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CHARACTERIZATION OF FUNGAL FIMBRIAE

by

Martina Celerin

Department of Plant Sciences

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
July, 1995

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ABSTRACT

Fungal fimbriae are surface appendages that were first described by Poon and Day (1974) on the haploid cells of Microbotryum violaceum. They are long (1 to 20 µm), narrow (7 nm) flexuous fibres that were implicated in important cellular functions such as mating and pathogenesis. Since this initial description, numerous other fungi from all five phyla have been shown to produce fimbriae on their extracellular surfaces. Because they are easy to purify, the fimbriae from M. violaceum have been investigated most extensively. The present study extends the earlier characterizations of M. violaceum fimbriae, and expands on the biochemical description of these unusual structures.

Fimbriae are complex structures that are shown here to be composed of three major components: protein, carbohydrate and RNA. The RNA component of fimbriae (f-RNA) is the first example of an extracellular RNA that has been well documented. It is a 30 base, single-stranded molecule that has a standard 5' phosphate. The f-RNAs constitute a homogeneous population of macromolecules that are present in fimbriae isolated from both a_1 and a_2 mating types. f-RNA is abundant (2.77×10^3 f-RNA molecules per 1 µm fimbrial length) and well-protected. The existence of this extracellular f-RNA suggests intriguing possible mechanisms that may exist for cell to cell communication.

The structural subunit of M. violaceum fimbriae is a 74 kDa glycoprotein. The carbohydrate component is a 'high mannose type' that is covalently linked to an asparagine residue on the 47 kDa aglycone via two N-acetylglucosamine residues. Unlike the protein epitopes which are conserved in at least four fungal divisions, the carbohydrate component of fimbriae is order specific and can be used to distinguish between members of the fungal orders Microbotryales and Ustilaginales.

The protein component of M. violaceum fimbriae was also analyzed extensively. The N-terminus and three internal amino acid sequences show a strong similarity to a single gene family, the collagens. Further, enzymatic digests and immuno-chemical analyses support this finding. Based on these results, it is suggested that the proteinaceous subunits of fimbriae should be termed fungal collagens. Fungal collagens may be examples of orthologous evolution. Thus collagen, the principal component of the animal extracellular matrix, may have evolved in a common ancestor that existed prior to the divergence of fungi and animals.

Finally, native fimbriae can function as mammalian extracellular matrix components. In particular, fimbriae can act as a substratum, permitting animal cells to adhere, spread, and proliferate.

It is concluded that fimbriae are multifarious structures, much more elaborate than was initially reported. Each of the components, the f-RNA, the carbohydrate, and the fungal collagen, has its own individual intricacies and the biochemistry of each is fascinating. However, the role of native fimbriae *in vivo* remains elusive. Possible functions are discussed.

DEDICATION

This thesis is dedicated to the memory of Dr. David E. Laudenbach. Dave was a inspiring supervisor, an outstanding scientist, and a true, dear friend. Thanks, Dave. Thanks for everything. I miss you and I wish you were here to share this with me.
MC.

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LIST OF ABBREVIATIONS

AcNeu	N-acetylneuraminic acid
AN	acetonitrile
ATP	adenosine triphosphate
BCIP/NBT	bromochloro-indolyl phosphate/nitroblue tetrazolium
BME	ß-mercaptoethanol
BSA	bovine serum albumin
c	centi-
C	celsius
Ci	curies
conc	concentration
cpm	counts per minute
CTP	cytidine triphosphate
Da	daltons
DEPC	diethyl pyrocarbonate
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ECM	extracellular matrix
EDTA-HCl	ethylene diamine tetra-acetate, pH adjusted with hydrochloric acid
EGTA-HCl	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid, pH adjusted with hydrochloric acid
ELISA	enzyme-linked immunosorbant assay
EM	electron microscope
f-RNA	fimbrial RNA
Fuc	fucose
g	times gravity; gram(s)
Gal	galactose
GalNAc	N-acetyl galactosamine
Glc	glucose
GlcNAc	N-acetyl glucosamine
GlcNeu	N-glycollylneuraminic acid
h	hour(s)
HPLC	high pressure liquid chromatography
k	kilo-
l	litre(s)
M	molar
m	milli-; metre(s)
min	minute(s)
mol	mole(s)
mRNA	messenger ribonucleic acid
mwco	molecular weight cut off
n	nano-

ORF	open reading frame
p	pico-
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse phase
rpm	revolutions per minute
RT	room temperature; reverse transcription
SDS	sodium dodecyl sulphate
sec	second(s)
SSC	tri-sodium citrate/sodium chloride buffer
TAE	Tris-HCl/acetate/EDTA-HCl buffer
TB	terrific broth
TBE	Tris-HCl/boric acid/EDTA-HCl buffer
TFA	trifluoroacetate
T-PBS	phosphate buffered saline with 0.1% tween-20
Tris-HCl	tris(hydroxymethyl)aminomethane, pH adjusted with hydrochloric acid
tRNA	transfer ribonucleic acid
U	units
UDP-GlcNAc	uridine-diphospho-N-acetylglucosamine
UTR	untranslated region
uv	ultraviolet
V	volts
vol	volume
v/v	volume/volume
w/v	weight/volume
YT	yeast extract/tryptone broth
µ	micro-
°	degree

CHAPTER 1 - INTRODUCTION

1.1 Microbotryum violaceum

Smut fungi constitute a group of basidiomycetes which share common life-cycles. They produce a parasitic, dikaryotic mycelium that grows intercellularly in the host plant, and forms large quantities of black to purple spore masses (teliospores) which resemble soot or smut.

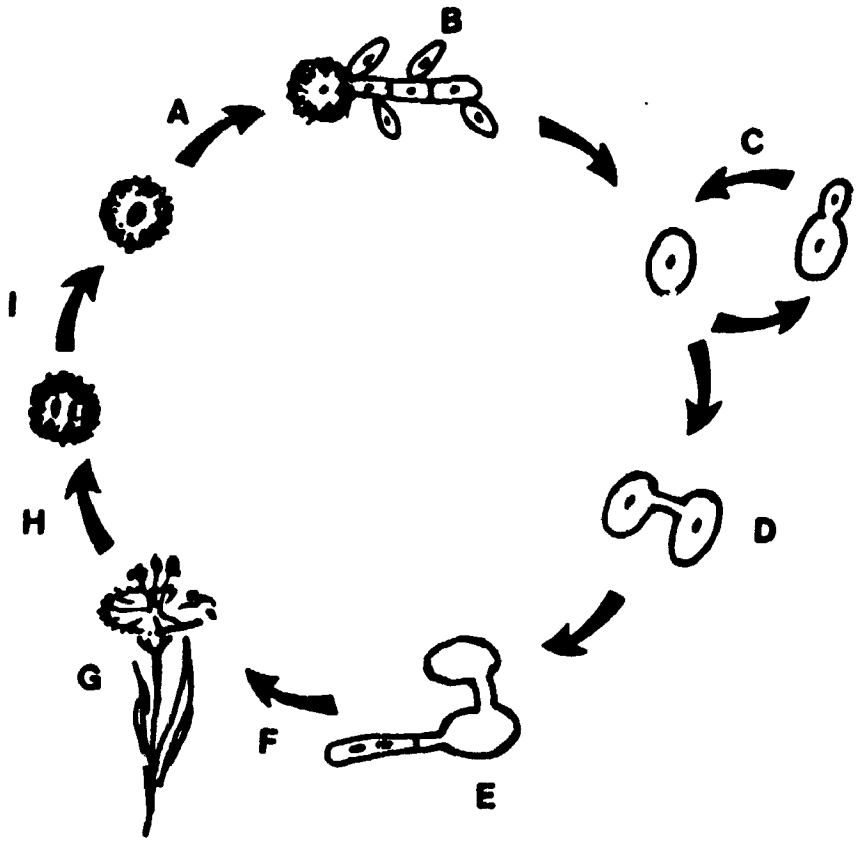
Fischer and Holton, (1957) recognized 34 genera of smuts. The smuts parasitize a diverse array of plant families. Several smuts, such as Ustilago maydis, U. hordeii, and U. nigra are pathogens of cereal crops and cause losses in both grain yield and quality. For this reason, much research has focused on this group of smuts. Others, such as Urocystis cepulae, Entyloma ellisii, and Ustilago scitaminea are crop pathogens of onions, spinach, and sugarcane, respectively. One of the pathogens of weeds and horticultural species, Microbotryum violaceum (Persoon:Persoon) G. Deml and Oberwinkler (syn. Ustilago violacea) is a smut fungus that can parasitize well over 100 species of Caryophyllaceae (Thrall et al., 1993).

The life cycle of M. violaceum is shown in Fig. 1.1. Under favourable conditions, the thick-walled teliospores germinate, undergo meiosis, and produce a three-celled promycelium. The nuclei divide mitotically and four haploid basidiospores (two a_1 and two a_2 mating types) bud from the promycelium. The basidiospores can proliferate by yeast-like budding. Under favourable conditions budding occurs every 5 h; thus numerous sporidia are formed in a short time.

Under appropriate conditions, a_1 and a_2 sporidia will pair and a conjugation tube will grow from the a_2 to the a_1 sporidium. On a suitable host, a dikaryotic infection hypha, an appressorium, and a penetration peg will form. After penetration, the dikaryotic hyphae will continue to grow intercellularly (Fischer and Holton, 1957). The entire plant can become infected systemically. However, infection can also be limited to only a portion of the plant (semi-systemic infection). Infected plants tend to exhibit subtle symptoms including a slightly shorter plant stature, a bushier appearance, and more flowers per plant (Alexander and Maltby, 1990). Infection can occur at anytime during the growing season of the host and the dikaryotic hyphae can overwinter in the roots. When the hyphae eventually reach the immature floral parts, either in the same or the next growing season, teliospore formation occurs.

Fig. 1.1 Life cycle of Microbotryum violaceum

A: Teliospore germination and meiosis
B: First mitosis from promycelium
C: Vegetative growth by budding cells
D: Conjugation between opposite mating type cells
E: Growth of dikaryotic mycelium
F: Infection of susceptible host
G: Intercellular growth until mycelium reaches immature floral structures
H: Dikaryotic teliospore formation
I: Karyogamy in the teliospore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Teliospore development begins with the formation of numerous globose dikaryotic cells, each surrounded by a thick gelatinous layer. The outer layer darkens and hardens. It is interesting that *in vivo,* teliospores exist almost exclusively in the anthers of plants. In male plants, the teliospores completely replace the pollen in the anthers. In female plants, most of the ovarian tissue aborts and the vestigial anthers enlarge and become filled with teliospores. However, some teliospore formation can also occur in the remaining ovarian tissue. Teliospores are disseminated mainly by pollinating insects (Baker, 1947).

The haploid sporidia can be cultured easily and maintained under laboratory conditions. Since they are well suited for mutational studies and are genetically stable, haploid sporidia of M. violaceum have been used extensively in classical genetic research.

Haploid sporidia of opposite types can be encouraged to undergo mating under certain laboratory conditions. During nutrient starvation (growth on water agar), haploid sporidia of opposite mating types that are in contact with each other will form short conjugation tubes and will mate (contact mating). In addition, distance mating has also been observed between opposite sporidium types. This involves the formation of a conjugation tube between cells that are up to 30 μm apart. In both cases, dikaryotic mycelial growth can be maintained under laboratory conditions in the presence of either host extracts or simply α-tocopherol (Castle, 1984).

1.2 The Fungal Extra-Cytoplasmic Region

1.2.1 Fungal Cell Walls - Composition

The fungal cell wall serves many functions including maintaining cellular shape by its rigidity, acting as a structural barrier and filter for molecular movement in either direction, serving as a site for various enzymes involved in nutrient uptake, and housing numerous macromolecules involved in recognition systems (Peberdy, 1990; Kuhn and Trinci, 1990; Ruiz-Herrera, 1992; Sentandreu et al., 1994). It is a highly dynamic structure that can change during different stages of growth and development of the organism. However, certain macromolecular components are always present.

Fungal cell walls are composed primarily of polysaccharides that constitute two groupings, based on their solubilities and their presumed

functions: skeletal polysaccharides and matrix components (Ruiz-Herrera, 1992; Gow and Gadd, 1995).

The water-insoluble β 1-4 chitin and ß-1-4 linked glucans are highly crystalline homopolymers that form the skeletal polysaccharides. All fungal cell walls contain chitin, although some, such as the Oomycetes have only small amounts (Gow and Gadd, 1995). Chitin synthases, present within the plasma membrane, transfer GlcNAc from UDP-GlcNAc at the cytoplasmic phase of the membrane to growing chitin chains at the outer surface of the membrane (Kuhn and Trinci, 1990). Hydrogen bonds link adjacent chains, thereby forming a dense crystalline lattice (Peberdy, 1990). In addition, the cell walls of all Ascomycetes, Basidiomycetes and Oomycetes contain (1-3)-ß-glucan. Like chitin synthases, (1-3)-ß-glucan synthases are also present in plasma membranes, although they catalyze the polymerization of glucose units from UDP-Glc (Girard and Fevre, 1984). Finally in mature cells, wall rigidity is augmented by the formation of alkali insoluble chitin - (1-3)-ß-glucan complexes (Wessels, 1986).

In contrast, the water-soluble α-glucans are amorphous or slightly crystalline, homo- and hetero-polymers, and even disaccharide complexes. These components, along with glycoproteins (especially mannan-protein complexes), form the matrix of the fungal cell wall (Peberdy. 1990; Gow and Gadd, 1995). Extensive research has been done on the mannoproteins present in the cell walls of several yeasts (Herrero et al., 1987; Elorza et al., 1985; Casanova et al., 1992). Up to 60 different mannoproteins have been released from cell walls and periplasmic regions of some yeasts, but in most cases their functions have not been determined.

In general, mannoproteins are biosynthesized in the secretory pathway of the cell and are released via secretory vesicles at the plasma membrane. They are associated with other cell wall components via hydrogen bonds, disulphide bonds, and by covalent linkages to ß-glucans (Peberdy, 1990). The composition and quantity of mannoproteins in the cell wall is not constant. Changes have been correlated with age, stage of growth cycle, and the switches to mycelial growth from yeast-like growth (Elorza et al., 1985).

Fungal cell walls are arranged in lamellae, and the components described above are organized in distinctive amounts in each of the layers (Ruiz-Herrera, 1992). In general, the inner layer of the wall is predominantly composed of the fibrous components (chitin and ß-[1-3]-glucan). The matrix components are

found throughout the fungal cell wall, but are the predominant components of the outer layer. Glycoproteins such as agglutinins and adhesins are found on the outer-most face of the wall, exposed to the environment (Gow and Gadd, 1995). In addition, other components that appear to be unique to certain fungi may also constitute layers of the wall. As an example, hydrophobins are hydrophobic proteins that form arrays of parallel rodlets on the outermost layer of the aerial hyphal walls of Schizophyllum commune (Wessels, 1992), Neurospora crassa, and Aspergillus nidulans (Gow and Gadd, 1995).

1.2.2 Fungal Cell Walls - Interactions

Cell to cell interactions are fundamental to the processes of fungal growth and development. In particular, cell to cell adhesions occur during mating (self-fusion), pathogenesis (fusion to a host cell), and symbiosis (fusion to a symbiont cell). The outer-most fungal cell wall surface functions as an interface in all three of these interactions, and thus it must contain key factors involved in cell to cell communication.

Mating fusions have been studied extensively in Saccharomyces cerevisiae. Contact between opposite mating types (a and α) is triggered by the secretion of reciprocal pheromones. When a-mating type cells are exposed to α -pheromone, the cell wall undergoes numerous changes. These include increased adhesiveness toward α-mating type cells and anisotropic cell wall synthesis which results in alterations of the cell's morphology (schmooing). The same response is observed in α-mating type cells exposed to a-factor. The increases in cell agglutinability are associated with the presence of new surface glycoproteins (a- and α-agglutinins; Sijmons et al., 1987). The changes in cell morphology are associated with alterations in cell wall architecture. In particular, the inner and outer cell wall layers are modified (Lipke et al., 1976). These two fungal cell wall changes are followed by the actual fusion of the two opposite mating type cell walls. Numerous biochemical components, including fimbriae (discussed below) have been implicated as having a role in this process. After this physical attachment event, chemical changes occur including highly localized lysis (Aylemore and Todd, 1986; Calleja et al., 1981).

The cell walls of pathogenic fungi must interact with host cell surfaces, especially during the initial adhesion. In addition to fungal fimbriae (discussed below), fungal cell wall polysaccharides and glycoproteins have roles as determinants in fungus-plant interactions (Peberdy, 1990), and fungus-animal

interactions (Douglas, 1987). The carbohydrate components may act as lectins and determine compatibility or incompatibility in plant-fungus interactions. In addition, fungal glycoproteins may elicit phytoalexin synthesis in infected plants.

In Candida albicans the switch from the yeast growth to hyphal growth is correlated with a change in mannoprotein cell wall composition and an increase in adhesiveness to epithelial cells (Elorza et al., 1985). Similar changes in glycoprotein cell wall components are observed in the traps of nematophagous fungi. The traps of Arthrobotrys oligospora contain a lectin-like glycoprotein that is absent in vegetative mycelium (Tunlid et al., 1992). In both of these examples, it is assumed that a complementary receptor must be present on the host cell surface.

Finally, the least understood interactions of fungal cell walls are those involving symbionic relationships, such as mycorrhizae and lichens. Bonfante-Fasolo et al. (1992) suggested that the fungal cell walls undergo characteristic modifications upon contact between the fungus and the plant. However, these changes have yet to be fully characterized. In addition, the recognition mechanisms between bionts leading to lichenization are also unknown (Hawksworth, 1988). However, ultrastructural studies of the cell walls demonstrate that the surface layers of both bionts are altered; both cell walls have tessellated patterns that mesh together when contact is made (Honegger, 1984). It has been suggested that lectin-mediated recognition between cell walls is used in the contact stages leading to lichenization (Hawksworth, 1988).

1.2.3 Fungal Fimbriae - An Overview

In the early 1950's, the first observations of bacterial fimbriae (Duguid et al., 1955), or pili (Brinton. 1959) were reported. Since then, isolated reports of surface fibrils on various eukaryotic organisms including fungi were published. However, these fibrils were never clearly defined biochemically nor were they correlated with the analogous bacterial appendages.

In 1974, Poon and Day published the first definitive report that described fungal fimbriae, structures that qualitatively resemble their bacterial counterparts. Based on EM observations, fungal fimbriae were characterized as flexible, 6-7 nm diameter structures that ranged in length from 0.5 to 20 μm (Fig. 1.2). Early biochemical analysis suggested that fimbriae are purely proteinaceous structures. It was also suggested that fimbriae are required for mating since it was noticed that haploid sporidia in the early stages of distant

Fig.1.2 Electron micrograph of fimbriae on the cell of M violaceum. Budding cells were shadowed with tungsten trioxide. Bar represents 1 μm. (adapted from Day and Garber, 1988).

mating (1.1) appeared to be connected by invisible threads. Subsequent EM examination of these cells revealed that in fact, they possessed long fibrils (termed fungal fimbriae) on their extracellular surfaces. Defimbriated cells only participated in mating at a distance after the fimbriae have begun to regenerate. Poon (1974) showed that the rate of regeneration of fimbriae is 2 μm/h.

Further studies by Gardiner (1985) showed that fimbriae of M. violaceum are composed of 74 kDa proteinaceous subunits that are able to re-assemble spontaneously into 7 nm fibrils. Based on the results of isoelectric focusing studies, Gardiner suggested that the subunits are modified post-translationally, being acetylated or phosphorylated, but not glycosylated. The fimbriae appear to be essential to cell survival. It is interesting that of the 22 races of M. violaceum examined, all were fimbriated. In addition, all of the induced fim^- mutants were unstable and reverted to fim^+. Gardiner proposed that this failure to isolate a stable fim^- mutant may indicate that mutation of the fim gene is lethal. Based on these findings and the apparent widespread distribution of fimbriae among fungi, Gardiner suggested that fimbriae have an essential role in fungal physiology. Since the fungal surface is involved in many vital processes, he proposed that fimbriae may be multi-functional, with possible roles in motility, adhesion, nutrient transport, and cell-to-cell communication.

Xu (1992) showed that the fimbrial subunits of M. violaceum are, in fact 74 kDa glycoproteins. In addition he showed that, at least in U. maydis, there appears to be more than one fimbrial type.

Research on fimbriae in general and on M. violaceum fimbriae in particular has been extensive. However, numerous questions still remain. This thesis attempts to examine both the biochemical composition of these structures, including the analysis of each of the components, and the function of the fimbrial components and native fimbriae.

1.3 Aims of Current Research

Three major aims were decided on at the beginning of this research.

1) To study the structure, location and biochemical composition of fimbriae associated with M. violaceum.

2) To ascertain the relatedness of the components of fungal fimbriae amongst members of the mycota.

3) To initiate investigations of possible biological functions of fungal fimbriae.

CHAPTER 2 - FIMBRIAL RNA

2.1 Introduction

RNA is an multi-functional macromolecule that has been implicated in many diverse biological functions. It can serve as a carrier of genetic information, an enzyme (Gilbert, 1986; Moore, 1992; Zaug and Cech, 1986) and a stimulator molecule (Wissler et al., 1988). Its presence in unexpected and diverse cellular activities has led to theories about its potential to be the archetypal functional biological unit (Joyce, 1989; Hoffman, 1991; Lewin, 1986). It has been suggested that this presence of RNA in such varied areas of biological activities is a remnant of its former fundamental functions. In effect, contemporary RNA is thought to be a biochemical fossil, exhibiting only certain aspects of its ancestral role. Thus the discovery of a novel RNA species that is involved in yet another facet of biological activity, namely mating and pathogenesis, is perhaps not surprising.

Fimbriae are involved in cell-to-cell communication during mating and pathogenesis. They are essential for conjugation, may be involved in the development of the conjugation tube (Day, 1976), and are responsible for transferring sex specific molecules between opposite mating types prior to conjugation tube growth (Day, 1976; Day and Poon, 1975). Fimbriae have also been implicated as carriers for the agglutination factors (Calleja, 1987; Crandall et al., 1977; Day, 1976) required specifically for sexual agglutination. In addition, fimbriae may play an important role in pathogenesis, in particular as adhesion structures responsible for attaching the pathogen to the host (Rghei et al., 1992).

The present study provides evidence that intact, native fimbriae of M. violaceum contain a nucleic acid component, consisting of single-stranded RNA molecules that are all approximately 30 bases in size. This research ropresents the first example of the isolation and characterization of an RNA molecule from an extracellular appendage in eukaryotes. This may, in fact, prove to be the anticipated factor involved in the cell-to-cell communication.

2.2 Methods and Materials

2.2.1 Stock Cultures and Growth Conditions

Stocks of M. violaceum, wild-type a_1 and a_2 strains (UWO-1 [a_1] and UWO-1 [a_2], ATCC 22,000 and 22,001; Gardiner et al., 1981) were stored either in liquid nitrogen or in silica at -20°C. Active cultures were maintained on Ustilago complete medium (Day and Jones, 1968) either in liquid culture or on solidified media (2% [w/v] agar) at 22°C.

2.2.2 Isolation and Purification of Fimbriae

Exponential-phase haploid cells from well-aerated liquid cultures were harvested by low speed centrifugation (Sorvall RC-3, HG-4 rotor, 4°C, 15 min, 2400 x g) (Poon and Day, 1975). The pelleted cells were kept on ice, resuspended in ddH_2O and mechanically defimbriated (Sorvall Omnimixer, maximum speed; Poon and Day, 1975). The defimbriated cells were pelleted by centrifugation (Sorvall RC-5B, SS-34 rotor, 4°C, 10 min, 6500 x g) and discarded after confirmation by microscopy that no cell breakage had taken place. The supernatant liquids containing the crude fimbriae were dialysed against distilled water (4°C, Spectra/Por membrane, mwco 3,500) and concentrated with polyethylene glycol 20 000 (Pohl, 1990).

The density of a cesium chloride solution (approx. 3.6 M) containing 2.5 mg ml^{-1} crude fimbriae (by dry weight) was adjusted using a diffractometer (Bausch & Lomb), to 1.36 g cm^{-3}. This solution was subjected to ultracentrifugation (Beckman L8-M ultracentrifuge, Ti40 rotor, 15°C, 19 h, 120,000 x g). The cesium chloride gradients generated were transilluminated with visible light (Fibre Lite, High intensity illuminator 170-D) and material present in the observed band was collected. The refractive index (RI) of the collected material was measured and the cesium chloride was removed by dialysis.

2.2.3 Electron Microscopy of Fimbriae

A drop of solution containing dialysed fimbriae was placed on a formvar coated (0.25%), carbon reinforced, 400 mesh copper grid (EMicron) and left for 30 sec. Excess fluid was blotted off and the sample was negatively stained with one drop of 2% [w/v] ammonium molybdate, pH 7.4 for 30 sec prior to drying (Poon and Day, 1974). The grids were drained, air dried, and viewed with an

EM. Material from the cesium chloride band, the top of the gradient, and pelleted material were negatively stained and examined in the EM (Philips EM 200).

2.2.4 Isolation of Glycoproteinaceous Subunits from Polyacrylamide Gels

Purified fimbriae were solubilized at 95°C for 10 min in sample buffer (10% [w/v] SDS, 60 mM Tris-HCl [pH 8], 20% [v/v] glycerol and 5% [v/v] BME). Fimbrial components were separated by electrophoresis on 10% polyacrylamide gels according to the method of Laemmli (1970). Vertical strips, cut from both sides of the gel were stained with 0.1% [w/v] Coomassie Blue R-250 (in 40% [v/v] methanol-10% [v/v] acetic acid and destained in the same solvent) and re-aligned with the gel. A horizontal strip containing the 74 kDa glycoprotein was cut from the gel and the contents were collected by electro-elution (100 V, 3-5 h, on ice, Little Blue Tank™, ISCO) using Laemmli (1970) buffer.

2.2.5 Assessment of Purity of Glycoprotein Components

Protein concentrations of purified fimbriae from M. violaceum were determined using the Bradford dye binding assay (Bio-Rad) with BSA as the standard. Crude fimbriae, purified fimbriae, and eluted subunit components were solubilized and separated by electrophoresis on 10% [w/v] polyacrylamide gels. Gels were stained either with Coomassie Blue R-250 (2.2.4) or silver (Bio-Rad, Silver Stain Plus).

CsCl purified fimbriae and eluted fimbrial subunits were analyzed spectrophotometrically (200 to 500 nm) using a Shimadzu UV-160 recording spectrophotometer.

2.2.6 Isolation of f-RNA

Purified fimbriae were concentrated using anhydrous 2-butanol and one of three methods was employed to isolate f-RNA. In all three methods, fimbriae were first subjected to DNase-free (Sambrook et al., 1989) RNase A digestion (Boehringer Mannheim, 5 µg/ml fimbriae, 37°C, 30 min) prior to f-RNA isolation.

The single step method of RNA isolation (Ausubel et al., 1989) using acid guanidinium thiocyanate-phenol-chloroform (AGPC) was used on intact, purified, RNase treated fimbriae. The resultant f-RNA was precipitated in isopropanol, washed with 70% [v/v] ethanol, and the pellet was dried and resuspended in DEPC treated water (Sambrook et al., 1989).

Alternately, f-RNA was isolated from purified fimbriae by digestion with Proteinase K (BRL, Bethesda Research Laboratories) based on a modification of the method described in Sambrook et al. (1989) (1 U/ml purified fimbriae, 10 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 0.5% [w/v] SDS, 1 mM $CaCl_2$, 50°C, 3h). The digestion was terminated with 2.2 mM EGTA-HCl (pH 8) and the solution was repeatedly extracted with phenol:chloroform:iso-amyl alcohol (25:24:1). Nucleic acids were precipitated with 3 vol of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). Pellets were dried and resuspended in DEPC treated water.

Finally, a method using guanidine hydrochloride (G-HCl) (A. White, pers. comm.) was employed to isolate f-RNA. Purified fimbriae were incubated on ice with 2.5 M G-HCl or 7 M G-HCl with 0.5 mM EDTA-HCl (pH 8). Precipitated material was centrifuged (12 000 X g, 15 min) and 100 µl of DEPC treated water was added to the pellet. The material was kept on ice for 30 min and the aqueous fraction, containing the nucleic acids, was collected. The nucleic acid component was precipitated and resuspended as described above.

2.2.7 Characterization of f-RNA

f-RNA samples and RNA size markers were denatured by heating to 95°C for 2 min in 80% (v/v) deionized formamide, followed by quick cooling on ice. Analysis by electrophoresis was performed in 1.5 mm thick, 6% [w/v] urea polyacrylamide gels (Hoeffer Mighty Small™; 1x TBE, 150 V, 25 min). These gels were stained for 5 min in ethidium bromide, transilluminated with uv, photographed, and subsequently digested with RNase A (Boehringer Mannheim 0.5 µg/ml, 37°C, 1 h). The gels were then re-stained in ethidium bromide and photographed a second time. In addition, f-RNA samples were subjected to high (0.3 M NaCl) or low (0.03 M NaCl) salt conditions during RNase A digestion (83 µg RNase A/µg f-RNA, 37°C, 30 min) in order to determine strandedness (Bozarth et al., 1971; Goodin et al., 1992).

2.2.8 Purification of f-RNA

f-RNA, visualized by ethidium bromide staining of a polyacrylamide gel, was excised and placed into 500 µl of 2 M ammonium acetate. The solution was warmed (35°C, 45 min) and stored for 5 h at 4°C. The aqueous phase was removed and the nucleic acid was precipitated (as described above).

Alternately, the isolated f-RNA was purified by HPLC using a Zorbax Bio Series Oligo Column (DuPont) in conjunction with System Gold (Beckman). The mobile phases (A = 20% acetonitrile, 80% 20 mM sodium phosphate [mono basic] pH 7.0, B = A + 1.0 M NaCl) were used to generate a gradient (15% B to 75% B) at a flow rate of 1 ml min^{-1} for 40 min. The effluent was monitored at 260 nm and peaks were analyzed spectrophotometrically, using diode array analysis, between 200 and 350 nm.

2.2.9 End-labelling of f-RNA

The 5' terminal phosphate residues of f-RNAs were hydrolysed using calf intestinal phosphatase (Boehringer Mannheim; Sambrook et al., 1989), and the resultant 5' hydroxyl groups were end-labelled radioactively using T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (>6000 Ci/mmol, NEN) as described by Sambrook et al. (1989). The radio-labelled f-RNAs were separated from the reaction mixture components by filtration through Sephadex G-25 columns, followed by precipitation with 3 vol of ethanol containing 0.3 M sodium acetate (pH 5.2). The dried pellets were resuspended in DEPC treated water and subjected to urea PAGE (as described above). Unlabelled f-RNA was analyzed on the same gel. After electrophoretic separation, the resultant gel was dried and the labelled f-RNA band was detected by autoradiography. Its size was confirmed by comparison with the unlabelled f-RNA and with the RNA size markers.

2.2.10 Attempts to Hybridize f-RNA to Genomic DNA

Total genomic DNA was isolated from M. violaceum using the method of Specht et al. (1982). Exponential-phase cells (2 g wet weight) were washed in 50 mM EDTA-HCl (pH 8), frozen to -80 °C,and lyophilized for 12 h. The dried cells were manually ground to a fine powder and the genomic DNA was slowly extracted from the ruptured cells in extraction buffer (10% [v/v] toluene, 2% [w/v] SDS, 150 mM NaCl, 100 mM EDTA-HCl [pH 8], 50 mM Tris-HCl [pH 7.9]). Following 48 h of agitation (200 rpms) at RT, sodium perchlorate (0.5 M final conc) was added to the slurry which was then extracted repeatedly with phenol:chloroform:isoamyl alcohol (25:24:1). The nucleic acids in the aqueous supernatant were precipitated with 2 vol of ethanol. Pellets were dried and resuspended at 4°C for one week. Genomic DNA was treated with RNase A, re-extracted, re-precipitated, and digested with restriction enzymes. DNA

fragments were separated by electrophoresis (0.8% [w/v] agarose gel, 1x TAE, 20 V), visualized with ethidium bromide, and transferred onto nitrocellulose membrane (Southern, 1975).

The specific activity of 5' end labelled f-RNA was determined using a liquid scintillation counter (Beckman, LS 6000 LL). Radiolabelled f-RNA (9×10^4 cpm mg^{-1} of ^{32}P-f-RNA) was added to RNase-free hybridization tubes containing M. violaceum genomic DNA immobilized onto nitrocellulose membranes. Annealing of the probe to the target DNA was carried out at various temperatures (22°C, 30°C, 42°C, 45°C, 48°C, 52°C). After 16 h of hybridization, the nitrocellulose membranes were washed (20x SSC, 0.1% [w/v] SDS, RT, 15 min) thrice and exposed to x-ray film.

2.3 Results

2.3.1 Isopycnic Centrifugation

A method was developed to isolate purified fimbriae of M. violaceum using isopycnic centrifugation in cesium chloride gradients. A single band was generated in the gradient tube (Fig. 2.1 A), while other material either floated on the surface of the cesium chloride gradient, or was found as a pellet at the bottom of the tube. EM examination of the material collected from this band showed only typical long, 7 nm diameter fimbriae (Fig. 2.1 B). Other material, located at the top of the gradient or as a pellet, was examined and did not contain fimbriae. Analysis of the material from the cesium chloride band (purified fimbriae) by SDS-PAGE (Fig. 2.1 C) revealed that the only proteinaceous material present was the 74 kDa subunit. Whereas, crude fimbrial preparations contained detectable contaminants. When the subunit glycoproteins were eluted from the polyacrylamide gels and re-separated on a second SDS-PAGE, the 74 kDa subunit remained evident and the band appeared similar to that generated by CsCl purification.

The density of the purified fimbriae was calculated, based on the observed refractive index of 1.368 (mean value of 15 purifications), and using the following equation (Ifft et al., 1961):

$$\rho^{25°C} = (10.8601 \times RI) - 13.4974$$

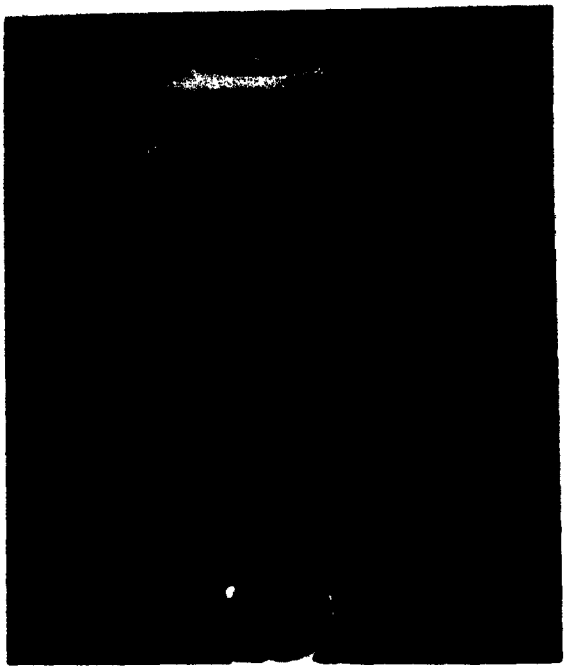
where $\rho^{25°C}$ = density at 25°C of a CsCl solution. The calculated density (1.363 g cm^{-3}) of fimbriae was used to approximate the percent composition of fimbriae based on the estimates that in CsCl solutions, nucleic acids and proteins have

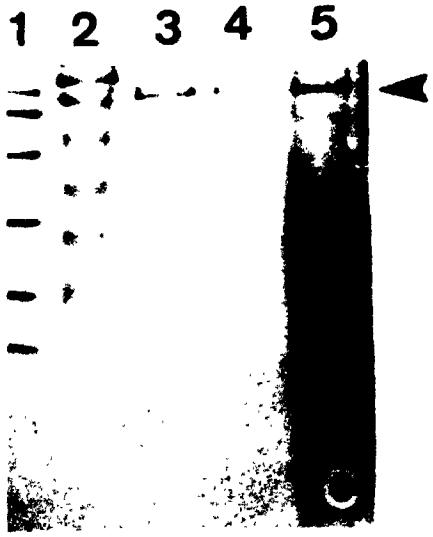
Fig. 2.1 Cesium chloride gradient centrifugation as a method to purify fungal fimbriae.

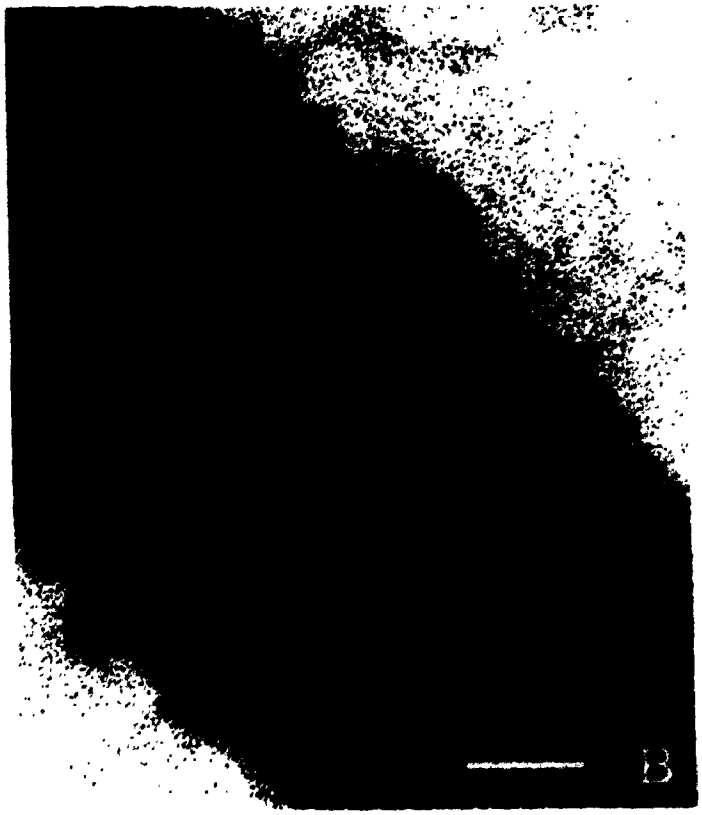
A: Photograph of isopycnic CsCl gradient used to purify fimbriae from M. violaceum. The tube was transilluminated using fibre optics. The density of band was 1.3683 g cm^{-3}.

B: Electron micrograph of negatively stained M. violaceum fimbriae purified by isopycnic CsCl gradient centrifugation. Fimbriae were found exclusively in the band generated in the gradient tube. The fimbriae of M. violaceum are 7 nm in diameter and range in length from 1 to 20 µm. Bar represents 0.1 µm.

C: Analysis of fimbrial subunits by SDS-PAGE. Gel was stained with Coomassie Blue R-250. Lanes 1: protein standards (kDa; Bio-Rad) 97, 68, 43, 30, 21, 14; lane 2: pre-stained protein standards (kDa; Bio-Rad), 107, 76, 52, 36.8, 27.2, 19; lane 3: solubilized fimbriae purified on cesium chloride density gradients; lane 4: solubilized, eluted glycoprotein subunits of fimbriae; lane 5: solubilized crude fimbrial preparation prior to purification. 74 kDa fimbrial subunit is indicated by arrow.




1 2 3 4 5
C


B

average densities of 1.70 g cm^{-3} and 1.30 g cm^{-3}, respectively Using the following equation (Freifelder, 1982):

$$\rho = N\,(1.7) + (1\text{-}N)\,(1.3)$$

where N = fraction of nucleic acid, and ρ = density, it was calculated that fimbriae contained approximately 15.7 % nucleic acid.

2.3.2 Spectral Changes Upon Depolymerization

Spectrophotometric examination of the purified fimbriae (Fig. 2.2, solid line) revealed a spectrum which resembled that of a nucleoprotein, with an absorption maximum at 258 nm and a minimum at 249 nm. The A_{280}/A_{260} ratios of purified fimbriae was calculated to be 0.68. Based on standard curves prepared by Paul (1959) for A_{280}/A_{260} ratios of simple plant viruses, it was estimated that intact fimbriae contain approximately 10 to 15% nucleic acid.

The uv spectrum of the eluted structural 74 kDa subunits is represented by the dashed line in Fig. 2.2. In contrast to intact, purified fimbriae (solid line), the uv absorption spectrum of the eluted 74 kDa glycoprotein subunits (dashed line) showed an absorption maximum at 274 nm and a minimum at 249 nm. This spectrum is typical of proteins containing <5% nucleic acid (Paul, 1959).

2.3.3 Analysis of f-RNA

Isolation of the nucleic acid component of fimbriae (f-RNA) was attempted by disrupting the proteinaceous components of fimbriae with chaotropic agents (AGPC method and G-HCl method) and proteolytic digestion (Proteinase K method). f-RNA was successfully isolated from the fimbriae using the AGPC method and the proteolytic digestion method. Both of these methods were effective in isolating the nucleic acid component, but the latter resulted in a higher yield (1 µg f-RNA/10 µg protein). The absorbance spectrum of the final product was consistent with that of a nucleic acid. The nucleic acid was estimated to contain approximately 30 residues by comparing its migration with that of RNAs of known sizes in a denaturing urea polyacrylamide gel. The nucleic acid preser. the gel was made visible by ethidium bromide staining and uv transillumination (Fig. 2.3 A), and then removed by subjecting the entire gel to digestion with RNase A (Fig 2.3 B). Digestion of all RNA polynucleotides present in this gel (and none of the DNA polynucleotides) indicated that the nucleic acid species present in fimbriae is in fact RNA. Since digestion occurred

Fig. 2.2 Spectrophotometric analysis of both purified fimbriae and eluted fimbrial subunits from M. violaceum. (——) indicates the uv spectrum of the intact fimbriae, while (- - -) shows the uv spectrum of the eluted 74 kDa glycoprotein subunits.

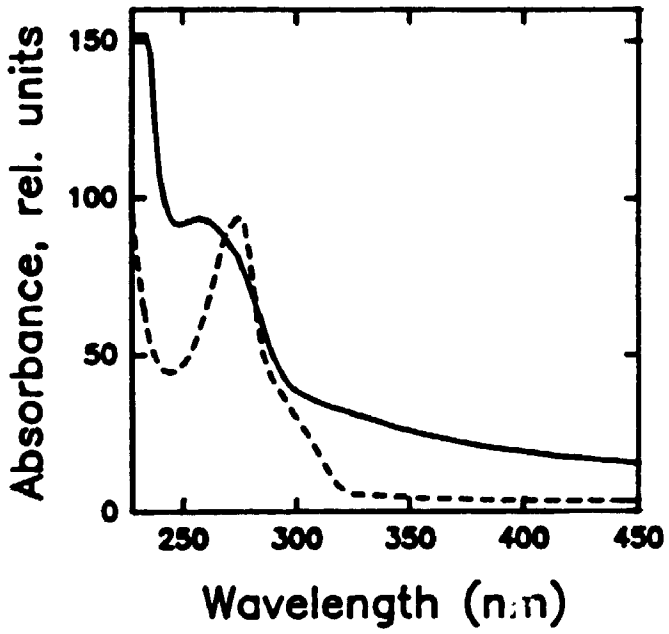
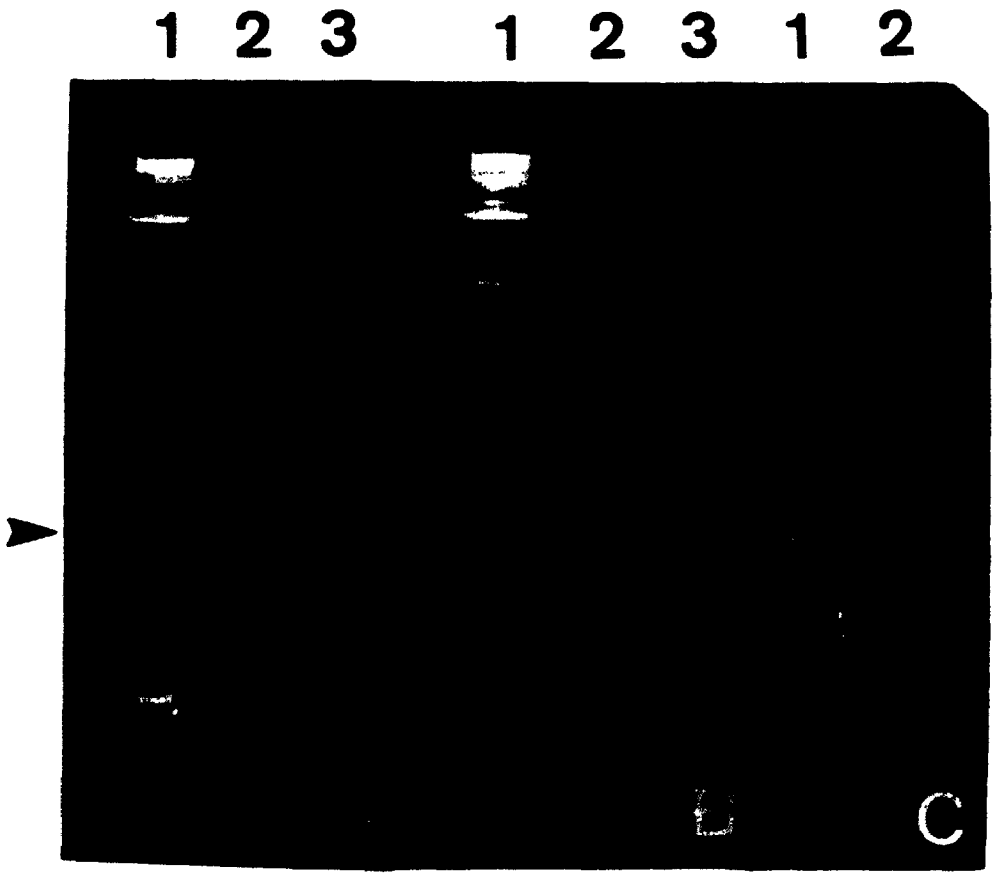

Absorbance, rel. units
150
100
50
0
250
300
350
400
450
Wavelength (nm)

Fig. 2.3 Urea-PAGE analysis of f-RNA.

A: Detection of f-RNA isolated from the fimbriae of M. violaceum, as analyzed by 6% urea PAGE, stained with ethidium bromide and uv transilluminated. Lane 1: partially denatured DNA size markers; lane 2: molecular RNA size markers (248, 153, 35 bases); lane 3: arrow marks the 30 base single-stranded f-RNA.

B: Same gel as panel A, but viewed after digestion with RNase A.

C: Determination of f-RNA strandedness by digestion with RNase A in the presence of high and low salt. Lane 1: in 0.3 M NaCl; lane 2: in 0.03 M NaCl.


1 2 3 1 2 3 1 2
C

in both 0.3 M NaCl and 0.03 M NaCl, the f-RNA is probably not double-stranded (Fig. 2.3 C).

Further, the 5' end of the f-RNA was successfully labelled using [γ-^{32}P]ATP (Fig. 2.4). This entailed enzymatic de-phosphorylation followed by enzymatic re-phosphorylation with a radiolabelled phosphate. The result indicates that the f-RNA has a standard 5' terminal phosphate.

2.3.4 Absorption Spectrum of f-RNA

The absorption spectrum of isolated f-RNA (Fig. 2.5) between 225 nm and 400 nm was consistent with that of most RNA species. That is, f-RNA had an absorption maximum at 258 nm and a minimum at 238 nm. In addition, f-RNA had a second maximum at 219 nm and a second minimum at 205 nm. f-RNA purified by both methods (GITC and Proteinase K) displayed this absorption pattern. f-RNA was purified by HPLC and the chromatogram is shown in Fig. 2.6 A. The absorption spectrum of the first peak (Fig 2.6 B) is consistent with that of f-RNA and it exhibits the same two maxima and minima. This result suggests that there is a functional group covalently linked to the RNA. Further studies are required to determine the identity of this material. In addition, the absorption spectrum of the second peak is shown in Fig. 2.6 C. Its identity has not yet been determined.

2.3.5 Hybridization Experiments

Radioactively labelled f-RNA was used as a probe in an attempt to locate the corresponding genomic DNA sequence. A range of hybridization conditions was attempted (see pg. 17), but the resulting autoradiographs failed to discern a unique band indicative of the complementary genomic DNA fragment (data not shown).

2.4 Discussion

Collectively, these results indicate that native fimbriae are composed of nucleic acid constituents in addition to glycoprotein components. The isolation of the nucleic acid component and its characterization both confirm its existence and show it to be a 30 base, single-stranded RNA.

The pre-digestions of the purified, intact fimbriae with RNase A prior to chaotropic disruption or proteolytic digestion did not affect f-RNA isolation. This indicates that 1) the f-RNA is not a contaminant which co-purifies with fimbriae,

Fig. 2.4 Radiolabelling f-RNA and analysis of the 5' terminus of f-RNA. RNAs were subjected to 6% urea-PAGE, stained with ethidium bromide and transilluminated with uv. Lane 1: molecular RNA size markers (248, 153, 35 bases); lane 2: unlabelled f-RNA; lane 3: autoradiograph of f-RNA 5' end-labelled with γ-^{32}P. A total of 75,000 cpms were loaded onto the gel. The exposure time was 1 h at -80°C with an intensifying screen.


1
2
3

Fig. 2.5 Spectrophotometric analysis of f-RNA from 220 to 350 nm. The absorption maximum and minimum are present at 258 nm and 239 nm, respectively.

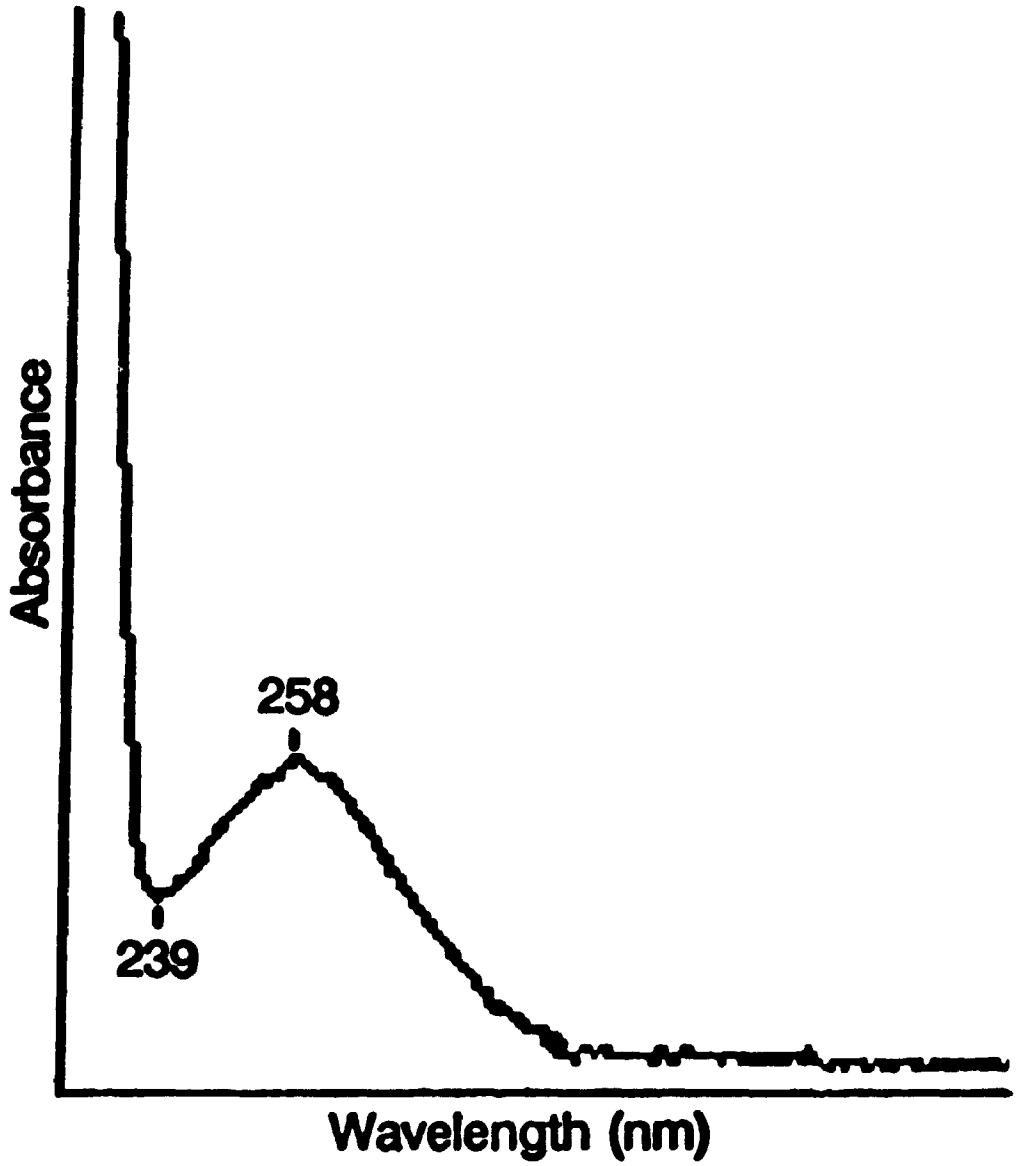
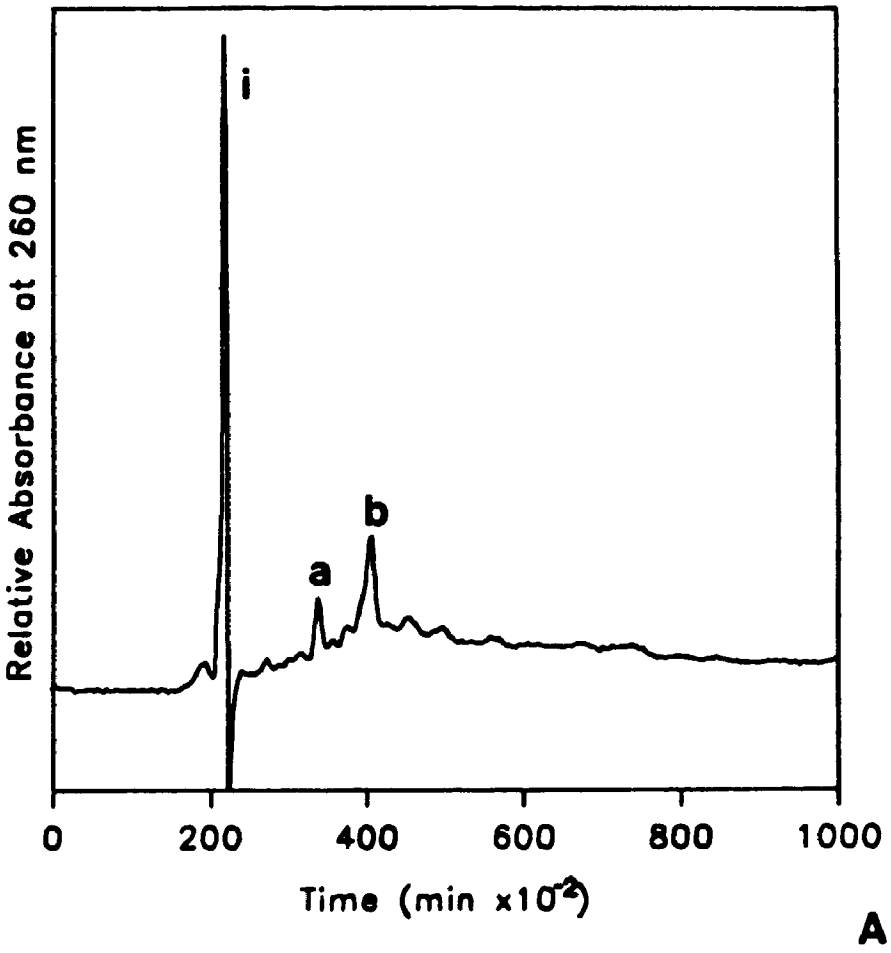

Absorbance
258
239
Wavelength (nm)

Fig. 2.6 HPLC analysis of f-RNA.

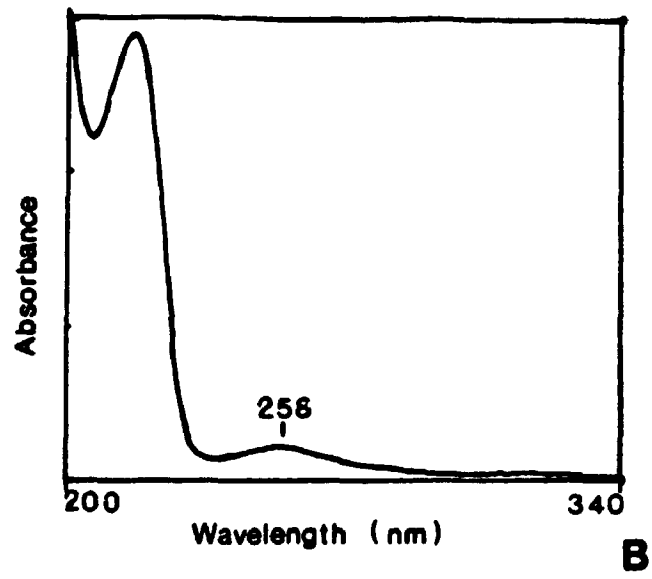
A: f-RNA, after isolation using the protease digestion method, was purified by HPLC. The HPLC plot consisted of the injection peak (i) and two additional peaks (a and b).

B: Uv spectrum of the first peak (a) of the f-RNA HPLC plot (shown in A). Spectrophotometric analysis is from 200 to 340 nm.

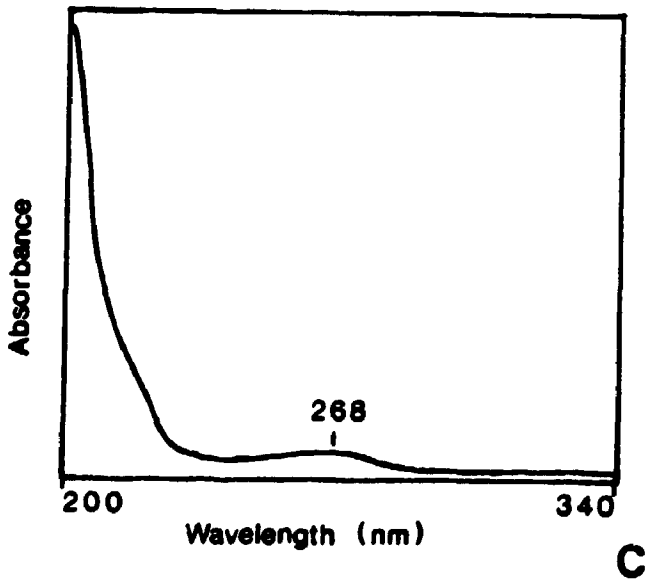
C: Uv spectrum of the second peak (b) of the f-RNA HPLC plot (shown in A). Spectrophotometric analysis from 200 to 340 nm. In this case the spectrum does not exhibit a conspicuous pattern which would permit identification of this component.


i
a
b
Relative Absorbance at 260 nm
0
200
400
600
800
1000
Time (min x10⁻²)

A


Absorbance
256
200
340
Wavelength (nm)

B


Absorbance
268
200
340
Wavelength (nm)

C

and 2) the f-RNA, when present in intact fimbriae, is resistant to RNase digestion. These results suggest that the fimbrial subunits, when present in their native form, must maintain a conformation which effectively protects the f-RNA from external enzymatic digestion. An unprotected RNA in the M. violaceum environment would be highly susceptible to degradation (Donly and Day, 1984).

The pronounced shift in the absorption maximum from 258 nm (purified fimbriae) to 274 nm (eluted 74 kDa glycoprotein subunits) indicates that the f-RNA is released upon depolymerization of the fimbriae into its subunits, thereby suggesting that f-RNA is probably not attached to the glycoprotein. This suggests that a non-covalent affiliation between the protein and nucleic acid components exists, which is the usual case with nucleoproteins. The nature and specificity of the attraction has yet to be determined, but probably involves electrostatic or hydrogen bonding.

The single peak of f-RNA in the HPLC chromatogram suggests that the f-RNA is of homogeneous size, since the methodology used would have separated RNAs of any other sizes. In addition, the absorption spectrum of purified f-RNA indicates that a chemical component is likely attached covalently to the f-RNA. This modification may be similar to that found on other RNA species that are challenged by harsh environments. Ovodov and Alakhov (1990) demonstrated that acetylated mRNAs were resistant to digestion by pancreatic RNases and that the modification does not interfere with translation. Also, Kowalak et al. (1994) found that the conformation of partially acetylated tRNA was more rigid, and thus more stable at high temperatures. To date, these acetyl-stabilized tRNAs have been found exclusively in archaeal thermophiles. Consequently, the modification of the f-RNA may have a role in protection from harsh extracellular conditions.

The chemical modification or a secondary structure (such as a hairpin loop) in the f-RNA could interfere with its ability to bind to the genomic DNA target sequence. This may explain the negative results obtained in the hybridization experiments. Conversely, the negative results may instead indicate that the f-RNA is not of genomic origin. Further analyses, such as direct RNA sequencing will be useful in both predicting any possible secondary structures and designing probes to re-screen the genomic DNA blots.

Since the ability of the 74 kDa subunits eluted from SDS-polyacrylamide gels to reassemble spontaneously into 7 nm diameter fibrils in the absence of any other component has been documented previously (Gardiner and Day,

1988), the nucleic acid is not required for the assembly of fibrils under certain conditions. This indicates that the f-RNA, at least under some conditions, is not required for the structural integrity of the fibrils. However, calculations indicate that a significant number of the f-RNA molecules are present in the intact fimbriae (approximately 2.7 X 10^3 f-RNA molecules in 1 μm length of fimbriae).

f-RNA is the first example of an extracellular RNA polynucleotide that has been localized, isolated and characterized. The existence of extracellular f-RNA suggests the possibility that intriguing mechanisms may be involved in cell-to-cell communication. f-RNA may also represent new evidence for the existence of an ancient RNA world.

CHAPTER 3 - CARBOHYDRATE COMPONENT OF FIMBRIAE

3.1 Introduction

Recently, a considerable amount of research has focused on the biological role of glycoproteins in fungi, and in particular, on the configuration and purpose of the carbohydrate components of these macromolecules. As extracellular constituents, glycoproteins have been implicated in many biological roles including recognition determinants in lectin mediated adhesion (Nordbring-Hertz and Chet, 1986; Muller and Gerisch, 1978), intermediaries of cell-to-cell interactions involved in mating (Weinstock and Ballou, 1986; Sijmons et al., 1987), and as adhesion molecules in pathogenesis (Hazen and Hazen, 1993; Casanova et al., 1992; Klotz et al., 1994). Glycosylation patterns for a given protein are often similar between different species, suggesting a conserved function. However, many protein glycosylation patterns are highly divergent among species, strains, and even tissues (in animal systems), implying a need for recognition specificity (Rademacher et al., 1988). The enormous pattern diversity found in the protein-associated sugars suggests they may confer a high degree of biological specificity upon a macromolecule (Paulson, 1989; Feizi, 1991).

Structures similar to fimbriae have been observed on many fungi and crude serological tests have confirmed that fungal surface epitopes are antigenically related (Gardiner et al., 1981, 1982; Castle and Boulianne, 1991; Benhamou and Ouellette, 1987; Rghei et al., 1992). Based on this evidence, it has been suggested that fungal fimbriae are composed of highly conserved glycoprotein subunits (Gardiner et al., 1981, 1982; Gardiner and Day, 1988). However, these initial studies failed to determine whether the protein, the carbohydrate or both of these components were highly conserved.

The present study was undertaken 1) to examine the accessible epitopic sites present on the surface of native fimbriae of M. violaceum which are exposed to the organism's extracellular environment, 2) to characterize further biochemically the carbohydrate moiety of the fimbrial subunits, and 3) to determine if fimbrial components are highly conserved amongst four groups of related fungi.

3.2 Methods and Materials

3.2.1 Enzymatic Deglycosylation

The purified fimbriae were subjected to digestion with four different glycosidases in an attempt to characterize the carbohydrate moiety of the fimbrial 74 kDa subunit.

ß-glucosidase cleaves terminal hexoses (Glc, Gal, or Fuc) which are β linked to a monosaccharide. ß-glucosidase (Mann Research Labs, 2 mg) was incubated at 35°C with 0.3 mg of fimbriae. Aliquots of the digest were taken at 30 min consecutive intervals for 3 h.

Neuraminidase from Clostridium perfringens cleaves terminal sialic acid residues that are α2,3-, α2,6-, or α2,8- linked to Gal, GlcNAc, GalNAc, AcNeu and GlcNeu. Neuraminidase (Sigma, 0.3 U) was incubated with 1 mg of fimbriae at 37°C for up to 16 h. Aliquots were taken every 30 min for the first 2 h.

Endoglycosidase F cleaves N-linked oligosaccharides of glycoproteins. Specifically, the enzyme cleaves between two adjacent GlcNAc residues, one of which is attached to the asparagine of the polypeptide. In contrast, N-glycosidase F cleaves the entire N-glycan chain from the polypeptide. However, two GlcNAcs must be proximal to the asparagine. Endoglycosidase F /N-glycosidase F (Boehringer Mannheim) is a mixed preparation of both enzymes. The activity of endoglycosidase F was selectively enhanced by digesting 20 µg of fimbriae with 0.3 U of enzyme at pH 5.6 (904 mM sodium acetate, pH adjusted with acetic acid). Conversely, the activity of N-glycosidase F was selectively enhanced by digesting 20 µg of fimbriae with 0.3 U of enzyme at pH 8.3 (400 mM ammonium bicarbonate). The latter two endoglycosidase digestions were carried out at 37°C for 12 h. An additional 0.3 U of enzyme was added to each and the digestions were continued for a further 5 h. In all four cases, the reactions were terminated by the addition of sample buffer and heating to 95°C.

Glycosidase-treated fimbriae were separated by gel electrophoresis (Laemmli, 1970) and stained for proteins (Coomassie Blue R-250) and/or for carbohydrates (periodic acid-Schiff's reagent, PAS; Segrest and Jackson 1972; Konat et al., 1984). Fimbrial preparations were also tested for the presence of glycosylation-specific epitopes using western blotting methods (Towbin et al., 1979).

3.2.2 Inhibition of Fimbrial Glycosylation

Exponential phase liquid cultures of M. violaceum were harvested by centrifugation (Sorvall HG-4L, 2400 g, 15 min, 4°C) and mechanically defimbriated (Gardiner et al., 1981) under aseptic conditions. Cells were resuspended in 20 ml of sterile ddH_2O, and approximately 2-3 g wet weight of cells were used to inoculate 200 ml liquid complete medium containing either 0, 15 mM, 30 mM or 60 mM salicylic acid (termed SA0, SA15, SA30, and SA60 respectively). Cultures were grown at RT for 4 days with mechanical aeration. Fimbriae were harvested as described above and samples were concentrated using the Centricon-10 system (Amicon). Samples were negatively stained and examined in the EM, analyzed by SDS-PAGE (Laemmli, 1970), and tested for the presence of antigenic components by western blot analysis (Towbin et al., 1979).

3.2.3 Production of Glycosylation-Specific Antibodies

New Zealand White rabbits were used to generate fimbrial glycosylation-specific antisera. Initial bleeds for pre-immune sera, labelled Pv-1 and Pv-2, were collected 2 weeks prior to antigen introduction and were stored at -20°C until required. Antibody production was based on the method of Matthews (1957) for creating anti-viral antibodies and modified as follows. Each rabbit was injected intravenously (iv) with 1.5 ml of purified M. violaceum fimbriae (0.5 mg ml^{-1} in 0.9% [w/v] NaCl, 10 mM Tris-HCl [pH 8]). The first boosting injection (3 ml, iv) was given to each rabbit 10 days later, and a second boosting injection (1.25 ml, iv) was given to each rabbit 18 days after the initial antigen introduction. Samples of blood were periodically collected and serum was tested for its ability to agglutinate intact cells of M. violaceum (Gardiner et al., 1981). Exsanguination was performed 25 days after the initial antigen introduction. Sera were separated from cells, labelled Av-1 and Av-2, and stored at -20°C until required. All following experiments were performed using Av-1. The antiserum directed against the purified subunits of fimbriae (AU) was isolated previously (Gardiner et al., 1981). Antibody titres were determined based on the method of Clark and Adams (1977). Purified fimbriae were applied to microtitre plates (Dynatech; 1 h, 37°C), rinsed with T-PBS, and the antibody dilutions were added and permitted to adsorb (3 h, 37°C). Plates were rinsed with T-PBS, and the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase; Bio-Rad) was added at a dilution of 1:5000 and allowed to adhere

(1 h, 37°C). Colourimetric development (p-nitrophenylphosphate in diethanolamine; Bio-Rad) was measured photometrically at 405 nm (Bio-Rad Model 2550, EIA Reader) after 30 min. Assays were repeated 4 times for each antibody. End-point absorption readings were compared and quantified as titre values for each antibody. The titre of the antiserum generated against the native fimbriae was 1/2048. The titre of the pre-immune serum was 1/4.

3.2.4 Analysis of Fungal Surface Components

Species of fungi used, and their sources are given in Table 3.1. Fungal stock cultures were maintained on either complete medium or YT, solidified with 2% [w/v] agar (Day and Jones, 1968). Surface components from fungi (listed in Table 3.1) were harvested according to Gardiner et al. (1981), Benhamou et al., (1986), Castle and Boulianne, (1991), Gardiner, (1985), and Rghei et al., (1992). To ensure that only extracellular or cell surface material was present in the samples, cells were examined microscopically to confirm that cell rupture had not occurred. Alternatively, spent culture medium was dialysed against water (12-14 K cutoff tubing, Spectra/por), and concentrated with polyethylene glycol 20 000 (Pohl, 1990).

Samples (1-5 µg) were separated by electrophoresis (Laemmli, 1970), and either stained with Coomassie Blue R-250 or transferred onto nitrocellulose membrane (Towbin et al., 1979). Polyacrylamide gels were stained with Coomassie Blue R-250 to ensure that proteins had transferred. Western blots were stained with Ponceau S, washed and blocked with gelatin, as previously described (Castle et al., 1992), and probed with Pv-1, Av-1 and AU at dilutions of 1:5000. All procedures for the Pv-1, Av-1 and AU were performed concurrently. Antigenic components were detected by incubating the primary antibody-labelled blots with secondary antibodies (goat anti-rabbit IgG conjugated to alkaline phosphatase; Bio-Rad) followed by colourimetric detection (BCIP/NBT; Bio-Rad).

Table 3.1: Survey of fungal surface proteins: Correlations between the presence of fimbriae and their antigenic relatedness using two different antisera AU (protein-specific epitopes) and Av-1 (glycosylation-specific epitopes).

Species	Source and Number	Fimbriae observed in EM	Agglutination with AU	Western Blot with AU	Apparent Molecular Mass (kDa)	Immunogold or Fluorescence with AU	Western Blot with Av-1	Apparent Molecular Mass (kDa)
Basidiomycetes								
dicot smuts								
Microbotryum violaceum	UWO 1a1	+[b]	+[c]	+[l]	74	+[l]	+[a]	74
M. violaceum	UWO 1a2	+[b]	+[c]	+[l]	74	+[l]	+[a]	74
M. violaceum	UWO 38a1	+[c]	+[c]	+[a]	105, 88, 72	NT	+[a]	72
M. violaceum	UWO 38a2	+[c]	+[c]	+[a]	105, 88, 69	NT	+[a]	69
M. scabiosae	CBS 176-24a1	+[c]	+[c]	+[a]	88, 59, 41, 34	+[k]	+[a]	92, 59
M. scabiosae	CBS 177-24a2	+[c]	+[c]	+[a]	88, 72, 55	+[k]	+[a]	72
Ustilago pinguicu	CBS 184-42	+[k]	+[k]	+[a]	105, 88, 76, 41, 34	+[k]	+[a]	88, 76
U. succisae	UWO DS2a2	NT	NT	+[a]	92, 67	NT	+[a]	92, 74
U. succisae	UWO DS2	NT	NT	+[a]	92, 67	NT	+[a]	92, 74
monocot smuts								
U. vaillantii	UWO MS2	NT	NT	NT	NT	+[k]	-[a]	-
U. maydis	UWO MS1	+[d]	+[c]	+[m]	76, 72, 50	NT	wa	60, 46
U. hordeii	JN race 4	+[c]	+[c]	w[a]	76	NT	-[a]	-
U. nigra	JN Ng301	+[c]	+[c]	+[a]	70, 63, 60, 54, 43, 40, 36	NT	-[a]	-
U. bullata	JN	+[c]	+[c]	+[a]	55	NT	-[a]	-
U. avenae	JN A60	+[c]	+[c]	+[a]	55	NT	-[a]	-
U. turcomanica	JN	+[c]	+[c]	+[a]	51, 33	NT	-[a]	-
U. cynodontis	JN	+[c]	+[c]	w[a]	28	NT	w[a]	48

Other Basidiomycetes								
Coprinus cinereus	BU201	+[e]	NT	+[e]	37	NT		
Schizophyllum commune	BU202	+[e]	NT	+[e]	50	NT		
Rhodotorula rubra	PS	+[c]	-[c]	-[a]		+[l]	+[a]	105, 78, 23
Ascomycetes								
Saccharomyces cerevisiae	GS 1274-4 a	+[d]	+[d]	+[a]	22	+[l]	-[a]	-
S. cerevisiae	GS 1274 α	+[d]	+[d]	NT		+[l]	-[a]	-
Ascocalyx abietina	GBO 1	+[f]	NT	NT		+[f]	NT	
Candida albicans	UWO CA1	+[g]	+[g]	+[a]	81, 84	NT	-[a]	-
Clavispora opuntiae	PS 83.827	NT	NT	NT		NT	-[a]	-
Sporopachydermia cereana	PS 87.487	+[a]	NT	+[a]	93, 75, 38	NT	-[a]	-
Botrytis cinerea	UWO 760	+[h]	NT	NT		+[h]	NT	
Oomycetes								
Peronospora hyoscyami	UWO ph1	+[i]	[illegible]	+[a]	80	+[i]	-[a]	-
Zygomycetes								
Mortiella pusilla	BU 100	+[j]	NT	+[j]	64	NT	-[a]	-
M. candelabrum	BU 101	+[j]	NT	+[j]	57, 60	NT	-[a]	-
Phascolomyces articulosus	BU 102	+[j]	NT	+[j]	64	NT	-[a]	-
Bacteria								
Synechococcus sp.	PCC 7002	+[a]	NT	NT		NT	-[a]	-

+: present, w: weak reaction, -: not detected, NT: not tested, NA: not applicable. a: this thesis, b: Poon and Day (1974), c: Gardiner et al. (1981), d: Poon and Day (1975), e: Castle and Boulianne (1991), f: Benhamou and Ouellette (1987), g: Gardiner et al. (1982), h: Svircev et al. (1986), i: Gardiner and Day (1988), j: Rghei et al. (1992), k: Gardiner (1985), l: Gardiner and Day (1985), m: Xu and Day (1992). CBS: Centraal Bureau voor Schimmel Cultures, Baarn, Netherlands, GS: Dr. G. G. Stewart, Labatts Breweries, London, Ontario, JN: Dr. J. Nielson, Can. Dept. Agriculture, Winnipeg, Manitoba, UWO: Microbial Collection, Department of Plant Sciences, University of Western Ontario, London, Ontario, PS: Department of Plant Sciences, Dr. M. A. Lachance, University of Western Ontario, London, Ontario, PCC: Pasteur Culture Collection, France, BU: Microbial Collection, Department of Biological Sciences, Brock University, St. Catharines, Ontario.
dicot smuts: smut fungi that infect dicotyledenous plants. monocot smuts: smut fungi that infect monocotyledenous plants

3.3 Results

3.3.1 Glycosylation of M. violaceum Fimbrial Subunits

Treatment of the M. violaceum fimbriae with the two exoglycosidases had no apparent effect on the molecular weight of the subunit (Table 3.2). This indicates that either the carbohydrate component does not have 1) an α2,3-, α 2,6-, or α2,8-to Gal, GlcNAc, GalNAc, AcNeu, or GlcNeu, or 2) a terminal hexose (Glc, Gal, or Fuc) ß-linked to a monosaccharide. However, if the quantity of terminal carbohydrate cleaved is low, a shift in the apparent molecular weight may be below detectable levels.

Treatment of the M. violaceum fimbriae with the endoglycosidase F and N-glycosidase F resulted in a change in the apparent molecular weight of the subunit (Table 3.2) from 74 kDa to 47 kDa. Unlike the 74 kDa fimbrial subunit, the 47 kDa material was not stained by PAS, which indicates that it is not glycosylated. Deglycosylation was also achieved using endoglycosidase F as indicated by the similar sized product (47 kDa) which was also not stained by PAS. Since endoglycosidase F digestion leaves only a terminal N-acetylglucosamine on the asparagine residue, the quantity of sugar present on the protein is below the level of detection for PAS. Therefore, even though the PAS stain results were negative, there must still be at least one sugar residue remaining on the protein.

The effect of glycosylation on the size of the fimbrial subunit was also analyzed by isolating fimbriae from M. violaceum that were grown in media containing salicylic acid (Table 3.2). The fimbriae were examined in the EM and no visible morphological differences between these fimbriae and those isolated from cells grown in standard growth conditions were noted. However, fimbriae were less numerous in isolations from higher concentrations of salicylic acid. The fimbriae from SA0, SA15, SA30, and SA60 were analyzed by SDS-PAGE and protein staining. Fimbriae from SA0 and SA15 had the expected 74 kDa subunit. However, fimbriae from SA30 and SA60 were composed of 67 kDa and 57 kDa proteins and did not contain any detectable amounts of the 74 kDa glycoprotein.

3.3.2 Western Blot Analysis of M. violaceum Fimbrial Protein

Two types of polyclonal antibodies were used in this study. One (AU) has been used in previous studies (Gardiner et al., 1981, 1982) and was created by injecting rabbits subcutaneously with denatured fimbrial subunits. Previous

Table 3.2: Comparison of enzymatic deglycosylation and chemical glycosylation inhibitors of fimbrial subunits

	SDS-PAGE Coo-R250 (kDa)	SDS-PAGE PAS	Western AV-1	Western AU	Electron Microscope
Enzymes					
ß-glucosidase	74	+	NT	NT	NT
neuraminidase	74	+	+	NT	+
endoglycosidase F	47	-	-	+	NT
N-glycosidase F	47	-	-	+	NT
α-mannosidase	67[1],47[1]	+[1]	NT	+[1]	NT
Inhibitors					
15 mM salicylic acid.	74	NT	+	NT	+
30 mM salicylic acid	67, 57	NT	-	NT	+
60 mM salicylic acid	67, 57	NT	-	NT	+
tunicamycin (4µg ml^{-1})	74, 67[2]	+/-[2]	NT	+[1]	+[3]

[1]A. Castle, pers. comm
[2]Castle et al. (1992)
[3]Gardiner and Day (1985)
+ detected
- not detected
+/- indeterminate
NT not tested

results showed that AU detected the 74 kDa fimbrial protein in M. violaceum (Castle et al., 1992). Castle et al. (1992) also showed that this antiserum reacted against the partially deglycosylated 67 kDa form of the fimbrial protein. Since the results of the present study demonstrated that AU detected the 47 kDa deglycosylated subunit (Fig. 3.1 A), it appears that AU antiserum is able to recognize protein-specific epitopes.

The second antiserum (Av-1) was obtained following intravenous injection of native intact purified fimbriae into rabbits. Av-1 detected the 74 kDa fimbrial glycoprotein subunits on western blot analysis (Fig 3.1). Av-1 also detected, on western blots, the fimbrial subunits (74 kDa) from SA0 and SA15 cells, but not the incompletely glycosylated 67 kDa and 57 kDa subunits obtained from SA30 and SA60 cells (data not shown). The 47 kDa deglycosylated subunit was also not detected by Av-1 (Fig 3.1). Thus, unlike the AU antiserum, Av-1 appears to recognize carbohydrate-specific epitopes.

3.3.3 Fimbrial Epitopes from Various Fungi

The two antisera (AU and Av-1) were used to screen total surface proteins from a number of different fungal species. AU antiserum detected one to seven bands on western blots of total surface proteins from most of the species tested (Table 3.1). The proteins detected are likely to be fimbrial proteins, but EM examinations of the material eluted from polyacrylamide gels would be necessary to confirm this. In contrast, the Av-1 antiserum detected antigens in western blots of total surface proteins only from species of smuts that infect dicotyledenous plants (dicot-infecting smuts) and R. rubra. Most other species, including all of the Ustilago species that infect monocotyledenous plants (monocot-infecting smuts) gave either weak or negative results. In the dicot-infecting smut group there were in each case one or two antigenically-related bands which ranged in size from 59-92 kDa (Table 3.1). These results indicated that all of the species tested in this group produce fimbriae that have a highly conserved glycosylation pattern.

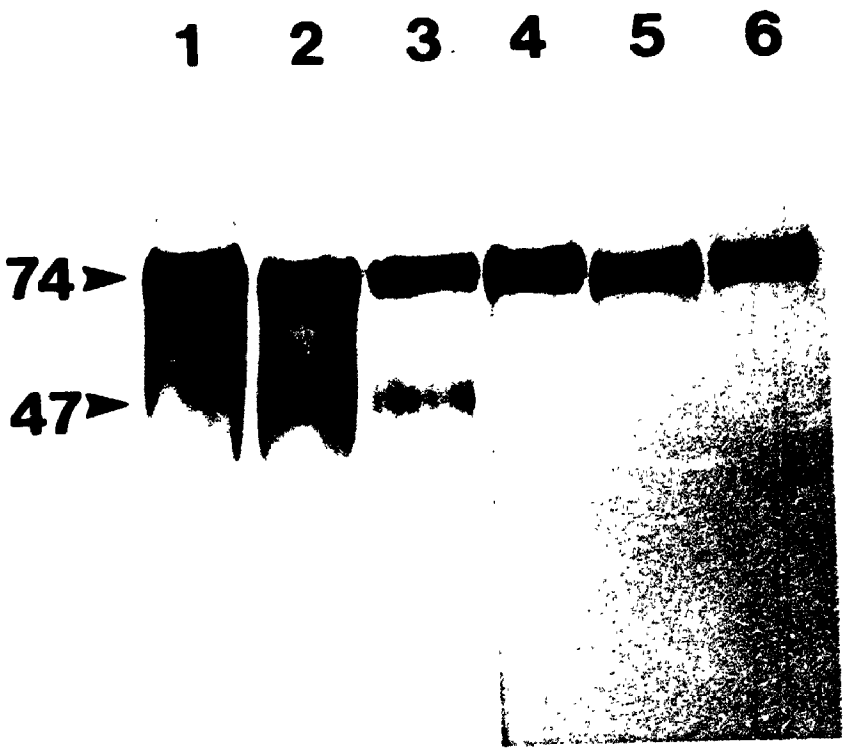
3.4 Discussion

Fimbrial subunits contain carbohydrates attached to the protein component (Castle et al., 1992; Xu, 1992). The aglycone of the fimbrial subunit was obtained using N-glycosidase F and was determined to be 47 kDa. This indicates that approximately 36% (by weight) of the fimbrial subunit is

Fig. 3.1 Western blot analysis of the fimbrial protein and the aglycone using two different antibodies. Fimbriae from M. violaceum digested with N-glycosidase F/endoglycosidase F. The products were separated by SDS-PAGE and electro-blotted onto nitrocellulose. Fimbrial subunits were visualized by immuno-detection with anti-fimbrial antibodies. Arrowheads indicate the apparent molecular weight of the fimbrial subunits.

Lanes 1, 2, and 3:
Western blot detected with the antibody AU. Lane 1: purified fimbrial protein; lane 2: fimbrial protein deglycosylated enzymatically with endoglycosidase F; lane 3: fimbrial protein deglycosylated enzymatically with N-glycosidase F.

Lanes 4, 5, and 6:
Western blot detected with the antibody Av-1. Lane 4: purified fimbrial protein; lane 5: fimbrial protein deglycosylated enzymatically with endoglycosidase F; lane 6: fimbrial protein deglycosylated enzymatically with N-glycosidase F.


1 2 3 4 5 6
74
47

carbohydrate. Previously published estimates (10-15%; Castle et al., 1992) probably account solely for the percent of mannose in the glycoprotein, since a 67 kDa partially glycosylated subunit was also obtained in this study. However, recent results (Castle, in prep.) showed that digestion with α-mannosidase generated a weak 47 kDa band in addition to the 67 kDa band.

Fimbriae produced by spores in the presence of tunicamycin are similar in appearance to those grown without tunicamycin (Gardiner and Day, 1985). However, it has recently been demonstrated that the fimbrial subunits isolated from cells grown in this glycosylation-inhibiting medium have a reduced apparent molecular mass (67 kDa; Castle et al., 1992). Tunicamycin inhibits one of the pathways to N-linked glycosylation by blocking the biosynthesis of dolichol pyrophosphate N-acetylglucosamine (Duksin and Mahoney, 1982). Studies employing a lectin-based glycan differentiation assay indicated that mannose is the major carbohydrate component of the 74 kDa subunits, and is not present in the partially deglycosylated 67 kDa subunits (Castle et al., 1992). However, mannose is not the sole sugar present on the subunits, since the 67 kDa protein generated by treatment with trifluoromethanesulphonic acid remains detectable with PAS staining (Castle et al., 1992). This indicates that other sugar residues are still associated with the protein component. Xu (1992) used lectins to analyze the carbohydrate component of native fimbriae from M. violaceum. The results of lectin agglutination tests using both sporidia and isolated fimbrial preparations suggested that α-D-mannose, α-D-glucose, ß-D-fructose, and methyl α-D-mannopyranoside may be present.

Salicylic acid (SA) is also a glycosylation inhibitor, but its mode of action differs from that of tunicamycin. SA inhibits acetyl-CoA synthetase activity which in turn inhibits sialic acid (AcNeu and GlcNeu) and hexosamine production (esp. glucosamine and galactosamine). The synthesis of neutral sugars is not affected by SA (Kent and Allen, 1968). Fungal cells grown in media containing SA have fimbriae that appear similar to cells grown without SA, as determined by EM examinations. However, analysis by SDS-PAGE indicates that these fimbriae are composed of incompletely glycosylated (67 and 57 kDa) subunits. These results suggest that acetylated sugars are probably components of the carbohydrate moiety of fimbrial subunits.

Previous studies on fimbriae have involved the use of a polyclonal antibody (AU) generated against the re-natured form of the protein (Gardiner et al., 1981). The antibodies were used in both crude testing for the presence of

antigenically related epitopes on the surface of other fungal species (agglutination tests, see Table 3.1) and in the localization of these epitopes (immuno-gold EM, see Table 3.1). These studies indicated that a highly conserved epitope (as detected by AU) is present on most of the fungal species that have been examined. Studies involving cells grown in tunicamycin, a glycosylation inhibitor, have shown that the partially deglycosylated subunits (67 kDa) are still detectable antigenically with AU (Castle et al., 1992), suggesting the AU antiserum is directed against a protein epitope of the fimbrial subunit. Results of this study indicate that a fimbrial subunit is detectable with the AU antiserum in all members of the four fungal divisions tested (Table 3.1).

Native structural glycoproteins have long been known to elicit a strong antigenic response (Robert et al., 1976). However, when most glycoproteins are introduced by subcutaneous, intramuscular or intraperitoneal injection into an animal host, they remain in the system for a prolonged time before significant levels of antibodies are created. Significant antigen degradation and alteration may result before antibody production is stimulated. Thus it is difficult to produce dependable, specific antibodies to proteins with limited stability or proteins that are readily modified. This problem is especially evident during long term exposures to antigens which are glycosylated. Since terminal sugars are readily cleaved, the antibodies created will not be to the original structural epitopes. Intravenous injection overcomes this problem since large quantities of antibodies are created quickly and specifically to the most prominent epitopic sites with minimal antigen modifications (Matthews, 1957). Intravenous injections are the preferred method for creating antibodies to large polymers, such as whole viruses which have limited stability. Antisera created by the above method to the native fimbriae were initially tested to confirm that the fimbrial subunits (74 kDa) of M. violaceum were indeed detected by western blot analysis. Neither of the deglycosylated fimbrial subunits made by enzymatic digestion (N-glycosidase F) nor by chemical inhibition (media containing SA) were detected by western blot analysis. This indicates that the antibodies detected the glycosylation pattern of the fimbrial subunits and not the polypeptide sequence (unlike AU).

Antibody screening (using AU) of the surface components of members from various fungal divisions indicated that fimbrial protein epitopes are highly conserved. This is, however not the case with the glycosylation pattern-specific antibody. Av-1 detected only the glycosylation pattern specific to the dicot-

infecting smuts and R. rubra. These fungi are members of a cohesive group, distinct from the phylogenic line Ustilaginales (Oberwinkler, 1995; Bauer et al., 1991; Prillinger et al., 1993; Deml, 1985, 1986, 1987; Moore 1992). Deml and Oberwinkler (1982) removed M. violaceum from the genus Ustilago based on physical characteristics (promycelium germination, teliospore ornamentation), and host range (dicotyledons). This alteration is supported by recent findings, namely physiological traits (siderophore type, enzymatic activities [Deml, 1987]) and cytoplasmic characteristics (subapical vesicles in prophase, lack of pores in septa [Berbee et al., 1991], spindle pole body morphology [Bauer et al., 1991]). In addition, M. violaceum and the other dicot-infecting smuts along with several other basidiomycetes including Rhodotorula spp., have been grouped into the order Microbotryales (Oberwinkler, 1995) based on 5S rRNA (Blanz and Gottschalk, 1984), 23S rRNA (G. Hagedorn, pers. comm), siderophore type (Hagedorn and Deml, 1994), DNA/DNA reassociation (Blanz, 1984), antibiotic response (Kimmich in Deml, 1987), cell wall carbohydrate composition (Prillinger et al., 1993) and sporidium formation (Deml, 1986).

Since glycosylation is a result of post-translational modification, the carbohydrate structures are determined as a result of glycosidase and transferase activities, one for each glycosidic linkage. Genus-specific motifs of sugar chain structures are the results of distinguishing patterns of expression of certain genes coding for these glycosyl processing enzymes (Bara and Oriol, 1993). Subsequent work is needed to identify these enzymes and the sequence of the cascade of modifications that occurs.

Further, it has yet to be determined if the unique glycosylation pattern identified in Microbotryales serves a biological purpose in recognition, or if it is simply an incidental result of the cellular glycosylation cascade. It has been established that the characteristics of oligosaccharides located on viral surface glycoproteins can determine both the efficiency and range of cellular infection (Rademacher et al., 1988).

Antibodies directed against specific glycosylation patterns present on proteins with conserved sequences have been used in several studies of surface component analysis. Glycosylation specific antibodies have been used, particularly in mammalian systems, to discriminate between normal and tumour cells (Kobata et al., 1990) and to detect other pathological conditions (Bara and Oriol, 1993).

To date, glycosylation pattern analysis has not been utilized as a conventional method for studying systematics. However, in this study conserved carbohydrate pattern analysis was a surprisingly effective tool in distinguishing between these superficially similar, but evidently very different orders of fungi.

CHAPTER 4

4.1 Introduction

Since fimbriae are extracellular structures, they are exposed to the environment which may be hostile. Gardiner (1986) in his characterization of the effects of physical and chemical treatments on fimbriae of M. violaceum, found that fimbriae were stable when exposed to high temperatures, pH extremes and many chemical treatments. The studies employed EM examinations of fimbriae in gross structure and serological assays (Ouchterlony) to determine if antigenic sites were preserved. However, the structural integrity of the glycoprotein subunits themselves was never examined. EM examinations of fimbriae may not show how the fimbrial subunits have been affected by the treatments. Thus, it is essential to analyze the glycoprotein components of fimbriae to determine the direct effect of enzymatic and chemical treatments on their integrity.

Because of their distinct location on the cell surface, fimbriae understandably have been associated with all extracellular activities including cell-to-cell communication, adhesion, mating and pathogenesis. Their position at the cell surface makes it vital to understand and classify not only the polypeptide sequence of the fimbrial subunit but also any post-translational modifications that lead to the formation of the mature protein.

Gardiner (1986) analyzed the amino acid composition of M. violaceum fimbrial protein and found that it showed no similarities to any known proteins. However, the amino acid sequence and/or the DNA sequence that codes for the polypeptide would be much more informative and would permit comparisons of the entire or partial polypeptide to all known protein polypeptide sequences. In addition, the polypeptide sequences can be useful tools for describing molecular evolution of a protein or protein family, and predicting phylogenetic relationships between organisms.

The present study characterizes the protein component of fimbriae. In particular, the study 1) appraises the effects of various enzymes and chemical treatments on the fimbrial subunits, 2) determines several partial amino acid sequences of the protein and compares them to known protein sequences, 3) uses the fimbrial protein epitopic sites and enzymatic recognition motifs to compare fimbrial protein to other structural proteins.

4.2 Methods and Materials

4.2.1 Chemical and Enzymatic Treatments of Fimbrial Glycoprotein

Intact purified fimbriae (50 µg) and fimbrial glycoprotein subunits (20 µg) were subjected to protease and nuclease digestions at 37°C (Table 4.1). In control experiments, fimbriae and fimbrial proteins were incubated under the same conditions as the digests, except that the enzymes were omitted. In the case of trypsin digests, purified fimbriae were solubilized in 6 M urea and 10 mM dithiothreitol and heated to 50°C for 15 min prior to digestion (Stone and Williams, 1993). Before their use, both RNase A and RNase B were pre-treated by heating to 95°C for 10 min followed by slow cooling to RT. This procedure inactivated contaminating proteases and DNases (Sambrook et al. 1989). All nuclease S1 digestions were done in the presence of 10 mM $ZnCl_2$.

Purified fimbriae were also subjected to chemical treatments (Table 4.2). Samples treated with NaOH and HCl were neutralized prior to further analysis. In addition, fimbrial proteins were solubilized with SDS both in the presence and absence of a reducing compound (5% [v/v] BME). Following both enzymatic and chemical treatments, products were separated by SDS-PAGE (Laemmli 1970) and visualized by staining with Coomassie Blue R-250 (2.2.4) and/or silver stain (Bio-Rad).

4.2.2. Amino Acid Sequencing of the Fimbrial Protein

Purified fimbrial protein was deglycosylated with endoglycosidase F as previously described (3.2.1), reduced (10 mM dithiothreitol, 50°C, 15 min) and carboxamidomethylated (20 mM iodoacetamide, RT, 15 min). The resulting product is referred to as the fimbrial polypeptide. Fimbrial oligopeptides were made by digesting the fimbrial polypeptide (20 µg) with endoproteinase Lys-C (Boehringer Mannheim; 1µg, 37°C, 15 h) by the method of Stone and Williams (1993). Both the fimbrial polypeptide (10 µg) and the oligopeptides (20 µg) were separated by RP-HPLC (Applied Biosystems Microbore) and eluates were monitored at 215 nm. The contents of the peaks were analyzed spectrophotometrically by diode array analysis and products consistent with protein spectra were collected. During both polypeptide and oligopeptide separations, buffer A was 0.1% TFA / 0% AN, and buffer B was 0.1% TFA / 80% AN. The runs were 0 to 100% B at flow rates of 1 ml min^{-1}. An Aquapore RP-300 (Brownlee) column (60 min run time) was used to purify the polypeptide and a Spheri-5 RP-18 (Brownlee) column (40 min run time) was used to separate

Table 4.1: Enzyme digests of the fimbrial glycoprotein subunits

Enzyme	Source	Quantity	Time	SDS-PAGE Results
Proteinase K	Me	3 U	0 h 1 h 2 h	U +/- D
Protease SA V8	Mi	1 µg	0 h 3 h 4 h 12 h	U +/- +/- D
Pronase E	Si	0.5 µg	0 h 1 h	U D
Trypsin	Si	0.5 µg	0 h 3 h 15 h	U +/- +/-
RNase A	BM	10 µg	0 h 1 h 12 h 48 h	U U U U
RNase B	Si	10 µg	0 h 1 h 12 h	U U U
DNase 1	BM	10 µg	0 h 1 h 12 h	U U U
Nuclease P1	Si	3 µg	0 h 12 h	U U
Nuclease S1	Se	500 U	0 h 12 h	U U

BM: Boehringer Mannheim
Me: Merck
Mi: Miles Laboratories
Si: Sigma
Se: Seikagaku Kogyo Co.

U: undigested; 74 kDa protein present
+/-: partially digested; 74 kDa protein partially degraded
D: completely digested; 74 kDa protein absent

Table 4.2: Chemical treatments of fimbrial glycoprotein subunits

Chemical Treatment	Quantity	Time (h)	SDS-PAGE Results
SDS, 95°C	2%	0	U
		1	U
		4	U
		6	+/-
		8	+/-
		12	D
HCl, RT	0 M	0.25	U
	0.01 M	0.25	U
	0.05 M	0.25	U
	0.10 M	0.25	U
	0.50 M	0.25	D
NaOH, RT	0 M	0	U
		7	U
	0.20 M	0.25	+/-
		1	+/-
		4	+/-
		7	D
	0.50 M	0.25	+/-
		1	+/-
		2	D
acetone, RT	0%	0.25	U
	5%	0.25	U
	20%	0.25	U
	40%	0.25	U
	50%	0.25	+/-
diethyl ether, RT	0%	0.25	U
	5%	0.25	U
	20%	0.25	U
	40%	0.25	U

U: unaffected, 74 kDa protein appears unchanged
+/-: 74 kDa protein partially degraded
D: completely degraded, 74 kDa protein is absent

the oligopeptides.

Automated amino acid sequencing by Edman degradation was preformed on the HPLC-purified polypeptide and three of the fimbrial oligopeptides by J. Lagueux (Service de Sequence des Proteines de l'Est du Quebec, Centre de Recherche du CHUL, Ste-Foy, Quebec) (Table 4.3).

4.2.3 Oligonucleotide Synthesis, PCR, and RT-PCR

The ORFs of all available Ustilago spp. and Microbotryum sp. genes were compiled using the data from Genetics Computer Group (GCG) and a codon usage chart was constructed (Table 4.4). The predicted codon preference was used to design oligonucleotides (Table 4.5) which were synthesized by Vetrogen Corp.

Synthetic oligonucleotides were radioactively labelled using T4 polynucleotide kinase (USB; 10 U/10 pmol oligonucleotide) and γ-^{32}P-ATP (Amersham, >5000 Ci/mmol; 50 µCi/10 pmol oligonucleotide) as per Sambrook et al. (1989). These radiolabelled oligonucleotides were used to probe the Southern blots of M. violaceum genomic DNA (2.2.10) at low stringency (37°C and 45°C; Sambrook et al., 1989).

The synthetic oligonucleotides were used as primers in PCR reactions. Taq polymerase (BRL; 1U/reaction) and Vent DNA polymerase (NEB, 1U/reaction) were used with all primer combinations (0.5 µM/reaction) in an attempt to amplify fimbrial sequences from genomic M. violaceum DNA (15 ng/reaction). Various annealing temperatures (45°C, 46°C, 47°C, 50°C, 54°C, 55°C, 56°C, 57°C, 62°C, 64°C, 65°C) and magnesium concentrations (2 to 6 mM) were used in combination with 2 min extension times. Products were separated on 6% (w/v) polyacrylamide gels (TBE, 150 V), stained with ethidium bromide, and visualized by uv transillumination.

The ends of the PCR products were filled in (T4 polymerase, Pharmacia, 10 U/reaction), phosphorylated (T4 polynucleotide kinase, BRL, 10 U/reaction), purified on low melt agarose gels (SeaPlaque GTG agarose, FMC), and ligated into the *Sma*I site of the plasmid pUC 18 (Sambrook et al., 1989). The plasmids were electroporated (Cell-Porator, BRL) into E. coli DH10B competent cells. Cells harbouring the plasmid containing an ampicillin resistance gene and an insert in the *lacZ* gene were selected for by plating on 2% (w/v) agar-LB supplemented with ampicillin (50 µg/ml) and X-Gal (1 mg/plate), respectively.

Table 4.3: Amino acid sequences of the fimbrial protein

Sequence Location	Amino Acid Sequence
N-terminus	GFPGLPGPXGE
Internal #1	GEPKPXGA
Internal #2	VLPGPMGPSGETGP
Internal #3	GFPGLPGXPAEPXGFKGENG

Table 4.4: Codon usage chart for M. violaceum

TTT-Phe 0.37	TCT-Ser 0.15	TAT-Tyr 0.35	TGT-Cys 0.34
TTC-Phe 0.63	TCC-Ser 0.16	TAC-Tyr 0.65	TGC-Cys 0.66
TTA-Leu 0.01	TCA-Ser 0.15	TAA-Stop 0.14	TGA-Stop 0.72
TTG-Leu 0.14	TCG-Ser 0.23	TAG-Stop 0.14	TGG-Trp 1.00
CCT-Leu 0.19	CCT-Pro 0.32	CAT-His 0.39	CGT-Arg 0.22
CTC-Leu 0.38	CCC-Pro 0.23	CAC-His 0.61	CGC-Arg 0.26
CTA-Leu 0.10	CCA-Pro 0.22	CAA-Gln 0.39	CGA-Arg 0.19
CTG-Leu 0.18	CCG-Pro 0.23	CAG-Gln 0.63	CGG-Arg 0.09
ATT-Ile 0.28	ACT-Thr 0.22	AAT-Asn 0.35	AGT-Ser 0.09
ATC-Ile 0.64	ACC-Thr 0.29	AAC-Asn 0.65	AGC-Ser 0.21
ATA-Ile 0.08	ACA-Thr 0.25	AAA-Lys 0.39	AGA-Arg 0.13
ATG-Met 1.00	ACG-Thr 0.24	AAG-Lys 0.61	AGG-Arg 0.11
GTT-Val 0.26	GCT-Ala 0.24	GAT-Asp 0.43	GGT-Gly 0.30
GTC-Val 0.44	GCC-Ala 0.30	GAC-Asp 0.57	GGC-Gly 0.37
GTA-Val 0.12	GCA-Ala 0.23	GAA-Glu 0.42	GGA-Gly 0.22
GTG-Val 0.18	GCG-Ala 0.23	GAG-Glu 0.58	GGG-Gly 0.10

Table 4.5: Synthetic oligonucleotides

Sequence ID	Amino Acid Sequence	Oligonucleotide Sequence
133	N-terminus	TTCCCCGGCCT(CG)CCCGGCCC
134	N-terminus	TTCCC(GCT)GG(CAT)CT(CG)CC(CGT)GG(CAT)CC
135	N-terminus	TTCCC(GCT)GG(CAT)TTGCC(GCT)GG(CAT)CC
136	Internal #2	CCCAT(CGA)GG(GTA)CC(CGA)GGGAG(CG)AC
137	Internal #2	CCCAT(CGA)GG(GTA)CC(CGA)GGCAA(CG)AC
138	Internal #3	CC(CGA)GGGAGGAT(CGA)GG(GA)AA(CG)GT
139	Internal #3	CCGTT(TC)TC(TGAC)CC(TC)TTGAA(TGCA)CC
140	Internal #3	GT(CT)TC(TGA)CC(CG)GA(CGA)GG(TGA)CCCAT
141	Internal #3	AC(GC)TT(CT)CC(GCT)ATCTCCC(GCT)GG
142	Internal #2	GT(GC)CTCCC(GCT)GG(CAT)CC(GCT)ATGGG

Selected E. coli colonies were amplified (5 ml TB, 50 µg/ml ampicillin, 37°C, 225 rpms, 10 h) and plasmids were purified (Qiagen, Qiaprep-8 Plasmid kits). Purified plasmids were denatured (Sambrook et al., 1989) and the sequences of the inserts were determined by dideoxy chain termination reactions (Sanger et al., 1977).

In addition, synthetic oligonucleotides were used as primers in RT-PCR reactions. Total RNA from M. violaceum was isolated based on modifications of the method of Kraig and Haber (1980). Exponential-phase cells of M. violaceum (2 g wet weight) were pelleted and resuspended in 2.5 ml lysis buffer (50 mM Tris-HCl [pH 6.8], 100 mM NaCl, 10 mM EDTA-HCl), Tris-HCl-saturated phenol (pH 8), and 11 g of acid washed glass beads (425-600 µm, Sigma). This slurry was alternately vortexed and cooled in an NaCl-ice bath at 30 sec intervals for 6 min. Subsequently, 2.5 ml of Tris-HCl-saturated phenol and 0.5 ml of 20% (w/v) SDS were added and the slurry was vortexed for 1 additional min. The phases were separated by centrifugation (10 000 x g, 5 min, 4°C) and the aqueous phase was collected. The interphase was re-extracted with 2.5 ml lysis buffer and pooled with the first aqueous phase. The pooled aqueous phases were repeatedly extracted with Tris-HCl-saturated phenol:chloroform:iso-amyl alcohol (25:24:1) and the nucleic acids were precipitated with 3 vol of ethanol at -20°C in the presence of 0.3 M ammonium acetate (pH 5.3).

cDNA was synthesized from M. violaceum total RNA based on the method of Valdimarsson et al. (1993), using an oligo(dT) 12-18 primer (BRL) and reverse transcriptase (SuperScript RT, BRL). The enzyme was removed by repeated extractions with phenol:chloroform:iso-amyl alcohol (25:24:1) and the nucleic acids were precipitated with 2 vol of ethanol and 0.3 M sodium acetate (pH 5.3). Nucleic acids, including RNA:DNA hybrids were resuspended in 100 µl of TE and the RNA components were digested with RNase A (10 µg, 37°C, 30 min). The enzyme was removed by repeatedly extracting with phenol:chloroform:iso-amyl alcohol and the cDNA was precipitated with 2 vol of ethanol and 0.3 M sodium acetate. The cDNA was used as the template, in combination with the synthetic oligonucleotides, in PCR reactions. The parameters and concentrations used in the RT-PCR were similar to those used in the PCR reactions described above. The resultant RT-PCR products were analyzed on polyacrylamide gels, cloned into pUC 18 and sequenced as described above.

4.2.4 Heterologous Collagen Probes

Collagen sequences from various organisms (Table 4.6) were radioactively labelled using T7 polymerase (QuickPrime kit, Pharmacia) and α-^{32}P-CTP (NEN, >3000 Ci/mmol). The radiolabelled collagen sequences were used as heterologous probes against Southern blots of genomic DNA from M. violaceum as described above (4.2.3 and 2.2.10). Hybridizations were done at 52°C for 16 h. Three stringency washes (2 x SSC, 0.1% [w/v] SDS, RT, 15 min) were performed and blots were exposed to x-ray film at -80°C.

4.2.5 Production of Antibodies Against Fimbrial Protein Epitopes

A New Zealand White rabbit was used to generate antiserum to fimbrial protein epitopes. Pre-immune serum was collected and stored as previously described (3.2.3). Antibody production was based on the method of Harlow and Lane (1988). Briefly, fimbriae were solubilized and the protein subunits were isolated by SDS-PAGE as previously described (2.2.4). However, fimbrial protein was not eluted from the polyacrylamide gels. Instead, gel strips containing fimbrial protein were frozen to -80°C and lyophilized (Multi-Dry, FTS Systems Inc.) for 12 h. The desiccated gel strips were ground to a fine powder and a slurry was made by mixing the powder with 2.5 ml milliQ water. The slurry, containing 100 µg of fimbrial protein, was homogenized by repeated passage through an 18 gauge needle attached to a 3 ml syringe. The samples were stored at -80°C. When required, the slurry was thawed and incubated at RT for 1 h prior to making a 1:1 (v/v) emulsification in Freunds incomplete adjuvant (BRL). 0.8 ml of this emulsified antigen was injected sub cutaneously (sc) into the rabbit. Boosting injections (0.8 ml, sc) were given after 35, 57, and 78 days. Exsanguination was performed 106 days after the initial antigen introduction. Serum was separated from cells, labelled Av-3 and stored at -20°C. End-point titres on both Av-3 and Pv-3 were determined to be 1:16 000 and 1:8 respectively, by ELISA as previously described (3.2.3).

4.2.6 Digestion of Fimbrial Protein with Collagenases

Three collagenases, gelatinase A (type IV collagenase, 72K GL), gelatinase B (type IV collagenase, 92K GL) and interstitial collagenase (FIB CL), were obtained from J. Ray (NIH) and were activated using organomercurials as described previously (Stetler-Stevenson et al., 1989). Zymograms, used to confirm that the enzymes were active, were performed as per manufacturers

Table 4.6: Heterologous collagen probes

Plasmid	Gene	Source Organism	Reference
pHC21	col2A1	Homo sapiens	Ramirez (pers. comm.)
pHC22	col2A2	H. sapiens	Ramirez (pers. comm.)
pHpl-14	col3A1	H. sapiens	Janeczko and Ramirez 1989
pHf-511	col5A2	H. sapiens	Weil et al. 1987
pα1R1	col1A1	Rat rattus	Rowe (pers. comm.)
pα2R2	col2A2	R. rattus	Rowe (pers. comm)
pJJ205	*sqt-1*	Caenorhabditis elegans	Kramer et al. 1988
pRF1	*rol-6*	C. elegans	Kramer et al. 1990
pCOL2	*col-2*	C. elegans	Kramer et al. 1982

instructions (Novex). Activated collagenases (20 ng of each) were separated by electrophoresis (Laemmli, 1970) in polyacrylamide gels which have gelatin incorporated in their matrices. Gels were stained with Coomassie Blue R-250 as previously described (2.2.4). In addition, native collagens (1 mg) were digested with activated collagenases (30 ng) and the products were separated by SDS-PAGE and visualized by staining with Coomassie Blue G-250 (Neuhoff et al., 1988).

Purified fimbriae (4.5 mg) were either pre-treated by heating (55°C, 30 min) and quick cooling on ice, or not pre-treated. Both pre-treated- and not pre-treated-fimbrial proteins were digested with 57 ng of each of the three collagenases for a maximum of 20 h. Aliquots of each digest were taken at various time intervals. Control experiments contained all of the components of the digests, and were subjected to the same conditions, except that the enzymes were omitted. Products were separated by SDS-PAGE (4-20% [w/v], Novex) and the bands were visualized by staining with Coomassie Blue G-250. Gels were analyzed using an LBK Ultroscan XL laser densitometer.

4.2.7 Immuno-chemistry of Structural Proteins

Fimbrial protein and other structural proteins, listed in Table 4.6, were solubilized, separated by SDS-PAGE, and transferred onto nitrocellulose membrane as previously described (3.2.4). Membranes were stained with Ponceau S to ensure that proteins had transferred. Western blots were blocked (5% [w/v} BSA-TTBS), and probed with various antibodies (Table 4.7) in 1% [w/v] BSA-TTBS. Antigenic components were detected by first incubating the primary antibody-labelled blots with secondary antibodies (goat anti-rabbit IgG conjugated to horseradish peroxidase, Sigma; or goat anti-mouse IgG conjugated to horseradish peroxidase, Bio-Rad) followed by detection using chemiluminescence (Amersham; ECL). Film (X-AR, Kodak) was exposed to fluorescing blots and developed as per manufacturers instructions.

In addition, collagen types I (rat tail, Sigma), III (calf skin, Sigma), and IV (mouse, H. Kleinmann) were analyzed for the presence of epitopes recognized by fimbrial protein-specific antibodies (Av-3) and compared to that of pre-immune serum (Pv-3). Fimbriae and the three types of collagen were adhered to microtitre plates and ELISAs were preformed as previously described (3.2.3).

4.3 Results

4.3.1 Chemical and Enzymatic Treatment of Fimbriae

Fimbrial proteins were susceptible to digestion by all four of the proteases listed in Table 4.1. As expected, there was considerable variation in the rates of digestion so that not all digestions went to completion. The rate differences are probably a reflection of the enzyme composition and specificity, and not necessarily the fimbrial protein's primary or secondary structure.

The fimbrial protein exhibited no detectable susceptibility to nucleases (Table 4.1). This indicates that either i) there is no nucleic acid covalently associated with the 74 kDa glycoprotein subunit or ii) the nucleic acid moiety quite small, such that its removal results in an undetectable mobility shift of the subunit as analyzed by SDS-PAGE.

Solubilization of fimbrial protein in the presence of BME results on SDS-PAGE in a band containing protein that has an apparent molecular mass of 74 kDa (Fig. 4.1, lane 2). In contrast, solubilization of fimbrial protein without a reducing agent results in a protein with an apparent molecular mass of 63 kDa (Fig. 4.1, lane 4). These results clearly indicate that fimbrial protein from M. violaceum contains at least one disulphide bridge. Heating fimbriae has no effect on the solubilization or disulphide bond reduction of the glycoprotein subunits (Fig. 4.1, lanes 3 and 5).

Fimbrial protein appears to be exceptionally thermostable as indicated by its resistance to degradation even after 4 h at 95°C. Nevertheless, complete degradation was evident after 12 h at 95°C (Table 4.2). Fimbrial protein also appears to be relatively acid stabile as indicated by its insusceptibility to acid degradation at concentrations up to 0.1 M HCl, at RT However, it is more resistant to degradation by a strong base since the protein evidently does not degrade fully until the NaOH concentration reaches 0.5 M for a 2 h exposure at RT.

4.3.2. HPLC and Amino Sequence of Fimbrial Protein

The fimbrial polypeptide was purified by HPLC, and a single peak containing material with a spectrum consistent with proteins was observed (Fig. 4.2 A). The protein peak was collected and analyzed by SDS-PAGE (Fig 4.2 B). In addition, the fimbrial oligopeptides were separated by HPLC and numerous

Fig. 4.1 Disulphide bond analysis of the fimbrial subunit using SDS-PAGE with and without a reducing agent. Gel was stained with Coomassie Blue R-250. Lane 1: protein standards (Bio Rad; 97, 68, 43, 32, 21, 14 kDa); lane 2: fimbrial protein solubilized with SDS and heat, lane 3: fimbrial protein solubilized with SDS only, lane 4: fimbrial protein solubilized with SDS and BME, lane 5: fimbrial protein solubilized with SDS, BME, and heat..

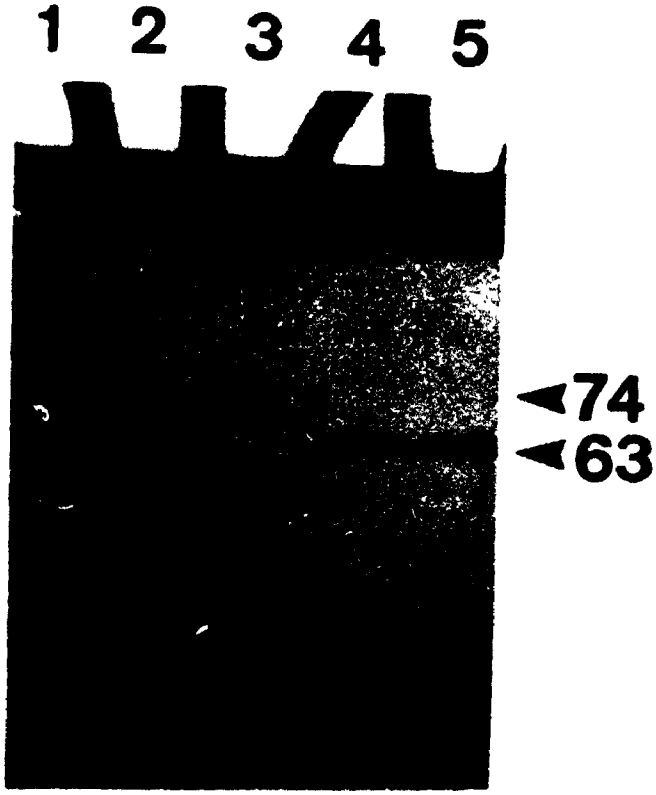
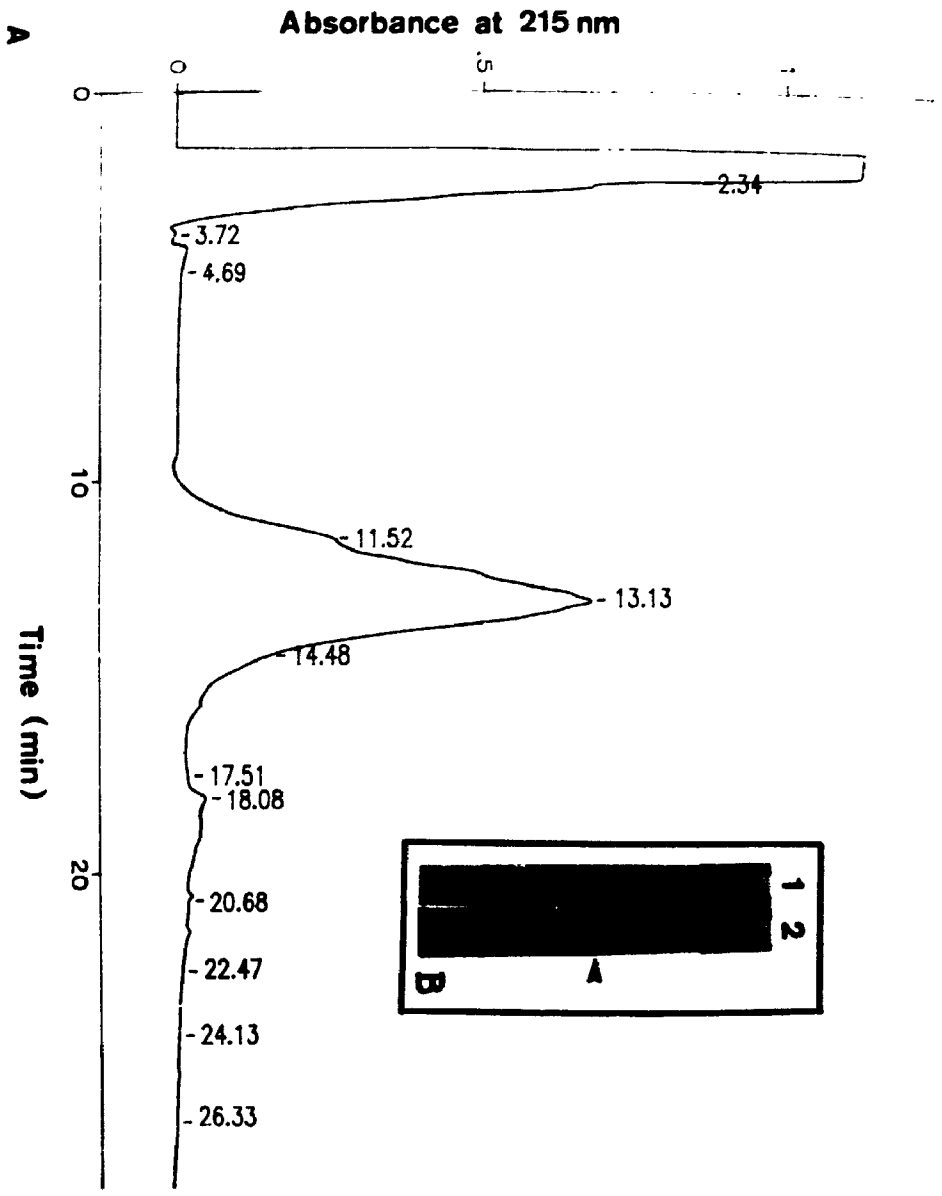

1 2 3 4 5
74
63

Fig. 4.2 Analysis by HPLC and SDS-PAGE of the reduced fimbrial aglycone.

A: HPLC chromatogram of deglycosylated and irreversibly reduced fimbrial polypeptide. A prominent peak is evident at 13.13 min. This material was collected and used in further studies including analysis by SDS-PAGE (panel B) and N-terminal sequencing (Table 4.3).

B: Analysis by SDS-PAGE of the HPLC purified deglycosylated and irreversibly reduced fimbrial polypeptide. Gel is stained with Coomassie Blue R-250. Lane 1: protein standards (Bio Rad; 97, 68, 43, 32, 21, 14 kDa); lane 2: CsCl purified fimbriae, solubilized with BME and SDS; lane 3: contents of 13.13 min peak (panel A).


A
Absorbance at 215 nm
0
5
0
2.34
3.72
4.69
10
11.52
13.13
14.48
Time (min)
17.51
18.08
20
20.68
22.47
24.13
26.33
1 2
B
A

peaks were observed (Fig. 4.3). Three peptides, represented as peaks marked 14.48, 17.6952, and 26.2672 (Fig. 4.3 and inset) were used for obtaining internal amino acid sequences. The N-terminal and three internal amino acid sequences of fimbrial protein are given in Table 4.2. Residues symbolized by an 'X' are not identifiable by conventional methods. Presumably they are modified. Their absorption spectra are consistent with that of hydroxylation (J. Lagueux, pers. comm). It is also noteworthy that both internal sequences 2 and 3 contain internal lysine residues even though the enzyme used to generate the oligopeptides was endopeptidase Lys C. This enzyme cleaves all lysylpeptide bonds efficiently with the exception of lysylproline (Sakiyama and Masaki, 1994).

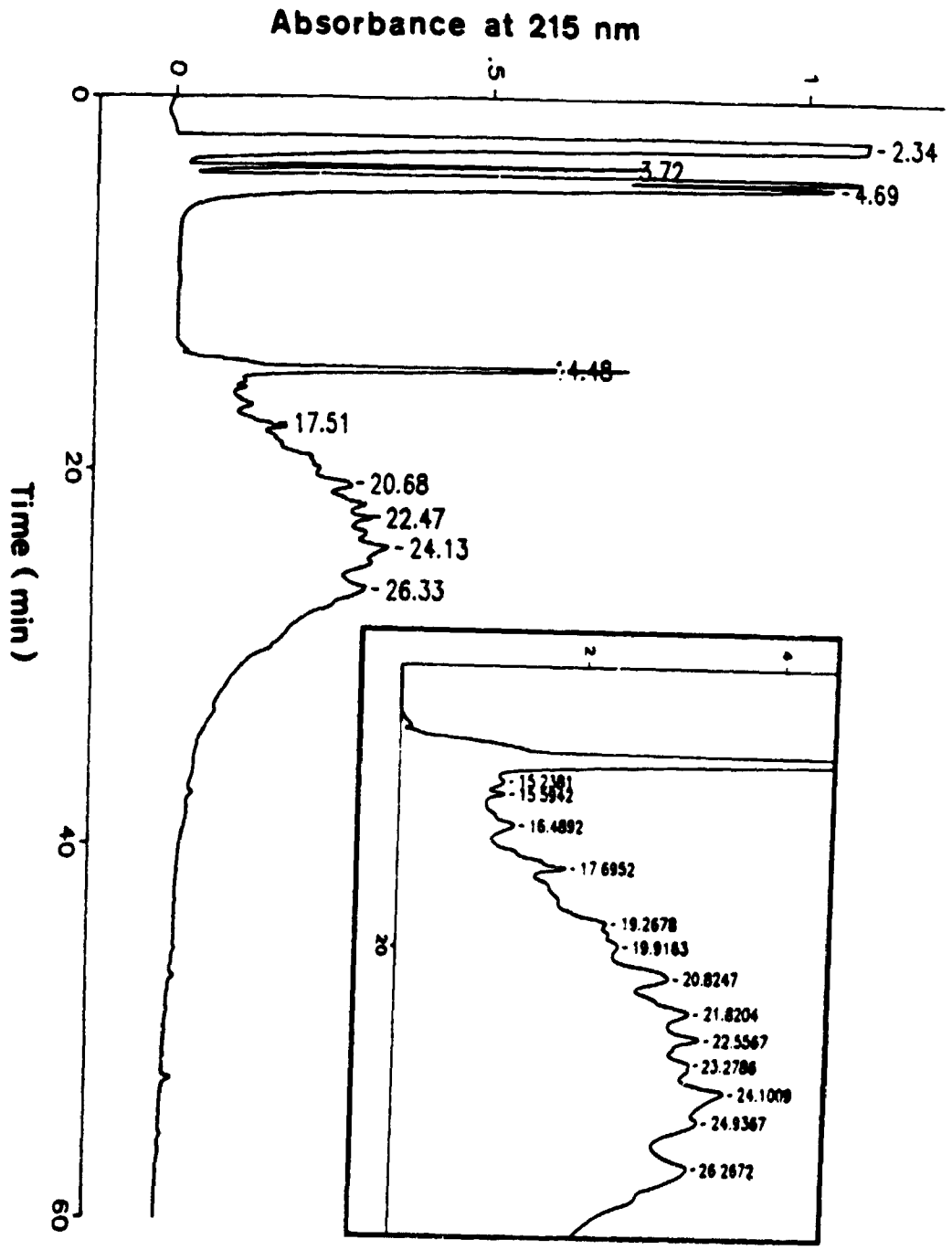
The four amino acid sequences were compared to all known protein sequences present in the Genbank database. All four showed a clear homology to a single protein family, collagen. For example, internal sequence #3 exhibits 63% identity and 63% similarity to human α2 (IV) collagen. Internal sequence #2 shows 72% identity and 81% similarity to human α1 (IV) collagen. In total, 49 residues of the fimbrial protein were determined by Edman degradation. The deglycosylated fimbrial protein has a molecular mass of 47 kDa (3.3.1). Since a 47 kDa protein contains approximately 358 residues (average of 136.75 Da per residue), direct amino acid sequencing determined approximately 14% of the total amino acids present in fimbriae. This indicates that at least 14% of the total fimbrial protein is similar to the collagen family of proteins.

4.3.3. Attempts to Clone the Fimbrial Gene

Four different approaches, low stringency oligonucleotide hybridizations, PCR, RT-PCR, and heterologous sequence hybridization, were used in attempts to clone the fimbrial gene. Low stringency oligonucleotide hybridizations produced autoradiographs which exhibited numerous (>20) bands of strong intensity (data not shown). This substantial number of bands precluded any attempt to clone the fimbrial gene using this methodology.

The sequences of the PCR products were determined, translated in all 3 reading frames and compared to all known protein sequences in Genbank. However, no significant homologies were found. In addition, with the exception of the region of the oligonucleotide primers, none of the subsequent nucleotides matched the predicted nucleotides based on the actual amino acid sequence of the fimbrial polypeptide, as determined by Edman degradation. However, when the nucleotide sequences of the PCR products were compared to all nucleotide

Fig. 4.3 HPLC analysis of fimbrial oligopeptides. The oligopeptides were generated by digesting the deglycosylated and irreversibly reduced fimbrial polypeptide with endopeptidase Lys C. The figure inset is an enlargement showing more detail of the peaks between 12 min and 28 min. The individual peptides were separated, collected and three (marked 14.48, 17.6952, and 26.2672 min,) were used to obtain internal amino acid sequences (Table 4.3).


Absorbance at 215 nm
0
5
1
0
2.34
3.72
4.69
4.48
17.51
20.68
22.47
24.13
26.33
20
40
60
Time (min)
20
15.2181
15.5942
16.4892
17.6952
19.2678
19.9163
20.8247
21.8204
22.5567
23.2786
24.1009
24.9367
26.2672

sequences in Genbank, an exact match to M. violaceum 5S ribosomal DNA (Blanz and Gottschalk, 1984) and strong homologies to other ribosomal DNA sequences were revealed.

The sequences of the RT-PCR products were also analyzed and compared to all known protein sequences. Again, these sequences did not show a significant homology to any known proteins. As for the sequences of the PCR products described above, the predicted amino acid sequences from the RT-PCR products did not match the anticipated amino acid sequence of the fimbrial polypeptide.

Various collagen genes (Table 4.6) were radiolabelled and used under moderate stringency conditions as heterologous probes in attempts to detect the fimbrial gene sequence in M. violaceum genomic DNA. The resultant autoradiographs exhibited numerous sized bands (>20) of various intensities (data not shown). These results again stifled attempts to clone the fimbrial gene using this methodology. In addition, the patterns produced by the hybridization of the numerous of collagen probes to M. violaceum genomic DNA were varied. They also did not match the patterns from the oligonucleotides. Therefore, even the patterns themselves could not be used as markers for cloning the fimbrial gene.

4.3.4. Collagenase Digestions of Fimbriae

The results of the zymograms (Fig. 4.4 A) confirmed that the three collagenases used were enzymatically active. The lack of Coomassie Blue R-250 staining at predicted locations in the gel indicated that the gelatin present in the gel matrix was effectively digested by the activated collagenases. In addition, native collagens were digested with the collagenases and the products were separated by SDS-PAGE (Fig. 4.4 B). The changes in the apparent molecular masses of the collagen subunits indicated that the digestion was successful. Figure 4.5 shows the results of the three collagenase digests of fimbrial protein. The products were separated by SDS-PAGE and appreciable quantities of some products are evident. In addition to visual inspection, laser densitometry scans were used to detect products that were generated in small quantities. Table 4.7 lists the molecular mass of the products obtained in the collagenase digests.

Fimbrial protein in the native fimbrial conformation was resistant to digestion with gelatinase B and interstitial collagenase as indicated by the

Fig. 4.4 Analysis using a zymogram and SDS-PAGE of the activated collagenases.

A: Activated collagenases were separated by electrophoresis in a polyacrylamide gel containing gelatin. This gel, known as a zymogram, was negatively stained with Coomassie Blue R-250 in order to visualize the region of digested gelatin (clear band) in the polyacrylamide matrix. Lane 1: gelatinase A; lane 2: gelatinase B; lane 3: interstitial gelatinase.

B: Analysis by SDS PAGE of collagen type I digested with activated collagenase A (lane 1). The control, lane 2, contains collagen type I without enzyme, but otherwise it was subjected to the same digestion conditions.

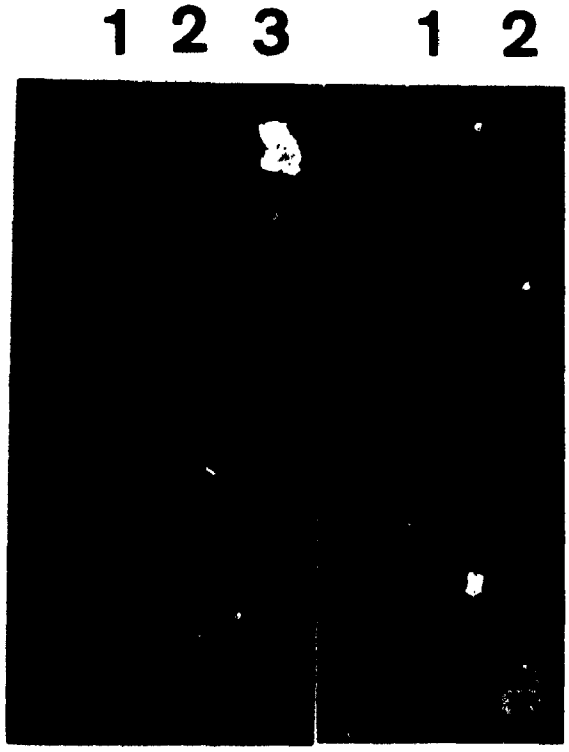

1 2 3
1 2

Fig. 4.5 Analysis by SDS PAGE of collagenase digested fimbrial subunits. CsCl purified fimbriae were subjected to digestion with gelatinase B (panels A and B), interstitial collagenase (panels C and D), and gelatinase A (panels E and F). In panels B, D, and F, fimbriae were heated briefly prior to enzymatic digestion. Control lanes (marked 'c') contain fimbriae subjected to the same conditions and for maximum length of the digest, however in each case the enzyme was omitted. Duration of the digests were as follows:

A: Lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 4 h.

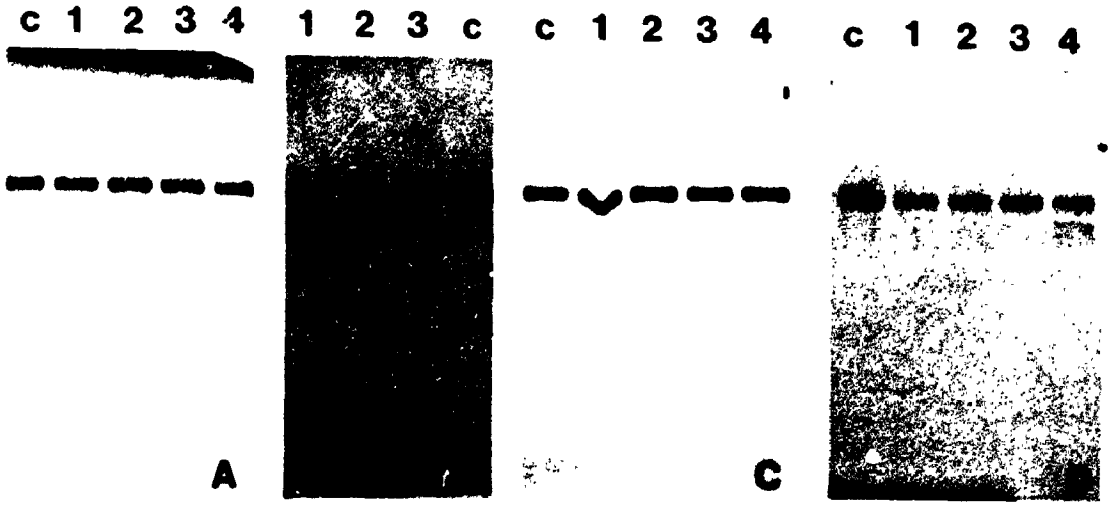
B: Lane 1, 2 h; lane 2, 1 h; lane 3, 0 h.

C: As per panel A.

D: As per panel A.

E: Lane 1, 4.5 h; lane 2, 2 h; lane 3, 1.5 h; lane 4, 1 h; lane 5, 0.5 h; lane 6, 0.25 h; lane 7, 0 h.

F: Lane 1, 0 h; lane 2, 0.25 h; lane 3, 0.5 h; lane 4, 1 h; lane 5, 1.5 h; lane 6, 4.5 h; lane 7, 20.5 h.


c 1 2 3 4
1 2 3 c
A
c 1 2 3 4
c 1 2 3 4
C

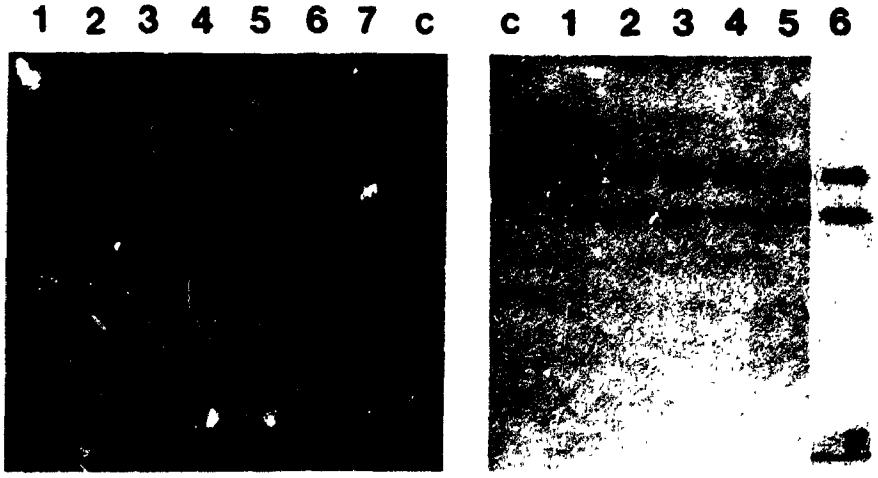

1 2 3 4 5 6 7 c
c 1 2 3 4 5 6

Table 4.7: Molecular weights of products obtained by digestion with collagenases: With and without heating the fimbriae prior to digestion

	Gelatinase A Digests	
Time (h)	Heated (kDa)	Not Heated (kDa)
C	74	74
0	74, 70, 56	74
0.25	74, 53, 36, 32, 6.8, 3.3	74
0.5	74, 53, 36, 32	74
1	74, 53, 32	74, 61, 56, 53
1.5	74, 53, 32	74, 56, 53
2	NT	74, 53
4.5	74, 53	74, 53
20.5	74, 53, 44, 9	NT

	Gelatinase B Digests		Interstitial Digests	
Time (h)	Heated (kDa)	Not Heated (kDa)	Heated (kDa)	Not Heated (kDa)
C	74	74	74	74
0	74, 60, 56, 50, 46, 45	74	74	74
1	74, 60, 56, 50, 46, 45	74	74	74
2	74, 60, 56, 50, 46, 45	74	74	74
4	NT	74	74, 62, 53	74

C: control, no enzyme
NT: not tested

seemingly unaffected 74 kDa protein in SDS-PAGEs (Fig. 4.5 A and 4.5 C), even after 20 h of incubation. However, fimbrial protein present in native fimbriae was susceptible to digestion with gelatinase A. Partial digestion was first evident after 1 h (Fig 4.5 E, lane 4). A relatively stable product (Mr = 53 kDa) was also first observed at this time, and eventually it emerged as the only stable product (Fig. 4.5 E, lane 1).

Partially denatured fimbrial protein, in contrast to native fimbrial protein, was significantly more susceptible to digestion by all three of the collagenases. Figure 4.5 B shows that the digestion of the partially denatured fimbrial protein with gelatinase B began almost immediately following the addition of the enzyme. With time, these products accumulated, indicating that they are stable.

The timed digests of the partially denatured fimbrial protein with interstitial collagenase were also analyzed by SDS-PAGE. The resultant gel is shown in figure 4.5 D. After 4 h of digestion, a considerable quantity of a stable product (Mr = 62 kDa) was formed.

Finally, the timed digests of partially denatured fimbriae with gelatinase A show the most dramatic effect. The digestion of fimbrial protein began as soon as the activated enzyme was added. However, these initial products (Mr = 70 and 56) were short-lived (Fig. 4.5 F, lane 1). A more stable, prominent product (Mr = 53 kDa) was evident after 15 min of digestion (Fig 4.5 F, lane 2), and it became the only significant product after 20 h of digestion (Fig. 4.5 F, lane 6).

The difference in the susceptibility of native compared to partially denatured fungal fimbriae to the collagenases suggests that there exists a digestion-resistant conformation which is disrupted with mild heating (50°C). This native, three dimensional structure of fimbrial protein is presumably important in its ability to resist digestion by the collagenases that are present in the fungus's external environment (Donly and Day, 1984; Iakovleva and Kozel'tsev, 1994). However, it is difficult to discern if the native fimbriae are truly resistant to collagenase digestion or if the digestion is retarded and a small quantity of product is not detected by these means.

4.3.5. Immuno-chemical Relatedness of Fimbriae and Collagens

Antibodies raised against numerous structural proteins, were used in western blot analyses to ascertain whether fimbrial proteins contain any regions which are epitopically-similar. The results of these experiments are summarized in Table 4.8. The only antibodies that detected fimbrial proteins were generated

Table 4.8: Immunodetections of structural proteins using various antibodies

Antibody	Source	Type	Fimbriae	Actin	Tubulin	Collagen I	Collagen III	Collagen IV	Ovalbumin	BSA
Pv-1	1	P	-	-	-	-	-	-	-	-
Pv-3	1	P	-	-	-	-	-	-	-	-
Av-1	1	P	+	-	-	-	-	-	-	-
Av-3	1	P	+	-	-	NT	NT	NT	-	-
Anti-actin	Si	P	-	+	-	NT	NT	NT	-	-
Anti-tubulin	Si	M	-	-	+	NT	NT	NT	-	-
Anti-vimentin	BM	M	-	-	NT	NT	NT	NT	-	-
Anti-collagen I, II, III, IV, V	CL	P	-	NT	NT	+	+	+	-	-
Anti-collagen IV	Si	M	-	NT	NT	-	-	+	-	-
Anti-collagen IV	HK	P	+	NT	NT	NT	-	+	-	-
Anti-collagen V	HK	P	+	NT	NT	NT	-	-	-	-

+: detected
-: not detected
NT: not tested
Si: Sigma
BM: Boehringer Mannhein
CL: Cedar Lane
HK: Hynda Kleinman
P: polyclonal
M: monoclonal

against collagens. Interestingly, the commercially available antibodies against collagens did not detect fimbrial protein. However the antibodies from H. Kleinmann, raised against collagens IV and V, were effective in identifying the fimbrial protein. The converse experiments, namely using fimbrial-specific antibodies in an attempt to detect various collagens, did not show any relationship.

The antigenic similarity of three collagens (I, III, and IV) to fimbriae was analyzed by ELISA using Av-3. The end-point values for the assays were determined to be 1:512, 1:265, 1:64, respectively. The pre-immune serum was tested synchronously and in all three cases the end-point titre was 1:8. Unlike the results of the western blot experiments listed in Table 4.7, these ELISAs clearly demonstrate that the fimbrial-specific antibodies are able to detect native collagens. However, the denatured collagens that were used in the western blot analyses were not detected with this same antibody.

4.4. Discussion

Early studies suggested that fungal fimbriae are primarily proteinaceous structures (Poon and Day, 1975) found on the surface of most fungi. Poon and Day (1975) showed that proteases degrade the fibrillar structures seen in the electron microscope, and Gardiner (1985) showed, using Ouchterlony plates, that the antigenic properties of fimbriae are lost after protease treatment. However, both of these studies failed to demonstrate explicitly that the 74 kDa subunits are susceptible to protease digestion. The present study establishes that this indeed is the case.

Fimbrial protein shows some degree of insusceptibility to degradation under certain harsh conditions (strong acids, strong bases and excessive heating). This resistance to degradation indicates that the fimbriae would survive in harsh environmental conditions. The fimbrial protein's durability may play a role in both self-preservation and the protection of the associated nucleic acid (f-RNA).

A nucleic acid associated with fimbriae has been characterized (f-RNA, 2.3.3). The nuclease digestion studies on the fimbrial subunits have established that there is probably not a second nucleic acid in fimbriae ie., associated directly with the 74 kDa glycoprotein. These results affirm that there is only one nucleic acid moiety associated with fimbriae, namely the 30 base, single

stranded f-RNA. The f-RNA is clearly not covalently linked to the fimbrial subunits.

One of the most surprising findings of this study was the unexpected homology of fimbrial protein to collagen. This resemblance is evident at the levels of the peptide sequence (direct amino acid sequencing), the epitopic sites present (ELISAs and western blot analyses), the presence of collagenase recognition motifs (collagenase digests), and the presence of modified residues consistent with hydroxylation (amino acid sequence). In combination, these findings conclusively demonstrate that fimbriae are members of the family of collagen proteins. As a consequence of these findings, the protein component of fungal fimbriae will now be referred to as a fungal collagen.

The collagen family of proteins are defined by distinctive common structural properties including 1) the presence of glycine residues every third amino acid, 2) an abundance of prolines and lysines, many of which are hydroxylated, 3) the characteristic configuration of the molecule, composed of three subunits which interact with each other to form a triple helical structure, and 4) the presence of many inter- and intramolecular crosslinks which result in a higher-order structural organization (Yamada et al., 1980). Collagens are initially subdivided into two main classes based on their macromolecular organization namely, fibril forming and non-fibril forming collagens. The former are visible as bundles of striated fibres (eg. tendons), while the latter are only discernable in the EM as individual narrow filaments, the smallest having a diameter of 8 nm. Additional features are then used to delineate 13 types or classes of collagen (Vuorio and de Crombrugghe, 1990) including sequence homology, subunit molecular weights, length of uninterrupted helical domain, intron-exon structure (Miller and Gay, 1987) and susceptibility to collagenase digestion.

Fungal collagen appears to fulfil the criteria required to be designated a member of the collagen family of proteins. All four of the amino acid sequences obtained exhibit the G-X-Y motif where G is glycine and X and Y are often prolines and lysines. In addition, the prolines are occasional hydroxylated. Castle et al. (1992) and Gardiner (1985) showed that as many as six variants of the subunit were found by isoelectric focusing prompting these authors to suggest that the polypeptide probably undergoes extensive post-translational modifications. Also, the apparent shift in the molecular mass of the subunit in the absence of a reducing agent (74 kDa to 63 kDa) conclusively indicates that

at least one disulphide bond is present in the native form of the subunit. Both the post-translational modifications and the intermolecular disulphide bonds are consistent with collagen proteins. EM examinations have shown that fimbriae are narrow (7 nm) individual strands whose surface appears coiled, or rope-like, consistent with the non-fibril forming collagens. In addition, fungal collagens are susceptible to digestion by trypsin, which is consistent with the non-fibril forming collagens. However, mammalian collagens have neither N-linked glycosylation nor RNA associated with them. All the same, fungal collagens fail to fit readily into one of the established 13 collagen types and hence they must constitute a novel, as yet undescribed class of collagens proteins.

DNA sequences that encode other proteins that contain collagen-like sequences have been identified in the genomes of non-animal organisms (Geck et al., 1990; Nurminskaya et al., 1993) although their expression into polypeptides has yet to be demonstrated. Moreover, these may simply be examples of lateral evolution and not truly orthologous evolution. Besides, certain proteins such as lung surfactants, complement factors and certain hydrolases contain short collagen-like regions (Bamford and Bamford, 1990; Johansson et al., 1994; Ichijo et al., 1993, Charalambous et al., 1988), even though they do not meet all of the criteria required to be considered true collagens. They may merely be examples of convergent evolution, or shuffling of specific domains, since their homology to the true collagens is considered unlikely.

All true collagenous proteins currently known are found exclusively in the kingdom Animalia (Garrone, 1978; Morris, 1993; Garrone et al., 1993). They have been used alone (Garrone, 1978) or in combination with other extracellular matrix (ECM) components (Morris, 1993) to argue that animals are a monophyletic group. It has been suggested that the ECM is a primitive feature of multicellularity in animals (Morris, 1993; Wainright et al., 1993; Garrone et al., 1993). However, all of these authors acknowledge that the ECM did not arise *de novo* with the first multicellular animal. Thus it is implied that components of the ECM evolved prior to the first Metazoan. This study suggests that one of the major components of the animal ECM, namely collagen, is much more primitive than has previously been documented, and probably evolved prior to the divergence of fungi and animals.

Cavalier-Smith (1987) used ultrastructural (flattened, non-discoidal cristae, lack of chloroplasts) and biochemical (chitin biosynthesis, glycogen for

carbohydrate storage, mitochondrial codon usage) characteristics to propose that animals and fungi are more closely related mutually than either is to plants. More recently, phylogenies based on 16S-like ribosomal RNA sequences (Wainright et al., 1993), amino acid sequences (Hasegawa et al., 1993; Baldauf and Palmer, 1993), and analysis of conserved oligopeptide insertions (Baldauf and Palmer, 1993) have all confirmed that indeed animals and fungi are sister groups while plants constitute an independent evolutionary lineage. Until the present data were obtained, a unique protein, limited in its range to organisms from only the two most closely related kingdoms, had yet to be described. The discovery of fungal collagen fills this gap and thereby supports what appears to be the most accurate phylogenetic relationship between plants, animals and fungi described to date.

Numerous attempts were made to isolate the M. violaceum gene that codes for fungal collagen. Each of the approaches was distinctive and had been used successfully previously to isolate other genes from various organisms. Heterologous probes have been used in numerous studies to isolate specific genes, including various collagens from the genomes of distantly related organisms (Brinker et al., 1985, Venkatesan et al., 1986). However, even amongst heterologous collagen hybridizations, difficulties have arisen. Kramer (pers. comm.) found that the isolation of specific collagen genes from particular organisms using a heterologous collagen probe has been hampered by dissimilar organismal codon usage. This is typified by failed attempts to isolate collagen genes from assorted nematodes using collagen genes from C. elegans as heterologous probes. Thus, the unsuccessful attempts to isolate the fungal collagen gene using the heterologous collagen probes are not completely unanticipated.

Both PCR and RT-PCR are useful methods for amplifying the DNA sequences that correspond to known protein sequences. RT-PCR of the triple helix region with degenerate oligonucleotides as primers has been used successfully to amplify a new collagen (Constantinou and Jimenez, 1991). Ideally, among other characteristics, the primer DNA should be 1) free of repeat regions that could result in false priming, thus 3' UTR and 5' UTR regions are preferred in structural proteins, and 2) should contain about 50% G+C. Unfortunately, these constraints could not be incorporated into the design of the oligonucleotide primers. The predicted amino acid sequence, translated from

the DNA of the PCR products, showed no similarity to the expected amino acid sequences of fimbrial collagens.

Although the fungal collagen gene has yet to be isolated, the evidence for the existence of fungal collagens is substantial and indisputable. The phylogenetic implications of the mere existence of fungal collagens are profound and of major significance to important phylogenetic questions.

CHAPTER 5 - LOCALIZATION AND FUNCTION OF NATIVE FIMBRIAE

5.1 Introduction

In chapter 5, two aspects of native fimbriae are presented, namely, fimbrial cellular localization and a possible role for fimbriae.

Intact, native fungal fimbriae were first described by Poon and Day (1974) on the surface of M. violaceum. Since then, various EM studies have examined these appendages using negative staining (Gardiner and Day, 1985; Rghei et al., 1992), freeze fracture (Tokunaga et al., 1986; Poon and Day, 1975), and immuno-gold labelling (Svircev et al., 1986b). These studies demonstrated the widespread distribution of the fungal fimbriae amongst the members of the Mycota and suggested that a fibrillar material, possibly fungal fimbriae, traversed the fungal cell wall. Still, an explicit demonstration of the localization and function of fungal fimbriae needs to be put forward.

Investigations described in Chapter 4 determined that the protein component of fungal fimbriae is in fact a fungal collagen. These studies used biochemical analyses to elucidate the composition of these extracellular appendages. However, *in vitro* studies are needed to determine if fungal fimbriae can function in roles usually restricted to animal collagens. In particular, collagens as components of the extracellular matrix form a substratum onto which animal cells can adhere and proliferate.

The present study was undertaken 1) to determine the localization of fimbrial epitopes in both mature and budding cells, 2) to learn if fimbriae can function in a manner similar to animal collagens.

5.2 Methods and Materials

5.2.1 Immuno-gold Detection of Fimbriae

A drop of purified fungal collagens was placed on a formvar coated (0.25%), carbon reinforced, 400 mesh copper grid (EMicron) and left for 30 sec. Excess liquid was removed and replaced with a drop of primary antibody diluted 1:500 in PTBN buffer (20 mM sodium phosphate [pH 7.4], 0.5% [v/v] Tween-20, 0.1% [w/v] BSA, 145 mM NaCl, 1 mM sodium azide). After 1 h, the drop was removed and replaced sequentially with four drops (1 min each) of PTBN. The final drop was replaced with secondary antibody (goat anti-rabbit IgG conjugated to gold particles 10 nm in diam) diluted 1:50. After 30 min, the drop was replaced with 4 consecutive drops of PTBN (1 min each). After the final drop was removed, the grid was negatively stained with ammonium molybdate as described previously (2.2.3).

5.2.2 Immuno-cytochemical Methods

Exponential-phase cells of M. violaceum were pelleted by centrifugation (1 000 x g, 1 min). The culture medium was removed and the cells were washed twice in ddH_2O. Cells were pre-embedded by resuspending them in water agar (2% [w/v] agar, 45°C) and cooling quickly to solidify. Agar cubes (1-2 mm^3) containing the M. violaceum cells were fixed for 1 h (1.5% [v/v] p-formaldehyde, 0.5% [v/v] gluteraldehyde) and post-fixed for 0.5 h (100 mM cacodylate buffer [pH 6.8], 2% [w/v] OsO_4) to enhance preservation and organelle visibility. Cubes were rinsed twice in ddH_2O, stained for 20 min in saturated aqueous uranyl acetate followed by 10 min exposures to an ascending series of ethanol concentrations (20% to 90%). Finally the cubes were incubated twice for 30 min in absolute ethanol followed by two 15 min transition steps through propylene oxide. Cubes were subsequently infiltrated for 1 h intervals through a graded series of propylene oxide:Epon-Araldite resin mixtures (3:1, 1:1 and 1:3). This was followed by an infiltration with slow end over end rotation overnight in pure resin, and 4-5 additional h in fresh, pure resin. Cubes were then placed in rectangular molds filled with fresh resin which was allowed to polymerize (45-60 h, 60°C).

Ultrathin sections, prepared using an ultramicrotome (Sorvall Porter-Blum MT2) equipped with a diamond knife, were lifted onto 400 mesh grids. Sections were etched to expose antigenic sites by floating them (section side down) in

saturated aqueous $NaIO_4$. Grids were blotted to remove excess $NaIO_4$, and washed 4 times, 1 min each time, by floating them on drops of ddH_2O. Sections were blocked in PTBN buffer for 10 min, and subsequently incubated overnight in a humidity chamber with either Av-1 (2.3.2), Av-3 (4.2.5) or AFMV (anti-foxtail mosaic virus; Rouleau, 1994) antisera at 1:500 diluted in PTBN. Grids were washed with 4 drop changes of PTBN (1 min) and then incubated with gold-conjugated goat anti-rabbit antibodies (30 min; 10 nm gold particles; 1:50 in PTBN) in a humidity chamber. Sections were washed 4 times (1 min) in ddH_2O, stained with saturated aqueous uranyl acetate (10 min), and washed with ddH_2O. Finally, grids were stained in lead citrate (35 mg lead citrate, 10 ml dd H_2O, 2 drops of 10N NaOH; 45 sec) in a Petri dish containing a few NaOH pellets to create a CO_2-free environment. Sections were subsequently observed with a Philips CM-10 electron microscope, operating at 60 or 80 kV.

5.2.3 Adhesion Assays

Purified fimbriae (0.1 to 5 µg) and fibronectin (Collaborative Research Inc., 0.2 µg) were permitted to adhere to microtitre wells (Nunc, RT, 2 h). Non-specific interactions were blocked with 1% (w/v) BSA (30 min, RT) and excess liquid was removed. A2058 human melanoma cells (Todaro et al., 1980) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum. Subconfluent monolayers of cells were treated with trypsin, resuspended in DMEM, counted, and allowed to recover for 1 h. Cells were pelleted and resuspended in DMEM to a concentration of 3×10^5 cells/ml. Resuspened cells (100 µl) were added to each well and incubated at 37°C in a humidity chamber. After 5.5 h, wells were rinsed with PBS and adhered cells were observed microscopically for changes in morphology (from globose to spreading). Cells were fixed and stained with Giemsa (Diff-Quik, Baxter), washed with PBS and photographed. The amount of adhesion was quantified by re-extracting stain from adhered, dried cells (10% [v/v] methanol, 5 min, RT) and read at 650 nm on an ELISA Plate Reader (Bio-Rad). Three replicates per experiment were performed and the results were averaged.

5.3 Results

5.3.1 Immuno-gold Detection of Fimbriae

Fimbriae were negatively stained and immuno-detected successfully with both Av-1 and Av-3. In control experiments, the pre-immune sera Pv-1, Pv-3, and an antibody to a plant virus (AFMV) were used as the primary antibodies. None of these antisera were able to detect fimbriae. In addition, a primary antibody was omitted and the detection was carried through as described. In all cases little or no gold labelling was apparent.

5.3.2 Immuno-localization of Fimbriae in M. violaceum

Thin sections of M. violaceum were immuno-gold labelled using Av-1 and Av-3, and examined in the EM. In both cases the immuno-gold particles were localized at the periphery of the cells, in particular the inner surface of the cell wall, the periplasmic space, and the most peripheral cytoplasm (Fig. 5.1). In addition, the gold labelling appeared to be continuous along the inner cell wall surface, including the neck region between the mother cell and budding daughter cell (inset, Fig. 5.2) and continued along the inner wall surface of the budding cell (Fig. 5.2). Also, it was evident in oblique sections of the cell wall (Fig 5.3) that the most pronounced gold labelling occured in three layers: both the outer and inner cell wall surfaces, and possibly the inner membrane surface. Low levels of gold labelling was detected in the extracellular region (Fig. 5.4).

5.3.3 Adhesion Assays

Cultured animal cell lines require the presence of specific ECM components for adhesion and proliferation. Adhesion of human melanoma cells to a fibronectin substratum resulted in changes in the cytoarchitecture. These alterations were manifested as spreading cells with numerous peripheral cell attachment sites (Fig 5.5 A). No characteristics of cell adhesion were observed in the absence of fibronectin or fimbriae (Fig 5.5 B). However, fimbriae were capable of functioning as an ECM for human melanoma cells (Fig 5.5 C). One hundred micrograms of fimbriae was sufficient to permit the adhesion and spreading of this cell line. Nevertheless, the interaction was more complex than expected, since apparently there was an inverse relationship between fimbrial concentration and cell adhesion. Increasing the concentration of fimbriae inhibited cell adhesion. This was evident as successively fewer and fewer cells

Fig. 5.1 Immuno-gold detection of fimbrial epitopes on thin-sectioned M. violaceum haploid cell. Av-1 is the primary antibody and it detects the fimbrial epitopes which appear to be localized in the cytoplasmic periphery and the inner cell wall layer. (Magnification 60 500 X)

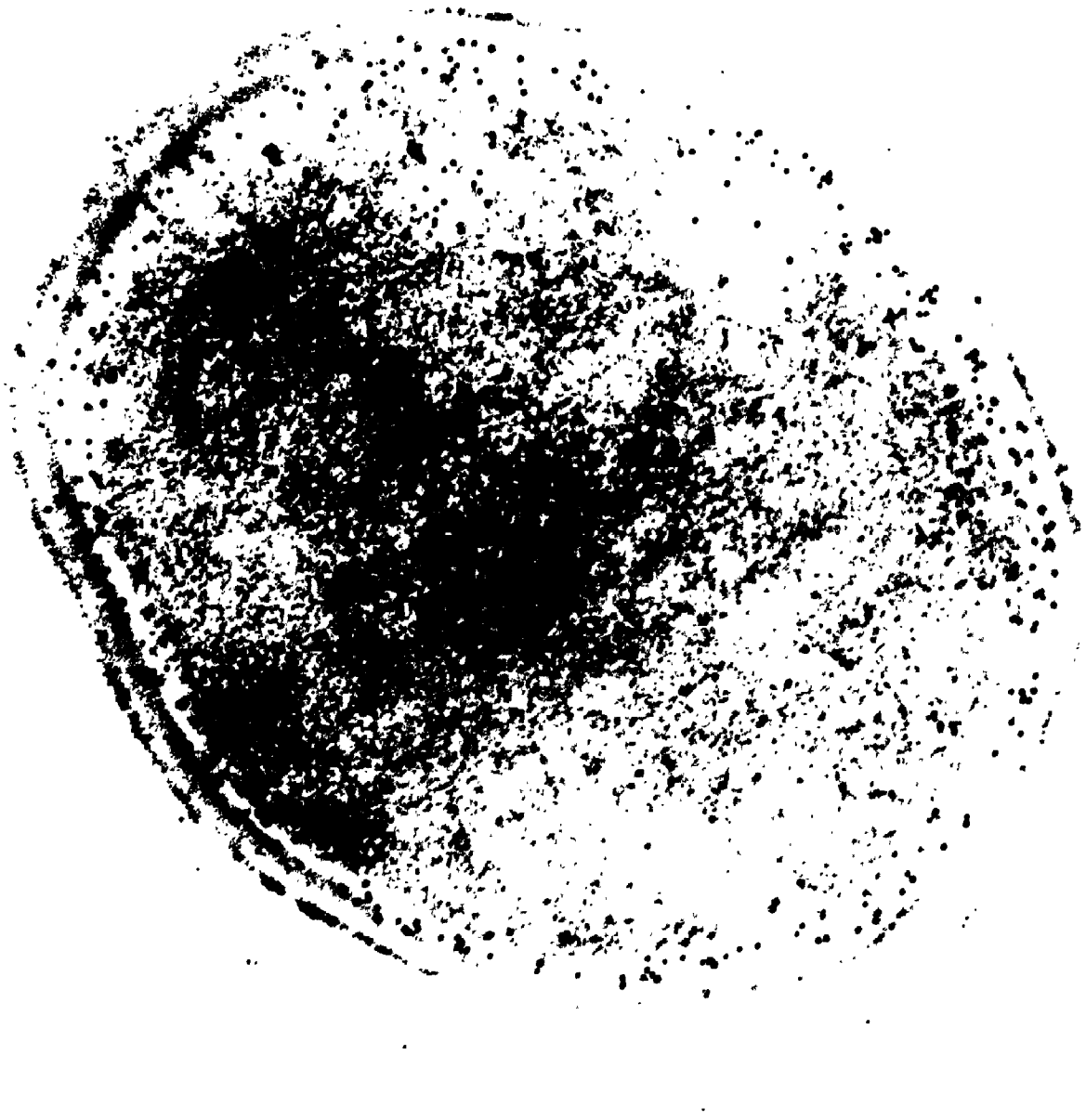


Fig. 5.2 Immuno-gold detection of fimbrial epitopes on thin-sectioned budding cell of M. violaceum. Immuno-gold particles using Av-1 as the primary antibody appear to form a single layer at the cell wall-cytoplasm interface. (Magnification 40 000 X). Inset: enlargement of mother-daughter neck region. Gold particles are also continuous throughout the cell wall-cytoplasm interphase of this region. (Magnification 72 000 X).

Fig. 5.3 Immuno-gold detection of fimbrial epitopes on an oblique, thin-sectioned cell of M. violaceum. Immuno-gold particles using Av-1 are localized to three distinct layers at the cell periphery. (Magnification 105 000 X). The inset diagram demonstrates these layers (a, b, and c).

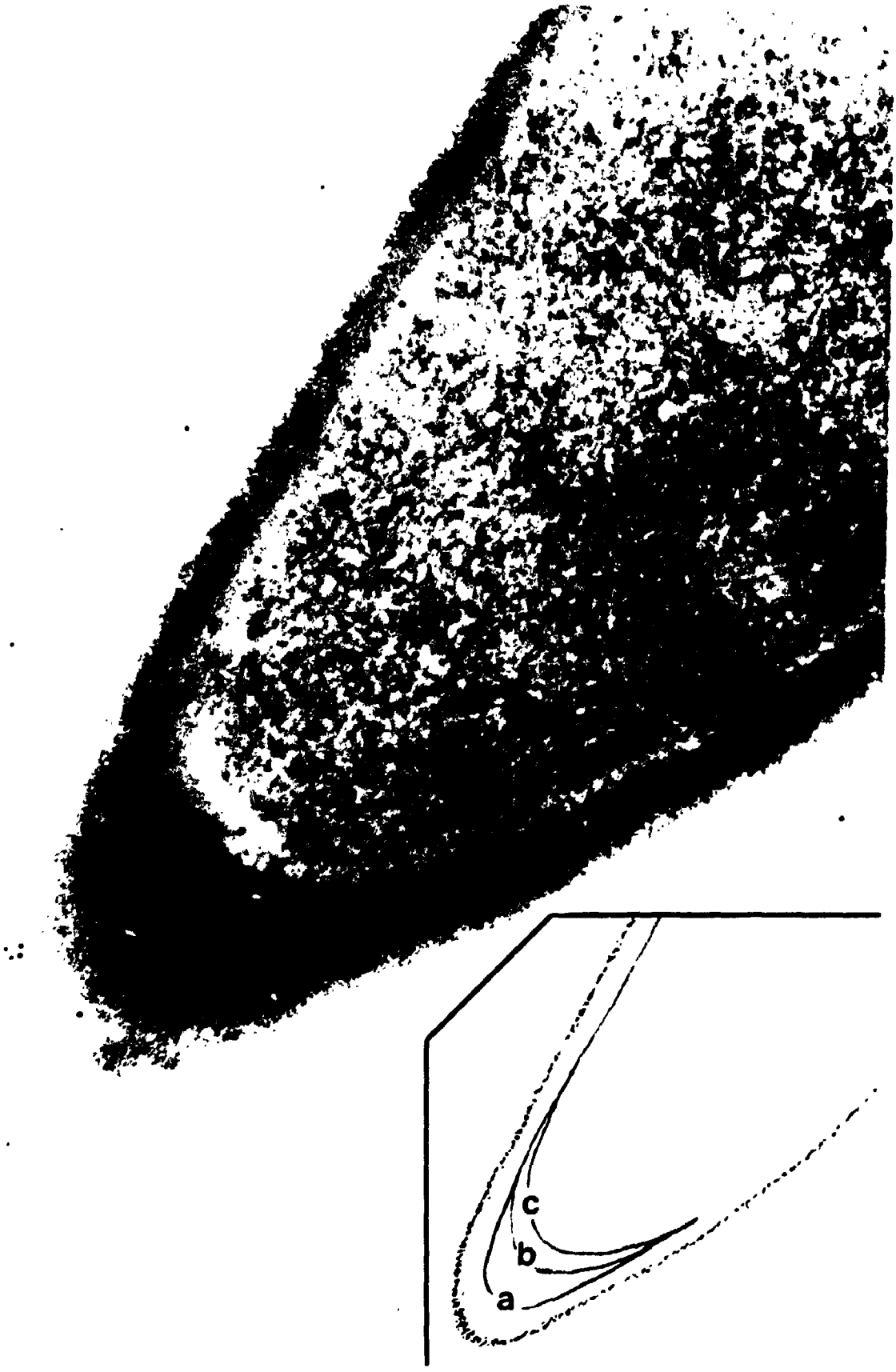

c
b
a

Fig. 5.4 Immuno-gold detection of fimbrial epitopes in thin-sections of the extracellular region between two adjacent cells of M. violaceum. Gold particles are evident in the extracellular region where mature fimbriae are known to be present. (Magnification 81 000 X).

Fig. 5.5 Adhesion of cultured human melanoma cells to fimbriae collagens. A-F are representative photographs of these animal cells adhering to fibronectin and fimbriae of various concentrations. (Magnification 500 X). An inverse relationship between the concentration of fimbriae and the number of cells adhered is evident.

A: Positive control: fibronectin adhered at 2.0 µg/ml.

B: Negative control: no protein.

C: Fimbriae adhered at 0.10 µg/ml.

D: Fimbriae adhered at 0.25 µg/ml.

E: Fimbriae adhered at 0.50 µg/ml.

F: Fimbriae adhered at 1.00 µg/ml.


B
C
D
E
F

attached with increasing amounts of fimbriae (Figs. 5.5 D, E, and F). This correlation was quantified as the amount of stain taken up by attached cells compared to amount of fimbriae present as substratum. Figure 5.7 clearly shows that a non-linear relationship exists between cell adhesion and the quantity of fimbriae used as substratum.

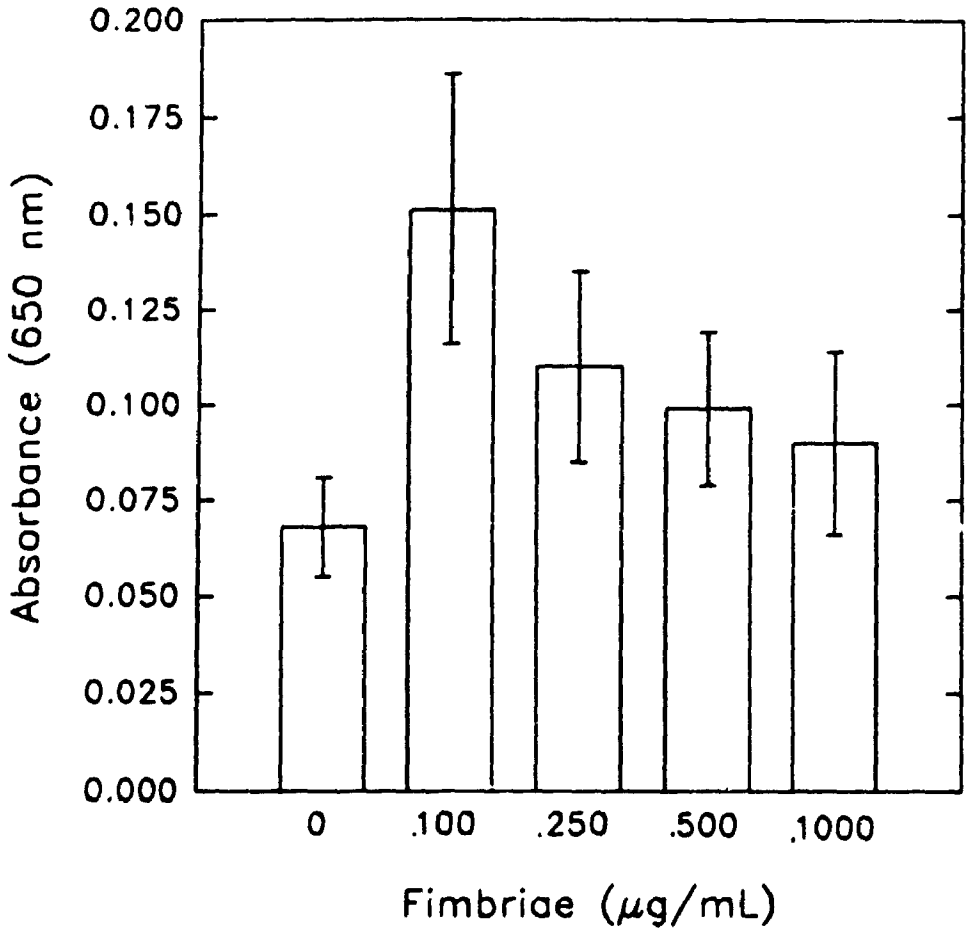
5.4 Discussion

ECM components play numerous roles in animal biology including cell-cell interactions, cell motility, cell shape changes, chemotaxis, and cell adhesion (Reddi, 1984). The ECM components form multiple interactions with each other, as well as with components on the animal cell surface. In many cases, collagens form the scaffolding onto which the other ECM components are assembled. Strong affinities exist between these collagens and other ECM components including fibronectin, glycosaminoglycans and proteoglycans (Reddi, 1984).

Cell adhesion is dependent on the presence of either an intact ECM or a solid substratum of certain ECM components such as collagen. Cell adhesion can trigger a multitude of biochemical cascades that can result in cell attachment and growth (Kleinman et al., 1981; Mann et al., 1992), differentiation (Zuk et al., 1989; Hauschka and Kornigsberg, 1966, Kleinman et al., 1993), and cell polarization (Reddi, 1984). *In vitro* studies have shown that certain ECM proteins alone, or in specific combinations, are sufficient to trigger a cascade of events. Current research indicates that each ECM component is involved in numerous and diverse interactions, although the precise role of each ECM component *in vivo* has yet to be determined.

Fimbriae are found throughout the Mycota (3.3.3). Although the function of fimbriae in general is not yet clear, a role in animal mycopathology can be predicted. The present investigation shows that fimbriae can mimic the roles ordinarily reserved for animal ECM proteins. Numerous studies have described ECM components such as fibronectin (Klotz and Smith, 1991; Casanova et al., 1992), entactin (Lopez-Ribot and Chaffin, 1994), laminin (Bouchara et al., 1990; Vicentini et al., 1994) and collagen (Ollert et al., 1993; Tsuchida et al., 1995) interacting with fungal surface components in order to mediate their adhesion (Vazquez-Juarez et al., 1993; Tronchin et al., 1991; Klotz, 1990). Independently, each of these interactions could be important in the adhesion of the fungus to the host tissues. However, Ollert et al. (1993) suggested that

Fig. 5.6 Graph depicting amount of human cells adhered versus concentration of fungal fimbriae. The greatest amount of adhesion is observed at 0.1 µg/ml of fimbriae. Increasing concentrations of fimbriae inhibit the adhesion of the cultured cells.


0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
Absorbance (650 nm)
0
.100
.250
.500
.1000
Fimbriae (μg/mL)

there exists multiple molecular mechanisms of fungal attachment to cultured cells. Nonetheless, all of these earlier studies suggested that the fungus has a receptor for ECM components. Conversely, the present study is the first to suggest that a potential host organism may recognize, and thus have a receptor for a familiar structure on a mycopathogen.

Plant cell walls and extracellular matrices have been studied extensively and some analogies to animal ECMs have been discovered. However, the evolution of these two ECMs differs significantly (Lord and Sanders, 1992) and consequently most studies suggest that they are examples of convergent evolution. Recently, an algal protein containing both an extensin-like domain and a fasciclin-like domain was described as the first plant CAM (cell adhesion molecule; Huber and Sumper, 1993). In addition, Zhu et al. (1994) have described a plant vitronectin-like protein which acts as an adhesin, but it shows no sequence similarity to vitronectin. Structures such as these are possible candidates for forming interactions with fimbriae, however additional studies are required to confirm this.

Immuno-gold labelling studies showed that fimbrial epitopes are present on both the inner and outer cell wall surfaces. Fimbriae are present and continuous along the mature cell surfaces and throughout the new bud cell surfaces. All stages of budding and non-budding cells have approximately the same amount of gold labelling. Thus, fimbrial epitopes are present exclusively at the cell wall, and equally at all times of the mitotic cell cycle.

The number of gold particles found on the inner surface of the cell walls exceeds that found on the outer surface. Earlier shadow-casting studies showed that fimbriae occur over the entire cell surface of both mature and budding cells (Day and Gardiner, 1988). Two explanations for the recent finding are possible. First, the fimbrial epitopes may have been dislodged during the fixation procedure. Additional studies using other EM preparative techniques such as progressive low temperature embedding and molecular distillation drying (L. Cole, pers. comm.) may confirm this. Second, the intercellular conformation of the fimbriae may differ, and thus may be more amenable to antibody recognition than the extracellular fungal collagen. This extracellular structural protein is synthesized inside the cell and may be maintained in a non-polymerized form (a pool) at the periphery of the cytoplasm. The members of this pool are transported to either 1) a growing tip, or 2) the bottom of the fimbriae, thereby pushing it through the cell wall

Sparse gold labelling is evident throughout the thickness of the cell wall and is significantly less than that at observed at either of the two surfaces. This may be a consequence of the fimbrial epitopes being less readily available to the antibodies when they are embedded in the cell wall, or simply that they are not present throughout the cell wall. Freeze fracture studies (Poon and Day, 1975) suggested that fimbriae may traverse the cell wall. However, the authors conceded that it was difficult to decide whether these were truly wall fibrils, fimbriae, or artifacts induced by etching. The present study indicates that mature, polymerized fimbriae may not traverse the cell wall, but are instead anchored to another structure present within the outer cell wall. The evidence for this observation is that the gold particles form 3 distict layers instead of being dispersed evenly throughout the cell wall. This suggests that fimbrial polymerization probably occurs at a growing tip, as described above. In this latter model, fimbriae would more closely resemble the collagen arrangement present in animal ECM systems. Further studies are necessary to support this idea.

CHAPTER 6 - CONCLUSIONS

6.1 Summary

This thesis has focused on elucidating the composition of the fimbriae of M. violaceum. M. violaceum was a useful organism for this study because it is maintained readily under laboratory conditions and it is known to produce ample, long fimbriae that are harvested easily. While establishing a method for purifying fimbriae, it was determined that they are composed of more than simply glycoproteinaceous subunits. Evidence indicated that fimbriae contain nucleic acid. This nucleic acid was subsequently purified and analyzed; it was a multiple copy, 30 base, single-stranded RNA that may be modified. The implications of an extracellular RNA were discussed, in view of the roles of fimbriae in pathogenesis and mating.

The carbohydrate moiety of the fimbrial glycoprotein was also examined. It constitutes approximately 36% by weight of the fimbrial subunit. The mannose-rich carbohydrate is attached to an asparagine residue within the polypeptide chain via two GlcNAc units. The pattern is conserved only among members of the Microbotryales.

In contrast to the carbohydrate structure, the protein component of the fimbrial subunit is conserved among members of four fungal phyla. Previous research suggested that fimbriae are widespread amongst the fungi. This is extremely interesting, given the results of the protein analysis. Three separate types of analysis (direct amino acid sequencing, immuno-chemical analysis, and substrate specific enzyme cleavage) all indicated that the fimbrial protein is, in fact, a fungal collagen. Unfortunately, neither reverse genetics, nor heterologous probes were useful in isolating the gene for fungal collagen. The evolutionary implications of the existence of fungal collagen, in light of the fact that known collagens have only been observed in members of the animal kingdom, were discussed. In addition, the relatedness of the animal ECM and fungal fimbriae, and the possibility that they both evolved from a common ancestral structure, was considered.

Fungal collagen appears to pool at the perifery of the cytoplasm. Immuno-gold EM suggests that the fibrils probably do not traverse the cell wall, but instead subunits polymerize onto a growing, extracellular fimbrial tip.

Finally, native fungal fimbriae were examined and were found to function as analogs of animal ECM components. They formed a viable substratum that

permitted the adhesion and proliferation of a cultured human cell line. The implications and possible roles for fimbriae in pathogenesis were discussed.

6.2 Future Studies

The discovery of f-RNA is intriguing. Its mere existence permits speculations on possible unconventional mechanisms of cell to cell communication. This novel extracellular nucleic acid should be characterized further in order to determine its role *in vivo*. The base composition of f-RNA is unknown and should be determined. Preliminary results suggest that some of the nucleotides are probably modified. Total hydrolysis, followed by HPLC and mass spectrum analysis will permit the identification of the component nucleotides of the f-RNA. The results of this study will determine if the next step of f-RNA analysis, namely determining the nucleotide sequence, is feasible. Analysis of the f-RNA sequence may provide the information to predict any secondary structures. Also, comparisons of the sequence to RNA motifs where structure-function relationships have been determined may be useful in suggesting a role for the f-RNA. Finally, fimbriae from other fungi need to be examined to determine if they too house an f-RNA. Preliminary results using S. commune and C. cinereus indicate that the densities of their fimbriae in CsCl gradients are also consistent with that of nucleoproteins (Castle, pers. comm.).

Conserved glycosylation patterns have been used in taxonomic analyses previously. However, they have never been used before to classify members of a fungal order. The present study differentiates members the Microbotryales, as proposed by Oberwinkler (1995), from the Ustilaginales. It is suspected that the converse of the present study is also true. Namely, that the members of the Ustilaginales *s. str.* also have a conserved glycosylation pattern on their fimbrial proteins. Fimbriae from fungi in this order could be used in a similar manner as described in this thesis to create glycosylation-specific antibodies. These antibodies may be useful in screening fimbriae of other smut fungi thereby determining the boundaries of another order.

Finally, further attempts to isolate the fungal collagen gene still should be performed. Two plausible approaches remain to be attempted. In both cases an expression library using mRNA from M. violaceum cells should be created. The mRNA should be harvested from exponential-phase cells since the latter appear to have the most fimbriae on their cell surfaces. Firstly, the antibody Av-3, described in this thesis, would be a useful tool for screening the expression

library since it was raised against the denatured fimbrial collagen. Secondly, radiolabelled f-RNA could be used to screen the library. Based on examples in the literature, it is expected that an attraction exists between the f-RNA and fungal collagen. Although reassociation studies have not been attempted in this thesis, preliminary studies using Western blots of denatured protein and labelled f-RNA will be done to confirm the feasibility of this second approach.

The importance of isolating the fungal collagen gene is multi-fold. The complete amino acid sequence of the polypeptide based on the cDNA sequence can be predicted. This information will either confirm that the entire fimbrial subunit is a true collagen, or it will indicate that a portion of the subunit has a collagen-like motif. Either way, the result will be useful for proposing the evolutionary history of fimbriae.

Protein motifs are well established, conserved amino acid sequences whose functions have been described throughout the literature. The predicted peptide sequence of the fimbrial subunit, based on the cDNA sequence, may be useful in predicting the location of the glycosylation(s) and any other post-translational modifications. In addition, the peptide sequence may contain motifs that would be useful in proposing how the f-RNA is associated with the polypeptide of the fimbrial subunit.

It is equally important to obtain the sequence of the genomic copy of the fungal collagen gene, but for different reasons. At the level of the protein, it is difficult to assign relatedness of the different collagen types or classes. However, at the DNA level an interesting observation has been made. Animal collagens appear to stem from two progenitor molecules. One of the collagen gene patterns contains exons of 54 bp or multiples thereof, separated by numerous large introns. It is speculated that this group of collagens arose by multiple duplications of a single genetic unit. In contrast, the other collagen gene pattern does not show any evidence of this 54 bp repeat, and the exons are separated by a few (<5) small introns. Based on these observations it has been suggested that two types of collagen arose by convergent evolution. The intron-exon arrangement of the fungal collagen gene will be interesting and hopefully useful in determining the latter's relatedness to one or both of the animal collagen gene patterns.

6.3 A Final Comment

It is often assumed that research should only be attempted after scholarly wisdom has been attained. When I began this thesis I thought that my research should build on solid, established knowledge. My greatest fear was failure due to a lack of knowledge which seemed, at the time, unattainable. Now I realize how fortunate I was not to have this knowledge, and to have naively blundered forward without the bias of pre-conceived dogmas. I truly hope that all researchers can begin there too. Its wonderful not knowing that something is impossible. And I also hope that I can keep the same naive open mind when I leave here. Extracellular RNA, tightly bundled in a fungal hair...who would have thought. And to top it off, the hair is actually collagen, the most abundant animal protein.

> The protein recognized as collagen occurs in all but the simplest of organisms....(Miller and Gay, 1987).

I'm delighted to know that fungi aren't simple.

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