Environmental Microbiology (2001) **3**(6), 380–396

Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures

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Summary

Dinoflagellates (Eukaryota; Alveolata; Dinophyceae) are single-cell eukaryotic microorganisms implicated in many toxic outbreaks in the marine and estuarine environment. Co-existing with dinoflagellate communities are bacterial assemblages that undergo changes in species composition, compete for nutrients and produce bioactive compounds, including toxins. As part of an investigation to understand the role of the bacteria in dinoflagellate physiology and toxigenesis, we have characterized the bacterial community associated with laboratory cultures of four '*Pfiesteria*-like' dinoflagellates isolated from 1997 fish killing events in Chesapeake Bay. A polymerase chain reaction with oligonucleotide primers specific to prokaryotic 16S rDNA gene sequences was used to characterize the total bacterial population, including culturable and non-culturable species, as well as possible endosymbiotic bacteria. The results indicate a diverse group of over 30 bacteria species co-existing in the dinoflagellate cultures. The broad phylogenetic types of dinoflagellate-associated bacteria were generally similar, although not identical, to those bacterial types found in association with other harmful algal species. Dinoflagellates were made axenic, and the culturable bacteria were added back to determine the contribution of the bacteria to dinoflagellate growth. Confocal scanning laser fluorescence microscopy with 16S rDNA probes was used to demonstrate a physical association of a subset of the bacteria and the dinoflagellate cells. These data point to a key component in the bacterial community being species in the marine alpha-proteobacteria group, most closely associated with the α-3 or SAR83 cluster.

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Received 20 February, 2001; revised 14 May, 2001; accepted 21 May, 2001.

Introduction

Reports of population increases in phytoplankton species, also known as harmful algal blooms (HABs), are increasing in frequency along the coastal regions of many oceans around the world. Many of these microorganisms are single-cell dinoflagellate species that produce toxic conditions or 'red tides' that may cause the death of aquatic animals, e.g. fish kills, and may be harmful to human health (Baden, 1983; Fleming *et al*., 1999).

Pfiesteria piscicida (Dinophyceae; Pfiesteriaceae) is a heterotrophic dinoflagellate (Steidinger *et al*., 1996) that has been implicated in fish deaths both in nature (Burkholder *et al*., 1992; 1995a) and in the laboratory (Noga *et al*., 1993). *P. piscicida* is considered a phantom ambush predator dinoflagellate, because it appears to lie in wait to prey on fish species (Burkholder *et al*., 1992). Reports have linked *P. piscicida* as the cause of massive fish deaths along the Atlantic Coast of the United States, especially in the estuaries of Pamlico Sound, North Carolina and the Chesapeake Bay of Maryland and Virginia (Burkholder *et al*., 1992; Burkholder, 1999). There are two hallmarks of the massive fish mortalities caused by *P. piscicida*. They tend to occur in populations of Atlantic menhaden (*Brevoortia tyrannus*), and affected fish frequently have large, external lesions and ulcers, presumably caused by an unknown toxin produced by the dinoflagellate (Glasgow *et al*., 1995; Burkholder and Glasgow, 1997a).

In addition to *P. piscicida*, other species of dinoflagellates have also been found associated with these fish kills. These dinoflagellates, which are similar in morphology and behaviour to *P. piscicida*, have been termed the *Pfiesteria* complex dinoflagellates or *Pfiesteria*-like dinoflagellates (Oldach *et al*., 1998). They include members of the recently named species *Pfiesteria shumwayae* and the genus *Cryptoperidiniopsis* (Steidinger *et al*., 1997a,b) as well as other, unclassified species with colloquial names such as the 'shepherd's crook' strain.

Human health problems, including respiratory problems, skin rashes or irritation and short-term memory loss, have also been linked to exposure to water with high levels of *P. piscicida* (Glasgow *et al*., 1995; Levin *et al*., 1997; 1999; Bever *et al*., 1998; Grattan *et al*., 1998a,b). This suggests that the same toxin or toxins affecting fish may also affect humans. Although the identity of the *P. piscicida* toxin(s) is still unknown, evidence suggests that

the toxins are synthesized or released in response to the presence of live fish (Burkholder *et al*., 1995b; Burkholder and Glasgow, 1997a). At least 24 different life forms or stages, ranging from flagellated zoospores and amoeboid forms to dormant cyst stages, have been reported for *P. piscicida and Pfiesteria*-like dinoflagellates (Burkholder *et al*., 1995b; Steidinger *et al*., 1996), some of which apparently do not produce toxin. Adding to a complicated life cycle are reports (Burkholder and Glasgow, 1997a) that, in the laboratory, cultures of *P. piscicida* lose toxicity over time, suggesting that some critical factor required for toxin production may be lost upon laboratory cultivation in fish bioassays (Marshall *et al*., 2000).

Dinoflagellate cells do not exist autonomously in the estuary, but rather co-exist with many other microorganisms that interact with the dinoflagellates in multiple ways to produce a dynamic microbial ecosystem. In shear numbers, bacterial assemblages are a major part of this microbial community, existing as epiphytes, endosymbionts and free-living bacteria that may be a food source for grazing dinoflagellates (Doucette, 1995). As part of the dinoflagellate community, natural bacterial assemblages change in species composition, compete for nutrients and produce bioactive compounds, which may be toxic to higher organisms. Indeed, one of the unresolved hypotheses in HAB research is the possibility that members of the bacterial community may be responsible for either directly producing some of the toxins or modifying those toxins of dinoflagellate origin (Sousa-Silva, 1990; Lafay *et al*., 1995; Gallacher *et al*., 1997).

Our laboratory is interested in understanding the impact of the bacterial community on the physiology and toxigenesis of *P. piscicida* and related dinoflagellates. In this report, we have characterized the community of bacteria associated with laboratory cultures of *P. piscicida* and three other *Pfiesteria*-like dinoflagellates from fish kill sites in the Chesapeake Bay area. Using two different approaches, we have demonstrated the specificity of the interaction between a subset of the bacterial population and the dinoflagellate host cell and demonstrate an apparent bacterial–dinoflagellate association that may benefit dinoflagellate growth and physiology.

Results

We first characterized the bacterial communities in four *Pfiesteria*-like dinoflagellate laboratory cultures. These cultures were maintained with axenic *Rhodomonas* prey algae, and all work with these cultures performed using laminar-flow Biosafety level III containment hoods to eliminate contamination from either airborne or human skin bacterial contaminants and preserve the stable bacterial community in these cultures that were originally derived from Chesapeake Bay.

The taxonomic type strain of *P. piscicida* was obtained as a gift from K. Steidinger (Florida Marine Research Institute) and isolated from a 1996 fish-killing event in a striped bass (*Morone saxatilis*) aquaculture pond near the Manokin River on the eastern shore of Chesapeake Bay. In addition to *P. piscicida*, three other dinoflagellate cultures were obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP; West Boothbay Harbor, ME, USA). CCMP1828 and CCMP1829 were collected from fish kill sites in Chesapeake Bay during the summer of 1997 *Pfiesteria* outbreaks. These cultures, as well as *P. piscicida*, are populated by a subset of bacteria derived from the initial sample sites, whereas CCMP1827 is an axenic dinoflagellate culture (i.e. free of bacteria) derived from CCMP1828. Although none of the three CCMP strains has been characterized taxonomically by thecal plate morphology (Steidinger *et al*., 1996), CCMP1827 and CCMP1828 are considered to be related to the genus *Cryptoperidiniopsis* (Steidinger *et al*., 1997a,b). CCMP1829, also known as 'shepherd's crook', is an unnamed genus of dinoflagellate that bears taxonomic similarity to *P. piscicida* (W. Coates, personal communication).

As indicated above, of the four *Pfiesteria*-like dinoflagellate cultures analysed in our study, only one (CCMP1827) was considered to be axenic by CCMP. The other three cultures, CCMP1828, CCMP1829 and *P. piscicida*, were bacterized, a term used to indicate that they contained a bacterial community derived from the original environmental samples, in this case from Chesapeake Bay water. Interestingly, we isolated bacterial colonies after inoculating 0.5× marine 2216 agar with samples of CCMP1827, the axenic strain of *Cryptoperidiniopsis*. These colonies grew very slowly and appeared after 10 days of incubation at 20°C, suggesting that they might have been overlooked in earlier evaluations. Because CCMP1827 was not axenic and derived from CCMP1828, we analysed all bacterial 16S rDNA clones from these two *Cryptoperidiniopsis* strains as one.

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the bacterial community

To obtain an unbiased characterization of the bacteria associated with these *Pfiesteria*-like dinoflagellates, we amplified the 16S rDNA genes from both the culturable bacteria (i.e. bacterial colonies obtained from incubation on 0.5× marine 2216 agar) and directly from the dinoflagellate culture (i.e. from cell pellets obtained after centrifugation). The 16S rDNA fragments were then cloned, and the resulting transformants were screened by digesting the plasmids separately with *Hae*III *and Hha*I

Table 1. RFLP patterns and 16S rDNA sequence similarities of 16S clones from *P. piscicida,* CCMP1827, CCMP1828, and CCMP1829 cultures.

RFLP group	Representative clone[a]	*Hae*III fragments (bp)	*Hha*I fragments (bp)	Nearest neighbour classification	Similarity (%)	GenBank accession no.
Alpha-Proteobacteria						
1	KE27A33*	19, 115, 158, 282, 295	59, 139, 276, 395	Marine bacterium SRF3	94	AF188164
2	KEPPIB5*	19, 158, 282, 410	59, 139, 276, 395	*Crassostrea virginica* symbiont CV919-312	96	AF188165
3	KEPPIB19	19, 34, 179, 282, 376	38, 61, 101, 184, 209, 297	Unidentified α-proteobacterium OM75	94	AF188166
4	KEPPIB37	19, 179, 279, 410	59, 115, 137, 280, 296	*Erythrobacter* str. MBIC 3019	99	AF188167
5	RSPPIC2*	19, 179, 279, 407	56, 253, 282, 296	*Devosia riboflavina*	97	AF190222
6	MA2801	18, 58, 179, 223, 408	139, 295, 452	RFLP 7/*Devosia riboflavina*	90	AF186697
7	MA2802*	19, 179, 281, 410	139, 296, 454	*RFLP 6*/Devosia riboflavina	89	AF186698
8	MA2805*	19, 79, 158, 199, 410	59, 135, 276, 395	*Roseobacter algicola*	97	AF186699
9	MA2824*	19, 158, 278, 410	135, 276, 454	*Sulfitobacter pontiacus*	99	AF186700
10	MA2827*	19, 34, 158, 283, 376	59, 110, 123, 167, 411	RFLP 15/*Hyphomonas jannaschiana*	93	AF186701
11	MA2830*	19, 179, 282, 409	59, 115, 139, 279, 297	*Phyllobacterium myrsinacearum*	99	AF186702
12	MA2832*	19, 179, 279, 410	58, 129, 137, 167, 396	'*Agrobacterium' sanguineum*	99	AF186704
13	RS29A8	19, 34, 179, 281, 375	139, 208, 245, 296	*Azospirillum lipoferum*	90	AF190209
14	RS29AI4*	19, 115, 158, 279, 295	59, 135, 277, 395	Unidentified α-proteobacterium GAI-5	97	AF190210
15	RSHD3E4	19, 34, 158, 283, 376	59, 123, 277, 411	RFLP 10/*Caulobacter* str. MCS33	91	AF190211
16	RSHD3S16*	19, 179, 282, 411	59, 116, 139 (2), 157, 281	*Sphingomonas subarctica*	98	AF190212
17	RSHD3S8*	19, 111, 171, 179, 410	59, 115, 139, 280, 297	*Agrobacterium* str. LMG 11915	98	AF190213
18	RSHD3S10*	19, 179, 282, 410	59, 115, 139, 280, 297	*Phyllobacterium myrsinacearum*	96	AF190214
Beta-Proteobacteria						
19	KEPPI37	9, 19, 59, 172, 218, 412	63, 298, 528	Unidentified β-proteobacterium OM43	94	AF188168
20	RS29AI-1*	19, 180, 308, 376	63, 292, 528	*Leptothrix discophora*	95	AF190215
21	RSHD3S6*	48, 149, 684	63, 290, 528	Uncultured proteobacterium OCS7	96	AF190216
Gamma-Proteobacteria						
22	RSHD3S12*	19, 180, 187, 224, 277	59, 239, 292, 297	*Alteromonas macleodii*	97	AF190217
23	KEPPIB7	19, 34, 68, 180, 212, 377	59, 298, 533	*Ornithidoros moubata* symbiote A	89	AF188169
24	MA2833*	9, 19, 59, 172, 220, 414	245, 300, 348	*Pseudomonas hibiscicola*	99	AF186705
25	RS29A27	9, 19, 34, 59, 70, 150, 171, 376	32, 59, 130, 137, 249, 281	*Marinobacter hydrocarbonoclasticus*	99	AF190218
26	MA2831*	19, 180, 195, 216, 279	59, 250, 281, 299	*Curacaobacter baltica*	100	AF186703
27	RSHD3S15*	9, 19, 34, 59, 171, 220, 377	59, 250, 281, 299	*Pseudomonas oleovorans*	99	AF190219
Cytophagales						
28	KEPPI47	19, 181, 277, 408	300, 585	RFLP 30/*Sphingobacterium spiritivorum*	86	AF188170
29	KEPPIB30	10, 19, 80, 101, 102, 115, 180, 277	103, 130, 169, 482	*Cytophaga* str. BD1-15	85	AF188171
30	KEPPIB35	19, 69, 112, 274, 408	300, 582	*RFLP 28*/*Sphingobacterium spiritivorum*	86	AF188172
31	KEPPIB22	200, 683	14, 38, 189, 642	*Microscilla furvescens*	89	AF188173
32	KEPPIB23	300, 336, 350	14, 38, 59, 78, 111, 586	Uncultured bacterium CLEAR 1	95	AF188174
33	KEPPIB45	15, 19, 20, 145, 337, 351	130, 169, 588	*Flexibacter maritimus*	90	AF188175
34	RSHD3B4	15, 19, 71, 81, 165, 195, 267	299, 587	'*Cytophaga' ulginosa*	94	AF190220
Gram-positive, high G+C group						
35	RSPPID3*	19, 34, 81, 146, 154, 225, 231	25, 53, 125, 182, 230, 275	*Curtobacterium luteum*	99	AF190221
36	KE27CII4*	19, 34, 59, 95, 146, 225, 313	182, 300, 409	*Microbacterium terrae*	96	AF188176

a. An asterisk indicates that this RFLP group clone is derived from a culturable bacterial species.

restriction endonucleases, producing a set of DNA fragments that could be used for RFLP analysis and the subsequent clustering of similar clones. The results of the RFLP analysis of the bacteria associated with these four *Pfiesteria*-like dinoflagellates are shown in Table 1. A total of 159 transformants was obtained from both gene clones and cell clones. From these, 36 clones (23% of the transformants) were identified as chloroplast DNA. This

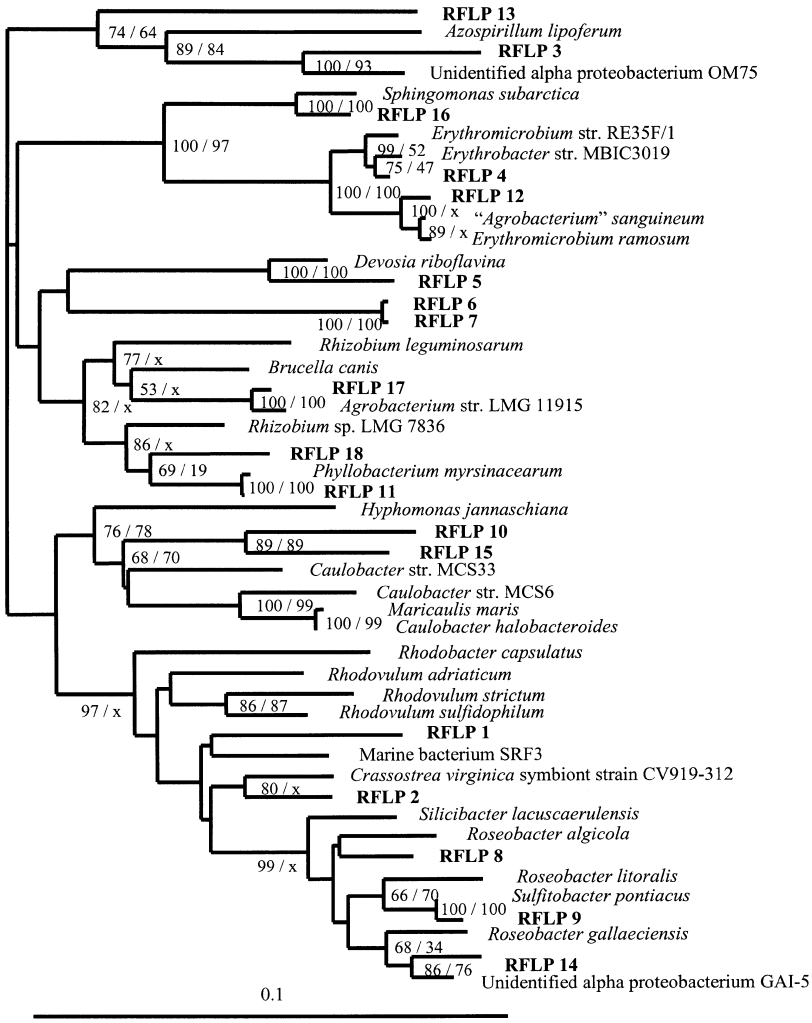

Fig. 1. Phylogenetic tree inferred from comparative sequence analysis of partial 16S rDNA generated by the neighbour-joining method and Jukes–Cantor distance algorithm showing the relationships between the bacteria associated with *Pfiesteria*-like dinoflagellates and sequences obtained from the GenBank database related to the α-Proteobacteria. Bootstrap values ($n = 100$ replicate resamplings) for branches are indicated for both neighbour-joining and parsimony methods (neighbour-joining bootstrap value/parsimony bootstrap value) as an aid to determining the efficacy of each node. Bootstrap values are only presented for neighbour-joining nodes of 50 or more. An 'x' represents a branch that was not present in the tree constructed through the parsimony method. The bar represents 0.1 U of evolutionary distance.

was expected, as the dinoflagellates are fed *Rhodomonas* algal prey. The chloroplast clones and another four clones determined to be chimeric sequences were culled from further consideration, resulting in a clone library of 119 clones (27 from CCMP1827/1828, 63 from CCMP1829 and 29 from *P. piscicida*), comprising 36 unique RFLP groups. As seen in Table 1, the most commonly found bacteria were members of the α-subdivision of the Proteobacteria accounting for 50% (18 out of 36) of the RFLP groups. The Cytophagales (*Flexibacter*, *Cytophaga* and *Bacteroides*) subdivision comprised 19% (seven out of 36) of the RFLP patterns, followed by the γ-Proteobacteria at 17% (six out of 36 RFLP groups), the β-Proteobacteria (three out of 36 RFLP groups) at 8% and members of the Gram-positive, high G+C bacterial phylum at 6% (two out of 36 RFLP groups). Some 58% of the RFLP groups represented culturable bacteria (21 of the 36 RFLP groups are indicated by an asterisk in Table 1).

In only one group, RFLP 26, did the nucleotide sequences have 100% similarity to sequences in the database identified as *Curacaobacter baltica*. However, several of the RFLP groups possess 98–99% similarity to 16S rDNA sequences in GenBank, suggesting that these bacteria are probably in the same genus, if not of the same species as the GenBank relative. Among the α-Proteobacteria (Fig. 1), RFLP groups 4, 9, 11, 12, 16 and 17 were 98–99% similar to published gene sequences of species of *Erythrobacter*, *Roseobacter*, *Sulfitobacter*, *Phyllobacterium*, *Agrobacterium* and *Sphingomonas*. Several clones from the γ-Proteobacteria (Fig. 3) subclass (RFLP 24, *Pseudomonas hibiscicola*; RFLP 25, *Marinobacter hydrocarbonoclasticus*; and RFLP 27, *Pseudomonas oleovorans*) and Gram-positive, high G+C group (Fig. 5; RFLP 35, *Curtobacterium luteum* and RFLP 36, *Microbacterium terrae*) also had at least 98% similarity to GenBank 16S rDNA sequences. On the other hand, none of the bacterial sequences that clustered with either the β-Proteobacteria subclass (Fig. 2) or the *Flexibacter*/*Cytophaga*/*Bacteroides* group (Fig. 4)

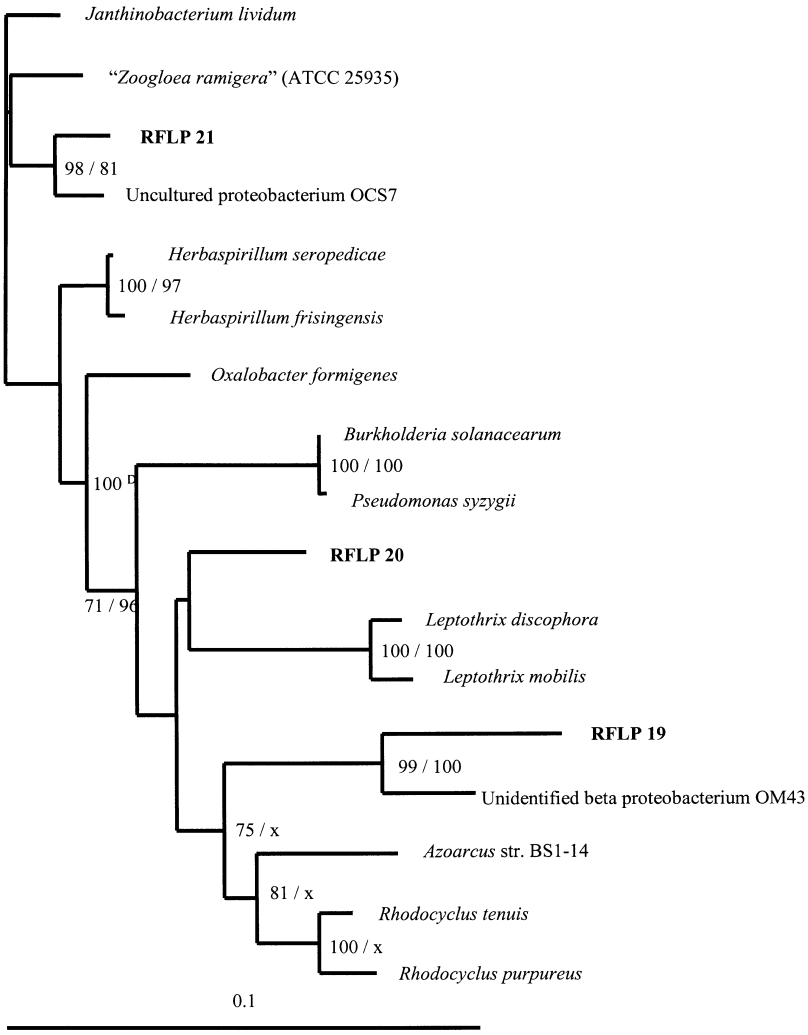

Fig. 2. Phylogenetic tree generated by the neighbour-joining method and Jukes–Cantor distance algorithm showing the relationships between the bacteria associated with *Pfiesteria*-like dinoflagellates and sequences obtained from the GenBank database within the β-subclass of Proteobacteria. Bootstrap values ($n = 100$ replicate resamplings) for branches are indicated for both neighbour-joining and parsimony methods (neighbour-joining bootstrap value/parsimony bootstrap value) as an aid to determining the efficacy of each node. Bootstrap values are only presented for neighbour-joining nodes of 50 or more. An 'x' represents a branch that was not present in the tree constructed through the parsimony method. The bar represents 0.1 U of evolutionary distance.

possessed high similarity to published sequences. The remaining bacteria displayed low nucleotide sequence homology to sequences in the databases (Table 1, % similarity data).

Phylogenetic analysis of the bacteria

The taxonomic relatedness of the bacteria associated with the *Pfiesteria*-like dinoflagellate cultures was deduced by the construction of phylogenetic trees inferred from comparative sequence analyses of the partial 16S rDNA sequence from each clone. Based on this comparison, the dinoflagellate-associated bacteria fell into either the α- (Fig. 1), β- (Fig. 2) or γ-Proteobacteria (Fig. 3) subclasses, the Cytophagales group (Fig. 4) or the Gram-positive, high G+C division (Fig. 5). The low bootstrapping value at many of the nodes in the phylogenetic trees represented in Figs 1–5 indicates ambiguity. For this reason, we used both the distance/neighbour-joining and parsimony programs in PHYLIP (Felsenstein, 1989) to help in the construction of the phylogenetic trees. This was done to aid the interpretation of phylogenetic branch points with low bootstrapping values. These values can be seen at the branch nodes in Figs 1–5, where the bootstrapping value obtained for the distance/neighbour-joining method is listed first followed by the bootstrap value for the parsimony algorithm. In both cases, a maximum bootstrap value of $n = 100$ replicates was used. Commonly, the same nodes were present in both distance/neighbour-joining and parsimony trees with bootstrapping values of > 50, and values between 90 and 100 were often observed for both methods, indicating the efficacy of that particular branch.

Frequency of obtaining bacterial clones

We next examined the frequency at which each clone was obtained. Of the total 119 clones resulting from all

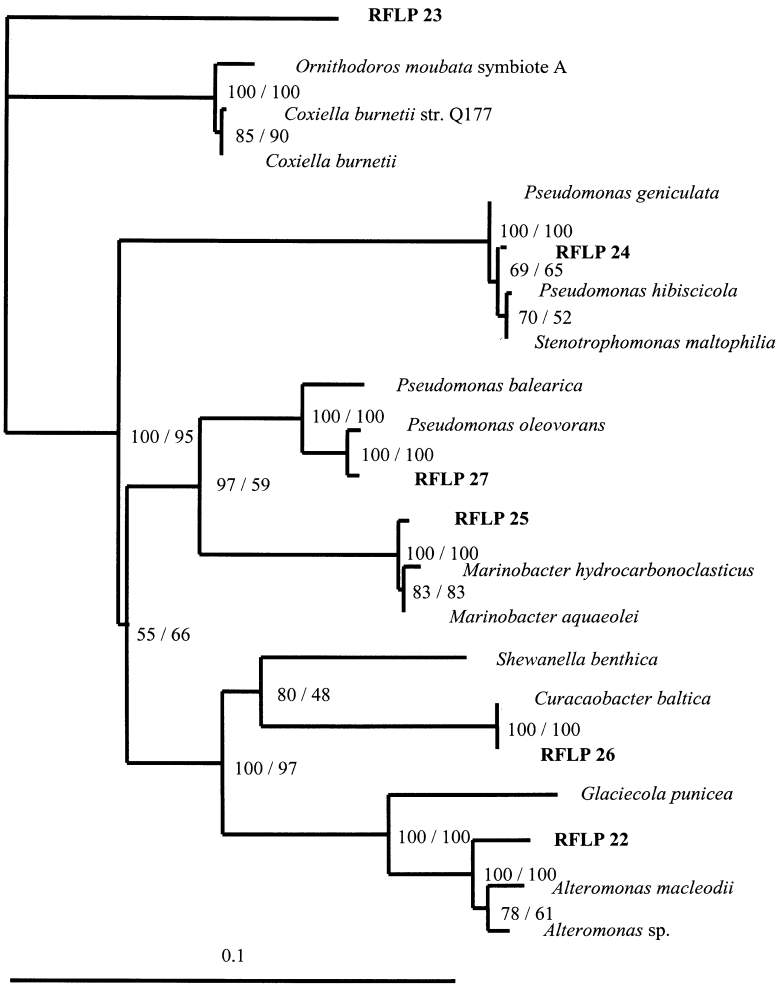

Fig. 3. Phylogenetic tree generated from comparative sequence analysis of 16S rDNA by the neighbour-joining method and Jukes–Cantor distance algorithm showing the relationships between the bacteria associated with *Pfiesteria*-like dinoflagellates and published 16S rDNA nucleotide sequences related to the γ-Proteobacteria. Bootstrap values ($n = 100$ replicate resamplings) for branches are indicated for both neighbour-joining and parsimony methods (neighbour-joining bootstrap value/parsimony bootstrap value) as an aid to determining the efficacy of each node. Bootstrap values are only presented for neighbour-joining nodes of 50 or more. An 'x' represents a branch that was not present in the tree constructed through the parsimony method. The bar represents 0.1 U of evolutionary distance.

dinoflagellate cultures, clones associated with RFLP 20 (*Leptothrix discophora*) were the most abundant at 18%. The sequences that followed were (in order of abundance) RFLP 8 (*Roseobacter algicola*, 10%), RFLP 7 (*Devosia riboflavina*, 10%), RFLP 10 (*Hyphomonas jannaschiana*, 9%), RFLP 1 (marine bacterium SRF3, 6%), RFLP 28 (*Sphingobacterium spiritivorum*, 6%) and RFLP 33 (*Flexibacter maritimus*, 6%). Each of the remaining 29 RFLP groups occurred at a frequency of 5% or less.

These data are informative and emphasize the diversity of these communities, but they also point to differences in the bacterial species between dinoflagellate cultures (Fig. 6). The sequences obtained from the *Cryptoperidiniopsis* cultures (CCMP1827 and CCMP1828) were (in order of abundance) *Devosia riboflavina* (RFLP 7, 44%), *Roseobacter algicola* (RFLP 8, 15%) and *Hyphomonas jannaschiana* (RFLP 10, 7%). The frequencies of the remaining nine RFLP groups that were found in this culture were $< 5\%$ each.

For CCMP1829, the *Pfiesteria*-like dinoflagellate referred to as 'shepherd's crook', the frequency of abundance was (in order) *Leptothrix discophora* (RFLP 20, 33%), *Hyphomonas jannaschiana* (RFLP 10, 14%), *Roseobacter algicola* (RFLP 8, 13%), unidentified α-Proteobacterium GAI-5 (RFLP 14, 10%) and *Sphingobacterium spiritivorum* (RFLP 28, 8%). Clones representing the remaining 12 RFLP groups each comprised $< 5\%$ of those sequences obtained.

The ordered abundance of bacterial clones obtained from the *P. piscicida* type strain was composed of only two RFLP groups, *Flexibacter maritimus* (RFLP 33, 24%) and marine bacterium SRF3 (RFLP 1, 17%). The remaining 17 RFLP groups each represented $< 5\%$ of the other bacterial clones obtained from *P. piscicida*.

Specificity of the association

Two different methods were used to demonstrate the specific association of the bacterial community with the dinoflagellate cells. The first approach used a gentle washing technique that used a unique characteristic of the

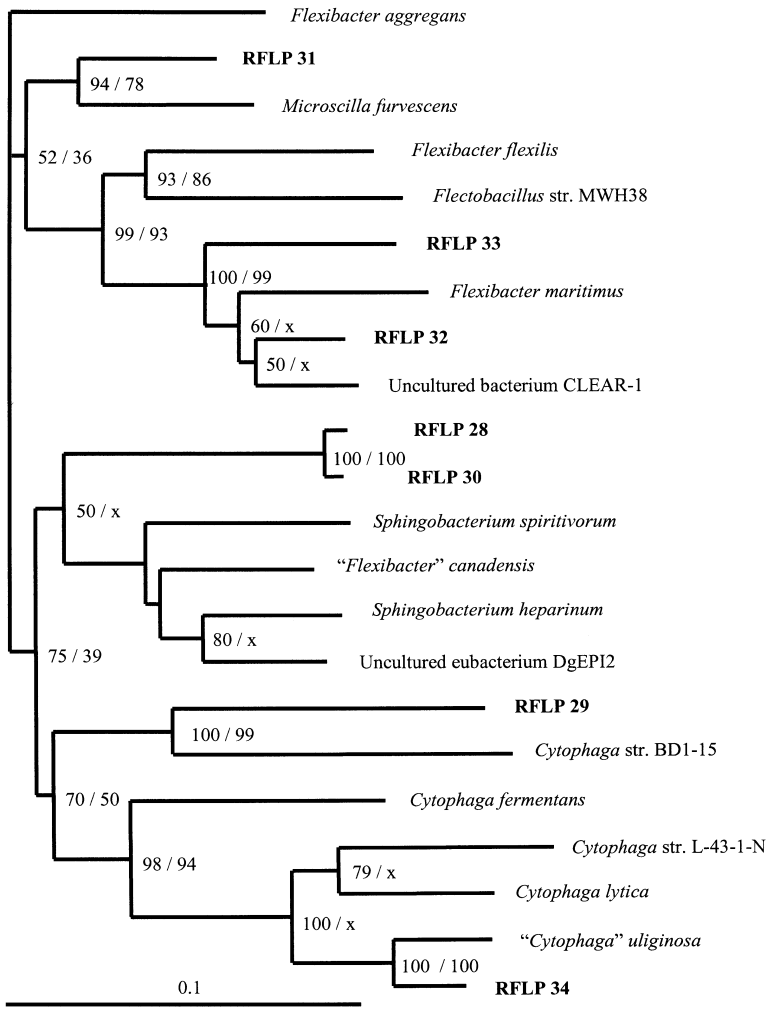

Fig. 4. Phylogenetic tree inferred by the neighbour-joining method and Jukes–Cantor distance algorithm showing the relationships between the bacteria associated with *Pfiesteria*-like dinoflagellates and gene sequences related to the Cytophagales division. Bootstrap values ($n = 100$ replicate resamplings) for branches are indicated for both neighbour-joining and parsimony methods (neighbour-joining bootstrap value/ parsimony bootstrap value) as an aid to determining the efficacy of each node. Bootstrap values are only presented for neighbour-joining nodes of 50 or more. An 'x' represents a branch that was not present in the tree constructed through the parsimony method. The bar represents 0.1 U of evolutionary distance.

behaviour of *Pfiesteria*-like dinoflagellates, whereby the dinoflagellates actively swim towards the bottom of the culture flask. The rate of swimming is greater than the swimming speed of motile bacteria and faster than the sedimentation of bacterial aggregates. Thus, once the population of dinoflagellates had migrated to the bottom of the flask, bacteria not physically associated (either epiphytic or intracellular/endosymbiotic) could be removed from the overlying supernatant. Repetitive washings were then used to ensure that unattached or loosely associated bacteria were removed from dinoflagellate cells (*Experimental procedures*).

Plate counts of the colony-forming units (cfus) obtained on 0.5× marine agar and confocal scanning laser fluorescence microscopy (CSLFM) and fluorescence *in situ* hybridization (FISH) with a fluorescently labelled eubacterial 16S ribosomal RNA-directed oligonucleotide probe were used to monitor the change in bacteria physically associated with the dinoflagellate cells. The CSLFM data obtained from washed CCMP1828 cells is shown in Fig. 7. Before washing, many bacteria could be observed surrounding the dinoflagellate cells (Fig. 7A–H), some of which appeared to be epiphytic or even intracellular. After washing, which reduced the number of bacteria per dinoflagellate cell, these epiphytic and intracellular bacteria were much more visible. Bacterial cells were observed to be attached to the dinoflagellates via their poles (Fig. 7I), in very close proximity to the outer surface of the dinoflagellate (Fig. 7P) and, apparently, in the cytoplasm of the dinoflagellate (Fig. 7L). These intracellular bacteria were frequently observed to co-localize with the dinoflagellate nucleus.

We observed a decrease in the cfus during the washing procedure that asymptotically levelled off to $\approx$10 bacteria per dinoflagellate cell (data not shown). Although a complete analysis of the bacteria attached to the *Pfiesteria*-like dinoflagellates has not been performed (manuscript in preparation), preliminary data obtained from one of the culturable bacteria indicate that it belongs to the α-Proteobacteria subdivision, *Roseobacter denitrificans* subgroup, and is identical to our RFLP group 9.

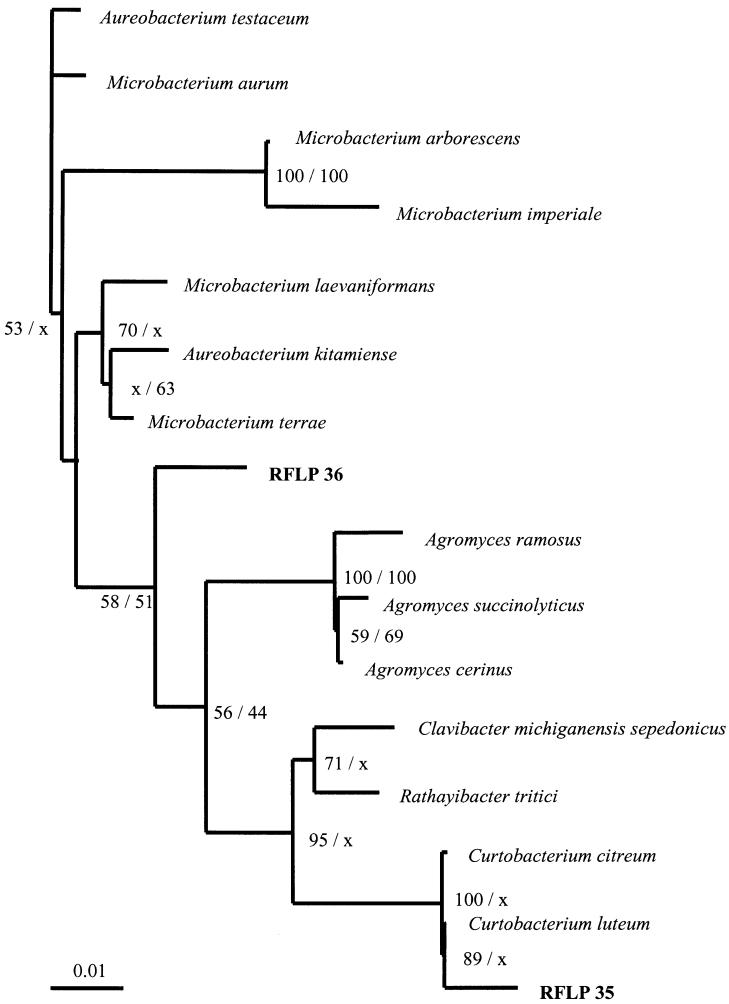

Fig. 5. Phylogenetic tree generated by the neighbour-joining method and Jukes–Cantor distance algorithm showing the relationships between the bacteria associated with *Pfiesteria*-like dinoflagellates and sequences obtained from the GenBank database related to the Gram-positive, high G+C group of bacteria. Bootstrap values ($n = 100$ replicate resamplings) for branches are indicated for both neighbour-joining and parsimony methods (neighbour-joining bootstrap value/ parsimony bootstrap value) as an aid to determining the efficacy of each node. Bootstrap values are only presented for neighbour-joining nodes of 50 or more. An 'x' represents a branch that was not present in the tree constructed through the parsimony method. The bar represents 0.01 U of evolutionary distance.

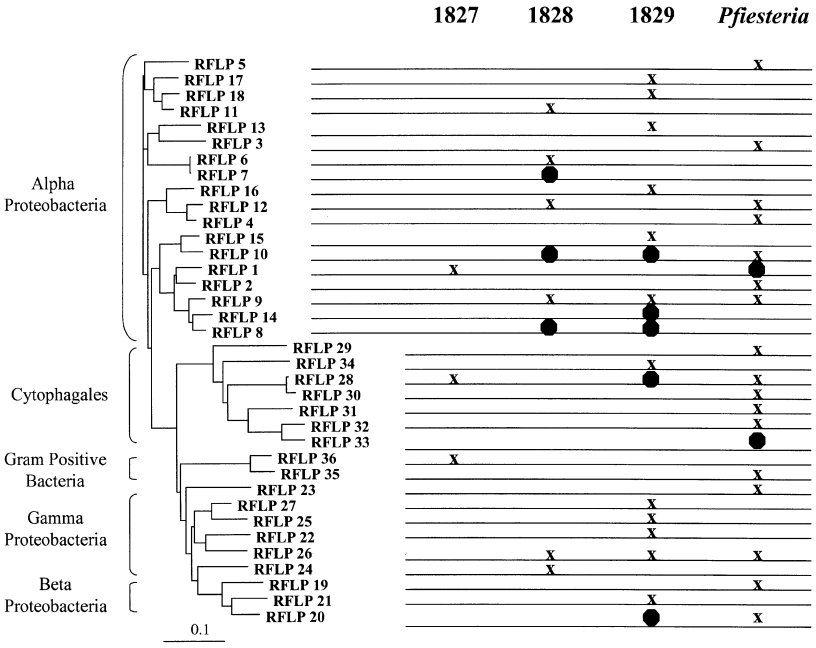

Fig. 6. Distribution of the bacterial clone RFLP groups compared with the dinoflagellate culture of origin, CCMP1827, CCMP1828, CCMP1829 or *P. piscicida*. The RFLP groups are organized in a phylogenetic tree inferred from 16S rDNA sequence data by the neighbour-joining analysis without bootstrapping. An 'x' indicates that a clone from that RFLP group was obtained from the respective dinoflagellate culture, and the filled octagonal is used to signify those RFLP groups with the highest frequency of clones as described in the text. The bar represents 0.1 U of evolutionary distance.

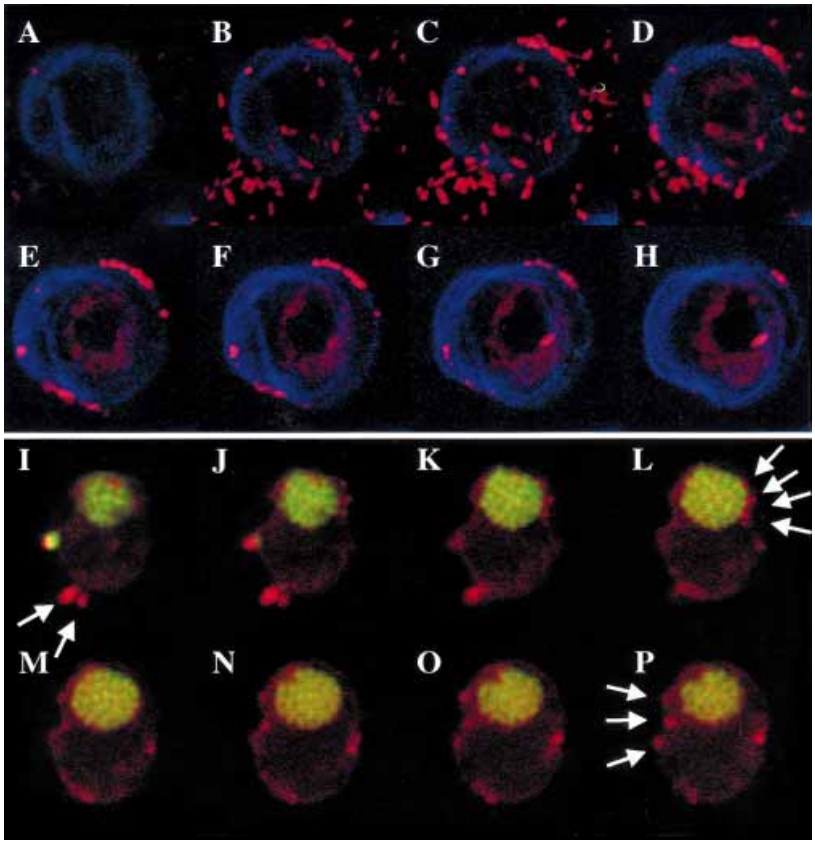

Fig. 7. Localization of attached and intracellular/endosymbiotic bacteria on and in washed dinoflagellate cells. Bacteria (false-coloured in red) were localized by FISH with a 16S rDNA eubacterial oligonucleotide probe and visualized using confocal laser scanning fluorescent microscopy (as described in *Experimental procedures*). Calcofluor background staining was used to highlight the thecal plates of the dinoflagellate (blue), whereas the dinoflagellate nucleus was stained with ethidium bromide and is depicted in green. Successive 0.5 μm optical sections through the *z*-axis of a fixed dinoflagellate before washing (A–H) or after washing (I–P) are shown. The arrows indicate bacteria that are epiphytic and polarly attached to the washed dinoflagellate (I), intracellular or endosymbiotic and located near the nuclear membrane (L) or epiphytic and closely associated with the outer surface of the dinoflagellate (P).

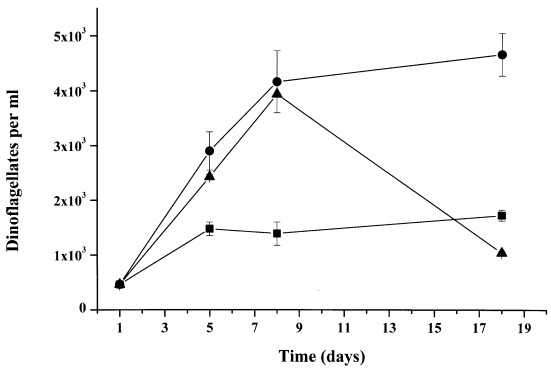

Fig. 8. Effect of the bacterial community on dinoflagellate growth. CCMP1828 was made axenic as described in *Experimental procedures*, and the culture was subdivided into three populations. One population was inoculated with a mixture of the culturable bacteria isolated from the parental (bacterized) culture of CCMP1828 and fed axenic prey algae (circles). The second culture was maintained without bacteria and fed axenic prey algae (squares). The third culture was inoculated with the culturable bacteria but not given algal prey (triangles). A comparison of dinoflagellate growth, as determined by cells ml^{-1}, indicates that axenic dinoflagellates (squares) fail to grow at comparable rates to the bacterized counterparts (circles). Dinoflagellates with bacteria, but no prey algae (triangles), fail to maintain an active growth rate when compared with the bacterized dinoflagellates fed axenic *Rhodomonas* (circles).

Bacterial association may benefit dinoflagellate growth and physiology

The second technique used to demonstrate the specificity of bacterial interactions with the dinoflagellates used axenic dinoflagellates and a series of 'add back' experiments, in which bacteria, isolated from the bacterized culture, were added back to the axenic dinoflagellate cells. Dinoflagellate cell numbers were then counted to determine the effect of the bacteria on the dinoflagellate population growth and physiology.

As shown in Fig. 8, the loss of the bacterial population results in poor growth of the dinoflagellates and a significant reduction in the rate of population increase. This effect does not result from a requirement for bacteria as a dinoflagellate food source, as axenic cultures to which bacteria, but not prey algae, are returned, only grow for a limited time, then diminish, despite the continued presence of bacteria during the duration of the experiment. Over longer periods, axenic cultures with only algal prey or bacteria eventually die unless given the missing counterpart (unpublished data). A preliminary characterization of the culturable bacteria responsible for dinoflagellate growth has demonstrated that a key component is a bacterium that is a member of the α-Proteobacteria subdivision

and may be related to the *Ruegeria* (*Roseobacter*) *algicola* subgroup (RFLP 8).

Discussion

The focus of this study was to gain knowledge about how bacteria affect *Pfiesteria*-like dinoflagellate physiology and toxin production. We have begun to answer these questions through taxonomically characterizing the bacterial population in four *Pfiesteria*-like dinoflagellate cultures and then providing evidence to show that a subset of these bacteria is physically associated with the dinoflagellate cells and is required for the growth of dinoflagellates.

Our study has used laboratory monocultures of *P. piscicida* and three *Pfiesteria*-like dinoflagellates. This has afforded an advantage in being able to control the culture conditions carefully to maintain homeostasis between the dinoflagellate and bacterial populations. These efforts have included the use of mammalian tissue culture techniques to ensure sterility and the use of axenic algal prey to prevent contamination by unwanted microorganisms. The ability to manipulate the cultures in defined ways and under controlled settings, e.g. production of axenic strains and the use of bacterial add back experiments, is a further advantage. Although these dinoflagellate cultures do not exactly duplicate natural samples, they are likely to be enrichment cultures in which a selection process has occurred to preserve and maintain those bacteria whose interaction with the dinoflagellates is beneficial (or at least not detrimental) to the eukaryote. Such a selection process would serve an additional advantage, as it would eliminate bacteria that may not directly interact with the dinoflagellate, yet retain those whose existence either requires the dinoflagellate or is required by the dinoflagellate.

The bacterial population in the dinoflagellate cultures is stable. The analyses reported in this study have been conducted over a period of >2 years. During this time, the bacterial population diversity, as measured by the 16S rDNA analysis, has had no dramatic changes. Increases in the density of certain bacterial species have been observed to occur after the addition of fresh prey algae, but these increases are from known bacterial species, are transient and have never resulted in the loss of a previously identified species or the *de novo* appearance of a new bacterium in the flasks (data not shown).

A total of 36 taxonomically distinct groups of bacteria was identified in the four *Pfiesteria*-like dinoflagellate cultures examined. Over half these bacteria (58%; 21 out of 36 16S rDNA clones) were culturable by our methods. The remainder was detected only through PCR amplification (Table 1) and is presumed to be non-culturable. Fifty per cent of the 16S rDNA species identified clustered in the α-Proteobacteria subdivision of eubacteria, with many of the clones phylogenetically similar to clones in the SAR83 clusters obtained from ocean and coastal 16S rDNA libraries (Rappe *et al*., 1997). Many of these clones, as shown in Fig. 1 for RFLP groups 1, 2, 8, 9 and 14, are in the α-3 subclass, which includes *Roseobacter algicola*, *Ruegeria* (formerly *Roseobacter*) *denitrificans*, *Roseobacter litoralis*, *Sulfitobacter pontiacus* and bacterial isolate GAI-5 (Gonzalez *et al*., 1999). These findings are in accordance with field data showing that bacteria in the marine alpha group, specifically in the α-3 subclass or the SAR83 cluster (Rappe *et al*., 1997), comprise a large fraction of the total bacterial community associated with an algal bloom in the north Atlantic (Gonzalez and Moran, 1997; Gonzalez *et al*., 1999; 2000). This observation further strengthens the idea that the bacterial populations in these dinoflagellate cultures are representative of the populations found in nature.

Many of the 16S rDNA sequences obtained from natural samples do not have 100% homology to sequences in the databases. Therefore, it is not surprising that only a few of the 16S rDNA nucleotide sequences obtained from the dinoflagellate-associated bacteria were closely related to the published nucleotide sequences contained in either GenBank or the RDPII databases (Table 1). In fact, only one of the 36 RFLP groups (RFLP 26) was taxonomically unambiguous. This group, RFLP 26 represented by clone MA2831 as shown in Table 1 and Fig. 3, appears to have identity to *C. baltica*, a *Pseudomonas*-like marine bacterium that has been isolated from the central Baltic (Strobowski *et al*., 1997). Unlike RFLP 26, most of the 16S clone sequences had only partial homology with counterparts in the databases. This can be seen from the similarity data presented in Table 1, as well as from the bootstrap values displayed at the branch nodes in Figs 2–5. For this reason, the assignment of a genus and species name to a particular RFLP group in this work serves only to help define possible physiological roles that the bacterium has in these cultures.

The culturable bacteria associated with toxic algal species have been isolated and characterized in previous studies (Tosteson *et al*., 1989; Doucette and Trick, 1995). The genera of bacteria frequently isolated from these toxic algae include *Nocardia*, *Pseudomonas*, *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, *Aeromonas*, *Flavobacterium*, *Cytophaga*, *Moraxella*, *Acinetobacter* and *Roseobacter*. These efforts have concentrated on the culturable bacteria and have not used a PCR-based approach to identify the bacterial community, including non-culturable species, as has been done in our investigation. Yet, consistent with the observations of others, 16S rDNA clones from the *Pfiesteria*-like dinoflagellate cultures match several species found in the

environmental samples. These species include several species of *Cytophaga* (RFLP 29 and 34), *Pseudomonas* (RFLP 24 and 27), *Alteromonas* (RFLP 22) and *Roseobacter* (RFLP 8).

In a recent study by Lu *et al*. (2000), the bacteria associated with the toxic dinoflagellate *Alexandrium minutum* were characterized and their location determined relative to the cell. *Pasteurella pneumotropica*, *Morganella wisconsensis*, *Flavobacterium oryzihabitans*, *Pseudomonas pseudomallei* and *Sphingomonas* sp. were determined to be extracellular to the dinoflagellate, whereas *Pasteurella haemolytica*, *Pseudomonas vesicularis* and a *Sphingomonas* sp. were determined to exist intracellularly. These data may suggest that the bacteria in our RFLP groups 24 and 27, phylogenetically aligned with *Pseudomonas* sp., could occur as intracellular bacteria in the *Pfiesteria*-like dinoflagellates. We are currently investigating this possibility through an analysis of the bacteria associated with the dinoflagellate cells after washing (manuscript in preparation).

Many of the clones obtained from the four *Pfiesteria*-like dinoflagellate cultures are phylogenetically similar to 16S rDNA sequences obtained from marine bacterioplankton obtained from both coastal and ocean habitats. For example, RFLP group 3, in the α-Proteobacteria subdivision (Fig. 1), is very similar to clone OM75 (Rappe *et al*., 1997) with affiliation to the *Rhodospirillum rubrum* assemblage. Two β-Proteobacteria clones, RFLP groups 19 and 21 (Fig. 2), align with a *Methylophilus* group clone (OM43) (Rappe *et al*., 1997) and a clone (OCS7) obtained from the Oregon coast and taxonomically similar to *Zoogloea* (Rappe *et al*., 1998) respectively.

RFLP 25, which is phylogenetically related to *Marinobacter hydrocarbonoclasticus* in the δ-Proteobacteria subdivision (Fig. 3), is also very interesting, as this bacterium has also been found associated with another toxic dinoflagellate, *Alexandrium fundyense* (J. Rooney-Varga, personal communication). Bacteria in this genus are capable of metabolizing complex or unusual hydrocarbon molecules, such as isoprenoid molecules (Rontani *et al*., 1997; Huu *et al*., 1999) and may represent species that use dinoflagellate secondary metabolites as carbon or energy sources. Dinoflagellate secondary metabolites can serve as the chemical precursors of certain dinoflagellate toxins (Baden, 1983), suggesting that RFLP 25 may have relevance in mediating the toxicity of these dinoflagellates.

The physiological and functional significance of most of the 36 groups of bacteria associated with these four *Pfiesteria*-like dinoflagellate cultures is not known and remains enigmatic. However, we can determine whether the interaction between a given bacterium and the dinoflagellate is specific. We chose to show this using two different approaches. First, a firm, close physical association of the bacterium and the dinoflagellate was chosen as one criterion by which the specificity of the interaction could be assessed. Bacteria that are firmly attached to the dinoflagellate or that live intracellularly as endosymbionts are likely to have developed specific interactions and physiological requirements provided by the dinoflagellate or its metabolites. Conversely, the dinoflagellate may require bacterial metabolites for its growth. Secondly, the ability of the bacteria to enhance the growth of the dinoflagellate population was used as another argument to support the specificity of the interaction. Specificity of interaction, in this case, is thus defined as either close physical contact or some physiological benefit imparted to the dinoflagellates by members of the bacterial community.

The dinoflagellate washing procedure used in this work is based upon a behaviour of the dinoflagellate cells to swim to the bottom of the flask. Using this behaviour, we avoided disturbing the dinoflagellates, which frequently leads to changes in the physiology of the cell, including cyst formation, loss of motility and lysis of the cells (Burkholder and Glasgow, 1997a). The washing removed most of the bacteria that were not attached or loosely attached to the dinoflagellate, e.g. we observed >100 bacteria per dinoflagellate cell before washing (Fig. 7A–H), but usually <10 bacteria per cell after washing (Fig. 7I–P). The use of FISH and confocal microscopy was invaluable in this effort, as the resolution of this method allows accurate determination of co-localization of the bacteria with the dinoflagellate. This was done by counterstaining key cellular features of the dinoflagellate with fluorescent chemicals that bound to the cellulose-containing thecal plates or chromosomes.

On many washed dinoflagellate cells, bacteria are observed that appear to be attached via the polar end (Fig. 7I). Morphologically, these bacteria resemble prosthecate bacteria of the genera *Caulobacter* and *Hyphomonas*. As shown in Fig. 1 and Table 1, we have at least three 16S clones, RFLP groups 10, 11 and 15, that align phylogenetically with known representatives of these genera, strengthening this idea.

Figure 7L shows several presumed bacteria (observed by 16S rDNA FISH and CSFLM) that are aligned with the nucleus or nuclear membrane of the dinoflagellate, based on the co-occurrence in the same optical section of the FISH signal and chromosomes. The presence of endosymbiotic bacteria has been reported previously in *P. piscicida* (Burkholder and Glasgow, 1997b), although few details have been published about the location or number of these endosymbionts.

A preliminary phylogenetic analysis of one of the culturable bacterial isolates obtained from the washed dinoflagellate cells suggests that the bacterium is taxonomically related to *Sulfitobacter pontiacus* (RFLP group

9). As described earlier, RFLP 9 is associated with the α-3 subclass or the SAR83 cluster of marine bacteria that are prevalent bacterioplankton in marine and coastal waters and includes the genus *Roseobacter*. Bacteria in this group are not known to possess holdfasts nor to be endosymbionts, so it is tantalizing to speculate that they may be epiphytic on the dinoflagellate surface similar to the bacteria shown in Fig. 7P. It will be exciting to determine the location of these bacteria by the development of species-specific 16S rDNA probes to use in FISH.

Although a specific requirement cannot be determined based upon the current data, the bacterial community appears to be beneficial for dinoflagellate growth and physiology. The growth patterns shown in Fig. 8 are consistently reproducible over a repetitive series of experiments, suggesting that this phenomenon is real. As seen in Fig. 8, the population of axenic zoospores plus algal prey remains near the same density over 18 days (Fig. 8, squares), unless both the prey algae and the bacteria are added back to the culture (Fig. 8, circles). When bacteria, but not algae, are returned to the axenic zoospores, the dinoflagellate population increases transiently over a period of 7–8 days, then declines precipitously (Fig. 8, triangles). This brief period of population growth may result from the consumption of a select set of bacteria by the zoospores, whose feeding depletes a bacterial food source to such low levels as to cause a crash in the dinoflagellate population. This idea is supported by the observations of others that *P. piscicida* consumes many types of unicellular microorganisms, including bacteria (Burkholder and Glasgow, 1997a). However, we have not been able to demonstrate active feeding on bacteria by these dinoflagellates, and the bacterial density remained stable or increased slightly throughout the period of these experiments under all three conditions (data not shown). An alternative explanation for the transient rise may be that the bacteria may do something to affect zoospore feeding on the prey algae, *Rhodomonas*. We are currently exploring these hypotheses.

Is there a specific bacterial species required by the zoospores for growth? Based on the preliminary data, a key component in the bacterial community that benefits axenic zoospore growth appears to be a species in the marine alpha-bacteria group, most closely associated with the α-3 or SAR83 cluster. *Sulfitobacter* species are also in this cluster suggesting that the two bacteria, identified as specifically interacting with the zoospores by separate methods, may share some common metabolic pathway that is important to the dinoflagellate cell. It is interesting to reflect that this group of bacteria appears to be closely associated with both coastal and oceanic algal blooms (Gonzalez and Moran, 1997; Gonzalez *et al*., 2000), underscoring their importance in nature as well as in the laboratory culture flask. Interpretation of the significance of these findings must, however, be tempered until further experiments are performed to understand the observed phenomena adequately.

The axenic dinoflagellate cells used in this study were produced by stimulating encystment of the zoospores artificially by the addition of the indoleamine, 5-methoxytryptamine (*Experimental procedures*). As the cysts withstand harsh treatment, bacteria on the outside of the cyst are killed by the addition of a dilute sodium hypochlorite (bleach) solution. Both plate counts of viable bacteria and 16S rDNA PCR analysis of the resulting cysts and zoospores have failed to show the presence of any bacteria or prokaryotic 16S rDNA, respectively, suggesting that these cells are devoid of bacteria. Yet, internal bacteria have been observed in untreated zoospores using CSLFM. It is possible that the internal bacteria observed in CSLFM are artifacts of the 16S rDNA FISH procedure, albeit the observations are reproducible from cell to cell and over multiple experiments. It is also possible that the 16S rDNA PCR methods used have failed to detect the presence of the endosymbiotic bacteria in the cysts and zoospores. Alternatively, but less likely, indoleamine encystment may cause expulsion of the intracellular bacteria leaving the cysts free of intracellular bacteria. We are currently investigating these and other possibilities.

One of the principal findings of this work is that bacteria related to the marine alpha-bacteria, e.g. *Roseobacter* spp., are significant members of this dinoflagellate culture community. As seen in Fig. 1, RFLP groups 8, 9 and 14 are taxonomically related to *R. algicola*, *R. litoralis* and *R. galaceciensis*, but not identical to these species, as can be seen by the bootstrap values at each node. The presence of *Roseobacter* species in the population may be significant to dinoflagellate toxigenesis. This genus is present in natural, environmental samples, as has been observed by others but, more importantly, the presence of *R. algicola* in the natural harmful algal community appears to be correlated with the production of some dinoflagellate toxins, particularly okadaic acid (Lafay *et al*., 1995; Gallacher *et al*., 1997). This suggests that one or more members of the dinoflagellate-associated bacteria related to *Roseobacter* may have the potential to synthesize one or more of the currently unidentified *Pfiesteria* toxins. Although it is exciting to think that there may be a connection between the identification of *R. algicola* and a bacterial origin of *Pfiesteria*-like dinoflagellate toxins, this idea requires much more thorough investigation. Our laboratory is currently examining each of the dinoflagellate-associated bacteria for toxin production in attempts to understand the role played by species of *Roseobacter* (and other genera) in dinoflagellate toxigenesis.

The findings presented here show that the bacterial

community associated with the *Pfiesteria*-like dinoflagellates in laboratory culture is similar in composition to the bacterial assemblages co-occurring in nature with other harmful algal species. The data suggest that some of the interactions between bacteria and dinoflagellates presumed to occur in nature also occur in these culture flasks. For example, members of this bacterial community are specifically associated by physical location, either epiphytic or intracellular, with the zoospores. There is also a suggestion that certain bacteria are beneficial to the normal growth and physiology of the dinoflagellate population. This is to be expected from a microbial community composed of both prokaryotic and eukaryotic members, which has evolved over time to interact with and become dependent upon one another in both physical and physiological ways. An analysis of these interactions in a defined laboratory setting in which reductive scientific approaches may be applied should provide a wealth of knowledge about the molecular nature of these interactions. That information can be developed into hypotheses that may be tested and applied to the bacterial communities existing alongside these dinoflagellates in nature.

Experimental procedures

Dinoflagellate strains and culturing conditions

Pfiesteria piscicida was a generous gift from Dr Karen Steidinger (Florida Department of Environmental Protection, Florida Marine Research Institute, St Petersburg, FL, USA). Axenic and bacterized strains of *Cryptoperidiniopsis* from a fish kill site in King's Creek, MD, USA (CCMP1827 and CCMP1828 respectively) and a bacterized strain of the *Pfiesteria*-like dinoflagellate from a fish kill site in 'shepherd's crook', MD, USA (CCMP1829) were obtained from the Provasoli–Guillard National Center for Culture of Marine Phytoplankton (CCMP). The dinoflagellates were cultured in f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) lacking silica and supplemented with 15 parts per thousand (p.p.t.) of artificial sea salts (Instant Ocean) at 20°C with a light–dark cycle of 14 h light (mean light intensity of 90–100 μM m^{-2} s^{-1}) and 10 h dark. The cultures were maintained by feeding with an axenic strain of a *Rhodomonas* sp. (Cryptophyceae, CCMP768) every 3–4 days with a change of spent culture medium. All handling of dinoflagellates and bacteria was conducted in a laminar flow chamber using sterile techniques in a Biosafety level 3 (BSL-3) laboratory. Axenic cultures were assayed for bacterial contaminants by direct culturing on marine nutrient agar and PCR amplification as described below.

Bacterial strains and culturing conditions

Culturable bacterial isolates obtained from dinoflagellate cultures were maintained on 0.5× 'Zobell' marine agar 2216 (18.7 g of Difco Marine broth 2216, 15 g of Difco Bacto agar and 1000 ml of dH_2O) at 20°C unless otherwise noted. *Escherichia coli* INVαF′ [F′ *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 λ^-] was grown on Luria broth (Sambrook *et al.*, 1989) at 37°C. Difco Bacto agar (15 g) l^{-1} Luria broth was added to make Luria agar medium. Kanamycin at a concentration of 80 μg ml^{-1} was added to media for selection of plasmid pCR2.1.

PCR amplification and cloning

PCR was used to amplify bacterial 16S rDNA from the mixed community composed of both prokaryotic and eukaryotic microorganisms. Universal primers 519F (5′-CAGCA/CG CCGCGGTAATA/TC-3′) and 1406R (5′-ACGGGCGGTGT GTA/GC-3′) were used for the amplification of bacterial 16S rDNA (Lane *et al.*, 1985). Template DNA for amplifications was obtained as follows. A 100 μl aliquot of a dinoflagellate culture with associated bacteria at a density of 2×10^4 to 4×10^4 cells ml^{-1} was centrifuged (14 000 *g* for 60 s), and the pellet was resuspended in 100 μl of sterile dH_2O. The genomic DNA was then extracted by boiling the sample for 5 min. The cellular debris was removed by centrifugation, and the supernatant containing the extracted DNA was either used immediately for PCR or stored at −20°C for later amplification.

To analyse the 16S rDNA sequences from culturable bacteria, a series of 10-fold dilutions of each dinoflagellate culture was made using 15 p.p.t. artificial sea water. Each dilution (100 μl) was spread on 0.5× marine broth 2216 (Difco) supplemented with 15 g l^{-1} agar. These cultures were incubated at 20°C for 3–5 days. Well-isolated bacterial colonies were then transferred to a microcentrifuge tube with a sterile toothpick and treated as described above.

PCR was carried out with *Taq* DNA polymerase (Perkin-Elmer) using a PTC200 thermal cycler (MJ Research) and a 50 μl reaction mixture containing 200 μM dNTP, 0.5 pmol μl^{-1} each primer and 10–100 ng of template DNA. Conditions for PCR were as follows: an initial denaturation step of 1.5 min at 94°C, 30 amplification cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C) and elongation (1.5 min at 72°C) and a final extension step of 5 min at 72°C. The PCR products were cloned into the TA cloning vector pCR2.1 (Invitrogen) using the ligation conditions recommended by the manufacturer and transformed into *E. coli* INVαF′ competent cells supplied with the Invitrogen Original TA cloning kit. Transformants were selected by overnight incubation at 37°C on Luria agar with kanamycin (80 μg ml^{-1}).

Library screening and RFLP analysis

A total of 159 clones (38 from CCMP1827/1828, 85 from CCMP1829 and 36 from *P. piscicida*) was selected randomly from transformations with total dinoflagellate culture DNA and grown overnight in Luria broth with kanamycin. Plasmid DNA was extracted from the overnight cultures using QIAprep Spin miniprep columns (Qiagen). The presence of a partial 16S rDNA insert was confirmed by *Eco*RI digestion of the recombinant plasmid and separation of the resulting DNA fragments by electrophoresis using a 1.0% agarose gel in 1×

Tris acetate–EDTA buffer (TAE; Sambrook *et al*., 1989). DNA bands in the agarose gel were visualized with SYBR green I nucleic acid gel stain (Molecular Probes) using a fluoroimager (Molecular Dynamics).

For RFLP analysis, the inserts from the selected plasmids were amplified using the oligonucleotide primers described previously, and the products were digested separately with *Hha*I and *Hae*III restriction endonucleases according to the procedure of Pulliam Holoman *et al*. (1998). The DNA fragments were resolved by electrophoresis on 2% TreviGel 500 (TreviGen) in 1× TAE and visualized using SYBR green staining and fluoroimagery. Clones were categorized according to their distinct RFLPs.

DNA sequencing and analysis

At least two representative 16S rDNA clones from each unique RFLP group were sequenced for comparative phylogenetic analysis, and the sequence was determined after dye terminator cycle sequencing on an ABI 373 automated sequencer (Applied Biosystems). Initially, the clones were sequenced from the flanking 5′ end with a T7 sequencing primer and from the flanking 3′ end with an M13 reverse sequencing primer, both located on the pCR2.1 vector, to obtain complete fragment sequence. As required, oligonucleotide primers to internal portions of the specific 16S rDNA clone were used to complete the sequence.

Chimeric sequence evaluation

Partial 16S rDNA sequences were evaluated by first analyzing the DNA with the CHECK CHIMERA program of the Ribosomal Database Project II (RDPII) (Maidak *et al*., 1997). In addition, short sequences (< 300 bp) of the 16S rDNA 5′ and 3′ flanking regions were submitted to both the SIM_RANK (Similarity rank; RDPII) and BLASTN (Altschul *et al*., 1990) programs from the National Center for Biotechnology Information (NCBI) for comparative phylogenetic analysis of whole and partial gene sequences.

Phylogenetic analyses

For each clone, a similarity search was preformed with the RDPII SIM-RANK program and also with BLASTN to obtain a preliminary list of closest phylogenetic neighbours. Sequences were then sorted according to phylum and subclass affiliation and were aligned manually with the 16S rDNA sequence editor PHYDIT (Chun, 1995) according to the author's instructions. In this step, nucleotides outside the DNA region amplified by the two universal prokaryotic 16S rDNA oligonucleotide primers were masked from consideration. The remaining sequences were matched to align both bases and secondary structure of each sequence to the bases and secondary structure of the template *E. coli* K-12 16S rDNA (J01695).

Phylogenetic trees were calculated with the neighbour-joining algorithm (Saitou and Nei, 1987) using the program NEIGHBOR of the PHYLIP software (Felsenstein, 1989). Evolutionary distances were calculated by the program DNADIST and the Jukes–Cantor algorithm (Jukes and Cantor, 1969). The output from this calculation was then used to construct both neighbour-joining and parsimony phylogenetic trees respectively. In each case, to check the consistency of the resulting tree, random resampling of the sequences (bootstrapping) was performed, and a tree representing a consensus of 100 trees (bootstrapping value of 100) was obtained for both methods. The distance/neighbour-joining consensus and parsimony consensus trees were then constructed ($n = 100$) to obtain bootstrapping values for any node presented in the distance/neighbour-joining tree. Any node with a value of 50 or higher was presented in the final trees. Final phylogenetic trees were produced and noted with both the neighbour-joining and parsimony bootstrapping values, if the bootstrapping was > 50. When bootstrapping values fell below 50, indicating a statistically unreliable node, no bootstrapping value was presented.

Behavioural washing of dinoflagellate zoospores

Dinoflagellate zoospores harbouring attached or intracellular bacteria were obtained by serial washing in 10 p.p.t. artificial sea water (ASW) as follows. A 10 ml sample of culture was slowly overlaid across 40 ml of sterile 10 p.p.t. ASW in a 50 ml culture flask. The flask was allowed to sit upright and undisturbed at room temperature, during which time actively swimming zoospores were rapidly observed to seek the bottom of the flask. Fifteen minutes later, the upper 40 ml was carefully removed by pipette aspiration and discarded. The bottom 10 ml, now containing the majority of zoospores, was removed and slowly overlaid across another 40 ml of sterile ASW. This procedure was repeated five more times for a total of six washes, which we have determined empirically to remove all the unattached or loosely attached bacteria (data not shown). Samples (1 ml) were taken before and after the washes for analysis of attached or intracellular bacteria.

Fixation of zoospores

Samples (1 ml) of washed and unwashed dinoflagellates were fixed for 30 min on ice in 3% paraformaldehyde (Sigma). The fixed dinoflagellates were pelleted at 10 000 *g* for 15 s and washed once in 10 p.p.t. ASW. The final pellet was resuspended in 100 μl of 10 p.p.t. ASW and pipetted to clean glass coverslips. The cells were allowed to adhere to the coverslips in a covered sterile Petri dish for 30 min at 42°C. The coverslips were then used immediately for FISH.

Fluorescent in situ *hybridization*

Procedures for FISH followed those of Amann *et al*. (1990; 1992) with the following modifications. A universal oligonucleotide probe complementary to the 16S rDNA of most eubacteria (EUB338, GCTGCCTCCCGTAGGAGT) was synthesized (Life Technologies) and fluorescently labelled at the 5′ end with Texas red (Molecular Probes). The probe was hybridized to fixed specimens by adding 1 ml of hybridization solution (50 mM Tris base, pH 7.5, 0.9 M NaCl, 20% formamide, 0.1% SDS) containing 5 ng of probe to coverslips in a six-well plate. The six-well plate was placed in a humidified chamber and incubated at 48°C for 2 h. To

remove excess probe after hybridization, each well was rinsed once with wash buffer (50 mM Tris base, pH 7.5, 0.9 M NaCl, 0.1% SDS), and the coverslips were incubated in 1 ml of wash buffer for 20 min at 50°C. The coverslips were then rinsed with 10 p.p.t. ASW, and the cells were stained with SYBR green (1:10 000; Molecular Probes) and/or calcofluor (1 mg ml^{-1} dH_2O; Sigma). Finally, coverslips were mounted on glass slides using Prolong mounting solution (Molecular Probes).

Confocal scanning laser fluorescence microscopy (CSLFM)

Dinoflagellates and attached bacteria were examined using a Zeiss LSM410 confocal laser scanning microscope with a 63×, NA 1.4 objective. Texas red and SYBR green were excited using the 568 and 488 nm lines of the 25 mW Kr/Ar laser and detected using the 590 nm longpass and 515–540 nm bandpass filters respectively. Calcafluor was excited using the 351 nm line of the 110 mW Ar laser and detected with the 403–435 nm bandpass filter. Individual dinoflagellates were optically sectioned in 0.5 μm sections across the *z*-axis. The resulting series of images from each fluorophore was artificially coloured and overlain into one image using IPLAB software (Scanalytics).

Preparation of axenic cultures

Cultures of *Cryptoperidiniopsis* (CCMP1828) were kept in 10 p.p.t. ASW and fed with axenic *Rhodomonas*, as described above at a zoospore–alga ratio of ≈1:30. A modification of the method described by Wong and Wong (1994) was used to prepare axenic cultures. Briefly, *Cryptoperidiniopsis* at a cell density of 10^4-10^5 were treated with 1 mM 5-methoxytryptamine (5-MOT; Sigma-Aldrich) for 4 h in the dark at room temperature to induce encystment. The bacteria were killed by treating the cysts with 0.21% sodium hypochlorite (dilution of Chlorox bleach) for 20 min. The cysts were then pelleted by centrifugation at 800 *g* for 5 min and a subsequent wash with sterile 10 p.p.t. ASW. After removal of 5-MOT and bleach by a second centrifugation step, the cysts were incubated in 10 p.p.t. ASW in a photoperiod room with light–dark cycle of 14:10 and allowed to excyst. The presence of bacteria in the recovered zoospores was checked by plating on 0.5 × marine 2216 agar and by PCR using the methods described above.

Adding back bacteria to axenic zoospore cultures

The culturable bacteria from CCMP1828 were isolated by plating on 0.5× marine 2216 agar as described above. After isolation of discrete colonies, a mixture of the total culturable bacteria was transferred from the plates and resuspended in 10 p.p.t. ASW. The cfus ml^{-1} of the bacterial suspension were obtained, and ≈10^5 bacteria were added to axenic CCMP1828 in a photoperiod room. The density of bacteria in each flask was monitored periodically throughout the experiment by plating aliquots of each culture on 0.5× marine 2216 agar and determining cfus ml^{-1}. At discrete times, 500 μl samples of the dinoflagellate culture were removed, and the zoospores were labelled with 2 μM fluorescent dye CellTracker (Molecular Probes) for 30 min. The zoospores were then concentrated by centrifugation at 800 *g* for 5 min and resuspended in 200 μl of 10 p.p.t. ASW. Labelled zoospores (100 μl per well) were added to the wells of a 96-well plate containing 0.03% SDS to lyse the *Rhodomonas* cells in the culture. An Olympus IX70 epifluorescent microscope was used to count the number of zoospores with a wide blue filter (450–470 nm excitation and 500–530 nm emission). To count the number of prey algae in cultures, 500 μl aliquots were removed and concentrated as described. The total number of cells containing chloroplasts was first determined by counting 100 μl aliquots with the epifluorescent microscope using a wide green filter (510–560 nm excitation and 573–640 nm emission). *Rhodomonas* cells were then lysed by the addition of 0.03% SDS, and the number of dinoflagellate cells containing chloroplasts from the prey was determined by the same fluorescent filter. This number was then subtracted from the total to obtain the number of *Rhodomonas* in the culture at each time interval.

Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences used to generate each phylogenetic tree are as follows: *Agrobacterium sanguineum*, AB021493; *Agrobacterium* str. LMG 11915, AJ130720; *Agromyces cerinus*, X77448; *Agromyces ramosus*, X77447; *Agromyces succinolyticus*, D45055; *Alteromonas macleodii*, X82145; *Alteromonas* sp., AB015135; *Aureobacterium kitamiense*, AB013907; *Aureobacterium testaceum*, X77445; *Azoarcus* str. BS1-14, AF011348; *Azospirillum lipoferum*, Z29619; *Brucella canis*, L37584; *Burkholderia solanacearum*, U28232; *Caulobacter halobacteroides*, AJ007804; *Caulobacter* str. MCS33, AJ227811; *Caulobacter* str. MCS6, M83811; *Clavibacter michiganensis sepedonicus*, U09764; *Coxiella burnetii*, D89798; *Coxiella burnetii* str. Q177, M21291; *Crassostrea virginica* symbiont strain CV919-312, AF114484; *Curacaobacter baltica*, AJ002006; *Curtobacterium citreum*, X77436; *Curtobacterium luteum*, X77437; *Cytophaga fermentans*, D12661; *Cytophaga lytica*, D12666; *Cytophaga* str. BD1-15, AB015524; *Cytophaga* str. L-43-1-N, D12675; *Cytophaga uliginosa*, M62799; *Devosia riboflavina*, D49423; *Erythrobacter* str. MBIC3019, AB012062; *Erythromicrobium ramosum*, AB013355; *Erythromicrobium* str. RE35F/1, AF118020; *Escherichia coli*, J01695; *Flectobacillus* str. MWH38, AJ011917; *Flexibacter aggregans*, M64628; *Flexibacter canadensis*, M62793; *Flexibacter flexilis*, M62794; *Flexibacter maritimus*, M62788; *Glaciecola punicea*, U85851; *Herbaspirillum frisingensis*, AJ238357; *Herbaspirillum seropedicae*, Y10146; *Hyphomonas jannaschiana*, AJ227814; *Janthinobacterium lividum*, Y08846; *Leptothrix discophora*, L33974; *Leptothrix mobilis*, X97071; *Maricaulis maris*, AJ227802; marine bacterium SRF3, AJ002565; *Marinobacter aquaeolei*, AJ000726; *Marinobacter hydrocarbonoclasticus*, X67022; *Microbacterium arborescens*, X77443; *Microbacterium aurum*, AB007418; *Microbacterium imperiale*, D21342; *Microbacterium laevaniformans*, D21344; *Microbacterium terrae*, Y17238; *Microscilla furvescens*, M58792; *Ornithodoros moubata* symbiote A, AB001521; *Oxalobacter formigenes*, U49753; *Phyllobacterium myrsinacearum*, AJ011330; *Pseudomonas balearica*,

AF054936; *Pseudomonas geniculata*, AB021404; *Pseudomonas hibiscicola*, AB021405; *Pseudomonas oleovorans*, D84018; *Pseudomonas syzygii*, U28237; *Rathayibacter tritici*, X77438; *Rhizobium leguminosarum*, D12782; *Rhizobium* sp. LMG 7836, X68389; *Rhodobacter capsulatus*, D16428; *Rhodocyclus purpureus*, M34132; *Rhodocyclus tenuis*, D16209; *Rhodovulum adriaticum*, D16418; *Rhodovulum strictum*, D16419; *Rhodovulum sulfidophilum*, D16423; *Roseobacter algicola*, X78315; *Roseobacter gallaeciensis*, Y13244; *Roseobacter litoralis*, X78312; *Shewanella benthica*, X82131; *Silicibacter lacuscaerulensis*, U77644; *Sphingobacterium heparinum*, M11657; *Sphingobacterium spiritivorum*, D14026; *Sphingomonas subarctica*, X94102; *Stenotrophomonas maltophilia*, AJ131114; *Sulfitobacter pontiacus*, Y13155; uncultured bacterium CLEAR-1, AF146228; uncultured eubacterium DgEPI2, AF059758; uncultured proteobacterium OCS7, AF001645; unidentified alpha-proteobacterium GAI-5, AF007256; unidentified alpha-proteobacterium OM75, U70683; unidentified beta-proteobacterium OM43, U70704; *Zoogloea ramigera* ATCC 25935, X74914.

Sequences of the partial 16S rDNA clones exhibiting unique RFLP patterns obtained from dinoflagellate-associated bacteria were submitted to GenBank under accession no. AF186697 to AF186705, AF188164 to AF188176 and AF190209 to AF190222 as indicated in Table 1.

Acknowledgements

The authors would like to thank Karen Steidinger for providing the *P. piscicida* type strain, Jongsik Chun for the gift of PHYDIT software for taxonomic analysis, and Russell Hill, Kevin Sowers, Juliette Rooney-Varga, as well as the members of the Belas laboratory, for their suggestions and comments regarding this manuscript. We also wish to thank our collaborators Allen Place, Gerardo Vasta and Yonathan Zohar at COMB for numerous and thought-provoking discussions. This work was supported by grants from NOAA ECOHAB (NA86OP0492), NIH NIEHS (PO1-ES9563) and The Knut and Alice Wallenberg Foundation. This is contribution number 8 for the ECOHAB programme.

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