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Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms†

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Most biofilms in their natural environments are likely to consist of consortia of species that influence each other in synergistic and antagonistic manners. However, few reports specifically address interactions within multispecies biofilms. In this study, 17 epiphytic bacterial strains, isolated from the surface of the marine alga *Ulva australis*, were screened for synergistic interactions within biofilms when present together in different combinations. Four isolates, *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii*, were found to interact synergistically in biofilms formed in 96-well microtiter plates: biofilm biomass was observed to increase by >167% in biofilms formed by the four strains compared to biofilms composed of single strains. When exposed to the antibacterial agent hydrogen peroxide or tetracycline, the relative activity (exposed versus nonexposed biofilms) of the four-species biofilm was markedly higher than that in any of the single-species biofilms. Moreover, in biofilms established on glass surfaces in flow cells and subjected to invasion by the antibacterial protein-producing *Pseudoalteromonas tunicata*, the four-species biofilms resisted invasion to a greater extent than did the biofilms formed by the single species. Replacement of each strain by its cell-free culture supernatant suggested that synergy was dependent both on species-specific physical interactions between cells and on extracellular secreted factors or less specific interactions. In summary, our data strongly indicate that synergistic effects promote biofilm biomass and resistance of the biofilm to antimicrobial agents and bacterial invasion in multispecies biofilms.

Bacteria in natural habitats commonly exist in biofilm consortia. In fact, it has been estimated that the majority of bacteria in natural aquatic ecosystems are organized in biofilms (8). In a biofilm, a microbial community is attached to a surface and embedded in a self-produced matrix composed of extracellular polymeric substances. This structure provides the bacteria present in the biofilm with several advantages compared to those living as planktonic cells. First, the bacteria are maintained in the selected microenvironment where population survival does not depend on rapid multiplication (24). This is especially advantageous in environments where the bacteria are exposed to constant liquid movements, as, for example, in aquatic environments. Additionally, the bacterial cells present in a biofilm have an increased resistance to desiccation, grazing, and antimicrobial agents compared to their planktonic counterparts (15, 24, 28, 32, 53). Also, biofilms offer enhanced opportunities for interactions such as horizontal gene transfer and cometabolism (24, 34, 51).

It is likely that most natural biofilms exist as multispecies consortia. While single-species biofilms have been studied extensively, we know very little about mixed-species biofilms and their interactions. However, in several studies, of which the majority focused on biofilms in the oral cavity, it has been demonstrated that different bacterial species in biofilms affect one another positively as well as negatively. Interactions beneficial to one or more strains or species include coaggregation of cells (37, 42, 47, 59), conjugation (17), and protection of one or several species from eradication when the biofilm is exposed to antimicrobial compounds (7, 13, 27). Such protection may be caused by a variety of factors, including enzyme complementation (48) and organized spatial distribution of the cells in the biofilm (7, 27). These and other mechanisms are likely to deliver synergistic effects that result in cooperative biofilm formation by strains that were unable to form a biofilm alone (14, 17, 38). Reports on negative interactions in biofilms include production of bacteriotoxins (41, 54) and lowering of pH (6, 49) by one member of the biofilm consortium. Generally, an important aspect of describing the interactions in multispecies biofilms is to evaluate whether individual species, or the multispecies consortium, gain any fitness advantages compared to single-species biofilms. A fitness advantage in this context is defined as the ability of the organism or biofilm to persist or grow in a given environment or under a particular environmental stress.

Bacteria on marine, living surfaces appear to form spatially structured, host-specific, temporally stable communities (4, 40, 55). Competition between bacteria, the ability to resist grazing, and host-derived factors such as surface-localized secondary metabolites are some factors likely to determine the final sur-

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† Supplemental material for this article may be found at http://aem.asm.org/.

face community composition (18, 30, 32, 41, 55). One system for studying bacteria associated with marine living surfaces is that of the marine alga *Ulva australis* (formerly known as *Ulva lactuca*) and its epiphytic microbial community. The composition of this epiphytic biofilm community is known to be essential for the normal growth and development of the alga and has also been suggested to assist the plant in its defense against colonization by fouling organisms (10, 12, 21).

The aim of this study was to evaluate whether synergistic interactions occur during multispecies biofilm formation by epiphytic marine bacteria isolated from *Ulva australis* and whether multispecies biofilms offer enhanced fitness compared to single-species biofilms.

MATERIALS AND METHODS

Isolation of bacterial strains. The bacterial strains were isolated from the surface of the marine alga *Ulva australis* as described by Rao et al. (41) and were subcultured at 25°C on complex Väätänen nine-salt solution (VNSS) marine medium (31) agar plates (containing 15 g agar/l) or in 5 to 10 ml of VNSS medium broths with shaking at 160 rpm.

Quantification of biofilm formation by use of CV. In order to identify and select epiphytic isolates with poor biofilm formation and subsequently determine whether the biofilm formation ability differed when these isolates were grown individually compared to in multispecies consortia, quantification of cell adhesion and biofilm formation by single- and multispecies consortia was performed. The method used was a modified version of that described by O'Toole and Kolter, based on staining biofilms with crystal violet (CV) (36). CV binds to negatively charged molecules, including nucleic acids and acidic polysaccharides, and therefore serves as an overall measure of the whole biofilm. Strains were subcultured from frozen glycerol (30%) stocks onto VNSS agar plates for 48 h, and from these, colonies were transferred to 9 ml of VNSS broth and incubated for 48 h. Strains were then harvested by centrifugation (6,000 × *g*, 5 min) and resuspended in 4.5 ml VNSS. The cell densities of all suspensions were adjusted to an optical density at 600 nm (OD_{600}) of 0.15 by dilution in VNSS medium. The strains were inoculated into flat-bottomed 96-well Costar microtiter plates (no. 3595, "96 wells Cell Culture Cluster" polystyrene; Corning Inc.). The final volume added to each well was 200 μl. To some wells, 200 μl of VNSS medium was added to serve as negative controls and to obtain a background value, which was subtracted from values obtained from the wells containing cells. Plates were then sealed with Parafilm and incubated with shaking (100 rpm) at room temperature for 24 h.

To correlate biofilm formation with planktonic cell growth in each well, the planktonic cell fraction was transferred to new microtiter plates and the OD_{600} was measured. The attached cells were rinsed three times with 200 μl phosphate-buffered saline (46) and stained with 200 μl of an aqueous CV solution (1%). After 20 min of staining, CV was removed and cells were rinsed once with 200 μl, once with 400 μl, and twice with 200 μl of PBS. To resuspend the CV, 200 μl of ethanol (96%) was added to each well, and the absorbance of CV at 600 nm was measured in a Wallac-Victor^2 1420 Multilabel Counter (Perkin-Elmer, Boston, MA). When the CV absorbance increased to an OD_{600} of above 1.5, the CV-ethanol was diluted 1:3 in 96% ethanol. Finally, the absorbance measurements obtained were related to the OD_{600} of the planktonic cell fraction.

16S rRNA gene-based identification of bacterial strains. On the basis of the results obtained in the screening of multispecies biofilm formation, four strains that interacted synergistically were chosen for species identification. Aliquots (1 ml) of overnight cultures of each of the isolates 2.04, 2.12, 2.3, and 2.34 were boiled for 10 min, chilled on ice, and centrifuged at 0°C and 14,000 g for 2 min. From the supernatants, 1 μl was used as the template in PCRs with primers designed to amplify the eubacterial 16S rRNA gene (27F and 1492R) (26). Sequencing reactions (DYEnamic ET dye terminator cycle sequencing kit [MegaBACE]) were performed using 0.1 μg purified PCR product and 10 pmol of primers (27F, 1100R, and 1492R) (26) in 10-μl reaction mixtures, and sequencing was performed using a MegaBACE 1000 sequencer (Molecular Dynamics, Sunnyvale, CA). Sequences obtained were compared to sequences available in the NCBI (National Center for Biotechnology Information) BLAST 2.0 database (Basic Local Alignment Search Tool) (2).

Resistance to antimicrobial agents in single- and multispecies biofilms. Examination of the activities of single- and four-species biofilms of strains 2.04, 2.12, 2.3, and 2.34, with and without the addition of antimicrobial agents, was performed by use of the respiratory indicator 2,3,5-triphenyltetrazolium chloride (TTC). This compound is soluble and colorless in its original state but forms a red, insoluble salt when it is reduced by the oxidative enzyme complexes in the bacterial cell (16). Microtiter plates were inoculated and incubated as described above. After 24 h, the VNSS medium and planktonic cells were discarded, and the wells were rinsed once (to remove planktonic cells) with fresh VNSS. Then, 200 μl VNSS containing either hydrogen peroxide (1,700 μg/ml) or tetracycline (20 μg/ml) was added to the wells. To some wells, VNSS without inhibitory compounds was added. These wells served as measures of the activity of the biofilms without inhibition, and the activity measurements for biofilms being inhibited were related to these to obtain the percentage of activity in the exposed biofilms. Each treatment was performed in four replicates. Plates were then sealed with Parafilm and incubated with shaking (100 rpm). After 1 hour, 20 μl of TTC (0.1%, prepared in sterile water) was added to each well, and the plates were sealed with Parafilm, wrapped in foil, and incubated again. To optimize the time course of the assay, the presence of reduced TTC was quantified by measuring the absorbance at 490 nm (EL 340 microplate reader; Bio-Tek Instruments, Winooski, Vermont) at various time points after the biofilms were exposed to the antimicrobial agents.

Resistance to invasion by *Pseudoalteromonas tunicata*. We examined the ability of preestablished single- and multispecies biofilms to resist invasion by the marine bacterium *Pseudoalteromonas tunicata*. This organism was selected for these studies because it aggressively colonizes and dominates biofilms on the surface of *U. australis* through the production of the potent antibacterial protein AlpP (41; unpublished data). Biofilms were grown in 20% VNSS broth in continuous-culture flow cells (channel dimensions, 1 by 4 by 40 mm) at room temperature as previously described (35). Biofilms were established by inoculating channels with 1 ml of overnight cultures (10^6 cells ml^{-1}) of either strain 2.04, 2.12, 2.3, or 2.34 (single-species biofilms) or with 250 μl of each strain (four-species biofilm), resulting in a total volume of 1 ml. Cultures were incubated without flow for 1 h to allow cell attachment, followed by maturation of biofilms in the presence of flow (flow rate of 150 μl min^{-1}) for 2 h. The biofilms were inoculated with 10^6 cells ml^{-1} of an overnight culture of green fluorescent protein-labeled *P. tunicata* (41), and the flow was stopped for 1 h. After resumption of the flow, the biofilms were monitored at regular intervals for a period of 44 h. Biofilms were stained with Syto 59 (diluted to 3 μl ml^{-1} in 20% VNSS) and visualized with a confocal laser scanning microscope (Olympus, Tokyo, Japan) using fluorescein isothiocyanate and tetramethyl rhodamine isocyanate optical filters. The flow cells were examined for red and green fluorescence, and the percent surface coverages of *P. tunicata* and of other cells were calculated using image analysis (ImageJ; NIH, Bethesda, Maryland). The flow cell experiments were repeated in three separate rounds with three independent flow cells running in parallel.

Effects of secreted compounds on biofilm synergy. The supernatants of isolates 2.04, 2.12, 2.3, and 2.34 were examined in order to evaluate the role of the secreted compounds in the observed multispecies biofilm synergy and whether compounds mediating *N*-acyl homoserine lactone (AHL)-dependent quorum sensing were produced by any of the isolates. Four- and single-species biofilm attachment and formation experiments with isolates 2.04, 2.12, 2.3, and 2.34 were performed as described above. Four variants of the four-species biofilms were prepared, in which one isolate was replaced by its filtered supernatant and the remaining three isolates were inoculated as described above. After centrifugation of the cells, the spent supernatants were filtered through 0.2-μm-pore-size Minisart filters (Sartorius, Hannover, Germany). To ensure that no cells were present in the filtrates, 100 μl was spread onto VNSS agar plates, and 200 μl was inoculated in separate wells in the microtiter plate.

The filtered supernatants were screened for presence of AHLs by the use of the *Agrobacterium tumefaciens* biosensor assay described by Zhu et al. (61). The AHL compound *N*-oxohexanoyl homoserine lactone at a final concentration of 10^{-7} M and sterile water were used as positive and negative controls, respectively.

Screening for plasmids. Attempts were made to purify plasmids from strains 2.04, 2.12, 2.3, and 2.34 by use of small-scale preparations of plasmid DNA (46), the QIAprep spin miniprep kit (QIAGEN), the Plasmid Midikit (QIAGEN), and the Plasmid Mini AX (A&A Biotechnology, Gdynia, Poland). The strains were grown for 48 h in appropriate volumes of VNSS, and the methods were used as recommended and further optimized. Plasmid purifications were visualized by agarose gel electrophoresis.

Nucleotide sequence accession numbers. The rRNA gene sequences of isolates 2.04, 2.12, 2.3, and 2.34 have been submitted to GenBank under accession numbers DQ328319 to DQ328322, respectively.

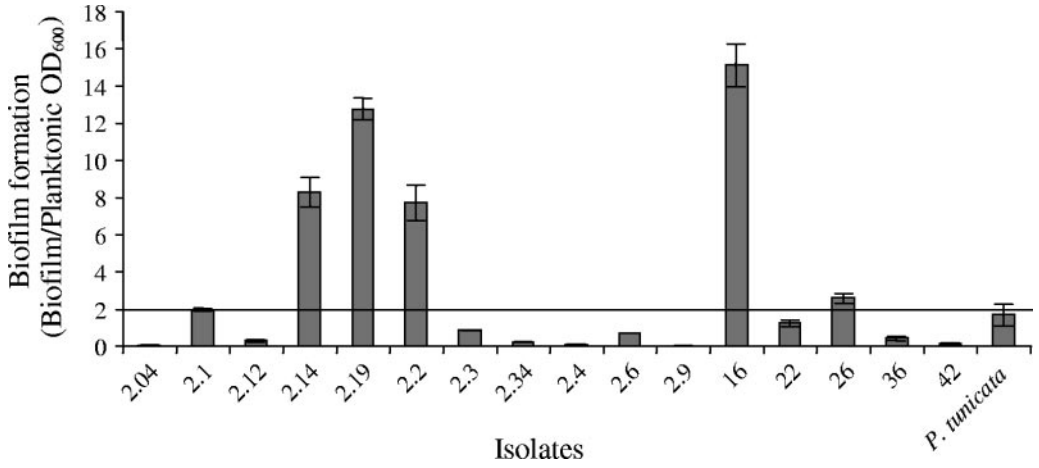

FIG. 1. Biofilm formation in microtiter wells by the 17 epiphytic isolates (including *Pseudoalteromonas tunicata*) isolated from the marine alga *Ulva australis*. After 24 h of incubation, the biofilm formation was quantified by staining with crystal violet followed by spectrophotometric absorbance measurements (OD_{600}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y* axis. Isolates with a biofilm formation of less than 2 (indicated by a line) were chosen for further studies on interactions in biofilms. Bars represent means ± standard errors for four replicates.

RESULTS

Biofilm formation by 17 epiphytic bacterial strains. In order to determine the extent to which 17 marine epiphytic isolates formed biofilms, the strains were incubated as single species in microtiter plates (replicates of four wells) and biofilm formation was measured after 24 h (Fig. 1). Due to the high absorbance of CV in some wells (containing isolates 2.14, 2.19, 2.2, and 16), the CV was diluted (1:3) several times. Ten isolates with a biofilm/planktonic OD_{600} value of less than 2 were categorized as poor biofilm formers. These strains were further examined for synergistic effects when coincubated with other isolates. A total of nine different combinations of three to six strains were grown in multispecies biofilm consortia and examined for synergy.

Synergistic interactions in a four-species biofilm. In one combination of four isolates (2.04, 2.12, 2.3, and 2.34), synergistic interactions were observed (Fig. 2). Each of these four isolates was grown as single-species biofilms and in all possible combinations of, two-, three-, and four-species biofilm consortia in replicates of four. With three combinations as exceptions, the biofilm formation by two or more species was significantly greater than the biofilm produced by any of the single species. When the four isolates coexisted in the biofilm, the biofilm biomass increased by >167% compared to that of the single isolates after 24 h of biofilm formation. The colony morphologies of the four isolates were distinct and were used to verify the presence of all of the strains in approximately equal numbers after 24 h of coincubation in the wells (see the supple-

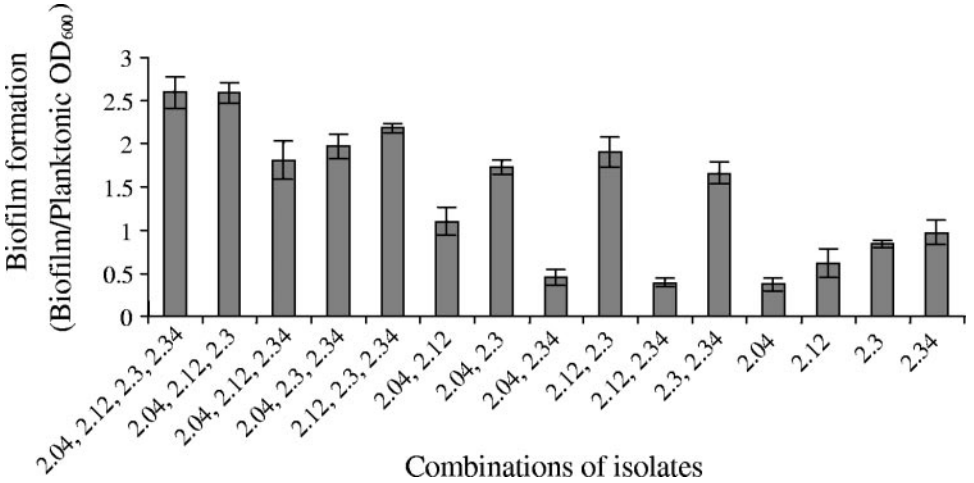

FIG. 2. Biofilm formed by the four epiphytic isolates, 2.04 (*Microbacterium phyllosphaerae*), 2.12 (*Shewanella japonica*), 2.3 (*Dokdonia donghaensis*), and 2.34 (*Acinetobacter lwoffii*), when incubated in microtiter wells in various combinations of one to four isolates. Equal total cell densities were inoculated in each well. The plate was incubated for 24 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{600}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y* axis. Bars represent means ± standard errors for four replicates.

TABLE 1. Identification of the four epiphytic isolates interacting synergistically in biofilms by 16S rRNA gene analysis

Strain	GenBank accession no.	No. of nucleotides subjected to Blast searches against sequences in GenBank[a]	Closest relative[b]
2.04	DQ328319	1,389	*Microbacterium phyllosphaerae*
2.12	DQ328320	1,321	*Shewanella japonica*
2.3	DQ328321	1,343	*Dokdonia donghaensis*
2.34	DQ328322	1,365	*Acinetobacter lwoffii*

[a] The sequences were obtained by sequencing of 16S rRNA genes.
[b] The sequences had 99 to 100% base identity to the closest relative in GenBank.

mental material). The experiment was repeated three times with similar outcome.

In two-species biofilms, each species interacted synergistically with at least one other organism. One isolate, strain 2.3, was able to cause synergy in all three dual-species combinations. In three-species biofilms, all combinations of strains, except that lacking strain 2.34, generated less biofilm biomass than that composed of the four isolates 2.04, 2.12, 2.3, and 2.34. While 2.34 appeared not to influence synergistic growth in four-species biofilms, we still included 2.34 in the four-species biofilm mix because it showed strong synergy with strain 2.3 in dual-species biofilms (Fig. 2).

Identification of the four biofilm-synergistic, epiphytic bacterial isolates. The 16S rRNA genes of each of the four isolates 2.04, 2.12, 2.3, and 2.34 were sequenced (Table 1) and found to be identical (99 to 100%) to those of previously sequenced marine bacteria. Sequences of 1,321 to 1,389 base pairs were obtained, and Blast searches against sequences available in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) were performed. The closest match of isolate 2.04 was the gram-positive *Microbacterium phyllosphaerae* (3), belonging to the class *Actinobacteria*. The other three matched gram-negative bacteria, two of which belong to the *Gammaproteobacteria*: isolate 2.12, with closest homology to *Shewanella japonica* (22), and isolate 2.34, which most closely matched *Acinetobacter lwoffii* (56) The closest relative of isolate 2.3 proved to be a recently defined species, *Dokdonia donghaensis* (60) (also known as *Dokdoa eastseensis*), which belongs to the family *Flexibacteriaceae* in the *Sphingobacteria* class. The strains are referred to as these species below.

Resistance to antimicrobial agents in single- and four-species biofilms. Hydrogen peroxide and tetracycline are common antimicrobial agents used to inhibit bacterial growth. Hydrogen peroxide causes oxidative stress in the bacterial cell, and the broad-spectrum antibiotic compound tetracycline inhibits protein synthesis. In order to assess the fitnesses of the single- and four-species biofilms composed of *M. phyllosphaerae*, *S. japonica*, *D. donghaensis*, and *A. lwoffii*, the activities of these biofilms when exposed to either hydrogen peroxide or tetracycline were examined. Biofilms were established in microtiter plate wells for 24 h and then exposed to hydrogen peroxide or tetracycline. The activities of these biofilms were determined by addition of the respiratory indicator TTC, and the activities obtained were related to those of the corresponding untreated biofilms. The data shown in Fig. 3 represent the TTC absorbance after 16 h of hydrogen peroxide or tetracycline exposure. A marked difference was observed in the relative activities (biofilms exposed to the antimicrobial agents versus nonexposed control biofilms) of the single- and four-species biofilms; the four-species biofilm was significantly more active, as deduced by the TTC measurements, in the presence of the inhibitory compounds than any of the single-species biofilms.

Resistance to invasion by *Pseudoalteromonas tunicata*. *The* marine epiphytic bacterium *P. tunicata* produces a range of biocidal compounds, including a broad-spectrum, antibacterial, high-molecular-weight protein, AlpP, that is effective against gram-negative and -positive isolates from various environments (9–11, 23). AlpP is expressed in *P. tunicata* biofilms and is known to play a role in the competitive dominance of the organism during mixed-species biofilm formation (41). Based on this as well as the recent finding that *P. tunicata* aggressively replaces *Cytophaga funicola* and an *Alteromonas* sp. in mixed-species marine biofilms (41), *P. tunicata* was included to determine resistance of established biofilms against invasion by another bacterial species. The four-species biofilm consortium was exposed to bacterial invasion by addition of a green fluorescent protein-expressing *P. tunicata* to preestablished single- and four-species biofilms. At various time points over 44 h, the degree of *P. tunicata* invasion was determined from confocal laser scanning microscope visualization and image analysis of invaded and noninvaded biofilms. Corresponding invaded and noninvaded biofilms were compared, and the results are presented as percent invasion in Fig. 4. At every time point, the *P. tunicata* invasion was significantly lower in the four-species biofilm, indicating that this biofilm resisted the invasion to a greater extent than did the biofilms composed of single species. Examples of confocal micrographs obtained during the invasion of mixed-species biofilms with *P. tunicata* are shown in the supplemental material.

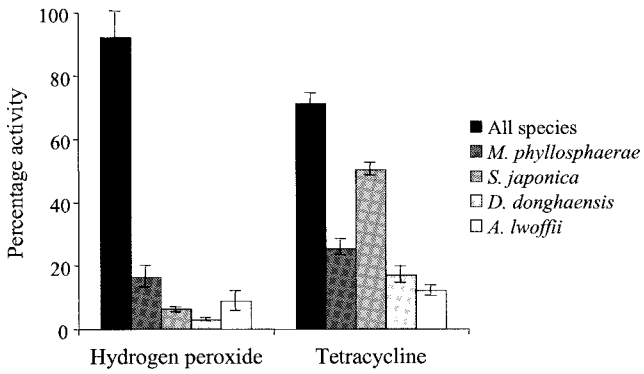

FIG. 3. Activities of biofilms composed of one or four strains of the epiphytic isolates, *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii*, when exposed to hydrogen peroxide (1,700 μg/ml) or tetracycline (20 μg/ml). After 24 h of incubation in microtiter wells, the biofilms were exposed to the antimicrobial agent in VNSS medium or to plain VNSS. After 1 h of exposure, the respiratory indicator TTC was added and the plates were further incubated for 15 h. The metabolic activity was determined by the absorbance of reduced (red) TTC at 490 nm. The activities of the hydrogen peroxide- or tetracycline-exposed biofilms were related to the activities of the corresponding, unexposed biofilms and are presented as "percentage activity". Bars represent means ± standard errors for four replicates.

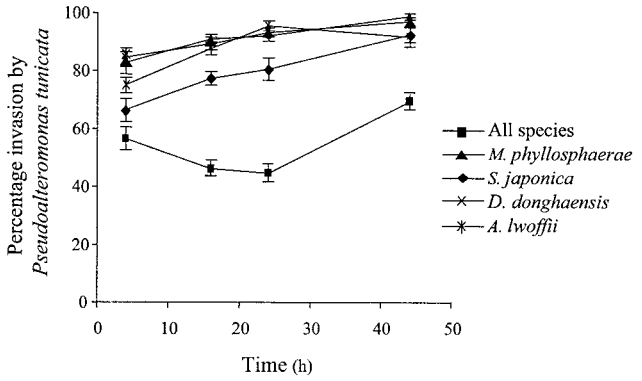

FIG. 4. Bacterial invasion of one- and four-species biofilms by *Pseudoalteromonas tunicata*. Biofilms composed of one or four strains of the epiphytic isolates, *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii*, were established in glass flow cells inoculated with equal total cell densities. After 2 h of growth in the presence of medium flow, the antibacterial protein-producing *P. tunicata* was introduced to the biofilms. This strain constitutively expressed green fluorescent protein. At various time points, the fraction of the surface covered by *P. tunicata* biofilm was determined by staining of the biofilm cells followed by confocal laser scanning microscopy, image analysis, and comparisons to corresponding biofilms not subjected to *P. tunicata* invasion. This is presented as "percentage invasion by *Pseudoalteromonas tunicata*". Bars represent means ± standard errors for eight replicates.

Effects of secreted compounds on biofilm synergy. In order to assess whether extracellular compounds produced by the epiphytic bacteria would induce biofilm synergistic effects, the supernatants from each of the four strains, *M. phyllosphaerae*, *S. japonica*, *D. donghaensis*, and *A. lwoffii*, were used in add-back experiments during the course of biofilm formation. The results are presented in Table 2. Significant reductions in biofilm formation, approximately 50%, were observed when *S. japonica* and *D. donghaensis* were replaced by their filtered supernatants. Only minor reductions were observed when *M. phyllosphaerae* and *A. lwoffii* supernatants were added instead of cells. Enhanced biofilm formation by supernatant addition was not detected.

None of the cell-free supernatants induced the AHL biosensor above the background level, indicating that these strains did not produce AHLs.

Add-back experiments with purified AlpP from *P. tunicata* were not performed because AlpP is a large, 190-kDa protein which exhibits limited diffusion through agar media (and likely also within cell-dense and polysaccharide matrix-encased biofilms). Our data also suggest that AlpP activity is principally cell associated and mediated by cell-cell contact with competing organisms (unpublished data). Exposure of biofilms to free exogenous AlpP is therefore unlikely to occur within the environment.

DISCUSSION

Synergy between species present in dual- or multispecies biofilms has been reported several times, mostly in descriptions of biofilm-forming bacterial isolates from the oral cavity (14, 38, 47, 59). In this study, a synergistic interaction in biofilm formation was observed when four epiphytic marine bacteria, which individually formed relatively poor biofilms, were combined. These bacterial strains, derived from the surface of the green alga *Ulva australis*, were identified as *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii* following 16S rRNA gene sequencing. All other combinations of the 17 epiphytic bacteria which were tested generated less biofilm biomass. The enhanced biofilm biomass formed in the four-species biofilm may be caused by enzyme complementation by some of the species. Alternatively, assuming that the isolates consume different nutrient sources in the culture medium, nutrient depletion in the medium will occur at lower cell densities in the biofilms composed of one species than in that composed of four.

In order to assess whether the observed synergy provided an increased fitness of the mixed biofilm, single- and four-species biofilms were exposed to the antimicrobial agents hydrogen peroxide and tetracycline, as well as to invasion by the epiphytic bacterium *P. tunicata*. This organism produces the antibacterial protein AlpP and effectively out-competes other bacteria during coincubation and biofilm formation (9–11, 23, 41). Hydrogen peroxide and tetracycline are both agents that are often used in disinfection and treatment of microbial infections. In natural systems bacteria may encounter detrimental oxidative stress caused by reactive intermediates in metabolism and degradation of organic matter or from hydrogen peroxide produced by other microorganisms, phagocytes, or predators present (25, 33). Likewise, tetracycline and other antibiotics with similar modes of action are produced by microorganisms in natural habitats (19) and are also widely used for treatment of infections in animals and humans (45). It may therefore be crucial for a microbial consortium to be able to survive exposure to such antimicrobial agents as well as bacterial invasion. We observed an increased protection of the cells present in the four-species biofilm during hydrogen peroxide and tetracycline exposure and *P. tunicata* invasion (Fig. 3 and 4), which is suggestive of an increased fitness provided by the mixed-species consortium. In particular, a pronounced difference was observed in the activity displayed by cells in the four-species biofilm in response to the oxidative stress agent hydrogen peroxide compared to any of the single-species biofilms, highlighting the difficulty in extrapolating observations from various single-species biofilms to the behavior of multispecies mixed biofilms. It is worth noting that the synergistic protective effect of the multispecies biofilm was observed under two different growth conditions of the biofilms: in microtiter plates and in flow cells. The shear forces and the substrata

TABLE 2. Four-species biofilm formation in the presence of cell-free supernatants

Isolate replaced by its supernatant	Biofilm formation[a]	Relative biofilm formation (%)
None	3.1 ± 0.18	100.0
Microbacterium phyllosphaerae (2.04)	2.8 ± 0.04	88.7
Shewanella japonica (2.12)	1.5 ± 0.28	46.8
Dokdonia donghaensis (2.3)	1.6 ± 0.23	51.9
Acinetobacter lwoffii (2.34)	3.0 ± 0.27	94.8

[a] The biofilm biomass was quantified by staining with crystal violet and absorbance measurements. Values represent means ± standard errors for four replicates (biofilm OD_{600}/planktonic OD_{600}).

vary considerably in the two experimental settings, emphasizing the validity of this observation. Moreover, studies of the colonization and competition behavior of bacteria isolated from the surface of *U. australis* have shown outcomes in laboratory experiments similar to those observed on the plant surface (41; D. Rao, J. Webb, and S. Kjelleberg, unpublished data). Thus, our findings of synergistic interactions in biofilms are likely also to be relevant in vivo on the *U. australis* surface.

The increased resistance to hydrogen peroxide, tetracycline, and *P. tunicata* invasion may result from changes in the mixed-species biofilm matrix, which would reduce the permeation of the antimicrobial agents and the antibacterial protein. It is possible that interactions between the different matrix polymers might result in a more viscous matrix. Increased matrix viscosity was suggested as a possible explanation for the enhanced resistance to disinfection of mixed-species biofilms of *Enterobacter agglomerans* and *Klebsiella pneumoniae* (50). Furthermore, interactions between polysaccharides from *Burkholderia cepacia* and *Pseudomonas aeruginosa* have also been shown to decrease the diffusion and antimicrobial activity of antibiotics (1). Thus, mixed-species biofilms may reduce the permeation and diffusion of inhibitory compounds. Moreover, a specific organized spatial distribution of the cells in the biofilm has been suggested to enable bacterial species to coexist (52). Also, protection of species by other species that are more resistant to elimination by an antimicrobial agent has been proposed (7, 27). Leriche et al. found that exposure to antimicrobial agents induced the cells in the biofilm to coexist in mixed structures, suggestive of protection by the more resistant species of other members of the biofilm (27). It is possible that the four isolates in this study responded to tetracycline exposure by spatial reorganization and that this enhanced the protection of the other species by *S. japonica* (Fig. 3). However, when exposed to hydrogen peroxide and *P. tunicata* invasion, all of the single-species biofilms were almost equally susceptible to the exposure, and in these cases it therefore seems unlikely that such protection occurs.

von Canstein et al. (57) also observed an increased fitness of a mixed biofilm consortium when examining mercury retention by several single-species biofilms and a multispecies biofilm consisting of seven different species. Those authors found that the multispecies biofilm showed higher retention efficiency than any of the single-species biofilms and that the bacteria in the multispecies biofilm were less affected by variations in the concentrations of incoming mercury (57). In contrast to these findings and the results obtained in the present study, Whiteley et al. (58), examining several single-species biofilms and a 20-species biofilm consortium exposed to Betadine, showed that while more biofilm biomass was formed in the multispecies biofilm, this did not lead to increased survival upon exposure to Betadine disinfection compared to that for the single species (58). Thus, synergistic effects from biofilm biomass development do not necessarily lead to a higher fitness of the biofilm.

The results from the experiments where spent supernatants rather than the cells of a species were added indicated that *S. japonica* and *D. donghaensis* were important for the synergistic effects (Table 2) and that this was due to physical properties of the cells rather than to excreted compounds. Such mechanisms could be caused by coaggregation of the cells (37, 42, 47, 59) or by pili encoded by conjugative plasmids (17). The four isolates interacting synergistically in this study were tested for their ability to coaggregate by use of two different methods (29, 43), but no coaggregation was observed (data not shown). Also, while several methods were used, it was not possible to isolate any plasmids from any of the four epiphytic isolates.

In contrast to the results obtained from *S. japonica* and *D. donghaensis* supernatant add-back experiments, the replacement of cells of *M. phyllosphaerae* and *A. lwoffii* by their supernatants resulted in only a small reduction in biofilm biomass (Table 2). This could indicate that excreted compounds, including quorum-sensing (QS) signal molecules, caused the synergistic effect observed in the four-species biofilm. The role of QS in biofilm development is species dependent and has been shown to affect several stages of biofilm formation in different species (39). Pertinent to the findings reported in this paper, biofilms of a *P. aeruginosa* QS mutant were found to be more susceptible to tobramycin, hydrogen peroxide, and macrophages (5), and in a dual-species biofilm, AHL QS signals produced by *P. aeruginosa* were shown to be perceived by *B. cepacia* (44). However, none of the four epiphytic isolates interacting synergistically in this study appeared to be AHL producers. Naturally, other classes of QS mediating compounds, such as AI-2 molecules mediating inter- as well as intraspecies bacterial communication (20), could contribute to the observed synergistic effect.

The finding that replacement of *M. phyllosphaerae* and *A. lwoffii* by their respective supernatants resulted in only very small reductions in the biofilm formation (Table 2) is in agreement with the results obtained while screening for synergistic increase in biofilm biomass (Fig. 2). In this experiment, a minor or no reduction in the biomass was observed in the three-species biofilms without *M. phyllosphaerae* or *A. lwoffii*, respectively, compared to that containing the four species. However, both of these isolates showed synergistic effects in two-species biofilms: *M. phyllosphaerae* with either *S. japonica* or *D. donghaensis* and *A. lwoffii* with *D. donghaensis* (Fig. 2). On the basis of these observations, we hypothesize that there are specific roles of *D. donghaensis* and *S. japonica* in the multispecies synergy. These roles rely on physical properties of *D. donghaensis* and *S. japonica*, as the synergy is abolished when the cells of these strains are replaced by supernatants (Table 2). However, the effect of *S. japonica* and *D. donghaensis* depends on the presence of other species to interact with, in this case *M. phyllosphaerae* and/or *A. lwoffii*. The roles of the latter partners are less specific, as their removal from the four-species biofilm does not affect the synergistic interaction (Fig. 2).

Bacteria are affected by the environment they live in and the variety of other species present. By performing studies on the interactions present in multispecies biofilms, basic knowledge on several aspects of sociomicrobiology can be gained (39). The results obtained in this study show that the biomass and fitness of a multispecies biofilm are not necessarily the sums of the characteristics of each single species. Hence, results obtained from single-species biofilm experiments cannot be extrapolated directly to multispecies consortia. From our observations, we suggest that bacterial species gain fitness advantages from residing in multispecies biofilm consortia compared to their biology as single-species biofilms. The use of defined consortia

such as the mixture of the epiphytic bacteria *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii* presented in this study may provide a powerful model system for understanding multispecies biofilm biology.

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