integrity, the morphology and size of the capsular envelope were investigated. In marine snow samples, only bacteria not embedded in the dense matrix of the flocs were considered in order to allow sizing of the capsule and to ensure that the fibrillar material forming the capsular envelope was actually derived from the bacterium. According to size, four categories of capsular envelopes were distinguished. (i) Extensive capsules cover the whole cell surface and are ≥1 cell diameter. (ii) Well-developed capsules are <1 cell diameter and cover more than half of the cell surface. (iii) Poorly developed capsules cover less than half of the cell surface. (iv) For the category of no capsules, cells lacking any capsular envelope were also counted.

The fixation method used in this study has been shown to prevent extraction of fibrillar material (26), therefore avoiding underestimation of the actual size of the capsule.

RESULTS

Intracellular integrity of bacterial cells. Examples of the different degrees of intracellular integrity of bacterioplankton are given in Fig. 1. Figure 1A shows an intact bacterium with all cell compartments, such as the cytoplasmic membrane, nucleoplasm, and cytoplasm. At higher magnification, ribosomes were detectable in all the intact cells. Damaged cells usually lacked an intact cytoplasmic membrane, which was in most cases shrunken or partly deteriorated (Fig. 1B). Empty cells lacked any recognizable plasm and usually a cytoplasmic membrane (Fig. 1C). Occasionally, small amorphous structures were detectable inside the otherwise empty cell wall as shown in Fig. 1C. These cells were also considered empty.

No significant differences were detectable between the two sampling methods for marine snow in terms of distribution of intact, damaged, and empty bacteria in the marine snow-associated bacterial community (Table 1). Differences were found, however, in the distribution of intact and damaged cells in marine snow-associated and free-living bacteria (Table 1).

FIG. 1. Examples of the three different categories used in this study based on the integrity of the internal structure of free-living marine bacteria. (A) Intact cells have an intact cytoplasmic membrane, cytoplasm (grainy structure), and nucleoplasm (fibrillar structure in the middle of the cell). (B) Damaged cell with a shrunken cytoplasmic membrane and only remnants of plasma. (C) In some of the otherwise empty cells, we found small remnants of amorphous structures which have no similarity to plasmatic structures. Scale bar, 100 nm.

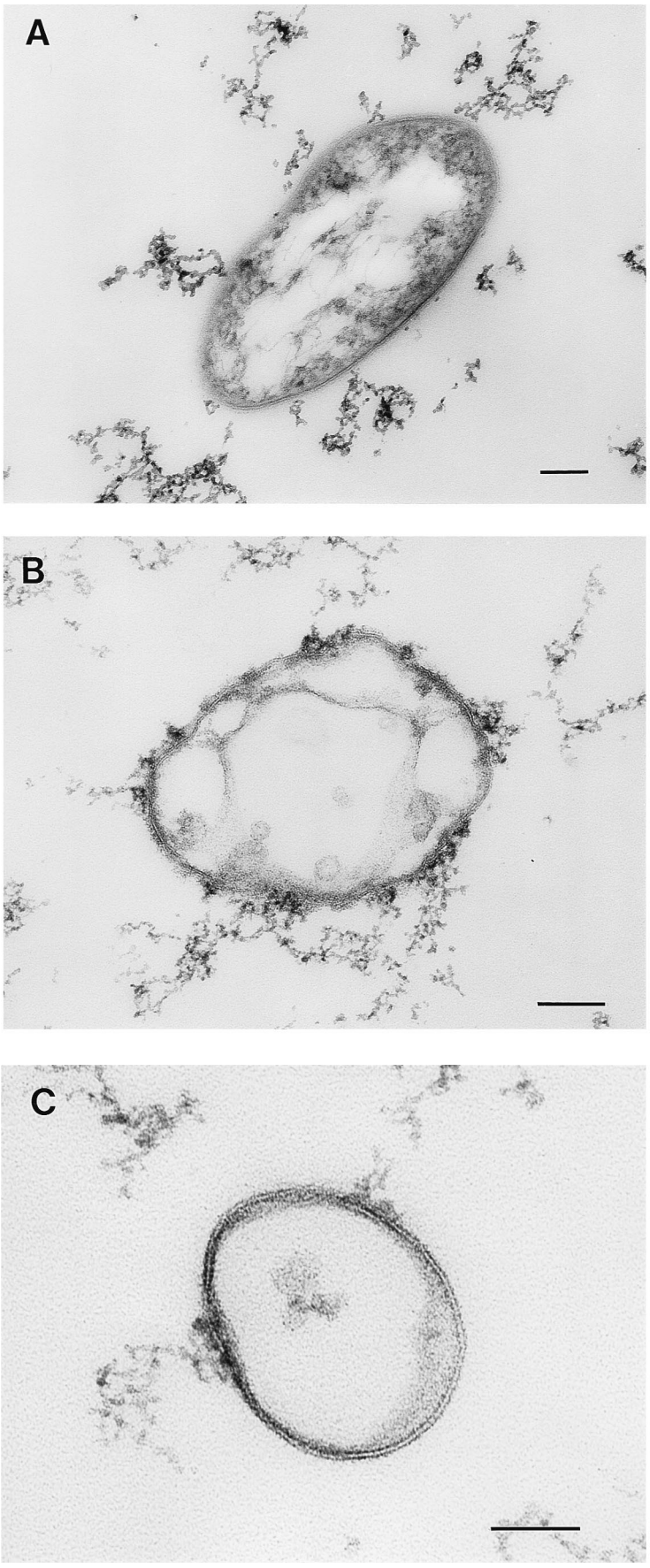
A
B
C

TABLE 1. Distribution of bacteria from natural samples according to their morphological state[a]

Sample	Value for morphological state:					
	Intact		Damaged		Empty	
	%	± SD	%	± SD	%	± SD
AW	34.1	9.8	42.0	5.2	23.9	5.0
MS (syringe)	52.5	2.8	24.0	5.9	23.5	4.5
MS (Niskin bottle)	48.8	4.8	28.7	0.5	22.5	5.3
MS (mean)	51.2	3.5	25.7	4.9	23.1	4.3

[a] For a detailed description of the criteria for morphological states, see Materials and Methods. MS, marine snow; AW, ambient water. Standard deviation is given for $n = 5$ (MS) and $n = 6$ (AW). The total numbers of bacteria counted were 350 for marine snow and 343 for ambient water. The two sampling methods for marine snow are also compared. There was no significant difference between sampling methods at the $P < 0.05$ level (Wilcoxon sign rank test).

While, on average, (51.2 ± 3.5)% of the marine snow-associated bacteria had an intact internal structure, the fraction in free-living bacteria with an intact internal structure was significantly lower ([34.1 ± 9.8]%, Wilcoxon sign rank test, $P < 0.05$). The fraction of damaged cells, however, was significantly lower in marine snow-associated bacteria ([25.7 ± 4.9]%, Wilcoxon sign rank test, $P < 0.05$) compared with the free-living bacteria ([42.0 ± 5.2]%). No significant difference was detectable between free-living and marine snow-associated bacteria in the fraction considered empty (Table 1).

Although the water column was stratified during sampling with a well-developed pycnocline at depths of 12 to 15 m, the internal integrity of the free-living bacterioplankton in different depth horizons of the water column showed only small fluctuations (Fig. 2A). With the exception of those at a depth of 10 m, bacteria with damaged internal structures composed the largest fraction of the bacterial community while the percentage of intact bacteria ranged from 28 to 37% of the total free-living bacterial community (Fig. 2A). Moreover, along the trophic gradient, the variation of the different categories of internal integrity was small (Fig. 2B). Only at the most eutrophic station, which is under the direct influence of the Po River, was the percentage of bacteria equally distributed among the three categories. With only one exception (station 4), damaged bacteria dominated the bacterioplankton community (Fig. 2B).

Capsular envelopes. Large variations in the extent of bacterial capsules and their morphology were detectable. Figure 3 shows some of the more frequently occurring types of capsules in free-living, intact marine bacteria. The extent of the capsular envelope ranges from larger than the actual cell (Fig. 3A) to only a thin coating as shown in Fig. 3F. The morphology of the capsular fibrils also varies considerably, ranging from a loose fibrillar mesh (Fig. 3A) to a dense capsular layer (Fig. 3B).

Remarkable differences in the expression of capsules were noticeable between marine snow-associated and free-living bacteria (Fig. 4) as well as among intact, damaged, and empty cells. While, on average, ≈48% of the intact marine snow-associated bacteria exhibited an extensive capsule (radius of ≥1 cell diameter [Fig. 4A]), only 18% of the intact free-living bacteria exhibited such an extensive capsule (Fig. 4B). The percentage of bacteria lacking any capsule increased from 18% in intact marine snow-associated bacteria to 54% in empty cells (Fig. 4A); in free-living bacterioplankton, the percentage of cells lacking a capsule increased from 34% in intact cells to 80% in empty cells (Fig. 4B).

DISCUSSION

In the present study, we determined the integrity of the internal structures by TEM. Based on our observations, 51.2% of the marine snow-associated bacteria were considered intact while only 34.1% of the free-living bacterioplankton were regarded as intact (Table 1). Most of the bacteria visible in the TEM could be easily assigned to one of the three categories of intracellular integrity. One major drawback of the method used is the time-consuming preparation and examination of the cells. This problem could be, at least partly, solved by concentrating the sample before embedding. Another drawback of this method is that it does not allow direct determination of single cell activity.

We assume that only bacteria with intact internal structures can be potentially active. Comparing the percentage of bacteria with an intact internal structure obtained in this study with other techniques to determine the active fraction of the bacterial community, our data are within the range reported in the literature (Table 2). Considerable variations exist; at present, it is unclear to what extent these differences are caused by using different methods and to what extent these fluctuations reflect the variability in the activity of natural bacterial communities. Douglas et al. (11) have shown that the same sample exhibits a variation of almost 300% depending on the substrate used for microautoradiography to assess the number of active cells. Using the recently reinvented method of rinsing DAPI-stained

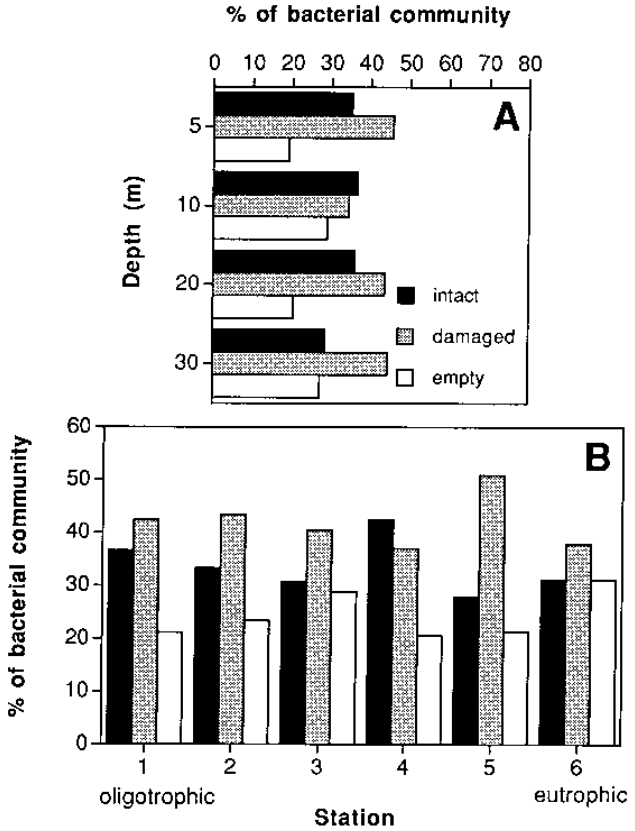

FIG. 2. Distribution of intact, damaged, and empty free-living bacteria in the northern Adriatic Sea. Samples were taken along a trophic gradient from the Croatian coast (station 1, oligotrophic) to the Po River estuary (station 6, eutrophic). Shown is percent distribution of the different categories of internal integrity of bacteria in different depth layers (A) and at different stations along the transect (B). A total of ≈200 bacteria were examined for each depth layer.

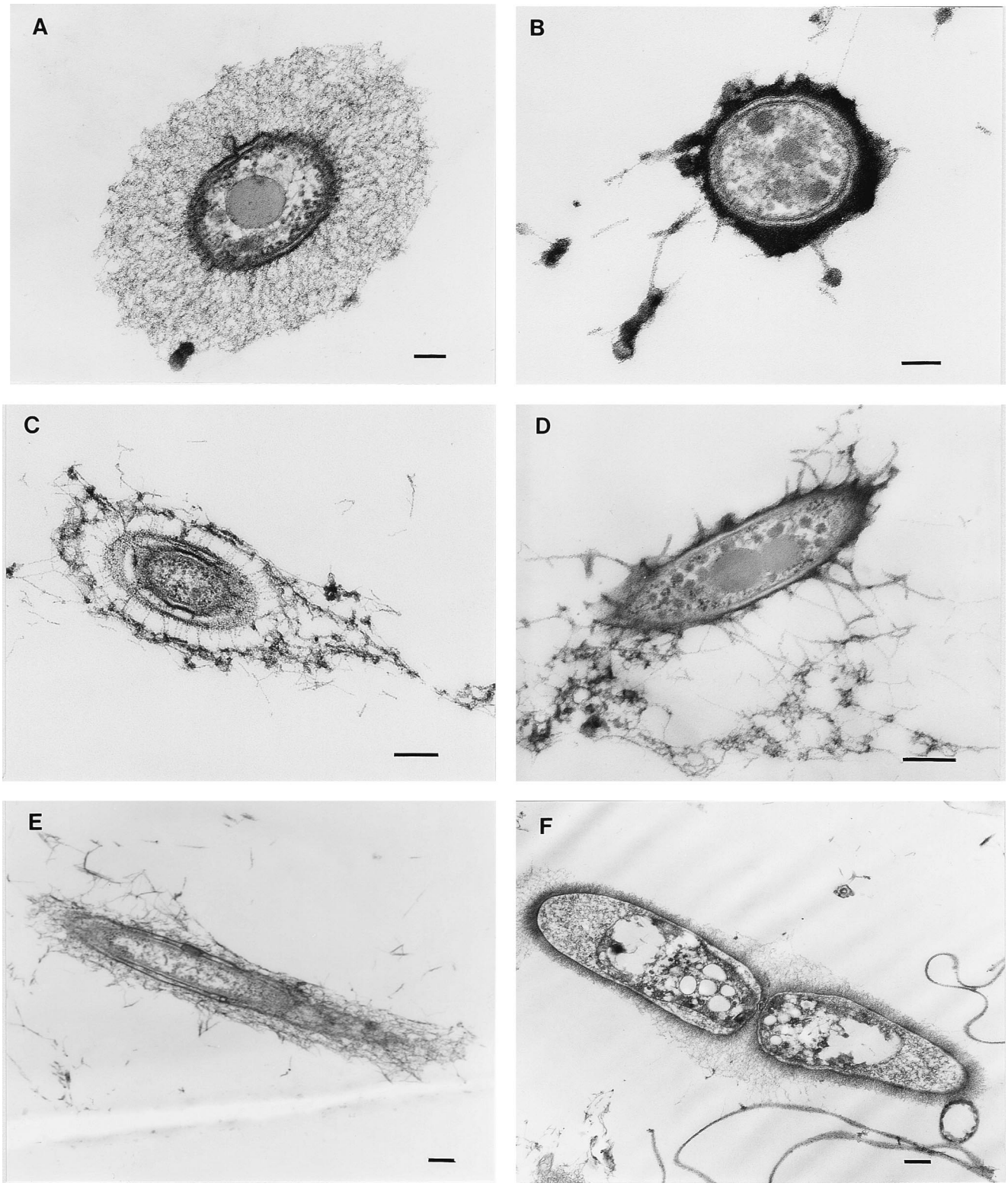

FIG. 3. Examples of the most common types of capsules found in free-living bacteria. Note that the morphology of the capsules varies considerably. The bacteria in panels D and F (a dividing cell) were observed near marine snow particles. Scale bars, 100 nm (A and B) and 200 nm (C to F).

bacterial cells with isopropanol to wash off the nonspecific binding of DAPI (48) in the same area as this study was performed, we arrived at similar numbers for active cells (unpublished data). Our TEM observations indicate that a considerable and remarkably constant fraction (≈23%) of the bacterial community consists of actually empty bacteria with basically only the cell wall remaining (Fig. 1C and 2; Table 1). These empty cells might be remnants from viral lysis or pro-

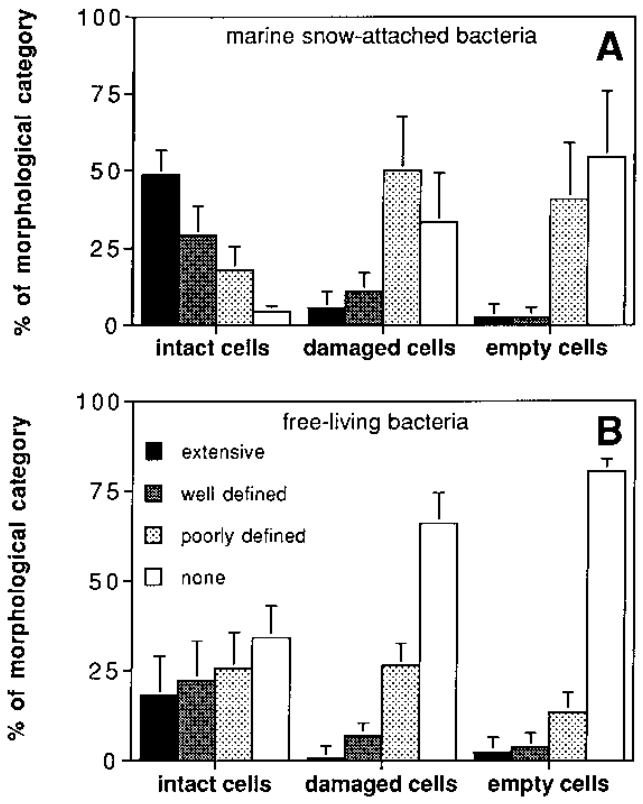

FIG. 4. Distribution of the capsule dimension in marine snow-associated (A) and free-living (B) bacteria among different categories of internal integrity of bacteria. There is a clear trend towards poorly developed capsules or a lack of a capsule in damaged and empty bacteria. Bars represent the mean ± 1 standard error ($n = 5$ for marine snow-associated bacteria; $n = 6$ for free-living bacteria). The total number of bacteria examined was ≈350 for marine snow-associated and free-living bacteria.

tistan grazing; both processes equally control bacterial production (14). Because of the refractory nature of the bacterial cell wall, this component of the bacterial cell will remain in a truly particulate stage for a comparatively long time. Considering the high growth rates of bacteria in aquatic systems, degradation processes related to this detrital matter in the form of empty bacteria deserve more attention. To our knowledge, no information is available on the degradation rates of bacterial cell walls under natural conditions. Only a negligible fraction of these empty cells of the free-living bacterial community are surrounded by a capsular envelope (Fig. 4B), indicating that the capsular material might be either released into the environment or solubilized directly at the cell wall as soon as the bacterial cell becomes inactive or damaged. Currently, experiments are being performed to elucidate the role of capsule production, the release into the environment, and the degradation rates of this exopolymeric material.

The formation of small aggregates called transparent exopolymer particles has received considerable attention (28, 34, 40). The production of exopolymers, which are the main component of transparent exopolymer particles, is attributed mainly to phytoplankton, particularly to diatoms. It has also been shown, however, that many marine particles partially consist of fibrils which are most likely produced by bacteria (33, 36) and that bacteria can produce copious amounts of exopolymers in natural systems (9, 20, 45). This exopolymer production can be induced by the presence of surfaces like inorganic or organic particles (46) and has been shown to be a major mechanism for bacterial attachment to surfaces (5, 7, 13). Measurements of the extent of the bacterial capsule clearly indicated that approximately half of the marine snow-associated bacterial community exhibits a bacterial capsule larger than the actual cell (Fig. 4A). The large extent of the bacterial capsule in marine snow-associated bacteria and the high abundance of bacteria in marine snow (31) might indicate that marine snow-associated bacteria might influence the physical and chemical properties of marine snow. Furthermore, we frequently found evidence that the capsular envelope of bacteria might be one of the initial steps in particle formation. Figure 5 shows the formation of fibrillar networks connecting particles (Fig. 5A) or bacterial cells (Fig. 5B). These microaggregates were frequently detectable in samples from the ambient water.

There is a clear tendency detectable from intact to empty cells (Fig. 4). While in free-living bacteria ≈65% of the intact cells exhibit a capsular envelope, <20% of the empty bacterioplankton cells exhibited a capsular envelope; more than 80% of the empty cells of the free-living bacteria have no capsule. In marine snow-associated bacteria, >95% of the intact cells are surrounded by a capsule while 55% of the empty

TABLE 2. Percentage of active bacteria determined by different techniques

Sample	Location	% Active/intact	Method[a]	Reference
Deep water (400 m)	Weddell Sea	49.2	MAR (leucine)	17
Open water	Weddell Sea	5.4	MAR (leucine)	17
Water above sandy sediments	Kjel Fjord	2.3–56.2	MAR (glucose)	29
Coastal water (8 m)	Nova Scotia	16.5	MAR (thymidine)	11
		46.3	MAR (glutamate)	11
Point B	Mediterranean Sea	20	NUCC	48
SR 50 to 100 m	Baltic Sea	12–27	NUCC	48
0 to 25 m	North Sea	2–5	NUCC	48
Coastal seawater	Newport Beach, Calif.	2.3	CTC	39
Coastal seawater	Mediterranean Sea	6–7	CTC	16
Seawater (8.5 m)	Chesapeake Bay	56–61	INT	44
Seawater (1 m)	Kiel Bight (Baltic Sea)	6–7	INT	47
Freshwater (1 m)	Westensee (North Germany)	23–36	INT	47
Marine snow	Northern Adriatic Sea	51.1	TEM	Present study
Ambient water	Northern Adriatic Sea	34.1	TEM	Present study

[a] MAR, microautoradiography (substrate used is in parentheses); INT, reduction of INT-CTC-formazan due to respiration; NUCC, nucleoid-containing cells determined by staining with DAPI.

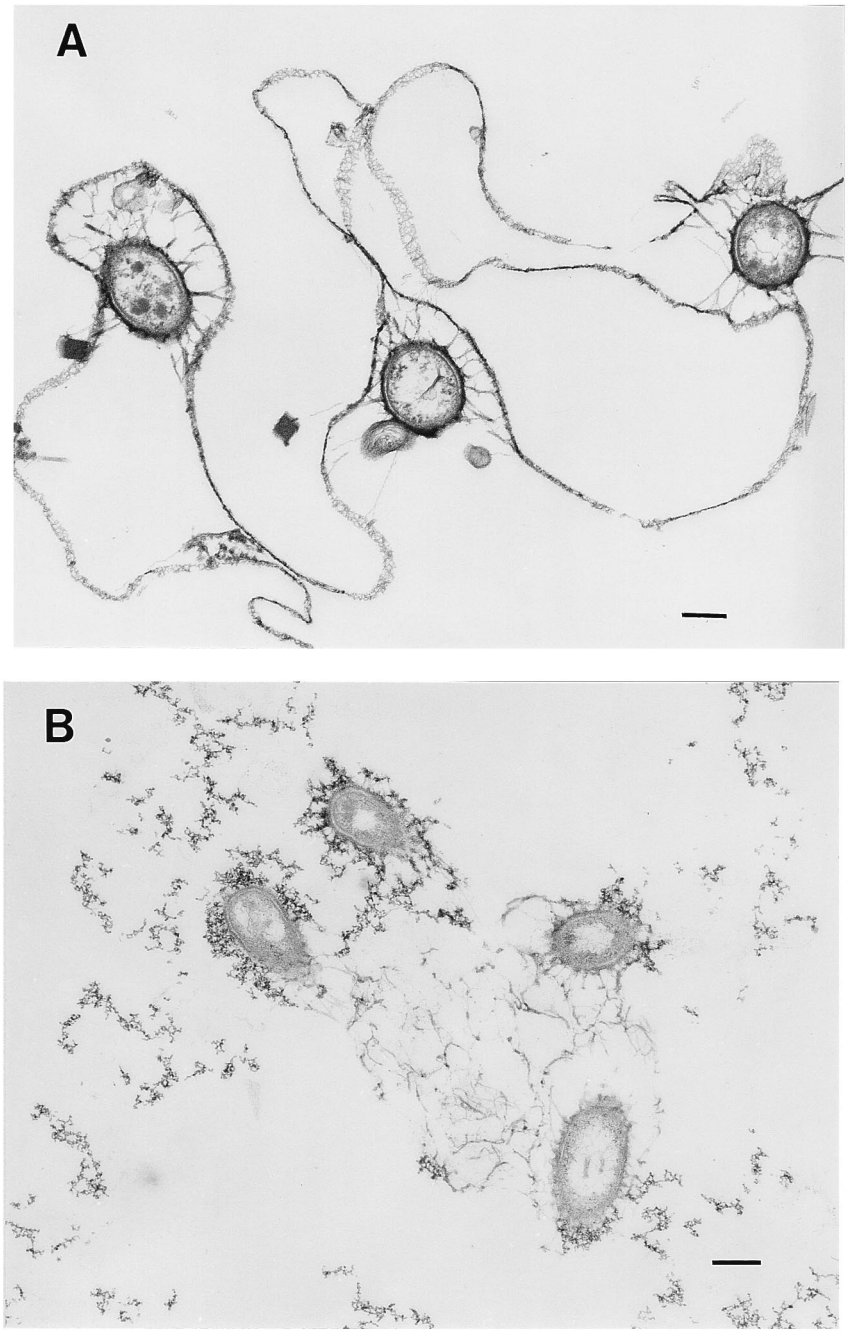

FIG. 5. Electron micrographs showing the ability of bacteria to use fibrillar exopolymers to attach to particles (A) or to other bacterial cells (B). This attachment leads to the formation of small particles extending the surface area of the individual cells. Both micrographs were taken from ambient water samples. Scale bars, 200 nm.

marine snow-associated cells have no capsule. This pattern indicates that (i) the capsular envelope is widely distributed in natural bacterial communities and (ii) upon damage or inactivity of the cell, the capsular layer is lost rapidly as indicated by the increase of bacteria lacking a capsule in the damaged and empty cell categories (Fig. 4).

In summary, we have shown that TEM observations are suitable to estimate the percentage of intact versus damaged or empty bacteria by carefully examining the internal structures and their morphology. The numbers of intact bacteria obtained with this method are comparable to the numbers of active bacteria determined by other methods. We have demonstrated that capsules are common among marine bacteria; particle-associated bacteria exhibit larger capsular envelopes than free-living bacteria. Since bacterioplankton represent the largest living surface in the ocean, we tentatively assume that the release of capsule-derived polysaccharides caused by either cell damage or death represents a major source of DOM especially below the euphotic zone. The fate and ecological significance of this material remain to be investigated.

ACKNOWLEDGMENTS

We are grateful to the staff of the Center for Marine Research (Rovinj, Croatia) and particularly to the captain and crew of the RV *Vila Velebita* for their hospitality and help during sampling. We thank M. Agis and G. Müller-Niklas (University of Vienna) for diving assistance; M. M. West, D. T. Flannigan, and J. Carson (McMaster University) for electron microscope services; and J. N. A. Lott (McMaster University) for providing electron microscope facilities.

Financial support was provided by the National Water Research Institute of Environment Canada to G. G. L., by the Austrian Science Foundation (FWF grants 8608, 9388, and 10974) to G. J. H., and by the European Community (contract no. MAS2-CT94-0090). A portion of this work was sponsored by the New Energy and Industrial Technology Development Organization (NEDO)/Research Institute of Innovative Technology for the Earth (RITE), both of Japan.

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