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Bacteria Associated with a Photosynthetic Dinoflagellate in Culture

C. RAUSCH DE TRAUBENBERG and M.O. SOYER-GOBILLARD*
Département de Biologie Cellulaire et Moléculaire, Observatoire Océanologique de Banyuls, Laboratoire Arago (URA CNRS No. 117), F-66650 Banyuls-sur-mer, France
Tel. 68 88 00 40, Telex 50 50 20 ARAGOBA, Fax 68 88 16 99

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Abstract

The interactions between the dinoflagellate *Prorocentrum micans* E. and its associated bacterial microbiota have been investigated in laboratory cultures. Bacteria attached outside to or located beneath the theca have been detected using scanning and transmission electron microscopy. A taxonomical analysis based on biochemical characterization of the associated microbiota indicates that all culture species belong to the family Pseudomonadaceae and that they are less diverse than seawater bacterial communities. This microbiota consisted of two populations: one is specific to the culture, in which it represents 17% of the bacteria, assimilating a wide range of sugars; the other is similar to seawater bacteria, representing 83% of the microbiota and assimilating only one sugar (glucose). This suggests that sugars are not major exudates released from *Prorocentrum micans*. The exudates which remain uncharacterized enhanced bacterial growth-rate. This suggests the hypothesis that *Prorocentrum micans* can control the bacterial cell density and thus the number attaching to its theca during growth in culture. This control through exudation is low at the beginning of the culture, increases during exponential phase and decreases as the cells age in stationary phase.

Keywords: bacteria, dinoflagellate, culture, exudates, growth-control

*The author to whom correspondence should be addressed

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1. Introduction

Interactions between phytoplankton and bacteria have been studied for many years. The means by which they interact is complex, but it is well known that living phytoplankton release soluble compounds during growth (Guillard and Wangersky, 1958; Chrost and Faust, 1983). These cellular exudates can constitute a significant fraction of marine dissolved organic carbon (Nalewajko et al., 1976) and be used by heterotrophic bacteria (Whittaker and Feeny, 1971; Bell and Lang, 1974; Wolter, 1982). Therefore some bacteria and photosynthetic cells associate together, but this interaction is more complex than a trophic relation from algae to bacteria because some photosynthetic organisms are able to produce antibiotics against bacteria (Sieburth, 1965, 1968; Berland et al., 1974). Also, bacteria release compounds which can be toxic (Berland et al., 1974; Delucca and McCracken, 1978) or beneficial for algae, the latter exemplified by the vitamin B12 that is required by many planktonic algae (Pringsheim, 1912) but that is synthesized by bacteria (Ericson and Lewis, 1953; Haines and Guillard, 1974). Furthermore, heterotrophic bacterial remineralization represents a major supply of limiting nutrients for algae (Golterman, 1972; Bloesh et al., 1977). Competition for these limiting nutrients (N or P) between bacteria and algae is also known: Rhee (1972) demonstrated a competition between *Pseudomonas* and *Scenedesmus* for phosphorus.

The cell biology of the Protoctist *Prorocentrum micans* Ehrenberg, a marine photosynthetic dinoflagellate (Sournia, 1986) is well-known (Herzog et al., 1984; Spector, 1984; Soyer and Herzog, 1985) and there is much molecular information about its genome organization (Maroteaux et al., 1989), phylogenetic position (Lenaers et al., 1989) and ecology (Taylor and Seliger, 1979; Taylor, 1987).

This species has been reported to release over 20% of its primary production as exudates, 90% of which is available for bacteria under natural conditions (Wolter, 1982). *Prorocentrum minimum* E. is also able to produce β dicetone, an antibiotic active against marine bacteria, which reduces their growth rate and inhibits heterotrophic uptake of glucose (Trick et al., 1984). A physical relationship between *Prorocentrum micans* and bacteria has also been observed by Silva (1982) who showed that bacteria may be incorporated into the dinoflagellate's theca. This was interpreted as a probable defensive reaction by the dinoflagellate against the bacteria since it effectively stops bacterial penetration into the cytoplasm.

Prorocentrum micans and its associated bacteria provide a good model for the study of phytoplankton/bacteria interactions. The purpose of this study

was to analyze the interactions between this photosynthetic Protoctist and the surrounding bacteria using a variety of methods in order to further our understanding of this complex relationship.

2. Material and Methods

Culture conditions

Prorocentrum micans strain obtained from the Botany School of Cambridge was grown in modified Erdschreiber's medium with added vitamins (Table 1), under a 12:12 hr light (2000 lux): dark cycle at 20°C. This culture was not axenic.

Preparation for SEM

Samples were collected during late exponential growth and concentrated by gentle centrifugation (800× g for 10 min) or by phototaxis using a light source (100 W at 1 m). Specimens were fixed in Erdschreiber medium with OsO_4 (final concentration 1%) according to the procedure of Soyer et al. (1982).

Preparation for TEM

Samples were collected during late exponential growth and concentrated by light as described above. One g/l of bactopeptone was then added to enhance bacterial growth for 12 hr before fixation. Pellets of specimens were fixed with 0.2 M PIPES-buffered, 2% paraformaldehyde + 1.25% glutaraldehyde and postfixed with 2% OsO_4 according to Karnovsky's procedure as modified by Soyer (1977).

Table 1. Composition of culture medium used for *P. micans* growth in 1000 ml of double-distilled water:

Biotin	0.2 mg
Cyanocobalamin	4.0 mg
Folic acid	0.4 mg
Inositol	1000.0 mg
Nicotinic acid	20.0 mg
Thiamin	100.0 mg
Thymin	600.0 mg

1 ml of the vitamin solution in 1000 ml of Erdschreiber's medium

Isolation and characterization of bacterial strains

Culture microbiota aseptically collected from the *Prorocentrum micans* culture were compared to aseptically collected Mediterranean seawater bacteria (Banyuls-sur-Mer, August 1988, long: 3°16′; lat: 42°23.3 N; at 15 m depth). After appropriate dilutions, bacteria were isolated by the spread plate technique with 2216E medium (Oppenheimer and Zobell, 1952). Twenty-four isolates (of each microbiota assemblage), obtained after an incubation of 7 days at 25°C, were subcultured on 2216E medium for 3 days until the purity and viability of each isolate were assured.

The 27 biochemical and morphological tests (Table 2) of the API 20B system (API system S.A. La Balm les Grottes, 38390 Montalieu Vercieu, France) were used in characterizing these isolates. This system was adapted for heterotrophic bacterial communities characterization as in Baleux (1977).

Numerical taxonomy of bacterial microbiota

Analyses, based on results from the API biochemical and morphological tests, was conducted using a MacIntosh Plus and the program "Progiciel R" written by P. Legendre (1985) according to the numerical taxonomy method of Sneath and Sokal (1963), in Legendre and Legendre (1984). Simple matching coefficients (S1) of Sokal and Michener (1963) were used to cluster the isolates according to the average association of Lance and William (1966a, 1967c) in Legendre and Legendre (1984) (Fig. 4).

Influence of P. micans *exudates on bacterial growth*

Growth of associated microbiota was studied in *P. micans* culture medium aged 5 and 30 days at 20°C in an incubator agitator (Fig. 5). Bacterial inoculum was collected from a late stationary phase *P. micans* culture (20 days after inoculation), after vigorous agitation with a vortex mixer to free attached bacteria. The culture was aseptically filtered through 5 μm membrane filters to eliminate all dinoflagellate cells. Bacteria were enumerated using the spread plate technique on 2216E medium after appropriate dilutions.

Bacterial growth during P. micans *development*

Samples, regularly collected for 60 days from a *P. micans* culture, were preserved with formaldehyde (8% final concentration). Bacteria were stained with DAPI (0.1 μg/ml) filtered onto 0.2 μm Nuclepore filters (Porter and Feig, 1980), observed and counted with an epifluorescence microscope (Polyvar

Table 2. Tests and abbreviations used in API 20B system

Abbreviations	Substrates	Test-reactions (Enzymes)
GEL	Gelatin	Hydrolysis (protease)
NIT	Potassium nitrate	Reduction to nitrites (nitrate reductase)
ONPG	Ortho-nitro-phenol-βgalactopyranosid	Hydrolysis (β galactosidase)
	CARBOHYDRATE UTILIZATION	Aerobic respiration
SAC	Sucrose	Acidification
ARA	L(+) Arabinose	Acidification
MAN	Mannose	Acidification
FRU	Fructose	Acidification
GLU	Glucose	Acidification
MAL	Maltose	Acidification
AMD	Starch	Acidification
RHA	Rhamnose	Acidification
GAL	Galactose	Acidification
MNE	Mannitol	Acidification
SOR	Sorbitol	Acidification
GLY	Glycerol	Acidification
URE	Urea	Urease
IND	Tryptophan	Indol formation
H_2S	Sulfur compounds + iron salts	Aerobic respiration-H_2S formation
VP	Pyruvic acid	Acetoïne production
CIT	Simmons citrate	Alcalinization
OX		Aerobic respiration-Cytochrome oxydase
CAT		Aerobic respiration-catalase
	COMPLEMENTARY TESTS	
OFFF	Glucose (Hugh and Leifson medium)	Oxidation or fermentation
MOB		Motility
GRAM		Gram coloration
OOCC		Morphology
SPOR		Spores present

Reichert). This visual enumeration distinguished free bacteria from those attached to *P. micans*.

3. Results and Discussion

Associated bacteria: EM observations

Bacteria more often appeared to be attached to *P. micans* theca (Fig. 1c) or agglomerated into the dinoflagellate's external mucus (Figs. 1a,b). All bacteria were rod-shaped and numbered, not more than 10 per *P. micans* half-cell (visible surface).

Some EM thin sections contained cross-sectioned bacteria located in 0.3 μm thecal depressions representing approximately half of the thecal thickness (Fig. 2a). The bacterial cell wall and the nucleoid are easily seen. Figures 2b and 2c show thin sections of several bacteria located into vesicles beneath the theca. The *P. micans* vesicular membrane surrounding these bacteria, clearly visible in Fig. 2c (arrows), demonstrates that bacteria are outside of the dense cytoplasm, but located beneath the dinoflagellate theca. These observations show that bacteria are either located upon or within the dinoflagellate theca (Fig. 2a), but seldom are intracellular.

Thin sections of the same pellet showing two different aspects of the bacteria associated with *P. micans* are presented in Fig. 3. The first (Fig. 3a) shows a well-developed nucleoid, abundant ribosomes located in the cytoplasm periphery and a smooth cell wall. The second (Figs. 3B and 3C) appears to have a very dense and granular cytoplasm in which the nucleoid is only visible in a narrow region and a wavy cell wall. Besides the differences of structural organization of the nucleoid and the cell wall in particular, these different bacterial characteristics could also reflect different physiological or metabolic states.

These SEM and TEM observations show the presence of bacteria within and under the dinoflagellate theca or located in a depression that could be interpreted as either a natural conformation or a result of lysis or digestion of the theca. Such depressions are common to the theca of *Prorocentrum* as seen in Fig. 1c. We have never observed bacteria in the dense *P. micans* cytoplasm but they are sometimes located in a clear vesicular region located beneath the theca, as seen in Figs. 2b,c. That vesicle is enclosed by a vesicle membrane (Figs. 2c, double arrow) which separates it from the dense cytoplasm as described in the dinoflagellate *Gyrodinium cohnii* (=*Crypthecodinium cohnii*) (Kubai and Ris, 1969). These observations complement those of Silva (1982) who had shown bacteria within *P. micans* theca. She interpreted this as bacteria partially inhibited by the dinoflagellate as a defense against bacterial penetration.

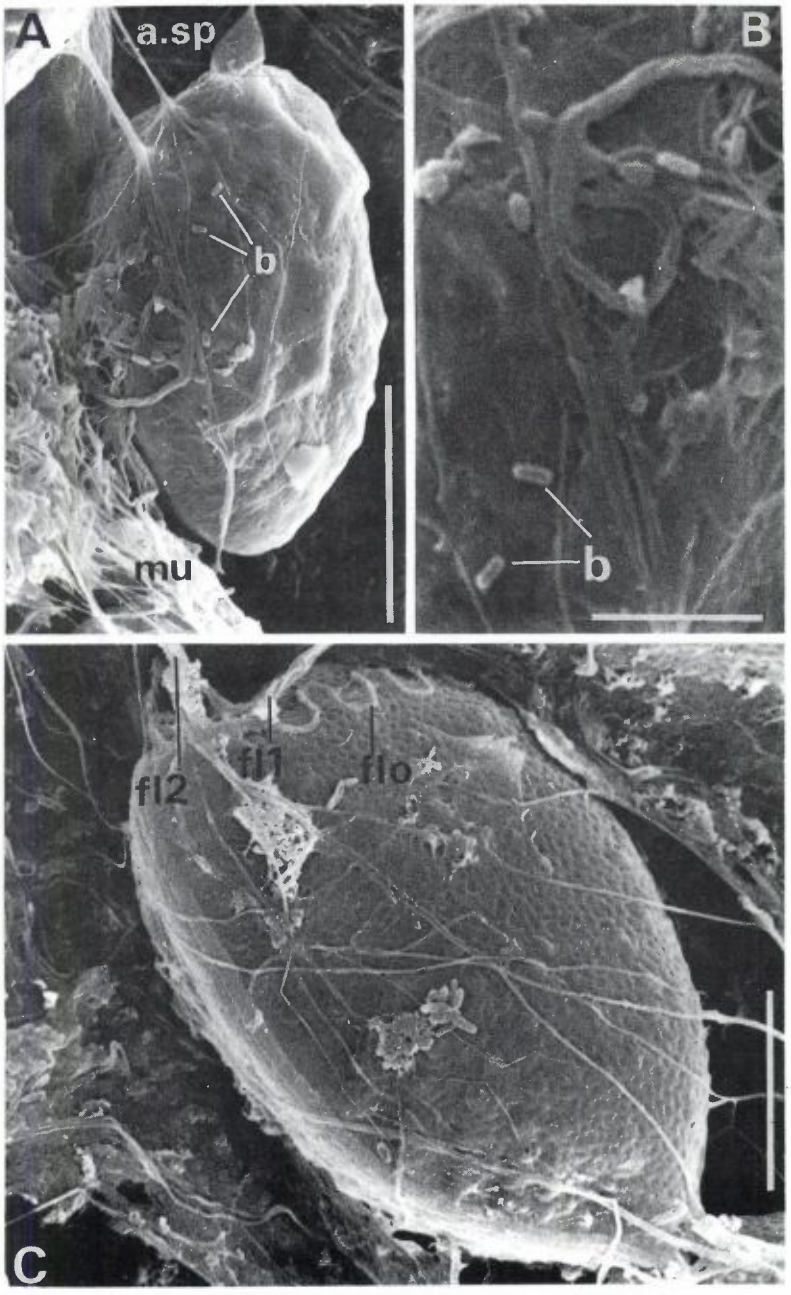


Figure 1. Bacteria fixed on *Prorocentrum micans* E., external SEM view.
(a) Bacteria agglomerated (b) on the dinoflagellate mucus (mu). The apical spine (a.sp) is visible. Bar = 10 μm.
(b) Detail of figure (a). Bar = 3 μm.
(c) *P. micans* theca and associated bacteria. Longitudinal flagella (fl1, fl2) and transverse flagellum (fl.o) are visible. Bar = 5 μm.

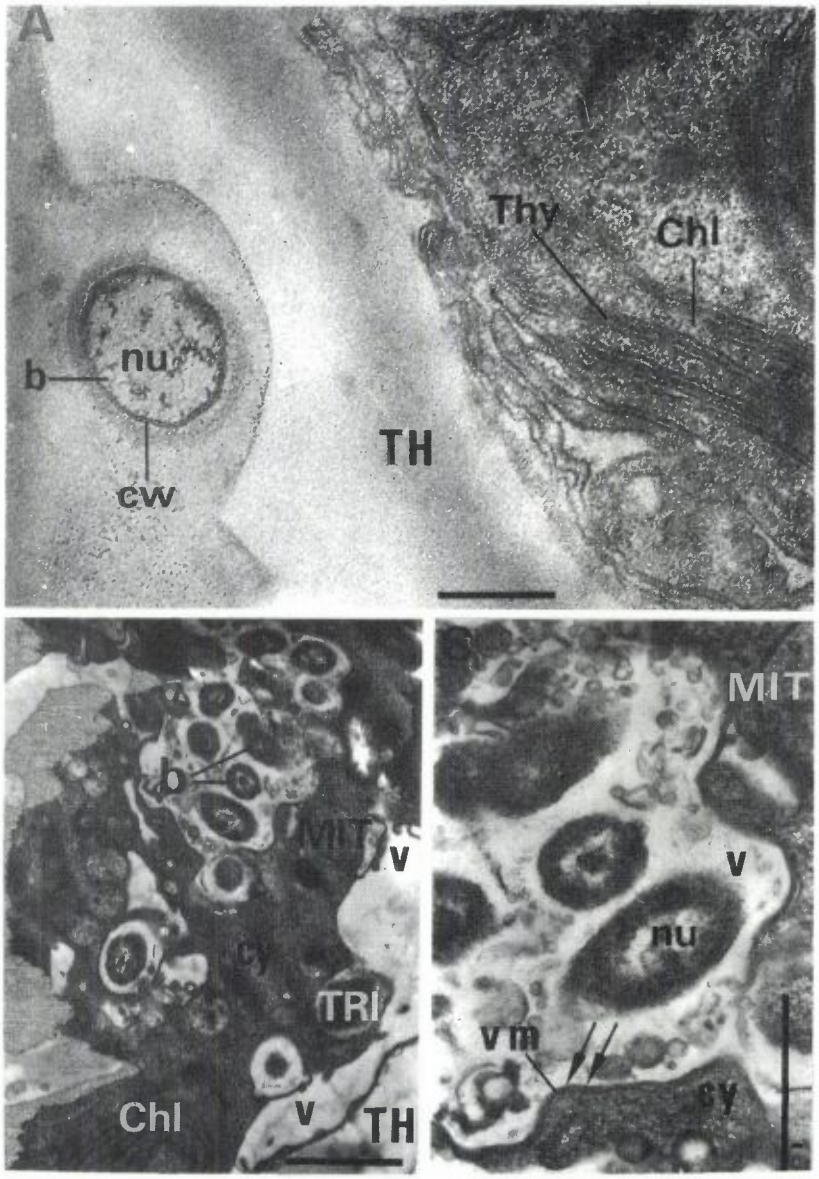


Figure 2. Bacteria associated to *Prorocentrum micans* cells. TEM thin sections
(a) Bacterium (b.) into a depression of the *P. micans* theca: nucleoid (nu), cell wall (cw) and clear cytoplasm. Theca (TH) and chloroplast (Chl) with its thylakoïds (Thy) are visible. Bar = 0.2 μm.
(b) and (c): Views of bacteria (b.) located in a clear vesicle region (v.) of *P. micans*, beneath the theca. Bacteria with their recognizable nucleoïds (nu) are grouped together into vesicle enclosed by a vesicle membrane (v.m., double arrows) and separated from the dark and dense cytoplasm (cy). Mitochondria (MIT), trichocysts (TRI) and chloroplast (Chl) are visible. Bar = 1 μm (b) and 0.5 μm (c).

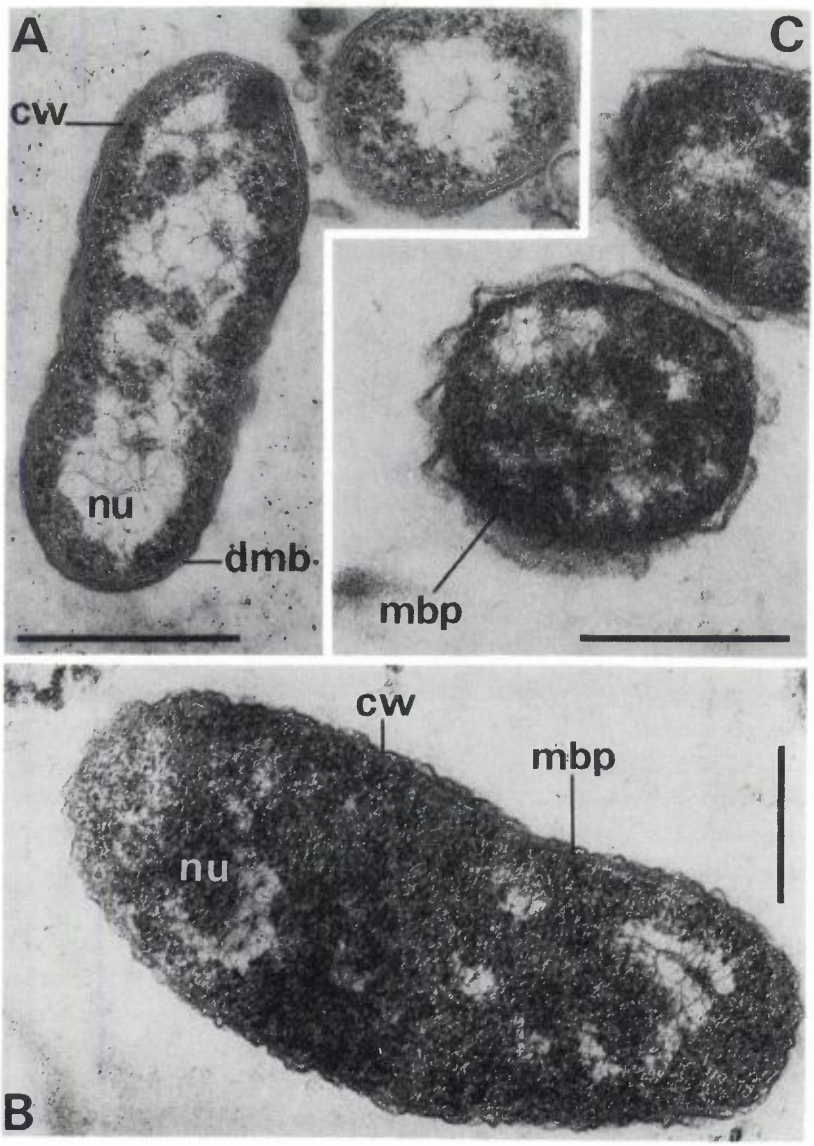


Figure 3. *Prorocentrum micans* free-living associated bacteria. TEM thin sections:
(a) Free-living bacteria with a well-developed nucleoid (nu), smooth cell wall (cw) and abundant ribosomes, dmb = double membrane. Bar = 1 μm.
(b) and (c) Free-living bacteria with a very granular cytoplasm, a less developed nucleoid (nu) and a cell-wall (cw) which appears to be wavy; mbp = plasmic membrane. Bar = 0.3 μm (b) and 0.4 μm (c).

Characterization and numerical taxonomy of the bacterial strains

API 20B tests have shown that all the *P. micans* culture bacterial isolates were Gram-negative rods, oxidase positive, motile, non glucose fermentative, which are all the *Pseudomonadaceae* family characteristics. This culture community, composed of 6 different strains, was less diverse than our seawater samples, which contained 18 different strains of bacteria.

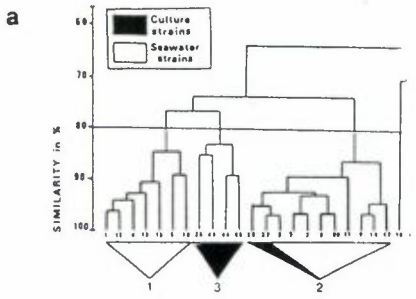
The cluster diagram in Fig. 4a clarifies the relationship between the two bacterial communities. A total of 96% of the isolates cluster at an 80% level of similarity, with only two seawater isolates (Refs. 18 and 19) failing to cluster at this level. Only *P. micans* associated bacteria are found in cluster 3; 2 other strains are grouped with seawater bacteria in cluster 2. Cluster 1 contains only seawater bacteria.

The biochemical characteristics of the three bacterial clusters are shown in Fig. 4b and sugar uptake seems to be the most discriminant character. Cluster 1 strains utilize sugars (100%) as mannose (MAN), fructose (FRU) and glucose (GLU); cluster 2 strains assimilate only glucose at a low level (20%) and cluster 3 isolates assimilate a wide range of sugars except starch (AMD).

Bacteria quantitative distribution into these different clusters is represented in Fig. 4c. This taxonomic analysis shows that *P. micans*-associated bacteria are less diverse than the surrounding seawater community. They belong to the Pseudomonadaceae family, the predominant family reported in temperate oceans (Bianchi, 1981). The *P. micans*-associated bacteria community grouped into two clusters. One (cluster 3) is composed of 17% of the 24 strains isolated from *P. micans* culture. These strains assimilated a wide range of sugars. The second cluster (2) contains strains more similar to seawater bacteria. These strains represented 83% of the remaining microbiota (i.e. 83% of the 24 strains isolated from the *P. micans* culture) and assimilate only glucose (GLU) as a sugar. If one accepts that there should be a good correlation between bacterial uptake capability and the nutrient supply to which they are adapted (Delille, 1990), these results suggest that sugars (aside from glucose) do not constitute a major portion of *P. micans* exudates.

Influence of P. micans *exudates on bacterial growth*

Figure 5 shows bacterial growth in three different media: sterile Erdschreiber medium with added vitamins and no *P. micans* exudates (culture A); *P. micans* culture medium-aged 5 days (culture B) and aged 30 days (culture C) are all represented by exponential curves. Growth curves of bacteria in medium



c

	CULTURE	SEAWATER
CLUSTER 1	0%	37.5%
CLUSTER 2	83%	54 %
CLUSTER 3	17%	0 %

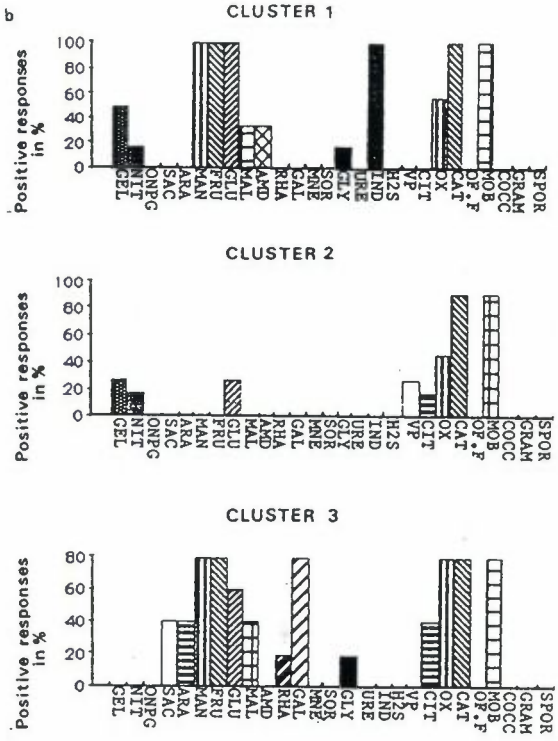


Figure 4. Taxonomic analysis based on API 20B system.

(a) Diagram representing clustering of the 6 different bacterial strains isolated from the 24 total *P. micans* isolates and the 18 different strains from the 24 total seawater isolates. Numbers 1,2 and 3 represent the different clusters.

(b) Biochemical characteristics of the three bacterial clusters: the percentage of positive reactions for each character in each cluster is noted.

(c) Quantitative distribution of 24 culture isolates and 24 isolates of sea water into the three clusters: Isolates of the culture are shared out in cluster 2 (83%) and in cluster 3 (17%). Isolates of seawater are shared out in cluster 1 (37.5%) and in cluster 2 (54%). The residual 8.5% are represented by the strains no. 18 and 19 (see Fig. 4a).

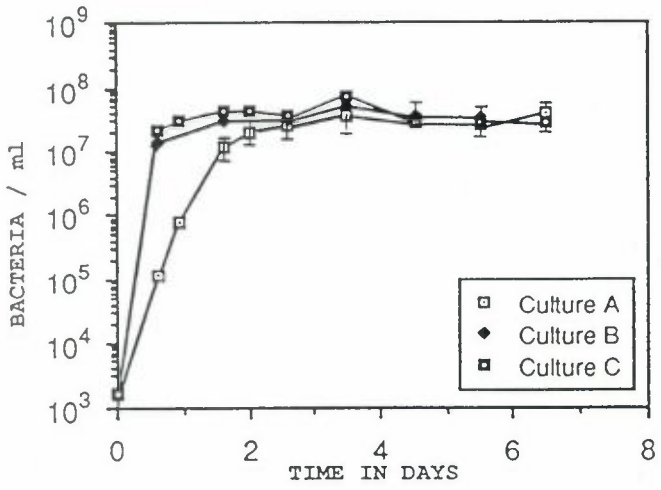


Figure 5. Influence of *Prorocentrum micans* exudates on the growth of associated bacteria. The culture media are: Sterile Erdschreiber medium with added vitamins (culture A); sterile 5 day old *P. micans* culture medium filtrate (culture B) and sterile 30 day old *P. micans* culture medium filtrate (culture C). Only culture B and C contain *P. micans* exudates. Culture A growth curve slope is 1/4 of cultures B and C, although the maximum bacterial density is similar for the three cultures, (n=3).

containing *P. micans* exudates (cultures B and C) are very similar. The growth curve of bacteria in the absence of these exudates (culture A) has a slope 1/4 as steep as the two others. Nevertheless, maximum bacterial density was very similar for the three culture conditions. Thus, *P. micans* exudates enhance the growth rate of associated bacteria, but have no effect on the maximum bacterial density.

Bacterial growth during P. micans *development*

As seen in the curves represented in Fig. 6, associated fluctuations in bacterial density seem to be correlated with the *P. micans* growth stage. During early exponential growth of *P. micans* (days 1–4), bacterial concentrations increased 6-fold (from 4×10^6 to 2.6×10^7 bacteria/ml). At the end of exponential growth of *P. micans*, bacterial density decreased continuously 5-fold (to 5.6×10^6 bacteria/ml) and remained constant through stationary stage. Only when *P. micans* entered senescence (days 40–60) bacterial concentrations increase again. This pattern suggests *P. micans* control of bacterial density. This control could be a defensive mechanism that reduces bacterial numbers

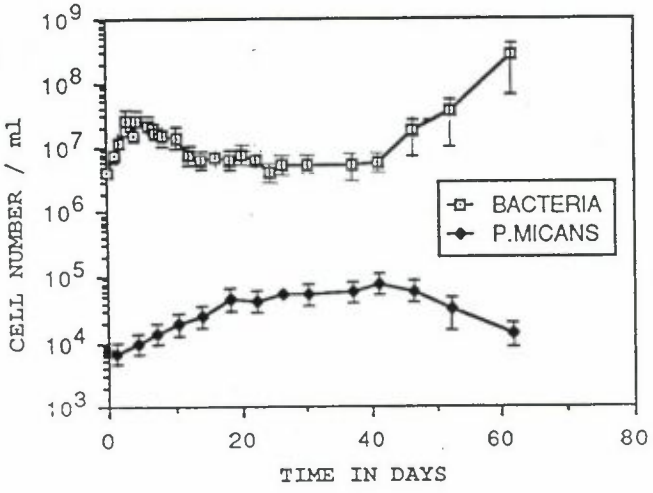


Figure 6. Bacterial growth during *Prorocentrum micans* development. Fluctuations in bacterial density seem to be correlated with *P. micans* growth phase, (n=3).

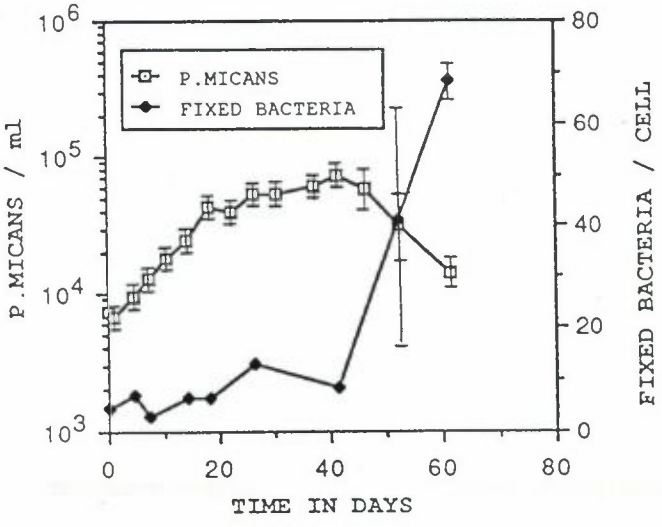


Figure 7. Bacterial colonization of *Prorocentrum micans* theca during dinoflagellate growth. The number of living bacteria attached to *P. micans* correlates to the *P. micans* growth phase, (n=3). The large standard deviation observed at 52 days (41 attached bacteria/cell) is due to an unwedging of the growth phase of one of the three *P. micans* cultures.

in order to limit natural infection and fouling (Sieburth, 1965). This regulatory property is low in early exponential growth (perhaps due to the low *P. micans* cell density) and then increases dramatically, stabilizing during stationary stage. As the *P. micans* cells age, the control is no longer effective. This hypothesis is in accordance with Trick et al. (1984) who demonstrated that *P. minimum* can produce an antibiotic (β dicetone) that can reduce bacterial abundance and then inhibit degradation of algae by associated bacteria. However, the hypothesis of a competition between *P. micans* and its associated bacteria cannot be excluded.

Bacterial colonization of P. micans *theca*

As seen in Fig. 7, direct counting permitted the enumeration of *P. micans* and attached bacteria. As before, fluctuations in the number of attached bacteria seem to correlate with the growth stage of the dinoflagellate: during exponential and stationary growth, colonization was low and approximately constant (5 to 10 bacterial/*P. micans* cell). As the senescence phase began, colonization increased and reached 70 bacteria/*P. micans* cell. This analysis leads to the same hypothesis presented above for dinoflagellate control of bacterial density: *P. micans* is able to limit bacterial attachment to its theca during exponential and stationary growth, but this ability decreases as the cells age.

Since *P. micans* seems to be controlling thecal attachment and cell penetration of its associated bacteria, the bacteria might be considered as parasites. However, it is well known that algae-associated bacteria are able to release vitamins (Ericson and Lewis, 1953; Haines and Guillard, 1974) and remineralize nutrients (Golterman, 1972; Bloesh et al., 1977; Axel et al., 1981) both of which directly benefit the algae. Thus, the interactions described above seem more to be a balance between inhibitions and stimulations with reciprocal exchanges that could be the first manifestation of symbiosis.

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