Biofouling is caused initially by bacterial growth and biofilm formation on natural and artificial surfaces. Bacterial biofilms facilitate the attachment and growth of a range of other fouling organisms, such as diatoms, invertebrate larvae, and algal spores (18). Strategies to prevent bacterial biofilms, therefore, represent a powerful approach to the prevention of biofouling.

Biofouling communities on surfaces of marine plants and animals can have detrimental effects on the host organism. Physical damage to the host results from the production of toxins, digestive enzymes, and waste products by the microbial community. However, the extent of biofouling on marine organisms is markedly less than the extent of biofouling on inanimate surfaces. Often the later stages of biofouling, such as the attachment of algae and barnacles, do not occur (2). In contrast to inanimate surfaces, which are colonized in a rapid and predictable manner by a diverse assemblage of marine microbes (62), biotic surfaces frequently harbor species-specific microbial communities (22, 61, 62). These communities can be variable and distinct from those found in the surrounding environment. Thus, many algal and invertebrate species are able to regulate the bacterial colonization of their surfaces (29, 32, 62).

Microalgal surfaces are typically covered by bacteria at densities of approximately 10^7 bacteria cm^{-2} (3). Studies of the epiphytic microbial communities present on macroalgae have emphasized the spatial distribution of bacteria, with specific parts of the thallus hosting specific bacterial populations (9). In some cases the bacterial populations change with the season or the age of the host (55, 56). Associations between algae and bacteria are common, and studies have generally focused on the benefits provided to the bacteria, such as support of bacterial growth by dissolved organic carbon released by algal cells (30, 34, 49).

Although some of the bacterium-alga interactions have been characterized, the ecological significance of most naturally occurring epiphytic bacterial communities is unclear, and in many cases the bacterial species involved have not been identified (17). It has been demonstrated that bacterial biofilms are present on the surface of Ulva australis (formerly known as Ulva lactuca) (54), and it has been speculated that the seaweed uses microbial defense to protect against fouling (24, 27). Ulva australis has no known physical or chemical defense systems against fouling organisms, and it has been suggested that the host may manipulate the bacterial community on its surface, which in turn protects the host by interfering with the development of a mature biofouling community. Such interactions are not uncommon in the marine environment (for reviews see references 4, 7, 15, 19, 26, and 44).

One important species of marine bacteria that is found in association with Ulva australis is Pseudoalteromonas tunicata. This bacterium produces a diverse range of biologically active compounds that specifically target marine fouling organisms (25). One of these compounds is a 190-kDa multisubunit an-
tibacterial protein designated AlpP which is effective against both gram-negative and gram-positive bacteria from a range of environments (28, 37). Recent work has demonstrated that AlpP can provide a competitive advantage to *P. tunicata* during biofilm growth in laboratory biofilm experiments (48).

*Roseobacter gallaeciensis* is also frequently isolated from the surface of *U. australis*. *Roseobacter* spp. are cosmopolitan and have been isolated, for example, from green seaweeds (53), marine snow particles (22), and dinoflagellates (1, 33, 39). *Roseobacter* spp. are able to metabolize dimethylsulfoniopropionate (DMSP), and their presence and activity on algal surfaces are significantly correlated with DMSP-producing algae, including dinoflagellates and prymnesiophytes (20).

The extensive, highly diverse microbial community associated with *U. australis* makes it an interesting study organism for addressing questions of surface colonization and host association. The dynamics of surface colonization in natural systems, particularly during the early stages of biofilm establishment, are poorly understood for marine bacteria. For example, an assessment of the ability of distinct bacteria to colonize algal surfaces would be useful for identifying bacterial traits that contribute to epiphytic fitness. We recently described competitive biofilm interactions of *P. tunicata* and other marine strains in a laboratory glass flow cell system (48). However, little is known about the ecology of colonization and competition on a living surface, including whether *P. tunicata* is a dominant competitor in ecologically relevant settings.

In this study we aimed to investigate, for the first time, the colonization biology of marine bacteria on the surface of a marine plant, in this case *U. australis*. We investigated the hypothesis that *P. tunicata* and *R. gallaeciensis* are effective colonizers of *U. australis* and are able to compete with and dominate other marine bacterial isolates during biofilm formation on the plant surface. We found that *P. tunicata* requires the presence of a natural seawater community to colonize effectively, whereas *R. gallaeciensis* is an aggressive colonizer under all conditions tested. Below we highlight the differences in colonization strategies exhibited by the epiphytic strains of *P. tunicata* and *R. gallaeciensis* and demonstrate that antibacterial compounds have a role in the colonization of *U. australis*.

This study demonstrated that microbial colonization of the plant surfaces is a dynamic process in which differences in attachment, colonization, and competitive biofilm formation can markedly affect the establishment and organization of epiphytic microbial communities.

**MATERIALS AND METHODS**

Collection of plants and generation of axenic *U. australis*. The common marine alga *U. australis* was collected from the rocky intertidal zone (latitude, 151.2572; longitude, −33.9121) near Sydney, Australia. The method used to make *U. australis* axenic was modified from the method described by Schell (51). Briefly, plants were rinsed in 50 ml autoclaved seawater and cut into 5-cm-long fragments. These fragments were swabbed with cotton buds, and 0.6-cm disks were punched out of the thallus using a cork borer. The disks were rinsed in autoclaved seawater and incubated in 0.012% NaOCl for 5 min. The disks were allowed to recover in sterile seawater for 1 h before they were incubated in an antibiotic cocktail (300 mg liter⁻¹ ampicillin, 30 mg liter⁻¹ polymixin B, and 60 mg liter⁻¹ gentamicin dissolved in sterile seawater) for 18 h in 24-well tissue culture plates (Sarstedt). Disks were then incubated in 0.008% NaOCl for 5 min. These disks were allowed to recover in sterile seawater for at least 3 h to remove traces of oxidants, suspended in 2 ml of sterile seawater, and agitated at room temperature at 60 rpm.

Plant tissue viability was examined by staining treated *U. australis* disks with 0.25% (wt/vol) Evans blue (60). This blue dye penetrates into dead and damaged plant cells, while intact cells are able to exclude the dye. All colonization and competition experiments were done in triplicate, and three to five individual disks were randomly sampled at each sampling time.

**Colonization experiments.** Bacterial strains were isolated from the surface of *U. lactuca* as described previously (48). Cultures were stored at −80°C in 50% (vol/vol) glycerol in VNSS medium (36) and maintained on VNSS medium plates. *P. tunicata* and *R. gallaeciensis* were labeled with a green fluorescent protein (GFP) color tag as described previously (48). Bacteria were cultured for 24 h at 25°C in VNSS broth for preparation of inocula. Cells were harvested by spinning down the culture and resuspending the pellet in seawater. The cell concentration was estimated by counting cells by epifluorescence microscopy using a hemocytometer and was adjusted to the desired level by dilution with seawater. Experiments were conducted in Sarstedt 24-well plates with axenic disks, and the bacteria were applied by immersing the disks in a suspension of *P. tunicata* and *R. gallaeciensis* for 3 h. The disks were then rinsed twice in filtered seawater and transferred to fresh Sarstedt 24-well plates containing 2 ml of filtered seawater. The plates were incubated on a shaker at 60 rpm and 25°C for 16 h in the presence of light.

**Factors affecting colonization.** In order to investigate some of the factors that were essential for colonization of *P. tunicata*, we tested a range of conditions.

(i) **Density of *P. tunicata* required for attachment.** *P. tunicata* was inoculated onto axenic *U. australis* disks in 24-well tissue culture plates at a range of concentrations (10⁴ to 10⁶ cells ml⁻¹). The disks were incubated for 3 h, rinsed twice in sterile seawater, and then incubated at 25°C and 60 rpm for the duration of the experiment.

(ii) **Axenic versus nonaxenic plant surfaces.** The attachment of *P. tunicata* cells which had been inoculated at a concentration of 10⁶ cells ml⁻¹ was compared on the both axenic and nonaxenic surfaces of *U. australis* disks.

(iii) **U. australis disks versus whole plants.** Attachment was tested on axenic disks in 24-well plates, as well as on axenic whole plants (which had been treated in the same way as the disks). The plants were oriented upright in beakers and immersed in filtered seawater.

(iv) **Dark versus light.** Preparations were inoculated in the dark at a concentration of 10⁷ cells ml⁻¹ and then incubated in the dark for 3 h. This procedure was compared to inoculation at the same density and incubation for 3 h in daylight.

(v) **Carbon source.** The standard culture conditions for marine strains included VNSS medium containing glucose. However, we also grew cells for 48 h in minimal medium (45) with celllobiose as the sole carbon source in a still culture. The cells were inoculated onto *U. australis* disks, and attachment was compared to the attachment of cells grown in minimal medium with trehalose and glucose as carbon sources.

(vi) **Incubation time.** Disks were inoculated with 10⁷ cells ml⁻¹ and incubated for 1, 3, 12, and 24 h.

(vii) **Effect of multispecies consortia on colonization.** Axenic algal disks were inoculated with overnight cultures of GFP-labeled *P. tunicata* and 17 other *P. tunicata* strains isolated from the surface of *U. australis* (48) in filtered seawater. *P. tunicata* was also inoculated into a natural seawater community. The resulting mixed-species biofilm was visualized by staining with acridine orange. Attachment and colonization of GFP-labeled *P. tunicata* within the mixed biofilm was visualized by observing the unstained biofilm with a confocal microscope.

To test all factors, a density of 10⁷ cells ml⁻¹ was used unless otherwise stated, and *P. tunicata* cells were suspended in filtered seawater. Attachment, biofilm formation, and microcolony development were monitored by epifluorescence microscopy. The times when preparations were examined were immediately after inoculation and 1, 3, and 7 days following inoculation. At each sampling time, three disks were randomly selected, and a total of 12 random fields of view were counted for each disk. Samples were viewed by epifluorescence microscopy with an Axiopt microscope equipped with a 10× 1.30-numerical-aperture objective (Leica).

Although preliminary experiments indicated that *R. gallaeciensis* was an aggressive colonizer of *U. australis*, we also tested attachment and colonization of this organism under all the experimental conditions used for *P. tunicata*.

**Competition in dual-species biofilms on *U. australis*.** Other marine organisms used in dual-species competition experiments were *Pseudoalteromonas gracilis, Alteromonas sp.*, and *Cellulophaga fuscicola*, all of which were isolated from *U. australis* as described in sterile previously (48). The strains were labeled with red fluorescent protein (DsRed) and GFP as described previously (48). The growth rates of wild-type marine isolates and their labeled derivatives on the surface of axenically treated *U. australis* did not differ significantly (data not shown). Overnight cultures of bacteria were inoculated onto *U. australis* at a density of
10^6 cells ml^-1 in filtered and natural seawater and incubated for 3 h without shaking at room temperature. Disks were rinsed three times with filtered seawater, transferred to sterile seawater in Sarstedt 24-well plates, and incubated at 25°C at 60 rpm for the duration of the experiment. Unlabeled biofilms were shaking at room temperature. Disks were rinsed three times with filtered seawater. In order to ensure that the initial ratio of attached cells of the two competing strains was 1:1, we first monitored the initial attachment of the mixed culture after a 3-h adhesion period. In most cases, the initial levels of attachment to the plant surface were deemed to be approximately equal, as determined by counting the numbers of red- and green-labeled cells by epifluorescence microscopy.

To investigate whether P. tunicata and R. gallaeciensis were able to invade and colonize an established biofilm, marine strains (P. tunicata, C. fucicola, and Alteromonas sp.) were allowed to preestablish a biofilm for 48 h on U. australis. The preformed biofilm was inoculated with ~10^6 cells of wild-type P. tunicata or R. gallaeciensis, incubated for 3 h without shaking, and then incubated at 60 rpm for the duration of the experiment. After the resumption of shaking, the biofilm was examined for red and green fluorescence. Experiments were repeated in three separate rounds with three independent 24-well plates run in parallel. The viability of bacterial cells was determined using a Live-Dead staining kit (Molecular Probes Inc., Eugene, Oreg.).

RESULTS

Obtaining axenic plant tissue. In order to conduct the attachment and colonization experiments, it was necessary to remove the majority of bacteria on the U. australis thallus surface. Observation of untreated algal tissue revealed that there was a complex community of epiphytic bacteria (approximately 460 cells cm^-2) (Fig. 1A). However, in treated axenic tissue there were negligible numbers (<50 cells cm^-2) of attached solitary cells on the plant surface (Fig. 1B). Antibiotic treatment resulted in removal of approximately 90% of the epiphytic bacteria, while there was minimal damage to the U. australis tissue (as determined by the Evans blue test for plant viability) (data not shown). Axenic disks that were not inoculated with bacteria in our experiments bleached rapidly and died within 1 week. However, our data suggest that plant death was probably not the direct result of the chemical treatment used to make the plants axenic. Rather, other factors appeared to play a role in the bleaching of plant material after treatment. This was shown by (i) Evans blue staining of plant tissue, which revealed that the tissue was viable and healthy after treatment, and (ii) the fact that treated plant disks that were reinoculated with bacteria in the course of the experiments did not bleach, suggesting that the microbial community itself may contribute to the fitness of the host.

Factors influencing attachment and colonization. Preliminary experiments showed that P. tunicata colonized the surface of U. australis poorly, which led us to test attachment under different conditions. Our data provide quantitative information concerning the attachment of P. tunicata on the surface of U. australis under a range of different environmental conditions (Table 1). P. tunicata harboring a GFP reporter gene was visualized by epifluorescence microscopy to differentiate P. tunicata cells from indigenous bacteria on the algal surface. We observed cells after a 3-h attachment period and found that they were not randomly scattered across the U. australis thallus surface. Rather, cells were distributed in patches on the plant surface. Observations after 24 h of growth showed that some of the attached cells had divided and formed microcolonies, which occurred in a wide range of sizes (5 to 50 μm). Observations after 3 days of incubation revealed that vast areas of U. australis thallus tissue remained uncolonized and that microcolonies were restricted to certain regions.

On the other hand, R. gallaeciensis formed microcolonies which were dispersed evenly over the surface of the thallus tissue. At 24 h, R. gallaeciensis had formed cell chains, which by day 3 had developed into small microcolonies (diameter, 10 to 20 μm). By day 7, these microcolonies had merged to form a

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of cells attached (cells cm^-2) (mean ± SE) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Density, 10^6 cells ml^-1</td>
<td>0</td>
</tr>
<tr>
<td>Density, 10^7 cells ml^-1</td>
<td>0</td>
</tr>
<tr>
<td>Density, 10^8 cells ml^-1</td>
<td>52 ± 3.0</td>
</tr>
<tr>
<td>Density, 10^9 cells ml^-1</td>
<td>224 ± 4.9</td>
</tr>
<tr>
<td>Density, 10^10 cells ml^-1</td>
<td>1,880 ± 58^b</td>
</tr>
<tr>
<td>Axenic disks</td>
<td>290 ± 11.4</td>
</tr>
<tr>
<td>Nonaxenic disks</td>
<td>270 ± 10</td>
</tr>
<tr>
<td>Carbon source, trehalose</td>
<td>250 ± 6.9</td>
</tr>
<tr>
<td>Carbon source, cellobiose</td>
<td>460 ± 7.7^b</td>
</tr>
<tr>
<td>Incubation time, 1 h</td>
<td>184 ± 6.5</td>
</tr>
<tr>
<td>Incubation time, 3 h</td>
<td>302 ± 9.3^b</td>
</tr>
<tr>
<td>Incubation time, 12 h</td>
<td>280 ± 11.4</td>
</tr>
<tr>
<td>Incubation time, 24 h</td>
<td>263 ± 9.2</td>
</tr>
<tr>
<td>Incubation in the light</td>
<td>250 ± 6.8</td>
</tr>
<tr>
<td>Incubation in the dark</td>
<td>374 ± 12.6^b</td>
</tr>
<tr>
<td>P. tunicata in autoclaved seawater</td>
<td>250 ± 6.6</td>
</tr>
<tr>
<td>P. tunicata in filtered seawater</td>
<td>263 ± 5.8</td>
</tr>
<tr>
<td>P. tunicata in natural seawater</td>
<td>539 ± 15.1^b</td>
</tr>
</tbody>
</table>

^a To test all factors apart from density, a density of 10^6 cells ml^-1 was used. The counts are the average values for 12 fields of view for each disk, which were converted to number of cells cm^-2.

^b Significant value.
relatively flat, mat-like biofilm, which covered a large proportion of the algal surface. Also in contrast to *P. tunicata*, *R. gallaeciensis* cells attached and colonized effectively under all conditions tested. At cell densities greater than $10^6$ cells ml$^{-1}$, colonization of *U. australis* by *R. gallaeciensis* appeared to be similar in all situations (data not shown). Furthermore, attached cells persisted indefinitely on algal disks. Compared to the analysis of *P. tunicata*, the smaller cell size and the high rates of cell attachment of *R. gallaeciensis* made counting difficult; hence, cell attachment was not quantified.

(i) Effects of inoculum cell density on colonization of *U. australis*. Direct in situ observation of epiphytic bacteria revealed that the density of cells in the inoculum affected microcolony formation. After an initial attachment period (3 h), we incubated disks for 24 h and then examined the plant surface for bacterial cells and microcolony formation. Surprisingly, no attachment took place when *P. tunicata* was inoculated at low densities ($10^4$ or $10^5$ cells ml$^{-1}$) (Table 1). At a density of $10^6$ cells ml$^{-1}$ we observed that few cells attached ($52$ cells cm$^{-2}$). These cells were mostly solitary; no microcolonies formed, and cells usually did not persist for more than 3 days. At a density of $10^7$ cells ml$^{-1}$ some of the attached cells were clustered together and formed small microcolonies consisting of about 15 to 20 cells after 24 h. At a density of $10^8$ cells ml$^{-1}$, microcolonies were considerably larger (up to 100 μm across) after the same growth period (24 h). In spite of the large sizes of microcolonies obtained with high cell densities, *P. tunicata* did not persist for more than 5 days. Although *R. gallaeciensis* was able to colonize at lower densities ($10^4$ and $10^5$ cells ml$^{-1}$), the persistence was much greater at higher densities ($10^6$ to $10^9$ cells ml$^{-1}$). At higher densities *R. gallaeciensis* rapidly formed microcolonies that survived and persisted indefinitely on the plant, compared to the colonies that formed at a low density, which did not remain for more than 5 or 6 days. Thus, both organisms exhibited density-dependent colonization and enhanced microcolony formation at high cell densities.

(ii) Colonization in the dark. On the basis of our observation of surprisingly low levels of attachment of *P. tunicata* on the plant surface, we investigated the possibility that *P. tunicata* attachment may be affected by the presence of DMSP on the plant surface. It is known that DMSP is released in senescent algae or when algae undergo oxidative stress, particularly under high light intensities (31). We therefore examined colonization of *U. australis* by *P. tunicata* in the dark. We observed that attachment of *P. tunicata* improved when cells were inoculated in the dark, and the number of cells that attached increased from 250 cells cm$^{-2}$ to 374 cells cm$^{-2}$ (Table 1). However, the cells did not persist and form biofilms. Most of the microcolonies formed were small, although the number was greater (about 16 to 20 microcolonies per cm$^{-2}$ with 12 to 25 cells in each microcolony, compared to about 3 to 6 microcolonies per cm$^{-2}$ with 50 to 90 cells in each microcolony for plant tissue incubated in the presence of light). We repeated these sets of experiments using the Live-Dead stain to establish whether cells in smaller microcolonies died or whether they simply failed to attach. However, the results were inconclusive as it was difficult to visualize the dead cells against the red autofluorescence of the algae. In contrast, *R. gallaeciensis* was able to attach and colonize at a density of $10^6$ cells ml$^{-1}$ regardless of whether the preparations were inoculated in the light or in the dark.

(iii) Carbon source. We found that *P. tunicata* cells pregrown in media containing cellobiose as a sole source of carbon were able to attach better on the surface of *U. australis*. As shown in Table 1 and Fig. 2A, the number of *P. tunicata* cells that were able to attach to the surface of the algae was much higher for cells grown in cellobiose than for cells grown in glucose or trehalose as a carbon source (Fig. 2B). However, similar to glucose- or treha-
lose-grown cells, the cellobiose-grown cells did not persist for more than 5 days on the plant surface.

(iv) Inoculation in natural seawater and mixed-species biofilm development. We compared colonization of *U. australis* by *P. tunicata* cells suspended in sterile filtered seawater, autoclaved seawater, and natural seawater. We observed that in sterile filtered water, *P. tunicata* did not persist for more than 5 days (Fig. 3A and Table 1). We also observed that colonization was most effective in natural seawater. Not only was there a higher level of attachment in this system, but cells also became established and persisted indefinitely on the surface of the algal thallus. Together, the bacteria formed a mixed biofilm, which was also able to persist for up to 3 weeks (C). GFP-labeled *P. tunicata* cells were enumerated under an epifluorescence microscopy (Table 1) before being stained with 0.01% acridine orange as shown here. Scale bars = 50 μm.

FIG. 3. Comparison of biofilm formation by mixed-species biofilms containing *P. tunicata* cells suspended sterile seawater, in natural seawater, or a mixture of 17 epiphytic strains. *P. tunicata* inoculated into filtered seawater did not persist for more than 7 days (A). *P. tunicata* inoculated into natural seawater was able to attach to *U. australis* and form biofilms that persisted for up to 3 weeks (B). *P. tunicata* inoculated with a mixture of 17 strains isolated from *U. australis* formed a complex biofilm, which was also able to persist for up to 3 weeks (C). GFP-labeled *P. tunicata* cells were enumerated under an epifluorescence microscopy (Table 1) before being stained with 0.01% acridine orange as shown here. Scale bars = 50 μm.

To investigate the role of the antibacterial protein AlpP of *P. tunicata* in competitive biofilm development on *U. australis*, we repeated the mixed-inoculum biofilm experiments described above, using the ΔalpP strain instead of the wild type. When competing strains were inoculated simultaneously, no difference in the outcome of the biofilm competition experiments with either the wild-type or ΔalpP strain was observed (data not shown). However, we found that AlpP can play an important role in the colonization of preestablished biofilms of other marine strains on *U. australis*. The ΔalpP strain had a greatly reduced ability to colonize and to establish a biofilm in 48-h-old biofilms of *P. gracilis*, *C. fucicola*, and *Alteromonas* sp. For example, the ΔalpP strain was unable to colonize and invade a
preestablished biofilm of *P. gracilis* (Fig. 4C). This mutant was unable to form microcolonies, and the few attached cells had little or no impact on the proliferation of competing strains.

**DISCUSSION**

Little is known about the establishment and ecological role of microbial communities on the surfaces of marine algae. However, the abundance of bacteria that produce extracellular inhibitory compounds on the surface of the marine alga *U. australis* has prompted speculation that these organisms may protect the alga against fouling (14, 26). In this study we explored some of the factors that influence attachment to and colonization of *U. australis* by epiphytic bacteria, as well as the competitive interactions that occur between bacterial strains on the plant surface.

**Effect of cell density on attachment.** One of the most important factors that influenced the attachment of *P. tunicata* suspended in filtered seawater was the density of cells in the inocula. We found, not surprisingly, that the number of cells attached to the surface was higher when inocula with higher concentrations of bacteria were used. However, we also observed that surprisingly high densities of cells were necessary for *P. tunicata* to become established and grow in microcolonies. Although *R. gallaeciensis* was able to attach at lower densities, biofilm formation and colonization were much improved at higher densities in a manner similar to the manner observed for *P. tunicata*, perhaps also because high-density inocula allowed the formation of larger numbers of microcolonies by this organism. The dependence on high cell densities for microcolony formation has been reported previously for biofilms on leaf surfaces (41, 42, 65). In these studies, there was evidence that cells in aggregates had a much greater ability to survive periodic desiccation stress, and increased tolerance was correlated with the size of aggregates (41). Presumably, cells in larger microcolonies are more tolerant of oxidative stress than cells located in smaller microcolonies are (10), and they may also be more capable of concentrating nutrients from dilute sources, which can have important consequences in a nutrient-poor environment.

The mechanism by which high cell densities allow enhanced microcolony formation is unclear, but quorum sensing may have a role in the colonization of *U. australis*. Quorum sensing is known to play a role in the formation of multicellular structures within biofilms (12, 23). To date, there is no evidence that *P. tunicata* produces signaling molecules (24), but recent studies in our laboratory have shown that *R. gallaeciensis* produces *N*-acyl-homoserine lactones, suggesting that it is capable of quorum sensing (R. Case, A. Low, and S. Kjelleberg, unpublished data). In some epiphytic bacteria, quorum sensing has previously been shown to play a role in colonization of the plant, and studies with *Pseudomonas syringae* have suggested that *N*-acyl-homoserine lactone production makes an important contribution to epiphytic fitness in the early stages of colonization of bean leaves (47).

**Attachment in the dark.** Inoculation in the dark clearly enhanced attachment of *P. tunicata* cells suspended in filtered seawater, indicating that this organism may be sensitive to DMSP or reactive oxygen species present on the surface of the
algal DMSP occurs in many diverse species of algae, including *Ulva australis* (14), and is released in senescent algae or under oxidative stress conditions, particularly under high light intensities (31, 59). There is evidence that DMSP provides protection against photooxidation for algae (59), but its effects on epiphytic bacterial communities on *Ulva* spp. are not fully understood (46). We observed that attachment of *P. tunicata* in the light resulted in fewer but larger microcolonies, whereas microcolonies established from cells inoculated in the dark were smaller and more numerous. One possibility is that cells in larger more resistant microcolonies survived exposure to oxidative stress, which increased with higher light intensities. *R. gallaeciensis* was able to attach and colonize effectively regardless of the light conditions, suggesting that it may be able to tolerate and/or metabolize DMSP (59). In studies of the diversity of dimethyl sulfide-producing bacteria from oceanic and estuarine waters it was found that all the isolates belonging to the *Roseobacter* group tested were dimethyl sulfide producers and therefore could utilize DMSP (21). Another proposed ecological role for DMSP is that it acts as a precursor of cues for chemosensory attraction between algae and certain specific bacteria (57). Thus, DMSP may be one of the chemical signals released by *U. australis* which is recognized by *R. gallaeciensis*. Furthermore, it has been proposed that the substrate for the sulfur-based inhibitory compounds produced by *R. gallaeciensis* (thiotropocin and tropodithietic acid) is DMSP (8).

**Effect of cellulose on attachment.** It is evident that *P. tunicata* attachment improved considerably when cells were grown in cellulose and then suspended in filtered seawater, but the cells did not persist on the surface of *U. australis*. Both *U. australis* and *Ciona intestinalis*, from which *P. tunicata* is most commonly isolated, contain accessible cellulose polymers in their cell walls (5, 13). A cellulose binding protein with a high binding affinity for microcrystalline cellulose was discovered in *P. tunicata* (11). Clearly, cellulose plays a role in attachment, but its effects may be restricted to the early stages of colonization, in which it has been suggested to function as an anchorage or cue for *P. tunicata* attachment (58). Recent studies have found that cellulose stimulated the production of pigment in this organism, which is known to be coregulated with expression of AlpP (58), and also induced the expression of mannose-sensitive hemagglutinin (type IV-like) pili in *P. tunicata* (11). Thus, cellulose appears to play an important role in the colonization and responses of *P. tunicata* on the surface of *U. australis*.

**Synergistic biofilm formation.** *P. tunicata* persisted on the surface of the algae only if it was inoculated in seawater that contained a natural seawater community or if it was inoculated in filtered seawater that contained a mixture of epiphytic strains isolated from *U. australis*. This suggests that *P. tunicata* requires the presence of diverse bacteria in the inoculum for effective colonization, which may allow succession and/or cooperative colonization of the surface. Cocolonizing bacteria could modify the habitat and create a “microenvironment” that encourages the attachment and growth of other colonizing microorganisms. We tested whether a preexisting biofilm enhances the attachment and persistence of *P. tunicata* by allowing it to colonize nonxenic *U. australis* disks with the intact natural community on the surface. However, *P. tunicata* was unable to colonize under these conditions, indicating that an established biofilm of other bacteria does not facilitate the colonization. Another possibility is that cooperative interactions during the process of colonization (for example, cometabolism or coaggregation) can occur between *P. tunicata* and other cocolonizing marine strains. The phenomenon of cooperative biofilm formation in mixed consortia has recently been described for other bacteria (16, 52). Of the 17 strains, it is not clear which strains or mixtures of strains are responsible for allowing *P. tunicata* to colonize. The underlying mechanisms of cooperative biofilm formation are the basis of ongoing experiments in our laboratory.

Attachment and colonization of *U. australis* by *P. tunicata* appears to be influenced by many factors. Attachment and colonization are particularly poor at low cell densities. The combination of environmental factors that appear to enhance colonization at a concentration of 10^7 cells ml\(^{-1}\) does not seem to be effective at lower cell densities (10^4 to 10^5 cells ml\(^{-1}\)). It appears that colonization is limited by nutrient availability, but it is also possible that unknown defensive chemicals produced by the plant play a role.

**Competition in biofilms on *U. australis***. In coinoculation competition studies, we observed that *P. tunicata* suspended in filtered seawater outcompeted all of the other marine isolates tested except *R. gallaeciensis*. The results are similar to our previous findings of competition on glass surfaces in a laboratory flow cell model. In that study, we demonstrated that competition was largely controlled by the production of inhibitory compounds and the relative sensitivity of competing strains to the inhibitors (48).

However, we did observe some important differences between bacterial competition on the surface of the marine plant and the competition in laboratory systems in the preestablished biofilm studies. Unlike laboratory flow cells, which allow a continuous flow of nutrients, the surface of algae likely presents nutrient-limited conditions. Our studies demonstrated that exogenous application of nutrients resulted in a rapid increase in the microcolony size of *P. tunicata* (data not shown), suggesting that microcolonies on the plant surface are indeed nutrient limited. Furthermore, our observation of the patchy distribution of microcolonies might reflect the spatial heterogeneity of nutrients available on algal surfaces. Several studies conducted with higher terrestrial plants suggested that most areas of a leaf harbor only small amounts of nutrients (35, 63), and on bean plants inoculated with *P. syringae* strain B728A aggregates of bacteria were distributed nonrandomly in a wide range of cluster sizes, which roughly corresponded to nutrient availability (36). Our observation that *P. tunicata* colonized a preestablished biofilm poorly suggest that the initial colonizers could deplete a large percentage of carbon sources and make it more difficult for the invading bacteria to sequester themselves in the existing biofilm community. This has been described for epiphytic bacteria on bean leaves, on which a preestablished biofilm can deplete resources, leading to preemptive exclusion (64).

In competition studies in which *P. tunicata* was allowed to invade a preestablished biofilm, it did not outcompete the other strains and coexisted with the competing strain for the duration of the experiment. When invading a preestablished biofilm, *P. tunicata* tends to colonize and establish microcolonies in areas which remain free from colonization by a prees-
established biofilm. This results in spatially segregated microcolonies of competing strains with limited interactions and leads to coexistence of the species. Our observations of competitive interactions on the surface of U. australis are supported by recent reports on spatial segregation of epiphytic bacteria on leaf surfaces (40, 43). The observations differ from the results of flow cell experiments in which P. tunicata was able to dominate and eventually remove certain competing strains (48). The evidence of a reduced ability to colonize a preestablished biofilm was even more pronounced for the AlpP mutant. The ability of the mutant to form microcolonies in a preestablished biofilm was impaired, and the mutant had minimal impact on the competing strain, which remained dominant. Thus, our results indicate that AlpP enhances colonization of a preestablished biofilm by P. tunicata.

In contrast, R. gallaeciensis did not seem to be constrained by nutrient limitations and formed a biofilm which eventually covered most of the surface of the alga. An ability to utilize a wide range of carbon sources (50) and an ability to metabolize DMSP may enable this organism to colonize regions of the plant that are inhospitable to other bacteria. Even when colonizing a preestablished biofilm, R. gallaeciensis might be able to access nutrients that are inaccessible to other epiphytic bacteria, and this ability might contribute to its success and epiphytic fitness (6).

Conclusions. P. tunicata and R. gallaeciensis were found to have different colonization and competition behaviors during colonization of U. australis. While R. gallaeciensis is capable of colonizing U. australis under a range of conditions, colonization by P. tunicata is enhanced by high cell densities, the presence of cellbiose in the precurcure, inoculation in the dark, and interactions with a natural seawater community to attach and persist on the surface of the alga. The epiphytic fitness of R. gallaeciensis may be attributed to several factors, including its versatility in utilizing a number of carbon sources (particularly those not available to competing strains) and the production of antibacterial compounds and signaling molecules.

Competition in which a preestablished biofilm is challenged with P. tunicata results in the coexistence of competitors. This may be due in part to the protective nature of microcolonies, which may resist invasion. There is a diffusion gradient in microcolonies, so that metabolically active cells at the outer edge of the microcolony may perish while cells in the deeper regions are likely to be protected from the antibacterial protein. Limited nutrients on the surface of U. australis may lead strains to inhabit distinct niches on the plant, and this may also result in the coexistence of competing strains. Microcolonies of competing strains are spatially separated, which may limit microbial interactions and provide refuges for competitors. R. gallaeciensis does not seem to be constrained by low-nutrient conditions and is able to invade and disperse competing strains, suggesting that its antibacterial protein is able to diffuse through microcolonies.

The environmental factors that influence nutrient accumulation on the surface of the seaweed and in turn microbial colonization and competitive interactions are complex. We propose that these factors may have a profound impact on the composition and activity of the epiphytic community.

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