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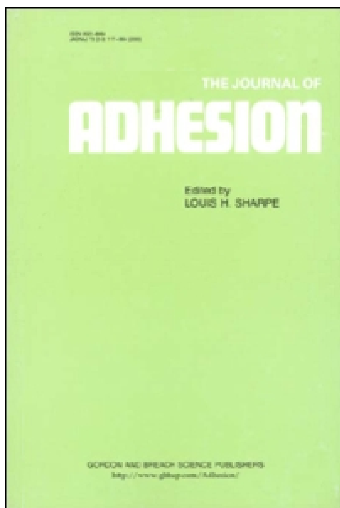

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Effect of Ellman's Reagent and Dithiothreitol on the Curing of the Spore Adhesive Glycoprotein of the Green Alga *Ulva*

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Green algae of the genus Ulva *(syn.* Enteromorpha*) are common, green macroalgae found throughout the world in the upper intertidal zone of seashores and as a fouling organism on a variety of man-made structures including ships' hulls. Adhesion of motile spores is achieved* via *the secretion of an adhesive, which is present in spores in highly condensed form within membrane-bound vesicles. The adhesive is initially liquid and displays a hydrogel-like behavior on release. It then starts to undergo "curing reactions," becoming progressively less soluble with time in anionic detergents, less sensitive to proteolysis, and less viscoelastic, which suggests that extensive cross-linking occurs. Spores also become progressively more difficult to detach from a surface. However, the nature of this adhesive curing process is totally unknown. In the present article we have tested the hypothesis that thiol cross-linking may be involved. We show that nontoxic concentrations of the thiol-capping reagent (Ellman's reagent) or thiol-reducing agent (dithiothreitol) effectively inhibit the time-dependent development of adhesive spore strength after attachment to a surface. Furthermore, we show by SDS-PAGE immunoblot analysis of extracted adhesive proteins that the major adhesive antigen retains solubility in the presence of these reagents, after release from spores, which suggests that cross-linking had been inhibited.*

Keywords: Adhesion; Curing; Dithiothreitol; Ellman's reagent; Green algae; Spore adhesive; *Ulva*

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INTRODUCTION

Green algae of the genus *Ulva* (syn. *Enteromorpha*) are common, green macroalgae found throughout the world in the upper intertidal zone of seashores and as a fouling organism on a variety of man-made structures including ships' hulls [1]. The genus *Enteromorpha* has recently been incorporated into the genus *Ulva* and the latter name is used forthwith [2]. Dispersal is achieved mainly through asexual zoospores: quadriflagellate, pear-shaped cells, 5–7 μm in length (Figure 1). Colonization of substrata involves the transition from a free-swimming spore to an adhered, nonmotile spore [3]. Adhesion is achieved *via* the secretion of an adhesive that is present in spores in highly condensed form within membrane-bound vesicles [4, 5]. The adhesive is initially liquid and displays a hydrogel-like behavior on release, swelling rapidly through the adsorption of water [6, 7] to form an adhesive pad that secures the settled spore to the substratum (Figure 1).

Initial biochemical studies on the zoospore adhesive of *Ulva* [5] have focused on the use of monoclonal antibodies raised against the

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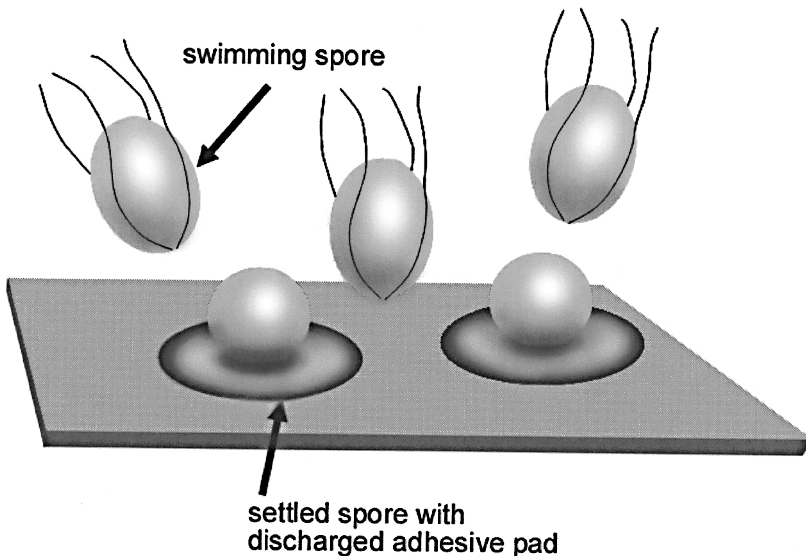

FIGURE 1 Cartoon depicting the transition between free-swimming zoospores of *Ulva* (showing the four flagella that generate motility) and attached spores that have shed their flagella and secreted pads of adhesive through which attachment to the surface is obtained. (See COLOR PLATE I)

contents of the adhesive vesicles. The antibodies labeled the zoospore adhesive both in immunohistochemical studies and on Western blots of SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulphate) gels of zoospore extracts. Under denaturing conditions (*i.e.*, in SDS detergent) Western blots revealed a family of antigens, the major component of which was a polydisperse protein band centered at ~110 kDa [5]. Under native (*i.e.*, nondenaturing) conditions the protein forms extensive aggregates of molecular mass >1000 kDa [8]. After release the adhesive proteins become progressively less soluble in SDS and less sensitive to proteolysis, suggesting, that extensive cross-linking occurs. This is consistent with the evidence from atomic-force microscope (AFM) force curves, which show that within minutes of release the viscoelastic properties of the adhesive change as the adhesive starts to "cure" [6]. Interfacial adhesion strength of whole spores increases over several hours as witnessed by the increased resistance to removal under hydrodynamic shear forces [9].

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However, the nature of this adhesive curing process is totally unknown. Recent advances in understanding the biochemistry of adhesive molecules in other marine species such as mussels [10, 11] and barnacles [12, 13] have illustrated both the versatility of these substances, with their ability to harden in saline aqueous environments, and the diversity of the adhesion chemistry employed. In *Mytilus*, a network of highly basic elongated proteins extensively interconnected by DOPA (dihydroxylphenylalanine)-quinone cross-links, forms an exquisitely tailored fibrous body, the byssus, which is responsible for anchoring the organism to the substratum [14]. By contrast, the adhesive proteins of the adult cement of barnacles are mainly hydrophobic species that owe their rigidity to extensive disulfide cross-linking [13, 15]. In marine algae the situation is less well understood. The existence of hydroxyproline-rich glycoproteins with some relationship to the extensins, a well-characterized family of cell-wall glycoproteins involved in the structural development of higher plants [16, 17], and in the cell wall of microalgae such as *Chlamydomonas* (for a review, see reference 18), have led some groups to postulate an extensin-like oxidative cross-linking mechanism as the key event in the curing of marine algal spore adhesives [19], but to date no clear evidence has been obtained to implicate extensin analogues as the key participants in adhesion. Most studies have been hampered by the propensity of the adhesives to aggregate and cross-link, rendering them intractable in laboratory conditions. More recently Ender *et al*. [20] characterized a series of hydroxyproline-rich proteins from the extracellular matrix of the colonial green alga *Volvox* that

undergo autocatalytic polymerization. The discovery that this polymerization is inhibited by the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid), or Ellman's reagent [21], prompted us to investigate the effect of this compound and the thiol-reducing agent dithiothreitol (DTT) on the time-dependent cross-linking behavior of the *Ulva* spore adhesive and the development of adhesion strength of settled spores.

MATERIALS AND METHODS

Release of *Ulva* Zoospores

Fertile plants of *Ulva linza* were collected from Wembury Beach, England (50°18′ N; 4°02′ W). Zoospores were released as described in Callow *et al.* [3]. Zoospore suspensions were filtered through four layers of muslin and the resulting green filtrate, containing zoospores, was transferred to an ice bucket. Migration of zoospores away from the light allowed concentrated suspensions of zoospores to be obtained by pipetting out of the filtrate. The concentrated suspensions were transferred to a glass beaker and kept on a magnetic stirrer at room temperature until needed (not more than 3 h after release).

For long-term storage, suspensions of zoospores were concentrated by centrifugation (2500 rpm, 10 min, 4°C). Supernatants were discarded. The pellets were flash-frozen in liquid nitrogen and transferred to a −25°C freezer.

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Water Jet Assays of Spore Adhesion Strength

Glass microscope slides used for zoospore settlement were acid-washed in a 1:1 methanol–2 M hydrochloric-acid mixture (2 h), followed by 2 M of hydrochloric acid (2 h) before the experiment. Solutions of the test compound (Ellman's reagent and dithiothreitol, both from Sigma-Aldrich, Poole, UK) were dissolved in artificial sea water (ASW) at twice the desired final concentration for the experiment, and the solution pH was adjusted to 8.2 prior to use. Freshly released zoospores (1–3 h after release) were used at a concentration of $1–3 \times 10^{-6}$ spores ml^{-1}, depending on individual releases (spore concentration was assessed by the absorbance at 660 nm as described in reference 3). Aliquots (5 ml) of zoospore suspension were added to individual microscope slide dishes (In Vitro Systems & Services GmbH, Göttingen, Germany) containing a glass microscope slide and 5 ml of either ASW (for controls) or 5 ml of test compound at double the final desired concentration. Dishes were swirled to ensure even mixing, and spores were settled in darkness. For each treatment six replicate

slides were prepared. After a settlement period of 1 h or 2 h (depending on the particular experiments; see legends to figures for details), all slides were gently washed in either ASW (for controls) or ASW containing the appropriate concentration of test reagent to remove residual unattached spores. Three replicate slides from each treatment were fixed in 2% glutaraldehyde in ASW and processed as described in Callow *et al*. [3]. These were used as reference slides to indicate spore settlement density before water-jet treatment. The remaining three replicates for each treatment were incubated in darkness for various times in the presence of ASW (controls) or the appropriate concentration of test reagent before exposure to the water jet as described by Finlay *et al*. [9]. Slides were then fixed in 2% glutaraldehyde. Adhered spores were visualized by autofluorescence of chlorophyll and quantified by image analysis as described in Callow *et al*. [22]. Thirty counts were taken at 1-mm intervals along the middle of the long axis of each of the three replicate slides. The mean numbers of spores remaining attached to the surface after exposure to the water jet were compared with the mean number before the slides were exposed. Because the level of settlement in each experiment is different (*e.g.*, because different spore batches are used), data are presented in terms of percent of spore removal compared with the controls, plus or minus standard errors calculated from arcsine-transformed data.

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Settlement of Zoospores for Immunoblot Analysis

Freshly released zoospores were diluted with ASW to 3×10^6 spores ml^{-1}. Spores were settled on 10.5-cm square Sterilin Petri dishes by transferring 12-ml of aliquots into dishes already containing either ASW (12 ml) or 12 ml of Ellman's reagent or dithiothreitol solution of twice the desired final concentration in ASW (pH 8.0–8.2). Dishes were swirled gently to ensure an even distribution of zoospores in the supernatant and transferred immediately to a dark cabinet. After 2 h of settlement, supernatants were decanted off and replaced with ASW or the appropriate concentration of Ellman's reagent or dithiothreitol in artificial seawater (pH 8.0–8.2). Dishes were then returned to the dark cabinet. After appropriate time intervals the supernatants were discarded, the dishes washed three times with ASW, and settling zoospores harvested by gentle brushing with a fine paintbrush and resuspension in 1.0–1.5 ml of ASW. Suspensions were transferred by pipette into 1.5-ml microcentrifuge tubes, and pelleted (13,000 rpm, 5 min). Pellets were air dried for 10–20 min and resuspended in 100 μl $1 \times$ SDS-reducing sample buffer [62.5 mM Tris-HCl buffer

(*Tris*[hydroxymethyl]aminomethane hydrochloride, Sigma-Aldrich, Poole, UK), pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) 2-mercaptoethanol, 0.0025% bromophenol blue] before heating at 95°C for 5 min. Suspensions were centrifuged (13,000 rpm, 5 min). Total protein concentration in the supernatants was determined using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA) performed on protein precipitated from 50-µl aliquots of supernatant using the PlusOne Protein Clean-Up Kit (Amersham Biosciences, Little Chalfont, UK) and redissolved in 50 µl of H_2O (Milli-Q). The remaining supernatants were diluted with 1 × SDS-reducing sample buffer to uniform total protein concentration.

Extraction of Nonsettled Zoospores for Immunoblot Analysis

Frozen pellets of unsettled zoospores (500-µl packed volume) were resuspended in 10 ml of buffer (0.1 M sodium phosphate, pH 7.0) before mechanical rupturing in a Retsch MM200 Mixer Mill (Glen Creston Ltd., Stanmore, UK) in a 25-ml zirconium vessel with ~8 ml of 1.5-mm-diameter zirconium grinding beads (30 Hz, 2 × 2 min). Suspensions were ice-cooled after milling and centrifuged, and supernatants stored at 4°C or −20°C in the presence of Complete Mini Protease Inhibitor (Roche, Lewes, UK). For electrophoresis, 4 volumes of supernatant (after centrifugation to remove cell debris) were treated with 1 volume of 5 × SDS-reducing sample buffer (312.5 mM Tris [pH 6.8], 10% w/v SDS, 50% v/v glycerol, 12.5% v/v mercaptoethanol, 0.0125% w/v bromophenol blue) and the solution was heater at 95°C for 10 min and ice-cooled before loading directly onto gels as a standard.

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SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli [23] as modified by Stafford *et al*. [24], was performed using a Mini Protean 3 vertical electrophoresis assembly (Bio-Rad, Hemel Hempstead, UK). SDS-PAGE gels were 0.75 mm or 1.5 mm thick, 10% acrylamide, 0.1% SDS gels poured in the laboratory using Prosieve 50 acrylamide mix (Flowgen Bioscience, Nottingham, UK). Stacking gels were 5% acrylamide containing 0.1% SDS. The SDS electrophoresis buffer employed was 25 mM of Tris base, 192 mM of glycine, 0.1% (w/v) of SDS. Prestained protein molecular-weight markers were obtained from New England Biolabs (Hitchin, UK). Electrophoresis was conducted at 100 V. Gels were developed

using Coomassie Brilliant Blue G or silver staining (Silverquest kit, Invitrogen, Paisley, UK).

Immunoblotting

Immuno- (Western) blotting was carried out using Western blotting apparatus (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. The blotting buffer employed was 25 mM of Tris base, 192 mM of glycine, and 20% (v/v) of methanol. Freshly run SDS-PAGE gels were equilibrated at room temperature with shaking for 20 min in a shallow dish containing the blotting buffer before blotting onto nitrocellulose membranes at 75 V for 90 min. Membranes were incubated overnight at 4°C in a solution of 5% (w/v) milk powder in Tris-buffered saline (TBS) (20 mM of Tris-HCl, 137 mM of NaCl, pH 7.6) containing 0.5% (v/v) TWEEN-20 (polyoxyethylene sorbitan monolaurate, Sigma-Aldrich, Poole, UK). Membranes were washed four times for 15 min with TBS and 0.5% TWEEN-20, and then incubated in Ent6 mAb tissue culture supernatant [5], diluted 1:5 in TBS, for 45 min. After a further four 15-min washes with TBS and 0.5% TWEEN-20 membranes were incubated for 1 h with AP-RAMIG (alkaline phosphatase conjugated rabbit antimouse immunoglobulins [Sigma-Aldrich, Poole, UK] diluted 1:1000 in TBS containing 10 mg/ml of bovine serum albumin). Membranes were washed twice for 15 min in TBS and 0.5% TWEEN-20, twice for 15 min TBS and 0.05% TWEEN-20, and developed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as described by Stafford *et al*. [24]. Typically, membranes were developed in foil-wrapped dishes for 1 h at room temperature, or overnight at 4°C.

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RESULTS AND DISCUSSION

Spore motility and settlement behavior were not affected by Ellman's reagent up to 1 mM but the time-dependent development of interfacial adhesion strength by settled spores was strongly inhibited (Figure 2). With time from settlement, as shown in previous reports [9], the proportion of spores detached from slides under hydrodynamic shear in the ASW control (*i.e.*, no Ellman's reagent) showed a clear decreasing trend as spores adhered more strongly to the surface with time. However, in the presence of 1 mM of Ellman's reagent this trend was not observed (Figure 2). Reduced adhesion strength was observed at concentrations as low as 0.25 mM of Ellman's reagent (Figure 3). Ellman's

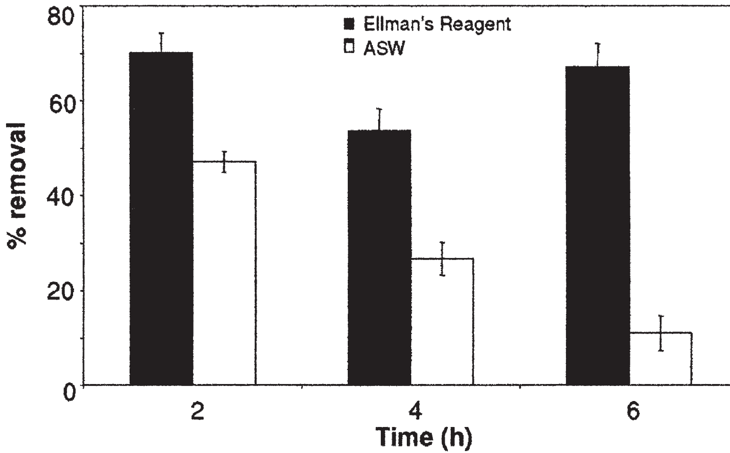

FIGURE 2 Effect of 1 mM of Ellman's reagent on percentage removal of attached spores (2, 4, and 6 h after settlement) after exposure to a surface pressure of 62 kPa. Spores were settled for 2 h, excess spores were removed by washing, and the attached spores were incubated for the period indicated. Percentage removal for each treatment is calculated as the proportion of spores left on slides after jet washing as compared with the non-jet-washed slide. Bars represent standard errors from arcsine-transformed data. The mean level of spore settlement in the experiment was 1100 spores mm^{-2}.

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reagent exerted no nonspecific toxic effects on the settled spores because after 24 h, spores germinated as normal in concentrations up to 1 mM.

Assays were also carried out with the thiol-reducing agent DTT. DTT strongly influenced the development of interfacial adhesion strength at concentrations between 0.05 mM and 0.5 mM (above this level there were noticeable effects on spore motility and settlement behavior). In control samples settled in ASW only 10% of spores could be removed by jet washing after 4 h of settlement time, but the extent of removal increased significantly with increasing concentration of DTT, with 86% removal at 0.5 mM (Figure 4). Time-course experiments with 0.25 mM of DTT showed reduced spore adhesion strength (*i.e.*, greater removal under shear) at all times tested during the 2–6 hour period, compared with control samples in ASW (Figure 5). As for Ellman's reagent, no nonspecific or toxic effects of DTT on spore germination were detected at these concentrations.

To explore the influence of these agents on the adhesive glycoprotein more directly, zoospores were settled on plastic Petri dishes

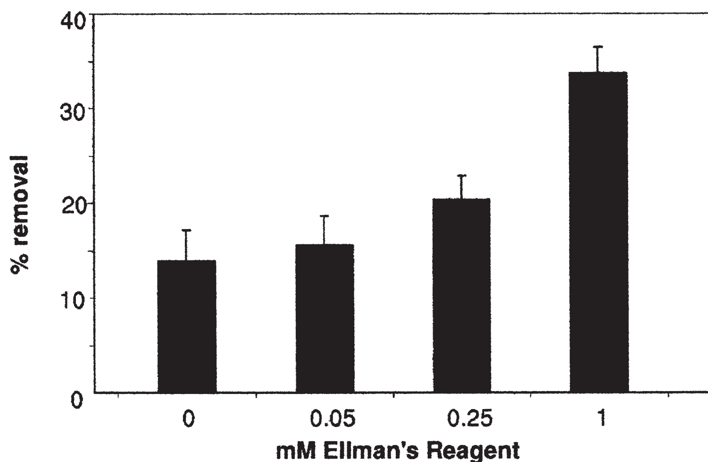

FIGURE 3 Effect of the concentration of Ellman's reagent on interfacial adhesion strength of attached spores. Spores were settled for 1 h in the presence of various concentrations of Ellman's reagent, and then excess unsettled spores were removed by washing. The attached spores were left for a further 3 h in Ellman's reagent (or ASW in the case of controls) before exposure to a water jet (65 kPa of surface pressure). Percentage removal is calculated as the proportion of spores remaining in each treatment as compared with the non-jet-washed slides. Bars represent standard errors from arcsine-transformed data. The mean level of spore settlement in the experiment was 555 spores mm^{-2}.

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in darkness over a series of time periods ranging from 2 to 24 h. Settled spores were harvested and extracted with reducing SDS buffer, total protein content determined by precipitation, and extracts diluted with reducing SDS buffer to uniform total protein concentration. After SDS-PAGE and transfer to nitrocellulose, blots were probed with the monoclonal antibody Ent6, which binds to the *Ulva* spore adhesive [5]. On immunoblots of extracts of swimming, unsettled spores (presented here as a "standard") the major 110-kDa adhesive antigen characterized by Stanley *et al.* [5] was clearly detected by the Ent 6 antibody (Figure 6, arrowed). In extracts of spores settled in plain sea water, the same antigen was present as a faint band at the first time point examined (4 h) but band intensity declined progressively with time of settlement and was almost undetectable after 16 h. In contrast, extracts of spores settled in the presence of 0.1 mM of DTT gave a strongly staining 110-kDa band, even after 20–24 h of settlement. A similar result was observed with immunoblots of spore

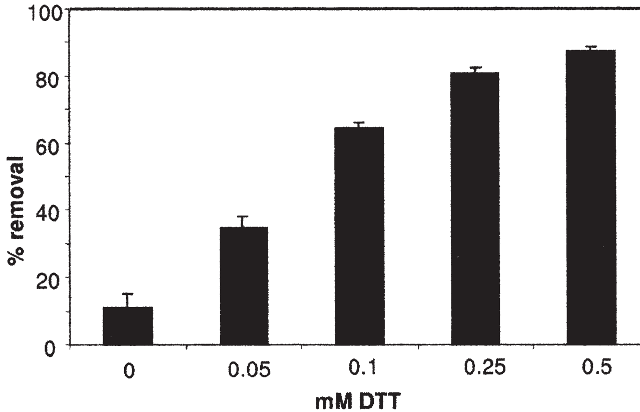

FIGURE 4 Effect of DTT concentration on interfacial-adhesion strength of attached spores. Spores were settled for 2 h in the presence of various concentrations of DTT, and then excess unsettled spores were removed by washing. The attached spores were left for a further 2 h in the presence of various concentrations of DTT before exposure to a water jet (125 kPa of surface pressure). Percentage removal is calculated as the proportion of spores remaining in each treatment as compared with the non-jet-washed slides. Bars represent standard errors from arcsine-transformed data. The mean level of spore settlement in the experiment was 1143 spores mm^{-2}.

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extracts settled in the presence of Ellman's reagent (Figure 7). In this case an earlier time point after settlement (2 h) was examined but showed no detectable difference between the treatment and the control. By 4 h there was a small but detectable difference in band intensity, which became more pronounced with time after settlement.

The Western blot assay indicates the relative proportions of adhesive antigens remaining in soluble form at a series of time points after settlement of the zoospores. In untreated zoospores, the gradual disappearance of the 110-kDa primary antigen band from the Western blots can be assumed to indicate either degradation of the antigenic epitope or the occurrence of a cross-linking or curing process that renders the adhesive completely insoluble within 24 h, even after boiling in 2% SDS. Curing of the adhesive is also implicated in the increasing strength of adhesion of the zoospore to a surface with time after settlement. The effect of both DTT and Ellman's reagent in inhibiting the development of adhesion strength, and in rendering the major adhesive antigen soluble over an extended period of settlement, is therefore consistent with an inhibition of adhesive curing by both reagents.

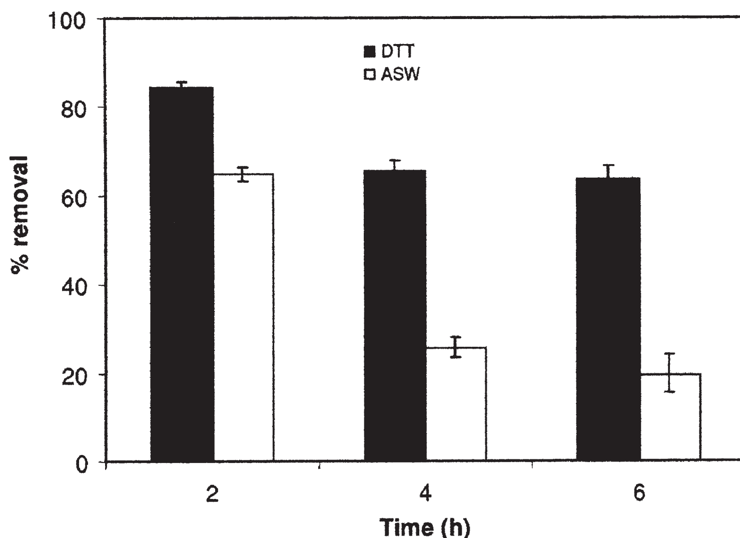

FIGURE 5 Effect of 0.25 mM of DTT on percentage removal of attached spores (2, 4, and 6 h after settlement) after exposure to a surface pressure of 65 kPa. Spores were settled for 1 h, excess spores were removed by washing, and the attached spores were incubated for the period indicated. Percentage removal for each treatment is calculated as the proportion of spores left on slides after jet washing as compared with the non-jet-washed slide. Bars represent standard errors from arcsine-transformed data. The mean level of spore settlement in the experiment was 552 spores mm^{-2}.

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Ellman's reagent is a sulfur-selective oxidizing agent that reacts with free sulfhydryl groups such as those present in reduced cysteine residues, forming mixed disulfide linkages. We hypothesize that the inhibition of adhesive curing by Ellman's reagent may, therefore, be due to the reagent "capping" key cysteine residues and preventing the formation of disulfide linkages, either in the adhesive protein itself or in any cross-linking enzyme(s) secreted with the adhesive glycoproteins. This interpretation is also consistent with the observation that a similar inhibition of adhesive curing occurs in the presence of dithiothreitol, a mild reducing agent used in protein biochemistry to maintain sulfhydryl groups in the reduced state.

Interestingly, pherophorin DZ1, a member of a family of extracellular proteins produced by the colonial green alga *Volvox*, catalyzes its own cross-linking, and this can be inhibited by Ellman's reagent [21]. DZ1 also has a monomer molecular mass of ~110 kDa, like the

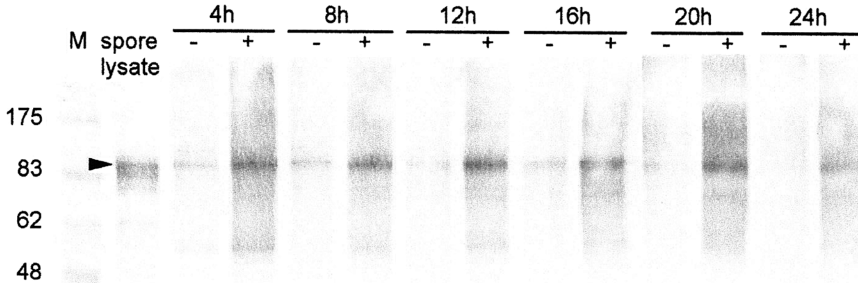

FIGURE 6 Immunoblots of extracts of spores of *Ulva* after settlement for various periods of time in the presence (+) or absence (–) of 0.1 mM of dithiothreitol. All extracts of spores were loaded onto 10% SDS-PAGE gels at the same protein concentration (6.8 μg per lane) to allow direct comparison of relative band intensity. After electrophoresis, gels were blotted to nitrocellulose and probed with the antibody Ent 6. An extract of unsettled spores ("spore lysate") was included for comparison. The prominent 110-kDa adhesive antigen is arrowed. The left-hand lane (M) shows the position of molecular mass markers (kDa).

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Ulva spore adhesive antigen. Without molecular characterization of the *Ulva* adhesive, it is not possible to say whether this similarity in certain properties is coincidental or indicative of a more fundamental homology.

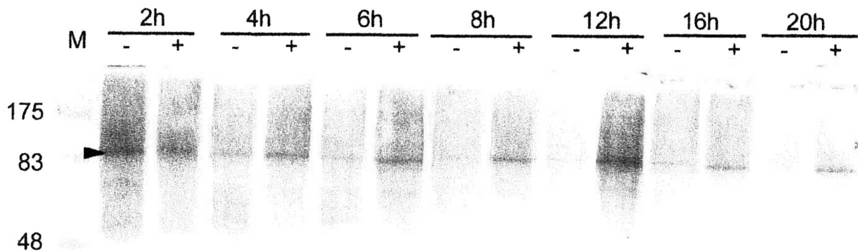

FIGURE 7 Immunoblots of extracts of spores of *Ulva* after settlement for various periods of time in the presence (+) or absence (–) of 1 mM of Ellman's reagent. All extracts of spores were loaded onto 10% SDS-PAGE gels at the same protein concentration (9 μg per lane) to allow direct comparison of relative band intensity. After electrophoresis, gels were blotted to nitrocellulose and probed with the antibody Ent 6. The prominent 110-kDa adhesive antigen is arrowed. The left-hand lane (M) shows the position of molecular mass markers (kDa).

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