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Rapid attachment of spores of the fouling alga *Ulva fasciata* on biofilms

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Abstract: *Attachment of spores of Ulva fasciata to natural biofilms was examined to probe the basis for specificity in settlement. Within 30 min from initial exposure in laboratory assays, spores attached to 1-, 3-, 6- or 9-day biofilms formed on acid-cleaned glass slides. The greatest number of spores attached to biofilms aged for 6-day (573.3 ± 45 spores mm^{-2}, $p<0.025$). The Morisita Index (MI) was used to investigate relationships between the spatial pattern of spores on natural biofilms, and was found to be random for 1-, 3- and 6-day biofilms (MI = 0.93, 0.98 and 0.95, respectively), but non-random on 9-day biofilms (0.82). In addition to the attachment of spores to natural biofilms, experimentally manipulated biofilms that provided potentially specific receptor sites were studied. Epifluorescence microscopy of 1-day biofilms confirmed that experimental sugar was incorporated into natural biofilms. The Jacalin galactose specific probe showed a homogeneous pattern of galactose incorporation on biofilms, whereas Concanavalin A probe (mannose) showed a discrete pattern for this sugar. Similarly, the addition of fetuin to a biofilm was detected as a heterogeneous pattern. Rapid spore attachment of U. fasciata found on natural biofilms was induced by fetuin (1-day: 1482 ± 46.6 spores mm^{-2}); specific molecules similar to fetuin may play a role in triggering settlement. Specific sugars and their analogs are important surface receptors and play an integral role in attachment of Ulva fasciata spores to aged biofilms. Biofilm age and the role of specific sugars on attachment are discussed.*

Key words: *Bioflim, Biofouling, Lectin, Spore attachment, Ulva fasciata*
PDF of full length paper is available with author ***(*hwshin@sch.ac.kr)***

Introduction

The economic consequence of biofouling is much significant because biofouling and biofilm formation are of great concern to many modern industries, including marine, food, water, mining and medical industries (Lappin-Scott *et al.,* 1992). Biofouling process is a sequence of events where during initial stages macromolecular film is colonized by biofilm (bacteria), to which motile spores of fouling marine algae settle and attach. Successful recruitment and development of planktonic stages of benthic plants and animals are crucial to the establishment of diverse marine benthic communities. A detailed ultrastructural aspect of spore settlement in *Enteromorpha* was investigated (Evans and Christie, 1970). Although many of the fouling organisms at planktonic stages show abilities to select their position in microenvironments through active selection of surface characteristics (Gaines and Roughgarden, 1985; Butman, 1989; Doherty and Fowler, 1994; Callow *et al*., 2000b; Patel *et al*., 2003; Ista *et al*., 2004; Callow *et al*., 2005), temporal and spatial variation in the planktonic stages are also results in variation in settling rates and recruitment pattern. Settlement cues (phototaxis; thigmotaxis; and chemotaxis) for *Enteromorpha* spore were indicated by Callow and Callow (2000). Further variation in settlement and attachment can be attributed to the attractiveness of the substratum that can have a positive or negative response for larval recruitment due to the presence of particular microbial films (Crisp and Ryland, 1960; Maki and Mitchell, 1986; Maki *et al.,* 1988; Unabia and Hadfield, 1999; Graham *et al*., 2000; Qian *et al*., 2003). Some studies also have documented behavioral responses of larvae to several physical factors such as light, gravity, fluid velocity and micro topography (Wethey, 1984; Walters and Wethey, 1991; Walters, 1992). Surface characteristics of all underwater substrata were greatly altered by bacterial biofilm (Mihm *et al.,* 1981; Holmstrom and Kjelleberg, 1994; Patel *et al.,* 2003). By and large, the most influential cues for active selection of a surface appear to emanate from biological sources.

Chemical cues have been implicated in the settlement and metamorphosis of a wide variety of invertebrates, in particular those that form nonspecific aggregations (Burke, 1986). Yet despite the detailed studies of larval settlement, specific interactions between the surface of algal cells and substrata, and the settling-stage of algal cells on different substrata are relatively less (Callow *et al*., 2000a; Joint *et al*., 2002). Larvae of *Janua* (*Dexiospira*) settle in response to particular polysaccharides or glycoproteins present in the bacterial films (Kirchman *et al.,* 1982), and it is thought that, surface recognition for algal spores may also be mediated by carbohydrates and lectins (Callow *et al.,* 1981).

Lectins are glycan-binding proteins or glycoproteins which have affinity to bind noncovalently to carbohydrate groups. Lectins of different sugar specificities were used to probe the carbohydrate moieties present on the surface. Lectins have been employed as probes for the cell-cell recognition during the fertilization in some species of algae (Bolwell *et al.,* 1979; Callow, 1985; Schmid, 1993; Kim and Fritz, 1993; Kim *et al.,* 1996; Kim and Kim, 1999). They have also been employed in the detection of cell surface glycan moieties in spores (Callow *et al.,* 1981). Glycoconjugates were reported to be present in the early stages of biofilms on inert surfaces in sufficient concentrations to allow their visualization and localization with lectin probes (Michael and Smith, 1995).

The cosmopolitan green macroalga, *Ulva fasciata*, is an important marine fouling organism. *Ulva* spp. have also been

implicated as 'greentide' algae (Fletcher, 1996; Taylor, 1999; Sidharthan *et al.,* 2004) and they have substantial reproductive potential (10^6 motile cells cm^{-2} fertile margin) and ability for rapid attachment to a variety of marine substrata. Upon attachment, motile cells lose flagella and discharge a glycoprotein - based adhesive to secure attachment and ultimately develop into germlings for related taxa (Fletcher and Callow, 1992). Based on earlier studies (Christie and Shaw, 1968; Tosteson and Corpe, 1975; Dillon *et al.,* 1989; Callow and Callow 2000; Callow *et al.,* 2002) with *Enteromorpha* species, suitable substrates are likely to be a major influence on the settlement of spores. However, few workers have examined the affinities of spores for various substrates. The influence of specific biochemical cues of a film on spore attachment has not been examined experimentally in detail to understand the attachment preferences of *Ulva* spores, except few (Holmstrom *et al.,* 1996) who reported inhibitory effect of certain bacterial strains on spore settlement. However, lectins of different specificities have been implicated in the detection of various sugar residues present in the biofilm matrix (Callow *et al.,* 1981; Kim and Kim, 1999).

The objectives of this study are: 1) to assess the attachment of spores on different ages of biofilms, and 2) to assess the attachment and distribution pattern (s) of spores on natural and sugar substrates incorporated biofilms.

Materials and Methods

Spore collection: Thalli of *U. fasciata* were collected randomly from Ka'alawai, O'ahu, Hawai'i, U.S.A and transported to the laboratory in a plastic bag with seawater. Individual plants were separated and several times cleaned with a soft brush in 0.2 μm filtered seawater. In order to accelerate the spore release, the algal material was blotted dry, and individual plants were placed in dry petri dishes under fluorescent lights (50 μmol m^{-2} s^{-1})° at room temperature (19.5°C). Twelve hours later the thalli were barely covered with filtered seawater (approximately 30 ml) to facilitate the release of motile cells, typically as a dense suspension of cells. One ml aliquots of spore suspension were preserved with 1 ml of 4% buffered formalin in vials for the determination of cell density. The density of motile cells in the initial suspensions was counted using a random field method (APHA, 1995). Counting and the identification of motile cells were accomplished with the phase contrast microscopy (Olympus model BH-2, Tokyo, Japan) at 200x magnification.

Natural biofilms: Marine biofilms were generated on replicates of glass slides (2.5 x 7.5 cm) that had previously been cleaned by immersion in 10% HCl for one day, followed by a one day rinse in deionized water (Hadfield *et al.,* 1994). Experimental slides kept in racks were suspended in fouling test site at Ford Island, Pearl Harbor, O'ahu, Hawai'i by a monofilament within 0.25 m of the water surface. Slide racks were collected after 1, 3, 6 and 9 day(s) of immersion and transported to the laboratory in Coplin jars filled with filtered seawater. Qualitative and quantitative aspects of biofim were not analyzed in this study. Since age of the biofilm was recognized as important factor in the settlement of various fouling organisms (Holmstrom *et al.,* 1996; Maki *et al.,* 2000; Joint *et al.,* 2002; Patel *et al.,* 2003; Tait *et al*., 2005; Callow and Callow, 2006), different ages of natural biofilm was used in this study to compare with the sugar-incorporated biofilms.

Spore attachment assay: Twenty five ml of motile cell suspension ($5.6 \times 10^6 \pm 1.2 \times 10^2$ cells ml^{-1} SD, n = 10) was delivered to Coplin jars containing 100 ml of 0.2 mm filtered seawater to test the attachment of spores on different ages of biofilms. All incubation procedures were performed in the dark at room temperature for 30 min.

Analysis of attachment pattern: Each glass slide was divided to three-unit area (5 mm^2) to examine the spatial patterns in the distribution of spore attachment. Spores were counted randomly in a microscopic field (1 mm^2) with 200x and 400x magnification. Morisita's similarity index (Morisita, 1959) was calculated using the following equation:

$$\text{Morisita's Index (MI)} = \frac{2 \Sigma XiYi}{(S1 + S2)\, N1N2}$$

$$\text{where } S1 = \frac{\Sigma Xi\,(Xi - 1)}{N1(N1-1)}, \quad S2 = \frac{\Sigma Yi\,(Yi - 1)}{N2\,(N2 - 1)}$$

and Xi is number of spores from microscopic field 1 in spore i, Yi is the number of spores from area of microscopic field 2 in spore i, N1 is Σ Xi and N2 is Σ Yi. For MI values range from 1 to 0.9, the objects are considered as randomly distributed. If the MI value range from 0.1 to 0.8, objects are contagious and < 0.1 objects are uniform.

Experimental biofilm: To assess the integration of biotin-labeled oligonucleotides like, haptens with the biofilms and to visualize the sugar incorporation in the films, a series of control experiments were conducted. Lectin standards used in this study, Jacalin, Concanavalin A (Con A), Wheat Germ Agglutin (WGA) and Limulin were purchased from Vector Laboratories and Sigma Co. One-day exposed films were stained with complementary fluorescein isothiocyanate (FITC)-labeled lectins under control conditions (native films) and other films were allowed to incubate with mannose, galactose or fetuin to integrate with the film. The integration of mannose was probed with Con A, galactose with Jacalin and fetuin with Con A, respectively. A set of slides was examined for each hapten following this procedure: i) a natural biofilm was stained with a complementary lectin and 4, 6-diamidino-2-phenylindole (DAPI), ii) a natural biofilm was pre-incubated for 30 min with the sugar or glycoprotein, then stained with a complementary lectin, iii) a biofilm was pre-incubated for 30 min with the sugar or glycoprotein, then incubated in seawater for 10 min or iv) a natural biofilm was pre-incubated for 30 min with the sugar or glycoprotein, then incubated in seawater for 20 min.

Epifluorescence microscopy: All lectins were applied at a minimal concentration of 200 μl ml^{-1}. Biofilms were incubated for 30 min with FITC-labeled lectins, rinsed twice with dilute formalin and distilled water. A drop of DAPI (10 μg ml^{-1}: Sigma Co., USA) was added during the last 5 min of the staining period. The slides were air-

Table - 1: Response of different organisms to specific sugars and its analogs

Sugar	Organism	Response	Reference
N-acetyl-D-glactosamine	*Bradyrhizobium japonicum*	Stimulation (adhesion)	Smith and Wollum (1993)
D-galactose	*B. japonicum*	Inhibition (adhesion)	Smith and Wollum (1993)
L-lysine	*B. japonicum*	Stimulation (adhesion)	Smith and Wollum (1993)
Mannose	*Mycobacterium avium*	Inhibition(adhesion)	Goswami *et al.* (1994)
Methyl α-D-mannose	*Antithamnion nipponicum*	Inhibition (fertilization)	Kim and Fritz (1993)
Fucose	*Nostoc* 2S9B	Excretion (adhesion)	Günter *et al.* (1995)

Table - 2: Spore attachment of *U. fasciata* on different pre-incubated biofilms exposed to seawater. Mean number of spores mm^{-2} ± SE (NS, not significant at p>0.01)

Sugar specificity	Exposure time (day)			
	1	3	6	9
Control	343.0 ± 26.3	467.0 ± 89.9	573.3 ± 45.0	557.5 ± 044.6
Galactosamine	105.8 ± 36.0	263.3 ± 56.1	563.3 ± 56.1	375.8 ± 036.0
Fucose	132.5 ± 39.9	42.5 ± 04.9 NS	330.8 ± 39.9 NS	315.0 ± 034.1
Mannose	150.8 ± 46.4	223.3 ± 57.8	593.3 ± 57.8	564.2 ± 046.4
Galactose	74.1 ± 52.6 NS	60.8 ± 16.8 NS	438.6 ± 45.9	196.4 ± 018.2 NS
Fetuin	1482.0 ± 46.6	453.3 ± 27.6	394.8 ± 41.4	1132.0 ± 100.0

dried and coverslips mounted with non-fluorescent immersion oil. The films were examined using a BHT Olympus microscope under blue (ca 475 nm max.) or ultraviolet (ca 365 nm max.) exciting beams in the epifluorescent mode. Attachment of spores of *U. fasciata* was examined on these experimental biofilms following presumed integration of sugars into the film matrix.

Biofilm pre-incubation with lectins: Glass slides with biofilm were treated with two ml of experimentally selected sugar solutions [N-acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose) 0.2 M; fetuin (glycoprotein) 200 mg ml^{-1}; L (-) fucose 0.1 M; methyl-α-D-mannopyranoside (α-methyl D-mannoside) 0.2 M; and D (+) galactose 0.2 M] for 30 min and incubated at room temperature and light. The concentration of sugars was based on the minimal level that inhibits agglutination of red blood cells. The sugar-incorporated biofilms were again incubated for 30 min at room temperature prior to exposure to spores. These slides were then placed vertically into Coplin jars containing 125 ml of spore suspension (5.6×10^6 cells ml^{-1}) and incubated in the dark at room temperature for 20 min. After 20 min, these experimental slides were preserved with 2% buffered formaldehyde in seawater. These slides were examined under microscope to estimate the number of spore attached (mm^{-2}) and their attachment patterns.

Statistical analysis: Statistical analyses were carried out using SAS statistical software (version 6.2). Comparisons of types of sugar biofilms and age of biofilms were analyzed with Duncan-Waller test. Normality was assessed with log transformation and $p < 0.05$. If normality was not found, the non-parametric Wilcoxon Scores test was employed.

Results and Discussion

Characterization of attachment on native biofilms of different ages: Spore attachment of *U. fasciata* was highest for 6- and 9-day old films (573.3 ± 45; 557.5 ± 44.6 spores mm^{-2}, SD, n = 30) while a mean of 343 ± 26.3 spores mm^{-2}, SD, n = 30 attached on 1-d old films (Fig. 1). Statistical analysis showed significant differences among spore attachment on 1-day biofilm and 3-, 6- or 9-day films ($p < 0.025$), (F value 3.34). This attachment pattern was analyzed by a saturation kinetic model: [Polynomial Regression: No. spores $mm^{-2} = 259.3 + (93.4 * \text{age of film}) - \{6.7 * (\text{age of film})^2\}$, n = 120, F = 12.12, $p < 0.001$]. The number of *Ulva* spores attached on biofilms increased in films developed for 0-3 day (s), whereas spore attachment did not increase on films developed for 6-day or longer. The intensity of biofilm on acid-cleaned glass slides generally increased with the length of immersion time.

Spore attachment and spatial distribution on natural and experimental biofilms: Distribution patterns of spores of *U. fasciata* were random (MI value³ 0.9) on all natural 1-, 3- and 6-day biofilms (MI = 0.93, 0.98 and 0.94 respectively, Fig. 2). In contrast, the spores on 9-day old biofilms were found to be contagiously distributed (MI = 0.82). Experimental 1-dday treated with galactosamine and for 3- and 9-day biofilms treated with galactose also demonstrated contagious spore distributions (MI = 0.53, 0.63 and 0.63, respectively). Experimental 1-, 3- and 9- day biofilms treated with mannose were found to be randomly distributed (MI = 0.94, 0.93 and 0.90, respectively). Thus, among the three experimental biofilms tested, settlement in these spores distribution was impacted by galactosamine and galactose (Fig. 2).

Lectins probe distributions of sugars in experimental one-day biofilms: The lectin Jacalin was used to probe controls for the experimentally introduced D (+) galactose in 1 day biofilms. Jacalin did not positively bind to the biofilm although some receptors were detected in epifluorescence microscopy around DAPI-labeled bacteria (Fig. 3A). Integration of D (+) galactose into the film, before immersion into the seawater was detected as clusters of lectin binding sites scattered in the film matrix (Fig. 3B). Following 10 and 20 min

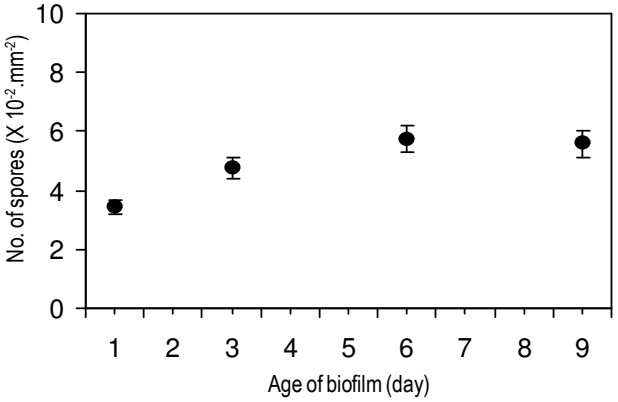

Fig. 1: Time course of *U. fasciata* spore attachment to biofilm exposed to sea water from 1 to 9 day

immersion in seawater to mimic the surface to which the spores were exposed, the galactose-specific lectin still localized and visualized galactose within the film. The pattern of binding sites appeared in a more homogeneous pattern than observed before immersion in seawater (Fig. 3C,D).

Con A was used to probe for experimentally introduced mannose in 1-day biofilms. In the absence of mannose, the film matrix bound some Con A at low levels (Fig. 3E); bacteria were detected by DAPI. After mannose addition, this probe of mannose integration into the film showed greater binding than the control film without the mannose. This signal was sustained through 10 and 20 min immersion in seawater (Fig. 3F).

Limulin- and WGA- were used to probe for experimentally introduced fetuin in 1-day biofilms. These lectins did not recognize receptors in the film prior to incubation. Integration of the glycoprotein into the film was demonstrated by the positive probe response following incubation (Fig. 3G). Following 10 min immersion, the film with fetuin appeared to be heterogeneous with granular (Fig. 3H) or fibrillar (Fig. 3I see arrow) configurations that were lectin positive. Following 20 min immersion, the film was more heterogeneous with patches of fetuin localized by the lectin probes (Fig. 3J and K).

Characterization of biofilms incubated with sugars: Spores favored biofilms pre-incubated with fetuin by attaching in substantially higher numbers (1482 ± 46.6 mm^{-2}) than to any other one-day experimental biofilms (Table 2). Spore attachment was inhibited in biofilms (1 day) pre-incubated with galactosamine, fucose and mannose (105.8 ± 36, 132.5 ± 39.9 and 150.8 ± 46.4 mm^{-2}, respectively) with the least number attaching to galactose (74.2 ± 52.6 mm^{-2}, df = 5, CHIQ = 61.6 p >0.01).

For 3 day biofilms, spores favored biofilms containing fetuin (453.3 ± 27.6 mm^{-2}) over any of the other experimental biofilms, and attached at levels equal to the control biofilms (Table 2). Spore attachment was slightly inhibited by galactosamine and mannose

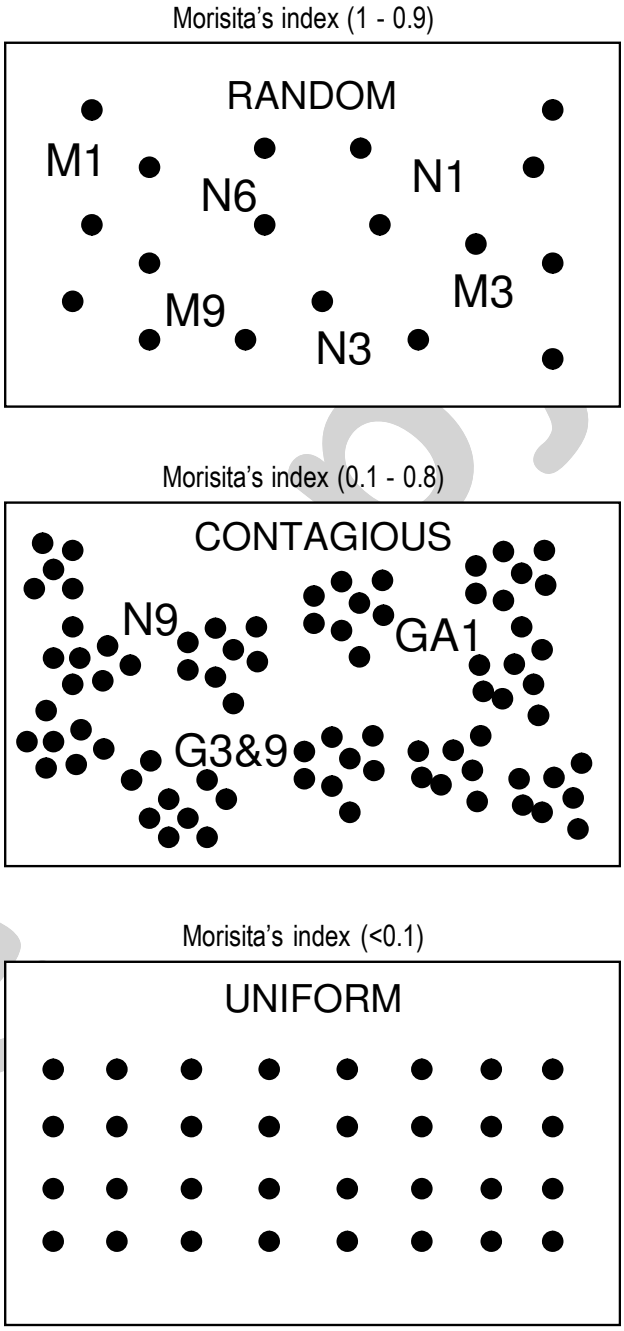

Fig. 2: Distribution pattern of *Ulva* spore attachment (Morisita's Index). Biofilms: N = Natural; M = Mannose; GA = Galactosamine; G = Galactose (following Nos. indicates respective day(s) of biofilm age)

(263.3 ± 56.1 mm^{-2} and 223.3 ± 57.8 mm^{-2}), while fucose and galactose had the greatest inhibitory effect with spore attachment of only 42.5 ± 5 mm^{-2} and 60.8 ± 16.8 mm^{-2}, respectively (df = 5, CHIQ = 63.0, p >0.01, Table 2).

For 6-d biofilms, spore attached to mannose treated biofilms in higher numbers (593.3 ± 57.8 mm^{-2}) than any of the other 6-d experimental biofilms. Spore attachment on galactosamine showed a greater degree of inhibition (563.3 ± 56.1 mm^{-2}, Table 2), with

fucose effectively inhibiting spore attachment more than any other 6-day biofilms (330.8 ± 39.9 mm^{-2}, df = 5, CHIQ = 13.3, p>0.01).

For 9-day biofilms, spore attachment was greatest with fetuin (1132 100 mm^{-2}, Table 2). For biofilms pre-incubated with mannose, spore attachment was equal to that of the control, while fucose and galactosamine showed a slight inhibition of spore attachment (315 ± 34.1 mm^{-2} and 375.8 ± 36 mm^{-2}, respectively). Minimum spore attachment among any 9-day biofilms was observed in galactose treated biofilms (196.4 ± 18.2 mm^{-2}, df = 5, CHIQ = 47.8, p >0.01).

During the planktonic stage, the quadriflagellate spores of *U. fasciata* actively select the surface film characteristics in natural biofilms. It is clear that spore attachment on various natural biofilms over different time courses shows a complex and dynamic response to biofilm age and chemical features. The patterns of spore settlement determined by MI indexes also demonstrate dynamic changes relating to film age. Positive cues inducing spore attachment may be provided by bacterial cell surfaces or by other suspended extracellular materials existing in the water medium. Similarly, an enhanced zoospore adhesion of *Enteromorpha* on mixed microbial biofilm was reported (Thomas and Allsopp, 1983; Dillon *et al.,*1989). Importance of bacterial biofilms in the development of algal communities was also recognized from the cell-to-cell communication between bacteria and motile zoospores of *Enteromorpha* (Joint *et al.,* 2002). Some marine bacteria have been demonstrated to produce specific molecules that enhance the attachment of the unicellular alga *Chlorella* on glass surfaces (Imam *et al.,* 1984). In other systems, sugar analogs tested were either reported as present on cell surfaces or cues on artificial sugar films (Michael and Smith, 1995). These studies show that a wide range of marine benthic organisms were stimulated in response to varied types of sugars or their analogs (Table 1). Similarly, in this study, biofilms pre-incubated in fetuin and exposed to seawater for 1 and 9 day showed 2-4 fold increase in spore attachment settlement over respective control levels (Table 2).

The surface chemistry of a biofilm and its influence on spore settlement can be quite complex and may not be solely dependent upon the number of attached bacteria. However, in an experimental study, a positive correlation was observed between the number of bacteria in natural marine biofilm on glass slides, and number of *Enteromorpha* spores that attached (Joint *et al.,* 2000). Similar observation with age of the biofilm was demonstrated by Patel *et al.,* 2003). This is in agreement with the present study in which spore attachment on natural biofilms rapidly increased (up to 6-day). In contrary, some strains of a biofilm bacterium, *Pseudomonas* reported to be inhibiting the settlement of *Enteromorpha* and *Ulva* (Holmstrom *et al.,* 1996; Holmstrom and Kjelleberg, 2000; Patel *et al.,* 2003). These studies clearly demonstrate the influence of biofilm on the spore attachment.

Substances (sugars?) on the surface of the substratum have been shown to play an important role in the settlement response of larvae from the barnacle, Cypris (Crisp and Meadows, 1962, 1963); however, these same substances in solution (the water column) do not elicit a settlement response (Rittschof *et al.,* 1985). These studies also suggest that chemicals actively produced by other marine organisms on the substrate may effectively prevent the recruitment and settlement of barnacle larvae, thus giving these other organisms a competitive advantage for limited space. Our study provides an example of this by showing that *Ulva* spore attachment on experimental biofilms with fucose is greatly reduced during all the exposure periods (Table 2).

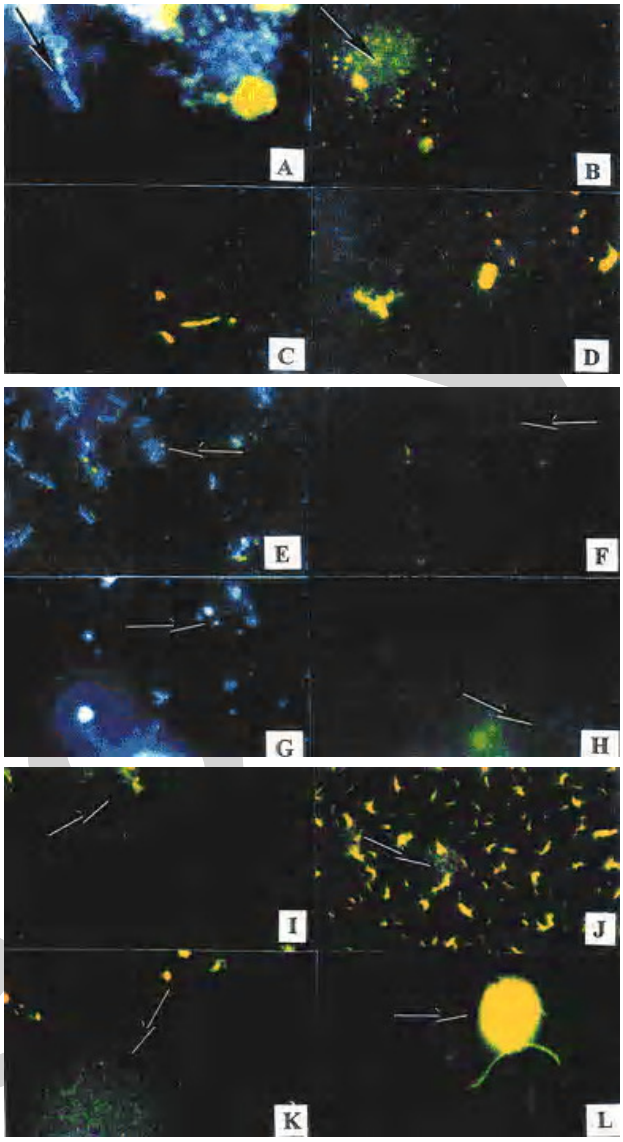

Fig. 3: (A-L) Autofluorescence from one-day biofilm with galactose, mannose and fetuin. Arrows indicate: A) organic matter and associated bacteria; B) clusters of lectin binding sites; C, D) homogeneous pattern of binding sites; E) low levels of film matrix bound lectin Con A; F) sustained signal binding subject to 10-20 min immersion in seawater; G) integration of the glycoprotein into the film; H) film with heterogeneous granular fetuin configurations; I) film with heterogeneous fibrillar fetuin configurations; J, K) heterogeneous patches of fetuin; L) typical lectin Con A response to the motile cell

Settlement of organisms on immersed surfaces during early stages of biofilm development has been a focus of research for

many years (Baier, 1973; Schrader *et al.,* 1988). Epifluorescence studies allow us to view biofilmed surface as a heterogeneous series of microhabitats, which could generate patterns in ultimate community development and its dynamics (Michael and Smith, 1995). In general, glycoconjugates on algal cell surfaces have been probed *via* microscopy with fluorescent chromophores to label the lectins. For *Dunaliella tertiolecta*, settlement and adhesion characteristics appear to be mediated by lectins, similar to those of invertebrate larvae (Maki and Mitchell, 1986). Attachment of dissolved organic matter along with bacteria observed on pre-incubated glass slides (Fig. 3A), integration of the glycoprotein in the biofilm (Fig. 3G) and Con A response to the motile cell shown in Fig. 3L, clearly demonstrate the influence of specific sugars in the early events of spore attachment of *U. fasciata* to biofilms. Adhesion of bacteria and its extracellular chemical derivatives (polysaccharides), greatly influence biofilm formation and the recruitment of successive macrofouling communities.

Glycoproteins and lectin sites on surfaces appear to act as recognition sites or attachment cues in algae (Evans and Christie, 1970; Stanley *et al*., 1999; Humphrey *et al*., 2005; Callow and Callow, 2006). In *U. fasciata,* for the minimum exposure period of 1-3 day(s), significant stimulation in spore attachment was seen in biofilms pre-incubated with galactosamine and mannose (Table 2). A variety of lectin and glycoprotein sites present in surface films may facilitate rapid attachment by acting as attachment cues.

A combination of specific biochemical cues that activate spore attachment receptors may be responsible for the enhanced attachment of *Ulva* spores. It was well demonstrated in the spore settlement of *Enteromorpha* (Callow *et al*., 2000a; Joint *et al.,* 2000, 2002). The experimental early aged-biofilms were chemically diverse and spatially dynamic even after a short period of immersion (Michael and Smith, 1995). They also suggested that a biological system could have a different time course, chemistry and spatial patterning for biofilm formation over a short period, 3 day or even less. Less attachment was seen in natural biofilms exposed to seawater for 1-3 day (Fig. 1) as early aged-biofilms were not as likely to be attractive to spores, perhaps because settlement stimuli were not present in great abundance. In an experimental study, similar observation with enhanced *Enteromorpha* spore settlement on 3 hr biofilm and less settlement on 1.5 hr or 48 hr biofilm was shown (Patel *et al.,* 2003). Reduction in the number spore attachment may also be due to the presence of certain bacterial strains that possess negative chemical cues as reported in *Ulva* spp. (Thomas and Allsopp, 1983; Holmstrom *et al.,* 1996; Holmstrom and Kjelleberg, 2000; Joint *et al.,* 2000; Patel *et al.,* 2003). This study helps to understand the essential role of chemical signals in the biofilms and their effect on the rapid attachment of *Ulva* spores.

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