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Histochemistry of Spore Mucilage and Inhibition of Spore Adhesion in *Champia parvula*, A Marine Alga

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HISTOCHEMISTRY OF SPORE MUCILAGE AND
INHIBITION OF SPORE ADHESION IN CHAMPIA PARVULA,
A MARINE RED ALGA.

BY
MARTHA E. APPLE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCES

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1994

DOCTOR OF PHILOSOPHY DISSERTATION

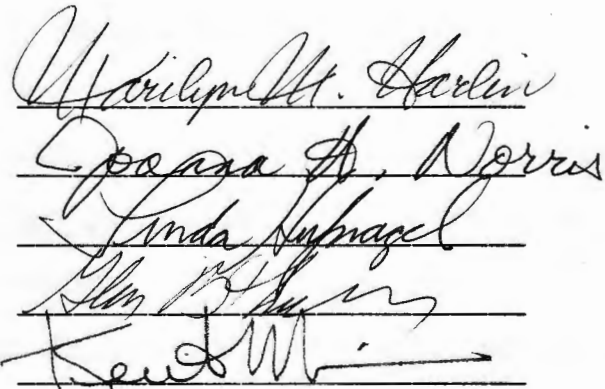
OF

MARTHA E. APPLE

APPROVED:

Dissertation Committee

Major Professor

The image shows five handwritten signatures, each written over a horizontal line. From top to bottom, the signatures are: 1. A cursive signature that appears to be 'William M. ...'. 2. A cursive signature that appears to be 'George H. Norris'. 3. A cursive signature that appears to be 'Linda ...'. 4. A cursive signature that appears to be 'John ...'. 5. A cursive signature that appears to be 'Kent ...'.

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1994

Abstract

Spores of the marine red alga, Champia parvula, attached initially to plastic or glass cover slips by extracellular mucilage. Adhesive rhizoids emerged from germinating spores, provided a further basis of attachment and rhizoidal division formed the holdfast. Mucilage of holdfasts and attached spores stained for sulfated and carboxylated polysaccharides. Rhizoids and holdfast cells but not mucilage stained for protein. Removal of holdfasts with HCl revealed protein anchors in holdfast cell remnants. Spores detached when incubated in the following enzymes: β -galactosidase, protease, cellulase, α -amylase, hyaluronidase, sulfatase, and mannosidase. The FITC-lectins Con A, LCA, PNA, SBA, and the lectin from Vicia villosa, were used to probe the mucilage of attached spores to detect the sugar haptens α -D-mannose, α -D-glucose, β -D-galactose, and N-acetylgalactosamine, whereas probing with WGA, Phytolacca americana mitogen (PWM), and UEA did not detect N-acetylglucosamine or α -L-fucose

Adhesion of newly released, floating tetraspores was inhibited by cycloheximide, tunicamycin, sodium molybdate, and Con A. These results indicate that proteins, glycoproteins, sulfated polysaccharides, and α -D-mannose or α -D-glucose, respectively, are all necessary for adhesion. Tetraspores remained attached in the presence of the inhibitors, suggesting that they do not maintain adhesion via synthesis of proteins, glycoproteins, or sulfated polysaccharides. Tetraspores killed with H₂SO₄ or sodium azide did not attach; therefore tetraspores must be alive to attach. Tetraspores did not detach when killed with sodium azide or DIH₂O. Death did not result in detachment unless the mucilage was damaged by H₂SO₄.

Glycoproteins with α -D-mannose and or α -D-glucose sugar moieties may detect the substrate upon contact and convey messages to the cytoplasm which translates spatial information about adhesion into germination and rhizoid production. The sugar moieties may recognize cellular surfaces of hosts on which C. parvula is epiphytic. These glycoproteins are probably embedded in a matrix of adhesive, sulfated polysaccharides which may be cross-linked with proteins, suggesting that several classes of molecules may interact to facilitate adhesion.

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HISTOCHEMISTRY OF SPORE MUCILAGE AND
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INTRODUCTION

Spores are dispersal and attachment units. They are the bridges between macroalgal life cycle phases. Establishment of attached adult plants depends on spore adhesion (Chamberlain and Evans 1981). Spores are highly vulnerable immediately after attachment; yet little is known about their survivorship during establishment (Brawley and Johnson 1991; Flavier and Zingmark 1993). Spore attachment must be strong enough to withstand wave action and spores must attach to substrata that receive enough light for photosynthesis (Norton and Mathieson 1983). The ability to be epiphytic as well as attached to other substrata confers the advantage of an increased range of possible habitats (Hay 1986). Even though spores are the primary reproductive unit of the macroalgae, little is known about their biology in general, and settlement in particular (Amsler and Neushul 1991). The mechanisms and histochemistry of red algal spore attachment are relatively unknown when compared to attachment of brown or green algae or of marine invertebrates. Knowledge gained from investigations of red algal spore attachment can be used to increase our understanding of algal spore biology (Pueschel 1990), host-epiphyte relationships, and biofouling. Such knowledge will also make a necessary contribution to the intriguing, significant, but as yet understudied field of red algal biology (Woelkerling 1990).

Red Algal Mucilage and Initial Spore Attachment

Red algal spores have what has been called "a ubiquitous mucilage" (Boney 1981). There is little argument that this encompassing mucilage functions in spore adhesion, especially because red algal spores are nonmotile (Fletcher and Callow 1992). They sink very slowly (Okuda and Neushul 1981) but do not have far to fall from their relatively small parent plants (Kain and Norton 1990) and the thicker the mucilage layer, the faster the spore settlement (Carrier 1984). The mucilage sticks to the substratum upon contact to provide initial, albeit insecure adhesion (Sawada et al. 1972). Attachment of almost all red algal spores becomes progressively firmer with time (Chamberlain 1976). Initial attachment of Ceramium spores is probably by the viscous mucilage envelope (Chamberlain and Evans 1981); attached spores are easily washed off their substrate at this stage. Adhesive secreted from golgi vesicles after spore release forms a resistant attachment pad and further anchorage is provided by rhizoid development.

Mucilage and vesicles which probably contain adhesives are formed during sporogenesis by a variety of red algae. Mucilage is produced by the golgi apparatus and sometimes by the endoplasmic reticulum. Starch accumulates and adhesive vesicles form during sporogenesis of Haliptilon cuvieri (Vesk and Borowitska 1984). Golgi vesicles change appearance from striated to fibrous and are involved in protein turnover during carposporogenesis in Polysiphonia novae-angliae (Wetherbee and West 1977). Proteinaceous crystalloids accumulate in vegetative cells but disappear upon sporogenesis in Wrangelia plumosa (Wetherbee et al. 1984).

Pueschel and Cole (1985) found that attached spores of Porphyra variegata did not have adhesive vesicles, but were coated with a substance that filled an indentation of the spore and resembled the vesicle contents; a true cell wall formed after initial attachment.

Attachment is mediated by sheaths of extracellular mucilage that develop after release to surround spores of Chondrus crispus (Chen and Taylor 1975). Within the sheath, attached carpospores and tetraspores divide and form disc-like sporelings that expand radially and form erect fronds. Without a sheath, sporelings form loosely attached semifilaments. Sheaths may provide physical conditions that are conducive to disc formation. Coalescence can occur between sporelings and between sheaths of adjacent sporelings (Tveter and Mathieson 1976), and may increase the chances for establishment of perenniating C. crispus holdfasts, which can regenerate up to 25% of their area if necessary (Taylor et al. 1981). Germinating spores also coalesce in Iridaea laminarioides, forming common basal discs for frond and stipe emergence, with vigorous growth in the center of the disc (Martinez and Santelices 1992).

Encrusting coralline red algae have a number of ways to ensure their tenacious attachment. When Phymatolithon lenormandii, P. laevigatum, P. polymorphum, Lithothamnion glaciale, Lithophyllum incrustans, and Lithophyllum sp., were cultured on glass slides, spore mucilage provided initial adhesion but was invisible after the algae became calcified (Walker and Moss 1984). As the algal crust developed, hypothallial filaments provided further attachment by flattening onto the smooth glass slides. On rough

substrate, hypothallial filaments moulded to and penetrated surface irregularities. Any spaces that remained or developed between the algal crust and the substrate were filled by a layer of aragonite crystals bound with an organic component, which was probably secreted by the cells. If the corallines were deliberately removed, material left behind stained positively for polysaccharides.

Spore mucilage and rhizoids are important in host-epiphyte and host-parasite interactions. Initial attachment of the epiphyte Polysiphonia lanosa onto Ascophyllum nodosum or Fucus vesiculosus is an interaction between spore and host mucilage (Pearson and Evans 1990). The epiphyte releases adhesive vesicles and penetrates the host with unicellular rhizoids. More spores survive on A. nodosum than on F. vesiculosus because lateral pit and axil sites provide shelter. Rhizoids deeply penetrate the host and remain attached after A. nodosum sheds its outer surface (Filion-Myklebust and Norton 1981). Choreocolax spores have enlarged vacuoles which may push the spore contents into the rhizoid when it penetrates the host (Goff and Coleman 1984). Some parasitic spores of Harveyella mirabilis attach with extensions of their outer coverings to walls of wounded cells of Odonthalia floccosa; others germinate in ruptured cytoplasm and attach with rhizoids that grow into the sulfated polysaccharide rich cortical cell walls (Goff and Cole 1976). Ultrastructural studies of parasitic red algal rhizoids revealed abundant rough and smooth endoplasmic reticulum that connects with the plasmalemma, numerous ribosomes, protein bodies, mitochondria, and protein staining microbody-like organelles (Goff 1982).

In addition to adhesion, a myriad of other functions are attributed to red algal spore mucilage. These include metal chelation (Tanaka et al. 1971), nutrient absorption (Percival and McDowell 1967), light absorption, resistance to abrasion, protection from physical and chemical changes (Boney 1981), protection from toxic compounds (Decho 1990), protection against mechanical injury (Davis 1972; Characklis and Cooksey 1983), antipredation (Percival 1979), antibiotic (Kugrens and West 1973), reproduction, and intercellular communication (Characklis and Cooksey 1983). Sulfated mucilage serves as an ionic regulator (Kloereg and Quatrano 1988). Deformation of the mucilage could dissipate the forces of waves, thereby maintaining adhesion (Boney 1975). The mucilage also functions in dispersal. For example, mucilage of Iridaea laminarioides carpospores sticks to legs of the amphipod Hyale sp., or the carpospores travel unharmed through the digestive tract (Buschmann and Bravo 1990, Buschmann and Vergara 1993).

Polysaccharides in Red Algal Cell Adhesion

Carpospores and tetraspores of Ceramium are thought to attach with a polysaccharide that becomes hydrated and forms a viscous, macro-molecular hydrocolloid that disperses upon contact with seawater (Chamberlain and Evans 1981). Spores detached when polysaccharides were degraded with periodic acid and sodium tetraborate. Although stains for proteins were positive in the mucilage of unreleased spores of Ceramium but negative following spore release, Chamberlain and Evans (1981) concluded that the adhesive was not proteinaceous because agents that disrupt proteins did not cause spore detachment. The mucilage of Hypnea musciformis, Polysiphonia

deusta, and Halarachnion ligulatum contains a highly sulfated acidic polymer (Diannelidis and Kristen 1988) which may be adhesive.

In addition to their role in adhesion, polysaccharides are important structural elements of cell walls of red algae (Craigie 1990). The viscous mucilage of Rhodella reticulata is thought to serve as protection; its production increased as growth stopped (Kroen and Ramus 1990). The polysaccharide component of this mucilage is synthesized and sulfated in the golgi apparatus, while its protein component is thought to be secreted through ducts in the endoplasmic reticulum that are fused to the plasma membrane (Callow and Evans 1981). Dessication is prevented by sulfated capsular polysaccharides of Porphyridium aerugineum (Ramus 1974); in which the golgi complex incorporates approximately half of the cellular sulfate into polysaccharides (Ramus and Groves 1972; Ramus and Groves 1974). Protein rich extracellular secretions of Nizymania australis that contain sulfated and carboxylated polysaccharides are thought to facilitate the release of spermatia and provide protection from epiphytes and pathogens (Bhatia and Vijayaraghavan 1990).

Glycoproteins in Red Algal Cell Adhesion

Glycoproteins have been shown to be involved in adhesion of red algal cells: in spore adhesion (Pueschel 1979), in reproduction (Kim and Fritz 1993a,b; Kaska et al.1988), and in wound healing (Kim and Fritz 1993c; Watson and Waaland 1983).

Little is known about the role of glycoproteins in red algal spore attachment. Red algae may have proteins or glycoproteins that are unique to and involved in spore adhesion. Spores may carry only those proteins or glycoproteins necessary to insure their attachment, germination, and viability. Glycoproteins, along with sulfated and acidic polysaccharides are probably adhesive molecules in tetraspores of Palmaria palmata (Pueschel 1979).

Glycoproteins are active in red algal reproduction. A glycoprotein with α -D-methyl mannose residues is found on the outer surfaces of spermatia of Antithamnion nipponicum, and binds with a receptor in the trichogyne to mediate gamete recognition (Kim and Fritz 1993b). Glycoproteins differ between male and female reproductive tissue in Porphyra perforata and in morphologically and functionally distinct regions of the thallus (Kaska et al. 1988).

Wound healing involves cell adhesion. A glycoprotein is thought to mediate wound healing in Antithamnion sparsum (Kim and Fritz 1993c). The hormone rhodomorphin is a glycoprotein with α -D-mannose and/or α -D-glucose sugar haptens that is necessary for wound healing in Griffithsia pacifica (Watson and Waaland 1983).

Interactions Between Adhesive Molecules

Adhesion may involve interactions between classes of molecules. Although red algal cell walls contain a proportionately smaller amount of proteins than carbohydrates (Craigie 1990), most algal adhesives studied

thus far are variations of polysaccharide-protein complexes that are at least partially synthesized in the golgi apparatus (Willey and Giancarlo 1986). There may be more than one adhesive or one adhesive may become functional when it is cross-linked (Wigglesworth-Cooksey and Cooksey 1992). Increases in the strength of red algal adhesion over time are thought to result from a "curing" process which may be polymerization or cross-linking (Bråten 1975, Chamberlain 1976, Heaney-Kieras et al. 1977), possibly between sulfated polysaccharides or glycoproteins and the divalent cations Ca^{2+} or Mg^{2+} (Jones et al. 1982; Craigie et al. 1992).

It appears that red algal adhesion is an interaction between sulfated and acidic polysaccharides, proteins, and glycoproteins. Golgi vesicles secrete mucilage rich in sulfated and acidic polysaccharides during tetraspore development of Palmaria palmata (Pueschel 1979). After mucilage deposition ceases, the golgi apparatus makes vesicles with glycoprotein rich contents. These vesicles are abundant in released tetraspores and probably have adhesive material for spore attachment. Both proteins and sulfated polysaccharides are found in vesicles produced by the Golgi apparatus in differentiating carposporangia of Chondria tenuissima (Tsekos 1985).

Adhesion of Brown Algae

The adhesion of brown algal zygotes has been investigated extensively (Crayton et al. 1974, Hardy and Moss 1978, 1979a,b,c; Evans et al. 1979; Quatrano et al. 1979; Brawley and Quatrano 1979a; Moss 1981; Brawley and Robinson 1985; Kropf et al. 1989; Wagner et al. 1992; Vreeland et al. 1992,

1993). Initial attachment of zygotes generally occurs with adhesive mucilage supplemented by adhesive zygotic walls, followed by secondary attachment with rhizoids (Hardy and Moss 1979a; Moss 1981; Vreeland et al.1993). Pelvetia canaliculata provides an example of such attachment. Zygotes are anchored with an enclosing mesochiton that can be said to correspond with the mucilage of red algal spores (Boney 1975) by countering water movements and assisting settlement. The mesochiton stains with alcian blue to suggest sulfated polysaccharides as initial adhesives; secondary adhesion occurs when the rhizoids provide further anchorage (Hardy and Moss 1979a). Initially, Halidrys siliquosa zygotes are attached by a rigid, alcian blue staining, adhesive wall that is secreted by the golgi apparatus and surrounds the zygote. Because the adhesive is also part of the wall, failure is likely to occur only at the adhesive-substrate interface, and not at the zygote-adhesive interface. The adhesive wall is shed once the rhizoids appear, which is not until several days after initial attachment. The four primary rhizoids and their derivatives have adhesive mucilage, and while the thallus grows their continued division eventually forms the holdfast (Hardy and Moss 1978). Adhesion is similar in Bifurcaria bifurcata (Hardy and Moss 1979b).

Zygotes of Fucus and Ascophyllum follow the pattern of attaching initially by exuding mucilage and secondarily by the primary rhizoid which produces alcian blue staining mucilage at its tip. If the substrate is smooth, this mucilage will spread to form an adhesive, suction-like "foot". New adhesive feet are produced in pulses as the rhizoid grows. On non-smooth surfaces the rhizoid grows down into the substratum through any available crevices. The basal embryo cells produce secondary rhizoids which fan out to provide further

anchorage (Moss 1981). Glass coverslips provide an artificial substrate that differs greatly from that supplied by intertidal rocks to which Fucus adheres in nature. Rhizoids of Fucus plants grown on glass were long, thin, and tapered while those grown on rocks were more stout for a greater contact area and firmer attachment (Hardy and Moss 1979c). Fucus zygotes attach more firmly when rhizoids can penetrate interstices in substrates such as porous rock and wood, and less firmly on smooth substrates such as glass where rhizoids cannot penetrate.

Fucus rhizoids are anchored by a highly sulfated fucan glycoprotein, (F2), (Brawley and Quatrano 1979a) which must be sulfated before adhesion will occur; Fucus embryos grown without sulfate formed rhizoids but did not adhere. Those grown with sulfate did adhere and sulfated polysaccharides were detected at the rhizoid tips with toluidine blue and the D-galactose specific lectin FITC-RCAI (Crayton et al. 1974). If embryos are grown without sulfate, F2 will not be sulfated or localized in the rhizoid (Quatrano et al. 1979). F2 is sulfated in the golgi apparatus, through which it is secreted (Evans et al. 1979) and then transported via an actin network to the rhizoid tip (Brawley and Robinson 1985; Kropf et al. 1989) with a vitronectin like glycoprotein, (Vn-F) (Wagner et al. 1992). Vn-F may begin its association with F2 during travel through the golgi apparatus. Vn-F is localized in the extracellular matrix of the elongating rhizoid tip, which anchors the zygote to the substrate. When two-celled Fucus embryos were cultured without sulfate, Vn-F was not localized in the rhizoid tip and the embryos did not adhere in the presence of the Vn antibody (Wagner et al. 1992).

It is noteworthy that a vitronectin-like glycoprotein has been found in the algae. Vitronectin and fibronectin are found in humans and other mammals (Singer et al. 1988; Underwood and Bennett 1989), and a homolog of vitronectin has been found in angiosperms (Sanders et al. 1991). A vitronectin-like protein on the surface of carrot cells is used by the pathogenic bacterium Agrobacterium tumefaciens as a receptor when it attaches to carrot cells (Wagner and Matthysse 1992). Vitronectin and fibronectin are adhesive proteins found on the external side of the plasma membrane in focal adhesions, which are membranous connections between the extracellular matrix and the cytoskeleton (Burrige 1988).

Adhesive formation increased with the addition of 1 μ M vanadate to Fucus zygotes cultured in artificial seawater (Vreeland et al. 1992). A peroxidase that requires vanadate may catalyze cross linkages between cell wall carbohydrates and phenolics to place the now cross linked phenolics at adhesive sites. Peroxidase activity and extracellular phenolics were found in the cell wall during initial adhesion and in the rhizoid tip after germination. Microspheres bound to the mucilage of Fucus gardneri zygotes to show localized patches of adhesive 3-6 h after fertilization (Vreeland et al. 1993); this corresponds with the localization of cross-linked phenolics at adhesive sites in the presence of vanadate. More microspheres bound as zygote development progressed, showing that more adhesive was being produced by the zygote. The patches of adhesive eventually grew to cover the hemisphere of the zygote oriented towards the substrate and the rhizoid upon its emergence. Because the adhesive appeared in patches, it was probably produced by cytoplasmic vesicles. The cell wall was anchored by strands of

adhesive polymers. Microspheres detached from rhizoids in the presence of calcium chelators, implying the necessity of divalent cations in adhesion. It appears that adhesion of Fucus rhizoids is an interaction between many factors: mucilage, phenolics, the sulfated fucan F2, the adhesive glycoprotein Vn-F, divalent cations, and positioning of these adhesive molecules through transport, cross-linking and enzyme catalysis.

Adhesion of Green Algae

Acidic polysaccharides (Bråten 1975; Bingham and Schiff 1979; Rogers Domozych et al. 1993), glycoproteins (Musgrave 1987; Sekimoto and Fujii 1992; Goodenough et al. 1993), mucopolysaccharides (Christie et al. 1970) and protein (Tosteson and Corpe 1975) have all been implicated as adhesives in the green algae. In addition, Ca^{2+} has been associated with adhesive events such as mucilage secretion (Rogers Domozych et al. 1993) and mating reactions (Pasquale and Goodenough 1987; Goodenough et al. 1993).

Zoospores of Enteromorpha intestinalis from different parent sources have different adhesive abilities but detach similarly when exposed to trypsin, pronase, and α -amylase (Christie et al. 1970). Because the effects of trypsin mirrored those of α -amylase, the adhesive is thought to be a mucopolysaccharide. Within minutes of substrate contact, the adhesive is secreted. Newly attached spores were most susceptible to enzyme induced detachment but became more resistant over time.

The initial adhesive of the zygote of Ulva mutabilis differs from that of the rhizoid, which provides secondary adhesion (Bråten 1975). Just before gametes are released, their cytoplasm contains small, electron dense vesicles. Once the zygotes form, their adhesive is probably secreted through these vesicles onto the substrate. This adhesive stains with ruthenium red for acidic polysaccharides and can be removed with pronase and α -amylase, but not with hyaluronidase or ruthenium red, which binds with acidic polysaccharides. The rhizoid cells differentiate several days after the initial adhesion of the zygote and produce adhesive continuously as they grow. The resistant adhesive of the rhizoid does not stain with ruthenium red or detach with enzymes and is therefore thought to have a different chemical composition than that of the initial, zygotic adhesive.

Ulva rigida and Enteromorpha compressa are often epiphytic on Gracilaria chilensis. Santelices and Varela (1993) found that exudates from the culture medium of G. chilensis stimulated settlement of spores of both green algal species. Upon analysis, the exudate was found to contain sulfated galactans. The mechanism of response by the epiphytes to the host exudate is not yet known. A high epiphyte load can be detrimental to the farming of G. chilensis (Buschmann and Gomez 1993), as the added weight can cause the host to detach. These epiphytes can compete for nutrients, light, and dissolved gases. They can also exude allelopathic substances that are either harmful or beneficial to the host (Harlin 1987).

Exudate containing protein and carbohydrate enhances adhesion of unicellular Chlorella vulgaris to glass (Tosteson and Corpe 1975). This non-

diffusible material originates from the following sources: 1) C. vulgaris, 2) fouled marine surfaces, 3) marine bacterial cultures and 4) natural seawater, with the latter two being the most effective. The exudate may induce adhesive polymer synthesis, stimulate secretion of the adhesive, stabilize the adhesive, or act as an adhesive substitute. C. vulgaris cells adhered less but began to agglutinate in a lectin induced manner when C. vulgaris exudate concentrations were above 0.2 ng/cell. More washed cells than unwashed cells adhered when exudate was added; washing may expose more adhesive sites or change the physiological state of the cell. Maximal adhesion of C. vulgaris occurs in the G2 period of interphase (Zaidi and Tosteson 1972), possibly because the cell surface may be altered by biochemical changes between cell cycle phases. Increased adhesion may be related to possible changes in the cell proteins within the cell cycle, which in turn may influence the composition of the proteinaceous exudate, which may be adhesive or have its synthesis catalyzed by a protein (Tosteson and Corpe 1975).

Agglutinating glycoproteins on the flagellar surfaces of Chlamydomonas eugametos gametes are intrinsic to the membrane and have differently shaped ends; one is knoblike and the other is hooklike (Musgrave 1987), which may facilitate their mediation of flagellar adhesion between + and - gametes. Adhesion of the flagellae initiates travel of a signal from the membrane to the cytoplasm of the paired cells, which fuse upon receipt of the signal. Ca^{2+} and cAMP interact as the messengers for this signal (Pasquale and Goodenough 1987; Goodenough et al. 1993). Upon adhesion, intracellular cAMP levels increase sharply. Gametes will mate if given di-buteryl-cAMP. This effect was blocked by inhibitors of Ca^{2+} transport or utilization.

Elongated cells of the desmid Closterium glide on substrates by mucilage that is secreted from the pole opposite the direction of motion (Rogers Domozych et al. 1993). The mucilage is an acidic polysaccharide that stains with Ruthenium red. Dense cored vesicles from the golgi apparatus associate with microtubules and are secreted out of the cell through flask-shaped pores in the cell wall (Domozych and Rogers Domozych 1993). Labeling with chlorotetracycline showed that these pores are associated with calcium rich areas on the cell surface (Rogers Domozych et al. 1993). Pores in the poles of Closterium ehrenbergii cell walls secrete receptors for the β -D-galactose specific lectin RCA₁₂₀ as part of the mucilage (v. Sengbusch et al. 1982); therefore carbohydrate recognition by lectins may be part of the adhesive process for these desmids, as lectins have been found in the Chlorophyta (Blunden and Rogers 1990).

Adhesion of Diatoms

The pennate marine diatom, Amphora coffeaformis, glides on adhesive mucilage containing acidic polysaccharides that is secreted from golgi vesicles through the raphe fissure (Drum and Hopkins 1966; Daniel et al. 1980; Webster et al. 1985). Binding of D-glucose to chemosensory receptors on the plasma membrane may signal release of inositol triphosphate as a transducer, which then binds to a further receptor to signal release of bound Ca^{2+} , opening of Ca^{2+} channels in the membrane, and an increase in cytoplasmic Ca^{2+} concentrations (Cooksey and Cooksey 1980; Cooksey 1981; Berridge and Irvine 1984; Cooksey and Cooksey 1986; Wigglesworth-

Cooksey and Cooksey 1992). Elevated cytoplasmic Ca^{2+} levels activate cytoplasmic transducers to translate the Ca^{2+} message into release of mucilage, hence adhesion. The mucilage probably remains attached to the cytoplasm through plasmalemma at the raphe; in this way it maintains contact with bundles of actin filaments that may translate adhesion into motility by moving the site of mucilage attachment along the raphe (Edgar and Pickett-Heaps 1984).

Adhesion and motility were prevented when *A. coffeaformis* was given podophyllotoxin to depolymerize microtubules (Cooksey and Cooksey 1988) and the energy uncoupler, carbonyl cyanide-3-chlorophenyl (CCCP), to indicate that adhesion requires cellular energy. When Ca^{2+} transport was blocked with α -isopropyl- α -[(N-methyl-N-homoveratryl)- α -amino propyl]-3,4,5-trimethoxyphenylacetonitrile (D-600), adhesion and motility were inhibited (Cooksey and Cooksey 1980; Cooksey 1981). Sr^{2+} could substitute for Ca^{2+} as a requirement for adhesion but not as a requirement for motility. These diatoms detached and left adhesive "footpads" behind in the presence of the Ca^{2+} chelator, ethylene glycol-bis(β -amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA), to suggest that external Ca^{2+} may maintain adhesion by cross-linking negative charges in the extracellular matrix (Cooksey and Cooksey 1986). Glycoproteins and proteins may be part of the adhesive or may synthesize the adhesive, as adhesion was reduced when glycoprotein and protein synthesis were inhibited with tunicamycin and cycloheximide, respectively. Because polysaccharides have been found repeatedly in the extracellular polymers of diatoms and proteins have not often been detected, it

is likely that the adhesive of diatoms is chiefly polysaccharide (Hoaglund et al. 1993).

The pennate marine diatom, Ardissonea crystallina, has a different means of motility (Pickett-Heaps et al. 1991). It can change direction as it glides on mucilage that adheres to the substrate, stains with alcian blue and is secreted through terminal grooves in the trailing end of the cell. The elastic mucilage may swell after release and push the cell in the direction of travel, which is similar to the effect of mucilage on desmids. A. crystallina can also attach with thick stalks that stain with alcian blue to indicate sulfated polysaccharides as adhesives in this diatom. Diatoms with stalks were found to be more abundant in the upper, well lit but more turbulent reaches of a reservoir than were diatoms without stalks (Hoaglund and Peterson 1990). Stalked diatoms were able to survive in both the upper and the lower reaches of the reservoir while unstalked diatoms did not fare well in the upper reaches.

Adhesion of Euglenoids

Colacium libellae migrates and attaches to freshwater arthropods. After travelling through the golgi apparatus, a polymer is extruded from pores in the anterior pellicle to form an adhesive disc which stains for sulfated polysaccharides with alcian blue but not for neutral polysaccharides with periodic acid-Schiff (PAS) (Willey and Giancarlo 1986). Next, a flexible stalk forms between the adhesive disc and the euglena cell. The core of the stalk stains with PAS and its periphery stains with alcian blue. Differential staining signifies different adhesive polymers. Both the adhesive disc and stalk resist

pronase digestion and remain attached to indicate polysaccharide in adhesion rather than the protein-polysaccharide complex generally found in algal cells (Chamberlain 1976).

Adhesion of Fungi

Ascospores of marine ascomycetes have a variety of attachment mechanisms (Hyde and Jones 1989). These include: 1) release of a mucilage drop from a polar end chamber to form an adhesive pad, 2) long, viscous threads which form when cap-like appendages uncoil, 3) sticky mucilagenous sheaths which may expand upon contact with water, 4) sticky vermiculate appendages, 5) tufts of fibrillar appendages which trap the spores on jagged substrate edges such as wood, 6) amorphous appendages which rely on contact with a large surface area of the substrate for adhesion, 7) adhesive spore walls with a sticky, fibrillar layer, and 8) combinations of the above. Marine fungi attach to substrata by chemical or physical forces and the greater the area of contact with the substrate, the greater the attachment. The appendages of marine fungi increase the area of substrate contact.

Spores of the plant pathogen, Nectria haematococca attach by their apices with "macroconidial tip mucilage" that labels with FITC-Con A only when the fungus is cultured in a medium that promotes adhesion (Kwon and Epstein 1988). Further investigation of the mucilage of adherent macroconidia with SDS-polyacrylamide gel electrophoresis revealed a 90 kDa glycoprotein that binds with Con A. Spores detached when exposed to protease and did not attach in the presence of Con A.

Adhesion of Marine Bacteria

Adhesive polysaccharides in bacterial mucilage attach to glycoproteins of the marine conditioning layer that becomes adsorbed to underwater marine surfaces (Baier 1980; Dempsey 1981). More bacteria attached to biofilms of previously attached bacteria of the same species than to biofilms of other species or to glass (Banks and Bryers 1992). Depending on the shape of the bacterium, adhesion will occur via mucilage to fill in the space between the substrate and the cell wall. Rods and cocci attach with strands and sheets, or less frequently with pads and capsules. Stalked bacteria attach with basal mucilage pads. Continuous colonial growth results in a film of bacteria with accompanying mucilage (Dempsey 1981).

Adhesion of Animals

Marine mussels, Mytilus spp., attach to surfaces by forming a byssus composed of a bundle of threads connecting the mussel to an adhesive plaque with a water resistant polyphenolic protein (Rzepecki et al. 1992) that attaches the plaque to the substrate (Benedict and Waite 1986b). The core of the byssal threads forms from collagenous secretions of the collagen gland (Vitellaro-Zuccarello 1980); the accessory gland then secretes a protective coating composed of a proteinaceous resin and a curing enzyme, catecholoxidase (Brown 1952; Vitellaro-Zuccarello 1981), which converts 3,4-dihydroxyphenylalanine (DOPA) in the resin to peptidyl DOP α -quinone, which probably acts as a cross-linker to harden the adhesive (Waite 1983;

Benedict and Waite 1986a; Waite 1990). M. californianus lives in exposed, turbulent places and has a less porous byssus, stronger attachment strength and an adhesive with a different protein composition than that of M. edulis, which inhabits calm, sheltered environs (Waite 1986).

Phragmatopoma californica, a marine polychaete, has a stable and insoluble proteinaceous adhesive (Jensen 1992). The adhesive connects hard particles so that a tube can be made for the polychaete to live in. Three percent of the adhesive's amino acid residues are 3,4-dihydroxyphenylalanine (DOPA). DOPA extracted from the adhesive signaled planktonic larvae of P. californica to induce attachment and metamorphosis, thereby expanding concretions of marine polychaetes.

Barnacles, Serni (Balanus) balanoides, disperse and explore the substrate during the cyprid stage of metamorphosis and use a temporary proteinaceous adhesive for this initial phase of attachment (Neal and Yule 1992). Settlement occurred with greater frequency on slate coated with proteins called arthropodins which were extracted from B. balanoides than on noncoated slate or slate coated with bovine serum albumin. Within 24 h of selection of a final settlement site they complete metamorphosis and a more permanent proteinaceous adhesive with a higher bonding strength is formed (Yule and Walker 1984). Barnacle adhesive is notable because of its tenacity (greater on slate than that of limpets or mussels on slate), ability to spread along fissures and attach to a variety of surfaces, and resistance to biodegradation. Although adhesion of barnacles is tenacious it is not instantaneous and enzymes

probably catalyze reactions that convert water-soluble adhesive proteins to an insoluble state (Yamamoto et al. 1989).

Chemical inducers of settlement and metamorphosis have been found in a variety of marine animals. It is of interest that the red algal pigment phycoerythrobilin contains structural analogs of the neurotransmitter gamma-aminobutyric acid (GABA), and that GABA induces metamorphosis in the red abalone Haliotis rubescens (Morse et al. 1979). Three crustose coralline red algal genera, Lithothamnium, Lithophyllum, and Hildenbrandia are specific substrata for the red abalone. Another crustose coralline red alga, Hydrolithon boergesenii, produces a specific morphogen that induces attachment and metamorphosis in the coral, Agarica humilis (Morse and Morse 1991). Lectin-like receptors on the coral probably bind with multiple N-acetylglucosamine and galactose residues on the morphogen, which is associated with a sulfated glycosaminoglycan. Adults of the oyster, Crassostrea virginica, and bacterial biofilms on oyster shells produce ammonium as a metabolite that induces settlement of oyster larvae (Tamburni et al. 1992).

Coelomocytes of the sea cucumber, Holothuria polii, require cations for adhesion to glass coverslips (Canicatti et al. 1992). Mg^{2+} ions were more effective than Ca^{2+} ions. Adhesion was not inhibited by the metabolic inhibitors potassium cyanide or sodium azide, but was inhibited by the inhibitor of microtubules, vinblastine. Adhesion was enhanced by coating glass coverslips with coelomic fluid or with purified 220 kDa coelomocyte aggregating factor, which is produced by coelomocytes and may be similar to fibronectin.

An elevation of intracellular pH by 0.2 - 0.3 units acts as a signal to the cytoplasm of mammalian cells that a substrate has been encountered and adhesion has taken place (Galkina et al. 1992). If the Na^+/H^+ antiport that facilitates the substrate dependent pH change in mouse fibroblasts or neutrophils (Margolis et al. 1988) is blocked by amiloride, mammalian cells will adhere but will not follow the sequence of events leading to proliferation (Galkina et al. 1992). In this sequence, cellular extensions known as lamellapodia develop and provide further anchorage, the round cells flatten and spread onto the substrate; spreading is then used as a signal for the cells to enter the S phase of mitosis and proliferate (Vasiliev 1985). Collisions between fibroblasts on the substrate will raise the pH, possibly because adhesive sites on the cell surface are occupied. The pH elevation was reversed when cells were detached with trypsin or EDTA. Adhesion of mammalian cells is mediated by integrins, which are transmembrane proteins that bind to the amino acid sequence Arg-Gly-Asp (RGD) on the adhesive glycoproteins vitronectin and fibronectin in the extracellular matrix (Hynes 1987). Binding of the RGD peptide to plasma membrane integrins will raise the pH in the same manner as will adhesion to a substrate (Galkina et al. 1992). Integrins in turn require the divalent cations Mg^{2+} or Ca^{2+} to bind to the RGD peptide (Gailit and Ruoslahti 1988) and chlorotetracycline, a fluorescent calcium chelator, was used to localize Ca^{2+} at attachment points of cell membranes in neural crest cells from the salamander, Ambystoma maculatum (Moran 1984).

Glycoproteins from mammalian cell membranes and extracellular matrices characterize and influence the development of different tissue types and are involved in cellular recognition (Moscona 1974). Glycoproteins of rat cells are supplied with mannose in the endoplasmic reticulum and with galactose and fucose in the golgi apparatus before being transported to the cell surface in vesicles that release their glycoproteins to fuse with the plasma membrane. In this way, glycoproteins reach the cell surface.

Changes in adhesives on the cell surface can be important factors in diseases of animals (Travis 1993). The adhesive molecules of metastatic cancer cells differ from those of nonmetastasizing cells and these adhesive changes may be what allows metastasizing cells to break away from primary tumors, travel through the bloodstream, and start new tumors. On a surface groove of HIV viral particles there is a glycoprotein which binds with CD4, a receptor protein of human cells (Moffat 1993). If the viral particles will bind to a mimic of CD4, infection could be prevented. Initial events in phagocytosis by disease fighting macrophages involve adhesion (Aggeler and Werb 1992) and adhesive glycoproteins have been found in macrophages of mice (Tomita and Ishikawa 1992).

Influence of the Substrate on Algal Spore Adhesion

Because adhesives must bond with the substrate to attach firmly, compatibility of the substrate and adhesive is crucial (Fletcher and Callow 1992). One characteristic of the substrate that probably influences spore adhesion is surface free energy. If a substrate is hydrophobic and has high

surface tension, it has low wettability and low surface free energy. On a hydrophobic Teflon surface, Fucus rhizoids were elongated and did not attach securely (Hardy and Moss 1979c). Spores of Enteromorpha settled on hydrophobic surfaces more readily when they were pressed with coverslips (Christie 1973). Surface tension varied between algal species and on different parts of a thallus; it may determine which epiphytes attach (Linskens 1963). Scirpus validus supported more algal epiphytes in the fall than earlier in the growing season, possibly because the necrotic culms had higher surface free energy due to the breakdown of the hydrophobic cuticle (Goldsborough and Hickman 1991).

Surface texture may also influence spore attachment (Harlin and Lindbergh 1977; Fletcher and Callow 1992). Spores may detect small differences in surface profile and attachment to one of the many planes of rough surfaces increases the chances of survival by providing shelter from waves and currents. Larger spores may require large pits on rough surfaces for attachment, while smaller spores may attach to small pits on smooth surfaces (Rees 1940) to avoid dislodgement by turbulent water beyond the boundary layer (Okuda and Neushul 1981). Pelvetia fastigata embryo survival rates were greater in small depressions of red algal turf than on ridges or flat areas where the embryos would not be constantly immersed (Brawley and Johnson 1991). Harlin and Lindbergh (1977) found that initial settlement of seaweeds did not differ on acrylic discs bolted to intertidal rocks and divided into quadrants; one was left smooth, while three were coated with 0.1-0.5 mm, 0.5-1.0 mm, or 1.0- 2.0 mm particles of hard silicon dioxide. Over time, patterns of species distribution developed on the quadrants. Chondrus crispus

and Ulva lactuca were abundant on the larger grades but scarce on the smooth surface. Polysiphonia harveyi was found equally on all three particle grades but was also scarce on the smooth quadrant. Corallina officinalis thrived on the smallest particles; when it appeared on the smooth surface it did not form upright, articulated branches.

Cell-substrate Contact Influences Differentiation

The act of adhering to a substrate has profound effects on the structure and behavior of cells (Hanein et al. 1993). Cell-substrate interactions trigger molecular interactions which determine and regulate cell morphology, motility, growth, and metabolism (Grinnell 1978, Ben-Ze'ev 1991). Increases in knowledge are necessary on the perception of surfaces by cells (Cooksey 1992). The cytoskeleton and proteins in the membrane may interact to detect the surface free energy and other characteristics of a substrate upon contact (Van Kooten 1992) to determine if adhesion and subsequent developmental changes take place (Hanein et al. 1993). Amphora coffeaformis, a pennate diatom, synthesizes small amounts of an extracellular, water-soluble, possibly acidic polysaccharide which diffuses into the water column and may act as a substrate detector by becoming more concentrated when diffusion is blocked as the cell approaches a substrate (Wigglesworth-Cooksey and Cooksey 1992). Swimmer cells of the bacterium Vibrio parahaemolyticus transform to swarmer cells if they are grown on or embedded in solid media, suggesting that contact with a surface or confinement are developmental cues which may induce bacteria to switch genes on or off to produce different phenotypes (McCarter et al. 1992).

Lectins Can Influence Adhesion

Lectins are proteins or glycoproteins that function in cell adhesion, cell-cell recognition, and cell agglutination by binding to complementary carbohydrates (Liener et al. 1986). The word lectin is from the latin *legere*, meaning to pick out or choose. Lectins have a greater affinity for oligosaccharides or glycoproteins than for their corresponding sugar haptens (von Sengbusch et al. 1982). All lectins that have been found in marine red algae will bind with glycoproteins (Blunden and Rogers 1990; Rogers and Hori 1993). Those with molecular weights above 60 kDa tend to have a requirement for divalent cations and will bind to monosaccharides such as D-galactose, N-acetylgalactosamine, N-acetylglucosamine, or D-fucose. Haemagglutination varied between lectins from tetrasporangial and cystocarpic plants of Chylocladia verticillata (Rogers et al. 1980). Because C. verticillata is in the same family as Champia parvula, lectins may be present in the mucilage of C. parvula spores. Most algal lectin literature does not focus on possible ecological or physiological functions of the lectins. Questions that arise are: 1) What is the role of marine red algal lectins?, 2) Might it be in substrate recognition during host-epiphyte interactions?, and 3) Might they be involved in spore adhesion in the intertidal realm?

An unknown chemical cue for parasitic spore attachment of Harveyella mirabilis originates with its host, Odonthalia floccosa (Goff and Cole 1976) and may involve enzymatic degradation of sulfated polysaccharides in cell walls of the host. A substance from the host may stimulate parasitic spores to

release the adhesive contents of their vesicles (Goff 1982). Spore attachment may be initiated by carbohydrates on host cells that bind with receptors on spores; this suggests that lectins are involved in attachment of this parasitic alga.

Baier (1980) suggested that there is a glycoproteinaceous conditioning layer that is adsorbed to all marine surfaces. If so, lectins or other plasma membrane receptors of adhesive organisms may interact with appropriate sugar haptens of glycoproteins in the conditioning layer to detect surfaces and then adhere (Wigglesworth-Cooksey and Cooksey 1992). Lectins may mediate in the settlement of larvae of Janua (Dexiospira) brasiliensis, a marine polychaete, by binding with bacterial polysaccharides or glycoproteins of the surface film (Kirchman et al. 1982). Enhanced tetraspore adhesion occurred in Champia parvula spores in seawater pre-conditioned by C. parvula tetrasporophytes (Dworetzky 1983), although a compound that enhances adhesion was not found. Perhaps the parent plants produce glycoproteins to form a marine conditioning layer that is recognized by lectins on C. parvula spore surfaces.

Fluorescein isothiocyanate (FITC) lectin binding reflected species-specific biochemical diversity expressed at cell surfaces in a variety of algal species (von Sengbusch and Muller 1983) and differentiated between clones of unicellular algae (Costas et al. 1993). Therefore, differences exist in cell surfaces that may contribute to lectin/receptor interactions. Callow et al. (1981) found that egg-sperm recognition in Fucus serratus is mediated by fucosyl and mannosyl containing ligands on the egg surface which bind with protein on

the sperm surface to suggest a lectin and sugar interaction. There is also evidence of intraspecific variation in lectin binding. In Fucus spiralis, FITC-lectins were used to demonstrate that the glycan moieties of the cell surface differ between spermatozoa, oocytes, zygotes, and embryos (Costas et al. 1994).

Lectins mediate adhesion in angiosperms, fungi, and bacteria. Lectin receptors in plants have been found to be glycoproteins, and glycolipids, phenolic glycosides, or glycosides of secondary metabolites (Etzler 1986). Gladiolus gandavensis stigmas contain β -glycosyl specific lectins which adhere to pollen grains and probably recognize the appropriate pollen so that self pollination can be avoided (Knox et al. 1976). The α -D-mannose/ α -D-glucose specific lectin concanavalin A, (Con A), from Canavalia ensiformis reacted with receptors on the stigma surface of G. gandavensis to inhibit penetration of the stigma by the pollen tube, possibly by blocking pollen-stigma interaction. Phaseolus vulgaris lectin and Con A stimulated *in vitro* germination of Lilium longiflorum pollen (Southworth 1975). Lectin from the potato plant inhibited hyphal extension and spore germination in Botrytis cinerea (Callow 1977) and wheat germ lectin bound to spores of chitinous fungi (Barkai-Golan et al. 1978). Lectins are involved in attachment of the symbiotic bacteria, Rhizobium leguminosarum, to root hair tips of peas (Kijine et al. 1988).

Inhibitors of Adhesion

Within an organism, disruption of many biosynthetic and metabolic processes can interfere with adhesion, as in Amphora coffeaformis (Cooksey 1981; Cooksey and Cooksey 1986,1988) where adhesion was inhibited by tunicamycin, a glycoprotein synthesis inhibitor; cycloheximide, a protein synthesis inhibitor; D-600, a Ca^{2+} transport inhibitor; podophyllotoxin, a depolymerizer of microtubules, and CCCP; an energy uncoupler. This implicated glycoproteins, proteins, calcium transport, the cytoskeleton, and energy as necessities for adhesion.

Biosynthesis inhibitors restricted adhesion and germination in other organisms. Chlorella vulgaris did not adhere when treated with actinomycin D, which inhibits DNA dependent RNA synthesis, and adhesion was reduced by 36% with puromycin, a protein synthesis inhibitor (Zaidi and Tosteson 1972). This suggests that protein synthesis is less important than production of messenger RNA in C. vulgaris (Zaidi and Tosteson 1972). Spore adhesion but not spore germination of the plant pathogenic fungus, Nectria haematococca was inhibited by Con A (Kwon and Epstein 1993). Spore germination of the freshwater fungus Aphanomyces astaci was inhibited by actinomycin D and by cycloheximide, which inhibits translation on cytoplasmic ribosomes (Soderhall and Cerenius 1983).

Cell-cell recognition leading to gamete pairing can also be inhibited. Chlamydomonas reinhardtii gametes did not form pairs when synthesis of glycoproteins on their flagellar surfaces was inhibited with tunicamycin,

although mutants that are resistant to tunicamycin will form pairs when exposed to tunicamycin (Dutcher and Gibbons 1988). In the dinophyte, Alexandrium catenella, gamete pairing was inhibited by tunicamycin and by Con A, although Con A inhibition was reversible with the addition of α -D-mannose or α -D-glucose (Sawayama et al. 1993)

Adhesion of animals can also be inhibited. The red abalone, Haliotis rubescens, did not undergo metamorphosis in the presence of Con A (Kirchman et al. 1982). This suggests that glucose or mannose haptens are involved in substrate identification, which must take place prior to adhesion and subsequent metamorphosis. Coelomocytes of the sea cucumber Holothuria polii attached in the presence of cytochalasin B, which disrupts microfilaments, but not in the presence of the anti-tubulin cytoskeletal inhibitor, vinblastine (Canicatti et al. 1992). Therefore, microtubules but not microfilaments may be involved in attachment of coelomocytes. Metabolic energy may not be required for coelomocyte attachment, as it was not inhibited by the metabolic inhibitors potassium cyanide and sodium azide. It is possible that the microtubules were in place before metabolism was blocked.

Molybdate is a competitive inhibitor of the ATP sulfurylase reaction, the first step in sulfate activation (Wilson and Bandurski 1958). Inside the cell, molybdate is transported by the sulfate carrier system to inhibit the formation of adenosine 3'-phosphate 5'-phosphosulfate, the activated donor for sulfate transfer reactions. In this way, molybdate blocks sulfate transfer to polysaccharides en route to the cell surface (Ramus 1974). In Porphyridium aeruginum, sulfation of polysaccharides was inhibited by molybdate, and

inhibition was reversed by the removal of molybdate (Ramus 1974). Adhesion was reduced when molybdate was added to mechanically separated cells of Prasiola stipitata, and was reversed by the addition of sulfate before or during molybdate addition (Bingham and Schiff 1979). Because molybdate competes with ATP sulfurylase, enzymes can be important catalysts of the synthesis of adhesives.

Sulfation is not the only process necessary for adhesion in Prasiola stipitata. Protein synthesis and photosynthesis are also required (Bingham and Schiff 1979). Cycloheximide inhibits translation on cytoplasmic ribosomes and chloramphenicol inhibits translation on plastid ribosomes. Addition of these inhibitors to Prasiola stipitata resulted in 90% and 40% inhibition of adhesion, respectively. Inhibition of photosynthesis can also reduce adhesion. Inhibition of photosynthesis with 3, (3,4 dichlorophenyl) 1,1 dimethyl urea (DCMU) resulted in reduced adhesion to implicate photosynthetic energy in attachment.

Biofouling

Biofouling can be defined as the attachment of marine organisms to anthropogenic structures placed in oceans (Dempsey 1981). Marine algae can attach to these structures to become biofouling organisms (Terry and Pickens 1986, Callow 1986, Oshurkov 1992). Biofouling communities may be established successionaly: first, a layer of non-living material becomes attached to submerged surfaces. Bacteria attach to this layer, followed by diatoms and other microorganisms (Floodgate 1971). The attachment and

development of algal spores and animal larvae forms the mature biofouling community, but the presence of seaweed and animal larvae can be delayed until a surface coated with an antifoulant loses toxicity (Dempsey 1981).

Biofouling increases the frictional resistance of ships, which reduces speed and decreases engine and fuel efficiency. Supertankers and other vessels must be drydocked every 1-2.5 years to remove biofouling organisms and to apply new antifouling paint (Dempsey 1981). In 1981 the U. S. Navy spent \$360 million on biofouling related drydocking and an additional \$100 million a year is spent by the U. S. Navy on hull cleaning, paint removal, repainting, and toxic water and grit disposal (Alberte et al. 1992). Time spent on biofouling control is also critical.

To control biofouling organisms, an agent must interfere with adhesion or other cellular processes of potential biofoulers. Unfortunately, such interference is often toxic to biofouling and other organisms. Traditionally, such compounds as tributyl tin have been used, but these are not environmentally acceptable (Cooksey 1992). By reducing surface free energy and weakening attachment strength, hydrophobic coatings have potential as antifoulants (Callow et al. 1986). The synergistic combination of silicone and fatty acid mixtures is now in wide use as an antifoulant (Goto et al. 1992). One course that may lead to safe, efficient biofouling control is that of using molecules similar to those that are perceived by cells as extracellular signals to adhere, but that would instead signal cells not to adhere (Wigglesworth-Cooksey and Cooksey 1992).

Another potential type of antifoulant is the non-leaching biocide that adsorbs to surfaces by ion exchange and kills potential foulers upon contact (Clarkson and Evans 1992). By definition, fouling organisms would be most likely to contact the coated surface and encounter the biocide. Therefore, untargeted organisms would not be effected by the biocide, provided that it did not leach into the water. One example of such a biocide is 3-(trimethoxysilyl)-propyloctadecyl-dimethyl ammonium chloride (Dow Corning 5700) which was found to be toxic to biofoulants. This "non-leaching" biocide had the disadvantage of leaching into solutions and reducing growth of test populations of Amphora and Dunaliella by 10% and 30%, respectively. An improved method of bonding the biocide to surfaces would prevent leaching.

More barnacles, Balanus balanoides, and blue mussels, Mytilus edulis, grew in tidepools dominated by Hildenbrandia prototypus than in those dominated by Ralfsia verrucosa (Conover and Sieburth 1966). Tannins isolated from Fucus vesiculosus and Ralfsia verrucosa were toxic to plankton. The branch tips of Sargassum natans and S. fluitans have antibacterial activity, are essentially free of epibiota, and contain tannin (Sieburth and Conover 1966). Epifauna, and especially hydroids, died when exposed to homogenates of the branch tips with 0.1 -0.8% tannic acid. Paint and varnish with 4-8% tannic acid on panels inhibited barnacles and algae. Seawater is an excellent medium for tannin toxicity because the alkaline pH favors algal tannin extraction and salt acts with the tannins to precipitate proteins (Conover and Sieburth 1966). Antifoulants with kelp or tannins were patented from 1880 to 1900 (Sieburth and Conover 1966). Algal tannins appear to be quite viable as marine antifoulants.

Extracts from marine animals may also become important controls of biofouling. The gorgonian coral, Pseudopterogorgia acerosa, is almost never host to biofouling organisms (Stochaj and Targett 1993). When polar organic metabolites were extracted from the coral, dissolved in methanol, and administered to the fouling diatom species, Nitzschia, for 48 h at 48 µg/ml, chlorophyll was lost, photosystems I and II were possibly disrupted, there was a decrease in cell carbohydrate, and no photosynthesis or growth took place. Fatty acids isolated from the marine sponge, Phyllospongia papyracea, have been shown to have antifouling activity against Mytilus edulis (Goto et al. 1992). This sponge also has few epibionts. Limpets may prove useful in biofouling control as they are grazers of attached algae and reduced biofouling cover by 80-90% when transplanted to submerged panels of shipping steel (Safriel et al. 1993).

Hopes for a biological and environmentally safe biofouling control agent have long been voiced (Sieburth 1965; Christie et al. 1970; Moss 1992; Fletcher and Callow 1992; Stochaj and Targett 1993) and an understanding of the biology of red algal spore adhesion will be useful in attaining this goal.

Champia parvula

The organism used in this study of spore adhesion was Champia parvula (C. Ag.) Harvey, a temperate and tropical marine red macroalga (Taylor 1957) that can be free floating, epiphytic, or attached. In the seagrass beds of Biscayne Bay, Florida, its most common substrate is Thalassia testudinum

(Humm 1964). It can attach to rocks or to anthropogenic marine surfaces (Norton and Mathieson 1983, Lethbridge et al. 1988) and can therefore be used as a means by which to determine the mechanisms of biofouling. It lives as an annual in Narragansett Bay, Rhode Island (Villalard-Bohnsack et al. 1988), where its habitat varies from open ocean to estuary to tidepool, although it appears to prefer oceanic water (Harlin and Rines 1993). Tetrasporophytes of C. parvula are found in summer (Davis 1892, Villalard-Bohnsack et al. 1988, Harlin et al. 1992a). This alga has been widely used in toxicity studies because its reproduction is as sensitive to the heavy metals Ag, Cd, Cu, and Pb as are the most sensitive marine animals, and it is also sensitive to cyanide, arsenite and arsenate (Steele and Thursby 1983, Thursby and Steele 1984, Thursby et al. 1985). Finally, C. parvula has potential for use in genetic studies (Steele et al. 1986) because of spontaneous mutations that affect morphology and pigmentation and follow Mendelian patterns of transmission.

As early as 1892, Davis published an illustration of a spore with its mucilage (Davis 1892). Champia parvula produces two sets of spores; carospores and tetraspores. Both sets of spores must attach before they can germinate (Dworetzky 1983) and establish adult plants; therefore spore adhesion is vital for completion of the life cycle. Attachment may act as a signal for germination and its accompanying physiological and morphological changes.

Because spore adhesion is a crucial stage in the life cycle of C. parvula, and because spores can be easily obtained in culture, this alga was selected

to examine mechanisms of spore adhesion. Light and scanning electron microscopy, histochemical studies, and interference with adhesion via inhibitors of biochemical pathways were used to investigate: 1) anatomical changes occurring during spore adhesion and germination, 2) the composition of the extracellular mucilage, and 3) the classes of molecules involved in initiation and maintenance of spore adhesion.

MATERIALS AND METHODS

Culture

Champia parvula was obtained from the Environmental Protection Agency and Science Applications International Corporation in Narragansett, Rhode Island. Champia parvula was cultured in Percival Growth Chambers at 20° C, + or - 2° C with cool white light at 60 - 80 microeinsteins illuminated from above and below on a 16:8 light-dark regime. The culture vessels were aerated 500 or 1000 ml flasks of filtered seawater from Narragansett Bay. The seawater was autoclaved (20 min, 121° C) to insure a unialgal culture. One ml modified 'f' medium per 100 ml seawater was added after autoclaving. Modified 'f' medium is also known as GP2 and consists of 6.35 g sodium nitrate, 0.64 g sodium phosphate, 133 mg EDTA, 51 mg sodium citrate, 9.75 mg iron, and 10 ml vitamin solution (2.0 g thiamine-HCl, 1.0 mg biotin, 1.0 mg B12 in 100 ml DIH₂O) in 1 liter DIH₂O (Guillard and Ryther 1962; Thursby and Steele 1986). Nutrients and seawater were changed weekly and excess algae discarded. Flasks were acid washed with 10 - 15% HCl or washed with RBS 35 (Pierce Co.), rinsed 5 min with DIH₂O to eliminate any lethal traces of detergent and autoclaved 20 min at 121° C.

Phases of the Life Cycle

Carpogonia of female gametophytes were fertilized by spermatia of male gametophytes to obtain carposporophytes, which grew within the female

gametophyte and produced cystocarps, inside of which were formed carpospores. These carpospores were released and attached to cover slips and germinated to form tetrasporophytes which underwent meiosis to produce tetraspores. After release, the tetraspores attached to cover slips and germinated to produce male and female gametophytes, thereby completing the life cycle.

Collection of Spores

Unattached spores were collected by placing spore bearing plants in petri dishes with seawater 5-10 min while they released spores. Attached spores were collected with their substratum - plastic cover slips placed in the bottom of culture flasks.

Spore Adhesion - Anatomy

SEM. Spores and germinated spores attached to plastic cover slips were fixed in 1.0% TEM grade glutaraldehyde and 0.1% osmium tetroxide with seawater as a buffer. Specimens were washed, dehydrated in an ethanol series, and dried in Peldri II (Ted Pella Company). Double stick mounting tape attached to mounted specimens was peeled off, inverted, and mounted. This enabled the bottoms of specimens detached by peeling the tape to be viewed (Bozzola and Russel 1991). Specimens were sputter coated with gold and viewed with a JEOL scanning electron microscope and photographed with Polaroid 55 Positive/Negative film.

LIGHT MICROSCOPY. While floating, newly released spores were examined with a Zeiss Universal light microscope for the presence of extracellular mucilage, and after attachment for adhesive pad and holdfast formation, germination, appearance of rhizoids, cell division, branching, and hair cell presence.

Spore Adhesion - Histochemistry

Composition of Mucilage

LECTINS. Spores and sporelings were incubated with 100 µg/ml FITC (fluorescein isothiocyanate) lectin in 0.6 M sorbitol with 10 mM CaCl₂ in DIH₂O for 1 h and rinsed with DIH₂O before viewing. As a control, FITC-lectin was added to 0.1 M competing monosaccharides with 0.2 M glucose, 0.4 M sorbitol, and 10 mM CaCl₂ in DIH₂O and preincubated 15 min before adding to spores in 0.6 M sorbitol/10 mM CaCl₂. Calcium was used in the incubation medium because certain lectins are known to have a high affinity metal binding site and to require divalent cations such as calcium for saccharide binding activity (Walko et al. 1987). Green fluorescence was visible when FITC-lectins bound with specific sugars in the mucilage and was observed with a Zeiss Universal epifluorescence microscope with mercury lamp illumination, a 436-520 nm filter. Spores were photographed with a Nikon FX-35A camera with Ektachrome T 160 or Vericolor 100 film. Lectins tested and their competing monosaccharides were: Con A - Canavalia ensiformis agglutinin, α-methyl-D-mannoside; LCA - Lens culinaris agglutinin, α-methyl-D-mannoside; UEA 1 - Ulex europaeus agglutinin, α-L-fucose; WGA -

Triticum vulgare agglutinin, N-acetyl-D-glucosamine; Phytolacca americana mitogen (PWM), N-acetyl-D-glucosamine; PNA - Arachis hypogaea agglutinin, D-galactose; Vicia villosa agglutinin, D-galactose; and SBA - Glycine max agglutinin, D-galactose. FITC-lectins and sugars were obtained from Sigma Chemical Co.

STAINS. Attached spores, germinated spores, and plants on plastic cover slips were stained with the following: 0.5% alcian blue HCl pH 0.5, 30 min, DIH₂O rinse, for sulfated polysaccharides; 0.5% alcian yellow HCl pH 2.5, 30 min, DIH₂O rinse, for carboxylated polysaccharides (Parker and Diboll 1966; McCully et al. 1980); 0.3% alcian blue pH 1.0 in 0.9 M MgCl₂, 30 min, DIH₂O rinse, for sulfated polysaccharides; 0.3% alcian blue pH 2.5 in 3% acetic acid, 30 min, DIH₂O rinse, for sulfated and carboxylated polysaccharides (Sheath and Cole 1990); 0.5% toluidine blue O pH 1.0, 1 min, DIH₂O rinse, for sulfated polysaccharides; and 0.1% Heath's neutral red, Heath's methylene blue, and Heath's toluidine blue, 5 min, for sulfated polysaccharides (Heath 1961). PAS (periodic acid-Schiff) stained for neutral polysaccharides (Cole et al. 1985). Coomassie blue (0.1%) G-250, 5 min, DIH₂O rinse, (Smith 1984) and 0.1% fast green, 1 min, DIH₂O rinse, stained for proteins (Klein and Klein 1970), and potassium iodine, IKI, 5-10 min stained for starch (Klein and Klein 1970). Blue fluorescence in algae cultured 24 h in 0.0025% Biofluor indicated cellulose, as did a blue stain resulting from placing algae in IKI 30 min, followed by 1 drop 65% H₂SO₄ between the cover slip and microscope slide (Klein and Klein 1970).

HCl. Attached spores were soaked in 7% HCl for 1 hr to detach branches from developing holdfasts. This concentration was used because it was high enough to detach branches but low enough to leave holdfast remnants behind. Holdfast remnants resulting from this treatment were stained with fast green and alcian blue as described above.

ENZYMES. Spores and germinated spores attached to plastic cover slips were incubated in a 1.0 mg/ml enzyme and DIH₂O or autoclaved seawater solution at 37° C for 1 h, following the methods of Bråten (1975). Enzymes tested were protease (0.7 - 1.0 units/mg), trypsin (10,000 units/mg), pepsin (3,200 - 4,500 units/mg), α -amylase (700 - 1,400 units/mg) and cellulase (1 - 10 units/mg) at pH 8.0, hyaluronidase (300 units/mg) at pH 5.5 and 8.0, polygalacturonase (500 - 2,000 units/mg) at pH 5.0, β -galactosidase (600 - 1,200 units/mg) at pH 7.3, sulfatase (5 - 15 units/mg) at pH 5.0 and 8.0, and α -mannosidase (20 units/mg) at pH 4.5 and 8.0 (Sigma Chemical Co.). The pH was adjusted with HCl and NaOH. A control group was incubated under similar conditions without the enzyme. If the algae remained attached after 1 h, incubation continued an additional 23 h. Cover slips were rinsed with DIH₂O or seawater and placed on a shaker at 100 rpm for 24 h and examined for remaining spores, germinated spores, adhesive pads, or holdfasts. Remaining algal material was stained with fast green, alcian blue, toluidine blue, or IKI and examined with a Zeiss Universal light microscope and photographed with Ektachrome T ASA 160 or Vericolor III ASA 100.

Spore Adhesion and Detachment

INHIBITORS. To determine their effects on spore adhesion and detachment, cycloheximide (a protein synthesis inhibitor), tunicamycin (a glycoprotein synthesis inhibitor), sodium molybdate (a polysaccharide sulfation inhibitor), the α -D-mannose/ α -D-glucose specific lectin Con A, sulfuric acid, deionized water, and 0.01% sodium azide (the latter three of which are lethal to *C. parvula*) were added to spores. Because cycloheximide and tunicamycin are insoluble in seawater, they were first dissolved in 100 μ l DMSO. Molybdate forms a precipitate in seawater, so molybdate assays were conducted in 1 ml GP2/100 ml DIH₂O. Inhibitors were added to either 1) free floating spores to assess inhibition of attachment, or to 2) spores attached to plastic cover slips to assess detachment of spores. Spores were placed in 10 X 10 mm gridded plastic petri dishes with 10 ml seawater and were counted through a dissecting microscope. A 24 h incubation (in light and at room temperature) followed; this timing is based on Dworetzky's 1983 finding that 90% of tetraspores of *C. parvula* adhered after 6 h and this percentage remained constant through 48 h. After 24 h, the incubation solution was decanted and the petri dish was rinsed with 10 ml seawater, which was then discarded. Five ml of new seawater was added. Attached spores were then counted and the percent of attached spores was calculated and compared with the percent of attached spores in the 100 μ l DMSO/10 ml seawater and 10 ml seawater controls. All inhibitors were tested over a range of concentrations to generate a dose/response curve. Four or five replicates of each concentration were tested in each of the experimental repetitions. As 50 μ g/ml cycloheximide inhibits algal growth in culture, (Zehnder and Hughes

1958), the concentrations of cycloheximide and tunicamycin ranged from 0.5 $\mu\text{g/ml}$ to 20 $\mu\text{g/ml}$ to minimize spore death. Spore viability after exposure to the above agents and in a control group was determined by assessing spores for color change from red to green or white, and using a dye exclusion assay by staining dead tissue with 0.5% trypan blue (Cooksey 1986) dissolved in seawater so that the trypan blue solution would not be lethal to the spores.

RESULTS

Anatomy of Spore Adhesion

Tetrads of tetraspores were released as a unit and did not separate upon adhesion. Extracellular mucilage was found on free floating tetraspores and carpospores immediately after spore release when observed with a dissecting or compound microscope. Within 24 h of release, spores attached with this mucilage. After initial attachment, the spores germinated and produced cellular rhizoids as a secondary means of attachment (Figs. 1 - 2). Tetraspores viewed with SEM had a mucilage pad extending 20-25 μm around the base of the tetrads, which were 40-45 μm in diameter. This smooth and sheetlike mucilage (Fig. 3) appeared to increase in diameter as cell division began. Each spore of the tetrad produced one rhizoid upon germination. In Fig. 4, a germinated spore was peeled off to reveal the positions of the rhizoids within the remaining pad of mucilage. Fig. 5 depicts a detached sporeling with mucilage, holdfast cells, and an upright branch with a hair cell. Fig. 6 is a basal view of the detached sporeling; it appears that the mucilage had adhered tightly to the substrate.

After germination, the spores began cell division and formed the first branch of the germling tetrasporophyte or gametophyte. While the branches formed, the rhizoids divided and began formation of the holdfast. Rhizoids and their derivative holdfast cells all had extracellular mucilage. Rhizoids were not directly visible with SEM because the mucilage became opaque when coated

with metal and because the rhizoids emerged and grew downwards from spore bases which were surrounded by mucilage. The holdfast cells continued to divide and provided a base of attachment as the germlings developed.

PLATE 1. Light micrographs of Champia parvula. Scale bars = 10 μm .
Fig. 1 - Tetrad attached with mucilage (M) as a unit of four tetraspores (T)
to a plastic cover slip. Fig. 2.- Spores of a tetrad that have germinated
and produced rhizoids (R).

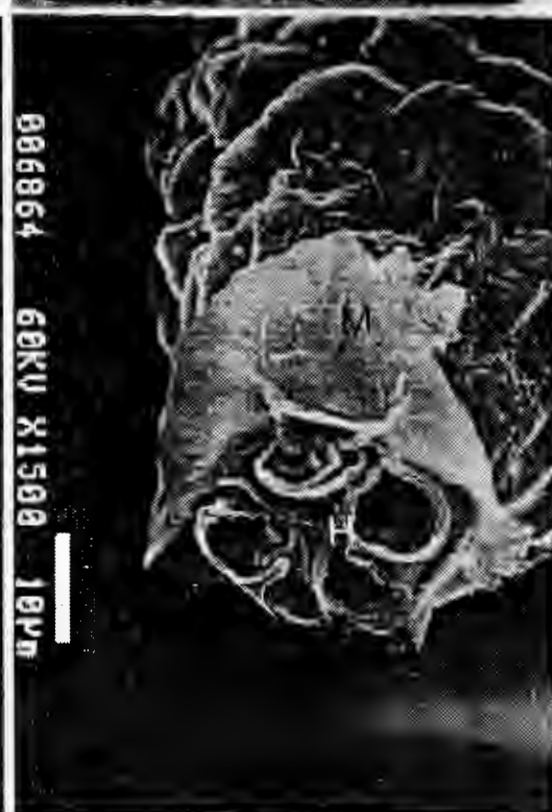
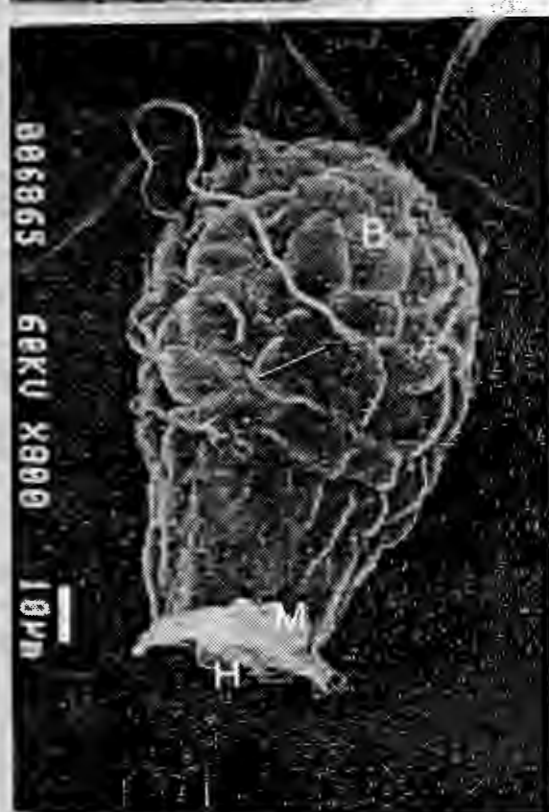
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2



PLATE 2. Scanning electron micrographs of Champia parvula. Scale bars = 10 μm . Fig. 3 - mucilage (M) of tetraspores (T) of attached tetrad ; Fig. 4 - positions of rhizoids (arrow) within mucilage (M) that remained on slide after removal of tetrad; Fig. 5 - mucilage (M), holdfast cells (H), upright branch (B) and hair cell (arrow) of a detached sporeling, Fig. 6 - basal view of the detached sporeling showing mucilage (M) and disrupted holdfast (H).



Biochemical Composition of Spore Mucilage and Rhizoids

FITC-LECTINS

To test the hypothesis that the mucilage of C. parvula spores and holdfasts contains a variety of sugar haptens, FITC-lectins were used as probes. Following incubation in FITC-lectins, the mucilage of C. parvula spores and holdfasts fluoresced with 5 of the 8 lectins tested (Table 1). Because fluorescence occurred with lectins of differing specificities, I conclude that there is a variety of sugar haptens in the mucilage of the spores and holdfasts.

Three of the lectins tested resulted in little to no fluorescence; indicating that their specific sugars are not present, are present in minimal quantities, or were inaccessible in the spore mucilage. The lectins that did not cause fluorescence are α -L-fucose specific UEA 1, (Fig. 7), and the (N-acetyl- β -(1-4)-D-glucosamine)₂ or N-acetylglucosamine specific lectins WGA, (Fig. 8) and Phytolacca americana mitogen (PWM). (Fig. 9).

In the mucilage of C. parvula α -D-mannose and α -D-glucose were abundant. The α -D-mannose and α -D-glucose specific lectin Con A produced green fluorescence in the mucilage around spores, sporelings, around the perimeters of holdfasts, and on branches. The mucilage of tetrads did not fluoresce before release (Fig. 10), but began to fluoresce just after release (Fig. 11). Mucilage of attached spores fluoresces (Fig. 12), but not in the control, (Fig. 13). In Fig. 14, also a control, the rhizoids have appeared. A

coverslip was inverted to reveal the bases of two developing holdfasts; fluorescent, adhesive mucilage of the rhizoids is visible in the holdfast on the right (Fig. 15). Fluorescent mucilage of a sporeling is illustrated in Fig. 16, and in Fig. 17, a basal view of the fluorescence of a holdfast. The spore mucilage fluoresced with an additional α -D-mannose and α -D-glucose specific lectin, LCA (Fig. 18).

Spore mucilage fluoresced in the presence of FITC-labelled peanut agglutinin (PNA), Vicia villosa lectin and soybean agglutinin (SBA), all of which are in the N-acetylgalactosamine/galactose group (Figs.19-21). Therefore, sugars in this group appear to be well represented in the mucilage of C. parvula spores.

Without FITC-lectin incubation, spore mucilage did not autofluoresce. Spores had orange-yellow autofluorescence (Fig. 22). Spores used as controls (preincubated in competing monosaccharides before exposure to FITC-lectins) autofluoresced but did not have autofluorescent mucilage.

Table 1
Fluorescence of the mucilage of *Champia parvula* spores with FITC-lectins
+ = fluorescence; - = no fluorescence

LECTIN	SPECIFICITY	FLUORESCENCE
Con A	α -D-mannose > α -D-glucose.> N-acetylglucosamine ¹	+
LCA	α -D-mannose > α -D-glucose > N-acetylglucosamine ¹	+
WGA	N-acetylglucosamine ² N-acetyl- β -(1-4)-D-glucosamine (N-acetyl- β -(1-4)-D-glucosamine) ^{2 3 4}	-
<u>Phytolacca</u>	β -(1-3)-galactose, N-acetyl- β -(1-4)-D-glucosamine (N-acetyl- β -(1-4)-D-glucosamine) ^{2 1}	-
PNA	galactosyl (β -1,3) N-acetylgalactosamine > α -D-galactose ¹	+
<u>Vicia</u>	N-acetylgalactosamine > α -D-galactose ¹	+
SBA	α or β N-acetylgalactosamine > α or β -D-galactose ¹	+
UEA 1	α -L-fucose ^{2 3}	-

¹ Goldstein and Poretz 1986

² Kaska et al. 1988

³ von Sengbusch 1982

⁴ Walko et al. 1987

PLATE 3. Fluorescent light micrographs of spores of Champia parvula, in which the mucilage surrounding the spores did not fluoresce following incubation in a. FITC-lectins or b. control (preincubation in competing monosaccharide followed by incubation in FITC-lectins). Scale bars = 50 μ m. Fig. 7 - a. UEA 1, b. α -L-fucose and UEA.1. Fig. 8 - a. WGA, b. N-Acetyl-D-glucosamine and WGA. Fig. 9 - a. Phytolacca americana (PWM) mitogen, b. D-galactose and Phytolacca americana (PWM) mitogen;

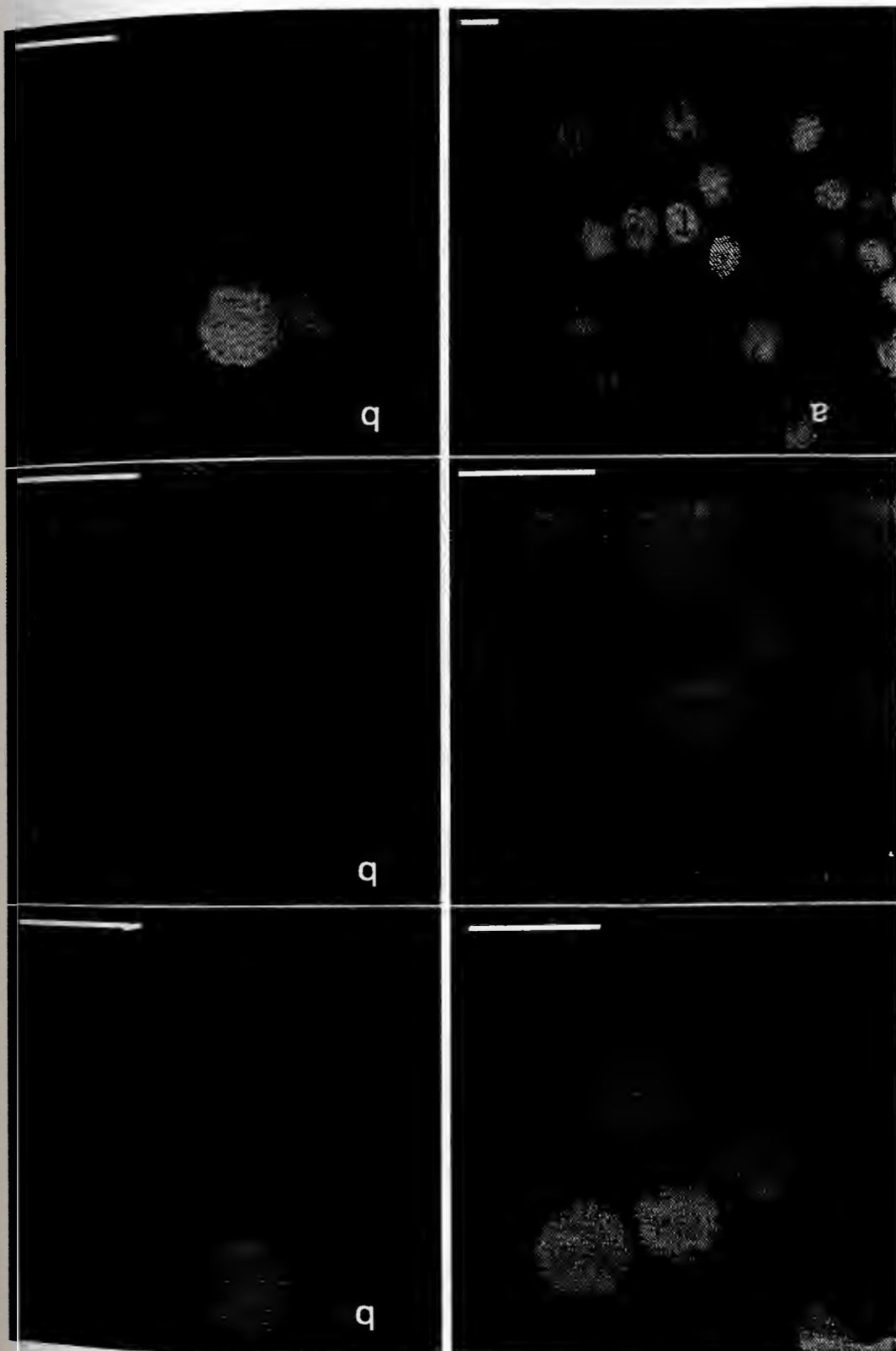


PLATE 4. Fluorescent light micrographs of spores of Champia parvula following incubation in FITC-Con A (Figs. 10 - 11, 13, and 15 - 17), or control, (Figs. 12 and 14), (preincubation in competing monosaccharide followed by incubation in FITC-Con A), green fluorescence (arrows). Scale bars = 50 μm . Fig. 10 - tetrads (T) before release from tetrasporophytic branch (B) Fig. 11 - release of tetrads (T), arrows point to green fluorescence at original sites of tetrads within tetrasporophytic branch (B) Fig. 12 - control, attached tetrads (T). Fig. 13 - attached tetrads. Fig. 14 - control, germinated tetrad with rhizoids (R) Fig. 15 - basal view of developing holdfasts with mucilage (M) and rhizoids (R) with adhesive mucilage Fig. 16 - detached sporeling (SL) with holdfast cells (H) and mucilage (M). Fig. 17 - basal view of a branch (B) and holdfast (H) with mucilage (M)..

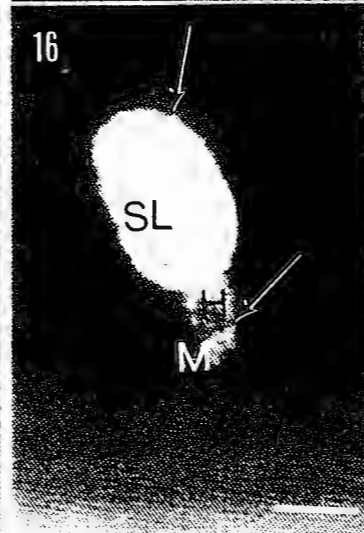
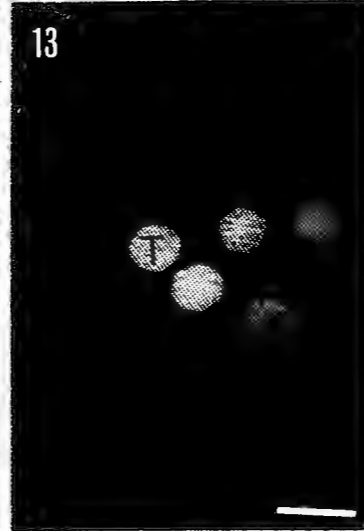
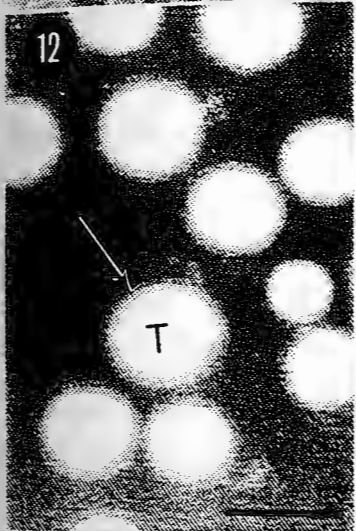
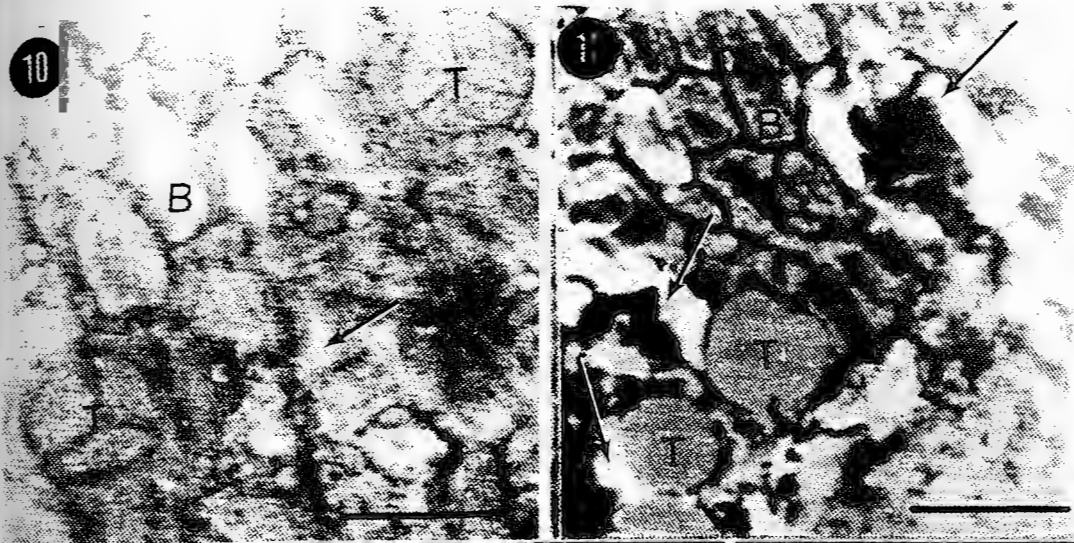
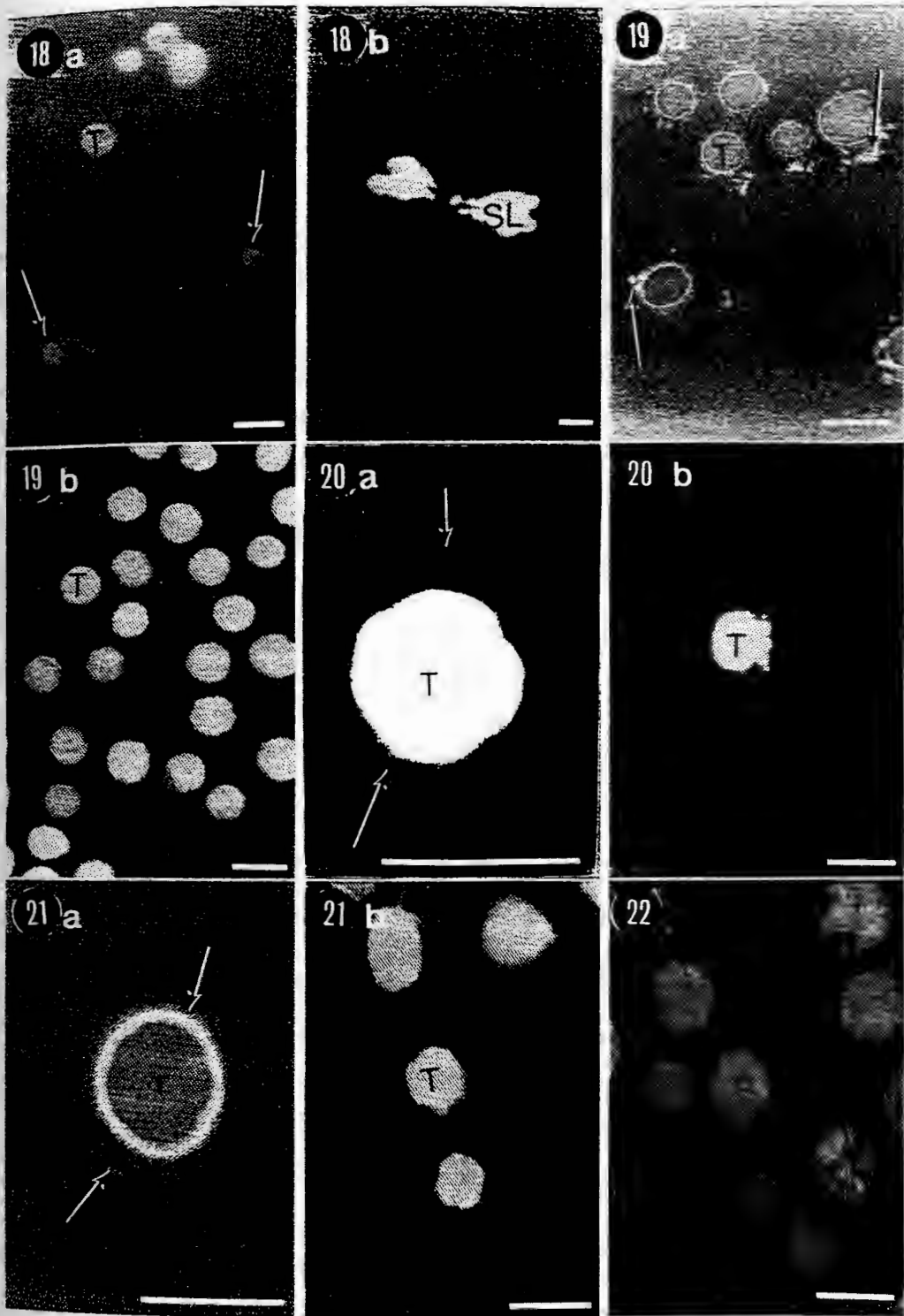


PLATE 5. Fluorescent light micrographs of spores of Champia parvula following incubation in a., FITC-lectins or b., control (preincubation in competing monosaccharide followed by incubation in FITC-lectins), green fluorescence (arrows). Scale bars =50 μ m. Fig. 18 - a. LCA, b. α -D-methyl-mannoside and LCA. Fig. 19 - a. PNA, b. D-galactose and PNA. Fig. 20 - a. Vicia villosa agglutinin, b. D-galactose and Vicia villosa agglutinin. Fig. 21 - a. SBA, b. D-galactose and SBA. Fig. 22. Autofluorescing spores.



Histochemical Stains of Spores, Rhizoids, and Mucilage

The results of histochemical staining of extracellular mucilage, spores, and rhizoids of *C. parvula* are summarized in Table 2. Staining of the rhizoids was consistent with that of the spores but different than that of the mucilage. One major difference was that the rhizoids and spores stained for protein with fast green (Fig. 23) and coomassie blue (Fig. 24) whereas the mucilage did not. In addition, the polysaccharide composition of the mucilage differed from that of the rhizoids and spores. Rhizoids and spores stained for neutral polysaccharides (Fig. 25) with PAS but did not stain for carboxylated polysaccharides with alcian yellow. Mucilage did not stain for neutral polysaccharides but did stain for carboxylated polysaccharides (Fig. 26).

Sulfated polysaccharides appear to be present in the spores, mucilage and rhizoids. The mucilage stained positively for sulfated polysaccharides with all seven of the stains (Figs. 27 - 33). The rhizoids did not stain as consistently for sulfated polysaccharides as did the mucilage. The three Heath's stains were positive in the rhizoids (Figs. 31 - 33) whereas alcian blue (HCl pH 0.5) and toluidine blue O did not detect polysaccharides in the rhizoids and spores.

Cellulose appears to be a component of the mucilage, spores and rhizoids, as it was detected with IKI-H₂SO₄ and Biofluor White in all three structures (Figs. 34 - 35). Starch was detected in the spores and rhizoids but not in the extracellular mucilage with IKI (Fig. 36).

Sporelings soaked in 7% HCl for 24 h detached intact from the substrate or broke at the branch-holdfast juncture. Holdfast remnants had fast green staining proteinaceous attachment points which appear to anchor the rhizoid and holdfast cells to the substrate. Protein anchors can be seen at the sites of rhizoid attachment (Fig. 37). The mucilaginous portion of the holdfast remnants stained for sulfated polysaccharides with toluidine blue (Fig. 38), or with alcian blue HCl pH 1.0.

Table 2
Staining of Champia parvula mucilage, spores, and rhizoids

STAIN	COMPOUND STAINED	MUCILAGE	SPORES/RHIZOIDS
PAS	neutral polysaccharide	-	+
Alcian Yellow	carboxylated polysaccharides	+	-
Alcian blue: HCl pH 0.5	sulfated polysaccharides	+	-
MgCl ₂ pH 1 acetic acid pH 2.5	sulfated polysaccharides sulfated/carboxylated polysaccharides	+	+
Alcian yellow	carboxylated polysaccharides	+	-
Toluidine blue O	sulfated polysaccharides	+	-
Heath's: neutral red	sulfated polysaccharides	+	+
methylene blue	sulfated polysaccharides	+	+
toluidine blue	sulfated polysaccharides	+	+
Biofluor white	cellulose	+	+
H ₂ SO ₄ -IKI	cellulose	+	+
IKI	starch	-	+
Fast green	protein	-	+
Coomassie blue	protein	-	+

PLATE 6. Histochemical staining of *Champia parvula*. Scale bars = 50 μ m. Fig. 23 - rhizoids (R) and spores (S) stained for protein with fast green; Fig. 24 - with coomassie blue. Fig. 25 - rhizoids (R) but not mucilage (M) stained for neutral polysaccharides with PAS. Fig. 26 - mucilage (M) of spores stained for carboxylated polysaccharides with alcian yellow. Fig. 27 - mucilage (M) of sporelings (SL) stained for sulfated polysaccharides with alcian blue HCl, pH 0.5; Fig. 28 - with alcian blue MgCl₂; Fig. 29 - with alcian blue acetic acid pH 2.5; Fig. 30 - with toluidine blue.

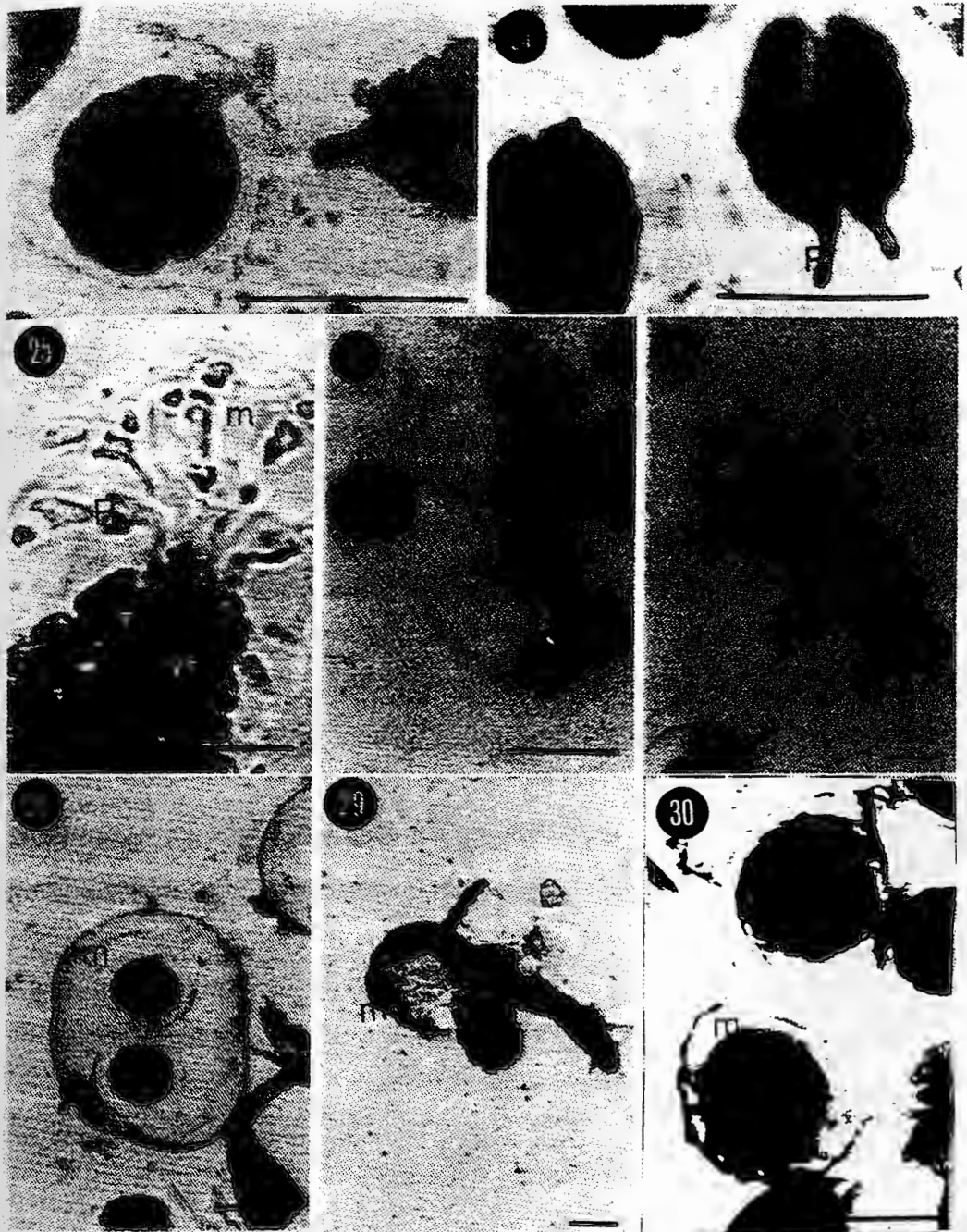
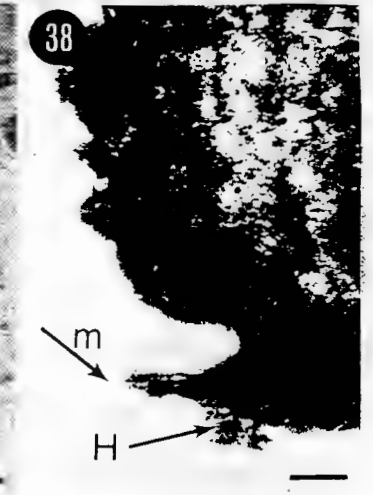
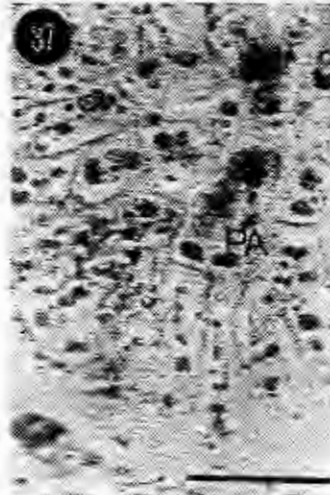
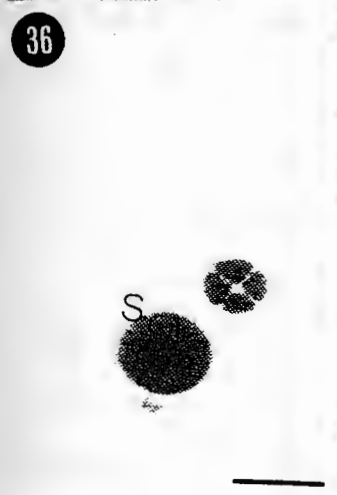
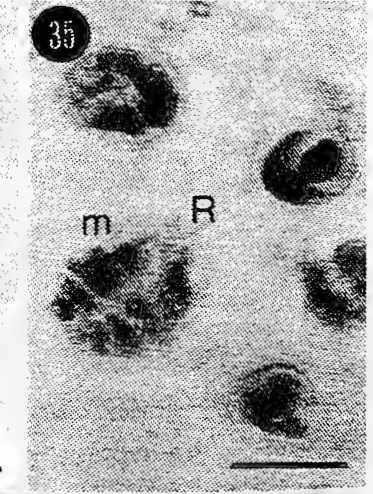
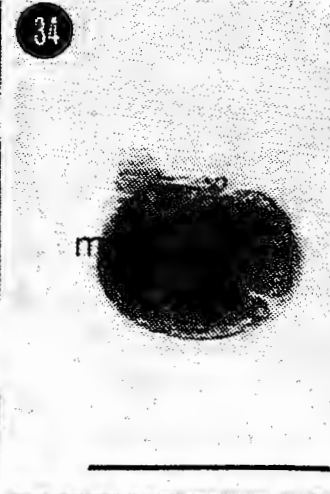
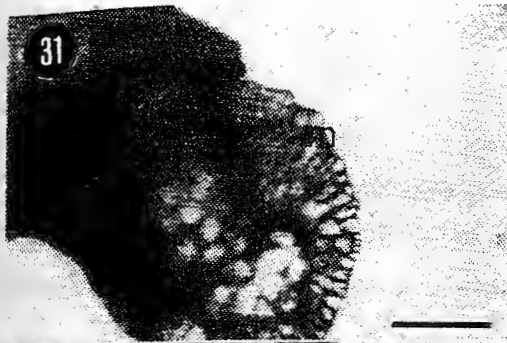


PLATE 7. Histochemical staining of *Champia parvula*. Scale bars = 50 μ m. Fig. 31 - mucilage (M) of holdfast (H) stained for sulfated polysaccharides with Heath's neutral red. Fig. 32 - mucilage (M), spores (S), and rhizoids (R) stained for sulfated polysaccharides with Heath's methylene blue. Fig. 33 - with Heath's toluidine blue. Fig. 34 - mucilage (M) of spores (S) stained for cellulose with IKI-H₂SO₄. Fig. 35 - with Biofluor White. Fig. 36 - spores (S) stained for starch with IKI. Fig. 37 - protein anchors (PA) staining with fast green in remnants of holdfast following its removal with a 24 h exposure to 7% HCl. Fig. 38 - Toluidine blue stained mucilage (M) and fast green stained holdfast cells (H) of sporeling detached with a 24 h exposure to 7% HCl.



Maintenance of Spore Adhesion

Enzymes were used to determine which classes of molecules play a role in maintaining adhesion. While exposure to all of the enzymes tested resulted in at least some spore detachment, the quantity and pattern of detachment varied among enzymes (Table 3). Spores detached at the mucilage/substrate interface or at the spore/mucilage interface to leave behind a spore socket, which appeared to be an adhesive disc of mucilage. There were no differences in detachment observed between tetraspores and carpospores. Control groups of spores in seawater or DIH₂O at pH 4.5, 5.0, 5.5, 7.3 or 8.0 had only 0-10% detachment following 24 h of 37° C incubation or shaking, or with incubation followed by shaking. Control groups of spores did not detach when touched with a dissecting needle.

The cell walls and mucilage of spores and sporelings were disrupted and destroyed after exposure to cellulase. Spores incubated in cellulase detached completely without being shaken and left no remnants of holdfasts on the cover slips. Cells of attached sporelings separated from each other.

Spores detached at the spore-mucilage interface following exposure to protease, trypsin, pepsin, α -amylase and polygalacturonase. The detachment pattern varied among these enzymes. With the proteolytic enzymes, adhesive discs which stained for sulfated polysaccharides with alcian or toluidine blue were left behind (Fig. 39). These discs and any remaining attached spores or sporelings detached after 24 h of shaking.

With α -amylase, adhesive discs were left on the cover slips when spores detached (Fig. 40). Sporelings often detached at the holdfast-branch juncture, leaving behind the developing holdfast. After shaking, only faint traces of spore mucilage or sporeling holdfasts remained and stained for sulfated polysaccharides with alcian blue. Branches of sporelings broke off of holdfasts and spores detached to leave behind their adhesive discs, or spore sockets, after polygalacturonase exposure. However, fewer than 25% of the spores detached and the spore sockets remained attached after shaking.

Of the enzymes which resulted in detachment at the mucilage-coverslip interface, β -galactosidase was the most effective. All spores and sporelings detached after incubation and shaking. Spores exposed to hyaluronidase at pH 8.0 had only limited detachment. Branches broke off sporelings and left behind the holdfasts, and spores detached at the spore-mucilage interface. The remaining spore sockets stained with alcian blue. At pH 5.5, they detached upon touch at the mucilage-substrate interface after exposure but before shaking. As with hyaluronidase, a decrease in the pH of mannosidase from 8.0 to 4.5 resulted in increased detachment of spores and sporelings. The algae detached at the mucilage-cover slip interface and did not detach unless touched.

Only 10-25% of the spores detached when exposed to 1.0 or 8.5 mg/ml sulfatase at pH 8.0. At pH 5.0, detachment did not increase to 50-75% until the concentration was increased to 8.5 mg/ml. Even so, the spores did not detach until after being shaken for 24 h and not unless they were touched. Spores

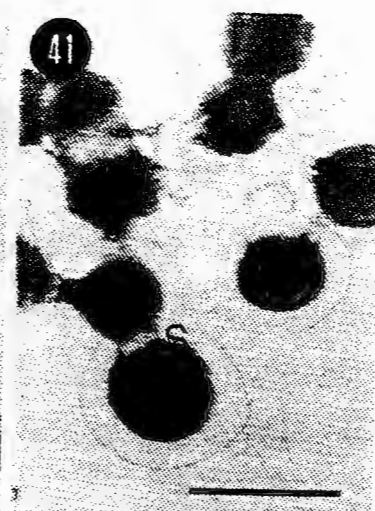
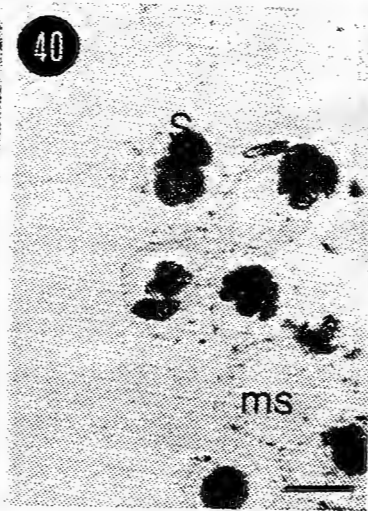
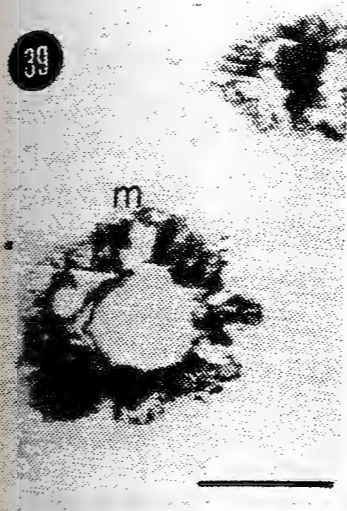
exposed to sulfatase at either pH detached at the mucilage-cover slip interface and not at the spore-mucilage interface (Fig. 41).

Enzyme	Source	Optimal pH	Optimal Temp
Cellulase	<i>Aspergillus niger</i>	5.0	30°C
Pectinase	<i>Aspergillus niger</i>	5.0	30°C
Ferrous	<i>Aspergillus niger</i>	5.0	30°C
α-amylase	<i>Aspergillus niger</i>	5.0	30°C
Hyaluronidase	<i>Aspergillus niger</i>	5.0	30°C
α-Amylase	<i>Aspergillus niger</i>	5.0	30°C
Deoxyribonuclease	<i>Aspergillus niger</i>	5.0	30°C
Polygalacturonase	<i>Aspergillus niger</i>	5.0	30°C
α-Mannosidase	<i>Aspergillus niger</i>	5.0	30°C
β-Galactosidase	<i>Aspergillus niger</i>	5.0	30°C
Sucrase	<i>Aspergillus niger</i>	5.0	30°C

Table 3
Detachment of C. parvula spores incubated in enzymes at 37° C

ENZYME: SOURCE & SIGMA #	pH	% DETACHMENT
Control	4.5-8.0	0-10
Protease <u>Streptomyces caespitosus</u> P0384	8.0	75-100
Trypsin Bovine pancreas T8003	8.0	75-100
Pepsin Porcine stomach mucosa P6887	8.0	50-75
Hyaluronidase Bovine testes H3506	5.5 8.0	75-100 10-25
α -Amylase Porcine pancreas A6255	8.0	75-100
Cellulase <u>Aspergillus niger</u> C2415	8.0	90-100
Polygalacturonase <u>Aspergillus niger</u> P3429	5.0	10-25
α -Mannosidase Jack beans M7257	4.5 8.0	50-75 0-10
β -galactosidase <u>E. coli</u> G5635	7.3	90-100
Sulfatase <u>Patella vulgaris</u> S8629	5.0 8.0	50-75 10-25

PLATE 8. Light micrographs of Champia parvula after exposure to enzymes. Scale bars = 50 μm . Fig. 39 - Alcian blue stained mucilage (M) that remained after 24 h incubation in 1 mg protease per ml DIH_2O , pH 8.0, 37° C. Fig. 40 - spores (S) detaching to leave behind mucilaginous sockets (MS) after 24 h incubation in 1 mg α -amylase per ml DIH_2O , pH 8.0, 37° C. Fig. 41 - spores (S) that detached upon touch after 24 h incubation in 8.5 mg sulfatase per ml DIH_2O , pH 5.0, 37° C.



Detachment of Tetraspores

Attached tetraspores did not detach in the presence of the biosynthesis inhibitors cycloheximide, tunicamycin, or sodium molybdate (N = 15), data not shown. Nor did tetraspores detach after exposure to Con A. The ranges of concentrations for tetraspore detachment assays were the same as those used in the tetraspore adhesion inhibition assays in the next unit..

Tetraspores soaked for 24 h in H₂SO₄ or HCl over a lethal pH range of 0.8 to 1.6 detached from plastic cover slips. The mucilage was visibly eroded after 24 h. Detachment because of cell death and detachment because of corrosion were indistinguishable in this case. Detachment occurred at the cover slip-mucilage interface and not at the spore-mucilage interface. At pH 1.4 or 1.6, holdfasts of sporelings remained attached but tetraspores detached. Therefore, holdfasts were more resistant to detachment than tetraspores were. Tetraspores remained attached in seawater from pH 4.5 to pH 7.5-8.0.

Tetraspores killed by soaking in DIH₂O for 24 h did not detach, (N = 30), data not shown. Tetraspores turned white but remained attached even after submersion in DIH₂O for 2 months. Tetraspores soaked in 0.01% sodium azide for 24 h were dead but did not detach. Therefore, tetraspores did not need to be living to maintain adhesion, although only viable tetraspores could initiate adhesion. Tetraspores did not attach after 24 h exposures to 0.01% sodium azide in seawater. This is evidence that tetraspores must be viable to attach. The acids H₂SO₄ or HCl (pH 0.8, 1.4, and 1.6) are lethal to tetraspores

and attachment was extremely limited in their presence. However, the acids are corrosive to the mucilage so treatment with acid did not differentiate between mucilage destruction and cell death. What little attachment that did occur probably took place before the acid could contact and damage the tetraspores, which were added to the seawater before the acid was so that parent plants would not be destroyed by the acid.

Inhibition of Tetraspore Adhesion

Tetraspore adhesion was inhibited following exposure to cycloheximide (Fig. 42), tunicamycin (Fig. 43), Con A (Fig. 44), and sodium molybdate (Fig. 45). The percentage of tetraspores that attached decreased with increasing concentrations of all four agents. Of the four, cycloheximide was the most potent inhibitor of adhesion. Tetraspore adhesion dropped from the control value of 50 - 60% to an average of below 20% at 0.5 μg cycloheximide per ml seawater. One μg of tunicamycin and 5.0-10.0 μg of Con A per ml seawater were required to reduce average tetraspore adhesion to less than 20%.

Much greater concentrations of sodium molybdate (0.1 M) were necessary to reduce adhesion to below 20%. Adhesion averaged 14% at 0.2 M, (48.0 mg) sodium molybdate per ml deionized water. Because sodium molybdate precipitates in seawater, the assays were conducted in 1 ml GP2/100 ml DIH_2O , in which the tetraspores remained viable for 24 h, the length of the assay. Although tetraspore adhesion was reduced from an average of 50 - 60% in seawater to 45 - 50% in 1 ml GP2/100 ml DIH_2O , spore adhesion averaged 26.0% less in 0.05 M sodium molybdate, the lowest concentration tested.

Because cycloheximide and tunicamycin are insoluble in seawater, they were first dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 1 mg inhibitor/ml DMSO. As a control, 100 μl DMSO per ml seawater was administered to free floating tetraspores. It did not interfere with attachment

and was not lethal to tetraspores. This is evidence that the inhibitors did not interfere with attachment solely by their physical presence.

To determine if the doses of cycloheximide, tunicamycin, Con A, and sodium molybdate that were administered in the adhesion assays were lethal to tetraspores, 0.5% trypan blue stain was used. Tetraspores killed with 0.01% sodium azide or DIH₂O stained with 0.5% trypan blue, whereas tetraspores that were exposed to the inhibitors did not stain with 0.5% trypan blue at the concentrations used in the adhesion assay. Nor did exposed tetraspores change color from red to green, an indication of viability. These compounds were therefore not lethal to tetraspores at the tested concentrations.

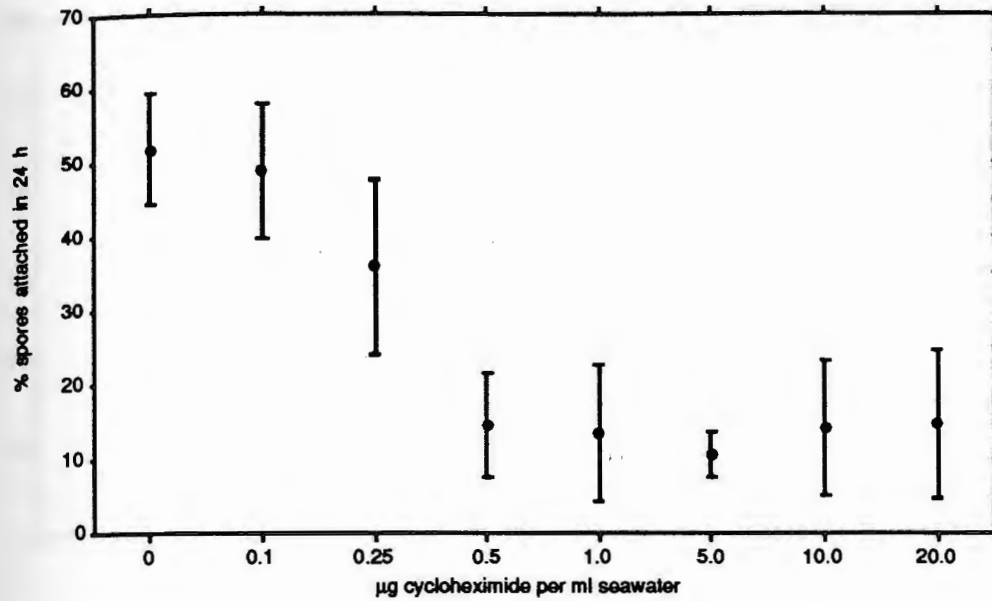


FIGURE 42. Average percent of adhesion (+/- standard deviation) of newly released, free floating *Champia parvula* tetraspores to plastic cover slips during 24 h incubations in cycloheximide, (N=15), a protein synthesis inhibitor.

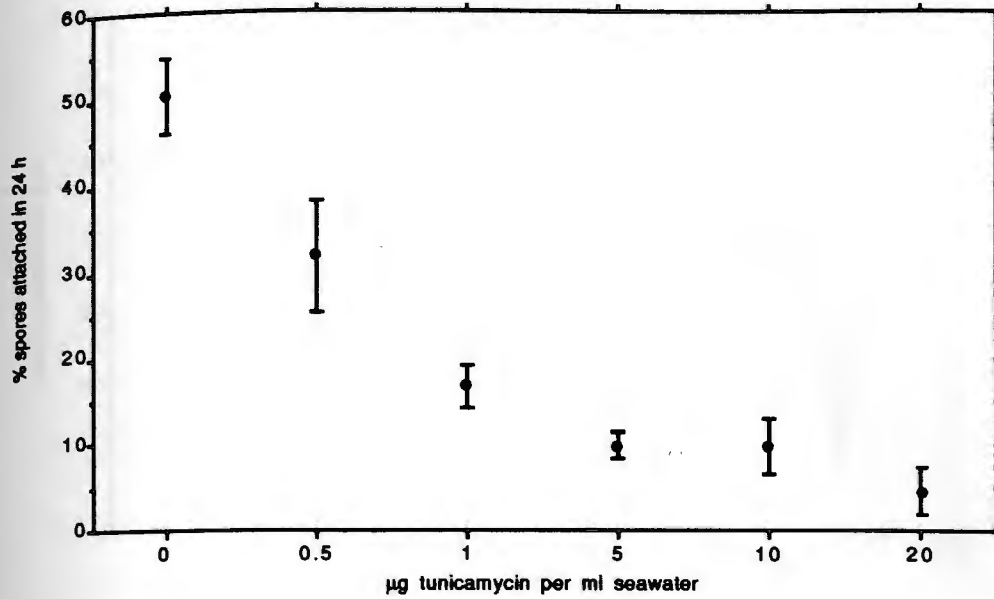


FIGURE 43. Average percent of adhesion (+/- standard deviation) of newly released, free floating Champia parvula tetraspores to plastic cover slips during a 24 h incubation in tunicamycin, (N = 15) a glycoprotein synthesis inhibitor.

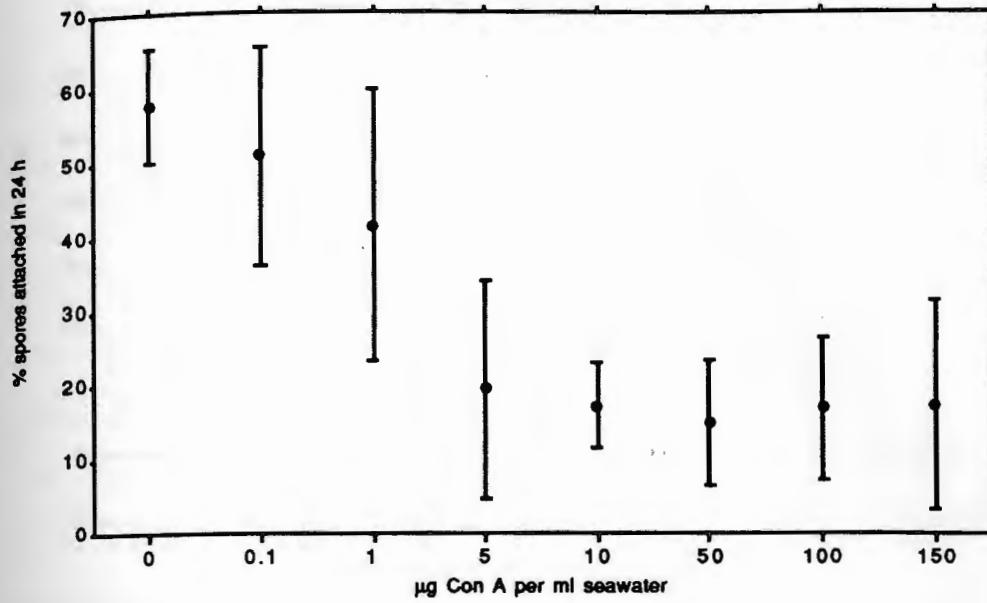


FIGURE 44. Average percent of adhesion (+/- standard deviation) of newly released, free floating Champia parvula tetraspores to plastic cover slips during a 24 h incubation in the α -D-mannose/ α -D-glucose specific lectin Con A, (N = 15).

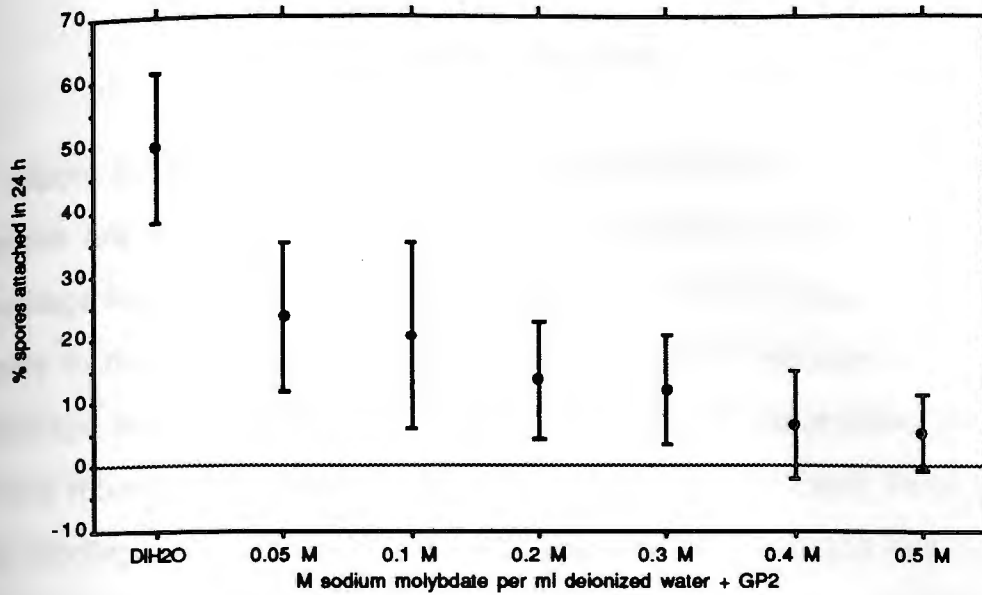


FIGURE 45. Average percent of adhesion (+/- standard deviation) of newly released, free floating Champia parvula tetraspores to plastic cover slips during a 24 h incubation in sodium molybdate, (N = 15) an inhibitor of sulfation of polysaccharides.

DISCUSSION

Spore attachment in *C. parvula* is mediated in a number of ways. First, the spores are surrounded by extracellular mucilage. Upon attachment, this mucilage forms a circular attachment pad with sides that slope down from the spore to the perimeter of the pad. Second, cellular rhizoids with their own mucilage are formed upon germination and provide secondary anchorage. These rhizoids will undergo cell division, forming the holdfast. Third, although the mucilage is smooth and uniform in appearance, it actually has a complex composition. Adhesion involves several classes of molecules which may interact and facilitate adhesion. These are sulfated polysaccharides, proteins, and glycoproteins. The sugar moieties α -D-mannose or α -D-glucose appear to be necessary for adhesion. Fourth, the spores must be living in order to attach. Therefore, adhesion is based on more than one characteristic of the spores.

Spore Adhesion - Anatomy

The mucilage of *C. parvula* spores is smooth and sheetlike; it forms the basis of initial attachment. The emergence of one cellular rhizoid per spore upon germination provides further anchorage. While the sporeling holdfast develops from rhizoidal cell division, upright branches form from non rhizoidal cells. This differs from the sporeling ontogeny of *Chondrus crispus*, in which the spore undergoes division and forms a multilayered discoid sporeling before branches are formed (Taylor and Chen 1975).

The edge of the mucilage appears to stick tightly to the substrate upon examination with SEM or light microscopy. It may be that the edge of the mucilage and its interaction with the cover slip are important in maintaining adhesion, possibly by a mechanism similar to that of a suction cup.

Substrate differences can influence spore adhesion in red algae. Boney (1978) found that spores of Porphyra schizophylla attached more slowly to negatively charged plastic cover slips than to uncharged cotton strings. Because spores of C. parvula can attach to plastic that is probably neutrally charged and hydrophobic, and to glass that is probably negatively charged and hydrophilic, to rocks, and because this alga can be epiphytic, C. parvula can attach to surfaces with a variety of chemical characteristics.

Tetrad Adhesion and Coalescence

In culture, the four spores composing C. parvula tetrads were released together and did not separate before germination. This observation supports those of Steele et al. (1986). Each spore within the tetrad germinated to produce one rhizoid. The cells derived from the original four spores coalesced to form a germling gametophyte. Sporeling coalescence occurs in Gracilaria verrucosa, Gigartina stellata, and Chondrus crispus (Tveter and Mathieson 1976). In parasitic marine red algae, genetically different vegetative filaments can fuse into pseudoparenchyma, resulting in different reproductive stages on the same thallus (Goff 1982). While the coalescence of C. parvula sporelings originates from non-separation of the four spores of the tetrad, C. crispus coalescence is a result of a high density of spores or sporelings growing

together. As C. crispus coalesced sporelings are enclosed by a common cuticle (Tvetter and Mathieson 1976), coalesced sporelings of C. parvula are surrounded by common extracellular mucilage. In C. crispus, juvenile tetrasporophytes coalesce more than sporeling gametophytes do. C. parvula carpospores are released singly and do not coalesce after germination into juvenile tetrasporophytes. Coalescence of gametophytic sporelings of C. parvula originates from non-separated tetrads and occurs frequently, if not always in culture. However, wave action in the intertidal zone probably separates the spores of the tetrad before they attach.

If the spores of the tetrad do not separate in the intertidal zone, then coalescence in C. parvula may confer advantages to sporelings as they establish themselves in intertidal marine habitats. One possibility is that an immediately larger sporeling size may contribute to faster growth. Adjacent, coalesced sporelings of C. crispus grow faster with more upright, branched fronds than non-coalesced sporelings do and also have secondary pit connections (Tvetter and Mathieson 1976). The mucilaginous spore coating in C. crispus has been thought to increase spore cohesiveness and longevity (Maggs and Cheney 1990). In the sand scoured habitat of G. verrucosa, Jones (1956) observed rafts of coalescing sporelings with the ecological advantages of speedier initiation and faster growth of fronds.

Composition of Spore Mucilage

FITC-lectins

FITC-lectins bound to a variety of sugars in the spore mucilage (Table 1). Because lectins have a greater affinity for sugar haptens of glycoproteins than for unincorporated sugars (von Sengbusch et al. 1982), it is likely that the FITC-lectins bound with sugar moieties of glycoproteins in the spore mucilage.

Con A and LCA are specific for α -D-mannose, and to a lesser extent, α -D-glucose (von Sengbusch 1982, Walko et al. 1987, Goldstein and Poretz 1986). Spore mucilage and the mucilage around the periphery of developing holdfasts fluoresced with both Con A and LCA. Compared to Con A, LCA has a weaker affinity for polysaccharides and a greater affinity for glycoproteins (Goldstein and Poretz 1986), suggesting that glycoproteins with α -D-mannose or α -D-glucose haptens are adhesive components of the spore mucilage in C. parvula. In other organisms, α -D-mannose and α -D-glucose have been found in outer cell walls and are involved in cell adhesion (Herth et al. 1982; Watson and Waaland 1983; Gingell and Owens 1992; Kim and Fritz 1993b; Kwon and Epstein 1993). Con A bound to cuticle, cell walls and intercellular mucilage of Polysiphonia (Diannelidis and Kristen 1988). In Antithamnion nipponicum, Con A can inhibit gametic binding (Kim and Fritz 1993b). Con A demonstrated that α -D-mannose and/or α -D-glucose are constituents of the red algal hormone rhodomorphin, found in Griffithsia pacifica (Watson and Waaland 1983). The spikes of Acanthosphaera zachariasii (Chlorococcales) are bundles of cellulosic microfibrils which are

extensions of the outer cell wall layer and bind with FITC-Con A at their bases (Herth et al. 1982). In Dictyostelium amoebae, Con A labelled membrane glycoproteins at the site of initial cell settling and spreading and on the edges or beneath the ultrathin lamellae (Gingell and Owens 1992).

Vicia villosa lectin and SBA are two lectins from the N-acetylgalactosamine/galactose group which gave positive results with C. parvula mucilage. It is of interest that the lectin from V. villosa is also specific for glycoproteins with one or two terminal N-acetyl- α -galactosamine groups linked to serine or threonine (Goldstein and Poretz 1986) and SBA has specificity for oligosaccharide molecules with terminal a or b linked N-acetylgalactosamine (Kaska et al. 1988). This is further evidence that glycoproteins are found in the mucilage of C. parvula spores, although polysaccharides are present.

FITC-lectins revealed no differences in fluorescence among the mucilage of spores, holdfasts, and branches, which can also adhere to plastic or glass. This differed from Antithamnion nipponicum, in which Kim and Fritz (1993a) used FITC-lectins and lectin gold labelling to find that N-acetyl-glucosamine, β -D-galactose, and α -L-fucose in the spermatial cell walls but not in the vegetative cell walls. Herth et al. (1982) found that cell walls and spike bases of Acanthosphaera zachariasi bound with the lectins Con A, RC α -1, PNA, to a lesser extent with WGA, and not with UEA, while the distal ends of the cellulosic spikes did not bind with any of the lectins. Spatial differences in sugars which bound to FITC-lectins were minimal in C. parvula, although PNA yielded scattered fluorescence near the periphery of the spore mucilage to

indicate patches of α -D-galactose or galactosyl (β -1,3) N-acetylgalactosamine. Fluorescence with all other FITC-lectins that were tested indicated that the variety of sugars present in the mucilage of C. parvula were otherwise distributed evenly within the mucilage.

C. parvula did not bind with the n-acetyl glucosamine specific lectin WGA, the (N-acetyl- β -(1-4)-D-glucosamine)₂ specific lectin from Phytolacca americana, or the α -L-fucose specific UEA. In another marine red alga, Porphyra perforata, extracted glycoproteins did not bind with biotinylated WGA but did bind with UEA (Kaska et al.1988), and glycoproteins differed between morphologically distinct regions of the thallus. While this work did not analyze spore mucilage, glycoproteins (18-68 kD) from the holdfast bound with Con A and an 18 kD glycoprotein bound to n-acetyl galactosamine specific SBA. If the glycoproteins were adhesive, then α -D-mannose, α -D-glucose and n-acetyl galactosamine may be adhesive sugar moieties in the holdfast of P. perforata.

The mucilage of C. parvula has a uniform appearance. However, it binds with lectins of different specificities, indicating a complex composition. This is in accordance with the findings of von Sengbusch and Muller (1983), who used lectin binding to demonstrate that many algal species with visually uniform sheaths or mucilage were composed of molecular mosaics and that variations were species-specific. Furthermore, lectin binding patterns varied between developmental states in some algae. von Sengbusch and Muller (1983) postulate that such variation is useful in intracellular communication and as a means for a cell to recognize "self and non self".

Histochemical Stains of Spore Mucilage and Rhizoids

In C. parvula, anchorage provided by the cellular rhizoids appears to be biochemically different from the initial attachment pad formed by the mucilage (Table 2.) Rhizoids of germinated spores stained for proteins; mucilage of ungerminated and germinated spores did not. The spore mucilage, and to a lesser extent the rhizoids, stained for sulfated and carboxylated polysaccharides. These polysaccharides may be the initial adhesive molecules. Protein anchors of rhizoids and cells of developing holdfasts (Fig. 37) provide further evidence of the importance of proteins in adhesion. Both the spore and the rhizoids are cells surrounded by adhesive mucilage. However, the cytoplasm of the spore does not contact the substrate upon initial adhesion, whereas protein anchors of rhizoid and holdfast cells do contact the substrate. Spore mucilage and rhizoids are temporally and spatially separated, as the spore mucilage is present before the rhizoids are formed and the rhizoids have their own mucilage.

It may be that small amounts of proteins and glycoproteins which were not detectable with fast green or coomassie blue acted within a matrix of polysaccharides to facilitate adhesion. The extracellular mucilage of Porphyridium cruentum consists largely of polysaccharide, but 1-2 % of the mucilage is protein covalently linked to the polysaccharide (Heaney-Kieras 1977). In three red algal species, Diannelidis and Kristen (1988) found that alcian blue stained the mucilage while coomassie blue did not. It is possible that protein-polysaccharide bonds or heavily glycosylated proteins obscured

the binding sites of the protein stains (Chamberlain and Evans 1981, Diannelidis and Kristen 1988). If proteins are indeed present in the mucilage, then rhizoid and mucilage adhesive composition may be more similar than suggested by histochemical stains. Because rhizoids and not mucilage stained with fast green and coomassie blue, proteins may be present in greater quantity in the rhizoids than in the mucilage. Another possibility is that proteins are not present in the mucilage but appear with the rhizoids upon germination when they participate in adhesion.

With PAS, the mucilage did not stain but the rhizoids did stain. It may be that the sulfated mucilage did not stain with PAS because sulfation blocks the relevant hydroxyl groups necessary for periodate oxidation (Gordon and McCandless 1973). Positive staining of the rhizoid with Heath's stains and with some of the alcian blue stains is evidence that the rhizoids do have sulfated polysaccharides. PAS staining was not blocked in the rhizoids. Sulfated polysaccharides of the mucilage would probably not have blocked PAS staining. Therefore, neutral polysaccharides would not be found in the mucilage. This is similar to the findings of Diannelidis and Kristen (1988), in which the mucilage of Hypnea musciformis did not stain with PAS but the cell walls did stain.

Alcian blue at pH 0.5, 1.0, or 2.5 stained spores but was also effective at detaching them. While low pH will detach spores, it may be that alcian blue detaches spores by binding with adhesive sulfated polysaccharides. Alcian blue also stained holdfast remnants and spore sockets following exposure to α -amylase or protease. Positive alcian blue stain, spore detachment with

sulfatase, and inhibition of sulfation and spore attachment with molybdate all implicate sulfated polysaccharides as adhesive molecules or as molecules that form connections with adhesive molecules that are necessary for adhesion

Proteins and polysaccharides appear to play a role in adhesion of C. parvula, but in Ceramium polysaccharides and not proteins are thought to be adhesive (Chamberlain and Evans 1981). The mucilage of unreleased Ceramium spores stained for polysaccharides and proteins; after spore release, polysaccharides but not proteins were detected in the mucilage. Chamberlain and Evans (1981) concluded that the lack of staining for proteins after spore release may be caused by 1) dilution of the proteins in seawater, 2) unavailability of the now cross-linked proteins, 3) artifacts of staining, or 4) absence of proteins. Agents that disrupt protein bonds did not detach Ceramium spores or did so to only a limited extent, whereas disruption of polysaccharides led to detachment of spores. Perhaps proteins of Ceramium spores play a non-adhesive role or are involved in initiating adhesion but not in maintenance of adhesion. Differential staining suggesting variation in adhesives has also been found within the stalk of the euglenoid flagellate Colacium (Willey and Giancarlo 1986) and in the green alga Ulva mutabilis, where staining differed between the zygote and rhizoids (Braten 1975).

The fibrillar component of algal cell walls is generally cellulose but can be xylan or mannan (Diannelidis and Kristen 1988). The composition of reproductive and vegetative cell walls sometimes differs, as in Antithamnion nipponicum, in which the vegetative cells but not the spermatia were labelled

with Calcofluor white (Kim and Fritz 1993a). While C. parvula spore, rhizoid, and branch cell walls stain for cellulose with H₂SO₄-IKI and Biofluor white, it is as yet unknown whether spermatia contain cellulose. Such information would be useful in determining the similarities between gametes, spores, and other reproductive structures in C. parvula.

Enzymatic Analysis of Adhesion

Enzyme, FITC-lectin, and stain data suggest that the mucilage of C. parvula is composed of a variety of molecules and that adhesion is mediated by more than one class of molecule. A variety of enzymes were effective in detaching spores (Table 3).

Proteins are instrumental in the maintenance of spore adhesion and their role in adhesion may include interactions with polysaccharides. Following exposure to protease or trypsin, spores detached from plastic cover slips only after 24 h of shaking. The adhesive was weakened but did not fail until an outside force was applied. This implies that spores have a means of attachment in addition to proteins. After shaking, spore sockets which stained for sulfated polysaccharides with alcian blue were left behind. Proteins may connect the spore with the mucilage or act as bridges between adhesive molecules contacting the substrate and the spore.

Proteins and polysaccharides play adhesive roles in other species of algae. The Ceramium adhesive often fails at the cell-adhesive interface (Chamberlain and Evans 1981). When the crustose coralline red alga,

Phymatolithon laevigatum, was removed from glass slides (Walker and Moss 1984) an inner circle which stained darkly with PAS was left behind. Loosely attached 3.5 h old Fucus zygotes detached from slides when shaken and left behind sticky rings which may contain the known mucilage components, alginate and fucan (Vreeland et al. 1993). In Colacium, cells were digested by pronase and trypsin but adhesive discs and stalks were left intact and stained with alcian blue (Willey and Giancarlo 1986). All of the above examples point to resistant adhesive discs in the algae.

Spores of C. parvula resisted detachment in sulfatase. This may be because: 1) sulfated polysaccharides in the mucilage were so abundant that the enzyme could not act upon enough of them to cause detachment, or 2) sulfated polysaccharides adhered to the substrate and were inaccessible to sulfatase. Sulfatase treated spores did detach at the mucilage-substrate interface, implying that sulfatase broke the contact at the outer edge of the mucilage-substrate interface. Spores were less resistant to β -galactosidase, which may have acted on the galactose portion of a sulfated galactan. Limpets graze on Gelidium sp. (Stephenson and Stephenson 1972), and the sulfatase used in this work was purified from the limpet Patella vulgata. Perhaps grazing limpets dissolve or digest red algae with sulfatase and red algae have evolved a resistance to sulfatase.

Hyaluronidase cleaves sulfated polysaccharide chains (Morse and Morse 1991) and binds with glycoproteins to render ruthenium red staining impossible (Trelease 1980). It was effective in detaching spores of C. parvula, possibly by disrupting sulfated polysaccharides or by binding with adhesive

proteins glycosylated with sulfated polysaccharide chains. Zygotes, but not rhizoids, of Ulva mutabilis detached in hyaluronidase (Braten 1975).

Spores of C. parvula detached in mannosidase, α -amylase, and cellulase. Detachment of spores in mannosidase, spore mucilage fluoresced with α -D-mannose/ α -D-glucose specific FITC-Con A, and Con A inhibited attachment of spores. This is evidence that α -D-mannose is present in the mucilage and necessary for adhesion and adhesion maintenance. Kim and Fritz (1993b) have found that α -D-methyl-mannose is necessary for sperm-trichogyne binding in Antithamnion nipponicum. Although spores detached at the mucilage - substrate interface in α -amylase, rhizoids and spores but not spore mucilage stained with IKI for starch. Starch appears to play a role in maintaining spore adhesion in an as yet unknown manner. Zoospores of the green alga Enteromorpha had reduced adhesion in the presence of α -amylase (Christie et al. 1970). In cellulase, spores detached completely without being shaken and left no remainders on the cover slips. It is likely that cellulase degraded the cellulose in the cell walls, facilitating detachment. In Gracilaria lemaneiformis, cellulase produced microcracks in the external thallus wall and collapsed the internal cell walls (San Martin et al. 1988). A mixture of enzymes from digestive tracts of the sea snail Littorina littorea and cellulase was used to degrade the thallus sheath and cell walls of Porphyra leucosticta (Chen 1987).

Spore Adhesion Initiation and Adhesion Maintenance

Spore Adhesion using Cycloheximide

Tetraspore adhesion in C. parvula was inhibited in the presence of 0.25 μ l cycloheximide per ml seawater (Fig. 42) which inhibits protein synthesis by inhibiting translation on cytoplasmic ribosomes (Soderhall and Cerenius 1983), thereby limiting the supply of new proteins. Proteins play a role in adhesion within 24 h of tetraspore release, the length of the incubation in cycloheximide. Adhesive proteins appear to be synthesized de novo after tetraspore release, which is when the cycloheximide contacted them to inhibit protein synthesis. This differs from Dictyostelium, where gene expression, early synthesis and storage of proteins which coat tetraspores occurs well in advance of use of the proteins (Fosnaugh and Loomis 1993).

If adhesive proteins had been present upon tetraspore release, adhesion probably would still have occurred because the cycloheximide would not have disrupted extant proteins. Another possibility is that cycloheximide would not inhibit adhesion if an adhesive protein was synthesized in advance and ubiquitinated upon or prior to spore adhesion. Ubiquitin is a regulatory protein that complexes with other cellular proteins and is found in all eukaryotic cells. One ubiquitinated protein became more abundant during gamete induction in Chlamydomonas in the presence of cycloheximide (Shimogawara and Muto 1991).

The vital stains neutral red and cresyl blue can enter red algal spores and leave the mucilage unaffected, which shows that the mucilage does not prevent substances from diffusing in and out of red algal spores (Boney 1975). This suggests that cycloheximide can diffuse into the cytoplasm of C. parvula tetraspores, as does the resultant reduced adhesion in the presence of cycloheximide.

Protein synthesis was also shown to play a role in the settlement of Haliotis rufescens larvae. Settlement occurred in the presence of cycloheximide but did not occur in the presence of two other protein synthesis inhibitors, emetine and anisomycin (Fenteany and Morse 1993), which by virtue of their lipophilic structure may have been better able to diffuse into larval tissues.

Spore adhesion using Tunicamycin

Adhesion decreased in newly released, free floating tetraspores when glycoprotein synthesis was inhibited with 0.5 µg tunicamycin per ml seawater (Fig. 43). Glycoproteins play a role in adhesion within 24 h of spore release, the length of the incubation in tunicamycin. Glycoproteins must be synthesized de novo after tetraspore release for adhesion to occur, which is when the tunicamycin was in contact with the tetraspores. If the glycoproteins had been synthesized before tunicamycin contact, the tetraspores probably would have adhered because tunicamycin would not have disrupted extant glycoproteins. Tunicamycin is a competitive inhibitor of UDP-N-acetylglucosamine-1-P-transferase mediated asparagine-linked glycosylation (Dutcher and Gibbons 1988). Asparagine linked glycosylation appears to be necessary for adhesion

in C. parvula tetraspores, and it is perhaps this step in glycoprotein synthesis which occurs after tetraspore release. Proteins to be glycosylated must be present and able to be glycosylated after tetraspore release, but they would not necessarily need to be synthesized after tetraspore release, although cycloheximide inhibition of protein synthesis shows that this is probably the case. The desmid Closterium has a glycoprotein which is involved in and synthesized prior to gamete release (Sekimoto and Fujii 1992), as shown by the addition of inhibitors of metabolism before release which then blocked subsequent gamete release. C. parvula appears to differ from Closterium in synthesizing its adhesive glycoproteins after tetraspore release.

Although the rhizoids and not the mucilage stained for proteins, spores can attach with their mucilage before germination and appearance of the rhizoids. Cycloheximide and tunicamycin interfered with attachment in tetraspores that had not yet formed rhizoids, therefore proteins and glycoproteins play an adhesive role in the mucilage. If rhizoidal proteins were the sole adhesive proteins, then cycloheximide and tunicamycin would have blocked adhesion only when the rhizoids appeared.

Spore Adhesion using Sodium Molybdate

Tetraspore adhesion in C. parvula was inhibited by the presence of sodium molybdate (Fig. 44). This is evidence that sulfation of polysaccharides is necessary within 24 h of tetraspore release for tetraspores to adhere. Tetraspores do not appear to have a sufficient store of sulfated polysaccharides before release, or they would be able to adhere in the

presence of molybdate. Tetraspores given molybdate in 1 ml GP2 per ml deionized water were red and did not stain with trypan blue, implying viability. Although molybdate did not cause tetraspore color change or spore death, it appears that it did enter the cytoplasm because tetraspore adhesion was reduced in its presence.

Spore Adhesion using Con A

In the presence of Con A, attachment of tetraspores of C. parvula was reduced (Fig. 45). As this lectin is α -D-mannose or α -D-glucose specific; it is possible that these sugars incorporated into glycoproteins are adhesive molecules. However, one must not overlook the possibility that Con A inhibited adhesion by altering a cellular function that was necessary for adhesion, as opposed to interfering with the actual adhesive. The inhibition of tetraspore adhesion by both Con A and tunicamycin points to glycoproteins as adhesive molecules with α -D-mannose and/or α -D-glucose sugar haptens. The sugars may contact the substrate and anchor the adhesive glycoproteins. In other organisms, α -D-mannose and/or α -D-glucose have been found to be necessary in reproduction and morphogenesis (Kirchman 1982; Kim and Fritz 1993b; Sawayama et. al. 1993; Kwon and Epstein 1993). Antithamnion nipponicum bases sperm-trichogyne recognition on an interaction between surface carbohydrates and receptors on the gametes. This recognition was inhibited by Con A, which blocked spermatial binding to trichogynes, and by the corresponding carbohydrate, α -D-methyl mannose, which blocked trichogyne receptors (Kim and Fritz 1993b). In the dinoflagellate Alexandrium catenella, Con A and tunicamycin inhibited sexual attachment in gamete pairs

and the addition of α -D-mannose or α -D-glucose overcame inhibition by Con A to indicate these sugars as components of cellular agglutinins (Sawayama et al. 1993). Con A inhibited larval settlement and subsequent metamorphosis in the marine polychaete Janua (Dexiospira) brasiliensis (Kirchman 1982). Macroconidial adhesion of the plant pathogenic fungus, Nectria haematococca, was blocked by Con A (Kwon and Epstein 1993). Such inhibition is not limited to Con A. Fusion between male and female gametes of the brown alga, Ectocarpus siliculosus, was inhibited when WGA, a lectin from Triticum vulgare, was added to female gametes or when the complementary sugar, N-acetylglucosamine was added to male gametes (Schmid 1993).

Spore Adhesion - Viability

Tetraspores of C. parvula must be alive in order to attach. Tetraspores killed with 0.01 % sodium azide did not attach. Sodium azide interferes with mitochondria but leaves the rest of the tetraspore relatively intact and does not disrupt the mucilage. Therefore, we know that tetraspore adhesion is an active process and does not rely solely on inherent stickiness of the mucilage.

In a multicellular organism, most cells are either attached to other cells within the organism or to a cellular or noncellular substrate (Galkina et al. 1992). Detection of substrata by attaching organisms is an area that deserves further exploration (Wigglesworth-Cooksey and Cooksey 1992). Spores may derive germination cues from the substrate. An as yet unknown molecular messenger may translate information about the substrate into adhesive

synthesis and subsequent spore germination. Polarity of rhizome emergence would be influenced by such a signal, as the rhizoids always germinate downward between the spore and the substrate. C. parvula spores sink through water to attach to a substrate; developmental signals to germinate may be cessation of sinking or a tactile response to contact with the substrate.

Spores will attach to and germinate on tetrasporophytes and gametophytes. This implies that there is no antagonism between spore adhesives and the mucilage surrounding the branches of the adult plants. Adjacent cells within a C. parvula plant do not germinate, so they do not derive germination cues from adjacent cells. When C. parvula branches come into contact with a substrate, they will produce rhizoids to form a holdfast. Therefore, the branch can perceive a cue from the substrate.

Spore Detachment

Tetraspores did not detach in the presence of cycloheximide, tunicamycin, molybdate, or Con A. Therefore, continued synthesis of proteins, glycoproteins, and sulfated polysaccharides are not necessary for adhesion maintenance. Synthesis inhibitors could not disrupt previously synthesized molecules that were already in place as adhesives. Alpha-D-mannose and/or α -D-glucose would not be available for binding with Con A if they were adhering to the substrate; therefore Con A was not effective in detaching tetraspores. The adhesive disc did not detach without physical disruption by scraping or chemical disruption with the acids H_2SO_4 and HCl or with enzyme exposure. Tetraspore viability is also not necessary for adhesion

maintainence, as attached tetraspores killed with 0.01% sodium azide or by immersion for 2 months in DIH₂O did not detach.

When treated with enzymes, spores sometimes detached and left behind the adhesive disc or remnants thereof. This implies that once the spore has adhered and the adhesive is firmly attached to the substrate, the adhesive may be inert and independent of the spore. Enteromorpha intestinalis zoospores which had settled for more than 5-10 min did not contain "settlement vesicles" whereas swimming, (pre-settlement) spores did have these vesicles (Christie et al. 1970). When inhibitors of trypsin were added to spores which were already in the presence of trypsin, no increase in the number of settled spores was seen when the inhibitors were added later than 5-10 min. after settlement. This suggests that adhesive synthesis occurs within minutes of settlement. Synthesis of sulfated polysaccharides is necessary for adhesion maintenance (Bingham and Schiff 1979) in Prasiola stipitata (Chlorophyta), in which the presence of molybdate led to cell detachment but was partially reduced by the addition of sulfate. It appears that in C. parvula, synthesis of the adhesive occurs after tetraspore release and diminishes after adhesion, as synthesis inhibitors did not cause detachment.

Spore sockets are formed when the spore detaches but leaves behind its adhesive material. Spore sockets can occur naturally when spores detach with no chemical treatment (Dworetzky 1983). Spore sockets may also result when the adhesive fails at the spore-mucilage interface. The adhesive-substrate interface may be maintained by interatomic forces (Chamberlain and Evans 1981). Such forces would explain why adhesive discs, or spore

sockets, can remain on the substrate after the spore is detached. Because the adhesive remains attached to the substrate, the adhesive is probably independent of the spore. This may allow the spore to remain attached to the substrate when it is no longer alive. One possibility is that remnants of the adhesive provide an attachment point for future spores of C. parvula.

Summary

Inhibition of protein synthesis with cycloheximide, of glycoprotein synthesis with tunicamycin, of polysaccharide sulfation with molybdate, and binding of Con A to α -D-mannose or α -D-glucose resulted in reduced tetraspore adhesion without cell death. The biosynthesis inhibitors and Con A were added to tetraspores immediately after tetraspore release, therefore adhesion probably relies on synthesis of proteins, glycoproteins, and sulfated polysaccharides and on the availability of α -D-mannose or α -D-glucose after release but before adhesion. If the inhibitors had not blocked adhesion, then adhesive molecules could be synthesized before tetraspore release.

Adhesion of C. parvula spores requires proteins, glycoproteins, sulfated polysaccharides, and α -D-mannose or α -D-glucose, which may be sugar moieties of adhesive glycoproteins. These classes of molecules may interact in as yet unknown ways to mediate spore attachment. Possible means of interaction include: 1) cross-linking of proteins and glycoproteins with polysaccharides, 2) embedding of proteins and glycoproteins in a matrix of sulfated polysaccharides, and 3) interpretation of and attachment to the substrate by α -D-mannose and α -D-glucose or other sugar moieties of

glycoproteins. These proposed means of interaction are not necessarily mutually exclusive.

Further Investigations

Furoidan (F2) is a highly sulfated fucan glycoprotein (Wagner et al. 1992) which must be sulfated for the rhizoids of Fucus embryos to adhere (Crayton et al. 1974; Brawley and Robinson 1985; Kropf et al. 1989). F2 sulfation may be necessary for F2 to associate and co-localize in the rhizoid tip with Vn-F, a vitronectin-like glycoprotein (Wagner et al. 1992) and this co-localization may be reflected in the changes in the cell surface of Fucus zygotes and rhizoids as detected with FITC-lectins by Costas et al. (1994). The next question is whether sulfated or vitronectin-like glycoproteins are involved in C. parvula rhizoid adhesion as they are in Fucus. While sulfation of polysaccharides and glycoprotein synthesis are important for C. parvula spore adhesion, it is not yet known whether sulfated glycoproteins are present and if so, adhesive. The rhizoids of germinated spores have 1) mucilage with sulfated polysaccharides and 2) anchor proteins. Further investigation may reveal an F2/Vn-F localization type of system in the rhizoids that is involved in anchoring rhizoids and securing spores to the substrate. Mechanisms of interactions between adhesive molecules await exploration.

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APPENDIX 1. PROTEINS IN THE LIFE CYCLE OF CHAMPIA PARVULA

ABSTRACT

Champia parvula, an intertidal marine red alga, was analyzed for proteins specific to stages in its life cycle. Electrophoresis did not reveal any changes in the proteins between male and female gametophytes, tetrasporophytes, and tetrasporophytic holdfasts. Unique proteins were not found in tetraspores. Glycoproteins were demonstrated in all phases of the life cycle with coomassie blue-silver dual staining of gels. It was concluded that shifts of proteins within the C. parvula life cycle were not detectable with the methods used. Stage-specific proteins may exist in quantities that are too small to be detected with one and two dimensional electrophoresis.

INTRODUCTION

The life cycle of Champia parvula, a temperate or tropical intertidal marine red alga (Taylor 1957), alternates isomorphic haploid and diploid generations which are connected by spores. When spermatia from the male gametophytes fertilize the carpogonia of the female gametophytes, carposporophytes form on the female gametophyte. The carposporophytes produce carpospores inside cystocarps. Upon germination, the carpospores form tetrasporophytes. These thalli undergo meiosis and create tetraspores, that germinate into male and female gametophytes, thereby completing the life cycle. In changing from one life cycle phase to the next, this alga undergoes morphological change. It is possible that there may be ecological or biochemical differences between the C. parvula life cycle phases as well, as such differences have been found between isomorphic stages of the marine red alga Iridaea laminarioides (Luxuro and Santelices 1989). Are the changes between life cycle stages accompanied by corresponding changes in the proteins or glycoproteins present in each life cycle stage of this alga? SDS PAGE electrophoresis and dual staining of the resultant gels with coomassie blue and silver were used to determine whether or not protein or glycoprotein change occurs between life cycle phases in this marine red alga.

Proteins and Glycoproteins in Algal Life Cycles

There is evidence to suggest that protein and glycoprotein composition may change within the life cycle of an alga. Glycoproteins of Porphyra

perforata, a marine red alga, were found to differ in male and female reproductive tissue and in morphologically and functionally distinct regions of the thallus (Kaska et al. 1988). Even though Porphyra blades appear to be simple they are morphologically and physiologically complex. Isolates of cells from functionally different areas of the blade regenerated to form different thalli (Polne-Fuller and Gibor 1984). Proteins from cuticles of Chondrus crispus tetrasporophytes were not the same as those from male and female gametophytes (Craigie 1992). Synthesis of a few specific proteins was found to change during early embryogenesis in Fucus sp., a brown alga (Kropf et al. 1989b). In Fucus serratus, monoclonal antibodies were used to show that glycoproteins on the egg surface are organized by differing composition and size (Stafford et al. 1993).

Black (1949) found seasonal fluctuations in the protein levels of Fucus sp., and Pelvetia canaliculata. Seasonal increases in Euchema nudum protein quantity were more closely related to growth or reproductive stages of the alga than to environment (Dawes 1981). Amino acid composition changes during cyst formation, storage and subsequent germination in the dinoflagellate Scrippsiella trochoidea (Lirdwitayaprasit et al. 1990). Haemagglutination, possibly caused by lectins (which are proteins or glycoproteins), differs between tetrasporophytic and cystocarpic plants of Chylocladia verticillata (Rogers et al. 1980). Therefore, it appears possible that C. parvula has temporal and stage-specific protein variation.

Algae may have proteins or glycoproteins that are unique to and involved in spore adhesion or reproductive events. Spores may carry only those

proteins or glycoproteins necessary to insure their attachment, germination, and viability. In spermatial vesicles and on the outer spermatangial surface, a glycoprotein with α -D-methyl mannose residues accumulates and is involved in sperm-trichogyne recognition in Antithamnion nipponicum (Kim and Fritz 1993b). Gametes and zoospores of Ulva mutabilis were found to have a 51.4 kDa protein that may represent tubulin (Guliksen et al. 1982). Ubiquitin is a protein found in all eukaryotic cells that conjugates with other proteins to regulate cellular processes such as development and differentiation. The 28 kDa ubiquitinated protein level was elevated during gamete induction in Chlamydomonas while other ubiquitinated protein levels decreased or remained the same (Shimogawara and Muto 1991). A glycoprotein induces the release of gametic protoplasts of the Closterium peracerosum-strigosum-littorale complex and its activity can be inhibited by metabolism inhibitors applied before the gametic protoplast release stage (Sekimoto and Fujii 1992).

Biochemical changes between cell cycle phases may alter cell surface characteristics, and in unicellular Chlorella vulgaris, adhesion varies at different points throughout the cell cycle, with maximum adhesion in the G2 period of interphase (Zaidi and Tosteson 1972). A proteinaceous adhesive or synthesis of an adhesive may reflect protein change in the cell cycle (Tosteson and Corpe 1975).

Change In Proteins and mRNAs of Angiosperms

Proteins and their mRNAs can have spatial and temporal variation in expression. For example, Sterk et al. (1991) found cell-specific gene expression of the carrot EP2 lipid transfer protein. With protein gel blots EP2 protein was found in cell walls and conditioned cell culture medium, and with RNA gel blots EP2 gene expression was found in embryogenic cell cultures, seedling shoot apices, developing flowers, and maturing seeds. In situ hybridization detected EP2 gene expression in somatic and zygotic embryo protoderm cells, and transient expression in leaf primordia epidermal cells and flower organs. In seeds of maize, lipid transfer protein mRNAs accumulated during both seed maturation and germination, and LTP mRNAs and most of the lipid transfer protein were localized in the outer epidermis of the coleoptile (Sossountov et al. 1991).

Change in Proteins and Glycoproteins During Life Cycles of Animals

Life cycle stage specific proteins and glycoproteins have been found in animals. The penaeid shrimp, Penaeus semisulcatus, was injected with ^{35}S - methionine through the thoracic cuticle in attempts to determine the fates of the lipoglycoprotein vitellin (Vt) of the oocyte cytoplasm, and the vitellin-immunoidentical protein vitellogenin (Vg) which is female-specific. 24 h after injection, free-labeled methionine remained in the hemolymph, ovary and hepatopancreas. This indicates that the shrimp had high endogeneous levels

of methionine which competed with ^{35}S -methionine labeling of proteins (Shafir et al. 1992).

Change in Proteins of Dictyostelium

Three major proteins compose the extracellular spore coat of Dictyostelium discoideum. Synthesis of these proteins is temporally and cell type specific, as it occurs in prespore cells shortly after aggregation. Spore coat proteins are stored in pre-spore vesicles during the slug stage, and finally secreted during spore encapsulation (Fosnaugh and Loomis 1993).

Cell Differentiation and Gene Expression

Swimming cells differentiate into swarmer cells upon surface contact in the bacterium Vibrio parahaemolyticus. Signals from the substrate must be detected by sensors which then control expression of specific genes (McCarter et al. 1992). Using *laf:lux* reporter gene fusions, McCarter et al. (1992) detected luminescence when the cell differentiation genes were activated upon swarmer cell surface contact, demonstrating that differentiation involves activation of specific genes. Bacteria associated with surfaces may have different phenotypes than aqueous bacterial inhabitants because contact with the surface may be instrumental in switching certain genes on or off.

In Porphyra tenera, polysaccharides and proteins differed between the thallus and conchocelis phases. The major amino acid of the conchocelis

phase is aspartic acid and in the thallus phase the major amino acids are glycine and alanine (Mukai et al. 1981). Amino acids are more acidic in the conchocelis. These biochemical differences may reflect different gene expression between the two life forms.

Studies of protein change during life cycles are valuable because changes in proteins signify the onset of developmental events.

MATERIALS AND METHODS

Culture

Champia parvula was obtained from the Environmental Protection Agency and Science Applications International Corporation in Narragansett, Rhode Island. Champia parvula was cultured in Percival Growth Chambers at 20° C with 75 microeinsteins illuminated from above and below on a 16:8 light-dark regime in aerated 500-1000 ml flasks of filtered, autoclaved (20 min, 121° C) seawater, with 1 ml GP2 growth medium per 100 ml seawater added after autoclaving. GP2 consists of 6.35 g sodium nitrate, 0.64 g sodium phosphate, 133 mg EDTA, 51 mg sodium citrate, 9.75 mg iron, and 10 ml vitamin solution (2.0 g thiamine-HCl, 1.0 mg biotin, 1.0 mg B12 in 100 ml DIH₂O) in 1 l DIH₂O (Thursby and Steele 1986). Nutrients and seawater were changed weekly and excess algae discarded. Flasks were acid washed or washed with RBS 35.

Phases of the Life Cycle

Carospores developed after female gametophytes were fertilized by male gametophytes. The carospores germinated and produced tetrasporophytes which underwent meiosis and produced tetraspores, which then germinated and developed into male and female gametophytes.

Electrophoresis - Protein Analysis

EXTRACTION. Each life cycle stage of Champia paryula was harvested immediately before protein extraction. 0.2 g samples (fresh weight, blotted dry) were placed in pre-chilled mortar and pestles filled with liquid nitrogen. After the liquid nitrogen boiled away, the frozen algae was ground to a powder and kept on ice. To each sample, 1.0 ml of protein extraction buffer consisting of 0.121 g Tris, 0.5 ml Triton X-100, 0.5 g SDS and 1.0 ml β -mercaptoethanol in 100 ml DIH₂O (Kropf et al. 1989b), with protease inhibitors (3.5 mg phenylmethylsulfonyl fluoride, PMSF, 7.0 mg N-tosyl-L-phenylalaninechloromethyl ketone,TPCK, and 2.5 mg N-ethyl maleimide) dissolved in 20 ml of buffer used in each extraction was added. After further grinding and upon thawing, 1 ml of protein extraction buffer was added. The extract was centrifuged at 10,000 rpm, 10 min, and the supernatant transferred to another centrifuge tube where 3-5 volumes of 4° C acetone were added, vortexed, and centrifuged at 10,000 rpm 10 min. The acetone was removed, fresh acetone added, and centrifugation repeated (Kropf et al. 1989a,b) until a pink pellet formed, which was homogenized and dissolved in SDS sample buffer and stored at -80° C.

ANALYSIS. Lowry protein determination (Lowry et al. 1951) yielded protein concentration in $\mu\text{g}/\mu\text{l}$ and was used to calculate sample volumes for electrophoresis. 10% SDS 1.5 mm polyacrylamide1-D gels (Laemmli 1970) were stained with 0.1% coomassie brilliant blue or dual stained with 0.1% coomassie and silver (De Moreno et al. 1985) to detect modified proteins (Dzandu et al. 1984). Molecular weights were determined using Sigma

molecular weight standards (kDa range) and the gels were dried in a Hoefer "Easy Breeze" gel drier and photographed. Alternatively, Champia parvula was labeled with 1 μ ci/ml seawater ^{35}S -Methionine or ^{14}C -L-amino acid mixture (NEN) for 2 - 4 hours and the labeled proteins underwent SDS PAGE electrophoresis or were isoelectrically focused (O'Farrell 1975) with 20,000 or 40,000 cpm in each lane or tube gel.

RESULTS

When protein samples from male and female gametophytes and tetrasporophytes were compared with SDS-PAGE electrophoresis, proteins were not demonstrated to change between stages of the life cycle. Fig. 1. is a gel stained with coomassie blue and Fig. 2 is a coomassie blue and silver dual stained gel of comparisons between stages of the life cycle. In Fig. 2, there is a protein of approximately 53 kDa in the tetrasporophytic tissue that appears as a thinner and more pale band in the male and female tissue. However, this is the only gel that showed this difference in concentration in the 53 kDa protein. SDS-PAGE electrophoresis and subsequent coomassie-silver dual staining of basal portions of free floating tetrasporophytes and male and female gametophytes did not reveal any differences between basal proteins and those from whole floating plants.

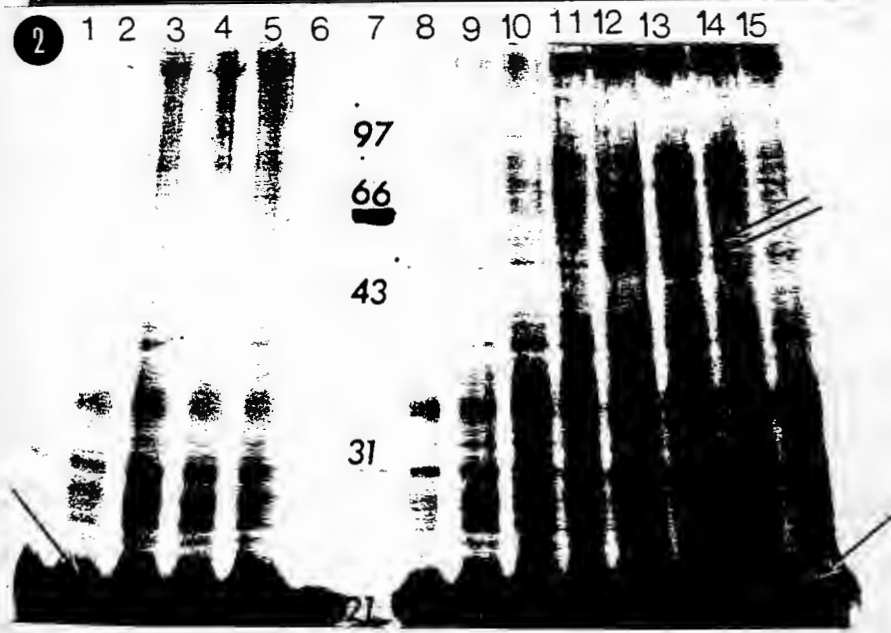
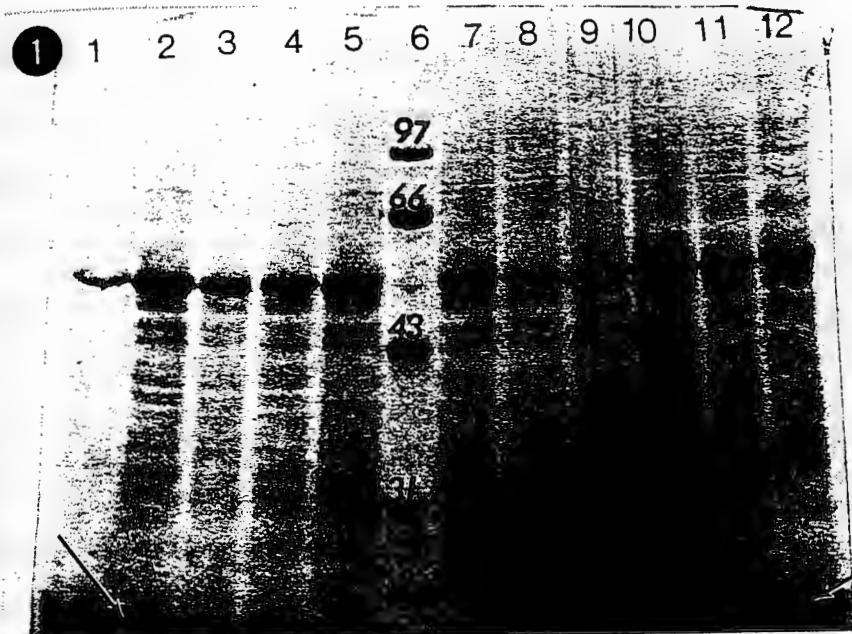
Glycoproteins were detected with silver staining in tetrasporophytes and in male and female gametophytes (Fig. 2). Bands on dual stained gels that turned brown are modified proteins, those that stained blue are not modified.

The most prevalent glycoproteins were 20 kDa, densely stained, thick bands.(Figs. 1 and 2).

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Plate 1. Fig. 1. Coomassie blue stained SDS polyacrylamide gel of protein extracted from male (lanes 1-3), female (lanes 4-5 and 7-8), and tetrasporophytic (lanes 9-12) tissue of *C. parvula*. Molecular weight standards are in lane 6. Fig. 2. Coomassie blue and silver dual stained SDS polyacrylamide gel of protein extracted from male (lanes 1-5), female (lanes 6 and 8-11), and tetrasporophytic (lanes 12-15) tissue of *C. parvula*. Molecular weight standards are in lane 7. Arrow indicates prevalent 20 kDa glycoprotein and double arrow indicates 53 kDa protein.



Discussion

One dimensional gel electrophoresis was more effective than isoelectric focusing in this work because the protein content of C. parvula is not sufficient to warrant the analysis of a single sample spread over an entire gel, as is required for isoelectric focusing. One dimensional gels were also more effective because samples from different stages of the life cycle could be directly compared within a single gel.

In C. parvula, ³⁵S-methionine labeling was weak, possibly because of the high level of sulfation in the extracellular mucilage. Apparently, the ³⁵S-methionine was incorporated into the extracellular mucilage. ¹⁴C-L-amino acid mixture also resulted in low levels of labelling in C. parvula, which resulted from most of the label going to the spiritoaqueous fraction. This is similar to what occurred in the red algae Gracilaria and Ahnfeltia, in which super ¹⁴C-galactose and super ¹⁴C-sodium bicarbonate labelling resulted in the following distribution of label: 1) spiritoaqueous fraction, 2) polysaccharides, 3) proteins, and 4) cellulose (Prozumenshchikova et al. 1989). In Rhodella (Callow and Evans 1981) used ¹⁴C-arginine to study synthesis of the protein component of the solubilized mucilage of this unicellular red alga because it is the only amino acid that Rhodella will use as a nitrogen source; Rhodella was grown without inorganic nitrogen. The labelled arginine was detectable in solubilized mucilage after 9-10 h. Methionine levels were higher in conchocelis phases of the red alga Porphyra tenera than in thallus phases (Mukai et al. 1981). Kropf et al. (1989b) did not meet with success in their attempts to label proteins of Fucus zygotes with the

radioactive amino acids ^3H -leucine and ^{35}S -methionine, ^3H labeled amino acid mixtures, and $\text{Na}_2^{35}\text{SO}_4$. Higher specific activity was achieved with $\text{Na}_2^{14}\text{CO}_3$, although different proteins may have been labeled with carbonate than would have been with radioactive amino acids

It would be interesting to study the protein composition of the spore and spore mucilage to determine if there are proteins unique to the spore stage of the life cycle. Such proteins could be involved in development or they could be related to spore adhesion, as the spores must attach before they can germinate to establish the next life cycle phase (Dworetzky 1983). However, it is difficult to obtain spores in sufficient numbers for electrophoresis. Callow and Evans (1981) used polyacrylamide gel disc electrophoresis to fractionate ^{14}C -L-arginine and $^{35}\text{SO}_4$ labelled Rhodella mucilage. The mucilage was recovered by freeze-drying and dissolving 0.2% mucilage in electrophoresis running buffer. The mucilage would not form a true solution so not all of the sample could enter the gel and be electrophoresed. C. parvula spore mucilage may be obtainable by freeze drying if sufficient quantities of spores were available. Electrophoresis of spore mucilage would be instrumental in determining the composition of the mucilage. Callow and Evans (1981) stained the 2.5% acrylamide Rhodella mucilage gels with PAS or toluidine blue for polysaccharides, amido black for protein, or assessed ^{14}C -arginine (for protein) and $^{35}\text{SO}_4$ (for polysaccharides) labelling results by scintillation counting.

Electrophoresis did not reveal any consistent protein or glycoprotein differences between male, female, and tetrasporophytic plants. There were

also no protein or glycoprotein differences found between basal and branch proteins of free floating male, female, and tetrasporophytic plants. Because SDS-PAGE electrophoresis reveals the 30-50 most common proteins, it could be that there are existing protein differences, but that the proteins differing between life cycle phases are not within the 30-50 most common. The most prevalent proteins in C. parvula have a molecular weight of approximately 20 kDa, which corresponds with the molecular weight of the phycobiliproteins, phycoerythrin, phycocyanin, and allophycocyanin (Gantt 1990).

While this work did not reveal any stage-specific proteins, it did provide a profile of the molecular weights of proteins and glycoproteins that are the most abundant in C. parvula. Although the same proteins and glycoproteins were found throughout the life cycle, the roles they play in completing the life cycle of Champia parvula have yet to be discovered.

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APPENDIX 2. PROTOCOLS

Culture

1. Obtain excised branch tips of male and female gametophytes and filtered seawater from SAIC or the EPA in Narragansett, Rhode Island.
2. Autoclave seawater 20 min at 121° C.
3. Wash 500 ml and 1 l Erlenmeyer flasks in Alconox, acid wash in 1:5 solution of HCl:DIH₂O, and rinse with DIH₂O. Alternatively, culture flasks may be washed in RBS 35 and rinsed with DIH₂O.
4. Add 300 ml seawater to 500 ml flask or 800 ml seawater to 1 l flask.
5. Add GP2 nutrients (10ml GP2/l seawater) and algae.
6. Cap flasks with foam stoppers and insert pipette between flask wall and stopper. Attach aeration tube to pipette at one end, and to aquarium pump at other end.
7. Place cultures in growth chamber at 20° C on a 16:8 light dark regime with 75 microeinsteins illuminated from above and below.
8. Change flasks, nutrients, and seawater weekly.
9. To obtain carpospores, place excised male and female gametophytic branches in same flask.
10. Tetrasporophytes are obtained from germinated carpospores.
11. Tetraspores are produced by tetrasporophytes almost continuously and germinate into male and female gametophytes.

12. Collect spores by placing plastic cover slips in flasks as substrates for spore adhesion. Free floating spores can be collected by placing spore bearing algae in a petri dish with seawater for 5-10 min with gentle agitation.

CULTURING RECIPES

GP2 nutrient solution

To 1 l DIH₂O add and stir in this order:

0.64 g sodium phosphate NaH₂PO₄-H₂O

133 mg sodium EDTA Na₂EDT α -2H₂O

*9.75 mg iron FeHCl

51 mg sodium citrate C₆H₅Na₃O₇-2H₂O

6.35 g sodium nitrate NaNO₃

*Place 9.75 mg iron powder in 25 ml graduated cylinder, add 2 ml HCl. Let sit 20 min, until iron dissolves and mixture is yellow. Bring volume to 19.5 ml with DIH₂O.

Autoclave 10 min at 121° C liquids, let cool and add 10 ml/LFS GP2 vitamin stock solution.

GP2 vitamin stock solution

To 100 ml DIH₂O add:

2.0 g Thiamine HCl (B1) C₁₂H₁₈C₁₂N₄O₅

1.0 mg Biotin C₁₀H₁₆N₂O₃S

1.0 mg Cyanocobalamin (B12) C₆₃H₈₈CoN₁₄O₁₄P

Place 10.0 ml aliquots in test tubes and autoclave. Bring to 121° C and drop temp gauge to 0° C. to stop incoming heat.

Spore Adhesion - Histochemistry

Inhibitors:

Spore Attachment

1. Place 10 ml seawater in 15 10 X 10 mm gridded plastic petri dishes.
2. Obtain free floating spores by swirling a tetraspore bearing branch in the water and waiting 5 min.
3. Count the free floating spores through a dissecting scope.
4. To 5 dishes, add the inhibitor being tested. To 5 more, add 100 μ l DMSO, and to the remaining 5 add nothing.
5. Incubate 24 h under a hood.in the light.
6. Decant liquid, fill and decant with 10 ml seawater to rinse, fill with 5 ml new seawater, and count #spores/attached per dish.
7. Compare # spores attached between experimental and control dishes.

Spore Detachment

1. Place 10 ml seawater in 15 10 X 10 mm gridded plastic petri dishes.
2. Put one plastic cover slip with attached spores in each dish.
3. Count the spores through a dissecting scope.
4. To five dishes, add the inhibitor being tested. To 5 more, add 100 μ l DMSO, and to the remaining 5 add nothing.
5. Incubate 24 h under a hood.
6. Decant liquid and count #spores remaining attached per dish.

7. Compare # spores remaining attached between experimental and control dishes.

Trypan Blue: Prepare a 0.5% solution in seawater. Stir 20 min. Stain will not dissolve completely.

Lectins:

1. Mix 100 µg/ml FITC-lectin into DIH₂O with 0.6 M sorbitol and 10 mM CaCl₂.
2. Incubate attached algae in fluorophore lectin conjugate (FITC-lectin) 1 hr with rotation.
3. Wash cover slips in DIH₂O.
4. Resuspend in the sorbitol medium to observe with a Zeiss Universal epifluorescence microscope with mercury lamp illumination, a 436-520 nm filter, and a Nikon FX-35A camera.
5. Concurrently with steps 1-4, preincubate FITC-lectins in 0.1 M competing monosaccharide with 0.2 M glucose, 0.4 M sorbitol, 10 mM CaCl₂ in DIH₂O for 15 min, then add to control attached algae in 0.6 M sorbitol, 10 mM CaCl₂ in DIH₂O, and follow steps 2-4. Competing monosaccharides were: Con-A (α-methyl-D-mannoside), PNA (D-galactose), WGA (N-acetyl-D-glucosamine), SBA (D-galactose), UEA 1 (L-fucose), Phytolacca americana agglutinin (D-galactose) and Vicia villosa agglutinin (D-galactose).
6. Photograph with Ektachrome 160 Tungsten or Vericolor 100.
7. Screen out autofluorescence with a chlorophyll filter.

Lectin Monosaccharide Specificity

Lectin	Monosaccharide
<u>Canavalia ensiformis</u> (Con-A)	α -D-mannose > α -D-glucose
<u>Triticum vulgare</u> (WGA)	(d-glcNAc) ₂ , Neu NAc
<u>Phytolacca americana</u>	(d-glcNAc) ₃
<u>Arachis hypogaea</u> (PNA)	β -D-galactose(1-3)-D-galNAc
<u>Glycine max</u> (SBA)	D-galNAc
<u>Vicia villosa</u>	D-galNAc
<u>Ulex europaeus</u> (UEA 1)	α -L-fucose

Stains:

Spores, germinated spores, and plants attached to cover slips were stained with the following and examined with a Zeiss light microscope.

1. 0.5% alcian blue adjusted to pH 0.5 with N HCl stains sulfated polysaccharides aqua. Immerse attached algae in AB for 30 min and rinse with DIH₂O.
2. 0.5% alcian yellow adjusted to pH 2.5 with N HCl stains carboxylated polysaccharides yellow. Immerse attached algae in AB pH 0.5, 30 min, rinse with DIH₂O, transfer to AY pH 2.5, 30 min and rinse in DIH₂O.
3. 0.3% alcian blue pH 1.0 with 0.9 M MgCl₂ stains sulfated polysaccharides aqua. Immerse algae in AB MgCl₂ 30 min and rinse with DIH₂O.
4. 0.3% alcian blue pH 2.5 in 3.0% acetic acid stains sulfated and carboxylated polysaccharides aqua. Immerse algae in AB acetic acid 30 min and rinse with DIH₂O.
5. 0.05% toluidine blue O pH 1.0 stains sulfated polysaccharides red-purple. Immerse algae 1 min and rinse with DIH₂O.

6. Periodic acid Schiff (PAS) stains neutral polysaccharides magenta. Fix attached algae 1 min in 5 ml formaldehyde, 45 ml 95% ethanol solution, rinse 1 min in slowly running DIH₂O. Immerse slides 5 min in periodic acid-schiff solution (1 g/dL in DIH₂O), rinse 1 min in slowly running DIH₂O, and immerse 15 min in Schiff's reagent. Wash 5 min in running DIH₂O and air dry.
7. Coomassie blue stains proteins blue. Immerse algae 5 min and rinse with DIH₂O.
8. Fast green stains protein green. Immerse algae 1 min or less and rinse with DIH₂O.
9. IKI stains starch blue-black. Immerse algae 5-10 min and rinse with DIH₂O.
10. IKI-H₂SO₄ stains cellulose blue. Immerse algae in IKI for 30 min, invert cover slip onto microscope slide and place a drop of 65% H₂SO₄ under the cover slip to diffuse into the tissues in 1-2 min. Observe quickly without rinsing.
11. Cellulose emits blue fluorescence when stained with Biofluor. Algae was cultured with 0.0025% Biofluor for 24 h and examined with fluorescence microscopy.
12. Heath's Neutral Red, Heath's Methylene Blue and Heath's Toluidine Blue - immerse algae in stain 5 min, rinse, and observe.

Enzymes:

1. Mix 1 mg/ml enzyme with DIH₂O, adjust pH.
2. Mix 1 mg/ml enzyme with autoclaved seawater, adjust pH.
3. Divide each solution into 4 aliquots, each in a 25 ml beaker.
4. Place the same volume of DIH₂O in 4 beakers, and the same volume of autoclaved seawater in 4 beakers.
5. Add algae attached to cover slips and cover with Parafilm.

6. Incubate in 37° C oven for 1 h.
7. If algae remains attached, continue incubation for 24 h total.
8. Check under microscope for detachment.
9. Place on shaker for 24 h.
10. Examine under microscope for detachment and for any remaining algal structures.
11. Stain with fast green, alcian blue, toluidine blue, or IKI to determine composition of any remainders.
12. Photograph with Ektachrome T 160 or Vericolor III.

Sources of Enzymes

Enzyme	Source
Protease	Bovine Pancreas, <i>Streptomyces caespitosus</i>
Trypsin	Bovine Pancreas
Pepsin	Porcine stomach mucosa
Cellulase	<i>Aspergillus niger</i>
α -amylase	Barley Malt, Porcine Pancreas
β -galactosidase	<i>E. coli</i>
Hyaluronidase	Bovine Testes
α -mannosidase	Jack Beans
Sulfatase	Limpets (<i>Patella vulgata</i>)
Polygalacturonase	<i>Aspergillus niger</i>

All enzymes were obtained from Sigma Chemical Company, St. Louis, MO.

Spore Adhesion - Anatomy

SEM:

1. Fix spores and germinated spores attached to plastic cover slips in 1.0% TEM grade gluteraldehyde in a seawater buffer overnight in refrigerator.
2. Rinse 3X 30 min in filtered seawater in refrigerator.
3. Place in 0.1% OsO₄ for 3 h in refrigerator.
4. Rinse 30 min in DH₂O.
5. Dehydrate in ethanol series: 30% - 1 h, 50% - 1 h, 75% - 1 h, 95% - 1 h, 100% - 2 overnights.
6. Equilibrate at 30° C for 1.5 h.
7. Dry in 1:1 100% Ethanol:Pell-Dri at 30° C for 1 h, followed by 2 1 h changes in 100% Pell-dri. Place specimens with Pell-dri in refrigerator 5 min to solidify.
8. Place under hood to sublimate Pell-dri. When sublimated, put samples in dessicator.
9. Mount samples by cutting a strip of their substrate, attaching it to double stick tape, and fastening this to a mount.
10. Attach double stick tape to mounted specimens, peel off, invert, and mount to expose bottoms of specimens.
11. Coat with metal.
11. View with a JEOL scanning electron microscope.
12. Photograph with Polaroid 55 Positive/Negative film.
13. Return samples to dessicator.

Light microscopy:

1. Culture as described above.

2. Collect free floating and attached spores.
3. Place free floating spores in petri dish of seawater and examine under a dissecting microscope.
4. Place a droplet of seawater with spores on microscope slide and examine under a Zeiss compound microscope.
5. Place cover slip with attached spores on microscope slide, add a drop of seawater, and examine under a compound microscope, or invert cover slip to examine basal part of attachment structures.

Electrophoresis - Protein Analysis

Protein Extraction:

1. Pre-chill mortars and pestles at -20°C .
2. Dissolve protease inhibitors in 20 ml extraction buffer (45 min).
3. Harvest 0.2 g of each life cycle stage to be sampled just before protein extraction and place in mortars and pestles.
4. Add liquid nitrogen and keep samples on ice.
5. When liquid nitrogen has boiled away, grind samples to a powder.
6. Add 1.0 ml extraction buffer to each sample and continue grinding.
7. Upon thawing, add 1.0 ml extraction buffer to sample and transfer the mixture to centrifuge tube.
8. Centrifuge 10 min at 10,000 rpm.
9. Transfer supernatant to another centrifuge tube.
10. Add 4°C acetone to within 2 cm of tube's top and vortex.
11. Centrifuge 10 min at 10,000.

12. Remove acetone, add fresh acetone, mix, centrifuge, and repeat until a pink pellet remains.
13. Dissolve and homogenize pellet in sample buffer and store at -80°C .

Lowry Protein Determination:

1. Make 0.1% BSA (bovine serum albumin) solution and place 0.1 ml/100 μg , 50 μl /50 μg , 25 μl /25 μg , and 10 μl /10 μg aliquots in glass tubes as standards.
2. Place 5, 10, and 20 μl aliquots of samples in glass tubes.
3. Place 5, 10, and 20 μl aliquots of buffer without sample in glass tubes as blanks.
4. Bring aliquot volume to 100 μl with DIH_2O .
5. Add 1.5 ml Lowry reagent C (made by adding 1 ml Lowry reagent B dropwise with stirring to 100 ml Lowry reagent A).
6. Mix and wait 10 minutes.
7. Add 300 μl Lowry reagent D and wait 30 minutes.
8. Read at A_{660} in spectrophotometer, calibrating with DIH_2O .
9. Make standard curve by plotting μg BSA on x axis against absorbance on y axis.
10. Subtract 5, 10 and 20 μl blank values from 5, 10, and 20 μl sample values and plot these values along standard curve.
11. Read protein content of sample in $\mu\text{g}/\mu\text{l}$ from graph and calculate sample volumes accordingly. For example, to load 35 μg of a 3.5 $\mu\text{g}/\mu\text{l}$ sample, volume should be 10 μl . C. parvula extractions yielded about 3.5 $\mu\text{g}/\mu\text{l}$, and the sharpest bands were obtained with 70-140 μg samples, or 20-40 μl .

1-D Gel:

Note: Acrylamide is a neurotoxin. Avoid contact.

1. Clean glass plates with 95% ethanol.
2. Orient any chipped plate surfaces toward the lower edge.
3. Place teflon spacers (0.75 or 1.5 mm) between plates, keeping the spacers below the top of the plates.
4. Clamp plates together, ensuring that the lower edge is straight, and fasten plate and clamp sandwich to electrophoresis unit.
5. Mix running gel solution in a side arm flask but do not add catalysts.
6. Withdraw 1.5 ml for plug and degas the remainder.
7. Add TEMED and ammonium persulfate to plug solution and quickly pour with pasteur pipet between glass plates into base of unit. After rapid polymerization, rinse the plug with DH_2O and absorb water with whatman paper.
8. Add 8 μl TEMED and 200 μl ammonium persulfate to the running gel solution and swirl.
9. Pour running gel between glass plates to app. 12 cm with a 10 ml pipette.
10. Slowly overlay top of gel with enough DH_2O to make a 2 mm even layer of water.
11. Allow 45 min to 1 hr for polymerization. If leaving overnight, wait for polymerization, remove DH_2O , add 1X lower tris as an overlay and cover with foil.
12. Rinse polymerized gel with DH_2O . Blot excess water with paper.
13. Position comb for lane formation.
14. Mix stacker gel.
15. Pour stacker gel almost to top of glass. Overlay with DH_2O and let polymerize.

16. Remove comb and rinse with DH_2O . Blot excess water with lane-sized strips of filter paper. Transfer glass plate and gel sandwich to running part of electrophoresis unit.
17. Thaw samples over ice and mix with vortex.
18. Calculate sample volume in μl based on Lowry protein determination.
19. Transfer sample volume to a microfuge tube. Pierce tube top with a needle to release pressure while boiling in #22.
20. Dilute sample with DH_2O to bring 3X sample buffer to 1X.
21. To 1.5 μl molecular weight standards (Sigma, type) add 13.5 μl 3X SDS sample buffer, and 15 μl DH_2O .
22. Boil samples for 3 min.
23. Spin in microcentrifuge in cold box for 1 min at 10,000 g.
24. Carefully load samples into lanes. Load molecular weight standards into lane 7.
25. Overlay samples with running buffer.
26. Clamp electrophoresis unit top into place.
27. Pour 500 ml running buffer into bottom chamber of electrophoresis unit. Add 2 or 3 drops bromophenol blue to remaining 500 ml, mix, and slowly pour into unit top.
28. Fasten unit front into place. Connect electrodes.
29. Turn on power supply. Run gels at 25 mAMPS until dye front reaches the bottom of the gel, app. 3-5 h.
30. In sink, remove front and top of electrophoresis unit. Separate glass plates by prying a flat spatula between spacer and plates. Have pyrex dish with solution ready and place glass plate with attached gel into solution. Gel should float off plate.

31. Clean electrophoresis unit and glass plates with DIH₂O.

Coomassie - Silver Dual Staining:

Put a 12.5% 1.5mm SDS polyacrylamide gel in a clean square pyrex dish. Avoid handling gel, as fingerprints or foldmarks will appear on stained gel. Use enough of each solution to cover gel and change solutions with an aspirator. Place pyrex dish with gel on shaker at 40 rpm for all solutions.

Omit steps 1.a., 1.b., and 1.c. if silver staining only.

1. Soak gel 3X 20 min in 40% methanol, 10% acetic acid. Gel can be left overnight in this solution.

a. Stain with 0.1% coomassie blue G-250 in 7% glacial acetic acid, 50% methanol 30 min.

b. Destain 3X 10 min with 5% TCA.

c. Soak 2X 10 min in 40% methanol, 10% acetic acid.

2. Soak 2X 10 min in 10% ethanol, 5% acetic acid.

3. Soak 10 min in 3.4 mM (0.5 g) potassium dichromate, 0.0032 N (0.1 ml) nitric acid in 500 ml DIH₂O at 25° C.

4. Wash in DIH₂O 3X 10 min at 25° C until water is clear.

5. Soak 30 min in 0.012 M (0.4 g) silver nitrate in 200 ml DIH₂O at 25° C.

Place close to uniform fluorescent room lighting for first 5 min.

6. Remove silver nitrate solution by rinsing 1 min or less with DIH₂O.

7. Develop in three rinses of well dissolved 0.28 M (26.71 g) sodium carbonate, 0.45 ml formalin, and DIH₂O to 0.9 l at 40° C. First rinse is rapid (1 min) and traces of brownish vapor should appear. Second rinse is 1 - 2 min, and the third rinse is watched closely until brown protein bands appear and

then quickly removed. If development is too rapid, reduce concentration of sodium carbonate or decrease developer temperature.

8. Stop development in 5% acetic acid 5 min.

9. Immerse gel in DIH₂O.

Coomassie Stain:

1. Place in 0.1% coomassie G-250 stain overnight and place on shaker.

2. Pour off stain, add destain, and place on shaker

3. Pour off destain and add fresh destain.

4. Repeat until destain remains clear.

Labeling:

1. Establish cultures with minimal volume of seawater.

2. Add 5 μ l (50 μ ci) thawed ³⁵S-methionine (NEN) to each culture to obtain 1 μ ci/ml (50 ml cultures).

3. Or add 100 μ l (10 μ ci) ¹⁴C-L-amino acid mixture (NEN) to each culture to obtain 1 μ ci/ml (10 ml cultures).

4. Incubate in growth chamber 4 h.

5. Pour culture into Buchler funnel on large flask with Whatman paper filter.

6. Wash with dH₂O to remove unincorporated label. Liquid goes to radioactive waste, as do all aeration pipettes and pipette tips. Monitor area with Geiger counter.

7. Freeze tissue in liquid N₂ in mortar and grind to a powder with pestle.

8. Continue grinding and add 0.5 ml extraction buffer with protease inhibitors.

9. Transfer sample to microfuge tube and rinse mortar with 0.1 ml extraction buffer and add to sample.

10. Microfuge 10 min at 12 k in cold box. Transfer supernatant to a new tube.
11. Spot 2 μ l sample on 1 cm² Whatman 3M filter paper.
12. Add 100% TCA (1 g TCA/1 ml H₂O) to supernatant to get a 1:9 TCA:supernatant ratio.
13. Refrigerate at 4° C overnight.
14. Microfuge 10 min at 12 k in cold box. Keep pellet.
15. Spot 2 μ l sample of supernatant on filter; remainder goes to radioactive waste.
16. Wash pellet with 5% cold TCA, vortex, microfuge 5 min at 12 k, and remove TCA.
17. Wash pellet in 1 ml cold acetone and vortex.
18. Microfuge 10 min at 12 k in cold box, resuspend pellet in acetone, and repeat. Remove acetone.
19. Dry on ice in hood for 1 h.
20. Resuspend in 50 μ l IEF focusing sample buffer (lysis buffer A) or in SDS-PAGE sample buffer.
21. Spot 1 μ l on filter.
22. Store sample at -20° C.
23. Allow all samples spotted onto filter paper to dry. Label each with pencil.
24. Drop in 10% TCA in 1 l beaker reserved for this purpose. Use 200 ml TCA for 10 filters.
25. Bring to a rolling boil in the hood.
26. After 10 min remove from heat, add ice to cause filters to drop to bottom of beaker.
27. Pour off TCA into radioactive sink. Rinse 2X with dH₂O, 2X with 95% ethanol, and 2X with acetone.

28. Place filters in scintillation vials and air dry.
29. Add 100 μ l dH₂O + 500 μ l Protosol. Heat in 45° C water bath 20 min, do not exceed 55° C.
30. Add 10 ml Econofluor using the hood and gloves. Chill.
31. Count as ¹⁴C Channel 9 in Beckman LS 7500 β spectrometer liquid scintillation counter.
32. Calculate cpm (counts per minute) per μ l sample by dividing cpm by volume in μ l of sample counted. 3242.00 cpm divided by 2 μ l = 1621cpm/ μ l sample, or 16,210 cpm/10 μ l sample. Load 20,000 or 40,000 counts per lane in 1-D gel wells or into tube gels for IEF.
33. After 1 or 2-D gels are run, place running gel in Enhance (Dupont) for 1 hr, wash in DIH₂O for 30 min on shaker, and dry gel. Place gel in contact with Kodak X-Omar-AR Diagnostic Film (Sigma) film in dark room, clamp folder shut and place in -80° C for 96 hours.
34. Remove gel from film in darkroom. Develop film in Dektol, fixer, (Kodak) and 3% acetic acid stop bath. Hang to dry.
35. Proteins will be represented as dark spots on film.

Isoelectric Focusing (IEF):

1st Dimension (Tube Gels)

1. Place glass tubes in Dichromate overnight.
2. Rinse with DIH₂O and dry in oven.
3. Mark tubes at 11 cm from bottom, wrap bottom with parafilm, and place tubes in rack.
4. Mix gels and degas for 2 minutes only. Add remaining ingredients.

5. Pour gels into tubes with long pasteur pipet. Dislodge bubbles trapped in tubes.
6. Overlay with 100 μ l thawed solution H. Let polymerize for at least 3 h.
7. Put 2 l of H_3PO_4 solution into lower tank and jiggle to eliminate bubbles.
8. Rinse gels, remove parafilm from tubes, and place on apparatus.
9. Load into tubes: 10,000-100,000 cpm (100 μ l or less) of samples in lysis buffer A.
10. Load 5 μ l IEF standards onto standard gel.
11. Overlay with at least 100 μ l solution K, making sure K is not heavier than samples.
12. Add 1 additional liter of H_3PO_4 solution to lower tank and about 750 ml NaOH solution to upper tank, keeping levels of tanks even to avoid pressure.
13. Run at 300 V overnight (at least 19 h), then turn up to 400 V 1.5-2 h.
14. Remove gels with syringe and dH_2O . Place in screw top tube with sample buffer O. Do not let gels fold over on themselves. Equilibrate 30 min on shaker and freeze in dry/ice Ethanol bath or carry them straight.
15. Do not place IEF standard in sample buffer O. Instead, fix in 10% TCA overnight, stain for 1 h in BioRad stain, and destain in BioRad destain.
16. Store at $-80^\circ C$.

2nd Dimension Gels (Slab gels):

1. Pour running gel and let polymerize.
2. Remove tube gels from $-80^\circ C$ freezer and thaw in dish of water.
3. Thaw ampersulfate.
4. Mix enough stacker gel solution for 2 gels.
5. Heat beaker of water in hood on hotplate.

6. If gels are thawed, place on shaker to mix gently.
7. Rinse tops of running gels with DIH₂O and blot dry.
8. Add 6 μ l TEMED to stacker mix, position comb teeth for molecular weight standards and pour stacker gel to almost top of plates with pasteur pipet.
9. Overlay with 200 μ l DIH₂O very carefully across stacker and check position of comb.
10. Melt agarose on hot plate.
11. Prepare SDS-PAGE molecular weight standard
12. Rinse top of stacker when polymerized and blot excess water.
13. Microfuge standards for 30 sec at 10 k in cold box.
14. Move gels to hood.
15. Load 16 μ l of standards in each gel.
16. Overlay with molten agar and remove bubbles with needle.
17. Fasten on upper chamber.
18. Position tube gel on concave spatula in dish.
19. Fill slot with agarose and remove bubbles. Carefully flip gel from spatula into slot, making sure gel stays straight. Do not tug on gel.
20. Allow agarose to harden in hood. Dish and tubes are rinsed in radioactive sink.
21. Move gels to lower chamber and fill 1/4 inch from top with electrode buffer.
22. Add 2-3 drops of Bromophenol blue to buffer, mix, and pour gently into upper chamber. Fasten on apparatus lid.
23. Run gels on constant current -24 mAMP, unlimited voltage beginning with 70 V for 5 1/2 h, or until dye front is 12.5 cm from top of slab.

ELECTROPHORESIS RECIPES

Extraction buffer: (Modified from Kropf et al. 1989)

0.121 g tris (10mM)

0.5 ml Triton X-100

0.5 g SDS

1.0 ml β -mercaptoethanol

Bring to 100 ml with DIH₂O.

Into 20 ml add the following protease inhibitors:

3.48 mg PMSF phenyl methyl sulfonyl fluoride (4° C)

7.04 mg TPCK N-tosyl L-phenyl alanine chloromethyl ketone (-20° C)

2.5 mg N-ethylmaleimide (4° C)

3X 2% SDS Sample Buffer:

7.5 ml glycerol 1.5 ml β -mercaptoethanol

7.5 ml 20% SDS 9.375 ml 4X upper tris

Bring to 25 ml with DIH₂O, store at 4° C, use at room temp.

20% SDS (w/v):

20 g SDS, bring to 100 ml with DIH₂O, store at room temp.

Lowry Reagent A:

2.0% Na₂CO₃, 0.02% Na Tartrate, 0.4% NaOH

Lowry Reagent B:

0.5% CuSO₄-H₂O

Lowry Reagent C:

(make fresh daily) To 100 ml Reagent A add 1 ml Reagent B, dropwise with stirring.

Lowry Reagent D:

0.5 N Folin Glacteau reagent (dilute 2 N stock 1/4)

4X Lower Tris pH 8.8:

18.17 g tris (1.5M)

2 ml 20% SDS

Bring to 100 ml with DIH₂O, pH, store at room temp.

4X Upper Tris pH 6.8:

6.06 g tris (0.5 M)

2 ml 20% SDS

Bring to 100 ml with DIH₂O, pH, store at room temp.

Acrylamide:

30 g acrylamide

0.8 g bis-acrylamide

Bring to 100 ml with DIH₂O, filter, store in dark at 4° C.

Ammonium Persulfate:

100 mg/ml DIH₂O. Make fresh daily.

10% Acrylamide Gel (2 gels, 1.5 mm spacers):

20 ml 4X lower tris

26.6 ml 30% acrylamide

33.2 ml DIH₂O

0.2 ml ammonium persulfate

Remove enough for plug, degas remainder.

Add 8 µl TEMED for each gel and pour.

Stacker gel (2 gels):

7.8 ml DIH₂O

3.8 ml 4X upper tris

2.5 ml 30% acrylamide

50-75 µl ammonium persulfate

6 µl TEMED

4X Tris-Glycine buffer pH 8.3:

12 g tris

57.6 g glycine

Bring to 1 liter with DIH₂O, check pH only, store at 4° C.

Electrode Buffer (Running Buffer):

250 ml Tris-Glycine buffer

5 ml 20% SDS

Bring to 1 liter with DIH₂O.

Stain:

2.0 g Coomassie Brilliant Blue (0.1%)

140 ml (7%) glacial acetic acid

1 L methanol

860 ml DIH₂O

Makes 2 liters.

Destain:

280 ml glacial acetic acid

800 ml methanol

120 ml glycerol

2800 ml DIH₂O

Makes 4 liters.

IEF Recipes:

Lysis Buffer A:

5.7 g Urea (9.5 M)

2 ml 10% NP-40 (2% w/v)

0.5 ml ampholines pH 5-8 (1.6%)

0.125 ml ampholines pH 3-10 (0.4%)

0.5 ml β-mercaptoethanol (5%)

Bring to 10 ml with DIH₂O and freeze.

Acrylamide D (30%):

7.1 g acrylamide

0.4 g bis-acrylamide

Bring to 25 ml with DIH₂O.

Gel Overlay H:

4.8 g Urea (8 M) in 10 ml DIH₂O and freeze.

Sample Overlay K:

4.8 g Urea (8 M)

0.25 ml ampholines pH 5-8

62.5 μ l ampholines pH 3-10

Bring to 10 ml with DIH₂O and freeze.

Solution O SDS Sample Buffer:

40 ml glycerol

20 ml β -mercaptoethanol (add last)

9.2 g SDS

50 ml 4X Upper tris or 3.03 g tris

288 ml DIH₂O

Bring to 400 ml with DIH₂O.

Add 0.7 ml agarose for gel embedding.

SDS-PAGE Standards for 2nd dimension gels:

4 μ l molecular weight standards

17 μ l 3X SDS dye

15 μ l DIH₂O

Boil for 3 minutes.

1st Dimension Gels (25 ml batch):

13.75 g Urea

3.32 ml Acrylamide D

5 ml 10% NP-40 (solution E - 10% w/v)

Make in side arm flask in 37° C water bath. Remove from water bath and add:

1.25 ml ampholines pH 5-8

0.31 ml ampholines pH 3-10

Degas 2 minutes only. Add:

125 µl 0.1% fresh riboflavin (10 mg/10 ml)

37.5 µl 10% ampersulfate

12.5 µl TEMED.

Anode Electrode Solution (+) (Bottom): 0.01 M H₃PO₄

0.68 ml 85% phosphoric acid/liter DIH₂O

Cathode Electrode Solution (-)(Top):

0.6 g of 0.02 M NaOH in 750 ml DIH₂O. Degas and store under vacuum.

BioRad Stain:

27 ml isopropanol

10 ml glacial acetic acid

40 mg coomassie R-250

0.5 g CuSO₄ or 0.68 g CuSO₄·5H₂O

63 ml DIH₂O

BioRad Destain:

12 ml isopropanol

APPENDIX 3. LIST OF ORGANISMS

Rhodophyta

- Ahnfeltia* sp.
- Antithamnion nipponicum*
- Antithamnion sparsum*
- Ceramium* sp.
- Champia parvula*
- Chondria tenuissima*
- Chondrus crispus*
- Chylocladia verticillata*
- Corallina officinalis*
- Coreocolax* sp.
- Gelidium* sp.
- Gigartina stellata*
- Gracilaria chilensis*
- Gracilaria lemanaeformis*
- Gracilaria verrucosa*
- Griffithsia pacifica*
- Halarachnion ligulatum*
- Haliptilon cuvieri*
- Harveyella mirabilis*
- Hildenbrandia* sp.
- Hydrolithon boergesenii*
- Hypnea musciformis*

Iridaea laminariodes
Lithophyllum incrustans
Lithophyllum sp.
Lithothamnion glaciale
Nizymeria australis
Odonthalia floccosa
Palmaria palmata
Phymatolithon laevigatum
Phymatolithon lenormandii
Phymatolithon polymorphum
Polysiphonia deusta
Polysiphonia harveyi
Polysiphonia lanosa
Polysiphonia novae-angliae
Porphyra leucosticta
Porphyra perforata
Porphyra schizophylla
Porphyra tenera
Porphyra variegata
Porphyridium aerugineum
Porphyridium cruentum
Rhodella reticulata
Wrangelia plumosa

Phaeophyta

Ascophyllum nodosum

Bifurcaria bifurcata

Ectocarpus siliculosus

Euchema nudum

Fucus gardneri

Fucus serratus

Fucus vesiculosus

Halidrys siliquosa

Hildenbrandia prototypus

Pelvetia canaliculata

Pelvetia fastigata

Ralfsia verrucosa

Sargassum fluitans

Sargassum natans

Chlorophyta

Acanthosphaera zachariasii

Chlorella vulgaris

Chlamydomonas eugametos

Chlamydomonas reinhardtii

Closterium ehrenbergii

Closterium peracerosum-strigosum-littorale

Enteromorpha compressa

Enteromorpha intestinalis

Prasiola stipitata

Ulva lactuca

Ulva mutabilis

Ulva rigida

Bacillariophyceae

Amphora coffeaformis

Ardissonea crystallina

Nitzschia sp.

Euglenophyceae

Colacium libellae

Dinophyceae

Alexandrium catenella

Scrippsiella trochoidea

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