Morphological, Biochemical and Molecular Characterization of Desiccation-Tolerance in Cyanobacterium *Nostoc commune* var. Vauch

by

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ABSTRACT

Filaments of the desiccation-tolerant cyanobacterium *Nostoc commune* are embedded within, and distributed throughout, a dense glycan sheath. Analysis of the glycan of field materials and of pure cultures of *N. commune* DRH1 through light and electron microscopy, immunogold-labelling and staining with dyes, revealed changes in the pattern of differentiation in glycan micro-structure, as well as localized shifts in pH, upon rehydration of desiccated field material. A Ca/Si rich external (pellicular) layer of the glycan acts as a physical barrier on the surface of *N. commune* colonies. A purified fraction (> 12 kDa) of an aqueous extract of the glycan from desiccated field material contained glucose, N-acetylglucosamine, glucosamine, mannose and galactosamine with ratios of 3.1 : 1.4 : 1 : 0.1 : 0.06, respectively. Ethanol extracts of *N. commune* contained trehalose and sucrose and the levels of both became undetectable following cell rehydration. Elemental analysis of glycan extracts showed

a flux in the concentrations of salts in the glycan matrix following rehydration of desiccated colonies. Intracellular cyanobacterial trehalase was identified using immunoblotting and its synthesis was detected upon rehydration of desiccated field cultures. Water-stress proteins (Wsp; molecular masses of 33, 37, and 39 kDa are the most abundant proteins in glycan), a water soluble UV-A/B-absorbing pigment, the lipid-soluble UV-protective pigment scytonemin, as well as two unidentified cyanobacterial glycoproteins (75 kDa and 110 kDa), were found within the glycan matrix. No evidence was found for either glycosylation, phosphorylation or acylation of Wsp polypeptides. NH_2-terminal sequence analysis of the three proteins of Wsp were identical: Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln-X-Ile-Gln-Asn-Pro-Ser-Asn-Pro-Ser-Asn-Gly-Lys-Gln. An unidentified 68-kDa protein, the second most abundant protein in aqueous extracts of the glycan, was isolated and its N-terminal sequenced was determined: Ala-Phe-Ile-Phe-Gly-Thr-Ile-Ser-Pro-Asn-Asn-Leu-Ser-Gly-Thr-Ser-Gly-Asn-Ser-Gly-Ile-Val-Gly-Ser-Ala. Gene bank searches with these sequences, and an internal sequence of Wsp (Glu-Ala-Arg-Val-Thr-Gly-Pro-Thr-Thr-Pro-Ile-Asp), identified homologies with various carbohydrate-modifying enzymes. Purified Wsp polypeptides associate with 1,4-β-D-xylanxylanohydrolase activity that was inhibited specifically by Wsp antiserum. In the absence of salt, Wsp polypeptides, and the water-soluble UV-A/B-absorbing pigments, form multimeric complexes through strong ionic interactions. The role of the glycan, and the protein and pigments that reside within it, in the desiccation tolerance of *N. commune* is discussed with respect to structure/function relationships.

To those people in my life who have inspired me to
wonder - Why? My teachers.
The greatest of whom was my mother,
Lydia Ellen Skirvin Hill.
30 November 1944 - 5 November 1991

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1° : primary

2° : secondary

A_{280} : absorbance at 280 nm

A_{312} : absorbance at 312 nm

ATTC: American Type Culture Collection

AP : Alkaline phosphatase

β : beta

BSA : Bovine serum albumin

BZA : Benzamidine

B&W : Black and white

°C: degree centigrade

CAPS : 3-[cyclohexylamino]-1-propanesulfonic acid

CBB : Coomassie Brillant blue

cm : Centimeter

DFP : Diisopropyl fluorophosphate

DIG : Digoxigenin

DNase : Deoxyriboneclease

DTT : Dithiothreitol

2D : Two dimensional

EDTA : ethylenediaminetetraacetic acid

EDX : Energy dispersive x-ray microanalysis

EPS : Extracellular polysaccharides

E. coli : *Escherichia coli*

Fab : Fragment having antigen binding site

FPLC : Fast performance liquid chromatography

g : Gravity

GC : Gas chromatography

gm : Gram

Glu-C : Endoproteinase Glu-C

HMDS : Hexamethylsisilazane

HRP : Horse radish peroxidase

ICP : Inductively coupled plasma spectrophotometry

IgG : Immunoglobulin G

Immobilon P : Polyvinylidene fluoride

kDa : Kilodalton

Lys-C : Endoproteinase Lys-C

max. : Maximum

m : Meter

MEGA 8 : Octanoyl-N-methylglucamide

µl : Microliter

µg : Microgram

µm : Micrometer

µmol : Micromoles

mA : Milliamphere

mg : Milligram

mm : Millimeter

mM : Millimolar

M : Molar, moles liter^{-1}

ml : Milliliter

MPa : Megapascal

M_r : Relative molecular weight

NAcGalN : N-acetyl galactosamine

ng : Nanogram

nm : Nanometer

NP-40 : Nonidet P-40

***N. commune* :** *Nostoc commune*

ONPX : o-nitrophenyl β-D-xylopyranoside

PAGE : Polyacrylamide gel electrophoresis

PAS : Periodic acid-Shiff reagent

PBS : Phosphate buffered saline

PGNase F : N-Glycosidase F

PMSF : Phenylmethylsulfonyl fluoride

PVDF : Polyvinylidene fluoride

RBB : Remazol Brillant blue

s : Seconds

SDS : Sodium dodecyl sulfate

SEM : Scanning electron microscopy

TBS : Tris buffered saline

TBST : Tris buffered saline with Tween

TEM : Transmission electron microscopy

TEMED : N-N-N'-N'-tetramethylethylene

TFA : Trifluoroacetic acid

TLC : Thin layer chromatography

TMS : Trimethylsilyl

Tris : Tri(hydroxymethyl)aminomethane

TWEEN 20 : Polyxyethylenesorbitan monolaurate

U : Units

UDP : Uridine diphosphate

UTEX : University of Texas Culture Collection

UV : Ultra-violet

v/v : Volume to volume ratio

w/v : Weight to volume ratio

Wsp : Water-stress protein

x : Times

1. Introduction

The removal of water from cells, the storage of cells in the air-dried state, and the rewetting of dried cells, impose physiological constraints which relatively few organisms can tolerate. The first reported observation of a dry organism reviving by the addition of water was in 1702 by Antony von Leeuwenhook, the father of "microscopic" biology. Because the organisms were thought to have been dead and were brought back to life, scholars and philosophers of the day termed the phenomenon "anabios" or "return to life". It was not until the 1950's that Keilen redirected this erroneous resurrectionist theory by coining the term "anhydrobiosis" or "life without water" (Keilen 1959). Today, the term "anhydrobiosis" in its simplest form means desiccation tolerance. Since Leeuwenhook's first account, a capacity for desiccation tolerance has been reported for a diverse assortment of prokaryotic and eukaryotic organisms (Clegg 1986; Crowe and Crowe 1992; Crowe et al. 1992).

Desiccation tolerance, in many respects, can be considered a primitive force, being more prevalent in prokaryotes than eukaryotes (Bewley 1979). The fact that the former have a simpler internal organization, as illustrated with their lack of compartmentalization, and less complex membrane structure may account for their desiccation tolerance abilities. The selective pressure of a water deficit is likely to have impinged upon prokaryotes at a very early stage in their evolution. The question of how the stresses of the environment limit the survival and proliferation of organisms can be addressed in two ways: What determines the

ability of an organism to live in that particular environment? and/or What mechanisms of adaptation were employed by that organism such that it can survive in that environment? More so than any other boundary/interface effect, the removal of cell-bound water through air-drying, and the addition of water to air-dried cells, are the predominant forces that influence the distribution and activities of bacterial communities. The reason how some bacterial species can cope with the problem of water deficits while others cannot is still unknown. Considerations of the water relations of bacteria dwell almost exclusively with the osmotic systems where cells are immersed in solvent-solute mixtures (Cayley et al. 1992; Csonka 1989; Csonka and Hanson 1991). The role of water in the structure-function relationships of cells has been inferred largely from biophysical studies with single purified proteins (Timasheff 1992, 1993) and from ecological studies. Unfortunately, to this date little light has been shed on the mechanisms of desiccation tolerance.

Water generally is considered to be indispensable for the preservation of the integrity of biological membranes, both for physical and physiological reasons (Tanford 1980). Mechanisms which maintain the structural integrity of membranes appear to be of importance to the survival of organisms to desiccation tolerance. Certain sugars, particularly trehalose, prevent damage from dehydration not only by inhibiting fusion between adjacent membrane vesicles during drying, but also by maintaining membrane lipids in a fluid phase in the absence of water (Crowe et al. 1987, 1992). The experimental evidence suggests that trehalose can stabilize cell membranes for short periods in air, but it can not do so for extended periods.

In the latter respect, other mechanisms must be important in the protection of cells from long-term dehydration stress. A still unexplained, and relatively unexplored, phenomena is the stabilizing effect divalent cations have in conjunction with certain sugars in the preservation of enzymatic activities during desiccation (Crowe et al. 1987).

Of those organisms which express desiccation tolerance, certain cyanobacteria have a particularly marked capacity to do so. One form, a filamentous, heterocystous, nitrogen-fixing obligate photoautotroph, *Nostoc commune*, has become a very useful model for the analysis of dehydration-induced stresses in photosynthetic cells (Potts et al. 1984; Potts 1985; Potts and Bowman 1985; Scherer et al. 1986; Stulp and Potts 1987; Peat et al. 1988; Scherer and Zong 1991; Tarranto et al. 1993). In these studies it has been discovered that a sequential reactivation of metabolic activities occurs upon rehydration. In order of recovery they are respiration, photosynthesis (which is limited by the recovery of adenine nucleotide pools as dictated by oxidative phosphorylation) and nitrogen fixation. Respiration and photosynthesis are both recovered within six hours and is explained by the fact that both activities precede growth and are exhibited by pre-existing vegetative cells. Nitrogen fixation, on the other hand, is dependent upon newly-differentiated heterocysts and requires approximately 150 hours of rehydration prior to its detection, with the acetylene reduction assay technique (Scherer et al. 1984) Cells of *N. commune* are subjected to acute water stress *in situ* through multiple, and often rapid, cycles of wetting and drying. These cycles interrupt often extended periods of desiccation during which the cells must tolerate further oxidative- and

radiation-induced stresses.

Extracellular polysaccharides (EPS) are a conspicuous feature of most bacterial cells. These investments may have the appearance of diffuse slimes, or may develop as rigid layers with a defined, complex ultrastructure (Potts et al. 1983). EPS layers are formed by the accumulation of various types of polymeric substances of high viscosity, they tend to be hygroscopic and, for aerophytic bacteria, often contain more water than the immediate environment. In view of the copious amounts of water trapped in these extracellular gels their structural analysis has proven to be difficult. Previous studies on bacterial extracellular polysaccharides have documented that they tend to have a very low affinity for various dyes and work using electron microscopy has established that in general they are less electron dense than the cell wall and the cytoplasm (Roth 1977). As a consequence, while analytical data are available, structural analyses of extracellular structures and elucidations of structure function relationships are few (Roth 1977).

Functions attributed to EPS include their participation in the anchorage of the bacterial cell to its substrate, and protection against desiccation, phagocytic predation, antibody recognition, and lysis by other bacteria and viruses (Tease and Walker 1987). The EPS of *Beijerinckia* may protect the cells against oxygen damage (Barbosa and Alterthum 1992). The EPS of bacteria represent an additional cell compartment and one that may contain the bulk of the water associated with a single cell. As such, it is widely believed that EPS provide bacterial cells with a means to survive drying, yet studies on the specific

response of polysaccharide synthesis to drying are few, and the mechanisms of sensing of water deficit, with subsequent induction and regulation of polysaccharide synthesis, remain poorly understood (Ernst et al. 1987; Roberson and Firestone 1992). The presence of proteins, uronic acids, pyruvic acid and O-methyl, O-acetyl and sulfate groups in EPS emphasizes the complexity of these extracellular layers and suggests that a number of enzymes are required to degrade (and synthesize) the polysaccharide structure. The occurrence of carbonyl, carboxyl, hydroxyl and sulfate groups provide a means to attach cations. In the latter respect these EPS may scavenge metals that may be used either in physiological processes such as nitrogen fixation, or as toxins to repel predators (Tease and Walker 1987).

The extracellular polysaccharides of cyanobacteria provide some of the most complex examples of bacterial sheath structures and are well-documented in the classical literature (Geitler 1932; Weckesser et al. 1988). For the most part cyanobacterial EPS are reminiscent of the glycocalyxes, slimes and capsules of other eubacteria (Nakagawa et al. 1987; Tease and Walker 1987), many of them have a complex ultrastructure (Pritzer et al. 1989) and, especially in communities growing *in situ*, they tend to be pigmented and sometimes heavily calcified (Potts and Whitton 1979).

Since the environmental conditions which give rise to water-stress and ultimately desiccation generally involve high exposure to solar radiation, it would not be surprising to discover an association of the mechanisms of desiccation tolerance with the components used

to shield an organism from harmful ultra-violet radiation. An UV-absorbing pigment, from *Nostoc commune*, with a absorption maximum of 312 nm and 335 nm recently has been isolated and characterized (Scherer et al. 1988; Böhm 1993). In field grown colonies, this UV-absorbing pigment accounts for up to 10% dry weight of the colonies of *Nostoc commune* miomass. Since the UV-pigment contains a polysaccharide and is localized in the extracellular glycan surrounding the cells, it has been postulated that it may play a role in water storage in conjunction with the glycan matrix. Besides the UV-absorbing pigments, the ultraviolet sunscreen pigment scytonemin is located in the sheath of cyanobacteria. It has been theorized that this sunscreen pigment aided cyanobacteria, by protecting them from fluxes in ultraviolet radiation, during their evolution to present day species (Proteau et al. 1993).

Brittle, dried and darkened crusts of one cyanobacterium, *Nostoc commune*, are a characteristic feature of limestone (Karst) regions from the Poles to the Equator, where they accumulate in shallow depressions in rock or become scattered over rocks and nutrient-depleted soils (see Fig. 1a in Whitton et al. 1979). The marked desiccation tolerance of *N. commune* reflects its ability to couple long-term structural and functional integrity in the air-dried state with a capacity to achieve instantaneous recovery of metabolic capacities upon cell rehydration. The basis for such desiccation tolerance likely reflects a complex array of interactions at the structural, physiological and molecular levels (Potts 1993). In a primary publication (Scherer and Potts 1989), it was documented that field colonies of *N. commune*

accumulate abundant amounts of water stress proteins (Wsp). These proteins are secreted from the cells together with the above mentioned water-soluble UV-A/B-absorbing pigment (Hill et al. 1994). During the past 5 years, great strides have been made in defining the components of the extracellular protein secretion machinery of gram-negative bacteria. However, knowledge of precisely how these components act together in extracellular secretion remains limited. What is speculated, is that the same mechanisms work in the import as well as the export of a wide range of substrates (Lorey 1992).

In order to decipher mechanisms that may permit *Nostoc commune* to survive the environmental stress of water deprivation, an understanding of the overall structural biology, physiology, biochemistry and molecular ecology of the organism was initiated. The present study documents a comprehensive analysis of Wsp and the components of its immediate environment, the glycan. In addition, data are presented on the structure and aggregation of components that reside within the EPS.

The immediate environment of the secreted components in desiccated field material of *Nostoc commune* is an extracellular glycan that constitutes the greater part of the colony bulk. Whether any of these secreted components are salvaged upon rehydration is unknown. While the role of water stress proteins remains cryptic, it is implausible to consider that they alone are responsible for the desiccation tolerance of the cyanobacterium *Nostoc commune*.

2. Materials and Methods

2.1. Microorganisms and Growth Conditions

Field materials of *Nostoc commune* were collected during the past 55 years from a wide range of geographic locations and all have been maintained in the air-dry state, in the dark, since their collection (Fig. 1). Further details for some of these materials are presented in Table 1. *Nostoc* samples from Germany were collected by Dr. Siegfried Scherer and Dr. T. W. Chen collected the samples located in the Hunan province of China. Colonies from rocks in a creek crossing Salt Sulfur Turnpike, Giles County, Virginia, were collected by John C. Strickland on August 9th, 1939. The latter sample was obtained from the herbarium of the Department of Biology, University of Richmond. All other samples studied were collected by Dr. Malcolm Potts. *Nostoc commune* strain DRH1 is derived from field materials of *N. commune* CHEN that can be grown under laboratory conditions as two distinct forms. On inert supports such as 1.2% w/v agar supplemented with BG ll_0 (Rippka et al. 1979) medium, or on nylon membranes subjected to isopiestic control of water potential (Potts et al. 1984) the strain forms spherical colonies of 1 mm to 1 cm diameter (Fig. 4). The strain forms a diffuse growth when grown in liquid BG 11_0, at 32 °C, under a photon flux density of approximately 50 µmol photons $m^{-2}s^{-1}$, with shaking. *Nostoc commune* UTEX 584 was grown in axenic culture in BG ll_0, at 32 °C in an airlift fermentor as described (Potts and

Bowman 1985).

2.2. Fixation, Embedding, Staining and Examination of Cells

A technique for the preparation of desiccated cells of *Nostoc commune* for electron microscopy study has been published (Peat and Potts, 1987). Samples of all materials were fixed in 2% w/v glutaraldehyde: 2% w/v paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.0, for 3 to 4 hours. Where necessary for membrane preservation for electron microscopy, 1% w/v osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.0, for 2 hours was employed for secondary fixation without rinsing between fixation steps. Samples were then dehydrated using a standard ethanol series and infiltrated with their respective resins, paraffin and Lowicryl, or EPON 812, for light and electron microscopy, respectively. Luft's recipe was used for the resin mixture of EPON 812 (Poly/Bed 812, 21 ml [22.17 gm]; DDSA, 13 ml [11.37 gm]; NMA, 11 ml [11.63 gm]; and 0.7 ml DMP-30 added just prior to use ; Polysciences, Inc. data sheet #233). The K4M recipe was used for the low temperature embedding resin Lowicryl (Crosslinker A, 2.70 gm; Monomer B, 17.30 gm; and Initiator C, 0.10 gm; Polysciences, Inc. data sheet #248). Resin for electron microscopy was changed at 24 hour intervals on each of seven successive days, using acetone as the solvent during infiltration with EPON 812 and 100% ethanol with Lowicryl. After transfer to gelatin capsules the samples in Lowicryl were cured for four days, under UV light, at 4 °C. Electron

microscopy samples embedded in the EPON 812 resin were cured under vacumn, in an oven at 60 °C for at least 18 hours. Light microscopy samples were vacumn infiltrated with paraffin and processed following a general paraffin protocol, xylene was used as the infiltration solvent (see Appendix 2).

Replicate paraffin sections, 15 μm thick, were used for scanning electron microscopy (SEM) and qualitative staining at the light microscopy level. Paraffin sections, adhered to glass coverslips, were cleared with xylene, critical point dried, carbon coated and placed on aluminum stubs for viewing with a Phillips 505 scanning electron microscope. Sections for energy dispersive x-ray microanalysis (EDX) were placed directly on carbon stubs, processed as described above and examined with an EDX 9900 system. Calibration for EDX analysis was performed on the element calcium and all scan counts were carried out for 330 seconds. Polaroid 55 Positive Negative 4X5 Instant Sheet Film was used for all SEM photographic data.

All staining protocols for qualitative light microscopy followed the instructions of Pearse (1980; see Appendix 2). Alcian blue staining was performed using a standard procedure and a pH of 2.5 to permit assessment of the acidity of the stained material. Periodic Acid-Schiff reagent (PAS) was used for the determination of the presence of glycoproteins. Stained and unstained sections were examined using an Olympus Model BH2-NIC microscope and photographed utilizing the PM-10ADS Olympus Automatic

Photomicrographic System and Kodak Ektachrome 160-T slide film.

Immunolabeling of ultra-thin sections for transmission electron microscopy (TEM) followed the protocol described in Hill et al (1994). Thin sections were transferred to nickel grids (400 mesh, 3.05 mm; Polysciences, Inc.) and the process of immunolabeling followed a modification of the technique of Titus and Becker (1985). All steps were performed at room temperature. The nickel grids were floated, section side down, for 10 minutes, on 20 µl droplets, on parafilm, of a solution of TBST (50 mM Tris-HCl at pH 7.2, 0.1 M NaCl, 0.3% TWEEN 20) which contained 1% w/v BSA (bovine serum albumin). Grids then were transferred, without rinsing, to 20 µl droplets of antiserum (1:100 dilution for both 1° [primary] antibodies used in this study) and incubated for 2 hours. After the incubation in 1° antibody, for the rabbit-Wsp antibody labelling, the grids were transferred to TBST-BSA for 10 minutes, gently washed under a stream of TBST, quickly blotted dry, and placed on the surface of a 20 µl droplet of a 1:20 dilution of Protein-A colloidal gold (G5 EM grade, 5 nm gold particles; Janssen Life Sciences, distributed by SIPI) in TBST and incubated for 45 minutes. The sheath antibody was generated in a mouse. Because Protein-A has less than half the affinity for a mouse generated IgG than it does for a rabbit IgG, an additional incubation step in 2° [secondary] antibody of Rabbit-Anti-Mouse, 1:100 dilution for 45 minutes, was performed before incubation in the Protein-A colloidal gold. Following the above Protein-A incubation step, the grids were placed on the surface of droplets of TBST,

left for 10 minutes, washed gently with TBST, and then rinsed thoroughly under a gently stream of sterile distilled water, and blotted dry.

In samples where the resin EPON 812 was used, etching of the resin with 3% v/v peroxide solution was necessary to expose the antigenic sites. Optimum conditions for the rabbit-Wsp antibodies was 5 minutes and for the mouse-sheath antibody 30 minutes (as determined by an etching series study, data not shown). No peroxide etching was necessary for the sections embedded in the Lowicryl resin. To check for non-specific staining, two different controls were run on the samples. One control was where the incubation stage with 1° antiserum was omitted and the other control employed the substitution of the 1° antiserum with the appropriate pre-immune serum. Post staining of non-immunolabeled ultra-thin sections was performed using 2% w/v ethanolic uranyl acetate for 15 minutes and then, after rinsing, for 5 minutes in a lead citrate solution. A Zeiss 10CA transmission electron microscope was used. Kodak Electron Microscopy film 4489 (ESTAR Thick Base) was utilized for all TEM photographic data (Appendix 7).

2.3 Isolation of the Extracellular Glycan

The extracellular glycan of desiccated field material was obtained following grinding of the sample, in liquid nitrogen, to a fine powder. The sample was then incubated in 2% w/v

SDS, 1% v/v NP-40, in 100 mM Tris, pH 7.5, at 65 °C overnight. The preparation was extracted three times with hot phenol/chloroform:isoamyl alcohol (24:1). The aqueous phase was collected and the glycan was recovered after precipitation with ethanol. The sample was reconstituted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4), treated with DNase, phenol/chloroform extracted, precipitated with ethanol, lyophilized to dryness and then weighed.

A 14-liter culture of *N. commune* DRH1 was grown as described (Hill et al. 1994). The cell-free supernatant fraction was recovered following harvesting of the cells and was concentrated using an Amicon concentrator with a YM10 membrane (10-12 kDa cut off; Amicon, Inc.). The filtrate was lyophilized to dryness and weighed. The retentate was extracted as described above, dried, and weighed.

2.4 Isolation of Wsp polypeptides from the Extracellular Glycan

Equivalent weights of desiccated colonies were suspended in sterile water with or without the addition of one or more protease inhibitors at the specified final concentration: 50 μM phenylmethylsulfonyl fluoride (PMSF), 25 μM diisopropyl fluorophosphate (DFP), 1 μM leupeptin, 0.07 μM benzamidine (BZA), 0.1 μM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), all prepared according to the specifications of the manufacturer (Sigma, St Louis, MO; see Appendix 3). Following different periods of

rehydration, the rehydration fluids, as well as the intact colonies, were recovered. Rehydration fluids were lyophilized and the dried residue was reconstituted with SDS-PAGE sample buffer (80 mM Tris-HCl, pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 10% v/v glycerol, 2% v/v β-mercaptoethanol, 0.001% w/v bromophenol blue) and boiled for 2 minutes prior to analysis by SDS-PAGE.

2.5 *Isolation of Native Wsp*

Between 5 and 20 gm of desiccated colonies were rehydrated in 200 to 600 ml of sterile distilled water for 30 minutes. Aliquots of the solution were centrifuged at 20,000x g, for 20 minutes, then the supernatant fraction was passed through a 0.2 μm filter (Millipore) and the filtrate was recovered. All further manipulations were performed at 4 °C. The filtrate was concentrated by ultrafiltration using Centriprep-10 cartridges (10 kDa cut-off; Amicon Inc., Beverly, MA).

Analytical liquid chromatography was performed using a Pharmacia FPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described in the figure legends. Two types of columns were used in the analysis of the sheath extracts: an anion exchange column (Mono Q HR 5/5) and a gel filtration column (Superose 12 HR 10/30), both acquired from Pharmacia, LKB.

2.6 *Cell Disruption and Isolation of Cellular Protein*

Desiccated (or lyophilized) colonies were suspended in grinding buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM KCl, 1 mM sodium azide, 1 mM β-mercaptoethanol), which also contained the above mentioned protease inhibitor cocktail. The mixtures were boiled for 5 minutes then frozen at -75 °C, thawed (3 cycles) and sonicated (Fisher Model 300 operated at 35% of maximum setting), for 10 minutes. Samples then were centrifuged at 1,000x g for 15 minutes, at 4 °C, to remove cellular debris. The supernatant fraction was then centrifuged at 12,000X g for 15 minutes, at 4 °C, recovered and stored at 4 °C until analyzis.

2.7 *Total Extraction of Wsp (preparative)*

Desiccated material was ground in liquid nitrogen and 5 gm (dry weight) aliquots were suspended in grinding buffer that contained 0.1 mM DFP, 1 mM PMSF and 5 mM EDTA. The suspension was subjected to four consecutive pressings (110 MPa) in a French pressure cell, with additions of fresh ice-cold grinding buffer. The slurry was mixed with a detergent buffer (final concentrations of 50 mM Tris-HCl, pH 7.8, 3 M urea, 2% w/v SDS, 1% v/v nonidet P-40 and 1 mM β-mercaptoethanol). The solution was incubated at 65 °C, for 48 hours, then centrifuged at 12,000x g for 15 minutes, at 4 °C, and the supernatant fraction was

retained for analysis.

2.8 Extraction and Analysis of Water- and Lipid-Soluble Components in the Glycan

Desiccated colonies were rehydrated with sterile distilled water, overnight, at 4 °C. The aqueous extract then was passed through a 2 μm acrodisc filter. The detergent-soluble fraction of the desiccated glycan was extracted using 8 M urea, 1% v/v NP-40 and 1 mM β-mercaptoethanol with gentle abrasive action employing a dab of alumina powder. The lipid soluble fraction was then passed through a 2 μm acrodisc filter and washed with 20 mM Tris buffer, pH 7.5 using a Centricon 10 microconcentrator (10 kDa cut off; Amicon, Inc.).

The water- and lipid-soluble extracts were analyzed using fast protein liquid chromatography (FPLC) as described in the figure legends. Fractions were subjected to spectrophotometric analysis and Western blotting as described (Hill et al. 1994).

Field material was rehydrated for a time course of rehydration with water. After recovery of the aqueous rehydration fluid, the remaining cellular material was lyophilized and extracted with 80% v/v ethanol, at 4 °C overnight. Analysis of the aqueous rehydration fluids as well as the filtered and concentrated lipid-soluble (ethanol) extract was performed via Spectral Analysis of Elements with inductively coupled plasma spectrometry (ICP) using a Jarrell-Ash ICAP 9000, at the Virginia Tech Soil Testing and Plant Analysis Laboratory. All

analyses were performed in duplicate.

2.9 Preparation of Antibodies and Immunoanalysis

A preparation of the purified glycan was used to immunize mice (3-4 week-old C3H-HEJ). The 39 kDa form of Wsp was purified via electroelution from SDS-PAGE gels, dialyzed against distilled water and was used to immunize rabbits (New Zealand white) and mice (as described above). The lyophilized shock fluid from a 10 liter culture of *E. coli* K12 strain pTRE1, overexpressing *treA*, was provided kindly by Dr. Winfried Boos. *E. coli* trehalase was purified using the procedure specified by Boos et al. (1987), after ammonium sulfate precipitation and using ion-exchange chromatography (Mono Q HR 5/5). The purified trehalase was recovered in a single peak from the salt elution profile and was used to immunize rabbits (New Zealand white).

For all three antigens the following general protocol was used to generate the antibodies. A solution of purified proteins or sheath extract was brought to 40 mM with phosphate buffer, pH 7.5, mixed 1:1 with complete Freund's adjuvant (ICN Biomedicals, Inc.), and injected into mice (approximately 10 μg of protein per mouse in 0.2 ml). After 2 weeks, a booster with the same amount of protein mixed 1:1 with incomplete Freund's adjuvant (ICN Biomedicals, Inc.) was given, and the mice were bled after an additional 10 days. For antibodies directed against rabbits a 0.1 mg sample was dissolved in 0.5 ml of

phosphate buffer, mixed with 0.5 ml of complete Freund's adjuvant, and injected subcutaneously at different sites. After 2 and 4 weeks, the immune response was stimulated by injecting 0.1 mg of protein dissolved in incomplete Freund's adjuvant and phosphate buffer. After precipitation of the blood clot, at room temperature, the serum was collected and used in the case of all three antibodies, without further treatment. Pre-immune serum was collected from all animals before injection.

2.10 Electrophoresis and Biochemical Analysis

Analytical SDS-PAGE (Laemmli 1970) was performed in 1.5 mm minigels, using a Mighty Small II SE 250 chamber (Hoefer Scientific Instruments, San Francisco). Proteins were separated out on 5% w/v stacking/15% w/v running SDS-PAGE, using a Tris-Glycine running buffer (25 mM Tris-HCl, 200 mM Glycine, and 3.5 mM Lauryl sulfate [SDS]). Pre-stained low-molecular weight range markers of Phosphorylase B [106 kDa], Bovine serum albumin [80 kDa], Ovalbumin [49.5 kDa], Carbonic anhydrase [32.5 kDa], Soybean trypsin inhibitor [27.5 kDa], and Lysozyme [18.5 kDa] (Bio-Rad Laboratories) were used for all molecular weight determinations of extract proteins. A colorometric dye-binding assay (Pierce, Rockford, IL, publication #23200) was used to estimate protein concentrations, using known dilutions of BSA to generate a standard curve (Appendix 3).

After separation on SDS-PAGE, proteins were transferred, using CAPS (3-

[Cyclohexylamino]-1-propanesulfonic acid) buffer at pH 10.3 to polyvinylidene difluoride membrane (Immobilon P; Millipore Corporation, Bedford, MA) at 450-500 mA for 25 minutes. Immunodetection was performed according to standard techniques. Immediately following transfer of the proteins to the Immobilon P membrane, the blot was incubated in 1% w/v BSA in TBST for 30 minutes, washed briefly in TBST (1-2 minutes), and then placed in 1° antibody. Only the Wsp (rabbit) and trehalase antisera were utilized in the Western analysis of the proteins. All Western blots were incubated overnight, at a dilution of 1:2,000, in TBST with the 1° antibodies. Prior to placing the blots in 2° antibody, they were washed 3 times in TBST for a minimum of 10 minutes each, then incubated in 1:1,000 of blotting grade affinity purified Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate (Bio-Rad) for 2 hours. Washing of the blots 2 more times in TBST, for 10 minutes, was followed by 2 washes in TBS for 10-15 minutes each (important to use TBS here because Tween interferes with the following color development step). Mixing of the color development reagent, solution A (50 ml TBS with 30 µl of 30% H_2O_2) plus solution B (10 ml of 100% v/v ice cold Methanol with 30 mg of HRP Chloro Development Reagent [4-chloro-1-napthol]; Bio-Rad) was done just prior to submerging the blots. The color development reaction took approximately 5 minutes at which time it was stopped by washing the blots in distilled water.

Protein sequence analysis was performed on proteins resolved in 15% w/v gels, transferred to Immobilon P membrane, stained with CBB stain (made up without acetic acid)

and washed extensively with sterile distilled water using the procedures described by Matsudaira (1987). Automated Edman degradation was carried out using a Applied Biosystems 477A Protein Sequencer, according to Hewick et al. (1981). Cysteine residues were not derivatized prior to analysis.

Visualization of proteins in SDS-PAGE gels was accomplished either by staining with Coomassie Brillant blue (CBB) or with silver stain. CBB staining used a 0.2% w/v Coomassie Brillant Blue R-250 (Sigma Chemical) solution in 45% v/v methanol and 10% v/v acetic acid, gels were typically stained for 30 minutes to 2 hours. To visualize the proteins, the gel was then destained with 50% v/v methanol and 10% v/v acetic acid, using kimwipes placed on the surface of the destaining solution to absorb excess stain released from the gel. Destaining of CBB stained gels generally required up to 12 to 18 hours.

Silver staining of gels followed the protocol of Merril et al. (1981). This staining protocol is approximately 100 fold more sensitive than CBB, making it useful for detecting proteins present in trace quantities (see Appendix 3 for the complete silver stain and general electrophoresis protocols used in this study).

2.11 Carbohydrate Analysis

Samples of purified extracellular glycan were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 4 hours, dried, reconstituted in water, and analyzed for neutral and amino

sugars. Hydrolysis for sialic acids required a separate reaction using 0.1 M hydrochloric acid at 80 ℃ for 1 hour, drying and reconstitution of the sample in water. Chromatography was performed on a Dionex BioLC using a Carbopac PA1 anion exchange column. A Rainin Dynamax data acquisition system was used to record and integrate the data. The addition of 2-deoxy glucose was used as an internal standard. All samples were analyzed, in duplicate, injections at the University of Michigan, Protein and Carbohydrate Structure Facility.

Low molecular mass carbohydrates were obtained from desiccated colonies following extraction with 80% v/v ethanol, in the dark, overnight at 4 ℃. Lyophilized aliqouts of the ethanol extracts were silylated using the Pierce HMDS-STOX reagent and the protocol of the supplier. Sugars were treated with hydroxylamine hydrochloride. The resulting oximes were converted to trimethylsilyl sugar (TMS) ethers. To accomplish this, the lyophilized ethanol extract was combined with 1.0 ml STOX reagent (pyridine solution containing 25 mg ml^{-1} hydroxylamine hydrochloride and 6 mg ml^{-1} phenyl-β-D-glucopyranoside as an internal standard) in a 3.0 ml Reacti-Vial (small thick walled vial with a Teflon lined cap). The vial was then capped and heated to 70-75 ℃ for 30 minutes. Digest was then allowed to cool to room temperature. The sugars then were silylated by the addition of 1.0 ml HMDS (hexamethyldisilazane). After mixing the solution, 0.1 ml of TFA (trifluoroacetic acid) was added and the solution was shaken thoroughly for 30 seconds. A white precipitate appeared which was allowed to settle out for 30 minutes. An aliquot of each silylated sugar solution

was then analyzed. Silylated sugars were separated and identified on a Varian 3700 gas chromatograph using a 3% OV17 Chromosorb W(HP) 80/100 mesh column. A program from 140-150 °C, with an initial 2-3 minute hold before programming, with a FID detector and a Hewlett Packard HP 3394A Integrator was used. Helium was used as the carrier gas, with a flow rate of 30 ml min^{-1}. The column was calibrated using standards of trehalose, glucose and sucrose, which were resolved in initial GC injections.

The presence of amino sugars in culture supernatents of *N. commune* DRH1 was confirmed using thin layer chromatography with silica gel plates and a solvent system of propanol:water (6:1) v/v followed by the staining of the plates with ninhydrin. A replicate plate was sprayed with sulfuric acid and charred at 120 °C (data not shown).

2.12 Endoglycosidase Digestion

Cells were treated with detergent buffer as described, in section *2.7*, except with the omission of urea and Nonidet P-40. The detergent, MEGA 8 (Sigma), was included to prevent denaturation of the endoglycosidase by SDS (Haselbeck and Hösel 1988). Aliquots of the supernatant fraction were diluted 1:20 with an endoglycosidase digestion buffer (80 mM Tris-HCl, pH 6.8, 1.7% v/v β-mercaptoethanol, 1% w/v MEGA 8) prior to the addition of 5 units of peptide-N-glycosidase F (PGNase F; 25,000 units mg^{-1} protein, Boehringer Mannheim Biochemica, Indianapolis, IN) in a final reaction volume of 50 µl. Rehydration

fluids (see above) were denatured before the addition of the PGNase. The solutions, including replicate controls lacking the PGNase, were incubated at 37 °C, for 12 hours.

2.13 Glycoconjugate Detection

Glycoconjugates were detected on Immobilon P membrane blots using a method developed by O'Shannessy et al. (1987) with a detection kit based upon a digoxigenin hydrazide-antidigoxigenin alkaline phosphatase conjugate colorimetric assay. Reagents were obtained from Boehringer Mannheim Biochemica and were used generally following the manufacturer's specifications but in accordance with the precautions and suggestions discussed by Fairchild et al. (1991). Protein blots were washed in 50 ml of PBS (50 mM potassium phosphate, 150 mM NaCl, pH 6.5) for 10 to 15 minutes. It was important not to use TBS at this time, because of the interference of Tris with the digoxigenin labeling. Oxidation with periodate of the proteins was accomplished by incubating the blot in 10 mM sodium metaperiodate in 100 mM sodium acetate buffer, pH 5.5, for 20 minutes at room temperature. The blots were then washed three times for 10 minutes each with PBS. The proteins were labelled with DIG-succinyl-ε-amidocaproic acid dissolved in dimethylformamide (vial 3, Boehringer Mannheim Biochemica Kit) by diluting 1 µl of vial 3 in 5 ml of sodium acetate buffer, pH 5.5, for 1 hour at room temperature. In order to eliminate non-specific staining using this protocol, it was critical that the periodate and DIG

labeling incubation times were strictly followed. TBS was now used to wash the blots three times for 10 minutes each. If desired, the blots were stained at this time with 0.2% w/v Ponceau S in 3% w/v acetic acid in order to visualize proteins on blot. However, even if the protein of interest was not present in a high enough concentration to visualize with Ponceau S it was still detected with the glycan staining protocol. The blots were now incubated, at room temperature, for 30 minutes with blocking solution (dissolve 0.5 gm of Boehringer Mannheim blocking reagent in 100 ml TBS, pH 7.5, by heating to 50-60 °C for 1 hour). After washing the blots three times for 10 minutes each in 50 ml TBS, they were incubated with 10 µl of polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase (750 U ml^{-1}) in 10 ml of TBS for 1 hour. The washing of the blots three more times, for 10 minutes each in 50 ml TBS, was performed prior to the color development. Filters were immersed without shaking in the staining solution (10 ml of 100 mM Tris-HCl, pH 9.5, 37.5 µl of a 5% w/v 5-bromo-4-chloro-3-indolyl-phosphate solution in 100% v/v dimethylformamide, and added just before use 50 µl of a 10% w/v 4-nitroblue tetrazolium chloride in dimethylformamide), the development of a grey to black color was observed after 5 minutes (prolonged color development can lead to non-specific staining with this protocol). Rinsing of the blots several times with copious amounts of distilled water stopped the color reaction. Transferrin was used as a positive control, and creatinase as a negative control, for the glycoprotein stain (data not shown).

2.14 *Peptide Mapping and Sequence Analysis*

Individual Wsp polypeptides were excised from CBB stained SDS-PAGE gels and electroeluted using the electroelution chamber of the Isco model 1750 electrophoretic concentrator (Isco, Inc., Lincoln, NE), using a 3.5 kDa cut off dialysis membrane (Spectra/Por 3, Baxter Diagnostics Inc., IL). Gels used for this procedure were stained 15-20 minutes and destained for approximately 1 hour. Electroelution was performed for 2-4 hours (with a cycling ice bath) in 200 mM glycine and 25 mM Tris-HCl, pH 8.3, at a constant current of 75 mA. Protein samples were dialyzed exhaustively against water using 3.5 kDa cut-off dialysis membrane. Peptide maps were obtained by using papain, chymotrypsin or *Staphylococcus* V8 protease (Sigma), under the conditions specified by Cleveland et al. (1977). After blotting to Immobilon P membrane the digestion products were visualized through immunodetection or by staining with CBB. Wsp polypeptides were treated with either endoproteinase Glu-C or endoproteinase Lys-C under the conditions specified by the manufacturer (Boehringer Mannheim). Treatment of Wsp polypeptides with cyanogen bromide was under a nitrogen gas phase, in the dark (Gross 1967; see Appendix 4 for all protein digest detailed protocols). Wsp polypeptides and peptide fragments were blotted to Immobilon P membrane (Matsudaira 1987) and subjected to automated Edman degradation using an Applied Biosystems 477A Protein Sequencer, at the Virginia Tech Protein Sequencing Facility. Cysteine residues were not derivatized prior to analysis.

2.15 *Microheterogenity of Wsp Polypeptides*

Individual native and denatured Wsp peptides were incubated at 37 °C with calf intestinal alkaline phosphatase (Sigma) in the buffer specified by Maniatis et al. (1989), for 3 hours. Samples were recovered at different time points and the phosphatase was inactivated through the addition of ethylenediaminetetraacetic acid (EDTA). Please refer to Appendix 4 for protein digest detailed protocol.

2.16 Enzyme Assays

The capacity of native extracts of Wsp to hydrolyze 4-O-methyl-D-glucurono-D-xylan -remazol brilliant blue R (RBB xylan, Sigma cat no. M-5019) was determined. Activities were quantitated using xylanase (1,4-β-D-xylanxylanohydrolase; EC 3.2.1.8) from *Trichoderma viride* (Sigma cat. no. X3876; 225 units mg protein^{-1}). One unit of this xylanase liberates 1 μmole of reducing sugar (xylose equivalent) from poly [β-D-xylopyranose (1→4)] min^{-1}, at pH 4.5, 30 °C. The determination of xylanase activity followed the protocol of Biely et al. (1985). One part native extract and one part RBB xylan substrate (5.75 mg ml^{-1} in 50 mM sodium acetate buffer, pH 5.4) were combined and allowed to incubate at room temperature (5 to 6 hours of incubation proved optimal for the native extract). The assay reaction was stopped by the addition of two parts of ice cold 95% ethanol. Once the assay

was stopped the reaction tubes were placed on ice and allowed to set undisturbed for 2 hours. The tubes were then centrifuged at 12,000x g for 5 minutes, the supernatant was collected and its absorbance was determined at 595 nm using a Shimadzu spectrophotometer Model UV/60U. Blanks for the above readings consisted of the dye-xylan solution mixed with the 95% ethanol prior to the addition of the native extract. Positive controls for the above assays contained 0.250 units of activity of a xylanase isolated from *Trichoderma viride*.

In order to determine the inherent absorbance of the "free" dyed-xylose generated by the xylanase activity the following experiment was performed. Multiple blanks consisting of only dyed-xylan substrate and 95% ethanol were placed on ice and centrifuged to collect any "free" xylose liberated from the substrate without enzymatic cleavage of the substrate. After the absorbance of the "free" dyed-xylose was determined (595 nm), an aliquot of the native extract was added to the supernatant, allowed to sit on ice for 1 hour and then centrifuged to re-collect the supernatant. The absorbance of the resulting supernatant was determined (595 nm).

Xylosidase activity was tested using o-nitrophenyl β-D-xylopyranoside (ONPX; Sigma cat. no. N3629) as substrate and β-xylosidase (exo-1,4-β-D-xylosidase; EC 3.2.1.37) from *Aspergillus niger* (Sigma cat. no. X 5375) as the positive control. The determination of xylosidase activity followed a modification of that described in Lee et al. (1987). Using the same native extract described above, 100 µl of extract was mixed with 900 µl of ONPX

40 mM OPNX in 50 mM citrate buffer, pH 4.5). Assay was stopped with the addition of 1 ml sodium carbonate solution. Blanks consisted of the substrate ONPX mixed with the sodium carbonate solution prior to the addition of the extract. Assay vials were placed uncapped in the dark until color change was detected.

Incubation of native gels with sugar phosphate substrates [D-fructose 1,6 diphosphate (sodium, trisodium and tetrasodium salt), pyridoxal 5-phosphate, α-D-glucose 1,6 diphosphate, uridine-5'-diphosphate, uridine-5'-diphosphate N-acetyl galactosamine, O-phospho-D-L-threonine, and O-phospho-D-L-serine (all used at 5 mM)] and the detection of the release of inorganic phosphate with malachite green followed the protocols of Zlotnick and Gottlieb (1986).

3. Results

3.1 Macroscopic and Microscopic Features of the Glycan

Colonies of *Nostoc commune* become visually conspicuous in terrestrial environments from the Tropics to the polar regions. Analysis of materials collected from all of the geographic locations (Fig. 1) indicated an overall consistency in their structural properties, composition and appearance. The analysis of desiccated colonies using light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) showed that cells can retain their structural integrity for decades in the air-dried state (see Fig. 3 and Table 1). Typically, a colony with a dry weight of 0.1 gm will become swollen, with a wet weight of approximately 20 gm, within 1 hour of rehydration. The specific data presented here are largely for materials of *N. commune* CHEN but can be considered representative of all the materials unless stated otherwise. Desiccated colonies of *N. commune* collected *in situ* appear black and they have the consistency of brittle parchment (Fig. 4A). In localities were the colonies adhere to soils, the lower face of the colonies i.e. the side in contact with the soil, tends to have a greener coloration and may retain water for longer periods than the exposed surface. In thick sections a yellow coloration is apparent that lends a specific banding pattern throughout the polymer, with the bands horizontal to the surface. This yellow-brown coloration is most intense at the exposed surface of the colony (Fig. 4E).

Table 1

Desiccated materials of N. commune and cultures of cyanobacteria

Desiccated materials were dry at the time of collection and were stored dry, in the dark until the time of analysis.

Acronymn or strain designation	Country	Locality	Year collected	Years dry at analysis
Desiccated materials:				
N. commune[g] CHEN	China	Hunan Province	1981	10-12
N. commune[h] HUN1	China	Hunan Province	1982	11
N. commune[i] HUN2	China	Hunan Province	1986	7
N. commune[a] TEN	USA	Knoxville, TN	1988	3
N. commune[d] TAG	Germany	Bodensee	1988	2
N. commune[n] 8122	Seychelles	Aldabra Atoll	1974	18
N. commune[j] ANT	Antarctica	Ross Ice Shelf	1979	11
N. commune[k] 776D	Seychelles	Aldabra Atoll	1974	18
N. commune[l] 779D	Seychelles	Aldabra Atoll	1974	18
N. commune var. *flagelliforme*[m] 857D	Seychelles	Aldabra Atoll	1974	18
N. commune[b] BBC	USA	Blacksburg, VA	1990	2
N. commune[e] BER	Germany	Bodensee	1988	2
N. commune[f] MAL	England	Malham Tarn	1989	3
N. commune[c] VA39	USA	Giles County, VA	1939	55

Cultures	notes/ref.
Cultures of cyanobacteria:	
N. commune UTEX 584	Potts and Bowman, 1985
N. commune DRH1	Isolate of *N. commune* CHEN
Anabaena sp. PCC 7118	Obtained from ATCC (no. 27892), Het$^-$
Anabaena sp. PCC 7119	Obtained from ATCC (no. 29151)
Anabaena sp. PCC 7120	Obtained from J. Elhai

[a-n] See Figures 1 and 2

As documented below, the yellow coloration is attributed to a UV-absorbing pigment, scytonemin. A 20 μm-thick layer encompasses the outer periphery of the thallus and this layer has a microstructure that includes fibrils perpendicular to the surface of the colony (Fig. 4H and 8D). The filaments of *N. commune* are embedded within, and distributed throughout, a dense glycan sheath (Figs. 4B, C, E, F, G and H). An envelope layer, with different structural and staining characteristics to the bulk polymer, surrounds each filament (Figs. 4B, C and G). PAS stain gave an intense reaction associated with heterocysts but not with the envelope layer that is constricted at heterocysts (Fig. 4G). In materials collected from Aldabra Atoll, some of the filaments are encased in a spherical, calcified deposit that appears laminated (Fig. 4F). These "oncolite"-like structures aggregate in clumps at the surface of colonies. During rehydration, the sheath takes on a vesicular, porous and honey comb-like appearance (Fig. 4C). After full rehydration, and then long-term incubation, numerous spherical colonies 1 mm to 1 cm in diameter, bud from the thallus (Fig. 4D and 7E). These spheres or "pearls," retain the yellow-brown coloration of the thallus, and develop conspicuous "stretch marks" if they start to dry (see arrow, Fig. 4D) . Upon complete desiccation pearls form flat sheets that swell upon rewetting to restore the spherical shape of the turgid pearl (data not shown).

3.2 Ultrastructural Analysis of the Glycan

In the desiccated thallus the filaments are separated from one another (Figs. 4B and E) and when examined in SEM appear to reside in narrow "tunnels" that permeate the glycan (Fig. 5). These "tunnels" have ribbed extensions when observed in transverse section in the SEM (Fig. 5B), are clearly cylindrical when observed in a plane parallel to the tunnel (Fig. 5D) and appear to be empty (Figs. 5A, B and D). In some materials these "tunnels" were less distinct or almost absent. Here, the glycan sheath at the periphery of the filaments appeared heavily pitted with a microporous structure that followed the contour of the "tunnels" observed in other materials (Fig. 5C). Magnesium, calcium, silicon, phosphorus and sulfur predominated the EDX spectrum of all materials that were studied (Fig. 5E). Relative to the glycan, cells were enriched in sulfur and phosphorus. The most obvious result of these analyses was the conspicuous accumulation of silicon and calcium in the external layer (Fig. 5E; compare with Figs. 4E and H).

Light microscopy and TEM resolved the immediate environment of each cell in desiccated colonies of field material as a homogenous envelope that was only weakly stained (Fig. 4B; Figs. 6A-B). This envelope layer, S_1, followed the contours of the individual cells and was constricted at their crosswalls (Figs. 6A and B). The envelope layer was appressed to the glycan where a dense staining membrane-like layer could be discerned, *IL* (Figs. 6B and C). The glycan, but not the envelope layer, was heavily labelled with antibodies prepared using a purified extracellular carbohydrate preparation (Fig. 6A). The pattern of labelling of

the glycan suggested that the apparently homogenous fibrous structure (Fig. 6B) was immunologically heterogeneous (Fig. 6A). When rehydrated cells of *N. commune* CHEN pearls or *N. commune* DRH1 were prepared under identical conditions of fixation, dehydration, infiltration and critical point drying, the envelope layer and glycan showed a different ultrastructure in comparison to the desiccated materials. Following short-term rehydration (30 min) portions of the envelope layer had the capacity to be post-stained (S_2). These areas appeared to consist of fibrils parallel to both the stained membrane interface layer (*IL*) and the contiguous fibrils of the glycan (Fig. 6C). After longer periods of rehydration (60 min), the developing layer, S_2, became more reticulate and stained with a greater intensity (Fig. 6D). In fully rehydrated material an envelope layer was either absent, or it was hardly discernible (Fig. 6E). Here, the glycan appeared to make contact with the cells (Fig. 6E, arrow; inset). The glycan sheath of pearls was more heterogeneous than that of desiccated materials (Fig. 6F). Immunogold-labelling of the glycan of pearls, with anti-sheath carbohydrate antibodies, revealed conspicuous accumulations of electron dense cross-reactive material while the bulk of the sheath had no accumulations of gold particles (Fig. 6F). A carbohydrate analysis was performed on the purified glycan that was used to generate the sheath antibody, the following sugars were present in pmoles (μg): glucosamine 22.9 (5.04), galactosamine 7.5 (1.65), glucose 110 (19.8), galactose 43.5 (7.8), and mannose 9.5 (1.7), which yields a ratio of 3:1:12:5:1 of the sugars, respectively.

The Ca/Si-rich external layer was conspicuous in cross sections of pearls (Figs. 7A,

B and C; compare Figs. 4H and 7B). Sheath layers comparable to those associated with cells of desiccated field material were difficult to discern (Fig. 7D). Although the "tunnel"-like structures (described above) were absent, Fig. 7D shows that filaments were encased in a layer that has different structural properties to the bulk glycan. Analysis through light microscopy also indicated different staining properties of this layer. Filaments, such as those with the appearance of the one in Fig. 7D, tend to occupy the outer portions of the colony where they form smaller pearls and packets (Fig. 7E), while non-encased filaments are present in the central portions of the pearl (Fig. 7F). During growth, minute pearls bud-off from the parent pearl at the periphery of the latter (Fig. 7E). These buds originate as encased filaments immediately below the surface of the pearl and at this stage an external layer is already apparent at the periphery of these small packets of filaments (*P*, Fig. 7E). The staining characteristics of the material in and surrounding these packets differ from those of the parent glycan (sheath) matrix (Fig. 7D). For example, with Alcian blue at pH 2.5 the small packets stain an intense dark blue in comparison to the light blue-green color of the surrounding glycan (data not shown).

The general features of the ultrastructure of the cells in desiccated colonies and pearls are similar and the data presented in Fig. 8 for pearls can be considered representative of both materials (with the qualifications discussed below). In pearls short filaments are often observed, e.g. Fig. 8F, and in some cases may consist only of one vegetative cell and a single heterocyst (data not shown). Heterocysts may be apical (Fig. 8A) or intercalary (Fig. 7F).

Honey comb configurations were noted at the poles of mature heterocysts (*hc*, Fig. 8B) and both proheterocycts and mature heterocysts had a non-staining layer characteristic of these differentiated cells (Figs. 8B and C). Filaments present in the central regions of pearls had a vesiculate nature in comparison to those present towards the periphery of the colonies (Figs. 8E and F). A feature of desiccated materials collected *in situ*, and of pearls grown under laboratory conditions, is that the interior of the colony is devoid of any other microorganism save for the filaments of *N. commune* (Fig. 8D). In addition we have never observed cyanophages or other bacteriophage-like structures within the colonies. As such there is a considerable layer of glycan that separates cells from the atmosphere (Fig. 8D), and this constitutes a barrier that appears to remain unbroken even during the budding process described above (Fig. 7E). In this context it can be noted that the exterior surface of the colonies is colonized by populations of microorganisms that vary in magnitude, and diversity, depending on the materials studied (Fig. 8G). Nevertheless, these organisms never penetrate the barrier of the silicon-rich external layer (Fig. 8D; note fibrillar structure of this layer and compare with Fig. 4H).

3.3 Features of N. commune Strains UTEX 584 and DRH1 in Liquid Culture

The life cycle of *N. commune* strain UTEX 584 (Hill and Potts, in preparation) includes one stage that results from the encasement of growing filaments in an envelope layer (Fig. 9A). The heterocysts in the filaments from which these structures arise are unable to

divide and remain between the packets formed by the encased filaments where the envelope is constricted. The filaments in these packets do, however, contain newly differentiated heterocysts. These structures are virtually identical to those observed in field materials of *N. commune* (compare Figs. 9A, 4B and 4G). A thickened envelope layer also surrounds single filaments of *N. commune* strain DRH1 (Fig. 9B) and these ultimately generate small packets wherein the filaments are encased (Fig. 9C).

3.4 Chemical and Elemental Analysis of the Glycan

The glycan of field material has a firm, rubbery consistency when wet, and is brittle when dry. In liquid cultures of *N. commune* DRH1 the glycan is dispersed and lends a viscous property to the cell-free liquid medium. In order of abundance the sugars detected in the purified hydrolysate of the glycan were glucose, N-acetylglucosamine, glucosamine, mannose, galactosamine and galactose (Fig. 10). Fucose was not detected in any sample. The quantitative amounts of the sugars in sample C and D (the amount of material used in the extract of C was approximately twice that of D) illustrates the heterogeneity of the field material. However, the ratio of the sugars with respect to each other is what is important to note. The ratios for these sugars in the glycan of desiccated field materials were the same for both samples: 3.1 : 1.4 : 1 : 0.1 : 0.06 : 0. Galactose was detected only in the small molecular weight (less than 12 kDa) fraction of the glycan from *N. commune* strain DRH1 and the ratio of sugars respectively was 1.3 : 0.5 : 1 : 0.2 : 0.3 : 0.1. The concentrations of glucosamine

and N-acetylgalactosamine in this low molecular mass fraction were almost two orders of magnitude less than in the fraction that was retained by a 12 kDa cut-off membrane. The ratio of sugars for the large molecular weight (greater than 12 kDa) fraction was 1.7 : 1.5 : 1 : 0.01 : 0 : 0, respectively. The concentrations of galactosamine and mannose were higher in the desiccated glycan than in the samples from liquid grown *N. commune* strain DRH1. In each sample a hydrolysis product, identified as sialic acid, was resolved during the carbohydrate analysis. Amounts of this compound in the field material were approximately 15 times greater than those found in the high molecular weight fraction of the glycan from *N. commune* strain DRH1 (2 pmoles versus 0.15 pmoles per μg dry wt, respectively). Only trace amounts (<0.03 pmoles) of sialic acid were detected in the small molecular weight fraction of the glycan from *N. commune* strain DRH1. Whether this compound is sialic acid and whether it is a true component of the glycan or attributable to the epiphytes on the surface of the colonies is still under investigation. Positive identification of this component must await GC-MS analysis.

In order of abundance trehalose, sucrose and glucose were detected in the cells following solvent extraction. The concentrations of each were greatest in desiccated material and decreased with time of cell rehydration. Initial measurements of trehalose in a desiccated sample were approximately 1 mg trehalose gm^{-1} dry weight of sample. After 30 minutes of rehydration the level of trehalose had dropped to 0.3 mg and by 6 hours the level was only one-tenth that present in the desiccated sample (this level remained constant through a further

48 hours of rehydration). The levels of sucrose followed the same trend as that for described for trehalose. The initial concentration of sucrose was approximately 0.8 mg gm^{-1} dry weight of sample. After 30 minutes the level was <0.3 mg and at 6 hours the level was less than one-tenth the original quantity (once again this level remained constant through 48 hours of rehydration). These data suggest turnover of trehalose and sucrose occurs during rehydration of the *N. commune* desiccated field material. The levels of glucose gradually increased during the time the levels of the disaccharides trehalose and sucrose decreased. Initial levels of glucose in the desiccated sample were less than one-fortieth the concentration of sucrose and trehalose (approximately 0.025 mg glucose gm^{-1} dry weight of sample extracted). After 30 minutes of rehydration the levels of glucose had risen four fold (highest concentration detected for glucose levels), then decreased during subsequent incubation and was barely detectable after 12 hours.

Aqueous extracts of field material were obtained, following different periods of rehydration, in order to gain some insight into the fluxes of dissolved salts that may occur *in situ* in response to wetting and drying. Potassium and calcium constituted the most abundant elements in aqueous extracts of *N. commune* CHEN obtained during a 6 hour period of wetting (Fig. 11A). Following the 6 hour period of aqueous extraction the cells were then recovered and extracted with 80% v/v ethanol for 60 minutes, a process that lead to leakage of cell contents but without disruption of the colony. The concentrations of elements in this case were reduced relative to those determined for the aqueous extract (Fig. 11B). The

elemental analysis of rehydration fluids of materials, from various geographic locations, were compared following 24 hours of extraction and were strikingly similar (Fig. 12). The extracts were substantially increased in K, Ca, Mg and Na relative to the quantities measured at 6 hours of wetting (compare Fig. 11A)

3.5 Wsp is Secreted Beyond the Outer Cell Membrane

Wsp polypeptides were distributed throughout the glycan (Figs. 3D and E). Very similar patterns of immunolabelling were obtained with rabbit antibodies generated against a preparation of three (33, 37, 39 kDa) purified Wsp polypeptides, and with mouse antibodies generated against the single purified 39-kDa Wsp polypeptide (data not shown).

3.6 Protein Composition of the Glycan

Wsp was extracted with both aqueous and non-polar solvent systems (Fig. 13, lanes 1 and 2), and was present in all materials studied save for two samples from Aldabra Atoll, which were collected in pools rather than on a terrestrial surface (Fig. 2). If present, Wsp represented the most abundant soluble protein. The abundance of Wsp in aqueous extracts of materials, which contained the protein, was estimated as 65 to 85% of the total protein in the aqueous extract (Fig. 2).

Experiments using light microscopy identified a conspicuous reaction of heterocysts with PAS, a stain used in glycoprotein detection (see Fig. 4G). Two polypeptides, that

showed a positive glycan reaction, were detected in aqueous extracts of desiccated samples from six geographic locations (Fig. 14). The reactions with the polypeptides were apparent within 5 minutes of the initiation of the color reaction, which was terminated after this time. The estimated molecular masses of the polypeptides were 75 kDa and 110 kDa, respectively. The most conspicuous reaction was associated with the 75 kDa polypeptide.

3.7 Wsp is Abundant in the Extracellular Glycan

Wsp was released from desiccated colonies by rehydrating them but without any form of physical disruption (Figs. 15A-D). In response to the first wetting the amounts of Wsp released from the colonies increased with time of rehydration (Figs 15A and B) and the use of protease inhibitors enhanced the recovery of Wsp (Fig. 15B). A similar, less-pronounced trend was evident for the second rehydration although some higher molecular mass cross-reactive bands were observed in extracts that lacked inhibitors (Figs. 15C and D). Signals from the 33, 37, and 39 kDa polypeptides were strongest following approximately 1hour of rehydration, irrespective of the presence or absence of inhibitors (Figs. 15A and B).

Rehydration fluids in which Wsp was abundant showed no UV-induced fluorescence in the visible region and they had a negligible spectrum in the 450-650 nm region confirming that soluble phycobiliproteins were absent (Fig. 16). The peak in absorbance at 312 nm was due to a secreted water-soluble UV-A/B absorbing pigment (Scherer, et al 1988; see below). Phycobiliproteins, as intracellular protein markers, were obtained only following cell

breakage. After cell breakage the corresponding samples were all found to have very similar concentrations of Wsp in the cytosolic fractions (data not shown). Because Wsp polypeptides may still have been present in the sheath despite multiple rounds of aqueous extraction (see Fig. 3E) it is difficult to assess with certainty the intracellular concentration of Wsp (in relation to total Wsp and/or total soluble protein). The amounts of protein released from colonies were also difficult to quantify in view of the presence of components that interferred with the dye-binding assay. However, the proteins which were resolved in the inset of Fig. 16 (measured as 10 μg total protein) represent 0.003% of the total amount obtained after the rehydration of 20 g dry weight of colonies. A similar value was estimated in a second trial.

3.8 Peptide Mapping of Wsp

The apparent molecular masses of the 33, 37 and 39 kDa polypeptides remained unchanged following treatment with cyanogen bromide for either 4 hours or overnight, at room temperature (data not presented). The peptide maps of the 39 kDa and 37 kDa polypeptides after treatment with *Staphylococcus* V8 protease or chymotrypsin shared many common bands (Fig. 17 lanes A and B, D and E). The maps of all three polypeptides were very similar following treatment with chymotrypsin (Fig. 17 lanes D-F).

3.9 Protein Sequence Analysis of Wsp

In addition to Wsp polypeptides, a protein with a molecular mass of 68 kDa, that did not cross react with Wsp antiserum, was conspicuous in concentrated rehydration fluids (see Fig. 4). This 68-kDa protein was the second most abundant protein in aqueous extracts of *N. commune* CHEN. The N-terminal sequence of this protein was AFIFGTISPNNLSGTSGNSGIVGSA. No obvious match of this N-terminal sequence was obtained following searches of the Swiss-Prot data base. The highest scores in the search (with homology values of between 36% and 44%) were with carbohydrate -modifying enzymes including: fructose-1, 6-bisphosphatase, glucan endo-1, 3-beta-glucosidase, mannose 6-phosphate isomerase, UDP-N-acetylglucosamine-dolichol-phosphate transferase, cyclomaltodextrin glucanotransferase and alpha-amylase.

The amino-terminal sequences of the three Wsp polypeptides (Fig. 17 lane I) were identical: Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln-X-Ile-Gln-Asn-Pro-Ser-Asn--Pro-Ser-Asn-Gly-Lys-Gln. Residue 11 could not be identified in any of the Wsp samples in multiple trials (Table 2). The amino-terminal sequence of the 68-kDa polypeptide showed no correspondence to the sequences of the Wsp polypeptides. Homology searches using the amino-terminal sequences of Wsp and the 68-kDa protein, revealed similarities to carbohydrate-modifying enzymes. The sequence at the amino-terminus of a 25 kDa proteolytic fragment of Wsp was Glu-Ala-Arg-Val-Thr-Gly-Pro-Thr-Thr-Pro-Ile-Asp (Fig. 17, lane H) and showed 66% sequence identity with a portion of the sequence of a

Table 2

Protein sequence analysis of the multiple bands of protein which represent Wsp.

Protein	Sequence
68 kDa	Ala-Phe-Ile-Phe-Gly-Thr-Ile-Ser-Pro-Asn-Asn-Leu-Ser-Gly-Thr-Ser-Gly-Asn-Ser-Gly-Ile-Val-Gly-Ser-Ala
Wsp 39 kDa	Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln- X -Ile-Gln-Asn-Pro-Ser-Asn-Gly-Lys-Gln
Wsp 37 kDa	Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln- X -Ile-Gln-Asn- X - X -Asn-Gly-Lys-Gln
Wsp 33 kDa	Ala-Leu-Tyr-Gly-Tyr-Thr-Ile-Gly-Glu-Gln- X -Ile- X -Asn-Pro-Ser- X -Gly- X -Gln

β-xylosidase (locus XYNB-CALSA in the Swiss-Prot 23 sequence library; Lüthi et al. 1990). Subsequently, quantitative Western analyses identified a strong antigenic response between the Wsp antiserum and a number of such enzymes including glycosidase F and β-D-galactoside galactohydrolase (pre-immune serum showed no cross reactions with either of these proteins, data not shown).

3.10 Enzyme Assays

In view of the results of homology searches native extracts of Wsp were tested for activity with a range of carbohydrate substrates. Rehydration fluids contained a weak xylosidase activity that was detected only after prolonged incubation (96 hours and greater) with o-nitrophenyl β-D-xylopyranoside as the substrate (the substrate showed no background hydrolysis during this time of incubation in control experiments).

Rehydration fluids hydrolysed RBB-xylan and the results from a typical experiment are presented as Fig. 18. The addition of Wsp antiserum to reaction mixtures caused a 10-fold reduction in xylanase activity while the Wsp antiserum had no inhibitory effect on the activity of a purified xylanase (Fig. 18; inset). In comparison, cell-free supernatants of liquid cultures of *Nostoc commune* DRH1 (approximately 0.1 gm dry wt l^{-1}) had low xylanase activities - approximately 0.01 μmol xylose released min^{-1} ml^{-1}. However excessive amounts of viscous polysaccharides synthesized by the cultures bound the products of the colorimetric assay and made it difficult to make reliable measurements. The amounts of Wsp secreted by

this culture after multiple transfers in liquid culture were detectable only through Western blotting.

Rehydration fluids did not hydrolyse p-nitrophenyl β-D-galactoside, 5-bromo 4-chloro 3-indolyl galactoside or 5-bromo 4-chloro 3-indolyl glucoside, nor did they show chitinase activity (using Remazol Brilliant Violet-poly-N-acetylglucosamine as the substrate, Sigma C 3020, and the method of Wirth and Wolf 1990; data not shown).

Malachite green staining revealed no release of inorganic phosphate in zymograms following incubations of Wsp preparations with either fructose-1,6-bisphosphate, uridine 5'-diphospho N-acetylglucosamine, uridine 5'-diphospho N-acetylgalactosamine or alpha-D-glucose 1,6 diphosphate (data not presented).

3.11 Association of Wsp with UV-A/B-absorbing Pigments

Rehydration fluids were subjected to analytical FPLC to further analyse interactions between Wsp and the UV-absorbing pigments. Two fractions of Wsp were identified through ionic exchange chromatography. One fraction failed to bind to Mono Q resin while the second fraction remained bound to the resin and was eluted only with salt concentrations of 1M KCl or higher. Fraction one was colorless, fraction two was pale yellow at pH 7.4, and both fractions contained UV-absorbing pigments (Fig. 19). In fraction one the pigments had a narrow absorption spectrum with a maximum at 312 nm; in fraction two the spectrum was broad and extended to 450 nm. We will document elsewhere that the pigments in fraction two

have the characteristics of scytonemin (Garcia-Pichel and Castenholz 1991). Pigments with the spectral characteristics of those in fraction one were eluted from the column at approximately 150 mM KCl, together with trace amounts of Wsp (Fig. 19). These data indicated that highly-charged components were present in the rehydration fluids. There was also some suggestion that the components of the rehydration fluids form high molecular mass complexes because native Wsp failed to traverse a 100 kDa cut-off dialysis membrane, and some material that cross reacted with the antiserum was resolved as a smear, of high apparent molecular mass, that migrated slower than Wsp polypeptides during SDS-PAGE (data not shown).

To study the sizes of these complexes rehydration fluids were analysed using gel filtration with simultaneous monitoring of the absorbance at 280nm and 312nm in the presence and absence of kosmotropic and chaotropic agents. Under native conditions (20mM Tris-HCl pH 7.4, 50 mM KCl) Wsp eluted in two fractions from a Superose 12 column with an apparent molecular mass between 35 and 45 kDa. Western blotting resolved two Wsp polypeptides in each fraction (Fig. 20; inset). Silver staining detected no other proteins in those fractions that contained Wsp (data not shown). These Wsp fractions contained a peak in absorbance at 312 nm with an A_{280}:A_{312} ratio of approximately 3:2. The principal peak in absorbance at 312 nm (apparent Mr = approximately 11 kDa) was present in a fraction that contained no Wsp, nor any proteins that could be detected through silver staining, and was attributed to free UV-absorbing pigments. The removal of salt, KCl, from the column buffer

caused a marked change in the elution profile of Wsp and the UV-absorbing pigments and both components underwent an apparent increase in molecular mass (Fig. 21). Wsp was present in fractions that contained one of the four principal peaks in absorbance at 312 nm and eluted with molecular masses between 60 and 80 kDa (Fig. 21). In these fractions the A_{280}:A_{312} ratio was approximately 1:1. Fractions that contained the other three peaks in absorbance at 312 nm contained no proteins, as judged by silver staining. The principal peak in absorbance at 312 nm (A_{280}:A_{312} = approximately 1:5) contained material that now eluted with a molecular mass of approximately 25 kDa (Fig. 21). These data were suggestive of ionic interactions between Wsp and the UV-absorbing pigments.

The elution profile of rehydration fluid in 50 mM KSCN, 20mM Tris-HCl, pH 7.4 was very similar to that obtained with 50mM KCl, 20 mM Tris-HCl, pH 7.4 but under these two conditions the major peak in absorbance at 312 nm appeared in fractions that eluted at 15.2 ml and 16.2 ml, respectively (data not shown). The elution profile, and distribution of Wsp, in the presence of 20 mM Tris-HCl, pH 7.4, 6.6 M urea, was very similar to that obtained under native conditions in the absence of salt (data not shown). These data confirmed that hydrophobic interactions may be of minor importance in the associations of Wsp and the UV-A/B-absorbing pigments.

3.12 Pigment Composition of the Glycan

Desiccated materials of *N. commune* TEN that contained conspicuous amounts of a yellow pigment (Fig. 4E) were subjected to extraction using either aqueous or detergent-soluble solvent systems. As discussed earlier, the aqueous extract contained a water soluble UV-A/B-absorbing pigment and the water stress protein, Wsp, as observed for *N. commune* CHEN (compare Figs. 13A and B with Figs. 16 and 19). Inclusion of NP-40 in the extraction buffer released the yellow pigment from the colonies as well as increased amounts of protein, notably phycobiliproteins that serve as markers of cell lysis (Fig. 22). The spectral scan of the detergent extract was enhanced in the region where the UV-absorbing pigment scytonemin absorbs (435 and 484 nm) Fig. 22A. Further analysis of the fractions that contained this pigment identified yellow (absorption max. 435 nm) and pink (absorption max. 435 nm and 493 nm) components. Through the use of TLC and spectrophotometric analysis, these pigments were identified as the oxidized (435 nm) and reduced (493 nm) forms of scytonemin, respectively (data not presented).

3.13 Co-purification of Wsp and Xylanase

The xylanase activity present in rehydration fluids (Fig. 18) eluted from a Superose 12 column in the presence of salt and was highest in one of the two fractions that contained Wsp (Fig. 20). In the second of these the xylanase activity was approximately 50% lower and xylanase activity was also found in fractions that contained no Wsp. Xylanase activites were

lower when fractions were eluted in the absence of salt and the highest of these activites were present in fractions that contained no Wsp, nor any other proteins as judged from silver staining (Fig. 21).

The elution profiles presented in Figs. 19, 20 and 21 are representative of those obtained in multiple trials with different batches of rehydration fluids.

3.14 Glycosylation Status of Wsp

Wsp preparations were resolved through SDS-PAGE and individual polypeptides were purified through electroelution. The polypeptides were freed of Coomassie blue dye through phenylsepharose chromatography and were found to have no absorbance at 312 nm (data not shown). Analysis of electroeluted Wsp polypeptides, of Wsp fractions obtained without cell disruption (rehydration fluids) and of Wsp preparations obtained through solubilization of cell wall fractions with detergents provided no evidence for glycosylation of Wsp using the glycoconjugate detection methods described in Materials and Methods. The apparent molecular masses of the proteins in these extracts remained unchanged following treatment with endoglycosidase F (data not presented).

3.15 Microheterogeneity of Wsp

Amino acid analysis of the three Wsp polypeptides indicated a combined serine/threonine content of approximately 20% in each one (Scherer and Potts 1989), and the

amino terminal sequence data presented here confirm the presence of tyrosine. As phosphorylation could account for the heterogeneity of Wsp in 2D gels, and in view of the recent identification of a secreted protein tyrosine/serine phosphatase in *N. commune* UTEX 584 (Potts et al. 1993), the mobilities of the Wsp polypeptides before, and at different times after, treatment with calf intestinal alkaline phosphatase using both native and SDS-PAGE electrophoresis were compared. These experiments provided no evidence for phosphorylation of Wsp, nor was free phosphate detected after termination of the assays (data not presented).

3.16 Presence of Wsp in Filamentous Cyanobacteria

Proteins that showed a cross reaction with the Wsp antiserum, including some in the size range 30 to 40 kDa, were detected in total cell extracts of cultures of *Nostoc commune* UTEX 584 and *Anabaena* spp. strains PCC 7118, 7119 and 7120 (data not presented). The cell-free culture fluids of these strains gave no positive signals in Western analysis. The cells of *N. commune* DRH1, and the cell-free, gel-like supernatant from cultures of this strain, gave a strong reaction in Western analysis (data not presented).

3.17 Cyanobacterial Trehalase

In view of the abundance of Wsp in aqueous extracts, its possible role as a carbohydrate modifying enzyme, and the immunoreactivity of Wsp serum to other carbohydrate-modifying enzymes, we tested whether trehalose antibodies showed any non-

specific cross-reaction with Wsp. No such cross reactions were observed (Fig. 23A). Two polypeptides in total cell extracts from *N. commune* CHEN, with molecular masses of 70 kDa and 106 kDa, cross reacted with the *E. coli* trehalase antibodies (Fig. 23B). The reactions with the 70 kDa protein became stronger as the time of rewetting of the cells prior to protein extraction was increased. The 106 kDa protein was first noticeable following 12 hours of rehydration and was most obvious after 48 hours of rewetting. Cyanobacterial trehalase was first noticeable in extracts following 30 minutes of rehydration, a time during which the sugar trehalose decreased in concentration in ethanol extracts of the rehydrated sheath, and increased in abundance with increased time of rehydration.

Trehalose antibodies did not cross react with any proteins present in the aqueous extracts (data not present). Therefore, while the glycoproteins and the trehalase cross-reactive proteins are of very similar size they cannot be the same protein.

Figure Legends

Key to figures: *H,* heterocyst; *tH,* terminal heterocyst; *F,* filament; S_1 , S_2 , S_3, differential layers of sheath; *S,* sheath; *O,* oncolite-like deposit; *V,* vegetative cell; *CM,* cell membrane; *PL,* peptidoglycan layer; *OM,* outer membrane; *CG,* cyanophycin granule; *C,* carboxysome; *PP,* polyphosphate granule; *PM,* polymorphic body; *P,* pearl bud;

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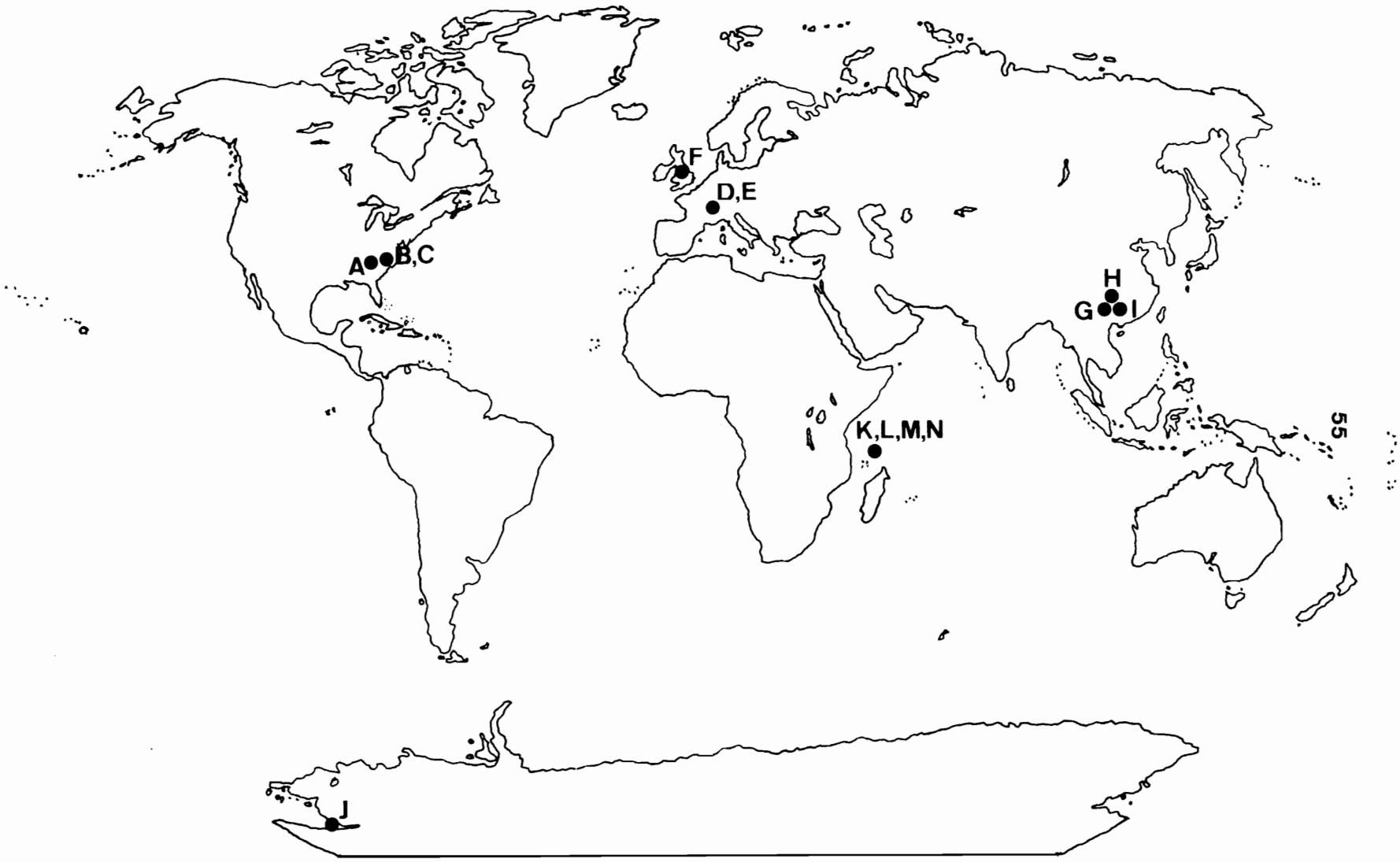
Figure 1. ***Nostoc commune* is cosmopolitan.** Refer to Table 1 for acronym descriptions. **A:** TEN, **B:** BBC, **C:** VA39, sample from USA, Giles County, VA, 1939, **D:** TAG, **E:** BER, **F:** MAL, **G:** CHEN, **H:** HUN1, **I:** HUN2, **J:** ANT, **K:** 776D, **L:** 779D, **M:** 857D, and **N:** 8122.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55

Figure 2. **WSP in aqueous extracts of *Nostoc commune* from various geographic locations.** Labeling of lanes corresponds to nomenclature used in figure 1. Western blot of aliquots of aqueous extracts, using the Wsp antibody. Approximately 1.5 μg of protein from the aqueous extract is present in each lane.

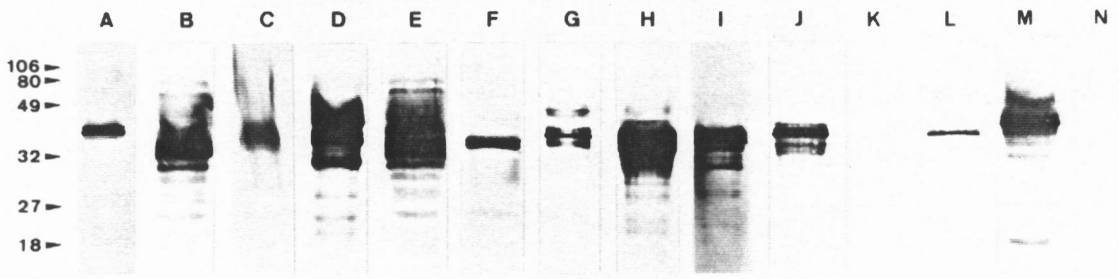
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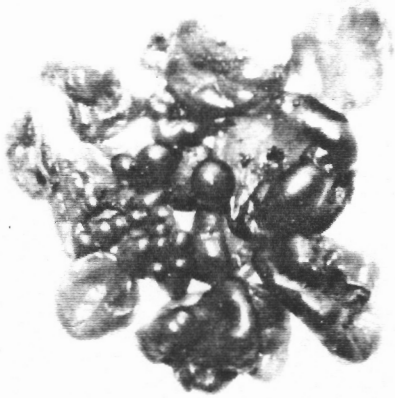
Figure 3. **Structure of the *N. commune* colony and immunolocalization of Wsp**
A: Rehydrated colonies (actual size). Colonies form black friable crusts when they are desiccated and an olive-green thallus, with the consistency of a rigid gel, when they are wetted. **B:** Cross section through one lobe of a desiccated colony (mag. 160X). The extracellular glycan (sheath) at the air/colony boundary appears striated and is often heavily pigmented (brown). The cells of *N. commune* develop unbranched filaments that are separated from one another and remain distributed throughout, and immobilized and embedded within, the extracellular glycan. **C:** Typical appearance of *N. commune* DRH1 and UTEX 584 grown in liquid culture. Vegetative cells form filaments of 7 μm diameter with differentiated (nitrogen-fixing) heterocysts (*H*) dispersed regularly along their length (mag. 430X). **D:** Immunolocalization of Wsp in the extracellular sheath(s) from a section through a desiccated colony (no post staining). The envelope of a single cell is visible in the top right hand corner (mag. 25,000X). **E:** Cross section (no post staining) through a single vegetative cell (*vc*) showing the thickened envelope and the sheath(s), S_1 and S_3. A discrete accumulation of Wsp (gold particles, 5 nm diameter) is apparent at the periphery of the sheath/cell envelope interface (arrows, mag. 20,000X; and inset, mag. 40,000X). **F:** Cross section (with post staining) through a single vegetative cell (*vc*) showing its characteristic ultrastructure, intact cell membranes (*OM, PL,* and *CM*) and the fibrous nature of the sheath, S_3; mag. 64,000X. **G:** SEM view of a cross section through a desiccated colony (showing a filament of vegetative cells (*vc*), the envelope "tunnel" within which the filament is located, and the extensive sheath, S_1 and S_3, 3,600X.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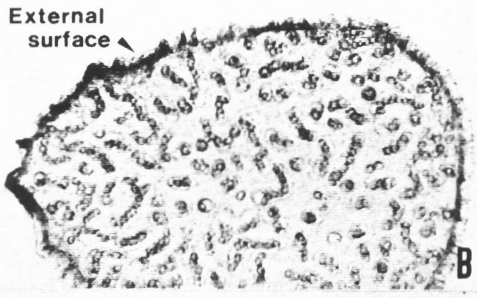
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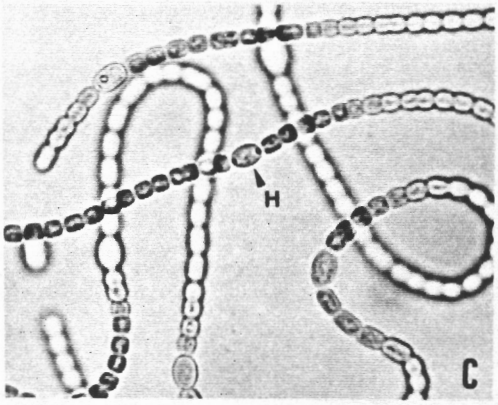
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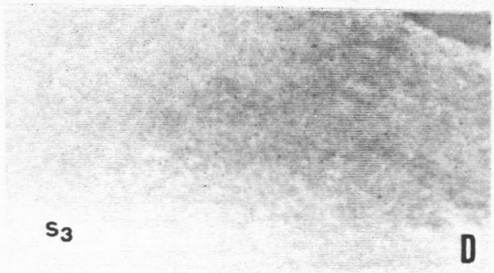
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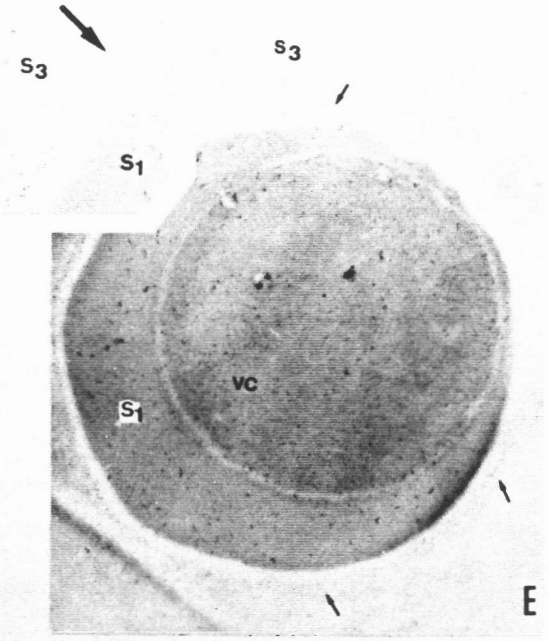
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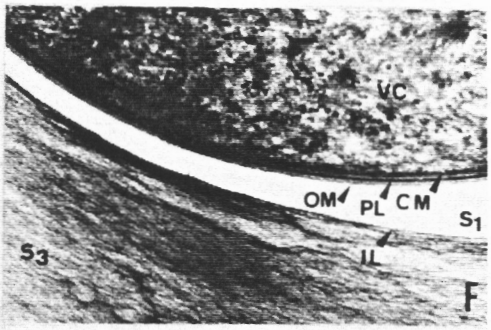
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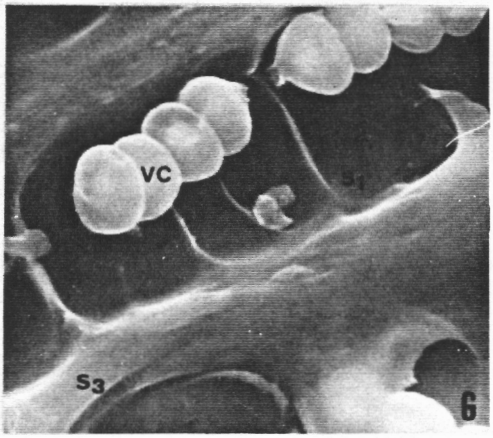
VC
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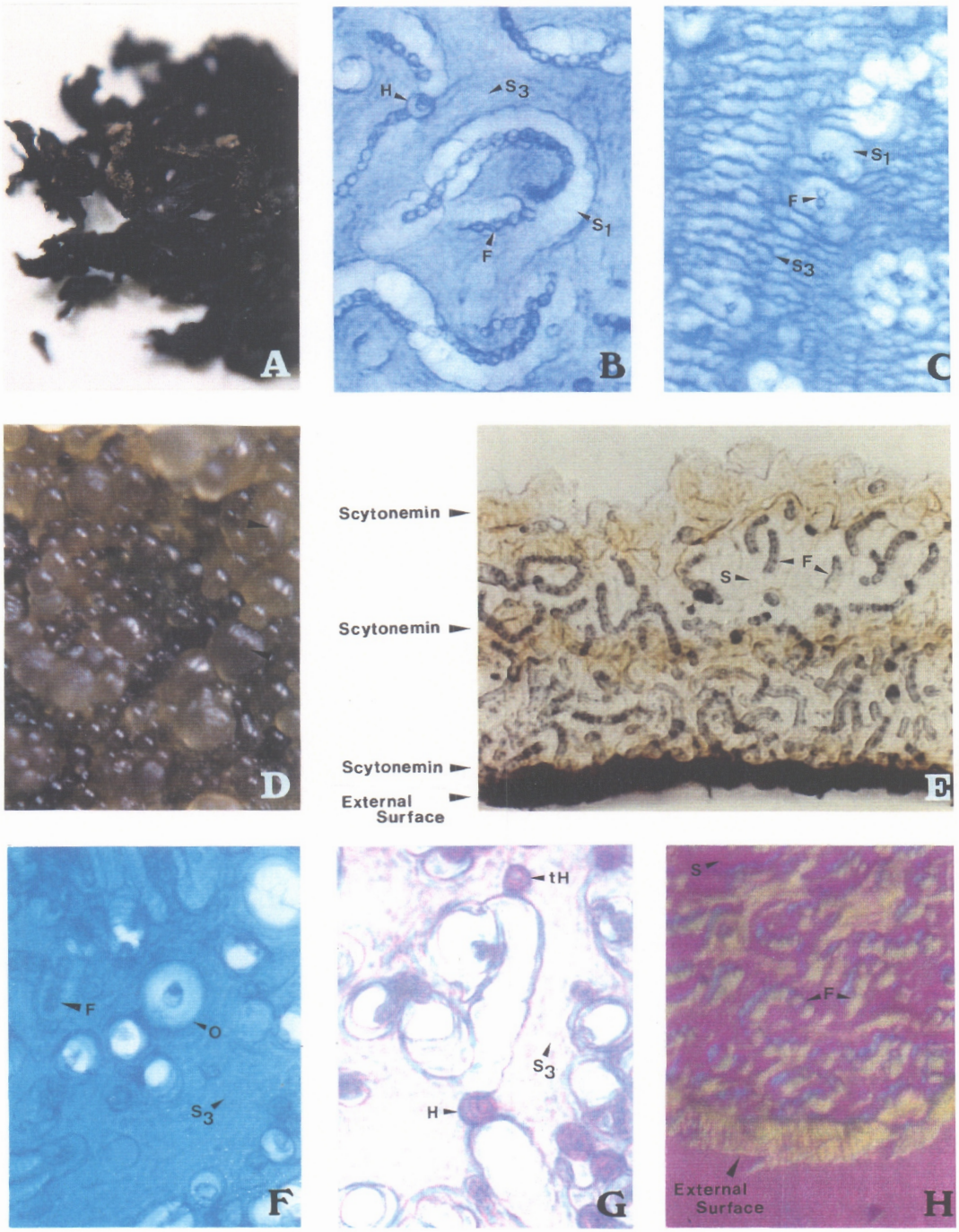
Figure 4. **Macroscopic and microscopic features of the glycan. A:** Desiccated field material *Nostoc commune* CHEN; 2X. **B:** Desiccated *Nostoc commune* CHEN stained with standard AB (Alcian blue) protocol; 40X. **C:** *Nostoc commune* CHEN, five minutes of rehydration, AB stained standard protocol; 40X. **D:** Pearls generated from rehydrated *Nostoc commune* CHEN; 2X. **E:** Unstained light microscopy section of *Nostoc commune* TEN; 20X. **F:** Desiccated *Nostoc commune* 8122 (Aldabra) stained with AB pH 2.5; 40X. **G:** Desiccated *Nostoc commune* 8122 stained with PAS; 60X. **H:** *Nostoc commune* TEN desiccated, stained with PAS, Normansky interference; 20X.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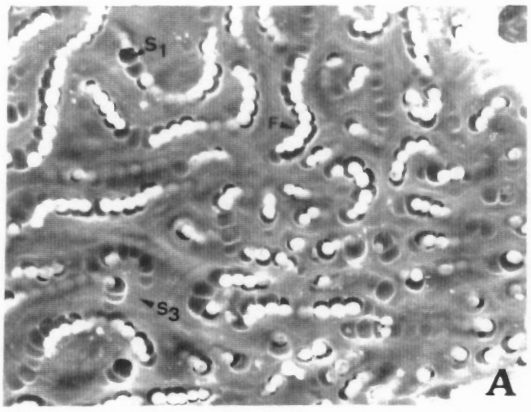
External Surface
H

Figure 5. **Scanning electron microscopy of desiccated field material. A:** *Nostoc commune* TAG; 900X. **B:** *Nostoc commune* TAG; 1,250X. **C:** *Nostoc commune* TEN, ▲▲ pitted areas of sheath following contour of S_1 sheath differentiation; 1,000X. **D:** *Nostoc commune* 8122 (Aldabra) with sites indicated where EDX analysis scans were performed [EXTERNAL, CELL, and SHEATH]; 1,000X. **E:** EDX elemental analysis scans on above indicated locations, all scans performed for 330 seconds, counts represent total counts during scan time. All three scans shown at same scale.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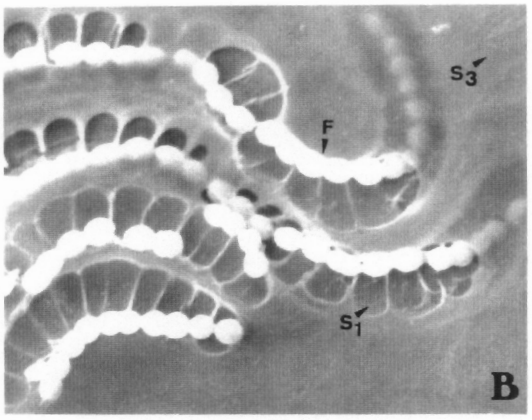
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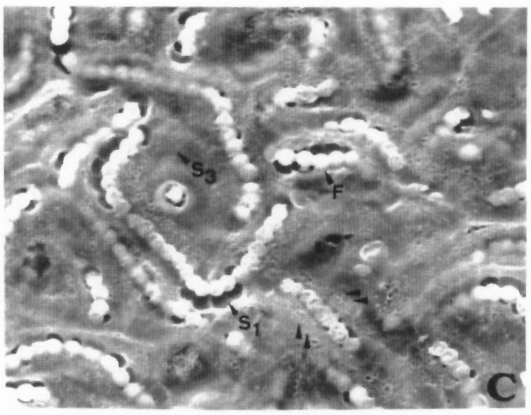
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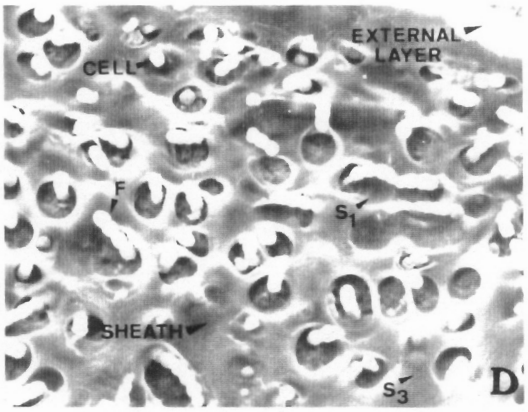
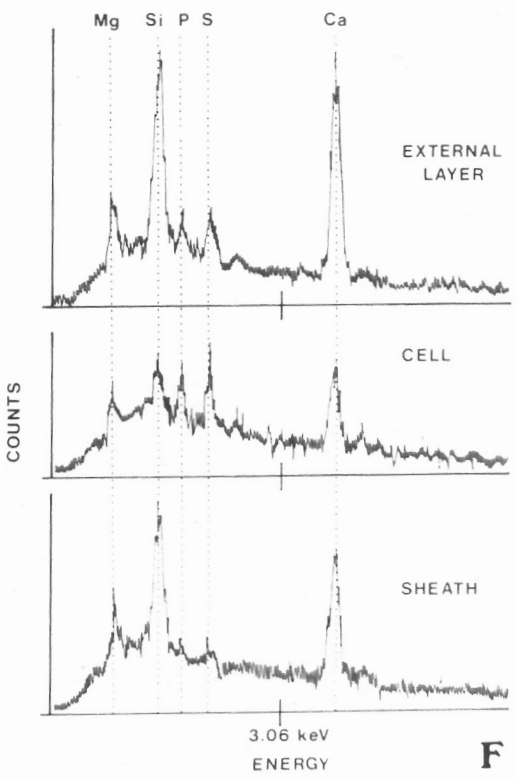

EXTERNAL LAYER
CELL
F
S1
SHEATH
S3
D


Mg
Si
P
S
Ca
EXTERNAL LAYER
CELL
SHEATH
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3.06 keV
ENERGY
F

Figure 6. **Transmission electron microscopy of *Nostoc commune* CHEN field material.** Specimens viewed in **A** and **F** were not exposed to secondary fixation with 2% w/v osmium tetroxide or post-stained with uranyl acetate/lead citrate, all other specimens in figure were treated with these steps. **A:** Desiccated field material immunolabeled with sheath antibody (5 nm gold particles), arrow indicates dense labeling areas within the sheath; 20,000X. **B:** Desiccated field material; 20,000X. **C:** Field material after 30 minutes of rehydration; 7,000X, inset 23,000X. **D:** Field material after 60 minutes rehydration; 12,500X, inset 25,000X. **E:** Field material after 12 hours of rehydration, arrow indicates an apparent junction between sheath and vegetative cell; 10,000X, inset 20,000X. **F:** Section of pearl generated through rehydrated field material and immunolabeled with sheath antibody; 17,500X.

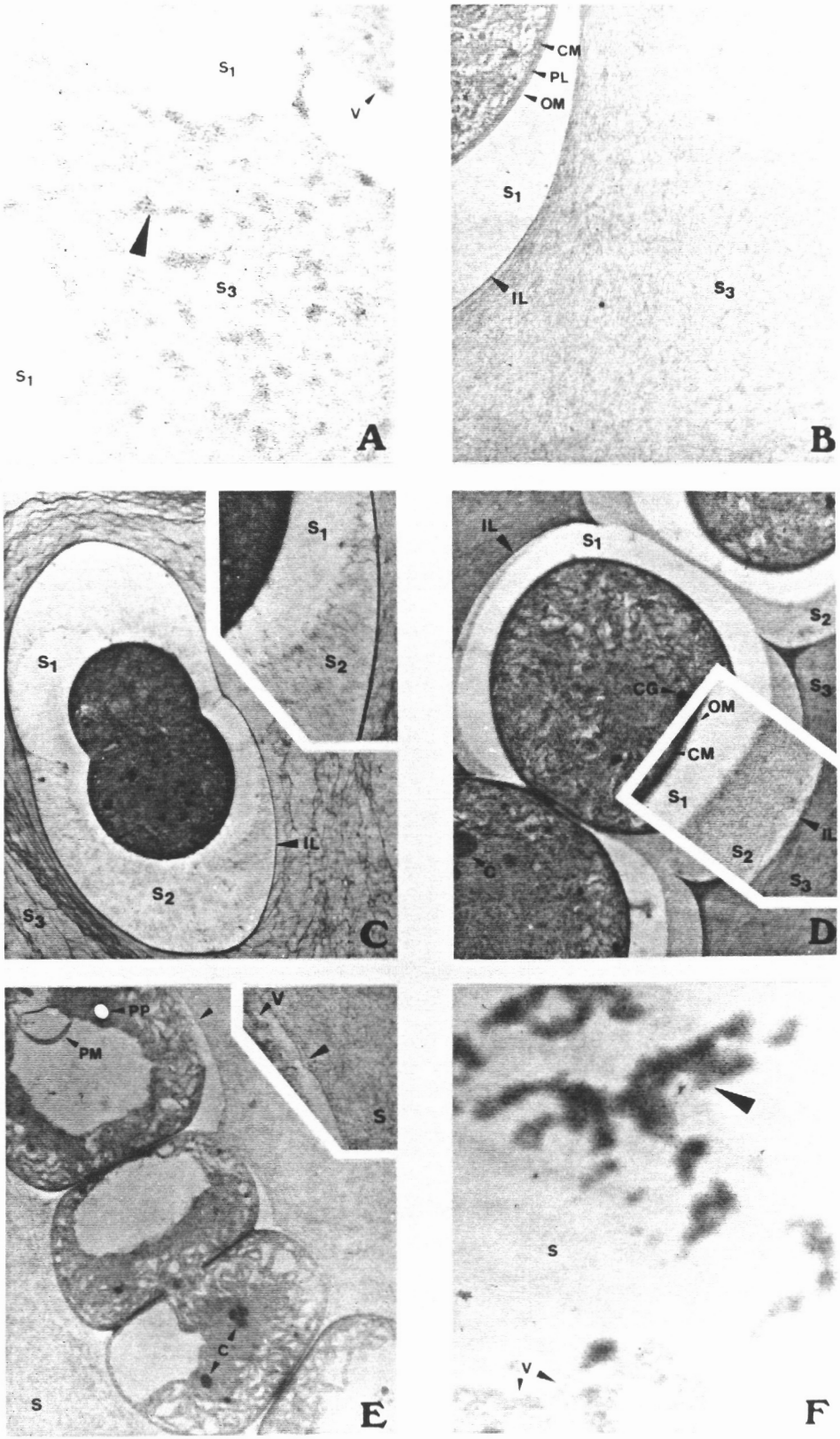

S1
V
S3
S1
A
CM
PL
OM
S1
IL
S3
B
S1
S2
S1
IL
S2
S3
C
IL
S1
S2
CG
OM
CM
S1
IL
S2
S3
C
D
PP
PM
V
S
C
S
E
S
V
F

Figure 7. **Scanning electron microscopy of pearl. A:** Cross section view through a pearl approximately 2 mm in diameter; 40X. **B:** View of filaments within the pearl; 150X. **C:** Outer sheath edge of pearl; 1,000X. **D:** Individual filament located towards the periphery of a pearl, arrow indicates differentiation of the sheath; 1,000X. **E:** Pearl buds on the outer periphery of a pearl; 200X. **F:** Individual filaments located in the central region of a pearl; 1,200X.

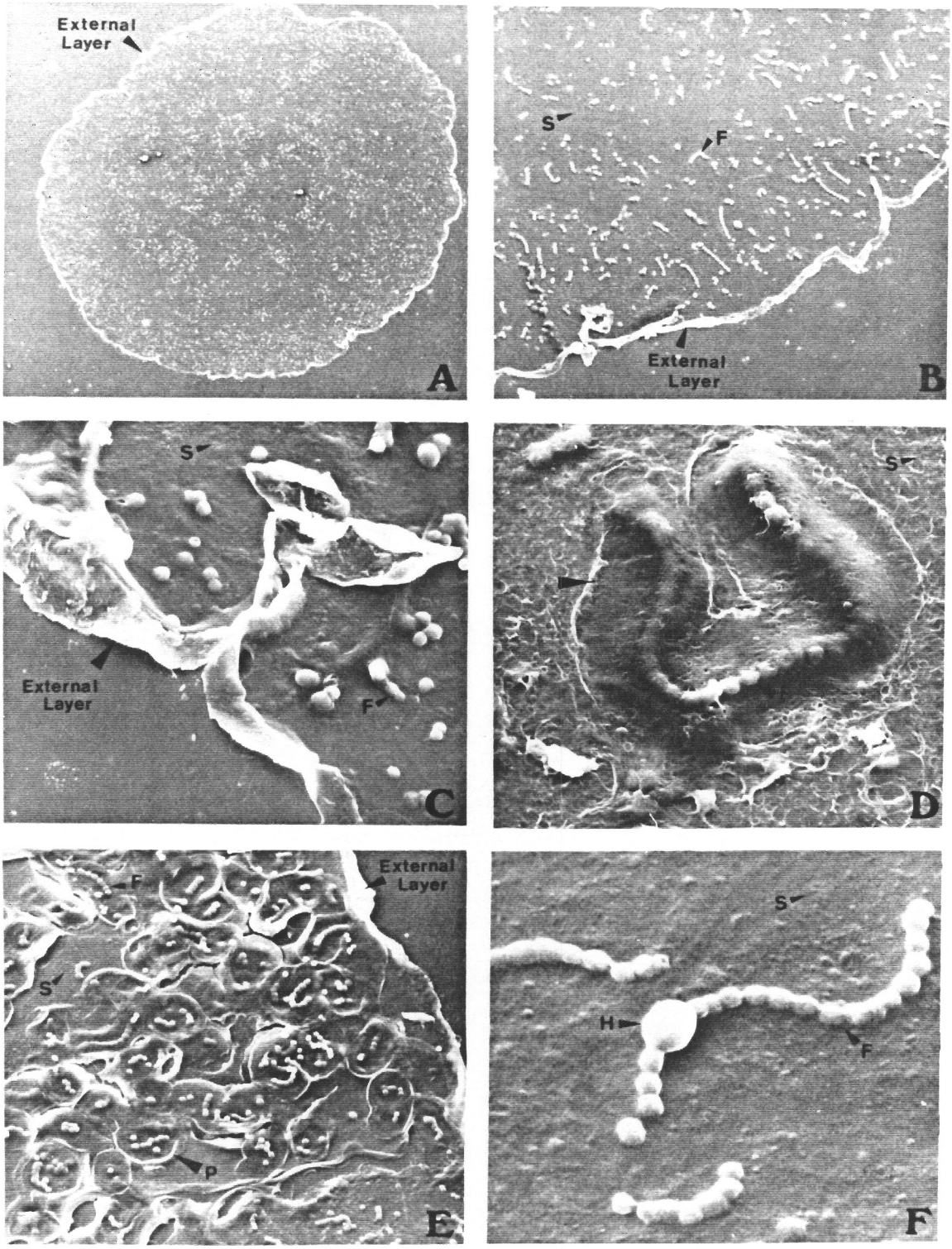

External Layer
A
S
F
External Layer
B
S
External Layer
F
C
S
D
F
S
External Layer
P
E
S
H
F
F

Figure 8. **Transmission electron micrographs of pearls. A:** Filament with a terminal heterocyst located towards the periphery of the pearl; 6,000X. **B:** Mature heterocyst, the fibrous structure of the outer layers of the heterocyst envelope indicated; 15,000X. **C:** Immature or developing heterocyst; 15,000X. **D:** External layer of the pearls appears striated; 9,450X. **E:** Filament located towards the center of the pearl, note the well defined and prolific thylakoid membranes present in cells; 6,000X. **F:** Filament present in the center of a pearl, note unusual configuration of thylakoid membranes; 3,200X. **G:** External layer of the pearl prevents the intrusion of epiphytic bacteria present on some areas of the pearl's surface; 9,450X.

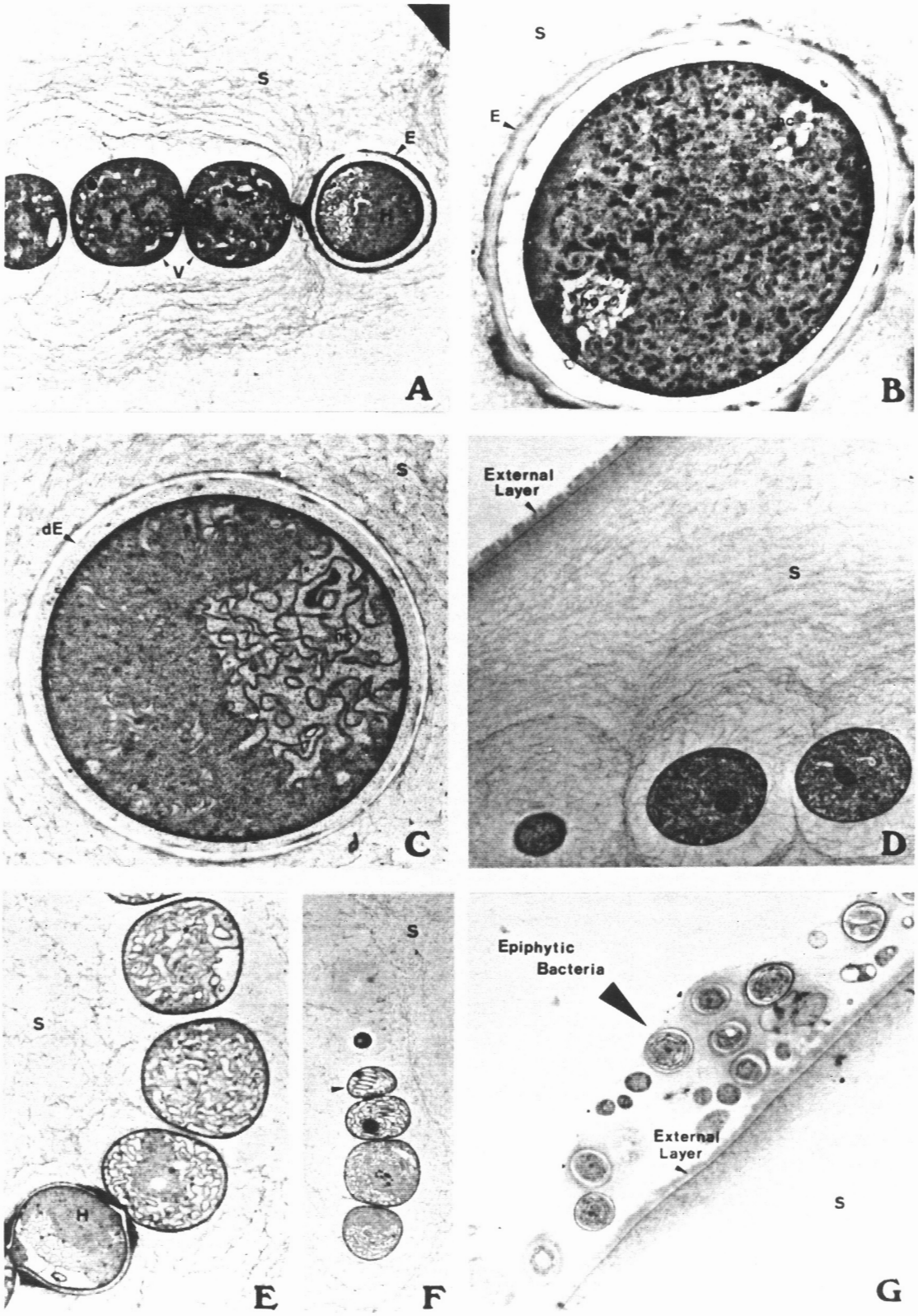

S
E
H
V
A
S
E
B
S
dE
C
External
Layer
S
D
S
H
E
S
F
Epiphytic
Bacteria
External
Layer
S
G

Figure 9. **Features of *Nostoc commune* strains UTEX 584 and DRH1 in liquid culture.** **A:** *Nostoc commune* strain UTEX 584 in a morphological form known as aseriate packets, ▲ sheath material which encases the packet of cells; 250X. **B:** Liquid culture of *Nostoc commune* strain DRH1, fresh inoculum, note sheath differentiation immediately surrounding filaments indicated by arrows ▲▲ at ridges of sheath; 750X. **C:** Older culture of *Nostoc commune* DRH1 where filaments have aggregated and become encased forming small versions of "pearls"; 200X.

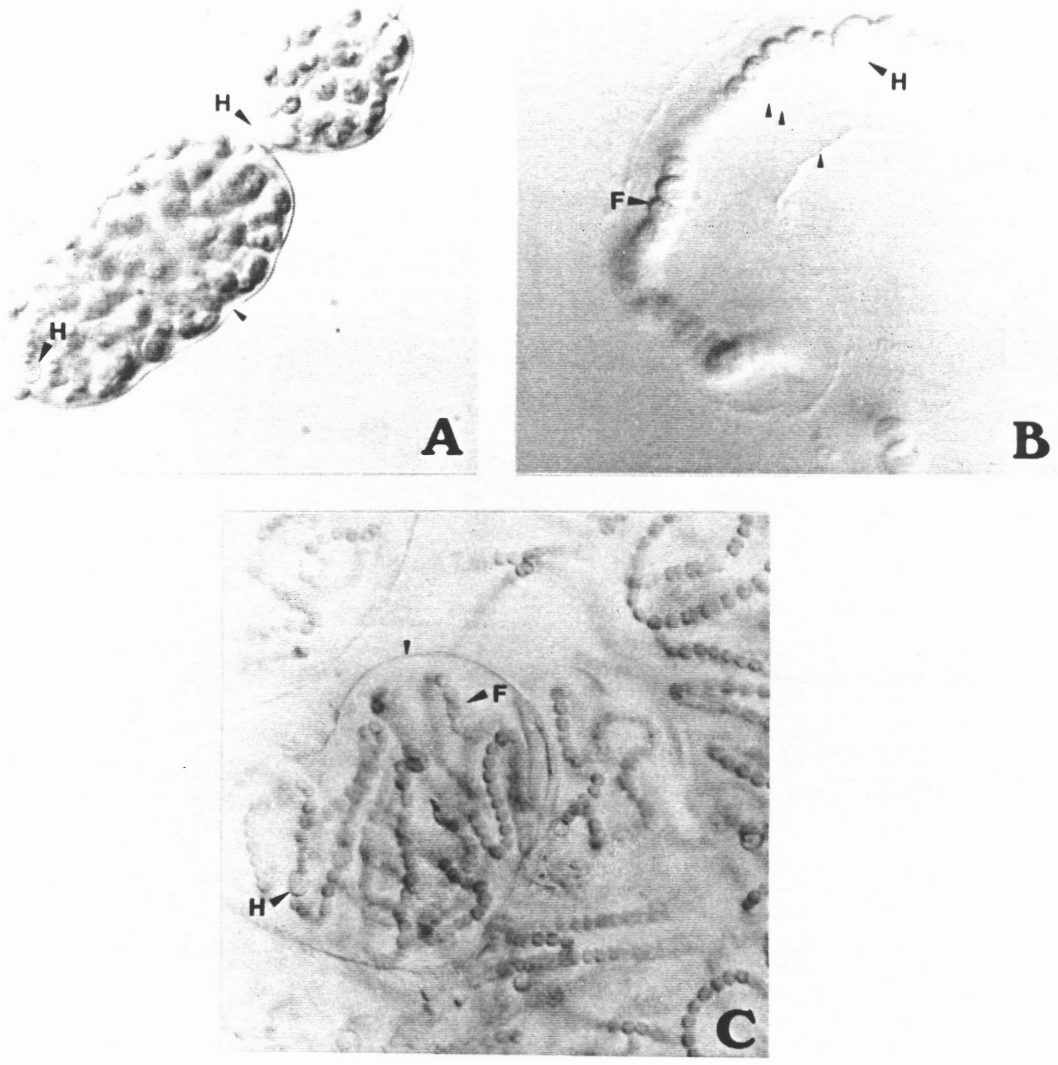
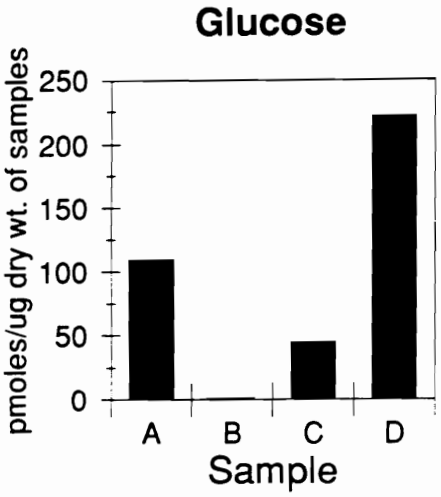
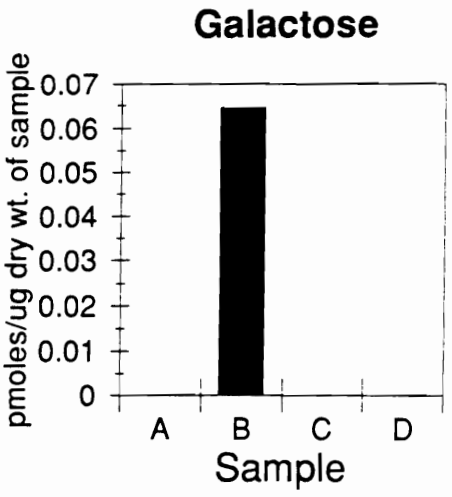
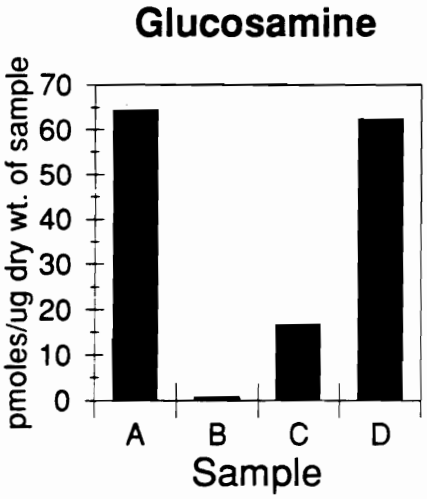
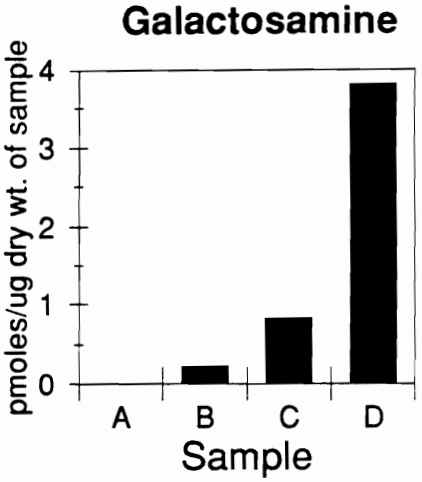

H
H
A
H
F
B
F
H
C

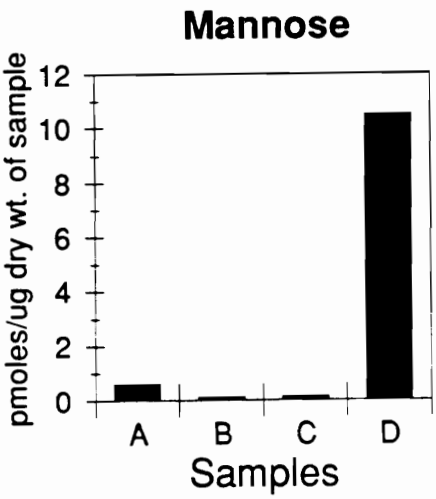
Figure 10. **Carbohydrate analysis of glycan fractions.** A, large molecular weight fraction (>12 kDa) of glycan extract from *Nostoc commune* DRH1 media; B, small molecular weight fraction of *Nostoc commune* DRH1 (<12 kDa). C and D, aqueous extracts of *Nostoc commune* CHEN. All sugar quantities are reported as pmoles of individual sugars μg^{-1} dry weight of sample extract of media or field material. Refer to Appendix 5.


Glucose
pmoles/ug dry wt. of samples
250
200
150
100
50
0
A
B
C
D
Sample


Galactose
pmoles/ug dry wt. of sample
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
A
B
C
D
Sample


Glucosamine
pmoles/ug dry wt. of sample
70
60
50
40
30
20
10
0
A
B
C
D
Sample


Galactosamine
pmoles/ug dry wt. of sample
4
3
2
1
0
A
B
C
D
Sample


Mannose
pmoles/ug dry wt. of sample
12
10
8
6
4
2
0
A
B
C
D
Samples

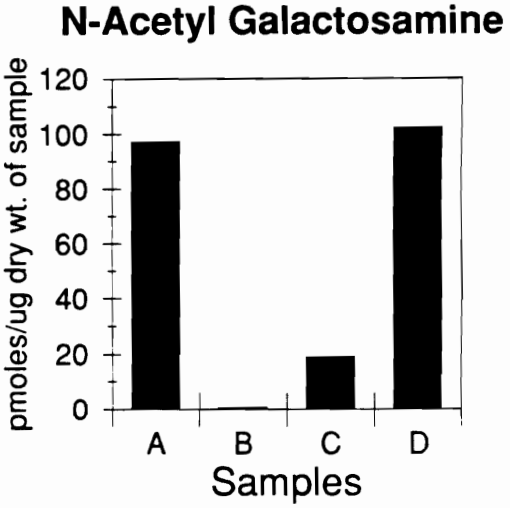
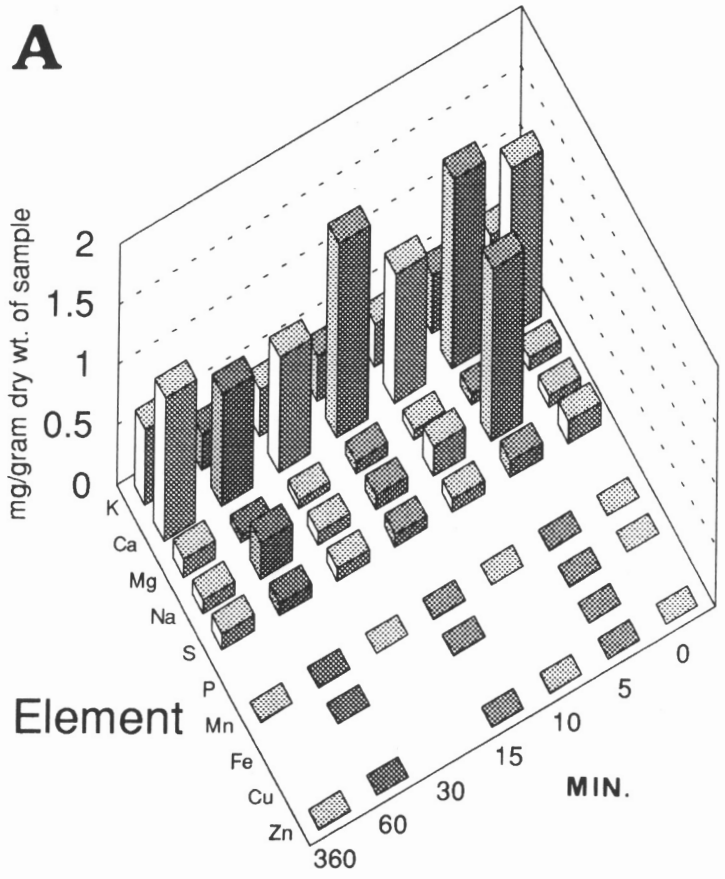

N-Acetyl Galactosamine
pmoles/ug dry wt. of sample
120
100
80
60
40
20
0
A
B
C
D
Samples

Figure 11. **Elemental analysis of *Nostoc commune* CHEN. A:** Analysis of aqueous extracts during a time course of rehydration from totally desiccated to 6 hours. **B:** Ethanolic extract of material in A which remained after aqueous extraction. Elemental quantities reported as mg of element per gm dry weight of desiccated material used in extract.


A
mg/gram dry wt. of sample
2
1.5
1
0.5
0
K
Ca
Mg
Na
S
P
Mn
Fe
Cu
Zn
Element
360
60
30
15
10
5
0
MIN.

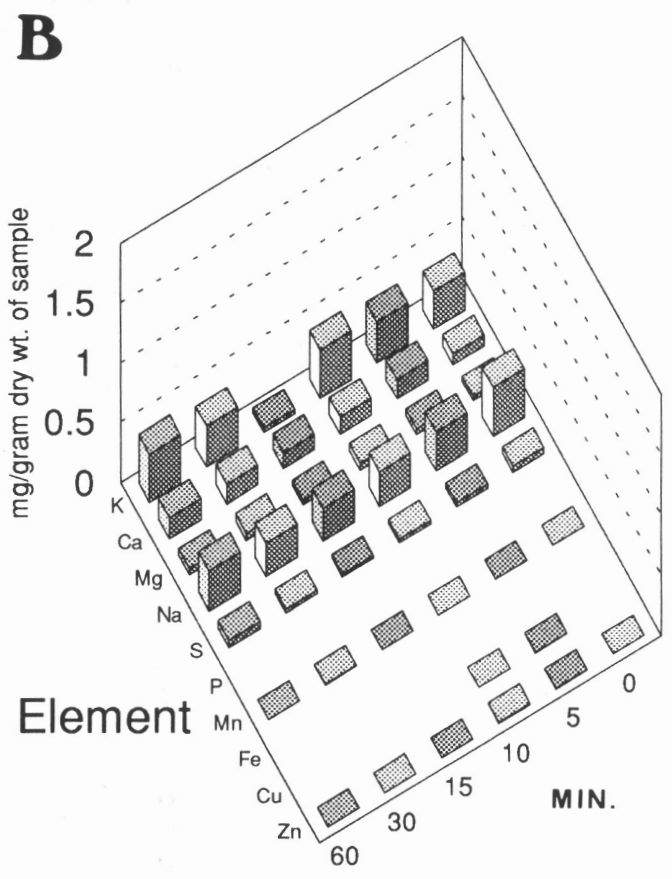
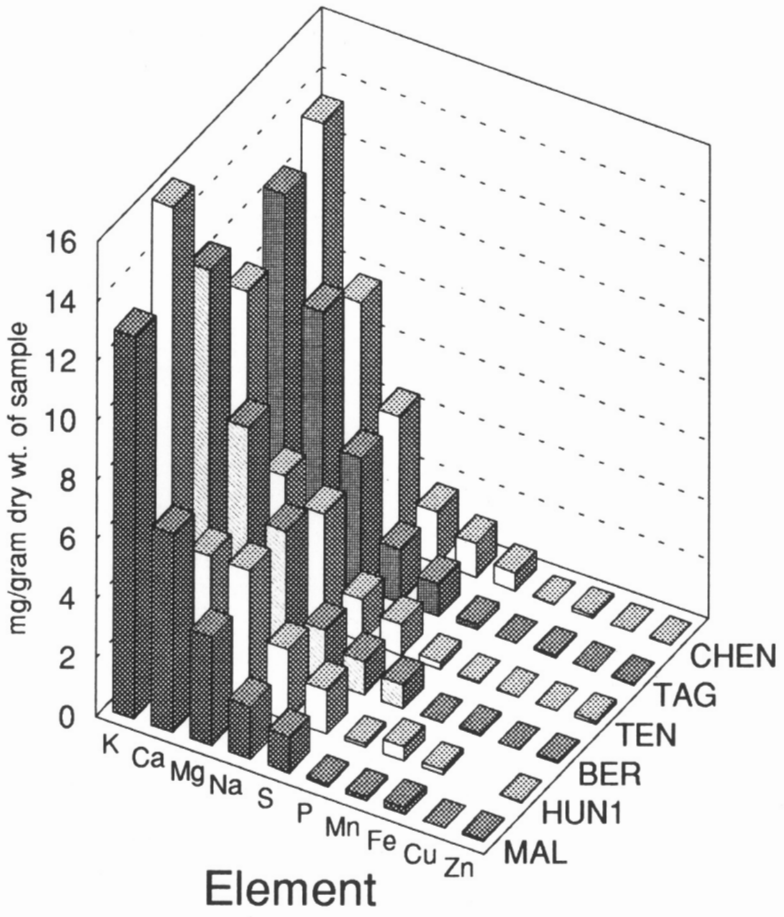

B
mg/gram dry wt. of sample
2
1.5
1
0.5
0
K
Ca
Mg
Na
S
P
Mn
Fe
Cu
Zn
Element
60
30
15
10
5
0
MIN.

Figure 12. **Elemental analysis of aqueous extracts of *Nostoc commune* from various geographic locations.** All quantities reported as in figure 11. Refer to Appendix 6.


mg/gram dry wt. of sample
16
14
12
10
8
6
4
2
0
K
Ca
Mg
Na
S
P
Mn
Fe
Cu
Zn
CHEN
TAG
TEN
BER
HUN1
MAL
Element

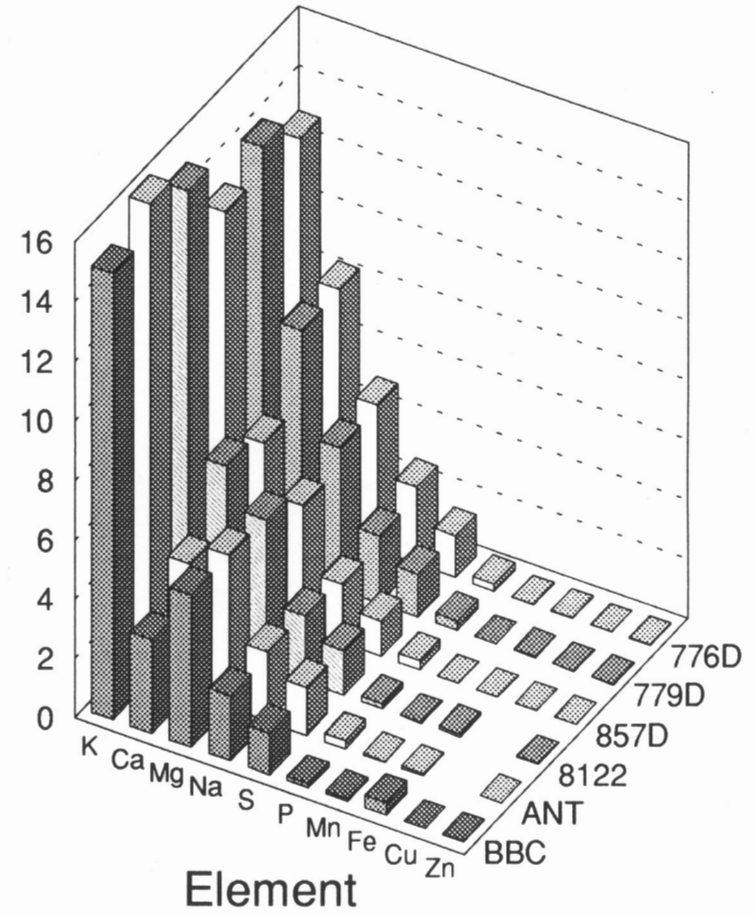

16
14
12
10
8
6
4
2
0
K
Ca
Mg
Na
S
P
Mn
Fe
Cu
Zn
776D
779D
857D
8122
ANT
BBC
Element

Figure 13. **Aqueous extract of *Nostoc commune* TEN contains Wsp and a UV-absorbing complex.** UV-visible spectrum of aqueous extract and Western blot of aqueous extract in lane 2 and non-aqueous extract in lane 1, using Wsp antibody. **B**. Elution profile of 2 ml of rehydration fluid (approximately 0.3 gm dry wt. of desiccated material) from a Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 7.5 (buffer A) with a flow rate of 1 ml min^{-1}. Profile was developed with a gradient buffer B (buffer A, 2 M KCl, ➖). Absorbance of each fraction was determined simultaneously on a Shimadzu spectrophotometer at 280 nm (⧖), 310 nm (▼), 435 nm (○) and 493 nm (■). Aliqouts from 1 ml fractions were subjected to Western blotting using the Wsp antibody (data not shown).

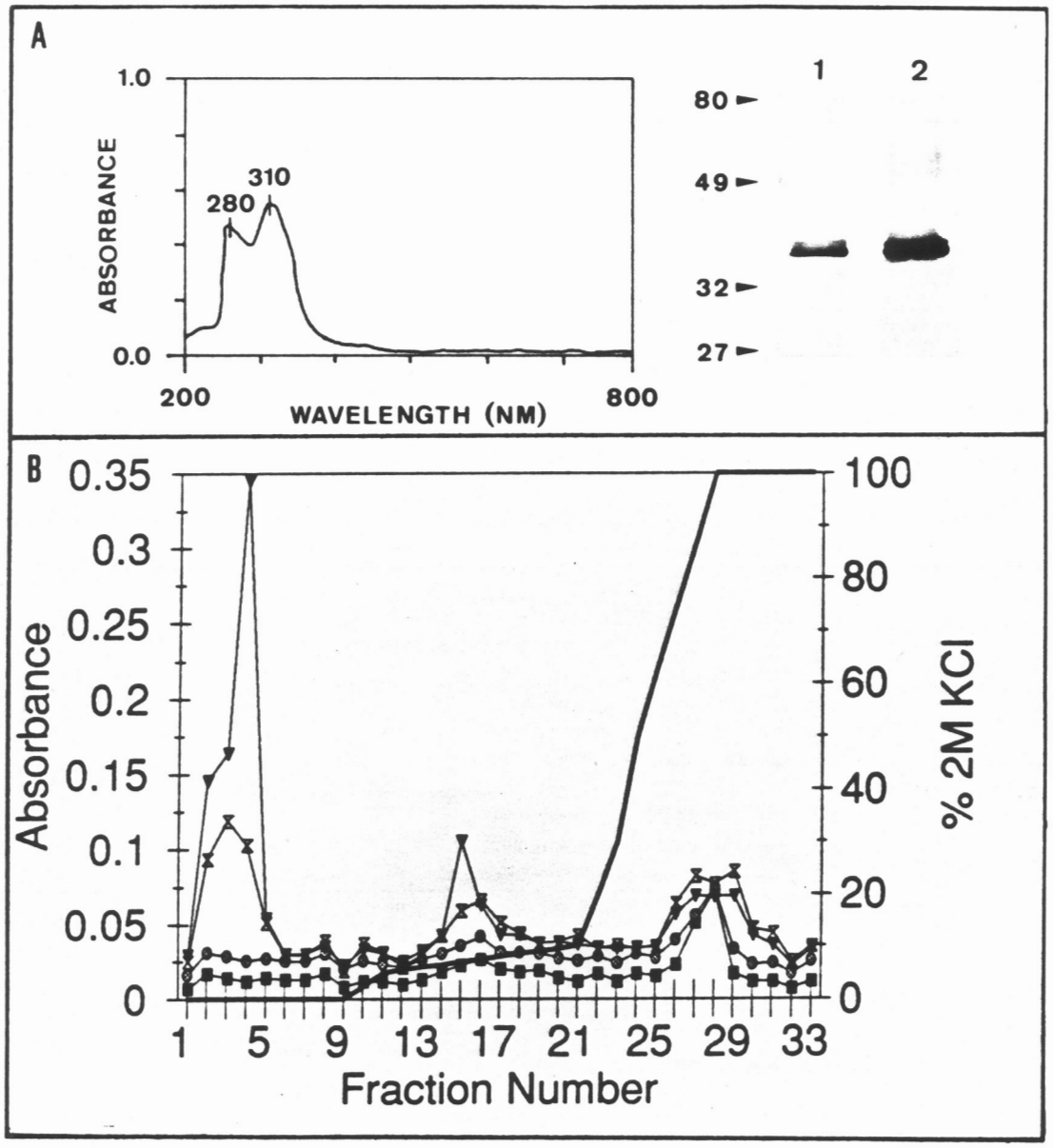

A
1.0
0.0
ABSORBANCE
280
310
200
800
WAVELENGTH (NM)
1
2
80
49
32
27
B
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance
100
80
60
40
20
0
% 2M KCl
1
5
9
13
17
21
25
29
33
Fraction Number

Figure 14. **Two Glycoproteins are secreted by *Nostoc commune*.** Glycan stain of selected locations, using same amount of protein as used in Western blot of figure 13. ● indicates the position of the glycoproteins. Refer to Table 1 for lane identifications.

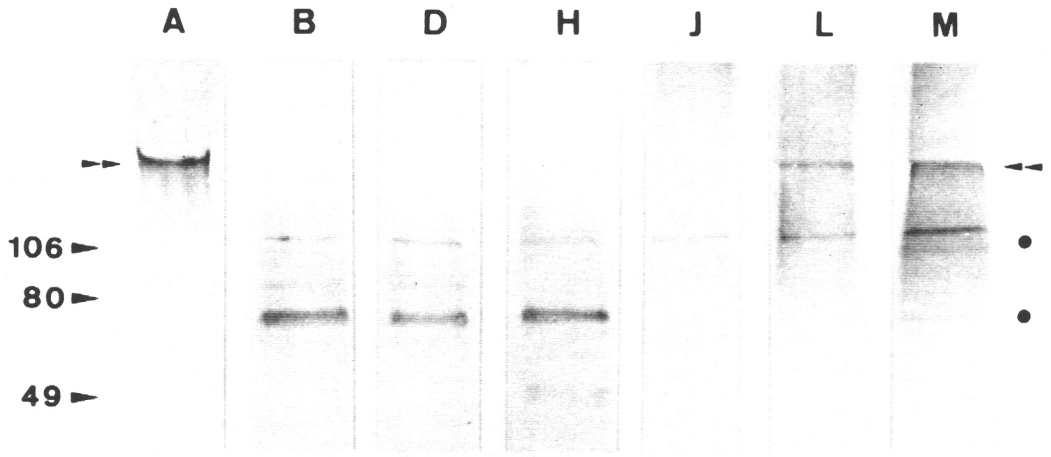

A
B
D
H
J
L
M
106
80
49

Figure 15. **Wsp polypeptides are released from desiccated colonies upon rehydration** Equivalent amounts (0.1 gm dry weight) of desiccated *N. commune* CHEN were suspended in 5 ml of sterile water, with or without the addition of protease inhibitors for periods between 5 minutes and 6 hours, at room temperature. The intact colonies were removed and lyophilized, and aliquots (3 μg total protein) of the supernatant fractions (1st rehydration fluids) were subjected to Western analysis (A and B). All of the lyophilized colonies were then rehydrated with a further 5 ml of sterile water for 48 hours, at 4 °C, and the 2nd rehydration fluids were similarly recovered and subjected to Western analysis (C and D). The first and second rehydrations released 0.50-0.55 and 0.51-0.64 μg ml^{-1} total protein, respectively. Dotted lines emphasize that no measurements are possible for the zero time points in the first rehydration series (desiccated colonies, O_o; desiccated/lyophilized colonies, O). Molecular mass markers in descending order are 106, 80, 49, 32 27, and 18 kDa.

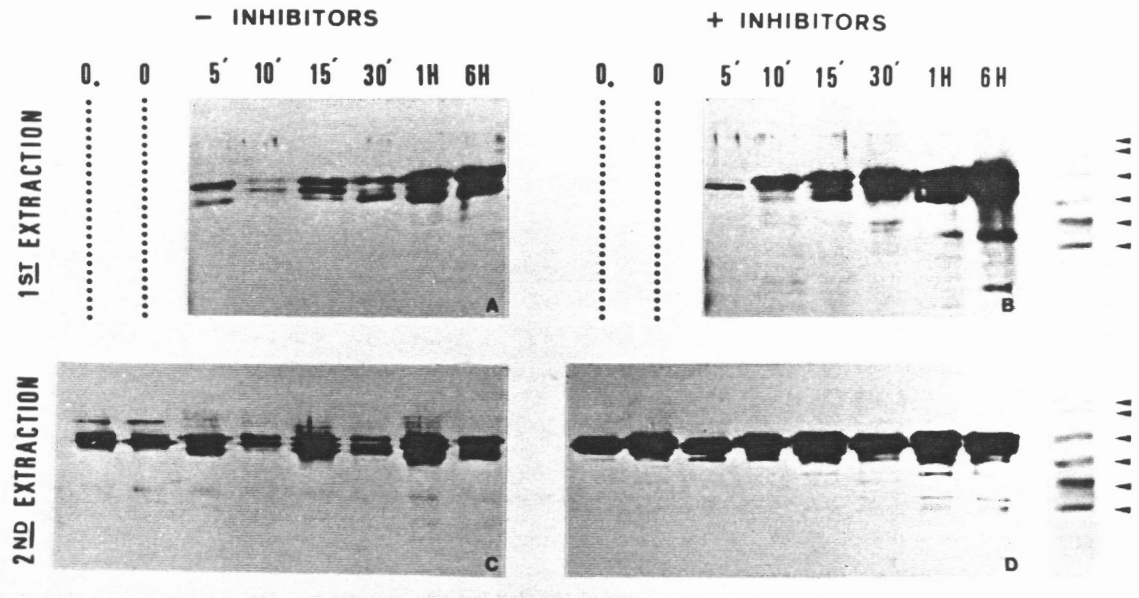

– INHIBITORS
+ INHIBITORS
0. 0 5′ 10′ 15′ 30′ 1H 6H
0. 0 5′ 10′ 15′ 30′ 1H 6H
1ST EXTRACTION
2ND EXTRACTION
A
B
C
D

Figure 16. **Wsp is secreted together with a UV-A/B-absorbing pigment** 100 µl aliquots of rehydration fluids from the second extraction (times 0_o and 15 minutes; see Fig. 15) were diluted in rehydration buffer and their spectra were collected using 1 cm pathlength cuvettes and a Shimadzu model UV 160U UV-visible recording spectrophotometer. *Insert*: 20 gm dry weight of desiccated material was rehydrated overnight, at 4°C, and Wsp was concentrated from the rehydration fluid following ammonium sulfate precipitation and ultrafiltration. Proteins were resolved by SDS-PAGE, silver staining (Wsp remain non-stained at these concentrations) then Coomassie Blue (right hand lane). Positions of Wsp polypeptides (Wsp) and a 68 kDa protein that was sequenced (Table 2) are indicated. Arrows in ascending order indicate the solvent front and molecular mass markers as Fig. 15, bottom arrow represents dye front..

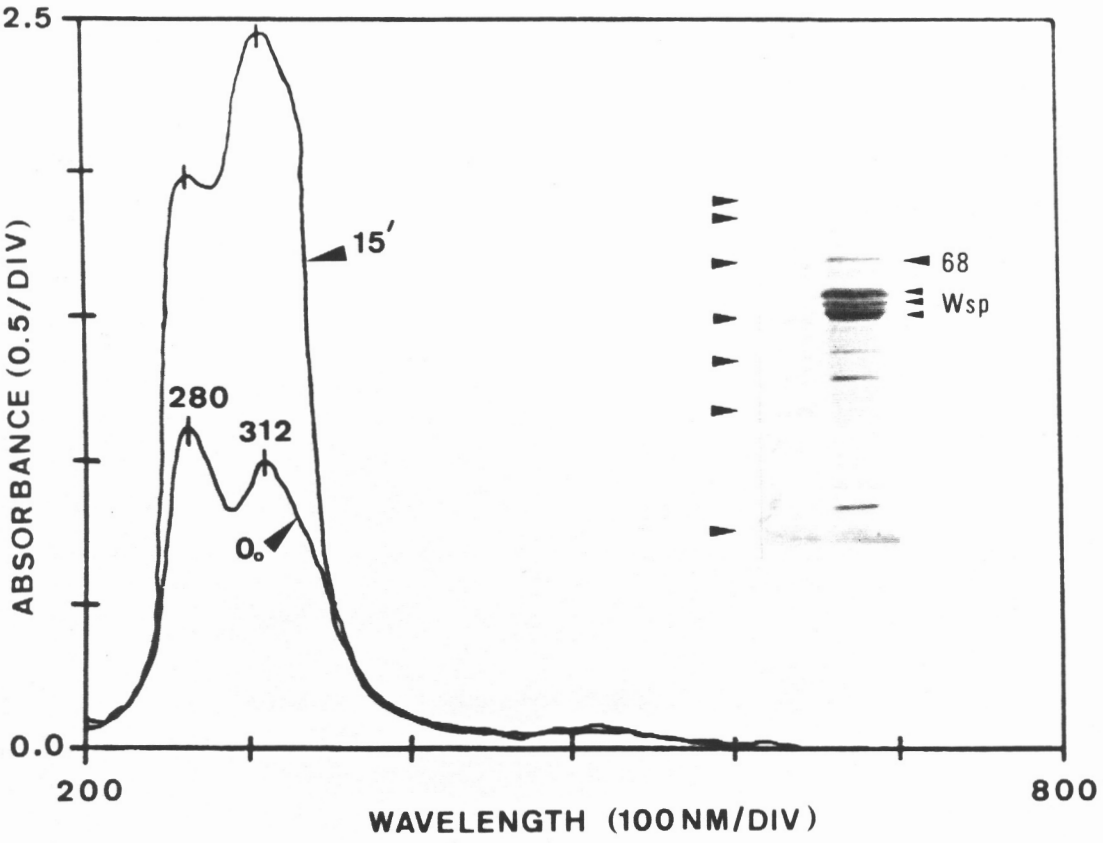

2.5
0.0
ABSORBANCE (0.5/DIV)
200
800
WAVELENGTH (100 NM/DIV)
15′
280
312
0
68
Wsp

Figure 17. **The peptide maps of Wsp polypeptides are very similar.** Western blots after treatment of purified 33, 37, and 39 kDa polypeptides with either V8 protease (lanes Lanes A, B and C, respectively), chymotrypsin (lanes D, E and F, respectively), endo Lys-C (lane G) or endo Glu-C (lane H; ● peptide sequenced). Lanes G and H represent digest of pooled 33, 37 and 39 kDa proteins. Lane I is a blot of the undigested Wsp polypeptides from which the individual polypeptides were purified through electroelution. Approximately 3 μg of polypeptide were loaded in each lane. Molecular mass markers as Fig. 15.

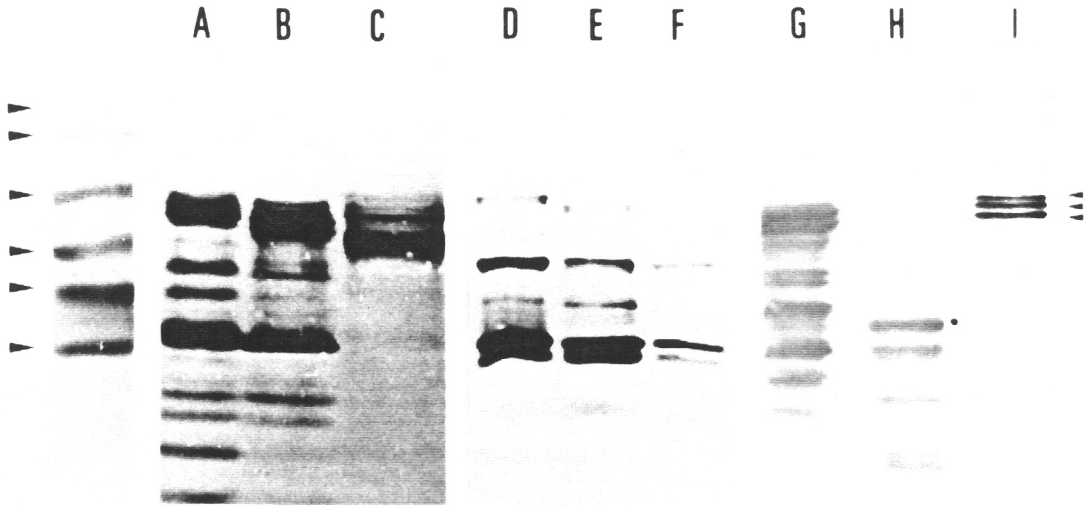

A
B
C
D
E
F
G
H
I

Figure 18. **Wsp preparations have xylanase activity.** Reaction mixtures contained 200 µl (approx 12 µg total protein ml^{-1}) of rehydration fluid and 200 µl of RBB substrate (see Materials and Methods). Each data point in the linear regression is the mean of three replicates. The data points at 60 minutes and 120 minutes (not shown) were 3.067 (± 0.307) and 6.82 (± 0.547) µmoles xylose released min^{-1}, respectively. Inset: specific activities (assayed after 25 minutes of incubation) of Wsp rehydration fluids and 0.25 units of purified *Trichoderma* xylanase incubated in the absence of Wsp antiserum (□), presence of Wsp antiserum (■) or presence of pre-immune serum (⊟). Sera were used at a final dilution of 1:200 in reaction mixtures. Reaction mixtures were incubated with the antiserum for 10 minutes prior to the addition of substrate.

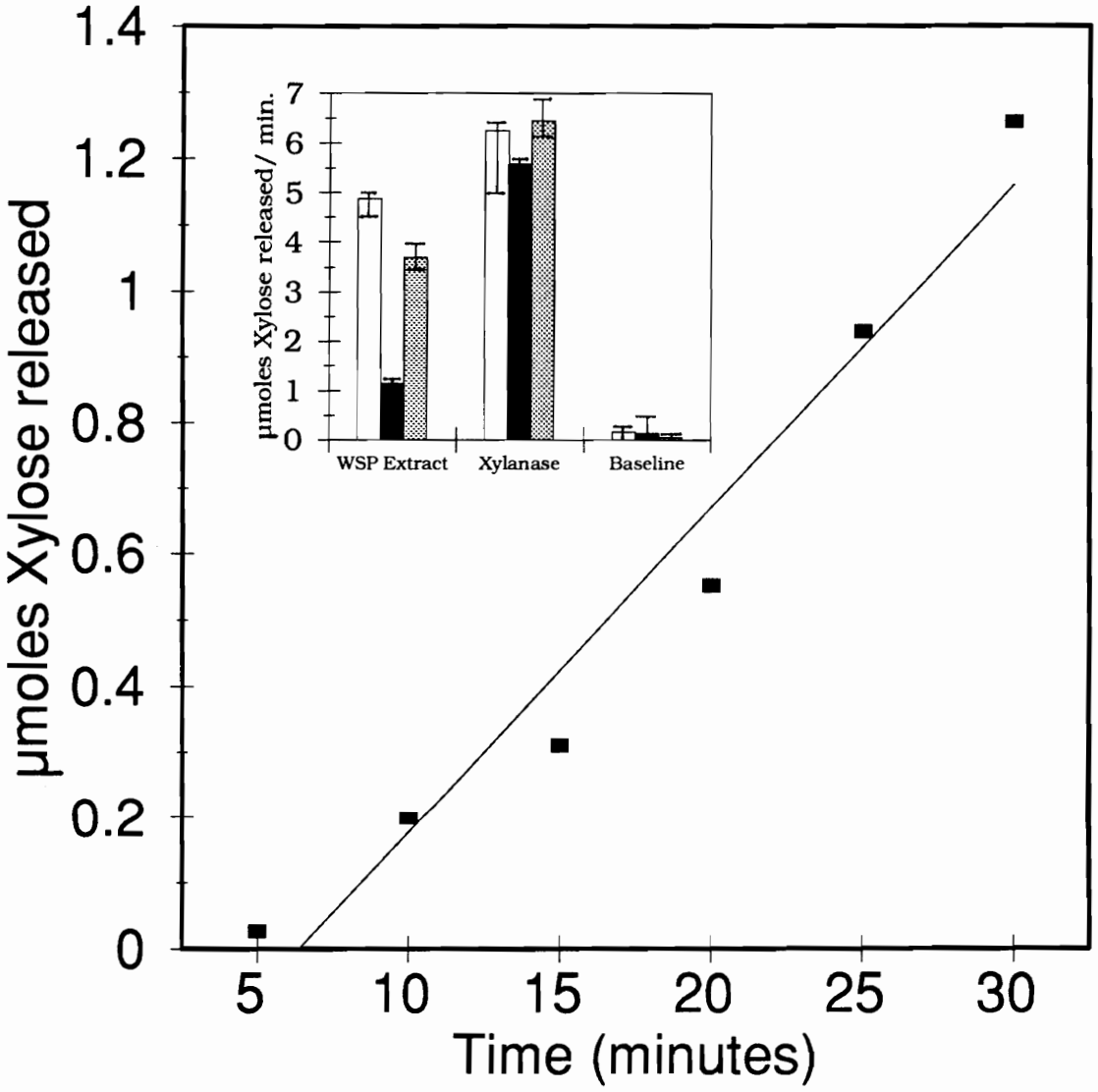

µmoles Xylose released
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Time (minutes)
µmoles Xylose released/ min.
7
6
5
4
3
2
1
0
WSP Extract
Xylanase
Baseline

Figure 19. **Resolution of WSP/UV-absorbing complexes.** Elution profile of 2 ml of concentrated rehydration fluid (approximately 100 µg total protein ml^{-1}) from a Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A) with a flow rate of 1 ml min^{-1}. Profile was developed with a gradient of buffer B (buffer A/1M KCl). Absorbance was monitored simultaneously at 280 nm (—) and 312 nm (---). Aliquots from 1 ml fractions were subjected to Western blotting (lower inset C = control, a-g = fractions analysed), and assays for xylanase activity (see text).

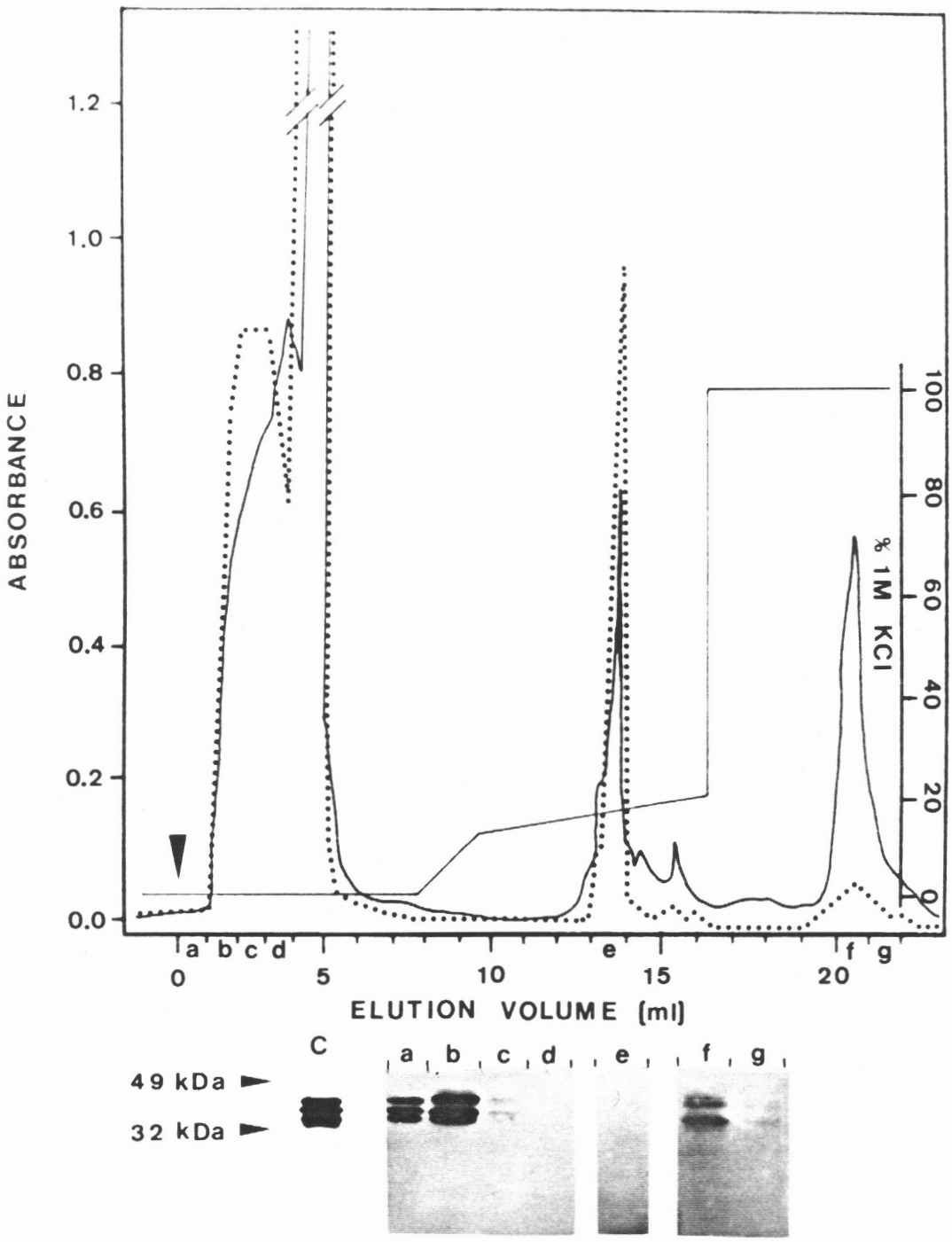

ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
% 1M KCl
100
80
60
40
20
0
a b c d
e
f g
0
5
10
15
20
ELUTION VOLUME (ml)
C
a b c d e f g
49 kDa
32 kDa

Figure 20. **Ionic interactions of Wsp UV-absorbing pigments, in presence of Salt.** Elution profile of 200 µl (approximately 100 µg total protein ml^{-1}) of concentrated rehydration fluid from Superose HR 10/30 column equilibrated in 20 mM Tris-HCl/50 mM KCl with a flow rate of 0.75 ml min^{-1}. Absorbance was monitored simultaneously at 280 nm (—) and 312 nm (---). Aliquots from 1 ml fractions were subjected to Western blotting (lower inset; see Fig. 19 legend), silver staining and assays for xylanase activity (--●--; see text and Fig. 18 for details). Column was calibrated using protein molecular mass markers (Boehringer Mannheim).

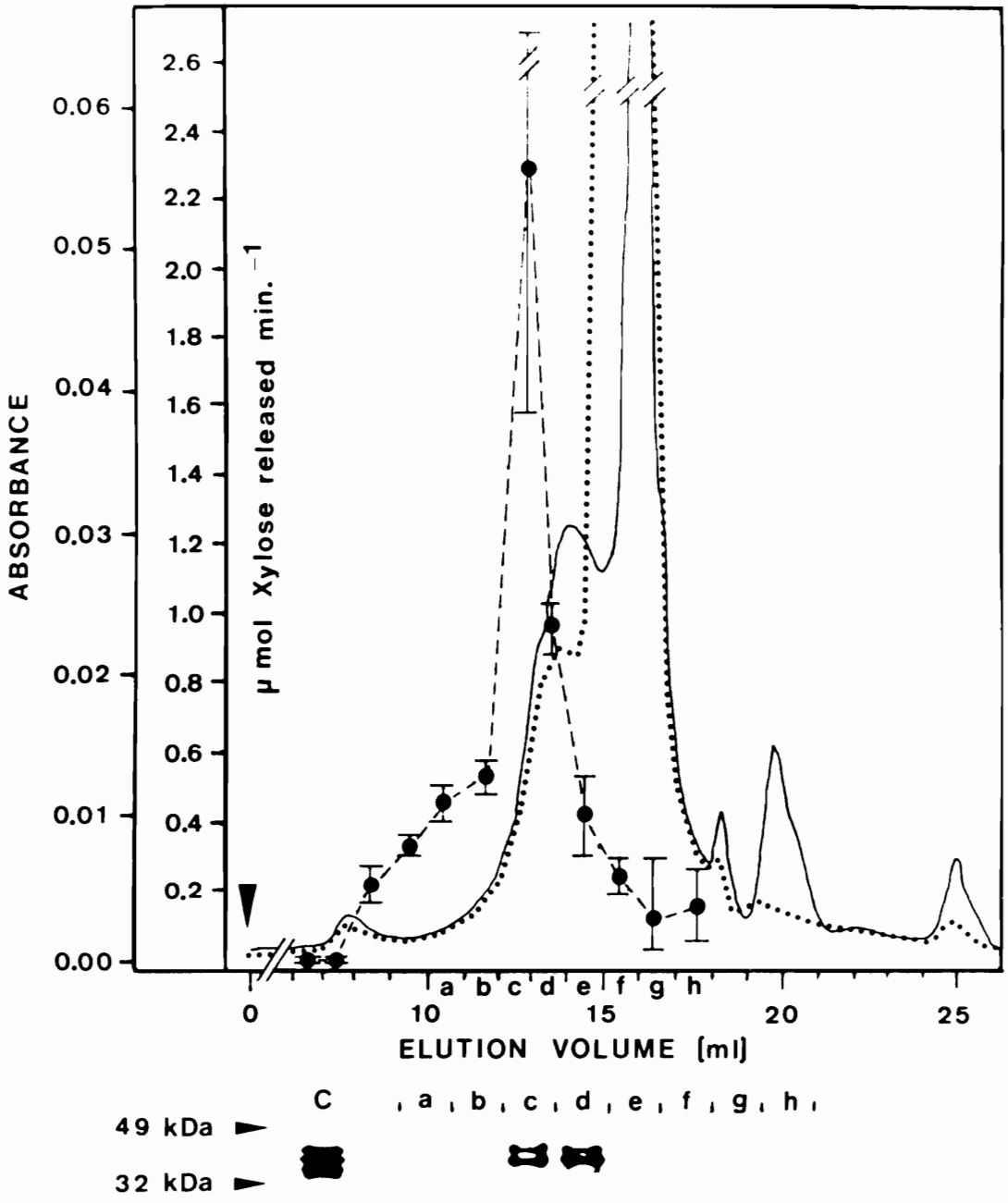

ABSORBANCE
0.06
0.05
0.04
0.03
0.02
0.01
0.00
μ mol Xylose released min.⁻¹
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a b c d e f g h
0
10
15
20
25
ELUTION VOLUME (ml)
C a b c d e f g h
49 kDa
32 kDa

Figure 21. **Ionic interactions of Wsp UV-absorbing pigments, in absence of Salt.** As in Figure 20; column buffer 20mM Tris-HCl, pH 7.4

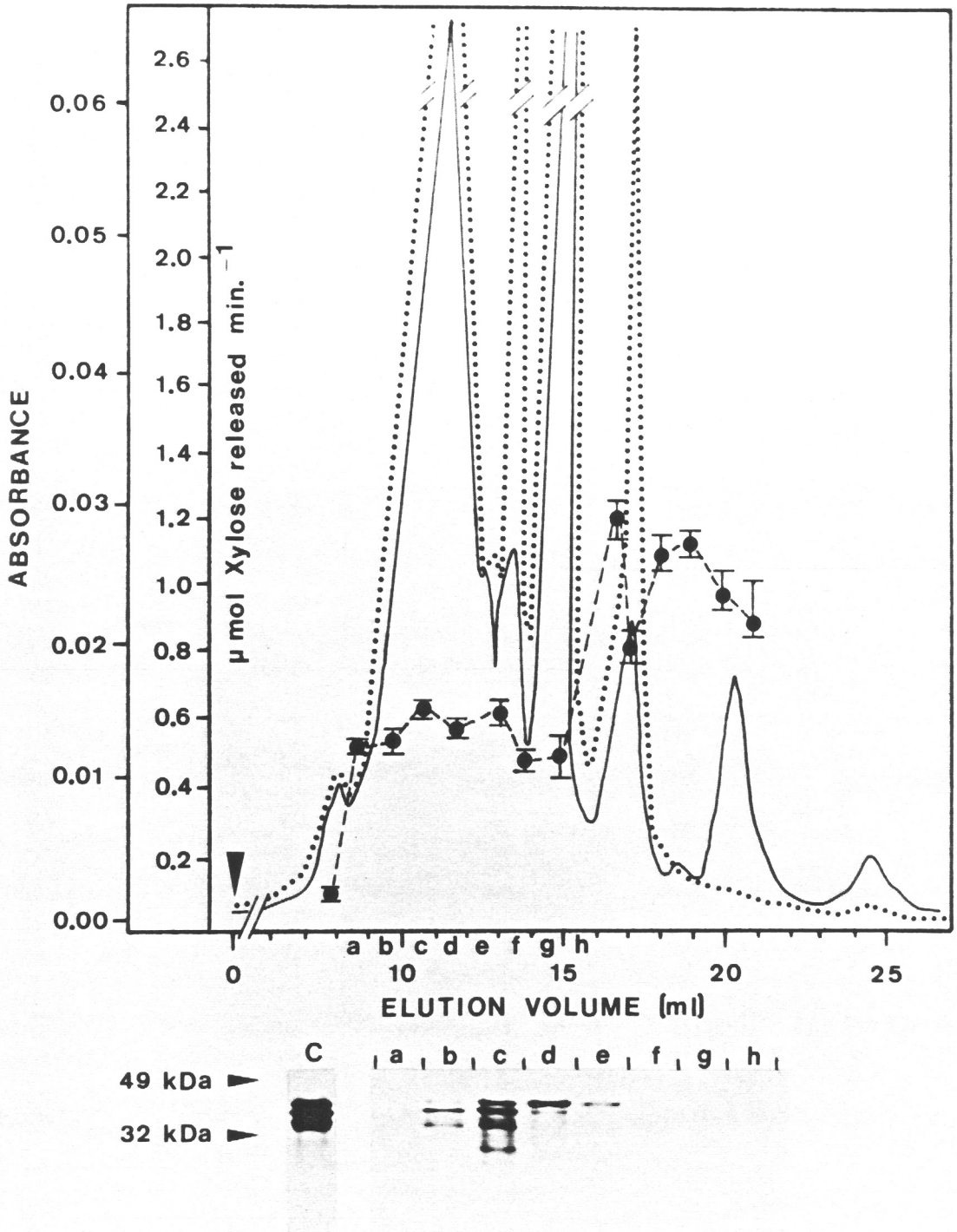

ABSORBANCE
0.06
0.05
0.04
0.03
0.02
0.01
0.00
µ mol Xylose released min.$^{-1}$
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
a b c d e f g h
0
10
15
20
25
ELUTION VOLUME (ml)
C
a b c d e f g h
49 kDa
32 kDa

Figure 22. **Urea NP-40 extract of desiccated *Nostoc commune* TEN contains WSP, UV-absorbing complex and the reduced and oxidized forms of scytonemin.** **A**. Spectral scan of Urea NP-40 extract indicating presence of protein (280 nm), UV-absorbing complex (310 nm), oxidized form of scytonemin (435 nm), reduced form of scytonemin (484 nm) and phycobiliproteins (668 nm). Western blot of aliquots from extract, *E*, and fractions *a*, *b*, and *c* from Mono Q profile. **B**. Elution profile of 2 ml of Urea NP-40 extract (approximately 0.15 gm dry wt. of desiccated material) from a Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A) with a flow rate of 1 ml min^{-1}. Profile developed with gradient buff B (buffer A, 1M LiCl, 1% NP-40, ▬). Absorbance of each fraction was determined simultaneously on a Shimadzu spectrophotometer at 280 nm (Ɪ), 310 nm (▼), 435 nm (⊙) and 493 nm (■). **C**. Silver stained SDS-PAGE gels of aliqouts from Mono Q profile. ● indicates location of phycobiliproteins.

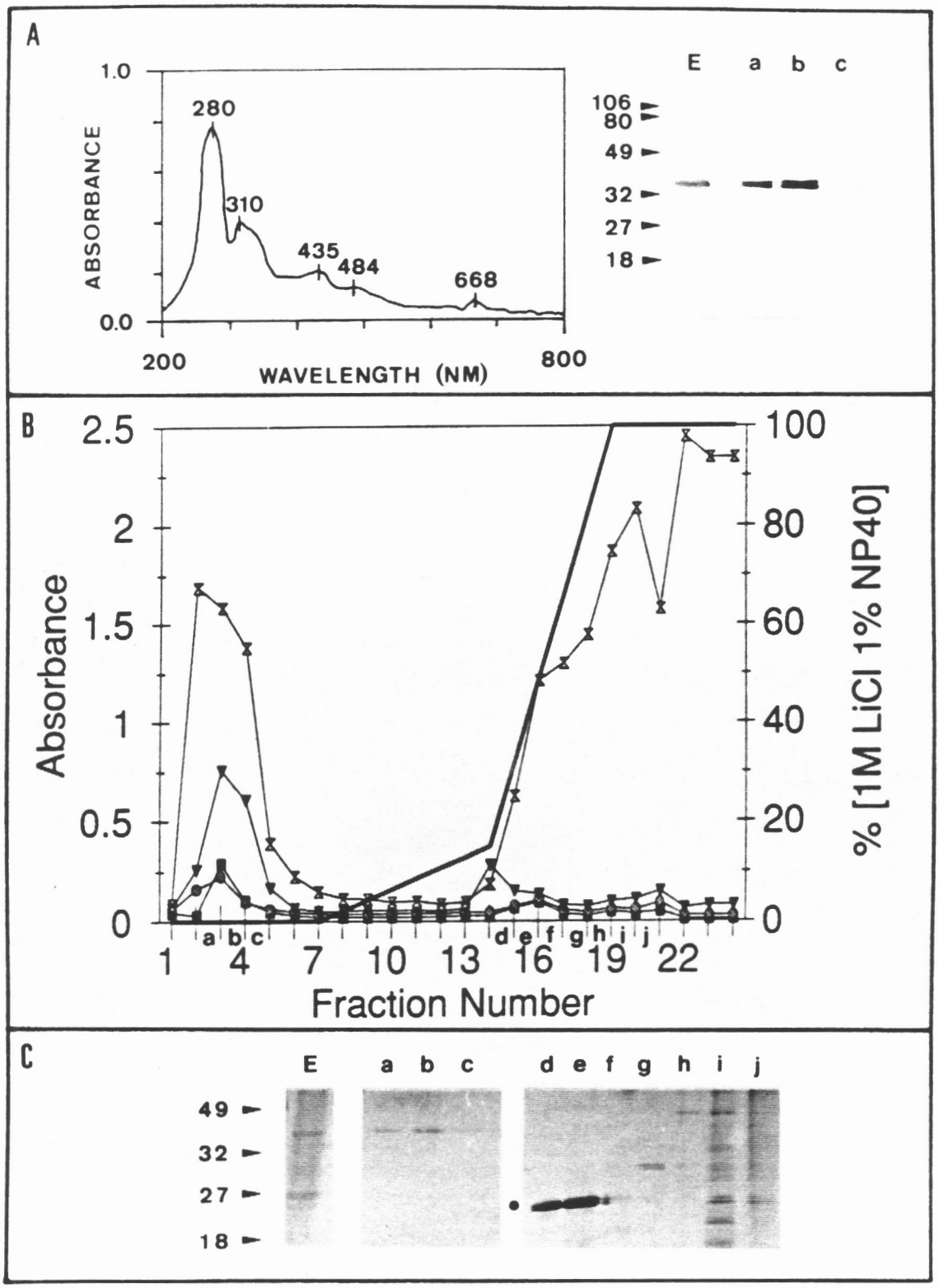
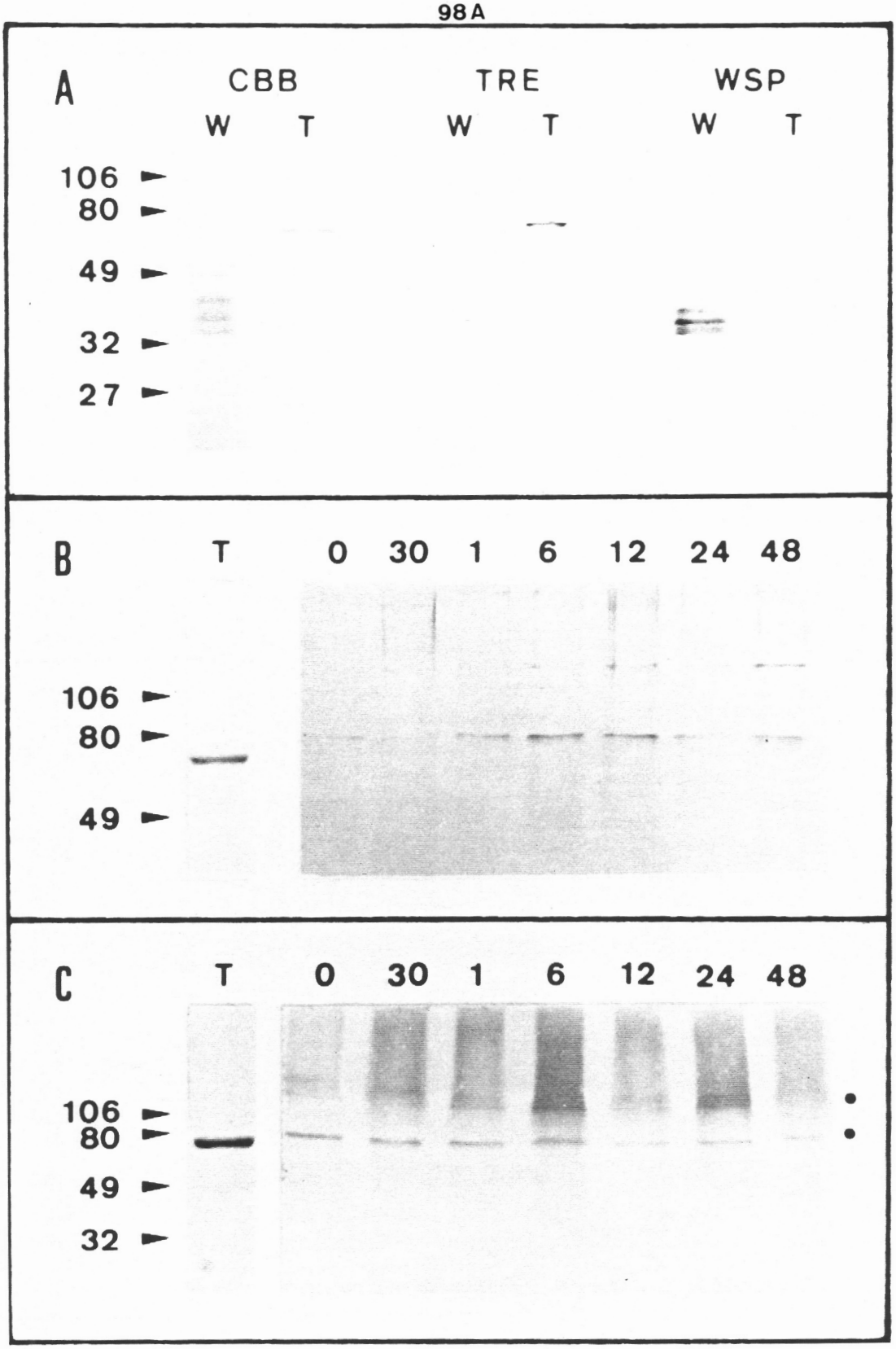

A
1.0
0.0
ABSORBANCE
280
310
435
484
668
200
800
WAVELENGTH (NM)
E a b c
106
80
49
32
27
18
B
2.5
2
1.5
1
0.5
0
Absorbance
100
80
60
40
20
0
% [1M LiCl 1% NP40]
a b c d e f g h i j
1 4 7 10 13 16 19 22
Fraction Number
C
E a b c d e f g h i j
49
32
27
18

Figure 23. **Detection of a Cyanobacterial Trehalase in *Nostoc commune*.** **A**. In corresponding CBB stained SDS-PAGE gels and Western blots using the trehalase and WSP antibodies, where W represents an aqueous extract from CHEN and T a purified trehalase from the shock fluid of *E. coli* K12 strain pTRE1 it is clear that WSP and Trehalase are not the same protein. **B**. Western blot using trehalase antibodies of total cellular extracts (3 μg of protein per lane) from a time course of rehydration (0, total desiccated; 30, 30 minutes rehydration; 1 through 48, 1 to 48 hours of rehydration). T is positive control of purified trehalase from *E. coli*. **C**. Glycan stain (lanes 0 through 48) of identical samples used for trehalase blot in panel **B**. The two glycoproteins from the total cellular extracts here are also present in the aqueous extracts in figure 14 (●), indicating that they are located in the aqueous soluble fraction of the glycan matrix. Lane T in **B** and **C** are identical and T is being used only as a reference point in **C**.


A
CBB
TRE
WSP
W
T
W
T
W
T
106
80
49
32
27
B
T
0
30
1
6
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24
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C
T
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4. DISCUSSION

4.1 Form and Structure of the Glycan.

The extracellular glycan of *N. commune* is abundant, it is both structurally- and chemically-complex, it effectively isolates the cells within the colony from their immediate environment, and it undergoes physical and biochemical changes some of which are marked and others subtle, in response to the principal environmental variable of water availability. What is the nature of this glycan and its components? The structures present in the immediate environment of each filament of *N. commune*, that is the S_1 (envelope) layer, requires a critical appraisal. Extracellular polysaccharides, such as capsules, tend to have a very low affinity for various dyes, and work using electron microscopy has established that in general they are less electron dense than the cell wall and the cytoplasm (Roth 1977). It is widely stated, largely on the basis of what seems to be a good deal of anecdotal evidence, that certain structures observed in the electron microscope are artifacts and the result of the "collapse" of cellular material during fixation and embedding. Particular controversy has surrounded the interpretation of the capsular polysaccharide (M antigen, colanic acid) of *Escherichia coli* (Roth 1977). The data of Schmid (1981), however, clearly document that in dehydrated and fixed cells the capsule is readily observable with the light microscope but when using the very same sections the capsule cannot be imagined with the electron microscope. The staining properties of the S_1 envelope layer of *N. commune* suggest that it lacks both lipid and protein.

In desiccated colonies the contents of the envelope are removed during the preparation of sections for examination by SEM but not during their preparation for analysis with light microscopy or TEM. Since the only major difference in these procedures is that SEM requires a critical point drying step these data suggest that the envelope layer contains material that is either lost or rearranged, most likely one or more carbohydrates, and it is acidic in nature as determined through Alcian blue staining. "The basis of the staining reaction of Alcian blue is debated. What is known is that staining with Alcian blue is blocked by methylation, the stain carries 2 or 4 positive charges that bind to polyanionic mucosubstances and, the more acidic the target, the less intense is the staining", Pearse 1980. The envelope contents were either not immunogenic in mice, and thus may be of low molecular mass, or they were not present in the antigen complex that was used to generate the "sheath" antibodies. Trehalose and sucrose were neither detected in the glycan aqueous extraction nor were they present in the antigen complex that was used to generate the "sheath" antibodies. It is conceivable, however, that the envelope space contains free sugars that are soluble only in a non-polar solvent.

Desiccated colonies contain only 4-5% water, yet during all the time of this study of *N. commune* shrunken cells or cells which show any degree of structural aberration or damage were never observed. Could the envelope layer perhaps be a glass? The glassy state arises through interactions between water and different solutes in response to temperature. Glasses are metastable, they are viscous, and they are either supercooled or supersaturated

(Burke 1986). Because glasses are viscous, they should impede or stop all chemical reactions that require molecular diffusion and thus they may contribute to dormancy and stability over time (Mackenzie 1977). The significance of glasses is that, in principal, the complete dehydration of bacterial cells may be avoided at temperatures below the melting point of the glass. Glasses are expected to have lower water vapor pressures than the corresponding crystalline solid and therefore they may add resistance to further dehydration of the system. The glass transition temperatures and phase relations for several saccharide-water systems have been reported (Green and Angell 1988), however the distribution and form of glasses in complex biological systems, such as bacterial cells, remain poorly understood. In reference to the previous speculation it is notable that both trehalose and sucrose have been identified as glass forming sugars.

The enhanced staining and fibrous appearance of the envelope layer following different periods of rehydration (e.g. Fig. 6C and D; Fig. 8D) may be interpreted in two ways. During rehydration either the contents of the envelope undergo some degree of polymerization or, there is some encroachment of the glycan into the envelope. The latter could result either from *de novo* synthesis of the glycan or through a swelling of shrunken and compacted layers at the periphery of the envelope (see Fig. 6). In the TEM the latter appears as a dark layer upon post-staining with gross appearance of a membrane (*IL*, Fig. 6C). In this study it is documented that this layer is associated with a discrete localization of Wsp polypeptides (Fig 3E). In fully rehydrated cells there is either no obvious envelope (interface) layer or only a

very thin one (Fig. 6E). However, in rehydrated cells a transition from loosely dispersed fibrils to densely aggregated fibrils marks a boundary that corresponds, in its dimensions, to the perimeter of the envelope layer of desiccated cells (see Figs. 7D and 8D). Observations, during this study, indicate that the conspicuous envelope layer, present in desiccated cells, develops during the drying of colonies, a process that may take several days to reach completion (Scherer et al. 1984).

In desiccated cells the bulk glycan is constricted at the cell cross walls but does not come into physical contact with the cells or filaments (Figs. 6A and 6B). In contrast, the glycan is closely appressed to the envelope layer. The envelope layer thus represents the last refuge of *N. commune* from its air-dried environment and it is the ultimate transducer of those forces which are caused by the shrinkage and/or swelling of the bulk glycan in response to water availability. Neither a retraction of the glycan from the envelope layer nor a retraction of the envelope layer from the cell outer membrane were oberved in this study (Fig. 6). These observations suggest that the envelope layer, the bulk glycan and the ribs of glycan that appear in a regular pattern around the inner surface of the "tunnels" and which are visualized in SEM (Fig. 5B), are components of a stress-bearing structure. The structure makes it possible to retain the spatial organization of the filaments throughout the glycan during air-drying and prevents cell separation and collapse of filaments in the air-dried glycan. As such, the glycan makes it possible to exercise a degree of control and uniformity over the rate of shrinkage and swelling of the glycan, and thus the extent to which pressure and shear

forces are imposed upon *N. commune*, in response to water availability. The pattern of ribs on the surface of each "tunnel" resembles the structures of sheath materials that have been described for free-living cyanobacteria and described in the classical phycological literature (e.g. see Figs. 507 and 527h in Geitler 1932). Also, the apparent constriction of the tunnels, i.e. their absence at heterocysts, forms structures (Fig. 8A) which are identical to the growth forms that arise during the life cycle of *N. commune* strain UTEX 584 in liquid culture (Fig. 9A; Potts and Bowman 1985; Hill and Potts in preparation). Furthermore, the envelope layer of *N. commune* cells is similar in its position, staining characteristics in TEM and in thickness, to other non-staining layers observed in vegetative cells, akinetes (Dauerzellen or resting stages) and mature heterocysts of other cyanobacteria (Sutherland et al. 1979; Bergman et al. 1985; Cox et al. 1981; Caiola et al. 1993). Differences in the ultrastructural appearance of the extracellular investments of *N. commune* have been commented by Bazzichelli et al. (1985, 1986, 1989) and have been used to define the different stages in the life cycle of this cyanobacterium. Two components of the investment were recognized from the perspective that the extracellular investment is a colloid system with fluid and fibrillar components. The terms "slime" and "sheath" were considered to represent, respectively, states of sol and gel with their clear connotations in structural analysis. The slime was defined as a structure with the characteristics of a corpuscular colloid in the state of sol, without structure and at unlimited dilution, and the sheath as a reticular colloid in the state of gel and at limited swelling.

4.2 Components of the Glycan

A colorless, water-soluble pigment with an absorption maximum at 312 nm, and a yellow lipid-soluble pigment with an absorption maximum at 435 nm, represented the two major classes of UV-absorbing components in the glycan. Both have been described in *N. commune* and in other cyanobacteria (Garcia-Pichel et al. 1991; Scherer et al. 1988; Proteau et al. 1993). The latter has the spectral properties of scytonemin, a 544 mol. wt. dimeric molecule of indolic and phenolic subunits, known only from the extracellular sheath materials of certain cyanobacteria (Proteau et al., 1993). The former is a complex mixture consisting of two chromophores linked to galactose, glucose, xylose and glucosamine (Böhm et al. in preparation). The 335 nm and 312 nm chromophores are 1,3-di-amino-cyclohexan and 3-amino-cyclohexan-1-on derivatives, respectively. Either alone, or in combination, scytonemin and mycosporines afford protection from incident solar irradiation (Garcia-Pichel and Castenholz 1991, 1993; Garcia-Pichel et al. 1993). The unique property of the *N. commune* mycosporine derivatives is that their chromophores are linked to carbohydrate (Böhm et al. in preparation). It is difficult to assess the degree of cross linking, if any, between the UV-absorbing pigments and the glycan, and this structural feature is presently under investigation. The mycosporine compounds may constitute up to 10% by dry weight of desiccated colonies and their release upon rehydration constitutes a significant loss of cellular carbon and nitrogen. In contrast, scytonemin is not lost upon aqueous extraction of desiccated material. The banding pattern of scytonemin, noted in colonies, may represent the

vestiges of the old surface of the colony as the colonies have grown, or some structural phenomenon associated with swelling and shrinkage of the colony. The strategy of synthesizing both water-soluble and lipid soluble components is clear. The former can saturate the glycan compartment rapidly upon rehydration but at the expense of a high loss of the pigment, while scytonemin provides a more localized screening with the advantage that the pigment is retained by the colonies. Scytonemin may likely have a much more important role than the aqueous UV-absorbing pigments during the protection of cells upon dispersal of colony fragments.

Sucrose and trehalose are employed as compatible solutes by a range of different organisms, including cyanobacteria, as a means to adjust intracellular water deficit (Hershkovitz et al. 1991). Sucrose and trehalose were both present in the non-polar extracts of desiccated field materials of *N. commune*, and reappear, albeit at much reduced levels, in desiccated pearls (data not shown). The disappearance of trehalose from cells matched the time at which a putative trehalase was detected in cell extracts using Western blotting of total cellular extracts. No trace of the protein trehalase or the sugar trehalose was found in any of the aqueous extracts. This is consistent with the facts that the enzyme trehalase is an intracellular enzyme (Boos et al. 1987). It is clear that there is a correlation between the disappearance of the sugar, trehalose, and the appearance of the enzyme, trehalase, upon rehydration of desiccated field material of *N. commune*. However, the significance of this to desiccation tolerance in cyanobacteria is unknown at this time.

The secretion of proteins by cyanobacteria has been documented in a number of studies but the functions and characteristics of these extracellular polypeptides remain poorly understood (Scanlan and Carr 1988). The criteria used to assess whether proteins are extracellular include whether the proteins are substantially and selectively enriched in cell-free media and, specifically for the cyanobacteria, whether the protein extract is completely devoid of phycobiliproteins (Scanlan and Carr 1988). The latter, in view of their general abundance, visible spectra and high extinction coefficients, provide an extremely sensitive measure of cell lysis. In addition to the complete lack of phycobiliproteins in aqueous extracts of desiccated colonies, it has been documented that filaments of *N. commune* retain both their integrity during desiccation and subsequent rehydration and constitute the only cells present in the glycan sheath (Peat et al. 1988). Scanning and transmission electron microscopy utilized in the present study confirmed the structural integrity of desiccated cells. As such, the data confirm that Wsp, the most abundant, water-soluble, protein in desiccated *N. commune*, is secreted and distributed throughout the extracellular glycan sheath. The patterns observed after immunogold-labelling of thin sections must, however, underestimate the actual extent of Wsp distribution because of the finite time required to fix the dehydrated material with aqueous glutaraldehyde. The latter procedure leads to leaching of Wsp from the colonies. However, as the cells were judged to be efficiently fixed under these conditions the discrete and reproducible labelling pattern observed around the periphery of cells may be evidence for a fraction of Wsp that is not removed from the sheath during rehydration.

The 33, 37, and 39 kDa Wsp polypeptides are immunologically-related, the amino acid sequences at their amino-termini are identical, their peptide maps are very similar, and they all appear to lack methionine residues (Table 2). Wsp polypeptides have very similar amino acid compositions (Scherer and Potts 1989). No evidence for glycosylation or phosphorylation of Wsp proteins was observed in this study. Fluorometric analysis indicated the carbohydrate content of electroeluted Wsp polyptides to be no greater than 1 mole glucose equivalent per mole of Wsp. The purified and denatured polypeptides showed no absorbance at 312 nm. Sequence analysis suggested that the amino-terminal residue of each polypeptide was unblocked, mild acid hydrolysis did not result in any change in mobility of the polypeptides, and all lacked a spectrum in the visible region. The possibility that the three polypeptides arise through the covalent modification of a single protein is, therefore, unlikely. The possibility that the 33 and 37 kDa polypeptides arise through the amino-terminal processing of the 39 kDa polypeptide is discounted on the basis of the protein sequence data. Our conclusion is that the Wsp polypeptides are either the products of separate genes (isoenzymes), or they arise through processing (proteolysis) at their carboxyl termini as has been described for the extracellular proteases of several Gram-negative bacteria (Terada et al 1990). An appraisal of the former possibility must await completion of DNA sequence analysis. Evidence for the latter possibility is the observation of a selective loss of the 39 kDa polypeptide in total cell extracts lacking protease inhibitors (see Fig. 5 in Hill et al. 1994). The three polypeptides may arise from a single precursor protein - a possibility given the fact

that Wsp is secreted - although no Wsp polypeptides greater than 39 kDa, other than aggregates of the monomers, have ever been observed in this study.

Accumulations of Wsp, the principal soluble protein, and the secreted UV-A/B absorbing pigment, discussed previously, represent major biochemical investments on the part of desiccation-tolerant *N. commune*. Both of these products are water-soluble, they accumulate in the extracellular glycan in substantial amounts, and they are released upon rehydration of desiccated colonies. The data indicate that strong ionic interactions influence the apparent molecular masses of Wsp- and UV-absorbing pigment-complexes in rehydration fluids. In the presence of salt Wsp polypeptides appear to exist as monomers. In the absence of salt both the Wsp polypeptides and UV-absorbing pigments undergo an apparent increase in molecular mass suggestive of some aggregation. There was an obvious stoichiometry in the aggregation (dimerization) and no evidence for precipitation of the complexes was observed. These interactions might involve negatively-charged components, and the presence of such components might explain why the greater fraction of Wsp polypeptides - with pI values of 4.3 to 4.8 (Scherer and Potts 1989) - were unable to bind to Mono Q resin at pH 7.4 (Fig. 19). Prokaryotic glycoproteins appear to be of limited occurrence and only for one example, the extracellular wall component of *Halobacterium* spp. (Lechner and Wieland 1989), has the glycoprotein identity been confirmed with certainty. Recent reports of intracellular glycoproteins (phycobilisome linker polypeptides) in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (Riethman et al. 1988) have been discounted after critical

scrutiny (Fairchild et al. 1991). No evidence for glycosylation or any other detectable modification of Wsp was obtained in this study.

Partial sequence analysis of Wsp polypeptides identified a region with conspicuous homology to a bacterial β-xylosidase (Lüthi et al. 1990). Rehydration fluids, enriched in Wsp, had a weak xylosidase activity but a pronounced xylanase activity. Following purification of the extracts by liquid column chromatography the xylanase activity was found in fractions that contained Wsp as well as fractions that lacked any immunogenic material. Aggregation of Wsp- and UV-pigment-complexes (in the absence of salt) lead to reduction in the xylanase activity and the resultant activity was then detected in fractions that contained or lacked Wsp. These data suggest that the xylanase activity cannot be not attributable to Wsp, rather they suggest that Wsp may form part of a protein complex that has the activity. Other proteins are present in rehydration fluids albeit at low concentrations. At least one of these, the 68-kDa protein, was partially characterized in this study, and also shows sequence homology with carbohydrate-modifying enzymes. The fact that Wsp antiserum did not inhibit the activity of a purified xylanase but caused a 10 fold decrease in the xylanase activity of rehydration fluids. We concluded that Wsp is associated with, but not directly responsible for, the xylanase activity. Purified Wsp polypeptides showed negligible hydrolysis of o-paranitrophenyl β-D-xylopyranoside although the possibility that a secondary xylosidase activity of Wsp may be present that was undetected under these assay conditions with the substrate in question cannot be discounted. Xylosidases and xylanases both contribute to the

hydrolysis of polysaccharides that contain 1,4-β-D-xylosyl residues, and they often exist in multiple enzyme forms (Wong et al. 1988). The basic repeating unit of polysaccharides from heterocysts and spore envelopes of the filamentous cyanobacterium *Anabaena* sp. ATCC 29414 contain xylose (Cardemil and Wolk 1979). Although a discrete localization of Wsp at the periphery of the envelope of both vegetative cells and heterocysts of *N. commune* is reported here (Fig. 3), the purified glycan of desiccated colonies, and that of cultures of *N. commune* DRH 1, contained no xylose (Fig. 10; Appendix 5). The UV-A/B-absorbing pigments of *N. commune* have a polysaccharide core and the structure of one of the chromophores has recently been discribed - it does contain xylose (Böhm et al., in preparation). While these data may suggest some relationship between Wsp and the UV-absorbing pigment, it is not possible to state at this juncture whether Wsp polypeptides and UV-absorbing pigments do aggregate specifically (bind one another), or whether these molecules simply have very similar size and charge properties under the different conditions used in our experiments. In addition these interactions may also involve other components of the glycan - the glycan and UV-absorbing pigment both contain amino sugars and Wsp clearly has similarities (both immunogenic and at the protein sequence level) to proteins that bind such residues.

4.3 Functions of the Glycan

One principal function of the glycan is that it provides a repository for water. The glycan represents a mixed system, the water and gel tend to mix as thoroughly as they can for thermodynamic reasons (Wiggins 1990). Work is required to remove water from the gel and this can be lost through the application of pressure, increased temperature, and through evaporation. This provides one explanation for the striking form of the colonies of *N. commune* DRH1. A sphere represents the minimum surface area for a given volume which would clearly provide a reduction, and uniformity, in the net rate of evaporation of water. Of course for the same considerations the spherical glycan surface provides a reduced capacity for gas uptake. Cyanobacterial sheath materials have been noted to retard gas exchange (Chang 1980). As such the outer layer of the colonies may act as a membrane which is under pressure from the bulk glycan.

Previous studies of cyanobacterial envelopes and sheath structures have suggested that these may serve to concentrate metals and thus may aid in excluding the colonies from predation by gastropods, and insects etc. (Tease and Walker 1987). Although microorganisms are certainly present at the surfaces of the *N. commune* colonies, the outer silicon-rich layer represents an impenetrable barrier for them. The silicon-rich layer must be made through physico-chemical precipitation as it is hard to account for a concerted synthesis of this layer on behalf of the cells. More likely the layer is the product of some oxygen/drying dependent effect on the peripheral sheath, although in liquid cultures of *N. commune* strain

DRH1 a discrete pellicular structure is also seen.

The glycan represents the bulk of the colony and constitutes a considerable diversion of the carbon and nitrogen budget (Ernst et al. 1987). The UV pigments represent another sizeable fraction of the dry weight, as well as the most abundant protein in the sheath, the water-soluble Wsp; both of which are secreted from the cells. Not surprisingly, the synthesis of Wsp and UV-pigment may be related. The finding of abundant Wsp in materials collected at different times from numerous locations over a period of some five decades implies that first, Wsp synthesis is constitutive *in situ* and second, the protein is very stable in desiccated colonies. One consequence of the secretion of Wsp by *N. commune* is that it must result in the loss of a substantial amount of cellular nitrogen and carbon during rehydration of colonies. This nutrient loss is surprising considering the extreme habitats colonized by this cyanobacterium (Whitton et al. 1979). It remains to be determined whether *N. commune* has scavenging mechanisms for any or all of these components or their degradation products. In the laboratory, liquid cultures of *N. commune* DRH1 secrete substantially less amounts of Wsp, and the protein was not detected in cell-free culture supernatants from a number of other cyanobacterial species including *N. commune* UTEX 584. Extracts from such cultures also fail to absorb in the region of the spectrum between 310 to 341 nm (Scherer et al. 1988). A proteolytic activity that was specific for the 39 kDa form of Wsp has been identified, although the suggestions are that this activity is intracellular in origin (Hill et al. 1994). One other protein isolated and characterized in the present study (68 kDa) is secreted, abundant

in aqueous extracts of the glycan and, like Wsp, appears to be related to carbohydrate-modifying enzymes.

There is striking uniformity in the glycan isolated from materials collected from a range of different climatic environments. These are characterized by extended periods of desiccation and often rapid, and intermittent, periods of rehydration. The extracellular glycan appears to represent a buffer zone between the atmosphere and the cells. The prodigious investments made in sheath synthesis and those components found within the sheath, and the interpretation of structure and composition reported here suggest the principal role for the glycan. It is a control component of the mechanisms used by *N. commune* to tolerate desiccation. These mechanisms will be uncovered through understanding of mode of synthesis of the glycan and of those components, such as Wsp and UV-absorbing pigments, present within it.

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APPENDIX 1

Growth Media Used for Nostoc commune.

For all cultures of *Nostoc commune* either BG ll or BG ll_0 media was used. In case of growth on plates either BG $ll_{(o)}$ in 1.2% w/v agar was used or BG $ll_{(o)}$ in 1.2% w/v agar calcium carbonate plates. For the calcium carbonate plates, add 0.5% w/v calcium carbonate and 0.5% w/v magnesium carbonate to media prior to autoclaving. Prior to pouring the calcium carbonate 1.2% w/v agar plates, add 5 ml of sterile filtered (2 µm acrodisc filter) potassium bicarbonate solution (0.05 gm ml^{-1}) to media, to give final bicarbonate concentration of 3 mM. Volume of stock solutions given for making one liter of BG ll or BG ll_0 media.

MEDIA COMPONENT	CONC	STOCK SOLN	VOLUME $liter^{-1}$
$MgSO_4$ - $7H_2O$	0.075 gm l^{-1}	4.5 gm 300 ml^{-1}	5 ml
$CaCl_2$ - $2H_2O$	0.04 gm l^{-1}	2.4 gm 300 ml^{-1}	5 ml
$NaNO_3$ **(omit for BG ll_0)**	1.5 gm l^{-1}	90.0 gm 300 ml^{-1}	5 ml
K_2HPO_4 anhydrous	0.031 gm l^{-1}	1.86 gm 300 ml^{-1}	5 ml
Citirc acid	0.006 gm l^{-1}	0.36 gm 300 ml^{-1}	5 ml
Ferric ammonium citrate	0.006 gm l^{-1}	0.36 gm 300 ml^{-1}	5 ml
Na_2Mg EDTA	0.001 gm l^{-1}	0.06 gm 300 ml^{-1}	5 ml
Na_2CO_3	0.02 gm l^{-1}	1.2 gm 300 ml^{-1}	5 ml
A-5 trace metals	1 ml	(see below)	1 ml
$CaCO_3$	0.5% w/v		
$MgCO_3$	0.5% w/v		

A-5 trace metals solution:	H_3BO_3	2.86 gm l^{-1}
	$MnCl_2$ - $4H_2O$	1.81 gm l^{-1}
	$ZnSO_4$ - $7H_2O$	0.22 gm l^{-1}
	Na_2MoO_4 - $2H_2O$	0.039 gm l^{-1}
	$CuSO_4$ - $5H_2O$	0.079 gm l^{-1}
	$Co(NO_3)_2$ - $6H_2O$	0.049 gm l^{-1}

APPENDIX 2

Microscopy Recipes and Protocols.

All chemicals and supplies purchased from Polysciences, Inc. or Sigma Chemical, CO., unless otherwise stated

Buffers and fixatives for microscopy.

Fixative - 2% w/v gluteraldhyde 2 % w/v paraformaldehyde in 0.2 M sodium cacodylate buffer, pH 7.0 (also known as Karnovsky fixative).

for 50 ml:

25% paraformaldehyde soln	1 ml
25% gluteraldehyde soln	1 ml
0.2 M Na Cacodylate buffer	9.5 ml
$CaCl_2$	0.125 mg

1) dissolve 1 gm paraformaldhyde in 20 ml d. H_2O by heating, clear by adding 1N NaOH dropwise, swirl (usually 1-2 drops).

2) filter solution into graduated cylinder containing
25 ml 0.2 M Na Cacodylate (1.07 gm) buffer, pH 7.0
2 ml 50% Gluteraldehyde (12.5 ml of 8%)
1 ml $CaCl_2$

<u>0.2 M Na Cacodylate buffer</u>
42.8 gm $liter^{-1}$ or 1.07 gm 25 ml^{-1}, pH to 7.0

<u>1% w/v Calcium chloride solution</u>
1 gm $CaCl_2$ 100 ml^{-1}

Fixative - 1% w/v Osmium tetroxide consist of 1 gm of OsO_4 in 100 ml of 0.1 M Na Cacodylate buffer, pH 7.0. ****Osmium tetroxide is very dangerous and should be handled with extreme caution****.

Resins for electron microscopy.

Poly/Bed 812 Resin Luft's recipe:

component	vol. measure	weight measure
Poly/Bed 812	21 ml	22.17 gm
DDSA (dodecenylsuccinic anhydride)	13 ml	11.37 gm
NMA (nadic methyl anhydride)	11 ml	11.63 gm
DMP-30	0.7 ml *add just before use	

Avoid contact. Prolonged and repeated contact of liquid or breathing vapors or mists may cause delayed and serious injury. Wash hands immediately after working with resin.

Lowicryls, low temperature embedding resin protocol:

A representative dehydration schedule for ethanol, all at 4 °C.

ethanol, vol. %	time	
15%	20 min.	
25%	20 min.	
40%	20 min.	
50%	20 min.	
70%	overnight	← for light microscopy sample
80%	1 hour	taken at this point.
90%	3 hours	
100%	1 hour	
100%	1 hour	

Resin: ethanol infiltration schedule, all at 4 °C.

vol.:vol.	time
1:1	12 hours
2:1	12 hours
Pure resin	12 hours

Resin cured for four days under UV light at 4 °C.

Resin recipe K4M:

component	weight measure
Crosslinker A	2.70 gm
Monomer B	17.30 gm
Initiator C	0.10 gm

Standard protocol for light microscopy tissue preparation.

Standard dehydration infiltration series (under vacumn using automatic processor):

component	vol. %	time
Ethanol ➡	70%	25 min.
	80%	10 min.
	95%	10 min.
	95%	10 min.
	100%	25 min.
	100%	10 min.
	100%	20 min.
Xylene ➡	100%	10 min.
	100%	15 min.
	100%	15 min.
Paraffin ➡	100%	20 min.
	100%	50 min.

All samples are then paraffin embedded into blocks by hand. Sections (15 μm) are cut with standard stainless steel razor knifes used with a hand cranked microtome.

Staining protocols and recipes for light microscopy.

In order to stain a sample in paraffin, the paraffin must first be cleared with xylene. Most dye binding stains require the presence of water. Therefore, the dehydrated sections must be rehydrated, stained and then dehydrated again to prevent degradation of the section. This is accomplished with the following protocol (slides, with sections adhered with chrom alum, are placed in glass racks and all steps are performed at room temperature in glass containers):

component	time	
Xylene 100%	5 min.	⇦ step which clears away paraffin
95% Ethanol	5 min.	
80% Ethanol	5 min.	
70% Ethanol	5 min.	
50% Ethanol	5 min.	
Water 100%	2 min.	

Appropriate staining protocol to be performed at this time.

50% Ethanol	5 min.

70% Ethanol	5 min.	
80% Ethanol	5 min.	
95% Ethanol	5 min.	
100% Ethanol	5 min.	
Xylene 100%	5 min.	⇦ sections can now be air dried and mounted with coverslip if desired

Alcian blue method for acid mucoploysaccarides:

1) bring sections to water
2) stain in freshly filtered 1.0% w/v solution of Alcian blue 8GX in 3% v/v acetic acid for 10 to 30 minutes.
3) rinse in distilled water
4) stain in Ehrlich's Haematoxylin for 5 to 10 minutes
5) differentiate in 1% ethanol
6) wash in running water for 10 to 20 minutes
7) dehydrate in ethanol, clear in xylene and mount

Results - acid mucopolysaccarides stain blue in color.

Alcian blue pH 2.5 procedure:

1) bring sections to water
2) stain in freshly filtered 1.0% w/v Alcian blue 8GX in 3% v/v acetic acid, pH to 2.5, for 30 minutes.
3) wash in running water for 5 minutes
4) counterstain in carmalum for 1 to 3 minutes
5) dehydrate in ethanol, clear in xylene and mount

Results - weakly acidic mucopolysaccharrides stain a dark blue, while strongly acidic mucopolysaccharrides stain weakly or not at all.

Periodic acid-Schiff reagent procedure:

1) bring sections to water
2) rinse in 70% ethanol
3) immerse in Periodic Acid solution for 5 minutes
4) rinse in 70% ethanol
5) immerse in Reducing bath for 1 minute
6) rinse in 70% ethanol
7) immerse in Schiff's reagent for 20 minutes
8) wash in running water for 10 minutes

9) stain with Celestin blue for 2 to 3 minutes, followed by Ehrlich's Haematoxylin for 2 to 3 minutes
10) differentiate in 1% v/v acid (HCl) ethanol
11) wash in running water for 30 minutes
12) dehydrate in alcohol, clear in xylene and mount

Results - hexose-containing mucosubstances (1,2-glycols) stain a deep purplish-red, nuclei a blue-black and acidophilic proteins a yellow color.

Periodic Acid

Dissolve 0.4 gm of periodic acid (HIO_4 - 2 H_2O) in 35 ml of reagent ethyl alcohol and add 5 ml of 0.2 M sodium acetate (27.2 gm of hydrated salt in 1 liter) and add 10 ml of distilled water. This solution should be kept in the dark at 17-22 °C and used at this temperature. Solution should be discarded if brown color appears.

Reducing Bath

Dissolve 1 gm of potassium iodide (KI) and 1 gm sodium thiosulphate ($Na_2S_2O_3$ - $5H_2O$) in 30 ml of reagent ethyl alcohol and 20 ml of distilled water. Add 0.5 ml of 2 N HCl (20% v/v of conc. HCl). A deposit of sulfur forms which can be ignored. Solution should be stored between 17-22 °C, the shelf life is 14 days (no longer).

Schiff's Reagent

Dissolve 1 gm basic fuschin in 200 ml of boiling distilled water. Shake for 5 minutes, cool to 50 °C and filter. To filtrate, add 1 ml of 1 N HCl. Cool to 25 °C and add 1 gm of sodium thiosulfate ($Na_2S_2O_3$). Stand in dark for 14-24 hours. Clear by shaking 1 minute after the addition of 2 gm of activated charcoal, filter. Store in dark at 0-4 °C. Use in dark at room temperature.

Celestin Blue Solution

Dissolve 2.5 gm of iron alum in 50 ml of distilled water by standing overnight at room temperature. Add 0.25 gm Celestin Blue B (C.I. 51050) and boil for 3 minutes. Filter when cool and add 7 ml of glycerol.

Ehrlich's Acid Alum Haematoxylin

Dissolve 2 gm of Haematoxylin in 100 ml of reagent ethyl alcohol and add 100 ml of distilled water. To this solution add 100 ml of glycerine and 10 ml of acetic acid. Add in excess aluminum potassium sulphate. To ripen, add 0.4 gm of potassium iodate.

APPENDIX 3

Electrophoresis Reagent Recipes and General Protein Gel/Blot Protocols.

All chemicals purchased from Sigma Chemical, Co. unless otherwise noted.

SDS-PAGE recipes

All quantities are given as dry weight of the respective compound. *Bis*-acrylamide is short for N-N'-methylene-*bis*-acrylamide, SDS is sodium dodecyl sulfate (also referred to as Lauryl sulfate) and TEMED is N-N-N'-N'-tetramethylethylene diamine. Amm. Per. is short for ammonium persulfate and should be made up fresh with $d.H_2O$ just before use.

To multicast 4 minigels in the Bio-Rad Mighty Small multi-gel caster:

Component	15%	12%	10%	5%
Acrylamide	7.25 gm	5.80 gm	4.84 gm	1.23 gm
Bis-acrylamide	0.25 gm	0.20 gm	0.17 gm	0.042 gm
Tris-HCl buffer -				
1.5 M, pH 8.0	12.5 ml	12.5 ml	12.5 ml	----------
0.5 M, pH 6.8	---------	---------	---------	3.5 ml
Bring up in $d.H_2O$ to	⇦	49.5 ml	⇨	24.5 ml
	▬▬▬▬▬ degass at this time ▬▬▬▬▬▬			
10% SDS	500 µl	500 µl	500 µl	250 µl
10% Amm. Per.	250 µl	250 µl	250 µl	125 µl
TEMED	25 µl	25 µl	25 µl	25 µl

All gels were run in a Bio-Rad Mighty small SE 250 electrophoresis chamber, using 1.5 mm thick gels. It is important to keep gels cool during electrophoresis with a circulating cold water bath. Typically, gels run at 50-80 mA for 2 to 4 hours.

Buffer recipes

1.5 M Tris-HCl, pH 8.0

Dissolve 181.65 gm Tris (Trizma base) in 1 liter $d.H_2O$, pH to 8.0 with HCl.

0.5 M Tris-HCl, pH 6.8

Dissolve 60.55 gm Tris (Trizma base) in 1 liter $d.H_2O$, pH to 6.8 with HCl.

5X SDS Running Buffer

Dissolve 15 gm of Tris (Trizma base), 72 gm of Glycine, and 5 gm of SDS up to 1 liter with distilled water. Store at room temperature and dilute 1:5 with $d.H_2O$ before use.

5X TBS (TBST), 10X TBS, 20X TBS

Component	5X	10X	20X
Trizma base	12.1 gm	24.2 gm	48.4 gm
NaCl	146.2 gm	292.4 gm	584.8 gm

For all concentrations, dissolve solid in $d.H_2O$, pH to 7.5 with HCl and bring up to 1 liter with $d.H_2O$.

Typically the 5X TBS solution is used the most. Prior to use, dilute 1:5 with $d.H_2O$. For TBST, add 250 µl of TWEEN 20 (polyoxyethylene sorbitan monolaurate) to a 1X TBS solution of 500 ml.

CAPS Buffer

To make 10 mM CAPS in 10% v/v methanol, dissolve 4.425 gm of CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 2 liters of distilled water, degass thoroughly (while proteins are being separated out on gel). Just before use, add 220 ml of 100% Methanol and pH to 10.3 (generally 13.5 ml of 1N NaOH is sufficient).

Extraction (Grinding) Buffer

Component	amount	final conc.
Trizma base	3.027 gm	50mM
$MgCl_2$	0.475 gm	10 mM
KCl	0.746 gm	20 mM
NaN_3	0.033 gm	1 mM
β-mercap	0.035 ml	1 mM

Adjust pH of solution to 7.8 and bring up to 500 ml with $d.H_2O$. Note β-mercap is short for β-mercaptoethanol (14.4 M). If desired can be used with the addition of the protease inhibitor cocktail.

Sample Buffer

Component	amount
β-mercaptoethanol	0.85 ml
10% SDS	10.0 ml
0.5 M Tris-HCl, pH 6.8	8.0 ml
Glycerol	10.0 ml
Bromphenol Blue*	120 µl

Bring the solution up to 50 ml with d.H_2O. *Bromphenol blue used is a 0.05% w/v solution in 100% methanol. Solution may be aliquoted and stored at -20 °C until needed.

Protease Inhibitor Cocktail

The following recipe is for a 200X solution, dilute with extraction (grinding) buffer before use. Store in the dark at 4 °C.

Component	amount	final conc 1:200
50 mM Leupeptin	120 µl	1 µM
1 mM BZA	480 µl	0.07 µM
1 M DTT	600 µl	0.1 µM
200 mM DFP	750 µl	25 µM
0.5 M EDTA	12 ml	1 mM
50 mM Tris-HCl, pH 7.2	15 ml	125 µM
200 mM PMSF	1.5 ml	50 µM

Leupeptin is a protease inhibitor with a FW of 493.6. BZA is Benzamidine, hydrochloride (FW 156.6). DTT is dithiothreitol (FW 154.2). DFP (**extremely hazardous**) is diisopropyl fluorophosphate (FW 184.1, density 1.06 gm ml^{-1}), is diluted in anhydrous propanol. EDTA is ethylenediaminetetraacetic acid (FW 292.2). PMSF is phenylmethylsulfonyl fluoride (FW 174.2).

10% SDS

Dissolve 100 gm of electrophoresis-grade SDS in 900 ml of distilled water. To assist dissolution, heat to 68 °C. Adjust the pH to 7.2 by the addition of a few drops of HCl. Adjust volume to 1 liter. Store at room temperature.

PBS (phosphate buffererd saline)

To make 50 mM potassium phosphate, pH 6.5, 150 mM NaCl, dissolve 8 gm sodium chloride, 0.2 gm of potassium chloride, 1.44 gm of sodium phosphate, dibasic (Na_2HPO_4 - $7H_2O$), and 0.24 gm potassium phosphate, monobasic (KH_2PO_4) in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add water to 1 liter, for storage it is best to autoclave the solution.

Sodium acetate Buffer

To make 100 mM sodium acetate buffer, dissolve 3.4 gm in distilled water. Adjust pH to 5.5 with acetic acid. Bring up to 250 ml with distilled water.

General protocol for PVDF and Western Blotting.

Activation of PVDF membrane

Being careful not to touch the membrane with your hands, activate the hydrophobic sites on the membrane by first wetting it with 100% v/v methanol for 5 seconds and then submerge the membrane in distilled water for 5 to 10 minutes. The membrane should then be equilibrated with the transfer buffer (CAPS) for at least 15 minutes prior to blotting.

Transfer of Proteins

All transfers performed in Hoeffer Scientific TE 22 Transphor electrophoresis unit. After the proteins have been separated (SDS-PAGE), gently rinse the gel in distilled water and then immediately place ("right" side down) on gel blotting paper (Schleicher & Schuell, Gel Blot Paper, no. GB 004) pre-equilibrated with CAPS buffer. Carefully place the equilibrated PVDF membrane on the back side of the gel, this is so after transfer the lanes will read on the blot as you loaded them on the gel. Avoid moving the membrane around after it has come into contact with the gel, proteins are already starting to be absorbed onto the PVDF membrane. Place several sheets of gel blot papers (pre-equilibrated with CAPS buffer) on top of the gel/blot sandwich. With the sandwich, and gel blot papers, submerged in CAPS buffer, gently knead out any air bubbles that may be trapped between the gel and the PVDF membrane. Now carefully place the transfer cassette into the transfer chamber, which contains the remaining CAPS buffer. REMEMBER to be sure that the gel side of the sandwich in the cassette is exposed to the black (negative charge) electrode, cathode. Proteins are going to be negatively charged and therefore move in the direction towards the anode, red (positive charge) electrode. It is important to keep transfer unit chamber cool with circulating cold water. To ensure a homogeneous dispersal of the buffer during transfer, place unit on stir plate and have small stir bar in bottom of chamber. In this present study with Wsp (33-39 kDa), approximately 25 minutes at 450-500 mA is required for complete transfer of the proteins. Smaller molecular weight proteins will require less transfer time, while larger molecular weight proteins will require longer times.

Western Blotting Protocol

After transfer to the PVDF membrane the proteins on the blot can now be screened with an antibody, using the following procedure:

1) Incubate the blot in 1 % w/v BSA in TBST. Note it may be possible to actually see the proteins on the blot as it is placed protein side up on the surface of the BSA solution. The proteins sometimes appear as a sheen on the surface of the PVDF membrane. After submerging the blot, incubate for 30 minutes.

2) Wash the blot in TBST for 5 minutes.

3) Incubate the blot in primary antibody, in this study rabbit-Wsp (675)

1:2000 dilution, overnight.

4) Wash the blot 3 times, for minimum of 10 minutes each, in TBST.

5) Incubate the blot in the secondary antibody, in this study GAR-HRP conjugate 1:1000 dilution in TBST, for 2 to 4 hours.

6) Wash blot 2 times, for minimum of 10 minutes each, in TBST.

7) Wash the blot in TBS (no TWEEN) twice for a minimum of 10 minutes each time. It is important to remove all the TWEEN from the blot because it will interfere with the color development reaction.

8) Start color development as follows -
Solution A: 50 ml TBS with 30 µl of 30% solution of hydrogen peroxide.
Solution B: 10 ml of ice cold 100% methanol with 30 mg of HRP color development reagent (4-chloro-1napthol)

Mix solution A with solution B and then submerge blot. Color development should take between 5 and 20 minutes. Note, if the blot is submerged in one solution or the other prior to the addition of the other solution, will get negative staining of all proteins on blot and background will be almost black. This mistake is irreversible. Proteins of interest should appear as black-brown bands on a white background. Stop color development by washing blot in distilled water several times over a 5 minute period. Place the blot between gel blotting paper to dry.
If it is desired to screen the blot with a different protein after the initial Western screen, simply strip the antibodies from the blot by submerging in 100% methanol for 5 minutes, wash in distilled water and proceed from initial step of Western (1).
It is important to keep a photographic record of all Westerns since the chloro-napthol color development is light sensitive and will fade over time (refer to Appendix 7).

Glycan Stain

After the proteins have been transferred to the PVDF membrane, the proteins on the blot can be screened for glycoproteins. For additional information contact Boehringer Mannheim, cat. no. 1142 372.
Incubate all blots by gently agitation at room temperature, except for color development which should be done without shaking. The procedure is as follows:

1) Wash the blot in ~50 ml PBS, pH 6.5. DO NOT USE TBS AT THIS TIME.

2) Oxidize the hydroxyl groups in sugars of the glycoconjugates to aldehydes by incubating in 10 mM sodium metaperiodate (0.04 gm in 20 ml of sodium acetate buffer, pH 5.5) for EXACTLY 20 minutes. Longer incubation times may lead to non-specific labelling.

3) Wash three times with PBS for 10 minutes each.

4) Label the aldehydes of the oxidized sugars by incubating the filter with 1 µl of the steroid hapten DIG-succinyl-ε-amidocaproic acid hydrazide (vial 3 of Boehringer Mannheim kit) dissolved in 5 ml sodium acetate buffer, pH 5.5, for 1 hour EXACTLY. Longer incubation times may lead to nonspecific labelling.

5) Wash the blot three times for 10 minutes each with TBS. NOW TBS CAN BE USED. If desired proteins on blot may be stained with Ponceau S at this time. Submerge the blot in undiluted Ponceau S solution for 10 to 15 minutes. Destain the blot by washing with distilled water.

6) Incubate the blot for 30 minutes in blocking solution. Blocking solution is made by dissolving 0.5 gm Blocking reagent in 100 ml of TBS by heating for 1 hour to 50 to 60 °C. The solution will remain turbid and should be allowed to cool before incubation of blot. If necessary, procedure can be interrupted here and blot stored at 4 °C.

7) Wash blot three times for 10 minutes each with TBS.

8) Now label the attached steroid hapten with an alkaline phosphatase conjugate. Incubate the blot with anti-digoxigenin-AP (10 µl of vial 4 dissolve in 10 ml TBS) for 1 hour.

9) Wash the blot three times for 10 minutes each with TBS.

10) Staining reaction usese the following solution, 10 ml 100 mM Tris-HCl, pH 9.5, 37.5 µl of a 5% w/v 5-bromo-4-chloro-3-indoyl-phosphate solution in 100 % dimethylformamide, and added just before use 50 µl of a 10% w/v 4-nitroblue tetrazolium chloride solution in dimethylformamide. The development of a gray to black color associated with the labelled glycoconjugates should appear in approximately 5 to 15 minutes, on a light pink background. Note prolonged color development can lead to non-specific staining, therefore, reaction should be stopped after approximately 15 minutes by washing the blot in distilled water.

11) The color reaction of the glycan stain is generally more stable than that of the chloro-napthol of the Western analysis, however, a photographic record of the blot should be made for future use (Appendix 7).

Protein Quantitation

All quantitative determinations of proteins in this study used the Pierce Protein Assay reagent (Coomassie Blue G250) and a one-half scale modification of the Micro Assay procedure.

Note: For determination of protein concentrations in the 1 µg to 25 µg ml^{-1} range.

1) Mix reagent solution well before use.

2) Prepare a known protein concentration series by diluting a stock BSA standard in the same diluent as that of the protein sample whose concentration is to be determined.

3) Pipet 500 µl of dilute standard or unknown protein sample directly into a 1 ml disposable microcuvette (UV range quality: Fisher Scientific, Semimicro Methacrylate). Do all assays in duplicate.

4) Add 500 µl of Protein Assay Reagent and mix well.

5) Read absorbance vs. distilled water at 595 nm.

6) Subtract absorbance at 595 nm of blank from each sample. Note: when using Shimadzu spectrophotometer use dual blanking system, which zeros out any non-specific absorbance from actual readings.

7) Prepare a standard curve by plotting known protein concentrations vs. absorbance (Shimadzu will do this for you in quantitation mode). Using this standard curve determine protein concentrations of unknowns.

Gel Staining Protocols

Coomassie Brillant blue (CBB)

0.2% w/v Coomassie Brillant Blue R-250 in 45% v/v methanol and 10% v/v acetic acid.

Component	amount
Coomassie Brillant Blue R-250	0.2 gm
Methanol 100%	45 ml
Acetic acid, conc.	10 ml
Distilled Water	45 ml

Typically CBB staining requires incubation in stain for 5 minutes to several hours depending on protein concentrations in gel. Gel is destained in 50% v/v Methanol and 10% v/v Acetic acid. Placing several Kimwipes (Kimberly-Clark, Co.) on the surface of the destaining solution, to absorb excess CBB dye, increases the speed of destaining and reduces the amount of destaining buffer used. Refer to Appendix 7 for photo specs.

Silver stain

After sufficient separation of proteins on SDS-PAGE, treat the gel as follows✪:

Step	Solutions	Time
1 Fix	50% Methanol 12% Acetic acid 500 µl of 37% Formaldehyde	≥ 1 hour
2 Wash	50% Ethanol	3 X 20 min.
3 Pretreat♧	Sodium thiosulfate, $Na_2S_2O_3$ - $5H_2O$ (0.2 gm l^{-1})	1 minute
4 Rinse	distilled water	2 X 20 sec.
5 Impregnate	Silver Nitrate, $AgNO_3$ (2 gm l^{-1}) 750 µl of 37% Formaldehyde	20 minutes
6 Rinse	distilled water	3 X 20 sec.
7 Develop	Sodium carbonate, Na_2CO_3 (60 gm l^{-1}) 500 µl of 37% Formaldehyde Sodium thiosulfate, [(4 mg l^{-1}) or 5 µl of 8 mg ml^{-1} stock soln]	10 minutes
8 Stop	50% Methanol, 12% Acetic acid	10 minutes
9 Wash	50% Methanol	≥ 20 minutes

✪ All steps carried out at room temperature, with glass containers on a shaker. Do not touch the gel with anything but a clean glass rod.

♧ If negative staining of the protein occurs, next time proteins are to be stained use an additional step here; pre-treat the gel in DTT (5 µg ml^{-1}) for 30 minutes.

☺ If gel should have heavy background staining or overstained, incubate in Kodak Rapid fix until desired. Destaining can be stopped by placing the gel in Kodak hypo clearing agent.

APPENDIX 4

Protein Digestion Protocols for Peptide Mapping.

All protease digest performed on protein samples which had been lyophilized down and reconstituted in 80 mM Tris-HCl, pH 6.8, 2% w/v SDS, 2% v/v glycerol and 0.001% w/v bromphenol blue. Samples were boiled for 2 minutes and then cooled on ice prior to initiation of digest. To stop protease digest 5 µl of sample buffer was added and reaction mixture was boiled for 5 minutes. Cyanogen bromide digest was stopped by re-lyophilization of the mixture (drives off formic acid which halts digest). Alkaline phosphatase digest stopped as described.

Papain - cleavage sites (-Arg↓-) and (-Lys↓-).

To 100 µl of protein sample add 100 µl of 0.2 mg ml^{-1} enzyme solution (in 100 mM Tris-HCl, pH 7.0, for all protease used, for final concentration of enzyme 0.1 mg ml^{-1}). Incubate for 10 minutes at 37 °C and then stop as described above.

S. aureus V8 (protease) - cleavage sites (-Glu↓-) and (-Asp↓-).

Add 20 µl of 1.1 mg ml^{-1} enzyme solution to 100 µl of protein sample. Incubate for 10 minutes at 37 °C and stop as above.

Chymotrypsin - cleavage sites (-Trp↓-), (-Tyr↓-) and (-Phe↓-).

Add 100 µl of 0.2 mg ml^{-1} enzyme solution to 100 µl of protein sample. Incubate for 10 minutes at 37 °C and stop as above.

Cyanogen bromide - cleavage site (-Met↓-).

To 100 µl protein sample add 200 µl of 100 mg ml^{-1} cyanogen bromide solution in 88% formic acid, flood with nitrogen and seal reaction tube with parafilm. Incubate digest at 65 °C for 12 hours. After reaction stopped, as described above, reconstitute in 200 µl of sample buffer.

Alkaline phosphatase - cleaves bound phosphate (PO_4) from proteins.

To 100 µl of protein sample add 40 µl of a 0.5 µunits μl^{-1} solution of calf intestinal alkaline phosphatase. Incubate digest at 37 °C for 18 hours. Stop the digest by quenching with 4 µl of 0.5 M EDTA (ethlenediaminetetraacetic acid), pH 7.0.

Endoproteinase Glu-C - cleaves specifically at (-Glu↓-).

Bring up the lyophilized (electro-eluted and copiously dialyzed) protein in 200 µl of 25 mM ammonium carbonate, pH 7.8 (sterile filtered). Bring up the tube of

lyophilized enzyme, endoproteinase Glu-C (50 μg; Boehringer Mannheim, No. 1420 399), in 50 μl of sterile distilled water for an enzyme concentration of 1 μg μl^{-1}. Add 20 μl of the Glu-C enzyme solution to the 200 μl of protein and incubate at room temperature (25 °C) for 12 hours. Lyophilize down the digest and bring up in sample buffer. Now digest fragments can be analyzed on a SDS-PAGE gel.

Endoproteinase Lys-C - cleaves specifically at (-Lys↓-).

The lyophilized (electro-eluted and copiously dialyzed) protein sample should be brought up in 200 μl of 25 mM Tris-HCl, pH 8.5 (EDTA 1 mM $liter^{-1}$). Bring the tube of lyophilized enzyme, endoproteinase Lys-C (5 μg; Boehringer Mannheim, No. 1420 429), in 50 μl of sterile distilled water (resulting enzyme buffer concentration: 50 mM Tricin, pH 8.0, and 10 mM EDTA) for an enzyme solution concentration of 0.1 μg μl^{-1}. Add 20 μl of the Lys-C enzyme solution to the 200 μl of protein and incubate at 37 °C for 12 hours. Lyophilize down the digest and bring up in sample buffer. Now digest fragments can be analyzed on a SDS-PAGE gel.

N-Glycosidase F (PNGase F) - deglycosylates proteins with N-linked sugars.

The peptide-N-glycosidase was isolated from *Flavobacterium meningosepticum*. Lyophilize down the native protein extract and bring up in 100 μl of digestion buffer (80 mM Tris-HCl, pH 6.8, 1.7% v/v β-mercaptoethanol, 1% w/v MEGA 8 [octanoyl-N-methylglucamide; Boehringer Mannheim, No. 988 600] or dilute 1:20 (aqueous extract, approximately 500 μl used for this digest) with the digestion buffer. Reconstitute the lyophilized PNGase F (20 U; Boehringer Mannheim, No. 903 337) enzyme in the above digestion buffer. Before adding the PNGase F enzyme, boil the protein for 5 minutes and use 50 μl of the protein sample in the digest. To the 50 μl of the boiled protein sample add 5 μl (1 U or 40 ng) of PNGase F. Incubate the digest at 37 °C for 12 hours. Lyophilize down the digest and bring up in sample buffer. Now digested and undigested fragments can be analyzed on a SDS-PAGE gel. PNGase runs at 35 kDa on an SDS-PAGE gel.

APPENDIX 5

Raw Data of Carbohydrate Composition.

Sample A is 65mg of DRH1 media conc down in 10kDa membrane and represents the retentate.
Sample B is 140mg of DRH1 media conc down and is flow thru of above conc of sample A.
Sample C is 65mg of aqueous extract from Chen field material which has been phenol/chloroform extracted delipitized and deproteinized.
Sample D is 33mg of aqueous extraction similar to sample C.

First hydrolysis was for neutral and amino sugars and used half of each sample and was ultimately brought up in 100ul .
Second hydrolysis was for sialic acid, the other half of each sample was used for this hydrolysis and was ultimately brought up in 100ul .

Sample A , 32.5mg used for digest brought to 100ul, 1:1000 dilution used and 25ul injected representing 0.325mg/ul, 1:1000 yields 0.325x10-3mg/ul, 25ul represents 8.125ug of dry weight of sample.

inject 1		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	0.000	0.000	0
glucosamine	574.000	70.646	1
glucose	974.400	119.926	1.7
mannose	6.500	0.800	0.01
NAcGalN	884.700	108.886	1.5

inject 2		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	0.000	0.000	0
glucosamine	471.100	57.982	1
glucose	798.400	98.265	1.7
mannose	3.000	0.369	0.01
NAcGalN	693.700	85.378	1.5

Sample B, 70mg used for each digest and each ultimately brought up to 100ul, which is 0.7mg/ul, for a 1:10 dilution this yields 0.07mg/ul for 25 ul injection is 1.75mg dry wt., for a 1:20 0.035mg/ul 25ul injection is 0.875mg. For injection 1, 1.75mg of dry weight per injection and for injection 2, 0.875mg of dry wt. per injection

inject1		pmole/ug	
sugar	pmole	dry wt. sample	**ratio**
galactosamine	375.000	0.375	0.3
glucosamine	1403.40	1.403	1
glucose	1837.90	1.838	1.3
mannose	236.000	0.236	0.2
NAcGalN	639.800	0.640	0.5

inject 2		pmole/ug	
sugar	pmole	dry wt. sample	**ratio**
galactosamine	198.900	0.199	0.3
glucosamine	674.800	0.675	1
glucose	1053.80	1.054	1.6
mannose	85.100	0.085	0.1
NAcGalN	406.100	0.406	0.6

Sample C, 32.5mg used for each digest and each ultimately brought up to 100ul, which is .325mg/ul, for a 1:250 dilution this yields 0.0013mg/ul or 1.3ug/ul for 25ul injection is 0.0325mg or 32.5ug dry wt of sample and for a 1:500 dilution this yields 0.00065mg/ul or 0.65ug/ul for a 25ul injection is 0.01625mg or 16.25 ug dry wt. of sample.
For injection 1, 1:250 dilution which is 0.0325mg or 32.5ug dry wt. sample and injection 2, 1:500 dilution which is 0.01625mg or 16.25ug dry wt. sample.

inject 1		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	14.600	0.015	0.1
glucosamine	273.500	0.274	1
galactose	0.000	0.000	0
mannose	2.700	0.003	0
NAcGalN	339.800	0.340	1.2

inject 2		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	19.400	0.019	0.05
glucosamine	410.300	0.410	1
galactose	0.000	0.000	0
mannose	3.200	0.003	0.01
NAcGalN	452.600	0.453	1.1

Sample D, 16.5mg used for each digest and each ultimately brought up to 100ul, which is 0.165mg/ul, for 1:500 dilution for a 25 ul injection there is 0.00825mg or 8.25ug dry wt. of sample
this yields 0.00033mg/ul or 0.33ug/ul.
For injection 1, 1:500 dil, 0.00825mg dry wt. per injection and inject 2, 1:500dil., 0.00825mg dry wt. per injection.

inject 1		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	62.900	0.063	0.1
glucosamine	513.400	0.513	1
galactose	0.000	0.000	0
glucose	1814.90	1.815	3.5
mannose	87.900	0.088	0.2
NAcGalN	803.400	0.803	1.5

inject 2		pmoles/ug	
sugar	pmoles	dry wt. sample	**ratio**
galactosamine	0.000	0.000	0
glucosamine	515.200	0.515	1
galactose	0.000	0.000	0
glucose	1845.80	1.846	3.6
mannose	85.100	0.085	0.2
NAcGalN	885.500	0.886	1.7

Average of injection 1 and injection 2 for each sugar of each sample.
avg. pmoles/ug dry wt. of sample

sample	galactosamine	glucosamine	galactose	glucose	mannose	NAcGalN
A	0.000	64.314	0.000	109.095	0.585	97.132
B	0.221	0.787	0.065	1.127	0.116	0.415
C	0.822	16.832	0.000	45.129	0.140	19.154
D	3.812	62.339	0.000	221.861	10.485	102.358

Sample A sialic acid, 32.5mg brought to 100ul, 25ul injected w/no dil., .325mg/ul times 25ul yields 8.125mg sample/injection.
Sample B sialic acid, 70mg brought to 100ul, 25ul injected w/no dil., 0.70mg/ul times 25ul yields 17.5mg sample/injectio
Sample C sialic acid, 32.5 mg brought to 100ul yields 0.325 mg/ul, dilutied1:10 yields 0.0325mg/ul, 25ul injection yield: 0.8125mg sample/injection.
Sample D sialic acid, 16.5mg brought to 100ul yielding 0.165mg/ul, diluted 1:10 yields 0.0165mg/ul, 25ul injection yiel 0.4125mg dry wt. sample.

Sialic acid detected in extracts.

sample	pmoles	pmoles/mg dry wt.	pmoles/ug dry wt.
A	1192.500	146.769	0.147
B	525.900	30.051	0.030
C	1540.200	1895.631	1.896
D	794.600	1926.303	1.926

TMS Sugar Analysis of Sheath (80% Ethanol) Extracts from Field Material *Nostoc commune*

(0.6 ul injections; sugar quantities are for total 2.1 ml TMS extract)

DWE represents dry weight extracted by TMS

sample	"dry" wt. (gm)	extract vol. (ml)	DWE (gm)
desiccated	0.286	6/8.5	0.202
5 min	0.196	4/8.5	0.092
10 min	0.172	4/9	0.076
15 min	0.331	4/9	0.147
30 min	1.066	4/8	0.533
1 hour	0.997	4/6	0.665
6 hours	2.772	4/9	1.232
12 hours	2.017	4/9	0.896
24 hours	1.636	4/8	0.818
48 hours	3.108	4/9	1.381
Pearls	0.837	6/9.5	0.529
des. Pearls	0.526	6/9.5	0.332

inject 1 sample	mg glucose per DWE	mg sucrose per DWE	mg trehalose per DWE
desiccated	0.0250	0.7420	0.9210
5 min	0.0540	0.9670	1.4560
10 min	0.9340	3.0390	2.6580
15 min	0.0340	0.7620	0.6260
30 min	0.0860	0.3090	0.3280
1 hour	0.0780	0.3160	0.2450
6 hours	0.0410	0.1870	0.1540
12 hours	0.0060	0.2180	0.1810
24 hours	0.0060	0.2470	0.1580
48 hours	0.0040	0.1040	0.1350
Pearls	<0.009	0.2850	0.0940
des. Pearls	<0.015	0.3010	0.5030

inject 2 sample	mg glucose per DWE	mg sucrose per DWE	mg trehalose per DWE
desiccated	0.1390	0.7030	1.3020
5 min	0.0540	0.5110	0.4890
10 min	1.0000	3.6970	2.3420
15 min	0.2860	2.2240	1.3200
30 min	0.0920	0.3150	0.2610
1 hour	0.0720	0.4250	0.2500
6 hours	0.0600	0.2890	0.1430
12 hours	0.0420	0.1370	0.1220
24 hours	<0.006	0.0820	0.0670
48 hours	0.0000	<0.029	0.0320
Pearls	0.0090	0.2680	0.0940
des. Pearls	0.0150	0.1170	0.4040

inject 3 sample	mg glucose per DWE	mg sucrose per DWE	mg trehalose per DWE
desiccated	0.1390	0.8370	1.2030
5 min	-	-	-
10 min	-	-	-
15 min	-	-	-
30 min	0.1050	0.3700	0.3000
1 hour	0.0800	0.4450	0.2510
6 hours	0.0590	0.3220	0.1800
12 hours	0.0420	0.1290	0.1050
24 hours	0.0270	0.0800	0.0680
48 hours	<0.014	0.0560	0.0800
Pearls	<0.038	0.2680	0.1190
des. Pearls	<0.060	0.2260	0.5990

Average sample	mg glucose per DWE	mg sucrose per DWE	mg trehalose per DWE
desiccated	0.1010	0.7607	1.1420
5 min	0.0540	0.7390	0.9725
10 min	0.9670	3.3680	2.5000
15 min	0.1600	1.4930	0.9730
30 min	0.0943	0.3313	0.2963
1 hour	0.0767	0.3953	0.2487
6 hours	0.0533	0.2660	0.1590
12 hours	0.0300	0.1613	0.1360
24 hours	0.0110	0.1363	0.0977
48 hours	0.0013	0.0533	0.0823
Pearls	0.0030	0.2737	0.1023
des. Pearls	0.0050	0.2147	0.5020

APPENDIX 6

Raw Data of Elemental Analysis.

		Values given for elements represents mg/gram dry wt. of sample (with dilution factor corrections).										Original	Extract	Analysis
	Sample	K	Ca	Mg	Na	S	P	Mn	Fe	Cu	Zn	dry wt (gm)	vol. (ml)	vol. used
Aqueous	0	0.527	1.383	0.105	0.094	0.181	ND	0.002	0.002	ND	0.002	0.2	2.4	1ml
Extracts	5	0.474	1.577	0.094	1.436	0.137	ND	0.001	0.002	0.001	0.004	0.2	5.8	1ml
	10	0.358	1.076	0.075	0.243	0.102	ND	0.004	ND	ND	0.005	0.2	6.25	1ml
	15	0.372	1.616	0.101	0.122	0.098	ND	0.005	0.004	ND	0.005	0.2	6	1ml
	30	0.380	0.954	0.064	0.101	0.105	ND	0.002	ND	ND	ND	0.2	5.3	1ml
	60	0.292	0.930	0.060	0.302	0.102	ND	0.005	0.003	ND	0.003	0.2	5	1ml
	360	0.615	1.180	0.146	0.109	0.131	ND	0.005	ND	ND	0.005	0.2	5	1ml
Ethanolic	0	0.322	0.091	0.033	0.413	0.050	ND	0.000	ND	ND	0.003	0.286	8.5	2ml
Extracts	5	0.344	0.165	0.053	0.323	0.037	ND	0.000	ND	0.001	0.006	0.196	8.5	2ml
	10	0.402	0.139	0.056	0.280	0.026	ND	0.001	ND	0.002	0.004	0.172	9	2ml
	15	0.035	0.125	0.042	0.271	0.025	ND	0.000	ND	ND	0.003	0.33	9	2ml
	30	0.342	0.162	0.060	0.273	0.028	ND	0.001	ND	ND	0.001	1.066	8	2ml
	60	0.399	0.174	0.054	0.329	0.060	ND	0.001	ND	ND	0.003	0.997	6	2ml
Geographic	CHEN	13.450	7.840	4.485	1.740	1.143	0.602	0.034	0.096	0.004	0.018	0.1	approx. 2.5	1ml
Extracts	TAG	12.360	8.840	4.393	1.789	1.102	0.198	0.009	0.085	0.001	0.033	0.1	approx. 2.5	1ml
	TEN	10.393	4.663	3.853	1.428	1.051	0.226	0.019	0.019	0.004	0.108	0.1	approx. 2.5	1ml
	BER	12.448	7.623	4.570	1.707	1.121	0.794	0.017	0.079	0.002	0.054	0.1	approx. 2.5	1ml
	HUN1	15.913	4.615	4.598	2.369	1.489	0.123	0.490	0.163	ND	0.019	0.1	approx. 2.5	1ml
	776D	12.898	8.270	4.865	2.570	1.389	0.235	0.009	0.024	0.001	0.035	0.1	approx. 2.5	1ml
	779D	13.938	8.218	4.755	2.255	1.445	0.316	0.014	0.031	0.001	0.030	0.1	approx. 2.5	1ml
	857D	13.075	5.770	4.120	1.944	1.190	0.306	0.002	0.007	0.001	0.011	0.1	approx. 2.5	1ml
	8122	15.110	6.343	4.990	2.211	1.506	0.182	0.012	0.089	ND	0.020	0.1	approx. 2.5	1ml
	MAL	12.845	6.685	3.648	1.787	1.223	0.097	0.116	0.206	0.001	0.059	0.1	approx. 2.5	1ml
	ANT	16.088	4.360	5.100	2.314	1.591	0.275	0.044	0.083	ND	0.004	0.1	approx. 2.5	1ml
	BBC	14.968	3.143	5.110	2.172	1.387	0.143	0.096	0.423	0.001	0.024	0.1	approx. 2.5	1ml

ND indicates element was either absent or below detection limits.

APPENDIX 7

Photographic Supplies and Procedures.

All chemicals and supplies from Kodak, unless otherwise stated.

General Photography

All general black and white photography used TMAX 100 B&W 35 mm negative film purchased in a bulk role of 100 ft. A Alden-74 Bulk film loader was used to load up to 36 exposures in 35 mm film cartriges. For all general photography shots, gels, blots, etc. a Canon A-1 camera with a Tamron telemacro-zoom lens (28 - 80 mm) was used. In all cases the built-in light meter of the camera was used for all exposure determinations.

The following procedure was used to develop the TMAX 100 35 mm film:

1) After removing film cassette from camera, open the cassette (in total darkness) and roll the film onto a developing real, place in tank and secure inset and lid (Paterson Super System 4 Film Developing Tank).

2) Make up the D-76 developer according to the specifications on the package and use at the following dilutions, temperatures and time (minutes) -

	18 ºC	**20 ºC**	21 ºC	22 ºC
D-76	10.5	**9**	8	7
D-76 (1:1)	14.5	**12**	11	10

Bold indicates recommended temperature and time. Gentle agitation should be used during time of development.

3) After the appropriate time of development, pour off developer and add Stop Bath to tank (approximately 5% v/v acetic acid solution) for 30 seconds.

4) Now pour off the Stop Bath and add Kodak Rapid Fix (prepared according to manufacture or purchased as a concentrate) and fix for 5 to 10 minutes.

5) After fixing, wash the film in copious amounts of running tap water for at least 20 minutes.

6) Before hanging negatives up to dry, rinse in Kodak Photo Flo solution.
All Polaroid Photography used the Polaroid Positive Negative 4X5 Instant Sheet Film. After taking the photo, the print was coated with the coater and the negative was developed by washing in 18% sodium sulfite solution, on stir plate, for 5 minutes and then in running water for 30 minutes. Negative was then hung to dry.

Exposure settings for taking Polaroid pictures of stained gels and blots (using a yellow filter) with the Fotodyne/Polaroid copy camera system with bottom illimunation using a light box are as follows:

	F-Stop	Shutter Speed
Stained Gel	8	1/8
PVDF Blot	11	1/2

All prints used Ilford Multigrade III, Resin Coated Glossy Paper. Standard development using Ilford chemicals and an automatic processing protocol was used for all prints. A general set of Ilford contrast filters was used for all printing.

For all prints of microscopy photographs a careful record of print magnification was kept in order to determine final magnification of print. This was acheived by simply measuring a clear rulers scale as projected from the negative holder onto the print surface.

Light Microscopy

All light microscopy employed the use of the Olympus Automatic Photomicrographic System (PM-10ASD), using an Olympus C-35AD-4 camera. For color shots Kodak Ektachrome 160T (Tungsten) color slide film was used, Speed 160 and Reciprocity 4. Color slide film was developed commercially. All black and white photographs were with the TMAX 100, Speed 32 and Reciprocity 2. A careful record of exposures and magnifications wer kept for all photographic shots.

Electron Microscopy

Transmission electron microscopy used Kodak Electron Microscopy Film 4489 (ESTAR Thick Base) and processed according to Kodak with D-19 developer. Scanning electron microscopy used Polaroid 55 Positive Negative 4X5 Instant Sheet Film and was treated as described above.

Negative Sleeves

All archival negative preservers were purchased from Print File, Inc. Those used are as follows:

Negative Type	Print File Style No.
Color Slides	2X2 - 20B
35 mm Negatives	35 - 6HB
TEM Negatives	EM - 6
Polaroid Negatives	45 - 4B

APPENDIX 8

Abbreviations and Molecular Weights for Amino Acids

Amino Acid	Three-Letter Abbrviation	One-Letter Symbol	Molecular Weight, kDa
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine or Aspartic acid	Asx	B	----
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic acid	Glu	E	147
Glutamine or Glutamic acid	Glx	Z	----
Glycine	Gly	G	75
Histidine	His	H	155
Isoleucine	Ile	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

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DATE & PLACE OF BIRTH

8th October 1961, Covington, Kentucky

EDUCATION

Ph.D., Biochemistry and Anaerobic Microbiology, April 1994
Virginia Polytechnic Institute and State University, Blacksburg, Virginia
Dissertation: Morphological, Biochemical and Molecular Characterization of Desiccation Tolerance in Cyanobacterium *Nostoc commune* var. Vauch
Advisor: Dr. Malcolm Potts, Professor.

M.S., Biology, May 1988
Western Kentucky University, Bowling Green, Kentucky
Thesis: Purification and Characterization of DNA Polymerase α and γ in Turnip and Soybean
Advisor: Dr. Val Dunham, Head Dept. Biology

B.S. Chemistry & Biology, Minors Biophysics & Technical Illustration, May 1986 Western Kentucky University, Bowling Green, Kentucky

RESEARCH INTEREST

Morphological and Biochemical/Molecular studies of the effects and responses to environmental stresses on prokaryotic organisms.

PUBLICATIONS

Water Stress proteins (WSP) of *Nostoc commune* (Cyanobacteria) are Secreted with UV-A/B Absorbing Pigments and are Associated with a 1,4-beta-D-Xylanxylanohydrolase (EC 3.2.1.8) Activity, Hill, D.R. ,S. Hladun, S. Scherer and M. Potts, Journal of Biological Chemistry, 269: 7726-7734.

Biochemistry and Structure of the Glycan Secreted by Desiccation-Tolerant *Nostoc commune* (Cyanobacterium). Hill, D.R., A. Peat and M. Potts, submitted to Protoplasma, April 1994.

Life Cycle of the Cyanobacterium *Nostoc commune*: Biochemical, Morphological and Molecular Analysis of the Changes During Growth of the Cyanobacterial Strains UTEX 584 and a New Isolate DRH1 and DRH2 [originally collected in the field in the Hunan Province of China], Hill, D.R., V. Joardar and M. Potts, in preparation to be submitted to the Proceedings of the Royal Society of London, Series B, tentative submission date Feb.1994.

Transformation of *Nostoc commune* strains UTEX 584 and DRH2 (field isolate) via electroporation, Joardar, V., D.R. Hill and M. Potts, in preparation.

Distribution, Subcellular Location and Regulation of Synthesis of Cyanoglobin and Immunologically-Related Proteins in Nitrogen-Fixing Cyanobacteria, Hill, D.R., V. Joardar, P. Böger, H. Pearl and M. Potts, in preparation.

TECHNICAL SKILLS

Morphology

Full range of microscopy techniques; tissue preparation to photography in transmission electron microscopy, scanning electron microscopy, general light microscopy, EDAX, immunolabelling and qualitative staining.

Proteins

Protein purification and characterization, FPLC, gel electrophoresis (1D and 2D), western blotting, enzyme assays, tissue culture, N-terminal protein sequencing, antibody generation and tittering, and purification of monoclonal antibodies by affinity chromatography.

DNA
DNA isolation and characterization, southern blotting, electroporation, restriction mapping and sequencing analysis.

Carbohydrates
Carbohydrate isolation and purification, acid hydrolysis, analysis by gas chromatography, mass spectroscopy, nuclear magnetic resonance, thin layer chromatography and specific staining for at the polyacrylamide gel and microscopy level.

RESEARCH EXPERIENCE

Research Associate, Department Biochemistry & Anaerobic Microbiology, Dr. M. Potts, Virginia Tech, Blacksburg, Virginia, April 1994 to present.

Graduate Research Assistant/Ph.D. Research, Department of Biochemistry & Anaerobic Microbiology, Dr. M. Potts, Virginia Tech, Blacksburg, Virginia, August 1988 - April 1994.

Graduate Research Assistant/M.S. Research, Department of Biology, Dr. V. Dunham, Western Kentucky University, Bowling Green, Kentucky, August 1986 - May 1988

Undergraduate Research Assistant, Department of Biology, Dr. R. McCurry, Electron Microscopy Lab, Western Kentucky University, Bowling Green, Kentucky, 1983 - 1985

Scientific Technical Illustrator, Ogden College, Deans Office, Western Kentucky University, Bowling Green, Kentucky, 1983-1985

Laboratory Research Assistant, Department of Anatomy & Cell Biology, Dr. R. Cardell, Electron Microscopy Lab, University of Cincinnati, Cincinnati, Ohio, Summer 1984

Laboratory Research Assistant, Department of Experimental Pathology, Dr. J. Primus, University of Kentucky, Lexington, Kentucky, 1982 - 1985

Undergraduate Research Assistant, Department of Chemistry, Dr. L. Tolbert,

University of Kentucky, Lexington, Kentucky, Summer 1982.

TEACHING EXPERIENCE

Teaching Assistant, Department of Biochemistry and Anaerobic Microbiology, Virginia Tech, Blacksburg, Virginia, Spring Semester 1990

- Supervised Juniors and Seniors during hands on laboratory techniques course.

Special Studies Student Teaching, Department of Biology, Western Kentucky University, Bowling Green, Kentucky, Spring Semester 1987

- Organized and presented a series of lectures (8 lectures) in Junior level Molecular Biology course.

Laboratory Instructor, Department of Biology, Western Kentucky University, Bowling Green, Kentucky, Fall 1986, Spring 1987 and Fall 1988

- Supervised and presented lectures to a Freshman level Introduction to Biology lab course.

PRESENTATIONS

International Cyanobacteria Workshop, Presented poster on initial dissertation research, University of Toronto, Toronto, Canada, June 1990

Graduate Student Seminars, Presented three one hour seminars on my research to the faculty and students in the Department of Biochemistry and Anaerobic Microbiology, Virginia Tech, Blacksburg, Virginia

PROFESSIONAL AFFILIATIONS

American Society of Microbiology, Student Affiliate

American Society of Biochemistry and Molecular Biology, Student Affiliate

Electron Microscopy Society of America, Student Affiliate