The red alga *Bonnemaisonia asparagoides* regulates epiphytic bacterial abundance and community composition by chemical defence

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Abstract

Ecological research on algal-derived metabolites with antimicrobial activity has recently received increased attention and is no longer only aimed at identifying novel natural compounds with potential use in applied perspectives. Despite this progress, few studies have so far demonstrated ecologically relevant antimicrobial roles of algal metabolites, and even fewer have utilized molecular tools to investigate the effects of these metabolites on the natural community composition of bacteria. In this study, we investigated whether the red alga *Bonnemaisonia asparagoides* is chemically defended against bacterial colonization of its surface by extracting surface-associated secondary metabolites and testing their antibacterial effects. Furthermore, we compared the associated bacterial abundance and community composition between *B. asparagoides* and two coexisting macroalgae. Surface extracts tested at natural concentrations had broad-spectrum effects on the growth of ecologically relevant bacteria, and consistent with this antibacterial activity, natural populations of *B. asparagoides* had significantly lower densities of epibacteria compared with the coexisting algae. Terminal restriction fragment length polymorphism analysis further showed that *B. asparagoides* harboured surface-associated bacteria with a community composition that was significantly different from those on coexisting macroalgae. Altogether, these findings demonstrate that *B. asparagoides* produces surface-bound antibacterial compounds with a significant impact on the abundance and composition of the associated bacterial community.

Introduction

Secondary metabolites in benthic marine algae (seaweeds) play a major role in defending the host against enemies such as herbivores (Paul *et al*., 2001). However, much less is known about the roles of algal metabolites as inhibitors of microbial surface colonization (Steinberg *et al*., 2001). Seaweeds provide nutrient-rich environments (Delille *et al*., 1997; Tyler & McGlathery, 2006), which render their surfaces favourable for bacterial colonization (Jaffray *et al*., 1997). This can have detrimental effects for the host (Littler & Littler, 1995; Sunairi *et al*., 1995; Sawabe *et al*., 1998; Vairappan *et al*., 2001) and should, therefore, exert selection for the development of defence mechanisms, such as chemical defence.

The limited knowledge of ecological roles of algal metabolites as microbial deterrents may partly be due to the difficulties in testing extracts or purified metabolites at ecologically relevant concentrations in bacterial assays. This requires information about the intraindividual distribution of secondary metabolites and how they are presented to interacting organisms, because these compounds need to be present on the surface of the host or released into the surrounding water in order to exert an effect on the colonizers (Davis *et al*., 1989). Recently, however, several investigations of the localization and delivery mechanisms...
of bioactive secondary metabolites have been conducted (Dworjanyn et al., 1999; Paul et al., 2006a; Nylund et al., 2008; Salgado et al., 2008). New methods also exist for extracting pertinent concentrations of surface metabolites, which facilitate the test of metabolites at concentrations that surface-colonizing bacteria encounter under natural conditions (Schmitt et al., 1995; de Nys et al., 1998; Nylund et al., 2007). Despite this progress, ecologically relevant roles of algal metabolites as inhibitors of microbial surface colonization have only been demonstrated for a few species (Maximilien et al., 1998; Kubanek et al., 2003; Paul et al., 2006b; Nylund et al., 2008). Evidence for the effects of algal metabolites on the composition of associated microbial communities is even more rare, indicating that the more general issue of the composition and specificity of bacterial communities on algae is still sparsely investigated (Longford et al., 2007). Two recent studies have, however, indicated the effects of waterborne algal metabolites on the community composition of associated bacteria (Dobretsov et al., 2006) as well as on the nearby pelagic bacteria (Lam & Harder, 2007).

The aim of this study was to investigate whether the red alga Bonnemaisonia asparagoides is chemically defended against bacterial colonization of its surface. Previous studies show that B. asparagoides produces a variety of polyhalogenated compounds, which are believed to be stored in numerous, surface-localized gland cells (McConnell & Fenical, 1977, 1980) (Fig. 1). Several of the halogenated metabolites show antibacterial activity (McConnell & Fenical, 1979), although their ecological roles are not known. We addressed this issue by comparing the abundance and community profile of epiphytic bacteria on B. asparagoides with two coexisting algae of similar morphology in a field survey. We also developed a protocol for the extraction of surface-associated metabolites and tested surface extracts from B. asparagoides on 12 ecologically relevant bacteria in laboratory assays. More specifically, we hypothesized that (1) the abundance and community richness of epiphytic bacteria are lower on B. asparagoides than on coexisting algae, (2) the community composition of bacteria on B. asparagoides is different from those on coexisting algae, (3) halogenated compounds are located on the surface of B. asparagoides and (4) surface extracts of B. asparagoides inhibit the growth of ecologically relevant bacteria at natural concentrations.

Materials and methods

Study organisms and collection of algal material

Bonnemaisonia asparagoides is a red alga with a heteromorphic diplohaplontic life cycle (Dixon & Irvine, 1977) (Fig. 1). The work presented in this study was carried out on the filamentous gametophytic phase, which occurs sparsely at exposed sites along the Swedish west coast at depths of around 5–25 m (Rueness, 1977; G.M. Nylund, pers. obs.). The collections of algal material were performed by scuba diving at depths around 12–15 m in the archipelago west of Tjärnö Marine Biological Laboratory. For surface extractions, several specimens of B. asparagoides were brought to the surface and transported to the laboratory, where they were kept in running seawater until used for extractions (within 3 days following collection). To reduce stress, the algae were placed in plastic bags with surrounding seawater during the scuba diving, and the bags were kept cool and in the dark during transportation. For bacterial analyses, 10 individuals of B. asparagoides, together with the coexisting red algae Lomentaria clavellosa and Polysiphonia stricta, were collected from two localities: Klavningarna and Våskär. The algal individuals were separately placed with a forceps in plastic bags, which were sealed underwater. Within 2 h after collection, the algae were rinsed in sterile-filtered seawater to remove loosely attached bacteria. Half of the material was then stored at −20 °C for subsequent analysis of bacterial community compositions and the other half was stored in

![Fig. 1. Bonnemaisonia asparagoides. The filamentous gametophytic stage (a) is considerably branched and has numerous gland cells located on the surface of the thallus (b, arrows). Scale bar: (a) 1.3 mm, (b) 50 μm.](image-url)
2.5% glutaraldehyde until used for determination of bacterial abundances.

**Bacterial abundance**

Bacterial abundances on the algal surfaces were quantified by direct counts of bacteria stained with 4′,6-diamidino-2-phenylindole (0.4 μg mL⁻¹). Individuals of the algal species (n = 6) were stained for approximately 10 min. Bacteria were enumerated by epifluorescence microscopy (Olympus BX 51 microscope, fluorescence mirror unit U-MNUA2) at a magnification of 1000 × in 15 randomly chosen unit fields (0.0002 mm²).

**DNA extraction**

Approximately 20 mg (wet weight) of algal material was extracted using the FastDNA spin kit for soil (Qbiogene) according to the manufacturer’s recommendations, with samples vortexed for 5 min (1600 r.p.m.) in the extraction tubes before and after processing in a FastPrep-24 instrument for 30 s at 5.5 m s⁻¹. For elution of DNA, 100 μL of DNase and pyrogen-free water were used.

**Terminal restriction fragment length polymorphism (T-RFLP) analysis**

PCR was carried out in 50 μL volume with 1.25 U HotStar-Taq DNA polymerase and 5 μL 10 × PCR buffer (Qiagen), 0.4 μM of primers 27F (6-FAM labelled) and 1492R (Lane, 1991), 200 μM of each dNTP and 2 μL DNA extract. The PCR protocol consisted of a hot start (95 °C, 5 min), 30 cycles of denaturation (94 °C, 45 s), annealing (52 °C, 45 s) and elongation (72 °C, 1 min 45 s), followed by a final elongation (72 °C, 7 min) using a Thermo Hybaid Px2 thermal cycler (Thermo Fisher Scientific). The amplified products were assessed by gel electrophoresis (1% agarose, 1 × TBE). PCR was performed in three to eight replicate reactions. The resulting PCR products were pooled and purified with QIAquick columns (Qiagen) before mung bean nuclease digestion (New England Biolabs) according to Egert & Friedrich (2003) for the removal of pseudoterminal fragments. QIAquick purification was used to terminate the mung bean nuclease digestion and concentrate the product. Restriction digestion was performed with 20 U of restriction enzyme (New England Biolabs) in 28-μL reactions with the recommended buffers at 37°C overnight. All samples were digested with Rsal, Hhal and Alul. Digestion was terminated at 65°C for 20 min. The digested product was purified with the Agencourt AMPure (Beckman Coulter) to remove salts and residual DNA polymerase activity (Grüntzig et al., 2002; Hartmann et al., 2007). Two microlitre of the purified product was mixed with 0.3 μL Genescan 1200 Liz ladder (Applied Biosystems) and 6.7 μL formamide. The mixture was denatured at 95 °C for 5 min, followed by rapid cooling to 4 °C. The samples were run on a 3730 DNA analyzer (Applied Biosystems), with an injection for 20 s at 9 kV. Terminal restriction fragments (TRFs) were sized and quantified with GENEMAPPER 3.7 (Applied Biosystems) using the local Southern method. Alignment of profiles was performed according to Dunbar et al. (2001) with a bin size of 1 bp. Fragments of sizes 50–1000 bp, with peak heights > 50 fluorescent units and with a percentage contribution of more than 1% of each total profile peak area, were selected for further data analysis. To avoid confusion between TRFs that originate from the bacterial community and TRFs that may originate from mitochondrial or chloroplast 16S rRNA gene sequences, an in silico T-RFLP analysis was performed using of MiCA Virtual Digest (http://mica.ibest.uidaho.edu/) with the primers and four restriction enzymes experimentally used on 160583 16S rRNA gene sequences > 1200 bp (Ribosomal Database Project 9.60). TRF patterns corresponding to mitochondrial or algal chloroplast sequences were identified and the corresponding experimentally obtained TRFs − 5 and +1 bp [based on the authors’ experience; see also e.g. Kitts (2001)] were excluded from the dataset.

**Extraction of surface-associated metabolites from B. asparagoides**

Surface-associated metabolites were extracted from B. asparagoides according to the dip method (de Nys et al., 1998; Nylund et al., 2007), which allows nonpolar metabolites at the surface of the algal thallus to be extracted without coextracting the interior of the algal cells. Several weighed individuals of B. asparagoides were separately extracted for 20 s in 6 mL of a 1 : 1 mixture of hexane and 0.5 M NaCl in deionized water, on a vortex. This combination of solvent and extraction time did not affect the lysis of surface cells (data not shown), which is necessary to avoid intracellular extraction (de Nys et al., 1998; Nylund et al., 2007). The algal tissues were removed by filtration and the filtrates of all extracted individuals were pooled together. The hexane phases were removed, combined and the resulting surface extract was reduced by rotary evaporation and stored at −20 °C until used in bioassays. Based on a surface area : wet weight ratio for B. asparagoides, the concentration of the resulting surface extract was calculated as the area of algal thalli per volume hexane.

**Chemical analyses**

The surface extract from B. asparagoides was analysed for the presence of previously described polyhalogenated compounds (McConnell & Fenical, 1977, 1980), using GC-MS
Inhibition of bacterial growth by the surface extracts of *B. asparagoides*

Bacterial isolates and culture media

The antibacterial effects of surface-associated metabolites from *B. asparagoides* were tested using 12 bacterial strains (Table 1), isolated previously from the surfaces of red algal species that live in the same habitat as *B. asparagoides* (Nylund et al., 2008). The autoclaved growth medium consisted of 5 g peptone and 0.5 g yeast extract in 1 L of glass wool-filtered seawater, with an amendment of 15 g agar for the agar plates.

Growth-inhibitory tests

The antibacterial effects of surface-associated metabolites from *B. asparagoides* were tested with a membrane bioassay, whereby bacteria were allowed to encounter surface-extracted metabolites on a surface rather than in a solution or an agar matrix (Nylund et al., 2008). According to Nylund et al. (2008), membranes (0.2 µm polycarbonate PVDF, diameter 25 mm, GE Osmonics) were coated with surface extracts to yield final surface concentrations of 0 (control), 10%, 40% and 100% of the amount of surface extract yielded from algal samples with the same surface area as the membranes (i.e. the mean natural concentration). The membranes were transferred to nutritive agar plates with sterile forceps, and droplets (5 µL) of each of the 12 bacterial strains selected, grown in nutrient broth to attain stable stationary growth phase, were added to the membranes in serial dilutions. The assay was performed in triplicate for every bacterial strain. The agar plates with bacterial strains on membranes were incubated at 20 °C and visually monitored for colony growth for 3 days.

Table 1. Planned comparisons of means for the effects of surface extract from *Bonnemaisonia asparagoides* on the growth of bacteria isolated from the three coexisting red algae *Ceramium virgatum* (Cv), *Polysiphonia fuucoides* (Pf) and *Rhodamea confervoides* (Rc) (Nylund et al., 2008).

<table>
<thead>
<tr>
<th>Identity (class)</th>
<th>Strain</th>
<th>Contrast</th>
<th>P-value</th>
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<td>Gammaproteobacteria</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.97</td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td>3</td>
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<tr>
<td></td>
<td>Pseudoalteromonas sp. Rc13</td>
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<td>0.0014</td>
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<tr>
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<td>Gammaproteobacterium Rc11a</td>
<td>4</td>
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<tr>
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<td>3</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>Sulfitobacter sp. Rc19b</td>
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The surface extracts were tested at four concentrations: 0 (control), 10%, 40% and 100% of the natural concentration of surface-associated metabolites in *Bonnemaisonia asparagoides*. To test for effects of the surface extract, three specific contrasts were included: highest concentration vs. control (4), intermediate concentration vs. control (3) and lowest concentration vs. control (2). Significant values in bold.

Statistical analyses

Multivariate analyses of the T-RFLP analyses were performed on data of the presence or absence of TRFs using Jaccard’s coefficient (e.g. Legendre & Legendre, 1998). Hypotheses about differences in the composition of bacterial communities among algal species were tested using a two-factorial, orthogonal permutational MANOVA (PERMANOVA) (Anderson, 2001, 2005). Tests were carried out using 999 unrestricted permutations. Similarities among samples and the importance of individual TRFs were visualized and evaluated using a canonical analysis of principal coordinates (Anderson & Willis, 2003; Anderson, 2004). This method of constrained ordination accounts for the information inherent in the experimental design by finding the canonical axes that maximize the separation among algal species and sampling locations. Thus, the interpretation of the first axis...
is that it best separates the communities among species and locations. Correlations between TRFs and canonical axis represent the individual contribution of a TRF to the particular axis. This type of analysis differs from unconstrained ordinations (e.g. multidimensional scaling and principal components analysis), in the sense that they find axes that maximize differences among groups rather than maximizing the variance explained, and thus allow a clearer focus on explicit hypotheses (Legendre & Legendre, 1998; Anderson & Willis, 2003).

The results from the field survey of bacterial abundances on algal surfaces and TRF numbers in the algal-derived bacterial communities were analysed by a two-factor ANOVA (Underwood, 1997). Planned comparisons using the control as a reference category were used to analyse the results from the membrane bioassays. Before ANOVA and planned comparison analyses, homogeneity of variances was tested with Cochran’s test (Underwood, 1997). Post hoc comparisons were made using the Student–Newman–Keuls test (SNK test), and interactions between factors that met the requirement for pooling (\( P > 0.25 \)) were consequently pooled (Winer et al., 1991; Underwood, 1997).

**Results**

**Bacterial abundance on *B. asparagoides* and the coexisting algae**

*Bonnemaisonia asparagoides* collected from Klåvningarna and Våskär in the archipelago west of Tjärnö Marine Biological Laboratory had significantly fewer surface-associated bacteria than the coexisting *L. clavellosa* and *P. stricta* (two-factor ANOVA, \( F_{2,30} = 51.286, P < 0.0001 \), followed by the SNK test, \( \alpha = 0.05 \)) (Fig. 2a). The significantly highest number of bacteria was found on *L. clavellosa* (two-factor ANOVA, \( F_{2,14} = 12.19, P = 0.0009 \), followed by the SNK test, \( \alpha = 0.05 \)) (Fig. 2b). A total of 59 TRFs were detected for the three algal species. PERMANOVA showed that there were significant differences in the composition of bacterial communities among the different algal species, but not between the two locations (Table 2). The lack of a significant interaction indicates that differences among species were consistent among locations. Further analyses of the nature of these patterns using constrained ordination revealed strong and significant differences among all algal species (Fig. 3). The first and the most important axis separates the communities in *B. asparagoides* from those of *L. clavellosa* and *P. stricta*. This

![Fig. 2](image-url)

**T-RFLP analysis of bacterial communities**

Four different combinations of TRFs corresponding to eukaryotic algal chloroplast 16S rRNA gene sequences were identified among the experimentally derived TRF patterns by *in silico* analysis with the four restriction enzymes MspI, HhaI, Rsal and AluI, chloroplasts of the red algae *Gracilaria tenuistipitata* (GenBank accession AY673996) and *Palmaria palmata*, (Z18289) and two uncultured picoplankton (U70721 and U32671) with sequences grouping with red algal chloroplasts (Rappé et al., 1998). These TRFs were omitted from the dataset before further analysis. No combinations of TRFs that corresponded to mitochondrial 16S rRNA gene sequences were observed among the experimentally derived TRF patterns.

The community richness, as the number of MspI-derived TRFs of the associated bacterial communities from each algal species, was significantly higher for *P. stricta* than the other algae, while there was no difference between *B. asparagoides* and *L. clavellosa* (two-factor ANOVA, \( F_{2,14} = 12.19, P = 0.0009 \), followed by the SNK test, \( \alpha = 0.05 \)) (Fig. 2b). A total of 59 TRFs were detected for the three algal species. PERMANOVA showed that there were significant differences in the composition of bacterial communities among the different algal species, but not between the two locations (Table 2). The lack of a significant interaction indicates that differences among species were consistent among locations. Further analyses of the nature of these patterns using constrained ordination revealed strong and significant differences among all algal species (Fig. 3). The first and the most important axis separates the communities in *B. asparagoides* from those of *L. clavellosa* and *P. stricta*. This
difference is mainly because of the presence of five conspicuous fragments from *B. asparagoides* (62, 162, 434, 450 and 943 bp). The second most important axis separates bacterial communities among all algal species. The bacterial community on *L. clavellosa* is primarily characterized by the presence of three TRFs (144, 431 and 433 bp), while the prevalence of seven TRFs distinguishes *P. stricta* (131, 186, 449, 482, 505, 516 and 623 bp). Consistent with PERMANOVA there was no separation between locations for any of the species. Out of the 59 TRFs observed, 14 contribute significantly to the separation along the first axis, while 13 contribute to the second. Besides the algal species-specific TRFs, general fragments were also observed for bacterial communities from the different algae. These TRFs, however, were not consistently distributed for the algal individuals sampled.

To confirm that the outcome of the T-RFLP analysis was not dependent on the method for data analysis and the choice of restriction enzyme (see e.g. Osborne et al., 2006; Schütte et al., 2008), the T-RFLP dataset was also analysed according to the normalization procedure suggested by Dunbar et al. (2001), and T-RFLP was performed with another restriction enzyme (RsaI) for a subset of the samples. In both cases, the trends of distinctly separated bacterial communities with significantly more TRFs from the bacterial community on *P. stricta* than on *L. clavellosa* and *B. asparagoides* were conserved (data not shown).

### Chemical analyses of the surface extract

Chemical analyses of the surface extract using GC-MS showed that *B. asparagoides* has polyhalogenated compounds located on its surface (Fig. 4). In total, four compounds showed mass spectra with bromine and chlorine isotopic patterns characteristic of the previously described polyhalogenated compounds in *B. asparagoides* (Fig. 4b–e). However, the identities of the compounds could not be determined.

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**Fig. 3.** Biplot of canonical analysis of principal coordinates of the epibiotic bacterial community T-RFLP dataset (restriction digestion with MspI). Vectors and numbers indicate individual TRFs significantly correlated to canonical axes (correlations are scaled by multiplying the original value by 0.5 to fit plot size). Black, *Bonnemaisonia asparagoides*; white, *Lomentaria clavellosa*; grey, *Polysiphonia stricta*; circles, locality Klävningarna; squares, locality Väskär.

**Fig. 4.** Chemical analyses of the surface extract from *Bonnemaisonia asparagoides*. GC chromatogram of the surface extract (a) and MS spectra of compounds showing chlorine and bromine isotopic patterns (b–e) characteristic of previously described polyhalogenated metabolites in *B. asparagoides* (McConnell & Fenical, 1977, 1980).
Effects of the surface extract on bacterial growth

The membrane bioassays show that the surface extract of *B. asparagoides* inhibited bacterial growth (Table 1, Fig. 5). At the highest concentration tested, the surface extract inhibited the growth of 10 of the 12 tested strains, encompassing all five phylogenetic classes of tested bacteria. The intermediate concentration significantly inhibited the growth of three bacterial strains, while the lowest concentration had no significant effect on bacterial growth (Table 1, Fig. 5).

Discussion

It has been suggested that metabolites with natural antifouling roles will primarily be nonpolar (surface adherent) and localized within the producing organism in a way that enables the metabolites to be released to the surface (Steinberg *et al.*, 2001). The polyhalogenated secondary metabolites of *B. asparagoides* are highly nonpolar and the surface extractions combined with the membrane bioassays show that antibacterial metabolites of *B. asparagoides* are found at effective concentrations on the algal surface, where they can interact with the colonizing bacteria. Many seaweed species store bioactive metabolites in specialized cells (Young *et al.*, 1980; Dworjanyn *et al.*, 1999; Schoenwaelder, 2002; Paul *et al.*, 2006a). *Bonemaisonia asparagoides* has numerous specialized gland cells on its surface from which metabolites could easily be released to the exterior of the thallus (Fig. 1). Based on our finding that the antibacterial compounds are found on the algal surface and on results from earlier structural and chemical studies on other members of *Bonemaisonia asparagoides* (Dworjanyn *et al.*, 1999; Paul *et al.*, 2006a; Nylund *et al.*, 2008) it seems very likely that the antibacterial metabolites in *B. asparagoides* are stored in these gland cells. The chemical analyses showed that the surface extract contained brominated and chlorinated compounds, which would correspond to the previously described polyhalogenated compounds of *B. asparagoides* (McConnell & Fenical, 1977, 1980). Definitive identification was unfortunately not possible in the present study due to insufficient algal material for extraction. However, the combined results from this and the previous work on antibacterial activities of *B. asparagoides* metabolites (McConnell & Fenical, 1979) suggest that the antibacterial effects of the surface extract observed were due to some of the previously described polyhalogenated compounds, although other nonhalogenated metabolites were also found in the surface extract. This would be in accordance with the findings in a study on the closely related *Bonemaisonia hamifera*, where the antibacterial activity of surface extracts was due to the major polyhalogenated metabolite (Nylund *et al.*, 2008).

As initially hypothesized, the surface extracts were inhibitory towards bacteria isolated from macroalgae living in the vicinity of *B. asparagoides*, which implies that the abundance of bacteria on the surface of this alga is controlled under natural conditions. The observation that *B. asparagoides* had significantly fewer bacteria on its surface compared with coexisting macroalgae supports this notion. A few other studies have also demonstrated a coupling between comparatively low densities of epiphytic bacteria and the production of antibacterial metabolites in macroalgae.

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**Fig. 5.** *Bonemaisonia asparagoides*. Growth-inhibiting effects of surface extracts (10%, 40% and 100% of the natural surface concentration) on bacteria isolated from red algae coexisting with *B. asparagoides*. Means±SE are shown (*n* = 3). A, Significant differences from controls at 100%; B, significant differences from controls at 40%; C, no significant differences from controls (planned comparisons, *α* = 0.05).
showed that the Statistical analyses of the results from the T-RFLP analyses community composition with (2) a low community richness. terial growth would result in (1) a distinct bacterial com-
associated metabolites with broad-spectrum effects on bac-
bacteria and defend itself against specific pathogenic bacteria. We also hypothesized that the production of surface-
associated metabolites with broad-spectrum effects on bact-
erial growth would result in (1) a distinct bacterial com-
nity composition with (2) a low community richness. Statistical analyses of the results from the T-RFLP analyses showed that the B. asparagoides bacterial community was more distantly separated from those on L. clavellosa and P. stricta, although the bacterial communities on all three algae were host specific, with no significant intraspecific differences between localities. The observed species specific-
city of the associated bacterial communities is in accordance with results from the few previous molecular studies of algal-associated bacterial communities, which indicate some degree of host specificity (Meusnier et al., 2001; Staufenberger et al., 2008) as well as large differences between algal species, at least at a detailed phylogenetic level (‘species’) (Longford et al., 2007). Substantial differences among alga-associated microbial communities have also been observed by traditional culture-based methods. How-
ever, the similar TRF numbers in bacterial communities on B. asparagoides and L. clavellosa, the alga with the highest total number of associated bacteria, contradict the second part of our hypothesis. One explanation is that the relation-
ship between stress, such as a natural chemical defence, and community richness, assessed here by the number of TRFs, is in fact not straightforward. This has also been indicated recently as the lack of effects of alga- and sponge-derived antibacterial chemicals on the numbers of bacterial community TRFs, although differences in the community com-
position were pronounced (Dobretsov et al., 2006). A hump-back model, where diversity peaks at moderate stress, has been proposed to better describe the stress-diversity interplay in microbial communities than a simple negative relationship (Giller et al., 1998), which may account for the results observed, although it was beyond the scope of this study to provide support for such a model. Other factors besides defence chemicals, such as the nutritional values of algal polysaccharides and other algal-derived organic com-
pounds, as well as the age and condition of the algal thallus, may also obviously affect the associated bacterial community composition (Hempel et al., 2008; Staufenberger et al., 2008).

In conclusion, this study has shown that the surface-
associated metabolites of B. asparagoides at natural concen-
trations exert broad-spectrum effects on the growth of ecologically relevant bacteria. Accordingly, the epibacteria of this alga are significantly less abundant and have a significantly different community composition, compared with the epibacteria on surrounding macroalgae. Thus, B. asparagoides seems to regulate its epiphytic bacteria by chemical defence.

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References


